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BILATERAL ASYMMETRY IN AVIAN TESTICULAR MORPHOLOGY AND FUNCTION

By

Pamela Paasché O'Hearn

A dissertation

submitted in partial fulfillment

of the requirements for the degree of

Doctor of Science in the Department of Biological Sciences

Idaho State University

May, 2017

To the Graduate Faculty:

The members of the committee appointed to examine the dissertation of Pamela Paasché O'Hearn find it satisfactory and recommend that it be accepted.

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Animal Welfare Research Committee Approval Page

29 January, 2016

Dr. David J. Delehanty
Department of Biological Sciences
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RE: Your application dated (3/21/2011) regarding study number (FRP-6350408): Left-Side Bias in Avian Testicular Morphology and Function

Dear Dr. Delehanty:

I have reviewed your request for expedited approval of the new study listed above. This is to confirm that I have approved your application. You may conduct your study as described in your application effective immediately. The study is subject to an annual review on or before 03/21/2012, unless closed before that date. Please note that any changes to the study as approved must be promptly reported and approved. Some changes may be approved by expedited review; others require full board review. Contact Tom Bailey (208-282-2179; fax 208-282-4723; email: anmlcare@isu.edu) if you have any questions or require further information.

Sincerely,

Curt Anderson, PhD

This dissertation is dedicated
in memory of
James Francis O'Hearn

ACKNOWLEDGMENTS

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ABSTRACT

In birds, sexual selection has strongly influenced physical and behavioral traits affecting transmission and receipt of gametes. Studies of avian physiology assume that the left and right testes of male birds are compositional analogues of one another and that both testes participate proportionally in androgen and sperm production. However, using a galliform species as a model, I tested the null hypothesis that the left and right testis are compositional analogues, and found that the left and right testes differ within a male. Both testes participate equally in androgen production, yet the left testis is enlarged relative to the right in spermatogenic cells. Using species within the monophyletic Galloanserae, I then tested the null hypothesis that testicular asymmetry is the primitive, or basal, condition versus the possibility that testicular asymmetry in spermatogenic cells is related to the presence or absence of a phallus. I found the evidence did not support a relationship of testicular asymmetry to presence or absence of a phallus, but that testicular asymmetry appears to be a basal condition. I then tested the hypothesis that testicular asymmetry was not influenced by mating system (and thus sperm competition), and found evidence to support a relationship between mating system and testicular asymmetry. Where sperm competition is more likely, the degree of testicular asymmetry is lower. I used the data regarding spermatogenic cell densities in the left versus right testes to test the null hypothesis that overall spermatogenic cell density was consistent between mating systems, and found evidence to support higher spermatogenic cell densities in polygamous species. Finally, I used the data regarding spermatogenic cell densities in the left versus right testes to test the null hypothesis that overall spermatogenic cell capacity in the testes was consistent throughout both the galliform and

anseriform branches of the Galloanserae and found that total testicular spermatogenic cell densities were higher in the Anseriformes than in the Galliformes.

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Introduction

Male birds generally exhibit testicular asymmetry in size and mass, most commonly with a larger left testis. Although this phenomenon has long been reported, to date testicular asymmetry has not received thorough attention. Investigating testicular asymmetry and its impacts is further complicated by the fact that avian testes do not reach a static mature size, but undergo an annual cycle of testis regression at the end of each breeding season followed by recrudescence preceding the subsequent breeding season. Additionally, testicular asymmetry increases with the age of the male (Graves 2004). This means that measures of males taken from the wild outside their peak breeding season may not reflect actual breeding season asymmetry, and that measures may be very different among the different age classes of males. Avian testis dynamics and bilateral asymmetry are important to understanding avian life history, breeding behavior, and evolution. Male birds are the product of substantial sexual selection, not only overt such as in ornamentation, but also cryptic sexual selection. Sperm competition is one strong form of cryptic sexual selection affecting male birds. Sexual selection can influence testicular morphology. The bilateral asymmetry found in avian testes means that studies that use one testis for androgen measures and the other for cytological measures may be inaccurately extrapolating values for both components. Artificial insemination has long been employed commercially in avian propagation, and is being used increasingly for conservation and species preservation. In fact, captive breeding has become essential to the maintenance and propagation of many species, but wild species reared in captivity across generations may be subject to intentional or unintentional

artificial selection due to semen harvesting and artificial insemination, potentially skewing testis development and annual reproductive dynamics.

At least three hypotheses have been advanced to attempt to explain why bird testes exhibit reproductive asymmetry. First, testes may be asymmetrical as an epiphenomenon related to female asymmetry, where male asymmetry results developmentally as a carry-over effect from female asymmetry with a species-wide tendency for germ cells to move asymmetrically to the gonads during differentiation to be driven as an outcome of female asymmetry (Venzke 1954). A second hypothesis proposes that possessing two equally large testes may have too great of a cost in terms of overall mass or immunosuppressive effects of high levels of androgens in the blood (Møller 1994). A third hypothesis explains avian testes asymmetry as fluctuating asymmetry, or deviation from perfect bilateral symmetry as a result of environmental factors (Kimball *et al.* 1997). However, to date, none of these hypotheses has been well supported by data. Neither are these hypotheses exhaustive of all possibilities. For example, testicular asymmetry may result from cryptic sexual selection on males to inseminate females and fertilize eggs.

One indication that avian testes may be subject to selection pressures different from those observed for other terrestrial vertebrates is the evolutionary loss of the phallus. In birds, fertilization is internal in all cases, but an intromittent phallus in males occurs in only approximately 3% of species. The phallus is present in the most basal avian lineages, the Paleognathae. Within the Neognathae, a phallus is present in only the basal, monophyletic Galloanserae, in which a transition from an intromittent phallus to an non-intromittent phallus occurs.

The Galloanserae are comprised of two avian orders, Anseriformes (waterfowl) and the Galliformes (gamefowl). Within Anseriformes, male and female reproductive tract morphology is the result of an extreme evolutionary arms race, with males developing large and complex intromittent phalluses that closely match the complex female reproductive anatomy (Brennan *et al.* 2007). It is believed that this has arisen as a result of high levels of male-male competition and forced extra-pair copulations (Birkhead *et al.* 1998, Brennan *et al.* 2010). In contrast, males within the Galliformes have greatly reduced phalluses that are non-intromittent.

Bilateral asymmetry between the left and right testes could be due to a simple size and mass difference between the two testes in which relative tissue constituents within each testis are constant. In this case, the right would simply be a smaller analogue of the left testis in males of species that exhibit a larger left testis. If the left testis is simply a larger analogue of the right, then the left testis would produce more sperm than the right simply because of greater size and mass. It is also possible that the left testis has been selected for greater sperm or androgen production, *per* se. The increased mass would be due to an increase in the proportion of sperm-producing or androgen-producing tissue contained in the testis. In either case, the left and right testes could be bilaterally symmetrical in terms of one function while being asymmetrical in terms of the other function.

The testis is composed primarily of sperm-producing tissues, and thus it is likely that any large-scale bilateral asymmetry involves sperm–producing tissues. For example, it may be that the left testis is larger than the right because it has a higher proportion of seminiferous tubules. This hypothesis is plausible given that germinal and endocrine

tissues originate at different times during gonadal development. Because the germinal and endocrine tissues originate at different times, there is no reason to assume that selection pressure for an increase in sperm producing tissue must result in selection for increased androgen cells within interstitial tissue. The existence of asymmetrical testes, in combination with the segregation of sperm on left and right sides until after ejaculation, opens the possibility that the left and right testes make unequal contributions to the total volume of sperm contained in an ejaculate.

It is also possible that the testicular asymmetry found in male reproductive systems of birds is reproductively advantageous in matching the unilateralism of females, and may maximize the efficiency of fertilization without an intromittent phallus. The reproductive asymmetry seen in males may have resulted from cascading effects emanating from a non-sexual adaptations in the avian body plan and female reproductive physiology including adaptations for flight.

Data from several species exists showing a left-side copulation bias. The argument for males copulating from the female's left side in order to access the left-of-center oviduct opening might also explain male gonadal asymmetry. In particular, cryptic sexual selection could favor males that copulate from the female's left side if the asymmetry of the female reproductive tract results in ejaculates being placed closer to the oviduct when cloacal contact occurs from the left rather than from the right. Also, during left-side copulation, if semen discharging from the left deferent duct of males is better positioned for insemination than semen from the right duct, then males may be under selection to allocate more resources to left testis development than to right testis development resulting in testicular asymmetry. In black coucals (*Centropus grillii*), a

polyandrous species with sole male parental care, males have the unusual condition of highly asymmetrical right-larger testes, and have a large right seminal papilla, perhaps to achieve more optimal placement of the semen into the female's left oviduct opening (Maurer 2008, Frey & Goymann 2009).

Testicular asymmetry, combined with a left-side bias in copulation, suggests that a complex system exists in which males may be able to transfer more sperm from their larger left testis into the vaginal opening of the female's oviduct if he copulates from her left side. Because male birds experience a high level of sperm competition and require a high level of female cooperation for insemination, males are under strong selection for effective gametic transfer. Traits allowing a male to increase his chances of fertilizing an egg should be strongly favored through increased fitness.

Thus, basal characteristics (intromittent phallus) may have been superseded by behavioral and internal morphological adaptations. It is hypothesized that the loss of the intromittent phallus may have arisen from female-driven selection pressures, such as avoiding forced extra-pair copulations. A fundamental question underlying sexual selection is mate choice. My research provides a new aspect of interplay of female choice and evolutionary arms race, where from a common ancestor that possessed an intromittent phallus, in one order (Anseriformes) males have developed large complicated phalluses which match complex, convoluted female reproductive tracts, yet in the sister order to this group, Galliformes, the phallus has become reduced, is non-intromittent, and the female vagina is simple. Examining these two groups, it appears that powerful adaptations are in play, but that these adaptations differ behaviorally and morphologically. This implies that the asymmetry in testis size in avian species may be

an evolutionary response to differences in fertilization success from the left versus the right testis, perhaps due to the asymmetry in the female reproductive tract.

I examined in detail bilateral testis asymmetry within the monophyletic basal neognathus birds known as the Galloanserae, i.e., the waterfowl and gamefowl. This group of birds exhibits well known gonadal asymmetry and also encompasses the evolutionary loss of an intromittent phallus among the neognathus birds. I selected this group to try to elucidate the relationship between testicular asymmetry and reproduction. In Chapter One, I provide an overview of avian reproduction with an emphasis on avian gonads but also provide an overview of avian mating systems and avian parental care systems in order to provide context for the cryptic sexual selection and sexual morphology that laid the foundation for this study.

In Chapter Two, I explore male testicular cytology and sperm production in depth, using the chukar (*Alectoris chukar*), a Eurasian partridge within the order Galliformes of the Galloanserae as a model species. Importantly, I find that the left testis, which is larger than the right testis in chukar, and right testes are not simple analogues of one another. The large left testis of chukar is larger because it is augmented with sperm-producing tissue but not correspondingly augmented with androgen-producing tissue. Furthermore, because the chukar testis is formed of two different embryological tissues that perform different functions, I recognize that evolutionary pressures can act on one tissue type within the testis without acting on the other tissue type. Androgens are released into general circulation and act on tissue throughout the male avian body, including the testes after having been delivered to tissues through general circulation. Release of androgens into general circulation nullifies any intrinsic benefit to bilateral

asymmetry in androgen production between the left and right testes. However, the sperm cells produced in the left and right testes are expelled through left and right deferent ducts, respectively. In most birds, sperm are emitted without prior merging in a phallus. Therefore, a unilateral bias in sperm production could be important, especially if efficiency in gamete production and emission increases with testis size and one deferent duct is better positioned than the other to inseminate a female effectively.

Using my findings from chukar as evidence that left and right testes within individual male birds are not histolological analogues of one another, I expanded the study to explore the left testis versus right testis asymmetry in spermatogenic production in the Galloanserae. Galloanserae is a monophyletic basal avian group consisting of the Anseriformes that possess an intromittent phallus employed in copulation, and the sister order Galliformes that have a small, non-intromittent phallus and where sperm that is emitted through a left and a right papilla. In Chapter Three, I explore the universality of the chukar findings, and possible relationships to the presence or absence of an intromittent phallus as well as the likelihood of a form cryptic sexual selection known as sperm competition. I found very strong evidence for previously unrecognized gonadal asymmetry in testis composition and spermatogenesis in the Galloanserae. As a group, the left testis of males in Galloanserae is strongly biased toward sperm production. This feature appears to be the primitive condition, occurring regardless of the presence or absence of an intromittent phallus. However, I identified a significant relationship between the degree of bilateral spermatogenic asymmetry and the likelihood that males are subject to sperm competition. Males of species with a high incidence of polygamous mating, and therefore high likelihood of sperm competition, exhibited a lower degree of

bilateral spermatogenic asymmetry than males of species within monogamous mating systems, regardless of phallus type or order. Males subject to sperm competition exhibited a high degree of left testis specialization for sperm production.

In the final chapter, I investigate more subtle differences in sperm production within the Galloanserae to try to discover differences in reproductive strategies that might be related to phallus loss in birds. Overall, I find evidence supporting greater sperm production in polygamous Galloanserae than monogamous Galloanserae, and greater sperm production in Anseriformes than in Galliformes. These findings, together, demonstrate a new dimension to spermatogenesis.

Phylogenetic Overview

The class Aves is a large and diverse class of vertebrates. First arising from the maniraptoran group of theropod dinosaurs in the Jurassic Period, there are approximately 10,000 species of birds present today, and an estimated 300 billion individuals. The order Crocodilia is the sister group to the Aves, sometimes named Neornithes, which together form the clade Archosauria.

Current molecular and morphological data suggests that the Neornthines are divided basally into the neognathous (Neognathae) and palaeognathous (Palaeognathae) birds, characterized by differences in skull and jaw morphology. The Paleognathae are comprised of the ratites, large flightless birds in the order Struthioniformes which include the ostriches (Struthionidae, 2 species), the rheas (Rhehidae, 3 species), emu (Dromaiidae, 1 species), cassowaries (Casuariidae, 3 species) kiwis (Apterygidae, 5 species), and the flighted tinamous (Tinamiformes, 47 species) found in Central and South America (Brennan *et al.* 2008, Boyd 2016).

The earliest divergence within the Neognathae likely occurred during the cretatceous period prior to the Cretaceous-Paleogene extinction event, and divides the Galloanserae from all remaining Neoaves (Figure I-1). The Galloanserae comprise a superorder containing the Anseriformes and Galliformes. The order Anseriformes contains approximately 180 species in 3 families: Anatidae (waterfowl, 170+ species), Anhimidae (screamers, 3 species), and Anseranatidae (magpie geese, 1 species). The order Galliformes comprises approximately 300 species in 5 families, the Phasianidae (pheasants, grouse and allies, 150+ species), Odontophoridae (New World quails, 34 species), Numididae (guineafowl, 6 species), Cracidae (guans, chachalacas and

curassows, 50 species), and the Megapodiidae (brush turkeys and moundbuilders, 20+ species) (Eo *et al.* 2009).

Literature Cited

Bertelli, S. (2016). Advances on tinamou phylogeny: an assembled cladistic study of the volant palaeognathous birds. *Cladistics* 32: 1-24.

Birkhead, T. R. (1998). Sperm competition in birds. *Reviews of Reproduction* 3: 123-129.

Birkhead, T. R., J. G. Martinez, T. Burke and D. P. Froman (1999). Sperm mobility determines the outcome of sperm competition in the domestic fowl. *Proceedings of the Royal Society of London, Series B: Biological Sciences* 266: 1759-1764.

Boyd, J. H. (2016). Taxonomy In Flux. http://jboyd.net/Taxo/List1.html.

Brennan P. L. R., R. O. Prum, K. G. McCracken, M. D. Sorenson, R. E. Wilson and T. R. Birkhead (2007). Coevolution of male and female genital morphology in waterfowl. *PLoS ONE* 2: e418. doi:10.1371/journal.pone.0000418.

Brennan, P. L. R., T. R. Birkhead, K. Zyskowski, J. van der Waag and R. O. Prum (2008). Independent evolutionary reductions of the phallus in basal birds. *Journal of Avian Biology* 39: 487-492.

Brennan, P. L. R., C. J. Clark and R. O. Prum (2010). Explosive eversion and functional morphology of the duck penis supports sexual conflict in waterfowl genitalia *Proceedings of the Royal Society of London, Series B: Biological Sciences* 277: 1309-1342.

Eo, S.H., O. R. Bininda-Emonds and J. P. Carroll (2009). A phylogenetic supertree of the fowls (Galloanserae, Aves). *Zoologica Scripta 38*: 465-481.

Frey, R. and W. Goymann (2009). A single functional testis and long deferent duct papillae: the peculiar male reproductive tract of the classically polyandrous, sex-

role reversed black coucal (*Centropus grillii*). *Journal of Ornithology 150:* 827-838.

Graves, G. R. (2004). Testicular volume and asymmetry are age-dependent in black-throated blue warblers (*Dendroica caerulescens*). *The Auk 121*: 473-485.

Hackett, S. J., R. T. Kimball, S. Reddy, R. C. K. Bowie, E. L. Braun, M. J. Braun, J. L. Chojnowski, W. A. Cox, K. L. Han, J. Harshman, C. J. Huddleston, B. D. Marks, K. J. Miglia, W. S. Moore, F. H. Sheldon, D. W. Steadman, C. C. Witt and T. Yuri. (2008). A phylogenomic study of birds reveals their evolutionary history. *Science* 320: 1763-1768.

Herrera, A.M., S. G. Shuster, C. L. Perriton and M. J. Cohn (2013). Developmental basis of phallus reduction during bird evolution. *Current Biology 23:* 1065-1074.

Kimball, R. T., J. D. Ligon and M. Merola-Zwartjes (1997). Testicular asymmetry and secondary sexual characters in red junglefowl. *The Auk* 114: 221-228.

King, A. S. (1981). Phallus. p107-148 *In:* A. S. King and J. McLelland [eds.] Form and Function in Birds. Vol. 2. Academic Press, New York.

Møller, A.P. (1994). Directional selection on directional asymmetry: testes size and secondary sexual characters in birds. *Proceedings of the Royal Society of London, Series B*: *Biological Sciences* 258: 147-151.

Peterson, A. A., M. P. Lombardo and H. W. Power (2001). Left-sided directional bias of cloacal contacts during tree swallow copulations. *Animal Behavior* 62: 739-741.

Prum, R. O., J. S. Berv, A. Dornburg, D. J. Field, J. P. Townsend, E. M. Lemmon and A. R. Lemmon (2015). A comprehensive phylogeny of birds (Aves) using targeted next-generation DNA sequencing *Nature* 526: 569-573.

Sibley, C. G. and J. E. Ahlquist (1990). *Phylogeny and Classification of Birds: A Study in Molecular Evolution*. Yale University Press, New Haven, CT.

Suh, A. (2016). The phylogenomic forest of bird trees contains a hard polytomy at the root of Neoaves. *Zoologica Scripta* 45: 50-62.

Venzke, W.G. (1954). The morphogenesis of the indifferent gonad of chick embryos. *American Journal of Veterinary Research* 15: 300-308.

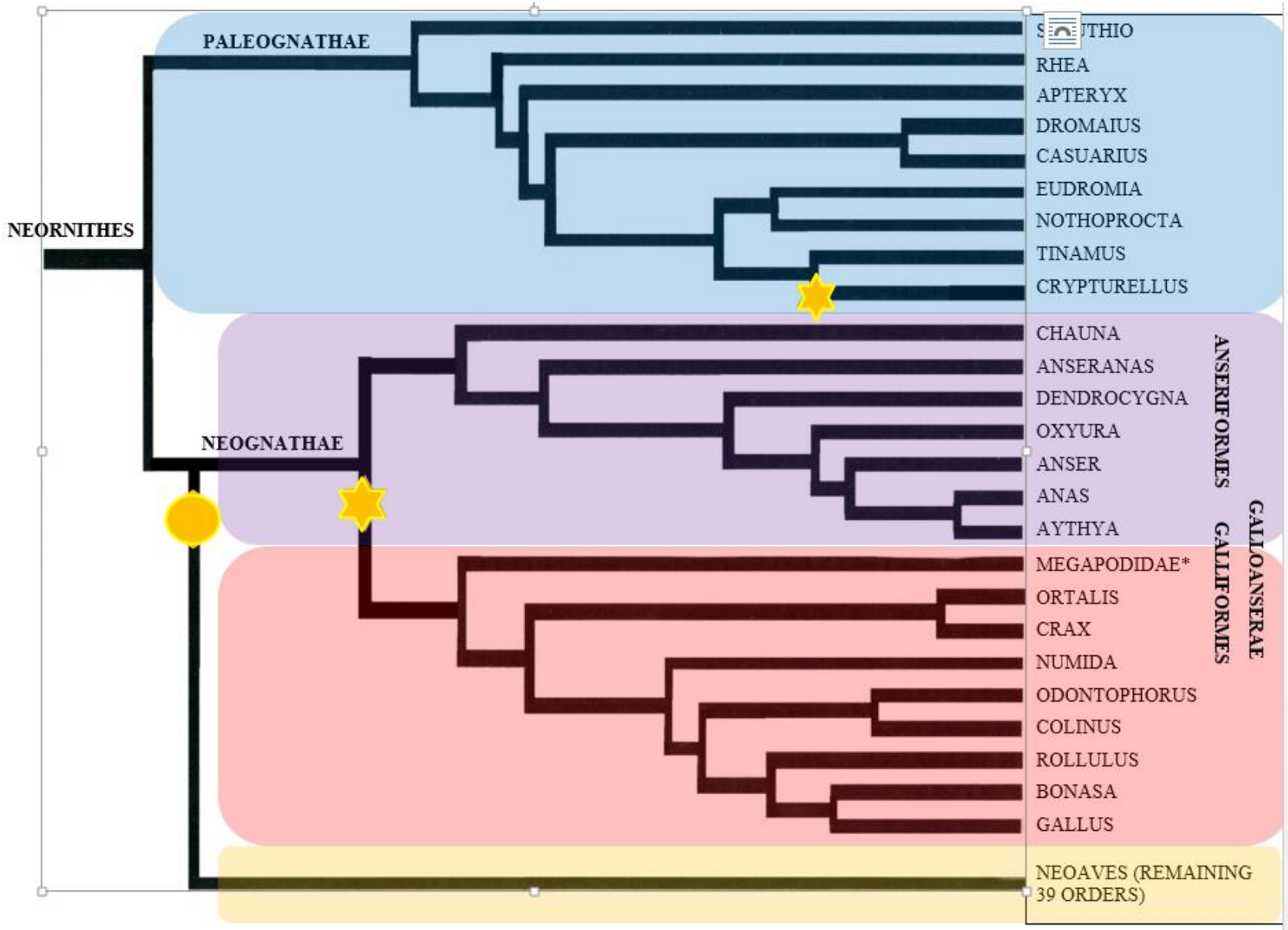

PALEOGNATHAE
NEORNITHES
NEOGNATHAE
STRUTHIO
RHEA
APTERYX
DROMAIUS
CASUARIUS
EUDROMIA
NOTHOPROCTA
TINAMUS
CRYPTURELLUS
CHAUNA
ANSERANAS
DENDROCYGNA
OXYURA
ANSER
ANAS
AYTHYA
MEGAPODIDAE*
ORTALIS
CRAX
NUMIDA
ODONTOPHORUS
COLINUS
ROLLULUS
BONASA
GALLUS
NEOAVES (REMAINING 39 ORDERS)
ANSERIFORMES
GALLIFORMES
GALLOANSERAE

Figure I-1. Basal avian phylogeny showing the relationship between the paleognathous and neognathous clades and the relationship between the Galloanserae and Neoaves within the Neognathae. Stars indicate points of reduction of the phallus from intromittent to non intromittent, occurring within *Crypturellus* in the Tinamiformes, and in the Galliformes. Orange dot marks the evolutionary point of complete phallus loss occurring in the Neoaves. In birds, a phallus is lacking in over 97% of extant species. (Phylogeny modified from Prum *et al*. 2015, also using Sibley & Ahlquist 1990, Hackett *et al*. 2009, Herrera *et al*. 2013, Bertelli 2016, Boyd 2016, Suh 2016).

CHAPTER ONE

SEXUAL REPRODUCTION IN BIRDS

Sex determination in birds

In birds, sex is determined at the time of fertilization through the inheritance of sex chromosomes (chromosomal sex determination) (Smith *et al.* 2007, Smith 2010). The female is the heterogametic sex, possessing a Z and a W chromosome, whereas the male is homogametic, possessing two Z chromosomes. Avian sex chromosomes derive from ancestral autosomal chromosomes. The Z sex chromosome is strongly conserved across all birds, while the W has undergone varying degrees of reduction among avian taxa (Shetty *et al.*1999, Nanda & Shmid 2002, Raudsepp *et al.* 2002, Berlin & Ellegren 2006).

The extant birds of the world are composed of two large clades, the Paleognathae and Neognathae. The classification is based anatomically on palatal form and supported phylogenetically. Paleognaths and neognaths share a typical avian karyotype (Takagi *et al.*1972, Takagi & Sasaki 1974, de Boer 1980, Belterman & de Boer 1984, Ansari *et al.*1988), except that sex chromosomes differ between the paleognaths and neognaths.

The paleognaths consist of the ratites, primarily southern hemispheric flightless species in the order Struthioniformes (families Struthionidae, Rhehidae, Dromaiidae, Casuariidae and Apterygidae) and the flighted Neotropical tinamous (order Tinamiformes) (Sibley & Ahlquist 1990, van Tuinen & Hedges 1998, 2000, Cracraft 2001, Bertelli 2016). In the ratites, sex chromosomes are largely homomorphic and euchromatic and show little differentiation (Takagi *et al.* 1972, de Boer 1980, Ansari *et al*. 1988). In tinamous, the W chromosomes are intermediate between the ratites and the

neognathous birds (Sasaki *et al.* 1980, Pigozzi & Solari 1999, 2005, Mank & Ellegren 2007, Tsuda *et al.* 2007, Itoh *et al.* 2008).

In the neognaths, W chromosomes are highly differentiated from the Z chromosomes. The W chromosomes are smaller than the Z chromosomes, conspicuously heterochromatin-rich and late replicating (Takagi *et al.* 1972, Schmid *et al.* 1989, Fridolfsson *et al.* 1998, Ogawa *et al.* 1998, Shetty *et al.* 1999). In the neognaths, the avian W chromosomes, like the mammalian Y chromosomes, have undergone considerable change during evolution, apparently due to suppression of genetic recombination between the proto-sex chromosomes (Ohno 1967). For example, in the chicken (*Gallus gallus*), a neognath in the basal order Galliformes, the Z sex chromosome has over 350 genes, while the smaller, heterochromatic W chromosome has fewer than 20 genes (Mizuno *et al.* 2002, Stiglec *et al.* 2007).

Chromosomal sex determination differs in birds relative to mammals. In birds, sex is determined autonomously within individual cells throughout the body, and not by circulating steroidal hormones arising in the developing gonads (Smith 2010). Gynandromorph birds are naturally occurring, but rare, birds which develop such that one side of their body exhibits male secondary sexual characteristics, and the other side female characteristics. In gynandromorph chickens, the cells on the male side contain mainly ZZ sex chromosomes, and the cells on the female side are predominantly ZW. Internally, the gonads reflected the relative contributions of ZZ and ZW cells. Testes are present if most cells are ZZ, ovaries if most cells are ZW, and ‘ovotestes’ if there is a mixture of ZZ and ZW cells. Environmental sex determination is not known in birds, although temperature dependent mortality has been found to be sex specific in some

species, such as the brush-turkeys (Megapodidae), resulting in skewed sex ratios (Goth 2005, 2007).

Embryonic Development of the Testes

Sex-determining genes are activated during embryonic development, and will induce the formation of either testes or ovaries and subsequently other primary and secondary sexual characteristics. Many of the genes responsible for sex-specific development of reproductive organs are shared between mammals and birds and are thought to be part of an ancestral sex-determining pathway. However, unlike mammals, there has been no avian *Sry*-like gene initiating male sexual development observed in birds. This implies that in birds, an ovary-determining gene may occur on the W chromosome, or a gene has been lost from the W chromosome creating a Z dosage mechanism for sex determination. The avian Z chromosome, like mammalian X chromosome, contains many androgen-expressed genes (Bellott *et al.* 2010). Recent work has indicated that Doublesex and Mab-3 Related Transcription factor #1 (DMRT1), has a key function in testis development and may represent a master avian testis determinant (Smith *et al.* 2009, Smith 2010), although this has yet to be confirmed.

In birds, the embryonic gonads of both sexes develop as thickenings of the coelomic epithelium on the ventromedial surface of the mesonephric kidneys. In the chicken, the gonads are apparent by day 3.5 of incubation (Hamburger-Hamilton stage 19; Hamburger & Hamilton 1951). At this stage, the gonads appear to be morphologically identical between the sexes and are considered ‘indifferent’ or ‘bipotential.’ The bipotential gonads comprise an outer epithelial layer of somatic cells (the cortex), and an underlying medulla of loose epithelial cords interspersed with mesenchymal cells. The medullary cords are thought to derive from proliferation and ingrowth of the overlying epithelial (cortical) layer (Merchant-Larios *et al.* 1984,

Rodemer-Lenz 1989). These medullary cords are critical to male gonadal development because they go on to form seminiferous tubules.

In the chicken embryo, the onset of morphological differentiation of the gonads into testes or ovaries occurs between days 5.5 and 6.5. In ZZ embryos, the disorganized medullary cords thicken and become organized into patent testis (seminiferous) cords. The organization of these cords is due to the differentiation of Sertoli cells. As in mammals, this process appears to be the key cellular event in the initiation of testis formation. The outer cortical zone becomes reduced to a monolayer of epithelium in males, and by day 10.5 bilateral testis development.

A fundamental divergence in reproductive morphology between avian and mammalian groups is the high degree of lateral asymmetry in the reproductive tracts of male and female birds. In birds, early in embryologic development, the reproductive system in both sexes develops bilaterally, but then reduction occurs on the right side (McCarrey & Abbott 1982, Bakst *et al.* 2007). The reduction of the developing right gonad is greater in females than in males. In females in most species, ultimately only the left ovary and oviduct will develop into a mature gonad and functional duct, respectively. In male birds, the mature left testis is often larger than the right testis, but the mature male gonadal condition does not show the extreme or consistent asymmetry seen in females (Calhim & Montgomerie 2015).

The avian embryonic (mesonephric) kidneys contribute cells to the developing testes (Rodemer *et al.*1986, Rodemer-Lenz 1989). In vitro experiments using embryonic chicken gonads cultured together with embryonic quail gonads show that mesenchymal cells migrate from the quail mesonephros specifically into the male chicken gonad at the

time of gonadal sexual differentiation (Smith *et al.* 2005). The immigrating cells contribute to the interstitial cell population of the testis. The interstitial cells are those distributed around the developing seminiferous cords. Interstitial cells give rise to at least three different cell types: the testosterone producing Leydig cells, peritubular myoid cells with muscle-cell type features, and vascular endothelial (blood) cells. It is unclear which of these interstitial cell types derive from the immigrating mesonephric cells, but it includes vascular cell precursors. The cell migration process has been shown to involve a conserved platelet-derived growth factor (PDGF) signaling pathway (Smith *et al.* 2005).

Primordial Germ Cells

Primordial germ cells (PGC's) will become the future gametes. They are diploid and originate outside the gonads, in the epiblast, an extra-embryonic region anterior to the head, in an area called the germinal crescent (Fujimoto *et al.* 1976). The PGC's subsequently migrate into the embryo and through the developing vascular system to the bipotential gonads, where they populate the outer cortical region (Meyer 1964, Fujimoto *et al.* 1976, Kuwana 1993, Petitte *et al.* 1997).

Primordial germ cells become asymmetrically distributed in the gonads before sexual differentiation, with more PGCs colonizing the left gonad in both sexes (Vallisneri *et al.* 1990, Zaccanti *et al.* 1990). Differences between males and females are apparent at the earliest stages of PGC development and migration. Primordial germ cells increase in number earlier in embryonic development in females than in males. Primordial germ cells are larger in female embryos than in males, at the point of earliest detection (Zaccanti *et al.* 1990, Smith 2010). Ultrastructurally, male versus female primordial germ cells differ primarily in the amount of rough and smooth endoplasmic reticulum, mitochondria, glycogen and lipid droplets, suggesting early differences between male and female germ cells (Zaccanti *et al.* 1990, El Jamil *et al.* 2008, DeFalco & Capel 2009). Gonadal sex differentiation can occur in both sexes in the absence of PGCs, as assessed by histology and hormonal output (McCarrey & Abbott 1978, 1982). Thus, testicular versus ovarian development is dependent upon the somatic tissues such as cortex or medullary cells.

Primordial germ cells can be identified early in embryogenesis, prior to their migration to and proliferation in the gonadal anlagen, across both invertebrate and vertebrate taxa (Tribe & Brambell 1932, Hubert, 1969, Fujimoto, 1979, Tsunekawa *et al.*

2000). In chickens, primordial germ cells can be first identified in stage X embryos (Kagami *et al.* 1995, 1997, Karagenc *et al.* 1996, Tsunekawa 2000, Nakamura *et al.* 2007). Embryos reach stage X at 20 hours uterine egg, or at laying. At this point, the formation of the area pellucida is completed, and clusters of cells form a mesh-like layer at the posterior end of the lower surface of the area pellucida. A transparent sickle-shaped belt can be seen posteriorly (Eyal-Giladi & Kochav 1976). At this stage, Nakamura *et al.* (2007) found approximately 130 PGC's, mainly in the area pellucida, particularly in the central zone of the blastoderm in chickens. The majority were localized on the ventral side of the epiblast layer, with only a few PGC's found on the dorsal side of the blastoderm. By stage 2, PGC's increase in number and move to the anterior region of the central zone. Primordial germ cells continue to move anteriorly, and by stage 3, PGC's have produced an arc-like distribution pattern, and are beginning to be found along the primitive streak. By stage 4, the PGC population increases nearly three-fold and concentrate around the anterior edge of the germinal crescent. In the domestic chicken nearly 300 PGC's were found in this region at stage 6. By stage 9 (after 30 hours of incubation), embryos exhibited PGC distribution at the anterior part of the extraembryonic region, including the proamnion, amniocardiac vesicle and along the neural groove, and the region of PGC colonization had expanded on both the dorsal and ventral sides (Karagenc *et al.* 1996, Tsunekawa *et al.* 2000, Nakamura *et al.* 2007).

In the chicken, PGC's are seen in the head region prior to stage 10. At this point, anteriorly scattered PGC's concentrate at the region anterior to the head on the dorsal side. By stage 11 (40 hrs of incubation), most PGC's are present at the region anterior to the head, and many began to appear in the blood vessels. At the latter half of stage 11, the

population of PGC's present at the anterior of the head decreases dramatically, while the PGC's in the vascular system increase greatly, indicating that the PGC's enter the blood vessels from the anterior region of the head (Kuwana & Fujimoto 1984, Ginsburg & Eyal-Gilaldi 1986, Tajima *et al.* 1999, 2000, Tsunekawa *et al.* 2000).

Beginning at stage 15, PGC's could be observed in the intermediate mesoderm, the future gonadal region. Importantly, by stage 15, PGC distribution is asymmetrical in both males and females, with more PGC's located on the left side of the intermediate mesoderm in both sexes, suggesting this uneven distribution of PGC's in intermediate mesoderm is independent of sexual difference (Zaccanti *et al.* 1990, Nakamura *et al.* 2007).

Avian Male Reproductive System

Testis

The testis is a dual-purpose organ, producing sperm and secreting androgens, and the structure and composition of the testis reflects its dual function. In adult male birds, the testes do not reach a static size, but instead testes undergo circannual cycles of growth and regression. The majority of the tissue of the avian testis consists of seminiferous tubules, comprising approximately 68% of the testis in the non-breeding season and 95% of the testis in the breeding season (Tae *et al.* 2005). The arrangement of the seminiferous tubules within the avian testis differ from those in the mammalian testis in that the avian seminiferous tubules anastomose, forming a complex network throughout the testis. Also, the tubules are not separated into lobules by septa originating from the testicular capsule as in the mammalian testis (Lake 1957, Budras & Schmidt 1976, Jones & Lin 1993). Instead, strands of connective tissue extend from the testicular capsule inward between the tubules to support the intertubule elements (Jones & Lin 1993). Seminiferous tubules are shaped by a basal lamina produced and covered by epithelial peritubular cells. These cells are myoid and drive the peristalsis necessary to transport the non-motile elongated secondary spermatozoa through tubules following their release from the apical seminiferous epithelium.

Spermatogenesis occurs within the seminiferous tubules. Androgen production and secretion occurs in the interstitial spaces between tubules, mainly in the Leydig cells. Leydig cells comprise approximately 1% of the testis mass during the breeding season, but comprise over 9% of the total mass of the reduced testis during the non-breeding season (Tae *et al.* 2005).

The remaining tissue within the testis consists of many types of supportive cells (Mendis-Handagama *et al.* 1988, Russell & de Franca 1995, Rey *et al.* 1996, Ariyatne & Mendis-Handagama 2000, Tae *et al.* 2005). Polarized Sertoli cells are attached to the basal lamina within the lumen of seminiferous tubules, where they provide the attached germ cells with structural and nutritive support and mediate androgenic signals from the outside into the propagating germ line. Germ cell differentiation takes place from the basal lamina, in which the undifferentiated spermatogonia are embedded in supporting Sertoli cells. Macrophages, which perform an immuno-regulatory function, act in concert with Sertoli cells in forming a blood–testis barrier. Additionally, testicular macrophages perform a role in Leydig cell steroidogenesis, and may act to inhibit leutenizing hormone (LH) secretion (Bryniarski *et al.* 2004). Myoid cells surround the seminiferous tubules in the testis, and are involved in the transport of spermatozoa and testicular fluid in the tubule. Furthermore, it has been reported that myoid cells contain androgen receptors and are involved in retinol processing. It is likely that peritubular myoid cells not only provide structural integrity to the tubule but also take part in the regulation of spermatogenesis and testicular function (Maekawa *et al.* 1996).

