NEONATAL ENVIRONMENT INFLUENCES BEHAVIOURAL AND
PHYSIOLOGICAL REACTIVITY TO STRESSORS, AND MAMMARY GLAND
DEVELOPMENT IN BALB/c MICE: IMPLICATIONS FOR BREAST CANCER RISK

A Thesis Submitted to the Committee on Graduate Studies in Partial Fulfilment of the
Requirements for the Degree of Master of Science in the Faculty of Arts and Science

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ABSTRACT

Neonatal Environment Influences Behavioural and Physiological Reactivity to Stressors, and Mammary Gland Development in BALB/c Mice: Implications for Breast Cancer Risk

Kyle Kenniphaas

Using rodent models, it is possible to study the behavioural and physiological outcomes of early life stress and the influences on normal mammary gland development and carcinogenic risk. Results demonstrate that the experience of three weeks of prolonged maternal separation (LMS; 4 hrs/day) increased the susceptibility of adult, but not pubertal, female BALB/c mice to engage in higher levels of depressive-related immobility behaviour and lower levels of active floating (a suggested adaptive coping behaviour) in the acute forced swim test, than offspring that experienced three weeks of brief separation (BMS; 15 min/day) events. Despite the increased immobility behaviour, adult LMS female offspring demonstrated lower basal corticosterone levels relative to BMS females. However, the experience of chronic early-life stress, regardless of the length, results in greater changes between non-stressed and stressed corticosterone levels (i.e. stressor reactivity) in adult females compared to their male counterparts. These changes were associated with decreased glucocorticoid receptor and coactivator-associated arginine methyltransferase 1 protein expression in mammary gland of female LMS mice at young adulthood, highlighting potential mechanisms underlying their heightened risk of mammary tumourigenesis. These data suggest that early life environments can induce behavioural and physiological alterations observed in adulthood, which may have an influence on the likelihood of malignancies developing in the breast.
Keywords: mother-infant interactions, early life stress, stressor reactivity, coping, mammary gland development, cancer etiology, corticosterone, steroid receptors, coactivators.
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<th>Description</th>
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<tbody>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropic hormone</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AVP</td>
<td>Arginine vasopressin</td>
</tr>
<tr>
<td>BMS</td>
<td>Mice experiencing brief maternal separation from dam for 15 min/day</td>
</tr>
<tr>
<td>BRCA1</td>
<td>Breast cancer type 1 susceptibility protein</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CARM1</td>
<td>Coactivator-associated arginine methyltransferase 1</td>
</tr>
<tr>
<td>cDNA</td>
<td>Copy deoxyribonucleic acid</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CORT</td>
<td>Corticosterone/cortisol</td>
</tr>
<tr>
<td>CRH</td>
<td>Corticotropin-releasing hormone</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3’-diaminobenzidine</td>
</tr>
<tr>
<td>DHEA</td>
<td>dehydroepiandrosterone</td>
</tr>
<tr>
<td>DMBA</td>
<td>7,12-diamethylbenz[a]anthracene</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxynucleotide triphosphate</td>
</tr>
<tr>
<td>E2</td>
<td>Estradiol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene-diamine-tetra-acetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>ERα</td>
<td>Estrogen receptor (α)</td>
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<tr>
<td>ERβ</td>
<td>Estrogen receptor (β)</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle-stimulating hormone</td>
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<tr>
<td>GH</td>
<td>Growth hormone</td>
</tr>
<tr>
<td>GR</td>
<td>Glucocorticoid receptor</td>
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<tr>
<td>HCl</td>
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<td>GH</td>
<td>Growth hormone</td>
</tr>
<tr>
<td>HPA</td>
<td>Hypothalamus-pituitary-adrenal</td>
</tr>
<tr>
<td>HPG</td>
<td>Hypothalamus-pituitary-gonadal</td>
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<td>LHPA</td>
<td>Limbic-hypothalamic-pituitary-adrenal</td>
</tr>
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<td>LMS</td>
<td>Mice experiencing long maternal separation from dam for 4 h/day</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>Magnesium chloride</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-(N-morpholino)propanesulfonic acid</td>
</tr>
<tr>
<td>MR</td>
<td>Mineralocorticoid receptor</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<tr>
<td>mtDNA</td>
<td>Mitochondrial deoxyribonucleic acid</td>
</tr>
<tr>
<td>ND2</td>
<td>NADH-Ubiquinone Oxireductase Chain 2</td>
</tr>
<tr>
<td>“No RT”</td>
<td>No reverse transcriptase</td>
</tr>
<tr>
<td>p53</td>
<td>Tumour suppressor gene p53</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PND</td>
<td>Postnatal days of age</td>
</tr>
<tr>
<td>PP5</td>
<td>Protein phosphate 5</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone receptor</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>Real-time quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>Ribonuclease</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SRC3</td>
<td>Nuclear receptor coactivator 3 (i.e. steroid receptor coactivator)</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-borate-EDTA</td>
</tr>
<tr>
<td>TEB</td>
<td>Terminal end bud</td>
</tr>
<tr>
<td>TR</td>
<td>Typically reared mice (animal facility reared)</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
</tbody>
</table>
Foreword

Both of the research chapters in this thesis either have been or will be submitted as manuscripts to peer-reviewed research journals. General methodology common to both chapters has been removed and placed in Chapter 2: General Methodology. Specific journal styles as well as contributors are provided below.

Chapter 3: Neonatal experiences, sex, and age interact to shape stressor reactivity, and depressive-like and coping behaviours in BALB/c mice.

In preparation for submission to Journal of Animal Behaviour.

Contributors:

Kyle Kenniphaas: Statistical analyses, manuscript writing

Dr. Leslie Kerr: Generation of research questions and hypotheses, overall supervision of project

Chapter 4: Neonatal experiences differentially influence gene and protein expression profiles in the adult mammary gland of BALB/c mice.

In preparation for submission to Journal of Mammary Gland Biology and Neoplasia.

Contributors:

Kyle Kenniphaas: RT-qPCR, immunohistochemistry, statistical analyses, manuscript writing

Brianna Beaver: immunohistochemistry (GR and MR)

Nikole Watson: immunohistochemistry (CARM1), animal rearing

Allison Boyd: Animal rearing

Dr. Leslie Kerr: Generation of research questions and hypotheses, overall supervision of project
Chapter 1: General Introduction

Biobehavioural Pathways and Breast Cancer Risk

According to the Canadian Cancer Society (2014), one in every nine women is expected to be diagnosed with breast cancer during her lifetime. Studies have identified several well-characterized risk factors, including having a family history of breast cancer, early age at menarche, late age at menopause, late age at first birth, alcohol consumption, having dense breast tissue on mammograms, and gene mutations (e.g., BRCA1) (Hankinson, Colditz, & Willett, 2004; Kelsey, Gammon, & John, 1993; Byrne et al., 1995; Hamajima et al., 2002; Tonin, 2000). Another extensively studied factor with respect to breast development and cancer risk is psychological stress; which is characterized by “the experience of a negative life event or the occurrence of such an event along with a subjective evaluation of inadequacy to effectively cope with it” (Costanzo, Sood, & Lutgendorf, 2011). Although exposure to stress has been generally related to an increased cancer initiation and progression, the manner and extent to which stress confers increased risk remains relatively unclear (Lutgendorf & Sood, 2011; Antonova, Aronson, & Mueller, 2011; Andersen, Kiecolt-glaser, & Glaser, 1994).

When a stressor (e.g., social isolation, job loss) in the environment is perceived as threatening, the central nervous system activates pathways of the autonomic nervous system and the hypothalamus pituitary adrenal (HPA) axis. In particular, the release of norepinephrine and epinephrine from the sympathetic nervous system and adrenal medulla are responsible for the effects of the autonomic nervous system on the cardiac, respiratory, and vascular system (Charmandari, Tsigos, & Chrousos, 2005). Further in response to stress, corticotrophin-releasing hormone and vasopressin are produced from
the paraventricular nucleus (PVN) of the hypothalamus, which then stimulate the anterior pituitary gland, resulting in the secretion of the adrenocorticotropic (ACTH) hormone. Subsequently, ACTH acts on the cells of the adrenal gland and this leads to the release of glucocorticoids (i.e., corticosterone in rodents and cortisol in humans). Glucocorticoids mediate the stress reactivity of several organ systems, as well as have a role in immune function (Lutgendorf, Sood, & Antoni, 2010; Charmandari, Tsigos, & Chrousos, 2005).

Although the activation of these pathways are required for adapting and surviving to the stressful environment, exposure to chronic stress can have negative effects on physiological systems due to the prolonged exposure to glucocorticoids and catecholamines (Thaker & Sood, 2008; McEwen, 2002). The physiological systems that chronic stress can negatively impact include the systems involved in the regulation of the stress response (e.g., HPA axis), and the cardiovascular and immune systems (McEwen, 2006). Furthermore, Antoni and Cole (2006) suggest that catecholamines function in a synergistic fashion with glucocorticoids to influence cellular and molecular events (e.g., DNA repair mechanisms) that not only can negatively affect stress-related systems by can also promote cancer growth in hormone responsive tissues, including the breast. Cortisol has also been known to suppress apoptosis in the mammary gland and reduce the ability of the immune system to detect and respond to tumour cells, indicating that prolonged exposure to stress may facilitate malignant mammary gland progression (Feng et al., 1995; Antonova, Aronson, & Mueller, 2011; Yang & Glaser, 2003).
The Rodent Model to Study Stress

It has been suggested that the maternal environment plays a critical role in affecting the development of mammals (Pryce & Feldon, 2003; Gutman & Nemeroff, 2002). In particular, aspects of the early environment, such as the parent-infant relationship, have a significant impact on the behavioural and physiological development of the offspring. Indeed, adverse experiences (e.g., loss of a parent, abuse, parental neglect) during development have been demonstrated to have long-lasting effects on emotionality and stress reactivity, and regulation of the hypothalamus-pituitary adrenal (HPA) axis (Plotsky et al., 2005; Tata, 2012; O’Mahony et al., 2009; Francis & Meaney, 1999; Miki et al., 2013). It also has been established that early stressful life events are risk factors for the development of an array of psychopathologies, and have been associated with increased vulnerability to different diseases in adulthood, such as diabetes, obesity, and cancer (Neumann et al., 2005; Paternain et al., 2012; Burdge, Lillycrop, & Jackson, 2009; Tacon, 2002).

Given children often endure several forms of adversity (e.g., loss of a parent, physical abuse) throughout development, inferring causal relationships between specific experiences and physiological and behavioural effects later in life is difficult (Loman & Gunnar, 2010; Tata, 2012). In order to overcome this limitation and study the impact of early life experiences on development, well-established animal models of ethologically relevant early life events, such as those manipulating mother-pup interactions, are used (George, Bordner, Elwafi, & Simen, 2010; Uchida et al., 2010; Bautista & Dueñas, 2012). For example, in the maternal separation paradigm, offspring are subjected to bouts of brief (e.g., 1-15 minutes/day; BMS), long (e.g., 2-6 hours/day: maternal separation;
LMS), or no (typical facility reared and used as an environmental control; TR) maternal separation from the dam over the first few (i.e., 1-3) weeks of life. This is ethologically relevant since it is typical in a natural setting for a rodent dam to leave the nest for short periods (e.g., 5-25 minutes) to forage (Kuhn & Schanberg, 1998). It is also common for lower ranking rodent females to leave their nests for prolonged periods of time (e.g., 2-3 hours) due to greater distance of their burrows from sources of food and water. The combination of relatively brief hypothermia and changes in dam-offspring interactions (including the mother’s feeding and nesting behaviors) that result, due to the short- and long-term maternal separation experiences, have been suggested to prepare the pups for the environment once they leave the relative safety of the nest by differentially altering LHPA axis development and reactivity. The influence of these experiences on brain peptide expression, stress hormone levels, and coping behaviors of the rodent offspring (Levine, 2000; Liu, et al., 1997; Pryce & Feldon, 2003) increases the likelihood of survival, if the environment to which they have vicariously adapted does not change substantially.

In particular, research has shown that the experience of long bouts of maternal separation results in increased behavioural and endocrine responses to stress, hyperactivity of the HPA axis, and increases in anxiety-like behaviours in adulthood (Sanders & Anticevic, 2007; Nishi, Horii-Hayashi, Sasagawa, & Matsunaga, 2013). On the other hand, it has been suggested that the experience of brief bouts of maternal separation reduces adulthood physiological and behavioural responses to stress, as well as reduces anxiety-like behaviours (Levine, 2005; Plotsky et al., 2005; Meany, Aitken, Sharma, Viau, & Sarrieau, 1990). Interestingly, it has also been demonstrated that
compared to female BMS mice adult female mice that experienced long bouts of maternal separation in the first three weeks of life have significantly higher tumour incidence following carcinogen (7,12-dimethylbenz(a)anthracene) administration, as well as significantly higher estrogen receptor alpha protein expression; a protein that is highly implicated in breast development and malignant mammary gland growth (Boyd et al., 2010).

**Normal Mammary Gland Development and Structure**

The development of the mammary gland commences during embryogenesis and continues to mature throughout puberty and pregnancy. Beginning on embryonic day 10-11 the ectoderm thickens, forming a mammary milk line/streak, which extends from the anterior to the posterior limb buds (Richert, Schwertfeger, Ryder, & Anderson, 2000; Hynes & Watson, 2010). Following approximately one day of its formation in rodents, the mammary line resolves into three thoracic pairs and two inguinal pairs of placodes, which invaginate into the underlying stroma. The five placode pairs then undergo symmetrical development (Cowin & Wysolmerski, 2010; Watson & Khaled, 2008). Although the female mouse mammary buds remain relatively inactive from embryonic day 14 to 16, in male mice the mesenchymal around the stalk expands and separates from the skin, followed by apoptosis of the mesenchyme and cells within the epithelial bud (Dunbar et al., 1999; Cowin & Wysolmerski, 2010). The next step in development for the female mammary bud consists of ductal branching morphogenesis on embryonic day 16. Epithelial cells grow from the mammary mesenchyme, giving rise to a second stromal compartment; the mammary fat pad (Hens & Wysolmerski, 2005). Branching begins
once the fat pad is reached by the primary cord, and this results in the formation of a ductal tree. After the initial round of branching that occurs from embryonic day 16 to the perinatal period, a primary duct is established. Before the conclusion of embryonic development, a ductal lumen is formed, as well as a nipple structure by modifying the skin overlying the primary mammary mesenchyme (Cowin & Wysolmerski, 2010; Watson & Khaled, 2008).

