Offspring sex ratio bias and sex related characteristics of eggs in chicken

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This research was conducted under the auspices of the Graduate School of Wageningen Institute of Animal Sciences (WIAS)

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Thesis
submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. Dr M.J. Kropff,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Monday 8 September 2014
at 11 a.m. in the Aula.

Muhammad Aamir Aslam
Offspring sex ratio bias and sex related characteristics of eggs in chicken,
192 pages.

PhD thesis, Wageningen University, Wageningen, NL (2014)
With references, with summaries in Dutch and English
ISBN 978-94-6257-075-7

Abstract

Aslam, M.A. (2014), Offspring sex ratio bias and sex related characteristics of eggs in chicken. PhD thesis, Wageningen University, The Netherlands

Understanding the factors influencing sex of egg and sex ratio in laying chicken may lead to finding potential solutions for the problem of killing of day old male chicks, which is the current practice in breeding of laying hens. In studies described in this thesis, it was investigated if the sex of the chicken egg can be predicted by measurable differences in male and female eggs at unincubated stage and if the female primary sex ratio can be induced in laying chicken using different experimental conditions such as feed restriction and corticosterone feeding. The method of sex determination in unincubated chicken eggs using PCR targeted to CHD1 gene was first developed. This method was subsequently used to study the primary sex ratio bias as well as relationship between egg sex and yolk hormones. No significant relationship of the sex of egg with concentrations of several hormones (testosterone, estradiol, androstenedione, progesterone, dihydrotestosterone) and glucose in yolk as well as of egg parameters (mass, width and length) was found. Effect of feed availability on sex ratio was tested in two separate studies. In one study, the rate of change of hen body mass between day of laying and day of laying minus 2 days (encompass time of meiosis completion) was a significant predictor for the sex of that egg, suggesting meiotic drive as mechanism of sex ratio bias. This relationship was not found in the later study. The difference in results could be due to the reason that hens decreased in body mass much less in the later study as compared to earlier study. Blood corticosterone concentrations were associated with sex ratio per hen in the earlier study. Effect of egg mass on egg sex was studied during the later experiment of feed restriction. The egg sex ratio per hen was negatively associated with the average egg mass per hen in the feed restriction group. Two groups of hens were selected from the feed restriction group i.e. male biased hens with low egg mass and female biased hens with high egg mass for microarray analysis of gene expression in the germinal disc of collected F1 follicle. The results did not show differential expression of genes between the groups. However, gene set enrichment analysis showed that a number of processes related to cell cycle progression, mitotic/meiotic apparatus and chromosomal movement were differently enriched between the groups, supporting meiotic drive as potential mechanisms underlying sex ratio determination. In another experiment,

blood circulating levels of corticosterone in hens were increased by feeding corticosterone mixed feed under ad libitum. The blood levels of corticosterone were significantly higher in treated hens but these levels were not associated with sex ratio. Treatment did not affect the overall sex ratio, but affected the sex ratio in interaction with hen body mass. In the corticosterone group, sex ratio, laying rate, and fertility rate per hen were decreased in heavy hens. These results suggest that three parameters (sex ratio, laying rate and fertility rate) are connected at the level of ovarian physiology. Interference with meiosis have been shown to affect these three parameters, suggesting the involvement of meiotic drive as mechanism of sex ratio bias.

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General Introduction

1.1Poultry breed specialisation and its consequences

The domestic chicken (*Gallus gallus domesticus*) is a domesticated fowl and one of the most common and widespread domestic animals with a world-wide population of around 20 billion. Chicken meat and eggs have contributed significantly to the well-being of humans by providing protein rich foods. In the second half of the previous century, special types of chicken breeds were developed meant to provide either meat or eggs. Chickens of the layer type breeds are fully geared towards egg production. This means that resources from the feed are largely funnelled towards the ovary to produce eggs, rather than being used for muscle growth. In contrast, in the broiler breeds, the physiology and biochemistry of the animal are fully geared towards muscle growth, rather than egg production. This has led to very efficient production of eggs and meat. The advantages are not only that consumers can enjoy low priced availability of meat and eggs, but also that the ecological footprint of meat and egg production is strongly reduced. To produce meat and eggs, the specialised breeds use much less feed and produce much less manure and other emissions compared with traditional breeds [1] and consequently, the use of resources (land, other resources) for producing feed is also much lower. Unfortunately there are also disadvantages of this practice. One major disadvantage is that the male chicks in animals of the layer breeds are not economically suitable for producing meat and are consequently considered as a useless by-product in the production of layer hens. In the current practise, male chicks of layer breeds are killed right after hatch. Approximately 45 million male chicks are killed annually in The Netherlands alone [2]. While most of these male chicks (85%) are used for pet food or feed for zoo animals, the killing of day old chicks is considered an ethical problem for animal rights and it is also a waste of resources for the poultry industry. Societal organisations, industry stakeholders, and Dutch parliament want to end the current practice of this killing and find solutions. In 2007 the Ministry of Agriculture (currently part of the Ministry of Economic Affairs) mandated research aimed at finding alternatives to this practice.

1.2 Potential solutions for killing of one day old male chicks in breeding of laying hens

In a desk study of 2007 and based on the knowledge available, a number of potential alternatives for killing of day old chicks were formulated [3]. It is only relevant to try and develop one or more of these potential solutions if they would represent an improvement on

the basis of ethical considerations, societal acceptation and consumer response. Therefore, research into societal acceptance of potential alternative solutions was conducted. First a series of 'Focus group' discussions were conducted to obtain insight into public awareness of and feelings about the current practice, and to gauge the arguments and considerations of the people in these groups. Secondly, an internet survey, with almost 1200 respondents, was performed to get also quantitative measures of the opinion of the larger public [4]. Apart from eight 'biotechnological' potential solutions the survey also included the option of the so-called 'dual purpose chicken', and 'accepting the current practise'. The latter is necessary to know whether a potential solution is considered as an improvement compared with the current practise. The three 'biotechnological' alternatives considered most favourable were: 1) In ovo sex determination of unincubated eggs through examining a sample from the yolk taken from freshly laid eggs; only the female eggs are subsequently incubated ; 2) Influencing the chicken so that a reduction of the proportion of male eggs in the total egg production would be obtained, inducing a female primary sex ratio bias ; 3) Genetically modifying the chickens such that eggs could be sexed before incubation based on genetic modification of the sex chromosomes. The work presented in this thesis is related to the first two alternatives. The third alternative was undertaken in a separate project.

Alternative 1) was proposed based on a number of studies in several birds species [5-8], indicating that various maternal steroid hormones could be present in the yolk in different concentrations in male and female eggs, and may play a role in mechanisms that influence egg sex and primary sex ratio. In addition, some studies indicated that male and female eggs may also differ in egg dimensions [9, 10]. Alternative 2) was proposed based on studies that indicated that birds have the ability to skew the sex ratio under natural as well as experimental conditions [11-22].

1.3 Sex allocation theory and sex ratio bias

Darwin was already intrigued by the observation that species tend to produce almost equal numbers of both sexes in a population [23]. The theory of sex allocation was not further explored until 1930 by Fisher, who explained how natural selection may lead to a sex ratio of 0.5 [24]. Fisher proposed that when the sex ratio is disturbed in a population, the mother that has the greatest reproductive variance of producing the rarer sex will have greater reproductive fitness and her offspring will be most successful. This would lead to an increase

in the frequency of the rare sex and, consequently, this will always result in an equilibrium in which the relative frequency of both sexes within a population are the same. Hamilton [25] further explored and supported Fisher's model through mathematical modelling and empirical testing of the sex allocation theory.

Some authors expanded the sex allocation theory by proposing that under certain conditions sex ratios may depart from parity, which may have selective advantage [26, 27]. According to the sex allocation theory, when the costs of rearing sons and daughters are different and the survival and reproductive success of one sex varies with the amount of parental resources transferred to offspring, the breeding females tend to produce more chicks of the sex which has more fitness returns under these conditions [26, 27].

There is abundant correlative and experimental evidence in a number of animal taxa (mammals: [28-33] reptiles: [34-37] and birds: [11, 14-16, 38-50]) that sex ratio biases can occur in relation to parental and environmental conditions, which supports the concepts of sex the allocation theory. However, the underlying mechanisms of sex ratio biases are not (completely) understood.

For the discussion of sex ratio bias, it is important to distinguish between the primary and secondary sex ratio. The primary sex ratio is the sex ratio resulting from the sex determined at meiosis or fertilization, while the secondary sex ratio is the sex ratio at later stages, often at birth (or hatching in birds). The primary and the secondary offspring sex ratio must be distinguished, as different mechanisms may be in place affecting one or the other. The secondary sex ratio may differ from the primary sex ratio, due to differential survival of oocytes depending on their sex, and sex dependent differential zygote or embryonic or postnatal offspring mortality [46]. The primary sex ratio is believed to be biologically more efficient as it is the most cost efficient way for a female to produce a higher number of the desired sex while minimising investment in the unwanted sex. For the same reason, this would be the most effective way of inducing female sex ratio bias in poultry industry as it would lead to a sex ratio skew without decreasing the number of eggs with viable embryos.

The mechanisms responsible for primary sex ratio bias are currently still an enigma in contrast to the mechanism responsible for a secondary sex ratio bias [20, 51]. There is a general biological interest in understanding the mechanism of primary sex ratio bias for several reasons. First, it could open up the possibility of manipulating sex ratio in the poultry industry in a favourable way. If it would become possible to induce a female sex ratio bias in

chicken (without negatively affecting health, welfare, or productivity) it could lead to finding ways and means to reduce the number of day old male chicks killed in the layer poultry industry [3, 52]. Secondly, it could also be relevant for applied sciences for species conservation programmes [53]. Thirdly, understanding these mechanisms in birds would help to understand the evolutionary and functional consequences of sex ratio bias.

1.4 Potential mechanisms of primary sex ratio bias in birds

In some taxa non-genetic mechanisms of sex determination occur. For instance, in a number of taxa such as in some fish, turtle and reptile species sex of the developing embryo can be determined by environmental factors such as the social environment or incubation temperature [54]. In mammals and birds, as far as we know, there are only genetic mechanisms of sex determination [55]. Birds are an interesting case as in contrast to mammals the female is the heterogametic sex, i.e. the males have two identical sex chromosomes (ZZ) and females have two different sex chromosomes (ZW). Thus, in birds, the sex of the offspring is determined in the female during meiosis, in which one sex chromosome (either Z chromosome giving rise to ‘male’ gametes or W chromosome giving rise to ‘female’ gametes) is retained in the developing ovum while the other chromosome is sequestered in the polar body. This opens the possibility that by manipulating the female we can manipulate the offspring sex ratio.

A number of pre-ovulatory and post-ovulatory mechanisms of the reported primary sex ratio and secondary sex ratio bias, respectively, have been proposed for birds [8, 46, 56, 57]. Several of the mechanisms related to an avian primary sex ratio bias will be described below in more detail. As these mechanisms relate to pre-ovulatory events, we first briefly describe the stages of follicle development in birds.

In birds, only the left ovary is functional, the other ovary is regressed. The functional ovary contains billions of developing pre-hierarchical follicles. Only a minority of these follicles mature up to the stage of ovulation [58, 59]. During each ovulatory cycle, a number of follicles are recruited for yolk deposition from a large pool of follicles with a diameter of less than 1 mm [60]. Until the follicles have grown up to a diameter of 8 mm, they can regress due to atresia. Once the follicles become 8 mm in diameter (large yolky follicles), these rarely undergo atresia and become established in a hierarchical order and grow up to 40 mm in diameter and are then ovulated [61, 62]. The meiosis, which is arrested in prophase I during

embryogenesis in females, resumes approximately six hours before the ovulation of the ovum in chicken [63, 64]. The final stages of meiosis, during which sex of an egg is determined, take place 2 to 4 hours before ovulation [18, 64]. The ovulated ovum is fertilized in the infundibulum, albumin and egg shell are then deposited while the egg passes through the oviduct and the egg is subsequently laid. The time between ovulation and oviposition is approximately 24 hours. The main proposed pre-ovulatory mechanisms for primary sex ratio bias in birds, suggested in the literature are the following:

1) *Asynchronous sex specific follicle development*. According to the asynchronous follicular development hypothesis there are already some factors in a follicle some time before meiosis that would predestine the follicle to become either male or female. Then, atresia during early stages of follicle recruitment could lead to resorption of follicles destined to become the un-preferred sex, or the growth rate could vary among recruited follicles destined to become either male or female. This mechanism would allow breeding mothers to control the production of eggs with preferred sex in a time dependent way [65]. This hypothesis has been put forward to explain sex ratio bias in the sequence of eggs in a clutch observed in several species, where one sex tends to be produced at the start or end of the season [39, 66-70]. However, the presumed factor that would already be present during follicle development and that would predestine the sex of the oocyte, has not been identified and remains speculative. It has been proposed [71] that the unknown factor could influence both atresia or growth rate and meiosis. Alternatively, the unknown factor could affect follicular growth rates during the phase of rapid yolk deposition, leading to different exposure of these follicles to biochemical factors present in the blood of breeding females. This could alter the yolk composition of the follicles which could in turn influence the segregation of sex chromosomes [72].

2) *Selective resorption of post-meiotic and pre-ovulatory follicle* [56, 57]. In this mechanism the breeding females could adjust the sex ratio by sex specific follicle abortion and subsequent resorption after the meiosis-I and before ovulation [57]. This mechanism is only conceivable if the mother could detect the sex of the ovum (i.e. whether it contains a Z or a W chromosome) before ovulation. The follicle abortion would lead to delay in clutch initiation and gaps in the intervals between oviposition of eggs of the same clutch and with these limitations, this mechanism is not likely to occur. One study in pigeons specifically addressed this and found no evidence for selective abortion for inducing sex ratio bias [73].

3) *Segregation distortion or meiotic drive*. A third suggested pre-ovulatory mechanisms is meiotic drive. Meiotic drive relates to mechanisms that influence the sex chromosomal segregation in a non-random fashion during meiosis-I. Hormones or other factors present in the yolk (perhaps in the outermost layer containing the germinal disc) or in blood around the time of completion of meiosis-I could affect the sex chromosome segregation. Meiotic drive would be the most efficient mechanism of adjusting the primary sex ratio, as it would avoid any loss of resources in the production of follicles and also it would not lead to any interruption of egg laying. Therefore, a number of authors have proposed that meiotic drive is the most likely mechanism for manipulation of the primary sex ratio [15, 21, 45-47, 56, 57, 72, 74].

Asymmetric segregation of sex chromosomes in itself may result from specific features of the meiotic division in birds. These include difference in the size and shape of the sex chromosome with Z being the larger chromosome, differences in the size of protein bodies attached to sex chromosomes, peripheral location of the germinal disc in the follicle, and variation in the position of centromeres of sex chromosomes (reviewed by Rutkowska & Badyaev [8]). Size difference in the sex chromosome should facilitate the non-random segregation of chromosome, as the polar wind- a force acting on the kinetochore of chromosomes moving towards the pole- is directly proportional to the size of chromosomes [75]. Avian sex chromosomes, especially from chicken, are known to differ in the epigenetic markings and telomere length [76]. The difference in telomere length, in the absence of compensatory mechanisms, has been proposed to facilitate biased segregation of sex chromosomes [8].

In addition, the expression of the telomerase enzyme, which controls the length of telomere in the developing avian follicle, is affected by steroid hormones such as oestrogens, progesterone and androgens [77]. Thus maternal hormonal status could affect the telomere length in a context dependent manner and thereby meiotic division. Furthermore, steroid hormones are actively involved in the modifications of the structure of tubulin (spindle) and tubulin and actin (cytoskeleton) filaments due to release of the intracellular Ca^{2+} [78, 79]. A steroid hormone gradient in the vicinity of the germinal vesicle could result in different activity of actin filaments on two sides of the meiotic plate, resulting in a different pulling force for the chromosomes on two sides of the pole.

The different number of microtubule filaments attached to kinetochores could also lead to a different chance for chromosome to be retained in the oocyte and this is called the 'centromere drive'. For example in the chicken chromosomes with higher number of microtubules attached to chromosomes preferentially segregate to the polar body [80]. Hormones such as oestradiol have been shown to directly bind to microtubules and affect the shape and functioning of the metaphase spindle [81]. It is known that teroid hormones can also act as transcription factors and may change the gene expression [82], which could indirectly affect chromosome segregation.

So it can be concluded that asymmetric chromosome segregation could occur and may be influenced by several factors. Thus meiotic drive may be considered a plausible mechanism of primary sex ratio bias in birds, However, at present these mechanisms remain hypothetical and the mechanism used by birds, and specifically by laying chickens, to influence the sex of an egg has yet to be elucidated. It could be possible that different mechanisms of sex ratio bias operate in different birds species due to differences in their life-history traits. For example, it would be conceivable that species with very small clutches could employ the mechanism of aborting ova of 'wrong' sex until an ovum of the 'right' sex is ovulated by manipulating only the first egg so that no laying gaps are induced. The abortion of ova might not be very efficient for species which lay larger clutches as it may induce such gaps which would lead to reduced egg production, counteracting achieving fitness returns from manipulating the sex ratio.

1.5 Experimental methods to investigate the mechanisms of sex ratio bias in birds

Different experimental methods and observed reproductive parameters of birds can help to get clues about the underlying mechanisms of sex ratio bias. For instance, increased laying gaps and decreased clutch sizes could indicate pre-ovulatory mechanisms such as follicle abortion [57, 83] or interference with progression of meiosis which had been reported to cause delays in ovulation [84-86]. Measuring growth rates of pre-ovulatory follicles and pattern of deposition of yolk layers could give information about sex specific follicular growth [57, 65, 87]. Decrease in fertility rates of eggs could indicate involvement of either pre-ovulatory mechanisms due to interference with meiotic progression and -fidelity, which has been reported to cause high infertility rates [88-94], or post-ovulatory mechanism such as sex

specific fertility [95]. Determining the fertility status and sex of the unincubated eggs could also provide clues regarding the possibility of embryonic mortality as a cause for the observed sex ratio bias. Eggs are said to be infertile when no development is seen during incubation. This does not necessarily mean that the oocyte had not been fertilized. Instead, early embryonic mortality could have occurred during the transit through the oviduct [46]. This can be resolved by staining the blastodisc of laid eggs with a DNA stain, such as Hoechst 33342, as absence of stained nuclei would indicate against embryonic death.

In addition, experiments could be done to specifically address potential mechanisms of meiotic drive, e.g. by studying gene expression in the germinal disc around the time of meiosis preferentially by the use of –omics-like approaches. If changes in the gene expression pattern can be found concomitantly to differences in sex bias ratios, such information can be used to identify functional proteins and/or processes potentially involved in the mechanisms underlying sex ratio bias. In a similar way, proteomics and metabolomics approaches may be used to get clues about pre-ovulatory mechanisms for primary sex ratio bias in birds. The germinal disc is the ‘animal pole’ of the follicle. It is visible as a whitish spot (approximately 3.5 mm diameter),on the surface of the pre-ovulatory follicle (F1) [96]. The important functional reproductive processes such as sex determining meiosis-I [72], fertilization and early embryonic development occur in the germinal disc [97, 98]. The germinal disc of the pre-ovulatory follicle in chicken (F1) contains large amount of RNA (2.1 µg/oocyte) [99], which may be used to regulate cellular processes including meiosis and chromosomal segregation. Meiosis-I occurs about 26 to 28 hours before oviposition [47, 64].

1.6 Food availability and other environmental factors may trigger sex ratio bias in birds

A number of previous studies have demonstrated the ability of birds to skew the sex ratio in response to a number of environmental cues under natural conditions, such as mate quality [42], season [40, 41], and habitat quality (grazing pressure by animals) [38]. In addition, quite a number of studies showed a sex ratio bias in response to variation in food availability under natural conditions [43, 44, 100, 101] or experimental conditions [49, 50]. Female body condition, which is directly influenced by the availability of food, was also shown to be associated with sex ratio bias under natural conditions [13, 102-105] or under experimental conditions [13, 48, 49, 53, 106, 107]. The results from these studies have often been

interpreted in the context of sex allocation theory, which predicts that mothers may adjust the brood sex ratio towards the sex which profits most from the given circumstances [26]. According to the predictions of this theory, under the conditions of decreased feed availability and decreasing body mass, a female sex ratio bias could be expected in species like chicken in which 1) sexes are dimorphic with the female being the smaller-less costly-sex [108] and 2) reproductive variance in males is larger than in females [5, 109]. The latter is due to the fact that in polygynous systems with intense male- male competition reproductive success is more quality dependent for the male sex than the female sex. Thus, a son of high quality/in better condition is expected to provide higher fitness return than a daughter, as such males can fertilize multiple females, whereas in poor conditions a daughter could provide higher fitness returns than a son as a daughter would produce fitness anyhow while poor quality males would be outcompeted by good quality males. Therefore, if offspring quality is correlated with maternal condition, mothers under conditions of less food availability and poor body conditions are expected to produce a female sex ratio bias. Plasma glucose levels are another can be considered to represent body condition. Plasma glucose levels were found to be associated with sex ratio in mice [29], horses [30] and voles (*Microtus agrestis*) [110] but not in pigeons [106]. Glucose levels in plasma and egg yolk have not yet been studied in relation to sex of eggs in birds.

The timing of recording maternal condition could be crucial and varies between different studies. The majority of previous studies measured body condition by taking into account the individual's body mass and skeleton size at one time point. That means that dynamic changes in body conditions (increase, decrease) are not considered (but see Goerlich et. al. [74], showing that this is a promising avenue). Changes of body condition reflect (periods of) negative energy balance and should therefore also be considered as a major parameter influencing the physiology of the hen. Therefore, measuring the changes of body condition or body mass over time may be important to study the influence of body condition on sex ratio bias.

In many avian species, egg mass has been reported to be positively associated with female body mass [111] and basal metabolic rate [112]. Furthermore, feed availability [113] and female body condition during egg production can affect the egg and yolk mass [114]. According to sex allocation theory, under certain conditions mothers may either shift the sex ratio or provide more resources towards the sex with more fitness return in a context

dependent manner [26]. Therefore, under conditions of variation in food availability and changing body conditions females could potentially manipulate the provision of resources differentially to male and female eggs to skew sex ratio of their offspring. Indeed, in birds, some of the previous studies reported that sex specific differential investment in offspring may arise at the level of egg formation [69, 115, 116]. These sex related adaptive differences between egg mass could be a mechanism through which females provide advantage to the smaller disadvantaged sex or favour the offspring with higher reproductive return. Some studies in various bird species showed that sex ratio in a clutch co-varied with egg mass [10, 117, 118]. Also, a number of studies indicated sexual dimorphism in the egg size in house sparrows [69, 116] or in mallards [9].These results suggest, but did not prove, that egg sex could be associated with egg physical parameters.

1.7 Hormones may mediate sex ratio bias in birds

Hormones, which serve as messengers between environment and maternal physiology, are important for diurnal cycles [119] and physiological response to long and short term environmental changes [120, 121]. In birds, the gonadal steroid hormones are produced by the ovarian follicular wall [122]. These steroid hormones which include estradiol, testosterone, and progesterone, play an important role in female reproductive physiology and regulate follicular maturation, resumption of meiosis and ovulation in birds [8, 123, 124] and also affect behaviour [125]. Other important steroid hormones are the corticosteroids, produced in the adrenal glands that also produce precursors for the gonadal steroids such as androstenedione. The corticosteroids are also influenced by environmental factors, social cues and maternal state, and could thus also potentially mediate signalling between environmental cues and physiological mechanisms of primary sex ratio bias. Indeed, in birds under field or experimental conditions, correlations were seen between sex ratio and maternal blood levels of corticosterone [11-13] and testosterone [12-14]. Furthermore, experimental manipulation of blood levels of steroid hormones such as progesterone [15], testosterone [13, 14, 16, 17, 47] and corticosterone [11, 12, 19-22, 45, 47] has been shown to induce sex ratio bias in various birds species.

The growing follicle is supplied with blood through arteries and veins which supplies the follicle with nutrients and hormones during follicular development [62, 126]. The corticosteroids are exclusively supplied via peripheral blood. The sex steroids, however, are

predominantly produced by the cells of the follicular wall (theca and granulosa cells), which are in direct contact with the oocyte [127]. These cells supply these hormones to the blood circulation and directly to the oocyte [126] and it is currently under discussion whether females can regulate the concentrations of gonadal hormones in blood and yolk independently (see review [126]). It can be envisaged that hormones in the yolk (or the germinal disc region) affect the sex of the egg directly, e.g. if these hormones were to be differentially enriched in the yolk, as has been reported previously [5, 13], and would somehow affect meiosis towards male or female bias depending on the concentration of the hormone in the yolk. In that case, the sex of an egg would correlate with the yolk hormone level. Alternatively, a hormone could perhaps affect sex determination indirectly, if a certain level of that hormone (in the blood, in the ovary, or in the oocyte) would trigger some other physiological or cellular factor that enhances the chance of one of the sex chromosomes to be segregated into the polar body during meiosis, leading to a sex ratio bias without having different concentrations for that hormone between eggs of different sex. One previous study in peafowl indicated that this may occur, as it was reported that a female sex ratio bias and a higher corticosterone concentration in the yolk of the eggs was induced by mating of a female with an unattractive male, without a difference in corticosterone concentration between male and female eggs [12]. Similarly, another study in pigeon associated high levels of testosterone with male sex ratio bias without difference of testosterone concentration between eggs bearing male and female embryos [14].

Various previous studies measured the concentrations of different steroid hormones in yolk of male and female eggs (e.g. testosterone, androstenedione, dihydrotestosterone and oestradiol) in different bird species [5-7, 12, 14, 128-137]. No sex specific differences for the concentrations of yolk testosterone were reported by some of the previous studies [12, 14, 128-132, 134-137], however, a few other studies reported sex specific differences when taking into account hen social status [5], mate quality (Groothuis, Boonekamp and Dijkstra in prep) or egg laying sequence [7, 133] and one study reported overall sex specific differences [6]. No sex specific differences for the concentrations of yolk androstenedione were reported previously [5, 128-131]. No sex specific differences for the dihydrotestosterone concentrations in yolk were reported by some of the previous studies [6, 129, 130, 132], and two other studies reported sex specific differences when taking into account egg laying sequence [7, 133]. Also for estradiol, no sex specific differences for the concentrations in yolk were reported in previous studies [12, 130], and one other study reported sex specific

differences when taking into account egg laying sequence [133]. Due to the inconsistent results from the previous studies, it is difficult to conclude about the overall associations of the yolk steroid hormones with sex of egg and to conclude about their role for influencing the sex determination process in birds. However, it could still be possible that yolk steroid hormones and other biochemical substances could interact to influence sex determination in birds. It is currently under discussion whether steroid hormones are the principal factors to affect sex of the avian egg.

Glucocorticoids are implicated in stress responses and are known to regulate reproductive physiology and follicular steroidogenesis [122, 124] in birds. Corticosterone as a catabolic hormone is related to conditions of decreased food availability [138, 139] and decreasing body condition [140, 141]. A previous study reported very low corticosterone concentrations to be present in the yolk of chicken eggs [142]. Other studies have reported detectable levels of corticosterone in yolk in other birds species [12, 143] but it is likely that this is due to cross reactivity of the antibody with gestagens [142, 144]. Blood corticosterone levels change in response to environmental factors that have been reported to influence sex ratio, such as habitat quality [43, 49, 145], and social dominance [146, 147] and partner quality [22]. The findings that the same environmental factors affect sex ratio and corticosteroid concentration suggests that maternal corticosteroids may be a key factor in the mechanism affecting sex ratio in birds.

Sex ratio studies focussing on corticosterone in different birds species and using different administration routes of the hormone have revealed varying results as both male and female biased offspring ratios have been reported in relation to elevated corticosterone. For instance, male biased primary sex ratios were reported in laying chicken (*Gallus gallus*) [47] and Zebra finches (*Taeniopygia guttata*) [45] after corticosterone injections five hours before the expected time of ovulation. In Gouldian finches (*Erythrura gouldiae*) male biased sex ratio was associated with chronically elevated blood corticosterone levels due to breeding with low-quality males [22]. In contrast, other studies associated female biased sex ratio with chronically and experimentally elevated maternal blood corticosterone, either in implantation studies in pigeons (*Columba livia domestica*) [74], white-crowned sparrows (*Zonotrichia leucophrys*) [11] and Japanese quail (*Coturnix coturnix japonica*) [21] or with elevated corticosterone levels in yolk in peafowl (*Pavo cristatus*) due to breeding of the female with low quality males [12]. These contradictory results, with different birds species and using

different methods of corticosterone treatment, demand further exploration. One explanation for the varying results may be that chronic elevation of corticosterone by means of implantation studies induces very unnatural profiles of the hormone, with a peak of the hormone early after implantation, often far outside the physiological range for the species, subsequently followed by a strong decrease [74]. Alternatively there may be species differences.

1.8 Using unincubated eggs for studying yolk hormones and sex ratio bias

In studies on sex ratio, the sex of a hatchling or embryo can be determined using PCR amplification of the CHD1 gene (chromodomain-helicase-DNA binding protein) [148]. This gene is present on the Z and W sex chromosomes, but the gene copy on the Z chromosome is larger than that on the W chromosome. The PCR amplified fragment of the W-chromosome associated CHD1 genes differs in length from the PCR amplified fragment of the Z-chromosome associated CHD1 gens and can be distinguished from each other by agarose gel electrophoresis.

The primary sex ratio is the sex ratio at fertilization, but to estimate the sex ratio of oocytes/ova just after fertilization would not be practical. One would need to collect and sex ova from the oviduct, which would mean one could probably obtain only one ovum per hen. All previous studies of sex ratio bias in birds were based on sexing eggs after oviposition or hatchlings. For sexing eggs, incubation for several days was required to obtain sufficient DNA material. However, it has been described that sex specific embryo mortality can occur [19, 20, 51]. Sex specific embryo mortality would lead to a secondary sex ratio bias. Distinguishing between primary and secondary sex ratio bias is, however, crucial for understanding the mechanisms for sex ratio bias. Previous studies on sex ratio bias investigated the primary sex ratio by either taking into account only those nests or broods in which all the laid eggs were successfully sexed or by concluding that embryo mortality was too low to explain the observed sex ratio bias. Sexing of freshly laid (unincubated) eggs would be a much better approximation of the primary sex ratio. However, a number of technical difficulties to sex unincubated eggs [149] should be addressed.

In addition, in all previous studies that investigated the relation between yolk hormone concentrations and sex of egg, yolk samples had also been obtained after incubation of the eggs for at least one day [15, 50, 150]. However, incubation can change the concentrations of

yolk hormones of maternal origin due to the metabolization of these hormones by the developing embryo, which can already be an issue during the early incubation days [151]. Secondly, hormone concentrations can change due to endogenous production of hormones by the developing embryo [152]. Furthermore, the yolk concentrations of hormones during incubation could also vary due to utilisation of yolk in the concentric yolk layers [153], and different yolk layers are known to vary in the concentrations of hormones [135, 154]. Indeed, several previous studies have shown that yolk hormone concentrations can change shortly after incubation [128, 155]. Therefore, for studying the sex specific concentrations of maternal yolk hormones or studying whether these hormones are related to the sex of the egg, sampling of egg yolk should be performed as early after fertilization as possible.

1.9 Aim and outline of thesis

The research presented in this thesis focuses on offspring sex ratio bias and sex related characteristics of eggs in chicken. The aim of this research was to contribute to reproductive biology research fields relevant to two potential alternatives for the current practice of killing day old male chicks in the layer poultry breeding industry. These two potential alternatives are: 1) In ovo sex determination through examining a sample taken from freshly laid eggs and not incubating the male eggs 2) Influencing the chicken hen by some factors so that fewer male eggs are laid i.e. inducing female primary sex ratio bias.

This chapter (chapter 1) provides the general background information about sex allocation theory and factors affecting sex ratio in birds, and discusses previous literature about associations of hormones in the yolk or in maternal blood with sex of egg as well as associations of physical egg parameters with sex of egg.

Chapter 2 of this thesis describes the development of a DNA based sexing method to sex unincubated chicken eggs. As described above it is important to be able to sex unincubated eggs because the sex ratio of freshly laid, unincubated eggs is a good approximation of the primary sex ratio and because egg yolk of freshly laid eggs can then be sampled to study the relation between maternal hormones in the yolk and the sex of the egg. We considered that the amount of DNA that can be isolated from the blastodiscs of fertile unincubated eggs should be sufficient for PCR-based sex determination, as the blastodisc of fertile laid eggs (stage X embryo) contains approximately between 30,000 to 60,000 cells. As described by Arnold et. al. [149], one problem of sexing unincubated eggs is that infertile eggs do not contain

embryonic DNA but are contaminated with maternal DNA (granulosa cells) or with paternal DNA (accessory sperm). We have addressed, and found solutions for this, and other technical difficulties, which resulted in an easy and reliable method for sexing unincubated eggs. This method was applied in all further studies described in this thesis (chapters 3, 4, 5, 6).

In chapter 3 we investigated whether it would be possible to sex eggs in a minimally invasive way by measuring differences between male and female eggs. We looked for differences between male and female eggs in various eggs parameters i.e. yolk concentrations of testosterone, estradiol, androstenedione, progesterone and dihydrotestosterone, or other egg characteristics such as yolk glucose concentrations, egg weight, egg length and egg width and also studied their potential interactions. If hormone or glucose levels in male and female eggs would be sufficiently different, this would potentially allow sexing of unincubated egg by sampling yolk by a fine needle and then measuring the concentrations of these compounds.

A further main topic of this thesis is the regulation of sex ratio. Our first goal was to establish that it is indeed possible to induce a sex ratio bias experimentally in layer chicken. If we could succeed in reproducibly inducing a sex ratio bias with a specific experimental treatment, this could be used as an animal experimental model for studying the mechanisms of primary sex ratio bias in chicken at physiological and molecular levels.

In chapters 4 and 5 we used feed restriction as treatment to induce a female sex ratio bias, as a female sex ratio bias would be predicted by the sex allocation theory. We studied whether a decrease in hen body mass//body condition (chapter 4 and 5) or a decrease of egg mass (chapter 5) due to feed restriction would lead to a female sex ratio bias.

As described in chapter 5, a significant negative association was found between egg mass per hen and sex ratio per hen after feed restriction treatment. Hens were sacrificed at the end of the feed restriction treatment at the time of occurrence of meiosis-I and the F1 pre-ovulatory follicles were collected. In order to study the underlying mechanisms of meiotic drive and sex ratio bias, genome wide transcriptome analysis was performed of the germinal discs of the follicles of two groups of hens i.e. male biased hens with low egg mass and female biased hens with high egg mass (chapter 5).

In chapter 6, we studied the effects of corticosterone on primary sex ratio bias in laying chicken. Hens had ad libitum access to either normal feed or feed with added corticosterone, with the aim to induce chronic elevation of circulating corticosterone concentrations. In most studies in various bird species, corticosterone treatment yielded a female sex ratio bias, but

some studies also found a male bias. It is uncertain whether the varying results are due to species differences or are related to the route of administration of corticosterone, with short transient or chronic elevation of corticosterone. Injection of corticosterone (short transient elevation) has been studied in chicken, yielding a male sex ratio bias [47], but chronic elevation has not been studied yet. Therefore the aim of our study was to see if chronic elevation of corticosterone in chicken would lead to a female sex ratio bias, as was found in most studies in other bird species. In order to avoid supra-physiological concentrations in blood followed by strong down regulation of the hormone concentration, as is the case in implantation studies, we used the technique of mixing the hormone through the feed, as this would provide a much more natural elevation [150]. In order to study the underlying proximate mechanisms of sex ratio bias, the associations among sex ratio per hen, laying rate, hen fertility rate, hen body mass and blood corticosterone concentrations were studied.

In chapter 7 the results from chapters 2-6 are discussed in a broader context. The results are discussed in the light of sex allocation theory and previous studies in different birds species on the effects of hormones on sex ratio bias. We discuss the possible future developments and recommendations to further elucidate the underlying mechanisms of sex ratio bias.

2

A reliable method for sexing unincubated bird eggs for studying primary sex ratio

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Molecular Ecology Resources, 2012 May;12(3):421-7.

