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A case study of sex-linkage in *Python regius* (Serpentes: Boidae), with new insights into sex determination in Henophidia

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Abstract

A case study of sex-linkage in *Python regius* (Serpentes: Boidae), with new insights into sex determination in Henophidia. In 2002, a herpetoculturist identified a unique phenotypic trait, Coral Glow (CG), in a Ball Python (*Python regius*), and bred the individual to produce offspring similar in appearance. Yet, as herpetoculturists subsequently discovered, the pattern of inheritance for this trait digressed from the expectations of simple Mendelian inheritance. Male CGs sired by male CGs primarily produced male CGs and female Wild Types (WT), and male CGs that were produced from female CGs primarily produced female CGs and male WTs. The current hypothesis to explain these observations is that CG is a sex-linked incomplete dominant trait subject to recombination in an XX/XY sex determination system in the Ball Python. Herein, we present data demonstrating the observed pattern of inheritance, and we subject these data to linkage analysis. We observe a logarithm of the odds (LOD) score >179.1, evidence that the CG phenotype and the sex phenotype are in a state of linkage disequilibrium. Despite previous investigators assuming that all snakes display female heterogamety, the sex determining mechanisms of boas and pythons have not been identified, and a review of relevant literature reveals that the hypothesis of male heterogamety in Henophidia is consistent with additional published observations. We offer an alternative interpretation of the findings of past case studies, wherein investigators reported to have confirmed the existence of viable WW *Epicrates maurus* and *Boa constrictor*, and we further discuss the implications of male heterogamety in Henophidia.

Keywords: Ball Python, Coral Glow, genetic recombination, herpetoculture, heterogamety, linkage disequilibrium, non-Mendelian inheritance, pseudoautosomal region, sex chromosomes.

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Resumo

Um estudo de caso ligado ao sexo em *Python regius* (Serpentes: Boidae), com novas ideias sobre a determinação sexual em Henophidia. Em 2002, um herpetoculturista identificou uma característica fenotípica única, o Brilho Coral (*Coral Glow* - CG), em um píton-bola (*Python regius*), e realizou cruzamento do indivíduo para produzir descendentes com aparência similar. Além disso, como os herpetoculturistas descobriram em seguida, o padrão de herança para esta característica não obedeceu a herança mendeliana simples esperada. Machos CG filhos de machos CG produziram principalmente machos CG e fêmeas tipo selvagem (WT), e machos CG produzidos a partir de fêmeas CG originaram principalmente fêmeas CG e machos WT. A hipótese atual para explicar essas observações é a de que CG é uma característica dominante incompleta ligada ao sexo sujeita a recombinação em um sistema de determinação sexual do tipo XX/XY em píton-bola. Apresentamos aqui dados que demonstram o padrão observado de herança e submetemos esses dados a uma análise de ligação. Observamos um valor de logaritmo de probabilidades (LOD) > 179,1, uma evidência de que o fenótipo CG e o fenótipo ligado ao sexo estão em um estado de desequilíbrio de ligação. Apesar de pesquisadores anteriores terem assumido que todas as serpentes exibem heterogamia feminina, os mecanismos de determinação sexual em jibóias e pítons não foram identificados, e uma revisão da literatura relevante revela que a hipótese de heterogamia masculina em Henophidia é consistente com observações adicionais publicadas. Oferecemos uma interpretação alternativa das descobertas dos estudos de caso passados, em que pesquisadores relataram ter confirmado a existência de indivíduos WW viáveis de *Epicrates maurus* e de *Boa constrictor*, e ainda discutimos as implicações da heterogamia masculina em Henophidia.

Palavras-chave: cromossomos sexuais, desequilíbrio de ligação, herpetocultura, heterogamia, herança não-mendeliana, píton-bola, recombinação genética, região pseudoautossômica.

Introduction

In 2002, a herpetoculturist in the United States imported and bred a wild caught Ball Python, *Python regius* (Shaw, 1802), with pale yellowish-orange and faded magenta-gray hues, deep red eyes, and small black spots, which altogether distinguished it from the black and brown Wild Type (WT; Figure 1). During the time the herpetoculturist established this phenotype to be genetically inherited, another herpetoculturist imported and bred a second specimen of the same phenotype; consequently, this trait became widely known by two names, Banana and Coral Glow (CG) (McCurley 2014). After many iterations of breeding CGs, herpetoculturists discovered that the sex ratios of animals being produced with and without the CG phenotype do not follow Mendel's laws of autosomal inheritance.

