MADRES SANAS, BEBES SANOS:
THE INTERGENERATIONAL EFFECTS OF MATERNAL STRESS IN THE GALAPAGOS ISLANDS

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ABSTRACT

Johanna R. Jahnke: *Madres Sanas, Bebés Sanos*: The Intergenerational Effects of Maternal Stress in the Galápagos Islands
(Under the direction of Amanda Thompson)

This research utilizes a longitudinal, mixed-methods design to analyze rich narrative interviews alongside psychosocial and physiological measures of stress to examine shifts in infant development over the course of the peripartum period. This work incorporates the understudied roles of the postpartum period, epigenetic regulation in the placenta, and the gut microbiome into existing models for infant HPA axis development that have continuously reported inconsistent findings. Since the infant HPA axis has consistently been associated with metabolic and neurobehavioral disorders in later life, disentangling the mechanisms that underpin early HPA axis dysregulation is essential. While other research has examined isolated mechanisms linking maternal stress to infant HPA axis dysregulation largely in wealthy, biomedical settings, this project investigates how various biological pathways work in tandem to shape HPA axis development in the Galápagos, a middle-income, ecological setting.

First, we find that maternal social support is a marker of distress in women in the Galápagos and that the postpartum period can attenuate prenatal insults to infant HPA axis development, thus providing support for a continuum of early development and emphasizing the importance of early life as a developmental niche. Second, we find that physiological stress during pregnancy, measured through maternal HPA axis dysregulation, is associated with lower placental HSD11B2 expression, which is associated with an exaggerated cortisol reactivity in infants. Further, maternal psychosocial distress during pregnancy was marginally associated with

more placental *HSD11B2* methylation and significantly associated with less HSD11B2 expression for the mothers of girls, but not boys. Evolutionarily, these results fit into a disrupted adaptive framework, in which the ability to upregulate expression in response to stress diminishes as maternal stress becomes chronic. Last, we find that maternal precarity and HPA axis dysregulation were associated with an increase in pathogenic bacteria in the infant microbiome, including *Enterobacteriaceae, Streptococcaceae,* and *Veillonellaceae*, and a decrease in protective bacteria, including *Bifidobacteriaceae* and *Lachnospiraceae*, as well as a decrease in overall microbiota diversity. Together, these findings contribute novel insights into early human development trajectories and reinforce the importance of using multidimensional measures of "stress" to investigate early environments.

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ACE	Adverse childhood experience
ACTH	Adrenocorticotropic hormone
BMI	Body mass index
C-section	Caesarean-section
CAR	Cortisol awakening response
CES-D	Center for Epidemiologic Studies Depression Scale
cm	Centimeters
CNS	Central nervous system
CpG	Cytosine-phosphate-guanine
CpG loci/site	Specific locations in the genome at which methylation of a cytosine is altered
CRH	Corticotropin-releasing hormone
CT	Cycle Threshold
DNA	Deoxyribonucleic acid; the carrier of the genetic code
DOHaD	Developmental origins of health and disease
E. coli	*Escherichia coli*
ELISA	Enzyme-linked immunosorbent assay
EPDS	Edinburgh Postnatal Depression Scale
g	grams
GSC	Galápagos Science Center
HOJ	Hospital Oskar Jandl
HOME	Infant/Toddler Home Observation for Measurement of Environment

HPA axis	Hypothalamic Pituitary Adrenal Axis
HSD11B2	11β-hydroxysteroid dehydrogenase type 2
IPV	Intimate partner violence
IUGR	Intrauterine growth restriction
kg	kilograms
MDD	Major depressive disorder
mg	Milligrams
mL	Milliliter
MPAS	Maternal Postnatal Attachment Scale
mRNA	messenger RNA; also known as transcript or gene expression
MSPSS	Multidimensional Scale of Perceived Social Support
n	Total number of observations
OTU	Operational taxonomic unit
PAR	Predictive adaptive response
PCoA	Principal Coordinate Analysis
PCR	Polymerase chain reaction
PHQ-8	Patient Health Questionnaire 8
PSS	Perceived Stress Scale
PSS-Fa	Perceived Social Support-Family
PSS-Fr	Perceived Social Support-Friends
RNA	Ribonucleic acid; the copy of DNA that is used to create proteins
SD	Standard deviation
SES	Socio-economic status

SSS	MacArthur Scale of Subjective Social Status: Community Standing
STAI	State-Trait Anxiety Inventory
STI	Sexually transmitted infection
UNC	University of North Carolina
UNESCO	United Nations Educational, Scientific and Cultural Organization
USFQ	Universidad San Francisco de Quito
UTI	Urinary tract infection
VBAC	Vaginal birth after delivery
WHO	World Health Organization

CHAPTER 1: INTRODUCTION

1.1 Study Summary

Globally, chronic diseases cause more deaths than all other causes of mortality combined. Low and middle-income countries disproportionately account for these deaths, bearing three quarters of the world's burden (WHO 2015). Research on the developmental origins of health and disease (DOHaD) has shown that early life environments, and particularly exposures to stress, shape long-term risk for a variety of chronic and metabolic diseases, including obesity, cardiovascular disease, hypertension, and diabetes (Barker, 2004; Barker, Osmond, Winter, Margetts, & Simmonds, 1989; Wells, 2010) as well as neurobehavioral disorders including mood and anxiety disorders, depression (Cryan & Dinan, 2012), attention deficit/hyperactivity (Talge, Neal, & Glover, 2007), and post-traumatic stress disorder (Yehuda et al., 2005) in offspring even when controlling for adverse birth outcomes.

The mechanisms by which prenatal maternal psychosocial stress is embodied in maternal and infant biology are not yet fully understood. Many animal models have linked prenatal stress exposure to HPA axis programming, but research on this relationship in humans has not consistently identified a mechanism for the relationship (O'Connor, Bergman, Sarkar, & Glover, 2012). The majority of the work in this field has focused on increased levels of maternal cortisol as the primary mechanism, but research has not fully explored other potential pathways, including the role of placental 11β-hydroxysteroid dehydrogenase type 2 (HSD11B2), an enzyme that metabolizes cortisol into inactive cortisone (B. E. P. Murphy, Clark, Donald, Pinsky, & Vedady, 1974); the role of the gut microbiome, which has been shown to communicate bi-

directionally with the central nervous system (CNS), and the HPA axis in particular (Cryan & Dinan, 2012; Rackers, Thomas, Williamson, Posey, & Kimmel, 2018); and the role of the postnatal period more broadly. Since HSD11B2 may buffer the amount of cortisol that reaches the developing fetus, it is hypothesized to have protective effects for fetal development (Edwards, Benediktsson, Lindsay, & Seckl, 1993). Nonetheless, little remains known about this enzyme and what influences its functioning and expression.

Further, unfavorable shifts in the infant gut microbiome, termed dysbiosis, have been associated with the same long-term disease risks as HPA axis dysregulation, namely increased risk for metabolic (Goulet, 2015) and neurobehavioral disorders (Cryan & Dinan, 2012), suggesting that the gut microbiome could be a candidate for involvement in this pathway. Analyzing how these mechanisms work together is critical for understanding the early development of an individual's HPA axis and thus their subsequent risk for metabolic disease and neurobehavioral disorders later in life.

This project aims to examine the mechanisms through which peripartum maternal stress shapes infant HPA axis development over the continuum of development from the third trimester of pregnancy through early infancy. The project employs semi-structured interviews as well as surveys on various measures of stress, depression, social experience, and economic status to assess maternal psychosocial precarity as well as biological and anthropometric measures to assess health and the embodiment of physiological stress for mother-infant dyads over the course of the peripartum period. In particular, this research incorporates the under-studied roles of the postpartum period, the placenta, and the gut microbiome into this pathway. While other research has examined isolated mechanisms linking maternal stress to infant HPA axis dysregulation largely in wealthy, biomedical settings, this project investigates how various biological pathways

work in tandem to shape HPA axis development in a middle-income, ecological setting. The goals of this study are:

Objective 1: To identify which factors contribute to psychosocial stress in peripartum women in the Galápagos and to assess how these exposures both during pregnancy and in the postpartum shape maternal and infant HPA axis regulation

Objective 2: To assess both the psychosocial and physiological relationships between maternal distress during pregnancy and the placental enzyme, HSD11B2, as well as the relationship between HSD11B2 and infant HPA axis development.

Objective 3: To analyze the relationships among maternal stress and HPA axis dysregulation during the peripartum period, infant gut microbiome composition, and infant HPA axis functioning

The Galápagos is an ideal site for this project due to the unique suite of stressors that life on the islands poses for residents and its simultaneous rapidly increasing rate of chronic disease, particularly obesity (Page, Bentley, & Waldrop, 2013; Waldrop, Page, & Bentley, 2016). Further, while Ecuador has recently been classified as a middle-income country, steep socioeconomic inequalities nonetheless persist in the Galápagos. Literature on DOHaD arose from a desire to understand how health disparities emerge in response to societal circumstances, but almost all studies on prenatal psychosocial stress have been conducted in high-income countries (Beijers et al. 2014). By following women over the course of approximately three

months and utilizing the home as the primary site of investigation, the current study was able to build trust with participants and better assess stress within the context that it is experienced. The peripartum period offers a particularly important opportunity to understand this process, since stress during this time has intergenerational effects on health, perpetuating existing disparities (Thayer and Kuzawa 2014; Wells 2010).

1.2 Article 1: Social support over the peripartum period shapes HPA axis development

Maternal distress during pregnancy has been shown to have long-term effects on infant hypothalamic-pituitary-adrenal axis (HPA axis) functioning, but hypotheses about underlying physiology and the role of the postpartum environment are poorly specified. We employ a biocultural approach to first qualitatively identify low social support as a central and culturally salient measure of distress for women in the Galápagos Islands, and then use it as an exposure to quantitatively test the effects of three models for infant HPA axis development. We test three propositions: 1) a direct effect of maternal social support (separately for pregnancy and the postpartum) on infant HPA axis regulation, 2) an additional indirect effect of social support on HPA axis regulation through maternal HPA axis regulation (separately for pregnancy and the postpartum), and 3) an indirect effect of social support during pregnancy on HPA axis regulation through postpartum support.

Data were collected on San Cristóbal island, Galápagos, in 2018 from 38 mother-infant dyads. We confirm our first hypothesis, that during pregnancy and the postpartum, low maternal social support is associated with infant HPA axis dysregulation. Our results do not support our second hypothesis, since during pregnancy maternal HPA axis functioning was not associated with infant HPA axis functioning, and in the postpartum maternal social support was not

associated with maternal HPA axis functioning. We confirm our third hypothesis, that postpartum support has an indirect effect on the relationship between prenatal support and infant HPA axis functioning, suggesting that postpartum experience can attenuate prenatal insults to infant development. By incorporating the culturally-salient role of social support during pregnancy and the postpartum into a model for infant HPA axis development, this study adds a critical component to the literature on the developmental origins of health and disease that will elucidate the pathways through which early environments shape development.

1.3 Article 2: Maternal distress influences placental 11β-hydroxysteroid dehydrogenase type 2: Psychosocial and physiological pathways

Background and objectives: Prenatal stress is known to influence fetal hypothalamic-pituitary-adrenal axis (HPA axis) development. Placental 11β-hydroxysteroid dehydrogenase type 2 (*HSD11B2*) is a central gene in this pathway, but little is known about what influences its functioning and expression. We aim to assess how maternal distress influences HSD11B2 functioning, and how HSD11B2 in turn, is associated with infant HPA axis development. *Methodology:* Data come from 26 mother-infant dyads on the Galápagos Islands. Using adjusted linear regression models, we assess the effects of maternal psychosocial (stress and depression symptoms) and physiological (HPA axis dysregulation) distress on *HSD11B2* methylation and expression and then test how these *HSD11B2* measures influence infant HPA axis development. *Results:* We find that higher *HSD11B2* methylation is associated with lower HSD11B2 expression ($p \leq 0.01$), and that maternal HPA axis dysregulation during pregnancy is associated with lower placental HSD11B2 expression, which is associated with an exaggerated cortisol reactivity in infants. Further, sex-specific analyses revealed that maternal depression symptoms

are marginally associated with more placental *HSD11B2* methylation and significantly associated with less HSD11B2 expression for the mothers of girls, but not boys. *Conclusions and implications:* Our results support a disrupted adaptive framework, in which the adaptive ability to upregulate HSD11B2 expression in response to acute stress diminishes as maternal stress becomes chronic. In this model, chronic stress may exhaust the protective mechanism of HSD11B2, leaving the infant vulnerable to high levels of maternal cortisol, which could injure the fetal HPA axis and disrupt neurobehavioral and metabolic development in the long-term. By incorporating both psychosocial and physiological measures of maternal distress into our model, as well as the role of infant HPA axis development in response to placental changes, this study adds a critical component to the literature on the fetal programming that will help illustrate the biological underpinnings of early life adaptations.

1.4 Article 3: Maternal precarity and HPA axis functioning shape infant gut microbiota and HPA axis development in humans

Background: Early life exposure to adverse environments, and maternal stress in particular, has been shown to increase risk for metabolic diseases and neurobehavioral disorders later in life. While many studies have examined the hypothalamic-pituitary-adrenal axis (HPA axis) as the primary mechanism for these relationships, emerging research on the brain-gut axis suggests that the microbiome may be a key piece of this mechanism. We test the relationships among maternal precarity and HPA axis dysregulation during the peripartum period, infant gut microbiome composition, and infant HPA axis functioning. *Methods:* Data come from 25 mother-infant dyads in the Galápagos, Ecuador. Women completed surveys on precarity measures (food insecurity, low social support, depression, and stress) and gave salivary cortisol

samples during and after pregnancy. Infant salivary cortisol was collected at 3 days postpartum and 2 months postpartum, and infant stool was collected at 2 months postpartum. Differences in microbial diversity and relative abundance were tested using adjusted linear regression models. *Results:* Measures of maternal precarity and maternal and infant HPA axis functioning were all associated with differences microbiome composition. Maternal precarity was associated with lower diversity and higher relative abundance of *Enterobacteriaceae* and *Streptococcaceae* and a lower relative abundance of *Bifidobacteriaceae* and *Lachnospiraceae*. These patterns of colonization for *Enterobacteriaceae* and *Bifidobacteriaceae* mirrored those found in infants with HPA axis dysregulation. Maternal HPA axis dysregulation during pregnancy was associated with a lower relative abundance of *Bacteroidaceae*, while the opposite was found for maternal HPA axis dysregulation in the postpartum. Maternal HPA axis dysregulation during pregnancy was also associated with a greater relative abundance of *Veillonellaceae. Conclusions:* Overall, exposures to precarity and HPA axis dysregulation were associated with an increase in pathogenic bacteria, including *Enterobacteriaceae, Streptococcaceae, and Veillonellaceae*, and a decrease in protective bacteria, including *Bifidobacteriaceae* and *Lachnospiraceae*, as well as a decrease in diversity.

CHAPTER 2: THE GALAPAGOS

2.1 Geography and Population

The Galápagos Islands, located 1000 km off the coast of Ecuador's mainland, make up Ecuador's archipelago province of over 20 islands (Walsh & Mena, 2016). According to the most recent census from 2015, the four populated islands are now home to of 25,000 residents, with roughly 15,700 residents on the island of Santa Cruz, 7,100 on San Cristóbal, 2,300 on Isabela, and 100 on Floreana (INEC, 2015). While Santa Cruz is the most populated island, San Cristóbal holds the provincial seat. The Galápagos does not have an indigenous population, and its human population remained minimal before the mid-twentieth century. Since then, the creation of the Galápagos National Park in 1959 and the designation of the Galápagos as a United Nations Educational, Scientific and Cultural Organization (UNESCO) World Heritage Site in 1978 incited vast population growth, and tourism and migration brought with it economic and urban development between the 1960s and 1990s (Hoyman & McCall, 2012; Walsh & Mena, 2013). Since 1950, the population has grown quickly, from approximately 1,300 residents to today's approximately 25,000 (INEC, 2015). Figure 2.1 illustrates the population growth from 1950 to 2015. Recent census data reports that among island residents, 36% were born in the Galápagos, 63% immigrated from Ecuador's mainland, and 1% immigrated from other nations (INEC, 2015).

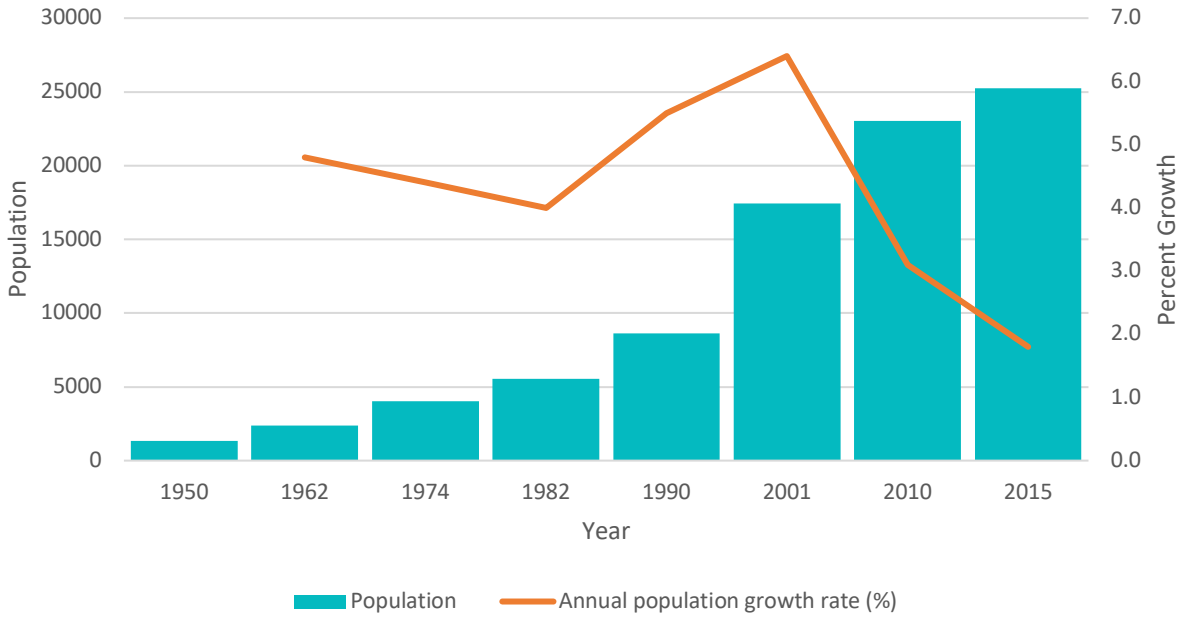
The Galápagos National Park, which covers ninety-seven percent of the geographic area of the Galápagos, attracts over 225,000 tourists every year, and despite efforts to control tourism and restrict immigration, the islands' population continues to grow (Walsh & Mena, 2016). In

response to an influx of migration to the Galápagos, the Special Law of the Galápagos, decreed in 1998, tightened regulations for immigration and labor markets by more strictly defining resident roles (Kerr, Cardenas, & Hendy, 2004). The law defined three groups of residents (permanent residents, temporary residents, and tourists) and regulated travel and work protocols for each in order to limit population growth, which the government feared could be detrimental to the Galápagos' fragile ecosystem. Through this decree, permanent residents were defined as those who had been residents of the islands for five years at any time before 1998 as well as people who married permanent residents and those who were born on the Galápagos (Kerr et al., 2004). Temporary residents were defined as Ecuadorian mainlanders or foreigners whose employers granted them temporary work on the islands, which is permitted when the employee has skills that cannot be found among the Galapaganean population. Temporary residencies can be renewed, and temporary residents may bring their spouses and children during their stay (Kerr et al., 2004). The third group, tourists and transients, are allowed short-term stays on the Galápagos for only up to 90 days within a one-year period.

The Special Law, which remains in place today, is meant to protect the Galápagos from rapid migration to the islands (particularly within the tourist industry) by providing preferential hiring to qualified permanent residents of the Galápagos. However, the law also provides generous government subsidies to workers on the Galápagos, so that wages on the islands must be at least 75% higher than the minimum wage for any particular job on the mainland (Kerr et al., 2004), drawing many Ecuadorian mainlanders to seek employment on the Galápagos, even if only temporarily (Waldrop, Sherwood, Ledford, Martinez, & Jahnke, n.d.). In the years since the law's passing, tensions have been growing between Galapaganeans and Ecuadorian mainlanders, who are ostensibly taking the jobs of Galapaganeans and whose presence changes the culture and

politics of the island. A major piece of this tension springs form the Galapaganeans' acute awareness that the best jobs on the islands require higher education and skilled training, which are out of reach for most islanders due to the islands' limited infrastructure and lack of higher education (Waldrop et al., n.d.).

Figure 2.1 Population and rates of growth according to the annual census, adapted from INEC Census (INEC, 2015)



2.2 The People of the Galápagos

Ethnicity. Ethnically, the Galápagos is largely homogenous, as 85% of the population identifies as Mestizo, 8% identify as Indigenous, 3% identify as Afro-Ecuadorian, 3% identify as White, and 1% identify as Montubio (INEC, 2015).

Education. Overall, education on the islands has been improving over the past few decades. The literacy rate rose from 97.1% in 2001 to 98.7% in 2015 (INEC, 2015). Nonetheless,

illiteracy persists at higher rates among women than men and, generationally, illiteracy is highest among residents over 65 years of age (8.9%) and lowest among individuals under 30 years of age (0.2%) (INEC, 2015). Despite differences in literacy by sex, in each census year, class attendance was higher among women than men, suggesting that literacy rates are better among younger generations of women. Overall, class attendance has also been improving over the past few decades, as class attendance for people 5 – 24 years old has risen from 64% in 2001, to 76% in 2010, to 78% in 2015 (INEC, 2015).

Housing. Since the 1990s, the Galápagos has undergone vast structural development, primarily in urban areas, doubling its number of households between 2001 and 2015. The census recorded the presence of roughly 5,700 households in 2001, 9,100 in 2010, and 12,000 in 2015 (INEC, 2015). Development is distributed approximately evenly relative to population size among Santa Cruz, San Cristóbal, and Isabela. Of the households surveyed in 2015, 44% were houses, 43% were apartments, 8% were rooms in a house, and 5% were other forms of housing (INEC, 2015). The average number of people living in each house was 3.2 people, while in apartments it was 2.8 people, and in rooms in a house it was 2.0 people (INEC, 2015). Houses on the Galápagos are most often constructed with concrete (97%), and most homes have tile floors (64%), while others have cement or dirt floors (INEC, 2015). Further, construction continues throughout the islands today, where many structures remain partially built as families wait to be able to afford completing projects and building is halted while permits are under review.

Employment. When people first settled on the islands, they lived in the highlands and worked in agriculture (Quiroga, 2014). After the 1950s, though, residents moved to the lowlands to pursue fishing, which became a lucrative career through the export of grouper in the 1960s, lobsters in the 1980s, and sea cucumbers in the 1990s (Quiroga, 2014). However, overfishing in

the 1990s and the rapid expansion of tourism in recent years has degraded the fisheries industry, and many residents have transitioned to other jobs, particularly within the tourism and conservation sectors, and the people of the Galápagos have embraced ecotourism as a solution to both environmental and economic concerns (Hoyman & McCall, 2012). Today, tourism is the backbone of the Galápagos' economy, and while the fishing industry has faced policy changes to meet environmental standards, the fishing industry continues to thrive as well (Hoyman & McCall, 2012).

2.3 Challenges in Context

Residents of the Galápagos face a unique suite of challenges in everyday life due to the islands' isolated geography and protection as a national park.

Food. For those who live on the islands, agricultural restrictions for the preservation of indigenous flora and fauna contribute to the changing social-ecological system by limiting local food production and increasing the residents' reliance on imported food from the mainland to sustain the needs of the growing population (Page et al., 2013). The majority of fruits and vegetables consumed on the islands are not grown on the islands, but instead must be imported by boat or plane. While the government is responsible for food provision on the Galápagos, private companies are hired to fill this need, and ships are often delayed for long periods, limiting food availability for weeks at a time (Jahnke, Thompson, & Archer, n.d.). When food does arrive, residents often report that fruits and vegetables have spoiled, and healthy options are limited. Consequently, traditional diets are being replaced by energy-dense foods, and particularly processed foods that cannot spoil (Jahnke, Thompson, et al., n.d.), which has contributed to the nutrition transition on the Galápagos (Page et al., 2013). In addition to

increased prices of food due to importation, the cost of food can be driven up quickly once on a small island with limited resources for re-stocking and ample tourists to feed. Consequently, residents worry about their ability to purchase healthy foods, and food security remains a major concern for residents.

Water. Water quality too, introduces health risks for residents, as fresh water scarcity places a burden on water and wastewater treatment on the islands (Walsh, McCleary, & Heumann, 2010). On San Cristóbal, where the present study was conducted, water quality testing has documented high levels of *Escherichia coli (E. coli)* in household tap water (Gerhard, Choi, Houck, & Stewart, 2017), and other studies have found common infectious morbidity from gastrointestinal, respiratory, and skin infections (Walsh et al., 2010). While San Cristóbal is the only island on the Galápagos whose piped water is sourced from fresh water, efforts to improve piped water quality are still ongoing. After a new drinking water treatment plant was implemented on the island, piped water quality improved through a decrease in both total coliforms and *E. coli*, but even after the implementation of the treatment plant, these pathogens have persisted in drinking water at some testing sites (Gerhard et al., 2017). Consequently, residents are hesitant to drink the piped water, and many opt to purchase large bottles of water for drinking, which is costly and can introduce a host of other water quality concerns, since residents frequently store this bulk water open in their homes, exposing it to environmental contamination.

Violence against women. Further, violence against women persists at high rates in the Galápagos and in Ecuador more broadly. In Ecuador, 61% of women report experiencing some kind of violence against women (INEC, 2011). In the national survey, 54% of women have reported experiencing psychological violence, 38% have experienced physical violence, and 26%

have experienced sexual violence (INEC, 2011). Of women who have experienced some type of violence, 76% report that the perpetrator was their current or ex-partner (INEC, 2011). On the Galápagos islands specifically, 55% of women have reported experiencing from some kind of violence, and 43% of women have reported being victims of violence in a relationship (INEC, 2011). According to a national survey, in the Galápagos, 50% of women reported experiencing psychological violence, 35% have experienced physical violence, and 23% have experienced sexual violence (INEC, 2011), demonstrating comparable rates to those elsewhere in Ecuador. Notably, violence against women is a sensitive and often fraught subject, and consequently, these data are likely under-reported.

Social isolation. The geographic isolation of the islands also leaves residents vulnerable to social isolation from family and friends on the mainland. Restrictive immigration laws prevent large-scale migration to the islands, and travelling to and from the mainland is costly for residents even with subsidized airfare provided by the government. Consequently, many residents who have married Galapaganeans born on the islands do not often have the chance to see their own families that remain on mainland Ecuador, limiting socials support, particularly for newcomers.

2.4 Health Care on the Galápagos

Over the past few decades, Ecuador has undergone a drastic restructuring of its health care system, transitioning from primarily private to increasingly public care (De Paepe, Tapia, Santacruz, & Unger, 2012; Rasch & Bywater, 2014). Since 2000, the Ministry of Health has increased spending on health care, and, in 2008, Ecuador's former President Rafael Correa adopted a new constitution that passed legislation making health care a right and guaranteeing

free care to all citizens (Aldulaimi & Mora, 2017; De Paepe et al., 2012). Despite this change, Ecuador's medical system has been and remains fragmented into a combination of public and private facilities, which are made up of both non-profit and for-profit institutions (López-Cevallos & Chi, 2010a). Nonetheless, public health care accounts for the vast majority of health facilities in Ecuador (Pan American Health Organization, 2008), and in many rural settings, public care is the only option (López-Cevallos & Chi, 2010a). Further, after the adoption of Ecuador's new constitution in 2008, former President Correa entered Ecuador into bilateral cooperation agreements on education, health, and social support with Cuba. Through these programs, Ecuador has invited over 1,000 Cuban doctors to work throughout Ecuador (Anderson, 2015), including in the Galápagos, where the doctors have been met with some resistance from the Galapaganean population who preferred Ecuadorian physicians (Jahnke, Archer, Thompson, Ocampo, & Bentley, n.d.). Recently though, in November 2019, Ecuador's current president, Lenín Moreno, who was elected in 2017, ended Ecuador's physician agreement with Cuba as a piece of his effort to pull away from his predecessor's commitment to "socialism of the 21st century" (Gámez Torres & Pentón, 2019). His actions both terminated existing contracts with Cuban physicians and halted further recruitment of Cuban physicians to Ecuador. Nonetheless, over the past decade, amid the major shifts in health care provision, Ecuadorians have reported being disappointed in public health care (De Paepe et al., 2012; Rasch & Bywater, 2014), which has influenced its utilization and ability to improve health among Ecuadorians (Adane, Mengistie, Mulat, Kloos, & Medhin, 2017).

In 2014, with funding from former President Correa's new legislation, Ecuador's Ministry of Health built a new hospital in Puerto Baquerizo Moreno, the capital city of the Galápagos, located on San Cristóbal Island. This free and public hospital, Hospital Oskar Jandl

(HOJ), replaced a smaller, older hospital on the island and is now the only hospital on San Cristóbal. Hospital Oskar Jandl contains both a hospital wing and a primary health care wing, which provides preventative services including vaccinations, dentistry, and counseling. The only other health care center on the island is a small public health clinic in the highlands of El Progreso for basic care, and the only other (much smaller) hospital on the Galápagos is on the island of Santa Cruz and is also run through Ecuador's Ministry of Health. Despite the Hospital Oskar Jandl's improved facilities and technologies, in 2016, health care providers at the hospital identified underutilization of health care services for both primary care and hospital services as a major concern. A qualitative analysis of health seeking behavior found that the community's perceptions that the hospital lacks specialists and is inefficient have deterred residents from seeking care at the hospital (Jahnke, Archer, et al., n.d.). Further, residents voiced concerns with provider trust and overall health care quality, motivating them to travel to the mainland for care, which poses financial burdens on individuals and potentially exacerbates health conditions during the waiting and travel time (Jahnke, Archer, et al., n.d.; Page et al., 2013). Despite the scarcity of specialists, island residents have been particularly impressed with the prenatal and labor and delivery care that they had received at Hospital Oskar Jandl (Jahnke, Archer, et al., n.d.), and travel to the mainland for delivery has decreased since the establishment of the new hospital.

Community members are not the only ones who are critical of the interaction between hospital staff and residents on the island. Healthcare providers at Hospital Oskar Jandl have reported feeling unwelcome by the community, citing their status as outsiders as the primary concern, since the majority of doctor and nursing staff at Hospital Oskar Jandl are from the mainland of Ecuador or other countries, including Cuba and Puerto Rico (Waldrop et al., n.d.).

The Special Law of the Galápagos prioritizes the recruitment of Galapaganean residents for staffing, but without applicants from qualified locals, positions open up to outsiders; however, non-resident staffing limits the terms of service, leading to high turnover at the hospital, which hinders residents' ability to form long-term relationships and develop trust with providers (Waldrop et al., n.d.). Further, the law requires that if someone from the community with the same qualifications later applies for the job, the non-resident staff must be asked to leave. Thus, many Galapaganeans view the presence of outsiders, and thus the hospital and its staff, as another means of political control imposed upon them from the continent. Much of their anger, though directed at hospital personnel, is rooted in past political injustices and current lack of control over many political and conservation-related efforts on the islands (Hoyman & McCall, 2012; Kerr et al., 2004).

The constant tension among the Ecuadorian government, the hospital staff, and the constituents of the Galápagos make health care provision fraught and complicated on the islands. Nonetheless, hospital personnel are hopeful that they can build trust with the community as more residents have good experiences with care.

2.5 Maternal and Child Health on the Galápagos

Overview

Alongside improvements to healthcare facilities in recent years, the Galápagos has seen improvements in health care utilization and health outcomes among women. In 2015, the vast majority of women on the Galápagos gave birth in a hospital (97.4%), and the rate of low birth weight on the islands (6.4%) is now comparable to that of the national average (6.8%). Despite this, the infant mortality rate on the islands (0.04%) remains higher than the national average

(0.013%) (Freire et al., 2015). On average, the fertility rate has been slowly declining over the past few decades from 2.3 children in 2001, to 2.2 in 2010 and 2.1 in 2015 (INEC, 2015). Nonetheless, the Galápagos still faces challenges to maternal and child health, specifically in regard to nutrition and obesity, infections, and overuse of Caesarean section, all of which can be detrimental to long-term health.

Nutrition and obesity

The Galápagos has the highest rate of overweight and obesity of all of Ecuador's provinces, with 12.7% of children under the age of five and 75.9% of adults between the ages of 10 and 60 qualifying as overweight or obese (Freire et al., 2018). Overweight and obesity put women at risk hypertension and gestational diabetes and obesity during pregnancy has been associated with for large for gestational age infants, who are more likely to be obese later in life (Pan et al., 2019).

The geographically isolated position of the Galápagos poses challenges not only for the availability and diversity of food, but also the cost of food, which can escalate quickly on a small island with limited resources. Food availability on the island is hindered by the designation of most of the islands as National Park (Walsh & Mena, 2016), which limits agriculture to a small area that cannot sustain the needs of the growing population. Consequently, the vast majority of fruits and vegetables are not grown on the islands, but instead imported from the mainland by boat or plane, adding to market costs of produce. Consequently, residents consume many ultra-processed and fried foods in the place of healthier, fresh foods (Freire et al., 2018). This substitution likely contributes to challenges in fulfilling micronutrient needs on Galápagos,

where 16.1 % of children under the age of 5 are anemic, while 6.4% are iron deficient, and 8.4% are Vitamin A deficient (Freire et al., 2014).

Infections

Both urinary tract infections and sexually transmitted infections are also of concern for island residents. The Galápagos has high rates of sexually transmitted diseases (STIs) and urinary tract infections (UTIs) (Jahnke, Thompson, et al., n.d.). Previous research has suggested that the high incidence of UTIs may be a consequence of bathing or showering in contaminated water (Houck et al., 2020; Walsh et al., 2010). These infections are of particular concern during pregnancy, since untreated UTIs during pregnancy have been associated with intrauterine growth restriction, low birthweight, and preterm delivery (R. Cohen, Gutvirtz, Wainstock, & Sheiner, 2019).

High rate of Caesarean delivery

Ecuador's Caesarean-section (C-Section) rate has been rising far beyond the 10-15% rate recommended by the World Health Organization (World Health Organization (WHO), 2015) over the past few decades (Jahnke, Houck, Bentley, & Thompson, 2019), and the C-section rate on the Galápagos is even higher than that on the mainland, at roughly 58% of all births (Thompson, Houck, & Jahnke, 2019). While C-sections can be necessary for the immediate health of a woman and her infant, unnecessary C-sections have a higher risk of morbidity and mortality for both the mother and the infant (Runmei et al., 2012; Villar et al., 2006), and less immediately, C-sections have been linked to a number of inflammatory conditions in childhood

and adolescence, including increased allergy and asthma and elevated risk of overweight and obesity (Blustein et al., 2013; Cho & Norman, 2013).

Infant growth

Children under the age of five on the Galápagos have a higher rate of overweight and obesity (12.7%) compared to children nationally in Ecuador (8.5%) (Freire et al., 2014). Galapagaean children also have a lower rate of underweight (1.2%) than those in Ecuador more generally (6.4%) (Freire et al., 2014). Nevertheless, children on the Galápagos have lower rates of stunting 10.6% and wasting (0%) than children in the national sample, whose rates of stunting are 25.2% and 3.9% respectively (Freire et al., 2014), suggesting that children on the Galápagos are more at risk for overnutrition than undernutrition. These differences may be due to the consumption of many high-calorie processed foods, but differences in underweight may be due in part to nutrient absorption as well, particularly since the rate of diarrhea in children on the Galápagos is marginally lower (10.2%) than that for Ecuadorian children in generally (11.8%) (Freire et al., 2015).

2.6 Summary

Overall, the Galápagos' geography, policies, and infrastructure have contributed to challenges for human health and well-being on the islands. While recent research has reported birth outcomes, growth measures, and other indicators of physical health, few studies have examined mental health on the islands, and provision of mental health care remains limited. Residents' everyday lives are complicated by food and water insecurity, poverty, limited connection to friends and family on the mainland, and access to high quality health care, among

other causes of distress. Together, these complex circumstances likely contribute to underdiagnosed stress, anxiety, and depression among island residents.

CHAPTER 3: STRESS AND THE BODY

3.1 Evolutionary Perspectives: The Developmental Origins of Health and Disease

The DOHaD hypothesis suggests that early life environments shape long-term disease risk. Robust evidence from epidemiological studies has shown associations between early life exposures to adverse environments and subsequent cardiovascular and metabolic disease, as well as a variety of neurobehavioral disorders (Barker et al., 1989; O'Connor, Heron, Golding, Beveridge, & Glover, 2002). In particular, prenatal maternal stress has been associated with negative birth outcomes in the forms of preterm birth, low birth weight, intrauterine growth restriction (IUGR), and impaired infant development (Copper et al., 1996; Dayan et al., 2002). At the same time, evidence from epidemiological studies suggests that these adverse birth outcomes are associated with a number of diseases in adulthood including metabolic disease, a suite of diseases including cardiovascular disease, hypertension, obesity, and diabetes (Barker, 2004; Barker et al., 1989); asthma (Mai et al., 2003); behavioral problems (Lagerstrom, Universio, Bremme, Eneroth, & Magnusson, 1990); and psychological dysfunctions (Bohnert & Breslau, 2008). Further, though many studies have found associations between low birth weight, preterm birth, and IUGR and disease in later life, these adverse birth outcomes constitute the extremes of fetal response to stressful contexts, while in fact, fetal environment shapes development across the range of normal birth weights, and modifications are not restricted to those born particularly small or early (Drake et al., 2012; Godfrey, Gluckman, & Hanson, 2010).

Within an evolutionary framework, some have employed a life history perspective to consider the drivers of these shifts in early development. Life history theory uses an adaptive

approach to investigate two fundamental evolutionary tradeoffs, that between investment in current or future reproduction, and that between the number of offspring and the fitness of offspring (Hill, 1993). Primarily focused on energetics and energy allocation (Hill 1993), life history theory recognizes that energy can be used for maintenance, growth, storage, and reproduction, and life history researchers investigate how humans navigate tradeoffs in energy investment in order to maximize reproductive effort (Hill, 1993). Further, reproduction is of particular interest within life history theory, since both during gestation and during the first few years of life, a child depends on its mother's energetic capacity. Thus, allocations of maternal energy must be made doubly—once within the mother for maintenance, growth and reproduction, and once within the child for maintenance, growth and reproduction. In this way, the perinatal period could provide insight into the energetic "decision-making" of mothers and how the interests and efforts of offspring may shape energy allocation.

Like life history theory, DOHaD has grown from the study of optimization of energy allocation for evolutionary success and can be considered to be a subset of developmental plasticity through which an individual modifies its phenotype in response to its environment throughout the life course (Gluckman, Hanson, & Beedle, 2007). Phenotypic plasticity, which extends from conception to after birth, enables individuals to modify their phenotypes in response to their environments in ways that are adaptive beyond their inherited genotypes (Gluckman, Cutfield, Hofman, & Hanson, 2005; Wells, 2010). The ability to retain some phenotypic plasticity during development has evolutionary benefits, allowing individuals flexibility for survival and therefore allowing genes to remain in surviving genomes through modification to existing (not just ancestral) environments. While authors agree that early life

plasticity is evolutionarily driven, many disagree about the specificities of how these early life modifications fit into evolutionary models.

Further, while much of the literature on DOHaD *in utero* has focused on the effects of energetic and nutritional stress, a growing body of literature has found that the effects of maternal psychosocial stress introduce additional burdens on energy expenditure and have long-term effects on the development of the fetal HPA axis (Nyberg et al., 2012; Pike, 2005; Wells, 2010). Prenatal psychosocial stress has been associated with dysregulated glucocorticoid function, which is known to underlie metabolic disorders (Reynolds et al., 2001), and with neurobehavioral disorders in offspring even when controlling for adverse birth outcomes including low birth weight and gestational age (Davis, Glynn, Waffarn, & Sandman, 2011; O'Connor et al., 2002; O'Donnell et al., 2013). Thus, both energetic and psychosocial stress can influence early life phenotypic modification. Physiologically, this makes sense, since it is the neuroendocrine system that shapes the physiologic mechanisms that affect changes to life history through resource allocation (Finch & Rose, 1995; Worthman & Kuzara, 2005).

Others suggest that the HPA axis of women experiencing stress during pregnancy may send hormonal cues of a stressful environment to the fetus, which can then modify its growth accordingly (Pike, 2005). Though these glucocorticoid fluctuations can shape fetal modifications throughout the range of normal development, some have suggested that in extreme cases of stress, small modifications may be insufficient, and hormonal cues may build, triggering a hormonal cascade that causes parturition, allowing the fetus early expulsion, or preterm birth (McLean M et al., 1995; Pike, 2005). Though preterm birth is an extreme response to poor environmental circumstance, it may be adaptive for both the mother and the infant, allowing the mother to limit her own costs in a stressful environment so that she can conserve resources for

future reproduction, and allowing a fetus to avoid direct competition for resources (Pike, 2005). This hypothesis mirrors life history theory, through which the mother and infant must carefully allocate and balance resources. Nonetheless, early parturition is not without trade-offs. Though potentially advantageous for survival in the short term, preterm birth, IUGR, and low birth weight leave the infant vulnerable to perinatal mortality and permanent changes in organ and metabolic functioning (Barker et al., 1989; Hales & Barker, 1992; Pike, 2005). Nonetheless, evolutionarily, this model could thrive through natural selection, since it confers an advantage in early life, and its detrimental ramifications often do not manifest until after an individual's primary reproductive years.