Following birth, but before puberty, the mammary ducts continue to slowly extend into the fat pad at a rate based on the growth of the animal. This rate is accelerated once puberty (approximately 3-6 weeks of age for mice) is started (Hennighausen & Robinson, 1998). In particular, the ducts grow and branch to form the secondary and tertiary ducts. However, the primary duct, which contains a layer of epithelial cells bordered by a thick layer of stroma, serves as a reservoir for milk during lactation, as well as provides a connection to the exterior nipple (Richert et al., 2000). The terminal end buds also appear at the beginning of puberty and are located on the tips of growing ducts. Terminal end buds are the primary epithelial structures that undergo the highest proliferation. Moreover, the two cell types (multilayered inner body cells and outer layer cap cells) of the terminal end buds form the mammary epithelial cells, as well as cap cells (pluripotent stems cells), which eventually give rise to the myoepithelial cells of the duct (Russo & Russo, 1996; Humphreys et al., 1996). By 10-12 weeks of age, the terminal end buds have reached the edge of the fat pad, and the development of lateral and alveolar buds is initiated in response to the cyclic secretion of estrogen (Andres & Stange, 1999). Lateral buds may form either branches with a layer of cap cells at the tip or alveolar buds.
Alveolar buds are capable of dividing into structures that secrete milk during pregnancy (Robinson, McKnight, Smith, & Hennighausen, 1995).

Unlike the rodent mammary gland, the human mammary gland consists of only two breasts on the anterior chest wall, but has a more complex structure (Brisken, 2013; Cardiff & Wellings, 1999). Specifically, the human nipple contains an outlet for five to 10 lactiferous ducts, which form triangular lobes with separate ductal systems. Conversely, the rodent mammary gland has an outlet for a single lactiferous duct that gives rise to five to 10 secondary ducts (Cardiff & Wellings, 1999). The ducts of the rodent mammary gland grow into the fat pad in a linear fashion. In humans, the functional site (source of milk production) of the mammary gland is referred to as the terminal ductal lobular unit (Petersen & Polyak, 2010; Cardiff, 1998). The terminal ductal lobular unit contains similar epithelial cells as the functional portion of the mouse mammary gland (lobulo-alveolar units or terminal end buds). Luminal and myoepithelial cells have also been recognized in both human and mouse mammary glands (Gusterson & Stein, 2012; Cardiff & Wellings, 1999). However, unlike the functional site of the mouse mammary gland, the human terminal ductal lobular unit is encased in a loose intra-lobular connective tissue surrounded by dense extra-lobular connective tissue (Wellings, 1980). Regardless, both the terminal ductal lobular unit and lobulo-alveolar unit are hormonally sensitive following development and are the origin for the majority of mammary cancers due to the high proliferative stem cell populations and sensitivity to somatic cell mutation (Visvader, 2009; Cardiff & Wellings, 1999; Russo et al., 1990).
Steroid Hormones Receptors and Coactivators in Mammary Gland Development and Carcinogenesis

Steroid hormones exert their effects primarily by binding to their cognate receptors. This process results in the recruitment of coactivators and transcriptional activation of the specific steroid-dependent genes. Therefore, there are many levels at which dysregulation can occur in the hormone signal, possibly increasing breast cancer risk.

Estrogen Receptor Alpha and Beta

Estrogen mediates its function through two specific intracellular receptors (estrogen receptor alpha [ERα] and estrogen receptor beta [ERβ]), and although both receptors share structural similarities, ERα and ERβ are transcripts of different genes (Gustafsson & Warner, 2000). When both receptors form dimers at specific DNA-elements (upon ligand activation), they are able to commence transcription activity by protein-protein interaction with other transcription factors or by binding directly to specific regions of DNA (Litwiniuk et al., 2008). The estrogen receptors are able to bind to the DNA indirectly as well, through alternative elements (e.g. DNA-response elements). In this case, transcription is influenced through recruitment of co-regulator proteins. When the receptors are unliganded, on the other hand, the estrogen receptors are associated within inhibitory protein complexes containing heat shock proteins (Hartman, Strom, & Gustafsson, 2009).

Complete mammary gland development occurs following puberty and is highly dependent on ERα (Mueller, Clark, Myers, & Korach, 2002; Ball, 1998). Indeed,
immunohistochemistry studies have found that ERα is present in both the mammary epithelial and stromal cells as early as day one of age (DiPaolo & Jones, 2000); however, its proliferative effects do not occur until four to five weeks of age (i.e. puberty for mice). Once puberty is reached, the ovaries produce estrogen which increases epithelial and stromal cell proliferation in the terminal ends buds (Fendrick, Raafat, & Haslam, 1998). A higher number of terminal end buds, as well as a higher relative proportion of undifferentiated terminal end buds to differentiated lobular alveolar units may predispose the developing mammary gland to breast cancer (Russo & Russo, 1996). Therefore, based on the role that ERα has in promoting normal breast development, including alterations of the mammary epithelial cells, ERα is also considered to play an important role in malignant mammary gland growth (Stingl, 2011).

In contrast to ERα, the effects of ERβ (the second main estrogen receptor isoform) on mammary gland development are believed to be subtle (Krege et al., 1998). While mammary gland development is significantly impaired when ERα is knocked out, only minimal morphological changes are noticed during mammary gland development when ERβ in knocked out. Specifically, there have been instances of impaired lobuloalveolar differentiation throughout pregnancy (Han, 2011). Nonetheless, research has confirmed that ERβ is expressed with ERα in both normal and malignant breasts (Roger, et al., 2001). In fact, Saji et al. (2000) found that approximately 60-70% of epithelial cell express ERβ at all stages of breast development. However, studies using ERβ knockout mice have indicated that ERβ may not be necessary for estrogen-induced proliferation of the mammary gland (Palmieri, 2002). Moreover, it has been well establish that ERβ may antagonize or have effects opposite to those of ERα in the
epithelium, such as inhibiting proliferation, migration, and invasion of cancerous breast cells (Madeira et al., 2013; Saji et al., 2000; Gustafsson & Warner, 2000). Research has also indicated that the two receptors also have different expression levels in breast cancer cells (Grober et al., 2011). In particular, higher ERα levels and lower ERβ have been discovered in malignant cells compared to normal mammary epithelial or benign tumour cells (Sugiura et al., 2007; Saji, Hirose, & Toi, 2005). The mechanism by which ERβ is downregulated though, still remains to be fully understood. Hartman, Strom, and Gustafsson (2009) propose that epigenetic changes could play an important role.

**Glucocorticoid Receptor**

Glucocorticoids are the primary hormone released in response to stress and their effects are mediated by the glucocorticoid receptor (GR) via activation or repression of gene expression (Beato, Herrlich, & Schutz, 1995). In particular, the GR acts as a ligand-dependent transcription factor and controls transcriptions by binding to glucocorticoid response elements (Reichardt et al., 2001). Importantly, the GR is involved in normal mammary gland development as GR staining can be seen in the nuclei of stromal, adipocytes, and luminal epithelial cells, as well as in the cytoplasm of luminal epithelial cells (Courtin et al., 2001; Lien et al., 2006; Buxant, Engohan-Aloghe, & Noel, 2010). In particular, GR is critically involved in cell proliferation during lobuloalveolar development and mammary lobular unit spatial formation (Wintemantel et al., 2005; Murtagh et al., 2004). It is well established that GR also contributes to milk protein synthesis by acting synergistically with prolactin, as well as lactation by controlling gene expression through protein-protein interaction with Stat5 in the absence of homodimeric DNA binding (Lippman, Bolan, & Huff, 1987).
Research has indicated that glucocorticoids have a role in the development of mammary carcinogenesis and that the GR may play a role in cancer development in the presence of chronic stress (Baschant & Tuckermann, 2010; Zhu, Jiang, & Thompson, 1998). Specifically, the GR likely interferes with cellular processes within mammary epithelial cells, such as control of apoptosis and cell proliferation. Glucocorticoids in conjunction with their receptors may promote cancer growth by acting as an anti-apoptotic factor in breast cancer cells, thus, inhibiting cell apoptosis by gene activation on glucocorticoid response elements (Mikosz et al., 2001; Vilasco et al., 2011). Although glucocorticoids have been shown to have anti-proliferative effects in cancerous breast cells, glucocorticoids and GRs may further be involved in the development of the malignant mammary gland given studies have also found that the intensity of GR nuclear staining actually decreases from normal breast tissue to invasive carcinomas (Lien et al., 2006; Buxant et al. 2010; Conde et al., 2008; Vilasco et al., 2011; Mattern, Buchler, & Herr, 2007).

Mineralocorticoid Receptor

The mineralocorticoid receptor (MR), like GRs also a member of the nuclear receptor family and binds directly to the DNA of a cell with the assistance of a ligand (Volden & Conzen, 2013). Moreover, MR has a high affinity for glucocorticoids; even higher (nearly 10 times) than that of the GR (Kolber, Wieczorek, & Muglia, 2008). Although the role of MR in normal and malignant mammary gland development is less well defined than GR, the results of a study conducted by Kingsley-Kallesen et al. (2002) is indicative that MR may compensate for the absence of GR at certain stages of mammary gland development. Specifically, MR may take on the role of regulating the
transcription of milk protein genes. It has been suggested that little information is available on the effect of mineralocorticoids because of the difficulty involved in distinguishing between a mineralocorticoid and glucocorticoid response (Cato & Weinmann, 1988). This is due to finding that MR has an equally high affinity to both mineralocorticoids (e.g., aldosterone) and glucocorticoids (e.g., corticosterone), whereas GR responds to glucocorticoids, but is mostly insensitive to mineralocorticoids (Savory et al., 2001).

**Coactivator-Associated Arginine Methyltransferase 1**

Coactivator-associated arginine methyltransferase 1 (CARM1) belongs to the type I protein arginine methyltransferase family and it is a multifunctional protein (Al-dhaheri et al., 2012). It is involved in several cellular processes related to tissue development, gene expression, regulating protein stability, and the coupling of transcription and mRNA processing (Cheng, Cote, Shaaban, & Bedford, 2007; Feng, Yi, Wong, & O’Malley, 2006; Yadav et al., 2003). Moreover, CARM1 enhances the ability of other coactivators to promote transcription (Monroy et al., 2003). Research has indicated that CARM1 is expressed in both the luminal and stromal tissue compartments of the normal mouse mammary gland and is required for the differentiation of adipocytes (Yadav, et al., 2008; Wang et al., 2013).

CARM1 may promote cell proliferation by enhancing ERα target gene expression, suggesting that CARM1 can play a role in the development of the malignant mammary gland (Teyssier et al., 2010). Indeed, CARM1 has been shown to regulate factors promoting progression of the estrogen-induced cell cycle and is a critical factor downstream of ERα, in the pathway of estrogen-stimulated breast cancer growth (Frietze,
Lupien, Silver, & Brown, 2008). According to research conducted by Al-dhaheri (2012), CARM1 expression was determined to be positively correlated with ERα levels in breast tumour samples. In addition, the overexpression of CARM1 has been implicated in grade-III breast cancers, and abnormal expression of CARM1 has been linked to human breast cancer cells (Messaoudi et al., 2006; Frietze et al., 2008; Cheng et al., 2013). These findings provide support for the role of CARM1 expression in ERα biology and malignant tumours. Furthermore, it has been determined that CARM1 is implicated in cancer cell proliferation and survival; therefore, aiding in the acceleration of tumour progression (Messaoudi et al., 2006). This knowledge has been gained through CARM1 knockdown studies demonstrating that reduced cellular proliferation and cell cycle progression have been observed in estrogen-treated MCF-7 human breast cancer cells (O’Brien et al., 2010).

**Nuclear Receptor Coactivator 3**

The nuclear receptor coactivator 3 (SRC3; also known as amplified in breast cancer 1) belongs to the p160 steroid receptor coactivator family and is required for the transcriptional activity of nuclear receptors (Torchia et al., 1997). Moreover, by recruiting chromatin modification enzymes (such as CARM1), SRC3 is able to amplify the transcriptional activities of transcription factors (Xu & Li, 2003). Research has shown that SRC3 is expressed in the epithelial tissue of the mammary gland and inactivation of this coactivator partially diminishes mammary ductal growth during puberty (Liao et al., 2002; Kuang et al., 2004). In particular, studies investigating the effects of SRC3 knockout have found that at four weeks of age the mammary gland of mice had limited growth and during the following rapid growth stage, ductal growth was significantly
lower. Once the mice reached two months of age, there was limited ductal branch penetration within the fat pad and full penetration did not occur until the eleventh week (Xu, 2000). It has suggested that this occurred because knockout of SRC3 significantly reduced levels of systemic estrogen (Hennighausen & Robinson, 2001). Other studies, using transgenic expression of SRC3 in mouse mammary epithelial cells, have found that overexpression leads to an increase in mammary epithelial proliferation (Rebbeck et al., 2001). Taken as a whole, these findings indicate that SRC3 is involved in the enhancement of mammary gland growth, and the effects occur in a stage specific manner during developmental (Xu et al., 2000; McKenna & O’Malley, 2002).

In a classic study by Bautista et al. (1998), 1157 breast tumours were analyzed for the amplification of SRC3 and it was discovered that this gene was amplified in nearly 5% of breast cancers, and the expression of SRC3 was positively correlated with tumour size. Since then, several groups have implicated SRC3 in several types of epithelial tumours (including breast) as it is the only steroid receptor coactivator that has been found to be overexpressed in these types of tumours (Fereshteh et al., 2008). Recent reports have claimed that the SRC3 gene is amplified in about 10% breast cancers and overexpressed in 30% to 60% breast cancers (Kuang et al., 2005). Interestingly, the overexpression of SRC3 has been determined to be sufficient to initiate mammary gland carcinogenesis. Overexpression in the mammary epithelial cells eventual leads to mammary hyperplasia and the development of malignant mammary glands (Planas-silva, Shang, & Donaher, 2001). Another study has shown that inactivating SRC3 in 7,12-dimethylbenz[a]-anthracene-(DMBA)-treated mice (DMBA is an established chemical
carcinogen) results in a reduction of the frequency and growth rate of mammary tumours (Kuang et al., 2004).

**NADH-Ubiquinone Oxioreductase Chain 2**

In general, abnormal mitochondrial functioning has long been suspected to contribute to the development and growth of cancer (Chatterjee, Dasgupta, & Sidransky, 2012). Mitochondrial DNA (mtDNA) play a critical role in the production of reactive oxygen species due to their role in producing energy via oxidative species (Jakupciak et al., 2005). A disturbance in the balance between the production of reactive oxygen species and antioxidant defenses results in excess reactive species, and reactive species can then react and damage nuclear and mtDNA (Klaunig & Kamendulis, 2004). mtDNA is susceptible to damage due to the lack of protective histones and efficient DNA repair mechanisms within the nuclease. The disturbance in balance is known as oxidative stress and this process has been implicated in carcinogenesis. Given mitochondria are involved in the metabolism of energy, production of reactive oxygen species, and the initiation of cell apoptosis, it has been proposed that mitochondrial damage may contribute to carcinogenesis by causing dysfunctional mitochondrial-induced apoptosis, and consequently, promoting cellular proliferation (Rohan et al., 2010).

