

SHORT COMMUNICATION

Incubation temperature causes skewed sex ratios in a precocial bird

Sarah E. DuRant^{1,*}, William A. Hopkins², Amanda W. Carter², Laila T. Kirkpatrick³, Kristin J. Navara⁴ and Dana M. Hawley³

ABSTRACT

Many animals with genetic sex determination are nonetheless capable of manipulating sex ratios via behavioral and physiological means, which can sometimes result in fitness benefits to the parent. Sex ratio manipulation in birds is not widely documented, and revealing the mechanisms for altered sex ratios in vertebrates remains a compelling area of research. Incubation temperature is a key component of the developmental environment for birds, but despite its well-documented effects on offspring phenotype it has rarely been considered as a factor in avian sex ratios. Using ecologically relevant manipulations of incubation temperature within the range 35.0–37.0°C, we found greater mortality of female embryos during incubation than males regardless of incubation temperature, and evidence that more female than male embryos die at the lowest incubation temperature (35.0°C). Our findings in conjunction with previous work in brush turkeys suggest incubation temperature is an important determinant of avian secondary sex ratios that requires additional study, and should be considered when estimating the impact of climate change on avian populations.

KEY WORDS: Parental effects, Secondary sex ratio, Wood duck, Incubation

INTRODUCTION

Many vertebrates, including those with genetic sex determination, can manipulate offspring sex ratios at various stages of development (Krackow, 1995; Pike and Petrie, 2003; Uller et al., 2007), including prior to fertilization, throughout embryonic development, and post-parturition or post-hatching (Pike and Petrie, 2003). In some cases, manipulation of sex ratios could be adaptive by producing more males or females when conditions are more favorable for one sex over the other (Charnov and Bull, 1977; Warner and Shine, 2008a,b; Pen et al., 2010). For instance, in parrot finches (*Erythrura trichroa*), mothers produce more sons under environmental conditions where daughters, but not sons, have high juvenile mortality (Pryke and Rollins, 2012). Although the mechanisms underlying deviations from balanced sex ratios are well studied in some vertebrate groups (e.g. reptiles), the mechanisms producing skewed ratios in birds, which exhibit genetic sex determination, remain largely unknown (Navara, 2013).

In birds, females are the heterogametic sex and there is evidence demonstrating that female birds are capable of manipulating primary sex ratios (sex ratio at fertilization) prior to fertilization (Krackow, 1995; Navara, 2013). However, birds exhibit considerable parental care post-oviposition and post-hatching, with both sexes contributing to care in many species. Thus, opportunities exist for parents to manipulate sex ratios after egg laying (i.e. secondary sex ratios). For example, incubation temperature could influence secondary sex ratios of birds via differential embryonic mortality between the sexes (Eiby et al., 2008; but see Collins et al., 2013). Avian temperature-dependent sex-biased embryonic mortality (TDSEM) was first documented in a Megapode species (Eiby et al., 2008), the Australian brush turkey (*Alectura lathami*). Unlike most other birds, which use contact incubation (i.e. transfer of heat from parents to eggs through physical contact), Megapodes use environmental heat from decomposing plant matter to incubate their eggs, and thus this avian group may be unique in showing TDSEM. Although not observed in an ecological context, three more recent studies in commercial poultry have demonstrated altered secondary sex ratios under differing incubation conditions (Tzschentke and Halle, 2009; Yilmaz et al., 2011; Piestun et al., 2013), and faster growth of male chicks post-hatch under conditions that produced more males (Tzschentke and Halle, 2009). However, these studies did not identify whether skewed sex ratios in poultry were the result of TDSEM. Thus, it remains unknown whether the occurrence of TDSEM is unique to Megapodes, and whether relatively large differences in incubation temperature are required to elicit sex-biased embryonic mortality in birds.

