Title
Sex Chromosomes and Testosterone: A Novel Mouse Model Sheds Light on the Etiology of Klinefelter Syndrome

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Publication Date
2016

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Los Angeles

Sex Chromosomes and Testosterone:

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A dissertation submitted in partial satisfaction

of the requirements for the degree

Doctor of Philosophy in Neuroscience

by

Shayna Marie Williams-Burris

2016

ABSTRACT OF THE DISSERTATION

Sex Chromosomes and Testosterone:

A Novel Mouse Model Sheds Light on the Etiology of Klinefelter Syndrome

by

Shayna Marie Williams-Burris

Doctor of Philosophy in Neuroscience

University of California, Los Angeles, 2016

Professor Arthur P. Arnold, Chair

Klinefelter syndrome (KS) occurs when boys are born with a second X chromosome so their cells are XXY instead of XY. Men with KS have low testosterone, infertility, obesity and metabolic dysfunction, and some conditions that typically affect women such as osteoporosis, lupus, and breast cancer. Notably, they exhibit cognitive differences such as speech delay in early childhood and difficulty with executive functions as adults. Historically, KS symptoms were thought to be primarily due to low testosterone levels, yet testosterone treatment does not cure KS. Therefore, we hypothesize that direct genetic effects of the second X chromosome play an essential role. Previously, it was impossible to separate the influence of genes vs. hormones, but our lab has established the novel Sex Chromosome Trisomy (SCT) mouse model, which can be used to distinguish the two factors. The SCT model produces mice with XY and XXY chromosomes that are either male or female. XXY males have low testosterone so differences between the male groups may be due either genetic or hormonal differences. However, both

female groups have low testosterone, so differences in females provide strong evidence for direct genetic effects.

We find that XXY mice have an obesity phenotype like that seen in human KS, and that sex chromosomes influence this trait, though the genetic effect may be facilitated by the presence of hormones. The mice also show an osteoporosis phenotype like humans that we find to be influenced by hormones only. Finally, a preliminary study in another mouse model of KS, the XY* model, show XXY* mice may have executive functions deficit like those seen in humans. Next steps should test the executive functioning in the SCT model, and to also measure pup vocalizations as a model of speech delay. Further research should expand the use of the model to gene expression studies to identify which genes are responsible for direct genetic effects in SCT mice. Ideally the SCT model will be increasingly characterized and lead to a better understanding of the genetic factors at play in KS, potentially leading to better treatment options for KS patients.

The dissertation of Shayna Marie Williams-Burris is approved.

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2016

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Acknowledgements

First, I would like to thank my advisor Arthur Arnold without whom this work would have been impossible. Art is a true mentor, and has taken seriously the charge to provide didactic guidance as well as constant support of my work and of my development as a scientist and as a person while I have been his student. Second, I would like to thank my committee members and collaborators Stephanie White, Karen Reue, David Jentsch, and Eric Vilain for their intellectual and practical support for this work and of me. I also thank my fellow lab members and coauthors over the years including Yuichiro Itoh, Xuqi Chen, Tuck Ngun, Negar Ghahramani, Hayk Barseghyan, Mariam Barseghyan, Shawn Aarde, Kathy Kampf, Rebecca McClusky, Maureen Ruiz-Sunstrom, Ryan Mackie, and Haley Hrncir for the vital roles they played in making this work possible. I must especially thank the large team of undergraduates who contributed to this work over the years, many substantially so, including Jessy Martinez, Gabriela Beroukhim, Caroline Arellano Garcia, Gal Daskal, Dorothy Yu, Yilei Zhang, Ernie Garcia, and Rylee Genner. And I thank the members of the neuroscience community at UCLA, most especially my fellow NSIDP students who have become beloved colleagues and friends, for their intellectual, social, and emotional support during my tenure in graduate school.

Looking back, I want to thank my undergraduate research mentors Barrie Robison and his former post-doc Robert Drew, and especially Melissa Coleman for the role they played in my scientific development leading up to graduate school including helping me come to the conclusion that I wanted to be a scientist at all. I want to acknowledge the support shown for my academic pursuits even before undergrad by my parents and siblings, who always saw my potential and believed me capable of achieving whatever I set out to do.

Finally, I also thank my husband Matthew Burris and my close friend Cristina Benitez for the immeasurable roles they have played in my life to help me be the best version of myself personally and professionally, and to overcome the obstacles I have faced in and out of academia during my time at UCLA, bringing me where I am today.

For funding I thank the UCLA Neuroendocrinology, Sex Differences, and Reproduction training grant (T32HD007228), the Hyde Fellowship via the UCLA Integrative Biology and Physiology department, and the Dissertation Year Fellowship through UCLA Graduate Division.

Chapter 2 is a version of the published article:

Chen X*, Williams-Burris SM*, McClusky R, Ngun TC, Ghahramani NM, Barseghyan H, Reue K, Vilain E, and Arnold AP. The Sex Chromosome Trisomy mouse model of XXY and XYY: metabolism and motor performance. Biology of sex differences. 2013 Aug 8;4(1):15. doi: 10.1186/2042-6410-4-15. PubMed Central PMCID: PMC3751353. * equal contribution

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Arnold AP, Reue K, Eghbali M, Vilain E, Chen X, Ghahramani N, Itoh Y, Li J, Link JC, Ngun T, **Williams-Burris SM**. The importance of having two X chromosomes. Philosophical Transactions of the Royal Society B: Biological Sciences. 2016 Feb 19;371(1688). doi: 10.1098/rstb.2015.0113. Epub 2016 Feb 1. Review. PubMed PMID: 26833834. Pubmed Central PMCID: PMC4785899.

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Chapter 1

The Sex Chromosome Trisomy Mouse Model of Klinefelter Syndrome

1.1 Introduction

1.1.1 Klinefelter syndrome

History and diagnostic features

Klinefelter syndrome (KS) is characterized by infertility due to absence of sperm, small testes, low testosterone levels, high gonadotropin levels, and gynecomastia. The first description of this syndrome in 1942 by Harry Klinefelter was based on these features as observed in a cohort of men who presented at a clinic for infertility [1].Then, in 1959 the association of the syndrome with the karyotype 47,XXY was discovered [2]. Other supernumerary X karyotypes in males (XXXY, XXXY, etc.) also cause Klinefelter syndrome with increasing severity with increasing X number, however XXY is the most common karyotype seen in KS [3]. Although the syndrome was characterized 70 years ago, little is known about the mechanism by which an extra X chromosome causes the symptoms of KS. Testicular dysfunction and resulting hormone dysregulation are defining features of the disorder and factor largely in the general health of men with KS, however they do not represent the full picture of the etiology of the syndrome. Especially in relation to prevalent non-gonadal health and

cognitive problems experienced by men with KS, it is likely that direct genetic effects of the second X chromosome play a role.

Increased risk of non-gonadal health problems

Men with KS experience increased prevalence of many health problems [4-6], some of which are usually more common in women than in men including breast cancer [7, 8], osteoporosis [9-11], and autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus [12-15]. Men with KS also have increased body fat, specifically abdominal fat, as well as increased rates of hyperinsulinemia, insulin resistance, type II diabetes, and metabolic syndrome [16-20]. In many cases low testosterone is thought to be the primary factor underlying these issues, and treatment with testosterone can provide improved outcomes for some KS phenotypes [21, 22]. However, at least in the case of metabolic dysfunction, testosterone does not seem to tell the whole story. Testosterone treatment is not sufficient to improve metabolic features [4], and an informative study comparing men with KS to men with another low-testosterone syndrome, idiopathic hypogonadotrophic hypogonadism (IHH), showed that men with KS had greater rates of diabetes than men with IHH before and after both groups received testosterone replacement therapy [20].

Cognitive features

The cognitive phenotype of KS patients has been studied extensively, sometimes yielding contradicting results. Despite the great variability among KS patients, a few traits appear consistent across methods and populations. First, one of the most robust findings is that up to 80% of XXY boys show speech and language deficits from an early age, and that these difficulties continue through the academic years and into adulthood [23-27]. Second, there is consistent evidence that XXY individuals have impairments in executive functioning such as impaired impulse inhibition, attention, and/or working memory [23-25, 28-31]. Third, a similarly

robust finding is that while most KS patients have an IQ in the normal range, on average the IQ of KS men is 10-15 points lower than siblings or peers, with a trend that verbal IQ is generally lower than performance IQ [23-26]. Fourth, a finding that is possibly related to language difficulties, XXY males show deficits in social functioning [32-35]. The observed combination of language deficits and social deficits has led researchers to hypothesize that XXY boys are at an increased risk of autism spectrum disorders [32-35]. Finally, there is also evidence for deficits in motor cognition and function [26, 36, 37].

Common, underdiagnosed, undertreated

KS occurs in 1:600 live male births, although it is estimated that up to 75% of men with KS are not diagnosed. The only treatment for KS is testosterone replacement therapy, otherwise symptoms are treated individually, and there is no direct treatment for any gene dose effects of the second X chromosome [3, 38, 39].

XYY

A related syndrome caused by the karyotype XYY occurs in 1:1000 live male births. Unlike KS, XYY does not cause infertility or low testosterone levels, however boys with the karyotype XYY do experience cognitive deficits similar to those seen in KS. XYY individuals have speech and language learning deficits, decreased verbal memory, and impaired motor skills [37]. XYY boys have also consistently been shown to have attention deficits; one study showed up to 76% of XYY boys have clinical attention deficit hyperactivity disorder (ADHD) [40].

1.1.2 Unanswered questions: gene dosage vs. hormone effects

Although the characteristics of KS patients are well defined, it is yet unclear how the supernumerary X chromosome results in physical and cognitive problems. Specifically, KS patients have low testosterone and high gonadotropin levels which are confounded by the presence of the extra X chromosome. These hormonal differences in KS men begin at or before

puberty. Although patient studies involving testosterone replacement therapy can yield clues to the relevance of genetic effects, it is impossible to cleanly separate hormonal from direct genetic effects in humans. By using a new mouse model of KS in which hormone levels are manipulated independently of X chromosome number, the goal of this research is to provide new understanding of the mechanism underlying the symptoms of KS. Specifically, the goal is to determine how direct genetic effects, hormone effects, or interactions of both underlie the physical health problems and cognitive differences that are experienced by men with KS.

1.1.3 The Sex Chromosome Trisomy model of Klinefelter syndrome

In this project a new mouse model of KS and XYY, the Sex Chromosome Trisomy (SCT) model, is presented and characterized for a range of phenotypes relevant to Klinefelter syndrome. This mouse model produces XX, XY, XXY, and XYY mice, each with either testes or ovaries, in the same litters. Thereby sex chromosome complement is varied independently of gonad type allowing for the study of direct genetic effects of the extra X chromosome in the absence of differing hormone levels. By comparing XXY to XY mice with or without testes, it is possible to observe effects of the supernumerary X chromosome that occur in the absence of differing testosterone levels. Specifically, differences that exist between XXY and XY mice that have ovaries point to direct genetic effects, which are not dependent on testicular hormones. This mouse model provides a novel method by which to understand the mechanisms underlying KS.

Preexisting mouse models of XXY

Previous to this new mouse model, two mouse models of KS existed [41-45]. The first mouse model is the XY* model, which is in one experiment of this project. In this mouse model there is an abnormal pseudoautosomal region causing the fusion of the X and Y chromosomes (forming the X^{Y*} chromosome). Litters are created with genotypes XY* (like XY males), XX^{Y*}

(like XXY males but with a fused X and Y chromosome), XY^{*X} female mice (like XO + psuedoautosomal region), and typical XX females [44, 45]. This mouse model is useful for the study of KS as the XX^{Y*} mice have smaller testes and infertility, and recapitulate other symptoms of men with KS [44, 45]. However, in this mouse model, as in humans, sex chromosome complement and testosterone levels are confounded, such that XX^{Y*} mice always have lower testosterone levels than XY* mice. Additionally, there are potential concerns as to whether gene expression on the X^{Y*} chromosome is completely similar to that of separate X and Y chromosomes. The second mouse model is the result of a four-generation breeding scheme stemming from the XY* model, which creates XY male, XXY male, XY^{*X} female, and XX female mice in the same litters [43, 46-48]. Again this mouse model exhibits phenotypes similar to those seen in KS humans, and has been used fruitfully to study KS. However, the disadvantage of this mouse model is again that the XXY mice have lower testosterone than the XY mice, confounding sex chromosome complement and testosterone levels. Even in studies where hormone levels are controlled in adulthood, differing hormone levels in XXY vs. XY mice early in development may cause changes in physical as well as cognitive phenotypes that can last a lifetime. A great deal of evidence indicates that permanent changes in physical and behavioral traits are caused by pre- and postnatal effects of testosterone, called organizational hormone effects [49].

Improved design

Our new SCT model of KS controls for this confound by producing mice that are XXY and XY with either testes or ovaries in the same litters. By studying XXY and XY gonadal female mice, which do not have differing testosterone levels, we control for the effects of differing testosterone levels which are seen in all other mouse models of KS. This mouse model is based on the Four Core Genotypes mouse model, in which the testis-determining gene *Sry* was spontaneously deleted from the Y chromosome (creating the Y^{-} chromosome) and inserted as a

transgene into an autosome (chromosome 3) [49-51]. Except for in the first experiment where the XXY$^-$ female breeders were produced using a more complicated breeding scheme, the SCT model is produced by first breeding XY$^-$ female mice with ovaries from the FCG model with XY males, which produces some XXY$^-$ female offspring. By breeding these XXY$^-$ females to XY$^-$*Sry* males, eight genotypes are produced: XX, XY$^-$, XXY$^-$, and XY$^-$Y$^-$ mice, each with or without the testis-determining gene *Sry*. These mice exist on an MF1 background, which is a robust outbred strain. This cross is not available on mice with an inbred background because in all currently studied FCG inbred mice the XY$^-$ females are infertile, making it difficult to create the SCT model in inbred strains. Our lab is currently attempting to backcross XXY female mice to the inbred strain C57-BL/6J in order to make inbred SCT mice available.

Similar to the Four Core Genotypes model, the SCT model allows not only detection of sex chromosome effects, but also of activational hormone effects as compared to organizational hormone effects. Activational hormone effects are those caused by circulating hormones that will be reversed when the hormone is removed. Phenotypes that are lost when a mouse is gonadectomized would be considered activational hormone effects. Organizational hormone effects cause a difference that persists even when the hormones are removed. Phenotypes that persist in gonadal males or gonadal females even after gonadectomy, but that require hormones during development, would be considered organizational hormone effects. This is in contrast to sex chromosome effects that are present in XX vs. XY (or XXY or XYY) regardless of gonad type.

1.2 **Approach**

Prior to this project the SCT model was completely uncharacterized in regard to any above traits including physical traits, metabolic features, or cognitive measures. Additionally, while previous mouse models have been used to study some traits including gonadal, bone

density and architecture, partner preference, and recognition memory phenotypes, they were unable to separate the effects of X gene dose from those caused by low testosterone levels. Here we replicate and extend some of those experiments, adding important novel insights into the independent contribution of sex chromosome effects versus organizational hormone effects.

1.2.1 Metabolism and motor performance in the Sex Chromosome Trisomy model of Klinefelter syndrome

In the first experiment, adult SCT mice of all 8 genotypes were gonadectomized (GDX) and implanted with testosterone releasing pellets for 3 weeks. Before and after GDX and testosterone treatment mice were measured for body weight and body composition, especially percent body fat and percent lean mass. At the end of the 3 weeks, mice were also tested for social recognition and sexual preference, and sexually dimorphic brain regions were collected to measure gene expression (data not presented here) [52]. Also at the end of the 3 week experiment, motor performance was measure, and metabolically relevant tissues were collected and weighed [53]. These data are presented in Chapter 2.

1.2.2 Testis, bone, and longitudinal metabolic measures in the Sex Chromosome Trisomy model of Klinefelter syndrome

In the second experiment adult SCT mice of all 8 genotypes were either GDX or left gonadally intact and weighed once a week and measured for body composition every 4 weeks for approximately 36 weeks. At the end of the experiment metabolic tissues were collected and weighed. For GDX mice, testes were weighed at the time of surgery, and for sham mice testes were weighed at the time of euthanasia and fixed, sliced, then stained for histological analysis. Femurs were collected and measured for length, bone mineral density (BMD) using DEXA, and morphology using μCT. These data are presented in Chapter 3.

1.2.3 Behavioral measures in the Sex Chromosome Trisomy and the XY* models of Klinefelter syndrome

For the third experiment the cognitive phenotype of SCT mice was studied. The goal of this study was to measure novel object recognition and reward response in adult GDX male and female XY and XXY SCT mice. Additionally, the XY* mouse model was measured for behavioral traits. Mice were either sham GDX (intact) or GDX. The tests for this experiment are identical to the SCT behavior experiment, except that after all other adult behavioral tests were completed, a reversal learning assay for executive function was also carried out. These data are presented in Chapter 4.

1.3 Summary

In this series of experiments, the SCT mouse model was characterized in regards to how it models features observed in boys and men with Klinefelter syndrome or in the related syndrome caused by the karyotype XYY. Several traits related to increased rates of physical illness were observed including testis, bone, and especially metabolic measures. Additionally, many behavioral traits were studied including motor performance, object recognition, and reward response. Previous to this work, none of these measures had been characterized in SCT mice. This is noteworthy because neither the study of male patients with sex chromosome trisomies nor the study of other mouse models of KS allows for the dissection of direct genetic effects from effects of gonadal hormones during development. Thereby, it was previously impossible to attribute phenotypes to one source or the other. In this body of work we show using the SCT mice that some traits are likely genetically modulated while others are likely caused by differing hormone levels. These findings are of importance because currently there is no treatment of KS aimed at targeting direct genetic effects of the second X chromosome. To move towards the goal of intelligent drug design, the first steps are to identify and separate the

genetic from hormonal contributions to KS symptoms, then to identify what gene pathways are involved, and eventually target those to change aberrant protein levels to manage KS symptoms. This work represents the first steps in this arduous process.

Additionally, the XY* model was tested for novel object recognition, reward response, and reversal learning. In this case, though it is impossible to separate organizational hormone effects from direct genetic effects, at least activational effects of hormones can be identified as relevant to a trait based on changes observed in gonadectomized mice. Here we repeat an experiment on object recognition that has been performed in these mice by another group [44], but we go further by adding a GDX group to the object recognition test, and by testing two previously unstudied phenotypes in these mice: reward response, and reversal learning.

Taken together these studies serve as key early steps necessary to ultimately better understand and better treat Klinefelter syndrome, which remains a prevalent, underdiagnosed, and under treated disorder that greatly affects the health and functioning of a significant population of boys and men worldwide.

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Chapter 2

The Sex Chromosome Trisomy Mouse Model of XXY and XYY: Metabolism and Motor Performance

2.1 Background

Klinefelter syndrome (KS), caused by the karyotype XXY, is characterized by small testes, azoospermia, low testosterone, hypergonadotropism, and gynecomastia [1, 2]. The prevalence of the KS karyotype is 1 in 600 live male births, making it the most common genetic cause of male infertility [3-6]. In addition to the gonadal phenotypes, KS men have non-gonadal traits and symptoms. For example, KS men are taller than XY siblings or peers and exhibit specific cognitive deficits – especially involving language, social, and executive functioning skills [7-15]. Another sex chromosome trisomy, XYY, does not cause infertility or hypogonadism, but XYY boys exhibit similar cognitive deficits to those seen in KS [9, 14].

KS men also experience an increased prevalence of several health problems [16, 17], some of which are usually more common in women than in men including breast cancer [18, 19], osteoporosis [20-22], and autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus [23-26]. Relative to XY males, KS men have increased body fat, specifically abdominal fat, as well as increased rates of hyperinsulinemia, insulin resistance, type II diabetes, and metabolic syndrome [16, 17, 27-31]. It is generally believed that hypogonadism is primarily responsible for the metabolic phenotype seen in KS patients.

Treatment with testosterone can provide improved outcomes for some KS phenotypes [32, 33], but is not sufficient to improve metabolic features [16]. Moreover, KS boys suffer from metabolic syndrome before puberty, when testosterone levels are similarly low in both KS boys and controls [30]. Additionally, a recent study comparing men with KS *vs.* men with idiopathic hypogonadotrophic hypogonadism (IHH) showed that men with KS had greater rates of diabetes than men with IHH both before and after both groups received testosterone replacement therapy [31]. Taken together these results suggest that there are likely to be direct genetic effects of the second X chromosome on metabolism, not exclusively mediated by reduced androgen levels in KS males.

Boys and men with KS also have impaired motor cognition and function. Several studies suggest that KS boys are clumsy and do not like sports [13]. KS boys show impairments in gross motor function [34-36], fine motor function [13, 34, 37], sensorimotor integration [35, 36], and overall strength and speed [36]. These deficits begin early in life [35], and persist into adulthood [38, 39]. Although XYY males are studied less often, they also show motor deficits similar to those seen in KS including fine motor impairment, gross motor impairment, decreased sensorimotor integration, and deficits in coordination [35, 36].

KS males may experience lower levels of testicular androgens at various life stages. These hormones are known to act pre- and post-natally to cause permanent ("organizational") effects on various tissues. They also act more acutely and reversibly at all life stages ("activational" effects) [40]. Although it is impossible to cleanly separate direct genetic effects from those caused by lower hormone levels in humans, here we offer a new mouse model of XXY and XYY with improved discrimination of hormonal *vs.* chromosomal effects, as well as their interactions.

The Sex Chromosome Trisomy (SCT) model produces XXY, XYY, XY, and XX mice, each genotype with either testes or ovaries, all within the same litters. The model allows

comparison of four different sex chromosome genotypes, and models both XXY and XYY human trisomies. The effects of sex chromosome complement are tested after exposure to ovarian or testicular secretions, and after removal of these hormones by adult gonadectomy, which allows for assessment of the hormonal independence of sex chromosome effects, as well as assessment of interactions of sex chromosomes and hormones. To validate the SCT model, we measured body composition and motor function, which are each known to be significantly different in KS men relative to XY males.

2.2 Methods

2.2.1 Mice

The Sex Chromosome Trisomy model, designed by Paul Burgoyne (personal communication; similar to mice used by Park et al. [41]), involves mating XXY^{-} females with the XY^{-}*(Sry+)* males (Figure 2-1A). The Y^{-} ("Y minus") chromosome is deleted for the testis-determining gene *Sry*, so that the Y chromosome no longer causes differentiation of testes [42]. The XY^{-}*(Sry+)* fathers possess an *Sry* transgene inserted into an autosome, which complements the lack of *Sry* on the Y chromosome and produces a fully functional male [40, 43, 44]. The mothers produce X and XY^{-} eggs, and the fathers produce X or Y^{-} sperm each with or without *Sry*. A total of 8 genotypes are produced: gonadal males XXY^{-}*(Sry+)*, $XY^{-}Y^{-}$*(Sry+),* XY^{-}*(Sry+)* and XX*(Sry+)*, as well as gonadal females XXY^{-}, $XY^{-}Y^{-}$, XY^{-} and XX (Figure 2-1A). Here we refer to these genotypes respectively as XXYM, XYYM, XYM, XXM, XXYF, XYYF, XYF, and XXF. For this study, the XXY^{-} mothers were produced by crossing XX females to $XY^{-}Y^{*X}$*(Sry+)* males, which are made by crossing XY^{*X} females to XY^{-}*(Sry+)* males (see references [11, 45-47] for discussion of the Y^{*X} chromosome). More recently we produce XXY^{-} mothers by breeding XY^{-} females to wild-type XY males, a more straightforward breeding

scheme in which about 30% of female offspring are XXY^{-}. To our knowledge the SCT model is easier to produce using outbred rather than inbred strains. Group sizes were 11 XXYF, 9 XXYM, 8 XYYF, 8 XYYM, 21 XYF, 14 XYM, 14 XXF, and 9 XXM. Measurements of brain gene expression and partner preference behavior of these mice are reported by Ngun et al. [48].

All mice were outbred MF1 strain. Although MF1 mice are random bred, and we attempt to maintain genetic diversity in our colony, the X chromosomes of all mice used here were identical and derived originally from mating an XY mouse with his XO dam to produce a line of mice with identical X chromosomes bred onto outbred stock (Paul Burgoyne, personal communication). Thus, in these experiments differences between mice with one *vs.* two X chromosomes were attributable to the number of X chromosomes, not to a difference in X alleles.

All experiments were approved by UCLA Animal Research Committee. Mice were group-housed based on their gonadal sex and maintained at 23°C on a 12:12 light:dark cycle. Mice were fed regular chow diet with 5% fat (LabDiet 5001).

2.2.2 Gonadectomy and hormonal implants

The SCT model primarily models XXY and XYY male genotypes of humans, which are normally only present in individuals with testes. Accordingly, the effects of these genotypes, relative to normal XY genotype, might be detected only when testosterone is present. Therefore, in the current study all mice were gonadectomized during adulthood at 97-124 days of age, and implanted with a Silastic testosterone pellet at the time of gonadectomy (Figure 2-1 B). The intent was to make circulating gonadal hormone levels as similar as possible, so that group differences could not be attributed to different levels of gonadal hormones in adulthood. Testosterone pellets contained 5mm of testosterone (Steraloids, Inc.) packed into a Silastic tube (1.57 mm ID x 2.41 mm OD, Dow Corning) and sealed at each end with 3mm of medical grade

Silastic adhesive (Dow Corning). Age of the mice did not differ across groups (overall ANOVA, $p>0.05$).

2.2.3 Genotyping

Karyotypes were determined from metaphase spreads prepared from tail or ear fibroblasts, using routine methods [49].

2.2.4 Measurement of plasma levels of testosterone

Plasma was collected from the carotid artery following decapitation, then stored at -20°C until radioimmunoassay was performed by the Ligand Assay and Analysis Core at the University of Virginia Center for Research in Reproduction (supported by NICHD (SCCPIR) Grant U54-HD28934). Measurements were performed in singlet reactions using Siemens Medical Solutions Diagnostics testosterone RIA with a reportable range of 0.72-111.00 ng/L.

2.2.5 Body weight, body composition and metabolic measures

Body weight and composition were measured just prior to gonadectomy, and at the end of the experiment (see Figure 1B). An NMR Mouse Minispec apparatus (Bruker Woodlands, TX) with Echo Medical Systems (Houston, TX) software was used to measure fat and lean mass [50] with coefficients of variation of less than 3%. Correlation between NMR and gravimetric measurements is better than 0.99. NMR was performed the day before GDX and 3 weeks after GDX.

At the end of the experiment (Figure 1B), the weights of two specific fat pads were measured, visceral fat of the peri-gonadal depot (referred to here as gonadal fat) and the subcutaneous fat depot from the femoral region (referred to as inguinal fat). Both depots were assessed because visceral and subcutaneous fat depots are differentially regulated and have diverse positive and negative effects on metabolic syndrome in humans and mice [51, 52].

2.2.6 Testis size

At the time of gonadectomy, testes were classified as small, medium, or normal (large) by a researcher who was blind to genotype. All XYM had normal testes, all XYYM had medium testes, and all XXM and XXYM had small testes, except for one XXYM that had medium testes.

2.2.7 Motor tests

At 18-22 days after GDX, motor function was assessed using two sensitive tests, the challenging beam traversal test and the pole test [53, 54]. The challenging beam test required mice to traverse a 1 m long beam made up of four 25 cm long sections of decreasing width (3.5 cm at its widest, and 0.5 cm at its narrowest, decreasing by 1 cm increments). Mice were trained to traverse the beam starting at the widest section and ending at their home cage, which was placed at the end of the narrowest section. Mice received two consecutive days of training, making 5 traversals each day, and were tested on the third day. On the testing day the beam traversal was made more challenging by adding a wire grid to the top of the beam so that mice had to grasp the wire to complete traversal, and errors could be calculated based on misses or slips while grasping. During testing, mice were video recorded making 5 traversals of the beam. The following variables were scored from videos of the behavior: time to traverse the beam, number of steps, and number of errors.

The pole test involved placing the mouse head-up at the top of a 50 cm long, 1 cm diameter vertical wooden pole with the base of the pole in the animal's home cage. Mice were trained to turn around to face down, and traverse down the pole into their home cage. Mice received 2 consecutive days of training, making 5 traversals each day. On the third day, the mice were video recorded making 5 traversals. The following variables were later scored from videos: time to turn around and time to reach the base of the pole.

Training for the challenging beam traversal test and the pole test were done consecutively for each mouse, such that each mouse completed training for the pole before training for the beam in a single session. Testing on the third day was done similarly, with each mouse completing the pole test then immediately completing the beam test.

2.2.8 Statistical analysis

An overall ANOVA was used to compare the 8 groups, with factors of gonadal sex (*Sry* present *vs.* absent) and genotype (four levels). In addition, our *a priori* hypotheses were that XY mice differed pairwise with XX, XXY, and XYY, and these comparisons were tested with three separate two-way ANOVAs with factors of gonadal sex and genotype (two levels). In two cases we used body weight as a covariate in an analysis of covariance (ANCOVA) to determine if group differences in the dependent variable were attributable to differences in body weight.