Others' hypotheses suggest that these developmental changes are driven by the prioritization of long-term adaptive advantages of early life modifications for offspring, suggesting that the development of the fetus is informed by the mother's external environment. Supporters of this model hypothesize through "predictive adaptive responses" (PARs), fetuses take cues from their environments and adjust their development, and thus adult phenotype, to be better suited for their own predicted future environment (Gluckman et al., 2005, 2007; Godfrey et al., 2010). In contrast, Wells (Wells, 2007, 2010) has expressed skepticism that adaptations to a mother's external environment during pregnancy, just a fleeting snapshot of her life history, would remain relevant over the life course. He presents another compelling hypothesis for the evolutionary advantage of poor fetal growth, proposing that the fetus takes cues not from its mother's external environment, but from its developmental "niche" within the maternal metabolism (Wells, 2003). He suggests that maternal metabolism may signal maternal phenotype not momentary environment, thereby giving clues about the mother's (and grandmother's) life experience. Wells argues that this type of cue conveys an entire life history, smoothing out short-

term perturbations in stress to reflect a reliable sense of postnatal experience that is not derailed by anomalies experienced during one pregnancy. While often this theory elaborates on maternal metabolism as a buffer, it was developed from work with animal models that demonstrates how maternal phenotype buffers offspring from exposure to psychological stressors, in turn buffering the offspring's HPA axis stress response (Hennessy, O'Leary, Hawke, & Wilson, 2002).

Kuzawa's hypothesis of intergenerational phenotypic inertia also prioritizes maternal physiology as a signal of the external environment over cues from the momentary external environment itself (Kuzawa, 2005). This hypothesis, which suggests that the fetus is able to use its mother's metabolism to discern and respond to long-term environmental quality, has been proposed as long-term phenotypic adaptation (Kuzawa, 2005). Kuzawa claims that this adaptation allows a fetus to adapt to environmental trends that may at once be too gradual to be detected through conventional developmental plasticity, which responds to cues from the maternal external environment, and also too rapid to be incorporated into the genome through natural selection. In this way, the fetus may "predict the future by seeing the past" (Kuzawa, 2005:13). These evolutionary hypotheses demonstrate the utility of phenotypic plasticity despite the fact that phenotypic modifications can lead to detrimental conditions later in life.

Despite debates on theoretical underpinnings, it is clear that early life modifications pose trade-offs over an individual's life course, prioritizing selective organ development for survival and evolutionary fitness over the development of other systems, which leaves the individual vulnerable to an array of pathologies, particularly those that develop in the post-reproductive years, like cardiovascular and metabolic disease (Godfrey et al., 2010; Hales & Barker, 1992). Further, many anthropologists agree that changes to biology early in life can be heritable, perpetuating disparities intergenerationally and leaving those with poor early environments

disproportionately at risk for pathology (Gluckman et al., 2007; Pike, 2005; Thayer & Kuzawa, 2011, 2014; Wells, 2010). Research on DOHaD has the potential to inform pathways of disease development, which could be used to mitigate future health disparities. By incorporating the role of the placenta, microbiome, and postnatal environment into research on DOHaD, this work provides a more comprehensive approach to understanding the mechanisms behind long-term development of disease. Early development constitutes a continuum of systemic alterations from the prenatal into the postnatal period. This study recognizes and emphasizes the importance of this often-divided period of development.

3.2 What is Stress?

Models of developmental plasticity often rely on "stress" as an exposure that shapes growth and development, but the definition of "stress" itself can be difficult to pinpoint, as it is constructed and utilized differently across studies. Nonetheless, Selye's original definition of stress as an acute threat to the homeostasis of an organism (Selye, 1936) remains a useful working definition. In this model, the threat may be physical, like energetic stress or an injury, or psychological, like the anticipation of a threat, both of which produce a cascade of physiological, emotional, and behavioral responses (Moloney, Desbonnet, Clarke, Dinan, & Cryan, 2014). Across disciplines, stress has been used to signify aspects of social and cultural environments, perception of anxiety or discomfort, as well as behavioral, social, or physical problems (McDade, 2002). To address "stress," this study, like many others in anthropology and across disciplines, builds on the conceptual foundations of the "General Adaptation Syndrome" (Selye, 1936), for which "stress" is a process built by four elements: stressors, responses, consequences, and moderators (McDade, 2002; Rudzik, Breakley, & Bribiescas, 2014)

In this framework, a *stressor* is the environmental (physical, social, or psychological) event or stimulus that alters normal functioning and creates an adaptive challenge and elicits a *stress response* from an individual (Chrousos, 2009; McDade, 2002). Throughout our lives, humans, like other organisms, must maintain a dynamic equilibrium, termed homeostasis, to survive. Stressors challenge homeostasis, and the stress response is the individual's attempt to restore homeostasis through behavioral or physiological adaptive responses (Chrousos, 2009; McEwen, 1998). Thus, stress occurs when homeostasis is threatened or is perceived to be threatened (Chrousos, 2009). In humans, basal homeostasis (also termed eustasis) and stress responses are controlled by the Central Nervous System (CNS) and peripheral organs and tissues and mediated largely through glucocorticoids like cortisol. In the face of a stressor, the body can produce either an appropriate response, returning the body to homeostasis, or it may produce an inappropriate response (inadequate or excessive), causing cacostasis (also termed allostasis), which can have *consequences* for an individual's health, particularly if the stressor is prolonged (Chrousos, 2009; McDade, 2002; McEwen, 1998). Such consequences may be physical, behavioral, or neuropsychiatric changes, including anxiety, depression, and cognitive dysfunction (Chrousos, 2009; McEwen, 1998). More specifically, chronic stress causes the continual or prolonged activation of the HPA axis, and thus the prolonged secretion of mediators, including corticotropin-releasing hormone (CRH), norepinephrine, and cortisol, which activate the fear system, causing a variety of conditions including anxiety, depression, and insomnia (Chrousos, 2009). The last component of the stress response, *moderators* are factors that could interfere with this pathway. Moderators may be biological, situational, or cultural factors that account for differences in vulnerability to stressors, stress responses, and their health consequences (McDade, 2002).

The stress response, which constitutes the immediate embodiment of the stressor, may be operationalized through mental processes, behavioral processes, and physiological processes (McDade, 2002). Mental processes are often operationalized through self-report surveys or symptom checklists, including self-rated perceived stress scores or symptoms of anxiety or depression. Behavioral processes could be operationalized through the analysis of many behaviors including of changes in diet, exercise, sleep patterns, smoking, or drinking. Last, physiological processes could be operationalized through measuring changes to nervous system activity, hormone activity, or cardiovascular function, all of which are shifted as the body attempts to re-establish homeostasis (Chrousos, 2009; McDade, 2002). While each of these processes provides insights into "stress," it is important to note that these mental, behavioral, and physiological processes are interconnected, making causal pathways difficult to articulate. For example, changes in sleep patterns could induce hormonal changes, and changes in self-perception of stress may alter someone's sleep patterns.

While the stress response can be operationalized in various ways, it is important to note that stress, and health more broadly, is situated within social, cultural, and political-economic contexts (Lock & Kaufert, 2001). Consequently, correctly interpreting reports of stress is dependent upon a shared understanding of "stress" cross-culturally (McDade, 2002).

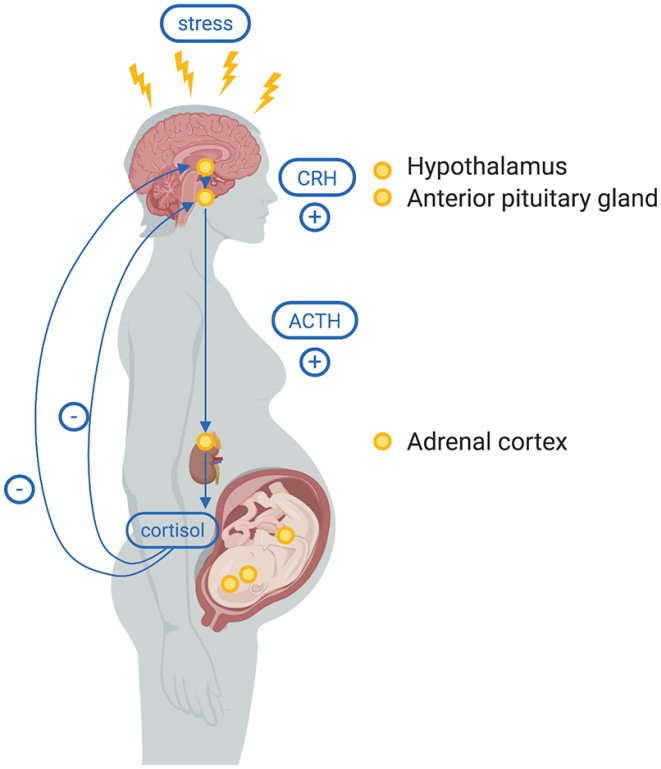
3.3 Intergenerational Stress Transfer and the Hypothalamic Pituitary Adrenal Axis

The stress system, which includes the Central Nervous System (CNS) and peripheral tissue, is responsible for maintaining basal homeostasis as well as managing the stress response in order to re-establish homeostasis in the face of a stressor (Chrousos, 2009; McEwen, 1998)(Chrousos 2009; McEwen 1998). As noted, chronic stress prolongs activation of the HPA

axis, thus prolonging the secretion of glucocorticoid mediators, including cortisol, among others (Chrousos, 2009). The exposure to stressors during critical periods of development, including the perinatal period and infancy, can induce prolonged cacostasis with lasting effects on the brain's stress response, establishing inappropriate basal activity or inappropriate responsiveness of the stress system that could last a lifetime (Chrousos, 2009). Inappropriate physiological management of stress, which may be established through insult during such critical periods can lead to an array of behavioral, physical, and neuropsychiatric conditions, including anxiety, depression, cognitive dysfunction, cardiovascular disease, and metabolic disease later in life (Chrousos, 2009).

In the body, the HPA axis plays a central role in regulating hormone systems, maintaining health, mobilizing energy stores, bolstering vigilance, and inhibiting inflammatory response during times of stress, both energetic and psychosocial (Talge et al., 2007). Biologically, stress stimulates the hypothalamus to release CRH, which binds to receptors in the anterior pituitary, releasing adrenocorticotropic hormone (ACTH). In turn, ACTH binds to receptors on the adrenal cortex, which stimulates the release of cortisol. Once a threshold of cortisol is reached, cortisol then downregulates the release of CRH from the hypothalamus, acting as a negative feedback loop (Figure 3.1). During pregnancy, the HPA axis takes on the additional role of regulating glucocorticoid feedback interactions among the mother, placenta, and fetus. As such, the activation of the HPA axis has been proposed to be the primary mechanism through which prenatal maternal stress shapes fetal development and subsequent long-term disease risk (Pike, 2005; Seckl, 2008).

Figure 3.1 The Hypothalamic Pituitary Adrenal Axis



While glucocorticoids play an essential role in the development and maturation of fetal tissue and metabolism (Drake et al., 2012), high levels of cortisol may alter fetal behavioral, immunological, and brain development, including areas of the brain that regulate the fetal HPA axis (Beijers, Buitelaar, & de Weerth, 2014). Theorists propose that frequent or prolonged maternal stressors activate the maternal HPA axis, increasing the production of cortisol, which could reach the infant through transfer across the placenta and/or trigger an increased production of placental CRH, thereby stimulating the fetal HPA axis to produce more fetal cortisol (Beijers et al., 2014). Increased fetal cortisol, in turn, has permanent effects on the fetal HPA axis, which could underlie subsequent long-term disease risk (Chrousos, 2009; Pike, 2005; Seckl, 2008). Table 3.1 provides a summary of many studies investigating maternal psychosocial stress and

maternal salivary cortisol in pregnant and postpartum women. These studies assess a variety of stressors, and provide mixed evidence as to whether chronic stress does indeed shape maternal salivary cortisol regulation.

Nonetheless, studies have found that maternal prenatal stress has significant effects on offspring HPA axis functioning as early as infancy (Brennan et al., 2008; Davis et al., 2011; Diego et al., 2004; Grant et al., 2009; Thayer & Kuzawa, 2014; Tollenaar, Beijers, Jansen, Riksen-Walraven, & De Weerth, 2011) into childhood (Gutteling, De Weerth, & Buitelaar, 2004, 2005; O'Connor et al., 2005), and through adolescence (Huizink et al., 2008; O'Donnell et al., 2013). Table 3.2 provides a summary of studies investigating prenatal stress and offspring HPA-axis functioning through salivary cortisol in humans. These studies suggest that prenatal psychosocial stress may lead to prolonged cacostasis through long-term HPA axis dysregulation in offspring, posing consequences on other systems that may persist throughout the life course. In fact, studies have found that early life changes in HPA axis regulation have long-term effects on growth, immune function, cognition, and cardiovascular and reproductive systems (Nepomnaschy, Vitzthum, & Flinn, 2009; Nyberg et al., 2012).

However, results from studies on the associations between prenatal psychosocial stress and maternal cortisol, as well as their effects on offspring, are inconsistent, suggesting that other mechanisms may play a role in the relationship between prenatal stress and infant HPA axis development. The present study explores the role of the HPA axis alongside potential placental, postnatal, and microbiome pathways to explain the varied outcomes in the literature.

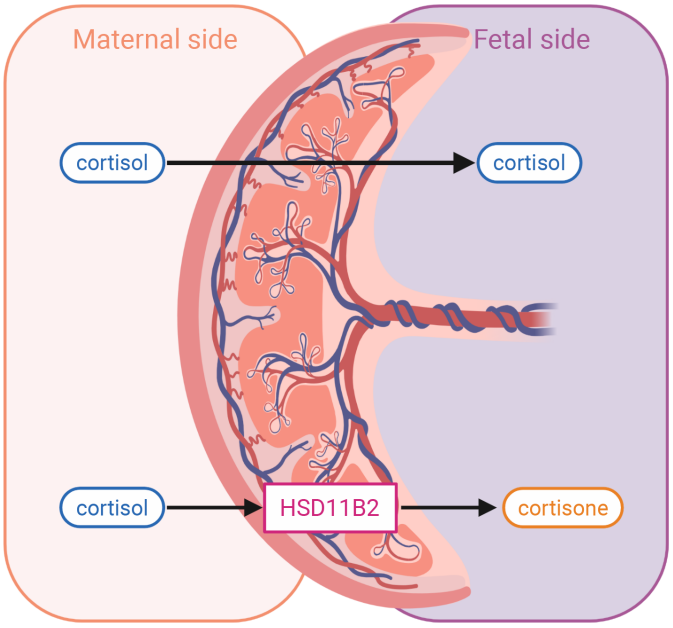
While a life history perspective suggests that up-regulation of cortisol may be adaptive in response to short-term challenges, chronic activation of the HPA axis strains energy demands, challenging energy allocation for the growth of other organs, and subsequently causing immune

deficiency, cognitive impairments, inhibited growth, damage to the hippocampus, and psychological maladjustment (Flinn & England, 1997; Nyberg et al., 2012). Work on fetal programming continues to investigate HPA axis programming as the primary mechanism (O'Donnell et al., 2013; Thayer & Kuzawa, 2014, 2015), but recently, other mechanisms have been proposed to underlie the relationship between maternal stress and adverse outcomes in offspring, including the roles of placental HSD11B2, catecholamines and the sympathoadrenal system, the maternal immune system, maternal microbiome microbiota, maternal behaviors, and maternal postnatal stress (Beijers et al., 2014).

3.4 Placental Regulation with HSD11B2

Contradictory results on the relationship between maternal cortisol and infant HPA axis development may be due to placental HSD11B2, an enzyme (protein) that buffers the amount of cortisol that reaches the fetus by catalyzing the reaction that metabolizes maternal cortisol into inert cortisone in the placenta (V. E. Murphy, Smith, Giles, & Clifton, 2006). This conversion is hypothesized to provide a protective effect to the fetus, (Edwards et al., 1993), and is illustrated in Figure 3.2.

Figure 3.2 Placental HSD11B2



The amount of HSD11B2 protein present in tissue is described as expression and is measured by messenger RNA (mRNA), which is regulated through epigenetic modifications. Epigenetic regulators respond to the environment in order to provide individual-level modifications by altering the way that deoxyribonucleic acid (DNA) is utilized in the body without changing the DNA sequence itself (Martin & Fry, 2018). Epigenetic regulators include histone modifications, noncoding RNA expression, and cytosine-phosphate-guanine (CpG) DNA methylation (Jablonka & Lamb, 2014, pp. 124–130), the last of which is of particular interest to this research. CpG DNA methylation is the process by which a methyl group attaches to a cytosine base in CpG islands, regions of the genome with a high density of cytosine and guanine bases (Martin & Fry, 2018). If the methylated CpG locus is part of a DNA sequence that codes for a protein, methylation of the region will not influence the amino acid sequence itself, but it will influence the likelihood that that the protein will be transcribed, and thus expressed

(Jablonka & Lamb, 2014, p. 126). Typically, genes in more densely methylated regions are less likely to be transcribed (Jablonka & Lamb, 2014, p. 126), which has been found to be the case for HSD11B2 (Alikhani-Koopaei, Fouladkou, Frey, & Frey, 2004; Marsit, Maccani, Padbury, & Lester, 2012). This research project investigates how maternal distress during pregnancy influences both the mRNA expression of placental HSD11B2 and the DNA methylation of its promoter region, thus assessing how epigenetic shifts in response to psychosocial exposures regulate the body's ability to produce HSD11B2 and thus protect the fetus from high maternal cortisol.

Expression of HSD11B2 has been shown to vary significantly among individuals (Welberg, Seckl, & Holmes, 2000) and to be lower in women who develop pre-eclampsia, have IUGR and low birth weight babies, and deliver preterm (O'Donnell, O'Connor, & Glover, 2009; Zhao et al., 2014). Low placental HSD11B2 would predispose the fetus to higher levels of maternal cortisol, but its relationship to maternal stress and infant HPA axis regulation has not been explored extensively in humans.

Table 3.3 provides a summary of the studies investigating prenatal maternal stress, measurements of HSD11B2, and infant HPA axis regulation, including both animal and human models.

In animal models, it appears that chronic stress and anxiety may diminish the protection of HSD11B2. One study found that acute stress up-regulated HSD11B2 activity, but chronic stress did not, and in fact, chronic stress hindered the ability of HSD11B2 to up-regulate in the face of an acute stressor (Welberg, Thrivikraman, & Plotsky, 2005). Another study found that prenatal stress increased the activity of HSD11B2 in low-anxiety rats, but not in high-anxiety rats (Lucassen et al., 2009). Other studies have found that higher prenatal stress is associated with an increase in methylation of the HSD11B2 gene promoter (Peña, Monk, & Champagne, 2012) and a decrease in the expression of HSD11B2 (Mairesse et al., 2007; Peña et al., 2012). These mechanisms suggest that chronic stress and anxiety decrease HSD11B2 expression and activity, and thus cannot protect against high cortisol transfer to the fetus.

Studies on these relationships in humans are limited and report mixed results. Like animal studies, many studies with humans have found that chronic maternal distress (including anxiety and/or depression) is associated with greater methylation of the HSD11B2 promoter region (Conradt, Lester, Appleton, Armstrong, & Marsit, 2013; Monk et al., 2016) as well as lower expression of HSD11B2 (O'Donnell et al., 2012; Seth, Lewis, Saffery, Lappas, & Galbally, 2015; Togher et al., 2014).

Further, other distress exposures have also been associated with these differences. In one such study, prenatal life events were associated with a downregulation in expression, though only in Caucasian women. In a few studies, though, the opposite effect is observed. One study found that women of low socioeconomic status (SES) had lower *HSD11B2* methylation

(Appleton et al., 2013). However, many studies report that anxiety (Capron, Ramchandani, & Glover, 2018) and depression (Capron et al., 2018; Conradt et al., 2013; Reynolds et al., 2015; Zhang et al., 2018) were not found to be associated with differences in HSD11B2 measures. Nonetheless, overall, the majority of results on this pathway suggest that maternal distress is associated with hypermethylation of the HSD11B2 CpG islands, causing transcriptional repression of the gene, ultimately downregulating HSD11B2 expression and leaving the infant more vulnerable to high levels of maternal cortisol, which could injure the fetal brain and HPA axis. Despite its central role as a gatekeeper for cortisol, the placenta remains an understudied component of this biological pathway. This research investigates the epigenetic regulation of HSD11B2 through both psychosocial and physiological stress pathways.

3.5 The Developmental Niche and Postnatal Environment

Though often left out of studies on prenatal maternal stress and offspring programming, the postnatal maternal environment plays a crucial role in the continued development of an infant's HPA axis. To account for individual differences in early development and health within the same community, Harkness and Super (Harkness & Super, 1994) proposed the theoretical framework of the developmental niche as a tool for analyzing the development of health at a more minute level—within the household. The developmental niche proposes that the household mediates the relationship between the environment and the child through three key components: (1) the child's physical and social setting, (2) customs of childcare, and (3) the psychology of caretakers. Using this model, an analysis of the infant's postnatal environment is essential for understanding early development.

The early postpartum period can be a time of particular maternal stress, which may be detrimental to a mother's mental health and caregiving behaviors (Grajeda & Perez-Escamilla, 2002; Rudzik et al., 2014). In turn, poor maternal mental health and patterns of care during early childrearing have each been found to be associated with HPA axis dysregulation in offspring later in life (Essex, Klein, Cho, & Kalin, 2002; Gunnar & Donzella, 2002; Tollenaar, Beijers, Jansen, Riksen-Walraven, & De Weerth, 2012; Wright, 2007). Postnatal maternal stress may also influence the infant directly through cortisol transfer during breastfeeding; Glynn and colleagues found that among breastfed infants, high maternal cortisol levels were associated with increased fearful temperament (Glynn et al., 2007). Improved postnatal stress may also work in the other direction, according to Bergman and colleagues, who found that the relationship between prenatal stress and infant health outcomes is moderated by mother-infant attachment, a marker of caregiving quality (Bergman, Sarkar, Glover, & O'Connor, 2010). These works suggest that postnatal environment is an important component of the mechanism through which the early HPA axis develops, and insufficient investigation of postnatal maternal stress may confound studies of prenatal stress and offspring development. By incorporating the role of postnatal stress into a study of prenatal stress and offspring HPA axis development, this research work adds a critical component to the literature on DOHaD that will elucidate the pathways through which early environment shapes development.

3.6 The Gut Microbiome

While HPA axis dysregulation is thought to be the primary mechanism through which prenatal maternal stress is linked to metabolic disease and neurobehavioral disorders later in life, dysbiosis of the infant gut microbiome has also been associated with risk for metabolic (Goulet,

2015) and neurobehavioral disorders (Cryan & Dinan, 2012), suggesting it could be a candidate for involvement in this pathway. Recent research has also found that microbiota communicate bidirectionally with the central nervous system (CNS) (Mayer, 2011), along the gut-brain axis, and thus gut microbiota may also influence and be influenced by brain function and behavior (Cryan & Dinan, 2012; Rackers et al., 2018). In other work, microbial composition has been linked to psychological disorders including anxiety, stress, autism, and depression (Dinan & Cryan, 2017). Recently, the gut microbiome has attracted great interest in the DOHaD literature, but its relationship to stress in pregnancy and infant health remains largely under-studied.

Gut microbiota, including bacteria, viruses, fungi, and other microorganisms, make up the diverse microbial communities that colonize the human gut (Rakers et al., 2017). Most microbial species develop a symbiotic relationship with their host that promotes healthy development, educates the immune system, supports the development of gut function, regulates intestinal barrier function, protects against infection, promotes food tolerance, and supports central nervous function and the neuroendocrine system including the HPA axis (Goulet, 2015; Rackers et al., 2018; Rakers et al., 2017). Generally, the healthy gut maintains a state of homeostasis, in which it balances microbial communities, epithelial tissue of the intestine, and the immune system (Matamoros, Gras-Leguen, Le Vacon, Potel, & De La Cochetiere, 2013). However, environmental disturbances, including changes in the immune system, diet, stress, and exposures to xenobiotics (antibiotics and anti-cancer medications), among others, can induce unfavorable changes in the composition of gut microbiota, termed dysbiosis (Matamoros et al., 2013), which has been associated with risk for obesity, metabolic disease, autoimmune disease and allergy, and intestinal inflammation (Cho & Norman, 2013; Goulet, 2015) in addition to psychological disorders.

3.7 Gut Microbiome Development

Though there is no consensus on an ideal healthy adult microbiome, the majority of microbiota in the adult gut is composed of two main phyla: Firmicutes and Bacteroidetes (Jandhyala et al., 2015; Tap et al., 2009). Other important phyla, including Actinobacteria, Proteobacteria, and Verrucomicrobia are not as well-represented, but nonetheless have important effects on health (Everard et al., 2011; Willing et al., 2010). In adults, the ratio of Firmicutes to Bacteroidetes remains a key measure of gut health and has been associated with a variety of metabolic disorders (DiBaise, Frank, & Mathur, 2012). Studies have also found that in adults, high microbial diversity has been associated with relatively more anti-inflammatory bacteria, while low microbial diversity has been associated with relatively more pro-inflammatory bacteria as well as higher adiposity and inflammation (Jandhyala et al., 2015).

The infant microbiome is distinct from that of healthy adults, and it is shaped by a variety of exposures, maturing over time until it eventually reaches an adult-like profile when a child is about three years old (Voreades, Kozil, & Weir, 2014). On the phylum level, the a newborn's gut microbiome predominantly consists of Actinobacteria and Proteobacteria (Dinan & Cryan, 2017). In the first few days of life, the microbiome transitions to the development of anaerobic bacteria such as *Bifidobacteria*, *Clostridia*, *Bacteroides*, and sometimes *Ruminococcus* (Matamoros et al., 2013). However, during early life, the infant gut microbiome is influenced by a variety of exposures including the prenatal environment, mode of delivery, antibiotic use, and infant feeding (Bäckhed et al., 2015; Goulet, 2015).

The long-held hypothesis that infants are born sterile (Mackie, Sghir, & Gaskins, 1999) has been challenged by recent research that demonstrates that the placenta (Aagaard et al., 2012)

and meconium (Jiménez et al., 2008) have microbial DNA, suggesting that the infant may encounter bacterial exposures before birth (Walker, Clemente, Peter, & Loos, 2017). The prenatal environment, including maternal body composition, diet, and other factors, have been associated with changes in the gut microbiome. Studies have found that both maternal body mass index (BMI) and consumption of a high-fat diet during pregnancy shape the neonatal gut microbiome and increase risk for obesity and metabolic diseases for the infant later in life (Friedman, 2018). Maternal obesity has also been associated with an increase in *Bifidobacterium* species as well as higher levels of *Staphylococcus* and *Enterobacteriaceae* including *E. coli* in the maternal gut microbiome, though more studies are needed to determine if these changes are mirrored in the infant microbiome (Calatayud, Koren, & Collado, 2019). Further, women's adverse childhood experiences have been associated with changes in their gut microbiome during pregnancy, such that women with more adverse experiences exhibit a differentially higher abundance of *Prevotella* than women with fewer adverse experiences in childhood (Hantsoo et al., 2019). Proponents of the hypothesis that infants are not born sterile suggest that maternal microbiota could be transferred to a developing fetus through the bloodstream and placenta (Borre et al., 2014), enabling shifts in a woman's microbiome to be passed to the fetus during pregnancy. While the question of newborn sterility remains open, perturbations in a woman's microbiota during pregnancy may, nonetheless, be transferred to the infant during birth.

Mode of delivery has been shown differentially shape the foundational gut microbiome of infants, which may alter energy harvesting and contribute to risk of obesity and overweight (Ajslev, Andersen, Gamborg, Sørensen, & Jess, 2011). Mode of delivery introduces bacteria to the infant gut through ingestion during birth (Guarner & Malagelada, 2003), such that infants born vaginally are exposed to and colonized by their mothers' vaginal and fecal bacteria, and

infants born by Caesarean are more often colonized by epithelial bacteria (Bäckhed et al., 2015; Dominguez-Bello et al., 2010). Infants born vaginally have been shown to have higher levels of *Bacteroides* species, which are associated with higher gut diversity and increased maturation (Stewart et al., 2018). Studies have also shown that infants born by Caesarean have elevated levels of *Clostridium difficile*, a species of gut microflora that has been associated with the development of asthma (Van Nimwegen et al., 2011). Notably, the microbiota of Caesarean-delivered newborns have a low abundance of Bifidobacteria (Biasucci et al., 2010), bacteria that has been associated with reduced risk for allergic disease (Björkstén, Sepp, Julge, Voor, & Mikelsaar, 2001; Kuitunen, Kukkonen, & Savilahti, 2012) and excessive weight gain (Dogra et al., 2015; Kalliomäki, Collado, Salminen, & Isolauri, 2008), and the infant's microbiome may not be colonized by Bifidobacteria until a few months after birth (Biasucci, Benenati, Morelli, Bessi, & Boehm, 2008).

Antibiotic treatment, too, can disturb intestinal microflora with effects lasting years (Jakobsson et al., 2010; Jernberg, Löfmark, Edlund, & Jansson, 2007). Antenatal antibiotic treatment has been shown to lower colonization of *Lactobacillus* and *Bifidobacteria* in the human neonate gut (Fouhy et al., 2012). Further, infants born by Caesarean and are more likely to be treated with antibiotics around the time of birth than infants delivered vaginally, and thus antibiotics may be the root cause of some associations found between Caesarean section and changes in the microbiome.

Infant feeding has been suggested to be the primary factor in the development of the infant gut microbiome (Stewart et al., 2018). Various components of breastmilk, including antibodies, cytokines, lactoferrin, lysosomes, and oligosaccharides are involved in shaping microbial communities (Bertelsen, Jensen, & Ringel-Kulka, 2016), and several studies have

found that there are distinct differences in infant gut microbiota based on feeding. Generally, breastfed infants have higher levels of *Bifidobacterium* species (Stewart et al., 2018) and have higher levels of probiotic taxa including *L. johnsonii* and *L. gasseri*, *L. paracasei* and *L. casei*, and *B. longum* (Bäckhed et al., 2015). Formula fed infants have higher levels of *Clostridium difficile*, *Granulicatella adiacens*, *Citrobacter spp*., *Enterobacter cloacae*, and *Bilophila wadsworthia* (Bäckhed et al., 2015). Further, the cessation of breastfeeding shifts the infant gut composition to a more adult-like state, with a higher abundance of the phylum Firmicutes (Stewart et al., 2018) as well as *Bacteroides, Bilophila, Roseburia, Clostridium*, and *Anaerostipes* (Bäckhed et al., 2015).

3.8 Stress and the Infant Microbiome

It has been known for some time that stress and consequent HPA axis activity can change the composition of gut microbiota throughout the life course and that stress early in life can pose long-term consequences on the composition of gut microbiota (Cryan & Dinan, 2012). Researchers hypothesize that stress activates the HPA axis and sympathetic system, increasing gut permeability and thus increasing the transfer of bacteria and bacterial antigens through the epithelial barrier (Cong, Henderson, Graf, McGrath, & Gregory, 2015). This could then activate a mucosal immune response that ultimately shifts the composition of the microbiome (Cong et al., 2015).

Research has shown that stress in the early postnatal period can alter the microbial composition of the infant gut (O'Mahony et al., 2009), and researchers are now considering that prenatal experience may also shape an individual's developing gut microbiome. Though this is a

new field of study, some research has shown that prenatal stress, the maternal gut microbiome, and the infant gut microbiome are all connected.

Studies have found that maternal stress during pregnancy influences maternal vaginal microbiota in animal models (Jašarević, Howard, Misic, Beiting, & Bale, 2017; Jašarević, Howerton, Howard, & Bale, 2015). In humans, maternal stress has been found to increase the risk of both bacterial (Culhane et al., 2001; Nansel et al., 2006) and fungal (Ehrström, Kornfeld, Thuresson, & Rylander, 2005) vaginosis, which may alter vaginal microbiota, and women with more adverse childhood experiences have been shown to have a differential abundance of some taxa (Hantsoo et al., 2019). Table 3.4 summarizes studies that have investigated the effects of stress on the maternal vaginal or gut microbiome. Since maternal microbiota are transferred to infant during parturition, stress-related perturbations in the maternal microbiome may be transferred to the infant, which could have long-term consequences on health (Rakers et al., 2017).

In fact, a few recent studies have found that maternal stress during pregnancy shapes the infant gut microbiome. Table 3.5 summarizes the studies that have examined perinatal stress and the development of the infant gut microbiome in animal and human models. In humans, one study found that high maternal stress, measured both by reported stress and by salivary cortisol, was strongly associated with infant microbiota composition at 16 weeks after birth (Zijlmans, Korpela, Riksen-Walraven, de Vos, & de Weerth, 2015). In this study, infants of stressed mothers had lower abundances of *Lactobacillus*, which exhibits anti-inflammatory properties and protects the body from pathogens (Martín et al., 2013), and *Bifidobacteria*, which has been associated with reduced risk for allergic disease (Björkstén et al., 2001), and higher abundances of Proteobacterial groups known to contain pathogens (Zijlmans, Korpela, et al., 2015). Overall,

the colonization pattern of infants of stressed mothers was associated with more reported infant gastrointestinal symptoms and allergic reactions (Zijlmans, Korpela, et al., 2015). Currently, this is the only study that has reported outcomes of prenatal maternal stress on infant gut microbiota in humans.

Though there are few studies that have examined this relationship in humans, similar results have been found in animal models (Walker et al., 2017), where offspring of monkeys stressed during pregnancy had significantly lower abundance of *Bifidobacteria* and *Lactobacillus* at 2 days post-birth (Bailey, Lubach, & Coe, 2004) and offspring of mice stressed during pregnancy had a significantly lower abundance of *Lactobacillus* (Jašarević et al., 2015). In the latter study, the abundance of *Lactobacillus* in the infant gut was positively associated with maternal vaginal *Lactobacillus*.

While evidence for this pathway is limited, together these data support the recent hypothesis that maternal stress during pregnancy shapes the composition of maternal vaginal microbiota, which is transferred to the infant. The evidence also suggests that infants born to stressed mothers may have a composition of microbiota with a lower abundance of protective bacteria and a higher abundance of harmful bacteria. This initial colonization may permanently alter the infant's neurodevelopment through changes to the synthesis of neuroinflammatory cytokines, neuromodulators, and neurotransmitters, leaving the individual more susceptible to neuropsychiatric disease later in life (Diaz Heijtz, 2016; Jašarević et al., 2015). Further, changes to an individual's foundational gut microbiome may increase risk of metabolic disease, autoimmune disease and allergy, and intestinal inflammation (Cho & Norman, 2013; Diaz Heijtz, 2016; Goulet, 2015).

3.9 Summary

In this biological model, the early development of the infant HPA axis could be influenced by stress and cortisol in mothers, placental HSD11B2 levels and the subsequent transfer of cortisol from mother to fetus, the postnatal environment, and microbiota. Studies addressing how these mechanisms function together are scarce. Further, many studies on maternal stress and offspring HPA axis development have relied on either maternal self-report and scales of stress or on physiological markers to measure maternal stress, but few have incorporated both measures (O'Connor et al., 2012). This research uses measures of both psychosocial and physiological stress to examine how these mechanisms work together to shape offspring HPA axis and gut microbiome development. Specifically, the project will analyze the role of perinatal maternal stress, the role of placental HSD11B2 expression, and the role of the infant gut microbiome to assess early infant development, which has long-term effects on health and well-being.

Table 3.1 Summary of studies investigating maternal psychosocial stress and maternal salivary cortisol in peripartum women

Study	Sample size	Psychosocial stress measure	Results
PRENATAL			
(van den Heuvel, van Assen, Glover, Claes, & Van den Bergh, 2018)	170	Anxiety (SCL-90) Depression	Significant relationship between high anxiety and low cortisol level at awakening
(Gilles et al., 2018)	405		Prenatal maternal stress was associated with altered diurnal cortisol pattern (flattened cortisol decline and higher evening cortisol) in moms
(Deligiannidis et al., 2016)	44	Anxiety (STAI-S) Depression (EPDS)	No significant relationship between mood disorder and cortisol
(Simon et al., 2016)	30	Stress	Higher levels of stress were associated with a smaller CAR
(Kane, Dunkel Schetter, Glynn, Hobel, & Sandman, 2014)	448	Anxiety	Higher mean levels of anxiety predicted steeper increases in cortisol after about 30 weeks of gestation.
(O'Connor et al., 2014)	101	Mood	There was a modest association between depression and elevated cortisol (measured by low morning level and diminished diurnal decline). Associations with anxiety and trauma were not significant.
(Field, Diego, Delgado, & Medina, 2013)	92	Depression Anxiety	Both interventions (yoga and social support) decreased depression and anxiety and cortisol immediately after intervention, but cortisol rose later
(Peer et al., 2013)	57	Mood, Stressful life events, Social support (MSPSS)	Women with high depressive symptoms had higher evening cortisol levels than those with low depressive symptoms. No significant difference for CAR.
(Voegtline et al., 2013)	112	Psychological measures	Women with more depressive symptoms between 30-32 weeks had higher cortisol levels than controls
(Giesbrecht, Campbell, Letourneau, Kooistra, & Kaplan, 2012)	83	Mood	Negative mood is associated with CAR
(Richter et al., 2012)	61	Subclinical depressive pathology (M-CIDI)	Intervention subjects had decreased CAR after 4-8 sessions of a manualized cognitive-behavioral group program for pregnant women with sub-clinically high stress, depression, and/or anxiety symptoms
(Tsubouchi et al., 2011)	69	Depression	Women with chronic stress had lower cortisol levels than controls in 2nd and 3rd trimesters, but not the 1st trimester

(Cheng & Pickler, 2010)	46	Stress Happiness Depression	No significant relationship between depression and cortisol
(Parcells, 2010)	59	Depression Anxiety Stress	No association between SCID diagnosis and cortisol, but cortisol significantly differed between women with BDII-II scores greater than 12 and less than 12
(Pluess, Bolten, Pirke, & Hellhammer, 2010)	66	Personality, Distress	High anxiety was associated with low baseline cortisol awakening levels in early pregnancy
(Harville, Savitz, Dole, Herring, & Thorp, 2009)	1587	Stress (PSS), Anxiety (STAI) Coping style Life events Social support (Social Support Survey) Pregnancy-specific anxiety	No significant relationship between psychological measures and cortisol at $r > 0.15$
(Taylor, Glover, Marks, & Kammerer, 2009)	51 total. 21 depressed, 30 non-depressed	Depression	Depressed women had a reduced morning CAR compared to controls, $R^2 = 0.34$
(Evans, Myers, & Monk, 2008)	182	Depression (CES-D, PES)	Comorbid depression and anxiety were associated with higher cortisol (at baseline, anticipation of task, and after task), but depressed and anxious cohorts did not differ from the control
(Kivlighan, DiPietro, Costigan, & Laudenslager, 2008)	98	Anxiety (STAI)	Higher trait anxiety was associated with a flatter afternoon decline for all mothers, and in primiparas, steeper morning declines were associated with lower infant birth weight
(Davis et al., 2007)	247	Depression	No significant relationship between depression and cortisol
(Nierop, Bratsikas, Zimmermann, & Ehlert, 2006)	57	Depressive symptoms	Greater increase in cortisol levels for group likely to develop depression
(Harris et al., 1996)	130	Depression	Depressed women had lower evening cortisol ($p < 0.05$)
(Harris et al., 1994)	130	Depression	No significant relationship between depression and cortisol
POSTPARTUM			
(De Rezende et al., 2016)	104	Depression (SCID, HDRS, EPDS)	Depressed women had significantly lower cortisol at awakening and half an hour and 3 hours after awakening.
(Iliadis et al., 2015)	365	Depressive symptoms (EPDS)	Women with an EPDS score greater than 10 had higher evening cortisol at 6 weeks postpartum

(Shimizu, Nishiumi, Okumura, & Watanabe, 2015)	65	Depression (EPDS) Health (GHQ)	No significant relationship between mood and cortisol
(Groer & Morgan, 2007)	200 (25 depressed, 175 non-depressed)	Depression	Depressed women had lower salivary cortisol levels than the control group
(Ehlert, Patalla, Kirschbaum, Piedmont, & Hellhammer, 1990)	70	Postpartum blues	Women with postpartum blues had higher cortisol levels in the mornings on days when they had symptoms
(Harris et al., 1989)	147	Depression	No significant relationship between mood disorders and cortisol
(Feksi, Harris, Walker, Riad-Fahmy, & Newcombe, 1984)	40	Maternity blues	No significant relationship between mood and cortisol

This table was adapted from the following publications: (Seth, Lewis, & Galbally, 2016; Szpunar & Parry, 2018)

Table 3.2 Summary of studies investigating prenatal stress and offspring HPA-axis functioning through salivary cortisol in humans, organized by infant age at assessment

