EFFECTS OF MATERNAL CHOLINE SUPPLEMENTATION ON OFFSPRING SELF-REGULATION: RESULTS OF TWO RANDOMIZED CONTROLLED TRIALS

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EFFECTS OF MATERNAL CHOLINE SUPPLEMENTATION ON OFFSPRING SELF-REGULATION: RESULTS OF TWO RANDOMIZED CONTROLLED TRIALS

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Background and Objective: Choline, an essential nutrient, has many important roles during pregnancy. However, most women do not consume the Adequate Intake (AI), and choline is not currently part of standard prenatal regimens. A large body of rodent research has demonstrated that maternal choline intake beyond amounts in standard rodent chow is important for offspring cognition throughout the lifespan, especially attention and memory. There is preliminary evidence to suggest the translation of these effects to humans, although important gaps in our knowledge remain, two of which are addressed by this dissertation. First, no prior studies have experimentally manipulated maternal choline intake and followed the children to school-age; the first chapter of this dissertation presents the results of a test of executive functioning administered to the 7-year-old children of women who participated in a controlled choline feeding study. Second, although a small body of rodent research suggests that maternal choline intake may be important for offspring socioemotional function, no human studies have evaluated the effects of maternal choline intake on infant socioemotional outcomes; chapters 2 and 3 of this dissertation, respectively, present results from a study of maternal choline supplementation on indices of infant temperament and affect regulation during the first year of life. **Methods:** Childhood Study: Third-trimester pregnant women were recruited to take part in a randomized controlled feeding trial. Women were randomized to consume either 480 mg/day (approximately the AI) or 930 mg/day choline until delivery. An ancillary follow-up study was conducted when

their children were 7 years old to assess attention, memory, and executive functioning. This thesis presents the results of the Tower of London, an executive function task of planning and problem-solving. Infancy Study: Second-trimester pregnant women were recruited to take part in a randomized controlled supplementation trial. Women were randomized to consume either 25 mg/day or 550 mg/day choline, plus usual diet, until delivery. An ancillary follow-up study was conducted when their infants were 5–13 months old to assess attention, memory, and socioemotional functioning. This thesis presents the results of the Infant and Early Childhood Behavior Questionnaires and the Face-to-Face Still Face Paradigm. **Results:** Childhood Study: In the childhood study (N = 20), children whose mothers consumed 930 mg/day choline performed better on a task of planning and problem-solving skills as compared to children whose mothers consumed 480 mg/day choline, indicative of superior executive functioning. Infancy Study: In the infancy study, there was no effect of maternal choline supplementation on parent-report measures of infant temperament (N = 25) across the first year of life or on a laboratory measure of affect regulation at 7 months of age (N = 16). **Conclusions:** Childhood Study: Maternal choline supplementation at approximately 2x the AI has significant beneficial effects on child executive functioning at 7 years of age compared to the AI. Infancy Study: Maternal choline supplementation in addition to usual diet does not have an effect on infant temperament or affect regulation. However, interesting patterns emerged indicative of a more adaptive affective response in the infants born to women in the higher choline supplementation group. These preliminary data indicate that the current AI for pregnant women may not be sufficient for offspring self-regulation and support the conclusion that choline should be added to a standard prenatal vitamin regimen.

BIOGRAPHICAL SKETCH

Dr. Kara A. Beckman was born in Rochester, New York, and raised in Downingtown, Pennsylvania. She is the daughter of Kathleen Callahan Beckman, J.D., and Stephen Beckman. Kara attended the College of Arts and Sciences at Cornell University, where she first joined the Cornell Choline Cognition Research Group as an undergraduate research assistant, studying the effect of maternal choline supplementation on biological markers of stress and tests of executive function in childhood, under the guidance of Dr. Julie Nevins, Dr. Barbara Strupp, and Dr. Rick Canfield. She received her Bachelor of Arts in Biological Sciences with a concentration in Nutritional Sciences in 2017. She then returned to Cornell University in the Fall of 2017 to obtain her Ph.D. in Human Nutrition in the Division of Nutritional Sciences. Under the advisement of Drs. Barbara Strupp and Rick Canfield, she conducted research to investigate the effects of maternal choline supplementation on child cognitive and affective outcomes. When not in the laboratory or the classroom, Kara enjoys cooking, virtual game nights with friends, and taking walks around Ithaca with her dog, Knightley.

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TABLE OF CONTENTS

Biographical Sketch iii
Acknowledgements iv
Table of Contents vi
List of Figures viii
List of Tables x
List of Abbreviations xi
Chapter 1: Introduction 1
1.1: Developmental Origins of Health and Disease 1
1.2: Choline: An Essential Nutrient for Fetal Neurodevelopment 4
1.3: Maternal Choline Intake: Recommendations and Practices 8
1.4: Animal and Human Evidence 9
1.5: Maternal Choline Intake and Executive Function 16
1.6 Maternal Choline Intake and Affect Regulation 18
1.7: Self-Regulation: Integrating Effects of MCS on Executive Function and Affect 20
1.8: Biological Substrates of Self-Regulation 21
1.9: Gaps in Knowledge 25
1.10: Assessing the Effects of Maternal Choline Supplementation on Self-Regulation 27
1.11: References 31
Chapter 2: Effects of Maternal Choline Supplementation on Child Executive Function at Seven Years of Age 46
2.1: Abstract 46
2.2: Introduction 47
2.3: Subjects and Methods 50
2.4: Results 57
2.5: Discussion 65
2.6: References 72
Chapter 3: Effects of Maternal Choline Supplementation on Parent-Report Measures of Infant Temperament 81
3.1: Abstract 81
3.2: Introduction 82
3.3: Subjects and Methods 86

3.4: Results....96
3.5: Discussion....109
3.6: References....119
Chapter 4: Effects of Maternal Choline Supplementation on Behavioral Measures of Infant Affect Regulation....128
4.1: Abstract....128
4.2: Introduction....129
4.3: Subjects and Methods....134
4.4: Results....142
4.5: Discussion....152
4.6: References....162
Chapter 5: Conclusions....171
5.1: Summary of Results....171
5.2: Significance of Findings....171
5.5: References....182
5.4: References....185
Appendix A: Full Testing Protocol, 7-Year Follow-up....188
Appendix B: Full Testing Protocol, Infancy Follow-up....189
Appendix C: Calculation of Problem Difficulty, Tower of London....191
Appendix D: Task Instructions, Tower of London....193
Appendix E: The Infant Behavior Questionnaire....195
Appendix F: The Early Childhood Behavior Questionnaire....202
Appendix G: Orienting/Regulation Factor, Infant Behavior Questionnaire....212
Appendix H: Maternal and Infant Demographic Characteristics, Infancy Study....214
Appendix I: Sensitivity Analyses, Behavior Questionnaires....216
Appendix J: Task Instructions, Face-to-Face Still-Face Paradigm....219
Appendix K: Coding Guide, Face-to-Face Still-Face Paradigm....222
Appendix L: Maternal and Infant Demographic Characteristics, FFSF Paradigm....232

LIST OF FIGURES

Figure 1.1: The hierarchical model of self-regulation 21
Figure 1.2: Study design and timeline, childhood study 27
Figure 1.3: Study design and timeline, infancy study 28
Figure 2.1: A three-move problem from the Tower of London task 54
Figure 2.2: Participant flow diagram, childhood study 57
Figure 2.3: Participant performance, Tower of London 62
Figure 2.4: Planning time, Tower of London 63
Figure 2.5: Execution time, Tower of London 64
Figure 3.1: Example question, Infant Behavior Questionnaire 90
Figure 3.2: Participant flow diagram, infancy study 97
Figure 3.3: Mean factor scores, IBQ 103
Figure 3.4: Mean Orienting/Regulation factor scores, IBQ 104
Figure 3.5: Mean Duration of Orienting scale scores 105
Figure 3.6: Mean factor scores, ECBQ 106
Figure 3.7: Mean Attentional Shifting scale scores, ECBQ 107
Figure 4.1: The phases of the Face-to-Face Still-Face Paradigm 138
Figure 4.2: Participant flow diagram, infancy study, FFSF task 143
Figure 4.3: Total negative affect for each group, FFSF 146
Figure 4.4: Proportion of infants who did not demonstrate negative affect 149
Figure 4.5: Kaplan-Meier curve for latency to negative affect 150
Figure 4.6: Regulatory behaviors for each group, FFSF 151

LIST OF TABLES

Table 1.1: The DRIs for choline across the life cycle 7
Table 2.1: Sample demographic characteristics, childhood study 58
Table 2.2: Mean problem score, Tower of London 59
Table 2.3: Descriptive data, Tower of London 60
Table 3.1: Scale and factor definitions, Infant Behavior Questionnaire 91
Table 3.2: Scale and factor definitions, Early Childhood Behavior Questionnaire 92
Table 3.3: Sample demographic characteristics, infancy study 98
Table 3.4: IBQ and ECBQ correlations, 5 to 13 months 99
Table 3.5: Mean ratings, IBQ scale and factor scores 100
Table 3.6: Mean ratings, ECBQ scale and factor scores 102
Table 4.1: Outcome measures assessed and reported for FFSF 140
Table 4.2: Sample demographic characteristics, infancy study, FFSF 144
Table 4.3: Total negative affect in each task phase, FFSF 147
Table 4.4: Regulatory behaviors for each group, FFSF 151

LIST OF ABBREVIATIONS

Term	Abbreviation
Acetylcholine	ACh
Adequate Intake	AI
Autonomic Nervous System	ANS
Behavior Assessment System for Children	BASC-3
Behavior Rating Inventory of Executive Function	BRIEF
Brain-Derived Neurotrophic Factor	BDNF
Body Mass Index	BMI
Child Behavior Checklist	CBCL
Child Behavior Questionnaire	CBQ
Corticotropin-Releasing Hormone	CRH
Developmental Origins of Health and Disease	DOHaD
Differential Reinforcement of Low Rate Responding	DRL
Estimated Average Requirement	EAR
Early Childhood Behavior Questionnaire	ECBQ
Executive Functions	EF
Electrocardiogram	ECG
Face-to-Face Still-Face Paradigm	FFSFP
Food Frequency Questionnaire	FFQ
Hypothalamus-Pituitary-Adrenal Axis	HPA
Human Metabolic Research Unit	HMRU
Infant Behavior Questionnaire-(Revised)	IBQ-(R)
Institute of Medicine	IOM
Maternal Choline Intake	MCI
Maternal Choline Supplementation	MCS
Mutual Regulation Model	MRM
Neural Tube Defect	NTD
National Health and Nutrition Examination Survey	NHANES
Prefrontal Cortex	PFC
Pediatric Review and Observation of Children's Environmental Support and Stimulation	PROCESS
Parasympathetic Nervous System	PNS
Phosphatidylethanolamine N-methyltransferase	PEMT
Randomized Control Trial	RCT
S-adenosylmethionine	SAM
Sustained Attention Task	SAT
Sympathetic Nervous System	SNS
Single Nucleotide Polymorphism	SNP
Tower of London	TOL
Tolerable Upper Limit	TUL
Very Low-Density Lipoprotein	VLDL
Wide Range Assessment of Memory and Learning	WRAML2
Wechsler Preschool and Primary Scale of Intelligence-Revised	WPPSI-R

CHAPTER 1

INTRODUCTION

1.1 Developmental Origins of Health and Disease

Developmental Origins of Health and Disease

The idea that what a pregnant woman eats, the physical and emotional stress she experiences, or her social and physical environment can impact her child's lifelong health and disease risk is an accepted scientific tenant of human development.[13–14,67] Since David Barker first published epidemiological data linking inadequate prenatal nutrition and low birth weight with coronary heart disease risk in adulthood, a large body of empirical data has provided strong support for his initial observations.[7] Originally the "Barker Hypothesis", and now widely known as the Developmental Origins of Health and Disease hypothesis (DOHaD), this idea posits that exposure to various environmental factors during key early periods of growth and development can have significant, lifelong influences on an individual's health and well-being, which are not always apparent at birth.[69,102] This was a significant shift from the prevailing theory of the twentieth century, in which the fetus was believed to be a "perfect parasite", whose development was unaffected by the mother's nutrition, health, or environment. This shift spurred a renewed interest in understanding the long-term health consequences of prenatal exposures that don't produce immediately observable clinical symptoms. Collectively, the research conducted on DOHaD has identified prenatal nutritional perturbations, prenatal chemical exposures, and prenatal maternal stress as three prominent early environmental inputs that impact disease susceptibility across the lifespan.[50]

DOHaD research initially focused on the deleterious effects of under-or over-nutrition in early life, identifying the effect of prenatal nutrition on birth weight as a risk factor for adulthood

diseases including hypertension, type two diabetes, and cardiovascular disease.[47–48,50,69,84] More recent research has demonstrated that small differences in a woman's intake of single nutrients such as vitamin D or iron, even within normal intake ranges, can have long-term consequences for her child's growth and physical health.[47–48,50,115] Several studies subsequent to the original Barker report demonstrated that disease risk varied across the birthweight continuum of his observed cohort, and was not limited to clinically-defined under- and overweight infants.[49,84] One observational cohort study found that small variations (~3 grams/day) in maternal carbohydrate intake during the first trimester of pregnancy were associated with child adiposity at 6 and 9 years old.[49] Further, the timing of nutritional perturbations during pregnancy has been shown to affect health outcomes. Data from the Dutch Famine Study showed that infants exposed to the famine in the first trimester had significant negative long-term health outcomes, including increased mortality from cardiovascular disease in adulthood, whereas those exposed to the famine in the third trimester had lower birth weights, but fewer long-term health problems.[101]

Nutritional deficiencies during pregnancy can also cause physical defects. In the mid-1900s, insufficient folate during early pregnancy was identified as a risk factor for neural tube defects (NTDs) in pregnant women of low socioeconomic status. Importantly, the amount of folate required to reduce the risk of NTDs was found to be relatively low, and supplementation with only 4 mg/day of folate during the first twelve weeks of pregnancy was sufficient to significantly reduce the risk of NTDs in the offspring of women who had already had a pregnancy affected by NTDs.[96] Prenatal choline supplementation has also been shown to play a role in the prevention of NTDS. In a case-control study, women who had choline intakes in the lowest quartile (< 300 mg/day) were four times more likely to have an infant with an NTD than

women with choline intakes in the highest quartile (> 500 mg/day), independent of folate intake.[96]

Epigenetics and Brain Development

One potential mechanism by which early developmental environmental perturbations (e.g., maternal nutrition or drug exposure) can cause lasting effects is epigenetic changes. The term epigenetics refers to alterations to gene expression or to products of gene expression, such as mRNA and proteins, that affect gene activity without changing the nucleotide sequence of the underlying DNA itself.[41,51] This in turn affects the course of development by altering cell growth, proliferation, and differentiation.[41] The field of epigenetics is broad, and many cellular processes can affect gene expression, but two epigenetic mechanisms that have been identified as important in early development are histone modifications and changes in DNA methylation.[41] DNA methylation, in which a methyl group (CH3) is added to a DNA sequence or a histone, typically blocking or reducing expression of a gene or DNA region, is an epigenetic mark that has been shown to play important roles in mammalian development.[63] Diet is a major source of methyl groups, and DNA methylation has been identified as one way by which early life nutrition can have long-term effects. One well-known example is a body of research in which agouti mice are supplemented with dietary methyl donors during pregnancy, which causes differential DNA methylation of the Avy allele and results in phenotypic differences in offspring coat colors and obesity.[113] Significantly, choline is the major source of methyl groups in the human diet (~30 mmol/day) and is crucial for the maintenance of one-carbon metabolism, which contributes to gene methylation.[9,80] In rats, choline supplementation beyond amounts seen in rodent chow during pregnancy has been shown to modify the methylation of both histones and genes in the brain and liver of the offspring, suggesting that epigenetic mechanisms may be one avenue by

which choline exerts lifelong effects on offspring cognition.[37]

The effects of early environmental inputs on a child's later physical disease risk have been extensively studied, although the subtler—but still functionally important—effects of early environment on later cognition, learning, and behavior are still being discovered. The brain, like most tissues, experiences rapid growth and expansion during the prenatal and early postnatal periods.[35] Although the brain continues to develop, and retains substantial plasticity throughout childhood and into early adulthood, much of the initial architecture on which later cognition, perception, and emotion are built is determined by patterns of gene expression and neural circuitry established during early development, which are influenced by early life experiences.[43] The brain is especially sensitive to nutrient input during pregnancy and early postnatal life.[35] Both animal and human research has shown that prenatal protein and/or energy insufficiences can harm later cognitive flexibility, learning, and memory[66,99], and insufficiencies in micronutrients such as iron, zinc, iodine, folate, B12, and choline have also been found to have long-term effects on neurodevelopment and cognition.[35] Choline, specifically, has been found in rodent models to alter the structure of the brain when given prenatally, and, given its many important roles during development, may be a key factor in the foundation of later cognition and socioemotional function.

1.2 Choline: An Essential Nutrient for Fetal Neurodevelopment

The Role of Choline in Fetal Development

Choline, recognized as an essential nutrient by the Institute of Medicine (IOM) in 1998[42,122], plays key roles in fetal growth and neurodevelopment, including providing constituents for the development of cell membranes, neurotransmitters, and epigenetic modifications. Choline-derived phospholipids, including sphingomyelin and

phosphatidylcholine, are key components of cell membranes (phosphatidylcholine comprises > 50% of phospholipids in mammalian cell membranes); thus, choline is key to the structural integrity of cells.[110,122] Phosphatidylcholine also acts as a key constituent of the lipoprotein very low-density lipoprotein (VLDL), which plays a main role in lipid metabolism and removes fat from the liver.[110]

Choline also acts as a required precursor to the neurotransmitter acetylcholine (ACh), a key neurotransmitter at neuromuscular junctions, in the visceral motor system, and in the central nervous system.[88] Increased dietary choline intake increases pools of choline stored in cholinergic neurons, which affects the rate of acetylcholine synthesis and sustained acetylcholine release.[21,123] Methyl groups from choline are available for one-carbon metabolism after the irreversible conversion of choline to betaine, and choline also serves as the primary dietary source of s-adenosylmethionine (SAM). These metabolites of choline play key roles in epigenetic modification of genes and histones, which affects gene expression.[122] During fetal development, choline influences brain development by influencing stem cell proliferation, migration, and apoptosis, specifically in the hippocampal septum.[5–6] Prenatal choline intake may also protect against the development of neural tube defects.[96]

Dietary and De Novo Sources of Choline and Requirements for Humans

Most choline is consumed via diet. Although choline is found in most foods that have membranes, animal foods are generally more choline-rich than plant foods.[110,122] Examples of foods that are high in choline include chicken liver, eggs, and wheat germ.[122] Choline is found in food as both free choline and in its esterified forms (glycerophosphocholine, phosphatidylcholine, etc.), and some research suggests that these forms may have different bioavailability, as water-soluble and fat-soluble forms are differentially taken up by the liver.[32]

In addition to dietary sources of choline, humans can also synthesize choline endogenously via the phosphatidylethanolamine N-methyltransferase (PEMT) pathway. This enzyme uses SAM as a methyl donor to synthesize choline.[124] PEMT -/- knockout mice have significantly decreased pools of liver phosphocholine, suggesting that this endogenously synthesized choline is an important supplement to dietary intake in meeting daily requirements; however, most people also require exogenous consumption of dietary choline to prevent deficiency.[110,124] Premenopausal women have an increased capacity to synthesize choline via the PEMT pathway, as gene expression of the PEMT enzyme is induced by estrogen.[40,110,124] However, many premenopausal women have a common single nucleotide polymorphism (SNP) in the gene that codes for the PEMT enzyme, which may increase susceptibility to choline deficiency and increase dietary needs for choline.[36] It is estimated that approximately 50% of people are affected by this polymorphism, raising the daily choline intake requirements of most premenopausal women to that of men and postmenopausal women.[110,123]

In 1998, the IOM released a report on dietary intake of B-vitamins and choline, setting the Adequate Intake (A1) for choline at 425 mg/day for premenopausal adult women and 550 mg/day for adult men (Table 1.1).[42] An AI is set based on either observed or experimentally determined estimates of nutrient intake by healthy people and is used when there is insufficient data to establish an Estimated Average Requirement (EAR).[54,82] The primary criterion used to set the AI was the prevention of liver dysfunction in adult men, assessed in a single study of fifteen men.[122] Due to the high nutritional needs of pregnancy and lactation, the AI was increased to 450 mg/day for pregnant women, and 550 mg/day for lactating women.[42]

Age Group	Adequate Intake (mg/d)	Tolerable Upper Limit (mg/d)
0–6 Months	125	Not possible to establish
7–12 Months	150	Not possible to establish
1–3 Years	200	1,000
4–8 Years	250	1,000
9–3 Years	375	2,000
≥ 14 Years (Men and Postmenopausal Women)	550	3,000
14–18 Years (Women)	400	3,000
≥ 19 Years (Women)	425	3,500
Pregnant Women	450	3,500
Lactating Women	500	3,500

Table 1.1: The DRIs for choline across the life cycle.[83] An Adequate Intake (AI) is a dietary intake recommendation set when there is insufficient data to set an Estimated Average Requirement.[54] Tolerable Upper Limit (TUL) is the maximum daily intake that can be safely consumed without risking serious side effects or overdose.[54,82]

Demands for Choline During Pregnancy

It is well established that choline is a nutrient in particularly high demand during pregnancy. In both animal models and humans, large amounts of choline are delivered to the fetus from the mother across the placental barrier, against its concentration gradient.[68,103] In unpublished observations, the choline concentration of amniotic fluid has been recorded as 10 times higher than in maternal blood.[122–123] Further, choline concentrations are 6–7 times higher in the fetus and newborn than in adults.[122] Much of this choline is stored in the placenta as acetylcholine (ACh), which makes the placenta the only non-nervous tissue to contain large stores of the neurotransmitter, and may indicate a storage pool designed to ensure delivery of choline to the fetus.[68] Throughout pregnancy, these increased fetal and placental demands for choline significantly reduce maternal stores in both rodents and humans.[119]

In rodent models, pregnant rats fed a choline-containing diet were found to have significantly lower hepatic choline stores than non-pregnant controls, and pregnant rats fed a choline-deficient diet had nearly depleted their hepatic choline stores.[123] Consistent with the

increased demand for choline during pregnancy, there is a pronounced depletion of maternal choline pools in pregnant women, even when they consume the AI.[56,71] Notably, in a recent study where the choline content of the diet was completely controlled, consumption of 930 mg/day of choline during pregnancy (approximately twice the AI) increased circulating levels of choline metabolites (v 480 mg/day) without affecting urinary choline excretion, suggesting that even greater intakes of choline are needed to meet pregnancy demands.[119]

1.3 Maternal Choline Intake: Recommendations and Practices

Despite the clear need for choline during pregnancy by both the mother and the developing fetus, pregnant women in the United States and Europe consume on average only 70% of the choline AI. In 2017, data from the National Health and Nutrition Examination Survey (NHANES) conducted from 2005–2014 showed that pregnant women had a usual dietary intake of 319 +/- 9.89 mg/day, a deficit of greater than 100 mg/day below the recommended amount. Further, only ~9% of pregnant women ages 13–44 years who were surveyed met the AI for choline consumption.[111–112] Analysis of the 2017–2018 update to the NHANES survey showed that this number is declining: on average, women ages 12–49 consumed ~270 mg/day of choline (+/- ~ 11.4 mg).[118] These data suggest that many pregnant women may be at risk for functional choline deficiency, with possible long-term consequences for their offspring.

Given the increased need for maternal choline intake during pregnancy, as well as choline's many important roles in fetal growth and development, the American Medical Association voted in 2017 to include evidence-based amounts of choline in all prenatal vitamins, and the American Academy of Pediatrics recognized choline as a key nutrient for early brain development in 2018.[8] Despite this scientific and professional support for standardized prenatal choline supplementation, choline remains absent from or present in only small amounts in

prenatal vitamins (~55 mg). This small amount, along with the average dietary intake of 350 mg/day, is not enough to meet the choline AI for pregnant women, which is set at 450 mg/day. Further, robust animal data and an emerging body of human data strongly suggest that supplementation well beyond the AI, which was set based on the preservation of liver function in adult men, and not on neurocognitive outcomes for infants, may be necessary for optimal development.[11,12,27,72,75,87]

1.4 Animal & Human Evidence

MCS and Cognition: Evidence from Rodent Models

In rodent models, perinatal choline supplementation beyond the amounts seen in standard rodent chow has been shown to improve offspring memory and attention, prevent age-related memory decline, and lessen cognitive dysfunction in both typically developing rodents and models of Down syndrome, autism, prenatal stress, prenatal alcohol exposure, and Alzheimer's disease.[72,75,87,108,116] For example, in a radial maze task, the adult offspring of dams supplemented with additional choline during pregnancy had superior performance to that of the adult offspring of dams fed a control (standard chow) diet.[72] In this task, eight of twelve maze arms were 'baited' with a food pellet. The task for the rat is to remember which arms they have already visited (and consumed a food reward in), so that they can obtain all 8 pieces of food as efficiently as possible. Both episodic memory (i.e., memory of which arms they have visited during the session) and reference memory (i.e., learning/memory of which arms are always baited) were superior in the offspring of dams fed additional choline during pregnancy.[72]

Perinatal choline supplementation has also been found to improve attention in rodents. In rats whose mothers were fed either choline-deficient, standard, or supplemented diets during pregnancy, the supplemented diet improved the offspring's attentional control in a one-hour

signal detection task during adulthood.[75] In another study of probability of attention (pA) to two cues, each presented for a different duration, the offspring of choline-supplemented dams were able to attend to both cues simultaneously, whereas offspring of dams fed the control diet or a choline-deficient diet were only able to attend to the briefer, easier cue, indicating improved attentional control in the supplemented offspring.[72] Further, although the control (standard) diet and choline-deficient rats showed age-related declines in attentional processing, the choline-supplemented rodents showed no such decline.[72] In a mouse model of Down syndrome (Ts65Dn), maternal choline supplementation during pregnancy and lactation ameliorated deficits in attention, such that choline-supplemented Ts65Dn mice performed better on a cue-detection task of attention than unsupplemented Ts65Dn mice.[87] Control disomic mice that received choline supplementation also performed better on the cue-detection task than unsupplemented control mice.

In rodent models, prenatal choline supplementation has also shown neuroprotective effects in a variety of prenatal insults, including maternal stress, infection, inflammation, and exposure to drugs and alcohol.[20,104–106] In particular, fetal alcohol exposure represents a significant public health problem, as even low-level maternal alcohol consumption may result in cognitive deficits in her offspring.[77] In one rodent study of fetal alcohol exposure, pregnant dams were randomized to receive an ethanol or control solution, as well as randomized to receive either a choline or saline solution.[105–106] The offspring were then tested on a number of physical and behavioral development tasks. Prenatal choline supplementation attenuated the effects of prenatal alcohol exposure on offspring birth and brain weight, as well as normalized offspring reflex responses to the level of control animals.[105] Prenatal choline supplementation also improved alcohol-exposed offspring performance on a Morris water maze task of working

memory, normalizing their performance to that of the control animals.[106]

In addition to the cognitive effects on memory and attention reported in rodent models, perinatal choline supplementation in rodent models also results in structural and functional changes to the brain—notably, in regions that subserve these improved areas of cognitive functioning. These structural and chemical changes in areas of the brain associated with memory, attention, and executive function may underlie the cognitive benefits of maternal choline supplementation that have been reported in rodent models. Choline supplementation has been shown to alter development of the hippocampus and septum, including progenitor cell mitosis and angiogenesis.[5,6,28–29,34,112,116] In a rodent model of Down Syndrome, maternal choline supplementation increased the number and size of basal forebrain cholinergic neurons.[10] Further, as noted above, choline and its metabolites are key constituents of phospholipid cell membranes, which are in high demand during fetal development to support the explosive proliferation of fetal neurons, glial cells, and myelin formation. It is also the precursor to acetylcholine, which directly and indirectly influences cell proliferation, differentiation, survival, morphology, and migration.[2,6,28–29]

MCS and Cognition: Evidence from Human Studies

Although the evidence for improved cognition with maternal choline supplementation is robust in rodent models, few studies have examined the effects of maternal choline intake on offspring cognition in humans. Nevertheless, preliminary data from human studies suggest that the effects seen in rodents translate to humans. Currently, there are four observational studies of maternal choline intake and offspring cognition, with two reporting significant correlations between maternal choline intake and offspring cognition.

<u>Observational Studies</u>

In one prospective cohort study of choline intake during pregnancy, women were administered a semi-quantitative food frequency questionnaire (FFQ) during first and second trimester visits to a maternity clinic.[22] When followed up at age three (N = 1210), there was no association between maternal choline intake and measures of child cognition, including the Peabody Picture Vocabulary Test and the Visual Motor Abilities Test.[109] The children were then followed up at age seven, and their visual memory and intelligence tested using the Wide Range Assessment of Memory and Learning (WRAML2) design and picture subtests and the Kaufman Brief Intelligence test. Of the 900 children tested, children of mothers in the highest quartile of estimated prenatal choline intake (N = 406 mg/day) scored significantly better on the WRAML2 measures of visual memory.[109] However, this study was limited in its assessment of maternal choline intake on offspring cognition, as food frequency questionnaires are prone to measurement error and are less precise than 24-hour dietary recalls.[100] Further, the highest quartile of choline intake was still below the current recommended intake level (406 mg/day vs. the AI of 450 mg/day), limiting assessment of the benefits of maternal choline supplementation beyond the AI on child cognition.

In the two observational studies that obtained serum measures of maternal choline, results were mixed. One prospective study in Alabama measured serum free and total choline from maternal blood samples collected at four timepoints during pregnancy (gestation weeks 16–18, 24–26, 30–32, and 36–38).[97] The offspring were followed up at five years of age and tested on IQ, spatial relation, and memory skills using the Wechsler Preschool and Primary Scale of Intelligence-Revised (WPPSI-R). The study found no effect of maternal choline serum concentrations on any of these measures of child cognition.[97] Another prospective study that measured serum free choline measures from blood samples collected at 16- and 36-weeks'

gestation assessed infant cognition at 18 months using the Bayley Scales of Infant Development.[117] Significant positive associations were found between scores on the cognitive tests and maternal serum choline levels at 16 weeks' gestation, but not 36 weeks' gestation. Although mixed, the conclusions drawn from these studies are limited, as serum choline levels are highly regulated and may be resistant to moderate changes in dietary choline intake.[1] Therefore, blood metabolite measures of choline status may not be very accurate, making them a bad measure of fetal exposure to choline.

Randomized Control Trials

Of the three experimental studies evaluating the effect of maternal choline supplementation on offspring cognition, two found benefits, while one did not. In one double-blind, randomized controlled trial, pregnant women were randomized at gestation week 18 to receive either placebo or 750 mg/day supplemental choline through delivery.[30] Infants were then assessed at 10 and 12 months of age on various aspects of cognition, including short-term visuospatial memory, episodic memory, language development, and global development (using the Mullen Scales of Early Learning). The study found no significant effect of choline supplementation on any tests of cognition at 10 or 12 months of age, though there was a non-significant trend toward group differences in episodic memory at 10 months.[30] However, this study was limited in its ability to detect an effect of maternal choline intake, as it had several methodological issues, including low adherence due to an onerous supplement dosing protocol (women were provided six large pills per day for supplementation).

In another double-blind, randomized controlled trial examining the effects of maternal choline supplementation on infant pathophysiology, women were randomized to receive either placebo or 900 mg/day of choline starting in the second trimester of pregnancy until delivery.[93]

As neonates, cerebral inhibition was measured via electrophysiological measurement of the inhibition of the P50 auditory evoked response during active sleep. Diminished amplitude of the P50 response may be linked to challenges in focused attention in schizophrenia patients, may be a risk factor for future psychopathology, and has also been shown to predict child behavior problems at 40 months of age.[53] The study demonstrated that the infants of women who were prenatally supplemented with choline had more suppressed P50 responses at five weeks postnatal, suggesting more timely delay of cerebral inhibition.[93] At six months, the infants' global development was assessed using the Mullen Scales of Early Learning, and at 40 months the Child Behavior Checklist (CBCL) was administered to assess child behavioral problems. There were no group differences found on any of the Mullen Scales, though the CBCL did indicate that children of supplemented mothers had fewer attentional problems and less social withdrawal compared to the children of unsupplemented mothers.[92] Although this study suggested some intriguing effects of maternal choline supplementation on cerebral inhibition and child behavior problems, it was limited in its ability to detect an effect on offspring cognition, as it lacked direct behavioral measures of memory or attention.

In one small but highly controlled feeding study, women consumed a standard diet during the third trimester of pregnancy and were randomized to receive a choline supplement that brought their total daily choline intake to either 480 mg/day (approximately the AI) or 930 mg/day.[27] Cognitive testing of their children showed that the infants of women in the higher choline group had better attentional orienting speeds across the first year of life. A follow-up study of the same cohort of children at 7 years of age found that children of women in the higher choline group (930 mg/day) had superior performance on the Sustained Attention Task (SAT).[12] Children in the 930 mg/day group earned a higher overall score on the task, as well as

demonstrated superior ability to maintain correct signal detections (also referred to as hits, or instances in which children correctly identified the presence of a stimulus on a computer screen). Of note, children in the 930 mg/day group were able to maintain correct signal detections for the briefest and most challenging (17 ms) signals, while children in the 480 mg/day group were not. Importantly, the SAT is a direct analog of the signal detection task used to detect effects of maternal choline supplementation in rodent models, providing strong evidence for a translation of the effects of MCS seen in rodent models to humans.[12]

There is also emerging evidence that the neuroprotective effects of choline seen in rodent models translate to humans. One randomized controlled trial of women who drank heavily during pregnancy (8–9 drinks per occasion, 1–2 times a week) assigned the women to receive either a choline supplement (2 g/day) or placebo from about mid-gestation to birth.[55] Choline supplementation resulted in better catch-up growth for alcohol-exposed infants at ages 6.5 and 12 months, as well as better performance on an eyeblink conditioning task at 6.5 months and the Fagan Infant Intelligence test of visual recognition memory at 6.5 months and 12 months.[55] Another randomized control trial assigned pregnant women who self-reported weekly binge drinking (five or more drinks) episodes to either a multivitamin/mineral supplement, multivitamin/mineral supplement and choline (750 mg/day), or control.[61] When the children were followed up at preschool age, maternal prenatal supplementation with choline improved performance on a reaction time test.[61]

These findings strongly suggest that the beneficial effects of choline intake found in animals may translate to humans and that increased choline in the maternal diet may have lifelong benefits for offspring cognition, although the work is still preliminary and larger studies are needed before obstetric recommendations for choline intake during pregnancy are changed.

In other words, across species, prenatal choline appears to exert a programming effect during a sensitive period for brain development, altering the architecture of various brain regions and the functioning of memory and attentional processing, resulting in long-term improvements in cognition.

1.5 Maternal Choline Intake and Executive Function

Although the rodent and human literature on maternal choline intake has thus far focused primarily on the effects of increased maternal choline intake (MCI) on offspring attention and memory, the pattern of results suggests that increased maternal choline intake results in improvements in child executive functioning. Executive functioning, a set of higher-order cognitive processes that includes cognitive skills such as working memory, attentional control, and inhibitory control, is integral to planning, problem-solving, and the execution of goal-directed behaviors.[60] The cognitive skills that scaffold later executive function begin to develop in infancy, and executive function develops rapidly during the first few years of life.[17–19] Although there is some debate around the definition and conceptualization of the cognitive skills that comprise executive functions, EF is generally defined as the ability to hold in mind information related to the task or goal at hand (working memory), the ability to resist the urge to achieve the goal using a prepotent response (response inhibition), and the ability to maintain or shift attention as needed to achieve a goal (attentional control).[4,52,60] Executive functions are key to the development of academic skills, including reading and mathematical reasoning.[19] Some studies have found that executive function is more important for school preparedness than general intelligence[19,38], and EF continues to predict competence in math and reading from elementary school through the early high school years.[38,45]

There are several reasons to believe that high maternal choline intake may result in

improved offspring executive function. Much like the original DOHaD hypothesis, which connected adverse prenatal exposures to risk for poor physical health outcomes later in life, an emerging body of literature has shown that adverse prenatal exposures are also predictive of risk for cognitive and behavioral deficits throughout the lifespan. Prior studies have demonstrated that executive functioning is uniquely sensitive to various prenatal insults, including exposure to cocaine, cannabis, and alcohol.[62,74,81]

A second line of evidence implicating an effect of maternal choline supplementation on offspring executive functioning is the numerous rodent studies (many of which are discussed above in Section 1.4) which have demonstrated that maternal choline supplementation improves performance in radial arm mazes.[72,87] While the nature of the improved performance exhibited by prenatally choline supplemented animals in this task is not entirely clear, it is known that the task requires that the rodent plans how to obtain all of the food in the maze most efficiently while it maintains memory of which arms of the maze it has already visited (working memory) and resists the impulse to return to those arms where it has already successfully found food (inhibitory control). Therefore, the rodent data seems to suggest that MCS results in improved offspring executive function.

Lastly, and most importantly, in our small but highly controlled feeding study (described above in Section 1.4), the infants born to mothers in the higher choline intake group (930 mg/day) had faster information processing speeds than those born to mothers consuming the lower intake level (480 mg/day) across the first year of life.[27] Previous research has found that infant processing speed at ages 7 and 12 months was predictive of child executive function at age 11 years.[91] Therefore, we would predict that the children of mothers in the higher choline intake group (930 mg/day) would also show superior executive function at school age, demonstrating

an effect of maternal choline supplementation on these foundational cognitive skills.

1.6 Maternal Choline Intake and Affect Regulation

Evidence from Rodent Models

Although the vast majority of research on maternal choline intake and offspring functioning has evaluated cognitive endpoints, there are preliminary animal and human data to suggest that prenatal maternal choline supplementation may impact affect regulation in the offspring as well. Affect regulation—the ability to modify the intensity and duration of physiological arousal and affective states to achieve a goal—underlies mental health and adaptive function throughout the lifespan. One rodent study focusing on prenatal stress found evidence that prenatal choline supplementation reduced offspring trait anxiety and social behavior problems in the offspring of stressed dams, although the effects varied somewhat by the sex of the offspring.[95] Female offspring of supplemented dams exposed to prenatal stress exhibited less anxiety than controls in the open-field task and elevated zero maze task, two tasks known to induce anxiety-like behaviors. For male offspring, the benefits of maternal choline supplementation were seen in a test of social behavior. Specifically, maternal choline supplementation normalized the social behavior of the prenatally stressed male offspring when confronted with a novel conspecific, increasing the amount of time that the mouse spent investigating the new social partner.[95]

Additional evidence for improved affect regulation following maternal choline supplementation is provided by a study involving an operant schedule called differential reinforcement of low rate responding (DRL).[31] Prenatal choline supplementation reduced offspring frustrative responding during this operant schedule. In DRL, rats are trained to wait to make a lever press for reward until a certain amount of time has elapsed. The duration to wait

changes throughout the task, increasing the likelihood of the rodents making an error and responding with frustration, measured as "burst responding" (repeated presses of the lever following a failure to receive the reinforcement). Prenatal choline supplementation reduced burst responding, suggesting a reduction in the amount of frustration expressed in response to an error.[31]

Studies have also found evidence that prenatal choline supplementation normalizes offspring affect regulation in rodent disease models characterized by aberrant emotional reactivity. In a murine model of Down syndrome, the trisomic animals exhibit an excessive affective reaction to making an error or not receiving an expected reward in an attention task; prenatal choline supplementation in this model normalized the aberrant emotional reaction to a task error or not receiving an expected reward, as measured by decreased hesitancy to begin the subsequent task trial, in comparison to unsupplemented trisomic animals.[76] In a mouse model of autism, which is characterized by behavioral deficiencies in social communication, prenatal choline supplementation normalized anxiety of BTBR (autism model) mice in an open-field test, measured as the amount of time spent exploring the field.[65] MCS also increased the amount of time spent in social approach to a strange mouse in comparison to unsupplemented BTBR mice.[65] Further, the MCS mice spent approximately as much time interacting with the strange mouse as the control strain of mice, suggesting normalization of social interaction with MCS.[65]

Evidence from Human Studies

Although few human studies have investigated the effects of maternal choline supplementation on offspring affect regulation, one double-blind placebo-controlled trial of maternal choline supplementation (~900 mg/day from gestation week 16 until delivery) found that MCS decreased social withdrawal in the children at three years of age, as measured by

parent report on the Child Behavior Checklist (CBCL).[92] This finding provides preliminary support for the rodent data on social interaction and anxiety and suggests the possibility of parallel effects of MCS on socioemotional functioning across species. Together, the rodent and human data on MCS provide preliminary data supporting the hypothesis that prenatal choline supplementation improves affect regulation in the offspring, in both normative and atypical populations.

1.7 Self-Regulation: Integrating Effects of MCS on Executive Function and Affect

Historically, cognition and affect have been treated as separate and distinct systems in the study of early exposures and brain development. However, emerging theories have begun to recognize the interconnectedness of these two systems, and the importance of understanding the ways in which they develop separately and in concert for understanding child outcomes. This line of thinking has led to the development of a scientific model that incorporates executive function and affect as two levels of a hierarchical system of self-regulation. At the most fundamental level, self-regulation may be understood as the exercise of control over oneself, by unconscious and conscious processes, in order to bring the self in line with a preferred or goal state.[15]

One way to characterize self-regulation, especially during early development, is as the integration of affect and executive function.[18] In this integrated, hierarchical model, affect and executive function represent two levels of a tiered system that also includes genetics, physiology, and behavior, with executive function at the highest level of the system (Figure 1.1). This system is both reciprocal and recursive.[18] Importantly, impairments in one level of the self-regulatory system can negatively impact another. Moderate affective arousal is needed to mobilize executive functions (e.g., feeling moderately stressed before a big exam may help to maintain

attentional focus on studying), but high levels of affective arousal may impair executive functioning (e.g., feeling so scared of doing poorly while taking the test that it is not possible to focus or retain pertinent information).

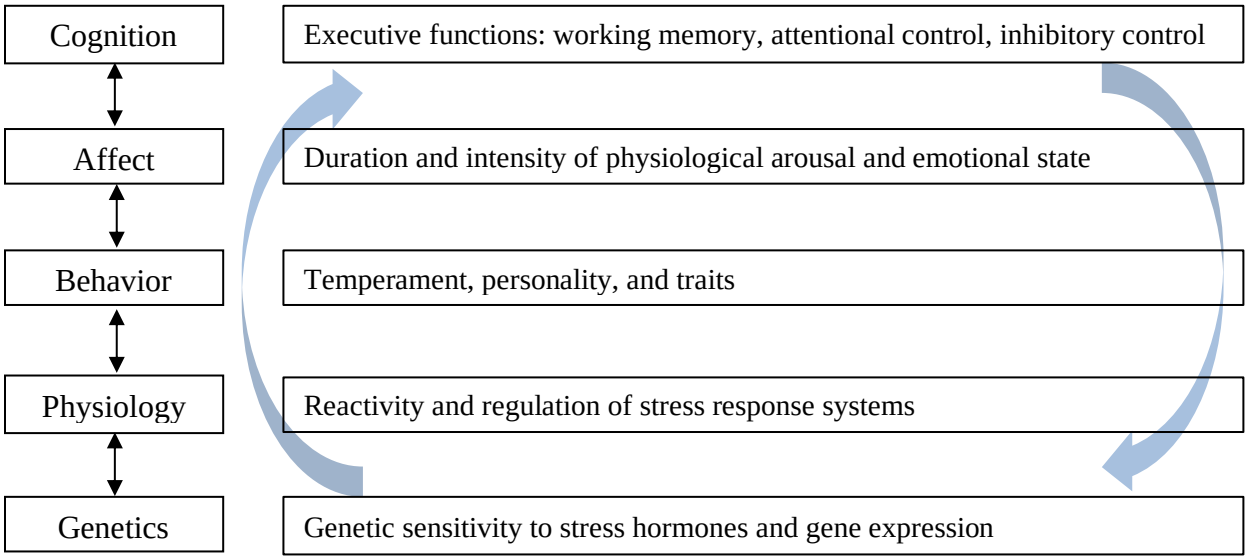


Figure 1.1: The hierarchical model of self-regulation. Adapted from Blair and Ku, 2022.

Early in development, the lower levels of this system—physiology, behavior, and affect—are more developmentally advanced than executive functions.[17,18] Very young infants are primarily reliant on activation of stress response systems (the autonomic nervous system and hypothalamus-pituitary-adrenal gland axis) to respond to environmental stimuli, and on their caregivers to regulate their response.[16,18] As an infant grows, their increased capacity for moderating their arousal and affective state scaffolds higher-order cognitive skills, such as the executive control of attention.[18] This self-regulatory system, along with input from environmental stimuli and feedback, sets the stage for children's social and academic success.

1.8 Biological Substrates of Self-Regulation

There are a number of biological and neural systems through which affect and executive function interact to produce successful self-regulation, and through which maternal choline

intake may exert an effect on offspring self-regulation. These are each discussed below.

The Autonomic Nervous System

Research on the biological substrates of self-regulation has identified the autonomic nervous system as an important player in the control of self.[25] The autonomic nervous system (ANS) is the branch of the peripheral nervous system (the components of the nervous system outside of the brain and spinal cord) that regulates the homeostatic function of internal organs (e.g., breathing, digestion).[85] The autonomic nervous system is comprised of two complementary branches: the parasympathetic (PNS) and sympathetic (SNS) nervous systems. The parasympathetic nervous system, often referred to as the 'rest and digest' system, maintains bodily functions and is responsible for the conservation of energy. The sympathetic nervous system, often referred to as the 'fight or flight' system, responds to environmental threats by alerting the body to potential dangers.[57,85] The PNS and SNS often have opposite effects on an organ or tissue.[114] However, that does not mean that the effects of these two systems are always counterposed, or have the same responses across all environmental contexts. In fact, the two branches of the ANS are more like well-trained co-pilots, constantly communicating with each other and with air traffic control (the brain) to gather information about bodily state and make small adjustments to respond adaptively to environmental inputs.[24,85]

A well-functioning autonomic nervous system is defined by the ability to assert the levels of PNS and SNS activation that are appropriate to the environmental context and to a particular goal. In general, a system that exerts high levels of PNS activation and low levels of SNS activation at baseline (a non-stressful environment) is considered optimal, though there are other patterns of autonomic activation.[90] The extent to which an individual shows different patterns of autonomic activity across different contexts is thought to modulate their ability to adjust their

affective state and achieve their desired goal in that context.[59,98] For example, research suggests that individuals with high PNS tone (greater influence of the PNS v the SNS on heart rate at rest), higher PNS withdrawal (removal of PNS influence on heart rate) in response to stress, and who return more quickly to their baseline PNS tone are better-regulated and have the greatest capacity for adaptation to different environmental contexts.[90] This is also known as the cardiac autonomic balance model, which allows for rest and restoration when environmental stress is absent, and for a wide range of responses to different contexts where environmental stress is present.[90] Individual patterns of autonomic activity develop early in life and have been found to be predictive of variations in psychopathology and risk for mental illness throughout the lifespan.[24,59] In one study, children who stably suppressed PNS activity in response to stress at age two were more likely to have better social and affect regulation skills, and fewer behavior problems at age four than children who were not able to suppress PNS activity in response to stress.[26] Autonomic activity reflects an individual's ability to appropriately regulate their affective state and represents an early marker of risk for behavior problems and psychopathology.

Although no prior studies have examined potential links between maternal choline intake during pregnancy and offspring autonomic reactivity, evidence of MCS-induced alterations in cholinergic system function suggests at least one possible mechanism. Acetylcholine is the primary neurotransmitter for the parasympathetic nervous system, and polymorphic variations in the gene which encodes the choline transporter (SLC5A7) have been found to influence parasympathetic reactivity.[58,78–79] The choline transporter 1 gene (CHT1) promotes choline uptake from the synaptic cleft[58], and genetic variations in CHT1 have also been associated with differences in risk of depression, which suggest a possible link between acetylcholine transport

and mood.[78–79]

Changes in neurotrophin levels due to MCS may also provide a mechanistic link between MCS and autonomic function: Prenatal choline supplementation has been shown to produce lasting increases in brain-derived neurotrophic factor (BDNF) activity.[33,46] Decreased BDNF expression has been shown to decrease choline acetyltransferase activity, and polymorphisms in the gene for BDNF that downregulate its activity have been shown to decrease parasympathetic function and are associated with anxiety.[120] Thus, increased BDNF activity may reduce the risk of affective dysfunction, possibly through alterations to parasympathetic activity.

Stress Hormones and the Hypothalamus-Pituitary-Adrenal Axis

When an individual experiences stress, the sympathetic nervous system releases the catecholamines epinephrine and norepinephrine. This triggers the hypothalamus-pituitary-adrenal (HPA) axis to release cortisol, which controls the body's long-term response to stress. Both norepinephrine and cortisol are neuromodulators.[18] The levels of these two neuromodulators affect in part how rapidly neurons fire in parts of the brain that are associated with emotional reactivity (the amygdala) and executive function (the prefrontal cortex or PFC). Importantly, when norepinephrine and cortisol are sustained at a high level in the brain, neural firing in the amygdala increases, and neural firing in the PFC decreases.[18] When this imbalance in activity across these two brain regions occurs during infancy and early development, it may result in lifelong patterns of connectivity that impair affect regulation and executive function.[18]

There are some studies that suggest that maternal choline intake during pregnancy may affect offspring HPA regulation and cortisol levels. In particular, one small but highly controlled feeding study (described in detail above in Section 1.4) found that higher maternal choline intake (930 mg/day v 480) resulted in increased promoter region methylation of the placental

corticotropin-releasing hormone (CRH) gene, resulting in reduced gene expression.[56] CRH is released by the hypothalamus in response to stress signals from the autonomic nervous system and stimulates the adrenal glands to produce cortisol.[64] Placental CRH can enter fetal circulation and activate the HPA axis.[64] In line with decreased expression of the CRH gene, cord blood samples of infants of mothers in the 930 mg/day group had lower cortisol concentrations than those in the 480 mg/day group.[56] CRH is a primary regulator of cortisol production and HPA axis reactivity.[56] Therefore, it is possible that increased maternal choline intake during pregnancy reduces neonatal cortisol production and/or HPA axis reactivity, allowing for appropriate rates of neuronal firing in the amygdala and PFC during early development.

1.9 Gaps in Knowledge

When viewed as a whole, the data linking supplemental prenatal choline to cognitive and affect regulation benefits in the offspring implicates improved self-regulation in the offspring of choline supplemented mothers, potentially via improvements in the function of the affect regulation and executive function systems. This has important implications for child development, as self-regulatory skills are key determinants of school readiness and academic achievement.[19] If maternal choline supplementation acts to improve functioning across multiple domains of self-regulation, there may be many long-term benefits for the physical health and social success of her child.

Research Questions

The rodent and human data collectively demonstrate that pregnancy increase the demand for choline, and that the amount of choline a mother consumes can have lifelong impacts on offspring neurobehavioral health. In rodent models, maternal choline deficiency during pregnancy adversely affects offspring cognitive function, whereas maternal choline

supplementation in both normative and atypical populations results in lifelong benefits in offspring cognitive function, reduces age-related memory decline, and is neuroprotective against a wide range of prenatal insults, including maternal alcohol and drug use and prenatal stress. Maternal choline supplementation has also been shown to produce lasting alterations in neural structure and function, which plausibly underlie the observed cognitive benefits. Although few studies have examined the effects of maternal choline intake on child outcomes in humans, results from a small but highly controlled choline feeding trial in pregnant women provide preliminary evidence that the types of long-term benefits of MCS seen in rodents are also seen in human infants and children. In this study, we demonstrated that infants of mothers who were supplemented with 930 mg/day choline (v 480 mg/day) during the third trimester were faster to orient to peripheral stimuli throughout the first year of life, indicating an enduring effect of MCS on infant attention.[27] When the children were tested again at age seven years, the children of mothers supplemented with 930 mg/day choline performed better on tasks of sustained attention and memory.[11–12] This study provides compelling support for the translation of the cognitive benefits of MCS seen in rodent models to humans. Together with the rodent data, this preliminary evidence offers a strong rationale for also investigating the translation of the affective benefits of MCS in humans. Investigating and understanding the effects of maternal choline intake on offspring outcomes in humans is critical, as 90% of pregnant women in the United States do not consume the recommended intake amount of choline—which itself may be inadequate—placing their children at risk for subtle, but functionally important cognitive and self-regulatory deficits. Our previous study demonstrated the benefits of choline supplementation at approximately twice the AI (930 mg/day) compared to the AI (480 mg/day) in a controlled feeding trial—however, questions remain:

1. The effects of maternal choline supplementation on aspects of executive function, including planning and problem solving, are not known.
2. The effects of maternal choline supplementation in the context of a typical diet are not known.
3. The effects of MCS on affect regulation in humans are not known and have not been assessed using behavioral measures.

1.10 Assessing the Effects of Maternal Choline Supplementation on Affective Outcomes During the First Year of Life and Executive Function at Seven Years of Age

This dissertation presents results from two studies conducted to address these gaps in knowledge. The first follow-up study, hereafter referred to as the childhood study, assessed the effect of third trimester choline supplementation on child cognition at seven years of age (Figure 1.2). Presented here are the results of one administered task for the childhood study: the Tower of London, a classic neuropsychological assessment of executive function.[4] The second study, hereafter referred to as the infancy study, expanded the findings of the randomized controlled feeding trial by investigating the effects of maternal choline intake during pregnancy on infant cognition and behavior when women were supplemented with choline in addition to their regular diet. Presented in this dissertation are the results of a parent-report survey of infant temperament using the Infant Behavior Questionnaire and the Early Childhood Behavior Questionnaire, both validated assessments of temperament during the first few years of life.[23,39,86,89,94] Lastly, this dissertation presents the results of one administered task for the infancy study: the Face-to-Face Still Face Paradigm (FFSF), a classic test of affect regulation that reliably produces increased negative affect and regulatory behaviors in children as young as a few hours old.[73,107]

The details of the randomized controlled feeding trial and subsequent childhood study have been published elsewhere.[11,12,27] Briefly, third trimester pregnant women were randomized

to consume either 480 mg/d or 930 mg/d of choline starting at gestational week 27 until delivery. Follow-up of the infants of supplemented mothers found that the children in the 930 mg/d group (v 480) had improved information processing speed across the first year of life.[27] The children were then invited to participate in a follow-up study to assess the effects of prenatal choline intake on child cognition when they were seven years of age.

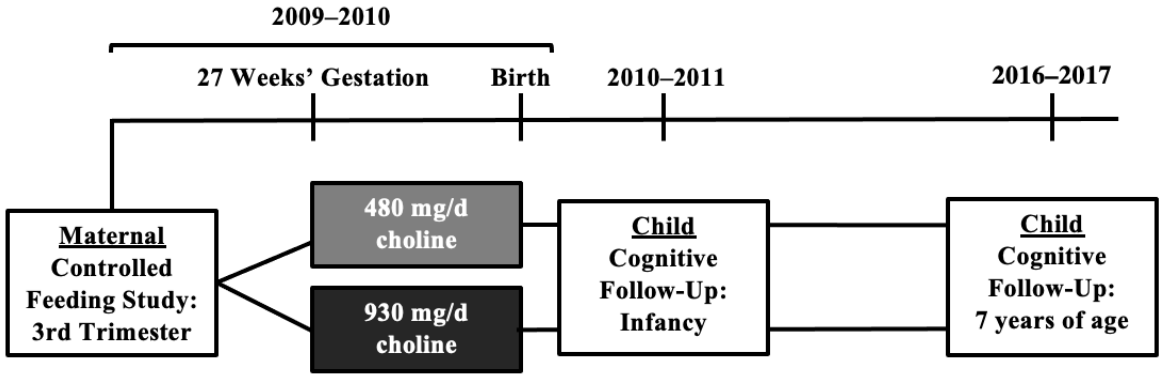


Figure 1.2: Study design and timeline of an ancillary follow-up to assess the effects of 3rd-trimester maternal choline supplementation on child cognition at seven years of age.