Unlike Megapodes, wood ducks (*Aix sponsa*) use contact incubation and nest in cavities, which is thought to be associated with relatively low variation in incubation temperature among nests compared with Megapodes (reviewed in DuRant et al., 2013a). However, substantial evidence indicates that even small variation in average incubation temperature (~1–2°C) can alter a suite of phenotypic traits (DuRant et al., 2013a,b) including evidence of skewed secondary sex ratios (this study). Specifically, more male wood duck hatchlings are produced at the lowest incubation temperature (35°C) compared with higher temperatures (Fig. 1). Based on this evidence, we determined the sex of the dead embryos to test whether TDSEM occurs in wood ducks, which, if present, could suggest that TDSEM occurs in other contact-incubating birds. Incubation temperatures used in this study represented the cooler, warmer and modal incubation temperature that a natural population of wood duck eggs experiences (Hepp et al., 2006).

MATERIALS AND METHODS

Study species

Wood ducks, *Aix sponsa* (Linnaeus), are a wide-ranging and common bird in North America. In our population, females nest from February to June, receive no aid from males during incubation,

¹Department of Integrative Biology, Oklahoma State University, Stillwater, OK 74075, USA. ²Department of Fish and Wildlife Conservation, Virginia Tech, Blacksburg, VA 24061, USA. ³Department of Biological Sciences, Virginia Tech, Blacksburg, VA 24061, USA. ⁴Poultry Science, University of Georgia, Athens, GA 30602, USA.

*Author for correspondence (Sarah.DuRant@okstate.edu)

 S.E.D., 0000-0003-0724-0878

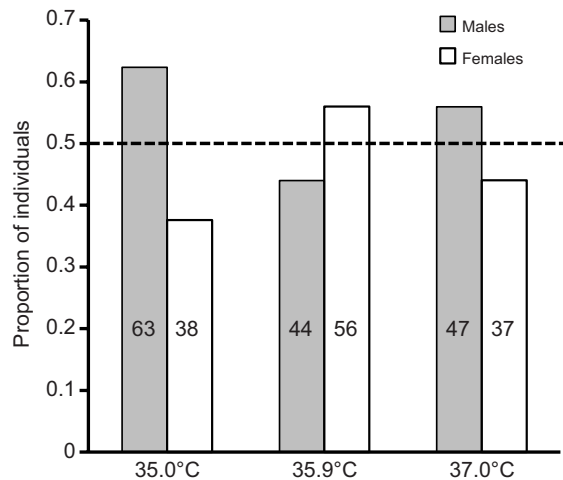


Fig. 1. Percentage of male and female wood ducks that hatched at each incubation temperature. Numbers inside bars represent the number of males or females that hatched.

and begin incubation after clutch completion (Hepp et al., 2006). Clutch size varies from 8 to 16 eggs, and nest parasitism can result in clutches of >25 eggs. Both the timing of nesting and clutch size can influence temperatures that eggs experience (reviewed in DuRant et al., 2013a).

Egg incubation

We collected eggs from wood duck nests on the Savannah River Site in Aiken, SC, USA, during the spring of 2011. We artificially incubated 380 eggs collected from 58 nests, which were evenly distributed across incubation temperature treatments to account for genetic and non-genetic parental effects. In many cases, eggs from a single nest were produced by multiple females, because wood ducks exhibit intraspecific brood parasitism. Eggs were incubated at overall average temperatures of 35.0, 35.9 or 37.0°C in Grumbach incubators (BSS 160) at Virginia Tech. Temperatures in incubators decreased twice daily by ~3°C to mimic recesses taken by females to forage (temperature reductions during recesses in field, 2–6°C). Temperatures in incubators were verified continuously throughout the study using i-button temperature loggers (model no. DS1923). Hatching success was 69–77% (hatching success in the wild is ~80% on average; range, 37.5–100%; S. F. Hope, S.E.D. and W.A.H., unpublished data) and did not differ among incubation temperatures ($P=0.27$). Further details on egg collection, incubation and treatment assignment in 2011 have been published elsewhere (DuRant et al., 2013b). All procedures were approved by the Virginia Tech Animal Care and Use Committee.

