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Maternal hypoxic insults during gestation may lead to an increased risk for neurodegenerative diseases, such as Parkinson's disease (PD), in progeny. Maternal hypoxic stress is a common consequence of many late-stage prenatal stressors (e.g., preeclampsia, eclampsia, inflammation, placental abruption).

It is unknown whether maternal hypoxic insults during late gestation have long-term effects on brain regions associated with PD, such as the nigrostriatal pathway. We hypothesized that late gestational maternal hypoxia would result in sustained nigrostriatal impairment in male progeny. To determine whether late-stage gestational hypoxia exposure induced PD-associated behaviors and oxidative stress in progeny, timed pregnant Long-Evans rats were exposed to five days (gestational days: 15-19) of chronic intermittent hypoxia (CIH) or room air normoxia. Progeny were tested during two developmental stages (pubertal and young adult) as late-stage gestational insults can impair the neuronal organization of the brain, which can impact pubertal and young adult functions. To examine PD-associated behavioral phenotype of motor dysfunction, we quantified fine and gross motor behaviors in an open field arena. To examine the integrity of the nigrostriatal pathway, we quantified ultrasonic vocalizations.

Our results showed that maternal CIH during late gestation did not impact gross or fine motor behaviors nor circulating oxidative stress. However, maternal CIH during late gestation did impair the nigrostriatal pathway integrity during puberty and young adulthood in both male and female progeny. Long-lasting consequences of maternal CIH during late gestation was most evident in young adult male progeny. Overall, we conclude that maternal hypoxia during late gestation induced sustained nigrostriatal pathway impairment in males more than females, which may underlie the increased risk for PD in men compared to women.

PRENATAL HYPOXIC INSULTS IMPACT BRAIN VULNERABILITY

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PRENATAL HYPOXIC INSULTS IMPACT BRAIN VULNERABILITY

THESIS

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Words cannot fully express my gratitude for all the help I received to complete this project, both physically and mentally. As such, I want to simply state my thanks here to a few select individuals, but of which does not fully cover the entirety of people to which I owe my profound gratitude.

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Advanced Oxidative Protein Products (AOPP)

Chronic intermittent hypoxia (CIH)

Corpus Striatum (STR)

Centimeters (cm)

Female hypoxic/CIH (FC)

Female normoxic (FN)

Gestational day (GD)

Male hypoxic/CIH (MC)

Male normoxic (MN)

Milliseconds (ms)

Normoxia (Norm)

Open field (OF)

Parkinson's disease (PD)

Post-natal day (PND)

Substantia nigra (SN)

Ultrasonic Vocalizations (USVs)

CHAPTER 1

INTRODUCTION

1.1 Developmental major milestones in humans

The human lifespan can be broken into eight different stages: prenatal/gestation, infancy and toddlerhood, early childhood, middle childhood, puberty, young adulthood, middle adulthood, and late adulthood. Throughout these different stages, maturation occurs at the different domains, such as physical, cognitive, and psychosocial. The physical domain is principally the biometrics of an individual, which can encompass biometrics, and fine and gross motor skills, among others. The cognitive domain includes learning, memory, reasoning, and other cognitive skills. Lastly, the psychosocial domain includes emotional intelligence, social interactive skills, personality, and other skills that are due to an interaction with their environment. These domains are interrelated, in which changes in one domain can impact changes in the other domains, even causing long-lasting changes. The brain is an important cornerstone in all these domains and plays an important role in how these domains interact [1] (**Figure 1**).

Development of the brain occurs early in pregnancy, specifically during the third week of gestation in which neural progenitor cells become differentiated and the neural tube is formed to make a primitive nervous system. As this primitive nervous system matures, the neural tube becomes the foundation for three brain regions: prosencephalon (embryonic forebrain), mesencephalon (embryonic midbrain), and rhombencephalon (embryonic hindbrain) [2-5] . By two months of pregnancy, the foundational structure of the brain is established, and neuron

production is initiated. The following months after gestational week 9, called the fetal period, consists of organizational structural changes, including cortical and subcortical regions. During this organizational period, newly produced neurons are migrating to different brain regions to make new connections and building neural pathways. Major neural pathways are completed by the end of gestation [6-8].

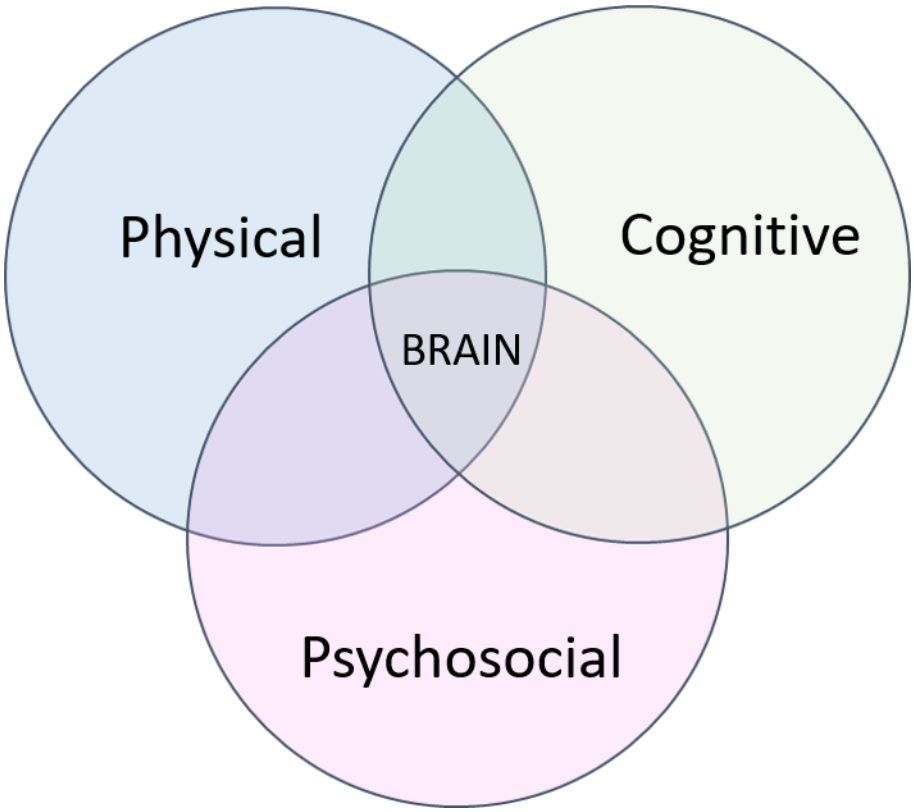

Figure 1: The developmental maturation encompasses physical, cognitive, and psychosocial domains. These domains are interrelated, due to the brain being at a focal period.

1.2 Sex differences in brain development

Sex differences in brain development occur during different stages of lifespan development that are called “critical periods”. These sex differences are under control of sex steroid hormones, such as testosterone and its metabolites, estradiol and dihydrotestosterone. According to the Organizational-Activational Hypothesis of sexual differentiation of the brain, steroid hormones permanently “organize” the brain into male or female phenotypes during *in utero* development, and then pubertal hormones “activate” these brain structures to result in phenotypic behaviors [9, 10] (**Figure 2**).

During this critical period of prenatal development, circulating testicular testosterone travels to the brain. In the brain, testosterone is metabolized to estradiol via the aromatase enzyme, and then estradiol masculinizes the brain into a male phenotype. It should be noted that both males and females have high levels of circulating estrogens but only males have a high level of circulating androgens (e.g., testosterone). For males, estradiol is metabolized from testicular testosterone. In contrast, ovarian estradiol is high in females due to maternal ovarian estrogen that can cross the placenta. However, circulating estradiol and other estrogens are not active due to being bound to binding protein globulin called alpha-fetoprotein that has a high affinity for estrogens but a low affinity for androgens. The binding of estrogens to alpha-fetoprotein keeps estrogens from entering the brain, which is how the organizational effects of estrogens are restricted to males, as only testosterone can cross the blood-brain-barrier to create the phenotypic male characteristics. In humans this period of organizational differentiation occurs roughly from the early second trimester through the mid- to late-third trimester, whereas this

period in rodent development occurs roughly in the late days of gestation though birth to the first few days of the perinatal period [11, 12].

The next critical period for sex hormone modulation of the brain is puberty, in which a surge of sex hormones can act on the organization of the brain to "activate" the brain to induce male and female behavioral phenotypes. Similar to prenatal organizational effects of sex hormones, long-term consequences of sex hormones during this critical period can occur. In rodents, puberty is typically determined to be roughly from post-natal day (PND) 40-50, aligning generally with humans' pubertal age of 9-14 years of age. Since critical periods during both prenatal development and puberty are essential for brain development, it is important to examine the impact of prenatal insults on both organizational and activational stages of brain development to understand sex differences [13, 14].

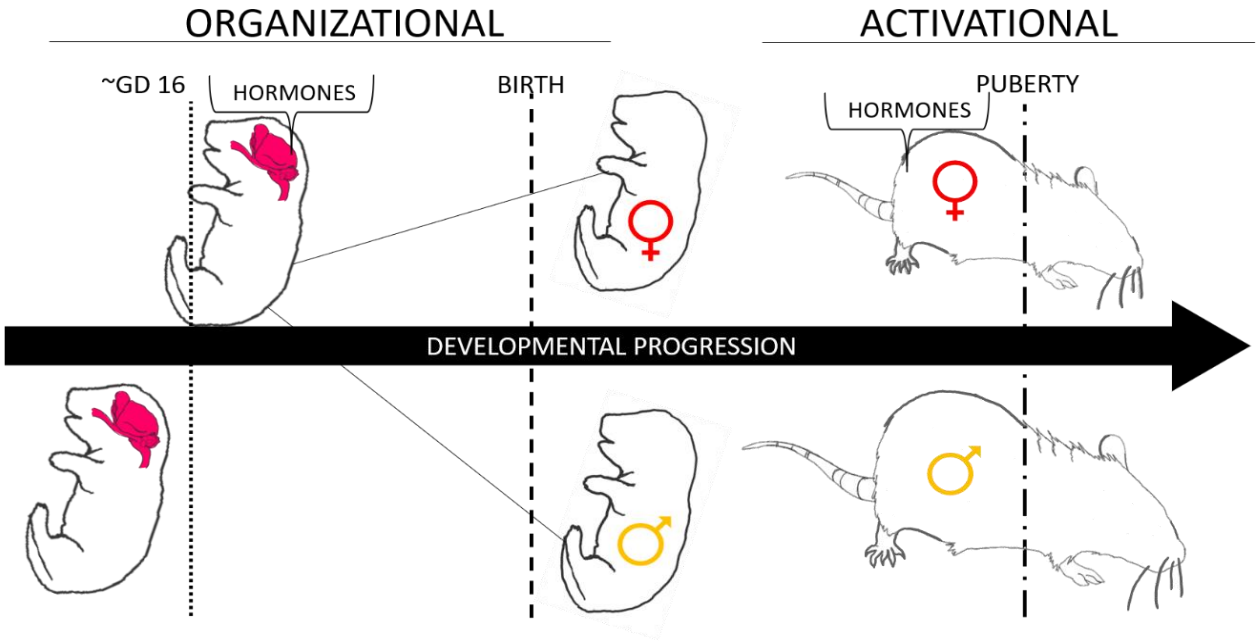

Figure 2: Critical periods of sex hormone mediated organizational and activational stages of brain maturation. Testicular testosterone crosses the blood-brain-barrier and is metabolized to estradiol, which "masculinizes" or "organizes" the brain. Pubertal hormones "activate" the brain to induce male and female behavioral phenotypes.

1.3 Major milestones comparisons between humans and rats

The rat lifespan is similar to humans with respect to developmental stages: prenatal/gestation, perinatal, prepubertal, puberty, young adulthood, middle adulthood, and late adulthood. Similarly, maturation occurs at physical, cognitive, and psychosocial domains. However, these stages are over a much shorter timeframe. The prenatal period in rats is approximately 21 days, compared to a roughly 9-month period in humans [2]. The perinatal period of rats (immediately before birth to postnatal week 2) is similar to infancy and toddlerhood in humans [15]. The prepubertal period for rats is postnatal day (PND) 22-40, which is equivalent to childhood in humans. Puberty for rats occurs PND 40-50. Young adulthood in rats is considered to be PND 60. Middle adulthood for rats is 9-12 months, and late adulthood is 24 months in rats [16, 17]. This shorter period allows many researchers to use this animal model in studies of fetal development and disease.

Brain development in rats occurs along a similar process as humans, but on a shorter time scale [18, 19] (**Figure 3**). The first 10 days of rat gestation is similar to the first three weeks of human gestation, in which neural progenitor cells become differentiated and the neural tube is formed [2]. The following week of rat gestation (days 10-16) is comparable to the first two months of human gestation, in which the foundational structure (cortical and subcortical regions) of the rat brain is established with neural production. During mid gestation or the embryonic stage, the rat brain is beginning to develop into specific regions. For example, one of the major brain pathways that is integral for gross and fine motor movements is the nigrostriatal pathway that consists of the substantia nigra (SN) and the corpus striatum (STR). The SN reaches peak formation around gestational day (GD) 14 in rats compared to around week 6 or 7 in humans

[20]. Distinct regions of the SN can be recognized by GD 20 [21]. Neural development of the striatum in the human brain is later than the SN, occurring at week 12 [22]. Consistent with this pattern in brain development, striatum development in rats occurs at GD 17 [23]. Lastly, the last week of rat gestation (GD 16-21) is roughly equivalent to week 9 through delivery in humans. This period allows for maturation of tissues and organs, as well as overall growth of the fetus.