Study	Sample size (dyads)	Prenatal stress measure	Maternal endocrine measure	Offspring age at assessment	Type of stressor if cortisol reactivity	Offspring outcome & Results
(Davis et al., 2011)	116	Maternal stress, anxiety and depression at 5 points across gestation (15, 19, 25, 31, 36+ weeks gestational age)	Prenatal plasma cortisol; Maternal anxiety, depression and stress scores not associated with maternal cortisol at any of the five prenatal assessments	24 hours	Heel-prick	Salivary cortisol;↑cortisol response in infants whose mothers have elevated cortisol concentrations in late second and third trimesters; no association between maternal stress, anxiety, or depression measures and infant cortisol response
(Stroud et al., 2016)	153	Prenatal depression, Pre-conception depression	None	1 month	NICU Network Neurobehavioral Scale (NNNS)	Daughters of mothers with prenatal MDD had 51% higher baseline cortisol and 64% higher cortisol stress reactivity than controls.
(Tollenaar et al., 2011)	173	General and pregnancy related feelings of stress and anxiety during last trimester	Salivary cortisol at 37 weeks; no association between stress and anxiety and cortisol	5 weeks, 8 weeks, 5 months, 12 months	Bathing session; vaccination; still-face procedure; maternal separation	Salivary cortisol; ↑cortisol in response to bathing in prenatal anxiety group; ↓ cortisol response to vaccination and maternal separation in prenatal anxiety group; maternal cortisol did not predict infant stress reactivity
(Thayer & Kuzawa, 2014)	55	SES	Salivary cortisol at 34-36 weeks gestation; ↑evening cortisol in women with greater material deprivation	6 weeks	Vaccination	Salivary cortisol; ↑cortisol response for infants of women with greater material deprivation
(Thayer & Kuzawa, 2015)	55	Discrimination/ prenatal stress at 34-36 weeks gestation	Salivary cortisol at 34-36 weeks gestation; ↑evening cortisol in	6 weeks	Vaccination	Salivary cortisol; ↑cortisol response for infants of women who report discrimination

			women who experience greater discrimination			
(Thomas, Letourneau, Campbell, & Giesbrecht, 2018)	243	Adverse Childhood Experiences (ACEs), Perceived Social Support (PSS)	Salivary cortisol at 6-22 weeks gestation and 27-37 weeks gestation	5-10 months	Laboratory stressor	Salivary cortisol; Prenatal maternal HPA axis function mediated the effects of maternal ACEs on infant HPA axis reactivity. Prenatal social support moderated the relationship between ACEs and maternal HPA axis regulation during pregnancy. Postnatal social support moderated the relationship between maternal HPA axis regulation and infant cortisol reactivity.
(Brennan et al., 2008)	189	Peri-partum depressive symptoms		6 months	Noise burst and arm restraint	Salivary cortisol; ↑cortisol response in maternal depression group
(Urizar & Muñoz, 2011)	86	Maternal mood screener (CES-D)	Salivary cortisol at 6 and 18 months postpartum	6 and 18 months	n/a	Intervention: Prenatal cognitive behavioral stress management (CBSM) and comparison group. Infants of women in the CBSM group had lower cortisol levels than infants in the control group
(Grant et al., 2009)	88	Maternal anxiety last 6 months of pregnancy		7 months	Still-face procedure	Salivary cortisol; ↑cortisol response in maternal anxiety group
(Levendosky et al., 2016)	182	Intimate partner violence (IPV)		1 year	Laboratory stressor	Prenatal, but not postnatal IPV was associated with infant cortisol reactivity (reactors vs. non-reactors)
(Yehuda et al., 2005)	38	PTSD symptoms resulting from 9/11 exposure in pregnancy	Salivary cortisol when baby is 1 year; ↓cortisol in mothers with PTSD	1 year	n/a	Salivary cortisol; ↓ at waking and bedtime in maternal PTSD group

(O'Connor et al., 2012)	125	Prospective longitudinal study. No psychosocial	Amniotic fluid at 17.2 weeks gestation (average)	17 months	Strange Separation: Separation-reunion stress	Salivary cortisol; ↑pre-stress cortisol and blunted response to stress exposure in infants who were exposed to higher levels of cortisol in utero
(De Bruijn, Van Bakel, Wijnen, Pop, & Van Baar, 2009)	103	Anxiety > 1 SD above mean at 12, 24, or 36 weeks gestation		3-3.5 years	Plastic barrier task	Salivary cortisol; higher baseline in prenatal stress group in girls only; no differences in reactivity
(Gutteling et al., 2004)	24	Daily hassles and fear of handicapped child at 16 weeks gestation		3-6 years	Vaccination	Salivary cortisol;↑cortisol response in maternal stress group
(Laurent et al., 2013)	192	Beck Depression Inventory for prenatal birth mother and postpartum adoptive mother and father		4.5 years	n/a	Salivary cortisol morning and evening; Prenatal maternal depression symptoms were associated with lower child cortisol (main effect), and adoptive parent postpartum depression symptoms provided interaction effects.
(Gutteling et al., 2005)	29	Fear of handicapped child at 16 weeks gestation		5 years	Response to first day of school	Salivary cortisol;↑cortisol level on school days and steeper diurnal slope on school day in maternal stress group
(O'Connor et al., 2005)	74	Anxiety at 18 and 32 weeks gestation		10-11 years	n/a	Salivary cortisol;↑CAR among group with mothers with high anxiety at 32 weeks gestation
(Van Den Bergh, Van Calster, Smits, Van Huffel, & Lagae, 2008)	58	Anxiety at three points in gestation		14-15 years	n/a	Salivary cortisol; flattened diurnal rhythm associated with ↑anxiety in early gestation
(Huizink et al., 2008)	556	Exposure to Chernobyl during pregnancy		14 years	n/a	Salivary cortisol;↑cortisol at mid-day only in children exposed during second trimester in pregnancy
(O'Donnell et al., 2013)	889	Anxiety and depression at 18 and 32 weeks gestation		15 years	n/a	Salivary cortisol; ↓CAR and flatter diurnal cortisol slope in maternal anxiety and

					depression group at both 18 and 32 weeks
(Entringer, Kumsta, Hellhammer, Wadhwa, & Wüst, 2009)	61	Negative life events in pregnancy	25 years	TSST	Salivary cortisol; Pre-TSST cortisol concentrations ↓ in prenatal stress group but cortisol response to TSST↑; no differences in diurnal cortisol

Table 3.3 Summary of studies investigating prenatal maternal stress, HSD11B2, and infant outcomes, animal and human models

Authors	Sample size	Stress measure	Biological stress measure	HSD11B2 Assay	Results
ANIMAL MODELS (RATS)					
(Peña et al., 2012)	12	Chronic restraint	None	DNA methylation, mRNA expression	Prenatal stress associated with decrease in placental HSD11B2 mRNA, and an increase DNA methylation at specific CpG sites within the HSD11B2 gene promoter
(Lucassen et al., 2009)		Exposure to an unfamiliar lactating resident & restraint	None	Activity	Prenatal stress significantly increased placental HSD11B2 activity in low anxiety-related behavior rats, but not high anxiety-related behavior rats (selectively bred)
(Mairesse et al., 2007)	20	Chronic restraint	None	mRNA expression, Activity	Prenatal stress was associated with reduced expression and activity of placental HSD11B2
(Welberg et al., 2005)	20	Restraint for chronic, anesthesia for acute	None	Activity	Acute stress up-regulates HSD11B2 activity by 160%, chronic stress did not alter HSD11B2 activity, but it diminished the capacity to up-regulate placental HSD11B2 by 90%. Suggests up-regulation in the face of an acute stressor but that chronic stress may diminish protection
HUMAN MODELS					
(Capron et al., 2018)	83	Anxiety (STAI), Depression (EPDS), Life events (LEQ)	None	mRNA expression	Prenatal anxiety and depression were not associated with changes in expression, but prenatal life events were associated with downregulation of HSD11B2, but only in Caucasians
(Zhang et al., 2018)	153	Depression (EPDS)	None	mRNA expression	Gene expression was not associated with prenatal or postnatal depression, but there was a significant interaction between depression and placental *HSD11B2* expression on infant negative affectivity
(Togher, Treacy, O'Keeffe, & Kenny, 2017)	121	Stress (PSS), Anxiety (STAI), Depression (EPDS)	None	mRNA expression	High maternal cumulative distress (composite of scores) was associated with lower expression of placental HSD11B2 mRNA
(Monk et al., 2016)	61	Mood, Stress (PSS)	Maternal salivary cortisol	DNA methylation	High PSS, but not cortisol, was associated more CpG methylation of HSD11B2
(Stroud et al., 2016)	153	Prenatal MDD, preconception MDD	Infant salivary cortisol	DNA methylation	HSD11B2 methylation moderated MDD and baseline infant cortisol. 1% methylation decreases were

			response at one month		associated with 9% increased baseline cortisol in infants of prenatal MDD moms.
(Reynolds et al., 2015)	56	Depression (CES-D)	None	mRNA expression	No association between depressive symptoms and HSD11B2 expression
(Seth et al., 2015)	33	Anxiety (STAI), Depression (EPDS)	None	mRNA expression	Results: negative correlations between HSD11B2 expression and both the EPDS and STAI
(Ghaemmaghami, Dainese, La Marca, Zimmermann, & Ehlert, 2014)	34	Acute stress	None	Activity	Amniocentesis (which is associated with increased anxiety) is associated with increased placental HSD11B2 activity
(Appleton et al., 2013)	444	Socioeconomic adversity	None	DNA methylation	Infants whose mothers have higher socioeconomic adversity have less methylation on the promoter region of the placental HSD11B2 gene, particularly for males
(Conradt et al., 2013)	482	Anxiety, Depression (medical records)	None	DNA methylation	Prenatal anxiety, but not depression, was associated with greater methylation of HSD11B2 CpG4
(O'Donnell et al., 2012)	56	Anxiety (STAI), Depression (EPDS)	None	mRNA expression	High prenatal Trait anxiety was negatively associated with placental HSD11B2 mRNA expression. No sex differentiation. High State anxiety were also significant. Results were weaker for depression (p=0.13). Preliminary analysis on subset suggests parallel for enzyme activity.
(Ponder et al., 2011)	164	Depression, Anxiety (self-report)	None	mRNA expression	Modest elevation in HSD11B2 mRNA expression in depression/anxiety group, but not significant

Table 3.4 Summary of studies on stress and maternal vaginal or gut microbiome

Authors	Sample size	Stress exposure	Biological stress measure	Results
ANIMAL MODELS				
(Gur et al., 2017)	15 mice	Chronic prenatal stress	IL-1β	Beta diversity of stool microbiome sample between stressed-exposed and non-stress-exposed differed significantly on PCoA plots.
(Jašarević et al., 2017)	30 breeding pairs of mice	Chronic prenatal stress	None	In maternal stool microbiome: Within the Bacteroidetes phylum: Stressed mice had more *Rikenellaceae* and *Odoribacter*, less *Bacteroides.* Stressed mice also had higher relative abundance of *Mucispirillum*. In maternal vaginal microbiome: Stressed mice had lower relative abundance of Firmicutes, Bacteroidetes, and *Lactobacillus* and a higher relative abundance of Proteobacteria (particularly the genus *Helicobacter*)
(Jašarević et al., 2015)	44 mice	Chronic prenatal stress	None	Vaginal lactobacillus abundance was lower in dams exposed to stress. Lactobacillus abundance was also lower in the gut microbiome of offspring of stressed dams
HUMAN MODELS				
(Hantsoo et al., 2019)	48 pregnant women	Adverse Childhood Experiences (ACE) Questionnaire, Trier Social Stress Test	IL-6, IL-1β, hsCRP, TNF-α, cortisol	Women reporting two or more ACEs had a larger abundance of *Prevotella* in gut than women with low ACEs.
(Paul, Boutain, Manhart, & Hitti, 2008)	400 women	Stressful life events	None	Stressful life events are associated with bacterial vaginosis
(Nansel et al., 2006)	3614 non-pregnant women	Perceived Stress Scale	None	Psychosocial stress is associated with an increase in bacterial vaginosis
(Ehrström et al., 2005)	70 (35 with vulvovaginal candida, 35 without)	Maternal salivary cortisol	Maternal salivary cortisol	Morning salivary cortisol rise is blunted in women with vulvovaginal candida, suggesting that chronic stress may impair immunity and have effects on the vaginal environment
(Culhane et al., 2001)	454 pregnant women at 14 weeks gestation	Perceived Stress Scale	None	Women experiencing moderate and high stress were statistically more likely to have bacterial vaginosis

Table 3.5 Summary of studies on perinatal stress and infant gut microbiome

Authors	Sample size	Stress exposure	Biological stress measure	Results
ANIMAL MODELS				
(Gur et al., 2017)	15 mice	Chronic prenatal stress	IL-1β	On the phylum level, offspring of stressed mice has higher relative abundance of Bacteroidetes and Firmicutes. On the family level, *Bifidobacteriaceae*, *Rikenellaceae*, and S24-7 were significantly higher in the offspring of stressed mice.
(Jašarević et al., 2017)	30 breeding pairs of mice	Chronic prenatal stress	None	Pups who were stress-exposed in utero had lower relative abundance of *Lactobacillus* and *Streptococcus*
(Golubeva et al., 2015)	11 rats	Restraint stress		Male offspring were assessed at four months of age. Offspring of stressed mothers had a lower abundance of *Lactobacillus* but a higher abundance of *Oscillibacter*, *Anaerotruncus*, and *Peptococcus*.
(Jašarević et al., 2015)	44 mice	Chronic prenatal stress	None	Vaginal *Lactobacillus* abundance was lower in dams exposed to stress. *Lactobacillus* abundance was also lower in the gut microbiome of offspring of stressed dams
(O'Mahony et al., 2009)	22 rats	Maternal separation from 2-12 days postpartum	None	DGGE profile of stressed and non-stressed pups differed significantly
(Bailey et al., 2004)	24 rhesus monkeys	Acoustical startle for pregnant monkeys	Maternal plasma cortisol	Prenatal stress reduced bifidobacteria and lactobacilli in infant stool
(Bailey & Coe, 1999)	20 rhesus monkeys	Maternal separation beginning at 6-9 months	Plasma cortisol	Decrease in *Lactobacilli* in separated monkeys 3 days postseparation
HUMAN MODELS				
(Zijlmans, Korpela, et al., 2015)	56	Reported stress and salivary cortisol	Maternal salivary cortisol	Infants of mothers with both high reported stress and high cortisol concentrations had higher relative abundance of Proteobacterial groups and lower relative abundance of lactic acid bacteria (*Lactobacillus*, *Lactoccus*, *Aerococcus*) and *Bifidobacteria*

CHAPTER 4: STUDY DESIGN, DATA COLLECTION, MEASURES, AND ANALYSIS

4.1 Study Aims

This project aims to examine the mechanisms through which peripartum maternal stress shapes infant HPA axis development and thus subsequent risk for metabolic and neurobehavioral disorders later in life. This project has three specific aims:

Aim 1: To identify which factors contribute to psychosocial stress in peripartum women in the Galápagos and to assess how these exposures both during pregnancy and in the postpartum shape maternal and infant HPA axis regulation

Aim 2: To assess both the psychosocial and physiological relationships between maternal stress during pregnancy and the placental enzyme, HSD11B2, as well as the relationship between HSD11B2 and infant HPA axis development

Aim 3: To analyze the relationships among maternal stress and HPA axis dysregulation during the peripartum period, infant gut microbiome composition, and infant HPA axis functioning

In alignment with these aims, this project employs mixed methods, including semi-structured and structured interviews; surveys for the measurement of stress, depression, social support, social status, infant attachment, home environment, and food security; biological

measurements including maternal and infant salivary cortisol for the measurement of HPA axis regulation, placental HSD11B2 methylation and expression, and infant stool; and anthropometry. Each measure is described in detail in this chapter.

4.2 Recruitment and Study Sample

The dissertation work was conducted over 12 months, from January 2018 through December 2018. Participants were recruited from Hospital Oskar Jandl (HOJ) and from the community. The study summary used for recruitment is available in APPENDIX A: . Visits with participants were conducted once during pregnancy and three times in the postpartum period. The prenatal visit was conducted at 34 – 36 weeks (Visit 1). Postpartum visits were conducted three days after delivery (Visit 2) (except the placental sample, which was collected at delivery), when the infant was one month old (Visit 3), and when the visit was two months old (Visit 4). For each mother-infant dyad, I collected semi-structured interviews, surveys, maternal and infant saliva samples, a placental sample, anthropometry, and an infant stool sample. A variety of validated surveys were used to measure stress, depression, social support from friends and family, subjective social status, maternal attachment, home environment, and food security. The period of observation for each mother-infant dyad was 12 – 14 weeks (34 – 36 weeks of pregnancy to two months postpartum). All visits were conducted in the participants' homes, places of work, the hospital, or the Galápagos Science Center (GSC), according to their preference. Each participant had at least one visit in the home, though most had all of their visits in the home. Figure 4.1 provides a data collection summary, and

provides a research timeline.

4.3 Ethical Considerations

All participants provided written informed consent prior to participation under appropriate protocols approved by the Institutional Review Boards for the University of North Carolina at Chapel Hill (UNC) and *Universidad San Francisco de Quito (USFQ)*. This project also received approval by Ecuador's Ministry of Public Health. Participants understood that they could discontinue participation at any time with no penalty. The UNC consent form can be found in APPENDIX B: .

4.4 Semi-Structured Interviews

Data Collection. Semi-structured interviews were utilized at 34 – 36 weeks gestation (Visit 1) and one month postpartum (Visit 3). In addition, the prenatal visit included a demographic survey that inquired about household size and composition, sociodemographics, employment, parity, smoking habits, medications, and other health issues. Perinatal stress and mental health are multidimensional concepts shaped by a variety of factors including socioeconomic status, ethnicity, social support, health, and discrimination (Rieger & Heaman, 2016; Thayer & Kuzawa, 2014, 2015). Thus, the semi-structured interviews generated narratives of stress and everyday life as they explored issues of stress, health, discrimination, family, support networks, joys, and concerns. The semi-structured format leaves questions open-ended, so that women could provide their own insights into stressors that may not be captured with stress surveys (Bernard, 2006: 212). All semi-structured interviews were audio-recorded.

Data Analysis. Semi-structured interviews from Visits 1 and 3, the prenatal visit and the one month postpartum visit respectively, were audio-recorded and then translated and transcribed directly into English by the interviewer and a second bilingual reviewer familiar with the idiosyncrasies of the Ecuadorian Spanish language, according to protocol by Regmi and colleagues (Regmi, Naidoo, & Pilkington, 2010). Non-verbal communication was included (eg. "[participant laughs]") according to protocol by McLellan and colleagues (McLellan, MacQueen, & Neidig, 2003). The majority of the interviews were transcribed verbatim, but local phrases whose verbatim translations are not meaningful were translated into their cultural equivalents. Pieces of audio recordings that were difficult to understand were reviewed by the scribes together to come to an agreement about the verbiage and meaning, according to Regmi and colleagues (Regmi et al., 2010). Utterances that could not be deciphered were marked "[inaudible]."

Transcribed interviews were then and analyzed using the qualitative data analysis software package NVivo® (version 12; QSR International; Melbourne, Australia). Each transcript was read once and coded for emerging central themes (nodes), generating Codebook 1. Then all interviews were re-read and re-coded using the completed Codebook 1 as well as new, more refined nodes, generating Codebook 2. Finally, the concurrence of nodes was analyzed to generate a node hierarchy both manually and through hierarchy charts and data visualization tools available in NVivo®. Some nodes were found to be both independent nodes from Codebook 1 as well as sub-nodes of one or more nodes under certain circumstances. Thus, some nodes may appear twice in Codebook 2 or both in Codebook 1 and Codebook 2. The progression from Codebook 1 to Codebook 2 can be visualized in

Table 4.1. Codebooks 1 and 2 were used together to interpret data from interviews.

4.5 Structured Sociodemographic Interview

Data Collection. In addition to semi-structured interviews about themes of stress and depression, we also collected general information on sociodemographic and other health data, including those on health and pregnancy, education, geographic history, food security, religiosity, neighborhood, and other themes. Gestational age at delivery, mode of delivery, infant sex, and pre-pregnancy maternal height and weight were recorded from paperwork distributed to the mothers from the hospital. A survey of household assets that was developed for this particular setting was used to approximate relative wealth. For this, participants were asked if their household owned a particular item and how many. A few items were observed instead of asked. The survey of household assets can be found in Appendix C: Household Survey of Assets.

4.6 Stress

Data Collection. The Perceived Stress Scale (PSS) (S. Cohen, Kamarck, & Mermelstein, 1983) was used to assess maternal chronic stress by measuring the degree to which situations are perceived as unpredictable, uncontrollable, and burdensome (S. Cohen et al., 1983). The PSS is particularly relevant for pregnancy, since it does not operationalize symptoms that occur frequently in both pregnancy and during times of stress (such as sleep disturbances) to assess chronic stress (Nast, Bolten, Meinlschmidt, & Hellhammer, 2013). We used a Spanish version of this scale that has been tested for reliability, validity, and sensitivity (Remor, 2006) in Spanish-speaking contexts. When analyzed categorically, the PSS is scored from 0 to 40, with scores of 0

– 13 indicating low stress, scores of 14 – 26 indicating moderate stress, and scores 27 – 40 indicating high stress. The PSS version used is available in Appendix C: Perceived Stress Scale.

4.7 Depression

Data Collection. Depression was measured by the Patient Health Questionnaire-8 (PHQ-8) (Kroenke, Spitzer, & Williams, 2001). We used a Spanish version of the PHQ-8 that has been validated (Baader et al., 2012) and tested for reliability (Cassiani-Miranda et al., 2017) in the Spanish language. The PHQ-8 is scored from 0 to 24, with a higher score indicating more depression symptoms. When analyzed categorically, depression was defined using the CDC's diagnostic cut-point for the PHQ-8, as scores greater than or equal to 10 (Kroenke et al., 2009).The PHQ-8 version used is available in Appendix C: Patient Health Questionnaire 8.

4.8 Social Support

Data Collection. Social support was measured by Spanish versions of the Perceived Social Support-Family (PSS-Family) scale and the Perceived Social Support-Friends (PSS-Friends) scale (Procidano & Heller, 1983), both of which have been previously validated in the Spanish language and in Latin American contexts (Espinosa, Menotti, Bravo, & Procidano, 2011). The PSS-Family is scored from 0 to 16, and the PSS-Friends is scored from 0 to 12. The PSS-Family is available in Appendix C: Perceived Social Support – Family, and the PSS-Friends is available in Appendix C: Perceived Social Support – Friends.

4.9 Subjective Social Status

Data Collection. The MacArthur Subjective Social Status (SSS) (Adler, Epel, Castellazzo, & Ickovics, 2000) was used to assess how participants view their place within their community. The MacArthur SSS has been validated in Spanish. The MacArthur SSS is available in Appendix C: MacArthur SSS.

4.10 Maternal-Infant Attachment

Data Collection. The Maternal Postnatal Attachment Scale (MPAS) (Condon & Corkindale, 1998) was used in Visit 3 and Visit 4 to assess maternal-infant attachment. The MPAS uses maternal dispositions toward the infant to measure maternal-infant attachment, which may affect maternal behavior toward the infant and thus the infant's stress (Van Bussel, Spitz, & Demyttenaere, 2010). The MPAS has been validated for use internationally, and during the summer of 2017, I held a focus group to determine any language changes that needed to be made for the MPAS. The MPAS is available in Appendix C: Maternal Postnatal Attachment Scale.

4.11 Home Environment

Data Collection. An adapted version of the Infant/Toddler Home Observation for Measurement of Environment (HOME) Inventory (Caldwell & Bradley 2003) was used at Visit 3. The HOME Inventory, which has been validated for use internationally, is designed to measure the stimulation and support available to a child in the home environment. The scale was adapted so that only questions relevant for infants under ten weeks of age will be used. The HOME Inventory relies on investigator observation to answer questions such as “Parent

spontaneously vocalizes to the child at least twice" and "Parent keeps child in visual range, looks at often" to discern aspects of the home environment are relevant to physical and psychosocial well-being. Data for the HOME that could not be observed, such as, "Father provides some care daily" or "Family visits relatives or receives visits once a month or so," were asked verbally. A full list of the HOME Inventory questions can be found in Appendix C: Infant/Toddler Home Observation for Measurement of Environment.

4.12 Food Security

Data Collection. Food security was assessed using the Latin American and Caribbean Food Security Scale (ELCSA) (Comité Científico de la ELCSA, 2012), with higher scores indicating a higher level of food insecurity. On this scale, a score of 0 indicates a food secure household, scores from 1 – 5 indicate mild household food insecurity, scores from 6 – 10 indicate moderate household food insecurity, and scores of 11 – 15 indicate severe household food insecurity (Comité Científico de la ELCSA, 2012).

4.13 Salivary Cortisol

Data Collection. Maternal cortisol was assessed through saliva samples. Salimetrics guides were used for maternal saliva collection and storage protocols (Salimetrics & SalivaBio, 2015). Maternal saliva samples were collected the day after Visits 1, 3, and 4. On each of these days, women provided four samples: one immediately upon awakening, one 30 minutes after awakening, one 60 minutes after awakening, and one prior to sleep. While previous research has often required two consecutive days of saliva collection, recent literature has shown that awakening salivary measurements over just one day provide reliable estimates of morning

cortisol in pregnant women (Vlenterie, Roeleveld, & van Gelder, 2016). Participants were instructed not to eat, drink, or brush their teeth in the 30 minutes prior to collecting samples and to record start and stop times for saliva collection. Participants were asked to store the samples in their own freezers until a study team member could retrieve them the next day. Samples were then transported to the GSC where they were frozen at -20° C until analysis. The instructions for maternal saliva collection are available in APPENDIX D: .

Infant salivary cortisol samples were collected when the infant was three days old (Visit 2) and two months old (Visit 4). Because the age that infants establish a diurnal rhythm in cortisol is up for debate (De Weerth, Zijl, & Buitelaar, 2003), many studies of HPA axis function in infancy have measured cortisol dysregulation through basal cortisol or stress reactivity, that is, in response to a stressor (O'Connor et al., 2012). At three days postpartum, basal cortisol samples were collected to capture variation due to the prenatal environment with little postnatal influence. At two months postpartum, infant basal cortisol and cortisol reactivity were measured. Cortisol reactivity was measured as the difference between salivary cortisol levels before and 20-25 minutes after a stressor per a previously published infant stress reactivity protocol (Tollenaar et al., 2011). Infant basal cortisol is determined from the first of these two samples. In order to apply a stressor to the infant to induce a stress reactivity, the research team placed the nude (except the diaper) infant on a metal tray for 60 seconds to mimic an infant's typical discontented response to being weighed in the hospital. The method for this stressor was developed by the research team and the nurses at HOJ in order to be non-invasive and culturally appropriate. All infant saliva samples were collected using Salimetrics Infant Swabs and placed into Salimetrics Swab Storage tubes and frozen at -20° C at the GSC until analysis. The instructions for infant saliva collection are available in APPENDIX E: .

Data Analysis. Saliva samples were thawed and assayed in duplicate for salivary cortisol using commercially available ELISA kits (Salimetrics, State College, PA) according to Salimetrics protocol (Salimetrics, 2016) at the GSC. Samples were run on a BioTek ELx808™ Absorbance Microplate Reader (BioTek Instruments, Inc, Winooski, VT). Difficulties in infant saliva collection limited the volume of saliva collected for infants, particularly for very young infants. Thus, we only had enough saliva for analysis from 18 infants from Visit 2 (3 days postpartum) and 25 infants at Visit 4 (two months postpartum). Inter-assay variability was 8.5%, and intra-assay variability was 7.11%.

4.14 HPA Axis Dysregulation

Maternal and infant HPA axis functioning were measured through salivary cortisol. Continuous, log-transformed cortisol concentrations were used to build HPA axis dysregulation variables for models.

Maternal HPA axis regulation was assessed through salivary cortisol at three timepoints: 34 – 36 weeks of pregnancy, one month postpartum, and two months postpartum. Though the level of cortisol rises throughout pregnancy, it maintains its diurnal rhythm, allowing circadian regulation in mothers to be assessed throughout pregnancy (Christian, 2012). Cortisol dysregulation in mothers was measured in four ways: elevated morning cortisol, elevated evening cortisol, a blunted cortisol awakening response at 30 minutes (CAR), and a poor daily cortisol decline. A blunted CAR was defined as a small difference in cortisol levels between waking and 30 minutes post-waking, and poor daily cortisol decline was defined as a large difference between evening cortisol and waking cortisol levels.

Cortisol dysregulation in infants was measured through elevated basal cortisol at three days old and two months old and a blunted or exaggerated cortisol reactivity at two months old. High basal cortisol (Stroud et al., 2016) and blunted (Tollenaar et al., 2011) and exaggerated (Davis et al., 2011) cortisol reactivity have been cited as evidence of infant cortisol dysregulation. The basal cortisol measure at three days is meant to serve as a proxy for HPA axis development in response to *prenatal* maternal stress alone in order to improve our understanding of how the postpartum period can then attenuate this prenatal effect. The measurements at two months are meant to reflect HPA axis development as a consequence of both prenatal and postpartum programming.

Infant HPA axis dysregulation serves as our outcome in some analyses, since it has been associated with metabolic (Reynolds et al., 2001) and neurobehavioral (Davis et al., 2011; O'Connor et al., 2002; O'Donnell et al., 2013) disorders in offspring and is thought to mediate the effects of the environment on child development (Thomas, Letourneau, Bryce, Campbell, & Giesbrecht, 2017).

4.15 Placental HSD11B2

Data Collection. Placental samples were collected in collaboration with doctors and nurses at Hospital Oskar Jandl. Healthy, intact placentas were collected immediately and dissected within 60 minutes of birth. Umbilical cords were removed, and placentas were weighed and measured. Maternal decidua was removed, and tissue samples were taken from four sampling sites on the fetal side of the placenta. The four sampling sites were selected in each of the four quadrants of the placenta that were at least 2 centimeters (cm) from the umbilical cord insertion site and at least 3 cm from the placental edge, following protocol by Burton and

colleagues (Burton et al., 2014). Samples were cut from each sampling site and pooled into one storage tube to control for intra-placental variation. The samples were stored in RNA*later* (Life Technologies, Grand Island, NY) and stored at -20°C at the hospital. Samples were then sent on ice to UNC-Chapel Hill and Duke University for analysis. During sample collection, collaborators as HOJ recorded important data about the birth and placenta, including placental length, height, and weight, time of birth, time of sample collection, infant birth weight, any medications received during the birth, duration of labor, and other observations. The placental collection instructions and data sheet is available in APPENDIX F: .

Placental HSD11B2 expression. The analysis of HSD11B2 expression was conducted at the Microbiome Core Facility at the University of North Carolina at Chapel Hill. Total RNA was isolated using Qiagen RNeasy Extraction Kit, with the addition of DNaseA digest per the manufacturer's guidelines. Total RNA was quantified and normalized to 50ng/uL prior to the synthesis of cDNA. 500ng total RNA was subject to cDNA synthesis via qScript cDNA synthesis kit. Expression of HSD11B2 was analyzed using the following primers (Capron et al., 2018): HSD11B2 forward: CTACTCATGGACACATTCAGCT, reverse: TCACTGACTCTGTCTTGAAGC.

Quantitative PCR was performed on QuantStudio Q6, using BioRad PowerSyber qPCR kit. The thermal cycling conditions were as follows: one cycle at 50°C for 20 sec, 95°C for 10 minutes, followed by 40 cycles of 15 seconds at 95°C, 1 minute at 60°C. Melting curve analysis was carried out using the continuous method from the Q6 Software (Applied Biosystems) conducted at 60°C, with increments of 1°C for 15 seconds. Data analysis was carried out with Q6 Software (Applied Biosystems). The auto threshold and baseline options were used for the calculations of cycle threshold (CT) values per well.

Placental HSD11B2 methylation. The analysis of *HSD11B2* methylation was conducted at the Murphy Lab at Duke University. Placental genomic DNA was extracted using the Lysing Matrix A from MP Biomedical with the FastPre24 for homogenization, followed by the Solid Tissues Protocol from Purgene. DNA samples were sodium bisulfite modified using the EZ DNA Methylation™ Kit (Zymo Research, Irvine, CA), and pyrosequencing was performed on PCR product amplified from bisulfite-modified DNA based on the region sequenced and displaying differential methylation in human placenta from Alikhani-Koopaei and colleagues (Alikhani-Koopaei et al., 2004). The extent of methylation at the HSD11B2 promoter region was examined with pyrosequencing using the Pyromark Pyrosequencing System (Qiagen Inc.) and the following forward and biotinylated reverse primers, Sequence (5'-3') were used for amplification (IDT Inc., Coralville, IA): HSD11B2-F2-AAGTTTTGGAAGGAAAGGGAAGA, HSD11B2-R2-[btn] ACAAAACCTACCTAAAACAAAAACTA, HSD11B2-S- GGGGT AGAGATTTTAAGAA.

The region analyzed contains four CpGs sites of interest (Alikhani-Koopaei et al., 2004) with reactions performed in duplicate. Sodium bisulfite–modified, fully methylated referent positive control and fully unmethylated (whole genome amplified) negative control DNA (Qiagen) were examined with each batch. The percent methylation at each CpG site was quantified using the PyroMark CpG software, version 1.0.11. (Qiagen). Methylation across each of the four HSD11B2 CpG sites was averaged to obtain an overall measure of methylation.

4.16 Infant Stool Sample

Data Collection. Infant stool samples were collected after Visit 4. For infant stool collection, mothers were given detailed oral and written instructions for the collection of the

stool sample, as well as a sample collection kit, including gloves, a small plastic spoon, a small plastic container. Mothers were asked to collect a small (roughly 400 mg) amount of stool from their infant's diaper and store it in the sealed plastic container in their own freezer until it could be collected by the research team later that day. Stool samples were then stored frozen at the Galápagos Science Center until they were transported to the Microbiome Core Facility at the University of North Carolina at Chapel Hill (UNC) for analysis. The stool sample collection instructions and data sheet are available in

APPENDIX G: .

DNA Isolation. Samples were transferred to a 2 mL tube containing 200 mg of ≤106 μm glass beads (Sigma, St. Louis, MO) and 0.3 mL of Qiagen ATL buffer (Valencia, CA), supplemented with 20 mg/mL lysozyme (Thermo Fisher Scientific, Grand Island, NY). The suspension was incubated at 37°C for 1 hour with occasional agitation. Subsequently the suspension was supplemented with 600IU of Qiagen proteinase K and incubated at 60°C for 1 hour. Finally, 0.3 mL of Qiagen AL buffer was added and a final incubation at 70°C for 10 minutes was carried out. Bead beating was then employed for 3 minutes in a Qiagen TissueLyser II at 30Hz. After a brief centrifugation, supernatants were aspirated and transferred to a new tube containing 0.3 mL of ethanol. DNA was purified using a standard on-column purification method with Qiagen buffers AW1 and AW2 as washing agents, and eluted in 10mM Tris (pH 8.0).

16S rRNA Amplicon Sequencing. 12.5 ng of total DNA were amplified using universal primers targeting the V4 region of the bacterial 16S rRNA gene (Caporaso et al., 2012; Kozich, Westcott, Baxter, Highlander, & Schloss, 2013). Primer sequences contained overhang adapters appended to the 5' end of each primer for compatibility with Illumina sequencing platform. Master mixes contained 12.5 ng of total DNA, 0.2 μM of each primer and 2x KAPA HiFi HotStart ReadyMix (KAPA Biosystems**,** Wilmington, MA). The thermal profile for the amplification of each sample had an initial denaturing step at 95°C for 3 minutes, followed by a cycling of denaturing of 95°C for 30 seconds, annealing at 55°C for 30 seconds and a 30 second extension at 72°C (25 cycles), a 5 minutes extension at 72°C and a final hold at 4°C. Each 16S amplicon was purified using the AMPure XP reagent (Beckman Coulter, Indianapolis, IN). In the next step each sample was amplified using a limited cycle PCR program, adding Illumina

sequencing adapters and dual-index barcodes (index 1(i7) and index 2(i5)) (Illumina, San Diego, CA) to the amplicon target. The thermal profile for the amplification of each sample had an initial denaturing step at 95°C for 3 minutes, followed by a denaturing cycle of 95°C for 30 seconds, annealing at 55°C for 30 seconds and a 30 second extension at 72°C (8 cycles), a 5 minutes extension at 72°C and a final hold at 4°C. The final libraries were again purified using the AMPure XP reagent (Beckman Coulter), quantified and normalized prior to pooling. The DNA library pool was then denatured with NaOH, diluted with hybridization buffer and heat denatured before loading on the MiSeq reagent cartridge (Illumina) and on the MiSeq instrument (Illumina). Automated cluster generation and paired–end sequencing with dual reads were performed according to the manufacturer's instructions.

Data Analysis. Sequencing output from the Illumina MiSeq platform were converted to fastq format and demultiplexed using Illumina Bcl2Fastq 2.18.0.12. The resulting paired-end reads were processed using QIIME 2 2018.11. Index and linker primer sequences were trimmed using the QIIME 2 invocation of cutadapt. The resulting paired-end reads were processed with DADA2 through QIIME 2 including merging paired ends, quality filtering, error correction, and chimera detection. Amplicon sequencing units from DADA2 were assigned taxonomic identifiers with respect to Green Genes release 13_08. Alpha diversity with respect to: Faith PD whole tree, Evenness (Shannon) index, and observed species number metrics was estimated using QIIME 2 at a rarefaction depth of 5,000 sequences per subsample. Beta diversity estimates were calculated within QIIME 2 using weighted and unweighted UniFrac distances as well as Bray-Curtis dissimilarity between samples at a subsampling depth of 5,000. Results were summarized, visualized through principal coordinate analysis, and significance was estimated as implemented in QIIME 2. Before analysis, microbiome data were cleaned for appropriate

sequence length and transformed from the 16sRNA gene sequences into operational taxonomic units (OTUs) in QIIME 2 (Bolyen et al., 2019).

4.17 Anthropometry

Data Collection. Infant birth date, weight in grams (g), length (cm), and head circumference (cm) were recorded from infant health paperwork distributed to the mothers from the hospital. Hospital staff measured weight to the nearest gram and length and head circumference to the nearest centimeter. Infant length (cm), weight (kg), arm circumference (cm), and waist circumference (cm) and maternal weight (kg) were measured at one month postpartum (Visit 3) and two months postpartum (Visit 4) by study staff. Infant length was measured to the nearest millimeter (mm) using the Seca® 417 Mobile Pediatric Measuring Board (Seca® Corporation, Hanover NH). Maternal and infant weight were recorded to the nearest 0.1 kg on a Seca® flat scale. Infant arm and waist circumference were measured with a Seca® measuring tape to the nearest mm. The anthropometry measurement form is available in APPENDIX H: .

Data Analysis. Infant growth data was used to calculate length-for-age, weight-for-age, and weight-for-length z-scores using the WHO growth standards (De Onis, 2006).

4.18 Study Schedule

A more detailed description of study visits is provided here:

Visit 1. Visit 1 was conducted when women were 34 – 36 weeks pregnant. Visits began with a sociodemographic survey and the ELCSA followed by the semi-structured interview, all administered verbally. Participants then filled out the PSS, PHQ-8, MacArthur SSS, PSS-

Friends, and PSS-Family on their own. Last, women were given vials and instructions for maternal salivary cortisol collection, which they were asked to take the next day. Maternal saliva samples were stored in participants' freezers until the investigator collected the samples two days after Visit 1.

Visit 2. Visit 2 was completed in two parts. The first part was the placental sample collection at the hospital right after the infant's birth. Placental sample collection was completed by the obstetrics and gynecology team at HOJ. The second component of this visit occurred three days postpartum, when the investigator visited the homes of the mother-infant dyads to collect a single, basal infant saliva sample.

Visit 3. Visit 3 was conducted when infants were one month old. Visits began with the verbal semi-structured interview. Next, participants filled out the PSS, PHQ-8, MacArthur SSS, PSS-Friends, PSS-Family, and the MPAS on their own. While participants filled out the surveys, the investigator collected data for the abbreviated HOME measurement. Data for the HOME that could not be observed were asked verbally. Last, maternal weight and infant anthropometrics, including weight, length, arm circumference, and weight circumference were measured and recorded. Before leaving, the investigator provided women with the vials and instructions for maternal salivary cortisol collection, which they were asked to take the next day. Maternal saliva samples were stored in participants' freezers until the investigator collected the samples two days after Visit 3.

Visit 4. Visit 4 was conducted when infants were two months old. Visits began with the baseline infant salivary cortisol sample. Immediately after, infants were disrobed (with the exception of the diaper) and placed on the metal tray for 60 seconds to induce a stress response. After the 60 seconds, the investigator started a 25-minute timer. When the timer went off, the

investigator paused the ongoing activity to take the infant's second salivary cortisol sample (needed to calculate stress reactivity), and then continued the visit. After the stressor was administered, the investigator conducted a brief structured interview verbally, and then participants filled out the PSS, PHQ-8, MacArthur SSS, PSS-Friends, PSS-Family, and the MPAS on their own. Maternal weight and infant anthropometrics, including weight, length, arm circumference, and weight circumference were measured and recorded. Before leaving, the investigator provided women with the vials and instructions for maternal salivary cortisol collection, which they were asked to take the next day. Women also received materials and instructions for infant stool sample collection, which they were asked to collect after the infant's next bowel movement. Infant stool and maternal saliva samples were stored in participants' freezers until the investigator collected the samples two days after Visit 4.

