ROLE OF PATERNAL PRECONCEPTION ENVIRONMENT IN ETHANOL- AND STRESS-RELATED PHENOTYPES

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While the importance of maternal health during pregnancy for proper offspring development is well-acknowledged, a potential role for preconception health -- especially that of the father -- is rarely considered. However, a recent surge of studies strongly implicates paternal experiences and environmental exposures prior to conception as causal drivers of complex neurobehavioral offspring phenotypes. Considering this work, my dissertation laboratory previously discovered that paternal chronic ethanol exposure increased ethanol sensitivity and decreased ethanol drinking preference selectively in male offspring. This dissertation builds off those preliminary studies with the hypothesis that paternal preconception environment alters ethanol- and stress-related phenotypes via epigenetic mechanisms in sperm. Initial experiments revealed that paternal chronic ethanol blunted the corticosterone response to acute stress, prevented stress-induced polydipsia, and altered genetic and epigenetic regulation of corticotropin-releasing factor in the hypothalamus in male offspring. In addition, paternal chronic stress reduced ethanol drinking behavior in male offspring and this phenotype was dependent on the vendor/shipping history of the sire. Subsequent experiments examined the effects of chronic ethanol on small noncoding RNA in sperm, an epigenetic mechanism causally implicated in the cross-generational effects of paternal preconception environment. The unique small noncoding RNA signature of sperm is shaped during epididymal transit by extracellular vesicles (i.e., epididymosomes). Small RNA sequencing revealed several ethanol-responsive small noncoding RNAs in sperm and some species were similarly affected in epididymosomes. Finally, the effects of epididymosomes on intergenerational

ethanol- and stress-related behaviors were directly tested. Normal sperm was incubated with epididymal extracellular vesicles from chronic ethanol (Ethanol EV-donor) or control-treated (Control EV-donor) mice prior to *in vitro* fertilization (IVF) and embryo transfer to produce adult progeny. While Ethanol EV-donor treatment did not recapitulate the ethanol- or stress-related intergenerational effects of paternal ethanol, Ethanol EV-donors did impart increased anxiety-like behavior to IVF-derived females and modestly increased limited access ethanol intake in IVF-derived males. In summary, paternal preconception environment impacts ethanol- and stress-related behavior in offspring, possibly via small noncoding RNAs in the germline and epididymal extracellular vesicles.

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LIST OF FREQUENTLY USED ABBREVIATIONS

ABBREVIATION	FULL TERMINOLOGY
ACTH	Adrenocorticotropic Hormone
AUD	Alcohol Use Disorder
BDNF	Brain-Derived Neurotropic Factor
BEC	Blood Ethanol Concentration
CEA	Central Amygdala
CIE	Chronic Intermittent Ethanol
CIVS	Chronic Intermittent Variable Stress
CORT	Corticosterone
CRF	Corticotropin-Releasing Factor
EV	Extracellular Vesicle
GR	Glucocorticoid Receptor
GWAS	Genome-Wide Association Study
HPA	Hypothalamic-Pituitary-Adrenal
IAP	Intracisternal A Particle
IHC	In-House Colony
IVF	*In vitro* Fertilization
miRNA	MicroRNA
mitosRNA	Mitochondrial Small RNAs
piRNA	Piwi-interacting RNA
PVN	Paraventricular Nucleus of the Hypothalamus
RT-qPCR	Reverse Transcription Quantitative PCR
SNP	Single Nucleotide Polymorphism
tDR	Transfer-RNA Derived Small RNAs
UTR	Untranslated Region
VBS	Vendor-Born and -Shipped

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PREFACE

Faced with a crystal ball-like foretelling at the onset of my graduate career, I would have been stunned by the trajectory of my dissertation research. I imagine scratching my head and wondering "what on Earth is an epididymosome?" That said, speaking as the man within the crystal ball, on the other side of this 5 ½ year endeavor, I am quite proud of my time with the Center for Neuroscience at the University of Pittsburgh (CNUP) and immensely grateful to many who have empowered my doctoral training experience.

First, I would like to thank my exemplary mentor, Dr. Gregg Homanics. I am forever grateful for his inquisitive and fearless research ambition, unshakable poise in the face of imbecilic blunders by my hand, and sincere commitment to my scientific and career development. Second, I want to thank past and present members of the lab. Carolyn Ferguson was a crucial contributor to many of my dissertation experiments and I cannot thank her enough for her scientific prowess, unrelenting kindness, and good humor. Dr. Andrey Finegersh was a brilliant graduate student and friend in the lab and I am thankful for his laboratory training and our countless academic discussions.

Beyond the lab, I want to thank my committee, the CNUP, and the Department of Molecular Pharmacology and Chemical Biology for providing a challenging and intellectually rewarding doctoral training environment. I also must acknowledge former mentors and colleagues: Dr. Kazu Nakazawa and Dr. Daniel Austin from my time at the National Institute of Mental Health and Dr. Robert Drugan from the University of New Hampshire. Their past guidance has proved invaluable. Finally, without the unwavering support of my friends and family – while I'd like to

think I could tough it out – I would have crumbled to nothing long ago. You are all amazing and have my eternal gratitude.

1.0 INTRODUCTION

1.1 HERITABLE EFFECTS OF PRECONCEPTION ALCOHOL

1.1.1 Missing heritability of alcohol use disorder (AUD)

Individual risk of developing most psychiatric disorders, including alcohol use disorder (AUD), is widely conceptualized as the product of gene × environment interactions (Meaney, 2017). That is, both heredity (i.e., familial factors presumed to be primarily genetic) and experiences throughout the lifetime (i.e., environmental factors) are considered to best define at risk populations for disease. For psychopathologists, the genetic component of the risk equation has been especially intriguing, given the implications for targeted disease treatment and prevention. Collectively, twin and adoption studies estimate that AUD is ~50% heritable (Prescott and Kendler, 1999; Young-Wolff et al., 2011; Ystrom et al., 2011); in other words, roughly half of the variation between individuals with AUD can be explained by heredity. Thus, to examine the genetic component of AUD, investigators throughout the world have employed genome wide association studies (GWAS) to survey diverse populations for genetic marks -- most often single nucleotide polymorphisms (SNPs) -- that predict alcohol-related phenotypic variation (e.g., AUD diagnosis).

The most well-recognized studies linking genetic variants and AUD risk, implicate genes for enzymes directly involved in alcohol metabolism -- alcohol dehydrogenase (ADH) and

acetaldehyde dehydrogenase (ALDH) enzymes (Higuchi et al., 1995; Birley et al., 2008; Bierut et al., 2012; Li et al., 2012). Identified SNPs on ADH1B and ALDH2 are associated with impaired clearance of acetaldehyde, the toxic byproduct of alcohol metabolism, and reduced AUD risk (Cederbaum, 2012), suggesting a function for alcohol metabolism-related genetic variants in AUD heritability. Nevertheless, although these risk variants are common in Asian populations, they are rarely identified in populations of central European origin (Bach et al., 2017). In addition, such studies have been the few exceptions in an otherwise conflicted field of study. The results of many GWAS efforts often fail to replicate across studies (Bierut et al., 2010; Treutlein and Rietschel, 2011). Moreover, one recent study estimated that none of the SNPs identified accounted for more than 0.1% of AUD heritability (Heath et al., 2011). Such outcomes are not unique to AUD. For example, Crohn's disease is estimated to have 80% heritability, but the additive effect of all SNPs associated with the disease account for only ~20% of that estimate (Park et al., 2010). This common discrepancy between estimated heritability of disease predicted by familial vs genetic variation, is referred to as the "missing" heritability problem.

There are numerous factors that may help explain missing AUD heritability. For example, current statistical models are not robust enough to reliably detect rare variants (Zuk et al., 2014) or more complex gene × gene interactions (Zuk et al., 2012) without working with much larger population data sets than are generally available. Moreover, the innumerable established and emerging variables that comprise the environmental component of the G x E interaction (e.g., cultural diversity, drug availability in addiction, early-life stress, environmental toxins, microbiota), as well as gender and age all severely compound the challenge of replication across heterogeneous sample populations (Ober and Vercelli, 2011). Lastly, given the complexity and heterogeneity in symptomology related to psychiatric disease states, collective efforts more focused on defining the

heritability of “intermediate” phenotypes (e.g., ethanol-induced body sway) could yield more consistent results (Blanco-Gomez et al., 2016). Therefore, genomic complexity, wide-ranging environmental variables, and experimental limitations may all contribute to the unexplained AUD heritability.

As an alternative explanation for missing heritability, there is an emerging interest in non-genomic mechanisms of inheritance that may accompany the DNA in the germline at conception. Among these “epigenetic” mechanisms, the most well-established are DNA methylation, histone modifications, and noncoding RNAs, that all function to influence gene expression [see Chapter 1.3.1 for a more detailed discussion of epigenetic mechanisms]. Unlike DNA where environmentally-induced nucleotide mutations in the germline are rare, these epigenetic mechanisms are sensitive to a myriad of environmental perturbations. Thus, at the time of conception, the germline may be shaped uniquely by lifetime experiences to drive phenotypic variation in the next generation.

1.1.2 Inheritance of paternal preconception experience

With the development of Mendelian inheritance and discovery of DNA in the mid-20th century, the genetic theory of evolution has been the unifying standard for modern biology. Largely parsimonious with Charles Darwin’s theory of natural selection, genetic theory posits that, for a given environment, success or fitness of the species will be determined by selective pressure for phenotypic traits that are genomic in origin (Orr, 2005). By this principle, the ancestral or parental environment has no targeted mechanism to influence the phenotype in subsequent generations. However, even preceding Darwin’s *On the Origin of Species*, the French biologist Jean-Baptiste Lamarck described a more flexible theory of heredity, positing that the ancestral environment is the

primary driver of phenotypic variation in posterity (Lamarck, 1802). While long-rejected in favor of a singular genetic-basis for heredity, over the past twenty years, a surfeit of clinical and preclinical evidence has reignited interest in Lamarckian theory and a potential complimentary role for parental environment in heredity (Skinner, 2015).

Nongenomic inheritance of a given phenotype is described as intergenerational if it is imparted from father (i.e., the F0 generation) to offspring (i.e., the F1 generation). If the given phenotype is observed in the F2 or subsequent generations, it is described as transgenerational. In the context of germline inheritance, the transgenerational terminology indicates that the given phenotype was transferred through germ cells never harbored by the affected F0 generation. Thus, for females, as the pregnant F0 females harbor the primordial germ cells from which the F2 generation will spawn, transgenerational effects are reserved for the F3 generation and beyond. This dissertation will focus largely on mechanisms of paternal intergenerational germline inheritance, though transgenerational effects will also be discussed in various sections.

Many of the initial epidemiological studies implicating a role for ancestral environmental exposures in intergenerational and transgenerational effects stem from the Överkalix population of northern Sweden which kept cross-generational historical records of harvest and food supply. For example, these studies reported that paternal grandfather and grandmother's prepubertal food supply was inversely related to male and female longevity (Bygren et al., 2001; Pembrey et al., 2006). Additionally, paternal smoking prior to puberty was associated with greater body-mass-index measures specifically in sons (Pembrey et al., 2006). More recent findings from Rachel Yehuda's group implicate parental stress in cross-generational effects. Both paternal and maternal post-traumatic stress disorder (PTSD) --from traumatic episodes preceding conception-- were associated with reduced basal cortisol levels and greater dexamethasone-suppression of cortisol in

adult offspring (Yehuda et al., 2007; Lehrner et al., 2014). Furthermore, offspring of women that survived the Holocaust prior to conception were at increased risk for the development of PTSD, depression, and anxiety disorders (Yehuda et al., 2008).

Determining whether there are heritable effects of preconception experience in the human population is challenged by the need for longitudinal data and the confounding influences of genetic diversity, familial interaction, socioeconomic status, and unique lifetime experiences of the population in question. Further impeding interpretation of cross-generational effects as germline-derived is the inherent difference between paternal and maternal preconception experience due to the lengthy *in-utero* period of female pregnancy. While alterations to the gestational environment may be due to preconception experience, they may also result from post-conception environmental perturbations. Thus, this dissertation focuses primarily on paternal exposures as most of the preconception environmental exposure studies with implications for nongenomic germline inheritance have been conducted with males.

To test the hypotheses inspired by cross-generational epidemiological data, preclinical investigators have utilized isogenic rodent strains under controlled laboratory conditions to directly test the effect of paternal preconception exposure to a vast range of environmental insults on an equally expansive number of biological and behavioral measures in offspring. Incredibly, several paternal preconception exposures, most prominently those to nutritional challenge (Rando and Simmons, 2015), behavioral stress (Bale, 2014), and various drugs of abuse (Vassoler and Sadri-Vakili, 2014), directly impact complex phenotypes in offspring. As sires are not involved in offspring gestation or rearing in these studies, the contribution of the male to offspring development is likely restricted to the germline. It is worth citing here research indicating that sire "fitness" can influence maternal care for offspring through social interaction during breeding (Mashoodh et al.,

2012). Nevertheless, several paternal exposure studies have partially or fully validated the germline origin of intergenerational effects using *in vitro* fertilization (Dietz et al., 2011; Dias and Ressler, 2014; Chen et al., 2016a; Huypens et al., 2016; Sharma et al., 2016). Taken together, these paternal exposure studies suggest that rodents are an excellent model system for examining whether preconception environment impacts cross-generational ethanol-related behaviors through the germline.

Finally, it is important to point out that paternal preconception exposure studies in mammals do not control for potential germline mutations induced by the environmental exposure. As some environmental toxins, such as ethanol, have mutagenic properties (Garaycoechea et al., 2018), this is an important limitation. However, unless the mutagenic effects are targeted to specific genomic loci, it is unlikely that they underlie emergence of unique intergenerational behavioral phenotypes detectable with the current sample sizes commonly employed. Intriguingly, epigenetic variation may promote local genomic instability in sperm (Skinner, 2015). Therefore, futures will need to more closely examine the interplay between genetic and epigenetic mechanisms of inheritance.

1.1.3 Cross-generational effects of preconception ethanol exposure

The risk of maternal alcohol abuse during pregnancy inducing fetal alcohol syndrome in offspring is well-recognized. Although a family history of AUD is strongly associated with several physiological and behavioral deficits in offspring (Finegersh et al., 2015b), in addition to increased risk for AUD (Schuckit, 1985b), these heritable effects are difficult to segregate from genetic, social, and environmental influences. In addition, given the long-held belief that only genetic information is passed through the germline, even the potential for germline-dependent effects of

preconception ethanol exposure has gone largely unconsidered. Nevertheless, there is over 30 years of preclinical research directly examining the impact of paternal preconception ethanol exposure on offspring development.

There have been over forty published paternal preconception ethanol exposure studies (reviewed in (Finegersh et al., 2015b)) in rodents, most varying in species, route of administration, and duration, making it challenging to compare outcomes across studies (see Table 1 for summary of results). Regardless, some intergenerational effects of paternal ethanol exposure have been consistent such as low birth weight (Ledig et al., 1998; Bielawski et al., 2002), altered organ weights (Abel, 1993b; Ledig et al., 1998; Lee et al., 2013; Chang et al., 2017), and increased number of runts (Bielawski and Abel, 1997; Bielawski et al., 2002). Furthermore, several studies have reported behavioral alterations including reduced spatiotemporal learning (Wozniak et al., 1991), increased anxiety- and impulsivity-like phenotypes (Kim et al., 2014; Liang et al., 2014), and increased sensitive to amphetamine-induced hyperlocomotion (Abel, 1993a). Thus, these preclinical studies confirm that paternal ethanol exposure has a causal effect on complex and varied behavioral phenotypes in offspring.

Table 1: Intergenerational effects of paternal ethanol exposure

(updated from Finegersh, Rompala, et. al, 2015)

REFERENCE	SPECIES	ROUTE	DURATION	PRIMARY FINDINGS IN OFFSPRING
Offspring weight and development				
(Anderson et al., 1981)	SW Mice	LD	4 weeks	↓ birth weight
(Mankes et al., 1982)	LE Rats	DW	8.5 weeks	↑ malformations, ↓ litter weight
(Randall et al., 1982)	C3H Mice	LD	4 weeks	No change in fetal weight
(Leichter, 1986)	Rats	LD	6 weeks	No change in fetal weight

(Abel and Moore, 1987)	SW Mice	LD	6 weeks	No change in fetal weight, mortality
(Abel and Tan, 1988)	SD Rats	LD	7.5 weeks	No change in birth or adult weight
(Abel, 1989b)	SW Mice	LD	7 weeks	No change in birth or adult weight
(Abel, 1989c)	LE Rats	LD	9 weeks	No change in fetal weight
(Abel, 1993b)	SD Rats	Gavage	9 weeks	↑ runts; no change in birth weight
(Abel, 1995)	SD Rats	Gavage	9 weeks	↑ fetal weight; no change in birth weight
(Bielawski and Abel, 1997)	SD Rats	Gavage	16 hours	↑ runts and malformations
(Ledig et al., 1998)	IW Rats	DW	13 weeks	↓ birth and adult weight in males
(Bielawski et al., 2002)	SD Rats	Gavage	9 weeks	↑ runts, ↓ fetal weight
(Knezovich and Ramsay, 2012)	C57 Mice	Gavage	5 weeks	↓ postnatal growth at day 35
(Lee et al., 2013)	CD1 Mice	Gavage	7 weeks	↑ fetal malformations
(Kim et al., 2014)	CD1 Mice	Gavage	7 weeks	No change in body weight
(Finegersh and Homanics, 2014)	C57 Mice	Vapor	5 weeks	↑ weight after weaning in males
(Chang et al., 2017)	C57 Mice	DID	10 weeks	↓ fetal weight, ↓ placental efficiency,
Learning and activity				
(Abel and Tan, 1988)	SD Rats	LD	7.5 weeks	↓ activity, ↓ learning in females
(Abel, 1989b)	SW Mice	LD	7 weeks	↓ activity prior to weaning
(Abel, 1989c)	LE/SD Rats	LD	4 weeks	Strain-dependent ↓ activity
(Abel, 1989a)	LE Rats	LD	9 weeks	↓ activity
(Wozniak et al., 1991)	SD Rats	LD	5.5 weeks	↓ learning in males
(Abel, 1993a)	SD Rats	Gavage	13 weeks	↑ amphetamine-induced activity
(Ledig et al., 1998)	IW Rats	DW	13 weeks	↑ activity and novelty seeking

(Kim et al., 2014)	CD1 Mice	Gavage	7 weeks	↑ activity and impulsivity
(Finegersh and Homanics, 2014)	129xB6 Mice	Vapor	5 weeks	No change in open field activity
Anxiety-related behaviors				
(Abel and Bilitzke, 1990)	Mice/Rats	LD	14 weeks	Species-dependent FST immobility
(Abel, 1991b)	SD Rats	LD	5.5 weeks	↓ grooming
(Abel, 1991a)	SD Rats	LD	30 weeks	↓ immobility on FST
(Ledig et al., 1998)	IW Rats	DW	13 weeks	↑ light-dark transitions
(Meek et al., 2007)	SW Mice	IP inj.	12 hours	↑ aggression and ↓ fear
(Liang et al., 2014)	KM Mice	Gavage	4 weeks	↑ anxiety-like behaviors
(Finegersh and Homanics, 2014)	129xB6 Mice	Vapor	5 weeks	No changes on basal anxiety tests
(Rompala et al., 2017) See Appendix A	B6 mice	Vapor	6 weeks	No changes on basal anxiety tests
Molecular and physiologic effects				
(Abel and Lee, 1988)	SW Mice	LD	7.5 weeks	↓ serum testosterone
(Nelson et al., 1988)	SD rats	Vapor	6 weeks	Altered neurotransmitter levels
(Berk et al., 1989)	SW Mice	LD	7 weeks	↑ ocular infections
(Hazlett et al., 1989)	SD Rats	LD	3 weeks	↑ severity of ocular infections
(Cicero et al., 1990)	SD Rats	LD	5.5 weeks	↓ sexual maturation in males
(Abel, 1993b)	SD Rats	Gavage	9 weeks	↑ adrenal and ↓ spleen weights
(Ledig et al., 1998)	IW Rats	DW	13 weeks	↓ glial enolase, SOD, GS
(Jamerson et al., 2004)	SD Rats	DW	7 weeks	↑ CCx thickness
(Knezovich and Ramsay, 2012)	C57 Mice	Gavage	5 weeks	↓ DNA methylation imprinting

(Liang et al., 2014)	KM Mice	Gavage	4 weeks	Altered imprinted gene expression
(Kim et al., 2014)	CD1 Mice	Gavage	7 weeks	↓ DAT, DNMT1, MeCP2 expression
(Finegersh and Homanics, 2014)	129×B6 Mice	Vapor	5 weeks	↑ BDNF, ↓ methylation in males
Drinking and alcohol-induced behaviors				
(Finegersh and Homanics, 2014)	129×B6 Mice	Vapor	5 weeks	↓ ethanol preference on 2BC, ↑ ethanol-induced anxiolysis on EPM in males
(Rompala et al., 2017) See Appendix A	B6 Mice	Vapor	6 weeks	↓ ethanol preference on 2BC, ↑ ethanol-induced anxiolysis on EPM in males
(Ceccanti et al., 2016)	CD1 Mice	LD	9 weeks	(dose-dependent) ↓↑, conditioned place preference for ethanol

Strains: LE = Long Evans; SW = Swiss Webster; SD = Sprague Dawley; IW = Italian Webster; KM = Kunming. Route of ethanol administration: LD = Liquid Diet; DW = Drinking Water; IP inj. = Intraperitoneal Injection. Other Abbreviations: FST = Forced Swim Test; EPM = Elevated Plus Maze; CCx = Cerebral Cortex; DAT = Dopamine Transporter; SOD = Superoxide Dismutase; GS = Glutamine Synthetase; DAT= Dopamine transporter; HPA = Hypothalamic-pituitary-adrenal; 2BC = 2-bottle choice; DID = Drinking in the dark.

In 2014, Finegersh and Homanics expanded the scope of published paternal preconception ethanol studies in rodents by examining a battery of ethanol-related behaviors in the next generation. They found that C57BL/6J (B6) adult male mice exposed intermittently to ethanol vapor over five weeks (average blood ethanol concentration ~160 mg/dL following each 8 hour exposure) sired hybrid B6 × Strain 129 male offspring with increased sensitivity to the anxiolytic effects of a low dose ethanol injection (1.0 g/kg) and decreased ethanol drinking preference (Finegersh and

Homanics, 2014). Since that original study, Rompala et al. replicated those key findings with mice on a pure B6 background (Rompala et al., 2017). Moreover, using a chronic ethanol liquid diet exposure, another group recently reported an effect of paternal ethanol exposure on conditioned place preference for ethanol in male offspring (Ceccanti et al., 2016). Taken together, these studies strongly suggest that paternal preconception ethanol exposure is a heritable factor capable of driving ethanol-related phenotypes in the next generation. Further investigation of the mechanisms driving intergenerational ethanol-related behaviors has significant implications for improving the presently limited understanding of AUD heritability. The next section of this introduction will review how both chronic ethanol exposure and ethanol drinking phenotypes are inextricable from neuroendocrine stress physiology.

1.2 CONVERGING MECHANISMS OF ALCOHOL AND STRESS

1.2.1 Alcohol impairs the hypothalamic-pituitary-adrenal (HPA) stress axis

Stress can be broadly defined as a disturbance in physiological homeostasis. The hypothalamic-pituitary-adrenal (HPA) axis is a complex neuroendocrine system in mammals that regulates the stress response to physiological or psychogenic (i.e. real or anticipated) challenge. The canonical HPA axis (see (Herman et al., 2016) for full review) begins with the parvocellular neurons of the paraventricular nucleus of the hypothalamus (PVN) which express the neuropeptide corticotropin-releasing factor (CRF). These PVN neurons send terminals to the median eminence to release CRF into the hypothalamic-pituitary portal vessels to bind with G protein-coupled CRF1 receptors on corticotropic cells in the anterior pituitary gland. CRF1 receptor binding stimulates the release of

adrenocorticotropic hormone (ACTH)-containing vesicles into systemic circulation and increases transcription of the ACTH-precursor, proopiomelanocortin (POMC). ACTH binds to melanocortin 2 receptors on zona fasciculata cells of the adrenal cortex to stimulate cholesterol production and synthesis into the glucocorticoid corticosterone (cortisol in humans). Corticosterone (CORT) is immediately released into circulation to act throughout the central and peripheral nervous systems. In addition to stimulating glucose production and suppressing immune function, glucocorticoid receptor (GR) activation completes a negative feedback loop by suppressing CRF-positive neurons of the PVN through limbic pathways such as the bed nucleus of the stria terminalis (BNST) routing axon terminals from the hippocampus and amygdala (Herman et al., 2012). Importantly, HPA axis stress responsivity is significantly regulated by several neurotransmitter systems including catecholamines, GABA, and various neuropeptides (Herman et al., 2016).

Clearly interpreting the acute effects of ethanol intoxication on the HPA axis has been challenging due to variability across studies and experimental conditions. Generally, higher doses of ethanol (i.e., those inducing blood ethanol concentrations (BEC) > 0.08 g/dL) are associated with increased ACTH and cortisol levels in blood (Mendelson and Stein, 1966; Valimaki et al., 1984; Schuckit et al., 1987). Conversely, lower ethanol doses (BEC < 0.06 g/dL) produce no change or reduce ACTH or cortisol levels (Waltman et al., 1993; Mick et al., 2013) and have also been found to inhibit HPA responsivity to pharmacological activation (Waltman et al., 1993) or social stress (Dai et al., 2002).

In rodents, ethanol is more consistently found to dose-responsively activate the HPA axis across all examined routes of administration (Rivier, 2014), including voluntary consumption (Richardson et al., 2008). The ability of ethanol to induce HPA activation is dependent on PVN-CRF signaling as ethanol induces CRF secretion from primary hypothalamic cells *in vitro (Li et al.,*

2005) and CRF antagonism blocks ethanol-induced ACTH and corticosterone release *in vivo* (Rivier, 1999). Furthermore, ethanol fails to induce ACTH release when applied directly to anterior pituitary cells *in vitro* (Rivier et al., 1984) and intracerebroventricular ethanol -- thereby, not directly accessing the pituitary -- was still sufficient to induce ACTH release (Lee et al., 2004).

The repeated binge intoxication and withdrawal cycles that typify the progression from alcohol abuse to dependence is frequently associated with HPA axis dysregulation (Stephens and Wand, 2012). This presents clinically as a sustained increase in tonic cortisol levels and blunted HPA axis responsivity to ethanol in alcohol dependent individuals (Sinha et al., 2009). After a spike in cortisol levels during acute withdrawal, HPA activity becomes suppressed relative to social drinkers during early abstinence from chronic ethanol abuse (Wand and Dobs, 1991; Esel et al., 2001). While basal cortisol levels normalize more quickly, blunted HPA responsivity to psychosocial or pharmacological stress has been observed months into withdrawal (Bernardy et al., 1996; Adinoff et al., 2005). Similar effects on the HPA axis have been reported in rodents undergoing repeated ethanol vapor exposure (Rivier et al., 1984; Allen et al., 2016) as well as prolonged ethanol self-administration (Rasmussen et al., 2000; Zorrilla et al., 2001; Richardson et al., 2008).

The severity of HPA axis hyporesponsivity during early abstinence in alcohol dependent individuals is associated with increased craving and predicted likelihood for relapse (Junghanns et al., 2005; Sinha et al., 2011). This supports the hypothesis that cortisol levels are associated with motivation for binge ethanol consumption in alcohol dependent individuals. That is, if cortisol levels are related to the ethanol craving, alcohol dependent individuals may need to drink in excess to achieve the same desired response as a social drinker (Blaine and Sinha, 2017). The next section will explore the role of glucocorticoids and stress in ethanol drinking behavior.

1.2.2 Role of stress and the HPA axis in ethanol drinking behavior

Many epidemiological studies support the "self-medication" or "tension reduction" hypothesis of stress-induced ethanol abuse. This theory posits that ethanol drinking alleviates the negative emotion from past, ongoing, or anticipated stressful life events and reinforces subsequent stress-responsive ethanol drinking (Cappell and Herman, 1972). Indeed, whereas cortisol levels only correlate with the stimulating effect of ethanol drinking when blood ethanol concentrations are rising, the anxiolytic effects of ethanol persist beyond peak blood ethanol concentrations (Cappell and Herman, 1972). However, the relationship between stress and alcohol drinking may be dependent on additional factors such as personality and past drinking experience. For instance, stress was found to account for variance in alcohol drinking habits selectively in men with internalizing traits (Cooper et al., 1992). Additionally, acute social discomfort stress, in a controlled-laboratory setting, increases ethanol drinking in alcohol-dependent individuals (Thomas et al., 2011), but not social drinkers (de Wit et al., 2003).

Rodent studies have similarly varied across experimental paradigms and studies. For instance, the model of ethanol drinking is important; a recent meta-analysis found that, whereas two-bottle free choice ethanol drinking is generally increased by stress, operant conditioning for ethanol self-administration is often unchanged (Noori et al., 2014). The common finding of a delayed-onset to stress-induced escalation of ethanol drinking behavior is hypothesized to underlie this discrepancy (Noori et al., 2014). Moreover, stress-escalated ethanol drinking is more frequently observed in mice with low baseline ethanol consumption (Becker et al., 2011). Conversely, mice with high baseline ethanol drinking preference, such as the C57BL/6J mouse strain, exhibit reduced or unchanged ethanol drinking behavior in response to several different stressors (Lopez et al., 2016). Finally, stress occurring during adolescence versus adulthood is more consistently

associated with increased stress-induced ethanol drinking continuing into adulthood (Chester et al., 2008; Lopez et al., 2011). This difference may be explained by the hyper-responsive state of the HPA axis during adolescence which is attributed to insufficient development of GR negative feedback mechanisms (Klein and Romeo, 2013).

As all stressors found to increase ethanol drinking behavior naturally engage the HPA axis, the primary mechanisms implicated in stress-escalated ethanol drinking are the HPA axis and GR signaling. Accordingly, adrenalectomy in rodents reduces ethanol drinking, an effect which can be rescued with CORT replacement (Fahlke et al., 1994; Fahlke and Eriksson, 2000). Furthermore, prolonged GR inhibition reduces ethanol drinking preference in high-preference animals and blocks acquisition of increased ethanol drinking preference in low-preferring animals (O'Callaghan et al., 2005). Thus, these studies suggest a causal role for glucocorticoids in promoting ethanol drinking behavior.

Corticosterone infusion into whole brain or ventral striatum, but not hippocampus or thalamus, increases ethanol drinking, suggesting a mechanism for glucocorticoids acting directly on brain reward systems (Fahlke et al., 1996; Fahlke and Hansen, 1999). Nearly all drugs of abuse, including ethanol, promote dopaminergic release in the nucleus accumbens (Volkow and Morales, 2015). Intriguingly, ventral striatal dopamine levels are also increased by local CORT infusion (Wheeler et al., 2017) as well as by social defeat stress (Han et al., 2015). Thus, glucocorticoids and stress may sensitize striatal dopaminergic pathways to increase reinforcement of ethanol drinking behavior(Spanagel et al., 2014).

Similar to the tension reduction theory for stress-motivated ethanol drinking, stress from acute ethanol withdrawal increases nausea and anxiety in alcohol dependent individuals that can be rapidly alleviated by ethanol drinking. Thus, ethanol-related stress and alterations in HPA function

also promote increased ethanol consumption. This transition from positive to negative reinforcement-motivated ethanol drinking is considered a hallmark of alcohol dependence (Koob et al., 2014). Although alcohol dependent individuals exhibit HPA axis tolerance, feed-forward mechanisms of GR signaling at extrahypothalamic brain regions (e.g., central amygdala (CeA), BNST) are sensitized by chronic stress (Rosenkranz et al., 2010) and chronic ethanol abuse (Makino et al., 1994; Cook, 2002). Induction of ethanol dependence by chronic vapor ethanol exposure escalates ethanol drinking behavior and increases GR expression and phosphorylation at Ser^{232} (marker of nuclear localization and transactivation) in CeA and BNST (Vendruscolo et al., 2012; Vendruscolo et al., 2015). Systemic or CeA-infusion of the GR antagonist, mifepristone, blocks escalation of ethanol drinking in dependent rats (Vendruscolo et al., 2012; Vendruscolo et al., 2015). This effect of antagonizing enhanced extra-HPA GR signaling is believed to underlie recent successes with mifepristone in treating alcohol dependence (Vendruscolo et al., 2015).

All together, these studies illustrate the convergent relationship between ethanol drinking and stress physiology. Ethanol is a stressor and, in turn, pharmacological or stress-induced HPA activation has causal effects on ethanol drinking behavior. Acute stressors may promote ethanol drinking through the direct effects of glucocorticoids in the brain reward circuitry and repeated stress such as chronic ethanol abuse may promote ethanol drinking via sensitization of the extrahypothalamic stress circuitry. Given the embedded role of stress and glucocorticoids in all aspects of ethanol drinking, many studies have examined how heritable alterations in HPA axis function associate with a family history of alcoholism and risk for alcohol dependence.

1.2.3 Family history of alcoholism and HPA axis function

Given that a family history of AUD is associated with increased risk for AUD (Windle, 1997) (Schuckit, 1985b), mechanisms that are common to this at-risk population have been intensively studied. Many of these studies have focused on HPA axis function. The initial and most well-replicated findings have reported no change in basal ACTH or cortisol levels, but revealed an enhanced cortisol response to the opioid antagonists naloxone and naltrexone in individuals with a family history of alcoholism (FHP) that are not alcohol dependent themselves (Wand et al., 1998; Wand et al., 2001; King et al., 2002). As β-endorphins inhibit hypothalamic CRF activity, this suggests differences in FHP hypothalamic opioid activity that may be unmasked by opioid antagonism (Wand et al., 2001). FHP men also exhibit increased cortisol in response to social stress (Zimmermann et al., 2004; Uhart et al., 2006). Interestingly, FHP subjects showed greater dampening of stress-induced ACTH and cortisol levels by mild ethanol intoxication (Zimmermann et al., 2004). Accordingly, FHP subjects report greater stress-related ethanol craving (Soderpalm Gordh and Soderpalm, 2011). Thus, FHP individuals show greater pharmacological and social stress sensitivity with a greater suppression of stress-induced HPA activity by ethanol.

In the absence of stress, mild intoxication dampened ACTH and cortisol levels in family history negative (FHN) subjects, but not FHP individuals (Mick et al., 2013). Furthermore, sons of alcoholics exhibit a blunted HPA axis response to heavy ethanol intoxication (Schuckit et al., 1987). Reduced basal ACTH (Dai et al., 2002) and cortisol (Schuckit et al., 1996) have also been documented in FHP individuals. Thus, there is a complex relationship between a family history of alcoholism and HPA axis function depending on stress state (basal or induced), treatment (pharmacological, stress, ethanol) and treatment dosage (e.g., ethanol intoxication).

Heritability accounts for ~62% of the etiological variance in basal cortisol levels (Bartels et al., 2003). Moreover, several polymorphisms have been identified in HPA regulatory genes such as *Nr3c1* and *Fkbp5*, which, respectively, encode GR and the GR co-chaperone FKBP5 that regulates GR affinity for cortisol (Stephens and Wand, 2012).

To date, no variants on HPA-regulatory genes have been directly linked to a family history of alcoholism. While this may be partially explained by the challenges to estimating heritable disease risk with GWAS (see Section 1.1.1), intriguingly, several recent cross-generational studies implicate parental preconception environment as a casual mechanism capable of driving HPA axis responsivity.

1.2.4 Effect of paternal experience on offspring stress responsivity

Various paternal preconception stress exposures impact HPA axis function and stress-related phenotypes in adult offspring. Adult male mice with social defeat-induced impairments in social behavior were found to sire male offspring with increased basal CORT levels and similar social behavioral deficits (Dietz et al., 2011). Using a comparatively less severe chronic variable stress paradigm, another group found that sires stressed during either adolescent or adulthood both conferred blunted HPA axis responsivity to acute restraint stress in male and female offspring (Rodgers et al., 2013). Finally, early-life maternal separation stress imparted increased active coping behaviors and increased hippocampal GR expression and methylation to male offspring (Gapp et al., 2016). These studies demonstrate that multiple paternal preconception stress exposures affect HPA axis responsivity in offspring, although the direction of the effects and associated behavioral alterations vary between paternal stress paradigms.

In addition to intergenerational effects of stress, some studies have discovered transgenerational germline inheritance of HPA axis function initiated by gestational exposure and transmitted through the male germline. For instance, a single exposure of gestating females to the fungicide vinclozolin increases stress responsivity in the F3 generation (Crews et al., 2012). Moreover, mothers treated with ethanol during gestation impart enhanced HPA axis responsivity to immune challenge to the F1 through F3 generations via the male germline (Govorko et al., 2012). Thus, not only parental preconception experience, but ancestral environmental exposures influence HPA axis function in rodents.

Finegersh and Homanics previously reported effects of paternal preconception ethanol on the ethanol drinking phenotype in male offspring (Finegersh and Homanics, 2014). The intergenerational effects of stress on HPA function raise two important possibilities that warrant direct investigation. First, as HPA function is sensitive to paternal preconception stressors, I propose that paternal preconception ethanol exposure alters HPA axis responsivity. Second, given that the paternal preconception ethanol vapor exposure reported to impart intergenerational ethanol-related behavior (Finegersh and Homanics, 2014) is a potent physiological stressor (Rivier, 2014), I propose that chronic variable stress is sufficient to similarly alter ethanol-related behavior in offspring. Thus, I propose that ethanol and stress have convergent intergenerational effects on HPA axis function and ethanol drinking behavior.

1.3 GERMLINE MECHANISMS OF EPIGENETIC INHERITANCE

Epigenetics is broadly defined as the study of molecular factors driving stable states in gene expression without changing the nucleotide sequence. Among the most well-understood epigenetic

mechanisms are cytosine methylation, histone post-translational modifications, and noncoding RNAs. Methylation at cytosine preceding guanine (CpG) dinucleotides within gene promoters is associated with transcriptional silencing by recruiting heterochromatic proteins and impeding transcription factor binding (Illingworth et al., 2008). Histone tail modifications regulate the affinity of positively-charged histone proteins for the phosphate-rich DNA, influencing gene expression by determining the "open" or "closed" state of the chromatin (Smith and Shilatifard, 2010). Finally, small and long noncoding RNAs function to regulating diverse transcriptional and translational processes, often targeting messenger RNAs with sequence homology (Cech and Steitz, 2014). Collectively, epigenetic mechanisms are the primary regulators of transcription and underlie the cellular diversity able to emerge from a single genome. This section will focus primarily on the unique epigenomic state of the male germline. Since DNA methylation and histone modifications are mitotically and meiotically heritable (Reik, 2007), and sperm RNA is delivered to the oocyte (Ostermeier et al., 2004), there has been intense interest in identifying "epialleles" or RNAs being passed through the germline to determine cross-generational gene expression.

1.3.1 Epigenetic mechanisms in sperm

Given the specialized function of the male germline in fertilization, sperm feature a unique nuclear structure. Most histones are replaced in the early stages of spermatogenesis with highly basic protamines that robustly neutralize the phosphodiesterase bond-rich DNA. The nucleus is further compressed by inter- and intramolecular disulfide-bonds, contributing to a condensed state at approximately 1/13 the volume of the oocyte nucleus (Martins and Krawetz, 2007). In this heavily neutralized state, and lacking major ribosomal machinery, mature sperm cells are transcriptionally quiescent (Kierszenbaum and Tres, 1975). Upon fertilization, most of the sperm genome is stripped

of DNA methylation and the epigenetic landscape is largely reset to facilitate pluripotency in early embryo development (Feng et al., 2010). Thus, until recently, the male gametes were presumed to deliver little to no epigenetic memory to the fertilized oocyte. However, with the advent of next generation sequencing methods at single nucleotide resolution, studies are beginning to characterize a unique, environmentally-responsive, and functional epigenetic landscape in sperm.

Not all genomic loci are stripped of DNA methylation at fertilization. For instance, large regions around intracisternal A particle (IAP) retrotransposons (Popp et al., 2010) and imprinting regions (Feng et al., 2010) are protected from global epigenetic reprogramming during embryogenesis. Additionally, a small percentage (1% in mouse, 10% in humans) of histones in sperm are retained in sperm (Bogliotti and Ross, 2012). Retained histones are concentrated at promoter regions of key developmental regulator genes, thereby well-positioned to influence zygotic gene expression (Hammoud et al., 2009; Brykczynska et al., 2010). Thus, sperm chromatin organization, maintenance, and function at fertilization remains an area of intense research.

In addition to chromatin, sperm contain RNA, though ~1% of that carried by somatic cells (Zhang et al., 2017). Due to the arrested transcriptional state of sperm, it was long assumed that this scant amount of RNA in sperm was purely comprised of degraded transcripts from the earlier transcriptionally-active stages of spermatogenesis in the testis. However, the discovery that sperm RNAs are delivered to the oocyte at fertilization raised the possibility that they could be functional epigenetic molecules in the early embryo (Ostermeier et al., 2004). Supporting this notion, subsequent deep sequencing studies over the last decade have helped reveal and characterize a diverse and unique population of noncoding RNA species in sperm. The majority of small noncoding RNAs (~18-45 nucleotides) represented in sperm are transfer RNA-derived small RNAs (tDR) and mitochondrial small RNAs (mitosRNAs) (Peng et al., 2012; Schuster et al., 2016b), while

microRNA (miRNA) and piwi-interacting (piRNA) species are expressed at much decreased levels (Chen et al., 2016b). The miRNAs are the most well-studied small noncoding RNA with a putative function in mRNA silencing through “seed”-sequence homology (Cai et al., 2009). The function of tDR is not well-understood in sperm though they have been implicated in various biological functions including miRNA-like mRNA silencing and translational inhibition (Kumar et al., 2016). The mitosRNA function is even less well studies, though several species were found to regulate expression of parent mitochondrial genes *in vitro* (Ro et al., 2013). Finally, piRNAs regulate expression of transposable elements during spermatogenesis and are the dominant noncoding RNA species in testis sperm (Ernst et al., 2017). The precise function of these small noncoding RNAs either in mature transcriptionally quiescent sperm, or upon delivery to the oocyte, remains to be determined.

Interestingly, while enriched in mature sperm, tDR are minimally expressed in testis sperm that has not passed through the epididymis (Sharma et al., 2016). Several recent studies in mice support a mechanism of tDR accumulation as sperm migrate from the caput to cauda regions of the epididymis via extracellular vesicles, known as “epididymosomes”, secreted by principle cells lining the epidydimal lumen. As proof of principle, *in vitro* coincubation of caput sperm with epididymosomes enriches the immature sperm cells for specific tDR and miRNA species (Reilly et al., 2016; Sharma et al., 2016). Furthermore, newly-synthesized 4-thiouridine (4-TU) labeled RNAs from the caput epididymis, but not liver or other somatic tissue, were found to accumulate in mature sperm cells *in vivo* (Sharma et al., 2017). Thus, the RNA profile of sperm is dramatically reshaped during epidydimal transit. However, this does not preclude the maintenance of small RNAs such as piRNAs from testis sperm in the mature spermatozoa. In fact, specific piRNAs were found to accumulate in cauda sperm relative to caput sperm, despite piRNA being completely absent in

epididymosomes. Thus, alternative biogenesis pathways for some noncoding RNAs in sperm such as *de novo* processing of long noncoding RNA species may come to light as this field progresses (Hutcheon et al., 2017).

1.3.2 Effects of environment insults on epigenetic mechanisms in sperm

The first example of a transgenerational phenotype associated with environmentally-induced epigenetic mechanisms in sperm showed that transient exposure of pregnant rats to endocrine disruptors (e.g., vinclozolin) imparts reduced fertility to males across multiple generations through the male germline (Anway et al., 2005; Nilsson and Skinner, 2015). This phenomenon was associated with altered DNA methylation patterns in sperm (Anway et al., 2005; Nilsson and Skinner, 2015). Additional studies have found that the male germline is sensitive to environmentally-induced changes in DNA methylation well beyond prenatal development. For instance, adult-onset prediabetic conditions (Wei et al., 2014), obesity (Fullston et al., 2013), and cocaine-seeking motivation (Le et al., 2017) all modify the DNA methylome in sperm.

Many studies have also reported loci-specific epigenetic alterations in sperm. Chronic ethanol exposure reduces DNA methylation at imprinting regions that may be more likely to escape epigenetic reprogramming (Knezovich and Ramsay, 2012; Finegersh and Homanics, 2014; Liang et al., 2014). In addition, low protein diet and hepatoxin-exposure induced altered levels of the repressive histone modification H3K27m3 in sperm at promoter regions for metabolic- or fibrosis-related genes, respectively (Carone et al., 2010; Zeybel et al., 2012). Other studies have found loci-specific epigenetic effects in sperm associated with gene expression in offspring, suggesting cross-generational epigenetic memory. Chronic cocaine self-administration resulted in increased H3 acetylation at brain-derived neurotropic factor (BDNF) promoter regions in sperm which was

associated with increased BDNF gene expression and a BDNF-dependent reduction in cocaine-seeking behavior in male offspring (Vassoler et al., 2013). In addition, olfactory fear conditioning to acetophenone induced hypomethylation of the odor receptor for acetophenone (Olfr151) in sperm and increased Olfr151-expressing olfactory sensory neurons in offspring brain (Dias and Ressler, 2014).

Finally, microarray and sequencing experiments have confirmed that various paternal preconception environmental exposures directly affect the small noncoding RNA milieu in sperm. Initial studies found that high fat diet, chronic stress, and early life stress impact the miRNA expression patterns in mouse sperm (Fullston et al., 2013; Rodgers et al., 2013; Gapp et al., 2014). Since then, RNA sequencing experiments have revealed effects of paternal high fat diet, low protein diet, and exercise on sperm tDR (Chen et al., 2016a; Sharma et al., 2016; Short et al., 2017). More recent studies have developed novel sequencing techniques for profiling long noncoding RNA in sperm which were affected by diabetic conditions in mice (Jiang et al., 2016; Zhang et al., 2017). Moreover, there is increasing interest in post-transcriptional RNA modifications (i.e., the epitranscriptome) as some of these modifications on sperm tDR (e.g. 5-methylcytidine, N7-methylguanosine) were found to be sensitive to paternal diet (Chen et al., 2016a). Absence of RNA modifications leads to rapid degradation of tDR oligos in fertilized oocytes, suggesting an essential role in RNA-mediated inheritance (Chen et al., 2016a). It is likely that many more novel epigenetic effects of environmental exposure will be revealed as the mammalian sperm epigenome is further characterized.

1.3.3 Causal role for sperm epigenetics in nongenomic inheritance

The first studies to implicate epigenetic mechanisms in the germline with inherited phenotypes in offspring were carried out by Emma Whitelaw and colleagues. Female mice with the A^{vy} allele of the *agouti* gene have an IAP inserted in the reverse orientation upstream of the transcriptional start site that acts as a pseudo-promoter. The degree of cytosine methylation at the IAP promoter determined agouti gene expression and coat color (Morgan et al., 1999). In particular, females with reduced methylation of the IAP promoter and more ectopic agouti gene expression were more likely to confer this euchromatic state of the IAP promoter and yellow coat color to offspring of the same genotype; this was inconsistent with inheritance rates predicted by Mendelian inheritance. Shortly after this original study, Whitelaw and colleagues discovered a similar paramutation associated with heritable methylation of an IAP upstream of the affected gene through both the maternal and paternal germline (Rakyan et al., 2003b). While this is compelling evidence for intergenerational inheritance that is dependent on DNA methylation in sperm, a complete causal examination of loci-specific epigenetic marks has been delayed by technological limitations, although such tools are rapidly developing (Heller et al., 2014).

It is also worth considering that while the *agouti* and *axin* examples suggest a causal role for DNA methylation in germline epigenetic inheritance, the potential for environmentally-induced changes in sperm DNA methylation to impact offspring phenotype have been strongly contested. Most studies examining DNA methylation in sperm report changes of ~10-20% in methylation at individual CpGs (Shea et al., 2015). Consequently if, for example, CpG methylation changes from 20 to 40%, methylation only increases from 1 of 5 sperm cells to 2 out of 5. This casts doubt over small changes in sperm DNA methylation to causally impact offspring phenotype with the sample sizes employed by most cross-generational inheritance studies to date (Shea et al., 2015). In

addition, many of the reported effects of environmental perturbations on sperm DNA methylation are not at genomic loci recognized to escape epigenetic reprogramming at fertilization (Shea et al., 2015). Consistently, environmentally-induced changes in DNA methylation at specific CpGs in sperm are rarely reproduced in the somatic tissue in offspring (Sharma and Rando, 2017).

In contrast to DNA methylation or histone modifications, techniques have been developed to directly test the role of sperm RNA in established animal models of intergenerational inheritance of preconception environment. Using IVF-produced embryos, sperm RNAs can be directly injected into the fertilized oocyte to examine resultant progeny and determine if sperm RNA is sufficient to recapitulate the cross-generational phenotypes in question. The pioneering work employing this methodology discovered that male mice heterozygosis for the *Kit* gene with a lacZ insertion on the inactive allele, conferred a white-tail phenotype to most wild type offspring (Rassoulzadegan et al., 2006). The lacZ insertion caused excessive fragmentation of *Kit* RNAs in somatic and developing germ cells that were maintained in mature sperm. Remarkably, by injecting RNA isolated from sperm or brain tissue of *Kit* heterozygotes into one cell embryos, the white tail phenotype was transmitted to near 50% of IVF-derived mice (Rassoulzadegan et al., 2006). Moreover, injecting two miRNAs that target the *Kit* gene into one cell embryos produced similar effects suggesting a miRNA-like role for *Kit* fragments and a plausible mechanism for sperm small noncoding RNAs in nongenomic inheritance (Rassoulzadegan et al., 2006). This study was soon followed by two more examples of RNA-induced cardiac and growth phenotypes in IVF mice by one cell embryo injections of miRNAs targeting the *Cdk9* and *Sox9* genes, respectively (Wagner et al., 2008; Grandjean et al., 2009). Collectively, this evidence of RNA-mediated inheritance supports a functional role for sperm-derived RNA in the early embryo.

In 2014, Isabella Mansuy and colleagues expanded on this work with the first evidence for RNA-mediated inheritance of paternal preconception experience in mice. Her group previously reported that male mice exposed to postnatal maternal separation stress conferred reduced anxiety- and increased depression-like behaviors to male offspring (Franklin et al., 2010). This early-life stress affected several miRNA in adult sperm and the intergenerational effects of maternal separation could be partially recapitulated in mice derived from one cell embryos injected with total sperm RNA from stressed males (Gapp et al., 2014). Subsequently, Tracey Bale and colleagues discovered that the intergenerational effect of paternal chronic stress on stress responsivity in offspring could be recapitulated in mice derived from embryos injected with synthetic oligos for nine stress-enriched sperm miRNAs (Rodgers et al., 2015). Another study examining the intergenerational effects of paternal high fat diet found that sperm tDR, but not miRNA or all sperm RNA > 40 nt could reproduce the diet-induced intergenerational effects on glucose tolerance in IVF-derived mice (Chen et al., 2016a). Finally, one low protein diet-enriched sperm tDR was sufficient and necessary for the intergenerational effects of high fat diet on embryonic gene expression (Sharma et al., 2016).

Indeed, treatment of one cell embryos with exogenous RNA is unlikely to completely reflect the endogenous RNA payload or match the temporal dynamics of endogenous sperm RNA function in the oocyte. However, these RNA-mediated inheritance studies causally implicate sperm noncoding RNAs in the intergenerational effects of paternal environment. Therefore, I propose that chronic ethanol impacts small noncoding RNAs in sperm and that this mechanism may contribute to the intergenerational effects of preconception ethanol exposure. In addition, as most sperm small noncoding RNAs are derived during epididymal transit, I hypothesize that chronic ethanol alters epididymosome trafficking to sperm to impart intergenerational phenotypes.

1.4 HYPOTHESIS AND SPECIFIC AIMS

While alcoholism has greater than 50% heritability, studies have had limited success identifying putative genetic risk factors that reliably associate with harmful drinking behavior and alcohol (ethanol) dependence. With ~13% of the US adult population afflicted by alcoholism (Haberstick et al., 2014), and ethanol abuse rendering ~$235 billion in societal costs annually (Rehm et al., 2009), novel research strategies are urgently needed to elucidate this "missing" genetic heritability. As an adjunct to traditional Mendelian genetics, epigenetic inheritance is re-emerging as a plausible mechanism for transmission of molecular and behavioral traits across generations. Multiple studies have demonstrated that paternal experiences, such as chronic stress or substance abuse, directly impact offspring behavior (Vassoler and Sadri-Vakili, 2014; Chan et al., 2017c). Previously, my colleague Dr. Andrey Finegersh and thesis mentor Dr. Gregg Homanics discovered that paternal preconception chronic ethanol exposure shapes ethanol-related behaviors in male offspring (Finegersh and Homanics, 2014). The experiments in this dissertation expand on their published work, examining the role of stress in intergenerational ethanol-related behaviors and the causal epigenetic mechanisms being transmitted through the male germline.