Abstract

In birds, offspring sex ratio manipulation by mothers is now well established with potentially important consequences for evolution and animal breeding. In most studies on primary sex ratio of birds, eggs are sexed after incubation by the use of PCR methods targeted to the sex-linked *CHD1* genes. Sexing of unincubated eggs would be preferred, but as fertile and infertile blastodiscs cannot be distinguished macroscopically, errors could arise from PCR amplifications of parental DNA associated to the vitelline membrane of infertile eggs. In this study, we stained blastodiscs without the vitelline membrane with Hoechst 33342. This allowed unequivocal distinction between fertile and infertile blastodiscs. Fertile blastodiscs contained thousands of fluorescent nuclei, whereas no nuclei were seen in infertile eggs. In addition, after nucleic acid analysis, fertile blastodiscs yielded much stronger chromosomal DNA and *CHD1* targeted PCR bands on agarose gels compared to infertile blastodiscs. These findings indicate that fertile blastodiscs contain much more embryonic DNA than parental DNA, allowing reliable sexing of the fertile eggs. The differences between fertile and infertile blastodiscs in chromosomal DNA and *CHD1* PCR banding intensities alone, could also be used to distinguish fertile from infertile eggs without using Hoechst staining. We conclude that identifying fertile blastodiscs either by Hoechst staining or by analyzing the yield of chromosomal DNA and *CHD1*-PCR products, combined with *CHD1*-targeted PCR amplification, presents an easy and reliable method to sex unincubated eggs.

Key words: Sexing; birds; unincubated eggs; primary ratio; *CHD1* genes

2.1 Introduction

Methods to determine the sex of birds using PCR amplification of sex chromosome specific sequences [156, 157] enable the sexing of early stage embryos, in principle also the sex of laid, unincubated fertile eggs. Such sexing methods have been used to study the primary sex ratio of birds, e.g. to test sex allocation theory [26] that implies that female birds may skew the sex ratio of their offspring under certain environmental conditions. Indeed, there is now ample evidence that avian mothers can skew the sex ratio of their offspring [11, 12, 14, 42, 46, 158], with potentially important consequences for evolutionary biology and animal breeding programs. However, such skews can be caused by skews at fertilization, the primary sex ratio, or by skews due to sex specific embryonic mortality during incubation or chick mortality at hatch, the secondary sex ratio. Primary and secondary sex ratio's may differ from each other. Distinguishing between the two is important for understanding both the mechanisms, function and evolutionary consequences of sex allocation.

As embryo mortality could be sex-specific [20, 51], the primary sex ratio, the ratio at fertilization, could be best approximated by measuring the sex ratio as early as possible in embryonic development. Moreover, it is suggested that avian mothers may allocate different amounts of hormones or other substances to the egg yolk in order to change the primary sex ratio [5, 130, 159]. Since concentrations of these substances change already during early incubation [155], also in such studies it would be important to know the sex of fertilized eggs at the earliest stages, preferably of unincubated eggs.

In blastodiscs of fertile unincubated eggs, the amount of DNA that can be isolated for PCR-based sex determination should be sufficient, as the blastodisc of fertile laid eggs (stage X embryo) contains approximately between 30,000 to 60,000 cells [52, 149]. However, fertile and infertile unincubated blastodiscs cannot be distinguished from each other macroscopically. In blastodiscs from unincubated infertile eggs, which are devoid of embryonic DNA, the presence of maternal DNA from granulosa cells and/or paternal DNA from sperm cells associated with the vitelline membrane may lead to invalid PCR-based sex results [149]. Therefore, in many studies, eggs were sexed after incubation for a few days [14, 15, 50], as fertile and infertile eggs can then be easily distinguished, and ample embryo tissue for isolation of DNA can be obtained.

The aim of this study was to establish a method for discriminating fertile or infertile unincubated eggs of laying hens and to sex the fertile eggs on the basis of their *CHD1-*

targeted PCR banding pattern. To this end, we used Hoechst 33342 staining or the yield of chromosomal DNA and *CHD1*-targeted PCR products and the quality of the isolated DNA/RNA in terms of fragmentation.

2.2 Materials and Methods

2.2.1 Experimental animals

In the part of the study in which we used Hoechst staining of the blastodiscs (see below), 30 mature laying hens and 4 roosters of layer breeding line ISA brown layer of 40 weeks of age were used. Hens and cocks were housed individually with a light:dark period of 18h:6h respectively at 25 °C and were fed normal layer diet. Following one week of acclimatization, the hens were inseminated twice weekly continuously with the pooled semen from the roosters, diluted with avian semen extender medium, and the 106 eggs used in this experiment were collected during third week after the start of the inseminations. Ten virgin hens of ISA brown layer were also used to get infertile eggs.

In a second part of the study, we used a total of 22 mature hens and 4 roosters of 45 weeks of age, of the layer breeding line Lohmann brown, using the same housing and feeding conditions. Twelve of these hens were inseminated twice weekly for two weeks with the pooled extended semen from the roosters, while 10 hens remained virgin hens. A total of 142 eggs (approximately 12 per hen) were collected from the inseminated hens on 12 consecutive days, starting on the second day after the 2nd artificial insemination. Ten eggs (one egg per hen) were collected from the virgin hens. The collected eggs were directly stored at 17 °C until use on the same or next day.

2.2.2 Isolation of blastodiscs

Eggs of two consecutive days were processed on every alternating day in such a way that eggs collected that day and the day before were processed. Eggshells were broken and contents of the eggs were poured in a culture disc keeping the yolk intact and separated from egg white. The blastodisc can be recognized as a whitish spot on the yolk. If necessary, a plastic pipette was used to carefully roll the yolk to position the blastodisc on top. Blastodiscs were isolated using a technique similar to that described by Chapman et al. [160]. A filter paper disc of approximately 1.2 cm diameter containing a hole in the center was placed on the blastodisc in

such a way that the blastodisc was present inside the hole of filter paper. The vitelline membrane was cut with surgical scissors along the outline of the filter paper disc, which was then carefully taken off the surface of the yolk and washed in phosphate buffer saline (PBS) in a petri dish. Under a binocular microscope the germinal discs were carefully separated from the vitelline membrane using a preparation of flexible horse hairs fixed onto small plastic sticks and transferred to a yolk-free area of the culture disc containing phosphate buffer saline (PBS) to remove adhering yolk as much as possible. Using a 1-ml pipette tip (with widened opening) the germinal disc was transferred to a second culture disc with fresh phosphate buffer saline (PBS) and washed again.

2.2.3 Hoechst 33342 staining of blastodiscs

Cleaned blastodiscs were carefully transferred to wells of 24-well plates containing 400 µl of PBS in each well with final concentration of 10 µM Hoechst 33342 DNA stain. The blastodiscs were incubated for 30 minutes at room temperature in the dark and observed under a fluorescence microscope with argon laser and using appropriate filters for Hoechst 33342 excitation and emission. Fertile blastodiscs were recognized by the presence of (large numbers of) cells with brightly fluorescing nuclei. After determining the fertilization status, the blastodisc samples were collected in 1.5-ml Eppendorf tubes and suspended in approximately 20 µl of buffer and frozen at -70 °C until nucleic acid extraction.

2.2.4 Isolation and analysis of nucleic acids

RNA and DNA from the blastodisc was isolated simultaneously using the proteinase K digestion method as described previously [161]. Prior to digestion, blastodiscs were suspended in 20µl PBS before 250µl of digestion buffer, containing 0.2 mg/mL RNase-free proteinase K, was added. After removal of protein contaminants by ammonium acetate precipitation, nucleic acids were recovered from the supernatant by alcohol precipitation. Pellets were washed with 70% (v/v) ethanol, air dried, and dissolved in 10µl of RNAse-free water.

For qualitative and quantitative analysis of isolated nucleic acids, an aliquot of 2 µl from the isolated sample was analyzed on 1.0 % (w/v) agarose gel to check the quantity and quality of intact chromosomal DNA and RNA. The RNA and DNA concentrations were measured on

NanoDrop ND-100 spectrophotometer assuming an extinction coefficient of 0.025 $(\mu g/ml)^{-1}$ cm^{-1}.

2.2.5 PCR analysis of the CHD1 gene

The method of PCR amplification of *CHD1* (chromodomain-helicase-DNA binding protein) gene sequences as described previously by Fridolfsson and Ellegren [148] was followed, using primer pair 2550F/2718R [148]. *CHD1* gene fragments of 450 bp (*CHD1*-W) and 600 bp (*CHD1*-Z) were amplified from the W and Z chromosome, respectively. For PCR amplification the Advantage® 2 PCR enzyme system (Clontech) was used. Briefly, for each nucleic acid extract (DNA and RNA) equal amount of nucleic acid (corresponding with ~20 ng RNA-DNA) was used as template in 30 µl of SA reaction buffer provided in the kit (final concentration of 2 mM $MgCl_2$). After 2 min of denaturation at 94 °C, *CHD1* fragments were amplified using a thermal "touchdown" scheme: 2 cycles of (94°C-30s/60°C-30s/68°C-60s), 2 cycles of (94°C-30s/57°C-30s/68°C-60s), 2 cycles of (94°C-30s/53°C-30s/68°C), and 30 of (94°C-30s/50°C-30s/68°C-60s), followed by an additional elongation period of 5 min at 68 °C. Six µl of the PCR reaction mix was analyzed on a 1.8 % (w/v) agarose gel.

2.3 Results

2.3.1 Macroscopic and microscopic observations

The procedure that we used to dissect and isolate the blastodisc under binocular microscopical inspection resulted in a clean preparation of the blastodisc free of yolk and separated from the vitelline membrane. Macroscopically, the blastodisc from fertile eggs (eggs from inseminated hens) and infertile eggs (from virgin hens) appeared similar, although infertile blastodiscs appeared to be more fragile. Also by binocular microscopy it was in virtually all cases not possible to distinguish fertile from infertile blastodiscs.

However, using fluorescence microscopy after staining the blastodiscs with Hoechst 33342, fertile blastodiscs could be easily recognized and distinguished from infertile blastodiscs (Fig. 2.1). Fertile blastodiscs showed large masses of cells with fluorescing nuclei (Fig. 2.1a). In the infertile eggs from virgin hens, the blastodisc contained no cells with fluorescing nuclei (Fig. 2.1b). A minority of the eggs from inseminated hens looked similar to the eggs of virgin hens, having no sign of any cellular development. These eggs had clearly not been fertilized

or perhaps had been fertilized but the ovum had died shortly after fertilization or after syngamy.

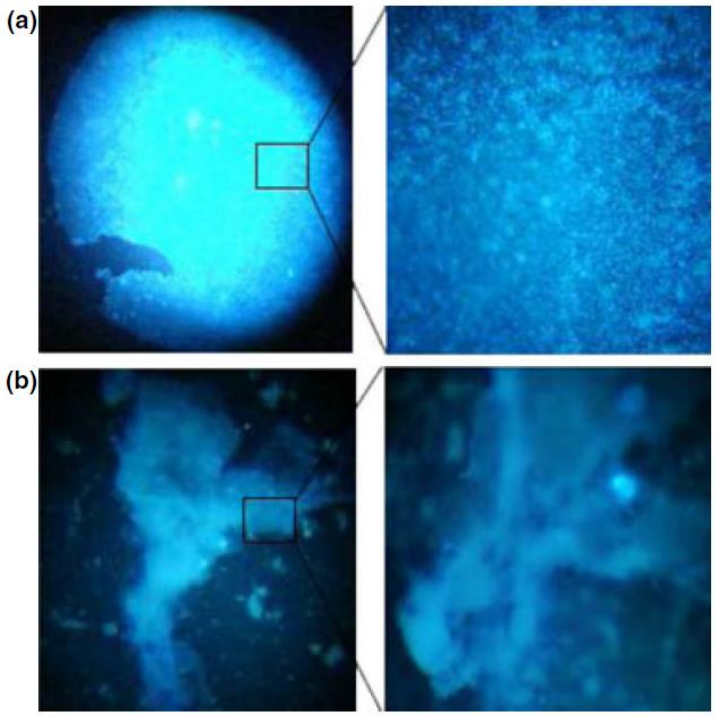

Fig. 2.1. Fluorescence microscopy of Hoechst 33342 stained blastodiscs from chicken eggs, showing a fertile blastodisc with thousands of stained nuclei (panel a) and an infertile blastodisc with no stained nuclei (panel b).

2.3.2 Nucleic acid based comparison of fertile and infertile eggs

Fertile and infertile blastodiscs could be unequivocally distinguished using the Hoechst fluorescence staining. In general, fertile blastodiscs gave a higher yield of nucleic acids but there was some overlap in the yields. The mean yield of nucleic acids isolated from fertile and infertile blastodiscs of H33342 stained eggs was 1.4±0.7 µg and 0.16±0.3 µg per blastodisc, respectively. On agarose gel, fertile eggs showed a very strong and distinct band of chromosomal DNA (Fig. 2.2a), whereas infertile eggs did not show such a band. In only some of the infertile eggs (Fig. 2.3, eggs from virgin hens) a very weak chromosomal DNA band could be detected. Fertile eggs showed clear and distinct bands for 28S and 18S ribosomal RNA, whereas infertile eggs showed less RNA, which appeared to be more degraded (Figs. 2.2a and 2.3).

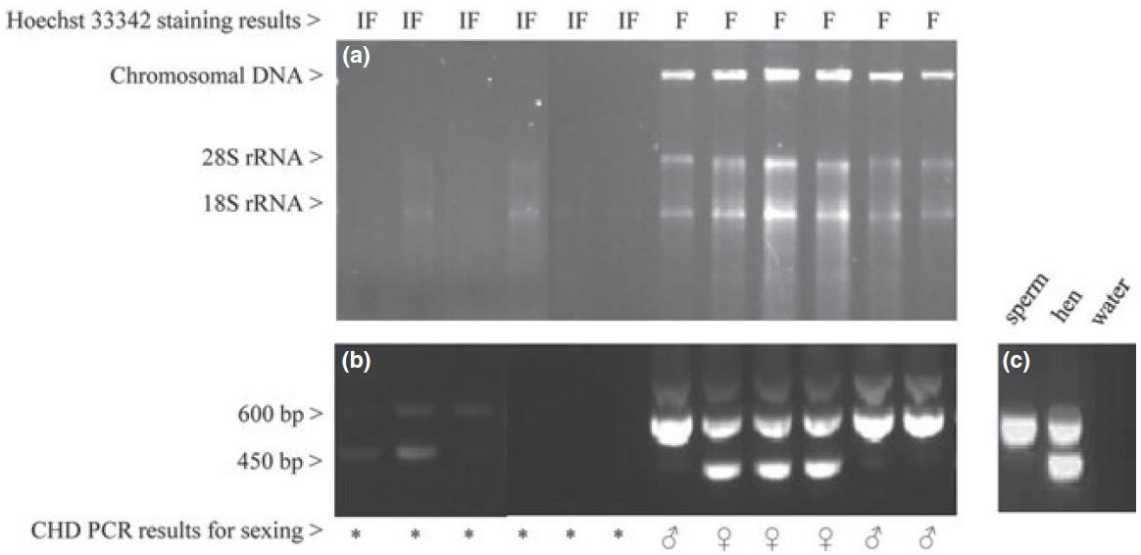

Fig 2.2. Panel a: Gel analysis of nucleic acid (DNA and RNA) isolated from blastodiscs from fertile and infertile eggs laid by inseminated hens. F and IF denote fertile and infertile blastodiscs respectively, as observed by fluorescence microscopy after H33342 staining. Panel b: *CHD1*-based PCR results from the same blastodiscs as in panel a. Interpretation of *CHD1*-based PCR products are indicated by ♂ (male), ♀ (female), and * (inconclusive). c) *CHD1*-based PCR products of positive and negative controls.

Furthermore, as shown in Fig 2.2b, the eggs that were identified as fertile after Hoechst 33342 staining, produced clear PCR products of either 600 or 600 and 450 base pairs, while the infertile eggs produced no or only very faint bands. In addition, the pattern of the *CHD1* targeted PCR products of the fertile eggs revealed a clear and unambiguous sex classification. Out of a total of 106 eggs, 85 eggs were identified as fertile while 21 were identified as infertile using H33342 staining. Male to female sex ratio in these fertile 85 eggs was 57:43.

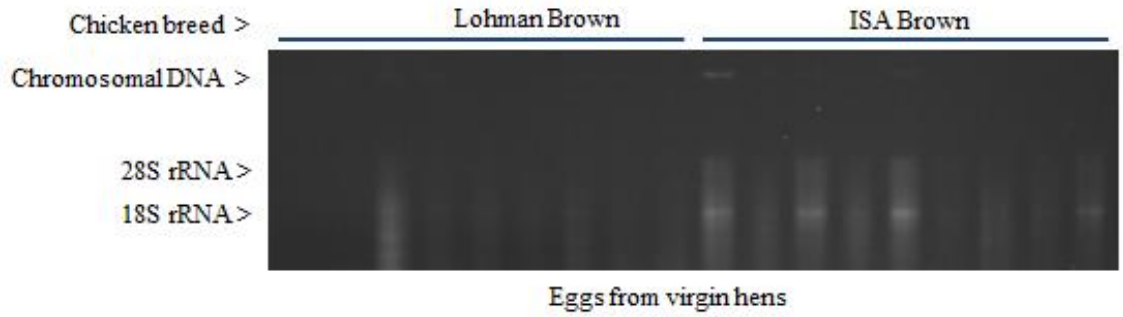

Fig 2.3. Agarose gel analysis of nucleic acid (DNA and RNA) isolated from blastodiscs of eggs from virgin hens.

2.3.3 Sexing of eggs without using fluorescence microscopy

In a series of 142 eggs, obtained in a separate experiment from 12 Lohmann brown hens that were inseminated twice weekly, we had not used Hoechst 33342 staining to distinguish fertile from infertile blastodiscs. A large majority (94%) of these eggs showed a very strong and distinct band of chromosomal DNA on agarose gels, while such a band was absent in the remainder 6% of the eggs.

For the 133 eggs that showed a distinct chromosomal DNA band, PCR amplification of the sex-linked *CHD1* genes resulted in clear and distinct PCR products, indicative for the Z (600 bp) or for the Z and W (600 and 450 bp) chromosomal genes, respectively. The 9 other eggs with no or a very faint band of chromosomal DNA produced either no PCR products, only the 450 bp PCR product, or faint Z and W specific PCR products. This PCR banding pattern did not allow a clear sex classification of the eggs, which is obvious for infertile eggs. On the other hand, on the basis of PCR banding pattern,133 fertile eggs could clearly be classified as male or female. The observed sex ratio of this group of eggs was running close to parity, 49:51 male to female.

2.4 Discussion

In the study of sex allocation it is essential to discriminate primary sex ratio from secondary sex ratio, both for understanding underlying causal mechanisms and function on evolution of sex allocation [46]. In studies of the primary sex ratio of birds and in studies aimed at measuring yolk constituents allocated by the mother in relation to the sex ratio, it is crucial to study eggs very early in embryonic development, preferably unincubated eggs. In this study, we established a method for sexing fertile unincubated eggs on the basis of their pattern of *CHD1*-targeted PCR amplification products, in which fertile and infertile eggs can be distinguished either by using Hoechst 33342 staining or by critical inspection of the quantitative and qualitative characteristics of the isolated nucleic acid samples, and by the clear and distinct presence of *CHD1* PCR products.

In the most of previous studies on avian primary sex ratio the sex of eggs or offspring was determined after several days of incubation of the eggs, or even after hatching of the chicks, rather than in unincubated eggs [12, 14, 15, 21, 42, 50, 107]. Incubated eggs offer the advantage that the developing embryo can be easily recognized and isolated, and ample

amounts of embryonic DNA can be readily obtained. In unincubated eggs, the blastodisc region can be readily isolated, but whether it contains blastoderm cells, i.e. whether it is a fertile or infertile blastodisc, cannot be recognized macroscopically. Moreover, it is often assumed that unincubated eggs would not deliver sufficient DNA for sexing.

Both fertile and infertile blastodiscs may carry various sources of paternal (Z) or maternal (ZW) DNA, confounding reliable DNA-based sexing. A minor source of contaminating DNA would be the presence of the female pronucleus (only in infertile eggs) and the polar bodies. In addition, both fertile and infertile eggs may contain the remains of up to 25 male pronuclei (Z) [162]. Larger sources of contamination may be presented by granulosa cells (ZW) and sperm (Z) attached to the vitelline membrane. Bobr et al. [163] showed that fertile and infertile eggs of inseminated hens have similar numbers of sperm attached to the vitelline membrane. The estimated numbers of sperm attached to the vitelline membrane vary in different studies [163-165], but taking the higher values [164, 165] one can assume that an excised piece of vitelline membrane of 5 x 5 mm overlying the blastodisc could contain as many as 2000 sperm cells. The number of granulosa cells appear to be lower than the sperm cells attached to vitelline membrane [164]. In fertile eggs, PCR amplification of contaminating maternal DNA could lead to a W-chromosomal associated *CHD1* PCR product of 450 bp, which could result in a male embryo to be erroneously sexed as female. Thus, it seems that contamination with DNA from granulosa cells could especially pose a contamination problem in fertile eggs. In infertile eggs, PCR amplification of contaminating DNA could result in inappropriately attributing a sex to infertile eggs.

In order to minimize the contaminating DNA, Arnold et al. [149] dislodged the vitelline membrane from the blastodisc proper, but still obtained Z and W specific PCR products after amplification of DNA from blastodiscs of infertile eggs from unmated hen [149], indicating that PCR products had been generated from contaminating DNA. In addition, these authors found equivocal PCR sexing results for a small proportion of eggs from mated hens [149]. However, as usually a proportion of eggs from mated hens is infertile and no attempt was made to discriminate between fertile and infertile blastodiscs, the few eggs that gave equivocal sexing results in the latter study may very well have been infertile.

In the current study we used a similar method as employed by Arnold et al. [149] to remove the vitelline membrane as much as possible to minimize contaminating DNA. As the amount of contaminating DNA would be reduced by removing the membrane and as the blastodisc of

fertile chicken eggs (i.e a ‘stage X’ embryo) contains approximately 30,000-60,000 blastoderm cells [52], we hypothesized that in fertile eggs, the amount of embryo DNA of the blastoderm cells would be sufficiently higher than that of the contaminating DNA to reliably sex the eggs. Thus, we hypothesized that sexing of unincubated eggs by the PCR method should be feasible provided that infertile blastodiscs can be excluded.

In this study we have used Hoechst 33342 staining and inspection of the blastodiscs by fluorescence microscopy to distinguish developed blastoderms from infertile blastodiscs. This way of staining and inspection of the blastodiscs gave very clear results and led in all cases to unequivocal discrimination of developed blastoderms from infertile blastodiscs. In addition, we have used eggs from virgin hens as a sure supply of unfertilized eggs. Fertile blastodiscs contained many fluorescing nuclei while the infertile blastodiscs only contained membranous structures with no fluorescing nuclei. The number of blastoderm cells of fertile eggs was much higher than the assumed maximum number of contaminating cells or nuclei, especially since we removed the vitelline membrane from the blastodisc proper. The nucleic acid yields of the fertile eggs were generally higher than those of infertile eggs, but did show some overlap. Furthermore, the intensities of chromosomal DNA bands on agarose gels were much higher for fertile eggs (representing embryo DNA plus possible contaminating DNA) as compared to infertile eggs (representing only contaminating DNA) that resulted in no or very weak chromosomal bands. Furthermore, fertile eggs also resulted in clear and distinct *CHD1* PCR amplification products on agarose gel while infertile eggs resulted in either no or only very faint *CHD1* PCR products. All these findings indicate that fertile blastodiscs indeed contain much higher quantities of embryonic DNA than contaminating DNA, allowing successful sexing of the fertile eggs with PCR amplification of the *CHD1* genes. Hence we could use the PCR method to sex unincubated eggs, provided that we can identify the eggs which contain a fertile blastodisc. Therefore, we conclude that the use of Hoechst 33342 staining to identify and exclude any infertile blastodiscs, combined with *CHD1*- targeted PCR sexing of the fertile eggs, presents an easy and reliable method to sex unincubated eggs.

There are a number of well-known pitfalls of PCR methods, most notably contamination of samples or solutions in the PCR laboratory, causing false positive readings, and PCR failure, causing false negative readings. Methods to avoid contamination of samples are elaborately reviewed by Aslanzadeh [166] and Borst et al. [167]. Inclusion of positive controls and internal PCR controls, as reviewed by Hoorfar et al. [168], can be used to monitor conditions

for successful PCR reaction. In our present study we have used negative and positive controls to detect any eventual carry-over laboratory contamination and monitor the right conditions for successful PCR reaction, respectively. In our study, we have used agarose gels to detect the *CHD1* PCR products. There are also other possible systems to detect PCR products, including non-gel based methods, for examples as described by Abd-Elsalam [169].

As discussed above, fertile and infertile blastodiscs differ in qualitative and quantitative aspects of the isolated nucleic acids and in the *CHD1* PCR results. Thus, as is shown in Fig 2.2, one can easily discriminate fertile from infertile eggs, just by visual inspection of nucleic acids on agarose gels. In all Hoechst stained106 eggs the fertile and infertile eggs could be correctly identified from the differences in nucleic acids in the agarose gels, independently from the results of the fertility status based on Hoechst staining. Hence we conclude that fertile and infertile blastodiscs can be distinguished from each other even without using Hoechst 33342 staining, and subsequently the fertile eggs can then be sexed on the basis of their *CHD1* banding pattern.

We have applied this method of identifying and sexing the fertile eggs from inseminated hens without using Hoechst 33342 staining on a sample of 142 eggs, obtained in a separate experiment from 12 Lohmann brown hens. In all cases, we could clearly determine the fertile (133) or infertile (9) status of the eggs. Subsequently, the sex of the 133 fertile eggs could be judged unequivocally from the *CHD1*- based banding pattern. The sex ratio was running close to parity in 133 eggs identified as fertile.

Thus, the overall conclusion is that the present method allows taking out the infertile eggs and sex the fertile eggs, either with or without use of Hoechst 33342 staining. This new method of sexing unincubated eggs provides for the first time a reliable, and relatively simple tool to determine sex in unincubated avian eggs. This is as close to fertilization as possible without invoking surgery and therefore currently the best method to study the primary sex ratio.

Acknowledgments

We thank Hendrix Genetics B.V., The Netherlands for providing ISA brown hens used during the experiment. We thank Ton G. G Groothuis for giving useful comments on this manuscript. We thank Ministry of Economic Affairs, Agriculture and Innovation, The Netherlands for funding this research under the project number BO-12.02.002.7.01.

3

Yolk concentrations of hormones and glucose and egg weight and egg dimensions in unincubated chicken eggs, in relation to egg sex and hen body weight

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Gen Comp Endocrinol. 2013 Jun 15;187:15-22.

Abstract

Birds can manipulate offspring sex ratio under natural and experimental conditions and maternal hormones have been shown to be involved in this process. Studies also provided evidence for the presence of sex specific concentrations of yolk hormones in avian eggs. These findings led to the suggestion that yolk hormones could influence genetic sex determination in birds. However, in previous studies, yolk hormone concentrations and egg sex were studied in incubated eggs, although incubation of the eggs and embryonic development can alter yolk hormone concentrations and measured sex ratio. This study is the first to determine a wide array of egg components and hen body weight in relation to the sex of the egg in unincubated eggs. Egg parameters studied were yolk concentrations of testosterone, estradiol, androstenedione, progesterone, dihydrotestosterone, and glucose, and egg weight and dimensions. In addition, we studied the associations among all measured parameters. Associations were found between a number of yolk hormones (progesterone associated with testosterone, estradiol and androstenedione; androstenedione with testosterone; dihydrotestosterone with estradiol and androstenedione) as well as between yolk testosterone and egg length and egg weight. There were no significant overall differences between male and female chicken eggs in any of the measured egg parameters. However, there were a few interactions such as the interaction of egg sex with dihydrotestosterone and with hen body weight which predicted estradiol levels and an interaction of estradiol levels with egg width for predicting sex of egg. Their biological relevance need, however, further study.

Keywords: Unincubated eggs; Maternal yolk hormones; Sex differences; Egg dimensions; Hen body weight

3.1 Introduction

In birds and other oviparous species, mothers transfer nutrients and hormones to the yolk of developing oocytes during vitellogenesis. Conditions experienced by the mother can affect the yolk concentrations of maternal hormones, which, in turn, may affect the phenotype of the offspring. Thus, yolk hormones can translate the environmental conditions experienced by the mother into adaptive phenotypic variation of the offspring [126, 134, 170, 171]. According to sex allocation theory [26], it could be beneficial for parents to allocate nutrients and hormones differently for the different sexes of the offspring, in response to certain environmental conditions when fitness benefits conferred by investment in sons and daughter offspring differ. The differential investment might lead to the manipulation of offspring sex ratio. In birds the female is the heterogametic sex and potentially in charge of determining the sex of the offspring. For a number of bird species it has indeed been reported that the female can manipulate the sex ratio of her offspring in response to environmental factors such as mate quality [12], habitat quality [38], seasonal rhythm [172] and maternal diet [49]. Also, it has been shown that the sex ratio can be manipulated experimentally by administration of hormones [11, 15, 16, 46]. A number of authors have suggested that the hen may be able to influence the segregation of Z and W chromosomes towards oocyte and polar body during meiosis, possibly by manipulating hormone levels or other characteristics of the yolk [5, 8, 15, 21, 159].

In addition to the biological relevance of yolk hormone concentrations with regard to possible mechanism of female manipulation of sex ratio bias, differences between male and female eggs could potentially be used as a method for sexing eggs. This could be relevant in the poultry industry for avoiding the killing of day old male chicks of layer breeds. A number of authors have studied concentrations of testosterone and androstenedione in the yolk of chicken eggs but found no overall differences between male and female eggs [5, 128, 130, 173]. In all studies mentioned above in chicken and other bird species, egg hormone concentrations and sex of eggs were determined after a few days of incubation of the eggs, because it was necessary to get sufficient embryonic tissue for accurate sexing of eggs. However, incubation of eggs can lead to changes in concentrations of yolk hormones during early embryonic development [128, 155]. In addition, sex specific differential mortality of avian embryos during incubation can occur [51], which may lead to a deviation of the observed sex ratio from the primary sex ratio. Therefore, in the present study we have studied

the yolk hormone and glucose concentrations of chicken eggs and the sex of the eggs without prior incubation of the eggs, by applying a recently developed method to sex unincubated chicken eggs [174]. In this study we included a large number of hormones, i.e. testosterone, estradiol, androstenedione, progesterone, dihydrotestosterone. We also included glucose measurements in the yolk for the following reasons. Female body condition [13, 48, 102, 105, 175] and nutrient availability [53, 176] have been associated with primary sex ratio in a number of previous studies. Change in body conditions can change several blood parameters including glucose [177, 178]. Plasma glucose levels were associated with sex ratio in mice [29], horses[30] and voles (*Microtus agrestis*) [110] but not in pigeons [106]. Moreover, the metabolite glucose in egg yolk has not yet been studied in relation to sex of eggs in previous studies. Also egg weight and dimensions (length and width) were included in the present studies as sex of egg was reported to be related to egg volume in mallards [9] or to egg mass in chicken [10].

3.2 Materials and Methods

3.2.1. Egg collection

Twelve hens and 2 cocks of Lohmann brown layer breeding line, 45 weeks of age, were obtained from a commercial hatchery and were housed individually in cages with ad lib access to normal layer feed and water and a light-dark programme of 18h-6h at 25 °C, and were acclimatized to the housing conditions for one week before starting inseminations. The cocks were trained for semen collection and the hens were artificially inseminated twice weekly with a volume of 0.2 ml of 4 times diluted semen with semen diluent. Egg collection began two days after the second insemination and a total of 142 eggs (approximately 12 per hen) were collected over a period of 12 days. The collected eggs were marked and directly stored at 17 °C until processing the same or the next day.

3.2.2. Collection of samples from eggs

Eggs were processed on every alternating day in such a way that eggs collected that day and the day before were processed. Yolks were cleared free from albumen and placed in a petri dish. Blastodiscs were isolated for sex determination as described by Aslam et. al., [174]. Isolated clean blastodiscs, free of yolk, were suspended in approximately 20 µl of PBS and frozen at -20 ℃ until use for nucleic acid extraction. Yolk samples were then collected from

whole yolk after thoroughly mixing the yolk, weighed and diluted three times with distilled water and frozen at -80 ℃ until further use.

3.2.3. Molecular sexing

The sex of the unincubated eggs was determined as described by Aslam et. al., [174]. In brief, nucleic acids were isolated from blastodisc samples using proteinase K digestion as described previously [161]. DNA pellets were washed in 70 % ethanol, air dried and dissolved in 10 µl of RNAse free water. Fertile eggs were distinguished from infertile eggs based on intensity of chromosomal DNA bands on agarose gel as described before [174] and 133 of the142 eggs (93.7%) were fertile. Subsequently, 20 ng of nucleic acid was used in the polymerase chain reaction to amplify the *CHD1* gene using primer pair 2550F/2718R [148]. *CHD1* gene fragments of 450 bp (*CHD1-W*) and 600 bp (*CHD1-Z*) were amplified from the W- and Z-chromosome specific CHD1 genes, respectively [148]. Amplified PCR products were run on 1.8 % agarose gel and the presence of two bands of equal intensity (for the *CHD1*-Z and *CHD1*-W gene, respectively) indicated female sex and the presence of only a single band (for the *CHD1*-Z gene) indicated male sex.

3.2.4. Radioimmunoassay

To determine yolk concentrations of the steroid hormones, yolk samples were extracted three times using diethyl ether / petroleum benzene 70:30 (vol/vol) (30–70%) allowing steroids to pass from the watery phase to the organic phase. The frozen three times diluted yolk samples were thawed and gravimetrically determined amounts of 250 mg were mixed with a small volume containing tritiated androstenedione, estradiol, testosterone or progesterone, respectively (equivalent of 2000 counts per minute; PerkinElmer Life and Analytical Sciences BV). Then, the samples were left to equilibrate for 2 h at room temperature after which they were three times extracted with 2.5 ml of diethyl ether / petroleum benzene 70:30 (vol/vol). The extraction procedure was as follows. After adding diethyl ether / petroleum benzene to samples, vigorous mixing was done on a vortex mixer and the tubes were centrifuged at 2000 rpm for 10 minute at 4 °C. The tubes were then snap frozen and the still liquid organic phase was decanted into fresh tubes. The extraction of the yolk sample was repeated for three times. The three volumes of organic phase from the three extraction round were pooled and then dried under a stream of nitrogen. Then 1 ml of 70% methanol was added, and the tubes were

vortexed and then stored overnight at −20 °C. The next day, samples were centrifuged at 4°C for 5 min at2000 rpm. The methanol was decanted into fresh tubes and dried off under nitrogen. The residue was dissolved in 200 µl of PBS buffer.

RIA for determination of yolk testosterone concentrations was performed according to Goerlich et al [14] using kit purchased from Diagnostic Systems Laboratories (Coated-Tube RIA 'DSL-4000'). RIA for determination of yolk androstenedione concentrations was performed according to Muller et al [5] using kit purchased from Diagnostic Systems Laboratories (RIA 'DSL-3800'). RIA for determination of yolk estradiol concentrations was performed according to Casagrande et al [179] using kit purchased from Diagnostic Systems Laboratories (ultra sensitive RIA 'DSL-4800'). RIA for determination of yolk progesterone concentrations was performed according to Coslovsky et. al. [180] using kit purchased from Diagnostic Systems Laboratories (RIA 'DSL-3900'). All the samples were measured in one RIA test for each of the hormone and only intra-assay CVs are given which were 4.3%, 9.7%, 3.4% and 5.3% for testosterone, estradiol, androstenedione and progesterone, respectively.

3.2.5. ELISA for determination of DHT concentrations

The dihydrotestosterone ELISA Kit, IBL international GmbH (DB52021), was used to determine the concentrations of DHT hormone in the yolk samples. The cross-reactivity of DHT ELISA kit with testosterone was reported to be 8.7 % by manufacturer of the kit. As the manufacturer of kit reports, this cross-reactivity would not influence the test results of this ELISA due to specific complexing buffer system which blocks the binding of the testosterone to the antibody. Protocol provided by manufacturer of ELISA Kit was used to perform ELISA assay. The sensitivity of the ELISA kit was 25 pg/ml. Two standard curves were used during the analysis, one provided with the kit and one home-made standard curve to obtain concentrations of 1000, 500, 250, 125, 62.5, 32.25, 16.12 and 8.62 pg/ml. The yolk samples were extracted using the extraction procedure described above. Extraction of yolk samples was done in five batches. Extracted samples were measured in four different ELISA plates. To estimate inter-batch variation during extraction, four yolk samples were used in all five extraction batches and measured in same ELISA test. Average CV for inter-batch variation during extraction was 7.8%. To estimate variation between different ELISA assays, aliquots from four extracted samples were used in each ELISA plate. Average CV for inter-assay of ELISA was 8.2%. For a number of the yolk samples, serial dilutions of the samples were

included to produce dilution curves to validate the ELISA. Dilution curves ran parallel to standard curves, indicating that this ELISA assay was suitable to measure dihydrotestosterone (DHT) concentrations in extracted yolk samples.

