Androgen and mineralocorticoid receptors are present on the germinal disc region in laying hens: Potential mediators of sex ratio adjustment in birds?

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Abstract

Female birds skew offspring sex ratios based on environmental and social stimuli; however, the mechanism mediating this phenomenon remains unknown. Growing evidence suggests that testosterone and corticosterone may influence meiosis, as they skew sex ratios when given immediately before chromosomal segregation. It is unclear if these hormones act on the germinal disc (GD) or through a downstream mediator. It is also unknown whether the GD contains receptors for these hormones. If testosterone and/or corticosterone act on the GD to skew sex ratios, then the GD should have receptors for them and that receptor levels should be higher in the GD regions compared to other follicular regions. Furthermore, fluctuations of receptor levels should occur near meiotic segregation. We collected ovarian follicles at 5h pre-ovulation (just before meiotic segregation) and 20h pre-ovulation (when sex chromosomes are arrested), and measured androgen receptor (AR) and mineralocorticoid receptor (MR) protein levels via Western blot. ARs and MRs were on the follicle in the GD and non-GD regions, and at 5h and 20h pre-ovulation. Both AR and MR protein levels were higher in the GD region than the non-GD region at both time points, but did not differ between time points. These results suggest that hen ovarian follicles have receptors for testosterone and corticosterone, and that the ability for testosterone to respond may be specifically higher in the GD-region, providing further support for the role of testosterone in the alteration of meiotic segregation.

Keywords: androgen receptor, mineralocorticoid receptor, testosterone, corticosterone, sex ratio, maternal effects, chickens, birds, avian, hormones

1. Introduction

Many studies have shown that female birds can control the sex ratios of their offspring in response to a wide range of environmental and social stimuli (Dijkstra et al., 1990; Ellegren et al., 1996; Nager et al., 1999). For example, Komdeur et al. (1997) showed that Seychelles warblers (*Acrocephalus sechellensis*) biased offspring sex ratios based on territory quality and the number of helpers present in the territory. In the great tit (*Parus major*), females that mated with large, high-quality males produced more sons (Kölliker et al., 1999). In kakapos (*Strigops habroptilus*), supplementary food provided to female birds in breeding sanctuaries resulted in male-skewed offspring sex ratios (Clout et al., 2002). The mechanism by which sex ratio manipulation occurs in birds, however, remains unknown. In birds, females are the heterogametic sex so it is the mother that determines offspring sex. Since, in most cases, sex ratio adjustment occurs without the loss of eggs or embryos in the laying sequence, it appears that birds can adjust their sex ratios before ovulation even occurs (reviewed in Pike and Petrie, 2003). Further, hormones are good candidates as mediators of sex ratio determination because they convert external stimuli that the mother experiences in her environment into physiological responses, and treatment with multiple hormones has been shown to skew sex ratios in birds (reviewed in Goerlich-Jansson et al., 2013 and Navara 2013).

There is mounting evidence that both testosterone and corticosterone may be potent modulators of offspring sex ratios in birds. Many studies have shown that mothers with higher levels of testosterone skew their offspring sex ratios towards males (Viega et al., 2004; Pike and Petrie, 2005; Rutkowska and Cichon, 2006; Goerlich et al., 2009; Pinson et al., 2011b). For instance, when plasma testosterone levels were artificially elevated during the breeding season in female spotless starlings (*Sturnus unicolor*), they produced significantly more male offspring