Approximately 0.5% of the testes during the breeding season and 3% during the non-breeding season is comprised of blood vessel tissue. Blood vessels are composed of two interacting cell types, endothelial cells that form the inner lining of the vessel wall, and pericytes, which are vascular smooth muscle cells that envelop the outer surface of the vessel (Berger & Song 2005). In addition to providing blood to the testicular tissues, recent work has indicated that cells of testicular blood vessels, specifically vascular smooth muscle cells and pericytes, may be the progenitors of Leydig cells (Davidoff *et al.*

2004). The seminiferous epithelium is comprised of Sertoli cells, non-germinal sustenacular cells, and the germ cells that will give rise to the spermatids. All of these cells are involved in the process of spermatogenesis.

Epididymis

In birds, the mature testes remain intra-abdominal, resting just anterior and proximal to the kidneys. Within the testes, spermatids begin development in the seminiferous epithelium and move to the lumen of the tubule. The seminiferous tubules converge at the hilus of the testis. Adjacent to the hilus of the testis lies the epididymis. The epididymis is generally spindle-shaped and attached to the medial border of the testis. The cranial end of the epididymis is incorporated into the adrenal gland capsule (Aire 2006). In birds, the epididymis is relatively small and consists of a series of three regions of tubules that receive and store sperm from the testis and ultimately deliver the sperm to the deferent duct. The tubule regions of the epididymis are the rete testis, the efferent ducts, and the epididymal tubules (Mehrotra 1964). The rete testis in birds develops from mesenchymal rete blastema and has an embryological origin different from the seminiferous tubules (Budras & Sauer 1975). The rete testis is located in birds on the dorsomedial aspect of the testis, and functions to transfer sperm from the seminiferous tubules to the efferent ducts of the epididymis (Aire 1979). Removal of the epididymis from the testis will separate the intratesticular and intracapsular regions from the extratesticular region of the rete testis with the intratesticular and intracapsular regions remaining attached to the testis and the extratesticular region remaining attached to the epididymis (Aire 1981).

The efferent ducts consist of two parts, the proximal and distal efferent ductules. Initially these ducts are wide, but gradually taper before forming the connecting ductules. The ductules progress toward the medial aspect of the epididymal region where they interconnect and once again become wider, eventually opening into the epididymal ducts (Tingari 1971). The efferent ducts function in adjusting the viscosity of the fluid moving from the testis to the epididymal ducts and ultimately to the deferent ducts (Aire *et al.* 1978, Aire 1979). In many taxa, the efferent ductules perform secretory functions. However, the avian system cellular structure indicates that the secretory function of this region is limited, and that absorption of fluids is more likely than secretion of additional fluids.

Epididymal Ducts

The networks of seminiferous tubules that unite in the avian efferent ductules pass through the region of the epididymal ducts before ultimately emptying into the deferent ducts. This region is shorter and smaller than that found in mammals (Mehrotra 1964, Tingari 1971). In birds, the epididymis is embedded within connective tissue at the attachment of the testis to the dorsal body wall (Aire 1979). The epididymal ducts appears as a short coiled white tubule in the juxtatesticular tissue located against the dorsomedial surface of the testis (Clulow & Jones 1987). The whole duct complex immediately adjacent to the testis is embedded in a fibrous stroma containing a few smooth muscle fibres (Tingari 1971). The epididymal tubules are lined with ciliated columnar epithelial cells bounded by a basement membrane. These cells are non-motile, but assist in the forming of a pathway for the secretion of the epididymal epithelium into

the lumen of the tubules. The epididymal tubules connect with the efferent ducts and finally collect into a single duct, the deferent duct, which opens into the cloaca.

Deferent Duct

The deferent ducts runs distally from the caudal border of the epididymis, toward the cloaca, into which it opens. There are no known accessory sex organs or glands in birds that are homologous or analogous to those found in mammals. In all birds, the deferent duct leaves the caudal end of the epididymis in a slightly wavy pathway, and becomes considerably convoluted, increases in diameter, and lies lateral to the relatively firm and regular ureters. The deferent duct then straightens before terminating in a spindle shaped or barrel shaped enlargement called a seminal vesicle, which is imbedded in the cloacal wall. In species that lack an intromittent organ, each seminal vesicle opens separately into the urodeum of the cloaca distally through a protruding seminal papilla. In species with an intromittent phallus, the papillae each empty into an ejaculatory groove, where semen from each side is thought to be mixed.

Phallus

The phallus in birds is homologous to the phallus of reptiles, and is retained in the paleognathous birds, *i.e.*, the ratites (orders Struthioniformes and Tinamiformes) as well as in the basal neognathus birds, a monophyletic group known as the Galloanserae and consisting of the waterfowl (Anseriformes) and gallinaceous gamefowl (Galliformes). Within these groups, the phallus may be intromittent, as in the ratites and Anseriformes, or non-intromittent as in the Galliformes. The avian phallus and reptilian phallus share both anatomical and histological characteristics (King 1981). The avian phallus is a single elongated structure of erectile tissue emerging from the ventral wall of

the proctodeum of the cloaca. In general, the true avian phallus is comprised of two fibroelastic bodies with a median ventral ejaculatory groove, or phallic sulcus, where these two bodies join. During phallic erection, the groove may become a closed channel through which the avian semen flows during ejaculation. Intromittent phalluses all have a fixed base of fibrous tissue and a free conical or tubular portion comprised of two fused fibrolymphatic bodies that are everted during erection and copulation (Montgomerie & Briskie 2007, Brennan *et al.* 2010). Such phalluses usually curve toward the male's left and thus will likely deposit sperm into the female's left oviduct. The fixed base of the phallus has lymphatic spaces that become dilated during erection, filling with lymph provided by vascular bodies in the floor of the urodeum. The base of the phallus also has glandular tissue that secretes mucus that lubricates the phallus and facilitates both eversion, and presumably copulation (Komarek & Marvan 1969, Oliveira & Mahecha 2000, Briskie & Montgomerie 2007).

In birds, the size, surface structures, shape, and histology of true phalluses varies widely, but study is limited. Within the paleognaths, the intromittent phallus is structurally similar to the crocodilian phallus in all species studied to date. The phallus is characterized by a conical base of fibrous tissue attached to the ventral floor of the proctodeum. The erect phallus bends to the male's left due to the fibroelastic bodies being laterally asymmetrical. In some species of paleognaths, there is a blind cavity located distally at the tip of the phallus. In some species, the phallus is helical when erect, and in some paleognaths, the phallus is large enough to require partial eversion during defecation (King 1981, Oliviera & Mahacha 2000).

Within the Galloanserae, the Anseriformes possess an intromittent phallus that can be very complex, whereas the Galliformes possess a non-intromittent phallus. In the Anseriformes, the mallard (*Anas platyrhynchos)* is the best studied. When flaccid, the phallus of the mallard is coiled within a peritoneal sac in the ventral wall of the proctodeum. During erection, the phallus fills the vent and the phallus extends approximately 4 cm. The left and right fibrolymphatic bodies are fused but are separated at the surface by a deep ejaculatory groove along which semen travels during ejaculation. The right fibrolymphatic body of the phallus is larger than the left, and this results in a phallus that twists in a left spiral three to four turns from the base to the tip. The phallus is smooth at the base but along the body becomes roughened by transverse ridges approximately 2mm apart (McCracken 2000, Coker *et al.* 2003, Montgomerie & Briskie 2007).

The non-intromittent phallus has not been well studied in birds except in the domesticated chicken and turkey. In those species, the phallus is a flattened oval or heart shaped protrusion on the internal wall of the cloaca with a median ejaculatory groove into which the paired deferent ducts both discharge semen. When the phallus is erect, both the vent and the floor of the proctodeum of the cloaca is everted, exposing the phallus externally briefly during copulation. In the chicken, the phallus vascular bodies are embedded in the cloacal wall in the same position as is found in the mallard (King 1981). The lymphatic channels of the vascular bodies connect with the vascular channels of the phallus, engorging the phallus and facilitating eversion. The phallus of the turkey is similar to that of the chicken, but differs in that it has two prominent humps separated by

a furrow. The lymphatic folds extend obliquely along the floor of the proctodeum and have three or four ridges on their surface (Briskie & Montgomerie 2007).

Evolution of the Avian Phallus

All birds employ internal fertilization, but a phallus capable of intromission is lacking in most species. The ancestral phallus has been retained in the basal avian groups, but lost in the majority of the neognathus species (Gerhardt 1933, Briskie & Montgomerie 1997, Brennan *et al.* 2008). Even within these basal lineages, in some taxa, the phallus may be reduced in size and non-intromittent. Because the reproductive anatomy of male birds has a direct effect on males' ability to transfer sperm to females, the lack of an intromittent phallus in 97% of the nearly 10,000 species of birds contradicts the assumptions that an intromittent phallus is necessary for effective internal fertilization (Briskie & Montgomerie 1997, Brennan *et al.* 2008).

Paleognathae

Within the basal lineages of birds, recent research implies that the transition from intromittent to non-intromittent to phallus loss has not been a single transition. An intromittent phallus is present in most, but not all, species (n=59) of Paleognathae (Brennan 2012). The phallus is not intromittent in some tinamous and megapodes. Within the tinamous, all 20 species in the genus *Crypturellus* possess a phallus that is non-intromittent, whereas the remaining 27 tinamou species in 8 genera likely retain an intromittent phallus (Brennan *et al.* 2008). Unfortunately, only a few tinamou species have been studied in any detail, but it seems likely that the evolutionary transition from intromittent to non-intromittent phallus has occurred at least once within the tinamous, in the lineage leading to *Crypturellus.*

Neognathae

Within the neognathous species, the phallus is present only in the basal monophyletic group known as the Galloanserae and composed of two orders, Anseriformes (waterfowl) and Galliformes (gamefowl). An intromittent phallus is present, and often well developed in the Anseriformes species (n=162), but is rudimentary, non-intromittent and variable among the Galliformes species (n=268). Within the galliforms, the reproductive tracts of male megapodes are understudied, but the male Australian brush turkey (*Alectura lathami*) has a non-intromittent phallus, and the male malleefowl (*Leipoa ocellata*) has no phallus (Brennan *et al.* 2008). The phallus also may be absent in the male orange-footed scrubfowl, based on casual observations, but this has yet to be confirmed (Brom & Dekker 1992, Brennan *et al.* 2008). The remaining 18 species of megapodes are thought to have an intromittent phallus, but the evidence for this is incomplete (Montgomerie & Briskie 2007). In the remaining galliform species studied, the phallus is present, but non-intromittent.

The remaining 9500+ extant bird species within the Neoaves do not possess a phallus, although a few species (i.e. *Notiomystis cincta*) have a secondarily derived phallus-like structure (Low *et al.* 2005).

Avian Sperm Formation

After a male bird hatches, germ cells enter a period of near-stasis until the male approaches sexual maturity. Upon sexual maturation, germ cells give rise to haploid spermatids through the process of spermatogenesis. Spermatogenesis is a conserved process across vertebrate taxa. In general, spermatogonia develop into spermatocytes that undergo meiosis to produce spermatids which enter spermiogenesis where they undergo morphological and biochemical transformation into spermatozoa. In all amniotes, spermatogenesis occurs in seminiferous tubules, which contain permanent populations of both Sertoli cells and spermatogonia that act as a germ cell reservoir for successive bouts of spermatogenesis (Pudney 1995).

The developing germ cells form intimate associations with Sertoli cells, and multiple germ cells of different stages will remain in contact with a single Sertoli cell. The various generations of germ cells are arranged in strictly defined cellular associations (Leblond & Clermont 1952). It is the cellular associations of these germ cells in concert with Sertoli cells that constitute a cycle of the seminiferous epithelium. Each particular association of germ cells is considered to be a stage. Thus, the number of stages of spermatogenesis in a particular species is defined by the number of morphologically recognizable germ cell associations within the testis (Russell *et al.* 1990).

The various germ cell types can be distinguished by their morphology and by the differential expression of proteins. Spermatogonia are present between Sertoli cells, close to the basement membrane of the tubule. Spermatogonia are the most immature germ cells in the testis, and in birds include both type A spermatogonia and type B spermatogonia. Both type A and type B spermatogonia have a number of sub-types. The

number of sub-types varies among species. The true stem cells of the germ cell population are considered to be subsets of the type A spermatogonia population, whereas it is the type B spermatogonia that further differentiate, although the identity of type A versus type B spermatogonia generally cannot be discerned on the basis of morphology, (Clermont 1972).

In the process of spermatogenesis, the spermatogonia first undergo numerous rounds of mitosis to produce a large number of germ cells that can then begin meiotic replication. Proliferation of spermatogonia provides allows for millions of sperm to be produced per day (Russell *et al.* 1990).

After the final mitotic round of type B spermatogonia, these spermatogonia will develop into primary spermatocytes (Russell *et al.* 1990). Primary spermatocytes are identifiable as the largest germinal cells in the avian testes, persisting for a relatively long period during spermatogenesis and possess a conspicuous nuclear structure (Jones & Lin 1993). Secondary spermatocytes have a relatively short lifespan and because of this are hard to image. However, research on Japanese quail (*Coturnix coturnix japonica)* (Yamomoto *et al.* 1967), domestic fowl (*Gallus gallus)* (Zlotnick 1947), mallard (*Anas platyrhynchos)* (Clermont 1958), and Guinea fowl (*Numida meleagris)* (Aire *et al.* 1980), describe spermatocytes as being only slightly larger than spermatogonia. Spermatocytes undergo meiosis to form haploid spermatids.

During prophase of the first meiotic division, spermatocytes undergo a number of morphological transitions each of which can be classified on the basis of nuclear size and morphology (Hess 1990). In the zygotene phase, pairing of homologous chromosomes occurs, and cells with completely paired chromosomes are termed

pachytene spermatocytes. After the pachytene phase, a brief diplotene phase follows in which the chromosome pairs partially separate, and the cells then undergo the first meiotic division to yield secondary spermatocytes (Clermont 1972). The secondary spermatocytes quickly undergo the second meiotic division to yield round, haploid primary spermatids. Immature primary spermatids transcribe high levels of mRNAs that are subject to translational delay until translation is required during spermatid elongation (Braun 1998). The differentiation of round primary spermatids into mature elongated secondary spermatids takes place with no further division during the process known as spermiogenesis. Spermiogenesis in birds is similar to that in mammals (Nagano 1962, Fawcett *et al.*1971, Tingari 1973, Jones & Lin 1993).

Twelve stages of spermatid development have been described, based mainly on the morphological development of the acrosome, nucleus and flagellum (Lin *et al.* 1990, Jones & Lin 1993, O'Donnell 2001). Spermiogenesis involves formation and development of the acrosome and flagellum, condensation of the chromatin, reshaping and elongation of the nucleus, and removal of the cytoplasm before release of the spermatid during spermiation (Leblond *et al.* 1990). After commencement of spermatid elongation, the highly condensed spermatid nucleus becomes incapable of transcription. Spermiation is the final step of spermiogenesis and involves the release of the mature, elongated secondary spermatid from the Sertoli cell into the lumen of the seminiferous tubule (Russell 1993, O'Donnell 2001).

Within the birds, details about the stages of spermatogenesis are known for only a small number of species. In domestic fowl (Lake 1956), two types of spermatogonia were identified, as in Guinea fowl (Aire *et al.*1980). In the Japanese quail, four stages in

two phases have been identified (Lin & Jones 1992). To maintain spermatogenesis, the spermatogonial stem cells must be renewed continuously. For Japanese quail, the first phase, the spermatogonial phase, involves not just renewal of the stem spermatogonial population by mitosis but also proliferation of spermatogonia via two or more mitotic divisions of one of the products of the renewal division of a spermatogonium. The other spermatogonium remains a stem cell. The second phase of spermatogenesis, the spermatocyte phase, involves meiosis. Spermatocytes are produced continuously during spermatogenesis as a result of the proliferation of spermatogonia. In the last step, spermiogenesis occurs in which the products of spermatocyte meiosis, i.e. haploid spermatids, differentiate to produce spermatozoa.

The cell membrane divisions by new proliferating spermatogonia are incomplete, meaning that each generation of germ cells forms a clone in which all members are joined together by cytoplasm until approximately the time of spermiation when spermatozoa migrate from the seminiferous epithelium (Fawcett & Slautterback 1959, Fawcett 1961, Dym & Fawcett 1971, Moens & Go 1972, Holstein & Roosen-Runge 1981, Jones & Lin 1993). Each clone is associated with a particular group of Sertoli cells within the tubule. These Sertoli cells can regulate the development of more than four successive generations of spermatogenic cells at a time as the spermatogenic cells migrate from the basal to the luminal side of the seminiferous epithelium. Thus, at any one time, a particular area of seminiferous epithelium is composed of several generations of germ cells all of which originate from the same stem spermatogonium. The time that any one generation is present is determined by the duration of its lifespan relative to the

other types of germ cells. Typically, numerous different cellular associations will exist simultaneously in the seminiferous epithelium (Lin & Jones 1992, Jones & Lin 1993).

In the avian species studied to date, the area of a seminiferous tubule which is occupied by spermatogenic cellular associations is smaller in birds than mammals (Clermont 1962, 1963, 1970, Heller & Clermont 1964, Leidl 1968, Chowdhury & Marshall 1980, Lin & Jones 1990). The sequences of stages that occur between two successive occurrences of the same stage of spermatogenesis is referred to as a cycle of the seminiferous epithelium. The duration of a cycle is the period between two successive occurrences of the same stage (Lin & Jones 1990, Jones & Lin 1993).

In the tubule, cycles generate a wave of spermatid production. In birds, only one report in Japanese quail has described this wave. Lin and Jones (1990) found that the stages of the cycle of the seminiferous epithelium were not distributed at random along the seminiferous tubule of the Japanese quail, but were arranged in a wave which spiraled in a helical plan along the seminiferous tubule.

In Japanese quail, adjacent stages in space formed complete spermatogenetic waves in which all 10 stages of the cycle occurred in sequential order. The arrangement of the stages of the seminiferous epithelium in the Japanese quail resembles that described for primates (Hilscher 1979, Schulze 1982, Hilscher 1983, Schulze & Rehder 1984, Dietrich *et al.* 1986, Schulze *et al.* 1986).

There are two reports on the timing of spermatogonial renewal and proliferation in birds. Clermont (1958) found that in the mallard, a type A stem spermatogonium divides at Stage V of the cycle of the seminiferous epithelium to produce one new type A stem spermatogonium and one differentiating type B spermatogonium. The B

spermatogonium then divides at Stage VIII of the cycle to form two differentiating (type C) spermatogonia; and the type C spermatogonia divide into four primary spermatocytes at StageVIII of the cycle. Lin and Jones (1992) studied the Japanese quail and concluded that each type A spermatogonium divides to produce one new type A spermatogonium and one type B spermatogonium during Stage IX of the cycle of the seminiferous epithelium. Further divisions occur and ultimately 32 spermatids can result from each division of a type A spermatogonium. These 32 spermatids produced from a single type A spermatogonium remain together as a bundle until step 11 of spermatid development, which is consistent with the findings by Swan (1985) of 32 spermatids per bundle in the canary (*Serinus canaries),* and European goldfinch (*Carduelis carduelis).*

These findings suggest that of avian spermatogenesis is simpler than for mammals. For example, rats (*Rattus norvegicus*) produce 128 times more spermatids from a single stem spermatogonium than quail and the area of a cellular association in the rat is 55 times the area in the quail (Lin & Jones 1990). Another major difference between avian and mammalian spermatogenesis is the duration of time from first division of stem cell spermatogonia until those cells are present in the ejaculate. Lin and Jones (1992) estimated that the duration of spermatogenesis in the Japanese quail is 12.8 days. In the domestic fowl, spermatozoa first appeared in the ejaculate 13-15 days after initial spermatogonial division (de Reviers 1968). In the Muscovy drake (*Cairina moschata*), the duration is approximately 12 days (Marchand *et al.* 1977, Jacquet & Sauveur 1995). Contrastingly, in mammals, spermatogenesis takes an average of 45.8 days, ranging from about 34 days in the boar (*Sus scrofa*), to 75 days in man (Roosen-Runge 1977).

Other significant differences in spermatogenesis exist between birds and mammals. Daily sperm production in quail is four times the rate of mammals (Jones & Lin 1993). Consequently, the difference between birds and mammals in the number of mitotic divisions during spermatogonial proliferation is not reflected in a difference in the efficiency of sperm production by the testes. Furthermore, quail have a shorter period of sperm transport through the male genital ducts and a limited capacity to store spermatozoa for a long period. However, due to production rate, Japanese quail have as many extragonadal spermatozoa, relative to body mass, as found in rat and ram (*Ovis aries*). Estimates of the number of ejaculates produced by the testis per day also support the concept of greater sperm production by the quail than rat and ram. It is uncertain how many extragonadal spermatozoa are immediately available for ejaculation in birds because there are no published findings on birds comparable to those described for mammals. Thus, if the avian deferent duct acts only as a delivery conduit for spermatozoa at the rate they are produced by the testis, then the number of sperm ejaculated per day would be limited to the rate of sperm production by the testis. However, if all of the extragonadal spermatozoa are available for ejaculation, there could be the equivalent of 25 ejaculates 'stored' in the eipididymal duct, deferent duct and seminal vesicles in the quail (Jones & Lin 1993).

Ultrastructural studies of fowl spermatozoa from the excurrent ducts of the testis indicate that spermatozoa are structurally mature when they leave the testis (Tingari 1973). Thus, it appears that unlike mammals, avian spermatozoa from the epididymal ducts are capable of fertilizing ova (Howarth 1983). Additionally, fowl spermatozoa are capable of becoming motile as they pass through extratesticular ducts of the epididymis

(Howarth 1983). This is correlated with developing the capacity to ascend the female's oviduct, or at least reach the sperm storage tubules, and be available for fertilization (Howarth 1983).

Ultrastructurally, avian spermatozoa display a number of ancestral characteristics also found in some reptiles. Avian sperm possess a conical acrosome shorter than the nucleus, and the nuclear rostrum slightly penetrates the acrosome. The endonuclear canal is deep and the tip granule is absent. There is a perforatorium present as well as a proximal centriole. The distal centriole is very long and contains singlets. The midpiece is short with a ribbed fibrous sheath, moderate dense fibers, and an annulus is present. Finally, there are several mitochondria present in the midpiece.

In comparison to mammalian sperm, avian spermatozoa have a long, cylindrical nucleus. Additionally, when present, the perforatorium is larger than in mammals, the spermatozoa have no post-acrosomal sheath attached to the plasmalemma, no striated columns forming a neck region at the junction of the head and tail, and no outer dense fibers extending along the principal piece of the tail.

Among avian species, there is considerable variation in the structure of avian spermatozoa. Avian sperm can be classified into three or four types, one associated with the most basal avian groups including the Galloanserae, the sperm of which diverge from the ancestral form in that they possess a simple, elongated head and perforatorium. Two or more derived groups of sperm are found in which the perforatorium is lost, and in the passerines a helical membrane may be present (Saita *et al.* 1982, Jones & Lin 1993).

Specifically, in the Galloanserae, the spermatozoa closely resemble that of the paleognaths and the crocodilians. The conical acrosome vesicle is shorter than the

nucleus and there is a perforatorium which penetrates the nucleus in an endonuclear canal. Mitochondria are present in tiers surrounding the distal centriole, and an annulus and a fibrous sheath are present. The Galloanserae spermatozoa differ from the paleognaths in that the perforatorium is shorter and stouter, the endonuclear canal is shorter, singlets are lost from the centrioles and the fibrous sheath is amorphous rather than ribbed. Anseriforms possess what appear to be the most primitive avian spermatozoa after the paleognaths. The spermatozoa of the Anseriformes are similar to those of the Galliformes but a notable distinction is that in anseriform sperm, as exemplified by the mallard, the perforatorium extends almost to the tip of the spermatozoa. Also, the acrosome vesicle is apically very narrow, whereas in Galliformes a large amount of material is present in the acrosome vesicle anterior to the tip of the perforatorium, correlated with a much smaller subacrosomal space.

Dynamics of Copulation

In females of most bird species, only the left ovary and oviduct develop fully and produce eggs (Kinsky 1971, King 1981a, Blackburn & Evans 1986, Proctor & Lynch 1993). One result of this unilateral development is that the vaginal opening to the single functional oviduct is not centered on the midline of the urodeum of the cloaca but instead is off-center, located on the left ventrolateral wall (King 1981a). Because the opening to the female's oviduct is located to the left of midline, a fundamental challenge for male birds is to achieve accurate placement of sperm into an off-center oviduct opening. In avian species that possess an intromittent phallus, one function of the phallus appears to be to place semen directly into the vagina of the female, and to sperm storage tubules if they are present. In ostriches, a paleognath that possesses a simple intromittent phallus, the phallus bends to the left, presumably to achieve better alignment with the left-of-center vagina of the female's oviduct (Lake 1981).

Male birds retain the primitive condition of having two independent orifices for ejaculation, the seminal papillae (King 1981b). The left and the right testis each independently connect to left and right seminal vesicles by way of a left and right deferent duct. Each vesicle has its own papilla located on the inner wall of the urodeum of the cloaca. In species which possess an intromittent phallus, it is assumed that semen emitting from each papilla is mixed in the phallus ejaculatory groove at ejaculation. In those species that lack an intromittent phallus, sperm exits each papilla separately. It is assumed that ejaculation occurs from both papillae but the degree of mixing of left and right ejaculates as males place ejaculate onto the female's everted vaginal opening has not been investigated.

Research has shown that copulation in many avian species is non-randomly biased to occur from the female's left side. Male ostrich normally copulate from the left side of the female (King 1981a,b). Tree swallows (*Tachycineta bicolor*) copulate significantly more often from the left side than from the right side (Peterson *et al.* 2001) as do house sparrows (*Passer domesticus*, Nyland *et al.* 2003) and chukar (*Alectoris chukar*, Delehanty & O'Hearn 2005). More recently, greater sage grouse (*Centrocercus urophasianus*), great-blue herons (*Ardea herodias*), American avocets (*Recurvirostra americana*) and Swainson's hawk (*Buteo swainsoni*) have been observed to copulate more frequently from the left side of the female (Turek 2007).

In chukar, breeding males engaging in left-side copulations have larger left testes relative to right testes and the copulating females have unilateral left-side reproductive tracts (Delehanty & O'Hearn 2005). One hypothesis for left-side copulation bias in species like chukar without an intromittent phallus and where the male's left testis is larger than the right and the female's reproductive tract is left-of-center is that the left ejaculatory papilla is especially well aligned to the vaginal opening when copulation occurs from the female's left side. Asymmetric testes, with a left-larger testis, is the predominant (Lake 1981), though not universal (Ligon 1997) condition in birds. Males may exhibit both behavioral and morphological left-side bias to accommodate left-side bias in female morphology resulting from the unilateral female reproductive tract.

Mating systems

Birds are unusual among vertebrates in that the vast majority of extant avian species (over 90%) are socially monogamous and exhibit biparental care of eggs or the young (Kendeigh 1952, Lack 1968, Ligon 1999). Parental care, in some form, is ubiquitous in birds. For example, even the moundbuilders (Family Megapodidae, Order Galliformes), which deposit eggs in mounds to be incubated environmentally and exhibit no post-hatch parental care, expend great effort in nest site preparation and egg care, a critical aspect of parental care (Jones *et al.* 1995, Burt *et al.* 2007). Eggs of all other birds are actively and directly nurtured by contact incubation within a nest.

Biparental care is found in over 90% of all modern bird species, and because of its predominance, is often assumed to represent the ancestral state for birds. Biparental care, however, is not seen in reptiles, and is uncommon in the basal avian lineages. It is unlikely that biparental care evolved directly from no parental care (Tullberg 2002). Several studies indicate that the theropod lineages most closely related to birds provided parental care. However, the sex of the adult providing nest care is not known (Norell *et al.* 1995, Varricchio *et al.* 1997, Meng *et al.* 2004, Xu & Norell 2004). There is phylogenetic support for the conclusion that the ancestor of birds showed male-only parental care (Burt *et al.* 2007). In the most basal branch of extant birds, the paleognaths, male parental care is the rule, with female nest attendance the exception.

The first step in the evolution of parental care in the lineage ancestral to birds likely involved reproductive and physiological patterns similar to typical reptiles. Ancestrally, ectothermic males may have defended resource-rich territories. These territories, as now, would have attracted females, which mated and deposited clutches of

eggs in nests buried in soil or vegetation within these territories. In this scenario, selective pressures would have favored females that chose males based on territory quality due to the importance of environmental resources for the development and survival of their young. In the process of territorial defense, males would have provided indirect nest defense to multiple nests and hatchlings.

As birds diverged from ancestral reptiles, selection appears to have favored female production of large eggs, presumably benefiting fitness. Production of larger eggs appears to have co-occurred with the evolution of sequential rather than simultaneous egg-laying. Sequential egg-laying is an efficient manner for a female bird to produce large, well-endowed eggs, while also retaining daily capacity for flight (Ligon 1999, Varricchio *et al.* 1999, Prum 2002, Burt *et al.* 2007). However, formation of a clutch of these eggs would require intense foraging effort and one hypothesis is that in early birds, females foraged and males tended nests (Williams 1966).

Additionally, production of large, hard-shelled eggs favored surface nesting due to the physical limitations of gas exchange across the shell surface. Avian eggs have a hard shell that provides protection from microbes and environmental desiccation, but were better laid in a surface nest because covering them would interfere with gas exchange (Burley & Johnson 2002).

Contact incubation allowed birds to place surface nests and eggs in sites that were better protected from discovery and predation (Kavanau 1987). Laying eggs in surface nests makes it possible for both male and female to contribute to post-laying parental care. Both sexes may participate in nest building, incubation, nest defense, and care of hatchlings. In many bird species, males contribute greatly to incubation success

by providing food for the female (Blackburn & Evans 1986, Ligon 1999). The first form of more active parental care may have been simple guarding of surface nests.

Most avian species exhibit a socially monogamous mating system in which two adults form a pair-bond, i.e., a prolonged social interaction for the purpose of reproduction. Usually the pair consists of one male and one female and often the sexes share in brood rearing. However, many species exhibit forms of polygyny, polyandry, and polygynandry and even in socially monogamous species sexual infidelity, i.e., extra-pair copulation (EPC), occurs regularly.

Breeding and mating systems of the Paleognathae

Among the many distinctive features of the Paleognathae is the apparent universality of male parental care. Nearly all Paleognathae exhibit male incubation of eggs exclusively (Handford & Mares 1985). Uniparental paternal care often is associated with polyandrous mating systems, but in ratites and tinamous it is associated with a variety of mating systems. Exclusive paternal care is rare among terrestrial vertebrates. It is found in nine amphibians and no other non-avian reptiles or mammals.

Breeding and mating systems of the Anseriformes.

Phylogenetic analysis indicates that the ancestor to the Anseriformes employed biparental care and biparental care persists in the basal branches of this order in the families Dendrocygnidae, Anhimidae and Anseranatidae (Ligon 1999). It is retained in swans and geese (genera *Cygnus* and *Branta*), but is lost in many ducks (genera *Anas, Aix, Oxyura and Melanitta*). Perennial social monogamy is found among the swans and geese (Anserini), and in the whistling ducks (Dendrocygninni) (Bolen 1971), and the shelducks, shelgeese and steamer ducks (Tadorninae) (Hori 1964, 1969; Weller 1976). In

virtually all species displaying long-term monogamy, male roles include vigilance and defense of females throughout incubation, as well as active help with brood rearing. Greater levels of parental care is demonstrated in some of the nine species of Dendrocygnini, and in the black swan (*Cygnus atratus*) in which males share incubation (Bolen & Smith 1979, Chronister 1985, Oring & Sayler 1992). Perennially socially monogamous waterfowl are less likely to participate in polygamous behaviors such as extra-pair copulations, promiscuous behaviors or polygyny, or in mate switching (Scott 1967, 1980, Minton 1968, Mineau & Cooke 1979, Patterson 1982, McKinney *et al.* 1983, Fabricius & Boyd 1985, MacInnes & Dunne 1988).

Dabbling ducks (Anatini) and pochards (Aythyini) generally are classified as strongly polygamous to seasonally monogamous. In most species for which data are available, individuals form new pair bonds each season and re-pairing of mates in subsequent years is rare. Females are strongly philopatric in many species. Pairing often occurs prior to arrival at breeding grounds, and males generally follow females to the breeding area. Male roles vary from simply defending the female from conspecifics, as in the northern pintail (*Anas acuta*), to defending a distinct breeding territory, as in the northern shoveler (*Anas clypeata*). Males play no direct role in incubation and often provide little or no help with brood rearing. The most prominent secondary mating strategy of males is forced copulation (McKinney *et al.* 1983). Phylogenetic analysis indicates 2 transitions to female dominated uniparental care, once in the Oxyura and occurring once again basal to the split between *Branta/Cygnus* (monogamous) and *Anas/Aix/Melanitta* (polygamous) (Tullberg & Temrin 2002).

Breeding and mating systems of the Galliformes

The Galliformes are a large order displaying a variety of parental care and a range of mating systems, including monogamy, polygyny, promiscuity and polygynandry. Pair bonds, if evident, may last only through copulation or may persist over multiple breeding seasons. In some species, dominance hierarchies exist, and high-ranking males often have greater mating success than lower ranking males. (Delacour & Amadon 1973, Johnsgard 1983, 1999, Campbell & Lack 1985, Stiles & Skutch 1991, Dickson, 1992, Jones, *et al.* 1995, Madge & McGowan 2002).

Parental care includes environmental (Australasian megapodes) or contact incubation (all other species). Contact incubation is performed predominantly by females. Brooding may be absent, be conducted primarily by the female, be shared, or, rarely, be conducted primarily by a male. Males may guard nest sites, territories, brooding females, and chicks. In all species of Galliformes, chicks are precocial to superprecocial, and are capable of leaving the nest and foraging shortly after hatching, but are tended by one or more adults (Delacour & Amadon 1973, Johnsgard 1983, 1999, Campbell & Lack 1985, Stiles & Skutch 1991, Dickson, 1992, Jones, *et al.* 1995, Madge & McGowan 2002).

Megapodidae

In the family Megapodidae, females lay eggs in mounds of soil and vegetation that serve to environmentally incubate eggs. Nest mounds are guarded by males, and multiple females may lay eggs in the nest or territory of one male. Females may also lay eggs in the mounds guarded by more than one male. Females in this family may be promiscuous or monogamous.

Cracidae, Odontophoridae, Numididae

In the family Cracidae (guans, currasows and chacalacas), most species raise young by some form of biparental care, though nesting and brood rearing data are scarce for this cryptic group. The mating systems of this family are understudied, but polygamous, promiscuous and monogamous species are known. However, monogamy is the most common mating system in this group. The Odontophoridae (new world quail), and Numididae (guineafowl) are predominantly biparental and socially monogamous.

Phasianidae

The Phasianidae are a large family encompassing over 200 species across a wide array of habitats and ecosystems. The ancestor to this family was most likely monogamous with biparental care (Tullberg *et al.* 2002). This is retained in most representatives among many taxa, such as in the jungle and wood partridges, the old world quail, spurfowl, francolins, partridges, and most of the subfamilies, but promiscuity, strong polygyny and female-dominant uniparental care is common in many of the more derived taxa, such as turkeys, the lekking grouse species, peafowl, pheasants and junglefowl (Johnsgard 1999, Madge & McGowan 2002, Tullberg *et al.* 2002).

Neoaves

In the Neoaves, which comprise all remaining avian taxa, there is a trend toward less investment in eggs and incubation, and greater investment in hatchlings. This increases the capacity for males to contribute, as well as the necessity, and rates of monogamy increase with decreasing independence of offspring.

Cryptic sexual selection

Sexual selection is a major force driving evolutionary change in sexually reproducing organisms. Sexual selection pressures often are distinguished as either intrasexual selection, in which individuals of the same sex compete for reproductive opportunities, or intersexual selection, in which opposite sexes interact, such as when one sex selects individuals of the opposite sex for reproductive purposes. Sexual selection drives secondary sexual characteristics such as sexual dimorphism between males and females, and also sex-specific characteristics such as male ornamentation. Sexual selection can also strongly influence primary sexual characteristics.

In birds, females are the 'limiting sex' (Emlen & Oring 1977) and variance in male reproductive success is high relative to females. Male competition for access to females is high and female choice place a fundamental role in male breeding opportunity. These factors open the way for cryptic sexual selection, both as inter- and intra- sexual selection.

Sexual selection can arise in response to either intersexual selection in which females choose males based upon desired characteristics, or intrasexual selection in which males compete for access to females or to acquire and maintain territories or other resources. These are both pre-copulatory selection pressures.

Post-copulatory sexual selection also occurs. An important example is male-male sexual selection in the form of sperm competition. Sperm competition arises when a female is inseminated by multiple males, yet fertilization results in differential, non-random, fertilization success among males. For males, these selective pressures often are referred to as a "lottery effect." The chances of fertilizing an egg are improved by

increasing the number of sperm transferred to females. Sexual selection results in many adaptations in males and females.

In males, adaptations to sexual selection, including sperm competition, may include increased proportional testis size to increase numbers of sperm, and sperm morphology. Across taxa, increased relative testis size is related to increased rates of sperm competition. In the avian family Maluridae, comprised of species which experience differing amounts of sperm competition, not only is increased testis size correlated with increased sperm competition, but the increased testis size is a result of an increase in the proportion of the testes that are comprised of spermatogenic tissues (Rowe & Pruett-Jones 2011).

An additional challenge to males is the presence of sperm storage tubules located in the vaginal opening of the oviduct of females (Birkhead & Møller 1992, Briskie & Montgomerie 1993). Sperm storage tubules are small invaginations in the wall of the uterovaginal junction of the oviduct where sperm can be stored for days or weeks (Freedman *et al.* 2001). Assuming it is beneficial for males to have this sperm available to females via being stored within females, one result is strong selection for traits which allow a male effectively and accurately to transfer a sufficient volume of sperm to a female when the opportunity arises.

LITERATURE CITED

Aire, T. A. (1979). The epididymal region of the Japanese quail (*Coturnix japonica*). *Acta Anatomica* 103: 305-312.

Aire, T. A. (1980). The ductuli efferentes of the epididymal region of birds. *Journal of Anatomy* 130: 707.

Aire, T. A. (1982). The rete testis of birds. *Journal of Anatomy* 135: 97.

Aire, T. A. (1997). The structure of the interstitial tissue of the active and resting avian testis. *The Onderstepoort Journal of Veterinary Research* 64: 291-299.