The infancy study is an ancillary cognitive-behavioral follow-up to a double-blind, randomized controlled clinical trial in which pregnant women were randomized to consume either supplemental choline (550 mg choline/day as choline chloride) or 25 mg choline day from gestation week 16 until delivery. Post-delivery, the infants were re-enrolled with maternal consent to participate in cognitive and behavioral assessments at four time points across the first year of life (Figure 1.3).

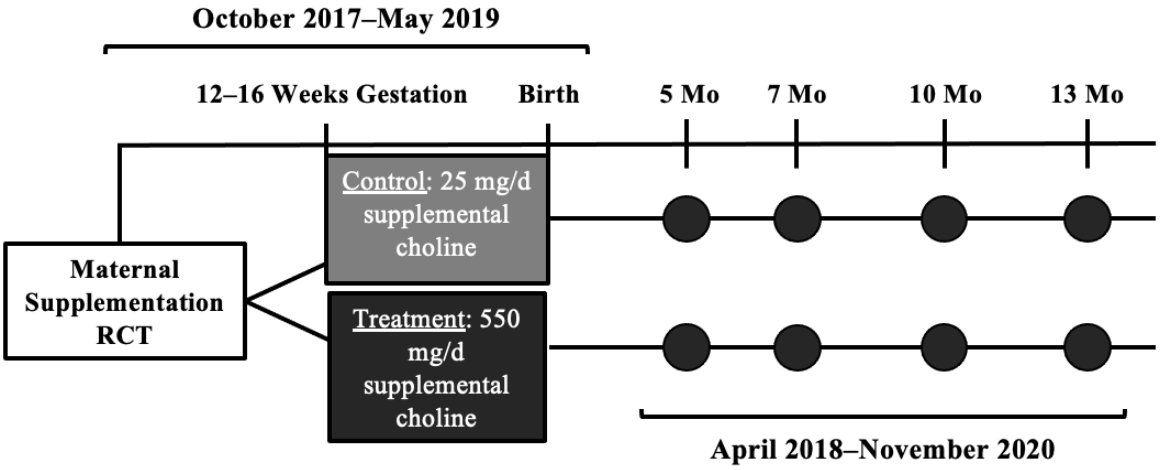


Figure 1.3: Study design and timeline of an ancillary follow-up to assess the effects of maternal choline supplementation on child cognitive and behavioral functioning across the first year of life.

For the childhood study, a battery of tasks was developed to evaluate child cognitive function at 7 years old. The tasks were selected because they assess the cognitive functions that were shown to be improved by maternal choline supplementation in rodents, including memory and attention.[72,75] In addition, tests of cognitive functions that could have plausibly been affected by maternal choline supplementation, including executive function and general intelligence, were administered. A description of the full testing protocol can be found in Appendix A.

For the infancy study, a battery of tasks was developed to evaluate infant cognitive functioning and affect regulation across the first year of life. The cognitive tasks were selected to assess the cognitive functions that were shown to be improved by maternal choline supplementation in rodents, including memory and attention[72,75], as well as to replicate our findings on infant information processing from the controlled feeding trial.[27] The rodent data on the effects of maternal choline supplementation on affect regulation is more limited, so the behavioral tasks selected assessed similar behaviors, including affective response to a violation of expectations in a social encounter.[3,107] A description of the full testing protocol can be found

in Appendix B.

These two studies provide a unique opportunity to assess the long-term effects of maternal choline intake on offspring cognition and affect regulation. The childhood study is an ancillary follow-up to a randomized control trial in which maternal choline intake was highly controlled. Women consumed their choline supplement with a meal on site every weekday, and all other food was provided by the study, resulting in high compliance. The follow-up was then designed to assess the domains of memory and attention that have been shown in rodent models to be affected by different levels of maternal choline intake, allowing for examination of the specific hypothesized benefits of increased maternal choline intake in our human sample.

The infancy study extends the findings of the controlled feeding trial by examining the effects of prenatal choline supplementation in the context of normal maternal diet. This makes the results of the study generalizable to the real-world scenario in which a pregnant woman may choose to or be prescribed to take a choline supplement as part of her prenatal regimen. The neurobehavioral follow-up was designed to assess domains of memory and attention that have been shown in both rodent models and our previous human study to be affected by different levels of maternal choline intake, as well as to include observational measures of affect that reliably elicit negative infant reactivity. Together, these two studies offer a high-quality investigation into the effects of increased maternal choline intake on offspring self-regulation from infancy through early childhood. These data offer key insight into the potential benefits of raising the recommended choline intake levels for pregnant women, with possible population-wide shifts towards improved memory, attention, and executive function, resulting in better health, socioemotional function, and economic success across the lifespan.[99]

1.11 References

1. Abratte, C. M., Wang, W., Li, R., Axume, J., Moriarty, D. J., & Caudill, M. A. (2009). Choline status is not a reliable indicator of moderate changes in dietary choline consumption in premenopausal women. *The Journal of Nutritional Biochemistry*, *20*(1), 62–69. https://doi.org/10.1016/j.jnutbio.2007.12.002

2. Abreu-Villaça, Y., Filgueiras, C. C., & Manhães, A. C. (2011). Developmental aspects of the cholinergic system. *Behavioural Brain Research*, *221*(2), 367–378. https://doi.org/10.1016/j.bbr.2009.12.049

3. Adamson, L. B., & Frick, J. E. (2003). The Still Face: A History of a Shared Experimental Paradigm. *Infancy*, *4*(4), 451–473. https://doi.org/10.1207/S15327078IN0404_01

4. Ahmed, S. F., Kuhfeld, M., Watts, T. W., Davis-Kean, P. E., & Vandell, D. L. (2021). Preschool executive function and adult outcomes: A developmental cascade model. *Developmental Psychology*, *57*(12), 2234–2249. https://doi.org/10.1037/dev0001270

5. Albright, C. D., Friedrich, C. B., Brown, E. C., Mar, M.-H., & Zeisel, S. H. (1999). Maternal dietary choline availability alters mitosis, apoptosis and the localization of TOAD-64 protein in the developing fetal rat septum. *Developmental Brain Research*, *115*(2), 123–129. https://doi.org/10.1016/S0165-3806(99)00057-7

6. Albright, C. D., Tsai, A. Y., Friedrich, C. B., Mar, M.-H., & Zeisel, S. H. (1999a). Choline availability alters embryonic development of the hippocampus and septum in the rat. *Developmental Brain Research*, *113*(1), 13–20. https://doi.org/10.1016/S0165-3806(98)00183-7

7. Almond, D., & Currie, J. (2011). Killing Me Softly: The Fetal Origins Hypothesis. *The Journal of Economic Perspectives : A Journal of the American Economic Association*, *25*(3), 153–172. https://doi.org/10.1257/jep.25.3.153

8. *AMA backs global health experts in calling infertility a disease*. (n.d.). American Medical Association. Retrieved January 11, 2022, from https://www.ama-assn.org/delivering-care/public-health/ama-backs-global-health-experts-calling-infertility-disease

9. Anderson, O. S., Sant, K. E., & Dolinoy, D. C. (2012). Nutrition and epigenetics: An interplay of dietary methyl donors, one-carbon metabolism and DNA methylation. *The Journal of*

Nutritional Biochemistry, *23*(8), 853–859. https://doi.org/10.1016/j.jnutbio.2012.03.003

10. Ash, J. A., Velazquez, R., Kelley, C. M., Powers, B. E., Ginsberg, S. D., Mufson, E. J., & Strupp, B. J. (2014). Maternal choline supplementation improves spatial mapping and increases basal forebrain cholinergic neuron number and size in aged Ts65Dn mice. *Neurobiology of Disease*, *70*, 32–42. https://doi.org/10.1016/j.nbd.2014.06.001

11. Bahnfleth, C., Canfield, R., Nevins, J., Caudill, M., & Strupp, B. (2019). Prenatal Choline Supplementation Improves Child Color-location Memory Task Performance at 7 Y of Age (FS05-01-19). *Current Developments in Nutrition*, *3*(Supplement_1). https://doi.org/10.1093/cdn/nzz048.FS05-01-19

12. Bahnfleth, C. L., Strupp, B. J., Caudill, M. A., & Canfield, R. L. (2022). Prenatal choline supplementation improves child sustained attention: A 7-year follow-up of a randomized controlled feeding trial. *The FASEB Journal*, *36*(1), e22054. https://doi.org/10.1096/fj.202101217R

13. Barouki, R., Gluckman, P. D., Grandjean, P., Hanson, M., & Heindel, J. J. (2012). Developmental origins of non-communicable disease: Implications for research and public health. *Environmental Health*, *11*(1), 42. https://doi.org/10.1186/1476-069X-11-42

14. Bateson, P., Barker, D., Clutton-Brock, T., Deb, D., D'Udine, B., Foley, R. A., Gluckman, P., Godfrey, K., Kirkwood, T., Lahr, M. M., McNamara, J., Metcalfe, N. B., Monaghan, P., Spencer, H. G., & Sultan, S. E. (2004). Developmental plasticity and human health. *Nature*, *430*(6998), 419–421. https://doi.org/10.1038/nature02725

15. Baumeister, R. F., & Vohs, K. D. (Eds.). (2004). *Handbook of self-regulation: Research, theory, and applications*. Guilford Press.

16. Bernier, A., Carlson, S. M., & Whipple, N. (2010). From External Regulation to Self-Regulation: Early Parenting Precursors of Young Children's Executive Functioning. *Child Development*, *81*(1), 326–339. https://doi.org/10.1111/j.1467-8624.2009.01397.x

17. Blair, C. (2010). Stress and the Development of Self-Regulation in Context: Stress and the Development of Self-Regulation. *Child Development Perspectives*, *4*(3), 181–188. https://doi.org/10.1111/j.1750-8606.2010.00145.x

18. Blair, C., & Ku, S. (2022). A Hierarchical Integrated Model of Self-Regulation. *Frontiers in Psychology*, *13*. https://www.frontiersin.org/article/10.3389/fpsyg.2022.725828

19. Blair, C., & Razza, R. P. (2007). Relating Effortful Control, Executive Function, and False Belief Understanding to Emerging Math and Literacy Ability in Kindergarten. *Child Development*, *78*(2), 647–663. https://doi.org/10.1111/j.1467-8624.2007.01019.x

20. Blusztajn, J. K., Slack, B. E., & Mellott, T. J. (2017). Neuroprotective Actions of Dietary Choline. *Nutrients*, *9*(8), 815. https://doi.org/10.3390/nu9080815

21. Blusztajn, J. K., & Wurtman, R. J. (1983). Choline and Cholinergic Neurons. *Science*, *221*(4611), 614–620. https://doi.org/10.1126/science.6867732

22. Boeke, C. E., Gillman, M. W., Hughes, M. D., Rifas-Shiman, S. L., Villamor, E., & Oken, E. (2013). Choline Intake During Pregnancy and Child Cognition at Age 7 Years. *American Journal of Epidemiology*, *177*(12), 1338–1347. https://doi.org/10.1093/aje/kws395

23. Bosquet Enlow, M., White, M. T., Hails, K., Cabrera, I., & Wright, R. J. (2016). The Infant Behavior Questionnaire-Revised: Factor structure in a culturally and sociodemographically diverse sample in the United States. *Infant Behavior and Development*, *43*, 24–35. https://doi.org/10.1016/j.infbeh.2016.04.001

24. Bush, N. R., Caron, Z. K., Blackburn, K. S., & Alkon, A. (2016). Measuring Cardiac Autonomic Nervous System (ANS) Activity in Toddlers—Resting and Developmental Challenges. *Journal of Visualized Experiments : JoVE*, *108*. https://doi.org/10.3791/53652

25. Calkins, S. D. (1997). Cardiac vagal tone indices of temperamental reactivity and behavioral regulation in young children. *Developmental Psychobiology*, *31*(2), 125–135. https://doi.org/10.1002/(SICI)1098-2302(199709)31:2<125::AID-DEV5>3.0.CO;2-M

26. Calkins, S. D., Graziano, P. A., & Keane, S. P. (2007). Cardiac vagal regulation differentiates among children at risk for behavior problems. *Biological Psychology*, *74*(2), 144–153. https://doi.org/10.1016/j.biopsycho.2006.09.005

27. Caudill, M. A., Strupp, B. J., Muscalu, L., Nevins, J. E. H., & Canfield, R. L. (2018a). Maternal choline supplementation during the third trimester of pregnancy improves infant information processing speed: A randomized, double-blind, controlled feeding study. *The*

FASEB Journal, *32*(4), 2172–2180. https://doi.org/10.1096/fj.201700692RR

28. Cermak, J. M., Blusztajn, J. K., Meck, W. H., Williams, C. L., Fitzgerald, C. M., Rosene, D. L., & Loy, R. (1999). Prenatal Availability of Choline Alters the Development of Acetylcholinesterase in the Rat Hippocampus. *Developmental Neuroscience*, *21*(2), 94–104. https://doi.org/10.1159/000017371

29. Cermak, J. M., Holler, T., Jackson, D. A., & Blusztajn, J. K. (1998). Prenatal availability of choline modifies development of the hippocampal cholinergic system. *The FASEB Journal*, *12*(3), 349–357. https://doi.org/10.1096/fasebj.12.3.349

30. Cheatham, C. L., Goldman, B. D., Fischer, L. M., da Costa, K.-A., Reznick, J. S., & Zeisel, S. H. (2012). Phosphatidylcholine supplementation in pregnant women consuming moderate-choline diets does not enhance infant cognitive function: A randomized, double-blind, placebo-controlled trial. *The American Journal of Clinical Nutrition*, *96*(6), 1465–1472. https://doi.org/10.3945/ajcn.112.037184

31. Cheng, R.-K., MacDonald, C. J., Williams, C. L., & Meck, W. H. (2008). Prenatal choline supplementation alters the timing, emotion, and memory performance (TEMP) of adult male and female rats as indexed by differential reinforcement of low-rate schedule behavior. *Learning & Memory*, *15*(3), 153–162. https://doi.org/10.1101/lm.729408

32. Cheng, W.-L., Holmes-McNary, M. Q., Mar, M.-H., Lien, E. L., & Zeisel, S. H. (1996a). Bioavailability of choline and choline esters from milk in rat pups. *The Journal of Nutritional Biochemistry*, *7*(8), 457–464. https://doi.org/10.1016/0955-2863(96)00079-4

33. Chourbaji, S., Hellweg, R., Brandis, D., Zörner, B., Zacher, C., Lang, U. E., Henn, F. A., Hörtnagl, H., & Gass, P. (2004). Mice with reduced brain-derived neurotrophic factor expression show decreased choline acetyltransferase activity, but regular brain monoamine levels and unaltered emotional behavior. *Molecular Brain Research*, *121*(1), 28–36. https://doi.org/10.1016/j.molbrainres.2003.11.002

34. Craciunescu, C. N., Albright, C. D., Mar, M.-H., Song, J., & Zeisel, S. H. (2003). Choline Availability During Embryonic Development Alters Progenitor Cell Mitosis in Developing Mouse Hippocampus. *The Journal of Nutrition*, *133*(11), 3614–3618. https://doi.org/10.1093/jn/133.11.3614

35. Cusick, S. E., & Georgieff, M. K. (2012). Nutrient Supplementation and Neurodevelopment:

Timing Is the Key. *Archives of Pediatrics & Adolescent Medicine*, *166*(5), 481–482. https://doi.org/10.1001/archpediatrics.2012.199

36. da Costa, K.-A., Kozyreva, O. G., Song, J., Galanko, J. A., Fischer, L. M., & Zeisel, S. H. (2006). Common genetic polymorphisms affect the human requirement for the nutrient choline. *The FASEB Journal*, *20*(9), 1336–1344. https://doi.org/10.1096/fj.06-5734com

37. Davison, J. M., Mellott, T. J., Kovacheva, V. P., & Blusztajn, J. K. (2009). Gestational Choline Supply Regulates Methylation of Histone H3, Expression of Histone Methyltransferases G9a (Kmt1c) and Suv39h1 (Kmt1a), and DNA Methylation of Their Genes in Rat Fetal Liver and Brain. *Journal of Biological Chemistry*, *284*(4), 1982–1989. https://doi.org/10.1074/jbc.M807651200

38. Diamond, A., & Lee, K. (2011). Interventions Shown to Aid Executive Function Development in Children 4 to 12 Years Old. *Science*, *333*(6045), 959–964. https://doi.org/10.1126/science.1204529

39. Dias, C. C., Costa, R., Pinto, T. M., & Figueiredo, B. (2021). The Infant Behavior Questionnaire – Revised: Psychometric properties at 2 weeks, 3, 6 and 12 months of life. *Early Human Development*, *153*, 105290. https://doi.org/10.1016/j.earlhumdev.2020.105290

40. DROUVA, S. V., LAPLANTE, E., LEBLANC, P., BECHET, J.-J., CLAUSER, H., & KORDON, C. (1986). Estradiol Activates Methylating Enzyme(s) Involved in the Conversion of Phosphatidylethanolamine to Phosphatidylcholine in Rat Pituitary Membranes*. *Endocrinology*, *119*(6), 2611–2622. https://doi.org/10.1210/endo-119-6-2611

41. Felsenfeld, G. (2014). A Brief History of Epigenetics. *Cold Spring Harbor Perspectives in Biology*, *6*(1). https://doi.org/10.1101/cshperspect.a018200

42. Folate, I. of M. (US) S. C. on the S. E. of D. R. I. and its P. on, Vitamins, O. B., & Choline, A. (1998). Choline. In *Dietary Reference Intakes for Thiamin, Riboflavin, Niacin, Vitamin B6, Folate, Vitamin B12, Pantothenic Acid, Biotin, and Choline*. National Academies Press (US). http://www.ncbi.nlm.nih.gov/books/NBK114308/

43. Fox, S. E., Levitt, P., & Nelson, C. A. (2010). How the timing and quality of early experiences influence the development of brain architecture. *Child Development*, *81*(1), 28–40. https://doi.org/10.1111/j.1467-8624.2009.01380.x

44. Gartstein, M. A., & Rothbart, M. K. (2003). Studying infant temperament via the Revised Infant Behavior Questionnaire. *Infant Behavior and Development*, *26*(1), 64–86. https://doi.org/10.1016/S0163-6383(02)00169-8

45. Gathercole, S. E., Pickering, S. J., Knight, C., & Stegmann, Z. (2004). Working memory skills and educational attainment: Evidence from national curriculum assessments at 7 and 14 years of age. *Applied Cognitive Psychology*, *18*(1), 1–16. https://doi.org/10.1002/acp.934

46. Glenn, M. J., Gibson, E. M., Kirby, E. D., Mellott, T. J., Blusztajn, J. K., & Williams, C. L. (2007). Prenatal choline availability modulates hippocampal neurogenesis and neurogenic responses to enriching experiences in adult female rats. *European Journal of Neuroscience*, *25*(8), 2473–2482. https://doi.org/10.1111/j.1460-9568.2007.05505.x

47. Gluckman, P. D., Hanson, M. A., Cooper, C., & Thornburg, K. L. (2008). Effect of In Utero and Early-Life Conditions on Adult Health and Disease. *New England Journal of Medicine*, *359*(1), 61–73. https://doi.org/10.1056/NEJMra0708473

48. Gluckman, P. D., Hanson, M. A., & Low, F. M. (2011). The role of developmental plasticity and epigenetics in human health. *Birth Defects Research Part C: Embryo Today: Reviews*, *93*(1), 12–18. https://doi.org/10.1002/bdrc.20198

49. Godfrey, K. M., Sheppard, A., Gluckman, P. D., Lillycrop, K. A., Burdge, G. C., McLean, C., Rodford, J., Slater-Jefferies, J. L., Garratt, E., Crozier, S. R., Emerald, B. S., Gale, C. R., Inskip, H. M., Cooper, C., & Hanson, M. A. (2011). Epigenetic Gene Promoter Methylation at Birth Is Associated With Child's Later Adiposity. *Diabetes*, *60*(5), 1528–1534. https://doi.org/10.2337/db10-0979

50. Heindel, J. J., Balbus, J., Birnbaum, L., Brune-Drisse, M. N., Grandjean, P., Gray, K., Landrigan, P. J., Sly, P. D., Suk, W., Slechta, D. C., Thompson, C., & Hanson, M. (2015). Developmental Origins of Health and Disease: Integrating Environmental Influences. *Endocrinology*, *156*(10), 3416–3421. https://doi.org/10.1210/en.2015-1394

51. Hochberg, Z., Feil, R., Constancia, M., Fraga, M., Junien, C., Carel, J.-C., Boileau, P., Le Bouc, Y., Deal, C. L., Lillycrop, K., Scharfmann, R., Sheppard, A., Skinner, M., Szyf, M., Waterland, R. A., Waxman, D. J., Whitelaw, E., Ong, K., & Albertsson-Wikland, K. (2011). Child Health, Developmental Plasticity, and Epigenetic Programming. *Endocrine Reviews*, *32*(2), 159–224. https://doi.org/10.1210/er.2009-0039

52. Howard, S. J., Vasseleu, E., Neilsen-Hewett, C., de Rosnay, M., Chan, A. Y. C., Johnstone, S., Mavilidi, M., Paas, F., & Melhuish, E. C. (2021). Executive Function and Self-Regulation: Bi-Directional Longitudinal Associations and Prediction of Early Academic Skills. *Frontiers in Psychology*, *12*. https://www.frontiersin.org/article/10.3389/fpsyg.2021.733328

53. Hutchison, A. K., Hunter, S. K., Wagner, B. D., Calvin, E. A., Zerbe, G. O., & Ross, R. G. (2017). Diminished Infant P50 Sensory Gating Predicts Increased 40-Month-Old Attention, Anxiety/Depression, and Externalizing Symptoms. *Journal of Attention Disorders*, *21*(3), 209–218. https://doi.org/10.1177/1087054713488824

54. Intakes, I. of M. (US) S. on I. and U. of D. R., & Intakes, I. of M. (US) S. C. on the S. E. of D. R. (2000). Using the Adequate Intake for Nutrient Assessment of Groups. In *DRI Dietary Reference Intakes: Applications in Dietary Assessment*. National Academies Press (US). http://www.ncbi.nlm.nih.gov/books/NBK222886/

55. Jacobson, S. W., Carter, R. C., Molteno, C. D., Stanton, M. E., Herbert, J. S., Lindinger, N. M., Lewis, C. E., Dodge, N. C., Hoyme, H. E., Zeisel, S. H., Meintjes, E. M., Duggan, C. P., & Jacobson, J. L. (2018). Efficacy of Maternal Choline Supplementation During Pregnancy in Mitigating Adverse Effects of Prenatal Alcohol Exposure on Growth and Cognitive Function: A Randomized, Double-Blind, Placebo-Controlled Clinical Trial. *Alcoholism: Clinical and Experimental Research*, *42*(7), 1327–1341. https://doi.org/10.1111/acer.13769

56. Jiang, X., Yan, J., West, A. A., Perry, C. A., Malysheva, O. V., Devapatla, S., Pressman, E., Vermeylen, F., & Caudill, M. A. (2012). Maternal choline intake alters the epigenetic state of fetal cortisol-regulating genes in humans. *The FASEB Journal*, *26*(8), 3563–3574. https://doi.org/10.1096/fj.12-207894

57. Johnson, J. O. (2013). Autonomic Nervous System Physiology. In *Pharmacology and Physiology for Anesthesia* (pp. 208–217). Elsevier. https://doi.org/10.1016/B978-1-4377-1679-5.00012-0

58. Jones, C. W., Gray, S. A. O., Theall, K. P., & Drury, S. S. (2018). Polymorphic variation in the SLC5A7 gene influences infant autonomic reactivity and self-regulation: A neurobiological model for ANS stress responsivity and infant temperament. *Psychoneuroendocrinology*, *97*, 28–36. https://doi.org/10.1016/j.psyneuen.2018.06.019

59. Jones-Mason, K. (n.d.). *Autonomic nervous system functioning assessed during the still-face paradigm_ A meta-analysis and systematic review of methods, approach and findings*. 27

60. Jurado, María Beatriz, & Rosselli, M. (2007). The Elusive Nature of Executive Functions: A Review of our Current Understanding. *Neuropsychology Review*, *17*, 213–233.

61. Kable, J. A., Coles, C. D., Keen, C. L., Uriu-Adams, J. Y., Jones, K. L., Yevtushok, L., Kulikovsky, Y., Zymak-Zakutnya, N., Dubchak, I., Akhmedzhanova, D., Wertelecki, W., & Chambers, C. D. (2022). The impact of micronutrient supplementation in alcohol-exposed pregnancies on reaction time responses of preschoolers in Ukraine. *Alcohol*, *99*, 49–58. https://doi.org/10.1016/j.alcohol.2021.12.002

62. Karpova, N., Zhang, D., Beckwith, A. M., Bennett, D. S., & Lewis, M. (2021). Prenatal drug exposure and executive function in early adolescence. *Neurotoxicology and Teratology*, *88*, 107036. https://doi.org/10.1016/j.ntt.2021.107036

63. Kim, B.-N., Kim, J.-W., Cummins, T., Bellgrove, M., Hawi, Z., Hong, S.-B., Yang, Y.-H., Kim, H.-J., Shin, M.-S., Cho, S.-C., Kim, J.-H., Son, J.-W., Shin, Y.-M., Chung, U.-S., & Han, D.-H. (2013). Norepinephrine Genes Predict Response Time Variability and Methylphenidate-Induced Changes in Neuropsychological Function in Attention Deficit Hyperactivity Disorder. *Journal of Clinical Psychopharmacology*, *33*(3), 356–362. https://doi.org/10.1097/JCP.0b013e31828f9fc3

64. Korsmo, H. W., Jiang, X., & Caudill, M. A. (2019). Choline: Exploring the Growing Science on Its Benefits for Moms and Babies. *Nutrients*, *11*(8), 1823. https://doi.org/10.3390/nu11081823

65. Langley, E. A., Krykbaeva, M., Blusztajn, J. K., & Mellott, T. J. (2015). High maternal choline consumption during pregnancy and nursing alleviates deficits in social interaction and improves anxiety-like behaviors in the BTBR T+Itpr3tf/J mouse model of autism. *Behavioural Brain Research*, *278*, 210–220. https://doi.org/10.1016/j.bbr.2014.09.043

66. Laus, M. F., Vales, L. D. M. F., Costa, T. M. B., & Almeida, S. S. (2011). Early Postnatal Protein-Calorie Malnutrition and Cognition: A Review of Human and Animal Studies. *International Journal of Environmental Research and Public Health*, *8*(2), 590–612. https://doi.org/10.3390/ijerph8020590

67. Lea, A. J., Tung, J., Archie, E. A., & Alberts, S. C. (2018). Developmental plasticity. *Evolution, Medicine, and Public Health*, *2017*(1), 162–175. https://doi.org/10.1093/emph/eox019

68. Leventer, S. M., & Rowell, P. P. (1984). Investigation of the rate-limiting step in the synthesis of acetylcholine by the human placenta. *Placenta*, *5*(3), 261–270. https://doi.org/10.1016/S0143-4004(84)80036-3

69. Mandy, M., & Nyirenda, M. (2018). Developmental Origins of Health and Disease: The relevance to developing nations. *International Health*, *10*(2), 66–70. https://doi.org/10.1093/inthealth/ihy006

70. McCann, J. C., Hudes, M., & Ames, B. N. (2006). An overview of evidence for a causal relationship between dietary availability of choline during development and cognitive function in offspring. *Neuroscience & Biobehavioral Reviews*, *30*(5), 696–712. https://doi.org/10.1016/j.neubiorev.2005.12.003

71. McMahon, K. E., & Farrell, P. M. (1985). Measurement of free choline concentrations in maternal and neonatal blood by micropyrolysis gas chromatography. *Clinica Chimica Acta*, *149*(1), 1–12. https://doi.org/10.1016/0009-8981(85)90267-0

72. Meck, W. H., & Williams, C. L. (2003). Metabolic imprinting of choline by its availability during gestation: Implications for memory and attentional processing across the lifespan. *Neuroscience & Biobehavioral Reviews*, *27*(4), 385–399. https://doi.org/10.1016/S0149-7634(03)00069-1

73. Mesman, J., van IJzendoorn, M. H., & Bakermans-Kranenburg, M. J. (2009). The many faces of the Still-Face Paradigm: A review and meta-analysis. *Developmental Review*, *29*(2), 120–162. https://doi.org/10.1016/j.dr.2009.02.001

74. Minnes, S., Min, M. O., Short, E. J., Wu, M., Lang, A., Yoon, S., & Singer, L. T. (2016). Executive function in children with prenatal cocaine exposure (12–15years). *Neurotoxicology and Teratology*, *57*, 79–86. https://doi.org/10.1016/j.ntt.2016.07.002

75. Mohler, E. G., Meck, W. H., & Williams, C. L. (n.d.). *Sustained Attention in Adult Mice is Modulated by Prenatal Choline Availability*. 16.

76. Moon, J., Chen, M., Gandhy, S. U., Strawderman, M., Levitsky, D. A., Maclean, K. N., & Strupp, B. J. (2010). Perinatal choline supplementation improves cognitive functioning and emotion regulation in the Ts65Dn mouse model of Down syndrome. *Behavioral Neuroscience*, *124*(3), 346–361. https://doi.org/10.1037/a0019590

77. Moreno, M. A. (2017). Prenatal Alcohol Exposure: No Safe Amount. *JAMA Pediatrics*, *171*(8), 820. https://doi.org/10.1001/jamapediatrics.2017.1093

78. Neumann, S. A., Brown, S. M., Ferrell, R. E., Flory, J. D., Manuck, S. B., & Hariri, A. R. (2006). Human Choline Transporter Gene Variation Is Associated with Corticolimbic Reactivity and Autonomic-Cholinergic Function. *Biological Psychiatry*, *60*(10), 1155–1162. https://doi.org/10.1016/j.biopsych.2006.03.059

79. Neumann, S. A., Lawrence, E. C., Jennings, J. R., Ferrell, R. E., & Manuck, S. B. (2005). Heart Rate Variability Is Associated With Polymorphic Variation in the Choline Transporter Gene: *Psychosomatic Medicine*, *67*(2), 168–171. https://doi.org/10.1097/01.psy.0000155671.90861.c2

80. Niculescu, M. D., & Zeisel, S. H. (2002). Diet, Methyl Donors and DNA Methylation: Interactions between Dietary Folate, Methionine and Choline. *The Journal of Nutrition*, *132*(8), 2333S-2335S. https://doi.org/10.1093/jn/132.8.2333S

81. Noland, J. S., Singer, L. T., Arendt, R. E., Minnes, S., Short, E. J., & Bearer, C. F. (2003). Executive Functioning in Preschool-Age Children Prenatally Exposed to Alcohol, Cocaine, and Marijuana. *Alcoholism: Clinical and Experimental Research*, *27*(4), 647–656. https://doi.org/10.1111/j.1530-0277.2003.tb04401.x

82. *Office of Dietary Supplements - Nutrient Recommendations: Dietary Reference Intakes (DRI)*. (n.d.). Retrieved January 11, 2022, from https://ods.od.nih.gov/HealthInformation/Dietary_Reference_Intakes.aspx

83. *Office of Dietary Supplements—Choline*. (n.d.). Retrieved May 25, 2022, from https://ods.od.nih.gov/factsheets/Choline-HealthProfessional/

84. Osmond, C., Barker, D. J., Winter, P. D., Fall, C. H., & Simmonds, S. J. (1993). Early growth and death from cardiovascular disease in women. *BMJ : British Medical Journal*, *307*(6918), 1519–1524.

85. *Peripheral Nervous System: Crash Course A&P #12*. (n.d.). Retrieved November 4, 2020, from https://www.youtube.com/watch?v=QY9NTVh-Awo&list=PL2vrmieg9tO1TE2BEft0UWG6lkMYCWXGY&index=11

86. Planalp, E. M., Van Hulle, C., Gagne, J. R., & Goldsmith, H. H. (2017). The Infant Version of the Laboratory Temperament Assessment Battery (Lab-TAB): Measurement Properties and Implications for Concepts of Temperament. *Frontiers in Psychology*, *8*. https://doi.org/10.3389/fpsyg.2017.00846

87. Powers, B. E., Kelley, C. M., Velazquez, R., Ash, J. A., Strawderman, M. S., Alldred, M. J., Ginsberg, S. D., Mufson, E. J., & Strupp, B. J. (2017). Maternal choline supplementation in a mouse model of Down syndrome: Effects on attention and nucleus basalis/substantia innominata neuron morphology in adult offspring. *Neuroscience*, *340*, 501–514. https://doi.org/10.1016/j.neuroscience.2016.11.001

88. Purves, D., Augustine, G. J., Fitzpatrick, D., Katz, L. C., LaMantia, A.-S., McNamara, J. O., & Williams, S. M. (2001). Acetylcholine. *Neuroscience. 2nd Edition*. http://www.ncbi.nlm.nih.gov/books/NBK11143/

89. Putnam, S. P., Gartstein, M. A., & Rothbart, M. K. (2006). Measurement of fine-grained aspects of toddler temperament: The Early Childhood Behavior Questionnaire. *Infant Behavior and Development*, *29*(3), 386–401. https://doi.org/10.1016/j.infbeh.2006.01.004

90. Quigley, K. M., & Moore, G. A. (2018). Development of cardiac autonomic balance in infancy and early childhood: A possible pathway to mental and physical health outcomes. *Developmental Review*. https://doi.org/10.1016/j.dr.2018.06.004

91. Rose, S. A., Feldman, J. F., & Jankowski, J. J. (2012). Implications of Infant Cognition for Executive Functions at Age 11. *Psychological Science*, *23*(11), 1345–1355. https://doi.org/10.1177/0956797612444902

92. Ross, R. G., Hunter, S. K., Hoffman, M. C., McCarthy, L., Chambers, B. M., Law, A. J., Leonard, S., Zerbe, G. O., & Freedman, R. (2016). Perinatal Phosphatidylcholine Supplementation and Early Childhood Behavior Problems: Evidence for CHRNA7 Moderation. *The American Journal of Psychiatry*, *173*(5), 509–516. https://doi.org/10.1176/appi.ajp.2015.15091188

93. Ross, R. G., Hunter, S. K., McCarthy, L., Beuler, J., Hutchison, A. K., Wagner, B. D., Leonard, S., Stevens, K. E., & Freedman, R. (2013). Perinatal Choline Effects on Neonatal Pathophysiology Related to Later Schizophrenia Risk. *American Journal of Psychiatry*, *170*(3), 290–298. https://doi.org/10.1176/appi.ajp.2012.12070940

94. Rothbart, M. K. (1981). Measurement of Temperament in Infancy. *Child Development*, *52*(2), 569–578. https://doi.org/10.2307/1129176

95. Schulz, K. M., Pearson, J. N., Gasparrini, M. E., Brooks, K. F., Drake-Frazier, C., Zajkowski, M. E., Kreisler, A. D., Adams, C. E., Leonard, S., & Stevens, K. E. (2014). Dietary choline supplementation to dams during pregnancy and lactation mitigates the effects of in utero stress exposure on adult anxiety-related behaviors. *Behavioural Brain Research*, *268*, 104–110. https://doi.org/10.1016/j.bbr.2014.03.031

96. Shaw, G. M., Carmichael, S. L., Yang, W., Selvin, S., & Schaffer, D. M. (2004). Periconceptional Dietary Intake of Choline and Betaine and Neural Tube Defects in Offspring. *American Journal of Epidemiology*, *160*(2), 102–109. https://doi.org/10.1093/aje/kwh187

97. Signore, C., Ueland, P. M., Troendle, J., & Mills, J. L. (2008). Choline concentrations in human maternal and cord blood and intelligence at 5 y of age. *The American Journal of Clinical Nutrition*, *87*(4), 896–902. https://doi.org/10.1093/ajcn/87.4.896

98. Stange, J. P., Hamilton, J. L., Olino, T. M., Fresco, D. M., & Alloy, L. B. (20161219). Autonomic reactivity and vulnerability to depression: A multi-wave study. *Emotion*, *17*(4), 602. https://doi.org/10.1037/emo0000254

99. Strupp, B. J., & Levitsky, D. A. (1995). Enduring Cognitive Effects of Early Malnutrition: A Theoretical Reappraisal. *The Journal of Nutrition*, *125*(suppl_8), 2221S-2232S. https://doi.org/10.1093/jn/125.suppl_8.2221S

100. Subar, A. F., Freedman, L. S., Tooze, J. A., Kirkpatrick, S. I., Boushey, C., Neuhouser, M. L., Thompson, F. E., Potischman, N., Guenther, P. M., Tarasuk, V., Reedy, J., & Krebs-Smith, S. M. (2015). Addressing Current Criticism Regarding the Value of Self-Report Dietary Data. *The Journal of Nutrition*, *145*(12), 2639–2645. https://doi.org/10.3945/jn.115.219634

101. Susser, M., & Stein, Z. (1994). Timing in Prenatal Nutrition: A Reprise of the Dutch Famine Study. *Nutrition Reviews*, *52*(3), 84–94. https://doi.org/10.1111/j.1753-4887.1994.tb01395.x

102. Suzuki, K. (2018). The developing world of DOHaD. *Journal of Developmental Origins of Health and Disease*, *9*(3), 266–269. https://doi.org/10.1017/S2040174417000691

103. Sweiry, J. H., Page, K. R., Dacke, C. G., Abramovich, D. R., & Yudilevich, D. L. (1986). Evidence of saturable uptake mechanisms at maternal and fetal sides of the perfused human placenta by rapid paired-tracer dilution: Studies with calcium and choline. *Journal of Developmental Physiology*, *8*(6), 435–445.

104. Thomas, J. D., Abou, E. J., & Dominguez, H. D. (2009). Prenatal choline supplementation mitigates the adverse effects of prenatal alcohol exposure on development in rats. *Neurotoxicology and Teratology*, *31*(5), 303–311. https://doi.org/10.1016/j.ntt.2009.07.002

105. Thomas, J. D., Idrus, N. M., Monk, B. R., & Dominguez, H. D. (2010). Prenatal choline supplementation mitigates behavioral alterations associated with prenatal alcohol exposure in rats. *Birth Defects Research Part A: Clinical and Molecular Teratology*, *88*(10), 827–837. https://doi.org/10.1002/bdra.20713

106. Thomas, J. D., La Fiette, M. H., Quinn, V. R. E., & Riley, E. P. (2000). Neonatal choline supplementation ameliorates the effects of prenatal alcohol exposure on a discrimination learning task in rats. *Neurotoxicology and Teratology*, *22*(5), 703–711. https://doi.org/10.1016/S0892-0362(00)00097-0

107. Tronick, E. Z. (2003). Things Still To Be Done on the Still-Face Effect. *Infancy*, *4*(4), 475–482. https://doi.org/10.1207/S15327078IN0404_02

108. Velazquez, R., Ash, J. A., Powers, B. E., Kelley, C. M., Strawderman, M., Luscher, Z. I., Ginsberg, S. D., Mufson, E. J., & Strupp, B. J. (2013). Maternal choline supplementation improves spatial learning and adult hippocampal neurogenesis in the Ts65Dn mouse model of Down syndrome. *Neurobiology of Disease*, *58*, 92–101. https://doi.org/10.1016/j.nbd.2013.04.016

109. Villamor, E., Rifas-Shiman, S. L., Gillman, M. W., & Oken, E. (2012). Maternal Intake of Methyl-Donor Nutrients and Child Cognition at 3 Years of Age. *Paediatric and Perinatal Epidemiology*, *26*(4), 328–335. https://doi.org/10.1111/j.1365-3016.2012.01264.x

110. Wallace, T. C., Blusztajn, J. K., Caudill, M. A., Klatt, K. C., & Zeisel, S. H. (2020). Choline: The Neurocognitive Essential Nutrient of Interest to Obstetricians and Gynecologists. *Journal of Dietary Supplements*, *17*(6), 733–752. https://doi.org/10.1080/19390211.2019.1639875

111. Wallace, T. C., & Fulgoni, V. L. (2016). Assessment of Total Choline Intakes in the United

States. *Journal of the American College of Nutrition*, *35*(2), 108–112. https://doi.org/10.1080/07315724.2015.1080127

112. Wallace, T. C., & Fulgoni, V. L. (2017). Usual Choline Intakes Are Associated with Egg and Protein Food Consumption in the United States. *Nutrients*, *9*(8), 839. https://doi.org/10.3390/nu9080839

113. Waterland, R. A., & Jirtle, R. L. (2003). Transposable Elements: Targets for Early Nutritional Effects on Epigenetic Gene Regulation. *Molecular and Cellular Biology*, *23*(15), 5293–5300. https://doi.org/10.1128/MCB.23.15.5293-5300.2003

114. Waxenbaum, J. A., Reddy, V., & Varacallo, M. (2020). Anatomy, Autonomic Nervous System. In *StatPearls*. StatPearls Publishing. http://www.ncbi.nlm.nih.gov/books/NBK539845/

115. Wei, S. Q. (2014). Vitamin D and pregnancy outcomes: *Current Opinion in Obstetrics and Gynecology*, *26*(6), 438–447. https://doi.org/10.1097/GCO.0000000000000117

116. Wong-Goodrich, S. J. E., Glenn, M. J., Mellott, T. J., Blusztajn, J. K., Meck, W. H., & Williams, C. L. (2008). Spatial memory and hippocampal plasticity are differentially sensitive to the availability of choline in adulthood as a function of choline supply in utero. *Brain Research*, *1237*, 153–166. https://doi.org/10.1016/j.brainres.2008.08.074

117. Wu, B. T. F., Dyer, R. A., King, D. J., Richardson, K. J., & Innis, S. M. (2012). Early Second Trimester Maternal Plasma Choline and Betaine Are Related to Measures of Early Cognitive Development in Term Infants. *PLOS ONE*, *7*(8), e43448. https://doi.org/10.1371/journal.pone.0043448

118. *WWEIA Usual Intake Data Tables: USDA ARS*. (n.d.). Retrieved May 25, 2022, from https://www.ars.usda.gov/northeast-area/beltsville-md-bhnrc/beltsville-human-nutrition-research-center/food-surveys-research-group/docs/wweia-usual-intake-data-tables/

119. Yan, J., Jiang, X., West, A. A., Perry, C. A., Malysheva, O. V., Devapatla, S., Pressman, E., Vermeylen, F., Stabler, S. P., Allen, R. H., & Caudill, M. A. (2012). Maternal choline intake modulates maternal and fetal biomarkers of choline metabolism in humans. *The American Journal of Clinical Nutrition*, *95*(5), 1060–1071. https://doi.org/10.3945/ajcn.111.022772

120. Yang, A. C., Chen, T.-J., Tsai, S.-J., Hong, C.-J., Kuo, C.-H., Yang, C.-H., & Kao, K.-P. (2010). BDNF Val66Met polymorphism alters sympathovagal balance in healthy subjects. *American Journal of Medical Genetics Part B: Neuropsychiatric Genetics*, *153B*(5), 1024–1030. https://doi.org/10.1002/ajmg.b.31069

121. Zeisel, S. H. (2006). Choline: Critical Role During Fetal Development and Dietary Requirements in Adults. *Annual Review of Nutrition*, *26*(1), 229–250. https://doi.org/10.1146/annurev.nutr.26.061505.111156

122. Zeisel, S. H., & da Costa, K.-A. (2009). Choline: An essential nutrient for public health. *Nutrition Reviews*, *67*(11), 615–623. https://doi.org/10.1111/j.1753-4887.2009.00246.x

123. Zeisel, S. H., & Niculescu, M. D. (2006). Perinatal Choline Influences Brain Structure and Function. *Nutrition Reviews*, *64*(4), 197–203. https://doi.org/10.1111/j.1753-4887.2006.tb00202.x

124. ZHU, X., SONG, J., MAR, M.-H., EDWARDS, L. J., & ZEISEL, S. H. (2003). Phosphatidylethanolamine N-methyltransferase (PEMT) knockout mice have hepatic steatosis and abnormal hepatic choline metabolite concentrations despite ingesting a recommended dietary intake of choline. *Biochemical Journal*, *370*(3), 987–993. https://doi.org/10.1042/bj20021523

CHAPTER TWO

EFFECTS OF MATERNAL CHOLINE SUPPLEMENTATION ON CHILD EXECUTIVE FUNCTION AT 7 YEARS OF AGE

2.1 Abstract

Objective: To test the efficacy of higher maternal choline intake (930 v 480 mg/day) in the third trimester of pregnancy on child performance on an executive function test of planning and problem solving at age 7 years. **Methods:** Third trimester pregnant women (N = 26) were randomized to receive either 480 mg/day [approximating the Adequate Intake (AI) level] or 930 mg/day choline in a controlled feeding trial. At age 7 years, children (N = 20) completed a battery of cognitive tests, including a computerized Tower of London test of planning and problem solving. Outcome measures included (1) total score, a measure of efficiency of performance, (2) proportion of problems solved on the first attempt, an index of performance accuracy, and (3) speed measures of performance. Statistical analyses included general and mixed linear models. **Results:** Total score did not vary by choline group. However, the children of mothers who consumed 930 mg/day (v 480) choline solved more problems on the first attempt ($p = 0.019$), a finding that was robust to sensitivity analyses and indicative of improved executive function. Although the groups did not differ in the time they took to plan and complete their first move, children in the 930 mg/day group completed the execution of their plan more quickly ($p = 0.026$). **Conclusions:** This study provides novel evidence that maternal choline intake during pregnancy at twice the AI improves child executive function at school age relative to intake at the AI level. These findings parallel a wealth of results showing enduring cognitive enhancement by maternal choline supplementation in rodents and emphasize the potential benefits of increasing choline intake among pregnant women—a population for which the typical intake is approximately 70% of the adequate intake level of 450 mg/day.

2.2 Introduction

The physiological need for choline, an essential nutrient with many roles in fetal development, is increased during pregnancy, where it provides constituents for the development of cell membranes, neurotransmitters, and epigenetic modifications.[67–69,72] During prenatal development, choline and its metabolites play key roles in brain development via several potential mechanisms, including effects on cellular proliferation, migration, and apoptosis, neurogenesis, and synaptic plasticity.[2,4,14–15,18,60,67,72] Choline-derived phospholipids, including sphingomyelin and phosphatidylcholine, are key components of cell membranes and help to maintain the structural integrity of cells.[68–70] Choline also acts as a required precursor to acetylcholine (ACh), a key neurotransmitter at neuromuscular junctions and in the central nervous system.[32,68–70] Further, choline is the major dietary source of methyl groups, and, through its conversion to the metabolites betaine and s-adenosylmethionine (SAM), provides methyl groups needed for DNA methylation.[68–70] These metabolites of choline play key roles in epigenetic modification of genes and histones, which can exert long-term effects via gene expression.[41,67–68]

Consistent with choline's many important roles, a robust body of rodent work has demonstrated the importance of maternal choline intake for the developmental programming of offspring cognition and behavior. In particular, rodent data demonstrate that maternal choline deficiency results in irreversible cognitive deficits in the offspring, and that conversely, maternal choline supplementation beyond amounts in standard rodent chow (which is designed to contain adequate choline) improves offspring memory, attention, and socioemotional function throughout the lifespan.[17,31,37,39–40,51,64] Further, prenatal maternal choline supplementation has been shown to lessen age-related cognitive decline and reduce cognitive dysfunction in rodent

models of several neurological disorders, including Down syndrome, autism, and Alzheimer's disease.[4,41,54,60] Finally, prenatal choline supplementation has been shown to lessen the dysfunction produced by a variety of prenatal insults, including maternal stress, infection, inflammation, and exposure to alcohol.[24,27,55–57]

Although these rodent data provide strong evidence that choline intake during pregnancy is critical for offspring brain development and cognitive functioning, relatively little is known about choline needs during pregnancy in humans, including the functional consequences for the child if maternal intake is insufficient.[12] In 1998, the IOM for the first time identified an Adequate Intake (AI) for choline at 425 mg/day for adult women, with a slight increase to 450 mg/day for pregnant and lactating women.[44] However, this recommendation was based on the amount of choline needed to prevent liver dysfunction in men (with a small increase for tissue expansion), not the more relevant outcome of child neurodevelopment.[12,44] Therefore, it is likely that the AI is insufficient for the demands of pregnancy. This is concerning in light of the fact that ~90% of pregnant women do not consume the AI, and most prenatal vitamins contain little to no choline (~55 mg).[62–63]

Few human studies have been conducted to assess the association between variations in maternal choline intake during pregnancy and offspring outcomes. Two observational studies found correlations between serum and/or dietary measures of maternal choline intake and offspring performance on tests of infant development and child memory[10,65], but two others found no association.[53,61] However, it is worth noting that observational studies do not allow for causal inferences due to risk of confounding with uncontrolled covariates.

The results of three randomized controlled trials (RCT) of maternal choline supplementation in typically developing infants have also been conducted. Of the three studies,

two found benefits indicative of improved offspring cognition, while the third did not. One of these studies found that maternal choline supplementation (v placebo) had beneficial effects on cerebral inhibition during infancy.[49–50] The second study, a controlled choline feeding trial comparing two levels of dietary choline intake, provided evidence of improved attentional orienting speed during infancy[13] and superior working memory and sustained attention at seven years of age.[5–6] The third trial detected no offspring cognitive benefits in the infants born to choline supplemented mothers (v placebo), based on assessments of memory and cognitive development.[16]

The present report describes the results of a test of executive functioning (The Tower of London) given to the 7-year-old offspring of women who participated in the choline feeding trial described above.[13] Executive functioning, a set of higher-order cognitive processes that includes planning, working memory, attentional control, and inhibitory control, is integral to the planning and execution of goal-directed behaviors when solving novel or difficult problems.[3,23] There were several reasons why a test of executive functioning was included in this 7 year follow-up. Most importantly, assessment of these same children during the first year of life had found that infants born to mothers in the higher choline intake group (930 mg/day) had faster information processing speeds than those born to mothers consuming the lower intake level (480 mg/day).[13] Notably, faster information processing speed during infancy has been shown to predict superior executive function in later childhood.[48] Second, numerous rodent studies have demonstrated that maternal choline supplementation improves spatial maze performance[54,60,64], indicative of improved working memory, impulse control, and planning. Third, prior studies have demonstrated that executive functioning is uniquely sensitive to various prenatal insults, including exposure to cocaine, cannabis, and alcohol.[29,38,43]

The present study tested the hypothesis that higher maternal choline intake (930 v 480 mg/day) intake during the third trimester of pregnancy will improve offspring executive functioning, as assessed by the Tower of London task, a classic neuropsychological measure of planning and problem solving—core elements of executive function.[11,19,26,33] Results of other cognitive tests are reported elsewhere.[5–6]

2.3 Subjects and Methods

Ethical Approval

Ethical approval for the present study was obtained from the Institutional Review Board for Human Participants at Cornell University in Ithaca, NY. Written parental consent and child assent was obtained from all study participants.

Study Design and Participants

Controlled Feeding Trial

The present study is a 7-year follow-up of a randomized, double-blind controlled choline feeding trial of women in their third trimester of pregnancy (NCT01127022). The original trial was powered to assess primary outcomes related to fetal and maternal biomarkers of choline metabolism.[25,66] Secondary outcomes included genomic expression, metabolomic profiling of plasma and placental tissues, and offspring cognition during infancy. This paper reports on an ancillary follow-up of the children of supplemented mothers at age 7 years to test for effects on child cognition, using pre-specified endpoints.

Details of the feeding trial have been published elsewhere.[25,66] Briefly, pregnant women in the third trimester (> 27 weeks' gestation) were recruited from the Ithaca area between January 2009 and October 2010. Eligibility to participate included healthy, singleton pregnancy, age ≥ 21, and willingness to comply with the study protocol. Exclusion criteria included current

tobacco or alcohol use, anemia, history of chronic disease (e.g., diabetes mellitus, gastrointestinal disorders, or cardiovascular disease), liver or kidney dysfunction, or use of prescription medication that affects liver function, or pregnancy complications or comorbidities.[66]

Enrolled women (N = 26) were entered into a 12-week controlled feeding study in which they were randomized to consume either 480 or 930 mg/day choline on a 7-day cyclical menu cycle. The meals contained an average of 380 mg of choline, and an additional supplement of either 100 mg or 550 mg as choline chloride was dissolved into cran-grape juice and consumed with one of the study meals. Both participants and study personnel were blinded to choline group assignments. At least one meal per day was consumed on-site at the Cornell University Human Metabolic Research Unit (HMRU). To monitor adherence, researchers communicated daily with study participants, who were asked to complete a daily checklist of food consumed. In addition to the study diet and choline supplement, all women consumed a daily prenatal multivitamin (Pregnancy Plus, Fairhaven Health LLC), a daily 200 mg docosahexaenoic acid (DHA; Neuromins, Nature's Way Products), and a 250 mg potassium and 250 mg magnesium supplement three times a week (General 37 Nutrition Corp.).

At six visits to the laboratory during study weeks 0, 3, 6, 9, 10, and 12, women provided blood and urine samples. At delivery, women were asked to provide a maternal blood and cord blood sample, as well as a sample of placental tissue.

Follow-Up Cognition Study

Beginning in August 2016, children of the mothers who participated in the feeding trial were invited to return to Cornell to participate in a longitudinal follow-up study to assess cognitive and behavioral outcomes of choline supplementation. The children (N = 20) were studied between the ages of 7–7.7 years old and participated in two days of testing by members

of the research team who were blinded to choline group assignment. The task discussed in this paper, the Tower of London (TOL), was used to assess executive function and planning, and was administered towards the end of the first day of testing. Other measures administered to the children included the Wechsler Preschool and Primary Scales of Intelligence (WPPSI-R) and tests of sustained attention and working memory, the results of which are reported elsewhere.[5,6]

Testing occurred in the Cornell University Human Metabolic Research Unit (HMRU, N = 16), or if travel to Ithaca was not possible, at an alternative location (N = 4). Participant and maternal characteristics, including race, ethnicity, child visual acuity, child grade in school, and maternal education were collected via parent report. Maternal characteristics at the time of the feeding study, including race, ethnicity, education, and age, were evaluated to assess bias from loss to follow-up. In addition, parents were asked to fill out several parent-report measures of child behavior, including the Behavior Assessment System for Children (BASC-3) and the Child Behavior Questionnaire (CBQ).

The Tower of London Task of Executive Function

The Tower of London (TOL) is a classic neuropsychological measure of planning and problem-solving skills, commonly included in batteries of executive functioning. This task was first developed in 1982 as an adaptation of the Tower of Hanoi.[52] Although the TOL was initially designed for clinical use in adults with frontal lobe lesions, many studies have established this task as a useful assessment of problem solving and planning skills across a wide age range in both clinical and nonclinical populations.[7,8,45] The TOL has several advantages that make it ideal for the assessment of executive function in children, including: (1) the task is challenging and engaging for children of many ages, while (2) incorporating several difficulty levels, and (3) can be administered within a short period of time, without placing excess demands on children's

attentional capacity.[3] The version of the Tower of London administered in this study was a computerized version adapted from the Krikorian et al.[30] procedure, and implemented using Inquisit software (Inquisit 5.0, Millisecond Software, Arlington, VA).[42]

The testing protocol included one practice problem with two moves, followed by thirteen test problems. The 13 test problems comprised 3 three-move problems, 4 four-move problems, and 6 five-move problems. The trials were administered in the same order for every child. At the start of each problem, the child was presented with three colored balls (one blue, one green, one red) distributed on three pegs in a starting configuration and asked to rearrange them into a displayed goal configuration (Figure 2.1). The child was instructed to complete the rearrangement in the minimum number of moves, with a move defined as taking a ball from one peg and successfully placing it on a different peg. The number of moves for each problem was presented on screen, and the experimenter verbally reminded the child of the number of moves allowed at the beginning of each problem.