3.2.6. Glucose measurements

Glucose concentrations were measured in diluted yolk samples using a FreeStyle Lite® electronic glucose measuring device and strips by applying a drop of diluted yolk. This method was validated using a home-made glucose standard curve and measuring serial dilutions of yolk samples prepared in PBS. The dilution curves ran parallel to the standard curve indicating that the used glucose meter was suitable for measuring glucose in yolk samples. Intra-assay CV was 8.0%.

3.2.7. Statistical analysis

Statistical analysis was performed using R software for mixed effects modelling, using the nlme (linear and nonlinear mixed effects) package to model data from different hens as multilevel and repeated measures [181].

For all egg characteristics it was tested whether this characteristic is predicted by sex of egg plus another factor (i.e other egg characteristic, or hen body weight) plus interaction sex of egg times other factor. Stepwise backward regression was performed. If the interaction with sex of egg was not significant, this interaction and the factor sex of egg were taken out of the model (sex of egg was never significant as main factor). T- and p-values for significant interactions are given. To provide an overview of only the main factors, simplest models for main factors without interactions were tested for all factors. Hen was used as random factor in all above models. To estimate the extent of contribution for associations between different egg factors in mixed models resulting from variation among hens and variation within hens, variance component for hen (random effect) and egg factor (fixed effect or main effect) for the mixed model were expressed as percentage of total variance present in all eggs from all hens.

Sex determination occurs after deposition of egg yolk [64], thus yolk composition could potentially affect which sex chromosome is retained in the ovum or withdrawn to the polar body (meiotic drive), perhaps by affecting the spindles that regulate their movement resulting in biased sex ratio among progeny [8, 72]. Therefore, using logit link function from lme4

package, we tested whether sex of the egg (response variable) was affected by other egg factors (explanatory variables). Combinations of any two egg factors and their interaction (testing also to what extent ratios of hormone concentrations could be of significance, such as the ratio between T and E2) were tested in separate models. Stepwise backward regression was performed. If the interaction between two egg factors was not significant, this interaction was taken out of the model. T- and p-values for significant interactions are given. To provide an overview of only the main factors, simplest models for main factors without interactions were tested for all factors. Hen was again used as random factor in all above models. Finally, it was tested to what extent the mean values per hen of hormone or glucose concentrations, egg weight or dimensions, or the hen body weight, is related to the proportion of male eggs per hen. Combinations of any two egg factors and their interaction (testing also to what extent ratios of hormone concentrations could be of significance, such as the ratio between T and E2) were tested in separate models. All the above described models were also tested using the data of hens which displayed high sex ratios (i.e two hens with 0.75 and 0.82) and low sex ratio's (i.e three hens with 0.33, 0.33 and 0.33). However, we find no evidence that in hens with apparently skewed sex ratio's the results were different. Therefore, we do not report these data.

3.3 Results

3.3.1 Differences in male and female eggs

Mean values of the concentrations of testosterone (T), estradiol (E2), androstenedione (A4), progesterone (P4), dihydrotestosterone (DHT) and glucose in the yolk of chicken eggs for all hens together are presented in Table 3.1 for male and female eggs and for values for each hen separately see supplementary Table 3.1. The mean values of egg weight, width, and length for all hens together are presented in Table 3.1 and for values for each hen separately see supplementary Table 3.2.

The mean values for each of the measured parameters were not different between male and female chicken eggs. Indeed, in mixed model analysis, for none of the measured parameters the effect of sex of egg was significant (see Table 3.1 for p values). However, the interaction of sex of egg times DHT concentrations was significant for prediction of yolk E2 concentrations ($p = 0.02$) (Fig. 3.1 and Table 3.1). The interaction of sex of egg times hen

body weight was also significant for the prediction of yolk E2 concentrations (p=0.009), i.e. relatively light hens tend to allocate more E2 to male eggs, while heavier hens tend to allocated more E2 to female eggs (Fig. 3.2 and Table 3.1).

Table 3.1: Associations between egg sex and egg parameters (hormones, glucose and egg physical parameters) and hen body weight. Associations were tested by using mixed effect modelling. Only significant interactions are shown. Average values of egg parameters (Mean ± SD) for all hens are given.

Main factors					**Mean ± SD** (hormones (pg/mg yolk), glucose (mM), egg weight (g), egg dimensions (mm))	
Factor	Models testing whether egg characteristic is predicted by sex of egg: Factor ~ Sex of egg		Models testing whether sex of egg is predicted by egg characteristic or hen body weight: Sex of egg ~ Factor		Male eggs	Female eggs
	t value	**p value**	**z value**	**p value**		
Testosterone	1.02	0.30	0.49	0.62	9.6±1.9	9.4±1.6
Estradiol	0.29	0.76	0.26	0.79	1.0±0.2	1.0±0.2
Androstenedione	1.20	0.22	-0.23	0.81	152±37	154±35
Progesterone	-0.02	0.98	-0.19	0.84	737±190	733±156
Dihydrotestosterone	0.84	0.40	0.51	0.60	3.1±0.7	3.0±0.8
Glucose	-0.37	0.71	0.16	0.84	9.0±1.3	9.0±1.2
Egg weight	-0.96	0.33	-0.31	0.74	63.8±1.9	64.2±1.8
Eggs length	-1.20	0.23	-0.56	0.56	58.1±1.0	58.3±0.9
Egg width	0.12	0.89	0.07	0.93	44.1±0.5	44.1±0.5
Hen body weight			-0.66	0.50		
Interactions (only significant interactions are shown)						
Dependent variable	Interaction		Interaction			
Estradiol	Sex of egg × DHT					
	-2.26	0.02				
Estradiol	Sex of egg × Hen body weight					
	-2.63	0.009				
Sex of egg			Estradiol × Egg width			
			2.28	0.02		

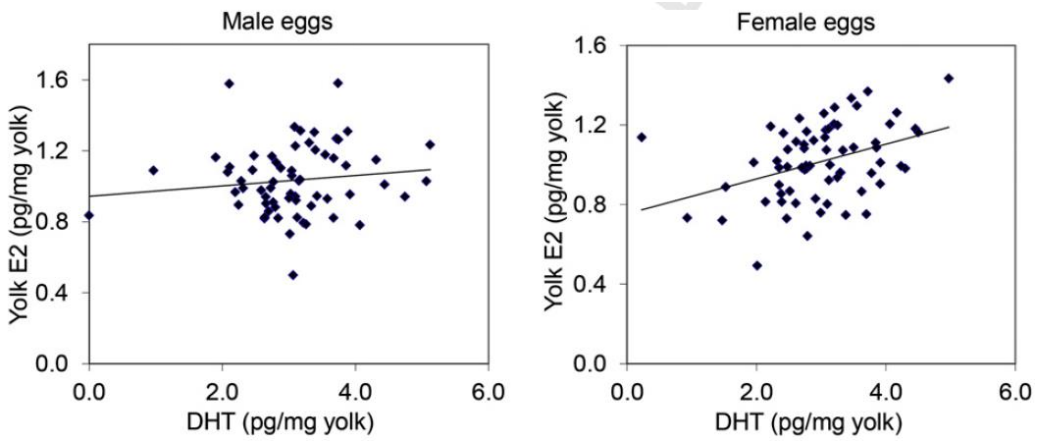

Fig. 3.1. Yolk concentration of E2 in relation to DHT for male eggs (left panel) and female eggs (right panel). In mixed model analysis, interaction of sex of egg with DHT was significant for yolk E2 concentrations (p=0.02).

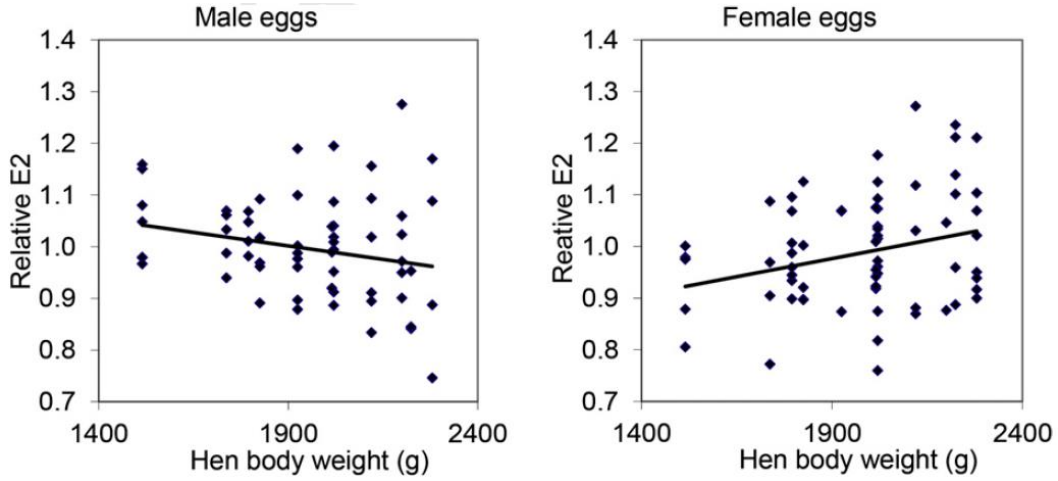

Fig. 3.2. Relative yolk concentration of E2 (E2 of an egg/ mean E2 per hen) in relation to hen body weight for male eggs (left panel) and female eggs (right panel). In mixed model analysis, interaction of sex of egg with hen body weight was significant for yolk E2 concentrations (p=0.009).

In another approach we tested whether the sex of an egg could be predicted from the egg characteristics or hen body weight. For none of the studied parameters prediction of sex of the egg was significant (see Table 3.1 for p-values). However, the interaction of yolk E2 level times egg width was significant for the prediction of sex of egg (p-value 0.02). Sex of egg was not predicted by hen body weight (p = 0.50).

3.3.2 Associations of egg characteristics with hen body weight

Hormone levels in yolk may differ between hens, and correlate with hen body weight. Mixed model analysis, using hen as random factor indicated that concentration of androstenedione (A4) in the yolk was significantly associated with hen body weight ($p = 0.005$, see Fig. 3.3 and Table 3.2). Other hormones T, P4 and DHT, all showed a similar trend, although these associations were not significant. As mentioned in the previous section there was a significant interaction of sex of egg times hen body weight for the prediction of yolk estradiol concentrations. Yolk glucose concentration was not associated with hen body weight.

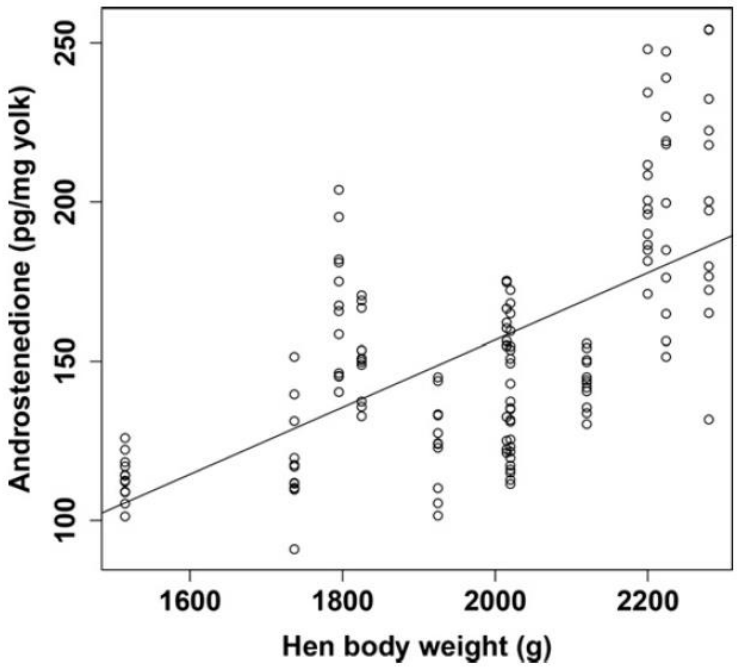

Fig. 3.3. Yolk concentrations of androstenedione in unincubated chicken eggs in relation to hen body weight. The trend line from the statistical model is shown.

The egg weight and dimensions did not have a significant association with, or even appeared to be associated with, hen body weight.

3.3.3 Associations between different egg parameters

In a mixed model analysis, using hen as random factor, there were a number of significant associations between yolk levels of various hormones (Table 3.2A). Also, there were significant associations between yolk testosterone and egg length and egg weight, and

between various egg dimension (Table 3.2A). The among hen and within hen variance component for these associations as percentage of total variance are given in Table 3.2B.

Table 3.2A. P-values and t-values of the significant associations between different egg parameters and between egg parameters and hen body weight tested in the mixed model using hen as random factor. Only significant effects are shown.

Variables in the model with hen included as a random factor		Parameters from the model			
Dependent variable	Independent variable	Estimate	Standard error	t-value	p-value
Egg Width	Egg Length	0.16	0.04	4.22	$p < 0.001$
Egg Weight	Egg Length	1.52	0.10	14.02	$p < 0.001$
Egg Weight	Egg Width	3.20	0.19	16.79	$p < 0.001$
Testosterone	Egg Length	0.18	0.08	2.16	$p = 0.03$
Testosterone	Egg Weight	0.09	0.04	2.17	$p = 0.03$
Androstenedione	Testosterone	0.06	0.007	8.22	$p < 0.001$
Androstenedione	Hen body weight	0.0006	0.0001	3.53	$p = 0.005$
Progesterone	Testosterone	48	7.94	6.04	$p < 0.001$
Progesterone	Androstenedione	344	77	4.46	$p < 0.001$
Progesterone	Estradiol	-261	96	-2.69	$p = 0.008$
Dihydrotestosterone	Androstenedione	0.89	0.35	2.52	$p = 0.01$
Dihydrotestosterone	Estradiol	0.74	0.38	1.96	$p = 0.05$

3.3.4 Proportion of male eggs per hen

The proportion of male eggs per hen, or the primary sex ratio, was not significantly associated with hen body weight, or with mean values per hen for all measured egg characteristics.

Table 3.2B. Among hen and within hen variance component for the associations given in Table 3.2A as percentage of total variance.

Variables in the model with hen included as a random factor		Partitioning of variance from the model	
Dependent variable	Independent variable	Among hen	Within hen
Egg Width	Egg Length	63.84	36.16
Egg Weight	Egg Length	67.99	32.01
Egg Weight	Egg Width	75.41	24.59
Testosterone	Egg Length	55.07	44.92
Testosterone	Egg Weight	57.79	42.21
Androstenedione	Testosterone	67.07	32.93
Androstenedione	Hen body weight	57.30	42.70
Progesterone	Testosterone	12.23	87.77
Progesterone	Androstenedione	73.59	26.41
Progesterone	Estradiol	36.20	63.39
Dihydrotestosterone	Androstenedione	53.81	46.19
Dihydrotestosterone	Estradiol	55.09	44.91

3.4 Discussion

3.4.1 Differences between male and female eggs

This study is the first to determine a wide array of egg components in relation to the sex of the egg in unincubated eggs. We found no significant differences between male and female chicken eggs in yolk concentrations of testosterone, estradiol, androstenedione, progesterone, dihydrotestosterone and glucose at laying. This indicates that chicken mothers do not allocate these egg components sex specifically. In addition, male and female eggs at laying did not differ in egg weight, egg length and egg width. The results also indicated that the apparent sex ratio per hen was not related to any of the measured egg characteristics, averaged per hen, or to hen body weight. Thus, sex of the egg cannot be predicted by measuring any of these egg characteristics.

It could be argued that the laying hen, after severe selection for egg production, may have lost the ability to manipulate offspring sex ratio, in contrast to wild bird species. However, in a follow up study in which we manipulated food and corticosterone levels (unpublished result), we did find effects on offspring sex ratio, making the suggestion obsolete. Alternatively, perhaps the females were not motivated to adjust offspring sex ratio. Although this may be relevant for the hens as a group, several individual hens produced skewed sex ratio and also in these hens we could not find any positive evidence.

Petrie et. al [159] reported for the first time that male and female peafowl eggs differed in concentrations of yolk steroid hormones estradiol, androstenedione, dihydrotestosterone and testosterone. Based on these observations, the authors suggested that yolk steroid hormones of maternal origin could play an important role in sex determination at the time of meiosis in birds. However, that study determined hormone concentrations at a time embryos produce hormones themselves and a later study on the same species did not replicate these findings [5]. Nevertheless, also other authors have suggested that the hen may be able to influence the segregation of Z and W chromosomes towards oocyte and polar body during meiosis, possibly by manipulating hormone levels or other characteristics of the yolk [5, 8, 15, 18, 21, 47, 72]. However, except for one study these studies did not determine embryo sex before incubation started and results from our study do not suggest that yolk hormones play a major role in sex determination in birds. Nevertheless, we can not exclude the role of hormones to affect sex ratio reported previously under various experimental conditions [11, 12, 18, 21, 45, 47].

The results from the present study are in agreement with previous studies by [5, 128], who also found no overall sex related differences for testosterone and androstenedione. However, when considering eggs from hens of different social status, Muller et al. [5] did find differences in yolk levels of testosterone between male and female eggs and reported that hen social status was associated with hen body weight. We did not look at social status, but we found an interaction of sex of egg with hen body weight with regard to yolk estradiol concentrations i.e. relatively light hens tended to allocate more estradiol to male eggs, while heavier hens tended to allocate more estradiol to female eggs. Such interactions with regard to body weight could be in line with the sex allocation theory [26] since other studies have also reported an interaction of sex and position of the egg in the lay sequence, or the amount of carotenoids, affecting androgen levels in the egg for a review see Groothuis et al.,[182]. Since laying hens do not produce well defined clutches we were not able to look at laying order effects. However, it may be questioned whether such interaction effects reveal real biological phenomena or are the results of multiple post-hoc testing [171].

With regard to egg size, Cunningham & Russell [9] reported that female mallards lay larger eggs for male embryos and Muller et al. [10] reported that proportion of male eggs was positively associated with egg mass. Other studies reported an effect of egg mass on the sex of the egg in yellow legged gull [118] and lizard (*Bassiana duperreyi*, Scincidae) [35]. Maternal

yolk estradiol levels did not influence the sex of eggs in the peafowl [12] but it was associated with sex of egg in turtles (*Chrysemys picta*) [183]. In our study there appeared to be an interaction of estradiol with egg width for the prediction of the sex of the eggs, but there was no direct association between sex of the egg and egg weight or dimensions. These previous studies in birds regarding egg weight and in reptiles regarding egg weight and yolk estradiol demonstrate that these could affect the sex of the egg but the physiological significance of the interaction between estradiol and sex of eggs in our study remains unknown and should be replicated.

3.4.2 Associations between different egg parameters and hen body weight

There was a significant positive association of yolk androstenedione concentration and hen body weight, and there was a similar trend for other hormones, testosterone, progesterone, and dihydrotestosterone. We have not measured maternal plasma concentrations of these hormones in this study, but the link between these concentrations and yolk hormone concentrations is unclear [126]. It has been suggested that females in better condition deposit more androgens into their eggs as this would be a costly process but so far evidence for this interpretation is lacking [126] and opposite patterns have been found too (see for a review [184]. Muller et al. [5] found an association between hen body weight and egg weight (i.e. heavier hens laid heavier eggs). We found no association between hen weight and egg weight or dimensions.

3.4.3 Associations between different egg parameters

Significant associations between yolk concentrations of different hormones were seen. These associations were partly, but not exclusively, due to variations between hens. For most of the cases of these significant associations, the calculated variance component from hens (random factor) was higher as compared to variance component from eggs within hen (main factor) contributing to these associations between two egg factors. These results are in agreement with a previous study which showed that Japanese quail display higher inter-female differences for yolk testosterone levels compared with intra-female variability [185]. Variance component among individuals is an estimate for repeatability of a character [186] which is often used as rough upper limit to the heritability [187, 188]. Another study in Japanese quail reported high heritability for yolk testosterone concentrations that was attributed to heritable

genetic variation among individuals [189], based on selection experiments. The quails selected for higher yolk testosterone concentrations also displayed higher androstenedione and lower estradiol concentrations as compared to quails selected for low yolk testosterone which showed that genetic variability and heritability could also affect the associations between yolk hormones [189]. Therefore, genetic variation among hens used in our study could contribute to associations between egg parameters observed in this study. Therefore, differences between hens could be important regarding associations among yolk hormones. The observed correlations between the yolk steroid hormones can be explained by the fact that pathways for synthesis and metabolism of different yolk steroid hormones are connected with each other [127]. The positive association of progesterone with testosterone, estradiol and androstenedione could be explained by the fact that synthesis of these hormones is connected through a cascade of enzymatic pathways during steroidogenesis via the Δ^4 pathway in ovarian cells. The association between androstenedione (A4) and testosterone can be explained by the fact that T can only be derived from A4 [127]. We also found associations between concentrations of DHT and estradiol and androstenedione that might be explained by the fact that both DHT and E2 are derived from A4 [127, 179, 190].

In the present study glucose was not at all associated with sex of the egg. We included glucose because it can be easily measured and there is evidence in birds that nutrient availability can affect sex ratio. In mammalian species, glucose was shown to influence sex ratio, but glucose affected the secondary sex ratio (early embryonic survival) rather than the primary sex ratio [29].

While we do not seem to find direct associations between hormone levels or other egg characteristics and sex of eggs, the finding that the interaction between estradiol and egg width predicted sex of the egg and interaction of egg sex with DHT and interaction of egg sex with hen body weight predicted estradiol concentrations may be reason for future research. However, a potential use of multiple parameters (e.g. combining E2 and DHT) for sexing of eggs will not be likely as the found differences are quite small compared with overall egg variation.

Acknowledgement

We thank Dr. Pieter van As for critically reading the manuscript. We thank the Ministry of Economic Affairs, Agriculture and Innovation, The Netherlands for funding this research under the project number BO-12.02.002.7.01.

Additional files

Supplementary Table 3.1. Concentrations of yolk hormones in unincubated female and male chicken eggs (mean per hen or mean overall ±s.d.).

		Number of eggs		T (pg/mg yolk)		DHT (pg/mg yolk)		E2 (pg/mg yolk)	
Hen	Sex ratio	♂	♀	♂	♀	♂	♀	♂	♀
1	0.75	6	2	13.1±1.7	12.3±0.9	3.6±0.3	3.8±0.2	1.2±0.1	1.1±0.1
2	0.33	4	8	8.7±0.6	8.4±0.6	2.5±0.4	2.2±0.9	1.0±0.1	1.0±0.1
3	0.45	5	6	9.1±1.0	9.6±0.8	3.0±0.2	2.6±0.6	0.99±0.1	0.91±0.1
4	0.75	9	3	8.1±0.7	7.1±1.0	2.7±0.3	2.7±0.3	0.93±0.1	0.93±0.1
5	0.33	4	8	10.1±0.7	9.2±1.0	3.4±0.2	3.3±0.4	1.0±0.2	1.0±0.1
6	0.58	7	5	7.5±0.6	7.9±0.9	2.5±0.4	2.1±0.4	1.0±0.1	0.93±0.1
7	0.54	6	5	10.2±0.7	10.1±1.4	2.7±1.0	3.2±0.3	1.0±0.1	1.0±0.1
8	0.33	4	8	11.4±1.9	10.8±1.1	3.5±0.4	3.1.±0.3	1.2±0.1	1.2±0.1
9	0.41	5	7	9.4±1.8	9.8±1.6	2.8±0.5	2.5±0.8	0.88±0.4	0.73±0.1
10	0.33	3	6	9.1±0.8	10.6±1.3	3.2±0.4	3.8±0.5	0.85±0.1	1.0±0.1
11	0.54	6	5	10.6±1.0	9.4±0.9	4.5±0.6	4.5±0.3	1.1±0.1	1.1±0.1
12	0.55	5	4	8.0±1.2	7.5±1.1	3.3±0.6	3.3±0.6	0.84±0.1	0.77±0.1
All	0.49	64	67	9.6±1.9	9.4±1.6	3.1±0.7	3.0±0.8	1.0±0.2	1.0±0.2

Supplementary Table 3.1, continued

	A4 (pg/mg yolk)		P4 (pg/mg yolk)		Glucose (mM)	
Hen	♂	♀	♂	♀	♂	♀
1	208±21	195±7	895±244	835±221	9.1±0.8	10.8±0.8
2	122±9	122±10	723±170	709±204	9.3±1.6	10.1±1.2
3	150±16	154±11	680±69	713±82	8.7±1.3	10.3±1.6
4	130±11	109±11	814±229	719±152	10.3±1.8	9.3±0.9
5	226±23	187±36	803±158	727±112	8.7±0.5	8.7±0.9
6	111±7	116±6	557±84	594±139	8.7±1.3	8.7±1.6
7	151±9	154±15	778±112	803±84	8.5±0.6	8.9±0.6
8	173±24	163±19	860±216	854±180	8.3±1.5	8.5±0.6
9	151±15	149±23	695±65	818±122	8.8±1.4	7.7±0.7
10	196±44	214±19	696±72	721±165	8.4±0.2	8.2±0.6
11	142±10	145±6	809±217	643±124	8.7±0.7	8.7±0.9
12	121±16	118±21	524±138	602±132	9±0.3	8.7±0.5
All	152±37	154±35	737±190	733±156	9.0±1.3	9.0±1.2

Supplementary Table 3.2. Egg physical dimension (Egg Weight, Width and Length) in female and male chicken eggs (mean per hen and mean overall±s.d.) and hen body weight and mean hen body weight±s.d.

	Hen body weight (g)	Egg Weight (g)		Egg Width (mm)		Egg Length (mm)	
Hen		♂	♀	♂	♀	♂	♀
1	2200	70.0±1.0	71.2±0.4	44.5±0.3	44.8±0.0	62.3±1.0	62.5±0.5
2	2020	65.4±1.0	64.5±1.5	44.4±0.7	43.9±0.6	58.9±1.1	59.4±1.0
3	2020	60.7±2.5	61.5±2.9	43.6±0.6	43.6±0.8	56.3±0.7	56.7±1.1
4	1925	58.8±2.3	57.4±2.0	42.9±0.7	42.7±0.6	56.5±1.0	55.5±1.2
5	2280	60.0±1.8	60.2±1.9	43.4±0.5	43.2±0.4	57.0±1.2	57.9±0.9
6	1515	65.9±1.2	67.2±0.6	44.5±0.5	44.9±0.3	58.7±0.7	58.8±0.4
7	1825	64.2±2.4	64.3±1.6	44.3±0.6	44.2±0.4	58.3±1.2	58.7±0.9
8	1795	61.5±1.3	62.7±3.0	43.0±0.4	43.0±0.7	58.6±1.4	59.2±1.7
9	2015	69.4±3.7	70.3±3.3	45.3±0.7	45.8±0.8	59.8±1.4	59.5±1.5
10	2224	62.8±1.0	63.6±1.4	44.5±0.5	44.6±0.4	56.7±0.3	57.0±0.5
11	2120	65.6±2.2	65.2±0.7	44.7±0.3	44.3±0.5	57.6±1.4	58.2±0.7
12	1737	60.9±1.9	61.8±1.8	44.1±0.5	44.1±0.6	56.8±0.6	56.8±0.9
All	1973±225	63.8±1.9	64.2±1.8	44.1±0.5	44.1±0.5	58.1±1.0	58.3±0.9

4

Feed restriction induces changes in primary sex ratio that correlate with changes in body mass and corticosterone levels in layer hens (*Gallus gallus*)

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Abstract

Female birds have been shown to manipulate offspring sex ratio under natural as well as experimental conditions. However, mechanisms of sex ratio bias are not well understood and it is often not clear whether a skewed sex ratio is due to primary or secondary sex ratio bias. In the current study, we experimentally manipulated food availability to laying chickens to study its effect on primary sex ratio by sexing unincubated eggs. The effect of feed restriction (upto to 70% of ad libitum) over time was significant for sex ratio (proportion of male eggs) per day in the feed restriction group. The rate of change of hen body mass between day of laying and day of laying minus 2 days (encompass time of meiosis completion) was a significant predictor for the sex of that egg. Feed restriction resulted in higher corticosterone concentrations in female blood plasma, which was associated with sex ratio. Also, corticosterone levels per hen were associated with the fraction of infertile eggs, but the egg sex ratio per hen was not at all associated with fertility or laying rate per hen. Change in hen body mass and negative energy balance due to feed restriction could perhaps affect meiotic drive via corticosterone or other hormonal or metabolic factors associated with a negative energy balance.

Key words: Primary sex ratio, Unincubated eggs, Feed restriction, Chicken, Body mass

4.1 Introduction

In species with chromosomal sex determination, Mendelian sex chromosome segregation leading to a sex ratio of 0.5 may be expected, provided that costs of producing and raising sons or daughters are equal [24]. However, according to sex allocation theory, under certain conditions sex ratios may depart from parity which may have selective advantage when costs of rearing of different sexes or fitness returns from different sexes differ [24, 26, 27]. Female birds are heterogametic and are therefore potentially in charge of determining the sex ratio of their offspring. A number of studies demonstrated the ability of birds to skew the sex ratio in response to a number of factors under natural conditions, such as habitat quality [38], laying sequence [39], season [40, 41], mate quality [42] and food availability [43, 44]. In addition, it has been shown that a sex ratio bias in birds can be induced under experimental conditions using a number of factors such as administration of hormones [11, 14-16, 45-47], changing female body condition [48] or female diet quality [49, 50].

A number of mechanisms have been proposed for explaining sex ratio bias in birds, which include biased sex chromosome segregation during meiosis, differential survival or fertilization success of oocytes depending on their sex, and sex dependent differential zygote or embryonic mortality until sex ratio is determined [46]. The first mechanism (relating to the primary sex ratio) would be more efficient, and therefore perhaps more likely, because resources provisioned to the eggs or offspring are not wasted [46]. The aims of the current study were to investigate if and how chicken hens are able to skew the primary sex ratio (the proportion of male eggs) under experimental conditions and, secondly, to develop an animal model to further study the physiology and molecular mechanisms of sex ratio regulation in birds.

The laying hen is an interesting study species for this research for two reasons. Improved understanding of mechanisms underlying sex ratio biases may have important implications for commercial poultry farming as it may lead to finding means to reduce the enormous number of male chicks born in the layer industry, which in the current practise are killed after hatch. The chicken is also an interesting model to study sex ratio bias in birds because chickens are sexually dimorphic; females are the smaller-less costly-sex [108] and have lower reproductive variance than males [5, 109]. According to sex allocation theory, in such species, a female sex ratio bias may be expected under conditions of low food availability [26]. Low food availability can lead to a decrease of body condition and elevated corticosterone concentration

in birds, and corticosterone has been shown to affect sex ratio in a number of bird studies [11, 21]. In the present study, our starting hypothesis was that feed restriction and decrease of hen body mass could lead to a female sex ratio bias in layer chicken concomitant with an increase in circulating corticosterone levels.

In all previous studies on sex ratio in birds, the sex ratio was determined by sexing embryos or hatchlings [12-15, 20, 21, 39, 42, 45, 47-50, 191] and bias in sex ratio could be due to both primary and secondary sex ratio bias in these studies. Distinguishing between primary and secondary sex ratio bias is crucial for understanding the mechanisms for sex ratio bias. Unlike previous studies, offspring sex ratio in the present study was determined in unincubated eggs to approximate the primary sex ratio by using a recently developed technique of sexing unincubated chicken eggs [174]. We studied the relations between body mass change, hen blood plasma corticosterone concentration, percentage of fertile eggs per hen, laying rate and sex of egg or hen sex ratio, comparing feed restricted and control hens, to analyse possible underlying mechanisms for sex ratio change. We were also interested whether the sex of the egg is predicted especially well by changes in estimated body mass around the calculated time of meiosis-I, which occurs about 26 to 28 hours before oviposition [47, 64] and determines sex of an egg [8, 15, 57].

4.2 Materials and Methods

4.2.1 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 2010076). Sixty hens and 15 cocks of a brown layer line, kindly provided by ISA BV, the layer breeding division of Hendrix Genetics, and 40 weeks of age, were housed individually in large ground cages, provided with litter and a roosting bar, with ad lib access to feed and water and a light-dark programme of 16h-8h at 25 °C. The feed used was a standard layer diet (11160 legmeel) from animal feed company Arkervaart, Nijkerk, the Netherlands. They were acclimatized to the housing conditions for 10 days before starting egg collection. The hens had visual and audio contact with each other and only audio contact with the cocks. The cocks were trained for semen collection and the hens were artificially inseminated twice weekly with a volume of 0.2 ml of 4 times diluted semen. Egg collection began two days after the third insemination and eggs were collected over a period of 26 days (from day 0^{th} to day 25^{th} of experiment). Eggs

were collected daily and marked with date and hen number and directly stored at 17 °C until processing the same day or the next day.

4.2.2 Feed restriction treatment

Hens were randomly assigned to the control and feed restriction groups, with 30 hens per group. The control group hens had ad libitum access to feed during the entire experiment. The feed restriction was implemented during days 0-16, after which they again received ad lib feed during days 17 to 25. Feed restriction started at 8:00 hours of 'day 0' and return to ad lib feeding was at 16:00 hours of day 16. Per hen, the daily feed rations during days 0-6 were 80% of the hen's daily ad lib consumption measured in the three days prior to start of treatment, and 70% of ad lib in days 7-16. Meals for the feed restriction group during feed restriction period were given once daily at 8:00h. The amount of feed given to these hens during that period was calculated for each hen based on its own ad libitum feed consumption measured during 4 days prior to starting the treatment. As we wanted to induce a steady decrease in hen body mass, for some birds (12 out of 30) feed rations were further deceased to 65% of ad lib in days 13-16, as these hens were not decreasing in body mass at this point in time. After this, ad lib feeding then continued until day 25.

4.2.3 Blastodisc isolation and sexing using PCR

In total, 1419 eggs were laid and all eggs were processed after being stored at 17 °C for a maximum of 1 day after laying. The fertilization status of all eggs except 9 was successfully determined and all fertile eggs were successfully sexed. Blastodiscs were isolated using a technique similar to that described by Chapman *et al.* [160]. Isolated clean blastodiscs free of yolk were stained with Hoechst 33342 for determining fertility of egg as described [174]. Then, the blastodiscs of fertile eggs were suspended in approximately 20 µl of PBS and frozen at -20 ℃ until use for nucleic acid extraction. The sex of the blastodiscs was determined according to the method described previously [174]. In brief, nucleic acids were isolated from blastodisc samples using proteinase K digestion as described previously [161]. DNA pellets were washed in 70 % ethanol, air dried and dissolved in 25 µl of RNAse free water. Subsequently 20 ng of nucleic acid was used in the polymerase chain reaction to amplify the *CHD1* gene using primer pair 2550F/2718R [148]. *CHD1* gene fragments of 450

bp (*CHD1-W*) and 600 bp (*CHD1-Z*) are amplified from the W- and Z- chromosome specific CHD1 genes, respectively [148].

4.2.4 Plasma samples collection and body mass measurements

Blood samples were taken from 10 hens per group on two time points (repeated measurement), day 8 and day 11 at 11:00h, for measurement of the plasma corticosterone concentration. From 10 other hens per group, blood samples for corticosterone measurement were taken on day 16 at 11:00h. Blood samples were collected by brachial venipuncture within 3 minutes after catching the birds to avoid elevations of corticosterone levels due to stress response. Approximately one ml of blood was collected in heparinized blood collection tubes and kept on ice until isolation of plasma. Plasma was separated from blood samples by spinning at 2000 rpm for 10 minutes. Isolated plasma samples were frozen at -70 °C until use for hormone concentrations determinations. In the feed restriction group a number of samples were not included in the statistical analysis of these samples: One of the ten hens sampled on days 8 and 11 stopped laying eggs at the start of experiment and was therefore not considered for statistical analysis, i.e. nine hens were considered for statistical analysis of corticosterone on days 8 and 11. Also, one of the day-16 samples was not considered for statistical analysis as the corticosterone value of that sample was an outlier (the value, 5.85, was more than two times the s.d. higher than the mean; mean ± s.d. including this hen = 1.89±1.55). The corticosterone level of another day-16 sample was not available due to technical reasons in the laboratory, i.e. eight hens were considered for statistical analysis of corticosterone on day 16.

