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Expression of Tumor Necrosis Factor-Alpha in a Chick Model of Stress Vulnerable, Treatment –
Resistant Depression

A Dissertation
Presented for the
Doctorate of Philosophy
Degree
University of Mississippi

By
Mary Katherine Jourdan

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Abstract

An increasing body of literature suggests a Major Depressive Disorder subpopulation, Treatment Resistant Depression (TRD), is associated with an increased immune response. More than a decade of research has identified and begun to validate a novel animal model of TRD. This study sought to determine whether TNF- α levels would be altered due to isolation stress and differentiated between stress-vulnerable and -resilient strains. Black Australorp and Production Red chicks were placed into isolation for varying times during which Distress Vocalization (dVoc) rates were calculated and transformed into entry into behavioral despair percent thresholds. Blood levels of TNF- α were quantified via ELISA either directly from the home cage and served as non-isolated controls or following isolation stress. Black Australorps entered into behavioral despair significantly more quickly than Production Reds. Non-isolated controls show no TNF- α level differences. Production Reds displayed an increase in TNF- α levels with a peak at 60 minutes in the isolation test period. Black Australorps displayed a low TNF- α response that remained stable across the isolation test period. These findings identify a blunted TNF- α response in stress-vulnerable Black Australorps that is influenced by a gene x environment interaction. These findings demonstrate the model may be useful for identifying novel cytokine targets for TRD.

Dedication

I dedicate this work to my family without whom none of this would be possible.

To my sweet, loving mother. Cindy, your constant encouragement, support, and kindness has been the light throughout these last few years. You have been my rock.

To my strong-willed, supportive father. Raymond, thank you for the constant encouragement to go where no Jourdan has gone before. Your constant encouragement has brought me here today.

To my encouraging, witty brother. Jay, thank you for reminding me to lighten up and live a little but focus and be productive when it is time to work.

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Thanks to my family and friends. Without their support and continuous “cheerleading” none of this would be possible.

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Chapter 1

1. Brief Overview of MDD

Major Depressive Disorder (MDD) is one of the leading causes of disability and affects 10% of the adult population in the United States (Global Burden of Disease Study, 2015; WHO, 2010). The Diagnostic and Statistical Manual of Mental Disorders 5th Edition (DSM-5) diagnosis for MDD requires 5 or more symptoms over a 2 week period with at least one symptom being (a) depressed mood or (b) anhedonia (American Psychiatric Association, 2013). Specific symptoms presented by patients determines MDD subtype diagnosis (i.e. dysthymia, bipolar depression, atypical depression, seasonal affective disorder) (American Psychiatric Association, 2013). MDD has a lifetime prevalence rate of 16.6% in the United States (Kessler et al., 2012). A recent World Health Organization study found MDD accounted for 8.3% of all U.S. years lived with disability (YLDs) (WHO, 2015). People diagnosed with MDD account for 38% productivity loss in the workplace (Beck et al., 2011). Current MDD treatments include psychotherapy, pharmacotherapy, or a combination of each. Pharmacological treatments typically take 4-6 weeks of continuous administration before beneficial effects present. Further, antidepressants are accompanied with unpleasant side effects that include, but are not limited to, nausea, sexual dysfunction, and insomnia (Dobson et al., 2008; Samuels et al., 2001; Khawam, Laurencic, & Malone, 2006). The poor efficacy of antidepressants may reflect an incomplete understanding of complex underlying neurophysiological sequelae of this stress related disorder.

Chapter 2

2.1 Overview of Theories

In this chapter, I describe the historical findings and current data of four biochemical and system alterations that underlie MDD. These systems include monoamines that regulate emotion in the limbic system, glucocorticoids that regulate stress response through the hypothalamus-pituitary-adrenal axis, neurotrophins that mediate neurogenesis in the hippocampus, and cytokines that regulate immune response through white blood cells. Supporting data for these theories are detailed separately, but all four systems are impacted by stressors and are likely interconnected in complex ways.

2.2 Monoamine Theory of Depression

The monoamine theory of depression was first proposed in 1950's following the observation of mood elevation in patients with tuberculosis (Freis, 1954). Tuberculosis is a debilitating condition caused by *Mycobacterium tuberculosis* that destroys lung tissue (Evans et al., 1996). This condition would typically confine patients to hospital wards often leading to altered mood states. Attempts to treat tuberculosis with iproniazid yielded serendipitous findings in that depressed patients showed elevated mood (Fox et al., 1952). The discovery that this pharmacological agent altered mood status prompted questions of how it acted on central nervous system targets to ameliorate depression. Iproniazid acts as a monoamine oxidase inhibitor (MAOI) (Ramachandria et al., 2011). MAOIs inhibit monoamine oxidase (MAO) from breaking down monoamine neurotransmitters (i.e. serotonin, norepinephrine, and dopamine).

This inhibition of monoamine neurotransmitter degradation allows for more synaptic serotonin, norepinephrine, and dopamine. The observation that increased levels of synaptic monoamines can elevate mood in tuberculosis populations with depression lends initial support for the monoamine theory.

Additional support that monoamines are involved in mood regulation comes from the use of reserpine in schizophrenic and hypertensive populations. Reserpine, derived from the *Rauwolfia serpentina* plant, has been shown to attenuate psychotic episodes in schizophrenics (Hollister et al., 1955) and to decrease cardiovascular signs associated with hypertension (Freis, 1954). Reserpine blocks vesicular monoamine transporter (VMAT) (Kim & Shore, 1963) that allows vesicles to sequester neurotransmitters in monoaminergic nerve terminals. Once neurotransmitters leave the vesicle and enter the cell cytoplasm, they are rapidly degraded by MAO leading to a depletion of dopamine, norepinephrine, and serotonin in the synapse. The therapeutic benefit of reserpine in schizophrenia and hypertension is mediated by the acute depletion of synaptic dopamine and norepinephrine, respectively. However, chronic administration of this drug produces major depression in both clinical populations, (Janicak et al., 1993; Feldman et al., 1997; Musselman et al., 1998). The observation that decreased levels of synaptic monoamines induce depression lends additional support that deficits in dopamine, norepinephrine, and serotonin signaling underlie MDD.

The use of MAOIs to treat MDD would soon reveal serious side effects of this class of therapeutics. MAO is necessary for the breakdown of tyramine found in foods such as aged cheeses and meats, and drinks such as wine. Patients prescribed MAOIs without dietary restrictions would experience a pressor reaction in which tyramine levels would increase thus elevating norepinephrine levels leading to stroke, and, in some cases, death (Lader, 1983;

Goldman, Alexander, & Luchins, 1986). The side effect profile of MAOIs would prompt pharmaceutical companies to the development the second-generation of antidepressant drugs that would affect monoamine levels through different and selective actions.

The second-generation of drugs to treat MDD belong to a class known as the tricyclic antidepressants (TCAs). These compounds are more selective on monoaminergic systems in that they target only norepinephrine and serotonin (Remick, 1988). The mechanism of action of TCAs is via inhibition of the presynaptic reuptake transporter, which leads to elevated norepinephrine and serotonin (Coppen, Shaw, & Farrell, 1963; Cuenca, Salva, & Valdecasas, 1964; Blier & De Montigny, 1994; Lopez-Munoz, Cuenca, & Alamo, 2007). Chronic administration of TCAs show therapeutic efficacy of around 65%, which is a similar rate to MAOIs. While TCAs do not possess dietary interactions, these compounds are not without side effects. These include cardiotoxicity through agonism of norepinephrine, sedation through antagonism of histamine, and an anticholinergic syndrome (i.e. dry mouth, urinary retention, constipation, and blurred vision) through antagonism of muscarinic receptors (Blier & Montigny, 1980; Spyraiki & Fibiger, 1981). During this time, a driving force in drug development was to develop compounds with greater selectivity in attempt to increase efficacy while reducing side effect profiles.

The third generation of antidepressants possessed high binding affinity for selective neurotransmitter systems at their presynaptic reuptake transporters (Dale, Bang-Andersen, & Sanchez, 2015). The first compounds introduced were the selective serotonin reuptake inhibitors (SSRIs). These target serotonin transporters with little activity elsewhere (Lambert & Bourin, 2002). Following SSRI administration, extracellular serotonin increases within minutes to hours (Bymaster et al., 2002; Rutter & Auerbach 1993). However, SSRI administration is associated

with a number of side effects including weight gain, sexual dysfunction, and drug interactions (Masand & Gupta; 2002).

Selective norepinephrine reuptake inhibitors (SNRIs) were introduced simultaneously. SNRIs were found to be highly selective on norepinephrine transporters with little activity elsewhere (Lambert & Bourin; 2002). SNRI administration is associated with sexual dysfunction as a side effect (Bostwick, 2010).

SSRIs tend to be most efficacious for severe MDD while SNRIs tend to be most efficacious with less severe MDD (Thase, 2008). Both SSRIs and SNRIs require chronic administration of 4-6 weeks to achieve therapeutic effects (Dobson et al., 2008; Samuels et al., 2001; Khawam, Laurencic, & Malone, 2006). Both SSRIs and SNRIs possess about a 65% efficacy rate (Locher et al., 2017).

The original monoamine theory of MDD focused on changes in synaptic neurotransmitter levels as the key factor in mood regulation. Here, the underlying mechanism of MDD was thought to reflect the depletion of synaptic norepinephrine and serotonin. However, antidepressants increase availability of neurotransmitters within hours of administration whereas clinical benefits typically require weeks of chronic administration (Andrade & Rao, 2010). These observations prompted researchers to re-examine alterations in monoamine functioning in a timeline that accompanies antidepressant clinical benefits.

A number of studies suggest that presentation of MDD may involve alterations in monoamine receptors rather than lowered synaptic neurotransmitter levels. Postmortem studies of suicide victims show increased (i.e. up-regulation) serotonin receptors in the frontal lobe (Arora & Meltzer, 1989, Mann et al., 1986; McKeith et al., 1987). This up-regulation is not only associated with MDD diagnosis but can be reversed with chronic antidepressant administration

in human clinical populations as well as in animal models of MDD (Stahl, 1992; Stahl, 1994; Sulser, Vetulani, & Mobley, 1978). Electroconvulsive therapy induces seizures to treat mental disorders, specifically treatment resistant depression, with a response rate of approximately 55% (Sackeim et al., 2001; Taylor, 2007). Studies have shown norepinephrine receptor turnover alterations following single electroconvulsive therapy (ECT) (Musacchio et al., 1969; Modigh, 1976; Ebert et al., 1973). Similarly, norepinephrine receptor turnover alterations following acute or long-term antidepressant administration (Charney, Menkes, & Heninger, 1981). These data suggest that MDD and its relief via antidepressants reflects downstream processes in monoamine receptor sensitivity rather than direct alterations of neurotransmitter levels.