Mendel's laws of autosomal inheritance predict that an autosomal trait will be passed on in equal ratios to male and female offspring, regardless of which parent carried the trait. Whereas the offspring of female CGs do not appear to deviate from the predictions of Mendel's laws, male CGs sired by male CGs primarily produce male CGs and female WTs, and male CGs that were produced from female CGs primarily produce female CGs and male WTs. In Ball Pythons, over 70 inheritable color, pattern, and morphology traits have been identified and widely produced by herpetoculturists, and, with the exception of CG, to our knowledge, none of these traits deviate from the predictions of simple Mendelian inheritance (with some appearing recessive, others incomplete dominant, and others dominant); although, some Ball Python traits appear to represent multiple alleles of the same genes, or possibly genes that are closely linked to one another, and some of these traits are known to be homozygous lethal, or cause non-lethal developmental maladies (McCurley 2014).

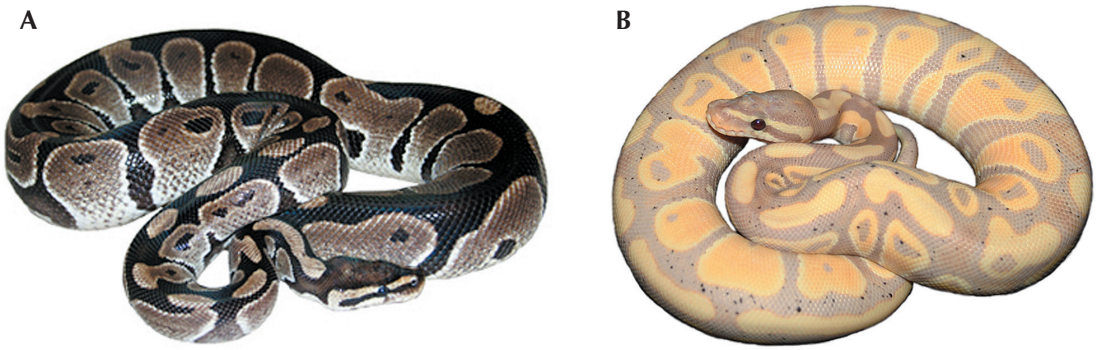

Figure 1. (**A**) Wild Type Ball Python (*Python regius*). (**B**) Coral Glow Ball Python. Photograph backgrounds have been removed, and photographs have been adjusted for brightness and contrast, by use of Adobe Photoshop CS3 Extended 10.0 (Adobe Systems Inc.). Photographed by Christopher S. Mallery Jr.

Mallery (2014) has previously hypothesized that CG is a sex-linked incomplete dominant trait subject to recombination between an X and a Y sex chromosome in Ball Pythons. Previous investigations have reported that all snakes have a ZZ/ZW sex determination system, but evidence indicating the presence of such a system is restricted to the highly derived, monophyletic group, Caenophidia (Matsubara *et al.* 2006, O'Meally *et al.* 2010, Vicoso *et al.* 2013, Ashman *et al.* 2014). Barker and Barker (2006) reported that Ball Pythons have homomorphic Z and W sex chromosomes. Pythons, however, belong to the clade Henophidia, which also includes Uropeltidae, Xenopeltidae, and Boidae, and as a whole, has been hypothesized to represent the living outgroup to the Caenophidia (Pyron *et al.* 2013). Mallery (2014) based his hypothesis about CG sex-linkage on preliminary observations, but did not publish evidence to support the hypothesis. If supported by sex-linkage data and not disputed by previously published evidence, the hypothesis that the henophidians have an XX/XY sex determination system will both introduce additional hypotheses to be tested and shed new light on the evolution of sex determination systems in Serpentes. Herein, we test Mallery's (2014) hypothesis that the CG phenotype is in a state of linkage disequilibrium with the sex phenotype, and we review the literature to further assess the validity of the hypothesis that the henophidians display male heterogamety (Mallery 2014).

Materials and Methods

Data Collection

We compiled clutch records from male CGs sired by male CGs, male CGs that were produced from female CGs, and from female CGs, as well as from exclusively WT clutches, to establish rates of hatching success. These clutch records represent accurately documented reproductive events between 2009 and 2015 at private facilities of several herpetoculturists who have maintained reliable records and the ability to accurately sex Ball Pythons, by means of probing or popping (i.e. manually everting the hemi-penes), as well as the ability to accurately identify the CG phenotype. There is no reason to suspect bias or dishonesty in reporting of these data, and observations appear consistent across breeders. In cases where multiple sired clutches occurred, we did not include data from any partially CG sired clutches. Individuals that did

not display the CG phenotype are herein described as WT, because they are WT with respect to the gene of interest, even if these individuals displayed additional traits that represent additional loci, which was a frequent occurrence, as herpetoculturists often pair Ball Pythons with the intent of combining multiple inheritable traits. Incubation temperatures ranged from 30.5–32.8°C, and there is no evidence that temperature has any influence on sex determination in pythons, nor on the presence or absence of the CG phenotype.