The problems presented to participants were chosen to represent a range of difficulty within each level of minimum moves. Problem difficulty was obtained from Unterrainer et al., who conducted the Tower of London task in a cohort of 6- through 9-year olds, and defined problem difficulty as one minus the proportion of problems solved on the first attempt across the entire sample (Appendix C).[58] Although minimum number of moves has traditionally been used to represent problem difficulty, studies have shown that problem characteristics other than number of moves can have significant influence on problem difficulty.[7,8,45] The Unterrainer et al. approach offers an empirical measure of problem difficulty for children in this age range.

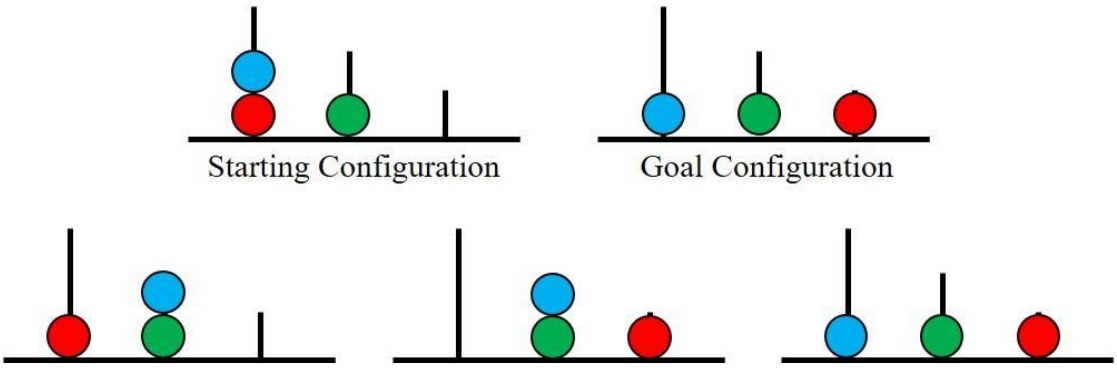


Figure 2.1: A sample three-move problem from our Tower of London problem set. Children were provided with the starting and goal configurations and asked to plan carefully before attempting to solve the problem in the minimum number of moves.

Our primary goal was to use this task to assess the putative benefit of increased maternal choline intake on children's planning ability. Because we were most interested in assessing planning, the instructions repeatedly encouraged the children to consider how to solve the problem before beginning to make moves. Specifically, the experimenter told the child to: *"Think carefully about how you can make the bottom pattern look like the top pattern using N moves. Wait until you think you know which moves to make. Then make your moves."* (Appendix D). These instructions were given before the practice trial and before each of the test problems. If the child failed to solve the problem on the first attempt, the experimenter restated the instructions emphasizing the importance of planning before starting their next attempt.

Participants had no time limit but were allowed only three attempts to solve each problem. An attempt was considered failed if the child did not solve the problem in the minimum number of moves. This procedure, by limiting the number of moves allowed, encourages the participant to focus on the need for careful planning.[7,8,45] After three failed attempts, the child was moved to the next problem in the sequence. All but one child were presented with all thirteen problems; in this one case, technical issues with the program prevented the presentation of one problem. Importantly, the problems presented only had one solution path that would allow

them to be solved in the minimum number of moves. This allowed us to better compare performance across children, as they were all required to solve each problem using the same solution path, and eliminated variance associated with differences in difficulty inherent in multiple solution paths.

We analyzed three outcome measures, assessing efficiency of problem solving, accuracy of problem solving, and speed of planning, respectively. The primary outcome was total score, defined as the score achieved across the whole set of test problems and computed as the sum of all individual problem scores.[42] Children earned three points if they solved the problem on the first attempt, with one point subtracted for each of the two subsequent attempts that were allowed. Children who did not solve the problem in three attempts received a score of zero. We selected total score, which takes into account sources of variance in problem solving and includes data from all attempts, as a sensitive measure of overall problem-solving performance. We also examined solution accuracy, defined as the number of problems solved in the minimum number of moves on the first of three attempts ("perfect" solutions), as a secondary outcome.[42]

Lastly, we examined the speed of planning using first move time, defined as the time between presentation of the test problem and completion of the participant's first move on the first attempt (i.e., when a ball is moved from one peg to another). This measure allows us to assess the extent to which the participants took the time to carefully consider a solution to the problem before beginning to solve it.[7,8,45]

Statistical Analyses

Maternal and child characteristics for the participants included in the final analytical sample were compared by treatment group using Student's t tests for continuous variables and Fisher's exact tests for categorical variables. The same approach was used to compare

participants included in the final analysis to the six children who did not provide cognitive and behavioral endpoint data (lost to follow-up, N = 5; data collection failure, N = 1. Figure 2.2).

Recognizing the limitations of estimating multiple statistical models in a small sample with multiple endpoints, our analysis plan (completed prior to unblinding) prespecified one basic generalized linear model for estimating the effect of third trimester choline intake on the total TOL score, and one basic linear mixed-effects model for estimating the effect of third trimester choline intake on the number of perfect solutions. Our *a priori* models included fixed effects for choline group status (930 mg/day v 480). A pre-specified fixed main effect of child sex was also included *a priori*, although the small number of females in both treatment groups precluded testing for interactions including sex. Random effects were specified for the individual children and for problem number. Speed of problem solving was assessed using the same mixed model described above. Models adjusting for problem difficulty included a fixed effect for problem difficulty, calculated empirically using the methods described above and in Appendix C.

Sensitivity Analyses

Sensitivity analyses were conducted to evaluate the robustness of the results from the primary analyses. Because power to detect differences in demographic characteristics is low in this small sample, we wanted to evaluate the possible existence of effects of even slight imbalances on our primary analyses. To assess the influence of possible imbalance in child and maternal demographic characteristics, we entered each variable presented in Table 2.1 as an individual covariate into the *a priori* models and estimated the change in treatment effect. Statistical analyses were completed using SAS 9.4 (SAS Institute, Inc., Cary, NC, USA). Statistical significance was set at $p < 0.05$ for main effects and $p < 0.10$ for interactions. All tests were t-tailed.

2.4 Results

Subject Characteristics

Of the 26 women who completed the feeding protocol, 21 of their children were successfully recruited for cognitive assessment at 7 years old (Figure 2.2). One child was successfully re-recruited but did not adhere to any task protocols in the battery and thus did not produce valid data for any of the tasks. Prior to unblinding the investigators to treatment group identity, the decision was made to exclude the data from this child from all analyses of cognitive endpoints. There were no statistically significant group differences between the children included in the final analytical sample (N = 20) and those who were not (N = 6) on child sex, maternal race or ethnicity, or maternal age or education level at conception.

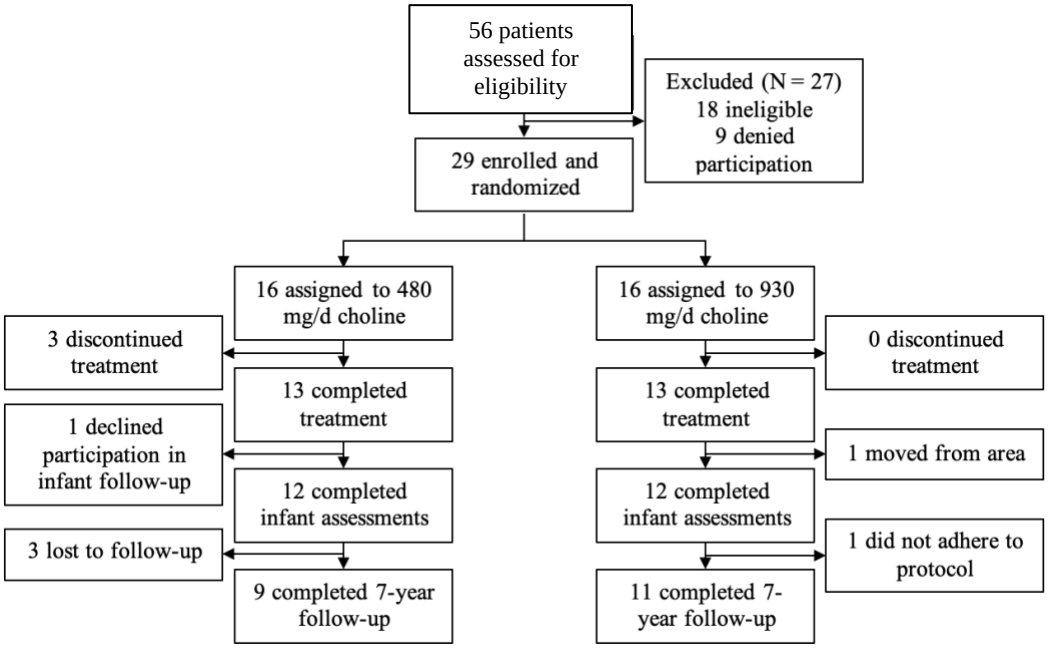


Figure 2.2: Participant flow diagram. Study screening, intervention, and infant and follow up assessments.

There were no statistically significant differences in sample characteristics by choline group (Table 2.1). The sample was 70% male, 76.5% white, and 80% non-Hispanic ethnicity. At the time of follow-up, mothers were on average 28 years old and 47.5% had an advanced degree,

making them older and more highly educated than the U.S. average.[43,46]

	3rd Trimester Maternal Choline Intake		
	480 mg/d (N = 9)	**930 mg/d (N = 11)**	**p**
Child Characteristics			
Sex, male (%)	6 (67)	8 (73)	1.0
Mean birthweight, grams (range)	3487 (2693.2–4252.4)	3467 (2920–4224.1)	0.93
Mean gestational age, weeks (range)	39.2 (36–41)	38.9 (37–41)	0.61
Mean breastfeeding duration, weeks (range)	16.6 (6–29)	15.4 (0–50)	0.82
Mean age at testing, years (range)	7.2 (7.01–7.62)	7.3 (7.03–7.6)	0.68
English not primary language (%)	1 (11)	3 (27)	0.59
Normal or corrected to normal vision (%)	9 (100)	11 (100)	1.00
Highest grade completed (%)			0.62
Kindergarten	3 (33)	2 (18)	
First Grade	6 (67)	9 (82)	
Keyboard experience (%)			0.81
None	1 (11)	0 (0)	
Minimal	5 (56)	7 (64)	
Frequent	3 (33)	4 (36)	
Race (%)			1.00
Asian	1 (11)	0 (0)	
Black	0 (0)	1 (9)	
Native American	0 (0)	1 (9)	
White	8 (89)	7 (64)	
Hispanic Ethnicity (%)	2 (22)	2 (18)	1.00
Maternal Characteristics			
Mean age at conception, years (range)	28.4 (25–33.1)	27.6 (21.6–33.5)	0.61
Education (%)			0.09
High School/Associate Degree	0 (0)	4 (36)	
Bachelor's Degree	3 (33)	4 (36)	
Masters/Doctoral Degree	6 (67)	3 (28)	
Family Income (per year)			0.67
<$50,000	0 (0)	2 (18)	
$50,000–<$100,000	4 (44)	4 (36)	
≥$100,000	5 (56)	5 (45)	

Table 2.1: Sample demographic characteristics by maternal choline intake group.

TOL Performance

Tables 2.2 and 2.3 provide an overview of performance on the TOL task by choline group, as well as problem and attempt-level descriptives of performance. As shown in Table 2.2, all participants were able to solve Problem 1 and Problem 6 in this problem set. One-way ANOVA, with total score as the outcome variable, revealed a significant effect of the minimum number of moves required to solve the problem [$F(1, 257) = 27.66$, $p < 0.001$]. Four-move problems were not significantly harder to solve than three-move problems [$F(1, 139) = 0.13$, $p = 0.72$] but five-move problems were significantly harder to solve than four-move problems [$F(1, 197) = 34.70$, $p < 0.0001$]

			3rd Trimester Maternal Choline Intake			
Problem Number	Number of Moves	Problem Difficulty (Kaller)	480 mg/d (N = 9)		930 mg/d (N = 11)	
			Mean Score (SD)	% Correct on First Attempt	Mean Score (SD)	% Correct on First Attempt
1	3	0.18	2.89 (0.33)	88.9%	2.82 (0.4)	81.8%
2	3	0.24	2.22 (0.44)	22.2%	2.18 (1.17)	54.6%
3	3	0.35	2.22 (1.2)	66.7%	2.91 (0.3)	90.9%
4	4	0.22	2.56 (1.01)	77.8%	2.73 (0.65)	81.8%
5	4	0.31	2.22 (1.09)	55.6%	3 (0)	100%
6	4	0.43	2.67 (0.5)	66.7%	2.73 (0.65)	81.8%
7	4	0.61	1.89 (1.05)	33.3%	2.09 (0.94)	45.5%
8	5	0.39	1.89 (1.27)	44.4%	2.82 (0.6)	90.9%
9	5	0.63	1.44 (1.13)	11.1%	1.73 (1.4)	45.5%
10	5	0.82	1.22 (1.3)	22.2%	1.55 (1.44)	45.5%
11*	5	0.82	1.5 (1.2)	22%	1.55 (1.13)	18.2%
12	5	0.92	1.22 (1.48)	33.3%	0.91 (1.04)	9.1%
13	5	0.8	0.88 (1.37)	33.3%	1.45 (1.44)	36.4%
Total Score			27.5 (5.16)		31.4 (5.07)	

Table 2.2: Descriptive data on task performance, including mean problem score, total score, and proportion of participants who solved each problem on the first attempt, by

choline group.
***Problem 11 was not presented to one child in the 480 mg/day group due to technical issues.**

	3rd Trimester Maternal Choline Intake							
Problem Number	480 mg/d (N = 9)				930 mg/d (N = 11)			
	First Attempt	Second Attempt	Third Attempt	Not Solved	First Attempt	Second Attempt	Third Attempt	Not Solved
1	8	1	0	0	9	2	0	0
2	2	7	0	0	6	3	0	2
3	6	0	2	1	10	1	0	0
4	7	1	0	1	9	1	1	0
5	5	2	1	1	11	0	0	0
6	6	3	0	0	9	1	1	0
7	3	3	2	1	5	2	4	0
8	4	2	1	2	10	0	1	0
9	1	5	0	3	5	2	0	4
10	2	2	1	4	5	0	2	4
11*	2	2	2	2	2	5	1	3
12	3	1	0	5	1	2	3	0
13	2	0	1	5	4	2	0	5

Table 2.3: Descriptive data on the number of children in each choline group who solved each problem on the first, second, or third attempt, or did not solve the problem at all.
***Problem 11 was not presented to one child in the 480 mg/day group due to technical issues.**

Efficiency of Problem Solving

The mean problem score (score on any individual problem) across the whole cohort was 2.07 (SD 1.17). In the 480 mg/day group, the mean score on any given problem ranged from 0.88–2.89. In the 930 mg/day group, the mean score on any given problem ranged from 0.91–3 (Table 2.2). The mean total score (sum of all individual problem scores for a participant) across the whole cohort was 29.6 (SD 5.58).

Children of mothers in the 930 mg/day group achieved a slightly higher total score than

children in the 480 mg/day group [31.4 (5.07) v 27.5 (5.16)]. In a general linear model adjusted *a priori* for child sex, this difference did not achieve statistical significance ($p = 0.13$).

Accuracy of Problem Solving

The total proportion of perfect solutions (problems solved on the first attempt) was 53% across all problems presented to all participants. In the 480 mg/day group, the proportion of participations who solved any given problem on the first attempt ranged from 11.1%–88.9%. In the 930 mg/day group, this proportion ranged from 9.1%–100% (Table 2.2).

In a mixed model adjusted *a priori* for child sex, children whose mothers consumed 930 mg/day (v 480 mg/day) choline solved significantly more problems on the first attempt ($p = 0.037$), indicating superior solution accuracy. In the mixed model adjusting for problem difficulty this difference remained significant, such that children in the 930 mg/day group (v 480) solved significantly more of the easier problems on the first attempt ($p = 0.019$) (Figure 2.3).

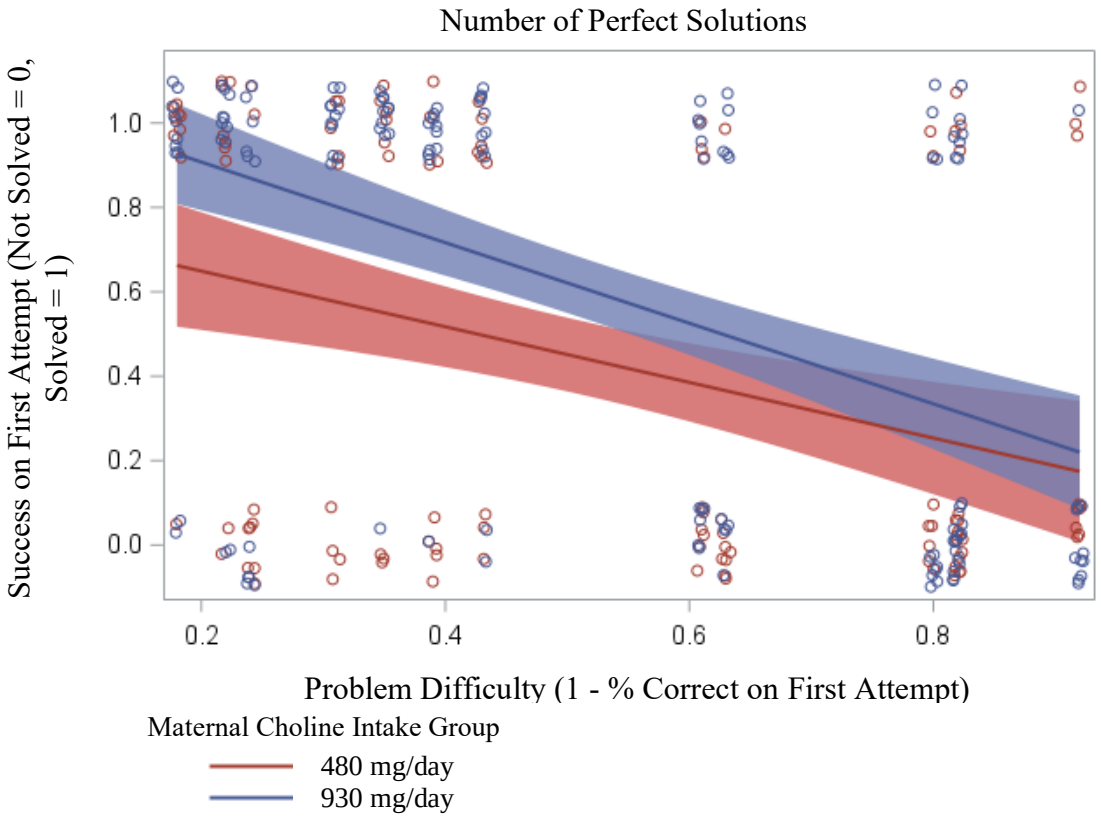


Figure 2.3: Participant performance on the first attempt at each problem, by problem difficulty and choline group. Bands represent 95% confidence intervals. Calculations for problem difficulty can be found in Appendix C.

Speed of Problem Solving

First move time on the first attempt did not differ by maternal choline group in univariate mixed models, adjusted *a priori* for sex (p = 0.64). Nor did first move time on the first attempt differ by maternal choline group in a mixed model adjusting for problem difficulty (Figure 2.4). In post-hoc analyses, we also examined execution time, defined as the time between the end of the participant's first move and completion of the first attempt at the problem. In a mixed model adjusting for child sex and problem difficulty, there was a significant interaction between choline group and problem difficulty (p = 0.026), such that children whose mothers consumed 930 mg/day choline (v 480) completed easier problems more quickly (Figure 2.5).

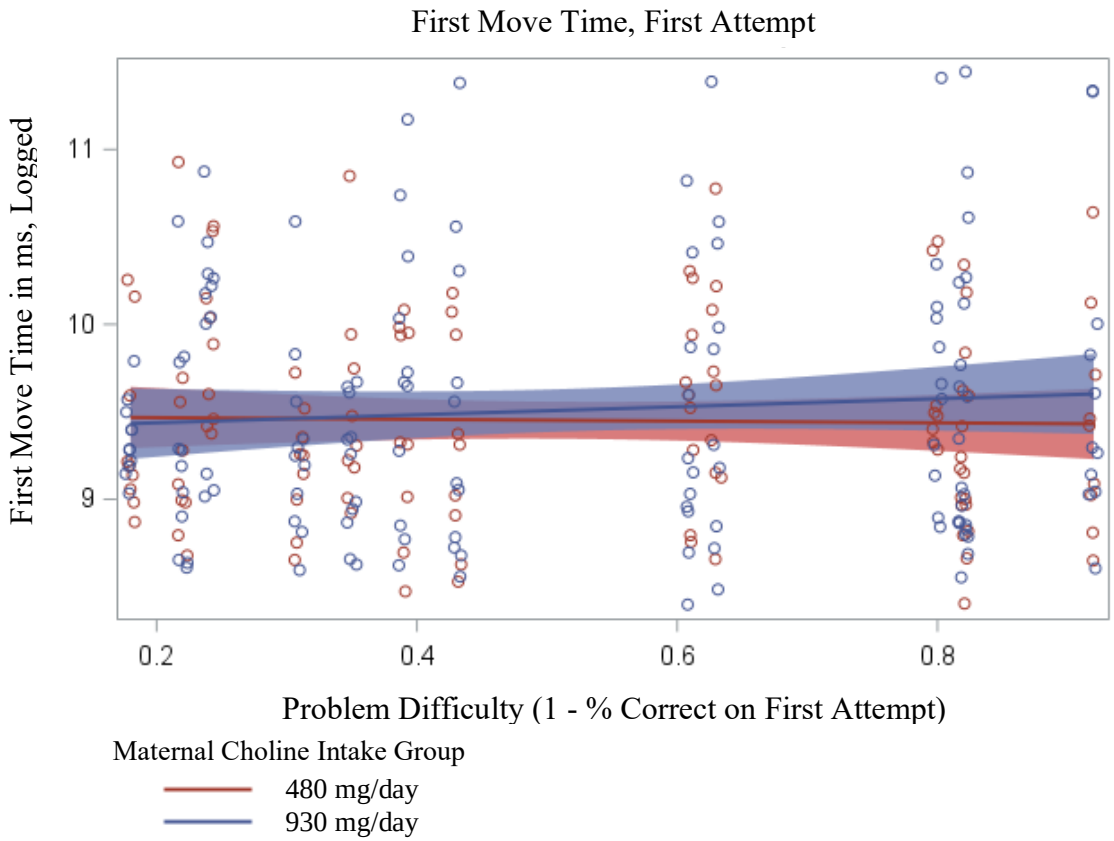


Figure 2.4: First move time (i.e., planning time) on the first attempt at each problem by choline group. Bands represent 95% confidence intervals. Calculations for problem difficulty can be found in Appendix C.

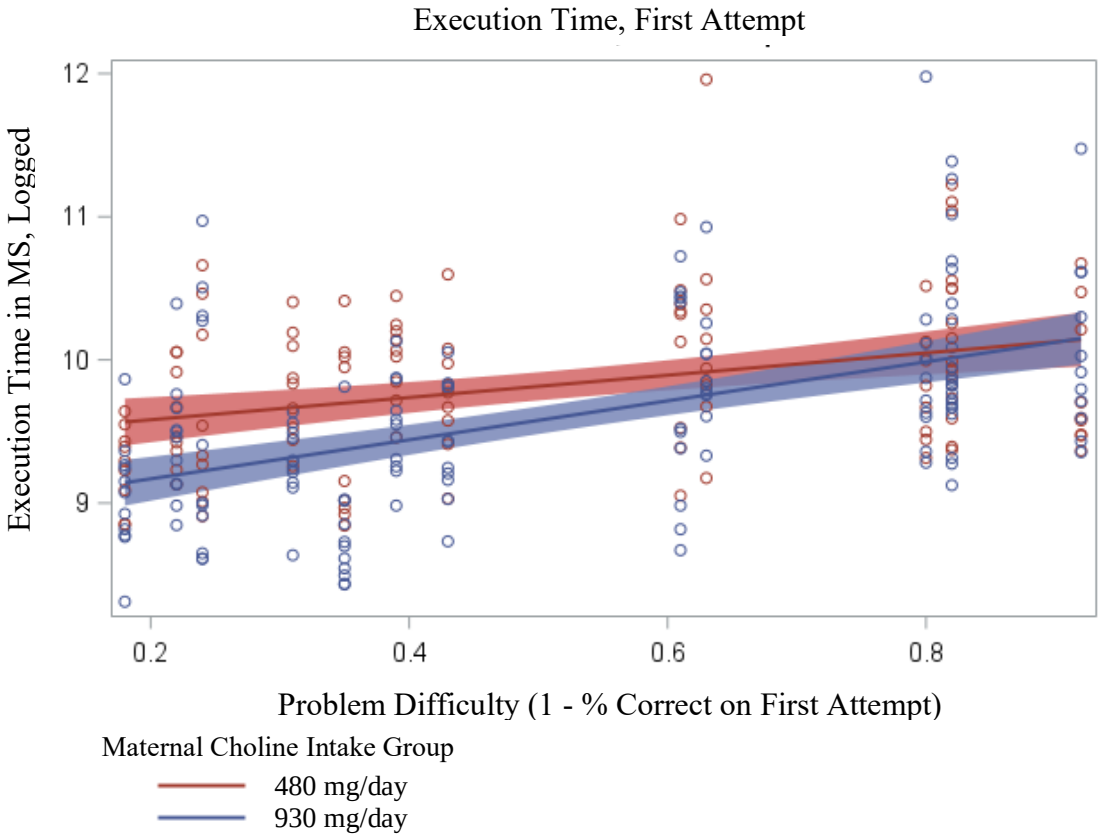


Figure 2.5: Execution time on the first attempt at each problem by choline group. Bands represent 95% confidence intervals. Calculations for problem difficulty can be found in Appendix C.

Sensitivity Analyses

Sensitivity analyses were conducted to test the robustness of the results. Each variable in Table 2.1 was entered as a single added covariate to the *a priori* models (child sex was included in all models). Problem difficulty was included in all models of solving the problem on the first attempt. For the total score, only one covariate altered the estimate of the choline effect by > 10%. Including gestational age in weeks decreased the estimate of the effect by 14.3% ($p = 0.16$). None of the covariates altered the p-value by much ($p = 0.12$ to $p = 0.16$). The mean change in the size of the estimate of the choline effect was -2.2% (range 0%–14.3%).

For the probability of solving the problem on the first attempt, no covariate altered the estimate of the effect of choline by more than 4.4%. Including gestational age in weeks increased

the estimate of the choline effect by 4%, while keyboard experience reduced the estimate of the effect by 4.4%. None of the covariates altered the statistical significance of the choline effect. The mean change in the size of the estimate of the choline effect was 0.06% (0%–4.4%), suggesting that the results of our *a priori* are quite robust to potentially influential covariates.

2.5 Discussion

Summary of Results

The findings of this study revealed that 7-year-old children born to women randomly assigned to 930 mg/day of choline during the 3rd trimester of pregnancy performed better on a classic test of executive functioning than children born to women assigned to 480 mg/day choline. Insight into the nature of this improved performance was provided by the pattern of group differences on various outcome measures.

Children in the 930 mg/day group scored four points higher on average (31.4 v 27.5) than children in the 480 mg/day group. Although this difference did not achieve significance in statistical models, it does indicate a trend towards a better score, and overall better performance on the task for the kids in the 930 mg/day group.

Given the importance of novelty for performance on the Tower of London task, we also examined group differences in ability to solve the problem on the first attempt. In contrast to our total score findings, we found that the children in the 930 mg/day group solved significantly more problems on the first attempt, when the problem was truly novel, than children in the 480 mg/day group ($p = 0.019$). This finding was robust even when demographic characteristics of the children and their mothers were controlled for in sensitivity analyses, which indicates that the difference between the groups can be attributed to the effect of the choline intervention.

Interpretation and Significance of Results

There are several theoretical and methodological reasons for why total score proved to be less sensitive in differentiating the groups than the number of problems solved on the first attempt. Executive function may be thought of as a complex set of cognitive processes that are needed to respond to a novel or challenging situation. In other words, executive functions are employed when a task requires controlled processes (i.e., actions that require the use of new response sequences, actions that require making a choice between several alternative responses, or tasks that require the need to overcome a habitual response), as opposed to automatic processes.[23] Therefore, novelty becomes an important element for activating the cognitive processes underlying executive function. Indeed, studies have found that, even within a single executive function task, re-administration does not tap executive function to the same extent as on the first presentation.[23] Because our protocol allowed for multiple attempts on each problem, attempting to solve the problem on the second or third attempt no longer represents planning on a novel problem. Therefore, the fact that the children in the 930 mg/day group solved significantly more problems on the first, novel, attempt is indicative of superior executive function in this group.

It is also likely that trial and error on the first attempt provides the child with certain information about the problem (i.e., "don't move the blue ball to the tallest peg"), which changes the approach that the child takes to solving the problem on subsequent attempts. As a result of the information gained from a wrong move made on the first attempt, the child can eliminate that move on their second attempt, resulting in both (1) an easier problem, as at least one incorrect move has been removed from the problem's solution path, and (2) the use of an automatic process ("don't move the blue ball to the tallest peg") to attempt to solve the problem. Therefore,

a measure that is inclusive of performance on all three attempts on each problem may be tapping both executive and non-executive cognitive skills, and not the specific executive skill of planning. This may also explain why, despite total score being a commonly used metric of Tower of London performance, this measure does not always reliably differentiate between groups.[36]

This interpretation is strengthened by our accuracy measures, captured as the number of problems solved on the first attempt. The children of mothers who consumed 930 mg/day of choline during the third trimester solved significantly more problems on the first attempt, when the problem was truly novel, and accurately solving the problems requires the use of new response sequences. This suggests that children in the 930 mg/day group were more successful in activating the cognitive processes underlying executive function, which translated to superior performance on their first attempt at solving the problems. Our accuracy results also suggest that children in the 930 mg/d group were more sensitive to experimenter directions to carefully plan before attempting to solve the problem, a strategy that may have contributed to their success at solving the problem on the first attempt.

This interpretation is also supported by our measures of problem-solving speed, which showed that there were no significant differences between groups in the time they took to plan their solution to the problem. However, once planning was complete, children in the 930 mg/day group executed their solution to the problem significantly more quickly ($p = 0.026$) than children in the 480 mg/day group on the easier problems. This suggests that while both groups listened to experimenter directions to wait before starting the problem, only children in the 930 mg/day group used that time to plan effectively, resulting in faster problem solving and more accurate results.

Notably, the results demonstrating superior accuracy and faster execution times for children in the 930 mg/day group were restricted to problems of easy and moderate difficulty. Successfully solving a problem requires integration of several executive function skills, including but not limited to planning, flexibility, working memory, and response inhibition.[11,19,26,33] It is possible that the most difficult problems in this task were very challenging for both groups, and therefore were more likely to be solved by trial and error by all of the children. It may be that only the easier problems were truly solved by superior planning and executive functioning, pointing to executive functioning as the skill that most differentiated the two groups.

Although it is not possible to determine from this small study which exact component of executive function this result measures, others have found that the ability to solve a problem on the first attempt may represent an ability to hold a mental representation of the task, involving working memory, inhibitory control, and planning skills.[33]

These findings confirm the prediction offered by our earlier data, which found that infants of mothers who consumed 930 mg/day choline during the third trimester of pregnancy had faster infant processing speeds across the first year of life.[13] Based on these findings, and other studies that have found infant information processing speed to be predictive of childhood executive function, we predicted that the children of women who consumed 930 mg/day would show superior executive functioning at age seven years. These data are also consistent with our other findings from this study, which showed that maternal choline supplementation with 930 (v 480) mg/d choline improved child sustained attention, a part of the attentional control component of executive function, at age seven years.[6,23] Better sustained attention in the children of the 930 mg/day group was illustrated by a superior ability to maintain signal detection performance

throughout the session, specifically for the briefest signals, which were the most difficult to detect.[6]

Although there is no direct measure of executive function in rodent models, these results do support the substantial body of animal literature that demonstrates lasting cognitive benefits of maternal choline supplementation. Notably, some of the strongest rodent data, in which the offspring of supplemented dams performed better on a radial maze task than the offspring of dams fed a standard diet, requires many cognitive skills that underpin executive function and that the Tower of London task purportedly measures, including working memory (maintaining a representation of which arms of the maze have already been visited) and inhibition (resisting the urge to return to an arm where food was previously found).[37,39]

This study also provides the first experimental evidence of the prenatal programming of executive function. Prenatal programming theories posit that prenatal experiences, especially during critical and sensitive periods, have long-lasting effects on fetal health and development, including the development of the brain and cognitive abilities.[1,11] Although a number of longitudinal prospective cohort studies have provided compelling evidence for a correlation between a number of adverse prenatal exposures (e.g. maternal obesity, stress) and risk for impaired executive function in childhood, these studies are confounded by other pre- and postnatal covariates and lack support for a causal pathway.[11] Due to the ethical challenges of randomizing human subjects to adverse prenatal experiences (e.g. stress, maternal tobacco use), experimental examination of the adverse prenatal factors that may predict later executive function is not possible. However, our highly controlled, randomized nutritional intervention provides clear causal links between a prenatal manipulation (maternal choline supplementation) and child executive function at school age.

Improved executive function may have important real-world implications for school-aged children. The higher-order cognitive processes that are encompassed in executive function skills, including planning, working memory, attentional control, and inhibitory control, are all key to the development of academic skills, including reading and mathematical reasoning. Some studies have found that executive function is more important for school preparedness than general intelligence[9,20], and EF continues to predict competence in math and reading from elementary school through the early high school years.[9,21] Notably, in our study, the total TOL score of the two groups approximates the total scores of children in different grades in a large cohort of elementary-school aged children.[30] Children in the 480 mg/day group scored at approximately a first-grade level, while the children in the 930 mg/day group scored at approximately a fifth grade level.[30] Therefore, maternal choline supplementation during pregnancy may result in population-wide shifts towards higher academic achievement and subsequent social and economic success.[21]

Strengths and Limitations

There are several strengths of the present study. Firstly, our study design allows for strong causal inferences. The highly-controlled randomized feeding trial encouraged high adherence to the study protocol, with total adherence > 70%.[66] Although the Tower of London is a classic neuropsychological measure of planning and problem solving, variations in administration and scoring of the task can affect the cognitive processes that are tested and measured.[3,7–8,19,23,28,30,45] In this study, we made intentional choices in our problem selection, procedure design, and instructions to the participants in order to specifically capture differences in planning ability. Specifically, we (1) selected problems with difficulty profiles that were found to best differentiate planning ability between different age groups, based on results from a large

sample[58]; (2) the tester instructed the participant before beginning the task to think about which moves they wanted to make before making their moves, and; (3) the tester reminded the participant at the beginning of each problem to wait until they knew which moves they wanted to make before attempting to solve the problem. Lastly, we did not impose a time limit, which allowed participants as much time as they wanted to plan before attempting to solve the problem. However, there are a few limitations to this research as well. Our small sample size increases the risk of chance findings—however, all our outcome variables were determined *a priori*, and findings were robust to sensitivity analyses. The homogeneous makeup of our sample (mostly male, white, and with highly educated mothers) limits the generalizability of these results.

Conclusions

It is crucial to continue to investigate the relationship between maternal choline supplementation and offspring outcomes in light of the low choline intake of most pregnant women in the United States. Currently, ~90% of pregnant women do not consume the recommended amount of choline. Importantly, these findings were in a sample in which women received either the AI (480 mg/day choline) or double the AI (930 mg/day choline), with benefits of supplementation beyond the AI demonstrated across several tasks. This suggests that the AI itself, which was originally set based on preventing liver dysfunction in healthy adult men, may be insufficient for optimal fetal cognitive development. This supports the urgent need for larger dose-response randomized controlled trials to establish appropriate recommendations for choline intake during pregnancy. The implications of raising the recommended choline intake levels for pregnant women could be considerable with population-wide shifts towards improved memory, attention, and executive function, resulting in better health, socioemotional function, and economic success across the lifespan.

2.6 References

1. Ahmed, S. F., Kuhfeld, M., Watts, T. W., Davis-Kean, P. E., & Vandell, D. L. (2021). Preschool executive function and adult outcomes: A developmental cascade model. Developmental Psychology, 57(12), 2234–2249. https://doi.org/10.1037/dev0001270

2. Albright, C. D., Tsai, A. Y., Friedrich, C. B., Mar, M.-H., & Zeisel, S. H. (1999). Choline availability alters embryonic development of the hippocampus and septum in the rat. Developmental Brain Research, 113(1), 13–20. https://doi.org/10.1016/S0165-3806(98)00183-7

3. Anderson, P., Anderson, V., & Lajoie, G. (1996). The tower of London test: Validation and standardization for pediatric populations. The Clinical Neuropsychologist, 10(1), 54–65. https://doi.org/10.1080/13854049608406663

4. Ash, J. A., Velazquez, R., Kelley, C. M., Powers, B. E., Ginsberg, S. D., Mufson, E. J., & Strupp, B. J. (2014). Maternal choline supplementation improves spatial mapping and increases basal forebrain cholinergic neuron number and size in aged Ts65Dn mice. Neurobiology of Disease, 70, 32–42. https://doi.org/10.1016/j.nbd.2014.06.001

5. Bahnfleth, C., Canfield, R., Nevins, J., Caudill, M., & Strupp, B. (2019). Prenatal Choline Supplementation Improves Child Color-location Memory Task Performance at 7 Y of Age (FS05-01-19). Current Developments in Nutrition, 3(Supplement_1), nzz048.FS05-01-19. https://doi.org/10.1093/cdn/nzz048.FS05-01-19

6. Bahnfleth, C. L., Strupp, B. J., Caudill, M. A., & Canfield, R. L. (2022). Prenatal choline supplementation improves child sustained attention: A 7-year follow-up of a randomized controlled feeding trial. The FASEB Journal, 36(1), e22054. https://doi.org/10.1096/fj.202101217R

7. Berg, W. K., & Byrd, D. L. (2002). The Tower of London Spatial Problem-Solving Task: Enhancing Clinical and Research Implementation. Journal of Clinical and Experimental Neuropsychology, 24(5), 586–604. https://doi.org/10.1076/jcen.24.5.586.1006

8. Berg, W. K., Byrd, D. L., McNamara, J. P. H., & Case, K. (2010). Deconstructing the tower: Parameters and predictors of problem difficulty on the Tower of London task. Brain and Cognition, 72(3), 472–482. https://doi.org/10.1016/j.bandc.2010.01.002

9. Blair, C., & Razza, R. P. (2007). Relating Effortful Control, Executive Function, and False Belief Understanding to Emerging Math and Literacy Ability in Kindergarten. Child Development, 78(2), 647–663. https://doi.org/10.1111/j.1467-8624.2007.01019.x

10. Boeke, C. E., Gillman, M. W., Hughes, M. D., Rifas-Shiman, S. L., Villamor, E., & Oken, E. (2013). Choline Intake During Pregnancy and Child Cognition at Age 7 Years. American Journal of Epidemiology, 177(12), 1338–1347. https://doi.org/10.1093/aje/kws395

11. Camerota, M., & Willoughby, M. T. (2020). Prenatal Risk Predicts Preschooler Executive Function: A Cascade Model. Child Development, 91(3), e682–e700. https://doi.org/10.1111/cdev.13271

12. Caudill, M. A. (2010). Pre- and Postnatal Health: Evidence of Increased Choline Needs. Journal of the American Dietetic Association, 110(8), 1198–1206. https://doi.org/10.1016/j.jada.2010.05.009

13. Caudill, M. A., Strupp, B. J., Muscalu, L., Nevins, J. E. H., & Canfield, R. L. (2018). Maternal choline supplementation during the third trimester of pregnancy improves infant information processing speed: A randomized, double-blind, controlled feeding study. The FASEB Journal, 32(4), 2172–2180. https://doi.org/10.1096/fj.201700692RR

14. Cermak, J. M., Blusztajn, J. K., Meck, W. H., Williams, C. L., Fitzgerald, C. M., Rosene, D. L., & Loy, R. (1999). Prenatal Availability of Choline Alters the Development of Acetylcholinesterase in the Rat Hippocampus. Developmental Neuroscience, 21(2), 94–104. https://doi.org/10.1159/000017371

15. Cermak, J. M., Holler, T., Jackson, D. A., & Blusztajn, J. K. (1998). Prenatal availability of choline modifies development of the hippocampal cholinergic system. The FASEB Journal, 12(3), 349–357. https://doi.org/10.1096/fasebj.12.3.349

16. Cheatham, C. L., Goldman, B. D., Fischer, L. M., da Costa, K.-A., Reznick, J. S., & Zeisel, S. H. (2012). Phosphatidylcholine supplementation in pregnant women consuming moderate-choline diets does not enhance infant cognitive function: A randomized, double-blind, placebo-controlled trial. The American Journal of Clinical Nutrition, 96(6), 1465–1472. https://doi.org/10.3945/ajcn.112.037184

17. Cheng, R.-K., MacDonald, C. J., Williams, C. L., & Meck, W. H. (2008). Prenatal choline supplementation alters the timing, emotion, and memory performance (TEMP) of adult male

and female rats as indexed by differential reinforcement of low-rate schedule behavior. Learning & Memory, 15(3), 153–162. https://doi.org/10.1101/lm.729408

18. Craciunescu, C. N., Albright, C. D., Mar, M.-H., Song, J., & Zeisel, S. H. (2003). Choline Availability During Embryonic Development Alters Progenitor Cell Mitosis in Developing Mouse Hippocampus. The Journal of Nutrition, 133(11), 3614–3618. https://doi.org/10.1093/jn/133.11.3614

19. Culbertson, W., & Eric, Z. (1998). The Tower of London: A Standardized Approach to Assessing Executive Functioning in Children. Clinical Neuropsychology, 13(3), 285–301.

20. Diamond, A., & Lee, K. (2011). Interventions Shown to Aid Executive Function Development in Children 4 to 12 Years Old. Science, 333(6045), 959–964. https://doi.org/10.1126/science.1204529

21. Gathercole, S. E., Pickering, S. J., Knight, C., & Stegmann, Z. (2004). Working memory skills and educational attainment: Evidence from national curriculum assessments at 7 and 14 years of age. Applied Cognitive Psychology, 18(1), 1–16. https://doi.org/10.1002/acp.93

22. Howard, S. J., Vasseleu, E., Neilsen-Hewett, C., de Rosnay, M., Chan, A. Y. C., Johnstone, S., Mavilidi, M., Paas, F., & Melhuish, E. C. (2021). Executive Function and Self-Regulation: Bi-Directional Longitudinal Associations and Prediction of Early Academic Skills. Frontiers in Psychology, 12. https://www.frontiersin.org/article/10.3389/fpsyg.2021.733328

23. Hughes, C., & Graham, A. (2002). Measuring Executive Functions in Childhood: Problems and Solutions? Child and Adolescent Mental Health, 7(3), 131–142. https://doi.org/10.1111/1475-3588.00024

24. Jacobson, S. W., Carter, R. C., Molteno, C. D., Stanton, M. E., Herbert, J. S., Lindinger, N. M., Lewis, C. E., Dodge, N. C., Hoyme, H. E., Zeisel, S. H., Meintjes, E. M., Duggan, C. P., & Jacobson, J. L. (2018). Efficacy of Maternal Choline Supplementation During Pregnancy in Mitigating Adverse Effects of Prenatal Alcohol Exposure on Growth and Cognitive Function: A Randomized, Double-Blind, Placebo-Controlled Clinical Trial. Alcoholism: Clinical and Experimental Research, 42(7), 1327–1341. https://doi.org/10.1111/acer.13769

25. Jiang, X., Yan, J., West, A. A., Perry, C. A., Malysheva, O. V., Devapatla, S., Pressman, E., Vermeylen, F., & Caudill, M. A. (2012). Maternal choline intake alters the epigenetic state of

fetal cortisol-regulating genes in humans. The FASEB Journal, 26(8), 3563–3574. https://doi.org/10.1096/fj.12-207894

26. Jurado, María Beatriz, & Rosselli, M. (2007). The Elusive Nature of Executive Functions: A Review of our Current Understanding. Neuropsychology Review, 17, 213–233.

27. Kable, J. A., Coles, C. D., Keen, C. L., Uriu-Adams, J. Y., Jones, K. L., Yevtushok, L., Kulikovsky, Y., Zymak-Zakutnya, N., Dubchak, I., Akhmedzhanova, D., Wertelecki, W., & Chambers, C. D. (2022). The impact of micronutrient supplementation in alcohol-exposed pregnancies on reaction time responses of preschoolers in Ukraine. Alcohol, 99, 49–58. https://doi.org/10.1016/j.alcohol.2021.12.002

28. Kaller, C. P., Unterrainer, J. M., & Stahl, C. (2012). Assessing planning ability with the Tower of London task: Psychometric properties of a structurally balanced problem set. Psychological Assessment, 24(1), 46–53. https://doi.org/10.1037/a0025174

29. Karpova, N., Zhang, D., Beckwith, A. M., Bennett, D. S., & Lewis, M. (2021). Prenatal drug exposure and executive function in early adolescence. Neurotoxicology and Teratology, 88, 107036. https://doi.org/10.1016/j.ntt.2021.107036

30. Krikorian, R., Bartok, J., & Gay, N. (1994). Tower of London procedure: A standard method and developmental data. Journal of Clinical and Experimental Neuropsychology, 16(6), 840–850.

31. Langley, E. A., Krykbaeva, M., Blusztajn, J. K., & Mellott, T. J. (2015). High maternal choline consumption during pregnancy and nursing alleviates deficits in social interaction and improves anxiety-like behaviors in the BTBR T+Itpr3tf/J mouse model of autism. Behavioural Brain Research, 278, 210–220. https://doi.org/10.1016/j.bbr.2014.09.043

32. Lauder, J. M., & Schambra, U. B. (1999). Morphogenetic roles of acetylcholine. Environmental Health Perspectives, 107(suppl 1), 65–69. https://doi.org/10.1289/ehp.99107s165

33. Levin, H. S., Fletcher, J. M., Kufera, J. A., Harward, H., Lilly, M. A., Mendelsohn, D., Bruce, D., & Eisenberg, H. M. (1996). Dimensions of cognition measured by the tower of London and other cognitive tasks in head-injured children and adolescents. Developmental Neuropsychology, 12(1), 17–34. https://doi.org/10.1080/87565649609540638

34. Levitsky, D. A., & Strupp, B. J. (1995). Malnutrition and the Brain: Changing Concepts, Changing Concerns. The Journal of Nutrition, 125(suppl_8), 2212S-2220S. https://doi.org/10.1093/jn/125.suppl_8.2212S

35. Mathews, T. J. (2016). Mean Age of Mothers is on the Rise: United States, 2000–2014. 232, 8.

36. McCormack, T., & Atance, C. M. (2011). Planning in young children: A review and synthesis. Developmental Review, 31(1), 1–31. https://doi.org/10.1016/j.dr.2011.02.002

37. Meck, W., Williams, C., Cermak, J., & Blusztajn, J. (2008). Developmental periods of choline sensitivity provide an ontogenetic mechanism for regulating memory capacity and age-related dementia. Frontiers in Integrative Neuroscience, 2. https://www.frontiersin.org/article/10.3389/neuro.07.007.2007

38. Minnes, S., Min, M. O., Short, E. J., Wu, M., Lang, A., Yoon, S., & Singer, L. T. (2016). Executive function in children with prenatal cocaine exposure (12–15years). Neurotoxicology and Teratology, 57, 79–86. https://doi.org/10.1016/j.ntt.2016.07.002

39. Mohler, E. G., Meck, W. H., & Williams, C. L. (n.d.). Sustained Attention in Adult Mice is Modulated by Prenatal Choline Availability. 16.

40. Moon, J., Chen, M., Gandhy, S. U., Strawderman, M., Levitsky, D. A., Maclean, K. N., & Strupp, B. J. (2010). Perinatal choline supplementation improves cognitive functioning and emotion regulation in the Ts65Dn mouse model of Down syndrome. Behavioral Neuroscience, 124(3), 346–361. https://doi.org/10.1037/a0019590

41. Niculescu, M. D., Craciunescu, C. N., & Zeisel, S. H. (2006). Dietary choline deficiency alters global and gene-specific DNA methylation in the developing hippocampus of mouse fetal brains. The FASEB Journal, 20(1), 43–49. https://doi.org/10.1096/fj.05-4707com

42. Nitz, D. (2016). Tower of London Task (TOL)—Krikorian et al. (1994) Version (Version 5) [Inquisit]. Millisecond Software.

43. Noland, J. S., Singer, L. T., Arendt, R. E., Minnes, S., Short, E. J., & Bearer, C. F. (2003). Executive Functioning in Preschool-Age Children Prenatally Exposed to Alcohol, Cocaine,

and Marijuana. Alcoholism: Clinical and Experimental Research, 27(4), 647–656. https://doi.org/10.1111/j.1530-0277.2003.tb04401.x

44. Opening Statement by Roy Pitkin on Dietary Reference Intakes for Thiamin, Riboflavin, Niacin, Vitamin B6, Folate, Vitamin B12, Pantothenic Acid, Biotin, and Choline. (n.d.). Retrieved March 28, 2022, from http://www8.nationalacademies.org/onpinews/newsitem.aspx?RecordID=s6015

45. Poreh, A. M. (Ed.). (2006). New indices of planning abilities using the Tower of London task. In The Quantified Process Approach to Neuropsychological Assessment. Psychology Press.

46. Products—Data Briefs—Number 332—February 2019. (2019, June 10). http://www.cdc.gov/nchs/products/databriefs/db332.htm

47. Robinson, S., Goddard, L., Dritschel, B., Wisley, M., & Howlin, P. (2009). Executive functions in children with Autism Spectrum Disorders. Brain and Cognition, 71, 362–368. https://doi.org/10.1016/j.bandc.2009.06.007

48. Rose, S. A., Feldman, J. F., & Jankowski, J. J. (2012). Implications of Infant Cognition for Executive Functions at Age 11. Psychological Science, 23(11), 1345–1355. https://doi.org/10.1177/0956797612444902

49. Ross, R. G., Hunter, S. K., Hoffman, M. C., McCarthy, L., Chambers, B. M., Law, A. J., Leonard, S., Zerbe, G. O., & Freedman, R. (2016). Perinatal Phosphatidylcholine Supplementation and Early Childhood Behavior Problems: Evidence for CHRNA7 Moderation. The American Journal of Psychiatry, 173(5), 509–516. https://doi.org/10.1176/appi.ajp.2015.15091188

50. Ross, R. G., Hunter, S. K., McCarthy, L., Beuler, J., Hutchison, A. K., Wagner, B. D., Leonard, S., Stevens, K. E., & Freedman, R. (2013). Perinatal Choline Effects on Neonatal Pathophysiology Related to Later Schizophrenia Risk. American Journal of Psychiatry, 170(3), 290–298. https://doi.org/10.1176/appi.ajp.2012.12070940

51. Schulz, K. M., Pearson, J. N., Gasparrini, M. E., Brooks, K. F., Drake-Frazier, C., Zajkowski, M. E., Kreisler, A. D., Adams, C. E., Leonard, S., & Stevens, K. E. (2014). Dietary choline supplementation to dams during pregnancy and lactation mitigates the effects

of in utero stress exposure on adult anxiety-related behaviors. Behavioural Brain Research, 268, 104–110. https://doi.org/10.1016/j.bbr.2014.03.031

52. Shallice, T. (1982). Specific Impairments of Planning. The Neuropsychology of Cognitive Function, 298(1089), 199–209.

53. Signore, C., Ueland, P. M., Troendle, J., & Mills, J. L. (2008). Choline concentrations in human maternal and cord blood and intelligence at 5 y of age. The American Journal of Clinical Nutrition, 87(4), 896–902. https://doi.org/10.1093/ajcn/87.4.896

54. Strupp, B. J., Powers, B. E., Velazquez, R., Ash, J. A., Kelley, C. M., Alldred, M. J., Strawderman, M., Caudill, M. A., Mufson, E. J., & Ginsberg, S. D. (2016a). Maternal choline supplementation: A potential prenatal treatment for Down syndrome and Alzheimer's disease. Current Alzheimer Research, 13(1), 97–106.

55. Thomas, J. D., Abou, E. J., & Dominguez, H. D. (2009). Prenatal choline supplementation mitigates the adverse effects of prenatal alcohol exposure on development in rats. Neurotoxicology and Teratology, 31(5), 303–311. https://doi.org/10.1016/j.ntt.2009.07.002

56. Thomas, J. D., Idrus, N. M., Monk, B. R., & Dominguez, H. D. (2010). Prenatal choline supplementation mitigates behavioral alterations associated with prenatal alcohol exposure in rats. Birth Defects Research Part A: Clinical and Molecular Teratology, 88(10), 827–837. https://doi.org/10.1002/bdra.20713

57. Thomas, J. D., La Fiette, M. H., Quinn, V. R. E., & Riley, E. P. (2000). Neonatal choline supplementation ameliorates the effects of prenatal alcohol exposure on a discrimination learning task in rats. Neurotoxicology and Teratology, 22(5), 703–711. https://doi.org/10.1016/S0892-0362(00)00097-0

58. Unterrainer, J. M., Kaller, C. P., Loosli, S. V., Heinze, K., Ruh, N., Paschke-Müller, M., Rauh, R., Biscaldi, M., & Rahm, B. (2015). Looking ahead from age 6 to 13: A deeper insight into the development of planning ability. British Journal of Psychology, 106(1), 46–67. https://doi.org/10.1111/bjop.12065

59. Unterrainer, J. M., Rahm, B., Kaller, C. P., Leonhart, R., Quiske, K., Hoppe-Seyler, K., Meier, C., Müller, C., & Halsband, U. (2004). Planning Abilities and the Tower of London: Is This Task Measuring a Discrete Cognitive Function? Journal of Clinical and Experimental Neuropsychology, 26(6), 846–856. https://doi.org/10.1080/13803390490509574

60. Velazquez, R., Ash, J. A., Powers, B. E., Kelley, C. M., Strawderman, M., Luscher, Z. I., Ginsberg, S. D., Mufson, E. J., & Strupp, B. J. (2013). Maternal choline supplementation improves spatial learning and adult hippocampal neurogenesis in the Ts65Dn mouse model of Down syndrome. Neurobiology of Disease, 58, 92–101. https://doi.org/10.1016/j.nbd.2013.04.016

61. Villamor, E., Rifas-Shiman, S. L., Gillman, M. W., & Oken, E. (2012). Maternal Intake of Methyl-Donor Nutrients and Child Cognition at 3 Years of Age. Paediatric and Perinatal Epidemiology, 26(4), 328–335. https://doi.org/10.1111/j.1365-3016.2012.01264.x

62. Wallace, T. C., & Fulgoni, V. L. (2016). Assessment of Total Choline Intakes in the United States. Journal of the American College of Nutrition, 35(2), 108–112. https://doi.org/10.1080/07315724.2015.1080127

63. Wallace, T. C., & Fulgoni, V. L. (2017). Usual Choline Intakes Are Associated with Egg and Protein Food Consumption in the United States. Nutrients, 9(8), 839. https://doi.org/10.3390/nu9080839

64. Wong-Goodrich, S. J. E., Glenn, M. J., Mellott, T. J., Blusztajn, J. K., Meck, W. H., & Williams, C. L. (2008). Spatial memory and hippocampal plasticity are differentially sensitive to the availability of choline in adulthood as a function of choline supply in utero. Brain Research, 1237, 153–166. https://doi.org/10.1016/j.brainres.2008.08.074

65. Wu, B. T. F., Dyer, R. A., King, D. J., Richardson, K. J., & Innis, S. M. (2012). Early Second Trimester Maternal Plasma Choline and Betaine Are Related to Measures of Early Cognitive Development in Term Infants. PLOS ONE, 7(8), e43448. https://doi.org/10.1371/journal.pone.0043448

66. Yan, J., Jiang, X., West, A. A., Perry, C. A., Malysheva, O. V., Devapatla, S., Pressman, E., Vermeylen, F., Stabler, S. P., Allen, R. H., & Caudill, M. A. (2012). Maternal choline intake modulates maternal and fetal biomarkers of choline metabolism in humans. The American Journal of Clinical Nutrition, 95(5), 1060–1071. https://doi.org/10.3945/ajcn.111.022772

67. Zeisel, S. H. (2004). Nutritional Importance of Choline for Brain Development. Journal of the American College of Nutrition, 23(sup6), 621S-626S. https://doi.org/10.1080/07315724.2004.10719433

68. Zeisel, S. H. (2006). Choline: Critical Role During Fetal Development and Dietary Requirements in Adults. Annual Review of Nutrition, 26(1), 229–250. https://doi.org/10.1146/annurev.nutr.26.061505.111156

69. Zeisel, S. H. (2009). Epigenetic mechanisms for nutrition determinants of later health outcomes. The American Journal of Clinical Nutrition, 89(5), 1488S-1493S. https://doi.org/10.3945/ajcn.2009.27113B

70. Zeisel, S. H., & da Costa, K.-A. (2009). Choline: An essential nutrient for public health. Nutrition Reviews, 67(11), 615–623. https://doi.org/10.1111/j.1753-4887.2009.00246.x

71. Zeisel, S. H., Mar, M. H., Zhou, Z., & da Costa, K. A. (1995). Pregnancy and lactation are associated with diminished concentrations of choline and its metabolites in rat liver. The Journal of Nutrition, 125(12), 3049–3054. https://doi.org/10.1093/jn/125.12.3049

72. Zeisel, S. H., & Niculescu, M. D. (2006). Perinatal Choline Influences Brain Structure and Function. Nutrition Reviews, 64(4), 197–203. https://doi.org/10.1111/j.1753-4887.2006.tb00202

CHAPTER THREE

EFFECTS OF MATERNAL CHOLINE SUPPLEMENTATION ON PARENT-REPORT MEASURES OF INFANT TEMPERAMENT

3.1 Abstract

Objective: To determine whether the children of mothers who received 550 mg/day choline (v 25 mg/day) from 12–16 weeks' gestation until delivery were rated by their parents as more attentive and easier to soothe on a parent-report measure of temperament throughout the first year of life. **Methods:** Pregnant women (N = 33) were randomized at gestation week 16 to receive either 25 mg/day or 550 mg/day choline until delivery. They returned with their infants (N = 26) at four times throughout the first postnatal year of life to complete a battery of cognitive and behavioral tests. We examined infant temperament using the Infant and Early Childhood Behavior Questionnaires. Outcomes included mean scores on the Orienting/Regulating, Effortful Control, and Negative Affectivity factors, as well as mean scores on the Duration of Orienting and Falling Reactivity scales. Statistical analyses included general and mixed linear models. **Results:** Orienting/Regulating, Effortful Control, Negative Affectivity, and Falling Reactivity scores did not differ by choline treatment group. Children of mothers who consumed 25 mg/d (v 550) were more likely to be rated as higher on the Duration of Orienting scale score on the Infant Behavior Questionnaire ($p = 0.038$). **Conclusions:** With the exception of one Infant Behavior Questionnaire scale, there was no effect of maternal choline supplementation on parent report measures of infant temperament across the first year of life.