Aire, T. A. (2007a). Anatomy of the testis and male reproductive tract. pp. 37 – 113 In [B. G. M. Jamieson, Ed] *Reproductive Biology and Phylogeny of Birds* Vol. 6A. New Hampshire, USA; Plymouth, UK: Science Publishers, Inc.

Aire, T. A. (2007b). Spermatogenesis and testicular cycles. pp. 279 – 347 In [B. G. M. Jamieson, ed] *Reproductive Biology and Phylogeny of Birds* Vol. 6A. New Hampshire, USA; Plymouth, UK: Science Publishers, Inc.

Aire, T. A., and M. Malmqvist (1979). Macrophages in the excurrent ducts of the testes of normal domestic fowl (*Gallus domesticus*). *Anatomia, Histologia, Embryologia 8*: 172-176.

Aire, T. A., J. S. Ayeni and M. O. Olowo-Okorun (1979). The structure of the excurrent ducts of the testis of the guinea-fowl (*Numida meleagris*). *Journal of Anatomy* 129: 633-643.

Aire, T. A., M. O. Olowo-Okorun and J. S. Ayeni (1980). The seminiferous epithelium in the guinea fowl (*Numida meleagris*). *Cell and Tissue Research* 205: 319-325.

Ando, Y., T. Kuwana and T. Fujimoto (1980). Chick primordial germ cells escaping from the blood-vessel to reach the gonadal primordium. *Development, Growth and Differentiation* 22: 707.

Ansari, H. A., N. Takagi and M. Sasaki (1988). Morphological differentiation of sex chromosomes in three species of ratite birds. *Cytogenetic and Genome Research* 47: 185-188.

Ariyaratne, H. B. S. and S. M. L. C. Mendis-Handagama (2000). Changes in the Testis Interstitium of Sprague Dawley Rats from Birth to Sexual Maturity. *Biology of Reproduction* 62: 680–690.

Bellott, D. W., H. Skaletsky, T. Pyntikova, E. R. Mardis, T. Graves, C. Kremitzki, L. G. Brown, S. Rozen, W. C. Warren, R. K. Wilson and D. C. Page (2010). Convergent evolution of chicken Z and human X chromosomes by expansion and gene acquisition. *Nature* 466: 612-616.

Belterman, R. H. R. and L. E. M. De Boer (1984). A karyological study of 55 species of birds, including karyotypes of 39 species new to cytology. *Genetica 65*: 39-82.

Bergers G. and S. Song (2005). The role of pericytes in blood-vessel formation and maintenance. *Neuro-Oncology* 7: 452-64.

Berlin, S. and H. Ellegren (2006). Fast accumulation of nonsynonymous mutations on the female-specific W chromosome in birds. *Journal of molecular evolution* 62: 66-72.

Bertelli, S. (2016). Advances on tinamou phylogeny: an assembled cladistic study of the volant palaeognathous birds. *Cladistics* 32: 1–24.

Birkhead, T. R. (1995). Sperm competition: evolutionary causes and consequences. *Reproduction, Fertility and Development* 7: 755-775.

Birkhead, T. R. (1998). Sperm Competition in Birds. *Reviews of Reproduction* 3: 123-129.

Birkhead, T. R., L. Atkin, and A. P. Møller (1987). Copulation Behavior in Birds. *Behaviour* 101: 101-133.

Birkhead, T. R. and J. D. Biggins (1998). Sperm competition mechanisms in birds: models and data. *Behavioral Ecology* 9: 253-260.

Birkhead, T. R., J. V. Briskie and A. P. Møller (1993). Male sperm reserves and copulation frequency in birds. *Behavioral Ecology and Sociobiology* 32: 85-93.

Birkhead, T. R., F. Fletcher and E. J. Pellatt (1998). Testes asymmetry, condition and sexual selection in birds: an experimental test. *Proceedings of the Royal Society of London, Series B: Biological Sciences* 265: 1185-1189.

Birkhead, T. R., B. J. Hatchwell and N.B. Davies (1991). Sperm competition and the reproductive organs of the male and female dunnock *Prunella modularis*. *Ibis* 133: 306-311.

Birkhead, T. R., F. M. Hunter and J. E. Pellatt (1989). Sperm competition in the zebra finch, *Taeniopygia guttata*. *Animal Behaviour* 38: 935-950.

Birkhead, T. R. and C. M. Lessels (1988). Copulation behavior of the Osprey *Pandion haliaetus*. *Animal Behaviour* 36: 1672-1682.

Birkhead, T. R., J. G. Martinez, T. Burke, and D. P. Froman (1999). Sperm mobility determines the outcome of sperm competition in the domestic fowl. *Proceedings of the Royal Society of London, Series B: Biological Sciences* 266: 1759-1764.

Birkhead, T. R. and A. P. Møller (1992). *Sperm Competition in Birds.* Academic Press, London.

Birkhead, T. R., J. Pellatt and F. M. Hunter (1988). Extra-pair copulation and sperm competition in the zebra finch. *Nature* 334: 60-62.

Blackburn, D. G. and H. E. Evans (1986). Why are there no viviparous birds? *American Naturalist* 128: 165-190.

Bolen, E. G. (1971). Pair-bond tenure in the Black-bellied Tree Duck. *The Journal of Wildlife Management* 35: 385-388.

Bolen, E. G., and E. N. Smith (1979). Notes on the incubation behavior of Black-bellied Whistling-Ducks. *Prairie Naturalist* 11: 119-123.

Boyd, J. H. (2016). Taxonomy In Flux. http://jboyd.net/Taxo/List1.html.

Braun, C. E. (1998). Sage grouse declines in western North America: what are the problems. In *Proceedings of the Western Association of State Fish and Wildlife Agencies* 78: 139-156.

Brennan, P. L. R., T. R. Birkhead, K. Zyskowski, J. van der Waag, and R. O. Prum (2008). Independent evolutionary reductions of the phallus in basal birds. *Journal of Avian Biology* 39: 487-492.

Brennan, P. L. R., C. J. Clark, and R. O. Prum (2010). Explosive eversion and functional morphology of the duck penis supports sexual conflict in waterfowl genitalia *Proceedings of the Royal Society of London, Series B: Biological Sciences* 277: 1309-1342.

Brennan P. L. R., R. O. Prum, K. G. McCracken, M. D. Sorenson, R. E. Wilson, and T. R. Birkhead (2007). Coevolution of Male and Female Genital Morphology in Waterfowl. *PLoS ONE* 2: e418.

Brillard, J. P. and M. R. Bakst (1990). Quantification of spermatozoa in the sperm-storage tubules of Turkey hens and the relation to sperm numbers in the perivitelline layer of eggs. *Biology of Reproduction* 43: 271-275.

Briskie, J. V. (1996a). Lack of sperm storage by female migrants and the significance of copulations en route. *Condor* 98: 414-417.

Briskie, J. V. (1996b). Spatiotemporal patterns of sperm storage and last-male sperm precedence in birds. *Functional Ecology.* 10: 375-383.

Briskie, J. V. and R. Montgomerie (1993). Patterns of sperm storage in relation to sperm competition in passerine birds. *Condor* 95: 442-454.

Briskie, J. V. and R. Montgomerie (1997). Sexual selection and the intromittent organ of birds. *Journal of Avian Biology* 28: 73-86.

Briskie, J. V. and R. Montgomerie (2001). Efficient copulation and the evolutionary loss of the avian intromittent organ. *Journal of Avian Biology* 32: 184-187.

Briskie, J. V. and R. Montgomerie (2007). Testis size, sperm size and sperm competition. pp 513 – 551 In [B. G. M. Jamieson, ed] *Reproductive Biology and Phylogeny of Birds* Vol. 6A. New Hampshire, USA; Plymouth, UK: Science Publishers, Inc.

Briskie, J. V., Montgomerie, R., and T. R. Birkhead (1997). The evolution of sperm size in birds. *Evolution* 51: 937-945.

Brom, T. G., and R. W. R. J. Dekker (1992). Current studies on megapode phylogeny. *Zoologische Verhandelingen* 278: 7-17.

Bryniarski, K, M. Szczepanik, K. Maresz., M. Ptak and W. Ptak (2004). Subpopulations of mouse testicular macrophages and their immunoregulatory function. *American Journal of Reproductive Immunology 52:* 27-35.

Budras, K. D. and T. Sauer (1975). Morphology of the epididymis of the cock (*Gallus domesticus*) and its effect upon the steroid sex hormone synthesis. I. Ontogenesis, morphology and distribution of the epididymis. *Anatomy and Embryology* 148: 175-196.

Budras, K. D. and F. G. Schmidt (1976). The early development of the gonads, and the ontogenesis of the rete testis and tubuli seminiferi recti in the chicken, *Gallus domesticus*. *Zentralblatt fuer Veterinaermedizin. Reihe C (Germany, FR).*

Burley, N. T. and K. Johnson (2002). The evolution of avian parental care. *Philosophical Transactions of the Royal Society of London, Series B: Biological Sciences* 357: 241-250.

Burt, D. W. (2007). Emergence of the chicken as a model organism: implications for agriculture and biology. *Poultry Science 86*: 1460-1471.

Campbell, B. and E. Lack (1985). A Dictionary of Birds. Vermilion, Buteo Books.

Chowdhury, A. K., and G. Marshall (1980). Irregular pattern of spermatogenesis in the baboon (*Papio anubis*) and its possible mechanism. *Testicular Development, Structure and Function* 129-137.

Chronister, C. D. (1985). Egg-laying and incubation behavior of black bellied whistling ducks. M.S. Thesis, University of Minnesota, Minneapolis.

Ciment, G., and J. A.Weston (1982). Early appearance in neural crest and crest-derived cells of an antigenic determinant present in avian neurons. *Developmental biology 93*: 355-367.

Clermont, Y. (1958). Contractile elements in the limiting membrane of the seminiferous tubules of the rat. *Experimental Cell Research* 15: 438-440.

Clermont, Y. (1962). Quantitative analysis of spermatogenesis of the rat: a revised model for the renewal of spermatogonia. *American Journal of Anatomy* 111: 111-129.

Clermont, Y. (1963). The cycle of seminiferous epithelium in man. *American Journal of Anatomy* 112: 35-52.

Clermont, Y. (1970). Dynamics of human spermatogenesis. pp 47 – 61. [Eds E. Rosemberg and C. A. Paulsen]. In *The Human Testis*. Plenum Press, Springer US. New York, London.

Clermont, Y. (1972). Kinetics of spermatogenesis in mammals: seminiferous epithelium cycle and spermatogonial renewal. *Physiology Reviews 52*: 198-236.

Coker, C. R., F. McKinney, H. Hays, S. V. Briggs and K. M. Cheng (2002). Intromittent organ morphology and testis size in relation to mating system in waterfowl. *The Auk* 119: 403-413.

Cracraft, J., F. K. Barker, M. Braun, J. Harshman, G. J. Dyke, J. Feinstein, S. Stanley, A. Cibois, P. Schikler, P. Beresford, J. Garcia-Moreno, M. D. Sorenson, T. Yuri and D. P. Mindell (2004). Phylogenetic relationships among modern birds (Neornithes). *Assembling the Tree of Life* 468-489.

Davidoff, M. S., R. Middendorff, G. Enikolopov, D. Riethmacher, A. F. Holstein and D. Müller (2004). Progenitor cells of the testosterone-producing Leydig cells revealed. *The Journal of Cell Biology* 167: 935-944.

De Boer, G. F., J. Van Vloten and L. Hartog (1980). Comparison of complement fixation and phenotypic mixing tests for the detection of lymphoid leukosis virus in egg albumen and embryos of individual eggs. *Avian Pathology* 9: 207-218.

DeFalco, T. and B. Capel (2009). Gonad morphogenesis in vertebrates: divergent means to a convergent end. *Annual Review of Cell and Developmental Biology 25:* 457-482.

Delehanty, D. J. and P. P. O'Hearn (2005). Behavioral and Morphological Asymmetries in Chukar *Alectoris chukar* Copulation. *Journal of Avian Biology* 36: 276 – 279.

Delacour, J. and D. Amadon (1973). *Curassows and Related Birds*. American Museum of Natural History.

De Melo Bernardo, A. (2016). Primordial Germ Cells and Amnion Development in the Avian Embryo. PhD dissertation, Department of Anatomy and Embryology, Faculty of Medicine, Leiden University Medical Center (LUMC), Leiden University.

de Reviers, M. D. (1968). Détermination de la durée des processus spermatogénetiques chez le coq à l'aide de thymidine tritiée. *International Congress on Animal Reproduction and Artificial Insemination [Proceedings]*.

Dubois, R. (1969). Le mecanisme d'entree des cellules germinale sprimordiales dans le reseau vasculaire, chez l'embryon de poulet. *Embryology and Experimental Morphology* 21: 255-270.

Dym, M., and D. W. Fawcett (1971). Further observations on the numbers of spermatogonia, spermatocytes, and spermatids connected by intercellular bridges in the mammalian testis. *Biology of Reproduction* 4: 195-215.

Ellegren, H. (2002). Dosage compensation: do birds do it as well? *TRENDS in Genetics* 18: 25-28.

El Jamil, A. S. Magre, A. Mazabraud and A. Penrad-Mobayed (2008). Early Aspects of Gonadal Sex Differentiation. *Journal of Experimental Zoology 309:* 127-137.

Ellegren, H. (2001). Hens, cocks and avian sex determination. *EMBO Reports* 2: 192-196.

Emlen, S. T. and L. W. Oring (1977). Ecology, sexual selection, and the evolution of mating systems. *Science* 197: 215-223.

Eo, S. H., O. R. Bininda-Emonds and J. P. Carroll (2009). A phylogenetic supertree of the fowls (Galloanserae, Aves). *Zoologica Scripta, 38*: 465-481.

Extavour, C. G. and M. Akam (2003). Mechanisms of germ cell specification across the metazoans: epigenesis and preformation. *Development* 130: 5869-5884.

Eyal-Giladi, H., and S. Kochav (1976). From cleavage to primitive streak formation: a complementary normal table and a new look at the first stages of the development of the chick: I. General morphology. *Developmental Biology* 49: 321-337.

Fawcett, D. W. (1961). Intercellular bridges. *Experimental Cell Research* 8: 174-187.

Fawcett, D. W., S. Ito and D. Slautterback (1959). The occurrence of intercellular bridges in groups of cells exhibiting synchronous differentiation. *The Journal of Biophysical and Biochemical Cytology* 5: 453-460.

Fawcett, D. W., W. A. Anderson and D. M. Phillips (1971). Morphogenetic factors influencing the shape of the sperm head. *Developmental Biology* 26: 220-251.

Freedman, S. L., V. G. Akuffo and M. R. Bakst (2001). Evidence for the innervation of sperm storage tubules in the oviduct of the turkey (*Meleagris gallopavo)*. *Reproduction* 121: 809-814.

Fridolfsson, A. K., H. Cheng, N. G. Copeland, N. A. Jenkins, H. C. Liu, T. Raudsepp, T. Woodage, B. Chowdhary, J. Halverson and H. Ellegren (1998). Evolution of the avian sex chromosomes from an ancestral pair of autosomes. *Proceedings of the National Academy of Sciences of the United States of America* 95: 8147–8152.

Fujimoto, T., A. Ukeshima, Y. Miyayama, F. Horio and E. Ninomiya (1979). Observations of primordial germ cells in the turtle embryo (*Caretta caretta*): light and electron microscopic studies. *Development, Growth and Differentiation 21:* 3-10.

Fujimoto, T., A. Ukeshima and R. Kiyofuji (1976). The origin, migration and morphology of the primordial germ cells in the chick embryo. *Anatomical Record* 185: 139–153.

Gerhardt, U. (1933). Cloaca and copulatory organs. pp. 267 – 350 In [Bolk L., E. Goppert, E. Kallius and W.Lubosch eds.] *Hanbuch der vergleichenden anatomie der wirbeltiere*. Vol 6. Urban and Schwarzenberg, Berlin.

Ginsburg, M. and H. Eyal-Giladi (1987). Primordial germ cells of the young chick blastoderm originate from the central zone of the area pellucida irrespective of the embryo-forming process. *Development* 101: 209-219.

Ginsburg, M. and H. Eyal-Giladi (1989). Primordial germ cell development in cultures of dispersed central disks of stage X chick blastoderms. *Gamete Research* 23: 421-427.

Göth, A. (2007). Incubation temperatures and sex ratios in Australian brush-turkey (*Alectura lathami*) mounds. *Austral Ecology 32*: 378-385.

Göth, A., and D. T. Booth (2005). Temperature-dependent sex ratio in a bird. *Biology Letters* 1: 31–33.

Govoroun, M. S., M. Pannetier, E. Pailhoux, J. Cocquet, J.-P. Brillard, I. Couty, F. Batellier and C. Cotinot (2004). Isolation of chicken homolog of the FOXL2 gene and comparison of its expression patterns with those of aromatase during ovarian development. *Developmental Dynamics* 231: 859-870.

Gray, J. G. (1937). The anatomy of the male genital ducts of the fowl. *Journal of Morphology* 60: 394-405.

Hackett, S. J. R. T. Kimball, S. Reddy, R. C. K. Bowie, E. L. Braun, M. J. Braun, J. L. Chojnowski, W. A. Cox, K. L. Han, J. Harshman, C. J. Huddleston, B. D. Marks, K. J. Miglia, W. S. Moore, F. H. Sheldon, D. W. Steadman, C. C. Witt and T. Yuri. (2008). A Phylogenomic Study of Birds Reveals Their Evolutionary History. *Science* 320: 1763-1768.

Hamburger, V. and H. L. Hamilton (1951). A series of normal stages in the development of the chick embryo. *Journal of Morphology* 88: 49–92.

Handford, P. and M. A. Mares (1985). The mating systems of ratites and tinamous: an evolutionary perspective. *Biological Journal of the Linnean Society*, *25*: 77-104.

Heller, C. H., and Y. Clermont (1963). Kinetics of the germinal epithelium in man. *Recent Progress in Hormone Research 20:* 545-575.

Herrera, A. M., S. G. Shuster, C. L. Perriton and M. J. Cohn (2013). Developmental basis of phallus reduction during bird evolution. *Current Biology 23:*1065-1074.

Hess, R. A. (1990). Quantitative and qualitative characteristics of the stages and transitions in the cycle of the rat seminiferous epithelium: light microscopic observations of perfusion-fixed and plastic-embedded testes. *Biology of Reproduction 43*: 525-542.

Hess, R. A., Thurston, R. J. and H. V. Biellier (1976). Morphology of the epididymal region and ductus deferens of the turkey (*Meleagris gallopavo*). *Journal of Anatomy* 122: 241-252.

Hilscher, W. (1983), Problems of the Keimbahm. *Bibliotheca Anatomica* 24: 1-21.

Hilscher, B. (1979). Spermatogenesis, an interacting proliferation process between stem cell spermatogonia and differentiating spermatogonia. *Fohringer Symposium, Midlum/Alker Sum.*

Holstein, A. F. and E. C. Roosen-Runge (1981). Spermatogonia pp. 33 – 57 in *Atlas of Human Spermatogenesis*. Grosse Verlag, Berlin.

Hong, Y. H., D. S. Seo, D. K. Jeong, K. D. Choi and J. Y. Han (1995). Migration of the primordial germ cells and gonad formation in the early chicken embryo. *Asian and Australasian Journal of Animal Sciences 8:* 557-562.

Hori, J. (1964). The breeding biology of the Shelduck *Tadorna*. *Ibis* 106: 333-360.

Hori, J. (1969). Social and population studies in the Shelduck. *Wildfowl* 20: 5-22.

Howarth, B. (1983). Fertilizing ability of cock spermatozoa from the testis epididymis and vas deferens following intramagnal insemination. *Biology of Reproduction* 28: 586-590.

Hubert, J. (1969). Origin and development of oocytes. In *Biology of the Reptilia*, vol. 14 (ed. C. Gans), pp. 43-50. New York: Wiley.

Itoh, Y., K. Kampf and A. P. Arnold (2008). Molecular cloning of zebra finch W chromosome repetitive sequences: evolution of the avian W chromosome. *Chromosoma* 117: 111–121.

Itoh, Y., and A. P. Arnold (2005). Chromosomal polymorphism and comparative painting analysis in the zebra finch. *Chromosome Research* 13: 47-56.

Jacquet, J. M. and B. Sauveur. (1995). Photoperiodic control of sexual maturation in muscovy drakes. *Domestic Animal Endocrinology 12*: 189-195.

Johnsgard, P. A. (1978). Review of The Pheasants of the World. *Auk* 95: 620-621.

Johnsgard, P.A. (1983). The Grouse of the World. Univeristy of Nebraska Press, Lincoln, NE, USA.

Johnsgard, P. A. (1999). The Pheasant of the World: Biology and Natural History, 2nd edition. Smithsonian Institution Press, Washington, DC.

Johnson, K., F. McKinney, R. Wilson and M. Sorenson (2000). The evolution of postcopulatory displays in dabbling ducks (Anatini): a phylogenetic perspective. *Animal Behaviour 59:* 953-963.

Jones, R. C. and M. Lin (1992). Spermatogenesis in birds. *Oxford Reviews of Reproductive Biology 15:* 233-264.

Jones, K. T., J. Carroll, J. A. Merriman, D. G. Whittingham and T. Kono (1995). Repetitive sperm-induced Ca2+ transients in mouse oocytes are cell cycle dependent. *Development 121*: 3259-3266.

Kagami, H., M. E. Clark, A. M. Gibbins and R. J. Etches (1995). Sexual differentiation of chimeric chickens containing ZZ and ZW cells in the germline. *Molecular Reproduction and Development 42*: 379-387.

Karagenç, L., Y. Cinnamon, M. Ginsburg and J. N. Petitte (1996). Origin of primordial germ cells in the prestreak chick embryo. *Developmental Genetics* 19: 290-301.

Kavanau, J. L. (1987). *Behavior and evolution: lovebirds, cockatiels, budgerigars*. Science Council of Canada.

Kimball, R. T. and J. D. Ligon (1999). Evolution of avian plumage dichromatism from a proximate perspective. *The American Naturalist* 154: 182-193.

Kimball, R. T., J. D. Ligon and M. Merola-Zwartjes (1997). Testicular asymmetry and secondary sexual characters in red junglefowl. *The Auk* 114: 221-228.

King, A. S. (1981a). Cloaca, p. 63-106. *In*: A. S. King and J. McLelland [eds.] Form and Function in Birds. Vol 2. Academic Press, New York.

King, A. S. (1981b). Phallus. P107-148 *In:* A. S. King and J. McLelland [eds.] Form and Function in Birds. Vol. 2. Academic Press, New York.

Kinsky, F. (1971). The consistent presence of paired ovaries in the kiwi (*Apteryx*) with some discussion of this condition in other birds. *Journal für Ornithologie* 112: 334-357.

Komárek, V. and F. Marvan (1969). Beitrag zur mikroskopischen Anatomie des Kopulationsorganes der Entenvögel. *Anat Anz, 124:* 467-476.

Kuwana, T. (1993). Migration of avian primordial germ cells toward the gonadal anlage. *Development, Growth and Differentiation 35*: 237-243.

Kuwana, T. and T. Fujimoto (1984). Locomotion and scanning electron microscopic observations of primordial germ cells from the embryonic chick blood in vitro. *The Anatomical Record 209*: 337-343.

Lack, D. L. (1968). Ecological adaptations for breeding in birds. FAO Agris Publication.

Lake, P. E. (1957). The male reproductive tract of the fowl. *Journal of Anatomy* 91: 116-129.

Lake, P. E. (1981). Male genital organs. p. 1-61. *In*: A. S. King and J. McLelland [eds.] Form and Function in Birds. Vol 2. Academic Press, New York.

Leblond, C. P. and Y. Clermont (1952). Definition of the stages of the cycle of the seminiferous epithelium in the rat. *Annals of the New York Academy of Science* 55: 548-573.

Leidl, W. (1968). Steurerung und beeinfussung der Samenbildungs-prozesse. *Zietschrift fur Tierzuchsung und Zuchtungbiologie.* 84: 273-289.

Ligon, J. D. (1997). A single functional testis as a unique proximate mechanism for promoting sex-role reversal in Coucals. *Auk* 114: 800-801.

Lin, M. and R. C. Jones (1990). Spatial arrangement of the stages of the cycle of the seminiferous epithelium in the Japanese quail (*Coturnix japonica*). *Journal of Reproduction and Fertility* 90: 361-367.

Lin, M. and R. C. Jones (1992). Renewal and proliferation of spermatogonia during spermatogenesis in the Japanese quail (*Coturnix japonica*). *Cell and Tissue Research* 267: 591-601.

Lin, M., Jones. R. C. and A. W. Blackshaw (1990). The cycle of the seminiferous epithelium in the Japanese quail (*Coturnix japonica*) and estimation of its duration. *Journal of Reproduction and Fertility* 88: 481-490.

Lin, M., M. H. Thorne, I. C. A. Martin, B. L. Sheldon and R. C. Jones (1995). Development of the gonads in the triploid (ZZW and ZZZ) fowl, *Gallus domesticus*, and comparison with normal diploid males (ZZ) and females (ZW). *Reproduction, Fertility and Development* 7: 1185–1197.

Low, M., I. Castro and A. Berggren (2005). Cloacal erection promotes vent apposition during forced copulation in the New Zealand stitchbird (hihi): implications for copulation efficiency in other species. *Behavioral Ecology and Sociobiology 58:* 247-255.

MacInnes, C. D. and E. H. Dunn (1988). Components of clutch size variation in Arctic-nesting Canada Geese. *Condor* 90: 83-89.

Madge, S. and P. McGowan (2002). Pheasants, Partridges and Grouse. *Christopher Helm, London.*

Mank, J. E., and H. Ellegren (2007). Parallel divergence and degradation of the avian W sex chromosome. *Trends in Ecology and Evolution* 22: 389–391.

Marchand, C. R., L. Gomote and M. de Reviers (1977). Contribution a l'etude par autoradiographe et marquage a la thymedine trite de la duree de la spematogenese du canard de barbarie (*Cairina moschata*) *Comptes Rendus des Seances, Society de Biologie* 21: 927-931.

McCarrey, J. R. and U. K. Abbott (1978). Chick gonad differentiation following excision of primordial germ cells. *Developmental biology 66*: 256-265.

McCarrey, J. R. and U. K. Abbott (1979). Mechanisms of genetic sex determination, gonadal sex differentiation, and germ-cell development in animals. *Advances in Genetics 20:* 217-290.

McCarrey, J. R. and U. K. Abbott (1982). Functional differentiation of chick gonads following depletion of primordial germ cells. *Journal of Embryology and Experimental Morphology 68*: 161-174.

McCracken, G. F. and G. S. Wilkinson (2000). Bat mating systems. *Reproductive Biology of Bats 12:* 3-5.

McKinney, F., S. R. Derrickson and P. Mineau (1983). Forced copulation in waterfowl. *Behaviour* 86: 250-294.

Mehrotra, P. N. (1964). On the Microscopic Anatomy of the Epididymis of *Anser melanotus* L. *Transactions of the American Microscopical Society:* 456-460.

Mendis-Handagama, S.M. L.C., G. P. Risbridger and D. M. de Krester (1987). Morphometric Analysis of the Components of the Neonatal and Adult Rat Testis Interstitium. *International Journal of Andrology* 10: 525-534.

Meng, Q., J. Liu, D. J. Varricchio, T. Huang and C. Gao (2004). Parental care in an ornithischian dinosaur. *Nature* 431:145–146.

Merchant-Larios, H., L. Popova and M. Reyss-Brion (1984). Early morphogenesis of chick gonad in the absence of mesonephros. *Development, Growth & Differentiation 26*: 403-417.

Merila, J. and B.C. Sheldon (1999). Testis size variation in the greenfinch *Carduelis chloris*: relevance for some recent models of sexual selection. *Behavior in Ecology and Sociobiology* 45: 115-123.

Meyer, D. B. (1964). The migration of the primordial germ cells in the chick embryo. *Developmental Biology* 10: 154-190.

Mineau, P. and F. Cooke (1979). Rape in the lesser snow goose. *Behaviour* 70: 280-291.

Minton, C. D. T. (1968). Pairing and breeding of mute swans. *Wildfowl* 19: 41-60.

Mizuno, S., R. Kunita, O. Nakabayashi, Y. Kuroda, N. Arai, M. Harata, A. Ogawa, Y. Itoh, M. Teranishi and T. Hori (2002). Z and W chromosomes of chickens: studies on their gene functions in sex determination and sex differentiation. *Cytogenetic and Genome Research* 99: 236–244.

Moens, P. B. and V. L. W. Go (1972). Intercellular bridges and division patterns of rat spermatogonia. *Zeitschrift für Zellforschung und Mikroskopische Anatomie 127*: 201-208.

Møller, A.P. (1994). Directional selection on directional asymmetry: testes size and secondary sexual characters in birds. *Proceedings of the Royal Society of London, Series B*: *Biological Sciences* 258: 147-151.

Montgomerie, R. and J. V. Briskie (2007). Anatomy and evolution of copulatory structures. *Reproductive Biology and Phylogeny of Birds Part A: Phylogeny, Morphology, Hormones and Fertilization Science Publishers*: 115-148.

Nagano, T. (1962). Observations on the fine structure of developing spermatid in the domestic chicken. *Journal of Cell Biology* 14: 193-205.

Nakamura, M. (2010). The mechanism of sex determination in vertebrates—are sex steroids the key-factor? *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology:* 313: 381-398.

Nakamura, Y., Y. Yamamoto, F. Usui, T. Mushika, T. Ono, A. R. Setioko, K. Takeda, K. Nirasawa, H. Kagami and T. Tagami (2007). Migration and proliferation of primordial germ cells in the early chicken embryo. *Poultry science* 86: 2182-2193.

Nanda, I., T. Haaf, M. Schartl, M. Schmid and D. W. Burt (2002). Comparative mapping of Z-orthologous genes in vertebrates: implications for the evolution of avian sex chromosomes. *Cytogenetic and Genome Research* 99: 178-184.

Noirault, J., J. P. Brillard and M. R. Bakst (2006). Effect of various photoperiods on testicular weight, weekly sperm output and plasma levels of LH and testosterone over the reproductive season in male turkeys. *Theriogenology 66*: 851-859.

Norell, M. A., J. M. Clark, L. M. Chiappe and D. Dashzeveg (1995). A nesting dinosaur. *Nature 378*.6559: 774-776.

Nyland, K.B., M. P. Lombardo and P.A. Thorpe (2003). Left-sided directional bias of cloacal contacts during house sparrow copulations. *Wilson Bulletin* 115: 470-473.

O'Donnell, L., K. M. Robertson, M. E. Jones and E. R. Simpson (2001). Estrogen and Spermatogenesis 1. *Endocrine Reviews 22*: 289-318.

Ogawa, S., S. Inoue, T. Watanabe, H. Hiroi, A. Orimo, T. Hosoi, Y. Yasuyoshi Ouchi and M. Muramatsu (1998). The Complete Primary Structure of Human Estrogen Receptor β (hERβ) and Its Heterodimerization with ER αin Vivoandin Vitro. *Biochemical and Biophysical Research Communications 243*: 122-126.

Ohno, S. (1967) Sex Chromosomes and Sex-linked Genes, p. 141. Springer-Verlag, New York.

de Oliveira, C. A. and G. A. B. Mahecha (2000). Morphology of the copulatory apparatus of the spotted tinamou *Nothura maculosa* (Aves: Tinamiformes). *Annals of Anatomy-Anatomischer Anzeiger 182*: 161-169.

Oring, L.W. and R. D. Sayler (1992). The mating systems of waterfowl. *Ecology and Management of Breeding Waterfowl:* 190-213.

Patterson, I. J. (1982). The Shelduck. Cambridge University press, London.

Patterson I. J. and M. Makepeace (1979). Mutual interference during nest prospecting in the Shelduck, *Tadorna tadorna. Animal Behavior* 27: 522-535.

Peterson, A. A., M. P. Lombardo and H. W. Power (2001). Left-sided directional bias of cloacal contacts during tree swallow copulations. *Animal Behavior* 62: 739-741.

Petitte, J. N., L. Karagenc and M. Ginsburg (1997). The origin of the avian germ line and transgenesis in birds. *Poultry science 76*: 1084-1092.

Pigozzi, M. I. and A. J. Solari (1999). The ZW pairs of two paleognath birds from two orders show transitional stages of sex chromosome differentiation.*Chromosome Research 7*: 541-551.

Pigozzi, M. I. and A. J. Solari (2005a). The germ-line-restricted chromosome in the zebra finch: recombination in females and elimination in males. *Chromosoma 114*: 403-409.

Pigozzi, M. I. and A. J. Solari (2005b). Meiotic recombination in the ZW pair of a tinamid bird shows a differential pattern compared with neognaths. *Genome 48*: 286-290.

Proctor, N. S. and P. J. Lynch (1993). Manual of Ornithology. Avian structure and function. Edwards Brothers Inc. *Ann Arbor, Michigan*.

Prum, R. O. (2002). Why ornithologists should care about the theropod origin of birds. *The Auk 119*: 1-17.

Prum, R. O., J. S. Berv, A. Dornburg, D. J. Field, J. P. Townsend, E. M. Lemmon and A. R. Lemmon (2015). A comprehensive phylogeny of birds (Aves) using targeted next-generation DNA sequencing. *Nature* 526: 569-573.

Pudney, J. (1995). Spermatogenesis in nonmammalian vertebrates. *Microscopy Research and Technique 32*: 459-497.

Raudsepp, T., S. R. Kata, F. Piumi, J. Swinburne, J. E. Womack, L. C. Skow and B. P. Chowdhary (2002). Conservation of gene order between horse and human X chromosomes as evidenced through radiation hybrid mapping. *Genomics 79*: 451-457.

Regnault, S., A. A. Pitsillides and J. R. Hutchinson (2014). Structure, ontogeny and evolution of the patellar tendon in emus (*Dromaius novaehollandiae*) and other palaeognath birds. *PeerJ* 2: 711.

Rey, R. A., C. A. Nagle and H. Chemes (1996). Morphometric study of the testicular interstitial tissue of the monkey *Cebus apella* during postnatal development. *Tissue and Cell 28*: 31-42.

Rey, R., L. Al-Attar, F. Louis, F. Jaubert, P. Barbet, C. Nihoul-Fékété, J. L. Chaussain and N. Josso (1996). Testicular dysgenesis does not affect expression of anti-müllerian hormone by Sertoli cells in premeiotic seminiferous tubules. *The American Journal of Pathology 148*: 1689.

Rodemer-Lenz, E. (1989). On cell contribution to gonadal soma formation in quail-chick chimeras during the indifferent stage of gonadal development. *Anatomy and Embryology 179:* 237-242.

Rodemer, E. S., A. Ihmer and H. Wartenberg (1986). Gonadal development of the chick embryo following microsurgically caused agenesis of the mesonephros and using interspecific quail—chick chimaeras. *Journal of Embryology and Experimental Morphology 98*: 269-285.

Roosen-Runge, E. C. (1977). Spermatogonia and kinetics of spermatogenesis. pp. 102 – 144 In *The Process of Spermatogenesis in Animals*, Cambridge University Press, Cambridge.

Rowe, M. and S. Pruett-Jones (2011). Sperm competition selects for sperm quantity and quality in the Australian Maluridae. *PLoS One*, *6*: e15720-e15720.

Russell, L. D. (1990). *Histological and Histopathological Evaluation of the Testis*. Cache River Press, Clearwater, FL.

Russell, L. D. and L. R. de França (1995). Building a testis. *Tissue and Cell 27*: 129-147.

Russell, L. D., H. P. Ren, I. S. Hikim, W. Schulze and A. P. S. Hikim (1990). A comparative study in twelve mammalian species of volume densities, volumes, and numerical densities of selected testis components, emphasizing those related to the Sertoli cell. *American Journal of Anatomy 188*: 21-30.

Saita, A, S. Tripepi and O. M. Longo (1982). Comparative observations on spermiogenesis. II Nucleus shaping in the absence of a microtubulas manchette in the spermatids of the bird *Crotophaga ani* (Cuculiformes). *Bolletino di Zoologia* 49: 115-124.

Sasaki, M., C. Nishida, N. Takagi and H. Hori (1980). Sex chromosomes of the elegant crested tinamou, *Eudromia elegans* (Aves: Tinamiformes: Tinamidae). *Chromosome Information Service* 29:19-21.

Schmid M., E. Enderle, D. Schindler and W. Schempp (1989) Chromosome banding and DNA replication patterns in bird karyotypes. *Cytogenetics and Cell Genetics* 52:139–146.

Schmid, M. E., I. Nanda, M. Guttenbach, C. Steinlein, H. Hoehn, M. Schartl, T. Haaf, S. Weigend, R. Fries, J-M Buerstedde, K. Wimmers, D. W. Burt, J. Smith, S. A'Hara, A. Law, D. K. Griffin, N. Bumstead, J. Kaufman, P. A. Thomson, T. Burke, M. A. M. Groenen, R. P. M. A. Crooijmans, A. Vignal, V. Fillon, M. Morisson, F. Pitel, M. Tixier-Boichard, K. Ladjali-Mohammedi, J. Hillel, A. Mäki-Tanila, H. H. Cheng, M. E. Delany, J. Burnside and S. Mizunou (2000). First report on chicken genes and chromosomes 2000. *Cytogenetic and Genome Research* 90: 169-218.

Schultz, M. C. and C. P. Leblond (1990). Nucleolar structure and synthetic activity during meiotic prophase and spermiogenesis in the rat. *American Journal of Anatomy 189*: 1-10.

Schulze, W. (1982). Evidence of a wave of spermatogenesis in human testis. *Andrologia* 14: 200-207.

Schulze, W. and U. Rehder (1984). Organization and morphogenesis of the human seminiferous epithelium. *Cell and Tissue Research* 237: 395-407.

Schulze, W., Riemer, M., Rehder, U. and K. H. Hohne (1986). Computer-aided three-dimensional reconstructions of the arrangement of primary spermatocytes inhuman seminiferous tubules *Cell and Tissue Research* 244: 1-8.

Scott, D. (1980). Functional aspects of the pair bond in winter in Bewick's swans (*Cygnus colombianus bewickii*). *Behavioral Ecology and Sociobiology* 7: 323-327.