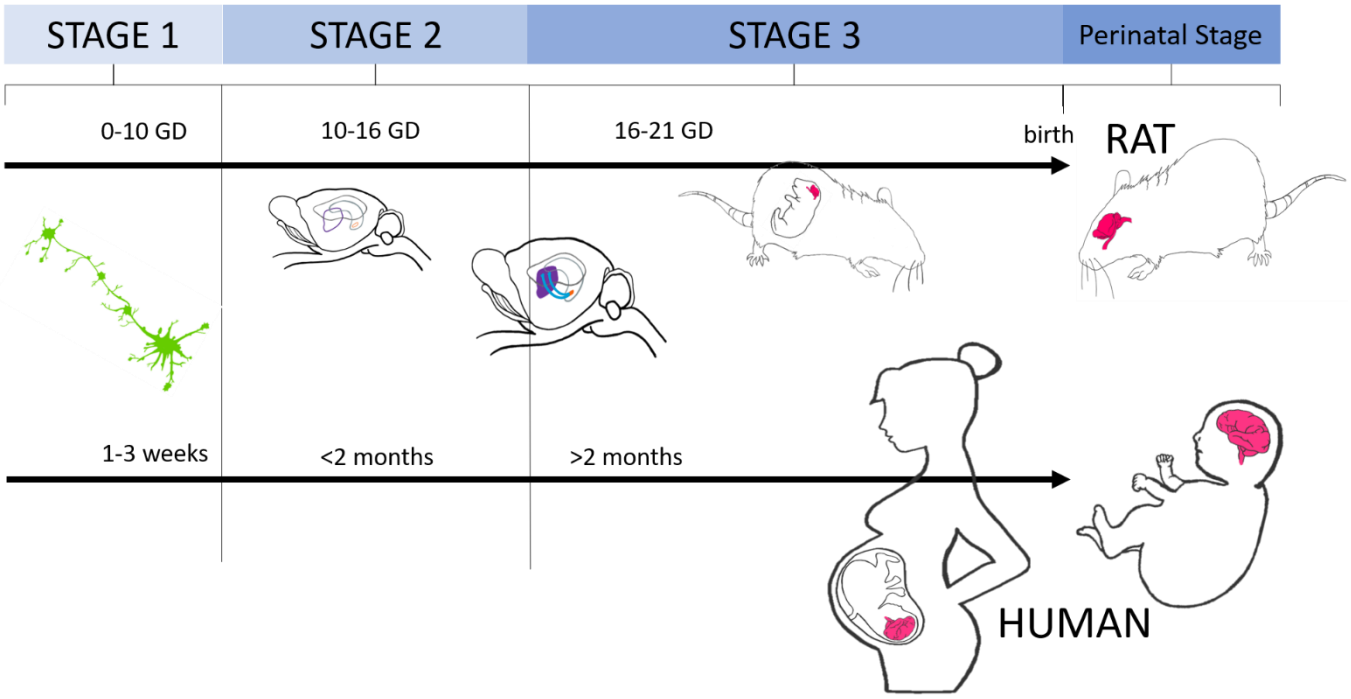

Figure 3: Developmental milestones of human and rat gestation. During stage one, neural progenitor cells become differentiated, and the neural tube is formed. During stage two, cortical and subcortical regions of the brain are established with neural production. Stage three is when maturation of tissues and organs occur. During the perinatal stage, astrocytes are produced (gliogenesis), which refine neuronal circuits via apoptosis of neurons to result in the loss of 20-80% of neurons.

1.4 Insults during brain development and prenatal programming

Gestational brain development is critical, as it can impact all the maturation stages of physical, cognitive, and psychosocial. Therefore, insults during any period of gestation could negatively impact the developing progeny [24]. Since the brain is maturing throughout gestation, insults can have different effects that are dependent on the time of the insult during gestation [25]. Insults during early pregnancy interfere with the development of the organizational structure of the brain, as this period is when the neural tube is developed [26-28]. However, insults during the human week 9 to delivery or rat GD 16-21 can have a significant impact on cortical and subcortical maturation, especially in neuronal cell types as astrocytes are developed during the perinatal stages of rat and human development [29-31]. Lastly, insults during the perinatal period can also impact brain maturation. During the perinatal period, astrocytes are produced (gliogenesis) and involved in modifying and refining neuronal circuits by inducing apoptosis of neurons, resulting in the loss of 20-80% of neurons [31] (**Figure 3**).

1.5 Sex differences prenatal vulnerability to insults

Studies have shown that male and female progeny respond differently to insults during gestation [32-35]. This is an active field of investigation. Males have a higher incidence of neurodevelopmental disorders than females, such as disorders that impact the psychosocial domain (e.g., autism, attention deficit/hyperactivity disorder), the cognitive domain (e.g., mental retardation), and physical domain (e.g., growth) of maturation [34, 36-43].

Prenatal insult rodent paradigms observed that male progeny, but not female progeny, exhibited impairments of all maturation domains: psychosocial domain (elevated brain regulated stress responses during adulthood [44, 45]), impaired social behavior [46], cognitive domain (learning and memory deficits [34, 46]), and physical domain (decreased growth following weaning [44, 45] and impaired motor function) [47]. Further, males have been shown to be more sensitive to common prenatal insults that can occur anytime during gestation, such as placental inflammation [36, 48], hypoxia [36, 48]; insults that can occur during mid pregnancy, such as placental abruption (~25 weeks human pregnancy) [47] and early preterm birth [49]; insults that occur during late pregnancy, such as term preeclampsia and eclampsia [47].

Although many studies have found that males may be more vulnerable to prenatal insults, recent studies have found that prenatal insults can also negatively impact female progeny. Animal studies that include females are not as extensively conducted, and thus the impact of prenatal insults on female progeny is understudied compared to male progeny [50-52]. A recent study using mid-gestation (GD 12.5) maternal inflammatory insults found that female progeny exhibited elevated pubertal social behaviors, increased anxiety behaviors, and growth impairments compared to males [47]. Therefore, the timing of the prenatal insult and the type of prenatal insult can impact male and female progeny differently.

1.6 Prenatal hypoxia and long-term consequences

A common consequence of many prenatal stressors (e.g., preeclampsia, eclampsia, inflammation, placental abruption) is hypoxia or decreased oxygen [25, 27, 29, 53, 54]. Generally, in instances of human pregnancy, stress that occurs from a decrease in oxygen to the mother or

fetus, is termed hypoxic stress. Hypoxic stress has been shown to impact male progeny more than female progeny [29, 36, 47, 48]. Generally, hypoxic stress occurs during late gestation, which is when cortical and subcortical maturation occurs (**Figure 3**). In rats the nigrostriatal pathway is established during late gestation at GD 16-20 [55]. Impairments of the nigrostriatal pathway can have long-term consequences and may even increase the risk for PD that results from an impaired nigrostriatal pathway [56, 57]. Studies have found that hypoxic insults during late gestation can negatively influence the nigrostriatal pathway in rats, with effects that persist to adulthood [58, 59].

Specifically, maternal hypoxic stress induced a multitude of long-term impairments across the physical, cognitive, and psychosocial domains, such as long-term memory problems [60-62] and motor impairments [63]. Nevertheless, discussions still exist over the mechanistic link between the hypoxic insult and the resulting effects from damage. However, few studies have examined the long-term impact of chronic hypoxic insults on the brain, much less in both male and female progeny. These investigations are needed as prenatal insults can increase the vulnerability of the brain, which may increase the risk for neurodegenerative disorders.

1.7 Neurodegeneration

Neurodegeneration, a process in which increased damage to neurons can lead to various abnormalities, is being vigorously studied as the causes for most neurodegenerative diseases are unknown. Recently, many scientists have proposed that prenatal insults may be involved in neurodegenerative disorders by increasing the vulnerability of specific brain regions to future

insults. This has led to the “two hits or multiple hit” hypothesis for neurodegenerative disorders, such as PD and Alzheimer’s disease. Specifically, the “first hit” occurs during early development which results in increasing vulnerability to a “second hit” later in life that will increase neurodegenerative disease risk [64-68].

Since the nigrostriatal pathway is sensitive to prenatal hypoxic insults [58, 59], it is possible that prenatal hypoxic insults can increase PD risk through the “multiple-hit” hypothesis. PD, an idiopathic disorder, has become the second leading neurodegenerative disease in the US. It was estimated that in the U.S. alone, 930,000 individuals would be living with PD as of 2020, with 1.2 million persons struggling with the disease by 2030 [69]. The annual economic cost of PD on both families and the federal government is approximately $52 billion per year [70]. This assessment included information on the total economic burden on patients, care partners, payers, employers, healthcare systems, and government programs, indicating a need for determining early risk factors for the disease.

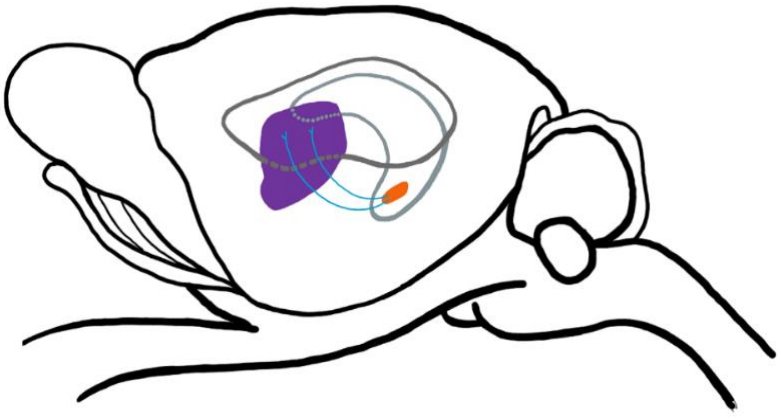

Figure 4: The nigrostriatal pathway is composed of the substantia nigra (orange) and the corpus striatum (purple), which are connected to each other by the medial forebrain bundle (blue). This pathway plays an important role in motor control.

1.8 Parkinson's disease

Neurodegeneration in Parkinson's disease is mostly the observed loss in dopaminergic neurons within the nigrostriatal pathway, mainly within the substantia nigra pars compacta (SN) [56, 71, 72] (**Figure 4**). Motor and non-motor related symptoms are typically observed once approximately 80% of SN is lost [56, 73]. Motor symptoms including tremors, 'pill-rolling' behavior, rigidity, akinesia (the loss of voluntary movement), postural abnormalities, and soft speech. Non-movement related symptoms include cognitive changes in memory or language, mood disorders like depression or anxiety, and decreased sense of taste or smell [74-76] (**Figure 5**). Although the cause of the loss of dopaminergic neurons within the nigrostriatal pathway in Parkinson's disease is unknown, oxidative stress is one of the key factors involved in this neurodegeneration [77-80].

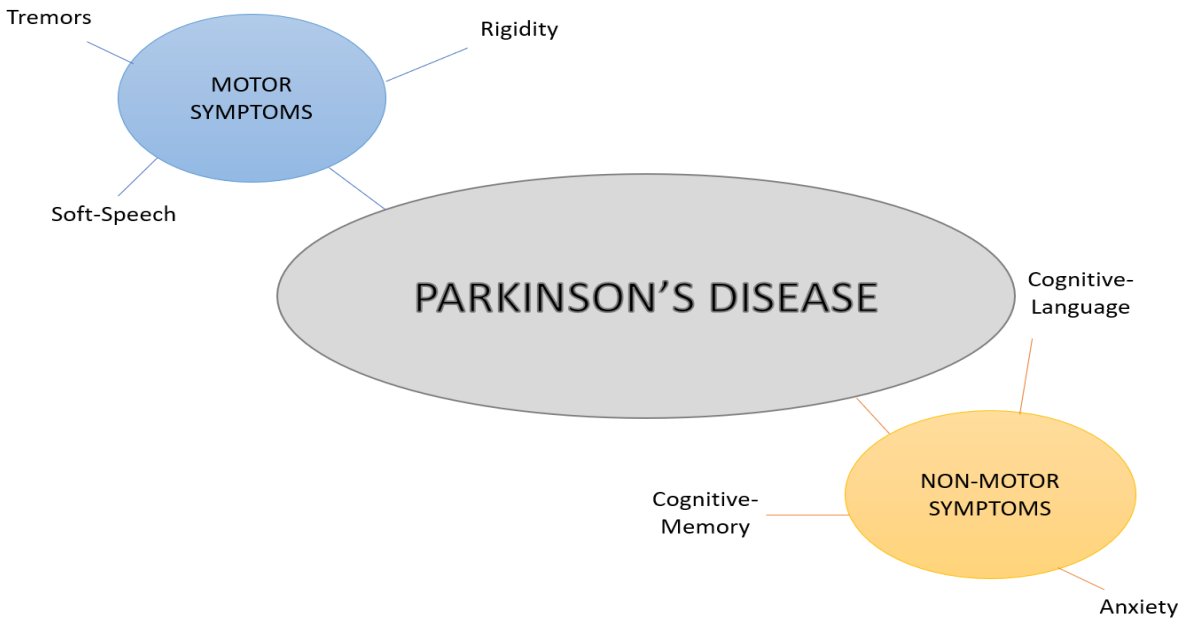

Figure 5: Clinical manifestations of Parkinson's disease include motor and non-motor symptoms. Motor symptoms can include tremors and soft speech, while non-motor symptoms include cognitive memory and language deficits.

Oxidative stress is the accumulation of free radical or reactive oxygen species. Accumulation of these free radicals can be detrimental and cause cell loss via apoptosis (**Figure 6**), especially to dopaminergic neural pathways such as the nigrostriatal pathway [81-83]. The impact of oxidative stress on the brain has led to the oxidative stress theory of aging, which proposes that cellular damage is due to a buildup of free radicals over time [84, 85]. Interestingly, sex differences in oxidative stress levels have been observed, in which oxidative stress is significantly higher in men compared to woman and accumulates with age [86, 87]. Similarly, this sex difference in oxidative stress has been observed in dopaminergic neurons of the SN male rats compared to female rats [88]. Further, hypoxic insults can increase oxidative stress within the nigrostriatal pathway in adult male rats [89, 90]. This sex difference in oxidative stress may mediate the male-bias sex difference observed in PD incidence [91, 92].

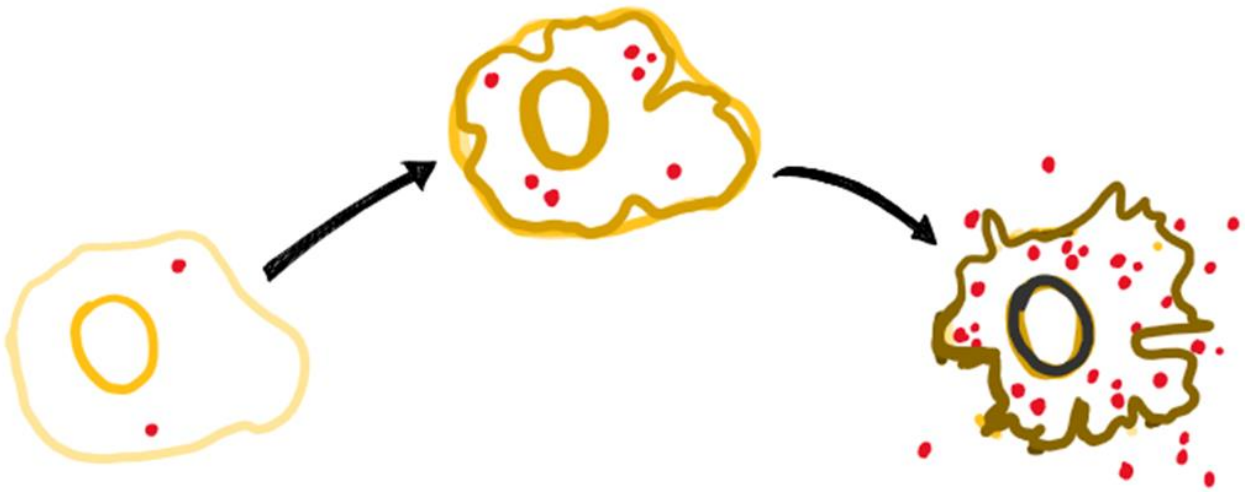

Figure 6: Oxidative stress involves the build-up of reactive oxygen species within a normal healthy cell (left to middle), which if not curbed, can result in the death or apoptosis of the cell (right).