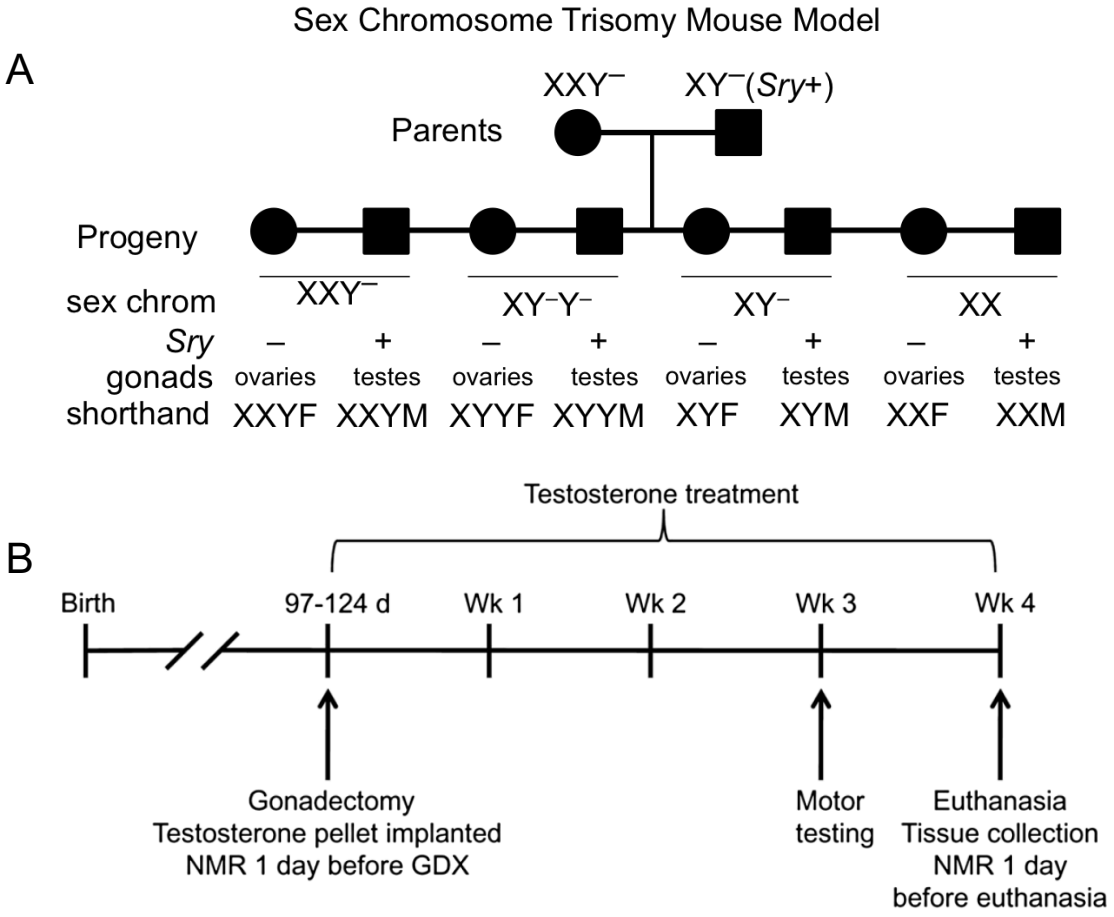

Figure 2-1: Diagram of the cross producing the SCT model and project timeline.
All mice were outbred MF1 strain. We attempt to maintain genetic diversity in our colony, but the X chromosomes of all mice used here were identical and derived originally from mating an XY mouse with his XO dam to produce a line of mice with identical X chromosomes bred onto outbred stock (P Burgoyne, personal communication). Thus, in these experiments, differences between mice with one vs. two X chromosomes were attributable to the number of X chromosomes, not to a difference in X alleles.

2.3 Results

2.3.1 Body weight and adiposity before GDX and testosterone treatment

In gonadally intact mice, XXY (but not XYY) sex chromosome complement was associated with greater body weight and adiposity

Mice were weighed just before gonadectomy. At this time, gonadal males were significantly heavier (25%) than gonadal females regardless of sex chromosome complement, and there was no overall effect of sex chromosome complement (Figure 2-2 A). In pairwise comparisons, XXY mice weighed significantly more (13%) than XY mice regardless of gonadal type, but XY did not differ from XX or XYY. Gonadal males also had significantly greater relative fat mass (32%, Figure 2-2 B), and significantly greater (70%) absolute fat mass than gonadal females (Supplementary Figure 2-7 A), but there was no overall effect of sex chromosome complement on either measure. In pairwise comparisons. XXY mice had significantly more (24%) relative fat mass than XY mice, but XY did not differ from XX or XYY, and there was no effect of sex chromosome complement on absolute fat mass. Relative lean mass was not affected by sex or sex chromosome complement in the overall analysis (Figure 2-2 C), but gonadal males had significantly greater (21%) absolute lean mass than gonadal females (Supplementary Figure 2-8 B), and there was a significant effect of sex chromosome complement on absolute lean mass. In pairwise comparisons for relative lean mass there was no significant effect of sex chromosome complement, but XXY mice had significantly more (10%) absolute lean mass than XY (Supplementary Figure 2-8 B), but XY did not differ from XX or XYY. Thus, the gonadally intact XXY mice resembled KS men in having increased body weight compared to XY mice, which was caused, in part, by increased proportional fat mass. These differences were independent of gonadal sex and suggest that XXY chromosome complement influences metabolism leading to altered body composition.

2.3.2 Body weight and adiposity 3 weeks after GDX and testosterone treatment

After GDX and testosterone treatment, XXY mice had greater body weight and adiposity than XY mice

To remove confounding effects of endogenous gonadal secretions, we performed GDX and restored testosterone in mice of all genotypes by testosterone pellet implantation. Three weeks after GDX and testosterone pellet implantation, all mice had testosterone levels in the physiological range (170-1440 ng/dL). There were no differences across genotypes, but gonadal females had significantly higher (14%) testosterone levels than males (Figure 2-3 A). The sex difference in level of testosterone is possibly explained by the larger body size of males, because using body weight as a covariate in an ANCOVA eliminated the significant sex difference in level of testosterone. Thus, we generated mice that do not differ in testosterone levels across genotype within sex. We assessed body weight and composition and related metabolic parameters in these animals.

Three weeks after GDX and testosterone pellet implantation, gonadal males were still significantly heavier (26%) than gonadal females (Figure 2-3 B), but there was no overall effect of sex chromosome complement. In pairwise comparisons, XXY mice weighed 13% more than XY, but XY did not differ from XX or XYY. Gonadal males had significantly greater relative (69%) and absolute (121%) fat mass than gonadal females (Figure 2-2 C, Supplemental Figure 2-8 A). In addition, sex chromosome complement had a significant overall effect on relative fat mass. In pairwise comparisons, XXY mice had 43% more relative fat mass than XY mice, and XX had 21% more than XY mice (Figure 2-3 C). Absolute fat mass was 60% higher in XXY compared to XY mice (Supplementary Figure 2-2 B). Fat mass was not different in XY relative to XX or XYY. Relative lean mass was 6% lower in gonadal males than in gonadal females (Figure 2-3 D), but absolute lean mass was 17% greater in gonadal males than females (Supplemental Figure 2-8 B). In pairwise comparisons XXY had 4% lower relative lean mass than XY, and XYY 7% more absolute lean mass than XY. XY did not differ from XX or XYY in

relative or absolute lean mass. Thus, the XXY genotype (but not XYY) was associated with increased body weight due to increases in both absolute fat and lean mass, and these animals had a higher proportion of their body mass as fat than XY mice.

GDX and testosterone treatment interact with sex and genotype to change body composition

To assess group differences in the effect of gonadectomy plus testosterone treatment, we used the change in body weight and composition (measurements at the end of the experiment minus measurements before GDX) as the dependent variable. There was no significant overall effect of sex or sex chromosome complement on the change in body weight. In pairwise comparisons, XYY gained more weight than XY (Figure 2-4 A). Females had a significantly greater loss in relative fat mass (Figure 2-4 B) and greater gain in relative lean mass (Figure 2-4 C) compared to males, but there was no overall effect of sex chromosome complement on these measures. There were significant interactions between sex chromosome complement and gonadal sex in pairwise comparisons of XY with all three other groups for body weight, and when comparing XY with XX for change in relative lean mass (Figures 2-4 A and 2-4 C). There were no significant effects of sex or sex chromosome complement on the change in absolute fat mass or lean mass (data not shown).

After GDX and testosterone treatment, XXY mice had heavier metabolic tissues than XY mice

To further define the basis for differences in body weight and composition among genotypes, we measured weights of key metabolic tissues, including representative visceral and subcutaneous fat pads (gonadal and inguinal fat depots, respectively), liver, spleen and kidney. Except for spleen, each of these tissues was significantly heavier in gonadal males than females (Figure 2-5). There was also a significant overall effect of sex chromosome complement on liver weight. In pairwise comparisons, XXY had significantly heavier liver,

gonadal fat pad, and inguinal fat pad compared to XY (Figure 2-5 A-C), and XY did not differ from XX or XYY. There were no group differences in kidney or spleen weights.

Differences in body weight and composition between XXY and XY occurred in gonadal female groups

Because XXY male mice may have lower testosterone levels than XY males during development [60,61], differences between XXY and XY mice could be caused by organizational effects of testicular hormones. To test whether differences exist under conditions in which the XXY vs. XY comparison is unlikely to be confounded by androgen levels in this manner, we assessed sex chromosome effects in females only, using one-way ANOVAs with sex chromosome complement as the factor. Before GDX (Figure 2-2 and Supplementary Figure 2-7), there was a significant effect of sex chromosome complement in females on body weight ($F(3,50) = 4.78$, $p = 0.005$, overall one-way ANOVA), relative fat mass ($F(3,50) = 2.79$, $p = 0.05$), absolute fat mass ($F(3,50) = 3.39$, $p = 0.025$), relative lean mass ($F(3,50) = 3.73$, $p = 0.017$), and absolute lean mass ($F(3,50) = 3.25$, $p = 0.029$). Notably, in pairwise comparisons XXYF differed from XYF in each of these measures (with greater body weight and fat, and less relative lean mass, $p < 0.009$, t-tests).

After GDX and testosterone treatment (Figure 2-3 and Supplementary Figure 2-8), there was a significant effect of sex chromosome complement on body weight ($F(3,48) = 2.82$, $p = 0.049$, overall one-way ANOVA), relative fat mass ($F(3,48) = 5.27$, $p = 0.003$), absolute fat mass ($F(3,48) = 5.66$, $p = 0.002$), and relative lean mass ($F(3,48) = 4.42$, $p = 0.008$). In pairwise comparisons XXYF differed from XYF in each of these measures (with greater body weight and relative fat, and less relative lean mass, $p < 0.022$, t-tests). XYF also differed from XXF in each of these measures ($p < 0.036$, t-test). In response to GDX and testosterone treatment, there was a significant effect of sex chromosome complement on change in body weight ($F(3,48) = 2.81$, $p = 0.049$, overall one-way ANOVA) (Figure 2-4A), but not in other measures. In pairwise comparisons, XXYF had less change in body weight than XYF ($p = 0.026$, t-test), and XYF had

greater change in body weight than XXF (p = 0.044, t-test)(Figure 2-4 A). In tissue weights (Figure 2-5), there was a significant effect of sex chromosome complement for inguinal fat pad weight (F(3,50) = 7.67, p = 0.0003, overall one-way ANOVA) and relative inguinal fat pad weight (F(3,50) = 7.58, p = 0.0003), but not for other tissues. In pairwise comparisons, XXYF differed from XYF for liver weight (p = 0.012, t-test), gonadal fat pad weight (p = 0.019, t-test), and inguinal fat pad weight (p < 0.000001, t-test). (Figure 2-5). XYF also differed from XXF for inguinal fat pad weight (p = 0.007, t-test). Thus, even under conditions in which mice have never had testes, the second X chromosome of the XXY genotype influences body composition and metabolic tissue weights.

2.3.3 Motor function

XYY mice differed from XY mice in tests of motor function

In the challenging beam test, gonadal males took significantly longer (38%) to cross the beam compared to females (Figure 2-6 A). In pairwise comparisons there was a significant sex by sex chromosome complement interaction for XY compared to XX such that XX gonadal males (but not gonadal females) took longer to cross the beam than XY. Gonadal males also took significantly more steps to cross the beam compared to gonadal females (Figure 2-6 B), and the effect of sex differed depending on sex chromosome complement. In pairwise comparisons there were sex by sex chromosome complement interactions such that XX took more steps than XY but only in gonadal males, and the XYY *vs.* XY difference was not consistently in the same direction for gonadal males and females.

For the pole test there was no overall effect of sex or sex chromosome complement (Figures 2-6 C and 2-6 D), but in pairwise comparisons there were significant sex by sex chromosome complement interactions such that XYY males (but not females) took longer to turn and complete the test than XY. Because the mice differed in body weight, and because

increased body weight could make completion of the pole test more difficult, we analyzed the pole data using an ANCOVA with weight as the covariate, but found that weight does not explain any of the group differences.

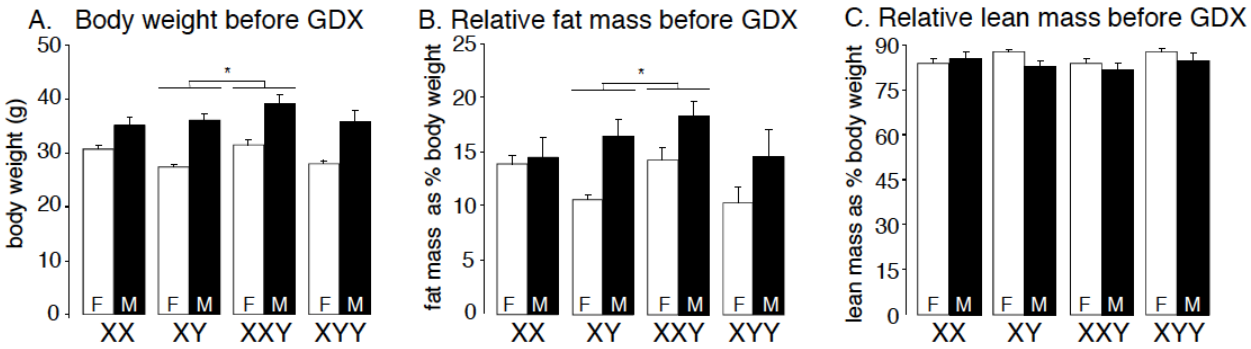

Figure 2-2: Body weight and composition just before GDX.
A. Gonadal males were about 25% heavier than gonadal females ($F(1,86) = 42.09$, $p < 0.000001$, overall ANOVA). In pairwise tests, XY mice weighed 13% less than XXY regardless of gonadal type ($F(1,51) = 6.65$, $p = 0.013$)(*). B. Gonadal males had on average about 32% more body fat as percent of body weight, compared to gonadal females ($F(1,86) = 6.05$, $p = 0.015$, overall ANOVA). In pairwise comparisons, XY had about 24% less body fat as percent of body weight than XXY irrespective of gonadal sex ($F(1,51) = 4.74$, $p = 0.034$)(*). C. Lean mass as percent body weight was unaffected by sex or sex chromosome complement. F, gonadal females, M, gonadal male. Values are mean + SEM.

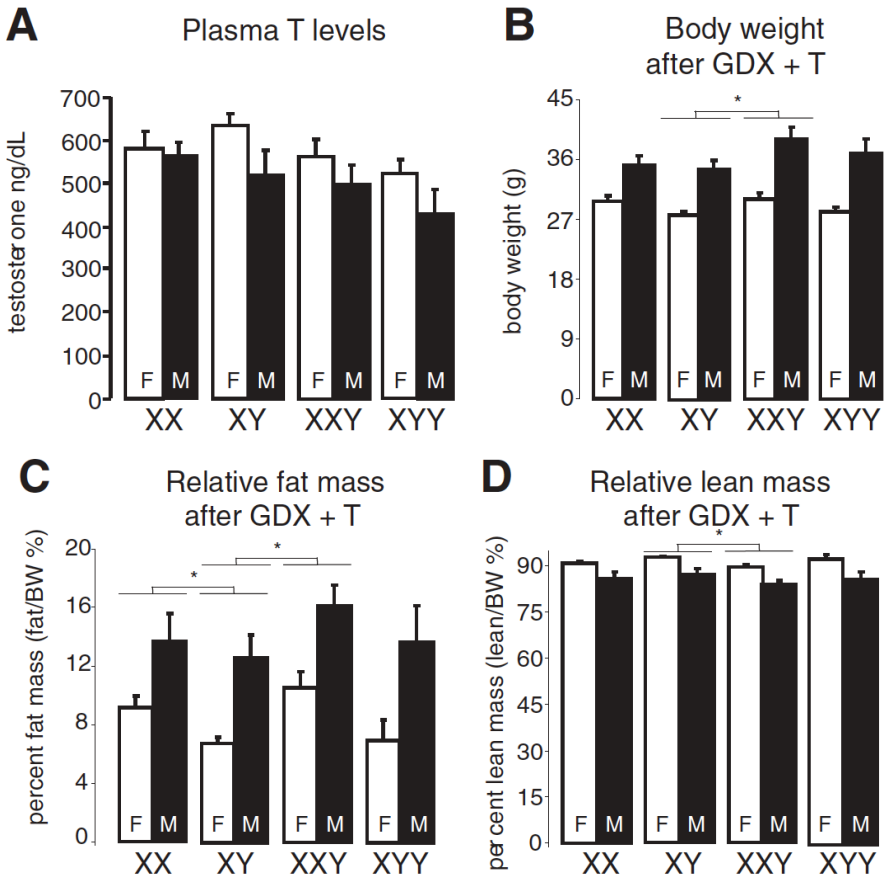

Figure 2-3: Plasma testosterone and body weight and composition after GDX and testosterone treatment.

A, Plasma testosterone did not differ as a function of sex chromosome complement within sex, but gonadal females had overall 14% higher levels of testosterone ($F(1,84) = 4.91$, $p=0.029$). B, Gonadal males were about 26% heavier than gonadal females ($F(1,84) = 63.13$, $p < 0.000001$), and XY mice 13% weighted less than XXY irrespective of gonadal sex ($F(1,51) = 7.40$, $p = 0.009$)(*). C, Gonadal males had 69% more relative fat mass than gonadal females ($F(1,84) = 37.75$, $p < 0.000001$), and there was a significant overall effect of sex chromosome complement ($F(3,84) = 4.30$, $p = 0.007$). XY mice had 43% less relative fat mass than XXY mice ($F(1,51) = 13.18$, $p < 0.0007$)(*), and 21% less than XX mice ($F(1,53) = 4.20$, $p = 0.045$)(*), irrespective of gonadal sex. D, Relative lean mass was 6% lower in gonadal males than females ($F(1,84) = 29.21$, $p = 0.000001$, overall ANOVA), In pairwise comparisons, XY had 4% greater relative lean mass than XXY ($F(1,51) = 6.63$, $p = 0.013$)(*). Values are mean + SEM.

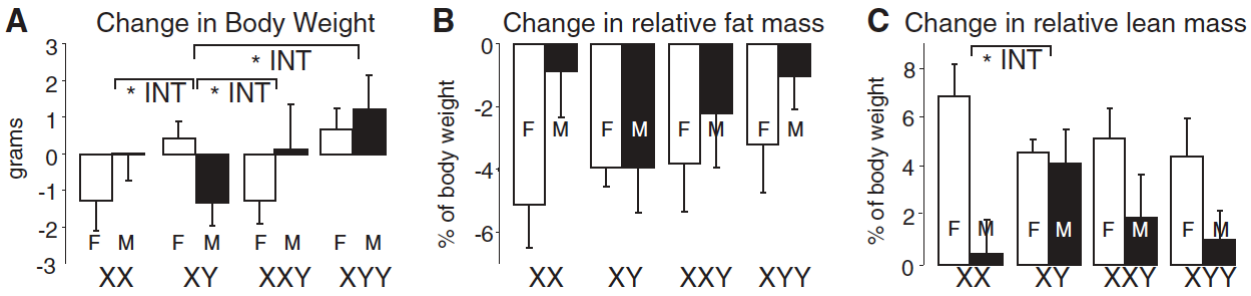

Figure 2-4: Change in body weight and composition caused by GDX and testosterone treatment.

Change in body weight and composition caused by gonadectomy and testosterone treatment. A, XYY mice gained more weight than XY ($F(1,46) = 4.58$, $p = 0.038$, main effect), In addition, the change in body weight in males vs. females depended on sex chromosome complement when comparing XY with XX ($F(1,53) = 0.020$, $p = 0.02$, significant interaction, *) and XY with XXY ($F(1,51) = 5.42$, $p = 0.024$, significant interaction, *). B, Gonadal females overall lost more relative fat mass than gonadal males ($F(1,84) = 4.37$, $p = 0.039$), but there was no significant effect of sex chromosome complement. C, Gonadal females overall gained more relative lean mass than gonadal males ($F(1,84) = 13.82$, $p = 0.0004$). The difference between XY and XX was different depending on their gonadal sex ($F(1,53) = 9.56$, $p = 0.003$, significant interaction, *). *INT indicates significant interactions of gonadal sex and sex chromosome complement. Values are mean + SEM.

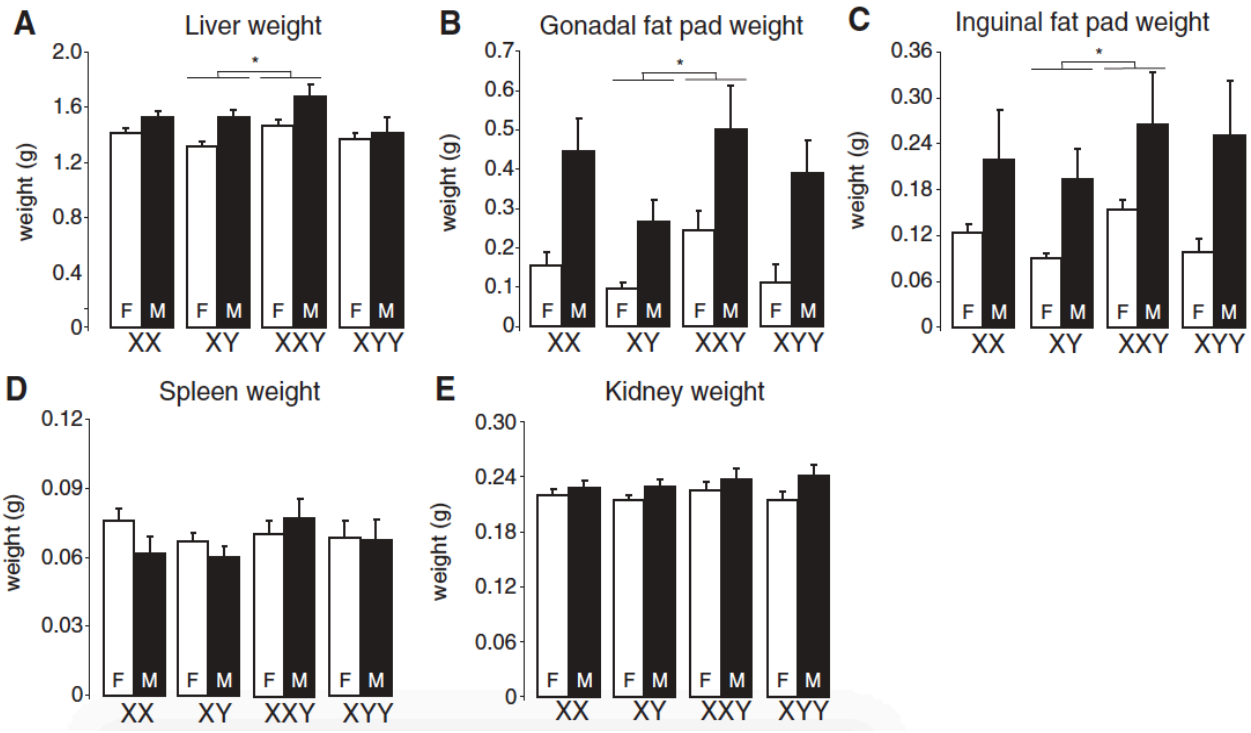

Figure 2-5: Tissue weights after GDX and testosterone treatment.
Tissue weights after gonadectomy and treatment with testosterone. All tissue weights were greater in gonadal males than females in the overall ANOVA (liver $F(1,85) = 13.05$, $p = 0.0005$; inguinal fat $F(1,86) = 19.41$, $p = 0.00003$; gonadal fat $F(1,50) = 23.29$, $p = 0.00001$; kidney $F(1,86) = 7.28$, $p = 0.008$), and sex chromosome complement affected liver weight in the overall ANOVA ($F(3,85) = 3.84$, $p = 0.012$). XXY mice had heavier livers, and gonadal and inguinal fat pads, compared to XY (liver $F(1,51) = 8.13$, $p = 0.006$; gonadal fat $F(1,29) = 7.61$, $p = 0.01$; inguinal fat $F(1,51) = 4.55$, $p = 0.038$)(*). There were no group differences in kidney or spleen weights. Values are mean + SEM

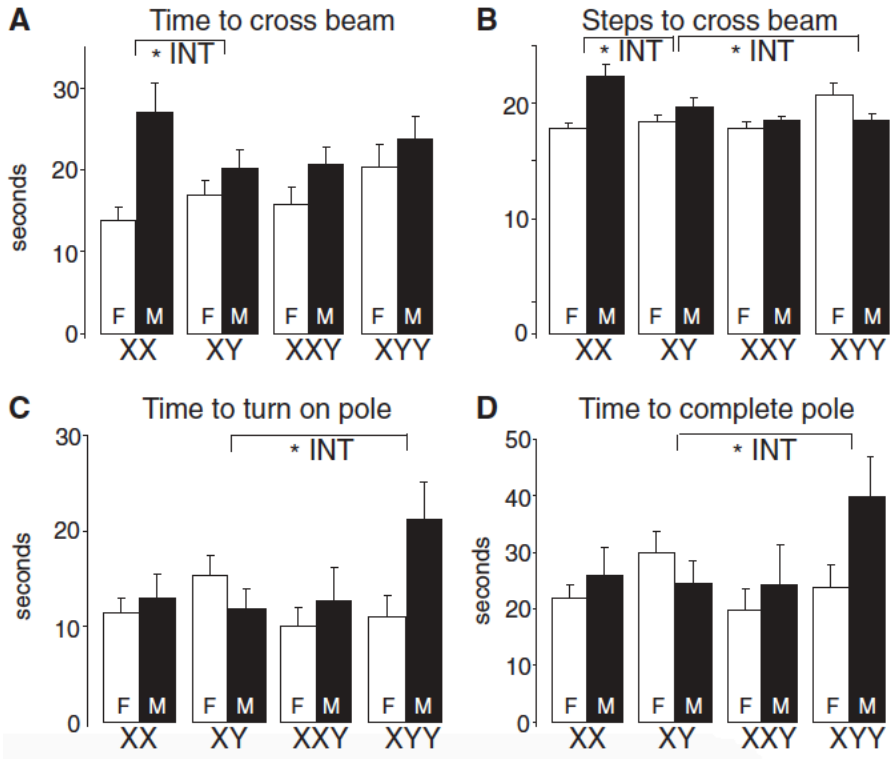

Figure 2-6: Motor performance.
Motor performance. A, In the challenging beam test, gonadal males took 38% longer to cross the beam compared to females ($F(1,85) = 12.45$, $p = 0.0007$, overall ANOVA) XX gonadal males took longer to cross the beam than XY males, but XX and XY females did not differ ($F(1,53) = 4.60$, $p = 0.037$, significant interaction, *). B, Gonadal males took 7% more steps to cross the beam compared to gonadal females ($F(1,85) = 4.41$, $p = 0.04$, overall ANOVA) but the effect of sex depended on genotype ($F(3,85) = 7.02$, $p = 0.0003$, significant interaction). In pairwise comparisons the difference between XY and XX, and XY and XYY, depended on sex ($F(1,53) = 5.25$, $p = 0.026$ and $F(1,46) = 4.78$, 0.034, respectively, significant interactions, *). C, In time to turn on the pole, the difference between XY and XYY mice depended on their sex ($F(1,46) = 6.80$, $p = 0.012$, significant interaction, *), D, In time to complete the pole test, the difference between XY and XYY mice depended on their sex ($F(1,46) = 4.62$, $p = 0.037$, significant interaction, *). *INT indicates significant interactions of gonadal sex and sex chromosome complement. Values are mean + SEM.

2.4 Discussion

Here we introduce a novel mouse model, the Sex Chromosome Trisomy Model, which compares XXY, XYY, XY, and XX mice within litters. Because gonadal sex is independent of sex chromosome complement, the effects of sex chromosome complement can be assessed independently of the exposure to testicular hormones. We find that XY and XXY mice differ in several measures of body composition and metabolic tissues including body weight, fat mass, lean mass, and metabolic tissue weights. Notably, these differences exist in XXY *vs.* XY gonadal females as well as in gonadal males, suggesting that these differences are caused, at least in part, by direct genetic effects of the second X chromosome acting outside of the gonads, rather than exclusively by lower levels of testicular androgens in XXY mice. The results several phenotypes that parallel the features of KS, and because the model unveils sex represent an initial validation of the SCT model, because XXY mice differ from XY mice in chromosome effects that would be difficult to study in humans or in previously described mouse models.

KS men have increased rates of obesity, especially abdominal obesity, and increased rates of type II diabetes [27-31, 55, 56]. In this study we show that XXY mice mimic the KS phenotype seen in humans because they also have increased body weight, increased body fat, and increased abdominal fat pad weight, relative to XY. Also similar to KS patients, the XXY mice differ in these measures both before and after manipulations of testosterone levels. Importantly, these group differences were seen in both gonadal male and female XXY mice, implicating direct genetic effects rather than hormone effects in these traits. These results supports a growing body of human literature that suggests that low testosterone levels do not explain the higher rates of metabolic disease in KS patients, and that testosterone treatment is not useful in preventing metabolic disease in these patients [30, 31, 55].

We hypothesized that XXY and XYY mice would have motor deficits compared to XY mice as seen in human patients with similar sex chromosome complement [35, 36]. We found that XXY gonadal males did not perform differently from XY males on two tests of motor function, but rather that the XYY males performed worse on the pole test. The XYY *vs.* XY differences were not found in gonadal females, suggesting that they represent the effects of an overdose of Y genes that require testicular hormones. For example, the overdose of Y genes may act on mechanisms that are masculinized by testosterone before adulthood. These motor impairments may model those seen in XYY humans, such as impairments in gross motor function and coordination [35, 36]. Our failure to detect motor impairments in XXY mice suggests either that XXY mice do not effectively model the motor deficits found in humans, or that we used tests that were not sensitive measures of the specific type of motor impairment seen in KS, such as fine motor dexterity.