(Veiga et al., 2004). Other studies have indicated that elevations in circulating levels of the stress hormone, corticosterone, over a long period of time result in a higher production of female offspring (Pike and Petrie, 2005; Pike and Petrie, 2006; Bonier et al., 2007). Bonier et al. (2007) found that female white-crowned sparrows (*Zonotrichia leucophrys*) with naturally higher levels of corticosterone had significantly more daughters. Additionally, females with time-release corticosterone pellets produced more female offspring than controls. Similarly, Pike and Petrie (2006) demonstrated that female Japanese quail (*Coturnix japonica*) with silastic corticosterone implants produced nearly 70% female offspring. While these studies illustrate that corticosterone treatment in the mother can skew offspring sex ratios, it is not always in the same direction. In zebra finches (*Taeniopygia guttata*) and domestic chickens, a pharmacological dose of corticosterone 5h before ovulation resulted in male-biased sex ratios (Gam et al., 2011; Pinson et al., 2011a). However, when chickens were treated with either a low or high physiological dose of corticosterone at 5h prior to ovulation, there was no influence on offspring sex ratios. But chickens that received the same corticosterone treatment one hour later (4h before ovulation) skewed sex ratios towards females (Pinson et al., 2015). Taken together, these results are very compelling because they show that (1) both hormones can stimulate sex ratio skews in multiple avian species, (2) the timing and dosage of hormones can influence the direction of the sex ratio skew, and (3) giving the hormones immediately prior to the segregation of the sex chromosomes can influence which sex chromosome the offspring inherits. This indicates that these hormones can exert control over sex chromosome movement. It remains unclear, however, whether these hormones are acting directly on the germinal disc during the time of meiosis to adjust sex ratios, or whether they are acting via another downstream mediator.

In the avian ovary, there are thousands of ovarian follicles that contain both W and Z sex chromosomes; these follicles eventually either undergo atrophy or are recruited to the pre-ovulatory follicle hierarchy (Fig. 1A) (Goerlich-Jansson et al., 2013). Towards the end of embryonic development, the follicles arrest halfway through meiosis I, where they are still in the diploid state and contain both sex chromosomes. They remain arrested throughout adulthood, and even after the follicles have been selected into the preovulatory hierarchy and are destined for ovulation. Preovulatory follicles are labeled according to size (F1 is the largest, F5 is the smallest), and in chickens, around 3-5 hours before the F1 ovulates, meiosis I completes in the germinal disc (GD) carried by that follicle, and one chromosome is retained in the oocyte and the other is discarded in the polar body (Fig. 1B) (Johnson 2000). It is at this point that the sex of the potential offspring is determined. Some have suggested that hormones may indirectly influence offspring sex by altering follicle growth and development (Young and Badyaev, 2004), though the evidence that a single injection immediately prior to ovulation can stimulate sex ratio skews indicates the potential for even more direct influences on the segregation of sex chromosomes. It has also been suggested that hormones may directly control sex ratio determination by acting on receptors on the GD region (Fig. 1C) to alter the expression of genes associated with sex chromosome segregation (Rutkowska and Badyaev, 2008). For this to happen, however, there must be receptors present on the GD that respond to these hormones. To our knowledge, whether receptors for testosterone and corticosterone are present on the germinal disc has never been tested for any avian species.

Androgen receptors (ARs) are the main receptors responsible for responding to testosterone (Chang et al., 1995) and mineralocorticoid receptors (MRs) are the receptors with the highest affinity for corticosterone (Eberwine 1999), thus we hypothesized that if testosterone

and/or corticosterone acts directly to influence sex chromosome segregation in birds, the germinal disc region of the F1 follicle would have both ARs and MRs present. Using the domestic chicken (*Gallus domesticus*) as our model system, we first examined if AR and MR protein levels differed between GD regions and non-GD regions collected from F1 follicles. We then compared AR and MR protein levels between GD regions collected right before the time of meiotic segregation (5h prior to ovulation) and GD regions collected at a time well before meiotic segregation was due to occur (20h prior to ovulation). We predicted that, if testosterone and/or corticosterone acts directly on the GD to influence offspring sex, then AR and/or MR numbers on the GD should be highest at the time right before meiotic segregation was due to occur (5h prior to ovulation) and that the GD regions should have higher receptor levels than non-GD regions. This study is the first step in helping us better understand the hormonal mechanism that controls offspring sex ratio determination.