Body mass of hens were measured on days -9, 6, 8, 10, 16 in the control group and on days -9, 6, 8, 10, 13, 15, 16, 20 and 25 in the feed restriction group, between 15:00h to 16:00h. Body mass between two measurements was estimated by linear interpolation.

4.2.5 Steroid hormone analysis

Corticosterone extraction from plasma samples was according to procedures as described by Goerlich *et al.* [14]. To calculate recoveries, 2500 cpm of tritiated corticosterone (Perkin Elmer, Product Number: NET399) was added to all samples. Briefly, double dichloromethane extraction was followed by single methanol extraction in which 1 ml of 70% methanol was added to extracts, vortexed and placed in –20°C overnight. The following morning, samples

were centrifuged for 5 minutes at 2000 rpm at 4°C and methanol phase was decanted and dried under a nitrogen evaporator. Extracted hormone was resuspended in 300 µl PBS. Recoveries were on average 77%±5.3. Corticosterone concentrations in plasma samples were measured using the ImmuChem Double Antibody Corticosterone radio immunoassay kit from MP Biomedicals, Germany (07–120102). The assay was validated for parallelism by measuring serial dilutions of plasma samples containing high concentrations of corticosterone. We calculated the coefficients of variation (CV) based on assay controls and standard curves. All the samples were measured in one RIA and average intra-assay CVs was 3.7 %.

4.2.6 Statistical analysis

Statistical analysis was performed using software R2.12.2. with lme4 and nlme packages applying generalized linear mixed modelling approach. All tests were two tailed with significance delimited by alpha = 0.05. Hens were used as random factor in all models to account for the fact that they contributed with more than one egg to the data sets. Egg sex data and egg sex ratio were analysed by logistic regression with binomial errors using logit link function.

The effect of treatment on sex of egg was tested in a model that included treatment and day (as the effect of treatment may increase over time), and their interaction. In all analyses pertaining to effect of treatment, or time, or corticosterone levels, on egg sex or egg sex ratio, we have used all relevant egg data as follows: With regard to the feed restriction period (day 0 to 16), the eggs laid between day 2 and day 17 were considered, as these would have had completion of meiosis-I during the treatment period (between day 0 and 16). Likewise, for the period of more severe feed restriction (70% of ad lib) the eggs laid between day 9 and day 17 were considered. The eggs of days 0 and 1 were used to calculate pre-treatment egg sex ratio which were 0.48 and 0.46 for control and feed restriction group, respectively.

In all analyses pertaining to the effect of hen body mass or body mass change, we have only used data from day six onward, as the body mass value of day 0 was not measured but rather estimated by interpolation. From day 6 onward (the period of more severe feed restriction) hen body mass was measured frequently on many days (specified above), although not on every day, while body mass between two measurements was estimated by linear interpolation. Therefore, we cannot exclude the effects of interpolating the hen body mass on the results

regarding associations of hen body mass and sex of egg. To address the relation between hen body mass and egg sex ratio over the time course of the experiment, we tested the effect of hen day body mass or relative hen day body mass (the latter tested by including hen body mass at day 6 as covariate in the model) on sex of egg two days later. In addition, to test the hypothesis that a negative energy balance or declining body condition either during yolk deposition prior to meiosis, or around the time of meiosis could affect sex ratio, we studied the effect of the rate of hen body mass change during appropriate time intervals on sex of egg. A factor or factors elicited by feed deprivation, possibly hormones or metabolic factors, will affect both a decline of body mass and meiosis, hence sex of egg. Both of these processes may occur gradually over time and possibly with different time courses and therefore we have tested three time intervals of 1, 2, and 3 days length that all encompass the calculated time of meiosis. With regard to time of meiosis-I, we tested if sex of egg was predicted by the change in body mass between the day an egg was laid and 2 or 3 days prior to that day, or by the body mass change between 2 days prior to egg laying and one day prior to egg laying. With regard to the time of yolk deposition prior to meiosis, we tested if sex of egg was predicted by the body mass change between 5 days prior to egg laying and 2 days prior to egg laying, and between 4 days prior to egg laying and 2 days prior to egg laying. Combined data of both feed restriction group and control group were used and treatment and interaction of body mass change with treatment were removed from the model as the interaction was not significant.

The feed restriction group contained a feed restriction period, followed by a subsequent ad libitum feeding phase. Therefore, the data of the feed restriction group was also analysed separately to test the effect of day on sex of egg using day square as a covariate in the model, as we expected a quadratic effect (first a decrease of egg sex ratio during the feed restriction period followed by an increase during the ad lib phase).

Effect of treatment on the concentration of corticosterone in the blood plasma of hens measured on day 8, 11 and 16 of the experiment and hen body mass change over the more severe (70% of ad lib) feed restriction period (days 6-16) was tested by mixed modelling using hen as random factor. The relationship between the change in hen body mass calculated over the more severe feed restriction period (days 6-16) and plasma corticosterone concentration was tested by linear regression. Associations of egg sex ratio per hen calculated over the period of feed restriction (eggs of days 2-17) with corticosterone concentrations were studied using logit function for corticosterone data obtained at day 8, 11 or 16 in models

including interaction of treatment times corticosterone concentrations. Associations of egg sex ratio per hen during the period of feed restriction (eggs of days 2-17) with fraction of fertile eggs per hen or laying rate per hen were also tested using logit function in models including interaction with treatment. In addition the associations of fraction of fertile eggs per hen and laying rate per hen with the day 8, 11, and 16 corticosterone concentrations were tested using logit function in models including interaction with treatment.

4.3 Results

4.3.1 Effect of treatment on hen body mass and sex of egg

Mean hen body mass (± s.d.) at the start of the experiment was 1815±157 g and 1802±121 g in feed restriction and control group respectively. Feed restriction resulted in a decrease in body mass of hens, with a mean body mass loss of 126±44 g during the period of more severe feed restriction from day 6 to day 16 ($df = 56$, $t = 3.38$, $p = 0.001$), while the mean change in body mass in the control group in that same period was less (26.8 g ± 31.56; $df = 58$, $t = 0.75$, $p = 0.45$). The body mass change of the treatment and the control hens during this period of feed restriction was significantly different (day 6 to 16, $df = 57$, $t = -10.02$, $p < 0.0001$). Body mass increased rapidly and significantly after returning to ad lib feeding from day 16 to day 25 with a mean body mass increase of 110±59 g during this 9 days period ($df = 56$, $t = -2.21$, $p = 0.03$). For unknown reasons, the hens in the control group tended to lose weight between day 10 and day 23, with a mean body mass decrease of 107±29 g (post hoc test, $df = 58$, $t = 1.51$, $p = 0.13$).

For analysing the effect of treatment on the sex of the eggs, the interaction of treatment times day number was first included in the model, because a potential effect of treatment may build up over time, but the interaction was not significant (day 2 to 17, no. of eggs = 721, variance hen random factor = 0.004, estimate = -0.03, $z = -1.06$, $p = 0.28$). After removing day and interaction of treatment times day from the model, the main factor treatment was not significant (no. of eggs = 721, variance hen random factor = 0.0005, estimate = -0.08, $z = -0.60$, $p = 0.54$). However, when only considering the eggs that had their meiosis during the period of more severe feed restriction (eggs of days 9-17), the interaction of treatment times day number was significant (no. of eggs = 421, variance hen random factor < 0.0001, estimate = 0.20, $z = 2.54$, $p = 0.01$). To estimate in this same sample the effect of the main factor

treatment alone, day and interaction of treatment times day were removed from the model showing that the main factor treatment displayed a trend (no. of eggs = 421, variance hen random factor < 0.0001, estimate = -0.33, z = -1.70, p = 0.08).

Within the feed restriction group, effects for day (day 2 to 25, no. of eggs = 487, variance hen random factor = 0.15, estimate = -0.15, z = -2.62, p = 0.008) and square of day (no. of eggs = 487, variance hen random factor = 0.15, estimate = 0.005, z = -2.41, p= 0.01) were significant for the prediction of the sex of the eggs. Also in the control group the same analyses were done for the control group, but day (no. of eggs = 526, variance random factor = 0.15, estimate = -0.04, z = -0.82, p = 0.41) and square of day (no. of eggs = 526, variance random factor = 0.15, estimate = 0.001, z = 0.54, p = 0.58) were non-significant for prediction of sex of egg.

4.3.2 Relation between hen body mass and sex of egg

The effect of day on sex of the eggs in the feed restriction group, representing changes in maternal body mass due to treatment (described in section above), could be due to hen body mass per se or the *change* in hen body mass. Sex of the eggs was not significantly predicted by hen day body mass at the day of laying (for days 9-17; no. of eggs = 421, variance hen random factor = 0.37, estimate = 0.0009, z = 0.60, p = 0.54) or by the relative hen body mass (the latter tested by including hen body mass at day 6 as covariate in the model, for days 9-17; no. of eggs = 421, variance hen random factor = 0.37, estimate = -0.003, z = -1.46, p = 0.14). However, potentially the change in body mass rather than body mass per se could affect the sex ratio. Indeed, the effect of rate of body mass change between the day an egg was laid and 3 days prior to that day on egg sex was significant (for days 9-17; no. of eggs =377, variance hen random factor < 0.0001, estimate = 0.009, z = 2.42, p = 0.01). The same was true for the time interval between the day an egg was laid and 2 days prior to that day (for days 9-17; no. of eggs = 377, variance hen random factor < 0.0001, estimate = 0.01, z = 2.37, p = 0.01). The effect of body mass change between 2 days prior to egg laying and one day prior to egg laying displayed a trend (for days 9-17; no. of eggs = 421, variance hen random factor < 0.0001, estimate = 0.01, z = 1.79, p = 0.07). Note that these different time intervals all encompass meiosis, 26 to 28 hours before oviposition [18, 64]. When testing the effect of body mass changes on sex of egg during the intervals that encompass yolk deposition just prior to meiosis, no significant effects were found: For body mass change between 5 days prior to egg

laying and 2 days prior to egg laying (for days 11-17; no. of eggs = 333, estimate = 0.003, variance hen random factor < 0.0001, z = 0.80, p = 0.42) and for body mass change between 4 days prior to egg laying and 2 days prior to egg laying (for days 10-17; no. of eggs = 383, variance hen random factor < 0.0001, estimate = 0.005, z = 0.98, p = 0.32).

4.3.3 Relation between body mass change, plasma corticosterone concentration, hen sex ratio, laying rate and fraction of fertile eggs

In addition to a stronger decrease of hen body mass in the feed restriction group than in the control group during the period of more severe feed restriction (day 6-16) (see first results section), hen blood plasma corticosterone concentrations (Mean ± SD) in the feed restriction group (1.39±0.43 ng/ml) were significantly higher than in the control group (1.06±0.34 ng/ml) when combining the corticosterone measurements of the three sampling days (days 8, 11, and 16; df = 37, t = 2.88, p = 0.006). Within the feed restriction group, there was a significant correlation between hen body mass decrease over more severe feed restriction period (days 6-16) and the corticosterone concentration on day 16 (Figure 4.1) (df = 6, estimate = -0.006, t = -3.08; p = 0.02), but not on day 8 (df = 7, estimate = -9.01×10^{-5}, t = -0.03, p = 0.97) or day 11 (df = 7, estimate = -0.003, t = -1.38, p = 0.20).

The interaction of treatment times day 8 hen blood plasma corticosterone concentration significantly explained variation in egg sex ratio per hen (df = 15, estimate = -1.81, z = -2.84, p = 0.004; see Figure 4.3A). After removing the interaction of treatment times blood plasma corticosterone concentration, sex ratio per hen was not associated with the corticosterone concentration on day 11 (df = 16, estimate = -0.04, z = -0.13, p = 0.89) and day 16 (df = 15, estimate = 0.30, z = 0.93, p = 0.35). The plasma corticosterone concentration per hen appeared to be quite dynamic. The change of the plasma corticosterone concentration between day 8 and day 11 was strongly and significantly (df = 17, estimate = -0.90, t = -4.98, p = 0.0001) inversely related to the day 8 corticosterone concentration in both feed restriction and control groups (R^2 = 0.62 for the two groups combined), i.e. hens high on day 8 went down and hens low on day 8 went up (see Figure 4.2).

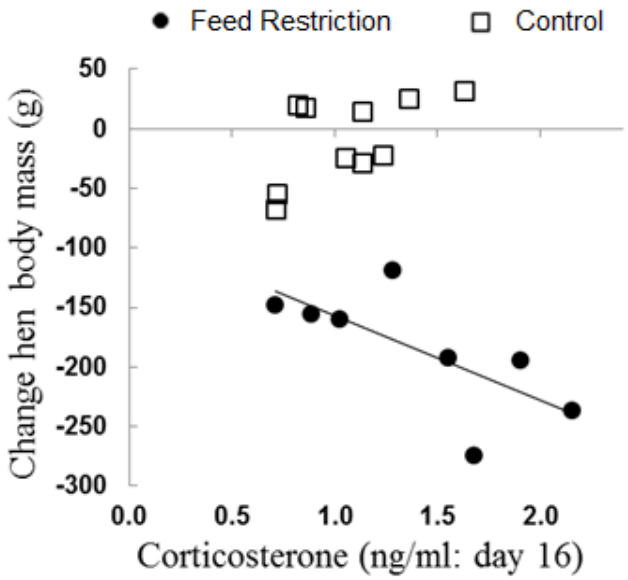

Figure 4.1. Body mass change between day 6 to 16 and the blood plasma corticosterone concentrations on day 16. The trend line shows the correlation between these two parameters in the feed restriction group (p = 0.02).

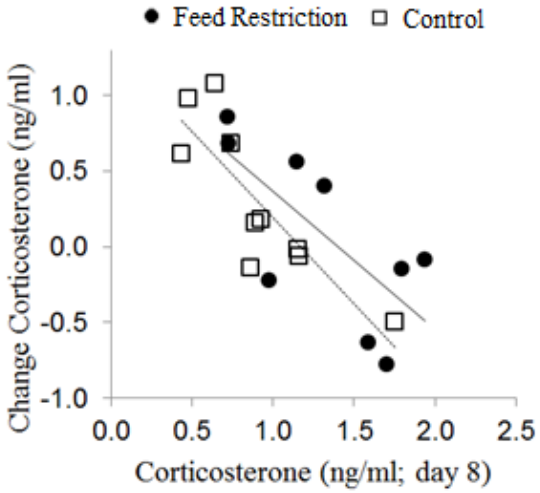

Figure 4.2. **Correlation between the change in corticosterone concentration (from day 8 to 11) and corticosterone concentrations on day 8.** The change of the plasma corticosterone concentration was inversely related to the day 8 corticosterone concentration in both feed restriction and control groups.

Laying rate per hen during the feed restriction phase (eggs of days 2-17) was not significantly different (df = 57, estimate = 0.57, z = 1.55, p = 0.12) between feed restriction (95%) and control group (97%). Sex ratio per hen was not significantly predicted by the interaction of

laying rate per hen times treatment (df = 55, estimate = 2.32, z = 0.68, p = 0.49). Laying rate per hen was not significantly associated with plasma corticosterone concentrations on day 8, 11, or 16 (df = 16-17, highest z = 1.06, lowest p = 0.28).

The fraction of fertile eggs laid per hen during the feed restriction phase (eggs of days 2-17) was not significantly different in the feed restriction group (0.80) and control group (0.83) (number of hens = 59, estimate = -0.13, z = -0.76, p = 0.44). Sex ratio per hen was not significantly predicted by the interaction of fraction of fertile eggs per hen times treatment (df = 55, estimate = 1.45, z = 1.48, p = 0.13). Overall, there appeared to be a negative association of hen blood plasma corticosterone concentration with the fraction of fertile eggs per hen (eggs of days 2-17). For the corticosterone concentration of day 8 (df = 15, estimate = 1.04, z = 1.42, p = 0.15) and day 16 (df = 14, estimate = 0.68, z = 0.67, p = 0.50), the interaction of treatment times corticosterone concentration was not significant. After removing the interaction, the fraction of fertile eggs per hen was significantly associated with corticosterone concentration (Figure 4.3B, day 8: df = 16, estimate = -1.18, z = -3.23, p = 0.001; day 16: df = 15, estimate = -0.93, z = -2.04, p = 0.04). For corticosterone concentrations of day 11, the interaction of treatment times corticosterone concentration was significant (df = 15, estimate = -2.31, z = -2.30, p = 0.02). In the feed restriction group (and not the control group), day 11 corticosterone concentrations were significantly associated with the fraction of fertile eggs per hen (df = 7, z = -2.27, p = 0.02). In addition, treatment times the change of body mass per hen during period of more severe feed restriction (between days 6-16) was significant for fraction of fertile eggs per hen (df = 55, estimate = 0.02, z = 5.17, p < 0.0001). In the feed restriction group (but not in the control group), there was a significant association between the fraction of fertile eggs per hen and the change of body mass per hen between day 6-16 (df = 27, estimate = 0.006, z = 3.34, p = 0.0008).

For the egg sex ratio per hen (eggs of day 2-17), the interaction of treatment times change of body mass per hen during period of severe feed restriction (between days 6-16) was not significant (df = 55, estimate = 0.001, z = 0.42, p = 0.66) and there was no significant association between the change of body mass per hen (between days 6-16) and sex ratio per hen (df = 57, estimate = 0.0002, z = 0.41, p = 0.68).

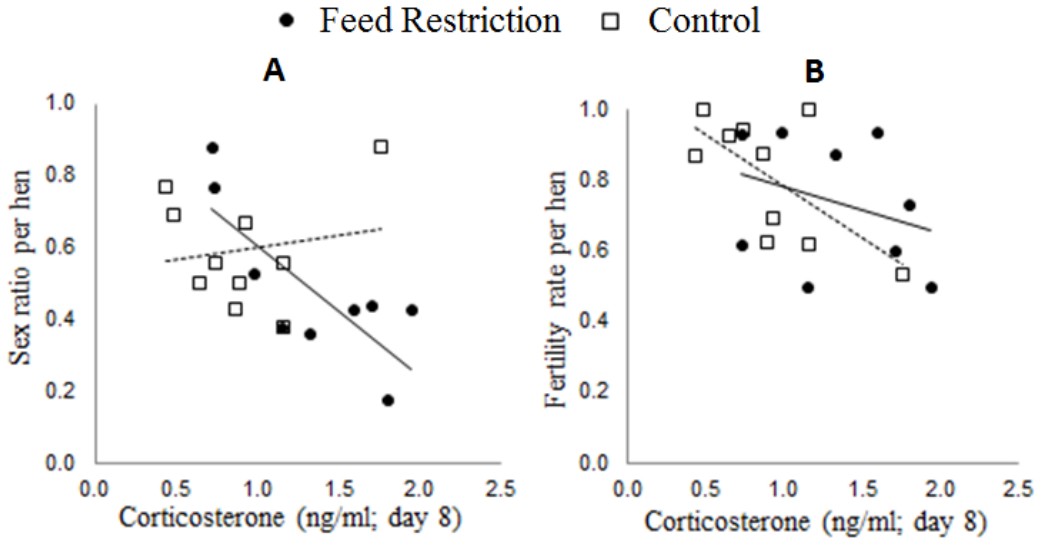

Figure 4.3. Sex ratio per hen and fraction of fertile eggs per hen in relation to plasma corticosterone on day 8. 3A: The interaction of treatment times day 8 hen blood plasma corticosterone concentration significantly explained variation in egg sex ratio per hen ($p = 0.004$). 3B: The association between fraction of fertile eggs per hen (eggs of days 2-17) and day 8 corticosterone concentration was significant in eggs from both control and feed restriction together ($p= 0.001$).

4.4 Discussion

The results from statistical model showed that sex ratio per day had a quadratic relationship with time in the feed restriction group i.e. sex ratio first decreased in during period of feed restriction followed by an increase during the subsequent period of *ab libitum* feeding. Treatment of feed restriction resulted in significant decrease in hen body mass during phase of feed restriction and after returning to ab lib feeding in subsequent phase there was a significant increase in the hen body mass. Thus it appears that changes in body mass caused by feed restriction can affect sex ratio. Treatment in interaction with day number of treatment was significant for sex ratio, suggesting that the effect of treatment builds up over time. However, body mass, or relative body mass per se did not significantly predict sex of egg, but the rate of change of hen body mass between day of laying and day of laying minus 2 days or minus 3 days (encompassing the period of meiosis for that particular egg) was a significant predictor for the sex of the eggs. Body mass changes in similar two- or three-day intervals, encompassing yolk deposition just prior to meiosis was not significant for the sex of the eggs. However, the associations of rate of change of hen body mass and sex of the eggs must be interpreted with care. Although body mass of hens were measured frequently, hen body mass

values between days of actual body mass measurements were estimated by linear interpolations. Therefore, effects of interpolating hen body mass data on the results cannot be excluded.

The feed restriction treatment also resulted in an increase of circulating levels of corticosterone. A significant association was seen between the sex ratio per hen (eggs of days 2-17) and the hen plasma corticosterone concentration on day 8, but this relation was not seen when considering the corticosterone values measured on day 11 and 16. Also, corticosterone levels per hen were negatively associated with the fraction of fertile eggs. This negative association was seen more consistently, i.e. on all three sampling days. However, the associations of corticosterone levels with fertility and sex ratio must be interpreted with care. In order to minimise any possible effect of stress due to blood sampling we have sampled only a subpopulation of the animals. Furthermore, it is known that corticosterone concentrations within an animal can vary over time, due to diurnal rhythms, feed intake, environmental (stress) factors, and physiological status [192, 193]. Indeed, our results indicated that the corticosterone levels in both the feed restriction and the control hens were highly dynamic, that is, hens that had a high level on day 8 had lower levels on day 11, and vice versa. For the feed restriction group, it would be conceivable that there are truly differences between hens in the dynamics of their response to the onset of feed restriction. However, since the apparent dynamic behaviour of corticosterone levels over time was equally seen in control hens, it seems more likely that this represents ‘regression to the mean’ resulting from noise. Thus, while the associations of corticosterone level with fertility and sex ratio could be plausible, more frequent blood sampling over time appears to be necessary.

The found decrease in sex ratio over time after start of feed restriction is in agreement with the theory of sex allocation [26], which predicts that a species may evolve mechanisms to adjust offspring sex ratio under conditions of restricted feed availability towards the sex that provides greater fitness returns under these conditions. For reasons explained in the introduction of this paper, in the chicken a female sex ratio bias could therefore be expected under conditions of low feed availability.

The results for the effect of feed restriction over time on sex of egg are in agreement with previous studies in various bird species showing that offspring sex ratio varied in relation to food availability [43, 44, 100, 101], or female body condition under natural conditions [13, 102-105] or under experimentally controlled conditions [13, 49, 53, 106, 107]. In addition,

our results are in line with a number of studies showing that the offspring sex ratio in birds may be affected by maternal corticosterone concentrations [11-13, 21]. Our study adds to both bodies of literature but with two new findings: we sexed the eggs at day zero of incubation, at the day the egg was laid, and therefore much closer to fertilization (the primary sex ratio) than all earlier studies in this field. Second, our analyses about the time window of body mass change in relation to egg sex gives a cue about its underlying mechanism.

With regard to the mechanism of sex ratio bias, a number of studies have shown sex specific embryonic death, i.e. the secondary sex ratio was changed [20, 57, 105, 107, 117]. A number of other studies have reported a primary sex ratio bias arguing that the incidence of embryonic death was too low to explain the observed sex ratio bias [12, 13, 43, 44, 100-103, 194]. Meiotic drive has been proposed to be the most likely mechanism for manipulation of the primary sex ratio [5, 8, 15, 21, 57, 159]. Mechanisms for asymmetric segregation of chromosomes during meiosis in relation to hormone levels have been proposed (reviewed in [8, 72]). In our current study, there was no significant effect of the overall decrease of body mass between days 6-16 on the sex ratio for eggs laid between days 2-17. Also, no significant effects were found when considering the rate of body mass change over 2- or 3-day time windows just prior to meiosis and including the major phase of yolk deposition. However, the sex of egg was significantly predicted by the rate of change of hen body mass in a specific time window (i.e between day of egg laying and day of laying minus 2 days or minus 3 days) that encompasses resumption of meiosis. This apparently critical time period not only encompasses meiosis, but also includes, ovulation, fertilization, and early development of the egg and embryo until laying. However, laying rate was close to 100% and was not associated with egg sex ratio per hen, and also fertility rate was not affected by feed restriction and not associated at all with sex ratio. Here, ‘fertility’ or ‘fertile egg’ is operationally defined to cover both fertilization of the oocyte by sperm and early embryonic development until laying. Thus, our results suggest that neither ovulation, nor fertilization or early embryonic development could explain the observed variation in sex ratio. Therefore, our results favour the mechanism proposed by various authors, that primary sex ratio bias in birds could be due to meiotic drive [11-13, 15, 21]. It has been proposed that nutrient availability could influence the rate of follicle growth, which in turn could influence meiosis [8, 56, 57, 65]. However, as we found no significant effect of body mass change over 2- and 3-day time periods just prior

to meiosis, other mechanisms involving metabolic or hormonal changes resulting from feed restriction may be more likely.

A number of studies have suggested that maternal corticosterone [13, 21, 22, 47] or other hormones [14, 15, 18, 45] could be involved in the mechanism of non-random sex chromosome segregation. Corticosterone is a hormone involved in conditions of catabolism (feed restriction, negative energy balance) and/or stress [121, 195, 196]. In our study we did find an association of hen blood plasma corticosterone concentration with sex ratio. However, it could be that both change in hen body mass and plasma corticosterone could explain part of variation in sex ratio, or that a change in plasma corticosterone represents only the effect of hen body mass change rather than the cause of sex ratio bias.

In conclusion, the results from the present study show that sex ratio can be influenced experimentally in chicken, and suggest meiotic drive as possible mechanism for reduction of the offspring sex ratio of layer hens under conditions of feed restriction. Change in hen body mass and negative energy balance due to feed restriction could perhaps affect meiotic drive via corticosterone or other hormonal or metabolic factors associated with a negative energy balance.

Acknowledgements

We thank Hendrix Genetics B.V., The Netherlands for providing hens used in the experiment. We thank Agnes de Wit and Rita Hoving-Bolink for expert technical assistance, and Martina Muller for assisting in statistical analysis of data. We thank Bonnie de Vries and Ilse Weites for determining corticosterone plasma concentrations. We thank Ministry of Economic Affairs, Agriculture and Innovation, The Netherlands for funding this project under the project number BO-12.02.002.7.01

5

Association of egg mass and egg sex: gene expression analysis from maternal RNA in the germinal disc region of layer hens (*Gallus gallus*)

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Abstract

Female birds have been shown to manipulate offspring sex ratio under natural as well as experimental conditions. 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 which could lead to skew in sex ratio. We employed feed restriction in laying chickens to induce a decrease in body condition and egg mass using 45 chicken hens in each of 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 sons) had a significant negative association with mean egg mass per hen. This association was largely due to differences between hens in egg mass decline during treatment. Based on this association, two groups of hens were selected from the feed restriction group i.e. hens producing a male bias with low egg mass and hens producing a female bias with high egg mass. Genome wide transcriptome analysis on the germinal discs of the F1 pre-ovulatory follicles of the two groups of hens 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 more enriched in female biased hens as compared with male biased hens. The differentially expressed gene sets may be involved in meiotic drive regulating sex ratio in chicken.

Keywords: primary sex ratio; egg mass; feed restriction; meiosis; gene expression; microarrays

5.1 Introduction

According to the sex allocation theory, mothers are expected to shift the offspring sex ratio towards 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 [24, 26, 27]. Sex ratio biases in relation to environmental or maternal factors have now been demonstrated in a wide variety of taxa, including many bird species [74]. Birds are an interesting case as 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 [43, 44, 100, 101], or female body condition under natural conditions [13, 102-105] or under experimentally controlled conditions [13, 49, 53, 106, 107]. Both the food availability [113] and female body condition during egg production have been reported to affect the mass of the egg and of the egg yolk [114] and in several species these correlate with the offspring sex ratio [69, 115, 116].

According to sex allocation theory, in sexually dimorphic species, a sex ratio bias towards the smaller -less costly- sex may be expected under conditions of low food availability [26]. 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 [108] having lower reproductive variance than males [5, 109] and lower energetic requirements [197]. 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 towards female offspring or the females would allocate more resources to the sex with low energetic requirements i.e. 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 [174].

The ovary of reproductively active hens recruit follicles in hierarchical manner (F1-F6), starting from smallest pre-hierarchical follicles (F6) to biggest pre-ovulatory follicle (F1) [98]. The pre-ovulatory follicle (F1) contains a large amount of yolk, as well as a small germinal disc (3.5 mm), visible in the form of a whitish spot on the surface of the follicle [96]. The important functional reproductive processes such as sex determining meiosis [72],

fertilization and early embryonic development occur in the germinal disc [97, 98]. The germinal disc of the pre-ovulatory follicle in chicken (F1) contains large amounts of RNA (2.1 µg/oocyte) [99]. This RNA serves to regulate the cellular processes of the oocyte as well as earliest development after fertilization. This means that also the cellular processes related to meiosis and chromosomal segregation are regulated by this RNA. Meiosis-I, during which sex of an egg is determined [8, 15, 57], occurs about 26 to 28 hours before oviposition [18, 64]. Asymmetric chromosomal segregation during occurrence of meiosis-I (meiotic drive) has been proposed to be the most likely mechanism of the primary sex ratio bias [5, 8, 15, 21, 57, 159, 198]. We hypothesized that the RNA from the germinal disc of the pre-ovulatory 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. As a significant negative association was found between egg mass and sex ratio, two groups of hens were selected from the feed restriction group i.e. male biased hens with low egg mass and female biased hens with high egg mass. The RNA from the germinal discs of the F1 pre-ovulatory follicles collected at the time of occurrence of meiosis-I from the two groups of selected hens sacrificed at the end of the feed restriction was compared in genome wide transcriptome analysis to study the underlying mechanisms of meiotic drive and sex ratio bias.

5.2 Materials and Methods

5.2.1 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 weeks 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 light-dark programme of 16h/8h 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 8:00h, of 80% of ad lib consumption during days 0-6 and 70% of ad lib in 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 15:00h to 16:00h

5.2.2 Collection of blastodiscs from pre-ovulatory F1 follicle

Hens were sacrificed 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, 17 and 18. During three days before planned euthanisation, the time of egg laying of each hen was recorded to predict the time of meiosis-I, which occurs about 26 to 28 hours before oviposition [47, 64]. Hens were sacrificed 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 disc region (GDR) was isolated from the F1 follicle as described previously [97, 199]. In brief, a small piece of filter paper with a round 6 mm diameter hole was placed over the germinal disc region to support the follicle membrane. The follicle membrane was cut on one side of the paper, allowing insertion of an artery forceps to clamp the paper plus germinal disc 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 disc 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 horse hair 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 disc was cut away and the germinal disc area (including oolemma and perivitelline layer and possibly some granulosa cells) was then transferred to clean RNAase free eppendorf tubes, frozen in liquid nitrogen and stored at −80 ℃ until the purification of the RNA.

5.2.3 Blastodisc isolation from eggs 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 day −5 to 18) using a technique similar to that described by Chapman *et. al.* [160]. Isolated clean blastodiscs free of yolk were stained with Hoechst 33342 for determining fertility as described [174]. 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 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 ℃ until use for nucleic acid extraction after a maximum storage of 3 weeks. The sex of the blastodiscs was determined by PCR amplification of the *CHD1* gene exactly as described by [174].

5.2.4 Sample sizes, statistical analysis and selection of hens for microarrays

Statistical analysis was performed using software R2.12.2. with lme4 and nlme package applying generalized linear mixed modelling approach. All 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 (≤ 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 of 43 hens of each group are used for statistical analysis. For practical reasons, egg mass was not determined on day 0 and 18. In addition 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 analysed by logistic regression with binomial errors using logit link function. In the feed restriction group, for analysing egg sex of hens while under the influence of feed restriction, the eggs laid between day 2 and day 18 were considered, as these would have had completion of meiosis-I during the treatment period (between day 0 and 18). Likewise, for the period of more severe feed restriction (70% of ad lib) the eggs laid between day 9 and day 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 day –1 and day 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 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 hens were included for analysing 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 Figure 5.1 for egg mass (mean of hens per group) over time). The effect of egg mass on egg sex in the control group was analysed using either all hens or using the two subpopulations of hens with negative and positive regression, respectively, considering eggs of days −5-18.

As a significant negative association was found between egg mass and sex ratio, two groups of hens were selected from the feed restriction group i.e. male biased hens with low egg mass and female biased hens with high egg mass for microarray analysis of gene expression in the F1 follicle (see Figure 5.2). Criteria for selecting the hens were 1) a negative regression of egg mass on treatment day, 2) Sex ratio per hen and mean egg mass per hen, for eggs of all treatment days (days 2-18) and for eggs of only the second phase of treatment (day 9-18), 3) the number of fertile eggs per hen (as estimates for sex ratio would be less reliable in the (few) hens with relatively low fertility rate), and 4) good quality of the RNA isolated from the germinal disc. The laying rates of the two selected groups (95% and 92% for male and female biased hens, respectively) did not differ significantly ($p = 0.38$). Also the fertility rate (100% and 96% for male and female biased hens, respectively) did not differ significantly ($p = 0.32$).

5.2.5 RNA purification and microarray analysis

5.2.5.1 RNA extraction

RNA from the blastodiscs of F1 follicles from the selected hens was purified using master pure RNA purification kit from the Epicentre (Cat. No. MCR85102) and following protocol as described by the manufacturer. All the 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 disc region per hen ranged from 1.5-5 µg.

5.2.5.2 Labelling, Hybridization, Scanning and Feature Extraction

Labelling was done as recommended by Agilent Technologies using the One-Color Microarray-Based Gene Expression Analysis Low input Quick Amp Labelling. The input was 10 ng of total RNA and 600 ng of labelled cRNA was used on the 8 pack array.

Hybridization was performed as described in the One-Color Microarray-Based Gene Expression Analysis Low input Quick Amp Labelling 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 hours. After 17 hours the arrays were washed as described in the One-Color Microarray-Based Gene Expression Analysis Low input Quick Amp Labelling protocol from Agilent.

The arrays were scanned using the DNA microarray scanner with Surescan high resolution Technology from Agilent Technologies. Agilent Scan Control with resolution of 2µ, 16 bits and PMT of 100%. Feature extraction was performed using protocol 10.7.3.1 (v10.7) for 1 colour gene expression.

5.2.5.3 Data Loading, Statistical and Functional Analysis

The files generated by the feature extraction software were loaded in GeneSpring GX 12 for quality control (QC). On the basis of aberrant values for the QC probes of one microarray, used for analysing transciptome of one female sex ratio bias hen, the data of this hen were taken out. All normalization and transforming of the data was performed within R 3.0.0 [200] using the LIMMA package. First a principle component analysis was performed to explore the data. Secondly, 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 [201, 202]). With DAVID Functional Annotation Clustering, enrichment is calculated for genes of interest across multiple databases and ranked accordingly to their enrichment score (ES). When a term has an ES above 1 it 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). Thirdly we performed a statistical analysis (t-test) using Benjamini-Hochberg correction for multiple testing to identify differentially expressed genes (FDR <0.05) between the two groups (hens with male and female sex ratio bias, respectively). Lastly, a Gene Set Enrichment Analysis (GSEA) [203] was performed to identify whether there were differences at 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 supplementary Table 5.1). Furthermore we assume that most processes are generic when comparing chicken and human, including cell cycle, spindle, as well as (cellular) metabolic processes.

5.3 Results

5.3.1 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 to 18, no. of eggs = 1162, variance of hen as random factor < 0.0001, estimate = 0.11, z = 0.96, p = 0.33).

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 nr. (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 Figure 5.1). In the control group during the same time period of days 1 to 17, the mean egg mass per day did not decline over time (i.e. effect of treatment day nr. 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 Figure 5.1).

5.3.2 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 2-15) 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, 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, day −5 to day 18), or in the two subpopulations of control hens (see chapter materials and methods).

