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Neurodevelopmental effects of Prenatal Maternal Stress in Chickens

Investigation on Allometric Scaling and Neuronal and Non-Neuronal Counts in the chicken brain

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1 Abstract

Prenatal maternal stress in animal models has shown significant impacts on altering the development of specific brain regions and causing long-term behavioural alterations in offspring. It is worth noting that prenatal maternal stress has been shown to affect neural density in rodents born relatively undeveloped. This makes it interesting to evaluate the same impact in precocial chickens since their brains develop significantly before birth, potentially making them more responsive to prenatal maternal stress. To investigate this further, in this study I analyse the effect of prenatal maternal stress on allometric scaling and neuronal and non-neuronal cell counts in offspring of an advanced intercross between red junglefowl and white leghorn. My results show that prenatal maternal stress has a marginal reducing effect on telencephalic non-neuronal counts. Moreover, I discovered a sex-dependent impact of prenatal maternal stress, which only in females leads to reduced telencephalic mass and reduced non-neurons in the cerebellum. Overall, my findings suggest an impact of prenatal maternal stress on avian gliogenesis, potentially shedding light on the reason why we observe some long-term behavioural effects and highlighting the importance of considering sex-dependent outcomes in future studies.

Keywords: prenatal maternal stress, neurodevelopment, gliogenesis, brain allometry

2 Introduction

2.1 What is Prenatal Maternal Stress?

The internal environments of both the pregnant mother and developing offspring are particularly interconnected and able to influence each other during embryonic development. Consequently, embryo exposure to specific maternal stimuli can result in physiological, metabolic, and epigenetic changes which have long-term implications (Lucas, 1994). In this perspective, we refer to prenatal maternal stress effects as that phenomenon in which the stress experienced by a mother during pregnancy or egg formation results in consequences on the phenotype of the offspring (Love et al., 2012). Prenatal maternal stress in animal models has shown significant impacts on altering the development of specific brain regions, which in turn cause long-term behavioural alterations in offspring.

Prenatal maternal stress has been observed to influence the proliferative potential of brain cells, as demonstrated in studies on humans (Buss et al., 2010) and mice (Zhang et al., 2021). However, to the best of my knowledge, no study has focused on similar effects in chickens, a precocial specie which at the time of birth has a higher degree of development and independence from the mother. Prenatal maternal stress may have a more pronounced impact on brain cell composition in this species since it has a larger neurodevelopmental time window to act upon. In this study, I examine the influence of prenatal maternal stress on brain cell composition by quantifying both neuronal and non-neuronal cell counts in chickens whose mothers underwent stress induction via exogenous corticosterone implantation. The findings presented herein provide evidence suggesting that prenatal maternal stress results in deficits which especially interest non-neuronal cell populations.

2.2 Why Chickens are a valuable animal model

Chickens are a valuable animal model to study prenatal maternal stress and its actions on neurodevelopment because of their precocial nature. Unlike altricial animals, in precocial species like chickens, a significant portion of neural development (including cell proliferation, neuronal differentiation, synapse formation, and myelination) occurs before hatching (Rogers, 1995 as cited in Henriksen 2011). This makes it possible to potentially observe enhanced prenatal maternal stress effects on neurodevelopment, aligning with the argument by Metcalfe and Monaghan (2001) that precocial animals might be more susceptible to prenatal deficits than altricial species, as they cannot compensate for any shortcomings during the crucial developmental period before birth. Another advantage of precocial species is the ability to minimize the influence of the post-natal environment. This is particularly evident in chickens, where the influence of maternal care can be neutralized during both incubation and post-hatch rearing.

Using chickens as an animal model for prenatal maternal stress also allows to more clearly elucidate the mechanisms of prenatal maternal stress mediation. In mammals the inaccessibility of the mammalian fetus to controlled experimental manipulations makes it difficult to investigate these processes, while in chickens the egg offers a readily and easily accessible fetal environment: it is produced in a short production window, and since it develops outside of the mother, it can be manipulated independently from her, facilitating investigations of the embryonic environment (Lay et al., 2002; Henriksen et al., 2011b).

Remarkably, the avian mechanistic functioning of mediation of prenatal maternal stress parallels to a certain extent the mammalian one. The stress axis is highly conserved across vertebrate taxa (Love et al., 2012): like in mammals, in birds the occurrence of a stressful event elicits hypothalamic neurons in the paraventricular nucleus to release corticotropin releasing-hormone which, after traveling through the hypophyseal portal system, act on the anterior pituitary to regulate secretion of adrenocorticotropic hormone (ACTH) in the bloodstream (Herman et al., 2003). ACTH then stimulates the adrenal cortex to synthesize glucocorticoids (GCs). Similarities also emerge when looking at prenatal maternal stress mediation to the embryo as the avian extraembryonic membranes do appear to share similar functions with the mammalian placenta and umbilical cord, being able to regulate respiration and excretion (Ferner & Mess, 2011), and notably steroid metabolism (Kumar et al., 2019). However, despite these similarities, significant differences exist between mammalian in-utero and avian in-ovo development. While avian embryos develop in eggs, where maternal effects are mainly established before incubation through the deposition of substances in the oocyte, in mammals, the maternal environment continuously influences the fetus through circulation throughout the whole gestation. In mammals, this can complicate the isolation of specific stress effects and their timing. For this reason, the insulation of avian embryos from continuous maternal influences once the egg is laid can allow researchers to isolate the impact of prenatal maternal stress and focus solely on the mechanisms of egg production. This makes the avian model unique for studying the effects of prenatal maternal stress associated with maternal substance allocation, but it also underscores the need for careful consideration when translating findings on prenatal maternal stress between avian and mammalian systems.

Overall, chickens can serve as valuable animal models to study prenatal maternal stress due to their precocial characteristics, which for instance can enable researchers to control for postnatal influences on neurodevelopment. Moreover, the chicken egg can allow for facilitated investigations of an embryonic environment and the complex processes of prenatal maternal stress mediation.

