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High progesterone during avian meiosis biases sex ratios toward females

Stephanie M. Correa[1,*], **Elizabeth Adkins-Regan**[1,2] **and Patricia A. Johnson**[3]

[1]*Department of Neurobiology & Behavior, Mudd Hall, Cornell University, Ithaca, NY 14853, USA*
[2]*Department of Psychology, Uris Hall, Cornell University, Ithaca, NY 14853, USA*
[3]*Department of Animal Science, Morrison Hall, Cornell University, Ithaca, NY 14853, USA*
**Author for correspondence (smc67@cornell.edu)*

Evidence of altered primary sex ratios in birds shows that mothers can manipulate the sex of their offspring before oviposition. In birds, females are the heterogametic sex (ZW) and males are homogametic (ZZ). Sex is determined in the first meiotic division, when one sex chromosome is retained in the oocyte and the other segregates to the polar body. Altered primary sex ratios suggest that birds may be capable of biasing the segregation of sex chromosomes during meiosis I. During the time of meiosis I, follicular steroid production is limited primarily to progesterone (P4). We experimentally manipulated the levels of P4 in female domestic chickens during the approximate time of meiosis I. We advanced the ovulation of the first egg of a sequence (or clutch) with a subcutaneous injection of P4. We found a significant effect of P4 dose on the sex of the resulting egg. The high progesterone group produced 25% males whereas the low progesterone group produced 61% males and the control group produced 63% males in the first ovulation of the sequence. We propose that variation in maternal progesterone during the critical time for genetic sex determination is the mechanism for primary sex ratio manipulation in birds.

Keywords: sex ratio; sex allocation; progesterone; meiosis; domestic chicken

1. INTRODUCTION

The Trivers–Willard hypothesis predicts that mothers in good condition should bias the sex ratio of their offspring toward males and mothers in poor condition should produce a bias toward females when the reproductive success of male offspring increases more from higher parental investment than the reproductive success of female offspring (Trivers & Willard 1973; Cameron & Linklater 2002). Recent evidence of altered primary sex ratios in birds, such as the Seychelles warbler (*Acrocephalus sechellensis*; Komdeur *et al.* 1997), suggests that females can manipulate the sex of their offspring before oviposition (reviewed in Pike & Petrie 2003).

In birds sex is determined in the first meiotic division (meiosis I), when one sex chromosome is retained in the oocyte and the other segregates to the polar body. Meiosis I occurs 2–4 h before ovulation (Olsen & Fraps 1950), when follicular steroid production is limited primarily to progesterone (Etches & Duke 1984). Petrie *et al.* (2001) suggested that maternal steroids could influence sex chromosome segregation. In the zebra finch (*Taeniopygia guttata*), von Engelhardt *et al.* (2004) investigated the relationship between maternal oestradiol and sex but did not find an effect of oestradiol injection, given before the initiation of a clutch (before pair formation), on the primary sex ratio. We chose to manipulate progesterone because it is the primary steroid produced by the avian preovulatory follicle during meiosis I, the critical time for genetic sex determination. We tested the hypothesis that increased progesterone can bias sex ratios in the domestic hen. We also measured circulating oestradiol (E2) in progesterone-injected hens to investigate the relationship between oestradiol and offspring sex, specifically during the time of meiosis I.

2. MATERIALS AND METHODS

(a) *Animals*

Single-comb White Leghorn hens (*Gallus gallus domesticus*) of the Babcock B300 strain were housed in individual cages on a 15 h L : 9 h D light schedule. Hens in their first year of egg production were inseminated every 3–5 days with pooled semen from 6 to 12 roosters to ensure egg fertilization. Progesterone (2 or 0.25 mg) was dissolved in 0.5 ml of sesame oil and injected subcutaneously 4 h before the end of the light phase. Hens in the control group were injected with 0.5 ml of sesame oil. Hens were randomly assigned to the treatment group to ensure that factors such as maternal condition were also randomly distributed. Twenty-two hens were assigned to the high progesterone group, 19 to the low progesterone group, and 22 to the control group (overall $n=63$). Simulation studies by Wilson & Hardy (2002) suggest that sample sizes of 20 per treatment group are sufficient when comparing single-egg samples with sex ratios of 0.25 and 0.65 ($\alpha<10\%$ and power$=70$–80%).

Blood samples were taken from the brachial vein immediately before injection (0 h) and 2, 4 and 6 h after injection to verify a surge of progesterone in P4-treated hens. A single egg, the first egg laid following injection, was collected from each hen and incubated at 37.5 °C and 85.5% humidity.