The differences between XXY and XY mice that we report could be the result of several genetic mechanisms that operate differently in mice with two *vs.* one X chromosome [57]. About 3% of mouse X genes escape inactivation, and are expressed from both X chromosomes [58]. Among these are six genes (*Ddx3x, Eif2s3x, Kdm5c, Kdm6a, Uba1, and Usp9x)* that are consistently reported to be expressed higher in one or more of the following patterns: XX>XO, XX>XY, XXY>XY, in numerous mouse tissues including brain [59-64]. These six genes are X-linked in both humans and mice, and most of them escape X inactivation in both species [58, 65-68]. We consider these genes as top candidates for differences between XXY and XY groups found here. Indeed, expression profiling from the mice used in the present study confirmed higher expression of two of these genes in XXY *vs.* XY (*Kdm6a* and *Eif2s3x*) [48]. A second category of candidate genes are those that receive a parental imprint, because of the presence of both parental imprints in XXY mice, relative to the exclusively maternal imprint in XY. This class of genes is not well described, but may include *Xlr3b* [69] and others [70, 71].

Although XXY karyotype in humans sometimes results from maternal non-disjunction of X genes, in which case both X chromosomes would have a maternal imprint, the SCT model produces only XXY mice with one X chromosome from each parent, a condition found also in some KS patients. A third category is more hypothetical, and includes genes that may be regulated indirectly by heterochromatizing factors that could be modulated by the presence of a large inactive X chromosome in cells with two X chromosomes [72, 73].

The findings that sex chromosome complement is a significant contributing factor to body weight and body composition is not unexpected considering that our previous work on the Four Core Genotypes mouse model has shown that there are large sex chromosome effects in mice such that mice with two X chromosomes are heavier than mice with one X chromosome, regardless of gonadal sex, in both C57BL/6J and MF1 backgrounds [64, 74]. In addition, in MF1 mice, a second sex chromosome of either type, X or Y, increases body weight and adiposity, relative to mice with one sex chromosome. In these previous reports, the sex chromosome effects were particularly large several months after removal of the gonads in adulthood. In the present study, we have also found similar sex chromosome effects both before and after adult gonadectomy with simultaneous treatment with testosterone. Unexpectedly, although the hormone treatments resulted in the same levels of testosterone in gonadal males, and the same level in gonadal females, the levels were higher in females than males. The sex difference in hormone levels are probably related to body size, such that equal-sized capsules produced higher hormone levels in the females, which were smaller. This inference is supported by the finding that the sex difference in testosterone levels was not significant when body weight was used as a covariate in an ANCOVA. Thus, the sex difference in level of testosterone at the end of the study could have contributed to the male-female differences found in some of the traits.

Several previous mouse models have been used to model KS [75-77]. One mouse model involves a four-generation breeding scheme that produces C57BL/6 XXY and XY mice in

the same litters [76, 78-80]. This model has been used fruitfully to discover important phenotypes associated with the XXY genotype in mice (*e.g.,* hypogonadism, osteoporosis, and learning and social behavioral phenotypes [76, 79, 80]), although another laboratory has had difficulty with this breeding scheme [81]. A second model, which involves breeding commercially available C57BL/6 XY* mice, is more tractable [81]. This model produces XY* gonadal males, and XX^{Y*} gonadal males. The X^{Y*} chromosome is a fusion of the non-pseudoautosomal regions of one X and one Y chromosome end-to-end, connected by an aberrant pseudoautosomal region. Thus, the XX^{Y*} gonadal male is similar to XXY, except that it has only two sex chromosomes. Comparison of XY* and XX^{Y*} indicates that XX^{Y*} have some phenotypes typical of KS men, including hypergonadotropic hypogonadism, infertility due to lack of sperm, Leydig cell hyperplasia, and behavioral deficits [81-83]. Therefore, the XY* model has advantages because of its easier breeding scheme and validated features similar to KS. All previous mouse models of KS have a similar disadvantage, which is that they produce XXY and XY gonadal males only, so that differences in sex chromosome complement are potentially confounded with lower levels of testicular hormones found in XXY mice [80, 82]. In addition, the XX^{Y*} mouse is not completely identical genetically to XXY [45]. Although a few studies on previous mouse models of KS have eliminated XXY *vs.* XY differences in the levels of testosterone in adulthood, by castrating mice and implanting them with testosterone pellets [41, 79, 83], the XXY and XY groups could nevertheless be different because of long-lasting (organizational) testosterone effects on phenotypes caused by lower androgen secretion in XXY mice during early critical periods of development, including prenatally. Thus, in mouse models comparing only XXY gonadal males with XY gonadal males, it is difficult to separate organizational hormone effects from the direct effects of sex chromosomes on non-gonadal tissues.

In the SCT model the gonadal sex of the animal is independent of sex chromosome complement, allowing for improved study of the individual contributions of direct genetic effects,

hormonal effects, and interactions of the two. The SCT model has the novel advantage of allowing for the study of the related chromosomal abnormality XYY, which causes a syndrome similar to KS but that does not involve low testosterone levels, and which has not been well-studied in animal models. The XYY animals are also a useful control for comparing the effects of an extra sex chromosome to the effects of an extra X chromosome specifically. Similarly, XX males serve as control for two X chromosomes without a third sex chromosome. The main disadvantages of the SCT model are the larger number of genotypes produced, and that the model is probably only viable on an outbred background such as MF1.

Some caveats are warranted. It is possible that the expression of *Sry* from the transgene is not identical to that from the endogenous *Sry* from wild-type (WT) mice. Phenotypically, XY^{-}*(Sry+)* males are identical to WT XY males in many but not all measured phenotypes [44]. Because *Sry* is expressed outside of the testes, in the brain, kidney, adrenals, and other tissues [84, 85], it is not possible to rule out differences between gonadal males in the present study relative to those carrying an endogenous *Sry* gene. Because the treatment of adult mice in the current study was for three weeks, it is possible that longer treatments with testosterone would have had more dramatic effects on phenotype.

Although the association between the XXY karyotype and KS was discovered nearly 60 years ago, the mechanisms by which the extra X chromosome causes the syndromic features of KS are still not well understood. Currently, the treatment of KS often involves testosterone replacement therapy, which is understudied but reported to have positive effects on language, intellectual, and motor skills of pre-school KS boys [32, 33]. Evidence suggests that in humans, not all KS phenotypes are the result of lower androgen levels [16, 30, 31]. Because direct sex chromosome effects are difficult to identify in patient populations or in previous animal models, they are poorly understood. The SCT model offers the opportunity to establish which phenotypic features of XXY and XYY are independent of, or dependent on, the gonadal effects of these

trisomies. The SCT model lays the foundation for using the power of mouse molecular genetics to discover the X genes that cause KS phenotypes, and the Y genes that make XYY mice different from XY.

2.5 Researchers' Contributions

Shayna Williams-Burris, Xuqi Chen, Tuck Ngun, Negar Ghahramani, and Hayk Barseghyan performed experiments and analyzed data. Shayna Williams-Burris performed literature review, and drafted the manuscript. Rebecca McClusky organized and implemented mouse breeding and genotyping. Karen Reue, Eric Vilain, and Arthur P. Arnold conceived of experiments, organized the research team, revised and edited the manuscript.

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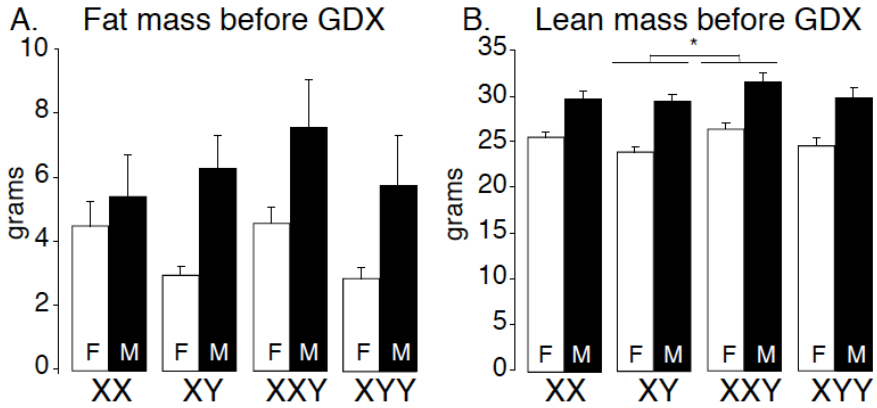
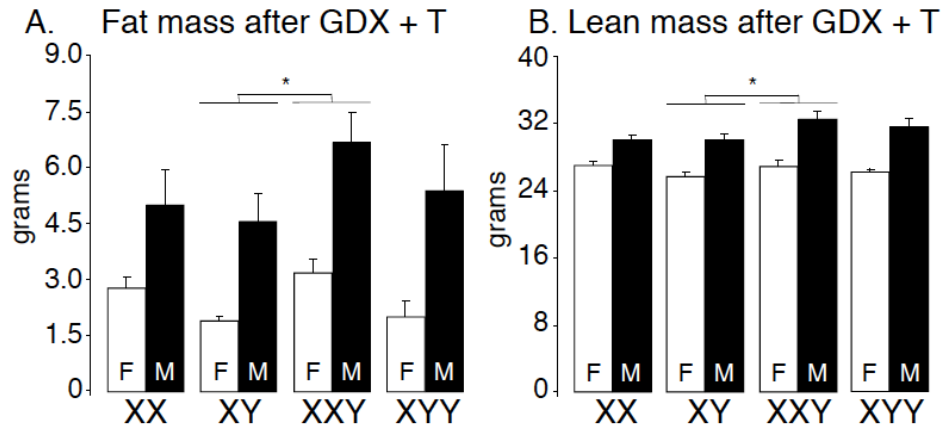
2.7 **Supplemental Materials**



Supplemental Figure 2-7: Body composition before GDX and testosterone treatment. A, Gonadal males had about 70% greater absolute fat mass than gonadal females, irrespective of sex chromosome complement ($F(1,86) = 14.92$, $p = 0.0002$, overall ANOVA). B, In the overall ANOVA, gonadal males had about 21% greater lean mass weight than gonadal females ($F(1,86) = 79.63$, $p < 0.000001$) irrespective of sex chromosome complement, and the effect of sex chromosome was significant ($F(3,86) = 2.83$, $p = 0.043$). In pairwise comparisons, XXY mice had about 10% more lean mass than XY ($F(1,51) = 8.88$, $p = 0.004$)(*). Values are mean + SEM.



Supplemental Figure 2-8: Body composition after GDX and testosterone treatment. Body composition after gonadectomy and treatment with testosterone. A, Gonadal males had about 121% greater fat mass weight than gonadal females, irrespective of sex chromosome complement ($F(1,84) = 34.42$, $p < 0.000001$). XXY mice had about 60% more body fat mass than XY ($F(1,51) = 7.08$, $p = 0.01$)(*). B, Gonadal males had 17% greater lean mass than gonadal females ($F(1,84) = 63.71$, $p < 0.000001$) irrespective of sex chromosome complement. XXY mice had 7% more lean mass than XY ($F(1,51) = 5.14$, $p = 0.028$)(*). Values are mean + SEM

Chapter 3

Testis, Bone, and Longitudinal Metabolic Measures in the Sex Chromosome Trisomy Model of Klinefelter Syndrome

3.1 Background

The karyotype XXY causes Klinefelter syndrome (KS) in humans. A defining feature of KS is small testes with seminiferous tubule degeneration, azoospermia, and Leydig cell hyperplasia. The testicular failure also leads to hormonal dysregulation such that men with KS have paradoxically low testosterone levels despite increased number of Leydig cells, the testosterone secreting cells of the testes. Men with KS also have chronic compensatory hypergonadotropism [1, 2]. KS occurs in 1:600 live male births, and is the most common genetic cause of male infertility [3-6].

In addition to the gonadal phenotype, KS men have several non-gonadal traits. KS men have increased rates of some illnesses that more frequently occur in women, such as breast cancer, lupus, and osteoporosis [7-15]. The increased frequency of osteoporosis is generally thought to be a result of hypogonadism. However, one paper using a mouse model suggests that there may be a genetic component because mice treated with testosterone for a long period of time in adulthood were not protected from bone loss as measured by micro computed

tomography (μCT) [16]. That study satisfactorily shows that correcting circulating hormone levels does not fully improve bone features, suggesting a direct genetic effect. The model used in this study, however, does not allow for the separation of direct genetic effects of the second X chromosome from the organizational effects of hormones levels during development that may lead the XXY mice to have permanently lower bone mineral density despite later testosterone treatment.

Another set of health problems that have increased incidence in KS men are obesity and obesity-related illnesses including hyperinsulinemia, insulin resistance, type II diabetes, and/or metabolic syndrome [17-23]. Treatment with testosterone can provide improved outcomes for some KS phenotypes in KS men, especially cognitive and motor problems [24, 25], but is not sufficient to improve metabolic features [18]. Additionally, KS boys begin to show metabolic syndrome before puberty, when testosterone levels are similarly low in both KS boys and controls [21]. Moreover a particularly informative study comparing men with KS with men who have idiopathic hypogonadotrophic hypogonadism (IHH) showed that men with KS had greater rates of diabetes than men with IHH both before and after both groups received testosterone replacement therapy [23]. Taken together, these results suggest that there may be direct genetic effects of the second X chromosome on metabolism, not exclusively mediated by reduced androgen levels in KS males. Nevertheless, it is not possible to cleanly distinguish X gene dose effects from hormone effects in human patient populations.

Indeed, in humans, as well as in all previously existing mouse models, it is impossible to separate the effects of low testosterone during development in XXY from direct genetic effects of the second X chromosome because the karyotype XXY induces low testosterone secretion from the testes. Here we use a unique mouse model, the Sex Chromosome Trisomy (SCT) model that produces XXY, XYY, XY, and XX mice, each genotype with either testes or ovaries, all within the same litters. By comparing the XXY and XY male and female mice it is possible to

identify KS features that are modeled by the mice, and to then identify which traits are likely genetic versus hormonal. More specifically, differences between XXY and XY mice that occur both in male and female groups do not require testicular hormones and are likely direct effects of the second X chromosome in XXY mice. In contrast, differences between XXY and XY mice that occur in male groups, but not in females, are more likely to be caused by possible group difference in hormone levels, or at least require the presence of testicular hormones. A previous study showed the SCT model to be useful for the study of sex chromosome effects versus hormone effects in body weight and composition, motor performance, social behavior, and gene expression in the brain [26, 27]. Here, we first describe the testicular phenotype of the SCT mice, expand the understanding of the osteoporotic phenotype of KS and its genetic vs. hormonal etiology, and follow up the previous SCT study to gain further insight into the metabolic phenotype of KS by measuring body weight and composition over time, and measuring several tissues at the time of dissection.

The SCT model, in addition to producing the KS comparison XXY and control XY male and female groups, produces XX and XYY male and female mice. These mice were also measured for most traits, except XX were not measured in the μCT experiment. There were few significant differences between groups when comparing XX and XYY to XY controls, and these findings were less relevant to the primary scientific question of whether genetic or hormonal effects are primarily at play in KS features, therefore these data are reported in supplemental materials only.

3.2 Methods

3.2.1 Animals

All mice were Sex Chromosome Trisomy mice on the MF1 outbred background strain as described in previous chapters. To summarize, XY^- female mice from the Four Core Genotypes (FCG) mouse model [28-30] were crossed with XY males to generate XXY^- female mice. XXY^- female mice were then crossed with XY^- *Sry* FCG male mice to create the 8 genotypes of the Sex Chromosome Trisomy model: XX, XY^-, XXY^-, and XY^-Y^- each either male (with the testes determining gene *Sry* inserted as a transgene on an autosome) or female (without *Sry*). Half of the mice were gonadectomized at 75 days after birth to create 16 total groups (Table 3-1). Note that data for XX and XYY groups in this experiment are presented in supplemental material (Supplemental Table 3-2).

All experiments were approved by the UCLA Institutional Animal Care and Use Committee. Mice were maintained at 23°C on a 12:12 light:dark cycle. Mice were fed regular chow diet with 5% fat (LabDiet 5001). Mice were single-housed because a food intake study was attempted, but the animals exhibited a behavior of shredding chow into powder without eating it, so it was impossible to measure how much was consumed. Animals were not re-housed into groups because of the high risk of fighting, and therefore the results presented here are from individually housed mice.

3.2.2 Genotyping

Mice were genotyped using fluorescent *in situ* hybridization with a specially designed probe mix that labels the RAB9B gene on the mouse X chromosome red and paints the Y chromosome green (Kreatech catalog number KI-30505, Kreatech Biotechnology, Amsterdam,

The Netherlands). FISH was performed by standard methods as instructed by the Kreatech protocol except 1 μL of probe mix was used instead of the 10 μL indicated by the protocol.

3.2.3 Longitudinal metabolic features

Body weight was measured weekly starting after weaning (21 days after birth) and ending just prior to euthanasia at 37 weeks after birth. Body composition was measured monthly during the same timespan using an NMR Mouse Minispec apparatus (Bruker Woodlands, TX) with Echo Medical Systems (Houston, TX) software with coefficients of variation of less than 3%. Correlation between NMR and gravimetric measurements is better than 0.99.

At the end of the experiment, the weights of two specific fat pads were measured: visceral fat of the peri-gonadal depot (referred to here as gonadal fat) and the subcutaneous fat depot from the femoral region (referred to as inguinal fat). Fasting blood glucose and the weights of liver, spleen, kidney, heart, and testes were also recorded (Figure 3-1).

3.2.4 Testes size and histology

Testes were collected and weighed either at gonadectomy for GDX mice, or at the time of dissection for intact mice. Testes that were collected at dissection were immediately placed in 10% formaldehyde overnight, embedded in paraffin, sliced into 4 μm sections, and hematoxylin and eosin stained for histological analysis.

3.2.5 Bone analysis

Whole femurs were collected after dissection into 100% ethanol and measured for bone length, bone mineral density, and bone architecture. Bone length was measured to the nearest 0.005 cm using calipers. Bone mineral density was measured in the whole femur and distal femur using a dual-energy x-ray absorption (DEXA) densitometer (Lunar PIXImus II Densitometer and associated software, GE Medical Systems).

μCT of the femur was performed using a Skyscan 1172 scanner (SkyScan, Kontich, Belgium). Scanning occurred at 100 kV and 100 μA with a 1-mm aluminum filter, and the exposure time was set to 590 ms. In total, 1800 projections were collected for each femur at a resolution of 6.93 μm/pixel. Sections were reconstructed using a modified Feldkamp cone-beam algorithm with beam-hardening correction set to 50. VGStudio MAX 1.2 software (Volume Graphics GmbH, Heidelberg, Germany) produced 3D visualizations from reconstructed sections. Trabecular morphometry of proximal femurs were quantified with CTAnalyser software (Version 1.02, SkyScan). The volume of interest for trabecular bone was selected within the endosteal borders from 0.1 to 1.1 mm above the proximal surface of the proximal growth plate. Trabecular morphology was described by bone volume fraction (percent BV/TV), trabecular number (Tn), trabecular separation (Ts), trabecular thickness (Tt), and bone surface to bone volume ratio (BS/BV).

3.2.6 Statistics

Statistics were calculated in R. In the main results section, only mice with the sex chromosome complements XY and XXY were included in analysis. The XY vs. XXY comparison in both gonadal males and gonadal females is most relevant to the question of whether sex chromosomes or hormonal effects are more influential in the observed KS related phonotype. In the supplemental data, only the mice with sex chromosome complements XY, XX, and XYY were included in analysis. For each measure, an omnibus test was first conducted to test for overall effects, then post hoc tests were conducted to identify group differences. For the longitudinal metabolic data, the omnibus test was a mixed model linear regression with the factors of sex chromosome complement, gonadal sex, gonadectomy (GDX), and age. The (lmer() function from package lmerTest in R), post-hoc tests were performed by stepwise backward elimination of all effects in the linear model (step() function from package lmerTest in

R). All data measured at a single age were analyzed by linear regression model (lm() function from package stats in R) and post hoc factor interaction tests were performed with false discovery rate (fdr) multiple testing correction (testInteractions() function from package phia in R) for lm() models.

In some cases, we had *a priori* hypotheses that we tested using pairwise tests irrespective of whether an interaction was first indicated by the omnibus test. This is valid because while the experimental design yields a three way repeated measures model, not all comparisons are biologically relevant. For example, differences between XX and XYY are not interpretable in the context of questions about the differences between KS and XY, or XYY vs. XY. However, some comparisons are biologically interesting even without a significant main interaction effect. For example, in bone length, the omnibus test did not show a significant GDX by sex chromosome complement by gonadal sex interaction. Nevertheless, because XY and XXY intact male mice have differing testosterone levels it is important to ask whether these two groups have differing bone length measures regardless of the outcome of the omnibus test, because the omnibus test includes biologically unimportant comparisons (e.g., XX vs.XYY) and decreases power to see biologically relevant differences. In the case of these *a priori* hypothesis tests, p-values were corrected for multiple testing by false discovery rate (p.adjust(,method="fdr") function in stats package in R).

Table 3-1: Experimental groups.

Gonadectomy	Genotype	Sex chromosome complement	Gonadal sex	Shorthand	Sample size
Intact	XY^{-}	XY	Female	Intact XY F	12
Intact	XXY^{-}	XXY	Female	Intact XXY F	8
Intact	XY^{-} *Sry*	XY	Male	Intact XY M	6
Intact	XXY^{-} *Sry*	XXY	Male	Intact XXY M	6
Gonadectomized	XY^{-}	XY	Female	GDX XY F	8
Gonadectomized	XXY^{-}	XXY	Female	GDX XXY F	8
Gonadectomized	XY^{-} *Sry*	XY	Male	GDX XY M	13
Gonadectomized	XXY^{-} *Sry*	XXY	Male	GDX XXY M	9

The SCT cross produces 8 genotypes (XX, XY, XXY, or XYY, each either male or female as determined by the presence or absence of *Sry*), and half of the animals were gonadectomized (GDX) while half were left intact yielding 16 experimental groups. To simplify results the XY and XXY mice, those most relevant for the study of Klinefelter syndrome, are presented here. Data for XX and XYY are presented in supplemental material.

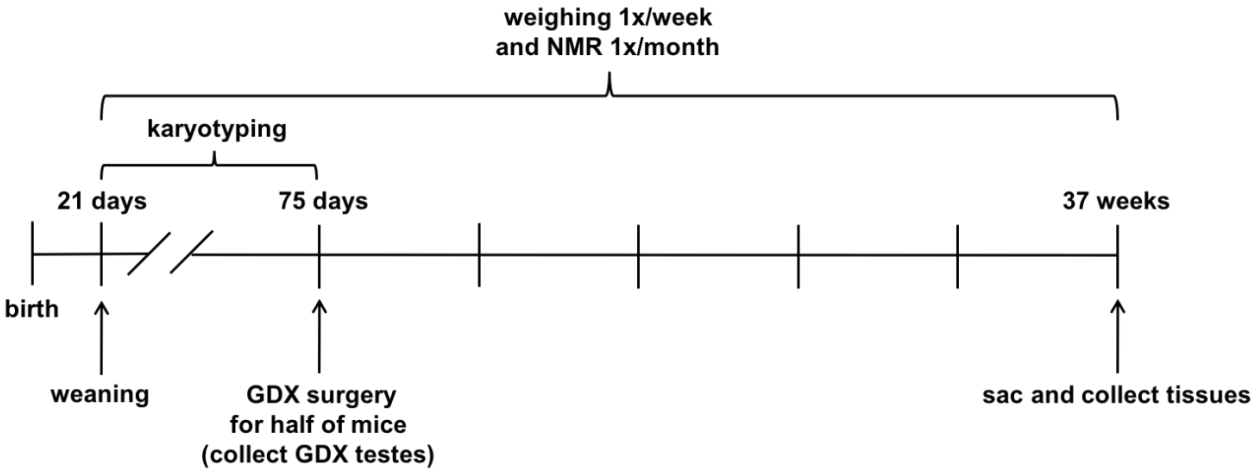


Figure 3-1: Experiment timeline.
Mice were weaned at 21 days and from that time forward were weighed every week and underwent body composition analysis every 4 weeks. At 75 days half of experimental animals underwent GDX surgery and testes were collected from males. When the mice reached 37 weeks of age they were euthanized and tissues were collected.

3.3 Results

3.3.1 Testes

XY mice have normal sized testes (mean weight = 0.11 g), while XXY mice have much smaller testes (mean weight = 0.02 g, $p < 0.0001$, Figure 3-2 A). Similar to men with KS, the XXY mice showed detrimental changes in histology including cell loss in seminiferous tubules and Leydig cell hyperplasia (Figure 3-2 B).

3.3.2 Bone

GDX mice had longer femurs than intact mice ($p = 0.0011$), and there was a gonadal sex by sex chromosome complement interaction ($p = 0.0220$). For this interaction post hoc tests did not show that there was a sex chromosome complement difference in either gonadal sex when combining GDX and intact animals (females $p = 0.1294$, males $p = 0.0698$), but there was a significant effect of sex chromosome complement when considering only intact males such that XXY had longer femurs than XY ($p = 0.0427$, Figure 3-3). For bone mineral density (BMD) in the whole femur, males had greater BMD than females ($p = 0.0069$), intact had greater BMD than GDX ($p = 0.0010$), and there was a GDX by sex chromosome complement interaction ($p = 0.0407$), and a gonadal sex by sex chromosome complement interaction ($p = 0.0260$) and a gonadal sex by sex chromosome complement by GDX interaction ($p = 0.0488$) such that the intact XY males had greater BMD than all other groups (Figure 3-4). Measurements of the distal femur showed similar group differences as for whole femur.

For the μCT measures; male mice had greater percent bone volume/tissue volume (percent BV/TV, $p = 0.0456$) and increased trabecular number (Tn, $p = 0.462$) compared to female mice, and intact animals had greater percent BV/TV ($p = 0.0027$) and increased Tn ($p = 0.0028$), but decreased trabecular separation (Ts, $p = 0.0122$) compared to GDX mice. GDX

and sex differences in bone surface over bone volume (BS/BV) failed to reach significance (Figure 3-5). Trabecular thickness was also measured but there were no group differences (data not shown).

3.3.3 Longitudinal metabolic measures

XXY mice weighed more than XY

In the overall linear regression for sex chromosome complement, gonadal sex, GDX, and age for body weight: XXY mice had greater body weight than XY ($p = 0.0002$), males had greater body weight than females ($p < 0.0001$), and GDX had greater body weight than intact ($p = 0.0458$). There was a gonadal sex by sex chromosome complement interaction ($p = 0.0426$) such that XXY males were significantly heavier than XY males ($p = 0.0002$), but this XXY vs. XY weight difference wasn't significant in females ($p = 0.1400$). There was also an effect of age such that all groups gained weight over the course of the experiment ($p < 0.0001$). At week 7 after birth there was predictably no effect of GDX group assignment because the mice had not been GDX'd yet ($p = 0.6584$), but males were already heavier than females ($p < 0.0001$), and XXY were heavier than XY ($p = 0.0001$). At the middle time point at 20 weeks, GDX had become heavier than intact ($p = 0.0069$), males were heavier than females ($p < 0.0001$), and XXY were heavier than XY ($p = 0.0064$). At the final time point at 32 weeks the GDX vs. intact difference had dissipated ($p = 0.0559$) probably due to increased variance within each group at that point of the experiment, males were still heavier than females ($p < 0.0001$), and XXY were heavier than XY ($p = 0.0498$). It is notable that there was a sex by genotype interaction in the overall linear regression but the interaction did not reach significance at any of the three independently tested time points (Figure 3-6).

While there was no significant gonadal sex by sex chromosome complement by GDX interaction pointing to a significant sex chromosome complement difference in just intact

females, results from previous studies on MF1 mice with assorted sex chromosomes suggest that the difference between XXY and XY mice in females may require the presence of gonadal hormones [31]. Therefore, it was interesting to independently test the *a priori* hypothesis that intact female XXY mice differ from intact female XY. In these groups, an overall linear regression for sex chromosome complement and time did not show a difference ($p = 0.3764$) between XXY and XY, but t-tests at the first, middle, and last measured time points showed that XXY intact females differed from XY intact females at 7 weeks ($p = 0.0220$) and 32 weeks ($p = 0.0404$), but not at 20 weeks ($p = 0.1250$); however, the differences at the first and last ages measured did not survive multiple testing correction by false discovery rate (both adjusted $p = 0.0638$).

XXY had greater percent body fat than XY at early ages but this difference disappeared at later ages

In the overall linear regression for sex chromosome complement, gonadal sex, GDX, and age for percent body fat: XXY mice had greater percent body fat than XY ($p = 0.0014$). There was no overall significant effect for gonadal sex or GDX but there was a gonadal sex by sex chromosome complement interaction ($p = 0.0110$) such that XXY males had significantly greater percent body fat than XY males ($p = 0.0001$), but XXY females were not significantly fatter than XY females ($p = 0.4860$). In stepwise post hoc tests, sex and GDX effects emerged such that males had greater percent body fat than females ($p = 0.04300$) and GDX mice had greater percent body fat than intact ($p < 0.0001$). At the first time point, month 1 (mice 75 days of age, before GDX), there was no effect of GDX ($p = 0.4495$), but sex chromosome complement was significant such that XXY mice had greater percent body fat than XY ($p = 0.0016$), and there was a gonadal sex by sex chromosome complement interaction ($p = 0.0012$) such that the XXY males were fatter than the XY males ($p = 0.0006$) but the XXY females were not fatter than the XY females ($p = 0.6503$). By 6 months later at age 7 months, the pattern had changed so that now there was a gonadal sex difference where males were fatter than females

(p = 0.00019), and GDX were fatter than intact (p < 0.0001), but there was no longer a sex chromosome complement difference between XXY and XY (p = 0.3333, Figure 3-7).

