Karyotype analysis and sex determination in Australian Brush-turkeys (*Alectura lathami*)

Madison T. Ortega¹,², Dustin J. Foote³,⁴, Nicholas Nees³, Jason C. Erdmann⁵, Charles D. Bangs⁵, Cheryl S. Rosenfeld¹,²,⁶*

¹ Bond Life Sciences Center, University of Missouri, Columbia, Missouri, United States of America, ² Biomedical Sciences, University of Missouri, Columbia, Missouri, United States of America, ³ Sylvan Heights Bird Park, Scotland Neck, North Carolina, United States of America, ⁴ Department of Biology, East Carolina University, Greenville, North Carolina, United States of America, ⁵ Cytogenetics Laboratory, Stanford Health Care, Palo Alto, California, United States of America, ⁶ Thompson Center for Autism and Neurobehavioral Disorders, University of Missouri, Columbia, Missouri, United States of America

* rosenfeldc@missouri.edu

Abstract

Sexual differentiation across taxa may be due to genetic sex determination (GSD) and/or temperature sex determination (TSD). In many mammals, males are heterogametic (XY); whereas females are homogametic (XX). In most birds, the opposite is the case with females being heterogametic (ZW) and males the homogametic sex (ZZ). Many reptile species lack sex chromosomes, and instead, sexual differentiation is influenced by temperature with specific temperatures promoting males or females varying across species possessing this form of sexual differentiation, although TSD has recently been shown to override GSD in Australian central beaded dragons (*Pogona vitticeps*). There has been speculation that Australian Brush-turkeys (*Alectura lathami*) exhibit TSD alone and/or in combination with GSD. Thus, we sought to determine if this species possesses sex chromosomes. Blood was collected from one sexually mature female and two sexually mature males residing at Sylvan Heights Bird Park (SHBP) and shipped for karyotype analysis. Karyotype analysis revealed that contrary to speculation, Australian Brush-turkeys possess the classic avian ZW/ZZ sex chromosomes. It remains a possibility that a biased primary sex ratio of Australian Brush-turkeys might be influenced by maternal condition prior to ovulation that result in her laying predominantly Z- or W-bearing eggs and/or sex-biased mortality due to higher sensitivity of one sex in environmental conditions. A better understanding of how maternal and extrinsic factors might differentially modulate ovulation of Z- or W-bearing eggs and hatching of developing chicks possessing ZW or ZZ sex chromosomes could be essential in conservation strategies used to save endangered members of Megapodiidae.

Introduction

Gonadal sexual differentiation during embryonic development may involve several genes. In mammals, these genes are for the most part located on sex chromosomes with females lacking male-promoting genes that reside on the Y chromosome. There are, however, notable
exceptions as two spiny rat species, Amami spiny rat (*Tokudaia osimensis*) and Tokunoshima spiny rat (*T. tokunoshimensis*) that reside on two islands off the coast of Okinawa, Japan, with derived system of sex determination [1, 2]. Sexual differentiation in this species is likely directed by different dosages of genes residing on autosomal chromosomes. Recently, Monica Ward’s group generated transgenic mice lacking a Y chromosome [3]. In these animals, two transgenes, *Sox9* and *Eif2s3x*, compensated for the absence of Y-chromosome encoded genes that gave rise to males who could sire offspring.

In contrast to mammals where males are heterogametic (XY), females are heterogametic (ZW) in most birds. Thus, the female can influence the sex of her offspring by differentially laying Z- or W-bearing eggs. Reptiles may exhibit genetic sex determination (GSD) via sex chromosomes and/or temperature sex determination (TSD). It has recently been shown that in Australian central beaded dragons (*Pogona vitticeps*), which typically demonstrate GSD, individual sex can be overriden at high incubation temperatures that gives rise to sex-reversed female offspring [4]. It has been suggested that one avian species, the megapode bird, Australian Brush-turkey (*Alectura lathami*), may be unique in demonstrating TSD alone or in combination with GSD, similar to Australian central beaded dragons [5, 6]. Example male and female Australian Brush-turkeys are shown in S1 Fig. In this species, females engage in mate choice by observing male activity prior to copulation, and males can have several females laying eggs at a time. As newly hatched brush-turkey chicks are precocial, the female’s investment in them ends after the eggs are laid. After the eggs are laid, the male will monitor the temperature of the nest with his tongue and can adjust it by removal or addition of nesting material (S1 Fig and S1 Video- times 00:13, 00:17, and 00.22 seconds show this male behavior). Thus, the evolution of TSD in this species has been postulated. It remains to be determined though whether the resulting offspring sex ratio is due to incubation temperature and/or interaction with sex chromosomes. One report cited a “personal communication” as evidence that this species may have sex chromosomes [5]. However, evidence of such has not been reported to date. ZZ/ZW sex chromosomes have been described in other species within the family Megapodiidae [7]. Thus, we sought to determine whether Australian Brush-turkeys possess heterogametic sex chromosomes.