The second generation of the monoamine theory of depression argued that up-regulation of postsynaptic norepinephrine and serotonin receptors underlies this disorder and is due to the lack of synaptic norepinephrine and serotonin transmission associated stress conditions (Taylor et al., 2005; Heninger, Delgado, & Charney, 1996). Antidepressant administration elevates norepinephrine and serotonin levels (Manji, Drevets, & Charney, 2001) and this causes increased activation of postsynaptic receptors. Over time, this activity on postsynaptic receptors leads to their down-regulation (Taylor et al., 2005) that coincides with the therapeutic benefits of antidepressants (Sulser, Vetulani, & Mobley, 1978).

2.3 Hypothalamic - Pituitary - Adrenal Axis Theory of Depression

The hypothalamic – pituitary – adrenal (HPA) axis theory posits that MDD is caused by HPA axis over activation. In healthy individuals, HPA axis activation is a physiological response to psychological and physical stressors (Burke, Davis, Otte, & Mohr, 2005). A stressor activates the HPA axis and initiates a cascade of biochemical events in which the hypothalamus releases

corticotrophin releasing factor (CRF). CRF acts on the pituitary gland to signal release of adrenocorticotrophic hormone (ACTH) that acts on the adrenal glands to signal the release of glucocorticoids (i.e. cortisol) (Jacobson and Sapolsky, 1991; Munck et al., 1984; Sapolsky, Krey, & McEwan, 1986; Sapolsky & Meaney, 1986).

In healthy individuals, the HPA axis utilizes a negative feedback loop in that once glucocorticoid levels reach a specific concentration, hypothalamic CRF and pituitary ACTH release is stopped (Jacobson and Sapolsky, 1991; Sapolsky, Krey, & McEwan, 1986). The decline in CRF and ACTH returns the body to homeostasis. However, individuals with MDD show an HPA axis over-activation as measured by increased cortisol levels in saliva, plasma, and urine (Nemeroff, 1996; Nemeroff & Vale, 2005). This finding lead researchers to believe increased cortisol levels is an MDD biomarker.

Several lines of research connect HPA axis over activity with MDD. HPA axis over activation was discovered while investigating Cushing's Syndrome and its association with MDD (Cohen, 1980; Halbreich et al., 1985; Asnis et al., 1987; Carroll et al., 1981; Geraciotti et al., 1992). This research found increased cortisol levels not only caused physical symptoms seen in Cushing's Syndrome, but was positively correlated with anxiety and depression (Cohen, 1980). Further research conducted in Cushing's Syndrome patients suggested increased cortisol levels correlate with a specific subtype of depression (i.e. endogenous depression or melancholia) (Gold et al., 1995; Du & Pang, 2015).

Evidence suggests HPA axis over activation cannot solely explain the MDD physiology. For example, not all MDD patients exhibit HPA activation and increased cortisol levels (Burke, Davis, Otte, & Mohr, 2005). Multiple factors, including age and gender, are known to influence cortisol response (Seeman and Robbins, 1994; Kudielka et al., 2004; Otte et al., 2005). For

example, older women tend to have elevated plasma cortisol levels in response to a stressor compared to younger men and women (Kudielka et al., 2004). Other factors that influence degree of HPA activation include depression subtype, depression severity, and type of stressors (Gold et al., 1995; Carroll et al., 1981; Meador-Woodruff et al., 1990; Heim et al., 2000; Yehuda et al., 2004; Dickerson & Kemeny, 2004). For example, increased negative HPA feedback is associated with Post Traumatic Stress Disorder (PTSD), atypical depression, and melancholic depression (Yehuda et al., 1991; Dickerson & Kemeny, 2004). HPA axis over activation is seen in individuals diagnosed with severe depression (Gold et al., 1995; Carroll et al., 1981; Meador-Woodruff et al., 1990; Nelson and Davis, 1997; Heim et al., 2000; Yehuda et al., 2004). Social stressors perceived as uncontrollable cause HPA axis over activation and an increased release of cortisol (Dickerson & Kemeny, 2004). Because the patterns of HPA activity is differentially affected by MDD subtypes, individuals, and stressors, the HPA axis theory does not fully explain the complex underlying pathophysiology of MDD.

2.4 Neurogenic Hypothesis of Depression

The neurogenic hypothesis posits that MDD presents because of altered brain derived neurotrophic factor (BDNF) and neurogenesis release in limbic structures (i.e. prefrontal cortex and hippocampus) responsible for mood regulation (Kempermann, 2002). BDNF release activates an intracellular signaling pathway that underlies neuronal development, survival, and function (Ghosh, Carnahan, & Greenberg, 1994; Poo, 2001; Duman & Aghajanian, 2012). Genetic factors contribute to neurogenic loss and induce structural changes in the hippocampus dentate gyrus (Kempermann, Kuhn, & Gage, 1997; Kempermann, Kuhn, & Gage, 1997; Jacobs, Van Praag, & Gage, 2000; D'sa & Duman, 2002; Duman et al., 2000; Jacobs, 2002; Jacobs et al.,

2000; Kempermann et al., 2002). Research shows a single nucleotide polymorphism (Val66Met) in the BDNF family occurs in a subset (20-30%) of MDD patients (Chen et al., 2006; Zhao et al., 2017) and is due to a methionine (Met) substitution for valine (Val) at codon 66 (Chen et al., 2006). This gene polymorphism controls release BDNF (Chen et al., 2006; Hwang et al., 2006; Gatt et al., 2009; Ozan et al., 2010). Human populations with the Met allele have smaller hippocampal volume and are more likely to be diagnosed with MDD (Bueller et al., 2006; Pezawas et al., 2004; Szeszko et al., 2005). This genetic predisposition is one of the many factors that contributes to loss of hippocampal volume.

There are conflicting ideas regarding mechanisms underlying hippocampal volume loss in MDD patients. One suggests decreased hippocampal volume in MDD is due to hippocampal progenitor cell loss. Hippocampal progenitor cells are located in the subgranular zone (SGZ) of the dentate gyrus (Jacobs et al., 2000). Progenitor cells divide a limited number of times with a predetermined development path (Jacobs et al., 2000). These progenitor cells remain dormant until activated and can produce either two neurons, or one glial cell and one progenitor cell (Boldrini et al., 2009; Lucassen et al., 2010). These findings suggest hippocampal progenitor cell loss is associated to volumetric changes in MDD pathophysiology.

A second perspective suggests hippocampal volumetric loss is caused by decreased dendritic structuring and neuronal cell death. These effects are due to disruption of multiple signaling pathways including the cyclic adenosine monophosphate (cAMP) cascade signaling that affects cAMP responsive element binding protein (CREB) and BDNF (Huang et al., 2015), and the BDNF-tropomyosin-related kinase B (TrkB) receptor signaling. Chronic stress and/or genetic predisposition causes cAMP-CREB signaling pathway dysfunction leading to decreased BDNF levels, atypical dendritic spine formation, and neuronal death (Smith et al., 1995;

Thoenen, 1995; Duman, Heninger, & Nestler, 1997; Chen et al., 2006; Sapolsky et al., 1996; Sheline et al., 1996; Duman et al., 1999; Santarelli et al., 2003; Duman & Aghajanian, 2012). Similarly, stress causes BDNF-TrkB pathway signaling reduction leading to decreased BDNF levels, and increased negative regulator, extracellular signal – regulated kinase (ERK) (Duric et al., 2010; Duman & Aghajanian, 2012). Alone, ERK increases cause depressive behaviors (i.e. anhedonia) (Duric et al., 2010). These two signaling pathways as well as decreases in BDNF levels and increases in ERK leads to hippocampal neurodegeneration and behaviors associated with MDD.

Although the neurogenesis theory of depression is becoming widely researched, it cannot solely account for all complexities associated with MDD. For example, in animal models, neurogenesis ablation does not consistently cause depressive-like symptoms (Jayatissa et al., 2010). Decreased neurogenesis is dependent on stressor type. For example, neurogenesis decreases following social defeat stress (Lagace et al., 2010). In order to include the physiological complexities that occur in MDD researchers suggest approaching the neurogenic hypothesis as a “neurogenic interactome” (Makhija & Karunakaran, 2013). This neurogenic interactome incorporates all MDD hypotheses (monoamine, HPA axis over activation, neurogenic, and neuroinflammation).

2.5 Neuroinflammation Theory of Depression

The neuroinflammation theory of depression posits immune system over activation and increased pro-inflammatory cytokines levels cause MDD (Maes et al., 1995). Pro-inflammatory cytokines linked with MDD include Interleukin-1 β (Il-1 β), Interleukin-6 (Il-6), Tumor Necrosis Factor- α (TNF- α), and C-reactive protein (CRP) (Young, Bruno, & Pomara, 2014). The role of

pro-inflammatory cytokines in mood regulation was made not from MDD patients but in patients infected with hepatitis C virus (Hauser et al., 2002; Loftis et al., 2004; Reichenberg, Gorman, & Dieterich, 2005). Interferons are a component of the body's immune system to fight viral infections (Samuel, 2001). Therapeutic Interferon- α (IFN- α) alleviates hepatitis C related symptoms; however, 30% of treated patients develop IFN- α induced MDD (Schafer et al., 2007; Su et al., 2010; Udina et al., 2012). The clinical presentation of depression in this population is linked to IFN- α –induced increase in pro-inflammatory cytokines (Hauser et al., 2002). Further support for the neuroinflammation theory of depression is provided by studies that show altered cytokines cause MDD symptoms such as anhedonia, fatigue, decreased appetite, insomnia, and cognitive impairments in both animal models of depression and clinical populations (Dantzer, et al., 2008; McAfoose & Baune, 2009; Dantzer, 2001; Misiak et al., 2018). These findings suggest increased immune system activation and cytokine release are linked to MDD.