2.3 Mechanisms of prenatal maternal stress mediation in chickens

Previous evidence on mammals has shown that maternal cortisol plasma levels correlate with fetal cortisol plasma levels (Van den Bergh et al., 2005 as cited in Gellisch et al., 2023). Elevated levels of GCs in fetal circulatory systems are a common event, and indeed they play a pivotal role in prenatal organ maturation (Fowden et al., 2015). GCs can also act indirectly

by instigating changes in maternal physiology, behaviour, thermoregulation, and the allocation of hormones and nutrients to developing embryos (Ensminger et al., 2018). The activation of the stress system involves sensitization of various neuroendocrine systems, including the gonadal axis (Ward, 1972). Notably, gonadal steroids (e.g. androgens, estrogens, and gestagens) have been shown to modulate the expression and activation of glucocorticoid receptors (Turner, 1997), suggesting their potential to indirectly modulate embryo GC exposure. Overall, during pregnancy, these hormones exert numerous programming effects that lead to changes in embryo morphology, metabolism, hormone sensitivity, and biochemical composition of fetal tissues, with widespread functional consequences at the whole-organ and systems levels (Fowden et al., 2015).

However, in oviparous species, part of fetal development occurs independently from the mother environment, and this distinction implies that some mechanisms of mediation of prenatal maternal stress may operate differently. Generally, the two main mechanisms of prenatal maternal stress mediation have been proposed to be fetal malnutrition and overexposure to GCs (Cottrell, 2009). in the next section evidence will be provided on how the egg mass (see 2.3.1) and hormonal composition (see 2.3.2) seem to parallel these mechanisms of stress mediation in avian species, Additionally, the role of epigenetics in mediating prenatal maternal stress effects will also be highlighted (see 2.3.3).

2.3.1 Prenatal Nutrition

Fetal malnutrition has been proposed as one of the major factors linking prenatal maternal stress and postnatal effects. In hens, this occurs when insufficient nutrient supplies are laid down for the embryo, and in on that aspect, there is evidence that both maternal nutritional stress and chronic stress influence such process.

Firstly, the hen's diet type (Lacin, et al., 2008) and nutritional status (de Haas et al., 2017) influence egg mass, a parameter that can be taken as an indicator of the amount of available nutrients. In this context, nutritional stress has been shown to diminish egg mass, a factor strongly correlated with chick mass (Finkler et al., 1998; Henriksen et al., 2013).

An additional factor is chronic stress since the process of egg formation is both an energy and nutrient intensive process (Nager, 2006), gestational stress may act as an adaptive response that facilitates energetic adaptation during stress by decreasing the investment in eggs (Henriksen et al., 2011b). Indeed, elevated plasma GCs have been demonstrated to alter the production of yolk precursors in the liver, thus diminishing the nutritional value of the egg

(Salvante and Williams, 2003). This holds significant implications for the embryo, as investment in egg yolk is necessary to produce more developed offspring (Hill, 1993, as cited in Henriksen et al., 2011a).

2.3.2 Egg Hormonal Composition

As previously mentioned, maternal hormones play a pivotal role in influencing embryo development, particularly in avian species where these molecules are transferred to the egg. Research has demonstrated how prenatal maternal stress can impact maternal allocation in the egg of various biological components such as testosterone and progesterone (Henriksen et al., 2011b), thyroid hormones (Hsu et al., 2017), antioxidants (Possenti et al., 2018), and immunoglobulins (Partecke et al., 2020).

Interestingly, GCs, known as the primary mediators of stress in mammals, seem to possess a different kind of importance in birds. Corticosterone (CORT) is the most secreted GC in birds, and although it has been shown to exert important effects when injected in substantial amounts into the egg (Henriksen et al., 2011b for review), the actual transfer rate from blood to egg and its amount in the yolk is low (Rettenbacher et al., 2009; Sas et al., 2006). Furthermore, GCs are restricted to some extent from entering the egg, leading to question the importance of direct CORT effect on post-natal effects, favouring consideration of its indirect impact which instead influences the concentrations of other steroid hormones in the egg. As a matter of fact, in chickens CORT has been found to inhibit the synthesis of gonadal steroids (Rettenbacher et al., 2009) and their allocation in the yolk (Henriksen et al., 2011a). Importantly, gonadal steroids may also influence embryo corticosterone exposure by determining the expression and activation of Glucocorticoid Receptors (Turner, 1997). Evidence shows that lower yolk androgens have effects on both physiology and behaviour (Groothuis et al., 2005; von Engelhardt et al., 2009), thus supporting the notion that gonadal steroids are indeed a key aspect of avian mediation of prenatal maternal stress effects. In such regard, an interesting notion comes from the role of estrogens: elevating CORT in hens through implants decreases yolk progesterone and testosterone but not estrogens (Henriksen et al., 2011a), which have been hypothesized to facilitate context-dependent epigenetic modifications (Kovács et al., 2020, Nätt, et al., 2009). Another class of hormones that could have a mediatory function are thyroid hormones (TH), which are fundamental for normal neurodevelopment, thermoregulation, and metabolism (McNabb, 2007). Indeed, recent studies on wild birds have shown that prenatal exposure to thyroid hormones affects offspring phenotype and embryonic survival, thus reinforcing the notion that androgen and TH-

mediated maternal effects should not be studied in isolation from each other (Hsu et al., 2017). Lesser-explored yolk molecules such as antioxidants (Possenti et al., 2018) and immunoglobulins (Partecke et al., 2020) also warrant attention. For instance, Vitamin E, an antioxidant molecule, has been observed to exert combined effects with other hormones on gull offspring phenotypes (Possenti et al., 2018). Lastly, it must be noted that female incubation behaviour may regulate embryo exposure to the previously egg-allocated hormone. Incubation temperature can influence neuroendocrine responses and chick behaviour (Bertin et al., 2018), and it is suggested that temperature-dependent enzymes in the egg can alter the hormonal composition, thus influencing embryo exposure to crucial mediatory hormones such as testosterone (Hernández et al., 2024).

2.3.3 Epigenetics

Epigenetic changes refer to the molecular or cellular alterations that influence patterns of gene expression without causing alterations to the DNA sequence itself (Hunter, 2012). For example, adding methyl groups to cytosines at CG dinucleotides in gene promoters typically suppresses gene expression (Moore et al., 2012), a process referred to as DNA methylation. Stressful interactions with the environment have a pronounced epigenetic impact that has the potential to initiate a cascade of effects in numerous behavioural and physiological pathways, with phenotypical changes that can persist through generations.