Statistics

For statistical analyses, we conducted Chi-squared test of goodness of fit with an expectation for CG:WT ratio of 1:1 in all cases, an expectation for male:female ratio of 1:1 in all cases, an expectation for hatching success in CG parented clutches based on that of exclusively WT clutches, and an expectation for male CG:female CG:male WT:female WT ratio of 1:1:1:1, as would be predicted by Mendel's laws of heredity, a model which assumes no (sex) linkage. We set the alpha level to 0.05 to delimit statistical significance.

We conducted a Chi-squared contingency analysis (Zar 1999) to determine if recombination rates were different between clutches sired by a male CG that was sired by a male CG, and clutches sired by a male CG that was produced from a female CG. We set the alpha level to 0.05 to delimit statistical significance. We also calculated a logarithm of the odds (LOD) score for linkage analysis, with an LOD score greater than 3.0 considered evidence supporting a hypothesis of linkage.

Results

We included data for a total of 1312 hatchlings from a total of 222 clutches parented by a CG, including 862 hatchlings from 142 clutches sired by male CGs that were sired by male CGs, 346 hatchlings from 58 clutches sired by male CGs that were produced from female CGs, and 104 hatchlings from 22 clutches produced by female CGs, as well as compiling records for 641 clutches not involving a CG parent, to establish expected hatching success rates. Hatching success in clutches sired by male CGs that were sired by male CGs was not significantly different from the expected ratio, as predicted based on exclusively WT clutches, (Chi-squared test of goodness of fit; $\chi^2 = 2.620$, df = 1, $p = 0.106$), hatching success in clutches sired by male CGs that were produced from female CGs was not significantly different from the expected ratio (Chi-squared test of goodness of fit; $\chi^2 = 0.010$, df = 1, $p = 0.920$), and hatching success in clutches produced by female CGs was not significantly different from the expected ratio (Chi-squared test of goodness of fit; $\chi^2 = 0.000$, df = 1, $p = 1.000$).

The number of CGs versus WTs did not differ significantly from an expected ratio of 1:1 amongst offspring sired by a male CG that was sired by a male CG (Chi-squared test of goodness of fit; $\chi^2 = 0.668$, df = 1, $p = 0.414$), nor amongst offspring sired by a male CG that was produced by a female CG (Chi-squared test of goodness of fit; $\chi^2 = 0.000$, df = 1, $p = 1.000$), nor amongst offspring produced from a female CG (Chi-squared test of goodness of fit; $\chi^2 = 3.115$, df = 1, $p = 0.078$). The number of males versus females did not differ significantly from an expected ratio of 1:1 amongst offspring sired by a male CG that was sired by a male CG (Chi-squared test of goodness of fit; $\chi^2 = 0.005$, df = 1, $p = 0.944$), nor amongst offspring sired by a male CG that was produced by a female CG (Chi-squared test of goodness of fit; $\chi^2 = 0.012$, df = 1, $p = 0.913$), nor amongst offspring produced from a female CG (Chi-squared test of goodness of fit; $\chi^2 = 0.038$, df = 1, $p = 0.845$), nor amongst offspring from exclusively WT clutches (Chi-squared test of goodness of fit; $\chi^2 = 0.303$, df = 1, $p = 0.582$).

The ratio of male CG:female CG:male WT:female WT was significantly different from the expected ratio of 1:1:1:1 (i.e. as would be

predicted by simple Mendelian inheritance) amongst offspring sired by a male CG that was sired by a male CG (Chi-squared test of goodness of fit; $\chi^2 = 629.090$, df = 3, $p < 0.001$), as well as amongst offspring sired by a male CG that was produced from a female CG (Chi-squared test of goodness of fit; $\chi^2 = 281.353$, df = 3, $p < 0.001$) (Figure 2). In clutches produced from female CGs, there is no evidence to suggest that the relative frequencies of male CG, female CG, male WT, and female WT depend on the parentage of the female CG, and the ratio of male CG:female CG:male WT:female WT did not significantly differ from 1:1:1:1 in these clutches (Chi-squared test of goodness of fit; $\chi^2 = 3.308$, df = 3, $p = 0.347$).

For offspring sired by male CGs that were sired by male CGs, we calculated an LOD score of 161.6, and a recombination rate of 7.3%. For offspring sired by male CGs that were produced from female CGs, we calculated an LOD score of 74.7, and a recombination rate of 4.9%. These rates of recombination in clutches from male CGs that were sired by male CGs versus in clutches from male CGs that were produced from female CGs were not significantly different from one another (Chi-squared contingency analysis; $\chi^2 = 2.291$, df = 1, $p = 0.130$). Therefore, we combined these data for further linkage analysis, accounting for the reversal of recombination. The combined LOD score was >179.1, and the combined recombination rate was 6.6%.