(b) *Molecular sexing*

Of the 63 eggs collected, 56 eggs showed embryonic development after 4–18 days of incubation. Seven eggs did not show visible development. The genetic sex of all 56 developed embryos was determined by PCR amplification of CHD alleles on the Z and W chromosomes (described in Griffiths *et al.* (1998)). DNA was extracted using DNeasy Tissue Kits (Qiagen, Valencia, CA). Of the 56 developed embryos, 44 were incubated for 10 days or more and dissected to verify normal gonadal development.

(c) *Radioimmunoassays*

Plasma samples were assayed in duplicate with Coat-A-Count Radioimmunoassay kits (Diagnostic Products Corporation, Los Angeles). The mean intra- and inter-assay coefficients of variation (CV) for progesterone were 8.1 and 10.9%, respectively. The progesterone kits have a cross-reactivity of less than 1% for 18 steroids tested, except 11-deoxycorticosterone (2.2%), hydroxyprogesterone (3.4%), 5α-pregnan-3,20-dione (9.0%) and 5β-pregnan-3,20-dione (3.2%). The mean intra- and inter-assay CV for oestradiol were 13.3 and 22.1%, respectively. The oestradiol kits measure 17β-oestradiol and have a cross-reactivity of less than 1% for 40 steroids tested, except *d*-equilenin (4.4%), oestrone (10.0%), oestrone -β-D-glucuronide (1.8%) and ethinyl oestradiol (1.8%).

(d) *Statistical analyses*

Logistic regression analyses were performed in the SAS Genmod Procedure (SAS Institute, Inc.). One analysis was performed with sex as the response variable (male$=1$) and progesterone dose as the only predictor variable. The second analysis included as predictor

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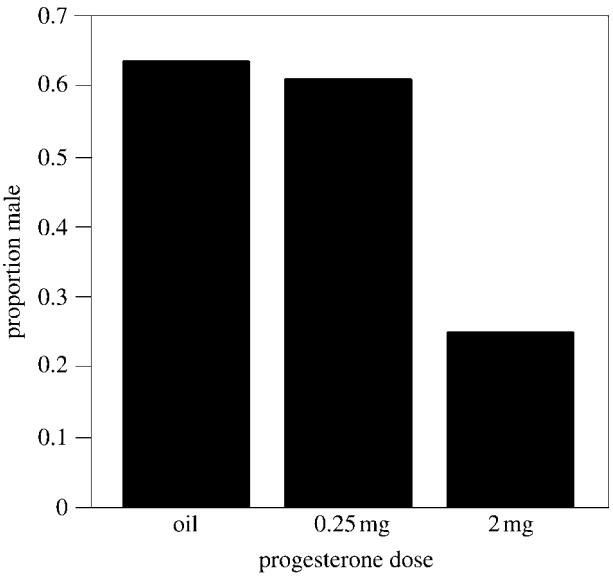

Figure 1. The sex ratio produced in each of three treatment groups: oil ($n=22$), 0.25 mg progesterone ($n=18$) and 2 mg progesterone ($n=16$). The effect of progesterone dose on sex is significant. (Likelihood ratio test, $\chi^2_2=6.67$, $p<0.036$.)

variables plasma P4 and E2 levels at 2 h after injection and sex as the response variable (male=1). *F*-tests were used to measure the change in deviance in stepwise backward elimination of non-significant terms ($p>0.05$).

3. RESULTS

We advanced the ovulation of the first egg of the sequence in hens with a low (0.25 mg) or high (2 mg) dose of progesterone. Hens in both P4-treated groups ovulated approximately 6 h after injection. The hens in the oil group ovulated spontaneously the next morning. Fifty-nine of 63 eggs were laid on the morning of predicted oviposition. Four eggs were laid 1–2 days after predicted oviposition, but all 63 eggs were tracked by palpation until laying, to exclude the possibility of egg resorption. Progesterone did not influence gonadal development, as genetic and gonadal sexes were identical in the 44 embryos for which these data were available.

We found a significant effect of progesterone treatment on the sex of the resulting egg (figure 1). The high progesterone group produced 25% males whereas the low progesterone and oil groups produced 61 and 63% males, respectively. The low progesterone and control groups produced similar sex ratios, showing that premature ovulation is not responsible for the bias toward female offspring.

Plasma samples taken at 0, 2, 4 and 6 h after injection show that a surge of progesterone was induced in the progesterone-treated hens (figure 2*a*). Only hormone levels taken 2 h after injection were analysed for an effect on sex because they coincide approximately with the initiation of meiosis I. Overall, hens that laid female eggs had higher plasma progesterone than hens that laid male eggs (figure 2*b*). This effect, however, is due exclusively to the high P4 group. In the low P4 and control groups, hens that laid female eggs did not tend to have higher plasma P4 than hens that laid male eggs.