Organisms are most susceptible to epigenetic modifications during early development (Nagy & Turecki, 2012) and prenatal maternal stress could potentially play a fundamental role in shaping the epigenome (Charrier et al., 2022). In this regard, there is a reliable inheritance of epigenetic variation in chickens (Natt et al., 2012), and mediation of such epigenetic changes could occur either through the egg environment or parental germline transmission (de Haas et al., 2021). Firstly, the egg environment has been shown to greatly affect the development of offspring, and it may do so through epigenetic mechanisms (Guerrero-Bosagna et al., 2018). Maternal hormones allocated in the egg might play a crucial part in causing epigenetic changes, as evidenced by studies on prenatal androgens where in-ovo testosterone injections caused DNA methylation of genes with long-term phenotypic action (Bentz et al., 2021, Bentz et al., 2016). On the other hand, parental germline transmission implies a contribution to the epigenome from both parents (Guerrero-Bosagna et al., 2018), so besides maternal epigenomic imprinting, males may be able to modify the offspring's phenotype through transmission of epigenetic marks (Jenkins et al., 2014, Immler, 2018; Xu et al., 2021),

opening up the interesting possibility of paternal stress effects (Henriksen et al., 2011, Groothuis et al., 2019).

2.4 How Prenatal Maternal Stress affects neurodevelopment in chickens

Previous research on avian prenatal maternal stress has reported neurodevelopmental consequences related to differential gene expression within the brain, particularly sub-telencephalic regions, which complete their neuronal expansion before hatching (Mezey et al., 2012). For instance, prenatal maternal stress has been linked to differential brain gene expression within the chicken hypothalamus (Nätt et al., 2009, Ahmed et al., 2014), a brain structure linked to the HPA axis and the stress response. Additionally, both prenatally stressed chickens and quails (whose mothers underwent environmental manipulation) demonstrate distinct patterns of gene expression and DNA methylation in the hypothalamus and amygdala (Nätt et al., 2009; Charrier et al., 2022). Similar to mammalian studies (Benoit, 2015), the hippocampus in birds appears susceptible to prenatal maternal stress, showing characteristic neuronal plasticity (Kumar et al., 2022). This sensitivity can lead to alterations in neurogenesis and synaptogenesis (Charrier et al., 2022; Gualtieri et al., 2019; Sanyal et al., 2013). The aforementioned brain structures collectively form the limbic system, responsible for emotion, behaviour, learning processes, and long-term memory, offering a potential explanation for observed behavioural alterations in affected animals. Prenatal maternal stress was also found to alter the expression of stress-related genes (e.g. tryptophan 5-hydroxylase 2) and dopamine receptor D1A in both parents and their chicks (Ericsson et al., 2016). Notably, dopamine (along with serotonin) plays a pivotal role as a behavioural modulator, and its activity can indeed be influenced by prenatal environmental conditions (de Haas et al., 2018).

2.5 Prenatal Maternal Stress Effects

In chickens, differential gene expression during neurodevelopment dictates a wide range of phenotypical changes. Alterations in physiological and behavioural traits of offspring have been brought up by a vast array of literature where avian mothers' stress levels were elevated directly by subcutaneous CORT-implantation or indirectly by exposure to environmental manipulations (e.g. Food frustration, social isolation, unpredictable light regimes). Additionally, many important findings emerged from egg injections of hormones at different stages of incubation, yet an important premise is that studies on avian maternal stress effects

are not always consistent. This may be because the effects are highly dependent on the timing and the modality through which the embryo is exposed to prenatal maternal stress (Henriksen et al., 2011b), as well as the genotype of the subjects (Peixoto et al., 2020).

While keeping these factors in mind, I will now briefly overview the major findings related to the effects of prenatal maternal stress on physiological, behavioural, and cognitive function.

2.5.1 Physiological and Morphological Modifications

One of the primary developmental changes caused by prenatal maternal stress in offspring is the alteration of hypothalamic function, along with its consequences on endocrine and neuroendocrine profiles. Altered HPA sensibility has been found both in quails (Hayward and Wingfield, 2004; Majer et al, 2023) and chickens (Ericson et al., 2016), and it is of particular relevance since an enhanced HPA axis not only can lead to more stress-sensitive offspring but may also produce effects on other endocrine systems. Indeed, prenatal maternal stress has demonstrated effects on the Hypothalamic-Pituitary-Gonadal (HPG) axis. Offspring from mothers with CORT-implantation exhibited heightened testosterone levels (Henriksen et al., 2013), and in ovo-CORT injection has been revealed to broadly suppress female reproductive performance, impacting both ovary and oviduct weights (Ahmed et al., 2014). Likewise, studies on quails have linked prenatal maternal stress to decreased reproductive function in males (Satterlee et al., 2007). As reviewed by Henriksen et al., 2011b, several of the studies have reported a decrease in body mass and growth as an outcome of avian prenatal maternal stress. In the case of chickens, there is additional evidence indicating a lower hatching mass, followed by catch-up growth to attain the mass of control offspring within 3-4 weeks (Henriksen et al., 2013). Moreover, prenatal maternal stress appears to influence the functioning of the immune system. Investigations into nutritional stress and CORT implantation in mothers have revealed associations with reduced antibody responses in offspring, potentially rendering them more susceptible to immune threats (Henriksen et al., 2013; Bowling et al., 2018).

Studies involving direct egg injection have also underscored how CORT can greatly disrupt normal developmental processes in the embryo. The findings reveal impairment in overall growth (Saino et al., 2005; Janczak et al., 2006; Eriksen et al., 2003), as well as compromised development of visual pathways (Rogers & Deng, 2005). Eriksen et al., 2003 demonstrated that in-ovo CORT injection can lead to greater fluctuating asymmetry, indicating developmental instability and thus causing asymmetry, abnormalities, or deviations from

normal morphological features. Prominent morphological changes further manifest as altered plumage (Saino et al., 2005) and skin development (Gellisch et al., 2023).

2.5.2 Behavioural Modifications

As noted earlier, avian prenatal maternal stress modifies brain areas responsible for mediating emotions, socio-cognitive processes, and memory. These neurodevelopmental changes are intricately connected to long-term effects on emotional and behavioural responses.