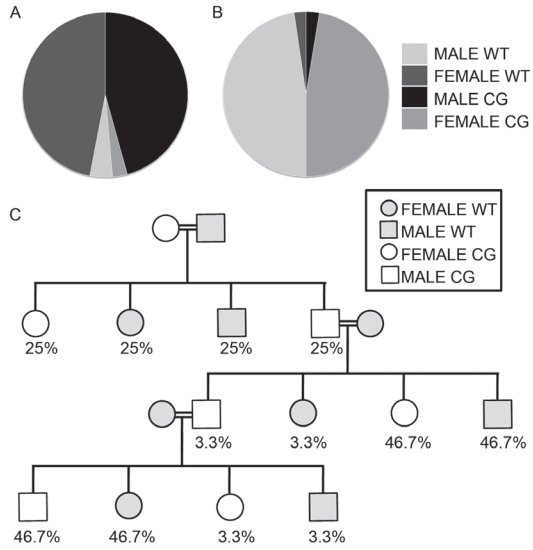

Figure 2. Pie graphs for observed data and hypothetical pedigree for Coral Glow (CG) X Wild Type (WT) crosses in Ball Pythons (*Python regius*). (**A**) Pie graph of observed phenotypes of offspring sired by male CGs that were sired by male CGs ($N = 862$). (**B**) Pie graph of observed phenotypes of offspring sired by male CGs that were produced by female CGs ($N = 346$). (**C**) A hypothetical pedigree demonstrating the observed inheritance pattern of the CG trait, with phenotypes of individuals labeled with observed overall recombination rates.

Discussion

Coral Glow

A few competing hypotheses may be considered to explain the pattern of inheritance observed for the CG trait in Ball Pythons. Hypotheses include sex biased mortality, as has documented in some snakes (Burger and Zappalorti 1988), or sex biased release of gametes, as has been documented in birds, even in response to an inheritable feather color trait carried by a mate (Pryke and Griffith 2009). With the observed hatching success rates not being significantly different from the expected, based on comparison to exclusively WT clutches, there is no reason to suspect that mortality of a particular genotypic combination has influenced the sex ratios of CG versus WT offspring. With the observed overall sex ratios not being significantly different from an expected ratio of 1:1, there is no reason to suspect that sex biased release of gametes has influenced the sex ratios of CG versus WT offspring. None of these models explains the sex biased digression from Mendelian inheritance observed across multiple

generations in the case of CG, but sex-linkage remains a viable explanation.

We have conducted linkage analysis between CG and sex, and we have demonstrated that CG is in a state of linkage disequilibrium with the sex phenotype. Coral Glow is inherited in a pattern that is not consistent with the ZZ/ZW sex determination system previously hypothesized for all snakes, but is consistent with an XX/XY sex determination system; we therefore posit that the gene underlying the CG phenotype is subject to genetic recombination between an X and a Y sex chromosome (Figure 3). Whereas some herpetoculturists consider CG a form of albinism, presumably a loss of function mutation affecting a melanin pathway protein, the gene (and specific allele) responsible for the CG phenotype has not been identified. The occurrence of both male and female homozygous CGs (super CGs) that are phenotypically distinguishable from the heterozygotes indicates that CG is incomplete dominant to the WT allele at the locus responsible, and is expressed similarly in both sexes. Our results suggest that the gene is part of a pseudoautosomal region (PAR) of the sex chromosomes, where the male and female sex chromosomes continue to exhibit genetic recombination between one another during gametogenesis.

Coral Glow is not unique in being a pseudoautosomal sex-linked trait that affects pigmentation, can recombine, and can be expressed in linkage with the male or the female sex chromosome. A similar example, with a nearly identical pattern of inheritance, has been observed in the Japanese Rice Fish (*Oryzias latipes*), with the dominant red trait and the recessive white trait occurring in linkage with both X and Y chromosomes (Aida 1921). Similar examples have also been documented for a few X and Y linked traits (e.g., red tail, black caudal-peduncle etc.) in the Guppy (*Poecilia reticulata*; Khoo *et al.* 1999).

Serpentes Sex Determination

Squamates display great evolutionary plasticity in sex determining mechanisms, including male heterogamety (XX/XY systems), female heterogamety (ZZ/ZW systems) and temperature-dependent sex determination (TSD), each of which occurs in multiple independently evolved Squamata lineages (Organ and Janes 2008, Ezaz *et al.* 2009, Pokorná and Kratochvíl 2009, Gamble *et al.* 2015, Ashman *et al.* 2014). All snakes in which reproduction has been studied utilize genotypic sex determination (GSD), and