3.2 Introduction

The physiological need for choline, an essential nutrient with many roles in fetal development, is increased during pregnancy, where it provides constituents for the development of cell membranes, neurotransmitters, and epigenetic modifications.[70–72,74] During prenatal development, choline and its metabolites play key roles in brain development and long-term offspring cognition via several potential mechanisms, including effects on cellular proliferation, migration, and apoptosis, neurogenesis, and synaptic plasticity.[1,2,16–17,21,65,68,74] Choline-derived phospholipids, including sphingomyelin and phosphatidylcholine, are key components of cell membranes and help to maintain the structural and functional integrity of cells.[71–72] Choline also acts as a required precursor to acetylcholine (ACh), the primary neurotransmitter of the parasympathetic nervous system and a prominent neuromodulator in the central nervous system.[33,71–72] Further, choline is the major dietary source of methyl groups, and, through its conversion to the metabolites betaine and s-adenosylmethionine (SAM), provides methyl groups needed for DNA methylation.[71–72] These metabolites of choline play key roles in epigenetic modification of genes and histones, which can exert long-term effects on brain and behavior via gene expression.[45,71–72]

Consistent with choline's many important roles, a robust body of rodent work has demonstrated the importance of maternal choline intake for the developmental programming of offspring cognition and behavior. Rodent data demonstrate that maternal choline deficiency results in irreversible cognitive deficits in the offspring, and that conversely, when compared to standard rodent chow (which is designed to contain adequate choline), maternal choline supplementation (MCS) improves offspring memory and attention throughout the lifespan.[36–42] Further, prenatal maternal choline supplementation has been shown to lessen age-related

cognitive decline and reduce cognitive dysfunction in rodent models of several neurological disorders, including Down syndrome, autism, and Alzheimer's disease.[2,32,43,65] Finally, prenatal choline supplementation has been shown to lessen the dysfunction produced by a variety of prenatal insults, including maternal stress, infection, and inflammation and exposure to alcohol.[27,61–63]

Several reports have also documented effects of maternal choline supplementation on socioemotional function in rodent models, although this body of research is more limited. One study of prenatal stress found evidence that prenatal choline supplementation reduced offspring anxiety-like behaviors and social behavior problems in the offspring of stressed dams, although the effects varied somewhat by the sex of the offspring.[57] Female offspring born to stressed but choline-supplemented dams exhibited less anxiety in the open-field task and elevated zero maze tasks compared to females born to stressed dams on a control diet. In contrast, male offspring born to stressed but choline-supplemented dams had normalized social behavior when confronted with a novel conspecific, increasing the amount of time that the mouse spent investigating a new social partner.[57]

Deficits in socioemotional function may also manifest as frustration, a negative affective response to goal blockage.[3] Studies have found that infants who are more easily frustrated may be more constrained in the development of self-regulatory behaviors.[3,12] Another rodent study found that prenatal choline supplementation reduced burst responding in response to task errors, suggesting a reduction in the amount of frustration expressed by the supplemented animals.[19] Studies have also found evidence that prenatal choline supplementation normalizes offspring socioemotional regulation in rodent disease models of Down syndrome and autism, adversities characterized by aberrant emotional reactivity.[32,43]

Although these rodent data provide strong evidence that choline intake during pregnancy is critical for offspring brain development and cognitive functioning, as well as preliminary evidence for beneficial effects of MCS on socioemotional functioning, relatively little is known about choline needs during pregnancy in humans, nor the functional consequences for the child if maternal intake is insufficient.[14] In 1998, the IOM for the first time identified an Adequate Intake (AI) for choline at 425 mg/day for adult women, with a slight increase to 450 mg/day for pregnant women.[46] However, this recommendation was based on the amount of choline needed to prevent liver dysfunction in men, not the more relevant outcome of child neurodevelopment.[14,46] Therefore, it is likely that the AI is insufficient for the demands of pregnancy. This is concerning in light of the fact that ~90% of pregnant women do not consume the AI, and most prenatal vitamins contain little or no choline (~55 mg).[66–67]

Few human studies have been conducted to assess the association between variations in maternal choline intake during pregnancy and offspring outcomes. Two observational studies found correlations between serum and/or dietary measures of maternal choline intake and offspring performance on tests of infant development and child memory[9,69], but two others found no association. However, observational studies do not allow for causal inferences due to risk of confounding with uncontrolled covariates. Further, none of these observational studies examined the association between variations in maternal choline intake during pregnancy and offspring socioemotional outcomes.

Three randomized control trials (RCT) of maternal choline supplementation in typically developing infants have been conducted. Of the three studies, two found benefits on measures of offspring cognition, including attentional orienting speed during infancy, and working memory and sustained attention at seven years of age.[4–5,15] The third trial detected no offspring cognitive

benefits, based on assessments of memory and cognitive ability in infants.[18]

Only one RCT examined the effect of maternal choline supplementation on offspring socioemotional function using the Child Behavior Checklist, a clinical parent-report measure used to detect behavioral and emotional problems in children.[52] This study found that MCS decreased social withdrawal, as measured by scores on the Social Problems subscale, in the children at three years of age. Children of women supplemented with choline were also rated as having fewer problems with anxiety, internalizing/externalizing behaviors, and fewer overall behavioral and emotional problems, although differences in these scores did not reach statistical significance in this sample of 49 children.[52]

One of the three RCTs described above is of particular relevance to the present report, which focuses on the effects of maternal choline supplementation on infant temperament. This highly controlled feeding trial randomized third trimester pregnant women to receive either 480 or 930 mg/day choline until delivery.[15] Follow-up of the infants of supplemented mothers found that infants of mothers in the 930 mg/day group had faster information processing speeds across the first year of life.[15] When followed up at 7 years of age, the children of mothers in the 930 mg/day group showed superior performance on tasks of sustained attention, working memory, and executive function (Chapter 2, this dissertation).[4–5] This study provides compelling support for the translation of the cognitive benefits of MCS seen in rodent models to humans. Together with the rodent data, this preliminary evidence offers a strong rationale for also investigating the translation of the socioemotional benefits of MCS in humans.

To do so, we took advantage of our ancillary cognitive-behavioral follow-up to a double-blind, randomized-controlled clinical trial. In this RCT, pregnant women were randomized to consume either supplemental choline (25 or 550 mg choline/day as choline chloride) from

gestation week 16 until delivery. The mothers were then re-recruited to return to the laboratory with their infants at four times throughout the first year of life to participate in assessments of offspring cognitive and socioemotional functioning. At each study visit, we examined the effects of MCS on infant temperament using the Infant and Early Childhood Behavior Questionnaires.

The Infant and Early Childhood Behavior Questionnaires (IB/ECBQ) are parent-report surveys that measure specific dimensions of temperament.[54–55,60] The conceptualization underlying these questionnaires defines temperament as the behavioral expression of enduring individual differences in reactivity and self-regulation, present from birth and stemming from the dynamic interactions between genetic, biological, and environmental factors across development.[22,49,54] Despite the changes in behavioral manifestations of temperament over time, the IB/ECBQ measure enduring traits of temperament, with studies reporting high rank-order stability of individual differences over time.[49]

The present study tested the hypothesis that infants of mothers who received a 550 mg/day choline supplement (v 25 mg/day control) plus usual diet during pregnancy will improve infant soothability and negative affect, as assessed by a parent-report measure of infant temperament at four time points across the first year of life.

3.3 Subjects and Methods

Ethical Approval

Ethical approval for the present study was obtained from the Institutional Review Board for Human Participants at Cornell University in Ithaca, NY. Written parental consent was obtained from all study participants.

Study Design and Participants

Supplementation Trial

The present study leveraged a clinical trial (NCT03194659) in which pregnant women (gestation week 12–16) were randomized to consume either supplemental choline (550 mg choline/day, as choline chloride) or control (25 mg choline/day), along with a once-daily prenatal vitamin/mineral supplement (Nature Made Prenatal Tablet; Pharmavite LLC; CA, USA) and a 200 mg/day DHA supplement, from enrollment until delivery (Nature's Way EfaGold Neuromins 200 mg DHA (plant source); DSM Nutritional Products; Netherlands). The 550 mg/day supplement was designed to achieve an average intake of ~900 mg/day of choline (~350 mg/day from diet + 550 mg supplemental choline = ~900 mg/day of choline). In our previous controlled choline feeding study, this amount of choline consumed throughout the third trimester was found to improve offspring information processing speed during infancy, and memory, attention, and executive function at 7 years of age.[4,5,15] An average daily intake of ~900 mg/day of choline is well below the IOM established upper tolerance limit of 3,500 mg/day.[14] The control supplement contained 25 mg/day choline, provided as deuterium-labeled choline, to investigate hypotheses related to the metabolism of choline (not presented here). Therefore, the 25 mg/day group does not represent a true placebo. However, total choline intake of the control group (350 mg/day from diet + 25 mg/day supplemental choline = ~375 mg/day of choline) approximates the average prenatal choline intake of most pregnant women. This small amount of supplemental choline, although similar to amounts found in a few prenatal vitamins, represents a trivial increase in choline intake over the average (7%), whereas the 550 mg/day choline supplement represents a substantial (~157%) increase over the average choline intake.

The sample size in this study was powered in relation to primary outcomes related to biomarkers of maternal/fetal choline and DHA metabolism.[31,58] Secondary outcomes included offspring cognition and affect regulation during infancy, genomic expression, and metabolomic

profiling of plasma and placental tissue. The current study is an ancillary follow-up of offspring from the initial pregnancy study at 5-, 7- 10- and 13-months postnatal age to assess effects on infant cognition and affect regulation, using pre-specified endpoints.

Details of the supplementation trial have been published elsewhere.[31,58] Briefly, second trimester pregnant women (12–16 weeks' gestation, N = 33) were recruited from the largest obstetrics practice in the Ithaca, NY area. Eligibility to participate included maternal age 21–40 healthy, singleton pregnancy, and willingness to comply with the study protocol. Exclusion criteria included a prepregnancy BMI of ≥ 32 kg/m^2 or current use of tobacco, alcohol, or drugs. Women with high habitual intakes of choline or omega-3 fatty acids, as assessed by self-report food frequency questionnaires at screening, were also ineligible to participate. Women who had pregnancy complications or comorbidities such as preeclampsia or gestational diabetes, either at enrollment or developed during the study, were ineligible to participate.

Supplementation began at the time of enrollment and continued until delivery. Supplements were administered as choline chloride dissolved in grape juice and provided to study participants in 13 mL test tubes, one for each daily dose. Supplements were provided at three visits to the laboratory at gestation weeks 12–16, 20–24, and 28–32. To monitor adherence, participants were instructed at each visit to return any unconsumed supplements at the following study visit. At these visits, women also provided blood, urine, and fecal samples, and completed a health questionnaire and 24-hour dietary recall. At delivery, women were asked to provide a placental tissue and cord blood sample.

Follow-Up Neurobehavioral Trial

When the infants were 4–6 weeks old, mothers were invited to bring their infants back to Cornell University to participate in the postnatal study on neurobehavioral functioning. Infants

(N = 26, Figure 3.2) and their mothers were recruited between April 2018 and October of 2019, and neurobehavioral testing occurred between April 2018 and November of 2020. Testing occurred at four ages postnatally at 5, 7, 10, and 13 months of age. Characteristics of the participants and their mothers were obtained via parent report at each follow-up visit and included infant age, sex, race, and ethnicity, recent illnesses, vision or motor problems, frequency and type of breast/bottle feeding and solid foods, and sleep habits of the infant. Maternal characteristics, including race, ethnicity, education, and age, were evaluated to assess possible bias arising from loss to follow-up.

Parent Report Measures of Infant Temperament

At each study visit, the attendant parent was asked to fill out either the Infant Behavior Questionnaire-Revised, short form (IBQ-R) or the Early Childhood Behavior Questionnaire (ECBQ), depending on the age of the child.

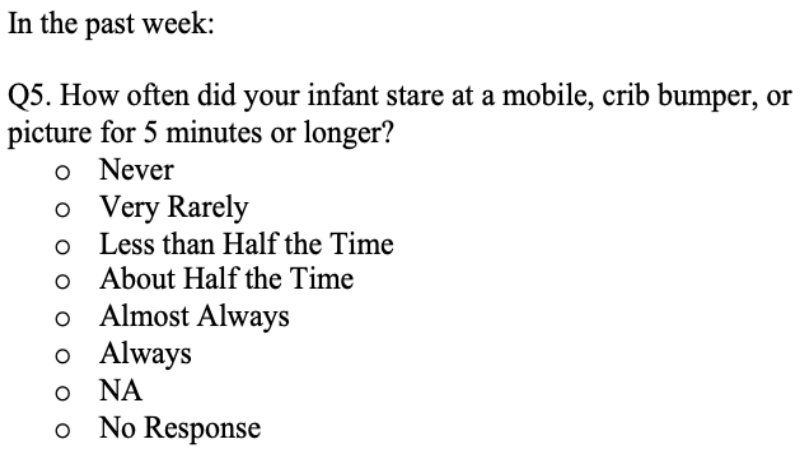
Infant Behavior Questionnaire

The Infant Behavior Questionnaire, developed in 1981, is one of the most widely used parent-report measures of infant temperament.[22,54] The instrument was revised (IBQ-R) in 1988 to refine and add several scales, and then again in 2008 to validate a short form (91 questions).[49,51] The Infant Behavior Questionnaire is designed to assess 14 specific dimensions of infant temperament, including attentional orienting, soothability, and rate of recovery from distress. Scores on these scales are used to calculate three superordinate factors—Surgency/Extraversion, Negative Affectivity, and Regulation/Orienting (Table 3.1). This factor structure is broadly consistent with dimensions of temperament reported in older children and has been used to predict child outcomes from infant temperament.[26] These factors are also broadly consistent with dimensions of personality in adults.[44,54–55] The inter-rater and internal

reliability, convergent and discriminant validity, and relative stability of scores have been demonstrated for the Infant Behavior Questionnaire with infants as young as 2 weeks of age.[26]

The Infant Behavior Questionnaire is designed to minimize several challenges associated with parent report measures of infant temperament, including recall bias and social desirability.[54] To reduce recall bias, the IBQ asks questions about concrete behaviors and situations that the parent is likely to have observed in the last one or two weeks, which aids in reducing recall bias. By asking about specific behaviors, rather than asking the parent to make abstract or comparative judgements, the questionnaire reduces bias due to perceptions of social desirability.[26]

For both instruments, the parent was asked to rate on a Likert scale the frequency with which their child engaged in specific, day-to-day behaviors in the last one to two weeks, and to answer specific questions about how frequently their baby responded in a particular manner (Figure 3.1). Scores ranged from Never (1) to Always (7). Parents could also select do not wish to respond) or NA (does not apply) for behaviors that they did not wish to report, or that their child did not engage in. The NA option is particularly important for behaviors that may not be observable in younger infants due to developmental constraints.[47]


In the past week:

Q5. How often did your infant stare at a mobile, crib bumper, or picture for 5 minutes or longer?

- Never
- Very Rarely
- Less than Half the Time
- About Half the Time
- Almost Always
- Always
- NA
- No Response

Figure 3.1: Example question from the Infant Behavior Questionnaire. The full questionnaire can be found in Appendix E.

Scale Label	Definition
Duration of Orienting	The baby's attention to and/or interaction with a single object for extended periods of time
Soothability	Baby's reduction of fussing, crying, or distress when the caregiver uses soothing techniques
Falling Reactivity/Rate of Recovery from Distress	Rate of recovery from peak distress, excitement, or general arousal; ease of falling asleep
Activity Level	Movement of arms and legs, squirming and locomotor activity
Distress to Limitations	Baby's fussing, crying, or showing distress while a) in a confining place or position; b) involved in caretaking activities; c) unable to perform a desired action
Approach	Rapid approach, excitement, and positive anticipation of pleasurable activities
Fear	The baby's startle or distress to sudden changes in stimulation, novel physical objects, or social stimuli; inhibited approach to novelty
Smiling and Laughter	Smiling or laughter from the child in general caretaking and play situations
Vocal Reactivity	Amount of vocalization exhibited by the baby in daily activities
Sadness	General low mood: lowered mood and activity specifically related to personal suffering, physical state, object loss, or inability to perform a desired action
Perceptual Sensitivity	Amount of detection of slight, low intensity stimuli from the external environment
High Intensity Pleasure	Amount of pleasure or enjoyment related to high stimulus intensity, rate, complexity, novelty, and incongruity
Low Intensity Pleasure	Amount of pleasure or enjoyment related to situations involving low stimulus intensity, rate, complexity, novelty, and incongruity
Cuddliness	The baby's expression of enjoyment and molding of the body to being held by a caregiver
Factor Label	**Definition**
Regulation/Orienting	Defined by scale scores of Low Intensity Pleasure, Cuddliness, Duration of Orienting, and Soothability
Surgency/Extraversion	Defined by scale scores of Approach, Vocal Reactivity, High Intensity Pleasure, Smiling and Laughter, Activity Level, and Perceptual Sensitivity
Negative Affectivity	Defined by scale scores of Sadness, Distress to Limitations, Fear, and Falling Reactivity/Rate of Recovery from Distress (loaded negatively)

Table 3.1: Scale and factor definitions for the Infant Behavior Questionnaire (IBQ).

Early Childhood Behavior Questionnaire (ECBQ)

The ECBQ was developed as an age-appropriate extension of the IBQ; it contains 107 questions appropriate for infants older than 12 months and was administered at the 13-month assessment.[50] The ECBQ measures 18 dimensions of toddler temperament and 3 broad factors similar to the IBQ factors (Surgency/Extraversion, Negative Affectivity, and Effortful Control, Table 3.2).

Scale Label	**Definition**
Attentional Focusing	Sustained duration of orienting on an object of attention; resisting distraction
Attentional Shifting	The ability to transfer attentional focus from one activity/task to another
Soothability	Rate of recovery from peak distress, excitement, or general arousal
Factor Label	**Definition**
Surgency/Extraversion	Defined by scale scores of Impulsivity, Activity Level, High Intensity Pleasure, Sociability, Positive Anticipation
Negative Affectivity	Defined by scale scores of Discomfort, Fear, Sadness, Frustration, Soothability (loaded negatively), Motor Activation, Shyness, and Perceptual Sensitivity
Effortful Control	Defined by scale scores of Inhibitory Control, Attentional Shifting, Low Intensity Pleasure, Cuddliness, and Attentional Focusing

Table 3.2: Early Childhood Behavior Questionnaire (ECBQ) scale and factor definitions for outcomes measured in this chapter. Other scale and factor definitions can be found in Appendix F.

The surveys were administered electronically via Qualtrics (Qualtrics, Provo, UT), and completed online within one week of each in-person laboratory visit. Item scores were summed according to IBQ and ECBQ guidelines to calculate the individual scale scores and the three factor scores for each measure.[54,60]

Statistical Analyses

Maternal and child characteristics for the participants included in the final analytical

sample were compared by treatment group using Student's t tests for continuous variables and Fisher's exact tests for categorical variables. The same approach was used to compare participants included in the final analysis to the 5 children who did not provide cognitive and behavioral endpoint data (lost to follow-up, N = 5. Figure 3.2).

The primary outcome for the IBQ in this study is the mean score on the Orienting/Regulation factor. The Orienting/Regulation factor estimates infants' ability to regulate negative affect in response to environmental stress (the individual scales and items that load onto the Orienting/Regulation factor can be found in Appendix G).[24,54] The selection of this endpoint as the primary outcome is based on the small body of rodent and human data that has examined the effects of maternal choline intake on offspring socioemotional outcomes.[32,42,52,53] These studies suggest that increased MCI results in reduced negative reactivity in the offspring in response to environmental stress, as well as improved attentional orienting.

Secondary outcomes included the mean score on the Duration of Orienting and Falling Reactivity/Rate of Recovery from Distress scales. The Duration of Orienting scale estimates infants' ability to maintain attention to or interaction with a single object for an extended period of time, and the Falling Reactivity/Rate of Recovery from Distress Scale estimates infants' ability to recover from strong feelings of distress, excitement, or general arousal (The individual items that load onto these scales can be found in Appendix G).[22,60] Secondary hypotheses regarding the mean factor score on the Negative Affectivity factor are also reported here.

The primary outcome for the ECBQ is the mean score on the Effortful Control factor, which represents an upward extension of the Orienting/Regulation factor from the IBQ. Secondary outcomes included the mean scores on the Attentional Focusing and Attentional Shifting scales, which represent an upward extension of the Attentional Orienting scale, and the

Soothability scale, which represents an upward extension of the Falling Reactivity/Rate of Recovery from Distress. Secondary hypotheses regarding the mean score on the Negative Affectivity factor are also reported here.

Recognizing the limitations of estimating multiple statistical models in a small sample with multiple endpoints, our analysis plan (completed prior to unblinding) prespecified one basic unadjusted mixed model for estimating the effects of treatment group on IBQ and ECBQ endpoints.

For the IBQ outcomes in this study, there are three repeated measures of temperament for each child, corresponding to the three assessment ages within the age range of the IBQ instrument (5, 7, and 10 months). The unadjusted mixed models for each IBQ factor score included as fixed classification effects child age, treatment group, and child identifier. Random effects were included for the intercept and for the individual child. Child sex was not included in the *a priori* model because the existence of gender differences for the IBQ has not been established.[22,49,54] In addition, the sex of the fetus was not known at the time of recruitment, and we were not able to stratify by sex to create groups with equal number of infants of each sex. This, in addition to the large number of female infants in our small sample, precluded analysis that controlled for infant sex.

Unadjusted models were also used to explore each of the 14 scales used to compute the three factor scores of the IBQ (see Table 3.1). We had *a priori* reasons to hypothesize a choline effect on two individual IBQ scales (Duration of Orienting and Falling Reactivity/Rate of Recovery from Distress), but models were estimated for all the other scales as exploratory analyses. These scale-specific unadjusted models included fixed effects for treatment group, age, and individual, and random effects for the intercept and of the individual child.

Because some studies have found an association between maternal serum choline levels during the 16th gestational week of pregnancy and scores on the IBQ[44–45], we conducted a post-hoc exploratory analysis adjusting for maternal serum choline levels measured at baseline (12–16 weeks' gestation), prior to when supplementation began. The mixed model adjusting for baseline serum choline levels included fixed effects for treatment group, age, and individual, and random effects for the intercept and of the individual child.

The same general statistical approach was applied to analysis of the three broad factors for the ECBQ: Surgency/Extraversion, Negative Affectivity, and Effortful Control (analogous to the Orienting/Regulation factor from the IBQ but measuring more mature self-regulation skills).[50] Each of the eighteen scales that comprise these three broad factors (see Table 3.2) were also explored using the same model. However, because the Early Childhood Behavior Questionnaire (ECBQ) was administered on only one occasion (13 months), treatment effects on the three factor scores and individual scale scores were estimated in general linear models that included only a fixed classification effect for treatment group and child identifier.

Sensitivity Analyses

Sensitivity analyses were conducted to evaluate the robustness of the results of the primary analyses. Because power to detect differences in demographic characteristics is low in this small sample, we chose to explore the possible existence of effects of even slight imbalances on our primary analyses. We examined the effects of infant sex, rater identity (mother or father), and maternal sensitivity, all characteristics that have been shown to associate with scores on the IBQ.[22,49,54] Maternal sensitivity was calculated as mean score on the Pediatric Review and Observation of Children's Environmental Support and Stimulation (PROCESS), a clinical assessment of maternal sensitivity completed by experimenters at each laboratory visit.[13]

Additionally, because the stay-at-home orders implemented at the beginning of the COVID-19 pandemic in March 2020 may have impacted parents' ability to observe some of the behaviors included on the IBQ and ECBQ (i.e., "*During the past two weeks when familiar relatives/friends came to visit, how often did your baby get excited?*), we also included the timing of the questionnaire as a binary covariate (0 = completed before COVID-19 lockdown began, 1 = completed after the COVID-19 lockdown began) in sensitivity analyses. To assess the possible influence of these variables, we entered each one as an individual covariate into the *a priori* models and estimated the change in treatment effect.

SAS 9.4 Software (SAS Institute, Cary, NC, USA) was used to conduct statistical analyses, including linear and logistic general and mixed-model methods. All tests were 2-tailed and statistical significance was set at $P < 0.05$ for main effects, and $P < 0.10$ for interactions.

3.4 Results

Subject Characteristics

Of the 33 women who completed the supplementation trial, 30 were eligible to return with their infants for follow-up testing. Three women (1 from the 25 mg/day group and 2 from the 550 mg/day group) developed gestational diabetes during the supplementation trial and were subsequently excluded from both the pregnancy and follow-up studies. 27 women were successfully recruited for their children to participate in the infant cognitive and behavioral assessments, but one infant was lost to follow-up after the 5-month visit, so cognitive and behavioral data was not available for this infant. (See Figure 3.2). There were no statistically significant group differences between the children included in the final analytical sample (N = 25) and those who were not (N = 5) on child sex, maternal race, or maternal age at conception (see Appendix H). Women included in the final analytical sample were significantly more likely

to have at least a master's degree (p = 0.02) than those who were not, and more women included in the final analytical sample identified as non-Hispanic than those who were not (p = 0.02).

One child was initially re-recruited, but only returned for one of the four follow-up visits, and the parent did not produce valid data for study questionnaires. Prior to unblinding the investigators to treatment group identity, the decision was made to exclude the data from this child from all analyses of cognitive and behavioral endpoints.

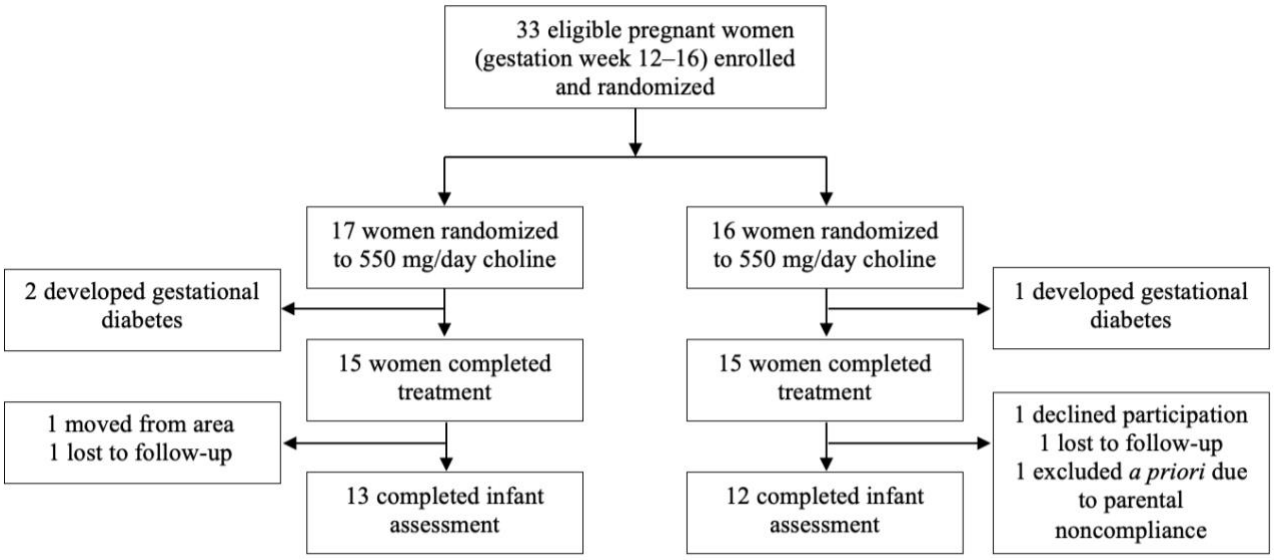


Figure 3.2: Participant flow diagram. Study screening, intervention, and infant and follow up assessments.

There were no statistically significant differences in sample characteristics by choline group (see Table 3.3). The infant sample was 72% female. At the time of follow-up, the mothers were on average 32.4 years old and 72% had an advanced degree, making them older and more highly educated than the U.S. average.[36,48] 88% of the mothers self-identified as white and 96% self-identified as non-Hispanic ethnicity.

	Maternal Choline Intake Group		
	25 mg/day (N = 12)	**550 mg/day (N = 13)**	**p**
Maternal Characteristics			
Mean age, years (range)	31.9 (27–39)	32.8 (24–38)	0.60
Mean BMI, kg/m^2 (range)	24.7 (18.5–31.8)	22.5 (18.5–26.3)	0.08
Education (%)			0.23
High School	1 (8.3)	1 (7.7)	
Bachelor's Degree	3 (25)	2 (15.4)	
Master's Degree	8 (66.7)	6 (46.1)	
Doctorate/Professional	0 (0)	4 (30.8)	
Race (%)			0.34
White	12 (100)	10 (76.9)	
Black	0 (0)	1 (7.7)	
Asian	0 (0)	2 (15.4)	
Ethnicity (%)			1.0
Non-Hispanic	12 (100)	12 (92.3)	
Other	0 (0)	1 (7.7)	
Pregnancy and Delivery			
Mean gestation length, days (range)	278.3 (263–287)	281 (253–299)	0.45
Pregnancy/labor complications (%)	5 (41.7)	4 (30.8)	0.69
Delivery method, vaginal (%)	9 (75)	12 (92.3)	0.32
Infant Characteristics			
Sex, female (%)	10 (83.3)	8 (61.5)	0.38
Mean birth length, inches (range)	19.4 (18–21)	19.4 (16–21)	0.90
Mean birth weight, grams (range)	3383.3 (2932–4194)	3325.2 (2550–3995)	0.73

Table 3.3: Sample demographic characteristics by maternal choline intake group.

Infant and Early Childhood Behavior Questionnaire Results

Overall Performance

		1	2	3	4	5	6	7	8	9	10	11	12
5 Mo	1. Orienting/Regulation	1											
	2. Negative Affectivity	-0.29	1										
	3. Surgency	0.51**	0.16	1									
7 Mo	4. Orienting/Regulation	**0.70*** **	-0.15	0.24	1								
	5. Negative Affectivity	-0.32	**0.60***	-0.12	-0.34	1							
	6. Surgency	0.54**	-0.06	**0.56***	0.39	-0.09	1						
10 Mo	7. Orienting/Regulation	**0.62****	-0.17	0.58**	**0.57***	-0.27	0.42*	1					
	8. Negative Affectivity	-0.15	**0.31**	-0.11	-0.15	**0.36**	-0.03	-0.21	1				
	9. Surgency	0.41*	-0.12	**0.62****	0.24	-0.30	**0.74*** **	0.48*	0.002	1			
13 Mo	10. Effortful Control	**0.44***	0.02	0.18	**0.55***	-0.22	0.23	**0.35**	0.18	0.06	1		
	11. Negative Affectivity	-0.03	**0.55***	0.04	0.16	**0.45***	0.27	-0.11	**0.47***	0.03	0.42	1	
	12. Surgency	0.06	-0.19	**0.22**	-0.12	0.03	**0.13**	0.16	-0.21	**0.25**	-0.26	-0.15	1

Table 3.4 Correlations between the IBQ-R and ECBQ factors from 5 months to 13 months of life.
***p < 0.05, **p < 0.01, ***p < 0.001**

	5 Months mean (range)		7 Months mean (range)		10 Months mean (range)		
	Maternal Choline Intake Group						
Scale Label	**25 mg/d (N = 12)**	**550 mg/d (N = 13)**	**25 mg/d (N = 12)**	**550 mg/d (N = 13)**	**25 mg/d (N = 12)**	**550 mg/d (N = 13)**	**p**
Activity Level	4.54 (2.86–5.71)	4.37 (3.29–5.29)	4.61 (3.14–6.0)	4.26 (1.71–5.86)	4.95 (4.0–6.43)	4.32 (2.33–5.86)	0.20
Distress to Limitations	3.94 (2.14–5.57)	4.02 (2.5–5.86)	3.82 (2.83–5.14)	4.29 (3.14–5.43)	4.38 (1.86–5.86)	4.19 (2.29–5.43)	0.70
Approach	5.30 (3.5–6.33)	5.10 (2.67–7.0)	5.74 (3.67–6.67)	5.29 (2.0–7.0)	6.10 (5.33–6.83)	5.85 (3.17–7.0)	0.36
Fear	2.61 (1.8–4.0)	2.57 (1.17–5.0)	3.15 (1.67–4.83)	3.19 (1.0–5.25)	3.27 (1.83–5.17)	3.85 (1.83–5.8)	0.43
Duration of Orienting	4.72 (3.0–5.67)	3.65 (2.20–4.83)	4.73 (3.67–6.17)	4.23 (1.5–6.33)	4.47 (3.33–6.0)	4.01 (2.17 – 5.83)	0.03*
Smiling and Laughter	4.40 (2.5–6.14)	5.06 (2.67–7.0)	4.7 (3.17–5.71)	5.15 (3.0–6.86)	4.96 (4.14–5.86)	4.92 (3.71–6.57)	0.30
Vocal Reactivity	5.10 (3.57–6.33)	4.99 (3.71–6.43)	5.22 (3.57–6.33)	5.33 (4.17–6.86)	5.61 (4.67–6.86)	5.32 (3.57–7.0)	0.73
Sadness	3.32 (1.27–4.83)	3.49 (1.83–6.60)	3.70 (2.5–5.0)	3.72 (2.5–5.5)	3.81 (2.0–5.0)	3.54 (2.0–4.67)	0.93
Perceptual Sensitivity	4.5 (3.5–6.5)	4.13 (1.0–7.0)	4.56 (1.67–6.33)	4.52 (2.4–6.33)	4.52 (1.67–6.33)	5.04 (3.33–6.75)	0.94

High Intensity Pleasure	5.64 (4.40–6.86)	6.79 (4.43–6.86)	5.73 (4.29–6.5)	6.11 (4.43–7.0)	6.16 (5.14–7.0)	5.93 (4.57–7.0)	0.67
Low Intensity Pleasure	5.54 (3.71–6.67)	5.57 (3.80–6.86)	5.64 (3.5–6.80)	5.79 (4.67–6.57)	5.1 (4.14–6.33)	5.59 (3.83–6.43)	0.36
Cuddliness	6.09 (4.67–7.0)	5.82 (4.50–7.0)	5.81 (4.5–7.0)	5.22 (3.0–6.83)	5.29 (4.0–6.5)	5.51 (4.33–6.67)	0.41
Soothability	5.85 (4.43–6.86)	5.68 (4.29–7.0)	6.1 (4.0–6.86)	5.36 (3.71–6.29)	5.75 (3.57–6.71)	5.51 (3.86–7.00)	0.29
Falling Reactivity/Rate of Recovery from Distress	5.42 (3.67–6.67)	5.33 (3.33–6.17)	5.62 (5.0–6.4)	4.87 (2.67–6.17)	5.10 (3.0–7.0)	5.13 (3.5–6.5)	0.36
Factor Label	**25 mg/d**	**550 mg/d**	**25 mg/d**	**550 mg/d**	**25 mg/d**	**550 mg/d**	**p**
Surgency/Extraversion	4.91 (3.78–5.88)	4.91 (3.53–6.31)	5.10 (3.37–6.15)	5.11 (3.92–6.37)	5.38 (4.56–6.11)	5.23 (3.84–6.33)	0.84
Negative Affectivity	3.11 (2.0–4.27)	3.19 (1.88–4.95)	3.26 (2.17–3.99)	3.58 (2.76–4.88)	3.59 (1.71–4.88)	3.61 (2.19–4.86)	0.55
Regulation/Orienting	5.55 (4.43–6.43)	5.18 (4.04–6.10)	5.57 (4.13–6.21)	5.15 (3.73–6.21)	5.15 (4.21–5.85)	5.16 (4.05–5.69)	0.19

Table 3.5: Mean ratings for the five-, seven-, and ten-month visits on IBQ scale and factor scores by choline group. *p < 0.05

Scale Label	Maternal Choline Intake Group		p
	25 mg/d (mean, range)	**550 mg/d (mean, range)**	
Attentional Focusing	4.35 (3.33–5.5)	4.38 (3.33–5.2)	0.90
Attentional Shifting	4.99 (3.71–6.25)	4.42 (2.67–6.13)	0.11
Soothability	5.43 (4.2–6.4)	5.3 (4.2–6.0)	0.63
Activity Level/Energy	5.36 (4.5–6.25)	5.29 (3.88–6.71)	0.89
Cuddliness	5.28 (4.5–6.5)	4.93 (3.17–6.33)	0.38
Discomfort	2.27 (1.0–3.6)	2.11 (1.0–3.14)	0.53
Fear	3.03 (1.43–5.25)	2.62 (1.0–3.5)	0.19
Frustration	3.67 (2.0–5.17)	3.57 (1.0–5.5)	0.86
High Intensity Pleasure	5.21 (4.17–6.5)	4.85 (3.25–6.2)	0.32
Impulsivity	4.75 (2.0–6.0)	4.27 (1.0–5.75)	0.37
Inhibitory Control	3.13 (2.0–4.5)	2.91 (1.0–5.0)	0.62
Low Intensity Pleasure	4.82 (4.0–5.67)	4.31 (2.33–5.5)	0.12
Motor Activation	3.28 (2.17–4.6)	3.0 (1.67–4.5)	0.46
Perceptual Sensitivity	4.66 (3.2–6.4)	4.58 (1.8–7.0)	0.86
Positive Anticipation	4.66 (2.6–7.0)	3.84 (1.0–6.33)	0.19
Sadness	3.08 (2.4–4.0)	3.41 (2.0–4.8)	0.19
Shyness	3.62 (2.0–7.0)	4.23 (2.8–5.6)	0.22
Sociability	5.63 (4.25–6.67)	5.51 (3.0–7.0)	0.82
Factor Label	**25 mg/day (mean, range)**	**550 mg/d (mean, range)**	**p-value**
Effortful Control	5.14 (4.59–5.72)	4.78 (3.86–5.83)	0.19
Negative Affectivity	3.27 (2.42–3.77)	3.28 (1.85–4.03)	0.94
Surgency/Extraversion	4.52 (3.88–5.25)	4.19 (3.06–4.94)	0.19

Table 3.6: Mean ratings for the thirteen month visit on ECBQ scale and factor scores by choline group.

Infant Behavior Questionnaire

The 550 mg/day group did not differ from the 25 mg/day group on any of the three broad factor scores [Surgency ($p = 0.84$), Negative Affectivity ($p = 0.55$), Orienting/Regulation ($p = 0.19$), Figure 3.3]. Nor did the groups differ on the Falling Reactivity/Rate of Recovery from Distress scale score ($p = 0.36$).

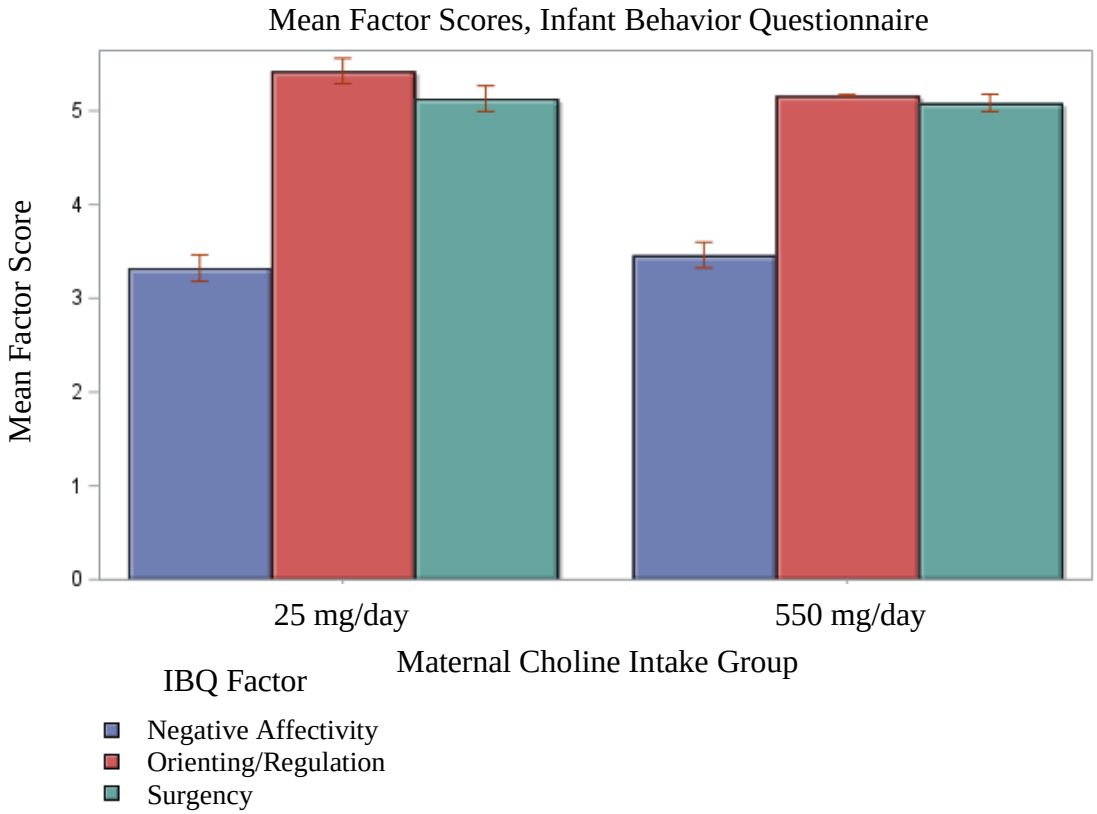


Figure 3.3: Mean factor scores for infants in both maternal choline supplementation groups across all three timepoints (5, 7, and 10 months) at which the IBQ was administered. Bars represent standard error.

However, examination of the least square means for the Orienting/Regulation factor in the repeated measures model showed that infants in the 25 mg/day group scored non-significantly higher on this factor at five and seven months (Figure 3.4).

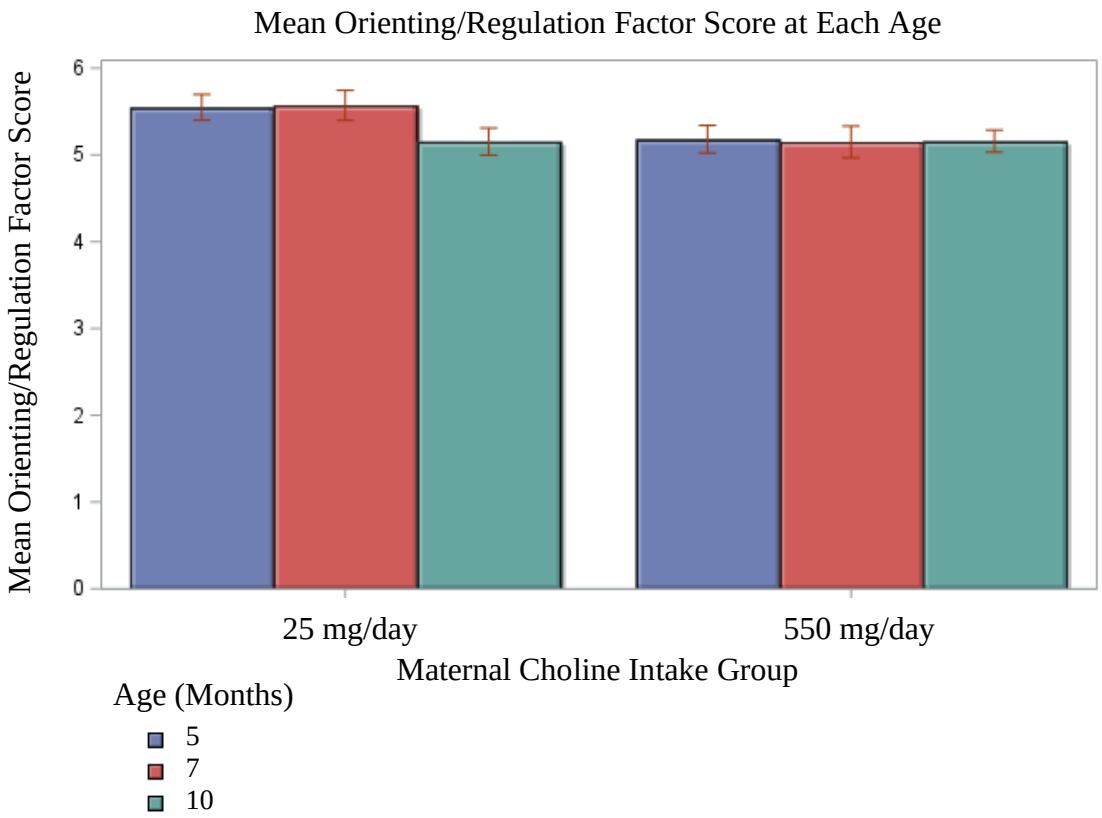


Figure 3.4: Mean Orienting/Regulation Factor scores for infants in both maternal choline supplementation groups for each time point at which the IBQ was administered. Bars represent standard error.

Analysis of the scales that loaded onto Orienting/Regulation showed that this trend was driven primarily by a significant main effect of choline on the Duration of Orienting scale ($p = 0.038$), with infants in the 25 mg/day group scoring non-significantly higher (v 550) on this scale at all three time points at which the IBQ was collected (Figure 3.5). The two groups did not differ in their scores on any of the other scales at any age (all $p > 0.2$).

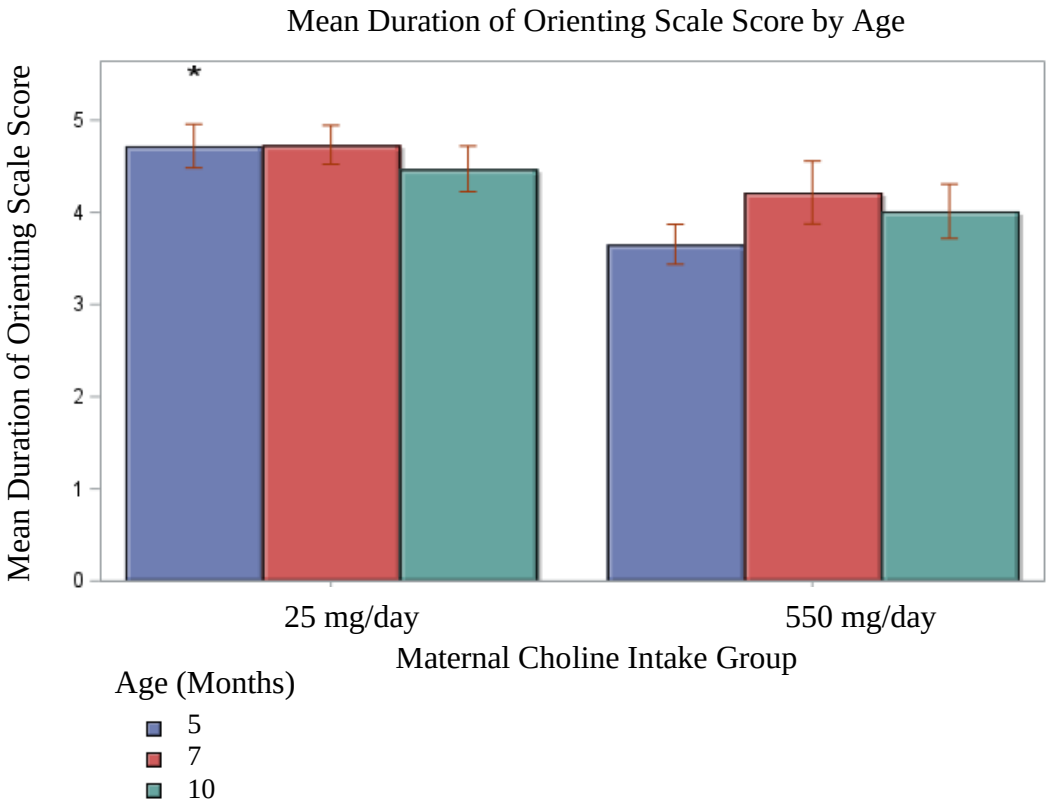


Figure 3.5: Mean Duration of Orienting scale scores for infants in both maternal choline supplementation groups for each time point at which the IBQ was administered (5, 7, and 10 months). Bars represent standard error. *p < 0.05

Maternal Serum Choline

The average maternal serum choline level at baseline was 6.45 μM (range 2.97–15.5) in this cohort (Appendix I). Mean serum choline levels did not differ by choline group, even with the inclusion of one extremely high baseline value (15.5 μM) in the 25 mg/day group.

Analyses adjusting for maternal serum choline levels at baseline did not reveal a significant difference between the groups on infant scores for any of the IBQ factors, overall or at any age [Surgency (p = 0.88), Negative Affectivity (p = 0.85), Orienting/Regulation (p = 0.35)]. Nor did including baseline serum choline in analyses noticeably change any of the estimated effects. There were no group differences on adjusted analyses for individual scales of interest either [Duration of Orienting (p = 0.05), Falling Reactivity/Rate of Recovery from Distress (p = 0.56)].

There was a significant main effect of baseline maternal serum choline level on the Orienting/Regulation factor score, such that higher serum choline levels were correlated with a higher score on this factor ($p = 0.02$). However, further analysis revealed that this effect was driven by the outlier noted above (one woman with a baseline serum choline level of 15.5 μM), and analyses excluding this dyad did not reveal a significant main effect of baseline maternal serum choline level ($p = 0.151$).

Early Childhood Behavior Questionnaire

As for the IBQ, the groups did not differ significantly on any of the three broad factor scores [Negative Affectivity ($p = 0.94$), Surgency ($p = 0.19$) or Effortful Control (0.19), Figure 3.6].

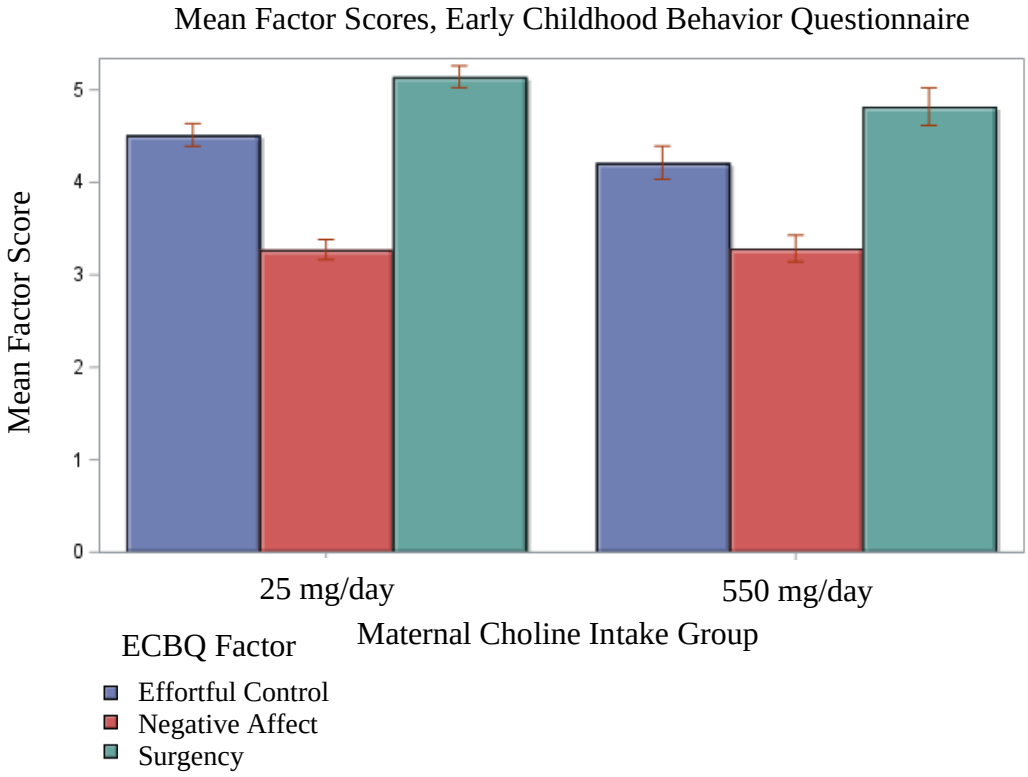


Figure 3.6: Mean ECBQ factor scores for infants in both maternal choline supplementation groups at thirteen months of age. Bars represent standard error.

At the individual scale level, we investigated the Attentional Focusing scale and the Attentional Shifting scale, which represent upward extensions of the IBQ Duration of Orienting scale. We also examined the ECBQ Soothability scale, which represents an upward extension of the Falling Reactivity/Rate of Recovery from Distress scale. The groups did not differ on the Attentional Focusing scale ($p = 0.90$) or the Soothability scale ($p = 0.63$), but on the Attentional Shifting scale we observed non-significant better attentional shifting by the infants in the 25 mg/day group ($p = 0.11$, Figure 3.7). However, further investigation revealed that this trend was driven primarily by an outlier value in the 550 mg/day group. Analysis of the scale excluding that outlier revealed no trend in group scores on the Attentional Shifting scale (25 mg/day group mean 4.99 v 550 mg/day group mean 4.59, $p = 0.21$).