TNF- α plays a major role in immune responses. TNF- α is an acute-phase protein and is released from macrophages and mast cells into the blood stream following an immunological threat detection (Dowlati, 2010). TNF- α release promotes inflammatory signaling, and stimulates pro-inflammatory cytokine release, leading to neural pruning (Wallach et al., 1999; Lee et al., 2000; Wajant, Pfizenmaier, & Scheurich, 2003; Naguib et al., 2004; Shen & Pervaiz, 2006; Dowlati, 2010). Increased TNF- α levels have been linked to “sickness behavior” symptoms of MDD (i.e. anhedonia, fatigue, and insomnia) (Suarez et al., 2002; Suarez et al., 2004; Motivala et al., 2005). Evidence shows acute and chronic administration of cytokine inducers (i.e. lipopolysaccharide or vaccinations) induces “sickness behaviors” or other MDD

symptoms, which can then be treated with antidepressants (Miller, 2009; Musselman et al., 2001; Capuron et al., 2002). Increased levels of TNF- α lead to symptoms seen in patients diagnosed with MDD.

A subset of MDD patients fail to respond to two or more classes of antidepressant medications and are referred to as having treatment-resistant depression (TRD). One consistent finding is increased pro-inflammatory cytokine levels are found in patients diagnosed with TRD. The Food and Drug Administration (FDA) defines TRD as MDD patients who do not respond to two or more antidepressant classes. Increased biomarkers of inflammatory mediators are associated with antidepressant non-responsiveness (Slavich & Irwin, 2014; Michopoulos et al., 2015; Rethorst et al., 2013). More specifically, individuals diagnosed with TRD display increased TNF- α levels compared to healthy populations (Feltes et al., 2017; Schiepers, et al., 2005; Clarke et al., 2017; Tuglu et al., 2003). The lack of treatment efficacy could be attributed to increased TNF- α levels.

2.6 Connecting the Theories of Depression

The four major theories of depression all suggested MDD is associated with decreased hippocampal volume and neuronal cell death. Stress activates the HPA axis, which releases glucocorticoids. Hippocampal stress-responsive adrenal-glucocorticoid receptors cause increased hippocampal glucocorticoid sensitivity (Medina et al., 2013; van Ast et al., 2013; Young et al., 1991). Increased levels of glucocorticoids slow neural stem/progenitor cell growth and neuronal differentiation, and prevent neurogenesis in the dentate gyrus (Bohn, 1980; Shlessinger, Cowan, & Gottlieb, 1975; McEwen, 1999; Saplosky, 2001; Holsboer and Barden, 1996; Gold and Chrousos, 2002). This, in turn, leads to BDNF level decreases, and decreased hippocampal

volume, ultimately contributing to HPA axis negative feedback dysregulation (Young et al., 1991; Saplosky, 2001; Gould et al., 1992; Heine et al., 2004; Fitzsimons et al., 2016).

Increased HPA axis activation also affects the immune system and inflammatory response. This increased HPA axis hyperactivation leads to loss of immune system repression, also called glucocorticoid resistance (Dinkel et al., 2003; MacPherson et al., 2005; Barrientos et al., 2015; Sorrells et al., 2009). Glucocorticoid resistance leads to increased pro-inflammatory cytokine release and can be seen in MDD subtypes (Miura et al., 2008; Gold, Licinio, Wong, & Chrousos, 1995). These findings demonstrate the HPA axis does not operate within itself but also affects other physiological processes.

Similarly, data suggests the neurogenesis process is not simply influenced by BDNF and the cAMP pathway, but could also be influenced by all systems and processes previously discussed. Research stemming from the Jacobs' lab (1999) suggests lack of neurogenesis is caused by the lack of synaptic serotonin, which hinders hippocampal progenitor cell proliferation and causes a decreased hippocampal volume (Gould, 1999). Increasing synaptic serotonin will stimulate hippocampal progenitor cell proliferation, and increase BDNF levels, as seen ~~in~~ following chronic antidepressant administration (Shaywitz & Greenberg, 1999; Czeh et al, 2001; Malber et al., 2000, Malberg et al, 2000). Lack of neurogenesis causes NMDA and glucocorticoid receptors to internalize (Holsboer & Barden, 1996; Gold & Chrousos, 2002). Lack of hippocampal glucocorticoid receptor binding availability can cause HPA axis dysregulation, which causes glucocorticoid sensitivity, and further inhibits neurogenesis (Herman et al., 1992; Herman et al., 1995; Webster et al., 2002; Medina et al., 2013). These data further prove the neurogenesis process is not only influenced by BDNF signaling but by a culmination of all four systems and processes.

Similar research suggests the monoaminergic system can influence other systems associated with MDD. Successful antidepressant therapy is shown to normalize the HPA axis hyperactivation by increasing glucocorticoid receptors, 5-HT_{1A} receptors, and normalizing cortisol levels (Holsboer, Liebl, & Hofschuster, 1982; Greden et al., 1983; Holsboer-Trachsler, Stohler, & Hatzinger, 1991; Barden, Reul, & Holsboer, 1995; Hanson, Owens & Nemroff, 2011). Four to six weeks of antidepressant administration normalizes the HPA axis and increases synaptic monoamines and neurogenesis (Barden, Reul, & Holsboer, 1995; Zhao et al., 2008; Andrade & Rao, 2010). These data show monoamine neurotransmission also affects the HPA axis and neurogenesis processes.

Finally, research suggests the proinflammatory cytokine immune response affects other neurobiological processes related to MDD. Increased cytokines levels have been directly linked to decreased monoamine levels via tryptophan depletion, which can be reversed with SSRI and SNRI administration (Capuron et al., 2003; Berthold-Losleben, & Himmerich, 2008; Jeon & Kim, 2016; Weidlocha et al., 2018; Galecki et al., 2018). TNF- α has been shown to stimulate the HPA axis and alter neurotransmitter networks that induce behaviors known as “sickness behaviors” (i.e. sleepiness, fatigue, loss of appetite) (Berthold-Losleben, & Himmerich, 2008). These findings show cytokine release affects multiple physiological systems that are associated with MDD.

Chapter 3

3.1 Ketamine

Current antidepressants work in approximately 40-60% of MDD patients and take approximately 4-6 weeks for therapeutic effects to occur (Schulberg et al., 1998; Fava & Davidson, 1996; Uher et al., 2011). This low efficacy and lengthy time to therapeutic outcomes has prompted decades of research into other potential antidepressant targets. Dr. Calvin Lee Stevens first synthesized ketamine in 1964, and was approved by the FDA for short-term anesthetic use in animals and humans (Hillhouse & Porter, 2015). Recently, ketamine is prescribed for treatment-resistant depression due to its quick onset of action and long lasting effects for up to 14 days (Zarate et al., 2006; Duman et al., 2014; Kiraly et al., 2017). Research regarding ketamine's antidepressant mechanistic effects on CNS remain unclear. Research shows ketamine acts as a non-competitive N-methyl-D-aspartate (NMDA) receptor antagonist (Newport et al., 2015; Zanos et al., 2016; Tyler et al., 2017). However, other NMDA receptor antagonists do not possess the rapid and long-lasting antidepressant effects compared to ketamine (Newport et al., 2015). Ketamine's antidepressant action was thought to somehow involve downstream action at AMPA receptors (Aleksandrova, Phillips, & Wang, 2017). Ketamine non-selectively potentiates AMPA receptor (Akinfiresoye, Luli, & Tizabi, 2013) and AMPA receptor antagonists block ketamine antidepressant effects (Maeng et al., 2008; Koike et al., 2011).

Ketamine's downstream MDD therapeutic effect most likely works via synaptogenesis. Synaptogenesis is defined as an intracellular signaling processes that underlies neuroplasticity;

neuroplasticity is the ability to process information from other neuronal inputs, store that information, and make appropriate future adaptive responses (Duman et al., 2012).

Synaptogenesis requires activation of AMPA receptors leading to mammalian target of rapamycin complex 1 (mTORC1) pathway activation, and increases in BDNF release (Maeng et al., 2008; Li et al., 2011). BDNF binds to TrkB receptors and activates a signaling cascade leading to increased transcription and neurogenesis (Ghosh, Carnahan, & Greenberg, 1994; Poo, 2001). The connection between ketamine, BDNF and MDD is still unclear but research shows ketamine's therapeutic effects against MDD is lost when BDNF release is blocked (Autrey et al., 2011; Lepack et al., 2015). The role of BDNF-mediated plasticity in MDD relief fits well with observations that chronic administration of traditional antidepressants ultimately regulate BDNF expression through downstream pathways (Duman & Aghajanian, 2012).

Ketamine not only increases BDNF and synaptogenesis, it decreases pro-inflammatory cytokines and may underlie the beneficial effects in TRD populations. For example, one rodent model of MDD shows chronic lipopolysaccharide (LPS) administration induces increased pro-inflammatory cytokine release, "sickness behaviors", and anhedonia. Ketamine administration attenuates increase in pro-inflammatory cytokine levels and depressive-like behaviors.

(Takenaka et al., 1994; Kawasaki et al., 1999; Loix, De Kock, & Henin, 2011). Ketamine administration alone decreases cytokine levels in MDD animal models and clinical populations (Kawasaki et al., 1999; Tan et al., 2017; Xie et al., 2017; Loix, De Knock, & Henin, 2011).

Moreover, ketamine administered in combination with traditional antidepressants further decreases pro-inflammatory cytokines and increases antidepressant activity (Réus et al., 2011).

These findings show ketamine's potential antidepressant effects could be due to decreases in pro-inflammatory cytokine effects.

Chapter 4

4.1 Translational Relevance

A number of concerns have been raised in recent years regarding the translational relevance of animal models utilized in drug discovery efforts (Garner, 2014; Miczek & de Wit, 2008; Nestler & Hyman, 2010; Mogil, Davis, & Derbyshire, 2010). Research suggests approximately 90% of drugs that pass animal pre-clinical efficacy screenings ultimately fail in clinical trials (Garner, 2014). This high failure rate could be attributed to the inability of animal models to fully mirror all aspects of a clinical syndrome.

One strategy to enhance the translational relevance of animal models is through endophenotypic mapping. This approach attempts to establish homologies between a syndrome's etiology, symptomatology, pathology, and treatment (Van der Staay, 2006) to that being modeled in an animal simulation. The rationale in this approach is that when an animal model simulation has a larger number of established homologies across these domains, the more predictive validity this model will possess in drug discovery.