XXY had decreased lean mass compared to XY

In the overall linear regression for sex chromosome complement, gonadal sex, GDX, and age for percent lean mass: XXY mice had lower percent lean mass than XY (p = 0.0027). There was no overall significant effect for gonadal sex or GDX but there was a gonadal sex by sex chromosome complement interaction (p = 0.0213) such that XXY males had significantly lower percent lean mass than XY males (p = 0.0002), but XXY females did not have significantly less lean mass than XY females (p = 0.4910). In stepwise post hoc tests it remained true that there was no gonadal sex difference (p = 0.112), but the GDX mice had lower percent lean mass than intact (p < 0.0001). At the first time point, month 1 (mice 75 days of age, before GDX), there was no effect of GDX (p = 0.2112), but sex chromosome complement was significant such that XXY mice had lower percent lean mass than XY (p = 0.0103), and there was a gonadal sex by sex chromosome complement interaction (p = 0.0324) such that the XXY males had less lean mass than XY males (p = 0.0065) but the XXY females did not have less lean mass than the XY females (p = 0.7779). By 6 months later at month 7 the pattern had changed so that now there was a gonadal sex difference where females has less lean mass than males (p = 0.0034), and intact had lower percent lean mass than GDX (p < 0.0001), but there was no longer a genotype difference between XXY and XY (p = 0.4196, Figure 3-8).

3.3.4 Fasting blood glucose and metabolic tissue weights

GDX mice had greater fasting blood glucose than intact (p < 0.0001). Male mice had larger gonadal fat pads than female mice (p = 0.0306), and GDX mice had larger gonadal fat pads than intact mice (0.0014). Male mice had larger inguinal fat pads than female mice (p =

0.0013), GDX mice had larger inguinal fat pads than intact mice ($p = 0.0096$), and XXY mice had larger inguinal fat pads than XY ($p = 0.0093$).

Male mice had larger livers than females ($p = 0.0001$), and there was a sex by GDX interaction for liver size such that intact male mice had greater liver weight female intact ($p < 0.0001$), but gonadectomy abolished the difference because GDX males did not have greater liver weight than GDX females ($p = 0.3688$). Male mice had larger kidneys than female mice ($p < 0.0001$), intact mice had larger kidneys than GDX mice ($p = 0.0118$), and there was a GDX by sex interaction such that intact males had larger kidneys than intact females ($p < 0.0001$), but GDX males did not have larger kidneys than GDX females ($p = 0.1677$). There were no group differences in spleen size. Male mice had larger hearts than female mice ($p = 0.0001$), and there was a GDX by sex interaction such that intact males had larger hearts than intact females ($p < 0.0001$), but GDX males did not have larger kidneys than GDX females ($p = 0.8196$).

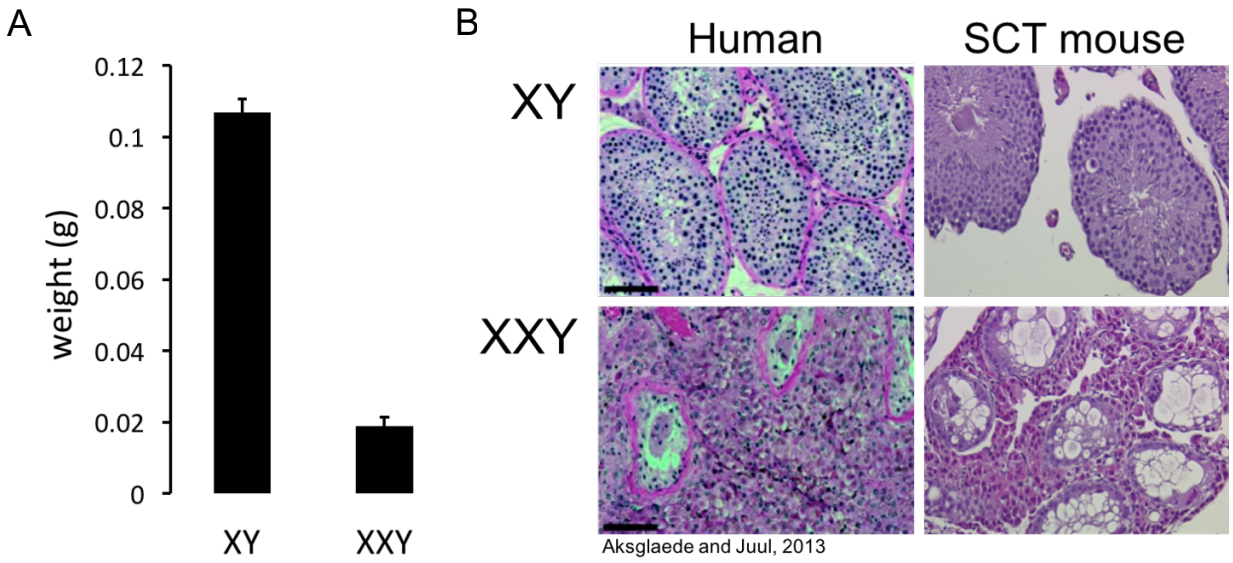


Figure 3-2: Testes histology and weight.
A) XY mice have normal sized testes, while XXY mice have much smaller testes ($p < 0.0001$), and B) histology shows XXY mouse testes to show signs if KS-like degradation including cell loss in seminiferous tubules and Leydig cell hyperplasia.

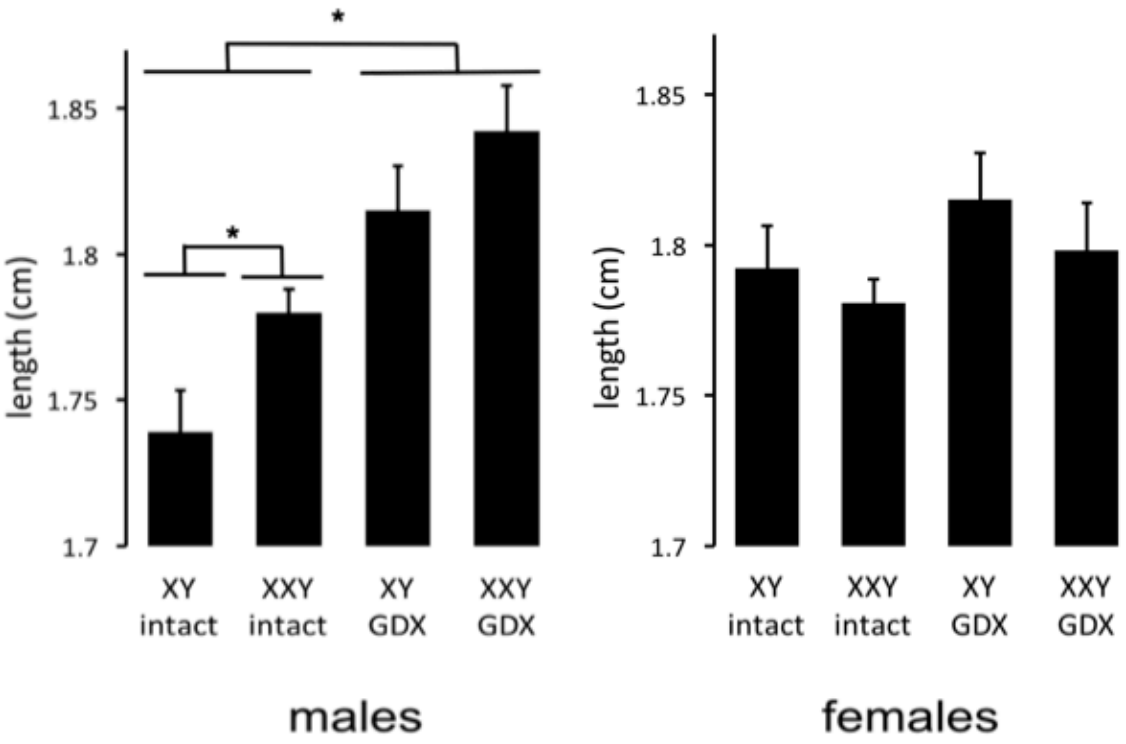


Figure 3-3: Bone length.
GDX mice had longer bones than intact mice (p = 0.0011), and there was a gonadal sex by sex chromosome complement interaction (p = 0.0220). For this interaction post hoc tests did not show that there was a sex chromosome complement difference in either gonadal sex when combining GDX and intact animals (females p =0.1294, males p = 0.0698), but there was a significant effect of sex chromosome complement when considering only intact males such that XXY had longer bones than XY (p = 0.0427, Figure 3-3).

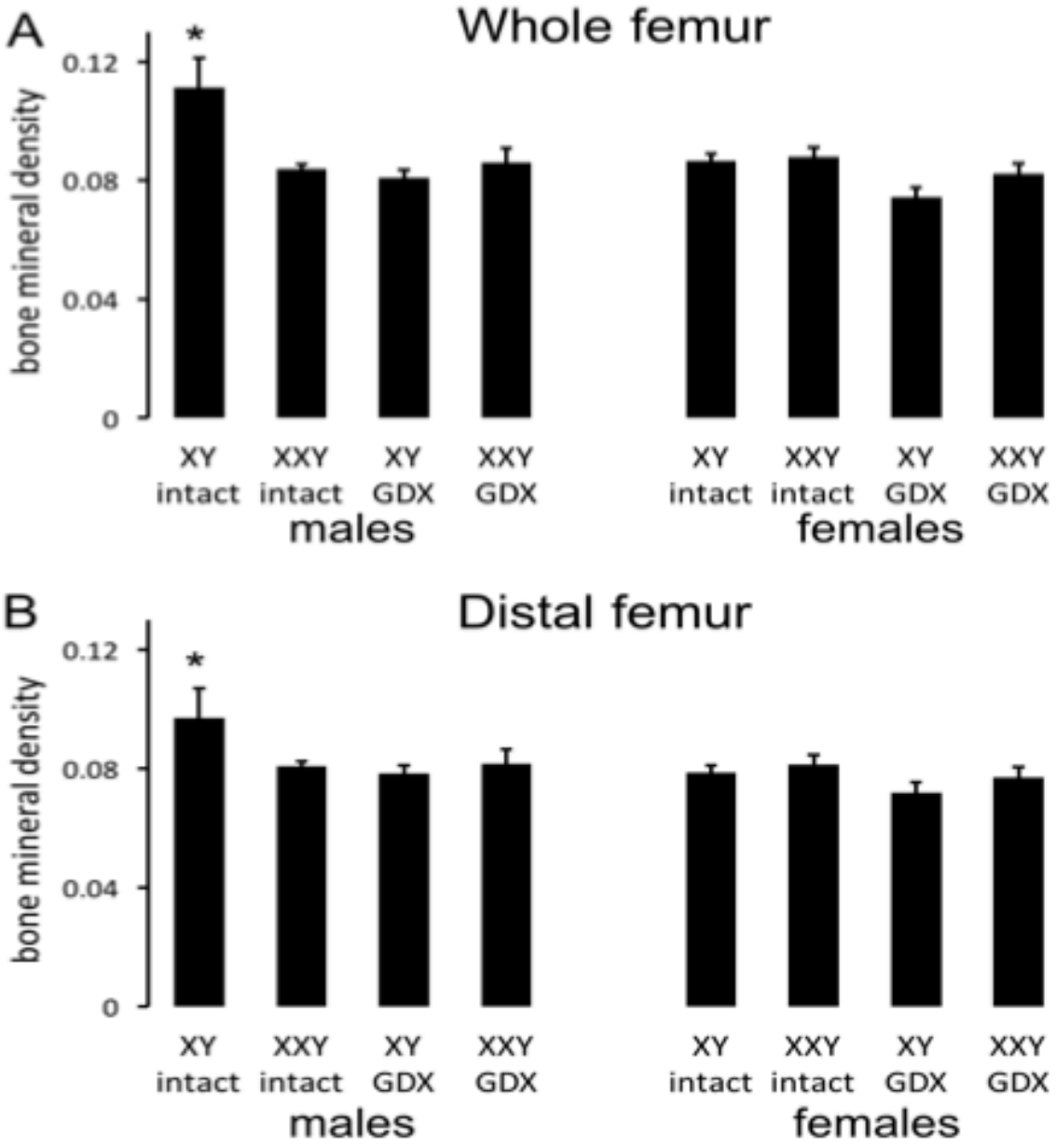


Figure 3-4: Bone mineral density.
A) Males had greater BMD than females ($p = 0.0069$), intact had greater BMD than GDX ($p = 0.0010$), and there was a GDX by sex chromosome complement interaction ($p = 0.0407$), and a gonadal sex by sex chromosome complement interaction ($p = 0.0260$) and a gonadal sex by sex chromosome complement by GDX interaction ($p = 0.0488$) such that the intact XY males had greater bone mineral density than all other groups. B) Measurements of the distal femur showed the same group differences as for whole femur.

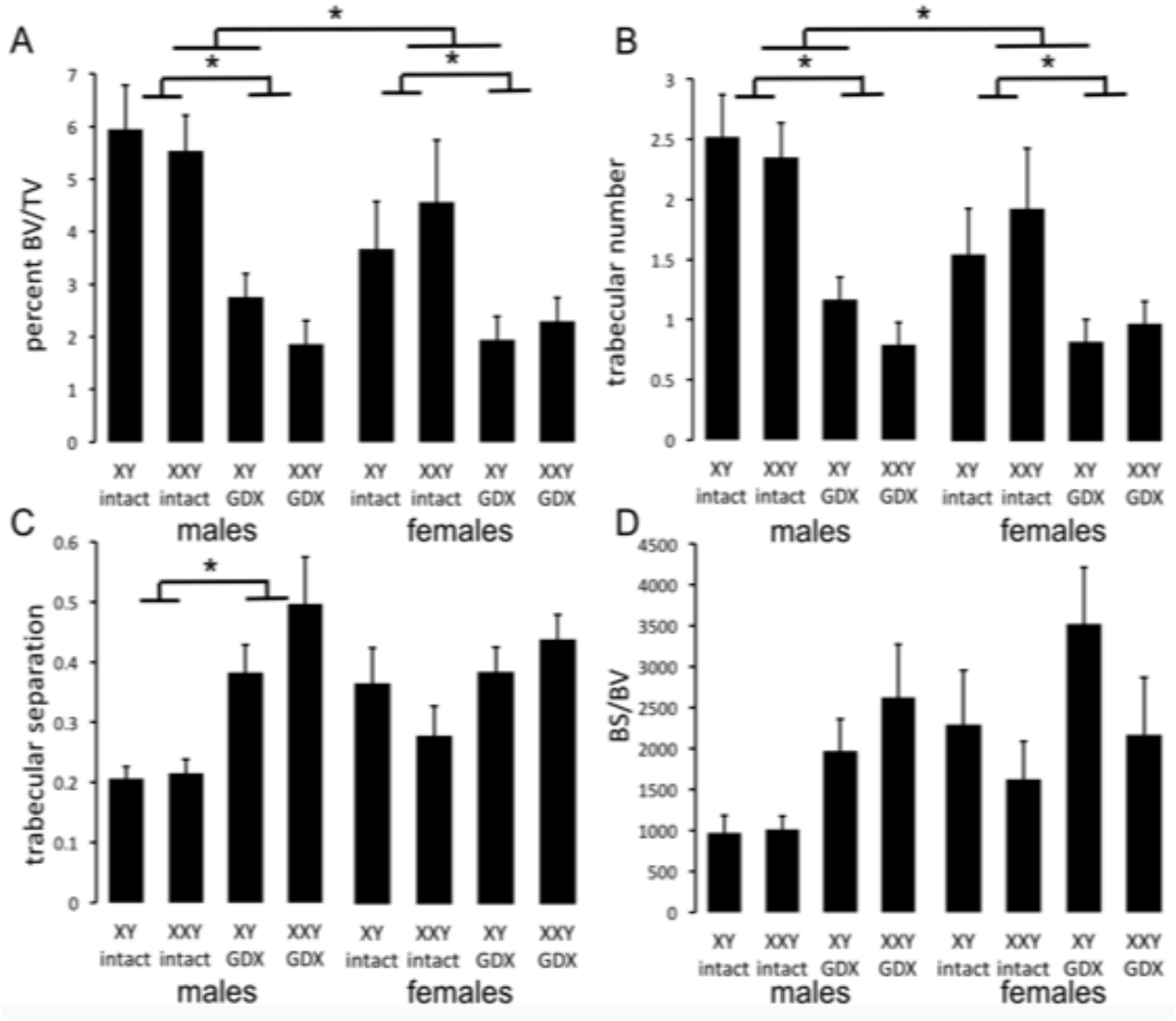


Figure 3-5: μCT data.
A) For percent bone volume/tissue volume (percent BV/TV), males had greater values than females ($p = 0.0456$), and intact had greater values than GDX ($p = 0.0027$). B) For trabecular number (Tn), males had greater values than females ($p = 0.462$), and intact had greater values than GDX ($p = 0.0028$). C) For trabecular separation, intact animals had lower values than GDX ($p = 0.0122$). D) GDX and sex differences in bone surface over bone volume (BS/BV) failed to reach significance. Trabecular thickness was also measured but there were no group differences (data not shown).

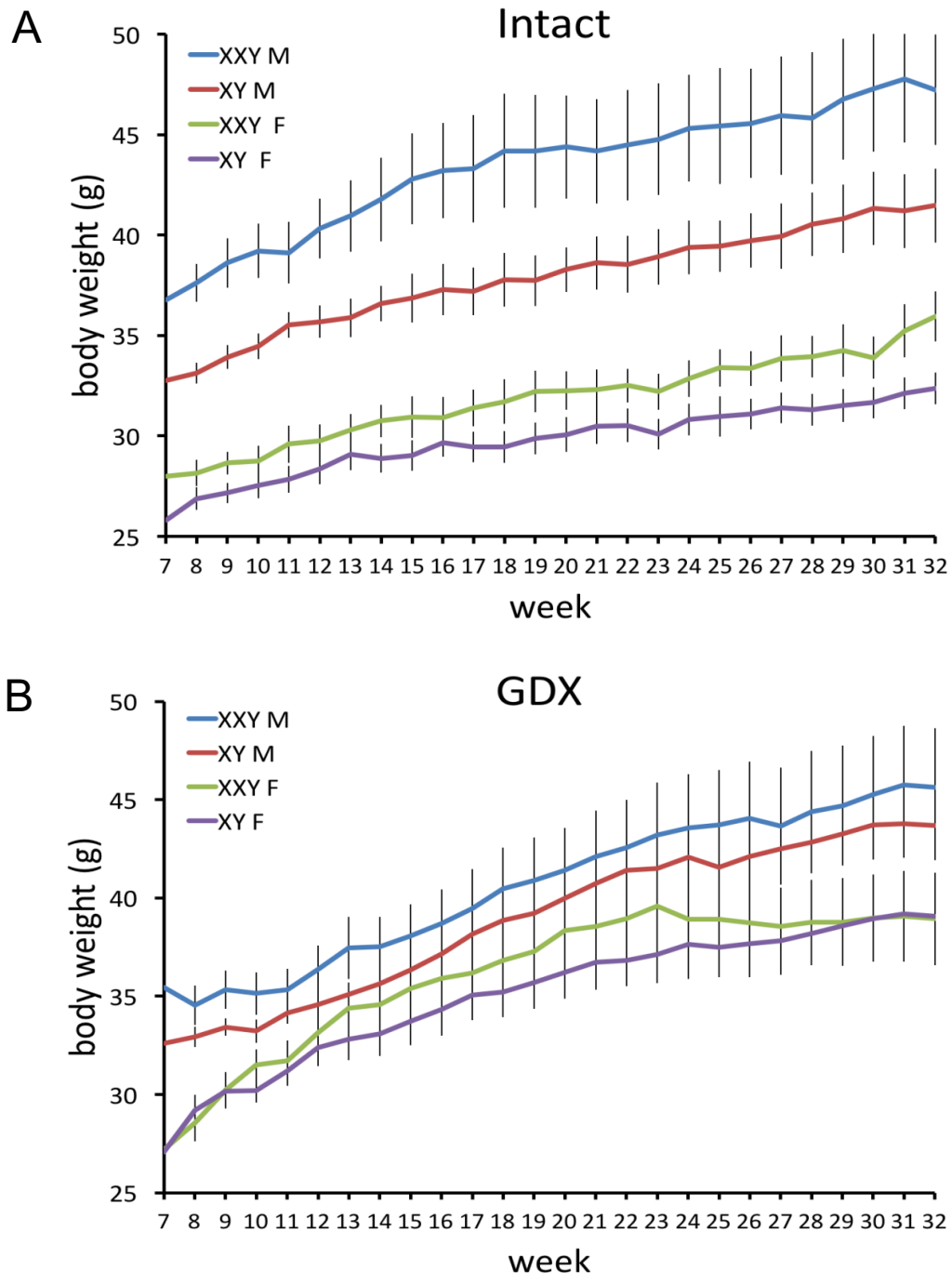


Figure 3-6: Body weight data.
XXY mice had greater body weight than XY ($p = 0.0002$), males had greater body weight than females ($p < 0.0001$), and GDX had greater body weight than intact ($p = 0.0458$). There was a gonadal sex by sex chromosome complement interaction ($p = 0.0426$) such that XXY males were significantly heavier than XY males ($p = 0.0002$), but this XXY vs. XY weight difference wasn't significant in females ($p = 0.1400$). There was also an effect of age such that all groups gained weight over the course of the experiment ($p < 0.0001$).

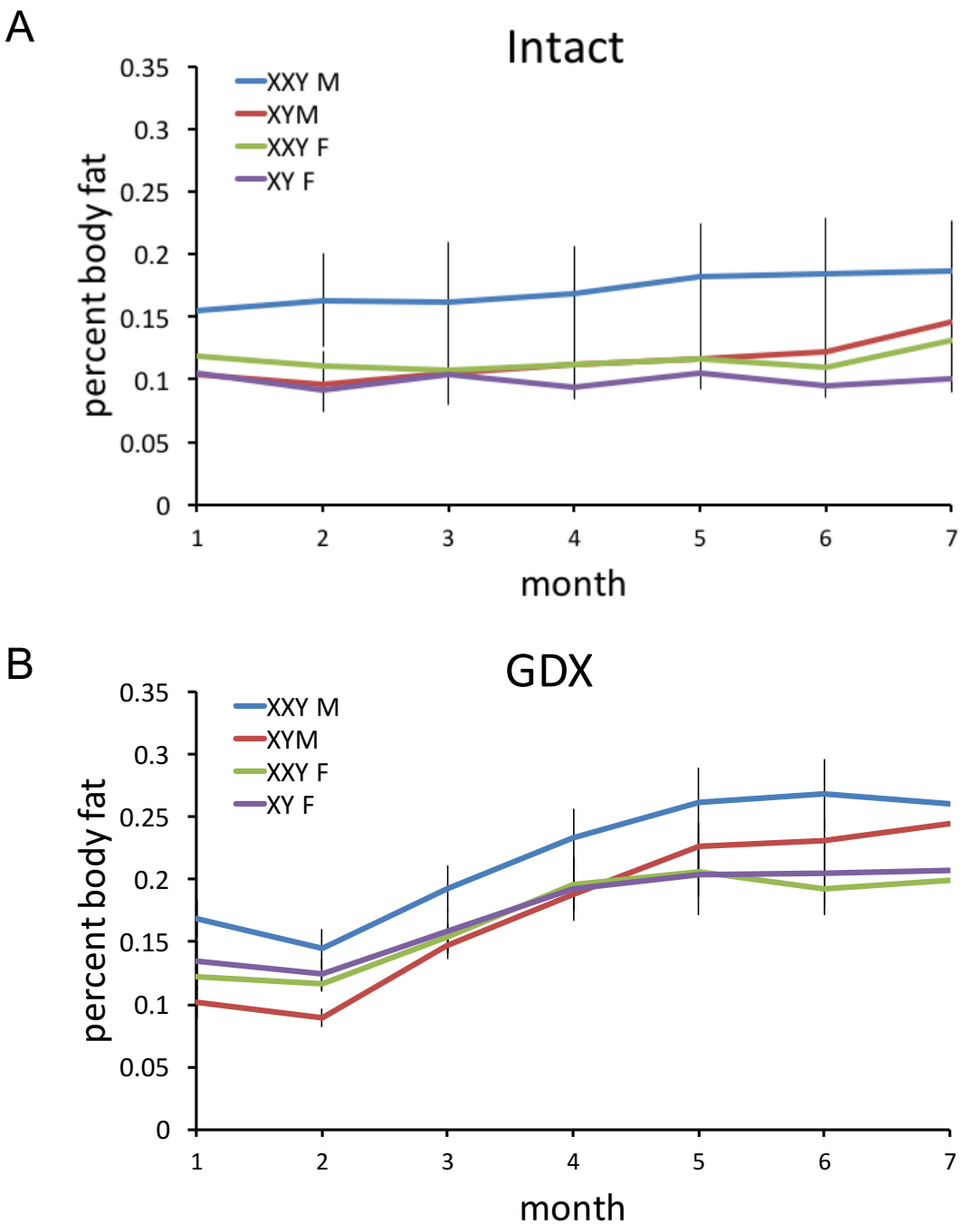


Figure 3-7: Percent body fat.
XXY mice had greater percent body fat than XY ($p = 0.0014$). There was no overall significant effect for gonadal sex or GDX but there was a gonadal sex by sex chromosome complement interaction ($p = 0.0110$) such that XXY males had significantly greater percent body fat than XY males ($p = 0.0001$), but XXY females were not significantly fatter than XY females ($p = 0.4860$). In stepwise post hoc tests, sex and GDX effects emerged such that males had greater percent body fat than females ($p = 0.04300$) and GDX mice had greater percent body fat than intact ($p < 0.0001$). At month 1 mice were 75 days of age, just before surgery in GDX animals.

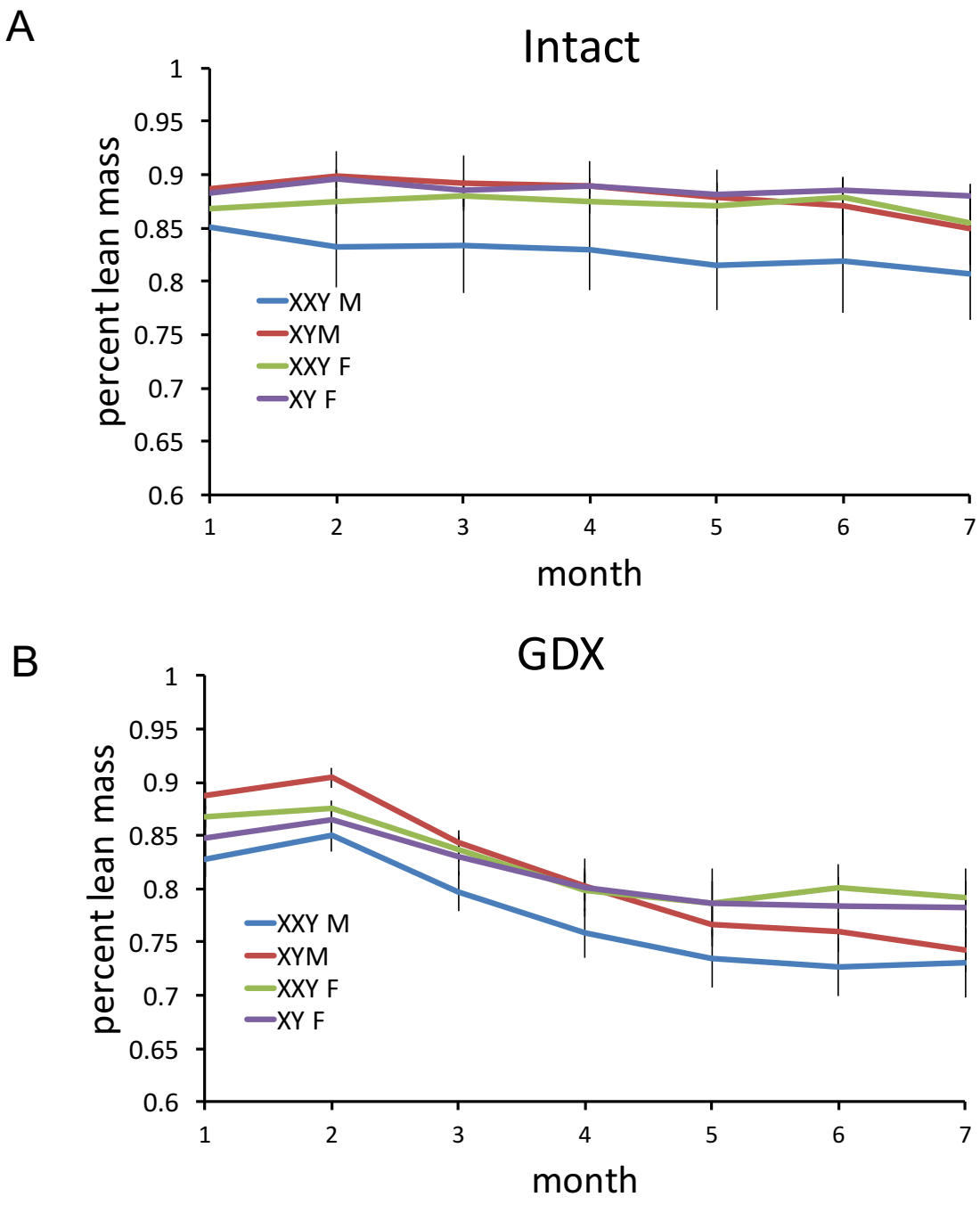


Figure 3-8: Percent lean mass.
XXY mice had lower percent lean mass than XY ($p = 0.0027$). There was no overall significant effect for gonadal sex or GDX but there was a gonadal sex by sex chromosome complement interaction ($p = 0.0213$) such that XXY males had significantly lower percent lean mass than XY males ($p = 0.0002$), but XXY females did not have significantly less lean mass than XY females ($p = 0.4910$). In stepwise post hoc tests it remained true that there was no gonadal sex difference ($p = 0.112$), but the GDX mice had lower percent lean mass than intact ($p < 0.0001$). At month 1 mice were 75 days of age, just before surgery in GDX animals.

3.4 Discussion

Previous to this study only one 3-week long experiment had been done to characterize the SCT model as a viable model for KS and XYY. Several interesting results came from that study including that this model of KS shows a qualitative difference in testes size, a sex chromosome effect on body weight and body composition that persists with testosterone treatment, and an impairment in motor function in XYY mice [26]. While yielding valuable information supporting the usefulness of SCT mice as a model, the first study left many questions unanswered in regards to the physical and cognitive traits of these mice and whether they model KS and XYY. The present study pursued some of the questions relating to the physical phenotype of this model, especially in search of sex chromosome effects that were outside of the scope of the first published experiment.