1.9 Parkinson's disease and sex hormones

Sex hormones, such as androgens and estrogens, have been proposed to mediate the sex differences observed in PD [87, 91]. Estrogens are considered the primary sex hormone for females, whereas androgens are the major male sex hormone. Several studies have hypothesized that estrogens may play a neuroprotective role, especially as PD incidence increases after menopause, a period of substantial estrogen loss in women [93-95]. Indeed, estrogens have anti-oxidative stress properties [96-98]. Although menopause is primarily considered a period of low estrogen, it is also a period of higher androgen to estrogen ratio as the ovaries continue to produce androgens [99-102]. In contrast to estrogens, androgens can act as an oxidative stressor [103, 104]. Therefore, it is possible that androgens and not estrogens drive PD risk in postmenopausal women and men.

1.10 Nigrostriatal impairment analysis in rodents

Determining the neural pathway impairments can be explored in animal studies. For example, the nigrostriatal pathway can be examined by several different behavior tests and biochemical assays. Oxidative stress damage and apoptosis can be examined by quantifying proteins in the nigrostriatal pathway that have undergone oxidative stress. Since oxidative stress in the nigrostriatal pathway can lead to cell loss and motor impairments, motor behaviors can be quantified.

In general, gross motor skills assess the ability of an individual to control and conduct broad limb movements, like walking, running, or standing, whereas fine motor skills include

grasping, reaching, and balance. There are various animal behavior tests that can examine these functions, such as locomotor behavior in an open field [105]. In this behavior task, a rodent is placed in an arena for a specific amount of time. Quantification of the rodent's gross locomotor activity, such as distanced traveled, is recorded. Fine motor activity can be assessed in the open field arena by examining balance via quantification of unassisted rears. Generally, gross motor activity is unaffected unless there is an 80% dopaminergic cell loss within the SN of the nigrostriatal pathway [56, 73]. However, fine motor impairments can be observed under conditions of less damage to the nigrostriatal pathway. If there is no cell loss in the nigrostriatal pathway but cellular dysfunction is present, these behavioral tasks may not be sensitive enough to quantify nigrostriatal impairment. However, there is one behavior assay that is sensitive enough to detect minor nigrostriatal impairment – ultrasonic vocalizations [106-111]. Ultrasonic vocalizations are calls made by rodents that are undetectable with human hearing. These calls can be characterized by the type of calls, intensity of calls, duration of calls, and the total number of calls [112].

1.11 Summary

It is unknown what the impact of chronic prenatal hypoxic insults during late gestation has on the nigrostriatal pathway and the long-lasting impact of this prenatal insult in male and females. Based on the "multiple-hit" hypothesis, hypoxic prenatal stress may increase the vulnerability of males more than females, which may be involved in the male-sex bias observed in PD.

1.12 Significance

The cause of PD is unknown. However, if prenatal stressors increase brain vulnerability and increase the risk for PD, then it may be possible to identify individuals at risk earlier in PD pathogenesis. If we can identify individuals and place them on treatments that can decrease oxidative stress (e.g., diet) prior to the manifestation of PD symptoms, such as motor impairment, then we may improve their quality of life and slow the progression of PD.

CHAPTER 2:

PRENATAL HYPOXIC INSULTS IMPACT

BRAIN VULNERABILITY

2.1 Specific Aims

Specific AIM 1: Determine the role of maternal hypoxic stress during late gestation on Parkinson's disease associated behaviors in pubertal and young adult male and female rats.

> **Hypothesis:** *Maternal exposure to hypoxic stressors while pregnant will increase PD associated behaviors in pubertal and adult male rats compared to female rats.*

Specific AIM 2: Determine the effects of maternal hypoxic stress during late gestation on oxidative stress in pubertal and young adult male and female rats.

> **Hypothesis:** *Maternal exposure to hypoxic stressors while pregnant will increase oxidative stress in pubertal and adult male rats compared to female rats.*

2.2 Methods

Animals

16 adult Long Evans first-time pregnant females were subjected to chronic intermittent hypoxia (CIH) or regular room air normoxia for a period of five days during the late gestational period of days 15 to 19. Animals then delivered naturally approximately on gestational day (GD) 21. Roughly 12-16 hours post-delivery, progeny animals were weighed, measured, and sexed by anogenital distance, and culled to approximately 4 males and 4 females per dam. The progeny were then allowed to mature with the mother without interruption until post-natal day (PND) 28, whereby the animals were weaned from their mothers.

Progeny rats were separated by sex and pair or triple housed with rats of similar weight and hypoxic treatment. Male and female rats were housed in separate rooms, in which each room was on a 12h reverse light cycle where lights were off at 900h. Reverse lighting allowed for behavioral testing to be conducted during animals wake phase of the circadian cycle.

Food and water were provided to all animals *ad libitum.* Progeny body weights were obtained weekly. To acclimatize rats to operator handling and reduce stress responses during behavior testing, animals were handled daily, beginning approximately 10 days prior to the start of behavior testing. All experiments were approved by IACUC at UNT Health Science Center and conducted in accordance with NIH guidelines on laboratory animals.

Chronic Intermittent Hypoxia (CIH)

Timed pregnant females were assigned to receive either CIH (n=8) or normoxic (NORM; n=8) treatments for 8 hours during their sleep phase of the circadian rhythm. To induce CIH, the home cages of the timed pregnant females were placed into Oxycycler chambers (76.2x50.8x50.8cm, BioSpherix, Lacona, NY, USA). Timed pregnant females were allowed to acclimatize to the Oxycycler under Normoxic (room air) conditions for a period of 4 days prior to starting the CIH protocol. The CIH protocol consists of oxygen reduction from 21% (room air) to 10%, then returned to 21% in 6 min cycles per hour (10 cycles/hour) over 8 hours/day for a period of 5 days, as previously described [89]. Once progeny were delivered, there were a total of 4 treatment groups: Female Normoxic (FN), Female CIH (FC), Male Normoxic (MN), and Male CIH (MC).

Behavioral Tasks

Behavioral studies were conducted either during puberty (PND 40-45) or young adulthood (PND 60-65) over two days during their wake period from 0945h to 1700h. Conducted behavior tests were randomized. Day one and day two studies were separated by a minimum of 18 hours. Male animals were behaviorally tested before females to avoid pheromone confounds on behavior. Behavioral apparatuses were thoroughly cleaned with 70% ethanol between each animal. One hour following behavior testing on males, female behaviors were examined. All behavior studies were conducted under red lighting and recorded for later analysis.

Open Field Test: Gross and fine motor activities were quantified using the San Diego Instruments Photobeam Activity System-Open field arena (40.64x40.64x38.1cm) using a unidirectional "rearing" bar located above a bi-directional main field bar. Open field behaviors of distanced traveled (cm), and the number of assisted and unassisted rears were tracked and analyzed for a period of 10-minutes. An assisted rear was classified when the animal stood on its hind limbs but used a forelimb to touch a wall, thus leaning or requiring aid to stand fully erect. If no such aid was used, the rear was considered 'unassisted'. To avoid confounds due to activity differences, rearing behaviors were normalized to distanced traveled (cm x 10^{-3}).

Ultrasonic Vocalizations: To examine nigrostriatal pathway integrity, ultrasonic vocalizations within the frequency of 50 kHz were quantified by using UltraSoundGate 116Hb, condenser microphones (CM16/CMPA) and Avisoft-Recorder USGH programming. To conduct this test, two cage mates were placed into a 50x25x30cm aquarium enclosed within a sound dampening chamber equipped with an Avisoft microphone. Once cage mates were placed into the aquarium, they were allowed to explore for two minutes [112, 113]. After two minutes, cage mates were placed into separate aquariums equipped with microphones to record ultrasonic vocalizations emitted in response to the separation of their cage mate for an additional five minutes. Separated cage mates will emit 50 kHz ultrasonic vocalizations, which is due to the separation and not the novelty of a new chamber [114]. This separation of cage mate protocol was used to avoid confounds of pheromone or hormonal elicitation of ultrasonic vocalizations that would be involved if opposite-sex conspecifics were used, as sex differences in opposite sex-elicited ultrasonic vocalizations occur [115-121]. Ultrasonic vocalizations were captured by Avisoft SASLab Pro (Version 5.2.12, Avisoft Bioacoustics, Berlin, Germany) recording equipment

and software. Spectrograms were made using a fast Fourier transform (FFT)-length of 256 points and an overlap of 50%. Spectrograms had a frequency resolution of 977 Hz and a time resolution of 0.5 ms. A threshold between -40 and -50 dB was chosen to remove background noise. If background noise interfered with call classification, those calls were not analyzed. Calls were manually selected within the spectrogram and categorized by call type.

Ultrasonic vocalizations were characterized based on type of calls, total calls made, call duration, call bandwidth, and call decibel levels. Call types included: chirp, simple, frequency modulated (FM), harmonic, and complex (**Figure 7**). All calls were selected for analysis based on those emitted at 50 kHz frequency, as these are described as vocalizations produced during conditions of reward, positive affect, or normal behaviors. Chirp type calls were defined as low intensity calls less than a 0.02ms duration, whereas simple calls were defined as calls emitted longer than 0.02ms durations visibly seen as a straight line. FM calls typically vary in duration and decibel level, but present with distinct waveforms that increase and decrease in frequency. Harmonic type calls were defined as one call with two separate, yet identifiable tones, creating a 'harmony', also varying in duration and decibel level. The final type was termed complex, defined as a combination of at least two identifiable call types (e.g., simple and FM), in which neither call could be separated into one distinct call.

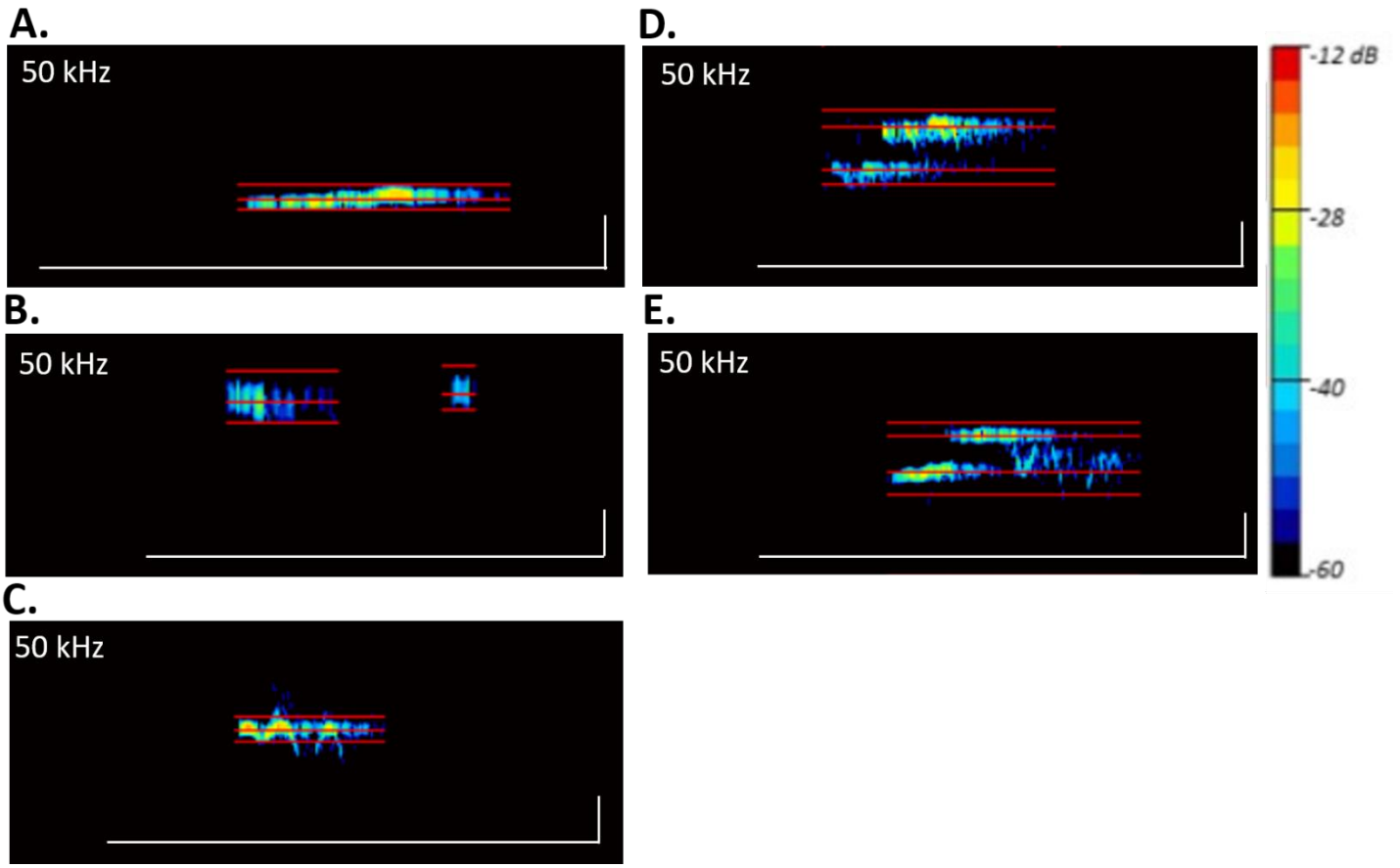

Figure 7. Categories of ultrasonic vocalizations. 50 kHz were analyzed, as these are considered within the normal frequency for social, reward, or positive conditions. A). 50 kHz simple calls are emitted typically as a straight line and greater than 0.02 milliseconds (ms). B). Chirps are short duration simple calls less than 0.02ms with a low intensity. C). Frequency modulated (FM) calls are waveforms that increase and decrease in frequency D). Harmonic calls are defined as one call separated into two tones, creating a 'harmony' and (E). complex calls are composed of more than one identifiable call (e.g., harmonic and FM), in which neither call can be separated into one distinct call. White bars are calibration bars: horizontal bars are time (10ms). The bar to the right indicates decibel level.

Biochemical Assays – Advanced Oxidative Protein Products (AOPP)

Circulating oxidative stress was assayed using Cell Biolabs, Inc. OxiSelect Advanced Oxidative Protein Products (AOPP) assay kit, according to our previously published protocol [89]. This kit measures the amount (uM) of all oxidized proteins in the plasma sample relative to a known standard. Chloramine in the kit reacts with oxidized proteins to produce a color change which can be read at 340nm. Assay results were reported as percent of control of the female normoxic animals (individual value / (average of normoxic control values) x 100).

Statistics

Significance was defined as $p \leq 0.05$, with a 95% confidence that results were not due to chance. In instances where results were greater than 0.05 but less than $p < 0.10$, the results were considered a trend with a 90% confidence that results were not due to chance. An interaction was defined when the significance of one independent variable was dependent on another independent variable. Main effects are defined when significance was seen in an independent variable of sex or CIH. Statistics were performed in a 2x2 ANOVA format using the factors of maternal exposure to hypoxic stressors during late pregnancy (normoxia, CIH) and sex of progeny (male, female) within the pubertal or young-adult groups. ANOVAs were performed for all behavior and biometric studies.