**Materials and methods**

**Life history of Australian Brush-turkeys included in the study**

All three sampled Australian Brush-turkeys are captive born specimens currently house at Sylvan Heights Bird Park (SHBP) in North Carolina, USA. They are undoubtedly decedents from the nominate race, *A. l. lathami* [8] based on body size, iris/wattle color, and importation records. SHBP facility identification numbers are K1380 (male), K1878 (female), and K1379 (male). Blood was withdrawn on April 11th, 2017 as staff moved all three birds from their indoor wintering aviaries to the summer breeding aviary. K1380 (male) and K1878 (female) are shown in S1 Fig and S1 Video. These studies were approved by the ethics board at the SHBP.

**Blood collection**

From the two males and one female, blood was collected from the basilic wing vein (located on the ventral surface of the proximal ulna) via an 18-gauge needle (Catalogue number: 305196, Becton, Dickinson and Company, Franklin Lakes, NJ) connected to a 3cc syringe (Catalogue number: 305196, Becton, Dickinson and Company). Avian blood sample collection protocol followed in accordance with Harrison’s Clinical Avian Medicine [9]. The samples were then
placed vertical in a rack on ice and shipped overnight to the Cytogenetics Laboratory at Stanford Health Care in Palo Alto, CA, where the samples were immediately processed.

Cytogenetic analysis

Using standard cytogenetic methodologies [10, 11], peripheral blood buffy coat obtained by centrifugation was inoculated into suspension culture using RPMI 1640 medium supplemented with 15% fetal bovine serum 50 μg/mL gentamycin sulfate and 2mM L-glutamine. Cultures were mitogenically stimulated with 1% Gibco phytohemagglutinin (Life Technologies Corp, Carlsbad, CA) and pokeweed 10 μg/mL mitogen (Sigma, St. Louis, MO) and incubated at 37˚C and 40˚C. Cultures were harvested at 72 hours following a two hour mitotic arrest with 0.05 μg/mL Colcemid and one hour addition of 10 μg/mL ethidium bromide using standard methodologies of hypotonic shock with 0.075 M KCl and fixation with methanol:acetic acid fixative (3:1) [12, 13]. Metaphase preparations were made by dropping fixed cell suspension onto wet microscope slides, flooding with fixative and air-drying. Slides were aged at 90˚C for 30 minutes and stained independently by trypsin/Giemsa G-banding and barium hydroxide C-banding [11, 12]. Metaphase cells were imaged and analyzed with an Olympus BX41 microscope (Olympus Corp., Center Valley, PA) 100x planapochromatic objective and Leica CytoVision image/karyotype system (Leica Microsystems Inc., Buffalo Grove, IL).

Results

G-banded chromosome analysis demonstrates an Australian Brush-turkey karyotype consisting of approximately 80 chromosomes. The karyotype is interpreted in reference to the standardized Domestic Chicken (Gallus gallus domesticus) [14] and other galliform lineage karyotypes [15, 16] as including 10 macrochromosome pairs and approximately sixty microchromosomes (Fig 1). Macrochromosomes include heteromorphic Z and W sex chromosomes. Imprecision regarding the exact chromosome number reflects the technical challenge of enumeration and classification of small microchromosomes in typical avian metaphase preparations. Based on G- and C-band analyses, chromosome #1 is morphologically sub-metacentric and chromosomes #2 through #9 are telocentric. Any Z chromosome C-band is nearly indiscernible, however the chromosome overall appears morphologically telocentric. Based on C-band staining, the W chromosome is largely heterochromatic. Comparative G-band analysis indicates that the chromosome #1 common to other published galliform karyotypes represents a fusion of the Australian Brush-turkey chromosomes #2 and #4, where chromosome #2 corresponds to a common galliform #1 long arm and #4 corresponds to the galliform #1 short arm. Consequently, the Australian Brush-turkey chromosome #1 corresponds to the G. g. domesticus chromosome #2, etc.

