ERE-INDEPENDENT ERα SIGNALLING IN FEEDING AND EXPLORATORY BEHAVIORS

BY

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ABSTRACT OF THE THESIS

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The reproductive steroid hormone, 17β-estradiol (E2), controls feeding and exploratory behaviors associated with mood disorders. The loss of circulating E2 puts menopausal women at an increased risk for developing obesity and mood disorders when compared to premenopausal women. Therefore, it is critically important to understand the role of sex steroids and their receptors in the neuroendocrine control of feeding and mood. The goal of this project is to understand the role of estrogen response Element (ERE)-dependent and ERE-independent ERα signaling on behavior by characterizing feeding patters and exploratory behaviors in male and female mice lacking either total ERα signaling or lacking ERE-dependent ERα signaling. We hypothesize that ERE-independent ERα is partially sufficient to restore feeding and exploratory behaviors that are lost in total ERα knockout mice. We tested three strains of mice: two ERα transgenic models, a total ERα knock out (ERKO) and a novel ERα knock in/knock out (KIKO) that lacks a functional DNA-binding domain) and their wild type (WT) C57 littermates using a real-time feeding behavior monitoring system and series of standard behavior tests (open field tests, elevated plus maze, forced swim test). To test our hypothesis FI and meal patterns were

observed while the animals were given *ad libitum* access to a LFD (Experiment 1a) followed by HFD (Experiment 1b). A separate set of animals the response to fasting was monitored for 24 h after caloric restriction (Experiment 1c). Exploratory, depressive, and locomotor behavior testing was conducted on mice from Experiments 1a/b (open field, elevated plus maze, forced swim test). Each experiment was initially done with intact animals and then again repeated in ovariectomized (OVX) animals split into either an oil treated control group or an E2-treated group. We observed ERE-dependent mechanisms are the main modulator of homeostatic LFD feeding meal patterns while ERE-independent ERα signaling was involved in the control of palatable, high-fat diet food intake. During refeeding, ERE-independent mechanisms contribute to a decreased fi rst meal food intake and slower rate of ingestion. When observed during a series of behav ior tests, WT animals explored more, regardless of treatment (differences could be attribu ted to higher levels of locomotor activity in WT). However, similarities between WT and KIKO females in the EPM indicate that ERE-independent pathways may contribute towa rds reducing anxiety measures, independent of locomotor activity. WT females were sho wn to have a decreased free float time, indicating ERE dependent signaling may be influencing despair like tendencies. Collectively, these suggest that both ERE-dependent and -independent ERα signaling are involved with both feeding and anxiety like homeostatic parameters.

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I for one am ready to close this chapter of my life and to get back to work..!

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Chapter 1: Literature Review

1.1 The role of estrogens in the female reproductive cycle

The role of estrogens in modulating key homeostatic and neurological functions led to the expression of "estrogens complicate everything." (Roepke, Malyala et al. 2007, Roepke, Xue et al. 2008, Mamounis, Yang et al. 2014, Yang, Mamounis et al. 2016). Science has long tended towards focusing first on the male model before eventually shifting focus onto the "more complicated" female model (Holdcroft 2007, Chilet-Rosell 2014, Liu and Mager 2016). The sophisticated machinery and hormonal circuitry associated with reproduction being the largest factor leading to the female model "complications." Estrogens, especially 17β-estradiol (E2), are involved in nearly every homeostatic process and estrogen receptor (ER) expression in non-reproductive organs in systems ranging from glucose homeostasis (Yasrebi, Rivera et al. 2017), thermoregulation, bone density, and feeding (Roepke, Bosch et al. 2010).

Reproduction relies on function of the Hypothalamic-Pituitary-Gonadal (HPG) axis. Gonadotropin Releasing Hormone (GnRH) is released to anterior pituitary where gonadotrophs are stimulated to secrete both luteinizing hormone (LH) and follicle Stimulating Hormone (FSH). In circulation, LH and FSH activate follicular cells in ovaries that lead to ovulation. E2, secreted from follicles into the blood stream, travels to the hypothalamus. During normal conditions, blood E2 levels are relatively low and only increase leading up to ovulation when E2 reaches peak secretion upon which GnRH neurons become stimulated (Sarkar, Chiappa et al. 1976, Caligioni 2009). Of the three types of physiological estrogen isoforms, E1 (Estrone), E2, and E3 (Estriol), E2 is the primary and most potent estrogen. E2, produced in the ovaries, is the primary driver for the sustained release of GnRH and LH, during the follicular phase of the estrous cycle.

E2 is also an active steroid in the control of the HPG axis in males (Schulster, Bernie et al. 2016, Cooke, Nanjappa et al. 2017).

Female mice exhibit a 4-7 day reproductive cycle known as an estrous cycle. In humans, the 28-day reproductive cycle is referred to as the menstrual cycle. Although much shorter than the human reproductive cycle, mouse estrous hormonal fluctuations react similarly to that of the human menstrual cycle. Menstrual cycle phases are driven either by ovarian or uterine events. There are two phases of the estrous cycle, follicular and luteal, punctuated by structural events in the ovary, ovulation and corpus luteal regression (Senger 2012). Within the estrous cycle the two phases are further broken down into four key stages: proestrus, and estrus (follicular), and metestrus and diestrus (luteal) (Senger 2012). In the menstrual cycle, the proliferative and secretory phases are considered uterine events. Menopause in higher primates and humans is signified by a decrease in ovarian hormone production of estrogen and progesterone leading to the cessation of menstruation.

Proestrus is categorized as the pre-ovulatory day when there is an increase of E2 being released from the ovary (Walmer, Wrona et al. 1992) and decease in circulating progesterone levels (as a direct result of the destruction of the CL). (Senger 2012). During this stage, antral follicles are maturing in preparation for ovulation (Senger 2012). At the start of the night cycle, LH from the anterior pituitary surges to initiate ovulation in the ovaries (Parkening, Collins et al. 1982). Estrus is determined by the advent of sexual behavior and receptivity in females following the LH surge. The start of proestrus signifies the point of the reproductive cycle where the dominant hormone shifts from being primarily progesterone to being E2 dominant (Senger 2012).

E2 is the dominant driving hormone with peak E2 secretion through estrus with levels elevated throughout the morning hours and decreasing into the nighttime (Walmer, Wrona et al. 1992, Senger 2012). In mice, ovulation occurs during the follicular phase after elevated LH when females show augmented behavior to signify peak sexual receptivity, vocal expression, attempts to mount, etc. (Bronson 1989, Kim, Son et al. 2016).

The stage in between ovulation and formation of the corpus luteum is known as metestrus, the start of the luteal phase. Early metestrus is classified as low circulating progesterone and E2 levels (Walmer, Wrona et al. 1992). Ovulated follicles change to become CL through a process known as luteinization. During diestrus, the newly formed CL matures into a fully functional ovarian structure and secretes large quantities of progesterone (Senger 2012). E2 levels typically remain low through diestrus. In a non-pregnant female, diestrus ends when the corpus luteum undergoes luteolysis and the production of progesterone decreases. In a pregnant female, the corpus luteum continues to produce progesterone to prepare the uterus for embryonic attachment and development (Senger 2012).

1.2 Estrogens and the control of energy balance and feeding behaviors

Obesity has become a world wide epidemic with some estimates putting adult obesity within the U.S. at a staggering 34%, with females having higher rates of obesity than males (Mitchell, Catenacci et al. 2011). Due to the decrease in circulating estrogens (17β-estradiol) brought about by menopause, post menopausal women are at a higher risk of positive weight gain eventually leading to obesity and its associated comorbidities.

(Moore 2004, Sullivan, Shearin et al. 2012). Additionally women have a higher prevalence of eating disorders, due in part to social and physiological factors. (Striegel-Moore, Rosselli et al. 2009) Food intake (FI) fluctuations have been reported over the course of a normal menstrual cycle (Buffenstein, Poppitt et al. 1995). Furthering that notion, sex steroids can affect feeding behaviors in a specialized manner. By itself, estrogen decreases FI and testosterone increases FI (through an increase in meal frequency); however when both progesterone and E2 are present in vivo, FI can be increased (Asarian and Geary 2006).

Energy balance is defined as energy intake (carbohydrates, fat, protein) minus energy being expended through heat release, physical activity or thermic effect of food (Stipanuk and Caudill 2013). Maintaining a proper energy balance is contingent on the CNS, central nervous system, detecting energy store information (through communication via peripheral nutrient and hormones) and matching expenditure to reflect energy intake (Ahima and Antwi 2008). Energy balance is a multi-pronged system involving the interaction of the CNS (brain and spinal cord), with many different afferent signals coming from many different portions of the digestive tract. These various afferent mechanoreceptor signals terminate at the nucleus of the solitary tract (NTS) located in the hindbrain. NTS projections then innervate with many hypothalamic endpoints such as the Arcuate (Arc), Paraventricular (PVN) and Lateral Hypothalamic Area (LHA) signals from which all eventually lead to the perception of GI fullness and satiety. (Farley, Cook et al. 2003).

The input of various mechanical and chemical receptors all interact with the CNS through vagal afferent nerve fibers (Stipanuk and Caudill 2013). A brain region of

particular importance to energy homeostasis is the hypothalamus. The hypothalamus processes signals that modulate food intake and energy expenditure and integrates that information to control other hypothalamic functions including the hypothalamic-pituitary-gonadal axis (HPG) and reproduction. The Arcuate nucleus of the hypothalamus is located directly inferior to the third vertical at the base of the hypothalamus and acts as a primary afferent signaling integration center, receiving input from other neural integration centers. Additionally, the Arcuate acts as a relay center sending satiety signals to the hindbrain (Stipanuk and Caudill 2013). The Arcuate contains two neuronal populations that act as "first order" neurons (Yasrebi, Hsieh et al. 2016) involved in feeding and energy homeostasis - Proopiomelanocortin (POMC) and Neuropeptide Y (NPY) neurons (Gao and Horvath 2007). POMC neurons are anorexigenic and co-express proopiomelanocortin and cocaine and amphetamine regulated transcript (CART). POMC activation releases α-melanocyte-stimulating hormone (aMSH) in the PVN and LH to act via melanocortin receptors, MC3 and MC4, leading to an inhibition of food intake (Seeley, Drazen et al. 2004, Stipanuk and Caudill 2013) by working in opposition to POMC are NPY neurons that secrete the neuropeptides, NPY and agouti-related protein (AgRP). AgRP acts as an agonist to MC4 to block the actions of αMSH (Stipanuk and Caudill 2013, Yasrebi, Hsieh et al. 2016).

Circulating peripheral peptide hormones, insulin, leptin and ghrelin, interact with both POMC and NPY neurons to control energy and glucose homeostasis (Austin and Marks 2009, Schaeffer, Langlet et al. 2013, Chabot, Caron et al. 2014). We and others have demonstrated that E2 impacts this signaling by regulating hormone receptors and mediators of signal transduction in these neurons (Kelly, Moss et al. 1977, Lagrange,

Ronnekleiv et al. 1997, Stincic, Grachev et al. 2018). Thus, the peptide hormone-hypothalamus relationship is further complicated by the addition of sex hormones. In a previous publication from our lab, we found that fasting increased *NPY, AgRP, GHSR, and UCP2* mRNA expression in male C57 Arc samples. When comparing fed vs fasted E2 treated females, we found genes implicated in the NPY pathway (*GHSR, CPT1c*, and *Foxo1*) were all elevated when compared to their oil treated counterparts (Yasrebi, Hsieh et al. 2016). Additionally, in the fed E2-treated group *Agrp* was also elevated. Similarly, fasting increased *Npy* and *Argp* gene expression in both oil- and E2-treated groups but fasting only increased *Ghsr* expression in fasted E2 treated group. (Yasrebi, Hsieh et al. 2016). Furthermore, E2 has also been shown to have effects on plasma peptide hormone level. Leptin levels were lowered in WT E2 administered mice when compared to their oil-treated counterparts. This trend extended to both low fat diet-fed and high fat diet-fed animals (Yasrebi, Rivera et al. 2017). There are also implications that E2 might have an effect on the actions of insulin (Gupte, Pownall et al. 2015), ghrelin (Yasrebi, Hsieh et al. 2016) and CCK (Asarian and Geary 2007, Asarian and Geary 2013).

The effects ovarian hormones play on feeding behaviors have been the focus of recent investigation. Through this research, evidence has been presented as to estrogens influence on feeding behaviors (augmented meal sizes, and affects on orexigenic neurons for instance) (Butera 2010). Food intake has been shown to be augmented to reflect ovarian hormone cyclicity in that intact rats have exhibited a reduction of meal size leading to a depression of overall feeding activity at times of elevated estrogen (at the peak of Proestrous leading into Estrous) (ter Haar 1972, Blaustein and Wade 1976). Additionally, decreased interest in feeding (by means of reduced FI) at times of peak

estrogen have been observed in several other animal models (Czaja 1975, Czaja, Butera et al. 1983).

Lack of estrogen, obtained through either ovariectomy (in animals) or in post menopausal women, leads to larger meal sizes and greater overall food intake in mice as well as leads to increased in body mass (Wade 1975, Blaustein and Wade 1976, Svendsen, Hassager et al. 1995, Asarian and Geary 2002), the effect of which was mitigated when dosed with physiologically relevant estradiol doses (Tarttelin and Gorski 1973, Wade 1975, Asarian and Geary 2002). Rats treated with progesterone alone saw now significant differences in FI, further confirming estrogens significance (Galletti and Klopper 1964, Wade 1975).

1.3 The central control of locomotor and anxiety-like behaviors

Anxiety:

Anxiety can affect a person both mentally and physically. The DSM5 classifies general anxiety disorder being accompanied with restlessness, fatigue, difficulty focusing, irritability, muscular tension, and sleep disturbance. (American Psychiatric Association and American Psychiatric Association DSM-5 Task Force. 2013) Diagnosing human anxiety or depression can be done by screening symptoms against known criteria; Diagnosing rodents of either anxiety or depression takes a more nuanced approach. Anxiety and depressive like states can demonstrate differently depending on factors such as sex, animal strain, and species. In order to detect either anxiety like or depressive like traits in animals, behaviors are monitored and scored for characteristic traits. (Bailey and Crawley 2009)

Brain regions involved in mood and anxiety:

No one "central" anxiety center exists in the brain but rather a wide network of corresponding regions are implicated to be involved with anxiety. Areas of interest include the extended amygdala and prefrontal cortex (Yassa, Hazlett et al. 2012). Furthermore the limbic system (encompassing many regions of the brain including the hypothalamus, amygdala, hippocampus, and regions of the prefrontal cortex (Bari, Niu et al. 2014) are involved in the processing of mood and emotional states.

It has been suggested fear and anxiety are both processed through two separate complimentary systems, the Amygdala and Bed Nucleus of the Stria Terminalis (BNST). The extended Amygdala encompasses several identified brain regions including but not limited to the central medial Amygdala, the Bed Nucleus of the Stria Terminalis (BNST), Nucleus Accumbens and the Sublenticular Sustancia Innominate (Nestler, Hyman et al. 2009). Further substantiating the integration of the two systems, the Basolateral Amygdala projects to the BNST and central nucleus of the Amygdala, both regions which then project to additional areas thought to be involved with anxiety (Walker, Toufexis et al. 2003). Human neuroimaging studies with functional magnetic resonance imaging (fMRI) demonstrate that neutral stimuli is less probable to cause Amygdala stimulus over emotional stimulus (Costafreda, Brammer et al. 2008). Additionally, human fMRI meta-analysis studies have also shown Amygdala activation preferred emotional state visual stimuli (Amygdala activated and responded to both positive and negative images) (Sergerie, Chochol et al. 2008).

The Amygdala has been shown to play a roll within the emotional stimulus interpreted pathway. In rodents, exposure to less predictable stimuli lead to basolateral

Amygdala activation, the activation of which lead to glutamate release stimulating both the central Amygdala (CeA) and separately the medial Amygdala (MeA). Additionally basolateral Amygdala activation causes the stress hormone, CRH, to act on the BNST which then further innervates the CeA (Davis 2006). Conditioned fear responses were abolished in rodents having been administered CeA legions. However, anxiolytic like behavior was still observed when the same rodents were exposed to unconditioned stimuli. When animals with BNST legions were exposed to a known stressor they did not exhibit anxiety like behaviors (Davis, Falls et al. 1993, Lee and Davis 1997). Estrogen administration was shown to produce modified behavioral responses. Upon administration of either physiologically relevant E2 doses or injecting estrogen directly into the Amygdala animals behaved in a more analgesic manner. When placed in a FST, immobility was also decreased. (Frye and Walf 2004, Walf and Frye 2006)

While the Amygdala is responsible for more short term responses to threatening stimuli (specific fear stimulus) the BNST has been shown to act to continue sustained anxiety like responses resulting in heightened behavioral response after the initial stimulus has dissipated (Gewirtz, McNish et al. 1998, Walker, Toufexis et al. 2003, Davis 2006, Yassa, Hazlett et al. 2012). Located in the ventral forebrain, the BNST has been implicated to be critical within stress response circuitry that also contributes roles in both anxiety and addiction (Avery, Clauss et al. 2016). BSNT has dense connections with the PVN of the hypothalamus. PVN is involved with the HPA axis in that it initiates cortisol secretions affecting stress/anxiety. Stress related cortisol levels have been shown to be altered post BNST legions further providing compelling evidence to the importance of BNST in the stress and anxiety response system (Sullivan, Apergis et al. 2004, Avery,

Clauss et al. 2016). Additionally, the Locus Coeruleus has been shown to be involved with arousal and can influence anxiety like behaviors from local noradrenergic neuron projection throughout the rest of the brain (Breton-Provencher and Sur 2019).

Depression:

It is suspected there are many variables contributing to the overall condition of depression. Regional brain abnormalities, hormonal fluctuations, neurotransmitter deregulation, disturbances in the Hypothalamic-Pituitary-Adrenal axis (HPA), the list goes on. The creation of specialized imagining technology (magnetic resonance imaging (MRI) and positron emission tomography (PET) for instance) (Pandya, Altinay et al. 2012) of the last 20 years have helped shed light on the regions of the brain implicated in human depression. Deceased blood flow and metabolism of the PFC have been found in patients suffering from Major depressive disorder, and both conditions have been found to be reversible upon regimented antidepressant interventions (Kimbrell, Ketter et al. 2002, Rigucci, Serafini et al. 2010) (Mayberg, Brannan et al. 2000) Abnormalities in the ventral Anterior Cingulate Cortex (vACC) have also been found in depressed patients; the vACC directly innervates the hypothalamus (a major endocrine secretion control center). (Critchley 2004)

The LC structure has many regions somehow tied in with animal behavior. For instance, the prelimbic (PLC), infralimbic Cortex (ILC) and medial Prefrontal Cortex (mPFC) have all been implicated in playing roles with animal sociability, of which when animals behave in more withdrawn sociable tendencies can be described as exhibiting depressive like behavior (Minami, Shimizu et al. 2017). When mPFC legion animals

were placed in an Elevated Plus Maze (EPM) they showed a reliance on coping behaviors and also behaved in a manner indicative of having increased anxiety when compared to intact animals (Jinks and McGregor 1997). The mPFC and PLC have directly innervating projections onto the Amygdala.

Although no specific depressive animal model has been developed, a few behavioral testing systems have been established. Seligman and Maier first described depressive like animal behavior as "learned helplessness" (Overmier and Seligman 1967, Seligman and Maier 1967). Dogs were exposed to an electric shock while enclosed in escapable or inescapable environments. Animals in the escapable environment were quickly able to avoid the shock and were otherwise unimpaired. Animals within the inescapable chamber did not resist and succumbed to the electric shock (Seligman and Maier 1967). The animals were said to have the appearance of having "given-up" (Seligman and Maier 1967)**.** Exhibiting a similar physical phenomena to when humans experience depressive like helplessness the phenomenon was proposed as a comparable depressive like state (Miller, Seligman et al. 1975). Further substantiating its comparative like state, it has been reported that when paired with a selective serotonin reuptake inhibitor (SSRI) treatment, some of the depressive like behavioral deficits were improved (Zazpe, Artaiz et al. 2007).

Learned helplessness has become the premise for many depressive state behavioral testing paradigms. The tests include behavioral despair (also know as the Porsolt test, or more commonly the Forced swim test) and tail suspension tests (Castagne, Moser et al. 2011, Belovicova, Bogi et al. 2017). The tests have been developed and are

regularly used to test the validity of antidepressants, as it has been found antidepressants increase the latency to immobility (Castagne, Moser et al. 2011)

The role of neurotransmitters and hormones on exploratory behaviors:

Allowing for neuronal communication, neurotransmitters act as a chemical link between mind and body. Impacting nearly every aspect of overall physiology, Serotonin, Dopamine, GABA and Norepinephrine are believed to regulate mood; Serotonin and GABA are thought to be inhibitory in nature whereas Dopamine and Norepinephrine ("the adrenalin hormone") are thought to be more excitatory. All four prominent neurotransmitters are in some way affected by ovarian hormones, either in an inhibitory or an excitatory nature (Barth, Villringer et al. 2015). It is not uncommon for progesterone to act in a manner counter to that of estrogen. (Smith, Waterhouse et al. 1987, Yokomaku, Numakawa et al. 2003, Bethea and Reddy 2012, Barth, Villringer et al. 2015)

Severe female depression has been reported immediately post parturition, when estrogen and progesterone levels drop, or after the onset of menopause, when ovarian hormone production abruptly decreases and eventually stops. The sudden decrease of estrogen and progesterone during the menstrual cycles late luteal phase may also contribute to Premenstrual syndrome (PMS). Associated with mood symptoms of anxiety, depression, and irritability (Schmidt et al., 1998), the mood-related symptoms associated with post-menopausal or women experiencing PMS are reversed when they are supplemented with pharmacological doses of estrogen (Shors and Leuner 2003). Additionally the sex differences in reported levels of depression disappear after the onset

of menopause when there is a cessation of ovarian hormones and decreased serotonin function (Halbreich, Rojansky et al. 1995, Sonnenberg, Beekman et al. 2000). This leads to the hypothesis of ovarian hormone fluctuations having a larger impact on depression than total depletion of gonadal steroids**.**

Corticotropin-Releasing Factor or Corticotropin-Releasing Hormone, CRF/CRH, has been found to be at elevated blood plasma levels (Banki, Bissette et al. 1987). Secreted from the PVN at the base of the hypothalamus, CRF can innervate with its receptors throughout the brain including the Amygdala, BNST, ARC/PVN of hypothalamus (Wong, Licinio et al. 1994). The CRH neurons within the PVN are directly influenced by the presence of Estradiol (E2). E2 suppressed the post synaptic voltage dependent potassium channel of CRH neurons (Hu, Liu et al. 2016). CRH mRNA expression in PVN was elevated at proestrous (time of increased circulating E2) (Bohler, Zoeller et al. 1990). Additionally, extraneous E2 administration to OVX rats caused a two-fold CRH mRNA gene expression in PVN tissue. Further complicating the mechanism, progesterone addition ameliorated the effect of estrogen alone (Roy, Reid et al. 1999). Additionally, CRF injection directly into the CNS of lab animals, have shown to replicate many depressive state symptoms (Nemeroff 1998). Antidepressant treatment has been shown to have a direct retarding impact on CRF containing neurons (Nemeroff 1998). These results emphasize the need to view depression as a set of behavioral symptoms that are manifested by interactions of hormonal and neurotransmitter systems in numerous brain regions. It is unlikely that any one molecule, but rather a commutation of variables, is responsible for such a phenomenon as depression.

1.4: 17β-estradiol signaling and the control of feeding and exploratory behaviors.

Tissue-specific ER and signaling:

Sex hormones, specifically estrogen, are crucial in ensuring proper cellular homeostasis. Once bound to either a nuclear or membrane estrogen receptor (ER), signaling is initiated which can act via multiple cellular pathways to regulate transcriptional processes. The binding of estrogen to its estrogen receptor results in translocation; when bound to a nuclear receptor the mechanism defined as a classic estrogen mechanism (Saczko, Michel et al. 2017). Classical nuclear receptors ERα and ERβ bind to nuclear ER receptors whereas membrane ER signaling occurs through membrane-attached nuclear receptors and G-protein coupled like receptors such as GRP30, Gq-mER, and ER-x receptors (Genazzani, Pluchino et al. 2007). Nuclear ERα/β directly binding to specific estrogen response elements (ERE) through nuclear translocation is known as genomic ("nuclear") signaling (Saczko, Michel et al. 2017), as well ER binding to other transcription factors (activator protein 1, AP-1; ER-SP-1 protein interactions (Heldring, Pike et al. 2007) to regulate ERE-independent transcription. Membrane-initiated estrogen signaling, also known as non-genomic ("non-nuclear pathways"), can directly influence cellular regulatory cascades (Saczko, Michel et al. 2017). Of the two signaling methods, the cellular mechanisms initiated through membrane-associated ER receptors occur at a much faster scale than those initiated through nuclear ERs (Cui, Shen et al. 2013).

Nuclear ERs- ERα and ERβ:

Nuclear estrogen receptors are spread throughout the body and in tissue-specific distribution. The nuclear receptor ERα is highly expressed in the gonads with lower levels of expression throughout other tissues, the heart, liver and skeletal muscles to name a few (Couse and Korach 1999). KO studies have shown ERα is required for negative and positive feedback of estradiol on GnRH firing (Cui, Shen et al. 2013). We have previously shown ERα, acting on hypothalamic regions, is heavily involved with maintaining energy homeostasis (Mamounis, Yang et al. 2014, Yang, Mamounis et al. 2016, Mamounis, Hernandez et al. 2017, Yasrebi, Rivera et al. 2017). Estrogen, acting through ERα, has been shown to augment locomotor activity in mice (Ogawa, Chan et al. 2003). ERβ is primarily expressed in secondary sex organs (breast tissue) and non gonadal tissue during development (bone marrow and lung alveoli) (Koehler, Helguero et al. 2005). Additionally ERβ has been shown to have protective cardiac tissue properties. Upon maturing, both nuclear receptors have been found to have distribution throughout the brain, especially within the hypothalamus (Couse and Korach 1999). Receptor and functionality overlap are both necessary for normal ovarian functioning (Krege, Hodgin et al. 1998).

Membrane ERs- GPR30, Gq-mER and ER-x:

GPR30 is a G-protein coupled receptor located on the cell and endoplasmic reticulum membrane (Shen and Shi 2015), structurally unrelated to ER, that functions through non classical ER binding. GPR30 is expressed throughout the body in both neural (forebrain and specific midbrain nucli) (Cui, Shen et al. 2013) and sex organs

(Thomas, Pang et al. 2005). Although higher E2 concentration are needed for GPER activation (Pedram, Razandi et al. 2006), GPR30 has been identified to trigger Estrogen signaling through non-genomic estrogen signaling independent of ERα/β (Roepke, Bosch et al. 2010). Another recently discovered G-protein coupled estrogen receptor is Gq-mER. Located primarily in the hypothalamus, once the membrane estrogen receptor is activated, it initiates the PLC–PKC–PKA pathway directly affecting POMC neurons (Lagrange, Ronnekleiv et al. 1997, Qiu, Bosch et al. 2003). A selective estrogen agonist, STX, can cause Gq-mER pathway activation without the use of estrogen (Vail and Roepke 2019). The less known membrane receptor ER-X expression occurs predominantly during development (Toran-Allerand, Guan et al. 2002) with relatively undetectable levels in healthy uninjured adults.

Signaling mechanisms:

ERE-dependent signaling is always Genomic while ERE-independent signaling can be both membrane-initiated or nuclear-initiated depending on its pathway conclusion ending with transcriptional regulation or not. Both Estrogen receptor α/β are nuclear ligand activated transcription factors which form dimers (hetero/homo) to bind with appropriate ERE sites in target gene promoter regions to increase or decrease genomic transcription (Couse and Korach 1999, Shen and Shi 2015) contingent on its different transcriptional gene target. ERα and ERβ can be broken down into five domains (A/b,c,d,e,f) from the N to C terminus ends respectively. Between the two receptors, of the five domains, the most varied domain is the N terminus end A/b domains. The N terminus can be bound at the N-terminus activation function-a (AF-1) domain which

initiates binding to primary transcriptional machinery. It is the primary transcriptional binding that ultimately leads to different intracellular pathway activation/inactivation (ERα can activate where ERβ can inhibit) (Liu, Albanese et al. 2002). Although the N-terminus contains the largest amount of receptor variation, the C-terminal (regions E/F) are important for nuclear ER activation by providing sites of ligand binding through the ligand binding (LBD) and AF-2 domains (Couse and Korach 1999). Upon activation, ERs translocate from plasma membrane to nucleus. Once bound to the nucleus, transcriptional machinery gets recruited to the ERE in Estrogen response gene promotor (Hewitt, Li et al. 2010). Direct DNA binding is not necessary for transcriptional regulation; approximately 35% of human E2 responsive genes begin genomic transcription via indirect estrogen receptor-DNA binding (O'Lone, Frith et al. 2004).

Membrane initiated pathway signaling:

An alternative to ER dependent signaling can be done through membrane initiated pathway signaling by use of predominate G-protein coupled receptors where the signaling is initiated at either the membrane or cytoplasm (Vasudevan and Pfaff 2008). Acting faster than classic transcriptional processes, estrogen binding through a nongenomic pathway acts to initiate a fast acting response upon which regulatory cascades involving MAPK, tyrosine cascades, growth factors, membrane associated ion channels, g-protein coupled receptors and other receptors such as insulin-like growth factor (IGF-1) (Kahlert, Nuedling et al. 2000, Saczko, Michel et al. 2017) are modified upon signaling initiation. Additionally the pathway can also be mediated through the

activation of several different protein kinase cascade pathways (P13K cascade for instance) (Hayashi and Yamaguchi 2008).

Summary and aims of thesis:

This thesis set out to investigate if ERE-independent ERα signaling was capable of restoring feeding and exploratory behaviors that are otherwise lost in total ERα knockout mice.

Aim 1: To determine the contribution of ERE-independent ERα signaling on feeding parameters in intact male and female and ovariectomized females with or without E2 replacement. Animals were monitored for homeostatic feeding (LFD), hedonic feeding (HFD) and refeeding parameters (Post 24 hour fast).

Aim 2: To explore the role of ERE-independent ERα signaling in exploratory, anxiety, and depressive-like mouse behaviors. Animals were run through a series of behavior tests (Open field test, Elevated plus maze, forced swim test) and scored based off of predetermined parameters.

Chapter 2: ERE-Independent ERα Signaling in Feeding And Exploratory Behaviors

2.1: Introduction

In addition to controlling reproduction and eliciting secondary sex characteristics, estrogen also provides many protective homeostatic properties. The drastic loss of estrogens at the onset of menopause eliminates the protective properties. Menopause is defined as the period at which there is a complete loss of ovarian hormone production. Post menopausal women are more susceptible to several different pathologies, coronary heart disease, obesity (Dalal and Agarwal 2015) and increased prevalence of mood disorders to name a few (Li, Ma et al. 2016). Intending to alleviate the symptoms and discomforts associated with estrogen withdrawal resulting from menopause, hormone replacement therapies are prescribed. However, the treatments may have adverse side effects and repercussions depending on treatment timing and stage of estrogen withdrawal (Hersh, Stefanick et al. 2004, Hodis, Collins et al. 2012, Pardini 2014). Some of these repercussions include increased risk of heart disease, cancer susceptibility and metabolic syndromes. Because of these outcomes, it is crucial to examine estrogens interaction with its many receptor subtypes, and the mechanisms of activation are a key target of investigation.