A wealth of studies has investigated the impact of prenatal maternal stress on emotional reactivity by studying its effects on behavioural indicators. One extensively examined behavioural marker is tonic immobility (TI), where an extended duration of immobility signifies heightened fearfulness. Studies have demonstrated higher fearful behaviour with an increase in the duration of TI in chicks from laying hens subjected to an unpredictable feeding schedule (Janczak et al., 2007a) and in chicks hatched from eggs injected with CORT during incubation (Ahmed et al., 2014). Other indicators of fear have also been investigated. In one study, CORT injection before incubation resulted in chicks that took a longer time to approach humans, and fewer chicks crossed a barrier to reach food, both indicative of higher fearfulness (Janczak et al., 2006). Another study found that chicks from hens exposed to different acute stressors vocalized less when socially isolated, signifying fearfulness and anxiety-like behaviour (Peixoto 2020). However, in this case, higher fearfulness was reported only in one of the five strains studied, specifically, a particular White hybrid. Consistent with this, investigations by de Haas et al., reported a correlation between mother CORT and offspring fearfulness and anxiety only for a white hybrid, but not brown hybrid (de Haas et al., 2014, de Haas et al., 2013). Anxiety-related behavioural traits are also associated with the tendency to develop severe feather pecking (de Haas et al., 2014), an injurious maladaptive behaviour that is suggested to also be correlated to prenatal maternal stress (de Haas et al., 2021). Overall, these findings underscore the importance of genetic strain in the development of fearful and anxious behaviour.

However, contrasting evidence exists, with some studies showing that progeny from corticosterone-implanted mothers exhibited no fear, anxiety, or even reduced fearfulness indicators (CORT-implanted mothers - Henriksen et al., 2013; environmentally manipulated mothers - Goerlich et al., 2012). This underscores the need for further investigation into the complex impact of prenatal maternal stress on the fearfulness and anxiety of chicks. Prenatal maternal stress has also been linked to higher aggression in offspring. Studies involving egg

injections have linked increased yolk CORT content to a significant rise in pecking frequency (Freire et al., 2006) and other aggressive behaviours such as grabbing (Ahmed et al., 2014). The heightened emotionality associated with prenatal maternal stress likely influences the social behavioural aspects of competition and dominance, areas that prenatal maternal stress has indeed been shown to impact.

Offspring of laying hens which underwent unpredictable lighting stress had increased competitiveness over feed than offspring from non-stressed hens (Natt et al., 2009; Lindqvist et al., 2007). However, no difference in competitive ability was found in offspring of red Jungle fowl exposed to the same stressor conditions (Lindqvist et al., 2007). Nevertheless, conflicting findings exist also in this domain, indicating that offspring of stressed mothers may exhibit decreased competitive ability compared to control offspring (CORT implants - Henriksen et al., 2013; unpredictable feeding - Janczak et al., 2007).

Overall, prenatal maternal stress can influence different aspects of behaviour, but conflicting findings exist, highlighting the importance of considering factors such as timing and modality of prenatal maternal stress induction, as well as the individual's genotype.

2.5.3 Cognition

Lastly, alteration of the neurodevelopmental process exerts profound effects on different cognitive capabilities of the offspring of stressed mothers. Notably, chicks injected with corticosterone during incubation exhibit weaker visual lateralization and an increased latency to detect predators (Freire et al., 2006). Consistently, Henriksen et al. (2013) reported reduced lateralization in the visual inspection of a novel object in the offspring of CORT-implanted mothers. Other important alterations involve cognitive processes associated with learning. In-ovo CORT injections have been associated with impaired filial imprinting (Nordgreen et al., 2006), as well as poorer performances in object discrimination tasks (Rodricks et al., 2006) and memory tasks (Sui et al., 1997; Rodricks et al., 2006). Moreover, unpredictable lighting schedules in mothers were linked to impaired spatial learning (Lindqvist et al., 2007). However, instances of cognitive improvement have been observed, as reported by Goerlich et al. (2012), where offspring of stressed mothers performed better in association tasks.

3 Material and methods

3.1 Subjects

Measurements were obtained from 82 individuals (37 females and 45 males) born from an advanced intercross (F19) that originated from one red junglefowl male native from Thailand (*Gallus gallus*) and three White Leghorn females (*Gallus gallus domesticus*) (Höglund et al., 2020). Prenatal maternal stress was artificially induced in 10 breeding hens by subcutaneous implantation of 7mg corticosterone slow-release pellet, while 10 other hens were implanted with control treatment. In the following 12 days post implantations their eggs were collected for incubation and hatching. The rearing environment was uniform for all individuals, with ad-libitum access to food, water, and perching. Both in the early (5 to 6 weeks of age) and late (26 to 27 weeks of age) stages of life, individuals were scored on a series of behavioural tests for future investigations. At 224 days of age, the individuals were weighted and euthanized via cervical neck dislocation and decapitation.

3.2 Brain Measurements

Immediately after culling, the brains of all the individuals were extracted and dissected into telencephalon, cerebellum (cut down in the vermis), optic tectum, and brain remainder (thalamus, remaining midbrain, and hindbrain). The regions were weighed and then further divided into left and right hemispheres. The left hemisphere was purposed for gene analysis, and thus flash-frozen in liquid nitrogen and stored at −80°C. The right hemispheres of the dissected brain regions were instead purposed for isotropic fractionation, and thus immersion fixed in 4% paraformaldehyde in 0.1 M phosphate buffer. The procedure of isotropic fractionation consists of transforming the brain into a suspension of free cell nuclei where the total number of cells and neurons can be estimated thereafter through fluorescent staining. Thus, brain tissue was mechanically dissociated in 40 mM sodium citrate with 1% Triton X-100 using Tenbroeck tissue homogenizers (Herculano-Houzel and Lent, 2005), until no tissue fragments were visible. Afterward, the addition of fluorescent DNA marker 40,6-Diamidine-20-phenylindole dihydrochloride (DAPI) makes possible estimation of total number of cells by transferring a defined volume of homogenized solution in a Neubauer improved chamber and counting cells under a fluorescent Nikon eclipse 80i microscope at 400 magnification. For the cell counts, at least four aliquots (10 mL) per individual were sampled and counted,

given that coefficients of variation among the four aliquots were lower than 15%. Cell quantification was automated using predefined calibrated criteria. Specifically, cells were recognized with a diameter ranging between 5,18 and 16,76 pixels and a degree of circularity ranging between 0.81 and 1.00. For the neuronal count, manual counting was used, and neurons were detected across samples using immunocytochemical detection of neuronal nuclear antigen NeuN, expressed in the nuclei of most neuronal populations within the brain apart from Purkinje Cells (Mullen et al., 1992). For each sample, a minimum of 500 nuclei were counted to estimate the proportion of neurons, which would then be multiplied by the total cell number to obtain the neuron number. The number of non-neuronal cells was then calculated by subtracting neurons from the total cells. The densities of neurons and non-neuronal cells were then derived by dividing their absolute number by the mass of the brain regions.