Discussion

The initial goal of the study was to confirm that Australian Brush-turkeys have sex chromosomes. Chromosome analysis demonstrates that in the limited sample size (two males and one female) Australian Brush-turkeys possess heteromorphic Z and W sex chromosomes consistent with other known galliform karyotypes. G-band analysis also indicates that the Australian Brush-turkey, as a representative megapode, has a karyotype distinct from other galliform lineages by virtue of two separate autosome pairs (#2 and #4) present in other galliform lineages as a single fused chromosome #1. Whether this represents an evolutionary process of chromosome fusion or fission is uncertain. One possibility is that this is a fission of a common ancestral chromosome #1 still extant in the other galliform lineages, or it may represent a fusion event occurring in an ancestral galliform subsequent to separation of the megapodes. Either
scenario is consistent with current phylogeny of the Galliformes, which separates Megapodiidae, who likely originated during the Cretaceous period, ancestrally from other Galliformes that were derived during Tertiary period [17–20]. However, the possibility that this represents a fission event from another galliform lineage rather than an ancestral galliform cannot be ruled out based on the current data. It is interesting to note that chromosome fusion as a speciation-associated karyotypic phenomenon is well-documented in primates where there is fusion of the chimpanzee and bonobo (Pan troglodytes, P. paniscus) chromosomes #12 and #13 to form the human chromosome #2 [21]. When comparing the Z chromosomes of the Australian Brush-turkey and G. gallus domesticus, the centromeric regions appear similar in that they
both lack a distinct centromere C-band unlike most autosomes in either karyotype. The main differences are morphologic: probable telocentric (Australian Brush-turkey) vs sub-metacentric (G. gallus domesticus). Additionally, G. gallus domesticus has a heterochromatic region not present in the Australian Brush-turkey. An inversion and a heterochromatic addition would account for the altered G. gallus domesticus Z chromosome relative to the Z chromosome of the Australian Brush-turkey.

With the documentation that the Australian Brush-turkey possesses sex chromosomes, it opens up several potential avenues by which this species can affect offspring sex ratio. For instance, the final sex ratio of Australian Brush-turkeys might vary based on interactions of offspring sex and nest temperature (i.e. temperature-dependent sex-biased embryonic mortality), as suggested by other reports [5, 6, 22]. Developing males appear to be more vulnerable at higher incubation temperatures; whereas, lower incubation temperatures tends to be lethal to females [22]. It is not clear why these sex-differences exist and whether they might relate to genes expressed on the now identified sex chromosomes (ZW) within this species. Incubation temperature can vary the dry mass of the yolk-free body and residual yolk of hatchlings in this species with elevated temperatures giving rise to chicks with reduced yolk-free body mass and greater residual yolk mass than those incubated at lower temperatures [23].

In most mammalian species, who possess sex chromosomes, a variety of maternal-associated mechanisms exist that can result in skewed offspring sex ratios [24–30]. In birds, skewed offspring sex ratio can result due to differential embryonic survival. However, as the heterogamic sex, females are the sex determining parent, and it could be that maternal factors differentially influence ovulation of Z- or W-bearing eggs. This has shown to be the case in the endangered flightless parrot located in New Zealand, the Kakapo (Strigops habroptila). By provisioning the females with additional nutrient supplements prior to ovulation, researchers were able to generate male-biased chick sex ratios, and thus, sex allocation theory might have practical importance in helping to vary the number of males and females available for breeding in this endangered species [31]. Further, a lek mating system is present in Kakapos where the males gather and show-off to the females who then select their reproductive partners. Males in the best body condition are likely successful in obtaining the best “booming sites” and thereby attract a greater number of females.

Studies with other avian species, including the Superb Starlings (Lamprotornis superbus), Homing Pigeons (Columba livia domestica), Meadow Pipits (Anthus pratensis), Gouldin Finches (Erythrura gouldiae), Tree Swallows (Tachycineta bicolor), Blue Tits (Cyanistes caeruleus), Red-capped Robins (Petroica goodenovii), Common Starlings (Sturnus vulgaris), and Lesser Black-backed Gulls (Larus fuscus) strongly indicate that maternal condition and surrounding environment can result in offspring sex ratio adjustments [32–41]. This maternal-induced offspring sex ratio skewing could be due to selective laying of Z- or W-bearing eggs or sex dependent differences in deposition of yolk proteins, hormones, or other nutrient factors within the egg. Variation in yolk androgen content has been previously identified in Australian Brush-turkeys [42].