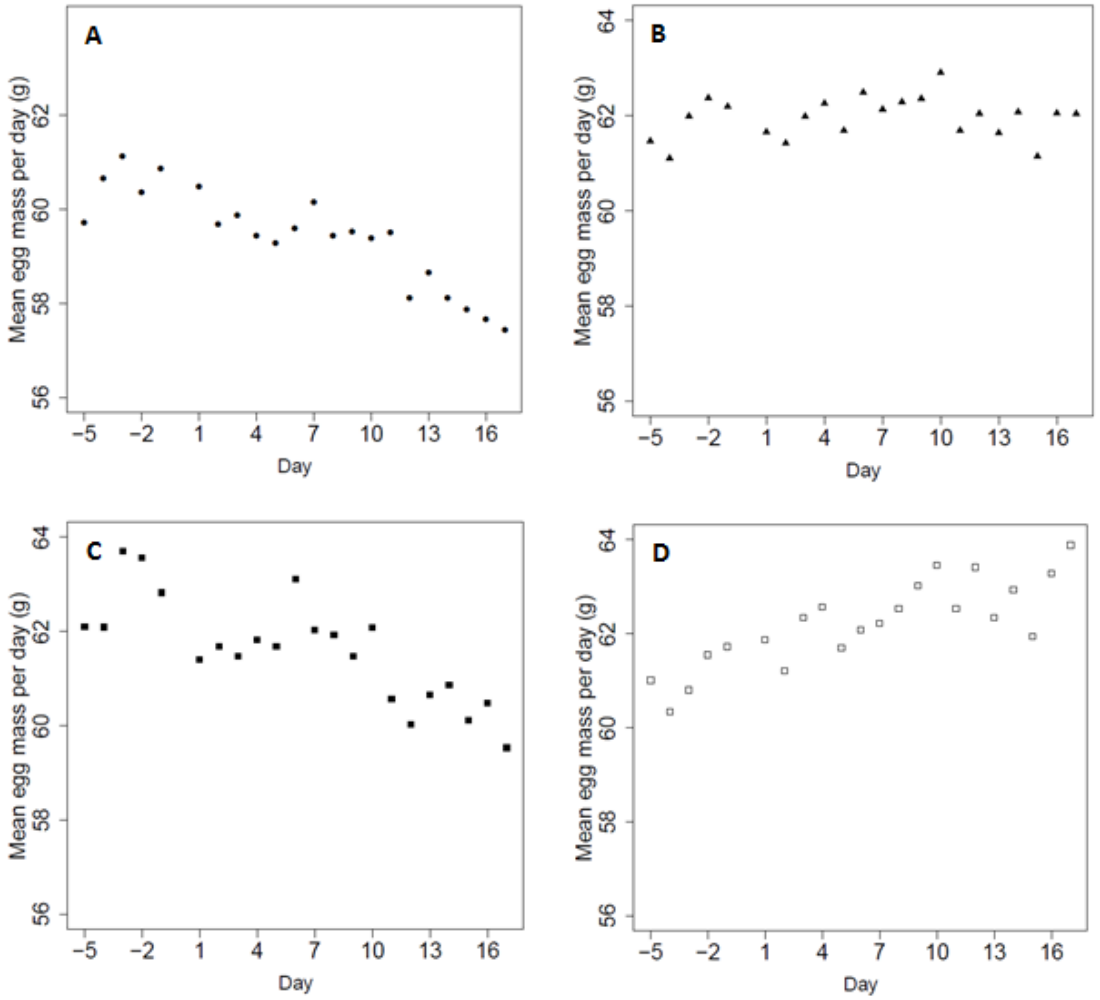


Figure 5.1. Mean egg mass per day (mean per group) as a function of day number. A) Feed restriction group (43 hens), B) Control group (43 hens). In the control group subpopulations were defined of hens showing a decrease (C, 18 hens) or increase (D, 25 hens) of egg mass over time.

5.3.3 Relationship between egg mass and egg sex

Statistical parameters for all tested effects and associations in this paragraph are shown in Table 5.1. In the feed restriction group, the effect of egg mass on sex of eggs was not significant for eggs laid in the treatment days (days 2-18) ($p = 0.16$), but was significant for eggs of days 9-18 (i.e. eggs that had meiosis in the period of more severe feed restriction) ($p = 0.02$). Likewise, the association of mean egg mass per hen with sex ratio per hen was not significant when considering all eggs (days 2-18) ($p = 0.26$), but was significantly negative for eggs laid on days 9-18 ($p = 0.009$, see Figure 5.2). The mean egg mass per hen before start of treatment (eggs laid on days –5 to –1) also tended towards 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 ($p = 0.14$). The decline of egg mass per hen (difference between the mean egg mass per hen of days −5 to −1 and the mean egg mass of days 9-18) was significantly negatively associated with the sex ratio per hen (for eggs laid on days 9-18) ($p = 0.01$). The association was not significant when relating the same measure of egg mass decline 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, $p = 0.50$).

In the control group, in the subpopulation of hens that showed a decrease of egg mass, there was a tendency towards a negative association between mean egg mass per hen and sex ratio per hen when considering all eggs (days −5 to18, $p = 0.06$), 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.

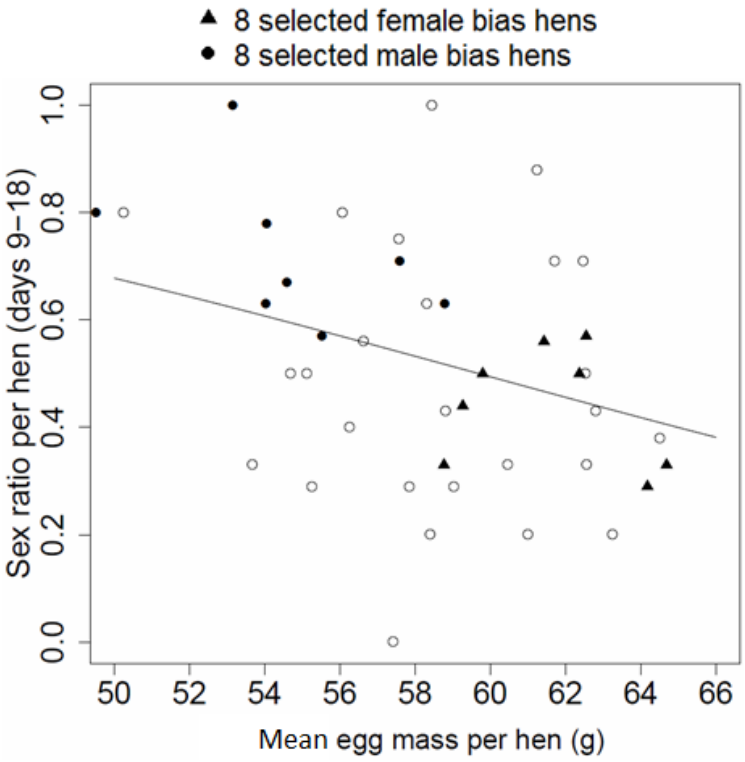


Figure 5.2: Graphical representation of the association of sex ratio (proportion of male eggs) per hen with mean egg mass per hen for the eggs of the last 10 days of treatment, days 9-18 (statistics given in Table 5.1), showing hens selected for the two groups to be compared in microarray transcriptome analysis, i.e. 'male biased hens with low egg mass' (solid circles) and 'female biased hens with high egg mass' (solid triangles).

5.3.4 Results from microarray studies

There were no significantly differentially expressed genes comparing the male sex ratio bias and female sex ratio bias groups of hens (results not shown). 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 5.2). The number of enriched processes (gene sets) found was much higher when the human database was used instead of the chicken database (Supplementary Table 5.1). Therefore the human database was used during the subsequent gene set enrichment analysis (GSEA). GSEA showed that cellular processes related to 'cell cycle progression', 'mitotic/meiotic apparatus' and 'chromosomal movement' had a significant (negative) normalized enrichment score (NES) (FDR < 0.25, (the default setting of GSEA)) for male sex ratio bias (Table 5.3). This means that these gene sets were enriched in genes that negatively correlated with sex ratio, i.e. the genes had a higher expression in hens

Table 5.1: Statistical parameters[1] of relations between egg mass and sex of egg or sex ratio. Upper panel: Relations within specific periods of treatment; prediction of sex of egg by egg mass, or associations between mean egg mass per hen and sex ratio per hen. Lower panel: Associations of sex ratio of eggs laid in specific periods with egg mass before treatment, or with decrease in egg mass during treatment. All data pertain to feed restriction group hens except where indicated otherwise.

	Sex of eggs predicted by egg mass					Sex ratio per hen associated with mean egg mass per hen			
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 18 [2]						16	−0.04	−1.82	0.06

	Sex ratio over period					
	days −5 to −1	days 2 to 18	days 9 to 18			
	p	p	df	est	z	p
Egg mass days −5 to −1			41	−0.04	−1.45	0.14
Δ egg mass [3]	0.50	0.10	**41**	**−0.14**	**−2.44**	**0.01**

[1] no. of observations (N), variance of the hen as random factor (var), degrees of freedom (df), estimate (est), z- and p- values of effect or association tested. Bold font indicates significance

[2] control group hens with decreasing egg mass

[3] (mean egg mass per hen days −5 to −1) − (mean egg mass per hen days 9 to18)

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 Supplementary Figure 5.1 showing 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 to 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 to 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 −5 to −1 and eggs of days 9 to 18). P-values for all correlations are given in supplementary Table 5.2, while

graphical representations are shown in Figure 5.3 (selected enriched gene sets) and in Supplementary Figure 5.2 (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 5.2) showed no correlation with sex ratio or mean egg mass per hen (Figure 5.4).

Table 5.2: Gene sets (general metabolic processes) significantly enriched in highly expressed (expression level >10) genes in the germinal discs of follicles obtained on the last day of the feed restriction treatment (day 16, 17 or 18).

Nr.[1]	Generalized Term[2]	Enrichment Score	p-value[3]
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

[1] Numbered in order of Enrichment Score. These numbers are used in the legend of Figure 5.4.

[2] DAVID functional annotation analysis using (default) human database, using data from all hens (male and female sex ratio bias groups together).

[3] p value for the comparison of mean gene expression per gene set of male biased versus female biased hens (no significant differences).

Table 5.3: Gene sets significantly enriched in genes that negatively correlated with sex ratio (significant negative NES score, FDR < 0.25), in the germinal discs of follicles obtained on the last day of the feed restriction treatment (day 16, 17 and 18).

Nr.[1]	Cellular process[2]	Size[3]	NES[4]	FDR[5]
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

[1] Numbered in order of FDR. These numbers are used in the legend of Supplementary Figure 5.2.

[2] Processes based on GO database unless KEGG database is indicated.

[3] Number of genes in the gene set.

[4] Normalized enrichment score.

[5] False discovery rate.

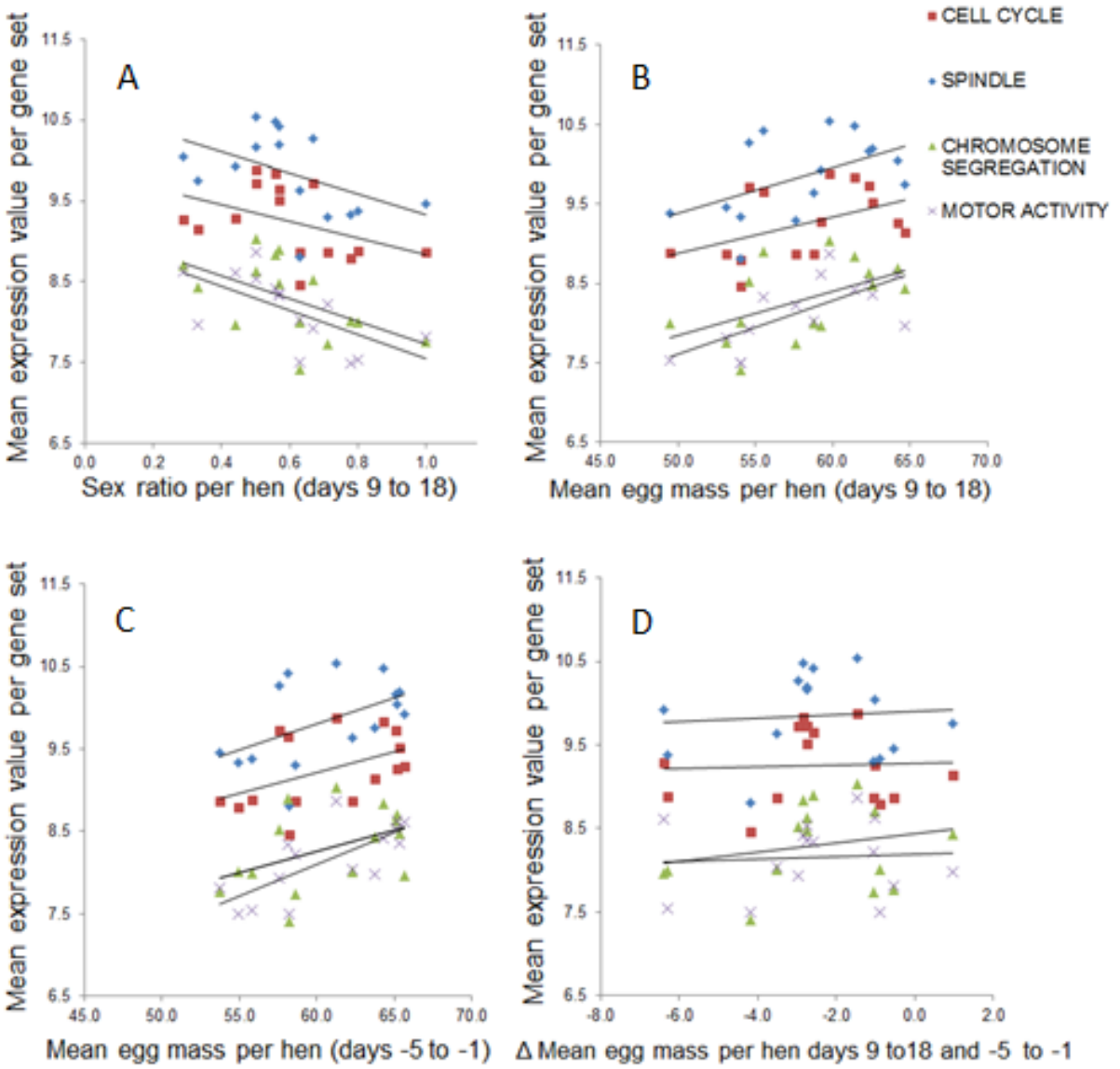


Figure 5.3: Mean expression levels per hen of gene sets that were significantly enriched with genes that negatively correlated with sex ratio (see Table 5.3) are shown as a function of the sex ratio (proportion of male eggs) per hen (eggs of days 9 to 18) (panel A), the mean egg mass per hen of eggs of days 9 to 18 (panel B) or of eggs of days −5 to −1 (panel C), or the mean decrease per hen of egg mass during feed restriction, i.e. between days −5 to −1 and days 9 to18 (panel D). All 15 significantly enriched gene sets show similar correlations but for clarity, four selected gene sets are shown. All gene sets are shown in Supplementary Figure 5.2.

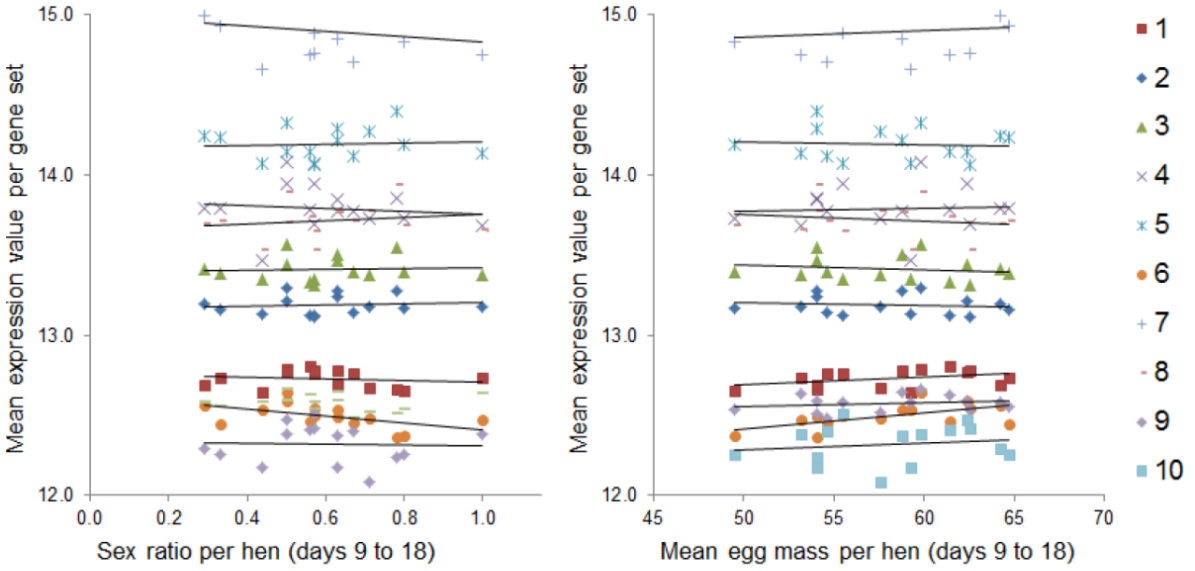


Figure 5.4: Mean expression levels per hen of gene sets of general metabolic processes (gene sets given in Table 5.2) are shown as a function of the proportion of male eggs per hen (eggs of days 9 to 18) (panel A), or the mean egg mass per hen of eggs of days 9 to 18 (panel B). Numbers 1 to 10 in the legend at the right side of the right panel identify the gene sets as listed in Table 5.2.

5.4 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 egg mass decline during treatment, which was significantly associated with the sex ratio of eggs of days 9-18. It may be that differences between hens in egg mass before start of treatment has contributed somewhat to this association as there was a tendency towards 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 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 [24, 26] 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 [24] the resulting shortage of males would make it more advantageous for the more 'economic' hens, i.e. 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 towards 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.

As explained in the chapter 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 sex ratio is determined (as reviewed by [46, 57, 74]. 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, as 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 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 [5, 8, 14, 15, 21, 57, 72, 74, 159, 198] 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 disc 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 disc 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 disc region. However, it is not self-evident that larger follicles would have more granulosa cells per surface area over the germinal disc. Also, granulosa cells stop proliferating and dividing (mitosis) toward completion of follicle growth [204]. Furthermore, gene expression of the highly expressed general metabolic processes was not correlated at all with either female bias or egg mass per hen. And lastly, the germinal disc of chicken and other avian oocytes contain very high amounts of RNA, several orders of magnitude (µg versus pg range, some five or six orders of magnitude) more than somatic cells [99, 199]. Therefore the contribution of granulosa cell mRNA to the germinal disc 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 [8, 72, 198]. 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. Although preliminary, the found differential enrichment of cell cycle related processes appears to be involved in meiotic drive, with a higher activity of these processes correlating with more female sex ratio bias.

5.5 Conclusion

Hens showed variation in their response to feed restriction; hens that responded by showing a stronger reduction of egg mass developed a male sex ratio bias during treatment and had relatively low expression of gene sets related to cell cycle in the follicle at the time of meiosis, while hens that showed less reduction of egg mass developed a female sex ratio bias

and had relatively high expression of these gene sets. The differentially expressed gene sets may be involved in meiotic drive regulating sex ratio in chicken in response to feed availability.

Additional files

Supplementary Table 5.1: Numbers of nodes and edges (interactions between genes) in gene networks constructed with BINGO[1,2], using either chicken or human annotation.

	Nodes	Edges
Chicken (gga)	162	285
Human (hsa)	415	684

[1] http://apps.cytoscape.org/apps/bingo

[2] Analyzing the 471 genes with highest gene expression (considering data from all 15 hens, i.e. both the female biased and the male biased hen group).

Supplementary Table 5.2: P values[1] of correlations (Pearson) between mean of the expression values of the core genes of significantly enriched cellular processes (see also Table 5.3) per hen and other egg parameters[2] per hen. These correlations are represented graphically in Supplementary Figure 5.2.

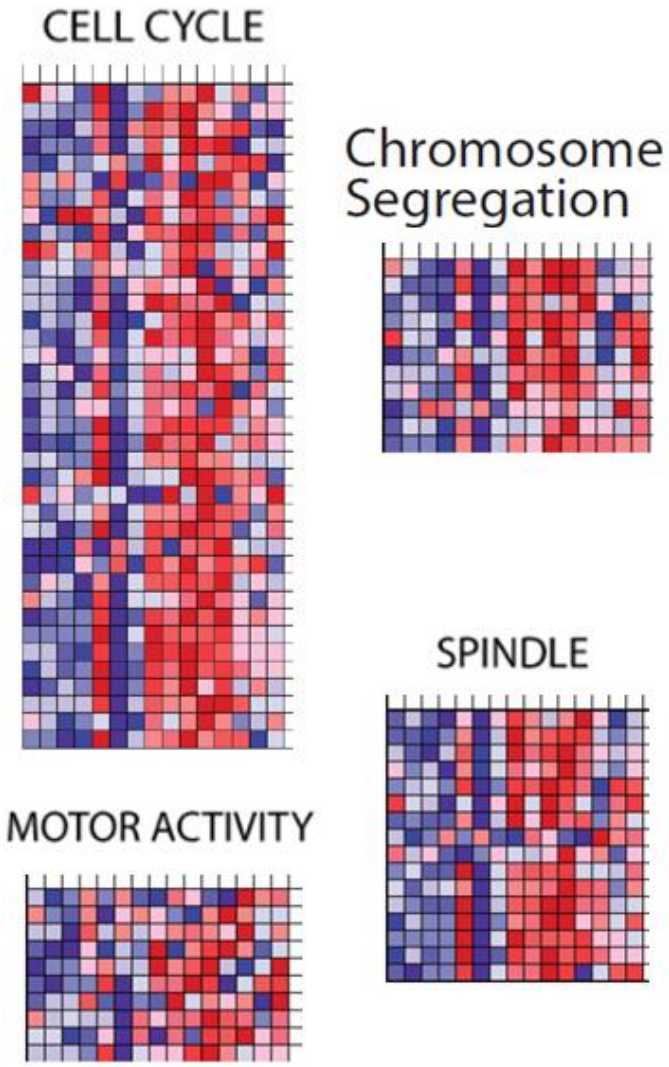
Nr.[3]	Cellular process[4]	S.R.	egg mass d9 to 19	egg mass d−5 to −1	Δ egg Mass
1	Cell cycle (KEGG)	0.11	0.08	0.07	0.86
2	M phase	**0.05**	**0.04**	**0.04**	0.75
3	Interphase	0.14	0.16	0.11	0.9
4	Cell division	0.08	**0.05**	**0.05**	0.85
5	Spindle	0.08	**0.05**	**0.05**	0.78
6	Cell cycle	**0.03**	**0.03**	**0.02**	0.9
7	Interphase of mitotic cell cycle	0.14	0.16	0.11	0.9
8	M phase of mitotic cell cycle	**0.02**	**0.02**	**0.02**	0.67
9	Motor activity	**0.01**	**0.003**	**0.002**	0.81
10	Small GTPase mediated signal transduction	**0.01**	**0.002**	**0.0005**	0.99
11	Homologous recombination (KEGG)	**0.03**	**0.04**	**0.01**	0.85
12	Regulation of mitosis	**0.05**	**0.04**	**0.04**	0.81
13	Cell cycle phase	**0.05**	**0.05**	**0.04**	0.84
14	Mitotic cell cycle	**0.03**	**0.04**	**0.03**	0.80
15	Chromosome segregation	**0.03**	**0.04**	**0.09**	0.40

[1] Bold P values are at or below 0.05

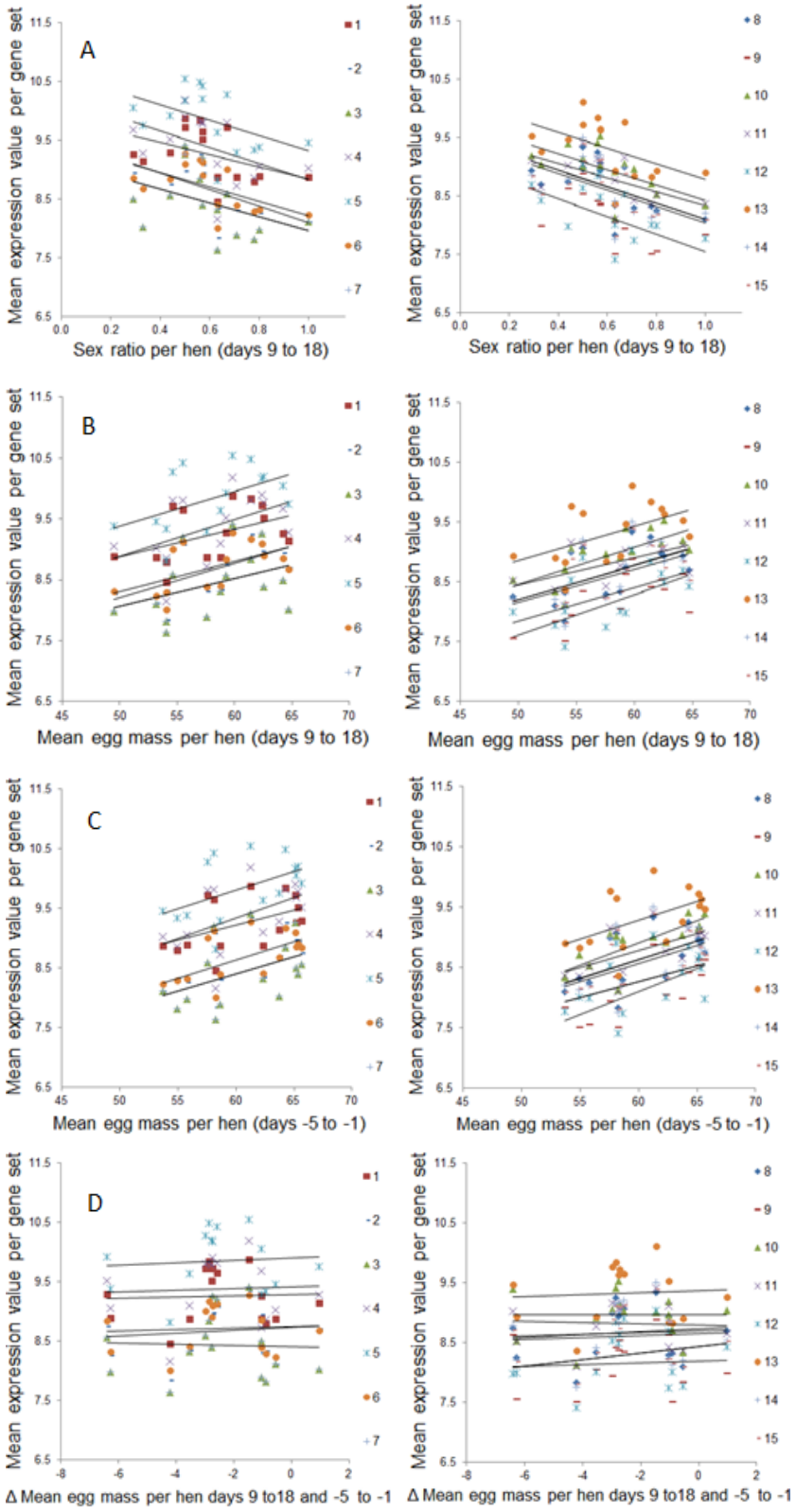
[2] S.R.: Sex ratio per hen (eggs of days 9 to 18); Mean egg mass per hen (days 9 to 18); Mean egg mass per hen (days −5 to −1); Δ egg mass: (mean egg mass per hen days −5 to −1) − (mean egg mass per hen days 9 to18)

[3] Ranked in order of FDR. These numbers are used in the legend of Supplementary Figure 5.2

[4] Processes based on GO database unless KEGG database is indicated.



Supplementary Figure 5.1: Heat maps showing expression levels of the core genes of selected significantly enriched gene sets (see Table 5.3), i.e. 'cell cycle', 'chromosome segregation', 'motor activity' and 'spindle'. Hens (columns) are arranged in decreasing order of sex ratio per hen, from left to right.



Supplementary Figure 5.2: Mean expression levels per hen of gene sets that were significantly enriched with genes that negatively correlated with sex ratio (see Table 5.3) are shown as a function of the sex ratio (proportion of male eggs) per hen (eggs of days 9 to 18) (panel A), the mean egg mass per hen of eggs of days 9 to 18 (panel

B) or of eggs of days −5 to −1 (panel C), or the mean decrease per hen of egg mass during feed restriction, i.e. between days −5 to −1 and days 9 to18 (panel D). Numbers in the figure (1-15) in the legend on the right side of each panel identify the processes as listed in Table 5.3.

Acknowledgements

We thank Hendrix Genetics B.V., The Netherlands for providing hens used in the experiment. We thank Rita Hoving-Bolink and Stephanie Vastenhouw for expert technical assistance. We thank Ministry of Economic Affairs, Agriculture and Innovation, The Netherlands for funding this project under the project number BO-12.02.002.7.01

6

Effect of corticosterone and hen body mass on primary sex ratio in laying hen (*Gallus gallus*), using unincubated eggs

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Biol Reprod. 2014 Apr 10;90(4):76.

Abstract

In various studies chronic elevation of corticosterone levels in female birds under natural or experimental conditions resulted in female biased offspring sex ratios. In chicken, one study with injected corticosterone resulted in a male sex ratio bias. In the current study, we chronically elevated blood plasma corticosterone levels through corticosterone feeding (20 mg/Kg feed) for 14 days using 30 chicken hens in each of treatment and control groups and studied the primary offspring sex ratio (here defined as the proportion of male fertile eggs determined in freshly laid eggs, i.e. without egg incubation). Mean plasma corticosterone concentrations were significantly higher in the treatment group but were not associated with sex ratio, laying rate, and fertility rate. Corticosterone treatment by itself did not affect egg sex, but affected sex ratio as well as laying rate and fertility rate in interaction with hen body mass. Body mass had a negative association with sex ratio, laying rate, and fertility rate per hen in the corticosterone group, but a positive association with sex ratio in untreated hens. These interactions were already seen when taking the body mass at the beginning of the experiment, indicating intrinsic differences between light and heavy hens with regard to their reaction to corticosterone treatment. The effects on laying rate, fertility rate, and sex ratio suggest that some factor related to body mass act together with corticosterone to modulate ovarian functions. We propose that corticosterone treatment in conjunction with hen body mass can interfere with meiosis, which can lead to meiotic drive and to chromosomal aberrations resulting in postponed ovulation or infertile ova.

Key words: Primary sex ratio, Female sex ratio bias, Unincubated eggs, Corticosterone feeding, Chicken, Body mass, Laying rate, Fertility rate

6.1 Introduction

In species with chromosomal sex determination, Mendelian sex chromosome segregation, leading to a primary sex ratio (the sex ratio after meiosis or fertilization) of 0.5, may be expected, provided that the costs of producing and raising sons or daughters are equal [24]. However, according to sex allocation theory, under certain conditions the sex ratio may depart from parity which may have selective advantage when costs of producing and rearing or the fitness returns from different sexes differ [24, 26, 27]. Sex ratio biases in relation to environmental or maternal factors have now been demonstrated in a wide variety of taxa (see references in [74].

Birds are an interesting case as the female is the heterogametic sex, therefore potentially in charge of determining the sex ratio of their offspring. Previous studies have demonstrated that the sex ratio in various birds species changed in response to several environmental cues such as diet quality and mate quality [12, 49, 50, 205] and maternal body condition [13, 43, 48, 102, 104, 105, 175] as predicted by sex allocation theory. Hormones could potentially mediate signalling between environmental cues and physiological mechanisms that lead to a primary sex ratio bias. Indeed, in birds under field or experimental conditions, correlations were seen between sex ratio and maternal blood levels of corticosterone [11-13] and testosterone [12-14]. In addition, other studies showed that experimental manipulation of progesterone [15], testosterone [13, 14, 16-18], and corticosterone [11, 12, 19-22, 45, 47, 74] induced sex ratio biases. Both corticosterone and body condition were shown to be associated with sex ratio either within the same experiment [13] or in different experiments (see references above). It is therefore not certain which of these two factors is the direct cause of sex ratio bias. The physiological mechanisms responsible for primary sex ratio bias in birds remain, however, unknown (see reviews [46, 57, 74, 206]). One of the proposed mechanisms is that these hormones influence sex chromosome segregation during meiosis (for more details see [8]).

Sex ratio studies focussing on corticosterone in birds have revealed inconsistent results as both male [22, 45, 47] and female [11, 12, 21, 74] biased offspring ratios have been reported in relation to elevated corticosterone. These contradictory results, with different birds species and using different methods of corticosterone treatment, demand further exploration. We used the domestic chicken for the following reasons. First, chronic treatment of corticosterone induced a female sex ratio bias in most studies (see references above). A short term

corticosterone treatment in chicken was found to induce a male bias sex ratio [47] but chronic corticosterone treatment was not studied in chicken. Thus, studying the effect of chronic treatment in chicken may help to disentangle whether the contradictory findings are due to treatment or species differences. Second, improved understanding of mechanisms underlying sex ratio biases in this species may have important implications for commercial poultry farming as it may lead to finding means to reduce the enormous number of male chicks born in the production of laying hens that in the current practise are killed after hatch. Third, studies in the chicken could generally give more insight in the biology of sex ratio regulation in birds since much is known about their physiology and genome.

Our study differs in several aspects from other studies. First, one explanation for the inconsistent results may be that chronic elevation of corticosterone by means of implantation studies induces very unnatural profiles of the hormone, with a peak of the hormone early after implantation, often far outside the physiological range for the species, subsequently followed by a strong decrease [74]. Corticosterone feeding in pigeons resulted in sustained elevated levels of blood corticosterone [150]. We therefore applied this method now in chicken using *ad libitum* access to feed mixed with corticosterone, with the aim to induce chronic elevation of circulating corticosterone concentrations, disrupting the natural correlation between maternal corticosterone and body condition.

Second, in all previous studies on corticosterone and sex ratio in birds, the sex ratio was determined by sexing embryos or hatchlings [11, 12, 15, 18, 21, 47, 48, 50, 191]. However, this means that effects on primary and secondary sex ratio cannot be distinguished in these studies. In our study we have looked at effects of corticosterone on the primary sex ratio (here defined as the proportion of male fertile eggs determined in freshly laid eggs, i.e. without egg incubation) using a recently developed technique of sexing unincubated chicken eggs [174]. To our knowledge, this is the first study to examine the effects of corticosterone on primary sex ratio determined in unincubated eggs of any birds species. We studied the relations between treatment, hen blood plasma corticosterone concentration, fertility rate, laying rate, hen body mass and egg sex ratio per hen to analyse possible underlying mechanisms for sex ratio bias.

6.2 Materials and Methods

6.2.1 Housing conditions, inseminations, corticosterone feeding, body mass measurements 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 2010076). Sixty hens and 15 cocks of a brown layer line, kindly provided by ISA BV, the layer breeding division of Hendrix Genetics, and 40 weeks of age, were housed individually in large ground cages, provided with litter and a roosting bar, with ad lib access to feed and water and a light-dark programme of 16h-8h at 25 °C. Hens were randomly assigned to the control and corticosterone feeding groups, with 30 hens per group. The hens had visual and audio contact with each other and only audio contact with the cocks. The cocks were trained for semen collection and the hens were artificially inseminated twice weekly with a volume of 0.2 ml of 4 times diluted semen during the entire experiment, starting after start of acclimatisation. This means that the third insemination occurred three days before start of egg collection, i.e. the sperm reservoir had been established, well before ovulation of the first collected egg. Defining the day of start of corticosterone treatment as day 0 (treatment started at 1600 h), acclimatization to individual housing began on day –9. Eggs laid on day 0 were not considered for analysis as the eggs were laid before starting the treatment. As it is assumed that corticosterone could affect the sex ratio by affecting meiosis [47], and the latter takes place and is completed about 26 to 28 hours before oviposition [47, 64], therefore, the egg laid on day 1 were not considered for analysis as those had meiosis before starting treatment. Treatment was implemented from day 0 to 13 (14 days) and eggs were collected from day 2 to 13 (12 days). The corticosterone group hens were fed corticosterone mixed feed (one kilogram feed contained 20 mg of corticosterone powder preparation from Sigma company, C2505-500MG) under ad libitum conditions. The dosage of corticosterone per kg feed was determined after a pilot experiment with different dosages (30 mg and 40 mg corticosterone per one kilogram feed). The dosage of corticosterone per kg feed was determined after a pilot experiment with different dosages (30 mg and 40 mg corticosterone per one kilogram feed) in the range of corticosterone values used in earlier studies (e.g. Goerlich et al. 2009 [150]). We found that 30 or 40 mg corticosterone per kg of feed did not affect the fertility rate but resulted in a decrease in laying rate of hens. Therefore, we chose a lower level of corticosterone (20 mg/per kilogram of feed) for the final experiment. Eggs were collected in

the morning and marked with date and hen number and directly stored at 17 °C until processing the same day or the next day. A total of 299 (83.06% laying rate) and 353 (98.06%) laying rate) eggs were laid by corticosterone and control group respectively during the 12 days period of egg collection (days 2-13). Laying rate in both groups during initial days of start of treatment was very high and of the same order of the laying rate of the control hens during the experiment (98%). In the corticosterone group, the laying rate was still high in days 2-5 (97%) gradually declining on later days to finally reach a laying rate of 67% in days 10-13. Fertility status and sex of all the eggs laid were successfully determined except for 7 eggs in the corticosterone group and 9 eggs in the control group. Fertility rates (fraction of fertile eggs i.e. number of fertile eggs divided by the total number of eggs; see section below for method to determine fertility of eggs) in the corticosterone and control group were 70% and 82% respectively.