Several studies have identified somatic alterations/mutations in mtDNA in relation to breast cancer (Parrella et al., 2001; Zhu et al., 2005). The mutations can result in the impairment of enhanced reactive oxygen species production and oxidative phosphorylation, and this can lead to a further increase in the frequency of DNA mutations. Penta et al. (2001) suggest that these mechanisms may influence the early stages of carcinogenesis. Importantly, most of the mutations occur in the displacement
D-loop (D-loop) region of the mtDNA. The D-loop is the noncoding area and control site for mtDNA expression. Furthermore, it is the location in which the replication of mtDNA occurs and where the major promoters for transcription are contained (Taanman, 1999). Thus, several studies have also investigated somatic mutations in the D-loop region, and in particular, the common 4977 base pair deletion. The location of this deletion is within the base pair span of the mitochondrially encoded NADH Dehydrogenase 2 (ND2) gene, which is located from base pair 4469 to 5510. Early studies demonstrated that this specific deletion was found in normal and malignant breast tissue of participants with breast abnormalities (Carew & Huang, 2002). In fact, the bulk of mutations in breast cancer occur in the D-loop region (Tseng et al., 2006; Tan, Bai, & Wong, 2002). Using direct DNA sequencing to screen the entire mitochondrial genome for mutations, it was discovered that nearly three-quarters of cancer patients had somatic mtDNA mutations, and 81.5% of those mutations were limited to the D-loop region (Tan et al., 2002; Carew & Huang, 2002). It is also worth noting that Tseng et al. (2006) found that the common deletion accumulates more in adjacent non-tumour tissue, rather than in cancerous cells. Nonetheless, it is clear that mutations of the ND2 gene are implicated in tumour growth and progression.

The present studies are designed to assess the impact of neonatal stress on the physiological and behavioural development of the offspring, as well as identify the molecular mechanisms underlying the differential effects of neonatal environment on mammary tumourigenesis. Research has demonstrated that the experience of prolonged maternal separation results in higher ERα protein levels in the normal mammary glands of adult female LMS mice, which has been related to increased mammary tumourigenesis
following adulthood carcinogen (7,12-dimethylbenz[a]anthracene; DMBA) administration (Boyd et al., 2010). GR, MR, CARM1, SRC3, and ND2 have been identified as potential mediators of these effects. These factors influence or are influenced by hormone-mediated activity in the mammary gland and have been shown, along with estrogen, to critically influence mammary gland development and differentiation as well as cancer initiation or growth.

**Objectives and Hypotheses**

**Objectives**

1. To assess behavioural and physiological reactivity to stressors (as measured by coping behaviours, and basal and stress-induced CORT levels, respectively) in pubertal (PND 30) and young adult (PND 60) male and female BALB/c mice exposed to the neonatal experiences of BMS and LMS.

2. To evaluate the gene expression profiles of GR, MR, CARM1, SRC3, and ND2, and protein expression profiles of GR, MR, and CARM1 in the normal mammary glands of young adult (PND 60) female BMS and LMS mice.

**Hypotheses**

It is expected that the chronic mild or moderate neonatal stressors of BMS and LMS, respectively, will differentially influence the behavioural and physiological reactivity to stressors. Specifically, it is anticipated that LMS mice will have higher basal and stress-induced CORT levels, as well as higher changes between non-stressed and stressed CORT levels, compared to BMS mice. LMS mice will also display more passive coping and anxiety-like behaviours relative to BMS mice. These finding should be
consistent across age (i.e. puberty to young adulthood) due to the influence of neonatal experiences on the emotional and physiological development of the offspring.

Furthermore, it is expected that BMS and LMS mice will have altered expression profiles of GR, MR, CARM1, SRC3, and ND2. In particular, the mammary glands of young adult (PND 60) LMS mice are expected to have higher transcript levels of GR, MR, CARM1, SRC3, and ND2, and higher protein expression of GR, MR, and CARM1, compared to BMS mice.

Chapter 2: General Methodology

Rearing of Animals and Neonatal Manipulations

Animal Breeding and Cross-Fostering

Upon arrival, adult female and male BALB/c mice (Charles River Laboratories, Saint-Constant, Quebec) were housed in same-sex cages of 3-4 mice/cage. Following 7-10 days of acclimatization in a temperature controlled room (22 ± 2°C) under artificial illumination (12: 12 h light/dark; lights on at 0700 h), 2-4 females were housed with 1 male in a polypropylene cage. Once females were pregnant, they were separated into individual cages and provided with nesting material. One day following birth, litters were randomly cross-fostered to reduce the possibility of litter effects, and cross-fostered litters were randomly assigned to brief (BMS) or long (LMS) maternal separation neonatal manipulation groups or to the unmanipulated, typically reared (TR) condition. Given some studies suggested that rodent mothers tend to spend more time caring for male than female pups (Moore and Morelli, 1979; Papaioannou et al., 2002), all litters were culled to a ratio of 3:2 females to males. Neonatal manipulations were performed between postnatal days (PND) 2-22. On PND 23-25, pups were weaned, ear punched, and housed
in same-sex, same-litter groups with 2-4 mice per cage. Cages and water bottles were cleaned weekly; food (Lab Diet 5015) and water were provided *ad libitum*. All procedures follow the guidelines and protocols approved by the Trent University Animal Care Committee (Canadian Council of Animal Care approved facility).

**Neonatal Manipulation**

Each day (starting between 1100 h and 1300 h) from postnatal day (PND) 2-22, mice in the LMS condition were exposed to protracted maternal separation. The start time of the BMS manipulation was dependent on the LMS start time, to allow for reunion to occur simultaneously between the two conditions. Mice in the TR condition were left undisturbed for the duration of the experiment, except for standard weekly cage and water changes. During the neonatal manipulation period, offspring in BMS cages were removed from their home cages and placed as a litter in paper towel-lined plastic containers for 15 minutes per day. Neonatal manipulation for LMS mice consisted of transferring the offspring to paper-towel lined plastic container for 4 hours per day.

The model of maternal separation used was similar to that used by other researchers (Zaharia et al., 1996; Romeo et al., 2003), which does not include maintaining the pups at a more nest-like temperature. In the wild, mice breed in temperatures ranging from 6°C (e.g., in Alberta, Canada) to 34°C (e.g., in Texas, USA), and it has been established that in the lab, neonates easily survive ambient temperatures (21-24°C) alone or as a litter for up to 6.5 hours (Barnett and Burn, 1967; Sun et al., 2007). The 4-hour separation of a litter from their dam in our study was designed to be moderately stressful, and would be typical of what pups would experience in a natural yet challenging environment (e.g., increased predation, limited food).
Following weaning between PND 23-25, normal housing resumed until behavioural tests or tissue collection at PND 30 or 60. Animal rearing and tissue collected were done over the course of several years, with animals being assigned to the following endpoint studies at birth:

1) PND 30, puberty onset endpoint (Chapter 3)
2) PND 60, early adulthood endpoint (Chapter 3-4)

Dissections

Beginning at either PND 27 or PND 57, the phase of estrous cycle was determined daily by cervicovaginal lavage. The mice were lavaged using a plastic transfer pipette with approximately 0.2 mL of physiological saline (0.9% NaCl), flushing the vaginal lumen, to obtain vaginal smears, which were observed under a light microscope. The stage of estrous was determined by evaluating the abundance of leukocytes and different types of epithelial cells in the smears. Mice were considered to be in estrus when majority of cells observed were cornified epithelial cells. All tissues were collected when mice were in estrous, to standardize fluctuations in hormones levels (Fata et al., 2001; Slotten et al., 2006).

At either PND 30 ± 3d or PND 60 ± 3d, female mice in estrus were terminated and trunk blood and mammary glands collected for future analyses. If a behavioural test was conducted (see Chapter 3), exanguination occurred immediately (within 2 minutes) following the behavioural test. Trunk blood was collected in a plastic weigh boat. A syringe was used to mix the blood with 0.2 mL heparin and to transfer the blood to a glass test tube that was kept on ice (for up to one hour) until centrifugation at 5000 x g
and 4°C for 10 minutes. Plasma was then stored at -80°C for future radioimmunoassay analyses.

Immediately following blood collection, the 2\textsuperscript{nd} and 3\textsuperscript{rd} thoracic mammary gland were dissected and placed in microcentrifuge tubes and immediately frozen in liquid nitrogen and later stored at -80°C for future use in protein analysis. The 7\textsuperscript{th} and 8\textsuperscript{th} thoracic mammary glands were dissected for gene expression analyses, and these were preserved in RNAlater (Ambion) at 4°C overnight, and then stored at -80°C until further use. The 4\textsuperscript{th} and 5\textsuperscript{th} inguinal mammary glands were collected for histological analysis. These mammary glands were spread out inside a tissue processing cassette and stored in 10% buffered formalin. The 9\textsuperscript{th} and 10\textsuperscript{th} inguinal mammary glands were used for morphological analyses. These mammary glands were spread on a glass slide and placed in a 25% glacial acetic acid/75% ethanol solution overnight, followed by carmine staining.

\textbf{Chapter 3: Neonatal experiences, sex, and age interact to shape stressor reactivity, and depressive-like and coping behaviours in BALB/c mice.}

\textit{Introduction}

The parent-offspring relationship helps to shape physiological and behavioural responses to the environment in adult offspring and may also influence the risk of developing depression or anxiety disorders. Human and animal studies have shown that exposure to stressful/neglectful early-life environments generally results in adult offspring with elevated or prolonged stress-induced secretions of hormones that govern the hypothalamic-pituitary-adrenal (HPA) axis including corticotrophin releasing
hormone (CRH), adrenocorticotrophic hormone (ACTH) and glucocorticoids (cortisol in humans and corticosterone in rodents) (Plotsky et al., 2005; Sanders & Anticevic, 2007; Nishi, Horii-Hayashi, Sasagawa, & Matsunaga, 2013). The levels of hormonal activity have been correlated with increased adaptive responses to stressful situations, including stress-induced anxiety and depressive behaviours (Calil & Marcondes, 2006; Liu et al., 1997; Pryce & Feldon, 2003; Romeo et al., 2006).

Although both men and women who experience adverse or neglectful childhood events appear to be at higher risk of developing depression following exposure to an acute stressor in adulthood (Heim, Newport, Mletzko, Miller, & Nemeroff, 2008), the overall risk of developing depression is approximately two times higher for women than for men (Kessler, 2003; Marcus, et al., 2005). The factors that underlie the increased risk of depression in women compared to men are complex, and have been suggested to depend on the type and severity of the adverse experiences, the timing of these events related to the infant’s development, genotype, gender differences in biological responses, self-concepts, and coping styles (Heim, et al., 2008; Nolen-Hoeksma, 2001).

Controlling for confounding and interactive variables that can contribute to depressive-like behaviours in human studies is challenging. Nevertheless, an established rodent model, that manipulates the early life environment and influences mother-neonate interactions, is widely used to analyze the effects of early-life experiences on brain development and subsequent behavioural repertoires, including coping behaviours in the adult offspring. As described in an earlier chapter, in this animal model, offspring are subjected to bouts of brief (e.g., 1-15 minutes/day; BMS), long (e.g., 2-6 hours/day: maternal separation; LMS), or no (typical facility reared and used as an environmental
control; TR) maternal separation from the dam over the first few (i.e., 1-3) weeks of life. Recall, these neonatal separations/experiences increase the likelihood of survival of the offspring, especially if the environment to which they have vicariously adapted does not change substantially.

Although studies have examined the influence of sex and age independently on the effects of neonatal environment manipulations on the behavioural and physiological phenotype of offspring, no study to-date has examined the interaction among sex, age, and neonatal experience on depressive-like and coping behaviours in the acute forced swim test and on plasma corticosterone levels following exposure to the forced swim test in BALB/c mice. In doing so, the subtleties among the interaction of these variables can be better addressed and understood. In the present study we examined the interactive effects of mild (brief maternal separation, BMS: 15min/day from PND 2-22) and moderate (long maternal separation, LMS: 4 hr/day from PND 2-22) chronic early-life stress, in the form of maternal separation, on basal (i.e. non-stressed) and stress-induced plasma corticosterone levels as well as on depressive-like and coping behaviours of pubertal (30-35 days of age) and young adult (55-65 days of age), male and female BALB/c mice. All female mice were tested during estrus, a time when both estrogen and progesterone levels are moderate relative to other stages of the murine estrous cycle (Fata, Chaudhary, & Khokha, 2001; Hiroi & Neumaier, 2006; Schedin, Mitrenga, & Kaeck, 2000). The ages at which we tested the mice are roughly similar to human adolescence and early adulthood (Laviola, Macri, Morley-Fletcher, & Adriani, 2003; Spear & Brake, 1983), and are typically the two developmental stages at which sex-related differences in depressive-like behaviours are suggested to occur in humans.
What is not known, however, is whether an animal that has a tendency to elicit a greater reactivity to a stressor in puberty also exhibits that same tendency in adulthood, and whether neonatal experiences differentially affect this tendency.

**Materials and Methods**

**Animals**

See Chapter 2 for general methods. One day following birth, litters were assigned to either brief (BMS; $n = 54$; 22 litters), prolonged (LMS; $n = 58$; 17 litters), or no, ‘typically reared’ (TR; $n = 57$; 17 litters) maternal separation experiences over the first three weeks of life. Neonatal manipulations were performed between PND 2-22. On PND 23-25, pups were weaned, ear-punched, and housed in same-sex, same-litter groups with 2-4 mice per cage. Approximately half of the mice from each neonatal condition were randomly assigned to be tested at puberty, while the remaining mice were tested in early adulthood. Cages and water bottles were cleaned weekly; food (Lab Diet 5015) and water were provided ad libitum.

Starting at PND 28, puberty onset was assessed in females. Male mice were handled in a similar manner to compensate for handling of females during estrous cycle testing. At puberty (i.e., first estrus: PND 30-35), female mice from each treatment group were subjected to an acute forced swim test ($n_s$: BMS = 13, LMS = 13, TR = 14). Age- and litter-matched males were subjected to the forced swim test at the same time as their female counterparts, between PND 32-37 ($n_s$: BMS = 11, LMS = 9, TR = 8). The
procedure was repeated at young adulthood (PND 55-65) with a subset of siblings from litters among the three treatment conditions \( (n): \) Female: BMS = 19, LMS = 17, TR = 17; Male: BMS = 10, LMS = 11, TR = 9). All procedures were in compliance with the Canadian Council on Animal Care Guidelines, and were approved by the Trent University Animal Care Committee.

**Neonatal manipulations**

See Chapter 2 for general methods.

**Acute forced swim test**

The forced swim test was administered between 0900h–1100h and at two distinct developmental stages, puberty (PND 30-35) or young adulthood (PND 55-65). All females were in estrus at the time of forced swim testing. Mice were individually placed in a clean, opaque cylindrical plastic container (22 cm in diameter) containing approximately 1300 mL of tap water at 24-26°C. After 1 minute of acclimatization to the water, mouse behaviour was recorded at 10 second intervals, for 5 minutes in pubertal mice and 10 minutes in young adult mice. The difference in the total swim time between pubertal and adult mice was based on previous studies (Hansen-Trench & Barron, 2005; Hefner & Holmes, 2007), and on our observations that the pubertal mice became fatigued after approximately eight minutes of swimming (e.g., swimming would become more erratic and many would sink). ‘Immobility’ was recorded if the mouse was not floating upright (i.e., floated more on its side) and made only slight movements of the forepaws in order to keep afloat. The amount of time spent immobile is a measure of the level of despair in the rodent; a high amount of time spent immobile is indicative of depressive behaviour and poor coping response (Hall, Sundstrom, Lerner, & Pert, 2001; Porsolt,
Bertin, & Jalfre, 1978). ‘Swimming’ was recorded if the mouse swam freely in the container with active use of forepaws and tail to propel it forward either around the sides or the centre of the cylinder (Hall, et al., 2001). ‘Climbing’ was recorded if the mouse was actively pawing the walls of the container. Both swimming and climbing are considered adaptive (coping) behaviours, high levels of which indicate adaptation/coping to a novel environment (Boccia & Pedersen, 2001). Finally, ‘active floating’ was recorded when the mouse actively maintained a level floating position (opposed to the immobile position where the mouse floated more on one side of its body, on an angle), while making small forepaw and/or tail movements. During active floating mice would periodically push off from the walls of the container thus positioning themselves more in the centre; in addition, whisker movement and visual environmental surveying were frequently observed. The active floating behaviour recorded in the present study is similar to that reported by other investigators (Belozertseva, Kos, Popik, Danysz, & Bespalov, 2007; Contreras, Gutierrez-Garcia, Bernal-Morales, Rodriguez-Landa, & Munoz-Lopez, 2008). The forced swim test was performed with the experimenters blind to the neonatal condition experienced by each mouse.