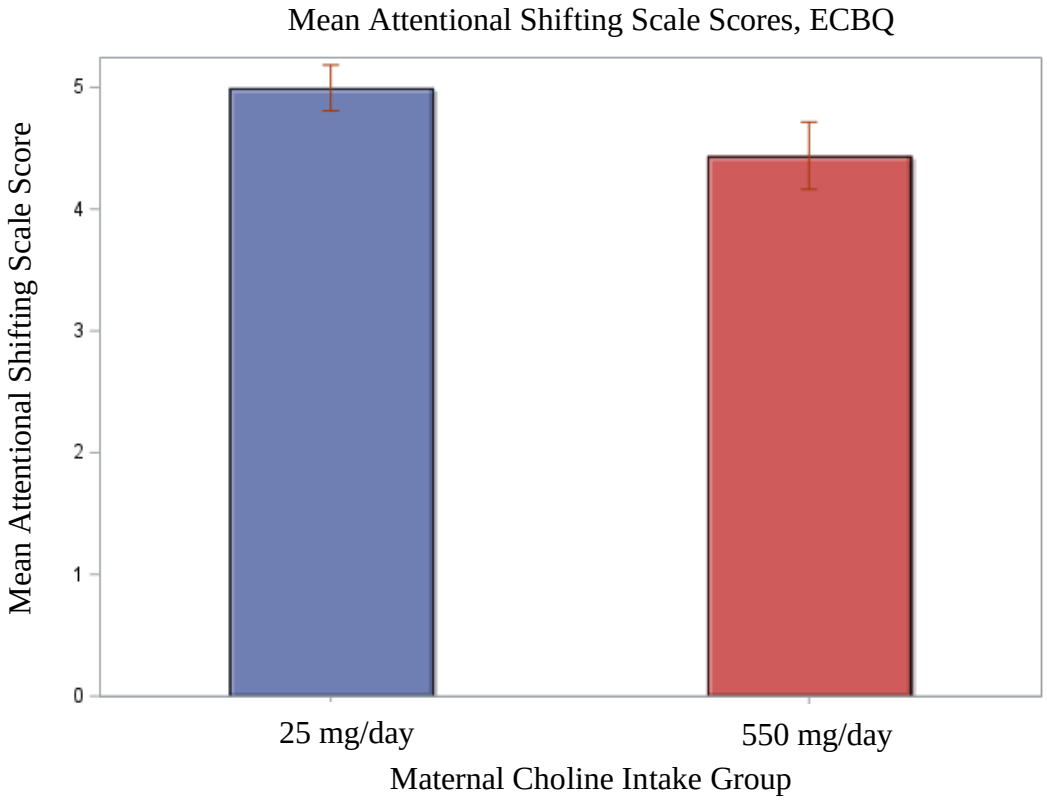


Figure 3.7: Mean scores on the Attentional Shifting scale of the ECBQ for infants in both maternal choline supplementation groups at thirteen months of age. Bars represent standard error.

We also investigated the other ECBQ scales (Fear, Low Intensity Pleasure, Positive Anticipation, and Sadness) with $p < 0.20$ to determine if there were any trends in the effect of maternal choline supplementation on these scale scores. For example, the 25 mg/day group scored slightly higher than the 550 mg/day group on the Fear scale (mean 3.03 v mean 2.62, $p = 0.19$). Post-hoc investigation found that this trend was driven by an outlier value. One infant in the 25 mg/day group was scored at a 5.25 on the fear scale, which was 1.5 points higher than the next highest score in that group (3.75) and 1.75 points higher than the highest score in the 550 mg/day group (3.5). Analysis of the scale excluding that outlier revealed no trend in group scores on the Fear scale (25 mg/day group mean 2.83 v 550 mg/day group mean 2.59, $p = 0.39$). Investigation of the other three scales with $p < 0.20$ found that these trends were also driven by outlier values. No other noteworthy group differences were observed at the scale level (all $p > 0.20$).

Maternal Serum Choline

Analyses adjusting for maternal serum choline levels at baseline did not reveal a significant difference between the groups on infant scores for any of the ECBQ factors [Surgency ($p = 0.25$), Negative Affectivity ($p = 0.94$), Effortful Control ($p = 0.31$)]. Nor did including baseline serum choline in analyses noticeably change any of the estimated effects. There were no group differences on adjusted analyses of individual scales of interest [Attentional Focusing ($p = 0.74$), Attentional Shifting ($p = 0.15$), Soothability ($p = 0.890$]. There was no significant main effect of baseline maternal serum choline levels on any of the ECBQ factors or scales.

Sensitivity Analyses

Sensitivity analyses were conducted to evaluate the robustness of the results from the primary analyses. Infant sex, rater identity (mother or father), maternal sensitivity, and timing of questionnaire completion (before or after the beginning of the COVID-19 lockdown in March 2020) were each entered, in turn, as a single added covariates added to the *a priori* models. The table showing changes in the estimates of the effect can be found in Appendix I. In brief, for the IBQ, none of the covariates changed the size of the effect such that it resulted in a significant effect of choline on any of the outcome measures. None of the covariates altered the statistical significance of the choline effect on the Duration of Orienting score on the IBQ. The mean change in the size of the estimate of the choline effect was 11.25%. For the ECBQ, none of the covariates changed the size of the effect such that it resulted in a significant effect of choline on any of the outcome measures. The mean change in the size of the estimate of the choline effect was 0.045%.

3.5 Discussion

Summary of Results

The findings of this study did not reveal an effect of maternal supplementation with 550 mg/day of choline (v 25 mg/day) on parent report measures of infant temperament throughout the first year of life.

The groups did not vary in their scores on any of the three overarching factors of the Infant Behavior Questionnaire. Children in the 25 mg/day group scored on average 0.40 points higher (v 550) on the Orienting/Regulation scales at 5 and 7 months, but this trend was nonsignificant ($p = 0.19$). Examination of the individual scales that load onto the Orienting/Regulation factor revealed that this trend was driven primarily by the Duration of

Orienting scale scores, where children in the 25 mg/day group were ranked 0.68 points higher on average than the children in the 550 mg/day group ($p = 0.038$).

On the ECBQ, as for the IBQ, the groups did not differ significantly on any of the three broad factor scores [Negative Affectivity ($p = 0.94$), Surgency ($p = 0.19$) or Effortful Control (0.19)]. The groups did not differ in their scores on any of the individual scales examined, although infants in the 25 mg/day group scored an average of 0.57 points higher ($p = 0.11$) on the Attentional Shifting scale, which measures the ability to transfer attentional focus from one activity/task to another and represents an upward extension of the Duration of Orienting scale.

There are several potential interpretations of our null results in this small ancillary follow-up study, both methodological and conceptual. The first, and most straightforward, is that there is no effect of maternal choline supplementation on infant temperament. However, based on several lines of evidence (discussed below), this is not the most parsimonious conclusion to derive from our results.

Methodological Interpretations

One possible threat to the validity of concluding that there is no effect of MCS on infant temperament and affect regulation is one or more methodological problems. To assess possible errors in implementing and administering the survey, we examined the overall patterns of our data. Given that the theory of infant temperament that underlies these questionnaires is that it is comprised of enduring characteristics, we would expect to see high longitudinal correlations in scores on the questionnaire scales and factors across time.[33,54] Table 3.4 provides some insight into the longitudinal stability of scores in this sample. We found that there were significant intercorrelations for each of the three IB/ECBQ factors across each of the four ages (r range 0.44–0.74, p's < 0.05). These correlations are in line with, and for some factors, higher than

other studies that have examined the longitudinal intercorrelations for these factors.[22] This provides some validation for these dimensions of temperament being relatively stable and enduring characteristics of the infants in our sample and leads to the conclusion that our failure to find an effect of the intervention was not due to technical or methodological error in administering these questionnaires. The high longitudinal correlations in our data demonstrate that we have successfully used the Infant and Early Childhood Behavior Questionnaires to assess our infant temperament outcomes in a way that fits with the theory underlying the measure.

The second methodological question that must be addressed is whether we failed to detect an effect of the intervention due to our small sample size. Because this is an ancillary follow-up study to a supplementation trial, which was powered to detect effects of the maternal choline intervention on metabolic outcomes[31,58], we were not able to conduct *a priori* power calculations in order to determine the sample size we would need to detect an effect of maternal choline supplementation on parent-report temperament measures. Although our sample size is smaller than many other studies that use the IBQ and ECBQ to measure infant temperament[26,44,47], it is large enough to detect the effects of maternal choline supplementation on other direct behavioral assessments of infant information processing speed.[11] Not only did we not find any statistically significant differences between our two groups (with the exception of the duration of orienting scale at 5 months of age), but the patterns of group means across age show no discernable patterns or trends that would indicate that there is an effect of maternal choline supplementation that would be revealed in a larger sample size (Table 3.5, Table 3.6). Therefore, sample size is not the primary challenge to our ability to detect effects of maternal choline supplementation.

Theoretical Interpretations

Given the statistical challenges discussed here, accuracy is not possible in the interpretation of these data. We should not conclude that there is no effect of maternal choline supplementation on infant temperament or affect regulation, but rather that our data are not conclusive. It may be better to consider the risks and benefits associated with different interpretations of the infant temperament and affect regulation data.

We did find one significant group difference, with children in the 25 mg/day group ranked on average 0.68 points higher on the Duration of Orienting Scale of the IBQ ($p = 0.038$). An initial interpretation of this result may be that infants in the 25 mg/day group have superior attentional control to those in the 550 mg/day group, suggesting that there is no benefit of supplementation (and potentially, even a detrimental effect). However, the Duration of Orienting scale may not measure attentional control, per se.

As noted above in Subjects and Methods, the Duration of Orienting scale estimates infants' ability to maintain attention to or interaction with a single object for an extended period. Studies have found that infants' sustained attention to single objects steadily declines over the first year of life.[24,34] A more flexible orienting reaction, which results in faster disengagement from stimuli and faster attention shifting, allows for the infant to engage more flexibly with environmental stimuli and may support the early development of executive function and self-regulation.[8,26]

This interpretation is supported by other findings from this study. At all four visits to the laboratory, the infants completed the Visual Expectation Paradigm (VeXP), a visual attention task. Infant information processing speed was determined by mean reaction time. Analysis revealed that infants in the 550 mg/day group had significantly faster reaction times than those in the 25 mg/day group.[11] These findings demonstrate that maternal choline supplementation

significantly improves infant information processing speed across the first year of life. Therefore, it is reasonable to hypothesize that infants in the 550 mg/day group received lower scores on the Duration of Orienting scale because they are able to process information about their environment more quickly and shift their attention to other stimuli.

This hypothesis would lead us to predict that infants in the 550 mg/day group would score higher on the Attentional Shifting scale on the ECBQ; however, we found a nonsignificant trend in the opposite direction, with infants in the 25 mg/day group scoring an average of 0.57 points higher than those in the 550 mg/day group ($p = 0.11$). However, further analysis showed that this trend was driven by an outlier value in the 550 mg/day group, and analysis of the scale excluding this outlier revealed no group differences on this scale (25 mg/day group mean 4.99 v 550 mg/day group mean 4.59, $p = 0.21$). This lack of group differences may be due to one or more of the challenges discussed below, including the possibility that parent-report questionnaires designed to capture broader differences in infant temperament may be insensitive to the specific effects of maternal choline supplementation on offspring attentional outcomes.

Further, the conclusion that there is no effect of MCS on infant temperament does not support the current, albeit limited, literature on the effects of maternal choline supplementation on offspring socioemotional function, which hints at a possible effect of MCS on emotion regulation. In a rodent model of typically developing offspring, MCS has been found to reduce burst responding, an expression of frustration, in response to task errors, suggesting a reduction in the amount of negative affect expressed by the supplemented animals.[43] Another rodent study examining the effect of MCS in the presence of adverse pregnancy exposures found evidence that supplementation reduced offspring trait anxiety and social behavior problems in the offspring of stressed dams.[57] Other studies have also found evidence that prenatal choline

supplementation normalizes offspring socioemotional regulation in rodent disease models characterized by aberrant emotional reactivity, including Down syndrome and autism.[32,43]

The majority of these studies examined the effects of maternal choline supplementation in populations that either experienced adverse exposures during pregnancy, or atypical populations of neurological diseases such as Down syndrome and autism.[32,43,52–53] Therefore, it is possible that maternal choline supplementation may only have an effect on offspring socioemotional function in populations for which it acts as a neuroprotective factor against adverse exposures during development. In this case, we would expect a reduction or normalization of aberrant socioemotional function, but not necessarily improvements in temperament beyond the normal range.

This interpretation is strengthened by human studies of maternal choline supplementation and effects on infant temperament. One study found that higher serum choline levels (>7 μM) measured during the 16th gestational week was neuroprotective against inflammation due to maternal infection, such that offspring of mothers who had an infection and higher serum choline levels had higher scores on the IBQ Regulation/Orienting Factor at age 1 than offspring of mothers who had an infection and serum choline levels < 7 μM.[24] In this population, higher maternal serum choline levels normalized the IBQ Regulation/Orienting Factor scores of offspring exposed to maternal inflammation at 16 weeks to approximately the same as the scores of offspring who were not exposed to maternal inflammation.[24]

However, the hypothesis that maternal choline supplementation has a beneficial effect only in populations where it exerts a neuroprotective effect against an adverse prenatal exposure would be in contrast to the current rodent and human data on the effects of maternal choline supplementation on offspring memory and attention. This body of literature has demonstrated

that MCS results in superior memory and attention in *both* atypical and normative populations—not just populations in which choline may be exerting a neuroprotective effect against adverse exposures.[4,5,11,15,3–40] Further, as noted above, maternal choline supplementation has been found to reduce expressions of frustration in typically-developing rodent models. Therefore, further research is needed to determine whether MCS may have a beneficial effect on socioemotional function only in atypical populations, or in both normative and atypical populations, as well as how best to assess these effects.

There are also some challenges presented by using parent-report measures for assessing infant temperament. Although the Infant and Early Childhood Behavior Questionnaires are specifically designed to reduce some of the biases inherent in parent-report measures[22,49,54] (recall bias, social desirability), the accuracy of these measures is still reliant on the parent's ability to recall instances of their child's specific behaviors in the last week to two weeks, and to accurately estimate the number of times they have seen those behaviors. Accuracy on these parent report measures relies not only on the parent's memory, but also the parent's access to the child and ability to view those behaviors.[56,61] In our sample, 23 of the 26 infants were cared for by at least one adult who was not a parent or primary caregiver for at least one hour a week. Therefore, the parent completing the survey is restricted to answering based only on the times in the past week when they were able to observe the infant, which may not reflect the entire range of the infant's behaviors in different contexts.

Given the challenges inherent in parent report measures, as well as the broad dimensions of temperament measured by the IBQ and ECBQ, it is also possible that maternal choline supplementation affects aspects of infant temperament, but that these more general parent-report measures are not as sensitive in revealing these effects as are direct behavioral measures. This is

because, while parent-report measures have the added variance of individual parents, who may potentially interpret and respond to questions different, direct behavioral measures in the laboratory setting control for variance by having a single experimenter taking measurements for all participants. Support for this hypothesis comes from a previous ancillary follow-up study of a controlled choline feeding trial conducted in our laboratory.[15] In that study, the children of mothers who were randomized to consume either 480 or 930 mg/day of choline during the third trimester of pregnancy were followed up for cognitive assessment at seven years of age. Analysis of one of the tasks, the Tower of London, a direct behavioral measure of child executive function, found that children of the mothers who consumed 930 mg/day (v 480) had superior problem solving and planning skills (Chapter 2, this dissertation). However, a parent report measure of child executive function, the Behavior Rating Inventory of Executive Function (BRIEF), administered concurrently to the parents of these children, revealed no group differences in executive functioning (unpublished data). Therefore, it is possible that especially in this small sample, the IBQ and ECBQ are not sensitive to the potentially subtle changes in infant temperament due to maternal choline supplementation, and that a direct behavioral measure of temperament may better assess these possible links (Chapter 4, this dissertation).

Lastly, it is possible that we found null results on the IB/ECBQ because these surveys measure temperament in a context where we would not expect the effects of maternal choline supplementation to be apparent. The IB/ECBQ are designed to assess everyday infant temperament and are not specific to measuring infant reactivity and regulation in response to stress. In other human studies of maternal choline supplementation, the effect of MCS is not apparent until the system that is being measured is presented with a challenge. For example, in our previous ancillary follow-up study discussed above, the children of women who consumed

930 (v 480) mg/day choline demonstrated superior performance on the Sustained Attention Task (SAT)—however, this improved performance was only significant on the shortest, most difficult trials.[4] Therefore, we may hypothesize that an effect of maternal choline supplementation on infant temperament (reduced reactivity or increased regulation) may only become apparent when the infant's emotional reactivity and regulation systems are challenged, such as in the behavioral assessment of the Still Face Paradigm, which elicits infant negative affect in response to a maternal violation of social expectations (Chapter 4, this dissertation).

Strengths and Limitations

There are several strengths of the present study. Firstly, our strong study design—a randomized controlled double-blind clinical trial—allows for strong causal inferences. Secondly, adherence to supplement intake was high among both groups in the pregnancy portion of the trial, which allows us to test our hypothesis with high confidence. Thirdly, the study design of providing a supplement in addition to usual diet makes the results of the study generalizable to the real-world scenario in which a pregnant woman may choose to or be prescribed to take a choline supplement as part of her prenatal regimen. We also used a standardized, validated, and widely used measure of infant temperament, which has been shown to have high convergent validity and longitudinal stability.[22,26,49–50,54] Although we did not find any effects of the choline intervention on these measures, this study still offers significant insight into the effect of choline on infant outcomes measured via parent-report questionnaires, and we have a robust longitudinal dataset that characterizes these infants at four times across the first year of life.

However, there are a few limitations to this research as well. Our small sample size, which was powered for metabolic outcomes not reported here, increases the risk of chance findings; however, all of our outcome variables were determined *a priori*, and findings were

robust to sensitivity analyses. The homogeneous makeup of our sample (mostly female, white, and with highly educated mothers) limits the generalizability of these results.

Conclusions and Future Directions

This study offers insight into how to conduct future research on the effects of maternal choline supplementation on infant temperament. Although parent-report questionnaires are an important measure of individual differences in infant temperament, and should continue to be included in study designs in order to collect longitudinal data on these enduring traits, laboratory measures of infant temperament in which infants must respond to a challenge presented to their affect regulation system (Still-Face Paradigm, Anger and Frustration LabTAB tasks) may be the most sensitive measures of changes in infant temperament due to maternal choline supplementation.

It is crucial to continue to investigate the relationship between maternal choline supplementation and offspring outcomes in light of the low choline intake of most pregnant women in the United States. Currently, ~90% of pregnant women do not consume the recommended amount of choline. Importantly, there is an urgent need for larger dose-response randomized controlled trials to establish appropriate recommendations for choline intake during pregnancy. Further research is needed to understand the effect of maternal supplementation on infant temperament. If MCS does indeed result in better offspring affective reactivity and regulation, then implications of raising the recommended choline intake levels for pregnant women could be considerable with population-wide shifts towards improved cognitive and emotional function, resulting in better health and economic success across the lifespan.

3.6 References

1. Albright, C. D., Tsai, A. Y., Friedrich, C. B., Mar, M.-H., & Zeisel, S. H. (1999). Choline availability alters embryonic development of the hippocampus and septum in the rat. *Developmental Brain Research*, *113*(1), 13–20. https://doi.org/10.1016/S0165-3806(98)00183-7

2. Ash, J. A., Velazquez, R., Kelley, C. M., Powers, B. E., Ginsberg, S. D., Mufson, E. J., & Strupp, B. J. (2014). Maternal choline supplementation improves spatial mapping and increases basal forebrain cholinergic neuron number and size in aged Ts65Dn mice. *Neurobiology of Disease*, *70*, 32–42. https://doi.org/10.1016/j.nbd.2014.06.001

3. Aksan, N., Goldsmith, H. H., Smider, N. A., Essex, M. J., Clark, R., Hyde, J. S., Klein, M. H., & Vandell, D. L. (1999). Derivation and prediction of temperamental types among preschoolers. *Developmental Psychology*, *35*(4), 958–971. https://doi.org/10.1037/0012-1649.35.4.958

4. Bahnfleth, C., Canfield, R., Nevins, J., Caudill, M., & Strupp, B. (2019). Prenatal Choline Supplementation Improves Child Color-location Memory Task Performance at 7 Y of Age (FS05-01-19). *Current Developments in Nutrition*, *3*(Supplement_1), nzz048.FS05-01-19. https://doi.org/10.1093/cdn/nzz048.FS05-01-19

5. Bahnfleth, C. L., Strupp, B. J., Caudill, M. A., & Canfield, R. L. (2022). Prenatal choline supplementation improves child sustained attention: A 7-year follow-up of a randomized controlled feeding trial. *The FASEB Journal*, *36*(1), e22054. https://doi.org/10.1096/fj.202101217R

6. Bates, J. E., & Bayles, K. (1984). Objective and Subjective Components in Mothers' Perceptions of Their Children from Age 6 Months to 3 Years. *Merrill-Palmer Quarterly*, *30*(2), 111–130.

7. *Blackwell Handbook of Early Childhood Development* (1st ed.). (2006). John Wiley & Sons, Ltd. https://doi.org/10.1002/9780470757703

8. Blair, C., & Ku, S. (2022). A Hierarchical Integrated Model of Self-Regulation. *Frontiers in Psychology*, *13*. https://www.frontiersin.org/article/10.3389/fpsyg.2022.725828

9. Boeke, C. E., Gillman, M. W., Hughes, M. D., Rifas-Shiman, S. L., Villamor, E., & Oken, E.

(2013). Choline Intake During Pregnancy and Child Cognition at Age 7 Years. *American Journal of Epidemiology*, *177*(12), 1338–1347. https://doi.org/10.1093/aje/kws395

10. Bosquet Enlow, M., White, M. T., Hails, K., Cabrera, I., & Wright, R. J. (2016). The Infant Behavior Questionnaire-Revised: Factor structure in a culturally and sociodemographically diverse sample in the United States. *Infant Behavior and Development*, *43*, 24–35. https://doi.org/10.1016/j.infbeh.2016.04.001

11. Brinkman, J. E. (2021). *Randomized Controlled Trial of Maternal Choline Supplementation: Effects on Infant Information Processing Speed*. https://doi.org/10.7298/w2e9-5n52

12. Casey, P. H., Bradley, R. H., Nelson, J. Y., & Whaley, S. A. (1988). The clinical assessment of a child's social and physical environment during health visits. *Journal of Developmental and Behavioral Pediatrics: JDBP*, *9*(6), 333–338.

13. Calkins, S. D., Dedmon, S. E., Gill, K. L., Lomax, L. E., & Johnson, L. M. (2002). Frustration in Infancy: Implications for Emotion Regulation, Physiological Processes, and Temperament. *Infancy*, *3*(2), 175–197. https://doi.org/10.1207/S15327078IN0302_4

14. Caudill, M. A. (2010). Pre- and Postnatal Health: Evidence of Increased Choline Needs. *Journal of the American Dietetic Association*, *110*(8), 1198–1206. https://doi.org/10.1016/j.jada.2010.05.009

15. Caudill, M. A., Strupp, B. J., Muscalu, L., Nevins, J. E. H., & Canfield, R. L. (2018). Maternal choline supplementation during the third trimester of pregnancy improves infant information processing speed: A randomized, double-blind, controlled feeding study. *The FASEB Journal*, *32*(4), 2172–2180. https://doi.org/10.1096/fj.201700692RR

16. Cermak, J. M., Blusztajn, J. K., Meck, W. H., Williams, C. L., Fitzgerald, C. M., Rosene, D. L., & Loy, R. (1999). Prenatal Availability of Choline Alters the Development of Acetylcholinesterase in the Rat Hippocampus. *Developmental Neuroscience*, *21*(2), 94–104. https://doi.org/10.1159/000017371

17. Cermak, J. M., Holler, T., Jackson, D. A., & Blusztajn, J. K. (1998). Prenatal availability of choline modifies development of the hippocampal cholinergic system. *The FASEB Journal*, *12*(3), 349–357.

18. Cheatham, C. L., Goldman, B. D., Fischer, L. M., da Costa, K.-A., Reznick, J. S., & Zeisel, S. H. (2012). Phosphatidylcholine supplementation in pregnant women consuming moderate-choline diets does not enhance infant cognitive function: A randomized, double-blind, placebo-controlled trial. *The American Journal of Clinical Nutrition*, *96*(6), 1465–1472. https://doi.org/10.3945/ajcn.112.037184

19. Cheng, R.-K., MacDonald, C. J., Williams, C. L., & Meck, W. H. (2008). Prenatal choline supplementation alters the timing, emotion, and memory performance (TEMP) of adult male and female rats as indexed by differential reinforcement of low-rate schedule behavior. *Learning & Memory*, *15*(3), 153–162. https://doi.org/10.1101/lm.729408

20. Cohen, L. B. (1972). Attention-Getting and Attention-Holding Processes of Infant Visual Preferences. *Child Development*, *43*(3), 869–879. https://doi.org/10.2307/1127638

21. Craciunescu, C. N., Albright, C. D., Mar, M.-H., Song, J., & Zeisel, S. H. (2003). Choline Availability During Embryonic Development Alters Progenitor Cell Mitosis in Developing Mouse Hippocampus. *The Journal of Nutrition*, *133*(11), 3614–3618. https://doi.org/10.1093/jn/133.11.3614

22. Dias, C. C., Costa, R., Pinto, T. M., & Figueiredo, B. (2021). The Infant Behavior Questionnaire – Revised: Psychometric properties at 2 weeks, 3, 6 and 12 months of life. *Early Human Development*, *153*, 105290. https://doi.org/10.1016/j.earlhumdev.2020.105290

23. Freedman, R., Hunter, S. K., Law, A. J., D'Alessandro, A., Noonan, K., Wyrwa, A., & Camille Hoffman, M. (2020). Maternal choline and respiratory coronavirus effects on fetal brain development. *Journal of Psychiatric Research*, *128*, 1–4. https://doi.org/10.1016/j.jpsychires.2020.05.019

24. Freedman, R., Hunter, S. K., Law, A. J., Wagner, B. D., D'Alessandro, A., Christians, U., Noonan, K., Wyrwa, A., & Hoffman, M. C. (2019). Higher Gestational Choline Levels in Maternal Infection Are Protective for Infant Brain Development. *The Journal of Pediatrics*, *208*, 198-206.e2. https://doi.org/10.1016/j.jpeds.2018.12.010

25. FREEDMAN, R., & ROSS, R. G. (2015). Prenatal choline and the development of schizophrenia. *Shanghai Archives of Psychiatry*, *27*(2), 90–102. https://doi.org/10.11919/j.issn.1002-0829.215006

26. Gartstein, M. A., & Rothbart, M. K. (2003). Studying infant temperament via the Revised Infant Behavior Questionnaire. *Infant Behavior and Development*, *26*(1), 64–86.

https://doi.org/10.1016/S0163-6383(02)00169-8

27. Hoffman, M. C., Hunter, S. K., D'Alessandro, A., Noonan, K., Wyrwa, A., & Freedman, R. (2020). Interaction of maternal choline levels and prenatal Marijuana's effects on the offspring. *Psychological Medicine*, *50*(10), 1716–1726. https://doi.org/10.1017/S003329171900179X

28. Hunter, S. K., Hoffman, M. C., D'Alessandro, A., Wyrwa, A., Noonan, K., Zeisel, S. H., Law, A. J., & Freedman, R. (2021). Prenatal choline, cannabis, and infection, and their association with offspring development of attention and social problems through 4 years of age. *Psychological Medicine*, 1–10. https://doi.org/10.1017/S0033291720005061

29. Jacobson, S. W., Carter, R. C., Molteno, C. D., Stanton, M. E., Herbert, J. S., Lindinger, N. M., Lewis, C. E., Dodge, N. C., Hoyme, H. E., Zeisel, S. H., Meintjes, E. M., Duggan, C. P., & Jacobson, J. L. (2018). Efficacy of Maternal Choline Supplementation During Pregnancy in Mitigating Adverse Effects of Prenatal Alcohol Exposure on Growth and Cognitive Function: A Randomized, Double-Blind, Placebo-Controlled Clinical Trial. *Alcoholism: Clinical and Experimental Research*, *42*(7), 1327–1341. https://doi.org/10.1111/acer.13769

30. Kable, J. A., Coles, C. D., Keen, C. L., Uriu-Adams, J. Y., Jones, K. L., Yevtushok, L., Kulikovsky, Y., Wertelecki, W., Pedersen, T. L., & Chambers, C. D. (2015). The impact of micronutrient supplementation in alcohol-exposed pregnancies on information processing skills in Ukrainian infants. *Alcohol*, *49*(7), 647–656. https://doi.org/10.1016/j.alcohol.2015.08.005

31. Klatt, K. C., McDougall, M. Q., Malysheva, O. V., Taesuwan, S., Loinard-González, A. (Alex) P., Nevins, J. E. H., Beckman, K., Bhawal, R., Anderson, E., Zhang, S., Bender, E., Jackson, K. H., King, D. J., Dyer, R. A., Devapatla, S., Vidavalur, R., Brenna, J. T., & Caudill, M. A. (2022). Prenatal choline supplementation improves biomarkers of maternal docosahexaenoic acid status among pregnant participants consuming supplemental DHA: A randomized controlled trial. *The American Journal of Clinical Nutrition*, nqac147. https://doi.org/10.1093/ajcn/nqac147

32. Langley, E. A., Krykbaeva, M., Blusztajn, J. K., & Mellott, T. J. (2015). High maternal choline consumption during pregnancy and nursing alleviates deficits in social interaction and improves anxiety-like behaviors in the BTBR T+Itpr3tf/J mouse model of autism. *Behavioural Brain Research*, *278*, 210–220. https://doi.org/10.1016/j.bbr.2014.09.043

33. Lauder, J. M., & Schambra, U. B. (1999). Morphogenetic roles of acetylcholine.

Environmental Health Perspectives, *107*, 5.

34. Lewis, M., Goldberg, S., & Campbell, H. (1969). A Developmental Study of Information Processing within the First Three Years of Life: Response Decrement to a Redundant Signal. *Monographs of the Society for Research in Child Development*, *34*(9), iii–41. https://doi.org/10.2307/1165696

35. *Longitudinal observation of infant temperament.: Articles & Full Text.* (n.d.). Retrieved April 18, 2022, from https://eds-p-ebscohost-com.proxy.library.cornell.edu/eds/pdfviewer/pdfviewer?vid=0&sid=bbaa09b2-9b0c-4a86-907b-21c3b37bbac8%40redis

36. Mathews, T. J. (2016). *Mean Age of Mothers is on the Rise: United States, 2000–2014*. *232*, 8.

37. McCann, J. C., Hudes, M., & Ames, B. N. (2006). An overview of evidence for a causal relationship between dietary availability of choline during development and cognitive function in offspring. *Neuroscience & Biobehavioral Reviews*, *30*(5), 696–712. https://doi.org/10.1016/j.neubiorev.2005.12.003

38. Meck, W. H., Smith, R. A., & Williams, C. L. (1988). Pre- and postnatal choline supplementation produces long-term facilitation of spatial memory. *Developmental Psychobiology*, *21*(4), 339–353. https://doi.org/10.1002/dev.420210405

39. Meck, W. H., Smith, R. A., & Williams, C. L. (1989). Organizational changes in cholinergic activity and enhanced visuospatial memory as a function of choline administered prenatally or postnatally or both. *Behavioral Neuroscience*, *103*(6), 1234–1241. https://doi.org/10.1037/0735-7044.103.6.1234

40. Meck, W. H., & Williams, C. L. (2003). Metabolic imprinting of choline by its availability during gestation: Implications for memory and attentional processing across the lifespan. *Neuroscience & Biobehavioral Reviews*, *27*(4), 385–399. https://doi.org/10.1016/S0149-7634(03)00069-1

41. Meck, W. H., Williams, C. L., Cermak, J. M., & Blusztajn, J. K. (2007). Developmental periods of choline sensitivity provide an ontogenetic mechanism for regulating memory capacity and age-related dementia. *Frontiers in Integrative Neuroscience*, *1*, 7. https://doi.org/10.3389/neuro.07.007.2007

42. Mohler, E. G., Meck, W. H., & Williams, C. L. (n.d.). *Sustained Attention in Adult Mice is Modulated by Prenatal Choline Availability*. 16.

43. Moon, J., Chen, M., Gandhy, S. U., Strawderman, M., Levitsky, D. A., Maclean, K. N., & Strupp, B. J. (2010). Perinatal choline supplementation improves cognitive functioning and emotion regulation in the Ts65Dn mouse model of Down syndrome. *Behavioral Neuroscience*, *124*(3), 346–361. https://doi.org/10.1037/a0019590

44. Neppl, T. K., Donnellan, M. B., Scaramella, L. V., Widaman, K. F., Spilman, S. K., Ontai, L. L., & Conger, R. D. (2010). Differential stability of temperament and personality from toddlerhood to middle childhood. *Journal of Research in Personality*, *44*(3), 386–396. https://doi.org/10.1016/j.jrp.2010.04.004

45. Niculescu, M. D., Craciunescu, C. N., & Zeisel, S. H. (2006). Dietary choline deficiency alters global and gene-specific DNA methylation in the developing hippocampus of mouse fetal brains. *The FASEB Journal*, *20*(1), 43–49. https://doi.org/10.1096/fj.05-4707com

46. *Opening Statement by Roy Pitkin on Dietary Reference Intakes for Thiamin, Riboflavin, Niacin, Vitamin B6, Folate, Vitamin B12, Pantothenic Acid, Biotin, and Choline*. (n.d.). Retrieved March 28, 2022, from http://www8.nationalacademies.org/onpinews/newsitem.aspx?RecordID=s6015

47. Parade, S. H., & Leerkes, E. M. (2008). The reliability and validity of the Infant Behavior Questionnaire-Revised. *Infant Behavior and Development*, *31*(4), 637–646. https://doi.org/10.1016/j.infbeh.2008.07.009

48. Products—Data Briefs—Number 332—February 2019. (2019, June 10). http://www.cdc.gov/nchs/products/databriefs/db332.htm

49. Putnam, S., Ellis, L., & Rothbart, M. (2001). *The structure of temperament from infancy through adolescence.* (pp. 165–182).

50. Putnam, S. P., Gartstein, M. A., & Rothbart, M. K. (2006). Measurement of fine-grained aspects of toddler temperament: The Early Childhood Behavior Questionnaire. *Infant Behavior and Development*, *29*(3), 386–401. https://doi.org/10.1016/j.infbeh.2006.01.004

51. Putnam, S. P., Helbig, A. L., Gartstein, M. A., Rothbart, M. K., & Leerkes, E. (2014). Development and Assessment of Short and Very Short Forms of the Infant Behavior Questionnaire–Revised. *Journal of Personality Assessment*, *96*(4), 445–458. https://doi.org/10.1080/00223891.2013.841171

52. Ross, R. G., Hunter, S. K., Hoffman, M. C., McCarthy, L., Chambers, B. M., Law, A. J., Leonard, S., Zerbe, G. O., & Freedman, R. (2016). Perinatal Phosphatidylcholine Supplementation and Early Childhood Behavior Problems: Evidence for CHRNA7 Moderation. *The American Journal of Psychiatry*, *173*(5), 509–516. https://doi.org/10.1176/appi.ajp.2015.15091188

53. Ross, R. G., Hunter, S. K., McCarthy, L., Beuler, J., Hutchison, A. K., Wagner, B. D., Leonard, S., Stevens, K. E., & Freedman, R. (2013). Perinatal Choline Effects on Neonatal Pathophysiology Related to Later Schizophrenia Risk. *American Journal of Psychiatry*, *170*(3), 290–298. https://doi.org/10.1176/appi.ajp.2012.12070940

54. Rothbart, M. K. (1981). Measurement of Temperament in Infancy. *Child Development*, *52*(2), 569–578. https://doi.org/10.2307/1129176

55. Rothbart, M. K., Posner, M. I., & Kieras, J. (2006). Temperament, Attention, and the Development of Self-Regulation. In *Blackwell Handbook of Early Childhood Development* (pp. 338–357). John Wiley & Sons, Ltd. https://doi.org/10.1002/9780470757703.ch17

56. Sameroff, A. J., Seifer, R., & Elias, P. K. (1982). Sociocultural Variability in Infant Temperament Ratings. *Child Development*, *53*(1), 164–173. https://doi.org/10.2307/1129649

57. Schulz, K. M., Pearson, J. N., Gasparrini, M. E., Brooks, K. F., Drake-Frazier, C., Zajkowski, M. E., Kreisler, A. D., Adams, C. E., Leonard, S., & Stevens, K. E. (2014). Dietary choline supplementation to dams during pregnancy and lactation mitigates the effects of in utero stress exposure on adult anxiety-related behaviors. *Behavioural Brain Research*, *268*, 104–110. https://doi.org/10.1016/j.bbr.2014.03.031

58. Taesuwan, S., McDougall, M. Q., Malysheva, O. V., Bender, E., Nevins, J. E. H., Devapatla, S., Vidavalur, R., Caudill, M. A., & Klatt, K. C. (2021). Choline metabolome response to prenatal choline supplementation across pregnancy: A randomized controlled trial. *The FASEB Journal*, *35*(12), e22063. https://doi.org/10.1096/fj.202101401RR

59. Talge, N. M., Mudd, L. M., Sikorskii, A., & Basso, O. (2014). United States Birth Weight

Reference Corrected for Implausible Gestational Age Estimates. *Pediatrics*, *133*(5), 844–853. https://doi.org/10.1542/peds.2013-3285

60. *The Infant Behavior Questionnaire (IBQ and IBQ-R) | Mary Rothbart's Temperament Questionnaires*. (n.d.). Retrieved March 31, 2022, from https://research.bowdoin.edu/rothbart-temperament-questionnaires/instrument-descriptions/the-infant-behavior-questionnaire/

61. Thomas, J. D., Abou, E. J., & Dominguez, H. D. (2009). Prenatal choline supplementation mitigates the adverse effects of prenatal alcohol exposure on development in rats. *Neurotoxicology and Teratology*, *31*(5), 303–311. https://doi.org/10.1016/j.ntt.2009.07.002

62. Thomas, J. D., Idrus, N. M., Monk, B. R., & Dominguez, H. D. (2010). Prenatal choline supplementation mitigates behavioral alterations associated with prenatal alcohol exposure in rats. *Birth Defects Research Part A: Clinical and Molecular Teratology*, *88*(10), 827–837. https://doi.org/10.1002/bdra.20713

63. Thomas, J. D., La Fiette, M. H., Quinn, V. R. E., & Riley, E. P. (2000). Neonatal choline supplementation ameliorates the effects of prenatal alcohol exposure on a discrimination learning task in rats. *Neurotoxicology and Teratology*, *22*(5), 703–711. https://doi.org/10.1016/S0892-0362(00)00097-0

64. Tikotzky, L., Chambers, A. S., Gaylor, E., & Manber, R. (2010). Maternal sleep and depressive symptoms: Links with infant Negative Affectivity. *Infant Behavior and Development*, *33*(4), 605–612. https://doi.org/10.1016/j.infbeh.2010.07.012

65. Velazquez, R., Ash, J. A., Powers, B. E., Kelley, C. M., Strawderman, M., Luscher, Z. I., Ginsberg, S. D., Mufson, E. J., & Strupp, B. J. (2013). Maternal choline supplementation improves spatial learning and adult hippocampal neurogenesis in the Ts65Dn mouse model of Down syndrome. *Neurobiology of Disease*, *58*, 92–101. https://doi.org/10.1016/j.nbd.2013.04.016

66. Wallace, T. C., & Fulgoni, V. L. (2016). Assessment of Total Choline Intakes in the United States. *Journal of the American College of Nutrition*, *35*(2), 108–112. https://doi.org/10.1080/07315724.2015.1080127

67. Wallace, T. C., & Fulgoni, V. L. (2017). Usual Choline Intakes Are Associated with Egg and Protein Food Consumption in the United States. *Nutrients*, *9*(8), 839. https://doi.org/10.3390/nu9080839

68. Wong-Goodrich, S. J. E., Glenn, M. J., Mellott, T. J., Blusztajn, J. K., Meck, W. H., & Williams, C. L. (2008). Spatial memory and hippocampal plasticity are differentially sensitive to the availability of choline in adulthood as a function of choline supply in utero. *Brain Research*, *1237*, 153–166. https://doi.org/10.1016/j.brainres.2008.08.074

69. Wu, B. T. F., Dyer, R. A., King, D. J., Richardson, K. J., & Innis, S. M. (2012). Early Second Trimester Maternal Plasma Choline and Betaine Are Related to Measures of Early Cognitive Development in Term Infants. PLOS ONE, 7(8), e43448. https://doi.org/10.1371/journal.pone.0043448

70. Yan, J., Jiang, X., West, A. A., Perry, C. A., Malysheva, O. V., Devapatla, S., Pressman, E., Vermeylen, F., Stabler, S. P., Allen, R. H., & Caudill, M. A. (2012). Maternal choline intake modulates maternal and fetal biomarkers of choline metabolism in humans. *The American Journal of Clinical Nutrition*, *95*(5), 1060–1071. https://doi.org/10.3945/ajcn.111.022772

71. Zeisel, S. H. (2006). Choline: Critical Role During Fetal Development and Dietary Requirements in Adults. *Annual Review of Nutrition*, *26*(1), 229–250. https://doi.org/10.1146/annurev.nutr.26.061505.111156

72. Zeisel, S. H. (2009). Epigenetic mechanisms for nutrition determinants of later health outcomes. *The American Journal of Clinical Nutrition*, *89*(5), 1488S-1493S. https://doi.org/10.3945/ajcn.2009.27113B

73. Zeisel, S. H., Mar, M. H., Zhou, Z., & da Costa, K. A. (1995). Pregnancy and lactation are associated with diminished concentrations of choline and its metabolites in rat liver. *The Journal of Nutrition*, *125*(12), 3049–3054. https://doi.org/10.1093/jn/125.12.3049

74. Zeisel, S. H., & Niculescu, M. D. (2006). Perinatal Choline Influences Brain Structure and Function. Nutrition Reviews, 64(4), 197–203. https://doi.org/10.1111/j.1753-4887.2006.tb00202

CHAPTER FOUR

EFFECTS OF MATERNAL CHOLINE SUPPLEMENTATION ON BEHAVIORAL MEASURES OF INFANT SELF-REGULATION

4.1 Abstract

Objective: To determine whether the children of mothers who received 550 mg/day choline (v 25 mg/day) during the second and third trimesters of pregnancy performed better on a task of social stress at age 7 months. **Methods**: Pregnant women (N = 33) were randomized at gestation week 16 to receive either 25 mg/d or 550 mg/d choline until delivery. They returned with their infants (N = 26) at four times throughout the first postnatal year of life to complete a battery of cognitive and behavioral tests. We examined infant affective and regulatory responses using the Face-to-Face Still-Face Paradigm, a measure of infant negative reactivity and affect regulation. Outcomes included decline in total negative affect between the distressing episode and the reunion episode, latency to negative affect during the distressing episode, and proportion of time in the distressing episode that infants engaged in attentional orienting as a self-regulatory strategy. Statistical analyses included general and mixed linear models. **Results**: The findings of this study did not reveal a significant effect of maternal supplementation with 550 mg/day of choline (v 25 mg/day) on infant affect regulation, reactivity, or regulatory behaviors in response to the Face-to-Face Still Face Paradigm at 7 months of age. However, patterns emerged indicative of a more adaptive affective response in the infants born to women in the higher choline supplementation group. **Conclusions**: Although there were no significant differences in infant affect regulation, reactivity, or regulatory behaviors, the results of this study suggest that 550 mg/day maternal choline supplementation (v 25) may produce a more appropriate affective response to a distressing episode. These data raise interesting questions for future studies of the effect of maternal choline supplementation on infant affect.

4.2 Introduction

The physiological need for choline, an essential nutrient with many roles in fetal development, is increased during pregnancy, where it provides constituents for the development of cell membranes, neurotransmitters, and epigenetic modifications.[64–66,68] During prenatal development, choline and its metabolites play key roles in brain development and long-term offspring cognition via several potential mechanisms, including effects on cellular proliferation, migration, and apoptosis, neurogenesis, and synaptic plasticity.[3–4,15–16,21,65,68] Choline-derived phospholipids, including sphingomyelin and phosphatidylcholine, are key components of cell membranes and help to maintain the structural and functional integrity of cells.[65,68] Choline also acts as a required precursor to acetylcholine (ACh), the primary neurotransmitter of the parasympathetic nervous system and a prominent neuromodulator in the central nervous system.[28,65,68] Further, choline is the major dietary source of methyl groups, and, through its conversion to the metabolites betaine and s-adenosylmethionine (SAM), provides methyl groups needed for DNA methylation.[65–66] These metabolites of choline play key roles in epigenetic modification of genes and histones, which can exert long-term effects on brain and behavior via gene expression.[41,65–66]

Consistent with choline's many important ontogenetic roles, a robust body of rodent work has demonstrated the importance of maternal choline intake for the developmental programming of offspring cognition and behavior. Rodent data demonstrate that maternal choline deficiency results in lasting cognitive deficits in the offspring, and that conversely, when compared to standard rodent chow (which is designed to contain adequate choline), maternal choline supplementation (MCS) improves offspring memory and attention throughout the lifespan.[32–36,38] Further, prenatal maternal choline supplementation has been shown to lessen age-

related cognitive decline and reduce cognitive dysfunction in rodent models of several neurological disorders, including Down syndrome, autism, and Alzheimer's disease.[4,39,55] Finally, prenatal choline supplementation has been shown to lessen the dysfunction produced by a variety of prenatal insults, including maternal stress, infection, and inflammation, as well as exposure to drug and alcohol products.[49–51]

Several reports have also documented effects of maternal choline supplementation on socioemotional function in rodent models, although this body of research is more limited. One study focusing on prenatal stress in a rodent model reported that prenatal choline supplementation reduced anxiety-like behaviors and social behavior problems in the offspring of stressed dams, although the effects varied somewhat by the sex of the offspring.[46] Female offspring born to stressed but choline-supplemented dams exhibited less anxiety in the open-field task and elevated zero maze tasks compared to females born to stressed dams on a control diet. In contrast, male offspring born to stressed but choline-supplemented dams had normalized social behavior when confronted with a novel conspecific, increasing the amount of time that the mouse spent investigating a new social partner.[46]

Deficits in socioemotional function may also manifest as frustration, a negative affective response to goal blockage.[12] Studies have found that infants who are more easily frustrated may be more constrained in the development of self-regulatory behaviors.[2,12] Another rodent study found that prenatal choline supplementation reduced burst responding in response to task errors, suggesting a reduction in the amount of frustration expressed by the supplemented animals.[18] Studies have also found evidence that prenatal choline supplementation normalizes offspring socioemotional regulation in rodent disease models of Down syndrome and autism, adversities characterized by aberrant emotional reactivity.[27,39,55]

Although these rodent data provide strong evidence that choline intake during pregnancy is critical for offspring brain development and cognitive functioning, as well as offering preliminary evidence for a beneficial effect of MCS on offspring socioemotional functioning, relatively little is known about choline needs during pregnancy in humans, nor the functional consequences for the child if maternal intake is insufficient.[13] In 1998, the IOM first identified an Adequate Intake (AI) for choline at 425 mg/day for adult women, with a slight increase to 450 mg/d for pregnant women.[42] However, the empirical basis for this recommendation for pregnant women was one small study of the amount of choline needed to prevent liver dysfunction in men, not the more relevant outcome of child neurodevelopment[13,42], and there is emerging evidence indicating that the current AI is insufficient for the demands of pregnancy.[5,6] This is concerning in light of the fact that ~90% of pregnant women do not consume even the AI, and most prenatal vitamins contain little or no choline (~55 mg).[57,58]

Few human studies have been conducted to assess the association between variations in maternal choline intake during pregnancy and offspring outcomes. Two observational studies found correlations between serum and/or dietary measures of maternal choline intake and offspring performance on tests of infant development and child memory,[9,62] but two others found no association.[47] However, observational studies do not allow for causal inferences due to risk of confounding with uncontrolled covariates. Further, none of these observational studies examined the association between variations in maternal choline intake during pregnancy and offspring socioemotional outcomes.

Three randomized control trials (RCT) of maternal choline supplementation in typically developing infants have been conducted. Of the three studies, two found benefits on measures of offspring cognition, including attentional orienting speed during infancy, and working memory,

sustained attention, and executive function at seven years of age (Chapter 2, this dissertation).[5,6,14,44] The third trial detected no offspring cognitive benefits, based on assessments of memory and cognitive ability in infants.[17]

Only one of these RCTs examined the effect of maternal choline supplementation on offspring socioemotional function; this study used the Child Behavior Checklist, a clinical parent-report measure used to detect behavioral and emotional problems in children.[44] This study found that MCS decreased social withdrawal, as measured by scores on the Social Problems subscale, in the children at 40 months of age. Children of women supplemented with choline were also rated as having fewer problems with anxiety, internalizing/externalizing behaviors, and fewer overall behavioral and emotional problems, although differences in these scores did not reach statistical significance in this sample of 49 children.[44]

One of the three RCTs described above is of particular relevance to the present report, which focuses on the effects of maternal choline supplementation on infant temperament. This highly controlled feeding trial randomized third trimester pregnant women to receive either 480 or 930 mg/day choline until delivery.[14] Follow-up of the infants of supplemented mothers found that infants of mothers in the 930 mg/day group had faster information processing speeds across the first year of life.[13] When followed up at 7 years of age, the children of mothers in the 930 mg/day group showed superior performance on tasks of sustained attention, working memory, and executive function (Chapter 2, this dissertation).[5,6] This study provides compelling support for the translation of the cognitive benefits of MCS seen in rodent models to humans. Together with the rodent data, this preliminary evidence offers a strong rationale for also investigating the translation of the socioemotional benefits of MCS in humans.

To do so, we took advantage of our ancillary cognitive-behavioral follow-up to a double-

blind, randomized-control clinical trial. In this RCT, pregnant women were randomized to consume supplemental choline at one of two doses (25 or 550 mg choline/day as choline chloride) from gestation week 16 until delivery. The mothers were then re-recruited to return with their infants to the laboratory at four times throughout the first year of life to participate in assessments of cognitive and socioemotional functioning. When the infants were seven months old, we examined the effects of MCS on infant affect and self-regulation using the Still-Face Paradigm.

The Face-to-Face Still-Face Paradigm (FFSF) is a procedure first introduced to examine the hypothesis that infants are active participants in social interaction.[37,54] The task consists of three phases: a play phase, in which the parent and infant are allowed to play and engage with each other; a still-face phase in which the parent looks at the infant with a neutral expression and does not respond to the infant's cues; and a reunion phase, in which the parent and infant can resume interaction. The Still-Face Paradigm is a useful measure of infant affect reactivity and regulation because it reliably produces a reaction from the infant known as the still-face effect—decreased positive affect, increased gaze aversion, and increased negative affect.[1,37] This measure has been used to investigate a wide variety of research questions in child development, including the effects of maternal prenatal depression on infant temperament, infant attachment, and the development of culturally specific differences in communication.[1,37] Infant affective and self-regulatory responses to the FFSF have been found to be predictive of attachment quality and behavioral problems during childhood.[11,40]

The present study tested the hypothesis that infants of mothers who received a 550 mg/day choline supplement (v 25 mg/day) plus usual diet during pregnancy will show superior affect regulation in response to the stress of the Face-to-Face Still Face Paradigm at 7 months of

age.

4.3 Subjects and Methods

Ethical Approval

Ethical approval for the present study was obtained from the Institutional Review Board for Human Participants at Cornell University in Ithaca, NY. Written parental consent was obtained from all study participants.

Study Design and Participants

Supplementation Trial

The present study leveraged a clinical trial (NCT03194659) in which pregnant women (gestation week 12–16) were randomized to consume one of two doses of choline (25 or 550 mg/day), along with a once-daily prenatal vitamin/mineral supplement (Nature Made Prenatal Tablet; Pharmavite LLC; CA, USA) and a 200 mg/day DHA supplement, from enrollment until delivery (Nature's Way EfaGold Neuromins 200 mg DHA (plant source); DSM Nutritional Products; Netherlands). The 550 mg/day supplement was designed to achieve an average intake of ~900 mg/day of choline (~350 mg/day from diet + 550 mg supplemental choline = ~900 mg/day of choline). In our previous controlled choline feeding study, this amount of choline consumed throughout the third trimester was found to improve offspring information processing speed during infancy, and memory, attention, and executive functioning at 7 years of age.[5,6,14] An average daily intake of ~900 mg/day of choline is well below the IOM established upper tolerance limit of 3,500 mg/day.[13] The 25 mg/day control is not a true placebo group, due to the need for each group to consume a small amount of tracer choline, administered as deuterium-labeled choline, to investigate hypotheses related to the metabolism of choline (not presented here). However, total choline intake of the control group (350 mg/day from diet + 25 mg/day

supplemental choline = ~375 mg/day of choline) approximates the average prenatal choline intake of most pregnant women. This small amount of supplemental choline, although similar to amounts found in a few prenatal vitamins, represents a trivial increase in choline intake over the average (7%), whereas the 550 mg/day choline supplement represents a substantial (~157%) increase over the average choline intake.

The sample size in this study was powered in relation to primary outcomes related to biomarkers of maternal/fetal choline and DHA metabolism.[26,48] Secondary outcomes included offspring cognition and affect regulation during infancy, genomic expression, and metabolomic profiling of plasma and placental tissue. The current study is an ancillary follow-up of offspring from the initial pregnancy study at 5-, 7- 10- and 13-months postnatal age to assess effects on infant cognition and affect regulation, using pre-specified endpoints.

Details of the supplementation trial have been published elsewhere.[26,48] Briefly, second trimester pregnant women (12–16 weeks' gestation, N = 33) were recruited from the largest obstetrics practice in the Ithaca, NY area. Eligibility to participate included maternal age 21–40, healthy singleton pregnancy, and willingness to comply with the study protocol. Exclusion criteria included a prepregnancy BMI of ≥ 32 kg/m^2 or current use of tobacco, alcohol, or drugs. Women with high habitual intakes of choline or omega-3 fatty acids, as assessed by self-report food frequency questionnaires at screening, were also ineligible to participate. Women who had pregnancy complications or comorbidities such as preeclampsia or gestational diabetes, either at enrollment or developed during the course of the study, were ineligible to participate.

Supplementation began at the time of enrollment and continued until delivery. Supplements were administered as choline chloride dissolved in grape juice and provided to study participants in 13 mL test tubes, one for each daily dose. Supplements were provided at

three visits to the laboratory at gestation weeks 12–16, 20–24, and 28–32. To monitor adherence, participants were instructed at each visit to return any unconsumed supplements at the following study visits. At these visits, women also provided blood, urine, and fecal samples, and completed a health questionnaire and 24-hour dietary recall. At delivery, women were asked to provide a placental tissue and cord blood sample.

Follow-Up Neurobehavioral Trial

Mothers were invited to bring their infants back to Cornell University to participate in the postnatal study on neurobehavioral functioning. Infants (N = 26, see Figure 4.2) and their mothers were recruited between April 2018 and October of 2019, and neurobehavioral testing occurred between April 2018 and November of 2020. Testing occurred at four ages postnatally at 5, 7, 10, and 13 months of age. Characteristics of the participants and their mothers were obtained via parent report at each follow-up visit and included infant age, sex, race, and ethnicity, recent illnesses, vision or motor problems, frequency and type of breast/bottle feeding and solid foods, and sleep habits of the infant. Maternal characteristics, including race, ethnicity, education, and age, were evaluated to assess possible bias arising from loss to follow-up.