Scott, D. (1967). The Bewick's swans at Slimbridge, 1966-67. *Wildfowl Trust Annual Report* 18: 24-27.

Shetty, S., D. K. Griffin and J. A. Marshall Graves (1999). Comparative painting reveals strong chromosome homology over 80 million years of bird evolution. *Chromosome Research* 7: 289–295.

Shetty, S., P. Kirby, D. Zarkower and J. A. Marshall-Graves (2002). *DMRT1*in a ratite bird: evidence for a role in sex determination and discovery of a putative regulatory element. *Cytogenetic and Genome Research* 99: 245–251.

Shoemaker, C., M. Ramsey, J. Queen and D. Crews (2007). Expression of Sox9, Mis, and DMRT1 in the gonad of a species with temperature-dependent sex determination. *Developmental Dynamics 236*: 1055-1063.

Sibley, C. G. and J. E. Ahlquist (1990). *Phylogeny and Classification of Birds: a Study in Molecular Evolution*. Yale University Press, New Haven, CT.

Simmons, L. W. and J. S. Kotiaho (2002). Evolution of Ejaculates: Patterns of Phenotypic and Genotypic Variation and Condition Dependence in Sperm Competition Traits. *Evolution* 56: 1622-1631.

Smith, C. A. (2010). Sex determination in birds: a review. *Emu* 110: 364–377.

Smith, C. A., J. E. Andrews and A. H. Sinclair (1997). Gonadal sex differentiation in chicken embryos: expression of estrogen receptor and aromatase genes. *Journal of Steroid Biochemistry and Molecular Biology* 60: 295–302.

Smith, C. A., P. J. McClive, Q. Hudson and A. H. Sinclair (2005). Male specific cell migration into the developing gonad is a conserved process involving PDGF signalling. *Developmental Biology* 284: 337–350.

Smith, C. A., P. J. McClive, P. S. Western, K. J. Reed and A. H. Sinclair (1999). Conservation of a sex-determining gene. *Nature* 402: 601–602.

Smith, C. A., K. N. Roeszler, Q. J. Hudson and A. H. Sinclair (2007). Avian sex determination: what, when and where? *Cytogenetic and Genome Research* 117: 165–173.

Smith, C. A., K. N. Roeszler, T. Ohnesorg, D. M. Cummins, P. G. Farlie, T. J. Doran and A. H. Sinclair (2009). The avian Z-linked gene DMRT1 is required for male sex determination in the chicken. *Nature 461*: 267-271.

Smith, C. A., K. N. Roeszler and A. H. Sinclair (2009). Genetic evidence against a role for W-linked histidine triad nucleotide binding protein (HINTW) in avian sex determination. *International Journal of Developmental Biology 53*: 59.

Smith, C. A., and A. H. Sinclair (2004). Sex determination: insights from the chicken. *BioEssays* 26: 120–132.

Stiglec, R., T. Ezaz and J. A. Marshall-Graves (2007). A new look at the evolution of avian sex chromosomes. *Cytogenetic and Genome Research* 117: 103–109.

Stiles, F. G., and A. F. Skutch (1989). A Guide to the Birds of Costa Rica. Cornell University Press, Ithaca, NY.

Suh, A. (2016). The phylogenomic forest of bird trees contains a hard polytomy at the root of Neoaves. *Zoologica Scripta* 45: 50-62.

Suh, A., L. Smeds and H. Ellegren (2015). The dynamics of incomplete lineage sorting across the ancient adaptive radiation of neoavian birds, *PLoS Biological Sciences* 13: e1002224.

Tae H. J., B. G. Jang, D. C. Ahn, E. Y. Choi, H. S. Kang, N. S. Kim, J. H. Lee, S. Y. Park, H. H. Yang and I. S. Kim (2005). Morphometric studies on the testis of Korean ring-necked pheasant (*Phasianus colchicus karpowi*) during the breeding and non-breeding seasons. *Veterinary Research Communications*. 29: 629-643.

Takagi, N., M. Itoh and M. Sasaki (1972). Chromosome studies in four species of ratites (Aves). *Chromosoma* 36: 281–291.

Takagi, N. and M. Sasaki (1974). A phylogenetic study of bird karyotypes. *Chromosoma* 46: 91–120.

Tingari, M. D. (1971). On the structure of the epididymal region and ductus deferens of the domestic fowl (*Gallus domesticus). Journal of Anatomy* 109: 423-435.

Tingari, M. D. (1972). The fine structure of the epithelial lining of the excurrent duct system of the testis of the domestic fowl (*Gallus domesticus*). *Quarterly Journal of Experimental Physiology* 57: 271-295.

Tingari, M. D. (1973). Observations on the fine structure of spermatozoa in the testis and excurrent ducts of the male fowl (*Gallus domesticus*). *Journal of Reproduction and Fertility* 34: 255-265.

Tribe, M. and F. Brambell (1932). The origin and migration of the primordial germ cells of sphenodon punctatus. *Quarterly Journal of Microscopy Science.* 75: 251-282.

Tsuda, Y., C. Nishida-Umehara, J. Ishijima, K. Yamada and Y. Matsuda (2007). Comparison of the Z and W sex chromosomal architectures in elegant crested tinamou (*Eudromia elegans*) and ostrich (*Struthio camelus*) and the process of sex chromosome differentiation in palaeognathous birds. *Chromosoma* 116: 159–173.

Tsunekawa, N., M. Naito, Y. Sakai, T. Nishida and T. Noce (2000). Isolation of chicken vasa homolog gene and tracing the origin of primordial germ cells. *Development 127*: 2741-2750.

Tullberg, B. S., M. Ah–King and H. Temrin (2002). Phylogenetic reconstruction of parental–care systems in the ancestors of birds. *Philosophical Transactions of the Royal Society of London, Series B: Biological Sciences 357*: 251-257.

Turek, N. K. (2007). Mechanisms and Motivations in Avian Copulation. MS Thesis. Idaho State University, Pocatello, ID.

Ukeshima, A. and T. Fujimoto (1978). Electron microscopy of primordial germ cells in early chick embryos. *Journal of Electron Microscopy 27:* 19-24.

Vakaet, L. (1970). Cinematographic investigations of gastrulation in the chick blastoderm. *Archives Biologie, Paris* 81: 387-426.

Vallisneri, M., A. Quaglia, A. M. Stagni and F. Zaccanti (1990). Differences between male and female protogonia in chick embryos before sex differentiation of the gonads. *Bollettino della Societa Italiana di Biologia Sperimentale* 66: 91–98.

van Tuinen, M. and S. B. Hedges (2001). Calibration of avian molecular clocks. *Molecular Biology and Evolution* 18: 206–213.

van Tuinen, M., C. G. Sibley and S. B. Hedges (1998). Phylogeny and biogeography of ratite birds inferred from DNA sequences of the mitochondrial ribosomal genes. *Molecular Biology and Evolution* 15: 370–376.

Varricchio D.J., F. Jackson., J. J. Borkowski and J. R. Horner (1997): Nest and egg clutches of the dinosaur *Troodon formosus* and the evolution of avian reproductive traits. *Nature* 385: 247–250.

Varricchio, D. J., F. Jackson and C. N. Trueman (1999). A nesting trace with eggs for the Cretaceous theropod dinosaur *Troodon formosus*. *Journal of Vertebrate Paleontology 19*: 91-100.

Venzke, W.G. (1954). The morphogenesis of the indifferent gonad of chick embryos. *American Journal of Veterinary Research* 15: 300-308.

Warwick, J. N. (2007). Assessing Functional Bilateral Asymmetry in Chukar (*Alectoris chukar*) Testes. MS Thesis. Idaho State University, Pocatello, ID.

Weller, M. W. (1976). Ecology and behaviour of steamer ducks. *Wildfowl* 27: 45-53.

Williams, G. C. (1966). Natural selection, the costs of reproduction, and a refinement of Lack's principle. *American Naturalist* 100: 687-690.

Xu, X. and M. A. Norell, (2004). A new troodontid dinosaur from China with avian-like sleeping posture. *Nature 431*: 838-841.

Yamomoto, S., H. Tamate and O. Itikawa (1967). Morphological studies on the sexual maturation in the male Japanese quail (*Coturnix japonica*) II The germ cell types and cellular associations during spermatogenesis. *Tohoku Journal of Agricultural Research* 18: 27-37.

Zaccanti, F., M. Vallisneri and A. Quaglia (1990). Early aspects of sex differentiation in the gonads of chick embryos. *Differentiation* 43: 71–80.

Zhang, S. O., S. Mathur, G. Hattem, O. Tassy and O. Pourquie (2010). Sex dimorphic gene expression and ineffective dosage compensation of Z-linked genes in gastrulating chicken embryos. *BMC Genomics* 11: 13.

Zhao, D., D. McBride, S. Nandi, H. A. McQueen, M. J. McGrew, P. M. Hocking, P. D. Lewis, H. M. Sang and M. Clinton (2010). Somatic sex identity is cell autonomous in the chicken. *Nature* 464: 237–242.

Zlotnick, I. (1947). The cytoplasmic components of germ-cells during spermatogenesis in the domestic fowl. *Quarterly Journal of Microscopical Science* 88: 353-365.

CHAPTER TWO

LEFT AND RIGHT TESTES WITHIN MALE CHUKAR (*ALECTORIS CHUKAR*) DIFFER FROM ONE ANOTHER IN COMPOSITION AND FUNCTION

ABSTRACT

Sperm production is central to avian reproduction and is an outcome of strong selection on male reproductive physiology and behavior. In birds, males of most species lack an intromittent phallus. Females of most species possess only a single (left) functioning ovary and oviduct. Males possess two functional testes, but in most species the left testis of males is larger than the right testis. Studies involving avian testicular function make a fundamental assumption that the left testis and right testis within individual male birds are compositional and functional analogues of one another. We tested this assumption using the testes of breeding adult male chukar (*Alectoris chukar*) and found it to be false. The left testis was not simply a larger analogue of the right testis. Rather, the left testis contains a higher proportion of spermatogenic cells per area of tissue than the right testis, whereas the total number of androgen-producing Leydig cells was equal between the testes despite the bilateral difference in size and mass. Chukar produce more sperm from their left testis, both due to greater size of the left testes relative to the right and also greater density of spermatogenic cells. We hypothesize this increased allocation of spermatogenic cells to the left testis is an effective adaptation for sperm production and also serves to deliver sperm efficiently to the female's left oviduct in the absence of an intromittent phallus.

INTRODUCTION

Sexual selection in birds results in modification of male traits including male morphology, physiology and sexual capacity. One consequence of the extreme anisogamy characterizing avian sexual reproduction is high variance in male reproductive success and strong selection on males to inseminate females effectively (Brown & Brown 1987), making sperm and androgen production by male birds central to avian reproduction. Studies involving avian testicular function make a fundamental assumption that the left and right testes within individual male birds are compositional and functional analogues of one another. We tested this assumption using chukar (*Alectoris chukar*, Order Galliformes), a Eurasian partridge, and found the assumption to be false. Here, we report that within male chukar, the left testis differs from the right testis in composition and function and not simply in size and mass.

All bird species employ internal fertilization exclusively. Evolutionarily, avian male reproductive morphology is characterized by the progressive loss of an intromittent phallus, i.e., a 'penis' that is inserted into the female's vagina during copulation. Among living birds, males of most species in the basal superorder Paleognathae (e.g., tinamous and ratites such as the ostrich, *Struthio camelus*) possess an intromittent phallus. Even within the Paleognathae, reduction and loss of an intromittent phallus occurs in a few species (Brennan *et al.* 2008). Within Neognathae, a group comprising >99% of living birds, the orders Anseriformes (waterfowl) and Galliformes (gamefowl) form a monophyletic basal clade collectively named Galloanserae. Male anseriforms possess an intromittent phallus. Male galliforms possess a small, non-intromittent phallus. The small galliform phallus is attached to the ventral aspect of the cloacal wall, but does not

penetrate the female vagina during copulation (King 1981a, Brennan *et al.* 2008). Barring a few species that have evolved phallus-like secondary structures, males of the remaining neognathous birds do not possess a phallus.

Clearly, an intromittent phallus is not essential to internal fertilization for most living birds (Gerhardt 1933, Briskie & Montgomerie 1997, Brennan *et al.* 2008). However, where it does occur, an intromittent phallus is thought to function in placing semen directly into the vagina of the female. Sperm within the lumen of the vagina is well positioned to be stored in sperm storage ducts which line the vaginal wall or to be transported up the oviduct to the infundibulum where fertilization occurs. In species without an intromittent phallus, presumably there are factors influencing sperm placement within the female to ensure these pre-fertilization functions.

Like other male vertebrates, male birds possess two testes. The testes are internal and bilateral, with the left and right testes located anterior-proximal to the left and right kidney, respectively. Importantly, male birds retain the primitive condition of having two ejaculatory orifices called seminal papillae (King 1981b), with one papilla serving the left testis and one serving right testis. On each side, sperm produced by the testis is transferred via a vas deferent duct to a seminal vesicle located within the wall of the urodeum of the cloaca. Seminal vesicles store mature sperm prior to ejaculation. Each seminal vesicle terminates distally as a seminal papilla and each papilla projects into the lumen of the urodeum. It is assumed, but untested, that during ejaculation semen is emitted from both the left and the right papillae.

Male birds characteristically emit a small volume of ejaculate (Anderson *et al.* 2002) relative to similarly sized mammals because unlike mammalian ejaculate, avian

ejaculate does not contain high volumes of accessory fluids. Female birds receive a small, viscous drop of semen (Fujihara 1992). Placement of semen at the vaginal opening of the oviduct increases the probability of fertilization (Biellier *et al.* 1961, Ogasawara & Rooney 1966).

Female birds typically have asymmetrical reproductive tracts, with the most common condition being a single functional ovary and oviduct located on the female's left side (King 1981b, Lake 1981). One consequence of this anatomical asymmetry is that the opening to the female's oviduct is located to the left of the midline of the urodeum of the female cloaca (King 1981b). Presumably, male birds without an intromittent phallus seek to ejaculate semen onto the small, off-center oviduct opening, access to which is under female control.

Female birds commonly mate with multiple males (Griffith *et al.* 2008) resulting in sperm competition (Birkhead *et al.* 1999) within the female. Evolutionarily, this cryptic sexual selection should act strongly on males to copulate in manner that most effectively transfers gametes to females when the opportunity arises.

The avian testis is a typical vertebrate testis insofar as it is a compound organ composed of germ cells which produce sperm and endocrine cells which produce androgens. Germ cells arise from germinal epithelium of seminiferous tubules which comprise most of the testis volume and mass. Spermatogenesis occurs within the seminiferous tubules. Upon maturation, sperm cells are advanced into the epididymis of each testis for storage prior to transport to seminal vesicles for ejaculation. At each stage, spermatic cells are contained within the tubule system and physically are segregated from the endocrine system of the testis by tubule walls. Endocrine cells (Leydig cells) occupy

interstitial spaces between the seminiferous tubules and produce androgens such as testosterone that are circulated throughout the body via blood. Precursors to germinal cells and precursors to endocrine cells differ in developmental origin and colonize the undifferentiated primordial gonad independently.

The separate embryological origins of the tissues that carry out gamete production versus androgen production means that evolutionary selection on one of these functions need not act on the other function. That is, sexual selection on sperm production within the left and right testes of males need not imply concordant selection on androgen production. In the avian family Maluridae, overall testis size is greater in species with higher incidence of sperm competition, and the increased size is a result of increased proportion of spermatogenic tissues, not equal increases of both endocrine and gametic tissues (Rowe & Pruett-Jones 2011).

Given inherent asymmetry of reproductive tracts of females and the bilateral ejaculatory papillae of males, evolution of male reproductive anatomy in response to sexual selection need not be symmetrical between left and right sides of males. If true, the bilateral testes may not be precise analogues of one another. Indeed, greater colonization by germ cell precursors into the left primordial testis than the right testis previously has been observed (Zaccanti *et al.* 1990, Naito *et al.* 2009). Males in many bird species exhibit a high degree of bilateral asymmetry in testis size and mass. The nearly universal pattern is for the left testis to be larger and heavier than the right testis, although in the coucals (Maurer 2007, Frey & Goymann 2009) the right testis is larger and heavier.

Three historical arguments seek to explain bilateral asymmetry of avian testes. (1) Asymmetry results from one testis being reduced evolutionarily to promote flight efficiency (Venzke 1954). (2) Male asymmetry is a carry-over effect of selection on females for left-bias in reproductive tracts that arose as an adaptation for flight efficiency in females (Kimball *et al.* 1997). (3) One testis has been reduced to decrease testosterone production to promote male parental care or to prevent deleterious physiological effects of excessive testosterone production in testes otherwise enlarged for sperm production (Wingfield *et al.* 1990, Møller 1994, Ligon 1997). In this scenario, male birds face a trade-off between increased testes mass to achieve paternity through high sperm production and high testosterone production that might inhibit male parental care, reducing survivorship of young secured through increased sperm production. This argument assumes that elevated testosterone production is an inevitable outcome of increased testis size.

Recognizing any bilateral asymmetry in avian testis composition has evolutionary, physiological, and conservation implications. Studies on avian testes assume that the left and right testes are analogues, despite size differences, and cytological measures often average the results from the left and right testes (Rowe & Pruett-Jones 2011, Calhim & Montgomerie 2015). Research investigating the physiological relationship between sperm production and testosterone production in birds employ protocols in which one testis is used to measure sperm production per unit testis and the opposite testis is used to measure androgen production per unit testis under the assumption that *sperm production:androgen production* is constant between left and right testes (Tae *et al.* 2005). Artificial insemination, which relies on harvesting sperm from

males, is used in the production of birds of commercial importance such as the domestic turkey (*Meleagris gallopavo*) and is increasingly being employed for zoological conservation (Lierz *et al.* 2013). To the extent that sperm production and harvest assumes that testes within a male bird are compositional analogues of one another, artificial insemination procedures are subject to potential error if left and right testes actually differ compositionally from one another.

To better understand bilateral asymmetry of avian testes, we measured and compared the spermatogenic, endocrine, and morphological characteristics of left and right testes within individual male chukar captured from the wild. The chukar is a monogamous, monomorphic, and biparental partridge within the order Galliformes native to Eurasia and widely introduced to the arid mountains of the American west. Male chukar possess a non-intromittent phallus, with separate seminal papillae to deliver sperm from the left and right testes. Previously, chukar were shown to exhibit left-biased testicular asymmetry in size and mass during the breeding season and a left-side bias in copulation (Delehanty & O'Hearn 2005) and it was hypothesized that the larger left testis and its deferent duct and ejaculatory papilla were especially well suited to deliver sperm to the left-side oriented female reproductive tract.

To test if left and right avian testes are analogues of one another, we measured year-round differences in left and right testis size and mass to assess inherent testis morphological asymmetry. We then quantified spermatogenic cell and Leydig cell densities of the left testis and right testis within individual males during the breeding season when testis activity is greatest. Lastly, we quantified whole testis sperm production per unit testis mass during the breeding season to quantify any side-bias in

sperm production in addition to the side-bias that would result simply from one testis being larger than the other.

METHODS

We measured four aspects of testicular form and function in adult male chukar: (1) the morphological asymmetry between the left testis and the right testis across the annual cycle of testicular recrudescence and regression; (2) left versus right sperm production capacity as represented by spermatogonia, spermatocyte, and spermatid densities within testes during the breeding season; (3) left versus right testis androgen production capacity as represented by Leydig cell density during the breeding season; and (4) left versus right testis overall production of sperm as represented by counts of mature secondary spermatids taken from whole testes collected during the breeding season.

Capture and handling of chukar (*Alectoris chukar*) previously has been described in detail (Delehanty and O'Hearn 2005, Warwick 2007) and was approved by the Idaho State University Animal Care and Use Committee protocols 0302439, 0605589, and 02142011. In August and September 2004, we captured wild chukar, individually marked them with uniquely numbered leg bands, and transferred them to $64m^2$ communal pens at an outdoor aviary at Idaho State University (Pocatello, ID). Prior to the onset of the study, we segregated male and female chukar and placed them in replicated, adjacent $64m^2$ pens that allowed sexes to see and hear one another but not to interact physically. Food and water were provided *ad libitum* throughout the study. For all portions of this study, we held male chukar in captivity for at least one year to ensure that each male had experienced at least one annual recrudescence/regression cycle prior to measurement.

Annual Morphological Asymmetry between Left and Right Testes

To assess if differences in left versus right testes persist outside the breeding season, we measured testis morphology across the annual cycle of testicular recrudescence and regression. We euthanized 2 male chukar per week from March 2006 through February 2007. Males were dissected immediately following euthanasia. Using handheld calipers, we measured length (mm) and width (mm) of the left and right testis at the longest and widest point *in situ*. We then removed each testis and measured length and width *in vitro* and wet mass (mg).

We analyzed the presence of bilateral asymmetry between left and right testis length, width, and mass using paired t-tests. Each data pair consisted of values from the left testis and the right testis from within an individual male. We used linear regression to evaluate the degree to which relative differences between the left and right testes were constant throughout the entire year. We also used regression to analyze differences between left versus right testis mass, length, and width across time.

Spermatogenic Cell Abundances in Left versus Right Testes

To measure the histological composition of sperm producing tissue in left versus right testes within sexually mature male chukar, we euthanized 4 adult male chukar on 21 June 2007. We bisected each fresh testis anterior to posterior along the ventral midline, leaving the tissue intact at the hilus, before progressively fixing the tissue through immersion for 5-10 min in neutral buffered formalin followed by 2-8 h immersion in 10% sucrose in phosphate-buffered saline (PBS) solution, followed by 8-24 h immersion in 30% sucrose PBS solution. When fully fixed, the testis sample was either processed and imaged immediately, or stored in 70% ethanol (ETOH) until imaged.

To image testis sections, we imbedded randomly selected ½ cm^2 testis subsamples in Tissue-Tek® O.C.T (optimum cutting temperature) Compound (Sakura Finetek, Torrance, CA) and froze the subsamples at -80° C. Subsamples then were brought to a cryostat temperature of -23 ° C for 24 h prior to sectioning. For each testis, we used 3 randomly selected testis subsamples, one from the anterior third, one from the middle third, and one from the posterior third of the testis. We cut sections to 10 μm using a Leica cryostat, and placed sections on precleaned glass slides.

For histological imaging and analysis, we stained slides with propidium iodide (PI) in glycerol. The propidium iodide powder was first diluted to 1 mg/ml in glycerol and stored protected from light at 4° C. To stain testes, a small amount of PI was freshly mixed to 1.5 μg diluted PI per ml glycerol. All diluted PI was protected from light within an aluminum foil wrapped amber bottle until used, and was used within 48 h of mixing. Stained slides were kept protected from light until imaged, and all were imaged within 24 h of staining.

We created digital images of seminiferous tubules using 400X magnification with a Leica™ DMRB compound microscope and SPOT digital microscope camera (Diagnostic Instruments, Sterling Heights, MI). We imaged 5 seminiferous tubules per section, for a total of 15 images within each testis. Seminiferous tubules were located haphazardly from tissue sections, but we used seminiferous tubules that were sectioned as orthogonal cross-sections with clearly visible cellular components.

We measured tubule diameter and circumference, and counted three types of spermatogenic cells from each imaged tubule (Figure 2-1). Using a high resolution, 24-inch, (60.96cm) touch-screen monitor, we measured the diameter and area in cross

section of seminiferous tubules, and counted the pooled total of spermatogonia (diploid) plus spermatocytes (primary, diploid; secondary, haploid); round primary spermatids (haploid); and elongate secondary spermatids (haploid) within each testis at 400X magnification. Using Image-J (NIH, Bethesda, MD) software (Abramoff *et al.* 2004, Rasband 2014), we overlaid a grid of 5000 μm^2 (3.83 pixels/ μm^2) line grid over the tubule. The grid could not be "randomly offset" due to unrepeatability. We created a polygon using the 'polygon' tool within image-J and generated a horizontally orientated polygon using the area of the grid closest to the midline of the tubule such that the top and bottom of the area to be counted was bordered by gridlines, and the sides encompassed the borders of the tubule. If two opposing edges of the tubule were not visible on the slide, or there were visual imperfections within the horizontal polygon, a vertical polygon as close to midline as possible was generated and used in the same manner. We calculated the area of each polygon using the 'area measure' function within Image-J. Within this area, we counted all spermatogonia and spermatocytes; round primary spermatids; and elongate secondary spermatids. Cell type was identified by size and shape (Figure 2-1).

We analyzed the difference in the numbers of diploid spermatogenic cells (spermatogonia + spermatocytes), round primary spermatids and elongate secondary spermatids, respectively, within the left testis and right testis of individual male chukar using paired-t-tests in which data pairs consisted of values from the left testis versus the right testis of individual male chukar.

Leydig Cell Abundance in Left Testis versus Right Testis

To quantify androgen-producing Leydig cells within each testis, we collected testes from 16 male chukar between 10 April and 19 June 2007 when testis mass was greatest based on the testis recrudescence/regression curve developed from testis morphology measurements taken across the annual cycle (Figure 2-2).

Testes were frozen, unfixed, prior to sectioning. We sectioned testes using a Leica cryostat held at -20°C. To standardize the region of each testis being analyzed, testes were thawed and bisected along the transverse plane, the anterior half affixed to a microtome mounting plate, and the first 25% sectioned and discarded. We then generated 20 sections of 12µm thickness. We placed these sections on subbed slides and allowed them to dry prior to staining.

We stained testis sections using a method first developed for mammalian testis tissues (Weibe 1976) to contrast Leydig cells (blue) with the other tissues (not blue) of the testis and adapted (Warwick 2007) for use on chukar (Figure 2-3). We stained samples for 120 min at room temperature using 36 ml 0.1 M Na-K Phosphate buffer (pH 8.0), 1.8 ml 0.138 µmol pregnenolone (Sigma-Aldrich, St. Louis, MO) in methanol, 2.25 ml 0.612 µmol nitroblue tetrazolium (NBT) (Thermo-Fisher Scientific, Waltham, MA) in Na-K buffer, and 4.5 ml 6.825 µmol NAD (Sigma-Aldrich, St. Louis, MO) in dH_20. After staining, the slides were washed with deionized water, allowed to dry completely and coverslips were mounted with Permount (Thermo-Fisher Scientific, Waltham, MA).

We imaged testis sections at 100x magnification using a Leica DMRB compound light microscope and obtained digital images using a SPOT digital microscope camera (Diagnostic Instruments, Inc., Sterling Heights, MI). Three digital images were taken of

four different randomly selected sections for each testis, for a total of 12 images per testis. Images were saved as raw TIFF files for later analysis (Figure 2-3).

We analyzed the TIFF file images of testis sections using the IPLab (Scanalytics, Inc, Milwaukee, WI) segmentation function. A separate segmentation threshold was determined for each chukar within IPLab, but thresholds were not varied between testes images from within the same chukar. IPLab generated the percent of the "region of interest" (ROI) stained. The ROI consisted of the entire field of view of a digital image analyzed by IPLab's segmentation tool. Thus, the percent region of interest (%ROI) represented the percent of pixels within the ROI that fell within segmentation colorimetric thresholds and thereby quantified the presence of Leydig cells within testis subsamples. We could then compare the %ROI blue between the left testis and right testis of individual males and determine the relative abundance of Leydig cells between left and right.

We also calculated the actual area of each section comprised of Leydig cells using an electronic caliper to measure to 0.01 mm the length and width of each section that had been digitally imaged. We calculated the area of each section using the equation for area of an ellipse [$(A = \pi (0.5D1) (0.5D2)$], where D1 and D2 represent section length (median-sagittal) and width (transverse), respectively. We then calculated the absolute abundance of Leydig cells within a given testicular section by multiplying %ROI blue by the total area of the section.

Differences in the % of testicular cells comprised of Leydig cells, as determined by the % ROI stained blue were analyzed using PROC MIXED maximum likelihood

procedures (SAS Institute Inc, Cary, N.C.). Comparisons of the % ROI in the left and % ROI in the right were analyzed by linear regression.

Sperm Production in Left Testis versus Right Testis

We measured sperm production in left versus right testes in 30 mature chukar euthanized on 21 June 2007, at maximal testicular recrudescence. We removed testes immediately upon euthanasia, trimmed of excess tissue and removed the epididymis, measured length, width and mass. We froze testes individually in liquid nitrogen and labeled and stored them at -80^{o} C until measurement. For measurement (Kirby *et al.* 1996, Kim & Yang 2000) we thawed and weighed each testis, placed it in an 236.6 ml (8 ounce) blender jar with 100 volumes (weight(g):volume(ml)) of 0.9% saline solution with 0.05% Triton 100 and 100 ppm merthiolate and homogenized it using 4-5 pulses of 30 s each in an Oster 6640 blender. Using 20 µl samples of homogenate placed on an improved Neubauer hemocytometer, we counted sperm cells (secondary spermatids) visually with a light microscope at 400X magnification (Figure 2-4). Following World Health Organization (WHO 1992) recommended procedures for high sperm counts, we counted sperm cells within 5 of the 25 0.04 mm^2 squares forming the central grid of the hemocytometer, using stratified random sampling. This procedure was performed four times per testis and measurements. To estimate spermatid concentration, we summed the 5 central grid square counts and calculated the concentration per mL of homogenate as:

Sperm concentration mL^{-1} = total spermatic count in 5 squares x 50,000 x dilution factor (1:100)

and estimated total sperm production as:

Sperm production of testis = Sperm concentration mL^{-1} x total volume of testis homogenate.

We analyzed bilateral asymmetry in spermatid concentration and total sperm production of the left testis and right testis of individual males using 2-tailed paired t-tests. Data pairs consisted of left testis versus right testis values for each male.

RESULTS

Annual Morphological Asymmetry between Left and Right Testes

In male chukar, the left-larger asymmetry persisted throughout the annual cycle (Figure 2-2). Mean left and right testes masses during quiescence was 0.069 ± 0.01g (n = 16) and 0.040 ± 0.01g (n = 16), respectively. By early April, the left testis had reached a mean peak mass of 1.51 ± 0.10g (n = 16) and the right had reached a peak mass of 1.01 ± 0.08g (n = 16). Measures of length and width were highly correlated with mass (Table 2-1).

Testis mass changed with seasons, including a smooth, rapid recrudescence at the onset of the breeding season, and regression of testis mass following the breeding season (Figure 2-5). The proportional mass difference between left and right testes mass was constant year round, as indicated by the absence of a significant regression of the right/left testes masses by ordinal date (R^2 = 0.016, t = -1.25, $d.f.$ = 98, p = 0.213; Figure 2-6). In chukar, right testis mass was consistently approximately 67% of left testis mass regardless of state of recrudescence. Relative (right/left) testes length *in situ* increased slightly across the year (R^2 = 0.059, t = 2.47, $d.f.$ = 98, p = 0.015; Figure 2-7). Relative (right/left) *in situ* width was constant across the year (R^2 = 0.012, t = -1.08, $d.f.$ = 98, p = 0.284; Figure 2-8).

Analysis of 16 male chukar during the peak of the breeding season (Table 2-1) showed the same pattern as the circannual pattern (Table 2-2). Left testis mass was

significantly greater than right testis mass (t = 10.25, $d.f.$ = 15, $p < 0.001$), left testis length exceeded the right (t = 6.75, $d.f.$ = 15, $p < 0.001$), and left testis width exceeded the right (t = 8.94, $d.f.$ = 15, $p < 0.001$). In all 16 male chukar sampled, left testis mass was greater than right testis mass.

Spermatogenic Cell Abundances in Left versus Right Testes

Cytological analysis of spermatogenic cells within the testis of chukar demonstrated that the left testis produced a higher quantity of spermatozoa. Mean cross-sectional areas of the left testis seminiferous tubules was significantly greater than the right testis tubules (paired-t test, t = 2.773, $d.f.$ = 3, p = 0.048) within individual males. Also, left testis tubules contained larger numbers of spermatogenic cells per unit area (μm^2) than tubules comprising the right testis. We were unable visually to distinguish diploid spermatogonia (spermatogonial stem cells) from diploid spermatocytes using fluorescence microscopy. We visually distinguished three classes of spermatogenic cell morphology: (1) the pooled cell count of spermatogonia and spermatocytes; (2) round haploid primary spermatids; and (3) elongate haploid secondary spermatids.

The seminiferous tubules within the left testis contained more pooled spermatogonia and spermatocytes per unit area than the tubules in the right testis (paired-t test, t = 3.828, $d.f.$ = 3, p = 0.031). Left testis tubules also contained higher densities of primary spermatids than right testis tubules (paired=t test, t = 7.936, $d.f.$ = 3, p = 0.004), and higher densities of secondary spermatids (paired-t test, t =3.398, $d.f.$ = 3, p = 0.042) than tubules in the right testis (Table 2-3).

When the greater mass and size of the left testis versus the right testis are coupled with the intrinsic differences in sperm production per unit area in left versus right testes, we calculate the left testis of chukar produces greater than 75% of total sperm.

Leydig Cell Abundance in Left versus Right Testes

Leydig cell analysis indicated that the left-larger size asymmetry in chukar testes could not be attributed to increased presence of endocrine tissues. The smaller right testes had a significantly higher percentage of their tissues made up of Leydig cells than did left testes ($F_{15, 367} = 7.29$, $p = 0.007$; Figure 2-9).

A regression of the left testis %ROI stained versus right %ROI stained indicated that the compositional difference was non-random, meaning that the right testis was not simply a small analogue of the left. The relationship of %ROI stained in the left versus right testis was significantly different from zero ($R^2 = 0.30$, $t = 2.45$, $d.f. = 15$, $p = 0.028$) and also significantly different from 1 ($R^2 = 0.30$, $t = 3.47$, $d.f. = 15$, $p < 0.01$) (Figure 2-10).

Importantly, the absolute area comprised of Leydig cells did not differ between left and right testes within a given male ($F_{15, 367} < 0.00$, $p = 0.965$; Figure 2-11). *Post hoc* analyses indicated that relationships of Leydig cell proportion and abundance between the left and right testes could not be explained simply by testis mass. The %ROI stained was not a function of mass *per se.* (left testis: $F_{14, 176} = 1.84$, $p = 0.177$; right testis: $F_{14, 176} = 0.05$, $p = 0.824$; Figure 2-12). Nor did *post hoc* analysis of the pooled effect of mass on the percent stained for the right and left testes indicate that mass determined percent stained ($F_{15, 367} = 0.36$, $p = 0.549$). The mass of the left testis was larger while the

percent of the testis tissue comprised of Leydig cells was lower in the left. The absolute presence of Leydig cells did not differ between left and right testes (Table 2-4).

Sperm Production in Left versus Right Testes

Total spermatid counts of homogenized testis using a hemocytometer from 29 male chukar also indicated greater sperm production per unit mass from the left testis than the right testis (paired-*t* test, $t = 0.091$, $d.f. = 57$, $p = 0.011$). Here, the left testis contributed approximately 61% of the total counted spermatids (Figure 2-13).

DISCUSSION

We tested the hypothesis that the asymmetrical left and right testes within chukar were simple analogues of one another, only differing in total size and mass. Chukar do exhibit bilateral asymmetry in testis size that persists throughout annual cycles of regression and recrudescence. However, we found the assumption that the testes were simple analogues to be false. Instead, the larger left testis contains larger seminiferous tubules and more spermatogenic cells per unit area than the small right testis. The left and right testes, however, contain equal total Leydig cell abundance. In other words, the larger left testis augments sperm production per se both through its greater size and the greater density of spermatogenic tissue while the left and right testes remain equal in androgen production. There appears to be selection for increased sperm production from the left testis. Endocrine tissues are from different embryologic origins than spermatogenic cells. Testosterone produced from these endocrine tissues are secreted into the bloodstream and circulated throughout the body, and is probably under independent selection forces from spermatogenic tissues.

We have found that, in chukar, the left and right testes maintain asymmetry throughout the annual cycle of recrudescence and regression, and that the asymmetry is not simply a function of differing mass, but a significant compositional difference between right and left testes exists. To our knowledge, this is the first time this has been found in birds, and potentially has significant ramifications to avian and endocrinological research. Throughout the year, the right testis is approximately 2/3 the mass of the left testis. However, our investigations found that the absolute amount of Leydig cells is statistically equal between the left and the right testis within an individual. This indicates that, although the testes are different in size, androgen production capacity is equal between left and right testes.

In contrast, the left testis produces substantially more spermatogenic cells than the right testis. Not only is there a size difference, but the tissues of the left testis exhibit a greater density per unit area than the right testis. The mean area of tubules in cross section in the left testis is significantly larger than tubules in the right testis. We found that the left tubules contained larger numbers of spermatogenic cells than the right, per unit area (per μm^2). The seminiferous tubules within the left testis contained more spermatogenic cells per unit area than the tubules in the right testis, which produced more spermatids than tubules in the right testis (Table 3). Finally, we found, significantly greater secondary spermatid production from the left testis in sperm counts of homogenized testis.

The left testis is functionally significantly different from the right testis. The androgen component of left and right testes is equal, but the left testis is greatly enhanced for sperm production. The increased size and mass of the left testis is comprised

predominantly of spermatogenic tissues, and the spermatogenic tissues on the left produce more sperm per unit area than the right testis.

The balanced androgen capacity and asymmetrical spermatogenic capacity are consistent with the mode of action of these two functions even under strong, cryptic sexual selection. Androgens enter the blood and circulate within the individual, acting on androgen-sensitive tissues. Testosterone, for example, need not be produced in the left testis for it to act on the left testis, and presumably, testosterone acting on sperm production is being delivered by blood, primarily, not diffusing from Leydig cells to the tubule environment. Sperm, on the other hand, serves its function by being expelled from the body to inseminate a female. Two cryptic selection forces would promote asymmetry: (1) a biomechanical advantage to emitting sperm from the left ejaculatory papilla such as might occur with female reproductive tract asymmetry, (2) greater sperm production efficiency in larger testes. In this scenario, birds subject to sperm competition may gain greatest sperm production capacity by investing into just one testis.

Our finding that the left and right testes are not functional analogues, but the left testis is producing more sperm per unit area, while the smaller right testis contains a greater density (but not total presence) of androgen producing Leydig cells means that studies that use one testis to calculate gametic production and the companion testis to calculate endocrine production may inadvertently skew assessment of the relationship between sperm production and androgen production in birds with asymmetrical testis.