2. Methods

2.1 General procedures

Single-comb Hy-Line W36 White Leghorn hens (n=400) were reared on the floor according to Hy-Line guidelines until they reached reproductive maturity. We then transferred them to individual layer cages in a single room, where they had *ad libitum* access to food and water throughout this study. They were maintained on a standard breeding light schedule of 16h light: 8h dark. Since ovulation occurs within 30 minutes of oviposition of the previous egg in laying hens (Johnson 1996), we used egg-laying patterns to predict the timing of ovulation of each individual bird used in the study. When hens reached 30 weeks of age (after egg production had maximized), oviposition times of all hens were monitored between 0830 AM – 1130 AM daily for 5 weeks, allowing us to predict the precise ovulation time for each hen. The 70 most

consistent layers were used for this experiment. From these layers, we collected tissue from ovarian follicles as outlined below for the measurement of AR and MR protein levels. On the day of collection the birds ovulating at the correct time were randomly assigned to a treatment group before beginning tissue collections.

2.2 Tissue Collection

Approximately 3-5 h before ovulation, the attachment of spindle fibers and segregation of sex chromosomes in the GD occurs (Johnson, 2000). We aimed to collect ovarian tissues right before this sex chromosome segregation was due to occur. At ~5h prior to ovulation, 70 hens were rapidly killed via lethal injection, and the F1 ovarian follicle was dissected out. An additional 70 hens were killed at ~20h before ovulation and F1 follicles were collected. The GD region was removed from the F1 follicle and briefly washed in Krebs buffer to remove any yolk material. It was then placed in 0.5mL reaction tube filled with 300µl of Arcturus® PicoPure® Extraction buffer and snap-frozen in liquid nitrogen. One GD region constituted each sample run in the Western Blot analyses. Samples were not pooled. From each follicle, a region of non-GD material was also collected from the area on the opposite side of the GD-region to compare chicken AR and MR protein levels.

2.3 Measurement of AR and MR Protein Levels

For measurement of AR and MR protein levels, tissues were homogenized in T-PER tissue protein extraction reagent (ThermoFisher Scientific) freshly supplemented with Halt protease inhibitor cocktail (catalog no. 78410; ThermoScientific). Tubes were centrifuged at 3000 rpm for 15 min at 4°C to remove cellular debris. Protein concentration was measured by Bio-Rad protein assay (Bio-Rad) using BSA as standard. Aliquots of 15ug protein were dissolved in 50µl of Laemmli buffer (catalog no. S3401; Sigma-Aldrich) containing 5% β-41

mercaptoethanol and boiled for 5 min at 95°C. Denatured protein lysates were run on 8% gels and then transferred to nitrocellulose membranes (catalog no. 1620168; Bio-Rad), which were subsequently incubated in Revert total protein stain following LICORs protocol and read on LICOR Odyssey Infrared Imaging System. Membranes where then incubated in Odyssey blocking buffer for 1 h at room temperature to reduce nonspecific binding by antibody and then incubated with primary antibodies in blocking buffer overnight at 4°C (Table 1). The next day, blots were washed three times in 0.1% Tween-20 PBS (TPBS) to remove unbound antibodies before incubation with the appropriate secondary antibody. Membranes were washed four times with 0.1% TPBS and exposed using the LICOR Odyssey Infrared Imaging System to detect protein presence. Relative protein amounts in identified immunoblots were measured as optical density of the bands using Image Studio analytical software. Proteins were normalized using total protein as a control. Total protein staining has been proven to be an efficient and even superior way to normalize western blots over housekeeping proteins (Aldridge et al., 2008; Eaton et al., 2013; Gilda and Gomes, 2013). Housekeeping proteins have been shown to be variable between animals and tissues. This is the reason we chose to go with total protein staining. This antibody-independent method corrects for variation in both sample protein loading, transfer efficiency, and monitors protein transfer across the blot at all molecular weights. After transfer but before immunodetection the membrane was treated with Revert® total protein stain to assess sample loading across the blot. Total protein stain is measured on the same blot and the same lane as each band it is being normalized to. The Western blot for AR protein abundance showed two bands near the location at which we expected the AR to be for both the GD and non-GD regions. This is common during measurement of AR levels using Western Blot because there are often multiple isoforms of androgen receptor present, so as done in previous studies (Pfaehler et