The Face-to-Face Still Face Paradigm

The Face-to-Face Still Face (FFSF) Paradigm is an experimental procedure first developed by Dr. Ed Tronick in 1975.[54] Since then, the FFSF has been used in a broad body of developmental research to measure infant self-regulation during a social interaction with their parent.[1,37] In this study, the FFSF task was administered at 7 and 13 months of age. Due to interruptions in research operations during the COVID-19 pandemic, only data from the 7-month administration of the task is presented in this dissertation.

Due to the possibility of distress caused by this task, it is conducted at the end of a

testing session containing various laboratory assessments of cognition and behavior. The infant was placed in a highchair, and the parent was asked to sit approximately one meter away, seated at a table and facing the infant. The task was monitored by a trained study experimenter (blinded to group assignment) from a separate room, hidden by a partition. Prior to the task, the parent was provided with verbal instructions for each of the three ordered stages of the interaction (Appendix K). Additionally, the parent was provided with several visual examples of a neutral expression from the NimStim Set of Facial Expressions (NimStim).[53] NimStim images were designed to be recognizable by untrained individuals (research participants), and parents participating in this study were presented with three color images of racially diverse women looking directly at the camera with a closed mouth and an emotionally neutral expression (Appendix K).[53] The parent was also given time to practice the expression and receive direction from the experimenter as needed.

As described in further detail in Figure 4.1, the Face-to-Face Still Face Paradigm consists of three ordered episodes, each lasting two minutes. In the play episode, the parent is instructed to engage normally in play with their infant without the use of toys. In the still-face episode, the parent is instructed to adopt a neutral expression, and to not touch or respond to their infant beyond maintaining eye contact. In the reunion period, the parent is allowed to resume interaction in whatever way they deem appropriate, without removing the infant from the highchair. The beginning and end of each period is indicated verbally by the experimenter. Parents were informed prior to the task that they could terminate the procedure at any time if they determined their infant was too upset to continue. In addition, if the infant exhibited continuous, hard crying for 15 seconds, the experimenter terminated the task. This rule was communicated to the parent prior to the task.

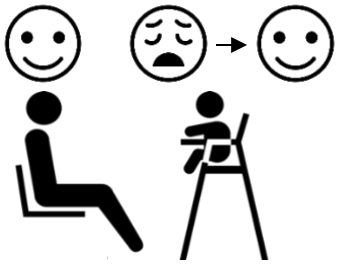
Play Episode (2 min)	Still-Face Episode (2 min)	Reunion Episode (2 min)
Normal play situation between parent and infant. Parent is encouraged to speak or sing to their baby, or to play games such as peek-a-boo. Toys are not provided, so all interaction is between the infant and the parent. Mostly positive affect.	The parent assumes a neutral, emotionless 'poker-face'. The parent is instructed not to engage with their infant beyond maintaining eye contact. 'Still-face effect' is seen in increased infant negative affect.	Parent resumes interaction with their infant in the same manner as the play episode, without removing the infant from the highchair. There may be some 'carry-over' of negative affect as the infant returns to their baseline affect.
		

Figure 4.1: The three stages of the Face-to-Face Still Face Paradigm. Adapted from Wolf et al., 2018[61]

The task was video- and audio recorded using GoPro cameras (GoPro Inc, San Mateo, California).

Behavioral Coding

Video and audio recordings were used to code for infant affect and self-regulatory behaviors using Behavioral Observation Research Interactive Software, version 7.12.2.[23] *Affect* was coded for all three phases and defined as non-negative, negative, or non-codable. Affect was considered non-codable if the infant expressed a brief (<2 second) change in affect that was too short to clearly be identified as negative or non-negative. *Latency to negative affect* was coded in each task phase. *Intensity of negative affect* was rated on five-second intervals on a Likert scale from 0 (no negative affect) to 3 (high negative affect) (Appendix L). Regulatory behaviors were coded in the still-face phase, and were categorized as *attentional orientation, social signaling, avoidance, or self-soothing* (Appendix L). *Infant gaze aversion*, defined as the duration of time

that the infant's gaze was oriented away from their social partner for any reason, was also coded during the play and still-face episodes.

The Still-Face Paradigm reliably produces the "still-face effect", defined as decreased positive affect, increased gaze aversion, and increased negative affect.[1,37] In order to assess if the task was successfully administered in this sample, we examined the duration of non-negative and negative affect in each task phase, as well as the proportion of time infants engaged in gaze aversion (calculated as duration of gaze aversion divided by the phase duration) during the play and still-face phases. We also examined the number of early task terminations in each phase.

Outcome Measures and Statistical Analyses

The small body of rodent and human data on the effects of maternal choline intake on offspring socioemotional outcomes suggests that increased MCI results in superior affect regulation, defined as modification of the intensity and duration of an affective state in response to environmental stress. Therefore, the primary outcome in this study is infant's affect regulation, defined as the magnitude of the decline in total negative affect from the still-face episode to the reunion episode (Table 4.1). Total negative affect, calculated separately for each phase, was calculated as the sum of negative affect intensity ratings in that phase. Secondary outcomes regarding affect reactivity (the speed of initial activation of affective responses to environmental stress) were also examined using the latency to negative affect during the still-face phase.

The rodent and human literature on the effects of increased maternal choline intake on offspring outcomes provides robust evidence for improved attention in these offspring.[32,38] As a self-regulatory strategy, attentional orienting may aid infants in the regulation of negative affect by directing attention away from a distressing stimulus.[45] Therefore, we also examined attentional orienting as a secondary outcome, using the proportion of time during the still-face

phase infants spent engaged in attentional orienting.

Construct	Definition	Outcome Measure	Operational Definition	Hypothesis
Affect regulation	Modification of the intensity and duration of an affective state and arousal in response to environmental stress	Total negative affect	Sum of negative affect intensity ratings in each task phase	Infants in the 550 mg/d group will have a greater decline in negative affect from the still-face to reunion phase
Affect reactivity	The speed of initial activation of affective responses to environmental stress	Latency to negative affect	Difference (in seconds) between first expression of negative affect and each phase start	Infants in the 550 mg/d group will have a longer latency to negative affect in the still-face phase
Regulatory behaviors	Behaviors that help the infant to reduce the intensity of affect in order to return to a more comfortable baseline state	Proportion of the still-face phase engaged in attentional orienting	Total duration of attentional orienting during the still-face phase divided by total duration of still-face phase	Infants in the 550 mg/d group will spend a greater proportion of time in the still-face phase engaged in attentional orienting

Table 4.1: Outcome measures assessed and reported in this paper.

Maternal and child characteristics for the participants included in the final analytical sample were compared by treatment group using Student's t tests for continuous variables and Fisher's exact tests for categorical variables. The same approach was used to compare the characteristics of participants included in the final analysis sample of the Still-Face Paradigm to the 9 children who were not able to provide data for this task due to COVID-19 closures (lost to follow-up, N = 9. Figure 4.2).

Our analysis plan (completed prior to unblinding) prespecified one basic unadjusted mixed model for estimating the effects of treatment group on infant affect regulation. There are three repeated measures of total negative affect for each infant, corresponding to the three phases

of the task (play, still-face, reunion). The unadjusted mixed model for negative affect included as fixed classification effects task phase, treatment group, and child identifier. Random effects were included for the intercept and for the individual child. In particular, we examined the difference in total negative affect between the still-face and reunion phases for both groups to assess infant affect regulation.

As latency can be defined as a "time to event" outcome variable (time until first expression of negative affect), and our hypothesis predicts a non-normal distribution of the latency results, we analyzed the latency data using a survival analysis. The event of interest was defined as first expression of negative affect, and an infant "survived" if they did not express negative affect by the end of the phase. In order to assess infant reactivity, we examined the survival analysis of latency to negative affect in the still-face phase. Because some infants did not start the still-face phase looking at their parent, we defined time zero as the first time the infant looked at the parent and noticed the change in demeanor (the still face) and calculated latency as the time between the infant's first look at the parent and their first expression of negative affect. Log-rank analysis was used to assess group differences in latency to negative affect.

Because self-regulatory behaviors, including attentional orienting, were coded only during the still-face phase of the task, treatment effects on proportion of the phase spent engaged in attentional orienting were estimated in a general linear model that included a fixed classification effect for treatment group and child identifier. The same statistical approach was used to examine the other self-regulatory behaviors measured.

Because our prespecified mixed model does not allow for an estimate of the effect of maternal choline supplementation group on total negative affect, we conducted a post-hoc

analysis of the difference of differences scores between the two groups from the still-face to the reunion episode. Difference scores for both groups were calculated as total negative affect during the still-face phase – total negative affect during the reunion phase, and a parametric t-test was used to compare the difference scores for both groups.

Child sex was not included in the *a priori* models because the existence of gender differences for the FFSF has not been established.[37] In addition, the sex of the fetus was not known at the time of recruitment, and we were not able to stratify by sex to create groups with equal number of infants of each sex. This, in addition to the large number of female infants in our small sample, precluded analysis that controlled for infant sex.

SAS 9.4 Software (SAS Institute, Cary, NC, USA) was used to conduct statistical analyses, including linear and logistic general and mixed-model methods. All tests were 2- tailed and statistical significance was set at $P< 0.05$ for main effects, and $P< 0.10$ for interactions.

4.4 Results

Subject Characteristics

Of the 33 women who completed the supplementation trial, 30 were eligible to return with their infants for follow-up testing. Three women (1 from the 25 mg/day group and 2 from the 550 mg/day group) developed gestational diabetes during the course of the supplementation trial and were subsequently excluded from both the pregnancy and follow-up studies. 26 women were successfully recruited for their children to participate in the infant cognitive and behavioral assessments (Figure 4.2). One child was initially re-recruited, but only returned for the five-month follow-up visit; thus, cognitive and behavioral data were not available for this infant at older ages. Prior to unblinding the investigators to treatment group identity, the decision was made to exclude the data from this child from all analyses of cognitive and behavioral endpoints.

On March 13th, 2020, all research involving human subjects at Cornell University was halted due to the COVID-19 pandemic. Our laboratory space was closed from March 13th until July 15th, during which time we were unable to conduct 7-month visits for 7 infants. Therefore, our analytical sample for the Still-Face Paradigm data presented in this chapter consists of those infants whose 7-month visits fell before or after the four-month pandemic closure (N = 16). Two infants in the 25 mg/day group were also excluded due to procedural or technical error. There were no statistically significant differences in sample characteristics between infants who were included in the analytical sample for the FFSF and those who were not (Appendix L).

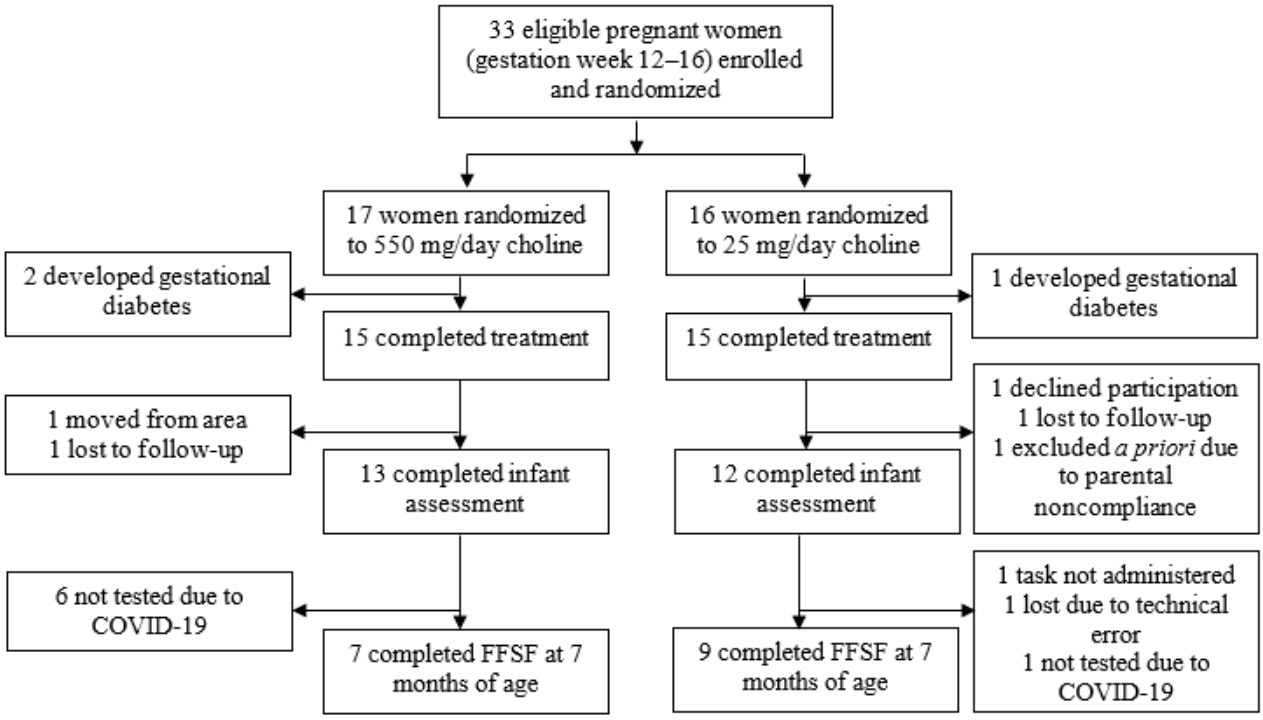


Figure 4.2: Participant flow diagram. Study screening, intervention, and infant and follow up assessments.

There were no statistically significant differences in sample characteristics by choline group (Table 4.2). The infant sample was 62.5% female. At the time of follow-up, the mothers were on average 31.2 years old and 68.8% had an advanced degree, making them older and more highly educated than the U.S. average.[30,43] 94% of the mothers self-identified as white and 94%

self-identified as non-Hispanic ethnicity.

	Maternal Choline Intake Group		
	25 mg/d (N = 7)	550 mg/d (N = 9)	p
Maternal Characteristics			
Mean age, years (range)	33.14 (24–38)	30 (27–36)	0.34
Mean BMI, kg/m2 (range)	22.2 (18.5–24.7)	23.8 (18.5–29)	0.28
Education (%)			0.28
High School	1 (14)	1 (11)	
Bachelor's Degree	0 (0)	3 (33)	
Master's Degree	4 (57)	5 (56)	
Doctorate/Professional	2 (29)	0 (0)	
Race (%)			0.44
White	6 (86)	9 (100)	
Black	1 (14)	0 (0)	
Asian	0 (0)	(0)	
Ethnicity (%)			0.44
Non-Hispanic	6 (86)	9 (100)	
Other	1 (14)	0 (0)	
Pregnancy and Delivery			
Mean gestation length, days (range)	282.4 (276–292)	277 (263–287)	0.16
Pregnancy/labor complications (%)	2 (29)	2 (22)	1.00
Delivery method, vaginal (%)	6 (86)	7 (78)	1.00
Infant Characteristics			
Sex, female (%)	3 (43)	7 (78)	0.30
Mean birth length, inches (range)	19.8 (18.5–21)	19.5 (18–21)	0.32
Mean birth weight, grams (range)	3482.4 (2860–3915)	3394.8 (2932–4194)	0.66

Table 4.2: Sample demographic characteristics by maternal choline intake group.

Results of the Face-to-Face Still Face Paradigm

Interpretation of data from infants participating in a complex emotion-elicitation protocol such as the Face-to-Face Still Face Paradigm requires validation that the protocol produced the expected pattern of infant behaviors as reported in the literature. The FFSF reliably produces the

"still-face" effect—decreased positive affect, increased negative affect, and increased gaze aversion from the play to still-face period.[37] Therefore, we would expect to see the same pattern in our data if the task was administered correctly.

The duration of non-negative affect was significantly shorter in the still-face phase (M = 89.1 seconds, SD = 41.3) than in the play phase (M = 113.4 seconds, SD = 12.3), $t(17.8) = 2.26$, $p = 0.036$. The duration of negative affect was significantly longer in the still-face phase (M = 19.1 seconds, SD = 25.8) than in the play phase (M = 4.32 seconds, SD = 9.13), $t(18.6) = -2.16$, $p = 0.044$. Lastly, the duration of gaze aversion was significantly longer in the still-face phase (M = 80.84 seconds, SD = 25.04) than in the play phase (M = 35 seconds, SD = 28.8), $t(30) = -4.85$, $p < 0.0001$. The magnitude of these differences corresponds closely to differences reported in a meta-analysis of infant responses to the FFSF.[37] Thus, our results demonstrate that the FFSF paradigm was successfully administered in this small sample.

We also examined the number of early terminations in each group. The two groups did not differ in the number of infants whose level of distress reached our prespecified termination criterion (15 seconds of continuous hard crying), resulting in early task termination, in any phase. The task was terminated early for one infant in each group during the still-face phase, and for one infant in each group during the reunion phase.

Total Negative Affect

Our primary outcome measure was total negative affect, defined as the sum of negative affect intensity ratings for each task phase. Intensity of negative affect was rated every five seconds on a Likert scale from 0 (no negative affect) to 3 (high negative affect). Each phase was 120 seconds long. The maximum possible intensity score in any one phase was 72 (24 intervals x the maximum intensity score of 3). However, because our criterion for task termination was 15

seconds of hard crying, it was not possible for infants to achieve this score. During the play phase, total negative affect ranged from a score of 0–14 During the still-face phase, total negative affect ranged from 0–27 (Table 4.3). During the reunion phase, total negative affect ranged from 0–26 (Table 4.3).

The two groups did not significantly differ (at $p < 0.05$) in total negative affect during the play period or the still-face period; however, infants in the 550 mg/day group (v 25 mg/day) scored on average four points higher on total negative affect during the still-face phase (Table 4.3). Infants in the 25 mg/day group expressed less negative affect during the reunion phase as compared to the 550 mg/day group, a difference that trended towards significance ($p = 0.08$, Table 4.3. P's not adjusted for multiple testing).

Total negative affect increased from the play to still-face phase for infants in the 550 mg/day group (+8.29, $p = 0.012$) but not for infants in the 25 mg/day group (+3.88, $p = 0.16$). Negative affect during the reunion phase did not differ significantly from the still-face phase for either group [25 mg/day = -2.98 ($p = 0.30$); 550 mg/day = +0.53 ($p = 0.87$)].

Play Phase			**Still-Face Phase**			**Reunion Phase**		
25 mg/d (N = 9)	**550 mg/d (N = 7)**	**p**	**25 mg/d (N = 9)**	**550 mg/d (N = 7)**	**p**	**25 mg/d (N = 8)**	**550 mg/d (N = 6)**	**p**
M = 1.67, SD = 4.64 Range: 0–14	M = 1.14 SD = 2.04 Range: 0–5	0.89	M = 5.56 SD = 10.6 Range: 0–27	M = 9.43 SD = 10.1 Range: 0–24	0.32	M = 2.57 SD = 9.65 Range: 0–10	M = 9.96 SD = 3.46 Range: 0–26	0.08

Table 4.3: Total negative affect score in each task phase. Group means, standard deviation, and ranges are presented here. P-values are from the mixed model and are not adjusted for multiple testing.

Our primary outcome was the decline in total negative affect score from the still-face to the reunion phase (Table 4.1). We tested this outcome using a prespecified unadjusted mixed model of total negative affect, specifically examining a single DF interaction contrast comparing

slope of total negative affect from still-face to reunion of the 550 mg/day group to the slope of total negative affect from still-face to reunion of the 25 mg/day group. The least square means estimate of the interaction contrast was $t(25.8) = -0.82$, $p = 0.42$, indicating that there was not a significant difference between the two groups in slope of total negative affect from the still-face to reunion phase.

Given our small sample size (N = 16), and therefore the likelihood of low power to detect an effect of the intervention, we calculated an estimate of the effect size associated with the group difference for our primary outcome (decline in total negative affect from the still-face to the reunion phase). This aids in clarifying whether the lack of statistical significance is due primarily to a small magnitude in the group difference, or our small sample size. Because there is no conventional definition of effect size for mixed effects models, we used a difference of differences t-test to estimate the effect size (Hedge's g) for our primary outcome. The results of the t-test closely approximated the results of the mixed model and were used to compute an effect size of $g = -0.45$. Thus, if the difference we observed was due to the choline intervention, this suggests that MCS has a medium-sized effect on decline in infant total negative affect from the still-face to reunion phases of the FFSF.

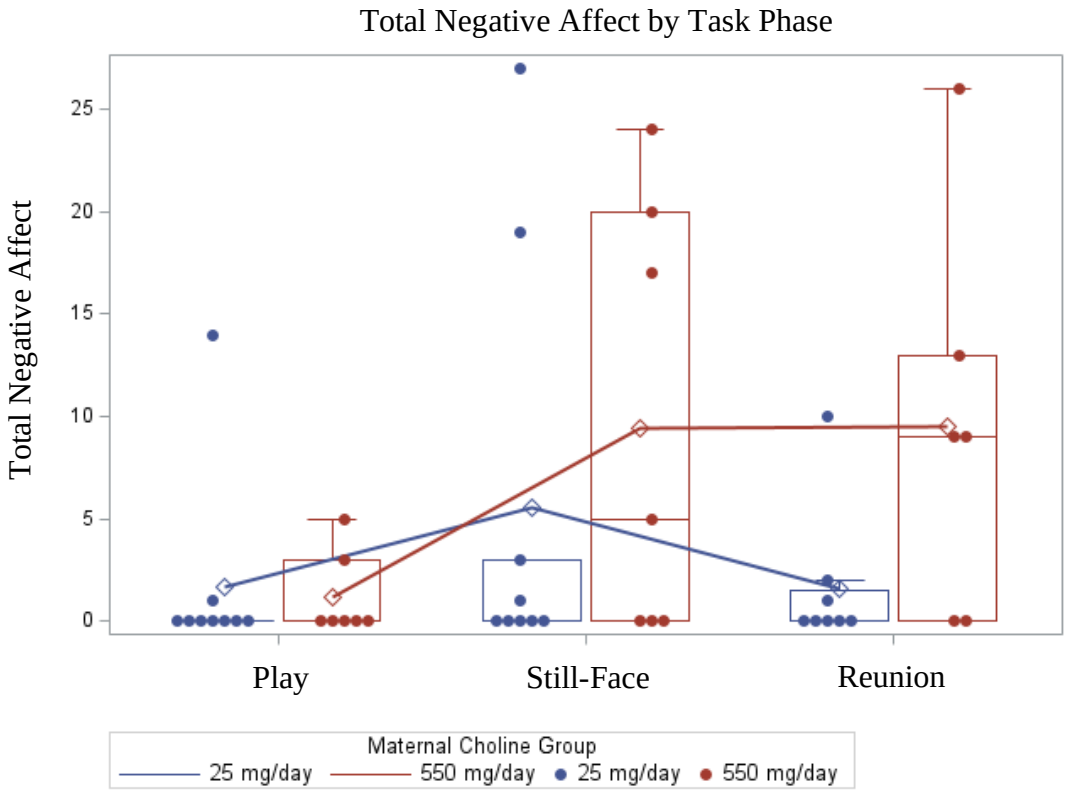


Figure 4.3: Total negative affect for each group across each task phase. Individual participant scores for each phase are represented as dots overlaying the box plots. Box plots display minimum and maximum scores for each group in each phase, as well as the interquartile ranges. Group means are represented by diamonds and are plotted across phase.

Another way to understand infant affect regulation is to observe total negative affect during the reunion phase as compared to the play phase.[10] Infants in the 550 mg/day group had significantly higher total negative affect in the reunion phase than the play phase (+8.82, p = 0.012). Total negative affect in the reunion phase did not significantly differ from total negative affect in the play phase for infants in the 25 mg/day group (+0.91, p = 0.75).

Another way to understand how negative affect differed between the two groups is to observe how the groups differed in the number of infants who did not express any negative affect during the still-face phase. This association was estimated using a *post-hoc* Chi-square analysis. This analysis showed that 55.6% of infants in the 25 mg/day group did not show any distress during this phase (Figure 4.4), while only 42.9% of infants in the 550 mg/day group did not get

upset at all during this phase, though this difference was not significant (p = 0.61).

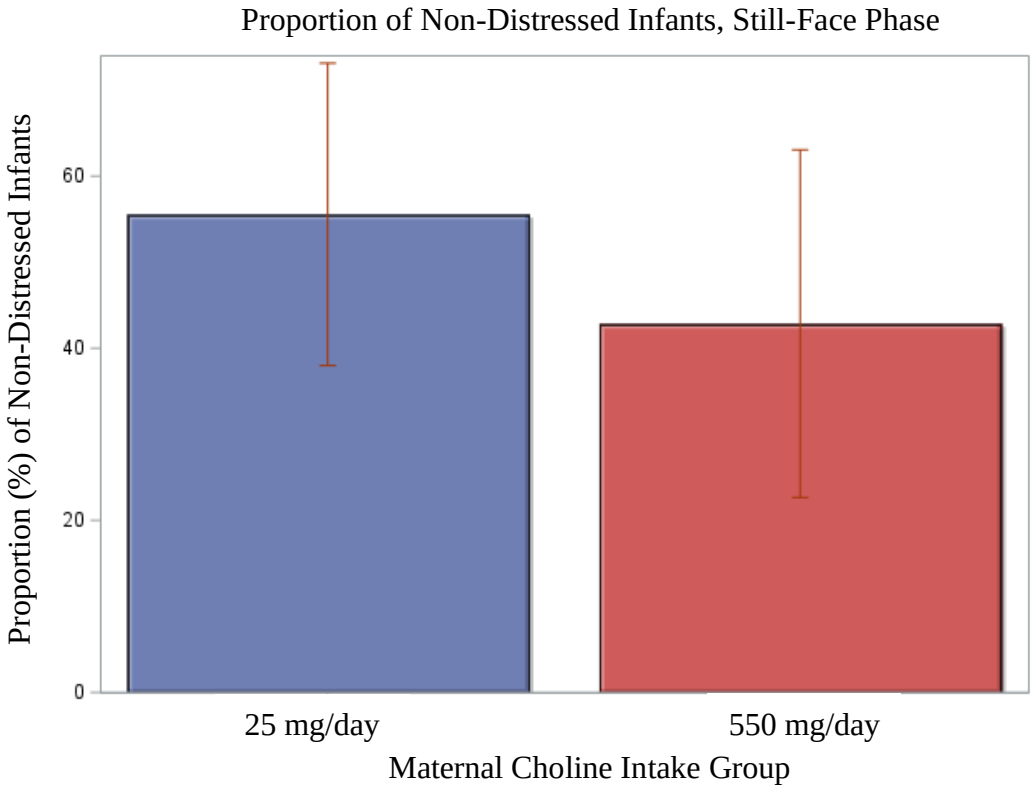


Figure 4.4: Proportion of infants in each group who did not display any negative affect during the Still-Face phase. Bars represent standard error.

Latency to Negative Affect

We used a survival analysis and associated log-rank test to examine our secondary hypothesis regarding latency to negative affect (Table 4.1). The two groups did not differ significantly in their latency to negative affect in the still-face phase in the log-rank analysis (p = 0.53). However, the Kaplan-Meier curves revealed a pattern that indicates infants in the 550 mg/day group had a lower probability of survival; that is, they were less likely to make it to the end of the phase without getting upset. Further, infants in the 550 mg/day group were more likely to get upset earlier in the phase.

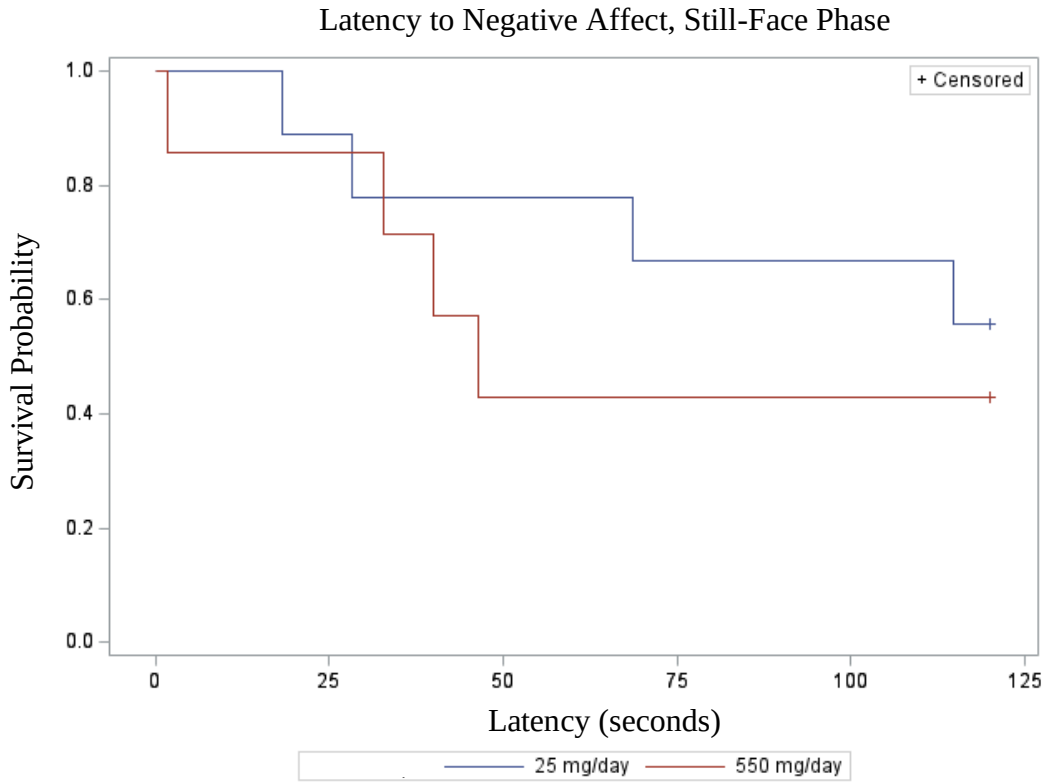


Figure 4.5: Kaplan-Meier curves for survival analysis of latency to negative affect in the still-face phase

Regulatory Behaviors

The two groups did not differ in the total proportion of time during the still-phase phase that they spent performing any type of regulatory behavior ($p = 0.65$), nor did they differ in the total proportion of time spent performing each regulatory behavior type (Table 4.4). However, two interesting trends emerged. First, infants in the 25 mg/day group (v 550) spent on average about 12% more time during the still-face phase engaged in attentional orienting towards something other than their social partner. Second, infants in the 550 mg/day group (v 25) spent on average about 10% more time during the still-face phase engaged in social signaling and attempting to re-engage the parent in social interaction. The groups were nearly identical in the proportion of time they spent performing either self-soothing or avoidance behaviors.

Regulatory Behavior	Maternal Choline Intake Group		p-value
	25 mg/day (N = 9)	550 mg/day (N = 7)	
%, range			
Attentional Orienting	42.3% (7.5–79.6)	27.7% (0–55.5)	0.21
Social Signaling	22.3% (0–64)	32.2% (0–69.5)	0.43
Avoidance	1.76 (0–13.8)	4.43% (0–26.8)	0.53
Self-Soothing	11.4 (0–32.7)	9.18% (0–26.7)	0.73

Table 4.4: Proportion of time each group spent engaged in each category of self-regulatory behavior during the still-face phase.

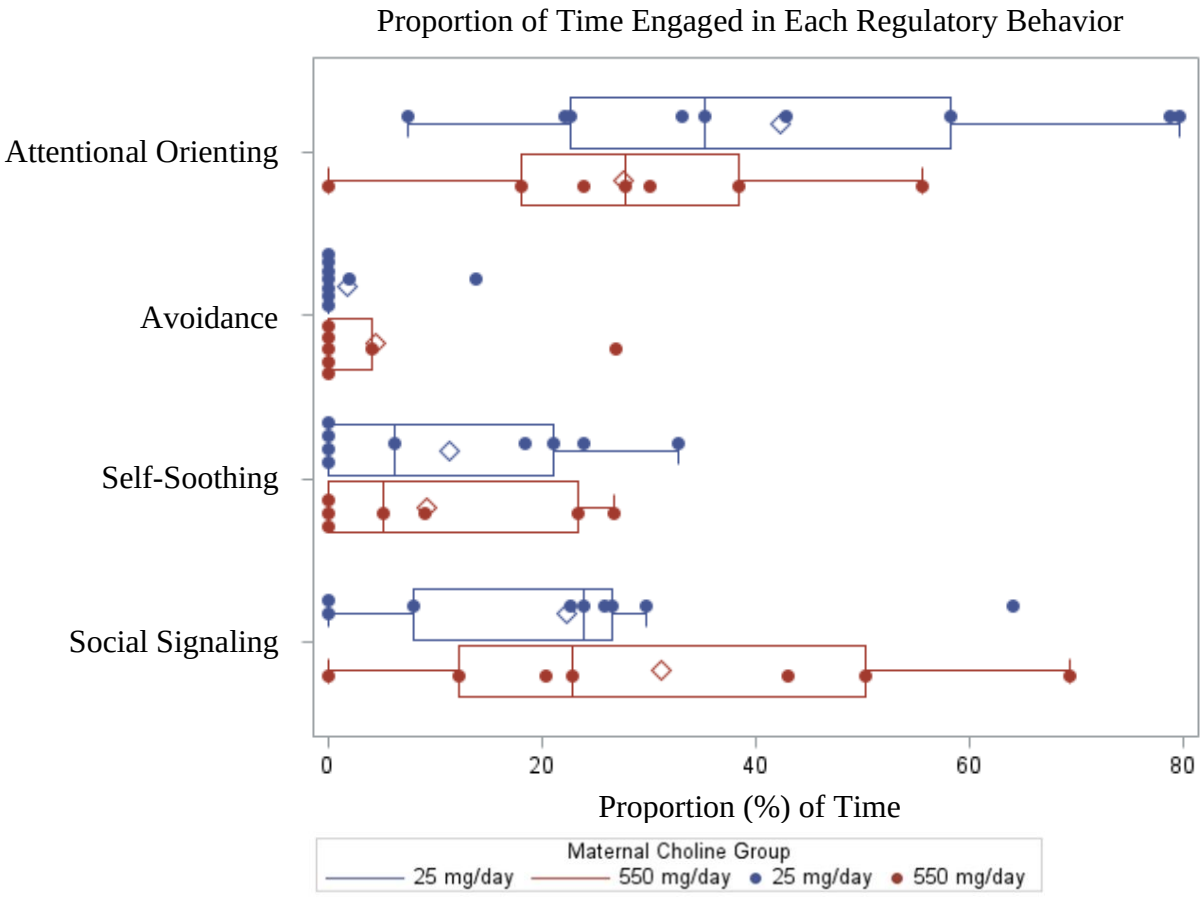


Figure 4.6: Proportion of time each group spent engaged in each category of self-regulatory behavior during the still-face phase. Individual participant data are represented as dots overlaying the box plots. Box plots display minimum and maximum scores for each group, as well as the interquartile ranges. Group means are represented by diamonds.

4.5 Discussion

Summary of Results

The findings of this small study did not reveal statistically significant (at $p < 0.05$) effects of maternal supplementation with 550 mg/day of choline (v 25 mg/day) on infant affect regulation, affect reactivity, or regulatory behaviors in response to the Face-to-Face Still Face Paradigm at 7 months of age.

The two groups did not differ in total negative affect during the play or still-face phases; however, infants in the 25 mg/day group expressed less negative affect during the reunion phase as compared to the 550 mg/day group, a difference that trended towards significance. Further, our primary outcome found that there was not a significant difference between the two groups in slope of total negative affect from the still-face to reunion phase.

The two groups did not differ significantly in their latency to negative affect in the still-face phase. However, infants in the 550 mg/day group had a lower probability of survival were less likely to make it to the end of the phase without getting upset and were more likely to get upset earlier in the phase, though this trend did not reach significance.

Lastly, neither group varied significantly in the duration or type of regulatory strategy employed during the still-phase phase, though several interesting trends emerged. Infants in the 25 mg/day group (v 550) spent on average about 12% more time during the still-face phase engaged in attentional orienting. Infants in the 550 mg/day group (v 25) spent on average about 10% more time during the still-face phase engaged in social signaling.

Interpretation of Results

Given the null results of the Face-to-Face Still Face Paradigm, it is reasonable to conclude that there is no effect of maternal choline supplementation on infant affect regulation.

If we assume that (1) the task was designed and implemented such that it successfully measured the outcomes of interest and (2) the results of the measure reflect the true effects in the population for randomly selected groups consuming one of the two supplements we provided, then we must conclude that there is no effect of MCS on the aspects of infant affect regulation and reactivity we measured. However, based on several lines of evidence (discussed below), this is not the most parsimonious conclusion to derive from our results.

First, we must examine whether we successfully administered the task such that it captured the outcomes of interest. To do so, we examined the overall patterns of our data for the "still-face" effect—decreased positive affect, increased negative affect, and increased gaze aversion from the play to still-face period. In our sample, we found that the duration of non-negative affect was significantly shorter in the still-face phase than in the play phase, and the duration of negative affect was significantly longer in the still-face phase than in the play phase. Further, the duration of gaze aversion was significantly longer in the still-face phase than in the play phase. The magnitude of these differences corresponds closely to differences reported in a meta-analysis of infant responses to the FFSF.[37] Thus, our results demonstrate that the FFSF paradigm was successfully administered in this small sample.

If methodological problems can't account for the absence of group differences, then should we conclude that maternal choline supplementation does not affect infant temperament or affect regulation? When one considers the issue of our small sample size, particularly as it affects our statistical power, the answer to this question is clearly no. Our original intended sample size was 30 infants (infants of all the mothers who participated in the pregnancy supplementation trial). The sample size for this study was not originally designed to assess subtle socioemotional outcomes in the offspring. Rather, the sample originated from a study of 30 pregnant women and

was powered to assess supplementation differences in biomarkers of maternal/fetal choline and DHA metabolism, not infant affect regulation. However, although the supplementation trial was not powered to detect an effect of MCS on infant outcomes, our previous ancillary follow-up to a controlled feeding trial produced significant effects of maternal choline supplementation on child cognitive functioning with group Ns of 9 and 11—including beneficial effects on child executive functioning, as demonstrated in Chapter 2 of this dissertation.[5,6,14]

Of this original sample of 30, only 26 agreed to participate in the postnatal follow-up study, and one infant's data was excluded *a priori* due to parental noncompliance with study protocols. Our sample size was then further compromised by COVID-19 related interruptions in research operations. Therefore, our analytical sample for the Still-Face Paradigm data consisted of only those infants whose 7-month visits fell before or after the two-month pandemic closure (N = 16).

Given this highly compromised sample, it is likely that we did not have sufficient power to detect an effect of maternal choline supplementation. To address this issue, we calculated the estimate of the effect for our primary outcome measure, decline in negative affect from the still-face to the reunion episode. The estimate of the effect associated with the group difference in decline in total negative affect, corrected for the different sample sizes of the two groups using Hedge's g, was -0.45, with a 95% confidence interval of -1.52–0.62.[21,52] Based on this estimate, future studies would require a minimum of 160 participants (80 per group) to achieve statistical significance for an estimated difference of this size.

Further, it is important to acknowledge that the variance in these data comes from a very small number of infants. 55.6% of infants in the 25 mg/day group and 42.9% of infants in the 550 mg/day did not express any negative affect during the still-face phase, resulting in total

negative affect scores of 0, with no variance in score within or between task phases. Therefore, the sampling distribution may be somewhat skewed in this sample. However, we investigated the possible effect of these scores on the results using descriptive statistics and t-tests, which supported the results of our main model.

Given the statistical challenges discussed here, accuracy is not possible in the interpretation of these data. We should not conclude that there is no effect of maternal choline supplementation on infant temperament or affect regulation, but rather that our data are not conclusive. It may be better to consider the risks and benefits associated with different interpretations of the infant affect regulation data.

First, we should explore the possible explanations for why we failed to detect an effect of maternal choline supplementation in this small sample. Our hypotheses were based on the small body of literature that has documented effects of maternal choline supplementation on socioemotional function in rodent models. However, in these studies, offspring of supplemented dams were evaluated when they were adults, not in early infancy.[18,27,39,46] This is an important distinction, as patterns of affect reactivity and regulation that are optimal for functioning in adulthood may not be the same as those that are optimal for functioning during infancy. Therefore, we may not have seen an effect of maternal choline supplementation because our outcome measures were not selected to find effects in the areas of infant socioemotional functioning where MCS may exert an effect.

It is also possible that the effects of maternal choline supplementation only become apparent not in a task of social stress, such as the FFSF, but rather in other areas of affect regulation, such as frustration, a negative affective response to goal blockage.[2,12] For example, one study of maternal choline supplementation in typically developing rats found that maternal

choline supplementation reduced burst responding in response to task errors, suggesting a reduction in the amount of frustration expressed by the supplemented animals.[18] While one could argue that the FFSF produces frustration in the infant as the result of goal blockage (continued interaction with the parent), the affective response to the still-face is more likely to elicit expressions of sadness than anger or frustration.[37] Therefore, behavioral measures that more specifically elicit frustration, such as the LabTAB anger/frustration tasks, may be more sensitive to the effects of maternal choline supplementation.[2] We administered the LabTAB anger/frustration tasks Gentle Arm Restraint and Attractive Toy Behind Barrier in this study, when the infants were 10 months old. However, analysis of these tasks is ongoing.

Lastly, it is possible that the null results found in this study are due to the timing of infant assessments. Although an infant's ability to self-regulate does mature over the first year of life, many of the affective and cognitive systems that underpin adaptive affect regulation are still immature at 7 months of age, and much of an infant's self-regulatory capacity during this time is contingent on external regulation by a parent or caregiver.[1,7–8,37] At this young age, behavioral signaling, including negative affect, acts as an important method by which an infant communicates their needs to the parent.[56] Therefore, the effects of maternal choline supplementation on offspring affective outcomes may become apparent as the child develops and gains more internal control of self-initiated regulatory processes[7–8] This sleeper effect has been seen in other studies of maternal prenatal supplementation and offspring outcomes. In a randomized controlled trial of prenatal supplementation with DHA, the offspring were followed up at 4, 6, 8, 12, and 18 months and assessed on attentional measures.[12] The results did not show differences between the two groups on attentional habituation at 8 months—however, at 18 months, children of mothers who received DHA supplementation showed superior attentional

focusing and reduced distractibility.[10] Therefore, if infants from our study were assessed study at an older age, effects of maternal choline supplementation may become more evident.

There are many possible explanations for our null findings; however, it is also worth investigating the patterns in our results, all of which were counter to our original hypotheses, which may be indicative of a more adaptive affective response in the infants born to women in the higher choline supplementation group. Although we did not have a specific *a priori* hypothesis about negative affect *during* the still-face phase, the pattern of our results is noteworthy. During the still-face phase, we found that infants in the 550 mg/day group had an average total negative affect score about 4 points higher than infants in the 25 mg/day group. Further, infants in the 25 mg/day group did not get significantly more upset in the still-face phase as compared to the play phase, while those in the 550 mg/day group did. It may be reasonable, therefore, to conclude that infants in the 25 mg/day group were actually hyporeactive in response to the still-face. Although negative affect, even in infants, is often associated with a "difficult" temperament, responding negatively to a perceived threat (such as a parent no longer responding to social cues) is a normative response and signal to the caregiver.[57] By signaling that something is wrong, the infant provides the caregiver the opportunity to respond in a synchronous and sensitive manner.[57] This concept underlies the Mutual Regulation Model (MRM), which proposes that infant-directed affective feedback to environmental stimuli, working together with sensitive caregiver responses, creates adaptive states of mutual regulation that support development of the infant's self-regulatory skills.[25,55] Therefore, not responding in a negative manner in a situation where negative affect provides a valuable signal to the caregiver is likely to increase the degree of mismatch in the parent-infant interaction.

This interpretation is supported by other studies of the FFSF in infancy. In one study of

typically developing infants, hyporeactivity in response to the still-face paradigm at 6 months of age predicted more oppositional defiance (ODD) behaviors later in childhood.[57] Although we cannot make a direct comparison to our results from this study, the authors found that infants who spent a greater proportion of the still-face phase directing gaze away from the mother, and who exhibited negative reactivity at 1 standard deviation below the sample mean, were significantly more likely to have problems with ODD-typic behaviors at 24, 30, and 36 months of age.[57] Another study of the FFSF found that infants who did not cry at all in response to the still-face at 6 months of age were rated as having more internalizing behaviors on the Child Behavior Checklist (CBCL) at 18 months of age.[41] Interestingly, the one human study that investigated the effects of maternal choline supplementation on infant affect regulation found that children of MCS mothers were rated as having fewer problems with social withdrawal, as well as fewer internalizing problems, at 40 months old.[45] Therefore, it is possible that maternal choline supplementation results in a more adaptive and socially oriented response to the distress of the still-face, and that lower choline intakes may place the offspring at increased risk for later behavior problems.

Evidence from the literature helps us to better understand our regulatory behavior data as well, and suggests that infants in the 550 mg/day group may have been engaging in a more adaptive form of self-regulation by attempting to elicit a response from their caregiver. For example, one study of typically developing infants found that failure to elicit the parent during the still-face phase at 6 months of age predicted avoidant attachment at 12 months of age.[19] Another study found that a greater proportion of time directing gaze away from the mother during the still-face phase (administered when infants were six months old) was associated with later oppositional and defiance behaviors in childhood.[57] Given that the FFSF paradigm is

characterized by a disruption in normal interaction between the infant and caregiver, social signaling may be a more adaptive regulatory behavior. Part of this may be because infants are not well-designed to self-regulate. Rather, they are designed to be regulated with the aid of a sensitive caregiver who is responsive to the infant's communicative signals.[8,10–11,19,23]

This hypothesis about the importance of social communication as a self-regulatory strategy may also help us to better understand our latency results. Our original hypothesis was that infants in the 550 mg/day group would have a longer latency to negative affect in the still-face phase—in fact, they had a shorter latency, though this trend was nonsignificant. This suggests that infants in the 550 mg/day group are more reactive to an environmental stressor; however, given that negative reactivity serves as an attempt to repair the social relationship in the context of the still-face, being quicker to respond to the caregiver's shift in behavior may reflect a superior ability to (1) recognize the disruption in the feedback loop between the infant and caregiver and (2) quickly signal to the caregiver in an attempt to repair that disruption.

Strengths and Limitations

There are several strengths of the present study. Firstly, our strong study design—a randomized controlled double-blind clinical trial—allows for strong causal inferences. Secondly, based on the number of supplement tubes returned by study participants, adherence to supplement intake appeared to be high among both groups in the pregnancy portion of the trial, which allows us to test our hypothesis with high confidence. Thirdly, the study design of providing a supplement in addition to usual diet makes the results of the study generalizable to the real-world scenario in which a pregnant woman may choose to or be prescribed to take a choline supplement as part of her prenatal regimen. We also used a widely used measure of infant reactivity and regulation, which has been demonstrated to reliably produce negative affect.

Although we did not find any effects of the choline intervention on these measures, this study still offers significant insight into the effect of choline on infant outcomes measured via the Face-to-Face Still-Face Paradigm.

However, there are a few limitations to this research as well. The small sample prevented us from being confident that our results reflect the results that would be found in a large, diverse reference population. We also lacked the statistical power to differentiate random variation across infants from systematic variation caused by the choline intervention.

There were also a few methodological challenges that may have limited our ability to detect effects in this study. Perhaps most important was our protocol for significant infant upset, which required experimenters to terminate the task after 15 seconds of continuous hard crying. This interval, which is shorter than intervals used in some other FFSF studies (~30 seconds)[37], meant that those infants who were most upset contributed less data to the task than those who were less distressed, which may have skewed our results towards less total negative affect.

Conclusions and Future Directions

This study offers insight into how to conduct future research on the effects of maternal choline supplementation on infant affect reactivity, regulation, and self-regulatory behaviors. First, as discussed above, taking a developmental perspective when adapting findings of the rodent literature, which is primarily conducted in adult offspring of supplemented dams, to human infants, may help researchers to distinguish between an ideal affective response pattern for a mature animal and an ideal affective response pattern for a human infant more reliant on external regulation. Second, methodological changes that allow for more of an opportunity to elicit negative affect—such as increasing the limit for duration of significant negative affect, or a repeated still-face protocol (in which a second still-face episode occurs after the first reunion

period)—may provide more information into patterns of negative reactivity and affect regulation.

It is crucial to continue to investigate the relationship between maternal choline supplementation and offspring outcomes in light of the low choline intake of most pregnant women in the United States. Currently, ~90% of pregnant women do not consume the recommended amount of choline. Importantly, there is an urgent need for larger dose-response randomized controlled trials to establish appropriate recommendations for choline intake during pregnancy. Further research is needed to understand the effect of maternal supplementation on infant temperament. If MCS does indeed result in better offspring emotional reactivity and regulation, then implications of raising the recommended choline intake levels for pregnant women could be considerable with population-wide shifts towards improved cognitive and emotional function, resulting in better health and economic success across the lifespan.

4.6 References

1. Adamson, L. B., & Frick, J. E. (2003). The Still Face: A History of a Shared Experimental Paradigm. *Infancy*, *4*(4), 451–473. https://doi.org/10.1207/S15327078IN0404_01

2. Aksan, N., Goldsmith, H. H., Smider, N. A., Essex, M. J., Clark, R., Hyde, J. S., Klein, M. H., & Vandell, D. L. (1999). Derivation and prediction of temperamental types among preschoolers. *Developmental Psychology*, *35*(4), 958–971. https://doi.org/10.1037/0012-1649.35.4.958

3. Albright, C. D., Tsai, A. Y., Friedrich, C. B., Mar, M.-H., & Zeisel, S. H. (1999). Choline availability alters embryonic development of the hippocampus and septum in the rat. *Developmental Brain Research*, *113*(1), 13–20. https://doi.org/10.1016/S0165-3806(98)00183-7

4. Ash, J. A., Velazquez, R., Kelley, C. M., Powers, B. E., Ginsberg, S. D., Mufson, E. J., & Strupp, B. J. (2014). Maternal choline supplementation improves spatial mapping and increases basal forebrain cholinergic neuron number and size in aged Ts65Dn mice. *Neurobiology of Disease*, *70*, 32–42. https://doi.org/10.1016/j.nbd.2014.06.001

5. Bahnfleth, C., Canfield, R., Nevins, J., Caudill, M., & Strupp, B. (2019). Prenatal Choline Supplementation Improves Child Color-location Memory Task Performance at 7 Y of Age (FS05-01-19). *Current Developments in Nutrition*, *3*(Supplement_1), nzz048.FS05-01-19. https://doi.org/10.1093/cdn/nzz048.FS05-01-19

6. Bahnfleth, C. L., Strupp, B. J., Caudill, M. A., & Canfield, R. L. (2022). Prenatal choline supplementation improves child sustained attention: A 7-year follow-up of a randomized controlled feeding trial. *The FASEB Journal*, *36*(1), e22054. https://doi.org/10.1096/fj.202101217R

7. Bernier, A., Carlson, S. M., & Whipple, N. (2010). From External Regulation to Self-Regulation: Early Parenting Precursors of Young Children's Executive Functioning. *Child Development*, *81*(1), 326–339. https://doi.org/10.1111/j.1467-8624.2009.01397.x

8. Blandon, A. Y., Calkins, S. D., Keane, S. P., & O'Brien, M. (2008). Individual differences in trajectories of emotion regulation processes: The effects of maternal depressive symptomatology and children's physiological regulation. *Developmental Psychology*, *44*(4), 1110–1123. https://doi.org/10.1037/0012-1649.44.4.1110

9. Boeke, C. E., Gillman, M. W., Hughes, M. D., Rifas-Shiman, S. L., Villamor, E., & Oken, E. (2013). Choline Intake During Pregnancy and Child Cognition at Age 7 Years. *American Journal of Epidemiology*, *177*(12), 1338–1347. https://doi.org/10.1093/aje/kws395

10. Bosquet Enlow, M., Kitts, R. L., Blood, E., Bizarro, A., Hofmeister, M., & Wright, R. J. (2011). Maternal posttraumatic stress symptoms and infant emotional reactivity and emotion regulation. *Infant Behavior and Development*, *34*(4), 487–503. https://doi.org/10.1016/j.infbeh.2011.07.007

11. Braungart-Rieker, J. M., Garwood, M. M., Powers, B. P., & Wang, X. (2001). Parental Sensitivity, Infant Affect, and Affect Regulation: Predictors of Later Attachment. *Child Development*, *72*(1), 252–270. https://doi.org/10.1111/1467-8624.00277

12. Calkins, S. D., Dedmon, S. E., Gill, K. L., Lomax, L. E., & Johnson, L. M. (2002). Frustration in Infancy: Implications for Emotion Regulation, Physiological Processes, and Temperament. *Infancy*, *3*(2), 175–197. https://doi.org/10.1207/S15327078IN0302_4

13. Caudill, M. A. (2010). Pre- and Postnatal Health: Evidence of Increased Choline Needs. *Journal of the American Dietetic Association*, *110*(8), 1198–1206. https://doi.org/10.1016/j.jada.2010.05.009

14. Caudill, M. A., Strupp, B. J., Muscalu, L., Nevins, J. E. H., & Canfield, R. L. (2018). Maternal choline supplementation during the third trimester of pregnancy improves infant information processing speed: A randomized, double-blind, controlled feeding study. *The FASEB Journal*, *32*(4), 2172–2180. https://doi.org/10.1096/fj.201700692RR

15. Cermak, J. M., Blusztajn, J. K., Meck, W. H., Williams, C. L., Fitzgerald, C. M., Rosene, D. L., & Loy, R. (1999). Prenatal Availability of Choline Alters the Development of Acetylcholinesterase in the Rat Hippocampus. *Developmental Neuroscience*, *21*(2), 94–104. https://doi.org/10.1159/000017371

16. Cermak, J. M., Holler, T., Jackson, D. A., & Blusztajn, J. K. (1998). Prenatal availability of choline modifies development of the hippocampal cholinergic system. *The FASEB Journal*, *12*(3), 349–357. https://doi.org/10.1096/fasebj.12.3.349

17. Cheatham, C. L., Goldman, B. D., Fischer, L. M., da Costa, K.-A., Reznick, J. S., & Zeisel, S. H. (2012). Phosphatidylcholine supplementation in pregnant women consuming moderate-choline diets does not enhance infant cognitive function: A randomized, double-blind,

placebo-controlled trial. *The American Journal of Clinical Nutrition*, *96*(6), 1465–1472. https://doi.org/10.3945/ajcn.112.037184

18. Cheng, R.-K., MacDonald, C. J., Williams, C. L., & Meck, W. H. (2008). Prenatal choline supplementation alters the timing, emotion, and memory performance (TEMP) of adult male and female rats as indexed by differential reinforcement of low-rate schedule behavior. *Learning & Memory*, *15*(3), 153–162. https://doi.org/10.1101/lm.729408

19. Cohn, J. F., Campbell, S. B., & Ross, S. (1991). Infant response in the still-face paradigm at 6 months predicts avoidant and secure attachment at 12 months. *Development and Psychopathology*, *3*(4), 367–376. https://doi.org/10.1017/S0954579400007574

20. Colombo, J., Kannass, K. N., Jill Shaddy, D., Kundurthi, S., Maikranz, J. M., Anderson, C. J., Blaga, O. M., & Carlson, S. E. (2004). Maternal DHA and the Development of Attention in Infancy and Toddlerhood. *Child Development*, *75*(4), 1254–1267. https://doi.org/10.1111/j.1467-8624.2004.00737.x

21. Craciunescu, C. N., Albright, C. D., Mar, M.-H., Song, J., & Zeisel, S. H. (2003). Choline Availability During Embryonic Development Alters Progenitor Cell Mitosis in Developing Mouse Hippocampus. *The Journal of Nutrition*, *133*(11), 3614–3618. https://doi.org/10.1093/jn/133.11.3614

22. *Effect Size Calculator (Cohen's D) for T-Test*. (n.d.). Retrieved June 29, 2022, from https://www.socscistatistics.com/effectsize/default3.aspx

23. Field, T., Vega-Lahr, N., Scafidi, F., & Goldstein, S. (1986). Effects of maternal unavailability on mother-infant interactions. *Infant Behavior and Development*, *9*(4), 473–478. https://doi.org/10.1016/0163-6383(86)90019-6

24. Friard, O., & Gamba, M. (2016). BORIS: A free, versatile open-source event-logging software for video/audio coding and live observations. *Methods in Ecology and Evolution*, *7*(11), 1325–1330. https://doi.org/10.1111/2041-210X.12584

25. Ham, J., & Tronick, E. (2006). Infant Resilience to the Stress of the Still-Face. *Annals of the New York Academy of Sciences*, *1094*(1), 297–302. https://doi.org/10.1196/annals.1376.038

26. Jahromi, L. B., Gulsrud, A., & Kasari, C. (2008). Emotional Competence in Children With

Down Syndrome: Negativity and Regulation. *American Journal on Mental Retardation*, *113*(1), 32–43. https://doi.org/10.1352/0895-8017(2008)113[32:ECICWD]2.0.CO;2

27. Klatt, K. C., McDougall, M. Q., Malysheva, O. V., Taesuwan, S., Loinard-González, A. (Alex) P., Nevins, J. E. H., Beckman, K., Bhawal, R., Anderson, E., Zhang, S., Bender, E., Jackson, K. H., King, D. J., Dyer, R. A., Devapatla, S., Vidavalur, R., Brenna, J. T., & Caudill, M. A. (2022). Prenatal choline supplementation improves biomarkers of maternal docosahexaenoic acid status among pregnant participants consuming supplemental DHA: A randomized controlled trial. *The American Journal of Clinical Nutrition*, nqac147. https://doi.org/10.1093/ajcn/nqac147

28. Langley, E. A., Krykbaeva, M., Blusztajn, J. K., & Mellott, T. J. (2015). High maternal choline consumption during pregnancy and nursing alleviates deficits in social interaction and improves anxiety-like behaviors in the BTBR T+Itpr3tf/J mouse model of autism. *Behavioural Brain Research*, *278*, 210–220. https://doi.org/10.1016/j.bbr.2014.09.043

29. Lauder, J. M., & Schambra, U. B. (1999). Morphogenetic roles of acetylcholine. *Environmental Health Perspectives*, *107*, 5.