Sex identification

For ducklings that hatched, we identified sex after they were euthanized by asphyxiation with CO₂. We dissected birds and examined the gonads, and checked for the presence or absence of a penis. For embryos, we identified sex using frozen liver tissue or the entire embryo depending on the developmental stage at death. DNA was extracted using a Qiagen DNeasy® Blood and Tissue Kit tissue protocol (Qiagen Inc., Valencia, CA, USA). Amplification of the CHD1W (450 bp) and CHD1Z (600 bp) gene fragments was done using primers 2550F (5'-GTTACTGATTCGCTACGAGA-3') and 2718R (5'-ATTGAAATGATCCAGTGCTTG-3') following previously established PCR conditions (Fridolfson and Ellegren, 1999) with minor modifications. Reactions were performed in 15 µl

volumes, and amplified with 3 µl of template DNA in a DNA Engine Dyad Peltier Thermal Cycler (Bio-Rad, Hercules, CA, USA) using an annealing temperature of 55°C. PCR products were separated using 3% agarose gels in SB buffer and visualized with SYBR® Safe DNA gel stain (Invitrogen Life Technologies, Grand Island, NY, USA).

Statistical analyses

Statistical analyses were performed using JMP and SAS (SAS Institute, Cary, NC, USA) software, and statistical significance was recognized at $\alpha=0.05$. To determine whether sex ratios of hatchlings differed across incubation temperatures, we used logistic regression. We also determined whether embryonic mortality of the sexes differed across incubation temperatures with a logistic regression model (JMP, SAS Institute) where mortality (survived or died) was the dependent variable and sex, temperature and their interaction were independent variables. We also tested for differences in embryonic mortality within incubation temperatures and within the sexes using a series of logistic regressions.

RESULTS AND DISCUSSION

We detected significant differences in secondary sex ratios of ducklings (Fig. 1; $\chi^2=6.9$, $P=0.03$), and provide evidence that skewing of the sex ratio may occur through TDSEM. Embryonic mortality significantly differed between the sexes (Fig. 2; $\chi^2=4.01$, $P=0.045$), and this difference appeared to be present only at the lowest incubation temperature (Fig. 2; individual effect test for females at 35.0°C versus 35.9°C: $P=0.047$), but we were unable to detect a statistically significant interaction between incubation temperature and sex on embryonic mortality (Fig. 2; temperature: $\chi^2=3.77$, $P=0.15$; sex×temperature: $\chi^2=4.23$, $P=0.12$). However, individual logistic regression analyses of embryonic mortality of males and females within each temperature support TDSEM; significantly more females died than males at 35.0°C ($\chi^2=3.96$, $P=0.046$), while a similar number of males and females died at 35.9 and 37.0°C ($P\geq 0.37$ in both cases). This is the first evidence that incubation temperature can lead to skewed sex ratios through

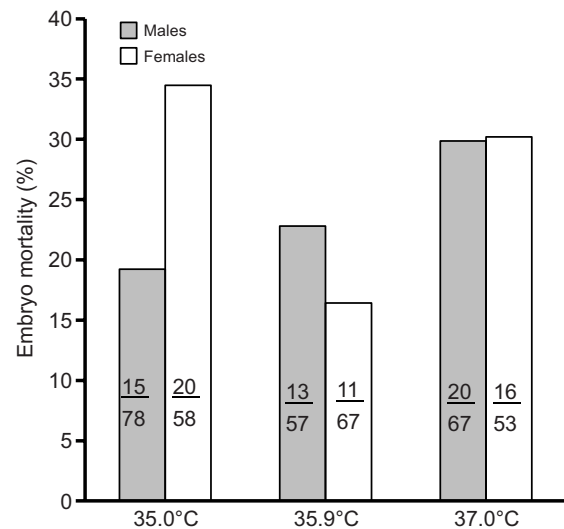


Fig. 2. Percentage of male and female wood duck embryos that died at each incubation temperature. Numbers inside bars represent the number of embryos that died (top number) and the number of embryos incubated (bottom number).

TDSEM in a wild bird exhibiting contact incubation, the predominant method of incubation among birds.