The current data provides definitive evidence that Australian Brush-turkeys possess sex chromosomes. Additionally, the potential fusion of autosomal pairs #2 and #4 of other Galliforms to form chromosome #1 in Australian Brush-turkeys is likely consistent with the previously identified earlier lineage of Megapodiidae relative to other Galliformes. While past studies have explored how adjustments in nest temperature by male Australian Brush-turkeys affects egg composition and offspring sex ratio, no studies to date have considered how maternal condition and environment might affect offspring sex ratio in this species. With the characterization of sex chromosomes in this species, it suggests that future studies should be directed at examining how maternal condition might influence laying of Z- or W-bearing
eggs. Additionally, genes expressed from the Z- or W- chromosome may interact with egg composition or incubation temperature to result in sexually dimorphic differences in survival under various intrinsic and extrinsic environments. Thus, the current studies that have definitively identified sex chromosomes in Australian Brush-turkeys may open up new avenues in research to examine how maternal condition, sex-chromosome expressed genes, and embryonic environment, interact to modulate primary offspring sex ratio, as appears to be the case with Australian central beaded dragons, where TSD can seemingly override GSD [4]. The main mechanisms that can affect offspring sex ratio in Australian Brush-turkeys, and likely other avian species, are summarized in S2 Fig. A better understanding of these complex interactions in Australian Brush-turkeys and other avian species may be critical in breeding-strategies designed to alter offspring sex ratio in species already genetically bottlenecked and on the brink of extinction.

Supporting information

S1 Fig. Male and female Australian Brush-turkeys. Comparison of example breeding male (Panels A and B) with an example breeding female (C and D) reveals that when the male is in full breeding mode, his wattle enlarges and become bright red in color. However, the female wattle, which is smaller, remains the same color and size from season to season. Females tend to be smaller than males, and the plumage of males is slightly darker. (TIF)

S2 Fig. Diagram of all the potential mechanisms that can result in skewing of primary sex ratio in Australian Brush-turkeys. A) As the sex-determining parent, females can selectively lay Z- or W-bearing eggs. She can also alter in a sex-dependent manner the amount of yolk proteins, hormones, or other nutritional factors within the egg. B) The male can affect offspring sex ratio by adjusting the temperature of the nest that may favor the survival of one sex over the other. C) It is also possible that both parents can affect primary offspring sex ratio by the collective methods shown in panels A and B. (TIF)

S1 Video. This video demonstrates how a male Australian Brush-turkey constructs a nest out of various materials. He will then proceed to check the temperature of it with his tongue and alter the amount of nesting material based on the perceived temperature. This behavior is demonstrated at 00:13, 00:17, and 00:22 seconds in the video. (MP4)

Acknowledgments

The authors are grateful to all of the staff at the Sylvan Heights Bird Park, Scotland Neck, NC, USA who assisted with these studies. The authors are also thankful to Donald L. Connor who drew the diagram depicting the primary mechanisms of how altered offspring sex ratio can occur in Australian Brush-turkeys, and to Marlys Houks, Holly Hobart and Dean Stock for their advice on the culture of avian blood for chromosome analysis.

Author Contributions

Conceptualization: Madison T. Ortega, Dustin J. Foote, Charles D. Bangs, Cheryl S. Rosenfeld.

Data curation: Dustin J. Foote, Jason C. Erdmann, Charles D. Bangs.
Formal analysis: Jason C. Erdmann, Charles D. Bangs, Cheryl S. Rosenfeld.

Investigation: Dustin J. Foote, Nicholas Nees, Jason C. Erdmann, Charles D. Bangs, Cheryl S. Rosenfeld.

Methodology: Madison T. Ortega, Dustin J. Foote, Nicholas Nees, Jason C. Erdmann, Charles D. Bangs.

Project administration: Dustin J. Foote, Charles D. Bangs, Cheryl S. Rosenfeld.

Resources: Dustin J. Foote, Nicholas Nees.