This lab has worked to enhance translational relevance in drug discovery research by employing an endophenotypic mapping approach in validating a novel animal model simulation for stress-related disorders. The chick anxiety-depression paradigm (Sufka et al., 2006) was initially developed in response to an increasing body of clinical literature showing high co-morbidities between anxiety and depressive disorders. This work essentially hybridized two existing chick social separation stress paradigms that model panic disorder (Warnick, Wicks, &

Sufka, 2006) and behavioral despair (Panksepp, 1991). This promising avian model of anxiety and depression has been widely validated and utilized in screening anxiolytic and antidepressant drugs and is described below.

The chick anxiety-depression model involves exposing socially-raised chicks (*Gallus gallus*) to an isolation stressor to elicit distress vocalizations (dVocs). DVocs are aimed at reestablishing social contact. Isolation produces an initial high rate of dVocs, which decreases by approximately 40-60% over the next 25-30 minutes, and plateaus for the remainder of the test period (up to 2 hrs). This overall pattern in dVocs is similar to other models of behavioral despair. Distress Vocalizations in the initial 0-5 min phases presents as an anxiety-like state while dVocs in the 30-120 min plateau phase presents as the depression-like phase.

Earlier validation studies explored anxiolytic and antidepressant sensitivity in the model (Feltenstein et al., 2004; Feltenstein & Sufka, 2005; Warnick, Wicks, & Sufka, 2008; Warnick et al., 2008; Sufka et al., 2009). All anxiolytics that are effective for treating panic disorder have screened positive in the assay, and those without anti-panic properties have screened negative. Further, the model successfully screened imipramine, maprotiline, and fluoxetine at various doses. (Warnick et al., 2008). This chick anxiety-depression model shows higher predictive validity than rodent assays by screening out two antidepressants that screened positive in those models of depression but subsequently failed in clinical trials (Sufka et al., 2009).

Additional validity work focused on examining whether a homology existed between the animal model simulation and cognitive symptoms in anxiety and MDD. In clinical populations, emotional states or traits can affect cognitive processes (Mineka & Sutton, 1992; Mathews et al., 1995; Warda & Bryant, 1998). These cognitive biases take the form as being more pessimistic and less optimistic about future outcomes with anxiety states showing the former and MDD

presenting both signs. Cognitive bias in the form of more pessimism has been assessed in rodents using avoidant stimulus discrimination assays (Matheson et al., 2008; Brilot et al., 2009). This lab was able to quantify both forms of cognitive bias by measuring the start and goal latencies to different ambiguous appetitive (i.e. mirror and 75% chick/ 25% owl morphed pictures) and ambiguous aversive (i.e. 50% chick/ 50% owl and 25% chick/ 75% owl morphed pictures, and owl) stimuli (Salmeto et al., 2011; Hymel & Sufka, 2011). Chicks not exposed to isolation displayed start and goal latencies increased with increased aversive stimuli. Chicks isolated for 5 minutes (anxiety phase) displayed slower start latencies when aversive stimuli were placed at the end of the maze. These results were interpreted as more pessimistic-like judgments. Chicks isolated for 60 minutes (depression phase) displayed slower start latencies when both appetitive and aversive stimuli were placed at the end of the maze. These results were interpreted as both more pessimistic-like and less optimistic-like judgments. These two forms of cognitive bias, as presented, further validate the chick anxiety-depression model as a simulation.

A follow up study sought to determine whether cognitive biases in the chick anxiety and depression could be reversed with pharmacological administration. Chicks were administered either clonidine and tested for 5 minutes (anxiety phase), imipramine and tested for 60 minutes (depression phase), or saline and tested for either 5 or 60 minutes. Following isolation, chicks were placed in a long alley maze with the same stimuli discussed above where start and goal latencies were measured. Clonidine did not reverse the more pessimistic-like judgments found in the anxiety phase; however, imipramine reversed the more pessimistic-like and less optimistic-like judgments found in the depression phase. These findings of pharmacological cognitive reversal further validate the chick anxiety-depression model.

Several studies have examined whether homologies in stress-related biomarkers exist in the chick anxiety-depression model. For example, cortisol is released in response to stress in humans (van Holland, Frings-Dresen & Sluiter, 2012). In the chick anxiety-depression model, isolation stress elevates corticosterone levels at the onset of the stressor (Sufka et al., 2006). Similarly, Il-6 levels are found elevated in patients with MDD (Haapakoski et al., 2015). In the chick anxiety-depression model, isolation stress elevates Il-6 levels but does so only in the depression phase (Warnick et al., 2008). Collectively, these two biomarkers help to establish construct validity of the stress model as an anxiety-depression simulation.

Environmental risk factors contribute to the presentation of stress-related disorders in humans. For example, children living in impoverished environments and/or are socially isolated have an increased risk for developing stress-related disorders (Southwick & Charney, 2012; Davidson & McEwan, 2012). Research to study this factor in the anxiety-depression model explored whether alterations in environmental housing affect patterns of entry into behavioral despair (Kim & Sufka, 2011). Separate groups of chicks were placed into one of four housing manipulations: continuous impoverished, continuous enriched, 3 days in impoverished followed by 3 days in enriched (late enriched), and 3 days in enriched followed by 3 days in impoverished (late impoverished). Chicks in a continuous impoverished and a late impoverished housing environment entered into behavioral despair more quickly than chicks housed in a continuous enriched environment and a late enriched environment. These data demonstrate positive effects of enriched housing (both continuous and late enrichment) on stress-related behaviors and adds another homology with which to validate the simulation.

In humans, genetic factors contribute to MDD risk. For example, published meta-analysis evaluating MDD heritability in biological and adopted families shows a 31%-42%

chance of diagnosis in biological families (Uher, 2014; Hyde et al., 2016; Sullivan, Neale, & Kendler, 2000). Recent research has explored genetic factors that may contribute to stress-resilience and –vulnerability in the chick anxiety and depression model (Hymel et al., 2013). Chicks from nine genetic lines raised in standard-impoverished housing were subjected to the social separation stressor and onset of behavioral despair was calculated. Two strains displayed contrasting responses relative to the seven other genetic lines. The stress-resilient Production Red line displayed a slower onset to behavioral despair while the stress-vulnerable Black Australorp line displayed a faster onset to behavioral despair compared to other lines. This pattern shows that genetic predispositions can affect social separation stress vulnerability (i.e., Black Australorp) and resiliency (i.e., Production Red) in the model and provides another homology with clinical features of MDD.

There is much interest in the role of BDNF in stress vulnerability. A follow up study explored whether social separation in the stress-vulnerable and stress-resilient strains would present in altered BDNF levels. Stress-vulnerable and stress-resilient chicks were placed into social separation for either 0, 30, 60, 90, or 120 minutes. Following social separation, animals were euthanized and hippocampal samples were taken for BDNF analysis. Stress-resilient chicks' BDNF levels remained stable across all test periods; however, stress-vulnerable chicks' BDNF levels showed a significant increase at 90 minutes of isolation. The finding that a stress-vulnerable chick line presents altered BDNF responses is homologous to studies in humans with MDD.

There are data to suggest that genetics not only contribute to stress vulnerability, they also influence antidepressant drug sensitivity (Tsai et al., 2003; Tansey et al., 2013). TRD is estimated to occur in approximately 15.8% of MDD patients (Cepeda et al., 2018). Having

identified stress-vulnerable and -resilient strains in the chick model, the next validation step was to determine whether these disparate genetic lines showed differential sensitivities to various classes of antidepressants (Sufka & White, 2013). Separate groups in each strain were administered a range of doses of imipramine, fluoxetine, maprotiline, and ketamine. Imipramine and maprotiline attenuated behavior despair in the stress-resilient Production Reds but fluoxetine and ketamine did not. Maprotiline and ketamine attenuated behavioral despair in the stress-vulnerable Black Australorps but imipramine and fluoxetine did not. This lack of pharmacological response to two classes of FDA-approved antidepressants in the stress-vulnerable Black Australorps mirrors that of the treatment-resistant clinical population. Moreover, that the Black Australorp line shows sensitivity to ketamine, a compound currently used for TRD in hospital emergency rooms, suggest strongly that this genetic line may model TRD.

In summary, previous research conducted in this lab identifying behavioral and pharmacological strain differences allows me to explore a causal relationship between TNF- α and social-separation stress. The involvement of pro-inflammatory cytokines in TRD, specifically TNF- α and the development of a TRD simulation in the chick social-separation stress paradigm prompts another set of validation experiments. One question is to determine whether an isolation stressor alters TNF- α levels and does so in a manner that differentiates stress-vulnerable and -resilient lines. Temporal patterns of TNF- α release will be quantified in these two chick lines in an attempt to not only establish an additional biomarker homology but also further validate the Black Australorp line as modeling TRD.

A related question is to determine whether effective pharmacological treatments in the two genetic lines influence patterns of TNF- α release. These data would elucidate the role of TNF- α as a key modulator of stress-related disorders and response to antidepressant therapeutics.

Chapter 5

5.1. Animals and ethical considerations

The following procedures were approved by the University of Mississippi Institutional Animal Care and Use Committee (Protocol #18-008) and were conducted in accordance with the principles of animal care as detailed in the Guide for Care and Use of Laboratory Animals (National Research Council, 1996).

Male Black Australorp and Production Red (*Gallus gallus*; Ideal Poultry, Cameron, TX, USA) chicks were obtained 1 day post-hatch and housed in stainless-steel cages (44 x 61 x 40 cm) with 12-14 chicks per cage. Food (Lab Diet, Chick Diet S-G, PMI Nutrition Intl., Brentwood, MO, USA) and water was provided ad-libitum through 1 quart gravity-fed feeders and waterers. Room temperature was maintained at 32 ± 1 °C and overhead illumination was maintained on a standard 12 h light-dark cycle.

5.2 Isolation apparatus

A six-unit test apparatus containing Plexiglas chambers (25 x 25 x 22 cm) surrounded by sound-attenuating media to reduce the transmission of sound to adjacent units was used to record dVocs. Each unit was illuminated by a 25-W light bulb and ventilated by an 8 cm diameter rotary fan (Model FP-10AX S1, Commonwealth Industrial Corp., Taipei, Taiwan). Chicks were monitored throughout isolation period by miniature video cameras (Model PC60XP, SuperCircuits, Inc., Liberty Hill, Texas, USA) mounted in the corner of the apparatus at floor-

level and routed through a multiplexor (Model PC47MC, SuperCircuits). DVocs were recorded via microphones [Radio Shack Omnidirectional Model 33-3013 (modified for AC current)] mounted at the top of each chamber and routed to a computer equipped with custom designed software for data collection.