**Blood collection**

Within 2-3 minutes following the forced swim test, trunk blood was collected from pubertal (postnatal day 30 ± 3) and young adult (postnatal day 60 ± 3) male and female mice. At the same time, trunk blood was collected from an additional cohort of postnatal day 30 ± 3 and 60 ± 3 mice that were not exposed to the forced swim test (for basal CORT levels). A syringe was used to mix the blood with approximately 0.03 mL heparin and transfer this mixture to a glass test tube which was kept on ice until
centrifugation for 10 minutes at 5000 x g and 4°C. Plasma was then stored at -80°C until use in CORT radioimmunoassays.

Corticosterone determination

Plasma CORT concentrations were measured by radioimmunoassays for samples from female and male mice immediately following exposure to the forced swim test, and from a separate cohort of male and females that were not exposed to the forced swim test. All assays were completed using an ImmunoChem Soluble Antibody Concentration125I radioimmunoassay kit (MP Biomedicals, New York), following the manufacturer’s instructions. Assays were performed at room temperature and counted on a Wizard 1470 Gamma Counter (Perkin Elmer). Each sample was run in a duplicate, in two different assays (four total replicated). Detection limits were 3.125-250 ng/mL, the intra-assay coefficients of variation were 7.3-8.1% and inter-assay coefficients of variation were 9.1-11.41%.

Results

Statistical analyses

Data from the acute forced swim test are expressed as the number of times a mouse exhibited each behaviour over the total number of behaviours exhibited within the measurement time. The change from basal to post-stress plasma CORT levels was calculated by rank ordering mice and subtracting the basal CORT levels from the levels immediately following exposure to the forced swim test. Thus, allowing for the determination of stressor reactivity. The effects of neonatal manipulations, age at testing, and sex on forced swim test behaviours (immobility, swimming, climbing, and active
floating), plasma CORT levels, and reactivity were analyzed by three-way (3x2x2) analysis of variance (ANOVA) (Treatment: BMS, LMS, TR; Age: pubertal, adult; Sex), three-way (3x2x2) ANOVA (Treatment; Stress: non-stressed, forced swim test; Sex), and two-way (3x2) ANOVA (Treatment; Sex), respectively. Where indicated, data were first log-transformed if parametric assumptions were violated; nonparametric two-tailed Mann-Whitney U tests were used if transformation was not possible. Statistical significance was set at \( p < .05 \) and moderate significance was set at \( p \leq .10 \). Significant effects as well as moderate effects were analyzed using Newman-Keuls post-hoc comparisons. All statistical analyses were conducted using the statistical package STATISTICA 10 (Statsoft, USA). Data are presented as means ± S.E.M.

Experiences of long bouts of maternal separation increase the tendency of adult, but not pubertal, female mice to engage in higher levels immobility and lower levels of active floating.

Immobility

The Treatment x Age x Sex ANOVA revealed significant main effects of Treatment \([F(2, 137) = 4.11, p < .05]\) and Age \([F(1, 137) = 39.90, p < .001]\), a significant interaction of Age x Sex \([F(1, 137) = 4.08, p < .5]\), and moderate interactions of Treatment x Age \([F(2, 137) = 2.97, p = .055]\) and Treatment x Age x Sex \([F(2, 137) = 2.41, p = .093]\). Examination of the significant Age x Sex effect indicated that when all neonatal conditions were considered together, immobility behaviour was significantly higher in male than in female pubertal mice \((p < .05; \text{Figure 1})\). The post hoc examination of the moderate three way interaction indicated that immobility behaviour in the forced swim test decreased with age for both male BMS \((p < .01)\) and LMS \((p < .05)\), as well as
for female BMS ($p < .05$) mice. Immobility behaviour also tended to decrease moderately with age in both male ($p = .107$) and female ($p = .075$) TR mice. Immobility in LMS female mice did not decrease from puberty to adulthood ($p = .727$). Accordingly, immobility in adult LMS female mice was significantly higher than in adult female BMS mice ($p < .01$) and tended to be slightly higher than that observed for adult male LMS mice ($p = .115$; Figure 1).

**Active Floating**

Significant main effects of Treatment [$F(2, 137) = 9.00, p < .001$] and Age [$F(1, 137) = 49.14, p < .001$], and significant interactions of Treatment x Sex [$F(2, 137) = 5.57, p < .01$] and Treatment x Age [$F(2, 137) = 5.18, p < .01$] were observed. Post hoc analyses indicated that active floating behaviour increased from puberty to adulthood for BMS and LMS mice ($p$’s < .001). Active floating was significantly higher in male than in female LMS mice ($p < .001$), and this effect was due primarily to differences in active floating behaviour in adult mice (male>female; $p < .01$; Figure 2).

*The active response of swimming was greatest in female mice that experienced long bouts of maternal separation – climbing behaviour was similar between males and females.*

**Swimming**

Significant main effects of Treatment [$F(2, 137) = 6.94, p = .001$] and Sex [$F(1, 137) = 4.28, p < .05$] and a significant interaction of Treatment x Sex [$F(2, 137) = 3.54, p < .05$] was observed for swimming behaviour. Overall, regardless of age, female LMS mice engaged in more swimming behaviour than male LMS mice ($p < .01$), whereas male TR mice engaged in significantly more swimming behaviour than male mice in either the
BMS ($p = .01$) or LMS ($p < .001$) conditions; swimming behaviour in female mice was similar across all neonatal conditions (Figure 3).

Climbing

Post hoc examination of the significant main effects of Treatment [$F(2, 137) = 4.03, p < .05$] and Age [$F(1, 137) = 7.37, p < .01$], revealed that climbing behaviour significantly decreased from puberty to adulthood. In general, climbing behaviour was observed significantly more in BMS mice than in TR ($p < .05$) mice (Figure 4).

Weight and puberty onset

Similar to other studies (Pryce, Bettschen, & Feldon, 2001; Rees, Steiner, & Fleming, 2006), neonatal conditions did not significantly affect weight of mice at puberty or at adulthood. Moreover, the timing of first estrus (i.e., puberty) in female mice was similar across all neonatal conditions (data not shown).

Non-stressed circulating CORT levels increased from puberty to adulthood in female offspring exposed to prolonged maternal separation relative to their male counterparts.

Data collected at puberty violated parametric assumptions following transformation, and consequently, Mann-Whitney U tests were used, however, data at adulthood met parametric assumptions and the Treatment x Stress x Sex ANOVA revealed significant main effects of Treatment [$F(2, 91) = 10.50, p < .001, \eta^2 = .19$], Stress [$F(1, 91) = 589.72, p < .001, \eta^2 = .87$], and Sex [$F(1, 91) = 132.50, p < .001, \eta^2 = .60$], and significant interactions of Treatment x Stress [$F(2, 91) = 6.60, p = .002, \eta^2 = .13$], Sex x Stress [$F(1, 91) = 42.10, p < .001, \eta^2 = .32$], and Treatment x Sex x Stress [$F(2, 91) = 5.40, p = .006, \eta^2 = .11$].
For non-stressed pubertal female mice, CORT levels were significantly higher in BMS ($U = 0, n_{BMSfemale} = 5, n_{BMSmale} = 6, p = .008$) and moderately higher in TR ($U = 4, n_{TRfemale} = 6, n_{TRmale} = 5, p = .055$) mice compared to those of their male BMS and TR counterparts. CORT levels were similar between pubertal female and male LMS mice. Non-stressed CORT levels of pubertal female BMS and LMS mice were similar, but significantly lower than those of TR mice ($U = 0, n_{LMS} = n_{TR} = 6, p = .005$; $U = 0, n_{TR} = 6, n_{BMS} = 5, p = .008$, respectively). CORT levels for non-stressed pubertal male BMS mice were moderately lower than male LMS and TR mice ($U = 6, n_{LMS} = n_{BMS} = 6, p = .066$; $U = 4, n_{TR} = 5, n_{BMS} = 6, p = .055$, respectively). At adulthood, circulating CORT levels of non-stressed female BMS, LMS, and TR mice were significantly higher than those of their male counterparts ($p$’s < .001). Unexpectedly, the non-stressed CORT levels of adult female LMS mice were significantly lower than CORT levels in adult female BMS and TR mice ($p$’s < .01). In non-stressed adult males, TR mice had moderately higher CORT levels than BMS and LMS mice ($p = .065$; $p = .059$, respectively).

Maternal separation results in greater reactivity to a stressor in adult female offspring whereas the opposite holds true for male offspring.

In general, pubertal female mice were more reactive to the forced swim test stressor than male mice as demonstrated by significantly higher differences between non-stressed and stressed CORT levels ($U = 77, n_{females} = n_{males} = 17, p = .021$). Both stress-induced CORT levels and CORT reactivity were significantly higher in pubertal female TR mice compared to those of their male counterparts ($U = 2, n_{TRfemale} = 7, n_{TRmale} = 6, p = .008$; $U = 1, n_{TRfemale} = 6, n_{TRmale} = 5, p = .014$, respectively). Interestingly, stress-induced CORT levels of pubertal female TR mice were also significantly higher than
those of female BMS and LMS mice ($U = 6, n_{TR} = n_{LMS} = 7, p = .02; U = 0, n_{TR} = 7, n_{BMS} = 5, p = .006,$ respectively). Similarly, CORT reactivity of pubertal TR female mice was higher than either BMS or LMS female mice ($U = 0, n_{BMS} = 5, n_{TR} = 6, p = .008; U = 6, n_{LMS} = n_{TR} = 5, p = .066,$ respectively). For pubertal male mice, neonatal manipulation had no significant influence on stress-induced CORT levels and reactivity.

Based on the Treatment x Sex ANOVA, there was a significant main effect of Sex [$F(1, 91) = 6.20, p = .017, \eta^2 = .14$] and a significant interaction of Treatment x Sex [$F(2, 91) = 12.35, p < .001, \eta^2 = .39$] on the changes between non-stressed and stressed CORT levels of adult mice. Adult female BMS and LMS mice were more reactive to the forced swim test than their male counterparts ($p = .051; p < .001,$ respectively). Furthermore, CORT levels immediately following exposure to the forced swim test were higher in adult female BMS and LMS mice compared to their male counterparts ($p = .042; p = .026,$ respectively)

Although the duration of maternal separation had no statistically significant influence on stress-induced CORT levels and CORT reactivity of adult female mice, adult female BMS and LMS mice had significantly higher CORT reactivity than adult female mice that did not experience maternal separation ($p = .028; p < .001,$ respectively). The duration of maternal separation also had no statistically significant influence on the stress-induced CORT levels and CORT reactivity of adult male mice. However, opposite to their female counterparts, adult male BMS and LMS mice were less reactive to the forced swim test stressor than male TR mice ($p’s < .05$).
Corticosterone and forced swim test behaviour correlations.

Since immobility reflects a depressive-like behaviour (Porsolt et al., 1978; Cryan, Markou, & Lucki, 2002), and depression is accompanied by hyperactive HPA axis (Heketh, Hogg, & Harbuz, 2005; Scott & Dinan, 2002), it would be expected that immobility would be related to an increase CORT levels (Kokras et al., 2012; Campbell, Lin, Devries, & Lambert, 2003; Craft, Kostick, Rogers, White, & Tsutsui, 2010; Pintér, Domokos, Mergl, Mikics, & Zelena, 2011). Indeed, increased immobility behaviour was related to increased CORT levels in adult male, but not female, BMS and LMS mice ($r = .90, p < .01; r = .71, p < .05$, respectively). Conversely, it may be expected that adaptive coping responses, such as swimming, climbing, and active floating (Kokras et al., 2012; Campbell et al., 2003; Craft et al., 2010), will result in a decreased CORT levels. This relationship was also treatment and sex specific. In particular, for adult male TR mice, the coping behaviour of active floating was found to negatively correlate with CORT levels ($r = -.89, p < .05$). Moreover, when the prevalence of swimming and active floating are combined, these behaviours negatively correlate with CORT levels of adult male LMS mice ($r = -.81, p < .05$). This finding suggests that active floating is an intentional behaviour that is similar to swimming, but allows for enhanced observation of the surrounding environment. On the other hand, in adulthood, the CORT levels of female LMS mice were found to moderately positively correlate with the active coping behaviour of swimming ($r = .67, p = .07$). Similarly, for pubertal female BMS mice, an increase in the active coping behaviour of climbing was related to higher CORT levels ($r = .90, p < .05$). Further highlighting the complexity of the relationship between sex, CORT, and forced swim test behaviours are the negative correlations between the active coping behaviour swimming and the CORT levels of pubertal female TR mice ($r = -.85,$
p < .05), and the active coping behaviour active floating and the CORT levels of adult female LMS mice (r = -.75, p < .05). It would appear that generalized relationships between coping responses and CORT levels should be made with caution. Future studies will examine ACTH levels in conjunction with CORT levels to provide a more accurate assessment of HPA functioning (Roper, Craighead, O’Carroll, & Lolait, 2010; Bhatnagar et al., 2004). See Table 1 for a list of all correlations between acute forced swim test behaviours and CORT levels.

Active floating and immobility: Two distinct behaviours

When comparing the correlations of the active coping behaviour of active floating and the passive behaviour of immobility, there are clear differences in the magnitude of the relationship of coping behaviours with CORT (e.g. adult female LMS mice: r = -.75; r = -.01, respectively), as well as more distinct directional differences (e.g. adult male BMS mice: r = -.31; r = .90, respectively). Although these correlations are not statistically significant, this pattern (i.e. differences in magnitude and direction) can be seen across all neonatal conditions and ages, with the exception of pubertal female LMS mice. Thus, this finding provides further support for the notion that, in addition to swimming and climbing, two distinct floating behaviours are being assessed during the forced swim test.
Table 1. Forced swim test behaviours and CORT correlations.