These findings also provide strong evidence that the cytologically and functionally different left and right testes of birds are consistent with sexual selection resulting in greater sperm production from the left side than the right side. Circulating

androgens are not subject to pressures resulting in side asymmetry. However, in response to the female left-of-center vaginal opening of the oviduct, significant selection pressures on males to efficiently place sperm in proximity to this opening likely influences the development of spermatogenic tissues in the testes. Previous work has shown a behavioral left-side bias by males in copulation in ostrich (Lake 1981), tree swallows (*Tachycineta bicolor*, Peterson *et al.* 2001), house sparrows (*Passer domesticus*, Nyland *et al.* 2003), and chukar (*Alectoris chukar*, Delehanty & O'Hearn 2005) and more recently, greater sage grouse (*Centrocercus urophasianus*), great-blue herons (*Ardea herodias*), American avocets (*Recurvirostra americana*) and Swainson's hawk (*Buteo swainsoni*) have been observed to copulate more frequently from the left side of the female (Turek 2007).

Seemingly, left-side copulation is providing better access to the left-of-midline oviduct and male birds are under strong sexual selection to copulate effectively. This study shows that the testes of males are perhaps also under cryptic selection pressures to deliver sperm from the left testis as a biomechanical adaptation to the position of the female oviduct.

LITERATURE CITED

Abramoff, M. D., P. J. Magalhaes and S. J. Ram (2004). Image processing with ImageJ. *Biophotonics International* 11: 36-42.

Anderson, S. J., D. M. Bird and M. D. Hagen (2002). Semen characteristics of the Quaker parakeet (*Myiopsitta monachus*). *Zoo Biology* 21: 507-512.

Biellier, H.V., R. Pashcange and M. Funk (1961). Effect of depth of insemination on fertility of broad breasted bronze turkey hens. *Poultry Science* 40: 1379.

Birkhead, T. R., J. G. Martínez, T. Burke, and D. P. Froman (1999). Sperm mobility determines the outcome of sperm competition in the domestic fowl. *Proceedings of the Royal Society of London, Series B: Biological Sciences* 266: 1759-1764.

Brennan, P. L. R., T. R. Birkhead, K. Zyskowski, J. van der Waag and R. P. Prum (2008). Independent evolutionary reductions of the phallus in basal birds. *Journal of Avian Biology* 39: 487-492.

Briskie, J. V. and R. Montgomerie (1997). Sexual selection and the intromittent organ of birds. *Journal of Avian Biology* 28: 73-86.

Brown, A. K. and J. H. Brown (1987). Anisogamy, sexual selection and the evolution and maintenance of sex. *Evolutionary Ecology* 1: 95-105.

Calhim S. and R. Montgomerie (2015). Testis asymmetry in birds: the influences of sexual and natural selection. *Journal of Avian Biology* 46: 175-85.

Delehanty D. J. and P. P. O'Hearn (2005). Behavioral and morphological asymmetries in chukar *Alectoris chukar* copulation. *Journal of Avian Biology* 36: 276-279.

Fujihara, N. (1992). Accessory reproductive fluids and organs in male domestic birds. *World's Poultry Science Journal* 48: 39-56.

Gerhardt, U. (1933). Kloake und Begattungsorgane. Pp 302 – 313 In [Bolk, L., E. Goppert, E. Kallius and W. Lubosch] Hanbuch der vergleichenden Anatomie der Wirbeltiere. Urban and Schwarzenberg, Berlin.

Griffith, S. C., I. P. F. Owens and K. A. Thuman (2008). Extra pair paternity in birds: a review of interspecific variation and adaptive function. *Molecular Ecology* 11: 2195-2212.

Kimball, R. T., J. D. Ligon and M. Merola-Zwartjes (1997). Testicular asymmetry and secondary sexual characters in red junglefowl. *The Auk* 114: 221-228.

King, A. S. (1981a). Phallus. pp. 107-148 *In* [A. S. King and J. McLelland eds.] Form and Function in Birds. Vol. 2. Academic Press, New York.

King, A. S. (1981b). Cloaca. pp. 63-106. *In* [A.S. King and J. McLelland eds.] Form and Function in Birds. Vol 2. Academic Press, New York.

Lake, P. E. (1981). Male Genital Organs. P1-61 *In:* A. S. King and J. McLelland [eds.] Form and Function in Birds. Vol. 2. Academic Press, New York.

Lierz, M., M. Reinschmidt, H. Müller, M. Ink, and D. Neumann (2013). A novel method for semen collection and artificial insemination in large parrots (Psittaciformes). *Scientific Reports 3*.

Ligon, J. D. (1997). A single functional testis as a unique proximate mechanism for promoting sex-role reversal in coucals. *The Auk* 114: 800-801.

Maurer, G. (2007). Love is in the air: Arboreal copulations in the pheasant coucal *Centropus phasianus*. *Northern Territory Naturalist* 19: 48-50.

Møller, A.P. (1994). Directional selection on directional asymmetry: testes size and secondary sexual characters in birds. *Proceedings of the Royal Society of London, Series B: Biological Sciences* 258: 147-151.

Naito, M., T. Minematsu, T. Harumi and T. Kuwana (2009). Preferential migration of transferred primordial germ cells to left germinal ridge of recipient embryos in chickens. *Journal of Poultry Science* 46: 40-45.

Nyland, K. B., M. P. Lombardo and P.A. Thorpe (2003). Left-sided directional bias of cloacal contacts during house sparrow copulations. *Wilson Bulletin* 115: 470-473.

Ogasawara, F. X, and W. F. Rooney (1966). Artificial insemination and fertility in Turkeys. *British Poultry Science* 7: 77-82.

Peterson, A. A., M. P. Lombardo, and H. W. Power (2001). Left-sided directional bias of cloacal contacts during tree swallow copulations. *Animal Behavior* 62: 739-741.

Rasband, W.S. (2004). ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2014.

Turek, N. C. (2007). Mechanisms and Motivations in Avian Copulation. MS Thesis, Idaho State University, Pocatello, ID.

Venzke, W.G. (1954). The morphogenesis of the indifferent gonad of chick embryos. *American Journal of Veterinary Research* 15: 300-308.

Warwick, J. N. (2007). Assessing functional bilateral asymmetry in chukar (*Alectoris chukar*) Testes. MS Thesis, Idaho State University, Pocatello, ID.

Wiebe, J. P. (1976). Steroidogenesis in rat Leydig cells: changes in activity of 5-ane and 5-ene 3β-hydroxysteroid dehydrogenases during sexual maturation. *Endocrinology* 98: 505.

Wingfield, J. C., R. E. Hegner, A. M. Dufty and G. F. Ball (1990). The "Challenge Hypothesis": Theoretical implications for patterns of testosterone secretion: mating systems, and breeding strategies. *The American Naturalist* 136: 829-846.

Wingfield, J. C., C. M. Vleck and M. C. Moore (1992). Seasonal changes in the adrenocortical response to stress in birds of the Sonoran Desert. *Journal of Experimental Zoology* 264: 419–428.

World Health Organization (1992). WHO laboratory manual for the examination of human semen and sperm-cervical mucus interaction. 3rd Edition. Cambridge University Press.

Zaccanti F., M. Vallisneri, and A. Quaglia (1990). Early aspects of sex differentiation in the gonads of chick embryos. *Differentiation* 43: 71-80.

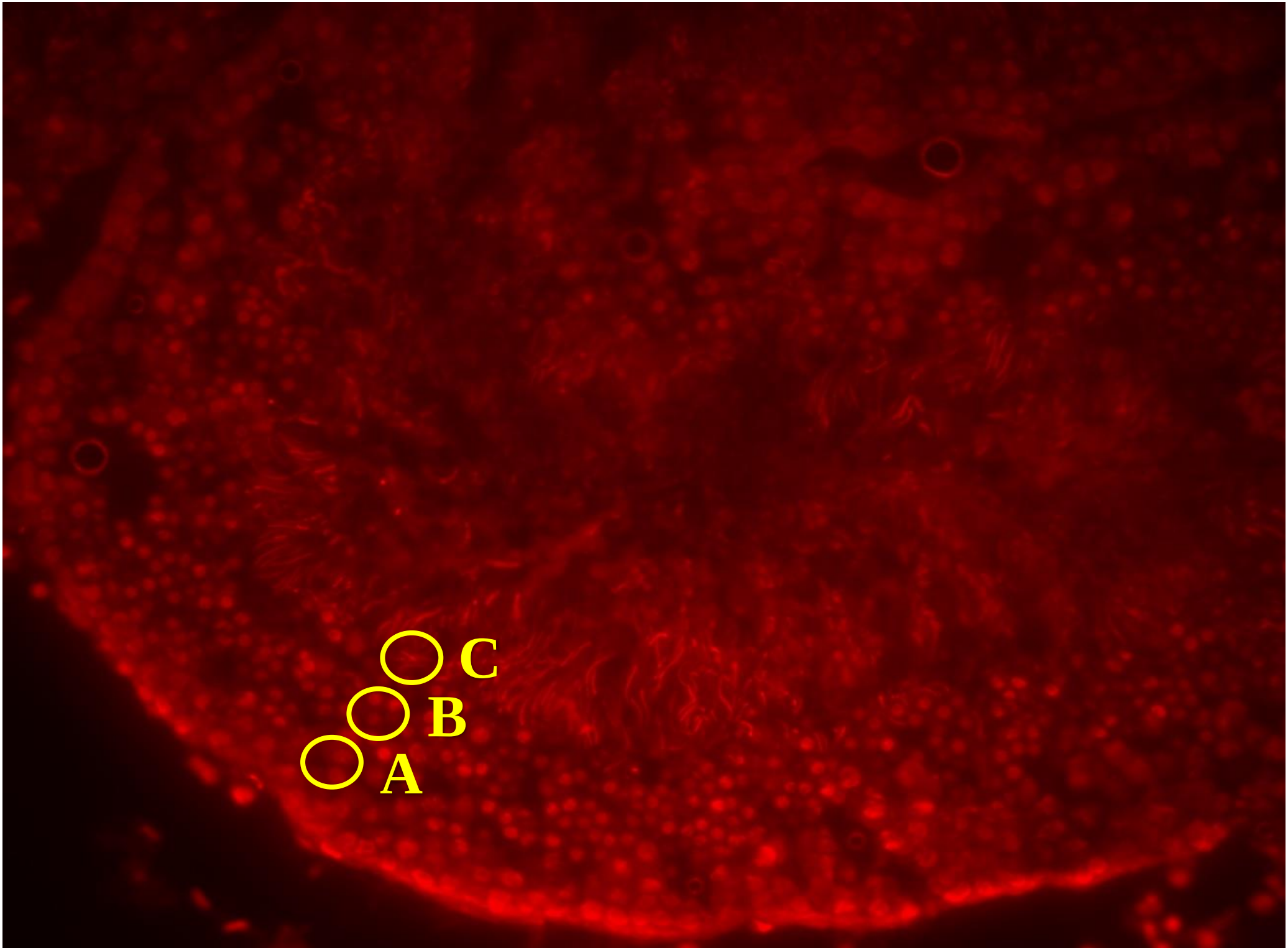
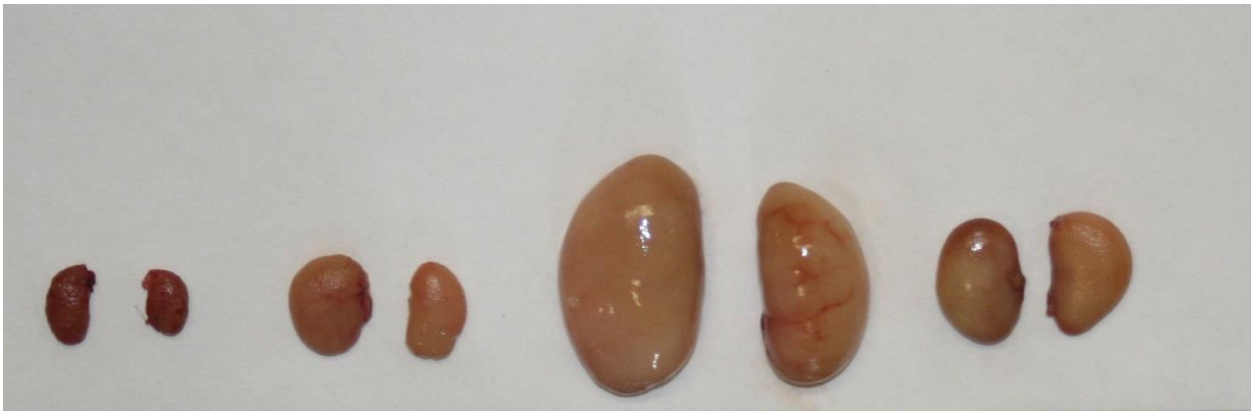

C
B
A

Figure 2-1: Cross section of seminiferous tubule stained with propidium idodide and imaged at 400X. We categorized three types of spermatogenic cells: A. Pooled spermatogonia and spermatocytes, B. round primary spermatids, and C. elongate secondary spermatids.



Maximal regression	Early recrudescence	Maximal recrudescence	Early regression
Oct-Feb	Mid Feb	Early Apr	Late Jun

Figure 2-2: Left and right testes, respectively, of 4 individual male chukar taken at stages of the annual cycle of recrudescence and regression. Chukar testes began recrudescing in mid-February, and by early April had reached peak mass. Regression of testes began at the end of June, and by the end of October testes had again reached quiescent state.

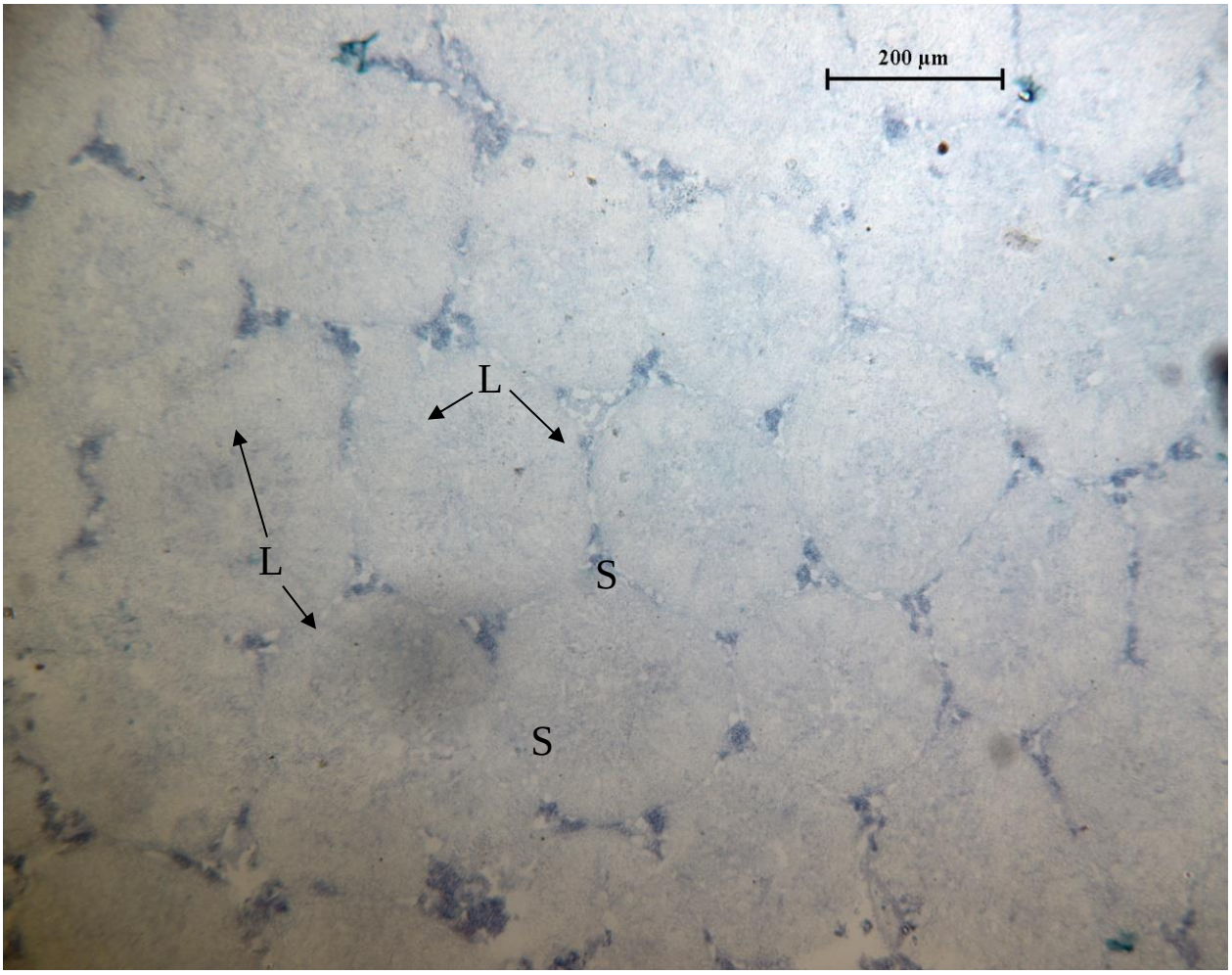


Figure 2-3. 100X image showing Nitroblue Tetrazolium (NBT) stained interstitial tissue (Leydig cells) of the chukar testis from an adult male chukar at maximal testicular recrudescence. S indicates seminiferous tubules, L refers to Leydig cell and arrows point to concentrations of Leydig cells (blue) in interstitial spaces.

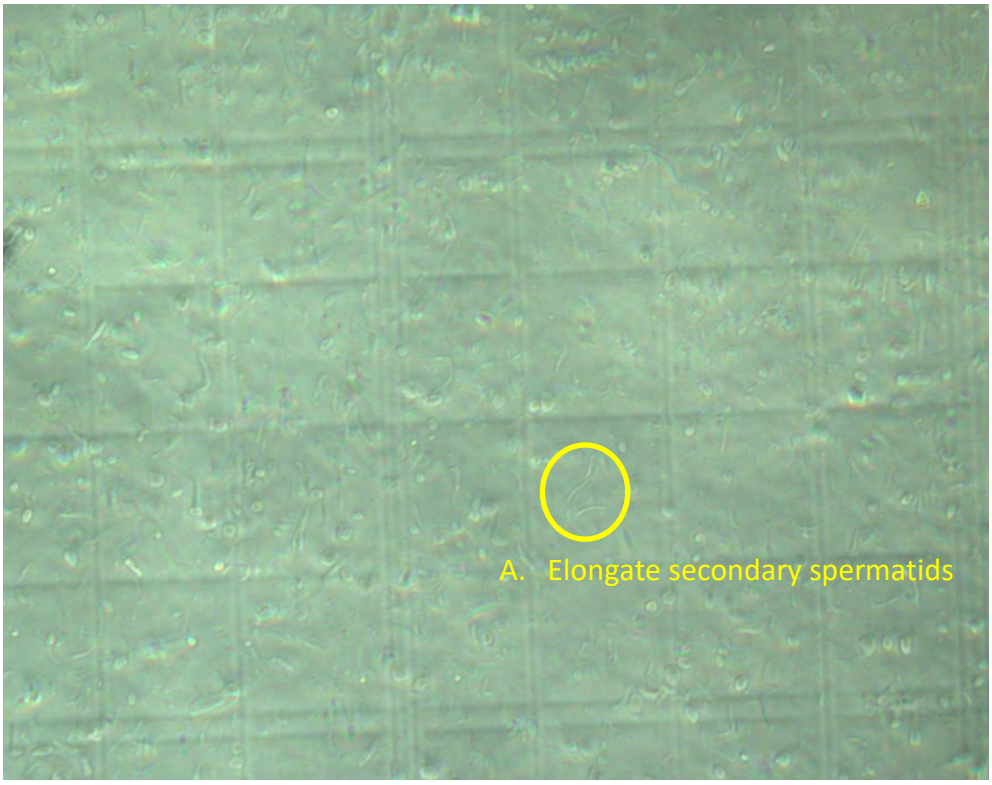


Figure 2-4: Elongate secondary spermatids of homogenized chukar (*Alectoris chukar*) testes imaged at 400X on an improved Neubauer hemocytometer. Left testes of chukar possessed greater numbers of spermatids than right testes.

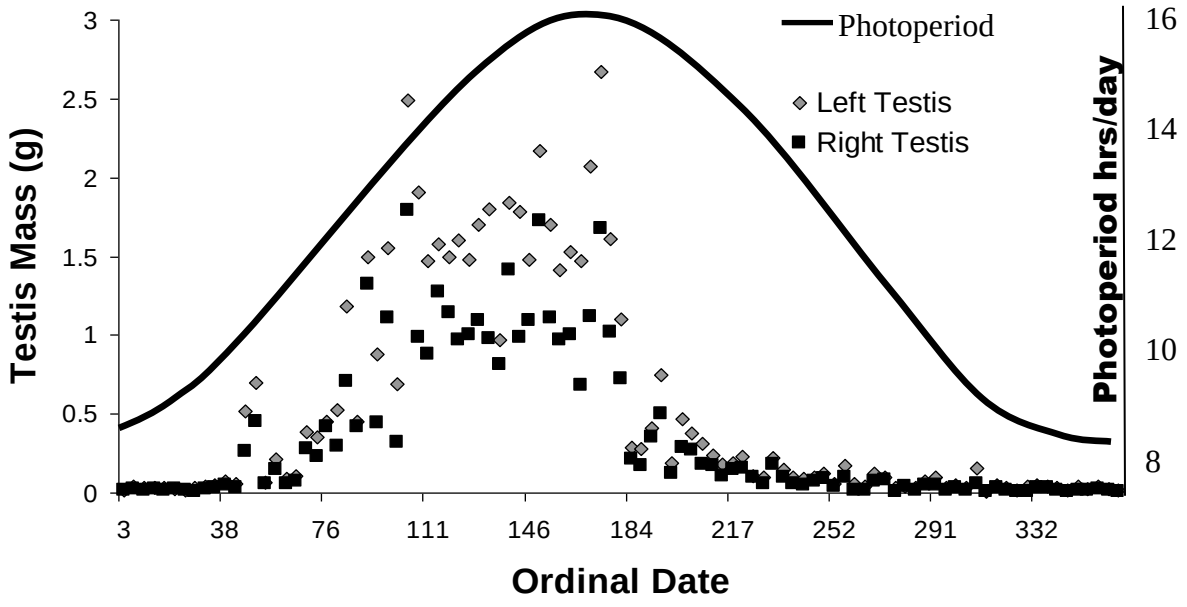


Figure 2-5. Mass of left and right testes of 99 male chukar euthanized weekly from 4 March 2006 to 20 February 2007. Left testes had greater mass than right testes in all birds sampled at all times of the year. Smooth curve indicates photoperiod throughout the year in hours of sunlight per day.

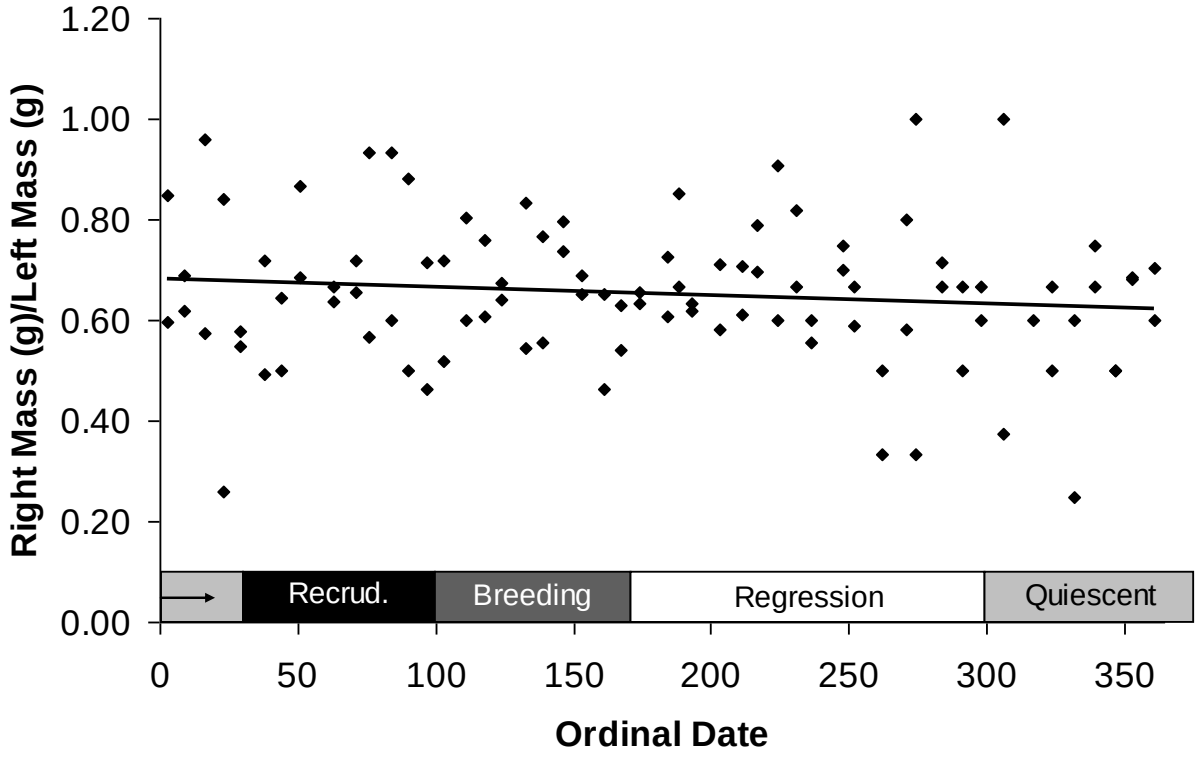


Figure 2-6. Linear regression of proportional mass of right versus left testes across 12 months for 99 wild-trapped adult male chukar held in an outdoor aviary at Idaho State University and measured between 4 March 2006 and 20 February 2007 (R^2 = 0.02, *d.f.* = 98, p = 0.213). Arrow indicates continuing quiescence.

Table 2-1. Mean testes mass, *in situ* length, and *in situ* width ± SE for 16 wild-trapped adult male chukar at maximal testicular recrudescence housed in an outdoor aviary at Idaho State University and measured between 4 March and 1 May 2006. *P*-values are based on paired-*t* tests (*d.f.* = 15 for all tests).

	Left	Right	Paired-*t* value	*p*-value
Mass (g)	1.51 ± 0.10	1.01 ± 0.08	10.25	< 0.001
Length (mm)	22.01 ± 0.56	19.27 ± 0.55	6.75	< 0.001
Width (mm)	12.82 ± 0.40	10.72 ± 0.40	8.94	< 0.001

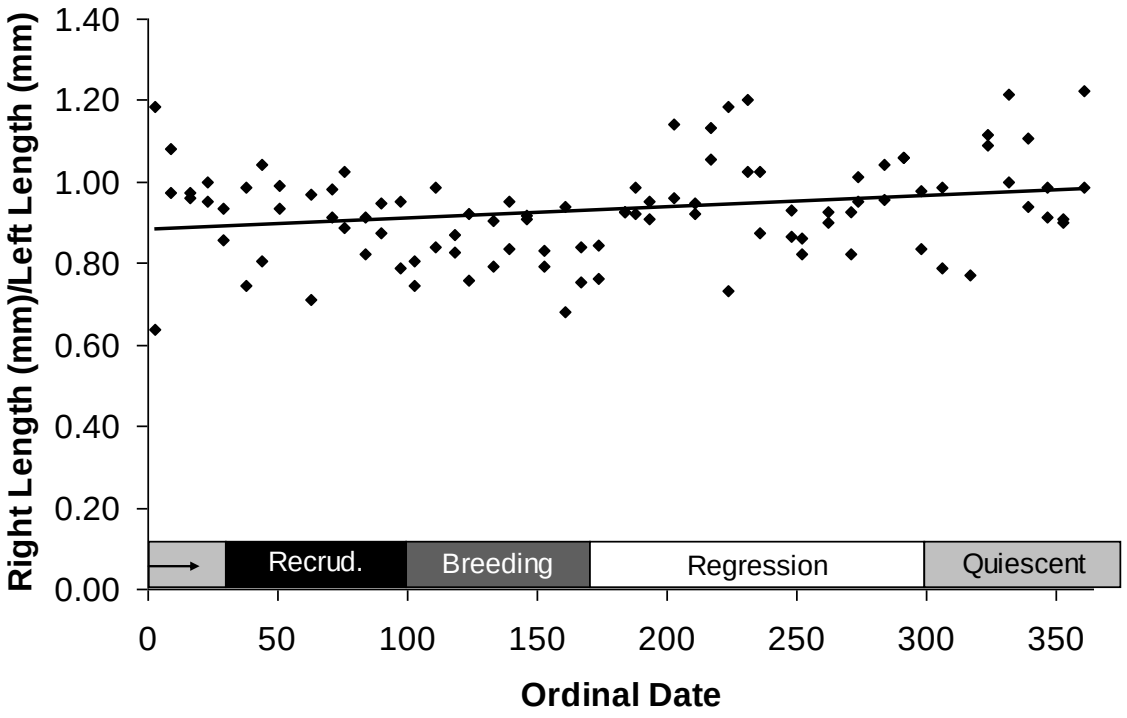


Figure 2-7. Linear regression of proportional length of right versus left testes across 12 months for 99 wild-trapped adult male chukar held in an outdoor aviary at Idaho State University and measured between 4 March 2006 and 20 February 2007 (R^2 = 0.06, *d.f.* = 98, p = 0.015). Arrow indicates continuing quiescence.

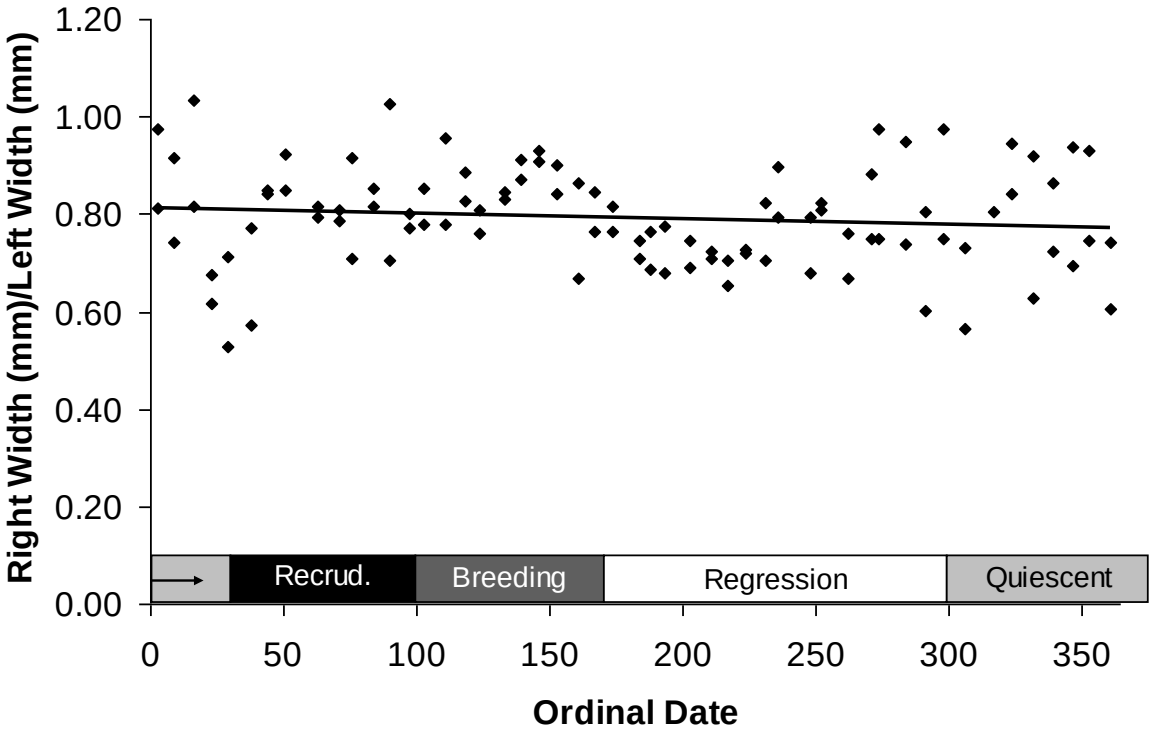


Figure 2-8. Linear regression of proportional width of right versus left testes across 12 months for 99 wild-trapped adult male chukar held in an outdoor aviary at Idaho State University and measured between 4 March 2006 and 20 February 2007 (R^2 = 0.01, *d.f.* = 98, p = 0.284). Arrow indicates continuing quiescence.

Table 2-2. Comparisons of left versus right testis for measured mass, length and width (by paired-*t* test) for 99 wild-trapped adult male chukar housed in an outdoor aviary at Idaho State University between 4 March 2006 and 20 February 2007.

	Paired-*t* value	*d.f.*	*p*-value
in vitro			
Mass (g)	7.19	98	< 0.001
Length (mm)	5.18	98	< 0.001
Width (mm)	10.88	98	< 0.001
in situ			
Length (mm)	6.57	98	< 0.001
Width (mm)	15.56	98	< 0.001

Table 2-3. Left versus right testis sperm production attributes in chukar. Left seminiferous tubule diameter numerically was larger than right tubule diameter, though not significantly so. Seminiferous tubules were significantly larger in cross sectional area in the left versus right testis. Spermatogenic cell abundance within seminiferous tubules differed significantly between left and right testis, with greater numbers of spermatogonia and spermatocytes; primary spermatids; and secondary spermatids per area in the left testes than the right testes.

	Paired *t*-value	*d.f.*	*p*-value
Seminiferous tubule diameter	2.7727	3	0.069
Seminiferous tubule area (in cross section)	2.7732	3	0.048
Pooled Spermatogonia & spermatocytes / area	3.8280	3	0.031
Primary spermatids/area	7.9363	3	0.004
Secondary spermatids/area	3.3978	3	0.042

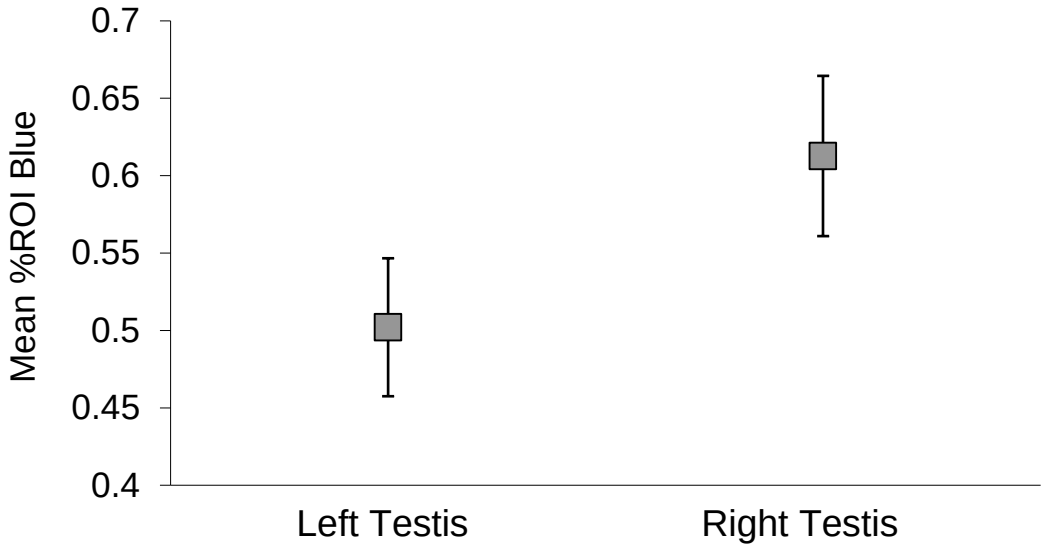


Figure 2-9. Mean percent region of interest blue (%ROI blue) representing the proportion of testicular cross sections stained blue by a Leydig cell-specific stain for left and right testes of 16 adult male chukar analyzed during maximal testicular recrudescence. Left and right testes are significantly different in relative Leydig cell density ($F_{15, 367}$, $p = 0.007$). Plot represents raw data, statistics based on log-transformed data. Bars indicate standard error.

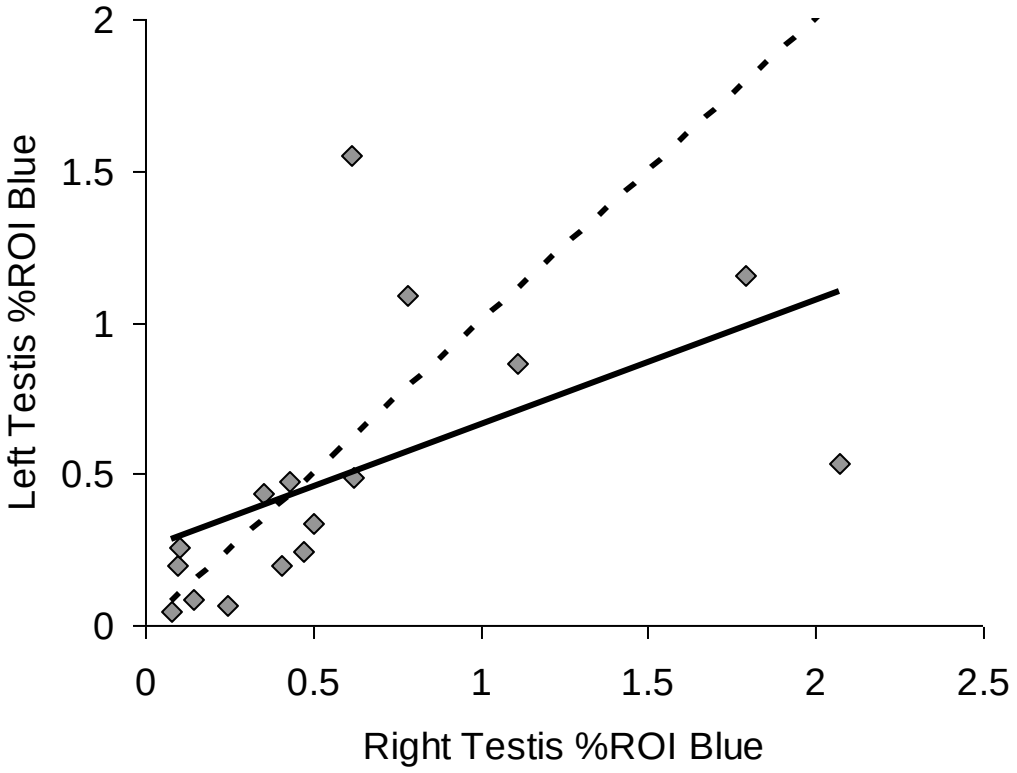


Figure 2-10. Regression (solid line) of relative mean percent region of interest blue for the left and right testes of 16 male chukar analyzed at maximal testicular recrudescence between 4 March 2006 and 20 February 2007. Dashed line indicates the null hypothesis that abundance of Leydig cells in relationship between left and right testes is constant (slope = 1). Actual relationship (y = 0.413x + 0.249) is significantly greater than 0 (R^2 = 0.30, *d.f.* = 15, p = 0.028) and also significantly less than 1 (R^2 = 0.30, *d.f.* = 15, p < 0.01).