al., 2012), we added the signal from the two bands for our analyses. For the AR analysis, we measured protein abundance in samples collected 5h pre-ovulation (26 GD and 24 non-GD), and at 20h before ovulation (24 GD and 24 non-GD). MR protein abundance was measured in 30 GD and 29 non-GD samples from 5h pre-ovulation, and in 28 GD and 29 non-GD samples collected at 20h pre-ovulation.

Table 1. Antibodies used in immunoblotting procedures.

Antibody	Dilution	Host Species	Catalog no.	Company
Androgen	1200	Rabbit polyclonal	A9853	Sigma-Aldrich
Mineralocorticoid	1000	Mouse monoclonal	ab2774	Abcam Inc.
IRDye® 800CW Goat anti-Rabbit	15000	Goat	926-32211	Li-Cor
IRDye® 800CW Goat anti-Mouse	10000	Goat	926-32210	Li-Cor

2.4 Data Analyses

Statistical comparisons were made with the two-way ANOVA followed by Tukey's test. We used JMP® Pro 12 for all data analyses. $P<0.05$ was considered statistically significant.

3. Results

We found that ARs and MRs are present on the hen ovarian F1 follicle, and in particular, on the GD-region. Androgen receptor protein levels varied significantly according to region (Two-Way ANOVA; model: $F_{2,94}=65.64$, $p<0.0001$). AR protein levels were significantly higher on GD regions than on non-GD regions, both at 5h prior to ovulation and at 20h prior to ovulation ($p<0.0001$, Fig. 2A,B). There was no significant difference in AR protein levels between GD regions collected 5h before ovulation and GD regions collected 20h before ovulation ($p=0.89$, Fig. 2A,B), and there was no interaction between location in relation to the GD and the time of collection ($p=0.89$).

MR protein was found on both GD and non-GD-regions, and, like AR protein levels, also differed significantly between the two regions (Two-Way ANOVA; model: $F_{2,111}$= 38.73, p<0.0001, Fig. 3A,B). MR protein levels did not differ between the two collection time points (p=0.31), and there was no significant interaction between the location in relation to the GD and the time of collection (=0.31)

4. Discussion

While there is still much to be learned about when and how hormones act to adjust offspring sex, this study has provided new insight into this process. First, we showed that there are receptors for both testosterone and corticosterone present on the GD region, which is where the segregation of the sex chromosomes takes place. Second, we showed that both AR and MR protein levels are highest in the GD region of the F1 follicle at both 5h and 20h before ovulation. This indicates that there are pathways available by which both hormones may influence the activities within the germinal disc, and that receptors are concentrated specifically within this region. When collecting the GD region, we purposely collected the layer of granulosa cells immediately surrounding the GD to get an assessment of AR and MR protein levels in the GD *region*. Multiple studies have demonstrated that granulosa cells located near the GD have an important role in regulating follicular growth and serves as a source of growth factors (Yokinori et al., 1994; Volentine et al., 1998). In future studies, it is now important to tease apart the granulosa layer from the GD to determine whether it is ARs and MRs in the granulosa layer or the GD itself that may be directing the impacts of androgens and glucocorticoids on the meiotic process.