Supervision: Dustin J. Foote, Charles D. Bangs, Cheryl S. Rosenfeld.

Validation: Jason C. Erdmann, Charles D. Bangs.

Writing – original draft: Madison T. Ortega, Dustin J. Foote, Nicholas Nees, Jason C. Erdmann, Charles D. Bangs, Cheryl S. Rosenfeld.

Writing – review & editing: Madison T. Ortega, Dustin J. Foote, Nicholas Nees, Jason C. Erdmann, Charles D. Bangs, Cheryl S. Rosenfeld.

References

1. Kuroiwa A, Handa S, Nishiyama C, Chiba E, Yamada F, Abe S, et al. Additional copies of CBX2 in the genomes of males of mammals lacking SRY, the Amami spiny rat (Tokudaia osimensis) and the Toku-noshima spiny rat (Tokudaia tokunoshimensis). Chromosome research: an international journal on the molecular, supramolecular and evolutionary aspects of chromosome biology. 2011; 19(5):635–44. Epub 2011/06/10. https://doi.org/10.1007/s10577-011-9223-6 PMID: 21656076.

2. Nakamura T, Kuroiwa A, Nishida-Umemura C, Matsubara K, Yamada F, Matsuda Y. Comparative chromosome painting map between two Ryukyu spiny rat species, Tokudaia osimensis and Tokudaia tokunoshimensis (Muridae, Rodentia). Chromosome research: an international journal on the molecular, supramolecular and evolutionary aspects of chromosome biology. 2007; 15(6):799–806. Epub 2007/09/18. https://doi.org/10.1007/s10577-007-1163-9 PMID: 17874214.

3. Yamauchi Y, Riel JM, Ruthig VA, Ortega EA, Mitchell MJ, Ward MA. Two genes substitute for the mouse Y chromosome for spermatogenesis and reproduction. Science (New York, NY). 2016; 351(6272):514–6. Epub 2016/01/30. https://doi.org/10.1126/science.aad1795 PMID: 26823431.

4. Deveson IW, Holleley CE, Blackburn J, Marshall Graves JA, Mattick JS, Waters PD, et al. Differential intron retention in Junonji chromatin modifier genes is implicated in reptile temperature-dependent sex determination. Science advances. 2017; 3(6):e1700731. https://doi.org/10.1126/sciadv.1700731 PMID: 28630932.

5. Göth ANN. Incubation temperatures and sex ratios in Australian brush-turkey (Alectura lathami) mounds. Austral Ecology. 2007; 32(4):378–85. https://doi.org/10.1111/j.1442-9993.2007.01709.x

6. Göth A, Booth DT. Temperature-dependent sex ratio in a bird. Biology Letters. 2005; 1(1):31–3. https://doi.org/10.1098/rsbl.2004.0247 PMID: 17148121.

7. Belterman RHR, De Boer LEM. A karyological study of 55 species of birds, including karyotypes of 39 species new to cytology. Genetica. 1984; 65(1):39–82. https://doi.org/10.1007/BF00056765.

8. Del Hoyo J, Elliot A., & Sargatak J. Handbook of the Birds of the World. Editions L, editor. Barcelona1992.

9. Harrison GJ L T. L. Clinical Avian Medicine. Spix, editor. Florida2006.

10. Hungerford DA. Leukocytes cultured from small inocula of whole blood and the preparation of metaphase chromosomes by treatment with hypotonic KCl. Stain technology. 1965; 40(6):333–8. Epub 1965/11/01. PMID: 5866557.

11. Houck ML, Lear T.L. & Charter S.J. Chapter 24 Animal Cytogenetics in The AGT Cytogenetics Laboratory Manual 4th Edition. Hoboken, New Jersey Wiley Blackwell; 2017.

12. Seabright M. A rapid banding technique for human chromosomes. Lancet (London, England). 1971; 2 (7731):971–2. Epub 1971/10/30. PMID: 4107917.

13. Arrighi FE, Hsu TC. Localization of heterochromatin in human chromosomes. Cytogenetics. 1971; 10(2):81–6. Epub 1971/01/01. PMID: 4106493.
14. Ladjiial-Mohamed K, Bitgood JJ, Tixier-Boichard M, Ponce De Leon FA. International system for standardized avian karyotypes (ISSAK): standardized banded karyotypes of the domestic fowl (Gallus domesticus). Cytogenetics and cell genetics. 1999; 86(3-4):271–6. Epub 1999/11/27. PMID: 10575225.