First, because of the interesting visible gross size difference in testes reported in the prior experiment, the testes of the SCT mice were more carefully examined in this cohort. Testes size among XY, XXY, and XYY show dramatic size differences that mirror differences seen in humans such that XY are larger than XYY, which are larger than XXY. Further, the testes of XXY mice show histological features very similar to those of KS men including degradation of seminiferous tubules, visible evidence of halted sperm production, and Leydig cell hyperplasia. Interestingly, the XYY mice exhibit an intermediate phenotype between XY and XXY mice including partial degradation of seminiferous tubules, and an increase in Leydig cell number compared to XY but not to the extent of that seen in XXY (Supplemental Figure 3-9). Some research suggests that sex chromosome trisomy in testes is detrimental to spermatogenesis and general testis health whether the supernumerary chromosome is X or Y [32]. Despite this genetically induced degeneration, both XXY and more so XYY men have some islands of healthy testicular tissue. It is thought that in these islands the supernumerary chromosome has

been spontaneously ejected, and that perhaps the smaller size of the Y chromosome allows for it to be more readily ejected than the X in KS men so that XYY men fare better in both fertility and healthy hormone levels compared to XXY [32, 33].

The impressive similarity between sex chromosome trisomy male testes phenotype in humans compared to that seen in the SCT mice is very promising for the study of testicular dysfunction. While it is obvious that the extra sex chromosome in germ cells is extremely detrimental to testis function, the mechanism by which the aberrant chromosome causes problems is poorly understood. A series of very informative studies in the XXY model as characterized by Lue et al. in 2005 and 2010 indicate that the supernumerary X in the germ cells, not in the other cell types in the testes, such as Sertoli cells and Leydig cells, is primary to testicular failure [34, 35]. The study of XXY SCT mice alone may not yield new information on that issue, but the ability to directly compare XY, XXY, and XYY mice in the same litters may allow better understanding of how sex chromosome trisomy affects testis function. The XX males, which are infertile like XXY mice but do not have low testosterone levels like XXY mice, though not shown here may provide a unique control group. These combined novel advantages of studying the SCT model for testes function could perhaps lead to improved therapeutic strategies to preserve spermatogenesis or hormonal regulation in KS men.

XXY (and XYY) men are taller than siblings and peers [36]. The SCT mice show a similar difference such that intact XXY males had longer femurs than intact XY males, and GDX males had longer femurs than intact males. However, there was no length difference in the intact XYY males, among the GDX male sex chromosome complement groups, or in female groups. These results point to circulating androgen levels as the probable factor primarily responsible for the bone length difference, at least in mice, but also possibly in humans. The testosterone surge during puberty signals closure of the growth plates of long bones [37], thereby lowered levels of testosterone may cause the growth plates to remain open longer and increase bone length in

KS teens. In this cohort, the mice with the highest circulating testosterone levels, the XY males, had the shortest bones, followed by the XXY males with intermediate hormone levels, and then the GDX male groups with the lowest levels. The result that there is no difference in the female groups, which have equally low testosterone levels, rules out sex chromosome complement as the primary factor in the bone length difference. However, there are also genetic factors that may play a role in height in humans with sex chromosome trisomies that are not relevant in mice. The SHOX genes on the X chromosome play a role in determining height in humans, and men with KS have two copies of these genes, so gene dose effects may play a role [36]. Further there are genes on the Y chromosome that modulate height [36], and thereby the height of XYY men could also be affected by genetic factors. Sex chromosome effects and hormone effects are virtually impossible to cleanly separate in humans or other mouse models, so prior to this study there was not clean evidence pointing to hormonal over direct genetic factors. The present evidence indicates that circulating testosterone levels, not gene dose effects, are probably primarily responsible for the difference in mice, and may play an important role in humans as well.

In SCT mice, intact XY males – those with the highest testosterone levels throughout development – have the greatest BMD and most positive morphological measures of all groups. Liu et al. 2010 previously conducted a similar study to the present study, measuring BMD DEXA and bone morphology with μCT, in GDX or testosterone treated adult XXY vs. XY mice [38]. They concluded that sex chromosome complement contributes to the bone loss in XXY mice compared to XY mice based on the result that XXY mice that were treated with testosterone for several weeks did not have improved bone health compared to untreated XXY males. Their study, however, does not consider the importance of organizational effects of hormones that may act during development to cause XXY males to never develop the same bone health as XY counterparts despite later testosterone treatment. For example, lower levels of androgens prior

to GDX may prevent XXY males from developing the same bone density and health as XY mice, for long periods of time even after later treatment with testosterone. The model of Liu et al. 2010 does not allow for the separation of organizational hormone from genetic factors. In SCT mice, measurement of the female groups helps to test if the sex chromosome effects require prior androgen effects. In the SCT model, males had greater bone health than females, and intact mice had greater bone health than GDX, strongly indicating a hormonal rather than genetic effect on bone health. These results are not surprising considering the already well-understood link between circulating steroid hormone levels and bone health such that androgens are more potent stimulators of bone mass than estrogens, and loss of either type of hormone rapidly results in bone loss [39], a result that Liu et al. 2010 also observe in their GDX group. The current results suggest that Liu et al. may have detected an organizational effect of androgens rather than direct genetic effects of the second X.

Finally, we report the longitudinal metabolic phenotype of SCT mice, which we had previously begun to characterize in the first published SCT study [26]. The current experiment was conducted on all 8 genotypes, either intact or GDX thus yielding 16 groups. In the present study the age at GDX was better controlled (75 days) than in the published study, which had a wider range (97-124 days). In the previous study, however, adult mice were GDX and treated immediately with testosterone, so there was no direct test of the effect of GDX. A highly consistent finding in the two studies was that XXY mice are heavier, fatter (greater percent body fat), and have lower percent lean mass than XY mice. However, although these findings were robust in males, it does not meet significance in females as it did in the previous study. The difference between XXY and XY females was larger in the previous study in intact mice than in GDX mice given testosterone. Based on those results, we hypothesized that the effect of sex chromosome complement may be facilitated by gonadal hormones. However, in the present study, although raw results of *a priori* hypotheses testing showed a significant difference in body

weight between XXY and XY intact females, the difference did not survive multiple testing correction. Thus, it is possible that this study was underpowered to see such a difference in females. In GDX females, the pattern of body weights is consistent with a sex chromosome effect, yet it does not reach significance even when tested as *a priori* hypotheses without correction. It is still possible that sex chromosome complement effect in females is at least partially facilitated by the presence of either ovarian secretions in intact females, or testosterone pellet treatment as in the previous study.

Embedded within the SCT experiment is a FCG experiment when only the XX and XY male and female groups are compared. Previous studies of FCG mice show that XX mice weigh more than XY when the strain background is C57BL6/J [28, 40]. Studies of FCG mice on an MF1 outbred background show more variable results. In one study measuring all GDX FCG and XY* mice on the MF1 background, XY sometimes weighed more than XX [31]. However, in the first published SCT study when all mice were GDX and treated with testosterone [26], XX mice weighed more than XY. In the current study, no overall effect of sex chromosome complement were present when analysis was narrowed to just XX and XY. Upon further inspection, however, in the intact groups XX weighed more than XY ($p = 0.04866$), but in the GDX groups the XY weigh more than the XX but this difference fails to reach significance. One possible explanation is that gonadal hormones may facilitate the X chromosome effect on body weight in the MF1 strain.

Though it is not assured to be successful, backcrossing the SCT model to the inbred C57BL6/J background strain may provide some resolution to these outstanding questions. For example, future research using SCT mice backcrossed on the C57BL6/J strain may allow for easier comparison of SCT studies to the majority of FCG metabolism studies. Also, the two other mouse models of KS – the XY* model and the XXY model – are also on the C57BL6/J, strain, therefore this backcross may yield greater interpretability to results across models. It is

an aim of this research to make the SCT model more widely available and easily utilized by a broader population of researchers to increase understanding of KS, as it is quite poorly understood and understudied for a prevalent genetic condition.

This large study demonstrates the utility of the SCT model for the study of Klinefelter syndrome in two significant ways. First, the SCT mice prove to model several traits seen in KS and XYY including the testicular phenotype, the bone loss phenotype, and the body weight and metabolism phenotypes experienced by human patients. Second, with one cohort of mice we identify sex chromosome effects, activational effects of gonadal hormones, organizational effects of gonadal hormones, and interactions of these factors to influence the measured phenotypes. This is significant because research on humans and on other mouse models of KS does not allow for the separation of these factors, especially organizational hormone effects versus sex chromosome complement effects. Usually, the presence of the second X chromosome in males decreases testosterone levels during development through critical periods that are difficult to experimentally control. The advantage of the SCT model is that organizational hormone effects are controlled for throughout development in the females without the need for invasive or time sensitive intervention. By utilizing the SCT model to detect sex chromosome complement effects especially, because they are not possible to study separately in other models, this research shows promise for improving the understanding of how genetic versus hormonal factors contribute to the health of men with KS. These are the first steps in spurring intelligent drug design to treat the non-hormonally regulated syndromic features of KS.

3.5 Researchers' Contributions

Shayna Williams-Burris contributed to experimental design, did all FISH genotyping, GDX surgeries, NMR, and dissections, as well as organized and helped carry out all other aspects of the experiments, did all statistical analysis, and drafted the manuscript. Gabriela Beroukhim

conducted bone measurements including bone length, DEXA, and μCT. Maureen Ruiz-Sundstrom assisted with breeding, dissections, weighing, and genotyping. Haley Hrncir, Gal Daskal, and Caroline Arellano-Garcia assisted with weighing and dissections. Richard Davis and his lab lent DEXA scanner equipment and expertise, and Sotirios Tetradis and his lab lent μCT scanner and expertise for bone experiments. The UCLA Institute for Digital Research and Education Statistical Consulting Group provided statistics consulting. Karen Reue and Arthur Arnold were involved with experimental design and interpretation of data. Arthur Arnold organized the research team and revised and edited the manuscript.

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3.7 **Supplemental Materials**

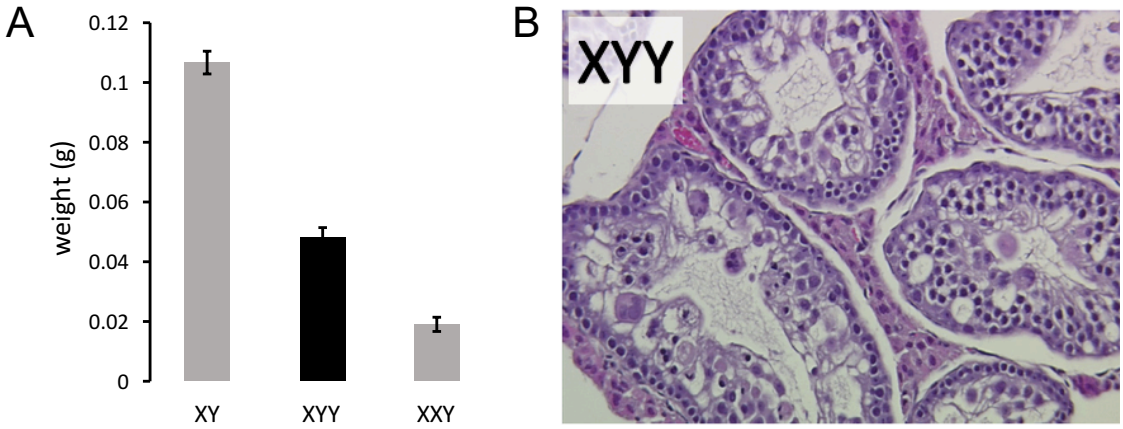
XX and XYY experimental groups

XX and XYY mice were measured in every experiment, except XX were excluded from the μCT experiment (Supplemental Table 3-2). XYY testes showed an interesting intermediate phenotype between the healthy XY phenotype and the degraded XXY phenotype (Supplemental Figure 3-9). There were no differences in bone length in XX or XYY compared to XY (Supplemental Figure 3-10). In bone mineral density XY had greater BMD than XYY suggesting XYY may have lower testosterone levels making them more like females or XXY males (Supplemental Figure 3-11). In μCT measures XYY did not differ from XY (Supplemental Figure 3-12). XX and XYY mice did not differ from XY mice in body weight, percent body fat, or percent lean mass (Supplemental Figures 3-13 and 3-14).

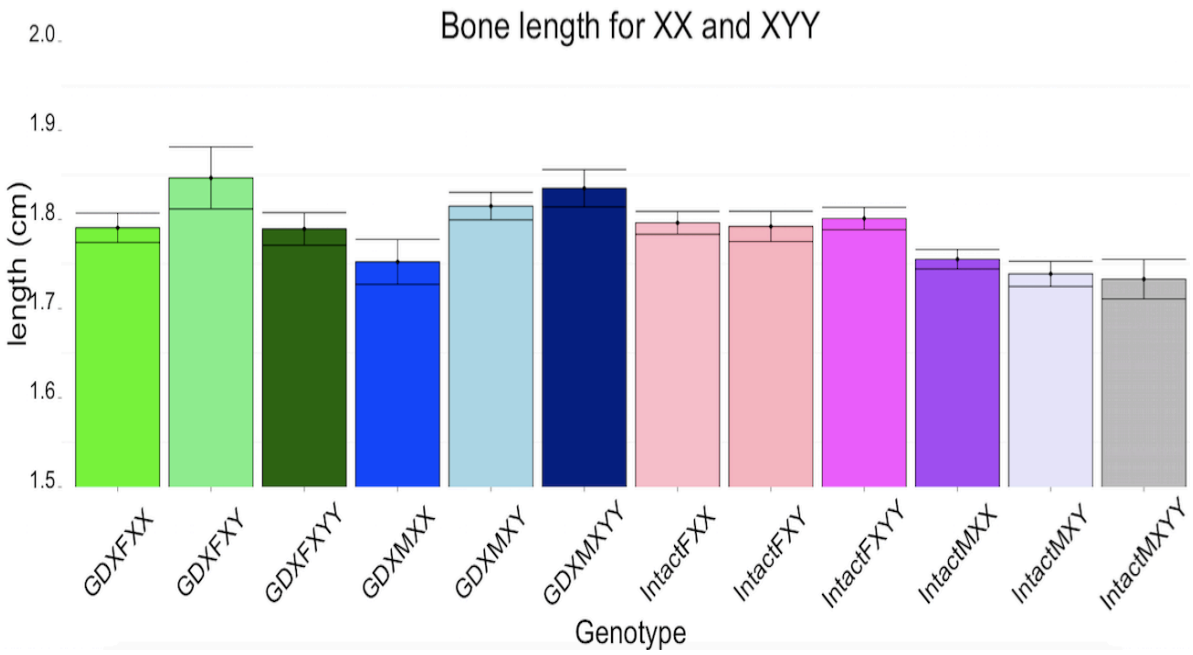
Supplemental Table 3-2: XX and XYY animals.

Gonadectomy	Genotype	Sex chromosome complement	Gonadal sex	Shorthand	Sample size
Intact	XX	XX	Female	Intact XX F	6
Intact	$XY^{-}Y^{-}$	XYY	Female	Intact XYY F	10
Intact	XX *Sry*	XX	Male	Intact XX M	13
Intact	$XY^{-}Y^{-}$ *Sry*	XYY	Male	Intact XYY M	8
Gonadectomized	XX	XX	Female	GDX XX F	15
Gonadectomized	$XY^{-}Y^{-}$	XYY	Female	GDX XYY F	10
Gonadectomized	XX *Sry*	XX	Male	GDX XX M	4
Gonadectomized	$XY^{-}Y^{-}$ *Sry*	XYY	Male	GDX XYY M	9

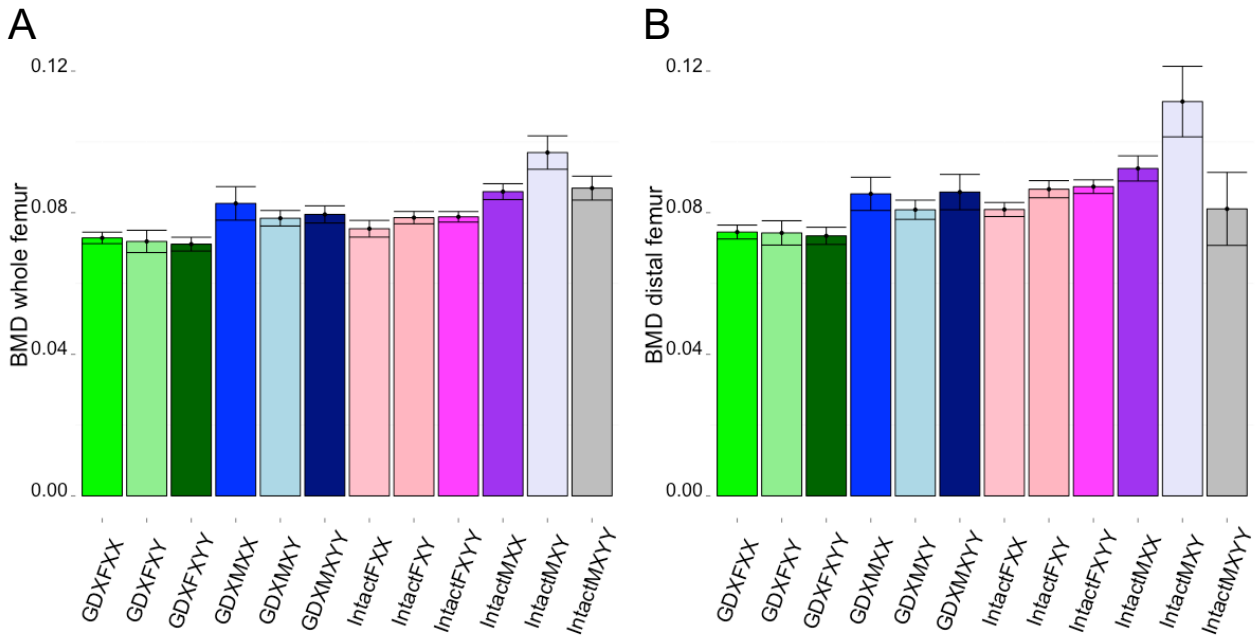
The SCT cross produces 8 genotypes (XX, XY, XXY, or XYY, each either male or female), and half of the animals were gonadectomized (GDX) while half were left intact yielding 16 experimental groups. While XY and XXY mice are most relevant for the study of Klinefelter syndrome, all groups were tested for all traits except XX were excluded from μCT experiments.



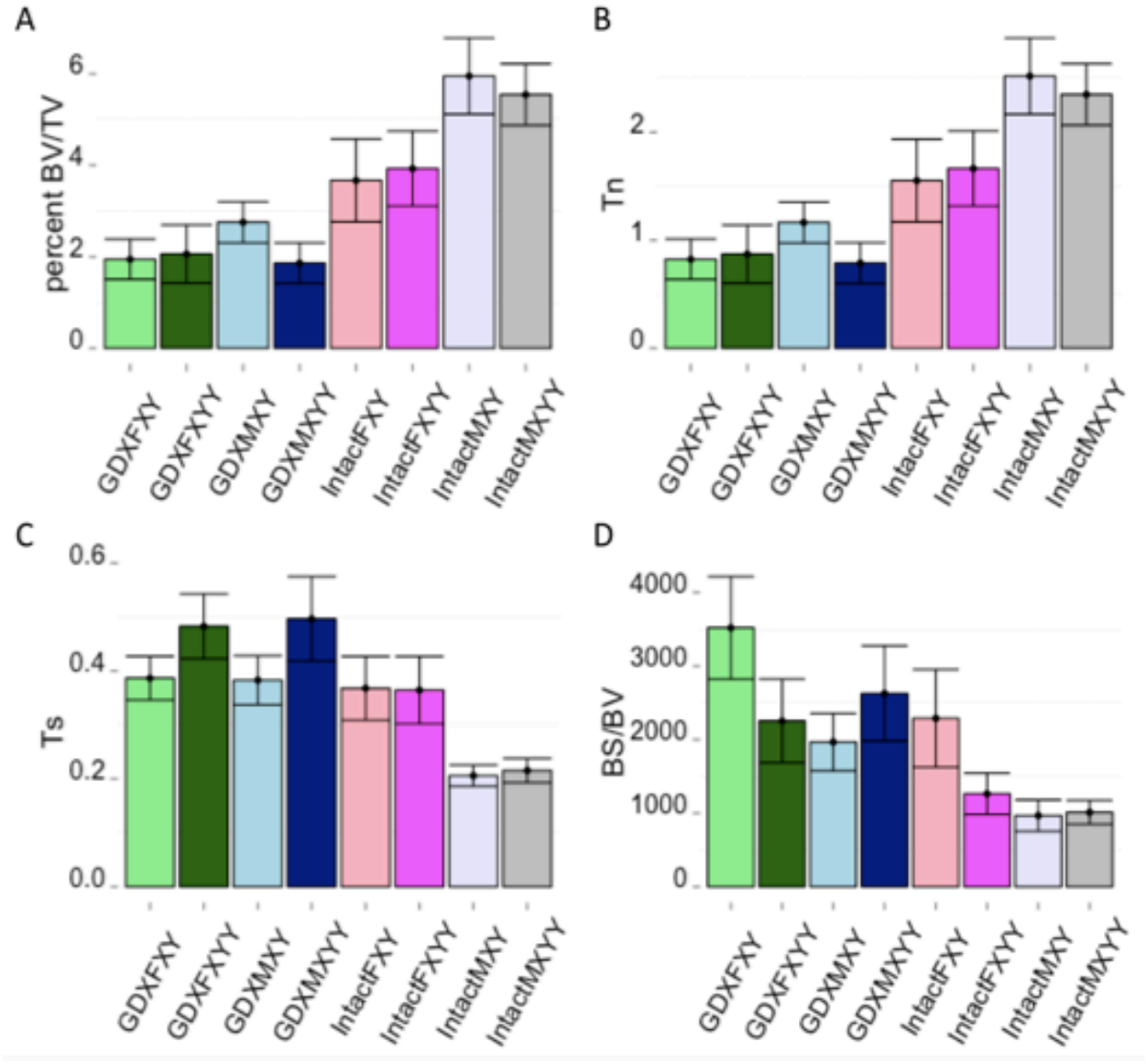
Supplemental Figure 3-9: XYY testes.
A) XYY testes are intermediate in size between XY testes and XXY testes (XYY significantly smaller than XY ($p < 0.0001$), XYY significantly larger than XXY ($p < 0.0001$)). B) XYY testes show intermediate phenotype between normal XY and degenerated XXY. XYY have some degradation of seminiferous tubules and some Leydig cell hyperplasia but not to the same extent as XXY. XX testes were the same size and had similar histology compared to XXY (data not shown).



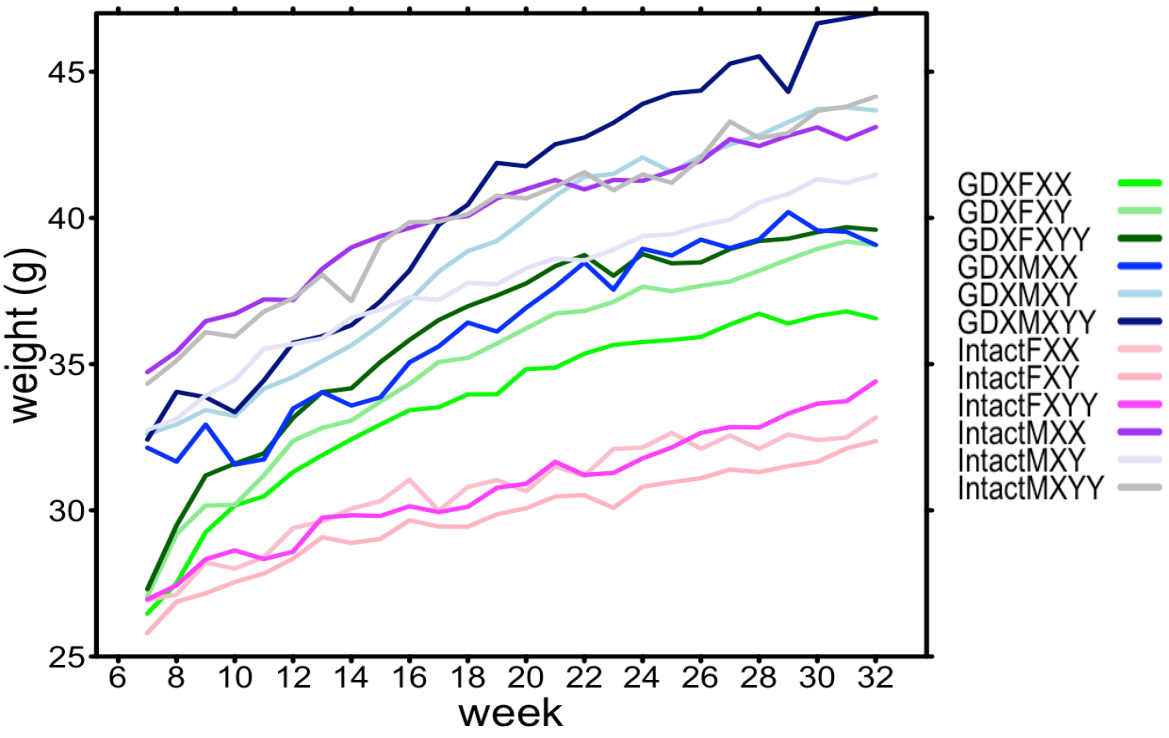
Supplemental Figure 3-10: XX and XYY bone length.
GDX groups had longer bones than intact (p = 0.0020), and there was a GDX by sex interaction (p = 0.0120) such that intact males had shorter bones than intact females (p = 0.0021) but GDX males did not differ from GDX females (p = 0.6352). There was no sex chromosome complement effect on bone length among XX, XY and XYY.



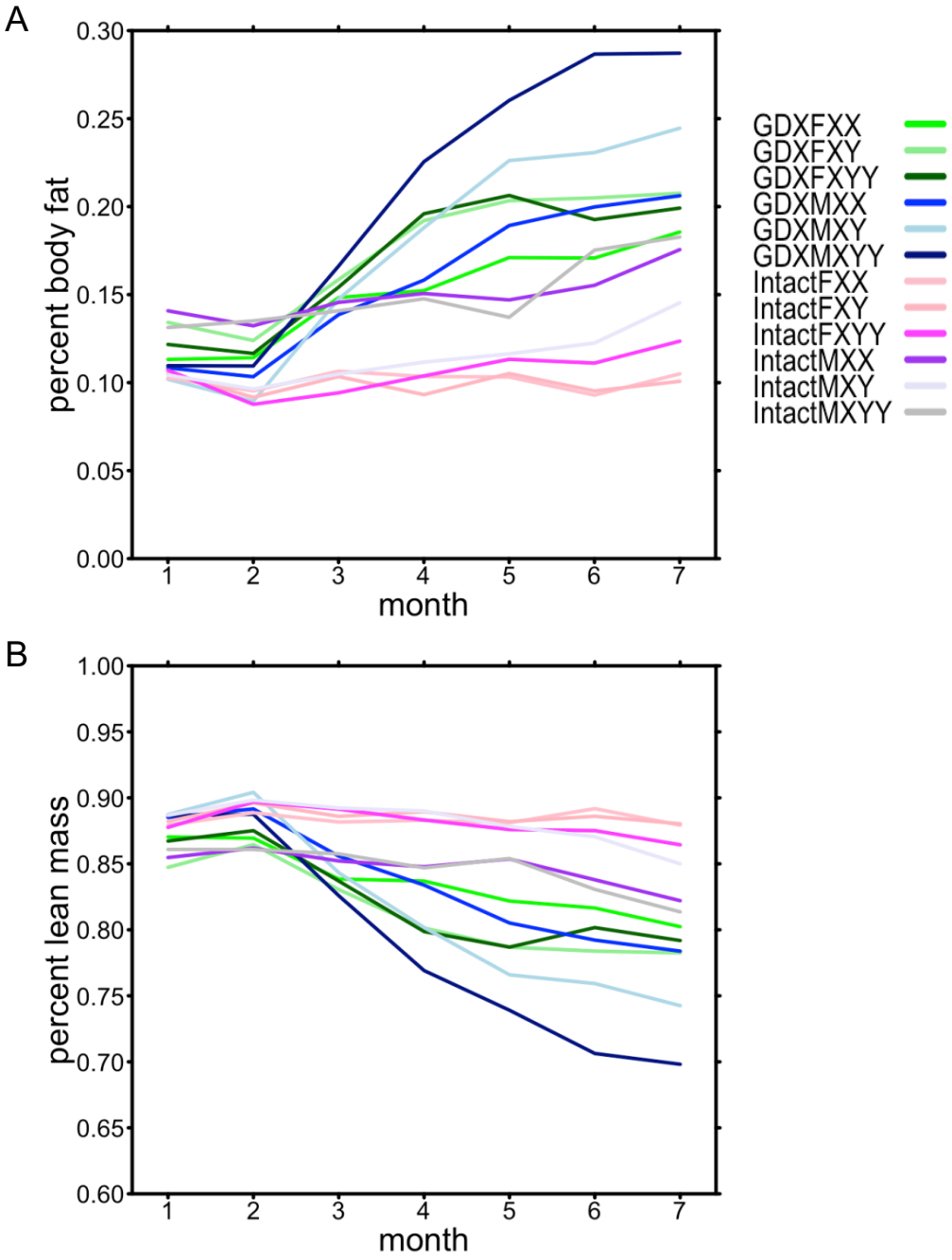
Supplemental Figure 3-11: XX and XYY bone density.
A) In the whole femur GDX had lower BMD than intact ($p < 0.0001$), and females had lower BMD than males ($p < 0.0001$) but there was no effect of sex chromosome complement ($p = 0.7176$). B) The distal femur showed the same pattern as the whole femur so that GDX had lower BMD than intact ($p < 0.0001$) and females had lower BMD than males ($p = 0.0009$), and there was no effect of sex chromosome complement ($p = 0.3463$). However, there was a sex by GDX by genotype interaction ($p = 0.0133$) such that intact XY males had higher bone mineral density than other groups ($p \leq 0.0454$).