Body mass of all hens were measured at the start (i.e. day –9 of treatment) and the end of the experiment (day 13 of treatment).

6.2.2 Blastodisc isolation and sexing using PCR

Blastodiscs were isolated using a technique similar to that described previously [160]. Isolated clean blastodiscs free of yolk were stained with Hoechst 33342 for determining fertility of egg as described previously [174]. Then, the blastodiscs of fertile eggs were suspended in approximately 20 µl of PBS and frozen at –20 ℃ until use for nucleic acid extraction. The sex of the blastodiscs was determined according to the method described previously [174]. In brief, nucleic acids were isolated from blastodisc samples using proteinase K digestion as described previously [161]. DNA pellets were washed in 70 % ethanol, air dried and dissolved in 25 µl of RNAse free water. Subsequently 20 ng of nucleic acid was used in the polymerase chain reaction to amplify the *CHD1* gene using primer pair 2550F/2718R [148]. *CHD1* gene fragments of 450 bp (*CHD1-W*) and 600 bp (*CHD1-Z*) are amplified from the W- and Z- chromosome specific CHD1 genes, respectively [148].

6.2.3 Blood plasma sample collection

Blood samples were obtained to determine circulating levels of corticosterone in corticosterone group hens on days 5, 7 and 10 and in the control group hens on day 10. To minimize handling stress, blood was obtained from different subsets of ten hens per sampling

day, so each hen was sampled only once. Blood samples were collected at 11:00 h AM by brachial venipuncture within 3 minutes after catching the birds to avoid elevations of corticosterone levels due to stress response. Approximately one ml of blood was collected in heparinized blood collection tubes and kept on ice until isolation of plasma. Plasma was separated from blood samples by spinning at 2000 rpm for 10 minutes. Isolated plasma samples were frozen at –70 °C until use for hormone concentrations determinations.

6.2.4 Corticosterone hormone analysis

Corticosterone extraction from plasma samples was according to procedures as described by Goerlich *et al.* [14]. To calculate recoveries, 2500 cpm of radioactive labelled corticosterone was added to all samples. Briefly, double dichloromethane extraction was followed by single methanol extraction in which 1 ml of 70% methanol was added to extracts, vortexed and placed at –20°C overnight. The following morning, samples were centrifuged for 5 minutes at 2000 rpm at 4°C and methanol phase was decanted and dried under a nitrogen evaporator. The residue was resuspended in 300 µl PBS. PBS used was prepared after mixing 5.3 g of NaH_2PO_4 H_2O, 16.35 g of Na_2HPO_4 $7H_2O$, 9 g of NaCl and 1 g of gelatin in upto 1000 ml of distilled water. One gram of NaN_3 was mixed in 1000 ml of prepared PBS and pH of the solution was adjusted to 7.10 using NaOH. Recoveries were on average 77%±5.3. Corticosterone concentrations in plasma samples were measured using enzyme immunoassay (EIA) as described previously [144] using kit 'ImmuChem Double Antibody Corticosterone (07-120102)' from MP Biomedicals Germany. The assay was validated for parallelism by measuring serial dilutions of plasma samples containing high concentrations of corticosterone. We calculated the coefficients of variation (CV) based on assay controls and standard curves. All the samples were measured in one RIA test and average intra-assay CVs was 3.7 %.

6.2.5 Statistical analysis

Statistical analysis was performed using software R2.12.2 developed by R Development Core Team, 2011 [200]. Normal distribution of data was confirmed. Differences in corticosterone concentration between the treatment and control group and between different days of sampling in the treatment group were tested using independent and paired t-tests respectively. The effect of treatment on the change in hen body mass between day of start (day –9) and end

(day 13) of the experiment was tested with mixed effects modelling and hen as random factor, using nlme (linear and nonlinear mixed effects) package.

Because the effect of the corticosterone treatment could build up over time, the interaction of treatment with day of treatment was tested for predicting the sex, laying status and fertility status of each egg using logistic regression and hen as random factor with lme4 package. To test the main effect of the treatment, the interaction term and day of treatment were removed from the model.

In addition to these analyses we analysed whether the egg parameters (see below) are affected by other predictors, such as body mass and corticosterone levels that were measured only at a particular day and the corticosterone treatment. Therefore, instead of predicting single egg parameters as described above, we had to use as dependent variables egg parameters averaged over all eggs per hen for all her eggs collected during the 12 days of the egg collection period (days 2 to 13). These included: Overall egg sex ratio per hen (fraction of male eggs per hen i.e. number of male eggs divided by number of eggs sexed), the overall laying rate per hen (fraction of laid eggs per hen i.e. number of egg laid divided by number of days of egg collection), the overall fertility rate per hen (fraction of fertile eggs per hen i.e. number of fertile eggs divided by the total number of eggs laid by that hen). Using these calculations, we first tested in stepwise backward regressions the interaction of the treatment times hen body mass (body mass at either day −9 or day 13 of experiment) for predicting overall egg sex ratio per hen, laying rate per hen and fertility rate per hen using logistic regression. Additionally, we used instead of body mass at day −9 or 13, the change in body mass between these days. We initially also tested for the three way interaction between treatment, time, and (change in) body mass, but these never approached significance and were left out from further analyses. To study the direction and strength of the associations for each treatment group, the association of (the change in) hen body mass with overall sex ratio per hen, laying rate, and fertility rate per hen were tested for data of each treatment group separately.

Hen body mass at day 13 significantly negatively predicted both the laying and fertility rate per hen as well as sex ratio per hen in the corticosterone group (see result section). This suggested that effects of hen body mass on sex ratio could be due to its effects on laying and fertility. Indeed it has been suggested that sex ratio biases might come about by sex specific follicle abortion or sex specific fertilization [46, 57]. Therefore, using stepwise backward logistic regression, in the corticosterone group we tested the interaction of hen body mass

with laying rate per hen and fertility rate per hen, respectively, for overall sex ratio per hen. To study the main effects of laying and fertility rates on sex ratio, the interaction term and hen body mass at day 13 were subsequently removed. To study these main effects in the control group, the associations of laying and fertility rate per hen itself with overall sex ratio per hen were also studied in the control group. Using the data of the corticosterone group, the interaction of change in hen body mass (between days –9 and 13) with laying and fertility rate per hen was also tested for explaining variation in sex ratio per hen.

To disentangle the effect of experimentally elevated circulating corticosterone concentration and body mass on sex ratio, laying rate and fertility rate, effects of plasma corticosterone concentrations of the corticosterone group hens sampled at day 7 and 10 (when elevated levels of corticosterone were seen) in interaction with hen body mass at the end of experiment (day 13) were studied on overall egg sex ratio per hen, laying rate per hen, and fertility rate per hen using logistic regression models that also included day of blood sampling as covariate. The effects of each of hen body mass and plasma corticosterone concentrations (with day of sampling as covariate) on egg sex ratio per hen, laying rate per hen, and fertility rate per hen were also tested separately in separate models. Additionally, using the data from same hens, we also tested for the effect of change in body mass (between days –9 and 13) by itself on egg sex ratio per hen, laying rate per hen, and fertility rate per hen or interaction with corticosterone concentrations.

6.3 Results

6.3.1 Effect of treatment on plasma corticosterone and change in body mass

Plasma corticosterone concentrations significantly increased during corticosterone feeding treatment. In the corticosterone group, the corticosterone concentrations were significantly higher at days 7 ($p = 0.02$) and 10 ($p = 0.008$) compared with day 5. The level of the corticosterone fed hens on day 5 was similar to that of the control hens (on day 10) (see Figure 6.1). The body mass of the hens at the start of the experiment (day –9) was 1802±119 and 1798±108 g for control and corticosterone groups, respectively (mean ± s.d.). The average increase in hen body mass (± s.d.) from day –9 to day 13 was 80.6±60.9g and 52.3±50.7g in the corticosterone and the control groups respectively, with a significant positive effect of treatment (No. of observations = 58, estimate = 28.27, $z = 1.92$, $p = 0.05$).

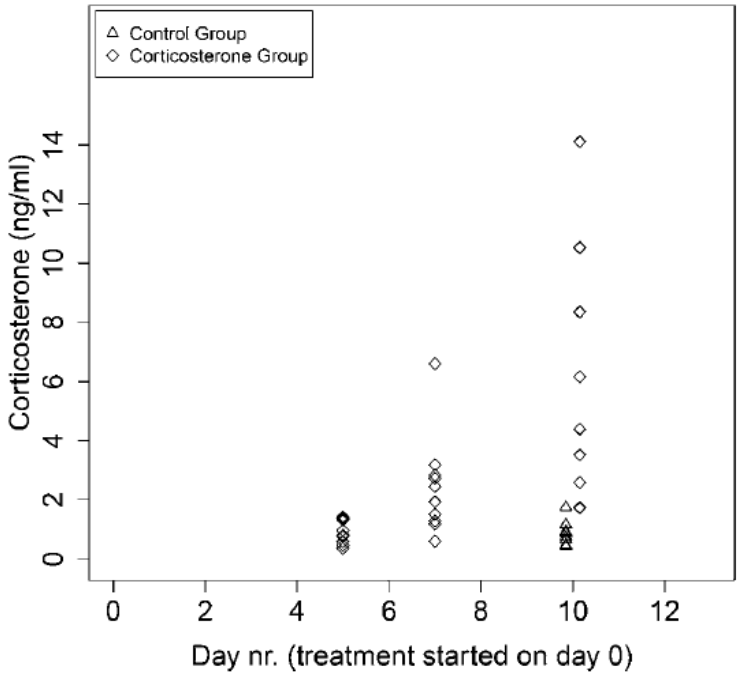

Figure 6.1. Blood plasma corticosterone concentrations in corticosterone group hens on days 5, 7 and 10, and in control group hens on day 10. To minimize handling stress, blood was obtained from different subsets of ten hens per sampling day. In the corticosterone group, the corticosterone concentrations were significantly higher at days 7 ($p = 0.02$) and 10 ($p = 0.008$) compared with day 5. The level of the corticosterone fed hens on day 5 was similar to that of the control hens (on day 10).

6.3.2 Effect of treatment in association with day of treatment on egg laying probability per day, and fertility status and sex of the eggs

6.3.2.1 Egg laying probability

The interaction of treatment times day of treatment significantly predicted egg laying probability per day (No. of observations = 706, estimate = –0.24, z = –1.96, p = 0.04). After removing day and interaction of day with treatment, treatment significantly negatively predicted the egg laying probability per day (No. of observations = 706, estimate = –2.14, z = –4.77, $p < 0.0001$).

6.3.2.2 Fertility status of egg

The interaction of treatment times day of treatment did not significantly predict fertility status of each egg (No. of observations = 636, estimate = –0.07, z = –1.21, p = 0.22). After removing day and interaction of day with treatment, treatment significantly negatively

predicted the fertility status of each egg (No. of observations = 636, estimate = –0.94, z = –2.15, p = 0.03).

6.3.2.3 Sex of egg

The sex ratio calculated over all the 12 days of egg collection of all 30 hens per group was 0.54 and 0.55 in the treatment and control groups respectively. Although corticosterone concentration in the treatment group increased over time (see above), the interaction of treatment times treatment day did not significantly predict sex of egg (No. of observations = 458, estimate = –0.02, z = –0.49, p = 0.62). After removing day and interaction of day with treatment, treatment did not significantly affect sex of egg (df = 58, estimate = –0.01, z = –0.07, p = 0.94).

6.3.3 Effect of treatment and body mass on overall laying rate, fertility rate and sex ratio per hen

6.3.3.1 Laying rate per hen

The interaction of treatment times hen body mass at the start (day –9, df = 56, estimate = –0.007, z = –2.12, p = 0.03) and body mass at the end of experiment (day 13, df = 56, estimate = –0.008, z = –2.61, p = 0.008) was significant for predicting the overall laying rate per hen (calculated over all eggs from days 2 to 13). Testing for both groups separately, hen body mass at day –9 was not significantly associated with overall laying rate per hen in the corticosterone group (df = 28, estimate = –0.001, z = –1.08, p = 0.27) and control group (df = 28, estimate = 0.005, z = 1.84, p = 0.06). While hen body mass at day 13 was significantly negatively associated with overall laying rate per hen in the corticosterone group (df = 28, estimate = –0.002, z = –2.72, p = 0.006), and not in the control group (df = 28, estimate = 0.005, z = 1.83, p = 0.06) (see Figure 6.2A for associations for body mass at day 13). Change in body mass per hen from day –9 to day 13 did not significantly affect overall laying rate per hen, neither in interaction with treatment nor by itself in any of the groups.

6.3.3.2 Fertility rate per hen

The interaction of treatment times hen body mass at the start of the experiment was almost significant (day –9, df = 56, estimate = –0.003, z = –1.79, p = 0.07) while significant for the

body mass at the end of the experiment (day 13, df = 56, estimate = –0.003, z = –2.25, p = 0.02) for predicting overall fertility rate per hen (calculated over all eggs from days 2 to 13). Testing for both groups separately, hen body mass was significantly negatively associated with overall laying rate per hen within the corticosterone group (for body mass day –9: df = 28, estimate = –0.004, z = –3.36, p = 0.0007 and for body mass day 13: df = 28, estimate = –0.004, z = –4.26, p < 0.0001) and this association was not significant in the control group (for body mass day –9: df = 28, estimate = –0.001, z = –0.94, p = 0.34 and for body mass day 13: df = 28, estimate = –0.0008, z = –0.82, p = 0.40) (see Figure 6.2B for associations for body mass day 13). Change in body mass per hen from day –9 to day 13 did not significantly affect overall fertility rate per hen, neither in interaction with treatment nor by itself in any of the groups.

6.3.3.3 Sex ratio per hen

The interaction of treatment times hen body mass at the start (day –9, df = 56, estimate = –0.004, z = –2.38, p = 0.01) and end of experiment (day 13, df = 56, estimate = –0.004, z = –2.86, p = 0.004) was significant for predicting the overall egg sex ratio per hen. Testing for both groups separately, hen body mass at the start of the experiment (day –9) was not significantly associated with overall egg sex ratio per hen in the corticosterone group (df = 28, estimate = –0.001, z = –1.13, p = 0.25) but significantly positively associated in the control group (df = 28, estimate = 0.002, z = 2.29, p = 0.02). For body mass at the end of the experiment (day13), this association was significantly negative in the corticosterone group (df = 28, estimate = –0.002, z = –2.39, p = 0.01) and not significant in the control group (df = 28, estimate = 0.001, z = 1.66, p = 0.09) (see Figure 6.2C for associations at the end of the experiment, day 13). Change in body mass per hen did not significantly affect overall egg sex ratio per hen, neither in interaction with treatment nor by itself in any of the groups.

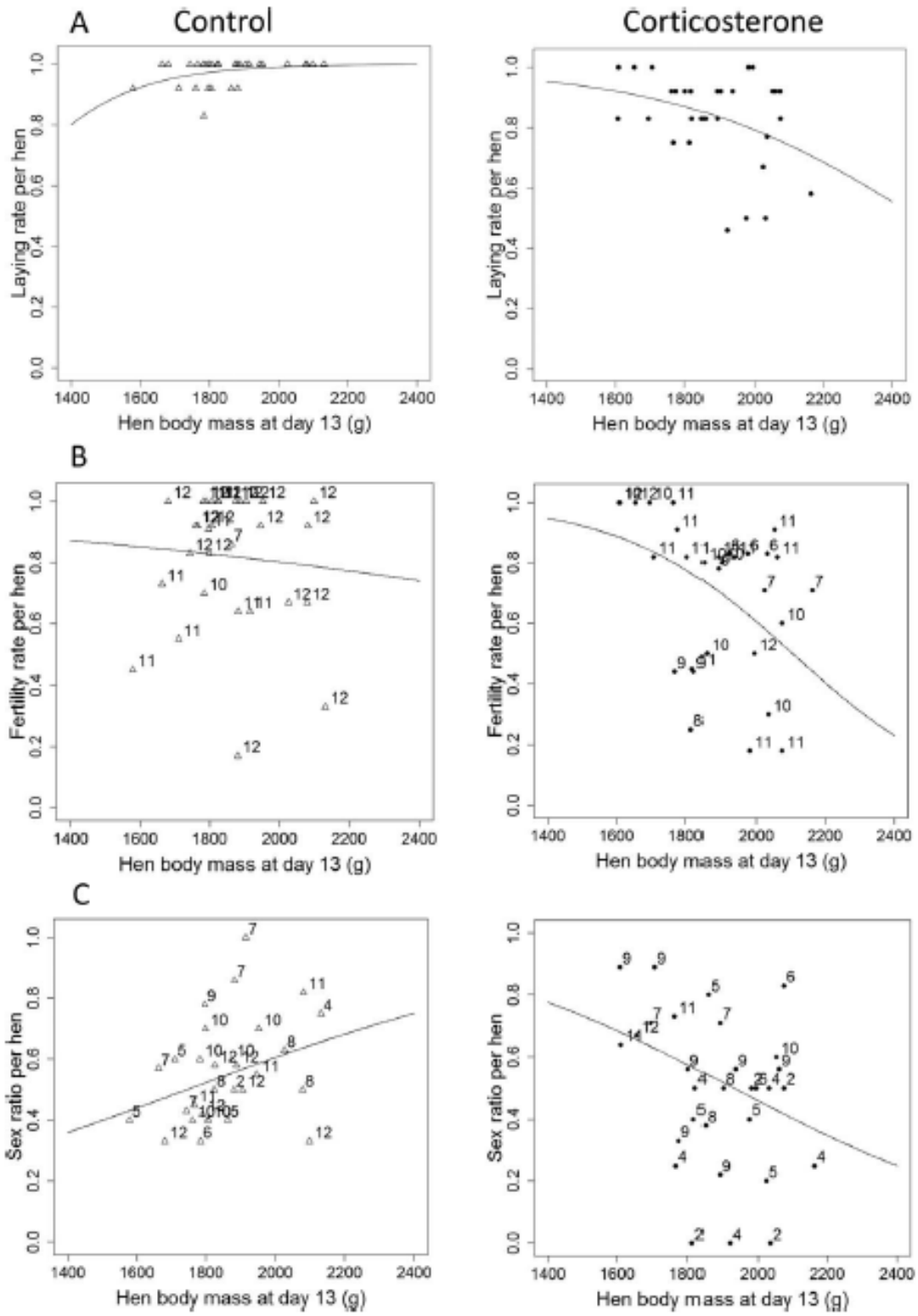

Figure 6.2. Graphs showing associations of hen body mass (day 13) with egg laying rate (A), fertility rate (B), and sex ratio per hen (C), in control (left panel) and corticosterone (right panel) groups. Lines in the graphs are trend lines from the statistical models. Eggs had been collected on 12 days (days 2 to 13). Numbers in figures B and C represent the numbers of eggs per hen with known fertility status and number of sexed eggs per hen, respectively.

6.3.4 Relation of laying rate and fertility rate with egg sex ratio in association with hen body mass

The fact that overall laying rate, fertility rate, and sex ratio per hen (see result section 3.3 above) were all three negatively associated with hen body mass at day 13 in the corticosterone group suggested that sex ratio is also directly associated with laying rate and fertility rate per hen. Therefore, sex ratio per hen in the corticosterone group was predicted by laying rate or fertility rate per hen and their interaction with body mass in separate stepwise backward regression models (Table 6.1). For both laying rate and fertility rate per hen the interaction was not significant. After removing the interaction from each model, the main effect of hen body mass was not significant in each model and a significant positive association was found for laying rate per hen. After further removing the hen body mass from each of the models, each of laying and fertility rate per hen was significantly positively associated with egg sex ratio per hen in the corticosterone group (see Table 6.1). For the control group we have separately tested the main effect of laying rate and fertility rate per hen on egg sex ratio per hen and these were non-significant (laying rate per hen: df = 28, estimate = 2.12, z = 0.67, p = 0.50 and fertility rate per hen: df = 28, estimate = –1.82, z = –1.28, p = 0.19). See Figure 6.3 for association of overall sex ratio per hen with overall laying rate (panel A) and fertility rate per hen (panel B) in the corticosterone and control groups. The interaction of the change in body mass per hen with either fertility rate per hen or laying rate per hen did not significantly predict the sex ratio per hen.

Table 6.1. Stepwise backward regression of statistical models to explain variation in sex ratio of the corticosterone group hens on the basis of hen body mass at day 13 (BM), and laying rate (LR) or fertility rate (FR).

Explaining model contains[1]	Explaining variable	Statistical parameters for effect of explaining variable on sex ratio			
		Estimate	df [2]	z	p
BM + LR + (BM x LR)	(BM x LR)	−0.0008	26	−0.10	0.91
BM + LR	BM	−0.001	27	−1.48	0.13
BM + LR	LR	2.39	27	1.93	0.05*
LR	LR	3.06	28	2.65	0.008**
BM + FR + (BM x FR)	(BM x FR)	−0.006	26	−1.25	0.21
BM + FR	BM	−0.001	27	−1.58	0.11
BM + FR	FR	1.05	27	1.28	0.19
FR	FR	1.63	28	0.74	0.02*

[1] The full (starting) models contain BM and LR and their interaction (BM x LR) (upper model), or BM and FR and their interaction (BM x FR) (lower model). Subsequently, simpler models are tested as well, after removing insignificant (p>0.05) items.

[2] Degrees of freedom

* Significant p <0.05

** Significant p <0.01

6.3.5 Disentangling effects of corticosterone and body mass

Using the data of day 7 and 10 blood sampled hens from the corticosterone group, the interaction of plasma corticosterone concentration times hen body mass (day 13), was not significant for predicting overall, laying rate, fertility rate, and sex ratio per hen (see Table 6.2). After removing the interaction, hen body mass significantly negatively predicted overall laying rate, fertility rate, and sex ratio per hen. Corticosterone plasma concentrations showed a marginally significant (positive) effect only on fertility rate, which would not hold when correcting for testing three dependent variables. When testing effects of each of the hen body mass and corticosterone concentration on overall laying rate, fertility rate, and sex ratio per hen in separate models, similar effects were seen as in the combined model (see Table 6.2). Using the data from the same hens, change in body mass per hen did not significantly affect overall laying rate, fertility rate, and sex ratio per hen, neither in interaction with corticosterone concentration nor by itself.

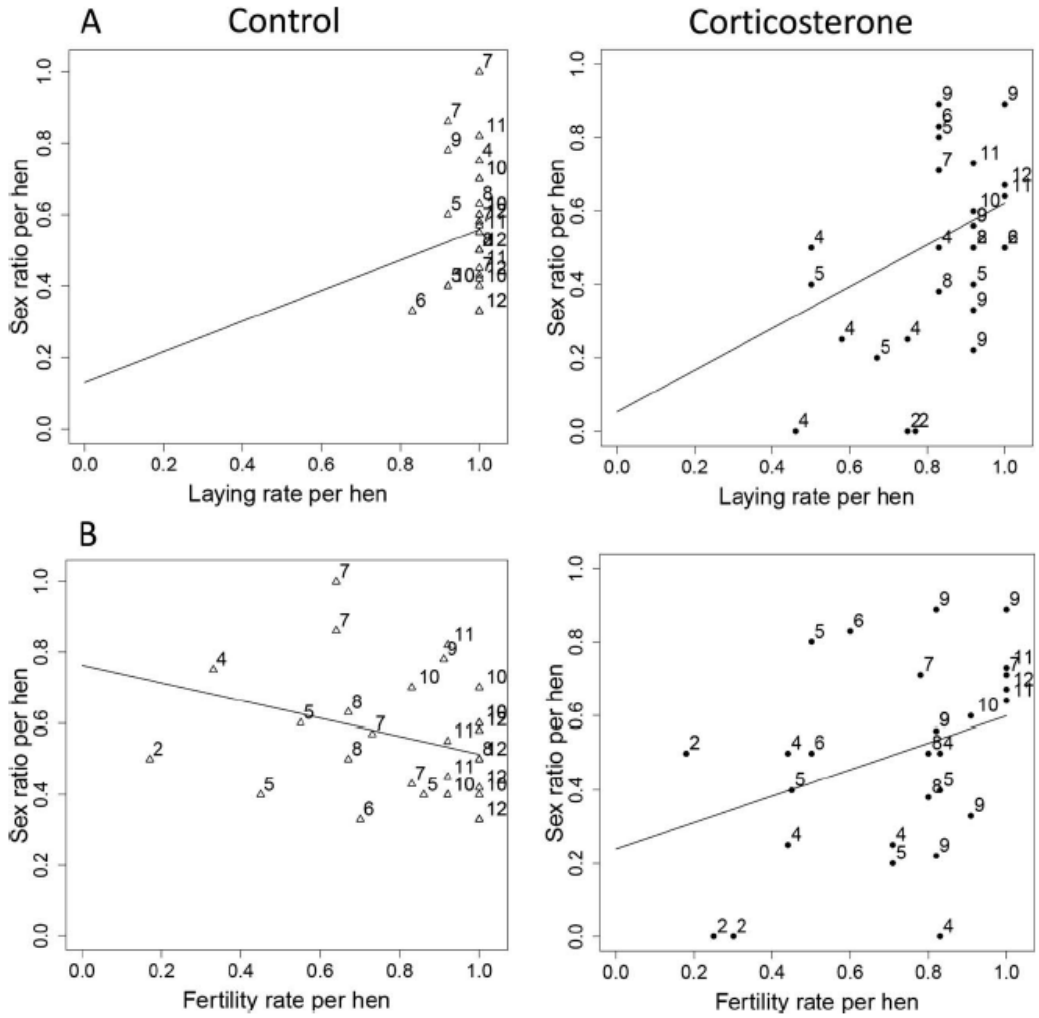

Figure 6.3. Graphs showing associations of egg sex ratio per hen with laying rate (A) and fertility rate per hen (B) in control (left panel) and corticosterone (right panel) groups. Lines in the graphs are trend lines from the statistical models. Numbers in the graph are total number of sexed eggs per hen.

Table 6.2. Stepwise backward regression of statistical models to explain variation in sex ratio (SR), laying rate (LR), or fertility rate (FR) of the corticosterone group hens on the basis of hen body mass at day 13 (BM), and plasma corticosterone concentration (Cort).

Explained variable	Explaining model contains[1]	Explaining variable	Statistical parameters for effect of explaining variable on explained variable			
			Estimate	df[2]	z	p
SR	BM + Cort + (BM x Cort)	(BM x Cort)	0.0002	15	0.54	0.58
	BM + Cort	BM	–0.003	16	–2.38	0.01*
	BM + Cort	Cort	0.02	16	0.48	0.63
	BM	BM	–0.003	18	–2.30	0.02*
	Cort	Cort	0.03	17	0.70	0.47
LR	BM + Cort + (BM x Cort)	(BM x Cort)	–0.0004	15	–0.63	0.52
	BM + Cort	BM	–0.003	16	–2.46	0.01*
	BM + Cort	Cort	0.06	16	0.91	0.36
	BM	BM	–0.003	18	–2.76	0.005**
	Cort	Cort	0.06	17	1.01	0.31
FR	BM + Cort + (BM x Cort)	(BM x Cort)	–0.0004	15	–1.23	0.21
	BM + Cort	BM	–0.003	16	–2.85	0.004**
	BM + Cort	Cort	0.10	16	1.94	0.05*
	BM	BM	–0.002	18	–2.48	0.01*
	Cort	Cort	0.11	17	2.05	0.03*

[1] The full (starting) model contained BM and Cort and their interaction (BM x Cort). Subsequently, simpler models were tested as well, after removing the interaction (as it was not significant). In addition, effects of BM and Cort were also tested in separate models (containing only BM or Cort as explaining variable).

[2] Degrees of freedom

* Significant p <0.05

** Significant p <0.01

6.4 Discussion

This is the first study in which the effect of corticosterone on primary sex ratio is tested using successfully a technique to sex unincubated eggs. In addition, in contrast to other studies, we manipulated corticosterone via food, resulting in a gradual increase of circulating levels of corticosterone within the physiological range. Overall, the sex ratio was not different in control and corticosterone groups, but the association of sex ratio with body mass was

different in the two groups (see Figure 6.2C), i.e. corticosterone treatment did affect sex ratio in interaction with hen body mass. In the corticosterone group, body mass had a negative association with sex ratio, laying rate, and fertility rate per hen (and laying rate and fertility rate were positively associated with sex ratio), i.e. heavy hens tend to perform poor with respect to laying rate and fertility rate, and tend to have a female sex ratio bias, when treated with corticosterone.

In our experiment, the corticosterone treatment by itself did not affect sex of egg. In a number of previous studies in other bird species a main effect of corticosterone was reported. In these studies, chronic corticosterone treatment by either silastic implants or by feeding corticosterone the way we did resulted in a lower sex ratio [74]. In addition, plasma corticosterone concentration was negatively associated with offspring sex ratio in manipulated or unmanipulated birds [11-13, 21, 150], but see [22]. Another difference between our study and these previous studies is that we found a decrease in laying and fertility rates as a main effect of corticosterone treatment, with a clear positive association of laying and fertility rate (per hen) with sex ratio per hen, which was not found in these previous studies. In our study we have used commercial layer hens. These animals have been intensively selected for laying very long series of eggs, with one egg a day, pushing events related to ovulation, fertilization and egg production towards the limit. Therefore any change of ovarian physiological function (discussed below) could perhaps more easily result in lowered laying rate and fertility rate in our laying hens than in the other species mentioned above.

In untreated hens (control) we found a positive relation between hen body mass and sex ratio. Also Parker [175] reported a positive association of sex ratio with hen body condition in chickens under unmanipulated conditions. The positive association of body condition with offspring sex ratio is in line with the sex allocation theory. The chicken is a sexually dimorphic species and females are the smaller -less costlier- sex [108] and have lower reproductive variance than males [5, 109]. According to the sex allocation theory, in such species, a positive association may be expected between female body conditions and sex ratio [26]. This was actually found in a number of bird species [13, 48, 53, 102, 104-106, 175, 194].

In our experiment, corticosterone treatment affected sex ratio as well as laying rate and fertility rate per hen in interaction with hen body mass. In this treatment group body mass was negatively associated with sex ratio, fertility rate and laying rate per hen, a negative main

factor effect of treatment was also found on the latter two. The interaction of treatment with hen body mass for predicting the sex ratio per hen was already seen when taking the body mass before the start of the treatment in the analyses (day −9). This indicates that there were intrinsic differences between light and heavy hens with regard to their reaction to the corticosterone treatment. This suggests that some factor related to body mass act together with corticosterone to modulate the ovarian functions, perhaps because both the hormone and body mass affect metabolic processes. Change in body mass between day of start and end of experiment was not associated with sex ratio, fertility rate and laying rate per hen, though treatment positively affected the change in body mass. However, interactions were stronger (lower p values) when taking body mass at end day 13 as compared to body mass at start of the experiment.

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 as described by [19, 20]. In the corticosterone group, body mass was negatively associated with sex ratio and also with laying rate and fertility rate per hen. This suggests that these three parameters are connected at the level of ovarian physiology. A hypothesis for the mechanism for the effect on sex ratio should explain at the same time also the effects on laying rate and fertility rate per hen. In mammals, effects of glucocorticoids were reported to interfere with meiosis in pigs [84] mouse [85] and sheep [86], while in the latter two studies more specifically it was reported that glucocorticosteroids interfere with microtubule organization and chromatin condensation during oocyte maturation. Increased cortisol levels due to acute restrain stress or due to injections in mice impaired developmental potential and ovulation of oocytes [207]. Stress in mice [88] and rats [89] due to restrain was shown to cause abnormal chromosomal segregation which resulted in aneuploidy. Aneuploidy is known to be a major cause of infertility in mammals [90-92]. It may be assumed that such effects of corticosteroids also apply to birds. Indeed, in a number of studies in birds, it was concluded that corticosterone could affect sex ratio [11-13, 21] and asymmetric segregation of sex chromosomes during meiosis (meiotic drive) has been proposed as mechanism of sex ratio bias [15, 21, 45-47, 56, 57, 72, 74]. We propose that in our study in chicken hens corticosterone treatment in conjunction with hen body mass affected sex ratio – by affecting allocation of sex chromosomes to oocyte or polar body, laying rate – by non-lethal arrest of meiosis leading to skipped ovulations, and fertility rate –by lethal chromosomal aberrations leading to infertile

eggs. One alternative hypothesis assumes that some factor during recruitment and the yolk deposition phase of follicles could affect both the growth rates of follicles and meiotic drive [8, 57, 65] such that follicles destined to become the preferred sex 'overtake' other follicles. This would not explain changed sex ratio in longer sequence of eggs such as in commercial laying chicken, which have been intensively selected for egg laying and do not produce small clutches. Also this hypothesis would not explain effects on laying rate and fertility rate. Another hypothesis assumes that some factor could lead to resorption of ova with unwanted sex after meiosis-I [57, 208], thus leading to a changed sex ratio and laying rate. This hypothesis would not explain the effects on fertility rate. Moreover, the resorption of follicles seems unlikely for commercial laying chicken hens, which are geared toward highly efficient production of ova each day. Also, remains of regressing follicles have never been described in chicken ovaries. Another proposed mechanism for sex ratio bias is that only follicles of wanted sex are fertilized by sperm, but this would not explain a change of laying rate and evidence of sex specific fertilization has not been reported. In view of all these considerations we favour the hypothesis as proposed above that interference with meiosis could at the same time lead to sex ratio bias and decrease in both laying rate and fertility rate of the chicken hens.

In conclusion, our results showed that body mass affected sex ratio in untreated hens. Corticosterone feeding of the chicken hens did not result in a major main factor effect on sex ratio, but affected the sex ratio in interaction with intrinsic differences between hens of different body mass. We propose that corticosterone treatment in conjunction with hen body mass can interfere with meiosis, which can lead to meiotic drive, or to chromosomal aberrations resulting in postponed ovulation or infertile ova.

Acknowledgements

We thank Hendrix Genetics B.V., The Netherlands for providing hens used in the experiment. We thank Agnes de Wit and Rita Hoving-Bolink for expert technical assistance. We thank Bonnie de Vries and Ilse Weites for determining corticosterone plasma concentrations. We thank Ministry of Economic Affairs, Agriculture and Innovation, The Netherlands for funding this project under the project number BO-12.02.002.7.01.

7

General Discussion

7.1 Aim of the study and major findings

7.1.1 Aim of the study

The purpose of the present study was to contribute to research fields relevant to two potential alternatives for the killing of day old male chicks in the laying poultry industry. These two potential alternatives had been shown in a public survey to have a reasonable level of societal acceptance, as they were considered more acceptable than maintaining the current practice (of killing male chicks). These two potential solutions were considered feasible, both from a technical and practical, as well as from a commercial (cost) perspective. The two alternatives are: 1) In ovo sex determination ('sexing' the eggs) through examining a sample taken from freshly laid eggs and not incubating the male eggs 2) Influencing the chicken by environmental or other factors so that fewer male eggs are laid i.e. inducing a female primary sex ratio bias. In all proposed alternatives for killing day old chicks, a strict boundary condition is that the method must not negatively affect animal welfare, health, and productivity, or food safety of the animal products. Furthermore, it must be easy and possible to implement the method in commercial practise. For sexing of eggs it had first to be demonstrated that there are reliable sex differences in egg components, which can readily be determined. If so, we would then investigate the potential negative effects on productivity, health and welfare, as taking a sample from the egg could pose a risk for future development of the embryo/chick. In this regard, it is important to note that research in the past [209] suggested that methods to sample eggs can be safe. For the second potential alternative, changing the sex ratio bias, it is not self-evident that hens can be treated for changing their egg sex ratio within the limits of the mentioned boundary conditions. Therefore, within the scope of this PhD research project, the objective with respect to the second alternative was to contribute to understanding of the physiological mechanisms of sex ratio adjustment in layer chicken. Such understanding could form the basis of further research into possible 'animal-friendly' interventions affecting the sex ratio regulation mechanism.