2.3 Results

Maternal hypoxic stress during late gestation increased body weight in young adult males

Final body weights were quantified at two different developmental time periods: puberty (P48) and young adult (P66). During puberty, no effect of maternal hypoxic stress was observed in male or female progeny ($F_{1, 59} = 0.0376$, $p > 0.10$) nor was there an interaction between these variables ($F_{1, 59} = 0.88$, $p > 0.10$). However, male pubertal progeny was significantly heavier than female pubertal progeny, regardless of maternal hypoxic stress ($F_{1, 59} = 109.699$, $p \leq 0.05$).

Male progeny were heavier than female progeny during young adulthood ($F_{1, 52} = 587.31$, $p \leq 0.05$), regardless of maternal hypoxic insults ($F_{1, 52} = 0.706$, $p > 0.10$). However, maternal hypoxic insult increased young adult male progeny weights, as evidenced by a significant interaction between sex and maternal hypoxic stress ($F_{1, 52} = 5.69$, $p \leq 0.05$) (**Figure 8**).

Progeny motor function was not impacted by maternal hypoxic stress during late gestation

Motor skills were assessed by using an open field behavior test. Gross motor function was quantified by examining distanced traveled (cm) and assisted rears normalized to distance traveled (cm x 10^{-3}) during a 10-minute test. Although not significant, a trend between the independent variables of sex and maternal hypoxic stress was observed on the distanced traveled during puberty ($F_{1, 28} = 3.001$, $p = 0.09$). Specifically, maternal hypoxic stress increased distance traveled in female pubertal progeny (FN: 3263.05 +/- 1426.18 s.d. versus FC: 5220.13 +/- 1881.04 s.d.) but not male pubertal progeny. There was no statistical significance due to either sex ($F_{1, 41} = 0.099$, $p > 0.10$), maternal hypoxic stress ($F_{1, 41} = 2.158$, $p > 0.10$), or an interaction between these variables on distance traveled during young adulthood. Further, differences were not

observed in pubertal progeny regarding assisted rears, regardless of sex ($F_{1, 28} = 1.93$, $p > 0.10$) or maternal hypoxia ($F_{1, 28} = 0.039$, $p > 0.10$). In the same way, no differences were found in the young adult progeny despite sex ($F_{1, 41} = 1.309$, $p > 0.10$) or maternal hypoxic stress (($F_{1, 41} = 0.022$, $p > 0.10$) of assisted rears (**Figure 9**).

Fine motor skills were assessed by quantifying unassisted rears normalized to distance traveled (cm x 10^{-3}) during a 10-minute test in the open field arena. Fine motor skills, as assessed by unassisted rears, were not impacted in pubertal progeny by maternal hypoxic stress ($F_{1, 29} = 0.485$, $p > 0.10$) or sex ($F_{1, 29} = 0.782$, $p > 0.10$). Although not significant, a trend was found in young adult animals regarding sex ($F_{1, 41} = 2.949$, $p = 0.09$) in unassisted rears with male progeny performing more rears than female progeny (**Figure 10**).

These results indicate that neither sex nor maternal hypoxic stress has long-term effects on gross and fine motor functions, as measured by distance travelled, assisted rears, and unassisted rears. These results are consistent with the nigrostriatal pathway not exhibiting severe degeneration, as loss of motor function is observed when 80% of dopaminergic neurons in the SN are lost [73].

Ultrasonic vocalizations are a measure of nigrostriatal pathway integrity

The integrity of the nigrostriatal pathway can be assessed by analyzing ultrasonic vocalizations [108-110]. Unlike impairments in gross motor function that are only observed when most of the SN is degenerated [73], ultrasonic vocalization impairments can be observed before substantial neurodegeneration of the SN is observed [108-110]. Ultrasonic vocalizations can be examined by quantifying the frequency of calls, the latency to call, call duration, call bandwidth,

call decibel level, along with the type of calls (e.g., chirp, simple, harmonic, frequency modulated, complex) (**Figure 7**).

Long-term effects of maternal hypoxic stress during late gestation on total calls only observed in young adult male progeny

Maternal hypoxic stress during late gestation significantly impacted the frequency of total calls (all call types) emitted ($F_{1,\ 58}$ = 8.634, $P \leq 0.05$). Specifically, maternal hypoxic insult suppressed the frequency of total calls made by progeny during puberty. However, this effect of maternal hypoxic stress on the frequency of total calls was lost during young adulthood ($F_{1,\ 55}$ = 0.264, $p > 0.10$). A sex effect on the frequency of total calls during young adulthood was observed, in which young adult female progeny did emit more total calls than young adult male progeny ($F_{1,55}$ = 4.617, $p \leq 0.05$) (**Figure 11**). In pubertal progeny, maternal hypoxic stress increased the latency or time to first call ($F_{1,\ 45}$ = 4.691, $p \leq 0.05$). This increase in call latency was only maintained in males during young adulthood but not females ($F_{1,\ 38}$ = 8.660, $p \leq 0.05$) (**Figure 12**).

Since total calls were impacted, investigation of specific call types was examined to determine if this effect of maternal hypoxia was due to a specific call. Ultrasonic vocalizations by rodents generally fall within the following types of calls: chirps, simple, harmonic, FM, and complex. In this study, the progeny emitted sufficient calls within the chirp, simple, and harmonic type calls for statistical analyses.

Long-term effects of maternal hypoxic stress during late gestation on simple calls observed in young adult progeny

Maternal hypoxic insults decreased the frequency of chirps emitted by pubertal female progeny ($F_{1, 43} = 6.697$, $p \leq 0.05$) (**Figure 13**). Further, maternal hypoxia decreased the duration of chirps in all pubertal progeny ($F_{1, 41} = 5.174$, $p \leq 0.05$) (**Figure 14**) but sex did not alter chirp duration ($F_{1, 41} = 0.001$, $p > 0.10$). No differences in chirp decibels were observed in pubertal progeny, regardless of maternal hypoxic insult ($F_{1, 43} = 0.596$, $p > 0.10$) or sex ($F_{1, 43} = 1.009$, $p > 0.10$). No effects of maternal hypoxia on frequency of chirps ($F_{1, 34} = 0.045$, $p > 0.10$), chirp duration ($F_{1, 34} = 1.348$, $p > 0.10$), or chirp decibel level ($F_{1, 34} = 0.542$, $p > 0.10$) were observed during young adulthood. Similarly, no effects of sex on frequency of chirps ($F_{1, 34} = 0.092$, $p > 0.10$), chirp duration ($F_{1, 34} = 1.430$, $p > 0.10$), or chirp decibel level ($F_{1, 34} = 0.433$, $p > 0.10$) were observed during young adulthood.

Maternal hypoxic insult decreased frequency of simple calls made by pubertal female progeny but did not impact male pubertal progeny ($F_{1, 28} = 3.746$, $p = 0.06$) (**Figure 15**). No effects of maternal hypoxic insult were observed on simple call duration ($F_{1, 28} = 0.484$, $p > 0.10$) or simple decibel level ($F_{1, 28} = 0.198$, $p > 0.10$) in pubertal progeny. Similarly, no effects of sex on simple call duration ($F_{1, 28} = 1.66$, $p > 0.10$) or simple decibel level ($F_{1, 28} = 0.805$, $p > 0.10$) were observed during puberty. During young adulthood, maternal hypoxic insult did not impact frequency of simple calls ($F_{1, 23} = 0.106$, $p > 0.10$), regardless of sex ($F_{1, 23} = 1.308$, $p > 0.10$). However, maternal hypoxic insult did increase the duration of simple calls in young adult females ($F_{1, 23} = 4.932$, $p \leq 0.05$) (**Figure 16**). Interestingly, maternal hypoxic insult decreased the loudness of simple calls by decreasing decibels in only young adult male progeny ($F_{1,23} = 10.79$, $p \leq 0.05$) (**Figure 17**).

Long-term effects of maternal hypoxic stress during late gestation on harmonic calls observed in young adult progeny

During puberty, maternal hypoxic insult did not affect the frequency of harmonic calls ($F_{1, 29} = 1.962$, $p > 0.10$), regardless of sex ($F_{1, 29} = 0.032$, $p > 0.10$). Neither maternal stress ($F_{1, 29} = 1.138$, $p > 0.10$) nor sex ($F_{1, 29} = 0.075$, $p > 0.10$) impacted harmonic call duration. Similarly, no effects of maternal stress ($F_{1, 29} = 0.044$, $p > 0.10$) or sex ($F_{1, 29} = 0.172$, $p > 0.10$) impacted harmonic call decibels. The bandwidth of harmonic calls by pubertal progeny was not affected by maternal hypoxic insult ($F_{1, 29} = 0.506$, $p > 0.10$) or sex ($F_{1, 29} = 0.607$, $p > 0.10$).

No effects of maternal hypoxia on the frequency of harmonic calls ($F_{1, 26} = 0.017$, $p > 0.10$), harmonic call duration ($F_{1, 26} = 0.096$, $p > 0.10$), or harmonic decibel level ($F_{1, 26} = 0.282$, $p > 0.10$) were observed during young adulthood. Similarly, no effects of sex on the frequency of harmonic calls ($F_{1, 26} = 0.193$, $p > 0.10$), harmonic call duration ($F_{1, 26} = 0.851$, $p > 0.10$), or harmonic decibel level ($F_{1, 26} = 0.603$, $p > 0.10$) were observed during young adulthood. However, both sex and maternal hypoxic stress impacted the bandwidth of harmonic calls. Maternal hypoxic stress increased the bandwidth of harmonic calls in young adult progeny ($F_{1, 26} = 5.517$, $p \leq 0.05$). Young adult males exhibited increased bandwidth in harmonic calls compared to young adult females ($F_{1, 26} = 4.631$, $p \leq 0.05$) (**Figure 18**).

Maternal hypoxic stress during late gestation did not impact plasma oxidative stress in progeny

Oxidative stress was quantified in progeny animals by measuring AOPP in the plasma. The oxidative stress marker was not increased in pubertal progeny regardless of sex ($F_{1, 57} = 0.378$, p

> 0.10) or maternal hypoxic stress ($F_{1, 57}$ = 0.000, p > 0.10). Further, there were no significant differences in plasma oxidative stress due to maternal hypoxic stress ($F_{1,46}$ = 0.068, p > 0.10) in young adult animals or sex (F1,46 = 0.103, p > 0.10) in young adult animals (**Figure 19).** Circulating oxidative stress (AOPP) was also measured in the timed pregnant females at GD20. AOPP was not elevated in any of the timed pregnant females ($F_{1, 10}$ = 0.065, p > 0.10) (**Figure 20**).

These results indicate that maternal hypoxic stress during late gestation did not impact circulating oxidative stress levels in the progeny, which is consistent with the lack of motor impairment in progeny exposed to maternal hypoxic stress during late gestation. Further, this was a mild hypoxic stress in the timed pregnant females, as evidenced by the lack of elevation in oxidative stress.

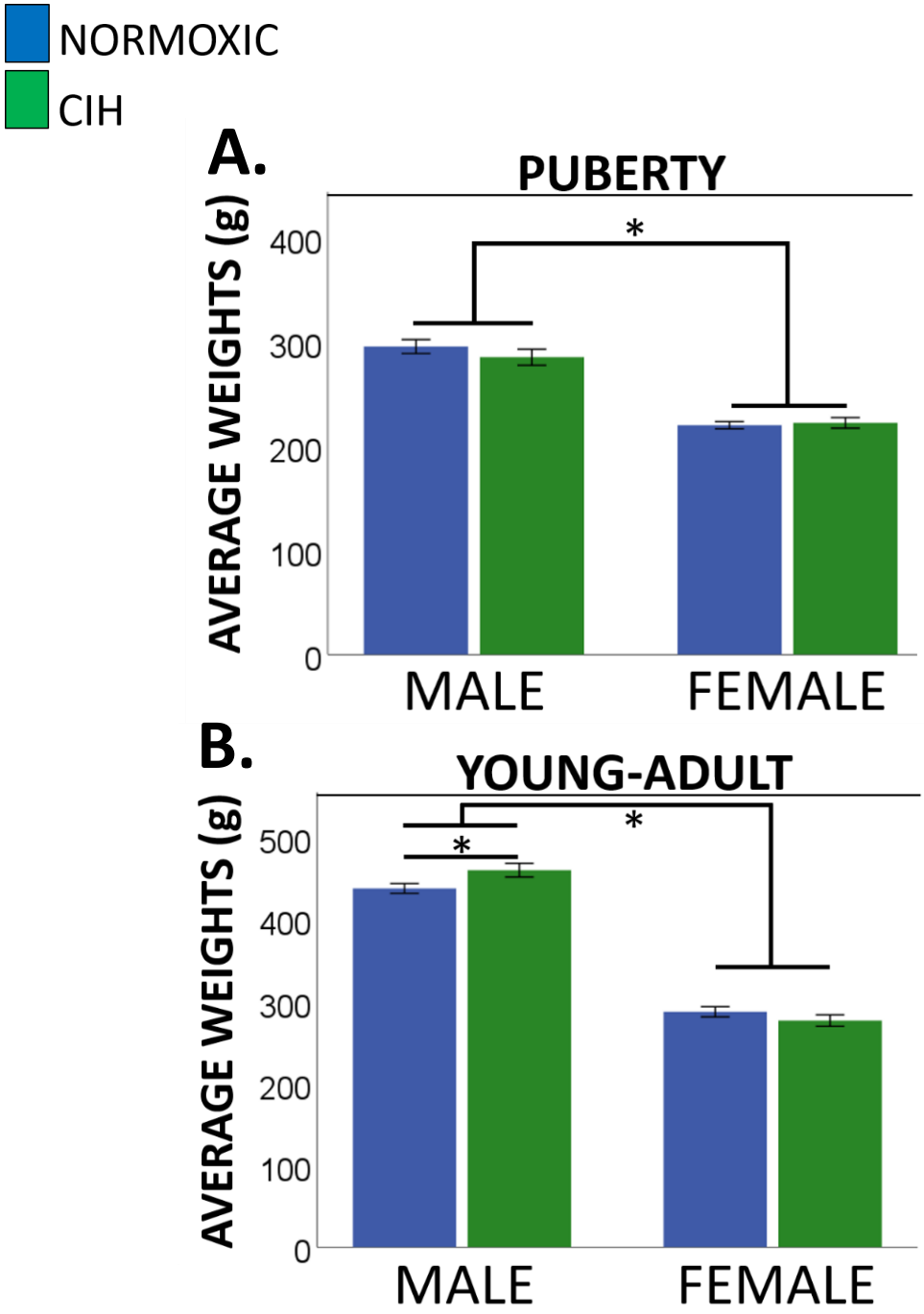

Figure 8: Final body weights at puberty (48d) and young adulthood (66d). A.) Puberty animals observed no effects of maternal hypoxic stress in male or female progeny (MN 16, MC 20, FN 14, FC 13). Male pubertal progeny were significantly heavier than female pubertal progeny, regardless of maternal hypoxic stress. B.) Male adult progeny were significantly heavier than female adult progeny, regardless of maternal hypoxic stress (MN 17, MC 13, FN 14, FC 12). However, maternal hypoxic insult increased young adult male progeny weights. * = $p \leq 0.05$.