Table 4.1 Qualitative analysis codebooks

Codebook 1	Codebook 2	
Anxiety	Baby's well-being The birth	Finances Housework
Baby's well-being	Antibiotics Colic	Growth
Depression		
Discrimination		
Domestic violence	Verbal abuse Physical abuse	Machismo
The home	Baby's needs Confinement/Quarantine	Housework
Husband/Partner	Support Machismo	
Infant feeding	Breastfeeding Formula Lactation group	Pain Work
Family	Distance from family Family tension In-laws tension My father	My mother My sister Support
Finances		
Food insecurity		
Friends	Distance from friends No friends	
Galápagos	Food insecurity Isolation	Teen pregnancy
Hospital	Health care	
Mainland	Distance from family Distance from friends	Health care
Mother's physical health	Antibiotics C-section	Infection
Neighborhood		
Religion	Church Community	God
Suggestions for improvement		
Work		

Figure 4.1 Data collection summary

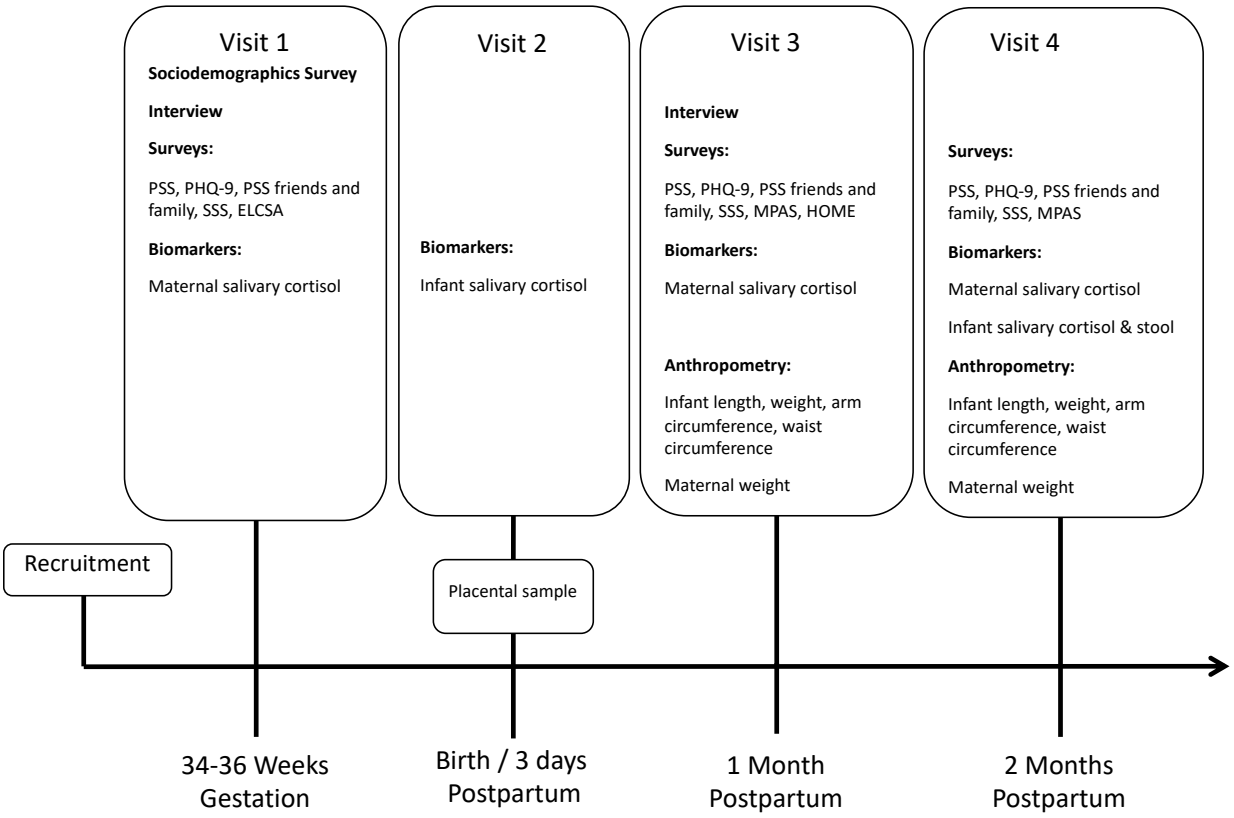


Table 4.2 Study timeline

Timeline (2018)	**Activity**
Jan. 1 – Jan. 31	Establish protocols with hospitals, train field assistants
Feb. 1 – Dec. 10	Recruit and enroll new participants
Feb. 1 – Dec. 10	Collect qualitative and quantitative data
Dec. 1 – Dec. 31	Laboratory data analysis

CHAPTER 5: THE FOURTH TRIMESTER MATTERS: PRENATAL AND POSTPARTUM SOCIAL SUPPORT PREDICT MATERNAL HPA AXIS DEVELOPMENT IN THE GALAPAGOS ISLANDS

5.1 Introduction

Robust evidence from epidemiological studies has shown associations between early life exposures to adverse environments and subsequent cardiovascular and metabolic disease as well as a variety of neurobehavioral disorders (Barker et al., 1989; O'Connor et al., 2002). While research on this phenomenon, termed the developmental origins of health and disease (DOHaD), originally focused on the effects of prenatal energetic and nutritional stress on infant health outcomes, a growing body of literature has found that maternal psychosocial distress introduces additional burdens that have long-term effects on fetal development, and specifically the hypothalamic-pituitary-adrenal axis (HPA axis) (Nyberg et al., 2012; Pike, 2005; Wells, 2010). Prenatal psychosocial stress has been associated with dysregulated glucocorticoid function in infants (Stroud et al., 2016; Thayer & Kuzawa, 2014, 2015), which is known to underlie metabolic (Reynolds et al., 2001) and neurobehavioral disorders in offspring even when controlling for adverse birth outcomes including low birth weight and gestational age (Davis et al., 2011; O'Connor et al., 2002; O'Donnell et al., 2013).

The prenatal activation of the HPA axis, which regulates glucocorticoid feedback interactions in the mother and the fetus, has been proposed as one mechanism through which prenatal maternal stress shapes fetal development and subsequent long-term disease risk (Pike, 2005; Seckl, 2008). Studies have found that prenatal stress in mothers has significant effects on offspring HPA axis regulation as early as infancy (Davis et al., 2011; Thayer & Kuzawa, 2014,

2015), into childhood (Gutteling et al., 2004, 2005), and through adolescence (O'Donnell et al., 2013) and that HPA axis imbalances underlie increased risk for metabolic, cardiovascular, immune, reproductive, and cognitive pathology later in life (Lupien, McEwen, Gunnar, & Heim, 2009; Thayer & Kuzawa, 2014). However, results from studies on the associations between prenatal psychosocial stress and biological measures of stress, as well as each of their effects on offspring development are inconsistent, suggesting that other mechanisms could play role in infant HPA axis development.

Most studies on maternal stress and subsequent infant development focus on exposures to maternal stress during pregnancy alone, while postpartum exposures are often omitted despite the fact that infants retain some developmental plasticity through the first few years of life (Gluckman et al., 2005). Studies that examine the longitudinal effects of psychosocial exposures both during and after pregnancy are even more rare. Using the developmental niche (Harkness & Super, 1994) as a framework, we hypothesize that the postpartum maternal environment plays a critical role in the continued development of an infant's HPA axis. The developmental niche framework proposes that the household mediates the relationship between the environment and the child through three key components: (1) the child's physical and social setting, (2) customs of childcare, and (3) the psychology of caretakers (Harkness & Super, 1994). In our analysis we consider this model in the justification for including maternal psychosocial distress both during pregnancy and in the postpartum into an analysis of infant HPA axis development.

The postpartum period can be a particularly stressful time for women, which may be detrimental to a mother's mental health and caregiving behaviors (Grajeda & Perez-Escamilla, 2002; Rudzik et al., 2014). In turn, poor maternal mental health and patterns of care during early childrearing have each been found to be associated with HPA axis dysregulation in offspring

later in life (Essex et al., 2002; Gunnar & Donzella, 2002; Tollenaar et al., 2012; Wright, 2007). It is also possible that postpartum maternal distress may influence the infant directly through cortisol transfer during breastfeeding (Hahn-Holbrook, Le, Chung, Davis, & Glynn, 2016). One study found that among breastfed infants, high maternal cortisol levels were associated with increased fearful temperament (Glynn et al., 2007). Further, studies have found that the relationship between prenatal stress and infant health outcomes is moderated by mother-infant attachment, a marker of caregiving quality (Bergman et al., 2010). These studies suggest that the infant's postnatal environment is an important component of the mechanism through which the young HPA axis develops, and insufficient investigation of postpartum maternal distress may account for the inconsistent results from studies of prenatal distress and offspring development. By incorporating both pregnancy and postpartum distress into a model for infant HPA axis development, we seek to add a critical component to the literature on DOHaD that will elucidate the pathways through which early environment shapes development.

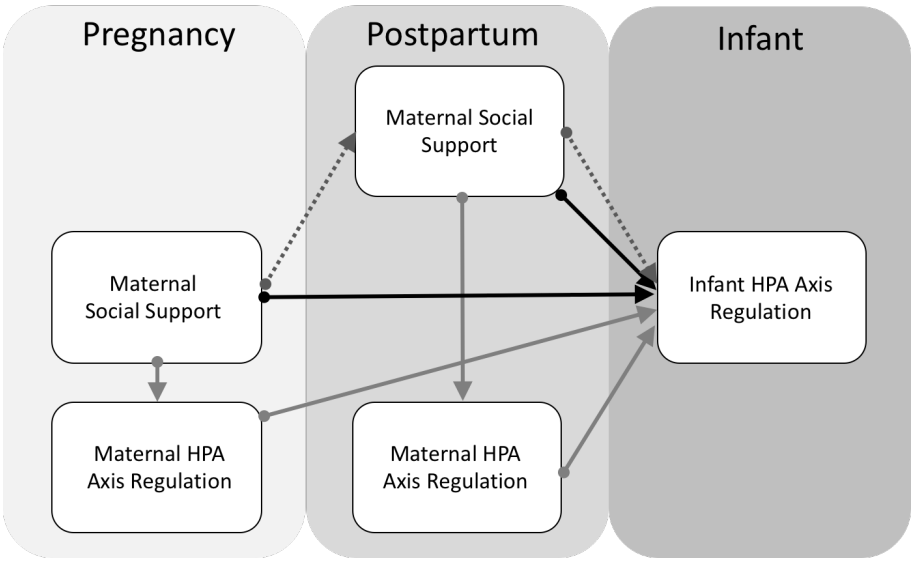
To investigate these understudied mechanisms, we use a biocultural approach first to qualitatively identify which factors contribute to maternal distress in the Galápagos Islands, and then to quantitatively assess how these factors may contribute to infant HPA axis dysregulation, which we assess through maternal and infant salivary cortisol. Essential to our investigation are the identification of salient distress exposures beyond stress and depression themselves and the inclusion of exposures into the postpartum period. After qualitatively identifying a low level of social support to be the central factor in women's distress, we utilize it as an exposure to test three propositions about infant HPA axis development (Figure 5.1).

1. That maternal social support has a direct effect on infant HPA axis regulation.

2. That maternal social support has an additional indirect effect on infant HPA axis regulation through maternal HPA axis regulation.
3. That maternal social support during pregnancy has an additional indirect effect on infant HPA axis regulation through postpartum social support.

Propositions 1 and 2 were modeled separately for prenatal and postpartum exposures.

Figure 5.1 Conceptual model



Proposition 1 is depicted with solid black lines, Proposition 2 is depicted with solid gray lines, and Proposition 3 is depicted with dashed gray lines.

5.2 Methods

Study setting

This investigation was done on the Galápagos Islands, which are best known for the Galápagos National Park and which are home to 30,000 residents (Walsh & Mena, 2016). As there is no indigenous population, the islands were only inhabited by humans within the last few

hundred years, and the population has grown quickly from approximately 3,500 in 1972 to today's approximately 30,000 (Walsh & Mena, 2016). The archipelago province of Ecuador lies 1000 km off the coast of the mainland (Walsh & Mena, 2016), leaving its residents both geographically and socially isolated. Further, its designation as a national park has restricted agriculture, putting pressure on its foodways, so that most food must be imported from the mainland to sustain the growing population (Page et al., 2013; Pera, Katz, & Bentley, 2019). Geographic isolation is also challenging for the islands' water systems, as fresh water scarcity restricts potable water availability (Walsh et al., 2010).

In terms of maternal and child health, most women on the Galápagos give birth in a hospital (97.4%), but the infant mortality rate on the islands (0.04%) is higher than the national average (0.013%) (Freire et al., 2015). Nonetheless, the rate of low birth weight on the islands (6.4%) is comparable to that of the national average (6.8%). While children under the age of five have a higher overweight and obesity rate (12.7%) than that of the national average (8.5%), these children have lower rates of stunting and wasting (Freire et al., 2014).

Study sample

The data were collected over 12 months, from January through December 2018. Participants were recruited either in-person or by phone from Hospital Oskar Jandl (HOJ) on the Galápagos' provincial capital island, San Cristóbal, using purposive sampling (N = 38). HOJ is a public hospital, free for all residents, and is the only hospital on the island, allowing the research team to screen all pregnant women with up-to-date contact information on the island within the recruitment period. Inclusion criteria limited participants to women between the ages of 18 and 50 years who planned to give birth on the island. Of those contacted who were ineligible, 12

planned to give birth on the mainland, four had already moved off the island, and three had serious pregnancy complications, and thus were excluded. Of those eligible, only four decided to not take part in the study. Visits with participants were conducted once during pregnancy and three times in the postpartum period. The prenatal visit was conducted at 34-36 weeks gestation (Visit 1 or V1). Postpartum visits were conducted three days after delivery (Visit 2 or V2), one month postpartum (Visit 3 or V3), and two months postpartum (Visit 4 or V4). Thus, the period of observation for each mother-infant dyad was 14 weeks. Of the original 38 mother-infant dyads, 38 participated in the prenatal visit, 27 participated in the 3-day postpartum visit, 28 participated in the one-month postpartum visit, and 25 participated in the two-month postpartum visit. For each mother-infant dyad, the research team collected semi-structured interviews; surveys on stress, depression, and social support; maternal saliva samples at 34-36 weeks gestation, one month postpartum, and two months postpartum; and infant saliva samples at 3 days postpartum and two months postpartum. All visits were conducted in the participants' homes, places of work, the hospital, or the Galápagos Science Center (GSC), according to participant preference.

Ethical approvals and informed consent

All participants provided written informed consent prior to participation under appropriate protocols approved by the Institutional Review Boards for the University of North Carolina at Chapel Hill and *Universidad San Francisco de Quito*. This project was also approved by Ecuador's Ministry of Public Health.

Measures

Semi-structured Interview. Semi-structured interviews were utilized at 34-36 weeks gestation (Visit 1) and one month postpartum (Visit 3) to investigate the central factors contributing to maternal distress in the Galápagos context. Perinatal distress and mental health are multidimensional concepts shaped by a variety of factors including socioeconomic status, ethnicity, social support, health, and discrimination (Rieger & Heaman, 2016; Thayer & Kuzawa, 2014, 2015). The semi-structured interviews generated narratives of distress and everyday life as they explored issues of stress, health, discrimination, family, support networks, joys, and concerns. The semi-structured format left questions open-ended, so that women could provide their own insights into stressors that may not be captured with stress surveys (Bernard, 2006: 212). All semi-structured interviews were audio-recorded.

Stress. The Perceived Stress Scale (PSS) (S. Cohen et al., 1983) was used to assess maternal chronic stress by measuring the degree to which situations are perceived as unpredictable, uncontrollable, and burdensome (S. Cohen et al., 1983). The PSS is particularly relevant for pregnancy, since it does not operationalize symptoms that occur frequently in both pregnancy and during times of stress (such as sleep disturbances) to assess chronic stress (Nast et al., 2013). We used a Spanish version of this scale that has been tested for reliability, validity, and sensitivity (Remor, 2006) in Spanish-speaking contexts. When analyzed categorically, the PSS is scored from 0 to 40, with scores of 0 – 13 indicating low stress, scores of 14 – 26 indicating moderate stress, and scores 27 – 40 indicating high stress.

Depression. Depression was measured by the Patient Health Questionnaire-8 (PHQ-8) (Kroenke et al., 2001). We used a Spanish version of the PHQ-8 that has been validated (Baader et al., 2012) and tested for reliability (Cassiani-Miranda et al., 2017) in the Spanish language.

The PHQ-8 is scored from 0 to 24, with a higher score indicating more depression symptoms. When analyzed categorically, depression was defined using the CDC's diagnostic cut-point for the PHQ-8, as scores greater than or equal to 10 (Kroenke et al., 2009).

Social Support. Social support was measured by Spanish versions of the Perceived Social Support-Family (PSS-Family) scale and the Perceived Social Support-Friends (PSS-Friends) scale (Procidano & Heller, 1983), both of which have been previously validated in the Spanish language and in Latin American contexts (Espinosa et al., 2011). The PSS-Family is scored from 0 to 16, and the PSS-Friends is scored from 0 to 12.

Maternal Salivary Cortisol Collection. Salimetrics guides were used for maternal saliva collection and storage protocols (Salimetrics & SalivaBio, 2015). Maternal saliva samples were collected at 34-36 weeks of pregnancy (Visit 1), at one month postpartum (Visit 3), and at two months postpartum (Visit 4). On each of these occasions, women provided three samples: one immediately upon waking, one 30 minutes after waking, and one prior to sleep (evening). Participants were instructed not to eat, drink, or brush their teeth in the 30 minutes prior to collecting samples and to record start and stop times for saliva collection. Participants stored samples in their own freezers until a study team member retrieved them the next day and transported them to the GSC where they were frozen at -20° C until analysis.

Infant Salivary Cortisol Collection. Infant salivary samples were collected when the infant was three days old (Visit 2) and two months old (Visit 4). Because the age that infants establish a diurnal rhythm in cortisol is up for debate (De Weerth et al., 2003), many studies of HPA axis function in infancy have measured cortisol dysregulation through basal cortisol or stress reactivity, that is, in response to a stressor (O'Connor et al., 2012). At three days postpartum, basal cortisol samples were collected to capture variation due to the prenatal

environment with little postnatal influence. At two months postpartum, infant basal cortisol and cortisol reactivity were measured. Cortisol reactivity was measured as the difference between salivary cortisol levels before and 20-25 minutes after a stressor per a previously published infant stress reactivity protocol (Tollenaar et al., 2011). Infant basal cortisol at this visit is determined from the first of these two samples. In order to apply a stressor to the infant to induce a stress response, the research team placed the diapered infant on a metal tray for 30 seconds to mimic an infant's typical discontented response to being weighed in the hospital. The method for this stressor was developed by the research team and the nurses at HOJ in order to be non-invasive and culturally appropriate. All infant saliva samples were collected using Salimetrics Infant Swabs and placed into Salimetrics Swab Storage tubes and frozen at -20° C until analysis.

Covariates. In addition to these data, we used a sociodemographic survey at Visit 1 that inquired about general sociodemographics, household size and composition, employment, parity, health behaviors, medications, education, geographic history, food security, religiosity, neighborhood, and other themes. Food security was assessed using the Latin American and Caribbean Food Security Scale (ELCSA) (Comité Científico de la ELCSA, 2012).

Qualitative analysis

Semi-structured interviews from Visits 1 and 3, the prenatal visit and the one month postpartum visit respectively, were audio-recorded and then translated and transcribed directly into English by the interviewer and a second bilingual reviewer familiar with the idiosyncrasies of the Ecuadorian Spanish language, according to protocol by Regmi et al. (Regmi et al., 2010). Non-verbal communication was included (eg. "[participant laughs]") following protocol by McLellan and colleagues (McLellan et al., 2003). The majority of the interviews were transcribed verbatim, but local phrases whose verbatim translations are not meaningful were

translated into their cultural equivalents. Pieces of audio recordings that were difficult to understand were reviewed by the scribes together to come to an agreement about the verbiage and meaning, following Regmi and colleagues (Regmi et al., 2010). Utterances that could not be deciphered were marked "[inaudible]."

Transcribed interviews were then and analyzed using the qualitative data analysis software package NVivo® (version 12; QSR International; Melbourne, Australia). Each transcript was read once and coded for emerging central themes (nodes), generating Codebook 1. Then all interviews were re-read and re-coded using the completed Codebook 1 as well as new, more finely tuned nodes, generating Codebook 2. Finally, the concurrence of nodes was analyzed for a node hierarchy both manually and through hierarchy charts and data visualization tools available in NVivo®. Codebooks 1 and 2 were used together to interpret data from interviews.

Laboratory analysis

Saliva samples were thawed and assayed in duplicate for salivary cortisol using commercially available ELISA kits (Salimetrics, State College, PA) according to Salimetrics protocol (Salimetrics, 2016) at the GSC. Difficulties in infant saliva collection limited the volume of saliva collected for infants, particularly for very young infants. Thus, we only had enough saliva for analysis from 18 infants from Visit 2 (3 days postpartum) and 25 infants at Visit 4 (two months postpartum). Inter-assay variability was 8.5%, and intra-assay variability was 7.11%.

HPA axis dysregulation

Maternal and infant HPA axis functioning were measured through salivary cortisol. Continuous, log-transformed cortisol concentrations were used to build HPA axis dysregulation variables for models.

Maternal HPA axis regulation was assessed through salivary cortisol at three timepoints: 34-36 weeks of pregnancy, one month postpartum, and two months postpartum. Cortisol dysregulation in mothers was measured in four ways: elevated morning cortisol, elevated evening cortisol, a blunted cortisol awakening response at 30 minutes (CAR), and a poor daily cortisol decline. A blunted CAR was defined as a small difference in cortisol levels between waking and 30 minutes post-waking, and poor daily cortisol decline was defined as a large difference between evening cortisol and waking cortisol levels.

Cortisol dysregulation in infants was measured through elevated basal cortisol at three days old and two months old and a blunted or exaggerated cortisol reactivity at two months old. High basal cortisol (Stroud et al., 2016) and blunted (Tollenaar et al., 2011) and exaggerated (Davis et al., 2011) cortisol reactivity have all been cited as evidence of infant cortisol dysregulation. The basal cortisol measure at three days is meant to serve as a proxy for HPA axis development in response to *prenatal* maternal stress alone in order to improve our understanding of how the postpartum period can then attenuate this prenatal effect. The measurements at two months are meant to reflect HPA axis development as a consequence of both prenatal and postnatal programming.

Infant HPA axis dysregulation serves as our outcome, since it has been associated with metabolic (Reynolds et al., 2001) and neurobehavioral (Davis et al., 2011; O'Connor et al., 2002;

O'Donnell et al., 2013) disorders in offspring and is thought to mediate the effects of the environment on child development (Thomas et al., 2017).

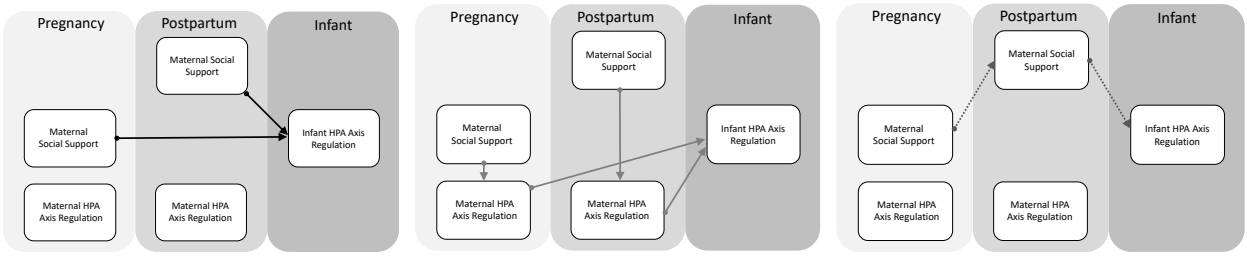
Analytic approach

All statistical analyses were performed using Stata Version 13.1 (StataCorp, College Station, TX). First, we present descriptive sociodemographic and health data for women and their infants, followed by descriptive data on distress variables including stress, depression, and social support, the last of which was determined to be the central factor contributing to women's distress and well-being through qualitative analysis. Next, we present bivariate associations between sociodemographic characteristics and distress data at each visit. Bivariate analyses were completed using Fisher's Exact, t-tests, one-way analysis of variance with a Bonferroni correction, and Pearson's *r* as appropriate. In bivariate tests, age, years married, parity, years living on Galápagos, food security, stress, depression, and friend and family social support were analyzed continuously.

To test Proposition 1 (Figure 5.2a), the direct effect of maternal social support on infant HPA axis regulation, we ran bivariate associations in which both social support and HPA axis dysregulation measures were analyzed continuously. We ran this test independently for prenatal social support and postpartum social support. Proposition 2 (Figure 5.2b), that maternal social support has an indirect effect on infant HPA axis regulation through maternal HPA axis regulation, was tested with path analysis using linear regression models. These paths were tested independently for prenatal and postpartum exposures, such that we examined the indirect effect of prenatal social support on infant HPA axis development through prenatal maternal HPA axis regulation, and we separately examined the indirect effect of postpartum social support on infant HPA axis development through postpartum maternal HPA axis regulation. Postpartum models

that examined Visit 3 social support only used Visit 3 maternal cortisol to measure HPA axis dysregulation, and models that examined Visit 4 social support only used Visit 4 maternal cortisol to measure HPA axis dysregulation. Proposition 3 (Figure 5.2c), that prenatal maternal social support has an indirect effect on infant HPA axis regulation through postpartum maternal social support, was also tested with path analysis using linear regression models. All three propositions were analyzed with social support and HPA axis dysregulation as continuous variables. Social support was examined both through support from family and support from friends.

Figure 5.2 Research propositions



a b c

5.3 Results

Demographic characteristics

Sample characteristics are presented in Table 5.1. At enrollment, the mean age of women was 27.9 (SD = 6.15), and 50% of women had been married for less than five years. Most women had a high school education (65.8%), and almost half (47.4%) were pregnant for the first time. Approximately one third of the sample had been living on the Galápagos for fewer than five years, while roughly a quarter of the sample had lived on the islands for over 20 years.

Nearly half (47.4%) of the population scored as having some degree of food insecurity within the past three months. On average, women were 38 weeks pregnant when they gave birth, and only 2 infants were born preterm (born at <37 weeks of gestation). The mean infant birth weight was 3382 grams, and only one infant in the study was low birth weight (<2500 grams). Just over half of infants were born by Caesarean, and 62.5% of infants were male. Ethnicity was largely homogenous (92% Mestizo).

Qualitative results

Qualitative analysis of interviews suggested that maternal loneliness, confinement to the home, and *machismo* are critical components contributing to the distress of pregnant and postpartum women on San Cristóbal island. Women often discussed their loneliness as stemming from distance from their families, many of which remain on mainland Ecuador. Our sociodemographic data demonstrated that many participants (47%) had moved to the Galápagos without their own families and married men from the islands, and of them, 32% had moved to the islands specifically for marriage, even joking that women's path to the Galápagos is "always the same story." However, due to the National Park's strict immigration laws and the expense of travel, women's families remain on the mainland and typically do not often have the opportunity to visit. Participants often discussed missing their own families or only confiding in their own family over the phone if they did not feel comfortable with their in-laws. Others reported keeping their problems to themselves, so they would not worry family that lives far away.

Further, women's loneliness is compounded by their limited interaction with the community, even before their pregnancies and the births of their infants. Many of the participants expressed feeling closed off from the community and not having many friends. When asked

about who they trust with their problems, women repeatedly suggested that they resolve them on their own or with their immediate family. When asked about their friends, women mostly reported that they had "none," "almost no one," and "almost none at all." Women new to the islands had a particularly difficult time forging close friendships as outsiders new to such a close-knit community. One woman explained:

> *"Here the people are very closed. Except to the tourists and to other Galapagueños. They don't like the people who live on the mainland…They don't like it. They want the islands only for them. So, they discriminate against the people from the mainland. It's a political thing too."*

Forging relationships with others on the island proved even more difficult during pregnancy and the postpartum. While 37% of participants were already full-time homemakers, participants who had previously worked outside the home often reported leaving their jobs when they found out they were pregnant. When asked if they feel connected to others in the community, many participants responded that they do not because they hardly ever leave their homes. When we asked one pregnant participant about how she feels compared to other women on the island, she said:

> *"I don't know because I never leave the house, but when I leave the house I see happy women."*

Further, the custom for a mother and her new baby to stay inside the home for forty days after the infant is born can be socially isolating for new mothers. Women reported that this custom is meant to ensure the young baby's safety by keeping it away from dangerous exposures of the outdoors, like extreme temperatures, the sun, and mosquitoes, among others. In their

narratives, it became clear that many women found this period of confinement and isolation to be distressing and lonely. Women said:

> *"That's what is stressing me out a little, because I want to go and I want to do so many things but I can't…My husband tells me to be patient and I can go back [to work] when the baby is four months old because right now she is too little."*
>
> *"I spend time alone and that stresses me out because, shoot, I don't have support in that regard in the same way [as my sister on the mainland]."*
>
> *"[The doctors said] just the first month, keep the baby in the house and then you can walk with him and all of that. But do not go to the beach. Because first the winter is starting, and maybe the baby can get sick. So, it's all for the baby. And I think sometimes, 'Oh my god, what did I do, I am so tired, it is too hard.' I am like, 'Oh my god, what have we done with our lives?'"*
>
> *"I used to go to the beach. Now I can't with him. The doctors told me that I can't go to the beach until he is 3 months old. He can feel the sun from in here, but he can't go outside."*

Another woman, who suggested that her confinement was causing her to feel depressed, reflected on the opportunity she had to leave the house because her own family was there to provide childcare for a day.

> *"A week ago, I was starting to be in depression. I was feeling so sad, I was taking care of my food and all of that, and I was doing a lot and I wanted to cry and I didn't want to touch the baby. And I told my husband, 'You have to take care of him. I don't want to touch him'… So I talked with my mom and my sister and they told me, 'Listen, you're not feeling good, you're not doing well, you have to get outside of the house, even just one day, come here and I will help you with the baby' … Getting outside was good. I couldn't sleep, but it was good because I got outside of all of this, the house. So today I woke up more animated so I started to clean and I started to do everything. Today I started to feel better."*

Last, strong *machista* sentiments were felt by many women who were stressed and overwhelmed by the amount of work they were expected to do around the house, often when

their partners did not contribute to household or childcare labor. Women frequently reported being stressed not only about their own health and that of their new babies, but also about keeping track of their older children's schoolwork, cooking for the family, doing housework, and managing their partners. Women said:

> *"For the great majority it's the husband that is causing the stress. Here it is like that because the men here are really machista. It's like every weekend. Men that don't drink are rare. The great majority here… they leave [their wives] alone with the kids, and they don't have support, and that causes stress. That's the main thing. The women need to do practically everything."*

Another participant, when asked about household labor, suggested at first that women have help because the husbands are around, but continued on to say that despite a husband's presence, he does not necessarily contribute to the home. She said:

> *"They have support, but for example, someone might have a husband here but the husband prefers to go out and play soccer."*

On the whole, the qualitative analysis provided the critical insight that social support was an important predictor of women's well-being on the Galápagos in many ways. All the central themes, including geographic isolation from family, inability to forge friendships, confinement to the home, and the *machismo* culture, resulted in loneliness and limited social support for pregnant and postpartum women. Based on these results, we used social support as a contextually salient variable for distress in our models of HPA axis development.

Maternal distress: social support, stress, and depression

Informed by our qualitative analysis, we examined maternal social support as our primary distress exposure alongside stress and depression. Table 5.2 shows the rates of each of these variables throughout the study period. Generally, support from family was slightly higher in the postpartum, and support from friends was lowest at one month postpartum. Notably, more women experienced high stress during the postpartum period than during pregnancy. Rates of depression were highest at one month postpartum but lowest at two months postpartum.

Next, we tested bivariate associations between sociodemographic characteristics and the distress variables at each time period as well as associations of distress co-occurrences (Table 5.3). All variables were analyzed continuously. Among the distress factors, low support during pregnancy, both from family and from friends, was often associated with low support in the postpartum as well. Low friend support during pregnancy was also associated with depression at that time, and low family support at two months postpartum was also associated with high stress at that time. In addition, prenatal stress was independently associated with postpartum stress at each time point and often with higher prenatal depression scores during pregnancy and the postpartum. High depression scores during pregnancy were independently associated with higher stress during and after pregnancy and higher depression scores in the postpartum.

Modeled results

Proposition 1: Does maternal social support have a direct effect on infant HPA axis regulation?

Bivariate associations between maternal distress variables and measures of maternal and infant HPA axis dysregulation are shown in Table 5.4. During pregnancy (Visit 1), low family support was significantly associated with higher infant basal cortisol at two months of age (Visit

4), and low friend support was associated with higher infant basal cortisol at 3 days postpartum (Visit 2) but not two months postpartum.

In the postpartum, low family support at one month postpartum (Visit 3) and at two months postpartum were independently associated with higher infant basal cortisol at two months postpartum. None of the social support exposures were significantly associated with differences in infant cortisol reactivity.

Proposition 2: Does maternal social support have an additional indirect effect on infant HPA axis regulation through maternal HPA axis regulation?

During pregnancy, low family support and low friend support were each independently associated with higher maternal morning cortisol and a blunted maternal CAR during pregnancy, indicating that low social support may dysregulate the maternal HPA axis. We then tested associations between prenatal maternal HPA axis dysregulation and infant HPA axis dysregulation to assess how cortisol dysregulation might be transferred biologically during pregnancy. Results for all associations between maternal and infant HPA axis regulation measures are shown in Table 5.5. No significant associations ($p \leq 0.05$) were found between maternal HPA axis dysregulation during pregnancy and infant HPA axis dysregulation.

In the postpartum period, no measure of social support was significantly associated with any measure of maternal HPA axis dysregulation. Nonetheless, we tested associations between postpartum maternal HPA axis dysregulation and infant HPA axis dysregulation. Results showed that elevated maternal evening cortisol at one month postpartum was independently associated with elevated infant basal cortisol and with a blunted infant cortisol reactivity at two months. Further, maternal cortisol decline at one month postpartum was also associated with a blunted

cortisol reactivity. These results provide evidence that maternal HPA axis dysregulation at one month postpartum is associated with infant HPA axis dysregulation.

To ensure that the correlations between maternal and infant HPA axis regulation were not merely a consequence of the fact that they are both caused by maternal support, we used linear regression models to assess whether maternal HPA axis regulation was associated with infant HPA axis regulation while controlling for postpartum social support. Results showed that these relationships remained significant in the following four models: 1) Elevated maternal evening cortisol at one month postpartum was associated with infant basal cortisol at two months postpartum while controlling for *family* support ($R^2 = 0.40$, $p \leq 0.01$), 2) Elevated maternal evening cortisol at one month postpartum was associated with infant cortisol reactivity at two months postpartum while controlling for *family* support ($R^2 = 0.30$, $p = 0.03$), 3) Elevated maternal evening cortisol at one month postpartum was associated with infant basal cortisol at two months postpartum while controlling for *friend* support ($R^2 = 0.26$, $p = 0.05$), 4) Elevated maternal evening cortisol at one month postpartum was associated with infant cortisol reactivity at two months postpartum while controlling for *friend* support ($R^2 = 0.33$, $p = 0.02$). A summary of significant results from proposition modeling is shown in Table 5.6.

Results from these analyses do not support the proposition that maternal social support has an indirect effect on infant HPA axis regulation through maternal HPA axis regulation during the pregnancy or the postpartum. The indirect effect during pregnancy was not supported because no significant associations were found between maternal HPA axis dysregulation during pregnancy and infant HPA axis dysregulation. The indirect effect in the postpartum was not supported because no significant associations were found between postpartum social support and maternal HPA axis dysregulation.

Proposition 3: Does maternal social support during pregnancy have an additional indirect effect on infant HPA axis regulation through postpartum social support?

First, bivariate analyses demonstrated that low maternal family support during pregnancy was associated with low family support at both postpartum time points and with low friend support at both postpartum time points. Low friend support during pregnancy was associated with low friend support at both postpartum time points. Previous tests from Proposition 1 showed that low family support at one month postpartum and at two months postpartum were independently correlated with higher infant basal cortisol at two months postpartum. Postpartum friend support was not associated with infant HPA axis measures.

To ensure that the association between postpartum support and infant HPA axis regulation was not a consequence of the fact that they are both caused by maternal support during pregnancy, we tested relevant models using linear regression to examine whether postpartum social support was associated with infant HPA axis regulation while controlling for social support during pregnancy. Results showed that association remained significant for the association between family postpartum support at one month and infant HPA axis dysregulation ($R^2 = 0.26$, $p=0.04$), but not for family postpartum support at two months and infant HPA axis dysregulation ($R^2 = 0.22$, $p=0.06$).

These analyses indicate that in some cases, postpartum family support has a significant indirect effect on the relationship between family social support during pregnancy and infant HPA axis dysregulation.

5.4 Discussion

In the present study, we used qualitative methods to identify social support as a contextually salient measure of distress for pregnant and postpartum women on the Galápagos Islands. Using a DOHaD and developmental niche framework, we then built conceptual models to examine how specifically maternal social support influenced maternal and infant biology both during and after pregnancy. Results demonstrate that maternal social support both during and after pregnancy is associated with infant HPA axis regulation (Proposition 1), and that postpartum social support has an additional indirect effect on the relationship between prenatal social support and infant HPA axis development (Proposition 3), suggesting that the HPA axis retains plasticity through the first few months of life. Nonetheless, the data did not support our hypothesis that maternal HPA axis regulation has an indirect effect on relationship between maternal social support and infant HPA axis regulation during pregnancy or in the postpartum (Proposition 2).

Maternal social support

Through recurrent themes of participants' distance from their families, inability to forge local friendships, confinement to the home, and the culture of *machismo*, our qualitative analyses informed the identification of social support as a central, context-specific driver of maternal mental health and well-being on the Galápagos Islands, a region isolated both geographically and socially from the rest of Ecuador. Social support is also a salient distress exposure conceptually, as many studies have found that social support buffers the HPA axis against insult from other maternal distress factors (Hostinar, Sullivan, & Gunnar, 2014).

Social support and infant HPA axis

Here, informed by our qualitative analyses, we expanded our understanding of distress beyond traditional measures of stress and depression by using low social support as the central distress exposure. Evidence provided support for Proposition 1, that low maternal social support was associated with infant HPA axis dysregulation independently during pregnancy and during the postpartum. Notably, neither stress nor depression scores were associated with infant HPA axis function, which lends support to our hypothesis that social support itself is a salient measure of maternal distress and does not merely moderate the effects of other distress exposures, as many other studies have shown (Hostinar et al., 2014; Jewell, Luecken, Gress-Smith, Crnic, & Gonzales, 2015; Thomas et al., 2018). Our results suggest that limited social support may dysregulate the infant HPA axis by chronically elevating infant basal cortisol but not by influencing the stress response. Maternal family support had more persistent effects on infant basal cortisol than friend support, which was not associated with infant basal cortisol by two months postpartum. Few other studies have examined the direct effects of social support on infant HPA axis functioning, but one recent study found that partner support during pregnancy is associated with lower infant cortisol reactivity through maternal depression (Thomas et al., 2017). More broadly, prenatal social support has been associated with higher birth weight (Feldman, Dunkel-Schetter, Sandman, & Wadhwa, 2000) and a positive effect on infant temperament (Stapleton et al., 2012).

Other studies have found that a variety of distress exposures during pregnancy may influence infant HPA axis dysregulation. For example, in one study, the infants of women with PTSD had lower cortisol at waking and bedtime (Yehuda et al., 2005), and studies have found an elevated cortisol response in infants whose mothers experience: material deprivation (Thayer &

Kuzawa, 2014), discrimination (Thayer & Kuzawa, 2015), depression (Brennan et al., 2008), and anxiety (Grant et al., 2009). In the postpartum, parental depression symptoms have been associated with decreased child cortisol (Laurent et al., 2013).

The indirect effect of maternal HPA axis regulation

Our models did not support Proposition 2, that maternal HPA axis regulation mediated the relationship between maternal social support and infant HPA axis function. Nonetheless, Thomas and colleagues did find support for this mediation (Thomas et al., 2018). In our analyses, the pregnancy model was rejected because no significant associations were found between maternal HPA axis dysregulation during pregnancy and infant HPA axis dysregulation. Other studies have found significant relationships between these variables, including associations between maternal elevated prenatal cortisol and blunted cortisol reactivity (O'Connor et al., 2012; Tollenaar et al., 2011), exaggerated cortisol reactivity (Davis et al., 2011; Gutteling et al., 2004; Tollenaar et al., 2011), and high basal cortisol (O'Connor et al., 2012). One study found an association between maternal CAR during pregnancy and blunted infant cortisol reactivity (Nazzari et al., 2019). Further, our analyses and others' (Harville et al., 2009) found that social support was associated with maternal HPA axis functioning during pregnancy, and many others have found similar associations between prenatal distress measures and dysregulated maternal cortisol (Diego et al., 2004; Thayer & Kuzawa, 2014, 2015; van den Heuvel et al., 2018). Together, these mixed results suggest that future research should continue to investigate this mediation pathway.

We did not find support for our postpartum model, because we did not find significant associations between postpartum social support and maternal HPA axis dysregulation. Jewell and

colleagues did find an association between low family support and high maternal cortisol in the postpartum (Jewell et al., 2015), but research on the association between other distress measures and HPA axis functioning in the postpartum is mixed. Some studies have found an association between postpartum blues or depression and maternal HPA axis functioning (Ehlert et al., 1990; Groer & Morgan, 2007; Parry et al., 2003; Taylor et al., 2009), while others have not (Shimizu et al., 2015), suggesting that more work on this question should be done to understand the biological underpinnings of postpartum distress.

Notably, though, the present study did find a significant association between postpartum maternal HPA axis regulation and infant HPA axis functioning, indicating the importance of untangling the biological mechanisms behind maternal HPA axis function. This association may be the result of maternal parenting behaviors, or more directly, a product of cortisol transfer through breastmilk (Glynn et al., 2007; Rudzik et al., 2014).

Postpartum social support as a mediator

Our analysis did lend support to our third proposition, that postpartum social support is a partial mediator for the relationship between prenatal social support and infant HPA axis development, suggesting that postpartum experience can attenuate prenatal insults to infant development. Few studies on the relationship between prenatal distress and infant HPA axis development have measured postpartum distress. Of those that have, one study found that despite an increase in offspring basal cortisol in response to both prenatal and postpartum depression, newborn physiology was more dependent on prenatal depression (Diego et al., 2004). Studies on other infant outcomes have found that postnatal mother-child relationship, which is likely shaped

by maternal psychosocial stress, mediated the relationship between prenatal cortisol and infant health outcomes (Bergman et al., 2010).