5.3 Experimental Procedures

Chicks were tested 5-6 days post hatch. Six chicks were removed from their home-cage, weighed, color-coded with a felt marker for identification, and then placed in the isolation apparatus where dVoc measurements were recorded for either 15, 30, 60, or 90 min (n = 24 chicks per condition). Following the test period, 4 from each squad in the social separation stress apparatus were returned to their home-cage while the other 2 were euthanized via rapid decapitation to collect blood samples. In total, 8 samples were collected per experimental condition. One group of 6 chicks were removed from their home-cage, weighed to identify potential outliers, and then euthanized via decapitation for blood collection. These 6 chicks served as a no test, non-isolated control group. Six samples per experimental condition were selected for further ELISA (enzyme-linked immunosorbent assay) analysis dependent upon weight of vial (i.e. amount of blood in vial).

A chicken TNF- α Emax Immunoassay ELISA kit (LifeSpan BioSciences, Seattle, WA, US) was utilized to analyze blood samples. A 96-well plate was coated with antibodies specific to chicken TNF- α . One hundred microliters of Standard, Blank, or Sample were added to each well then sealed with a plate sealer and incubated for 90 min at 37° C. Liquid was then be aspirated out of each well. One hundred microliters of 1x Biotinylated Detection Antibody were added to each well then sealed with a plate sealer and incubated for one hour at 37° C. Liquid was then be

aspirated out of each well and washed 3 times. One hundred microliters of 1x HRP Conjugate were added into each well then sealed with a plate sealer and incubated for 30 min at 37° C. Liquid was aspirated from each well and washed 5 times. Ninety microliters of TMB Substrate solution was added to each well then sealed with a plate sealer and incubated for 15 min at 37° C. Fifty microliters of Stop Solution were added to each well. Finally, the plate was placed in a microplate reader set to 450 nm.

5.4 Statistical Analysis

Data collection software provided rate of dVocs/min. To determine onset of behavioral despair (latency to depression phase), the difference in dVoc rate from the anxiety phase (0-1 dVocs per minute rate) to the depression phase (30-90) minute period with dVoc number/min) was calculated and a threshold value at which the dVoc rate declines 25%, 50%, 75%, and 95% of this difference was determined. The time point at which the average of three consecutive single minute periods equal or are less than this threshold value was determined. Behavioral data were analyzed via a repeated measure ANOVA. One-way ANOVAs and Bonferroni comparisons were utilized to identify group differences for TNF- α levels.

Predictions:

- Black Australorps will enter into behavioral despair more quickly compared to Production Reds.
- Production Reds will show stable levels of TNF- α throughout test period

- Black Australorps will have a time-dependent elevation in TNF- α that exceeds that of Production Reds at 60, and 90 minutes.

Chapter 6

6.1 Results

Temporal patterns of dVocs across isolation conditions (15, 30, 60, and 90) in Black Australorps and Production Reds are summarized in Figure 1A and B, respectively. DVoc rates were relatively high in the initial 3-minute period, decreased approximately 40-60% during the next 15-25 minutes, and then plateaued for the remainder of the test period. This temporal pattern of dVoc rates has previously been described as an anxiety phase (0-5 min) and a depression (i.e. behavioral despair) phase (30-90 min). To determine whether dVoc rates decreased significantly across the test session, one-way repeated measures ANOVAs were performed for the 15, 30, 60, and 90 treatment conditions for each strain. One-way repeated measures ANOVAs for Black Australorps revealed a significant main effect for Time at each time point, $F(4, 92)=19.490$, $p<0.001$, $F(9,207)=10.688$, $p<0.001$, $F(19,437)=6.701$, $p<0.001$, $F(29, 667)=6.049$, $p<0.001$. One-way repeated measures ANOVAs for Production Reds revealed a significant main effect for Time at each time point, $F(4, 92)=19.490$, $p<0.001$, $F(9,207)=34.789$, $p<0.001$, $F(19,437)=13.395$, $p<0.001$, $F(29, 667)=15.835$, $p<0.001$. In both strains, this pattern in dVoc rates across the isolation test period illustrates the presence of behavioral despair in each strain and further replicates two phases of the Anxiety-Depression Model.

To determine onset of behavioral despair, the difference in dVoc rate from the anxiety phase (0-1 dVocs per minute rate) to the depression phase (30-90) minute period was calculated and a threshold value at which the dVoc rate declines 25%, 50%, 75%, and 95% was determined

for each bird. Because only two groups of animals could have those calculations performed, albeit, with different amount of time determining that average, the data will be presented in two data sets for 60 and 90 minutes in Figure 2A and B, respectively. Black Australorps entered into behavioral despair more quickly at each threshold than Production Reds in both 60- and 90-minute data sets. A two-way repeated measures ANOVA was conducted for animals in isolation for 60 minutes and revealed a significant main effect for Despair Threshold, $F(3,44) = 32.550$, $p < 0.001$, and Strain, $F(1,46) = 8.547$, $p < 0.05$, terms. However, the Despair Threshold x Strain interaction $F(3,44) = 1.915$, $p = 0.141$, term was not significant. A two-way repeated measures ANOVA was conducted for animals in isolation for 90 minutes and revealed a significant main effect for Despair Threshold, $F(3,44) = 30.622$, $p < 0.001$, and Strain, $F(1,46) = 10.613$, $p < 0.05$, terms. However, the Despair Threshold x Strain interaction, $F(3,44) = 1.582$, $p = 0.207$, term was not significant. This pattern, whereby Black Australorp enters into behavioral despair more quickly than Production Reds replicates that of previous findings in this lab (Kim & Sufka, 2011; Loria et al., 2013; Sufka & White, 2013) and illustrates Black Australorps show stress vulnerability relative to Production Reds.

Animals were euthanized at various times (0, 15, 30, 60, and 90 minutes) throughout isolation and blood samples were collected for TNF- α level quantification are summarized in Figure 3. TNF- α levels in both non-isolated strains were similar. TNF- α levels in Black Australorps remained stable across the test period. However, TNF- α levels in Production Reds showed an inverted u-shaped function with a peak at 60 minutes compared to the non-isolated Production Reds. A two-way ANOVA was conducted on TNF- α levels in both strains following 0, 15, 30, 60, and 90 minutes of isolation and revealed a significant main effect for Strain, $F(1,45) = 4.549$, $p < 0.05$. However, the main effect for Time, $F(4, 45) = 0.628$, $p = 0.645$, and Time x

Strain interaction, $F(4,45)=0.837$, $p=0.509$, terms were not significant. Bonferroni pairwise comparisons on Production Reds at each time point compared to non-isolated Production Reds were not statistically significant ($p_s > 0.358$). Bonferroni pairwise comparisons on time point 0, 15, 30, 60, and 90 minutes revealed TNF- α levels at 60 minutes were significantly higher in Production Reds compared to Black Australorps ($p < 0.028$). At time points 0, 15, 30, and 90, TNF- α levels were not significantly different between strains ($p_s > 0.181$).

Chapter 7

7.1 Discussion

The goal of this research was to investigate whether TNF- α , a pro-inflammatory cytokine implicated in stress-vulnerable treatment-resistant populations, is expressed in two outlier strains that represent stress-vulnerability and -resiliency to isolation stress in the Chick Anxiety-Depression Model. Black Australorps and Production Reds were socially-housed and then isolated at various times ranging from 0-90 minutes in which dVocs were recorded. Chicks were taken either directly from home cage or following the social-separation test session, euthanized, and blood collected for TNF- α quantification via ELISA analysis. TNF- α samples in chicks taken directly from home cage served as non-isolated controls.

As predicted, both strains of chicks in all test conditions displayed initially high dVocs until approximately 5 minutes into the test session followed by a 40-60% decrease during the next 30 minutes and remained stable thereafter. This pattern of dVocs has been described previously as representing an anxiety phase (0-3 minutes) and a depression phase (30-91 minutes) and these two phases have been pharmacologically validated in studies examining the effects of anxiolytic and antidepressant compounds in the model (Sufka et al., 2006; Warnick et al., 2009). For example, Sufka and colleagues (2006) selected two probes with different clinical activity in an attempt to dissociate the two phases. One compound was chlordiazepoxide which shows anxiolytic (i.e., anti-panic) effects but not antidepressant effects in clinical populations. Chlordiazepoxide attenuated chick dVocs in the anxiety phase without affecting behavioral despair in the depression phase. The second compound was imipramine which shows both

anxiolytic and antidepressant effects in clinical populations. High imipramine doses attenuated the high rates of dVocs into the anxiety phase and the decrease into the depression phase. A follow up study conducted by Warnick and colleagues (2009) further explored this pharmacological dissociation between phases showed compounds from different classes with anxiolytic and antidepressant properties. Chicks administered chlordiazepoxide and clonidine (i.e. a benzodiazepine and an $\alpha 2$ agonist) displayed decreased dVocs during the anxiety phase; however, these drugs did not affect dVoc rates in the depression phase. Chicks administered imipramine, maprotiline, and fluoxetine (i.e. tricyclic, SNRI, and SSRIs) displayed an attenuation of behavioral despair in the depression phase. Of these three compounds, imipramine and maprotiline possessed anxiolytic activity in clinical populations whereas fluoxetine does not. Interestingly, chicks administered high doses of imipramine or maprotiline displayed decreased dVocs during anxiety phase. These data established that these phases represent two parts of the stress-induced anxiety-depression continuum. This stress continuum seems to mirror the newer conceptualization of the temporal relationship of anxiety and depressive disorders as per the DSM- 5 with anxiety symptoms preceding and then becoming co-morbid with depressive symptoms in stress-related disorders (American Psychiatric Association, 2013).

The pattern of behavioral despair in this avian model of depression is homologous to that of rodent models of depression. For example, two common assays to model depression are the forced swim test (FST) and the tail suspension test (TST). In the former, rodents are placed into a pool of water in which escape is impossible. Animals engage in a treading/escape behavior that, over the 15-minute test session, transitions into a floating behavior described as behavioral despair (Willner, 1984; Porsolt, 1989). Porsolt, creator of the FST, defines behavioral despair as

“the recognizable immobile posture observed in rats or mice forced to swim in a restricted space from which there is no escape (1989).” In the TST, rodents are suspended by their tails and will struggle to right themselves over the 6-minute test session. This struggle/escape behavior diminishes over time similar to that seen in the FST. In the chick social-separation procedure, dVocs are initially produced in order to reestablish social contact. In the absence of locating social companions, this high level of behavior begins to decrease. The behavioral end point of behavioral despair is useful in drug discovery in that in both rodent and chick models, antidepressant drugs attenuate behavioral despair.