3.3 Statistical Analysis

Statistical analyses were performed in the base package of R 4.3.2 (R Core Team, 2021).

To investigate the potential effect of treatment in relation to allometric relationships, linear regression analysis was conducted. Afterward, whenever a significant interaction between sex and treatment was discovered, a post-hoc analysis using a t-test was performed to examine how the effects of treatment varied depending on sex.

The studied allometric relationships included absolute and relative brain mass against body mass, total number of cells against brain mass, and neuronal and non-neuronal counts against brain mass. Such relationships were investigated both for the overall brain, as well as taking singularly the telencephalon and cerebellum regions.

Sex, Treatment, and their potential interaction were included in the linear models. Furthermore, as neuron number in telencephalon was counted by two different researchers, to safely avoid researcher biases, whenever telencephalon neuron and non-neuron numbers were involved in the investigation, the analysis involved the Researcher as a Fixed Factor.

Furthermore, when analysing for Treatment effects, the dataset was restricted only to the 54 individuals hatched from eggs that were laid 4 days after CORT-implantation due to the reported influence of this condition on prenatal maternal stress treatment effects (Henriksen et al., 2011a). Lastly, correlational analysis was also conducted to elucidate trends in the studied allometric relationships.

4 Results

4.1Allometric variables

4.1.1 Absolute Brain Mass

A linear regression analysis was conducted to examine the influence of body weight, sex, and treatment on total brain mass, telencephalon mass, and cerebellum (see Supplementary Material 10.1.1).

Treatment did not have a significant effect on total brain mass (p =1.336) or cerebellum mass (p=0.168) but showed a significant effect on telencephalic mass (p = 0.0458).

Post-hoc analysis on Sex and Treatment interaction indicated that this significant reduction affected only prenatally stressed females, marking a significant tendency to exhibit lower telencephalic mass than controls (t(19.67)=2.2, p= 0.040)

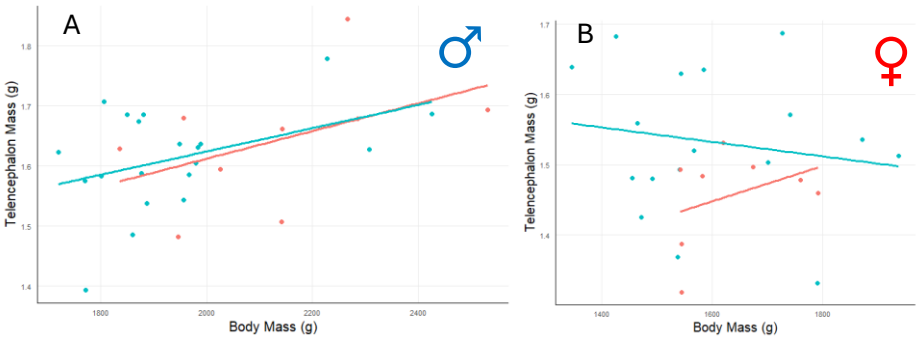

Figure 1. Scatterplots of telencephalic absolute brain masses (g) of (A) Males and (B) Females against the mass (g) of the body. Prenatally stressed individuals are shown in orange and controls in cyan. Prenatal maternal stress causes marginal reduction of telencephalic mass in females.

In the total brain and both regions, sex explains differences in respective region masses, with males having greater absolute brain masses compared to females (total brain: p= 0.038; telencephalon: p=0.0321; cerebellum: p=0.078), corroborating with previous evidence (Cunha et al., 2022).

Correlational analysis was additionally performed for each brain region's absolute mass in relation to body mass (Fig 2), and the detailed results are provided in the supplementary material section (see 10.1.2).

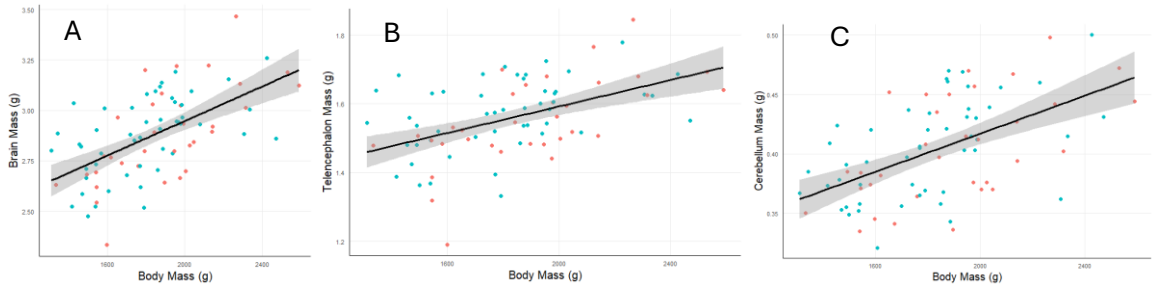

Figure 2. Scatterplots of absolute brain masses (g) of (A) Total Brain Mass (B) Telencephalon (C) Cerebellum against the mass (g) of the body. Across all regions, absolute brain mass positively correlates with body mass (See Supplementary Material). Prenatally stressed individuals are shown in orange and controls in cyan.

4.1.2 Relative Brain Mass

A linear regression analysis was conducted to examine the influence of body weight, sex, and treatment on relative brain mass in the whole brain, telencephalon, and cerebellum (see Supplementary Material 10.2.1).

Interaction of Sex and Treatment showed a marginal effect on relative total brain mass ($p = 0.0516$), indicating that prenatally stressed females have a tendency to exhibit lower relative brain mass than controls (Fig 3B, $p= 0.094$).

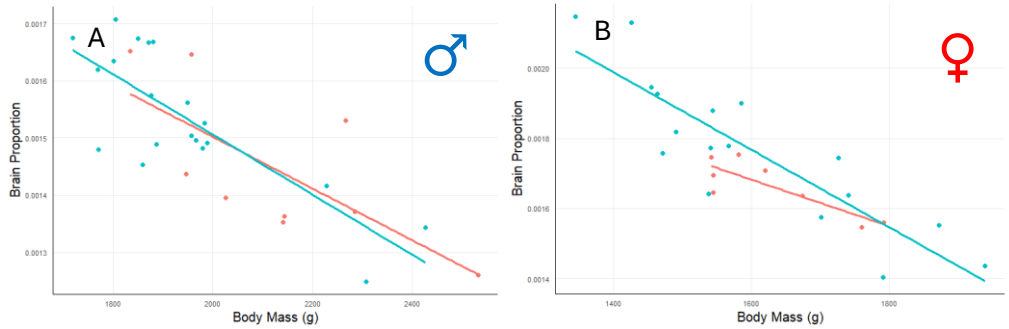

Figure 3. Scatterplots of relative brain masses (g) of (A) Males and (B) Females against the mass (g) of the body. Prenatally stressed individuals are shown in orange and controls in cyan. Prenatal maternal stress causes a marginal reduction of relative brain mass in females.