Consistent with findings in Megapodes, female embryonic mortality was highest at the lowest incubation temperature in wood ducks. However, Megapodes also experience higher male embryonic mortality at the highest incubation temperature (Eiby et al., 2008), which we did not detect. However, Megapode embryos were incubated across a 4°C (32–36°C) temperature range (Eiby et al., 2008), twice the narrow temperature range used in the current study. Further, in wood ducks, when comparing the primary sex ratio with the secondary sex ratio at the lowest incubation temperature, the ratio becomes more skewed towards males by 5%. This pattern diminishes in a step-wise pattern with increasing temperature (35.9°C, 2%; 37.0°C, –0.2%), suggesting that at higher incubation temperatures secondary sex ratios may eventually be skewed towards females in wood ducks. Because average incubation temperature of wood duck nests can exceed 37°C and fall below 35°C (S. F. Hope, S.E.D. and W.A.H., unpublished data), it remains possible that effects of incubation temperature on TDSEM in wood ducks may be even more apparent if eggs are incubated at temperatures closer to the thermal extremes. Across avian species, TDSEM may be less likely to occur in species exhibiting little variation in incubation temperature (Collins et al., 2013), but more likely in species exhibiting a broader range of incubation temperatures (Coe et al., 2015).

It is unclear why more female embryos died during incubation in this study relative to male embryos, or why more females died at the lower incubation temperature in both wood ducks and Megapodes. Male and female survival can vary differentially across ontogeny (Orzack et al., 2015). Further, males and females can respond differently to developmental conditions and environmental stressors (e.g. Trivers and Willard, 1973; Love et al., 2005; Sockman et al., 2008), and some studies in reptiles suggest that sexes produced at temperatures more conducive to their development experience greater survival and reproduction (Burger and Zappalorti, 1988; Warner and Shine, 2008a). Research in reptiles exhibiting temperature-dependent sex determination suggests that differences in maternally derived sex hormones and changes in embryonic gene expression are both important in sex determination (Crews et al., 1994; Bowden et al., 2014). Whether these factors or other maternally derived egg constituents (e.g. corticosterone; Love et al., 2005) can also contribute to sex-biased embryonic mortality in birds is unknown (Alonso-Alvarez, 2006), but warrants investigation. In addition, investigating when embryonic mortality occurs may help reveal mechanisms for sex-biased mortality.

It remains unknown whether TDSEM could be adaptive, or is simply an artifact of sex-specific differences in embryonic physiology. It is possible that like some reptiles (Warner and Shine, 2008a), more robust males are produced at temperatures conducive to male embryonic development and more robust females are produced at temperatures conducive to female embryonic development. This question has not been explicitly tested in birds. There is, however, some indirect evidence that incubation temperature results in skewed avian sex ratios that could result in fitness benefits to the parent (Tzschentke and Halle, 2009; Graham et al., 2011; Bowers et al., 2013; Piestun et al., 2013). For example, in Lincoln sparrows (*Melospiza lincolnii*), more male offspring are produced early in the nesting season when male offspring tend to develop traits associated with high-quality adults (Graham et al., 2011). Although not measured in the study on sparrows, nest temperatures could have produced the observed skewed sex ratios, whereby the abiotic environment (changing temperature) affected

nest temperatures (DuRant et al., 2013a) and embryonic mortality. Alternatively, female sparrows may actively alter primary sex ratio (Komdeur, 2013) during the part of the season that is more conducive to each sex's embryonic development.

Regardless of whether birds can adaptively skew secondary sex ratios via incubation temperature, our findings in conjunction with the work in brush turkeys suggest that temperature warrants further investigation as a factor that influences avian sex ratios. Manipulation of avian sex ratios during incubation offers research opportunities to investigate how incubation temperature causes sex-biased embryonic mortality, whether physiology (e.g. brood patch temperature; Lea and Klandorf, 2002) and behavior of incubating parents influence sex ratios, and whether parents modulate behavior or reproductive physiology based on environmental factors that result in beneficial skewing of sex ratios. Further, given that our findings are based on temperature differences of less than 1°C and that environmental temperature influences nest temperature, our findings should be considered when estimating the effects of climate change and other anthropogenic disturbances on avian populations (DuRant et al., 2013a).