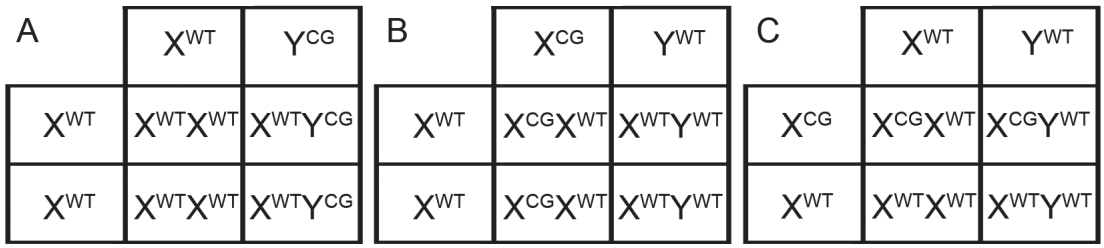

Figure 3. Punnett squares depicting predicted outcomes from crosses between a Coral Glow (CG) and a Wild Type (WT) Ball Python (*Python regius*). (**A**) Punnett square representing a male CG (with no recombination occurring), sired by a male CG, crossed to a female WT. This Punnett square also represents the recombinant Punnett square for a male CG, produced by a female CG, crossed to a female WT. (**B**) Punnett square representing a male CG (with no recombination occurring), produced by a female CG, crossed to a female WT. This Punnett square also represents the recombinant Punnett square for a male CG, sired by a male CG, crossed to a female WT. (**C**) Punnett square representing a male WT crossed to a female CG.

cytogenetic techniques have revealed the presence of heteromorphic Z and W sex chromosomes, with a degenerated or at least partially heterochromatic W chromosome, in many caenophidians (e.g., see Beçak *et al.* 1964, Trinco and Smith 1971, Baker *et al.* 1972, Singh 1972, Vicoso *et al.* 2013). However, despite sex determination studies including many Henophidia and non-Alethinophidia snakes, evidence of sex chromosomes and sex determining sequences has not been identified in any snakes outside of the Caenophidia; studies include: (1) karyotyping of eight python species (Singh 1972, Singh *et al.* 1976, Mengden and Stock 1980, Barker and Barker 2006), 13 boa species (Beçak *et al.* 1964, Gorman and Gress 1970, Singh 1972), eight blindsnake species (Ruiz Garcia and Hernando 2007), and a Sunbeam Snake (*Xenopeltis unicolor*) (Singh *et al.* 1976); (2) fluorescent *in situ* hybridization (FISH) mapping of the Burmese Python (*Python bivittatus*) (Matsubara *et al.* 2006) and the Water Python (*Liasis fuscus*) (O'Meally *et al.* 2010); (3) genome sequencing of the Boa Constrictor (Vicoso *et al.* 2013) and the Burmese Python (Castoe *et al.* 2011, 2013); and (4) mutation rate and gene expression analyses in Boa Constrictors (Vicoso *et al.* 2013). Mengden and Stock (1980) reportedly identified heteromorphic sex chromosomes in one Dumeril's Boa (*Acrantophis dumereli*) karyotype, but the images published are poor quality, and, moreover, the investigators were not aware of the phenotypic sex of the individual that was karyotyped. Despite this lack of evidence, investigators have repeatedly assumed that all snakes possess female heterogamety (e.g., Organ and Janes 2008, Pokorná and Kratochvíl 2009, Booth *et al.* 2011a, b, Ashman *et al.* 2014). Even when reporting on karyotypes, FISH mapping, genomes, and gene expression analyses that have all failed to identify sex chromosomes in Henophidia, researchers have interpreted the fourth largest chromosomes of henophidians to be homomorphic sex chromosomes, based on homology to the Z chromosome of the caenophidians (Beçak *et al.* 1964, Ray-Chaudhuri *et al.* 1971, Matsubara *et al.* 2006, O'Meally *et al.* 2010, Vicoso *et al.* 2013). In fact, this assumption formed part of the basis for Ohno's hypothesis that sex chromosomes evolved from autosomes (Ohno 1967).

As snakes are nested within the Squamates, and the living outgroup (including Anguimorpha and Iguania) to Serpentes employs all above mentioned sex determination systems, the ZZ/ZW system utilized by Caenophidia has been hypothesized to be a derived state (Organ and Janes 2008, Pokorná and Kratochvíl 2009, Gamble *et al.* 2015). It is possible that the Caenophidia ZZ/ZW system evolved within the stem lineage to all Episquamata, to all Toxicofera, to all Serpentes, or within the Serpentes lineage (Organ and Janes 2008, Pokorná and Kratochvíl 2009). However, whereas there is much genome-wide chromosomal homology between Anguimorpha, Iguania, and Serpentes, Anguimorpha and Iguania are not currently known to utilize a ZZ/ZW system homologous to that of Caenophidia (O'Meally *et al.* 2012, Ashman *et al.* 2014, Gamble and Zarkower 2014, Rovatsos *et al.* 2014a, b); this suggests that the ZZ/ZW sex determination system present in caenophidians may be of independent evolutionary origins from the ZZ/ZW systems of other Squamates.