Specifically, I test the hypothesis that paternal chronic ethanol and chronic stress exposures impart similar ethanol-related and stress-related behaviors to male offspring via soma-to-germline epigenetic mechanisms.

Specific Aim 1 tests the hypothesis that paternal preconception chronic ethanol exposure mitigates stress-related phenotypes in male offspring.

Specific Aim 2 tests the hypothesis that paternal chronic variable stress reduces ethanol drinking behavior and increases ethanol sensitivity in male offspring.

Specific Aim 3 will test the hypothesis that chronic ethanol exposure reshapes the small noncoding RNA profile in sperm and imparts ethanol- and stress-related phenotypes to male offspring through exosomal trafficking to sperm.

2.0 PATERNAL ETHANOL IMPARTS STRESS HYPORESPONSIVITY

Adapted from: Rompala, G.R., Finegersh, A., and Homanics, G.E. (2016). *Paternal preconception ethanol exposure blunts hypothalamic-pituitary-adrenal axis responsivity and stress-induced excessive fluid intake in male mice*. Alcohol 53, 19-25. doi: 10.1016/j.alcohol.2016.03.006.

2.1 INTRODUCTION

Epigenetic inheritance has been gaining acceptance as a plausible explanation for transmission of complex behavioral traits across generations (Bohacek and Mansuy, 2013; Vassoler and Sadri-Vakili, 2014). Several studies have shown that paternal preconception exposures to stress (Dietz et al., 2011; Rodgers et al., 2013; Gapp et al., 2014) or addictive substances (Vassoler et al., 2013; Dai et al., 2017) can impart adaptive behavioral phenotypes to offspring. Similarly, various chronic paternal ethanol exposures induce intergenerational phenotypes (see (Finegersh et al., 2015b) for review). Recently, Finegersh and Homanics reported that exposing adult male mice to vapor ethanol over five weeks prior to mating with ethanol-naïve females conferred attenuated two-bottle choice ethanol-drinking behavior and increased ethanol-induced anxiolysis selectively in male offspring (Finegersh and Homanics, 2014). The mechanisms underlying these effects of paternal ethanol on intergenerational ethanol-related behaviors are unknown.

Ethanol acutely engages the hypothalamic-pituitary-adrenal (HPA) axis (Rivier, 2014) and the transition to ethanol dependence is characterized by sustained HPA axis tolerance to ethanol and other stressors (Stephens and Wand, 2012). Interestingly, non-ethanol dependent individuals with a family history of alcoholism have aberrant HPA axis responsivity to pharmacological stimulation, acute stress, and ethanol exposures (Schuckit et al., 1988; Wand et al., 2001; Dai et al.,

2002; Sorocco and Ferrell, 2006; Evans et al., 2012). While maternal ethanol exposure during gestation or prior to conception impacts HPA axis responsivity in offspring (Govorko et al., 2012; Jabbar et al., 2016), it is not known whether paternal preconception ethanol exposure impacts stress responsivity in the next generation. Notably, paternal chronic stress exposure reduces HPA axis responsivity in offspring (Rodgers et al., 2013).

The experiments in Chapter 2 test the hypothesis that paternal ethanol exposure blunts HPA axis responsivity to acute stress and alters stress-induced ethanol-drinking behaviors. The results suggest that paternal ethanol exposure prior to conception may have an underappreciated impact on stress responsivity in the next generation. As stress is a major risk factor for excessive and problematic ethanol drinking (Becker et al., 2011; Koob et al., 2014), these results have significant implications for intergenerational ethanol-drinking behavior.

2.2 MATERIALS AND METHODS

2.2.1 Animals

All experiments were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh and were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Eight-week-old, ethanol-naïve, C57BL/6J (B6) and Strain 129S1/SvImJ (Strain 129) mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and CD-1 mice were purchased from Charles River Laboratories (Burlington, MA). Unless otherwise specified, specific pathogen-free mice were group-housed in individually

ventilated micro-isolater cages under 12-h light/dark cycles (0700-1900) and had *ad libitum* access to food (irradiated 5P76 ProLab IsoPro RMH 3000, [LabDiet, St. Louis, MO]) and water.

2.2.2 Chronic intermittent ethanol vapor inhalation (CIE)

The methods for chronic intermittent ethanol vapor inhalation (CIE) were adopted from previously published work (Finegersh and Homanics, 2014). Briefly, group-housed eight-week-old, B6 male mice were exposed to ethanol vapor (E) or room air control conditions (C) for 8 hours/day (0900 to 1700), 5 days/week (M-F) for 5 weeks. Notably, this method has been optimized to induce stable blood ethanol concentrations (BECs) in mice without the use of an alcohol dehydrogenase inhibitor. Sires were weighed at the beginning of each week and BECs were measured following the final exposure of each week. To measure BECs, <10 µL tail vein blood was collected using heparin-coated capillary tubes (Drummond, Broomall, PA) and centrifuged at 2000 × g for 5 min to separate plasma. Ethanol content was measured using an Analox Ethanol Analyzer (AM1, Analox Instruments, London, UK) according to the manufacturer's protocol.

2.2.3 Breeding scheme and offspring rearing

Immediately after the final day of CIE, each E- and C-exposed male mouse was mated in the home cage of two 8-week-old Strain 129 ethanol-naïve female mice for 48 hours. Breeding was limited to 48 hours to minimize the influence of paternal ethanol exposure on maternal care. Strain 129 mice were chosen for mothers in accordance with published methods utilizing the same paternal CIE paradigm (Finegersh and Homanics, 2014).

2.2.4 Acute restraint stress and measurement of corticosterone

Twelve-week-old male and female E- and C-sired offspring were subjected to a 15-min restraint stress exposure. All animals were tested between 10:00-13:00 of the light cycle. Briefly, mice were removed from group-housing and restrained in 50 ml conical plastic tubes (Cat #:525-0158, VWR, Radnor, PA) with several air hole perforations near the animal's head and an opening for the tail. After the 15-min restraint, each mouse was placed in a single novel cage. Only one mouse was tested per group-housed cage to avoid pre-stressing any test animals. Tail blood (<10 μL) was collected with heparin-coated capillary tubes (Drummond, Broomall, PA) at time points 0, 15, 30, and 90 min from the onset of restraint. After the 90-min blood draw, mice were returned to the home cage. Blood samples were centrifuged for 10 min at 2000 × g to separate plasma for measurement of corticosterone (CORT) with an enzyme immunoassay (Cat #: ADI-900-097; Enzo Life Sciences, Farmingdale, NY). For both males and females, all test mice were derived from 6 E-sired and 6 C-sired litters with no more than two mice selected per litter.

2.2.5 Chronic intermittent and variable stress (CIVS) and two-bottle choice ethanol drinking

Adult eight-week-old male mice were first acclimated to two-bottle choice ethanol drinking. Sipper tubes were designed by fitting ball-bearing sippers (Cat# TD-99; Ancare Corp, Bellmore, NY) into modified 25 ml polystyrene serological pipets (Cat # 357525; Corning Incorporated, Durham, NC) and securing the fit with heat-shrink and parafilm. E- and C-sired male offspring were single-housed and habituated to two sipper tubes filled with water. After one week, one tube was filled with escalating ethanol concentrations of 2 and 4% for 4 days at each concentration, followed by 8%

ethanol for the remainder of testing. Baseline two-bottle choice ethanol drinking continued at 8% for 3 weeks before the onset of stress. Baseline drinking measures used in the study were obtained over the final 8 days preceding stress. Tube position was changed daily to control for side preference.

Following acclimation to two-bottle choice ethanol drinking, mice were exposed to CIVS while daily home cage drinking behavior was continuously monitored. Over the four-week CIVS period, each week began with 3 consecutive days of the same unique stress exposure (described in detail below). Each stress exposure occurred between 1400 and 1700 h during the light cycle. All male mice used in this test were derived from 6 E-sired litters and 6 C-sired litters with no more than two mice selected per litter.

Social defeat stress

Test mice were introduced to the home cage of a ten-month-old outbred CD-1 male aggressor mouse. All aggressors were retired breeders and screened for reliable attack behavior prior to use with test mice as advised in published methods (Golden et al., 2011). Body weights for aggressors were at least 25% greater than those of each test mouse. After the aggressor mouse completed one 3–5 sec attack, the test mouse was isolated in a wire cup within the aggressor cage for another 30 min before being returned to the home cage, where ongoing two-bottle choice ethanol drinking was continued. The social-defeat procedure was repeated for two additional days, each time with new pairings of aggressor and test mice.

Forced Swim Stress

The forced swim stressor was completed in a 12-cm diameter 1000 ml glass cylinder filled with 900 ml of 23º C water. Each mouse was placed in the cylinder for a 5-min period. Following

the test, mice were briefly dried and placed under a heating lamp for 3 min before being returned to their home cages.

Predator Odor Exposure

The predator odor stress test was performed in the home cage within a fume hood with the cage cover removed. Four single-housed mice were tested simultaneously for 15 min with two folded filter papers soaked with 1 mL of fox urine (Tink's Red Fox-P ®, Covington, GA) placed just outside each cage, flanking each side.

Restraint Stress

Restraint stress was conducted as described in section (2.2.4) except the stress lasted for 30 min and no tail blood samples were collected.

2.2.6 Isolation of RNA from paraventricular nucleus of the hypothalamus (PVN) and amygdala and DNA from PVN

Tissue was collected from E- and C-sired male mice at the termination of CIVS and 72 hours following the termination of two-bottle choice ethanol drinking in the home cage. Mice were sacrificed between 1200 and 1600 hr during the light cycle. Brains were dissected and frozen with dried ice before being sectioned with a Microm HM 550 cryostat (Thermo Fisher, Waltham, MA). Using a 1 mm tissue micropunch, 300 micron punches were collected from the paraventricular nucleus of the hypothalamus (PVN) (-0.58 to -1.18 mm relative to bregma) and central amygdala (-0.82 to -1.72 mm relative to bregma) into Trizol LS Reagent (Thermo Fisher) diluted 3:4 in nuclease free water. Tissue punches were then lysed with a douche homogenizer for RNA extraction using phenol-chloroform separation. Samples were further processed with DNase I (Thermo Fisher)

treatment, followed by column-based washes with Zymo RNA Clean and Concentrator (Zymo Research, Irving, CA) and elution into 14 µL nuclease free water.

DNA was recovered from PVN by back extraction from the organic phase of Trizol LS. After removal of the aqueous phase following phenol-chloroform extraction, 50 µl of back extraction buffer (4M Guanidine Thiocyanate, 50 mM NaCi, 1M Tris) was added to the organic phase and samples were mixed on a shaker for 10 min. Samples were then centrifuged at 12,000 × g for 30 min at room temperature and the aqueous phase was collected. Next, 400 µL isopropanol was mixed with the aqueous phase and incubated for 5 min at room temperature. Samples were the centrifuged at 12,000 × g for 15 min at 4 ºC. Supernatant was removed and the DNA pellet was washed once with 500 µL of 70% ethanol and re-pelleted at 12,000 × g for 15 min at 4 ºC, dissolved in 50 µL and stored at -80 ºC.

2.2.7 Reverse transcription quantitative PCR (RT-qPCR)

Reverse transcription of total RNA was performed with iScript cDNA synthesis kit (BioRad, Hercules, CA). The cDNA product was diluted 1:10 and qPCR was performed using SYBR green fluorescent master mix (BioRad) on an iCycler [15 sec at 95 ºC, 30 sec at 59 ºC, 30 sec at 72 ºC; 40 cycles] real-time PCR detection system (BioRad). All oligos used for RT-qPCR are listed in Table 1. Specificity of all qPCR primers was verified with melt curve analysis and gel electrophoresis. Fold change in gene expression was computed by normalizing cycle threshold (Ct) values within sample to β-actin Ct values and then between groups using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008).

Table 2. List of all oligos used for RT-qPCR experiments in Chapter 2

SYBR Green qPCR primers	Oligo sequence
NR3C1 forward	5'-AGC TCC CCC TGG TAG AGA C-3'
NR3C1 reverse	5'-GGT GAA GAC GCA GAA ACC TT-3'
CRF forward	5'-CCG GGC AGA GCA GTT AGC-3'
CRF reverse	5'-CAA CAT TTC ATT TCC CGA TAA TCT C-3'
FKBP5 forward	5'-CGG AAA GGC GAG GGA TAC TC-3'
FKBP5 reverse	5'-TTC CCC AAC AAC GAA CAC CA-3'
AVP forward	5'-CGC CAG GAT GCT CAA CAC TA-3'
AVP reverse	5'-AAA AAC CGT CGT GGC ACT CG-3'
BDNF exon IX forward	5'-AGC CTC CTC TAC TCT TTC TGC TG-3'
BDNF exon IX reverse	5'-GTG CCT TTT GTC TAT GCC CCT G-3'
β-actin forward	5'- CGT TGA CAT CCG TAA AGA CC-3'
β-actin reverse	5'-AAC AGT CCG CCT AGA AGC AC-3'

2.2.8 Melt curve analysis for quantitation of DNA methylation

DNA from the PVN was treated with bisulfite using the EZ DNA Methylation-Gold Kit (Zymo Research). This was followed with nested PCR on the bisulfite-converted DNA to amplify the CRF promoter region using custom forward (5'-TTT AAA AAT TTT TGT TAA TGG ATA AGT TAT-3') and reverse (5'-ACC TCC TAC AAA TTT TCT TCC TCT T-3') primers and 40 cycles of PCR (15 sec at 94 ºC, 30 sec at 53 ºC, 30 sec at 68 ºC). PCR products were examined for specificity and successful amplification on a 1.0% agarose gel and then purified with QIAquick PCR Purification Kit (Qiagen, Valencia, CA). These nested PCR products were quantified with a NanoDrop instrument and used as input for SYBR green PCR (94 ºC at 15 sec, 55 ºC at 30 sec and 72 ºC at 30 sec) with CRF forward (5'-TTT GGT AGG GTT TTA TTA TTT ATG TAG GA-3') and reverse (5'-CTA AAT TTC TCC ACA CCA AAA CCT A-3') primers. PCR products were then melted gradually from a 55 ºC start point, stepping up the temperature 0.1 ºC every 10 sec up to 85º C. Melt curve normalization and quantitation of the T50 melting point (i.e., the point at which 50% the PCR products are melted) was performed according to published methods (Smith et al., 2009).

2.2.9 Statistical analysis

Unpaired two-way Student's tests were used for melt curve analysis, RT-qPCR experiments, and area under the curve analysis for HPA axis responsivity with Bonferroni correction for multiple comparisons where appropriate. Two-way repeated measures analysis of variance (ANOVA) was used for corticosterone levels and body weights and ethanol drinking during chronic intermittent and variable stress (factors of paternal ethanol and time point). For significant paternal ethanol × time point interactions, Fisher's least significant difference (LSD) *post-hoc* tests were used to examine effects of paternal ethanol at specific time points.

2.3 RESULTS

2.3.1 Paternal preconception chronic intermittent ethanol (CIE) exposure

Adult B6 males were exposed to CIE or room air conditions for 5 weeks, 5 consecutive days/week, and 8 hours/day (Figure 1A) prior to breeding to produce offspring of chronic ethanol- (E-sired) and control-exposed sires (C-sired). The average blood ethanol concentration (BEC) over the five weeks of ethanol exposure was 179.3 $\pm$ 69.31 mg/dL $\pm$ standard error of the mean (SEM) (Figure 1B). Consistent with published results using this CIE paradigm (Finegersh and Homanics, 2014), there was no effect of CIE on body weight (Figure 1C).

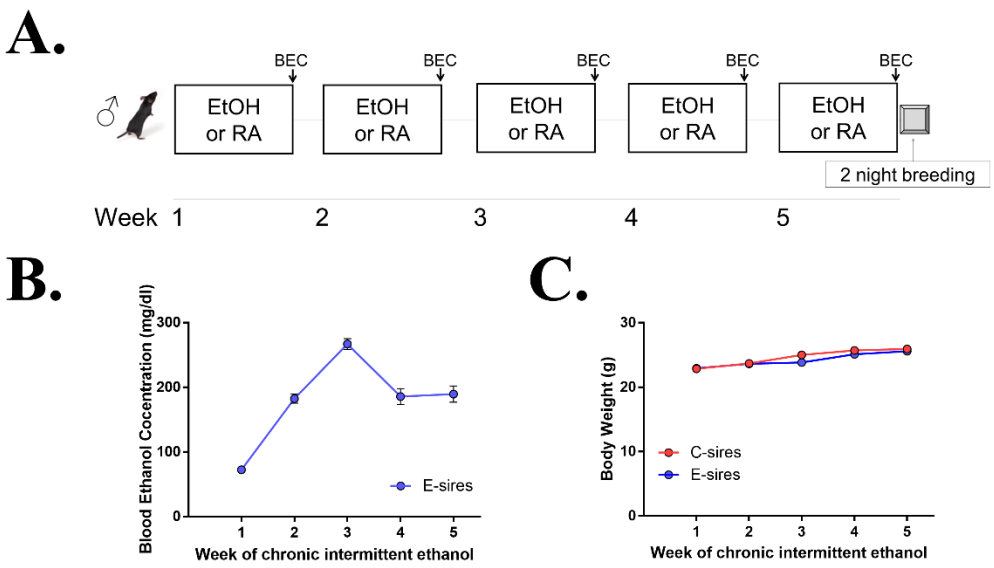


Figure 1. Paternal preconception chronic ethanol exposure

(A) Adult male B6 mice were exposed to chronic intermittent ethanol (EtOH) or room air control conditions (RA) over five consecutive days (8 hours/day) each week for five weeks total. Blood ethanol concentrations (BEC) were measured after the final ethanol exposure each week. Following the final CIE exposure, males were housed with two Strain 129 females for two nights to produce offspring for behavioral testing. **(B)** BECs each week for CIE-exposed sires (E-sires) (N=12/group). **(C)** Body weights of E-sires (N=12/group) and room air-exposed control sires (C-sires) (N=12/group). Data presented as μ ± SEM. In panel C, error bars are obscured by data points.

2.3.2 Paternal chronic ethanol increases body weight selectively in males

Body weights of E- and C-sired offspring were measured at three and eight weeks postnatal. For male offspring, there was a significant effect of paternal ethanol ($F_{(1, 18)} = 9.82$, $p<0.01$; Figure 2A) and paternal ethanol × postnatal age ($F_{(1, 18)} = 12.40$, $p<0.01$; Figure 2A). *Post-hoc* analysis revealed a significant increase in E-sired male body weight at 8 weeks postnatal ($p<0.001$). In contrast, there was no effect of paternal ethanol or paternal ethanol × postnatal age on the body weights of female offspring (Figure 2B).

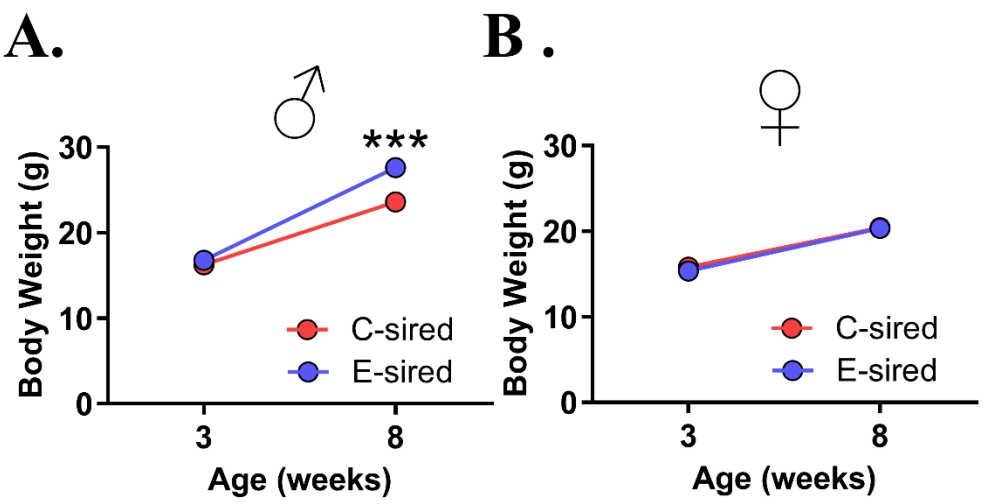


Figure 2. Paternal chronic ethanol increases body weight selectively in males

(A) Increased body weights at 8 weeks postnatal for E-sired vs C-sired males (N=10/group). **(B)** No difference in body weight in E-sired vs C-sired females (N=10-20/group). ***=p<0.001. Data presented as μ ± SEM. Error bars obscured by data points.

2.3.3 Paternal ethanol blunts HPA axis responsivity selectively in males

HPA axis responsivity was assayed following 15 min of acute restraint stress to measure plasma corticosterone (CORT) levels collected from tail blood. In male mice, there was a significant effect of paternal ethanol ($F_{(1, 13)} = 7.41$, $p<0.05$; Figure 3A) and paternal ethanol × time ($F_{(3, 39)} = 2.86$, $p<0.05$; Figure 3A). *Post-hoc* analysis revealed that E-sired males had reduced plasma CORT at 30 ($p<0.001$) and 90 ($p<0.05$) min from the onset of stress. Area under the curve (AUC) analysis further supported the reduction of CORT levels in E-sired vs C-sired males ($t_{(13)} = 2.71$, $p<0.05$; Figure 3B) There was no effect of paternal ethanol or paternal ethanol × time in female offspring (Figure 3C) and no difference in AUC between groups (Figure 3D).

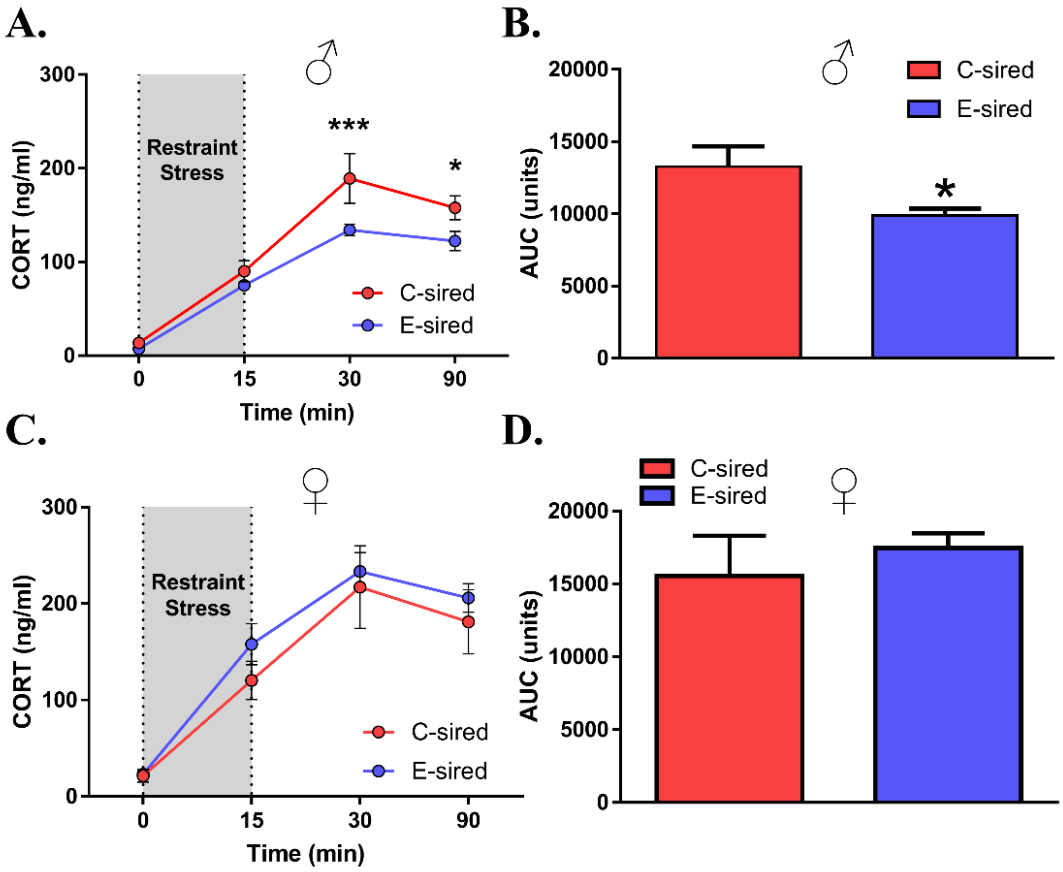


Figure 3. Paternal chronic ethanol blunts HPA axis responsivity selectively in males

(A) Decreased plasma corticosterone (CORT) levels in E-sired vs C-sired males at 30 and 90 min after the onset of 15 min restraint stress (shaded bars). **(B)** Area under the curve (AUC) analysis revealed a significant reduction in stress responsivity in E-sired males. **(C)** No change in plasma CORT levels in response to restraint stress and **(D)** no difference in AUC in E-sired vs C-sired females. *=$p<0.05$, ***=$p<0.001$. Data presented as $\mu \pm$ SEM.

2.3.4 Paternal chronic ethanol prevents stress-induced polydipsia in males

Previously, paternal chronic ethanol vapor exposure reduced two-bottle choice ethanol drinking preference in male offspring (Finegersh and Homanics, 2014). Importantly, no single animal model

of ethanol drinking can reflect all the motivational and context-related aspects that influence human drinking behavior. For instance, the mechanisms underlying stress-induced ethanol drinking differ from those influencing basal two-bottle choice ethanol preference and consumption (Spanagel et al., 2014). Thus, experiments were designed to examine whether paternal ethanol additionally alters ethanol drinking behavior in response to chronic intermittent and variable stress (CIVS). Thus, males were habituated to sipper tubes containing 8% (w/vol) ethanol and water in the home cage for several weeks to establish equal baseline drinking preference between groups. Test mice were subsequently exposed to four weeks of CIVS with each week comprised of three consecutive daily exposures to a unique stressor (Figure 4A). Surprisingly, there was no significant effect of sire or sire × week of stress on ethanol drinking preference (Figure 4B) or ethanol consumption (Figure 4C). Interestingly, there was a significant sire × week of stress interaction for total fluid intake ($F_{(4, 68)} = 3.95$, $p<0.01$; Figure 4D). *Post-hoc* analysis revealed significantly reduced total fluid intake in E-sired vs C-sired males during CIVS weeks one ($p<0.05$) and two ($p<0.05$). When examining the percent change in total fluid intake from baseline levels, there was a significant effect of sire ($F_{(1, 17)} = 7.30$, $p<0.05$; Figure 4E) and sire × week of stress ($F_{(3, 51)} = 3.61$, $p<0.05$; Figure 4E). *Post-hoc* analysis revealed significantly reduced percent change in total fluid intake from baseline levels in E-sired vs C-sired males during weeks one ($p<0.01$) and two ($p<0.01$).

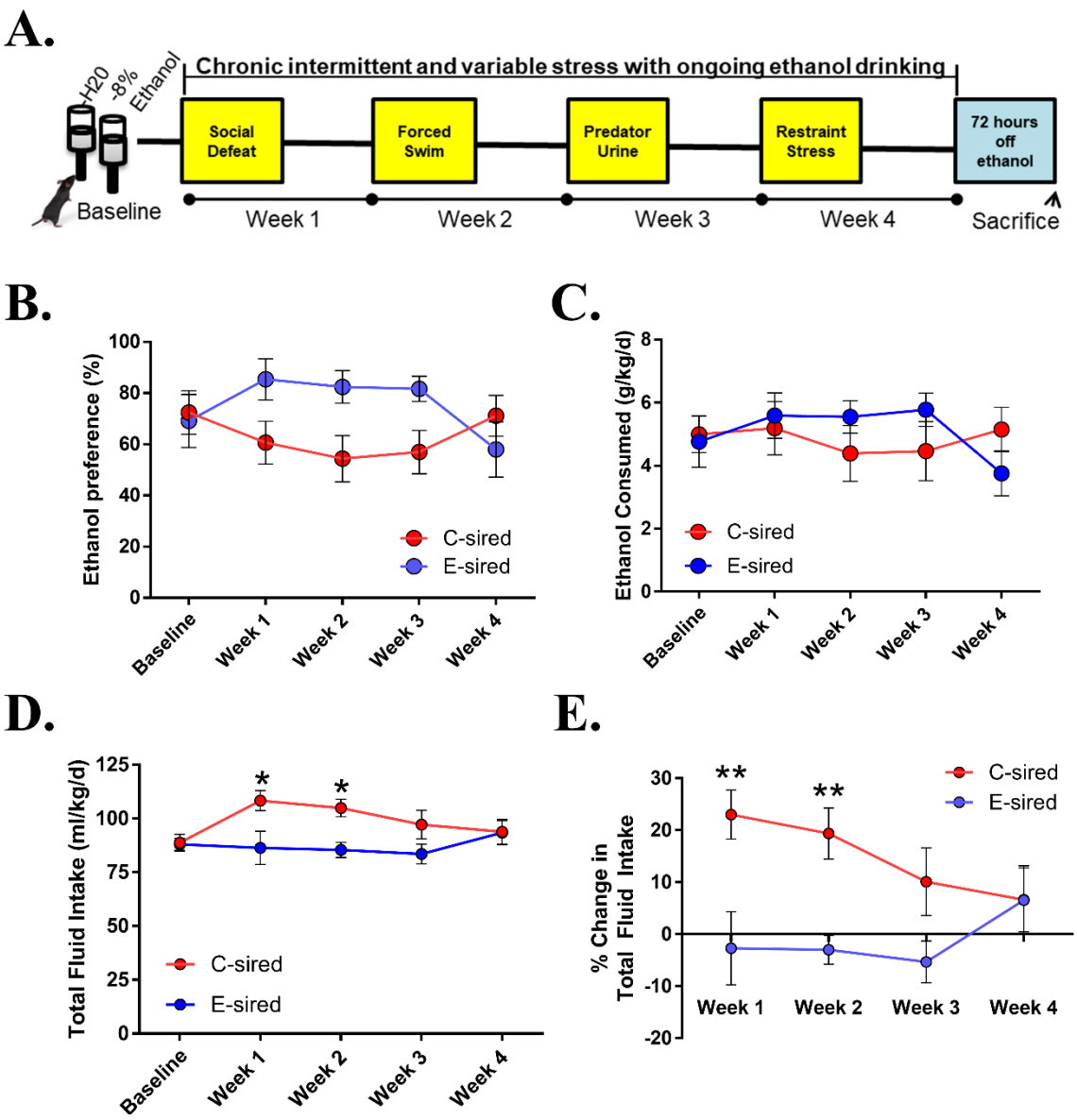


Figure 4. Paternal chronic ethanol prevents stress-induced excessive fluid intake in males

(A) After establishing stable baseline ethanol drinking preference in the home cage, mice were exposed to CIVS over a four-week period while continuing two-bottle choice drinking in the home cage. There was no effect of paternal ethanol on **(B)** ethanol drinking preference or **(C)** ethanol consumption for E-sired vs C-sired males (N=10/group). E-sired males had reduced **(D)** total fluid intake and **(E)** percent change in total fluid intake from baseline vs C-sired males (N=10/group). *=p<0.05, **=p<0.01. Data presented as μ± SEM.

2.3.5 Reduced CRF gene expression in stressed E-sired males

There was no effect of paternal ethanol on expression of several stress-regulatory genes in the paraventricular nucleus of the hypothalamus (PVN) (Figure 5A) or amygdala (Figure 5B). However, after CIVS with ethanol drinking (Figure 4A), CRF mRNA expression were reduced in the PVN of E-sired vs. C-sired males ($t_{(12)}$=3.21, Bonferroni-corrected $p<0.05$; Figure 5C). Comparatively, CRF was not altered in the amygdala of CIVS-exposed E-sired vs C-sired males (Figure 5D).

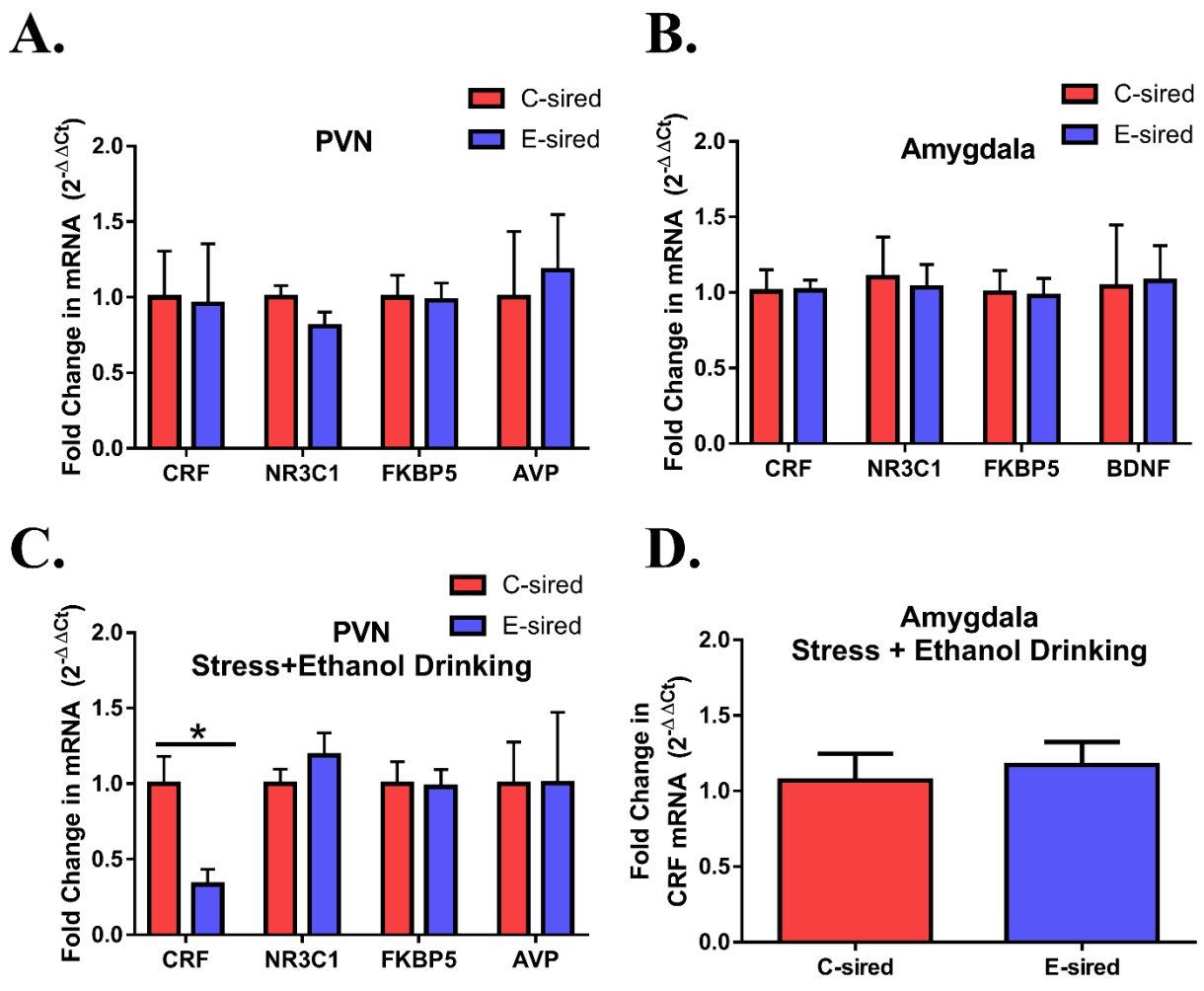


Figure 5. Reduced CRF gene expression in CIVS E-sired males

No effect of paternal ethanol on expression of stress-regulatory genes in **(A)** PVN and **(B)** amygdala in unstressed C-sired and E-sired mice. **(C)** CRF expression was reduced in PVN of CIVS-exposed E-sired vs C-sired males. **(D)** No difference in CRF expression in the amygdala of CIVS-exposed E-sired vs. C-sired males. *=p<0.05. Data presented as μ ± SEM.

2.3.6 Increased CRF promoter methylation in stressed E-sired males

To examine epigenetic regulation of CRF in the PVN of CIVS-exposed E-sired males, melt curve analysis was performed with bisulfite-treated DNA to assess cytosine methylation of the CRF promoter (Figure 6A). CRF methylation was increased in CIVS E-sired vs C-sired males after CIVS ($t_{(12)} = 3.21$, $p<0.01$; Figure 6B). Moreover, there was a significant correlation between CRF gene expression and methylation in CIVS-exposed C-sired males ($r = -0.92$, $p<0.05$; Figure 6C).

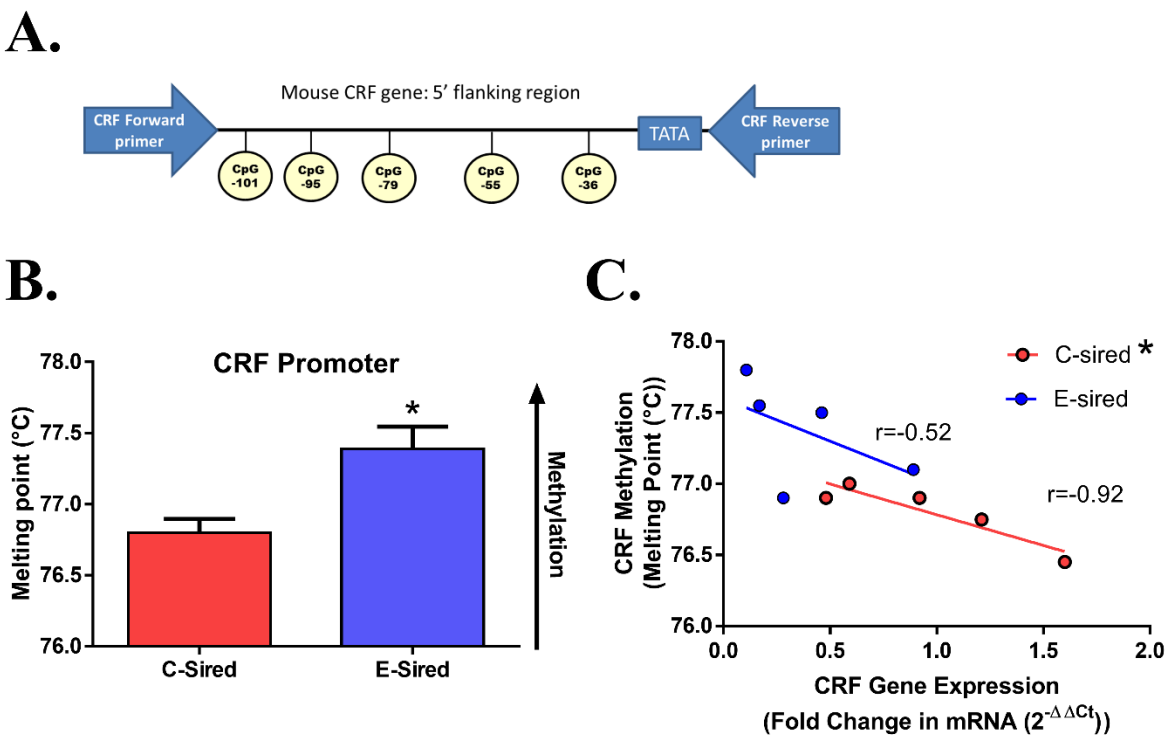


Figure 6. Differential methylation of the CRF promoter in CIVS E-sired males

(A) CRF primers flanking several cytosine phosphate guanine (CpG) dinucleotides at denoted positions upstream of the TATA box for melt curve analysis of cytosine methylation using bisulfite-treated DNA. **(B)** Increased CRF promoter methylation in CIVS E-sired vs C-sired males. **(C)** Correlation between CRF gene expression and methylation in CIVS E- and C-sired males. *=$p<0.05$, **=$p<0.01$. r= Pearson correlation coefficient. Data in panel B presented as $\mu \pm$ SEM.

2.4 DISCUSSION

Paternal preconception chronic ethanol exposure confers various phenotypes to offspring including reduced testosterone levels (Abel, 1989c), decreased grooming (Abel, 1991b), reduced organ weight (Abel, 1993b), increased immobility time in a forced-swim test (Abel and Bilitzke, 1990), thickening of the cerebral cortex (Jamerson et al., 2004), impaired working memory (Kim et al., 2014) and, most recently, alterations in ethanol sensitivity and drinking preference (Finegersh and Homanics, 2014). In the current chapter, the results add to this literature with the novel finding that paternal ethanol exposure imparts attenuated stress responsivity to adult male offspring. Paternal ethanol exposure resulted in reduced plasma CORT in response to acute restraint stress, resistance to stress-induced excessive fluid intake, and epigenetic remodeling of the CRF promoter in PVN of male offspring. Altered HPA-axis function is involved in both stress- and addiction-related disorders (Clarke et al., 2008; Pariante and Lightman, 2008). Thus, the observed effects of paternal ethanol exposure on stress responsivity in the next generation may have broad human health implications.

The hypothesis that paternal ethanol exposure would alter HPA-axis function in offspring was based on two important lines of evidence. First, the HPA axis is strongly implicated in the neuropathophysiology of alcoholism, and deficits are frequently observed in individuals with a

family history of alcoholism (Schuckit et al., 1987; Dai et al., 2002; Stephens and Wand, 2012). For instance, sons of alcoholics had greater basal cortisol levels that showed significant attenuation by ethanol vs family history negative controls (Zimmermann et al., 2004). Secondly, paternal pharmacological and behavioral stress exposures have been shown to impact stress-related behavior in offspring (Dietz et al., 2011; Crews et al., 2012; Pisu et al., 2013; Gapp et al., 2014). Indeed, the finding that paternal ethanol exposure blunts HPA axis responsivity in male offspring is remarkably similar to a recent study that found the same blunted CORT phenotype following acute restraint stress in offspring of fathers exposed to chronic variable stress (Rodgers et al., 2013). Ethanol is a potent physiological stressor, activating the HPA axis in rodents during both forced ethanol exposures as well as during voluntary ethanol drinking (reviewed in (Rivier, 2014)). Therefore, it is possible that the stress associated with chronic ethanol exposure may be important for the intergenerational phenotypes observed in E-sired male offspring. Additional studies are needed to explore whether other stress exposures, such as chronic variable stress, can similarly impact intergenerational ethanol-drinking behavior and sensitivity to ethanol-induced anxiolysis. This is examined directly in Chapter 3.

Notably, the effects of paternal ethanol exposure on HPA axis responsivity were sex-specific, consistent with the previous findings for ethanol-related phenotypes (Finegersh and Homanics, 2014). Indeed, similar intergenerational and transgenerational studies have found sex-specific effects of paternal preconception exposures (Franklin et al., 2010; Vassoler et al., 2013). The complex epigenetic mechanisms underlying sex-specific vs. sex-independent intergenerational phenotypes remain to be elucidated. One limitation to the current study is females were not monitored for estrus cycle stage. As sex-steroids influence HPA-axis activity (Kalil et al., 2013),

future experiments are needed to determine if sex-specific effects of paternal ethanol can be explained by natural variations in estrus.

Acute CORT escalates ethanol-drinking behavior, and inhibition of CORT by adrenalectomy decreases ethanol consumption (Fahlke et al., 1996; Fahlke and Hansen, 1999). Therefore, stress hyporesponsive E-sired males were hypothesized to show reduced ethanol-drinking behavior vs C-sired males in response to chronic stress. Surprisingly, stressed E-sired males did not differ in ethanol-drinking preference or consumption vs. stressed C-sired males. However, there was a large difference between E-sired and C-sired males in total fluid intake. Specifically, C-sired males exhibited a robust polydipsia-like phenotype (i.e., excessive fluid intake) during weeks 1 and 2 of CIVS that was absent in E-sired male offspring.

Preclinical studies examining the relationship between stress and ethanol drinking have produced inconsistent results (Becker et al., 2011). In the present study, C-sired males did not show a significant stress-evoked increase in ethanol-drinking behavior. The relationship between stress and ethanol drinking is complex and several variables including type of stress, strain, sex, and time course can ultimately influence the direction and magnitude of ethanol-drinking behavior (Spanagel et al., 2014). Future experiments will aim to validate and employ a specific model for stress-escalated ethanol drinking, such as chronic stress preceding ethanol consumption (Lopez et al., 2011; Rodriguez-Arias et al., 2016).

Increased total fluid intake following chronic stress such as social defeat is an adjunctive phenotype in mice referred to as stress-induced polydipsia (Golden et al., 2011), a hallmark behavioral phenotype in mice exposed to chronic or subchronic social defeat (Golden et al., 2011; Goto et al., 2014). CORT inhibition blocks polydipsia-induced excessive water intake, suggesting an important role for HPA axis activity (Strekalova et al., 2011). Therefore, resistance to stress-

induced polydipsia-like drinking further supports the conclusion that E-sired males are hyporesponsive to stress.

In addition to protection from stress-induced polydipsia, E-sired males had reduced CRF gene expression in the PVN following the CIVS with two bottle choice ethanol drinking experiments. This change in CRF expression was not observed at baseline. Moreover, stressed E-sired mice had greater DNA methylation at the CRF promoter. The specific region of the CRF promoter targeted by melt curve analysis in the current study was previously found to undergo epigenetic remodeling in response to chronic stress (Elliott et al., 2010; Sterrenburg et al., 2011). Thus, the differences in methylation may have emerged in response to CIVS. There was a strong negative correlation between CRF gene expression and methylation in C-sired controls, supporting the notion that epigenetic regulation of the CRF gene may be driving differences in gene expression between E-sired and C-sired mice after CIVS. Given that chronic stress increases CRF mRNA expression in the PVN (Gomez et al., 1996; Elliott et al., 2010; Sterrenburg et al., 2011), it is likely that CRF is potentiated in C-sired males after CIVS and that this effect is mitigated in E-sired males. Furthermore, chronic ethanol diet treatments and acute withdrawal did not alter CRF protein levels in the PVN (Wills et al., 2010), supporting a more critical role for stress vs ethanol drinking in hypothalamic CRF gene expression. However, future experiments concurrently examining stressed and unstressed groups of ethanol-naïve mice are needed to confirm that E-sired males are protected from stress-enhanced CRF gene expression.

It is worth discussing certain limitations to the current study. It is possible that maternal care differed between groups based on altered maternal-paternal interactions during mating (Mashoodh et al., 2012). Indeed, maternal care can directly influence HPA-axis responsivity (Champagne and Meaney, 2001). However, in the current study, the breeding period was limited to just two nights

and there were no differences in E-sired and C-sired male body weights at the time of weaning, suggesting that maternal care was not significantly different between E-sired and C-sired litters. Another potential confound in the current study is that breeding occurred immediately after ethanol exposure. Thus, it is unclear whether the intergenerational effects of paternal ethanol exposure are specific to preconception chronic ethanol exposure and not, in part, dependent on acute intoxication or withdrawal at the time of mating. Therefore, future experiments will delay the period between paternal ethanol exposure and breeding.

How might paternal ethanol exposure impart stress-related phenotypes to offspring? Several studies have found changes in DNA methylation (Govorko et al., 2012; Dias and Ressler, 2014) or histone modifications (Vassoler et al., 2013) in sperm that are associated with altered complex phenotypes in offspring. Interestingly, two recent studies have implicated stress-responsive sperm RNAs as epigenetic drivers of intergenerational stress-related behaviors (Rodgers et al., 2013; Gapp et al., 2014). Remarkably, the intergenerational effects of paternal stress were recapitulated by injecting sperm RNAs from stressed males into fertilized oocytes (Gapp et al., 2014; Rodgers et al., 2015), suggesting this epigenetic mechanism may underlie inheritance of paternal preconception environment. It is conceivable that paternal ethanol may similarly alter epigenetic marks in sperm to causally impact stress responsivity in the next generation.

In summary, paternal ethanol exposure confers stress hyporesponsivity to male offspring. Alterations in stress responsivity were observed at the endocrine, behavioral, genetic, and epigenetic levels. Identifying heritable mechanisms that mediate stress resilience or vulnerability has major implications for the development of novel prevention and treatment strategies for psychiatric disease and addiction. Therefore, the experiments in Chapter 4 of this dissertation aim

to identify ethanol-induced epigenetic factors in sperm that confer altered stress-related phenotypes to male offspring.

3.0 PATERNAL STRESS REDUCES ETHANOL DRINKING IN MALES

3.1 INTRODUCTION

Recent epidemiological findings have spurred increased interest in the potential for preconception stress to affect offspring development and health outcomes (Bowers and Yehuda, 2016). This hypothesis is supported by wide-ranging preclinical studies illustrating that the paternal preconception environment imparts diverse behavioral phenotypes to offspring (Chan et al., 2017c). As these studies are largely carried out with isogenic mice or rats, the intergenerational effects are unlikely to be explained by genetic variation. Recently adding to this literature, Finegersh and Homanics discovered that paternal chronic intermittent ethanol exposure confers reduced ethanol drinking preference and increased ethanol sensitivity to male offspring (Finegersh and Homanics, 2014). Given the high prevalence and societal costs associated with alcohol use disorder (Rehm et al., 2009; Haberstick et al., 2014), the potential for paternal preconception environment to causally effect intergenerational ethanol-related behaviors warrants further investigation.

Ethanol intoxication (with blood ethanol concentrations (BEC) > 80 mg/dL), as well as acute ethanol withdrawal activates the hypothalamic-pituitary-adrenal (HPA) stress axis (Blaine et al., 2016). Furthermore, in animal models, ethanol engages the HPA axis via all examined routes of ethanol administration (Rivier, 2014), including voluntary drinking (Richardson et al., 2008). Particularly relevant to the previous intergenerational studies, vapor ethanol exposure robustly increases HPA activity over the course of ethanol inhalation (Lee and Rivier, 2003). Thus, if the HPA axis-engaging mechanism of ethanol underlies the effects of paternal ethanol exposure, other

forms of chronic stress may similarly be able to influence intergenerational ethanol-related behaviors.

Several animal models of early life and chronic stress including maternal separation (Franklin et al., 2010), social defeat (Dietz et al., 2011), social isolation (Pisu et al., 2013), and chronic variable stress (Rodgers et al., 2013) impact complex neurobehavioral phenotypes in offspring. In addition, paternal chronic corticosterone (CORT) exposure increases anxiety-related behaviors in offspring (Short et al., 2016). Interestingly, many paternal stress exposures alter HPA axis function in offspring (Dietz et al., 2011; Pisu et al., 2013; Rodgers et al., 2013; Gapp et al., 2016). For instance, chronic variable stress blunts HPA axis responsivity in male and female offspring (Rodgers et al., 2013). Experiments in Chapter 2 revealed the same blunted HPA responsivity phenotype in males sired by chronic ethanol-exposed fathers, suggesting there may be convergent intergenerational effects of ethanol and stress.

Given that ethanol engages the HPA stress axis and that ethanol and stress have comparable intergenerational effects on HPA function, the intergenerational effects of stress and ethanol may similarly converge on ethanol-related behaviors. Thus, Chapter 3 examines the hypothesis that paternal chronic variable stress alters ethanol-related behaviors in offspring.

3.2 MATERIALS AND METHODS

3.2.1 Animals

All experiments were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh and were conducted in accordance with the National Institutes of Health

Guidelines for the Care and Use of Laboratory Animals. Seven-week-old, ethanol-naïve, C57BL/6J (B6) and Strain 129S1/SvImJ (Strain 129) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Purchased B6 male mice were either habituated for one week before chronic stress or control treatment (these mice were to be used in the vender born and shipped (VBS)-sire cohort) or housed for six weeks before breeding for one week to eight-week-old B6 females to produce the in-house colony (IHC)-sire cohort. For rearing of eventual IHC-sires, after the one week breeding period, male breeders were removed and each pregnant female was moved to individual housing. At three weeks post-natal, eventual IHC-sires were weaned to four males per cage. Unless otherwise specified, specific pathogen-free mice were group-housed in individually ventilated micro-isolater cages under 12-h light/dark cycles (lights on at 07:00) and had *ad libitum* access to food (irradiated 5P76 ProLab IsoPro RMH 3000, [LabDiet, St.Louis, MO]) and water.

3.2.2 Paternal preconception chronic variable stress

Eight-week-old adult male B6 group-housed mice were exposed to six weeks of chronic variable stress or control conditions. The chronic variable stress exposure was based on published methods (Rodgers et al., 2013; Rodgers et al., 2015). Briefly, chronic variable stress consisted of daily exposure to one of seven stressors (each described below) on a randomized schedule with each stressor utilized six times total. The seven stressors employed are described below.