The effect of oestradiol on sex was not significant and was eliminated from the full model (figure 2*d*). However, when oestradiol is in the model alone, the effect of oestradiol on sex is significant. (Likelihood ratio test $\chi^2_1=5.01$, $p<0.026$.) Progesterone dose had an effect on the plasma concentration of oestradiol (figure 2*c*; ANOVA, $F_{2,56}=8.19$, $p<0.0008$). Oestradiol was significantly higher in the high P4 group than in the control ($t_{36}=4.02$, $p<0.0002$) but not higher in the low P4 group than in the control ($t_{38}=-1.83$, $p<0.0722$).

4. DISCUSSION

Experimental elevation of progesterone resulted in a decrease in the proportion of males produced by the first egg of the sequence (high P4: 25%; low P4: 61%; control: 63%). The progesterone levels induced in the high P4 group, but not the low P4 group, are higher than previously published reports for the first ovulation of a sequence (Johnson & van Tienhoven 1980). High progesterone may be necessary to bias offspring sex ratios in the domestic chicken (*Gallus gallus domesticus*), since biased primary sex ratios have not been reported in this species (Müller *et al.* 2002). Parker (2002) found a relationship between maternal mass and chick sex ratios in jungle fowl (*Gallus gallus*), but the sex ratio was measured weeks after hatching and may be attributable to sex-specific mortality.

We hypothesize that in those species that facultatively bias the primary sex ratio, high progesterone during meiosis is the mechanism for manipulating offspring sex. As in domestic chickens, relatively low peak concentrations of P4 have been reported in prelaying female yellow-eyed penguins (≤ 0.5 ng ml^{-1}; Cockrem & Sneddon 1994), white ibises (≤ 0.9 ng ml^{-1}; Heath *et al.* 2003), and northern mockingbirds (≤ 2 ng ml^{-1}; Logan & Wingfield 1995). However, peak progesterone concentrations similar to those of the high P4 hens in this study have been reported in laying female mallards (≤ 6 ng ml^{-1}; Bluhm *et al.* 1983*a*), canvasback ducks (≤ 5.5 ng ml^{-1}; Bluhm *et al.* 1983*b*), ring doves (≤ 10.7 ng ml^{-1}; Lea *et al.* 1986) king penguins (≤ 8 ng ml^{-1}; Mauget *et al.* 1994), American kestrels (≤ 6 ng ml^{-1}; Rehder *et al.* 1986), Bengalese finches (≤ 7.5 ng ml^{-1}; Seiler *et al.* 1992), turkeys (≤ 8 ng ml^{-1}; Sharp *et al.* 1981) and western gulls (≤ 5 ng ml^{-1}; Wingfield & Farner 1993). Species with high plasma progesterone during egg laying may be more likely than other birds to manipulate the sex of their offspring under ecological and social circumstances that favour such manipulation.

High progesterone may bias offspring sex by producing non-random segregation of the Z and W chromosomes during meiosis I. Progesterone has been shown to alter spindle formation in dividing liver cells in the rat (Boada *et al.* 2002) and evidence in female XO mice suggests that spindle structure can mediate the non-random segregation of sex chromosomes (LeMaire-Adkins & Hunt 2000). High progesterone may promote the formation of a functionally asymmetric meiotic spindle, resulting in