These results, and our own findings, demonstrate the importance of the postpartum period for infant development, and specifically the importance social support. Further, these results give support to theoretical model for the developmental niche, which proposes that the child's social setting, customs of childcare, and psychology of caretakers are all critically important for early development (Harkness & Super, 1994). Notably, each of these factors are likely influenced by a mother's social support, which may protect her health through two physiological mechanisms. First, emotional social support may increase a woman's confidence in her ability to cope with stressors, allowing her to respond in a more biologically well-regulated way, and thus avoiding chronic stress (Jewell et al., 2015; Uchino, 2006). Second, physical social support may grant women trusted allies with whom they can leave their child to regroup, sleep, or just get out of the house, as demonstrated through our qualitative analysis. Maternal social support can also be protective for infant development since cortisol may be passed from mother to child through breastmilk (Glynn et al., 2007) and women with more social support may have differences in parenting behaviors and infant attachment (Spieker & Bensley, 1994).

Strengths and limitations

This study utilizes a longitudinal, mixed-methods design to analyze rich narrative interviews in order to identify the complex factors that are most important to the well-being of pregnant and postpartum women in the Galápagos. Using a truly biocultural design, this study uses qualitative results to build quantitative models and better understand how maternal experience is embodied in mothers and infants in this context. Further, this is one of the first

studies to address the experience of peripartum distress and its effects on infant development in a middle-income country. While many studies have investigated either prenatal or postpartum distress and development, this work is one of the few to integrate the pre- and postpartum period into a continuum of development.

Nonetheless, this work is not without limitations. The study's small sample size limits its statistical power, but we estimate that we captured more than 50% of births on the island in 2018 based on annual birth rates. Second, some participants were lost to follow-up, primarily due to participant travel to the mainland to visit family. We also faced challenges collecting a sufficient amount of infant saliva for analysis, particularly at 3 days postpartum, when infants are not producing much saliva. These challenges limit our statistical power and the generalizability of results. Nonetheless, this study does detect clear and significant relationships between social support and HPA axis function, and thus it may serve as foundational exploratory research for larger projects that seek to investigate the relationships assessed here. More integrative research is needed to investigate how specifically maternal social support and the postpartum period continue to shape infant HPA axis development after birth.

5.5 Conclusions

Overall, we find that maternal social support both during and after pregnancy is associated with infant HPA axis regulation, and that postpartum social support has an additional indirect effect on the relationship between prenatal social support and infant HPA axis development. These results suggest that the young HPA axis retains plasticity through the first few months of life, and that postnatal experience can attenuate prenatal insults to infant development. This understudied pathway is particularly important for informing interventions on

maternal well-being and early infant HPA axis development, which has long-term consequences on cardiometabolic and neurobehavioral systems (Davis et al., 2011; O'Connor et al., 2002; O'Donnell et al., 2013). Nonetheless, our results did not support our hypothesis that maternal HPA axis regulation has an indirect effect on the relationship between maternal social support and infant HPA axis regulation during pregnancy or in the postpartum, suggesting that the mechanism for this relationship may be due more to shifts in mothers' parenting behavior and less to the transfer of cortisol biologically. By incorporating the culturally-relevant role of social support during both pregnancy and the postpartum into a model for infant HPA axis development, this study adds a critical component to the literature on the developmental origins of health and disease that will elucidate the pathways through which early environment shapes development.

Table 5.1 Sociodemographics and infant characteristics

Demographic Characteristics	
Age, mean (SD)	27.89 (6.15)
Married	89.5%
Years married	
0 – 2	23.5%
3 – 5	26.5%
6 – 9	32.4%
10 – 15	17.7%
Highest education	
Completed high school	65.8%
Completed college	26.3%
Primipara	47.4%
Parity before birth, mean (SD)	0.76 (0.85)
Born and raised on Galápagos	36.8%
Years living on Galápagos	
0 – 4.9 years	31.6%
5 – 9.9 years	10.5%
10 – 14.9 years	21.1%
15 – 19.9 years	10.5%
$\geq$ 20 years	26.3%
Food security	
Secure	52.6%
Mild food insecurity	36.9%
Moderate food insecurity	7.9%
High food insecurity	2.6%
Pregnancy and Infant Characteristics	
Weeks pregnant at delivery, mean (SD)	38.33 (1.32)
Infant birth weight (g), mean, (SD)	3382.59 (366.61)
Caesarean delivery	51.7%
Male offspring	62.5%
Weight-for-length z-score at 2 months pp	0.31 (1.74)
Length-for-age z-score at 2 months pp	0.17 (1.06)

Table 5.2 Distress throughout the peripartum period

	Visit 1 (N = 38)	**Visit 3 (N = 28)**	**Visit 4 (N = 25)**
Family support, mean (SD)	13.11 (2.99)	13.37 (3.16)	13.20 (3.16)
Friend support, mean (SD)	8.16 (2.90)	7.33 (2.95)	8.32 (2.69)
Depression	26.3%	28.6%	16.0%
Stress			
Low	34.2%	36.8%	42.1%
Moderate	65.8%	36.8%	21.1%
High	0%	26.3%	36.8%

V1, V3, and V4 refer to Visits 1, 3, and 4, and reflect data from the visits at 34-36 of weeks gestation, one month postpartum and two months postpartum respectively

Table 5.3 Predictors of social support, stress, and depression, represented as correlation coefficients (*r*)

Characteristic		Stress			Depression			Family Support			Friend Support		
		V1	V3	V4	V1	V3	V4	V1	V3	V4	V1	V3	V4
Demographics													
Age		0.24	0.06	0	-0.09	-0.08	0.09	-0.04	0.12	0.01	0.18	0.32	0
Years married		-0.02	0.28	0.04	-0.03	0.11	0.37	-0.05	0.28	0.23	-0.06	-0.01	-0.18
Education		0.17	0.12	-0.12	-0.14	0.05	-0.09	0.12	0.15	0.01	0.12	0.11	0.09
Parity		-0.07	0.17	0.03	0.19	0.07	0.14	-0.27	-0.25	-0.23	-0.18	0.07	-0.15
Years living on Galápagos		-0.13	0.13	-0.03	-0.28	-0.16	-0.06	-0.13	0.19	0.14	-0.05	0.21	-0.01
Food Insecurity		0.29	**0.51***	**0.41***	**0.40***	0.33	**0.58***	-0.22	-0.08	-0.11	-0.18	-0.01	0.06
Distress Surveys													
Stress	**V1**		**0.43***	**0.55***	**0.40***	0.35	0.35	-0.14	-0.09	-0.22	-0.15	-0.01	-0.10
	V3			**0.53***	0.32	**0.73***	**0.58***	0.06	-0.04	-0.16	-0.07	-0.15	-0.21
	V4				**0.65***	**0.43***	**0.63***	-0.26	-0.32	**-0.41***	-0.05	-0.33	-0.28
Depression	**V1**					**0.51***	**0.63***	-0.27	-0.34	**-0.38***	**-0.40***	-0.34	-0.29
	V3						**0.71***	-0.04	-0.12	-0.25	-0.31	-0.28	-0.31
	V4							0.10	0.05	-0.06	-0.07	-0.17	-0.20
Family support	**V1**								**0.82***	**0.82***	**0.53***	**0.46***	**0.52***
	V3									**0.94***	0.24	**0.40***	0.34
	V4										0.15	0.33	0.37
Friend support	**V1**											**0.79***	**0.80***
	V3												**0.86***
	V4												

* Indicates $p \leq 0.05$

V1, V3, and V4 refer to Visits 1, 3, and 4, and reflect data from the visits at 34-36 of weeks gestation, one month postpartum and two months postpartum respectively

Table 5.4 Bivariate associations between distress and HPA axis dysregulation in mothers and infants, represented as correlation coefficients (*r*)

		Maternal morning cortisol			Maternal evening cortisol			Maternal cortisol awakening response (CAR)			Maternal cortisol decline			Infant basal cortisol		Infant cortisol response
		V1	**V3**	**V4**	**V1**	**V3**	**V4**	**V1**	**V3**	**V4**	**V1**	**V3**	**V4**	**V2**	**V4**	**V4**
Family support	**V1**	**-0.36***	-0.09	-0.26	-0.22	-0.32	-0.23	**0.34***	-0.02	0.11	0.10	-0.21	-0.01	0.02	**-0.42***	0.35
	V3		0.02	-0.14		-0.30	-0.02		-0.03	0.03		-0.28	0.10		**-0.51***	0.18
	V4			-0.13			-0.06			0.03			0.06		**-0.47***	0.18
Friend support	**V1**	**-0.38***	0.29	-0.06	-0.14	-0.20	-0.26	**0.46***	-0.04	0.13	0.18	**-0.38***	-0.23	**-0.45***	-0.11	0.25
	V3		0.37	0.04		-0.02	0.08		-0.19	0.18		-0.28	0.03		-0.12	0
	V4			0.09			-0.13			0.28			-0.20		-0.25	0.20
Stress	**V1**	0.08	-0.03	-0.03	-0.13	0.34	0.17	-0.04	-0.07	0.25	-0.16	0.32	0.17	-0.10	0.19	-0.20
	V3		-0.11	-0.19		0.09	0.07		0.23	0.15		0.16	0.21		-0.26	0.40
	V4			-0.23			0.06			0.13			0.25		0.30	0.01
Depression	**V1**	0.27	0.15	-0.29	0.14	0.30	0.18	**-0.36***	-0.24	0.20	-0.09	0.16	0.39	0.07	0.18	0.01
	V3		-0.34	-0.38		0.12	-0.03		0.26	0.08		0.35	0.27		-0.18	0.15
	V4			**-0.44***			-0.03			0.10			0.32		-0.14	0.15

* Indicates $p \leq 0.05$

V1, V2, V3, and V4 refer to Visits 1, 2, 3, and 4, and reflect data from the visits at 34-36-weeks of gestation, 3 days postpartum, one month postpartum, and two months postpartum respectively

Table 5.5 Bivariate associations between maternal and infant HPA axis dysregulation, represented as correlation coefficients (*r*)

		Infant basal cortisol		Infant cortisol response
		V2	**V4**	**V4**
Morning cortisol	**V1**	0.43	0.13	-0.07
	V3	-0.13	0.24	-0.16
	V4	-0.25	0.22	-0.25
Evening cortisol	**V1**	-0.04	0.28	-0.02
	V3	0.11	**0.51***	**-0.55***
	V4	0.22	0.17	-0.18
CAR	**V1**	-0.39	-0.18	0.16
	V3	-0.11	0.11	-0.11
	V4	0.07	-0.24	0.28
Cortisol decline	**V1**	-0.40	0.13	0.04
	V3	0.21	0.33	**-0.42***
	V4	0.39	0.02	-0.01

* Indicates $p \leq 0.05$

V1, V2, V3, and V4 refer to Visits 1, 2, 3, and 4, and reflect data from the visits at 34-36 weeks of gestation, 3 days postpartum, one month postpartum, and two months postpartum respectively

Table 5.6 Significant associations in propositions models

Proposition 1			
Social Support	**Infant HPA Axis Dysregulation**	**Coeff. (*r*)**	**p-value**
↓ V1 Family	↑ V4 Basal cortisol	-0.42	0.04
↓ V1 Friend	↑ V2 Basal cortisol	-0.45	0.05
↓ V3 Family	↑ V4 Basal cortisol	-0.51	0.01
↓ V4 Family	↑ V4 Basal cortisol	-0.47	0.02
Proposition 2			
Social Support	**Maternal HPA Axis Dysregulation**	**Coeff. (*r*)**	**p-value**
↓ V1 Family	↑ V1 morning cortisol	-0.36	0.04
↓ V1 Family	↓ CAR	0.38	0.05
↓ V1 Friend	↑ V1 morning cortisol	-0.38	0.03
↓ V1 Friend	↓ CAR	0.46	≤ 0.01
Maternal HPA Axis Dysregulation	**Infant HPA Axis Dysregulation**		
↑ V3 Evening cortisol	↑ V4 Basal cortisol	0.51	0.01
↑ V3 Evening cortisol	↓ V4 Reactivity	-0.55	≤ 0.01
↑ V3 Cortisol decline	↓ V4 Reactivity	-0.42	0.05
Maternal HPA Axis Dysregulation	**Infant HPA Axis Dysregulation**	**Coeff. (adj R^2)**	
[1]↑ V3 Evening cortisol	↑ V4 Basal cortisol	0.40	≤ 0.01
[1]↑ V3 Evening cortisol	↓ V4 Reactivity	0.30	0.03
[2]↑ V3 Evening cortisol	↑ V4 Basal cortisol	0.26	0.05
[2]↑ V3 Evening cortisol	↓ V4 Reactivity	0.33	0.02
Proposition 3			
Prenatal Support	**Postpartum Support**	**Coeff. (*r*)**	**p-value**
↓ V1 Family	↓ V3 Family	0.82	≤ 0.01
↓ V1 Family	↓ V4 Family	0.82	≤ 0.01
↓ V1 Family	↓ V3 Friend	0.46	0.02
↓ V1 Family	↓ V4 Friend	0.52	≤ 0.01
↓ V1 Friend	↓ V3 Friend	0.79	≤ 0.01
↓ V1 Friend	↓ V4 Friend	0.80	≤ 0.01
Postpartum Support	**Infant HPA Axis Dysregulation**		
↓ V3 Family	↑ V4 Basal cortisol	-0.51	0.01
↓ V4 Family	↑ V4 Basal cortisol	-0.47	0.02
Postpartum Support	**Infant HPA Axis Dysregulation**	**Coeff. (adj R^2)**	
[3]↓ V3 Family	↑ V4 Basal cortisol	0.26	0.04

Associations presented here are bivariate unless otherwise indicated with superscripts 1, 2, and 3.
[1] Controlling for family support at V3, [2] Controlling for friend support at V3, [3] Controlling for family support at V1

CHAPTER 6: MATERNAL STRESS DURING PREGNANCY IS ASSOCIATED WITH PLACENTAL 11β-HYDROXYSTEROID DEHYDROGENASE TYPE 2 METHYLATION AND EXPRESSION IN HUMANS: PSYCHOSOCIAL AND PHYSIOLOGICAL PATHWAYS

6.1 Introduction

The developmental origins hypothesis suggests that early life environments can shape long-term disease risk (Gluckman et al., 2005). A growing body of literature supportive of this hypothesis has found that maternal stress during pregnancy, including stress, anxiety, depression, and other factors, have been associated with long-term effects on metabolic functioning and neurobehavioral disorders in offspring (Reynolds, 2013). Specifically, prenatal stressors have been associated with increased risk for schizophrenia (Khashan et al., 2008), autism, ADHD (Ronald, Happé, Dworzynski, Bolton, & Plomin, 2010), and impaired cognitive development (King & Laplante, 2005). Despite strong epidemiological evidence for these associations, the underlying biological mechanisms remain unclear.

The activation of the hypothalamic-pituitary-adrenal axis (HPA axis), which regulates glucocorticoid feedback interactions among the mother, placenta, and fetus during pregnancy, has been proposed to be the primary mechanism through which prenatal maternal stress shapes fetal development and subsequent long-term disease risk (Pike, 2005; Seckl, 2008). While glucocorticoids play an essential role in fetal development (Drake et al., 2012), high levels of cortisol may alter fetal behavioral, immunological, and neurological development, including areas of the brain that regulate the fetal HPA axis (Beijers et al., 2014). Theorists propose that frequent or prolonged maternal stressors activate the maternal HPA axis, increasing the

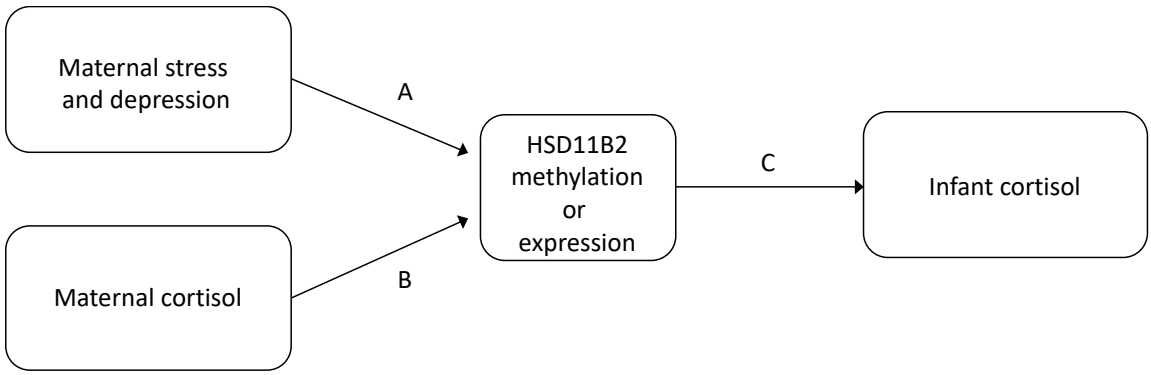
production of cortisol, which could be transferred to the infant through the placenta and/or trigger an increased production of placental corticotropin-releasing hormone (CRH), thereby stimulating the fetal HPA axis to produce more fetal cortisol (Beijers et al., 2014). Increased fetal cortisol, in turn, has permanent effects on fetal HPA axis development, which could underlie subsequent long-term disease risk (Chrousos, 2009; Pike, 2005; Seckl, 2008). However, results from studies on prenatal maternal distress and offspring development have demonstrated mixed results, suggesting that other mechanisms may play a role in the relationship between prenatal stress and infant HPA axis development.

One such mechanism may be that of the placental enzyme, 11β-hydroxysteroid dehydrogenase type 2 (HSD11B2), which buffers the level of cortisol that reaches the fetus by catalyzing a reaction that converts active cortisol to inert cortisone in the placenta (V. E. Murphy et al., 2006). In this way, HSD11B2 is hypothesized to have protective effects for the fetus by minimizing glucocorticoid exposure (Edwards et al., 1993). Recently, researchers have begun to utilize both animal and human models to investigate the relationship between maternal stress and various measures of HSD11B2, hypothesizing that maternal distress itself may up-regulate the enzyme as an adaptive, protective measure for the fetus or down-regulate the enzyme due to energetic or other limitations. Nonetheless, studies have reported mixed results, and often sex-specific results, but little remains known about this enzyme and what influences its functioning and expression.

In the present study, we assess how prenatal distress influences the methylation and expression of placental HSD11B2 and how these differences shape infant HPA axis development. Our conceptual model is shown in Figure 1. Specifically, we investigate: Path A) how prenatal maternal psychosocial distress, measured through stress and depression symptoms,

influences placental HSD11B2; Path B) how prenatal maternal physiological distress, measured through maternal cortisol regulation, influences HSD11B2; and Path C) how differences in HSD11B2 are associated with infant cortisol regulation. Analyses were run independently for placental DNA methylation of the *HSD11B2* gene and mRNA expression of HSD11B2 on each path.

Figure 6.1 Conceptual model



6.2 Methods

Participants

The data were collected over 12 months in 2018 on the Galápagos Islands, where limited infrastructure and geographic isolation contribute to daily distress for residents. Participants were recruited from Hospital Oskar Jandl (HOJ), a public hospital on the Galápagos' provincial capital island San Cristóbal, using purposive sampling (N = 26). HOJ is free for all residents and is the only hospital on the island, allowing the research team to screen all pregnant women on the island within the recruitment period. Inclusion criteria included women between the ages of 18 and 50 years who planned to give birth on the island. Visits with participants were conducted

once during pregnancy, at 34 – 36 weeks gestation, and once in the postpartum, when the infant was two months old. For each mother-infant dyad, the research team collected surveys on stress and depression symptoms, placental samples, and maternal and infant saliva samples. All visits were conducted in the participants' homes, places of work, the hospital, or the Galápagos Science Center (GSC). All participants provided written informed consent prior to participation under appropriate protocols approved by the Institutional Review Boards for the University of North Carolina at Chapel Hill (UNC-Chapel Hill) and *Universidad San Francisco de Quito*. This project was also approved by Ecuador's Ministry of Public Health.

Maternal stress and depression

Stress. The Perceived Stress Scale (PSS) (S. Cohen et al., 1983) was used to assess maternal chronic stress by measuring the degree to which situations are perceived as unpredictable, uncontrollable, and burdensome (S. Cohen et al., 1983). The PSS is particularly relevant for pregnancy, since it does not operationalize symptoms that occur frequently in both pregnancy and during times of stress (such as sleep disturbances) to assess chronic stress (Nast et al., 2013). We used a Spanish version of this scale that has been tested for reliability, validity, and sensitivity (Remor, 2006) in Spanish-speaking contexts. When analyzed categorically, the PSS is scored from 0 to 40, with scores of 0 – 13 indicating low stress, scores of 14 – 26 indicating moderate stress, and scores 27 – 40 indicating high stress. In adjusted regression models, stress is analyzed continuously.

Depression. Depression symptoms were measured by the Patient Health Questionnaire-8 (PHQ-8) (Kroenke et al., 2001). We used a Spanish version of the PHQ-8 that has been validated (Baader et al., 2012) and tested for reliability (Cassiani-Miranda et al., 2017) in the Spanish

language. The PHQ-8 is scored from 0 to 24, with a higher score indicating more depression symptoms. When analyzed categorically, depression was defined using the CDC's diagnostic cut-point for the PHQ-8, as scores greater than or equal to 10 (Kroenke et al., 2009). In adjusted regression models, depression symptoms are analyzed both continuously and categorically (depressed vs. not depressed).

Maternal salivary cortisol

Salimetrics guides were used for maternal saliva collection and storage protocols (Salimetrics & SalivaBio, 2015). Maternal saliva samples were collected at 34 – 36 weeks of pregnancy. Over the course of one day, women provided three samples: one immediately upon awakening, one 30 minutes after awakening, and one prior to sleep. Participants were instructed not to eat, drink, or brush their teeth in the 30 minutes prior to collecting samples and to record start and stop times for saliva collection. Participants stored samples in their own freezers until a study team member retrieved them the next day and transported them to the Galápagos Science Center (GSC) where they were frozen at -20° C until analysis.

Cortisol dysregulation in mothers was measured in four ways: elevated morning cortisol, a blunted cortisol awakening response (CAR) at 30 minutes after waking, elevated evening cortisol, and a poor daily cortisol decline. A blunted CAR was defined as a small difference in cortisol levels between waking and 30 minutes post-waking, and poor daily cortisol decline was defined as a large difference between evening cortisol and waking cortisol levels.

Infant salivary cortisol

Infant cortisol was measured when the infant was two months old. At this visit, a saliva sample was collected before and 20-25 minutes after a stressor per a previously published infant stress reactivity protocol (Tollenaar et al., 2011). In our analyses, baseline cortisol is the first of these measures, and cortisol reactivity is the difference between these two measures. All infant saliva samples were collected using Salimetrics Infant Swabs and placed into Salimetrics Swab Storage tubes and frozen at -20° C until analysis.

Cortisol dysregulation in infants was measured as elevated baseline cortisol and as a blunted or exaggerated cortisol reactivity at two months old. High baseline cortisol (Stroud et al., 2016) and blunted (Tollenaar et al., 2011) and exaggerated (Davis et al., 2011) cortisol reactivity have all been cited as evidence of infant cortisol dysregulation (see Chapter 5).

Demographic and obstetric characteristics

Maternal demographics and health history were collected at the initial visit, and obstetric and infant characteristics including gestational age, infant sex, infant birth weight, mode of delivery, and placental weight were recorded by hospital staff at HOJ during the birth.

Placental collection

Placental samples were collected in collaboration with doctors and nurses at Hospital Oskar Jandl. Healthy, intact placentas were collected immediately and dissected within one hour of birth. Umbilical cords were removed, and placentas were weighed and measured. Maternal decidua was removed, and tissue samples were taken from four sampling sites on the fetal side of the placenta. The four sampling sites were selected in each of the four quadrants of the placenta

that were at least 2 cm from the umbilical cord insertion site and at least 3 cm from the placental edge, following protocol by Burton and colleagues (Burton et al., 2014). Samples were cut from each sampling site and pooled into one storage tube to control for intra-placental variation. The samples were stored in RNA*later* (Life Technologies, Grand Island, NY) and stored at -20°C at the hospital. Samples were then sent on ice to UNC-Chapel Hill and Duke University for analysis.

DNA Methylation analysis

The analysis of *HSD11B2* methylation was conducted at the Murphy Lab at Duke University. Placental genomic DNA was extracted using the Lysing Matrix A from MP Biomedical with the FastPre24 for homogenization, followed by the Solid Tissues Protocol from Purgene. DNA samples were sodium bisulfite modified using the EZ DNA Methylation™ Kit (Zymo Research, Irvine, CA), and pyrosequencing was performed on PCR product amplified from bisulfite-modified DNA based on the region sequenced and displaying differential methylation in human placenta from Alikhani-Koopaei and colleagues (Alikhani-Koopaei et al., 2004). The extent of methylation at the HSD11B2 promoter region was examined with pyrosequencing using the Pyromark Pyrosequencing System (Qiagen Inc.) using the following forward and biotinylated reverse primers for amplificiation, Sequence (5'-3') (IDT Inc., Coralville, IA): HSD11B2-F2-AAGTTTTGGAAGGAAAGGGAAGA, HSD11B2-R2-[btn] ACAAAACCTACCTAAAACAAAAACTA, HSD11B2-S- GGGGTAGAGATTTTAA GAA.

The region analyzed contains four CpGs sites of interest (Alikhani-Koopaei et al., 2004) with reactions performed in duplicate. Sodium bisulfite–modified, fully methylated referent positive control and fully unmethylated (whole genome amplified) negative control DNA

(Qiagen) were examined with each batch. The percent methylation at each CpG site was quantified using the PyroMark CpG software, version 1.0.11. (Qiagen). Methylation across each of the four HSD11B2 CpG sites was averaged to obtain an overall measure of methylation. *HSD11B2* methylation was treated as a continuous variable in analyses.

mRNA gene expression analysis

The analysis of HSD11B2 expression was conducted at the Microbiome Core Facility at the University of North Carolina at Chapel Hill. Total RNA was isolated using Qiagen RNeasy Extraction Kit, with the addition of DNaseA digest per the manufacturer's guidelines. Total RNA was quantified and normalized to 50ng/ul prior to the synthesis of cDNA. 500 ng total RNA was subject to cDNA synthesis via qScript cDNA synthesis kit. Expression of HSD11B2 was analyzed using the following primers (Capron et al., 2018): HSD11B2 forward: CTACTCATGGACACATTCAGCT, reverse: TCACTGACTCTGTCTTGAAGC.

Quantitative PCR was performed on QuantStudio Q6, using BioRad PowerSyber qPCR kit. The thermal cycling conditions were as follows: one cycle at 50°C for 20 sec, 95°C for 10 minutes, followed by 40 cycles of 15 seconds at 95°C, 1 minute at 60°C. Melting curve analysis was carried out using the continuous method from the Q6 Software (Applied Biosystems) conducted at 60°C, with increments of 1°C for 15 seconds. Data analysis was carried out with Q6 Software (Applied Biosystems). The auto threshold and baseline options were used for the calculations of cycle threshold (CT) values per well. Gene expression within placental tissue was calculated using ΔCT to correct for internal variation between the placentae by adjusting for a well-regulated and stable housekeeping gene, RPL19. In order to ease interpretation of models,

ΔCT scores have been inverted [$x(-1)$], so that a higher ΔCT value indicates higher HSD11B2 gene expression. HSD11B2 expression was treated as a continuous variable in analyses.

Statistical analysis

Stress and depression scores were normally distributed. Neither placental *HSD11B2* methylation nor expression were normally distributed, so non-parametric tests, including Spearman's correlation and Mann-Whitney U tests, were used for preliminary analyses of these data. Statistical analyses were conducted using Stata version Stata/MP 16.0 (StataCorp, College Station, TX).

First, we assessed the relationship between placental *HSD11B2* methylation and expression using linear regression with robust standard errors to account for concerns about normality in expression. We then tested associations on each pathway using adjusted linear regression models with robust standard errors. All paths were first assessed for *HSD11B2* methylation. A second round of analyses was then done for HSD11B2 expression. Analyses were adjusted for each pathway individually. Covariates were selected as they were significantly associated with either the predictor or the outcome variables at $p < 0.10$ on each pathway. We assessed gestational age, maternal age, birth weight, placental weight, infant sex as potential covariates for all paths. Ultimately, in Paths A and B, models for DNA methylation were adjusted for infant sex, which was associated with DNA methylation ($p = 0.04$), and models for mRNA expression were unadjusted. On Path C, models for both DNA methylation and mRNA expression were adjusted for gestational age, which was marginally associated with infant cortisol response ($p = 0.07$), and infant sex. Sex-specific associations were assessed via stratification of adjusted linear regression models.

6.3 Results

Sample characteristics

Maternal, infant, and placental characteristics are detailed in Table 1. Participants ranged in age from 18 to 39 years, and the vast majority (92%) identified as ethnically *Mestizo*. The majority of women were married (87.5%), and 20.8% had completed college. On psychosocial distress measures, 29.2% of the women in the study scored as depressed on the PHQ-8, and 58.3% scored as moderately or highly stressed on the PSS. The average gestational age was 38.4 weeks, and 58.3% of infants were born by Caesarean. Just over half of the infants (54%) were male, and the average birth weight was 3380 grams.

Placental HSD11B2 characteristics

Quantitative bisulfite sequencing was used to determine methylation of a CpG island region in the promoter of the *HSD11B2* gene. The average methylation across the four CpG loci was 11.7%. In order to assess the functional signfiicance of variation of methylation, we also quantified HSD11B2 gene expression using PCR. High DNA % methylation was significantly associated with lower mRNA gene expression ($\beta = -0.16$, $p \leq 0.01$) (Figure 2).

Maternal distress and HSD11B2 outcomes

The results of all adjusted linear regression models for our conceptual model are shown in Table 2. In our analyses, neither maternal stress nor depression symptoms were significantly associated with differences in *HSD11B2* methylation or gene expression.

While maternal cortisol dysregulation during pregnancy was not associated with differences in *HSD11B2* methylation, two measures of maternal cortisol dysregulation were associated with HSD11B2 expression. High maternal morning cortisol ($\beta = -0.98$, $p = 0.01$) and a blunted maternal CAR ($\beta = 0.82$, $p = 0.05$), which indicates an individual's inability to properly cortisol, were associated with lower mRNA expression of HSD11B2. These associations are shown in Figure 3. Neither evening cortisol nor daily cortisol decline were associated with differences in HSD11B2 methylation or expression.

HSD11B2 exposures and infant cortisol

DNA methylation was not associated with either measure of infant cortisol regulation, but lower mRNA expression of HSD11B2 was associated with a higher cortisol reactivity ($\beta = -0.32$ $p = 0.04$) (Figure 4), but not with higher baseline cortisol when infants were two months old.

Sex-specific associations

Infant sex was associated with differences in placental *HSD11B2* methylation ($p = 0.04$), but not expression. In girls, the average percent methylation of the *HSD11B2* gene in the placenta was 14.2%, and in boys it was 9.5%. Due to these results, and others' findings of sexually dimorphic responses to glucocorticoids in the fetus and the placenta (Clifton, 2010; Gabory, Roseboom, Moore, Moore, & Junien, 2013), we stratified our models by infant sex to examine these pathways. While maternal depression symptoms were not associated with differences in methylation or expression of HSD11B2 with the whole sample, we did observe associations in sex-specific models. When the sample was limited to just girls, maternal

depression symptoms were marginally associated with higher methylation of the *HSD11B2* gene (β = 6.74, $p = 0.06$) and it was significantly associated with lower HSD11B2 expression (β = -1.74, $p = 0.01$). These differences were not observed in the boys sample, where depression symptoms were neither associated with methylation (β = -2.4, $p = 0.15$) nor expression (β = 0.19, $p = 0.76$). No other paths demonstrated significant differences in stratified models.

6.4 Discussion

To our knowledge, this is the first study in humans to examine the effects of maternal distress on both *HSD11B2* methylation and expression as well as offspring HPA axis development. This study aimed to assess how maternal psychosocial and physiological distress (measured through cortisol) influence epigenetic regulation of the *HSD11B2* gene in the placenta, and how these differences then can shape HPA axis regulation in infants, which has long-term consequences for health (Chrousos, 2009; Pike, 2005; Seckl, 2008). Our results show an inverse association between DNA methylation of the *HSD11B2* gene promoter region and HSD11B2 gene expression in the placenta. Further, results show that maternal HPA axis dysregulation during pregnancy is associated with lower expression of HSD11B2 in the placenta, which in turn, is associated with an exaggerated cortisol reactivity in infants. Sex-specific analyses revealed that maternal depression symptoms during pregnancy is associated with lower HSD11B2 gene expression for the mothers of girls, but not boys. Nonetheless, we did not observe differences in HSD11B2 measures by maternal psychosocial stress or depression symptoms during pregnancy in the full sample, nor did we observe differences in HSD11B2 measures by other measures of maternal HPA axis dysregulation, including evening cortisol and daily cortisol decline.

Maternal distress and HSD11B2 measures

Our finding that that higher placental *HSD11B2* methylation is associated with lower HSD11B2 expression is consistent with the results from other studies on this relationship (Alikhani-Koopaei et al., 2004; Marsit et al., 2012). In the full sample, we did not observe differences in placental *HSD11B2* methylation or HSD11B2 expression based on psychosocial indicators of distress (stress and depression symptoms). Nonetheless, we did observe associations between maternal physiological distress and HSD11B2 expression. Two measurements of HPA axis dysregulation in particular, high morning cortisol and a low (blunted) cortisol awakening response, were each associated with lower HSD11B2 expression. Further, in sex-specific analyses, prenatal maternal depression symptoms were marginally associated with higher *HSD11B2* methylation and significantly lower HSD11B2 expression for girls, but not for boys.

While these results are seemingly contradictory to an adaptive framework, where maternal distress would decrease HSD11B2 methylation thus increasing HSD11B2 expression to provide a protective effect to the infant in times adversity, our results are consistent with findings from many other studies and may fit into a broader physiological model where adaptive responses become disrupted through overuse. Animal models have found that chronic stress and anxiety may diminish the protection of placental HSD11B2. One study found that prenatal stress increased the activity of HSD11B2 in low-anxiety rats, but not in high-anxiety rats (Lucassen et al., 2009), while another study found that while acute maternal stress up-regulated HSD11B2 activity, chronic stress diminished the capacity of placental HSD11B2 to up-regulate in the face of an acute stressor (Welberg et al., 2005). Other studies on chronic prenatal stress in rats have

found that chronic restraint is associated with lower placental HSD11B2 expression (Mairesse et al., 2007; Peña et al., 2012) as well as increased methylation of specific CpG sites of the *HSD11B2* gene promoter (Peña et al., 2012).

Studies on this pathway in humans report mixed findings. Like in animal studies, many studies in humans have found that chronic maternal distress (including anxiety and/or depression) is associated with greater methylation of the *HSD11B2* promoter region (Conradt et al., 2013; Monk et al., 2016) as well as lower expression of HSD11B2 (O'Donnell et al., 2012; Seth et al., 2015; Togher et al., 2014). Other distress exposures have also been associated with these differences. In one study, prenatal life events were associated with a downregulation in HSD11B2 expression, though only in Caucasian women (Capron et al., 2018). In a few studies, though, the opposite effect is observed. One study found that women of low socioeconomic status (SES) had lower *HSD11B2* methylation (Appleton et al., 2013), which would promote HSD11B2 expression. Nonetheless, some studies report no differences in placental HSD11B2 measures based on anxiety (Capron et al., 2018), depression (Capron et al., 2018; Conradt et al., 2013; Reynolds et al., 2015; Zhang et al., 2018), or prenatal natural disasters (St-Pierre et al., 2018). While results are mixed, findings from animal and human models most often suggest that in healthy, low-stress individuals, the placenta increases HSD11B2 expression in the face of distress, protecting the fetus from excess cortisol, but that in chronically-stressed individuals, this protection may diminish, exposing the infant to high levels of cortisol that have long-term consequences for neurological development (Welberg et al., 2005).

HSD11B2 measures and infant cortisol

Our analyses of the effects of HSD11B2 measures on infant cortisol found that lower HSD11B2 expression is associated with an higher cortisol reactivity in infants, which has been cited as evidence of infant HPA axis dysregulation (Davis et al., 2011). Nonetheless, we do not observe differences in baseline cortisol when infants are two months old. While this result may indicate that HSD11B2 expression does not have an effect on infant baseline cortisol, it is also possible that postpartum exposures have attenuated prenatal insults to HPA axis development (see Chapter 5). Other studies have reported mixed results along this pathway. One study found that *HSD11B2* methylation moderated the relationship between prenatal depression and infant baseline cortisol at one month postpartum, such that a 1% decrease in methylation was associated with a 9% increase in baseline cortisol in infants whose mothers were depressed during pregnancy (Stroud et al., 2016).

Few studies in humans have assessed the relationship between placental HSD11B2 measures and infant cortisol, but many have assessed the relationship between HSD11B2 measures and neurobehavioral outcomes in infants. One such study, which used factor analysis, found that high scores of the factor characterized by *HSD11B2* methylation reduced the risk that infants were characterized into a reactive, poorly regulated neurobehavioral profile (Paquette et al., 2015). Another study found that higher maternal depression scores during pregnancy were significantly associated with higher levels of negative affectivity among infants with low placental HSD11B2 expression, but not among infants with high HSD11B2 expression (Zhang et al., 2018). Our results fit into these frameworks, where higher levels of placental *HSD11B2* methylation and lower levels of HSD11B2 expression may contribute to neurological changes in infants.

Sex-specific placental HSD11B2 measures

Our sex-specific findings reveal that on average, the placentas of girls had significantly more *HSD11B2* methylation than those of boys. Further, maternal depression symptoms were marginally associated with higher *HSD11B2* methylation and significantly associated with lower HSD11B2 expression in the placentas of girls, but not of boys. These results are consistent with others' findings that infant sex is often associated with differences in placental function and physiology (Burton et al., 2014). Further, our finding that girls have lower HSD11B2 expression than boys aligns with the general finding that girls are typically more susceptible to insults to the HPA axis in early life (Carpenter, Grecian, & Reynolds, 2017), since higher HSD11B2 expression could provide a protective effect against excess maternal cortisol. Though not all studies find sex differences in placental measures (Demendi et al., 2012), a few others have observed similar findings. One study found that among small for gestational age infants, the placentas of girls exhibit lower HSD11B2 activity than those of boys (Mericq et al., 2009), and another found that among placentas of women with untreated asthma, those of girls exhibit lower HSD11B2 activity than those of boys (V. E. Murphy, Gibson, Giles, & Zakar, 2003). While the mechanisms behind sex differences in fetal programming remain unknown, others have suggested that these differences in early life may be the consequence of a "viability-vulnerability tradeoff" (Sandman, Glynn, & Davis, 2013), in which males do not adjust to early life adversity, and thus only the most fit survive, while females modify their growth in response to adversity, improving their viability but increasing their vulnerability to the deleterious effects of these adjustments later in life (Sandman et al., 2013).

Strengths and limitations

This is the first study to examine how both *HSD11B2* methylation and expression in the placenta respond to maternal distress and shape infant cortisol. This study significantly adds to the literature on prenatal stress and HSD11B2 functioning by expanding the scope of maternal stress to include both psychosocial and physiological measures of distress, offering a more refined understanding of the biological underpinnings behind these relationships. Further, after Stroud and colleagues (Stroud et al., 2016), this is only the second study to examine how differences in HSD11B2 measures shape infant HPA axis regulation in humans. Last, while the majority of studies on *HSD11B2* methylation and expression have focused on Caucasian populations, our study examines these pathways with a majority *Mestizo* population from Ecuador. This distinction is especially important since others have found differences in HSD11B2 measures based on ethnicity (Capron et al., 2018).

Despite these strengths, this study has a few limitations that should be noted. First, we are limited by our small sample size, which decreases our statistical power. Further, while we assess the epigenetic profile and expression of *HSD11B2*, which plays a central role in the conversion of cortisol to cortisone, we do not test methylation or expression of *NRC31,* a glucocorticoid receptor in the placenta that may be an upstream regulator placental *HSD11B2* (Capron et al., 2018). Further studies should assess this gene alongside HSD11B2 in order to better understand how epigenetic changes in the placenta shape infant HPA axis regulation. Despite these limitations, our study shows clear and significant relationships between maternal distress, HSD11B2 regulation, and infant HPA axis development, contributing to evolutionary theories of early life adaptation and serving as foundational exploratory research on the understudied placental influence on fetal programming.

6.5 Conclusions

Our findings indicate that maternal physiological distress during pregnancy, measured through HPA axis dysregulation, is associated with lower placental HSD11B2 expression, which in turn, is associated with an exaggerated cortisol reactivity in infants. In addition, sex-specific analyses revealed that maternal psychosocial distress during pregnancy, measured through depression symptoms, is marginally associated with more placental *HSD11B2* methylation and significantly associated with less HSD11B2 expression for the mothers of girls, but not boys. Together with others' findings, our results support a disrupted adaptive framework, in which the adaptive ability to upregulate HSD11B2 expression in response to acute stress diminishes as maternal stress becomes chronic. In these cases, chronic stress, and potentially the overuse of this biological mechanism, can cause the hypermethylation of *HSD11B2* and thus transcriptional repression of the gene, which downregulates HSD11B2 expression, leaving the infant vulnerable to high levels of maternal cortisol. In turn, overexposure to maternal cortisol injures the fetal brain and HPA axis (O'Donnell & Meaney, 2017), which could permanently alter the infant's neurobehavioral and metabolic pathways (Reynolds, 2013). As results from some similar studies has been inconsistent, further research on these important pathways are necessary to better understand the protective role of HSD11B2 in infant development.