Supplemental Figure 3-12: XYY μCT measures.
For all four measures there was a significant effect of GDX ($p \leq 0.0255$) such that GDX mice had poorer bone health than intact in A) percent BV/TV, B) Tn, C) Ts, and D) BS/BV. There was a trend for a sex difference in percent BV/TV, Tn, and Ts ($p \leq 0.05618$) such that females had poorer bone health than males. There was no sex chromosome complement difference in any measure.



Supplemental Figure 3-13: XX and XYY body weight.
In the overall linear regression there were effects of age ($p < 0.0001$) such that all groups gained weight over time, sex ($p < 0.0001$) such that males weighted more than females, and GDX ($p = 0.0069$) such that GDX weighed more than intact, but there was no sex chromosome complement effect ($p = 0.3907$) on body weight.



Supplemental Figure 3-14: XX and XYY percent fat mass and percent lean mass.
A) For percent fat mass there was an effect of age such that mice gained fat over the course of the study ($p < 0.0001$), but there was no effect of sex ($p = 0.1885$), GDX ($p = 0.0671$), or sex chromosome complement ($p = 0.4249$). B) For percent lean mass, there was an effect of age such that overall mice lost percent lean mass the course of the study ($p < 0.0001$), and males lost more lean mass than females ($p = 0.0155$), but there was no effect of GDX ($p = 0.0591$), or sex chromosome complement ($p = 0.3616$).

Chapter 4

Behavioral Measures in the Sex Chromosome Trisomy and XY* Mouse Models of Klinefelter Syndrome

4.1 Background

Men with Klinefelter syndrome (KS), caused by the karyotype XXY, experience a unique cognitive phenotype that has been well-documented in the past 70 years since the syndrome was first described. The patient population is diverse and has varying degrees of cognitive impairment. Cognitive issues in KS are in general subtle, so much so that men with KS are usually not identified or diagnosed based on cognitive traits. However, group differences are measurable and some specific traits are repeatedly revealed as different in boys and men with KS compared to age matched neurotypical controls.

Cognitive differences begin at an early age. Delayed speech often is the first sign of KS. Speech delay or language impairment are so common in KS, occurring in up to 80% of KS boys, that it has been suggested that any boy presenting with these traits should be screened for KS [1]. Similarly, boys with KS have increased rates of learning disability that often put them behind grade level in reading ability in elementary school. These early disadvantages, though sometimes so small that they are not identified without special screening, are thought to accumulate throughout primary education so that KS boys fall increasingly behind and contribute to poor socioeconomic outcomes found in the KS population [2].

In adulthood, men with KS have slightly decreased IQ within the normal range, compared to siblings or peers. This small drop in IQ likely does not have far-reaching effects. Rather, more specific cognitive deficits measured in KS men may have a greater impact on quality of life. For example, men with KS experience well-documented and robust cognitive deficits in executive functioning; these deficits include cognitive inflexibility, impaired inhibition, attention deficits, and/or problems with working memory [3-9]. It is thought that these deficits affect the ability of boys and men with KS to regulate their emotions and behavior, perhaps increasing difficulty with academic and professional functioning.

A fundamental question in the study of KS is whether the symptoms are caused by low androgen levels experienced by XXY boys and men especially after puberty, or by direct genetic effects of the second X chromosome. There is some evidence that testosterone levels are primarily at play in influencing the cognitive aspects of KS. For example, treating KS boys with testosterone improves cognitive measures [10, 11]. Nevertheless, there are hints at genetic factors as well. First, cognitive issues begin early in development, before the testosterone levels are dramatically different in KS vs. control boys. Second, boys with idiopathic hypogonadotropic hypogonadism do not exhibit the same cognitive issues as boys with KS. Third, girls with trisomy X (caused by the karyotype XXX) exhibit executive functioning deficits similar to, or even worse than, those of boys with KS [12].

However, one mouse study suggests testosterone levels directly modulate recognition memory. In the novel object recognition test using the XY* model of KS, XY* mice perform better on recognition memory than XXY* littermates, and within the XXY* group the performance on the task directly correlates with circulating androgen levels [13]. However, the previous study could not rule out genetic effects because low testosterone is always confounded with the presence of the second X chromosome in KS patients.

Here we first aimed to replicate the XY* novel object recognition result and expand that experiment to include gonadectomized (GDX) animals. Next, we use the same animals to test for a measure of executive function. Utilizing operant behavior chambers to conduct a reversal learning test that measures impulse inhibition and behavioral flexibility, we aim to contribute the first evidence for executive function deficits in an animal model of KS.

Inclusion of the GDX groups allows for comparison of XY* and XXY* when circulating testosterone levels have been equalized so that a result showing an XY* vs. XXY* difference in the intact could be either genetic or hormonal. If the difference was abolished in the GDX groups, however, that would be evidence that the difference is actually caused by circulating testosterone levels. If, however the opposite were true and the GDX groups continued to show a difference between groups, the cause could be either a genetic effect or an organizational effects of low testosterone in the XXY* males during development. In the XY* model of KS these two effects are always confounded. Although, the XY* model can be used to glean some valuable information regarding the etiology of KS, it cannot fully answer the question of whether a trait is genetic or hormonal.

Therefore, to try to better separate the independent contributions of X gene dose versus low testosterone, we also utilized another mouse model of KS, the Sex Chromosome Trisomy model, in which the effects of sex chromosome complement are studied both in gonadal females and gonadal males. The addition of XXY and XY female groups allows the assessment of the effects of a second X chromosome in mice that lack testes altogether, and therefore assesses the XY vs. XXY difference in different endocrine conditions. This expansion of group comparisons provides additional evidence to support or undermine confidence that the effects of the second X chromosome are mediated by specific hormonal differences. In this model, the testis determining gene *Sry* is not located on the Y chromosome, but it is instead on an autosome (chromosome 3) [14]. Thereby XX and XY animals can each be either gonadally male

or female depending on whether they have *Sry* or not. By crossing XXY females with XY males (with *Sry)* we can produce 8 types of mice: XX, XY, XXY, and XYY animals, each either male or female. If a KS trait differs between XY males to XXY males, the culprit could again be either genetic or hormonal because XXY male SCT mice, like men with KS and XXY* male mice, have low circulating testosterone levels that may cause long lasting effects. If the same trait also differs between XY and XXY females, however, the trait is more likely caused by genetic effects because XY and XXY females are expected to have similarly low testosterone levels during development and thereby differences are not caused by group differences in levels of testicular hormones.

Establishing animal models of behavioral traits is the first step to understanding the mechanisms underlying the cognitive issues experienced by KS patients. One reason is that in animals, unlike humans, genetic contributions can be separated from hormonal influences by either directly manipulating hormone levels, as by GDX, or by using models such as the SCT model where lifetime hormone exposure is altered due to genetic makeup. Another reason is that if genetic factors are found to play a role, studies to understand what genes are involved and how, are possible in mice that are not possible in humans. The combined utilization of these two mouse models of KS to understand behavioral traits is a strategy to uncover better treatments for the KS patient population.

4.2 Methods

4.2.1 Animals

XY model animals*

The first group of mice tested were XY* model mice on the C57BL6/J inbred background strain (Figure 4-1). XY* male mice have a normal X chromosome, plus a unique Y chromosome

with an abnormal pseudoautosomal region that allows it to recombine with the X chromosome during meiosis. Breeding an XY* male with a normal XX female produces 4 genotypes: XX females, XY* males, plus XY*^{X} females, and XXY* males. XY*^{X} females have a normal X chromosome plus a small chromosome called Y*^{X} that is almost exclusively a pseudoautosomal region and contains few genes. XY*^{X} mice can be thought of as females with only 1 X chromosome, and are sometimes used as a model for Turner syndrome (45, XO in humans). XXY* mice have a normal X chromosome plus a long chromosome that is a fusion of the X and Y*. Although these mice only have 2 sex chromosomes, they have all of the non-pseudoautosomal genes as in 2 X chromosomes and a Y, and therefore are a model of Klinefelter syndrome. All 4 genotypes were tested in all experiments. Mice were either GDX or sham GDX in adulthood to measure the contribution of activational effects of circulating gonadal hormones, creating 8 experimental groups. Again, because we expected behavioral differences may be subtle, we tried to have groups of about 15 animals per genotype for each the intact and GDX cohorts to provide sufficient power. The slight unbalance of group sizes is because we kept all offspring from breeding pairs so some random variation in genotype ratios is to be expected.

Table 4-1: XY* model experimental groups.

Gonadectomy	Sex chromosome complement	Gonadal sex	Similar to	Sample size
Intact	XY^{*X}	Female	XO (Turner syndrome)	10
Intact	XX	Female	XX (typical female)	16
Intact	XY*	Male	XY (typical male)	15
Intact	XXY*	Male	XXY (Klinefelter	16
Gonadectomized	XY^{*X}	Female	XO	17
Gonadectomized	XX	Female	XX	9
Gonadectomized	XY*	Male	XY	16
Gonadectomized	XXY*	Male	XXY	13

The XY* cross produces 4 genotypes (XY^{*X}, XX, XY*, and XXY*), and mice were either sham GDX (intact) or GDX in adulthood to create 8 experimental groups.

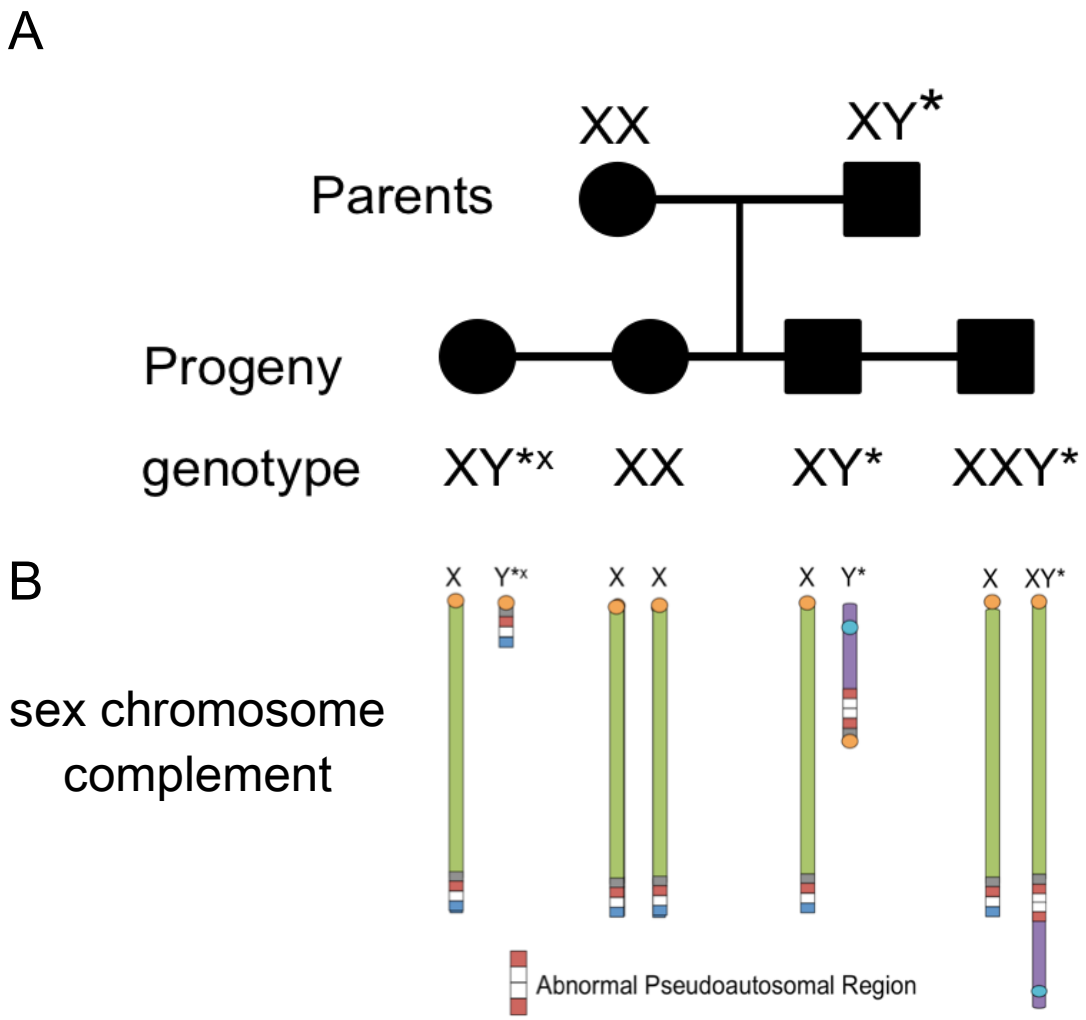


Figure 4-1: XY* model.
A) Breeding an XY* male with a normal XX female produces 4 genotypes: XX females, XY* males, plus XY*^{X} females, and XXY* males. B) XY*^{X} females have a normal X chromosome plus a small chromosome called Y*^{X} that is essentially a pseudoautosomal region and contains few genes. XX females only have two normal X chromsomes and no abnormal chromsomes. XY* male mice have a normal X chromosome, plus a unique Y chromosome with an abnormal pseudoautosomal region that allows it to recombine with the X chromosome during meiosis. XXY* mice have a normal X chromosome plus a long chromosome that is a fusion of the X and Y,* and are a model of Klinefelter syndrome

SCT model animals

The other mouse model tested was the Sex Chromosome Trisomy mouse model on the MF1 outbred background strain, as described in previous chapters. To summarize, SCT mice are first produced by breeding XY^- female mice from the Four Core Genotypes (FCG) mouse model, where the Y^- chromosome is a normal Y chromosome with a spontaneous deletion of the testis determining gene *Sry* [14-16]. The XY^- females were crossed with XY males to generate XXY^- female mice. XXY^- female mice were then crossed with XY^- *Sry* FCG male mice, where *Sry* has been inserted as a transgene on chromosome 3, to create the 8 genotypes of the Sex Chromosome Trisomy model: XX, XY^-, XXY^-, and XY^-Y^- each either male (with the testes determining gene *Sry* inserted as a transgene on an autosome) or female (without *Sry*). Because of the labor intensive nature of the behavioral tests, for this experiment only male and female XY and XXY mice, the most relevant genotypes to Klinefelter syndrome, were tested. All SCT mice were GDX in adulthood to equalize the level of gonadal hormones, which eliminates activational effects of those hormones. Because we expect behavioral results might be subtle, we again aimed for group sizes of about 15 animals to have enough power to see differences. We had more XY mice than XXY in both sexes. We have recently found that the XXY^- female cross with XY^- *Sry* males consistently produce more XX and XY than XXY or XYY animals (unpublished data). However, despite this we were able to have nearly balanced group sizes (Table 4-2).

Table 4-2: SCT model experimental groups.

Genotype	Sex chromosome complement	Gonadal sex	Shorthand	Sample size
XY^{-}	XY	Female	XY F	22
XXY^{-}	XXY	Female	XXY F	15
XY^{-} *Sry*	XY	Male	XY M	20
XXY^{-} *Sry*	XXY	Male	XXY M	14

The SCT cross produces 8 genotypes (XX, XY, XXY, or XYY, each either gonadal male or female). To conserve resources and focus on the most relevant groups to Klinefelter syndrome, only XY and XXY males and females were tested. All mice in this experiment were gonadectomized in adulthood

Animal care

Animals in both groups were treated identically except after novel object recognition and reward response tests XY* mice were food restricted and then also tested for reversal learning. At weaning, mice were group housed according to gonadal sex. Mice were either gonadectomized (GDX) or sham GDX at 75 days. Mice were allowed to recover for 1 month after gonadectomy before beginning behavioral testing. All experiments were approved by UCLA Institutional Animal Care and Use Committee. Mice were maintained at 23°C on a 14:10 light:dark cycle and were fed regular chow diet (LabDiet 5001).

4.2.2 Genotyping

Mice were genotyped with PCR to detect the presence of the Y chromosome, followed by FISH using a specially designed probe mix that labels the RAB9B gene on the mouse X chromosome red and paints the Y chromosome green (Kreatech catalog number KI-30505, Kreatech Biotechnology, Amsterdam, The Netherlands). FISH was performed by standard methods as instructed by the Kreatech protocol except 1 μL of probe mix was used instead of the 10 μL indicated by the protocol.

4.2.3 Novel object recognition

Mice were habituated for 3 days and tested on the fourth day. On the first day of habituation each mouse was placed in an empty Plexiglas cube chamber for 20 minutes in a quiet, dimly lit room. For the following two days of habituation the procedure was repeated but with two black plastic pipes placed in either corner of the chamber. On the test day, each mouse was placed in the empty chamber for 5 minutes, presented with two metal pipes for 5 minutes, and then removed from the chamber for 1 hour. After one hour the mouse returned to the chamber and presented with one metal pipe (old object) and one white pipe (new object) and video recorded for 5 minutes (Figure 4-2 A). Videos were coded by trained reliable scorers that

were blind to the group of the animals. Time spent interacting with each object was defined as instances when the mouse was within 3 cm of the object and oriented towards it, when at least one forepaw was on the object, or when the mouse was licking or sniffing the object. Because the SCT mice exhibited a behavior of pushing the objects around the box, the objects were anchored to the bottom of the box by screws.

4.2.4 Reward response test

For the reward response test, there were two measures: free consumption and dose response. For both tests mice were temporarily housed individually for two hours and presented with two bottles, one with sweetened condensed milk (SCM) and one with water (Figure 4-2 B). For free consumption, mice were presented with 10% SCM for 8 consecutive days. For dose response, mice were presented with 3%, 10%, or 32% SCM for two consecutive days each (order of concentration presentation was counterbalanced). Bottles were weighed before and after presentation to measure SCM consumption in grams.

4.2.5 Food restriction for XY* mice

Mice were food restricted for reversal learning to 85-90% baseline body weight to increase motivation to work for SCM as a palatable food reward. For food restriction, mice were weighed daily and their percent body weight was calculated. If they weighed within 85-90% of their baseline weight they were given the same amount of food as the previous day. If mice were below or above the 85-90% range they were given 0.1 g more or less food than the previous day. Mice typically required 1.5 to 3 g of food per day to maintain appropriate body weight.

4.2.6 Reversal learning for XY* mice

Reversal learning was only tested in XY* model mice, not SCT mice. 10% SCM solution was used as a reinforcer in the reversal learning task. Mice were tested in an operant conditioning chamber containing an array of 5 nose poke apertures on one side and a reinforcer delivery magazine on the opposite side. Mice were trained first to poke in the central nose poke with increasing hold times where a correct response was rewarded with 10 µL 10% SCM. Next, the task was changed so that a response to one of two apertures (left or right of center) was rewarded. Once the mouse successfully poked the correct hole at performance criterion, the task was reversed such that a poke to the opposite side was correct, and trials to criterion were measured. Mice passed when they scored 16 out of the past 20 trails correct in a sliding window (Figure 4-2 C).

4.2.7 Statistics

For novel object recognition, to calculate inter- and intra-rater reliability each scorer recorded time spent interacting with each object for the same 9 videos twice each, not scoring the same video consecutively. For both inter- and intra-rater reliabilities the scorers were extremely consistent: for inter-rater reliability Kendall's $W \geq 0.97$ (function kendall() in package irr in R); for intra-rater reliability Pearson's $r \geq 0.99$ (function rcorr() in package Hmisc in R). Two measures of recognition memory were calculated. First, just the raw ratio of time spent with the new object over time spent with the old object was compared. Second, recognition index was calculated as the ratio of time spent with the new object over total time spent with both objects. This measure can help correct for extreme ratios created by mice that spent little time with either object. Statistical analyses for each measure were performed using the lm() function from package stats in R, and post hoc factor interaction tests were performed with false discovery rate (fdr) multiple testing correction testInteractions() function from package phia in R.

For reward response, an omnibus test was first conducted to test for overall effects, then post hoc tests were conducted to identify group differences. For the longitudinal metabolic data the omnibus test was a mixed model linear regression with the factors of genotype, gonadectomy, and day or dose (lmer() function from package lmerTest in R), post-hoc tests were performed by stepwise backward elimination of all effects in the linear model (step() function from package lmerTest in R). All data measured at a single time point was analyzed by linear regression model (lm() function from package stats in R) and post hoc factor interaction tests were performed with false discovery rate (fdr) multiple testing correction (testInteractions() function from package phia in R) for lm() models.

For reversal learning, the variance was large in all groups for the dependent variable mean trails to criterion, and the distribution of scores showed an extreme skew to the right. Because of the non-normal distribution, the data were log2 transformed. This transform was sufficient to establish normality of the data (Shapiro-Wilk normality test $p = 0.3911$, shapiro.test() function from stats package in R), therefore statistical analyses were run on the transformed values using the lm() function from package stats in R.

In some cases, we had *a priori* hypotheses that we tested using pairwise tests irrespective of whether an interaction was first indicated by the omnibus test. This is valid because although the experimental design yields a three way repeated measures model not all comparisons are biologically relevant. For example, differences between XY^{*X} and XXY* are not interpretable in the context of KS. However, some comparisons are biologically interesting even without a significant main interaction effect. For example, in reversal learning the omnibus test did not show a significant GDX by genotype interaction. However, because XY* and XXY* intact male mice have differing testosterone levels it is interesting to ask whether these two groups have differing reversal learning. This question can be answered regardless of the outcome of the omnibus test, because the omnibus test includes biologically unimportant comparisons and

decreases power to see biologically relevant differences. In the case of these *a priori* hypothesis tests, p-vaues were corrected for multiple testing by false discovery rate (p.adjust(,method="fdr") function in stats package in R).

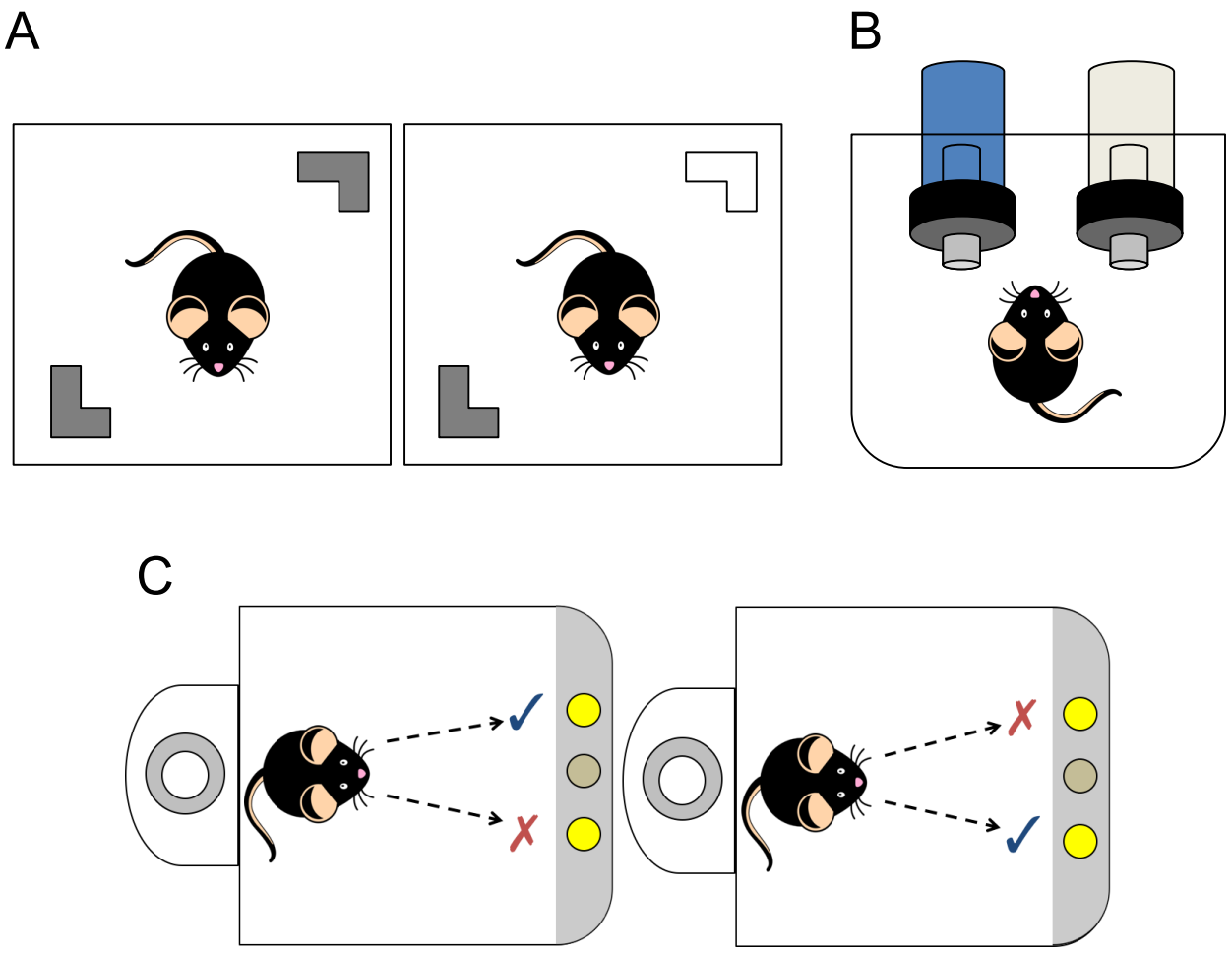


Figure 4-2: Adult behavior tests.
A) For the novel object recognition test mice were first habituated then presented with two identical objects for 5 minutes. Mice were then returned to their home cage for 1 hour and then returned to the testing chamber this time with one old object and one new object, and recorded for 5 minutes. Videos were scored to determine time spent investigating each object. B) For reward response, there were two measures: free consumption and dose response. For both tests mice were temporarily single housed for two hours and presented with two bottles, one with sweetened condensed milk (SCM) and one with water (Figure 4-2 B). For free consumption, mice were presented with 10% SCM for 8 consecutive days. For the reward response test, mice were presented with 3%, 10%, or 32% SCM for two consecutive days each (order of concentration presentation was counterbalanced). Bottles were weighed before and after presentation to measure SCM consumption in grams. C) For reversal learning, mice were tested in an operant conditioning chamber containing an array of 5 nose poke apertures on one side and a reinforcer delivery magazine on the opposite side. Mice were trained to respond to one of two apertures (left or right of center) across trials and sessions. Once the mouse successfully poked the correct hole at performance criterion, the task was reversed such that a poke to the opposite side was correct, and trials to criterion were measured. Mice passed when they scored 16 out of the past 20 trails correct in a sliding window

4.3 Results

4.3.1 Novel object recognition in XY* mice

In the XY* mice, for the raw ratio there was no overall effect of GDX ($p = 0.4035$) or genotype ($p = 0.5721$), and there was no interaction between these two factors ($p = 0.4805$, Figure 4-3 A and C). Second, for recognition index, again there was no significant overall effect of GDX ($p = 0.1061$) or genotype ($p = 0. 7729$), and there was no interaction between these two factors ($p = 0.1317$, Figure 4-3 B and C). Post hoc tests for *a priori* hypotheses did not show any significant group differences.

4.3.2 Reward response in the XY* model

In the XY* model mice, for free consumption there was a main effect of GDX such that GDX mice drank more overall than intact mice ($p < 0.0001$), and there was a main effect of genotype ($p = 0.0072$), and post hoc tests indicated that it was such that XY* mice drank more milk than all other groups ($p \leq 0.0120$, Figure 4-4 A and C). There was a significant main effect of day such that mice drank more milk on later days than on the first days ($p < 0.0001$). This result was expected because previous studies using this method have noted the same result. The change over days is thought to occur because mice require repeated exposure to the milk to overcome their natural neophobia towards novel foods [17]. Because a goal of the reward response experiment in the XY* was to determine whether 10% SCM is an appropriate reinforcer for the reversal learning task, the genotype effect on milk consumption could be a concern. However, the genotype difference was in acquisition of milk drinking behavior and by day 8 there was no longer a genotype effect ($p = 0.4549$). For dose response, again the GDX drank more than the intact ($p = 0.0022$), but there was no overall genotype difference (Figure 4-4 B and D). Pairwise post hoc tests showed that GDX XY* had greater consumption of 3% SCM

than GDX XXY*, but the significance of this difference did not survive multiple testing correction by false discovery rate. The GDX vs. intact difference in consumption of 10% SCM in both free consumption and dose response is notable, but in both the day 8 of free consumption and the 10% dose of dose response there was no genotype difference within GDX or within intact. Therefore, although the results may not be generalizable across GDX groups, differences found between genotypes within GDX groups or within intact groups would not be attributable to differences in milk preference.

4.3.3 Reversal learning in XY* mice

For reversal learning the mean trails to criterion for all groups was 273.5. However, the variance was very large with the values ranging from 41 at the lowest to 1017 at the highest, and the overall distribution showed an extreme skew to the right. Because of the non-normal distribution, the data were log2 transformed (Figure 4-5). This transform was sufficient to make the data normally distributed, and statistical analyses were run on transformed values.

In the overall linear regression for $\log_2$ trials to criterion, there was no significant main effect of genotype ($p = 0.6120$) or gonadectomy ($p = 0.1867$) and there was no interaction of these effects ($p = 0.1652$, Figure 4-6). However, we had *a priori* hypotheses that XY* mice would differ from XXY* mice independent of the results of the female groups, and we hypothesized that this may occur across GDX groups, or within just intact or GDX. In pairwise tests, there was no difference in XY* vs. XXY* across GDX groups ($p = 0.1855$). Within just the intact, XXY took significantly more trials to criterion than XY* ($p = 0.0305$), but this difference did not survive multiple test correction by false discovery rate (corrected $p = 0.0916$). Further this difference was not seen in the GDX mice ($p = 0.7645$).