Of the receptors estrogen interacts with, estrogen receptor alpha (ERα) has been found in brain regions associated with energy homeostasis (Arcuate nucleus of the hypothalamus), ventromedial hypothalamus (VMH), and areas involved with mood disorders (Amygdala). ERα signaling occurs through estrogen response element (ERE), protein-protein interactions with transcription factors, or via membrane-initiated signaling events. The latter two types of signaling are collectively called ERE-independent pathways.

One novel means of investigation is to discern ERα activation and mechanistic pathways. Until recently, studies of the two pathway differentiations were only possible through the use of ERα agonists/antagonists or use of estrogen receptor blockers limiting many studies to cell culture. The development of ER knockout mice by Dr. Ken Korach and colleagues added an additional tool to study the ER-mediated pathway that control reproduction, homeostasis, and behaviors. To further differentiate between ERE-dependent and -independent signaling, another model was developed that expressed an ERα lacking a functional DNA binding domain and thus ERE-independent signaling (called ERα, knockin/knockout or KIKO). This model is not fertile (Couse, Dixon et al. 2001, Hewitt, Winuthayanon et al. 2016), but exhibits WT-like energy and glucose homeostasis. We have previously shown that the ERα KIKO animal model does not respond to ovariectomy nor are as sensitive to E2 as WT (Mamounis, Yang et al. 2014).

We have also observed differences between WT, KIKO and KO in terms of gross feeding, metabolism, and locomotor activity. Intact wild type and KIKO females were shown to have similar blood estrogen levels; however the recorded uterine mass of intact KIKO females was shown to average between both WT and KO counterparts (Mamounis, Yang et al. 2014). Cumulative body mass gain in oil treated (via IP injection) OVX females (fed a standard diet) showed WT gained more body mass than KIKO which gained more than KO counterparts over the course of a month. KIKO and KO groups body mass gain trended one another (Mamounis, Yang et al. 2014). Similarly when additional OVX animals were placed on either a low fat diet (LFD) or a matched high fat diet (HFD) chow and treated with either oil or E2 (orally) OVX KIKO animals gained body mass less than comparable WT females but more than comparable KO

females (Yasrebi, Rivera et al. 2017). OVX oil/E2 treated KIKO and KO females had significantly lower day and night V.O2 and RER differences when compared to their WT counterparts (Mamounis, Yang et al. 2014).

Food chewing was seen across all three genotypes. The intact WT females however had an excessive amount leading to a significantly higher rate/amount of food chewing compared to their KIKO or KO littermates. The trend was also seen across E2 treated OVX mice. WT and KIKO females trended towards lower food chewing when compared to OVX KO mice regardless of treatment (Mamounis, Yang et al. 2014). These differences in activity, metabolism, crude food intake, and chow chewing lead us to hypothesize that the lack of ERE-dependent signaling during development and adulthood substantially alters the feeding, motivational, and mood neural circuits. To investigate this hypothesis, we examined feeding behaviors in both male and female mice of all three genotypes use of a real-time food intake monitoring system (Research diets BIODAQ) as well as locomotor and exploratory behaviors using open field, elevated plus maze, and forced swim test.

2.2: Materials and Methods

2.2.1: Animals

All animal procedures were in compliance with standards established by NIH. Animals were handled in accordance to institutional Animal Care and Use Committee approval at Rutgers University (Protocol: 11-003). Animals were allowed water *ad libium* and housed under a consistent controlled climate (phototropic light cycle (12/12 light/dark cycle); 23° Celsius). Two different breeding paradigms were used to produce

target genotypes. Originally provided courtesy of Dr. Ken Korach, NIEHS, heterozygous C57BL/6 ERα WT/KI males were bred with heterozygous C57BL/6 ERα WT/KO females to produce full ERα WT/WT and ERα KI/KO pups. Heterozygous C57BL/6 ERα WT/KO males were bred with heterozygous C57BL/6 ERα WT/KO females to produce full ERα WT/WT and ERα KO/KO pups. The pups were weaned at PND 21, DNA was extracted from a small ear clipping and the animals genotypes were determined using PCR (Hewitt, O'Brien et al. 2009). Once identified all animals were group housed through adulthood and only separated for food intake data collection.

2.2.2: Estrogen

17β-estradiol benzoate (E2B) was purchased from Steraloids (Newport, RI, USA). E2B prepared for injections was dissolved in ethanol (1mg/ml) prior to mixing in sesame oil (Sigma-Aldrich) to obtain a low (0.25μg) and high (1.5μg) E2B concentration mixtures. E2B prepared for oral dosing was dissolved in ethanol (8mg/ml) prior to mixing in sesame oil (Sigma-Aldrich) to obtain a final dosing concentration of 300μg/kg (Yasrebi, Rivera et al. 2017).

2.2.3: Diet and meal pattern analysis

All chow was obtained from Research diets (New Brunswick, NJ). Animals were given standard chow (13% kCal fat, Lab Diet 5V75; 3.48 kcal/g; low phytoestrogen, <75 ppm) until experimentation using the Biological Data Acquisition (BioDAQ) system (Research diets). All real time food intake monitoring was done on a BioDAQ system (monitor version 2.3.13, data viewer version 2.3.14) on loan from Research Diets. The

BioDAQ system is an automated machine capable of providing researchers with real time Bout and Meal information. All animals were provided chow in a deep hopper designed with an enclosed back. Among other features, the BioDAQ provides a swing door which when closed restricts chow access.

2.2.4: Surgery

Adult female mice (>3months) were sedated under general anesthesia (Isoflurane, 2% in O2) and given a local injection of Marcaine (2mg/kg; Henry Schein) at the site of incision. The ovaries were removed via a single ventral incision. Immediately upon completion, mice were injected with Rimadyl (4mg/kg; Henry Schein) and again 24 hours post-op (Yasrebi, Rivera et al. 2017).

2.2.5: Experiment 1a/b:

Male and female ERα WT/KIKO/KO littermates of >3 months of age were bred from our main ERα colony (as previously described in (Mamounis, Yang et al. 2014)). All animals were group housed and fed a standard chow diet (13% kCal fat, Lab Diet 5V75; 3.48 kcal/g; low phytoestrogen, <75 ppm). Prior to running the animals through the BioDAQ, the animals were separated into individually housed cages and were allowed to acclimate to the BioDAQ monitoring room for one week. Ovariectomized (OVX) animals were housed singly post-surgery and allowed one-week recovery in single housed cages prior to being moved into BioDAQ cages. To mitigate any additional stressors, post ovariectomy female mice were not injected with estrogen but rather orally dosed with either a blank (sesame oil 30 ml + dehydrated peanut butter) or E2B+dehydrated peanut butter mixture QOD (Yasrebi, Rivera et al. 2017). We have not

previously observed any feeding differences resulting from the ~40mg dehydrated peanut butter administration. The BioDAQ is a comprehensive system designed to monitor and analyze meal patterns and feeding behavior in rodents. Through the BioDAQ system, it is possible to assess real time meal number, meal duration, and meal size. Upon loading the animals into their respective BioDAQ cages, all animals were initially given a controlled LFD (D12450H, 10% kcal fat, Research diets) and allowed an additional three days of further habituation prior to any data collection. If necessary, any animal handling was done between 1000-1400 hours. All animals LFD meal patters were monitored and recorded for 96 h. Immediately following, the animals were fed HFD (D12451, 45% kcal fat, Research diets) for 72 h. All animal chow was monitored daily; animals that exhibited excessive food chowing were given additional chow once chewed pellet mass was obtained. Animal mass was recorded at four different time points; 1) at the initial 1-week single housing habituation (post-op day 1 for OVX mice), 2) at the initial start of their LFD exposure, 3) upon initial HFD exposure, 4) upon HFD completion.

2.2.6: Experiment 1c:

Individually housed adult male and female ERα WT/KIKO/KO littermates were exposed to a 24 h fast and allowed a 24 h LFD refeed under continuous FI monitoring in the BioDAQ monitoring system. Females were OVX one week prior to fasting/refeed window exposure. OVX females were subcutaneously injected with a low dose (250 ng) of E2B 24 hours prior to fasting and a high dose of E2B (1.5 ug) at the time of fasting (Yang, Mamounis et al. 2016). All animal handling was done between 1700-1800 h. All injections and subsequent chow modifications (fasting/granting food access) were done upon completion of any necessary handling. To aid in BioDAQ habituation, all animals

were housed in the BioDAQ during the time of fast and allowed food access immediately 24 h afterwards with standard light-dark cycle.

2.2.7: Experiment 2:

Experimental design

Following their BioDAQ run, the females were group housed and allowed to recover for two weeks prior to any behavioral test administration. The WT males were left singly housed due to territorial conflict between adult males, while the ERKO and KIKO males were returned to group housing for two weeks prior to behavioral testing. All animals were moved into the behavioral testing room 24 h prior to exposure of either stress or depression test. Prior to the start of either chamber test, the main room lights were shut off and the testing arena was slightly illuminated with a white-60 watt incandescent bulb. The animals were exposed to Open Field Maze (10 min), Elevated Plus maze (5 min), and lastly a Forced Swim Test (6 min) with 1-week intermission in between tests; all behavior tests were recorded for future scoring. Upon completion all males were sacrificed while all intact females were allowed two weeks recovery time prior to OVX. Post-OVX the females were housed individually and orally dosed oil or E2B every other day indefinitely (dosing was administered on a weight specific basis). After their use for Aim 1 BioDAQ testing, OVX females were tested on all three behavioral tests on days corresponding to their scheduled dosing. Upon FST completion all oil/E2 females were sacrificed and blood was collected for serum estradiol analysis. All three behavior tests were scored via the Hindsight (version 1.3) time-sampling computer software using three blinded viewers where each viewer watched recordings of each test and appropriately scored based off of predetermined criteria (Verpeut, DiCicco-

Bloom et al. 2016). Each zone was scored for number of frequencies entered (with entrance being defined as two paws and half torso crossings) and length of exploration.

Open field test

Mice were gently placed into the middle of a 100 cm X 100 cm black acrylic 5 sided box. The floor of the box was partitioned into 10 x 10 cm squares with the middle 8 x 8 boxes outlined in a slightly bolder line representing the middle portion of the box. The animals were allowed 10 uninterrupted minutes to explore the novel space; their movements were recorded from above for future analysis. The apparatus was sanitized prior and post every new run. Upon analysis the box was broken down into two scoring zones; Outer-most perimeter and center inner square.

Elevated plus maze

Mice were placed into the middle most portion of a raised platform containing adjacent open and closed 50 cm arms. Each animal was allowed 5 min to freely explore the novel plane while being recorded from above for future analysis. Upon analysis, the EPM was broken into two differen scoring zones - closed arm, or open arm. The apparatus was sanitized prior to every new run.

Forced swim test

Mice were dropped into a 23 cm diameter tank containing 15 cm deep 24-30 °C tap water. Animals were allowed a total of six min to explore their tank space prior to completion. Trials were cut prematurely short if animals exhibited signs indicating excessive struggling or sinking. Upon completion, animals were retrieved, thoroughly

dried and allowed to recover in a recovery cage placed directly on top of a heating pad prior to being returned to their home cage (Can, Dao et al. 2012). The apparatus was rinsed thoroughly and filled with fresh tap water prior to every new run. The apparatus was sanitized both before and after the initial FST exposure.

2.2.8: Statistical Analysis

Analytical comparison between treatment groups will be made using a two-way ANOVA with *post-hoc* (Newman-Keuls) paired analysis. Outliers were identified and excluded using the Grubbs statistical analysis test. All statistical analysis were performed using Graphpad software with a significance set to $\alpha < 0.05$. Data will be analyzed using the appropriate ANOVA with post-hoc analysis and tests for normality and distribution ($\alpha < 0.05$). (Yasrebi, Rivera et al. 2017)Investigators for the behavioral measurements will be blind to the treatments, and order of animals will be randomly assigned.

Results

Adult mice (>3 months) were acclimated to the BioDAQ food monitoring room for one full week. After acclimation, mice were placed into the individual BioDAQ cages and allowed to acclimate an additional three days on a low-fat diet (LFD). Data was collected for four consecutive days on a LFD followed by three days on a high-fat diet (HFD). Mice were removed from the BioDAQ chamber, fed a standard chow for two weeks, and exposed to the three behavior tests with one-week interval between each test. The animals were first exposed to a Open Field Test (OFT) chamber (measuring 100 cm x 100 cm) for 10 full min, then an Elevated Plus Maze (EPM) exposure (each arm measuring 50 cm) for five min, and finally a Forced Swim Test (FST) chamber (a 24cm diameter chamber was

filled with 13 cm water ranging from 24-30°C) for a total of six min, the first two minutes of which were attributed towards acclimation and not analyzed. Upon completion of the FST, males were euthanized for blood collection. All intact females were given a two-week rest, followed by ovariectomy (OVX), and a second pass through the BioDAQ and each behavior test. All OVX females were orally dosed on a bi-daily basis with either oil or EB (300 μg/kg). Dosing was scheduled in a manner where each female was dosed twice per each diet treatment in the BioDAQ and dosed on the morning prior to any behavior testing. Data was analyzed by a two-way ANOVA with Newman-Keuls multiple comparison tests.

Experiment 1a: The effects of sex, genotype, and steroid on feeding behaviors on a LFD

Body weight gain was affected by sex on a low-fat diet (LFD) after seven days ($F(1, 75) = 4.080$, $P<0.05$). WT, KIKO, and KO males lost body mass where females did not (Fig. 2A). On a high-fat diet (HFD; Fig. 2B), body weight was affected by sex ($F(1, 75) = 4.945$, $P<0.05$) and an interaction of genotype and sex ($F(2, 75) = 3.278$, $P<0.05$). WT, KIKO, and KO males all gained similar body mass while in the intact females, KO females gained more body mass than both WT ($P<0.05$) and KIKO ($P<0.01$). Furthermore, WT females gained less than their WT male counterparts ($P<0.05$). When OVX female mice were fed a LFD, body weight was affected by genotype ($F(2, 57) = 7.303$, $P<0.01$) and an interaction of steroid and genotype ($F(2, 57) = 4.532$, $P<0.05$; Fig. 2C). Oil-treated WT females gained significantly more body weight than oil-treated KIKO and KO with oil-treated KIKO and KO females not gaining in body weight. E2-treated WT and KIKO females

gained more body weight on LFD than E2-treated KO females. All OVX groups gained body weight when placed on a HFD, regardless of steroid condition, although oil-treated females did gain more than E2-treated ($F(1, 57) = 4.415$, $P<0.05$; Fig. 2D).

During the last 96 h of recorded LFD ingestion, total food ingested was dependent on genotype ($F(2, 74) = 13.52$, $P<0.0001$) and an interaction of genotype and sex ($F(2, 74) = 5.032$, $P<0.01$) in intact males and females (Fig. 3A). WT males ingested more than KIKO ($P<0.01$) or KO ($P<0.001$); respectively. Intact KIKO consumed less than both WT ($P<0.01$) and KO ($P<0.05$) females. In Fig. 3B, total food ingested was also dependent on genotype ($F(92, 56) =11.01$, $P<0.0001$). Oil-treated WT females ingested more than KIKO ($P<0.01$) and KO ($P<0.01$) and E2-treated OVX WT females consumed more than their KO counterparts ($P<0.05$).

During the last 96 h, total LFD ingestion in intact males was dependent on genotype ($F(2, 117) = 23.55$, $P<0.0001$) and an interaction of genotype and day ($F(6, 117) = 2.385$, $P<0.05$; Fig. 4A). Furthermore, intact WT males consistently ingested more than their KIKO (day 3: $P<0.001$; day 4: $P<0.05$) and KO (day 1: $P<0.0001$; day 2: $P<0.01$; day 3: $P<0.001$) counterparts. KIKO males also consumed more on day 1 than KO males ($P<0.01$). For intact females, total LFD ingestion was dependent only on genotype ($F(2, 179) = 11.27$, $P<0.0001$; Fig. 4B). WT and KO females ingested more than KIKO on day 1 (WT: $P<0.001$; KO: $P<0.01$) and day 2 (WT: $P<0.05$; KO: $P<0.05$). KO females continued to ingest more than KIKO females on day 4 ($P<0.05$). In oil-treated, OVX females, total LFD ingestion was dependent on genotype ($F(2, 100) = 13.31$, $P<0.0001$) and an interaction of genotype and day ($F(6, 100) = 2.251$, $P<0.05$; Fig. 4C). WT females ingested more than KO on day 1 and day 2 ($P<0.01$) and more than

KIKO on day 2 ($P<0.001$). In E2-treated, OVX females, total LFD ingestion was dependent only on genotype ($F(2, 123) = 12.31$, $P<0.0001$; Fig. 4D). WT females ingested more than KO on day 1 ($P<0.001$), day 2 ($P<0.05$), and day 3 ($P<0.05$) and KIKO on day 3 ($P<0.05$). KIKO ingested more than KO on day 1 ($P<0.05$). During the 96 h LFD feeding, there was no consistent effect of E2 on total ingested food except in WT females on day 2 ($P<0.05$; Fig. 5).

We further analyzed LFD consumption on an hourly basis. Intact males of all genotypes exhibited a similar diurnal meal pattern although during the nighttime hours when WT males consumed more food than both KIKO and KO (Fig. 6A). Hourly food intake was dependent on time ($F(23, 694) = 23.75$, $P<0.0001$), genotype ($F(2, 694) = 8.298$, $P<0.001$), and an interaction of genotype and time ($F(46, 694) = 3.227$, $P<0.0001$). The average food intake across all three genotypes during lights-on was less than 0.05 g/h. However, after lights-out, all genotypes increased food intake to 0.1-0.4 g/h. Similar to their male counterparts, intact females exhibited minimal average food intake difference between 700-1900 h and an increase in food intake at lights-out (Fig. 6B). WT females ingested more chow than KIKO and KO during the nighttime, which was dependent on time ($F(23, 694) = 23.75$, $P<0.0001$), genotype ($F(2, 694) = 8.298$, $P<0.001$), and an interaction of genotype and time ($F(46, 694) = 3.227$, $P<0.0001$). When oil-treated, OVX females were fed a LFD, both WT and KIKO females had a noticeable shift in food intake when compared to their intact states (Fig. 6C) with effects of time ($F(23, 600) = 9.880$, $P<0.0001$), genotype ($F(2, 600) = 4.400$, $P<0.05$), and an interaction of genotype and time ($F(46, 600) = 2.180$, $P<0.0001$). Although the females exhibited a period of time with minimal food intake, the duration was much shorter than observed in

intact females. For example, WT females exhibited a switch to a crepuscular circadian rhythm of food ingestion, with a higher rate of food intake during the early morning and early evening hours that was not sustained during the nighttime. E2-treated, OVX females exhibited a more exaggerated alteration of their food intake compared to their oil-treated counterparts (Fig. 6D). All three genotypes increased food intake immediately at lights-out and directly before lights-on with effects of time ($F(23, 744) = 13.48$, $P<0.0001$), genotype ($F(2, 744) = 3.550$, $P<0.05$), and an interaction of genotype and time ($F(46, 744) = 3.162$, $P<0.0001$). WT females, both oil- and E2-treated, ingested more than their KIKO and KO counterparts during the nighttime.

When comparing across all WT female groups, hourly food intake was dependent on time ($F(23, 1176) = 41.49$, $P<0.0001$), steroid ($F(2,1176) = 5.949$, $P<0.01$), and a steroid and time interaction ($F(46, 1176) = 2.314$, $P<0.0001$). Oil-treated, WT females ingested more ($P<0.05$) than intact WT females (Fig. 7A). There was no specific hourly difference in KIKO females, although hourly food ingestion was altered by time ($F(23, 480) = 8.415$, $P<0.0001$), steroid ($F(2, 480) = 3.562$, $P<0.05$), and a steroid and time interaction ($F(46, 480) = 1.416$, $P<0.05$) effect (Fig. 7B). In KO females, intact and oil-treated females differed at 2100 h ($P<0.01$), Fig. 7C) with only an effect of time ($F(23, 768) = 11.52$, $P<0.0001$). Additionally, we observed in KIKO females a crepuscular peak similar to their WT counterparts; however, this pattern was not observed in KO females.

During 96 h of recorded LFD administration, no significant differences in total number of meals was observed in either intact male and female groups. (Fig. 8A). However, in OVX females, meal number was altered by genotype ($F (2, 55) = 18.85$,

P<0.0001) steroid (F (1, 55) = 10.68, P<0.01) and an interaction of genotype and steroid (F (2, 55) = 6.874, P<0.01; Fig. 8B). E2-treated, WT females consumed a higher number of meals than KIKO (P<0.0001) and KO (P<0.0001). Additionally, E2-treated, WT females ingested more than oil-treated, WT females (P<0.0001). In OVX females, meal size was impacted by an interaction of genotype and steroid (F (2, 54) = 4.071, P<0.0226; Fig. 8D). E2-treated, WT females consumed smaller meal sizes than their KIKO (P<0.05) and KO (P<0.05) and surprisingly their oil-treated, WT counterparts (P<0.001). An opposite pattern was observed in the meal frequency of the E2-treated females (Fig. 8F) in which genotype (F (2, 55) = 18.85, P<0.0001), steroid (F (1, 55) = 10.68, P< 0.01), and a genotype-steroid interaction (F (2, 55) = 6.874, P<0.01). E2-treated, WT females consumed meals at higher frequency than their E2-treated, KIKO (P<0.0001) and KO (P<0.0001) and their oil-treated, WT counterparts (P<0.0001). Interestingly, meal duration in intact mice were altered by genotype (F (2, 74) = 13.68, P<0.0001; Fig. 8G). Intact WT males consumed food for a longer duration than their KIKO littermates (P<0.01). Intact WT females had consumed food for a longer duration times than both KIKO (P<0.05) and KO (P<0.0001). There was no effect of genotype or steroid on meal duration in OVX females.

Experiment 1b: The effects of sex, genotype, and steroid on feeding behaviors on a HFD

After LFD ingestion, all mice were switched to HFD for 72 h. In intact mice, HFD consumption was dependent on genotype (F(2, 51) = 12.83, P<0.0001) and an interaction of genotype and sex (F(2,51) = 3.243, P<0.05; Fig. 9A). Intact WT males

ingested significantly more than KO ($P<0.05$) and KIKO ($P<0.001$) males respectively. For intact females, WT ($P<0.05$) and KO ($P<0.001$) females ingested significantly more HFD than their KIKO. For OVX females, HFD ingestion was dependent on genotype ($F(2,46) = 5.198$, $P<0.01$) and the interaction of genotype and steroid ($F(2,46) = 4.619$, $P<0.05$; Fig. 9B). Oil-treated KO ingested significantly less than their WT ($P<0.01$) and KIKO ($P<0.5$). This pattern was not observed in E2-treated OVX females.

HFD ingestion in intact males was dependent on genotype ($F(2, 68) = 11.26$, $P<0.0001$; Fig. 10A). Additionally, WT males consistently ingested more than their KIKO (day 1: $P<0.05$; Day 2: $P<0.05$; day 3: $P<0.05$) and KO male counterparts (day 3: $P<0.05$). Intact KO males ingested more than intact KIKO males ($P<0.05$) during the first day of HFD feeding (Fig. 10A). For intact females, total HFD ingestion as dependent on genotype ($F(2, 87) = 19.86$, $P<0.0001$) and day ($F(2, 87) = 5.027$, $P<0.01$; Fig. 10B). Intact KO females ingested more ($P<0.05$) than intact WT females during the first 24 h HFD. Intact KIKO females, however, ingested less than intact WT (day 2: $P<0.05$, day 3: $P<0.01$) and intact KO females (day 1: $P<0.05$; day 2: $P<0.001$, day 3: $P<0.001$) across all three days of HFD. Additionally, KIKO females ingested less on day 1 compared to day 3 ($P<0.01$), KO females ingested more on day 1 and 2 ($P<0.01$), than day 3 ($P<0.05$). Intact male and female groups had genotype ($F(2, 49) = 11.958$, $P<0.0001$) and day ($F(2, 98) = 7.2440$, $P<0.01$). In oil-treated, OVX females, total HFD ingestion was dependent on genotype ($F(2, 75) = 8.386$, $P<0.001$), day ($F(2, 75) = 15.28$, $P<0.0001$) and an interaction of genotype and day ($F(4, 75) = 6.557$, $P<0.0001$; Fig. 10C). Both WT and KIKO females ingested more on their first day of HFD administration ($P<0.001$) than KO counterparts. Furthermore, both WT and KIKO

groups ingested more on their first day than day 2 (WT: $P<0.01$, KIKO: $P<0.0001$) or day three (WT: $P<0.01$, KIKO: $P<0.0001$). In E2-treated, OVX females, total HFD ingestion was dependent on genotype ($F(2, 62) = 3.521$, $P<0.05$) and day ($F(2, 62) = 6.006$, $P<0.01$; Fig. 10D). KIKO females ingested more on day 1 than day 2 and day 3 ($P<0.05$). Similarly, KO females ingested more on day 1 than day 2 ($P<0.05$) and day 3 ($P<0.01$). Over the courses of 72 hours HFD data collection, the only consistent effect of E2 on total ingestion happened on day 1. Both WT and KIKO groups, E2 decreased HFD ingestion (WT: $P<0.05$, KIKO: $P<0.05$). E2-treated KO females ingested more ($P<0.05$) than their oil counterparts (Fig. 11). Overall, there were genotype ($F(2, 54) = 14.731$, $P<0.0001$), day ($F(3, 162) = 3.2958$, $P<0.05$), and day and genotype interaction ($F(6, 162) = 5.1734$, $P<0.0001$) effects.

Hourly HFD intake was dependent on hour ($F(23, 624) = 11.30$, $P<0.0001$), genotype ($F(2, 624) = 5.019$, $P<0.01$) and interaction of genotype and time ($F(46, 624) = 1.812$, $P<0.01$). The average food intake across all three genotypes during lights-on was less than 0.1 g/h, increasing to 0.2-0.3g/h after lights-out with WT males continuing to have a higher rate of ingestion than their KIKO counterparts throughout the nighttime (Fig. 12A). With the exception of a peak at lights on (700-900), intact females exhibited minimal average food intake differences between 700-1900h and an increase in food intake at lights-out (Fig. 12B). Hourly ingestion in intact females was dependent on genotype ($F(2, 840) = 5.662$, $P<0.01$), time ($F(23, 840) = 8.188$, $P<0.0001$) and an interaction of genotype and time ($F(46, 840) = 2.421$, $P<0.0001$). WT and KO females ingested more chow than KIKO littermates immediately after lights-off which continued through the night. When oil-treated, OVX females were switched from LFD to HFD, all

three genotypes saw a noticeable increase in FI when compared to their intact states (Fig. 12C) with effects of time ($F(23, 522) = 10.26$, $P<0.0001$) and an interaction of genotype and time ($F(46, 552) = 2.207$, $P<0.0001$). All three genotypes saw a sharp spike in FI at lights on (0700) and again at lights off (1900) with relatively continuous FI in between. KIKO females increased their HFD ingestion leading up to and tapering back down to low levels at lights off. E2-treated, OVX females exhibited sever fluctuations in FI when compared to oil-treated counterparts (Fig. 12D). All three genotypes saw increased food intake leading up to lights out with effects of time ($F(23, 552) = 5.468$, $P<0.0001$), genotype ($F(2, 552) = 13.66$, $P< 0.0001$) and an interaction of genotype and time ($F(46, 552) = 1.560$, $P<0.05$). Additionally, E2-treated, WT females maintaining a sustained elevated FI through lights off leading up to lights on.

When comparing across all WT female groups, hourly HFD intake was dependent on time ($F(23, 696) = 7.732$, $P<0.0001$), steroid ($F(2, 696) = 29.36$, $P<0.0001$), and steroid and time interaction ($F(46, 696) = 2.363$, $P<0.0001$; Fig. 13A). E2-treated WT females ingested more ($P<0.05$) at lights off than their counterparts. KIKO females exhibited hourly food intake that was altered by time ($F(23, 504) = 4.302$, $P<0.0001$), steroid ($F(2, 504) = 27.44$, $P<0.0001$) and steroid and time interaction ($F(46, 504) = 1.674$, $P<0.01$; Fig. 13B). All three treatments saw an increase in food ingestion at lights-on that continued for several hours with E2-treated females continuing the longest. Similar to WT females, E2-treated KIKO females had an initial higher rate of ingestion at lights off. KO females experienced a substantial FI spike at lights off but returned to lower consistent levels leading to lights on (0700) with effects of time ($F(23, 744) = 13.79$, $P<0.0001$) and steroid and time interaction ($F(46, 744) = 3.510$,

P<0.0001; Fig. 13C). Overall KO females had a subdued eating pattern when compared to their genotypic littermates with the only deviations occurring directly after lights off when steroid treatment groups ingested more HFD than intact counterpart.

During recorded HFD feeding, intact WT males ingested a larger number of meals (P<0.01) than KIKO and KO counterparts (genotype: F (2, 53) = 14.95, P<0.0001). Intact WT females ingested larger number of meals than KIKO (P<0.001) and KO (P<0.05) counterparts while KIKO females had less numbers of meals than KO (P<0.05) littermates (Fig. 14A). In OVX females, meal number was altered by genotype (F (2, 43) =21.13, P<0.0001), steroid (F(1, 43) = 25.50, P< 0.0001), and a genotype*steroid interaction (F(2, 43) = 7.430, P = 0.01; Fig. 14B). Oil-treated KIKO females had fewer number of meals than both oil-treated KO females (P<0.05) and oil-treated WT females (P<0.001; Fig. 14B). Interestingly, E2-treated, WT females had higher number of meals than both KIKO and KO females (P<0.0001). Meal size in intact mice was altered by genotype (F (2, 61) = 3.948, P<0.05) and sex (F (1, 61) = 6.552, P<0.05; Fig. 14C). Intact WT females consumed larger meal sizes than both intact KO females (P<0.01) and intact KIKO females (P<0.05). Average meal sizes in OVX females were dependent on genotype (F (2, 44) = 3.311, P<0.05) and steroid (F (1,44) = 6.276, P< 0.05; Fig. 14D). E2-treated, WT females consumed smaller meal sizes than E2-treated KIKO and KO females (P<0.05). Intact HFD meal frequency was altered by genotype (F (2, 62) = 20.84, P<0.0001; Fig. 14E). Intact WT males exhibited higher meal frequency (P<0.05) than KIKO and KO littermates. Intact WT females exhibited a higher meal frequency than KIKO and KO littermates (P<0.0001). Additionally, intact KIKO females consumed HFD less frequently than intact KO female littermates (P<0.05). In

OVX females, meal frequency was dependent on genotype ($F(2,45) = 14.03$, $P<0.0001$), steroid ($F(1,45) = 16.44$, $P<0.001$) and a genotype*steroid interaction ($F(2, 45) = 3.743$, $P<0.05$; Fig. 14F). E2-treated, WT females had higher meal frequencies than their KIKO ($P<0.0001$) and KO ($P<0.0001$) counterparts. (Fig. 14F). Meal seconds in intact mice was altered by genotype ($F(2,61) = 7.175$, $P<0.01$, Fig. 14G). Intact WT females had a higher meal seconds average than KO counterparts ($P<0.05$). In OVX females, meal seconds was dependent on steroid treatment ($F(1, 45) = 6.159$, $P<0.05$; Fig. 14H) with no other observable differences.