As the current case study of CG is consistent with the hypothesis that Ball Pythons have an XX/XY sex determination system, and there is no published evidence that refutes male heterogamety in the henophidians, nor any non-caenophidians, additional questions arise. Is there any additional published evidence of sex chromosome system type in snakes outside of the caenophidians? If there is any additional evidence specifically of male heterogamety in the snakes, how widespread is this system, phylogenetically, and in what lineage did this sex determination system evolve?

Parthenogenesis and Sex Determination

Booth *et al.* (2011a, b, 2014) documented the occurrence of facultative parthenogenetic repro-

duction in the Boa Constrictor, the Colombian Rainbow Boa (*Epicrates maurus*), the Ball Python, and the Reticulated Python (*Malayopython reticulatus*), and Kinney *et al.* (2013) documented facultative parthenogenesis in the Brazilian Rainbow Boa (*Epicrates cenchria*). In these studies, Booth *et al.* (2011a, b, 2014) and Kinney *et al.* (2013) genotyped multiple loci of the offspring and demonstrated that the offspring are homozygous throughout the majority of these loci, which the investigators interpreted as indicative of terminal fusion automictic parthenogenesis (TFAP). The parthenogens studied in these case studies were all females; Booth *et al.* (2011a, b) and Kinney *et al.* (2013) interpreted this to mean that these offspring were homozygous for a W sex chromosome. Booth *et al.* (2011a, b) hypothesized that only female parthenogens resulted (rather than 50% females, genotype WW, and 50% males, genotype ZZ, as would be expected from a ZW female displaying TFAP) because the mother is hemizygous, with genotype WØ (i.e. aneuploidy, presumably because of nondisjunction of sex chromosomes during spermatogenesis in her father). However, neither Booth *et al.* (2011a, b), nor Kinney *et al.* (2013), conducted any tests to confirm the presence or absence of any sex chromosomes, nor sex determining regions, in either the mothers, nor in the offspring. Therefore, the explanation given by Booth *et al.* (2011a, b) and Kinney *et al.* (2013) builds upon the assumption that the boas generally bear a ZZ/ZW sex determination system, and assumes that both WØ and WW individuals naturally occur, and are viable.

In amphibians that have minimally differentiated sex chromosomes, homogametic WW females, resulting from experimental manipulation, are viable (Mikamo and Witschi 1964, Ohno 1967). Yet, many species of Caenophidia snakes, as well as lizards and birds, that are well documented as having ZZ/ZW sex determination systems appear to produce only male offspring when reproducing by TFAP, indicating that WW is a non-viable combination in these amniotes (e.g., Olsen and Marsden 1954, Schuett *et al.* 1998, Watts *et al.* 2006, Booth *et al.* 2012, Reynolds *et al.* 2012). With no substantiated evidence of a ZZ/ZW sex determination system in boas and pythons, a more parsimonious interpretation of Booth *et al.'s* (2011a, b, 2014) and Kinney *et al.*'s (2013) observations is that the boas and pythons observed to reproduce by TFAP have an XX/XY sex determination system, and that the half clone parthenogens have the homozygous XX genotype, as does the mother; this would also explain why no males result from these TFAP cases.

Booth *et al.* (2011a) also reported observing that male Boa Constrictors produce both male and female gonads during development, whereas females only produce female gonads. Although sex determination systems are subject to a high degree of evolutionary plasticity (Bachtrog *et al.* 2014), Booth *et al.*'s (2011a) observation may be interpreted as evidence that the male is the heterogametic sex, because it demonstrates that the male has the genetic underpinnings necessary to express male and female gonads, whereas, there is no evidence to suggest that the female is capable of producing male gonads.

Candidate Henophidian Sex Chromosomes

Bergero and Charlesworth (2008) took note that some snakes may have an XX/XY sex chromosome system with only a small non-recombining region, which has not yet been detected, and this hypothesis is consistent with the case study of CG presented herein, as well as with the cases of TFAP thus far documented in Henophidia (Booth *et al.* 2011a, b, 2014, Kinney *et al.* 2013). A few candidate sex chromosomes exist for Henophidia, based on genomic comparison to sex determining systems in other vertebrates. Caenophidians and henophidians may bear homologous sex chromosomes (henophidian chromosome four), except that a transition occurred wherein an ancestral X and Y chromosome gave rise to a W and Z chromosome, respectively, or vice versa (for discussion on sex

chromosome transitions, see Marshall Graves and Shetty 2001, Ezaz *et al.* 2006, Pokorná and Kratochvíl 2016). Additional candidate sex chromosomes include henophidian chromosome six, which may display variable gene expression, as compared to other henophidian chromosomes, as a result of current or historical sex-linkage (see Vicoso *et al.* 2013), or chromosome two, the location of genes (*DMRT1* and *SOX9*) that are known to be involved in sex differentiation in other vertebrate lineages (Matsubara *et al.* 2006).