Novel object exposure: 30 glass marbles (10 mm diameter) were placed in the home cage for 12 hours during the dark cycle.

Saturated cage bedding overnight: At the onset of the dark cycle, ~200-600 ml (depending on the amount of bedding in the cage) of autoclaved water (~23º C) was applied to the home cage.

The exposure was terminated at the onset of the light cycle when the home cage was changed and mice were gently dried with a towel or briefly with a heat lamp.

White noise overnight: From the onset to the termination of the dark cycle (12 hr +/- 30 min), home cages were moved to sound-controlled chambers in an animal behavior room (same room each exposure) fitted with a ventilation fan and computer-operated speakers programmed with Audacity 2.2.1 free software to emit continuous 100 db white noise.

Multiple cage changes: Throughout the 12 hr light cycle, home cages were changed 3-5 times at randomized time points.

Constant light exposure: Home cages were placed in an air-controlled and ventilated fan-equipped chamber with room lights left on from the onset to the termination of one dark cycle.

Restraint stress: Animals were restrained for 15 min between 3 and 5 hours after lights-on (10:00-12:00). Mice were restrained in 50 ml conical plastic tubes (VWR) with several air hole perforations near the animal's head. All mice in the group-housed cage were restrained within the home cage simultaneously in a fume hood.

Predator odor exposure: The predator odor exposure was performed in the home cage within a fume hood with the cage cover removed. All stress-treatment cages were exposed simultaneously for 15 min with 3 × 3 inch paper towel strips soaked with 1 mL of fox urine (Cat # W2645, Tink's Red Fox-P ®, Covington, GA) placed just outside each cage, flanking each length-wise side of the cage.

For both control and stress exposure groups, body weights were measured and cages were changed simultaneously and weekly at the same time of day (09:00-11:00).

3.2.3 Breeding scheme and offspring rearing

Following the final chronic variable stress exposure, mice were pair housed with eight-week-old B6 females for two weeks. CORT levels were measured one week following the final stressor and two hours before the start of the dark cycle (17:00) using the commercial enzyme-linked immunosorbent assay (ELISA) described in section 3.2.7. After the two-week post-stress period, all males were moved to housing with two stress-naïve Strain 129 eight-week-old females for 48 hours before males were removed and pregnant dams were single-housed for rearing of stress (S) and control (C)-sired offspring. Offspring were weaned at three weeks postnatal and group- housed (3-4/cage) with same sex littermates of the same treatment group. Importantly, for all behavioral testing, no more than two mice of the same sex were examined per litter and per sire.

3.2.4 Elevated plus maze and acute ethanol treatment

The elevated plus maze was conducted with different conditions for IHC- vs VBS-sired mice where indicated. Adult mice were singe-housed and habituated to the test room for one hour prior to testing. The elevated plus maze apparatus featured two closed and open arms with both floors and arm enclosures made of opaque white plexiglass. Light intensity was 100 lux for IHC-sired mice and 35 lux for VBS-sired mice. Ten min (IHC-sired) or thirty min (VBS-sired) prior to testing, mice received an intraperitoneal injection of 5% (w/vol) ethanol (1.0 or 1.5 g/kg IHC-sired/VBS-sired) or physiological saline (0.9% NaCl) and were placed back into the home cage. After ten or thirty min, mice were placed in the center of the elevated plus maze with head always facing the same closed arm. Scoring of time spent in the arms was performed using LimeLight tracking software (Coulbourn Instruments, Holliston, MA).

3.2.5 Twenty-four hour, two-bottle free choice drinking behavior

Eight-week-old adult mice were single-housed for one week while habituating to two 25 ml sipper tubes filled with autoclaved water. Sipper tubes were designed by fitting ball-bearing sippers (Cat# TD-99; Ancare Corp) into modified 25 ml polystyrene serological pipets (Cat # 357525; Corning Incorporated) and securing the fit with heat-shrink and parafilm. After the one week, ethanol drinking behavior was assessed by filling one tube with ethanol. Consumption of ethanol and water was measured daily and the position of the ethanol and water tubes was rotated each day. Ethanol concentrations started at 3% (w/vol) and was increased every four days to 6, 9, 12, and 15% successively. Cages were changed and animals were weighted every four days when the ethanol concentrations were adjusted. After the final day of ethanol drinking, there was a one week washout period, during which mice had access to two sipper tubes filled with water. After the one week, one tube was filled with 0.06 % (g/ml) saccharin (Cat# 240931; Sigma-Aldrich) and consumption was measured for four days with tube position rotated daily. After the final trial, there was another one week washout before one tube was filled with 0.06 mM quinine (Cat# 145904; Sigma-Aldrich) and two bottle consumption measures were taken over four days with tube position rotated daily.

3.2.6 Drinking in the dark assay

The drinking in the dark assay was performed based on published methods (Thiele et al., 2014). For four nights, mice were habituated in the home cage to one 25 ml sipper tube filled with water that replaced their regular water bottle two hours into the animal's dark cycle. After the final habituation trial, sipper tubes were filled with 20% (w/vol) ethanol and consumption was measured for two hours over three consecutive nights and four hours on the fourth night.

To examine blood ethanol concentrations, immediately following the four-hour trial, ≤10 µL tail vein blood was collected from each animal using heparin-coated capillary tubes (Drummond). Tail blood was centrifuged at 2000 × g for 10 min and plasma was stored at -80º C prior to being measured for ethanol (mg/dL) using an Analox Ethanol analyzer (AM1, Analox Instruments, London, UK).

3.2.7 Hypothalamic-pituitary-adrenal (HPA) axis responsivity

During the animal's light cycle between 10:00-13:00, single-housed mice were restrained for fifteen min in modified 50 ml conical tubes with the cone endings removed and an aperture added to the cap for the tail. Tail blood was collected with heparin-coated capillary tubes (Drummond) at time points 0,15,30, and 90 min from the onset of restraint stress. Plasma was collected from blood by centrifugation at 2000 × g for 10 min. CORT levels were measured in plasma using Corticosterone ELISA kit (Cat # ADI-900-097; Enzo Life Sciences, Farmingdale, NY). Using 5 µL of plasma, samples were prepared and analyzed in duplicate on a 96-well plate following manufacturer's protocol.

3.2.8 Statistical analysis

Elevated plus maze measures and saccharine and quinine drinking preference were analyzed using two-way unpaired Student's t test. Body weights, two-bottle choice ethanol drinking, drinking in the dark, and BEC results were analyzed with two or three-way (i.e., paternal stress × sire source × ethanol concentration or trial number) repeated measures analysis of variance (ANOVA). Drinking in the dark results were analyzed by averaging the three two hour trials to produce two conditions

for trial length (two and four hours). Litter size results were analyzed using Student's t-tests or two-way ANOVA (factors of paternal stress and sire source). Significant interactions were further analyzed using Fisher's least significant difference (LSD) *post-hoc* tests. Basal CORT levels were compared using two-way ANOVA (factors of paternal stress and sire source) and HPA axis responsivity was assessed using repeated measures two-way ANOVA (factors of paternal stress and time point).

3.3 RESULTS

3.3.1 Paternal preconception chronic variable stress

Adult B6 male mice were either exposed to six weeks of daily variable stress or control conditions (Figure 7A). Body weight was significantly reduced by stress ($F_{(1, 7)} = 63.16$; $p<0.001$, Figure 7B). Two weeks after stress or control conditions, males were briefly mated with stress-naïve Strain 129 females to produce stress (S)-sired and control (C)-sired male and female offspring (Figure 7C). Upon birth of C- and S-sired offspring, there was no effect of paternal stress on litter size (Figure 7D). There was a significant effect of paternal stress × postnatal age on both male ($F_{(1, 35)} = 8.122$; $p<0.01$, Figure 7E) and female ($F_{(1, 36)} = 4.624$, $p<0.05$, Figure 7F) offspring body weight. *Post-hoc* analysis revealed that stress did not affect body weigh at 3 weeks postnatal, but increased S-sired male ($p<0.05$) and female ($p<0.01$) weights at 8 weeks postnatal.

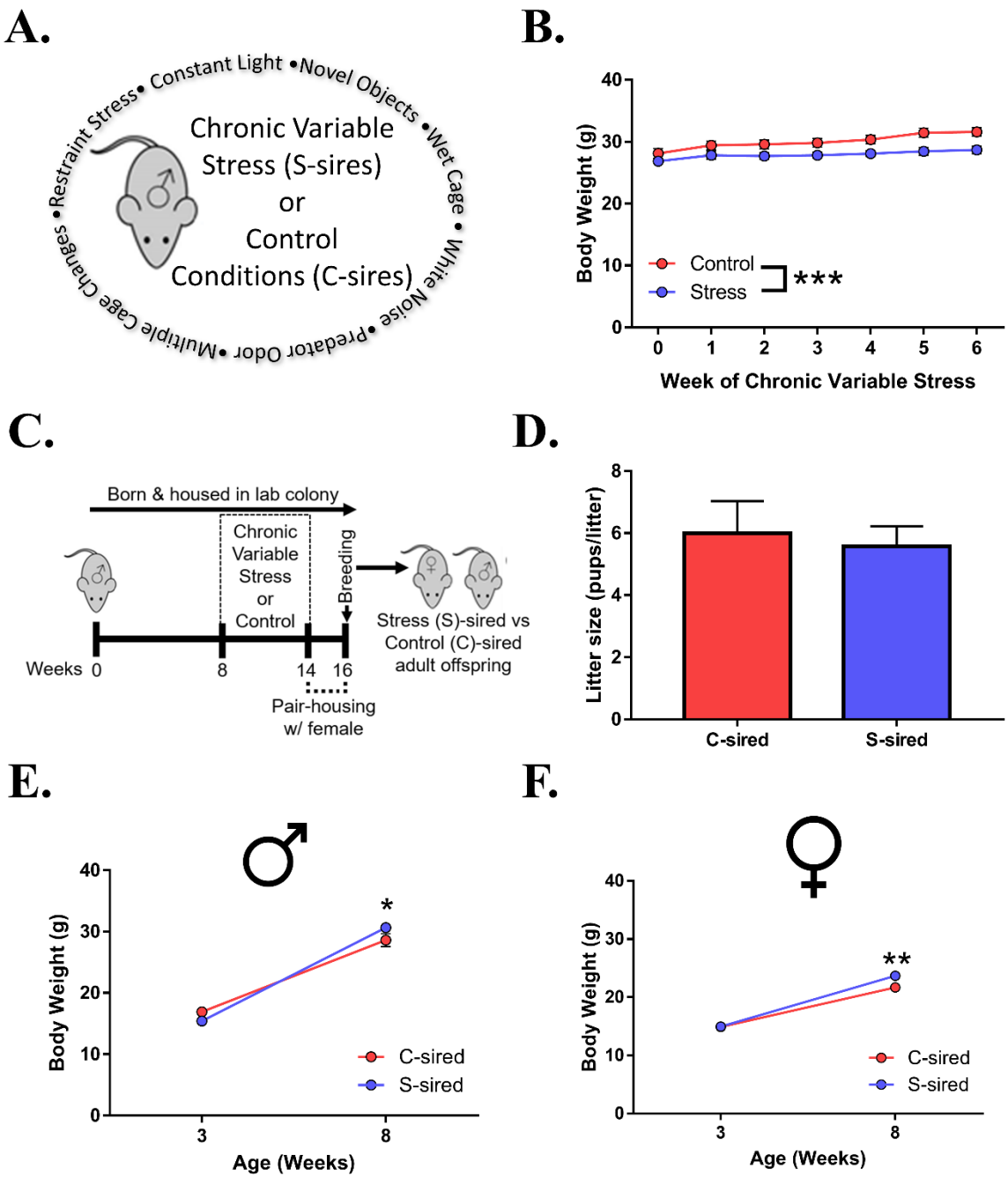


Figure 7. Examining the intergenerational effects of paternal chronic variable stress

(A) Eight-week-old male mice were exposed to six weeks of stress comprised of daily exposures to one of seven different stressors (listed in the illustration) on a randomized schedule or control conditions. **(B)** Significantly reduced body weight in S-sires vs C-sires (N=8/group). **(C)**

Experimental timeline. Two weeks after chronic variable stress or control conditions, males were bred with stress-naïve females for two nights to produce male and female S-sired and C-sired offspring. (D) No effect of paternal stress on litter sizes (N=6-7/group). **(D)** Increased body weight at 8 weeks postnatal in male offspring (N=17-20/group). **(E)** Increased body weight at 8 weeks postnatal in female offspring (N=19/group). *=p<0.05, **=p<0.01, ***=p<0.001. Data presented as mean (μ) ± standard error of the mean (SEM). Error bars in panels C, E, and F are obscured by symbols.

3.3.2 Paternal stress reduces ethanol drinking preference in males

The initial experiments with S-sired and C-sired mice examined ethanol drinking preference in the two-bottle free choice test at ethanol concentrations of 3, 6, 9, 12, and 15% (w/vol) for four days each. For males, paternal stress dramatically reduced ethanol preference ($F_{(1, 35)}$ = 37, p<0.001, Figure 8A) and ethanol consumption ($F_{(1, 14)}$ = 11.24, p<0.01, Figure 8B) and had no effect on total fluid intake (p>0.05, Figure 8C). There was no interaction between paternal stress × ethanol concentration for ethanol preference, ethanol consumption, or total fluid intake. In contrast, for females, there was no effect of paternal stress on ethanol preference (p>0.05, Figure 8D) or ethanol consumption (p>0.05, Figure 8E), although paternal stress significantly decreased total fluid intake ($F_{(1, 14)}$ = 8.591, p<0.05, Figure 8F). There was no significant effect of paternal stress × ethanol concentration on ethanol preference, ethanol consumption, and total fluid intake.

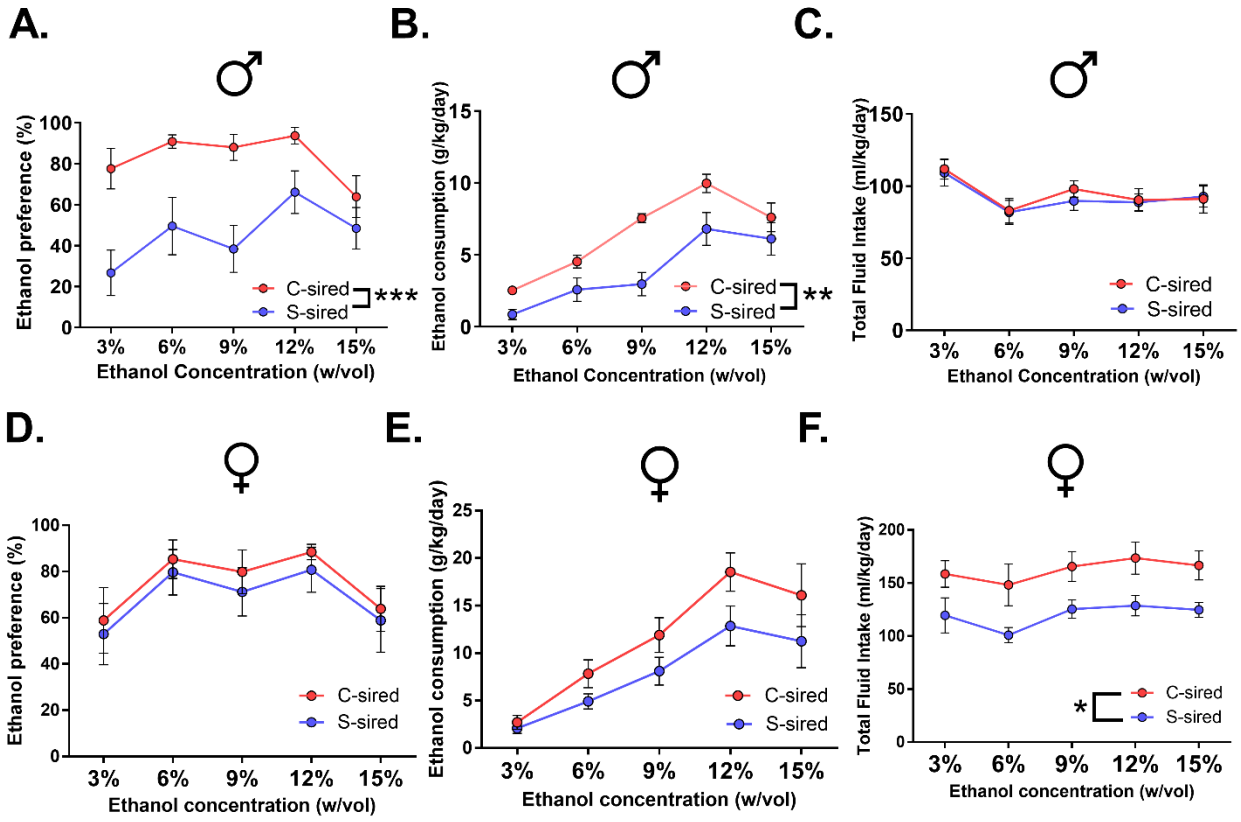


Figure 8. Paternal stress reduces ethanol drinking behavior in males

For male mice (N=8/group), paternal stress significantly reduced **(A)** ethanol preference and **(B)** ethanol consumption and had no effect on **(C)** total fluid intake. For female mice (N=8/group), there was no effect of paternal stress on **(D)** ethanol preference or **(E)** ethanol consumption, and a significant reduction in **(F)** total fluid intake. *=p<0.05, **=p<0.01, ***=p<0.001. Data presented as μ ± SEM. Each data point represents the daily average calculated from four 24 hour trials.

3.3.3 No effect of paternal stress on saccharin or quinine preference

There was no effect of paternal stress on male or female drinking preference for saccharin or quinine tastants (p>0.05; Figure 9A-B).

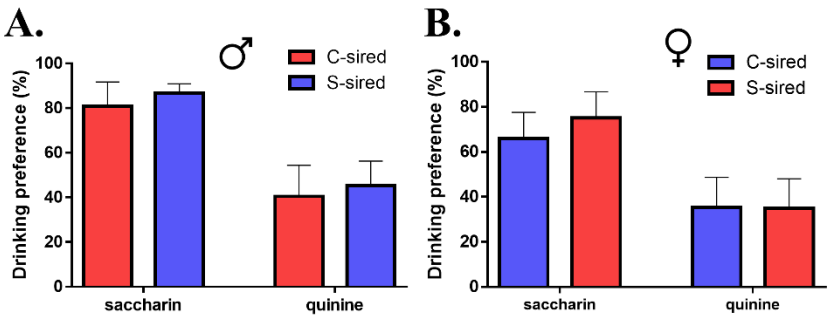


Figure 9. No effect of paternal stress on saccharin and quinine preference

(A) No effect of paternal stress on saccharin or quinine drinking preference in males (N=8/group). **(B)** No effect of paternal stress on saccharin and quinine preference in females (N=8/group). Data presented as μ ± SEM.

3.3.4 Paternal stress reduces binge-like ethanol drinking in males

The effects of paternal stress on ethanol drinking behavior were further assessed using the drinking in the dark model for "binge-like" ethanol intake (Thiele et al., 2014). For ethanol consumption in males, paternal stress significantly reduced ethanol consumption ($F_{(1,13)}$ = 13.12, $p<0.01$; Figure 10A). There was no ethanol consumption × test day interaction. For ethanol consumption in females, there was no effect of paternal stress on ethanol consumption ($p>0.05$, Figure 10B). There was no effect of paternal stress × test day.

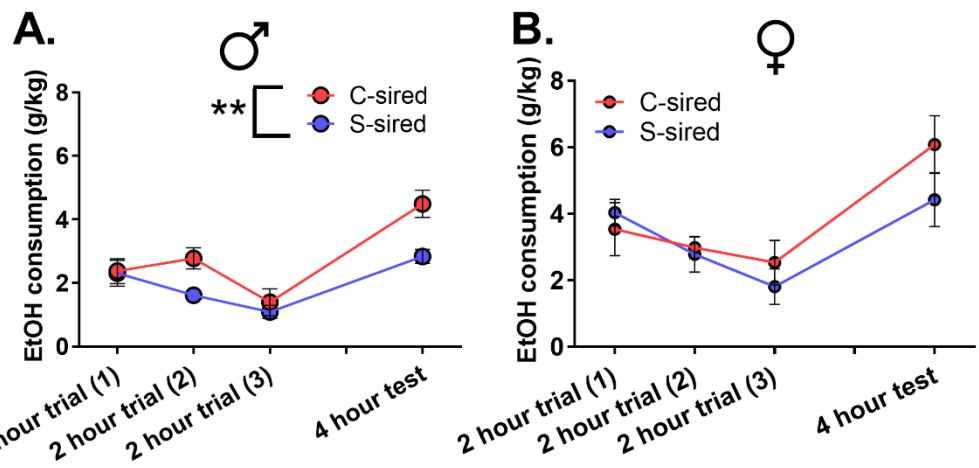


Figure 10. Paternal stress reduces binge-like ethanol consumption in males

(A) Reduced ethanol consumption in the drinking in the dark assay in S-sired males (N=8/group).

(B) No effect of paternal stress on ethanol consumption in females (N=8/group). ***=p<0.001.

Data presented as μ ± SEM.

3.3.5 No effect of paternal stress on ethanol-induced anxiolysis

Offspring were examined for sensitivity to the anxiolytic effects of ethanol injection in the elevated plus maze. For male offspring, there was no effect of ethanol, paternal stress, or paternal stress × ethanol injection on open arm time or total arm entries (Figure 11A-B). For female offspring, there was a significant effect of ethanol injection ($F_{(1, 26)} = 7.41$, $p<0.05$, Figure 11C), but not paternal stress or paternal stress × ethanol injection on open arm time and no effect of ethanol injection, paternal stress or paternal stress × ethanol injection on total arm entries (Figure 11D).

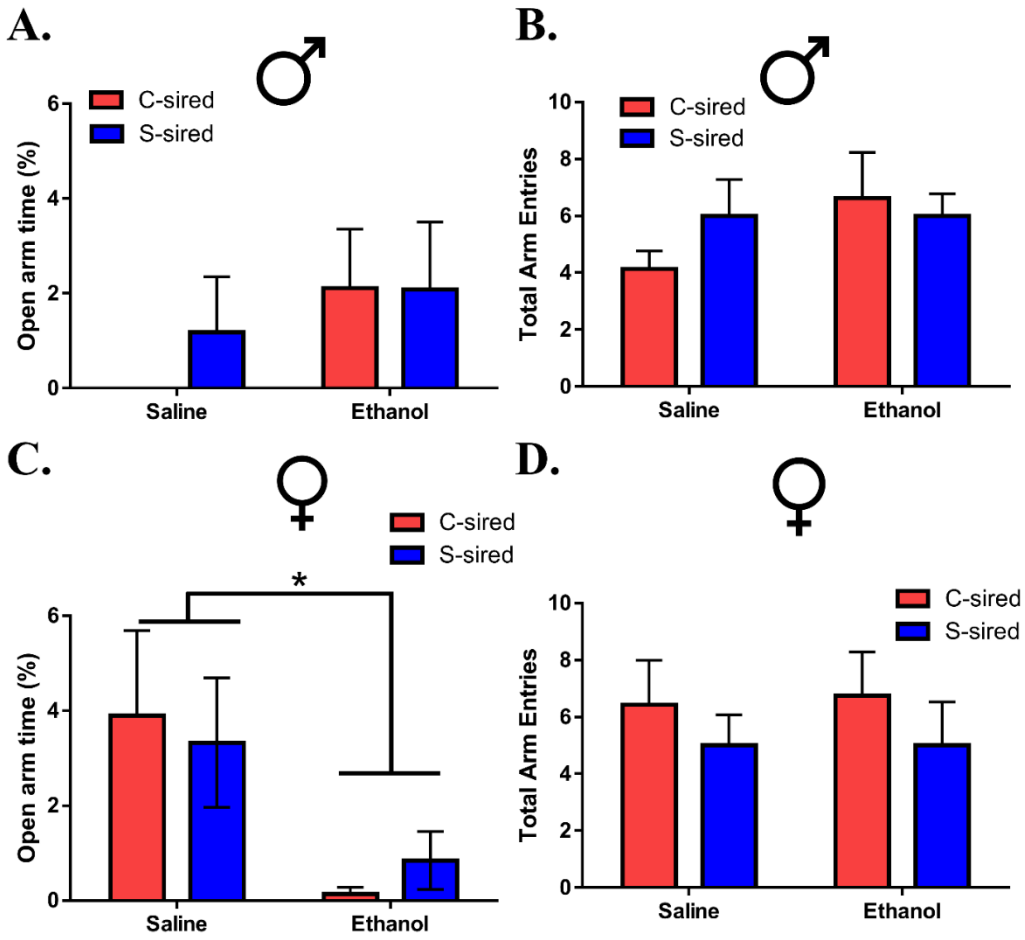


Figure 11. No effect of paternal stress on ethanol-induced anxiolysis

For **(A-B)** males (N=8-10/each represented column) and **(C-D)** females (N=8/each represented column), there was no effect of paternal stress on open arm time or total arm entries. *=p<0.05. Data presented as μ ± SEM.

3.3.6 No effect of paternal stress on HPA axis responsivity

There was no significant effect of paternal stress or paternal stress × time on CORT levels in response to 15 min of restraint stress in male (Figure 12A) or female (Figure 12B) mice.

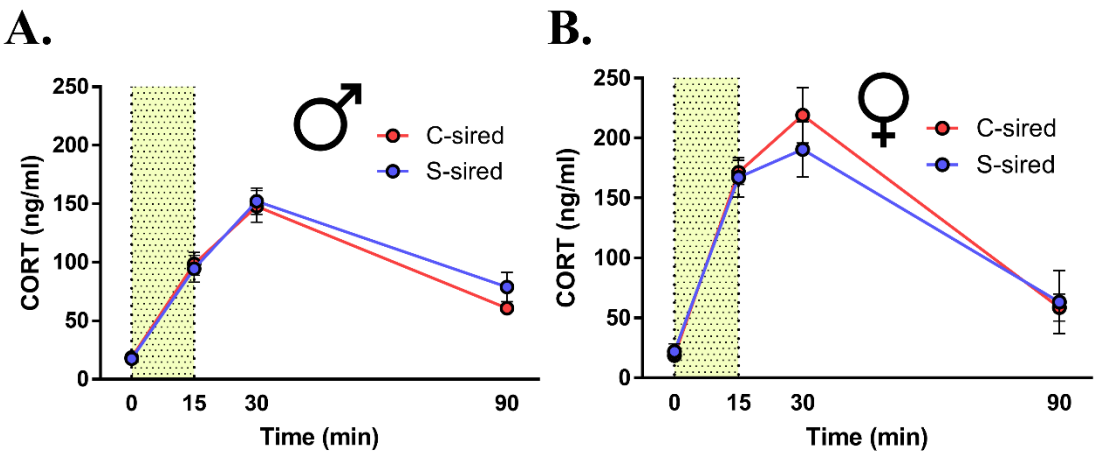


Figure 12. No effect of paternal stress on HPA responsivity

No effect of paternal stress on **(A)** male (N=8/group) or **(B)** female (N=8/group) plasma CORT levels at 0, 15, 30, and 90 min from the onset of 15-min restraint stress (represented by shaded bar). Data presented as μ ± SEM.

3.3.7 No effect of paternal stress with vendor-born and -shipped (VBS) sires on ethanol drinking behavior

Importantly, the first cohort of paternal stress sires was the second generation of an in-house colony (IHC), born within the animal vivarium and within the same facility as the animal behavioral core (see Figure 7C). As animal vivarium conditions can vary, in order to determine the reproducibility of our findings across laboratory settings, we examined a second cohort using sires born with the vendor (The Jackson Laboratory, Bar Harbor, ME) and shipped (VBS) to the animal vivarium one week prior to the onset of chronic stress or control conditions (Figure 13A). With this cohort, there

was no effect of stress or stress × week of exposure on body weight (Figure 13B, p>0.05). There was no effect of paternal stress on litter size (Figure 13C, p>0.05). For VBS-sired offspring, there was no effect of paternal stress or paternal stress × postnatal age on male and female offspring body weights (Figures 13D-E, p>0.05).

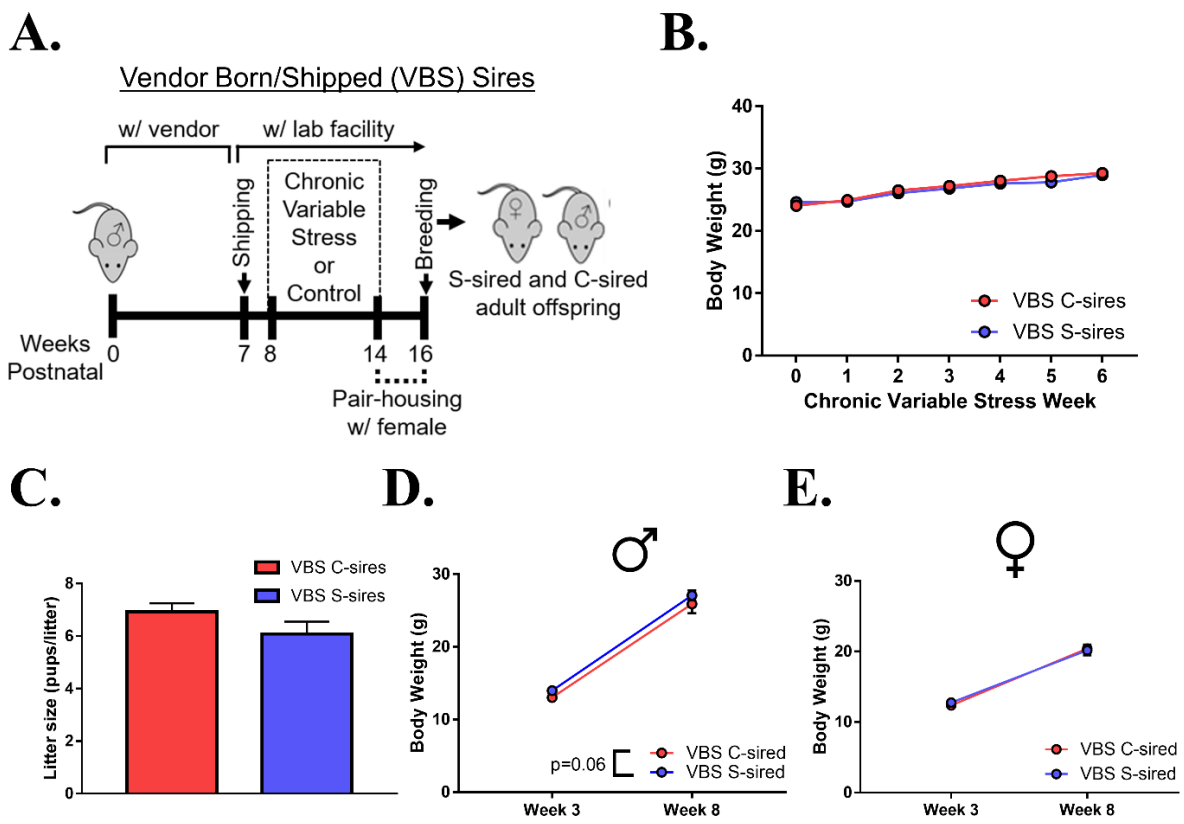


Figure 13. Paternal stress with VBS sires

(A) Sires were shipped from the vendor at 7 weeks postnatal to the animal vivarium before beginning chronic stress from 8 to 14 weeks postnatal and, at 16 weeks, breeding to produce VBS S-sired and C-sired males and females. **(B)** No effect of chronic stress on body weight (N=16/group). **(C)** No effect of paternal stress on litter sizes (N=13-14/group). **(D)** No effect of paternal stress on body weights of VBS-sired males (N=39-50/group) or **(E)** VBS-sired females (N=29-51/group). Data presented as μ ± SEM and error bars are obscured in panels B, D, E.

Examining the effect of paternal stress with VBS-sires on two-bottle free choice ethanol drinking behavior there was no effect of paternal stress or paternal stress × ethanol concentration on male (Figure 14A-C, all $p>0.05$) or female (Figure 14D-F, all $p>0.05$) ethanol preference, ethanol consumption, or total fluid intake.

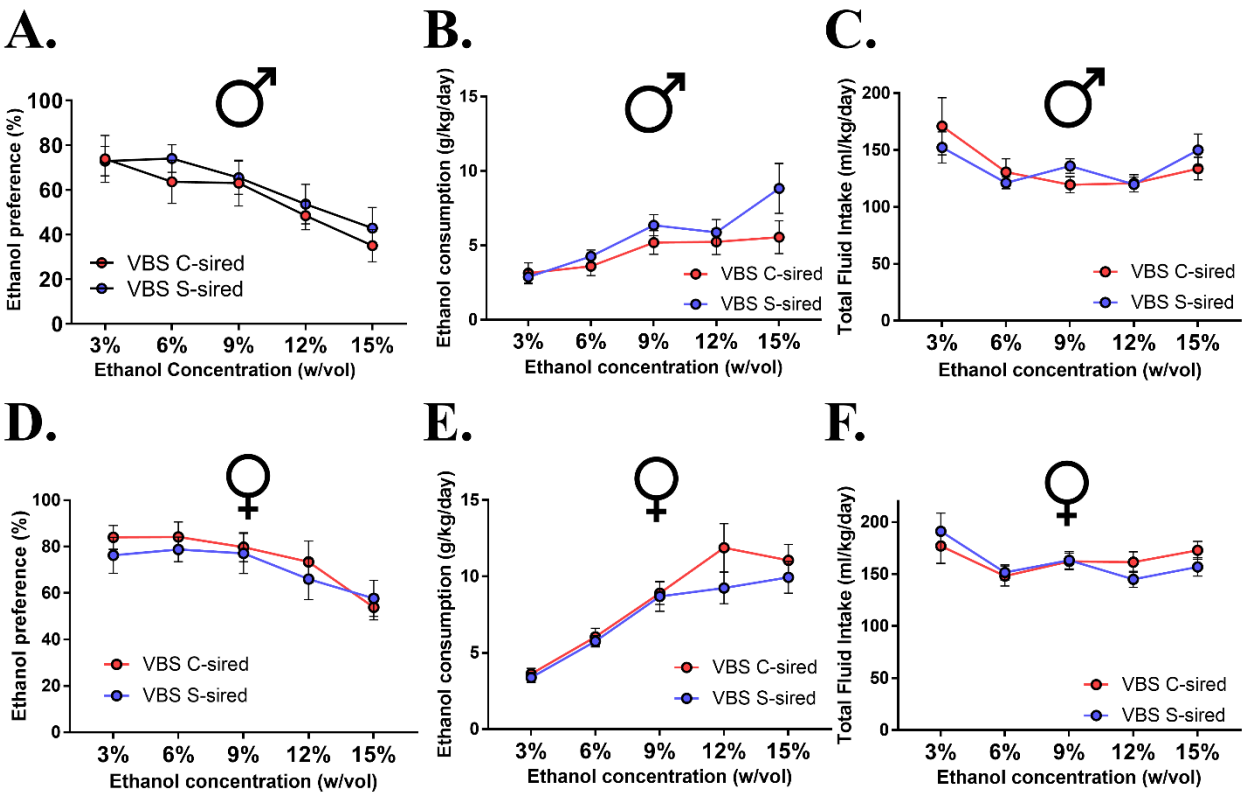


Figure 14. No effect of paternal stress with VBS sire on ethanol drinking

No effect of paternal stress on ethanol preference, ethanol consumption, and total fluid intake of **(A-C)** males (N=10/group) or **(D-F)** females (N=10/group). Data presented as μ ± SEM.

In the drinking in the dark assay with VBS-sired offspring, there was no effect of paternal stress or paternal stress × test day on male (Figure 15A, $p>0.05$) or female (Figure 15B, $p>0.05$) ethanol consumption

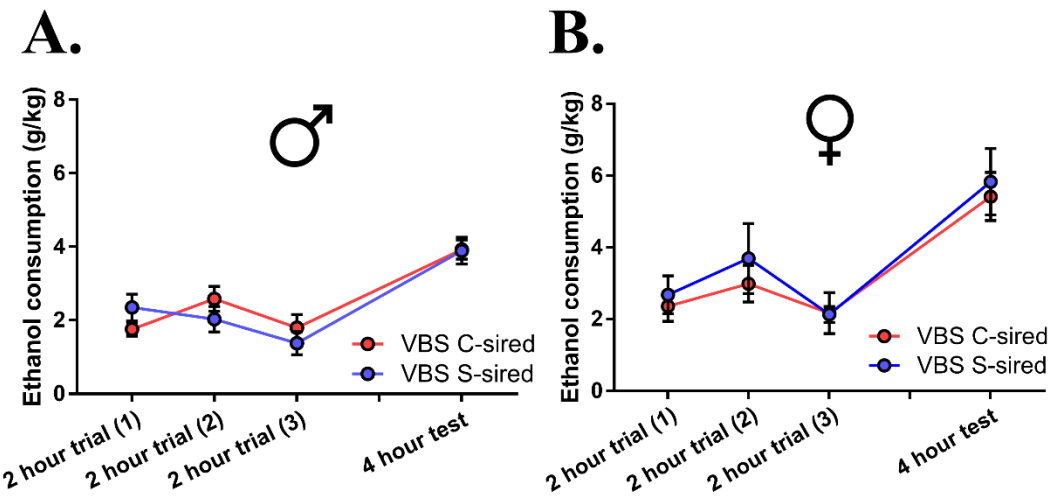


Figure 15. No effect of paternal stress with VBS sires on binge-like ethanol drinking

No effect of paternal stress with VBS sires on ethanol consumption of (A) males (N=9-10/group) or (B) females (N=10/group). Data presented as μ ± SEM.

3.3.8 No effect of paternal chronic stress with VBS-sires on ethanol-induced anxiolysis in offspring

For VBS-sired males, there was a significant effect of ethanol injection on open arm time ($F_{(1, 32)}$ = 5.35, $p<0.05$, Figure 16A), but no effect of paternal stress or paternal stress × ethanol injection. There was no effect of ethanol injection, paternal stress, or paternal stress × ethanol injection on open arm entries or total arm entries (Figure 16B-C). For VBS-sired females, there was a significant effect of ethanol injection on open arm time ($F_{(1, 33)}$ = 6.39, $p<0.05$, Figure 16D), but no effect of paternal stress or paternal stress × ethanol injection. There was no effect of ethanol injection, paternal stress, or paternal stress × ethanol injection on open arm entries or total arm entries (Figure 16E-F).

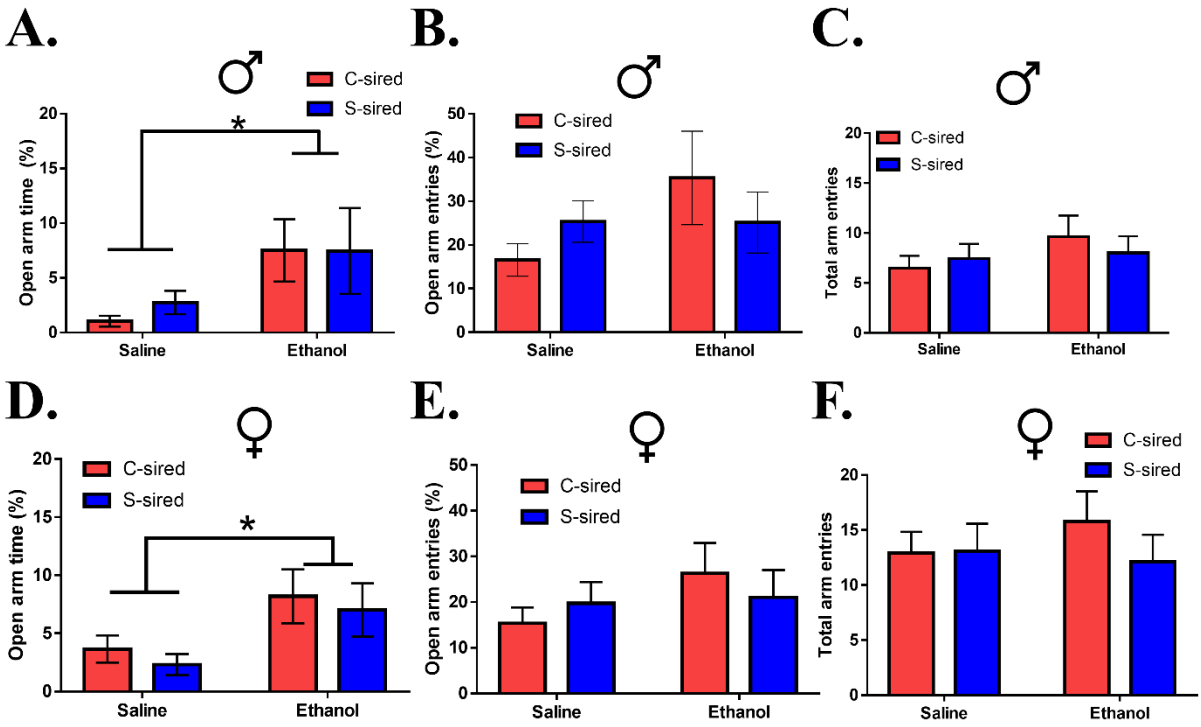


Figure 16. No effect of paternal stress on ethanol-induced anxiolysis in VBS-sired mice

No effect of paternal stress on open arm time, open arm entries, or total arm entries for (A-C) male (N=8-10/represented column) or (D-F) female (N=10/represented column). Data presented as μ ± SEM.

3.3.9 Role for sire source in intergenerational effects of stress

Given the contrasting effects of paternal stress on ethanol drinking preference and consumption in males with either IHC or VBS sires, the effect of sire source on intergenerational ethanol drinking behaviors was examined directly in a third cohort of males (females not included due to cohort size limitations and the absence of ethanol phenotypes in the first two cohorts). For sire body weights, there were significant main effects of stress ($F_{(1,5)} = 12.41$, $p<0.01$; Figure 17A) and sire source

($F_{(1,5)}$=24.33, $p<0.001$) with no significant effects of stress × sire source and no significant interaction with week of exposure ($p>0.05$).

One week after chronic variable stress, there was no effect of stress, but a significant effect of sire source ($F_{(1, 32)} = 6.0$, $p<0.05$, Figure 17B) and a trending effect of sire source × stress ($F_{(1, 32)} = 3.9$, $p<0.06$; Figure 17B) on basal CORT levels. *Post-hoc* analysis of the sire source × stress trend revealed that chronic variable stress significantly increased plasma CORT levels in VBS S-sires vs C-sires ($p<0.05$), but not IHC S-sires vs. C-sires ($p>0.05$). Paternal stress significantly reduced litter size ($F_{(1, 36)} = 5.83$, $p<0.05$; Figure 17C) and there was no effect of sire source or paternal stress × sire source ($p>0.05$). For offspring body weights, there was no effect of paternal stress, sire source, paternal stress × sire source and no significant interaction with postnatal week ($p>0.05$, Fig. 17D).

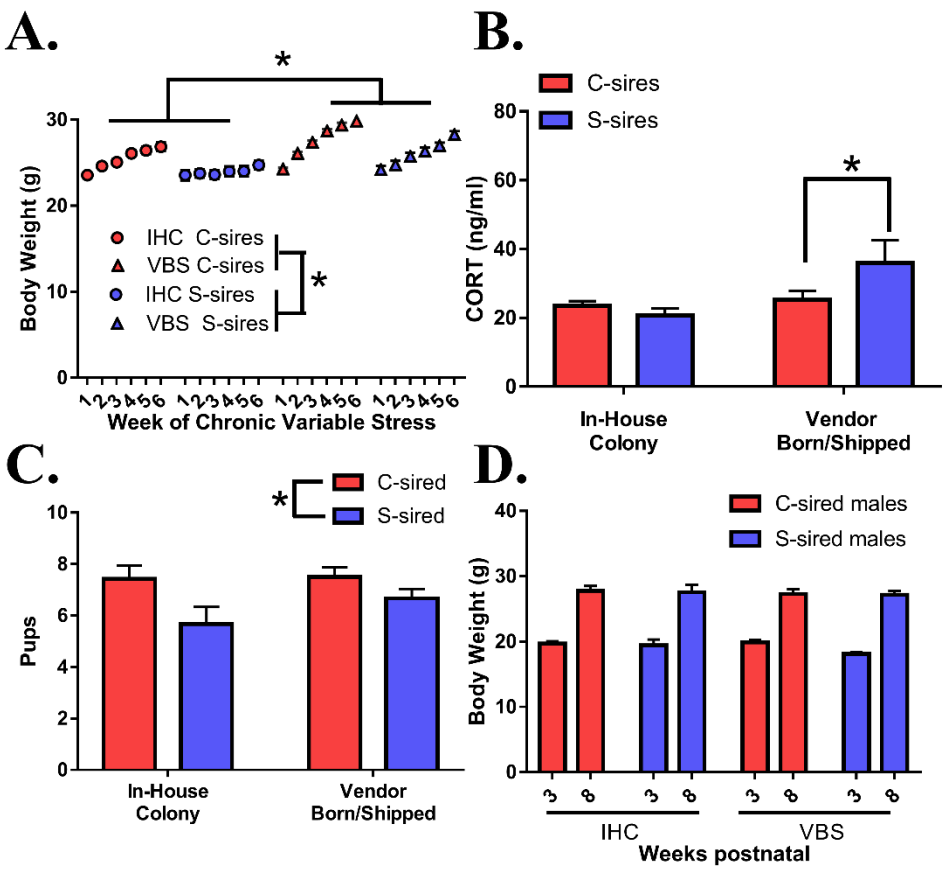


Figure 17. Examining role for sire-source in the intergenerational effects of stress

(A) Stress significantly reduced body weights and IHC sires showed reduced body weights vs VBS sires (N=8-10/group). **(B)** Significant increase in basal plasma CORT in VBS S-sires (N=8-10/group). **(C)** Significant effect of paternal stress on litter sizes (N=8-14/group). **(D)** No effect of paternal stress on body weights at 3 and 8 weeks postnatal (N=7-19/group). *=p<0.05. Data presented as μ ± SEM. Error bars are obscured by data points in panel A.

3.3.10 Sire-source dependent paternal chronic stress reduces ethanol drinking in male offspring

In the two-bottle free choice ethanol drinking test, there was a significant effect of paternal stress × sire source on both ethanol preference ($F_{(1,39)}$ =5.68; p<0.05) and ethanol consumption ($F_{(1,39)}$ =

5.36; $p<0.05$). Therefore, the effects of paternal stress were examined independently by sire source (IHC or VBS). For IHC-sired males, there was no effect of paternal stress or paternal stress × ethanol concentration on ethanol preference. There was a significant effect of paternal stress ($F_{(1, 17)} = 4.66$, $p<0.05$) and paternal stress × ethanol concentration ($F_{(4, 68)} = 3.22$, $p<0.05$, Figure 18B) on ethanol consumption. *Post-hoc* analysis revealed reduced ethanol consumption at 12% ($p<0.05$) and 15% ($p<0.001$) ethanol concentrations in IHC S-sired males. There was no effect of paternal stress or paternal stress × ethanol concentration on total fluid intake for IHC-sired offspring (Figure 18C). For VBS-sired males, there was no effect of paternal stress or paternal stress × ethanol concentration on ethanol preference (Figure 18D) or consumption (Figures 18E). For total fluid intake, there was a significant effect of paternal stress ($F_{(1, 22)} = 9.20$, $p<0.01$) and paternal stress × ethanol concentration ($F_{(4, 88)} = 2.80$, $p<0.05$) on total fluid intake (Figure 18F). *Post-hoc* analysis revealed a significant reduction in total fluid intake at ethanol concentrations of 6, 9, 12, and 15% in VBS S-sired vs C-sired males ($p<0.05$ for 6 and 9%, $p<0.01$ for 12%, $p<0.001$ for 15%).

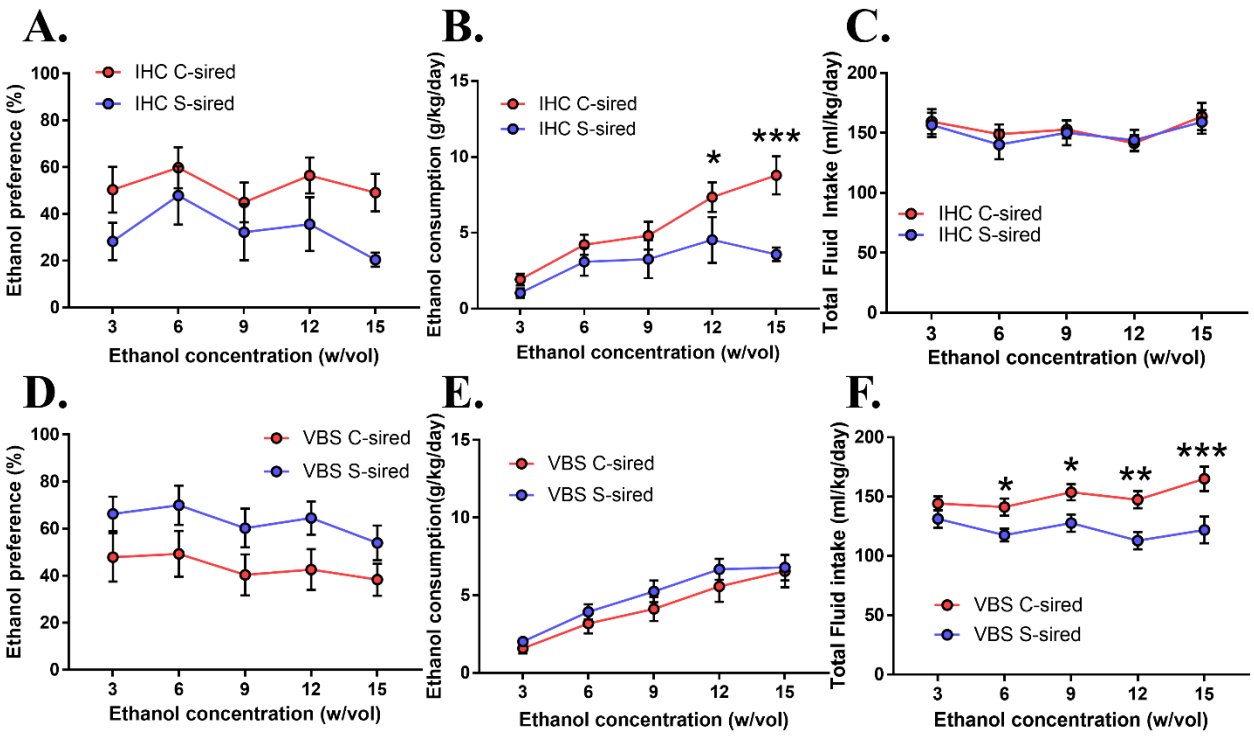


Figure 18. Paternal stress selectively with IHC-sires reduces ethanol drinking in males

(A) No change in ethanol preference of IHC S-sired males. **(B)** Significant reduction in ethanol consumption in IHC S-sired males. **(C)** No effect of paternal stress on total fluid intake in IHC-sired males. **(D-E)** No effect of paternal stress on ethanol preference or consumption in VBS-sired males. **(F)** Significant reduction in total fluid intake in VBS S-sired males (N=12/VBS groups and 7-12/IHC groups). *=p<0.05, **=p<0.01, ***=p>0.001. Data presented as μ ± SEM.

3.3.11 Sire-source dependent paternal chronic stress reduces binge-like ethanol drinking

In the drinking in the dark assay, there was a significant effect for paternal stress ($F_{(1,38)}$=7.05, p<0.05), paternal stress × sire-source ($F_{(1,38)}$=4.65, p<0.05), and paternal stress × sire-source × test day ($F_{(1,38)}$=5.75, p<0.05) on ethanol consumption. Therefore, the IHC- and VBS-sired mice were again analyzed separately. For IHC-sired males, there was a significant effect of paternal stress ($F_{(1,}$

$_{17)}$ = 10.91, p<0.01) and paternal stress × test day ($F_{(1, 17)}$ = 6.12, p<0.05; Figure 19A). *Post-hoc* analysis revealed significantly reduced ethanol consumption during the four-hour test in S-sired vs C-sired males (p<0.001). For VBS-sired offspring, there was no effect of paternal stress or paternal stress × test day on ethanol consumption (Figure 19B).

For blood ethanol concentrations (BECs) after the four-hour test, there was no effect of paternal stress or sire source, but there was a significant paternal stress × sire-source interaction ($F_{(1, 26)}$ = 4.78; p<0.05, Fig. 19C). *Post-hoc* analyses revealed reduced BECs in IHC S-sired vs C-sired males (p<0.05) and no difference in BECs for VBS S-sired vs C-sired males.