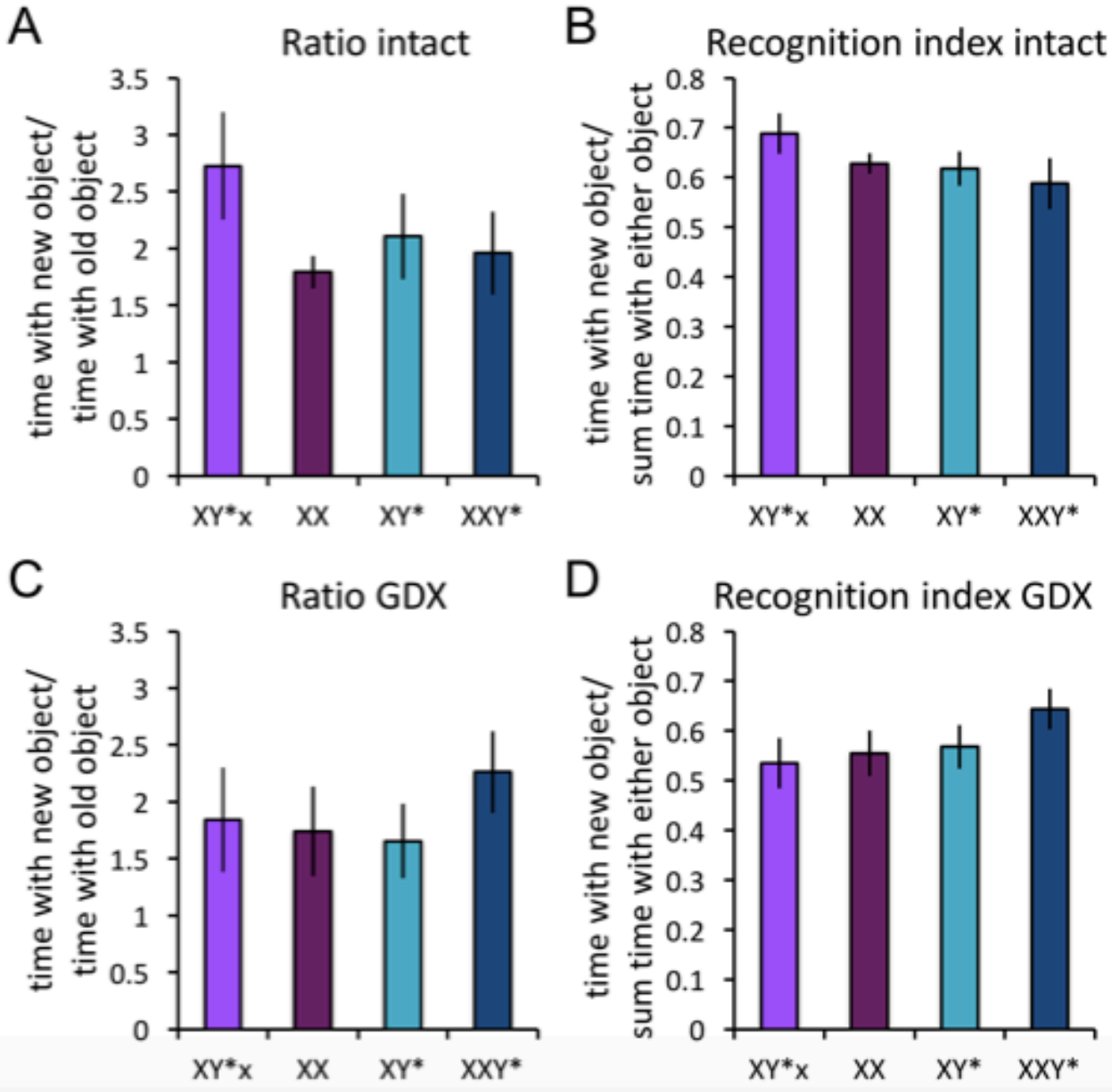


Figure 4-3: Novel object recognition in XY* mice.
A and C) For the raw ratio there was no main effect of GDX (p = 0.4035) or genotype (p = 0.5721), and there was no interaction between these two factors (p = 0.4805). B and D) For recognition index, again there was no significant main effect of GDX (p = 0.1061) or genotype (p = 0. 7729), and there was no interaction between these two factors (p = 0.1317).

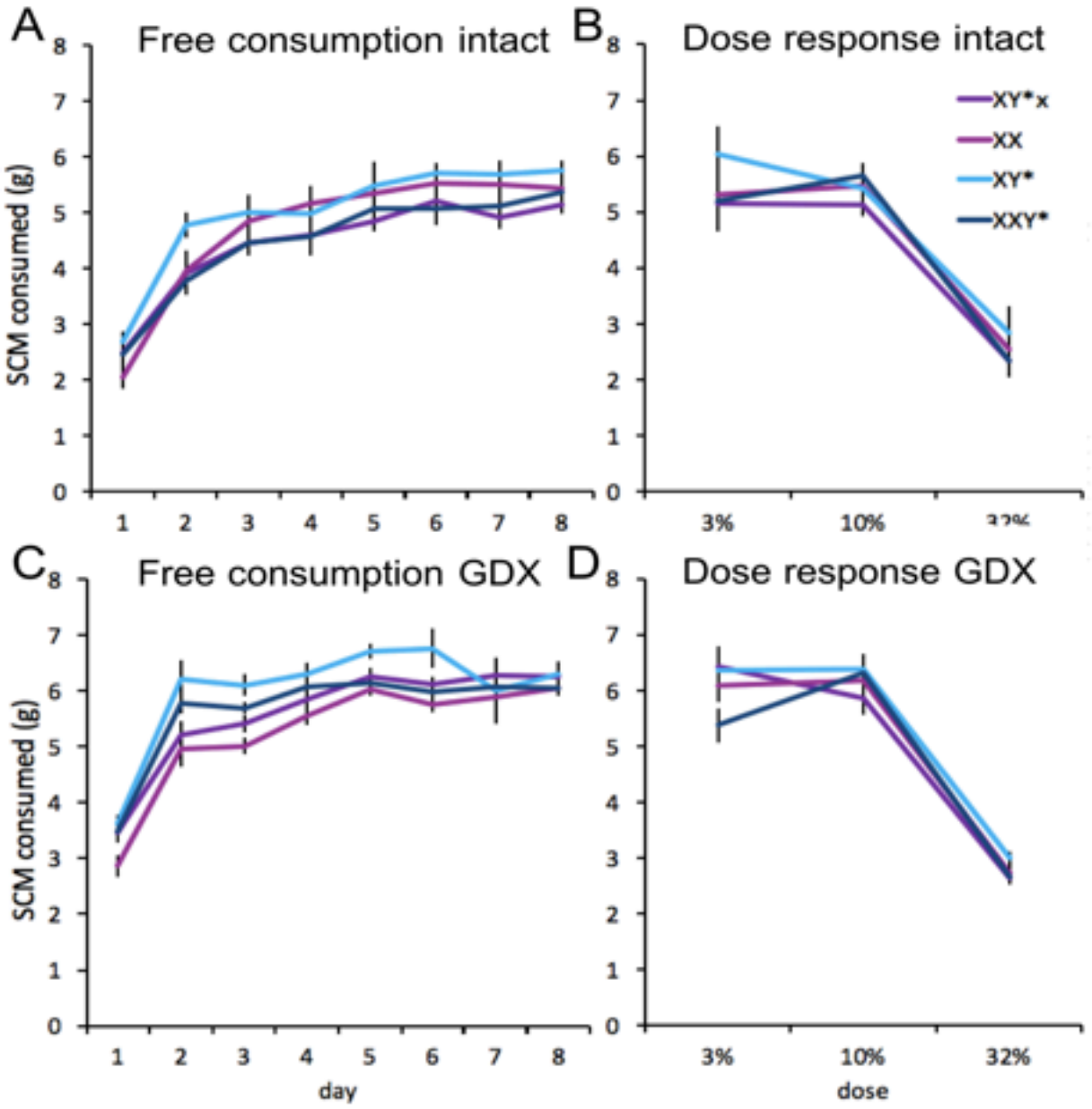


Figure 4-4: Reward response in XY* mice.
A and C) For free consumption there was a significant effect of day such that mice drank more milk on later days than on the first days ($p < 0.0001$). There was also an effect of GDX such that GDX mice drank more overall than intact mice ($p < 0.0001$), and there was an effect of genotype ($p = 0.0072$) such that XY* mice drank more milk than all other groups ($p \leq 0.0120$). B and D) For dose response, again the GDX drank more than the intact ($p = 0.0022$), but there was no overall genotype difference.

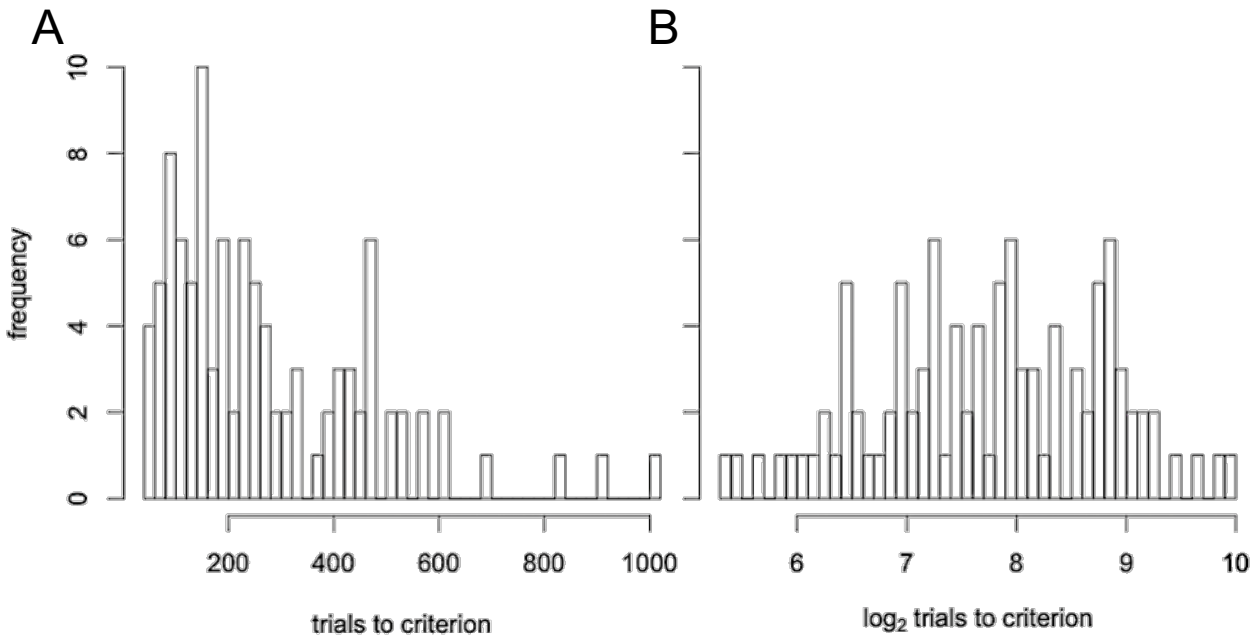


Figure 4-5: Histograms of raw and log_2 transformed reversal data distributions.
A) For reversal learning the mean trails to criterion for all groups was 273.5, however the variance was very large with the values ranging from 41 at the lowest to 1017 at the highest, and the overall distribution showed an extreme skew to the right (Shapiro-Wilk normality test $p > 0.0001$). B) To address this, the data were log_2 transformed. This transform was sufficient to make the data normally distributed, and statistical analyses were run on transformed values Shapiro-Wilk normality test $p = 0.3911$). Later statistical tests used log_2 transformed values.

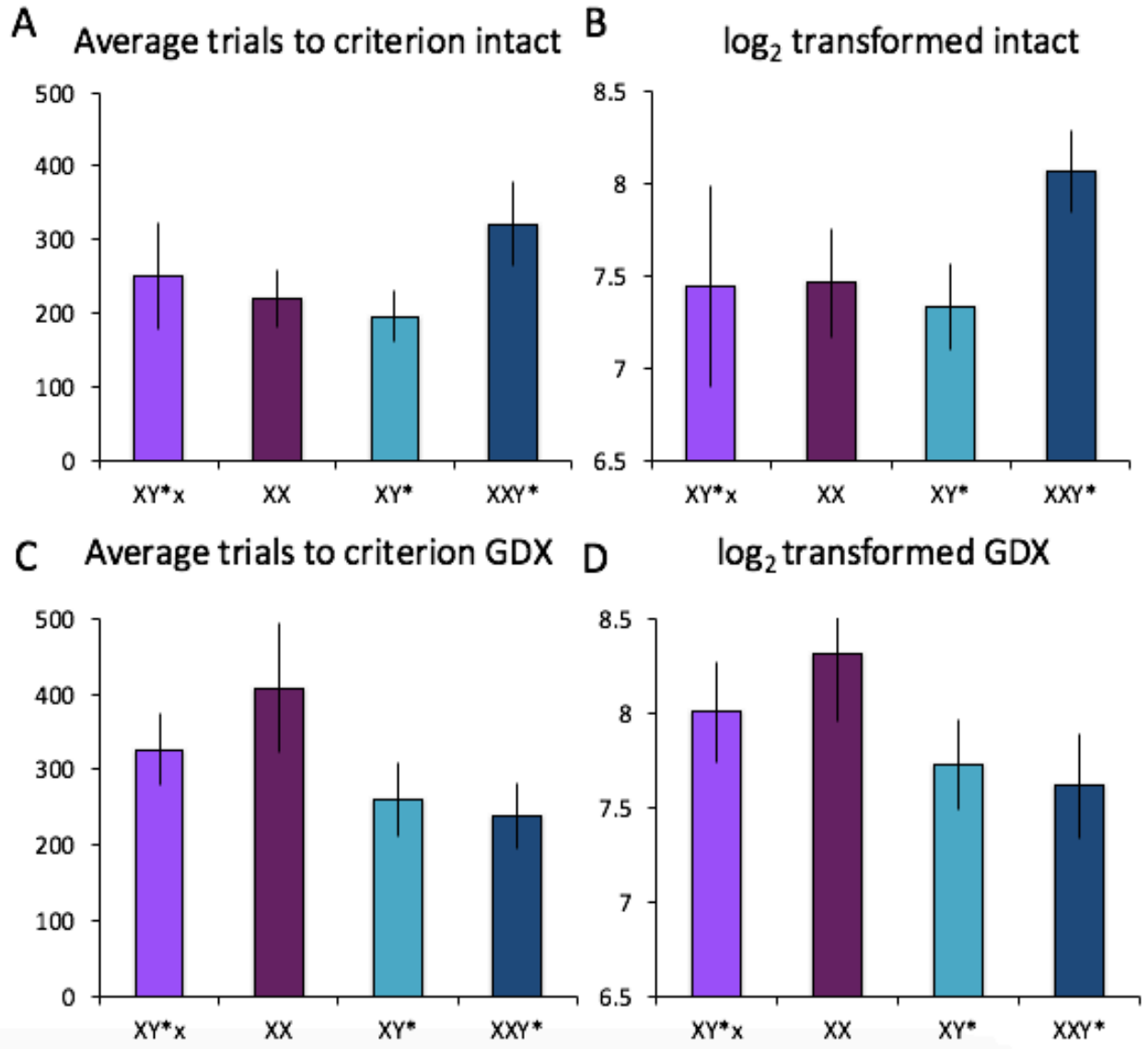


Figure 4-6: Reversal learning in XY* mice.
For $\log_2$ transformed trails to criterion, there was no significant effect of genotype ($p = 0.6120$) or gonadectomy ($p = 0.1867$) and there was no interaction of these effects ($p = 0.1652$). However, we had *a priori* hypotheses that XY* mice would differ from XXY* mice independent of the results of the female groups, and we hypothesized that this may occur across GDX groups, or within just intact or GDX. In pairwise tests, there was no difference in XY* vs. XXY* across GDX groups ($p = 0.1855$). Within just the intact, XXY took significantly more trials to criterion than XY* ($p = 0.0305$), but this difference did not survive multiple test correction by false discovery rate (corrected $p = 0.0916$), but this difference was not seen in the GDX mice ($p = 0.7645$).

4.3.4 Novel object recognition in SCT mice

For the raw ratio there was a pattern such that XY spent more time with the new object compared to the old object compared to XXY, but this difference did not reach significance ($p = 0.2052$), and there was no sex effect (0.2672), or interaction between these factors ($p = 0.9286$, Figure 4-7 A). For recognition index, a measure that is less sensitive to extreme values created by mice that spend little time with either object, there was again no sex chromosome complement main effect ($p = 0.1822$), sex main effect ($p = 0.1110$), or interaction ($p = 0.4379$, Figure 4-7 B). Post hoc tests for *a priori* hypotheses did not show any significant group differences.

4.3.5 Reward response test in SCT mice

For free consumption there was no main effect of sex ($p = 0.1257$) or sex chromosome complement ($p = 0.43918$), but there was a sex by sex chromosome complement interaction ($p=0.0317$). However, in post hoc tests there was no sex chromosome complement effect in either males or females. There was a significant effect of day such that mice consumed more milk on later days than on the first days of testing ($p < 0.0001$, Figure 4-8 A). For dose response there was no significant effect of sex or sex chromosome complement, but there was an effect of dose ($p < 0.0001$) such that mice preferred 10% SCM over the other two doses (Figure 4-8 B). The finding that there were no group differences in milk consumption in either free consumption or in dose response is notable because it suggests that 10% SCM may be a good reinforcer for later operant behavior testing in SCT mice.

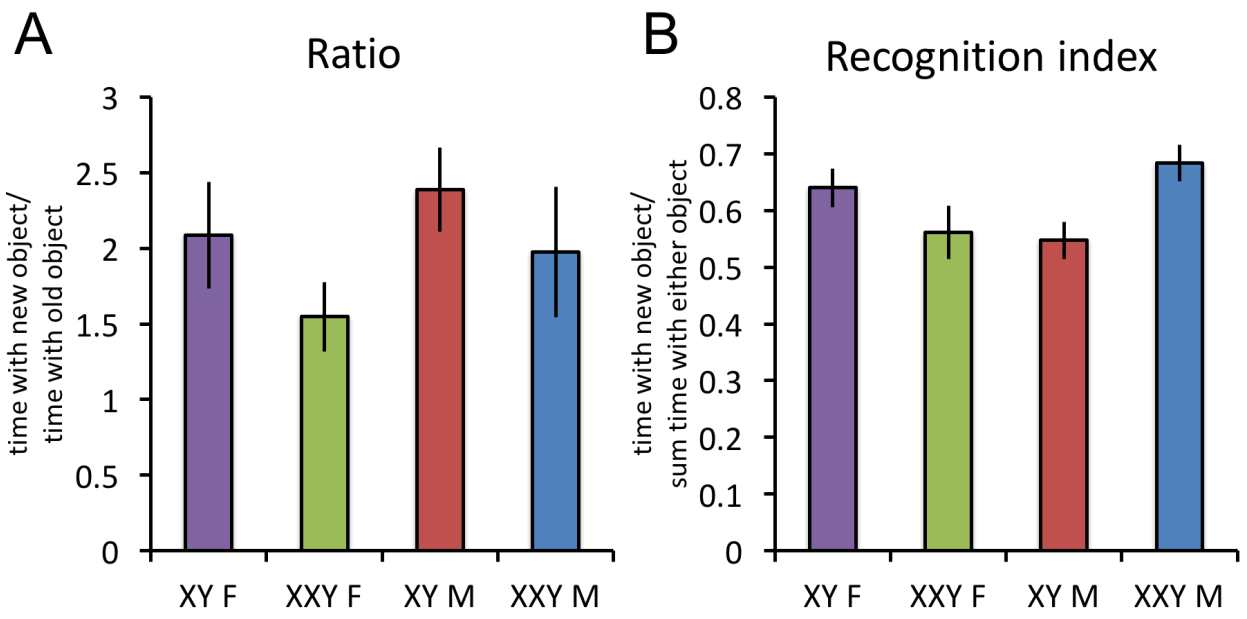


Figure 4-7: Novel object recognition in SCT mice.
A) For ratio there was a pattern such that XY spent more time with the new object compared to the old object compared to XXY, but this difference did not reach significance ($p = 0.2052$), and there was no sex effect (0.2672), or interaction between these factors ($p = 0.9286$). B) For recognition index, a measure that is less sensitive to extreme values created by mice that spend little time with either object, there was again no sex chromosome complement effect ($p = 0.1822$), sex effect ($p = 0.1110$), or interaction ($p = 0.4379$). Post hoc tests for *a priori* hypotheses did not show any significant group differences.

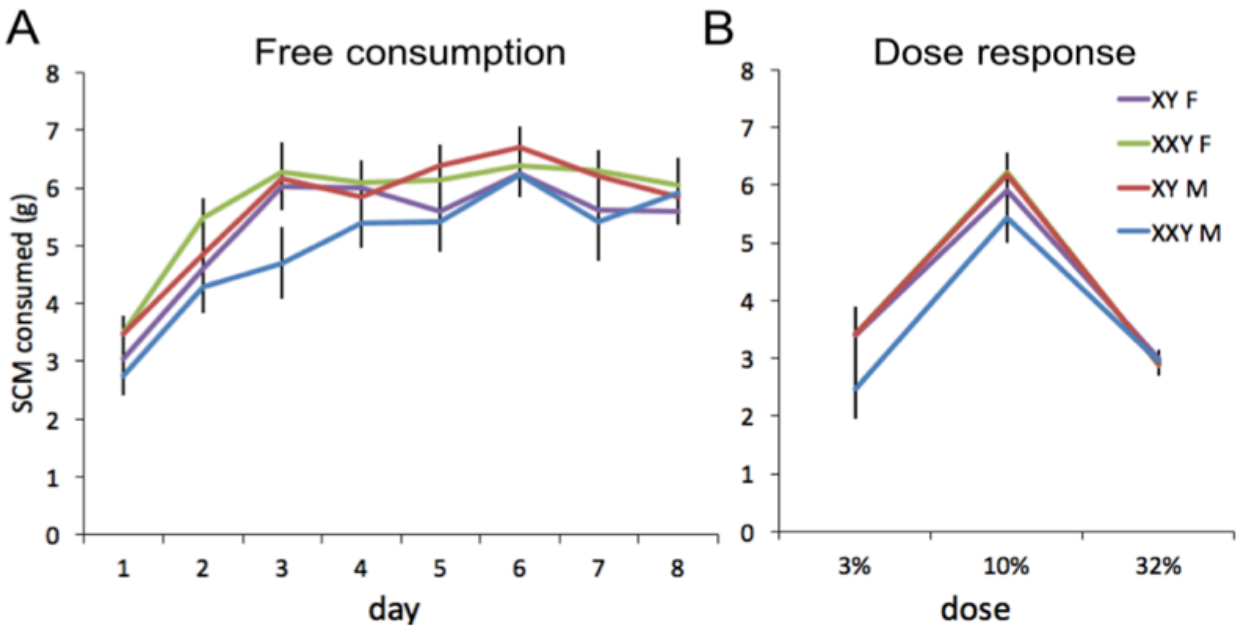


Figure 4-8: Reward response in SCT mice.
A) For free consumption there was no effect of sex ($p = 0.1257$) or sex chromosome complement ($p = 0.43918$), but there was a sex by sex chromosome complement interaction ($p=0.0317$), however in post hoc tests there was no sex chromosome complement effect in either males or females. There was a significant effect of day such that mice consumed more milk on later days than on the first days of testing ($p < 0.0001$). B) For dose response there was no significant effect of sex or sex chromosome complement, but there was an effect of dose ($p < 0.0001$) such that mice preferred 10% SCM over the other two doses.

4.4 Discussion

In the novel object recognition test, in our hands, XXY* male mice do not perform worse than XY* mice in either intact or GDX animals. This finding is surprising considering the previous study by Lewejohann et al. 2009 found a robust result that intact XXY* male animals perform worse on the task than intact XY*. Because Lewejohann et al. used XY* mice in the same background strain and with group size comparable to our study, we first considered that our animals were not performing on the test at all – perhaps due to experimenter error. However, XY* mice did in fact perform the task. Rather, the XXY* mice in our study performed equally well (Supplementary Figures 4-9 A and 4-10 A). Similarly, the GDX animals performed as well as the intact animals, and there were no group differences across genotype in the GDX animals (Supplementary Figures 4-9 B and 4-10 B). Barring experimental differences that may have helped impaired mice perform better, these data suggest that the novel object recognition task does not consistently reveal difference in XY vs. XXY mice and the XY* model.

For the SCT mice, the groups do not differ by sex or by genotype. In this case, however, it is not clear – especially when looking at individual results in the females – that the majority of the mice are performing the task correctly. Instead, a subset of the mice seem to have opposite behavior as would be expected of healthy mice such that they spend more time with the old object than the new object (Supplementary Figures 4-11 and 4-12). Because XY* and SCT mice were tested and scored the same way, there may be a strain difference in this behavior. In this case too, however, these results suggest that this test is not ideal for differentiating the cognitive phenotype of XY vs. XXY mice across models. These findings do not conflict with results from human KS research, because in many measures of cognitive function KS boys and men score within the normal range, and there are no specific studies that indicate a problem with recognition memory.

In the reward response test results indicate that within both strains, and across all groups, all mice prefer 10% SCM and will consume 5 or more grams of milk over the course of 2 hours when given free access. The one group difference is that the intact XY* drink more milk in the first days of free consumption testing than other groups. This finding is interesting on its own because it is consistent with another paper that showed that XY male mice drank more milk and would work harder for milk as a reinforcer, than XX male mice or female mice in a study using the Four Core Genotypes mouse model [17]. The difference in XY* consumption is a potential concern for using SCM as a reinforcer for reversal learning, because in case that group differed in behavior it would be difficult to interpret a difference in cognition versus a difference in motivation. However, by day 8 of free consumption as well as during the following dose response test the other groups consumed the same amount of milk as the XY* group.

The reversal learning task is specifically a measure of executive function rather than general cognition, and so is more relevant to the study of Klinefelter syndrome than the novel object recognition test. It is exciting, then, that in this test there is a strong trend that the intact XXY* mice perform worse than the XY* mice. This result does not survive multiple testing correction, and so must be interpreted with caution, but the direction of the difference is promising as an indication that KS model mice may have a deficit in executive function. Replication of this study including more animals may be sufficient to increase power to more readily detect a difference. Because the difference is present only in intact, but not in GDX mice, this trend points to an effect of circulating testosterone on executive function rather than organizational effects of hormones or direct genetic effects. Further studies will be needed to discriminate between two possibilities. One is that the XXY vs. XY difference is the result of lower testosterone levels in XXY mice in adulthood or earlier in life. The second is that the XXY vs. XY difference is an effect of the second X chromosome, but that the effect requires the presence of androgens in adulthood and at the time of testing.

Because the results could point to an activational effect of testicular hormones, a vital follow up experiment will be to test whether testosterone replacement is sufficient to bring XXY* intact male mice up to the same level as the XY* mice. The two primary possibilities are that testosterone does improve executive function in the XXY* mice, in which case it is further evidence that testosterone replacement therapy is effective in treating the cognitive features of KS. If, however, testosterone is insufficient to bring XXY* up to the level of XY* mice, there is likely an interaction between activational hormone effects and either organizational hormone effects or direct genetic effects. In that case further research to characterize the difference in the SCT model would be called for.

The unique ability of the SCT model to distinguish organizational hormone effects from direct genetic effects of the second X chromosome in XXY mice increases the attractiveness of this model to understand cognitive traits of KS. Because of potential strain differences between XY* and SCT mice, even in the event that XY* experiments point overwhelmingly to activational hormone effects, there could still be masked effects of organizational hormones or sex chromosome complement that are measurable in SCT mice. In part due to technical issues, testing reversal learning in the SCT model was beyond the scope of this project, however that approach remains promising for improving understanding of executive functioning deficits in KS.

4.5 **Researchers' Contributions**

Shayna Williams-Burris contributed to experimental design, collected data for all aspects of the project and trained and organized others to assist with data collection for all other aspects of the experiments as well as did all data analysis and drafted the manuscript. Jessy Martinez and Haley Hrncir helped collect data for novel object recognition and reward response experiments. Jessy Martinez, Haley Hrncir, Dorothy Yu, Ernie Garcia, Rylee Genner, and Sheyda Mesgarzadeh assisted with novel object recognition video scoring. Jessy Martinez,

Gabriela Beroukhim conducted FISH genotyping. Haley Hrncir and Ryan Mackey conducted breeding and GDX surgeries. David Jentsch and Arthur Arnold helped conceive of experiments, organized the research team, and revised and edited the manuscript.