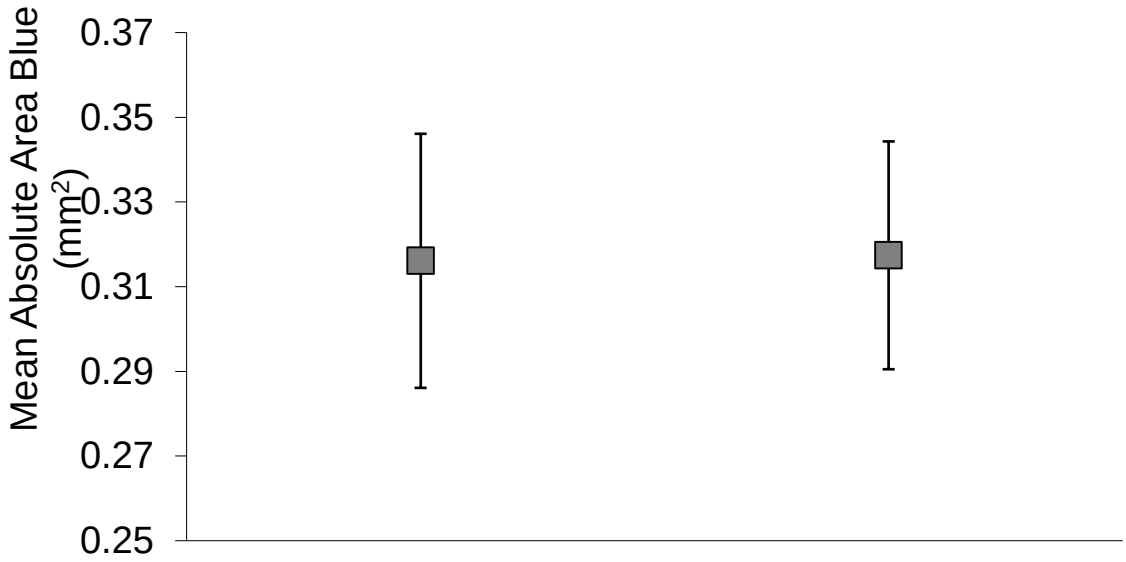


Figure 2-11. Mean absolute area stained blue by a Leydig cell-specific stain for left and right testes from 16 adult male chukar housed in an outdoor aviary at Idaho State University between 4 March 2006 and 20 February 2007. Left and right testes did not differ significantly ($F_{15, 367} = 0.00$, $p = 0.965$). Plot represents raw data, statistics based on log-transformed data. Bars indicate standard error.

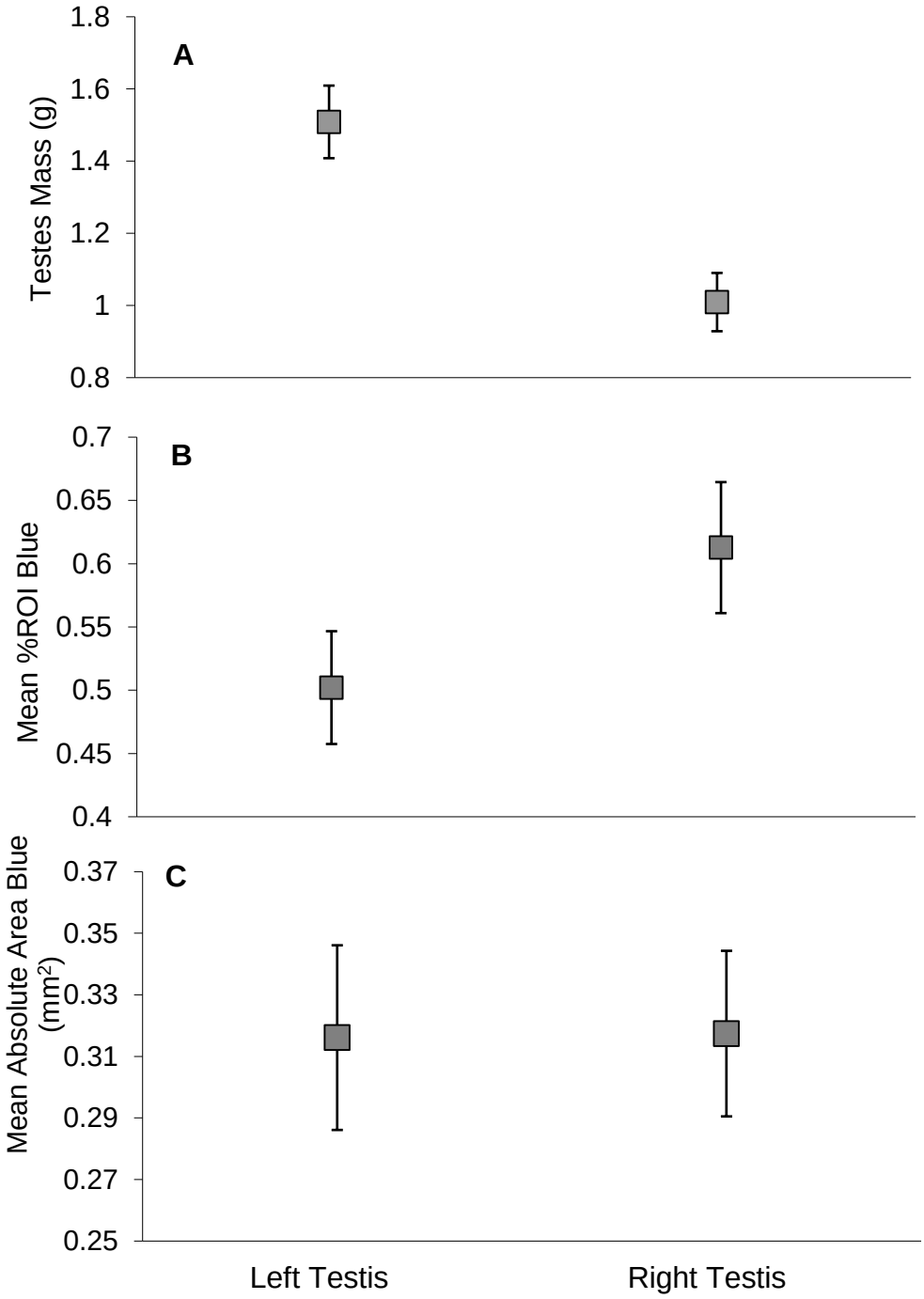
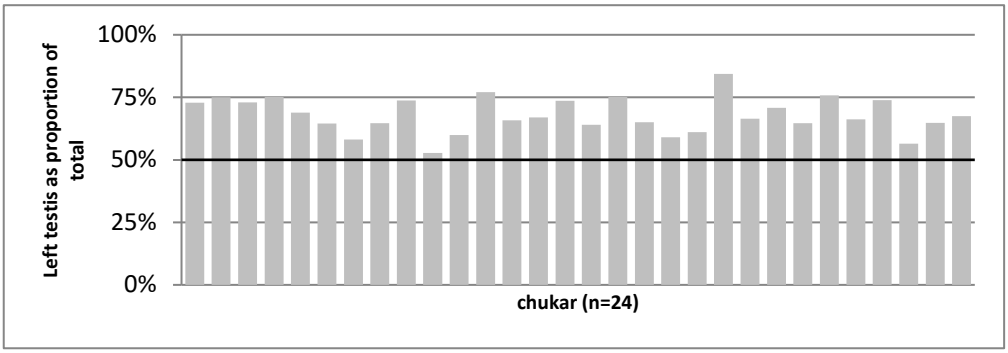


Figure 2-12. Mean testes mass (A), Mean %ROI blue (B) and mean absolute area stained blue (C) for 16 adult male chukar at maximal testicular recrudescence held in an outdoor aviary at Idaho State University. Error bars indicate standard error.

Table 2-4. Mean mass, mean %ROI blue, and mean absolute area blue ± SE for left and right testes of 16 adult male chukar at maximal recrudescence housed in an outdoor aviary at Idaho State University and measured between 4 March 2006 and 20 February 2007. %ROI Blue and absolute area blue statistical results based on log-transformed data.

	Left	Right	Statistical Value	*P*-value
Mass (g)	1.51 ± 0.10	1.01 ± 0.08	Paired-t = 10.25	< 0.001
%ROI Blue	0.502 ± 0.04	0.613 ± 0.05	$F_{15, 367}$ = 7.29	0.007
Abs. Area Blue (mm^2)	0.316 ± 0.03	0.317 ± 0.02	$F_{15, 367}$ = 0.00	0.965



Percent contribution of sperm production by left testis in male chukar. In each case, the left testis produced greater than 50% of total sperm production.

Figure 2-13. Relative amounts of spermatozoa in each the left versus right testes in adult (AHY) male chukar during the breeding season. We found, using a paired-sample, 2-tailed, *t*-test a significant difference in the sperm production of the left versus right testis, with greater sperm production from the left testis ($p = 0.011$, *d.f.* 57). In each case, the left testis produced greater than 50% of the total sperm production for the individual.

CHAPTER THREE

Bilateral Asymmetry in Sperm Production within Male Birds

Spermatozoa (sperm) are haploid cells that carry the paternal portion of the nuclear genome and serve to fertilize ova to create zygotes. Sperm function similarly across all animal taxa, yet sperm are the most diverse animal cell type (Pitnick *et al.* 2009a, Albrecht *et al.* 2015). Although only a single sperm is required to fertilize an ovum, the sperm cells emitted in just one ejaculation by a male greatly outnumber the lifetime production of ova by a female.

One outcome of avian anisogamy, in which males produce a multitude of tiny sperm that vastly outnumber the few, large ova produced by females, is that it creates avenues for the expression of sexual selection. Sexual selection sometimes takes the form of intrasexual selection in which individuals of the same sex compete with one another for reproductive opportunity, such as male x male competition for access to females or to acquire and maintain territories or other resources. Sexual selection also can be intersexual in which interactions occur across sexes. Mate choice in which females choose male mating partners based upon male characteristics, or forced matings in which males use coercion to copulate with unwilling females are examples of intersexual selection.

While sexual selection often occurs prior to copulation, many forms of post-copulatory sexual selection also occur, including cryptic events that occur within an individual. An important example of this kind of sexual selection in birds is sperm

competition. Sperm competition arises when a female has been inseminated by multiple males, putting the sperm of one male in competition with the sperm of another male for fertilization. Because sexual infidelity is common in birds, sperm competition appears to exert strong effects on male reproductive biology. Under these circumstances, a male's chance of fertilizing an ovum may be improved by increasing the number of sperm transferred to females, sometimes referred to as a "lottery effect." Sperm competition makes sperm production capacity of testes particularly important because fertilization success may be determined by the relative number and quality of sperm between the ejaculates of rival male (Fisher *et al.* 2016). One male adaptation to sperm competition is increased testis size resulting in greater sperm production. Across taxa, increased relative testis size is related to increased rates of sperm competition (Rowe & Pruett-Jones 2011). Furthermore, when the ejaculates of 2 or more males compete to fertilize an ovum, sperm quality also becomes important (Santiago-Moreno *et al.* 2015). Under sperm competition, males face a complex physiological and behavioral problem in which sperm number and sperm quality as well as the timing of copulation and the effectiveness of the transfer of ejaculate to females all influence male reproductive success.

Under high levels of sperm competition, males are predicted to produce large quantities of high quality sperm, but various morphological and physiological constraints may limit male investment in sperm production or trade-off sperm quantity against quality (Martin *et al.* 1974, Birkhead *et al.* 1999, Gage *et al.* 2004, Snook 2005). For example, larger sperm may take longer to mature, making fewer sperm available when a reproductive opportunity arises. To maximize fitness, males probably must strike a balance between the spermatogonial stem cells producing enough differentiating daughter

cells to meet current sperm production demand and maintaining a pool of undifferentiated spermatogonial stem cells to produce enough sperm in the future.

Further complicating a male's efforts to reproduce, female birds can store sperm in vaginal sperm storage tubules for two to three weeks. A male's chance of fathering offspring depend not just on pre-copulatory success in achieving reproductive access among rival males, but also in post-copulatory attributes of sperm that are able to outlast and outcompete rival sperm. Ultimately, a competitive advantage for the male can be achieved by a combination of factors, such as controlling the overall number of matings, controlling the number of one male's matings in comparison to rival males' matings, by increasing the number of sperm in a single ejaculate, or by the quality of sperm displayed in myriad characteristics such as the ability of sperm to fit into storage tubules, remain viable in storage, and traverse the distance to the ovum. These issues make the process of testicular spermatogenesis important to understanding avian reproduction under sexual selection.

Spermatogonia, the stem cells from which spermatids are derived, populate the testis early during embryonic development. During initial colonization, more germ cell precursors colonize the left testis (Vallisneri *et al.* 1990, Zaccanti *et al.* 1990). Upon sexual maturity, spermatogonia undergo mitosis. Some of the mitotically replicated cells remain as spermatogonia and are available for future sperm production. Other mitotically replicated cells become spermatocytes that proceed to meiosis and the formation of haploid spermatids and ultimately mature, haploid sperm. There is variation among taxa in the number of mitotic replications that spermatogonia undergo, as well as the number of spermatogonia that are retained in the germ cell population versus the number that

develop to form spermatocytes and spermatids (Russell *et al.* 1990, Lin & Jones 1992, Jones & Lin 1993). The rate at which spermatids mature and are released from the lumen of the seminiferous tubules of the testes to the testicular epididymis for use in ejaculation also varies among species studied, and possibly within individuals of a single species under varying levels of sperm competiton (Fawcett & Slautterback 1959, Fawcett 1961, de Reviers 1968, Dym & Fawcett 1971, Moens & Go 1972, Marchand *et al.* 1977, Holstein & Roosen-Runge 1981, Lin & Jones 1992, Jones & Lin 1993, Jacquet & Sauveur 1995, Giannarka *et al.* 2016).

Avian Testis

The avian testis is a dual-function gonad, producing sperm and also secreting androgenic steroid hormones, primarily testosterone. Testicular structure reflects these dual functions. Most of the mass of the avian testis is comprised of seminiferous tubules, within which sperm production (spermatogenesis) occurs. Testosterone production and secretion occur mainly in the Leydig cells, which occupy interstitial spaces between the seminiferous tubules of the testis. Spermatogenic tissues and endocrinological tissues each are of different embryologic origin.

Early in embryologic development, the reproductive system in both sexes of birds develops bilaterally, but then reduction occurs on the right side (King 1981a,b, Lake 1981, Montgomerie & Bakst 2007). In female Galloanserae, the reduction is functionally complete, but partial in males, with both testes remaining functional. Testicular asymmetry is found widely among bird species, with a large left testis relative to the right testis a common, but not exclusive pattern among asymmetrical species. (Lake 1981, Ligon 1997, Montgomerie & Bakst 2007, Calhim & Montgomerie 2015).

Recent studies in chukar (*Alectoris chukar)* indicate that testicular asymmetry in chukar is due to greater presence of spermatogenic seminiferous tubules on the left side, rather than from an increase in all tissue types within the testis. For chukar, the larger left testis of the male contains a lower proportion of Leydig cells per unit mass than the smaller right testis, whereas the two testes contain the same absolute amount of steroidogenic Leydig cells (Warwick 2007). It appears that the 33% greater mass of the left testis is comprised of spermatogenic seminiferous tubules. A larger amount of seminiferous tubules in the left testis would be expected to produce a larger quantity of spermatozoa than the right testis.

Multiple arguments have been raised seeking to explain bilateral asymmetry in avian testes. Testicular asymmetry may be a carry-over result of female reproductive asymmetry (Kimball *et al.* 1997), a result of space constraints in the abdominal cavity, evolutionary reduction to reduce and centralize mass and promote flight efficiency (Venzke 1954), to decrease androgen production to prevent deleterious physiological effects of increased production as a result of testes enlarged for sperm production, or decreased testosterone production to promote male parental care (Wingfield *et al.* 1990, Møller 1994, Ligon 1997). In this scenario, male birds face a trade-off between increased testes mass to achieve paternity through high sperm production and high testosterone production that might inhibit male parental care, reducing survivorship of young secured through increased sperm production. This argument assumes that elevated testosterone production is an inevitable outcome of increased testis size. This argument also assumes that male parental care is the derived state, and not the ancestral state. To date, each of the hypotheses regarding asymmetry in testis size in avian species assume testes to be

functionally analogous, possessing the same proportions of both endocrinological and spermatogenic tissues in each testis. Here, we test that assumption. If the assumption of testicular analogy is false, explanations for avian testicular bilateral asymmetry will need to be modified.

Avian Phallus

In most living species of birds, males do not possess an intromittent phallus (penis), although the presence of an intromittent phallus is the ancestral condition. An intromittent phallus is present among the paleognathus birds (tinamous and ratites) as well as among reptiles from which birds arose. The Anseriformes and the Galliformes together form the Galloanserae, a monophyletic group basal to all neognathus birds. The Anseriformes (waterfowl such as ducks, geese, and swans) possess an intromittent true phallus. The Galliformes (gamefowl), the sister taxon to the Anseriformes, possess a non-intromittent true phallus the function of which has received little investigation.

Within the Anseriformes, there appears to be an evolutionary arms race between males and females as evidenced by the extreme morphological characteristics of male and female reproductive organs, and is most visible in those species in which females are exposed to high rates of forced extra-pair copulations (Brennan *et al.* 2010). In the Anseriformes, males possess intromittent copulatory organs that are often large and complex in structure, closely matched to the female's reproductive anatomy (Coker *et al.* 2002, Brennan *et al.* 2007). In the Galliformes, however, the phallus is reduced and non-intromittent, indicating diverging pathways to respond to the selective pressures of reproduction. It may be that responses to sexual selection are manifested in more subtle physiological and behavioral mechanisms in species lacking copulatory organs.

In avian males, little accessory fluid is added to semen in the male reproductive tract. Males transmit a small volume of viscous, sperm-rich semen to females during copulation. In species that possess an intromittent phallus, the ejaculate from each vas deferens merges in the duct of the phallus and is transmitted by the phallus to the opening of the single oviduct of the female. In species that lack an intromittent phallus, the semen exits through separate left or right seminal papillae when transmitted to the female, possibly merging within a cloacal groove.

Avian Sperm Transfer

In studies of artificial insemination success in turkeys, placing sperm directly into the oviduct opening is important to ensure fertilization success (Biellier *et al.* 1961). We hypothesize that in males that lack an intromittent phallus, a larger left testis may be advantageous in placing a greater number of sperm at the opening to the left oviduct of females. If semen discharging from the left deferent duct of males is better positioned for insemination than semen from the right duct, then males may be under selection to allocate more resources to left testis development than to right testis development resulting in testicular asymmetry. This would be greatest in species with simple phalluses and non-intromittent phalluses. Because the evolutionary loss of an intromittent phallus occurs with Galloanserae, the Galloanserae are an ideal group to study sperm production and its relationship to bilateral testicular asymmetry, presence or absence of an intromittent phallus, and mating system.

Because gametic transmission is central to fitness, it is reasonable to expect selection to act strongly on the events of copulation. For example, given female

reproductive tract asymmetry, selection may act on male morphological and behavioral traits to accommodate female asymmetry.

Such manifestations would have ramifications beyond those currently appreciated in the ornithological and reproductive physiology literature. Specifically, presence of compositional differences between left and right testes within male birds would mean that some physiological models would need to be adjusted. For example, the practice of using one testis to measure steroid production and the other to measure sperm production will produce skewed ratios if spermatogenesis per unit volume is unequal between testes. Also, our understanding of gametic transmission both in the wild in the form of courtship and copulation, and in captivity as related to artificial insemination may fail to appreciate the complexities of bilateral asymmetry. Finally, a broader and more dynamic evolutionary arms race between males and females may be in play in birds than we have previously appreciated.

We investigated bilateral testicular asymmetry in a number of species in the Galloanserae to examine gonadal morphology and sperm production in birds under the lens of sexual selection, particularly cryptic sexual selection. Specifically, we examined the sperm production capacity of the left testis in comparison to the right testis, to test the widespread assumption that, despite any difference in overall size, the left and right testes are cytologically analogous.

To do this, we assessed the difference in mass between left and right testes of males of 10 species within the Galloanserae. We then measured the densities of total spermatogenic cells, pooled spermatogonia and spermatocytes, primary spermatids and secondary spermatids within each individual to determine the sperm production capacity

of left vs right testes within birds. Finally, we tested whether differences existed in the testicular asymmetry in relation to phallus (order) and/or the risk of sperm competition (mating system).

METHODS

We measured sperm production in relation to testicular asymmetry in males of ten species within the Galliformes and Anseriformes (Appendix 1). We selected species such that we also were able to evaluate potential effects of presence or absence of and intromittent phallus and monogamous versus polygamous mating systems. We harvested all individuals under ISU Institutional Animal Care and Use Committee (IACUC) protocol# FRP-6350408. Additional chukar testis mass measures were obtained from 27 chukar obtained under ISU Institutional Animal Care and Use Committee (IACUC) protocols# 302439, 0605589, and 02142011. During the breeding season, from approximately 15 March through 30 June of 2008 through 2012, we collected males in breeding condition by lethal collection (firearm). All collecting was conducted under permits granted by Idaho Department of Fish and Game permit # 080327, Rhode Island Division of Fish and Wildlife #2012-1, and US Fish and Wildlife Service permit # MB180278-1 in accordance with all state and federal laws regulating the take of wildlife for research purposes.

We weighed each bird immediately upon collection. We then opened the bird with an abdominal incision, and removed organs to access the testes. Using handheld calipers, we measured length (mm) and width (mm) of the left and right testis at the longest and widest point *in situ*. We then removed each testis and measured length and width *in vitro* and wet mass (mg).

We measured the histological composition of sperm producing tissue in left versus right testes within sexually mature male birds. We bisected each fresh testis anterior to posterior along the ventral midline to just prior to the hilus before progressively fixing the tissue through immersion for 5-10 min in neutral buffered formalin followed by 2-8 h immersion in 10% sucrose PBS for 2-8 h, followed by 8-24 h immersion in 30% sucrose PBS solution. When fully fixed, the testis was either processed and imaged immediately, or stored in 70% ETOH until imaged.

To image testis sections, we embedded randomly selected ½ cm^2 testis sections in Tissue-Tek® O.C.T (optimum cutting temperature) Compound (Sakura Finetek, Torrance, CA) and froze the subsamples at -80^o C in preparation for creating tissue slides for histological analysis using a Leica cryostat. Subsamples were brought to a cryostat temperature of -23 ^{o} C for 24 h prior to sectioning. For each testis, we used 3 randomly selected testis subsamples, one from the anterior third, one from the middle third, and one from the posterior third of the testis. We cut sections to 10 μm, and placed sections on precleaned glass slides.

For histological imaging and analysis, we stained slides with propidium iodide in glycerol. The propidium iodide powder was first diluted to 1 mg/ml in glycerol and stored protected from light at 4^o C. To stain testes, a small amount of propidium iodide was freshly mixed to 1.5 μg diluted PI per ml glycerol. All diluted PI was protected from light within an aluminum foil wrapped amber bottle until used, and was used within 48 h of mixing. Stained slides were kept protected from light until imaged, and all were imaged within 24 h of staining.

We created digital images of seminiferous tubules using 400x magnification with a Leica™ DMRB compound microscope and SPOT digital microscope camera (Diagnostic Instruments, Sterling Heights, MI). We imaged 5 seminiferous tubules per section, for a total of 15 images within each testis. Seminiferous tubules were located haphazardly from tissue sections, but we used seminiferous tubules that were sectioned as orthogonal cross-sections with clearly visible cellular components.

We measured tubule diameter and circumference, and counted three types of spermatogenic cells from each imaged tubule (Figure 3-1). Using a high resolution, 24-inch, (60.96cm) touch-screen monitor, we measured the diameter and area in cross section of seminiferous tubules, and counted the number of pooled spermatogonia & spermatocytes, round primary spermatids, and elongate secondary spermatids within each testis at 400X magnification. Using Image-J (NIH, Bethesda, MD) software (Abramoff *et al.* 2004, Rasband 2014), we overlaid a grid of 5000 μm^2 (3.83 pixels/ μm^2) line grid over the tubule. The grid could not be "randomly offset" due to unrepeatability. We created a polygon using the 'polygon' tool within image-J. We generated a horizontally orientated polygon using the area of the grid closest to the midline of the tubule such that the top and bottom of the area to be counted was bordered by gridlines, and the sides encompassed the borders of the tubule. If two opposing edges of the tubule were not visible on the slide, or there were visual imperfections within the horizontal polygon, a vertical polygon as close to midline as possible was generated and used in the same manner. We calculated the area of each polygon using the 'area measure' function within Image-J. Within this area, we counted all spermatogonia, spermatocytes and spermatids. Cell type was identified by size and shape, as illustrated in Figure 3-2.

Statistical analysis

Testis Mass

We assessed differences in length, width, and mass between the left and right testes using paired t-tests. Data pairs consisted of values from left and right testes from a given bird.

Seminiferous Tubule Size

We measured the circumference of tubules within the left and right testes, and calculated the area of tubules in cross-section. We compared differences in the cross-sectional area by paired t-test. Data pairs consisted of values from left and right testes from a given individual male bird.

Spermatogenic cell counts

We measured the difference in the densities (number per unit volume of testicular tissue) of spermatogonia, spermatocytes, and spermatids counted within the left and right testes of male birds by cytological analysis using a paired, t-tests. Each data pair consisted of the value from left testis and right testis from within an individual male.

RESULTS

Left vs right testis mass

Male Galloanserae exhibited significant bilateral asymmetry in testis mass. Mean relative mass of left versus right testes within an individual pooled across all males showed a strong pattern of greater relative mass of the left testis within Galloanserae males (Table 3-1). Most males within most species exhibited greater left testis mass and for most, but not all, species we observed a significant mean difference (Table 3-1). The left testis mass was greater than the right testis mass in both Anseriformes and

Galliformes, and in both monogamous and polygamous species (Table 3-1). The magnitude by which the left testis mass exceeded the right testis mass did not differ between Anseriformes and Galliformes, but the magnitude of asymmetry did differ significantly between monogamous and polygamous species (Table 3-2). Males of monogamous species exhibited a significantly greater degree of bilateral asymmetry in testis mass than did males in polygamous species (Table 3-2).

Seminiferous tubule size between left versus right testes

Overall, seminiferous tubules within left testis of males were greater in area in cross-section than the seminiferous tubules of the right testis. When data from all males were pooled, this difference was highly significant (Table 3-3). At the species level, most individuals of all species had seminiferous tubules that were larger in the left testis than in the right (Table 3-3). The larger area in cross-section of the left seminiferous tubules was found in both Anseriformes and Galliformes, and both monogamous and polygamous species. The degree of left-larger seminiferous tubules was not significantly different between Anseriformes and Galliformes, nor between monogamous versus polygamous species (Table 3-2).

Spermatogenic cell density in the left testis versus the right testis

When considering the entire population of spermatogenic cells, i.e., spermatogonia, primary and secondary spermatocytes, and primary and secondary spermatids that comprise the cell series that produces mature, haploid sperm, we found that spermatogenic cell densities differed between a male's left testis and its right testis. We observed significantly higher densities of spermatogenic cells per μm^3 testicular tissue in the left testis than in the right testis across Galloanserae (Table 3-4). Significant bilateral

asymmetry in spermatogenic cell density was present in both Anseriformes and Galliformes (Table 3-4) and we did not observe a difference in magnitude of the bilateral asymmetry between these two orders (Table 3-2). We did observe a strong numeric trend ($p = 0.056$; Table 3-2) in bilateral asymmetry of total spermatogenic cell density between monogamous versus polygamous species. Numerically, males of monogamous species exhibited higher spermatogenic cell densities within their left testis to a greater degree than did males of polygamous species.

Spermatogonia & Spermatocytes

Spermatogonia (diploid stem cells) and spermatocytes (both primary and secondary spermatocytes) are spermatogenic precursor cells from which sperm will be produced. We observed significant bilateral asymmetry in density of the pooled population of spermatogonia and spermatocytes between the left testis and the right testis of Galloanserae males (Table 3-5). These spermatogenic precursor cells were found in significantly greater density in the left testis of males throughout Galloanserae. While significant bilateral asymmetry was present in both Anseriformes and Galliformes and within many species in these orders (Table 3-5), we did not observe a difference in the magnitude of bilateral asymmetry in spermatogonia and spermatocyte cell density between Anseriformes versus Galliformes nor between males grouped as monogamous versus polygamous (Table 3-2).

Spermatids

The meiosis II division of secondary spermatocytes produces round primary spermatids that develop into elongate secondary spermatids. The left testis of

Galloanserae males exhibited higher densities of total spermatids (primary and secondary spermatids pooled) per μm^3 testicular tissue than the corresponding right testis within males (Table 3-6). This bilateral asymmetry in density of spermatids was significant in Anseriformes and Galliformes and for several species within these orders (Table 3-6). We did not observe a difference in the magnitude of bilateral asymmetry in spermatid density between Anseriformes and Galliformes (Table 3-2). However, the magnitude of bilateral asymmetry did differ significantly between males of monogamous species versus males of polygamous species (Table 3-2). Males of monogamous species, on average, exhibited a greater degree of bilateral asymmetry in spermatid cell density.

Primary spermatids

When considering primary (round) spermatids exclusively, the left testis of male Galloanserae exhibited a higher primary spermatid density per μm^3 than the corresponding right testis (Table 3-7). Significant bilateral asymmetry was present within Anseriformes and Galliformes and was observable in several individual species (Table 3-7). As with total spermatids, we did not observe a difference in the magnitude of bilateral asymmetry between male Anseriformes and Galliformes but we did observe a significantly greater magnitude of asymmetry between monogamous versus polygamous males (Table 3-2). Monogamous males exhibited a greater degree of bilateral asymmetry in primary spermatid cell density asymmetry than did polygamous males.

Secondary spermatids

When considering secondary spermatids exclusively, the left testis of male Galloanserae exhibited a higher density of secondary spermatids than the corresponding right testis (Table 3-8). This pattern was true within Anseriformes and within Galliformes

and several individual species (Table 3-8). However, we did not detect a difference in the magnitude of the asymmetry between Anseriformes and Galliformes nor between monogamous versus polygamous males.

Testis-wide presence of secondary spermatids

Incorporating both the significant bilateral asymmetry in testis size (volume) and the significant bilateral asymmetry in secondary spermatid density (Table 3-8), we calculated the number of secondary spermatids present in whole left testes versus whole right testes for 55 individuals for which testis volumes could be calculated accurately. Among these individuals (n=55), we found that the left testis produced significantly more secondary spermatids than the right testis. Bilateral asymmetry in secondary spermatids between the left testis versus the right testis was highly significant for male Galloanserae as a group and for males within Anseriformes and Galliformes, respectively, as well as for males within monogamous and polygamous mating systems, respectively (Table 3-9). Numerically, all species exhibited greater secondary spermatid populations with left testes, significantly so for several species despite low sample sizes (Table 3-9). We did not detect differences in the magnitude of the bilateral asymmetry in left testis versus right testis populations of secondary spermatids between Anseriformes and Galliformes. However, the differences in the magnitude of bilateral asymmetry in left testis vs right testis populations of secondary spermatids was highly significant between monogamous and polygamous males (Table 3-2).

DISCUSSION

Using male Galloanserae, the waterfowl and gamefowl that form the monophyletic basal group of neognathus birds, we tested the assumption that the left

testis and right testis within a male bird are compositional analogues of one another. We found this assumption to be false. The left testis of male Galloanserae birds collected during the breeding season exhibited significantly greater spermatogenic capacity per unit volume of testicular tissue than the right testis within the males as evidenced by nonrandom elevated densities of a suite of spermatogenic cells within the left testis relative to the right testis. The phenomenon of bilateral asymmetry in testicular composition and spermatogenic activity has not been described previously for birds or, to our knowledge, for any other vertebrate. It presents a new aspect of sexual reproduction in birds and its evolutionary basis and biological implications remain to be developed.

The compositional asymmetry we describe is in addition to previously recognized avian bilateral asymmetry in testis size and mass. We, too, found greater size and mass of the left testis relative to the right testis to be a regular feature within male waterfowl and gamefowl. One implication of greater spermatogenic cell densities in the left testis of males is that the left testis produces more sperm not only as a result of being larger; the left testis also produces more sperm because of a higher density of spermatogenic cells within its testicular tissue.

The strong patterns of bilateral asymmetry in testis mass (Table 3-1) and spermatogenic cell densities (Table 3-4) occurred within and across the two avian orders comprising Galloanserae and indicates that left and right testes in Galloanserae males are not compositional analogues of one another. The left testis of males within this basal group of neognathus birds exhibits greater spermatogenic activity than the right testis of males. Physiological studies and evolutionary hypotheses that assume the left and right testes of Galloanserae males are compositional and functional analogues may need to be

reevaluated. Because males of many other neognathus orders also express bilateral asymmetry in testis size, our findings suggest benefit to careful examination of bilateral testis composition throughout neognathus birds.

Not only did we see overall asymmetry in testis mass and spermatogenic cells densities, we found the seminiferous tubules within the left testis to be larger than in the right testis (Table 3-3). Perhaps greater surface area within the left testis seminiferous tubule lumen allows for increased spermatogenic cell capacity within the left testis. This is reasonable considering that spermatogenesis occurs within the inner surface seminiferous tubules.

The degree of bilateral asymmetry of testis mass did not differ between Anseriformes versus Galliformes (Table 3-2). Males of both orders exhibited asymmetry. However, degree of asymmetry in testis mass was significantly greater in monogamous species than in polygamous species ($p < 0.001$, Table 3-2). Degree of bilateral asymmetry in seminiferous tubules did not differ by order or mating system. However, the degree of bilateral asymmetry in total spermatogenic cell density exhibited a strong trend toward monogamy ($p < 0.056$, Table 3-2). Thus, male Galloanserae as a group employ strong bilateral asymmetry in testicular attributes, with larger left testes comprised of larger seminiferous tubules that contain greater spermatogenic cell densities than right testes. While these attributes are universal, they show a tendency to be expressed to a higher degree in monogamous species regardless of taxonomic order. Our data do not distinguish between greater or lesser degree of bilateral asymmetry as being the evolutionarily derived state. However, our data suggest that while bilateral asymmetry is

the norm within Galloanserae, the degree of asymmetry may in some way be sensitive to mating system.

The pattern of a higher density of spermatogenic cells within the tubules of the left testis generally was observable when various types of spermatogenic cells were considered separately. Within the pool of all males, the left testis had a higher density of spermatogonia and spermatocytes than the right (Table 3-6). Spermatogonia and primary spermatocytes are diploid spermatogenic stem cells while secondary spermatocytes, which we could not visually distinguish from primary spermatocytes, are haploid precursors to spermatids. The higher density of spermatogonia and spermatocytes in the left testis versus the right testis was significant in the Galliformes, but not in the Anseriformes. However, we did not detect a significant difference in the degree of bilateral asymmetry between orders or between mating systems (Table 3-2). During spermatogenesis, spermatogonia undergo mitosis, creating two daughter cells. Some of these daughter cells retain their condition as spermatogonia, i.e., they 'recycle' to maintain the spermatogonia population. Other daughter cells become primary spermatocytes, so called because they proceed to phase one of meiosis, dividing to create two haploid secondary spermatocytes. The rate at which mitotic daughter cells of spermatogonia recycle back into the spermatogonia cell population is thought to vary among species. This may account for our inability to detect clear patterns in bilateral asymmetry of spermatogonia and spermatocyte cell densities within seminiferous tubules. Cinnamon teal, ring-necked pheasants, and chukar stood out as species exhibiting clear signals of bilateral asymmetry in the population of spermatogonia and spermatocytes (Table 3-5). Our data do not allow us to distinguish between embryonic colonization of

primordial germ cells versus spermatogonial cell recycling as mechanisms underlying strong asymmetry in some species relative to others.

Total spermatid (primary + secondary) density exhibited pronounced bilateral asymmetry throughout Galloanserae with higher density within the lumens of the seminiferous tubules of the left testis (Table 3-6). Spermatids are the haploid products of meiosis II of secondary spermatocytes. Essentially, they are immature sperm cells without tails that are still developing within the lumen of seminiferous tubules. During early development spermatids are round and are called primary spermatids. As they develop, the primary spermatids elongate and become secondary spermatids.

We were able to detect significant bilateral asymmetry in spermatid density throughout Galloanserae: within both Anseriformes and Galliformes and within monogamous and polygamous species. In fact, the bilateral asymmetry was detectable within most individual species (Table 3-6), lending support to the hypothesis that the left testis produces more sperm per unit volume than the right testis. The degree of bilateral asymmetry was greater among monogamous species ($p = 0.052$, Table 3-2) than polygamous species and was not associated with avian order.

To better understand this pattern, we evaluated primary and secondary spermatids separately. Primary spermatids occurred at greater densities within left testes throughout Galloanserae, including within orders and mating systems (Table 3-7). This observation is important because primary spermatids are close to the spermatogenic end product, but are not known to be shed from the testis at that stage of development. The high degree of bilateral asymmetry in density is strong evidence of heightened sperm production from

the left side of males. Intriguingly, here too greater degree of bilateral asymmetry was associated with monogamous species rather than polygamous species (Table 3-2).