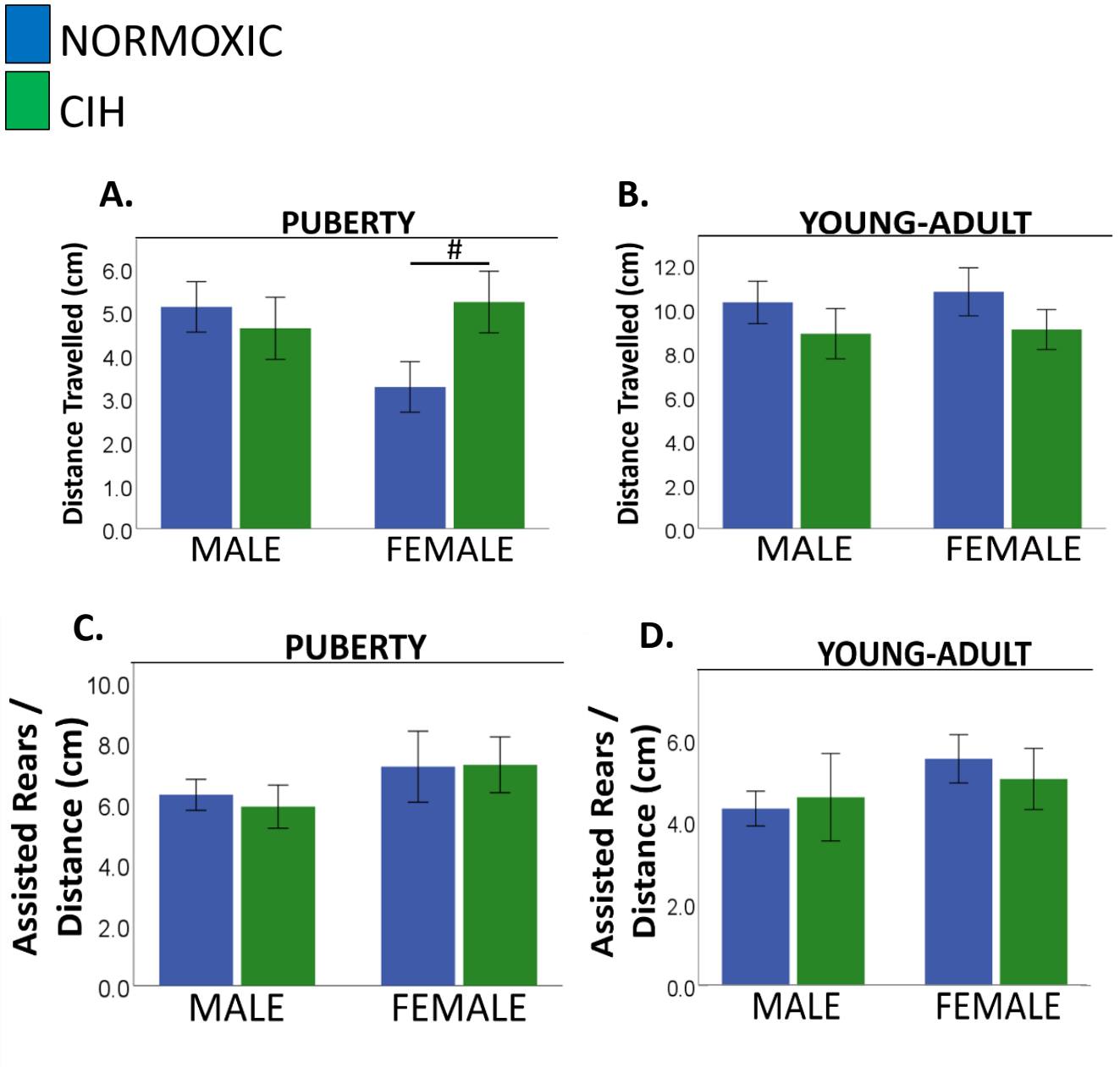

Figure 9: Gross motor behavior in an open field apparatus. A.) A trend in distance traveled by pubertal females exposed to maternal hypoxic stress during late gestation was observed **(**MN 8, MC 11, FN 6, FC 7). B.) No effects of maternal hypoxic stress during late gestation on distance traveled was observed in young adult progeny (MN 15, MC 11, FN 10, FC 9). C.) No effects of maternal hypoxic stress or sex was observed on assisted rears in pubertal progeny (MN 8, MC 11, FN 6, FC 7) or D.) adult progeny (MN 15, MC 11, FN 10, FC 9). # = $p < 0.10$.

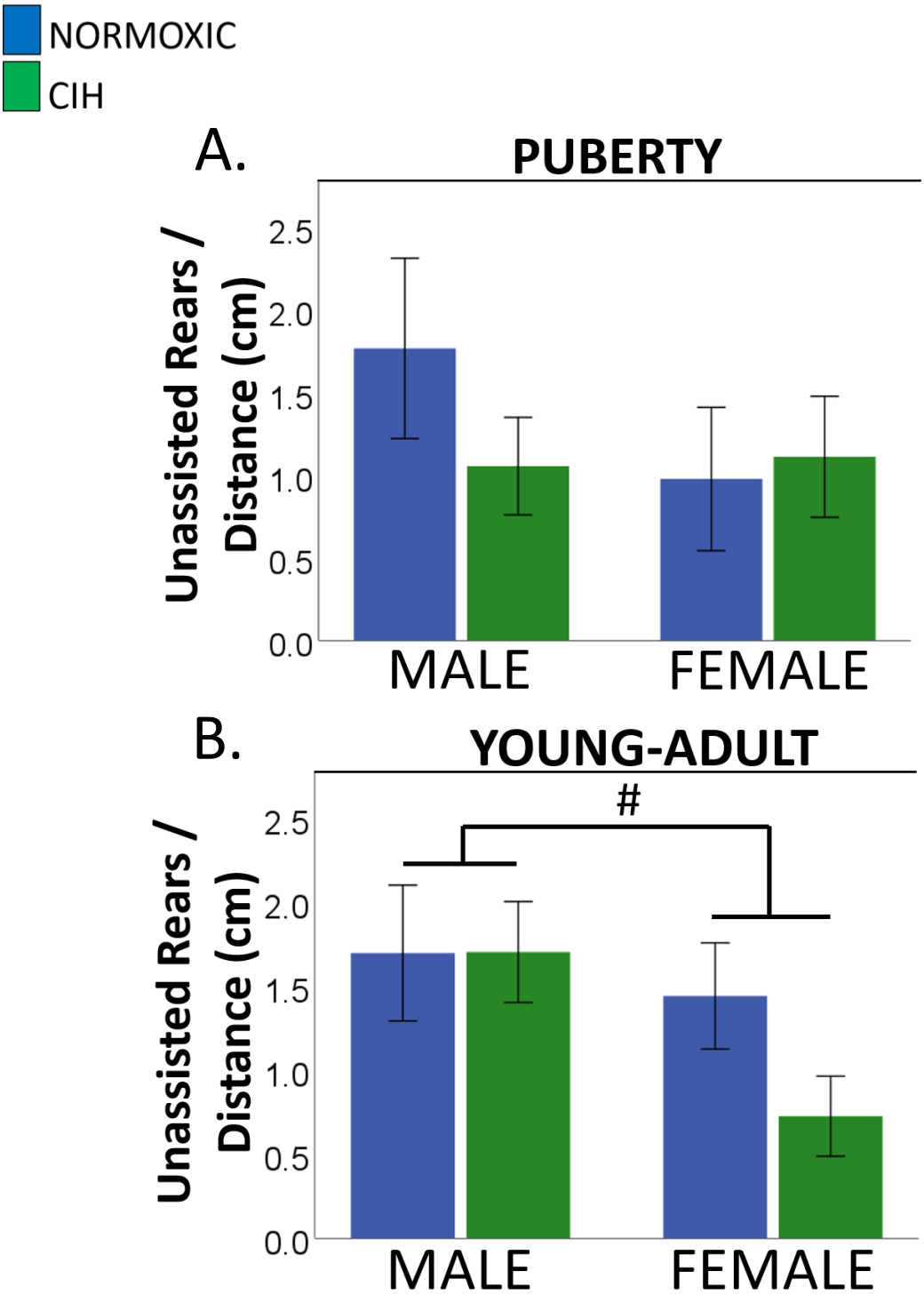

Figure 10: Fine motor behavior in an open field apparatus. A.) Unassisted rears were not impacted in pubertal animals, regardless of maternal hypoxic stress or (MN 8, MC 11, FN 7, FC 7). B.) A trend in unassisted rears was observed in young adult male progeny compared to young adult female progeny (MN 15, MC 11, FN 10, FC 9). # = $p < 0.10$.

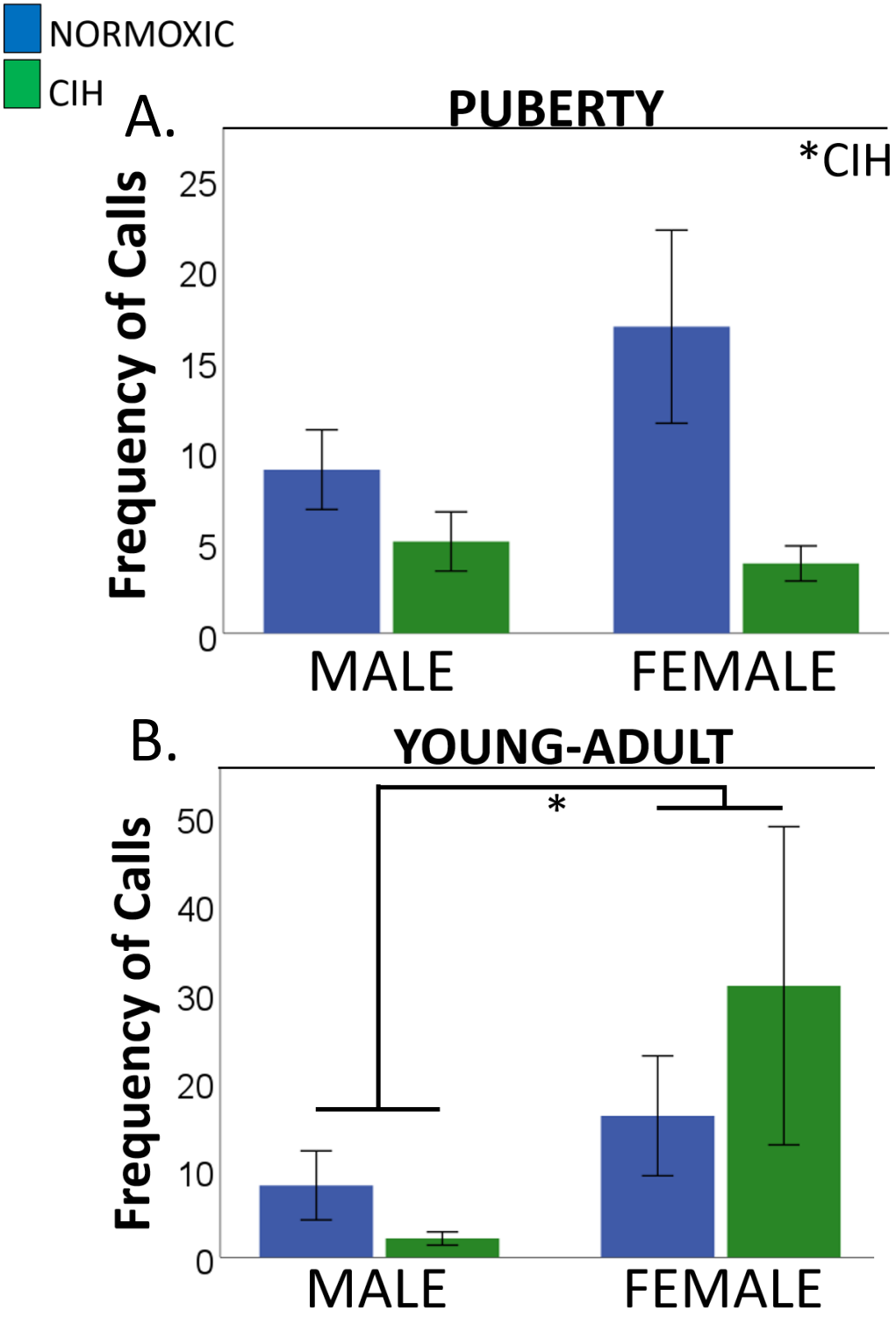

Figure 11: Frequency of total calls. A.) Maternal hypoxic stress decreased the frequency of calls produced by pubertal progeny **(**MN 16, MC 19, FN 14, FC 13). B.) No effects of maternal hypoxic stress observed in adult animals. Adult female progeny did emit more calls than adult male (MN 18, MC 14, FN 15, FC 12). * = $p \leq 0.05$.

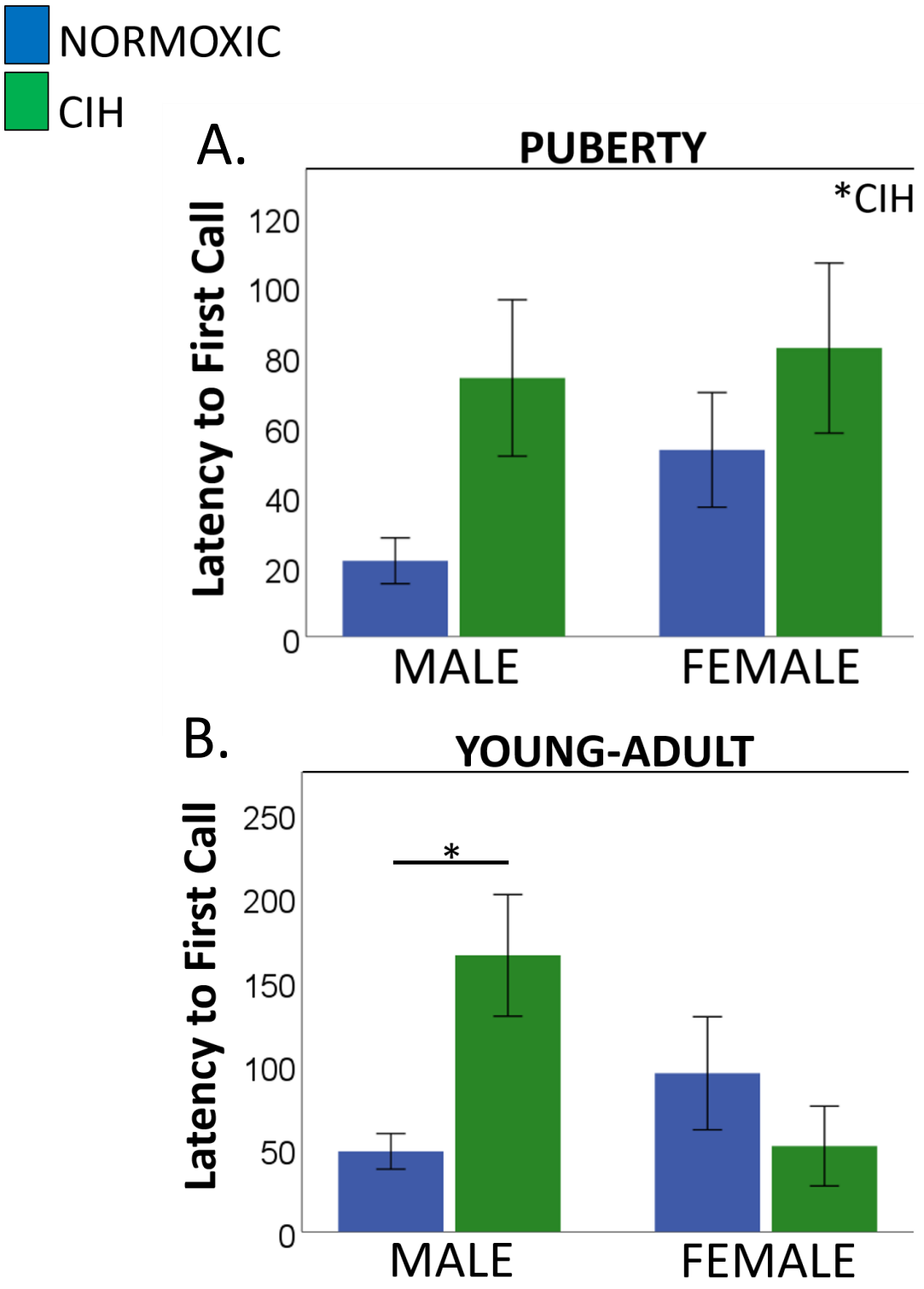

Figure 12: Latency to first call. A.) Maternal hypoxic stress increased the latency to first call in pubertal progeny (MN 13, MC 14, FN 11, FC 11). B.) This increase in call latency was only maintained in male progeny during young adulthood but not females (MN 11, MC 10, FN 10, FC 11). * = $p \leq 0.05$.