In the present study, it was predicted that Black Australorps would enter into behavioral despair more quickly than Production Reds and do so at one or more of the thresholds calculated. This pattern is consistent with earlier research comparing these two lines in the chick anxiety-depression model (Hymel et al., 2013; Loria et al., 2013; Sufka & White, 2013). In one study, Hymel et al. (2013) evaluated genetic differences to isolated stress sensitivity. Nine genetic lines were selected based on their feather pigmentation diversity and hatch availability. These researchers found 7 genetic lines were not significantly different from one another when calculating their time to enter into behavioral despair. However, the Black Australorp line entered behavioral despair more quickly and the Production Reds entered more slowly compared to all other lines. Two additional studies examining biomarkers and drug sensitivity between the two strains replicated the same pattern of entry into behavioral despair between those two lines.

Entry into behavioral despair, as calculated in the chick model, may be a more sensitive measure for screening antidepressant drugs in rodent models than total time in behavioral despair across a test session. Typically, rodent behavioral despair is measured by total immobility time throughout a test session (Yoshikawa et al., 2002; El Yacoubi et al., 2003; Baker et al., 2018;

Koh et al., 2007). However, a recent study found entry into behavioral despair was a more sensitive endpoint in distinguishing between drug classes where one is prone to false positives. False positives occur when a model screens a compound to be clinically effective, but these effects do not translate into clinical populations. As an example, psychostimulants are prone to false positives in rodent depression models. For example, Castagne and colleagues (2009) administered various antidepressants and psychostimulants in mice to test in the FST. Mice were administered various drug probes from various classes (i.e. imipramine, desipramine, fluoxetine, duloxetine, escitalopram, venlafaxine, amphetamine, modafinil) then placed in FST. Total immobility time and latency into behavioral despair was calculated. Researchers found latency into behavioral despair able to screen out false positive effects that are screened in by total time in behavioral despair, and ultimately, dissociating antidepressant and psychostimulant effects. Latency into behavioral despair has only been used in the chick model to compare strain and environmental housing manipulations (i.e., impoverished- versus enriched-housing) on stress sensitivity. However, this endpoint has not been used in examining activity of anxiolytic and antidepressant drugs. Chick dVoc rates has consistently shown pharmacological sensitivity including avoiding false positives screened in rodent models (Warnick et al., 2009). It may be that measures of ongoing behavior such as dVoc rates or latency to reach behavioral despair will prove to be a better endpoint in antidepressant drug screening than total time in behavioral despair.

In the present study, the first prediction was TNF- α levels in non-isolated controls would not differ between Black Australorps and Production Reds. These control groups showed similar levels of TNF- α that were in the range of 20-50 pg/ml. The finding that basal levels of this stress-related biomarker is similar between strains prior to separation stress is consistent with previous

work examining strain differences in neurotrophic factors, another stress-related biomarker. Loria and colleagues (2013) sought to determine patterns of BDNF levels during the isolation period and found that in non-isolated Black Australorps and Production Reds, basal levels of BDNF were not significantly different from one another. Like BDNF, the findings of the current study indicate that basal TNF- α levels do not account for stress-vulnerability and -resiliency of these two lines in the model.

One goal of this project was to determine whether an isolation stressor elicits TNF- α alterations. The second prediction was that TNF- α levels in stress-resilient Production Reds would increase across the test session and peak around 60-minutes. Consistent with that prediction, Production Reds showed a gradual increase in TNF- α at the 30-minute mark that peaked at the 60-minute mark, and declined by the test 90-minute time point. This inverted U-shaped function of TNF- α release has been shown in rodent models of depression utilizing an LPS stressor induction (Taffett et al., 1989; Givalois et al., 1994). For example, Givalois and colleagues (1994) sought to investigate the immune response when administered LPS in rats. Researchers found rats administered LPS displayed peak TNF- α levels at 60 minutes. These findings add an additional biomarker validation study to the chick anxiety and depression model.

The third predication was that TNF- α levels in stress-vulnerable Black Australorps would show an elevated response across the test period compared to Production Reds. In contrast to this prediction, TNF- α levels in Black Australorps were unaffected by isolation stress across the entire test period. Indeed, TNF- α levels never increased above 50 pg/ml across the test session. The prediction that TNF- α levels would be higher than Production Reds was due to 1) this stress-vulnerable genetic line modeling treatment resistant depression and 2) findings that increased TNF- α levels are found in clinical populations with Treatment Resistant Depression (Feltes et al.,

2017; Schiepers, et al., 2005; Tuglu et al., 2003; Mikova et al., 2001 Lanquillon et a., 2000). However, there is a literature that describes a number of altered patterns of stress biomarkers, one of which is detailed below that dovetails with this chick simulation of TRD.

The absence of a stress biomarker production whereby, biological levels are unusually low, has been called either blunted or depleted response in both clinical populations and animal models of depression (Holsboer et al., 1984; Gold et al., 1986; Young et al., 1993; Peeters et al., 2003; Carroll et al., 1981; Maes et al., 1994; Burke et al., 2005; Cohen et al., 2006). For example, Burke and colleagues (2005) sought to determine the association of depressive symptoms and cortisol levels in high-risk clinical populations (i.e. participants from low income areas). Participants were administered the Center for Epidemiologic Studies-Depression Scale (CES-D) and saliva samples were taken upon arrival, 25 minutes, and 50 minutes after arrival. Researchers found participants with lower CES-D scores showed a significantly increased cortisol response; however, participants with elevated CES-D scores failed to show a cortisol response. These findings suggest populations living in low-income areas show a depletion in the stress-related biomarker cortisol.

Such gene x environment interaction may explain the TNF- α response in these outlier strains. Multiple studies have reliably shown the genetic component of this model in which stress-vulnerable Black Australorps enter into behavioral despair more quickly than stress-resilient Production Reds (Loria et al., 2013, Hymel et al., 2013; Sufka & White, 2013). Another line of research has shown environmental manipulations in housing affect entry into behavioral despair. Enriched housing with perches, mirrors, dust baths, and string delays entry into behavioral despair relative to standard “impoverished” housing conditions (Kim & Sufka, 2011). In the present study, chicks were housed under a standard “impoverished” housing condition.

Impoverished housing is often used as a chronic mild stressor that is known to affect acute stress-related behavior. It appears these stressors have a differential effect on TNF- α in the two outlier genetic lines. Stress-resilient Production Reds raised in standard impoverished housing show an inverted U-shaped TNF- α response to an acute stressor that is interpreted as a normal cytokine response. Stress-vulnerable Black Australorps raised in standard impoverished housing show a blunted TNF- α response to an acute stressor. These findings suggest standard impoverished housing as an environmental stressor is less impactful on cytokine release patterns in Product Reds compared to Black Australorps.

For this gene x environment interaction theory on a blunted cytokine response and increased entry into behavioral despair to be true, it would follow that environmental enrichment during housing in Black Australorps would positively affect these two endpoints. The prediction is that enriched housing would produce a more normalized response (i.e. inverted u-shaped function) on TNF- α release and slow their entry into behavioral despair compared to their impoverished counterparts. However, neither endpoints in Black Australorps are likely to reach the respective levels of Production Reds given the latter line represent an outlier strain in terms of stress resilience. A more appropriate comparison might be one of the other seven genetic lines that was tested in the model and showed an intermediate latency into behavioral despair to isolation stress (Hymel et al., 2013).

Existing data from a different study offers predictions for how enriched environments will affect these two endpoints in the stress-resilient Production Reds. This work, being prepared for publication, has shown stress-resilient Production Reds raised in an impoverished environment enter into behavioral despair more slowly and display lower BDNF levels compared to those housed in an enriched environment. These unexpected findings were interpreted to

suggest impoverished housing in a stress-resilient strain may inoculate, to some degree, the effects of a subsequent acute stressor. If this interpretation is correct, then Production Reds housed in an enriched environment will enter behavioral despair more quickly and TNF- α levels will display a smaller inverted u-shaped function response compared to their impoverished counterparts.

This gene x environment interaction theory suggests standard impoverished housing is chronic mild stress that differentially affect TNF- α release patterns and onset times into behavioral despair of an acute stressor in these genetic lines. Chronic mild stress leads to TNF- α elevation. TNF- α then binds to a TNF- α receptor and starts a biological cascade that initiates neural pruning to a variety of systems that release TNF- α and other systems that could be responsible for behavioral despair (i.e. the hippocampus and limbic pathway). The neural pruning of TNF- α target systems leads the loss of TNF- α release and, ultimately, the blunted TNF- α response in the stress-vulnerable Black Australorps. The use of agonists and antagonists would help better understand the mechanism in which TNF- α is released and leads to the blunted TNF- α response. As with enrichment, agonist and antagonist drug probes might have differential effects on TNF- α production and entry into behavioral despair on these outlier strains. In Black Australorps, if chronic mild stress causes a blunted TNF- α response from chronic TNF- α release and neural pruning then etanercept, a TNF- α inhibitor that acts as a decoy receptor, should prevent neural pruning by TNF- α binding to it and, ultimately, increase TNF- α to a small inverted u-shaped function. In Production Reds with chronic mild stress, if chronic mild stress causes a normal TNF- α response are due to typical TNF- α release, then an agonist should decrease TNF- α and display a blunted response. However, both approaches present challenges

for determining TNF- α release patterns in Black Australorps and Production Reds. These approaches, along with their respective confounds and predictions, are discussed below.