The Z chromosome of Caenophidia is homologous to chromosome six (a macrochromosome) in the Green Anole (*Anolis carolinensis*), a pleurodont iguanian (Vicoso *et al.* 2013). However, the pleurodont X and Y sex chromosomes are microchromosomes, and several (currently unplaced) genomic scaffolds of Burmese Pythons (e.g., NW_006532210.1, NW_006532455.1, NW_006534197.1, NW_006532240.1, NW_006534297.1) share synteny and sequence level homology with the pleurodont X chromosome (Castoe *et al.* 2013, Rovatsos *et al.* 2014a–c); incidentally, this pleurodont sex chromosome maps to a chicken microchromosome, an autosome assigned as chromosome 15, which also shares homology with the documented Z and W sex chromosomes of the Chinese Soft-shelled Turtle (*Pelodiscus sinensis*) (Kawagoshi *et al.* 2009, Rovatsos *et al.* 2014a–c).

Ball Pythons have a diploid set of 36 chromosomes (as do most snakes thus far studied), 18 pairs, including 17 pairs of autosomes and two sex chromosomes (Trinco and Smith 1971, Barker and Barker 2006, Bachtrog *et al.* 2014). Of the haploid set of 18 chromosomes, eight are macrochromosomes, which account for over 80% of the genome, and 10 are microchromosomes, which account for the remainder (Barker and Barker 2006). In Ball Pythons, there are over 70 inheritable color, pattern, and morphology traits known (as well as many more in other Henophidia species) (McCurley 2014), which, accounting for redundancy (i.e. traits that appear to represent multiple alleles of the same gene), we estimate to represent manifestations of over 40 different genes, all of which are housed on the 18 Ball Python chromosomes, and many of which are likely located on the eight macrochromosomes. With the exception of CG, none of these traits are known to be in linkage diseqilibrium with sex, and, as our linkage analysis suggests that CG is closely linked with the sex determining region, it is possible that the sex chromosomes of the henophidians are microchromosomes, as are the X and Y sex chromosomes of the pleurodont iguanians (Rovatsos *et al.* 2014a–c). Testing the hypothesis that the henophidian sex chromosomes share homology with the X and Y sex chromosomes of the pleurodont iguanians, and identifying the molecular underpinnings of the henophidian sex determination system, will require further investigation [e.g., identification of sex specific markers via RAD-seq, as demonstrated for *Anolis* in Gamble and Zarkower (2014) and for several gecko species in Gamble *et al.* (2015)].

Evolutionary Perspectives

Henophidia are hypothesized to represent the living sister group to the Caenophidia (Pyron *et al.* 2013), and the common ancestor to the henophidians diverged from the caenophidians approximately 103.7 million years ago (MYA) [Vidal *et al.* 2009, although Hsiang *et al.* (2015) have recently hypothesized this and other divergence times discussed herein to be more recent than previous estimates]. Before these lineages diverged, their common ancestor diverged from their lizard outgroup approximately 179 MYA (Vidal and Hedges 2005). By use of parsimony based ancestral state reconstructions, Organ and Janes (2008) hypothesized the ancestral character state for the stem lineage to all Squamata to be XX/XY, as well as for Episquamata; however, in this model, findings are equivocal between TSD, male heterogamety, and female heterogamety for Toxicofera, under the former assumption that all Serpentes display female heterogamety (Organ and Janes 2008).

Alternatively, Pokorná and Kratochvíl (2009) conducted parsimony based ancestral state reconstructions, finding support for the hypothesis that TSD was likely the ancestral state for all Squamata, as well as for Gekkota, and Pokorná and Kratochvíl (2009) suggested that TSD is unlikely to evolve from GSD, with GSD functioning as an evolutionary trap. Gamble *et al.* (2015) used this evolutionary trap model to construct a maximum-likelihood ancestral state reconstruction for sex determination in Squamata, and Gamble *et al.*'s (2015) reconstruction supported TSD as the ancestral state for Squamata, Episquamata, and Toxicofera, suggesting that the XX/XY sex determination system of the pleurodont iguanians is a synapomorphy of the pleurodont clade, and that the ZZ/ZW sex determination system of the Serpentes is a synapomorphy of the Serpentes clade (under the assumption that all snakes possess a ZZ/ZW system). Pokorná and Kratochvíl (2016) further argue in favor of the evolutionary trap hypothesis, and suggest that transitions between one GSD system and another (e.g., replacement of a ZZ/ZW system with an XX/XY system, or vice versa) may be adaptive and easily evolved.