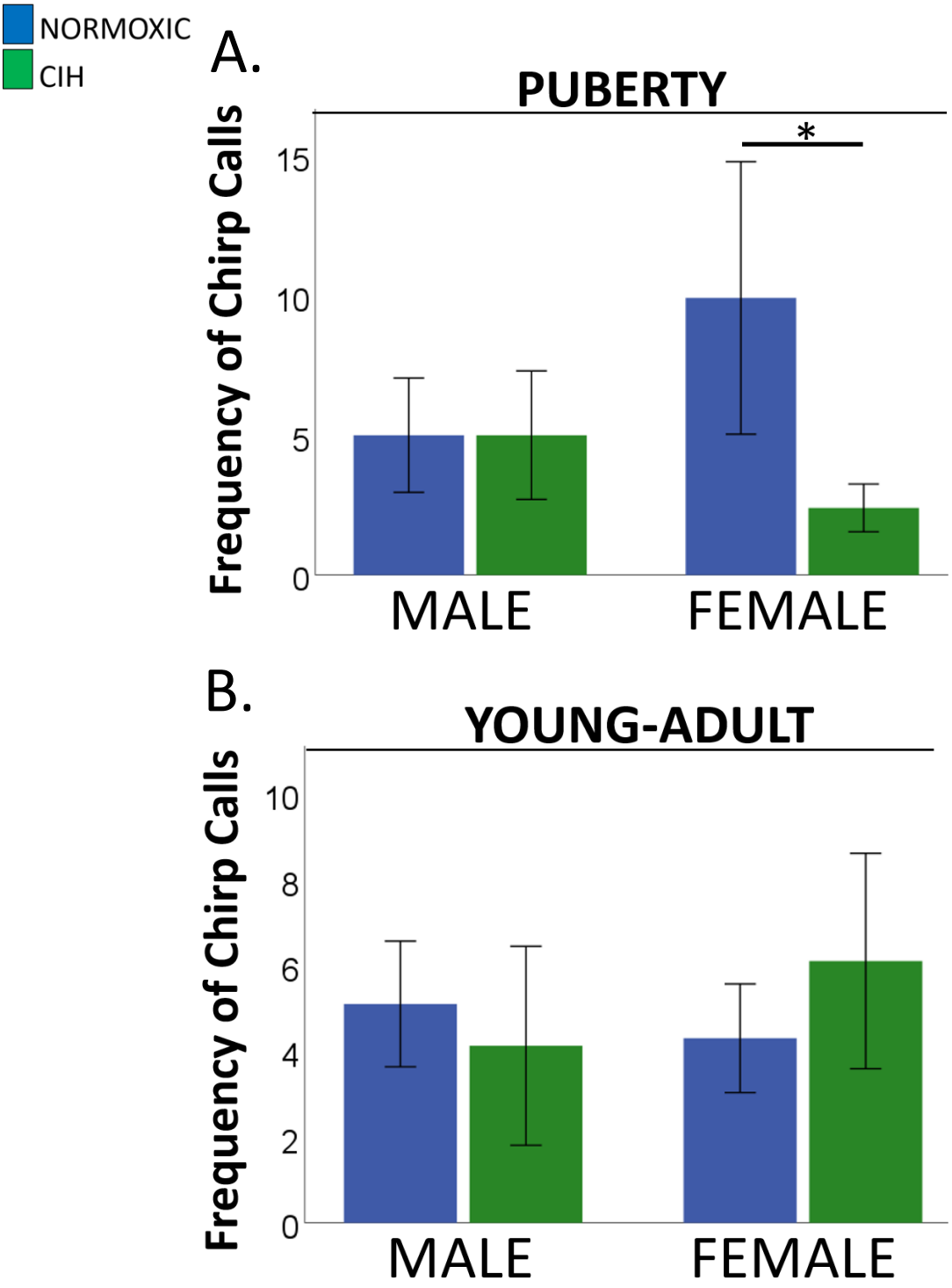

Figure 13: Frequency of chirp calls. A.) Maternal hypoxic insults decreased the frequency of chirps emitted by pubertal female progeny (MN 12, MC 14, FN 11, FC 10) compared to normoxic animals. B.) However, these effects were not observed in young adult animals (MN 10, MC 8, FN 10, FC 10). * = $p \leq 0.05$.

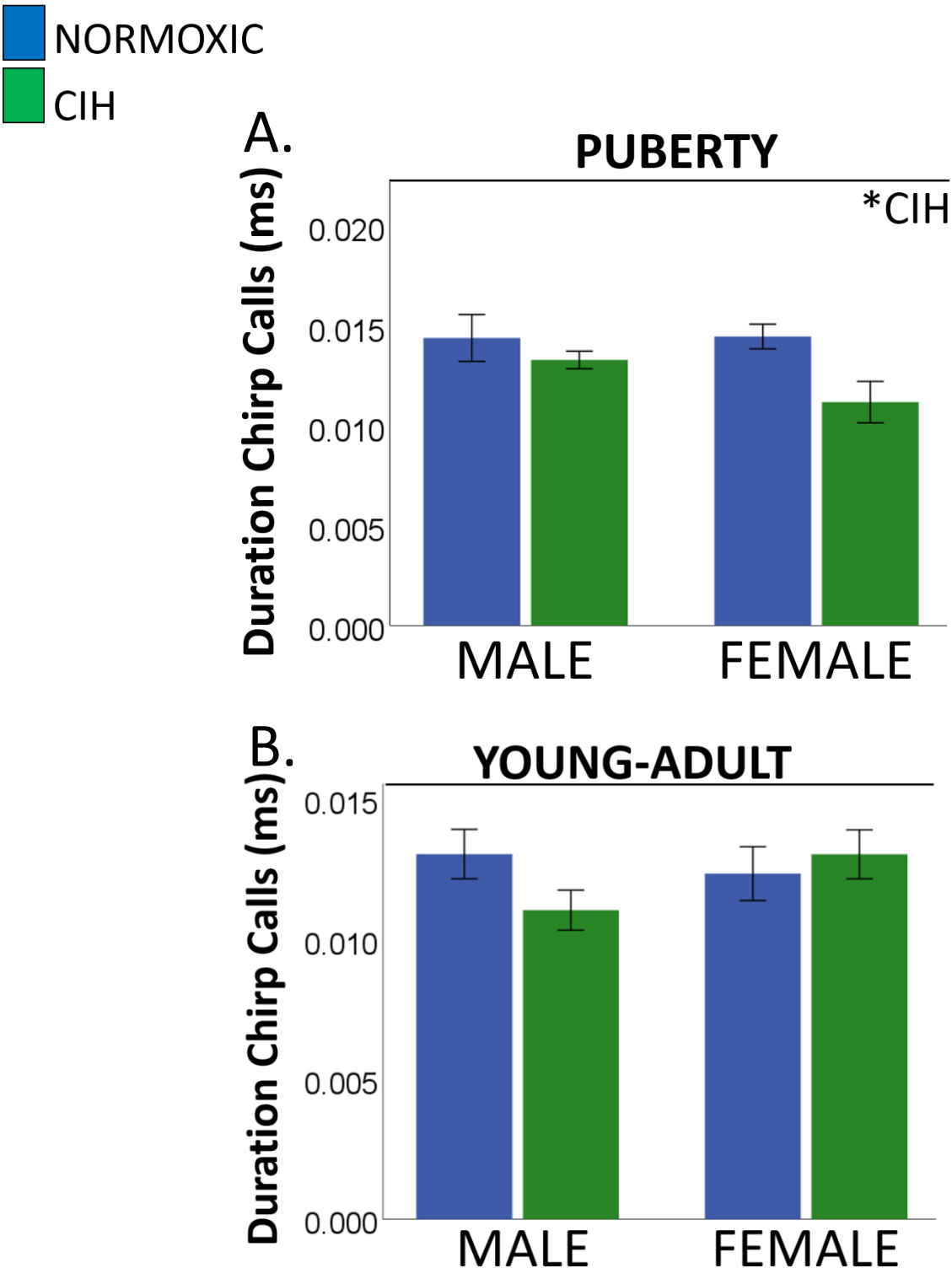

Figure 14: Duration of chirp calls. **A.)** Maternal hypoxia decreased the duration of chirps in all pubertal progeny (MN 12, MC 14, FN 11, FC 10). B.) No effects of hypoxia or sex on chirp duration observed in young adult animals (MN 10, MC 8, FN 10, FC 10). * = $p \leq 0.05$.

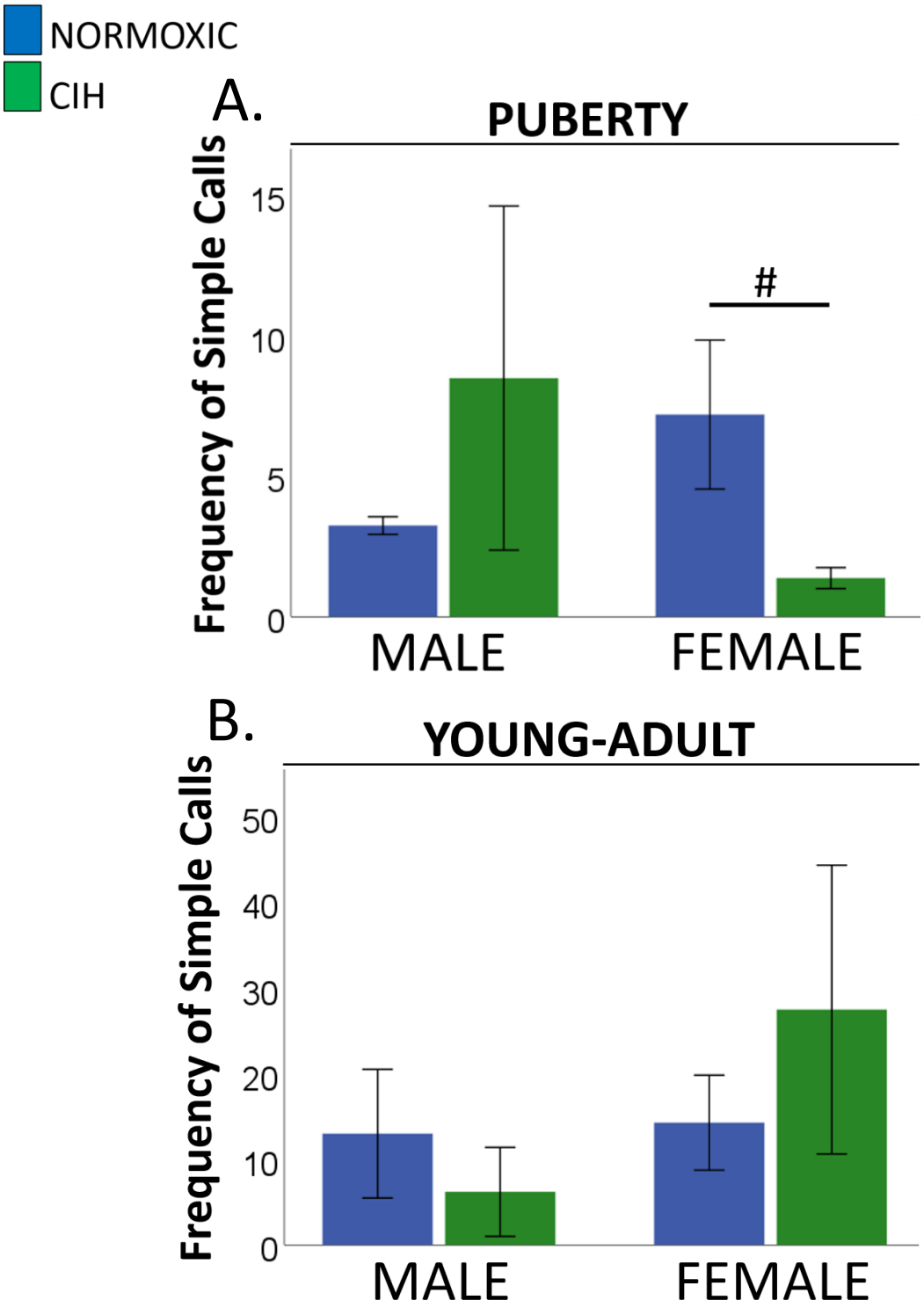

Figure 15: Frequency of simple calls. A.) Maternal hypoxic stress decreased the frequency of simple calls emitted by pubertal females (MN 8, MC 6, FN 10, FC 8). B.) During young adulthood, maternal hypoxic insult did not impact frequency of simple calls (MN 9, MC 5, FN 7, FC 6). * = $p \leq 0.05$, # = $p = 0.06$.