30. Li, W., Woudstra, M. J., Branger, M. C. E., Wang, L., Alink, L. R. A., Mesman, J., & Emmen, R. A. G. (2019). The effect of the still-face paradigm on infant behavior: A cross-cultural comparison between mothers and fathers. *Infancy*, *24*(6), 893–910. https://doi.org/10.1111/infa.12313

31. Mathews, T. J. (2016). *Mean Age of Mothers is on the Rise: United States, 2000–2014*. *232*, 8.

32. Mayes, L. C., & Carter, A. S. (1990). Emerging Social Regulatory Capacities as Seen in the Still-Face Situation. *Child Development*, *61*(3), 754–763. https://doi.org/10.2307/1130960

33. McCann, J. C., Hudes, M., & Ames, B. N. (2006). An overview of evidence for a causal relationship between dietary availability of choline during development and cognitive function in offspring. *Neuroscience & Biobehavioral Reviews*, *30*(5), 696–712. https://doi.org/10.1016/j.neubiorev.2005.12.003

34. Meck, W. H., Smith, R. A., & Williams, C. L. (1988). Pre- and postnatal choline supplementation produces long-term facilitation of spatial memory. *Developmental Psychobiology*, *21*(4), 339–353. https://doi.org/10.1002/dev.420210405

35. Meck, W. H., Smith, R. A., & Williams, C. L. (1989). Organizational changes in cholinergic activity and enhanced visuospatial memory as a function of choline administered prenatally or postnatally or both. *Behavioral Neuroscience*, *103*(6), 1234–1241. https://doi.org/10.1037/0735-7044.103.6.1234

36. Meck, W. H., & Williams, C. L. (2003). Metabolic imprinting of choline by its availability during gestation: Implications for memory and attentional processing across the lifespan. *Neuroscience & Biobehavioral Reviews*, *27*(4), 385–399. https://doi.org/10.1016/S0149-7634(03)00069-1

37. Meck, W., Williams, C., Cermak, J., & Blusztajn, J. (2008). Developmental periods of choline sensitivity provide an ontogenetic mechanism for regulating memory capacity and age-related dementia. *Frontiers in Integrative Neuroscience*, *2*. https://www.frontiersin.org/article/10.3389/neuro.07.007.2007

38. Mesman, J., van IJzendoorn, M. H., & Bakermans-Kranenburg, M. J. (2009a). The many faces of the Still-Face Paradigm: A review and meta-analysis. *Developmental Review*, *29*(2), 120–162. https://doi.org/10.1016/j.dr.2009.02.001

39. Mohler, E. G., Meck, W. H., & Williams, C. L. (n.d.). *Sustained Attention in Adult Mice is Modulated by Prenatal Choline Availability*. 16.

40. Moon, J., Chen, M., Gandhy, S. U., Strawderman, M., Levitsky, D. A., Maclean, K. N., & Strupp, B. J. (2010). Perinatal choline supplementation improves cognitive functioning and emotion regulation in the Ts65Dn mouse model of Down syndrome. *Behavioral Neuroscience*, *124*(3), 346–361. https://doi.org/10.1037/a0019590

41. Moore, G. A., Cohn, J. F., & Campbell, S. B. (2001b). Infant affective responses to mother's still face at 6 months differentially predict externalizing and internalizing behaviors at 18 months. *Developmental Psychology*, *37*(5), 706–714. https://doi.org/10.1037/0012-1649.37.5.706

42. Niculescu, M. D., Craciunescu, C. N., & Zeisel, S. H. (2006). Dietary choline deficiency alters global and gene-specific DNA methylation in the developing hippocampus of mouse fetal brains. *The FASEB Journal*, *20*(1), 43–49. https://doi.org/10.1096/fj.05-4707com

43. *Opening Statement by Roy Pitkin on Dietary Reference Intakes for Thiamin, Riboflavin,*

Niacin, Vitamin B6, Folate, Vitamin B12, Pantothenic Acid, Biotin, and Choline. (n.d.). Retrieved March 28, 2022, from http://www8.nationalacademies.org/onpinews/newsitem.aspx?RecordID=s6015

44. Products—Data Briefs—Number 332—February 2019. (2019, June 10). http://www.cdc.gov/nchs/products/databriefs/db332.htm

45. Ross, R. G., Hunter, S. K., Hoffman, M. C., McCarthy, L., Chambers, B. M., Law, A. J., Leonard, S., Zerbe, G. O., & Freedman, R. (2016). Perinatal Phosphatidylcholine Supplementation and Early Childhood Behavior Problems: Evidence for CHRNA7 Moderation. *The American Journal of Psychiatry*, *173*(5), 509–516. https://doi.org/10.1176/appi.ajp.2015.15091188

46. Rueda, M., Posner, M., & Rothbart, M. (2005). The Development of Executive Attention: Contributions to the Emergence of Self-Regulation. *Developmental Neuropsychology*, *28*, 573–594. https://doi.org/10.1207/s15326942dn2802_2

47. Schulz, K. M., Pearson, J. N., Gasparrini, M. E., Brooks, K. F., Drake-Frazier, C., Zajkowski, M. E., Kreisler, A. D., Adams, C. E., Leonard, S., & Stevens, K. E. (2014). Dietary choline supplementation to dams during pregnancy and lactation mitigates the effects of in utero stress exposure on adult anxiety-related behaviors. *Behavioural Brain Research*, *268*, 104–110. https://doi.org/10.1016/j.bbr.2014.03.031

48. Signore, C., Ueland, P. M., Troendle, J., & Mills, J. L. (2008). Choline concentrations in human maternal and cord blood and intelligence at 5 y of age. *The American Journal of Clinical Nutrition*, *87*(4), 896–902. https://doi.org/10.1093/ajcn/87.4.896

49. Taesuwan, S., McDougall, M. Q., Malysheva, O. V., Bender, E., Nevins, J. E. H., Devapatla, S., Vidavalur, R., Caudill, M. A., & Klatt, K. C. (2021). Choline metabolome response to prenatal choline supplementation across pregnancy: A randomized controlled trial. *The FASEB Journal*, *35*(12), e22063. https://doi.org/10.1096/fj.202101401RR

50. Thomas, J. D., Abou, E. J., & Dominguez, H. D. (2009). Prenatal choline supplementation mitigates the adverse effects of prenatal alcohol exposure on development in rats. *Neurotoxicology and Teratology*, *31*(5), 303–311. https://doi.org/10.1016/j.ntt.2009.07.002

51. Thomas, J. D., Idrus, N. M., Monk, B. R., & Dominguez, H. D. (2010). Prenatal choline supplementation mitigates behavioral alterations associated with prenatal alcohol exposure in

rats. *Birth Defects Research Part A: Clinical and Molecular Teratology*, *88*(10), 827–837. https://doi.org/10.1002/bdra.20713

52. Thomas, J. D., La Fiette, M. H., Quinn, V. R. E., & Riley, E. P. (2000). Neonatal choline supplementation ameliorates the effects of prenatal alcohol exposure on a discrimination learning task in rats. *Neurotoxicology and Teratology*, *22*(5), 703–711. https://doi.org/10.1016/S0892-0362(00)00097-0

53. Thompson, B. (2007). Effect sizes, confidence intervals, and confidence intervals for effect sizes. *Psychology in the Schools*, *44*(5), 423–432. https://doi.org/10.1002/pits.20234

54. Tottenham, N., Tanaka, J. W., Leon, A. C., McCarry, T., Nurse, M., Hare, T. A., Marcus, D. J., Westerlund, A., Casey, B., & Nelson, C. (2009). The NimStim set of facial expressions: Judgments from untrained research participants. *Psychiatry Research*, *168*(3), 242–249. https://doi.org/10.1016/j.psychres.2008.05.006

55. Tronick, E., & Beeghly, M. (2011). Infants' Meaning-Making and the Development of Mental Health Problems. *The American Psychologist*, *66*(2), 107–119. https://doi.org/10.1037/a0021631

56. Velazquez, R., Ash, J. A., Powers, B. E., Kelley, C. M., Strawderman, M., Luscher, Z. I., Ginsberg, S. D., Mufson, E. J., & Strupp, B. J. (2013). Maternal choline supplementation improves spatial learning and adult hippocampal neurogenesis in the Ts65Dn mouse model of Down syndrome. *Neurobiology of Disease*, *58*, 92–101. https://doi.org/10.1016/j.nbd.2013.04.016

57. Wagner, N. J., Mills-Koonce, W. R., Propper, C. B., Willoughby, M. T., Rehder, P. D., Moore, G. A., & Cox, M. J. (2016). Associations between Infant Behaviors during the Face-To-Face Still-Face Paradigm and Oppositional Defiant and Callous-Unemotional Behaviors in Early Childhood. *Journal of Abnormal Child Psychology*, *44*(8), 1439–1453. https://doi.org/10.1007/s10802-016-0141-0

58. Wallace, T. C., & Fulgoni, V. L. (2016). Assessment of Total Choline Intakes in the United States. *Journal of the American College of Nutrition*, *35*(2), 108–112. https://doi.org/10.1080/07315724.2015.1080127

59. Wallace, T. C., & Fulgoni, V. L. (2017). Usual Choline Intakes Are Associated with Egg and Protein Food Consumption in the United States. *Nutrients*, *9*(8), 839.

https://doi.org/10.3390/nu9080839

60. Weinberg, M. K., & Tronick, E. Z. (1996). Infant Affective Reactions to the Resumption of Maternal Interaction after the Still-Face. *Child Development*, *67*(3), 905–914. https://doi.org/10.2307/1131869

61. Wong-Goodrich, S. J. E., Glenn, M. J., Mellott, T. J., Blusztajn, J. K., Meck, W. H., & Williams, C. L. (2008). Spatial memory and hippocampal plasticity are differentially sensitive to the availability of choline in adulthood as a function of choline supply in utero. *Brain Research*, *1237*, 153–166. https://doi.org/10.1016/j.brainres.2008.08.074

62. Wolf, I., Gilles, M., Peus, V., Scharnholz, B., Seibert, J., Jennen-Steinmetz, C., Krumm, B., Rietschel, M., Deuschle, M., & Laucht, M. (2018). Impact of prenatal stress on mother-infant dyadic behavior during the still-face paradigm. *Borderline Personality Disorder and Emotion Dysregulation*, *5*. https://doi.org/10.1186/s40479-018-0078-8

63. Wu, B. T. F., Dyer, R. A., King, D. J., Richardson, K. J., & Innis, S. M. (2012). Early Second Trimester Maternal Plasma Choline and Betaine Are Related to Measures of Early Cognitive Development in Term Infants. *PLOS ONE*, *7*(8), e43448. https://doi.org/10.1371/journal.pone.0043448

64. Yaari, M., Rotzak, N. L., Mankuta, D., Harel-Gadassi, A., Friedlander, E., Eventov-Friedman, S., Bar-Oz, B., Zucker, D., Shinar, O., & Yirmiya, N. (2018). Preterm-infant emotion regulation during the still-face interaction. *Infant Behavior and Development*, *52*, 56–65. https://doi.org/10.1016/j.infbeh.2018.05.008

65. Yan, J., Jiang, X., West, A. A., Perry, C. A., Malysheva, O. V., Devapatla, S., Pressman, E., Vermeylen, F., Stabler, S. P., Allen, R. H., & Caudill, M. A. (2012). Maternal choline intake modulates maternal and fetal biomarkers of choline metabolism in humans. *The American Journal of Clinical Nutrition*, *95*(5), 1060–1071. https://doi.org/10.3945/ajcn.111.022772

66. Zeisel, S. H. (2006). Choline: Critical Role During Fetal Development and Dietary Requirements in Adults. *Annual Review of Nutrition*, *26*(1), 229–250. https://doi.org/10.1146/annurev.nutr.26.061505.111156

67. Zeisel, S. H. (2009). Epigenetic mechanisms for nutrition determinants of later health outcomes. *The American Journal of Clinical Nutrition*, *89*(5), 1488S-1493S. https://doi.org/10.3945/ajcn.2009.27113B

68. Zeisel, S. H., Mar, M. H., Zhou, Z., & da Costa, K. A. (1995). Pregnancy and lactation are associated with diminished concentrations of choline and its metabolites in rat liver. *The Journal of Nutrition*, *125*(12), 3049–3054. https://doi.org/10.1093/jn/125.12.3049

69. Zeisel, S. H., & Niculescu, M. D. (2006). Perinatal Choline Influences Brain Structure and Function. *Nutrition Reviews*, *64*(4), 197–203. https://doi.org/10.1111/j.1753-4887.2006.tb00202.x

CHAPTER 5

CONCLUSIONS

5.1 Summary of Results

This doctoral work reports on the findings of two randomized controlled trials conducted to address gaps in our knowledge about the effects of prenatal choline supplementation on offspring cognitive and affective functioning.

Chapter Two presents data from our childhood study, an ancillary follow-up of a randomized controlled feeding trial, in which third trimester pregnant women were randomized to consume either 480 or 930 mg/day of choline until delivery. The results of this study provide evidence for the hypothesis that maternal choline supplementation during the 3rd trimester of pregnancy statistically significantly (at $p < 0.05$) improves child executive functioning at age 7 years as assessed by the Tower of London, a classic neuropsychological test of planning and problem-solving skills.[18,23]

Chapters Three and **Four** present data from our infancy study, an ancillary follow-up of a randomized controlled trial, in which pregnant women were randomized to consume either 550 mg/day or control (25 mg/day) choline in addition to usual diet from gestation week 12–16 until delivery. The results did not suggest a statistically significant (at $p < 0.05$) benefit of maternal choline supplementation on infant temperament or affect regulation. However, our small sample size does not allow for definitive conclusions, and indeed, interesting patterns emerged which may help to guide future research. The implications of each of these findings is discussed below.

5.2 Significance of Findings

Childhood Study

As a result of this doctoral work, we have gained important preliminary understanding of

the effects of increased maternal choline intake on child executive functioning during school age. Few human studies have been conducted to examine the effects of maternal choline intake on child cognitive outcomes and notably, this is the first study to demonstrate the benefit of maternal choline supplementation on child cognitive functioning at school age using direct behavioral measures. The present study adds to the growing literature supporting the idea that the amount of choline in the maternal diet has a causal role in the quality of her child's cognitive functioning.

Specifically, the pattern of results from the Tower of London task indicates that the children of women who consumed 930 mg/day choline during the third trimester were more successful in activating the cognitive processes underlying executive function, which translated to superior performance on their first attempt at solving the problems. Because executive functions are employed when a task requires controlled processes (i.e., new or non-habitual response sequences)[1], the fact that the children in the 930 mg/day group solved significantly more problems on the first attempt is indicative of superior executive function in this group. The pattern of results found in our measures of problem-solving speed support this interpretation. Children in the 930 mg/day group executed their solution to the problem significantly more quickly than children in the 480 mg/day group on the easier problems. This suggests that while both groups listened to experimenter directions to wait before starting the problem, only children in the 930 mg/day group used that time to plan effectively, resulting in faster problem solving and more accurate results.

This study also provides the first experimental evidence of the prenatal programming of executive function. Prenatal programming theories posit that prenatal experiences, especially during critical and sensitive periods, have long-lasting effects on fetal health and development,

including development of the brain and cognitive abilities.[11] Although a number of longitudinal prospective cohort studies have provided compelling evidence for a correlation between a number of adverse prenatal exposures (e.g., maternal obesity, stress) and risk for impaired executive function in childhood, these studies are confounded by other pre- and postnatal covariates and lack support for a causal pathway.[11] Due to the ethical challenges of randomizing human subjects to adverse prenatal experiences (e.g., stress, maternal tobacco use), experimental examination of the adverse prenatal factors that may predict later executive function is not possible. However, our highly controlled, randomized nutritional intervention provides clear evidence for a causal link between a prenatal manipulation (maternal choline supplementation) and child executive function at school age.

Improved executive functioning may have important real-world implications for school-aged children. The higher-order cognitive processes that are encompassed in executive function skills, including planning, working memory, attentional control, and inhibitory control, are all key to the development of academic skills, including reading and mathematical reasoning.[1,7,11,13,17] Some studies have found that the quality of early executive functioning is more important for school preparedness than general intelligence, and EF continues to predict competence in math and reading from elementary school through the early high school years.[17] Notably, in our study, the total TOL score of the two groups approximates the total scores of children in different grades in a large cohort of elementary-school aged children. Children in the 480 mg/day group scored at approximately a first-grade level, while the children in the 930 mg/day group scored at approximately a fifth-grade level. Therefore, maternal choline supplementation during pregnancy may result in population-wide shifts towards higher academic achievement and subsequent social and economic success.[17]

Infancy Study

Given the null results of both the Infant and Early Childhood Behavior Questionnaires and the Face-to-Face Still Face Paradigm, one must squarely face the straightforward interpretation of there being no effect of maternal choline supplementation on infant temperament or affect regulation. If we assume that (1) the tasks and questionnaires were designed and implemented such that they successfully measured the outcomes of interest and (2) the results of these measures reflect the true effects in the population for randomly selected groups consuming one of the two supplements we provided, then we must conclude that there is no effect of MCS on the aspects of infant temperament and affect regulation we measured. However, based on several lines of evidence (discussed below), this is not the most parsimonious conclusion to derive from our results.

One possible threat to the validity of concluding that there is no effect of MCS on infant temperament and affect regulation is one or more methodological problems. For example, it is possible that the way in which we administered the IB/ECBQ produced invalid results. In **Chapter 3**, to assess possible errors in administering the Infant Behavior and Early Childhood behavior questionnaires, we examined the cross-age correlations in each of the three factor scores. Given that the theory of infant temperament that underlies these questionnaires is that it is comprised of enduring characteristics, we would expect to see high longitudinal correlations in scores on the questionnaire scales and factors across time.[16] We found that there were significant intercorrelations for each of the three IBQ/ECBQ factors across each of the four ages (r range 0.44–0.74, p's < 0.05). These correlations are in line with and for some factors, higher than other studies that have examined the longitudinal intercorrelations for these factors.[14] This provides some validation for these dimensions of temperament being relatively stable and enduring

characteristics of the infants in our sample and leads to the conclusion that our failure to find an effect of the intervention was not due to technical or methodological error in administering these questionnaires.

It is also possible that we did not administer the FFSF properly, resulting in infant behavior that was abnormal for the paradigm. In **Chapter 4**, we examined overall patterns of our data to assess the success of task administration. The FFSF reliably produces the "still-face" effect—decreased positive affect, increased negative affect, and increased gaze aversion from the play to still-face period. Therefore, we would expect to see the same pattern in our data if the task was administered correctly. The duration of non-negative affect was significantly shorter in the still-face phase than in the play phase ($p = 0.036$), and the duration of negative affect was significantly longer in the still-face phase than in the play phase ($p = 0.04$). Further, the duration of gaze aversion was significantly longer in the still-face phase than in the play phase ($p < 0.0001$). The magnitude of these differences corresponds closely to differences reported in a meta-analysis of infant responses to the FFSF.[20] Thus, our results demonstrate that the FFSF paradigm was successfully administered in this small sample.

If methodological problems can't account for the absence of group differences, then should we conclude that maternal choline supplementation does not affect infant temperament or affect regulation? When one considers the issue of our small sample size, particularly as it affects our statistical power, the answer to this question is clearly no. The sample size for this study was not originally designed to assess subtle socioemotional outcomes in the offspring. Rather, the sample originated from a study of 30 pregnant women and was powered to assess supplementation differences in biomarkers of maternal/fetal choline and DHA metabolism, not infant temperament and affect regulation. Of this original sample of 30, only 26 agreed to

participate in the postnatal follow-up study, and one infant's data was excluded *a priori* due to parental noncompliance with study protocols. Our sample size was then further compromised by COVID-19 related interruptions in research operations. As noted in **Chapter 4**, our laboratory space was closed from March 13th until July 15th, during which time we were unable to conduct 7-month visits for 7 infants. Therefore, our analytical sample for the Still-Face Paradigm data consisted of only those infants whose 7-month visits fell before or after the two-month pandemic closure (N = 16).

Given this highly compromised sample, it is likely that we did not have sufficient power to detect an effect of maternal choline supplementation. Post-hoc power analyses were run to assess our ability to assess the effects of MCS on our primary outcome measures for the parent-report questionnaires and the FFSF (mean score on the Orienting/Regulation factor of the IBQ and decline in total negative affect from the still-face to reunion phase). These analyses revealed that with our sample of 12/13 per group, the power to detect a statistically significant effect for the mean score on the Orienting/Regulation factor of the IBQ was 21.5%. The power to detect a statistically significant effect for decline in total negative affect during the FFSF, with our sample of 7/9 per group, was 4.1%.[15] Clearly, our study was inadequately powered to detect effects of maternal choline supplementation on these outcome measures.

However, recognizing the significant hazards of using post-hoc power analyses to claim insufficient power, we also examined the 95% confidence intervals of the effect size for the two primary outcome measures listed above. For the mean score on the Orienting/Regulation factor of the IBQ, the effect-size estimate, corrected for the different sample sizes of the two groups using Hedge's g, was -0.45, with a confidence interval of -1.24–0.35.[24] Based on this estimate, future studies would require a minimum of 160 participants (80 per group) to achieve statistical

significance for an estimated difference of this size. Using Cohen's d guidelines for the magnitude of the effect size, the effect-size estimate we found is moderate (~0.5).[24] However, this translates to about a half-point difference in mean Orienting/Regulation factor score between the two groups. It is not possible to know from this small sample whether this is a meaningful difference in scores—in other words, if a half-point difference in score on the Orienting/Regulation factor represents individual differences in temperament that may impact the infant's development or risk of later behavioral problems. Future research investigating score differences of this size in a large, diverse sample, as well as following up with children at older ages (where behavioral problems may be more measurable) may help to elucidate the meaning of this effect.

We also calculated the estimate of the effect for our FFSF data. For decline in total negative affect during the FFSF, the estimate of the effect, corrected for the different sample sizes of the two groups using Hedge's g, was also -0.45, with a 95% confidence interval of -1.52–0.62.[15,24] Based on this estimate, future studies would require a minimum of 160 participants (80 per group) to achieve statistical significance for an estimated difference of this size. Using Cohen's d guidelines for the magnitude of the effect size, the effect-size estimate we found is moderate (~0.5). Again, it is not possible to know from this small sample if this is a meaningful difference in decline in negative affect: future research may help to elucidate the meaning of this effect.

Given the statistical challenges discussed here, accuracy is not possible in the interpretation of these data. We should not conclude that there is no effect of maternal choline supplementation on infant temperament or affect regulation, but rather that our data are not conclusive. It may be better to consider the risks and benefits associated with different

interpretations of the infant temperament and affect regulation data.

First, we should explore the possible explanations for why we failed to detect an effect of maternal choline supplementation in this small sample. One potential explanation may be that maternal choline supplementation only has an effect on infant temperament and affect regulation in populations where it exerts a neuroprotective effect in the face of a prenatal or developmental insult. In this case, we would expect a reduction or normalization of aberrant socioemotional function, but not necessarily improvements in temperament within the normal range.

There's some evidence in the literature to suggest this may be the case. For example, studies have found evidence that prenatal choline supplementation normalizes offspring socioemotional regulation in rodent disease models characterized by aberrant emotional reactivity, including Down syndrome.[21,25] In one study in mice, maternal choline supplementation normalized the emotional response of trisomic (Ts65Dn mouse model of Down syndrome) supplemented offspring to the level of the disomic mice, as compared to the unsupplemented trisomic animals.[21] However, MCS had no effect on the emotional response of disomic mice.[21]

This interpretation is strengthened by human studies of maternal choline supplementation and effects on infant temperament. In the one randomized control trial that examined the effects of maternal choline supplementation on offspring socioemotional outcomes, the offspring of prenatally supplemented mothers were assessed using the Child Behavior Checklist, a parent-report measure of offspring behavior, at age 40 months.[22] There was a significant interaction of schizophrenia risk allele and MCS on the Withdrawn scale of the CBCL, such that children in both groups (MCS and placebo) who did not have the risk allele did not differ significantly on their Withdrawn scores—however, for children who did have the risk allele, children in the

placebo group had significantly higher Withdrawn scores than those in the MCS group, indicating more behavioral problems related to social withdrawal.[22] This suggests that, for those offspring who may be at higher risk for later development of schizophrenia, prenatal choline supplementation may exert a protective effect on socioemotional outcomes. Although we do not have data from this study related to potential risk alleles for later mental illness, nor data on levels of prenatal maternal stress, we collected data on the demographics of the mothers and the health history of the infants in our sample. Our mothers were majority white and highly educated, with healthy, typically developing infants (Chapter 3, Chapter 4). It is reasonable to conclude that this was a sample of infants who were at low risk for exposure to prenatal or developmental insults, and therefore not likely to benefit from the neuroprotective effects of maternal choline supplementation.

Further, it's possible that we found null results on the IB/ECBQ because these measures assess temperament in a context where we would not expect the effects of maternal choline supplementation to be apparent. In particular, the IB/ECBQ are designed to assess everyday infant temperament and are not specific to measuring infant reactivity and regulation in response to stress.[16] In other human studies of maternal choline supplementation, the effect of MCS is not apparent until the system that is being measured is presented with a challenge. For example, in our previous ancillary follow-up study (Chapter 2), the children of women who consumed 930 (v 480) mg/day choline demonstrated superior performance on the Tower of London test of executive functioning when the problems were novel and most demanding of their problem-solving and planning abilities (Chapter 2). This pattern was consistent with another task from that same study, the Sustained Attention Task (SAT), on which children of women who consumed 930 (v 480) mg/day choline demonstrated superior performance, a difference that was

only significant on the shortest, most difficult trials.[4] Therefore, we may hypothesize that an effect of maternal choline supplementation on infant temperament (reduced reactivity or increased regulation) may only become apparent when the infant's affective reactivity and regulation systems are challenged, such as in the behavioral assessment of the Face-to-Face Still-Face paradigm.

It is also possible that the effects of maternal choline supplementation only become apparent not in a task of social stress, such as the FFSF, but rather in other areas of affect regulation, such as frustration, a negative affective response to goal blockage.[2,10] For example, one study of maternal choline supplementation in typically developing rats found that maternal choline supplementation reduced burst responding in response to task errors, suggesting a reduction in the amount of frustration expressed by the supplemented animals.[21] While one could argue that the FFSF produces frustration in the infant as the result of goal blockage (continued interaction with the parent), the affective response to the still-face is more likely to elicit expressions of sadness than anger or frustration.[20] Therefore, behavioral measures that more specifically elicit frustration, such as the LabTAB anger/frustration tasks, may be more sensitive to the effects of maternal choline supplementation.[5] We administered the LabTAB anger/frustration tasks Gentle Arm Restraint and Attractive Toy Behind Barrier in this study, when the infants were 10 months old. However, analysis of these tasks is ongoing.

Lastly, it is possible that the null results found in this study are due to the timing of infant assessments. Although an infant's ability to self-regulate does mature over the first year of life, many of the affective and cognitive systems that underpin adaptive affect regulation are still immature at 7 months of age, and much of an infant's self-regulatory capacity during this time is contingent on external regulation by a parent or caregiver.[5,6,20] Therefore, the effects of maternal

choline supplementation on offspring affective outcomes may become apparent as the child develops and gains more internal control of self-initiated regulatory processes.[10] This sleeper effect has been seen in other studies of maternal prenatal supplementation and offspring outcomes. In a randomized controlled trial of prenatal supplementation with DHA, the offspring were followed up at 4, 6, 8, 12, and 18 months and assessed on attentional measures.[12] The results did not show differences between the two groups on attentional habituation at 8 months—however, at 18 months, children of mothers who received DHA supplementation showed superior attentional focusing and reduced distractibility.[10] Therefore, if infants from our study were assessed study at an older age, effects of maternal choline supplementation may become more evident.

There are many possible explanations for our null findings; however, it is also worth investigating the patterns in our results which may be indicative of a more adaptive affective response in the infants born to women in the higher choline supplementation group. We did find one significant group difference on the parent-report measures of infant temperament (Chapter 3), with children in the 25 mg/day group ranked on average 0.68 points higher on the Duration of Orienting Scale of the IBQ ($p = 0.038$). An initial interpretation of this result may be that infants in the 25 mg/day group have superior attentional control to those in the 550 mg/day group, suggesting that there is no benefit of supplementation (and potentially, even a detrimental effect). However, the Duration of Orienting scale may not measure attentional control, per se.

The Duration of Orienting scale estimates infants' ability to maintain attention to or interaction with a single object for an extended period of time. Studies have found that infants' duration of engagement with single objects steadily declines over the first year of life.[19] A more flexible orienting reaction, which results in faster disengagement from stimuli and faster

attention shifting, allows for the infant to engage with a wider array of environmental stimuli and some evidence suggests it is an early predictor of child executive function and self-regulation.[5,6] This interpretation is supported by other results from this study, which found that infants in the 550 mg/day group had significantly faster reaction times than those in in the 25 mg/day group on the Visual Expectation Paradigm task of information processing and attentional orienting.[9]

For the Face-to-Face Still-Face Paradigm, although we did not find any significant differences in the groups for any of our outcome measures, there were some intriguing patterns in the data that may warrant further investigation. Infants in the 550 mg/day group were more likely than those in the 25 mg/day group to express any negative affect in response to the still-face, as well as had higher total negative affect in both the still-face and reunion episodes. Additionally, they spent more time during the still-face phase engaged in social signaling than infants in the 25 mg/day group. Although higher negative reactivity may seem like a poor outcome, or an indication of failure to regulate, it may represent a functionally valuable response by signaling to the caregiver in the context of the disruption in social feedback between the infant and their parent (**Chapter 4**). Therefore, it is possible that maternal choline supplementation results in a more adaptive and socially oriented response to the distress of the still-face, and that lower choline intakes, which result in a hyporeactive response, may place the offspring at increased risk for later behavior problems.

5.3 Future Directions

Future research into the effects of maternal choline supplementation on offspring self-regulatory outcomes may be able to pursue several interesting questions. First, it is important to confirm the findings of our childhood study, an ancillary follow-up of a controlled feeding trial, using a large RCT. Although our results provide compelling evidence for the effects of maternal

choline supplementation on offspring executive function at age 7 years, as well as effects on information processing speed in infancy and sustained attention and memory at age seven years[3–4], the data from this small, relatively homogenous sample is not generalizable to a larger, more diverse population. Further, this feeding trial highly controlled mothers' prenatal choline intake. Future RCTs should examine maternal choline supplementation in addition to usual diet (much like our infancy study) in order to better understand the effects of maternal choline supplementation on offspring executive function in a more real-world context (i.e., to assess the effectiveness of maternal choline supplementation).

This doctoral work also helps to identify gaps in our knowledge of the effects of maternal choline supplementation on offspring affect regulation. There are a number of questions that we were not able to address in this study. Some of these are listed below:

1. **Timing of Supplementation:** Timing of prenatal supplementation may be particularly important for understanding the effects of maternal choline supplementation on infant temperament and affect regulation. In the infancy study, supplementation began between gestation weeks 12–16, which are approximately at the beginning of the second trimester of pregnancy. However, affect is, from an evolutionary perspective, "older" than higher-order cognition, which means that the neural circuits that underly it begin to develop fairly early in gestation. Indeed, neural circuits of the limbic system, which includes many structures related to the development and control of affect (such as the amygdala), begin to develop as early as two weeks' gestation.[6] Therefore, it is plausible that maternal choline supplementation beginning as early as the first trimester of pregnancy may produce more pronounced effects on offspring affect.
2. **Timing of Postnatal Assessment:** As discussed above, postnatal assessment at an older

age, when affect regulation is more mature, using age-appropriate measures of temperament and affect reactivity and regulation, may make the potential effects of maternal choline supplementation more evident. Assessment across a broader range of childhood may help to elucidate any effects of maternal choline supplementation on the long-term development of self-regulation.

It is crucial to continue to investigate the relationship between maternal choline supplementation and offspring outcomes in light of the low choline intake of most pregnant women in the United States. Currently, ~90% of pregnant women do not consume the recommended amount of choline. Importantly, there is an urgent need for large dose-response randomized controlled trials to establish appropriate recommendations for choline intake during pregnancy. Based on the estimated effect sizes calculated from our infancy data, future studies should enroll 160 participants (80 per group) at a minimum. Further research is needed to understand the effect of maternal supplementation on infant temperament. If MCS does indeed result in better offspring emotional reactivity and regulation, then implications of raising the recommended choline intake levels for pregnant women could be considerable with population-wide shifts towards improved cognitive and emotional function, resulting in better health and economic success across the lifespan.

5.4 References

1. Ahmed, S. F., Kuhfeld, M., Watts, T. W., Davis-Kean, P. E., & Vandell, D. L. (2021). Preschool executive function and adult outcomes: A developmental cascade model. *Developmental Psychology*, *57*(12), 2234–2249. https://doi.org/10.1037/dev0001270

2. Aksan, N., Goldsmith, H. H., Smider, N. A., Essex, M. J., Clark, R., Hyde, J. S., Klein, M. H., & Vandell, D. L. (1999). Derivation and prediction of temperamental types among preschoolers. *Developmental Psychology*, *35*(4), 958–971. https://doi.org/10.1037/0012-1649.35.4.958

3. Bahnfleth, C., Canfield, R., Nevins, J., Caudill, M., & Strupp, B. (2019). Prenatal Choline Supplementation Improves Child Color-location Memory Task Performance at 7 Y of Age (FS05-01-19). *Current Developments in Nutrition*, *3*(Supplement_1), nzz048.FS05-01-19. https://doi.org/10.1093/cdn/nzz048.FS05-01-19

4. Bahnfleth, C. L., Strupp, B. J., Caudill, M. A., & Canfield, R. L. (2022). Prenatal choline supplementation improves child sustained attention: A 7-year follow-up of a randomized controlled feeding trial. *The FASEB Journal*, *36*(1), e22054. https://doi.org/10.1096/fj.202101217R

5. Baumeister, R. F., & Vohs, K. D. (Eds.). (2004). *Handbook of self-regulation: Research, theory, and applications*. Guilford Press.

6. Blair, C., & Ku, S. (2022). A Hierarchical Integrated Model of Self-Regulation. *Frontiers in Psychology*, *13*. https://www.frontiersin.org/article/10.3389/fpsyg.2022.725828

7. Blair, C., & Razza, R. P. (2007). Relating Effortful Control, Executive Function, and False Belief Understanding to Emerging Math and Literacy Ability in Kindergarten. *Child Development*, *78*(2), 647–663. https://doi.org/10.1111/j.1467-8624.2007.01019.x

8. Braungart-Rieker, J. M., Garwood, M. M., Powers, B. P., & Wang, X. (2001). Parental Sensitivity, Infant Affect, and Affect Regulation: Predictors of Later Attachment. *Child Development*, *72*(1), 252–270. https://doi.org/10.1111/1467-8624.00277

9. Brinkman, J. E. (2021). *Randomized Controlled Trial of Maternal Choline Supplementation: Effects on Infant Information Processing Speed*. https://doi.org/10.7298/w2e9-5n52

10. Calkins, S. D., Dedmon, S. E., Gill, K. L., Lomax, L. E., & Johnson, L. M. (2002). Frustration in Infancy: Implications for Emotion Regulation, Physiological Processes, and Temperament. *Infancy*, *3*(2), 175–197. https://doi.org/10.1207/S15327078IN0302_4

11. Camerota, M., & Willoughby, M. T. (2020). Prenatal Risk Predicts Preschooler Executive Function: A Cascade Model. *Child Development*, *91*(3), e682–e700. https://doi.org/10.1111/cdev.13271

12. Colombo, J., Kannass, K. N., Jill Shaddy, D., Kundurthi, S., Maikranz, J. M., Anderson, C. J., Blaga, O. M., & Carlson, S. E. (2004). Maternal DHA and the Development of Attention in Infancy and Toddlerhood. *Child Development*, *75*(4), 1254–1267. https://doi.org/10.1111/j.1467-8624.2004.00737.x

13. Diamond, A., & Lee, K. (2011). Interventions Shown to Aid Executive Function Development in Children 4 to 12 Years Old. *Science*, *333*(6045), 959–964. https://doi.org/10.1126/science.1204529

14. Dias, C. C., Costa, R., Pinto, T. M., & Figueiredo, B. (2021). The Infant Behavior Questionnaire – Revised: Psychometric properties at 2 weeks, 3, 6 and 12 months of life. *Early Human Development*, *153*, 105290. https://doi.org/10.1016/j.earlhumdev.2020.105290

15. *Effect Size Calculator (Cohen's D) for T-Test*. (n.d.). Retrieved June 29, 2022, from https://www.socscistatistics.com/effectsize/default3.aspx

16. Gartstein, M. A., & Rothbart, M. K. (2003). Studying infant temperament via the Revised Infant Behavior Questionnaire. *Infant Behavior and Development*, *26*(1), 64–86. https://doi.org/10.1016/S0163-6383(02)00169-8

17. Gathercole, S. E., Pickering, S. J., Knight, C., & Stegmann, Z. (2004). Working memory skills and educational attainment: Evidence from national curriculum assessments at 7 and 14 years of age. *Applied Cognitive Psychology*, *18*(1), 1–16. https://doi.org/10.1002/acp.934

18. Krikorian, R., Bartok, J., & Gay, N. (1994). Tower of london procedure: A standard method and developmental data. *Journal of Clinical and Experimental Neuropsychology*, *16*(6), 840–850.

19. Lewis, M., Goldberg, S., & Campbell, H. (1969). A Developmental Study of Information

Processing within the First Three Years of Life: Response Decrement to a Redundant Signal. *Monographs of the Society for Research in Child Development*, *34*(9), iii–41. https://doi.org/10.2307/1165696

20. Mesman, J., van IJzendoorn, M. H., & Bakermans-Kranenburg, M. J. (2009). The many faces of the Still-Face Paradigm: A review and meta-analysis. *Developmental Review*, *29*(2), 120–162. https://doi.org/10.1016/j.dr.2009.02.001

21. Moon, J., Chen, M., Gandhy, S. U., Strawderman, M., Levitsky, D. A., Maclean, K. N., & Strupp, B. J. (2010). Perinatal choline supplementation improves cognitive functioning and emotion regulation in the Ts65Dn mouse model of Down syndrome. *Behavioral Neuroscience*, *124*(3), 346–361. https://doi.org/10.1037/a0019590

22. Ross, R. G., Hunter, S. K., Hoffman, M. C., McCarthy, L., Chambers, B. M., Law, A. J., Leonard, S., Zerbe, G. O., & Freedman, R. (2016). Perinatal Phosphatidylcholine Supplementation and Early Childhood Behavior Problems: Evidence for CHRNA7 Moderation. *The American Journal of Psychiatry*, *173*(5), 509–516. https://doi.org/10.1176/appi.ajp.2015.15091188

23. Shallice, T. (1982). Specific Impairments of Planning. *The Neuropsychology of Cognitive Function*, *298*(1089), 199–209.

24. Thompson, B. (2007). Effect sizes, confidence intervals, and confidence intervals for effect sizes. *Psychology in the Schools*, *44*(5), 423–432. https://doi.org/10.1002/pits.20234

25. Velazquez, R., Ash, J. A., Powers, B. E., Kelley, C. M., Strawderman, M., Luscher, Z. I., Ginsberg, S. D., Mufson, E. J., & Strupp, B. J. (2013). Maternal choline supplementation improves spatial learning and adult hippocampal neurogenesis in the Ts65Dn mouse model of Down syndrome. *Neurobiology of Disease*, *58*, 92–101. https://doi.org/10.1016/j.nbd.2013.04.016

APPENDIX A: FULL TESTING PROTOCOL, CHILDHOOD STUDY

A rigorous, two-day testing protocol was designed to assess the effects of maternal choline supplementation on child cognition and hypothalamus-pituitary-adrenal (HPA) axis function at 7 years of age. Children participated in 3 hours of cognitive testing, split into two 90-minute sessions across 2 consecutive days. A summary of the tasks completed by the children is presented in Table A.1.

Testing Day 1	**Testing Day 2**
Parent Consent and Child Assent	Picture Memory
Zoo Locations	Attention Network Task
Sustained Attention Task	Saliva Sample 1 and Break
Saliva Sample 1 and Break	Cancellation
Bug Search	Similarities
Information	Mr. Peanut, 8 Second
Mr. Peanut, 1 Second	Weight Measurement and Break
Height Measurement and Break	Block Design
Tower of London	Matrix Reasoning
Continuous Performance Function	Continuous Performance Function, Delayed
Saliva Sample 2	Saliva Sample 2

Table A.1: Summary of tasks completed by children in the childhood study. Bolded tasks are discussed in this dissertation.

Testing was conducted by two trained graduate students blinded to maternal choline group assignment. Testers were randomized to participants as they enrolled in the study. To ensure consistency between testers and adherence to task protocols, all testing sessions were audio- and video-recorded.

APPENDIX B: FULL TESTING PROTOCOL, INFANCY STUDY

A comprehensive protocol was designed to assess the effects of maternal choline supplementation on child cognition and emotion across the first year of life. Tasks were designed to be both developmentally appropriate and to assess the cognitive functions that were shown to be improved by maternal choline supplementation in rodents, including memory and attention[2,3], as well as to replicate our findings on infant information processing from the controlled feeding trial.[1] The rodent data on the effects of maternal choline supplementation on affect regulation is more limited, so the behavioral tasks selected assessed similar behaviors, including affective response to a violation of expectations in a social encounter. Children participated in about 45 minutes to 1 hour of cognitive and behavioral testing. A summary of the tasks completed by the children is presented in Table B.1.

Construct	5 Months	7 Months	10 Months	13 Months
Memory	Visual Paired Comparison	Visual Paired Comparison	Visual Paired Comparison	Visual Paired Comparison
Attention Orienting Speed	Visual Expectation Paradigm	Visual Expectation Paradigm	Visual Expectation Paradigm	Visual Expectation Paradigm
Focused Attention	Free-play with Toy	Free-play with Toy	Free-play with Toy	Free-play with Toy
Affect Regulation	NA	**Face-to-Face Still-Face Paradigm (FFSF)**	Lab-TAB: Gentle Arm Restraint and Barrier	Face-to-Face Still-Face Paradigm (FFSF)
Autonomic Regulation	ECG during FFSF	ECG during FFSF	ECG during FFSF	ECG during FFSF

Table B.1: Summary of tasks completed by children in the infancy study. Bolded tasks are discussed in this dissertation.

While infants were participating in cognitive and behavioral tasks, parents were asked to fill out several questionnaires, including a health history questionnaire, sleep questionnaire, and

food-frequency questionnaire (FFQ). A summary of the questionnaires administered is in Table B.2.

5 Months	7 Months	10 Months	13 Months
Infant Behavior Questionnaire	**Infant Behavior Questionnaire**	**Infant Behavior Questionnaire**	**Early Childhood Behavior Questionnaire**
Health History Questionnaire	Health History Questionnaire	Health History Questionnaire	Health History Questionnaire
Brief Infant Sleep Questionnaire	Brief Infant Sleep Questionnaire	Brief Infant Sleep Questionnaire	Brief Infant Sleep Questionnaire
Food-Frequency Questionnaire	Food-Frequency Questionnaire	Food-Frequency Questionnaire	Food-Frequency Questionnaire

Table B.2: Summary of questionnaires completed by parents in the infancy study. Bolded questionnaires are discussed in this dissertation.

Testing was conducted by two trained graduate students blinded to maternal choline group assignment. Testers were randomized to participants as they enrolled in the study. To ensure consistency between testers and adherence to task protocols, all testing sessions were audio- and video-recorded.

References, Appendix B

125. Caudill, M. A., Strupp, B. J., Muscalu, L., Nevins, J. E. H., & Canfield, R. L. (2018a). Maternal choline supplementation during the third trimester of pregnancy improves infant information processing speed: A randomized, double-blind, controlled feeding study. *The FASEB Journal*, *32*(4), 2172–2180. https://doi.org/10.1096/fj.201700692RR

126. Meck, W. H., & Williams, C. L. (2003). Metabolic imprinting of choline by its availability during gestation: Implications for memory and attentional processing across the lifespan. *Neuroscience & Biobehavioral Reviews*, *27*(4), 385–399. https://doi.org/10.1016/S0149-7634(03)00069-1

127. Mohler, E. G., Meck, W. H., & Williams, C. L. (n.d.). *Sustained Attention in Adult Mice is Modulated by Prenatal Choline Availability*. 16.

APPENDIX C: CALCULATION OF PROBLEM DIFFICULTY AND PROBLEM CHARACTERISTICS, TOWER OF LONDON

Problem difficulty was obtained from Unterrainer et al.[1], who conducted the Tower of London task in a cohort of 6- through 9-year-olds, and defined problem difficulty as one minus the proportion of problems solved on the first attempt across the entire sample. We selected a subset of problems from those administered to the Unterrainer et al. cohort to represent a range of difficulty within each level of minimum moves. We calculated problem difficulty as one minus the proportion of problems solved on the first attempt in the subset of 6-7-year olds in the Unterrainer et al. cohort. Problem characteristics and calculations are in Table C.1. The start and end positions of all task problems are in Figure C.1.

Problem Number: Cholkids Study	Problem Number: Unterrainer et al. Study	Number of Moves	% Correct: 6-7 y [(N= 48, M=7.0 (6.2-7.8 y)]	Problem Difficulty (1 - % Correct)
1	5	3	0.82	0.18
2	4	3	0.76	0.24
3	3	3	0.65	0.35
4	15	4	0.78	0.22
5	16	4	0.69	0.31
6	14	4	0.57	0.43
7	10	4	0.39	0.61
8	19	5	0.61	0.39
9	20	5	0.37	0.63
10	17	5	0.18	0.82
11	23	5	0.18	0.82
12	22	5	0.08	0.92
13	24	5	0.20	0.80

Table C.1: Problem characteristics from the Unterrainer et al.[1] cohort used to calculate the measure of problem difficulty used in our analysis of the Tower of London task.

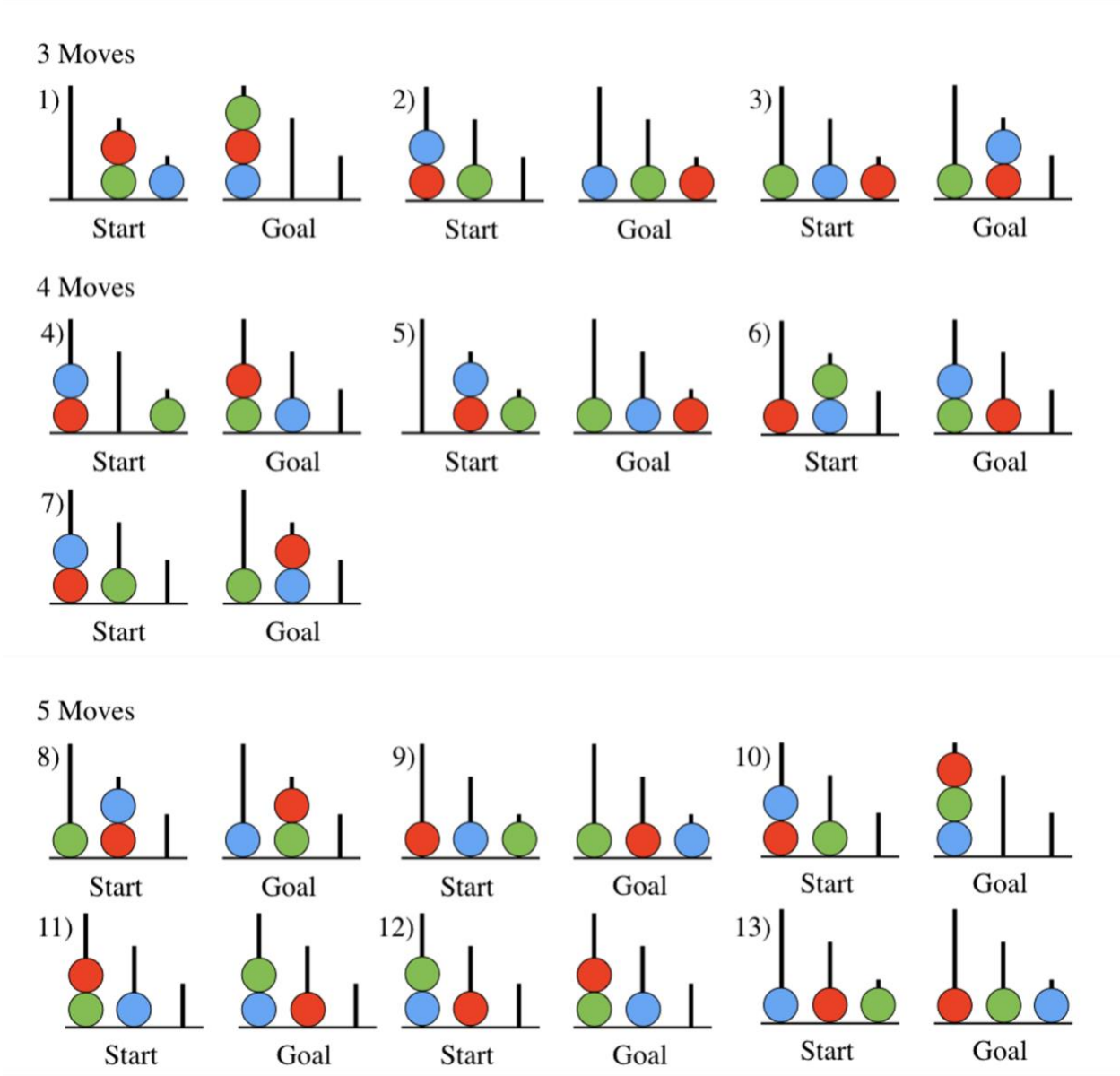


Figure C.1: Start and goal configurations of problems presented in the Tower of London task. The problems presented only had one solution path that would allow them to be solved in the minimum number of moves.

References, Appendix C

1. Unterrainer, J. M., Kaller, C. P., Loosli, S. V., Heinze, K., Ruh, N., Paschke-Müller, M., Rauh, R., Biscaldi, M., & Rahm, B. (2015). Looking ahead from age 6 to 13: A deeper insight into the development of planning ability. British Journal of Psychology, 106(1), 46–67. https://doi.org/10.1111/bjop.12065

APPENDIX D: TASK INSTRUCTIONS, TOWER OF LONDON

The full script for the Tower of London task is presented below. Because we were most interested in assessing planning, the instructions repeatedly encouraged the children to consider how to solve the problem before beginning to make moves. These instructions were given before the practice trial and before each of the test problems.

Demonstration slide:

"Here you can see two different patterns of colored balls on wooden pegs. The way you play the game is to make the bottom pattern ***(point to bottom pattern)*** look like the top pattern ***(point to the top pattern)***. Before we start the game, there are some rules that you need to remember to play the game."

1. **First**, you can't move a ball that is underneath another ball, like this green one ***(point to green ball)***. If you want to move that ball, you have to move the one on top to another peg first.
2. **Second**, you can only put a certain number of balls on each peg. Three balls can fit on the left peg, two can fit on the middle peg, and one can fit on the right peg.
3. **Finally,** the goal is to make the new pattern in the smallest number of moves possible. A move is any time you pick up a ball and put it on a new peg. The smallest number of moves possible will be shown in the upper right corner, but I will remind you each time how many moves you should make.

Practice Slide:

"Now look at the bottom pattern and think carefully about how you can make it look like the top pattern in just two moves. Before you start, make sure you know which moves you need to make. Click the ball you want to move and drag it to where you want to move it."

Repeat for each problem:

"This is an N move problem. Think carefully about how you can make the bottom pattern look like the top pattern using N moves. Wait until you think you know which moves to make. Then make your moves."

APPENDIX E: THE INFANT BEHAVIOR QUESTIONNAIRE-REVISED

The Infant Behavior Questionnaire (IBQ) was developed in 1981 by Dr. Mary Rothbart[1] and revised and refined by Dr. Rothbart and her colleague Dr. Marsha Gartstein in 1998 (IBQ-R).[2] In 2008, a short form (91 items) and very short form (37 items) version of the IBQ was developed by Dr. Sam Putnam.[3] The short form of the IBQ-R was administered to parents in this study.

Instructions:

As you read each description of the baby's behavior below, please indicate how often the baby did this during the LAST WEEK (the past seven days) by circling one of the numbers in the left column. These numbers indicate how often you observed the behavior described during the last week.

(0) Do Not Wish to Answer
(1) Never
(2) Very Rarely
(3) Less than Half the Time
(4) About Half the Time
(5) More than Half the Time
(6) Almost Always
(7) Always
(X) Does Not Apply

The Does Not Apply (X) column is used when you did not see the baby in the situation described during the last week. For example, if the situation mentions the baby having to wait for food or liquids and there was no time during the last week when the baby had to wait, circle the (X) column. Does Not Apply is different from Never (1). Never is used when you saw the baby in the situation, but the baby never engaged in the behavior listed during the last week. For example, if the baby did have to wait for food or liquids at least once but never cried loudly while waiting, circle the (1) column. Please be sure to circle a number for every item.

Questionnaire Items:

Q1: During the past week, how often did your baby make talking sounds when they were ready for more food?

Q2: During the past week, how often did your baby seem angry (crying and fussing) when you left him/her in the crib?

Q3: During the past week, how often did your baby seem contented when left in the crib?

Q4: During the past week, how often did your baby cry or fuss before going to sleep for naps?

Q5: During the past week, how often did your baby look at pictures in books and/or magazines for 5 minutes or longer at a time

Q6: During the past week, how often did your baby stare at a mobile, crib bumper, or picture for 5 minutes or longer?

Q7: During the past week, how often did your baby play with one toy or object for 5 – 10 minutes?

Q8: During the past week, how often did your baby play with one toy or object for 10 minutes or longer?

Q9: During the past week, how often did your baby laugh aloud in play?