4.6 References

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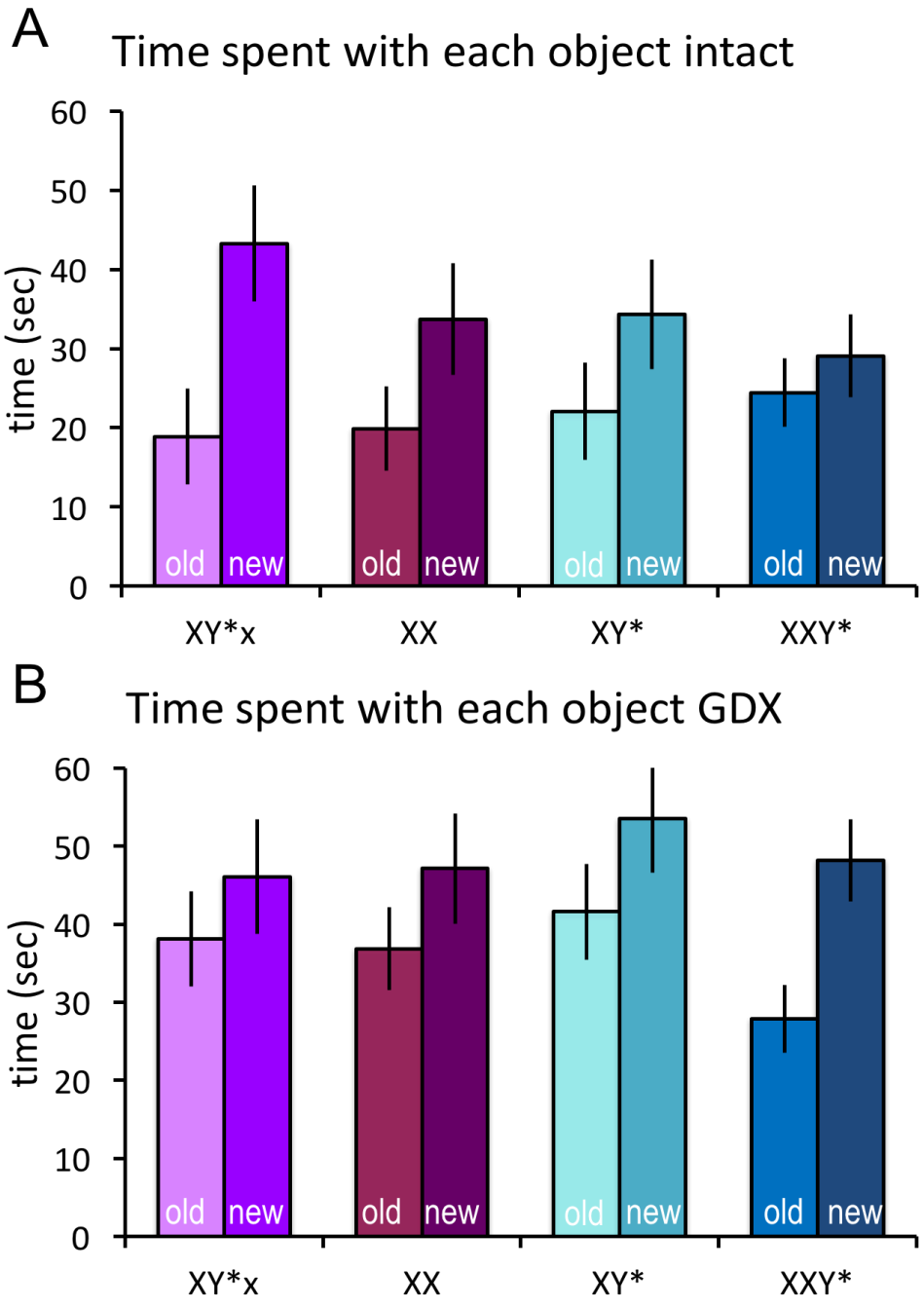
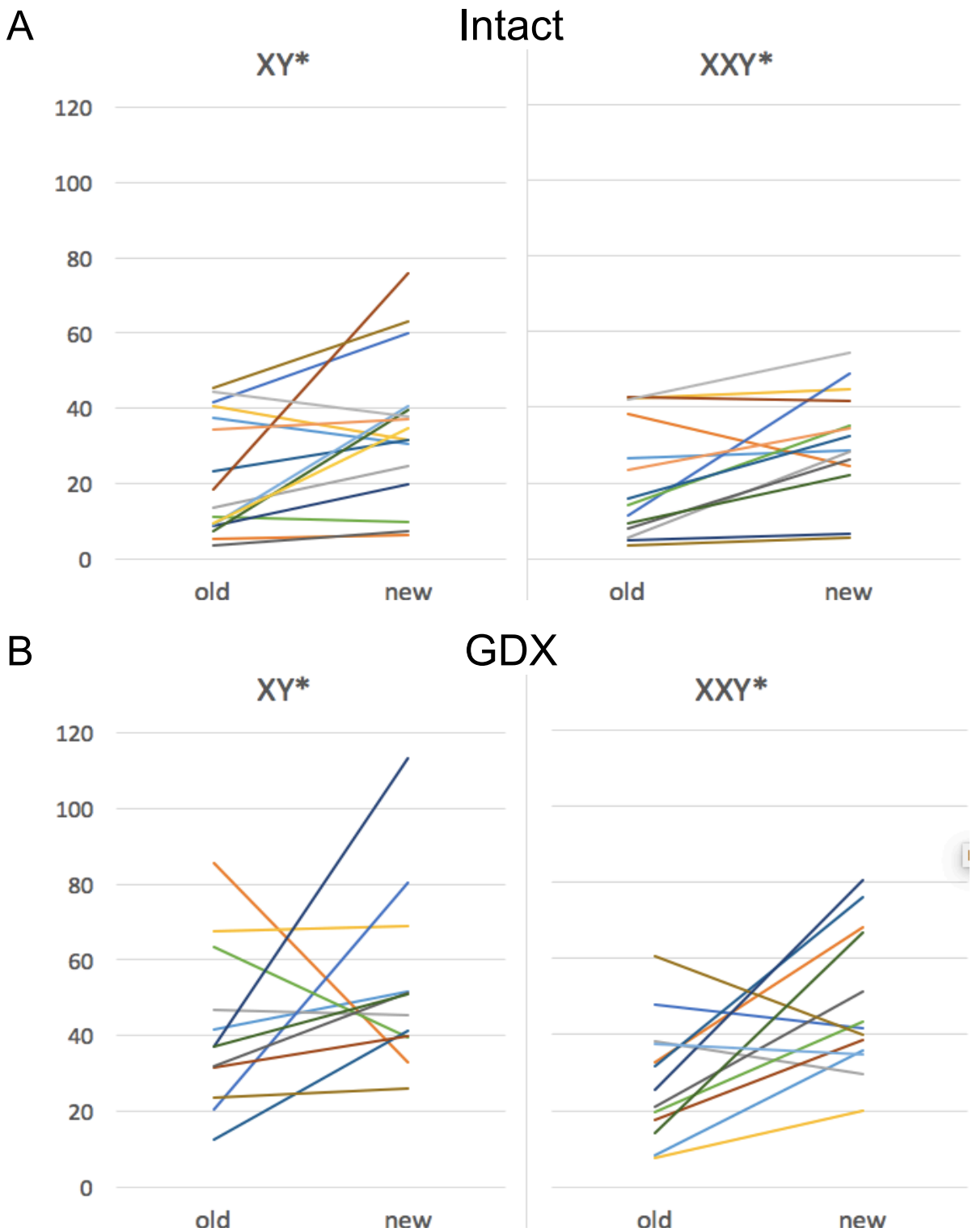
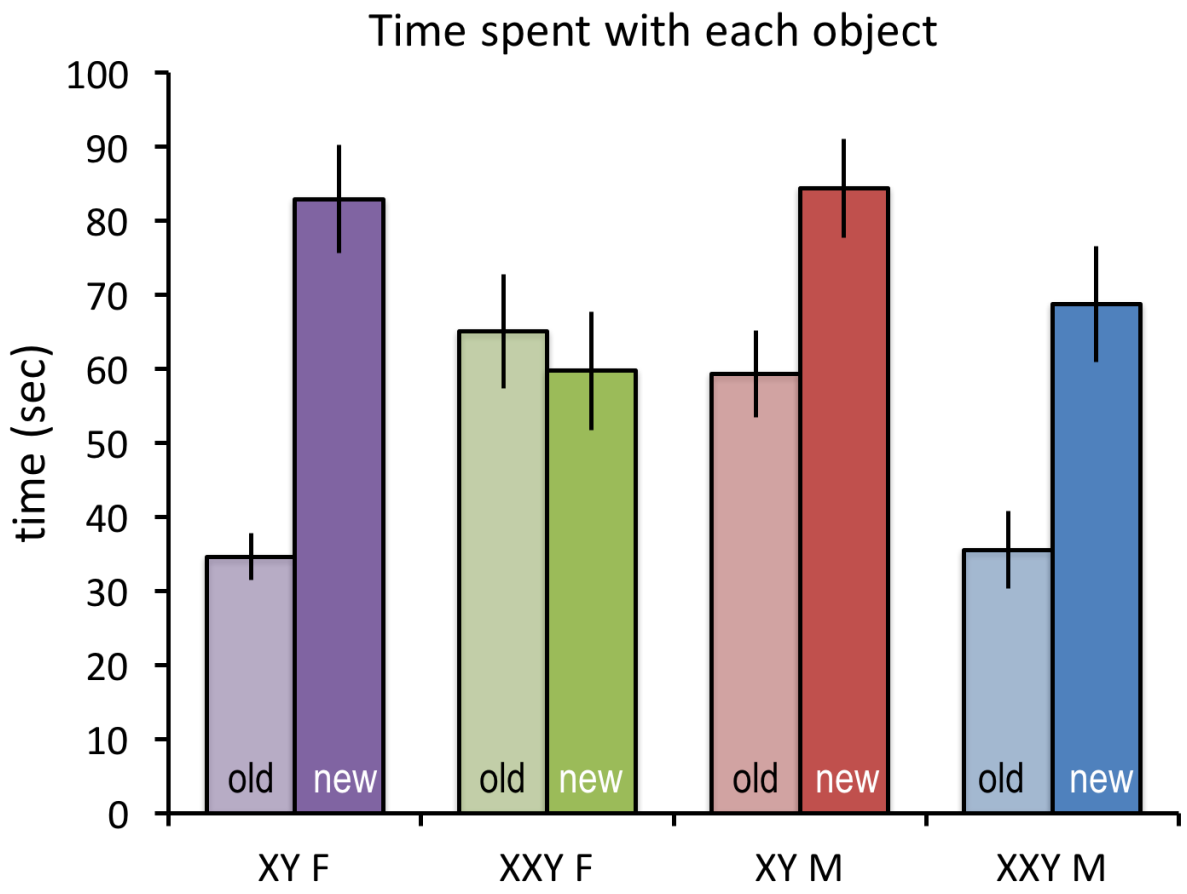
4.7 **Supplemental Materials**



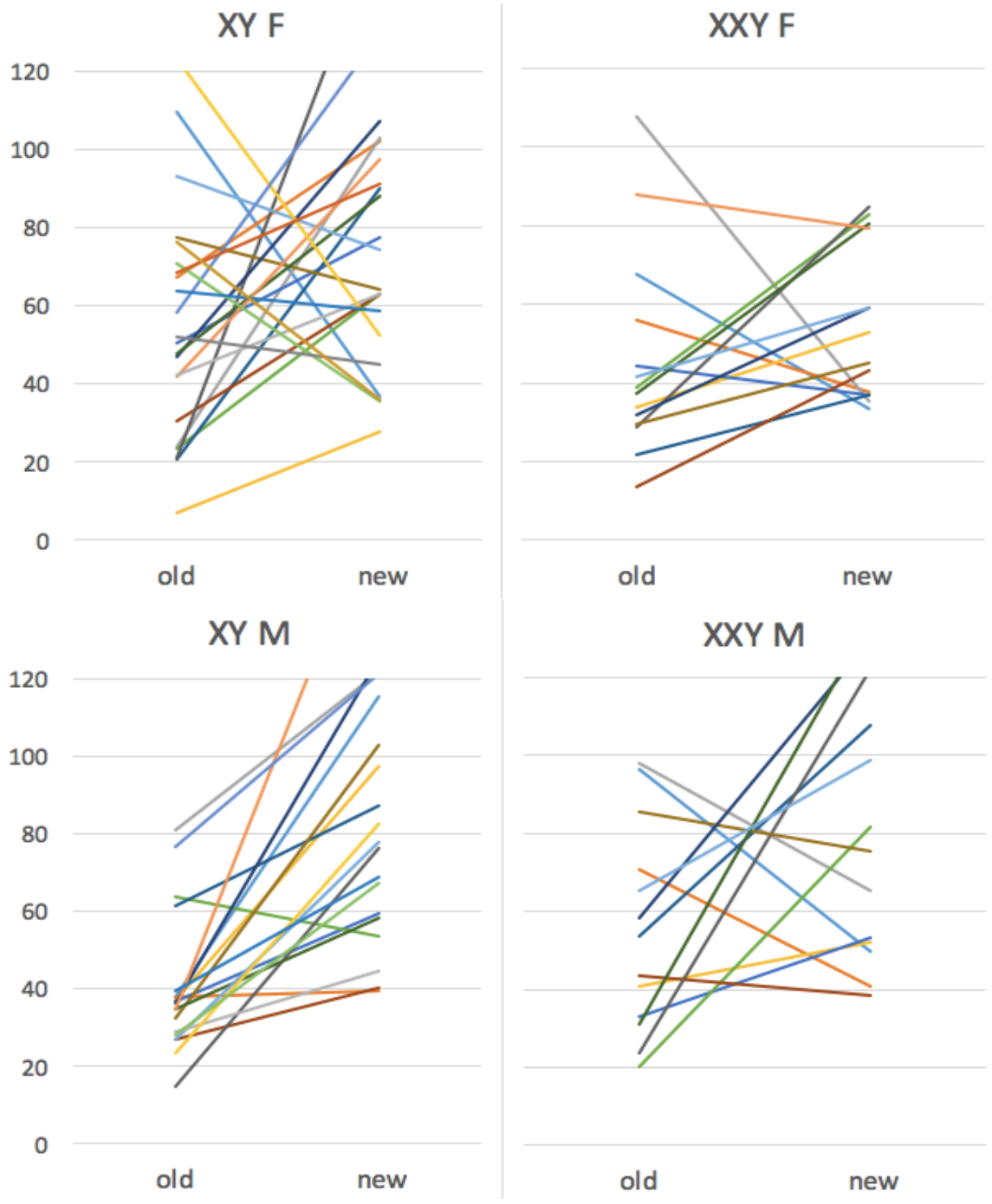
Supplemental Figure 4-9: XY* breakdown of time spent for novel object recognition. In all groups, either A) intact or B) GDX, mice spent more time with the new object than old object



Supplementary Figure 4-10: Individual mouse results for male XY* object recognition. On an individual level both XY* and XXY* mice generally perform well on the novel object recognition task both in A) intact and b) GDX conditions. XY^{*X} and XX had similar results (data not shown).



Supplemental Figure 4-11: SCT breakdown of time spent for novel object recognition.
In all groups except the XXY F, SCT mice spent more time with the new object than old object.



Supplementary Figure 4-12: individual mouse results for SCT object recognition.
On an individual level the SCT mice had variable performance on the novel object recognition test.

Chapter 5

Conclusions, Future Directions, and Closing Remarks

5.1 Summary

This dissertation presents the Sex Chromosome Trisomy (SCT) model of Klinefelter syndrome (KS) in a series of three studies. In the first study, the metabolic phenotype was first described and a motor test was carried out. In the second study, the metabolic phenotype was more carefully examined under more conditions and for a longer period of time, and the testes and bone phenotype was characterized. In the last study, the cognitive phenotype was characterized in both the SCT model and in another model of KS, the XY* mouse model.

5.2 Conclusions and Next Steps

5.2.1 Sex chromosome complement, not low testosterone alone, contributes to metabolic differences in men with Klinefelter syndrome

Two major studies characterizing the metabolic phenotype of the SCT mice show a consistent result that XXY mice are heavier and have greater percent body fat than XY mice. In the first study mice were gonadectomized (GDX) and treated with testosterone at around 100 days of age. Results of that study show XXY weigh more and have greater percent body fat both before GDX and 3 weeks after GDX and testosterone treatment in both males and

females. The result that the females differ in the same direction as the males provides strong evidence for a sex chromosome effect on metabolic measures, not just a hormone difference.

In the second study animals were either left intact or GDX at 75 days of age (more closely controlled and earlier than in the previous study) and measured for metabolic traits over the course of several months. In that study the XXY males weighed more and had greater percent body fat than XY males throughout the study in both intact and GDX animals. In the female groups the difference went in the same direction but was not as robust. In fact, while there was a trend for a sex chromosome effect in the intact females, the difference was not at all statistically significant in the GDX females. These results point to an interaction such that, at least in females, gonadal hormones must be present to facilitate a sex chromosome complement effect on body weight and percent body fat. Thus, while it is common practice to control for the activational effects of gonadal hormones by gonadectomizing groups, it may be more appropriate for the study of metabolic measures in SCT mice to bring all mice to equal circulating hormone levels with GDX and replacement, not GDX alone.

The present results are in general agreement with studies of the effects of one vs. two X chromsomes on metabolic parameters of FCG and XY* model mice in a C57BL/6J background [1]. Those studies show that XX mice are heavier and fatter than XY mice, regardless of their gonadal sex. A high fat diet exacerbates the XX vs. XY difference, causes greater dyslipidemia, and causes much more liver steatosis in XX than XY mice. The sex chromosome effect is attributed to the number of X chromosomes, not the presence / absence of the Y chromosome, because in the XY* model XX mice are heavier than XO, and XXY* mice are heavier than XY*. The latter comparison is the classic KS comparison used in the present studies.

The present studies also are in general agreement with studies of effects of sex chromosome complement on mice on an MF1 background[2]. Those studies show that in comparison of GDX mice with two sex chromsomes, those with a second sex chromosome,

either X or Y, weigh more and have greater body fat than those with only one X chromosome. Because either a second X or Y chromosome serves a similar function to increase body weight and fat, the results imply that the two sex chromosome may encode factors that have a similar effect on body weight. The X and Y chromosomes contain “X-Y gene pairs”, which are highly homologous genes that derive from an ancient precursor gene on the undifferentiated X chromosome, prior to separate evolution of the X and Y chromosome. In the gene pairs, the X gene typically escapes inactivation, and is expressed higher in XX than XY cells. The Y paralog is thought to provide dosage compensation, so that XX have two copies of the X gene, and XY cells have one copy each of the X and Y genes. Thus, although the X-Y gene pairs overlap in their function, the Y paralog may have diverge somewhat and evolved male-specific functions [3, 4].

The results in MF1 mice point to X-Y genes as possible mediators of sex chromosome effects on metabolism. These same genes may account for the effects of a third sex chromosome in KS mice. However, the effects of adding a third sex chromosome in XXY mice, compared to XY mice, is more modest than the addition of a second sex chromosome relative to XO mice. Although XXY mice are generally heavier than XY mice, that difference is not always statistically significant, as found in the present study and in Chen et al, 2012.

5.2.2 XXY and XYY male mice may be a good model for testes function in men with sex chromosome trisomies

A promising finding in the second study using the SCT mice is that the testicular phenotype in XY, XXY, and XYY seems to closely resemble that in humans. The XY have normal sized testes with normal histology including signs of spermatogenesis. XXY mice, on the other hand, have much smaller testes and obvious signs of degeneration as seen in KS men, including seminiferous tubules lacking spermatogonia and spermatocytes, and Leydig cell

hyperplasia, and no signs of healthy spermatogenesis. Interestingly, the XYY mice show an intermediate phenotype in both size and in cellular characteristics, including some seminiferous tubule degradation and Leydig cell hyperplasia but not to the same extent as seen in XXY. In all three sex chromosome complements, the SCT mouse testes are remarkably similar to their human counterparts. Notably, XX males had small testes and are known to be infertile like XXY, but do not have low testosterone levels as seen in KS, so these mice – also produced in the SCT cross – may prove an informative control group for the study of XXY. Further research is necessary to determine how similar the SCT testes phenotype is to humans in regard to function and the mechanism of degradation, but because this model uniquely allows for the study of XY, XXY, XYY, as well as XX male mice in the same litters may, it may prove advantageous to understanding how the supernumerary sex chromosome in the testes causes dysfunction. The genes on the supernumerary sex chromosomes that interfere with normal testis function and fertility have not been identified. Study of mouse models may help overcome this lack of information.

5.2.3 Bone health problems in men with Klinefelter syndrome are likely primarily due to hormonal, not genetic influence

The bone phenotype of the SCT was characterized in the second study; bone length, bone mineral density, and bone morphology were quantified. In humans, KS men have longer long bones than controls and have increased rates of osteoporosis[5]. It had been previously proposed that a sex chromosome effect on bone mineral density and bone morphology exists in the XXY mouse model of KS because long term treatment with testosterone in adulthood was not sufficient to equalize bone measures in XXY compared to XY male mice [6]. However, that study did not adequately take into consideration the possibility of long-lasting organizational hormone effects, acting prior to GDX in adulthood, that may be irreversible even with

testosterone treatment after GDX. In the present study we show convincingly that in the SCT model the bone phenotype is more likely caused by differing testosterone levels during development, not sex chromosome complement. We find that within intact and GDX XY and XXY male mice, only the intact XY mice, the group with the highest testosterone levels, had elevated and presumably normal bone mineral density. More informatively, the XY and XXY females showed uniform bone mineral density regardless of GDX. Therefore, only in the presence of differing testosterone levels do XY differ from XXY in bone mineral density. One interesting follow-up study might be to treat female SCT mice with testosterone for a prolonged period in adulthood, as was done in the previous study. Alternatively, females could be treated perinatally with testosterone, or during adolescence, to test for long-lasting (organizational) effects of androgens. By using females (that do not have differing levels during development), instead of males (in which the XXY have lower testosterone levels than XY that might lead to permanent differences between groups), the interaction effect of sex chromosome complement with artificially increased circulating testosterone levels could be more cleanly observed.

Finally, the current results are compatible with the idea that sex chromosome complement (XY vs. XXY) causes differences in bone parameters, but only when testicular hormones are present. In this scenario the XY vs. XXY difference is not caused by differences in androgen levels, but by a sex chromosome effect that operates only when androgens are elevated as in males, not females. This idea could also be tested by treatment of female XY and XXY groups with androgens at specific ages.

5.2.4 Further research on the behavioral features of SCT mice could shed light on the etiology of cognitive problems experienced by men with Klinefelter syndrome

The third study aimed to characterize the behavioral phenotype in SCT mice in novel object recognition and reward response, but also tested the same measures as well as reversal

learning in intact and GDX XY* model mice. A previous study in intact XY* mice with methods very similar to our study found a difference between XY* and XXY* males in novel object recognition memory [7], but we were unable to confirm this difference in those groups, in GDX XY* mice, or in SCT mice. We found that all groups performed the test equally well, and that they were showing apparently normal behavior rather than perhaps showing a fear response or other altered behavior due to technical error. One possibility is that small differences in experimental methods account for the difference. Another explanation is that the novel object recognition test is not sensitive enough to consistently measure differences in mouse models of KS. This may not be unexpected considering that cognitive deficits seen in men with KS are usually limited to specific abilities – especially language and executive functioning – and not to general cognition tasks such as memory. Because of this, the final behavior measured, reversal learning, which is a measure of executive function, may be a more sensitive and relevant behavior for understanding the cognitive phenotype of Klinefelter syndrome.

The reversal learning experiment showed that XXY* intact mice take more trials to reverse a learned behavior than XY* intact controls. This result was only significant when not corrected for multiple testing, so further use of the reversal learning task – perhaps with larger sample sizes to detect subtle differences – is needed to carefully characterize this model. The difference was not found in the GDX XXY* and XY* animals, perhaps pointing to circulating testosterone levels as the underlying cause of the difference. An important follow up experiment will be to treat XY* and XXY* mice with testosterone to determine whether increasing testosterone in the XXY* is sufficient to improve function in the task. There is some research that suggests that testosterone treatment improves the cognitive features of KS in humans [8], so a result in that direction would be interesting. The alternative result, that XXY* are not improved to the level of XY* even with testosterone treatment would also be an interesting result and indicate that either organizational effects of testosterone or sex chromosome effects

underlie the difference. Some evidence in humans suggests that sex chromosome effects are implicit in the deficits seen in KS patients, especially when considering another low testosterone syndrome, idiopathic hypogonadotropic hypogonadism, does not cause the same deficits, whereas women with trisomy X do have similar deficits [9]. However, it is impossible to cleanly separate organizational hormone effects from those of sex chromosome complement in human KS patients or in the XY* or other mouse models of KS. The study of SCT model mice would be integral to improving the understanding of executive functioning deficits in KS.

5.3 Future Directions

Besides following up the current research with the proposed immediate experiments for each measure as suggested above, greater horizons still exist for the study of the SCT model.

5.3.1 Ultrasonic pup vocalization as a model for speech delay

Klinefelter boys have impaired speech and language development from an early age, and these difficulties persist into adulthood. The evidence is robust suggesting KS boys have a primary speech learning disability, and research shows that up to 80% of boys have language impairment. These deficits include delays in expressive speech milestones, impaired language learning, difficulty with articulation, impairments in verbal memory and fluency, and reading disability. Disability due to problems with language have been shown to accrue with increasing age, causing KS boys to be several grade levels below age-matched controls by the age of 18-20. [10-14] In one study XXY men were shown to perform worse on a verbal ability test compared to non-XXY hypogonadal men, pointing to a possible direct genetic effect rather than a hormonal effect causing deficits in speech and language [15].

Although these language deficits have been described extensively in the human literature, there have been no studies on KS mouse models regarding vocalizations [16].

Although language is not learned in mice, the speech impairment seen in KS is significant enough that a comparable difference in vocalization could be measurable in the SCT model. When pups are isolated from their nest they emit isolation calls to promote retrieval by their mother. These calls have been shown to be disrupted in other models of speech and language impairment in mice, including autism models, which may exhibit similar phenotypes to KS mice [17-19]. Additionally, isolation calls have been shown to be sexually dimorphic in rats and mice, indicating already that sex-biasing factors are in play in this behavior [20-22]. Finding differences in pup vocalizations between XY and XXY animals, especially in the SCT model where it will also be possible to identify the underlying mechanism as either testosterone exposure or gene dose, would make an exciting novel contribution to the understanding of speech pathology in KS and open up previously inaccessible avenues of research into the etiology of a trait that greatly influences the lives of KS patients.

5.3.2 Gene expression analysis

In the case of traits that are identified by the SCT model to be due to direct genetic effects, either independent of or in interaction with hormone effects, a next step would be to identify what genes or gene networks are involved in the pathogenesis of the symptoms of KS. Because most of the X chromosome is silenced, genes that escape X inactivation are primary candidates for causing direct genetic effects seen in KS. The six primary candidate X-inactivation escapee genes are *Ddx3x, Eif2s3x, Kdm5c, Uba1, Kdm6a,* and *Usp9x.* These genes have often been shown to be expressed higher in XX compared to XY cells in mice and humans. To date, it has not been shown in SCT mice that these genes are expressed higher in XXY compared to XY, so an important first step would be to confirm expression levels in SCT mouse tissues in general. Next experiments should be to a) determine whether dose of these genes correlates directly with any traits that show a sex chromosome complement effect, and 2)

determine what gene networks are downstream of the X inactivation escapee genes that may be directly involved in those traits.

The most accessible and comprehensive approach to address these issues would be to measure genome-wide gene expression with microarray analysis or RNAseq in metabolic tissues collected in the first two studies of this dissertation. The animals in those experiments show that sex chromosome complement influences body weight and body composition, so these metabolic traits can be used as a model for how X inactivation escapee genes may act to influence KS phenotypes in general. Although a candidate gene approach using qPCR could establish a correlation between X inactivation escapee gene expression and the metabolic traits, it is to be expected that the X inactivation escapee genes will be higher expressed in animals with two X chromosomes. Therefore, a potentially more enlightening approach would be to measure genome wide expression using microarray analysis or RNAseq and then use network analysis to identify gene networks and potential pathways by which the X inactivation escapee genes may act to influence the metabolic traits. Especially because many of the X inactivation escapee genes are epigenetic regulators, there are a range of potential mechanisms of action for how they may influence not just metabolic traits, but any trait that is affected by sex chromosome complement. A whole genome approach with careful analysis using modern statistical techniques such as weighted gene co-expression network analysis could be a highly fruitful avenue of research.

5.3.3 Backcross SCT model to inbred strain

The SCT model, though a powerful tool already, is limited by the facts that 1) the MF1 background strain is outbred, 2) the MF1 strain is not widely used outside of the sex chromosome research field, and 3) MF1 mice are not commercially available in the US. The reason for this is necessity: the model is created by breeding XY^{-} females to produce XXY^{-}

females, but XY^- females are infertile in the inbred strain most commonly used by our lab, C57BL/6J (B6), as well as each of the few other inbred strains that have been tested. But even though the SCT model cannot be produced directly from XY^- females in the B6 strain, it may be possible to back cross the SCT model onto the B6 strain. There is evidence, at least in MF1, that XXY^- females are more fertile than XY^- females [23], so this may also be the case in B6. Whether or not that is the case, the backcross may still be successful because by definition at each generation genes that preserve fertility will be selected for, so if only a small number of MF1 genes are necessary to preserve fertility these may be able to be maintained generationally even as each generation becomes increasingly predominantly the B6 background, thereby maintaining fertility of the SCT model on the B6 background.

Backcrossing the SCT model to the inbred BJ background strain may provide some resolution to outstanding questions. For example, future research using SCT mice backcrossed on the B6 strain may allow for easier comparison of SCT studies to the majority of other metabolism studies implicating sex chromosomes in metabolic traits. Also, the two other mouse models of KS – the XY* model and the XXY model – are also on the B6 strain, therefore this backcross may yield greater interpretability to results across models. Perhaps most importantly, Klinefelter syndrome is poorly understood and understudied considering that it is a prevalent genetic condition, and the SCT model provides a powerful tool for the study of the syndrome. It would be ideal to make the SCT model readily available to a broader base of researchers, but currently the main source of SCT mice is our lab. The successful backcross to a common inbred strain such as B6 would allow for the model to be made commercially available, an integral step in spreading the SCT model as a tool to study KS.

5.4 Closing Remarks

The Sex Chromosome Trisomy model represents a novel and powerful tool for the study of Klinefelter syndrome. As presented in this dissertation, three large studies to characterize the model have been carried out with promising results. First, the model shows several features that model health concerns in men with KS including obesity, testis function, and osteoporosis. Further, the model has shown its usefulness by helping to determine the etiology of some features: the metabolic phenotype appears to be influenced by both sex chromosome complement and hormone levels, whereas the bone phenotype seems to be primarily organized by hormone levels. The model also shows promise for the study of cognitive features of KS, though further work in this area – especially using more sensitive measures of cognitive function such as the reversal learning task for executive function – is necessary. Other future work should expand the use of the model to examine other KS traits that might be modeled in the SCT mice (such as ultrasonic vocalizations), and to more fully utilize the power of the model as a genetic tool to understand how the XXY karyotype may alter gene expression leading to KS traits. Backcrossing the model to an inbred strain may prove a pivotal step in making the model more widely used, but whether that venture is successful or not, ideally the SCT model will be further studied and prove fundamental for increasing the understanding of Klinefelter syndrome, ultimately leading to better treatments for the patient population.

5.5 References

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