If the GD is more sensitive to androgen and glucocorticoid activity throughout the ovulatory cycle, then testosterone and corticosterone may be able to manipulate offspring sex by

influencing meiosis. Hormones may be able to act on the GD region right before the sex chromosomes start to separate, which would influence which chromosome is retained in the GD and which one is discarded in the polar body. In the GD of the preovulatory follicle, two pairs of homologous chromosomes are maintained as bivalents. A few hours prior to ovulation these bivalents travel to the meiotic plate. Here, spindle fibers attach to the bivalents and pull the chromosomes towards opposite sides of the follicle. Chromosomes pulled to the top are discarded into the polar body while those at the bottom are kept in the oocyte (Rutkowska and Badyaev, 2008). Hormones could influence the movement of the chromosomes by interacting with cellular machinery. Alternatively, they may adjust calcium gradients to influence the actin filament network to control meiotic segregation (reviewed in Rutkowska and Badyaev, 2008). Androgen receptors are known to interact with intracellular calcium regulatory mechanisms to modulate intracellular ion concentrations (Foradori et al., 2007). Also, Axelsson et al. (2010) showed that centromeres and telomeres potentially play a role in non-random chromosome segregation by controlling chromosome movement in chickens. We do know that sex steroids and glucocorticoids are capable of influencing telomere length. Stier et al. (2015) showed that testosterone may mediate telomere erosion in free-living great tit nestlings (*Parus major*). Higher baseline corticosterone levels were associated with shorter telomeres in thorn-tailed rayadito nestlings (*Aphrastura spinicauda*) (Quirici et al., 2016). However, in all studies examining influences of steroid hormones on telomere lengths to date, the effects were long-term effects. It is unclear whether steroid hormones can influence telomere lengths within a matter of hours, as would be required here if this were the mechanism responsible for sex ratio adjustment in birds.

At this point, whether testosterone and/or glucocorticoids do indeed act through androgen and mineralocorticoid receptors to influence sex ratio adjustment in the GD, and through what

mechanism this may occur, remains speculative. It is also possible that these hormones are acting in an indirect manner, through other potential mediators. For example, elevations in corticosterone concentrations lead to changes in levels of blood glucose, circulating fatty acids, and many other potentially relevant physiological components (Remage-Healey and Romero, 2001). In addition, it has been suggested that these hormones may act to influence rates of yolk deposition, which then impacts meiotic segregation. Since yolk deposition stops about 24h prior to ovulation, this would not likely explain the influences that these hormones can exert just hours prior to ovulation, but it is possible that more than one mechanism exists to skew sex ratios. This would explain why MR and AR protein levels do not appear to fluctuate over time.

We predicted that levels of AR and MR protein would be highest immediately prior to ovulation, when sex chromosomes are segregating, and lower at a time distant from ovulation. We did not see this. Instead, AR and MR protein levels were similar whether they were collected at 5h or 20h prior to ovulation. This may not be particularly surprising, however, because both androgens and glucocorticoids likely influence developing follicles in many ways. For example, a study in house finches indicates that testosterone may be related to rates of yolk accumulation during rapid yolk deposition (Young and Badyaev 2004). In addition, it is possible that the effects of hormones on the germinal disc are not regulated through fluctuations in receptor concentrations, but instead are completely dependent on levels of hormones. Johnson and van Tienhoven found in chickens that corticosterone levels rise significantly at the time of ovulation, and this elevation of corticosterone is critical for the process of ovulation (Johnson and van Tienhoven, 1980, Johnson and van Tienhoven 1981, Etches and Cunningham, 1976, Etches, 1977).

While MRs are the receptors with the highest affinity for corticosterone, it is also important in the future at measure glucocorticoid receptors (GRs). The MR has a 10-fold higher binding affinity for glucocorticoids than the GR and baseline levels of corticosterone bind with high affinity to MRs (Breuner and Orchinik, 2009). If corticosterone starts to elevate in response to a stressor, however, MR receptor densities become saturated and the hormone will then bind to GRs (Krause et al., 2015). Given this, it is possible that GRs rather than MRs are the direct link between corticosterone and sex ratio adjustment when birds are experiencing chronic stress and Johnson and Van Tienhoven (1980) documented that corticosterone is elevated above baseline when meiotic segregation is occurring. Thus, in future studies, it should be explored whether GR regulation in the GD varies near meiotic segregation.