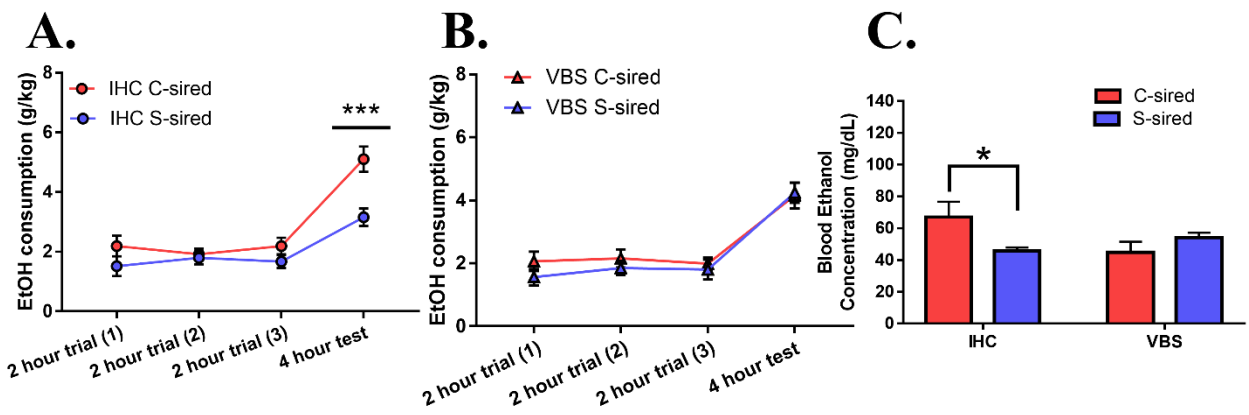


Figure 19. Sire-source dependent paternal stress reduces binge-like ethanol drinking

(A) Significant effect of paternal chronic variable stress on ethanol consumption in IHC-sired offspring. (B) No effect of VBS pre-stress sire conditions on ethanol consumption. (C) Significant effect of paternal chronic stress on blood ethanol concentrations following the four-hour drinking in the dark test trial for IHC, but not VBS S-sired vs. C-sired male offspring (N= 7-12/group for IHC-sired males and 12/group for VBS-sired males). *=p<0.05, ***=p<0.001. Data presented as μ ± SEM.

3.4 DISCUSSION

The results from Chapter 3 revealed that paternal chronic variable stress exposure suppressed ethanol drinking behavior selectively in male offspring. However, this intergenerational effect was specific to sires born within the in-house colony and was not reproducible with sires born and shipped from the vendor during adulthood. In addition, paternal chronic variable stress did not alter ethanol-induced anxiolysis or HPA axis responsivity in offspring. Overall, the present results support the hypothesis that chronic stress imparts unique ethanol drinking phenotypes to male offspring, although this intergenerational effect is uniquely dependent on sire source.

Paternal chronic variable stress significantly reduced ethanol drinking behavior in two distinct ethanol drinking paradigms, the two-bottle choice ethanol drinking test with continuous access and the limited access drinking in the dark test. The reduction in two-bottle choice ethanol drinking in males, and the increase in adult body weight, was strikingly similar to the intergenerational effects of paternal chronic intermittent ethanol exposure (Finegersh and Homanics, 2014; Rompala et al., 2017). As in those studies, reduced ethanol drinking behavior was selective to male offspring and specific to ethanol, as saccharine and quinine drinking preference were unaltered.

It is unclear exactly how chronic variable stress and chronic intermittent ethanol exposure impart convergent intergenerational effects on ethanol drinking behavior. Each chronic variable stressor and the ethanol vapor exposure significantly increase CORT levels (Lee and Rivier, 2003; Willner, 2017). Moreover, both chronic stress and ethanol exposure reshape glucocorticoid receptor expression throughout the central nervous system (Vendruscolo et al., 2012; Guidotti et al., 2013; Willner, 2017). Fittingly, chronic variable stress and chronic intermittent ethanol exposures have similar effects on HPA responsivity to acute restraint stress in the next generation (Rodgers et al.,

2013; Rompala et al., 2016), although the present study failed to reproduce the results from Rodgers et al. (discussed below). As CORT increases ethanol drinking behavior in rodents (Fahlke et al., 1994; Fahlke et al., 1996; Fahlke and Eriksson, 2000), HPA axis hyporesponsivity may contribute to intergenerational ethanol drinking behavior in both paternal exposure paradigms. Thus, further study of these two unique sire exposures may be advantageous for identifying shared epigenetic alterations in offspring brain driving reduced intergenerational ethanol drinking behavior.

The other major finding in Chapter 3 was the dependence of intergenerational ethanol drinking behaviors on sire source. Specifically, while all utilized mouse lines were originally sourced to The Jackson Laboratory, one cohort of sires was the second generation of an in-house colony (IHC) and the other cohort was the first generation, born with the vendor and shipped (VBS) one week prior to the onset of chronic variable stress. Remarkably, stressed IHC-sires imparted reduced ethanol drinking behaviors to male offspring, while stressed VBS-sires did not. This finding suggests that IHC and VBS sires differentially respond to chronic variable stress. Supporting this notion, chronic stress increased basal CORT levels in VBS-sires, but did not effect IHC-sires. Relatedly, paternal preconception social defeat stress differentially affects offspring CORT and social behavior depending on whether fathers were determined to be susceptible or resilient to social defeat (Dietz et al., 2011). Thus, differences in the sire environment prior to chronic stress may have shaped resilience or vulnerability to stress that, in turn, impart disparate ethanol drinking behaviors to offspring.

There are several potential effects of animal shipping and vendor history that may conceivably mediate sensitivity to chronic variable stress. For instance, shipping stress increased blood pressure for up to three weeks after shipping in mice (Hoorn et al., 2011). Additionally, HPA axis responsivity was found to vary between animal vendors (Turnbull and Rivier, 1999; Pecoraro

et al., 2006; Olfe et al., 2010). Finally, vendor history plays a causal role in shaping the fecal microbiota of mice (Ericsson et al., 2015), and microbiota alterations influence stress-related behaviors (Foster and McVey Neufeld, 2013). Thus, future studies will need to directly examine IHC vs VBS mice for various predisposing adaptations in stress physiology that may predict chronic variable stress vulnerability and intergenerational phenotypes.

There was no effect of paternal preconception stress on ethanol-induced anxiolysis in experiments with both IHC and VBS sire cohorts. As there was no effect of paternal stress on IHC or VBS offspring behavior, ethanol-induced anxiolysis was not analyzed further with the mixed IHC- and VBS-sire cohort. Previously, paternal preconception ethanol exposure was found to increase ethanol-induced anxiolysis in the elevated plus maze. Thus, the intergenerational effects of paternal stress and paternal ethanol on ethanol-related behaviors do not entirely overlap.

Surprisingly, there was no effect of paternal chronic variable stress on male and female offspring HPA axis responsivity, as previously reported (Rodgers et al., 2013). Due to mouse number limitations, HPA axis responsivity was examined in mice following two other behavioral tasks prior to testing (i.e., elevated plus maze with ethanol injection and drinking in the dark with two weeks between each test). Thus, it is possible that the effects of paternal chronic variable stress on HPA axis responsivity were masked by the preceding behavioral battery. In addition, in the present study, B6 sires were bred with Strain 129 females to produce B6 × Strain 129 hybrid offspring. In Rodgers et al., both sires and breeder females were on a B6 × Strain 129 mixed background. Therefore, the effects of chronic variable stress may differ between B6 and B6 × 129 males given the differences in stress responsivity between these two strains (van Bogaert et al., 2006; Chan et al., 2017b). Moreover, B6 and Strain 129 females exhibit different levels of maternal

care (Champagne et al., 2007). Therefore, direct comparison between the present study and Rodgers et al. must be carefully considered.

Many studies have implicated germline epigenetic alterations in the intergenerational effects of paternal preconception environment. For instance, postnatal maternal separation and chronic variable stress alter several sperm microRNAs (Rodgers et al., 2013; Gapp et al., 2014). Remarkably, in Rodgers et al, chronic variable stress increased nine miRNAs in sperm that, when injected into normal fertilized embryos, recapitulate the intergenerational effects of paternal stress on HPA axis responsivity (Rodgers et al., 2015). Whether the same microRNAs are enriched in stressed IHC or VBS sires in the present study remains to be determined. Notably, chronic ethanol vapor exposure does not affect any of the nine stress-enriched miRNAs (see Section 4.3.1). Thus, other small noncoding RNA types, such as tRNA-derived small noncoding RNAs (Chen et al., 2016a; Sharma et al., 2016), or alternative epigenetic mechanisms such as DNA methylation and histone modifications are more likely to underlie intergenerational ethanol drinking behaviors.

The results from Chapter 3 expand the rapidly growing number of effects associated with paternal preconception stress to include reduced ethanol drinking behavior. These results suggest the interwoven mechanisms of stress and ethanol extend across generations. By illuminating the significance of paternal preconception environment in ethanol drinking behavior, these findings have major implications for determining familial risk of addiction disorders with complex behavioral symptomology.

4.0 CHRONIC ETHANOL ALTERS SMALL NONCODING RNAS IN SPERM

4.1 INTRODUCTION

Studies examining the cross generational effects of alcohol have primarily focused on maternal alcohol abuse during pregnancy given the severe risk of inducing developmental deficits that typify fetal alcohol syndrome in offspring. Given the long-held belief that fathers only contribute genomic information through the germline, the preconception health of the father has historically been viewed as inconsequential to offspring development. However, a surge of recent preclinical research has triggered a growing interest in how various paternal factors such as stress, diet, and alcohol prior to conception can also affect the offspring phenotype, presumably via epigenetic mechanisms in sperm (Finegersh et al., 2015b; Stuppia et al., 2015; Schagdarsurengin and Steger, 2016a).

Various forms of chronic ethanol treatment in male rodents prior to conception have been found to directly affect diverse phenotypes such as body weight, cortical thickness, and even behavioral sensitivity to drugs like amphetamine in the next generation (reviewed in Finegersh et al., 2015b). In addition, males exposed to chronic intermittent ethanol vapor produce male offspring with reduced ethanol drinking behavior, increased ethanol sensitivity and attenuated stress responsivity (see Chapter 2) (Finegersh and Homanics, 2014; Rompala et al., 2016; Rompala et al., 2017). Since these studies were performed using isogenic sires that played no role in offspring rearing and development, paternal preconception ethanol may be driving unique changes in offspring behavior through nongenomic mechanisms in sperm. Therefore, greater emphasis should

be put on understanding the consequences of paternal alcohol abuse prior to conception and identifying potential epigenetic mechanisms in the germline.

Although sperm DNA is densely packed in the nucleus, sperm are not solely passive carriers of genetic material, but also feature a complex epigenetic machinery. As most histones in sperm are exchanged for protamines during spermatogenesis, and sperm DNA loses most of its methylation at fertilization, identifying sperm-based mechanisms of epigenetic inheritance has been challenging (Heard and Martienssen, 2014). However, in addition to chromatin, sperm have a unique RNA profile enriched with diverse small noncoding RNA species (Ostermeier et al., 2002; Krawetz et al., 2011). These include well-described small RNA classes like microRNA (miRNA) and piwi-interacting RNA (piRNA) as well as under-studied groups like tRNA- and mitochondria-derived small RNAs that are overrepresented in sperm (Peng et al., 2012; Schuster et al., 2016b). As the sperm genome is thought to be transcriptionally quiescent (Kierszenbaum and Tres, 1975), these small noncoding RNAs may instead function during the earliest stages of embryogenesis. Indeed, sperm RNA is delivered to the oocyte (Ostermeier et al., 2004) and recent studies have found that sperm-derived small noncoding RNAs are required for normal embryonic development (Liu et al., 2012; Yuan et al., 2016; Guo et al., 2017).

The earliest evidence for RNA-mediated inheritance demonstrated that a mutation-induced white tail color phenotype in mice could be transmitted to wild type offspring via altered sperm RNA (Rassoulzadegan et al., 2006). Since then, numerous studies have found that sperm small noncoding RNAs are sensitive to various paternal environmental factors including stress, diet and exercise (Rodgers et al., 2013; Gapp et al., 2014; Chen et al., 2016a; de Castro Barbosa et al., 2016; Sharma et al., 2016; Short et al., 2016; Short et al., 2017). Moreover, in humans, alterations in sperm small noncoding RNAs have been associated with obesity (Donkin et al., 2016) and smoking

history (Marczylo et al., 2012). Finally, recent intergenerational studies have shown that cross generational effects of stress and diet can be recapitulated in offspring derived from embryos injected with stress- or diet- altered sperm RNAs, respectively, suggesting a causal role in paternal epigenetic inheritance (Gapp et al., 2014; Grandjean et al., 2015; Rodgers et al., 2015; Chen et al., 2016a).

Ethanol has deleterious effects on several measures of sperm quality in mice such as sperm count, circulating testosterone levels, and overall fertility, and similar effects have been found in alcoholic men (reviewed in La Vignera et al. 2013). Additionally, ethanol has been shown to impact epigenetic mechanisms in sperm. For instance, DNA methylation at imprinting gene loci is reduced in chronic ethanol-treated mice (Knezovich and Ramsay, 2012; Finegersh and Homanics, 2014; Liang et al., 2014) and men with alcohol use disorder (Ouko et al., 2009). However, whether ethanol directly affects small noncoding RNAs in sperm is entirely unknown. This is an important question given the prevalence of alcohol use disorder and the implication of small noncoding RNAs as a causal factor in paternally-linked epigenetic inheritance of complex behavior. Therefore, given the evidence that paternal preconception ethanol exposure has intergenerational effects, the current chapter tests the hypothesis that ethanol causes epigenetic reprogramming of sperm small noncoding RNAs.

4.2 MATERIALS AND METHODS

4.2.1 Animals

All experiments were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh and conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Specific pathogen free C57BL/6J (B6) and CD-1 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were habituated to the University of Pittsburgh animal facility for at least one week prior to initiation of experiments. Mice were housed under 12 h light/dark cycles (0700-1900) and had *ad libitum* access to food (irradiated 5P76 ProLab IsoPro RMH 3000, [LabDiet, St. Louis, MO]) and water.

4.2.2 Chronic intermittent ethanol vapor inhalation

Chronic intermittent ethanol vapor exposure was performed as previously described (Finegersh and Homanics, 2014; Finegersh et al., 2015a; Rompala et al., 2016; Rompala et al., 2017). Briefly, eight-week-old male B6 mice were randomly assigned to one of two treatments: half of the mice were exposed to ethanol inhalation chambers in the home cage with water and food for five weeks from 09:00-17:00 over five consecutive day blocks with two days in between blocks. The other half of mice were assigned to the room air control group in identical chamber conditions without ethanol vapor. All animals were group-housed throughout the experiment and cages, food, and water were all changed routinely after the final exposure of each week. Blood ethanol concentration was measured after the final ethanol exposure of each week by extracting tail vein blood ($\leq$10 µl) using heparin-coated capillary tubes (Drummond, Broomall, PA) and running plasma samples (extracted

from blood by centrifugation at 2300 × g for 10 min) on an Analox Ethanol analyzer (AM1, Analox Instruments, London, UK). Tail blood was drawn from all groups to control for stress from the extraction procedure. Ethanol content in the ethanol inhalation chambers was monitored using a custom sensor generously provided by Brian McCool and flow rates in the chambers were adjusted weekly based on blood ethanol concentration measurements made during the preceding week. Importantly, animals do not lose significant body weight (defined as >10%). In addition, the effects of ethanol vapor on lungs, heart, and liver are comparable to those associated with other chronic ethanol exposure models (Mouton et al., 2016).

4.2.3 Isolation of motile sperm from cauda epididymis

Sperm samples were isolated from adult male mice sacrificed ~16-19 hours following the final ethanol or room air exposure during the light cycle (08:00-11:00). Briefly, after euthanasia by CO2 asphyxiation, left and right cauda epididymides were dissected into 1.5 ml of EmbryoMax Human Tubal Fluid (HTF) (Cat# MR-070-D; Sigma-Aldrich, St. Louis, MO) at 37 ºC. Several small cuts were made in each epididymis to release the sperm into solution. The sperm solution was then transferred to a 1.5 ml Eppendorf tube and motile sperm were disperse in the media for 20 min at 37 ºC. The top 1.2 ml of supernatant was carefully collected for further processing while the settled epididymal tissue was stored at -80º C for later RNA extraction. Next, the recovered supernatant was centrifuged at 2000 × g for 5 min to pellet the sperm. The supernatant from this step was saved for epididymosome isolation and the pelleted sperm was then gently resuspended by pipetting in 1.0 ml of somatic cell lysis buffer (0.1% SDS (Cat# L3771, Sigma-Aldrich), 0.5% Triton X-100 (Cat# IB07100, IBI Scientific, Peosta, IA) which was placed on ice for 20 min. This step is also critical for lysis and removal of adherent RNA-containing extracellular vesicles (Sharma et al.,

2016). Next, the sperm was re-pelleted and washed twice with ice cold 1X PBS. After the final wash, the sperm pellet was lysed in 1.0 ml Trizol (Cat# 15596026; Thermo Fisher, Waltham, MA) supplemented with 200 mM β-mercaptoethanol (Cat# 516732; Sigma-Aldrich) to facilitate lysis of disulfide-bond enriched sperm cells. Lysis was performed using a 2.0 ml Dounce glass tissue homogenizer (Cat# 885302, Kimble Chase, Vineland, NJ) to break up the sperm pellet and further homogenized with a mechanical homogenizer on ice followed by brief heating at 65 ºC for 5 min before moving the sample back to ice. Complete lysis of the sperm nucleus was confirmed with light microscopy.

4.2.4 Isolation of extracellular vesicles from epididymis

Following the pelleting of motile cauda sperm, epididymosomes were isolated from the supernatant by filtration and ultracentrifugation. First, the epididymosome-containing media was centrifuged at 10,000 × g for 30 min at 4 ºC before being passed through a 0.2 µm syringe filter. Finally, epididymosomes were pelleted on a table top ultracentrifuge at 120,000 × g for 2 hours at 4 ºC, washed once with ice cold 1.5 ml PBS to remove excess protein aggregates, centrifuged again at 120,000 × g for 2 hours at 4 ºC and snap frozen with liquid nitrogen.

4.2.5 Enrichment for sperm from caput epididymis

Caput sperm were extracted from caput epididymis into 1.5 ml HTF at 37 ºC. Since caput sperm are not fully motile, sperm were centrifuged at 300 × g for three min to discard larger tissue pieces (while the partially motile sperm remained in suspension) and treated with somatic cell lysis buffer (described in Section 4.2.3) for 30 min to enrich for caput sperm and remove adherent

epididymosomes. Sperm were then recentrifuged at 2000 × g for 5 min and washed twice with 1X PBS. Sample purity was confirmed using light microscopy.

4.2.6 RNA isolation

All samples were lysed in Trizol (note the additional steps used for sperm described above) using phenol-chloroform separation. The aqueous phase was then processed with Zymo RNA Clean and Concentrator Kit with DNAse1 on-column treatment (Zymo Research, Irving, CA). Final sperm RNA concentrations were determined with Qubit RNA HS assay (Thermo Fisher) and RNA Analysis ScreenTape (Agilent, Santa Clara, CA) was used to confirm absence of 18S and 28S ribosomal peaks that are indicative of somatic cell contamination.

4.2.7 Small RNA sequencing

Barcoded small RNA libraries were prepared from 100 ng total RNA using NEBNext Small RNA for Illumina Kit (New England Biolabs, Ipswich, MA) per manufacturer's instructions. To prevent carry over of adapter dimers and nonspecific amplicons into the sequencing run, cDNA libraries were size-selected using 2% agarose gel electrophoresis with a Pippin Prep system (Sage Science, Beverley, MA). cDNA libraries were multiplexed and sequenced to an average depth of 9 million reads/sample on a NextSeq500 (Illumina, San Diego, CA) at the John G. Rangos Sr. Research Center at Children's Hospital of Pittsburgh of UPMC (Pittsburgh, PA). Investigators were blinded to treatment for both library preparation and sequencing.

4.2.8 Bioinformatics

Small RNA sequencing fastq files were filtered for read quality and trimmed with Cutadapt (Martin, 2011) which removed library preparation adapters and sequences outside the 15-45 nt range. For alignment to the mouse genome (GRCm38/mm10 assembly), Bowtie2 (Langmead and Salzberg, 2012) was used with standard parameters (-n 1,-l 18, -e 70). Mapped reads were annotated to small noncoding RNA features [provided in (Tang et al., 2017)] and summated with FeatureCounts (Liao et al., 2014). Final normalized counts were extracted and analyzed for differential expression using DESeq2 (Love et al., 2014). For tDR analysis, all sized fragments mapping to a single species (e.g. tDR Glu-CTC) were summed to a single data point. The program tDRmapper (Selitsky and Sethupathy, 2015) was used to determine the size distribution of tDR reads and to further classify tDR species by type of fragmentation (e.g. 5'-tRH). Predicted genes with 3'UTRs targeted by miRNAs were determined using TargetScan Mouse Custom ver. 5.2 (Lewis et al., 2005). For an unbiased prediction of genes with 5'UTR, coding, or 3'UTR regions targeted by tDR, RNAhybrid (Kruger and Rehmsmeier, 2006) was employed with established parameters (Schuster et al., 2016a). For analysis of gene promoter regions targeted by sperm tDR, the UCSC Blat alignment tool (Kent, 2002) was used with standard parameters (-stepSize=5, -repMatch=2253, -minScore=0, -minIdentity=0) and ethanol-responsive tDR sequences were queried against a database of promoter sequences for mouse genome obtained as 2000 bp stretches upstream the transcriptional start site of each gene (source: http://hgdownload.cse.ucsc.edu/goldenPath/mm10/bigZips/). Only tDR with >16 nt sequence homology with promoter regions were maintained. Gene ontology analysis was performed on all predicted target gene lists using DAVID Bioinformatics Resources ver. 6.8 (Huang da et al., 2009).

4.2.9 RT-qPCR

For cDNA preparation of tDRs and mRNAs, cDNA was produced from 100 ng of total RNA using with RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher, Waltham, MA) with gene-specific RT primers (see (Kramer, 2011) for detailed stem-loop primer design methodology) for tDR and oligo-dT RT primers for mRNA. For miRNA, cDNA was produced from 50 ng of total RNA using miScript II RT Kit (Qiagen, Valencia, CA). Diluted cDNA was used for qPCR with iScript SYBR green (BioRad, Hercules, CA) on a BioRad iCycler real-time PCR detection system. Expression was calculated from cycle threshold values (Ct) using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008). Small RNAs and mRNAs were normalized to U6 and β-Actin, respectively. All qPCR amplicons were validated by melt curve analysis, electrophoresis, and, for tDRs, additionally with Sanger sequencing. See table for a full list of RT-qPCR oligos.

Table 3. RT-qPCR Oligos used in Chapter 4

Gene-specific RT primers	**Oligo sequence**
tDR Ser-AGA	5'-GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACC CTT AA-3'
tDR His-GTG	5'-GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACC AAC GC-3'
tDR Glu-CTC	5'-GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACA GAG CG-3'
tDR Pro-AGG	5'-GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACC GAG AA-3'
U6	5'-AAC GCT TCA CGA ATT TGC GTG-3'
SYBR Green qPCR primers	**Oligo sequence**
U6 forward	5'-GCT CGC TTC GGC AGC ACA-3'
U6 reverse	5'-AAC GCT TCA CGA ATT TGC GTG-3'
tDR Pro-AGG	5'-GGC-TCG-TTG-GTC-TAG-GGG-TAT-G-3'
miR-99b	5'-CAC CCG TAG AAC CGA CCT TGC G-3'
tDR Ser-AGA	5'-GTA GTC GTG GCC GAG TGG TTA AGG-3'
tDR His-GTG	5'-GCC GTG ATC GTA TAG TGG TTA GTA C-3'
miR-10a	5'-TAC CCT GTA GAT CCG AAT TTG TG-3'
tDR Glu-CTC	5'-CAC ACA TCC CTG GTG GTC TAG TG-3'
Universal reverse primer for tDR	5'-CCA GTG CAG GGT CCG AGG TA-3'
Universal reverse primer for miRNA	10x miScript Universal Primer
NSun2 F'	5'-TAC CAT GTT CCC ACC AAC GG-3'
NSun2 R'	5'-ACG TTT GTT CCA CGG CAT TG-3'
Dnmt2 F'	5'-AGC CTG TGG CTT TCA GTA TCA-3'
Dnmt2 R'	5'-TTG GCT GAC TTT CTT CAA CTA CTG C-3'
β-Actin F'	5'-CGT TGA CAT CCG TAA AGA CC-3'
β-Actin R'	5'-AAC AGT CCG CCT AGA AGC AC-3'

4.2.10 Ultra-high-performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) analysis of sperm small noncoding RNA modifications

Sperm total RNA was pooled from 4-8 mice (3 pooled samples/group), loaded (~ 1 µg/lane) on Novex TBE-Urea 15% polyacrylamide gels (Thermo Fisher) and electrophoresed at 180 V for one hour. Under UV light, the ~30-40 nt band of RNA was recovered using ZR small-RNA PAGE Recovery Kit (Zymo Research). For each sample, 100 ng of the recovered small RNA was digested and prepared for UHPLC-MS/MS at the University at Albany RNA Mass Spectrometry Core (Albany, NY) using established methods (Basanta-Sanchez et al., 2016). Briefly, prior to UHPLC-MS/MS analysis, each sample was diluted to 10 ng/µl in 10 µl volume prior to enzymatic hydrolysis. This process involved the use of two enzymes. Nuclease P1 at 37 ºC overnight first followed by the addition of bacterial alkaline phosphatase at 37 ºC for 2-h. Resultant nucleoside mixtures were lyophilized and reconstitute to final concentration of 1 ng/µl in RNase-free water, 0.1% formic acid for subsequent UHPLC-MS/MS analysis. A total of 3 instrument replicates were processed per sample. To quantify RNA modified nucleosides, calibration curves were prepared for 42 modified nucleosides including adenosine, cytidine, guanosine and uridine. [13C15N]-Guanosine was used as an internal standard. Several processing software scaffolds including MassLynx and Targetlynx (Waters, Milford, MA) were used for the post processing of UHPLC-MS/MS data. Python script / Production of calibration curves and the Originlab software suite (Northampton, MA) were used to quantify RNA modified nucleosides. Investigators were blinded to treatment throughout UHPLC-MS/MS procedures and analysis.

4.2.11 *In vitro* sperm and epididymosome coincubation experiments

The coincubation of epididymosomes with sperm was adapted from previously established methods (Reilly et al., 2016; Sharma et al., 2016; Sharma et al., 2017).

Carboxyfluorescein succinimidyl ester (CFSE)-labelled protein transfer from epididymosomes to sperm

For this experiment, purified caput and cauda epididymosomes (as described in Section 4.2.4) were pooled from three adult B6 mice (age $\geq$ 12 weeks). This epididymosome pool and an equal volume of the epididymosome media (50 µl) was then treated with Exo-Glow (Cat # EXOG200A-1, System Biosciences, Palo Alto, CA) according to manufacturer's protocol. The epididymosome and epididymosome-depleted media were then co-incubated with ~1 $\times 10^6$ purified cauda sperm (see Section 4.2.3). Quantified sperm was pelleted and resuspended in 600 µL HTF (supplemented with 1 mM $ZnCl_2$ and pH adjusted to 6.5). Sperm samples were incubated for three hours at 37 ºC with either the Exo-Glow treated epididymosome pool or the epididymosome-depleted media. Following coincubation, sperm were washed three times at 2000 × g before imaging on a fluorescent microscope using standard GFP filter settings (excitation ~ 494 nm).

In vitro epididymosome trafficking of RNA to immature sperm

For each paired testis sample, three ~20-month-old adult male mice were sacrificed and each testis was dissected out by removing the tunica and placing the seminiferous tubules in 3 ml HTF media at 37 ºC. The tissue was finely minced and gently pipetted up and down to release spermatozoa and spermatogenic cells. After incubating further for 15 min at 37 ºC, the sperm cell suspension was run through a 100 µm cell strainer and centrifuged for three min at 300 × g to minimize somatic cell contamination. This testicular spermatozoa-enriched preparation was centrifuged at 1000 × g and washed once in PBS. The sperm pellet was resuspended in 600 µL HTF (supplemented with 1

mM $ZnCl_2$ and pH adjusted to 6.5) and half the sample was incubated for three hours at 37 ºC with epididymosomes isolated from the whole epididymis of one mouse and the other half with an equal volume (50 µL) of epididymosome-depleted media from ultracentrifugation. Following coincubation, sperm were washed twice at 2000 × g with PBS and immediately processed for RNA extraction.

4.2.12 *In vitro* fertilization

All media was equilibrated with mineral oil (Cat# M5310, Sigma-Aldrich) and kept at 37 ºC in a CO2 incubator. Six-week-old B6 oocyte donor females (habituated to the animal colony for at least one week) were superovulated by intraperitoneal (ip) injection with 5 IU pregnant mare serum gonadotropin (Cat# G4877; Sigma-Aldrich) and 5 IU human chorionic gonadotropin (hCG) (Cat# CG5-1V; Sigma-Aldrich) 48 hr later. The following day, 10.5 hours after the hCG injection, one 10-week-old B6 donor male (habituated to the mouse colony for two weeks) was sacrificed for rapid collection of cauda epididymis into HTF. The left and right epididymis were split into separate 500 µL HTF preparations. Small cuts were made to release sperm into solution and sperm were incubated for 2 min. Next, the epididymal tissue was removed and the sperm suspension was centrifuged at 300 × g for 1 min. The supernatant was discarded and the remaining sperm pellet was resuspended in 500 µL HTF. Sperm concentration was quantified with a hemocytometer and 6×10^5 sperm were added to 190 µg of pooled epididymal extracellular vesicles (EV) (4 mice from same group-housed home cage/pool) in HTF supplemented with 1 mM $ZnCl_2$ and adjusted to pH 6.5 at a final volume of 40 µL and incubated for three hours. For each IVF culture, 3 oocyte donor females were sacrificed for rapid collection of oviducts into HTF media supplemented with 1 mM glutathione (GSH) (Cat# G4251; Sigma-Aldrich) to increase zona pellucida permeability. For each

oocyte donor, oviducts were teared at the ampulla to release oocyte masses into solution and moved to a 300 µL HTF+GSH drop on the IVF culture dish. Finally, 20 µL of the epididymosome-mixed sperm was added to each IVF dish and incubated for 6 hours. Following IVF, oocytes were washed in 3 different 100 µL HTF drops to remove debris and excess sperm. Presumptive zygotes were then cultured overnight. The next morning, two cell embryos were counted and separated from unfertilized or degenerating oocytes and cultured in KSOM media (Cat# MR-121-D; Sigma-Aldrich) for 1-3 hours prior to selection for transfer to pseudopregnant CD-1 foster mothers.

4.2.13 Embryo transfer

CD-1 females (Charles River Labs) at 8-12 weeks old were mated naturally to CD-1 vasectomized males (Charles River Labs). The following morning, females were checked for vaginal plugs; plug-positive (pseudopregnant) females were segregated to be used as recipients. Two-cell embryos (15-30 embryos per recipient) were surgically transferred to both oviducts of anesthetized (10 mg/kg ketamine (Zoetis, Kalamazoo, MI) and 1mg/kg zylazine (Akron, Lake Forest, IL)) recipients. The incisions were closed with wound clips and recipients were monitored under a heat lamp and treated with 2.5 mg/kg banamine (Bimeda-MTC Animal Health, Cambridge, ON) for pain for 48 hours. Embryos derived from different EV-donor pools of the same treatment group were not mixed for embryo transfer so that all pups in a litter were derived from the same EV-donor pool. Pregnant dams were maintained in single housing and were housed with offspring until weaning at three weeks postnatal. Importantly, for all behavioral testing, no more than two mice of the same sex were examined per litter.

4.2.14 Elevated plus maze

Adult mice were single-housed and habituated to the test room for one hour in the home cage prior to the test trial. The elevated plus maze apparatus is fitted with two closed and open arms and both the floors and walls were made of opaque white plexiglass. Light intensity directly over the apparatus was set to 35 lux. Ten min prior to the test trial, mice received IP injections of 5% (w/vol) ethanol (1.0 g/kg) or saline (0.9% NaCl) and returned to the home cage. This ethanol dosage has been shown to produce BECs ~90 mg/dL in mice 10 minutes after IP injection (Becker et al., 2004). After 10 min, mice were placed in the center of the elevated plus maze, always positioned with the snout-end facing the same closed arm. After five min, animals were returned to the home cage. Scoring of time spent in the open and closed arms was performed automatically using LimeLight tracking software (Coulbourn Instruments, Holliston, MA).

4.2.15 Light dark box

The light-dark box features adjacent light and dark compartments that the test mouse can move freely between through an aperture in the dividing wall. The dark region features black plexiglass flooring and walls with a removeable cover to place the animal inside (light intensity of 2 lux). The light region has transparent flooring and walls with no roof (light intensity of 390 lux). One hour preceding the trial, single-housed mice were habituated to the test room. At the beginning of the 5-min trial, test mice were placed into the dark region of the apparatus and latency to enter and time spent in the light region were recorded with an overhead camera and scored manually. To be scored as in the light region, all four paws needed to be visible in the light region of the box.

4.2.16 Open field test

Mice were placed in the corner of a 43.2 x 43.2 x 30.5 cm open field box with a white floor and clear plexiglass walls (Med Associates Inc., St. Albans, VT). One hour preceding the trial, single-housed mice were habituated to the test room. The open field box was illuminated to ~100 lux. Total distance traveled was scored automatically with an overhead camera using LimeLight tracking software over a 5-min trial.

4.2.17 Two-bottle free choice ethanol drinking test

Mice were single-housed for one week while habituating to two 25 ml sipper tubes filled with autoclaved water. After the one week, ethanol drinking behavior was assessed by filling one tube with ethanol. Consumption of ethanol and water was measured daily and time the position of the ethanol and water tubes were rotated each day. Ethanol concentrations started at 3% (w/vol) and was increased every four days to 6,9,12, and 15% successively. Cages were changed and animals were weighted every four days.

4.2.18 Drinking in the dark assay

The drinking in the dark assay was performed based on published methods (Thiele et al., 2014). For four nights, mice were habituated to a 10-ml sipper tube filled with water that replaced their regular water bottle two hours into the animal's dark cycle. Sipper tubes were designed by fitting ball-bearing sippers into modified 10 ml serological pipets (Cat #357551; Corning Incorporated, Durham, NC) sawed off at the tip and securing the fit with heat-shrink and parafilm.

After the final habituation trial, the 10-ml sipper tube was then filled with 20% (w/vol) ethanol and consumption was measured for two hours over three consecutive nights and finally four hours on the fourth and final night. Tail blood was collected immediately following the four-hour trial to measure BECs as described in Section 4.2.2. As a control measure, one week after the four-hour trial, saccharine consumption was examined two hours into the dark cycle in four hour trials over two consecutive days.

4.2.19 Acute HPA axis responsivity

Sixteen-week-old male and female mice were subjected to a 15-min restraint stress exposure. All animals were tested between 10:00-13:00 of the light cycle. Briefly, mice were removed from group-housing and restrained in 50 ml conical plastic tubes (VWR) with several air hole perforations near the animal's head and an opening for the tail. After the 15-min restraint, each mouse was housed in a single novel cage under a fume hood for another 75 min. Only one mouse was tested per group-housed cage to avoid pre-stressing any test animals. Tail blood was collected (as described in Section 4.2.2) at time points 0, 15, 30, and 90 min from the onset of restraint stress. Blood samples were centrifuged for 10 min at 2300 × g to separate plasma for measurement of corticosterone with an enzyme immunoassay (Cat # ADI-900-097; Enzo Life Sciences, Farmingdale, NY).

4.2.20 Statistical analysis

Unpaired student's t-tests were used to compare control and ethanol group means [body weight and all RT-qPCR experiments] and paired t-tests were used for the sperm-epididymosome coincubation

experiment. Two-way analysis of variance (ANOVA) was used to compare distribution of tDR reads between control and ethanol for group effects or interactions. Bonferroni post-hoc analysis was used to analyze specific group effects in the event of a significant interaction. Sequencing data was corrected for false discovery rate ($q<0.1$). Pearson's r was used to analyze all correlations between sperm and epididymosome tDRs.

For IVF experiments, unpaired student's t-test was used to compare Control EV-donor and Ethanol EV-donor groups means for IVF efficiency, litter size, light dark box measures, open field measures, and BECs. Two-way ANOVA was used for elevated plus maze measures (factors of ethanol injection and EV-donor), two-bottle choice ethanol drinking, drinking in the dark, and HPA axis responsivity (factors of EV-donor and trial). Fisher's least significant difference (LSD) *post-hoc* test was used to examine significant interactions from ANOVA.

4.3 RESULTS

4.3.1 Small noncoding RNA profile of chronic ethanol-treated mouse sperm

Adult male B6 mice were exposed to vapor ethanol or room air conditions for 8 hours/day, 5 days/week over 5 weeks. This chronic ethanol exposure induced an average blood ethanol concentration of ~160 mg/dL and there was no effect of chronic ethanol on body weight at the end of the five-week exposure (Figure 20A) as previously reported (Finegersh and Homanics, 2014). Twenty-four hours following the final ethanol or control exposure, motile sperm were collected from each cauda epididymis for small RNA sequencing to analyze various small noncoding RNA

species. Consistent with other studies in mice (Peng et al., 2012; Sharma et al., 2016), the majority (>60%) of 15-45 nucleotide (nt) sequencing reads were transfer RNA (tRNA)-derived small RNAs (tDR) in sperm from both control and ethanol-treated mice while the remaining reads were classified as mitochondrial small RNA (mitosRNA), piRNA, microRNA (miRNA), ribosomal RNA (rRNA), small nucleolar RNA (snoRNA) and small nuclear RNA (snRNA) (Figure 20B). The vast majority of tDR are ~30-35 nt halves (Figure 21C) cleaved from the 5' end of whole length tRNA at or near the anticodon loop (see Discussion for mechanisms of tRNA halves production). Interestingly, there was a significant interaction between chronic ethanol exposure and the size distribution of tDR reads ($F_{(20,336)} = 4.2$; $p<0.001$). *Post-hoc* analysis revealed that chronic ethanol exposure reduced 30 ($p<0.001$) and 31 nt tDR ($p<0.01$) while increasing 35 nt tDR ($p<0.001$) (Figure 20C). When tDR were sorted by their amino acid and anticodon sequence, two tDR species, Gly-GCC and Glu-CTC, accounted for >70% of all tDR sequencing reads as previously reported (Peng et al., 2012; Chen et al., 2016a; Cropley et al., 2016; Sharma et al., 2016). Notably, the 30-31 nt tDR were dominated by Gly-GCC while Glu-CTC accounted for the majority of 33-35 nt reads (Figure 20D).

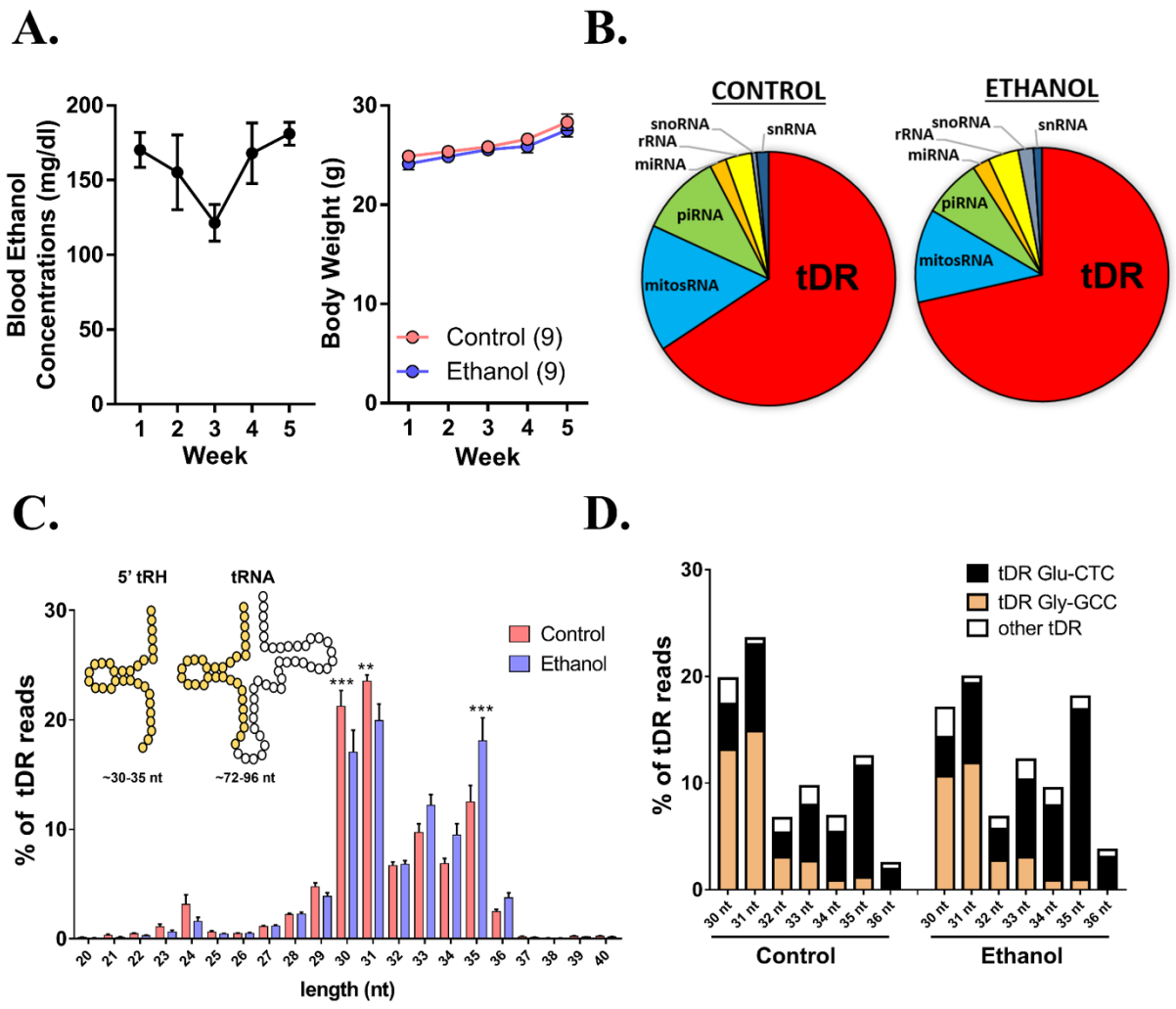


Figure 20. Chronic ethanol shifts the tDR profile of small noncoding RNA in sperm

(**A**) Chronic intermittent ethanol vapor (left panel) induced an average blood ethanol concentration of 159.2 ± 9.2 mg/dL (mean (μ) ± standard error of the mean (SEM)) over the five weeks of exposure. There was no effect of chronic ethanol on body weight (right panel) compared to the control group ($p>0.05$). (**B)** Pie charts displaying the percentage of each small RNA class represented in sperm from control and ethanol treatment groups. (**C)** Most tDR are 30-35 nt 5'-derived tRNA halves (5'-tRH) (see insert) and chronic ethanol significantly altered the percentage of 30, 31, and 35 nt tDR reads. (**D)** Most 30-36 nt tDR reads map to Glu-CTC and Gly-GCC relative to all other tDR species. **= $p<0.01$. ***=$p<0.001$. N=9/group. Data in panels A and C presented as μ ± SEM and μ in panel B.

4.3.2 Chronic ethanol alters the small noncoding RNA profile of sperm

Examining the effect of chronic ethanol exposure on the four most enriched small noncoding RNA classes in sperm, small RNA sequencing revealed 15 tDRs (Figure 21A), 8 miRNAs (Figure 21B), 5 mitosRNAs (Figure 21C), and 0 piRNA that were significantly affected by ethanol after adjusting for false discovery rate ($q \leq 0.1$, Figure 21D, see Appendix B for complete table of results for all detected small RNAs). Subsequently, several altered small noncoding RNAs with high endogenous expression were chosen for RT-qPCR validation in an independent cohort of mice. Here, five of the six analyzed small noncoding RNAs were significantly altered by chronic ethanol exposure [tDR Glu-CTC ($t_{(14)} = 2.33$, $p<0.05$); tDR His-GTG ($t_{(14)} = 3.14$, $p<0.01$); tDR Ser-AGA ($t_{(14)} = 2.08$, $p<0.05$); tDR Pro-AGG ($p>0.05$); miR-10a ($t_{(18)} = 2.41$, $p<0.05$); miR-99b ($t_{(20)} = 2.79$, $p<0.05$)] (Figure 22E), supporting the validity of the sequencing results.

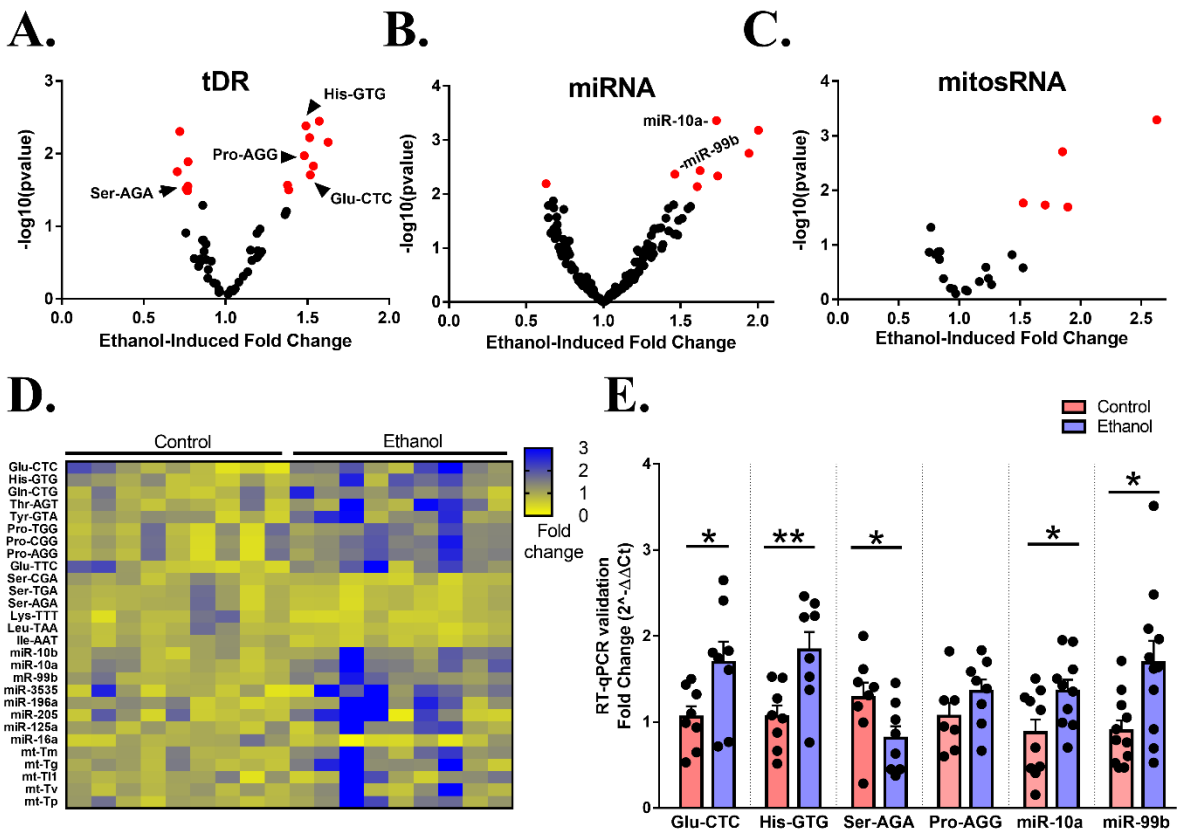


Figure 21. Chronic ethanol alters several tDR, miRNA, and mitosRNA species in sperm

Volcano plots depicting fold change and log-transformed p value for sperm (**A**) tDR, (**B**) miRNA, and (**C**) mitosRNA. Red dots indicate false discovery rate adjusted significance ($q \leq 0.1$). (**D**) Heat map of differentially expressed sperm small noncoding RNAs representing ethanol-induced fold change in normalized counts for each small RNA sequencing sample (N=9/group). (**E**) RT-qPCR validation of sequencing results revealed a significant effect of chronic ethanol on sperm tDRs Glu-CTC ($p<0.05$), His-GTG ($p<0.01$), Ser-AGA ($p<0.05$), with no change in Pro-AGG ($p>0.05$) and significantly increased miR-10a ($p<0.05$) and miR-99b ($p<0.05$), N=7-11/group. RT-qPCR data presented as $\mu \pm$ SEM with black dots representing a single data point.

4.3.3 Predicting gene targets of ethanol-responsive small noncoding RNAs

Given the evidence that sperm miRNA and tDR have been causally-linked to paternal epigenetic inheritance, follow-up target prediction and gene ontology analysis on these ethanol-responsive small noncoding RNAs to infer at a potential function upon fertilization. The primary function attributed to miRNAs is RNA silencing through post-transcriptional regulation of the 3'-untranslated region (UTR). Analyzing predicted 3'-UTR targets of the 7 miRNA that were increased by chronic ethanol exposure for common targets revealed 37 genes targeted by at least 3 miRNAs (see Table 3 for full list) and 3 genes (*Lcor*, *Nr6a*, *Rora*) that were targeted by > 4 ethanol-enriched miRNAs (Figure 22A). Gene ontology analysis of the predicted 3'-UTR targets of $\geq$ 3 sperm miRNA revealed enrichment for activators (i.e. transcription factors), transcription-regulators, and Ubl conjugation genes ($q<0.01$, Figure 22B).

Table 4. List of genes with predicted 3'-UTR targets of ≥ 3 ethanol-enriched miRNA

Ortholog of target gene	Gene name	Number of miRNA
LCOR	ligand dependent nuclear receptor corepressor	5
NR6A1	nuclear receptor subfamily 6, group A, member 1	4
RORA	RAR-related orphan receptor A	4
ELOVL6	ELOVL fatty acid elongase 6	3
ESRRG	estrogen-related receptor gamma	3
CADM2	cell adhesion molecule 2	3
GATA3	GATA binding protein 3	3
BLZF1	basic leucine zipper nuclear factor 1	3
RYBP	RING1 and YY1 binding protein	3
FOSL2	FOS-like antigen 2	3
TNRC6B	trinucleotide repeat containing 6B	3
CTDSPL	CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) small phosphatase-like	3
MBNL3	muscleblind-like splicing regulator 3	3
CREB1	cAMP responsive element binding protein 1	3
MTMR3	myotubularin related protein 3	3
THRA	thyroid hormone receptor, alpha	3
KCNC3	potassium voltage-gated channel, Shaw-related subfamily, member 3	3
ZDHHC21	zinc finger, DHHC-type containing 21	3
LUZP1	leucine zipper protein 1	3
NT5DC1	5'-nucleotidase domain containing 1	3
NFAT5	nuclear factor of activated T-cells 5, tonicity-responsive	3
DVL3	dishevelled segment polarity protein 3	3
LRRC8B	leucine rich repeat containing 8 family, member B	3
TRIM71	tripartite motif containing 71, E3 ubiquitin protein ligase	3
TAOK1	TAO kinase 1	3
TMEM136	transmembrane protein 136	3
DUSP7	dual specificity phosphatase 7	3
CPM	carboxypeptidase M	3
CPEB3	cytoplasmic polyadenylation element binding protein 3	3
KLF3	Kruppel-like factor 3 (basic)	3
ZNF281	zinc finger protein 281	3
PDE7A	phosphodiesterase 7A	3
ERBB4	v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 4	3
QKI	QKI, KH domain containing, RNA binding	3
HNRNPR	heterogeneous nuclear ribonucleoprotein R	3
NFIB	nuclear factor I/B	3
CCNJ	cyclin J	3

Although many studies have found that some tDR species can play a similar role to miRNA in post-transcriptional regulation of gene expression (Keam and Hutvagner, 2015), the specific mechanisms involved are unknown. Recent studies that employed rigorous target prediction analysis for all tDR species suggest that most tDR are more likely to act on the 5'-UTR of transcripts through complementary sequence-based gene regulation (Schuster et al., 2016a; Schuster et al., 2016b). Consistently, ethanol-responsive tDR had substantially more genes with predicted 5'-UTR targets relative to the coding and 3'-UTR regions (Figure 22C). Strikingly, the number of genes with predicted 5'-UTR targets was more than 14 times greater for one tDR, Glu-CTC, relative to

all other ethanol-responsive tDR examined (Figure 22C). Given the magnitude of tDR Glu-CTC mapping reads in sperm (Figure 20D), in addition to the surfeit of predicted targets, follow-up gene ontology analysis was performed for predicted 5'UTR targets of tDR Glu-CTC, focusing on high confidence results. This revealed enrichment for gene targets associated with the signal transduction (i.e. phosphoproteins, acetylation), alternative splicing, and the cytoplasm (q<0.01, Figure 22D).