Table 6.1 Characteristics of the study sample

Maternal Characteristics	**Mean (SD)/range or no. (%)**
Age (years)	29.2 (5.9) / 18 – 39
Ethnicity	
Mestizo	22 (91.7%)
Indigenous	1 (4.2%)
Afro-Ecuadorian	1 (4.2%)
Parity	0.92 (0.78)/ 0 – 2
Married	21 (87.5%)
Education	
Less than high school	3 (12.5%)
Completed high school	16 (66.7%)
Completed college	5 (20.8%)
Born and raised on Galápagos	9 (37.5%)
Food security	
Secure	13 (54.2%)
Mild food insecurity	8 (33.3%)
Moderate – High food insecurity	3 (12.5%
Depressed (PHQ-8)	7 (29.2%)
Moderate – High stress (PSS)	15 (62.5%)
Obstetric and Infant Characteristics	
Gestational age at delivery (weeks)	38.4 (1.2) / 36 – 41
Caesarean delivery	14 (58.3%)
APGAR 5 min	9 (0) / 9 – 9
Male offspring	13 (54%)
Infant birth weight (g)	3380 (383.8) / 2465 – 3900
Placental Characteristics	
Placental weight (g)	530.8 (101.5) / 398 – 896
% methylation, average all *HSD11B2* CpG sites	11.7 (4.9)
CpG1, % methylated	7.9 (3.6)
CpG2, % methylated	17.6 (7.0)
CpG3, % methylated	7.8 (3.7)
CpG4, % methylated	13.4 (5.4)

Figure 6.2 Placental *HSD11B2* methylation predicts HSD11B2 expression

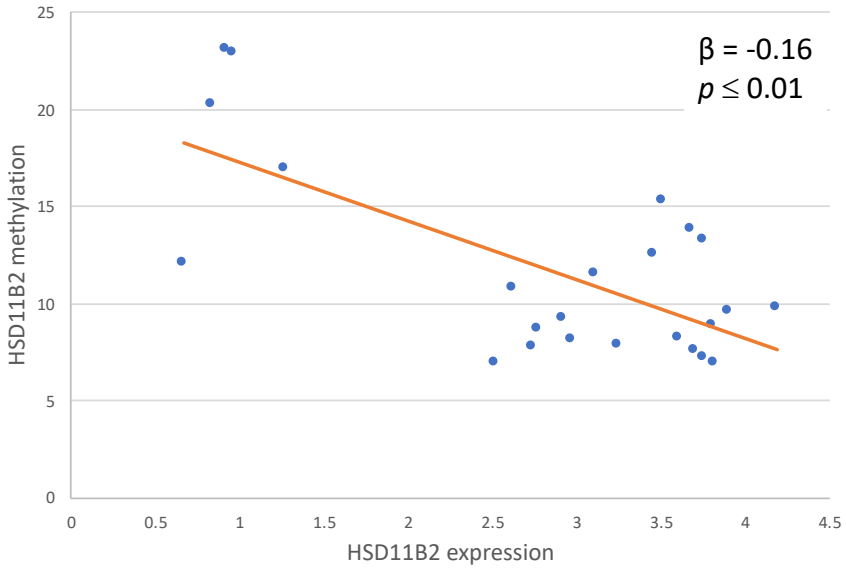


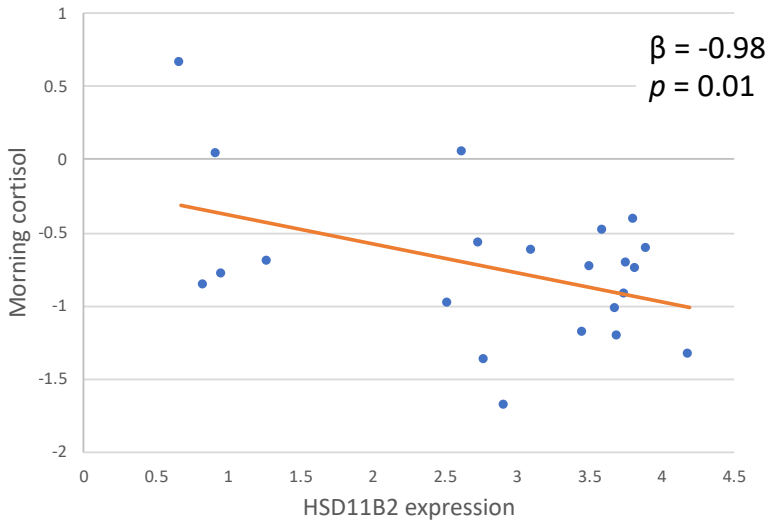
Table 6.2 Adjusted regression analyses for paths in conceptual model

	Exposure	Outcome	Model p-value	DNA Methylation	Model p-value	mRNA Expression
Path A	Stress	HSD11B2	0.06	β = -2.1 , p = 0.31	0.77	β = 0.01 , p = 0.77
	Depression, continuous	HSD11B2	0.06	β = 0.15 , p = 0.41	0.13	β = -0.07, p = 0.13
	Depression, categorical	HSD11B2	0.06	β = 2.40, p = 0.32	0.09	β = -0.92, p = 0.09
Path B	Maternal morning cortisol	HSD11B2	0.09	β = 1.38 , p = 0.37	**0.01***	**β = -0.98, p = 0.01***
	Maternal CAR	HSD11B2	0.11	β = 0.15 , p = 0.94	**0.05***	**β = 0.82, p = 0.05***
	Maternal evening cortisol	HSD11B2	0.07	β = -0.67, p = 0.77	0.78	β = -0.13, p = 0.78
	Maternal daily cortisol decline	HSD11B2	**0.05***	β =-1.07, p = 0.29	0.18	β = 0.44, p = 0.18
Path C	HSD11B2	Infant baseline cortisol	0.25	β = 0.00 , p =0.97	**0.04***	β = 0.18 , p = 0.18
	HSD11B2	Infant cortisol reactivity	0.28	β = 0.03 , p = 0.54	**0.05***	**β = -0.32, p = 0.04***

For DNA methylation, Path A and Path B were adjusted for infant sex. Path C was adjusted for gestational age and infant sex.
For mRNA expression, Path A and Path B were unadjusted. Path C was adjusted for gestational age and infant sex.
*Indicates a p-value of ≤0.05

Figure 6.3 Maternal cortisol regulation is associated with differences in placental HSD11B2 expression

a) Maternal morning cortisol



b) Maternal cortisol awakening response

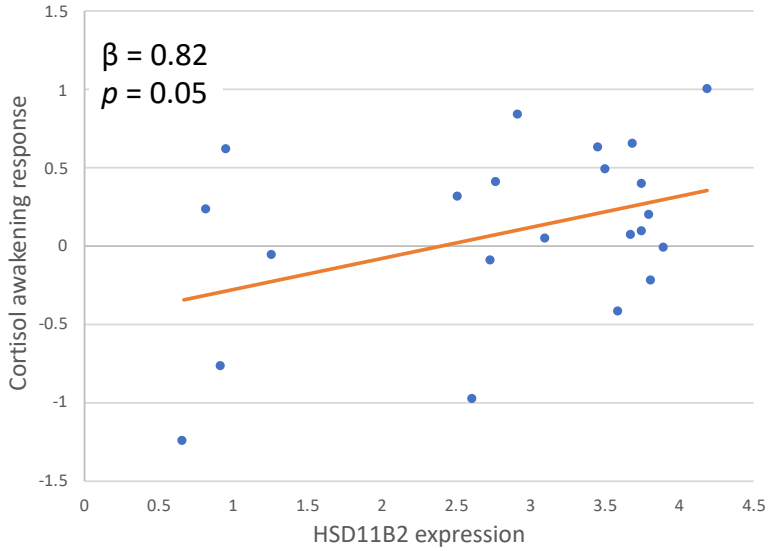
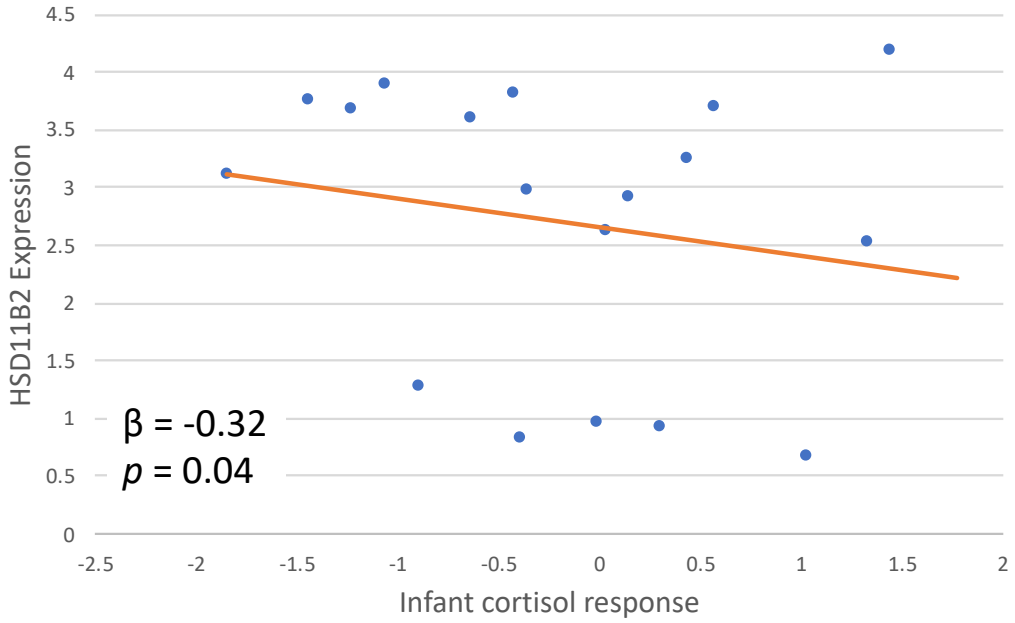


Figure 6.4 Placental HSD11B2 expression is associated with infant cortisol regulation



CHAPTER 7: MATERNAL PRECARITY AND HPA AXIS FUNCTIONING SHAPE INFANT GUT MICROBIOTA AND HPA AXIS DEVELOPMENT IN HUMANS

7.1 Introduction

Important both developmentally and evolutionarily, growth within the first 1000 days, the period from conception through the second year of life, constitutes a sensitive period during which an individual's phenotype is plastic. Research on the developmental origins of health and disease (DOHaD) has shown that stress experienced *in utero* and in early life shapes long-term risk for metabolic diseases, including obesity, cardiovascular disease, and diabetes (Wells, 2010) as well as neurobehavioral disorders in offspring even when controlling for adverse birth outcomes (Cryan & Dinan, 2012; O'Mahony et al., 2009).

Nonetheless, the mechanisms by which perinatal stress is embodied within mother-infant dyads are not yet fully understood, and studies have used various measures of maternal precarity (stress, depression, socioeconomic status, etc.) to assess this question. Many animal models have primarily linked prenatal stress exposure to hypothalamic-pituitary-adrenal axis (HPA axis) dysregulation (Thayer & Kuzawa, 2014), but studies with humans have not consistently identified a mechanism for the relationship, and research has not fully explored other pathways for these changes in development, including the role of the gut microbiome. Further, unfavorable shifts in the infant gut microbiome, termed dysbiosis, have been associated with the same long-term disease risks as HPA axis dysregulation, namely increased risk for metabolic (Goulet, 2015) and neurobehavioral disorders (Cryan & Dinan, 2012), suggesting that the gut microbiome could be a candidate for involvement in this pathway.

Most microbial species develop a symbiotic relationship with their host that promotes healthy development, educates the immune system, supports the development of gut function, regulates intestinal barrier function, protects against infection, promotes food tolerance, and supports central nervous function and the neuroendocrine system including the HPA axis (Goulet, 2015; Rackers et al., 2018; Rakers et al., 2017). Generally, the healthy gut maintains a state of homeostasis, in which it balances microbial communities, epithelial tissue of the intestine, and the immune system (Matamoros et al., 2013). However, environmental disturbances, including changes in the immune system, diet, stress, and exposures to xenobiotics (antibiotics and anti-cancer medications), among other exposures, can induce dysbiosis (Matamoros et al., 2013), which has been associated with risk for obesity, metabolic disease, autoimmune disease and allergy, and intestinal inflammation (Cho & Norman, 2013; Goulet, 2015).

Further, recent research has shown that microbiota communicate bidirectionally with the central nervous system (CNS) (Mayer, 2011) and thus gut microbiota may both influence and be influenced by brain function (Cryan & Dinan, 2012; Rackers et al., 2018). The HPA axis, in particular, has been at the center of much of this work, and studies have found that differences in HPA axis function are associated with differences in gut microbiome composition (Foster & McVey Neufeld, 2013). In particular, studies have found that germ-free mice (those with no commensal microbiota) have a higher stress response than house-specific pathogen-free mice (Sudo et al., 2004), and that pre-treating rats with probiotics reduces hyper-reactivity of the HPA axis (Ait-Belgnaoui et al., 2012). Other research on this pathway has found that psychological disorders that influence the HPA axis, including anxiety, stress, autism, and depression, are associated with differences in gut microbiome composition (Dinan & Cryan, 2017).

The peripartum period offers a unique opportunity to assess how maternal stress may be embodied in offspring through the microbiome, conferring microbiome dysbiosis with long-term health consequences intergenerationally. During pregnancy, maternal stress has been shown to alter maternal vaginal (Jašarević et al., 2017, 2015) and gut microbiota (Gur et al., 2017; Hantsoo et al., 2019), and stress-induced changes to maternal microbiota could be transferred to offspring *in utero*, during parturition, or both. The long-held hypothesis that infants are born sterile (Mackie et al., 1999) has been challenged by recent research that demonstrates that the placenta (Aagaard et al., 2012) and meconium (Jiménez et al., 2008) contain fragments of bacterial DNA, suggesting that infants may encounter bacterial exposures before birth (Walker et al., 2017). Proponents of this hypothesis suggest that maternal microbiota can be transferred to a developing fetus through the bloodstream and placenta (Borre et al., 2014), enabling shifts in a woman's microbiome to be passed to the fetus during pregnancy.

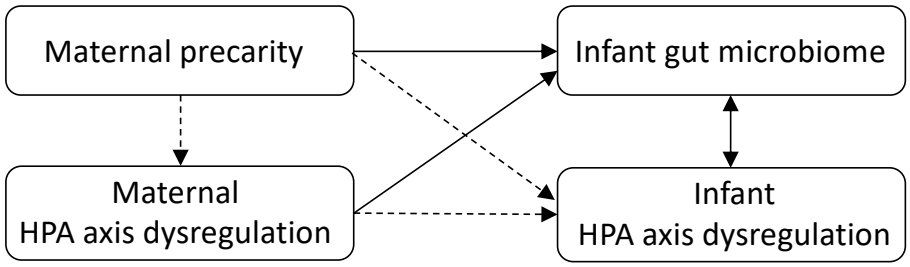
While the question of newborn sterility remains open, perturbations in a woman's microbiota during pregnancy may, nonetheless, be transferred vertically to the infant during parturition, and thus serve as foundational microbial communities (Jašarević et al., 2017, 2015; Rakers et al., 2017). Research on these pathways has found evidence that maternal stress during pregnancy influences offspring microbiome composition in both animal (Gur et al., 2017; Jašarević et al., 2017) and human models (Zijlmans, Korpela, et al., 2015).

Infant stress in the postpartum period has also been associated with differences in infant gut microbiome development. Early life stress has been found to influence the composition of bacterial microbiota in rhesus monkeys (Bailey & Coe, 1999) and have long-term effects on the gut microbiota of rats (O'Mahony et al., 2009). Further, postpartum maternal stress may influence infant stress, and thus infant microbiome composition through a variety of pathways.

First, postpartum maternal stress can compromise a mother's mental health and caregiving behaviors (Grajeda & Perez-Escamilla, 2002; Rudzik et al., 2014), which have each been found to be associated with HPA axis dysregulation in offspring (Essex et al., 2002; Gunnar & Donzella, 2002; Tollenaar et al., 2012; Wright, 2007). Second, high postpartum maternal cortisol (Glynn et al., 2007; Hahn-Holbrook et al., 2016) and microbial shifts (Moossavi et al., 2019) could be transferred to the infant during breastfeeding (Moossavi et al., 2019). In addition to shaping the infant HPA axis, which communicates with the infant's microbiome, postpartum maternal stress could shape an infant's physical environment, which has been shown to influence the gut microbiome (Borre et al., 2014). These studies demonstrate how stress during the peripartum period can have significant and long-term effects on the development of the gut microbiome, but few studies have directly addressed these questions in humans.

In the present study, we examine the relationships among measures of maternal well-being during and after pregnancy and the development of the infant gut microbiome in humans. Specifically, we ask: a) Does peripartum maternal precarity, measured through food insecurity, depression symptoms, stress, and low social support, shape differences in infant gut microbiota diversity and predominant taxa? b) Does peripartum maternal HPA axis dysregulation influence infant gut microbiota diversity and taxa? And, c) Are differences in infant gut microbiome composition associated with differences in infant HPA axis regulation? Our conceptual model is shown in Figure 7.1. The relationship between maternal precarity and infant cortisol and the relationship between maternal cortisol and infant cortisol have been examined previously (Chapter 5).

Figure 7.1 Conceptual model



Pathways on solid lines will be tested in this chapter. Pathways with dotted lines have been assessed in Chapter 5.

7.2 Materials and Methods

Setting and sample

The data were collected over 12 months from January through December 2018. Participants were recruited from a public hospital on the Galápagos' San Cristóbal island using purposive sampling (N = 25). The hospital is free for all residents and is the only hospital on the island, allowing the research team to screen all pregnant women in the community within the recruitment period. Inclusion criteria required that women be between the ages of 18 and 50 years old and plan to give birth on the island. Visits with participants were conducted once during pregnancy and three times in the postpartum period. The prenatal visit was conducted at 34-36 weeks. Postpartum visits were conducted at three days postpartum, one month postpartum, and two months postpartum. For each mother-infant dyad, the research team collected semi-structured interviews; surveys on stress, depression, social support, and food insecurity; maternal and infant saliva samples; and infant stool samples at 2 months of age. All visits were conducted in the participants' homes, places of work, the hospital, or the Galápagos Science Center (GSC). All participants provided written informed consent prior to participation under appropriate

protocols approved by the Institutional Review Boards for the University of North Carolina at Chapel Hill and *Universidad San Francisco de Quito*. This project was also approved by Ecuador's Ministry of Public Health.

Measures

Infant stool collection. Infant stool samples were collected when the infant was two months old. The mean (SD) infant age at stool collection was 59.8 (5.2) days. For infant stool collection, mothers were given detailed oral and written instructions for the collection of the stool sample, as well as a sample collection kit, including gloves, a small plastic spoon, a small plastic container. Mothers were asked to collect a small (roughly 400 mg) amount of stool from their infant's diaper and store it in the sealed plastic container in their own freezer until it could be collected by the research team later that day. Stool samples were then stored frozen at the Galápagos Science Center until they were transported to the Microbiome Core Facility at the University of North Carolina at Chapel Hill (UNC) for analysis.

Various measures of maternal precarity were taken during and after pregnancy. In this analysis, we included measures of food insecurity, stress, depression, and low social support as maternal precarity exposures. Scores on each of the surveys were analyzed categorically for ease of analysis with microbiome data.

Food insecurity. Food insecurity was assessed using the Latin American and Caribbean Food Security Scale (ELCSA) (Comité Científico de la ELCSA, 2012), with higher scores indicating a higher level of food insecurity. On this scale, a score of 0 indicates a food secure household, scores from 1 – 5 indicate mild household food insecurity, scores from 6 – 10 indicate moderate household food insecurity, and scores of 11 – 15 indicate severe household food insecurity (Comité Científico de la ELCSA, 2012). In our analyses, we grouped food secure

and low food insecurity into one category, which will be referred to as "food secure" (88% of sample) and we grouped moderate and high food insecurity into another category that will be referred to as "food insecure" (12% of sample).

Stress. The Perceived Stress Scale (PSS) (S. Cohen et al., 1983) was used to assess maternal chronic stress by measuring the degree to which situations are perceived as unpredictable, uncontrollable, and burdensome (S. Cohen et al., 1983). We used a Spanish version of this scale that has been tested for reliability, validity, and sensitivity in Spanish-speaking contexts (Remor, 2006). The PSS is scored from 0 to 40, with scores of 0 – 13 indicating low stress, scores of 14 – 26 indicating moderate stress, and scores 27 – 40 indicating high stress. For this analysis, scores were dichotomized so that scores of 0 – 13 will be referred to as "low stress" and scores of 14 – 40 will be referred to as "high stress."

Depression. Depression was measured by the Patient Health Questionnaire-8 (PHQ-8) (Kroenke et al., 2001). We used a Spanish version of the PHQ-8 that has been validated (Baader et al., 2012) and tested for reliability (Cassiani-Miranda et al., 2017) in the Spanish language. The PHQ-8 is scored from 0 to 24, with a higher score indicating more depression symptoms. When analyzed categorically, depression was defined using the CDC's diagnostic cut-point for the PHQ-8, as scores greater than or equal to 10 (Kroenke et al., 2009).

Social support. Social support was measured by Spanish versions of the Perceived Social Support-Family (PSS-Family) scale and the Perceived Social Support-Friends (PSS-Friends) scale (Procidano & Heller, 1983), both of which have been previously validated in the Spanish language and in Latin American contexts (Espinosa et al., 2011). The PSS-Family is scored from 0 to 16, and the PSS-Friends is scored from 0 to 12, with higher scores indicating a higher level

of support. Support questionnaires were dichotomized into low support and high support based on the distribution of the data.

Salivary cortisol. Salimetrics guides were used for maternal and infant saliva collection and storage protocols (Salimetrics & SalivaBio, 2015). Maternal saliva samples were collected at 34-36 weeks of pregnancy and at one month postpartum. On both of these occasions, women provided three samples: one immediately upon waking (sample 1), one 30 minutes after waking (sample 2), and one prior to sleep (sample 3). Women stored their samples in their freezers until they were collected by the study team the following day. Saliva samples were then stored at -20° C until analysis. Infant salivary samples were collected when the infant was three days old and two months old. At three days old, basal cortisol samples were collected to capture variation due to the prenatal environment with little postnatal influence. At two months of age, infant basal cortisol and cortisol reactivity were measured. Cortisol reactivity was measured as the difference between salivary cortisol levels before and 20-25 minutes after a stressor per a previously published infant stress reactivity protocol (Tollenaar et al., 2011). All infant saliva samples were collected using Salimetrics Infant Swabs and placed into Salimetrics Swab Storage tubes and frozen at -20° C until analysis.

HPA axis dysregulation. Maternal HPA axis dysregulation was assessed through morning cortisol and through cortisol awakening response (CAR), the difference between the cortisol concentrations of sample 2 and sample 1. In our analyses, high morning cortisol and a blunted (low) CAR were considered to be measures of maternal HPA axis dysregulation. Cortisol dysregulation in infants was measured through elevated basal cortisol at three days old and two months old and a blunted or exaggerated cortisol reactivity at two months old. High basal cortisol (Stroud et al., 2016) and blunted (Tollenaar et al., 2011) and exaggerated (Davis et al.,

2011) cortisol reactivity have been cited as evidence of infant cortisol dysregulation (see Chapter 5).

Covariates. In addition to the scales, saliva samples, and stool samples, we used a sociodemographic survey at baseline that inquired about general sociodemographics, household size and composition, employment, parity, health behaviors, medications, education, geographic history, and other themes. Data on obstetric and infant characteristics were collected at postpartum visits.

Laboratory analysis

DNA isolation. Samples were transferred to a 2 mL tube containing 200 mg of ≤106 μm glass beads (Sigma, St. Louis, MO) and 0.3 mL of Qiagen ATL buffer (Valencia, CA), supplemented with 20 mg/mL lysozyme (Thermo Fisher Scientific, Grand Island, NY). The suspension was incubated at 37°C for 1 h with occasional agitation. Subsequently the suspension was supplemented with 600IU of Qiagen proteinase K and incubated at 60°C for 1 h. Finally, 0.3 mL of Qiagen AL buffer was added and a final incubation at 70°C for 10 minutes was carried out. Bead beating was then employed for 3 minutes in a Qiagen TissueLyser II at 30Hz. After a brief centrifugation, supernatants were aspirated and transferred to a new tube containing 0.3 mL of ethanol. DNA was purified using a standard on-column purification method with Qiagen buffers AW1 and AW2 as washing agents, and eluted in 10mM Tris (pH 8.0).

16S rRNA amplicon sequencing. 12.5 ng of total DNA were amplified using universal primers targeting the V4 region of the bacterial 16S rRNA gene (Caporaso et al., 2012; Kozich et al., 2013). Primer sequences contained overhang adapters appended to the 5' end of each primer for compatibility with Illumina sequencing platform.

Master mixes contained 12.5 ng of total DNA, 0.2 μM of each primer and 2x KAPA HiFi HotStart ReadyMix (KAPA Biosystems**,** Wilmington, MA). The thermal profile for the amplification of each sample had an initial denaturing step at 95°C for 3 minutes, followed by a cycling of denaturing of 95°C for 30 seconds, annealing at 55°C for 30 seconds and a 30 second extension at 72°C (25 cycles), a 5 minutes extension at 72°C and a final hold at 4°C. Each 16S amplicon was purified using the AMPure XP reagent (Beckman Coulter, Indianapolis, IN). In the next step each sample was amplified using a limited cycle PCR program, adding Illumina sequencing adapters and dual-index barcodes (index 1(i7) and index 2(i5)) (Illumina, San Diego, CA) to the amplicon target. The thermal profile for the amplification of each sample had an initial denaturing step at 95°C for 3 minutes, followed by a denaturing cycle of 95°C for 30 seconds, annealing at 55°C for 30 seconds and a 30 second extension at 72°C (8 cycles), a 5 minutes extension at 72°C and a final hold at 4°C. The final libraries were again purified using the AMPure XP reagent (Beckman Coulter), quantified and normalized prior to pooling. The DNA library pool was then denatured with NaOH, diluted with hybridization buffer and heat denatured before loading on the MiSeq reagent cartridge (Illumina) and on the MiSeq instrument (Illumina). Automated cluster generation and paired–end sequencing with dual reads were performed according to the manufacturer's instructions.

Salivary cortisol. Saliva samples were thawed and assayed in duplicate for salivary cortisol using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Salimetrics, State College, PA) according to Salimetrics protocol (Salimetrics, 2016). Cortisol concentrations were log-transformed prior to analysis.

Statistical analysis

Sequencing output from the Illumina MiSeq platform were converted to fastq format and demultiplexed using Illumina Bcl2Fastq 2.18.0.12. The resulting paired-end reads were processed using QIIME 2 2018.11. Index and linker primer sequences were trimmed using the QIIME 2 invocation of cutadapt. The resulting paired-end reads were processed with DADA2 through QIIME 2 including merging paired ends, quality filtering, error correction, and chimera detection.

Amplicon sequencing units from DADA2 were assigned taxonomic identifiers with respect to Green Genes release 13_08. Alpha diversity with was measured with respect to Faith PD whole tree, Evenness (Shannon) index, and observed species number metrics and it was estimated using QIIME 2 at a rarefaction depth of 5,000 sequences per subsample. Beta diversity estimates were calculated within QIIME 2 using weighted UniFrac distances as well as Bray-Curtis dissimilarity between samples at a subsampling depth of 5,000.

Before analysis, microbiome data were cleaned for appropriate sequence length and transformed from the 16sRNA gene sequences into operational taxonomic units (OTUs) in QIIME 2 (Bolyen et al., 2019). Then, to examine each proposed pathway, we tested differences in alpha and beta diversity as well as differences in relative abundance of taxa.

First, we assessed the samples for richness and diversity at the taxonomic levels of interest (phyla and family) within QIIME2. Mann-Whitney U-tests were used to assess differences in alpha diversity (measured by Shannon Entropy) based on precarity and cortisol measures. Beta diversity was measured in two ways: weighted UniFrac distances and Bray-Curtis dissimilarity. Principal Coordinates Analysis (PCoA) of weighted UniFrac distances

matrices was used to determine clustering between two groups (eg. not depressed vs. depressed). Both measures of beta diversity were computed from the sequence data within QIIME2.

Next, to examine the taxa distribution for the entire sample, we calculated relative abundances of bacteria within each taxon. Taxa that constituted less than 1% average relative abundance of the samples were excluded. Next, we conducted taxon-specific analysis to test for relationships between maternal precarity exposures and patterns of colonization using non-parametric Mann-Whitney U-tests, since relative abundances of taxa were not normally distributed. The same was done for relationships between maternal cortisol and patterns of colonization as well as infant cortisol and patterns of colonization. Finally, adjusted linear regression models with robust standard errors were conducted to test for significant differences between phylum and family groups controlling for mode of delivery, infant feeding, and pre- or postpartum exposure to the exposure of interest in the model. For example, the model assessing the effects of friend support during pregnancy on relative abundance of taxa was adjusted for mode of delivery, infant feeding, and postpartum friend support, and the model assessing the effects of postpartum depression on relative abundance of taxa was adjusted for mode of delivery, infant feeding, and depression during pregnancy, etc. Infant feeding was a dichotomous variable defined by either exclusive breastfeeding or ever having been fed formula at 2 months of age. These controls were selected based on their persistent association with gut microbiome differentiation in the literature (Bäckhed et al., 2015; Dominguez-Bello et al., 2010; Stewart et al., 2018). Additional analyses using the same covariates were run for selected exposures at the genus level. All regression analyses were conducted using Stata 16.

7.3 Results

Precarity and cortisol descriptive data

In this sample, 12% (n = 3) of women were food insecure (Table 7.1). The majority of women experienced high stress during pregnancy (60%), but the experience of high stress decreased over time in the postpartum, so that by 2 months postpartum only 36% of women reported experiencing high stress. The rate of depression peaked at 1 month postpartum (29.2%), and decreased to only 16% by 2 months postpartum. While the rates of both low family and low friend support were least common in during pregnancy, each at 36%, low family support was most common at 1 month postpartum (46%), and low friend support was most common at 2 months postpartum (48%).

Over half (56%) of the infants in the sample were born by Caesarean section, and 36% had received formula by 2 months postpartum. Infants born by Caesarean had significantly different beta diversity than infants born vaginally on measures of weighted UniFrac distances and Bray Curtis dissimilarity. Infants born by Caesarean also had a significantly higher abundance of Bacteroidetes at the phylum level and *Lachnospiraceae* and *Enterobacteriaceae* at the family level and a lower abundance of Bacteroidetes at the phylum level and *Coriobacteriaceae* and *Bacteroidaceae* at the family level than infants born vaginally. Infants who had ever received formula by 2 months postpartum did not exhibit differences in diversity, but did have a marginally higher abundance of Veillonellaceae ($p = 0.07$) than those who were exclusively breastfed. Mode of delivery and formula feeding were not associated with one another, and both of these measures were included as covariates in regression analyses. Cutoffs for high and low cortisol concentrations were based on the distributions of each variable, so that roughly half of the participants had high and half had low cortisol measures.

Alpha diversity

In the postpartum, maternal depression ($p = 0.04$), stress ($p = 0.01$), and high morning cortisol ($p = 0.04$) were all significantly associated with a lower alpha diversity measured by Shannon Entropy in infant stool at 2 months of age (Figure 7.2). Pre-partum measures of depression, stress, and maternal cortisol were not associated with differences in alpha diversity in infant stool at 2 months of age. Social support and food security in the peripartum period were also not associated with differences in alpha diversity in infant stool. Shannon Entropy was not associated with either mode of delivery or infant feeding at $p \leq 0.05$.

Beta diversity with PCoA plots

When weighted UniFrac distances and Bray-Curtis dissimilarities were plotted on PCoA plots, samples from infants whose mothers were depressed at two months postpartum clustered separately from samples of infants whose mothers were not depressed at two months postpartum (Figure 7.3). These differences were statistically significant for both weighted UniFrac distance ($p = 0.03$) and Bray-Curtis dissimilarity ($p < 0.01$). Measures of beta diversity in infant stool were not significantly different for any other precarity measures or for maternal depression experienced during pregnancy. Analyses of differences in beta diversity for maternal cortisol measures revealed that samples from infants whose mothers had a low postpartum CAR plotted separately from samples of infants whose mothers had a high CAR on PCoA plots of weighted UniFrac distances ($p = 0.02$), but not of Bray-Curtis dissimilarity ($p = 0.31$). No other measures of maternal cortisol were associated with significantly different beta diversity in infant stool samples taken at 2 months of age. Last, when we tested differences in beta diversity of the infant

gut microbiome based on infant cortisol, only basal infant cortisol at 3 days old was significantly associated with differences for weighted UniFrac distance ($p = 0.02$) and Bray-Curtis dissimilarity ($p = 0.03$).

Relative abundance of predominant taxa

Four phyla, Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria accounted for 99.9% of the average total composition of infant gut microbiota (Table 7.2). Twelve families, each of whose average relative abundance was >1%, accounted for 94.7% of the average total composition of infant gut microbiota at the family level.

Mann-Whitney U tests and adjusted regression models were run for precarity and cortisol exposures with each of these phyla and families. Mann-Whitney U-tests that were significantly associated ($p \leq 0.05$) with differences in abundance are shown in Table 7.3, and adjusted regression models that were significantly associated with differences in taxa abundance are shown in Table 7.4. Relative abundance plots for maternal precarity measures are shown in Figure 7.4, and relative abundance plots for maternal and infant cortisol measures are shown in Figure 7.5.

Of the measures of maternal precarity, food insecurity, low friend support during pregnancy, and postpartum depression were significantly associated with differences in taxa abundance in infant stool in adjusted models. Food insecurity was associated with a higher relative abundance of Proteobacteria ($p = 0.05$), specifically *Enterobacteriacea*e ($p = 0.05$), and a lower relative abundance of *Lachnospiraceae* ($p = 0.01$). Low friend support during pregnancy was associated with a higher relative abundance of Proteobacteria ($p = 0.01$), specifically *Enterobacteriaceae* ($p < 0.01$). Postpartum depression was associated with a lower relative

abundance of Actinobacteria ($p = 0.04$), specifically *Bifidobacteriaceae* ($p = 0.04$). It was also associated with a lower abundance of *Lachnospiraceae* ($p = 0.01$) and a higher relative abundance of *Streptococcaceae* ($p = 0.01$). Adjusting for the same covariates, postpartum depression was associated with lower relative abundance of the genus *Bifidobacteria* in particular, ($\beta = -29.06$, $p = 0.04$). Stress and family support were not associated with differences in taxa.

In adjusted models, maternal cortisol concentrations were associated with differences in taxa both during and after pregnancy. Notably, maternal CAR during and after pregnancy were each associated with differences in Bacteroidetes, and particularly the family *Bacteroidaceae*, but in different directions. A low maternal CAR during pregnancy was associated with a lower abundance of Bacteroidetes ($p = 0.03$) and *Bacteroidaceae* ($p = 0.01$), and a low maternal CAR in the postpartum was associated with a higher abundance of these taxa ($p < 0.01$ for each) as well as a higher abundance of *Veillonellaceae* ($p = 0.01$). In models at the genus level, which adjusted for the same covariates, low CAR during pregnancy was associated with lower abundance of *Bacteroides* at the genus level ($\beta = 27.88$, $p < 0.01$), and a low CAR in the postpartum was associated with a higher abundance of *Bacteroides* ($\beta = -21.37$, $p < 0.01$). Maternal morning cortisol during pregnancy was not associated with differences in infant gut taxa, but high postpartum morning cortisol was associated with a greater abundance of Bacteroidetes ($p = 0.03$), *Bacteroidaceae* ($p = 0.01$), and *Bacteroides* ($p = 0.01$).

Infant cortisol concentrations at three days postpartum, but not two months postpartum, were associated with differences in infant gut taxa in adjusted models. High infant basal cortisol at three days postpartum was associated with a lower abundance of Actinobacteria ($p < 0.01$),

and specifically *Bifidobacteriaceae* ($p < 0.01$), and a higher abundance of Proteobacteria ($p = 0.03$), particularly *Enterobacteriaceae* ($p = 0.04$).

7.4 Discussion

In this study, we examined how multiple facets of women's precarity and HPA axis functioning in the peripartum period contribute to the development of their infants' gut microbiome communities and associated HPA axis functioning. We found support for all three of our proposed pathways, suggesting that: 1) maternal peripartum precarity does shape differences in infant gut microbiota diversity and taxa abundance, 2) peripartum maternal HPA axis functioning also influences the development of the infant gut, and 3) differences in relative abundance of taxa in the infant gut are associated with differences in infant HPA axis functioning. Together, these findings provide support for the intergenerational transmission of stress from mother to infant and for communication between the gut and the brain through the HPA axis.

Our results show support for our first proposed pathway, that maternal precarity during and after pregnancy shapes differences in infant gut microbiota diversity and predominant taxa. Overall, women's experiences of precarity during the peripartum period primed their infants to be colonized less diverse microbiota, and more pathogenic and less protective bacteria on the whole. Prenatal exposures to precarity, including food insecurity and low social support, were associated with higher relative abundance of Proteobacteria, and specifically, a higher relative abundance of *Enterobacteriacea*e, and a lower relative abundance of *Lachnospiraceae.*

Prenatal exposures to precarity were associated with a higher relative abundance of Proteobacteria, a phylum which is known to contain pathogens (Zijlmans, Korpela, et al., 2015),

in infant stool. Within the Proteobacteria phylum, these infants had a higher relative abundance of *Enterobacteriacea*e, a family of bacteria that includes the pathogenic *Escherichia* and *Enterobacter*, and specifically *Escherichia coli* and *Salmonella* (Zijlmans, Korpela, et al., 2015). Further, *Enterobacteriacea*e are known to produce lipopolysaccharides (LPS), endotoxins that stimulate the HPA axis (Black, 2002) and have been associated with inflammation in a variety of metabolic diseases (Cani, Osto, Geurts, & Everard, 2012). A high relative abundance of *Enterobacteriaceae* in early infancy has been associated with risk for allergy and eczema (Gosalbes et al., 2013). Prenatal precarity was also associated with a lower abundance of *Lachnospiraceae*, a family of bacteria that promote gut health and have been shown to be protective against obesity and insulin resistance in mice (Truax et al., 2018) and decrease risk for heart failure in humans (Kummen et al., 2018). In contrast to other studies that have examined this pathway, we did not find that offspring exposed prenatally to maternal precarity have lower abundances of *Lactobacillus and Bifidobacteria*. One study found these associations in humans (Zijlmans, Riksen-Walraven, & de Weerth, 2015), while others have found similar results in animal models (Walker et al., 2017), where offspring of monkeys stressed during pregnancy had significantly lower abundance of *Bifidobacteria* and *Lactobacillus* at two days after birth (Bailey et al., 2004) and offspring of mice stressed during pregnancy had a significantly lower abundance of *Lactobacillus* (Jašarević et al., 2015).

Nonetheless, we did observe that postpartum maternal precarity measures were associated with a lower relative abundance *Bifidobacteria*, as well as a lower abundance of *Lachnospiraceae* and *Streptococcaceae* in infant stool. This abatement in *Bifidobacteria* in response to precarity is of particular interest since *Bifidobacteria* are anaerobic, anti-inflammatory bacteria known to be one of the cornerstones of a healthy infant gut microbiome

(Fallani et al., 2010). A high abundance of *Bifidobacteria* has been associated with reduced risk for allergic disease (Björkstén et al., 2001; Kuitunen et al., 2012) and excessive weight gain (Dogra et al., 2015; Kalliomäki et al., 2008), and a low abundance has been associated with increased crying in infants (De Weerth, Fuentes, Puylaert, & De Vos, 2013). The observed difference in *Bifidobacteria* abundance in our study is consistent with other literature that has found that *Bifidobacteria* is sensitive to environmental perturbations (Zijlmans, Korpela, et al., 2015), including preterm birth (Normann, Fahlén, Engstrand, & Lilja, 2013), antibiotic exposure (Fouhy et al., 2012), and Caesarean section (Biasucci et al., 2010), which have all been associated with a lower abundance of *Bifidobacteria* in the infant gut. Further, in addition to a lower abundance of *Lachnospiraceae*, whose benefits are discussed above, postpartum precarity was associated with a higher relative abundance of *Streptococcaceae*, whose genus *Streptococcus* has been associated with higher waist circumference (Fei et al., 2019), cardiometabolic diseases (Jackson et al., 2016), and inflammatory diseases (Echchannaoui et al., 2002). In general, maternal peripartum precarity was associated with a lower abundance of protective bacteria including *Bifidobacteria* and *Lachnospiraceae*, and a higher abundance of pathogenic bacteria, including *Enterobacteriacea*e and *Streptococcaceae*, in infant stool. Together, these results suggest that infants born to women who experienced precarity in the peripartum period are at a higher risk for adverse long-term health outcomes, including allergy, overweight, and cardiometabolic and inflammatory diseases.

Our analyses demonstrate mixed results for our second pathway, which assessed whether peripartum maternal cortisol influences infant gut microbiota composition. Our results demonstrate contradictory microbial colonization patterns between maternal cortisol dysregulation during pregnancy and in the postpartum. Specifically, a low maternal CAR (an

indicator of HPA axis dysregulation) during pregnancy was associated with a lower abundance of Bacteroidetes, and specifically the *Bacteroides* genus, while a low maternal CAR in the postpartum was associated with a higher abundance of these taxa. This difference in colonization based on the timing of maternal HPA axis dysregulation is likely due to the mechanism of colonization. Prenatal stress (measured through a low CAR) may have decreased Bacteroidetes in the mother's own microbiota, which the infant was exposed to *in utero* and during birth, while postpartum maternal stress may have shaped infant biology through physical environmental exposures, breastfeeding, or parenting behaviors. Other work has found that *Bacteroidaceae* (Thompson et al., 2019) and *Bacteroides* (Biasucci et al., 2010) are higher in infants born vaginally and those who are formula-fed. Low maternal CAR in the postpartum was also associated with a higher abundance of *Veillonellaceae*, which has been associated with more cardiometabolic risk factors in adults (Fei et al., 2019).