7.1.2 *Major findings*

The first research topic of this project was focussed on sexing unincubated eggs by using any measurable difference between female and male eggs. As described in chapter 3, we found no overall differences between male and female unincubated eggs for measured egg parameters (yolk concentrations of testosterone, estradiol, androstenedione, progesterone,

dihydrotestosterone, and glucose, and egg weight and dimensions). We concluded that the sex of unincubated eggs cannot be predicted based on these parameters (chapter 3).

Regarding the second topic (changing the sex ratio), we have shown that sex ratio bias can indeed be experimentally induced in layer chickens. In one study (chapter 4) we observed that due to a feed restriction treatment, sex ratio of eggs decreased over time during phase of feed restriction and increased after switching to ad lib feeding. The rate of change of hen body mass over 2 to 3 days before egg laying and encompassing meiosis, significantly predicted the sex of egg. In this study, the concentration of blood corticosterone was negatively associated with sex ratio in the feed restriction group. In a follow up study in which feed restriction was implemented under the same experimental conditions, the rate of change of hen body mass during the same time window was not associated with egg sex (chapter 5). This was probably due to the fact that in the latter experiment there was much lesser decrease in hen body mass.

In the later study (chapter 5) it was found that hens, in response to the feed restriction, differed in the magnitude of decrease in egg mass. In the control group, the average egg mass of hens stayed approximately constant, but the eggs of some hens decreased while those of other hens increased in egg mass over time. For the feed restricted hens and for the control hens that showed a declining egg mass over time, the egg sex ratio per hen was negatively associated with the average egg mass per hen. In the feed restriction group, we analysed gene expression patterns of the germinal disc of oocytes collected at the end of the feed restriction treatment and compared hens displaying male sex ratio bias and low egg mass with hens displaying female sex ratio bias and high egg mass. The results did not show differential expression of genes between the groups. However, gene set enrichment analysis showed that a number of processes related to cell cycle progression, mitotic/meiotic apparatus and chromosomal movement were differently enriched between the groups, pointing towards potential mechanisms underlying sex ratio determination.

In another study (chapter 6), corticosterone feeding treatment did not affect the overall sex ratio, but affected the sex ratio in interaction with hen body mass. In the corticosterone group, sex ratio, laying rate, and fertility rate per hen were decreased in heavy hens. In line with this, the overall laying rate and fertility rate per hen were positively associated with overall sex ratio per hen in the corticosterone group. In the study described in chapter 4, in hens treated with feed restriction, a negative association was found between the serum corticosterone concentration and sex ratio. In the hormone manipulation study (chapter 6) serum

corticosterone concentration was not significantly associated with sex ratio and hen laying rate and was positively associated with hen fertility rate in corticosterone fed hens.
Thus, the results of our studies indicate relationships between feed availability, change in body mass (energy balance), change in egg mass, corticosterone levels, and ovarian function, potentially affecting sex ratio, fertility rate, and laying rate.

7.2 Importance of studying yolk hormones and sex ratio bias in unincubated eggs

In previous studies on the primary offspring sex ratio in birds, the sex ratio was determined by sexing embryos or hatchlings [12-15, 20, 21, 39, 42, 45, 47-50, 191]. It has been described that sex specific embryo mortality can occur [19, 20, 51]. If this would indeed occur, the observed sex ratio would in fact be a 'secondary sex ratio' deviating from the primary sex ratio. It was reported that the percentage of early embryonic mortality (occurring roughly in the first 5-7 days of incubation) is up to 33% in traditional chicken [210] and 8% in commercial broiler chickens [211, 212]. In studies of sex ratio, one may try and sex also the dead embryos, provided that development had proceeded to a stage in which the embryos could be readily sexed. However, this would probably not be possible for embryos that died within the first 2 days of incubation. In our own experience with a commercial breed, the percentage of eggs of which the blastodisc or embryo showed no progress beyond a day-2 embryo was roughly 6% of all incubated eggs (Woelders, in preparation). For traditional breeds of chicken this percentage may be higher. These eggs may have either been not fertilized at all, or the embryo may have died on day 0, 1 or 2 of incubation. In the worst case scenario that all embryos that died very early were of one sex, e.g. male, and assuming very early embryonic death of 6%, this would lead to an observed sex ratio of 0.47. When assuming very early embryonic death of 33%, the observed sex ratio would drop to 0.35. It is not very likely that early embryonic death will be limited to one sex only, and also an assumed percentage of earliest embryonic death of 33% may not be likely. Thus the deviation from the primary sex ratio due to embryonic mortality may be relatively small but could at least contribute to some extent to an observed sex ratio bias. As explained in the chapter Introduction, in studies of the relation between maternal hormones in yolk and sex of egg, a second problem of using incubated eggs is that yolk hormone concentrations are reported to change during incubation as they may be metabolized in the yolk or by the embryo, or

produced by the embryo [128, 155]. If this change during incubation would be sex specific, the use of unincubated eggs could lead to incorrect assessment of the relation between maternal allocation of hormones to the yolk and the sex of the egg.

These two reasons motivated our efforts to try and develop a method of sexing unincubated eggs. The reason to sex incubated eggs is that after a few days of incubation, a developed embryo can clearly be recognized and sufficient amounts of embryonic DNA can be obtained for application of DNA-based sexing technologies. In unincubated eggs an embryo if present is still in the blastodisc stage and it is difficult to discriminate between the stage X embryo of a fertile egg and the germinal disc of an infertile (not fertilized) egg. In eggs that do contain a healthy stage X embryo (blastodisc), there is smaller amount of embryonic DNA than in incubated eggs. This means that a smaller amount of DNA must be amplified more strongly by PCR. A chicken stage X embryo contains approximately 30,000-60,000 blastoderm cells [52]. In the blastodisc of unincubated eggs it cannot be prevented that the embryo DNA is contaminated with maternal (granulosa cells) and paternal (sperm) DNA. It is reported that approximately 2000 sperm cells may remain attached to the 5 x 5 mm area of perivitelline layer overlying the blastodisc [164, 165]. In addition, blastodisc may contain the remains of up to 25 male pronuclei (Z) [162]. PCR amplification of contaminating maternal DNA could also lead to a W-chromosomal associated *CHD1* PCR product of 450 bp, which could result in a male embryo to be erroneously sexed as female but would not have consequences for a female embryo. In fertile eggs, paternal contamination (sperm, Z) is not expected to lead to errors. In infertile eggs there is no (or very little) embryonic DNA. Therefore, in infertile eggs there is a huge risk of erroneous result. It may be sexed either as male or female on the basis of maternal or paternal contaminated DNA, thus inappropriately attributing a sex to infertile eggs.

In a commercial breed and best practise situation there may be approximately 5% infertile eggs (here defined as absence of any apparent development beyond the germinal disc or blastodisc stage, i.e. these eggs may not have been fertilized at all, or embryo may have died before, at, or shortly after stage X embryonic development). But in other breeds or less good practice perhaps, the percentage of eggs defined as infertile may be higher, e.g. 10% or perhaps even 20%. If all 20% infertile eggs were erroneously sexed as female and the true primary sex ratio were in fact 0.50, the observed sex ratio would be 0.40. Likewise if all 20% infertile eggs were erroneously sexed as male, the observed sex ratio would be 0.6. But in best

practice situation the deviation of the observed sex ratio due to inappropriately attributing a sex to infertile eggs would be smaller.

As we have shown in chapter 2, the risks of erroneous sexing of fertile and infertile eggs can be sufficiently reduced or prevented in two ways. One way is to discriminate the fertile from infertile blastodiscs by Hoechst 33342 staining. Absence of stained nuclei in isolated germinal discs shows that the germinal disc was truly not fertilized or that the zygote failed development in the first division, and these eggs were not sexed.

The other way is to minimise the contamination with maternal DNA from granulosa cells, which can, as said above, pose a risk even for sexing fertile eggs. As was reported previously [97, 199, 204], the granulosa cells can easily be detached from the blastodisc region by gentle manipulation as the junctions between the granulosa cells and the underlying blastodisc region become loose after the ovulation as the embryo develops in the oviduct (i.e. up to Stage X) [97, 199, 204]. As a result of removing the extra- and peri-vitelline layers with the granulosa cells, the amount of contaminating maternal DNA is strongly reduced. Indeed, using our method of sexing unincubated chicken eggs (chapter 2), we found either two equally strong *CHD-1* gene bands for the W and Z specific genes, respectively, indicating a female embryo, or only one single Z chromosomal band, without any W-specific PCR product on the agarose gel, indicating a male embryo. Thus it appears that any contamination of maternal DNA was not sufficient to generate a detectable W-specific band in the agarose gel for male embryos.

So the developed method allows to study the primary sex ratio as well as the relation between sex of egg and maternal hormones in yolk using unincubated eggs, preventing the errors associated with using incubated eggs. We have applied this method throughout the studies in this thesis.

7.3 Relationship of hormones to egg sex in birds

As described in the chapter Introduction, a large body of evidence exists indicating that in birds, hormones in peripheral blood or in egg yolk may be involved in sex determination, and may thus affect sex of the egg or the offspring sex ratio. Gonadal steroid hormones are produced locally by the cells of the follicular wall (theca and granulosa cells), which are in direct contact with the developing ovum and its germinal disc. These hormones are supplied directly to the oocyte and are also released into blood [127]. In contrast, the corticosteroids,

which have also been implicated in affecting the sex of avian egg, are produced by the adrenal gland and can enter the developing growing ovum from peripheral blood [62, 126]. Regardless of the production site of hormones, two ways can be envisaged through which the hormones could affect the sex of the egg. One possibility is that these hormones accumulate more in some of the developing oocytes than in other, and the chance of developing one sex or the other during meiosis is determined by the level of that hormone in the germinal disc. In that case female and male oocyte would differ in the yolk hormone concentration. Another possibility is that these hormones, if present at a certain level, would switch on some other factor in the ovary or perhaps in an entirely different organ of the hen, which would subsequently lead to a signal to the follicle to affect the chance of obtaining one sex or the other during meiosis. In that case there need not be a direct association between sex of egg and yolk hormone concentration but there would be a relation between hormone concentrations and the sex ratio of the entire clutch. Therefore, we analysed the relationship of hormones to sex of egg in two ways. First, the direct relationship of the concentrations of several hormones in yolk with sex of the egg was studied (chapter 3). Second, the association of overall sex ratio per hen with the mean concentrations of several hormones in the yolk of all eggs per hen was studied (chapter 3), as well as the association of overall sex ratio per hen with the concentration of corticosterone in peripheral blood of the hen (chapter 4 and 6). As explained in section 7.2., in all our studies we determined yolk hormone concentrations and sex of the eggs in unincubated eggs, using a new technique that we had developed (see chapter 2).

It has been shown previously that it is possible to withdraw small yolk samples from eggs with a high throughput, without affecting the viability of the embryo [209]. In case that we would find a real difference in the concentration of yolk hormones, glucose or other egg characteristics between male and female unincubated eggs, this could potentially be used to sex the unincubated eggs in-ovo in the layer or broiler industry. This could potentially solve the problem of killing one-day old chicks in layer production. Unfortunately, in the present study, no differences between male and female unincubated chicken eggs were observed in the concentrations of the maternally derived sex steroid hormones (testosterone, estradiol, androstenedione, progesterone, dihydrotestosterone) or glucose in the yolk, nor in the ratio of androgens and estrogen (chapter 3). We also did not find an association of the mean concentration of the yolk hormones or glucose per hen with sex ratio per hen (chapter 3).

These findings do not suggest a simple obvious role of these substances in the sex determining process in chicken. In addition, there seems to be no possibility to sex unincubated eggs based on the measured yolk hormones or glucose.

A previous study with captive peafowl (*Pavo cristatus*) showed that eggs from females mated with unattractive males did not differ in the sex related differences of yolk corticosterone but the treated females had higher yolk concentration of corticosterone and displayed a female sex ratio bias [12]. However, there is much debate about the reliability of measurement of corticosterone in bird eggs [144, 213]. Although after experimental administration of radioactive corticosterone and using HPLC techniques, detectable levels of corticosterone were reported in chicken yolk [154], these concentrations were much lower than previously reported. In our study we could not measure appreciable concentrations of corticosterone in chicken egg yolk, suggesting that the concentration was below the detection limit of the used RIA. It has been shown previously that concentrations of yolk hormones could vary in the different layers of yolk [154, 214].

It is possible that hormone concentrations in the outermost layer of yolk are more relevant for sex determining process, as the blastodisc is present in the outermost layer of the yolk follicle. In the present study we have measured the concentrations of the yolk steroid hormones in whole yolk and none of the previous studies investigated the concentrations of yolk hormones in the outer most layer of yolk in relation to sex of the egg. Although this remains to be investigated, our finding that there appear to be no sex-specific differences in yolk concentrations of the studied hormones, makes it less likely that hormones are differentially allocated into yolk of different eggs and that this would influence the sex determining process.

Regarding the relation between hormone concentrations in peripheral blood and the offspring sex ratio, a complicating factor is that various hormones may interact with each other. For instance corticosterone and testosterone compete for binding to the same plasma hormone binding protein called corticosterone binding protein (CBG) [215]. The CBG concentration in blood can themselves change in birds under various environmental conditions [216-219]. Thus, changes in concentration of one hormone may change the free concentration (and consequently the biological activity and the clearance rate) of the other hormone, which makes it difficult to directly relate the blood concentrations of one hormone to reproductive parameters such as sex ratio, laying and fertility rates (see also next section 7.4). This could be one of the reasons that the observed associations of corticosterone blood levels with sex

ratio (chapters 4 and 6) were not constant and that the results varied between the two studies described in these chapters. However, our observation that blood levels of steroid hormone corticosterone in feed restricted hens are related to sex ratio bias in chicken (chapter 4), is in line with (a number of) previous studies which implicated different steroid hormones in blood (including corticosterone) to sex determination in various birds species. In the study described in chapter 6, where hens were fed with feed containing corticosterone, no association between blood concentrations of corticosterone and sex ratio per hen was found. However, as will be discussed in more detail in the next section, also in the study in chapter 6 there were clear effects of corticosterone, but in interaction with hen body mass.

7.4 A special case for corticosterone; interplay with energy balance in the regulation of sex ratio

Corticosterone as a catabolic hormone is related to conditions of decreased food availability [138, 139] and decreasing body condition [140, 141]. Supplying ad libitum access to feed containing corticosterone led to increase in both circulating corticosterone levels and hen body mass, thus disrupting the natural negative correlation that exist between maternal corticosterone concentrations and body condition/body mass (chapter 6). As described in chapter 6, we observed no main effect of corticosterone feeding on sex ratio. However, we observed a clear effect of the corticosterone treatment in interaction with hen body mass such that corticosterone treatment resulted in lower sex ratio, laying rate and fertility rates in heavier hens (chapter 6). This interaction was already seen when taking the hen body mass before the start of the treatment, which suggests that lighter and heavier hens were already intrinsically different regarding their reaction to corticosterone feeding treatment.

Chronic treatment of corticosterone induced a female sex ratio bias in most of the previous studies in different bird species [11, 12, 21, 74]. One previous study in chicken resulted in male sex ratio bias due to acute elevation of blood corticosterone concentrations through injections around the time of meiosis [47]. The effects of chronic corticosterone treatment were not studied in chicken. We hypothesized that the experimental chronic elevation of blood corticosterone concentrations in chicken could lead, like in most other bird studies (but see Pryke et. al. 2011), to female sex ratio bias [22]. Previously, both corticosterone and body condition were shown to be associated with sex ratio either within the same experiment [13]

or in different experiments[11-13, 43, 48, 102, 104, 105, 175]. It has also been described that corticosterone levels may also change in response to other environmental factors that have been reported to influence sex ratio, such as habitat quality [43, 49, 145], and social dominance [146, 147] and partner quality [12, 22].

We found a negative association of rate of decrease in female body mass with sex of egg in the hens subjected to feed restriction (chapter 4), i.e. hens which decreased more in body mass had lower sex ratio. In untreated hens (control) we found a positive relation between hen body mass and sex ratio, i.e. lighter hens have lower sex ratio (chapter 6). These results are in agreement with previous studies, which showed a similar association of sex ratio with female body condition under natural conditions [13, 102-105] or under experimentally controlled conditions [13, 49, 53, 106, 107]. Chickens are sexually dimorphic: females are the smaller-less costly-sex with lower energetic requirements [108] and have lower reproductive variance than males [5, 109]. Therefore, our results are in agreement with the sex allocation theory which predicts that in species such as chicken, a female sex ratio bias may be expected under conditions of low food availability and decreasing body mass [26]. The association between rate of decrease in female body mass and the sex of eggs, as observed in the experiment described in chapter 4, was not found in the follow up experiment in which the hens were again subjected to feed restriction under similar experimental conditions (chapter 5). In the latter experiment we observed a much smaller decrease in body mass of hens (61.8±43.4 g, chapter 5) as compared to first experiment (186±60 g, chapter 4), which could be a reason for disparity in the results. The observed lower laying rate in the later experiment (87%, chapter 5) as compared to the earlier experiment (95%, chapter 4) in feed restricted hens could be a reason for the smaller decrease in hen body mass. We do not know why there was a difference in the laying rates between the feed-restricted hens of the two experiments.

We observed that plasma corticosterone concentrations were negatively associated with both the sex ratio and change in hen body mass during our feed restriction treatment (chapter 4). These results are in line with previous results from various authors which suggested that the effects of food shortage or body mass condition on sex ratio are mediated by corticosterone [138-141]. Corticosterone concentrations appeared to be dynamic, as different concentrations were observed at different time points in both feed restriction and control hens during the experiment (chapter 4). In addition, the association of corticosterone levels with sex ratio was not constant over time. In the corticosterone feeding experiment (chapter 6), we hypothesized

that corticosterone would negatively affect sex ratio, laying and fertility rate. However, we observed that blood corticosterone concentrations were not associated with sex ratio per hen and laying rate per hen but were positively associated with the fertility rate per hen. There were large differences in the blood corticosterone concentrations of the treated hens. Towards the end of the experiment some hens displayed levels similar to control hens while other hens showed 10-20 times higher levels that control hens. We assume that individual and time differences in feed intake and in the absorption of the hormone from the feed is unlikely to be so substantial and to be large enough to explain the results. Instead, these results may be explained as follows, Corticosterone binding globulins (CBG) bind to corticosterone in blood, thus regulating bioavailability and metabolic clearance of corticosterone [220-222]. Our assay measures both free and bound corticosterone hormone together. However, the free hormone is the relevant biologically active fraction (for the gonadal hormones this is a much less relevant issue as only 10% of this hormone is bound to CBG). A further complication is that the release of corticosterone from CBG might be tissue specific affecting local concentration and binding to glucocorticoid receptors. It is known that birds regulate plasma CBG levels to regulate the free concentration of plasma corticosterone and that this can be condition specific [216-219]. It is possible that the hens displaying lower levels of blood corticosterone had lower levels of blood corticosterone binding globulins (CBG), resulting in high concentrations of free corticosterone and thus higher clearance rates from the blood (the half life of free hormone is less than 30 minutes). In other hens, higher levels of CBG could have resulted in low free concentrations of blood hormones and therefore also low clearance rates, but measured as high circulating levels of total corticosterone in our assay. The interaction of corticosterone with CBG would explain the observed variation of corticosterone concentration between different hens in the corticosterone group, positive relationship of corticosterone with fertility rate and lack of association of corticosterone with sex ratio in the treated hens (chapter 6). For the same reason, variations in the CBG concentrations could also explain the dynamic behaviour of the corticosterone concentrations observed in the hens during the feed restriction experiment (chapter 4). Corticosterone might also interact with other hormones which have been shown to be associated with both sex ratio and hen body mass. For example testosterone has been shown to be positively associated with sex ratio [14, 18, 106] and with hen body mass [223]. Changing blood corticosterone concentrations could potentially affect the free concentrations of testosterone because both these hormones have been reported to

compete for binding to same plasma protein called corticosterone binding protein (CBG) [215].

Egg mass constitutes another component of the energy balance axis apart from hen body mass. We studied the effect of egg mass in chapters 3 and 5. Both food availability [43, 44, 100, 101] and female body condition [13, 102-105] had been shown in previous studies to be associated with sex ratio and also both these had been shown to affect the mass of the egg and of the egg yolk [113, 114]. The effect of egg mass on sex of egg was studied in chapter 3 and 5. In contrast to our findings from an earlier study in which a low number of eggs from hens under unmanipulated conditions were studied (133 eggs, chapter 3), sex ratio per hen was associated with the average egg mass per hen (282 eggs, chapter 5) under conditions of feed restriction in a larger sample size. There were differences among the hens regarding the magnitude of decrease in egg mass in response to feed restriction and this resulted in greater differences between hens for the mean egg mass per hen due to feed restriction (chapter 5). Therefore the association of sex ratio per hen became more strong during the second half of experiment during which a more severe feed restriction was implemented. The rate of decline of egg mass per hen was significantly negatively associated with the sex ratio per hen. These results suggested that both mean egg mass per hen and the magnitude of the decrease of egg mass in response to feed restriction play a role in sex ratio regulation.

We speculate that the hens of our experimental population differed from each other in 'coping styles' to feed restriction. On the one hand, the hens that generally lay smaller eggs and responded towards a shortage of feed by a stronger decrease in egg mass tended towards a male sex ratio bias. On the other hand, the hens that generally lay larger eggs and responded to feed restriction with a less clear decrease in egg mass tended towards a female sex ratio bias. Sex allocation theory [24, 26] predicts that large mothers, laying large eggs, and producing offspring that may have genetic predisposition for being large, i.e. more costly, would tend to produce less male (more costly sex) offspring in periods of reduced availability of resources. Then, according to Fisher's principle [24] the resulting shortage of males would make it more advantageous for the more 'economic' hens, i.e. 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 tended

towards 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.

7.5 Mechanism of sex ratio bias

Bias in the sex ratio as determined in embryos in incubated eggs or in hatchlings could be due to both primary sex ratio bias and secondary sex ratio bias. In our study we have determined the sex ratio in unincubated eggs, therefore sex ratio bias in our studies is due to events occurring before oviposition. 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 sex ratio is determined [46]. We have ruled out sex specific embryonic death during incubation as a cause for sex ratio bias as we have sexed unincubated eggs. Hoechst 33342 staining was used to determine fertility status of the germinal disc regions, which resulted in unequivocal distinction between ‘infertile’ eggs or ‘fertile’ eggs (see for details [174]).

In the corticosterone feeding group, body mass was negatively associated with sex ratio and also with laying rate and fertility rate per hen (chapter 6). Sex specific infertility has not been described in birds. In the feed restriction experiments, the observed effects on sex ratio cannot be due to sex specific fertilization of follicles as fertility rate per hen was not associated with egg sex ratio per hen (chapter 4 and 5). Also, sex specific follicle resorption, or selective ovulation cannot be the cause of sex ratio bias as the laying rate per hen was not associated with sex ratio per hen in the feed restriction experiments (chapter 4 and 5). But sex ratio was predicted significantly by laying rate and fertility rate per hen in corticosterone feeding group (chapter 6). However, there appears to be a discrepancy of the results described in chapter 6 with results described in chapters 4 and 5, where feed restriction (which elevates blood corticosterone levels) did affect sex ratio but not laying rate and fertility rate. We assume that this apparent discrepancy may be due to the fact that corticosterone elevation in chapter 6 was obtained in a un-physiological way leading to supra-physiological levels of blood corticosterone. It is very well possible that after feed restriction, increased corticosterone levels do also affect laying rate and fertility rate to some extent, but the latter two may not be changed strong enough to be significant. Interestingly, in chapter 5, the selected hens with

female sex ratio bias did happen to have numerically lower (but not significantly lower) values of laying rate and fertility rate than the male biased hens. But clearly, the results of chapter 6 do indicate effects of corticosterone treatment on all three parameters (sex ratio, laying rate and fertility rate). This suggest that the three parameters (sex ratio, laying rate and fertility rate) are connected at the level of ovarian physiology. A hypothesis for the mechanism for the effect of corticosterone feeding treatment on sex ratio should explain at the same time also its effects on laying rate and fertility rate per hen. In mammals, glucocorticoids have been reported to interfere with meiosis [84-86], ovulation of oocyte [207], and abnormal chromosomal segregation resulting in aneuploidy [88, 89]. Aneuploidy is known to be a major cause of infertility in mammals [90-92]. It may be assumed that corticosterone similarly effects the ovum resulting in decreased ovulation and fertility of the ovum. A number of previous studies in birds showed that corticosterone could affect sex ratio [11-13, 21] and asymmetric segregation of sex chromosomes during meiosis (meiotic drive) has been proposed as mechanism of sex ratio bias [15, 21, 45-47, 56, 57, 72, 74]. I propose that corticosterone feeding treatment in chicken hens in interaction with some factor related to hen body mass affects sex ratio – by affecting allocation of sex chromosomes to the oocyte or polar body, laying rate – by non-lethal arrest of meiosis leading to skipped ovulations, and fertility rate –by lethal chromosomal aberrations leading to infertile eggs. Our studies provide further evidence for meiotic drive. For instance, the sex of egg was significantly predicted by the rate of change of hen body mass in a specific time window (i.e between day of egg laying and day of laying minus 2 days or minus 3 days) that encompasses resumption of meiosis and was not significantly predicted during the time window which encompasses rapid yolk deposition phase before occurrence of meiosis (chapter 4).

Further insight into the mechanism of sex ratio regulation was obtained by comparing hens with low mean egg mass and high sex ratio to hens with high mean egg mass and low sex ratio in genome wide gene expression analysis in germinal discs of F1 follicles collected at the time of meiosis-I (chapter 5). By analysing the differences between the groups at the individual gene level, no significant differences were found in these two groups of hens. However by analysing the differences at the gene-set level, we observed that several gene-sets differed between the groups. Functional analysis of these gene-sets suggested that a number of processes in the germinal discs of the two groups differed from each other, including processes related to cell cycle progression, mitotic/meiotic apparatus and chromosomal

movement . These processes were significantly enriched in genes that negatively correlated with sex ratio, and the mean expression level of these processes correlated negatively with sex ratio (and positively with mean egg mass per hen). In other words, these processes (gene sets) were more strongly expressed in female biased hens compared with male biased hens. In contrast, gene expression levels of general cellular processes such as general membrane transport, cellular protein metabolism and cellular respiration were not associated with the mean egg mass or sex ratio per hen.

Contamination of granulosa cells cannot be avoided while isolating the germinal disc region from the pre-ovulatory F1 follicles [97, 199, 204]. It could be argued that the found differences in processes could be related to granulosa cells rather than the ovum, e.g. by assuming that follicles from hens that lay heavier eggs may be larger and have more granulosa cells or a higher rate of granulosa proliferation, hence more expression of cell-cycle related processes. However, it is not self-evident that the density of granulosa cells over the germinal disc (cells per square mm) is related to follicle size. Also, the granulosa cells stop proliferating and dividing (mitosis) toward completion of follicle growth [224]. Furthermore, gene expression of the highly expressed general metabolic processes was not correlated at all with either female bias or egg mass per hen. And lastly, the germinal disc of chicken and other avian oocytes contain several orders of magnitude more mRNA than somatic cells [99, 199]. The contribution of granulosa cell mRNA to the germinal disc mRNA preparation is likely to be small. Therefore it is not likely that granulosa RNA contamination could explain the differential expression of cell cycle related processes. Instead, our results indicate that there is a difference between male and females sex ratio biased hens at the level of processes in the ovum during occurrence of meiosis for the processes related to cell cycle progression, mitotic/meiotic apparatus and chromosomal movement. We propose that regulation of these cell cycle related processes is involved in meiotic drive, with a higher activity of these processes leading to more female sex ratio bias.

As described in the chapter Introduction, there may be a number of features of the two sex chromosomes and the occurrence of meiosis that could lead to meiotic drive in birds. These include Z being the larger chromosome, differences in the size of protein bodies attached to sex chromosomes, peripheral location of the germinal disc in the follicle, variation in the position of centromeres of sex chromosomes (reviewed by Rutkowska & Badyaev [8]). Telomere length, which has been shown to be affected by steroid hormones [77], could affect

the asymmetric sex chromosome segregation [8]. The elements of spindle structure which are directly involved in chromosome segregation during meiosis have been shown to be modified by various hormones [79]. In chicken it was shown that chromosomes with higher number of centromeres preferentially segregate to the polar body [80]. Steroid hormones act as transcription factors and change gene expression [82]. Specifically, we found that gene sets related to cell cycle, motor activity, spindle and chromosome segregation were differentially expressed in female biased hens compared with male bias hens. We think that the different expression levels of the gene sets relates to the mechanism of meiotic drive. This could be confirmed if, in future experiments, using a different method of inducing sex ratio bias, e.g. corticosterone treatment, changed expression levels of the same gene sets would be seen.

7.6 Conclusions and future perspectives

Results from this thesis showed that laying hens could potentially change the primary sex ratio under certain conditions such as feed restriction or mimicking stress by experimentally elevating blood levels of corticosterone stress hormone. However, the methods described in this thesis for inducing sex ratio bias in laying hens would not be suitable for use in commercial poultry farming. Implementing the feed restriction or mimicking stress by feeding corticosterone would lead to another ethical problem of stressing birds and this could also lead to severe compromise for the egg production of hens. The results suggest that mechanism of sex ratio bias involve meiotic drive. The animal experimental models used in this thesis can be further used to elucidate the underlying mechanisms of primary sex ratio bias at molecular level. Studying the metabolomic profile of the blood plasma and germinal disc region by techniques such as nuclear magnetic resonance (NMR) could help to further understand these mechanisms. This could lead to finding ways and means to induce the sex ratio bias in poultry industry in a favourable way without compromising on the welfare and productivity of hens. This could potentially not only lead to reducing the number of day old male chicks killed in poultry industry but it would also lead to increase the production of more profitable and desired sex i.e. females in egg layer industry and males in broiler industry. Generally, understanding these mechanisms is also of interest for applied sciences for species conservation programmes [53] and for various fields of biology as it would help to understand the evolutionary and functional consequences of sex ratio bias.

Our results showed that unincubated eggs do not differ for the measured hormones and glucose concentrations, and therefore in-ovo sex determination is not possible based on these parameters. After incubation of 7-14 days, concentrations of oestradiol in the allantoic fluid of chicken have been reported to differ significantly to allow identification of male and female eggs [225, 226]. In-ovo sex determination during incubation of eggs can be considered after determination of oestradiol in allantoic fluid of incubated eggs. This alternative was not favourable from the point of societal acceptability as the male embryos would be killed after considerable development [4]. Genetic modification can also be performed such that male embryos die at a very early stage in the oviduct or during early incubation. This alternative was also societally less acceptable [4], but if embryonic mortality would be strictly confined to very early stages of development, i.e. if effectively development doesn't actually occur in male embryos, this alternative could be reconsidered. Genetic modification can also be performed to insert green fluorescent protein (GFP) gene on Z chromosome in females of purebred lines poultry stock such that expression of GFP can be detected in male eggs at the unincubated stage or at an early stage of incubation. Our results indicated that sex ratio per hen was associated with both the average egg mass per hen and hen body mass. The heritability of sex ratio in conjunction with other traits, such as egg mass or hen body mass, could be studied. Preferably one would like to be able to select for one trait without affecting too much other traits. Along a completely different line, the development and use of improved dual purpose breeds of the chickens may reduce the killing of day-old chicks, but this may only be a possibility for a limited part of the market.

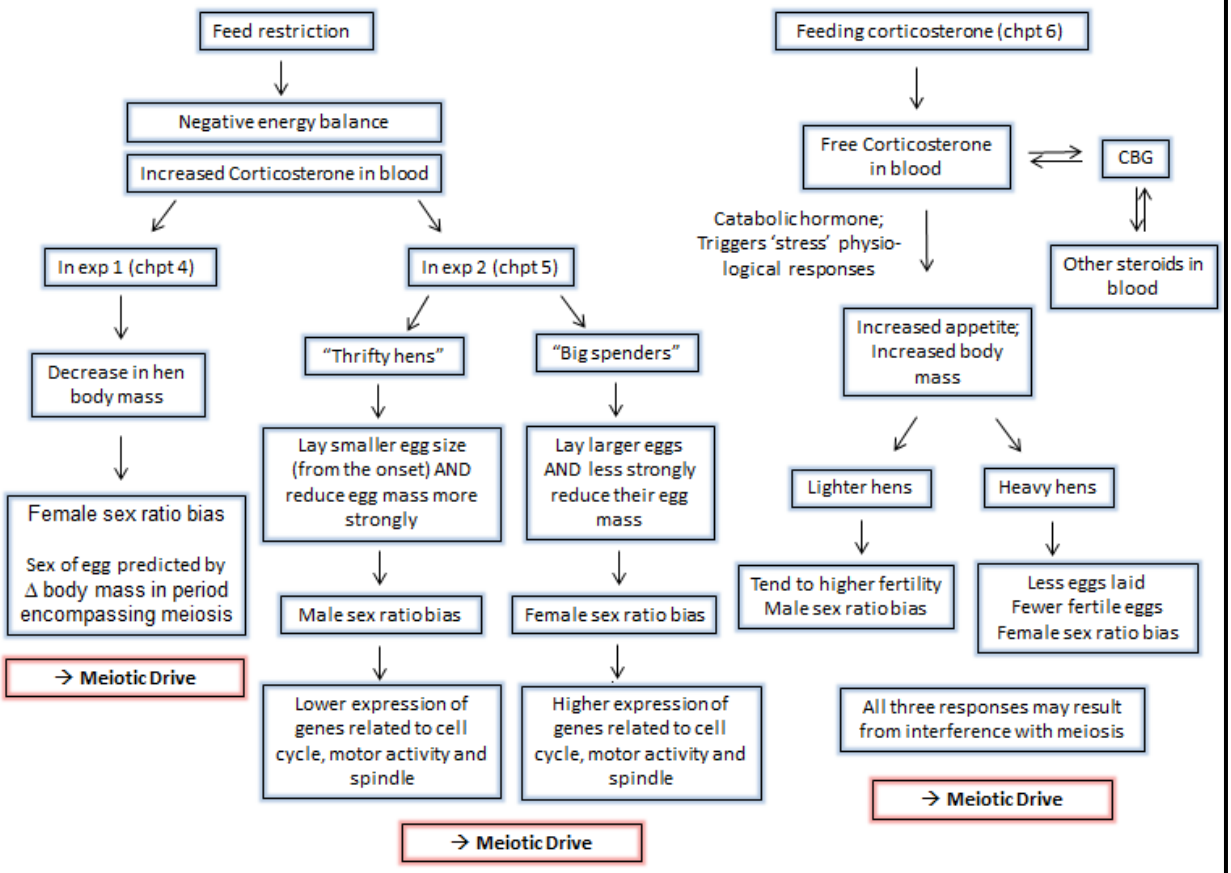

Figure 7.1. Schematic overview summarizing the main findings and conclusions as described in sections 7.4 and 7.5. Some parts of this scheme may be speculative or less directly supported by our own empirical observations. For instance, we have not measured CBG content or free corticosterone concentrations, but we did see an increase of total corticosterone in blood in response to corticosterone feeding and we saw very strong effects of corticosterone feeding in interaction with hen body mass. As described in chapter 4, we also found an increase of the total corticosterone levels in blood in response to feed restriction and we found an association between corticosterone levels on day 8 of the feed restriction treatment and the sex ratio. On the basis of chapters 4 and 6 we speculate that corticosterone may be the actual trigger for physiological mechanisms in the ovary that lead to primary sex ratio bias. The responses are however different in different 'types' of hens. It seems that hens that are less economical in terms of energy expenditure (lay larger eggs, reduce their egg mass less in response to feed restriction, have larger body mass, and increase their body mass stronger in response to ad libitum corticosterone feeding) tend to have a female offspring sex ratio bias in the eggs laid towards the end of the feed restriction or corticosterone feeding treatments, while the opposite is seen for more economical hens. The summarizing scheme shows three independent pieces of evidence supporting that the sex ratio bias may result from asymmetric sex chromosome segregation during meiosis, or 'meiotic drive'.