However, when looking in detail at the subdivided brain regions, Treatment did not affect their relative brain mass (telencephalon: $p= 0.119$, cerebellum: $p= 0.566$). In the total brain and both regions, also Sex wasn't found to be a significant predictor of relative brain mass (total brain: $p=0.994$, telencephalon: $p= 0.996$, cerebellum: $p=0.921$).

Correlational analysis was additionally performed for each brain region relative to brain mass in relation to body mass (Fig 4), and the detailed results are provided in the supplementary material section (see 10.2.2).

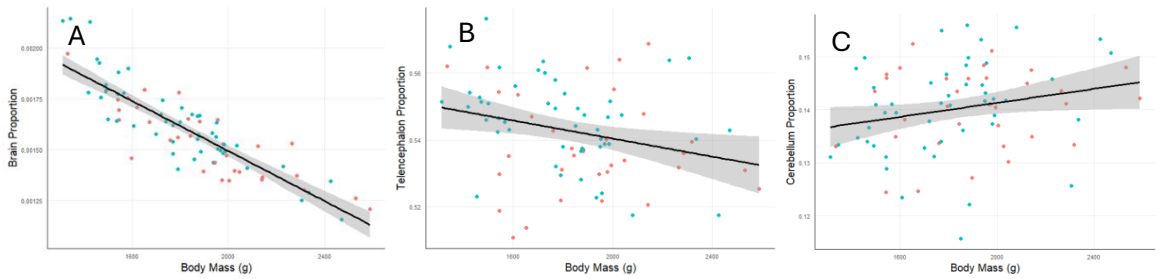

Figure 4. Scatterplots of relative brain mass (%) of (A) Total Brain (B) Telencephalon (C) Cerebellum against the mass (g) of the body. Prenatally stressed individuals are shown in orange and controls in cyan.

4.2 Brain Cell Counts

4.2.1 Telencephalon Cell Counts

A linear regression analysis was conducted to examine the influence of brain mass, sex, and treatment on total cell count, neurons, and non-neurons in the telencephalon (see Supplementary Material 10.3.1).

Prenatal maternal stress induced a marginal reduction in total telencephalic cell counts in prenatally stressed individuals compared with controls (p= 0.098). Treatment had no significant effect on telencephalic neuron counts (p= 0.79912), but it did on non-neuronal ones, where prenatal stress induced a marginal reduction in telencephalon non-neurons in prenatally stressed individuals compared with controls (Fig 5, p=0.061).

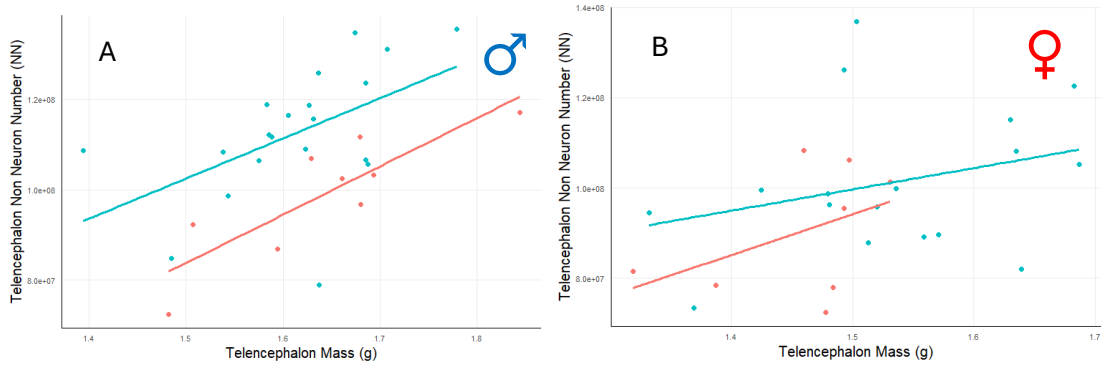

Figure 5. Scatterplots of telencephalic non-neuron number of (A) Males and (B) Females against the mass (g) of the telencephalon. Prenatally stressed individuals are shown in orange and controls in cyan. Prenatal maternal stress reduces telencephalic non-neuron number in both males and females.

Correlational analysis was additionally performed for each counting type in relation to telencephalic mass, and the detailed results are provided in the supplementary material section (see 10.3.2). The correlational analysis didn't show a significant correlation between telencephalon neuron number and

telencephalon mass (Figure 6A, slope = -0.075, $p > 0.05$). However, the telencephalon non-neuron number does exhibit a significant positive correlation with telencephalon mass (Figure 6B, slope = 0.500, $p < 0.01$). This indicates that individuals with greater telencephalons tend also to have higher non-neuronal numbers in them, but the number of neurons tends to remain constant.

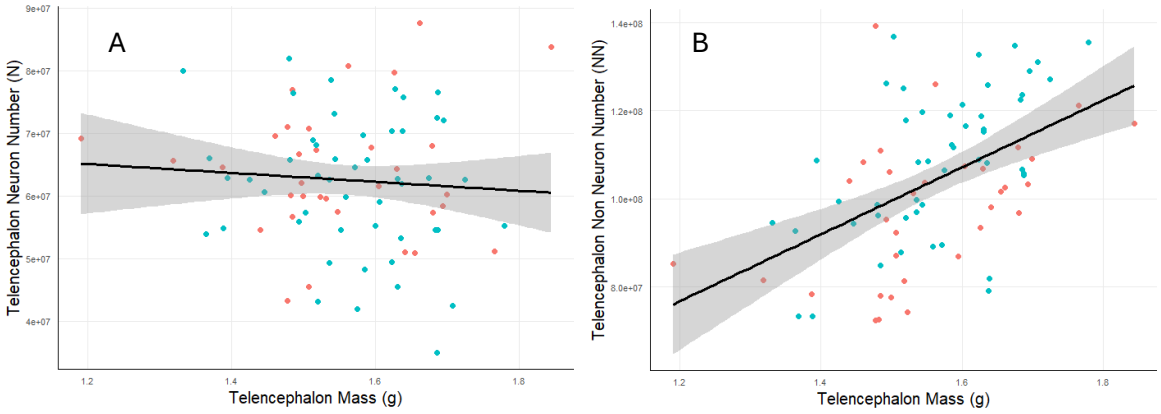

Figure 6. Scatterplots of telencephalon counting of (A) neurons, and (B) non-neurons against the mass (g) of the telencephalon. Prenatally stressed individuals are shown in orange and controls in cyan.