Last, our results demonstrate support for our third pathway, which analyzed whether infant gut microbiota composition was associated with differences in infant cortisol in the postpartum. Notably, our results showed that shifts in microbiota colonization in response to infant HPA axis dysregulation mirror major microbial shifts associated with maternal precarity, including a lower abundance of Actinobacteria, specifically *Bifidobacteriaceae*, and a higher abundance of Proteobacteria, specifically *Enterobacteriaceae*. Other research has also found that *Bifidobacteria* are intricately involved with the HPA axis. One study found that the reconstitution of *Bifidobacteria* decreased stress responses in mice (Sudo et al., 2004). Further, this association is of particular interest since prenatal exposure to maternal stress has often been associated with a lower abundance of *Bifidobacteria* in the infant gut (Bailey et al., 2004; Zijlmans, Korpela, et al., 2015). Together, this evidence, along with our own results, suggests

that maternal stress may influence infant HPA axis activity through microbial agents, though research is limited.

Beyond differences in taxa abundances, we found that in the postpartum, two measures of maternal precarity (depression and stress) and one measure of maternal HPA axis dysregulation (high morning cortisol) were associated with significantly lower alpha diversity. Studies have found that in adults, high microbial diversity has been associated with relatively more anti-inflammatory bacteria, while low microbial diversity has been associated with relatively more pro-inflammatory bacteria as well as higher adiposity and inflammation (Jandhyala et al., 2015), suggesting that these stress-related differences in microbial diversity may lay the foundation for unfavorable consequences in the long-term. Notably, we did not observe differences in alpha diversity for any maternal precarity or HPA axis dysregulation exposure during pregnancy. Since alpha diversity continues to develop throughout infancy (Bäckhed et al., 2015), this observation could be a consequence of postpartum exposures on infant gut microbiome development.

Despite the fact that we found support for some measures in each of our proposed pathways, other measures within each pathway were not significantly associated with differences in gut microbiome development, demonstrating the complexity of measuring stress. Notably, maternal "stress" itself, measured on the PSS, was not associated with any differences in taxa abundance either during or after pregnancy, though postpartum stress was associated with lower alpha diversity. Our results are in contrast to other work that has found that infants of mothers with high reported stress have a higher relative abundance of Proteobacterial groups and a lower relative abundance *Bifidobacteria* (Zijlmans, Korpela, et al., 2015). Our results may be due to the fact that we used the PSS to measure stress, while other studies have utilized measures of stress and anxiety to indicate stress. Nonetheless our analyses show similar results borne out through

other measures of maternal precarity, including maternal food insecurity, low social support, and depression.

Our results demonstrate the importance of using a multi-faceted approach to interrogate these complex biological pathways. By using four measures of maternal precarity, food insecurity, low social support, depression, and stress, we were able to examine women's experiences more fully. Further, many differences in microbiota composition were consistent across measures of precarity, suggesting an internal consistency among measures. For example, both postpartum stress and depression were associated with lower alpha diversity, and food insecurity, low friend support, and depression were all associated with lower *Bifidobacteriaceae* and higher *Enterobacteriaceae* (though not always at a significance of $p < 0.05$). Notably, this same pattern of colonization was observed in infants whose HPA axis was dysregulated, lending support for hypotheses regarding communication between of the brain and the gut and reinforcing the importance of examining these intertwined pathways with a variety of both psychosocial and biological measures. To our knowledge, this is only the second study to assess the effects of maternal prenatal stress on infant gut microbiome development and associated HPA axis function in humans, and it is the first study to include both prenatal and postnatal stress in these models. Despite the fact that postnatal environments are known to contribute to infant gut composition (Bäckhed et al., 2015; Thompson et al., 2019), many studies on early gut development focus only on prenatal stress exposures. A few studies with animal models have tested the effects of infant postnatal stress on gut microbiome development (Bailey & Coe, 1999; O'Mahony et al., 2009), but to our knowledge, none of have tested this relationship in humans, which may be due to methodological limitations of examining stress in infants. Nonetheless, in the present study, we use both maternal precarity and HPA axis function measures that could

affect infant stress as well as measures of infant HPA functioning to examine infant stress in the postnatal period. Our results lend support to the intergenerational transmission of stress through the microbiome in the peripartum period in humans.

Nonetheless, these results have several important limitations. First, our sample size (n = 25) allows us to test associations between variables, but not causation. Specifically, while pathways between infant gut microbiome composition and infant HPA axis functioning are evident, our analysis cannot distinguish directionality. Further, the pathways in our models would be elucidated more clearly with a measurement of maternal gut or vaginal microbiome composition, which we did not have in this study. Despite these limitations, significant and persistent differences in infant gut microbiota composition are associated with measures of maternal precarity, and this work lays a foundation for further testing of these pathways in human models. Last, the limitations of two covariates, mode of delivery and infant feeding are important to consider. First, mode of delivery included the categories of vaginal birth or Caesarean section, but we did not have data for membrane rupture, which could limit our understanding of which bacteria infants exposed to during birth. Second, infant feeding was split into two categories, "exclusive breastfeeding" and "ever received formula." While formula feeding does induce important differences to the infant gut microbiome (Stewart et al., 2018), we did not have data on how often infants in the "ever formula" category were formula fed, and therefore we could not assess dose effects. Nonetheless, other work has suggested that infants who have ever received formula do have significant differences in their gut microbiome compositions than infants who are exclusively breastfed (Ho et al., 2018; Thompson, Monteagudo-Mera, Cadenas, Lampl, & Azcarate-Peril, 2015), suggesting that this distinction is important.

7.5 Conclusions

Overall, we find that exposures to maternal precarity and HPA axis dysregulation are associated with an increase in pathogenic bacteria, including *Enterobacteriaceae, Streptococcaceae, and Veillonellaceae*, and a decrease in protective bacteria, including *Bifidobacteriaceae*, *Lachnospiraceae*, as well as a decrease in diversity. This initial colonization may permanently alter the infant's neurodevelopment through changes to the synthesis of neuroinflammatory cytokines, neuromodulators, neurotransmitters, and the HPA axis, leaving the individual more susceptible to neuropsychiatric disease later in life (Diaz Heijtz, 2016; Jašarević et al., 2015). Further, unfavorable changes to an individual's foundational gut microbiome may increase risk of metabolic disease, autoimmune disease and allergy, and intestinal inflammation (Cho & Norman, 2013; Diaz Heijtz, 2016; Goulet, 2015). Our results suggest that maternal stress is an important driver of early infant gut microbiome composition, and that patterns of infant gut colonization may cause or respond to differences in infant HPA axis development. Further research is needed to further elucidate the relationships among these important pathways.

Table 7.1 Maternal and infant characteristics

Maternal Characteristics	**Mean (SD) or no. (%)**		
Age (years)	27.9 (5.9)		
Parity	0.96 (0.89)		
Married	22 (88%)		
Education			
Less than high school	3 (12%)		
Completed high school	16 (64%)		
Completed college	6 (24%)		
Born on Galápagos	9 (36%)		
Obstetric Data			
Gestational age (weeks)	38.3 (1.4)		
Caesarean delivery	14 (56%)		
Infant Characteristics			
Male offspring	15 (60%)		
Infant birth weight (g)	3346.4 (370.7)		
Formula feeding [a]	9 (36%)		
Precarity Measures	**Pre-partum**	**Postpartum, 1 month**	**Postpartum, 2 months**
Food Insecure	12.0%	--	--
High stress	60.0%	50.0%	36.0%
High depression	24.0%	29.2%	16.0%
Low family support	36.0%	46.0%	40.0%
Low friend support	36.0%	38.0%	48.0%

[a] Measures taken when the infant was 2 months old

Figure 7.2 High postpartum maternal depression, stress, and morning cortisol concentration significantly decrease Shannon's Entropy in infant stool

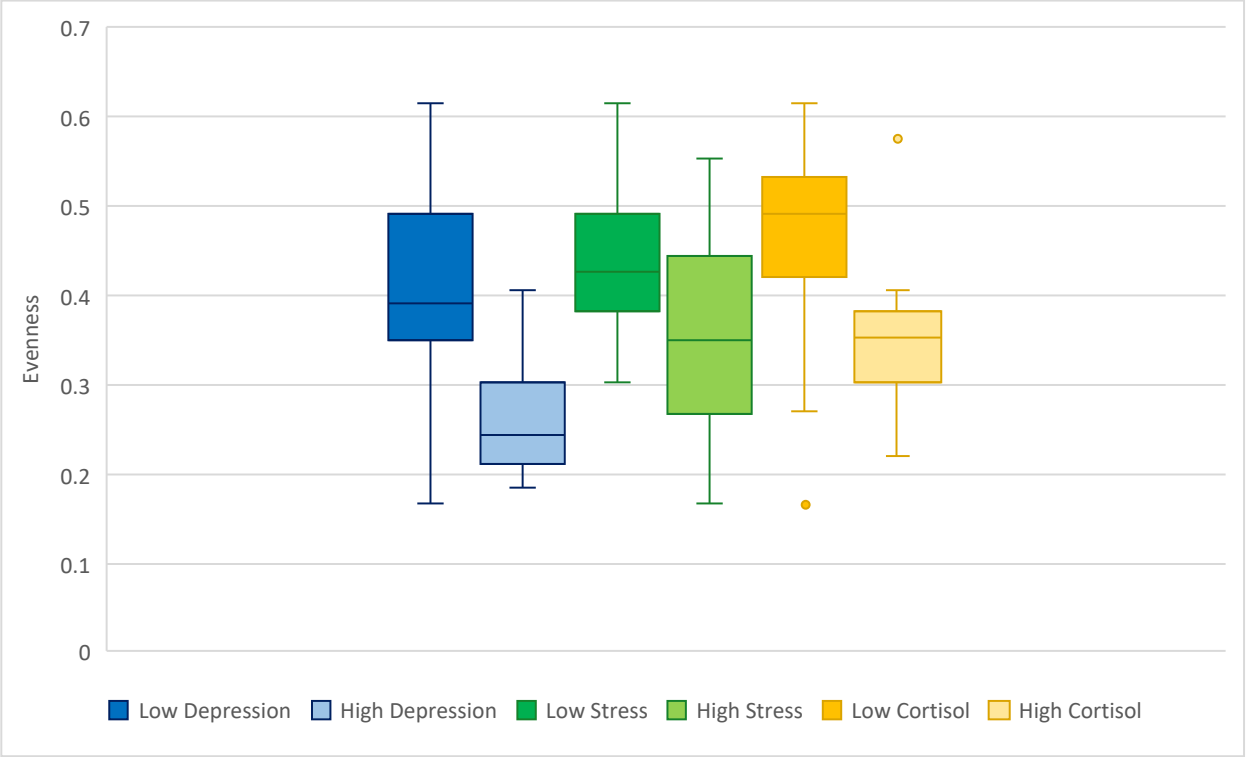


Figure 7.3 Stool sample sequences from infants cluster separately by maternal postpartum depression, maternal postpartum cortisol awakening response, and infant cortisol on a Principal Coordinates Analysis (PCoA)

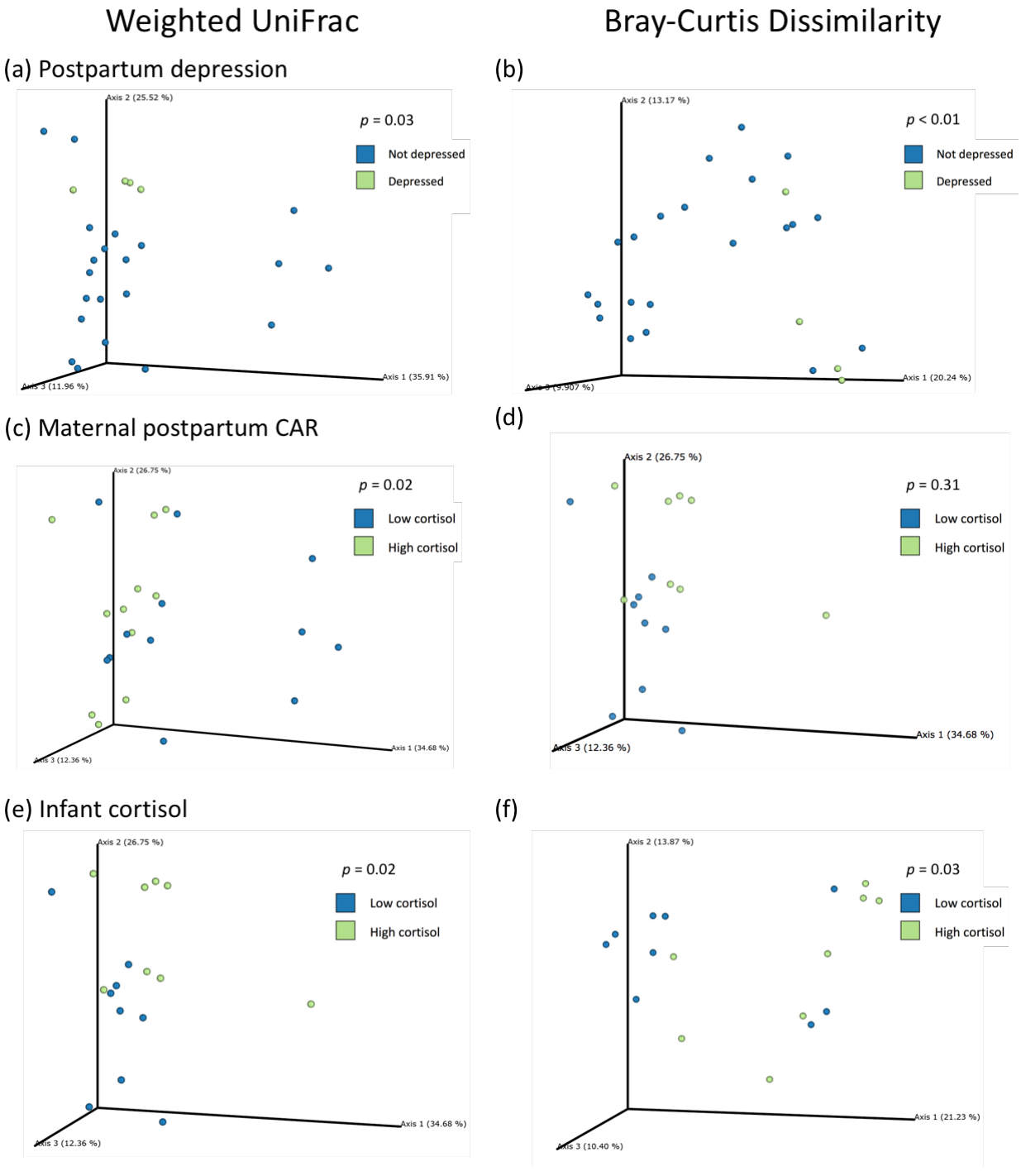


Table 7.2 Average infant gut microbiota composition at the phylum and family levels

Phylum	**% abundance**	***Family***	**% abundance**
Actinobacteria	30.8%	*Bifidobacteriaceae*	27.8%
		Coriobacteriaceae	2.0%
Bacteroidetes	13.6%	*Bacteroidaceae*	11.6%
Firmicutes	32.1%	*Enterococcaceae*	2.4%
		Lactobacillaceae	2.9%
		Streptococcaceae	4.0%
		Clostridiaceae	5.2%
		Lachnospiraceae	4.4%
		Peptostreptococcaceae	4.3%
		Veillonellaceae	1.1%
		Erysipelotrichaceae	6.0%
Proteobacteria	23.4%	*Enterobacteriaceae*	23.0%
Other	0.1%	*Other*	5.3%

Table 7.3 Maternal precarity and maternal and infant HPA axis dysregulation are associated with differences in taxa abundance in bivariate analyses

Exposure	Outcome	p-value
Maternal Precarity		
Phylum		
Food Insecurity, pregnancy	Proteobacteria	0.02
Family support, 1 month postpartum	Proteobacteria	0.05
Depression, 1 month postpartum	Proteobacteria	0.04
Depression, 2 months postpartum	Actinobacteria	0.01
Family		
Food Insecurity, pregnancy	*Enterobacteriaceae*	0.02
Family support, pregnancy	*Coriobacteriaceae*	0.04
Family support, 2 months postpartum	*Enterococcaceae*	0.04
Friend support, pregnancy	*Streptococcaceae*	0.02
Friend support, pregnancy	*Lachnospiraceae*	0.05
Friend support, 1 month postpartum	*Coriobacteriaceae*	0.02
Stress, pregnancy	*Clostridiaceae*	0.02
Stress, 2 months postpartum	*Clostridiaceae*	<0.01
Depression, 1 month postpartum	*Streptococcaceae*	0.01
Depression, 1 month postpartum	*Enterobacteriaceae*	0.05
Depression, 2 months postpartum	*Bifidobacteriaceae*	0.01
HPA Axis Dysregulation		
Phylum		
Maternal CAR, postpartum	Bacteroidetes	0.02
Infant basal cortisol	Actinobacteria	0.01
Family		
Maternal CAR, pregnancy	*Streptococcaceae*	0.02
Maternal CAR, postpartum	*Veillonellaceae*	0.04
Maternal morning cortisol, pregnancy	*Erysipelotrichaceae*	0.01
Maternal morning cortisol, postpartum	*Peptostreptococcaceae*	0.05
Infant basal cortisol	*Bifidobacteriaceae*	0.01
Infant basal cortisol	*Streptococcaceae*	0.03

Table shows all significant results for precarity measures and relative abundance of phylum and family taxa at $p \leq 0.05$

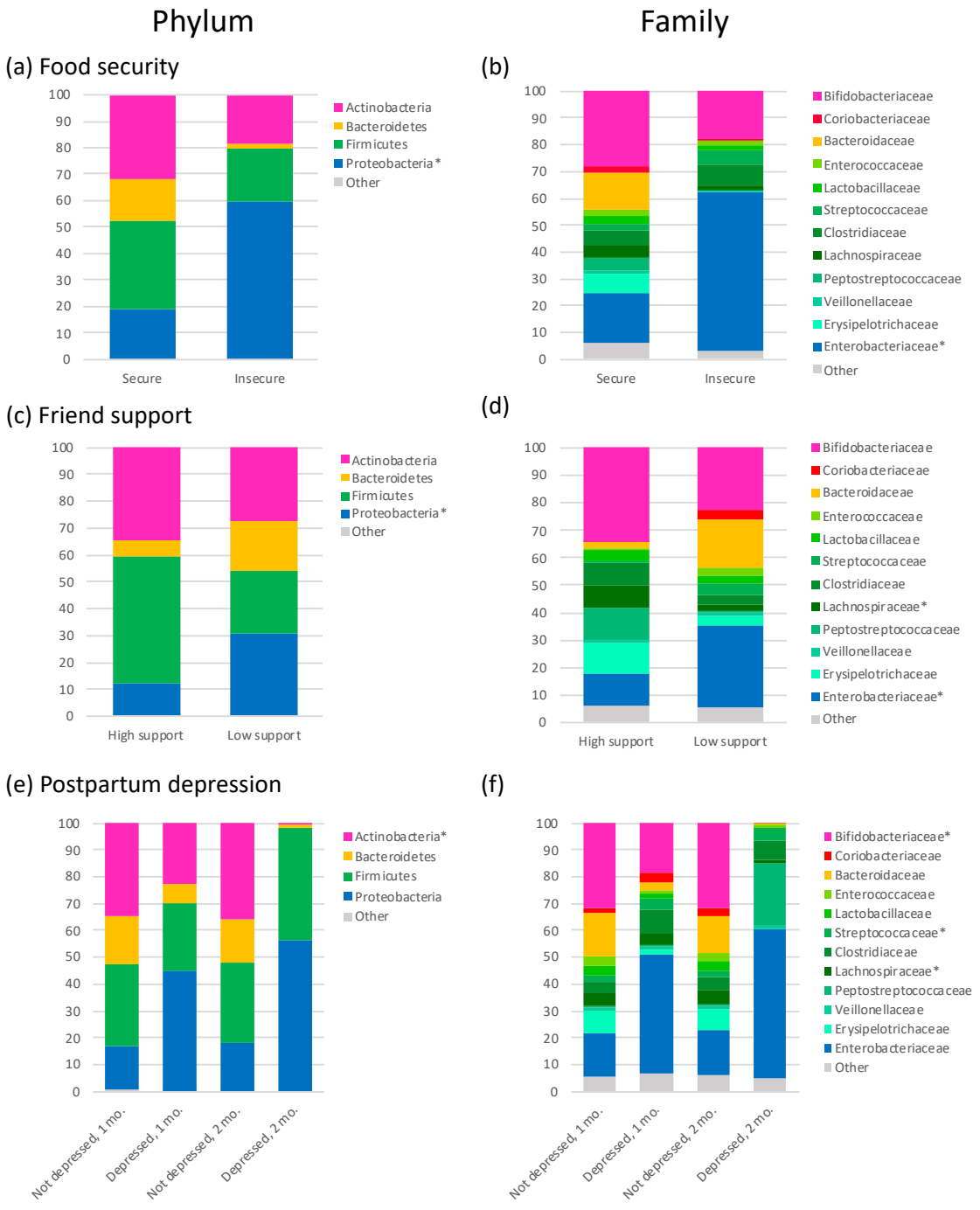
Table 7.4 Maternal precarity and maternal and infant HPA axis dysregulation are associated with differences in taxa abundance in adjusted models

Exposure	Outcome	Model p-value	Variable β	Variable p-value
Maternal Precarity				
Phylum				
↑ Food Insecurity, pregnancy	↑ Proteobacteria	0.02	33.67	0.05
↓ Friend support, pregnancy	↑ Proteobacteria	0.02	-29.02	0.01
↑ Depression, 2 months postpartum	↓ Actinobacteria	<0.01	-31.20	0.04
Family				
↑ Food Insecurity, pregnancy	↑ *Enterobacteriaceae*	0.02	33.63	0.05
↑ Food Insecurity, pregnancy	↓ *Lachnospiraceae*	0.03	-6.63	0.01
↓ Friend support, pregnancy	↑ *Enterobacteriaceae*	0.02	-28.97	<0.01
↑ Depression, 1 month postpartum	↑ *Streptococcaceae*	<0.01	3.53	0.01
↑ Depression, 2 months postpartum	↓ *Bifidobacteriaceae*	<0.01	-29.16	0.04
↑ Depression, 2 months postpartum	↓ *Lachnospiraceae*	<0.01	-7.21	0.01
HPA Axis Dysregulation				
Phylum				
↓ Maternal CAR, pregnancy	↓ Bacteroidetes	<0.01	22.32	0.03
↓ Maternal CAR, postpartum	↑ Bacteroidetes	<0.01	-23.05	<0.01
↑ Maternal morning cortisol, postpartum	↑ Bacteroidetes	0.01	24.76	0.03
↑ Infant basal cortisol	↓ Actinobacteria	<0.01	-35.69	<0.01
↑ Infant basal cortisol	↑ Proteobacteria	0.04	26.29	0.03
Family				
↓ Maternal CAR, pregnancy	↓ *Bacteroidaceae*	0.01	24.65	0.01
↓ Maternal CAR, postpartum	↑ *Bacteroidaceae*	<0.01	-21.37	<0.01
↓ Maternal CAR, postpartum	↑ *Veillonellaceae*	<0.01	-1.39	0.01
↑ Maternal morning cortisol, postpartum	↑ *Bacteroidaceae*	<0.01	27.79	0.01
↑ Infant basal cortisol	↓ *Bifidobacteriaceae*	<0.01	-34.65	<0.01
↑ Infant basal cortisol	↑ *Enterobacteriaceae*	0.04	26.15	0.04

Table shows all significant results for precarity measures and relative abundance of phylum and family taxa at $p \leq 0.05$

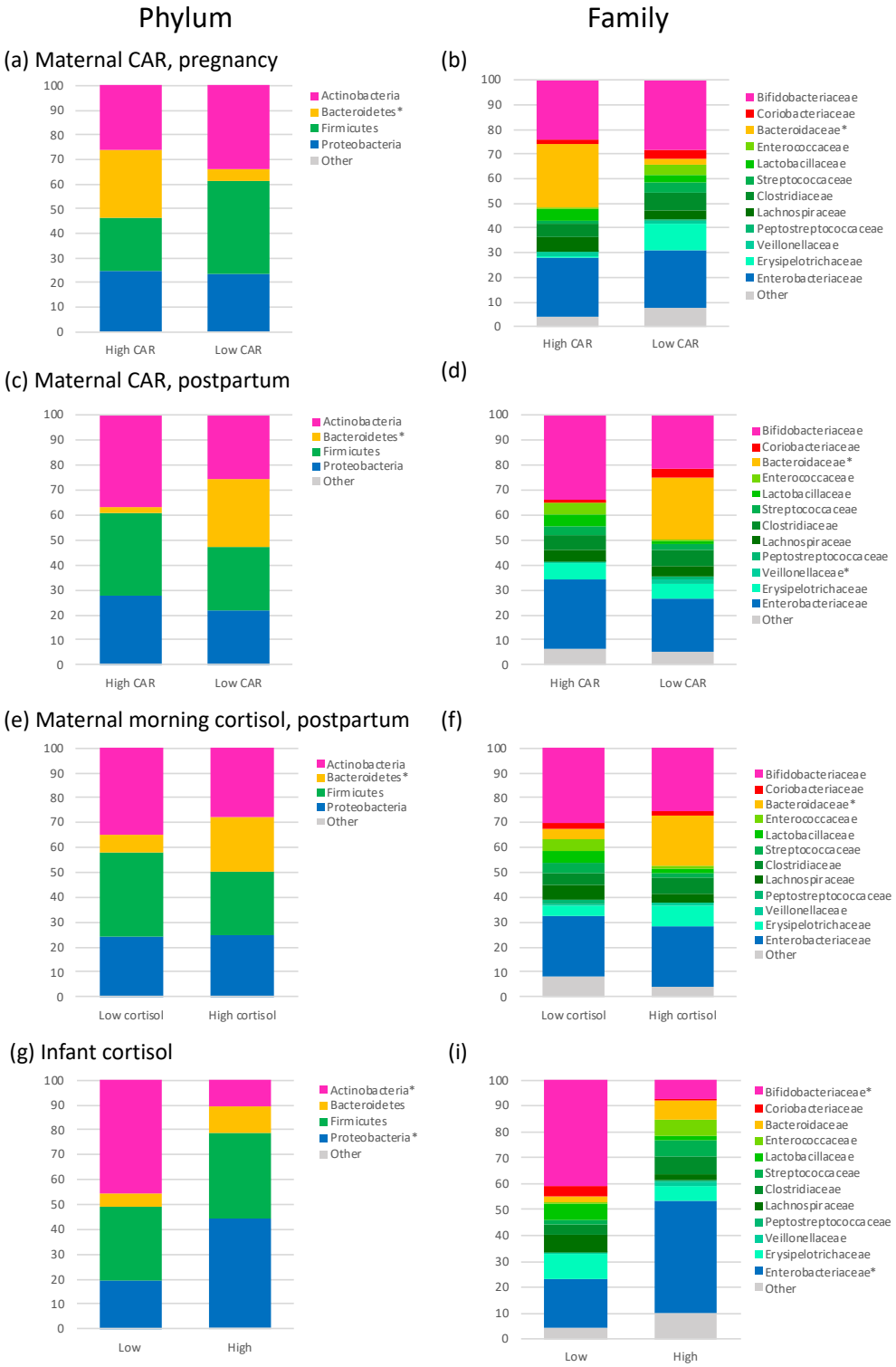
All models control for mode of delivery and infant feeding. Pre-partum models control for postpartum precarity measures. Postpartum models control for pre-partum precarity measures.

Figure 7.4 Food security, social support, and postpartum depression contribute to differential relative abundance of the phylum and family taxonomic level in infant stool



*Indicates a significant difference in relative abundance of taxa while controlling for mode of delivery, infant feeding, and pre- or postpartum exposure to the same precarity measure.

Figure 7.5 Maternal and infant cortisol are associated with differential relative abundance of the phylum and family taxonomic level in infant stool



*Indicates a significant difference in relative abundance of taxa while controlling for mode of delivery, infant feeding, and pre- or postpartum exposure the relevant cortisol measure

CHAPTER 8: THE EMBODIMENT OF STRESS IN THE GALAPAGOS: CONCLUSIONS AND IMPLICATIONS

8.1 Summary and Significance of Research Findings

This project investigates the mechanisms through which both psychosocial and physiological maternal stress contribute to shifts in infant development over the course of the peripartum period. In particular, this research incorporates the understudied roles of the postpartum period, epigenetic regulation in the placenta, and the gut microbiome into existing models for infant HPA axis development that have continuously reported inconsistent findings. Since the infant HPA axis has consistently been associated with metabolic (Reynolds et al., 2001) and neurobehavioral (Davis et al., 2011; O'Connor et al., 2002; O'Donnell et al., 2013) disorders in later life, disentangling the mechanisms that underpin early HPA axis dysregulation is essential. The results of each specific aim are below.

This project's first aim, to identify which factors contribute to psychosocial stress in peripartum women in the Galápagos and to assess how these exposures shape maternal and infant HPA axis regulation, is addressed in Chapter 5. Previous research has shown that maternal distress during pregnancy is associated with long-term effects on infant HPA axis regulation, but the underlying physiology and the role of the postpartum period are not well understood. In this chapter, we used a biocultural approach to first qualitatively identify a central and culturally relevant source of distress for women on the Galápagos Islands (low social support), and then use it as an exposure to quantitatively test its effect on maternal and infant HPA axis development. We tested three propositions: 1) a direct effect of maternal social support

(separately for pregnancy and the postpartum) on infant HPA axis regulation, 2) an additional indirect effect of social support on HPA axis regulation through maternal HPA axis regulation (separately for pregnancy and the postpartum), and 3) an indirect effect of social support during pregnancy on HPA axis regulation through postpartum support. Through our analysis, we confirm our first hypothesis, that during pregnancy and the postpartum, low social support is associated with infant HPA axis dysregulation. Our results do not support our second hypothesis that maternal HPA axis regulation has an indirect effect on relationship between maternal social support and infant HPA axis regulation during pregnancy or in the postpartum. We confirm our third hypothesis, that postpartum support has an indirect effect on the relationship between prenatal support and infant HPA axis functioning, suggesting that postpartum experience can attenuate prenatal insults to infant development. By incorporating the culturally-salient role of social support during pregnancy and the postpartum into a model for infant HPA axis development, this study adds a critical component to the literature on the developmental origins of health and disease that will elucidate the pathways through which early environments shape development. Further, this study is one of the first to investigate the longitudinal influence of maternal distress throughout the peripartum period on infant HPA axis development, and its primary result, that the postpartum period continues to influence infant HPA axis functioning, can be used to develop programming that emphasizes continual, pre- and postpartum mental health support for women.

This project's second aim, to assess both the psychosocial and physiological relationships between maternal distress during pregnancy and the placental enzyme, HSD11B2, as well as the relationship between HSD11B2 and infant HPA axis development, was assessed in Chapter 6. The placental enzyme, HSD11B2, catalyzes the metabolism of cortisol to inert

cortisone in the placenta, and thus is a central component of the pathway through which maternal cortisol reaches a developing fetus. Nonetheless, little is known about what influences this enzyme's functioning and expression. Using adjusted linear regression models, we assessed the effects of maternal psychosocial (stress and depression) and physiological (HPA axis dysregulation) distress on *HSD11B2* methylation and expression and then tested how these measures influence infant HPA axis development. Our results show that that higher *HSD11B2* methylation is associated with lower HSD11B2 expression, and that maternal HPA axis dysregulation during pregnancy is associated with lower placental HSD11B2 expression, which is associated with an exaggerated cortisol reactivity in infants. Sex-specific analyses found that maternal depression was marginally associated with more placental *HSD11B2* methylation and significantly associated with less HSD11B2 expression for the mothers of girls, but not boys. Our results support a disrupted adaptive framework, in which the ability to upregulate HSD11B2 expression in response to acute stress diminishes as maternal stress becomes chronic. In this model, it is possible that chronic stress exhausts the protective mechanism of HSD11B2, leaving the infant vulnerable to high levels of maternal cortisol, which could injure the fetal HPA axis and disrupt neurobehavioral and metabolic development. By incorporating both psychosocial and physiological measures of maternal distress into our model, as well as the role of infant HPA axis development in response to placental changes, this study adds a critical component to the literature on the fetal programming that will help illustrate the biological underpinnings of early life adaptations.

This project's third aim, to analyze the relationships among maternal stress and HPA axis dysregulation during the peripartum period, infant gut microbiome composition, and infant HPA axis functioning, was addressed in Chapter 7. While many studies have examined the HPA

axis as the primary mechanism for the relationship between maternal stress and metabolic diseases and neurobehavioral disorders in offspring, recent research on the brain-gut axis suggests that the microbiome may play an important role in this pathway. In this chapter, we examined food insecurity, low social support, depression, and stress as measures of precarity that may contribute to physiological changes. Measures of maternal precarity and maternal and infant HPA axis functioning were all associated with differences microbiome composition. Maternal precarity was associated with lower diversity and higher relative abundance of *Enterobacteriaceae* and *Streptococcaceae* and a lower relative abundance of *Bifidobacteriaceae* and *Lachnospiraceae*. These patterns of colonization for *Enterobacteriaceae* and *Bifidobacteriaceae* mirrored those found in infants with HPA axis dysregulation. Maternal HPA axis dysregulation during pregnancy was associated with a lower relative abundance of *Bacteroidaceae*, while the opposite was found for maternal HPA axis dysregulation in the postpartum. Maternal HPA axis dysregulation during pregnancy was also associated with a greater relative abundance of *Veillonellaceae.* Overall, exposures to precarity and HPA axis dysregulation were associated with an increase in pathogenic bacteria, including *Enterobacteriaceae, Streptococcaceae,* and *Veillonellaceae*, and a decrease in protective bacteria, including *Bifidobacteriaceae* and *Lachnospiraceae*, as well as a decrease in overall microbiota diversity. Our results suggest that the gut microbiome is intricately intertwined with both maternal stress and infant HPA axis development and that the gut microbiome likely plays an important role in the relationship between peripartum maternal stress and long-term shifts in infant development.

8.2 Implications for Maternal and Child Health in the Galápagos

This research demonstrates the intergenerational effects of maternal stress, depression, food insecurity, and low social support on infant development. While the results of this work have implications for the mechanisms underpinning the intergenerational inheritance of stress, they also have implications for the people of the Galápagos Islands. The Galápagos Islands were selected as the setting for this research in part for their uniquely stressful environment. Unbeknownst to most visitors of the islands, everyday life for residents poses a variety of geographic, political, and infrastructural challenges. Food and water insecurity contribute to detrimental health consequences and economic hardship, strict migration policies limit connections with friends and family on the mainland, inaccessibility of trusted health care exacerbates existing health conditions, and steep socioeconomic inequalities hinder access to everyday needs in the islands' tourist economy. Together, these factors also impair overall well-being and contribute to mental health concerns including stress, anxiety, and depression, which have not been adequately addressed by the healthcare system on the island.

Interviews with participants revealed that although there is one psychologist appointed at HOJ, he has not been able to help with participants' concerns about their well-being, and particularly, their concerns about depression. Even participants who had a history of depression (diagnosed on the mainland), did not feel comfortable discussing these concerns with the psychologist on the island, which could stem from a variety of factors. First, as discussed in Chapter 2, residents are still building trust with the new, government-built hospital (HOJ), and many are skeptical of the quality of physicians. Second, since clinical appointments for health care providers on the islands are contracted for only a few years at a time, the community has not had the opportunity to build trust with most providers, making intimate conversations about

mental health and depression difficult. In the same vein, in interviews, participants often discussed their hesitance to confide in anyone outside their own families due to the proclivity for gossip on the small island of only 7,000 people. In interviews, health care providers at HOJ have also suggested that doctor-patient confidentiality is not always upheld, which prevents patients from receiving the care they may need.

In providing evidence for how domains of distress are embodied in the health of women and their children, this research emphasizes the importance of addressing mental health burdens for residents of the Galápagos. Building psychological service capacity at HOJ will be integral to this process, but within the hospital, best practices guidelines for doctor-patient confidentiality will need to be developed and followed for these psychological services to be effective. This in itself will require a cultural shift in the hospital, which will take time. Further, this research provides evidence that infant HPA axis development continues after birth and that postpartum relief from distress can ameliorate some of the consequences of prenatal adversity. This finding may be used to develop programs for maternal and child health on the islands. Last, as many women discussed experiencing a lack of social support, programs may consider implementing partner or group work, so that women have the opportunity to develop strong relationships with their partners or with other women on the island, which could improve their own well-being as well as the health of their children. Including fathers in infant development workshops would also ameliorate some of the tensions expressed by women regarding *machismo* on the island and provide caregivers with the knowledge and resources they need to work together in childrearing.

8.3 Overall Strengths and Limitations

This research utilizes a longitudinal, mixed-methods design to analyze rich narrative

interviews alongside psychosocial and physiological measures of stress to examine how stress contributes to the health and well-being of mothers and their children on the Galápagos Islands. This project incorporates three under-studied concepts, the postpartum period, the role of the placenta, and communication with the gut microbiome, into the traditional study of maternal stress and infant HPA axis development. Further, this work uses anthropological and evolutionary theories to situate these results within anthropology and epidemiology.

One key strength of this research its multi-faceted approach used to interrogate the concept of psychosocial stress. In Chapter 5, low social support is identified as a central and culturally-relevant distress exposure through the qualitative analysis of interview data. In Chapter 6, stress and depression are used to examine psychosocial stress, and in Chapter 7, food insecurity, low social support, depression, and stress, were each used as a measure of precarity to investigate differences in microbiome development. Examining stress in this multidimensional way provides a deeper understanding of an individual's well-being that moves beyond traditional measures of "stress." Using varied measures of psychosocial distress also defines finely articulated concepts, which can be intervened upon in different ways.

This study's longitudinal design is another key strength. Mother-infant dyads were enrolled in the study for approximately three months, during which time they each participated in four visits. This design allowed for the collection of maternal psychosocial and physiological (salivary cortisol) stress data from one prenatal and two postpartum time points. This data was essential to demonstrating the importance of the postpartum period in shifting infant HPA axis development (Chapter 5). Further, the longitudinal design was also central to the foundational model used throughout each chapter, as it assesses how experiences in pregnancy and the postpartum shape infant development.

More broadly, this project provides novel evidence for various biological pathways in humans. The role of both placental HSD11B2 and the gut microbiome are only recently being considered as mechanisms involved in the intergenerational transfer of stress. Consequently, Chapter 6 constitutes the first study to assess how placental *HSD11B2* methylation and expression respond to maternal distress and shape infant cortisol in humans. After Stroud and colleagues (Stroud et al., 2016), this is only the second study to examine how differences in HSD11B2 measures shape infant HPA axis regulation in humans. Further, to my knowledge, Chapter 7 is only the second study to examine the effects of maternal prenatal stress on infant gut microbiome development and associated HPA axis function in humans and is the first study to include both prenatal and postnatal stress in these models.

Last, while the majority of studies on intergenerational inheritance of stress have been conducted with Caucasian populations in high-income countries (particularly the United States and Europe), this is one of the first studies to assess these relationships in a primarily *Mestizo* population in South America. This distinction is particularly important for Chapter 6, since recent work has found differences in HSD11B2 measures based on ethnicity (Capron et al., 2018).

Despite these strengths, this research is not without limitations. The study's small sample size limits its statistical power, but overall, we estimate that we were enrolled over half of all births on San Cristóbal in 2018 based on annual birth rates. Second, some participants were lost to follow-up, primarily due to participant travel to the mainland to visit family. We also faced challenges collecting a sufficient amount of infant saliva for analysis, particularly at 3 days postpartum, when infants do not produce much saliva. These challenges limit our statistical power and the generalizability of results. Nonetheless, our studies do detect clear, consistent, and

significant relationships between measures of distress and our outcomes, and as novel contributions to biological pathways in humans, these studies may serve as foundational exploratory research for larger projects.

8.4 Directions for Future Research

Future research should examine these pathways on a larger scale, particularly in non-Caucasian populations in low- and middle-income contexts. In regard to the role of the continuum of early development in particular, future research should assess how maternal mental health interventions during the peripartum period influence shifts in maternal and infant physiology. This research could inform evolutionary theory by providing evidence for the adaptive mechanisms behind early life modifications to development. For example, this work may be able provide evidence for either predictive adaptive responses, through which fetuses adjust their development according to their mother's current environment (Gluckman et al., 2005, 2007; Godfrey et al., 2010), and others' hypotheses that early life shifts in development respond to cues from maternal physiology, which has built up over her lifetime (Kuzawa, 2005; Wells, 2007, 2010). This work would also be useful in developing interventional mental health programs during this important period of growth and development for both mother and infant.

Future research on the HSD11B2 pathway should incorporate a variety of epigenetic measures. For example, several studies have found that another protein, NRC31, serves as a placental glucocorticoid receptor that may be an upstream regulator placental *HSD11B2* (Capron et al., 2018). Assessing how this protein, and others, are influenced by epigenetic changes will be essential to building a better understanding of placental physiology, which has been largely understudied.

Further research on the gut microbiome's role in this pathway should incorporate maternal vaginal and gut microbiome measures in order to articulate biological pathways more clearly. Other research should investigate the utility of vaginal seeding, the process through which a swab is used to transfer maternal vaginal fluids to the mouth, nose, or skin of the infant in order to transfer maternal bacteria to an infant. While this practice is currently being investigated (but not recommended) for infants born by Caesarean, its central principle could also be used in other contexts, like priming an infant with more beneficial and less pathogenic bacteria if its mother's microbiota suggest that dysbiosis. More research is needed in this area to determine intervention protocols for the gut microbiome.