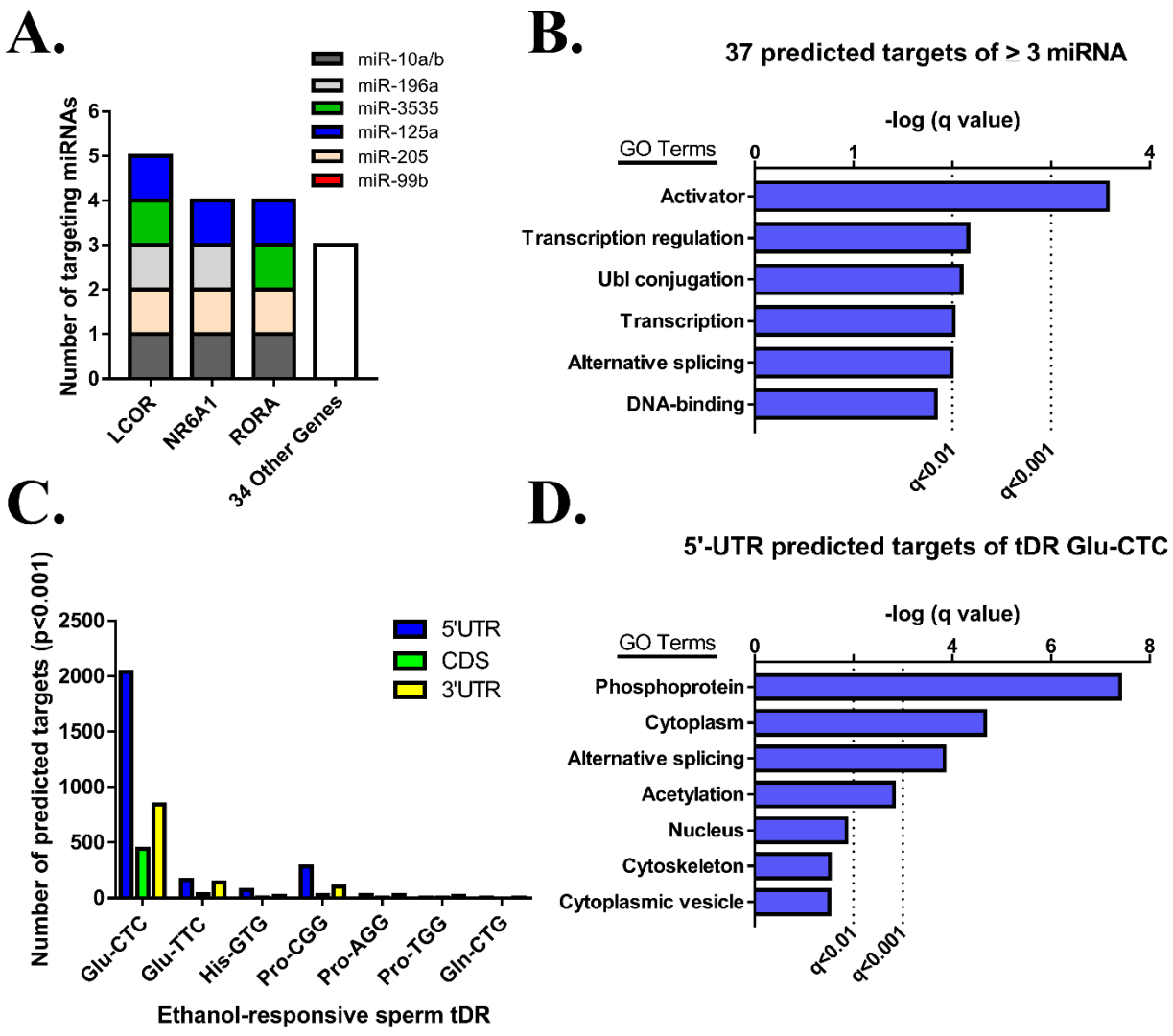


Figure 22. Analyzing predicted gene targets of ethanol-responsive sperm small RNAs

(A) Genes with 3'-UTRs targeted by three or more miRNAs**. (B)** Gene ontology analysis of predicted target genes of ≥ 3 miRNA. (**C**) Number of genes with predicted 5'-UTR, coding, or

3’-UTR targets of ethanol-responsive sperm tDR. (**D**) Gene ontology analysis for genes with predicted 5’-UTR targets of tDR Glu-CTC.

Table 5. List of predicted gene promoter targets of ethanol-responsive sperm tDR

(yellow/blue = increased/decreased by ethanol)

tDR species	Targeted promoter-gene symbol	Gene name	Chromosome
Glu-CTC	*Acp6*	acid phosphatase 6, lysophosphatidic(Acp6)	3
His-GTG	*Acp6*	acid phosphatase 6, lysophosphatidic(Acp6)	3
Glu-TTC	*Wbp4*	WW domain binding protein 4(Wbp4)	14
Pro-AGG	*Rnase4*	ribonuclease, RNase A family 4(Rnase4)	14
Pro-AGG	*Ang*	angiogenin, ribonuclease, RNase A family, 5(Ang)	14
Gln-CTG	*Rab11a*	Ras-related protein Rab-11A	9
Gln-CTG	*Aloxe3*	arachidonate lipoxygenase 3(Aloxe3)	11
Pro-CGG	*Rnase4*	ribonuclease, RNase A family 4(Rnase4)	14
Pro-CGG	*Ang*	angiogenin, ribonuclease, RNase A family, 5(Ang)	14
Ser-AGA	*2610301B20Rik*	RIKEN cDNA 2610301B20 gene(2610301B20Rik)	4
Ser-AGA	*Casc5*	Kinetochore-Null Protein	2
Ser-TGA	*Ctnna3*	catenin (cadherin associated protein), alpha 3(Ctnna3)	10
Ser-TGA	*Casc5*	Kinetochore-Null Protein	2
Ser-CGA	*Smarcc2*	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily c, member 2	10
Ser-CGA	*Casc5*	Kinetochore-Null Protein	2
Lys-TTT	*Aloxe3*	arachidonate lipoxygenase 3(Aloxe3)	11
Leu-TAA	*Casc5*	Kinetochore-Null Protein	2
Tyr-GTA	*Rnase4*	ribonuclease, RNase A family 4(Rnase4)	14
Tyr-GTA	*Ang*	angiogenin, ribonuclease, RNase A family, 5(Ang)	14
Ile-TAT	*Plekhg2*	pleckstrin homology domain containing, family G member 2(Plekhg2)	7

In addition to post-transcriptional regulation, tDR have been proposed to function as transcriptional regulators given that some tDR species overlap with gene promoter regions (Chen et al., 2016a). Analysis of the ethanol-responsive tDR targets revealed a small number of genes with very high sequence homology within regions 2000 base pairs upstream of transcriptional start sites (Table 7).

Notably, none of the predicted promoter targets were on the Y chromosome (Table 7). Moreover, 0 of the 414 predicted mRNA targets of $\geq$2 miRNAs and just 1 of all significant 5’-UTR targets of ethanol-responsive tDR (*Uba1y*) was transcribed from Y chromosome genes.

4.3.4 Chronic ethanol alters select sperm small RNA modifications

Recent evidence suggests a functional role for post-transcriptional nucleoside modifications on small noncoding RNAs in sperm, particularly on tDR, as tRNA is the most heavily modified RNA class (Kirchner and Ignatova, 2015). For instance, whereas native sperm tDR is stable in the fertilized oocyte for several hours, synthetic tDR lacking endogenous nucleoside modifications are rapidly degraded (Chen et al., 2016a). Thus, whether chronic ethanol exposure affects nucleoside modifications in the tDR-enriched ~30-40 nt fraction of sperm RNA was examined directly using ultra performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) (Basanta-Sanchez et al., 2016). The analysis focused on 22 post-transcriptional modifications previously identified in eukaryotic species (Table 5) (Machnicka et al., 2013). This revealed two significantly increased nucleoside modifications: the uridine modification, 5'-methylaminomethyl-2-thiouridine (mnm^5s^2U) ($q<0.1$; Figure 23A) and the cytosine modification, formylcytidine (f^5C) ($q<0.01$; Figure 23B). There were no alterations to adenosine (Figure 23C) or guanosine (Figure 23D) base modifications.

Table 6. List of all RNA modifications analyzed in sperm small noncoding RNA

SYMBOL	TYPE OF RNA MODIFICATION	PARENT NUCELOSIDE
ac^4C	N4-acetylcytidine	cytidine

Cm	2-O'-methylcytidine	
f^5C	5-formylcytidine	
m^5C	5-methylcytidine	
m^3C	3-methylcytidine	
Am	2-O'-methyladenosine	adenosine
m^1A	1-methyladenosine	
m^6A	N6-methyladenosine	
Um	2-O'-methyluridine	uridine
m^3U	3-methyluridine	
m^5U	5-methyluridine	
s^2U	2-thiouridine	
mnm^5U	5-methylaminomethyluridine	
mcm^5U	5-methoxycarbonylmethyluridine	
mnm^5s^2U	5-methylaminomethyl-2-thiouridine	
$cmnm^5U$	5-carboxymethylaminomethyluridine	
y	pseudouridine	guanosine
Gm	2-O'-methylguanosine	
$m^2{}_2G$	N2-dimethylguanosine	
m^2G	N2-methylguanosine	
m^7G	7-methylguanosine	

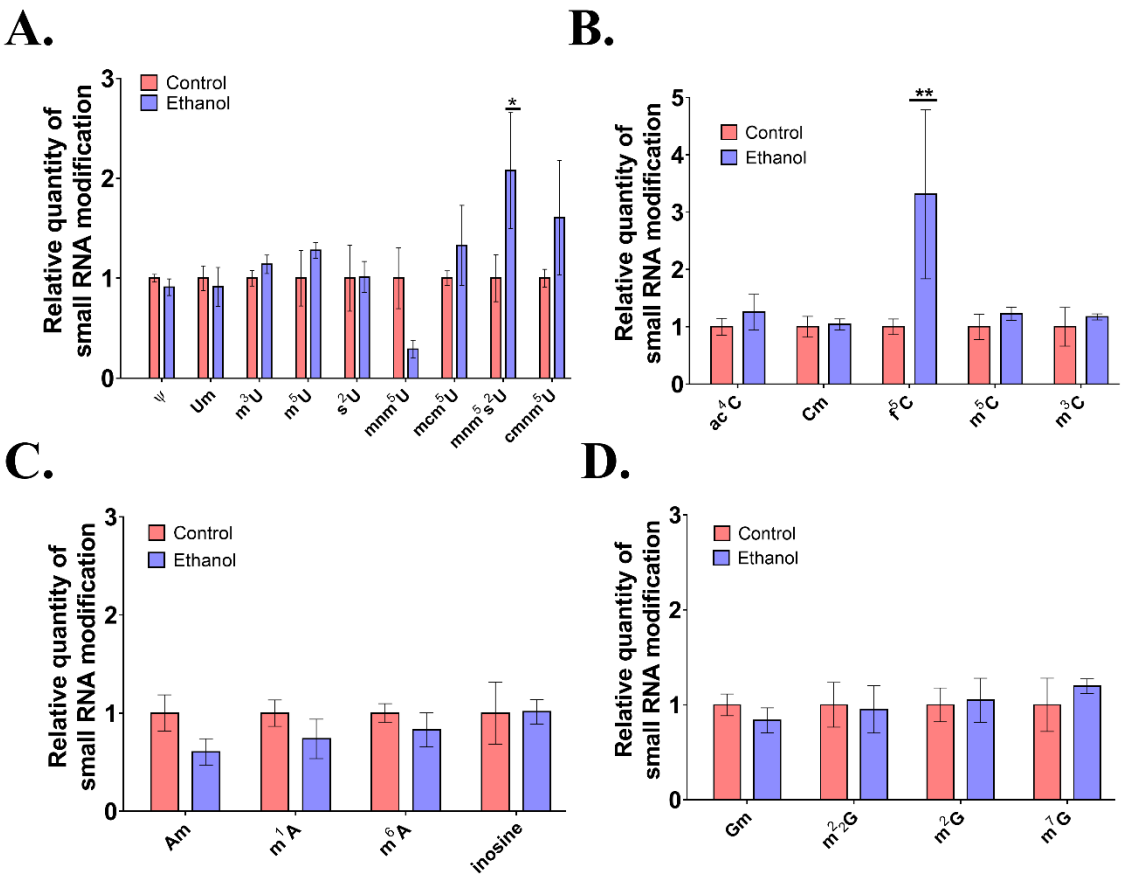


Figure 23. Chronic ethanol alters select modifications of small RNA in sperm

UHPLC-MS/MS was performed on the ~30-40 nt fraction of sperm RNA from chronic ethanol and control exposed groups. Post-transcriptional modifications were examined for each of the parent nucleosides, (**A**) uridine, (**B**) cytidine, (**C**) adenosine, and (**D**) guanosine. Chronic ethanol increased the uridine modification, 5-methylaminomethyl-2-thiouridine (mnm^5s^2U) ($q<0.1$) and the cytidine modification formylcytidine (f^5C) ($q<0.01$). Data presented as $\mu \pm$ SEM. N= 3 pooled samples/group. *=$q<0.1$, **=$q<0.01$.

4.3.5 Effects of ethanol on sperm tDR are reflected in epididymosomes

Following spermatogenesis in the testis, newly developed spermatozoa enter the epididymis, gaining motility while migrating from the caput to cauda segment where mature sperm are stored prior to ejaculation (Figure 24A). Interestingly, for sperm isolated from the caput segment, there was no effect of chronic ethanol exposure on the tDR species altered by ethanol in cauda sperm (Figure 24B), suggesting the tDR alterations in mature sperm emerge during epidydimal transit or storage. This is consistent with recent evidence suggesting that sperm tDR are nearly absent in testis and become the dominant small RNA type through interactions with tDR-enriched extracellular vesicles or "epididymosomes" (Figure 24C) in the epididymal lumen (Reilly et al., 2016; Sharma et al., 2016). Supporting a direct role for epididymosomes in sperm maturation, epididymosomes deliver carboxyfluorescein succinimidyl ester (CFSE)-labelled protein cargo to epididymal sperm *in vitro*(Reilly et al., 2016)(see Figure 24D). Furthermore, immature sperm from testis were enriched for major tDR species following *in vitro* coincubation with epididymosomes (tDR Gly-GCC: $t_{(4)} = 3.67$, $p<0.05$; Figure 24E).

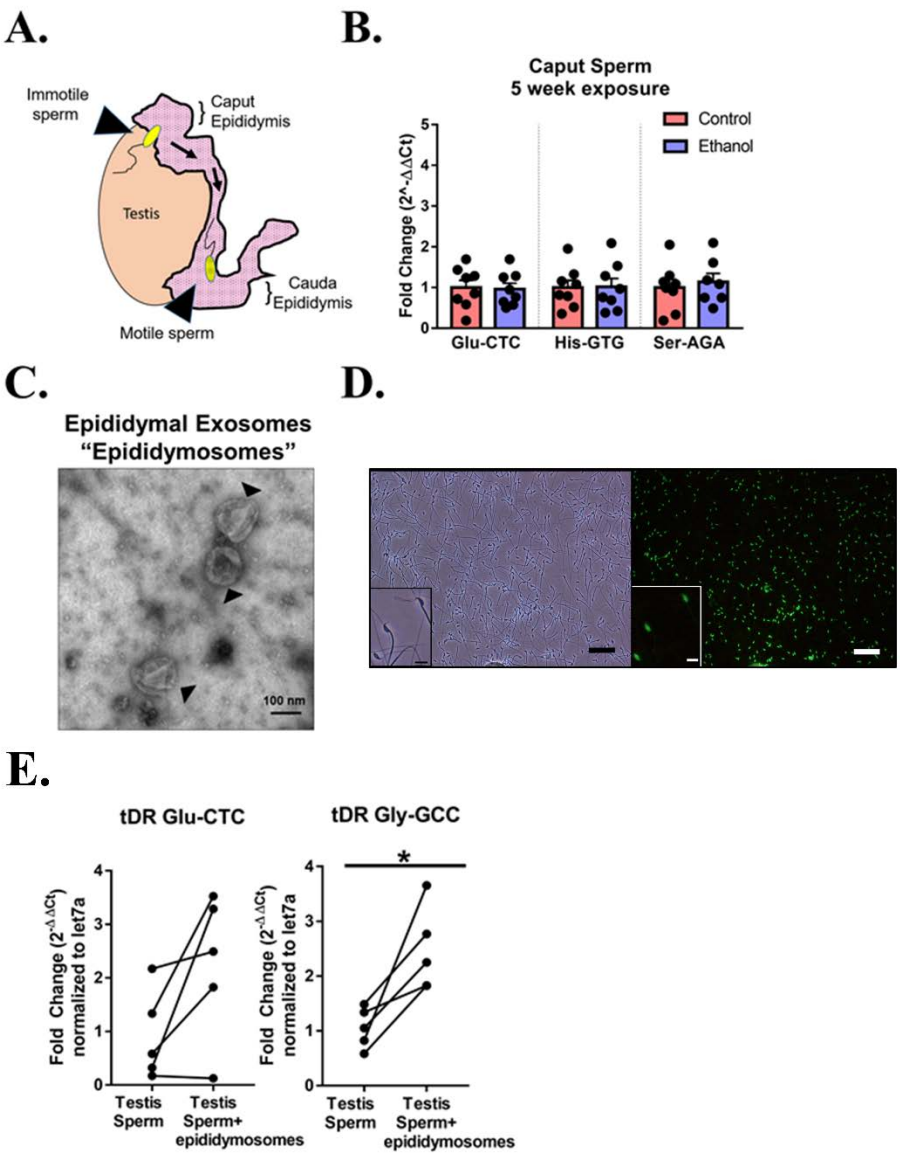


Figure 24. Sperm RNA profile is shaped during epididymal transit

(A) Following spermatogenesis in testis, immature sperm gain motility travel from the caput to cauda end of the epididymis (B) RT-qPCR showing no effect of chronic ethanol on tDR Glu-CTC, His-GTG, and Ser-AGA in caput epididymal sperm ($p>0.05$). (C) Representative transmission electron microscopy image of epididymosomes (arrows) isolated from adult mouse cauda epididymis. (D) (left panel) Bright field image of sperm after three hours co-incubated with epididymosomes. (right panel) Fluorescent microscopy of sperm after co-incubation with CFSE-labelled epididymosomes. (E) RT-qPCR with paired testis sperm samples after *in vitro* coincubation with control media or epididymosomes for (left) tDR Glu-CTC and (right) tDR Gly-

GCC. *= p<0.05. Data presented as μ ± SEM with black dots representing each data point. Panel D bar lengths = (inserts) 8 μm, (large image) 100 μm.

Remarkably, isolating cauda epididymosomes from control- and ethanol-treated mice to examine the same tDR species that were altered in sperm revealed increased tDR Glu-CTC after two weeks of ethanol treatment ($t_{(18)} = 2.41$, $p<0.05$; Figure 25A) and tDR His-GTG was increased after five weeks ($t_{(13)} = 2.20$, $p<0.05$; Figure 25B) with no change in tDR Ser-AGA ($p>0.05$) at either time point. Expression of each tDR was not correlated between cauda sperm and cauda epididymosomes at five weeks (Figure 26A-C, $p>0.05$), although there was a significant correlation at two weeks for tDR Glu-CTC in the ethanol group (Figure 26D, $p<0.05$).

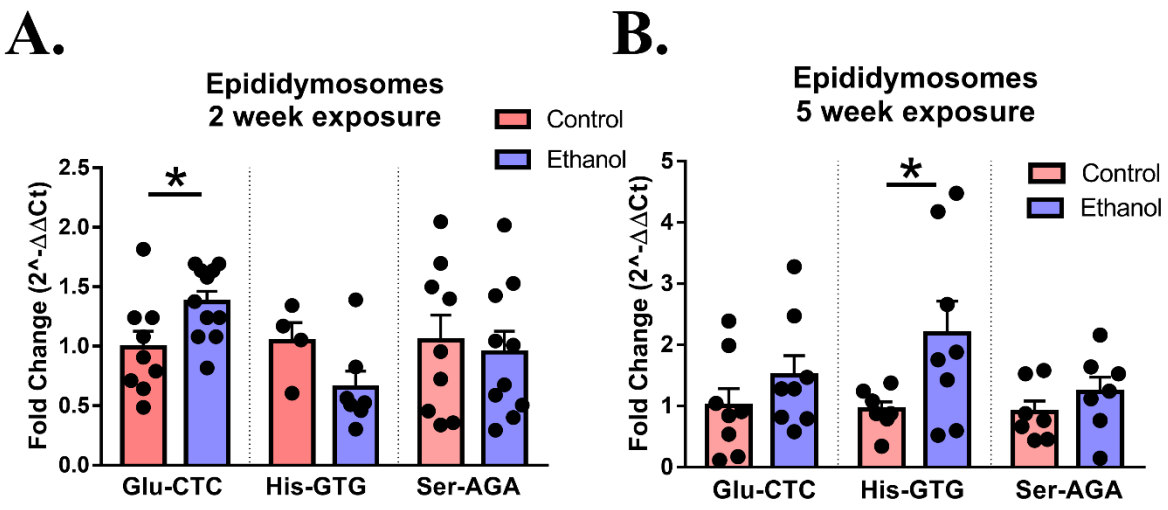


Figure 25. Effects of chronic ethanol on sperm tDR are reflected in epididymosomes

(A) RT-qPCR reveled a significant effect of chronic ethanol on tDR Glu-CTC with no change in His-GTG or Ser-AGA after two weeks of ethanol exposure. (B) RT-qPCR showing increased tDR His-GTG with no change in Glu-CTC or Ser-AGA with five weeks ethanol exposure. N=4-11/group. *=p<0.05. Data presented as μ ± SEM with black dots representing each data point.

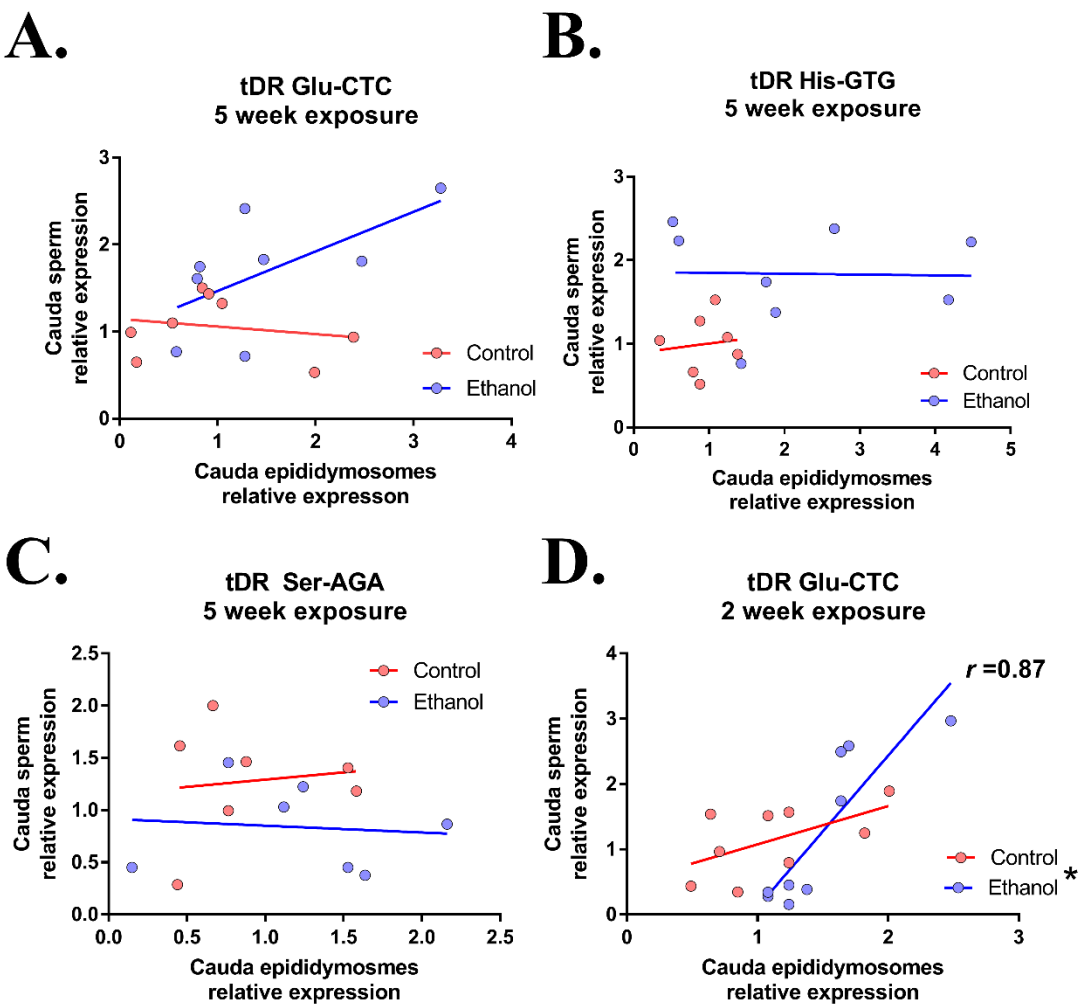


Figure 26. Relationship between tDR species expression in sperm vs epididymosomes

Scatterplots showing no correlation for levels of tDRs (**A**) Glu-CTC, (**B**) His-GTG, and (**C**) Ser-AGA expression between cauda sperm and cauda epididymosomes at five weeks and (**D**) a significant correlation at two weeks for tDR Glu-CTC in the ethanol group. *=p<0.05. Plotted lines for control and ethanol groups represent linear regression analysis.

4.3.6 Examining the cross-generational effects of epididymosomes

Given the evidence supporting epididymosomes as a plausible origin of RNA in mature sperm (Reilly et al., 2016; Sharma et al., 2017), *in vitro* fertilization (IVF) experiments were designed to

test the hypothesis that epididymosomes from chronic ethanol exposed-males incubated with sperm preceding IVF is sufficient to alter ethanol- and stress-related behaviors in offspring (Figure 27A). Epididymosomes were pooled from four chronic ethanol-exposed (Ethanol EV-donor) or control (Control EV-donor) mice. This pooling was repeated for 4 pooled samples/group. Average BEC during chronic ethanol exposure of all Ethanol EV-donor pools was ~150-175 mg/dL (Figure 27B). There was no effect of EV-donor on IVF efficiency (Figure 27C) or litter sizes (Figure 27D).

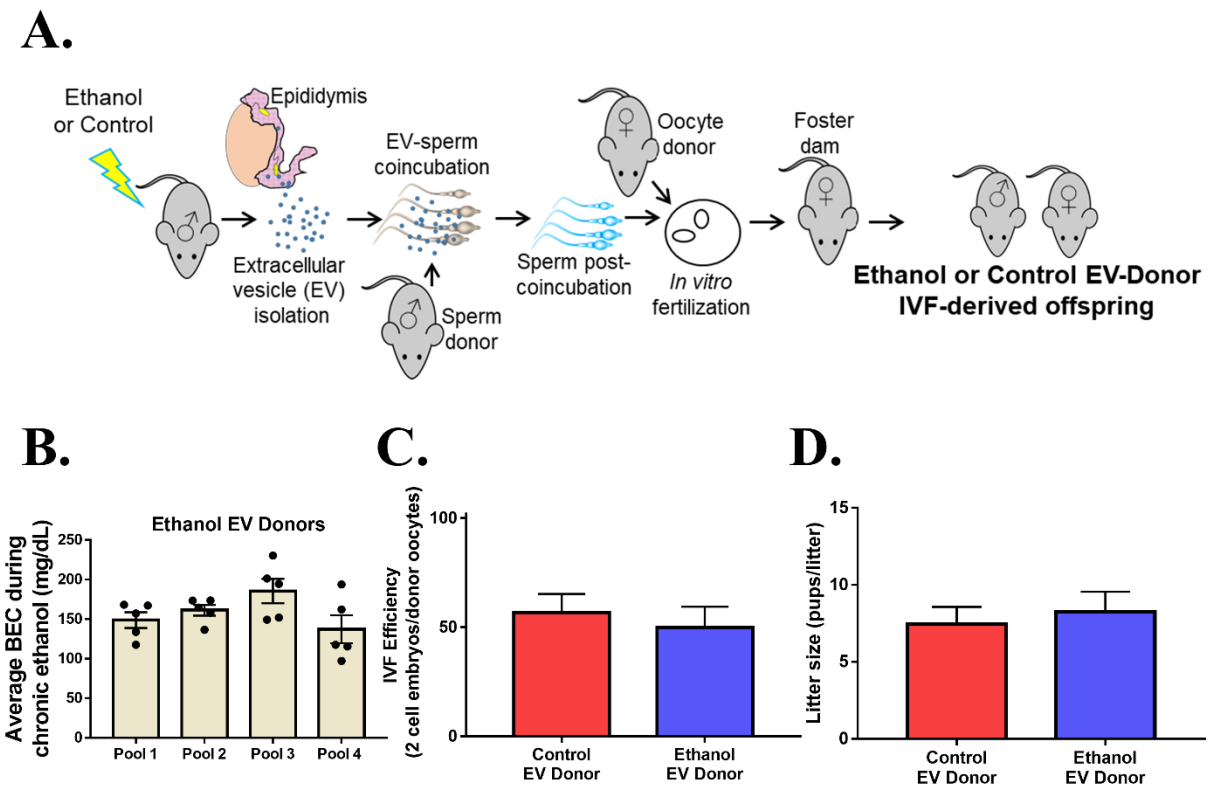


Figure 27. Examining the effects of Ethanol EV-donors on IVF-derived mice

(A) Experimental design for examining the effect of epididymosomes on intergenerational ethanol- and stress-related behaviors. After adult males were exposed to chronic ethanol or control treatment, mice were sacrificed and extracellular vesicles (EV) were isolated from epididymis. For each Ethanol and Control EV-donors, EV were pooled from four mice and incubated with sperm during capacitation immediately preceding in vitro fertilization. Fertilized oocytes were implanted in foster dams and the adult progeny was phenotyped for

ethanol- and stress-related behaviors. (B) Blood ethanol concentrations (BEC) for each ethanol EV pool (N=4 mice/pool). (C) No difference in efficiency of IVF for Control and Ethanol EV-donor groups (N=11/group). (D) No difference in litter sizes for Control or Ethanol EV-donor groups (N=9/group). Data presented as μ + SEM.

4.3.7 Ethanol EV-donors confer reduced body weight selectively to males

Analysis of IVF-derived males and females at weaning (3 weeks postnatal) and early adulthood (8 weeks postnatal), revealed a significant effect of EV-donor ($F_{(1, 72)} = 16.48$, $p<0.001$) and EV-donor × postnatal age ($F_{(1,72)} = 6.25$, $p<0.05$; Figure 28A) on male body weight. *Post-hoc* analysis revealed that Ethanol EV-donor males had reduced body weight at three weeks postnatal (Ethanol EV-donor: 12.62 ± 0.33 grams vs Control EV-donor: 14.33 ± 0.2 grams, $p<0.001$), but no effect at eight weeks postnatal ($p>0.05$). There was no effect of EV-donor, but a significant effect of EV-donor × postnatal age on body weights in females (Figure 28B). *Post-hoc* test revealed no significant difference for female body weights between Control and Ethanol EV-donor groups at 3 or 8 weeks postnatal.

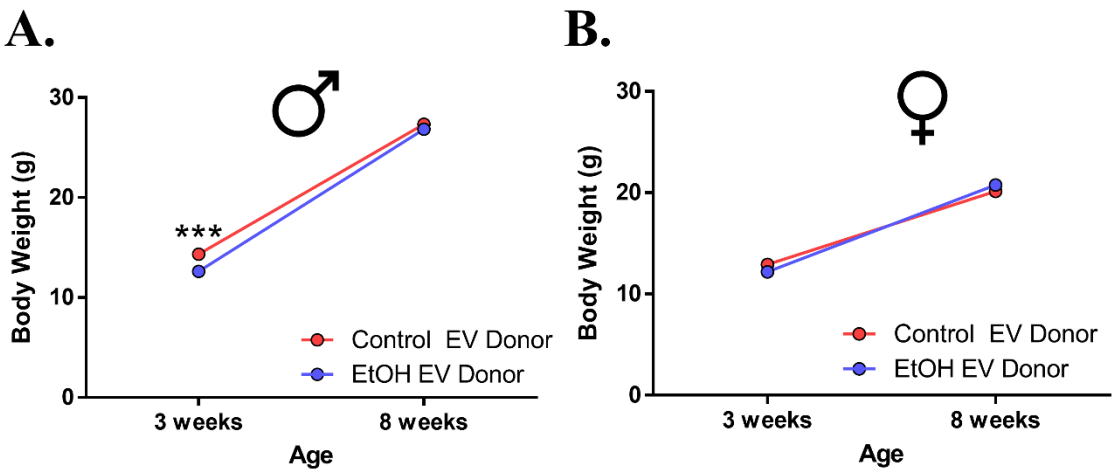


Figure 28. Ethanol EV-donors confer reduced body weight to males

(A) Ethanol EV-donors conferred reduced body weight to males (N=31/Ethanol EV-donor and 43/Control EV-donor) at three weeks postnatal. (B) There was no effect of EV-donor on body weights in females (N=24 Control EV-donor and N=35 Ethanol EV-donor). ***=p<0.001. Data presented as μ with SEM obscured by data points.

4.3.8 Ethanol EV-donors confer increased anxiety-like behavior to females

IVF-derived males and females were examined for ethanol-induced anxiolysis in the elevated plus maze. In males, there was a trending effect of ethanol injection (1.0 g/kg, IP) on open arm time ($F_{(1, 46)} = 3.75$, $p<0.06$; Figure 29A), a significant effect of ethanol on open arm entries ($F_{(1, 47)} = 31.27$, $p<0.001$; Figure 29B), and no effect on total arm entries (Figure 29C). There was no effect of EV-donor or EV-donor × ethanol injection on any measure in males. For females, there was a significant effect of ethanol injection on open arm time ($F_{(1, 40)} = 28.56$, $p<0.001$), open arm entries ($F_{(1, 40)} = 15.43$, $p<0.001$; Figure 29E), and total arm entries $F_{(1, 40)} = 10.54$, $p<0.01$; Figure 29F). In addition, there was a significant effect of EV-donor on open arm time ($F_{(1, 40)} = 10.65$, $p<0.01$; Figure 29D) and open arm entries ($F_{(1, 40)} = 11.44$, $p<0.01$; Figure 29E), but not total arm entries (Figure 29F). There was no effect of EV-donor × ethanol injection on any measure for females.

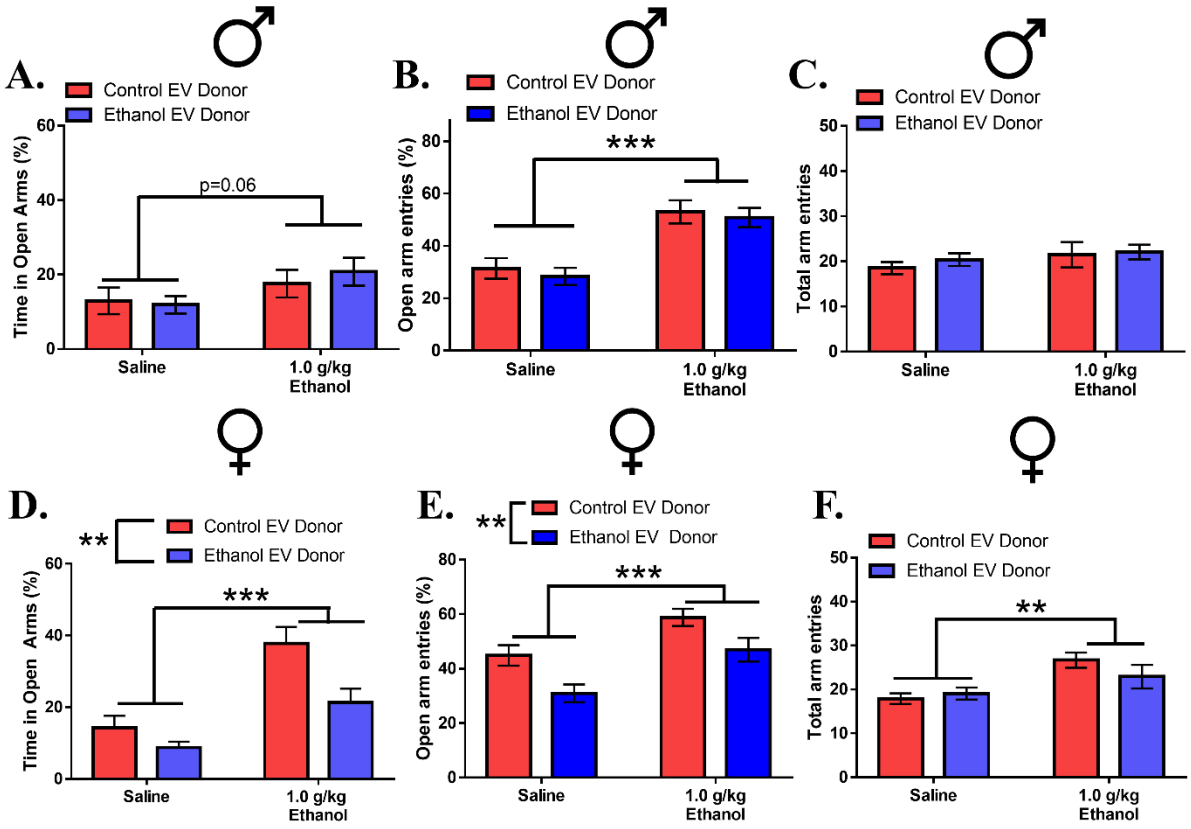


Figure 29. Effects of Ethanol EV-donors on ethanol-induced anxiolysis in IVF mice

No effect of EV-donors on (A) open arm time, (B) open arm entries, and (C) total arm entries in males. (D) Reduced open arm time in Ethanol EV-donor females. (E) Reduced open arm entries in Ethanol EV-donor females. (F) No effect of EV-donor on total arm entries in females. N=12-15/male group and 10-14/female groups. **=p<0.01, ***=p<0.001. Data presented as $\mu \pm$ SEM.

To further examine basal anxiety-like behavior, males and females were tested in the light/dark transition test. Here, there was no effect of EV-donor on time spent in the light region or latency to enter the light in males (Figure 30A-B). For females, there was no effect of EV-donor for time spent in the light region (Figure 30C), but a significant increase in latency to enter the light region for Ethanol EV-donor vs Control EV-donor females ($t_{(22)} = 2.99$; Figure 30D). Females were further examined for activity in the open field test and there was no effect of Ethanol EV-donor on total

distance traveled [Ethanol EV-donor (N=6): 1866 ± 125.8 cm (μ ± SEM) and Control EV-donor (N=8): 1925 ± 125.8 cm].

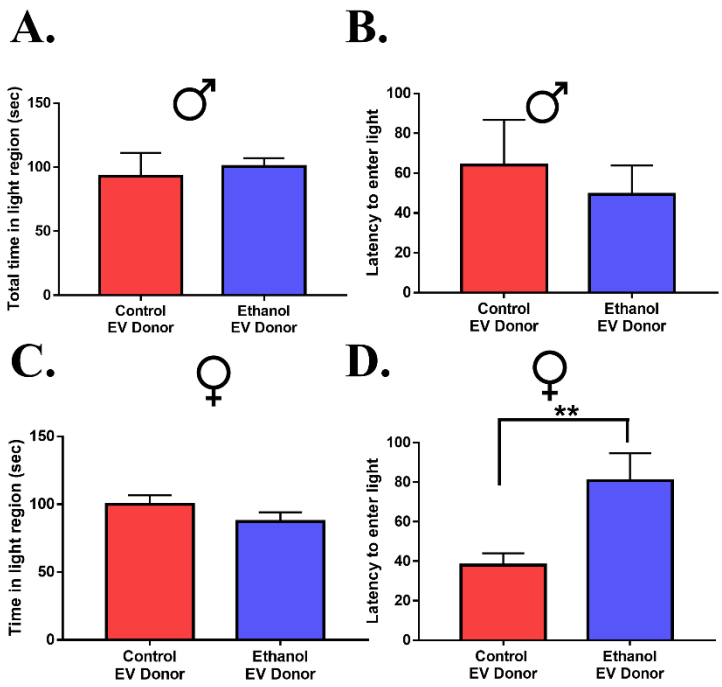


Figure 30. Ethanol EV-donors confer increased anxiety-like behavior to IVF females

(A) No effect of Ethanol EV-donor on total time spent in the light region and (B) latency to enter light in males. (C) No effect of Ethanol EV-donor on time in light region for females. (D) Increased latency to enter the light region in Ethanol EV-donor females. N= 6-7/male groups and 11-12/female groups. **=p<0.01. Data presented as μ ± SEM.

4.3.9 No effect of Ethanol EV-donor on two-bottle choice ethanol drinking

In the two-bottle choice test, there was a significant effect of ethanol concentration on ethanol consumption ($F_{(4,100)}$ = 95.96, p<0.001) and total fluid intake ($F_{(4,100)}$ = 9.73, p<0.001) in IVF-derived males. There was no effect of EV-donor, or EV-donor × ethanol concentration on ethanol

drinking preference, ethanol consumption, or total fluid intake in IVF-derived males (Figure 31A-C). For IVF-derived females, there was a significant effect of ethanol concentration on ethanol consumption ($F_{(4,40)}$= 51.75, p<0.001). There was no effect of EV-donor, or EV-donor × ethanol concentration on ethanol preference, ethanol consumption, or total fluid intake (Figure 31D-F).

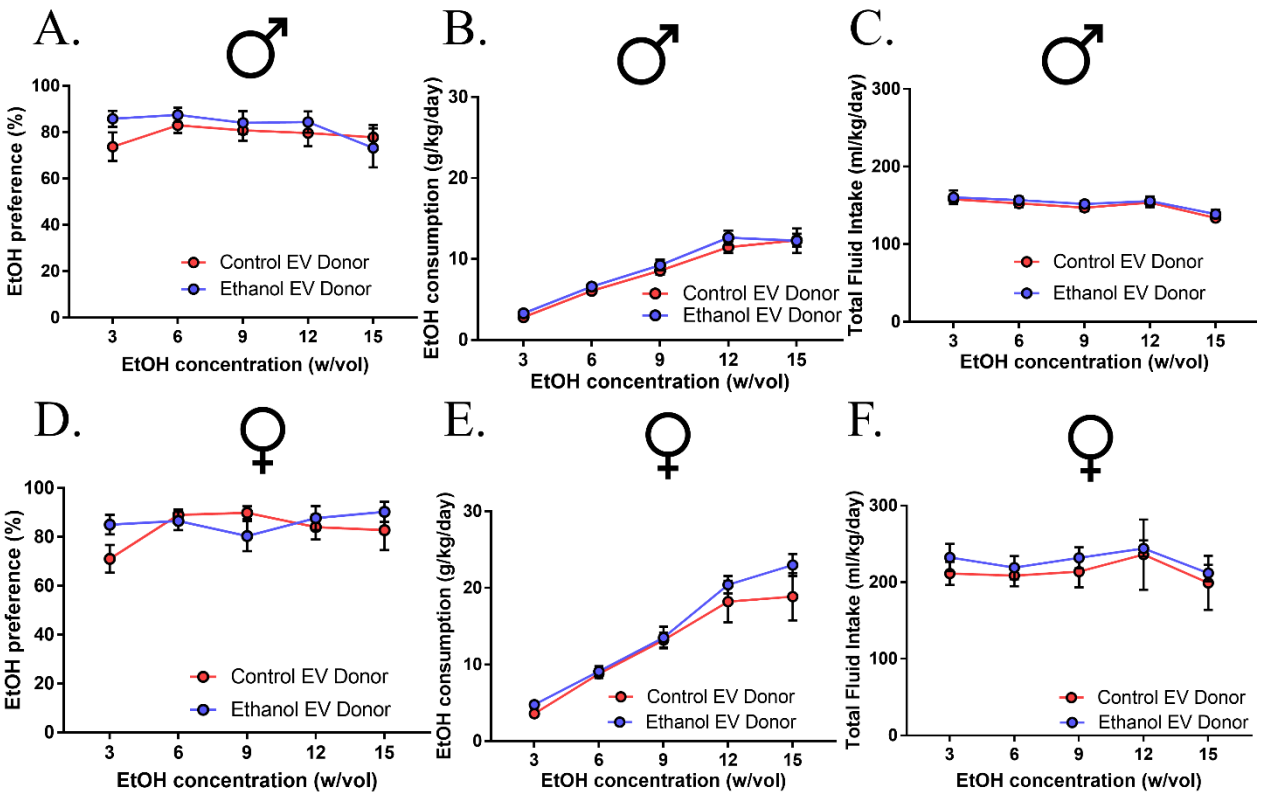


Figure 31. No effect of Ethanol EV-donors on two bottle-choice ethanol drinking

No effect of Ethanol EV-donor on ethanol preference, ethanol consumption, and total fluid intake in the two-bottle choice test for males (A-C, respectively) or females (D-F, respectively) mice. N= 12-15/male group and 10-12/female group. Data presented as μ ± SEM.

4.3.10 Ethanol EV-donors increase binge-like ethanol consumption in males

In the drinking in the dark assay, for IVF-derived males, there was a significant effect of trial day on ethanol consumption ($F_{(1,25)}$ = 131.5, p<0.001). There was no effect of EV-donor, but a

significant effect of EV-donor × test day on ethanol consumption ($F_{(3, 75)} = 3.68$, $p<0.05$; Figure 32A). *Post-hoc* analysis revealed significantly increased ethanol consumption in Ethanol EV-donor males during the four-hour test compared to Control EV-donor males ($p<0.05$). For females, there was a significant effect of test day ($F_{(1,23)} = 61.74$), but no effect of EV-donor or EV-donor × test day on ethanol consumption (Figure 32B). There was no effect of Ethanol EV-donor on BECs measured following the four-hour trial in males or females (Figure 32C). In addition, there was no effect of EV-donor or EV-donor × trial on saccharine consumption in males (Figure 32D) or females (Figure 32E).

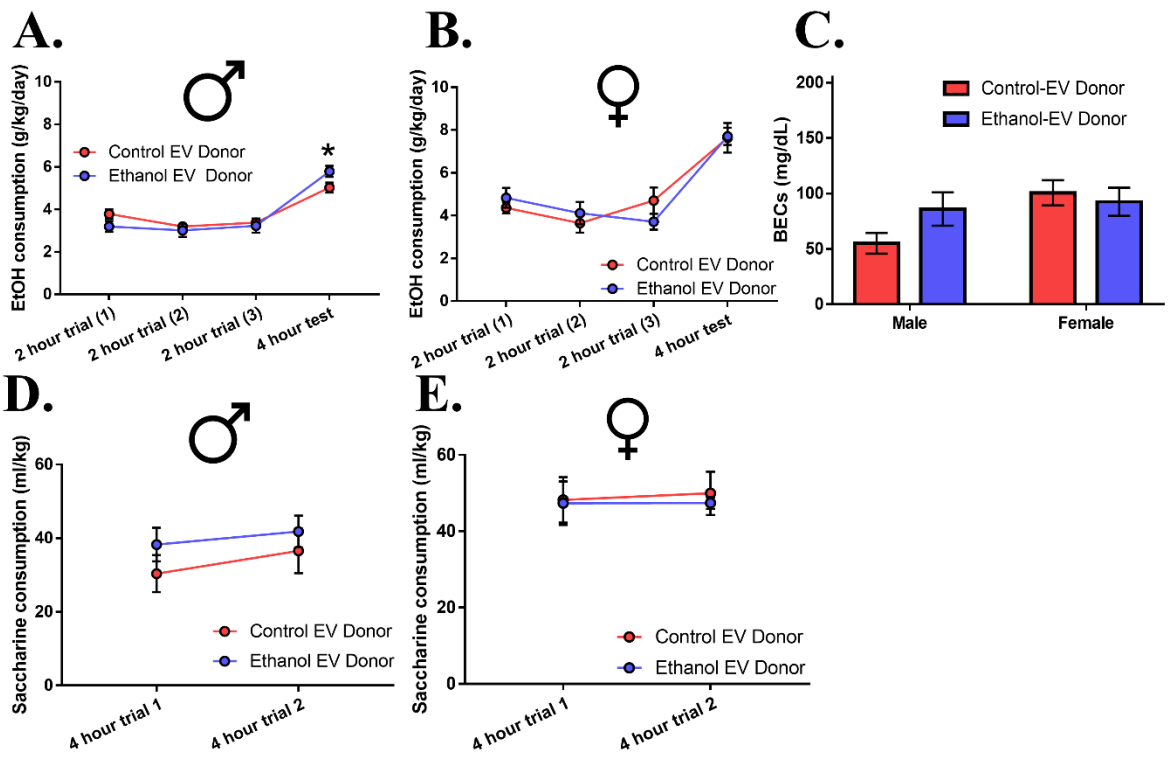


Figure 32. Ethanol EV-donors confer increased binge-like ethanol drinking to males

(A) Ethanol EV-donors confer increased ethanol consumption to males during the drinking the dark four-hour trial (N=12-14/group). (B) There was no effect of Ethanol EV-donor on ethanol consumption in females (N=12-13/group). (C) No effect of EV-donor on blood ethanol concentrations (BECs) in males or females. No

effect of Ethanol EV-donor on saccharine consumption in (D) males or (E) females. *=p<0.05. Data presented as μ ± SEM.

4.3.11 No effect of Ethanol EV-donors on HPA axis responsivity

When examined for HPA responsivity to 15 min of acute restraint stress, there was no effect of EV-donor or EV-donor × time on corticosterone levels in males (Figure 33A) or females (Figure 33B).

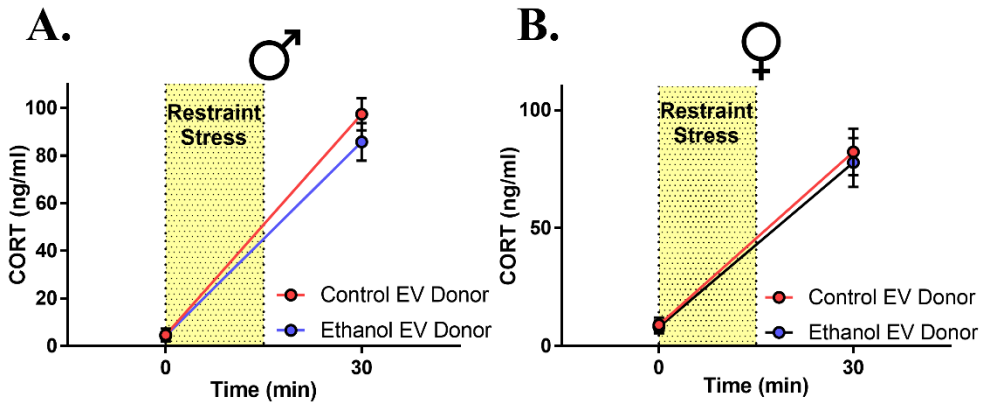


Figure 33. No effect of Ethanol EV-donors on HPA axis responsivity

No effect of Ethanol EV-donors on plasma corticosterone (CORT) levels at 0 and 30 min from the onset of 15-min restraint stress (shaded bar) in **(A)** males (N=9/group) or **(B)** females (N=9-10/group). Data presented as μ ± SEM.

4.4 DISCUSSION

Several studies suggest that small noncoding RNAs are functional epigenetic regulators in sperm, capable of directing gene expression in the early embryo and ultimately impacting offspring behavior into adulthood. Chronic intermittent ethanol exposure altered the expression of several sperm tDR, mitosRNAs, and miRNAs. In addition, chronic ethanol increased specific posttranscriptional nucleoside modifications on sperm small noncoding RNAs. Gene ontology analysis of predicted ethanol-responsive miRNA and tDR targets revealed enrichment for gene sets involved in diverse biological functions, most robustly transcriptional factors and phosphoproteins. Ethanol-responsive sperm tDR were similarly affected in epididymal extracellular vesicles (i.e. epididymosomes), suggesting a somatic origin to altered small noncoding RNAs in the male germline. Finally, incubating epididymosomes from ethanol-treated males with sperm had very modest effects on body weights and at three weeks postnatal and limited access ethanol consumption in IVF-derived males and increased anxiety-like behavior in IVF-derived females.