5. Conclusions

This experiment is the first step towards understanding how testosterone and corticosterone may interact with the GD to control sex ratio adjustment in birds. Our results suggest that there is potential for both testosterone and corticosterone to act directly at the level of the GD to influence offspring sex. However, there is still much to be learned about how testosterone and corticosterone ultimately skew offspring sex. The next step is to examine if administering hormone injections to laying hens at the time meiosis influences the expression of genes involved in that process. In the long term, a full understanding of this mechanism could allow for purposeful manipulation of avian sex ratios in both poultry industry and conservation contexts.

List of abbreviations

GD = germinal disc; AR = androgen receptor; MR = mineralocorticoid receptor; NGD = non-GD region

Declarations

Ethics approval and consent to participate

This work was approved by the University of Georgia Institutional Animal Care and Use Committee (PRN #A2017 10-019-Y1-A0). All procedures performed in this study involving animals were done in accordance with the ethical standards of the University of Georgia.

Availability of data and material

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Figure captions

Figure 1. (A) The gross morphology of a chicken ovary. It contains a hierarchy of pre-ovulatory follicles (F1-F5), pre-hierarchical follicles (SYF), and post-ovulatory follicles (POF). (B) The ovarian follicle contains both a W and Z chromosome, which will ultimately segregate with one being retained in the oocyte and the other into a polar body. This could result in either a W chromosome in the oocyte with a Z polar body or a Z chromosome in the oocyte with a W polar body. (C) The germinal disc (GD) is located on the periphery of the oocyte, contains the genetic material of the cell, and can be seen with the naked eye as a white circle

Figure 2. (A) Quantity of chicken AR protein levels in GD and non-GD regions collected at a time right before meiotic segregation (5h prior to ovulation) and at a time well before meiotic segregation (20h prior to ovulation). **(B)** Quantification of the AR Western blot analysis. Each column is mean±standard error. The dots represent individual samples.

Figure 3. (A) Western blots of chicken MR protein levels in GD and non-GD regions collected at a time right before meiotic segregation (5h prior to ovulation) and at a time well before meiotic segregation (20h prior to ovulation). **(B)** Quantification of the MR Western blot analysis. Each column is mean±standard error. The dots represent individual samples.