Secondary spermatids (elongate, haploid cells without tails) also occurred with bilaterally asymmetrical densities in Galloanserae as a group and within mating systems and orders (Table 3-8). These represent sperm cells that are close to maturity and throughout Galloanserae they are occurring at higher densities per unit volume of testicular tissue in the left testes of males than the right testes of males. We did not detect a difference in the degree of asymmetry of secondary spermatids between mating systems or between orders. This is puzzling considering the greater degree of bilateral asymmetry in primary spermatids previously seen in monogamous species. Understanding these dynamics may require greater knowledge of the clearance rate of maturing spermatids or other cell population dynamics. Despite not understanding this nuance, the overall occurrence of bilateral asymmetry in secondary spermatid density provides a strong indication of signal left testis predominance in sperm production.

We accounted for bilateral asymmetry in testis mass as well as bilateral asymmetry in secondary spermatid density to calculate an overall weighted mean of secondary spermatid cell density per unit of testicular tissue for each male. This allowed us to compare secondary spermatid densities at the scale of total testicular capacity, i.e., left and right testis effects together. The combined effects of a larger left testis and greater left testis secondary spermatid density was clearly observable throughout Galloanserae (Table 3-9). Using secondary spermatids as a proxy for mature sperm, the left testis of a Galloanserae male produces significantly more sperm than the right testis of the male. We observed this across Galloanserae as well as within mating systems and

orders and within most individual species. Male Galloanserae are highly bilaterally asymmetrical in sperm production. The degree of bilateral asymmetry in sperm production is significantly greater among monogamous species ($p < 0.001$, Table 3-2) versus polygamous species and is not associated with avian order. This finding yields two important conclusions. (1) The degree of asymmetry in sperm production is strongly and positively associated with monogamy within Galloanserae. (2) Because the degree of bilateral asymmetry in sperm production in Galloanserae males is not associated with avian order, the presence of an intromittent phallus (Anseriformes) or its absence (Galliformes) does not appear to drive bilateral asymmetry in sperm production.

The asymmetry in the mass of left vs right testes was significantly different between monogamous and polygamous species, with the degree of asymmetry in testis mass resulting in a larger left testis more pronounced in monogamous species than in polygamous species. It may be that sperm competition in polygamous species, which encourages greater sperm production and thus proportionally larger testes, leads to greater production from both testes and reduces the trend toward asymmetry. Seminiferous tubules in the left testis were larger than those in the right testis, but no difference in the degree of asymmetry was seen between orders or mating system.

When looking at spermatogenic cells, we did find a marginally significant difference between monogamous and polygamous species, with monogamous species having a higher degree of asymmetry in the density of the total spermatogenic cells between the left and right seminiferous tubules.

We found no difference in the density of spermatogonia and spermatocytes in the left testis vs right testis between monogamous and polygamous species. We did find a

significant difference in the density of total spermatids (both primary and secondary combined) in the left testes vs right testes of monogamous vs polygamous species. The left testes are denser than the right testes in total spermatids, and this increased density in the left testis is more pronounced in monogamous species than in polygamous species. This difference persisted when we counted only round primary spermatids, with monogamous species, which were more asymmetrical in testis size, also possessing a higher density of round primary spermatids in the left testis than the right. We did not detect a difference in the density of maturing secondary spermatids between mating systems. It is possible that the number of secondary spermatids that have elongated, but not yet passed to the deferent ducts is less plastic due to their placement within the lumen, and that differences in their rate of passage to and through the deferent ducts, as well as how many ejaculates can be stored in the extra-testicular reproductive tract exist between mating systems. However, the total numbers of secondary spermatids present in the left testis were much greater than those in the right testis, and this was seen more so in monogamous species than in polygamous species.

The magnitude of bilateral asymmetry in testis attributes we observed was not a function of avian order. We did not detect differences in magnitude of asymmetry between the left testis and right testis when comparing Anseriformes (waterfowl) to Galliformes (gamefowl). This implies that the attribute of intromittent phallus presence (Anseriformes) or absence (Galliformes) is not central to the magnitude of bilateral asymmetry in Galloanserae. Galloanserae males exhibit strong but similar levels of bilateral asymmetry in testis composition whether or not males place sperm into females via an intromittent phallus.

Overall, the magnitude of bilateral asymmetry in testis attributes frequently was associated with mating system (Table 3-2) with greater magnitude of bilateral asymmetry for monogamous males than for polygamous males for testis mass, total spermatid cell density, primary spermatid cell density, and testis-wide populations of secondary spermatids, as well as a strong statistical trends of greater magnitude for total spermatogenic cell density. In all cases, the left testis exhibited greater values for the attribute in question than the right testis, forming a 'left-bias' bilateral asymmetry.

Several explanations for heightened bilateral asymmetry among monogamous males are plausible. One possibility is that monogamous males exhibited greater asymmetry due to male age, itself an artifact of experimental design. In domestic fowl, testicular asymmetry increases with age (Lake 1981, Kimball *et al.* 1997). All males were wild and free ranging, collected from the wild during the breeding season. Males of monogamous species often were tending females. Presumably, these were mature, successful males and male gonadal size asymmetry may be positively correlated to male age. Mute swans are an example of this. Males likely were 5-15 years old and exhibited great bilateral asymmetry. Male mallards, on the other hand, often are outside the presence of females and their age and breeding status could not be ascertained with certainty. It may be that initial asymmetrical colonization of primordial germ cells, with more colonizing the left testis than the right testis, lays the groundwork for increased asymmetry with increasing age as Sertoli cell secretions in the left testis act to reduce the size of the right testis, as is seen in mammalian Sertoli cell tumors.

Perhaps the association of heightened asymmetry with monogamy is a direct result of mating system. Monogamous males with prolonged pair bonds and cooperative

copulation behavior evolutionarily may be investing in efficient sperm production by specializing a larger left testis while concurrently reducing the right testis. Alternatively, polygamous males with competitive copulations, including forced copulations in waterfowl, may be maximizing sperm production by developing otherwise reduced right testes. It may be illuminating to measure the right testis as a proportion of total body mass to try to elucidate if the right testis is increasing in polygamous species, or the left is increasing in monogamous species. Testing and distinguishing between these competing hypotheses likely will require a better understanding of the efficiency of sperm production in birds, a question that itself will benefit from the new understanding that left and right testes are not analogues in sperm production. Ultimately, determining differing fertilization success from the left testis versus the right testis might provide the greatest insight into the adaptive significance of testis asymmetry in birds.

LITERATURE CITED

Abramoff, M. D., P. J. Magalhaes and S. J. Ram (2004). Image Processing with ImageJ. *Biophotonics International* 11: 36-42.

Bakst, M. R., V. Akuffo, P. Trefil and J. P. Brillard (2007). Morphological and histochemical characterization of the seminiferous epithelial and Leydig cells of the turkey. *Animal Reproduction Science 97*: 303-313.

Bennison, C., N. Hemmings, J. Slate and T. Birkhead (2015). Long sperm fertilize more eggs in a bird. In *Proceedings of the Royal Society, London, Series B: Biological Sciences* 282: 1897.

Biellier, H.V., R. Pashcange and M. Funk (1961). Effect of depth of insemination on fertility of broad breasted bronze turkey hens. *Poultry Science* 40: 1379.

Birkhead, T. R. (1998). Sperm competition in birds. *Reviews of Reproduction* 3: 123-129.

Birkhead, T. R., J. G. Martinez, T. Burke and D. P. Froman (1999). Sperm mobility determines the outcome of sperm competition in the domestic fowl. *Proceedings of the Royal Society of London, Series B: Biological Sciences* 266: 1759-1764.

Brennan, P. L. R., C. J. Clark and R. O. Prum (2010). Explosive eversion and functional morphology of the duck penis supports sexual conflict in waterfowl genitalia. *Proceedings of the Royal Society of London B: Biological Sciences* 277: 1309-1342.

Brennan P. L. R., R. O. Prum, K. G. McCracken, M. D. Sorenson, R. E. Wilson and T. R. Birkhead (2007). Coevolution of Male and Female Genital Morphology in Waterfowl. *PLoS ONE* 2: e418.

Calhim S. and R. Montgomerie (2015). Testis asymmetry in birds: the influences of sexual and natural selection. *Journal of Avian Biology* 46: 175-85.

Coker, C. R., F. McKinney, H. Hays, S. V. Briggs and K. M. Cheng (2002). Intromittent organ morphology and testis size in relation to mating system in waterfowl. *The Auk* 119: 403-413.

Cramer, E. R. A., E. Stensrud, G. Marthinsen, S. Hogner, L. E. Johannessen, T. Laskemoen, M.-C. Eybert, T. Slagsvold, J. T. Lifjeld and A. Johnsen (2016). Sperm performance in conspecific and heterospecific female fluid. *Ecology and Evolution* 6: 1363–1377.

Gage, M. J., C. P. Macfarlane, S. Yeates, R. G. Ward, J. B. Searle and G. A. Parker (2004). Spermatozoal traits and sperm competition in Atlantic salmon: relative sperm velocity is the primary determinant of fertilization success. *Current Biology* 14: 44-47.

Giannarka, A., L. Sharer and S. A. Ramm (2016). Sperm competition-induced plasticity in the speed of spermatogenesis. *BMC Evolutionary Biology* 16: 1-10.

Dym, M., and D. W. Fawcett (1971). Further observations on the numbers of spermatogonia, spermatocytes, and spermatids connected by intercellular bridges in the mammalian testis. *Biology of Reproduction* 4: 195-215.

Fawcett, D. W. (1961). Intercellular bridges. *Experimental Cell Research* 8: 174-187.

Fawcett, D. W., S. Ito and D. Slautterback (1959). The occurrence of intercellular bridges in groups of cells exhibiting synchronous differentiation. *The Journal of Biophysical and Biochemical Cytology* 5: 453-460.

Holstein, A. F. and E. C. Roosen-Runge (1981). Spermatogonia, pp. 33-57 in *Atlas of Human Spermatogenesis*. Grosse Verlag, Berlin.

Jacquet, J. M. and B. Sauveur (1995). Photoperiodic control of sexual maturation in Muscovy drakes. *Domestic Animal Endocrinology 12*: 189-195.

Jones, R. C. and M. Lin (1993). Spermatogenesis in birds. *Oxford Reviews of Reproductive Biology 15:* 233-264.

Lin, M. and R. C. Jones (1992). Renewal and proliferation of spermatogonia during spermatogenesis in the Japanese quail (*Coturnix japonica*). *Cell and Tissue Research* 267: 591-601.

Marchand, C. R., L. Gomote and M. de Reviers (1977). Contribution a l'etude par autoradiographe et marquage a la thymedine trite de la duree de la spematogenese du canard de barbarie (Cairina moschata) *Comptes Rendus des Seances, Society de Biologie* 21: 927-931.

Moens, P. B. and V. L. W. Go (1972). Intercellular bridges and division patterns of rat spermatogonia. *Zeitschrift für Zellforschung und Mikroskopische Anatomie 127*: 201-208.

de Reviers, M. D. (1968). Détermination de la durée des processus spermatogénetiques chez le coq à l'aide de thymidine tritiée. *International Congress on Animal Reproduction and Artificial Insemination [Proc]*.

Fisher, D. N., R. Rodríguez-Muñoz and T. Tregenza (2016). Comparing pre- and post-copulatory mate competition using social network analysis in wild crickets. *Behavioral Ecology* 27: 912-919.

Graves, G.R. (2004). Testicular volume and asymmetry are age-dependent in black-throated blue warblers (*Dendroica caerulescens*). *The Auk 121*: 473-485.

Kimball, R. T., J. D. Ligon and M. Merola-Zwartjes (1997). Testicular asymmetry and secondary sexual characters in red junglefowl. *The Auk* 114: 221-228.

King, A. S. (1981a). Cloaca, p. 63-106. *In*: [A. S. King and J. McLelland, Eds.] Form and Function in Birds. Vol 2. Academic Press, New York.

King, A. S. (1981b). Phallus. pp. 107-148 *In:* [A. S. King and J. McLelland, Eds.] Form and Function in Birds. Vol. 2. Academic Press, New York.

Lake, P. E. (1981). Male genital organs. p. 1-61. *In*: [A. S. King and J. McLelland, Eds.] Form and Function in Birds. Vol 2. Academic Press, New York.

Ligon, J. D. (1997). A single functional testis as a unique proximate mechanism for promoting sex-role reversal in Coucals. *The Auk* 114: 800-801.

McCarrey, J. R. and U. K. Abbott (1982). Functional differentiation of chick gonads following depletion of primordial germ cells. *Journal of Embryology and Experimental Morphology 68*: 161-174.

Martin, P. A., T. J Reimers, J. R. Lodge and P. J. Dzuik (1974). The effect of ratios and numbers of spermatozoa mixed from two males on proportions of offspring. *Journal of Reproduction and Fertility*. 39: 251-258.

Merila, J. and B. C. Sheldon (1999). Testis size variation in the greenfinch *Carduelis chloris*: relevance for some recent models of sexual selection. *Behavior in Ecology and Sociobiology* 45: 115-123.

Møller, A.P. (1994). Directional selection on directional asymmetry: testes size and secondary sexual characters in birds. *Proceedings of the Royal Society of London, Series B*: *Biological Sciences* 258: 147-151.

Montgomerie, R. and J. V. Briskie (2007). Anatomy and evolution of copulatory structures. pp. 115 – 148 in [B. G. M. Jamieson, Ed.]. *Reproductive Biology and Phylogeny of Birds Part A: Phylogeny, Morphology, Hormones and Fertilization* Science Publishers.

Omotoriogun, T. C., T. Laskemoen, M. Rowe, T. Albrecht, R. C. K. Bowie, O. Sedláček, D. Hořák, U. Ottosson and J. T. Lifjeld (2016). Variation in sperm morphology among Afrotropical sunbirds. *Ibis* 158: 155–166.

Parker, G.A. (1990). Sperm competition games: raffles and roles. *Proceedings of the Royal Society of London, Series B: Biological Sciences 242*: 120-126.

Peterson, A. A., M. P. Lombardo and H. W. Power (2001). Left-sided directional bias of cloacal contacts during tree swallow copulations. *Animal Behavior* 62: 739-741.

Pitnick, S., D. J. Hosken and T. R. Birkhead (2008). Sperm morphological diversity. p. 69 in [Birkhead, T. R., D. J.Hosken and S. Pitnick, Eds.] *Sperm Biology: an Evolutionary Perspective:* Academic Press, Elsevier, Oxford, UK.

Rasband, W.S., (2008). ImageJ. *http://rsbweb. nih. gov/ij/*.

Rasband, W.S. (2012). ImageJ: Image processing and analysis in Java. *Astrophysics Source Code Library*, *1*, p.06013.

Rasband, W.S. (2014) ImageJ, U.S. National Institutes of Health, Bethesda. *Maryland, USA*.

Rowe, M., T. Albrecht, E. R. A. Cramer, A. Johnsen, T. Laskemoen, J. T. Weir and T. J. Lifjeld (2015). Postcopulatory sexual selection is associated with accelerated evolution of sperm morphology. *Evolution* 69: 1044–1052. doi: 10.1111/evo.12620

Santiago-Moreno, J., M. C. Esteso, C. Castaño, A. Toledano-Díaz and A. López-Sebastián (2015). Post-coital sperm competence in polygamous animals: the role of sperm traits in species–specific strategies. *Andrology S1:* 2167-0250.

Simmons, L.W. and J. S. Kotiaho (2002). Evolution of ejaculates: patterns of phenotypic and genotypic variation and condition dependence in sperm competition traits. *Evolution 56*: 1622-1631.

Snook, R.R. (2005). Sperm in competition: not playing by the numbers. *Trends in Ecology & Evolution 20*: 46-53.

Vallisneri, M., A. Quaglia, A. M Stagni and F. Zaccanti (1990). Differences between male and female protogonia in chick embryos before sex differentiation of the gonads. *Bollettino della Societa Italiana di Biologia Sperimentale* 66: 91–98.

Venzke, W.G. (1954). The morphogenesis of the indifferent gonad of chick embryos. *American Journal of Veterinary Research* 15: 300-308.

Warwick, J. N. (2007). Assessing Functional Bilateral Asymmetry in Chukar (*Alectoris chukar*) Testes. MS Thesis, Idaho State University, Pocatello, ID.

Wingfield, J. C., R. E. Hegner, A. M. Dufty and G. F. Ball. (1990). The "Challenge Hypothesis": Theoretical implications for patterns of testosterone secretion: mating systems, and breeding strategies. *The American Naturalist* 136: 829-846.

Zaccanti, F., Vallisneri, M., and A. Quaglia (1990). Early aspects of sex differentiation in the gonads of chick embryos. *Differentiation* 43: 71–80.

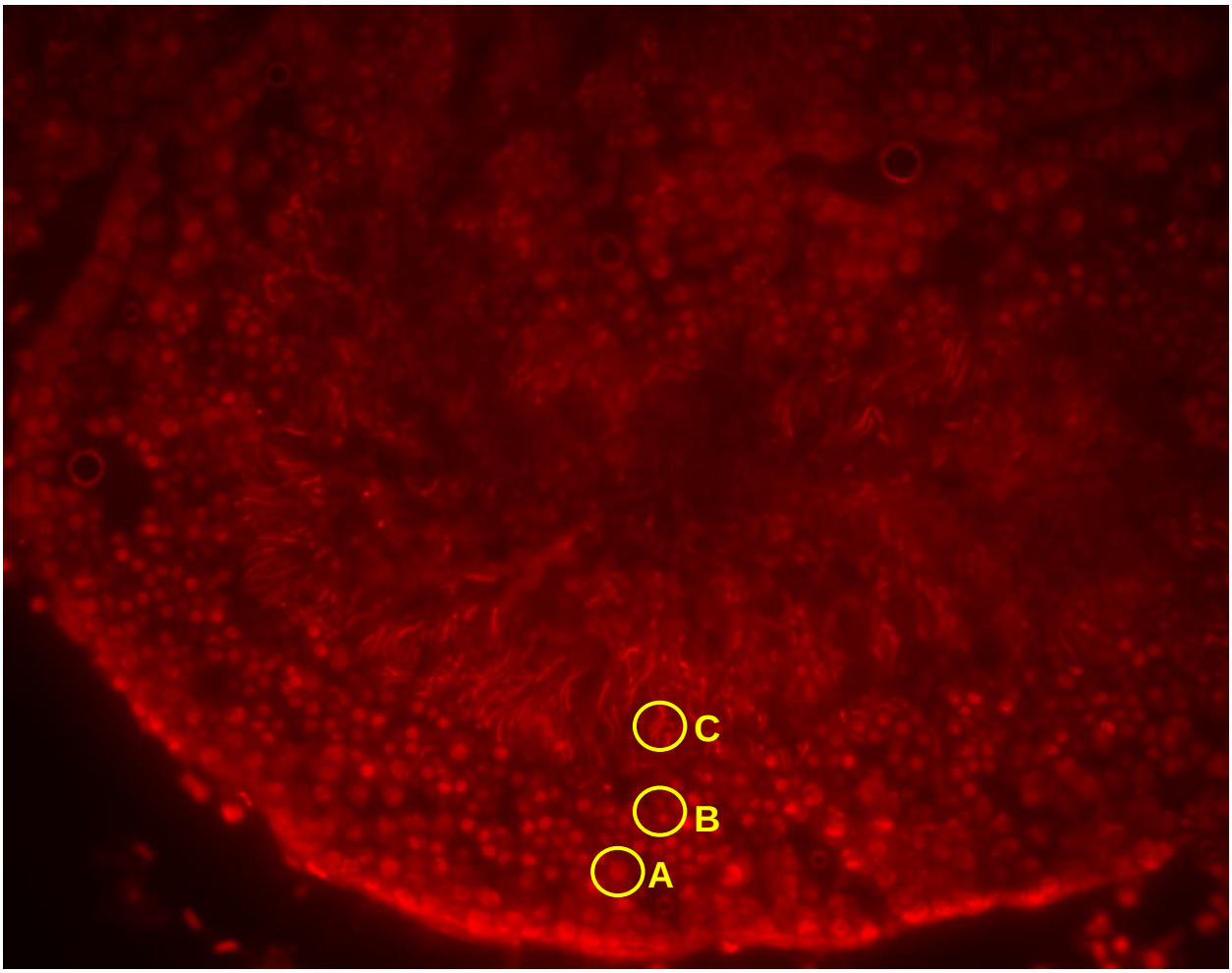


Figure 3-1: Cross section of Galloanserae male seminiferous tubule stained with propidium iodide and imaged at 400X. We were able to consistently categorize three types of spermatogenic cells: (A) spermatogenic precursor cells, the pool of spermatogonia and spermatocytes, found closest to the outer wall of the tubule; (B) round primary spermatids; and (C) elongate secondary spermatids within the lumen of the tubule.

Appendix 1. Species harvested, their Order and Mating System designation and the number sampled.

Species	**Order**	**Mating System**	**Number in Sample**
Mallard (*Anas platyrhynchos)*	Anseriformes	Polygamous	12
Cinnamon teal (*Anas cyanoptera*)	Anseriformes	Polygamous	10
Gadwall (*Anas strepera*)	Anseriformes	Polygamous	3
Northern shoveler (*Anas clypeata*)	Anseriformes	Polygamous	2
Mute swan (*Cygnus olor*)	Anseriformes	Monogamous	4
Wild turkey (*Meleagris gallopavo*)	Galliformes	Polygamous	11
Ring-necked pheasant (*Phasianus colchicus*)	Galliformes	Polygamous	7
Greater sage-grouse (*Centrocercus urophasianus*)	Galliformes	Polygamous	4
Chukar (*Alectoris chukar*)	Galliformes	Monogamous	4
California quail (*Callipepla californica*)	Galliformes	Monogamous	2

Table 3-1. Relative mass (g) of left testis versus right testis of individual males organized by mating systems and taxonomic groups of interest. P-values represent paired *t*-tests of data pairs consisting of the left testis and the right testis within individual males such that positive t-values with significant p-values indicate greater relative mass of the left testis within males. Specifically, for each male within a group of interest, we calculated (*left testis mass - right testis mass*)*/*(*left testis mass + right testis mass*) and compared the resulting group mean to a null hypothesis mean of zero representing no relative difference between the left testis and right testis of males.

TAXONOMIC STATUS AND MATING SYSTEM	N[1]	DF[2]	MEAN	SE[3]	t-value	p-value
GALLOANSERAE						
All Males Pooled	54	53	0.107	0.109	5.553	**<0.001**
Polygamous Waterfowl and Gamefowl	44	43	0.068	0.018	3.770	**<0.001**
Monogamous Waterfowl and Gamefowl	10	9	0.277	0.031	8.979	**<0.001**
ANSERIFORMES						
All Males Pooled	29	28	0.093	0.030	3.135	**0.004**
Polygamous Waterfowl						
Mallard	12	11	0.044	0.035	1.242	0.240
Cinnamon Teal	8	7	0.047	0.048	0.983	0.358
Gadwall	3	2	0.120	0.105	1.147	0.370
Northern Shoveler	2	1	0.011	0.001	1.000	0.500
Monogamous Waterfowl						
Mute Swan	4	3	0.351	0.045	7.795	**0.004**
GALLIFORMES						
All Males Pooled	25	24	0.123	0.024	5.204	**<0.001**
Polygamous Gamefowl						
Wild Turkey	11	10	0.142	0.033	4.334	<**0.001**
Ring-necked Pheasant	4	3	0.081	0.035	2.309	0.082
Greater Sage-Grouse	4	3	-0.019	0.033	-0.585	0.600
Monogamous Gamefowl						
Chukar	4	3	0.172	0.016	10.608	**<0.001**
California Quail	2	1	0.171	0.029	6.000	0.105

[1]N: Number in sample
[2]DF: Degrees of Freedom
[3]SE: Standard Error

Table 3-2. Degree of bilateral asymmetry in testicular attributes between left and right testes within Galloanserae males as functions of avian order and mating system. Values represent 1-way ANOVA comparisons between groups of interest. For avian orders, positive values indicate a greater degree of bilateral asymmetry within Anseriformes relative to Galliformes. For mating systems, positive values indicate greater degree of bilateral asymmetry within monogamous species relative to polygamous species.

			Anseriformes versus Galliformes			Monogamous versus Polygamous		
Testicular Attribute	**N[1]**	**DF[2]**	**MSF[3]**	**F[4]**	**P[5]**	**MSF[3]**	**F[4]**	**P[5]**
Testis Mass[a]	54	1, 52	0.012	0.615	0.436	0.355	26.178	**<0.001**
Seminiferous Tubule Area[b]	59	1, 57	0.019	0.437	0.511	0.020	0.055	0.815
Total Spermatogenic Cell Density[c]	59	1, 57	0.020	1.174	0.283	0.060	3.791	0.056
Spermatogonia & Spermatocyte Density[d]	59	1, 57	<0.001	1.391	0.243	<0.001	1.690	0.199
Total Spermatid Cell Density[e]	59	1, 57	0.020	1.797	0.185	0.050	3.841	**0.052**
Primary Spermatid Cell Density[f]	59	1, 57	<0.001	0.141	0.709	<0.001	4.739	**0.034**
Secondary Spermatid Cell Density[g]	59	1, 57	0.020	2.767	0.102	0.010	1.519	0.223
Testis-wide Secondary Spermatids[h]	54	1, 52	0.002	0.049	0.825	0.584	15.992	**<0.001**

[a] mass (g)
[b] area generated by measured linear distance (mm) traced around tubule boundary, area calculated within ImageJ program *(MSF = X x 10^3)*
[c] all spermatogenic cells / measured polygon volume (μm^3) *(MSF = X x 10^3)*
[d] pooled spermatogonia and spermatocytes / measured polygon volume (μm^3) *(MSF = X x 10^3)*

[e] total (primary + secondary) spermatids / measured polygon volume (μm^3) *(MSF = X x 10^3)*
[f] primary spermatids / measured polygon volume (μm^3) *(MSF = X x 10^3)*
[g] secondary spermatids / measured polygon volume (μm^3) *(MSF = X x 10^3)*
[h] testis volume ($4/3\pi(width)^2$ x length) in mm^3 x (secondary spermatids per mm^3) (*cells/testis*)
[1]N: Number in sample
[2]DF: Degrees of Freedom
[3]MSF: Mean Squares Factor
[4]F: Fisher statistic
[5]P: Probability statistic

Table 3-3. Mean seminiferous tubule area (mm^2) difference between left versus right testes within individual males organized by mating systems and taxonomic groups of interest. P-values represent paired *t*-tests of data pairs consisting of the left testis mean tubule area versus the right testis mean tubule area of individual males such that positive t-values with significant p-values indicate greater seminiferous tubule area within the left testis of males. Specifically, for each male within a group of interest, we calculated the ((*left testis mean seminiferous tubule area - right testis mean seminiferous tubule area*) / (*left testis mean seminiferous tubule area + right testis mean seminiferous tubule area* / 2)) and compared the resulting group mean to a null hypothesis mean of zero representing no relative difference between the left testis and right testis of males.

TAXONOMIC STATUS AND MATING SYSTEM	N	DF	MEAN	SE	t-value	p-value
GALLOANSERAE						
All Males Pooled	59	58	0.199	0.027	7.27	**<0.001**
Polygamous Waterfowl and Gamefowl	49	48	0.196	0.031	6.37	**<0.001**
Monogamous Waterfowl and Gamefowl	10	9	0.213	0.061	3.50	**0.007**
ANSERIFORMES						
All Males Pooled	31	30	0.216	0.040	5.39	<**0.001**
Polygamous Waterfowl						
Mallard	12	11	0.343	0.054	6.30	**<0.001**
Cinnamon Teal	10	9	0.101	0.034	2.94	**0.016**
Gadwall	3	2	0.141	0.077	1.83	0.209
Northern Shoveler	2	1	-0.166	0.212	-0.78	0.577
Monogamous Waterfowl						
Mute Swan	4	3	0.370	0.108	3.42	**0.042**
GALLIFORMES						
All Males Pooled	28	27	0.180	0.037	4.83	**<0.001**
Polygamous Gamefowl						
Wild Turkey	11	10	0.222	0.088	2.52	**0.030**
Ring-necked Pheasant	7	6	0.107	0.023	4.72	**0.003**
Greater Sage-Grouse	4	3	0.111	0.150	7.19	**0.006**
Monogamous Gamefowl						
Chukar	4	3	0.131	0.044	2.96	0.060
California Quail	2	1	0.065	0.017	3.72	0.167

Table 3-4. Mean difference in density (cells per μm^3) of total spermatogenic cells between the left testis versus the right testis within individual males organized by mating systems and taxonomic groups of interest. P-values represent paired *t*-tests of data pairs consisting of the left testis mean total spermatogenic cell density versus the right testis mean total spermatogenic cell density of individual males such that positive t-values with significant p-values indicate greater total spermatogenic cell density within the left testis of males. Specifically, for each male within a group of interest, we calculated the (*left testis mean total spermatogenic cell density*) - *right testis mean total spermatogenic cell density (cells per* μm^3) and compared the resulting group mean to a null hypothesis mean of zero representing no relative difference between the left testis and right testis of males.

TAXONOMIC STATUS AND MATING SYSTEM	N	DF	MEAN	SE	t-value	p-value
GALLOANSERAE						
All Males Pooled	59	58	3.90	0.52	7.505	**<0.001**
Polygamous Waterfowl and Gamefowl	49	48	3.26	0.59	5.527	**<0.001**
Monogamous Waterfowl and Gamefowl	10	9	6.15	0.98	6.262	<**0.001**
ANSERIFORMES						
All Males Pooled	31	30	4.16	0.85	4.887	<**0.001**
Polygamous Waterfowl						
Mallard	12	11	2.85	1.53	1.860	0.089
Cinnamon Teal	10	9	7.31	0.61	12.048	<**0.001**
Gadwall	3	2	-1.10	2.59	-0.423	0.713
Northern Shoveler	2	1	4.11	1.65	2.487	0.243
Monogamous Waterfowl						
Mute Swan	4	3	6.31	1.04	6.077	**0.009**
GALLIFORMES						
All Males Pooled	28	27	3.29	0.62	5.326	**<0.001**
Polygamous Gamefowl						
Wild Turkey	11	10	1.27	0.61	2.068	0.065
Ring-necked Pheasant	7	6	3.55	0.98	3.626	**0.011**
Greater Sage-Grouse	4	3	4.57	1.59	1.860	0.089
Monogamous Gamefowl						
Chukar	4	3	8.12	0.13	6.266	**0.008**
California Quail	2	1	1.60	0.11	1.439	0.387

[1] Mean and SE expressed as density (cells per μm^3) x 10^3

Table 3-5. Mean difference in density (cells per μm^3) of spermatogonia and spermatocytes between the left testis versus the right testis within individual males organized by mating systems and taxonomic groups of interest. P-values represent paired *t*-tests of data pairs consisting of the left testis mean of spermatogonia and spermatocytes cell density versus the right testis mean spermatogonia and spermatocyte cell density of individual males such that positive *t*-values with significant p-values indicate greater density of spermatogonia and spermatocytes within the left testis of males. Specifically, for each male within a group of interest, we calculated the (*left testis mean* (spermatogonia + spermatocytes cells per μm^3)) - (*right testis mean* (spermatogonia + spermatocytes cells per μm^3)) and compared the resulting group mean to a null hypothesis mean of zero representing no relative difference between the left testis and right testis of males.

TAXONOMIC STATUS AND MATING SYSTEM	N	DF	MEAN[1]	SE[1]	t-value	p-value
GALLOANSERAE						
All Males Pooled	59	58	0.44	0.16	2.846	**0.006**
Polygamous Waterfowl and Gamefowl	49	48	0.35	0.18	1.997	**0.052**
Monogamous Waterfowl and Gamefowl	10	9	0.89	0.29	3.101	**0.013**
ANSERIFORMES						
All Males Pooled	31	30	0.27	0.22	1.223	0.231
Polygamous Waterfowl						
Mallard	12	11	-0.30	0.48	-0.618	0.549
Cinnamon Teal	10	9	0.92	0.13	7.197	<**0.001**
Gadwall	3	2	-0.28	0.38	-0.732	0.541
Northern Shoveler	2	1	0.59	0.53	1.113	0.466
Monogamous Waterfowl						
Mute Swan	4	3	0.58	0.38	1.549	0.219
GALLIFORMES						
All Males Pooled	28	27	0.63	0.22	2.917	<**0.001**
Polygamous Gamefowl						
Wild Turkey	11	10	0.15	0.37	0.419	0.065
Ring-necked Pheasant	7	6	1.15	0.47	2.434	**0.011**
Greater Sage-Grouse	4	3	0.36	0.17	2.069	0.089
Monogamous Gamefowl						
Chukar	4	3	1.58	0.41	3.828	**0.008**
California Quail	2	1	0.34	0.27	1.257	0.387

[1] Mean and SE expressed as density (cells per μm^3) x 10^3

Table 3-6. Mean difference in density (cells per μm^3) of total spermatids (round haploid spermatids and elongate secondary spermatids) between the left testis versus the right testis within individual males organized by mating systems and taxonomic groups of interest. P-values represent paired *t*-tests of data pairs consisting of the left testis mean total spermatid cell density versus the right testis mean total spermatid cell density of individual males such that positive t-values with significant p-values indicate greater total spermatid cell density within the left testis of males. Specifically, for each male within a group of interest, we calculated **(***left testis mean total spermatids* (μm^3)) – (*right testis mean total spermatids* (μm^3)) and compared the resulting group mean to a null hypothesis mean of zero representing no relative difference between the left testis and right testis of males.

TAXONOMIC STATUS AND MATING SYSTEM	N	DF	MEAN[1]	SE[1]	t-value	p-value
GALLOANSERAE						
All Males Pooled	59	58	0.21	0.03	7.535	<**0.001**
Polygamous Waterfowl and Gamefowl	49	48	0.18	0.03	5.858	<**0.001**
Monogamous Waterfowl and Gamefowl	10	9	0.38	0.05	7.972	<**0.001**
ANSERIFORMES						
All Males Pooled	31	30	0.23	0.04	5.479	**<0.001**
Polygamous Waterfowl						
Mallard	12	11	0.16	0.08	1.940	0.078
Cinnamon Teal	10	9	0.34	0.03	10.829	<**0.001**
Gadwall	3	2	-0.05	0.12	-0.412	0.720
Northern Shoveler	2	1	0.16	0.04	3.882	0.160
Monogamous Waterfowl						
Mute Swan	4	3	0.41	0.04	10.726	**0.002**
GALLIFORMES						
All Males Pooled	28	27	0.20	0.04	5.122	**<0.001**
Polygamous Gamefowl						
Wild Turkey	11	10	0.09	0.05	1.811	0.100
Ring-necked Pheasant	7	6	0.15	0.06	2.377	**0.055**
Greater Sage-Grouse	4	3	0.34	0.11	2.242	**0.048**
Monogamous Gamefowl						
Chukar	4	3	0.45	0.07	6.368	**0.008**
California Quail	2	1	0.18	0.10	1.824	0.319

[1] Mean and SE expressed as density (cells per μm^3) x 10^3

Table 3-7. Mean difference in density (cells per μm^3) of primary spermatids (round haploid spermatids) between the left testis versus the right testis within individual males organized by mating systems and taxonomic groups of interest. P-values represent paired *t*-tests of data pairs consisting of the left testis mean primary spermatid cell density versus the right testis mean primary spermatid cell density of individual males such that positive t-values with significant p-values indicate greater primary spermatid cell density within the left testis of males. Specifically, for each male within a group of interest, we calculated (*left testis mean primary spermatids* (μm^3)) –(*right testis mean primary spermatids* (μm^3)) and compared the resulting group mean to a null hypothesis mean of zero representing no relative difference between the left testis and right testis of males.

TAXONOMIC STATUS AND MATING SYSTEM	N	DF	MEAN[1]	SE[1]	t-value	p-value
GALLOANSERAE						
All Males Pooled	59	58	1.27	0.23	5.480	<**0.001**
Polygamous Waterfowl and Gamefowl	49	48	6.66	0.31	23.646	<**0.001**
Monogamous Waterfowl and Gamefowl	10	9	8.47	0.84	10.105	<**0.001**
ANSERIFORMES						
All Males Pooled	31	30	0.65	0.33	4.149	**<0.001**
Polygamous Waterfowl						
Mallard	12	11	0.95	0.67	1.429	0.181
Cinnamon Teal	10	9	0.97	0.48	4.365	**0.002**
Gadwall	3	2	0.09	0.65	0.145	0.890
Northern Shoveler	2	1	1.14	0.43	2.687	0.227
Monogamous Waterfowl						
Mute Swan	4	3	1.73	0.55	3.166	**0.051**
GALLIFORMES						
All Males Pooled	28	27	1.18	0.33	3.525	**0.002**
Polygamous Gamefowl						
Wild Turkey	11	10	0.04	0.40	0.092	0.928
Ring-necked Pheasant	7	6	1.49	0.65	2.308	0.060
Greater Sage-Grouse	4	3	1.38	0.43	3.252	**0.047**
Monogamous Gamefowl						
Chukar	4	3	3.84	0.48	7.936	**0.004**
California Quail	2	1	0.63	0.59	1.078	0.476

[1] Mean and SE expressed as density (cells per μm^3) x 10^3

Table 3-8. Mean difference in density (cells per μm^3) of secondary spermatids (elongate secondary spermatids) between the left testis versus the right testis within individual males organized by mating systems and taxonomic groups of interest. P-values represent paired *t*-tests of data pairs consisting of the left testis mean secondary spermatid cell density versus the right testis mean secondary spermatid cell density of individual males such that positive t-values with significant p-values indicate greater secondary spermatid cell density within the left testis of males. Specifically, for each male within a group of interest, we calculated (*left testis mean secondary spermatids* (elongate secondary spermatids per μm^3)) - *right testis mean secondary spermatids* (elongate secondary spermatids per μm^3)) and compared the resulting group mean to a null hypothesis mean of zero representing no relative difference between the left testis and right testis of males.