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Summary

Summary

This thesis describes research on factors influencing sex of egg and sex ratio in chickens. Understanding the factors influencing sex of egg and sex ratio in birds is of general interest for various fields of biology. Particularly it is of interest to poultry layer industry as it may lead to finding potential solutions for the problem of killing of day old male chicks, which is the current practice in breeding of laying hens. In studies described in this thesis, it was investigated if the sex of the chicken egg can be predicted by measurable differences in male and female eggs at unincubated stage. It was also investigated if the female primary sex ratio can be induced in laying chicken using different experimental conditions such as feed restriction and corticosterone feeding. After establishing the animal experimental model for manipulating the primary sex ratio bias, the underlying physiological and molecular mechanism of primary sex ratio bias were investigated.

Chapter 1 provides the general background information about sex allocation theory and factors affecting sex ratio in birds, and discusses previous literature about associations of hormones in the yolk or in maternal blood with sex of egg as well as associations of physical egg parameters with sex of egg.

Chapter 2 describes the method of sex determination in unincubated chicken eggs using PCR targeted to CHD1 gene. In most of the previous studies on primary sex ratio of birds, eggs are sexed after incubation by the use of PCR methods targeted to the sex-linked CHD1 genes. If there is sex specific embryo mortality during incubation, the primary sex ratio can deviate from the observed sex ratio called 'secondary sex ratio'. In studies of the relation between maternal hormones in yolk and sex of egg, change in the concentrations of yolk hormone can occur during incubation. If this change during incubation would be sex specific, the use of unincubated eggs might lead to incorrect assessment of the relation between maternal allocation of hormones to the yolk and the sex of the egg. These two reasons motivated our efforts to try and develop a method of sexing unincubated eggs. During sexing of unincubated eggs fertile and infertile blastodiscs cannot be distinguished macroscopically, and errors could arise from PCR amplifications of parental DNA associated to the vitelline membrane of infertile eggs. The risks of erroneous sexing of unincubated eggs was prevented in two ways. First the contamination with maternal DNA from granulosa cells was minimized after removing the extra- and peri-vitelline layers along with the granulosa cells from the blastodisc. Secondly, only fertile eggs were sexed after distinction between fertile and

infertile blastodiscs using Hoechst 33342, which showed thousands of fluorescent nuclei in fertile blastodisc, whereas no nuclei were seen in infertile eggs. In addition, after nucleic acid analysis, fertile blastodiscs yielded much stronger chromosomal DNA and CHD1 targeted PCR bands on agarose gels compared to infertile blastodiscs. These findings indicate that fertile blastodiscs contain much more embryonic DNA than parental DNA, allowing reliable sexing of the fertile eggs. This method of sexing unincubated eggs was applied throughout the studies in this thesis.

Hormones both in the peripheral blood or in egg yolk have been shown in previous studies to influence sex of the egg or the offspring sex ratio. In chapter 3 the direct relationship of the concentrations of several hormones (testosterone, estradiol, androstenedione, progesterone, dihydrotestosterone) and glucose in yolk as well as of egg parameters (mass, width and length) with sex of the egg was studied. No significant relationships were found. The association of overall sex ratio per hen with the mean of all eggs per hen of the above described parameters was also not significant (chapter 3). Therefore in ovo sex determination at unincubated stage is not possible based on measurements of these egg parameters. This suggests that hormones do not play a direct role in the sex dertermination process in birds.

Female birds have been shown to manipulate offspring sex ratio under natural as well as experimental conditions due to various factors such as variation in food availability. Effect of feed availability on sex ratio was tested in chapter 4. Thirty laying hens were randomly allocated to each of control and feed restriction groups. Hens were inseminated twice a week using artificial insemination and eggs were sexed without incubation. Feed restriction (upto 80% of pre-treatment ad libitum for first 7 days and upto 70% for next 10 days) was implemented for a period of 17 days and after the end of feed restriction period hens were subsequently fed ad libitum for additional 9 days. The effect of feed restriction over time was significant for sex ratio (proportion of male eggs) per day in the feed restriction group. The rate of change of hen body mass between day of laying and day of laying minus 2 days (encompass time of meiosis completion) was a significant predictor for the sex of that egg. Feed restriction resulted in higher corticosterone concentrations in female blood plasma. Blood corticosterone concentrations were associated with both the fertility rate and sex ratio per hen. These associations of blood corticosterone concenttrations were found only at one day out of three different sampling days of blood. The egg sex ratio per hen was not at associated with fertility or laying rate per hen.

Experiments described in chapter 5 were aimed to reproduce the results described in chapter 4. Forty five laying hens were randomly allocated to each of control and feed restriction groups. Feed restriction hens received 80% of ad lib for 7 days and 70% of ad lib for an additional 9-11 days under same experimental conditions as in the previous experiments (chapter 4). Hens were inseminated twice a week using artificial insemination and eggs were sexed without incubation. In addition to studying the effects of feed restriction on sex ratio bias, effects of feed restriction on egg mass and associations of egg mass with sex ratio were studied. The association between rate of decrease in female body mass and the sex of eggs, as observed in the experiment described in chapter 4, was not found in this follow up experiment (chapter 5). In the latter experiment a much smaller decrease in body mass of hens (61.8±43.4 g, chapter 5) was observed as compared to first experiment (186±60 g, chapter 4), which could be a reason for disparity in the results. The observed lower laying rate in the later experiment (87%, chapter 5) as compared to the earlier experiment (95%, chapter 4) in feed restricted hens could be a reason for the smaller decrease in hen body mass. The reason for the difference in the laying rates between the feed-restricted hens of the two experiments remain unknown.

During feed restriction experiment described in chapter 5, the F1 follicle around the time of meiosis-I (i.e. around 26-28 hours before predicted egg laying) were collected after sacrificing the hens during last three days of experiment. The germinal disc region (GDR) was isolated from the F1 follicle and RNA was isolated for performing microarray analysis for gene expression studies. In response to the feed restriction, hens differed in the magnitude of decrease in egg mass. In the control group, the average egg mass of hens stayed approximately constant, but the eggs of some hens decreased while those of other hens increased in egg mass over time. The effects of feed restriction on sex ratio could potentially be due to its effects on egg mass. For the feed restricted hens and for the control hens that showed a declining egg mass over time, the egg sex ratio per hen was negatively associated with the average egg mass per hen. As a significant negative association was found between egg mass and sex ratio, two groups of hens were selected from the feed restriction group i.e. male biased hens with low egg mass and female biased hens with high egg mass for microarray analysis of gene expression in the germinal disc of collected F1 follicle. The results did not show differential expression of genes between the groups. However, gene set enrichment analysis showed that a number of processes related to cell cycle progression,

mitotic/meiotic apparatus and chromosomal movement were differently enriched between the groups, pointing towards potential mechanisms underlying sex ratio determination.

Corticosterone as a catabolic hormone is related to conditions of decreased food availability and decreasing body condition. Therefore, the effects of feed restriction on sex ratio could be potentially be mediated through corticosterone. In experiments described in chapter 6, blood circulating levels of corticosterone in hens were increased by feeding corticosterone mixed feed (20 mg/per kilogram of feed) under ad libitum conditions for 13 days. Thirty hens were allocated to each of control and treatment groups. Hens were inseminated twice a week using artificial insemination and eggs were sexed without incubation. This resulted in a gradual increase of circulating levels of corticosterone. This also disrupted the negative natural correlation between hen body mass and blood corticosterone concentrations. Corticosterone feeding treatment did not affect the overall sex ratio, but affected the sex ratio in interaction with hen body mass. In the corticosterone group, sex ratio, laying rate, and fertility rate per hen were decreased in heavy hens. In line with this, the overall laying rate and fertility rate per hen were positively associated with overall sex ratio per hen in the corticosterone group. Blood serum corticosterone concentration was not significantly associated with sex ratio and hen laying rate and was positively associated with hen fertility rate in corticosterone fed hens.

During studies described in this thesis, following experimental observations and results support meiotic drive as a mechanism of primary sex ratio bias. In the feed restriction experiments (chapter 4 and 5), treatment did not significantly affect the laying and fertility rates and the latter two were not associated with sex ratio. This suggests that follicle resorption, or selective ovulation or sex specific fertility cannot be the cause of the sex ratio bias by themselves. However, in corticosterone feeding experiment (chapter 6), treatment did significantly affect both the laying and fertility rates per hen and both these later two were significantly positively associated with sex ratio in the treatment group. Discrepancy of the results could be due to the fact that corticosterone feeding resulted in unnatural and supra-physiological blood corticosterone concentrations as compared to those achieved during feed restriction experiments. The results from corticosterone feeding experiment (chapter 6) suggest that the three parameters (sex ratio, laying rate and fertility rate) are connected at the level of ovarian physiology. In mammals, corticosterone had been previously shown to interfere with meiosis resulting in delayed ovulation and chromosomal abnormalities resulting in infertility. I propose that similar effects of corticosterone for interference with meiosis and

cell cycle could be present in the corticosterone feeding hens. This resulted in lower laying and fertility rates as well as lower sex ratio in heavy hens. This supports the sex specific chromosomal segregation ‘meiotic drive’ as a mechanism for the sex ratio bias which had already been proposed previously. In chapter 5 processes related to cell cycle progression, mitotic/meiotic apparatus and chromosomal movement were differently enriched between the male and female biased hen groups. Involvement of these processes appears to be involved in meiotic drive, with a higher activity of these processes leading to more female sex ratio bias. Further evidence for ‘meiotic drive’ comes from chapter 4, the sex of egg was significantly predicted by the rate of change of hen body mass in a specific time window (i.e between day of egg laying and day of laying minus 2 days or minus 3 days) that encompasses resumption of meiosis and was not significantly predicted during the time window which encompasses rapid yolk deposition phase before occurrence of meiosis (chapter 4).

Chapter 7 of this thesis discusses the results described above. In conclusion the results showed that unincubated eggs do not differ for the measured hormones and glucose concentrations, and therefore in-ovo sex determination is not possible based on these parameters. Laying hens could potentially change the primary sex ratio under certain conditions such as feed restriction or mimicking stress by experimentally elevating blood levels of corticosterone stress hormone. Meiotic drive is the most likely mechanism of sex ratio bias. The animal experimental models used in this thesis can be further used to elucidate the underlying mechanisms of primary sex ratio bias at molecular level. This could lead to finding ways and means to induce the sex ratio bias in poultry industry in a favourable way without compromising on the welfare and productivity of hens.

Samenvatting

Samenvatting

Dit proefschrift beschrijft onderzoek naar een aantal factoren die van invloed zijn op de geslachtsverhouding van kippeneieren. Beter inzicht in deze factoren is van algemeen belang voor verschillende gebieden van de biologie. Het is ook van belang voor de legkippen-sector met name als dit inzicht tot mogelijke oplossingen leidt voor het probleem van het doden van één dag oude mannelijke kuikens. Het doden van ééndagshaantjes in de pluimee sector roept maatschappelijke weerstand op. In de studies die in dit proefschrift beschreven zijn werd onderzocht of het geslacht van een kippenei kan worden voorspeld aan de hand van meetbare verschillen in mannelijke en vrouwelijke niet-bebroede eieren. Er werd ook onderzocht of de primaire geslachtsverhouding kan worden beïnvloed door leghennen bloot te stellen aan verschillende experimentele condities zoals voerbeperking en een behandeling met corticosteron. Nadat een experimenteel model was opgezet voor het moduleren van de primaire geslachtsverhouding, kon een begin gemaakt worden met het bestuderen van de onderliggende fysiologische en moleculaire mechanismen.

Hoofdstuk 1 bevat algemene achtergrondinformatie over de “sex allocation theory” en over factoren die van invloed zijn op de geslachtsverhouding bij vogels. Vervolgens wordt ingegaan op de relatie tussen bepaalde hormonen in de dooier of in het bloed van moederdieren met het geslacht van de gelegde eieren. Ook wordt de relatie tussen fysieke ei parameters met het geslacht van eieren besproken.

Hoofdstuk 2 beschrijft een methode van geslachtsbepaling van niet-bebroede kippeneieren met behulp van een PCR-test gericht op de geslachtsgebonden CHD1 genen. In de meeste eerdere studies over de geslachtsverhouding bij vogels wordt deze PCR-test pas ingezet nádat de eieren voor een bepaalde periode bebroed zijn. Echter, als er geslachtsspecifieke embryosterfte optreedt tijdens deze broedperiode kan de primaire geslachtsverhouding afwijken van de waargenomen (secundaire) geslachtsverhouding. Ook is het mogelijk dat bij eerder onderzoek naar de relatie tussen hormonen in de dooier en het geslacht van eieren de hormoonconcentraties tijdens het broedproces gewijzigd zijn. Als zulke veranderingen tijdens het broedproces geslachtsspecifiek zijn, dan zou het gebruik van bebroede eieren tot onjuiste conclusies kunnen leiden over de relatie tussen maternale hormonen in de dooier en het geslacht van het ei. Dit waren de twee belangrijkste redenen om een methode te ontwikkelen voor geslachtbepaling in niet-bebroede eieren. Tijdens de geslachtsbepaling van niet-bebroede eieren kan geen macroscopisch onderscheid gemaakt worden tussen een bevruchte of een

onbevruchte kiemschijf. Hierdoor kunnen fouten ontstaan door een PCR-amplificatie van het ouderlijke DNA dat geassocieerd is aan de vitelline membraan van onbevruchte eieren. Het risico op fouten werd echter op twee verschillende manieren tegengegaan. Allereerst werd de kiemschijf zoveel mogelijk ontdaan van granulosacellen door het verwijderen van de extra- en peri-vitelline lagen. Vervolgens werd onderscheid gemaakt tussen bevruchte en onbevruchte kiemschijven door aankleuring met Hoechst 33342. Hierbij zijn duizenden fluorescerende kernen zichtbaar in bevruchte eieren terwijl er geen kernen aankleuren in onbevruchte eieren. Bovendien werd uit de bevruchte eieren meer DNA geïsoleerd en waren de CHD1 PCR producten veel intenser op agarose-gel vergeleken met onbevruchte eieren. Dit wijst erop dat bevruchte kiemschijven veel meer (embryonaal plus ouderlijk) DNA bevatten dan niet-bevruchte kiemschijven (alleen ouderlijk DNA). Deze methode van geslachtsbepaling in niet-bebroede eieren werd toegepast voor alle overige studies die in dit proefschrift beschreven zijn.

Het is eerder aangetoond dat hormonen in het perifere bloed of in eidooiers het geslacht van eieren en de geslachtsverhouding van de nakomelingen kunnen beïnvloeden. In hoofdstuk 3 is onderzocht of er een directe relatie is tussen het geslacht van eieren en de concentraties van verschillende hormonen (testosteron, estradiol, androsteendion, progesteron, dihydrotestosteron) en glucose in eidooiers. Ook werd hierbij gekeken naar parameters zoals massa, breedte en lengte van het ei. Er werden in deze studie geen significante relaties gevonden. Er werden ook geen significante relaties gevonden tussen de totale geslachtsverhouding per hen en het gemiddelde van al haar eieren voor de gemeten parameters (hoofdstuk 3). Daarom achten we in-ovo geslachtsbepaling van niet-bebroede eieren met een of meerdere van de genoemde parameters op dit moment niet haalbaar. De resultaten suggereren ook dat de gemeten ei hormonen geen directe rol spelen bij de geslachtsbepaling van vogels.

Voor vrouwelijke vogels is aangetoond dat ze onder zowel experimentele als natuurlijke omstandigheden de geslachtsverhouding van hun nakomelingen kunnen moduleren als gevolg van verschillende externe factoren zoals bijvoorbeeld de beschikbaarheid van voedsel. Het effect van de beschikbaarheid van voeder op de geslachtsverhouding bij kippen werd in hoofdstuk 4 getest. Dertig legkippen werden beperkt gevoerd en eenzelfde aantal fungeerde als controle dieren. Leghennen werden tweemaal per week bevrucht via kunstmatige inseminatie en het geslacht van hun niet-bebroede eieren werd bepaald. Het restrictieve

voerbeleid werd over een periode van 17 dagen uitgevoerd, de eerste 7 dagen tot 80% en de volgende 10 dagen tot 70% van de "ad libitum" inname voorafgaand aan de voerrestrictie. Na afloop van de voeder restrictieperiode werden de hennen weer "ad libitum" gevoerd voor een periode van 9 dagen. In de voerrestrictie groep vonden we een significant effect van de voerbeperking in de tijd op de geslachtsverhouding van de eieren. De mate van de verandering van het lichaamsgewicht van de dieren gedurende de twee dagen voordat het ei werd gelegd, was een significante voorspeller voor het geslacht van dat specifieke ei. Voerrestrictie resulteerde in hogere corticosteron concentraties in het bloedplasma van de leghennen. Deze corticosteron concentraties waren geassocieerd met zowel het percentage bevruchte eieren als de geslachtsverhouding van de eieren per leghen. Overigens werd deze relatie maar op één van de drie dagen waarop bloed werd afgenomen gevonden. De geslachtverhouding van de eieren per leghen was niet geassocieerd met de vruchtbaarheid van hun eieren of met hun leg frequentie.

Het dierexperiment dat in hoofdstuk 5 is beschreven was erop gericht om de reproduceerbaarheid van het dierexperiment in hoofdstuk 4 te onderzoeken. Vijfenveertig legkippen werden willekeurig toegewezen aan een controle groep of aan een voerrestrictie groep. De laatste groep ontving gedurende 7 dagen 80% van de voorafgaande "ad libitum" inname en gedurende de daaropvolgende 9-11 dagen 70% van deze "ad libitum" inname onder dezelfde experimentele omstandigheden als in het experiment beschreven in hoofdstuk 4. De dieren werden tweemaal per week bevrucht met behulp van kunstmatige inseminatie en het geslacht van hun niet-bebroede eieren werd bepaald. In dit experiment werd niet alleen het effect van voorrestrictie op de geslachtverhouding van eieren bestudeerd maar ook het effect van voerrestrictie op het gewicht van eieren en de relatie tussen ei-gewichten en hun geslachtsverhoudingen. In dit experiment werd de associatie tussen de snelheid van gewichtsafname van de dieren en het geslacht van hun eieren, zoals waargenomen in hoofdstuk 4, niet meer gevonden. In vergelijking met het experiment van hoofdstuk 4 werd een veel kleinere afname in lichaamsgewicht gevonden (61,8 ± 43,4 g, hoofdstuk 5 versus 186 ± 60 g, hoofdstuk 4). Mogelijk is dit de reden waarom dit keer geen associatie werd gevonden tussen gewichtsafname en het geslacht van eieren. De lage leg frequentie in het experiment beschreven in hoofdstuk 5 (87%) ten opzichte van het experiment beschreven in hoofdstuk 4 (95%) in de voerrestrictie groepen kan een reden zijn voor de lagere gewichtsafname van de

leghennen. Waarom er verschil is tussen de twee experimenten in leg frequentie blijft onduidelijk.

Gedurende het dierexperiment beschreven in hoofdstuk 5 werden rond de tijd van meiose-I, ongeveer 26-28 uur vóór de verwachte leg, F1 follikels verzameld. Uit de kiemschijf regio's van deze follikels werd RNA geïsoleerd voor het uitvoeren van genexpressie studies m.b.v. microarray analyses. De eieren van leghennen uit de voerrestrictie groep waren lichter van gewicht dan die van de controle dieren. In de controle dieren bleven de ei-gewichten vrijwel constant, de ei-gewichten van sommige hennen nam af terwijl dat van andere hennen toenam. De effecten van voerrestricties op de geslachtsverhouding van eieren zou te wijten kunnen zijn aan de effecten van voerrestrictie op het gewicht van de eieren. In leghennen waarvan de ei-gewichten daalden in de tijd, van zowel controle als voerrestrictie dieren, werd inderdaad een significante negatieve correlatie gevonden tussen de geslachtsverhouding van de eieren per hen en het gemiddeld gewicht van de eieren per hen. Omdat een dergelijke negatieve correlatie werd gevonden, werden twee groepen leghennen geselecteerd uit de voerrestrictie groep: leghennen die lichte eieren produceren met een "bias" voor het mannelijke geslacht en leghennen die zware eieren produceren met een "bias" voor het vrouwelijke geslacht. Het RNA uit de kiemschijf van de F1 follikel van deze twee groepen leghennen werd gebruikt voor profilering van de genexpressie. Hoewel er tussen de groepen leghennen geen significante verschillen in de expressie van individuele genen werd gevonden, bleken een aantal processen wel degelijk te verschillen tussen de twee groepen dieren. De processen die verschillend waren hebben betrekking op de voortgang van de cel cyclus, op processen betrokken bij mitose en meiose en bij het bewegen van chromosomen van en naar het evenaarsvlak in delende cellen. Dit wijst op een mogelijk mechanisme dat ten grondslag ligt aan de geslachtbepaling van eieren.

Corticosteron is als een katabool hormoon gerelateerd aan condities die optreden bij een verminderde beschikbaarheid van voeder en bij verminderde lichaamscondities. Hierdoor zouden de effecten van voerrestrictie bewerkstelligt kunnen worden via tussenkomst van corticosteron. In experimenten beschreven in hoofdstuk 6 werden de corticosteron bloedwaarden in leghennen verhoogd door toevoeging van corticosteron aan het voer (20 mg / per kilogram voeder) gedurende 13 dagen. De controle en behandelgroep, elk bestaande uit 30 leghennen, werden twee keer per week bevrucht met behulp van kunstmatige inseminatie en het geslacht van hun niet-bebroede eieren werd vastgesteld. De behandeling resulteerde in een

geleidelijke verhoging van de corticosteron bloedwaarden en verstoorde de natuurlijke negatieve correlatie tussen lichaamsgewicht en corticosteron concentraties in het bloed. De corticosteron behandeling had geen invloed op de totale geslachtsverhouding, maar had wel invloed op de geslachtsverhouding in interactie met het lichaamsgewicht. In de corticosteron groep namen bij de zwaardere leghennen de geslachtsverhouding, de leg frequentie en het percentage bevruchte eieren per hen af. De positieve associatie tussen leg frequentie en de vruchtbaarheid per hen met de totale geslachtsverhouding per hen in de corticosteron groep, is hiermee in overeenstemming. In de behandel groep waren de corticosteron concentraties in het bloed niet significant geassocieerd met geslachtsverhoudingen in de eieren en met de leg frequentie per hen. De corticosteron concentraties in het bloed waren echter significant positief geassocieerd met het percentage bevruchte eieren per hen.

De resultaten van de experimenten die in dit proefschrift beschreven zijn, ondersteunen de hypothese dat het mechanisme voor de primaire geslachtsverhouding is gebaseerd op meiotische “drive”. In de voerrestrictie experimenten (hoofdstuk 4 en 5) had de behandeling geen significant effecten op de leg frequentie en op het percentage bevruchte eieren, terwijl deze twee parameters niet geassocieerd zijn met de geslachtsverhouding van eieren. Dit suggereert dat follikel resorptie, selectieve ovulatie of geslachtspecifieke vruchtbaarheid op zichzelf niet de oorzaken kunnen zijn van veranderingen in de geslachtsverhouding. Echter, de behandeling met corticosteron (hoofdstuk 6) had een significant effect op de leg frequentie en op het percentage bevruchte eieren, terwijl beide parameters significant positief geassocieerd zijn met de geslachtsverhouding van eieren in de behandelde groep. Dit verschil in resultaten kan te wijten zijn aan het feit dat de toediening van corticosteron tot een onnatuurlijk en supra-fysiologische concentratie van corticosteron in het bloed leidt vergeleken met de corticosteron bloedwaarden die bereikt worden in de voerrestrictie experimenten. De resultaten van de corticosteron behandeling (hoofdstuk 6) suggereren dat de drie parameters, geslachtsverhouding, leg frequentie en percentage bevruchte eieren op de een of andere manier gekoppeld zijn aan fysiologische processen in de ovaria. Voor zoogdieren is aangetoond dat corticosteron interfereert met de meiose en dat het leidt tot verlate ovulatie en chromosomale afwijkingen en uiteindelijk tot onvruchtbaarheid. Ik stel voor dat vergelijkbare effecten van corticosteron bij leghennen plaatsvinden en dat in de dieren, die blootgesteld worden aan een voerrestrictie, corticosteron interfereert met de meiose en de cel cyclus. Dit resulteert in lagere leg-frequenties en lagere percentages bevruchte eieren en in lagere

geslachtsverhoudingen in de eieren van zware leghennen. Deze gedachtenlijn ondersteunt het idee dat geslachtspecifieke segregatie van chromosomen, oftewel meiotische ‘drive”, ten grondslag ligt aan de modulatie van geslachtsverhoudingen van eieren. De cellulaire processen die verschillen tussen leghennen met een voorkeur voor mannelijke dan wel vrouwelijke nakomelingen (hoofdstuk 5), met name de processen die betrekking hebben op de voortgang van de celcyclus, op het mitose en meiose apparaat en op chromosomale bewegingen, wijzen in dezelfde richting. Al deze processen lijken betrokken te zijn bij de meiotische “drive”, met een hogere activiteit van deze processen in leghennen met een voorkeur voor vrouwelijke nakomelingen. Additionele ondersteuning voor het meiotisch “drive” principe komt uit hoofdstuk 4. Hier werd gevonden dat het geslacht van het ei nauw samenhangt met de mate van veranderingen in het lichaamsgewicht van de leghennen over een bepaald tijdsbestek: met name gedurende de twee dagen vóór het leggen van het ei. Dit tijdsbestek bestrijkt juist de periode waarin de meiose plaatsvindt. Opvallend hierbij is dat in het tijdinterval vóór de meiose waarin dooier afzetting plaatsvindt, er geen verband werd gevonden tussen de veranderingen in het lichaamsgewicht en de geslachtsverhouding in eieren.

Hoofdstuk 7 bediscussieert de resultaten en belangrijkste conclusies van dit proefschrift. 1) Een in-ovo geslachtsbepaling in niet-bebroede eieren is niet mogelijk met de hormonen en glucose die in dit hoofdstuk onderzocht zijn. 2) Leghennen kunnen de primaire geslachtsverhouding van hun nakomelingen onder bepaalde omstandigheden veranderen, bijvoorbeeld door voerrestrictie of door het op een of andere manier verhogen van de bloedwaarden van het stresshormoon corticosteron. 3) Meiotische “drive” is het meest waarschijnlijke mechanisme voor de modulatie van geslachtsverhoudingen door leghennen. 4) Het experimentele model dat in dit proefschrift beschreven is, kan toegepast worden voor het verder ontrafelen van de moleculaire mechanismen die hieraan ten grondslag liggen. Dit vervolgonderzoek kan mogelijk bijdragen aan het vinden van nieuwe manieren om in de pluimvee sector de geslachtsverhouding van eieren in een gunstige richting te sturen zonder afbreuk te doen aan het welzijn en productiviteit van de dieren.

Curriculum Vitae

About the Author

Muhammad Aamir Aslam was born on 1ST July 1983 in Faisalabad. In 2005, he graduated from the University of Agriculture, Faisalabad obtaining the degree of Doctor of Veterinary Medicine (DVM). In 2007, he also did his master from the same university in "Veterinary Microbiology" during which he investigated the antimicrobial effects of few selected herbal extracts on 3 bacterial species. In 2007, he won a scholarship from Higher Education Commission of Pakistan to pursue his higher education in The Netherlands. In 2007, he started his master degree "Molecular Mechanisms of Diseases" in Nijmegen Centre for Molecular Life Sciences (NCMLS), Radboud University, Nijmegen. During this master, he performed two research internships. During the first research internship at NCMLS, he investigated the role of post translational epigenetic histone modifications in the DNA damage repair response in yeast. During the second research internship at Erasmus Medical Centre in Rotterdam, he investigated the immunomodulatory effects of the recombinant Hepatitis C virus core protein on the innate immune cells (macrophages and dendritic cells) obtained from healthy human donors. In 2009, he started his PhD at Wageningen University during which he investigated different factors affecting sex of egg and sex ratio in commercial laying chicken. The results of his PhD research are presented in this thesis.

Peer reviewed publications

M. A. Aslam, Hulst M, Hoving-Bolink RA, de Wit AA, Smits MA, Woelders H. A reliable method for sexing unincubated bird eggs for studying primary sex ratio, Molecular Ecology Resources (2012) 12, 421–427.

M. A. Aslam, Hulst M, Hoving-Bolink RA, Smits MA, de Vries B, Weites I, Groothuis TG, Woelders H.Yolk concentrations of hormones and glucose and egg weight and egg dimensions in unincubated chicken eggs, in relation to egg sex and hen body weight, General and Comparative Endocrinology , 187, 15 June 2013, Pages 15–22.

M. A. Aslam, Smits MA, Groothuis TG, Woelders H Effect of corticosterone and hen body mass on primary sex ratio in laying hen (Gallus gallus), using unincubated eggs, Biology of Reproduction. 2014 Apr 10;90(4):76.

Conference proceedings

M. A. Aslam, Hulst M, Smits MA, and Woelders, H. Sexing of day zero unincubated chicken eggs (Gallus gallus), IFRG meeting, Ede, 30 – 31st August 2011.

M. A. Aslam, Hoving-Bolink RA, Smits MA, Groothuis TG and Woelders, H. Levels of maternally allocated steroid hormones and glucose in the yolk of unincubated male and female chicken eggs, PDP workshop, Wageningen, 1-2 September, 2011.

M. A. Aslam, Hoving-Bolink RA, de Wit AA, Smits MA, Groothuis TG, Woelders H. Decrease in body weight of laying hen due to feed restriction leads to a female biased primary sex ratio, XXIV international poultry symposium PB WPSA, Poland, 12-14 September 2012.

M. A. Aslam, Groothuis TG, Hoving-Bolink RA, de Wit AA, Dijkstra C, Smits MA, Woelders H, Feed restriction induced change in body mass affects offspring sex ratio in laying hens, NVG annual meeting, Soesterberg, 28-30 Nov 2012.

M. A. Aslam, Hulst M, Hoving-Bolink RA, de Wit AA, Dijkstra C, Groothuis TG, Smits MA, Woelders H, Association of maternal hormones with sex of egg and sex ratio in chicken: towards potential solution for killing one-day old male chicks, WIAS Science day, Wageningen, 28 Feburary, 2013.

M. A. Aslam, Schokker D, de Wit AA, Groothuis TG, Smits MA and Woelders H, Association of egg mass with egg sex in layer hens (*Gallus gallus*): gene expression analysis from germinal disc region of F1 follicle around time of meiosis, NVG annual meeting, Soesterberg, 28-29 November, 2013.

Training and supervision plan

Training and Supervision Plan

The Basic Package (3 ECTS)

WIAS Introduction Course	2010
Ethics and Philosophy of Animal Science	2010

Scientific Exposure (9 ECTS)

International Conferences (2.5 ECTS)

The 5th workshop on fundamental physiology and perinatal development in poultry, Wageningen	2011
Incubation and fertility research group (WPSA working Group 6) Meeting – EDE	2011
XXIV international poultry symposium PB WPSA, Poland	2012
Annual meeting of the 184etherlands society for behavioural biology, Soesterberg (2x)	2012-2013

Seminars and Workshops (0.5 ECTS)

WIAS science day (2x)

Presentations (6 ECTS)

Levels of maternally allocated steroid hormones and glucose in the yolk of unincubated male and female chicken eggs, PDP workshop, Wageningen (oral)	2011
Sexing of day zero unincubated chicken eggs (Gallus gallus), IFRG meeting, Ede (oral)	2011
Decrease in body weight of laying hen due to feed restriction leads to a female biased primary sex ratio, XXIV international poultry symposium PB WPSA, Poland (oral)	2012
Feed restriction induced change in body mass affects offspring sex ratio in laying hens, NVG annual meeting, Soesterberg (poster)	2012
Association of maternal hormones with sex of egg and sex ratio in chicken: towards potential solution for killing one-day old male chicks, WIAS Science day, Wageningen (oral)	2012
Association of egg mass with egg sex in layer hens (*Gallus gallus*): gene expression analysis from germinal disc region of F1 follicle around time of meiosis, NVG annual meeting, Soesterberg (poster)	2012

In-Depth Studies (6 ECTS)

Disciplinary and interdisciplinary courses (1.2 ECTS)

Analysis of microarray gene expression data, Leiden University	2010

Advanced statistics courses (4.5 ECTS)

Statistics for life sciences, Wageningen University, The Netherlands	2011
Introduction to Multilevel Analysis, Utretch University, The Netherlands	2013
Advanced Statistics course Design of Experiments, Wageningen University, The Netherlands	2013

Statutory Courses (1 ECTS)

Laboratory Use of Isotopes, Wageningen University, The Netherlands	2010

Professional Skills Support Courses (3 ECTS)	
Techniques for writing and presenting a scientific paper, Wageningen University, The Netherlands	2011
Project and time management, Wageningen University, The Netherlands	2011
Information literacy PhD including Endnote introduction, Wageningen University, The Netherlands	2012
PhD Competence assessment or Job assessment, Wageningen University, The Netherlands	2012
Research Skills Training (9 ECTS)	
Preparing own PhD research proposal	2010
External training period at University of Groningen, The Netherlands	2010
Introduction to R for Statistical Analysis, Wageningen University, The Netherlands	2012
Education and Training Total	**31 ECT**

Acknowledgements

Acknowledgements

The adventure towards getting a PhD degree started in Sep, 2007 by arriving in Nijmegen in an adventurous manner. At that day the Dutch railway company stopped the Nijmegen-destined train in Arnhem and by not understanding the announcement in the Dutch language, I became locked in a train compartment. A Dutch boy who also caged himself in the train while sleeping set both of us free from the locked train after calling to police. During my journey towards getting a PhD degree there were also some ups and downs, but finally I succeeded in finishing my thesis. Successful completion of this journey was not possible without the continuous support of many people around me. Thanks to all those people.

First of all I would like to thank my daily supervisor Dr. Henri Woelders for continuous support and guidance throughout my PhD studies. Thank you for your discussions on the data, neither I nor you was a statistician, but your continuous discussions with me on different aspects of the data made me more like a statistician than a biologist. Thank you for your time for improving my critical thinking and writing skills. I am very grateful to you for your enthusiasm and time investment in me. Thanks to my supervisors Prof. Ton Groothuis and Prof. Mari Smits for their time and guidance. Ton, thank you for giving me the opportunity to work in your lab and thank you for your time investment. Thank you for often coming to Lelystad for discussions and thanks for your critical comments on the research work and research papers. Mari, thank you for your investment of time during our meetings and for your critical comments on my work and papers.

I am thankful to Agnes de Wit for her continuous support during the laboratory work and her participation and help during the animal experiments. Thanks to Rita Hoving for organizing and taking part in meetings and guidance during this project. I am thankful to Dr. Marcel Hulst for his support and guidance during the laboratory work.

I am thankful to Cor Dijkstra and Martina Muller for their help with data analysis. I am grateful to Bonnie de Vries and Ilse Weites for guiding me during the laboratory work in Groningen and for their investment of time in me. I am thankful to all my colleagues at Lelystad for giving nice time at social gatherings and coffee breaks. Thanks to Arun Kommadath, Han Mulder and Mario Calus for giving tips and guidance on R software and statistics. I am thankful to Dirkjan Schokker for his tips on R and help in microarray data analysis.

I am thankful to all my friends in The Netherlands for organizing social gathering. Thanks to Waseem, Faisal, Tayyab, Khurshid, Fraz, Muzamil, Yahya, Tariq, Rubbaniy, Kaleem, Ali, Shahbaz, Zeeshan, Imran, Riaz, Shoaib and Saboor for their support. Again thanks to Waseem for providing me with books on R.

I am thankful to my family members for their continuous moral support. I am very grateful to my mother and father for their love, affection and support. I am thankful to my brother and sisters for always supporting me and remembering me in their good thoughts. I am thankful to my wife for her support during my stay in the Netherlands.

Colophon

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The research described in this thesis was supported by Ministry of Economic Affairs, The Netherlands under the project number BO-12.02.002.7.01.

The printing of this thesis was funded by the Animal Breeding and Genomics Centre, Wageningen University, Netherlands.

The cover of this thesis was designed by Muhammad Aamir Aslam.

This thesis was printed by GVO drukkers & vormgevers B.V. | Ponsen & Looijen, Ede, Netherlands.