Acknowledgements

We thank S. Chin, M. Hepner and T. Lombardi for assistance in the field and lab, and G. R. Hepp, R. A. Kennamer and the Savannah River Ecology Laboratory for making this work possible.

Competing interests

The authors declare no competing or financial interests.

Author contributions

S.E.D. and W.A.H. contributed to all aspects of the study. A.W.C. contributed to data collection, interpretation and manuscript preparation. D.M.H., L.T.K., K.J.N. contributed to sample analysis, data interpretation and manuscript preparation.

Funding

Funding came from Directorate for Biological Sciences grant IOB-0615361 to W.A.H., Directorate for Biological Sciences DDIG (DEB-1110386) to S.E.D. and W.A.H., Directorate for Biological Sciences grant IOS-1054675 to D.M.H., and the Fralin Life Sciences Institute.

References

- Alonso-Alvarez, C. (2006). Manipulation of primary sex-ratio: an updated review. *Av. Poult. Biol. Rev.* **17**, 1–20.
- Bowden, R. M., Carter, A. W. and Paitz, R. T. (2014). Constancy in an inconstant world: moving beyond constant temperatures in the study of reptilian incubation. *Integr. Comp. Biol.* **54**, 830–840.
- Bowers, E. K., Munclinger, P., Bureš, S., Kučerová, L., Nádvořník, P. and Krist, M. (2013). Cross-fostering eggs reveals that female collared flycatchers adjust clutch sex ratios according to parental ability to invest in offspring. *Mol. Ecol.* **22**, 215–228.
- Burger, J. and Zappalorti, R. T. (1988). Effects of incubation temperature on sex ratios in pine snakes: differential vulnerability of males and females. *Am. Nat.* **132**, 492–505.
- Charnov, E. L. and Bull, J. (1977). When is sex environmentally determined? *Nature* **266**, 828–830.
- Coe, B. H., Beck, M. L., Chin, S. Y., Jachowski, C. M. B. and Hopkins, W. A. (2015). Local variation in weather conditions influences incubation behavior and temperature in a passerine bird. *J. Avian Biol.* **46**, 385–394.
- Collins, K. E., Jordan, B. J., McLendon, B. L., Navara, K. J., Beckstead, R. B. and Wilson, J. L. (2013). No evidence of temperature-dependent sex determination or sex-biased embryo mortality in the chicken. *Poult. Sci.* **92**, 3096–3102.
- Crews, D., Bergeron, J. M., Bull, J. J., Flores, D., Tousignant, A., Skipper, J. K. and Wibbels, T. (1994). Temperature-dependent sex determination in reptiles: proximate mechanisms, ultimate outcomes, and practical applications. *Dev. Genet.* **15**, 297–312.
- DuRant, S. E., Hopkins, W. A., Hepp, G. R. and Walters, J. R. (2013a). Ecological, evolutionary, and conservation implications of incubation temperature-dependent phenotypes in birds. *Biol. Rev.* **88**, 499–509.
- DuRant, S. E., Hopkins, W. A., Carter, A. W., Stachowiak, C. M. and Hepp, G. R. (2013b). Incubation conditions are more important in determining early