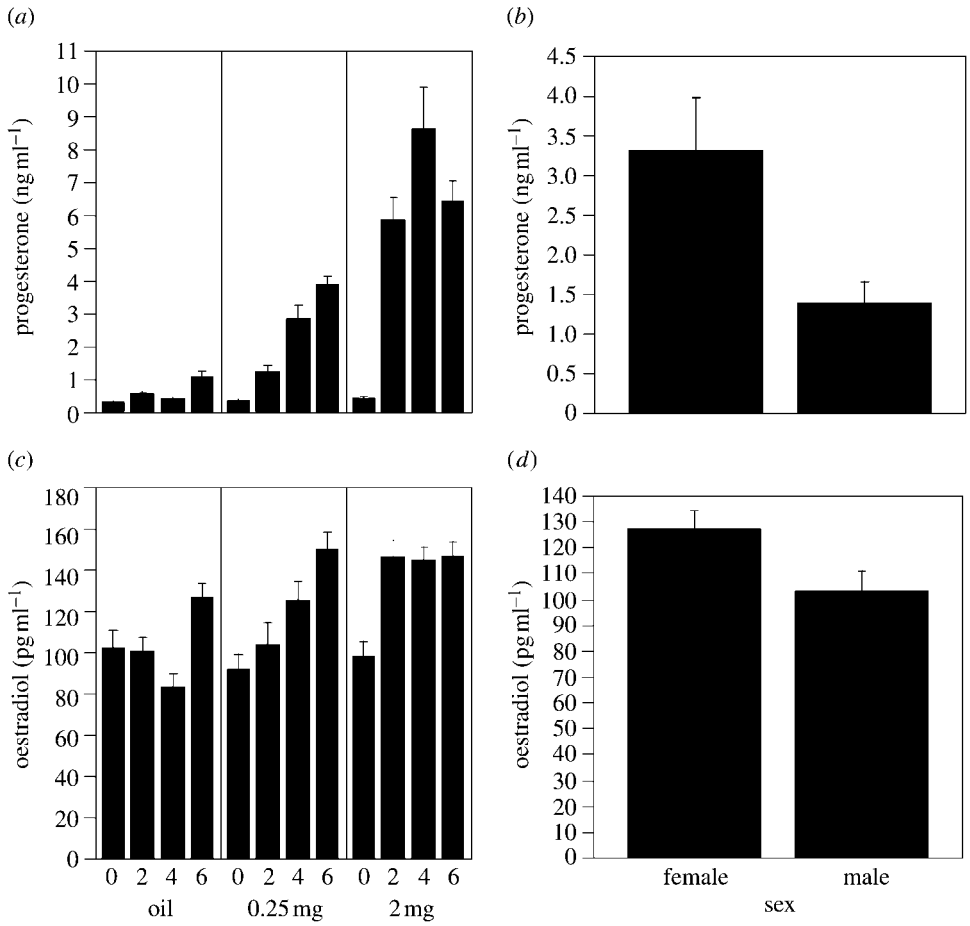

Figure 2. Plasma hormone concentrations (mean+standard error) for hens injected with oil ($n=22$), 0.25 mg of progesterone ($n=18$) or 2 mg of progesterone ($n=16$). (*a*) Progesterone concentrations 0, 2, 4 and 6 h after injection. (*b*) Progesterone was higher in hens that laid female eggs ($n=27$) than in hens that laid male eggs ($n=29$; likelihood ratio test $\chi^2_1=7.72$, $p<0.006$) in samples taken 2 h after injection, the approximate time of meiosis I for the progesterone-treated groups. (*c*) Oestradiol concentrations 0, 2, 4 and 6 h after injection. (*d*) Oestradiol tended to be higher in hens that laid female eggs ($n=27$) than in hens that laid male eggs ($n=29$) in samples taken 2 h after injection, but not significantly so. (Likelihood ratio test $\chi^2_1=1.24$, $p<0.265$.)

non-random sex chromosome segregation, whereas low progesterone could produce a functionally symmetric spindle, resulting in Mendelian segregation of sex chromosomes (see Pardo-Manuel de Villena & Sapienza (2001) for discussion of spindle asymmetry and non-random segregation).

Progesterone may have also decreased the incidence of egg fertilization. Seven of the 63 ovulations resulted in eggs with no visible development after 4–16 days of incubation, which were not included in the sex ratio analyses (0 in the oil group, 1 in the low P4 group and 6 in the high P4 group). High progesterone may have interfered with sperm release from sperm-storage sites in the infundibulum and/or uterovaginal junction, preventing egg fertilization. Although the mechanism of sperm release is unknown, sperm-storage tubules of laying hens contain progesterone receptors (Yoshimura *et al.* 2000). It is unlikely that only Z-containing eggs were unfertilized but we cannot exclude this possibility.

Alternatively, high progesterone could be toxic to embryos in the very early stages of development. However, we suggest that progesterone does not reduce early male survival for several reasons. First, the only embryonic death that we were able to confirm, which occurred after the development of a circulatory system, was in the high P4 group and was sexed as a female by PCR. Second, progesterone administration on day 4 of incubation results in retarded growth but it is not sex specific and does not increase embryonic mortality (Renden & Benoff 1980). Third, progesterone treatment on days 7 or 10 of incubation promotes male but not female growth (Ahmad & Zamenhof 1979).

If we assume that the undeveloped eggs were indeed male then the proportion of males in the high P4 group increases to 62%, but we do not have any evidence to suggest that the bias toward females in the high P4 group is owing to differential mortality of male embryos. Instead, our evidence suggests that high progesterone affected the primary sex ratio. This provides a plausible mechanism for sex ratio manipulation in birds.

The relationship between E2 and sex is not statistically significant (when P4 is in the model) but warrants further investigation. E2 was elevated in the high progesterone group, raising the possibility that progesterone acted through E2 to produce the female bias.

We show a relationship between the genetic sex of offspring and maternal plasma hormones during the time of genetic sex determination. High progesterone may promote non-random segregation of sex chromosomes in meiosis I to cause the retention of the W chromosome in the oocyte and the production of females. The effect of maternal progesterone on fertilization success may (i) reduce the fertilization rate of Z-containing ova to bias the primary sex ratio, or (ii) may not be sex specific and represent a potential cost of sex ratio manipulation that limits the strategy to certain species or to individuals of a certain quality.

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