4.2.2 Cerebellum Cell Counts

A linear regression analysis was conducted to examine the influence of brain mass, sex, and treatment on total cell count in the cerebellum (see Supplementary Material 10.4.1).

Treatment did not have a significant effect on cerebellum total cell counts ($p = 0.578$). Regarding cerebellum neuron number, no significant differences in intercepts were observed concerning treatment ($p = 0.586$). However, in the case of non-neuron numbers, significant differences in intercepts were identified and were attributed to the interaction between sex and treatment ($p= 0.0497$). Specifically, prenatally stressed females have decreased numbers of non-neuronal cells in the cerebellum, compared to controls (Fig 7B, $p= 0.069$), but not in males (Fig 7A, $p=0.249$).

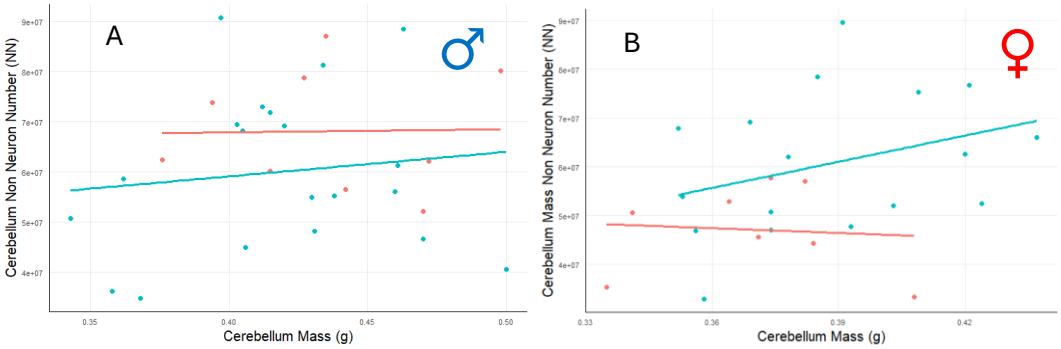

Figure 7. Scatterplots of cerebellum non-neuron number of (A) Males and (B) Females against the mass (g) of the cerebellum. Prenatally stressed individuals are shown in orange and controls in cyan. Prenatal maternal stress reduces cerebellar non-neuron number in females.

Correlational analysis was additionally performed for each counting type in relation to cerebellar mass, and the detailed results are provided in the supplementary material section (see 10.4.2). Furthermore, correlational analysis indicated a significant correlation between cerebellum neuronal and non-neuronal numbers and cerebellum mass (neuron: Figure 8A, slope = 0.606, $p < 0.05$; non-neuron: Figure 8B, slope = 0.310, $p < 0.05$;). This indicates that individuals with greater cerebellum tend also to have higher numbers of neurons and non-neurons.

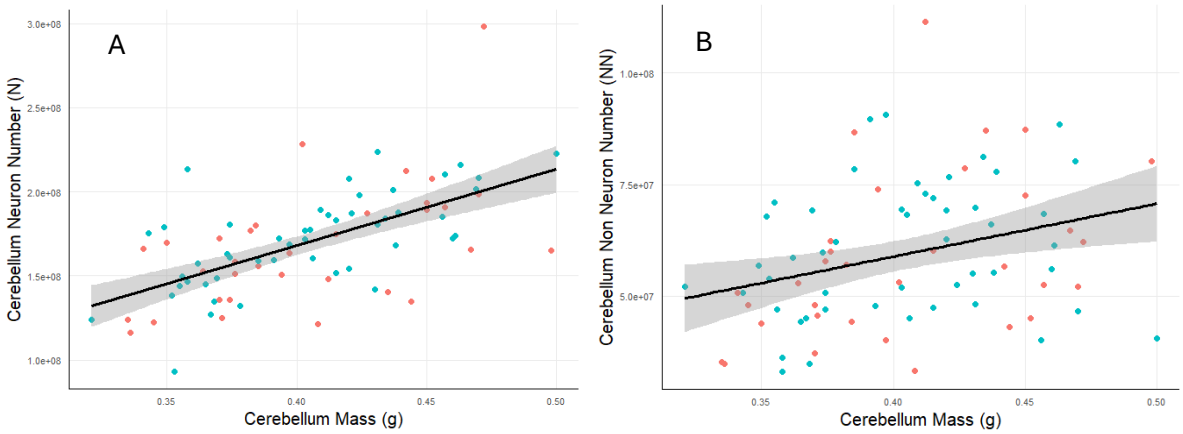

Figure 8. Scatterplots of cerebellum counting of (A) neurons, (B) non-neurons against the mass (g) of the cerebellum. Prenatally stressed individuals are shown in orange and controls in cyan.

5 Discussion

In the present study, I sought to provide evidence that prenatal maternal stress influences the neurodevelopment of chickens, a good model species for the investigation of such effects. Linear regression models were used to analyse various allometric relationships involving brain sizes and cell counting, seeking for potential influence of prenatal maternal stress.

Maternal Stress reduces non-neuron numbers in telencephalons and the female cerebellum.

Results showed an influence of prenatal maternal stress on chicken brain development. However, while previous studies on mice reported an effect of prenatal maternal stress on neurogenesis (Zhang et al., 2021), our findings reveal a predominant impact on non-neuronal cell development or gliogenesis. In the telencephalon, prenatally stressed individuals exhibited trends of reduced telencephalic non-neuronal counts. Moreover, prenatally stressed females showed significantly lower brain proportion and telencephalon masses, without concurrent changes in neuronal counts. This reduction in mass could thus likely be due to alterations in non-neuronal cell populations.