<table>
<thead>
<tr>
<th>Behaviours</th>
<th>Pubertal Female</th>
<th>Pubertal Male</th>
<th>Young Adult Female</th>
<th>Young Adult Male</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BMS</td>
<td>LMS</td>
<td>TR</td>
<td>BMS</td>
</tr>
<tr>
<td>SWIM</td>
<td>.52</td>
<td>-.67</td>
<td>-.85*</td>
<td>-.43</td>
</tr>
<tr>
<td>IMM</td>
<td>-.66</td>
<td>.22</td>
<td>.34</td>
<td>-.15</td>
</tr>
<tr>
<td>ACTFLT</td>
<td>.12</td>
<td>.40</td>
<td>.02</td>
<td>.38</td>
</tr>
<tr>
<td>STRGL</td>
<td>.90*</td>
<td>.64</td>
<td>.31</td>
<td>.05</td>
</tr>
<tr>
<td>ALLFLOAT</td>
<td>-.77</td>
<td>.50</td>
<td>.66</td>
<td>.33</td>
</tr>
<tr>
<td>SWIM&amp;ACTFLT</td>
<td>.40</td>
<td>-.47</td>
<td>-.50</td>
<td>.05</td>
</tr>
</tbody>
</table>

Note. BMS = brief maternal separation; LMS = long maternal separation; TR = typically reared; SWIM = swimming; IMM = immobility; ACTFLT = active floating; STRGL = struggling; ALLFLOAT = immobility + active floating; SWIM&ACTFLT = swimming + active floating. Significance symbol, \( p < .05 \): (*).
Figure 1. Mean (± S.E.M.) occurrences of immobility behaviour relative to all other behaviours exhibited of BMS (brief separation), LMS (prolonged separation), and TR (typically laboratory reared) pubertal and adult male and female BALB/c mice subjected to a 5 minute (pubertal; \(n_s: 8-14\)) or 10 minute (adult; \(n_s: 9-19\)) acute forced swim test. Pubertal males displayed significantly more immobility behaviour than their age-matched female counterparts. At adulthood, LMS females displayed the greatest amount of immobility behaviour when compared to female BMS mice and moderately higher levels of immobility than LMS males. Significance symbol, \(p < .05\): (*). Trend symbol, \(p < .1\): (#).
Figure 2. Mean (± S.E.M.) occurrences of active floating behaviour relative to all other behaviours exhibited of BMS (brief separation), LMS (prolonged separation) and TR (typically reared) pubertal and adult male and female BALB/c mice subjected to a 5 minute (pubertal; ns: 8-14) or 10 minute (adult; ns: 9-19) acute forced swim test. Adult male LMS mice engaged in significantly more active floating, a suggested passive coping/ risk assessment behaviour, than adult female LMS mice. Significance symbol, $p \ < .05$: (*).
Figure 3. Mean (± S.E.M.) occurrences of swimming behaviour relative to all other behaviours exhibited of BMS (brief separation), LMS (prolonged separation) and TR (typically reared) pubertal and adult male and female BALB/c mice subjected to a 5 minute (pubertal; $n_s$: 8-14) or 10 minute (adult; $n_s$: 9-19) acute forced swim test. Regardless of age, LMS female mice engaged in swimming behaviour significantly more than their male counterparts. Significance symbol, $p < .05$: (*).
Figure 4. Mean (± S.E.M.) occurrences of climbing behaviour relative to all other behaviours exhibited of BMS (brief separation), LMS (prolonged separation), and TR (typically reared) pubertal and adult male and female BALB/c mice subjected to a 5 minute (pubertal; $n_s$: 8-14) or 10 minute (adult; $n_s$: 9-19) acute forced swim test. Pubertal mice engaged in climbing behaviour significantly more than adult mice.
Figure 5. Mean (± S.E.M.) plasma corticosterone concentration (ng/mL) of BMS (brief separation), LMS (prolonged separation), and TR (typically laboratory reared) pubertal male and female BALB/c mice prior to (i.e. non-stressed), and immediately following (i.e. stressed), exposure to a 10 minute (adult; \( n_s: 5-7 \)) acute forced swim test. Non-stressed and stressed pubertal female TR mice had moderately and significantly (respectively) higher CORT levels compared to their age-matched male counterparts. Moreover, stressed pubertal female BMS mice had significantly higher CORT levels than stressed pubertal male BMS mice. For pubertal female mice, non-stressed and stressed CORT levels of both BMS and LMS mice were significantly lower than TR mice. However, for pubertal males, the non-stressed CORT levels of BMS mice were moderately lower compared to LMS and TR mice. Significance symbol, \( p < .05 \): (*). Trend symbol, \( p < .1 \): (#).
Figure 6. Mean (± S.E.M.) plasma corticosterone concentration (ng/mL) of BMS (brief separation), LMS (prolonged separation), and TR (typically laboratory reared) adult male and female BALB/c mice prior to (i.e. non-stressed), and immediately following (i.e. stressed), exposure to a 10 minute (adult; \(n_c\): 5-11) acute forced swim test. Overall, non-stressed and stressed CORT levels were significantly higher for adult female mice exposed to maternal separation (regardless of the length) compared to their age-matched male counterparts. For adult female mice, non-stressed CORT levels of LMS mice were the lowest, whereas for male mice, non-stressed CORT levels of both BMS and LMS mice were moderately lower than TR mice. Significance symbol, \(p < .05\): (*). Trend symbol, \(p < .1\): (#).
Discussion

The development of depressive-like behaviours from puberty to adulthood between male and female is not well described, and the impact of early life experiences on that development is even less understood. The present study examined the interactive effects of brief and prolonged maternal separation on depressive-like and coping behaviours, as well as basal and stress-induced CORT levels of pubertal and young adult, male and female BALB/c mice. The results indicate that the length of maternal separation, sex, and age at testing interact to differentially influence both behavioural and physiological coping responses elicited by exposure to an acute forced swim test. At puberty, sex differences in depressive-like behaviour were apparent, such that regardless of neonatal experience, immobility behaviour was significantly lower in female than in male pubertal mice. Interestingly, however, there were no statistically significant differences between non-stressed and stressed CORT levels between pubertal female and male mice. Although the differential effects of neonatal experiences were not apparent at puberty, in adulthood differences in physiological reactivity, and depressive-like and coping behaviours were found. Specifically, females that were exposed to long bouts of neonatal maternal separation (LMS) displayed significantly lower levels of active floating and moderately greater immobility in adulthood compared to LMS males. LMS females also had higher stressor-induced increases in CORT levels compared to their male LMS counterparts. These findings suggest that the effects of neonatal experiences are triggered by the developmental changes that females undergo during puberty, which lead to an increase in stressor reactivity, and subsequently, a higher risk of depression in adulthood relative to males.
Previous research has yielded inconsistent findings regarding the effect of neonatal stress on basal and stress-induced CORT levels. Specifically, between mice that experienced repeated prolonged maternal separation (i.e. 3 hours) and mice that did not experience neonatal stress (regardless of sex), there have been reports of no significant differences in basal CORT levels at puberty and adulthood (Sachs et al., 2013; Own & Patel, 2013, respectively), as well as no difference in stress-induced CORT levels at adulthood (Own & Patel, 2013). On the other hand, it has been found that the basal CORT levels of pubertal (i.e. PND 45) male mice are lower than that of non-separated controls (Pinheiro et al., 2011), but not at adulthood (Kawakami, Quadros, Takahashi, & Suchecki, 2007), and higher in pubertal and adult female LMS mice compared to BMS mice (Meagher et al., 2010). Furthermore, it has been found that maternally separated adult male (van Heerden, Russell, Korff, Stein, & Illing, 2010) and female (Kiank, Mundt, & Schuett, 2009) mice have significantly lower stress-induced CORT levels than control mice. Unlike previous research, our findings indicate that prolonged maternal separation may produce females with a stress-resistant phenotype (Kiank et al., 2009; Panagiotaropoulos et al., 2004), given pubertal and adult female LMS basal and pubertal female LMS stress-induced CORT levels were lower than female TR mice. However, when the adulthood stressor reactivity was considered, it was discovered that female LMS and BMS mice have significantly higher changes between non-stressed and stressed CORT levels than adult female TR mice. Moreover, there is an increased presence of the passive coping behaviour immobility of adult female LMS mice, and this is indicative of depressive-like symptomology. Overall, these results suggest that the adaptive response to early life stress observed at puberty is not maintained into adulthood, at least in female
mice that experience the chronic moderate neonatal stress of prolonged maternal separation. Taken as a whole, these findings highlight the complexity of the physiological outcomes to neonatal stress, as well as the importance of analyzing the stress-induced changes in CORT levels in addition to basal and stress-induced levels. Future studies assessing complementary stress hormone levels downstream of the pituitary, such as adrenocorticotropic hormone (ACTH), will provide further valuable insight into stress-induced HPA functioning as secretion of ACTH triggers the secretion and synthesis of corticosterone (Reynolds et al., 2013; Groenink, Gugten, Zethof, Heyden, & Olivier, 1994).

In addition to the typically reported forced swim test behaviours of swimming, climbing, and immobility, we describe here another behaviour which we termed “active floating”. Two other papers (Belozertseva, et al., 2007; Contreras, et al., 2008) have also characterized a similar behaviour, which the authors referred to as an active/mobile behaviour in rats subjected to a classical forced swim test. In the present study, although limited movements by mice were observed in both active floating and immobility, there were distinct differences between the two actions. Active floating was characterized by slight forepaw and tail movements made by the mouse in order to maintain an upright and stable floating position. Also during active floating, whisker movement and visual surveying of the environment were typically observed, and mice would periodically gently push off from the walls of the container and position themselves more in the center. On the other hand, a mouse was scored as immobile when it made only enough movements to stay afloat, its body position was not upright (i.e., floating more on one
side of its body), its tail was rigid, and it was not observed to actively survey the environment.

Analysis of the correlations among the active and passive coping behaviours with CORT is supportive of the notion that two distinct floating types of behaviours are being assessed. In particular, a pattern emerged that can be seen across all neonatal conditions and ages (with the exception of pubertal female LMS mice) such that there are magnitude and directional differences with respect to the association between CORT and the behaviours of active floating and immobility. For example, the correlation between active floating and CORT was -.75, whereas the correlation between immobility and CORT was much lower at -.01 for adult female LMS mice; underlining the difference in the magnitude of the relationships. On the other hand, the correlation between active floating and CORT was -.31, whereas the correlation between immobility and CORT was .90 for adult male BMS mice; emphasizing the directional differences in the relationships. Importantly, studies examining rodent behaviours during the forced swim test describe immobility as a minimalist, non-active behaviour as we also have. However, it is not clear whether a behaviour similar to what we termed active floating was also occurring in these other studies. Given active floating appears to involve goal-directed energy expenditure (albeit limited) in order to actively survey environment, I believe that active floating, like climbing and swimming, may be a form of adaptive coping or risk assessment behaviour that conserves energy and increases the chances of escape from a stressful and novel situation (Boccia & Pedersen, 2001). Coping strategies that represent active engagement with the environment are represented by those behaviours that are characterized by increased somatomotor activity, increased vigilance, hyper-reactivity,
and related physiological changes allowing the animal to effectively escape or avoid a stressor. Alternatively, coping strategies that demonstrate disengagement with the environment include those with the characteristics of reduced somatomotor activity and decreased vigilance (Keay & Bandler, 2001). It appears that in active floating, both of the conditions for active and passive coping strategies are met and compared to immobility, it seems to be more of an adaptive, goal-directed behaviour. The specific effects of indolamine or catecholamine selective antidepressants on active floating behaviour in the forced swim test remain to be examined.

Neonatal experience and sex interacted to differentially influence the development of both active and passive coping behaviours in the present study. Specifically, LMS female mice exhibited significantly more active coping (higher levels of swimming) and less passive coping (lower levels of active floating) behaviours compared to LMS males. Interestingly, in addition to an increased tendency to engage in active coping behaviours, adult female LMS mice also exhibited significantly higher levels of immobility compared to BMS females and moderately higher than LMS males, suggesting increased predisposition to engage in despair-related behaviours among the LMS females. Differences in stress-induced emotionality and coping behaviours leading to behavioural despair have been suggested to be involved in the etiology of depression in humans (Goel & Bale, 2009; Steimer & Driscoll, 2003). Furthermore, rodent studies have suggested that animals that are more fearful/emotional tend to use passive coping strategies in stressful situations (e.g., Steimer, la Fleur, & Schulz, 1997), and have increased tendency to engage in immobility behaviour in the forced swim test (Piras, Giorgi, & Corda, 2010). Indeed, this combination of emotionality and behavioural
coping style is typically described in the highly reactive BALB/c mouse strain (Griebel, Belzung, Perrault, & Sanger, 2000). However, these data demonstrated that neonatal experiences can alter the development of this ‘typical’ coping style demonstrating that in addition to genotype, the environment also shapes the phenotype. Moreover, we also demonstrate that the chronic moderate stress of protracted maternal separation impacts the greatest on female BALB/c offspring resulting in an adult phenotype that, when exposed to the novel stress of the acute forced swim test, engages in a more active coping style, but appears to “give up” faster (i.e., engaged in higher frequency of immobility behaviour).

The present study demonstrates that similar to other rodent studies (Llorente et al., 2007; Toledo-Rodriguez & Sandi, 2007), and also consistent with the human literature (Seeman, 1997), pubertal male mice engaged in significantly higher levels of immobility behaviour than pubertal females. We extend these findings and show that the development of adult behaviours is differentially influenced by sex and neonatal experiences such that immobility behaviour decreased from puberty to adulthood in all mice except in LMS females. The general trend in decreased despair or depressive-like behaviours from puberty to adulthood in our study might be expected, as other studies have shown that compared to their adult counterparts, pubertal mice generally exhibit increased emotional reactivity and risk-taking behaviours (Laviola, et al., 2003; Ray & Hansen, 2005; Romeo, Lee, & McEwen, 2004). However, the chronic, moderate stressor of protracted maternal separation experienced by mice in the LMS condition appeared to have a greater impact on the development of depressive-like and coping behaviours of female mice. Consequently, adult LMS females engaged in significantly higher levels of
depressive- or despair-like (immobility) behaviours than their BMS counterparts and moderately higher than adult male LMS mice. Alternatively, protracted maternal separation shaped the development of the physiological reactivity to stressors. Pubertal LMS females had lower changes between non-stressed and stressed CORT levels than those of TR females, but similar changes to pubertal male counterparts. On the other hand, adult LMS females had a significantly higher physiological reactivity than those of TR females, and higher than their adult male counterparts. Thus, neonatal manipulations produced not only sex-dependent behavioural and physiological reactivity differences in males and females exposed to the acute forced swim test, but these neonatal experiences also interacted with sex to differentially affect the development of stress-induced behaviours and physiological responses to stressors from puberty to adulthood. Cumulatively, these findings also suggest that behaviour is not always predictive of general physiological changes, and vice versa.

Several explanations have been proposed for the adaptation of the immobility response that occurs during the FST. Although immobility was originally considered to indicate diminished efforts to escape (i.e., ‘giving up’), it has also been suggested to represent a persevered coping strategy that is beneficial to the rodent (Cryan & Mombereau, 2004; Willner, 1990; Thierry, Steru, Chermat, & Simon, 1984). In particular, immobility has been thought of as a behavioural posture that allows for energy conservation and increases the rodent’s chance of survival in the inescapable environment brought on by the FST (Calil & Marcondes, 2006; Binik & Sullivan, 1983). Although there are gains of preserving energy in a stressful environment, the findings demonstrate that there is also a detrimental cost of engaging in immobility; an increased
HPA response (e.g., physiological reactivity). Moreover, given the increased complexity of the human environment, the advantage of engaging in passive coping styles may be further reduced. Taken as whole, I am not suggesting that immobility represents a failure to cope, but rather, highlighting that immobility represents a less proactive form of coping than previously believed (Calil & Marcondes, 2006).