Experiment 1b.2.: The effects of sex, genotype, and steroid on feeding behaviors attributed with the first HFD meal

After 96 hours of LFD, all mice were switched to a HFD for the following 72 hours. Fig. 15-17 are reflective of the initial 24 hours post diet change. No significant differences were detected within intact males or females first meal FI (Fig. 15A). First meal food intake was dependent on genotype ($F(2,49) = 4.402$, $P<0.05$) and the interaction of steroid and genotype ($F(2, 49) = 4.415$, $P<0.05$; Fig. 15B). Oil-treated, KIKO females consumed more food than oil-treated WT ($P<0.05$) and KO ($P<0.001$) littermates and E2-treated, KIKO females ($P<0.05$). The only observed differences in first meal duration within intact animals were a longer first meal duration in WT females than WT males (Fig. 15C). No significant differences in meal duration were observed in first meal duration within steroid treated groups however a genotype-treatment interaction was detected ($F(2,46) = 3.343$, $P<0.05$; Fig. 15D).

Latency to HFD FI was dependent on sex ($F(1,64) = 4.601$, $P<0.05$) and the interaction of sex and genotype ($F(2,64) = 3.693$, $P<0.05$) within intact males and

females. (Fig. 16A). Intact KO females had a longer latency to FI than intact WT females ($P<0.01$), intact KIKO females ($P<0.01$), and intact KO males ($P<0.01$). Latency to food intake in OVX females was dependent on steroid ($F(2,47) = 6.462$, $P<0.01$) and an interaction of steroid and genotype ($F(2,47) = 5.876$, $P<0.01$; Fig. 16B). Oil-treated, KO females had a longer latency to Fi than oil-treated, WT females ($P<0.01$), oil-treated, KIKO females ($P<.0001$) and E2-treated, KO females ($P<0.05$). Additionally, E2-treated, KIKO females had a longer latency to FI than both E2-treated, WT females and oil-treated, KIKO females ($P<0.05$). Meal rate in intact animals was dependent on the interaction of sex and genotype ($F(2,64) = 4.585$, $P<0.05$; Fig. 16C). Intact KO females had a higher meal rate than WT ($P<0.01$) and KIKO ($P<0.01$) littermates. No differences were detected in meal rate with in OVX females (Fig. 16D). Inter-meal interval time differences were dependent on sex ($F(1,65) = 8.287$, $P<0.01$; Fig. 16E) in intact animals and steroid ($F(1,49)=4.085$, $P<0.05$; Fig. 16F) in OVX animals.

Hourly food intake in intact males was dependent on time ($F(23, 600) = 3.876$, $P<0.0001$; Fig. 17A). Overall, intact KIKO males ingested less than WT and KO males just prior to lights on ($P<0.05$). Conversely the trend was flipped a few hours into lights on when KIKO males experienced an increased FI ingesting more than WT and KO males ($P<0.05$). Similar to when compared to their overall HFD eating patters, intact females exhibited a slightly subdued eating pattern when compared to their male counterparts. HFD FI was dependent on time ($F(23, 840) = 3.702$, $P<0.0001$) and interaction of genotype and time ($F(46, 840) = 1.479$, $P<0.05$; Fig. 17B). Overall oil-treated animals had similar FI patters during their first 24 hours of HFD administration as they did during the entire 72-h HFD period with hourly FI depending on genotype (F

(2, 528) = 6.168, P<0.01), time (F(23, 528) = 5.052, P<0.0001) and genotype and time interaction (F(46, 528) = 1.869, P<0.001; Fig. 17C). WT and KIKO animals had higher HFD intake fluctuations when compared to oil-treated KO females (P<0.05) with separations at 1200,17-2000 hours. E2-treated females hourly FI was dependent on time (F(23, 552) = 5.166, P<0.0001; Fig. 17D). E2-treated females had a slightly subdued HFD FI patters at lights off during their initial 24-h exposure than when compared to the remaining 72 hours on HFD. Compared to their oil-treated counterparts E2-treated females had a more consistent rate of ingestion in between genotypes leading to a tighter sigmoidal curve (WT and KIKO separations of P<0.05 occurred at 1600).

When comparing across all WT female groups, the first 24 h HFD intact was affected by time (F(23, 672) = 4.035, P<0.0001; Fig. 18A). Both oil and E2-treated WT females had a similar overall FI ingestion pattern (excluding the 1200 outlier time point). Intact WT females exhibited a slightly subdued FI pattern at lights on and conversely had a slightly higher rate of FI immediately after lights off. In KIKO females, FI in the first 24 h on a HFD intact was affected by steroid (F(2, 504) = 6.853, P<0.0001), time (F(23, 504) = 4.154, P<0.0001) and interaction of steroid and time (F (46, 504) = 2.218, P<0.0001; Fig. 18B). Overall. KIKO females exhibited a crepuscular pattern of FI with increased FI immediately after lights on (0.4g), subsiding mid day (0.1-0.2g), and again increasing (0.3-0.6g) leading to lights off. KIKO females had higher continual rates of HFD ingestion leading to lights off both their WT and KO counterparts. Hourly HFD FI in KO females was dependent on steroid (F(2, 744) = 3.389, P<0.05), time (F(23, 744) = 6.070, P<0.0001) and interaction of steroid and time (F(46, 744) =

2.031, P<0.0001; Fig. 18C). Interestingly, E2-treated KO females had an increase in FI immediately after lights on (consistent with both E2 WT and KIKO treated groups).

Experiment 1c: The effects of sex, genotype, and steroid on feeding behaviors after a 24 h fast

Individually housed adult (>3 months) male and female ERα WT/KIKO/KO littermates were administered a 24 hour fast and allowed a 24 hour LFD refeed under continuous FI monitoring in the Biodaq monitoring system. All intact animals were relocated into the Biodaq room six days prior to the 24 hour fast and subsequent refeed period. OVX mice were relocated immediately post-op (day zero) and allowed to recover in the Biodaq room. The OVX animals were split into either an oil or E2 treated group. At day six the animals were administered the first of two IP injections, either a sesame oil blank, or a 0.25 ng E2B dose. The animals were injected with either a second sesame oil blank or a 1.5 ng E2B dose on day seven. All injections occurred at 1800 hours. The animals were fasted for 24 hours immediately post-injection and allowed a Biodaq monitored 24 hour LFD refeed the following day at 1800. Upon refeed completion all OVX animals were euthanized for blood and uterus collected.

When fasted for 24 h, intact animals body weigh loss was affected by sex ($F(1, 69) = 24.21$, $P<0.0001$), and genotype ($F(2, 69) = 12.11$, $P<0.0001$; Fig. 20A). Although all genotypes experienced weigh loss across both sexes, KIKO males and females lost less BW than WT counterparts (males: $P<0.0001$, female: $P<0.05$). Additionally, KIKO males lost less than KO males ($P<0.0001$). WT and KO females lost less BW than respective males (WT: $P<0.05$, KO: $P<0.0001$). Furthermore, BW loss within OVX

females was affected by genotype ($F(2, 54) = 9.753$, $P<0.001$) and treatment ($F(1, 54) = 9.968$, $P<0.01$; Fig. 20B). E2-treated, WT females lost more BW than KIKO ($P<0.01$) and KO ($P<0.0001$) and oil-treated WT ($P<0.0001$). When allowed to refeed on standard chow for 24 h, there was no difference in BW fluctuations across sexes or genotypes (Fig. 20C). BW gain was affected by genotype ($F(2, 54) = 5.300$, $P = 0.0079$; Fig. 20D) in OVX females. E2-treated, WT females gained more BW than KIKO and KO counterparts ($P<0.05$).

During the 24 h chow refeed, total food ingested in intact mice was dependent on genotype ($F(2, 69) = 3.788$, $P<0.05$) and sex ($F(1, 69) = 4.951$, $P<0.05$; Fig. 21A). Intact WT females ingested more than KIKO and KO littermates ($P<0.01$) and intact males ($P<0.001$) counterparts. Total chow ingested in OVX females was dependent on steroid ($F(2, 53) = 8.843$, $P<0.001$; Fig. 21B). Upon refeed both oil- and E2-treated, KIKO females ingested less than WT counterparts ($P<0.05$). Furthermore, E2-treated, KO females also ingested less than E2-treated, WT females ($P<0.01$).

Upon LFD refeed intact males did not exhibit the same severity to their sigmoidal pattern observed when intact males were on a non-refeed LFD regiment. LFD refeed FI in intact males was dependent on time ($F(23, 984) = 11.10$, $P<0.0001$; Fig. 22A). Males had continual chow intake (0.1-0.2g) throughout lights off hours with a gradual tapering down leading to lights on. Refeed FI in intact females was dependent on genotype ($F(2, 648) = 22.33$, $P<0.0001$), time ($F(23, 648) = 12.78$, $P<0.0001$) and interaction between genotype and time ($F(46, 648) = 1.452$, $P<0.05$; Fig. 22B). Intact WT females exhibited higher initial refeed FI than both KIKO and KO littermates for several hours after initial lights off. All OVX oil-treated groups maintained

a consistently elevated level of FI during lights off when compared to non-refeed LFD groups (Fig. 22C) Oil-treated females feeding was dependent on genotype ($F(2, 648) = 4.760$, $P < 0.01$) and time ($F(23, 648) = 13.05$, $P<0.0001$). In E2-treated females, there was an effect of genotype ($F(2, 624) = 9.218$, $P<0.0001$) and time ($F(23, 624) = 12.94$, $P<0.0001$) on hourly FI after a 24-h fast (Fig. 22D). E2-treated females saw a separation between WT and KO females ($P<0.05$) and KIKO and KO females ($P<0.05$) during the lights off hours. FI peaked at the onset of initial LFD access (0.4 g) and slowly decreased throughout lights off eventually subsiding during the lights on hours. Consistent with continual LFD administration, E2-treated females again began FI around 1600.

In WT females, refeed FI was dependent on time ($F(23, 696) = 16.78$, $P<0.0001$; Fig. 23A). When re-fed WT females were compared across treatments, oil-treated females exhibited a delayed response to the timing of FI compared to both intact and E2 females ($P<0.05$) leading up to lights off (16 h), which contrary to when animals are kept on a LFD (Fig. 7). In re-fed KIKO females, hourly LFD FI was dependent on time ($F(23, 648) = 14.76$, $P<0.0001$), treatment ($F(2, 648) = 11.80$, $P<0.0001$), and interaction of time and treatment ($F(46, 648) = 1.567$, $P<0.05$; Fig. 23B) effects. KIKO females exhibited feeding differences immediately upon refeed, between all three groups at hours 1800 and 1900. Hourly food intake in KO females was dependent on time ($F(23, 576) = 7.611$, $P<0.0001$) and treatment ($F(2, 576) = 8.093$, $P<0.001$; Fig. 23C). Oil-treated, KO females separated from intact and E2-treated, females ($P<0.05$) throughout the lights off hours and returning to a WT-like pattern during lights on hours.

No differences in total number of meals were observed in either intact mice (Fig. 24A) or OVX females (Fig. 24B). Overall, meal size was affected by sex ($F(1, 68) = 5.750$, $P<0.05$), genotype ($F(2, 68) = 6.707$, $P<0.01$), and interaction of sex and genotype ($F(2, 68) = 5.609$, $P<0.01$); Fig. 24C). Meal size in intact males was stable across all genotypes. However, in intact females, WT females consumed larger meal than KIKO ($P<0.01$), KO ($P<0.001$) littermates and intact WT males ($P<0.001$). Meal size in OVX females was dependent on steroid ($F(2, 53) = 12.43$, $P<0.0001$; Fig. 24D). There were no differences in meal frequency in intact or OVX groups. Oil- and E2-treated, WT females had significantly larger meal sizes than their KIKO (oil: $P<0.01$, E2: $P<0.01$) and KO (oil: $P<0.01$, E2: $P<0.01$) counterparts. Intact mice experienced differences in meal duration dependent on sex ($F(1, 67) = 4.536$, $P<0.05$) and genotype ($F(2, 67) = 5.576$, $P<0.01$; Fig. 24G). Intact WT females exhibited longer meal durations than their KIKO ($P<0.05$) or KO ($P<0.05$) littermates and intact WT males ingested less than intact WT female ($P<0.05$). Meal duration of OVX females was dependent on steroid ($F(2, 52) = 6.548$, $P<0.01$; Fig. 24H). E2-treated WT females exhibited longer LFD meal duration times than either E2-treated KIKO ($P<0.01$) or KO ($P<0.05$) treated counterparts.

In intact mice, post refeed FI was dependent on sex ($F(2,67) = 6.380$, $P<0.01$; Fig. 25A). Intact WT males consumed less than intact WT females ($P<0.001$). Intact WT females also displayed a higher FI than their KIKO ($P<0.05$) or KO ($P<0.01$) littermates. In OVX females, first meal FI was dependent on genotype ($F(2,52) = 5.535$, $P<0.01$; Fig. 25B). Oil-treated, WT females exhibited a greater FI than their KIKO counterparts ($P<0.05$). Within intact groups meal duration was dependent on sex ($F(1,65)$

= 7.840, P<0.01) and the interaction of sex and genotype (F(2,65) = 3.555, P<0.05; Fig. 25C). Intact WT females maintained longer first meal duration than their KIKO (P<0.05) and KO (P<0.05) littermates and intact WT males (P<0.001). There was no effect of steroid or genotype on duration of first meal in OVX females (Fig. 25D). No differences were detected in latency to first meal FI in either intact mice (Fig. 26A) or OVX females (Fig. 26B). In OVX females, meal rate was dependent on genotype (F(2,52) = 4.798, P<0.05; Fig. 26D). Oil-treated, WT females had a higher first meal rate their KIKO counterparts (P<0.01).

Experiment 2: The role of sex, genotype, and steroid on anxiety- and depressive-like behaviors

The open field test (OFT) is split into two measuring areas, outer farthest most perimeter and center (the inner 25% of the chamber). When placed in an OFT, percent of exploration in the inner arena was dependent on sex (F(1, 90) = 20.66, P<0.0001; Fig 27A.) in intact mice. Intact KO females spent less time exploring the center than either intact WT (P<0.01) or KIKO (P<0.01) littermates. Additionally, intact WT and KIKO females spent longer exploring the center than their male WT (P<0.001) or KIKO (P<0.01) counterparts. The time spent exploring the center arena in OVX females was dependent on genotype (F(2, 57) = 3.579, P<0.05; Fig. 26B). E2-treated KO females explored less (P<0.05) than their E2-treated WT littermates. Intact KO females had a higher rate of outer perimeter exploration than both intact WT (P<0.001) or KIKO (P<0.001) littermates (Fig. 26C), both of which spent less time exploring the perimeter than their male counterparts (WT: P<0.001; KIKO: P<0.0001) leading to a sex-dependent effect dependent (F (1, 88) = 25.10, P<0.0001) and interaction of genotype and

sex ($F(2, 88) = 6.324$, $P = 0.0027$). Perimeter exploration was dependent on both steroid ($F(1, 57) = 10.79$, $P = 0.0017$) and genotype ($F(2, 57) = 5.297$, $P = 0.0078$; Fig. 27D) in OVX females. E2-treated, KO females spent more time in the perimeter of the OFT than both their WT ($P<0.01$) and KIKO ($P<0.05$) littermates. E2 treatment in KIKO females reduced perimeter field exploration compared to oil-treated KIKO ($P<0.05$).

Frequency of center arena entrance was dependent on sex ($F(1, 91) = 15.76$, $P<0.0001$; Fig. 28A) in intact mice. Intact females entered the center of the arena at a higher frequency than their KIKO and KO ($P<0.01$) littermates, respectively. Both intact WT and KIKO females had higher frequencies of entrance into the center than their male counterparts (WT: $P<0.001$; KIKO: $P<0.05$). Both oil- and E2-treated WT females had higher rates of entrance into center than their KIKO (oil: $P<0.01$; E2: $P<0.05$) and KO (oil: $P<0.01$; E2: $P<0.001$) littermates, respectively, resulting in a genotype effect ($F(2, 55) = 19.22$, $P<0.0001$; Fig. 28B). Outer perimeter exploration frequency within intact mice was dependent on both sex ($F(1, 90) = 6.416$, $P<0.05$) and interaction of sex and genotype ($F(2, 90) = 3.653$, $P<0.05$; Fig. 28C). Both intact WT and KIKO females entered the outer perimeter at higher frequency than their male counterparts ($P<0.05$). Additionally both WT and KIKO female groups also had higher rates of outer perimeter entrance than their KO littermates with WT females having more than KO ($P<0.05$). Frequency of perimeter entrances was dependent on genotype ($F(2, 55) = 27.63$, $P<0.0001$; Fig. 28D) in OVX females. Oil- and E2-treated, WT females had a higher rate of entrance than both their KIKO (oil: $P<0.001$; E2: $P<0.05$) and KO (oil: $P<0.0001$; E2: $P<0.001$). Additionally, KO

females entered the outer perimeter at a rate less than that of their KIKO littermates ($P<0.05$).

When placed into an elevated plus maze (EPM), open arm exploration was dependent on an overall interaction ($F(2, 88) = 6.731$, $P<0.01$; Fig. 29A) in intact mice. Intact WT females explored the open arms at a higher rate than both their KIKO ($P<0.01$) and KO ($P<0.001$) littermates. WT females also explored the open arm for longer than their intact WT male counterparts ($P<0.001$). Conversely, closed arm exploration was dependent on genotype ($F(2, 88) = 6.871$, $P<0.01$; Fig. 29C). Both intact KIKO and KO females explored the closed arm at a higher rate than intact WT (KIKO: $P<0.01$, KO: $P<0.001$) females. Additionally, WT females explored the closed arm less than their intact male counterparts ($P<0001$). Open and closed arm exploration was dependent on a genotype*interaction effect (open: $F(2, 61) = 14.73$, $P<0.0001$; Fig. 29B; closed: $(2, 61) = 15.66$, $P<0.0001$, Fig 29D) in OVX females. Both oil and E2-treated, WT and KIKO OVX females explored the open arm longer than KO littermates (oil- WT: $P<0.01$, KIKO: $P<0.01$; E2- WT: $P<0.001$, KIKO: $P<0.05$). The trend was reversed in closed arm exploration; both oil- and E2-treated KO females exhibited longer durations of closed arm exploration than their littermates (oil- WT: $P<0.01$, KIKO: $P<0.01$; E2- WT: $P<0.001$, KIKO: $P<0.05$; Fig. 29D).

In intact animals, open arm entrance frequency was dependent on sex ($F(1, 90) = 13.81$, $P<0.001$) and genotype-sex interaction ($F(2, 90) = 7.539$, $P<0.001$; Fig. 30A). Closed arm exploration frequency was dependent on sex ($F(1, 90) = 12.32$, $P<0.001$) and genotype-sex interaction ($F(2, 90) = 6.115$, $P<0.01$; Fig. 30C). Intact WT females had higher rates of open and closed arm entries than either of their intact KIKO

($P<0.05$) or KO ($P<0.0001$) littermates, respectively. KIKO females entered both the open and closed arm more frequently than intact KO ($P<0.05$) but less than intact WT littermates. Additionally, intact WT females also had a higher rate of entrance than intact WT males ($P<0.0001$). Open and closed arm exploration were dependent on a genotype (open: $F(2, 61) = 17.10$, $P<0.0001$; Fig. 30B; closed: ($F(2, 60) = 16.61$, $P<0.0001$; Fig. 30D) in OVX females. Both groups of oil- and E2-treated, KO females had lower rates of entries than either their WT ($P<0.001$) or KIKO ($P<0.01$) littermates, respectively.

The forced swim test (FST) was scored on three criteria, free float, passive paddle, and vigorous paddle. In intact animals, free floating was dependent on genotype ($F(2, 89) = 3.322$, $P<0.05$; Fig. 31A). Intact KO females remained in the state of free float longer than their WT littermates ($P<0.01$). Within steroid-treated mice, free floating was also dependent on a genotype effect ($F(2, 54) = 18.28$, $P<0.0001$; Fig. 31B). The initial trend seen within intact female mice was not only seen but also slightly exaggerated in E2-treated, OVX mice with both KIKO and KO mice remaining in free float longer than their WT counterparts (oil, KIKO: $P<0.05$, KO: $P<0.05$; E2, KIKO: $P<0.01$, KO: $P<0.0001$). In intact animals, the passive paddle state was dependent on a genotype effect ($F(2, 89) = 4.598$, $P<0.05$; Fig. 31C). Both intact WT and KO males remained in passive paddle for longer than their KIKO counterparts. Intact WT females remained in passive paddle longer ($P<0.5$) than their KO littermates. The passive paddle state in steroid-treated mice was dependent on a genotype ($F(2, 56) = 5.938$, $P<0.01$; Fig. 31D) effect. E2-treated, WT females remained in passive paddle for significantly longer ($P<0.05$) than their E2 treated counterparts. No differences in vigorous paddle

were seen within intact animals. However, steroid-treated females had a genotype effect ($F(2, 51) = 8.023$, $P< 0.001$; Fig. 31F). E2-treated, WT females remaining in a state of vigorous paddle for longer than either their KIKO ($P<0.05$) or KO ($P<0.05$) counterparts.

No effects were seen within intact animals entering the free float stage (Fig. 32A), the vigorous paddle stage (Fig. 32E) or steroid animals entering the passive paddle stage (Fig. 32D). Steroid treated animals entered the free float phase with a steroid effect ($F(1, 55) = 4.395$, $P<0.05$; Fig. 32B). In intact mice, the transition to passive paddle phase was dependent on a sex ($F(1, 89) = 5.382$, $P<0.05$; Fig. 32C). Intact KO females entered passive paddle less than both WT and KIKO ($P<0.05$) females. Additionally, intact KIKO females entered the passive paddle stage more frequently ($P<0.05$) than intact male counterparts. Lastly, vigorous paddle frequency in steroid-treated females was dependent on a genotype ($F(2, 55) = 4.426$, $P<0.05$; Fig 32F). E2-treated WT females entered vigorous paddle more frequently than E2-treated KIKO counterparts.

Discussion

Continuous Food intake, LFD

In humans, the sensations associated with hunger usually stop upon eating until satiated. Social and environmental cues aside, physiologically the feeling of satiety can rely on many factors ranging from micronutrients profile as well as hormones that control meal patterns (rate, size, frequency, and caloric intake duration) (Hirschberg 2012). The Arcuate neurons of the hypothalamus, play a key role in appetite and appetite regulation. The medial preoptic area, Arcuate neurons, and neurons of the dorsal raphe have all been

shown to contribute towards decreases in FI upon estradiol administration (Santollo, Torregrossa et al. 2011). With a robust population of NPY/AgRP and POMC neurons, projections from Arcuate innervate other key appetite regulating centers including the PVN, VMH, and LH. NPY/AgRP neurons exert oerexogenic effects, increasing appetite and decreasing energy expenditure. Co-expressing CART, POMC neurons exert anorexigenic effects, decreasing appetite and increasing energy expenditure). Additionally, the release of gut peptides (CCK, PYY) in conjunction with mechano-stretch receptor activation signal satiety to the brain. Released from white adipose cells, leptin also acts as a satiety signal on the brain. The interaction of gut peptides, neurotransmitters, and sex hormones are necessary for optimal homeostatic energy regulation and appetite control (Asarian and Geary 2006).

BW fluctuations

The weight gain seen in KO females when on HFD, and more specifically the lack of weight gain in KIKO females, recapitulate our previous findings where we have shown ERE-independent signaling was able to partially protect against DIO (Mamounis, Yang et al. 2014)**.** Estrogens protective traits were seen in intact WT females having gained less body mass than male counterparts. Furthermore, the observation of OVX animals weight fluctuations being higher in oil-treated WT than E2B-treated counterparts further shows estrogens protective qualities.

LFD Food intake/Meal measurements

Overall, both male and female WT animals have higher rates of FI with select genotype and sex specific differences. KIKO animals trended towards having

intermediate FI in between WT and KO animals regardless of treatment. Estradiol modulated meal pattern but total overall effects of FI remain the same. The same amount in total overall ingestion (no difference in between steroid treatment groups) but differences in meal pattern were observed. E2B-treated WT females had smaller meal sizes but higher compensatory total number of meals and meal frequency than E2B-treated KIKO/KO females and oil treated WT counterparts. Interestingly no effects were seen in meal duration within OVX groups.

The reduction of FI seen in intact WT females compared to WT males can be explained in part by the higher levels of circulating estrogen found in females. Estradiol (acting through ERα signaling) inhibits food intake by enhancing intestinal secretagogue CCK satiety (Asarian and Geary 2007). Estradiol did not capitulate the satiety effects of CCK in female ERKO mice (Geary, Asarian et al. 2001). Leptin sensitivity has been shown to increase in E2-treated female mice (Clegg, Brown et al. 2006). Furthermore, E2B-treated mice did not show an increase in FI potentially due to a diminished effect of ghrelin's orexogenic actions by estrogen (Clegg, Brown et al. 2007). This is potentially due to the expression of GHSR in KNDy neurons, which modulate the POMC-NPY/AgRP circuit to control energy balance (Conde et al., in progress). These differences potentially justify the increased meal size seen in KIKO and ERKO mice over WT. Additionally, it has been suggested estrogens effect on food intake regulation could be occurring through actions in both the hypothalamus and the NTS (Asarian and Geary 2006). Meal size, rather than number or frequency are influenced by the HPG axis (Asarian and Geary 2002). Estrogen has also been shown to augment the actions of

feedbacks pertaining to changer in secretion of glucagon (Geary and Asarian 2001) and ghrelin (Clegg, Brown et al. 2007).

Independent of CCK signaling, estradiol alone, acting on ERα and not ERβ, has been shown to decrease meal size (Geary 2001). E2B- treated OVX rats had decreased FI compared to vehicle treated rats (Santollo, Katzenellenbogen et al. 2010). Estradiol increases the activity of CCK satiation signaling pathway to decrease meal size and food intake at times of high estrogen associated ovarian cycle (estrous/ovulatory). The pathway of action is unknown but E2B-treated OVX rats had an increase of CCK induced c-FOS expression in hypothalamic brain regions highly expressing ERα (NTS, PNV, amygdala) suggesting ERα pathway involvement (Eckel and Geary 1999, Geary 2001, Eckel, Houpt et al. 2002). Our data suggests that these mechanisms are primarily through ERE-dependent signaling. Furthermore, when administered MPrP, an ERα antagnost, the food intake reducing actions of E2B-treated OVX and intact cycling rats were negated (Santollo, Katzenellenbogen et al. 2010). We predicted that food intake and meal patterns would be different in oil- and E2-treated WT females, but no effect of E2 on total LFD FI were observed, although genotype effects were seen in OVX females. These genotype effects indicate that the loss of ERE-dependent signaling during some stage of development or growth alters the central neurocircuits that control feeding.

We previously reported the ERKO phenotype being associated with decreased FI and whole-body physiological characteristics when compared to WT littermates (Mamounis, Yang et al. 2014). Further substantiating our claim, treatment of ERα agonist (PPT) has been shown to decrease FI in WT OVX mice but not in ERKO mice (Thammacharoen, Geary et al. 2009). We have shown that estradiol modulated meal

patterns but total effect of FI remains the same. Similar to what was previously shown, OVX ERKO mice did respond to E2 administration in food intake or meal patterns when compared to oil-treated. Contrary to the findings from many studies of E2 replacement in OVX WT rodents, we did not observe any estradiol-induced changes to FI or meal patterns (Richard 1986, Geary, Asarian et al. 2001, Toth, Poehlman et al. 2001).

Estrogen increases the activity of POMC neurons (Thornton, Loose et al. 1994, Pelletier, Li et al. 2007), while decreasing the activation and expression of NPY neurons (Pelletier, Li et al. 2007, Santollo and Eckel 2008). In fact, estrogen decrease NPY neurons orexigenic properties by affecting meal size but not overall meal number (Santollo and Eckel 2008). Estrogen inhibits NPY neurons and reduces their orexigenic properties through membrane-initiated ER mechanisms (Smith, Bosch et al. 2013). Therefore, ERE-independent ERα signaling in KIKO still retain ERα signaling which may be partially responsible for food intake differences seen between KIKO and KO animal models.

LFD Hourly intake patterns

Overall, intact WT males and females were measured as having higher rates of hourly FI than their counterparts during the lights off hours. Although they shared similar meal sizes, meal durations were longer than KIKO and KO (only in female) counterparts. One possible explanation is that KIKO and KO animals have more depressed nocturnal activity patterns than WT counterparts (Mamounis, Yang et al. 2014). We reported ERKO mice having lower metabolic activity (as measured by oxygen consumption) and

lower overall rates of FI. Therefore, the reduction in metabolism is offset by the lower caloric intake effect.

Interestingly, E2B administration modified meal patterns but the total overall effect of FI remains the same. OVX animals exhibited a pronounced crepuscular FI pattern than intact animals. Overall WT E2B-treated animals exhibited higher rates of FI during nocturnal hours. OVX oil WT and KIKO females ingested significantly higher LFD than comparable KO females. The trend was virtually reversed in E2B-treated females. Matching previous findings (Asarian and Geary 2002) when compared across treatments, OVX oil-treated WT mice exhibited a crepuscular trend where there were separations in FI from 0700 through 0900 corresponding to lights on (0700) and again leading up to lights off.

OVX E2B-treated WT decrease in meal size was counteracted with an increase in meal frequency and total number of meals. This suggests estrogen influences satiety signals either directly or indirectly, leading to lower food intake (Eckel 2004). Estrogen replacement corresponding with physiologically relevant cycling estrogen concentrations were shown to produce normal feeding and weight augmentation parameters in OVX rats (Asarian and Geary 2002). The earlier onset of FI in OVX animals compared to intact has been shown to be attributed to sex chromosome compliment interactions on food intake rhythms observed in gonadectomized animals (Chen, Wang et al. 2015).

A possible explanation for the shift in earlier FI seen in males could be a result of sex differences in circadian rhythm controlled by the suprachiasmatic nucleus (SCN) of the hypothalamus. Ovarian hormone influence on circadian rhythm is less clear (Sellix and Menaker 2010). The SCN does acts on the HPG to influence preovulatory LH surge

timing (de la Iglesia and Schwartz 2006, Kriegsfeld and Silver 2006). Due to the low overall expression of classical ER's within the SCN itself, the overall consensus is that estradiol does not directly act upon the SCN, but rather act on neurons that project to the SCN from ER-rich regions (De La Iglesia, Blaustein et al. 1999). For instance, estradiol has shown to change the peak expression time of *Per1* in SCN while biphasic patterns of gene expression were observed in uterine tissue. These findings suggest the reproductive clock and central clock can be differentially controlled by E2. (Nakamura, Moriya et al. 2005). Regardless, the loss of E2B in OVX females alters the timing of food intake, through unknown pathways in the hypothalamus.

Continuous Food intake, HFD

HFD Food intake/Meal measurements

Not surprisingly, mice are known to spontaneously choose more palatable (higher lipid and sucrose) foods over lesser (Takeda, Imaizumi et al. 2000). The mesolimbic dopamine system acting on hypothalamic regions of the midbrain and hindbrain (nucleus accumbens), both areas high in ERα expression, are heavily responsible for the reward system involved in the increased FI associated with pleasurable (non homeostatic) feeding (Hirschberg 2012). Additionally, estrogen acts on the NTS to decrease FI (Asarian and Geary 2007). The hypothalamus has a higher level of activation from high fat diet over low fat diet ingestion stimuli (Grabenhorst, Rolls et al. 2010). NPY has also shown to be innervated with increasing FI during exposure to highly palatable foods (Pandit, Luijendijk et al. 2014). Arcuate inflammation affecting NPY/POMC cells resulting from fat ingestion leads to leptin and insulin resistance development (De Souza,

Araujo et al. 2005, Enriori, Evans et al. 2007). Upon exposure to fatty foods, oral and small intestinal receptors signal the release of gut peptide (CCK, PPY, GLP-1) and leads to slower gastric emptying and overall suppression of FI (Little and Feinle-Bisset 2011).