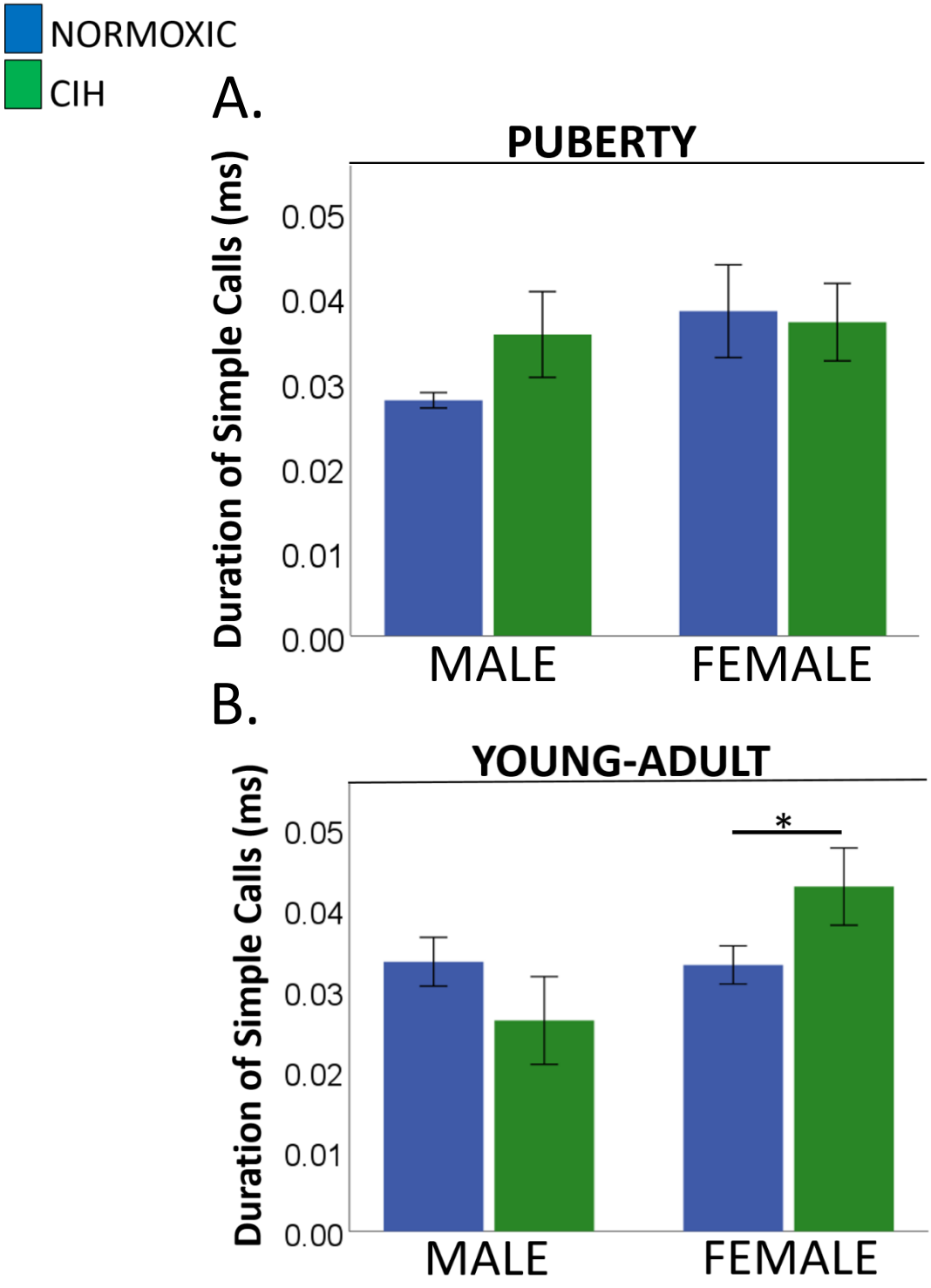

Figure 16: Duration of simple calls. A.) maternal hypoxic insult did not impact simple call duration in pubertal progeny (MN 8, MC 6, FN 10, FC 8). (B). Maternal hypoxic insult increased the duration of simple calls in young adult females (MN 9, MC 5, FN 7, FC 6) compared to females exposed to normoxia during gestation. * = $p \leq 0.05$.

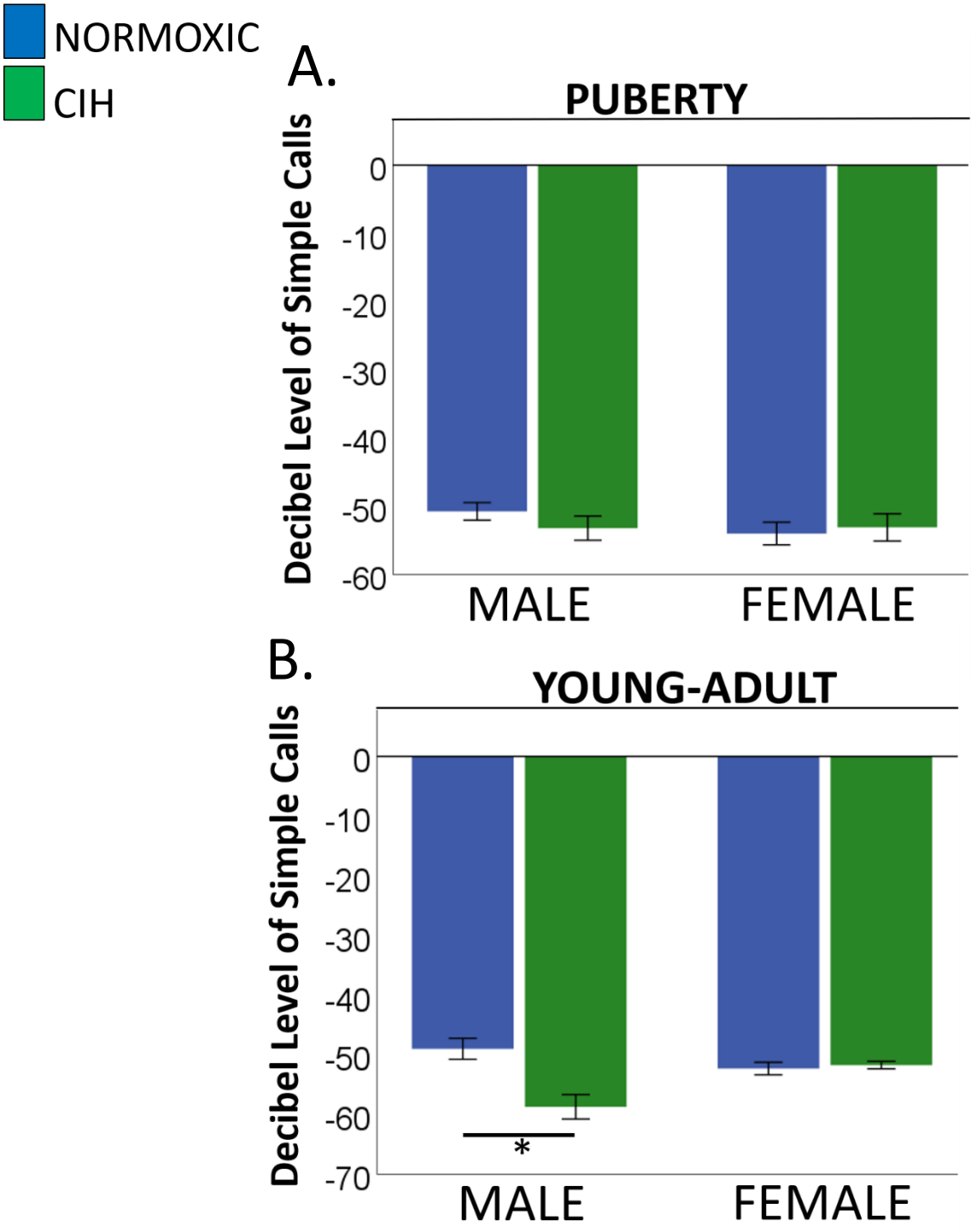

Figure 17: Loudness of simple calls. A.) No effects of maternal hypoxic insult or sex were observed on the decibel level of simple type calls by pubertal progeny (MN 8, MC 6, FN 10, FC 8). B.) Maternal hypoxic insult decreased the loudness of simple calls by decreasing decibels in young adult male progeny (MN 9, MC 5, FN 7, FC 6). * = $p \leq 0.05$.

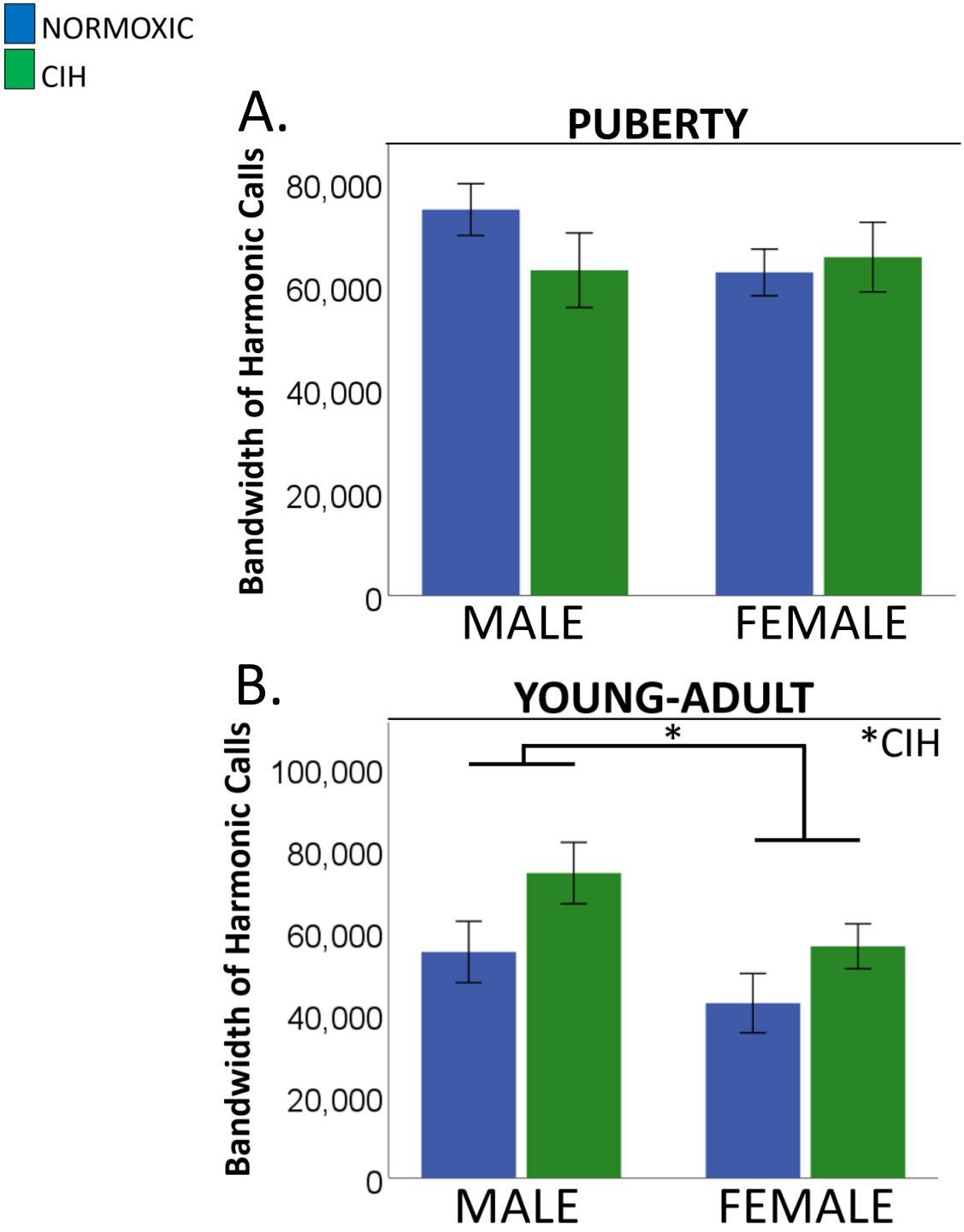

Figure 18: Harmonic call bandwidth. A.) The bandwidth of harmonic calls by pubertal progeny was not affected by maternal hypoxic insult or sex (MN 12, MC 8, FN 8, FC 5). B.) However, both sex and maternal hypoxic stress impacted the bandwidth of harmonic calls in young adult animals. Maternal hypoxic stress increased the bandwidth of harmonic calls in young adult progeny and males exhibited increased bandwidth compared to young adult females (MN 7, MC 5, FN 8, FC 10). * = $p < 0.05$.

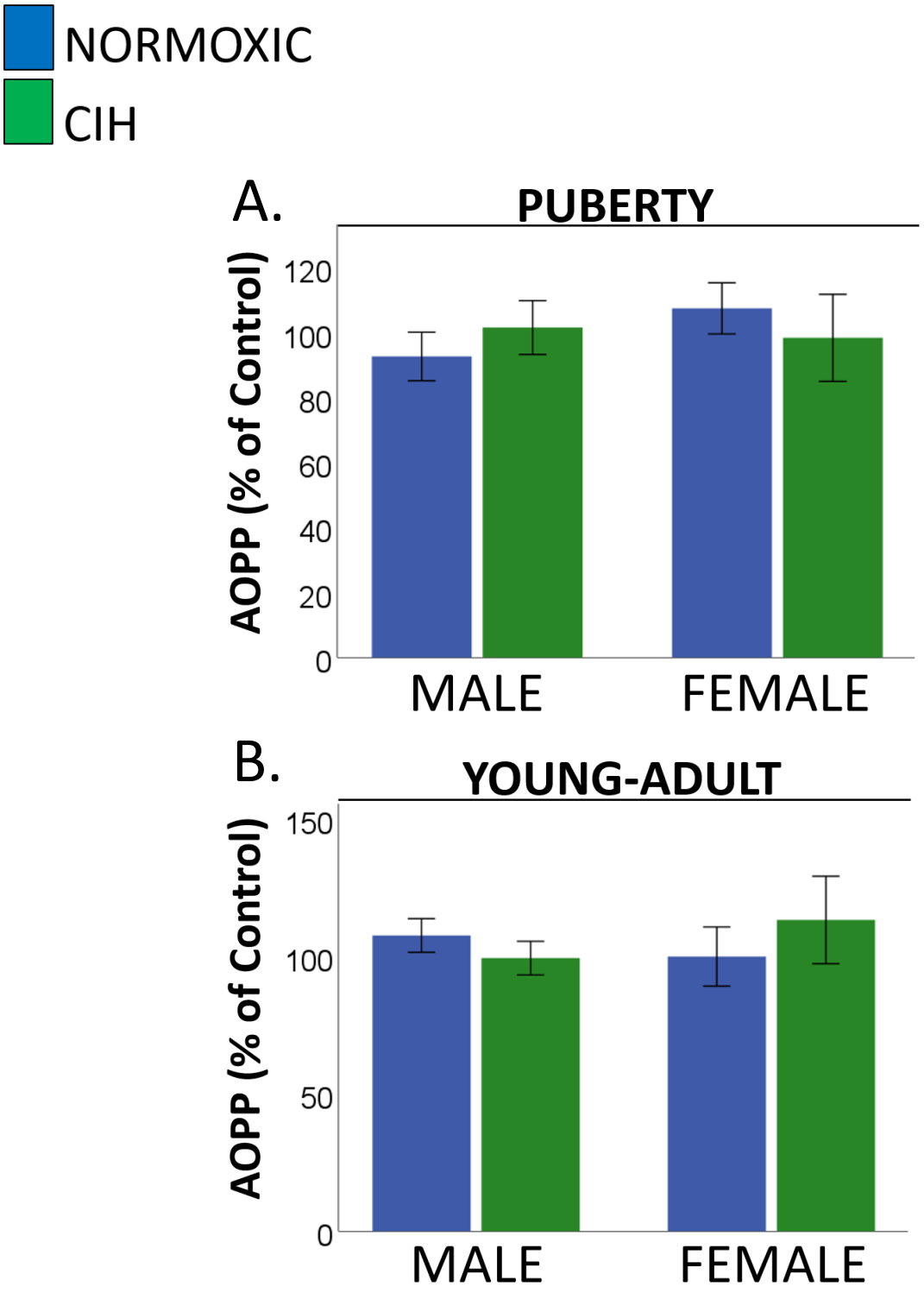

Figure 19: Plasma oxidative stress (AOPP) in progeny. A.) Maternal hypoxic stress nor sex affected AOPP in pubertal (MN 15, MC 20, FN 13, FC 13). B.) No effect of maternal hypoxic stress or sex on AOPP observed in young adult progeny (MN 17, MC 11, FN 12, FC 10).