In the present study, female mice were tested during estrus, a time when, in the mouse, circulating estrogen levels are falling and progesterone levels are also moderate relative to the other stages in the cycle (Fata et al., 2001; Hiroi & Neumaier, 2006; Schedin et al., 2000). In general, both estrogen and progesterone have antianxiety and antidepressant effects in females (Estrada-Camarena, Fernandez-Guasti, & Lopez-Rubalcava, 2003; Frye & Walf, 2009; Walf & Frye, 2006), but these effects may be dependent on the perceived threat of the situation (Boccia & Pedersen, 2001; Wigger & Neumann, 1999). Relevant to the present study, the behavioural differences that were observed between pubertal and adult mice may be due to the stimulatory effects of estrogen and progesterone on neural activity which may have more of an effect in adult than in pubertal female mice. That is, because pubertal females were tested at their first estrus and therefore have just begun to experience estrous cycles, they may not have been exposed to elevated levels of estrogen and progesterone for a long enough time to cause neural and behavioural/affect changes (Neumann et al., 2005; Walf & Frye, 2006). The specific neural and hormonal mechanisms underlying the differences in depressive-like behaviours among mice at the different developmental stages remain to be identified.

The findings of the present study add to those of previous studies by concurrently examining across age and sex the effects of mild and moderate stressful early-life
experiences on the development of hormonal and behavioural phenotypes. Specifically, the study demonstrated that the experience of protracted maternal separation increased the susceptibility of adult, but not pubertal, female mice to engage in higher levels of depressive-related immobility behaviour in the acute forced swim test, as well as lower levels of active floating - a suggested adaptive coping behaviour. The present study also revealed that the experience of chronic early-life stress, regardless of the length, results in greater stressor reactivity in adult females compared to their male counterparts. Taken as a whole, these findings suggest that adaptation in the form of engaging in depressive-related immobility behaviour, although conserves energy and increases the rodents chance of survival in an inescapable situation (Morley-Fletcher, 2004; Calil & Marcondes, 2006), results in an increased corticosterone response. These results parallel those of human studies of the etiology of depression, and suggest that this animal model may be useful in helping to better elucidate the mechanisms involved in the environmentally mediated, sex-dependent risk of depression.

Chapter 4: Neonatal experiences differentially influence gene and protein expression profiles in the adult mammary gland of BALB/c mice.

Introduction

Elaboration of the factors underlying normal breast development will contribute to breast cancer prevention efforts. Since the mammary gland completes its development postnatally, it may be sensitive to alterations in the hormonal and molecular environments during development. Indeed studies have indicated that the neonatal environment can influence mammary gland development and the risk of carcinogenesis.
For example, the experience of the chronic moderate stressor of long bouts (i.e. four hours) of maternal separation (LMS) over the first three weeks of life resulted in accelerated mammary gland development, higher incidence of mammary tumourigenesis following adulthood carcinogen [7,12-dimethylbenz(a)anthracene] administration, and higher estrogen receptor alpha (ERα) levels in the mammary gland (Boyd et al., 2010). ERα is a protein that has important roles in normal and malignant mammary gland growth such that high levels may be indicative of a predisposition to mammary tumourigenesis (Shoker et al., 1999; Boyd et al., 2010; Hewitt et al., 2002).

Within the mammary gland, estrogen and glucocorticoids have opposing effects. Specifically, estrogens promote cell growth and proliferation, whereas glucocorticoids exert anti-proliferative effects (Sutherland, Prall, Watts, & Musgrove, 1998; Zhou, Bouillard, Pharaboz-Joly, & Andre, 1989). The anti-proliferative effects of glucocorticoids have been confirmed in *in-vitro* studies of human breast cancer cells (Krishnan, Swami, & Feldman, 2001), and the anti-proliferative effects may be related to the promotion of tissue differentiation in rodent cancers (Lee et al., 1998).

Research focusing on the cross-talk between ER and GR has used the MCF-7 breast cancer cell lines. The findings indicate that estrogen agonists down regulate GR protein levels via protein degradation, and in particular, through the proteasome degradation pathway (Kinyamu & Archer, 2003; Krishnan et al., 2001). Studies have also identified protein phosphate 5 (PP5) to be involved in the inhibition of GR activity by estrogen. Specifically, it was shown that estrogen increases expression of PP5, mediating the dephosphorylation of GR at Ser211 (Zhang, Leung, Nordeen, S., & Goleva, 2009). Residues within GR (e.g. Ser211) require phosphorylation for the GR to gain full
transcriptional activity (Galliher-Beckley & Cidlowski, 2009). Phosphorylation (i.e. the addition of a phosphate group to a protein) is a post-translational modification central to cancer biology and treatment (Reimand, Wagih, & Bader, 2013). When PP5 is knocked down, estrogen loses its ability to suppress GR phosphorylation, and a significant reduction in estrogen-promoted cell proliferation by glucocorticoids is observed (Zhang, Leung, Nordeen, S., & Goleva, 2009). These findings provide a novel mechanism of cross-talk between estrogen and glucocorticoids. Alternatively, glucocorticoids may act to indirectly increase ER levels due to a reduction in posttranslational modifications of ERα due to glucocorticoid-induced decreases in breast cancer type 1 susceptibility protein (BRCA1) levels (Antonova & Mueller, 2008; Ma et al., 2010).

The mineralocorticoid receptor (MR) also has a high affinity for glucocorticoids; much higher than that of the GR (Kolber, Wieczorek, & Muglia, 2008). Although the role of MR in normal and malignant mammary gland development is less well defined than GR, the results of a study conducted by Kingsley-Kallesen et al. (2002) is indicative that MR may compensate for the absence of GR at certain stages of mammary gland development. For example, MR may take on the role of regulating the transcription of milk protein genes. It has been suggested that little information is available on the effect of mineralocorticoids because of the difficulty involved in distinguishing between a mineralocorticoid and glucocorticoid response (Cato & Weinmann, 1988). This is due to finding that MR has an equally high affinity to both mineralocorticoids (e.g., aldosterone) and glucocorticoids (e.g., corticosterone), while GR responds to glucocorticoids, but is mostly insensitive to mineralocorticoids (Savory et al., 2001).
The transcriptional functions of ERα have also been found to be influenced by coactivator-associated arginine methyltransferase 1 (CARM1) (Mann, Cortez, & Vadlamudi, 2011). CARM1 is recruited to ERα target genes, and subsequently, activates ER-dependent transcription (Xu et al., 2004). In particular, through cyclic adenosine monophosphate signalling, CARM1 activates ERα and this leads to ERα phosphorylation. Once this occurs, CARM1 and the estrogen receptor interact and can bind to DNA to regulate target genes (Tanos, Rojo, Echeverria, & Brisken, 2012).

Interestingly, studies exploring the impact of mouse embryos with a targeted disruption of CARM1 have found that the loss of CARM1 results in the abolishment of the estrogen receptor response, and the expression of estrogen-regulated genes were greatly reduced (Yadav et al., 2003). Furthermore, the effects of glucocorticoids, another steroid hormone, on gene transcription may be dependent on CARM1. Although this has yet to be shown in the mammary gland, this finding has been demonstrated in pulmonary epithelial cells. Using CARM1 and GR knockout lungs, O’Brien et al. (2010) analyzed gene expression of cell cycle genes and markers of differentiation. Firstly, hyperproliferation of pulmonary epithelial cells during embryonic development in CARM1 knockout was observed. Secondly, there was an overlap between CARM1 and GR expression signatures. In particular, both knockouts resulted in similar dysregulation of cell cycle genes and markers of differentiation in the lung, suggesting that the loss of CARM1 may disrupt GR signaling. Thus, the hyperproliferation and lack of maturation of the alveolar cells are potentially caused by attenuation of glucocorticoid mediated signaling. These findings are consistent with previous reports that CARM1 functions as a coactivator of GR transcription (O’Brien et al., 2010). CARM1 has also been found to
synergistically function to increase GR transcriptional activity by binding to the glucocorticoid-interacting protein (Lee, Teyssier, Strahl, & Stallcup, 2005; Liu, Hsieh, Chou, & Huang, 2006; Teyssier, Ou, Khetchoumian, Losson, & Stallcup, 2006). Taken as a whole, this suggests that CARM1 is a main determinant of ERα and GR target gene expression (Wang, 2013).

Another coactivator for the transcription of ERα is the nuclear receptor coactivator 3 (SRC3; also known as amplified in breast cancer 1). It was initially believed that SRC3 was involved in the proliferation, and thus, promotion of hormone-dependent cancers (e.g. estrogen receptor positive) by acting as a transcriptional coactivator for nuclear receptors such as the estrogen receptor (Ma, Ren, Wang, & He, 2011; Chang & Wu, 2012). Support for this notion is provided by studies exploring the effects of SRC3 depletion which have demonstrated that SCR3 inhibits estrogen-stimulated cell proliferation in estrogen receptor positive MCF-7 breast cancer cells, and eventually leads to a decrease in growth of MCF-7 cancerous cells when this tissue is transplanted into mice (Karmakar, Foster, & Smith, 2009; List et al., 2001). Furthermore, when mice undergo ovariectomy at puberty (thereby blocking production of a significant proportion of estrogens), transgenic SRC3 mice do not develop mammary gland tumours (Torres-Arzayus, Zhao, Bronson, & Brown, 2010). However, it has also been confirmed that ERα-negative breast cancers are associated with high mRNA expression levels of SRC3 (Lahusen, Henke, Kagan, Wellstein, & Riegel, 2009). It has become clear that SRC3 has other significant roles in cancer, in addition to being a nuclear receptor coactivator, such as coactivating transcription factors (e.g. p53). Therefore, SRC3 may also be involved in the progression of both hormone-dependent and hormone-independent cancer cells (Ma,
Ren, Wang, & He, 2011; Chang & Wu, 2012). Thus, SRC3 is not only essential for the normal mammary development, but also plays an important role in mammary tumourigenesis (Gao & Nawaz, 2002).

Mitochondrial DNA (mtDNA) may also alter expression of genes that stimulate cell proliferation and differentiation (Rohan et al., 2010). It has been well-established that estrogens induce growth in the mammary gland via proliferation of epithelial cells. Estrogens have also been shown to increase cell proliferation of breast cancer cells (Mahalingaiah, & Singh, 2014). High rates of proliferation can increase vulnerability to mutations throughout DNA replication, which may lead to tumourigenesis (Roca, Oliver, Sastre-Serra, & Nadal-Serrano, 2007; Gonzalez, Bonnard, & Grienenberger, 1993). Although direct links have not necessarily been made between ND2 and ER, several groups have identified relationships between mitochondrial DNA and estrogen.

Interestingly, it has been shown that mitochondria are significant targets of estrogens (Chen, Delannoy, Cooke, & Yager, 2004). Research examining whether estradiol-induced reactive oxygen species control is involved in the growth of breast cancer cells has revealed that estrogen regulates the earlier stages of the cell cycle (i.e. G1 stage) through mitochondrial reactive oxygen species. Secondly, it was shown that the modulation of mitochondrial function, transcription/replication, or protein synthesis, block estrogen-induced cell cycle progression. Lastly, it was confirmed that reactive oxygen species do indeed play a role in the progression of estrogen-stimulated breast epithelial cancer cells (Felty, Singh, & Roy, 2005). A study has also demonstrated estrogen-dependent presence of ERα and ERβ within the mitochondrial matrix (using immunohistochemistry) of MCF-7 human breast cancer cells. The authors proposed that
this presence may directly enhance the levels of mtDNA-encoded transcripts (by binding to estrogen response elements); a finding similar to what has been observed for the glucocorticoid receptor (Chen & Yager, 2004).

The objective of the present study was to further assess the impact of early-life experiences on the molecular profiles of the female mammary gland at adulthood. A well-characterized neonatal manipulation of daily brief (BMS; 15 minutes) or long (LMS; 4 hours) maternal separations for the first 3 weeks of life, as well as mice that did not experience maternal separation [typically reared (TR)], was used to model mild and moderate chronic early-life stress. We previously demonstrated that neonatal experiences differentially influence ERα protein levels (but not p53) in normal mammary glands of adult mice in a transcript-independent manner. Moreover, higher ERα protein levels in mammary glands of LMS mice were related to increased mammary tumourigenesis following adulthood carcinogen (7,12-dimethylbenz[a] anthracene; DMBA) administration (Boyd et al., 2010). To further build on the biobehavioural pathways that may be involved in the effect of the experience of long maternal separation on increased mammary tumour incidence, mRNA levels of GR, MR, CARM1, SRC3, and ND2, and protein levels of GR, MR, and CARM1 in normal mammary glands of young adult (postnatal day 60) female BALB/c mice reared in each environmental condition were measured. These molecular factors were chosen as they interact with ERα, and thus, either directly or indirectly influences normal and malignant mammary gland growth.
**Materials and Methods**

*Tissue collection*

Mammary glands were collected at approximately PND 60 (± 2 PND), and only when mice were in estrus. Mammary glands used for analyses of RNA (seventh and eighth thoracic glands) were stored at -80°C until required. Mammary glands for immunohistochemical analyses of protein (fourth and ninth inguinal glands) were fixed overnight and then underwent immunohistochemical procedures.

*Immunohistochemistry*

*Tissue Preparation*

Immunohistochemical staining for glucocorticoid receptor (GR), mineralocorticoid receptor (MR), and coactivator associated arginine methyltransferase 1 (CARM1) was performed on paraffin embedded inguinal mammary gland sections from neonatally manipulated young adult mice. The 4th and 5th right inguinal glands of each mouse were dissected, placed in a cassette and fixed in a 4% neutral buffered formalin solution overnight. Mammary glands were then washed in distilled water (3 x 15 minutes each), followed by a 30 minute wash in 50% ethanol, and immersed in 70% ethanol at 4°C for at least two days before tissues were processed. Tissues were then paraffin embedded. Embedded mammary glands were sectioned using a microtome to a thickness of 4-6μm and two serial sections were placed on each Superfrost Plus charged slide. Slides were baked at 65°C for two to three hours to soften the wax for removal and to further facilitate the adhesion of tissue sections to the slide surface.
Colourmetric immunohistochemical staining

Sections were de-paraffinized in xylene (3 x 10 minutes), followed by a gradual re-hydration in 95% ethanol (3 x 4 minutes) and a three minute wash each in 75% ethanol and 50% ethanol before a distilled water rinse. Sections were then washed for ten minutes with a 3% hydrogen peroxide solution, and rinsed with distilled water. Following this re-hydration process, tissue sections were boiled for 10 minutes at 95-100°C in 0.5M sodium citrate buffer (pH 6.0), cooled to room temperature by immersion in a distilled water bath, and rinsed in 1X phosphate buffered saline (PBS) (pH 7.0-7.4). To reduce non-specific binding, sections were incubated for 30 minutes at 25°C with 7% goat serum prepared in 1% bovine serum albumin (BSA). Tissue sections were then incubated with rabbit polyclonal [GR (M-20) and MR (H-300), Santa Cruz Biotechnologies CA, U.S. CARM1, a generous gift from Dr. Wei Xu (University of Madison, Wisconsin) at 4°C overnight for 19 hours and then for two hours at 25°C. Primary antibody concentrations were 1:200 for GR, 1:35 for MR, and 1:125 for CARM1; each diluted in 1% BSA.