Dietary fat ingestion has been shown to cause an inhibition of ghrelin release without any changes to blood glucose of insulin levels (Heath, Jones et al. 2004). The differences seen in average meal size/meal duration of HFD (compared to LFD) could in part be due to satiety factor of CCK/Leptin/GLP-1 secretion. GLP-1 released from NTS acts on the midbrain to reduce dopamine neuron excitability on the hypothalamus, VTA, and nucleus accumbens, thus causing a FI suppression (Gu, Roland et al. 2013, Wang, Liu et al. 2015).

The same overall treads seen when intact males and females were fed LFD were recapitulated once given HFD; intact WT males ingested more overall HFD than KIKO and KO counterparts whereas both intact WT and KO females ingested more overall HFD than their KIKO counterparts. Intact KO males have a higher FI than their KO counterparts upon initial administration but lose interest by the second day. Intact WT and KIKO females ingested less HFD than KO counterparts upon initial HFD administration. The trend was lost by the second day where intact KIKO females ingest less HFD than both WT and KO groups. Interestingly however, once ovariectomized, both oil WT and KIKO females ingested more overall HFD than oil WT females. No differences were seen in E2B treated animals. The stark differences can be seen much clearer by looking at the daily FI breakdown; both oil WT and KIKO females ingested much higher levels of HFD on day one before returning to similar levels for day two-

three. This suggests OVX KIKO females may have an increase in hedonic eating when in an E2-free physiological state.

Although no difference in average meal size or duration were seen within Intact male groups, WT males had a higher number of total meals and meal frequency than either KIKO/KO. The increases in total number of meals and meal duration with the lack of differences in meal size and duration suggest although WT males are getting satiated as the same rate as KIKO and KO males, their interest in hedonic eating seems to be stronger. Although no differences were seen in intact female meal patterns on LFD, on a HFD many meal pattern differences appear to become apparent. Intact WT females had smaller average meal sizes than their KIKO and KO counterparts. Additionally the smaller meal sizes were recapitulated by having an increased total number of meals, meal frequency, and meal duration than intact KO animals. By having smaller meal sizes but longer meal durations, WT females ingested HFD at a slower rate than their counterparts. Of note, KIKO females had less number of overall meals and meal frequency than both intact WT and KO females. This suggests although intact KO females are becoming satiated as quickly as KIKO females, they are returning and ingesting HFD at a higher frequency than KIKO animals. Interestingly, KO meal durations are not different than KIKO animals suggesting although the animals are becoming satiated at the same rate, they are exhibiting a higher level of interest in the chow and returning for more often. Oil-treated KIKO animals ingested HFD less often than oil-treated WT and KO counterparts. E2B-treated animal meal patterns on HFD were similar to when the animals were on a LFD; WT animals had smaller meal sizes than their KIKO and KO

counterparts. Additionally, E2B-treated WT animals had a higher number of meals and frequency than their counterparts as well as oil-treated WT females.

HFD Hourly intake patterns

WT males had an increase in FI leading up to lights off (17-1800) where it would plateau but remained elevated through the lights off cycle (22-0200). All three genotypes peaked and then settled down with KIKO/KO having lower rates of HFD FI than WT from 22-0200. The change in FI could possibly be due to lower levels of night-time activity reported in KIKO and KO animals (Mamounis, Yang et al. 2014). Intact females did not exhibit as high of a FI fluctuation leading up to lights off. KO females had a pattern of higher FI than KIKO females into lights off (1900-2200) with WT females ingesting higher HFD from 0100. This finding is consistent and supportive of KIKO females having lower overall rates of HFD ingestion.

Oil-treated animals had an overall sustained increase in HFD FI. All three genotypes began having increases in FI around 1700 and continued through 2100. Both KIKO and KO animals had a dramatic increase in FI prior to lights out at 1700-1800 tapering off and lowering at lights off. Oil-treated WT animals did not begin their drastic FI climb until lights off (1800-2000). WT and KIKO animals again had an increase in FI immediately after lights on (0700-0900). Unexpectedly, E2B-treated animals had very few points of diminished FI and the tight corpuscular curves seen in previous graphs are no longer defined. E2B-treated WT and KIKO animals had higher overall levels of FI than KO females, especially exaggerated in post lights off hours. When steroid-treated females were compared, oil-treated WT and KIKO had higher rates of FI than E2B

counterparts. Interestingly, the opposite was seen in KO animals. This data suggests E2B may act on ERE-independent mechanism to protect against the increase palatability of HFD by affecting gut peptides known to affect satiety.

First HFD meal intake

Hedonic feeding can be classified as eating out of pleasure rather than nutritional desire (Lowe and Butryn 2007). The parts of the brain associated with hedonic feeding, overlap with physiological components of the brain associated with depressive and addictive like phenotypes. In depressive phenotypes there is a dysregulation of serotonin. The dorsal raphe in the hindbrain (directly involved in hedonic eating) has direct serotonin projections and has been shown to have direct influence in latency to FI (Santollo, Torregrossa et al. 2011). Intact WT females had an increased meal duration compared to intact WT males. The difference in meal duration are lost in the same animals once they are OVX. This leads us to believe the effect may be due to an increase in organization effects, rather than activation effect. Not seeing differences between steroid treated animals is a sign of not having activation effects but rather potential developmental effects. The presence, or lack of presence of other ovarian hormones may also be a contributing factor. When analyzing latency to start of a palatable meal, the longer a latency to first meal is directly correlate with an increased measure of anhedonia. Conversely, a decreased latency to start implies a higher state of pleasure seeking and an increased measure of hedonia.

In intact WT females, the presence or absence of circulating ovarian hormones had no difference from male counterpart groups. Additionally, no hedonic effects of

steroid treatment were seen in WT steroid treated animals. These combined further contribute to effects within WT animals being organizational effects (Phoenix, Goy et al. 1959, Arnold 2009). Intact KO females had a longer latency to FI than all other intact female and intact KO male counterparts. The lack of functional ERα receptor within the KO females lead to an increased rate of anhedonic behavior. Once the animals began FI they ingested HFD at a rate faster than intact female counterparts, suggesting a biological drive to eat quickly rather than eating for pleasure. However, when the animals were OVX E2B treated, the protective nature of estrogen seems to be partially contributing against anhedonic behaviors within the KO as seen by the decrease in latency of first mean intake in E2B treated animals. The different densities of estrogen receptors that control hedonic behaviors in the Dorsal Raphe on serotonin receptors may be responsible and warrants further investigation (Sheng, Kawano et al. 2004)**.** Intact KIKO females had a similar latency to FI as that of WT females. Again, OVX oil-treated KIKO females did not have a significant difference from OVX oil WT females. Interestingly, OVX E2B KIKO females had a significant delay in FI over WT females. When E2B is introduced they behave in an anhedonic behavior signifying anhedonic tendencies potentially working through ERE-independent mechanisms.

Hourly intake

The loss of recognizable corpuscular curves seen in the first 24 hours of FI in steroid treated females could be in part due to the nature of the highly palatable foods the animals were exposed to for the first time. Additionally, another explanation could be a result of the dysregulation of gut peptide pathways when on HFD (Duca, Sakar et al.

2013). Overall intact male and females animals did not experience any trends in genotype differences. OVX oil-treated animals saw much more variation in FI than E2B-treated animals, in part most likely due to the lack of estrogens protective qualities. E2B-treated WT animals separated from KIKO animals at 1600, and 24-0100 hours. Of note, the large peak in separation of OVX oil-treated WT and KIKO animals is suspected to have been due to an interference in recording conditions vs. animal behaviors. When females were organized by genotype across treatments no desirable trends were observed within WT or KIKO females. Intact KO females however exhibited a familiar corpuscular curve seen in LFD hourly monitoring, the trend was lost upon OVX.

24 hour fast

The role of ERα signaling in the response to fasting and refeeding

Our lab has previously shown GHSR signaling pathways in NPY neurons are regulated in a sex dependent manner in which fasted state, DIO and estrogen levels were found to be influential (Yasrebi, Hsieh et al. 2016). We also shown fasted males had increased GHSR expression in NPY cells and E2B-treated fasted females had an increase in FOX01 expression. Involved in the GHSR pathway, FOX01 has been shown to decrease adipogenesis regulation which could affect hepatic glucose secretion and insulin sensitivity (Farmer 2003). In times of calorie restriction, orexigenic NPY/AgRP expression increases while the anorexigenic POMC decreases expression and firing rate (Sohn 2015). Additionally the gut peptides CCK, Leptin decrease and Ghrelin and PYY expression increases increased. CCK, Leptin, and Ghrelin have all been shown to be effected by ERα expression (Asarian and Geary 2007, Fusco, Galgani et al. 2010, Choi,

Lee et al. 2011). After a 36 hr fast, leptin levels have been shown to significantly decrease in both men and women with women having a larger reduction (Maccario, Aimaretti et al. 2000). Although considered a physiological stressor, the resulting cortisol increase from a single 24 hr fast did not prove to be enough of a stressor to affect LH suppression in monkeys (Helmreich, Mattern et al. 1993).

Produced both by the stomach and in the CNS, the endogenous hormone Ghrelin acts on the GH secretagogue receptor to enact antianorexic sensations (Sakata, Tanaka et al. 2006). Others have also shown ERα plays an important part in ghrelin expression. (Sakata, Tanaka et al. 2006). Densely populated by GHSR, we have shown NPY neuron expression is augmented by estrogen through ERE-independent pathways (Yasrebi, Hsieh et al. 2016). Ghrelin has been shown to cause an increase in FI in low estrogen state animals (males/OVX females) (Nakazato, Murakami et al. 2001, Wren, Seal et al. 2001, Wren, Small et al. 2001, Davidson, Kanoski et al. 2005) more so than when animals are in late state Proestrous estrogen levels (Clegg, Brown et al. 2007) suggesting estradiol may affect ghrelins orexigenic actions.

Body Weight fluctuations

Because weight loss can alter feeding behaviors (less bodyfat will results in lower levels of leptin secretion, resulting in higher ghrelin expression) we monitored body weight during the study. Intact KIKO male and female mice had smaller BW fluctuations resulting from a 24 hr fast than their counterparts. As expected, intact WT females lost less body mass than WT male counterparts. Surprisingly, and contrary to estrogens protective qualities, E2B-treated animals were not protected against weight loss

compared to oil-treated counterparts. Unexpectedly and countering intact WT female findings, intact KO females lost less mass than intact KO male counterparts. E2B-treated WT animals lost more BW but also rebounded higher than KIKO and KO counterparts.

Total food intake

Aligned with what we have previously reported (Mamounis, Yang et al. 2014), intact KIKO females had similar levels of FI to KO counterparts. Oil and E2B-treated WT animals ingested more than both steroid treated KIKO animals with E2B-treated WT female also ingesting more than E2B-treated KO animals. Interestingly, all but the (intact and oil-treated) KO refeed groups had reduced 24 hr refeed ingestion compared to LFD fed groups (experiment 1). While we did not measure corticosterone levels in these mice, it is known that men exhibit elevated cortisol levels from physiological stress resulting from a short term fast (Bergendahl, Vance et al. 1996). PVN neurons located in the hypothalamus react to elevated levels of CRH to decreased levels of FI (Krahn, Gosnell et al. 1988). Although low levels of ERα have been detected in the PVN, this suggests ERα may be influenced indirectly by PVN activity (Suzuki and Handa 2005) to affect FI.

Intact WT females meal sizes and durations were longer than WT male and female counterparts. Previously reported data reaffirms estrogen (either from naturally cycling or E2B-treated post OVX), was necessary for normal meal size patterns in rats (Blaustein and Wade 1976, Asarian and Geary 2002). OVX WT had larger meal sizes than counterparts but interestingly oil-treated WT mice ingested their chow at a higher rate and in larger portion sizes suggesting ERE-dependent mitigated pathways may be at play to control meal ingestion rate. Additionally, except for E2B-treated WT animals,

average refeed animal meal sizes were also decreased. Interestingly, E2B-treated WT animals had an increased meal duration, not otherwise seen in standard LFD feeding, explained partly due to Ghrelins anorexic properties. These data suggest estrogen, through ERE-dependent mechanisms, contributing protection against fasted state meal pattern differences.

Post refeed hourly FI

Upon initial refeed KIKO males had an increase in FI which quickly dissipated through the dark cycle, intact females had very little initial FI. Consistent with having larger first meal durations and intake, WT females had an immediate group separation that lasted several hours into lights off (1800-2300) with a secondary peak in FI leading up to lights off (16-1700). The initial trend of high WT intake and low intact KIKO/KO intake may be a result of Ghrelin acting to increase meal initiation in negative energy state animals (Klok, Jakobsdottir et al. 2007). Interestingly however, very few first meal differences were seen.

OVX animals had slightly higher variation in overall hourly FI trends. Consistent with having smaller measured meal sizes compared to WT littermates, oil-treated KO animals experienced decreased in FI (@2000, 2300) when compared to oil-treated WT animals. Interestingly however, oil-treated WT animals had higher first meal FI than KIKO, and not KO counterparts. Ghrelin's orexigenic influences can be inhibited in E2B-treated mice suggesting oil-treated OVX mice may have higher ghrelin mediated rates of FI (Clegg, Brown et al. 2007). Contrary to expectations, E2B-treated animals had longer periods of sustained FI with levels eventually decreasing at lights on. E2B-treated KIKO

animals trended at higher levels of FI for the first two-three hours of refeed before immediately falling down to KO levels. This suggests estrogen may be acting on CCK satiety to contribute to the intermittent drop in FI seen within E2B-treated KIKO females.

The crepusular curve trend seen when compared within genotype of LFD fed mice were not exhibited in post fasted-refeed animals, but rather all three genotypes experienced increased FI at refeeding, and again leading up to nocturnal hours. Intact KIKO females consistently ingested less than OVX counterparts during the first refeed hours. This was reflected in intact KIKO females having longer latencies to food intake and inter-meal interval times compared to OVX counterparts. Oil-treated KO females frequently had spikes in FI separating from intact and E2B treated counterparts. Although KO animals do not have any functioning ERα receptors, the spikes seen in oil-treated animals suggest when KO animals are in an estrogen free state their feeding pathways may be less inhibited by estrogens influence on other estrogen receptor mechanisms.

Behavior testing

Hypothalamic regions (Amygdala, DR, and PFC) rich with ERα activity have been implicated in emotional response, emphasizing anxious and depressive like states. In response to stressful/anxious conditions, corticotrophin hormones are released into circulation and act on the CNS. Males rats have been observed to have a short lived cortisoterone response when compared to females during chronic stressor exposure (Galea, McEwen et al. 1997). CRF receptors (CRFR-1) have been shown to be present at high concentrations in female PVN neurons (areas high in ERα expression) with extremely low expression found in males (Rosinger, Jacobskind et al. 2019). Rats in high

estrogen states (intact late P, high estrogen low progesterone, or OVX-E2 treated animals) had higher rates of adrenocorticotropin and corticosterone responses when exposed to stressful situations compared to male counterparts (Le Mevel, Abitbol et al. 1979, Viau and Meaney 1991). Additionally, ERα activation within the medical preoptic area have been shown to contribute anxiogenic effects (Spiteri, Ogawa et al. 2012).

Exploratory and locomotor behavior

Sex-specific anxiolytic like behaviors are well known. Male rats are less active than females in OFT tests (Blizard, Lippman et al. 1975). Intact WT and KIKO females spent a longer duration of the test in the center of the OFT than KO littermates and male KIKO/KO counterparts. As expected, the trends were reversed in outer perimeter exploration. Previous reporting have shown similar results, untreated OVX KO females were less active during OFT than WT; it wasn't until animals were treated with E2B that increases in OFT activity were observed (Ogawa, Chan et al. 2003). Additionally, acting through ER receptors in the medial preoptic area (mPOA), E2B-treated WT animals were shown to have an increase wheel running activity compared to KO counterparts (Ogawa, Chan et al. 2003). This suggests locomotor activity may be influenced by ERα mediated mechanisms.

E2B-treated KO females explored the center space less than WT counterparts while spending more time in the outer perimeter than both E2B-treated WT and KIKO. These findings suggest there was a main anxiolytic effect of estrogen on OVX WT animals. Interestingly, E2B-treated KIKO animals spent less time in the outer perimeter than oil-treated KIKO animals suggesting ERE-independent mechanism may be involved

in anti-anxiolytic behaviors. We suspect a contributing factor to the animals spending such little time in the middle of the chamber may be resulting from the sheer size of the OFT apparatus.

Animals transitioned from center to outer perimeters in a predictable manner, animals having spent longer in the center also had a higher rate of center perimeter entrance frequency. However, OVX WT animals had higher entrance frequencies into both center and outer perimeters when compared to their OVX steroid treated counterparts. E2B-treated KIKO animals entered the outer perimeter area less frequently than E2B-treated WT but more than comparable KO. No steroid differences were observed suggesting a developmental genotype effect may be contributing to WT animals behaving in more anxiolytic manner. These findings are similar to previously studies in female rats with elevated ovarian hormone performing in an anxiolytic-like manner when compared to low estrogen state counterparts (Frye, Petralia et al. 2000). E2B-treated, OVX rats entered the center square of an OFT more often than oil-treated counterparts (Walf, Paris et al. 2009).

While the OFT can be used for multiple behavioral parameters (locomotor, and exploratory behaviors), the EPM tend to measure more anxiety like tendencies. Estrogens protective qualities are again seen within intact EPM results. As expected, due to increased prevalence of estrogen receptors, intact WT females spent longer in the open arm, and conversely, less in the closed arm, than both KIKO and KO female littermates as well as WT males. Interestingly, post-OVX KIKO animals behaved similarly to WT animals spending more time in open and less time in closed arms than OVX KO animals. This suggests either involvement of ERE-independent mechanisms of ERα or possibly

other estrogen receptor activity in conjunction with the influence of other ovarian hormones on anxiolytic behaviors. Intact WT and KIKO females had higher frequencies of entries into the center and outer perimeter when compared to male counterparts. This is consistent with previous reports of females having higher activity levels than male counterparts (Valle and Gorzalka 1980).

Polosk test

Previous reports have shown females having decreased immobility, and increases in swimming behavior in intact females over intact males (Brotto, Barr et al. 2000). Unexpectedly, we did not witness any sex-dependent effects in FST (Alonso, Castellano et al. 1991, Barros and Ferigolo 1998). Since CRF expression in the PVN and BSNT are influenced by sex-steroids (Broad, Keverne et al. 1995), we had expected to see sex differences. KO females, regardless of treatment, spent longer in "free float" like immobility than WT counterparts. Upon OVX, steroid treated KIKO animals also spend longer in free float than WT counterparts. By binding on the ERE of the CRH gene promoter, estrogen is able to direct stimulatory actions on CRH expression (Stratakis and Chrousos 1995).

E2B-treated WT females swam for longer than E2B-treated KO animals. This is consistent with previous reports of E2 being shown to decrease immobility and increase struggling duration (Walf, Paris et al. 2009). Additionally, recent reports have shown rats having E2 injected into their Amygdala have reductions in depressive like behavior (Frye and Walf 2004). We speculate ERα activation in the Amygdala may be contributing toward E2B-treated WT decrease in immobility and increase in swim time. Additionally

lack of ovarian hormones have been shown to increase depressive and anxiety like states which can be reversed with reintroduction of an estrogen paradigm (Marcondes, Miguel et al. 2001, Estrada-Camarena, Fernandez-Guasti et al. 2003).

Chapter 3: Conclusion and Future Directions

Our current study demonstrates the contribution of ERα to anxiolytic behaviors and further substantiates the role estrogen plays on ERα activation pertaining to meal patterns. We hypothesized that the direct effects of E2 actions interacts with gut peptides via regulation of receptor or signaling molecule expression. We have shown ERE-independent signaling is substantial enough to partially restored basic homeostatic feeding parameters but not others.

Future experiments will characterize food intake and meal specific parameter differences results from exogenous administration of gut peptides (CCK, Leptin, Ghrelin), using our novel animal models. We will investigate if ERE-independent mechanisms are sufficient to restore sensitivity to orexigenic gut-peptide actions.

3.1.1: Experiment 1a, LFD conclusions

WT animals had an increased FI compared to KIKO and KO littermates. The mice were influenced in a manner where ERE-dependent mechanisms were the main modulator of homeostatic LFD feeding meal parameters. Intact WT male and female mice both exhibited increases in meal duration but not in meal size or frequency. In OVX mice, E2B increased the frequency for FI but reduced the overall meal size in E2B-treated WT animals. OVX induced an augmentation in the daily FI patterns, possibly due a circadian rhythm effect.

3.1.2: Experiment 1b, HFD conclusions

Intact WT male and OVX WT and KIKO animals exhibited more overall HFD FI than KO animals, a trend that extended into the initial nocturnal hourly intake. Intact

animals exhibited similar hourly HFD feeding parameters while OVX WT and KIKO circadian FI patterns were disrupted. Contrary to when placed on a LFD, once on a HFD ERE-independent ERα signaling was involved in the control of palatable hedonic food intake patterns.

3.1.3: Experiment 1c, Refeed conclusions

No differences were seen within male groups. Intact females experienced differences in meal duration and size in an ERE-dependent manner. When animals were placed in a hypoestrogenic state, KIKO and WT females responded differenced to fasting. Differences in KIKO females exhibited ERE-independent mechanisms contribute to a decreased first meal FI and slower rate of ingestion.

3.2.1: Experiment 2, Behavior testing

While in some behavior tests ERE-independent signaling was sufficient enough to augment anxiolytic behavior it was not ubiquitous. No differences were seen within male exploration of the OFT of EPM. WT females, in estrogenic states, spent more time exploring the center of the OFT than their KO counterparts. Both OVX WT and KIKO animals behaved similar in the EPM; ERE-independent pathways may contribute towards reducing anxiety measures, independent of locomotor activity in an ovariectomized state. WT females, across all treatments, exhibited longer bouts of free float and longer durations of activity then their KIKO and KO counterparts suggesting ERE-dependent mechanisms may be at play to help reduce “despair” like tendencies.

3.3: Future directions

Upon completion of our initial behavior testing experiments, we have acquired several mouse-sized behavior testing apparatuses (OFT, EPM, LDB, 3 chamber test, Y-maze) as well as automated scoring software (ANY-maze software; Stoelting Co.) which is capable of providing precise and detailed information pertaining to animal behaviors and locomotor tendencies. With the addition of new autonomous scoring software and appropriately sized behavior chambers, we are currently rerunning and analyzing intact and steroid-treated OVX WT, KIKO, KO mice through these testing paradigms to determine if we can further substantiate our findings in the current study. In addition, we are also examining the role of the E2-responsive Gq-coupled mER using its selective ligand, STX, in exploratory and locomotor behaviors (Roepke, Bosch et al. 2010). A future line of experimentation our lab is interested in investigating are the impacts of activation of ERE-dependent and independent signaling through out different stages of development to see if developmental timing has any impact on anyxiolytic or feeding behaviors and tendencies

Figure legends

Figure 1: Adult mice (>3 months) were acclimated to the BioDAQ food monitoring room for one full week. After acclimation, mice were placed into the individual BioDAQ cages and allowed to acclimate an additional 3 days on a low-fat diet (LFD). Data was collected for 4 consecutive days on a LFD followed by three days on a high-fat diet (HFD). Mice were removed from the BioDAQ chamber, fed a standard chow for two weeks, and exposed to the 3 behavior tests with one-week interval between each test. The animals were first exposed to a Open Field Test (OFT) chamber (measuring 100cm x 100cm) for 10 full min, then an Elevated Plus Maze (EPM) exposure (each arm measuring 50cm) for 5 min, and finally a Forced Swim Test (FST) chamber (a 24cm diameter chamber was filled with 13cm water ranging from 24-30 C) for a total of 6 min, the first two minutes of which were not analyzed. Upon completion of the FST, males were euthanized for blood collection. All intact females were given a 2-week rest, followed by ovariectomy (OVX), and a second pass through the BioDAQ and each behavior test. All OVX females were orally dosed on a bidaily basis with either oil or EB (300 μ g/kg). Dosing was scheduled in a manner where each female was dosed twice per each diet treatment in the BioDAQ and dosed on the morning prior to any behavior testing. Data was analyzed by a two-way ANOVA with Newman-Keuls multiple comparison tests.

Figure 2: Change in body weight over the 7 days of LFD feeding in the BioDAQ for **A:** Intact male and female mice and **B:** OVX oil- or E2-treated females. Change in body weight over the 3 days of HFD feeding in the BioDAQ for **C**: intact male and female mice and **D**: OVX oil- or E2-treated females. All OVX females were dosed on the

first and third day of monitoring. Data were analyzed by a two-way ANOVA with post-hoc Newman-Keuls multiple comparison tests. Capped lines with asterisks denote significance between genotypes within sex or steroid treatment and letters denote significance between sex or steroid treatment groups (a,*=P<0.05; **=P<0.01).

Figure 3: Total LFD ingested over 96 hours for **A**: Intact male and female mice and for **B**: OVX oil- or E2-treated females. Data were analyzed by a two-way ANOVA with post-hoc Newman-Keuls multiple comparison tests. Capped lines with asterisks denote significance between genotypes within sex or steroid treatment and letters denote significance between sex or steroid treatment groups (a,*=P<0.05; **=P<0.01; ***=P<0.001).

Figure 4: Daily LFD ingestion for **A**: Intact males; **B**: Intact females; **C**: OVX oil-treated females; and **D**: OVX E2 treated females. All OVX females were dosed on the first and third day of monitoring (represented by boxes). Data were analyzed by a two-way ANOVA with post-hoc Newman-Keuls multiple comparison tests. Capped lines with asterisks denote significance between genotypes within sex or treatment (*=P<0.05; **=P<0.01; ***=P<0.001; ****=P<0.0001).

Figure 5: Daily food ingestion during the last 96 h of LFD. All OVX females were dosed on the first and third day of monitoring (represented by boxes). Data were analyzed by a two-way ANOVA with post-hoc Newman-Keuls multiple comparison tests. Capped lines

with asterisks denote significance between genotypes within treatment (*=P<0.05; **=P<0.01; ***=P<0.001; ****=P<0.0001).

Figure 6: Averaged hourly LFD ingestion over 96 hours for **A**: Intact males; **B**: Intact females; **C**: OVX oil-treated females; and **D**: OVX E2-treated females. Animals experienced a 12:12 light cycle (lights on from 700-1900, lights off from 1900-700) as denoted by the underscore. All OVX females were dosed on the first and third day of monitoring at approximately 1000. Data were analyzed by a two-way ANOVA with post-hoc Newman-Keuls multiple comparison tests. Letters denote a significance between genotypes (a = P<0.05 between WT & KIKO; b = P<0.05 between WT & KO; c = P<0.05 between KIKO & KO).

Figure 7: Average hourly LFD ingestion over 96 hours in **A**: WT females; **B**: KIKO females; and **C**: KO females. Animals experienced a 12:12 light cycle (lights on from 700-1900, lights off from 1900-700) as denoted by the underscore. OVX females were dosed on the first and third day of monitoring at approximately 1000. Data were analyzed by a two-way ANOVA with post-hoc Newman-Keuls multiple comparison tests. (b = P<0.05 Intact vs Oil).

Figure 8: Total number of meals in **A**: Intact mice and **B**: OVX females. Average meal size in **C**: Intact mice and **D**: OVX females. Meal frequency in **E**: Intact mice and **F**: OVX females. Average meal duration in **G**: Intact mice and H: OVX females. Data was collected from the last 96 h of a LFD BioDAQ run. All OVX females were dosed on the

first and third day of monitoring at approximately 1000. Data were analyzed by a two-way ANOVA with post-hoc Newman-Keuls multiple comparison tests. Capped lines with asterisks denote significance between genotypes within sex and treatment. Letters denote significance between sex or steroid treatment within genotypes (* = P<0.05; ** = P<0.01; *** = P<0.001; d,**** = P<0.0001).

Figure 9: Total HFD ingested over 72 hours for **A**: Intact male and female mice and for **B**: OVX oil- or E2-treated females. Data were analyzed by a two-way ANOVA with post-hoc Newman-Keuls multiple comparison tests. Capped lines with asterisks denote significance between genotypes within sex or steroid treatment and letters denote significance between sex or steroid treatment groups (*=P<0.05; **=P<0.01; ***=P<0.001).

Figure 10: Daily HFD ingestion for **A**: Intact males; **B**: Intact females; **C**: OVX oil-treated females; and **D**: OVX E2-treated females. All OVX females were dosed on the first and third day of monitoring (represented by boxes). Data were analyzed by a two-way ANOVA with post-hoc Newman-Keuls multiple comparison tests. Capped lines with asterisks denote significance between genotypes within sex or treatment (*=P<0.05; **=P<0.01; ***=P<0.001; ****=P<0.0001).

Figure 11: Daily food ingestion during 72 hours of HFD. All OVX females were dosed on the first and third day of monitoring (represented by boxes). Data were analyzed by a two-way ANOVA with post-hoc Newman-Keuls multiple comparison tests. Capped lines

with asterisks denote significance between genotypes within treatment (*=P<0.05; **=P<0.01; ***=P<0.001; ****=P<0.0001).

Figure 12: Averaged hourly HFD ingestion over 72 hours for **A**: Intact males; **B**: Intact females; **C**: OVX oil-treated females; and **D**: OVX E2-treated females. Animals experienced a 12:12 light cycle (lights on from 700-1900, lights off from 1900-700) as denoted by the underscore. If required, any animal handling was done between 1000-1400 hours. All OVX females were dosed on the first and third day of monitoring at approximately 1000. Data were analyzed by a two-way ANOVA with post-hoc Newman-Keuls multiple comparison tests. Letters denote significance between genotypes (a = P<0.05 between WT & KIKO; b = P<0.05 between WT & KO; c = P<0.05 between KIKO & KO).

Figure 13: Average hourly HFD ingestion over 72 hours in **A**: WT females; **B**: KIKO females; and **C**: KO females. Animals experienced a 12:12 light cycle (lights on from 700-1900, lights off from 1900-700) as denoted by the underscore. All OVX females were dosed on the first and third day of monitoring at approximately 1000. Data were analyzed by a two-way ANOVA with post-hoc Newman-Keuls multiple comparison tests. (a = P<0.05 oil vs E2, b = P<0.05 Intact vs oil, c = P<0.05 E2 vs Intact).

Figure 14: Total number of meals in **A**: Intact mice and **B**: OVX females. Average meal size in **C**: Intact mice and **D**: OVX females. Meal frequency in **E**: Intact mice and **F**: OVX females. Average meal duration in **G**: Intact mice and H: OVX females. Data was

collected from a 72 h HFD BioDAQ run. All OVX females were dosed on the first and third day of monitoring at approximately 1000. Data were analyzed by a two-way ANOVA with post-hoc Newman-Keuls multiple comparison tests. Capped lines with asterisks denote significance between genotypes within sex and treatment. Letters denote significance between sex or steroid treatment within genotypes (a,* = $P<0.05$; ** = $P<0.01$; *** = $P<0.001$).

Figure 15: Total grams of HFD ingested during the first meal upon initial HFD administration in **A**: Intact mice and **B**: OVX females. Average first HFD meal length in **C**: Intact mice and **D**: OVX females. All OVX females were dosed at the same time as initial HFD administration. Data were analyzed by a two-way ANOVA with post-hoc Newman-Keuls multiple comparison tests. Capped lines with asterisks denote significance between genotypes within sex and treatment. Letters denote significance between sex or steroid treatment within genotypes (a,* = $P<0.05$; ** = $P<0.01$).