- thermoregulatory ability than post-hatch resource conditions in a precocial bird. *Physiol. Biochem. Zool.* **86**, 410-420.
- Eiby, Y. A., Wilmer, J. W. and Booth, D. T.** (2008). Temperature-dependent sex-biased embryo mortality in a bird. *Proc. R. Soc. B Biol. Sci.* **275**, 2703-2706.
- Fridolfson, A.-K. and Ellegren, H.** (1999). A simple and universal method for molecular sexing of non-ratite birds. *J. Avian Biol.* **30**, 116-121.
- Graham, E. B., Caro, S. P. and Sockman, K. W.** (2011). Change in offspring sex ratio over a very short season in Lincoln's sparrows: the potential role of bill development. *J. Field Ornithol.* **82**, 44-51.
- Hepp, G. R., Kennamer, R. A. and Johnson, M. H.** (2006). Maternal effects in Wood Ducks: incubation temperature influences incubation period and neonate phenotype. *Funct. Ecol.* **20**, 307-314.
- Komdeur, J.** (2013). Daughters on request: about helpers and egg sexes in the Seychelles warbler. *Proc. R. Soc. B Biol. Sci.* **270**, 3-11.
- Krackow, S.** (1995). Potential mechanisms for sex ratio adjustment in mammals and birds. *Biol. Rev.* **70**, 225-241.
- Lea, R. W. and Klandorf, H.** (2002). The brood patch. In *Avian Incubation: Behaviour, Environment, and Evolution* (ed. D. C. Deeming), pp 100-118. Oxford: Oxford University Press.
- Love, O. P., Chin, E. H., Wynne-Edwards, K. E. and Williams, T. D.** (2005). Stress hormones: a link between maternal condition and sex-biased reproductive investment. *Am. Nat.* **166**, 751-766.
- Navara, K. J.** (2013). The role of steroid hormones in the adjustment of primary sex ratio in birds: compiling the pieces of the puzzle. *Integr. Comp. Biol.* **53**, 923-937.
- Orzack, S. H., Stubblefield, J. W., Viatcheslav, R. A., Colls, P., Munné, S., Scholl, T., Steinsaltz, D. and Zuckerman, J. E.** (2015). The human sex ratio from conception to birth. *Proc. Natl. Acad. Sci. USA* **112**, E2102-E2111.
- Pen, I., Uller, T., Feldmeyer, B., Harts, A., While, G. M. and Wapstra, E.** (2010). Climate-driven population divergence in sex-determining systems. *Nature* **468**, 436-438.
- Piestun, Y., Druyan, S., Brake, J. and Yahav, S.** (2013). Thermal treatments prior to and during the beginning of incubation affect phenotypic characteristics of broiler chickens posthatching. *Poult. Sci.* **92**, 882-889.
- Pike, T. W. and Petrie, M.** (2003). Potential mechanisms of avian sex manipulation. *Biol. Rev.* **78**, 553-574.
- Pryke, S. R. and Rollins, L. A.** (2012). Mothers adjust offspring sex to match the quality of the rearing environment. *Proc. R. Soc. B Biol. Sci.* **279**, 4051-4057.
- Sockman, K. W., Weiss, J., Webster, M. S., Talbott, V. and Schwabl, H.** (2008). Sex-specific effects of yolk-androgens on growth of nestling American kestrels. *Behav. Ecol. Sociobiol.* **62**, 617-625.
- Trivers, R. L. and Willard, D. E.** (1973). Natural selection of parental ability to vary the sex ratio of offspring. *Science* **179**, 90-92.
- Tzschentke, B. and Halle, I.** (2009). Influence of temperature stimulation during the last 4 days of incubation on secondary sex ratio and later performance in male and female broiler chicks. *Br. J. Poult. Sci.* **50**, 634-640.
- Uller, T., Pen, I., Wapstra, E., Beukeboom, L. W. and Komdeur, J.** (2007). The evolution of sex ratios and sex-determining systems. *Trends Ecol. Evol.* **22**, 292-297.
- Warner, D. A. and Shine, R.** (2008a). The adaptive significance of temperature-dependent sex determination in a reptile. *Nature* **451**, 566-568.
- Warner, D. A. and Shine, R.** (2008b). Interactions among thermal parameters determine offspring sex under temperature-dependent sex determination. *Proc. R. Soc. B Biol. Sci.* **278**, 256-265.
- Yilmaz, A., Tepeli, C., Garip, M. and Çağlayan, T.** (2011). The effects of incubation temperature on the sex of Japanese quail chicks. *Poult. Sci.* **90**, 2402-2406.