TAXONOMIC STATUS AND MATING SYSTEM	N	DF	MEAN[1]	SE[1]	t-value	p-value
GALLOANSERAE						
All Males Pooled	59	58	2.04	0.32	6.332	<**0.001**
Polygamous Waterfowl and Gamefowl	49	48	1.86	0.37	5.044	<**0.001**
Monogamous Waterfowl and Gamefowl	10	9	2.91	0.53	5.453	<**0.001**
ANSERIFORMES						
All Males Pooled	31	30	2.54	0.53	4.793	**<0.001**
Polygamous Waterfowl						
Mallard	12	11	1.52	0.90	1.691	0.119
Cinnamon Teal	10	9	4.30	0.60	7.164	<**0.001**
Gadwall	3	2	-1.10	1.94	-0.567	0.628
Northern Shoveler	2	1	2.31	1.47	1.569	0.361
Monogamous Waterfowl						
Mute Swan	4	3	4.01	0.42	9.458	**0.002**
GALLIFORMES						
All Males Pooled	28	27	1.48	0.32	4.643	<**0.001**
Polygamous Gamefowl						
Wild Turkey	11	10	0.97	0.35	2.760	**0.020**
Ring-necked Pheasant	7	6	0.92	0.70	1.327	0.233
Greater Sage-Grouse	4	3	2.82	1.02	2.762	0.070
Monogamous Gamefowl						
Chukar	4	3	2.70	0.79	3.398	**0.043**
California Quail	2	1	1.13	1.50	0.750	0.590

[1] Mean and SE expressed as density (cells per μm^3) x 10^3

Table 3-9. Testis-wide differences in number of secondary spermatids (elongate secondary spermatids) between the left testis versus the right testis within individual males organized by mating systems and taxonomic groups of interest. *P*-values represent paired *t*-tests of data pairs consisting of the estimated secondary spermatid population of the left testis versus the estimated secondary spermatid population of the right testis of individual males such that positive t-values with significant p-values indicate a greater secondary spermatid population within the left testis of males. For each male within a group of interest, we calculated ((*left testis secondary spermatid density)/*(*left testis volume/volume measured) – (right testis secondary spermatid density/(right testis volume/volume measured))* and compared the resulting group mean to a null hypothesis mean of zero representing no relative difference between the left testis and right testis of males.

TAXONOMIC STATUS AND MATING SYSTEM	N	DF	MEAN	SE	t-value	p-value
GALLOANSERAE						
All Males Pooled	54	53	0.241	0.029	8.178	<**0.001**
Polygamous Waterfowl and Gamefowl	44	43	0.191	0.029	6.576	<**0.001**
Monogamous Waterfowl and Gamefowl	10	9	0.459	0.058	7.978	**<0.001**
ANSERIFORMES						
All Males Pooled	29	28	0.235	0.047	4.994	**<0.001**
Polygamous Waterfowl						
Mallard	12	11	0.191	0.091	2.093	0.060
Cinnamon Teal	8	7	0.237	0.030	7.939	<**0.001**
Gadwall	3	2	0.121	0.114	1.065	0.480
Northern Shoveler	2	1	0.045	0.141	0.317	0.804
Monogamous Waterfowl						
Mute Swan	4	3	0.544	0.072	7.534	**0.005**
GALLIFORMES						
All Males Pooled	25	24	0.248	0.034	7.331	<**0.001**
Polygamous Gamefowl						
Wild Turkey	11	10	0.211	0.035	6.088	<**0.001**
Ring-necked Pheasant	4	3	0.182	0.064	2.827	0.066
Greater Sage-Grouse	4	3	0.185	0.103	1.805	0.169
Monogamous Gamefowl						
Chukar	4	3	0.511	0.055	9.210	**0.003**
California Quail	2	1	0.186	0.071	2.616	0.232

[1] *Mean and SE expressed as density (cells per μm^3) x 10^3*

CHAPTER FOUR

SPERM PRODUCTION IN WATERFOWL AND GAMEFOWL AND ITS RELATIONSHIP TO MATING SYSTEM AND PRESENCE OF AN INTROMITTENT PHALLUS

The transmission of sperm from males to females through copulation is a critical event in avian reproduction and subject to diverse behavioral, morphological, and physiological influences that determine the paternity of subsequent offspring. Even after copulation, strong sexual selection can persist. For example, sperm competition in which sperm from two or more males is present simultaneously within a female appears to be a common condition in birds based on the high frequency of mixed paternity observed across avian taxa (Martin & Hannon 1988, Lifjeld & Robertson 1992, Sheldon & Burke 1994, Wagner *et al.* 1996, Griffith *et al.* 2002, Hunter *et al.* 2000, Abbott *et al.* 2006, van Oers *et al.* 2008). Recent evidence indicates that male sperm availability is subject to important constraints, especially under conditions of sperm competition (Moller 1991, Hunter *et al.* 2000, Torok *et al.* 2003, Smith 2012). Time and physiological investment are necessary to produce sperm. Sperm depletion can occur when breeding success requires high copulation frequency or high numbers of sperm to be transmitted. Underlying these evolutionary dynamics is testicular sperm production. Quantifying testicular sperm production among species that vary in the likelihood of sperm competition and vary in male reproductive morphology could yield insight into evolutionary responses of male birds subject to sexual selection.

Male birds compete for reproduction in complex ways that serve to thwart sperm competition. For example, males may seek to monopolize sexual access to females (Emlen *et al.* 1977, Briskie 1992, Currie *et al.* 1998, Wagner 1998), time copulation to coincide with female fertility (Birkhead *et al.* 1993), transfer greater numbers of sperm during copulation relative to rival males (Lifjeld *et al.* 1994, Krokene *et al.* 1996), deliver sperm to vaginal sperm storage ducts (Biellier *et al.* 1961, Christensen & Bagley 1989), and deliver sperm capable of fertilizing ova under conditions of sperm competition (Birkhead 2000). Female birds may overtly or surreptitiously seek copulation with preferred males or repel copulation from unwanted males (Eberhard 1998). Females also may thwart the effects of sperm transfer by expelling ejaculate (Davies 1983) or causing ejaculate to be deposited into diverticula within the vagina where it is poorly positioned for fertilization (Brennan *et al.* 2007).

In most, but not all, avian species, females appear to be able to control whether or not copulation occurs, probably as an outcome of the evolutionary loss of the intromittent phallus among neognathus birds (Briskie & Montgomorie 1998, 2001, Brennan *et al.* 2008). An important exception to female control of copulation occurs in the waterfowl (Anseriformes). Waterfowl, together with the gamefowl (Galliformes), form a monophyletic group (Galloanserae) basal to the neognathous birds that comprise approximately 98% of living species (Briskie & Montgomerie 2001, Hackett *et al.* 2008). Waterfowl males retain the ancestral condition of possessing an intromittent phallus and these males are known to engage in forced copulation with females (McKinney *et al.* 1983, Gowaty *et al.* 1998). Male gamefowl possess a small, non-intromittent phallus and are not known to engage in forced copulation.

Galloanserae not only exhibits important variation in the phallus, the taxon contains both socially polygamous and socially monogamous species providing an opportunity to examine testicular sperm production relative to social mating system. Overall, polygamous mating systems may present a greater likelihood of sperm competition than monogamy simply because multiple sexual partners are intrinsic to polygamy. However, many factors influence the prevalence of sperm competition. Female birds that are highly dependent on male assistance for incubation or care of young are more likely to exhibit sexual fidelity to males (Gowaty & Buschhaus 1998). Degree of sexual fidelity varies widely between species, but low levels of extra-pair paternity can be found in monogamous (Petrie & Kempenaers 1998, Westneat 1987) polygynous (Hasselquist *et al.* 1996), and even serially polyandrous species (Delehanty *et al.* 1998). Sperm production within Galloanserae males that form long-term, monogamous pairbonds provides in interesting comparison to males that form multiple consortships characteristic of polygamy in Galloanserae.

Recent analysis of the testes of monogamous and polygamous male waterfowl and gamefowl indicates that the left and right testes within Galloanserae males are not simple analogues of each other. Within Galloanserae males, the left testis has a higher density of spermatogenic cells than the right testis. This compositional difference is in addition to previously described size and mass differences between the left and right testes within male birds. For example, we observed that in Galloanserae males the left testis is on average 30% larger than the right testis in mass and volume. Combining the newly recognized bilateral asymmetry in spermatogenic cell density between the left and right testes of Galloanserae males with the previously recognized bilateral asymmetry in

size and mass of testes provides an opportunity to make precise estimates of net bilateral sperm production capacity within the basal taxon of neognathous birds where the evolutionarily transition to copulation without an intromittent phallus occurred.

Our aim was to incorporate the bilateral differences in testicular density of spermatogenic cells with the bilateral differences in testes size and mass in order to calculate the mean spermatogenic cell density per unit volume of the testes within individual Galloanserae males. This measurement of sperm production capacity per unit of testicular tissue would then allow us to compare sperm production between Galliformes and Anseriformes species and between monogamous and polygamous species. Because the Galloanserae represent the point of transition to new male copulatory morphology, namely the absence of an intromittent phallus, Galloanserae is a relevant group to study the relationship between sperm production and sexual selection. We hypothesized that overall sperm production capacity would be related to mating system, with polygamous species having higher spermatogenic cell densities, and also by presence or absence of an intromittent phallus, hypothesizing that spermatogenic cell density would increase with reduction of phallus and thus be greater in Galliformes than Anseriformes.

Methods

To investigate differences in spermatogenic cell density between mating systems and between those species that possess an intromittent copulatory phallus and those that do not, we collected 54 males of 20 species within the Anseriformes (intromittent copulatory phallus) and Galliformes (non-intromittent phallus), such that within each order we had representative monogamous and polygamous species. We have described study methods in detail previously (Chapter 3). Briefly, we harvested all individuals

under ISU Institutional Animal Care and Use Committee (IACUC) protocol# FRP-6350408. During the breeding season, from approximately 15 March through 30 June of 2008 through 2012, we collected males in breeding condition by lethal collection (firearm).

Immediately upon collection, we weighed each bird, measured *in situ* and *in vitro* length (mm) and width (mm) of the left and right testis at the longest and widest and weighed each testis for wet mass (mg).

We measured the histological composition of sperm producing tissue in left versus right testes within sexually mature male birds. We progressively fixed the testicular tissue by immersion neutral buffered formalin followed by immersion in 10% sucrose PBS and finally 30% sucrose PBS solution. When fully fixed, the testis was either processed and imaged immediately, or stored in 70% ETOH until imaged.

To image testis sections, we imbedded randomly selected ½ cm^2 testis sections in Tissue-Tek® O.C.T (optimum cutting temperature) Compound (Sakura Finetek, Torrance, CA) to create tissue slides for histological analysis. Subsamples, one each from the anterior third, one from the middle third, and one from the posterior third of the testis, were cut to 10 μm, and placed on precleaned glass slides.

For histological imaging and analysis, we stained slides with propidium iodide in glycerol. We created digital images of seminiferous tubules using 400x magnification. We imaged 5 seminiferous tubules per section, for a total of 15 images within each testis. Seminiferous tubules were located haphazardly from tissue sections, but we used seminiferous tubules that were sectioned as orthogonal cross-sections with clearly visible cellular components.

Using Image-J (NIH, Bethesda, MD) software (Abramoff *et al.* 2004, Rasband 2014), we measured diameter, circumference and area in cross section of tubules. We then overlaid a grid of 5000 μm^2 (3.83 pixels/ μm^2) line grid over the tubule, from which we generated a polygon using the area of the grid closest to the midline of the tubule such that the top and bottom of the area to be counted was bordered by gridlines, and the sides encompassed the borders of the tubule. We calculated the area of each polygon, and counted three categories of reproductive cells within the polygon boundary. Cell type was identified by size and shape. Spermatogonia and spermatocytes (primary and secondary) were large, round cells found toward the outer margin of the tubule. Primary spermatids were small, round cells located well within the tubule, and secondary spermatids were elongate cells found closest to the lumen of the tubule.

Statistics

We determined the densities of each spermatogenic cell type for each testis calculated as the number of cells per um^3. We then calculated the proportion each testis contributed to the total testis size (single testis volume / total volume of both testes). The mean density of spermatogenic cells of interest could then be determined by weighting each testis by its size and the density of each cell type within that testis.

We generated comparisons of total reproductive cell densities within seminiferous tubules between monogamous and polygamous species, and between Anseriformes and Galliformes by one-way ANOVA.

Results

Total spermatogenic cell density, composed of spermatogenic cells and primary and secondary spermatids, was significantly greater in waterfowl (Anseriformes) males

than gamefowl (Galliformes) males (Table 4-1). Waterfowl males exhibited a mean density of 0.0225 ± 0.0152 spermatogenic cells per μm^3 versus a mean density of 0.0179 ± 0.0083 spermatogenic cells per μm^3 for game bird males. This overall difference in spermatogenic cell density was driven by significantly greater density of spermatids in waterfowl than in gamefowl (Table 4-1) and particularly by significantly greater density of secondary spermatids in waterfowl relative to gamefowl (Table 4-1). Mean total spermatid cell density/μm^3 was 0.0183 ± 0.0104 for waterfowl versus 0.0139 ± 0.0051spermatid cells per μm^3 for gamefowl. Difference in secondary spermatid densities between waterfowl and gamefowl was even more pronounced with densities of 0.0111 ± 0.004 secondary spermatids per μm^3 versus 0.0075 ± 0.0015 secondary spermatids per μm^3, respectively. We did not detect significant differences between waterfowl and gamefowl in densities of precursor spermatogenic cells (spermatogonia and primary and secondary spermatocytes), nor did we detect a significant difference between waterfowl and gamefowl in primary spermatids. However, in all cases we tallied numerically greater densities among waterfowl than gamefowl (Table 4-1).

Total spermatogenic cell density was also significantly greater in polygamous than monogamous species (Table 4-1). Polygamous species had a mean density of 0.0208 ± 0.0203 spermatogenic cells per μm^3, whereas monogamous species had a mean density of 0.0177 ± 0.0032 spermatogenic cells per μm^3. This overall difference in spermatogenic cell density was driven by significantly greater density of secondary spermatids in polygamous (0.0101± 0.0051 secondary spermatids per μm^3) than in monogamous (0.0066 ± 0.0004 secondary spermatids per μm^3) species (Table 4-1). We did not detect significant differences between monogamous species and polygamous

species in densities of precursor spermatogenic cells (spermatogonia and primary and secondary spermatocytes). Nor did we detect a significant difference between mating systems in primary spermatids. However, in all cases we tallied numerically greater densities among polygamous species than in monogamous species.

Discussion

We measured the total bilateral mean spermatogenic cell densities per unit testicular tissue of male Galloanserae birds, incorporating new information that the left and right testes of Galloanserae males do not simply differ in size, but also differ between the left and right testes in density of spermatogenic cells. We used Galloanserae males because the taxon forms the monophyletic basal group of neognathus birds and also encompasses the evolutionary loss of the intromittent male phallus. Galloanserae is composed exclusively of the waterfowl (Anseriformes) for which males possess an intromittent phallus and the gamefowl (Galliformes) for which males possess a small non-intromittent phallus. Because Galloanserae also contains both monogamous and polygamous waterfowl and gamefowl, we were able to examine mean spermatic cell densities relative to social mating system as well as relative to the evolutionary loss of an intromittent copulatory phallus.

We found greater sperm production to be associated with waterfowl relative to gamefowl and with polygamy relative to monogamy. In both cases, higher densities of spermatogenic cells appeared to be driven particularly by significantly greater densities of secondary spermatids within seminiferous tubules. Secondary spermatids are haploid gametic cells in which the gametic cell body has begun to elongate to take the form of a mature sperm cell. Developmentally, secondary spermatids are close to being shed from the lining of the seminiferous tubule and transferred to epididymis to be stored for future

ejaculation. In other words, waterfowl, which retain an intromittent phallus and place sperm deep into vaginal portion of the female oviduct during copulation, produce more sperm per unit of testicular tissue than do gamefowl, which do not possess an intromittent phallus and place sperm more superficially onto the vaginal orifice of the oviduct. We also found that polygamous species regardless of taxonomic order, produced more sperm per unit of testicular tissue than did monogamous species.

The complex reproductive morphology of male and female waterfowl has been well documented and clearly many waterfowl species are subject to strong sexual selection and sperm competition. But we observed high spermatic cell densities not only within the so-called 'puddle ducks' known for complex intromittent phalluses among males, forced extra-pair copulations, and serial polygamy, but also among mute swans (*Cygnus olor*) known for their long-term monogamous pairbonds and high degree of parental cooperation. Relative to the closely related gamefowl, high sperm production appears to be intrinsic to waterfowl despite a morphological capacity to place sperm into the female oviduct. This raises the interesting question of whether gamefowl achieve a degree of efficiency in sperm transfer in the absence of an intromittent phallus resulting in relatively lower sperm production. Such an outcome could occur through cooperative copulation behavior or an efficiency in copulatory morphology that has not been explained.

Our observation that greater sperm production is associated with polygamy relative to monogamy is consistent with a large body of literature on sperm competition. Assuming that polygamy results in a generally higher frequency of sperm competition than monogamy as a result of higher frequency of multiple sexual partners by females,

males may benefit from elevated sperm production in order to transmit high numbers of sperm during copulation. Our study contributes to this overall finding but additionally yields evidence of this phenomenon occurring across very different copulatory morphologies among closely related species. In other words, our results speak to the strength of selection for high sperm counts in the face of sperm competition regardless of evolutionary innovations in copulatory morphology or behavior.

Male birds characteristically transmit high numbers of sperm in ejaculates. High sperm count in male ejaculate is thought to promote male fitness not only by ensuring sufficient sperm availability for fertilization but also to defend against sperm competition by a "lottery effect" (Møller & Briskie 1995) to increase the probability that the male's sperm fertilizes ova in the face of sperm competition. Physiologically, males also may produce sperm with competitively advantageous attributes, although males face important tradeoffs in doing so. For example, longer sperm are more successful at fertilizing ova, but take time and physiological investment to produce and may reduce the total numbers of sperm available in an ejaculate or reduce the total number of sperm that can occupy female sperm storage tubules (Gomendio & Roldan 1991, Briskie & Montgomerie 1992, Bennison *et al.* 2015). Additionally, in the process of producing mature sperm from precursor spermatogonia, errors and damage occur. To produce only high quality sperm, sperm number is reduced and time to produce enough sperm for a large ejaculate increased. Presumably, males balance sperm count and sperm quality to be competitive under conditions of sperm competition. The difference that we observed in testicular sperm production are expressions of these evolutionary and physiological dynamics.

Literature Cited

Abbott, C. L., M. C. Double, R. Gales and A. Cockburn (2006). Copulation behaviour and paternity in shy albatrosses (*Thalassarche cauta*). *Journal of Zoology* 270: 628-635.

Alatalo, R. V., K. Gottlander and A. Lundberg (1984). Extra-pair copulations and mate guarding in the polyterritorial Pied Flycatcher, *Ficedula hypoleuca*. *Behaviour* 101: 139-155.

Bennison, C., N. Hemmings, J. Slate and T. Birkhead (2015). Long sperm fertilize more eggs in a bird. In *Proceedings of the Royal Society of London, Series B: Biological Sciences 282.1799 :1897.*

Biellier, H. V., R. Pashcange and M. Funk (1961). Effect of depth of insemination on fertility of Broad Breasted Turkey hens. *British Poultry Science* 40: 1937.

Birkhead, T. (2000). *Promiscuity: an Evolutionary History of Sperm Competition*. Harvard University Press, Boston.

Birkhead T. R and A. P. Møller (1992*). Sperm Competition in Birds: Evolutionary Causes and Consequences*. Academic Press, San Diego.

Birkhead, T. R., F. Fletcher, and E. J. Pellatt (1998). Testes asymmetry, condition and sexual selection in birds: an experimental test. *Proceedings of the Royal Society of London, Series B: Biological Sciences* 265: 1185-1189.

Birkhead T. R., J. E. Pellat and F. M. Hunter (1988). Extra-pair copulation and sperm competition in the zebra finch. *Nature* 334: 60–62.

Blackburn, D. G. and H. E. Evans (1986). Why are there no viviparous birds? *American Naturalist* 128:165-190.

Briskie, J. V. and R. Montgomerie (1992). Sperm size and sperm competition in birds. *Proceedings of the Royal Society of London, Series B: Biological Sciences 247*: 89-95.

Briskie J. V. and R. Montgomerie (1993). Patterns of sperm storage in relation to sperm competition in passerine birds. *Condor* 95: 442–454

Christensen, V. L. and L. G. Bagley (1989). Efficacy of fertilization in artificially inseminated turkey hens. *Poultry Science 68*: 724-729.

Currie, D. R., T. Burke, R. L. Whitney and D. B. A. Thompson (1998). Male and female behaviour and extra-pair paternity in the wheatear. *Animal Behaviour 55*: 689-703.

Davies, N. B. (1983). Polyandry, cloaca-pecking and sperm competition in dunnocks. *Nature 302*: 334-336.

Delehanty, D. J. and P. P. O'Hearn (2005). Behavioral and morphological asymmetries in chukar *Alectoris chukar c*opulation. *Journal of Avian Biology* 36: 276 – 279.

Delehanty, D. J., R. C. Fleischer, M. A. Colwell and L. W. Oring (1998). Sex-role reversal and the absence of extra-pair fertilization in Wilson's phalaropes. *Animal Behaviour* 55: 995-102.

Eberhard, W. G. (1998). Female Roles in Sperm Competition. pp. 91-116 in [T.R. Birkhead and A.P. Møller, Eds]. *Sperm Competition and Sexual Selection. Academic Press, London.*.

Griffith, S. C., I. P. F. Owens and K. A. Thuman (2002). Extra-pair paternity in birds: a review of interspecific variation and adaptive function. *Molecular Ecology* 11: 2195-2212.

Gomendio, M. and E. R. Roldan (1991). Sperm competition influences sperm size in mammals. *Proceedings of the Royal Society of London, Series B: Biological Sciences 243*: 181-185.

Hackett, S. J., R. T. Kimball, S. Reddy, R. C. K. Bowie, E. L. Braun, M. J. Braun, J. L. Chojnowski, W. A. Cox, K. L. Han, J. Harshman, C. J. Huddleston, B. D. Marks, K. J. Miglia, W.S. Moore, F. H. Sheldon, D. W. Steadman, C. C. Witt, and T. Yuri (2008). A phylogenomic study of birds reveals their evolutionary history. *Science* 320: 1763-1768.

Hasselquist, D., S. Bensch and T. von Schantz (1996). Correlation between male song repertoire, extra-pair paternity and offspring survival in the great reed warbler. *Nature 381*: 229-232.

Hunter, F. M., R. Harcourt, M. Wright and L. S. Davis (2000). Strategic allocation of ejaculates by male Adélie penguins. *Proceedings of the Royal Society London, Series B: Biological Sciences* 267: 1541-1545.

Kimball, R. T., J. D. Ligon and M. Merola-Zwartjes (1997). Testicular asymmetry and secondary sexual characters in red junglefowl. *Auk* 114: 221-228.

King, A. S. (1981a). Cloaca, p. 63-106. *In*: [A. S. King and J. McLelland, Eds.] *Form and Function in Birds. Vol 2.* Academic Press, New York.

King, A. S. (1981b). Phallus, p. 107-148. *In*: A. S. King and J. McLelland [eds.] *Form and Function in Birds. Vol 2.* Academic Press, New York.

Kinsky, F. (1971). The consistent presence of paired ovaries in the kiwi (*Apteryx*) with some discussion of this condition in other birds. *Journal für Ornithologie* 112: 334-357.

Krokene, C., K. Anthonisen, J. T. Lifjeld and T. Amundsen (1996). Paternity and paternity assurance behaviour in the bluethroat, *Luscinia s. svecica*. *Animal Behaviour 52*: 405-417.

Lake, P. E. 1981. Male genital organs. p. 1-61. *In*: [A. S. King and J. McLelland, Eds.] *Form and Function in Birds. Vol 2.* Academic Press, New York.

Lifjeld, J. T. and R. J. Robertson. 1992. Female control of extra-pair fertilization in tree swallows. *Behavioral Ecology and Sociobiology* 31: 89-96.

Lifjeld, J. T., P. O. Dunn and D. F. Westneat (1994). Sexual selection by sperm competition in birds: male-male competition or female choice? *Journal of Avian Biology* 25: 244-250.

Ligon, J. D. (1997). A single functional testis as a unique proximate mechanism for promoting sex-role reversal in Coucals. *Auk* 114: 800-801.

Martin, K., and S. J. Hannon (1988). Early pair and extra-pair copulations in willow ptarmigan. *Condor* 90: 245-246.

McKinney, F., S. R. Derrickson and P. Mineau (1983). Forced copulation in waterfowl. *Behaviour 86*: 250-293.

Merila, J. and B. C. Sheldon (1999). Testis size variation in the greenfinch *Carduelis chloris*: relevance for some recent models of sexual selection. *Behavior in Ecology and Sociobiology* 45: 115-123.

Møller, A. P. (1994). Directional selection on directional asymmetry: testes size and secondary sexual characters in birds. *Proceedings of the Royal Society of London Series B: Biological Sciences* 258: 147-151.

Moller, A.P. (1991). Sperm competition, sperm depletion, paternal care, and relative testis size in birds. *American Naturalist* 137: 882-906.

Nyland, K.B., M. P. Lombardo and P.A. Thorpe (2003). Left-sided directional bias of cloacal contacts during house sparrow copulations. *Wilson Bulletin* 115: 470-473.

Petersen, A. A., M. P. Lombardo and H. W. Power (2001). Left–sided directional bias of cloacal contacts during tree swallow copulations. *Animal Behavior* 62: 739-741.

Petrie, M. and B. Kempenaers (1998). Extra-pair paternity in birds: explaining variation between species and populations. *Trends in Ecology & Evolution* 13: 52-58.

Santiago-Moreno, J., M. C. Esteso, C. Castaño, A. Toledano-Díaz and A. López-Sebastián (2015). Post-coital sperm competence in polygamous animals: the role of sperm traits in species–specific strategies. *Andrology* S1: 2167-0250.

Sheldon, B. C. and T. Burke (1994). Copulation behavior and paternity in the chaffinch. *Behavioral Ecology and Sociobiology* 34: 149-156.

Simmons, L. W. and J. S. Kotiaho (2002). Evolution of ejaculates: patterns of phenotypic and genotypic variation and condition dependence in sperm competition traits. *Evolution* 56: 1622-1631.

Smith, R. L. ed., (2012). *Sperm Competition and the Evolution of Animal Mating Systems*. Academic Press, Elsevier, London.

Tae H. J., B. G. Jang, D. C. Ahn, E. Y. Choi, H. S. Kang, N. S., Kim, J. H. Lee, S. Y. Park, H. H. Yang and I. S. Kim (2005). Morphometric studies on the testis of

Korean ring-necked pheasant (*Phasianus colchicus karpowi*) during the breeding and non-breeding seasons. *Veterinary Research Communications*. 29: 629 – 643.

Török, J., G. Michl, L. Z. Garamszegi and J. Barna (2003). Repeated inseminations required for natural fertility in a wild bird population. *Proceedings of the Royal Society of London, Series B: Biological Sciences* 270: 641-647.

Turek, N. K. (2007). Mechanisms and Motivations in Avian Copulation. MS Thesis. Idaho State University, Pocatello, ID.

Van Oers, K., P. J. Drent, N. J. Dingemanse and B. Kempenaers (2008). Personality is associated with extrapair paternity in great tits, *Parus major*. *Animal Behaviour 76*: 555-563.

Wagner, R. H. (1998). Hidden leks: sexual selection and the clustering of avian territories. *Ornithological Monographs:* 123-145.

Wagner, R. H., M. D. Schug and E. S. Morton (1996). Condition-dependent control of paternity by female purple martins: implications for coloniality. *Behavioral Ecology and Sociobiology* 38: 379-389.

Warwick, J. N. (2007). Assessing Functional Bilateral Asymmetry in Chukar (*Alectoris chukar*) Testes. MS Thesis. Idaho State University. Pocatello, ID.

Westneat, D. F., A. B. Clark and K. C. Rambo (1995). Within-brood patterns of paternity and paternal behavior in red-winged blackbirds *Behavioral Ecology and Sociobiology* 37: 349-356.

Table 4-1. Comparisons of total mean spermatogenic cell densities across the bilateral testes of wild male Galloanserae harvested during the breeding season as functions of avian order and mating system. Values represent 1-way ANOVA comparisons between groups of interest. For avian orders, positive values indicate a greater degree of spermatogenic cell density within Anseriformes relative to Galliformes. For mating systems, positive values indicate greater degree of spermatogenic cell density within polygamous species relative to monogamous species.

			Anseriformes versus Galliformes			**Polygamous versus Monogamous**		
Testicular Attribute	**N[1]**	**DF[2]**	**MSF[3]**	**F[4]**	**P[5]**	**MSF[3]**	**F[4]**	**P[5]**
Total Spermatogenic Cell Density[a]	54	1, 52	<0.001	17.160	**<0.001**	0.090	4.298	**0.043**
Diploid Spermatogenic Cell Density[b]	54	1, 52	<0.001	0.397	0.531	<0.001	1.439	0.236
Total Spermatid Cell Density[c]	54	1, 52	<0.001	15.77	**<0.001**	<0.001	2.890	0.095
Primary Spermatid Cell Density[d]	54	1, 52	<0.001	2.146	0.149	<0.001	1.971	0.166
Secondary Spermatid Cell Density[e]	54	1, 52	<0.001	17.115	**<0.001**	0.010	8.850	**0.004**

[a] all spermatogenic cells / (μm^3)
[b] pooled spermatogonia and spermatocytes / (μm^3)
[c] total (primary + secondary) spermatids / (μm^3)
[d] primary spermatids / (μm^3)
[e] secondary spermatids / (μm^3)

[1]N: Number in sample
[2]DF: Degrees of Freedom
[3]MSF: Mean Squares Factor
[4]F: Fisher statistic
[5]P: Probability statistic

SUMMARY

Sexual reproduction and sexual selection creates selective pressure that modifies morphology, physiology and behavior. In birds, males generally exhibit testicular asymmetry, usually with a larger left testis (Calhim & Birkhead 2007, Calhim & Montgomerie 2015). The degree and side-consistency of testicular asymmetry is greater in birds than in other taxa (Yu 1998). Understanding spermatogenesis within asymmentrical testes is relevant to understanding avian breeding biology and life history. Male birds are the product of substantial sexual selection, not only overt such as in mate choice, but also cryptic sexual selection. Sperm competition is a well described form of cryptic sexual selection affecting male birds. Undoubtedly, overt and cryptic sexual selection can influence testicular morphology and spermatogenesis. I carefully investigated the composition of left and right testes of male birds as an outcome of cryptic sexual selection on males to inseminate females and fertilize eggs.

It long has been assumed that bilateral asymmetry between the left and right testes was due to a simple size and mass difference between the two testes in which relative tissue constituents within each testis are constant. However, the testis is a compound gland in which spermatogenic tissues and endocrine tissues arise from different progenitor cells during embryonic gonadal development. Specifically, the cells that eventually become steroidogenic Leydig cells migrate from the mesonephric kidneys and do so prior to testicular colonization by extra-cephalic stem cells that develop into spermatogenic testicular tissue. As a result, selection that acts on sperm production need not necessarily act on the androgen producing tissue of the testis.

In chapter 2, I tested the hypothesis that the asymmetrical left and right testes within chukar were simple analogues of one another, only differing in total size and mass, and found the larger left testis contains larger seminiferous tubules and more spermatogenic cells per unit area than the small right testis. The left and right testes, however, contain equal total Leydig cell abundance. In other words, the larger left testis augments sperm production both through its greater size and the greater density of spermatogenic tissue while the left and right testes remain equal in androgen production. There appears to be selection for increased sperm production from the left testis but no concordant alteration of steroidogenic tissue.

My findings also provide strong evidence that the cytologically and functionally different left and right testes of birds are consistent with sexual selection, which may result in greater sperm production from the left side than the right side. Males, in response to the avian female reproductive tract, experience selection pressures to efficiently place sperm into the oviduct opening, and this likely influences the development of spermatogenic tissues in the testes.

In chapter 3, I expanded my investigation of asymmetry in testicular spermatogenic cell capacity to the avian superorder Galloanserae. The Galloanserae are a monophyletic basal group of birds that also mark the evolutionary point of avian phallus loss. Using the Galloanserae, I investigated the hypothesis that the increased spermatogenic capacity of the left testis arises as an outcome of cryptic selection pressures to deliver sperm. I did so understanding that left-side investment into gonads may be the ancestral condition in birds but also was aware that the left testis, in concert with left-side biased copulation, may be better positioned to place sperm at the opening

of the female's oviduct. Additionally, mating system can result in significant sexual selection on sperm production as a result of sperm competition risk. Namely, mating systems with which females predictably mate with two or more males result in sperm competition.

To investigate the relationship between the differing spermatogenic capacities of left versus right testes, I assessed the difference in mass between left and right testes of males of 10 species within the Galloanserae. I then measured the densities of total spermatogenic cells, pooled spermatogonia and spermatocytes, primary spermatids, and secondary spermatids within each individual to determine the sperm production capacity of left versus right testes within birds and tested whether differences existed in the testicular asymmetry in relation to phallus (order) and/or the risk of sperm competition (mating system).

I found strong evidence for previously unrecognized gonadal asymmetry in testis composition throughout the Galloanserae. As in chukar, the left testis of males in Galloanserae appears to be biased toward sperm production, occurring in both orders, regardless of the presence or absence of an intromittent phallus. However, I found a significant relationship between the degree of bilateral spermatogenic asymmetry and the likelihood that males are subject to sperm competition. Males of species with a high incidence of polygamous mating, and therefore high likelihood of sperm competition, exhibited a greater degree of testis asymmetry in the density of spermatogenic cells than males of species with monogamous mating and a correspondingly lower probability of sperm competition regardless of phallus type or order. Males subject to sperm competition exhibited a high degree of left testis specialization for sperm production.

In the final chapter, I investigated more subtle differences in sperm production within the Galloanserae to try to identify any differences in reproductive strategies that might be related to phallus loss in Galloanserae. I used my findings of different spermatogenic cell densities in the left and right testes to calculate the total bilateral mean spermatogenic cell densities per unit testicular tissue of male Galloanserae. I found overall greater sperm production in male waterfowl relative to male gamefowl and greater overall sperm production in males of polygamous species relative to males of monogamous species. In both cases, higher densities of spermatogenic cells appeared to be driven particularly by significantly greater densities of secondary spermatids within seminiferous tubules. I found waterfowl, which retain an intromittent phallus, produce more sperm per unit of testicular tissue than do gamefowl, which do not possess an intromittent phallus. I also found that polygamous species (regardless of taxonomic order) produced more sperm per unit of testicular tissue than did monogamous species.

This research indicates that high sperm production appears to be intrinsic to waterfowl despite a morphological capacity to place sperm into the female oviduct. This raises the interesting question of whether gamefowl achieve a degree of efficiency in sperm transfer in the absence of an intromittent phallus resulting in relatively lower sperm production. Such an outcome could occur through cooperative copulation behavior or another aspect of reproductive morphology that has not yet been explained.

This work also found that there is greater sperm production in polygamous species relative to monogamous species, which is consistent with previous work on sperm competition. Assuming that polygamy results in a generally higher frequency of sperm competition than monogamy, males benefit from elevated sperm production in

order to transmit high numbers of sperm during copulation. This research not only supports this overall finding but importantly provides evidence of this occurring among closely related species with intromittent and non-intromittent phalluses. It may be that in polygamous species, males have proportionally increased the right testis. Also, the initial asymmetrical colonization of primordial germ cells, with more colonizing the left testis than the right testis, may lay the groundwork for asymmetry which increases with increasing age as a result of Sertoli cell activity. In some of the monogamous species studied, onset of breeding age is delayed, and birds may be four or five years old before successfully breeding.

Perhaps the association of heightened asymmetry with monogamy is a direct result of mating system. Monogamous males with prolonged pair bonds and cooperative copulation behavior evolutionarily may be investing in efficient sperm production by specializing a larger left testis while concurrently reducing the right testis. Alternatively, polygamous males with competitive copulations may be maximizing sperm production by developing otherwise reduced right testes. It may be illuminating to measure the right testis as a proportion of total body mass to try to elucidate if the right testis is increasing in polygamous species, or the left is increasing in monogamous species.

This study found that asymmetry is both testicular size and spermatogenic production. There may be subtle aspects of sperm production generating further asymmetry. Male birds not only transmit high numbers of sperm in ejaculates, but physiologically, males also must produce sperm with competitively advantageous attributes. However, males face important tradeoffs in doing so. For example, longer sperm are more successful at fertilizing ova, but take time and physiological investment

to produce and may reduce the total numbers of sperm available in an ejaculate or reduce the total number of sperm that can occupy female sperm storage tubules. Additionally, in the process of producing mature sperm from precursor spermatogonia, errors and damage occur. To produce only high quality sperm, sperm number is reduced and time to produce enough sperm for a large ejaculate increased. Presumably, males balance sperm count and sperm quality to be competitive under conditions of sperm competition. Here too, testicular sperm production underlies these dynamics.

In short, my findings demonstrate a new level of cryptic sexual selection in birds. In birds, the testes have long been known to exhibit asymmetry in size, with the left testis often the larger of the paired testes. I found that not only a size asymmetry, but an asymmetry in composition that indicates the left testis is higher in spermatogenic capacity. Little is known of the details of spermatogenesis in most bird species. Rate of production, or the time from first division of the spermatogonia and the onset of spermatogenesis to the point of spermiation, is known to be variable among species and can greatly influence the amount of available sperm in an ejaculate. Additionally, recent work by Giannakara *et al.* (2016) has found that risk of sperm competition can alter the speed of spermatogenesis in an individual. Furthermore, little is known about how many ejaculates are immediately available within the reproductive tract of a male. More work is needed investigating the endocrine and juxtacrine production of both Leydig cells and Sertoli cells within the avian testes. Combining these variables with the unknown differences in contribution from the left versus right testis to fertilization success, there are myriad avenues for further study, such as examining the fertilization success of sperm originating from the left testis versus the right testis in symmetric and asymmetric

species, and in those species with a phallus versus those without. In addition, combining bilateral asymmetry in testis spermatogenic composition with potential differences in rates of production of sperm, may elucidate more subtle differences in sperm production strategies related to cryptic sexual selection.

Literature Cited

Calhim, S. and T. R. Birkhead (2007). Testes size in birds: quality versus quantity – assumptions, errors, and estimates. *Behavioral Ecology* 18: 271-275.

Calhim S. and R. Montgomerie (2015). Testis asymmetry in birds: the influences of sexual and natural selection. *Journal of Avian Biology* 46: 175-85.

Yu, Z. H. (1998). Asymmetrical testicular weights in mammals, birds, reptiles and amphibia. *International Journal of Andrology* 21: 53-55.