Figure 16: Length of latency to first meal initiation upon HFD administration in **A**: Intact mice and **B**: OVX females. First HFD meal rate (mG/min) **C**: Intact mice and **D**: OVX females. Number of seconds between the conclusion of the first meal and start of the second meal (Inter-Meal Interval) **E**: Intact mice and **F**: OVX females. All OVX females were dosed at the same time as initial HFD administration. Data were analyzed by a two-way ANOVA with post-hoc Newman-Keuls multiple comparison tests. Capped lines with asterisks denote significance between genotypes within sex and treatment. Letters

denote significance between sex or steroid treatment within genotypes (a,* = P<0.05; ** = P<0.01; *** = P<0.001).

Figure 17: Hourly HFD ingestion over the initial 24 hour period of the 72 hours HFD administration for **A**: Intact males; **B**: Intact females; **C**: OVX oil-treated females; and **D**: OVX E2-treated females. Animals experienced a 12:12 light cycle (lights on from 700-1900, lights off from 1900-700) as denoted by the underscore. All OVX females were dosed at the onset of initial HFD administration. Data were analyzed by a two-way ANOVA with post-hoc Newman-Keuls multiple comparison tests. Letters denote a significance between genotypes (a = P<0.05 between WT & KIKO; b = P<0.05 between WT & KO; c = P<0.05 between KIKO & KO).

Figure 18: Hourly HFD ingestion over the initial 24 hour period of the 72 hour HFD administration s in **A**: WT females; **B**: KIKO females; and **C**: KO females. Animals experienced a 12:12 light cycle (lights on from 700-1900, lights off from 1900-700) as denoted by the underscore. All OVX females were dosed at the onset of initial HFD administration. Data were analyzed by a two-way ANOVA with post-hoc Newman-Keuls multiple comparison tests. (a = P<0.05 Oil vs E2, b = P<0.05 Intact vs Oil, c = P<0.05 E2 vs Intact).

Figure 19. Individually housed adult (>3 months) male and female ERα WT/KIKO/KO littermates were administered a 24 hour fast and allowed a 24 hour LFD refeed under continuous FI monitoring in the Biodaq monitoring system. All intact animals

were relocated into the Biodaq room 6 days prior to the 24 hour fast and subsequent refeed period. OVX mice were relocated immediately post-op (day 0) and allowed to recover in the Biodaq room. The OVX animals were split into either an oil or E2 treated group. At day 6 the animals were administered the first of two IP injections, either a sesame oil blank, or a 0.25ng E2B dose. The animals were injected with either a second sesame oil blank or a 1.5ng E2B dose on day 7. All injections occurred at 1800 hours. The animals were fasted for 24 hours immediately post-injection and allowed a Biodaq monitored 24 hour LFD refeed the following day at 1800. Upon refeed completion all OVX animals were euthanized for blood and uterus collected.

Figure 20: Change in body weight after a 24 hour fast in **A:** Intact mice (male and female) and **B:** OVX oil- or E2-treated females. Change in body weight immediately after a 24 hour ad lib LFD refeed in the BioDAQ for **C**: intact male and female mice and **D**: OVX oil- or E2-treated females. All OVX females were dosed via two injections; 24 hour prior to the fast animals were IP injected with either a sesame oil blank or a low priming dose of E2B. Directly before the initiation of the 24 hour fast the animals were again injected with either a sesame oil blank or a high dose of E2B. Data were analyzed by a two-way ANOVA with post-hoc Newman-Keuls multiple comparison tests. Capped lines with asterisks denote significance between genotypes within sex or steroid treatment and letters denote significance between sex or steroid treatment groups (a,*=$P<0.05$; b,**=$P<0.01$; ***=$P<0.001$; d, ****=$P<0.0001$).

Figure 21: Total LFD ingested over 24 hour refeed period **A**: Intact male and female mice and for **B**: OVX oil- or E2-treated females. OVX female mice were injected with either a second sesame oil injection or a high E2 dose at the time of 24 hour fast. Data were analyzed by a two-way ANOVA with post-hoc Newman-Keuls multiple comparison tests. Capped lines with asterisks denote significance between genotypes within sex or steroid treatment and letters denote significance between sex or steroid treatment groups (*=P<0.05; **=P<0.01; c, ***=P<0.001).

Figure 22: total hourly LFD ingestion over 24 hour refeed period for **A**: Intact males; **B**: Intact females; **C**: OVX oil-treated females; and **D**: OVX E2-treated females. Animals experienced a 12:12 light cycle (lights on from 700-1900, lights off from 1900-700) as denoted by the underscore. OVX female mice were injected with either a second sesame oil injection or a high E2 dose at the time of 24 hour fast onset (6pm). Animals were allowed to refeed at 6pm the start of the following day. Data were analyzed by a two-way ANOVA with post-hoc Newman-Keuls multiple comparison tests. Letters denote a significance between genotypes (a = P<0.05 between WT & KIKO; b = P<0.05 between WT & KO; c = P<0.05 between KIKO & KO).

Figure 23: Average hourly LFD ingestion over 24 hour refeed period in **A**: WT females; **B**: KIKO females; and **C**: KO females. Animals experienced a 12:12 light cycle (lights on from 700-1900, lights off from 1900-700) as denoted by the underscore. OVX female mice were injected with either a second sesame oil injection or a high E2 dose at the time of 24 hour fast onset (6pm). Animals were allowed to refeed at 6pm the start of

the following day. Data were analyzed by a two-way ANOVA with post-hoc Newman-Keuls multiple comparison tests. Letters denote a significance between genotypes (a = P<0.05 Oil vs E2, b = P<0.05 Intact vs Oil, c = P<0.05 E2 vs Intact)

Figure 24: Total number of meals in **A**: Intact mice and **B**: OVX females. Average meal size in **C**: Intact mice and **D**: OVX females. Meal frequency in **E**: Intact mice and **F**: OVX females. Average meal duration in **G**: Intact mice and **H**: OVX females. Data was collected during a 24 hour LFD refeed BioDAQ run. OVX female mice were injected with either a second sesame oil injection or a high E2 dose at the time of 24 hour fast onset (6pm). Data were analyzed by a two-way ANOVA with post-hoc Newman-Keuls multiple comparison tests. Capped lines with asterisks denote significance between genotypes within sex and treatment. Letters denote significance between sex or steroid treatment within genotypes (* = P<0.05; ** = P<0.01; *** = P<0.001).

Figure 25: Total grams of LFD ingested during the first meal upon initial LFD refeed in **A**: Intact mice and **B**: OVX females. Average first LFD meal length in **C**: Intact mice and **D**: OVX females. All OVX females were dosed via injection 24 hour prior to LFD administration. Data were analyzed by a two-way ANOVA with post-hoc Newman-Keuls multiple comparison tests. Capped lines with asterisks denote significance between genotypes within sex and treatment. Letters denote significance between sex or steroid treatment within genotypes (* = P<0.05; b,** = P<0.01).

Figure 26: Length of latency to first meal initiation upon LFD refeed in A: Intact mice and B: OVX females. First HFD meal rate (G/min) C: Intact mice and D: OVX females. Number of seconds between the conclusion of the first meal and start of the second meal (Inter-Meal Interval) E: Intact mice and F: OVX females. All OVX females were dosed via injection 24 hours prior to LFD refeed. Data were analyzed by a two-way ANOVA with post-hoc Newman-Keuls multiple comparison tests. Capped lines with asterisks denote significance between genotypes within sex and treatment. Letters denote significance between sex or steroid treatment within genotypes (* = $P<0.05$; b,** = $P<0.01$).

Figure 27: Total percent exploration of "center" chamber exploration in **A**: Intact mice and **B**: OVX females. Total percent exploration of "perimeter" exploration in **C**: Intact mice and **D**: OVX females. All OVX females were dosed prior to start of Open field test. Data were analyzed by a two-way ANOVA with post-hoc Newman-Keuls multiple comparison tests. Capped lines with asterisks denote significance between genotypes within sex and treatment. Letters denote significance between sex or steroid treatment within genotypes (a,* = $P<0.05$; b,** = $P<0.01$; c,*** = $P<0.001$; d,**** = $P<0.0001$).

Figure 28: Total number of crossings into "center" OF chamber in **A**: Intact mice and **B**: OVX females. Total number of crossings into "perimeter" OF in **C**: Intact mice and **D**: OVX females. All OVX females were dosed prior to start of Open field test. Data were analyzed by a two-way ANOVA with post-hoc Newman-Keuls multiple comparison tests. Capped lines with asterisks denote significance between genotypes within sex and

treatment. Letters denote significance between sex or steroid treatment within genotypes (a,* = P<0.05; ** = P<0.01; c,*** = P<0.001; **** = P<0.0001).

Figure 29: Percent total time exploration of "Open arm" chamber in **A**: Intact mice and **B**: OVX females. Total percent exploration of "Closed arm" exploration in **C**: Intact mice and **D**: OVX females. All OVX females were dosed prior to start of Elevated Plus Maze test. Data were analyzed by a two-way ANOVA with post-hoc Newman-Keuls multiple comparison tests. Capped lines with asterisks denote significance between genotypes within sex and treatment. Letters denote significance between sex or steroid treatment within genotypes (* = P<0.05; ** = P<0.01; c,*** = P<0.001; d,**** = P<0.0001).

Figure 30: Total number of crossings into "Open arm" chamber in A: Intact mice and B: OVX females. Total number of crossings into "Closed arm" exploration in C: Intact mice and D: OVX females. All OVX females were dosed prior to start of Elevated Plus Maze test. Data were analyzed by a two-way ANOVA with post-hoc Newman-Keuls multiple comparison tests. Capped lines with asterisks denote significance between genotypes within sex and treatment. Letters denote significance between sex or steroid treatment within genotypes (* = P<0.05; ** = P<0.01; *** = P<0.001; d,**** = P<0.0001).

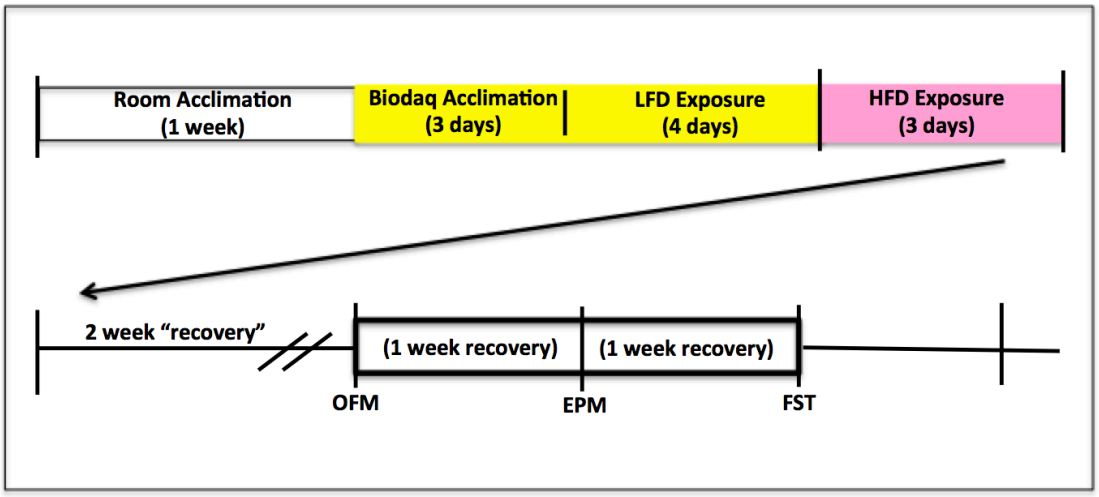
Figure 31: Total percent time spent in Free floating in **A**: Intact mice and **B**: OVX females. Percent total of seconds of Passive paddling in **C**: Intact mice and **D**: OVX females. Percent time spent in states of Vigorous paddling in **E**: Intact mice and **F**: OVX

females. The FST spanned a total of six minutes, with the first two minutes being discarded prior to data analysis. All OVX females were dosed prior to start of stress test. Data were analyzed by a two-way ANOVA with post-hoc Newman-Keuls multiple comparison tests. Capped lines with asterisks denote significance between genotypes within sex and treatment. Letters denote significance between sex or steroid treatment within genotypes (* = P<0.05; ** = P<0.01; *** = P<0.001; **** = P<0.0001).

Figure 32: Total number of transitions to Free floating in A: Intact mice and B: OVX females. Total number of transitions to Passive paddling in C: Intact mice and D: OVX females. Total number of transitions to Vigorous paddling in E: Intact mice and F: OVX females. The FST spanned a total of 6 minutes, with the first two minutes being discarded prior to data analysis. All OVX females were dosed prior to start of stress test. Data were analyzed by a two-way ANOVA with post-hoc Newman-Keuls multiple comparison tests. Capped lines with asterisks denote significance between genotypes within sex and treatment. Letters denote significance between sex or steroid treatment within genotypes (* = P<0.05; ** = P<0.01; *** = P<0.001; **** = P<0.0001).

Figures

Figure 1



Experiment 1a: The effects of sex, genotype, and steroid on feeding behaviors on a LFD

Figure 2

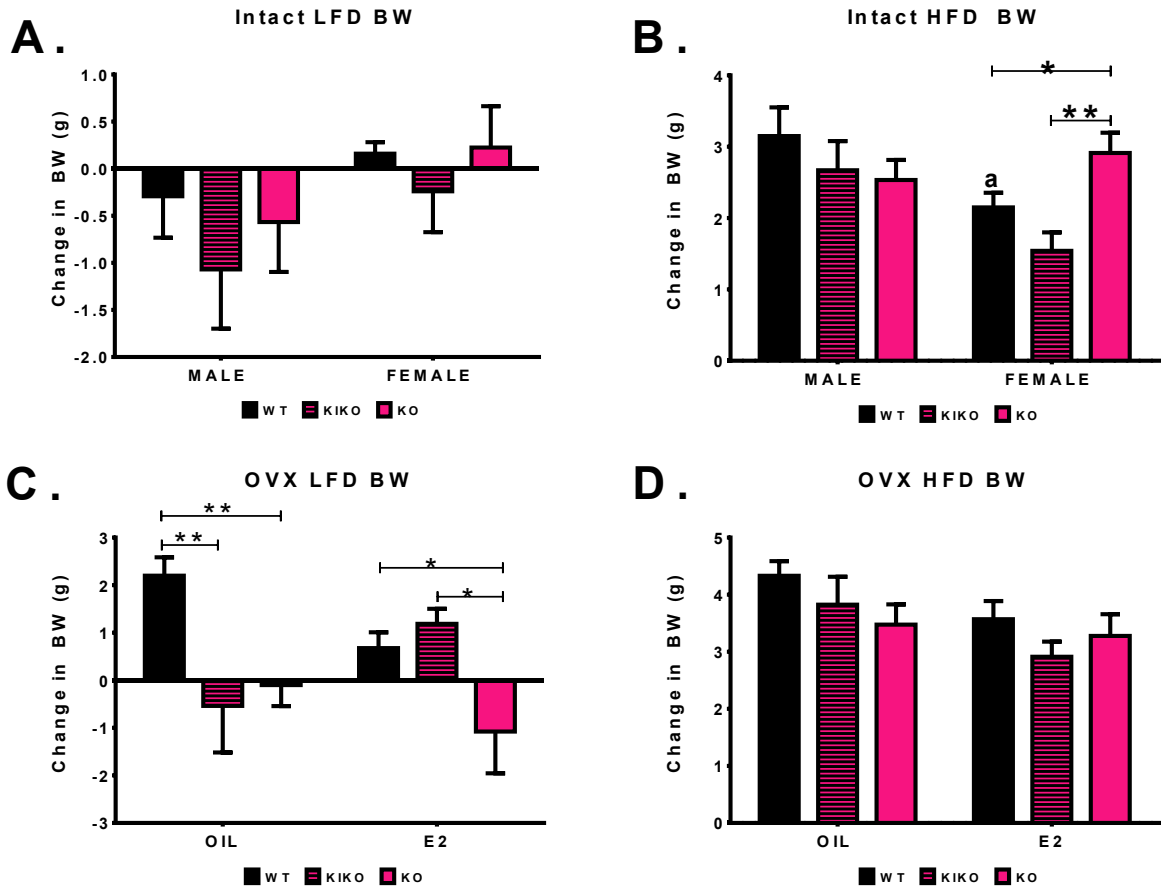


Figure 3

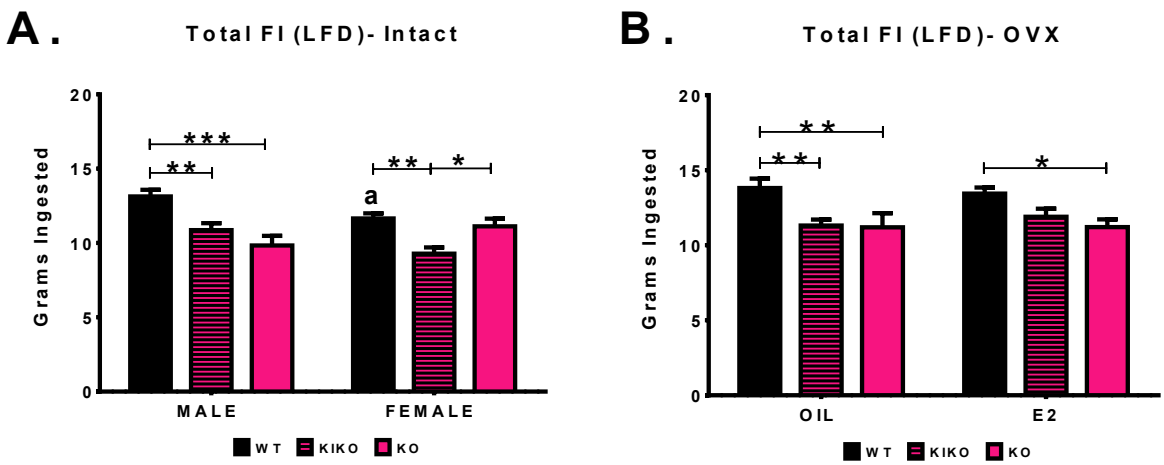

A.
Total FI (LFD)- Intact
Grams Ingested
20
15
10
5
0
***
**
**
*
a
MALE
FEMALE
WT
KIKO
KO
B.
Total FI (LFD)- OVX
Grams Ingested
20
15
10
5
0
**
**
*
OIL
E2
WT
KIKO
KO

Figure 4

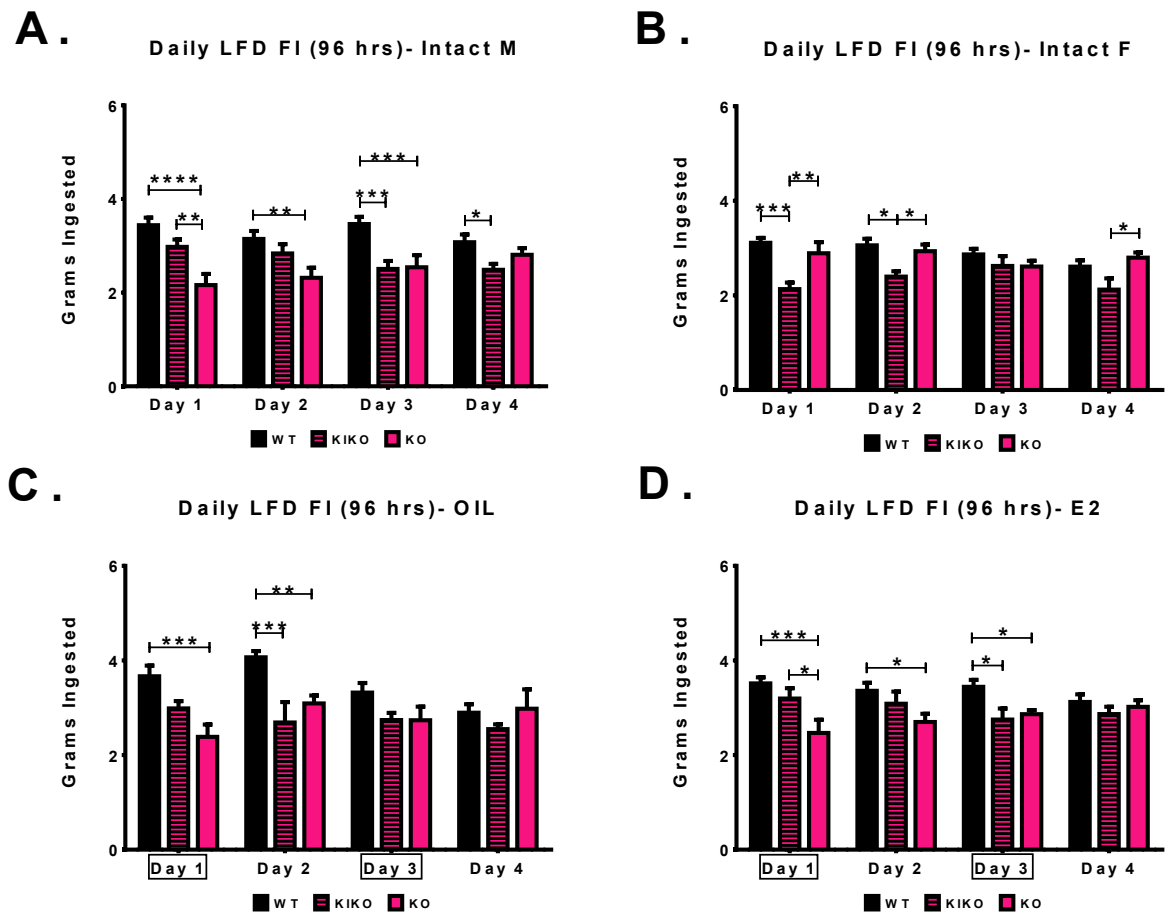

A.
Daily LFD FI (96 hrs)- Intact M
Grams Ingested
Day 1
Day 2
Day 3
Day 4
WT
KIKO
KO
B.
Daily LFD FI (96 hrs)- Intact F
C.
Daily LFD FI (96 hrs)- OIL
D.
Daily LFD FI (96 hrs)- E2

Figure 5

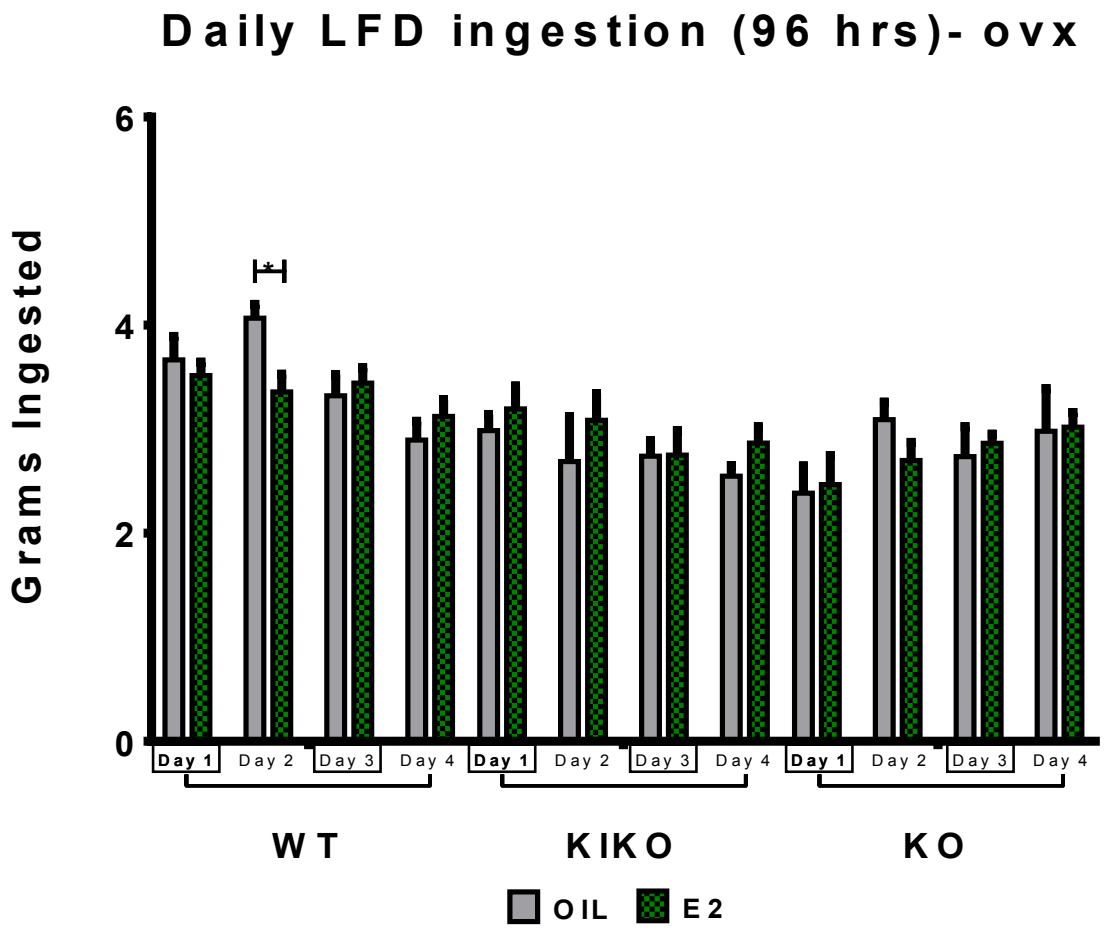

Daily LFD ingestion (96 hrs)- ovx
Grams Ingested
6
4
2
0
*
Day 1
Day 2
Day 3
Day 4
Day 1
Day 2
Day 3
Day 4
Day 1
Day 2
Day 3
Day 4
WT
KIKO
KO
OIL
E2

Figure 6

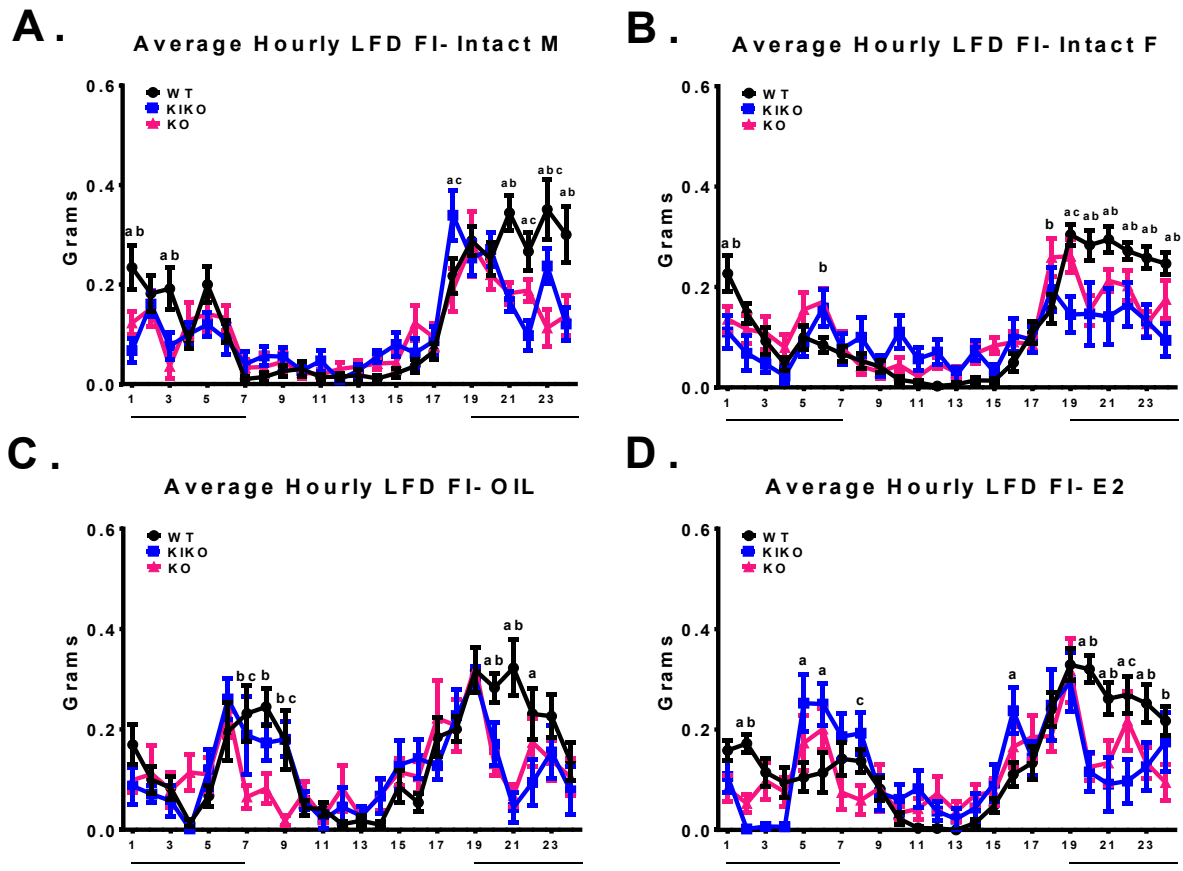

A.
Average Hourly LFD FI- Intact M
B.
Average Hourly LFD FI- Intact F
C.
Average Hourly LFD FI- OIL
D.
Average Hourly LFD FI- E2
WT
KIKO
KO
Grams
0.0
0.2
0.4
0.6
1 3 5 7 9 11 13 15 17 19 21 23