15. Stock AD, Bunch TD. The evolutionary implications of chromosome banding pattern homologies in the bird order Galliformes. Cytogenetics and cell genetics. 1982; 34(1-2):136–48. Epub 1982/01/01. PMID: 7151485.

16. De Boer LEM, Belterman RHR. Chromosome banding studies of the razor-billed curassow, Crax mitu (Aves: Galliformes: Cracidae). Genetica. 1981; 54(3):225–32. https://doi.org/10.1007/bf00135038

17. Kan XZ, Yang JK, Li XF, Chen L, Lei ZP, Wang M, et al. Phylogeny of major lineages of galliform birds (Aves: Galliformes) based on complete mitochondrial genomes. Genetics and molecular research: GMR. 2010; 9(3):1625–33. Epub 2010/08/24. https://doi.org/10.4238/vol9-3gmr898 PMID: 20730714.

18. Pereira SL, Baker AJ. A molecular timescale for galliform birds accounting for uncertainty in time estimates and heterogeneity of rates of DNA substitutions across lineages and sites. Molecular phylogenetics and evolution. 2006; 38(2):499–509. Epub 2005/08/23. https://doi.org/10.1016/j.ympev.2005.07.007 PMID: 16112881.

19. Cracraft J. Toward a phylogenetic classification of the recent birds of the world (class Aves). Auk. 1981; 98:681–714.

20. Groth JG, Barrowclough GF. Basal divergences in birds and the phylogenetic utility of the nuclear RAG-1 gene. Molecular phylogenetics and evolution. 1999; 12(2):115–23. Epub 1999/06/25. https://doi.org/10.1006/mpev.1998.0603 PMID: 10381315.

21. Dutrillaux B, Rethore MO, Lejeune J. [Analysis of the karyotype of Pan paniscus. Comparison with other Pongidae and man (author's transl)]. Humanangenetik. 1975; 28(2):113–9. Epub 1975/06/19. PMID: 50278.

22. Eiby YA, Wilmer JW, Booth DT. Temperature-dependent sex-biased embryo mortality in a bird. Proceedings Biological sciences. 2008; 275(1652):2703–6. Epub 2008/08/30. https://doi.org/10.1098/rspb.2008.0954 PMID: 18755669.

23. Eiby YA, Booth DT. The effects of incubation temperature on the morphology and composition of Australian Brush-turkey (Alectura lathami) chicks. Journal of comparative physiology B, Biochemical, systemic, and environmental physiology. 2009; 179(7):875–82. Epub 2009/05/28. https://doi.org/10.1007/s00360-009-0370-4 PMID: 19471897.

24. Rosenfeld CS, Grimm KM, Livingston KA, Brokman AM, Lamber son WE, Roberts RM. Striking variation in the sex ratio of pups born to mice according to whether maternal diet is high in fat or carbohydrate. Proceedings of the National Academy of Sciences of the United States of America. 2003; 100(8):4628–32. https://doi.org/10.1073/pnas.0330808100 PMID: 12672968.

25. Rosenfeld CS, Roberts RM. Maternal diet and other factors affecting offspring sex ratio: a review. Biology of reproduction. 2004; 71(4):1063–70. https://doi.org/10.1095/biolreprod.104.030890 PMID: 15229140.

26. Rosenfeld CS. Periconceptional influences on offspring sex ratio and placental responses. Reproduction, fertility, and development. 2011; 24(1):45–58. Epub 2012/03/08. https://doi.org/10.1071/RD11906 PMID: 22394717.

27. Bowers EK, Thompson CF, Sakaluk SK. Maternal natal environment and breeding territory predict the condition and sex ratio of offspring. Evolutionary biology. 2017; 44(1):11–20. Epub 2017/03/14. https://doi.org/10.1007/s11692-016-9380-9 PMID: 28286350.

28. Douhard M, Festa-Bianchet M, Pelletier F. Maternal condition and previous reproduction interact to affect offspring sex in a wild mammal. Biol Lett. 2016; 12(8). Epub 2016/08/12. https://doi.org/10.1098/rsbl.2016.0510 PMID: 27512136.