8.5 Conclusions

This dissertation aimed to better understand the complex psychosocial and physiological pathways through which stress is embodied in women and their infants on the Galápagos Islands. This work employed both biocultural and evolutionary models to investigate these pathways and incorporated three under-studied concepts into traditional frameworks for intergenerational stress transfer: the role of the postpartum period, the role of the placenta, and the role of the gut microbiome.

The results contribute novel findings to human research on DOHaD. First, we find that the postpartum period can attenuate prenatal insults to infant HPA axis development, thus providing support for a continuum of early development and emphasizing the importance of early life as a developmental niche in which a child's physical and social setting, customs of childcare, and psychology of caretakers can affect infant health and development. Second, we find that physiological stress during pregnancy, measured through maternal HPA axis

dysregulation, is associated with lower placental HSD11B2 expression, which is associated with an exaggerated cortisol reactivity in infants. Further, maternal psychosocial distress during pregnancy, measured through depression was marginally associated with more placental *HSD11B2* methylation and significantly associated with less HSD11B2 expression for the mothers of girls, but not boys. Evolutionarily, these results fit into a disrupted adaptive framework, in which the ability to upregulate HSD11B2 expression in response to acute stress diminishes as maternal stress becomes chronic. In this framework, which is supported by previous research with animal models, it is possible that chronic stress exhausts the protective mechanism of HSD11B2, leaving the infant vulnerable to high levels of maternal cortisol, which could injure the fetal HPA axis and disrupt neurobehavioral and metabolic development. Notably, this is the first study to assess how placental *HSD11B2* methylation and expression respond to maternal distress and shape infant cortisol in humans. Last, in our microbiome pathway, we find that maternal precarity and HPA axis dysregulation were associated with an increase in pathogenic bacteria, including *Enterobacteriaceae, Streptococcaceae, and Veillonellaceae*, and a decrease in protective bacteria, including *Bifidobacteriaceae* and *Lachnospiraceae*, as well as a decrease in overall microbiota diversity. These results suggest that the gut microbiome likely plays an important role in the relationship between peripartum maternal stress and long-term shifts in infant development. This is the second study to assess the effects of maternal prenatal stress on infant gut microbiome development and HPA axis function in humans, and it is the first study to include both prenatal and postnatal stress in these models. Overall, these findings contribute novel insights into early life development trajectories and reinforce the importance of using multidimensional measures of "stress" to investigate early

adverse environments. Results from this work can be used to inform both maternal mental health and early infant development interventions.

APPENDIX A: RECRUITMENT STUDY SUMMARY

Esquema del Estudio
Estudio: *Madres sanas, bebés sanos*
Investigador: Hannah Jahnke, [phone number redacted], hjahnke@live.unc.edu

Visita 1:

- **¿Cuándo?:** Esta visita se realizará cuando tenga entre 34 y 36 semanas de embarazo.
- **¿Dónde?:** Esta visita se realizará en su casa o en cualquier otro lugar que elija.
- **¿Cuánto tiempo?:** Durará aproximadamente una hora.
- **¿Qué?:** Consistirá en:
    - Cuestionarios
    - Muestra de saliva de la mamá
    - Muestra de cabello de mamá (solo si usted está de acuerdo)

Visita 2:

- **¿Cuándo?:** Se realizará en la primera semana después del nacimiento de su bebé.
- **¿Dónde?:** Esta visita se realizará en su casa o en cualquier otro lugar que elija.
- **¿Cuánto tiempo?:** Durará aproximadamente diez minutos.
- **¿Qué?:** Consistirá en:
    - Muestra de saliva del bebé

Visita 3:

- **¿Cuándo?:** Esta visita se realizará cuando tenga aproximadamente 4 semanas.
- **¿Dónde?:** Esta visita se realizará en su casa o en cualquier otro lugar que elija.
- **¿Cuánto tiempo?:** Durará aproximadamente una hora.
- **¿Qué?:** Consistirá en:
    - Cuestionarios
    - Muestra de saliva de la mamá
    - Del bebé: medidas

Visita 4:

- **¿Cuándo?:** Esta visita se realizará cuando tenga aproximadamente 8 semanas.
- **¿Dónde?:** Esta visita se realizará en su casa o en cualquier otro lugar que elija.
- **¿Cuánto tiempo?:** Durará aproximadamente una hora.
- **¿Qué?:** Consistirá en:
    - Cuestionarios
    - Muestra de saliva de la mamá
    - Muestra de cabello de mamá (solo si usted está de acuerdo)
    - Del bebé: Muestra de saliva, muestra de heces, y medidas

APPENDIX B: INFORMED CONSENT DOCUMENTS

Universidad Carolina del Norte en Chapel Hill
Consentimiento de los adultos y el permiso de los padres para que un menor participe en un estudio de investigación

Fecha de la versión del formulario de consentimiento: 11/13/17
Nº de estudio del IRB 17-0272
Título del estudio: Efectos intergeneracionales del estrés materno en las islas Galápagos
Investigador principal: Hannah Jahnke
Departamento de la UNC-Chapel Hill: Antropología
Número telefónico de la UNC-Chapel Hill: +1-508-397-9329
Dirección de correo electrónico: hjahnke@live.unc.edu
Asesor facultativo: Amanda Thompson
Número telefónico de asesor facultativo: (919) 843-2060

Origen del financiamiento: National Science Foundation

¿Cuáles son algunas de las cuestiones generales que usted debe saber sobre los estudios de investigación?
Se le pedirá su consentimiento para participar en el estudio de investigación. Al dar su aprobación o consentimiento significa que usted aprueba o da el permiso para participar. La participación en este estudio es voluntaria. Si no desea participar o dar permiso para que su hijo participe, usted no tiene que hacerlo, y usted y su niño no será incluido en el estudio. Usted y su hijo puede dejar de participar en cualquier momento, por cualquier razón, sin ninguna penalidad.

Los estudios de investigación han sido diseñados para obtener información nueva. Es posible que esta nueva información ayude a las personas en el futuro. Pueda que no reciba ningún beneficio directo de la participación en este estudio de investigación. También puede haber riesgos asociados con la participación en estudios de investigación.

Los detalles de este estudio se discuten a continuación. Es importante que usted entienda esta información para que pueda tomar una decisión informada sobre la participación en este estudio de investigación. Se le dará una copia de este formulario de consentimiento. Usted debe preguntar a los investigadores mencionados anteriormente, o a los miembros del personal que los asisten, cualquier pregunta que tenga acerca de este estudio en cualquier momento.

¿Cuál es el objetivo de este estudio?
El propósito del estudio es comprender mejor los factores que influyen en el estrés materno durante y después del embarazo en las Islas Galápagos y cómo este estrés da forma a la salud infantil. Para evaluar la salud materna e infantil, este estudio analizará los niveles de hormonas maternas e infantiles, una enzima placentaria y la salud intestinal del bebé.
Hay hormonas que regulan el estrés en nuestros cuerpos. Cuando el estrés es demasiado alto, los niveles de estas hormonas del estrés pueden aumentar, lo que puede conducir a otros problemas

de salud. El alto estrés materno durante y después del embarazo puede aumentar los niveles de estrés en el bebé en desarrollo de la madre. Se tomarán muestras de saliva una vez durante el embarazo y dos veces después del embarazo. También se le extraerá una pequeña muestra de su cabello una vez durante su embarazo y una vez después. También se recolectarán muestras de saliva de su hijo en la primera semana de su vida y antes y después de un pinchón del talón cuando su hijo tiene ocho semanas de edad. Las muestras de saliva y pelo se llevarán al Centro de Ciencias de Galápagos y se analizarán para determinar los niveles de estrés.

También hay una enzima en la placenta que regula las hormonas del estrés durante el embarazo. Se tomará una pequeña muestra de la placenta después del nacimiento del niño. La muestra se examinará en USFQ para medir los niveles de esta enzima.

Existen bacterias beneficiosas en los intestinos que ayudan a digerir los alimentos. Ellos son responsables de mantener intestinos sanos. Se recogerá una pequeña muestra de heces de su hijo cuando tengan ocho semanas. La muestra se llevará a la Universidad de Carolina del Norte para determinar si los intestinos están sanos mediante el examen de bacterias beneficiosas.

Se le harán preguntas sobre su salud general y la salud de su hijo. También se le harán preguntas sobre su estrés durante y después del embarazo y sobre su interacción con su hijo después de que nazca. También mediremos su altura y peso. Se le pedirá permiso para que su hijo participe en este estudio porque usted es la madre o el cuidador del niño.

Le solicitan que participe en el estudio porque está embarazada y vive en las Islas Galápagos.

¿Existe algún motivo por el que usted no deba participar en este estudio?
Usted no debe dar permiso si usted no quiere que su hijo menor de 1 año participe. Usted puede negarse a dejarlos participar si no quiere que ellos participen.

¿Cuántas personas participarán en este estudio?
Si usted participa y decide permitir que su hijo participe en este estudio, usted y su niño será uno de los aproximadamente 100 niños y 100 madres en este estudio de investigación.

¿Cuánto tiempo participará en este estudio?
Usted y su hijo serán contactados aproximadamente ocho veces en aproximadamente cuatro meses. Te visitaremos una vez durante tu embarazo y tres veces después de haber dado a luz. Todas las visitas durarán aproximadamente una hora con la excepción de la segunda visita, que solo tomará unos diez minutos. Recibirá recordatorios de llamadas telefónicas antes de cada visita. Las muestras de saliva, cabello, placenta y heces recolectadas a lo largo del estudio serán almacenadas y analizadas dentro de dos años.

¿Qué ocurrirá si participa en este estudio?

Durante cuatro meses, se le visitará cuatro veces, una durante su embarazo y tres veces después del nacimiento. Todas las visitas ocurrirán en su casa. La visita prenatal ocurrirá cuando tenga 34-36 semanas de embarazo. En la visita prenatal, será entrevistado, responderá cuestionarios sobre estrés, ansiedad y depresión, y proporcionará muestras de saliva y una pequeña muestra de cabello. En la Visita 1, se le darán instrucciones detalladas y materiales de recolección para proporcionar una muestra de saliva después de las visitas. Después de las visitas 1, 3 y 4, volveremos más tarde en la semana para recoger su muestra de saliva.

La visita 2 estará una semana después del nacimiento, cuando el personal del estudio la visitará para recolectar una muestra de saliva de su bebé. La visita 3 ocurrirá cuando su hijo tenga cuatro semanas y estará en su casa o en su ubicación preferida, donde será entrevistado, responderá preguntas sobre el estrés, la ansiedad, la depresión y la interacción con su bebé y le brindará una muestra de saliva. La visita 3 ocurrirá cuando su hijo tenga cuatro semanas y estará en su casa o en su ubicación preferida y procederá de la misma manera que en la Visita 3, pero también incluirá muestras de saliva de su hijo antes y después de un pinchazo en el talón. El pinchazo en el talón servirá para proporcionar niveles de hierro para evaluar la anemia en los bebés y servirá como un estrés para que se pueda evaluar la reactividad del cortisol salival. El pinchazo en el talón se realizará con un microlanceta desechable y estéril, del mismo tipo que se usa de manera rutinaria en las visitas de detección neonatal. Además, en la Visita 4, recogeremos una segunda muestra pequeña de su pelo y una muestra de materia fecal de su bebé. Cada visita durará aproximadamente una hora, con la excepción de la Visita 2, que tomará aproximadamente diez minutos. Se realizarán recordatorios antes de cada visita de seguimiento.

El personal del estudio recogerá sus muestras de placenta del hospital con su permiso. Las muestras de saliva y placenta se analizarán en el Centro de Ciencias de Galápagos y los laboratorios de la USFQ dentro de dos años. Las muestras de pelo y heces se analizarán en UNC-Chapel Hill dentro de dos años. Las muestras no se analizarán para el análisis de infecciones, enfermedades o genética específicas. No recibirás estos resultados.

Puede optar por no responder una pregunta durante las entrevistas por cualquier motivo. Puede optar por abandonar el estudio en cualquier momento sin penalización.

¿Cuáles son los posibles beneficios por participar en este estudio?

La investigación está diseñada para beneficiar a la sociedad mediante la obtención de nuevos conocimientos. Es posible que no se beneficie personalmente por su participación en este estudio de investigación.

¿Cuáles son los posibles riesgos o molestias que implica la participación en este estudio?
Aunque es raro, hablando acerca de su salud y la salud de sus hijos puede causar cierta angustia emocional para la madre (o cuidador). Por lo tanto, estas preguntas se pedirán con compasión y comprensión. Si el investigador ve evidencia de angustia emocional, la madre se le dará tiempo para recomponerse y se preguntará si le gustaría continuar o saltar a otras cuestiones menos angustiantes.

¿De qué manera se protegerá su privacidad?
En este estudio se protegerá la privacidad y la confidencialidad de los participantes. Los participantes no serán identificados en los informes o publicaciones sobre este estudio. Los nombres serán codificados usando un sistema numérico. La información obtenida a partir del cuestionario y las muestras se almacenarán en un fichero automatizado en la oficina del investigador.

Los participantes *no serán* identificados en informes o publicaciones sobre este estudio. Aunque se realizarán todos los esfuerzos por conservar los registros de investigación en forma privada, podrá ocurrir que la ley federal o estatal exija que tales registros, incluida la información personal, sean revelados. Esto es muy poco probable, pero si alguna vez se pide que sean revelados, UNC-Chapel Hill tomará las medidas permitidas por ley para proteger la privacidad de la información personal. En algunos casos, su información reunida en este estudio de investigación podría ser examinada por representantes de la Universidad, patrocinadores de la investigación u organismos gubernamentales con fines tales como el control de calidad o la seguridad.

Las grabaciones de audio se almacenarán como archivos protegidos con contraseña. Después de la transcripción, los archivos de audio serán destruidos. Los participantes que no deseen ser grabados pueden solicitar que se apaguen las grabaciones de audio.

Compruebe la línea que mejor se adapte a su elección:

_____ Está bien grabarme durante la investigación

_____ No está bien grabarme durante la investigación

¿Recibirá algo por participar en este estudio?
Usted no recibirá nada por participar en este estudio.

¿Le costará algo la participación en este estudio?
No existirá ningún costo por participar en este estudio.

¿Qué sucede si desea formular preguntas sobre este estudio?
Tiene el derecho de preguntar, y que le respondan, cualquier duda que tenga acerca de esta investigación. Si tienen preguntas o inquietudes, deben ponerse en contacto con los investigadores mencionados en la primera página de este formulario.

¿Qué sucede si usted desea formular preguntas sobre sus derechos como participante de una investigación?

Toda investigación realizada con voluntarios humanos es examinada por un comité que trabaja para proteger sus derechos y su bienestar. Si tiene preguntas o inquietudes acerca de sus derechos como sujeto de una investigación, puede ponerse en contacto, de manera anónima si lo desea, con el Institutional Review Board de Universidad de Carolina del Norte (Comité de revisión institucional, IRB por sus siglas en inglés) al 1+919-966-3113 o por correo electrónico a IRB_subjects@unc.edu o puede contactar al Dr. William F. Waters, Presidente del Comité de Bioética de la USFQ, al siguiente correo electrónico: comitebioetica@usfq.edu.ec.

Acuerdo del participante:

He leído la información proporcionada más arriba. He realizado todas las preguntas que tengo en este momento. Yo voluntariamente estoy de acuerdo para mí y por mi hijo (o pupilo legal) , _____ , a participar en este estudio de investigación.

_____ _____
Firma de la madre del niño participante Fecha

_____
Nombre de la madre

_____ _____
Firma de la madre para participar Fecha

_____
Nombre de la madre

_____ _____
Firma de la persona que obtiene el consentimiento Fecha

_____
Nombre de la persona que obtiene el consentimiento en imprenta

APPENDIX C: SURVEYS AND SCALES

Household Survey of Assets

¿Tiene su hogar alguno de los siguientes elementos?

Posesión	Cantidad	Notas
Electricidad		
Panel Solar		
Internet		
Wifi		
Aire acondicionado		
Coche/Carro		
Moto		
Bicicleta		
Televisión		
Computadora		
Teléfono tipo de iPhone/Android		
Teléfono móvil no “smart”/antiguo		
Cocina eléctrica		
Cocina de gas		
Licuadora		
Microonda		
Refrigeradora		
Horno		
Ducha		
Baño		
Lavadora		
Secadora		
Lancha		
Empleada doméstica		

By Observation:

1. What is the material used for the floors? _____
2. Is the furniture primarily wooden or plastic? _____
3. Matching wooden living room set? _____
4. Matching wooden dining room set? _____

Perceived Stress Scale

Escala de Estrés Percibido

Las siguientes preguntas se refieren a sus sentimientos y pensamientos durante EL ÚLTIMO MES. En cada pregunta, se le pedirá. Aunque algunas de las preguntas son similares, hay pequeñas diferencias entre ellas y debe tratar cada una como una pregunta separada. El mejor enfoque es responder con bastante rapidez. Es decir, no trate de contar el número exacto de veces que se sintió de una manera particular, pero dígame la respuesta que en general parece la mejor. Por cada afirmación, por favor dígame si usted ha tenido estos pensamientos o sentimientos: nunca, casi nunca, de vez en cuando, a menudo, o muy a menudo. (Lea todas las opciones de respuesta cada vez)

	Nunca	Casi nunca	De vez en cuando	A menudo	Muy a menudo
1. En el último mes, ¿con qué frecuencia ha estado afectado por algo que ha ocurrido inesperadamente?	0	1	2	3	4
2. En el último mes, ¿con qué frecuencia se ha sentido incapaz de controlar las cosas importantes en su vida?	0	1	2	3	4
3. En el último mes, ¿con qué frecuencia se ha sentido nervioso o estresado?	0	1	2	3	4
4. En el último mes, ¿con qué frecuencia ha estado seguro sobre su capacidad para manejar sus problemas personales?	0	1	2	3	4
5. En el último mes, ¿con qué frecuencia ha sentido que las cosas le van bien?	0	1	2	3	4
6. En el último mes, ¿con qué frecuencia ha sentido que no podía afrontar todas las cosas que tenía que hacer?	0	1	2	3	4
7. En el último mes, ¿con qué frecuencia ha podido controlar las dificultades de su vida?	0	1	2	3	4
8. En el ultimo mes, ¿con que frecuencia se ha sentido que tenia todo bajo control?	0	1	2	3	4
9. En el último mes, ¿con qué frecuencia ha estado enfadado porque las cosas que le han ocurrido estaban fuera de su control?	0	1	2	3	4
10. En el último mes, ¿con qué frecuencia ha sentido que las dificultades se acumulan tanto que no puede superarlas?	0	1	2	3	4

Patient Health Questionnaire 8

CUESTIONARIO SOBRE LA SALUD DEL PACIENTE-8 (PHQ-8)

Durante las <u>últimas 2 semanas</u>, ¿qué tan seguido ha tenido molestias debido a los siguientes problemas? *(Marque con un "□" para indicar su respuesta)*	**Ningún día**	**Varios días**	**Más de la mitad de los días**	**Casi todos los días**
1. Poco interés o placer en hacer cosas	0	1	2	3
2. Se ha sentido decaído(a), deprimido(a) o sin esperanzas	0	1	2	3
3. Ha tenido dificultad para quedarse o permanecer dormido(a), o ha dormido demasiado	0	1	2	3
4. Se ha sentido cansado(a) o con poca energía	0	1	2	3
5. Sin apetito o ha comido en exceso	0	1	2	3
6. Se ha sentido mal con usted mismo(a) – o que es un fracaso o que ha quedado mal con usted mismo(a) o con su familia	0	1	2	3
7. Ha tenido dificultad para concentrarse en ciertas actividades, tales como leer el periódico o ver la televisión	0	1	2	3
8. ¿Se ha movido o hablado tan lento que otras personas podrían haberlo notado? o lo contrario – muy inquieto(a) o agitado(a) que ha estado moviéndose mucho más de lo normal	0	1	2	3

FOR OFFICE CODING 0 + _____ + _____ + _____

=Total Score: _____

Perceived Social Support – Family

PSS-Fa (16 ítems)

1.	Mi familia me da mucho ánimo.	1. Sí	No
2.	Yo recibo consejos prácticos de mi familia.	2. Sí	No
3.	La mayoría de la gente es más cercana a su familia que yo a la mía.*	3. Sí	No
4.	Cuando comparto mis opiniones y sentimientos personales con mis familiares más cercanos, me da la impresión que los hace sentir incómodos.*	4. Sí	No
5.	A mi familia le gusta escuchar lo que pienso.	5. Sí	No
6.	Los miembros de mi familia comparten muchos de mis gustos e intereses.	6. Sí	No
7.	Algunos de mis familiares se acercan a mí cuando tienen problemas o necesitan ser aconsejados.	7. Sí	No
8.	Dependo de mi familia para apoyo emocional.	8. Sí	No
9.	Cuando me siento triste o decepcionado(a), puedo contárselo a alguien de mi familia sin arrepentirme de ello después.	9. Sí	No
10.	Mi familia y yo expresamos abiertamente nuestras opiniones.	10. Sí	No
11.	Mi familia está consciente de mis necesidades personales.	11. Sí	No
12.	Mis familiares hablan conmigo cuando se sienten mal.	12. Sí	No
13.	Mi familia es de gran utilidad para ayudarme a resolver mis problemas.	13. Sí	No
14.	Tengo un vínculo muy cercano con varios de mis familiares.	14. Sí	No
15.	Le doy a mis familiares consejos útiles y prácticos.	15. Sí	No
16.	Mis familiares dicen que soy útil ayudándoles a resolver sus problemas.	16. Sí	No

Perceived Social Support – Friends

PSS-Fr (12 ítems)

1.	Mis amigos me dan muchos ánimos.	1. Sí	No
2.	La mayoría de la gente es más cercana a sus amigos que yo a los míos.*	2. Sí	No
3.	A mis amigos les gusta escuchar lo que pienso.	3. Sí	No
4.	Dependo de mis amigos para apoyo emocional.	4. Sí	No
5.	Siento que encajo un poco mal en mi círculo de amigos.*	5. Sí	No
6.	Cuando me siento triste o decepcionado(a), puedo contárselo a alguno de mis amigos sin arrepentirme de ello después.	6. Sí	No
7.	Mis amigos están conscientes de mis necesidades personales.	7. Sí	No
8.	Mis amigos son de gran utilidad para ayudarme a resolver mis problemas.	8. Sí	No
9.	Le doy a mis amigos consejos útiles y prácticos.	9. Sí	No
10.	Mis amigos dicen que soy útil ayudándoles a resolver sus problemas.	10. Sí	No
11.	Los amigos de otros muestran más cariño y preocupación entre ellos, que los míos por mí.*	11. Sí	No
12.	Desearía que mis amigos fueran muy diferentes.*	12. Sí	No

MacArthur SSS

Piense en esto escalera como una representación de las personas en sus comunidades.
Las personas definen la comunidad de diferentes maneras; por favor defínalo de la forma que sea más significativa para usted. En la parte **superior** de la escalera están las personas que tienen la posición más alta en su comunidad. En la parte **inferior** están las personas que tienen la posición más baja en su comunidad.
¿Dónde se colocaría usted en esta escalera?
Coloque una "**X**" grande en el escalón donde cree que se encuentra en este momento de su vida, en relación con otra gente en su comunidad.

Maternal Postnatal Attachment Scale

Escala de Apego Maternal Postparto

Estas preguntas tratan acerca de los pensamientos y sentimientos que usted tiene acerca de su bebé. Por favor, elija una sola opción en cada pregunta.

1. Cuando estoy cuidando al bebé, me siento fastidiada o molesta:
   a. Muy Frecuentemente
   b. Frecuentemente
   c. Ocasionalmente
   d. Muy rara vez
   e. Nunca

2. Cuando estoy cuidando al bebé, siento que se está portando mal a propósito o que lo hace por molestarme:
   a. Muy Frecuentemente
   b. Frecuentemente
   c. Ocasionalmente
   d. Muy rara vez
   e. Nunca

3. En las dos últimas semanas, puedo describir mis sentimientos hacia el bebé como:
   a. Desapego
   b. No tengo sentimientos fuertes hacia el bebé
   c. Algo de cariño
   d. Cariño fuerte
   e. Muchísimo cariño

4. Al pensar en el nivel de involucramiento con el bebé:
   a. Me siento muy culpable de no estar más involucrada
   b. Me siento culpable de no estar más involucrada
   c. Me siento un poco culpable de no estar involucrada
   d. No tengo sentimientos de culpa con relación a este aspecto

5. Cuando yo interactúo con el bebé me siento:
   a. Muy incompetente e insegura
   b. Algo incompetente e insegura
   c. Medianamente competente y segura
   d. Muy competente y segura

6. Cuando estoy con el bebé me siento tensa y angustiada:
   a. Muy Frecuentemente
   b. Frecuentemente
   c. Ocasionalmente
   d. Casi nunca

7. Cuando estoy con el bebé y hay otras personas conmigo, yo me siento orgullosa del bebé:
   a. Muy Frecuentemente
   b. Frecuentemente
   c. Ocasionalmente
   d. Casi nunca

8. Intento relacionarme lo más que pueda al JUGAR con el bebé:
   a. Esta afirmación es verdadera
   b. Esta afirmación es falsa

9. Cuando tengo que dejar al bebé:
   a. Normalmente me siento triste (o siento que es difícil dejarlo)
   b. A veces me siento triste (o a veces siento que es difícil dejarlo)
   c. Siento al mismo tiempo tristeza y alivio
   d. A veces me siento aliviada (y se me hace fácil dejarlo)
   e. Normalmente me siento aliviada (y se me hace fácil dejarlo)

10. Cuando estoy con el bebé:
   a. Siempre estoy alegre/ siento satisfacción
   b. Frecuentemente estoy alegre/ siento satisfacción
   c. Ocasionalmente estoy alegre / siento satisfacción
   d. Muy rara vez estoy alegre /siento satisfacción

11. Cuando no estoy con el bebé, me encuentro pensando en el bebé:
   a. Casi todo el tiempo
   b. Muy frecuentemente
   c. Frecuentemente
   d. Ocasionalmente
   e. Nunca

12. Cuando estoy con el bebé:
   a. Normalmente trato de alargar el tiempo que paso con él/ella
   b. Normalmente trato de acortar el tiempo que paso con él/ella

13. Luego de haber estado separada del bebé por un rato, al saber que va a estar de nuevo con él/ella, yo me siento:
   a. Emocionada con la idea
   b. Medianamente emocionada
   c. Un poco emocionada
   d. No siento nada
   e. Me siento angustiada/nerviosa/no me siento bien

14. Ahora, cuando pienso en el bebé:
   a. Pienso que sin lugar a dudas es mi bebé
   b. Me cuesta darme cuenta que es mi bebé
   c. No pienso en él como mi bebé

15. Al pensar en las cosas que he tenido que dejar de lado por el bebé :
    a. Me siento realmente frustrada
    b. Me siento medianamente frustrada
    c. Me siento algo frustrada
    d. No me siento frustrada

16. En los últimos tres meses, siento que no he tenido tiempo para mí o para hacer las cosas que me interesan:
    a. Casi todo el tiempo
    b. Muy frecuentemente
    c. Ocasionalmente
    d. Nunca

17. Hacerme cargo de este bebé es una responsabilidad demasiado grande. Pienso que esta afirmación es:
    a. Realmente cierta
    b. Más o menos cierta
    c. Algo cierta
    d. Para nada cierta

18. Confío en que sé identificar lo que el bebé necesita:
    a. Casi nunca
    b. A veces
    c. Muchas veces
    d. Casi todo el tiempo

19. Normalmente cuando estoy con el bebé:
    a. Me siento muy impaciente
    b. Me siento algo impaciente
    c. Me siento medianamente paciente
    d. Me siento realmente paciente

Infant/Toddler Home Observation for Measurement of Environment

Note: Questions in grayed out boxes were deemed inappropriate for infants under 10 weeks of age and thus were excluded.

HOME Inventory

1. Parent permit child to engage in "messy" play.		
2. Parent spontaneously vocalizes to the child at least twice.	NO	YES
3. Parent responds verbally to the child's vocalizations or verbalizations.	NO	YES
4. Parent tells child name of object or person during visit.	NO	YES
5. Parent's speech is distinct, clear, and audible.	NO	YES
6. Parent initiate verbal interchanges with visitor.	NO	YES
7. Parent converses freely and easily.	NO	YES
8. Parent spontaneously praises child at least twice.	NO	YES
9. Parent's voice conveys positive feelings towards child.	NO	YES
10. Parent caresses or kisses child at least once.	NO	YES
11. Parent responds positively to praise of child offered by visitor.	NO	YES

12. No more than one instance of physical punishment during past week.		
13. Family has a pet.	NO	YES
14. Parent does not shout at child.	NO	YES
15. Parent does not express overt annoyance with or hostility to child.	NO	YES
16. Parent neither slaps nor spanks child during visit.	NO	YES
17. Parent does not scold or criticize child during visit.	NO	YES
18. Parent does not interfere with or restrict child more than three times during visit.	NO	YES
19. At least 3-4 books are present and visible.	NO	YES
20. Child care, if used, is provided by one of three regular substitutes.	NO	YES
21. Child is taken to grocery store at least once a week.		
22. Child gets out of house at least four times a week.	NO	YES

23. Child is taken regularly to doctor's office or clinic.	NO YES
24. Child has a special place for toys and treasures.	
25. Child's play environment is safe.	NO YES

26. Muscle activity toys or equipment.	
27. Push or pull toys.	
28. Stroller or walker, kiddie car, scooter, or tricycle	NO YES
29. Cuddly toys or role- playing toys.	NO YES
30. Learning facilitators-mobile, table, and chair, high chair, play pen.	NO YES
31. Simple hand-eye coordination toys.	
32. Complex hand-eye coordination toys.	
33. Toys for literature and music.	
34. Parent provides toys for child to play with during visit.	

35. Parent talks to child while doing household work.	NO YES
36. Parent consciously encourages developmental advance.	
37. Parent invests maturing toys with value via personal attention.	
38. Parent structures child's play period.	
39. Parent provides toys that challenge child to develop new skills.	
40. Parent keeps child in visual range, looks at often.	NO YES

41. Father provides some care daily.	NO YES
42. Parent reads stories to child at least three times weekly.	
43. Child eats at least one meal a day with mother and father.	
44. Family visits relatives or receives visits once a month or so.	NO YES

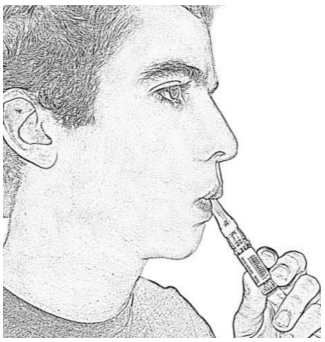
APPENDIX D: MATERNAL SALIVA COLLECTION INSTRUCTIONS

Instrucciones de la colección de Saliva:
Le pedimos que por favor recoja una muestra de saliva cuatro veces en durante un día. Consulte las siguientes instrucciones o contacte a las investigadoras si usted tiene alguna pregunta. Por favor, tome las muestras en los siguientes horarios:

Muestra 1: Inmediatamente cuando usted se despierta
Muestra 2: 30 minutos después del despertar
Muestra 3: 60 minutos después del despertar
Muestra 4: Justo antes de cepillarse los dientes y dormirse

Colección:

1. Por favor, no coma, beba ni cepille los dientes durante 30 minutos antes de recoger la saliva.
2. Retire una pajita y un tubo de la bolsa de plástico con la etiqueta "tubos no utilizados."
3. Permita que la saliva se acumule en su boca (si usted necesita ayuda, ¡imagine comer su delicia favorita!)
4. Inclina la cabeza hacia adelante y babea por la paja y dentro del tubo. Mientras que algunas burbujas o espuma son normales, no fuerce la saliva en el tubo: simplemente déjese babear, como se muestra en la imagen a continuación.
5. Continúe hasta que el tubo esté al menos 1/2 lleno.
6. Cierre la tapa en el tubo y coloque el tubo tapado en la bolsa de plástico etiquetada como "viales llenos." Deseche la paja.
7. Coloque la bolsa de "tubos llenos" en su congelador. Si usted no tiene acceso a un congelador, también puede colocar muestras en el refrigerador.
8. Por favor, recoja las muestras usando los tubos etiquetados en el orden en que proporciona las muestras.
9. Complete la hoja a continuación a medida que se recoge cada muestra.
10. Cuando se hayan recolectado las cuatro muestras, la bolsa etiquetada como "tubos llenos" debe tener cuatro tubos llenos y la bolsa "tubos no utilizados" debe estar vacía. Continúe almacenando la bolsa de "tubos llenos" en el congelador o refrigerador hasta que se reúna con la investigadora.



Section A: Identification

1 Mother Case ID_____
2 Child Case ID_____
3 Interview date_____
4 Location_____
5 Visit #_____

Seguimiento de la colección Saliva:

Por favor, complete esta tabla a medida que proporciona muestras:

Número del tubo	Fecha (DD/MM/YY)	Tiempo iniciado	Tiempo terminado	¿Cómo se siente ahora?
1				
2				
3				
4				

Nota: los números de los viales deben corresponderse con los siguientes tiempos de recopilación:

Muestra 1: Inmediatamente cuando se despierta
Muestra 2: 30 minutos después del despertar
Muestra 3: 60 minutos después del despertar
Muestra 4: Justo antes de cepillarse los dientes y dormirse

Por favor responda las siguientes preguntas sobre su día:

1. ¿Como durmió anoche? _____

2. ¿Pasó algo inusual hoy? Si es así, ¿a qué hora?

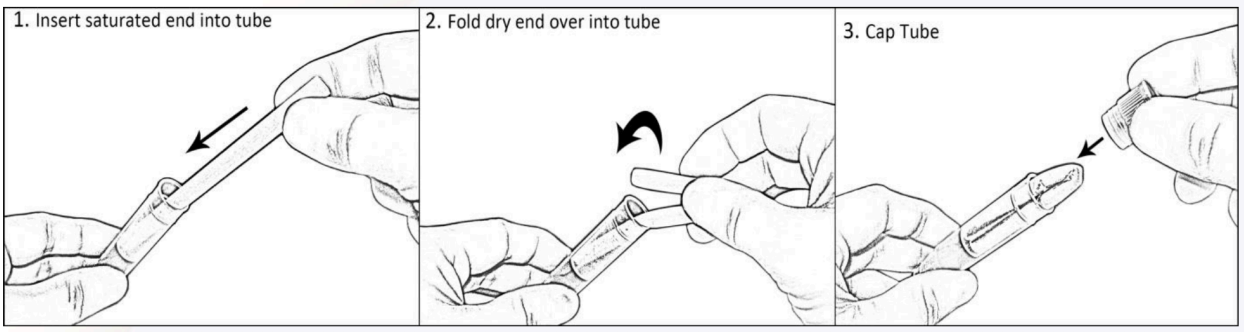
APPENDIX E: INFANT SALIVA SAMPLE COLLECTION

Instructions for Use
(Instructions taken directly from Salimetrics Saliva Collection and Handling Advice)

1. For the SCS (SalivaBio Child Swab) or SIS (SalivaBio Infant Swab), peel open the outer package and remove the device.
2. Securely hold one end of the device and try to place the other end under the child's tongue. With infants it may only be possible to collect pooling saliva (often at the corners of the mouth or under the tongue). You can try to collect for the full 60-90 seconds at once by resting the swab inside the mouth, or collect in intervals by re-introducing the swab into the mouth as needed until the lower third of the swab is saturated (60-90 seconds total).
3. Place the saturated SIS or SCS into the Swab Storage Tube for recovery by centrifugation, or use a 3-5 mL syringe for immediate compression.

The compression method allows the researcher to determine if sufficient saliva has been collected on the first attempt, and the procedure can be repeated if necessary. Some researchers prefer to cut free the saturated portion of the swab before placing it in the centrifuge tube or syringe.

If the swab is used to collect samples for analytes that are affected by saliva flow, however, we advise placing the entire swab into the tube or syringe, in order to estimate saliva flow rates, as described above under Effects of Mouth Location and Flow Rate on Salivary Analytes. The entire swab may be placed in the Swab Storage Tube by inserting the saturated end first, followed by doubling over the dry end into the opening, and finally using the cap or plunger to push the entire swab into the interior space.



SCS/SIS Cautions:

- These devices are packaged clean, not sterile.
- Adult assistance and supervision is required during use.
- Inspect device for tears or imperfections. DO NOT USE if cuts or tears are present.
- When not used as directed these devices may represent a choking hazard for children.
- Store out of reach of children. These devices are not toys and are intended for collection of saliva.

Section A: Identification

6 Mother Case ID_____
7 Child Case ID_____
8 Interview date_____
9 Location_____
10 Visit #_____

Saliva Collection Tracking:
Please fill in this table as you provide samples:

Vial Number	Time started	Time finished
1		
2		

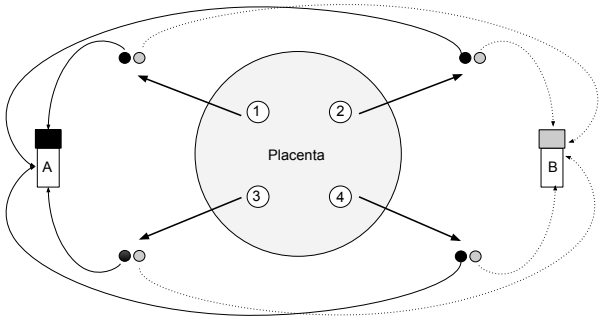
Time of stressor: _____

Did mother feed baby after stress? If yes, how?

APPENDIX F: PLACENTAL SAMPLE COLLECTION INSTRUCTIONS

Las muestras de placenta se deben recolectar dentro de una hora después del alumbramiento de la placenta. Por favor, siga las siguientes instrucciones:

1. Registre la información en "Recolección de datos" abajo.
2. La placenta debe estar fresca antes de la recolección del tejido y no debe tratarse con conservantes o reactivos (por ejemplo, formalina o etanol).
3. Mida la placenta desde los aspectos coriónicos y basales con la regla provista.
4. Retire las membranas amnióticas, tanto como sea posible (idealmente 2 cm de ancho) desde el sitio de su ruptura hasta el margen placentario.
5. Corte el cordón umbilical hasta un centímetro de su origen y asegure que quede atado.
6. Tome el peso de la placenta, utilizando la balanza específica para ello.
7. Coloque la placenta con la membrana basal en la parte superior, e identifique 4 sitios para muestreo, que se encuentren al menos a 2 cm del sitio de inserción del cordón umbilical y al menos a 3 cm del borde de la placenta (ver la figura). En cada sitio, quite la membrana basal cortando con el bisturí provisto. Luego, en cada sitio, use el sacabocados (punch) para obtener dos muestras del tejido velloso expuesto, evitando cualquier área de evidente patología. Por favor usa solo un sacabocados (punch) por cada madre.



8. Inmediatamente, con la pinza provista, ponga las cuatro muestras de cada sitio en el vial criogénico A (que contiene RNAlater), y ponga las otras cuatro muestras (uno de cada sita) en el vial criogénico B (que contiene RNAlater).
9. Rotule tanto el vial A como B con la identificación de la madre.
10. Guarde los viales criogénicos A y B en refrigeración (4°C) durante 72 horas (3 días). Luego de las 72 horas, se debe llevar los dos viales criogénicos a congelación a -20°C.
11. El investigador principal o su ayudante va a venir al hospital cada semana para recolectar las muestras de placenta.

Recolección de los datos

Identificador de caso		Peso del recién nacido	
Fecha		APGAR 5 min	
Peso de la placenta		Duración de la labor	
Tamaño de la placenta (cm)	**x**	Medicación recibida	
Hora de expulsión de placenta		Otras observaciones	
Hora de toma de las muestras		Responsable	

APPENDIX G: STOOL SAMPLE INSTRUCTIONS

Section A: Identification

1. Mother Case ID_____
2. Child Case ID_____
3. Interview date_____
4. Location_____
5. Visit #_____

Muestra de heces
Fecal Sample
Ahora le voy a dar instrucciones para recoger una muestra de heces de su hijo. Por favor, recoja la muestra antes de la segunda visita, una semana a partir de hoy. La muestra será llevada a la Universidad de Carolina del Norte para determinar si el intestino está sano mediante el examen de los productos de las bacterias beneficiosas. Después de que se ha completado la prueba, la muestra será destruida y desechada. Usted no recibirá estos resultados.

Now I am going to give you instructions to collect a sample fecal sample from you child. Please collect the sample before the second visit, one week from today. The sample will be taken to the University of North Carolina to determine if the gut is healthy by examining beneficial bacteria products. After the test is completed the sample will be destroyed and discarded. You will not receive these results.

Suministros *Supplies:*
3 hojas de plástico, *3 sheets of plastic*
Guantes, *gloves*
Pequeña cuchara de plástico, *small plastic spoon*
Recipiente cerrado con líquido preservativo, *sealed container with liquid preservative*
Bolsa de plástico cerrada, *plastic sealed bag*

APPENDIX H: INFANT ANTHROPOMETRY

Section A: Identification

1. Mother Case ID_____
2. Child Case ID_____
3. Interview date_____
4. Location_____
5. Visit #_____

1. A - F

a. Edad:	b. Talla (cm):
c. Peso (kg):	d. Circunferencia de cinturón (cm):
e. Pliegue de piel del brazo (mm):	f. Circunferencia de brazo (cm):

2. ¿En las últimas dos semanas, su hijo(a) ha estado enfermo? Si es así, complete la tabla.

Enfermedad: Nombre y síntomas	Fecha de inicio	Duración	Tratamiento

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