Figure 7

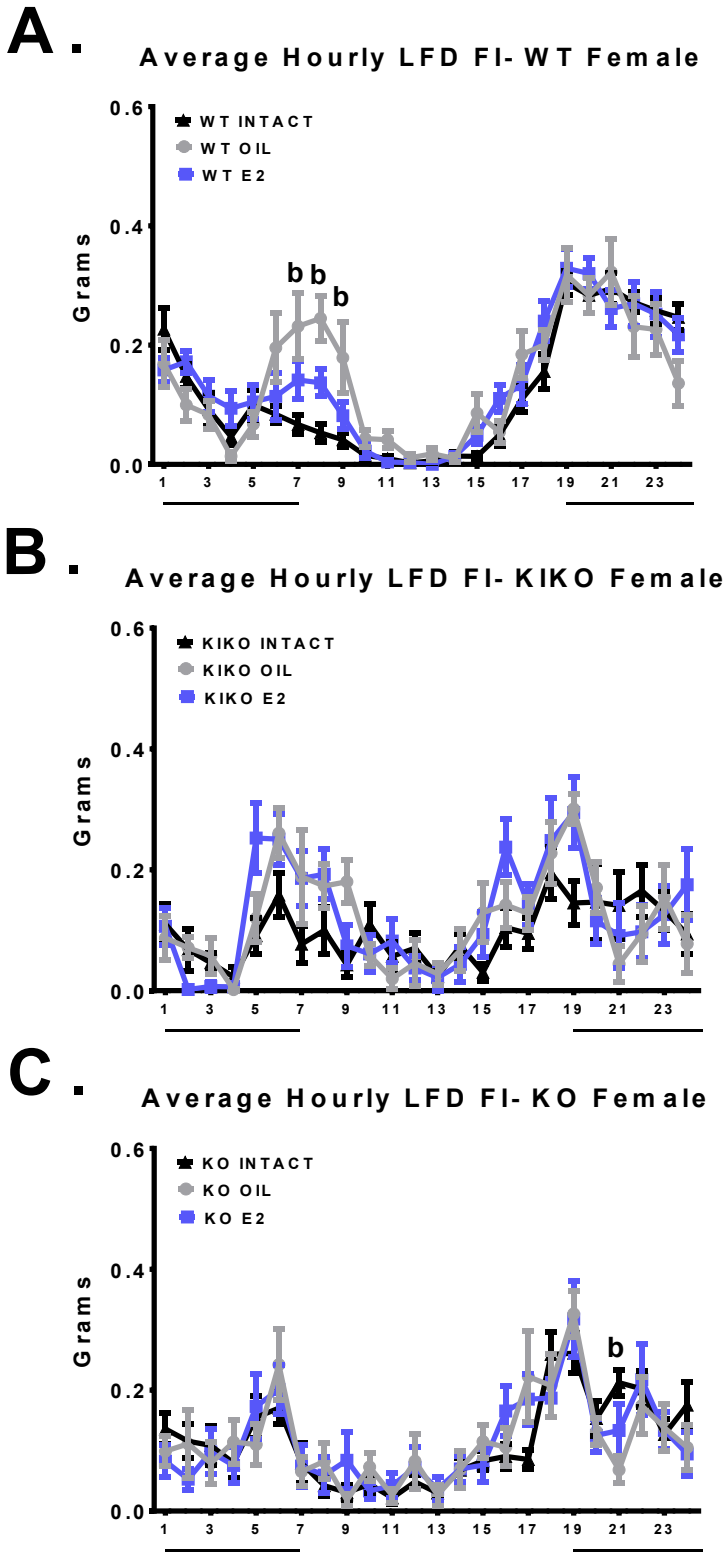
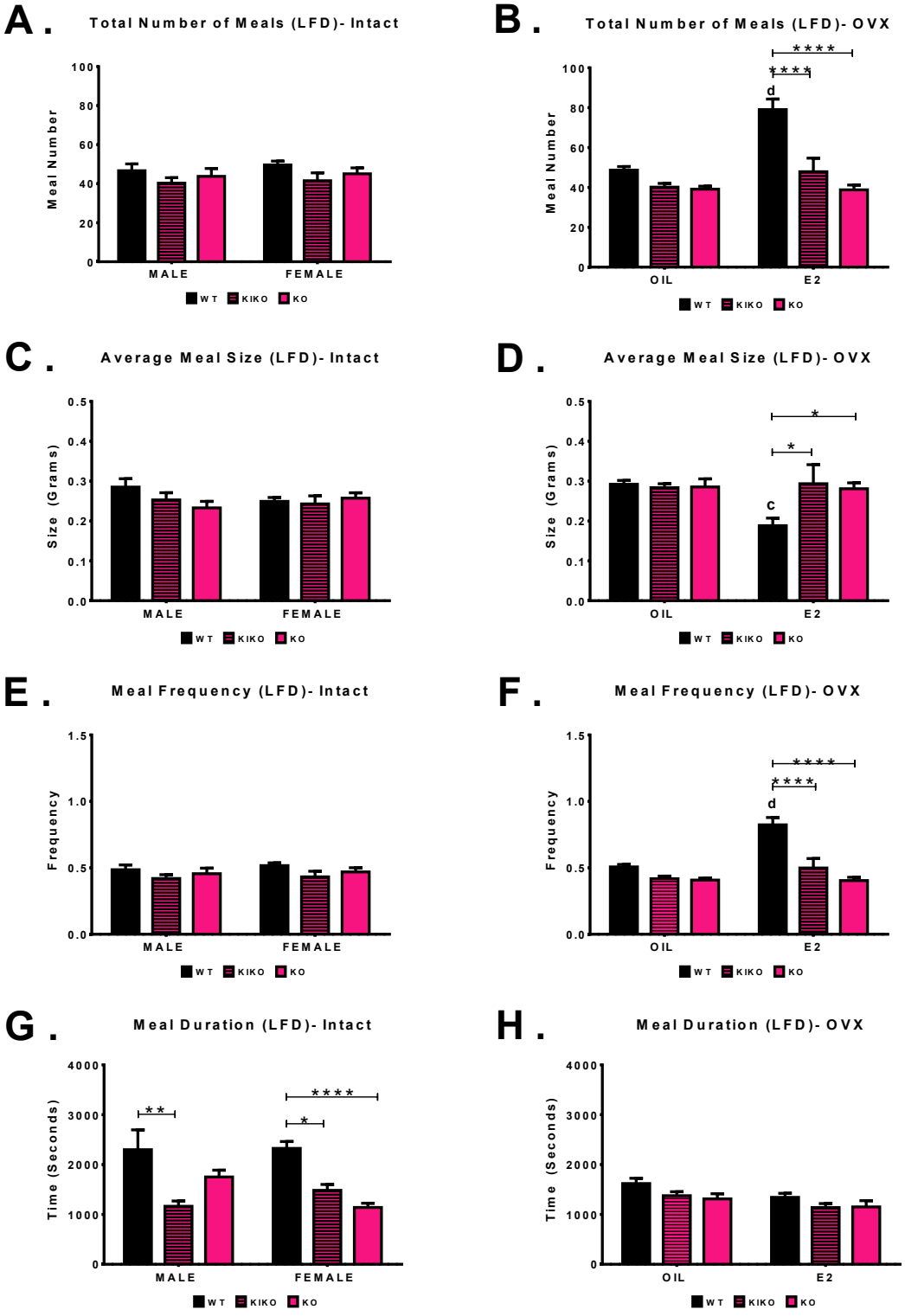

A.
Average Hourly LFD FI- WT Female
WT INTACT
WT OIL
WT E2
Grams
0.6
0.4
0.2
0.0
b b b
1 3 5 7 9 11 13 15 17 19 21 23
B.
Average Hourly LFD FI- KIKO Female
KIKO INTACT
KIKO OIL
KIKO E2
Grams
0.6
0.4
0.2
0.0
1 3 5 7 9 11 13 15 17 19 21 23
C.
Average Hourly LFD FI- KO Female
KO INTACT
KO OIL
KO E2
Grams
0.6
0.4
0.2
0.0
b
1 3 5 7 9 11 13 15 17 19 21 23

Figure 8


A.
Total Number of Meals (LFD)- Intact
Meal Number
MALE
FEMALE
WT KIKO KO
B.
Total Number of Meals (LFD)- OVX
****
****
d
OIL
E2
C.
Average Meal Size (LFD)- Intact
Size (Grams)
D.
Average Meal Size (LFD)- OVX
*
*
c
E.
Meal Frequency (LFD)- Intact
Frequency
F.
Meal Frequency (LFD)- OVX
G.
Meal Duration (LFD)- Intact
Time (Seconds)
**
*
H.
Meal Duration (LFD)- OVX

Experiment 1b: The effects of sex, genotype, and steroid on feeding behaviors on a HFD

Figure 9

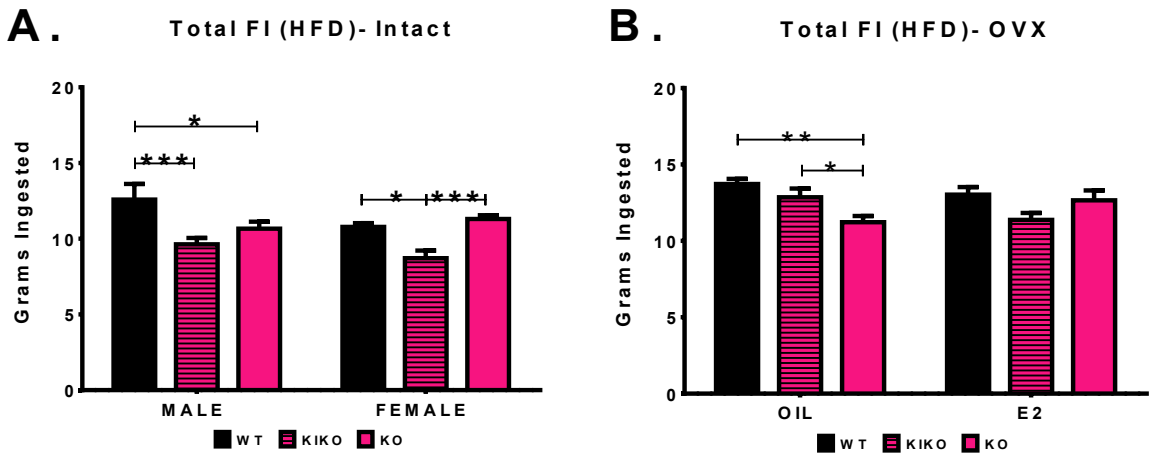


Figure 10

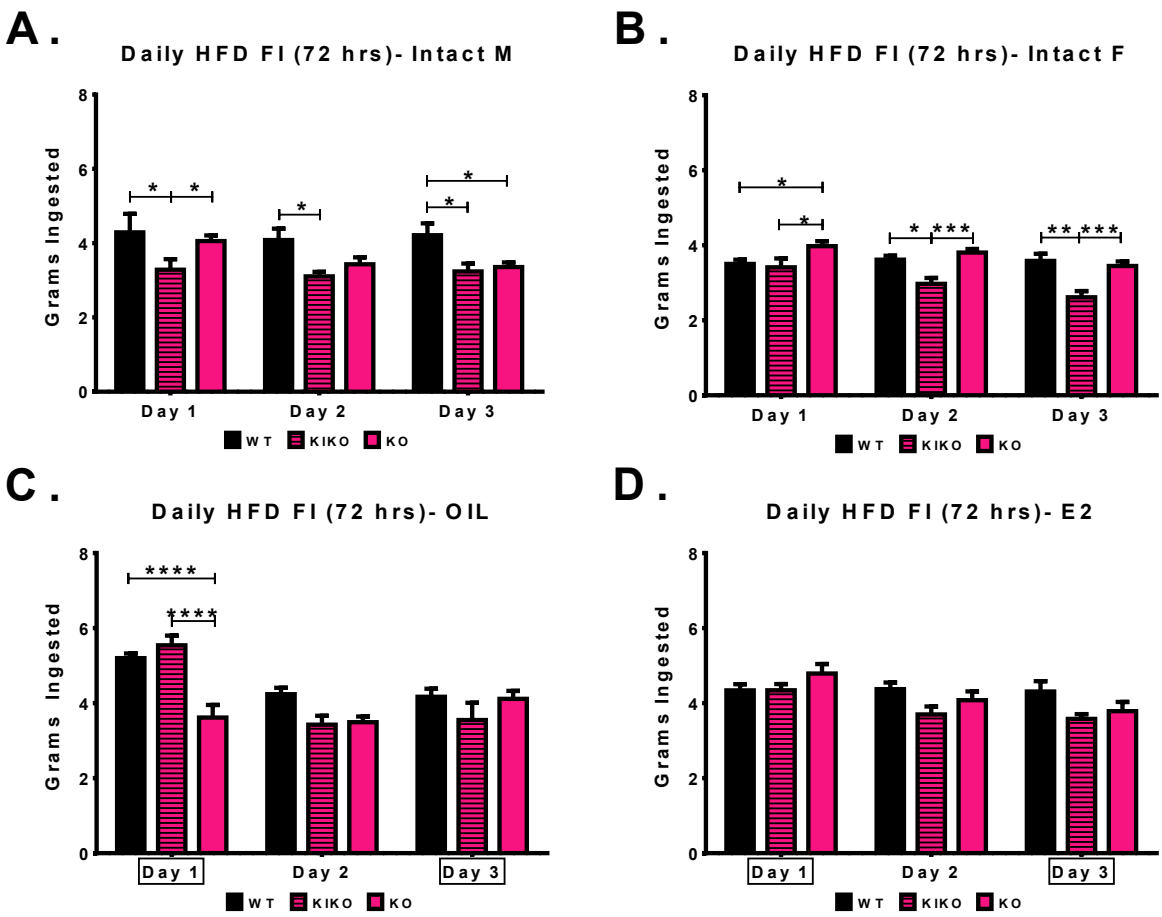

A.
Daily HFD FI (72 hrs)- Intact M
Grams Ingested
Day 1
Day 2
Day 3
WT
KIKO
KO
B.
Daily HFD FI (72 hrs)- Intact F
C.
Daily HFD FI (72 hrs)- OIL
D.
Daily HFD FI (72 hrs)- E2

Figure 11

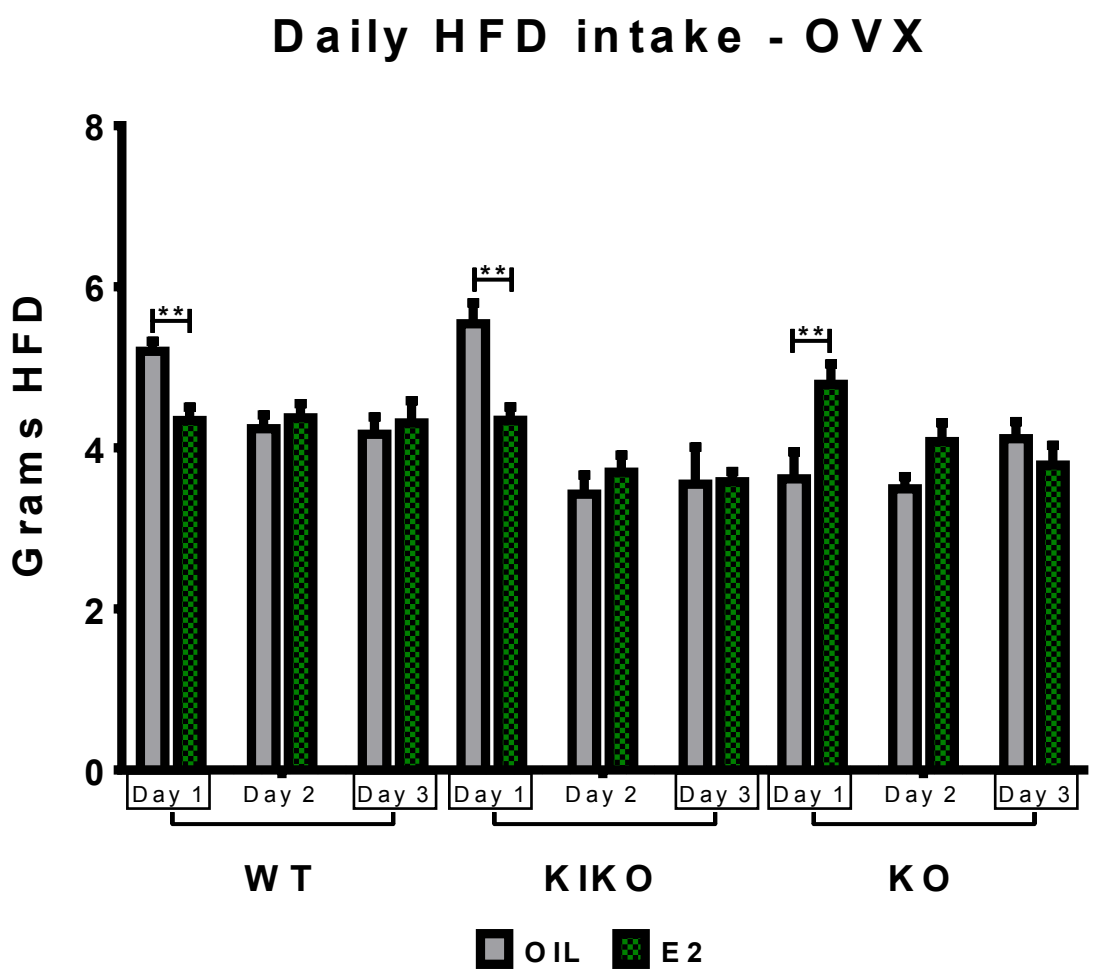

Daily HFD intake - OVX
Grams HFD
8
6
4
2
0
**
**
**
Day 1
Day 2
Day 3
Day 1
Day 2
Day 3
Day 1
Day 2
Day 3
WT
KIKO
KO
OIL
E2

Figure 12

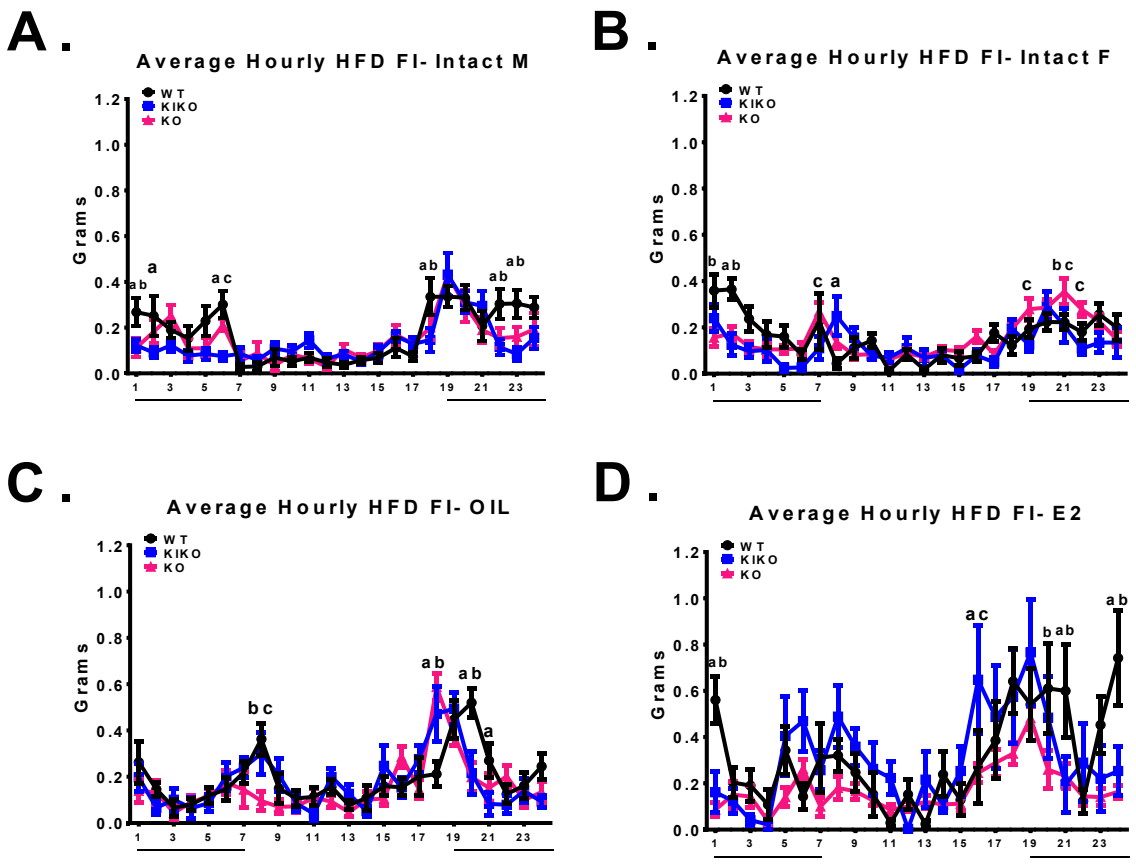

A.
Average Hourly HFD FI- Intact M
B.
Average Hourly HFD FI- Intact F
C.
Average Hourly HFD FI- OIL
D.
Average Hourly HFD FI- E2
WT
KIKO
KO
Grams

Figure 13

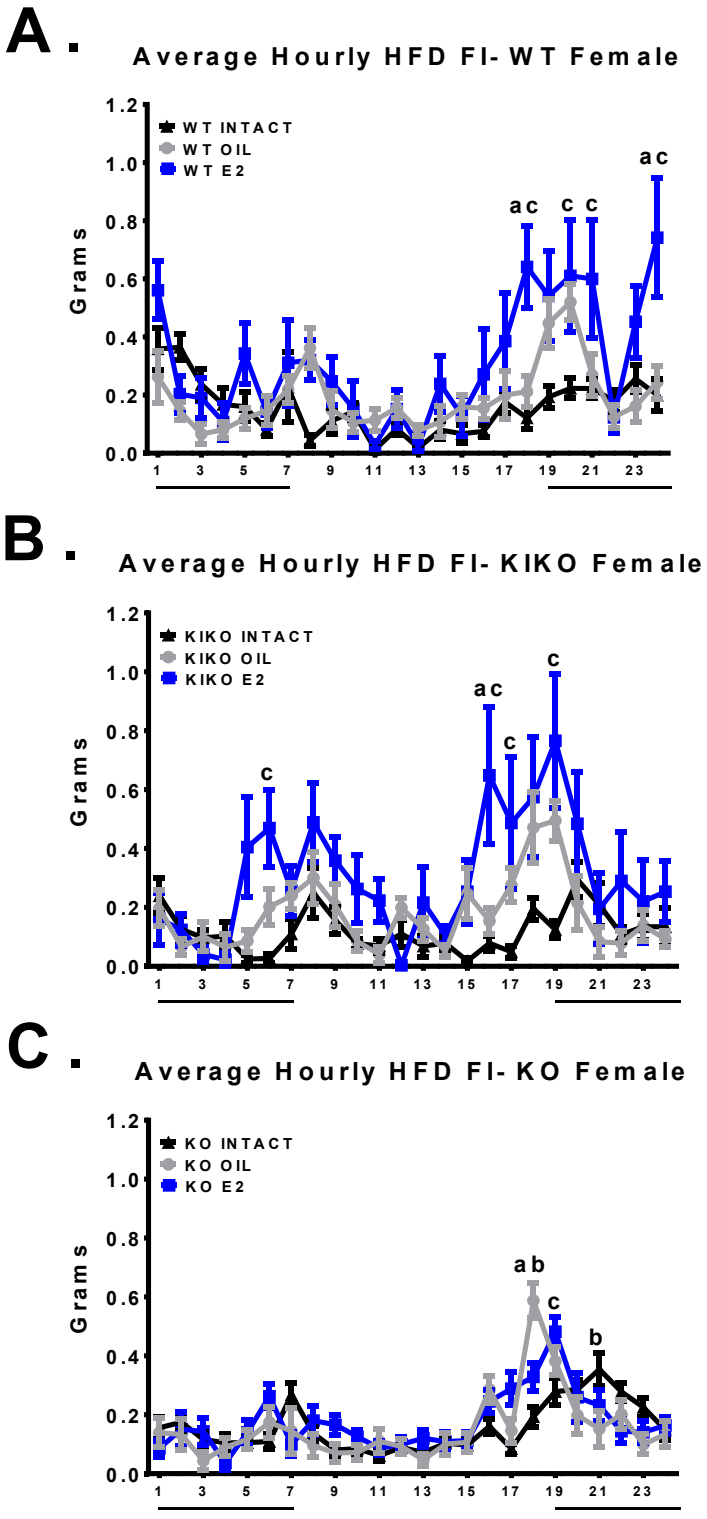
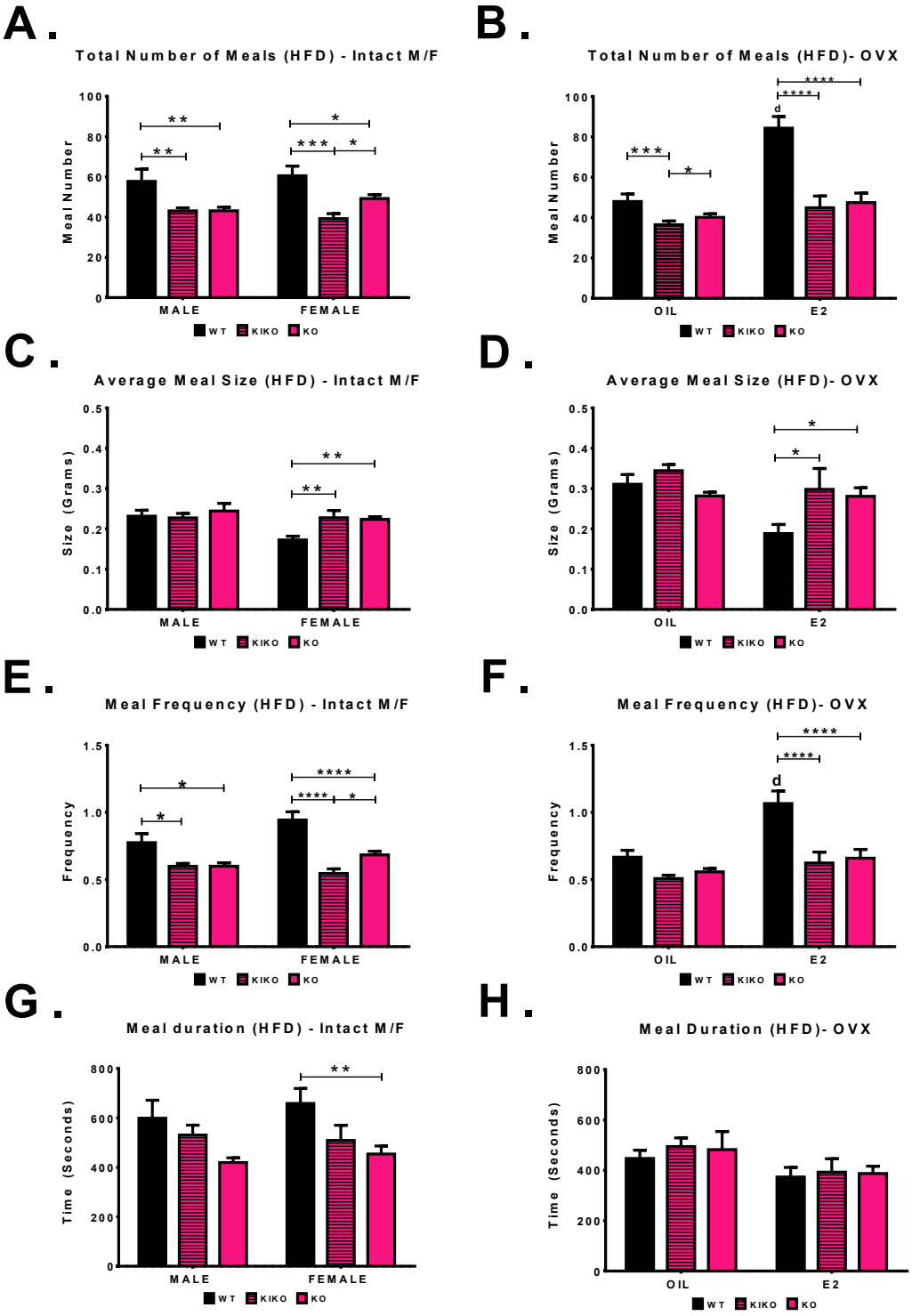

A.
Average Hourly HFD FI- WT Female
WT INTACT
WT OIL
WT E2
Grams
ac
ac c c
B.
Average Hourly HFD FI- KIKO Female
KIKO INTACT
KIKO OIL
KIKO E2
Grams
c
ac
c
C.
Average Hourly HFD FI- KO Female
KO INTACT
KO OIL
KO E2
Grams
ab
c
b

Figure 14


A.
Total Number of Meals (HFD) - Intact M/F
Meal Number
MALE
FEMALE
WT KIKO KO
B.
Total Number of Meals (HFD)- OVX
Meal Number
OIL
E2
WT KIKO KO
C.
Average Meal Size (HFD) - Intact M/F
Size (Grams)
MALE
FEMALE
WT KIKO KO
D.
Average Meal Size (HFD)- OVX
Size (Grams)
OIL
E2
WT KIKO KO
E.
Meal Frequency (HFD) - Intact M/F
Frequency
MALE
FEMALE
WT KIKO KO
F.
Meal Frequency (HFD)- OVX
Frequency
OIL
E2
WT KIKO KO
G.
Meal duration (HFD) - Intact M/F
Time (Seconds)
MALE
FEMALE
WT KIKO KO
H.
Meal Duration (HFD)- OVX
Time (Seconds)
OIL
E2
WT KIKO KO

Experiment 1b.2: The effects of sex, genotype, and steroid on feeding behaviors attributed with the first HFD meal

Figure 15

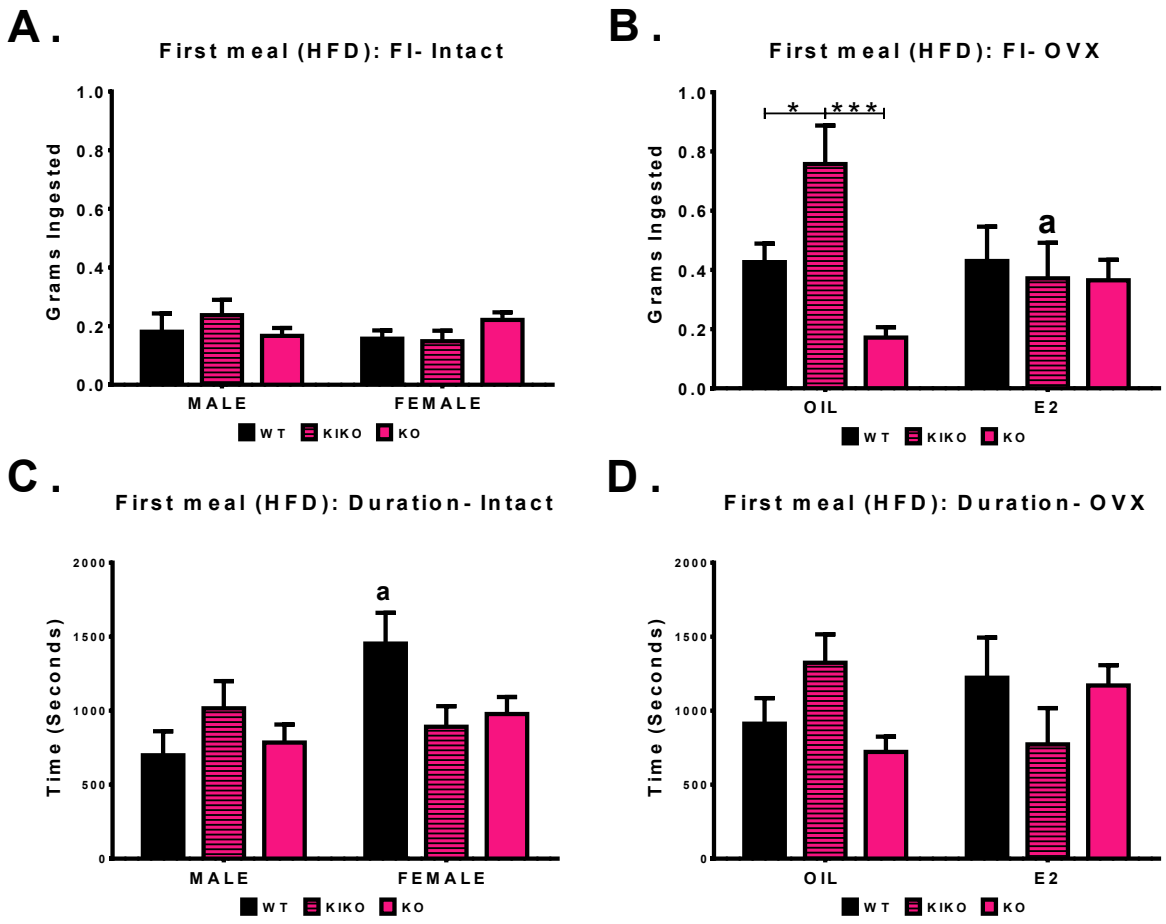


Figure 16

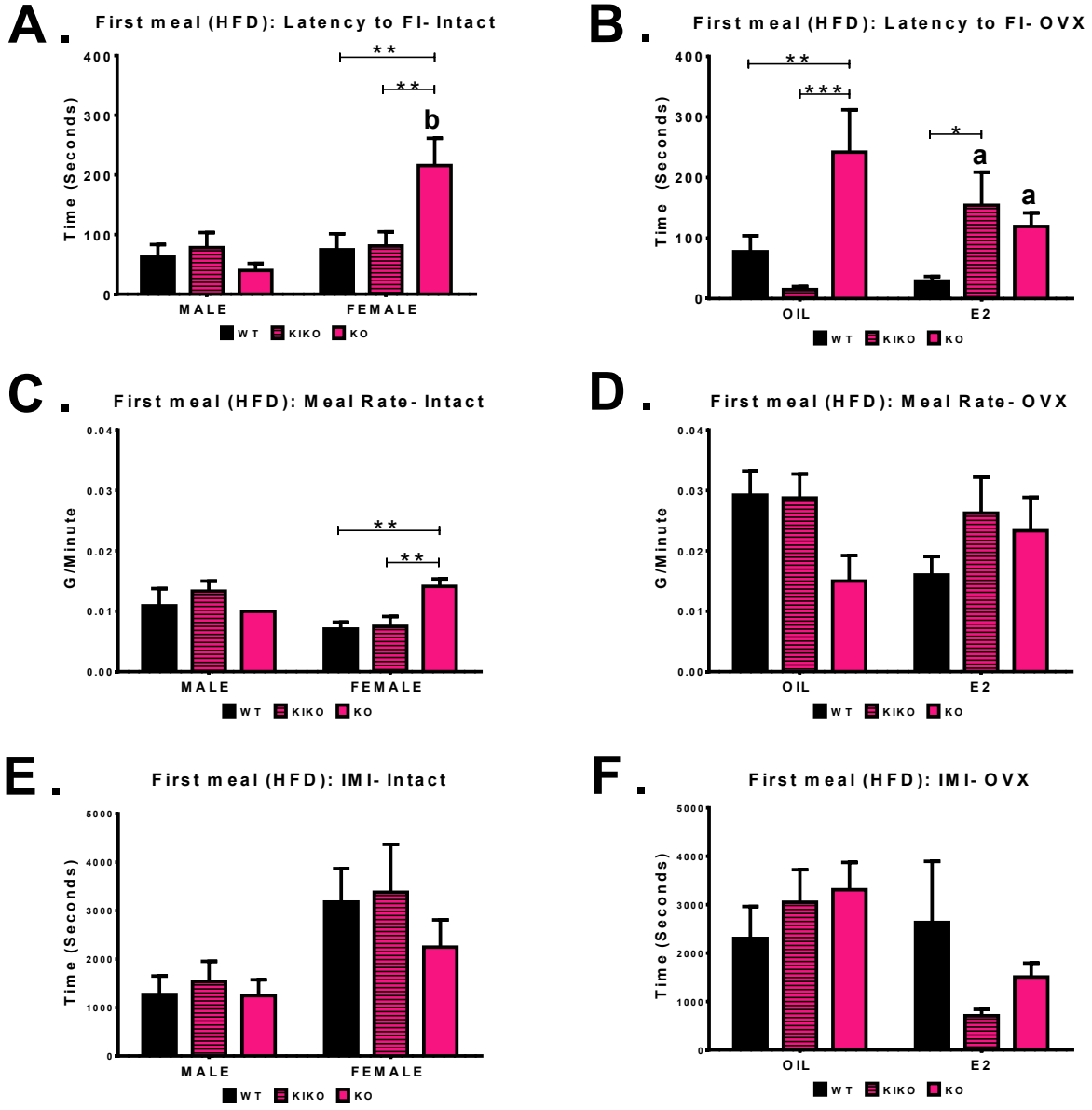

A.
First meal (HFD): Latency to FI- Intact
Time (Seconds)
400
300
200
100
0
**
**
b
MALE
FEMALE
WT
KIKO
KO
B.
First meal (HFD): Latency to FI- OVX
Time (Seconds)
400
300
200
100
0
**
***
*
a
a
OIL
E2
WT
KIKO
KO
C.
First meal (HFD): Meal Rate- Intact
G/Minute
0.04
0.03
0.02
0.01
0.00
**
**
MALE
FEMALE
WT
KIKO
KO
D.
First meal (HFD): Meal Rate- OVX
G/Minute
0.04
0.03
0.02
0.01
0.00
OIL
E2
WT
KIKO
KO
E.
First meal (HFD): IMI- Intact
Time (Seconds)
5000
4000
3000
2000
1000
0
MALE
FEMALE
WT
KIKO
KO
F.
First meal (HFD): IMI- OVX
Time (Seconds)
5000
4000
3000
2000
1000
0
OIL
E2
WT
KIKO
KO