29. Ryan CP, Anderson WG, Berkvens CN, Hare JF. Maternal gestational cortisol and testosterone are associated with trade-offs in offspring sex and number in a free-living rodent (Urocitellus richardsonii). PloS one. 2014; 9(10):e111052. Epub 2014/10/30. https://doi.org/10.1371/journal.pone.0111052 PMID: 25353347.

30. Schindler S, Gaillard JM, Gruning A, Neuhaus P, Traill LW, Tuljapurkar S, et al. Sex-specific demography and generalization of the Trivers-Willard theory. Nature. 2015; 526(7572):249–52. Epub 2015/08/22. https://doi.org/10.1038/nature14968 PMID: 26390152.

31. Robertson BC, Elliott GP, Eason DK, Clout MN, Gemmell NJ. Sex allocation theory aids species conservation. Biol Lett. 2006; 2(2):229–31. https://doi.org/10.1098/rsbl.2005.0430 PMID: 17148369.

32. Baeta R, Bellisle M, Garant D. Importance of breeding season and maternal investment in studies of sex-ratio adjustment: a case study using tree swallows. Biol Lett. 2012; 8(3):401–4. Epub 2011/12/02. https://doi.org/10.1098/rsbl.2011.1009 PMID: 22130173.
33. Dowling DK, Mulder RA. Combined influence of maternal and paternal quality on sex allocation in red-capped robins. Journal of evolutionary biology. 2006; 19(2):440–9. Epub 2006/04/08. https://doi.org/10.1111/j.1420-9101.2005.01017.x PMID: 16599920.

34. Goerlich VC, Dijkstra C, Boonekamp JJ, Groothuis TG. Change in body mass can overrule the effects of maternal testosterone on primary offspring sex ratio of first eggs in homing pigeons. Physiological and biochemical zoology: PBZ. 2010; 83(3):490–500. Epub 2010/03/30. https://doi.org/10.1086/651315 PMID: 20345244.

35. Goerlich-Jansson VC, Muller MS, Groothuis TG. Manipulation of primary sex ratio in birds: lessons from the homing pigeon (Columbia livia domestica). Integrative and comparative biology. 2013; 53(6):902–12. Epub 2013/05/31. https://doi.org/10.1093/icb/icct056 PMID: 23720529.

36. Henderson LJ, Evans NP, Heidinger BJ, Adam A, Arnold KE. Maternal condition but not corticosterone is linked to offspring sex ratio in a passerine bird. PloS one. 2014; 9(10):e110858. Epub 2014/10/28. https://doi.org/10.1371/journal.pone.0110858 PMID: 25347532.

37. Love OP, Chin EH, Wynne-Edwards KE, Williams TD. Stress hormones: a link between maternal condition and sex-biased reproductive investment. The American naturalist. 2005; 166(6):751–66. Epub 2006/02/14. https://doi.org/10.1086/497440 PMID: 1675090.

38. Nager RG, Monaghan P, Griffiths R, Houston DC, Dawson R. Experimental demonstration that offspring sex ratio varies with maternal condition. Proceedings of the National Academy of Sciences of the United States of America. 1999; 96(2):570–3. Epub 1999/01/20. PMID: 9892674.

39. Prior GL, Evans DM, Redpath S, Thirgood SJ, Monaghan P. Birds bias offspring sex ratio in response to livestock grazing. Biol Lett. 2011; 7(6):958–60. Epub 2011/05/13. https://doi.org/10.1098/rsbl.2011.0264 PMID: 21561962.

40. Pryke SR, Rollins LA, Griffith SC. Context-dependent sex allocation: constraints on the expression and evolution of maternal effects. Evolution; international journal of organic evolution. 2011; 65(10):2792–9. Epub 2011/10/05. https://doi.org/10.1111/j.1558-5646.2011.01391.x PMID: 21967422.

41. Rubenstein DR. Temporal but not spatial environmental variation drives adaptive offspring sex allocation in a plural cooperative breeder. The American naturalist. 2007; 170(1):155–65. Epub 2007/09/15. https://doi.org/10.1086/518671 PMID: 17853999.

42. Goth A, Eising CM, Herberstein ME, Groothuis TG. Consistent variation in yolk androgens in the Australian Brush-turkey, a species without sibling competition or parental care. General and comparative endocrinology. 2008; 155(3):742–8. Epub 2007/12/19. https://doi.org/10.1016/j.ygcen.2007.11.004 PMID: 18086471.