Following three washes with PBS, appropriate biotinylated secondary IgG antibodies (BA-1000 Vector Laboratories, ON, Canada for GR; SC-2040, Santa Cruz Biotechnologies, CA, U.S. for MR and CARM1) were applied at a concentration of 1:250 for GR, 1:200 for MR, and 1:200 for CARM1 for two hours at 25°C. As a negative control, primary antibodies were omitted for one mammary gland section per slide. GR, MR, and CARM1 immunoreactivity were visualized using an avidin-biotin complex (Vectastain ABC Kit PK-6100, Vector Laboratories, CA, U.S.), followed by an application of diaminobenzidine (DAB). Tissue sections were then counterstained with a 20% solution of Harris’ Haematoxylin (Sigma-Aldrich, Canada). Following staining, slides were de-hydrated in five serial ethanol solutions followed by three washes of
xylene. After processing, slides were mounted with Permount (Fisher Biotechnology, Canada) and coverslipped.

**Quantification of GR, MR, and CARM1 protein expression**

Tissue sections were examined under a light microscope (Leica DM 6000B) attached to a digital camera (Leica DFC 420) to determine positive staining for GR, MR, and CARM1 proteins. Positive cells are indicated by the presence of brown stained nuclei in the mammary tissue. Three areas for GR and MR and five areas for CARM1, representative of the entire organ from each tissue section and its corresponding negative control were photographed at 40x magnification and the digital images were analyzed using image software (Adobe Photoshop Version 5.5, USA). Positive and negative stained nuclei in mammary stroma and epithelia were counted and expressed as the percentage of positively stained nuclei relative to the total number of nuclei in each of three or five random areas. For each neonatal manipulation group, tissue sections from four mice per treatment group were used for GR and MR, and seven to 11 mice per treatment group were used for CARM1. Approximately 200-2000 nuclei were counted per tissue section, with a total of 2000-5000 nuclei for each mouse.

*Quantitative Real Time-PCR*

**Primer Design**

Specific forward and reverse primers were generated for genes of interest by inputting *Mus musculus* mRNA sequences obtained from the National Centre for Biotechnology Information (NCBI) into NCBI Primer-BLAST software (see Table 2 for accession numbers and Table 3 for primer sequences and cycling parameters). The following specifications were used: primer length of 18-22 base pairs, product length of
90-200 base pairs, annealing temperature of 58-62°C, and percentage of GC content from 45% to 55%. For all primer pairs, each of the two primers were designed to bind to sequences in different exons, or one of the two primers spanned an exon-exon junction to avoid amplification of genomic DNA. A Primer-BLAST search was conducted to ensure that selected primer pairs would not bind to any unintended sequences (see Supplementary Materials for primer sequences).

Homogenization and RNA Isolation

RNA later-preserved normal mammary glands \( n = 3 \) for BMS, LMS, and TR were pulverized for total RNA extraction by flash freezing tinfoil-wrapped tissue in liquid nitrogen and hammering. Total RNA was then extracted using the RNeasy Plus Universal Mini Kit (Qiagen). The pulverized tissues were transferred to RNase-free microcentrifuge tubes and tissues were homogenized by immersion in 900 \( \mu \)L of phenol/guanidine-based Qiazol lysis reagent (Qiagen) and blending with a motorized Teflon pestle tissue grinder. After homogenization, tissue remained in the lysis reagent for five minutes at room temperature to promote dissociation of nucleoprotein complexes, following which 100 \( \mu \)L of genomic DNA eliminator solution was added to each tube. The tubes were shaken for 15 seconds and 200 \( \mu \)L of chloroform was added to create an organic/aqueous phase separation. Once chloroform was added, tubes were shaken, incubated for two minutes at room temperature and centrifuged at 12 000 x g for 15 minutes at 4°C. The aqueous, RNA-containing phase was transferred to a new tube after the centrifugation step, and the organic phase and interphase were discarded (containing proteins and DNA, respectively). To create appropriate conditions for binding total RNA to silica membrane, 70% ethanol was added to the extracted aqueous phase, in equal
volume. This mixture was immediately transferred to an RNeasy spin column with a silica membrane for RNA purification. A series of four washes in aqueous RW1 and RPE buffers (Qiagen) were then conducted to eliminate contaminants. Between each wash, samples were briefly centrifuged (for approximately 15 seconds) at 12 000 x g at room temperature to draw the wash buffers through the membrane. An additional centrifugation step was carried out after discarding the flow-through from all washed to ensure that there was no residual ethanol. Finally, RNA was eluted in 20 µL of nuclease-free water.

Assessment of RNA Quality and Quantity

Isolated RNA for each sample was quantified with a NanoDrop Spectrophotometer (ThermoFisher Scientific, Pittsburgh, PA) and RNA contamination was assessed using optical density 260/280 nm readings (acceptable range: 1.9-2.1, which indicates that a sample is free of DNA and protein contamination). Formaldehyde denaturing agarose gel electrophoresis was also used to evaluate RNA integrity. 1% formaldehyde denaturing agarose gels were prepared by combining 0.5 g of agarose with 36 mL deionized water and 5 mL of 10X MOPS buffer (0.4 M 3-(N-morpholino)-propanesulfonic acid (pH 7.0), 0.01 M ethylene-diamine-tetra-acetic acid (pH 8.0) and 0.1 M sodium acetate). This mixture was heated by microwave and 9 mL of 37% formaldehyde was added before pouring the gel. RNA samples (0.5 µg total) were mixed with 2X RNA loading dye (Fermentas), in equal volumes. The loading dye contains both ethidium bromide and formamide (a denaturing agent). Gels were run at 55 volts for approximately two hours, immersed in 1X MOPS buffer. Ultraviolet (UV) visualization of gels was performed to ensure that each sample has two sharp bands representing the
28S and 18S ribosomal RNA bands (with the 28S ribosomal RNA band being approximately twice as intense as the 18S ribosomal RNA band). This is indicative that RNA degradation has not occurred.

First Strand cDNA Synthesis

A reverse-transcription reaction was performed using 25 µg/µL RNA from each sample (500 ng of RNA added per 20 µL final reaction volume) using a First Strand cDNA Synthesis Kit (Fermentas) with RevertAid BMS Minus Moloney Murine Leukemia Virus reverse transcriptase and a non-specific poly (T) primer. First, RNA was combined with oligo(dT)-18 primer (at a final concentration of 25 ng/µ) and nuclease-free water heated to 70°C for five minutes in a water bath to denature RNA. Next, 5X Reaction buffer, RiboLock ribonuclease inhibitor (final concentration of 1 unit/µL) and deoxynucleotide triphosphate (dNTP) mix (final concentration of 1 mM) were added, and this new mixture was heated at 37°C for five minutes in a water bath. Finally, RevertAid BMS Minus M-MulV reverse transcriptase was added (final concentration of 1 unit/µL), the mixture was heated by water bath at 42°C for 60 minutes and reaction was stopped by raising the temperature to 70°C for five minutes. “No reverse transcriptase” (No RT) controls were prepared using the exact same procedure but substituting nuclease-free water for reverse transcriptase.

Preparation of Standards from Universal cDNA

Standards were prepared from Universal cDNA Reverse Transcribed by Random Hexamer: Mouse Normal Tissue (Cedarlane). Following nanodrop spectrophotometry to obtain the concentration of universal cDNA, a standard 2-fold serial dilution was
prepared with nuclease free water for the following concentrations: 100 ng/µL, 50 ng/µL, 25 ng/µL, 12.5 ng/µL.

Quantitative Real Time Polymerase Chain Reaction

A Stratagene Mx3000P real-time PCR machine was used to amplify sample cDNA using mRNA transcript-specific primer pairs (see Table 2 for accession numbers and Table 3 for primer sequences and cycling parameters). Each 25 µL reaction well contained the following, in final concentrations: 25 ng cDNA, 1X Promega PCR buffer, 2.5 µM MgCl$_2$, 400 nM Promega dNTP mix, 8% glycerol (to increase thermal stability of the polymerase enzyme), 0.5X Invitrogen Rox reference dye, 0.17X Invitrogen SYBR Green I dye, 0.1 U/µL Promega GoTaq Flexi Polymerase, 500 nM forward primer, 500 nM reverse primer, with the remaining volume made up with nuclease-free water. Two plates were run for each biological replicate for each gene transcript, with biological replicates run in triplicate on each plate. Correct product formation was verified used melting curve analysis, agarose gel electrophoresis, and RT-qPCR product sequencing. Relative gene expression was calculated using the $\Delta\Delta$CT method. The expression of the $\beta$-actin gene for each sample was used as the normalizer, and the gene expression in the TR sample was used as the calibrator. Data from qPCR runs were only analyzed if the calculated efficiencies were found to be between 97-103%.
**Table 2.** NCBI Accession numbers for *Mus musculus* mRNA sequences used in primer design.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucocorticoid receptor (GR)</td>
<td>NC_000084.6</td>
</tr>
<tr>
<td>Mineralocorticoid receptor (MR)</td>
<td>NC_000074.6</td>
</tr>
<tr>
<td>Coactivator-associated arginine methyltransferase 1 (CARM1)</td>
<td>NC_000075.6</td>
</tr>
<tr>
<td>Nuclear receptor coactivator 3 (SRC3)</td>
<td>NC_000068.7</td>
</tr>
<tr>
<td>NADH-Ubiquinone oxioreductase chain 2 (ND2)</td>
<td>NC_005089.1</td>
</tr>
<tr>
<td>β-actin</td>
<td>NC_000071.6</td>
</tr>
</tbody>
</table>

**Table 3.** Primer pairs used in RT-qPCR experiments.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
<th>Cycling Parameters</th>
</tr>
</thead>
</table>
| GR   | F: 5'-CAAAGCCGTTTCACTGTCC-3'  
      | R: 5'-ACAATTTTCACACTGCAACC-3' | 95°C: 10 min; 45X  
      |                          | (95°C: 15s, 58°C: 60s) |
| MR   | F: 5'-GGCCAAGGTTACTCCAGTGT-3'  
      | R: 5'-CCCTGGCAACGCCATCAT-3' | 95°C: 10 min; 45X  
      |                          | (95°C: 15s, 60°C: 60s) |
| CARM1| F: 5'-ATGCAGAGGTGTCTGGTGAAG-3'  
      | R: 5'-TCATCGGTCAAGGAGTCCGT-3' | 95°C: 10 min; 45X  
      |                          | (95°C: 15s, 58°C: 60s) |
| SRC3 | F: 5'-CTGGCAGTCTGATGAGGAG-3'  
      | R: 5'-AGCCATTGGGCAATTTAAG-3' | 95°C: 10 min; 45X  
      |                          | (95°C: 15s, 58°C: 60s) |
| ND2  | F: 5'-CGCCCCATTCCACCTTCTT-3'  
      | R: 5'-TTAAGTCTCTCCTATGCCCT-3' | 95°C: 10 min; 45X  
      |                          | (95°C: 15s, 58°C: 60s) |
| β-actin | F: 5'-AGCCTTCTTTCTTTGGGTATG-3'  
       | R: 5'-GGTCTTTACGGGATGTCAAC-3' | See parameters for other genes; this housekeeping gene was chosen because of high efficiencies at a wide range of cycling parameters |
**Results**

*Statistical Analyses*

Statistical comparisons were completed using repeated measures ANOVAs (RT-qPCR data, using replicate experimental runs as a within-subjects factor) or factorial ANOVAs (immunohistochemistry data using tissue compartment as a factor). Where indicated, data were first log-transformed if parametric assumptions were violated; nonparametric two-tailed Mann-Whitney U tests were used if transformation was not possible. Statistical significance was set at $p < .05$ and moderate significance was set at $p \leq .10$. Significant effects as well as moderate effects were analyzed using Newman-Keuls post-hoc comparisons. All statistical analyses were conducted using the statistical package STATISTICA 10 (Statsoft, USA). Data are presented as means ± S.E.M.

*Transcript levels of GR, but not MR, CARM1, SRC3, and ND2, is increased in mammary glands of adult female LMS mice.*

GR transcript expression levels were found to be significantly higher in mammary glands of young adult female LMS mice than BMS mice ($p = .017$) (See Figure 7). Conversely, the transcript levels of MR, CARM1, SRC3, and ND2 in the mammary glands of young adult female BMS and LMS mice were similar (See Figure 9, 11, and 13, respectively).

*Protein expression of GR, but not MR and CARM1, is decreased in mammary glands of adult female LMS mice.*

Although a main effect of tissue compartment in GR protein expression pattern was observed (stromal > epithelial, $p = .0084$), the expression patterns were similar
across neonatal conditions. In particular, examination of the significant main effect of treatment \([F(2, 27) = 3.78, p = .036]\) indicated that at young adulthood (PND 60), GR protein expression was significantly higher in the mammary glands of female BMS mice than female LMS \((p = .035)\) and TR \((p = .047)\) mice (See Figure 7). In contrast to GR protein levels in mammary glands of young adult female mice, MR protein expression was similar across neonatal conditions (See Figure 9). The length of maternal separation had no significant impact on CARM1 protein expression, given CARM1 levels were significantly lower in the mammary glands of both young adult female LMS \((U = 230, p < .001)\) and BMS \((U = 230, p < .01)\) mice than female TR mice (See Figure 11).
Figure 7. Relative GR mRNA transcript and protein expression in mammary glands of young adult (PND 60±5) BMS, LMS, and TR mice. A, relative GR mRNA transcript expression as determined by RT-qPCR; \( n = 3 \) BMS, \( n = 3 \) LMS, \( n = 3 \) TR. Transcript levels are expressed relative to β actin, as well as relative to the transcript levels of TR controls (dashed line). B, relative GR protein expression as determined by IHC; \( n = 4 \) BMS, \( n = 4 \) LMS, \( n = 4 \) TR. At young adulthood, GR protein expression was highest in the mammary glands of BMS mice compared to LMS and TR mice. GR transcript expression, however, was higher in the mammary glands of LMS mice compared with BMS mice. Data are presented as means ± SEM. *, \( p < .05 \).

Figure 8. Representative IHC images of GR protein expression in the mammary glands of young adult (PND 60±5) female mice. A, BMS; B, LMS; C, TR. Brown represents positive staining and blue represents negative staining. Inset pictures show negative controls.
Figure 9. Relative MR mRNA transcript and protein expression in mammary glands of young adult (PND 60±5) BMS, LMS, and TR mice. A, relative MR mRNA transcript expression as determined by RT-qPCR; \( n = 3 \) BMS, \( n = 3 \) LMS, \( n = 3 \) TR. Transcript levels are expressed relative to β actin, as well as relative to the transcript levels of TR controls (dashed line). B, relative MR protein expression as determined by IHC; \( n = 4 \) BMS, \( n = 4 \) LMS, \( n = 4 \) TR. At young adulthood, MR protein and transcript expression was not significantly different across neonatal conditions. Data are presented as means ± SEM.