The finding that chronic ethanol exposure alters sperm small noncoding RNAs adds to a growing literature demonstrating that a diverse range of paternal preconception exposures with cross-generational effects are associated with altered small noncoding RNAs in sperm (Rodgers et al., 2013; Gapp et al., 2014; Chen et al., 2016a; de Castro Barbosa et al., 2016; Sharma et al., 2016; Short et al., 2016; Short et al., 2017). Remarkably, recent studies identified a causal relationship between altered small noncoding RNA, specifically miRNA and tDR, and intergenerational phenotypes (Rodgers et al., 2015; Chen et al., 2016a). Chronic preconception ethanol exposure alters various complex behaviors in male offspring including ethanol drinking preference and consumption (Finegersh and Homanics, 2014; Rompala et al., 2016; Rompala et al., 2017). Here, the same chronic ethanol exposure directly affects small noncoding RNA in sperm. Thus, additional

studies are needed to directly test the role of sperm ethanol-responsive small noncoding RNAs in the intergenerational effects of paternal preconception chronic ethanol exposure.

Among the different small noncoding RNA types, tDR were most affected by chronic ethanol treatment. Given the abundance of tDR in sperm and emerging evidence of their functional significance, tDR have become a major focus as a potential causal mechanism for paternally-linked epigenetic inheritance. High fat diet, low protein diet, and increased exercise have all been shown to directly confer changes in sperm tDR (Chen et al., 2016a; Sharma et al., 2016; Short et al., 2017) while obesity and vinclozolin exposure affect sperm tDRs transgenerationally (Cropley et al., 2016; Schuster et al., 2016a). Remarkably, one recent study found that the effects of paternal high fat diet on glucose tolerance in offspring were recapitulated in mice derived from fertilized embryos injected with sperm tDR, but not when the embryos were injected with all other sperm RNA classes except tDR (Chen et al., 2016a). This illustrates a specific causal role for tDR in RNA-mediated epigenetic inheritance.

In addition to affecting tDR, chronic ethanol exposure increased several of the second most abundant small noncoding RNA type, mitosRNA. While little is known about these small noncoding RNAs, they are derived from mitochondrial genes for rRNA and tRNA and have been shown to increase the expression of their parent genes *in vitro* (Ro et al., 2013). They are also cleaved into precise small RNA species (mostly ~40-45 nt) by an as yet unidentified mitochondrial ribonuclease (Ro et al., 2013). Given that mitosRNA are enriched in total sperm and yet barely detected in the sperm head (Schuster et al., 2016b), they are likely confined to the sperm mitochondrial sheath. Several parameters of mitochondrial function appear critical for sperm motility and fertilization capacity, including control of reactive oxygen species production, apoptotic pathways, and calcium homeostasis (Amaral et al., 2013). As chronic ethanol has been

shown to affect several measures of sperm quality including reduced motility and increased apoptosis (Rahimipour et al., 2013), it is possible that ethanol directly affects sperm mitochondrial function. While paternal mitochondria do enter the oocyte at fertilization, the mitochondria and its DNA are rapidly degraded (Politi et al., 2014). It is unknown whether the mitochondrial RNA and mitosRNAs are similarly degraded or if they may serve some function in the early embryo.

While less expressed in sperm relative to other small noncoding RNA species, miRNA have been found to play a critical role in fertilization and preimplantation development (Liu et al., 2012; Yuan et al., 2016). Furthermore, altered sperm miRNA have been associated with the greatest range of environmental factors (although this may be due to the greater use of miRNA-specific analysis strategies such as microarray). Most notable among these studies, chronic variable stress was shown to increase nine sperm miRNAs and offspring of stressed sires have a blunted stress responsivity phenotype (Rodgers et al., 2013). That same phenotype could be elicited in mice derived from control fertilized embryos injected with the nine stress-enriched miRNAs, suggesting a causal role for environmentally-responsive sperm miRNA (Rodgers et al., 2015). Interestingly, although chronic ethanol exposure similarly blunted stress responsivity in male offspring (see Chapter 2), none of the stress-enriched miRNAs reported by Rodgers et al. were affected in the current study, suggesting the intergenerational effects of the current chronic ethanol exposure are likely conferred through a different constellation of small noncoding RNAs or an alternative epigenetic pathway.

Although the specific function of sperm small noncoding RNAs is unknown, the two species directly implicated in intergenerational inheritance, miRNA and tDR, are both associated with post-transcriptional regulation and may function in the fertilized oocyte by regulating mRNA target stability and/or translation (Chen et al., 2016b). As, miRNA have been found to predominantly target the 3'-UTR of mRNAs, sequence homology target prediction was utilized to identify

common 3'-UTRs targeted by ethanol-responsive sperm miRNAs. Gene ontology analysis of common targets revealed enrichment for transcription factors and transcriptional regulators. Three genes, *Lcor*, *Nr6a1*, and *Rora*, were targeted by five or more of the seven ethanol-enriched miRNAs. *Lcor* binds with various steroid receptors including estrogen, progesterone, and glucocorticoid receptors (Palijan et al., 2009), has been found to directly attenuate progesterone regulated gene expression (Fernandes et al., 2003), and is highly expressed in two-cell embryos (Fernandes et al., 2003) suggesting a critical role in steroid-hormone receptor mediated gene expression during embryogenesis. In addition, loss of *Nr6a1*, also known as germ cell nuclear factor, results in lethality during embryonic development (Wang and Cooney, 2013). Thus, future studies are warranted to investigate the effects of paternal chronic ethanol exposure on predicted targets of ethanol-responsive miRNA during embryogenesis.

While the specific role of sperm tDR is unknown, most evidence suggests tDR act similarly to miRNA via post-transcriptional regulation of mRNA stability or translation. Notably, while miRNA function is primarily associated with regulation at 3'-UTRs, tDR are more likely to target 5'-UTRs (Schuster et al., 2016a; Schuster et al., 2016b). Remarkably, the number of predicted 5'-UTR targets was substantially greater for one tDR, Glu-CTC, relative to all other analyzed species. This was striking considering it is also enriched several hundred-fold relative to nearly all other small noncoding RNAs in sperm. Gene ontology analysis of predicted 5'-UTR targets of tDR Glu-CTC revealed greatest enrichment for genes related to phosphoprotein, alternative splicing, cytoplasm, and acetylation. Therefore, Glu-CTC is well-positioned to be functionally significant in the fertilized oocyte. Supporting this notion, one study found that approximately half of the predicted targets of tDR Glu-CTC were sharply reduced from the oocyte to four cell-stage of embryonic development (Cropley et al., 2016). Furthermore, injecting the other equally-enriched

tDR species in sperm, Gly-GCC, into fertilized oocytes dramatically altered gene expression while an equal amount of endogenously less-expressed sperm tDRs were comparatively ineffective (Sharma et al., 2016), suggesting a potentially greater role for sperm small RNAs with robust endogenous expression such as Glu-CTC. Future studies will need to examine the effect of ethanol-sensitive tDR Glu-CTC on gene expression in the early embryo.

As the intergenerational effects of the chronic ethanol exposure used in this chapter are selective for male offspring(Finegersh and Homanics, 2014; Rompala et al., 2017), the heritable phenotype could be a result of unique epigenetic regulation of the Y chromosome in sperm. However, in the current study among the predicted mRNA targets of ethanol-responsive miRNA and mRNA and gene promoter targets of ethanol-responsive tDR, Y-chromosome expressing genes are sparsely represented. This suggests that other non-Y chromosome-encoded targets of ethanol-responsive small RNAs, such as *Lcor* (see Figure 22), which is critical for sex-hormone signaling(Palijan et al., 2009), may be more important for the sex-specific intergenerational effects. Alternatively, other epigenetic factors at the Y chromosome such as DNA methylation and histone modifications may be more sensitive to chronic ethanol exposure.

In other tissues, the production of 5'-derived tRNA halves results from cellular stress-induced cleavage at the anticodon loop by the RNase angiogenin (Fu et al., 2009). This tRNA cleavage is increased in the absence of cytosine-5 methylation (Tuorto et al., 2012). The major cytosine-5 tRNA methyltransferase enzymes are *Nsun2* and *Dnmt2* and chronic ethanol exposure reduced expression of *Nsun2* in cauda epididymis. Loss of *Nsun2*-dependent tRNA methylation results in dramatically increased cleavage of tRNAs into ~30-35 nt halves by angiogenin (Blanco et al., 2014). Although it is unclear whether angiogenin-mediated cleavage and tRNA cytosine-5 methylation similarly regulate tDR production in epididymis and sperm, *Nsun2* and *Dnmt2* are

highly expressed in both testis and epididymis and *Nsun2* is critical for proper germ cell differentiation (Hussain et al., 2013). Furthermore, a recent study found that maternal and paternal *Dnmt2* expression is essential in two separate animal models of RNA-mediated inheritance (Kiani et al., 2013). Thus, tRNA cytosine-5 methyltransferase activity may be important for sperm tDR biogenesis and RNA-mediated epigenetic inheritance. More studies are needed to investigate the mechanistic role of tRNA cytosine-5 methyltransferase enzymes specifically in sperm tDR production and function.

There is growing interest in the role of post-transcriptional nucleoside modifications in RNA function. Small noncoding RNAs also feature these modifications which are important for stability in the oocyte and even the ability of small noncoding RNAs to induce intergenerational phenotypes (Chen et al., 2016a). Using HPLC-MS/MS to examine the tDR enriched ~30-40 nt sperm RNA fraction directly for nucleoside modifications, there was a significant effect of chronic ethanol exposure on two modifications, f^5C and mnm^5s^2U. Each of these nucleoside modifications have been identified previously on intact mitochondrial-encoded tRNAs at the wobble position of the anticodon loop (Yan and Guan, 2004; Machnicka et al., 2013; Nakano et al., 2016), critical to tRNA structure and codon recognition. Two pathogenic point mutations have been associated with the inability to form f^5C modifications (Nakano et al., 2016), suggesting functional significance. Whether these nucleoside modifications reflect alterations to the parent mitochondrial tRNA in sperm or if they also serve a specific function on mitosRNA such as stability or target recognition is unknown. Interestingly, f^5C is found on mt-Tm, the mitochondrial tRNA for methionine (Nakano et al., 2016), and mitosRNAs mapping to mt-Tm were increased by chronic ethanol exposure (Figure 2D). Increased f^5C may be a consequence of increased mt-Tm small noncoding RNAs or it is also possible that f^5C stabilizes mt-Tm-mapping mitosRNAs. Overall, these findings further

support the notion that in addition to sperm small RNA abundance, post-transcriptional modifications are sensitive to environmental insults such as chronic ethanol exposure.

Given that DNA in sperm is condensed by highly alkaline protamines, there is minimal transcriptional activity in mature sperm. Thus, environmentally-induced changes to the sperm RNA profile are likely driven by extracellular factors. Supporting this notion, the epididymis is enriched with principal secretory cells that release extracellular vesicles (i.e. epididymosomes) capable of fusing with the sperm membrane. In addition, some fraction of these extracellular vesicles and RNAs may originate from distal organ systems including the brain (Cossetti et al., 2014; Marre et al., 2016). Many studies have characterized epididymosome-mediated protein exchange with sperm (see (Sullivan, 2016) for review). More recently, it was found that epididymosomes carry a tDR-enriched small RNA milieu that is similar to sperm (Sharma et al., 2016) and epididymosomes can directly transfer small noncoding RNAs to immature sperm *in vitro* (Reilly et al., 2016; Sharma et al., 2016). Here, caput sperm did not have the same ethanol-induced changes to sperm tDR seen in cauda sperm, suggesting ethanol-sensitive sperm tDRs are altered during epididymal transit. Indeed, examining tDR from epididymosome revealed that the effects of chronic ethanol exposure on sperm tDRs Glu-CTC and His-GTG were reflected in epididymosomes.

While there was only a correlation between tDR and epididymosomes for tDR Glu-CTC and only following two, but not five weeks of ethanol exposure, several factors may contribute to differences between the RNA cargo in epididymosomes vs sperm housed in the cauda epididymis at a given time point. For instance, after epididymal transit, mature rodent sperm are estimated to remain motile in the cauda epididymis for one month (Jones, 1999). Moreover, the majority of epididymosomes have been found to target dead sperm while a subtype of CD9-positive epididymosomes show increased preference for live sperm (Caballero et al., 2013). Thus, a better

understanding of the temporal and subtype-specific dynamics of *in vivo* epididymosome to sperm RNA transfer is needed in future investigations of this novel soma to germline mechanism.

Finally, incubating epididymosomes from ethanol-exposed donors with sperm differentially affected body weight, anxiety-like behavior and binge-like ethanol drinking in IVF-derived mice. Notably, these phenotypes, aside from reduced body weight in males, were inconsistent with the effects of paternal ethanol on offspring—increased ethanol-induced anxiolysis, decreased ethanol drinking, and blunted HPA axis responsivity selectively in male offspring (Finegersh and Homanics, 2014; Rompala et al., 2016). Moreover, the significant effects of ethanol EV-donor on ethanol drinking in males were very modest, limited to the drinking in the dark paradigm and not reflected by BEC following the drinking in the dark test. Nevertheless, while these results do not directly support epididymosomes as conferring the intergenerational effects of ethanol, they are the first proof of principle evidence supporting a causal role for epididymosomes in epigenetic inheritance of paternal preconception environment.

There are several limitations to the epididymosome donor IVF experiments. While the hypothesized cross-generational mechanism for epididymosomes is RNA trafficking, it is also possible that ethanol-exposed epididymosomes uniquely affected the internal and surface protein content of sperm (Martin-DeLeon, 2015). Epididymosome-derived sperm proteins influence immunoprotection, capacitation, and acrosomal exocytosis, all of which may conceivably affect embryonic development (Martin-DeLeon, 2015). Moreover, the ethanol-exposed epididymosomes may have affected sperm survival as most epididymosomes have been found to target dead sperm for protein-trafficking. Finally, the ultracentrifugation of extracellular vesicles may not have been sufficient to remove contaminants such as protein aggregates, ribonucleoprotein complexes, and DNA-fragments (Li et al., 2017; Shurtleff et al., 2017). Thus, additional mechanistic studies are

needed to determine whether the cross-generational effects of Ethanol EV-donors were specific to epididymosomal RNA trafficking.

In summary, the findings in Chapter 4 provide the first evidence that chronic ethanol exposure alters small noncoding RNA abundance and nucleoside modifications in sperm. Additionally, ethanol has similar effects on small noncoding RNAs in epididymosomes and treating sperm with ethanol-exposed epididymosome differentially affects complex behavioral phenotypes in IVF mice. These results support the hypothesis that epigenetic reprogramming of sperm RNA may be downstream of environmentally-induced changes in extracellular vesicle trafficking in the epididymal lumen. These findings have significant public health implications as they suggest that paternal ethanol exposure significantly affects offspring behavior via epigenetic mechanisms in the epididymis and sperm. Future studies are needed to directly interrogate the effects of ethanol-sensitive small noncoding RNAs in sperm on embryo and offspring development.

5.0 FINAL DISCUSSION

The experiments completed in this dissertation build off the initial work of (Finegersh and Homanics, 2014) which established that the heritable effects of preconception ethanol exposure include altered ethanol-related behaviors -- most notably, ethanol drinking behavior (Finegersh and Homanics, 2014; Rompala et al., 2017). I have completed three separate, but complimentary experiments, all aimed at elucidating mechanisms of intergenerational ethanol-related behavior (see Table 6). The experiments in Chapter 2 demonstrate that paternal chronic ethanol exposure blunts HPA axis responsivity in male. In Chapter 3, paternal chronic variable stress was sufficient to reduce ethanol drinking in males. Finally, the experiments in Chapter 4 reveal that chronic ethanol exposure alters small noncoding RNA in sperm and implicate epididymal extracellular vesicles as a plausible soma-to-germline mechanism for the effects of paternal preconception environment on ethanol- and stress-related behavior. Overall, this dissertation reveals several novel effects of paternal environment on behavior and epigenetic mechanisms in sperm. Several future lines of study examining the systems- and generations- spanning mechanisms of epigenetic inheritance (i.e., the intricate passage of epigenetic memory from paternal environment to soma to germline to soma to behavior) are warranted.

Table 7. Dissertation results summary

Chapter	Paternal Exposure	Intergenerational Effects (B6 × Strain 129 offspring)	Sex
2	Chronic Ethanol	Blunted acute stress-induced CORT	♂
		Resistance to stress-induced polydipsia	♂
		Reduced chronic stress-induced CRF gene expression in PVN	♂
		Increased chronic stress-responsive CRF methylation in PVN	♂
3	Chronic Stress	Reduced two-bottle choice ethanol drinking (IHC-sire dependent)	♂
		Reduced binge-like ethanol drinking (IHC-sire dependent)	♂
		Epigenetic Effects in Sperm	
4	Chronic Ethanol	Differential expression of 15 tDR, 8 miRNAs, and 5 mitosRNAs	
		Differential expression of two small RNA modifications	
		Ethanol-responsive sperm tDR affected in epididymal EVs	
	Germline Exposure	**Effects on IVF-derived B6 mice**	
4	Ethanol-Derived EVs	Increased anxiety-like behavior	♀
		Increase binge-like drinking in IVF-males	♂

5.1 CHAPTERS 2 AND 3: SIGNIFICANCE AND FUTURE DIRECTIONS

5.1.1 Paternal ethanol imparts stress hyporesponsivity to males

The finding that paternal ethanol imparts stress hyporesponsivity to male offspring is comparable to the cross-generation effects of paternal stress. Strikingly, the effect of paternal ethanol on the CORT response to acute restraint in males is very similarto the effects of paternal chronic variable stress on HPA responsivity in male and female offspring (Rodgers et al., 2013). In addition, parental social isolation stress blunts the CORT response to foot-shock stress in offspring and reduces CRF gene expression in the PVN (Pisu et al., 2013). Epidemiological studies have also reported similar intergenerational effects such as suppression of basal cortisol levels and increased dexamethasone suppression of cortisol levels in offspring of parents with preconception-diagnosed posttraumatic

stress disorder (PTSD), and decreased GR methylation in offspring of Holocaust survivors (Bowers and Yehuda, 2016). Therefore, the effects of paternal ethanol on HPA axis responsivity reflect these collective findings, suggesting that exposures that induce chronic hyperactivation of the HPA axis drive intergenerational epigenetic reprogramming of stress responsivity.

Future Directions: **What are the epigenetic mechanisms underlying intergenerational stress hyporesponsivity?**

The inherited epigenetic mechanisms responsible for stress hyporesponsivity in ethanol-sired males are unknown. In Chapter 2 of this dissertation, there was reduced CRF gene expression in the PVN associated with greater methylation of the CRF promoter in stressed ethanol-sired vs stressed control-sired males. However, in unstressed males, there was no effect of paternal ethanol on CRF gene expression and it is unclear whether differences in CRF promoter methylation were present prior to stress exposure. One relevant study found that social defeat stress induces loss of CRF methylation, presumably by active demethylation mechanism (Elliott et al., 2010). This suggests the difference in CRF methylation between ethanol- and control-sired males is explained by loss of CRF methylation in controls in response to chronic intermittent variable stress. Nevertheless, future studies are warranted to examine DNA methylation and histone modification in ethanol-sired males at baseline which could explain protection from stress-potentiated CRF expression in control males. In addition, while the current study only examined stress-regulatory genes in PVN and CeA, several studies have demonstrated a crucial role for epigenetic regulation of the glucocorticoid receptor (GR) in HPA responsivity (Turecki and Meaney, 2016). Indeed, paternal chronic variable stress increased expression of GR-responsive genes in the PVN (Rodgers et al., 2013). Moreover, paternal early life stress decreased GR methylation in hippocampus of adult male offspring which coincided with increased active coping behaviors (Gapp et al., 2016). Thus,

additional characterization of stress-regulatory genes and brain regions may reveal other candidates for paternal preconception ethanol-induced epigenetic reprogramming of offspring brain.

5.1.2 Is intergenerational stress hyporesponsivity adaptive?

Given, that a family history of stress-related disorders is a major risk factor for psychiatric illness (Lohoff, 2010), the finding that paternal ethanol exposures blunt HPA responsivity appears counter-intuitive. Nevertheless, the results from Chapter 2 are supported by various rodent and human studies examining the cross-generational effects of chronic stress (Pisu et al., 2013; Rodgers et al., 2013; Bowers and Yehuda, 2016). Other examples of intergenerational "resilience-like" phenotypes have been reported for paternal ethanol and paternal cocaine exposures which conferred reduced ethanol drinking preference and resistance to cocaine reinforcement, respectively, in male offspring (Vassoler et al., 2013; Finegersh and Homanics, 2014; Rompala et al., 2017). Therefore, parental environmental perturbations may promote the transmission of adaptive epigenetic memory to prime the next generation for environmental exposure to stressors or drugs of abuse. Further supporting such a mechanism, paternal olfactory fear conditioning to foot shock lead to enhanced cross-generational fear conditioning specifically to the foot shock-paired odorant (Dias and Ressler, 2014).

On the contrary, there is no clear evidence that reduced HPA stress responsivity underlies stress resilience in rodents or humans. In Rodgers et al, while chronic variable stress reduced HPA stress responsivity in male and female offspring, there was no effect on anxiety- or depression-related phenotypes (Rodgers et al., 2013). Additionally, in humans, parental PTSD is associated with reduced HPA responsivity in offspring and concurrent increased PTSD risk. This observation undergirds the popular theory that premature termination of cortisol in response to severe stress

disinhibits the sympathetic nervous system response, leading to persistent catecholamine dysregulation in PTSD (Bowers and Yehuda, 2016). Thus, whether blunted stress responsivity in ethanol- and stress-sired male offspring is adaptive or maladaptive may be context-dependent.

5.1.3 Relationship between ethanol and stress-related mechanisms in F0 and F1 generations

Paternal exposures to chronic ethanol or chronic stress induced similar changes in ethanol drinking behavior (Finegersh and Homanics, 2014; Rompala et al., 2017)(Chapter 3) and stress responsivity (Rodgers et al., 2013)(Chapter 2) in male offspring. Ethanol acts as an HPA activator (Rivier, 2014), especially under the forced conditions employed in these dissertation experiments. These results suggest corticosterone may be a critical regulator of reduced ethanol drinking and blunted stress responsivity intergenerational phenotypes. Notably, one recent study found that paternal corticosterone resulted in increased anxiety-like behavior in adult male offspring, but ethanol and HPA responsivity phenotypes were not examined (Short et al., 2016). In addition, it will be intriguing to examine whether paternal voluntary ethanol consumption imparts similarly ethanol and stress related behaviors to male offspring as a recent study found opposing intergenerational effects of paternal voluntary vs forced cocaine exposure (Le et al., 2017).

Whether aberrant HPA axis function in ethanol-sired male offspring contributes to the coinciding reduced ethanol drinking phenotype (Finegersh and Homanics, 2014; Rompala et al., 2017) remains to be examined. CORT is sufficient and necessary for ethanol preference and consumption in rodents that have established baseline ethanol drinking (Fahlke et al., 1994; Fahlke et al., 1996; Fahlke and Eriksson, 2000). However, the role for CORT in establishing initial low concentration ethanol drinking preference -- which would be hypothesized in the case of

intergenerational ethanol drinking behavior -- has not been directly explored. Notably, CORT levels in response to various acute stressors does not predict subsequent ethanol drinking preference or consumption in B6 male mice (Cozzoli et al., 2014). Accordingly, using mice on a pure B6 background, paternal ethanol increased ethanol sensitivity and decreased ethanol drinking in males, with no coinciding effect on HPA axis responsivity (see Appendix A) (Rompala et al., 2017). In addition, as presented in Chapter 3, paternal chronic variable stress reduced ethanol drinking in B6 × 129 hybrid male offspring, but not HPA axis responsivity in the same cohort. Thus, intergenerational reduced ethanol drinking behavior has been observed in two separate studies in the absence of stress hyporesponsivity. Nevertheless, as stress and the HPA axis are integral to alcoholism pathophysiology and a wide range of ethanol drinking behaviors in rodents (Koob, 2015), further investigation of the mechanistic relationship between paternal environment-imparted intergenerational stress- and ethanol-related phenotypes is warranted.

<u>Future directions</u>: Does stress hyporesponsivity underlie intergenerational ethanol drinking behavior?

To directly examine the role of glucocorticoids in reduced ethanol drinking, future studies should examine whether CORT administration can rescue the paternal ethanol-imparted reduced ethanol drinking phenotype in males. In addition, failure to induce adequate CRF gene expression in response to stress could be upstream of the CORT deficit in ethanol-sired males as CRF was reduced in the PVN of ethanol-sired males with a history of chronic stress and ethanol drinking. Indeed, the HPA-stimulating effects of ethanol are dependent on hypothalamic CRF (Rivier, 2014). Nevertheless, compared with B6 × Strain 129 males, there was no effect of paternal ethanol on HPA axis responsivity in B6 mice. This may be explained by a strain-specific mechanism or inherent challenges to studying the stress physiology of mice on a B6 background (Chan et al.,

2017b). As a result, the HPA axis may not be the preferred target for rescuing reduced ethanol drinking in ethanol-sired males. Alternatively, BDNF gene expression was increased in the VTA of both hybrid and pure background ethanol-sired males (Finegersh and Homanics, 2014; Rompala et al., 2017). The dopaminergic neurons of the VTA are integral to encoding drug reward and glucocorticoid signaling in ventral striatum facilitates ethanol drinking behavior (Spanagel et al., 2014). Moreover, GR directly reduces BDNF transcription by binding to the activity-dependent BDNF promoter region (Chen et al., 2017). Therefore, further examination of VTA and related brain reward systems may undercover a plausible link between stress hyporesponsivity and reduced ethanol drinking phenotypes.

5.1.4 Determining causality of inherited epigenetic mechanisms in intergenerational ethanol-and stress-related behaviors

Paternal ethanol exposure alters DNA methylation of BDNF (Finegersh and Homanics, 2014) and CRF promoters (Chapter 2), but the role of these genes or the specific epigenetic modifications in intergenerational behavior is unknown. There is no putative evidence for a paternal environment-induced epigenetic mark in offspring somatic cells that casually-induces an intergenerational behavior in mammals. However, there is compelling evidence for intergenerational behaviors closely associating with epigenetic remodeling at specific loci. For instance, paternal cocaine taking reduced object memory retention, increased hippocampal expression of DAO1 (which metabolizes D-serine) and increased permissive histone marks near the DAO1 transcriptional start site in male offspring (Wimmer et al., 2017). The memory retention deficit in cocaine-sired males was ameliorated with D-serine treatment (Wimmer et al., 2017). In a similar study, paternal cocaine taking increased BDNF gene expression in medial prefrontal cortex (mPFC), increased permissive

histone modifications at BDNF promoter regions, and conferred a BDNF-dependent reduction in cocaine self-administration behavior to male offspring (Vassoler et al., 2013). However, in these studies it remains unknown whether the euchromatic state of the *Dao1* locus in hippocampus or *Bdnf* locus in the mPFC was sufficient or necessary for the related intergenerational behavioral phenotypes. Consequently, while such studies strongly implicate epigenetic regulation of gene expression in cross-generational phenotype, technical limitations have delayed causal testing. In addition, while an epigenetic modification may be important for cross-generational adult behavior, it is unknown whether implicated histone modifications and DNA methylation are stable through postnatal and adolescent development, especially as these mechanisms are responsive to a variety of early life environmental perturbations including maternal care (Champagne, 2013).

Future directions: Identifying epigenetic mechanisms underlying ethanol-related phenotypes

If the gene targets presently implicated by paternal ethanol-- BDNF in the VTA or CRF in the PVN-- are found to drive intergenerational ethanol- and stress-related phenotypes, the DNA methylation and/or histone modification signature at these loci should be characterized and ultimately causally examined for a direct role in intergenerational behavior. Indeed, techniques are being rapidly developed and utilized that allow for delivery of DNA methyltransferase and histone deacetylase enzymes to specific genomic targets in adult animals (Heller et al., 2013; Heller et al., 2014; Heller et al., 2016).

To date, the examination of epigenetic alterations in ethanol-sired males has been limited to candidate genes found to have altered gene expression. Indeed, a more complete characterization of gene expression with RNA sequencing in brain regions, such as PVN or VTA, followed by high throughput epigenetic profiling with classical (i.e., chromatin immunoprecipitation sequencing) or emerging techniques (e.g., ATAC-sequencing for DNA accessibility, or CRISPR-CAPTURE (Liu

et al., 2017) for locus-specific RNA and protein extraction) could expedite the discovery of hundreds of genetic and epigenetic alterations in ethanol-sired offspring.

5.1.5 Sex-specificity of intergenerational phenotypes

Several studies have revealed sex-specific effects of paternal preconception exposures. Remarkably, both paternal ethanol in Chapter 2 and paternal stress in Chapter 3 induced intergenerational effects only in males. Importantly, this outcome could result from the confounding influence of the female estrus cycle. For instance, ovarian hormones mediate HPA axis responsivity to acute restraint stress (Kalil et al., 2013). In contrast, the significance of estrus cycle variation for ethanol drinking behavior is unsupported (Becker and Koob, 2016). In addition, as the sex-specific intergenerational ethanol drinking phenotype has been observed now across two published studies (Finegersh and Homanics, 2014; Rompala et al., 2017), and again in Chapter 3, the possibility that estrus cycle variation is masking the effects of paternal environment on female drinking behavior appears unlikely.

While the differential epigenetic regulation of the Y-chromosome in sperm is a plausible mechanism for male-specific intergenerational effects, we found that ethanol-responsive small RNAs have minimal Y-chromosome gene or mRNA targets. Thus, if small RNAs underlie the sex specific intergenerational effects of paternal ethanol, it is likely through regulation of non-Y encoded sexual dimorphic genes.

<u>Future Directions</u>: What is the epigenetic signature of sex-specific cross-generational phenotypes?

Sex differences in epigenetic programming could be set in motion at fertilization by oestridiol and testosterone, which are potent epigenetic regulators of the sexually dimorphic brain

(Bale, 2015). Relatedly, there is an emerging hypothesis that the parental preconception environment alters epigenetic regulation of Y chromosome transcription factors (e.g., SRY) and X-chromosome silencing in females (Sarkar, 2016). While we did not discover candidate Y chromosome genes targeted by ethanol-responsive small RNAs, other epigenetic factors such as DNA methylation or histone modifications may be involved. Thus, future studies should examine whether Y chromosome encoded genes are affected by chronic ethanol in the early embryo; thereafter, attractive candidate genes could be examined for loci-specific epigenetic effects in sperm. Additionally, it will be of great interest to separate X and Y chromosome bearing sperm with flow cytometry (Garner et al., 2013; Hashimoto et al., 2013). Comparing epigenetic marks (e.g., small noncoding RNAs) between these sperm populations may facilitate discovery of sex-specific sperm epigenetic marks or could increase resolution for identifying mechanisms of sex-specific intergenerational inheritance.

5.2 CHAPTER 4: SIGNIFICANCE AND FUTURE DIRECTIONS

5.2.1 Chronic ethanol induces epigenetic reprogramming of small noncoding RNAs in sperm

Among the small noncoding RNAs types in sperm, tDR were the most responsive to chronic ethanol exposure. Sperm tDR comprise the majority of small noncoding RNA in sperm (Peng et al., 2012; Sharma et al., 2017). While no published studies have examined the effects of stress or drugs of abuse on sperm tDR, paternal diet and exercise studies have directly implicated this small RNA type in intergenerational inheritance (Chen et al., 2016a; Sharma et al., 2016; Short et al., 2017).

Embryo injections with the tDR-enriched fraction (~30-40 nt) of high fat diet-treated mouse sperm RNA, but not other sized sperm RNAs, recapitulated the effects of paternal high fat diet on glucose tolerance in resultant offspring (Chen et al., 2016a). Moreover, the low protein diet enriched sperm tDR Gly-GCC is sufficient to recapitulate the effects of paternal low protein diet on embryonic gene expression (Sharma et al., 2016). Together, these studies strongly implicate tDR in nongenomic germline inheritance.

Despite the numerous studies demonstrating a causal role for sperm RNA in intergenerational effects of paternal environment (Gapp et al., 2014; Grandjean et al., 2015; Rodgers et al., 2015; Chen et al., 2016a; Sharma et al., 2016), it is not clear how sperm RNA function in the fertilized oocyte. Injection of miRNA mimics induced primarily a reduction in gene expression, consistent with a canonical miRNA silencing mechanism, though only 8 of 75 predicted targets were reduced in one cell embryos (Rodgers et al., 2015). By comparison, injection of a tDR mimic for the endogenously-enriched tDR Gly-GCC suppressed embryonic expression of genes regulated by the muERV-L/MERVL transposable element that plays a critical role in preimplantation development (Sharma et al., 2016). These studies support the hypothesis that sperm small noncoding RNAs are post-transcriptional regulators in the embryo. In theory, this transient transcriptional regulation during embryogenesis could induce lasting epigenetic alterations such as *de novo* DNA methylation that persists into adulthood (Greenberg et al., 2017). It is possible that sperm RNAs acquire unique functionality by forming as-yet unidentified protein-RNA effector complexes or through RNA modifications (Kiani et al., 2013). In addition, if the RNA cargo from epididymosomes adhering to the sperm exterior is also trafficked to the embryo cytoplasm, this would greatly increase the paternal RNA contribution (Sharma and Rando, 2017). Finally, sperm-derived RNAs may be reverse-transcribed and amplified in the early embryo as transcriptionally-

competent cDNA (Spadafora, 2017). Undoubtedly, additional studies are needed to uncover potential mechanisms of RNA-mediated inheritance.

Future directions: How does sperm RNA function in the embryo?

Directly testing the effects of sperm tDR at fertilization on ethanol- and stress-related phenotypes in the resultant adult offspring would be a crucial step forward in establishing a causal role for ethanol-responsive sperm small noncoding RNAs in epigenetic inheritance of paternal environment. The ethanol-enriched sperm tDR Glu-CTC may be a good candidate as it has substantially more predicted mRNA targets than other ethanol-responsive tDR (Figure 22). Moreover, it is one of the two extremely-enriched tDR species; the other is Gly-GCC, which was sufficient to recapitulate the effects of paternal high fat diet on embryonic gene expression (Sharma et al., 2016). Conversely, multiple or all ethanol-responsive sperm RNAs may be critical for the intergenerational phenotypes, as was discovered in a recent study examining the intergenerational effects of chronic stress-enriched sperm miRNAs (Rodgers and Bale, 2015). However, experiments injecting RNA into fertilized embryos to examine sperm RNA function have important caveats that currently limit the interpretation of results. In addition, the sperm RNA payload is minuscule (100 fg in rodents) and even the contribution of sperm-enriched tDR and miRNA species is negligible relative to the amount of pre-existing copies in the oocyte (~1 ng) (Yang et al., 2016). Therefore, studies to date examining the function of specific RNAs in fertilized embryos do not reflect physiological conditions ($\geq$10 × RNA from one sperm)(Gapp et al., 2014; Rodgers et al., 2015; Chen et al., 2016a), especially as any exogenously-delivered RNA is in surplus to the endogenous sperm RNA payload delivered at fertilization. To address these limitations, subsequent experiments could test whether candidate ethanol responsive sperm RNAs are both sufficient and necessary for intergenerational inheritance (e.g., testing necessity of candidate RNAs by injecting antisense

oligos into fertilized embryos for targeted inhibition). Finally, given that two distinct paternal exposures (i.e., ethanol and stress) effect ethanol drinking behavior in males, comparing the sperm RNA milieu between these groups could improve detection of candidate RNAs for causal examination.

5.2.2 Soma-to-germline trafficking of epigenetic memory

Incredibly, while incubating sperm with ethanol-donor epididymosomes prior to IVF did not recapitulate the intergenerational effects of paternal ethanol, this *in vitro* manipulation did impact body weight, anxiety-like behavior, and modestly affected ethanol-related phenotypes in the IVF progeny. This unexpected result can likely be explained by the inability of a three hour *in vitro* sperm and epididymosome coincubation to reflect the *in vivo* mechanisms occurring throughout epididymal transit and storage that can span over a month in rodents (Jones, 1999). Considering these results, substantially more work is needed to elucidate how sperm were altered by epididymosomes in this experiment. Although the ability of epididymosomes to deliver RNA cargo to immature testis and caput sperm *in vitro* has been demonstrated previously (Figure 24E)(Reilly et al., 2016; Sharma et al., 2016; Sharma et al., 2017), this remains to be validated in cauda sperm.

<u>Future Directions</u>: How do environmental perturbations affect epididymosomal trafficking to sperm?

Given the evidence implicating epididymosomes as the source of major RNA species in sperm, there is a fast-growing interest in characterizing gonadal and neuroendocrine regulation of epididymosome production and/or function. For instance, testosterone levels mediate epididymosome protein trafficking to the sperm membrane (Suryawanshi et al., 2012). Moreover, a recent study found that chronic variable stress increases GR expression in caput epididymis (Chan

et al., 2017a). In principle epididymal cell cultures, chronic CORT treatment altered the miRNA cargo of epididymosomes (Chan et al., 2017a). It will be fascinating to see if GR upregulation in caput epididymis *in vivo* is sufficient and necessary for the intergenerational effects of chronic variable stress. Future studies should further investigate how adaptive changes in epididymal gene expression effect epididymosome RNA cargo sorting and delivery to sperm. One powerful tool for such experiments was introduced in a recent study utilizing a caput-specific Cre recombinase mouse line crossed with a LoxP line for uracil phosphoribosyltransferase (UPRT) which incorporates traceable 4-TU into newly synthesized RNA (Sharma et al., 2017). As a result, when these mice are treated with 4-TU, caput-synthesized RNAs can be detected as U>C mutations with downstream sequencing methods. This methodology can be used to examine the effect of various environmental perturbations directly on soma-to-germline RNA trafficking. Moreover, many studies have shown that extracellular vesicles can travel between distal organ systems such as brain and liver (Ridder et al., 2014). While one study did not find liver or testis synthesized RNAs in mature sperm, the the analysis may not have been sufficiently sensitive to detect small contributions relative to epididymis-derived RNAs (Sharma et al., 2017). In addition, this distal RNA trafficking mechanism could be uniquely engaged under aberrant physiological conditions. For instance, mice xenografted with tumor cells were found to express tumor-cell specific RNAs in sperm likely via long-range extracellular vesicle trafficking (Cossetti et al., 2014).

5.3 CONCLUSION

In summary, this dissertation has advanced several lines of inquiry into the varied and systems-spanning mechanisms of intergenerational ethanol- and stress-related behaviors. Paternal chronic ethanol and chronic stress exposures impart similar ethanol- and stress-related behaviors to male offspring. Moreover, paternal ethanol alters several small noncoding RNAs, an epigenetic mechanism causally implicated in intergenerational inheritance. Finally, incubating epididymosomes from ethanol-exposed donors with sperm was sufficient to confer complex phenotypes to IVF-derived mice, implicating soma-to-germline communication as a causal mechanism in cross-generational inheritance. These findings lend themselves to several specific follow-up studies as described throughout Chapter 5. Broadly, future intergenerational studies are needed to elucidate: a) how paternal environment influences soma-to-germline communication, b) how sperm drive epigenetic reprogramming in the early embryo, and c) how paternal epigenetic memory inherited in the early embryo shapes the adult brain and behavioral phenotype. Overall, this dissertation work has profound public health implications, demanding serious consideration of the paternal preconception environment as an active mechanism in offspring development.

Appendix A

PATERNAL PRECONCEPTION ALCOHOL EXPOSURE IMPARTS INTERGENERATIONAL ALCOHOL-RELATED BEHAVIORS TO MALE OFFSPRING ON A PURE C57BL/6J BACKGROUND

Adapted from:
Rompala, G.R., Finegersh, A., Slater, M., and Homanics, G.E. (2017). Paternal preconception alcohol exposure imparts intergenerational alcohol-related behaviors to male offspring on a pure C57BL/6J background. *Alcohol* 60**,** 169-177. doi: 10.1016/j.alcohol.2016.11.001.

Abstract

While alcohol use disorder (AUD) is a highly heritable condition, the basis of AUD in families with a history of alcoholism is difficult to explain by genetic variation alone. Emerging evidence suggests that parental experience prior to conception can impact inheritance of complex behaviors in offspring via non-genomic (epigenetic) mechanisms. For instance, male C57BL/6J (B6) mice exposed to chronic intermittent vapor ethanol (CIE) prior to mating with Strain 129S1/SvImJ ethanol-naïve females produce male offspring with reduced ethanol drinking preference, increased ethanol sensitivity, and increased BDNF expression in the ventral tegmental area (VTA). In the present study, we tested the hypothesis that these intergenerational effects of paternal CIE are reproducible in male offspring on an inbred B6 background. To this end, B6 males were exposed to six weeks of CIE (or room air as a control) before mating with ethanol-naïve B6 females to produce ethanol (E)-sired and control (C)-sired male and female offspring. We observed a sex-

specific effect, as E-sired males exhibited decreased two bottle free-choice ethanol drinking preference, increased sensitivity to the anxiolytic effects of ethanol, and increased VTA BDNF expression; no differences were observed in female offspring. These findings confirm and extend our previous results by demonstrating that the effects of paternal preconception ethanol are reproducible using genetically identical, inbred B6 animals.

Introduction

Twin and adoption studies suggest that alcoholism has a heritability of ~50% (Prescott and Kendler, 1999; Young-Wolff et al., 2011; Ystrom et al., 2011). Indeed, genome wide association studies (GWAS) have identified several DNA variants associated with a family history of alcoholism, suggesting a significant role for genotype in determining risk for AUD. However, despite the abundant evidence that alcoholism is highly heritable, to date, identified genetic variants only account for a small fraction of AUD heritability (Treutlein and Rietschel, 2011). While this may be due to technical and experimental limitations, it is also possible that alternative biological mechanisms may mediate and explain this "missing" AUD heritability.

Remarkably, many recent studies have found that paternal experience in rodents can drive inheritance of complex phenotypes in offspring. For example, exposing male mice to high fat diet, cocaine, or stress prior to conception has intergenerational effects on glucose tolerance, cocaine preference, or stress responsivity, respectively (Rodgers et al., 2013; Vassoler et al., 2013; Chen et al., 2016a). The results from paternal exposure studies are provocative as the inherited phenotypes must be mediated through the germline rather than factors such as *in utero* physiology that are difficult to control with maternal perturbations. Furthermore, as many of these studies have been performed with animals on an identical genetic background, inheritance cannot be due to transmission of DNA variants across generations. Instead, inherited behavioral phenotypes are hypothesized to be governed through epigenetic mechanisms, defined here as environmentally-induced changes to non-genomic factors such as DNA methylation, histone modifications, or small noncoding RNAs that are transmitted by the paternal germline at fertilization (Bohacek and Mansuy, 2015). Thus, studying animal models of experience-driven epigenetic inheritance may

have major implications for elucidating novel mechanisms that contribute to the heritability of a wide range of human health conditions such as AUD.

Indeed, several studies have demonstrated that paternal preconception exposure to alcohol leads to developmental and behavioral alterations in offspring (see Finegersh et al., 2015 for review). Expanding on this literature, we previously investigated if paternal ethanol exposure impacts ethanol preference and sensitivity. We found that adult male mice exposed to chronic intermittent vapor ethanol (CIE) prior to mating with ethanol-naïve females produced male offspring with attenuated ethanol drinking behavior, increased sensitivity to the anxiolytic effects of ethanol, and increased brain-derived neurotropic factor (BDNF) gene expression in the ventral tegmental area (VTA) (Finegersh and Homanics, 2014). Furthermore, we found that paternal CIE (E)-sired males exhibit blunted acute and chronic stress-related phenotypes (Rompala et al., 2016). These results have major implications for AUD heritability, as they show that chronic exposure to ethanol prior to conception directly influences the inheritance of ethanol- and stress-related behaviors in offspring.

While these effects of paternal CIE are consistent with the surge of recent findings that show paternal experience can shape the behavioral phenotype of offspring, whether such intergenerational phenotypes are reproducible and sustained across various mouse strains is largely unknown. Indeed, changes in breeding strategy, such as the use of intercrossing vs outcrossing, have been shown to greatly affect the penetrance of paramutations in rodents (Yuan et al., 2015). As humans have a diverse genetic make-up, establishing whether models of intergenerational epigenetic inheritance are maintained across various rodent strains will be important for determining the translational implications of such findings.

Our previous two studies utilized CIE-exposed C57BL/6J (B6) male sires mated with Strain 129S1/SvImJ females to produce hybrid F1 male offspring that exhibited altered ethanol- and stress-related behaviors (Finegersh and Homanics, 2014; Rompala et al., 2016). In the current study, we tested the hypothesis that the effects of paternal CIE on ethanol- and stress-related behaviors would generalize to male offspring on an inbred genetic background. To this end, B6 males were exposed to CIE and mated with ethanol naïve B6 females to produce genetically identical F1 offspring for assessment of ethanol drinking behavior, sensitivity to acute ethanol injection and acute HPA axis responsivity. Our results confirm that paternal CIE produces decreased ethanol drinking behavior and increased ethanol sensitivity in inbred males of the next generation. Conversely, we did not see an effect of paternal CIE on HPA axis responsivity in male offspring. These results indicate that many, but not all effects of paternal preconception ethanol exposure are reproducible on inbred B6 animals.

Materials and Methods

All experiments were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh and conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Eight-week-old, ethanol-naïve, specific pathogen free B6 and Strain 129S1/SvImJ mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Unless otherwise specified, mice were group-housed in individually ventilated microisolater cages under 12 hour light/dark cycles and had ad libitum access to food and water.

Chronic intermittent vapor ethanol exposure (CIE)

The CIE paradigm used to model paternal preconception ethanol exposure was modified only slightly from previously published methods (Finegersh and Homanics, 2014; Rompala et al.,

2016). Briefly, group housed eight-week-old, B6 male mice were exposed to vapor ethanol (E) or room air control conditions (C) for 8 hours/day (0900 to 1700), 5 days/week (M-F) for six weeks. Sires were weighted weekly and blood ethanol concentrations (BECs) were measured following the final exposure of each week. Following the fifth week of exposure, mice were mated with one eight-week-old Strain 129S1/SvImJ females for two nights for the purpose of eliminating mature sperm that were not exposed to ethanol during all stages of spermatogenesis.

Immediately after the final ethanol exposure, E- and C-exposed males were bred in the home cage of two eight-week-old ethanol-naïve female B6 mice for 48 hours to produce F1 male and female offspring. Each C-sired and E-sired mouse was only used for one experiment (i.e. there was no repeated testing of any one mouse). For all experiments, no more than two mice of the same sex were used per litter. Offspring body weight measurements in Fig. 2 were only recorded from mice used in the two bottle free choice and acute ethanol injection experiments.

Two bottle free choice ethanol drinking

Two bottle, free choice ethanol drinking behavior was performed as previously described (Finegersh and Homanics, 2014). Briefly, at 8 weeks age, ethanol-naïve male and female E- and C-sired B6 offspring were single housed and habituated to two ball-bearing sipper-fitted 25 ml falcon tubes filled with water for one week. Following habituation, one tube was filled with ethanol at escalating concentrations of 3, 6, 9, 12, 15%, with each concentration tested for four days. Tube position and cages were changed every four days. Male mice used in the two bottle free choice drinking experiments were derived from 6 E-sired litters and 8 C-sired litters. Female mice were derived from 8 E-sired litters and 8 C-sired litters.

Two-Bottle Free Choice Saccharine and Quinine Drinking

Following testing at 15% ethanol, and a one-week washout period where only water was available, mice were tested for saccharin and quinine preference at two concentrations each with a one-week washout between tastants to control for sweet and bitter taste preferences, respectively.

Acute ethanol injection and successive elevated plus maze, open field, and accelerating rotarod tests

Eight-week-old male and female E- and C-sired B6 offspring were tested on the elevated plus maze, open field, and accelerating rotarod tasks all on the same day and in succession 10, 20, and 35 min, respectively, following an acute intraperitoneal injection of 0.9% saline (0.02 ml/g body weight) or 1.00 g/kg ethanol (0.02 ml/g of 5% ethanol in saline) as previously described (Finegersh and Homanics, 2014). Male mice used were derived from 10 E-sired litters and 7 C-sired litters for both saline and ethanol treatments. Female mice used in this experiment were derived from 6 E-sired litters and 5 C-sired litters for both saline and ethanol treatments.

Brain tissue processing and reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR)

Tissue was collected from eight-week old behaviorally-naïve E- and C-sired male mice. Mice were sacrificed between 1200 and 1600 hr during the light cycle. Brains were dissected and frozen with dried ice before being sectioned with a Microm HM 550 cryostat (Thermo Scientific, Waltham, MA). Using a 1 mm diameter micropuncher, three 300 micron thick tissue punches were collected from the VTA (approximately -5.2 to -6.1 mm, relative to bregma) (Paxinos and Franklin, 2001) into Trizol (Ambion, Grand Island, NY). Tissue was then lysed with a dounce homogenizer for RNA extraction using phenol-chloroform separation. Samples were further processed for RT-qPCR by DNAse I (Ambion) treatment, followed by final purification with RNA Clean and

Concentrator (Zymo Research, Irvine, CA) and elution into 14 µl nuclease free water. Reverse transcription of RNA was performed with the iScript cDNA Synthesis Kit (BioRad Laboratories, Hercules, CA) according to manufacturer's protocol. The cDNA product was diluted 1:10 before qPCR using BioRad SYBR Green Fluorescent Master Mix and a BioRad iCycler. Oligo sequences were: brain derived neurotropic factor (BDNF) exon IX Forward (F): 5'-AGC CTC CTC TAC TCT TTC TGC TG-3' and BDNF exon IX reverse (R): 5'-GTG CCT TTT GTC TAT GCC CCT G; β-actin F: 5'-CGT TGA CAT CCG TAA AGA CC-3' and R: 5'-AAC AGT CCG CCT AGA AGC AC-3'. Threshold cycle values for each gene were normalized within sample to β-actin and then between groups for computation of delta delta cycle threshold (ΔΔCt) to calculate fold change in mRNA expression. Due to limited animal availability, we restricted this experiment to male offspring. E- and C-sired males used in this experiment were derived from 4 E-sired litters and 4 C-sired litters.

Acute restraint stress and measurement of plasma corticosterone (CORT)

Eight-week-old male E- and C-sired offspring were subjected to a 15 min restraint stress exposure. All animals were tested between three and five hours after lights on (1000-1200 hr). Briefly, mice were restrained in conical plastic tubes with several air hole perforations near the animal's head and an opening for the tail. After the 15 minute restraint, each mouse was returned to its home cage. Tail blood (<10ul) was collected with heparin-coated capillary tubes (Drummond, Broomall, PA) at time points 0, 15, 30, and 90 minutes from the onset of restraint. Blood samples were centrifuged for 10 minutes at 4500 × g to separate plasma for measurement of CORT with an enzyme immunoassay (Enzo Life Sciences, Farmingdale, NY). Samples were diluted 1:40 and run in duplicate. The correlation coefficient for duplicate measures in our assay was r= 0.99. The reported sensitivity of this kit for detecting CORT concentrations ranges from 0.032-20 ng/ml. Due

to limited animal available, we restricted this experiment to male offspring. Male mice used in this experiment were derived from 8 E-sired litters and 9 C-sired litters.

Statistical Analysis

Behavioral and HPA axis responsivity experiments were analyzed using two way ANOVAs with or without repeated measures where appropriate. For ANOVA results reaching statistical significance ($p<0.05$), post-hoc pairwise comparisons were made using Fisher's LSD test. For rt-qPCR results, analysis was performed using Student's t-test.

Results

Paternal preconception CIE exposure

B6 males were exposed to CIE or room air conditions for six weeks. The average BEC (Fig. 34A) across all weeks of paternal CIE was 180.2 ± 14.7 mg/dl (mean ± S.E.M.). There was a significant effect of time on sire body weight ($F_{(5,110)} = 92.6$, $p <0.001$; Fig. 34B) but no effect of ethanol exposure or ethanol exposure × time interaction. These results indicate that animals gained weight and there was no difference in body weight between E-sires and C-sires over the course of the exposure period.

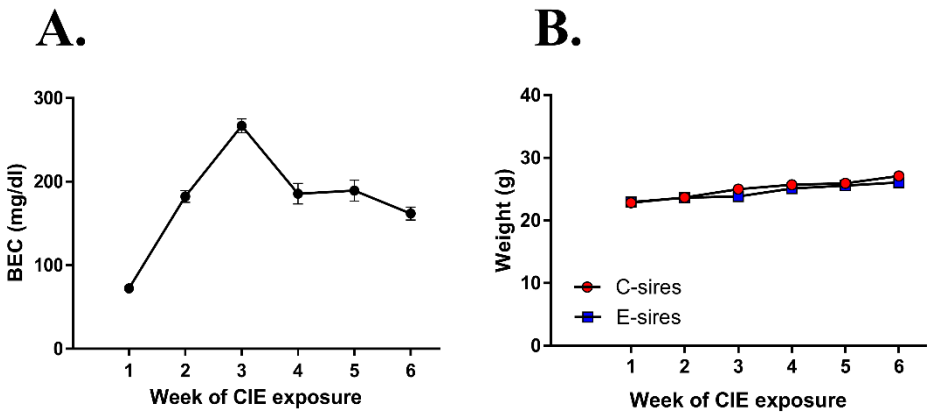


Figure 34. Paternal preconception CIE exposure

(A) Mean blood ethanol concentrations (BEC) are shown for E-sires. (B) There was no effect of CIE on the body weights of E-sires vs C-sires (n=12, 12; E-sires, C-sires). Data presented as mean ± SEM.