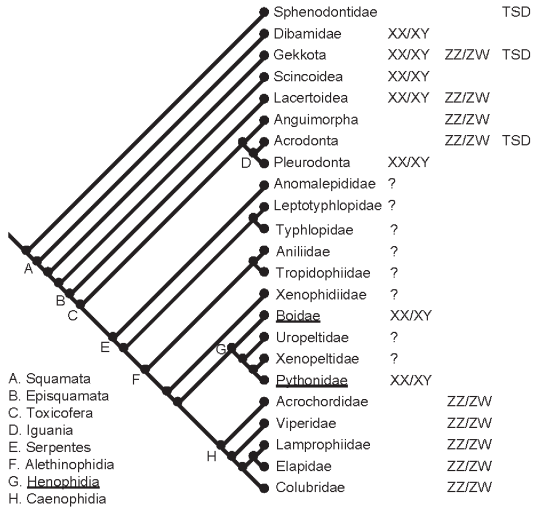

Figure 4. Phylogenetic tree of Squamata, with sex determining mechanisms indicated, with emphasis on Serpentes; clades of particular interest in this study are underlined; tree based on Pyron *et al.* (2013); sex determining mechanisms taken from Organ and Janes (2008), Pokorná and Kratochvíl (2009), Gamble *et al.* (2015), Ashman *et al.* (2014), and the current study. Tree generated in Mesquite 2.75 (Maddison and Maddison 2011).

Considering that many pleurodont iguanians (part of the outgroup to the Serpentes) display male heterogamety (Rovatsos *et al.* 2014a–c), and at least some henophidians appear to display male heterogamety, it is possible that the trait is symplesiomorphic, and that all non-caenophidian snake lineages (approximately 600 species, or one sixth of the currently known living snake species, including the paraphyletic lineages of Anomalepididae, Typhlopidae, Xenophidiidae, etc.) may also display male heterogamety (Figure 4); as there is no data currently available on sex chromosomes in these lineages, this hypothesis will require further investigation. However, this raises the question as to whether the most deeply divergent lineage of caenophidians may display male heterogamety. The sister groups Acrochordidae and Xenodermatidae, together, represent the most deeply divergent living monophyletic lineage within the caenophidians, having diverged from the remaining caenophidians approximately 90.7 MYA (Vidal *et al.* 2009, Pyron *et al.* 2013). Facultative parthenogenesis has been observed in an acrochordid, the Arafuran Filesnake (*Acrochordus arafurae*), and in this event, the parthenogens were males, which is consistent with a ZZ/ZW system of sex determination (Dubach *et al.* 1997). If female heterogamety does not occur in non-Caenophidia snakes, then this observation could be interpreted as evidence that the common ancestor to all Caenophidia evolved a ZZ/ZW sex determination system between approximately 103.7 and 90.7 MYA, prior to the divergence of this early branch of caenophidian, but after the divergence from Henophidia, making female heterogamety a synapomorphy of the caenophidian stem

lineage and its descendants. Alternatively, although female heterogamety has not been detected in snakes outside of Caenophidia, it is possible that female heterogamety is more widespread in Serpentes than has been documented, and that male heterogamety is a synapomorphy of the Henophidia stem lineage and its descendants, as male heterogamety appears to be present in deeply divergent lineages within the Henophidia, but there is currently no evidence of male heterogamety in snakes outside of Henophidia; we note, however, that Anguimorpha and some acrodont iguanians (members of the outgroup to the Serpentes) do possess a ZZ/ZW sex determination system (Johnson Pokorná *et al.* 2014, Gamble *et al.* 2015, Rovatsos *et al.* 2015). However, Gamble *et al.*'s (2015) research does not support a common evolutionary origin for the sex chromosomes of snakes and their lizard outgroup, but instead suggests that each of the systems of GSD in these lineages (Anguimorpha, Acrodonta, Pleurodonta, and Serpentes) has evolved independently from an ancestral state of TSD. Overall, much more work on sex determination in non-caenophidian snakes will be needed to resolve these questions, and future studies in this area should test for homology of sex chromosomes within snakes and between snakes and their living outgroup.

Conclusions

Upon conducting linkage analysis and reviewing the relevant literature, we have failed to identify any evidence disputing the hypothesis that CG is a sex-linked trait that is subject to recombination between an X and a Y sex chromosome in the Ball Python. Conversely, we have found CG to be in a state of linkage disequilibrium with the sex phenotype and we have identified additional published evidence consistent with the hypothesis of male heterogamety in the henophidians. The sex-linkage demonstrated herein serves as a prime case study for lessons in inheritance, and may serve as a useful example for teaching a variety of concepts in basic biology and genetics classrooms, as well as contributing to what is known of reptilian sex and trait evolution.

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