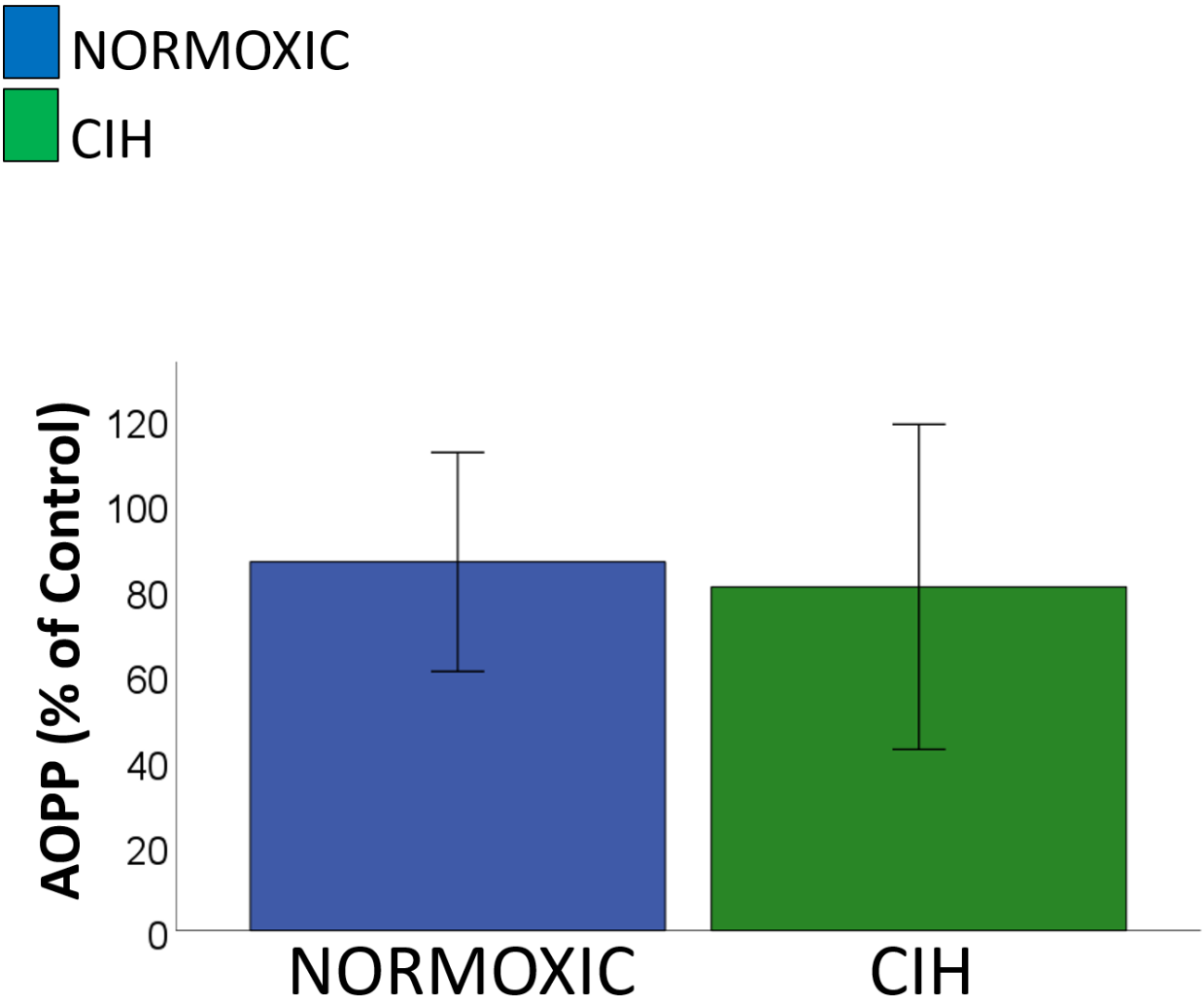

Figure 20: Plasma oxidative stress (AOPP) in dams. Oxidative stress levels were measured as AOPP in time pregnant females on gestational day 20, immediately following CIH. No effects of hypoxia during late gestations were observed in exposed dams (Norm: 6, CIH: 6).

CHAPTER 3:

DISCUSSSION AND CONCLUSIONS

The hypothesis for this study was that exposure to a maternal hypoxic stressor during late gestation would impair the nigrostriatal pathway in male progeny compared to female progeny. Impaired nigrostriatal pathway during early life in males could be a possible mechanism for the two-fold increased risk for PD in men. To examine the nigrostriatal pathway in this project, plasma oxidative stress, fine and gross motor functions, and ultrasonic vocalizations were quantified during two development periods: puberty and young adulthood. These time periods were chosen to examine whether prenatal insults during late gestation and the neural organization of brain impacted hormonal activation of brain circuits during puberty (**Figure 2**), along with the long-term consequences of prenatal hypoxic insults on the nigrostriatal period as prenatal insults can program or "organize" the brain.

The major findings of this study are that maternal hypoxic stress during late gestation have both pubertal and long-term (adult) consequences. In both male and female pubertal progeny, maternal hypoxic insult during late gestation suppressed the frequency of total calls, increased the latency to first call, decreased the duration of chirp calls, and decreased the frequency of chirps emitted by females. Generally, ultrasonic vocalizations were impacted by maternal hypoxic stress during late gestation in both sexes. This lack of pubertal sex differences indicates that this stressor did not impair the hormone "activational" stage of puberty.

Sex differences were observed in the long-term consequences of maternal hypoxic insult during late gestation. In adult male progeny, maternal hypoxic *in utero* insult increased body

weight, increased latency to vocalize, and decreased simple call loudness. In contrast, this gestational insult only increased simple call duration in females, while it increased harmonic call bandwidth in males and females. Overall, male progeny were impacted more by maternal hypoxic stress during late gestation than female progeny, which indicates that male progeny are more sensitive to the insults during the organizational period of brain development that could increase the risk for neurological disorders.

Sex differences that were unrelated to maternal hypoxic stress during late gestation were found in this study. As expected, male rats weighed more than female rats. No sex differences in gross or fine motor functions were observed. No pubertal sex differences were observed in ultrasonic vocalizations, which is consistent with other studies [122]. However, sex differences in ultrasonic vocalizations during young adulthood were observed in which adult males had increased harmonic bandwidth compared to female rats, which is similar to Warren et.al., 2018 that found male mice have a larger bandwidth in complex calls than female mice [123]. In the current study, adult females vocalized more than males. This result is interesting as most studies show that males emit more ultrasonic vocalizations than females [119, 123-126]. It should be noted that these studies were conducted under different parameters than the current study, in which ultrasonic vocalizations were quantified during male-female interactions. Changing the parameters or the environment that the ultrasonic vocalizations are collected can dramatically impact ultrasonic vocalizations. Indeed, no sex differences in ultrasonic vocalizations are observed under conditions of same-sex interactions in mice [127]. Female mice emit more ultrasonic vocalizations in the presence of novel female conspecifics than familiar female conspecifics [128]. The current study used the cage mate separation protocol which avoids the

confounds of hormone or novelty elicited ultrasonic vocalizations [114], and thus unmasked a previously unknown sex difference. Few studies have examined sex differences on ultrasonic vocalizations in adult rodents, as the estrous cycle can alter the frequency of calls in females [121, 129] and the separation of cage mates protocol to elicit ultrasonic vocalizations has only been used in male rats [114, 130, 131].

Ultrasonic vocalizations are innate behaviors and not learned [132, 133]. 50 kHz ultrasonic vocalizations were first described in the early 1970's, and they were associated with rodent mating behaviors [134]. However, 50 kHz ultrasonic vocalizations are not restricted to mating behaviors [120, 135, 136], as they are emitted under several conditions (e.g., mother-pup retrieval, juvenile play interactions, same-sex interactions, and opposite-sex interactions) [125, 137, 138]. The function of each ultrasonic vocalization call type is not well-understood [139]. Chirps and simple calls are the most common ultrasonic vocalization call type [125], which are generally associated with social coordination functions (e.g., re-establishing social contact) [112, 114, 135, 140] and not reward or pleasure [135]. Chirps and simple are the most common ultrasonic vocalization call types (FM calls not observed) in the cage mate separation protocol [112, 114], which was observed in the current study. Few complex calls, except for harmonic calls, were observed. The more complex ultrasonic vocalizations, such as FM and harmonic call types, are associated with sex behaviors, group housing, juvenile play, and other pleasurable behaviors (e.g., tickling) [115-117, 130, 141-144].

Different stressors have been shown to impair ultrasonic vocalizations, such as restraint stress suppression of ultrasonic vocalizations [125], social isolation of pups decreased ultrasonic vocalizations call diversity [137, 145], and ischemic stroke-induced decrease in vocalization by

males compare to females. Few studies have examined the impact of early life stressors on ultrasonic vocalizations [146]. Isolation of rodent pups resulted in changes to ultrasonic vocalizations, in which longer call duration and more ultrasonic vocalizations were produced by isolated female pups compared to isolated male pups [147-149]. Hypoxic injury during the perinatal stage (PND 10) in mice using temporary ligation of the carotid artery decreased ultrasonic vocalizations in males and females on PND 12 [150]. Doran et.al. 2019 examined this association in hypoxic injury at PND 10 in male mice on ultrasonic vocalizations during puberty and found that vocalizations were suppressed; female mice were not investigated [151]. The current study is the only study that examined the impact of maternal hypoxic stress during late gestation on ultrasonic vocalizations emitted by progeny, and the only study to examine the impact of early life stress on ultrasonic vocalizations produced during puberty and young adulthood.

Ultrasonic vocalizations can be used to examine the integrity of dopaminergic neurotransmission in the nigrostriatal pathway in the brain [108-110]. Dopamine agonists (e.g., as amphetamine, cocaine, apomorphine) can increase 50 kHz ultrasonic vocalizations [112, 152-158] and even increase the complexity of ultrasonic vocalizations [112]. Further, lesioning dopaminergic neurons in the nigrostriatal pathway can decrease ultrasonic vocalizations, including complex calls (e.g., FM calls), decrease amplitude or loudness, and decrease bandwidth of calls [106, 109, 110, 152] . Pharmacological inhibition of dopamine neurotransmission using dopamine receptor (D2) antagonists can also decrease call bandwidth, amplitude/loudness, and call complexity [109, 110]. These dopaminergic deficit effects on ultrasonic vocalizations are

similar to what is observed in individuals diagnosed with PD, which show decreased vocal loudness and speech complexity or monotone [108, 159-164].

PD is characterized by the loss of dopaminergic neurons and increased oxidative stress in the nigrostriatal pathway. One of the major behavioral phenotypes of PD is motor dysfunction that occurs when 80% of the SN is lost [73], and oxidative stress has been proposed to mediate the early stages of neurodegeneration [79, 165-167]. Hypoxic insults during adulthood in male rats increased oxidative stress damage to the nigrostriatal pathway and increased circulating oxidative stress [89]. Further, hypoxic insults (1 minute hypoxic-normoxic cycle/hour for 8 hours during the sleep phase) through pregnancy (GD 3-19) in rats resulted in growth restriction and oxidative stress [168]. Hypoxia-induced weight loss was observed during young adulthood in male and female progeny but not during puberty. A sex difference in oxidative stress was observed, in which increased oxidative stress was observed in young adult male progeny but not in young adult female progeny stress [168].

No elevation in oxidative stress or motor impairments in progeny exposed to maternal hypoxic insult during late gestation were observed in the current study. This is not surprising as the maternal hypoxic insult used was different than the Chen 2018 study [168] in multiple parameters: hypoxic insult was only during the last 5 days of gestation (GD 15-19) in the current study instead of 16 days (GD 3-19) and a milder hypoxia (10 hypoxic cycles/hour over 8 hours) was used instead of 60 hypoxic cycles/hour over 8 hours. This mild hypoxia protocol was evidenced by the lack of circulating oxidative stress in dams at GD 20 compared to dams exposed to room air (**Figure 20**). The milder hypoxia protocol during late gestation was chosen to more closely model hypoxic stressors that occur during late gestation in humans, which is when

organization of the neural circuits (e.g., nigrostriatal pathway) occurs [23, 55]. Consistent with human literature showing prenatal hypoxic stress impacts male progeny more than female progeny [36, 48], late gestation hypoxic stress impaired the nigrostriatal pathway more in male progeny than female progeny and this effect was long-lasting into young adulthood. This impaired nigrostriatal pathway in male progeny may be one mechanism that could underlie the increased risk for PD in men [169-172].

CHAPTER 4:

FUTURE DIRECTIONS

The current studies did not show fine motor dysfunction in progeny exposed to maternal hypoxic stress during late gestation. However, it is possible that fine motor impairments could be present. To further examine this, we plan to use the modified open field behavior test, which has an elevated wire mesh grid that increases the difficulty for rats to perform unassisted rears. Other behavioral tests for fine motor skills in rats that could be used include the rotarod test that examines balance on a rotating rod or reaching through a maze for food.

We plan to examine local oxidative stress generation in the nigrostriatal pathway by examining oxidative stress damage to the SN and the striatum. Oxidative stress damage to proteins can be quantified through different approaches. Electrophoresis followed by western blotting can be used to quantify enzymatic oxidative stress reactions (Calpain-cleavage of Spectrin), oxidative stress damage to proteins (3-nitrotyrosine, carbonyls), lipid peroxidase (4-HNA, 8-isoprostane) and oxidative stress damage to DNA (hydroxy-deoxyguanosine). Examining local oxidative stress will allow us to determine the vulnerability of the nigrostriatal pathway in response to maternal hypoxic insult during late gestation and how this prenatal insult can increase risk for PD later in life.

To determine if maternal hypoxic insult during late gestation increased the vulnerability of the nigrostriatal system, we plan to expose progeny to another hypoxic stressor during young adulthood. This experimental paradigm is based on the "multiple hits" hypothesis of neurodegenerative diseases (e.g., PD and Alzheimer's disease), in which the "first hit" occurs

during early development and increases the vulnerability to a “second hit” later in life to increase disease risk [64, 68, 173, 174]. We plan to examine the same behavior endpoints of motor function, ultrasonic vocalizations, and oxidative stress measures.

CHAPTER 5:

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