Paternal CIE reduces post-weaning body weight selectively in B6 male offspring

There was a significant effect of age ($F_{(4,128)}$=1177, p<0.001) and sire ($F_{(1,32)}$=9.93; p<0.01; Fig. 35A) on male offspring body weight indicating that E-sired males weighted significantly less vs C-sired males. Fisher's LSD post-hoc test revealed that E-sired males had significantly decreased body weight at postnatal weeks 4-5 (p<0.05), 6-7 (p<0.01) and 8 (p<0.001) vs C-sired males. Analysis of female offspring body weights revealed a significant effect for age ($F_{(4,180)}$=650.7, p<0.0001; Fig. 35B), but no effect of sire and no age × sire interaction.

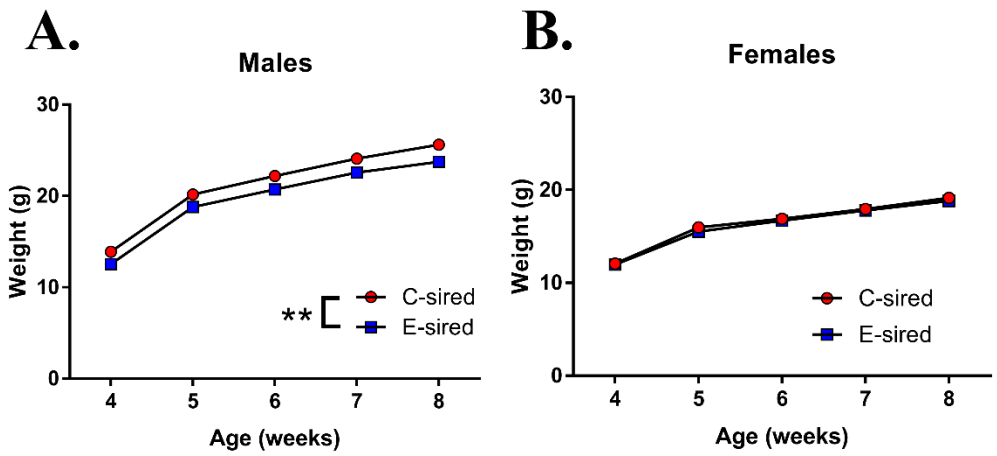


Figure 35. Paternal CIE reduces body weight selectively in male offspring.

(A) B6 E-sired males (n=19) showed decreased body weight vs C-sired males (n=15) at ages 4- 8 weeks. (B) B6 E-sired females (n=23) showed no significant difference in body weight vs C-sired females (n= 24). Data presented as mean ± SEM. *=p<0.05, **=p<0.01, and ***=p<0.001.

Paternal CIE reduces ethanol drinking behaviors selectively in male offspring

E- and C-sired adult male and female offspring were tested for ethanol drinking behavior in a two bottle choice test at sequential ethanol concentrations of 3, 6, 9, 12, and 15 % (w/vol) for 4 days each. Analysis of ethanol preference in males revealed a significant effect of ethanol

concentration ($F_{(19,266)}$=3.165, $p<0.001$) with no effect of sire, and a significant sire × ethanol concentration interaction ($F_{(19,266)}$= 1.8, $p<0.05$; Fig. 36A); Fisher's LSD post-hoc analysis revealed significantly reduced ethanol preference during three days each at the 3 and 6% ethanol concentrations ($p<0.05$ and 0.01). In addition, there was a significant effect for ethanol concentration ($F_{(19, 266)}$=46.57, $p<0.001$) and sire ($F_{(1,14)}$ =5.8, $p<0.05$; Fig. 36B) with no ethanol concentration × sire interaction for ethanol consumption. Fisher's LSD post-hoc analysis of ethanol consumption over individual days revealed significantly reduced ethanol consumption by E-sired vs C-sired males on a single day each of 9% and 15% ethanol concentrations ($p<0.05$). There was no effect of ethanol concentration, sire, or ethanol concentration × sire interaction on total fluid intake (Fig. 36C).

In contrast to males, female offspring showed no effect of ethanol concentration, sire, or ethanol concentration x sire interaction on ethanol preference (Fig. 36D). For ethanol consumption, there was a significant effect of ethanol concentration ($F_{(19,266)}$=26.84, $p<0.001$) with consumption increasing at higher concentrations, but no effect of sire or ethanol concentration × sire interaction (Fig. 36E). Finally, there was no effect of ethanol concentration, sire, or ethanol concentration × sire interaction on total fluid intake (Fig. 36F).

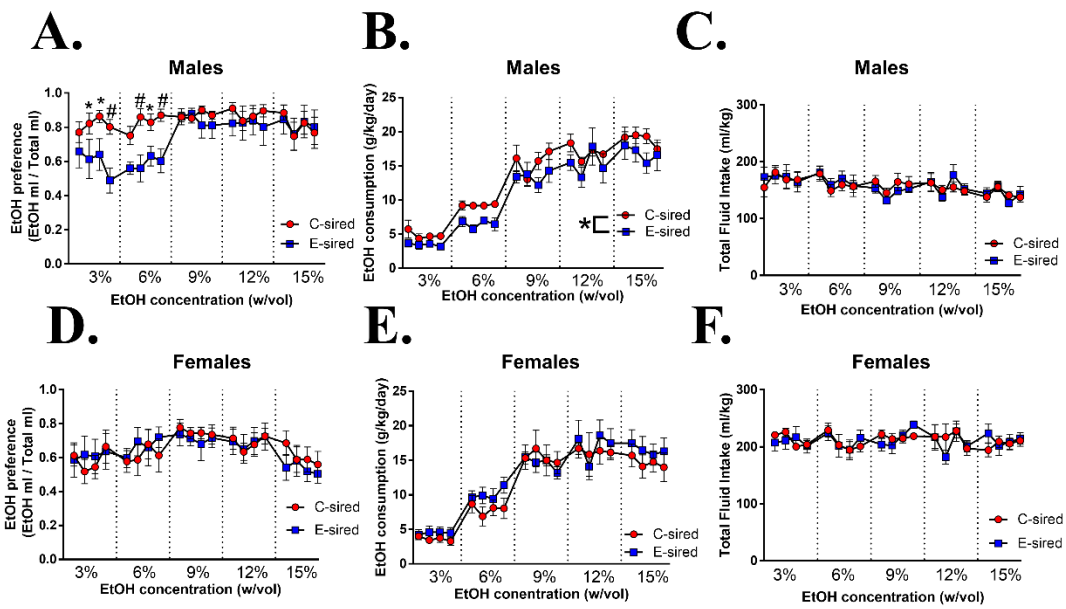


Figure 36. Paternal CIE attenuates ethanol drinking behavior selectively in male offspring

(A) E-sired males (n=8) showed reduced ethanol preference and (B) ethanol consumption over multiple days of the two bottle choice test vs C-sired males (n=8). (C) There was no difference in total fluid intake for E-sired vs C-sired males. (D) E-sired (n=8) and C-sired (n=8) females showed no significant difference in ethanol drinking preference, (E) ethanol consumption, or (F) total fluid intake. Data presented as mean + SEM. *=p<0.05. #=p<0.01.

Paternal CIE does not affect offspring drinking preference for sweet and bitter solutions

Following a one-week washout period, male and female offspring were tested in a two bottle choice paradigm with saccharine or quinine, controlling for preference of sweet and bitter tastants, respectively. In males, there was no effect of tastant concentration, sire, or their interaction on saccharin or quinine preference (Figs. 37A-B). In female offspring, no effect of concentration, sire, or their interaction was observed for saccharin preference (Fig. 37C). For quinine preference, there was no effect for concentration or sire, but a significant concentration × sire interaction ($F_{(1,}$

$_{14)}$=7.17, p<0.05; Fig. 37D); Fisher's LSD post-hoc test revealed increased quinine preference at the 0.033 mM concentration for E-sired vs C-sired females (p<0.05).

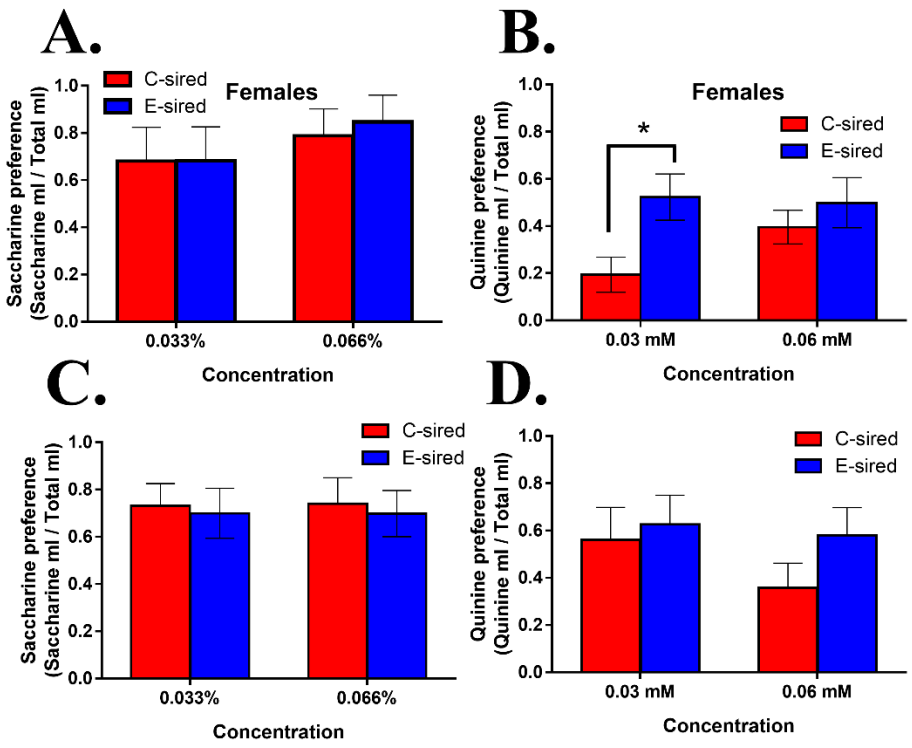


Figure 37. Effects of paternal CIE on offspring saccharin and quinine preference

In control tests for (A) saccharin and (B) quinine drinking preference, there was no significant difference for E-sired (n=8) vs C-sired males (n=8). In females, (C) saccharin preference was not different for E-sired (n=8) vs C-sired (n=8) groups, but E-sired females did have greater (D) quinine preference specifically at the 0.03 mM concentration. Data presented as mean ± SEM. *=p<0.05.

Paternal CIE increased sensitivity to the anxiolytic effects of ethanol selectively in male offspring

Both male and female offspring were assessed for sensitivity to an acute low dose of ethanol (1.0 g/kg) or saline in a sequential three test behavioral battery (elevated plus maze, open field, rotarod). Ten minutes following ethanol or saline injections, mice were assessed for exploratory behavior of open and closed arms on the elevated plus maze. For male offspring, there was a

significant effect of treatment ($F_{(1, 40)}$ = 8.226, $p<0.01$) and a treatment × sire interaction ($F_{(1,40)}$ =5.358, $p<0.05$) on percent time spent in the open arms (Fig. 38A); Fisher's LSD post-hoc analysis revealed that E-sired males showed a significant increase in open arm time vs saline-injected E-sired males ($p<0.001$) and vs ethanol-injected C-sired males ($p<0.01$). For open arm entries (Fig. 38B), there was an increase with ethanol treatment ($F_{(1, 41)}$ = 6.53, $p<0.05$) but no effect of sire or treatment × sire interaction; Fisher's LSD post hoc tests revealed significantly increased open arm entries in E-sired vs C-sired males after ethanol injection ($p<0.05$). For total arm entries, there was a significant increase with ethanol treatment ($F_{(1,41)}$=37.74, $p<0.001$, Fig. 38C), but no significant effect for sire or sire × treatment interaction.

Testing of female offspring on the elevated plus maze revealed a significant increase in open arm time following ethanol treatment ($F_{(1, 22)}$ = 15.11; $p<0.001$, Fig. 38D), but no effect of sire or sire × treatment interaction. For open arm entries, there was a significant increase with ethanol treatment ($F_{(1, 22)}$ = 8.805, $p<0.01$, Fig. 38E), but no effect of sire and no treatment × sire interaction. For total arm entries, there was a significant increase with ethanol treatment ($F_{(1,22)}$=6.832, $p<0.05$; Fig. 38F), but no effect for sire or sire × treatment interaction.

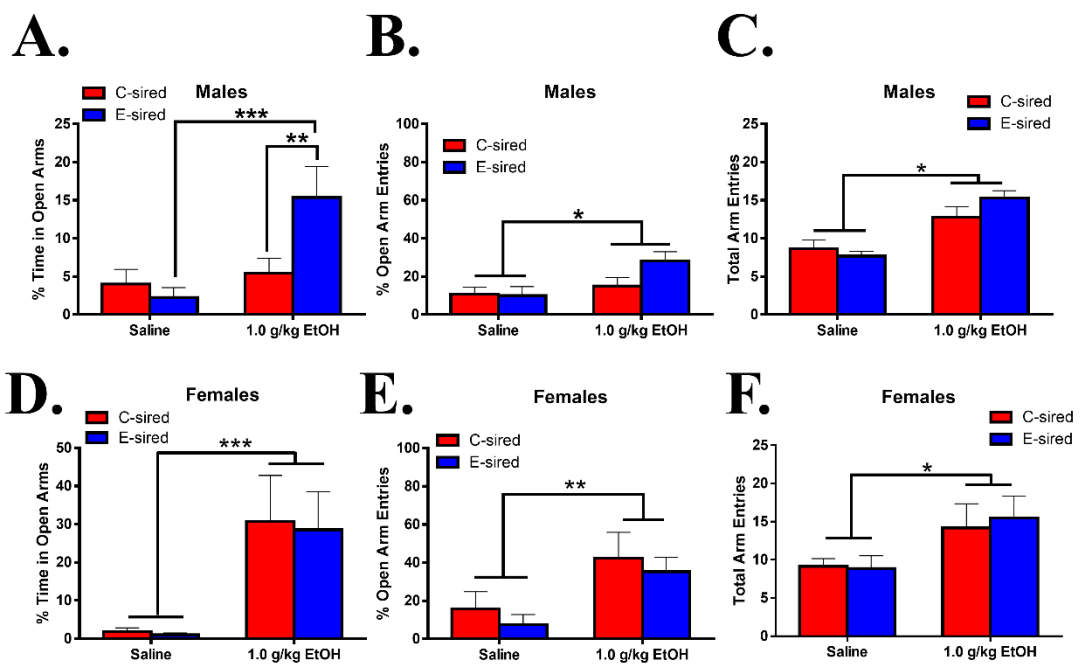


Figure 38. Paternal CIE increases sensitivity to acute ethanol selectively in male offspring

(A) In the elevated plus maze, following an acute injection with 1 g/kg ethanol, E-sired males (n=11) spent more time in the open arm (% of total time) and made more (B) open arm entries (% of total entries) vs C-sired males (n=10). (C) ethanol injection increased total arm entries, but there was no difference between E- and C-sired males. There was no difference between E-sired and C-sired males for all elevated plus maze measures following saline injection (n=12 for E-sired; n=11 for C-sired). (D) There was no difference between E-sired (n=6) and C-sired (n=6) female mice for time in the open arm, (E) open arm entries, or (F) total arm entries following acute ethanol injection. Saline-treated E-sired and C-sired females (n=7 for E-sired; n=7 for C-sired) did not differ across all measures in the elevated plus maze. Data presented as mean ± SEM. *=p<0.05, **=p<0.01, ***=p<0.001.

Five minutes after the elevated plus maze (i.e., 20 min following ethanol or saline injection), mice were examined during a 10 min open field test for ethanol-induced locomotor activity. For males, there was a significant increase in distance traveled in the open field test following ethanol

treatment ($F_{(1,40)}$=16.84, p<0.001; Fig. 39A), but no effect of sire and no treatment × sire interaction. For females, there was no effect of treatment, sire, or treatment × sire interaction (Fig. 39B).

Five minutes following the open field test (i.e., 35 min following ethanol or saline injection), mice underwent five trials on an accelerating rotarod test to assess basal motor coordination and ethanol-induced ataxia. Males showed a significant improvement in time spent on the rotarod over the five trials ($F_{(4,156)}$=10.83, p<0.001; Fig. 39C), but no effect of treatment or trial × treatment interaction. Similarly, female mice showed a significant effect for trial ($F_{(4,88)}$=9.04, p<0.001; Fig. 39D), with no effect for treatment or interaction.

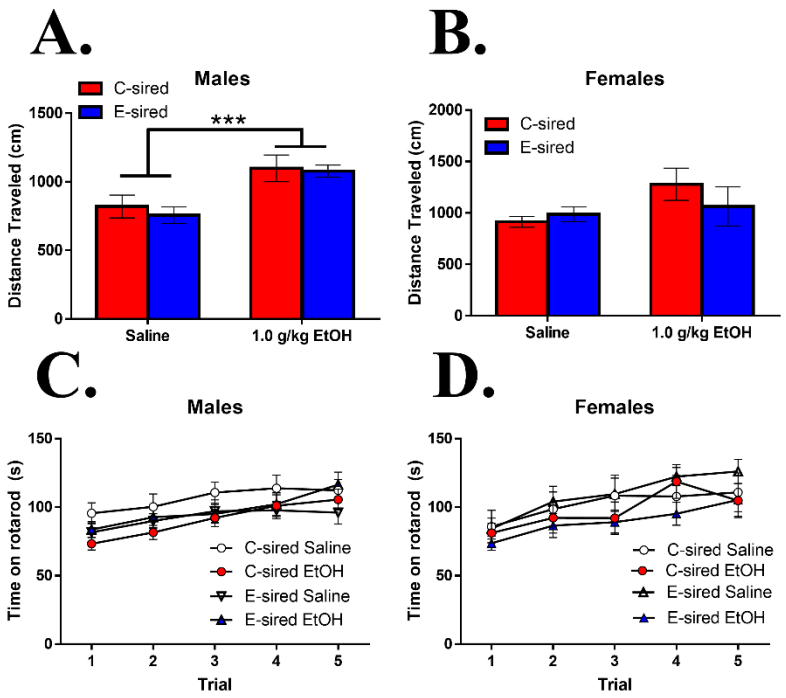


Figure 39. No effects of paternal CIE on exploratory behavior or accelerating rotarod performance after acute ethanol injection

(A) E-sired (ethanol, n=11; saline, n=12) and C-sired (ethanol, n=10; saline, n=11) male mice performed similarly in the open field test following an acute ethanol injection. In the accelerating rotarod task, (B) E-sired (ethanol, n=5; saline, n=7) and C-sired (ethanol, n=6; saline, n=7) females responded similarly to acute

ethanol in the open field test. (C) C-sired males (ethanol, n=10; saline, n=11) and E-sired males (ethanol, n=11; saline, n=12) did not show a significant effect for ethanol injection on rotarod performance. (D) There was no effect of ethanol on rotarod performance in C-sired females (ethanol, n=6; saline, n=7) or in E-sired females (ethanol, n=6; saline, n=7). Data presented as mean + SEM. ***=p<0.001.

Paternal CIE increases BDNF gene expression in the VTA of male offspring

Comparing adult E- and C-sired male offspring, we found that BDNF gene expression was increased in the VTA of E-sired vs C-sired males ($t(6)= 2.94$, $p<0.05$; Fig. 40A). In addition, when comparing E- and C-sired males for acute CORT responses to 15 min restraint stress, there was an effect for time of measurement, reflected by a sharp increase in corticosterone levels at 15 and 30 minutes from the onset of restraint stress ($F_{(3,45)}=110.2$, $p<0.001$; Fig. 40B); however, there was no effect for sire and no sire × time interaction.

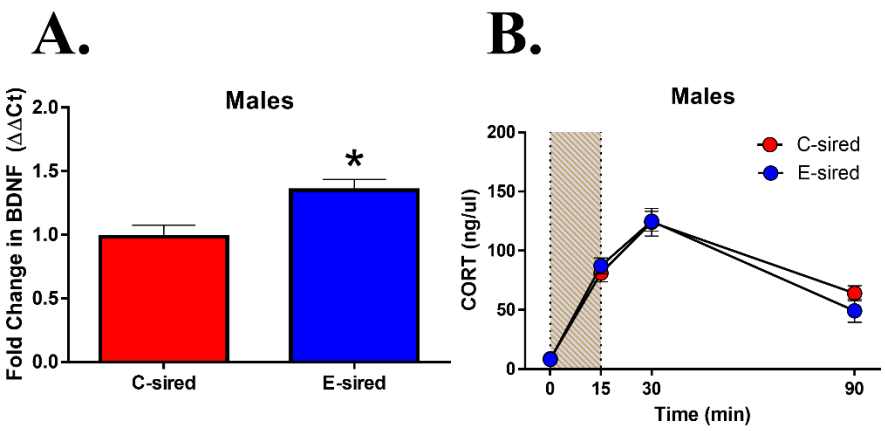


Figure 40. Paternal CIE increases BDNF mRNA expression VTA of male offspring

(A) There was a significant increase in BDNF mRNA expression in the VTA of E-sired males vs C-sired males (n= 4/4, C-sired, E-Sired). (B) No difference in corticosterone levels was observed between groups following 15 minutes of acute restraint stress (shaded bar) (n= 9/8, C-sired, E-sired). Data presented as mean ± SEM. *=p<0.05.

Discussion

Here, we report that paternal preconception chronic ethanol exposure imparts decreased ethanol drinking preference at low concentrations, increased sensitivity to the anxiolytic effects of ethanol, and increased BDNF gene expression in the VTA to adult male offspring. Whereas our prior study illustrated that E-sired F1 offspring on a Strain 129S1/SvImJ x B6 hybrid genetic background exhibited altered ethanol-related behaviors (Finegersh and Homanics, 2014), the current study extends those observations and demonstrates the same intergenerational phenotype using genetically identical, inbred B6 animals. Furthermore, the male offspring-specific intergenerational effects of paternal CIE are also consistent with our prior two studies (see Finegersh & Homanics 2014 or Rompala et al. 2016 for discussion of sex-specific effects of paternal CIE). There is an increasing emphasis from the National Institutes of Health for investigators to demonstrate that significant findings are in fact reproducible. Moreover, there is some concern that high profile epigenetic inheritance studies are vulnerable to reporting results with inflated statistical significance (Francis, 2014). Therefore, our results show that intergenerational effects of paternal CIE on ethanol-related behaviors in male offspring are in fact reproducible and also observable on an inbred genetic background.

The consistent results between this study and Finegersh & Homanics, 2014 are remarkable considering the various dissimilarities between the two maternal mouse strains used. Strain 129 and B6 mice differ on measures of stress reactivity (van Bogaert et al., 2006), taste perception (Bachmanov et al., 1996), and two bottle choice ethanol drinking (Rhodes et al., 2007). Moreover, Strain 129 and B6 dams provide different levels of maternal care (Gabriel and Cunningham, 2008) and each strain differentially regulates the maintenance of paternally inherited methylation at intracisternal A particles *in utero* (Rakyan et al., 2003a). Therefore, despite the heterogeneous

behavioral and epigenetic profile of these two mouse strains, our findings demonstrate a reproducible effect for paternal CIE on ethanol-related behaviors in male offspring.

While our finding that paternal CIE confers an attenuated ethanol drinking phenotype to male offspring was originally unanticipated given the tendency for alcoholism to run in families, the outcome is similar to other recent paternal preconception studies in rodents. For instance, male mice exposed to chronic cocaine or stress were found to sire offspring with decreased cocaine preference and blunted stress responsivity, respectively (Rodgers et al., 2013; Vassoler et al., 2013). Therefore, it is possible that paternal CIE promotes the inheritance of reduced ethanol preference, protecting male offspring against excessive ethanol consumption.

While we have remarkably replicated our previous finding that paternal CIE reduces ethanol drinking preference selectively in male offspring, it is worth noting certain limitations. For instance, both the current study and our results from Finegersh & Homanics, 2014 only report effects of paternal CIE on ethanol drinking preference in male offspring at the lower ethanol concentrations tested (primarily 3 and 6 percent). It is unclear whether this effect is specific to low concentrations of ethanol or the sequence of concentrations tested (ascending from 3 to 15 percent). In addition, although we have observed sex-specific effects of paternal CIE, it is conceivable that effects on female offspring are confounded or masked by factors such as estrus cycle (Meziane et al., 2007) or altered tastant sensitivity as reflected by our present finding of reduced quinine preference in E-sired females (Fig. 4). Thus, to further define the ethanol drinking phenotype in E-sired offspring, additional experiments and drinking paradigms need to be considered (e.g. drinking in the dark, operant self-administration). Likewise, alternative paternal preconception ethanol exposure models may be necessary as it is unknown whether paternal voluntary ethanol consumption would have the

same intergenerational effects as our forced vapor exposure that produces higher BECs and HPA stress axis activation (Rivier, 2014).

In addition to decreased ethanol drinking preference, and again consistent with our original findings from Finegersh & Homanics, 2014, we found that E-sired male offspring exhibited heightened sensitivity to an anxiolytic dose of ethanol in the elevated plus maze. Decreased subjective response to ethanol is associated with increased risk for AUD (Schuckit, 1985a; Schuckit and Smith, 1996), suggesting that ethanol sensitivity is inversely associated with AUD. This would suggest that increased ethanol sensitivity in E-sired males is consistent with the reduced ethanol drinking behavior phenotype. However, we did not find an effect for paternal CIE on basal or ethanol-induced locomotor activity and motor coordination. Hence, only a subset of ethanol-induced behavioral measures are impacted in E-sired male offspring. One notable limitation to these experiments is the timing of the behavioral battery and fixed sequence of experiments. The elevated plus maze was conducted 10 min after ethanol or saline injection and then sequentially followed by the open field test (20 min post injection) and accelerating rotarod (35 min post injection). As a result, we cannot rule out that the intergenerational effect of paternal CIE exclusively in the elevated plus maze may have been due to the timing and/or sequence of experimentation.

In addition to altered intergenerational ethanol-related behaviors, there was a significant increase in BDNF gene expression in the VTA of E-sired males, in accordance with our previous findings (Finegersh and Homanics, 2014). Expression of BDNF in various brain regions has been found to mediate alcohol drinking behavior in rodents (Pandey, 2016). Indeed, innate BDNF expression is increased in the VTA of alcohol-avoiding rats (Raivio et al., 2014). Infusion of BDNF into the VTA is sufficient to shift conditioned place preference for alcohol from a dopamine-

dependent to dopamine-independent behavior (Ting et al., 2013). Thus, mechanisms governing ethanol motivation may differ between ethanol-sired and control male mice and BDNF may be an attractive target for further elucidating the neurobiological substrates involved.

Lastly, we did find that some of the effects of paternal CIE on male offspring varied with maternal strain. First, contrary to our findings in Finegersh & Homanics 2014 that paternal CIE increases postweaning body weight in hybrid male offspring, here, there was a small, but significant reduction of postweaning body weight in E-sired male offspring on an inbred background. However, this outcome is not surprising given that many paternal ethanol exposure studies have found either increased or decreased offspring body weight using different rodent strains or exposure paradigms (Mankes et al., 1982; Ledig et al., 1998; Knezovich and Ramsay, 2012). The second inconsistency between studies is the absence of an effect of paternal CIE on acute HPA axis responsivity in F1 inbred male offspring, counter to our published findings showing that paternal CIE produced stress hyporesponsivity phenotypes in F1 hybrid male offspring (Rompala et al., 2016). Indeed, the different outcome may be due to aforementioned differences between B6 and Strain 129 dams. For instance, variations in maternal behavior, as seen with B6 and Strain 129 dams, has a significant effect on adult HPA axis responsivity in adult offspring (Caldji et al., 2000). Thus, additional experiments, such as employing a cross-fostering strategy, may be necessary to determine whether the strain-dependent effect of paternal CIE on body weight and intergenerational HPA axis responsivity is explained by differences in maternal biology or maternal care.

Between this study and the results from Finegersh & Homanics, 2014, we have established a reproducible model of paternal preconception ethanol exposure that stably impacts ethanol drinking behavior and ethanol sensitivity selectively in male offspring. This model will facilitate future experiments attempting to identify the causal mechanisms in sperm that drive heritable

changes in complex ethanol-related behaviors. Along with the paternal genome, sperm transmit epigenetic mechanisms including but not limited to DNA methylation, histone modifications, and small noncoding RNAs to the oocyte at fertilization (Schagdarsurengin and Steger, 2016b) (Rando, 2016). Small noncoding RNAs are an attractive mechanism for epigenetic inheritance as recent studies have shown they can be transmitted from the somatic cells of the central nervous system to the germline (Devanapally et al., 2015), possibly through exosome signaling (Cossetti et al., 2014; Sharma et al., 2016). Moreover, paternal experience such as chronic stress alters sperm miRNA expression (Rodgers et al., 2013; Gapp et al., 2014) and injection of the most altered miRNAs into fertilized oocytes from normal donor mice can recapitulate the intergenerational effects of paternal stress in adult progeny (Rodgers et al., 2015). In addition, other studies have shown that paternal experience alters DNA methylation (Govorko et al., 2012; Dias and Ressler, 2014; Finegersh and Homanics, 2014) and histone modifications (Vassoler et al., 2013; Siklenka et al., 2015) in sperm although technical limitations have complicated identifying a causal role for these mechanisms in epigenetic inheritance of paternal experience. The germline function of such mechanisms will likely become more delineated with the advancement of novel technologies for targeted chromatin remodeling such as zinc-finger protein or CRISPR/Cas systems (Thakore et al., 2016).

In summary, paternal preconception ethanol exposure confers reduced ethanol drinking behavior, increased sensitivity to ethanol, and increased BDNF gene expression in the VTA to male offspring. The evidence for these intergenerational phenotypes is robust as we have now observed effects of paternal CIE on both hybrid and inbred male offspring. Identifying heritable epigenetic mechanisms that confer resistance to excessive ethanol drinking behavior has major implications for the development of novel AUD prevention and treatment strategies. Therefore, future studies

will aim to identify sperm-borne epigenetic mechanisms with a causal role in intergenerational ethanol-related behaviors.

Appendix B

DIFFERENTIAL EXPRESSION ANALYSIS OF SMALL NONCODING RNAS IN ETHANOL-TREATED MOUSE SPERM

*=padj<0.1

Validated differentially-expressed small noncoding RNAs are denoted in bold

tDR species	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj
Ala-AGC	2082.98	-0.05	0.20	-0.27	0.79	0.82
Ala-CGC	5309.45	0.28	0.17	1.60	0.11	0.30
Ala-TGC	6111.80	-0.06	0.25	-0.24	0.81	0.82
Arg-ACG	167.37	0.22	0.21	1.05	0.30	0.45
Arg-CCG	3133.02	0.27	0.23	1.18	0.24	0.43
Arg-CCT	2875.57	0.15	0.21	0.69	0.49	0.63
Arg-TCG	155.54	0.11	0.21	0.54	0.59	0.73
Arg-TCT	53.52	0.26	0.21	1.24	0.22	0.41
Asn-GTT	1177.28	-0.16	0.19	-0.85	0.40	0.54
Asp-GTC	12702.20	0.46	0.24	1.86	0.06	0.19
Cys-GCA	1021.75	-0.06	0.18	-0.33	0.75	0.82
*Gln-CTG	2352.13	0.46	0.21	2.21	0.03	0.11
Gln-TTG	410.17	0.25	0.17	1.53	0.13	0.31
***Glu-CTC**	**1108041.84**	**0.60**	**0.26**	**2.33**	**0.02**	**0.10**
*Glu-TTC	62456.89	0.62	0.25	2.44	0.01	0.10
Gly-ACC	366.93	-0.40	0.26	-1.54	0.12	0.31
Gly-CCC	163038.27	0.18	0.23	0.80	0.42	0.56
Gly-GCC	627356.91	0.25	0.23	1.11	0.27	0.45
Gly-TTC	3254.73	-0.22	0.11	-1.95	0.05	0.17
His-ATG	0.58	-0.17	0.26	-0.65	0.52	0.65
***His-GTG**	**11874.96**	**0.58**	**0.20**	**2.87**	**0.00**	**0.07**
*Ile-AAT	279.06	-0.38	0.15	-2.49	0.01	0.10
Ile-GAT	1.17	-0.31	0.28	-1.09	0.28	0.45
Ile-TAT	36.21	0.29	0.23	1.22	0.22	0.41
Leu-AAG	2972.72	-0.13	0.13	-1.03	0.30	0.45
Leu-CAA	1186.41	-0.22	0.15	-1.42	0.16	0.35

Leu-CAG	2780.76	0.04	0.14	0.32	0.75	0.82
*Leu-TAA	1161.17	-0.47	0.17	-2.81	0.00	0.07
Leu-TAG	2302.86	-0.18	0.13	-1.35	0.18	0.38
Lys-CTT	11489.27	0.45	0.25	1.82	0.07	0.20
*Lys-TTT	1844.78	-0.50	0.21	-2.37	0.02	0.10
Met-CAT	3788.59	-0.05	0.22	-0.25	0.80	0.82
Phe-GAA	118.11	-0.20	0.17	-1.22	0.22	0.41
*Pro-AGG	1511.66	0.57	0.22	2.55	0.01	0.09
*Pro-CGG	1601.31	0.60	0.22	2.75	0.01	0.07
*Pro-TGG	2766.96	0.47	0.22	2.15	0.03	0.11
SeC	5027.35	-0.21	0.15	-1.42	0.15	0.35
***Ser-AGA**	**8946.59**	**-0.40**	**0.18**	**-2.16**	**0.03**	**0.11**
*Ser-CGA	344.20	-0.38	0.17	-2.20	0.03	0.11
Ser-GCT	15136.55	-0.24	0.23	-1.07	0.28	0.45
Ser-GGA	1.33	-0.26	0.28	-0.92	0.36	0.50
*Ser-TGA	4993.83	-0.38	0.18	-2.14	0.03	0.11
Sup-TTA	0.47	-0.24	0.24	-1.01	0.31	0.45
*Thr-AGT	33.73	0.70	0.26	2.70	0.01	0.07
Thr-CGT	105.97	-0.08	0.17	-0.50	0.62	0.73
Thr-TGT	225.29	-0.18	0.17	-1.06	0.29	0.45
Trp-CCA	1638.55	0.20	0.16	1.24	0.21	0.41
*Tyr-GTA	55.70	0.65	0.22	2.91	0.00	0.07
Val-AAC	23440.31	0.06	0.23	0.28	0.78	0.82
Val-CAC	32203.02	0.08	0.23	0.36	0.72	0.82
Val-GAC	0.07	0.02	0.12	0.18	0.85	0.85
Val-TAC	2244.72	-0.11	0.20	-0.52	0.60	0.73

miRNA	**baseMean**	**log2FoldChange**	**lfcSE**	**stat**	**pvalue**	**padj**
mmu-let-7a-5p	1502.57	0.11	0.17	0.64	0.52	0.77
mmu-let-7b-5p	320.25	0.49	0.22	2.20	0.03	0.23
mmu-let-7c-5p	1669.09	0.51	0.21	2.36	0.02	0.20
mmu-let-7d-3p	24.44	0.05	0.23	0.21	0.83	0.91
mmu-let-7d-5p	192.91	0.36	0.23	1.54	0.12	0.34
mmu-let-7e-5p	63.44	0.40	0.24	1.67	0.09	0.32
mmu-let-7f-5p	2435.22	0.39	0.21	1.90	0.06	0.25
mmu-let-7g-5p	1812.02	0.05	0.17	0.27	0.78	0.87
mmu-let-7i-5p	762.52	0.36	0.21	1.73	0.08	0.32
mmu-let-7j	370.85	0.37	0.22	1.70	0.09	0.32
mmu-miR-100-5p	241.58	0.54	0.22	2.41	0.02	0.20
mmu-miR-103-3p	225.05	-0.10	0.19	-0.51	0.61	0.80
mmu-miR-106b-3p	334.00	-0.18	0.26	-0.70	0.48	0.75
***mmu-miR-10a-5p**	**3556.55**	**0.79**	**0.23**	**3.52**	**0.00**	**0.05**
*mmu-miR-10b-5p	3582.56	0.70	0.24	2.91	0.00	0.13
mmu-miR-1198-5p	9.45	0.22	0.28	0.81	0.42	0.72

*mmu-miR-125a-5p	76.65	0.68	0.26	2.68	0.01	0.14
mmu-miR-125b-5p	212.39	0.06	0.15	0.37	0.71	0.85
mmu-miR-126a-3p	44.02	0.11	0.19	0.60	0.55	0.79
mmu-miR-128-3p	109.15	-0.01	0.27	-0.04	0.97	0.98
mmu-miR-130b-5p	12.20	-0.21	0.30	-0.68	0.50	0.75
mmu-miR-140-3p	64.52	-0.40	0.25	-1.58	0.11	0.34
mmu-miR-143-3p	2452.09	0.12	0.21	0.56	0.58	0.80
mmu-miR-145a-3p	9.57	0.09	0.28	0.31	0.75	0.85
mmu-miR-145a-5p	429.55	0.63	0.27	2.35	0.02	0.20
mmu-miR-146a-5p	34.25	0.12	0.24	0.50	0.62	0.81
mmu-miR-148a-3p	1090.26	0.27	0.20	1.34	0.18	0.42
mmu-miR-148a-5p	6.22	-0.64	0.29	-2.20	0.03	0.23
mmu-miR-148b-3p	144.50	-0.02	0.20	-0.12	0.90	0.96
mmu-miR-151-3p	100.23	0.29	0.18	1.61	0.11	0.34
mmu-miR-151-5p	13.96	-0.40	0.27	-1.46	0.15	0.36
mmu-miR-152-3p	23.12	0.11	0.27	0.42	0.67	0.84
mmu-miR-15b-5p	129.71	-0.44	0.24	-1.82	0.07	0.27
mmu-miR-16-5p	143.16	-0.67	0.25	-2.73	0.01	0.15
mmu-miR-17-5p	8.22	-0.02	0.28	-0.08	0.94	0.97
mmu-miR-181a-5p	33.41	0.35	0.21	1.62	0.11	0.34
mmu-miR-181b-5p	10.81	0.47	0.27	1.72	0.09	0.32
mmu-miR-181d-5p	15.13	0.40	0.27	1.52	0.13	0.34
mmu-miR-182-5p	252.75	0.44	0.22	2.02	0.04	0.25
mmu-miR-183-5p	113.95	0.08	0.21	0.37	0.71	0.85
mmu-miR-1839-5p	20.95	0.60	0.27	2.20	0.03	0.23
mmu-miR-184-3p	1254.25	-0.11	0.26	-0.42	0.68	0.84
mmu-miR-191-3p	8.61	-0.22	0.30	-0.74	0.46	0.73
mmu-miR-191-5p	1858.58	-0.43	0.18	-2.34	0.02	0.20
mmu-miR-192-5p	16.36	-0.52	0.26	-1.98	0.05	0.25
mmu-miR-194-5p	7.73	-0.54	0.29	-1.83	0.07	0.27
mmu-miR-196a-5p	43.19	1.00	0.29	3.41	0.00	0.05
mmu-miR-196b-5p	55.50	0.54	0.28	1.92	0.05	0.25
mmu-miR-1981-5p	21.40	0.64	0.27	2.39	0.02	0.20
mmu-miR-199a-3p	145.21	0.08	0.23	0.34	0.73	0.85
mmu-miR-199a-5p	24.20	0.57	0.26	2.16	0.03	0.24
mmu-miR-1a-3p	62.93	0.55	0.29	1.92	0.05	0.25
mmu-miR-200a-3p	110.97	0.39	0.25	1.54	0.12	0.34
mmu-miR-200a-5p	8.07	0.02	0.28	0.07	0.94	0.97
mmu-miR-200b-3p	225.01	0.51	0.26	1.98	0.05	0.25
mmu-miR-200b-5p	14.56	0.19	0.25	0.77	0.44	0.73
mmu-miR-200c-3p	856.70	0.08	0.19	0.44	0.66	0.84
mmu-miR-203-3p	28.80	-0.03	0.25	-0.12	0.90	0.96
mmu-miR-204-5p	30.93	0.29	0.21	1.35	0.18	0.42

*mmu-miR-205-5p	26.29	0.96	0.31	3.13	0.00	0.10
mmu-miR-20a-5p	89.67	-0.57	0.23	-2.47	0.01	0.20
mmu-miR-2137	14.23	0.01	0.31	0.04	0.97	0.98
mmu-miR-21a-5p	252.75	0.07	0.23	0.31	0.76	0.85
mmu-miR-221-3p	47.48	0.34	0.27	1.25	0.21	0.47
mmu-miR-221-5p	11.76	0.16	0.29	0.57	0.57	0.80
mmu-miR-22-3p	89.15	-0.36	0.20	-1.79	0.07	0.29
mmu-miR-23a-3p	120.66	0.41	0.20	2.02	0.04	0.25
mmu-miR-23b-3p	79.24	0.33	0.23	1.43	0.15	0.37
mmu-miR-24-3p	36.42	0.39	0.27	1.44	0.15	0.36
mmu-miR-25-3p	2043.04	-0.19	0.25	-0.78	0.43	0.73
mmu-miR-26a-5p	418.02	0.22	0.24	0.95	0.34	0.64
mmu-miR-26b-5p	30.46	0.00	0.23	0.00	1.00	1.00
mmu-miR-27a-3p	21.64	-0.06	0.26	-0.22	0.83	0.91
mmu-miR-27b-3p	226.24	-0.10	0.18	-0.55	0.58	0.80
mmu-miR-28a-3p	16.24	0.30	0.26	1.14	0.25	0.53
mmu-miR-292a-5p	10.16	0.03	0.30	0.10	0.92	0.96
mmu-miR-296-5p	10.74	-0.21	0.29	-0.72	0.47	0.73
mmu-miR-298-5p	9.05	-0.56	0.28	-2.01	0.04	0.25
mmu-miR-29a-3p	102.19	0.10	0.20	0.52	0.60	0.80
mmu-miR-3074-5p	16.81	0.25	0.27	0.92	0.35	0.65
mmu-miR-30a-3p	390.48	-0.03	0.17	-0.19	0.85	0.92
mmu-miR-30a-5p	144.44	-0.12	0.25	-0.50	0.62	0.81
mmu-miR-30b-5p	405.94	-0.10	0.18	-0.52	0.60	0.80
mmu-miR-30c-2-3p	30.29	-0.08	0.23	-0.33	0.74	0.85
mmu-miR-30c-5p	632.01	-0.12	0.16	-0.76	0.45	0.73
mmu-miR-30d-5p	618.67	-0.17	0.17	-0.95	0.34	0.64
mmu-miR-30e-3p	241.55	-0.14	0.20	-0.67	0.50	0.75
mmu-miR-30e-5p	6.32	-0.40	0.31	-1.30	0.19	0.45
mmu-miR-30f	28.12	-0.30	0.26	-1.15	0.25	0.53
mmu-miR-320-3p	38.15	0.41	0.20	2.00	0.05	0.25
mmu-miR-328-3p	60.36	-0.07	0.21	-0.34	0.73	0.85
mmu-miR-340-5p	30.71	-0.39	0.25	-1.52	0.13	0.34
mmu-miR-342-3p	43.59	0.07	0.23	0.30	0.76	0.85
mmu-miR-3473b	7.39	-0.45	0.30	-1.48	0.14	0.35
mmu-miR-34b-3p	1119.03	-0.24	0.27	-0.92	0.36	0.65
mmu-miR-34b-5p	58.83	-0.34	0.27	-1.24	0.21	0.47
mmu-miR-34c-3p	285.35	-0.34	0.27	-1.27	0.20	0.47
mmu-miR-34c-5p	3044.03	-0.33	0.27	-1.22	0.22	0.49
mmu-miR-350-3p	14.11	-0.49	0.29	-1.67	0.10	0.32
*mmu-miR-3535	61.54	0.80	0.28	2.83	0.00	0.13
mmu-miR-361-3p	30.00	0.30	0.27	1.10	0.27	0.56
mmu-miR-365-3p	10.66	0.27	0.28	0.98	0.33	0.64

mmu-miR-375-3p	932.27	-0.15	0.27	-0.54	0.59	0.80
mmu-miR-378a-3p	17.33	0.26	0.24	1.06	0.29	0.59
mmu-miR-378d	45.45	0.18	0.24	0.75	0.45	0.73
mmu-miR-379-5p	47.82	0.19	0.20	0.98	0.33	0.64
mmu-miR-382-5p	9.07	0.19	0.27	0.72	0.47	0.73
mmu-miR-423-3p	24.91	0.21	0.23	0.89	0.37	0.67
mmu-miR-423-5p	65.00	0.24	0.22	1.06	0.29	0.59
mmu-miR-425-5p	90.30	-0.23	0.24	-0.95	0.34	0.64
mmu-miR-429-3p	14.16	0.14	0.27	0.52	0.60	0.80
mmu-miR-434-3p	9.34	0.12	0.28	0.44	0.66	0.84
mmu-miR-449a-5p	26.41	-0.54	0.28	-1.91	0.06	0.25
mmu-miR-463-5p	6.81	-0.60	0.31	-1.93	0.05	0.25
mmu-miR-465a-3p	299.50	-0.36	0.24	-1.51	0.13	0.35
mmu-miR-465a-5p	351.68	-0.10	0.25	-0.41	0.68	0.84
mmu-miR-465b-5p	412.68	-0.14	0.26	-0.54	0.59	0.80
mmu-miR-465c-5p	692.17	-0.19	0.25	-0.76	0.45	0.73
mmu-miR-465d-3p	57.11	-0.40	0.24	-1.67	0.10	0.32
mmu-miR-465d-5p	225.07	0.09	0.27	0.33	0.74	0.85
mmu-miR-467a-5p	12.03	-0.54	0.28	-1.95	0.05	0.25
mmu-miR-470-5p	944.05	-0.03	0.26	-0.10	0.92	0.96
mmu-miR-471-3p	21.73	0.01	0.29	0.03	0.97	0.99
mmu-miR-471-5p	16.73	-0.33	0.29	-1.15	0.25	0.53
mmu-miR-486a-5p	24.92	0.10	0.26	0.40	0.69	0.84
mmu-miR-501-3p	45.71	0.22	0.21	1.01	0.31	0.62
mmu-miR-5126	10.20	-0.11	0.29	-0.38	0.71	0.85
mmu-miR-532-5p	87.23	-0.02	0.20	-0.10	0.92	0.96
mmu-miR-541-5p	58.13	0.30	0.19	1.57	0.12	0.34
mmu-miR-6238	125.26	0.10	0.27	0.38	0.70	0.85
mmu-miR-6239	35.94	0.19	0.29	0.68	0.50	0.75
mmu-miR-6240	11.29	0.26	0.30	0.88	0.38	0.68
mmu-miR-6395	9.35	-0.46	0.27	-1.67	0.09	0.32
mmu-miR-6412	17.35	-0.22	0.29	-0.77	0.44	0.73
mmu-miR-652-3p	16.14	0.22	0.29	0.76	0.45	0.73
mmu-miR-6538	15.99	-0.27	0.28	-0.95	0.34	0.64
mmu-miR-669a-5p	8.26	-0.45	0.30	-1.49	0.14	0.35
mmu-miR-669c-5p	109.03	0.01	0.27	0.02	0.98	0.99
mmu-miR-672-5p	43.61	0.46	0.22	2.04	0.04	0.25
mmu-miR-676-3p	19.39	0.20	0.24	0.83	0.41	0.71
mmu-miR-690	11.92	0.19	0.29	0.67	0.50	0.75
mmu-miR-6937-5p	17.31	0.11	0.29	0.36	0.72	0.85
mmu-miR-7210-5p	35.77	-0.47	0.30	-1.58	0.11	0.34
mmu-miR-741-3p	95.69	-0.52	0.23	-2.22	0.03	0.23
mmu-miR-743a-3p	109.83	-0.64	0.26	-2.40	0.02	0.20

mmu-miR-743a-5p	44.56	-0.17	0.27	-0.64	0.52	0.77
mmu-miR-743b-3p	81.28	-0.51	0.26	-1.94	0.05	0.25
mmu-miR-744-5p	33.51	0.33	0.22	1.50	0.13	0.35
mmu-miR-7a-5p	466.39	-0.08	0.23	-0.33	0.74	0.85
mmu-miR-871-3p	373.02	0.16	0.25	0.62	0.54	0.78
mmu-miR-871-5p	15.64	-0.03	0.29	-0.11	0.91	0.96
mmu-miR-872-5p	101.58	-0.38	0.24	-1.60	0.11	0.34
mmu-miR-878-3p	8.11	-0.26	0.30	-0.84	0.40	0.71
mmu-miR-878-5p	71.56	-0.16	0.28	-0.57	0.57	0.80
mmu-miR-880-3p	7.92	-0.60	0.31	-1.94	0.05	0.25
mmu-miR-881-3p	928.71	-0.13	0.28	-0.45	0.65	0.84
mmu-miR-883a-3p	25.70	-0.52	0.28	-1.88	0.06	0.25
mmu-miR-92a-3p	134.03	-0.37	0.24	-1.55	0.12	0.34
mmu-miR-92b-3p	214.00	-0.10	0.22	-0.44	0.66	0.84
mmu-miR-93-5p	219.02	-0.56	0.24	-2.36	0.02	0.20
mmu-miR-9-5p	108.82	-0.51	0.25	-2.09	0.04	0.25
mmu-miR-98-5p	13.87	0.46	0.28	1.64	0.10	0.33
mmu-miR-99a-5p	364.28	0.57	0.30	1.90	0.06	0.25
***mmu-miR-99b-5p**	**235.95**	**0.55**	**0.19**	**2.86**	**0.00**	**0.13**
mitosRNA species	**baseMean**	**log2FoldChange**	**lfcSE**	**stat**	**pvalue**	**padj**
mt-Rnr1	3823.70	-0.39	0.19	-1.98	0.05	0.19
mt-Rnr2	92411.83	-0.07	0.16	-0.47	0.64	0.73
mt-Ta	1264.86	0.22	0.30	0.72	0.47	0.63
mt-Tc	30.82	-0.11	0.22	-0.49	0.62	0.73
mt-Td	3.08	0.34	0.54	0.63	0.53	0.67
mt-Te	183.47	0.29	0.25	1.14	0.26	0.42
mt-Tf	2.68	0.61	0.54	1.12	0.26	0.42
*mt-Tg	1418.14	1.39	0.40	3.48	0.00	0.01
mt-Th	236734.95	0.09	0.24	0.38	0.70	0.73
mt-Ti	814.00	-0.26	0.19	-1.33	0.18	0.34
mt-Tk	1335.26	-0.28	0.19	-1.50	0.13	0.30
*mt-Tl1	304.18	0.92	0.40	2.32	0.02	0.10
mt-Tl2	54.71	-0.29	0.20	-1.46	0.15	0.30
*mt-Tm	326.50	0.89	0.29	3.10	0.00	0.02
mt-Tn	442.08	0.07	0.17	0.43	0.67	0.73
*mt-Tp	82.28	0.61	0.26	2.39	0.02	0.10
mt-Tq	17512.91	0.52	0.36	1.44	0.15	0.30
mt-Tr	536.55	-0.32	0.22	-1.44	0.15	0.30
mt-Ts1	5798.86	-0.41	0.27	-1.49	0.14	0.30
mt-Ts2	1553.48	-0.05	0.16	-0.28	0.78	0.78
mt-Tt	2032.37	-0.25	0.17	-1.51	0.13	0.30
*mt-Tv	392.69	0.77	0.33	2.35	0.02	0.10
mt-Tw	11.83	0.31	0.38	0.83	0.41	0.58

mt-Ty	14.73	-0.20	0.25	-0.82	0.41	0.58
piRNA	**baseMean**	**log2FoldChange**	**lfcSE**	**stat**	**pvalue**	**padj**
10008-1%piRNA	140.42	0.01	0.17	0.04	0.97	0.99
10134-1%piRNA	127.63	0.19	0.12	1.57	0.12	0.58
10150-1%piRNA	109.70	-0.01	0.11	-0.06	0.95	0.99
10179-1%piRNA	1273.18	0.18	0.11	1.54	0.12	0.58
10197-1%piRNA	331.55	-0.02	0.12	-0.20	0.84	0.99
10220-1%piRNA	394.02	0.08	0.14	0.57	0.57	0.87
10352-1%piRNA	94.85	0.14	0.12	1.17	0.24	0.71
10370-1%piRNA	101.06	-0.01	0.15	-0.07	0.95	0.99
10423-1%piRNA	189.83	0.09	0.13	0.70	0.48	0.83
10617-1%piRNA	201.51	0.09	0.12	0.78	0.44	0.79
10638-1%piRNA	111.48	0.36	0.15	2.48	0.01	0.40
10723-1%piRNA	276.00	0.24	0.18	1.35	0.18	0.67
10945-1%piRNA	81.95	0.01	0.13	0.06	0.96	0.99
1096-6%piRNA	1234.05	0.05	0.19	0.26	0.80	0.99
11023-1%piRNA	115.48	-0.01	0.13	-0.05	0.96	0.99
11036-1%piRNA	197.68	-0.02	0.11	-0.16	0.87	0.99
11151-1%piRNA	123.25	0.13	0.15	0.89	0.37	0.77
11196-1%piRNA	124.94	-0.23	0.12	-1.82	0.07	0.51
11257-1%piRNA	402.12	0.13	0.16	0.80	0.43	0.79
11306-1%piRNA	159.50	-0.25	0.13	-1.91	0.06	0.51
11310-1%piRNA	89.48	0.11	0.13	0.80	0.43	0.79
11503-1%piRNA	167.64	0.10	0.16	0.63	0.53	0.85
11673-1%piRNA	183.05	-0.11	0.12	-0.96	0.33	0.74
1189-5%piRNA	118.92	0.22	0.17	1.30	0.19	0.67
11906-1%piRNA	127.71	-0.10	0.10	-1.01	0.31	0.73
11962-1%piRNA	160.32	-0.12	0.14	-0.91	0.36	0.77
11978-1%piRNA	180.73	0.03	0.14	0.18	0.85	0.99
12049-1%piRNA	97.35	-0.24	0.17	-1.38	0.17	0.66
12053-1%piRNA	137.47	-0.06	0.13	-0.47	0.64	0.92
1209-5%piRNA	398.42	0.13	0.12	1.13	0.26	0.71
12250-1%piRNA	1243.62	0.19	0.13	1.47	0.14	0.61
12319-1%piRNA	73.91	-0.27	0.15	-1.74	0.08	0.51
12526-1%piRNA	130.25	0.21	0.12	1.68	0.09	0.53
12573-1%piRNA	114.61	-0.08	0.14	-0.59	0.56	0.86
12643-1%piRNA	273.27	0.00	0.14	-0.01	0.99	0.99
12753-1%piRNA	243.43	0.00	0.16	-0.01	0.99	1.00
12765-1%piRNA	114.52	-0.27	0.17	-1.60	0.11	0.58
128-1614%piRNA	199.24	0.22	0.13	1.71	0.09	0.52
1284-4%piRNA	980.89	0.24	0.19	1.28	0.20	0.67
13026-1%piRNA	9346.59	0.05	0.19	0.25	0.81	0.99
13096-1%piRNA	130.23	0.05	0.14	0.37	0.71	0.95