Figure 17

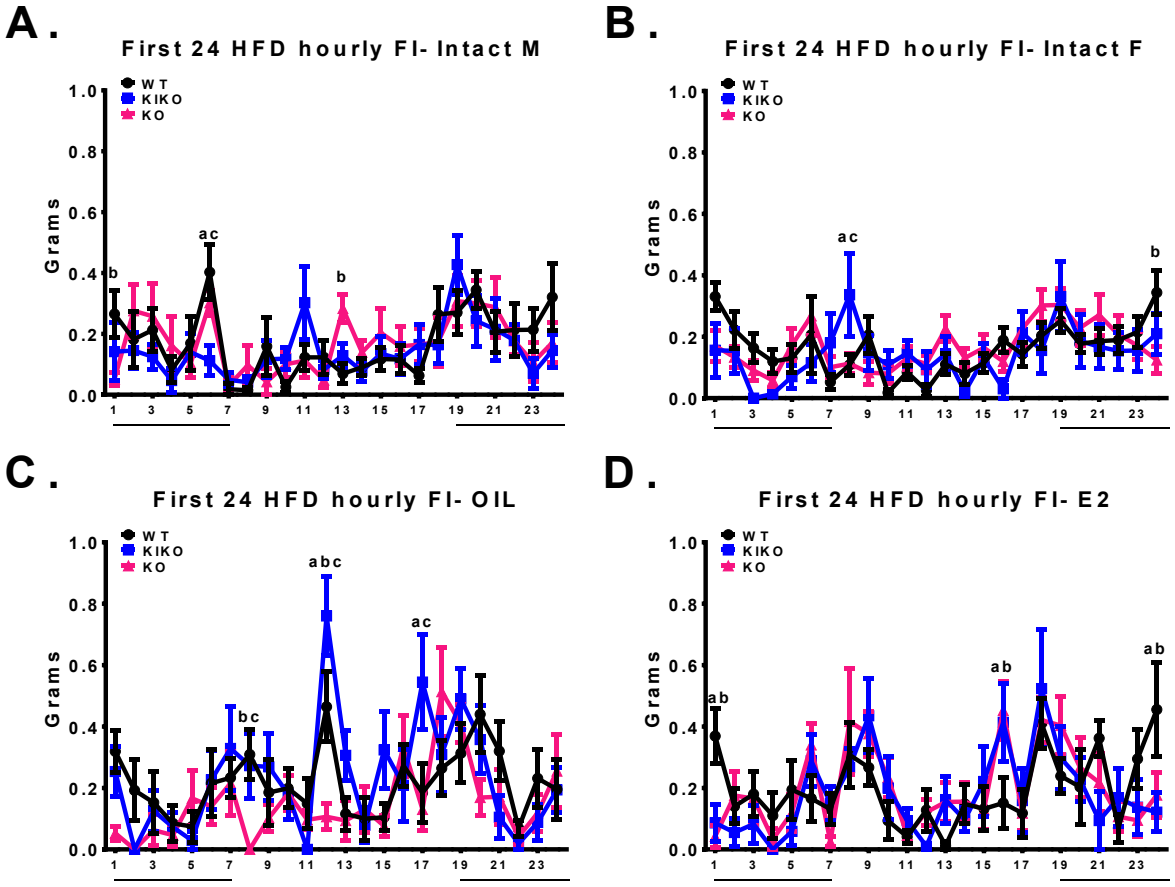
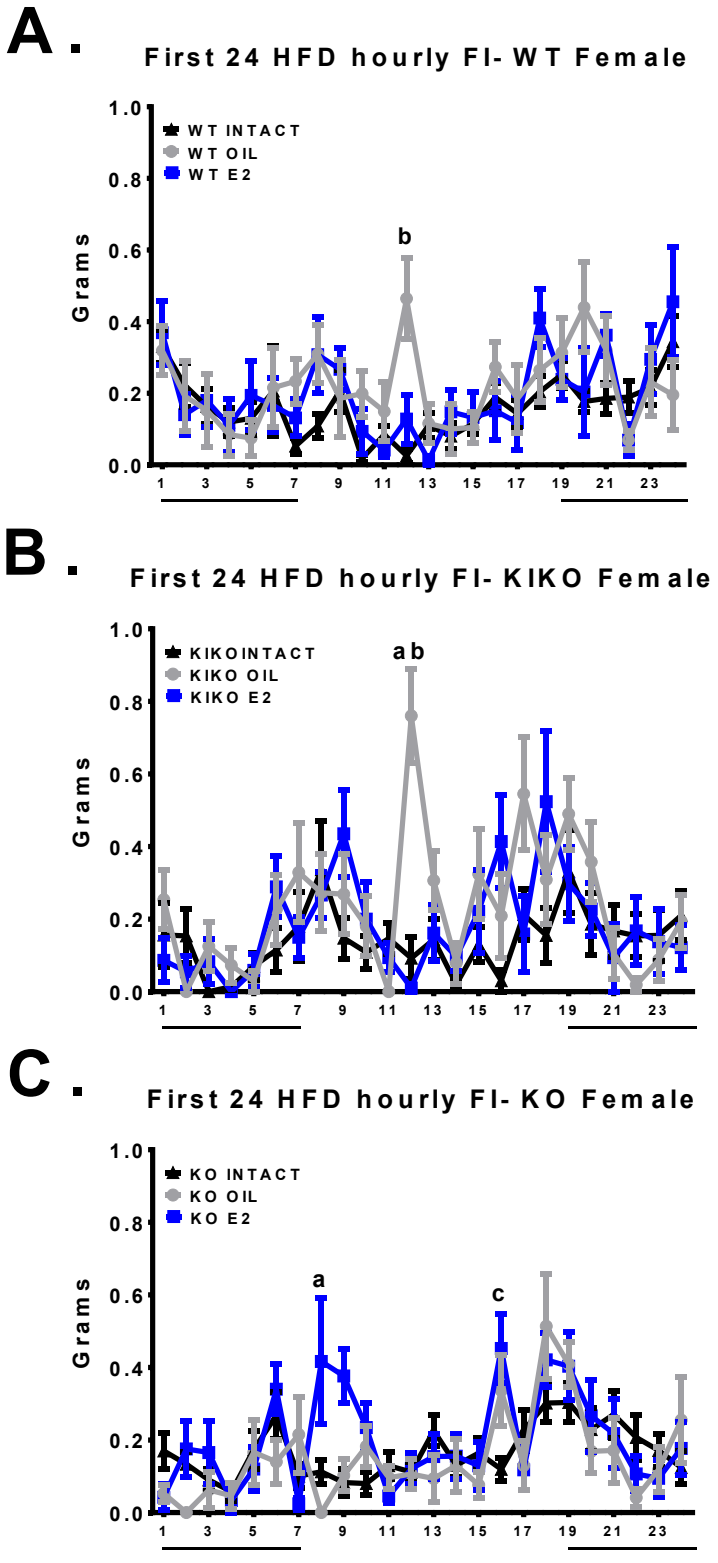

A.
First 24 HFD hourly FI- Intact M
B.
First 24 HFD hourly FI- Intact F
C.
First 24 HFD hourly FI- OIL
D.
First 24 HFD hourly FI- E2
WT
KIKO
KO
Grams
1.0
0.8
0.6
0.4
0.2
0.0
1 3 5 7 9 11 13 15 17 19 21 23
b
ac
b
ac
b
bc
abc
ac
ab
ab
ab

Figure 18


A.
First 24 HFD hourly FI- WT Female
WT INTACT
WT OIL
WT E2
Grams
b
B.
First 24 HFD hourly FI- KIKO Female
KIKOINTACT
KIKO OIL
KIKO E2
Grams
ab
C.
First 24 HFD hourly FI- KO Female
KO INTACT
KO OIL
KO E2
Grams
a
c

Experiment 1c: The effects of sex, genotype, and steroid on feeding behaviors after a 24 h fast

Figure 19

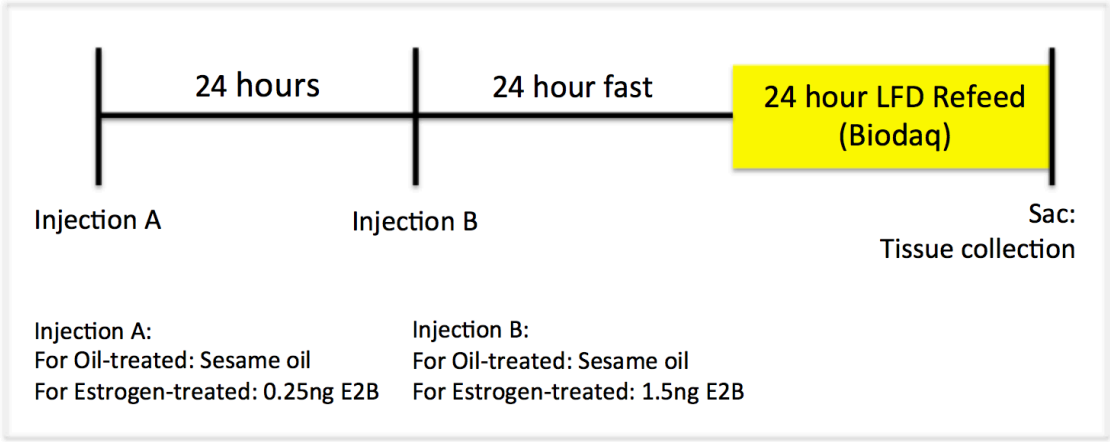


Figure 20

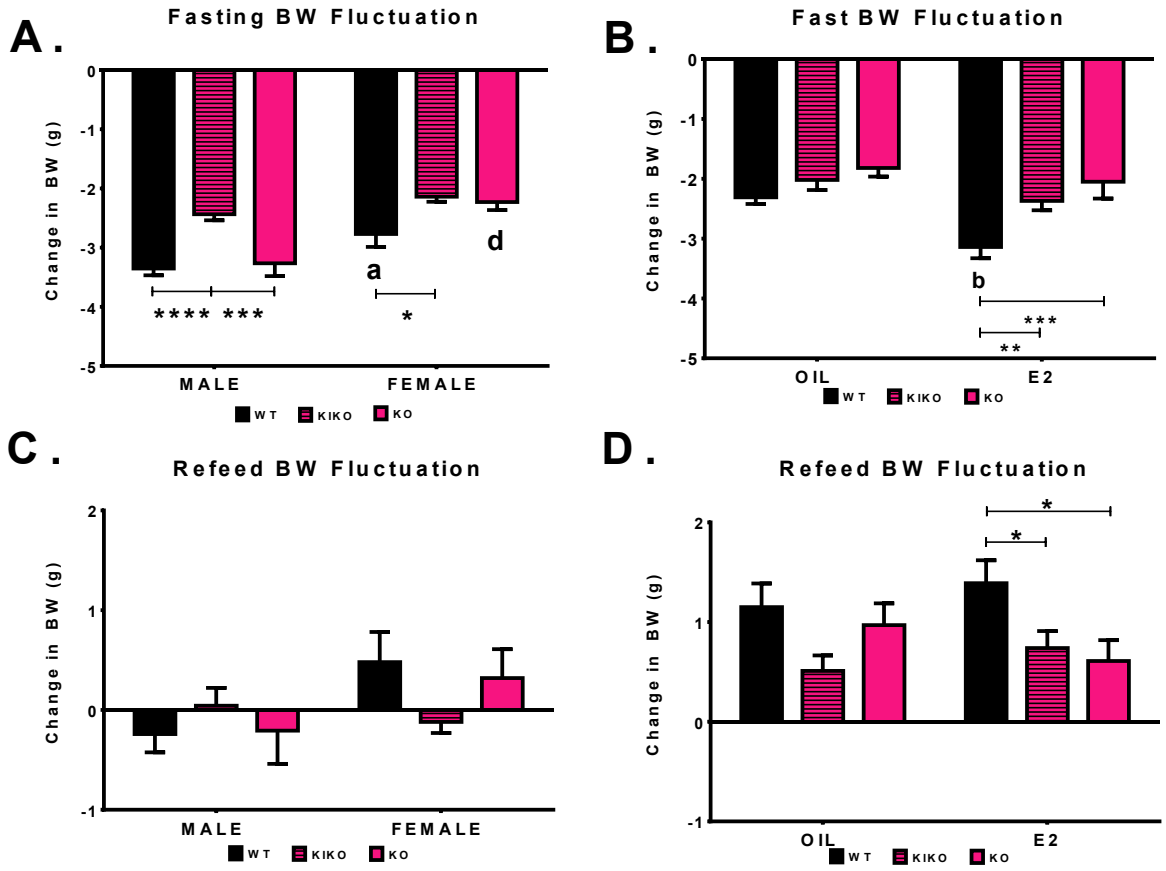

A.
Fasting BW Fluctuation
Change in BW (g)
0
-1
-2
-3
-4
-5
**** ***
*
a
d
MALE
FEMALE
WT
KIKO
KO
B.
Fast BW Fluctuation
Change in BW (g)
b
***
**
OIL
E2
WT
KIKO
KO
C.
Refeed BW Fluctuation
Change in BW (g)
2
1
0
-1
MALE
FEMALE
WT
KIKO
KO
D.
Refeed BW Fluctuation
Change in BW (g)
*
*
OIL
E2
WT
KIKO
KO

Figure 21

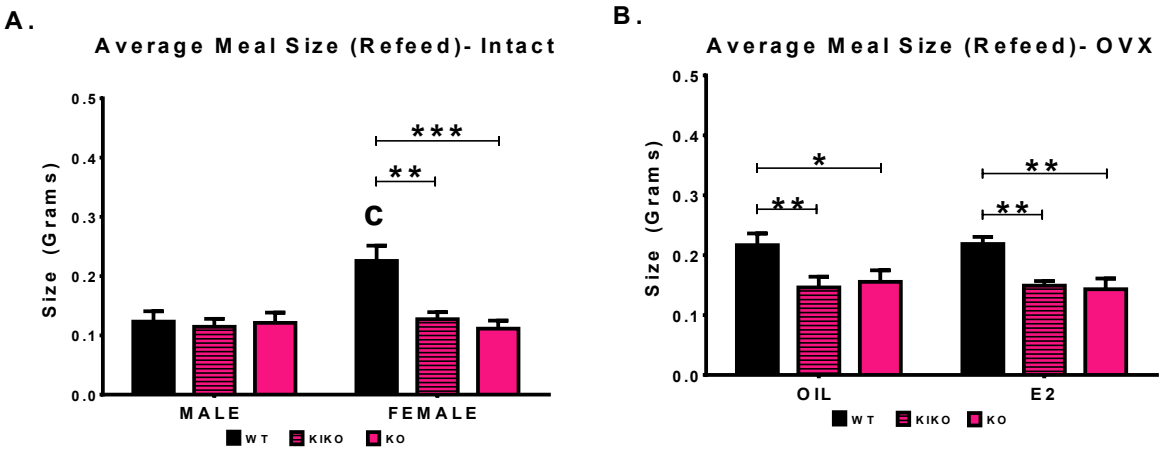

A.
Average Meal Size (Refeed)- Intact
Size (Grams)
0.5
0.4
0.3
0.2
0.1
0.0
***
**
C
MALE
FEMALE
WT
KIKO
KO
B.
Average Meal Size (Refeed)- OVX
Size (Grams)
0.5
0.4
0.3
0.2
0.1
0.0
*
**
**
**
OIL
E2
WT
KIKO
KO

Figure 22

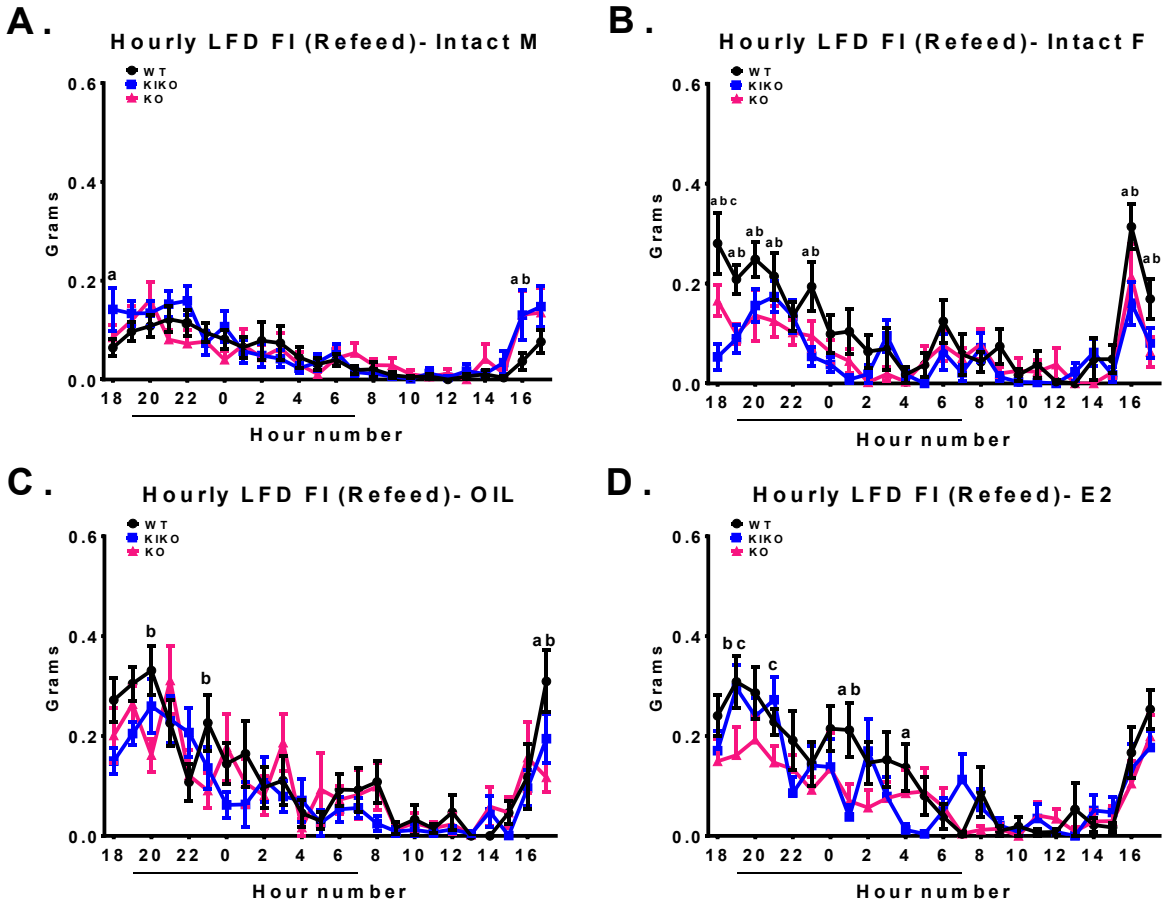

A.
Hourly LFD FI (Refeed)- Intact M
B.
Hourly LFD FI (Refeed)- Intact F
C.
Hourly LFD FI (Refeed)- OIL
D.
Hourly LFD FI (Refeed)- E2
WT
KIKO
KO
Grams
Hour number
18 20 22 0 2 4 6 8 10 12 14 16
0.0 0.2 0.4 0.6

Figure 23

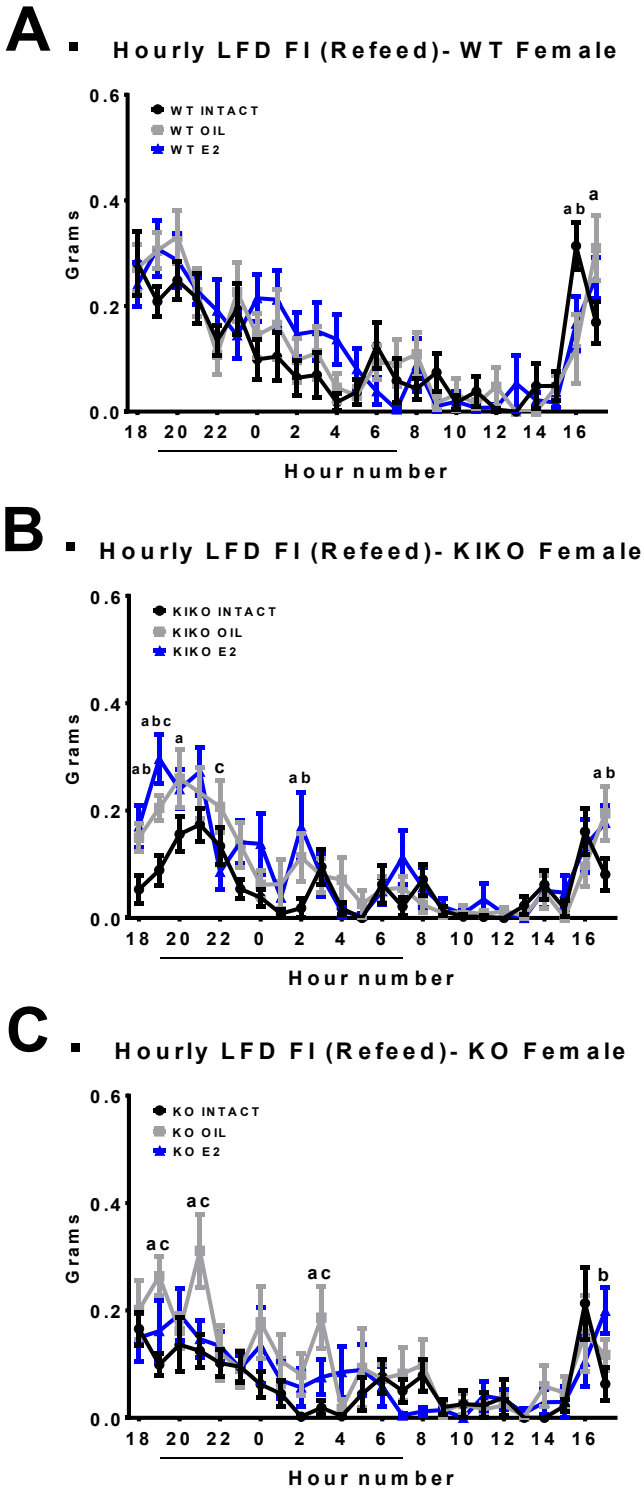

A
Hourly LFD FI (Refeed)- WT Female
WT INTACT
WT OIL
WT E2
Grams
0.6
0.4
0.2
0.0
ab
a
18 20 22 0 2 4 6 8 10 12 14 16
Hour number
B
Hourly LFD FI (Refeed)- KIKO Female
KIKO INTACT
KIKO OIL
KIKO E2
Grams
0.6
0.4
0.2
0.0
abc
a
ab
c
ab
ab
18 20 22 0 2 4 6 8 10 12 14 16
Hour number
C
Hourly LFD FI (Refeed)- KO Female
KO INTACT
KO OIL
KO E2
Grams
0.6
0.4
0.2
0.0
ac
ac
ac
b
18 20 22 0 2 4 6 8 10 12 14 16
Hour number

Figure 24

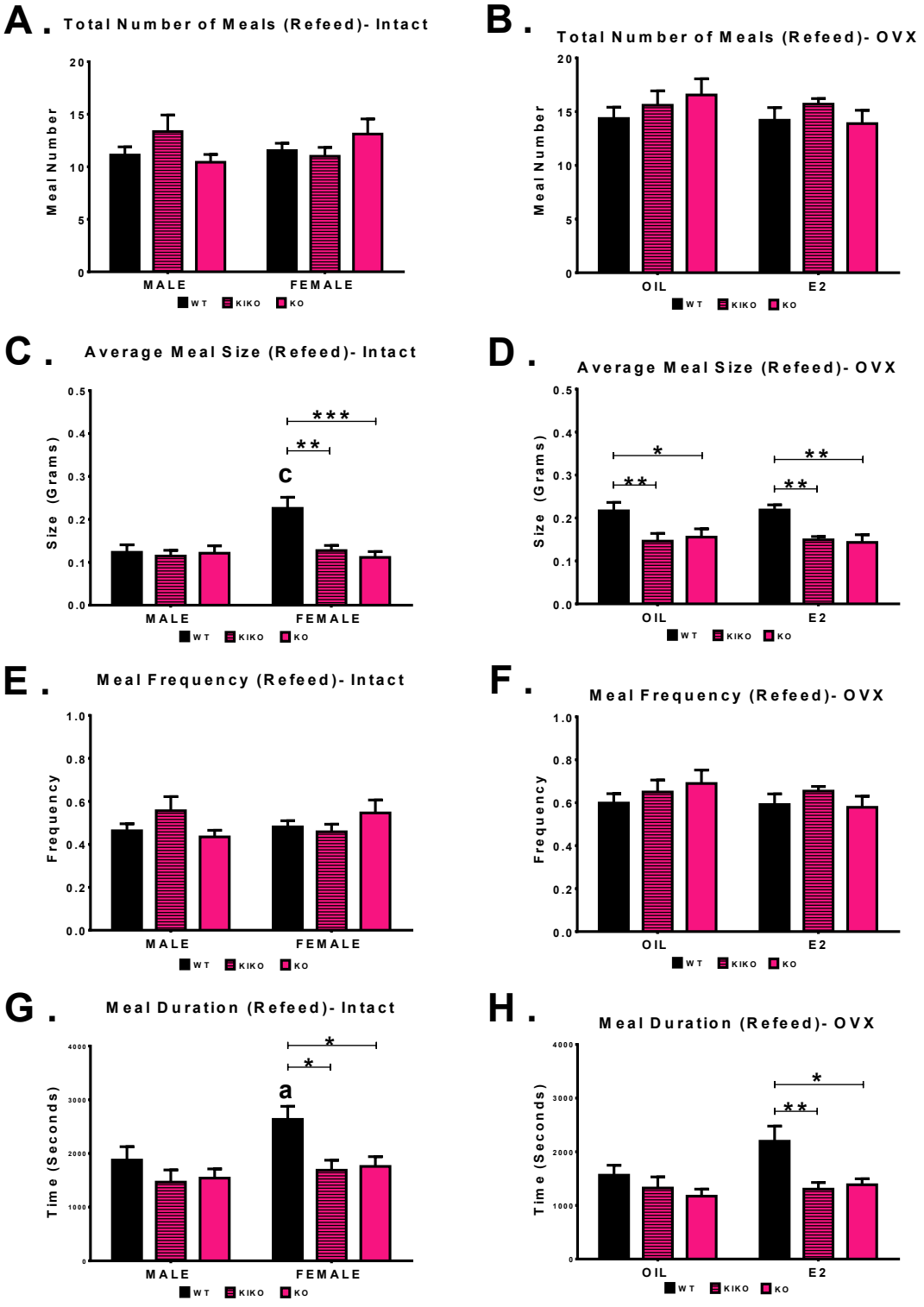

A.
Total Number of Meals (Refeed)- Intact
Meal Number
MALE
FEMALE
WT KIKO KO
B.
Total Number of Meals (Refeed)- OVX
Meal Number
OIL
E2
WT KIKO KO
C.
Average Meal Size (Refeed)- Intact
Size (Grams)
***
**
c
MALE
FEMALE
WT KIKO KO
D.
Average Meal Size (Refeed)- OVX
Size (Grams)
*
**
**
**
OIL
E2
WT KIKO KO
E.
Meal Frequency (Refeed)- Intact
Frequency
MALE
FEMALE
WT KIKO KO
F.
Meal Frequency (Refeed)- OVX
Frequency
OIL
E2
WT KIKO KO
G.
Meal Duration (Refeed)- Intact
Time (Seconds)
*
*
a
MALE
FEMALE
WT KIKO KO
H.
Meal Duration (Refeed)- OVX
Time (Seconds)
*
**
OIL
E2
WT KIKO KO