Figures

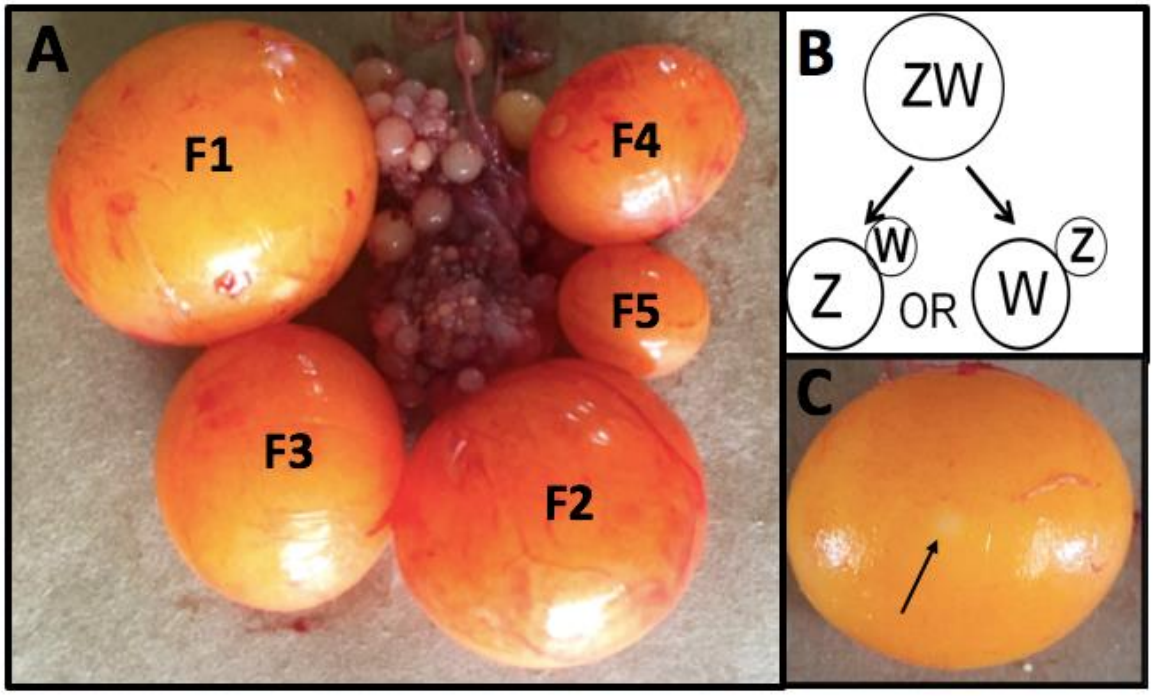

Fig. 1

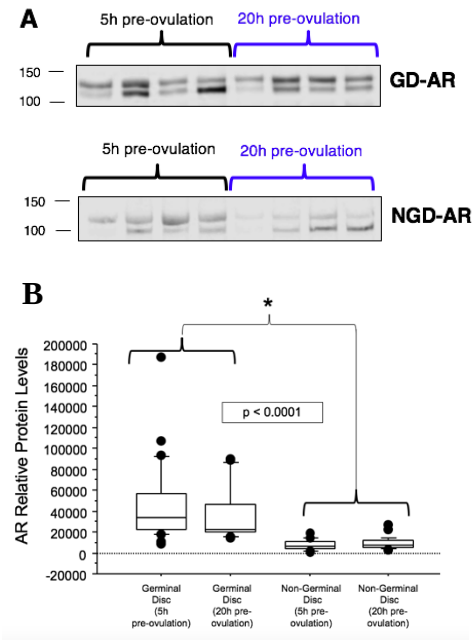

Fig. 2

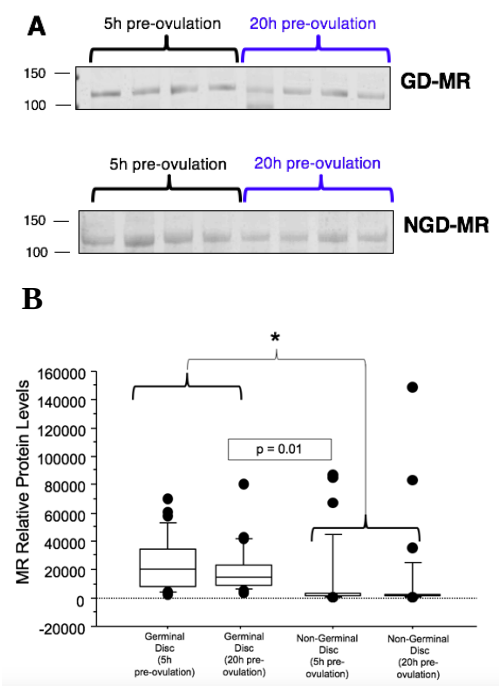
A
5h pre-ovulation
20h pre-ovulation
150
100
GD-MR
5h pre-ovulation
20h pre-ovulation
150
100
NGD-MR
B
*
MR Relative Protein Levels
160000
140000
120000
100000
80000
60000
40000
20000
0
-20000
p = 0.01
Germinal Disc (5h pre-ovulation)
Germinal Disc (20h pre-ovulation)
Non-Germinal Disc (5h pre-ovulation)
Non-Germinal Disc (20h pre-ovulation)

Fig 3.