One pharmacological approach to determining the mechanisms of TNF- α blunted response in Black Australorps is to antagonize the target system of interest. Etanercept is a TNF- α inhibitor and a large body of literature has studied its effects in behavioral despair models (Inglis et al., 2005; Venegas-Pont et al., 2010; Krugel et al., 2013; Bayramgurler et al., 2013). Current cytokine-related depression animal models utilize repeated TNF- α administration and show an attenuation of behavioral despair (Inglis et al., 2005; Venegas-Pont et al., 2010; Krugel et al., 2013; Bayramgurler et al., 2013). Bayramgurler and colleagues (2013) sought to determine whether long-term etanercept administration would affect anxiety- and depression-like behaviors in rats. Rats were chronically administered either saline or etanercept then enrolled in the elevated plus maze and forced swim test. Researchers found chronic etanercept administration caused a significant increase in time spent in open arm in the elevated plus maze and a significant decrease in immobility time in the forced swim test. These findings suggest chronic etanercept administration possesses anxiolytic and antidepressant effects. The same pattern of effects is found in chronic mild stress paradigms.

Similarly, Krugel and colleagues (2013) sought to determine whether repeated etanercept administration would alter, depression-like behaviors in rats. Animals either received no restraint stress or repeated restraint stress and repeated saline, imipramine, or etanercept at various doses. Following restraint and repeated administration, animals were enrolled in the forced swim test. Similarly, rats administered etanercept and imipramine showed significant immobility time decreases compared to animals in the restraint-saline group. These findings lead

researchers to suggest that repeated administration of etanercept possessed antidepressant-like properties similar to that of imipramine.

In summary, the previous studies show chronic etanercept administration antidepressant effects in rodent models of depression. However, it is important to note the method in which etanercept is administered. For example, Bayramgurler et al., (2013) and Krugel et al., (2013) both utilize chronic etanercept administration in rodents. Bayramgurler et al., (2013) administered etanercept daily for eight weeks to rats. Similarly, Krugel et al., (2013) administered etanercept twice weekly for three weeks to rats. In both studies, chronic etanercept administration produces antidepressant-like properties. Researchers utilize the chronic etanercept administration to not only achieve drug efficacy but also mirror chronic antidepressant administration required for efficacy in clinical populations and rodent models of depression.

Chronic etanercept administration in our chick model, in a manner that would replicate that in a rodent model, is not possible. Chronic drug administration in rodent models occurs over several weeks. The social separation stress test in chicks occurs at one week post hatch due to decreased isolation sensitivity seen in older chicks. Due to this limiting window of isolation sensitivity, animals would not be able to receive the length of chronic drug administration as seen in clinical populations nor rodent models of depression. However, chronic administration in the chick model is possible when drug probes are administered every day from arrival to testing. We would predict that chronic agonist and antagonist drug administration would differentially affect behavioral despair and TNF- α levels in each genetic line.

To further test whether patterns of blunted TNF- α response in Black Australorps occurs as a byproduct of the gene x environment interaction, a TNF- α inhibitor (etanercept) could be chronically administered during standard impoverished housing. There is evidence that suggests

chronically high TNF- α levels lead to glucocorticoid receptor resistance and glucocorticoid depletion (Miller, Pariante, & Pearce, 1999). If TNF- α depletion is due to chronic mild stressors in which elevated TNF- α has excessive activity on a regulatory negative feedback system, then chronic etanercept administration should prevent chronic TNF- α release during chronic mild stress and thus preventing the inhibition of the negative feedback system. If impoverished housing causes the blunted TNF- α response, in Black Australorps, then chronic etanercept administration would prevent any downstream TNF- α effect and, ultimately, increase TNF- α . Although these endpoints are unlikely to reach the respective levels of the stress-resilient Production Reds. A more appropriate comparison would be one of the other seven genetic lines that showed an immediate response to isolation stress (Hymel et al., 2013).

Another pharmacological approach to understand the mechanisms of TNF- α release in light of the gene x environment interaction is to agonize the target system. One approach to agonize TNF- α is via administration of lipopolysaccharide (LPS). While LPS causes animals to enter into behavioral despair it is also the case that LPS not only affects TNF- α but also IL-6 and IL-10 (Ji et al., 2014; Ohgi et al., 2013). LPS can affect behavioral endpoints in rodent models but it does not show the specificity to test the expression of TNF- α in the stress-resilient Production Reds. LPS administration would not be able to answer the question the gene x environment interaction and TNF- α mechanism due to the lack of cytokine specificity.

The best way to investigate the mechanism of TNF- α in relation to a gene x environment interaction is to administer TNF- α itself. For example, Biesmans and colleagues (2015) sought to understand the role of peripherally administered TNF- α on cytokine activity and stress-related behaviors. One set of animals had blood and brain tissue harvested at various time points for cytokine quantification. Peripherally administered TNF- α produced a dose dependent increase

in IL-6 and TNF- α at 2 and 6 hour time points in both blood and brain. In addition, TNF- α increased IL-10 at the 6 hour time point in blood. A second set of animals received TNF- α and was enrolled in repeated behavioral assays of forced swim, open field, and sucrose preference tests across at 2, 6, 24 and 48 hours. TNF- α caused alterations in open field and sucrose preference behavior indicative of anxiety and depression, respectively. However, TNF- α failed to enhance behavioral despair on the forced swim test. It remains to be determined how TNF- α affects the dependent measure of latency into behavioral despair in the chick anxiety and depression model. Further, TNF- α reduced locomotor behavior, and decreased fluid intake and body weight each of which are indices commonly used to quantify “sickness-behavior” (i.e. weight changes, decreased fluid intake, and reduced motor activity). However, sickness has been shown to go away within 24 hours of administration.

To further understand the differential patterns of TNF- α driven by a gene x environment interaction in Production Reds in the chick anxiety and depression model, an agonist (TNF- α) would be chronically administered in standard impoverished housing. If TNF- α depletion is due to chronic mild stressors in which elevated TNF- α has excessive activity on a regulatory negative feedback system, then chronic TNF- α administration during chronic mild housing stress should exacerbating the effects of the negative feedback system. If impoverished housing produces a “normal” inverted u-shaped function TNF- α response in Production Reds, then chronic TNF- α administration prior to isolation stress would decrease TNF- α levels to mirror a blunted response seen in Black Australorps. Similarly, if impoverished housing decreases entry into behavioral despair, then chronic TNF- α administration prior to isolation stress would further decrease entry into behavioral despair compared to their saline administered counterparts.

There are a number of major findings and implications from this study. First, in general, this chick stress assay presents TNF- α response in a manner similar to clinical populations. TNF- α represents a fourth stress-related biomarker, along with corticosterone, IL-6, and BDNF, quantified in predictable patterns in this anxiety-depression simulation. The finding of multiple stress-related biomarker homologies represents significant progress in validating this animal model simulation. Second, TNF- α 's response pattern was dependent upon the strain being evaluated in the model. TNF- α presents itself as an inverted u-shaped function in the stress-resilient Production Reds. However, TNF- α presents itself in a rather unusual blunted pattern in the stress-vulnerable Black Australorps that appears to be driven by a gene x environment interaction. This pattern of blunted stress-related biomarkers in chicks mirrors populations diagnosed with depression living in low socioeconomic conditions. This additional homology represents an important etiological validation step in developing this as an animal model simulation.

These two genetic lines and use of impoverished/enriched housing might be useful in parsing out the relative contributions of genetics and environments in a model of stress-related disorders. For example, this model could answer questions about the degree to which environmental enrichment modifiers might overcome genetic predisposition to stress-vulnerability and -resiliency. Moreover, this model may be useful for identifying efficacious novel pharmacotherapies targeting cytokine systems for treatment resistant depression. For example, TNF- α has also shown to affect downstream targets on pro- and anti-inflammatory cytokines and glucocorticoids that could potentially play a role in stress-related disorders. Whether these and other targets prove efficacious as antidepressant treatments remain to be determined.

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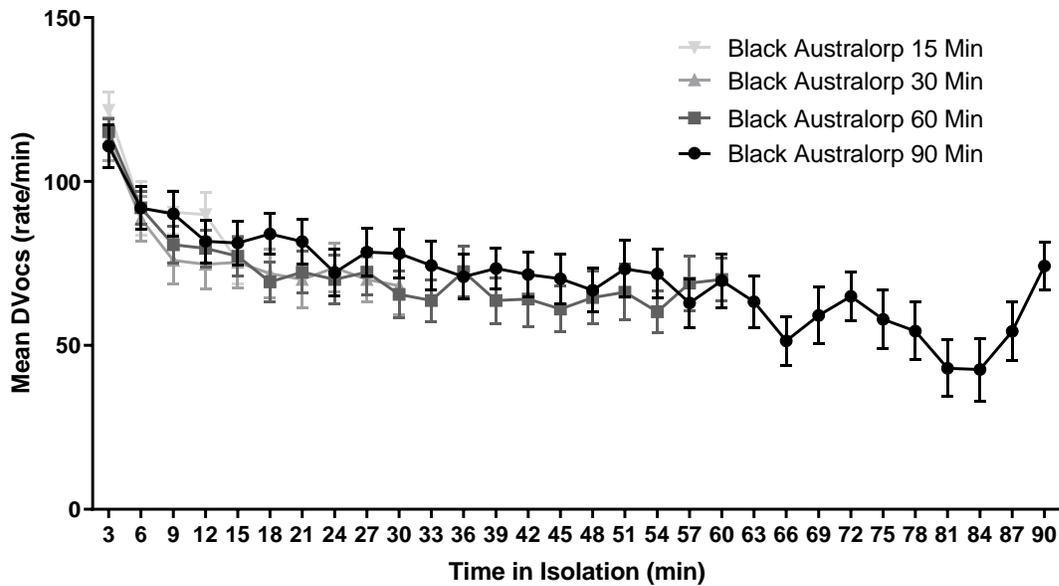
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List of Appendices