Figure 10. Representative IHC images of MR protein expression in the mammary glands of young adult (PND 60±5) female mice. A, BMS; B, LMS; C, TR. Brown represents positive staining and blue represents negative staining. Inset pictures show negative controls.
**Figure 11.** Relative CARM1 mRNA transcript and protein expression in mammary glands of young adult (PND 60±5) BMS, LMS, and TR mice. A, relative CARM1 mRNA transcript expression as determined by RT-qPCR; \( n = 3 \) BMS, \( n = 3 \) LMS, \( n = 3 \) TR. Transcript levels are expressed relative to \( \beta \) actin, as well as relative to the transcript levels of TR controls (dashed line). B, relative CARM1 protein expression as determined by IHC; \( n = 9 \) BMS, \( n = 7 \) LMS, \( n = 11 \) TR. At young adulthood, CARM1 protein expression was significantly lower in LMS and BMS mice compared to TR mice. However, CARM1 transcript expression was not significantly different across neonatal conditions. Data are presented as means ± SEM. *, \( p < .05 \).

**Figure 12.** Representative IHC images of CARM1 protein expression in the mammary glands of young adult (PND 60±5) female mice. A, BMS; B, LMS; C, TR. Brown represents positive staining and blue represents negative staining. Inset pictures show negative controls.
Figure 13. Relative SRC3 and ND2 mRNA transcript expression in mammary glands of young adult (PND 60±5) BMS, LMS, and TR mice. A, relative SRC3 mRNA transcript expression as determined by RT-qPCR; \( n = 3 \) BMS, \( n = 3 \) LMS, \( n = 3 \) TR. B, relative ND2 mRNA transcript expression as determined by RT-qPCR; \( n = 3 \) BMS, \( n = 3 \) LMS, \( n = 3 \) TR. Transcript levels are expressed relative to \( \beta \) actin, as well as relative to the transcript levels of TR controls (dashed line). At young adulthood, SRC3 and ND2 transcript expression was not significantly different across neonatal conditions. Data are presented as means ± SEM.
**Discussion**

The present study has demonstrated that the early-life experiences of either brief or prolonged maternal separation differentially influence the gene and protein expression profiles of the female mammary gland at young adulthood. Specifically, GR protein expression was significantly increased in mice that experienced brief maternal separation relative to mice that experienced prolonged or no maternal separation. However, the study has also shown that LMS mice have a significantly higher GR transcript expression compared to BMS mice. Despite not reaching statistical significance, MR protein and transcript expression followed the same pattern as GR. Although no significant differences were observed across neonatal condition in CARM1, SRC3, and ND2 transcript expression, an interesting finding was with respect to CARM1 protein expression. The experience of maternal separation, regardless of duration, significantly reduced CARM1 protein levels compared with mice that did not experience maternal separation. Taken as a whole, these findings suggest that neonatal experience influences the expression of genes and proteins involved in development of the normal mammary gland.

It has been established that the experience of prolonged maternal separation promotes mammary tumourigenesis through an increase in the expression of ERα protein (Boyd et al., 2010). However, the finding that LMS mice have reduced GR protein levels relative to BMS mice provides a complementary explanation for the increased risk. In particular, reduced expression of GR results in an inability for the receptor to exert anti-proliferative effects within the mammary gland (Sutherland et al., 1998; Zhou et al., 1989; Kishnan et al., 2001). Lower GR levels may be due to estrogen down-regulating
GR protein levels through protein degradation via the proteasome degradation pathway or by estrogen-induced increases in PP5; ultimately contributing to a reduction in the ability for GR to gain full transcriptional activity (Kinyamu & Archer, 2003; Krishnan et al., 2001; Zhang et al., 2009; Galliher-Beckley & Cidlowski, 2009). Furthermore, reduced CARM1 protein expression of LMS mice (compared to TR mice) may also disrupt GR signaling, resulting in the reduced GR protein expression of LMS mice (O’Brien et al., 2010).

Similar to previous literature, this study also highlights that transcript levels should not be used as a major determinant of protein expression (Gygi, Rochon, Franzia, & Aebersold, 1999). The results of the present study indicate that the experience of prolonged maternal separation increases GR transcript expression, but decreases GR protein expression, compared to BMS mice. Furthermore, we have previously shown that LMS mice have higher ERα protein, but not transcript expression than BMS mice (Boyd et al., 2010). Nuclear receptors, such as ER and GR, are regulated by post-translational modifications and are susceptible to a wide variety capable of impacting receptor function (Anbalagan, Huderson, Murphy, & Rowan, 2012). Common modifications of GR include phosphorylation and acetylation, and there are at least six serine residues that are phosphorylated on GR (Oakley & Cidlowski, 2011). Given estrogen indirectly influences the dephosphorylation of GR at the N terminus site Ser211, the increased ER levels of LMS mice may reduce the likelihood of residues within GR (i.e. Ser211) to receive the addition of a phosphate group in order to gain full transcriptional activity (Chen et al., 2008; Galliher-Beckley & Cidlowski, 2009; Zhang et al., 2009). This may explain the reduced GR protein expression, yet increased transcript expression, observed
in LMS mice. Future immunohistochemical phosphoprotein analyses will provide insight into the phosphorylation levels of the GR protein. Interestingly, post-translational modifications are also involved in the onset and progression of disease such as cancer (Reimand et al., 2013).

Given the low predictive value of GR transcript levels on GR protein expression, future studies should assess the protein expression of SRC3 and ND2 within the mammary gland of mice exposed to neonatal manipulations. Although no statistically significant differences were found among neonatal conditions with respect to SRC3 and ND2 transcript levels, this may not be the case for their protein profiles, because mitochondrial DNA are a significant target of estrogens, and SRC3 is required for estrogen-stimulated cell proliferation (Chen et al., 2004; Karmakar et al., 2009; List et al., 2001). Interestingly, a pattern emerged such that LMS mice had higher ND2 transcript expression than BMS mice. This may be related to the finding that reactive oxygen species play a role in the progression of estrogen-stimulated breast cancers (Felty et al., 2005). However, increasing the sample size of the LMS mice for ND2 transcript expression may decrease the particularly high variability of that group and allow for the difference between LMS and BMS mice to reach statistical significance.

Given CARM1 is known to be recruited to ERα, activates ER-dependent transcription, and is a main determinant for the expression of estrogen-regulated genes (Xu et al., 2004; Yadav et al., 2003), it was unexpected that LMS and BMS mice would have comparable CARM1 protein expression given differences in ERα protein expression. This suggests that there are other equally important determinants for the expression of estrogen-regulated genes, such as GR. Nonetheless, the reduced CARM1
expression relative to TR mice may result in an inability to block cell proliferation and induce differentiation (Al-Dhaheri et al., 2012), at least in LMS mice, and thus further promote mammary tumourigenesis in mice exposed to prolonged maternal separation.

Improved awareness of the factors underlying normal breast development will ultimately contribute to breast cancer prevention. The results from this study indicate that the experience of chronic, moderate early-life stress in the form of prolonged maternal separation causes molecular changes in the mammary glands of adult mice, which are related to a higher incidence of carcinogen-induced mammary tumours. Specifically, alterations in the expression or activity of GR, MR, CARM1, SRC3, and ND2 as a result of early-life stress could be a later consequence of other neoplastic changes in the mammary gland. Therefore, this study contributes to our understanding of the mechanisms by which early-life environments influence the development of the mammary gland and subsequent carcinogenic risk.
Chapter 5: General Discussion

Stressful early-life environments can influence the susceptibility to breast cancer risk by altering behavioural, emotional, hormonal, and physiological development. The studies presented here have demonstrated the influence that the neonatal environment has on the development of physiological and behavioural reactivity to stressors at puberty and young adulthood, as well as on gene and protein expression profiles related to mammary gland development and carcinogenic risk in BALB/c mice. Specifically, the length of maternal separation, sex, and age at testing interacted to differentially influence both behavioural and physiological coping responses elicited by exposure to an acute forced swim test, with adult female mice showing greater reactivity to stressors than males, regardless of the length of separation. Moreover, it was discovered that adult female LMS mice have significantly lower basal circulating CORT levels, but exhibit moderately higher levels of immobility during the forced swim test, than female BMS mice. The experience of prolonged maternal separation was also found to decrease GR and CARM1 expression in the mammary gland, highlighting potential mechanisms underlying the heightened risk of mammary tumourigenesis for LMS mice.

The data presented in Chapter 3 indicate that females exposed to long bouts of neonatal maternal separation had significantly lower levels of active floating and moderately greater immobility in adulthood compared to LMS males. LMS females also had higher stressor-induced increases in CORT levels compared to their male LMS counterparts. Although these differential effects of neonatal experiences were not apparent at puberty, these findings suggest that the effects of neonatal experiences are triggered by the developmental changes that females undergo during puberty. These
changes lead to an increase in stressor reactivity for females, and subsequently, a higher risk of depression in adulthood relative to males. Taken as a whole, these results parallel those of human studies of the etiology of depression and suggest that this animal model may be useful in helping to better elucidate the mechanisms involved in the environmentally mediated, sex-dependent risk of depression.

The results from Chapter 3 can also be related to tumour production and growth. Interestingly, young adult female LMS mice were found to have lower basal circulating CORT levels than female BMS mice. This may suggest that LMS females have successfully adapted to their earlier-life environment because lower CORT levels are often indicative of high relative fitness (Bonier, Martin, Moore, & Wingfield, 2009). However, the finding that young adult female LMS mice exhibit higher emotional reactivity to stressors, such as increases in the passive coping behaviour immobility during the forced swim test, than female BMS mice, may provide insight into a biological pathway explaining the greater risk of breast cancer for LMS females. In humans, higher emotional stressor reactivity (i.e., greater increases in anxiety following laboratory stressor exposure) of postmenopausal women has been associated with higher levels of the androgen dehydroepiandrosterone (DHEA) (Fang et al., 2014). DHEA has been shown to directly activate ERα expression in breast cancer cells and higher levels have been related to increased risk of breast cancer (Tworoger et al., 2006; Feng et al., 2014; Maggiolini et al., 1999). Thus, future research assessing DHEA levels in female mice exposed to the neonatal conditions may shed light on an additional pathway by which early-life stress exposure is associated with breast cancer risk.
The data presented in Chapter 4 indicated that GR protein expression was significantly decreased in mammary glands of mice that experienced prolonged maternal separation relative to mice that experienced brief maternal separation. However, it was also shown that LMS mice have a significantly higher GR transcript expression compared to BMS mice. Despite not reaching statistical significance, MR protein and transcript expression followed the same pattern as GR. This pattern was as expected as, similar to GR, MR has a high affinity for glucocorticoids (Kolber, Wieczorek, & Muglia, 2008). Moreover, MR may even take on the role of GR during certain stages of mammary gland development when GR is absent (Kingsley-Kallesen et al., 2002). Although no significant differences were observed across neonatal conditions in CARM1, SRC3, and ND2 transcript expression, an interesting finding was with respect to CARM1 protein expression. The experience of maternal separation significant reduced CARM1 protein levels compared with mice that did not experience maternal separation, and this finding was irrespective of the length of separation.

The discovery that young adult female LMS mice had significantly lower GR protein expression than BMS mice has important implications for carcinogenesis. GR plays a role in cancer development in the presence of chronic stress through mechanisms such as the inhibition of cell apoptosis and exertion of anti-proliferative effects (Baschant & Tuckermann, 2010; Zhu et al., 1998; Sutherland et al., 1998; Zhou et al., 1989; Kishnan et al., 2001). The lower levels of GR in the mammary glands of LMS mice is likely associated with the increased incidence of carcinogen-induced mammary tumours of LMS mice (Boyd et al., 2010), due to reduced anti-proliferative activity. Therefore, lower GR levels, coupled with higher ERα levels (and thus, higher rates of proliferation)
(Boyd et al., 2010), may point to mechanisms through which the experience of chronic moderate early-life stress in the form of prolonged maternal separation contributes to a higher risk of breast cancer (Boyd et al., 2010). Although the present studies have demonstrated that GR transcript and protein expression is differentially influenced by neonatal manipulations, further investigation of the role of post-translational modifications surrounding GR will help elucidate the role of early life stress in influencing cancer risk.

Another interesting candidate for study in the context of the current research paradigm is growth hormone (GH). It is well-established that prolonged maternal separation suppresses GH secretion, whereas brief maternal separation results in an increase of GH (Jurcovicova & Dobrakovova, 1998; Kuhn, Pauk, & Schanberg, 1990). The suppression of GH secretion is believed to be a result of a reduction in growth-releasing factor through noradrenergic neurons (Katz, Nathan, Kuhn, & Schanberg, 1996). Interestingly, earlier studies using mutant rodents with reduced secretion of GH have demonstrated incomplete mammary ductal growth similar to that of mice with the estrogen receptor knocked out (Bocchinfuso & Korach, 1997). It was further discovered that GH is involved in the differentiation of an immature ductal tree into more mature terminal end buds and alveolar structures; a process also requiring the production of estradiol (Walden, Ruan, Feldman, & Kleinberg, 1998; Kleinberg, 1997). Moreover, GH acts on the mammary epithelial cells to induce cell proliferation and development through stromal growth hormone receptor activation (Kleinberg, Feldman, & Ruan, 2000; Divisova et al., 2006). In addition to having a role in normal mammary gland development, research has also implicated GH in breast cancer development and
progression (Friend, 2001). Specifically, it has been shown that rodents lacking functional GH are resistant to carcinogen-induced mammary tumours, whereas GH treatment restores susceptibility to carcinogenesis (Swanson & Unterman, 2002; Thordarson et al., 2004). Although the role of GH in mammary gland development is clear, it is yet to be determined if the suppressing effects of maternal separation on GH are long-lasting, and whether the effect of chronic early-life stress is prevalent in circulating GH levels and GH receptor expression in adulthood. Thus, this future avenue of research would provide insight into a potential therapeutic target.

Taken as a whole, the findings from these studies suggest that the neonatal experience influences circulating hormone levels, subsequently altering the development of the mammary gland, and resulting in changes in the expression of genes and/or proteins involved in tumourigenesis. By highlighting differences in the transcript and protein expression in the mammary gland at adulthood between mice exposed to mild and moderate neonatal stressors, awareness has been brought to the mechanisms of action that explain how early life stress influences an individual’s risk of breast cancer. Thus, the maternal separation model has proved to be highly useful in studying the mechanisms influencing physiological development as related to breast cancer development. Even though the steroid hormone receptor and coactivator profiles have been studied in the normal mammary glands of BMS and LMS mice (Chapter 4), their profiles within the malignant mammary gland have yet to be elucidated. However, by understanding the influence of chronic early life stress on the development of behavioural and physiological stressor reactivity, and molecular profiles in the normal breast, awareness is brought to
the impact of stress with respect to breast development, cancer risk, and consequently, being able to identify the behavioural and physiological attributes of at risk individuals.
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