Q10: During the past week, how often did your baby repeat the same movement with an object for 2 minutes or longer (e.g., putting a block in a cup, kicking or hitting a mobile)?

Q11: During the past week, how often did your baby smile or laugh after accomplishing something (e.g. stacking blocks, etc.)?

Q12: During the past week, how often did your baby smile or laugh when given a toy?

Q13: During the past week, how often did your baby enjoy being read to?

Q14: During the past week, how often did your baby enjoy hearing the sound of words, as in nursery rhymes?

Q15: During the past week, how often did your baby enjoy gentle rhythmic activities, such as rocking or swaying?

Q16: During the past week, how often did your baby enjoy being tickled by you or someone else in your family?

Q17: During the past week, how often did your baby enjoy the feel of soft blankets?
Q18: During the past week, how often did your baby enjoy being rolled up in a warm blanket?

Q19: During the past week, how often did your baby enjoy listening to a musical toy in a crib?

Q20: During the past week, how often did your baby look up from playing when the telephone rang?

Q21: During the past week, how often did your baby protest being placed in a confining space (infant seat, play pen, car seat, etc.)

Q22: During the past week, how often did your baby startle at a sudden change in body position (for example, when moved suddenly?)

Q23: During the past week, how often did your baby move quickly towards new objects?

Q24: During the past week, how often did your baby show a strong desire for something they wanted?

Q25: During the past week, how often did your baby watch adults performing household activities (e.g., cooking, etc.) for more than five minutes?

Q26: During the past week, how often did your baby squeal or shout when excited?

Q27: During the past week, how often did your baby notice low-pitched noises (e.g. air conditioner, heating system, or refrigerator running or starting up)?

Q28: During the past week, how often did your baby notice a change in light when a cloud passed over the sun?

Q29: During the past week, how often did your baby notice the sound of an airplane passing overhead?

Q30: During the past week, how often did your baby notice a bird or a squirrel up in a tree?

Q31: During the past week, how often did your baby notice fabrics with scratchy texture (e.g. wool?)

Q32: During the past week, how often did your baby appear sad for no apparent reason?

Q33: During feeding in the past week, how often did your baby lie or sit quietly?

Q34: During feeding in the past week, how often did your baby squirm or kick?

Q35: During feeding in the past week, how often did your baby wave his/her arms?

Q36: When going to sleep at night during the past week, how often did your baby fall asleep within two minutes?

Q37: During the past week, when going to sleep at night during the past week, how often did your baby have a hard time settling down to sleep?

Q38: During the past week, when going to sleep at night during the past week, how often did your baby settle down to sleep easily?

Q39: When being dressed or undressed during the past week, how often did your baby squirm and/or try to roll away?

Q40: When being dressed or undressed during the past week, how often did your baby smile or laugh?

Q41: When being undressed or dressed during the past week, how often did your baby coo or vocalize?

Q42: When put into the bath water during the past week, how often did your baby smile?

Q43: When put into the bath water during the past week, how often did your baby laugh?

Q44: When tossed around playfully during the past week, how often did your baby smile?

Q45: When tossed around playfully during the past week, how often did your baby laugh?

Q46: During a peekaboo game in the past week, how often did your baby smile?

Q47: During a peekaboo game in the past week, how often did your baby laugh?

Q48: During the past week, how often did your baby enjoy bouncing up and down while on your lap?

Q49: During the past week, how often did your baby enjoy bouncing up and down on an object, such as a bed, bouncer chair, or toy?

Q50: When being held during the past week, how often did your baby pull away or kick?

Q51: When being held during the past week, how often did your baby seem to enjoy herself?

Q52: When your baby wanted something during the past week, how often did he/she become upset when he/she could not get what he/she wanted?

Q53: When your baby wanted something during the past week, how often did he/she have tantrums (crying, screaming, face red) when he/she could not get what he/she wanted?

Q54: When placed in an infant seat or car set during the past week, how often did your baby wave arms and kick?

Q55: When placed in an infant seat or car set during the past week, how often did your baby squirm and turn his/her body?

Q56: During the past week, how often did your baby make talking sounds when riding in a car?

Q57: During the past week, how often did your baby make talking sounds when riding in a shopping cart?

Q58: During the past week, how often did your baby make talking sounds when you talked to them?

Q59: When rocked or hugged during the past week, how often did your baby seem to enjoy herself?

Q60: When rocked or hugged during the past week, how often did your baby seem eager to get away?

Q61: During the past week, while being fed in your lap, how often did the baby seem eager to get away as soon as the feeding was over?

Q62: During the past week, after sleeping, how often did the baby cry if someone didn't come within a few minutes?

Q63: During the past week, when put down for a nap, how often did your baby settle down quickly?

Q64: During the past week, when it was time for bed or a nap and your baby did not want to go, how often did they whimper or sob?

Q65: During the past week, when face was washed, how often did the baby smile or laugh?

Q66: During the past week, when hair was washed, how often did the baby vocalize?

Q67: During the past week, when playing quietly with one of his/her favorite toys, how often did your baby enjoy lying in the crib for more than 5 minutes?

Q68: During the past week, when your baby saw a toy they wanted, how often did they get very excited about getting?

Q69: During the past week, when given a new toy, how often did your baby immediately go after it?

Q70: During the past week, when placed on his/her back, how often did your baby squirm and/or turn their body?

Q71: During the pat week, when frustrated with something, how often did your baby calm down within 5 minutes?

Q72: During the past week, when your baby was upset about something, how often did they stay upset for up to 20 minutes or longer?

Q73: During the past week, when being carried, how often did your baby push against you until put down?

Q74: During the past week, when tired, how often did your baby show distress?

Q75: During the past week, at the end of an exciting day, how often did your baby become tearful

Q76: During the past TWO WEEKS: when introduced to an unfamiliar adult, how often did your baby cling to a parent?

Q77: During the past TWO WEEKS: when introduced to an unfamiliar adult, how often did your baby refuse to go to the familiar person?

Q78: During the past TWO WEEKS: when introduced to an unfamiliar adult, how often did your baby never "warm up" to the unfamiliar adult?

Q79: During the past TWO WEEKS, when you were busy with another activity and your baby was not able to get your attention, how often did they become sad?

Q80: During the past TWO WEEKS, when you were busy with another activity and your baby was not able to get your attention, how often did they cry?

Q81: During the past TWO WEEKS, when singing or talking to your baby, how often did your baby soothe immediately

Q82: During the past TWO WEEKS, when singing or talking to your baby, how often did your baby take more than 10 minutes to soothe?

Q83: During the past TWO WEEKS, when showing your baby something to look at, how often did your baby soothe immediately?

Q84: During the past TWO WEEKS, when showing your baby something to look at, how often did your baby take more than 10 minutes to soothe?

Q85: During the past TWO WEEKS, when patting or gently rubbing some part of your baby's body, how often did your baby soothe immediately?

Q86: During the past TWO WEEKS, when patting or gently rubbing some part of your baby's body, how often did your baby take more than 10 minutes to soothe?

Q87: During the past TWO WEEKS, when in the presence of several unfamiliar adults, how often did the baby continue to be upset for 10 minutes or longer?

Q88: During the past TWO WEEKS, when visiting a new place, how often did the baby get excited about exploring new surrounds?

Q89: During the past TWO WEEKS, when an unfamiliar adult came to your home or apartment, how often did your baby cry when the visitor attempted to pick him/her up?

Q90: During the past TWO WEEKS, when familiar relatives/friends came to visit, how often did your baby get excited?

Q91: During the past TWO WEEKS, when rocking your baby, how often did they take more than 10 minutes to soothe?

References, Appendix E

1. Rothbart, M. K. (1981). Measurement of Temperament in Infancy. *Child Development*, *52*(2), 569–578. https://doi.org/10.2307/1129176

2. Gartstein, M. A., & Rothbart, M. K. (2003). Studying infant temperament via the Revised Infant Behavior Questionnaire. *Infant Behavior and Development*, *26*(1), 64–86. https://doi.org/10.1016/S0163-6383(02)00169-8

3. Putnam, S. P., Helbig, A. L., Gartstein, M. A., Rothbart, M. K., & Leerkes, E. (2014). Development and Assessment of Short and Very Short Forms of the Infant Behavior Questionnaire–Revised. *Journal of Personality Assessment*, *96*(4), 445–458. https://doi.org/10.1080/00223891.2013.841171

APPENDIX F: THE EARLY CHILDHOOD BEHAVIOR QUESTIONNAIRE

The Early Childhood Behavior Questionnaire (ECBQ) was developed in 1998 by Dr. Mary Rothbart and Dr. Sam Putnam.[1] In 2009, a short form (107 items) and very short form (36 items) version of the ECBQ was developed. The short form of the ECBQ was administered to parents in this study.

Instructions:

As you read each description of the baby's behavior below, please indicate how often the baby did this during the LAST TWO WEEKS by circling one of the numbers in the left column. These numbers indicate how often you observed the behavior described during the last two weeks.

(0) Do Not Wish to Answer
(1) Never
(2) Very Rarely
(3) Less than Half the Time
(4) About Half the Time
(5) More than Half the Time
(6) Almost Always
(7) Always
(X) Does Not Apply

The Does Not Apply (X) column is used when you did not see the baby in the situation described during the last two weeks. For example, if the situation mentions the baby having to wait for food or liquids and there was no time during the last two weeks when the baby had to wait, circle the (X) column. Does Not Apply is different from Never (1). Never is used when you saw the baby in the situation, but the baby never engaged in the behavior listed during the last two weeks. For example, if the baby did have to wait for food or liquids at least once but never cried loudly while waiting, circle the (1) column. Please be sure to circle a number for every item.

Questionnaire Items:

Q1: During the past two weeks, when told that it was time for bed or a nap, how often did your child get irritable?

Q2: During the past two weeks, when approached by an unfamiliar person in a public place, how often did your child pull back and avoid the person?

Q3: During the past two weeks, when approached by an unfamiliar person in a public place, how often did your child cling to a parent?

Q4: During the past two weeks, during everyday activities, how often did your child tap or drum with fingers on tables or other objects?

Q5: During the past two weeks, during everyday activities, how often did your child become uncomfortable when his/her socks were not aligned properly on his/her feet?

Q6: During the past two weeks, during everyday activities, how often did your child become distress when his/her hands were dirty and/or sticky?

Q7: During the past two weeks, during everyday activities, how often did your child notice low-pitched noises such as the air-conditioner, heater, or refrigerator running or starting up?

Q8: During the past two weeks, during everyday activities, how often did your child blink a lot?

Q9: During the past two weeks, while playing outdoors, how often did your child enjoy sitting quietly in the sunshine?

Q10: During the past two weeks, while playing outdoors, how often did your child look immediately when you pointed at something?

Q11: During the past two weeks, while playing outdoors, how often did your child choose to take chances for the fun and excitement of it?

Q12: During the past two weeks, while playing outdoors, how often did your child seem to be one of the most active children?

Q13: During the past two weeks, when she was carried, how often did your child push against you until put down?

Q14: During the past two weeks, when she was carried, how often did your child snuggle up next to you?

Q15: During the past two weeks, while having trouble completing a task (e.g. building, drawing, dressing), how often did your child get easily irritable?

Q16: During the past two weeks, when a familiar child came to your home, how often did your child seek out the company of the child?

Q17: During the past two weeks, when offered a choice of activities, how often did your child stop and think before deciding?

Q18: During the past two weeks, when offered a choice of activities, how often did your child decide what to do very quickly and go after it?

Q19: During the past two weeks, when asked not to, how often did your child touch an attractive item anyways?

Q20: During the past two weeks, during daily or evening quiet time with you and your child, how often did your child enjoy just being quietly sung to?

Q21: During the past two weeks, during daily or evening quiet time with you and your child, how often did your child smile at the sound of words, as in nursery rhymes?

Q22: During the past two weeks, during daily or evening quiet time with you and your child, how often did your child enjoy just being talked to?

Q23: During the past two weeks, during daily or evening quiet time with you and your child, how often did your child enjoy rhythmic activities, such as rocking or swaying?

Q24: During the past two weeks, during daily or evening quiet time with you and your child, how often did your child want to be cuddled?

Q25: During the past two weeks, while at home, how often did your child show fear at a loud sound?

Q26: During the past two weeks, while at home, how often did your child seem afraid of the dark?

Q27: During the past two weeks, while bathing, how often did your child sit quietly?

Q28: During the past two weeks, when she was upset, how often did your child change to feeling better within a few minutes?

Q29: During the past two weeks, when engaged in play with his favorite toy, how often did your child play for more than 10 minutes?

Q30: During the past two weeks, when engaged in play with his favorite toy, how often did your child continue to play while at the same time responding to your remarks or questions?

Q31: During the past two weeks, when approaching unfamiliar children playing, how often did your child watch rather than join in?

Q32: During the past two weeks, when approaching unfamiliar children playing, how often did your child seem uncomfortable?

Q33: During the past two weeks, during everyday activities, how often did your child move quickly from one place to another?

Q34: During the past two weeks, during everyday activities, how often did your child notice the smoothness or roughness of objects she touched?

Q35: During the past two weeks, during everyday activities, how often did your child become sad or blue for no apparent reason?

Q36: During the past two weeks, during everyday activities, how often did your child pay attention to you right away when you called to her?

Q37: During the past two weeks, during everyday activities, how often did your child seem to be disturbed by loud sounds?

Q38: During the past two weeks, during everyday activities, how often did your child seem frightened for no apparent reason?

Q39: During the past two weeks, during everyday activities, how often did your child seem to be irritated by tags in her clothes?

Q40: During the past two weeks, after having been interrupted, how often did your child return to a previous activity?

Q41: During the past two weeks, after having been interrupted, how often did your child have difficulty returning to the previous activity?

Q42: During the past two weeks, when told that loved adults would visit, how often did your child get very excited?

Q43: During the past two weeks, when told that loved adults would visit, how often did your child become very happy?

Q44: During the past two weeks, during quiet activities such as reading a story, how often did your child swing or tap his foot?

Q45: During the past two weeks, during quiet activities such as reading a story, how often did your child fiddle with his hair, clothing, etc.?

Q46: During the past two weeks, during quiet activities such as reading a story, how often did your child show repeated movements like squinting, hunching up the shoulders, or twitching the facial muscles?

Q47: During the past two weeks, while playing indoors, how often did your child like rough and rowdy games?

Q48: During the past two weeks, while playing indoors, how often did your child enjoy playing boisterous games like chase?

Q49: During the past two weeks, while playing indoors, how often did your child enjoy vigorously jumping on the couch or bed?

Q50: During the past two weeks, in situations where she is meeting new people, how often did your child turn away?

Q51: During the past two weeks, when being gently rocked or hugged, how often did your child seem eager to get away?

Q52: During the past two weeks, when encountering a new activity, how often did your child sit on the sidelines and observe before joining in?

Q53: During the past two weeks, when encountering a new activity, how often did your child get involved immediately?

Q54: During the past two weeks, when visiting the home of a familiar child, how often did your child engage in an activity with the child?

Q55: During the past two weeks, when engaged in an activity requiring attention, how often did your child move quickly to another activity?

Q56: During the past two weeks, when engaged in an activity requiring attention, how often did your child tire of the activity relatively quickly?

Q57: During the past two weeks, when in a public place, how often did your child seem uneasy about approaching an elevator or escalator?

Q58: During the past two weeks, when in a public place, how often did your child cry or show distress when approached by an unfamiliar animal?

Q59: During the past two weeks, when in a public place, how often did your child seem afraid of large, noisy vehicles?

Q60: During the past two weeks, when in a public place, how often did your child show fear when the caregiver stepped out of sight?

Q61: During the past two weeks, when being dressed or undressed, how often did your child squirm and try to get away?

Q62: During the past two weeks, when being dressed or undressed, how often did your child stay still?

Q63: During the past two weeks, when told no, how often did your child stop the forbidden activity?

Q64: During the past two weeks, when told no, how often did your child become sadly tearful?

Q65: During the past two weeks, following an exciting activity or event, how often did your child calm down quickly?

Q66: During the past two weeks, following an exciting activity or event, how often did your child have a hard time settling down?

Q67: During the past two weeks, following an exciting activity or event, how often did your child seem to feel down or blue?

Q68: During the past two weeks, during everyday activities, how often did your child easily shift attention from one activity to another?

Q69: During the past two weeks, during everyday activities, how often did your child become bothered by sounds while in noisy environments?

Q70: During the past two weeks, during everyday activities, how often did your child become bothered by scratchy materials like wool?

Q71: During the past two weeks, during everyday activities, how often did your child notice changes in your appearance?

Q72: During the past two weeks, during everyday activities, how often did your child appear to listen to even very quiet sounds?

Q73: During the past two weeks, during everyday activities, how often did your child seem full of energy, even in the evening?

Q74: During the past two weeks, during everyday activities, how often did your child become irritated when his clothes were tight?

Q75: During the past two weeks, while playing indoors, how often did your child run through the house?

Q76: During the past TWO WEEKS: while playing indoors, how often did your child climb over furniture?

Q77: During the past TWO WEEKS: while playing indoors, how often did your child enjoy activities such as being spun?

Q78: During the past TWO WEEKS: when playing alone, how often did your child become easily distracted?

Q79: During the past TWO WEEKS: When playing alone, how often did your child play with a set of objects for 5 minutes or longer at a time?

Q80: During the past TWO WEEKS: When playing alone, how often did your child tear materials close at hand?

Q81: During the past TWO WEEKS: before an exciting event, how often did your child get very excited about getting it?

Q82: During the past TWO WEEKS: before an exciting event, how often did your child remain pretty calm?

Q83: During the past TWO WEEKS: when she asked for something and you said no, how often did your child become frustrated?

Q84: During the past TWO WEEKS: When she asked for something and you said no, how often did your child protest with anger?

Q85: During the past TWO WEEKS: when she asked for something and you said no, how often did your child have a temper tantrum?

Q86: During the past TWO WEEKS: When she asked for something and you said no, how often did your child become sad?

Q87: During the past TWO WEEKS when playing or walking outdoors, how often did your child notice sights or sounds?

Q88: During the past TWO WEEKS when asked to wait for a desirable item, how often did your child go after it anyway?

Q89: During the past TWO WEEKS When asked to wait for a desirable item, how often did your child wait patiently?

Q90: During the past TWO WEEKS, when being gently rocked, how often did your child smile?

Q91: During the past TWO WEEKS When you removed something he should not have been playing with, how often did your child become sad?

Q92: During the past two weeks, while being held on your lap, how often did your child seem to enjoy herself?

Q93: During the past two weeks, while being held on your lap, how often did your child mold to your body?

Q94: During the past two weeks, when hearing about a future family outing, how often did your child look forward to it?

Q95: During the past two weeks, while looking at picture books on his own, how often did your child become easily distracted?

Q96: During the past two weeks, when a familiar adult visited your home, how often did your child want to interact with the adult?

Q97: During the past two weeks, when asked to do so, how often was your child able to stop an ongoing activity?

Q98: During the past two weeks, when asked to do so, how often was your child able to be careful with something breakable?

Q99: During the past two weeks, when visiting a new place, how often did your child not want to enter?

Q100: During the past two weeks, while you were talking with someone else, how often did your child easily switch attention from speaker to speaker?

Q101: During the past two weeks, when you mildly criticized or corrected his behavior, how often did your child get mad?

Q102: During the past two weeks, when she was upset, how often did your child cry for more than 3 minutes, even when being comforted?

Q103: During the past two weeks, when she was upset, how often did your child become easily soothed?

Q104: During the past two weeks, when you were busy, how often did your child find another activity to do when asked?

Q105: During the past two weeks, while playing outdoors, how often did your child want to jump from heights?

Q106: During the past two weeks, when around large gatherings of familiar adults or children, how often did your child enjoy playing with a large number of different people?

Q107: During the past two weeks, when she was asked to share her toys, how often did your child become sad?

Scale Label	Definition
Activity Level/Energy	Level (rate and intensity) of gross motor activity, including rate and extent of locomotion
Attentional Focusing	Sustained duration of orienting on an object of attention; resisting distraction
Attentional Shifting	The ability to transfer attentional focus from one activity/task to another
Cuddliness	Child's expression of enjoyment in and molding of the body to being held by a caregiver
Discomfort	Amount of negative affect related to sensory qualities of stimulation, including intensity, rate or complexity of light, sound, texture
Fear	Negative affect, including unease, worry, or nervousness related to anticipated pain or distress and/or potentially threatening situations; startle to sudden events
Frustration	Negative affect related to interruption of ongoing tasks or goal blocking
High Intensity Pleasure	Amount of pleasure or enjoyment related to high stimulus intensity, rate, complexity, novelty, and incongruity
Impulsivity	Speed of response initiation
Inhibitory Control	The capacity to stop, moderate, or refrain from a behavior under instruction
Low Intensity Pleasure	Amount of pleasure or enjoyment related to situations involving low stimulus intensity, rate, complexity, novelty, and incongruity
Motor Activation	Repetitive small-motor movements; fidgeting
Perceptual Sensitivity	Amount of detection of slight, low intensity stimuli from the external environment
Positive Anticipation	Excitement about expected pleasurable activities
Sadness	Tearfulness or lowered mood related to exposure to personal suffering, disappointment, object loss, loss of approval, or response to other's suffering
Shyness	Slow or inhibited approach and/or discomfort in social situations involving novelty or uncertainty
Sociability	Seeking and taking pleasure in interactions with other
Soothability	Rate of recovery from peak distress, excitement, or general arousal
Factor Label	**Definition**
Surgency/Extraversion	Defined by scale scores of Impulsivity, Activity Level, High Intensity Pleasure, Sociability, Positive Anticipation
Negative Affectivity	Defined by scale scores of Discomfort, Fear, Sadness, Frustration, Soothability (loaded negatively), Motor Activation, Shyness, and

	Perceptual Sensitivity
Effortful Control	Defined by scale scores of Inhibitory Control, Attentional Shifting, Low Intensity Pleasure, Cuddliness, and Attentional Focusing

Table F.2: Full list of scales and factors on the Early Childhood Behavior Questionnaire

References, Appendix F

1. Putnam, S. P., Gartstein, M. A., & Rothbart, M. K. (2006). Measurement of fine-grained aspects of toddler temperament: The Early Childhood Behavior Questionnaire. *Infant Behavior and Development*, *29*(3), 386–401. https://doi.org/10.1016/j.infbeh.2006.01.004

QUESTIONNAIRE

The individual items and scales that load onto the Orienting/Regulation Factor of the Infant Behavior Questionnaire-Revised, Short are listed below in Table G.1.

Duration of Orienting: The baby's attention to and/or interaction with a single object for extended periods of time	
Question Number	Question Text:
5	"During the past week, how often did your baby look at pictures in books and/or magazines for 5 minutes or longer at a time?"
6	"During the past week, how often did your baby stare at a mobile, crib bumper, or picture for 5 minutes or longer?"
7	"During the past week, how often did your baby play with one toy or object for 5–10 minutes?"
8	"During the past week, how often did your baby play with one toy or object for 10 minutes or longer?"
10	"During the past week, how often did your baby repeat the same movement with an object for 2 minutes or longer (e.g., putting a block in a cup; kicking or hitting a mobile)?"
25	"During the past week, how often did your baby watch adults performing household activities (e.g., cooking, etc.) for more than five minutes?"
Low-Intensity Pleasure: Amount of pleasure or enjoyment related to situations involving low stimulus intensity, rate, complexity, novelty, and incongruity	
13	"During the past week, how often did your baby enjoy being read to?"
14	"During the past week, how often did your baby enjoy hearing the sound of words, as in nursery rhymes?"
15	"During the past week, how often did your baby enjoy gentle rhythmic activities, such as rocking or swaying?"
17	"During the past week, how often did your baby enjoy the feel of soft blankets?"
18	"During the past week, how often did your baby enjoy being rolled up in a warm blanket?"
19	"During the past week, how often did your baby enjoy listening to a musical toy in a crib?"
67	"During the past week, when playing quietly with one of his/her favorite toys, how often did your baby enjoy lying in the crib for more than 5 minutes?"
Cuddliness: The baby's expression of enjoyment and molding of the body to being held by a caregiver	
50	"When being held during the past week, how often did your baby pull away or kick?"

51	"When being held during the past week, how often did your baby seem to enjoy herself?"
59	"When rocked or hugged during the past week, how often did your baby seem to enjoy herself?"
60	"When rocked or hugged during the past week, how often did your baby seem eager to get away?"
61	"During the past week, while being fed in your lap, how often did the baby seem eager to get away as soon as the feeding was over?"
73	"During the past week, when being carried, how often did your baby push against you until put down?"
Soothability: Baby's reduction of fussing, crying, or distress when the caretaker uses soothing techniques	
81	"During the past TWO WEEKS, when singing or talking to your baby, how often did your baby soothe immediately"
82	"During the past TWO WEEKS, when singing or talking to your baby, how often did your baby take more than 10 minutes to soothe?"
83	"During the past TWO WEEKS, when showing your baby something to look at, how often did your baby soothe immediately?"
84	"During the past TWO WEEKS, when showing your baby something to look at, how often did your baby take more than 10 minutes to soothe?"
85	"During the past TWO WEEKS, when patting or gently rubbing some part of your baby's body, how often did your baby soothe immediately?"
86	"During the past TWO WEEKS, when patting or gently rubbing some part of your baby's body, how often did your baby take more than 10 minutes to soothe?"
91	"During the past TWO WEEKS, when rocking your baby, how often did they take more than 10 minutes to soothe?"

References, Appendix G

1. Gartstein, M. A., & Rothbart, M. K. (2003). Studying infant temperament via the Revised Infant Behavior Questionnaire. *Infant Behavior and Development*, *26*(1), 64–86. https://doi.org/10.1016/S0163-6383(02)00169-8

2. Putnam, S. P., Helbig, A. L., Gartstein, M. A., Rothbart, M. K., & Leerkes, E. (2014). Development and Assessment of Short and Very Short Forms of the Infant Behavior Questionnaire–Revised. *Journal of Personality Assessment*, *96*(4), 445–458. https://doi.org/10.1080/00223891.2013.841171

APPENDIX H: MATERNAL AND INFANT DEMOGRAPHIC CHARACTERISTICS,

INFANCY STUDY

Of the 33 women who completed the supplementation trial, 30 were eligible to return with their infants for follow-up testing. Three women (1 from the 25 mg/day group and 2 from the 550 mg/day group) developed gestational diabetes during the course of the supplementation trial and were subsequently excluded from both the pregnancy and follow-up studies. 27 women were successfully recruited for their children to participate in the infant cognitive and behavioral assessments, but one infant was lost to follow-up after the 5-month visit, so cognitive and behavioral data was not available for this infant. Group differences in maternal and infant characteristics for infants that were included in the analytical sample (v those who were not) are below in Table H.1.

	Included in Analytical Sample (N = 25)	Not Included in Analytical Sample (N = 5)	p
Maternal Characteristics			
Mean age, years (range)	32.1 (24–38)	30 (23–36)	0.28
Mean BMI, kg/m^2 (range)	23.8 (20–32.4)	23.6 (18.5–31.8)	0.92
Education			*0.02
High School	2 (8)	2 (40)	
Bachelor's Degree	5 (20)	3 (60)	
Master's Degree	14 (56)	0 (0)	
Doctorate/Professional	4 (16)	0 (0)	
Race (%)			1.00
White	23 (92)	5 (100)	
Black	1 (4)	0 (0)	
Asian	1 (4)	0 (0)	
Ethnicity			*0.02
Non-Hispanic	24 (96)	3 (60)	
Hispanic	0 (0)	2 (40)	
Other	1 (4)	0 (0)	
Pregnancy and Delivery			
Mean gestation length, days (range)	279.8 (253.0–299.0)	271.6 (258.0–286.0)	0.10
Pregnancy complications (%)	3 (60)	8 (32)	0.42
Delivery method, vaginal (%)	3 (60)	21 (84)	0.25
Infant Characteristics			
Gender, female (%)	18 (72)	2 (40)	0.30
Mean birth length, inches (range)	19.41 (16–21)	19.15 (18–20.5)	0.64
Mean birth weight, grams (range)	3353.1 (2550–4194)	3458.9 (3020–3940.6)	0.59

Table H.1: Demographic characteristics of infants included in the final analytical sample and those who were not.

APPENDIX I: MATERNAL SERUM CHOLINE DATA AND SENSITIVITY ANALYSES, INFANT AND EARLY CHILDHOOD BEHAVIOR QUESTIONNAIRES

The average maternal serum choline level at baseline was 6.45 μM (range 2.97–15.5) in this cohort. Mean serum choline levels did not differ by choline group, even with the inclusion of one extremely high baseline value (15.5 μM) in the 25 mg/day group.

	Maternal Choline Intake Group		
Blood Metabolite	**25 mg/d (mean, range)**	**550 mg/d (mean, range)**	**p**
Serum Choline (umol/L)	6.95 (4.04–15.5)	5.98 (2.97–9.36)	0.336
Serum Betaine (umol/L)	12.44 (8.38–22.57)	14.59 (8.46–32.35)	0.325
Serum Dimethylglycine (umol/L)	1.40 (0.75–2.28)	1.69 (0.90–3.74)	0.303

Table I.1: Maternal choline levels at baseline

Sensitivity analyses were conducted to evaluate the robustness of the results from the primary analyses. Infant sex, rater identity (mother or father), maternal sensitivity, and timing of questionnaire completion (before or after the beginning of the COVID-19 lockdown in March 2020) were each entered, in turn, as a single added covariates added to the *a priori* models.

Scale/Factor	Covariate	Unadjusted Effect Size	Adjusted Effect Size	% Change
Duration of Orienting	Infant Sex	0.6814	0.5205	-23.61%
	Rater Identity	0.6814	0.661	-2.99%
	Maternal Sensitivity	0.6814	0.6469	-5.06%
	COVID-19	0.6814	0.6867	0.78%
Falling Reactivity/Rate of Recovery from Distress	Infant Sex	0.2676	0.3461	29.33%
	Rater Identity	0.2676	0.2575	-3.77%
	Maternal Sensitivity	0.2676	-0.0253	-109.45%
	COVID-19	0.2676	0.3154	17.86%
Regulation/Orienting	Infant Sex	0.2614	0.1904	-27.16%
	Rater Identity	0.2614	0.258	-1.30%
	Maternal Sensitivity	0.2614	0.1695	-35.16%
	COVID-19	0.2614	0.2791	6.77%
Negative Affect	Infant Sex	-0.1384	-0.2024	46.24%
	Rater Identity	-0.1384	-0.1106	-20.09%
	Maternal Sensitivity	-0.1384	0.03851	-127.83%
	COVID-19	-0.1384	-0.1621	17.12%
Surgency	Infant Sex	0.0453	-0.0905	-299.96%
	Rater Identity	0.0453	0.04552	0.53%
	Maternal Sensitivity	0.0453	0.01653	-136.51%
	COVID-19	0.0453	0.05904	30.39%

Table I.2: Sensitivity analyses, Infant Behavior Questionnaire

Scale/Factor	Covariate	Unadjusted Effect Size	Adjusted Effect Size	% Change
Attentional Shifting	Infant Sex	0.5566	0.4862	-12.65%
	Rater Identity	0.5566	0.5566	0.00%
	Maternal Sensitivity	0.5566	0.388	-30.29%
	COVID-19	0.5566	0.6019	8.14%
Soothability	Infant Sex	0.141	0.2454	74.04%
	Rater Identity	0.141	0.141	0.00%
	Maternal Sensitivity	0.141	-0.028	-119.86%
	COVID-19	0.141	0.1536	8.94%
Effortful Control	Infant Sex	0.2998	0.263	-12.27%
	Rater Identity	0.2998	0.2998	0.00%
	Maternal Sensitivity	0.2998	0.2818	-6.00%
	COVID-19	0.2998	0.357	19.08%
Negative Affect	Infant Sex	-0.0122	-04214	-73.52%
	Rater Identity	-0.0122	-0.0122	0.00%
	Maternal Sensitivity	-0.0122	-0.0582	-8.26%
	COVID-19	-0.0122	0.3604	66.94%
Surgency	Infant Sex	0.3228	0.3045	-5.67%
	Rater Identity	0.3228	0.3228	0.00%
	Maternal Sensitivity	0.3228	0.3211	-0.53%
	COVID-19	0.3228	0.3267	1.21%

Table I.3: Sensitivity analyses, Early Childhood Behavior Questionnaire

APPENDIX J: TASK PROTOCOL, FACE TO FACE STILL-FACE PARADIGM

IMPORTANT: The Still-Face paradigm is likely to cause distress to both the infant and the parent, so it is imperative that the rationale behind this task is clearly explained to the parent before the task begins, and that it is made clear to her that she can stop the task at any time. Due to the stressful nature of this task, it is administered at the end of the session, after data from all the other tasks has been collected.

Verbal Instructions:

After the free play task has ended, the parent and infant should be given the opportunity to take a short break if necessary. Once the parent indicates that they are ready for the next task, use the following script to introduce the SFP :

"We are now going to do a task called the Still-Face task. Developmental psychologists have used this procedure for several decades to study infant emotion. In this exercise, we will ask you to sit across from your baby for three two-minute periods. In the first two minutes, you will interact normally with your infant. In the second two minutes, we will ask you to sit across from your baby and look at them with a "neutral" face, and to not respond as you normally would if your infant expresses distress. This task is designed to assess how your baby reacts to a social stress, so a sad expression or crying is completely normal. After the two minutes are complete, you will be allowed to re-engage with your infant while they remain in the infant seat for the last two minutes. We will give you as much time as you need to soothe [baby's name],and of course we will stop the task at any time if you or I feel that your baby is too distressed. This task might remind you of times in your daily life where your infant is upset, and you can't immediately soothe them (such as when you are driving a car, cooking dinner, etc.). The task will proceed in four steps, the first of which is a quiet baseline where your infant will be sitting in the infant seat next to you while you read some written instructions and practice making a neutral face."

- Before the task begins, be sure to give the parents a chance to observe the example 'neutral' faces and ask any questions he or she might have (Figure K.1).
- **IMPORTANT:** During this task, it is imperative that the tester remain out of sight of the infant. The primary tester can remain in the testing area during the task to keep time and monitor infant distress, but they should be out of view of the infant during this time. Closing the screen between the second and third testing

area is required.

Play Episode

- The first of three "episodes" in the SFP task is a play episode, in which parents are asked to play with their infant as they normally would, without toys
- Have the parent sit in a chair facing their infant across the table and provide them the following instructions:

"First, we're going to ask you to play with your baby for two minutes, as you normally would at home. You can talk to and interact with him/her in any way you like, but you cannot take him/her out of the chair. When the timer goes off, we will ask you to immediately move into the neutral face episode."

- Once the parent has initiated play, set the timer for two minutes and step out of the infant's visual range.

Still Face Episode

- As soon as the timer indicates the end of the play episode, indicate to the parent to move into the neutral face episode.
- The parent should immediately adopt and maintain a neutral expression
- During this time, the parent should be still, and not touch or respond to the infant in any way.
- As soon as the parent has moved to the appropriate location and adopted a neutral expression, the timer should be set for two minutes.

Reunion Episode

- Immediately after the timer indicates the end of the still face episode, indicate to the parent that they can now resume play and respond to their baby in any way they feel appropriate, without removing their infant from the chair.
- Once the parent has resumed play, set the timer for two minutes.
- After two minutes, the parent may remove their infant from the chair and soothe them in any way they deem appropriate for as long as they would like.

In the Event of Significant Infant Distress

- While this task is designed to elicit an emotional response, we want to avoid serious upset or potential loss-to-follow-up due to study procedures.
- It is important to emphasize to the parent on several occasions before the task starts that they have the right to terminate the task at any point when they determine their infant is too upset to continue.
- The experimenter also has a cutoff point of 15 continuous seconds of hard crying, at which point the experimenter should make the call to terminate the task and allow the parent to soothe their infant.

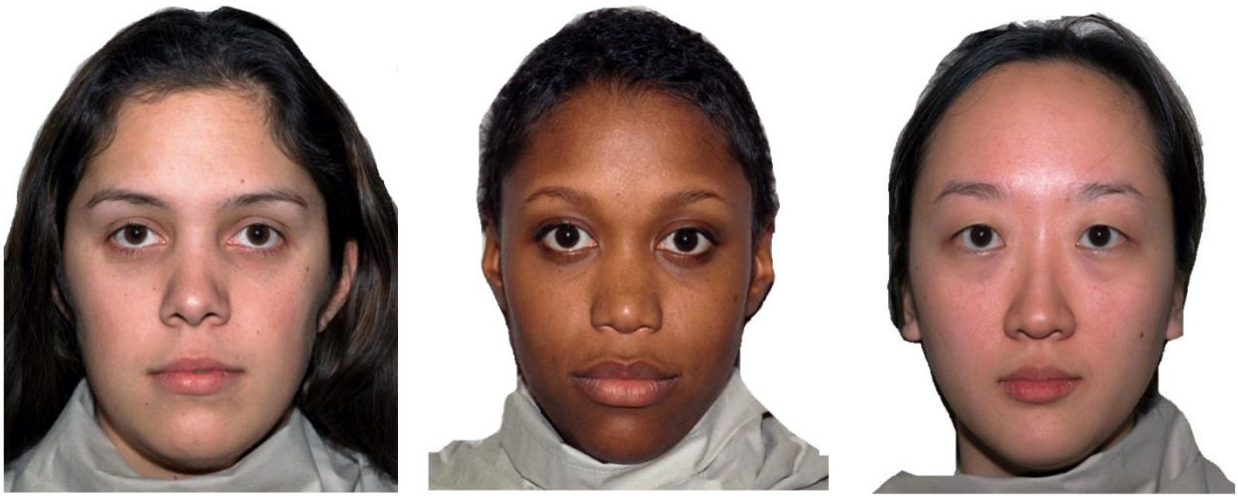


Figure K.1: Examples of a neutral expression (from NimStim) shown to parents prior to FFSF

APPENDIX K: CODING GUIDE, FACE TO FACE STILL-FACE PARADIGM

Cholbabies Coding Guide for the Face-To-Face Still Face Paradigm

General Instructions for Coding:

All infant behavioral coding should be conducted on the laboratory computers using BORIS coding software. Instructions for opening BORIS and loading an observation are as follows:

- Log on to the laboratory computer (using instructions for remote access if necessary, or otherwise using your login information for the physical computers).
- Open BORIS: Go to the search bar at the bottom left hand side of the screen and type BORIS—click on the application once it pops up.
    - **Remember:** You cannot open BORIS directly from your ethogram! Clicking on your ethogram directly will just open it in PDF form.
- Once BORIS is open, select File > Open Project. Your individual ethogram can be found at the following file path:
    - \\canfieldnas\Lab_NAS\CholBabies\Emotion Coding
    - The file name will be FFSF_YourName
- This will open your ethogram – you should see it on the left-hand side of the application.
- **To start an observation:**
    - Go to Observations > New Observation.
    - In Observation ID, name the observation CholbabiesID-Month (e.g., 202-7).
    - Select "Add Media". The video files can be found at the following file path:
        - \\canfieldnas\Lab_NAS\CholBabies\Videos\Participants\Processed Video
        - Select the folder for the correct infant and age.
    - **IMPORTANT:** You should be coding using the side-by-side video of the infant and the parent. These are labeled as CholbabiesID_Month_SFP_BOTH.
    - Click "Start" at the bottom right of the pop-up screen.
    - You can now begin coding!

The multiple-pass method:

- The recommended way to code the still-face procedure is in a multiple-pass method. This entails watching the full video through multiple times and coding separate items on each watch. For example:

    - Pass 1: Watch the full video and code all task components (task start/stop, phase start/stops, infant look, etc.).
    - Pass 2: Watch the full video and code for negative affect (anger/sadness). Anger and sadness are separate codes, so if you find it easier you can also code these two forms of negative affect separately.
    - Pass 3: Watch the full video and code for positive/neutral affect.
    - Pass 4 – 7: Watch the still-face period (which you can now easily jump to because it's in your events!) through four times, coding for a different regulatory behavior category each time.
- This may sound like a lot of extra work – but by carefully taking the time to think about one code at a time while watching a video, you will actually increase your accuracy and decrease the amount of time you spend making judgements while watching.

How to save your observation – Practice good BORIS hygiene!:

- You want to make sure that you save the observation in your current project file, and not as a separate project. To do this, you should always go through the File menu:
    - Go to File >
        - Save the observation first.
        - Save Project. **DO NOT** select "Save Project As" – this will save the observation in a separate project file, which you do not want.
        - Close the program through the file menu.
- If you would like to return to an observation you've already completed, follow the above steps to open your project file, then go to Observations > Edit Observation and select the video you'd like to review.

<u>Coding Negative Affect</u>

Definition: Negative affect is a broad concept that can be summarized as feelings of emotional distress that occur when one has failed to achieve a goal or when one is not satisfied with the current state of affairs. It is an overarching construct that includes specific negative emotions, including sadness, anger, guilt, and shame. Importantly, there is a difference between the <u>psychic experience</u> of negative affect (emotion) and the <u>physical expression</u> of that negative affect (behavior).

- Emotion: A state of being or feeling; differ in valence (positive/negative) and intensity; change in response to environmental stimuli. Has a physiological component.
- Behavior: Physical expression of or response to an emotion. May be used to regulate an intense emotion, or to change the environment in order to stimulate a more positive emotion.

Summary:

Negative affect in an infant can be broadly categorized into either externalizing or internalizing emotions and behaviors – or, more simply, anger and sadness.

- **Externalizing emotions/behaviors (anger)** are directed outwards, towards the environment or others. Externalizing behaviors usually manifest as the child's outward behavior, and result in the child acting negatively on the external environment around them. In older children, this can be expressed as a number of behaviors and emotional disorders, including aggression, conduct disorders, and hyperactivity. In infants, externalizing emotions are expressed as anger and/or active protest. When coding emotion, look for the following:
    - Facial expression of anger, including furrowed or lowered eyebrows, tense or squinted eyes, and an open or squarish mouth. Infant's attention may be oriented towards partner or away at the environment.
    - Vocal expressions of anger, including strong crying, screaming, or sounds of protest.
    - Physical motions, including straining against restraint and/or attempts to orient body, head, and/or eyes away from their parent.
- **Internalizing emotions/behaviors (sadness)** are directed inwards, towards the child's own internal psychological environment. Internalizing emotions result in more anxious, inhibited, or depressed behaviors. In infants, internalizing emotions are expressed as sadness and/or withdrawal. When coding emotion, look for:
    - Facial expression of sadness, including raised or furrowed eyebrows, droopy or lowered cheeks, lips drawn down or pushed out by chin. Infant is grimacing, pouting, or frowning. The infant's attention is likely oriented away from the partner and towards the self or an object.
    - Vocal expressions of sadness, including whimpering, fussing noises, or crying.

- Physical motions, including partial or complete turning of the body, head or eyes away from the partner. Attention may be downcast onto the self, or an object such as a strap or the side of the infant seat.

Coding Intensity of Negative Affect

Definition: Affect intensity reflects the strength of an individual's experience and expression of an emotional response.

Summary: We are coding intensity of negative affect on a four-point Likert scale.

0 = No intensity (i.e., no presence of negative affect).

1 = Mild negative affect. Mild negative affect may look like a mild vocalization of protest or intermittent fussing fussing and/or whimpering, some facial expression of sadness/anger (furrowed brow, pout or wide, open mouth), some mild movements (gentle kicking, reaching for parent).

2 = Moderate negative affect. Moderate negative affect may look like crying, sobbing, or whining, more intense contortions of the face into a frown, furrowed brow, or open mouth, increased tension in the body (slight arching of back, rubbing hands or feet together, some mild attempt at escaping the high chair).

3 = High negative affect. High negative affect may look like shrieking and or hysterical crying, gulping, or losing air from crying, intense contortion of the face (face screwed up in anger or sadness), thrusting or pulling to get out of the chair, forcefully arching body away, slamming feet or hands.

Each period of negative affect is split into five second intervals, and each interval receives an intensity score that represents the mean intensity of the infant's negative affect during that interval. If a period or interval is less than five seconds long, it receives one score representing the mean intensity of the infant's negative affect during that time.

Coding Positive Affect/Neutral Affect

Note: We are coding positive and neutral affect together – i.e., if the child is not demonstrating negative affect and is in either a positive, neutral, or otherwise non-negative affective state, you should code that with a single code (see the ethogram for more!).

Definition: Positive Affect

Positive affect is a broad concept that can be summarized as feelings of emotional satisfaction that occur when one is moving towards attaining a goal or is engaged with the environmental in a

way that is interesting or joyful. It is an overarching construct that includes specific positive emotions, including happiness, joy, excitement, enthusiasm, calm, and contentment. Importantly, there is a difference between the psychic experience of positive affect (emotion) and the physical expression of that positive affect (behavior).

- Emotion: A state of being or feeling; differ in valence (positive/negative) and intensity; change in response to environmental stimuli. Has a physiological component.
- Behavior: Physical expression of or response to an emotion. May be used to regulate an intense emotion, or to change the environment in order to stimulate a more positive emotion.

Summary:

Positive affect in infants can look like facial and vocal expressions of pleasure, interest or joy, increased activity, or positive approach towards a social partner. It can be categorized broadly as emotions and behaviors of joy and/or pleasure.

- **Emotions and Behaviors Indicative of Joy/Pleasure:** Behaviors of joy are directed towards the environment or the social partner. When coding joy/pleasure, look for the following:
    - The infant looks at the object of joy/pleasure. Facial expressions of joy, including smiling, crinkling around the eyes. Mouth may be open in a wide grin and cheeks may be bulging.
    - Vocal expressions of joy/pleasure, including positively toned giggling, squealing, and/or laughter. Laughter may have a rhythmic quality to it.
    - Positive motor activity, including gleeful banging hands on table, waving arms in excitement, reaching towards social partner.

Definition: Neutral Affect

Neutral affect is coded when the affective state cannot be clearly coded as negative or positive. In neutral affect, the infant may be engaged with an object, the environment, or the parent, but displays only a neutral facial expression and no facial, physical, or vocal indicators of distress or joy.

Coding Regulatory Behaviors

Note: It is important to code regulatory behaviors only when they are infant-initiated – in other

words, the parent is not directing the attention, engaging socially, or playing with the baby's hands or feet. For this reason, we will currently only be coding regulatory behaviors of the infant **during the still-face period**.

Definition: Affect regulation is the ability to modify the intensity and duration of physiological arousal, attention, and affective states to achieve a goal. Affect regulation underlies psychological developmental pathways, mental health and adaptive function throughout the lifespan. It is an overarching construct that can include unconscious regulatory systems (autonomic nervous system, cortisol stress response) and conscious behaviors. Regulatory behaviors help the infant to reduce the intensity of affect (usually negative: fear, anger, frustration) in order to return to a more comfortable baseline state, engage in social communication, match their state with their environment, or achieve a goal or attain a need.
Summary: When coding, you should note the presence of regulatory behaviors regardless of the infant's current affective state (positive or negative). Broadly, regulatory behaviors can be categorized in to attentional, self-soothing, communicative, or avoidance strategies. You may also see autonomic indicators of self-regulation. When coding for regulatory behaviors, look for (adapted from IRSS):

- Attentional Orienting:
    - Attentional orienting behaviors are coded if one or more of the following is observed for an extended duration (2 or more seconds):
        - The infant looks at or manipulates an **object of interest** (strap, infant chair, camera) or person of interest with a neutral or interested expression (open mouth, raised eyebrows, wide eyes).
        - The infant's attention and body language are directed towards the **object of interest.**
        - The infant glances with interest at objects or around the laboratory without focusing on an object.
- Social Signaling:
    - Social signaling behaviors are coded if one or more of the following is observed:
        - The infant vocalizes with (1) neutral/positive, (2) fussy, or (3) crying vocalizations while looking at and/or reaching for the parent.

        - The infant (1) gestures to be picked up or (2) moves his or her arms or legs in an organized manner in the direction of the parent (e.g., reaching)
        - The child is physically and/or visually oriented towards the parent.
- Avoidance:
    - Avoidance behaviors are coded if one or more of the following is observed:
        - The infant attempts to distance him or herself from the parent by turning, twisting, or arching his or her body in the infant seat.
        - The infant averts his/her gaze away from the parent without interest in any specific object around the room.
- Self-Soothing:
    - Self-soothing behaviors are coded if one or more of the following is observed:
        - The infant (1) sucks on his or her body (e.g. thumb-sucking) or (2) sucks on an object (e.g. the chair strap).
        - The child coos or babbles to oneself while looking at or away from the parent.
        - The child blows bubbles or tongues (makes movements/shapes with their tongue).

BORIS Ethogram

Below is a copy of the ethogram found in the behavioral coding software, BORIS (Figure K.1).

Behavior Code	Behavior Type		Key
Negative Affect	State event	Negative affect is coded as: **Facial** expression of anger, including furrowed or lowered eyebrows, tense or squinted eyes, and an open or squarish mouth. Infant's attention may be oriented towards partner or away at the environment; **Facial** expression of sadness, including raised or furrowed eyebrows, droopy or lowered cheeks, lips drawn down or pushed out by chin. Infant is grimacing, pouting, or frowning. The infant's attention is likely oriented away from the partner and towards the self or an object. **Vocal** expressions of anger, including strong crying, screaming, or sounds of protest; **Vocal** expressions of sadness, including whimpering, fussing noises, or crying. **Physical** motions, including	a

		straining against restraint and/or attempts to orient body, head, and/or eyes away from their partner. **Physical** motion**s**, including partial or complete turning of the body, head or eyes away from the partner. Attention may be downcast onto the self, or an object such as a strap or the side of the infant seat.	
Positive/Neutral Affect	State event	**Positive** affect in infants can look like facial and vocal expressions of pleasure, interest or joy, increased activity, or positive approach towards a social partner. **Neutral** affect is coded when the affective state of the infant cannot be clearly coded as negative or positive. The infant may be engaged with an object, the environment, or the parent, but displays only a neutral facial expression and no facial, physical, or vocal indications of either distress or joy.	e
Pause in Affect	State event	A brief pause in expression of affect (crying, fussing, moving, etc.) that lasts <5 seconds and does not indicate shifting to another affective state.	w
Task Start/Stop	State event	Tester indicates the beginning of the task (may hear verbal instructions to begin or beep from the timer). Ends when tester indicates the end of the task (may hear verbal instructions to begin or beep from the timer).	t
Play Phase	State event	Parent begins the first phase (play) by engaging with the student.	p
Still-Face Phase	State event	Tester indicates the beginning of the still-face phase (may hear verbal instructions to begin or beep from the timer).	f
Infant Look	Point event	The infant makes their look at the parent during the still-face phase (unofficial start of still-face) ***Coding Note**: When you identify the "Infant Look", please add a comment noting whether it is just a brief glance or an actual look encoding the change in mother's behavior	l
Reunion Phase	State event	Tester indicates the beginning of the reunion phase (may hear verbal instructions to begin or beep from the timer).	r
Early Termination	Point event	Task is terminated early due to infant distress.	h
Anomaly	State event	The anomaly code represents deviations from the task paradigm that may affect the infant's affect or behavior. For example, the parent may break the still-face and the infant responds by shifting from negative to positive affect, or the parent accidentally pinches	n

		the infant too hard during the play period and they become upset. Any time you see a behavior or event that deviates from the "ideal" or could interfere with coding, it should be marked here. Be sure to add a comment noting the behavior and your assessment of how it changes infant affect	
Attentional Orienting	State event	Attentional orienting behaviors are coded if one or more of the following is observed: The infant looks at or manipulates an object for 2 sec or more (the infant looks at an object of interest (strap, infant chair, camera) or person of interest with a neutral or interested expression (open mouth, raised eyebrows, wide eyes); the infant's attention and body language is directed towards the object of interest for an extended duration, > 2 seconds); the infant glances with interest at objects or around the laboratory without focusing on an object for more than two seconds.	o
Social Signaling	State event	Social signaling behaviors are coded if one or more of the following is observed: Vocalizations: The infant vocalizes with (1) neutral/positive, (2) fussy, or (3) crying vocalizations while looking at and/or reaching for the parent; the infant (1) gestures to be picked up or (2) moves his or her arms or legs in an organized manner in the direction of the parent (e.g., reaching); the child is visually oriented towards the parent.	c
Avoidance	State event	Avoidance behaviors are coded if one or more of the following is observed: The infant attempts to distance him or herself from the parent by turning, twisting, or arching his or her body in the infant seat; the infant averts his/her gaze from the parent without interest (i.e., there is no specific object away from the parent towards which they have directed their attention).	v
Self-Soothing	State event	Self-soothing behaviors are coded if one or more of the following is observed: The infant self-soothes by (1) sucking on his or her body (e.g. thumb-sucking) or (2) sucking on an object (e.g. the chair strap); the child is cooing or babbling to oneself (while looking at or away from the parent); the child is blowing bubbles or tonguing.	b
Gaze Aversion	State event	Infant looks away from parent for any reason during play or still face period	g
Negative Affect	State event	**Positive** affect in infants can look like facial and vocal expressions of pleasure, interest or joy, increased	

		activity, or positive approach towards a social partner. **Neutral** affect is coded when the affective state of the infant cannot be clearly coded as negative or positive. The infant may be engaged with an object, the environment, or the parent, but displays only a neutral facial expression and no facial, physical, or vocal indications of either distress or joy.	
Anger/Protest	State event	A brief pause in expression of affect (crying, fussing, moving, etc.) that lasts <5 seconds and does not indicate shifting to another affective state.	a
Sadness/Withdrawal	State event	Tester indicates the beginning of the task (may hear verbal instructions to begin or beep from the timer). Ends when tester indicates the end of the task (may hear verbal instructions to begin or beep from the timer).	s
Positive/Neutral Affect	State event	Parent begins the first phase (play) by engaging with the student.	e
Pause in Affect	State event	Tester indicates the beginning of the still-face phase (may hear verbal instructions to begin or beep from the timer).	w
Task Start/Stop	State event	The infant makes their look at the parent during the still-face phase (unofficial start of still-face) ***Coding Note**: When you identify the "Infant Look", please add a comment noting whether it is just a brief glance or an actual look encoding the change in mother's behavior	t

APPENDIX L: MATERNAL AND INFANT DEMOGRAPHIC CHARACTERISTICS, FACE TO FACE STILL-FACE PARADIGM

	Included in FFSF Analytical Sample (N = 16)	**Not Included in FFSF Analytical Sample (N = 9)**	**p**
Maternal Characteristics			
Mean age, years (range)	31.4 (24–38)	33.3 (30–38)	0.21
Mean BMI, kg/m^2 (range)	23.3 (18.5–29)	24.4 (19.7–31.8)	0.51
Education (%)			0.06
High School	2 (12.5)	0 (0)	
Bachelor's Degree	3 (18.8)	2 (22.2)	
Master's Degree	9 (56.3)	5 (55.6)	
Doctorate/Professional	2 (12.5)	2 (22.2)	
Race (%)			0.24
White	15 (93.8)	8 (88.9)	
Black	0 (0)	1 (11.1)	
Asian	2 (6.2)	0 (0)	
Ethnicity (%)			0.64
Non-Hispanic	15 (93.8)	9 (100)	
Hispanic	0 (0)	0 (0)	
Other	1 (6.2)	0 (0)	
Pregnancy and Delivery			
Mean gestation length, days (range)	279.5 (263–292)	280.3 (253–299)	0.84
Pregnancy complications (%)	6 (37.5)	2 (22.2)	0.79
Delivery method, vaginal (%)	13 (81.3)	8 (88.9)	1.00
Infant Characteristics			
Gender, female (%)	10 (55.6)	8 (44.4)	0.35
Mean birth length, inches (range)	19.7 (18–21)	18.9 (16–20.5)	0.14
Mean birth weight, grams (range)	3433.1 (2863.3–4194.0)	3210.8 (2550.0–3995.0)	0.19

Table L.1: Demographic characteristics of infants included in the final analytical sample for the Face-to-Face Still Face Paradigm and those who were not.