Appendix A: Figures and Captions

A. Black Australorps



B. Production Reds

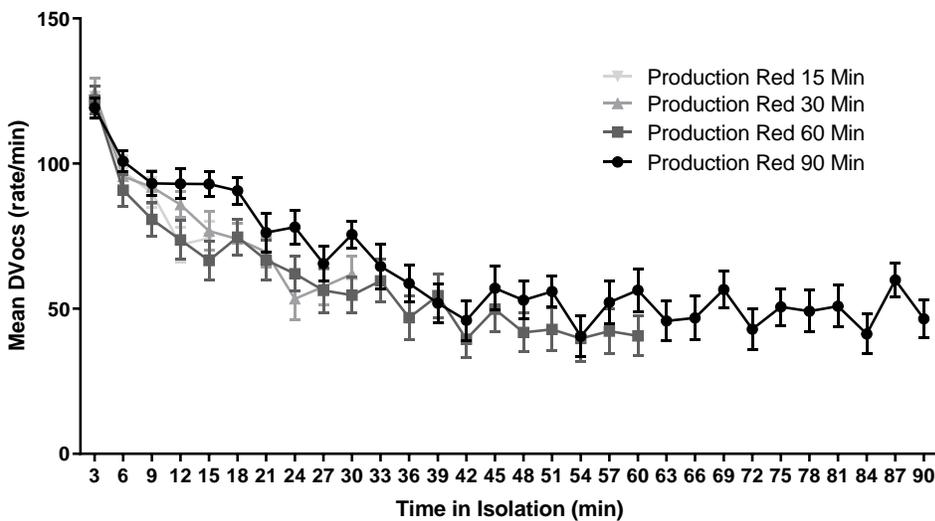
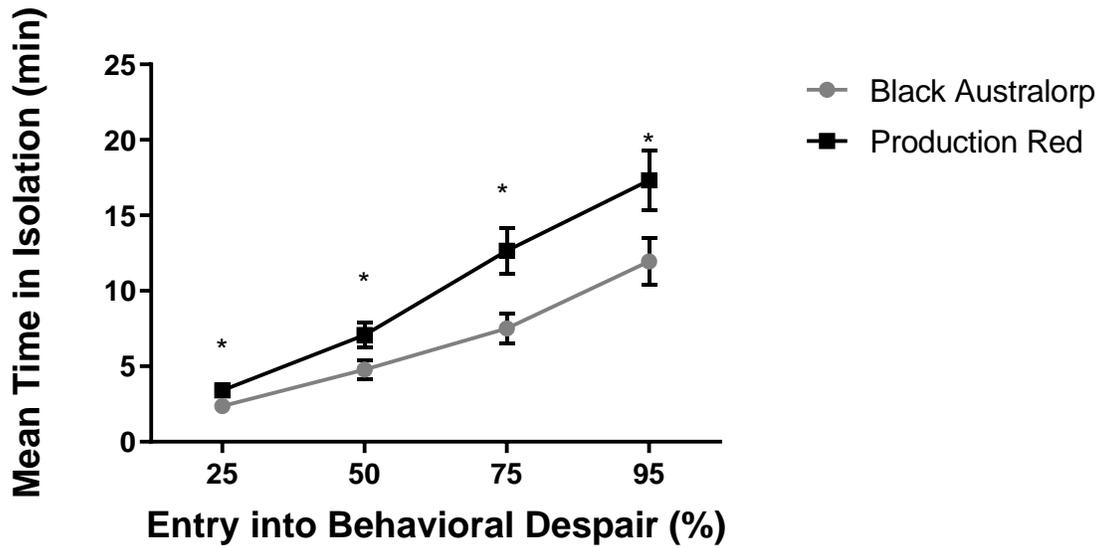


Figure 1. The effects of social separation stress in Black Australorps (panel A) and Production Reds (panel B). Values represent mean DVoc rates (\pm SEM) in 3 min blocks across various times in isolation. Sample sizes were $n = 24$.

A. 60 Minutes



B. 90 Minutes

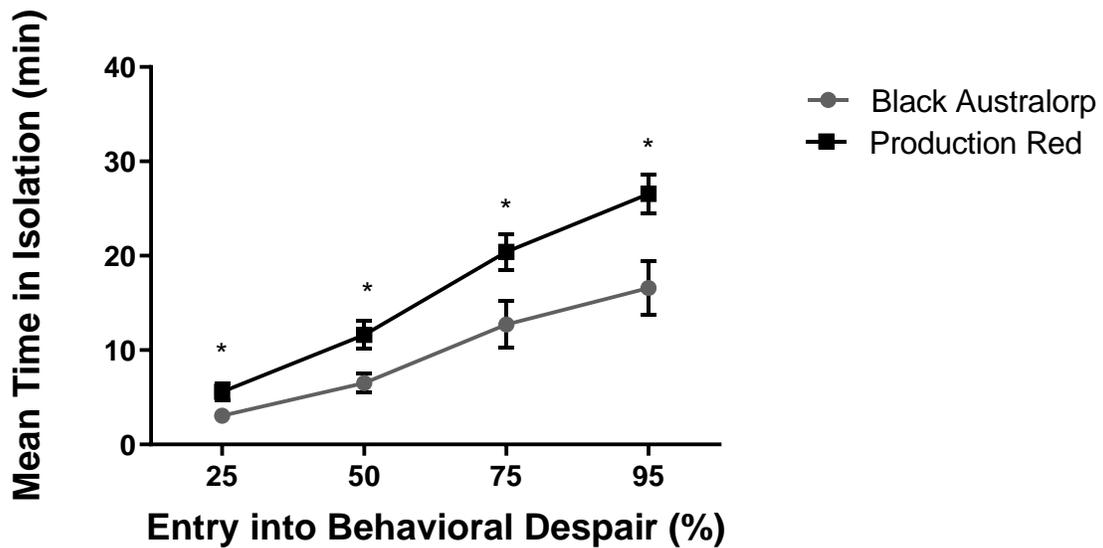


Figure 2. Mean depression onset thresholds for Black Australorps and Production Reds isolated for 60 minute test period (panel A) and 90 minutes (panel B). Values represent mean (\pm SEM). Asterisk (*) indicates significantly shorter depression threshold latencies in Black Australorps. Sample sizes were $n=24$.

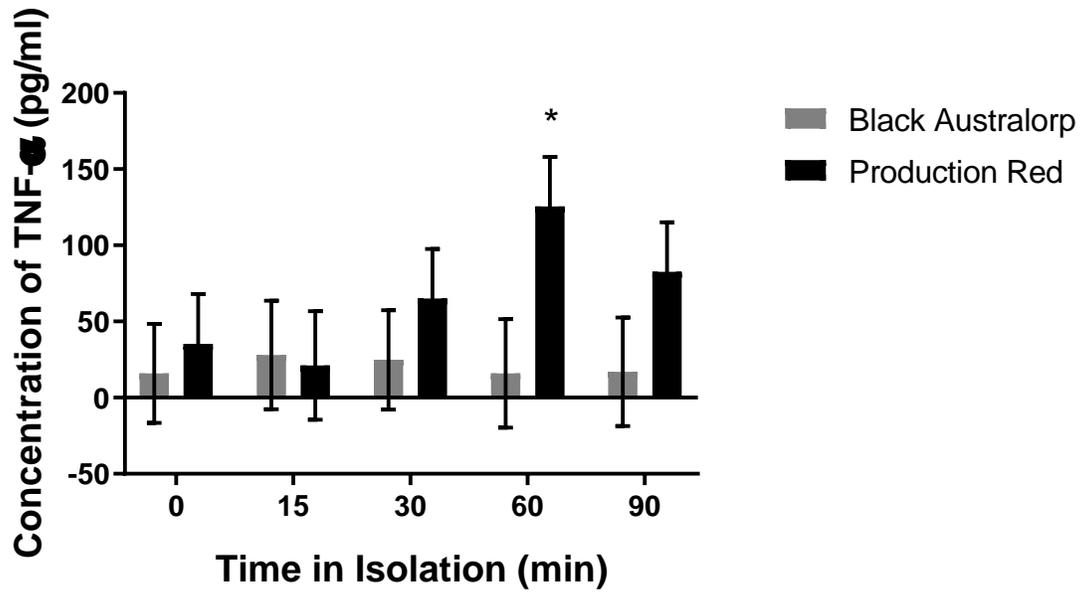


Figure 3. The effects of social separation stress on serum TNF- α levels in Black Australorp and Production Red Strains. Values represent mean TNF- α in pg/ml (\pm SEM). Serum samples were collected following 0, 15, 30, 60, or 90 minutes of isolation. Asterisk (*) indicates significantly higher TNF- α levels in Production Reds. Sample sizes were n=24.

VITA

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EDUCATION

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Carpenter JM, Jourdan MK, Fountain EM, Ali Z, Abe N, Khan IA, Sufka KJ (2016) The effects of *Sceletium tortuosum* (L.) N.E. Br. extract fraction in the chick anxiety-depression model. *Journal of Ethnopharmacology*

MANUSCRIPTS UNDER REVIEW, SUBMITTED, OR IN PREPARATION

White SW, Jourdan MK, Anchor, SM, Sharma PK, Murthy SN, Sufka KJ. (In Preparation). Biochemical and behavioral effects of environmental enrichment on strain-dependent vulnerability to anxiety and depression in the chick separation stress paradigm.

INTERNATIONAL CONFERENCES PAPERS AND ABSTRACTS

Jourdan MK, Anchor, SM, White SW, Sharma PK, Murthy SN, Sufka KJ. (November, 2017). Biochemical and behavioral effects of environmental enrichment on strain-dependent vulnerability to anxiety and depression in the chick separation stress paradigm, Society for Neuroscience, Washington D.C.

Jourdan, Mary K., Carpenter, Jessica M., Fountain, Emily M., Ali, Zulfiqar, Abe, Naohito, Khan, Ikhlal A., and Sufka, Kenneth J., (November, 2016). The Effects of *Sceletium tortuosum* extracts in the Chick Anxiety-Depression Model, Society for Neuroscience, San Diego, CA.

Salmeto, Amy L., Jourdan, Mary K., and Sufka, Kenneth J. (October 2015). Measurement of Anhedonia in the Chick Anxiety-Depression Model, Society for Neuroscience, Chicago, IL.

REGIONAL AND LOCAL CONFERENCE PAPERS AND ABSTRACTS

Anchor SM, Jourdan MK, White SW, and Sufka KJ. Biochemical and behavioral effects of environmental enrichment on strain-dependent vulnerability to anxiety and depression in the chick separation stress paradigm. (April 2017), Psychology Department Research Day, Oxford, MS.

Fountain, Emily M., Jourdan, Mary K., Carpenter, Jessica M., Ali, Zulfiqar, Abe, Naohito, Khan, Ikhlas A., and Sufka, Kenneth J., (April, 2016). The Effects of *Sceletium tortuosum* Extracts in the Chick Anxiety-Depression Model, Psychology Department Research Day, Oxford, MS.

Anchor SM, Jourdan MK, White SW, and Sufka KJ. Biochemical and behavioral effects of environmental enrichment on strain-dependent vulnerability to anxiety and depression in the chick separation stress paradigm. (April 2016). Annual UM Neuroscience Minor Research Showcase.

PRESENTATIONS

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