Figure 25

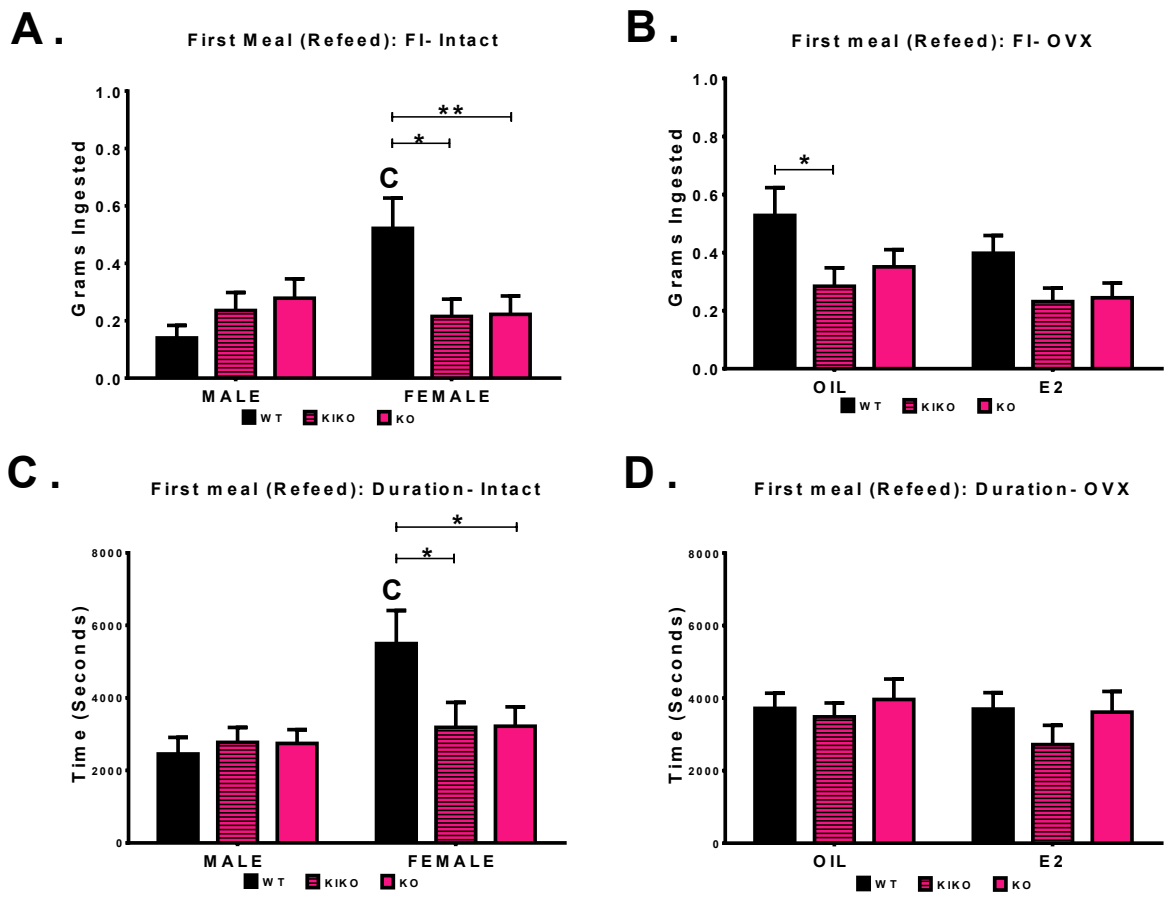
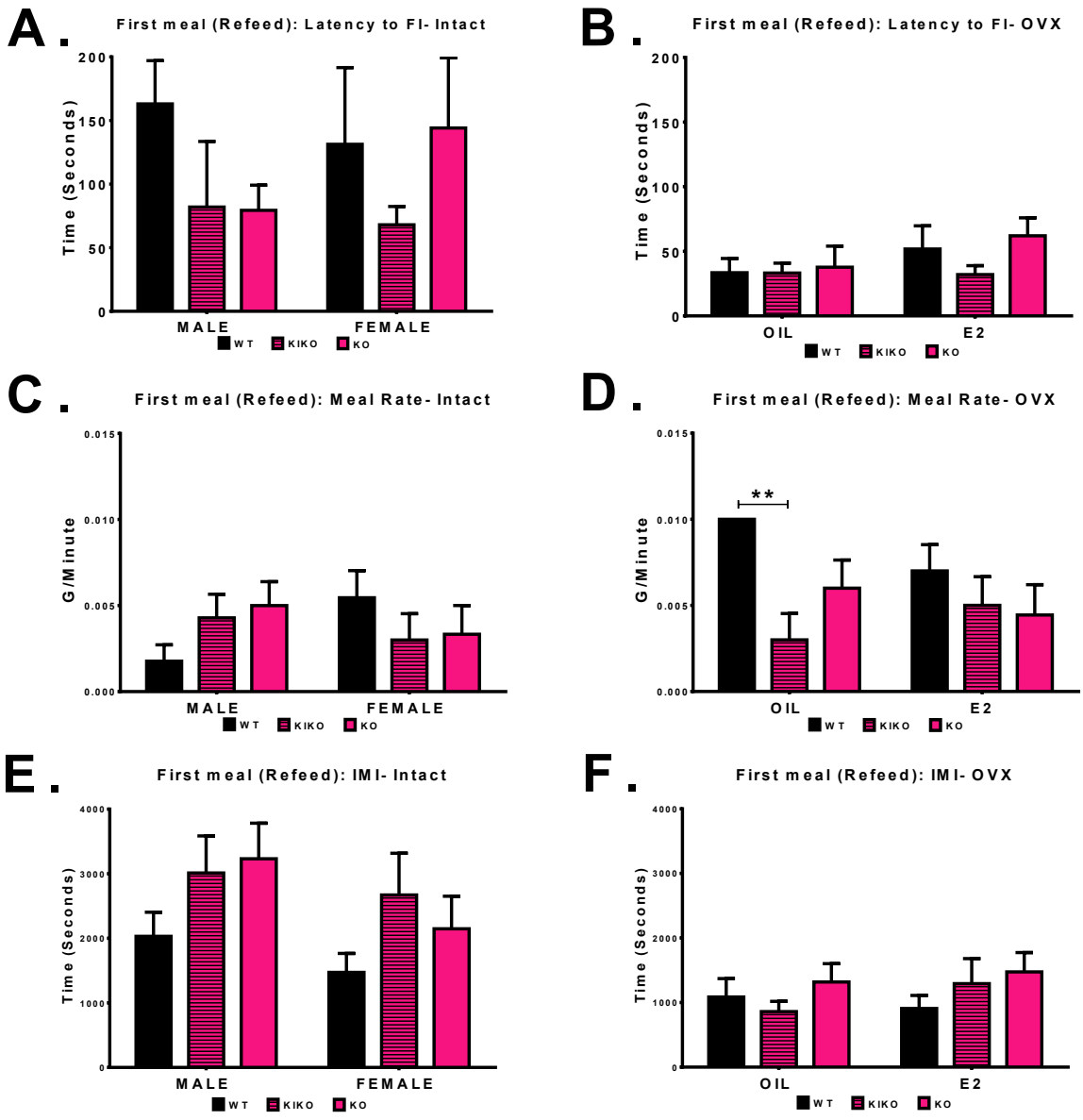

A.
First Meal (Refeed): FI- Intact
Grams Ingested
1.0
0.8
0.6
0.4
0.2
0.0
**
*
C
MALE
FEMALE
WT
KIKO
KO
B.
First meal (Refeed): FI- OVX
Grams Ingested
1.0
0.8
0.6
0.4
0.2
0.0
*
OIL
E2
WT
KIKO
KO
C.
First meal (Refeed): Duration- Intact
Time (Seconds)
8000
6000
4000
2000
0
*
*
C
MALE
FEMALE
WT
KIKO
KO
D.
First meal (Refeed): Duration- OVX
Time (Seconds)
8000
6000
4000
2000
0
OIL
E2
WT
KIKO
KO

Figure 26


A.
First meal (Refeed): Latency to FI- Intact
Time (Seconds)
200
150
100
50
0
MALE
FEMALE
WT
KIKO
KO
B.
First meal (Refeed): Latency to FI- OVX
Time (Seconds)
200
150
100
50
0
OIL
E2
WT
KIKO
KO
C.
First meal (Refeed): Meal Rate- Intact
G/Minute
0.015
0.010
0.005
0.000
MALE
FEMALE
WT
KIKO
KO
D.
First meal (Refeed): Meal Rate- OVX
G/Minute
0.015
0.010
0.005
0.000
**
OIL
E2
WT
KIKO
KO
E.
First meal (Refeed): IMI- Intact
Time (Seconds)
4000
3000
2000
1000
0
MALE
FEMALE
WT
KIKO
KO
F.
First meal (Refeed): IMI- OVX
Time (Seconds)
4000
3000
2000
1000
0
OIL
E2
WT
KIKO
KO

Experiment 2: The role of sex, genotype, and steroid on anxiety- and depressive-like behaviors

Figure 27

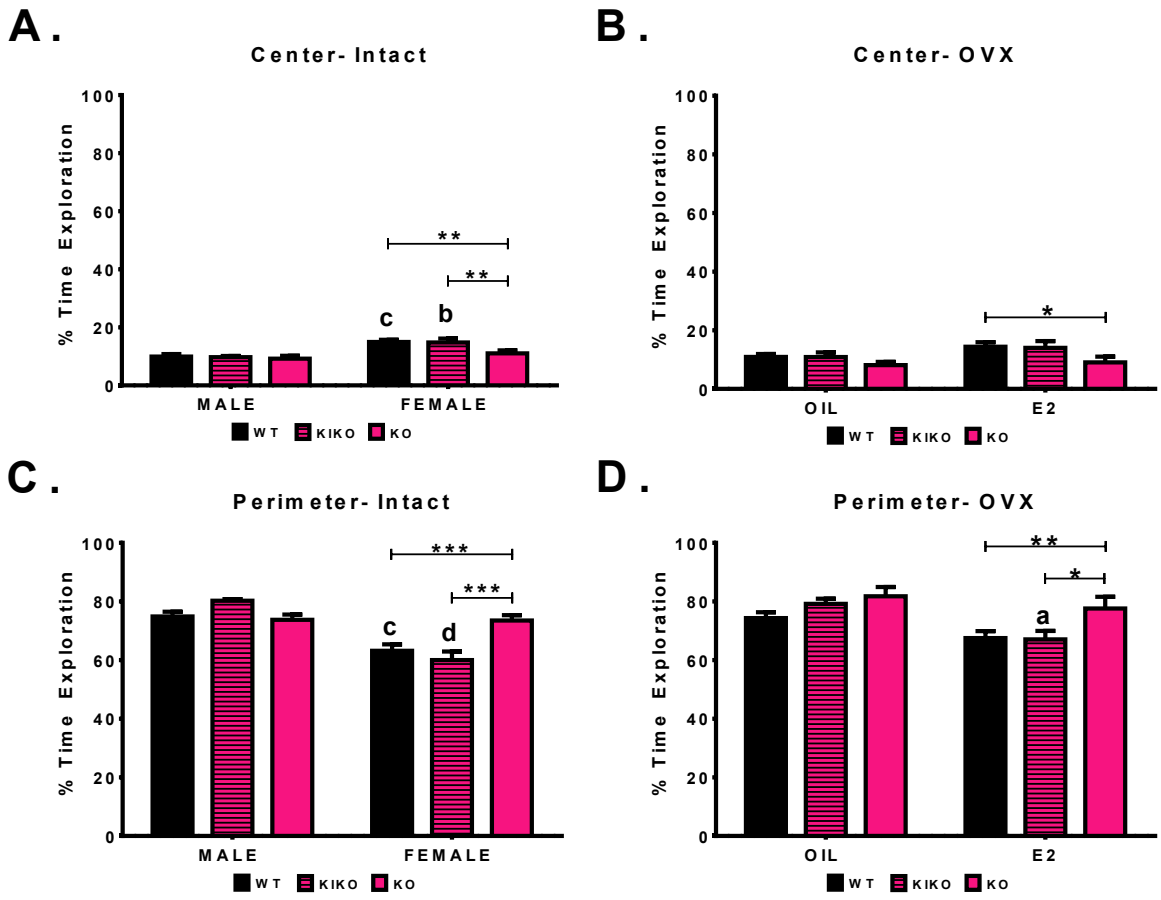


Figure 28

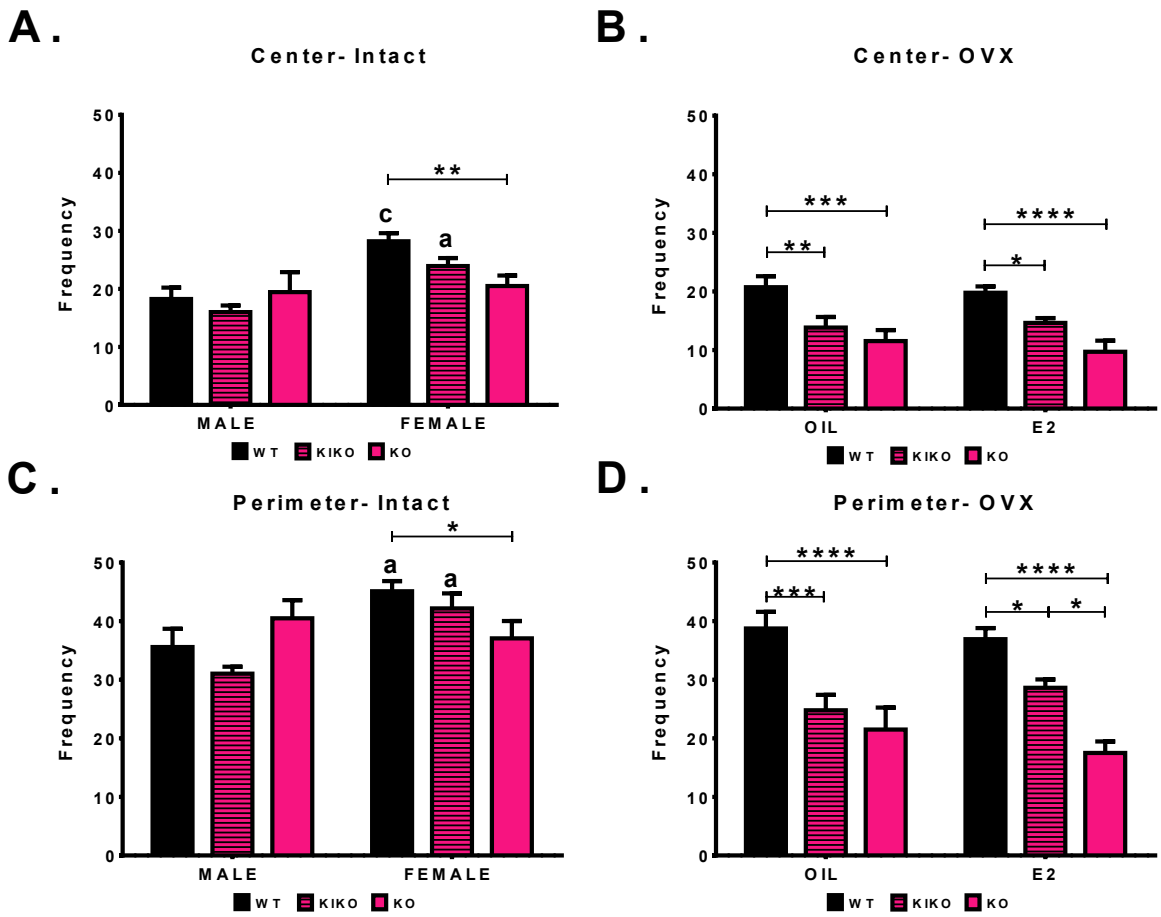

A.
Center- Intact
Frequency
50
40
30
20
10
0
**
c
a
MALE
FEMALE
WT
KIKO
KO
B.
Center- OVX
***
**
****
*
OIL
E2
C.
Perimeter- Intact
*
a
a
D.
Perimeter- OVX
****
***
****
*
*

Figure 29

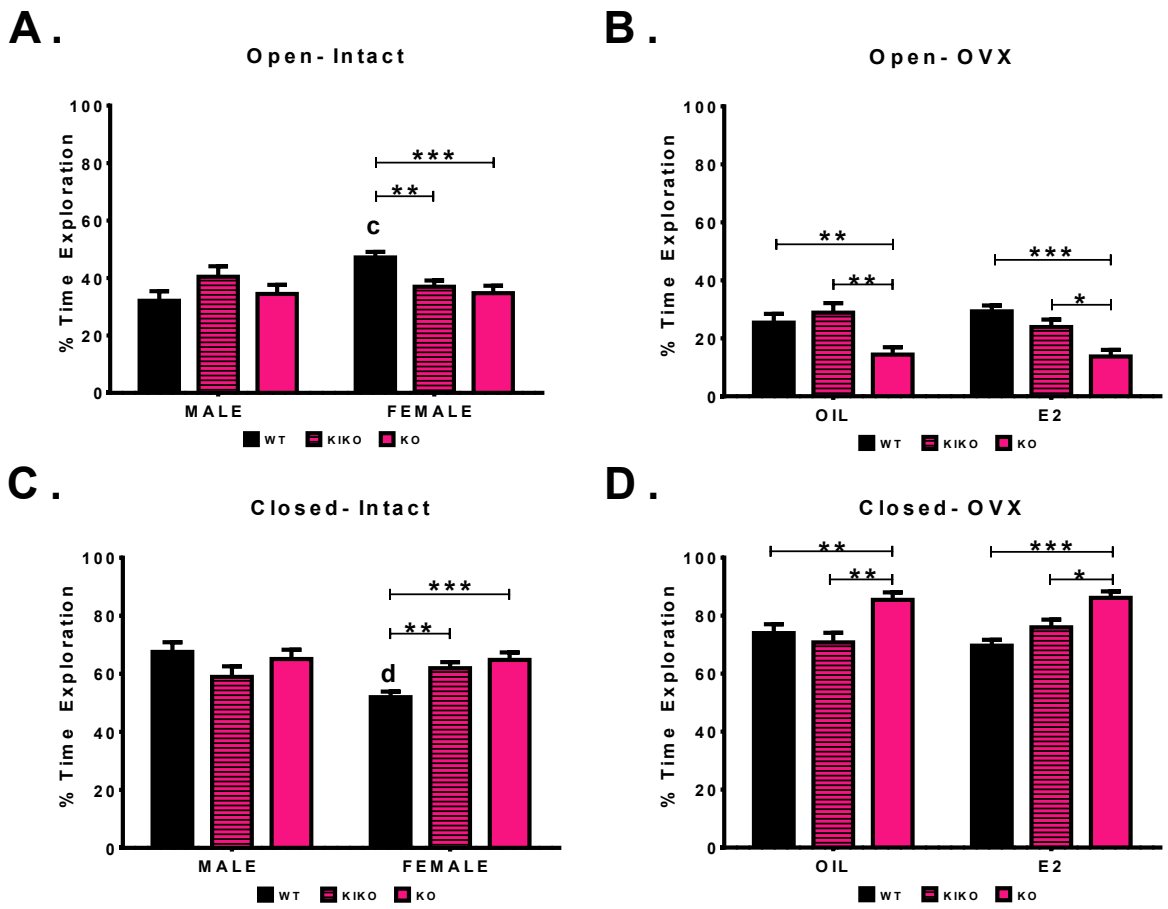

A.
Open- Intact
% Time Exploration
100
80
60
40
20
0
***
**
c
MALE
FEMALE
WT
KIKO
KO
B.
Open- OVX
% Time Exploration
**
**
***
*
OIL
E2
WT
KIKO
KO
C.
Closed- Intact
% Time Exploration
***
**
d
MALE
FEMALE
WT
KIKO
KO
D.
Closed- OVX
% Time Exploration
**
**
***
*
OIL
E2
WT
KIKO
KO

Figure 30

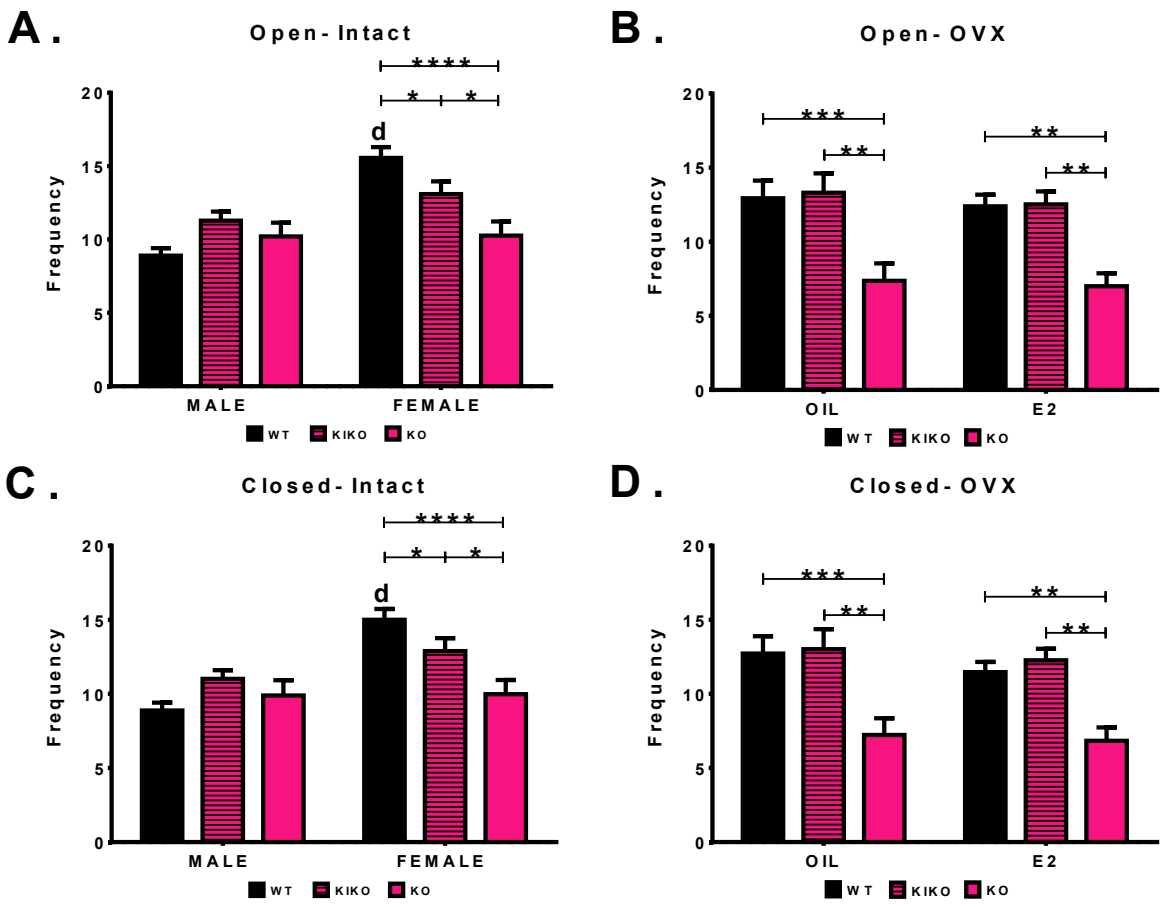

A.
Open- Intact
Frequency
MALE
FEMALE
d
****
*
*
WT
KIKO
KO
B.
Open- OVX
OIL
E2
***
**
**
**
C.
Closed- Intact
D.
Closed- OVX

Figure 31

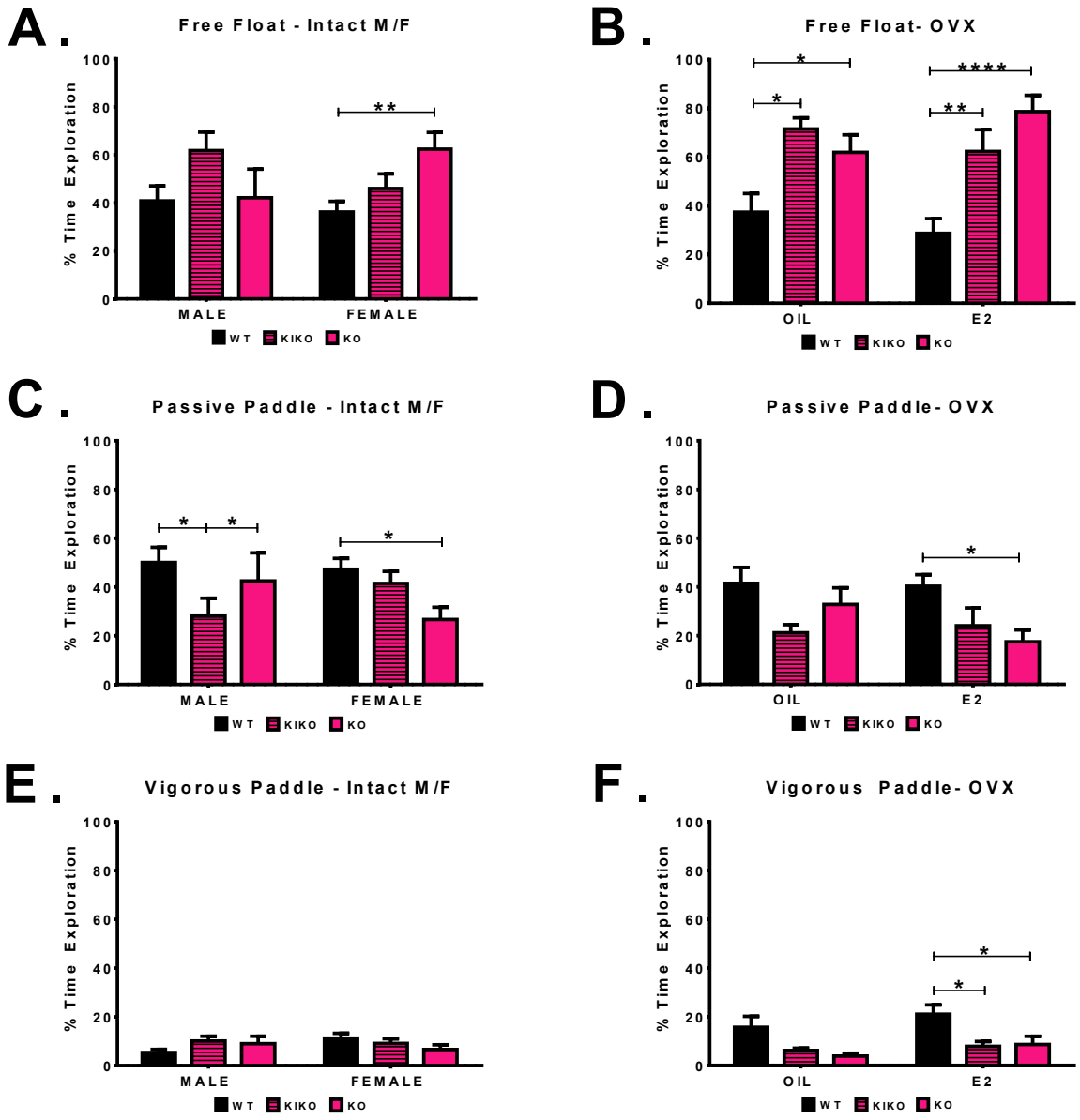
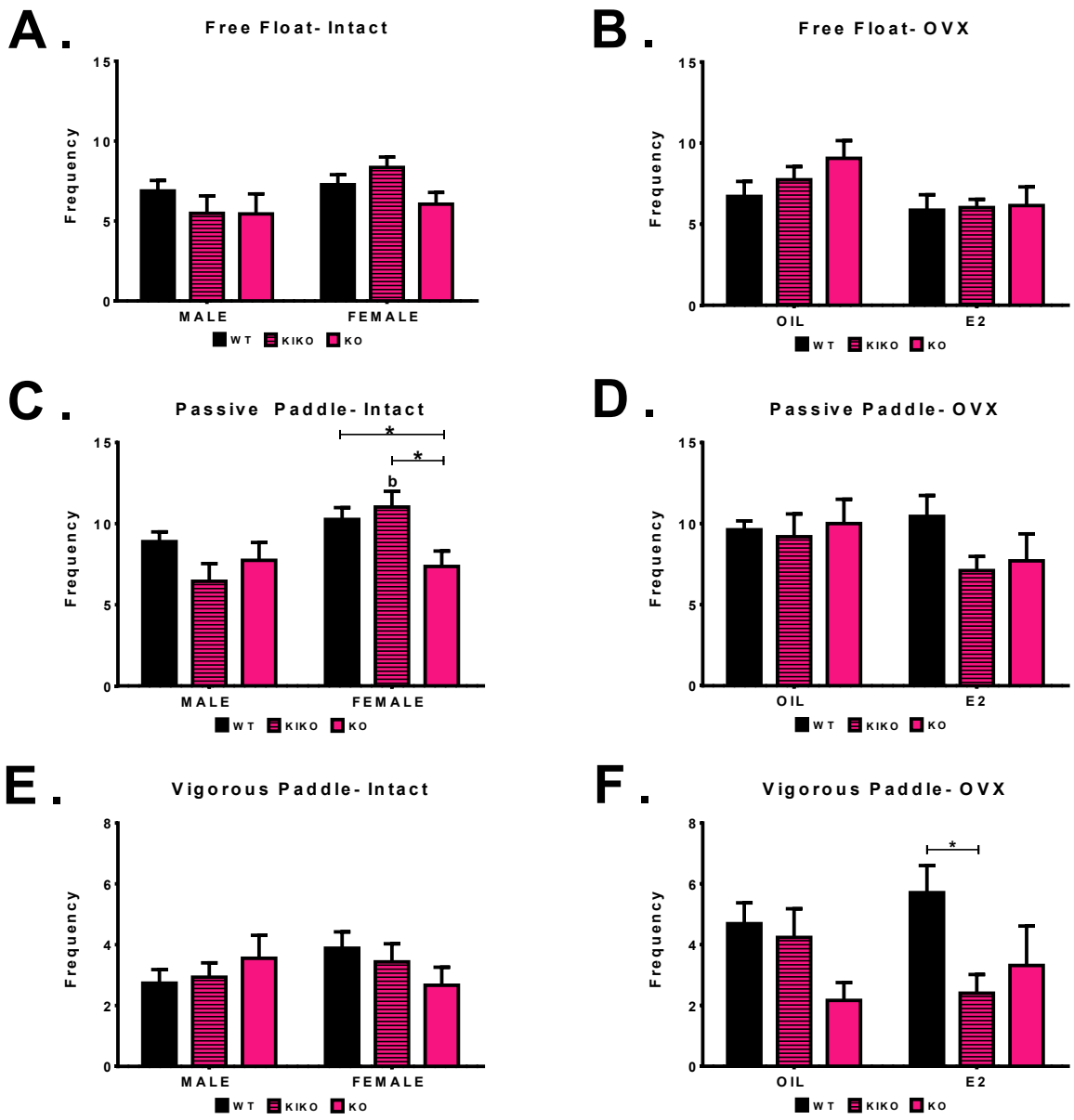

A.
Free Float - Intact M/F
% Time Exploration
MALE
FEMALE
WT KIKO KO
B.
Free Float- OVX
OIL
E2
C.
Passive Paddle - Intact M/F
D.
Passive Paddle- OVX
E.
Vigorous Paddle - Intact M/F
F.
Vigorous Paddle- OVX

Figure 32


A.
Free Float- Intact
Frequency
MALE
FEMALE
WT KIKO KO
B.
Free Float- OVX
Frequency
OIL
E2
WT KIKO KO
C.
Passive Paddle- Intact
*
*
b
Frequency
MALE
FEMALE
WT KIKO KO
D.
Passive Paddle- OVX
Frequency
OIL
E2
WT KIKO KO
E.
Vigorous Paddle- Intact
Frequency
MALE
FEMALE
WT KIKO KO
F.
Vigorous Paddle- OVX
*
Frequency
OIL
E2
WT KIKO KO

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Taylor & Francis Group, LLC.

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