13135-1%piRNA	113.37	-0.01	0.14	-0.08	0.94	0.99
13160-1%piRNA	95.13	0.02	0.10	0.16	0.87	0.99
13255-1%piRNA	148.24	-0.13	0.12	-1.03	0.30	0.73
13317-1%piRNA	268.60	0.34	0.17	2.03	0.04	0.51
13562-1%piRNA	136.80	0.25	0.13	1.99	0.05	0.51
13645-1%piRNA	111.91	0.01	0.11	0.10	0.92	0.99
13713-1%piRNA	185.23	0.01	0.16	0.05	0.96	0.99
13840-1%piRNA	124.20	-0.04	0.13	-0.30	0.76	0.96
13874-1%piRNA	140.48	0.01	0.10	0.13	0.89	0.99
1391-4%piRNA	142.09	0.14	0.18	0.75	0.45	0.80
1403-4%piRNA	90.76	-0.03	0.13	-0.20	0.84	0.99
14061-1%piRNA	89.18	0.10	0.13	0.78	0.44	0.79
14104-1%piRNA	82.59	-0.07	0.12	-0.60	0.55	0.86
1453-4%piRNA	78.50	-0.01	0.14	-0.05	0.96	0.99
14736-1%piRNA	104.47	-0.01	0.12	-0.11	0.91	0.99
1492-4%piRNA	522.59	0.18	0.19	0.93	0.35	0.76
1495-4%piRNA	254.14	0.14	0.19	0.76	0.45	0.79
14980-1%piRNA	148.06	-0.12	0.14	-0.84	0.40	0.79
15371-1%piRNA	152.86	-0.12	0.14	-0.84	0.40	0.79
15463-1%piRNA	118.08	-0.07	0.12	-0.60	0.55	0.86
15594-1%piRNA	85.76	-0.17	0.14	-1.22	0.22	0.69
15595-1%piRNA	78.75	0.03	0.15	0.21	0.83	0.99
15634-1%piRNA	135.53	-0.19	0.13	-1.47	0.14	0.61
15639-1%piRNA	156.54	0.01	0.12	0.08	0.94	0.99
15910-1%piRNA	276.97	-0.01	0.12	-0.11	0.91	0.99
15943-1%piRNA	417.73	-0.15	0.14	-1.08	0.28	0.73
15974-1%piRNA	112.59	-0.13	0.12	-1.09	0.28	0.73
16046-1%piRNA	347.51	-0.07	0.11	-0.59	0.56	0.86
16056-1%piRNA	148.03	0.14	0.12	1.11	0.26	0.71
16156-1%piRNA	97.59	-0.04	0.12	-0.38	0.70	0.95
16400-1%piRNA	9418.69	0.22	0.19	1.16	0.25	0.71
16614-1%piRNA	135.87	0.26	0.13	1.96	0.05	0.51
16666-1%piRNA	133.76	0.13	0.10	1.38	0.17	0.66
16730-1%piRNA	101.65	0.19	0.13	1.53	0.13	0.58
17154-1%piRNA	94.72	0.15	0.10	1.52	0.13	0.58
17159-1%piRNA	105.62	0.03	0.10	0.33	0.74	0.95
17213-1%piRNA	133.55	0.19	0.13	1.39	0.17	0.66
17287-1%piRNA	167.08	0.05	0.13	0.34	0.73	0.95
17302-1%piRNA	63.80	-0.19	0.18	-1.06	0.29	0.73
17406-1%piRNA	215.61	0.11	0.14	0.76	0.45	0.79
17512-1%piRNA	546.80	-0.04	0.11	-0.34	0.73	0.95
17712-1%piRNA	134.52	0.07	0.17	0.42	0.68	0.94
17779-1%piRNA	84.67	0.12	0.12	0.94	0.35	0.76

17787-1%piRNA	108.56	0.06	0.11	0.51	0.61	0.90
17806-1%piRNA	106.51	-0.02	0.14	-0.16	0.87	0.99
17986-1%piRNA	107.97	-0.29	0.13	-2.17	0.03	0.51
1809-3%piRNA	1437.05	0.17	0.19	0.87	0.38	0.77
18128-1%piRNA	363.42	0.00	0.10	0.00	1.00	1.00
18257-1%piRNA	72.11	-0.12	0.15	-0.82	0.41	0.79
1835-3%piRNA	89.85	-0.27	0.12	-2.27	0.02	0.51
18360-1%piRNA	104.13	0.07	0.11	0.65	0.51	0.85
18393-1%piRNA	257.77	-0.13	0.11	-1.15	0.25	0.71
18429-1%piRNA	93.37	0.13	0.15	0.84	0.40	0.79
18533-1%piRNA	240.10	-0.02	0.18	-0.09	0.93	0.99
18700-1%piRNA	3438.63	0.12	0.19	0.64	0.52	0.85
18743-1%piRNA	131.28	0.15	0.19	0.78	0.43	0.79
18833-1%piRNA	132.67	0.04	0.11	0.38	0.70	0.95
19061-1%piRNA	132.87	0.05	0.14	0.40	0.69	0.95
19066-1%piRNA	142.36	0.23	0.13	1.73	0.08	0.51
19100-1%piRNA	99.25	-0.17	0.17	-1.00	0.32	0.73
19244-1%piRNA	79.68	-0.31	0.16	-1.91	0.06	0.51
19431-1%piRNA	993.38	-0.18	0.11	-1.60	0.11	0.58
19482-1%piRNA	180.65	-0.02	0.12	-0.18	0.86	0.99
19540-1%piRNA	145.01	0.07	0.16	0.48	0.63	0.92
19716-1%piRNA	295.10	-0.14	0.14	-1.07	0.29	0.73
1999-3%piRNA	99.69	0.26	0.15	1.75	0.08	0.51
20002-1%piRNA	164.15	0.05	0.15	0.35	0.73	0.95
20118-1%piRNA	130.30	0.23	0.15	1.57	0.12	0.58
20185-1%piRNA	189.07	0.17	0.14	1.21	0.23	0.69
20332-1%piRNA	155.54	0.27	0.17	1.57	0.12	0.58
20350-1%piRNA	643.22	0.18	0.18	0.99	0.32	0.73
20417-1%piRNA	564.08	0.14	0.10	1.32	0.19	0.67
20449-1%piRNA	112.09	0.06	0.11	0.56	0.58	0.87
20543-1%piRNA	135.02	0.14	0.12	1.11	0.27	0.71
20804-1%piRNA	143.76	-0.20	0.13	-1.53	0.13	0.58
2081-3%piRNA	185.69	0.07	0.12	0.62	0.53	0.85
20862-1%piRNA	370.70	0.34	0.18	1.87	0.06	0.51
21010-1%piRNA	85.19	-0.12	0.12	-0.99	0.32	0.73
21011-1%piRNA	94.86	-0.04	0.11	-0.33	0.74	0.95
21050-1%piRNA	131.71	0.10	0.13	0.79	0.43	0.79
21116-1%piRNA	202.02	0.18	0.13	1.40	0.16	0.66
21283-1%piRNA	121.46	0.03	0.14	0.23	0.82	0.99
21336-1%piRNA	312.08	0.15	0.12	1.34	0.18	0.67
21344-1%piRNA	959.68	0.07	0.12	0.58	0.56	0.87
2140-3%piRNA	99.57	-0.23	0.16	-1.45	0.15	0.62
21602-1%piRNA	83.37	0.12	0.13	0.99	0.32	0.73

21704-1%piRNA	92.05	0.23	0.12	2.01	0.04	0.51
21815-1%piRNA	198.59	0.27	0.14	1.86	0.06	0.51
21875-1%piRNA	228.67	0.15	0.12	1.27	0.20	0.67
21954-1%piRNA	110.72	-0.04	0.14	-0.31	0.75	0.96
22089-1%piRNA	67.45	-0.19	0.15	-1.23	0.22	0.69
2255-3%piRNA	162.52	0.21	0.12	1.79	0.07	0.51
22580-1%piRNA	192.08	-0.02	0.12	-0.16	0.87	0.99
22666-1%piRNA	146.77	-0.01	0.13	-0.08	0.93	0.99
23124-1%piRNA	93.37	0.05	0.14	0.31	0.75	0.96
23145-1%piRNA	140.05	-0.13	0.13	-1.02	0.31	0.73
2320-3%piRNA	366.11	0.12	0.19	0.63	0.53	0.85
23244-1%piRNA	138.11	-0.07	0.11	-0.57	0.57	0.87
23325-1%piRNA	97.69	0.06	0.15	0.43	0.67	0.94
23367-1%piRNA	171.22	0.15	0.15	0.99	0.32	0.73
23382-1%piRNA	181.92	0.25	0.12	2.03	0.04	0.51
2347-3%piRNA	94.28	-0.13	0.19	-0.67	0.50	0.84
23485-1%piRNA	219.01	0.06	0.12	0.48	0.63	0.92
2360-3%piRNA	93.66	-0.25	0.13	-1.85	0.06	0.51
23676-1%piRNA	580.05	0.03	0.13	0.24	0.81	0.99
23691-1%piRNA	84.42	-0.10	0.13	-0.79	0.43	0.79
2378-2%piRNA	118.77	-0.27	0.15	-1.75	0.08	0.51
23814-1%piRNA	126.06	-0.09	0.14	-0.62	0.54	0.85
2387-2%piRNA	125.16	-0.22	0.19	-1.19	0.23	0.70
2392-2%piRNA	167.58	0.10	0.14	0.74	0.46	0.80
24068-1%piRNA	87.11	-0.41	0.16	-2.58	0.01	0.36
24114-1%piRNA	168.15	0.12	0.11	1.11	0.27	0.71
24163-1%piRNA	93.70	-0.14	0.16	-0.90	0.37	0.77
24236-1%piRNA	111.43	-0.20	0.17	-1.19	0.23	0.70
2446-2%piRNA	132.40	-0.09	0.11	-0.81	0.42	0.79
24605-1%piRNA	104.89	-0.33	0.15	-2.18	0.03	0.51
2464-2%piRNA	166.34	0.30	0.19	1.57	0.12	0.58
24741-1%piRNA	79.83	-0.16	0.12	-1.30	0.19	0.67
24846-1%piRNA	110.33	0.02	0.14	0.16	0.87	0.99
24906-1%piRNA	380.04	-0.09	0.11	-0.86	0.39	0.79
2499-2%piRNA	131.79	0.15	0.10	1.51	0.13	0.59
25179-1%piRNA	239.97	0.23	0.11	2.06	0.04	0.51
25219-1%piRNA	82.86	0.00	0.14	-0.02	0.98	0.99
25550-1%piRNA	1116.20	0.17	0.11	1.57	0.12	0.58
25555-1%piRNA	427.52	-0.25	0.12	-2.05	0.04	0.51
25955-1%piRNA	201.49	-0.01	0.11	-0.12	0.90	0.99
26054-1%piRNA	150.16	-0.04	0.13	-0.29	0.77	0.97
26068-1%piRNA	109.48	-0.18	0.15	-1.22	0.22	0.69
26181-1%piRNA	629.61	-0.11	0.12	-0.96	0.34	0.74

26190-1%piRNA	87.96	0.04	0.12	0.31	0.76	0.96
26200-1%piRNA	148.46	-0.09	0.11	-0.79	0.43	0.79
2620-2%piRNA	85.13	-0.28	0.15	-1.79	0.07	0.51
26257-1%piRNA	103.10	0.05	0.14	0.36	0.72	0.95
26332-1%piRNA	91.43	0.00	0.12	0.01	0.99	0.99
26464-1%piRNA	1770.42	0.25	0.19	1.32	0.19	0.67
26532-1%piRNA	304.24	0.03	0.13	0.23	0.82	0.99
2668-2%piRNA	95.64	-0.01	0.13	-0.05	0.96	0.99
26726-1%piRNA	106.62	-0.09	0.10	-0.90	0.37	0.77
26759-1%piRNA	89.32	0.20	0.16	1.23	0.22	0.69
26829-1%piRNA	799.78	0.03	0.13	0.20	0.84	0.99
27248-1%piRNA	159.96	-0.09	0.11	-0.75	0.46	0.80
27607-1%piRNA	111.69	-0.40	0.15	-2.59	0.01	0.36
28150-1%piRNA	82.26	-0.16	0.15	-1.09	0.28	0.73
28181-1%piRNA	1039.01	-0.01	0.13	-0.06	0.95	0.99
28315-1%piRNA	250.43	0.23	0.12	1.91	0.06	0.51
28357-1%piRNA	82.60	-0.04	0.13	-0.33	0.74	0.95
28435-1%piRNA	80.49	-0.13	0.12	-1.06	0.29	0.73
28669-1%piRNA	145.69	-0.38	0.13	-2.92	0.00	0.32
28855-1%piRNA	554.85	-0.09	0.11	-0.78	0.44	0.79
28878-1%piRNA	116.52	0.05	0.13	0.35	0.73	0.95
2897-2%piRNA	94.48	0.23	0.15	1.55	0.12	0.58
29098-1%piRNA	234.55	0.05	0.12	0.41	0.68	0.94
29349-1%piRNA	143.23	0.04	0.15	0.29	0.77	0.97
2954-2%piRNA	83.85	-0.08	0.13	-0.59	0.55	0.86
29575-1%piRNA	168.64	0.06	0.14	0.40	0.69	0.94
29719-1%piRNA	183.92	0.05	0.13	0.35	0.73	0.95
29796-1%piRNA	270.08	0.09	0.13	0.74	0.46	0.80
2981-2%piRNA	146.94	0.06	0.11	0.54	0.59	0.88
29911-1%piRNA	80.94	-0.01	0.14	-0.04	0.97	0.99
30025-1%piRNA	97.59	0.13	0.13	1.00	0.32	0.73
30066-1%piRNA	981.07	0.20	0.18	1.14	0.25	0.71
30117-1%piRNA	375.23	-0.49	0.17	-2.95	0.00	0.32
30164-1%piRNA	98.43	-0.11	0.15	-0.73	0.46	0.80
30256-1%piRNA	106.84	0.10	0.10	0.95	0.34	0.75
30313-1%piRNA	136.66	0.16	0.13	1.28	0.20	0.67
30489-1%piRNA	255.36	-0.13	0.15	-0.84	0.40	0.79
30699-1%piRNA	198.95	0.13	0.19	0.67	0.50	0.84
3082-2%piRNA	1048.27	0.11	0.18	0.63	0.53	0.85
30846-1%piRNA	142.31	-0.09	0.14	-0.65	0.52	0.85
31047-1%piRNA	277.65	0.03	0.11	0.29	0.77	0.97
31088-1%piRNA	108.71	-0.13	0.13	-1.01	0.31	0.73
31209-1%piRNA	645.12	0.14	0.14	0.99	0.32	0.73

31463-1%piRNA	212.17	0.25	0.11	2.16	0.03	0.51
31504-1%piRNA	93.07	0.03	0.14	0.24	0.81	0.99
31527-1%piRNA	881.79	-0.12	0.11	-1.16	0.25	0.71
31651-1%piRNA	93.28	-0.09	0.12	-0.82	0.41	0.79
31652-1%piRNA	451.53	0.41	0.15	2.70	0.01	0.36
31662-1%piRNA	176.55	-0.01	0.10	-0.14	0.89	0.99
31762-1%piRNA	97.75	-0.13	0.12	-1.08	0.28	0.73
31781-1%piRNA	89.01	-0.25	0.14	-1.86	0.06	0.51
32091-1%piRNA	372.41	0.10	0.10	1.02	0.31	0.73
32297-1%piRNA	148.68	-0.17	0.12	-1.40	0.16	0.66
32379-1%piRNA	101.89	-0.12	0.12	-1.00	0.32	0.73
32463-1%piRNA	125.42	0.19	0.14	1.35	0.18	0.67
32812-1%piRNA	111.29	0.05	0.13	0.42	0.67	0.94
32851-1%piRNA	116.89	-0.01	0.12	-0.07	0.94	0.99
3294-2%piRNA	289.60	0.02	0.14	0.16	0.87	0.99
3303-2%piRNA	207.89	0.17	0.19	0.92	0.36	0.77
33074-1%piRNA	114.45	-0.26	0.15	-1.79	0.07	0.51
33104-1%piRNA	120.30	-0.45	0.14	-3.28	0.00	0.32
33266-1%piRNA	635.98	0.13	0.18	0.74	0.46	0.80
33431-1%piRNA	84.10	-0.03	0.11	-0.26	0.79	0.99
33553-1%piRNA	76.73	0.03	0.15	0.20	0.84	0.99
33557-1%piRNA	243.97	0.02	0.10	0.16	0.88	0.99
33741-1%piRNA	111.29	0.15	0.12	1.27	0.21	0.67
34138-1%piRNA	133.12	-0.04	0.11	-0.37	0.71	0.95
34336-1%piRNA	371.25	0.19	0.11	1.67	0.10	0.53
34381-1%piRNA	81.25	0.06	0.12	0.48	0.63	0.92
34424-1%piRNA	82.15	-0.23	0.17	-1.41	0.16	0.66
34487-1%piRNA	123.56	-0.06	0.14	-0.45	0.66	0.93
34500-1%piRNA	101.40	-0.05	0.14	-0.35	0.73	0.95
34960-1%piRNA	1217.52	0.20	0.18	1.11	0.26	0.71
35406-1%piRNA	74.57	-0.38	0.17	-2.27	0.02	0.51
35456-1%piRNA	94.74	0.21	0.12	1.72	0.08	0.52
35484-1%piRNA	90.64	-0.01	0.12	-0.05	0.96	0.99
35505-1%piRNA	110.81	0.01	0.14	0.10	0.92	0.99
35516-1%piRNA	456.68	0.16	0.18	0.89	0.38	0.77
35541-1%piRNA	102.95	-0.17	0.14	-1.25	0.21	0.68
3559-2%piRNA	230.55	0.37	0.19	1.94	0.05	0.51
35615-1%piRNA	108.16	0.34	0.11	2.97	0.00	0.32
35659-1%piRNA	74.51	-0.03	0.15	-0.18	0.86	0.99
3566-2%piRNA	170.42	0.23	0.12	1.92	0.05	0.51
35682-1%piRNA	1814.19	0.17	0.13	1.28	0.20	0.67
35794-1%piRNA	82.49	-0.06	0.13	-0.43	0.67	0.94
35818-1%piRNA	178.07	-0.04	0.13	-0.35	0.73	0.95

3589-2%piRNA	86.00	-0.07	0.13	-0.54	0.59	0.88
35981-1%piRNA	176.35	0.04	0.14	0.26	0.80	0.99
36028-1%piRNA	132.80	-0.01	0.12	-0.12	0.91	0.99
36100-1%piRNA	246.67	-0.06	0.14	-0.40	0.69	0.94
36159-1%piRNA	342.51	-0.08	0.14	-0.56	0.57	0.87
36186-1%piRNA	92.70	-0.04	0.13	-0.34	0.73	0.95
36269-1%piRNA	131.17	-0.29	0.14	-2.09	0.04	0.51
36361-1%piRNA	85.92	-0.10	0.11	-0.89	0.37	0.77
36381-1%piRNA	237.27	0.27	0.14	1.93	0.05	0.51
3639-2%piRNA	549.92	0.20	0.18	1.13	0.26	0.71
36401-1%piRNA	171.51	-0.11	0.12	-0.94	0.35	0.76
36488-1%piRNA	167.84	0.29	0.11	2.54	0.01	0.37
36513-1%piRNA	136.86	0.09	0.14	0.68	0.50	0.84
36573-1%piRNA	119.66	0.01	0.16	0.08	0.93	0.99
36749-1%piRNA	185.50	-0.09	0.11	-0.83	0.41	0.79
36818-1%piRNA	117.53	0.23	0.12	1.95	0.05	0.51
36994-1%piRNA	85.49	-0.15	0.12	-1.28	0.20	0.67
37321-1%piRNA	84.23	-0.30	0.17	-1.83	0.07	0.51
37441-1%piRNA	94.62	-0.01	0.14	-0.10	0.92	0.99
37550-1%piRNA	74.13	-0.07	0.15	-0.48	0.63	0.92
3762-2%piRNA	240.52	-0.17	0.13	-1.26	0.21	0.67
37751-1%piRNA	101.44	-0.02	0.13	-0.15	0.88	0.99
38049-1%piRNA	132.07	0.07	0.12	0.56	0.58	0.87
38137-1%piRNA	628.96	-0.02	0.11	-0.19	0.85	0.99
3828-2%piRNA	128.79	0.03	0.13	0.23	0.82	0.99
38288-1%piRNA	183.71	0.02	0.14	0.13	0.90	0.99
38362-1%piRNA	278.77	0.13	0.12	1.09	0.28	0.73
38578-1%piRNA	82.27	-0.42	0.15	-2.71	0.01	0.36
38764-1%piRNA	77.48	-0.06	0.13	-0.43	0.67	0.94
38852-1%piRNA	69.93	-0.27	0.15	-1.79	0.07	0.51
39067-1%piRNA	101.66	-0.11	0.13	-0.82	0.41	0.79
39248-1%piRNA	188.67	-0.07	0.14	-0.53	0.59	0.88
39375-1%piRNA	90.66	0.16	0.13	1.20	0.23	0.70
39489-1%piRNA	235.13	0.02	0.11	0.19	0.85	0.99
39522-1%piRNA	221.95	0.01	0.12	0.12	0.91	0.99
39578-1%piRNA	89.13	-0.26	0.15	-1.82	0.07	0.51
3974-2%piRNA	547.74	0.23	0.13	1.75	0.08	0.51
39883-1%piRNA	107.87	0.08	0.13	0.66	0.51	0.84
39891-1%piRNA	127.78	-0.20	0.12	-1.69	0.09	0.53
39952-1%piRNA	188.62	0.35	0.19	1.88	0.06	0.51
4115-2%piRNA	350.50	0.31	0.19	1.67	0.09	0.53
4163-2%piRNA	147.44	-0.37	0.18	-2.07	0.04	0.51
4333-1%piRNA	90.27	0.06	0.12	0.50	0.62	0.91

4377-1%piRNA	158.78	-0.08	0.12	-0.65	0.52	0.85
4469-1%piRNA	99.12	0.03	0.13	0.25	0.80	0.99
4669-1%piRNA	99.16	-0.05	0.13	-0.43	0.67	0.94
4683-1%piRNA	86.25	-0.02	0.13	-0.18	0.86	0.99
4805-1%piRNA	131.44	0.08	0.12	0.66	0.51	0.84
4876-1%piRNA	88.67	-0.02	0.14	-0.14	0.89	0.99
5010-1%piRNA	104.66	0.19	0.13	1.49	0.14	0.61
5029-1%piRNA	81.79	-0.03	0.14	-0.20	0.85	0.99
5042-1%piRNA	98.10	0.11	0.12	0.90	0.37	0.77
5174-1%piRNA	97.59	-0.50	0.19	-2.72	0.01	0.36
533-53%piRNA	8781.28	0.32	0.19	1.67	0.10	0.53
5511-1%piRNA	150.62	-0.15	0.13	-1.16	0.24	0.71
5542-1%piRNA	99.53	0.26	0.14	1.82	0.07	0.51
5584-1%piRNA	191.02	0.02	0.11	0.16	0.87	0.99
5597-1%piRNA	193.22	0.01	0.10	0.05	0.96	0.99
5632-1%piRNA	181.58	0.00	0.12	0.01	0.99	0.99
563-41%piRNA	128.43	0.13	0.14	0.90	0.37	0.77
5755-1%piRNA	105.92	-0.30	0.19	-1.61	0.11	0.58
5758-1%piRNA	76.60	-0.15	0.15	-1.04	0.30	0.73
5775-1%piRNA	104.36	-0.37	0.19	-1.98	0.05	0.51
5787-1%piRNA	103.12	0.06	0.13	0.45	0.66	0.93
6089-1%piRNA	88.57	0.03	0.11	0.31	0.76	0.96
6206-1%piRNA	157.72	-0.10	0.12	-0.84	0.40	0.79
6322-1%piRNA	113.01	0.15	0.12	1.28	0.20	0.67
6372-1%piRNA	76.41	-0.05	0.16	-0.33	0.74	0.95
6378-1%piRNA	218.63	0.14	0.18	0.78	0.43	0.79
6713-1%piRNA	333.41	-0.28	0.13	-2.08	0.04	0.51
6772-1%piRNA	175.06	-0.19	0.14	-1.31	0.19	0.67
6775-1%piRNA	1618.61	-0.06	0.14	-0.48	0.63	0.92
6793-1%piRNA	68.79	-0.42	0.17	-2.43	0.02	0.43
6901-1%piRNA	113.69	-0.15	0.14	-1.02	0.31	0.73
6995-1%piRNA	110.39	-0.01	0.12	-0.08	0.94	0.99
7147-1%piRNA	111.31	0.00	0.13	-0.02	0.98	0.99
7208-1%piRNA	86.22	-0.16	0.12	-1.32	0.19	0.67
7341-1%piRNA	3619.19	0.21	0.14	1.55	0.12	0.58
7447-1%piRNA	147.45	0.17	0.13	1.26	0.21	0.67
7613-1%piRNA	158.02	-0.02	0.15	-0.13	0.89	0.99
7730-1%piRNA	91.63	-0.14	0.15	-0.97	0.33	0.74
7739-1%piRNA	78.11	-0.18	0.14	-1.24	0.22	0.69
7759-1%piRNA	110.62	0.09	0.14	0.68	0.49	0.84
7801-1%piRNA	78.39	-0.06	0.12	-0.46	0.64	0.92
7918-1%piRNA	329.98	-0.15	0.12	-1.30	0.19	0.67
7971-1%piRNA	90.12	-0.19	0.11	-1.74	0.08	0.51

8040-1%piRNA	148.76	0.16	0.09	1.74	0.08	0.51
8142-1%piRNA	90.29	0.10	0.14	0.66	0.51	0.84
8151-1%piRNA	4258.04	0.19	0.19	1.01	0.31	0.73
8283-1%piRNA	258.17	0.14	0.15	0.89	0.38	0.77
8601-1%piRNA	126.73	-0.11	0.14	-0.77	0.44	0.79
8613-1%piRNA	99.82	0.10	0.12	0.82	0.41	0.79
8659-1%piRNA	97.66	0.08	0.13	0.59	0.56	0.86
8686-1%piRNA	150.49	0.19	0.19	1.01	0.31	0.73
8733-1%piRNA	121.52	0.32	0.12	2.62	0.01	0.36
897-11%piRNA	1026.33	0.41	0.19	2.20	0.03	0.51
9026-1%piRNA	87.59	-0.16	0.14	-1.15	0.25	0.71
9065-1%piRNA	145.64	0.11	0.19	0.56	0.57	0.87
9237-1%piRNA	309.31	-0.24	0.13	-1.77	0.08	0.51
9-24721%piRNA	93.01	-0.09	0.15	-0.62	0.54	0.85
9337-1%piRNA	163.57	-0.13	0.13	-1.00	0.32	0.73
9365-1%piRNA	80.11	-0.02	0.14	-0.16	0.87	0.99
9392-1%piRNA	229.34	-0.01	0.13	-0.05	0.96	0.99
9467-1%piRNA	562.42	-0.17	0.13	-1.34	0.18	0.67
9531-1%piRNA	123.70	-0.22	0.15	-1.46	0.14	0.61
9568-1%piRNA	306.36	0.02	0.13	0.16	0.87	0.99
9612-1%piRNA	213.15	-0.10	0.12	-0.84	0.40	0.79
9686-1%piRNA	214.05	0.11	0.09	1.21	0.23	0.69
9852-1%piRNA	88.41	-0.33	0.15	-2.21	0.03	0.51

Appendix C

SMALL RNA SEQUENCING: METHODS FOR LIBRARY PREPARATION AND DATA ANALYSIS

Preparation of cDNA Libraries for Small RNA Sequencing

1. Start with 100 ng of total RNA in 6 µl in nuclease free water for each sample
2. Procure the NEBNext Multiplex Small RNA Library Prep Kit for Illumina (Cat # E7330, New England BioLabs, Ipswich, MA)

   3' Adaptor Ligation to RNAs
3. Dilute the 3' SR Adaptor 1:2 in nuclease free water
4. Mix the following components in a 0.2 ml PCR tube
   - 6 µl RNA
   - 1 µl diluted 3' SR Adaptor
5. Incubate the RNA-3'SR Adaptor mix for 2 min at 70 °C in a thermal cycler. Immediately after, transfer the tube to ice
6. Add the following components:
   - 3' Ligation Reactor Buffer (2X) 10 µl
   - 3' Ligation Enzyme Mix 3 µl

   Total volume in the tube will now be 20 µl
7. Incubate the 20 µl sample for 18 hours at 16 °C

   Remove excess 3' Adaptor
8. Dilute the SR RT Primer 1:2 in nuclease free water
9. Add the following components to the 20 µl samples:
   - Nuclease-free water 4.5 µl
   - SR RT Primer 1 µl

   Total volume in the tube will now be 25.5 µl
10. Heat samples in a thermal cycler for 5 min at 75 °C, followed by 15 min at 37 °C, and 15 min at 25 °C.

    Ligate the 5' Adaptor
11. Resuspend the 5' SR Adaptor in 120 µl of nuclease free water.
12. Dilute the 5' SR Adaptor 1:2 with nuclease free water for working solution
13. Incubate the diluted 5' SR Adaptor at 70 °C for 2 min to denature and then immediately place on ice. Keep the tube on ice and use within 30 minutes.
14. Add the following components to the samples:
    - Denatured 5' SR Adaptor 1 µl
    - 5' Ligation Reaction Buffer (10X) 1 µl

- 5’ Ligase Enzyme Mix 2.5 µl

  Total volume in the tube is now 30 µl

15. Incubate for 1 hour at 25 °C in a thermal cycler

Perform Reverse Transcription

16. Mix the following components in a separate nuclease-free 200 µl PCR tube:
    - Adaptor-ligated sample from step 15—30 µl
    - First Strand Synthesis Reaction Buffer—8 µl
    - Murine RNase Inhibitor—1 µl
    - Protoscript II Reverse Transcription—1 µl

    Total volume is now 40 µl
17. Incubate for 60 min at 50 °C
18. Immediately proceed to PCR steps

Perform PCR Amplification

19. Add the following components to the sample from step 17 and mix well:
    - LongAmp Taq 2X Master Mix 50 µl
    - SR Primer 2.5 µl
    - Unique Index Primer for each sample 2.5 µl
    - Nuclease free water 5 µl

      Total volume in the tube is now 100 µl
20. Incubate the samples in a thermal cycler with the following PCR conditions:

Cycle Step	Temp	Time	# of Cycles
Initial Denaturation	94 °C	30 sec	1
Denaturation	94 °C	15 sec	15
Annealing	62 °C	30 sec	
Extension	70 °C	15 sec	
Final Extension	70 °C	5 min	1
Hold	4 °C		

PCR Purification

21. Utilize the QIAquick PCR Purification Kit (Cat #: 28104, Germantown, MD)
22. Add 500 µl of Buffer PB to the 100 µl sample and mix.
23. Place a QIAquick column in a 2 ml collection tube
24. Apply the 600 µl mixture from step 22 to the QIAquick column
25. Centrifuge the column at 16,000 ×g for 30 sec and discard the flow through
26. Apply 750 µl Buffer PE to the QIAquick sample column and repeat centrifugation at 16,000 × g for 30 sec and discard the flow through
27. Repeat centrifugation at 16,000 × g for 1 min to remove residual Buffer PE
28. To elute the purified PCR product, add 35 µl of nuclease free water to the QIAquick column. Let sit for 1 min and then centrifuge at 16,000 × g for 1 min.
29. Store samples at -80 °C until library size selection step.

Size-Selection of cDNA Libraries for Small RNA Sequencing

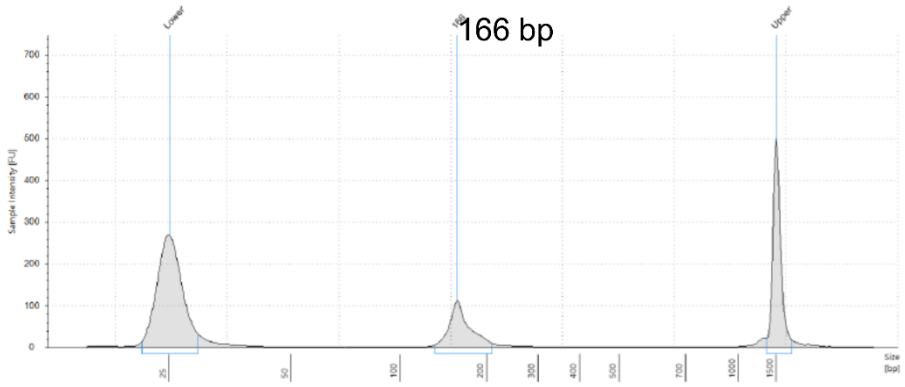
Materials:

- Pippin Prep System (Cat # PIP0001, Sage Science, Beverly, MA)

- 2% Agarose Gel Cassettes (5 wells/cassette) with ethidium bromide (Cat # CSD2010, Sage Science)

The Pippin Prep electrophoresis device is run with provided software on a desktop computer.

1. Open the Pippin Prep software and go to the Protocol Editor Tab.
2. Click the "Cassette" folder and select "3 % DF Marker F"
3. Select the "Range" collection mode and enter the following size selection parameters. Base Pair (BP) start (105) and BP end (155). The BP Range should be indicated as "Broad". These settings were optimized to isolate cDNA ~170 bp, with minimal carry over of non-specific adapter dimers that runs near ~125 bp (see attached figure)
4. Click the "Use of Internal Standards" button
5. Press "Save As" and save these settings for future use
6. Bring DNA marker "F" (provided with 2% gels) to room temperature
7. Combine 30 µl of the sample from library preparation with 10 µl of DNA marker "F"
8. Mix samples by vortexing and centrifuge briefly to collect
9. Load the 40 µl cDNA library +marker mix into each well of the 2% agarose gel cassette (See:http://www.sagescience.com/wp-content/uploads/2016/09/Pippin-Prep-Operations-Manual-460010-Rev-B.pdf for proper loading and orientation of cassette)
10. Seal the cassette with provided sealant and load into the Pippin Prep device.
11. Run the prepared program. This programs takes ~45 minutes to complete automated elution of size-selected libraries
12. Collect the 40 sample from the elution well
13. Save 1 µl for bioanalyzer analysis -- for small RNA sequencing, the recovered peak should be at a concentration of >2 nM (as indicated in the bioanalyzer report) with <2% contamination from the ~125 bp adapter dimer or other non-specific peaks (see Figure 41).
14. Samples are then stored at -80 °C before submission for sequencing with an Illumina NextSeq 500 (for the experiments in Chapter 4, sequencing was performed at the John G. Rangos Sr. Research Center, Pittsburgh PA).



Sample Table

Well	Conc. [pg/µl]	Sample Description	Alert	Observations
B1	136	C3		

Peak Table

Size [bp]	Calibrated Conc. [pg/µl]	Assigned Conc. [pg/µl]	Peak Molarity [pmol/l]	% Integrated Area	Peak Comment	Observations
25	422	-	26000	-		Lower Marker
166	136	-	1260	100.00		
1500	250	250	256	-		Upper Marker

Figure 41. Bioanalyzer trace of cDNA library after size selection.

Example of a cDNA library after size-selection with Pippin Prep. The desired peak indicated at 166 bp. Note the peak molarity measure of 1260 pM or 1.26 nM. As the samples are diluted 1/20 for bioanalyzer analysis, the actual concentration of this sample is ~25 nM and sufficient for small RNA sequencing (>2 nM).

Small RNA Sequencing Data Analysis

All files necessary for small RNA sequencing analysis (aside from the raw sequencing data) can be accessed from this RESOURCES folder:
https://drive.google.com/drive/folders/15JEHtPI79mz8GRIDPDnhRwvmks4rFa1N?usp=sharing

Unprocessed fastq files from the experiments in Chapter 4 can be accessed from: https://www.ncbi.nlm.nih.gov/bioproject/414349

To begin, access or acquire a Linux OS that can connect to the University of Pittsburgh (Pitt) HTC Cluster: For Mac, it is possible to use the command line already on your macOS—this is the preferred option as it requires no installations. For PC users, download the latest versions of Oracle VM Virtual Box and Ubuntu. VirtualBox is needed to open the Ubuntu OS that can run from within the standard Windows OS. See https://www.lifewire.com/run-ubuntu-within-windows-virtualbox-2202098 for step-by-step installation instructions. The University of Pittsburgh Tech Support can assist if there are difficulties, available by appointment and free of charge. (http://technology.pitt.edu/help-desk/technical-support-walk-locations)

Acquire access to the Pitt Computing Cluster: As the amount of memory and space needed to perform many of the bioinformatic analysis involved is too great for the standard personal

computing set up, you will need to access the Pitt High Throughput Computing (HTC) Cluster. To get access, go to: https://crc.pitt.edu/apply/. For non-Pitt users, identify and utilize the high throughput computing resources at your disposal.

Getting into the Pitt HTC Cluster:

- Pull up your command line terminal in Apple or Ubuntu

Type each of the following commands individually: EXACTLY AS WRITTEN (EVERYTHING IS SPACE/CASE SENSITIVE)

- sudo vpnc
- Enter Linux/Ubuntu or Apple OS password as prompted
- Enter vpnc.pitt.edu for the prompted address
- sam_users <<< is the user name when prompted
- ooL6ohho <<< is the password when prompted
- Type **your** exact PittUserName as prompted
- Type **your** exact PittPassWord as prompted
- Complete duel identification log-in with your phone or other appropriate device
- If this worked, you should see 'vpn running in background' and you can proceed with the next couple of steps:
- Type ssh PittUserName@htc.sam.pitt.edu
- Type your exact PittPassWord as prompted

If successful, you should see Welcome to HTC cluster prompt

Getting raw data files (fasta/fastq):

- Download the raw sequencing files into the desired folder in Apple OS or Ubuntu
- Note then the name of the path to that folder you've created: for instance, if I have a folder called RawSequencingData on my desktop, if I right click on it and select properties, I see that the path is /home/greg/Desktop/RawSequencingData <<< this is important for subsequent steps

Moving the raw data files to your HTC cluster folder: Open a SEPARATE terminal that is NOT connected to the HTC cluster as done in previous steps (you can keep multiple terminals open at once)**.** In this terminal, type the following command to move files from your computer folders to the HTC cluster folder:

scp -r /home/greg/RawSequencingData **PittUserName**@htc.sam.pitt.edu:/ihome/ghomanics/**PittUserName**

Note again that all code is case and space sensitive. This scp function will be handy so note format (the -r specifies that RawSequencingData is a folder, if we wanted to move, one file, you don't need the -r).

Confirming the files have been moved to the HTC cluster folder:

- Go into your terminal where you've logged into the HTC cluster
- Type **ls** and it will list the files in your HTC cluster folder—there, you should see the folder "RawSequencingData"
- It is worth getting familiar with the various commands for moving between folders and directories—these are available with a quick Google search—you can solve many basic problems this way. Some example commands are **cd** to enter a folder/directory (used interchangeably) and **pwd** tells you what folder you are in

Building and submitting the "script" or string of programming commands for: TRIMMING RAW SEQUENCES, **ALIGNING** TRIMMED SEQUENCES TO SMALL RNA REFERENCE LIBRARY, AND **COUNTING** THE ABUNDANCE OF TRIMMED SEQUENCES THAT ALIGN TO EACH UNIQUE SMALL RNA REFERENCE

Understanding the bioinformatic programs utilized:

cutadapt is for **trimming** of adapter sequences from the 3'-end of sequencing reads as the raw data contains 50 base reads for each small RNA read. After this step, only 18-45 base sequences will remain and only those that had at least the first 5 bases of the adapter sequence detected on the 3'-end.

Bowtie2 is for mapping/**aligning** your reads against a list of genomic sequences. Here, the list of genomic sequences is all known small RNA species (various miRNA, trna fragments, piRNA, etc.). Often there are parameters such as those used here where we're allowing for a certain amount of mismatched nucleotides for a given length of nucleotides (for a stretch of 18 nucleotides we are allowing for no more than one mismatch between our small RNAs and any of the known small RNA sequences we're matching to

featureCounts: Bowtie aligned all sequences to the list of small RNA features-- this program is **counting** those sequences that map to the same small RNA features. This outputs a text file with counts for each small RNA feature (e.g. number of reads mapping to miR-16, miR-10, tRNA Glu-CTC, etc.)

For far more in depth reference information, see the websites and associated manuals for these programs (listed at end of Appendix C). I strongly recommend this for ANY and ALL bioinformatic programs ever used. Refer to the manuals for each program to better understand the commands used in the script.

Examine the contents of a slurm file (script). The .slurm file is a script that will be used to call all three bioinfomatic programs in succession to process the raw sequencing data. This can be edited easily with a standard text editor in Ubuntu or Apple. You will need to AT MINIMUM, update the script for your specific raw sequencing files. You shouldn't need to modify any of the # lines (see the file provided in the resources folder).

Below is the RNASEQ.slurm script. See my bold # notes following each set of commands for each of the 3 programs providing explanations for the important lines of code
Note for this example, we are processing 4 files for 4 unique samples:

- Sample 1
- Sample 2
- Sample 3
- Sample 4

RNASEQ.slurm script:

#!/bin/bash

#

#SBATCH -N 1 # Ensure that all cores are on one machine

#SBATCH -t 0-08:00 # Runtime in D-HH:MM

#SBATCH --cpus-per-task=1 # Request that ncpus be allocated per process.

#SBATCH --mem=10g # Memory pool for all cores (see also --mem-per-cpu)

This job requires 1 CPUs (4 CPUs per task). Allocate 4 CPUs from 1 node in the default partition.

Change to the directory that the script was launched from. This is the default for SLURM.

module load cutadapt/1.12 **#this loads cutadapt**

cutadapt -a AACTGTAGGCACCATCAAT -m 18 -M 45 -o Sample1_trim.fastq.gz Sample1.fastq.gz

cutadapt -a AACTGTAGGCACCATCAAT -m 18 -M 45 -o Sample2_trim.fastq.gz Sample2.fastq.gz

cutadapt -a AACTGTAGGCACCATCAAT -m 18 -M 45 -o Sample3_trim.fastq.gz Sample3.fastq.gz

cutadapt -a AACTGTAGGCACCATCAAT -m 18 -M 45 -o Sample4_trim.fastq.gz Sample4.fastq.gz

#the m and M in cutadapt refer to trimming size...everything <18 or >45 is discarded. -a is the 3' end adapter (MAKE SURE THIS SEQUENCE IS CORRECT BASED ON THE LIBRARY PREPARATION USED). -o is the output file name….your input is the last file Sample4.fastq.gz with the gz meaning it is compressed

module load bowtie2/2.3.2-gcc5.2.0

bowtie2-build edited_mouse_smallRNA_reference.fa mouse_smallRNA

bowtie2 -x ~/mouse_smallRNA -U Sample1_trim.fastq.gz -S Sample1.sam -N 1 -L 18

bowtie2 -x ~/mouse_smallRNA -U Sample2_trim.fastq.gz -S Sample2.sam -N 1 -L 18

bowtie2 -x ~/mouse_smallRNA -U Sample3_trim.fasta.gz -S Sample3.sam -N 1 -L 18

bowtie2 -x ~/mouse_smallRNA -U Sample4_trim.fasta.gz -S Sample4.sam -N 1 -L 18

#the bowtie build function is just setting up the reference library from the list of small rna sequences (the edited_mouse….fa file) and the last part -- mouse_smallRNA -- is just the name for your new reference library

#-x is the new reference library -U is your trimmed sequences file and -S is your output file in sam format -N is number of mismatches allowed in the alignment and -L is the length for which one mismatch is allowed (1 mismatch allowed for every 18 nucleotides aligned)

module load subread/1.5.0-p2

featureCounts -a RNASEQ.saf -F SAF -M -o RNASEQ_COUNTS.txt Sample1.sam Sample2.sam Sample3.sam Sample4.sam

#the -a this is the same as your reference library, but with genomic coordinate information It is worth noting if you ever use a different reference library in Bowtie2, you will need a different SAF or GTF/GFF file for featureCounts as well. For the purposes of small RNA sequencing, the files provided will work fine. -F represents the file format used in -a and is CASE SENSITIVE. -M means reads that map to multiple parts of the genome will be counted (as tRNA and piRNA are multi-mapping small RNAs). -o is the name of your output file (i.e., COUNTS for each unique small RNA). Finally, the next elements are the

aligned sequence files to be included in the output (Samples 1-4 as the SAM files outputted by Bowtie2).

From looking at the above script, you can more files need to be in your HTC cluster folder before you can run the RNASEQ.slurm script: all raw files to be processed, RNASEQ.saf, edited_mouse_smallRNA_reference.fa, and RNASEQ.slurm

Running the script:

Once logged into the HTC cluster with all the necessary files in the HTC cluster folder (including the script!), simply enter the following in terminal:

sbatch RNASEQ.slurm

This will submit the script to the HTC cluster as a job—once the job is submitted, you do not need to stay logged in—all of the processing will be done within the cluster and your computer/internet connection and/or user input is not needed. Refer to http://core.sam.pitt.edu/HTCCluster for helpful commands regarding checking the status of your script processing (commonly referred to as status of your "job"). Among the files produced by your job should be an RNASEQ.out file that you can open to see how the job performed and find out what error is being reported if the job failed.

Processing your counts for differential expression analysis:

1. Open the RNASEQ.txt counts file in Excel
2. Remove columns "Chr" through "Length"
3. Remove the first row with "# Program..."
4. Your data should now look like "phase 1" in my attached example
5. Let's say Samples 1-2 are Group X and Samples 3-4 are Group Y
6. Change column names from Sample1 Sample2 Sample3 Sampl4 to X1 X2 Y3 Y4
7. Make sure order of columns from left to right is X1 X2 Y3 Y4
8. Keep only the small RNAs you want to analyze. For instance, if only analyzing miRNA, just keep miRNA columns
9. Select all columns and use the FIND AND REPLACE function--- find all cells with "0" value and make sure to specify you are looking for exact "0" value, not any cell that has a 0 in it

10. It is standard to remove any small RNA that had a zero for ≥1 sample. Here's some guidance in the link: you want to select all cells than that have that 0 value and then you'll right click and delete, selecting to delete entire row. i had to do this twice to get it to actually get rid of all zero values.. you should be left with what you see in Phase 4--281 miRNA rows https://www.extendoffice.com/documents/excel/815-excel-remove-rows-based-on-cell-value.html#a1
11. Save the file now as a .csv file (e.g. COUNTS.csv) and move this file to your HTC cluster folder.
12. *See example COUNTS.csv in Resources folder to compare formatting.

Differential Expression Analysis:

Use the files DE_analysis.slurm and DE_analysis.R scripts for differential expression analysis. DE_analysis.R will have to be customized for your experiment. Once you have the COUNTS.csv, DE_analysis.slurm, and DE_analysis.R in the HTC cluster folder, enter the following into the command line terminal:

sbatch DE_analysis.slurm

This job should complete quickly and you should have a file in your folder called miRNA_DEtable.csv that can be easily viewed/sorted/organized in Excel. Also, this step can be done outside of HTC cluster if you have the program R on your desktop as the computing demands are very low for this step.

Contents of DE_analysis.slurm (this doesn't have to be changed unless changing name of the DE_analysis.R file)

```
#!/bin/bash
#
#SBATCH -N 1 # Ensure that all cores are on one machine
#SBATCH -t 3-00:00 # Runtime in D-HH:MM
#SBATCH -J DE_analysis
#SBATCH --output=COUNTS_DE.out

#SBATCH --cpus-per-task=1 # Request that ncpus be allocated per process.

module load R/3.2.2-gcc5.2.0

R CMD BATCH DE_analysis.R  #DE_analysis.R is R SCRIPT EMBEDDED IN THE SLURM—that is, the .slurm script is primarily needed just to call/run the R script with the major analysis details (see below)

# or
#Rscript script.R > $SLURM_JOBID.out
```

Contents of the DE_analysis R script ---needs to be customized for your specific analysis!

```
library(DESeq2)   #enters the program DESeq2
```

miRNAreads<-read.csv("COUNTS.csv",header=TRUE,row.names=1) **#uploads your counts as miRNAreads**

samples<-
data.frame(row.names=c("X1,"X2","Y3","Y4"),condition=as.factor(c(rep("X",2),rep("Y",2)))) **#enter your sample information --- enter all sample as shown and the groups for the as.factor section group X with 2 samples, group Y with 2 samples**

matrix<-
DESeqDataSetFromMatrix(countData=miRNAreads,colData=samples,design=~condition) **# just need to put in the name used for your uploaded counts (miRNAreads) and name used for your sample information (samples)—all else stays the same**

analysis<-DESeq(matrix**)** **#runs DESeq for matrix (which comprises your count/sample info)**

results<-results(analysis) **#generates results**

write.csv(results,file="miRNA_DEtable.csv") **#writes results to your HTC cluster folder**

*This link may be helpful for understanding this step of DESeq2 analysis:

http://seqanswers.com/forums/showthread.php?t=35010

*Also see the DESeq2 manual.

Completed analysis:

At this point, you should have the excel .csv file as a table with p values and FDR-adjusted p values for every small RNA analyzed (see miRNA_DEtable.csv in the resources folder as an example of completed analysis file).

General helpful links:

Gregory Rompala- grrompala@gmail.com

Cutadapt: http://cutadapt.readthedocs.io/en/stable/guide.html

Bowtie2: http://bowtie-bio.sourceforge.net/bowtie2/index.shtml

featureCounts: http://bioinf.wehi.edu.au/featureCounts/

DESeq2:

http://www.bioconductor.org/packages/3.7/bioc/vignettes/DESeq2/inst/doc/DESeq2.html

Google: 95% of the time, someone has asked your specific question before within a bioinformatics message board such that often your question can be nicely answered with just a little bit of sleuthing.

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