Interestingly, our findings suggest a similar but sex-dependent impact in the cerebellum where only prenatally stressed female chickens exhibited reduced numbers of non-neuronal cells. Chicken maternal stress research has long been suggested to have sex-dependent effects on physiology and behaviour (Henriksen et al., 2011b for review), and this study provides evidence for a similar influence on neurodevelopment. These sex-dependent effects on phenotype might be linked by the fact that avian prenatal maternal stress has been suggested to not be mediated solely by CORT concentration in the egg, but also its sex hormone composition (Groothuis et al., 2005). In this study, the influence of sex hormones on gliogenesis and myelination could be noteworthy, as these processes begin quite early in gestation (Rogers, 1995, Norkutė et al., 2010, Lever et al., 2013) and thus could potentially be affected by the indirect mediatory action of gonadal hormones in the egg. Moreover, although present in an alternative stress model, sex-dependent decreased gliogenesis has been previously reported in female mice where prenatal maternal stress has been suggested to impair such developmental processes in the hippocampus (Behan et al.,2011).

Considering the critical role of non-neuronal cells in supporting neuronal function and synaptic plasticity, these findings might hold implications for understanding the long-term consequences of prenatal maternal stress on brain function. Indeed, decreased or impaired glial functioning, as observed in our study, has been associated with affective pathology (Soe et al., 2016), suggesting potential pathways through which prenatal maternal stress may exert lasting effects on neurophysiology and behaviour. These associations and the current findings highlight the potential role of sex hormones in modulating nervous development and the importance of considering sex-dependent outcomes in research on chicken prenatal maternal stress.

Our findings also highlight the vulnerability of the female cerebellum and telencephalon to prenatal maternal stress. The telencephalon is recognized for being involved in higher cognitive functions, while historically, the cerebellum has been predominantly associated with sensory-motor control (Strick et al., 2009). However, emerging evidence suggests that the cerebellum also plays a role in higher cognitive functions such as associative learning, memory, and emotional processing (Schmahmann, 1991; Buckner, 2013; Adamaszek et al., 2017). Thus, future integration of already recorded behavioural data into our findings could offer further insights into the consequences of impaired non-neuronal functioning on cognitive and emotional development, aligning with previously documented effects of avian prenatal maternal stress (Henriksen et al., 2011b for Review).

Contrasting allometric findings

Our dataset presents allometric findings that both align and contrast with previous research on brain cell scaling by Cunha et al., 2022 in chickens of the same breed.

Consistent trends are present when examining the relationship between absolute brain mass and body weight, and all cell counts and brain mass, but contrasting evidence emerges when investigating neuronal and non-neuronal counts.

Unlike previous evidence which indicated a proportional increase in telencephalon neurons with its mass, we observed no such correlation in our dataset. Instead, our findings suggest that increases in telencephalon cell counts, and mass are driven primarily by non-neuronal cells. This is in line with mammalian and other avian literature where increasing forebrain masses are associated with lower neural densities and higher non-neural ones (mammals: Herculano et al., 2014, avian: Olkowicz et al.,2016).a

Discrepancies arise also when looking at the cerebellum non-neuronal counts. Whereas previous findings by Cunha et al., 2022 report no concurrent increase of non-neurons along with cerebellar mass, our data sets present contrasting results, revealing a positive correlation between non-neurons and cerebellar mass. These contrasting findings raise questions regarding what factors influence brain cellular composition and their implications.

Limitations

Limitations to the study must be considered. For example, I find that prenatal maternal stress decreased relative brain mass in females, and it might be linked to the more pronounced reduction of telencephalic mass observed in females, which in turn could be related to the decrease in telencephalic non-neuronal cells. However, I am limited in drawing such conclusions because the procedure does not allow us to discern the different types of non-neuronal cells within the brain we examined, nor does it enable us to analyse their size, structure, or composition, which are factors that could contribute to mass differently, and thus affect measures dependent on mass such as brain proportions. For example, oligodendrocytes are cells that might contribute more to brain mass by putting more myelin on neuronal axons. Hence, a reduction of non-neuron numbers might not necessarily be correlated with a reduction in brain size and so it cannot be necessarily correlated to less brain proportions. Discriminating between the types of non-neuronal cells and the size of the cells could provide precious insights into how prenatal maternal stress affects brain cellular composition.

Another limitation that has to be addressed is that the isotropic fractionator technique does not allow to include Purkinje cells in counting, and although these cells do not represent a large fraction of the total cerebellar neurons (Cunha et al., 2020, 2021), we cannot know if treatment had an effect on this neuronal population.

Furthermore, physiological investigations on hormonal allocations in the egg may allow to gain valuable information on how egg composition influences observed outcomes. Previous studies indicate that after day 4 of implantation, laid eggs have an increase in hormonal allocation in the egg and we can start to see discernible effects of treatment in offspring (Henriksen et al., 2011a). In my results, I amalgamate individual hormonal values into a comprehensive "post4 treatment" category. While this approach still allows to discern treatment effects between subjects receiving treatment and controls, having the means to consider hormonal concentrations in the egg could ensure a direct link between prenatal maternal stress effects and egg hormonal allocations. This approach would not only allow to

account for hormonal allocation as a contributing factor but also provide more understanding of how egg hormonal allocations influence neurodevelopmental outcomes.

Lastly, to confirm sex differences and to study more reliably sex-dependent effects, future studies would be improved by a larger sample size with a higher representation of both sexes.

6 Conclusion

Overall, I found that prenatal maternal stress influences chicken brain development, particularly affecting non-neuronal cell populations in the telencephalon and cerebellum. In the telencephalon, prenatally stressed individuals exhibited reduced telencephalic masses and non-neuronal counts. In the cerebellum, prenatal maternal stress was also linked to reduced non-neuronal counts, but only in females. These findings thus highlight the importance of considering sex-specific responses to prenatal maternal stress when looking at neurodevelopment. However, my study has limitations, such as the inability to discriminate between different types of non-neuronal cells and to include Purkinje cells in our neuron counting. We must also note that our study also presents contrasting findings regarding brain cellular composition compared to previous research, raising questions about the factors influencing these discrepancies.

Future research would be encouraged to investigate the effects of prenatal maternal stress with the ability to discriminate between non-neuronal cell subtypes, to also understand the effect of prenatal maternal stress on brain cellular compositions. It would also be interesting to investigate the specific effects of hormonal allocations in the egg on brain development to deepen our understanding of how hormones are involved in prenatal maternal stress mediation.

Finally, understanding these mechanisms and integrating behavioural data could provide valuable insights into the long-term consequences of prenatal maternal stress on brain function and behaviour.

7 Societal / ethical considerations

In the present study, to investigate the consequences of prenatal maternal stress, hens were implanted with corticosterone slow-release pellet, and their offsprings were then subjected to a series of behavioural tests aimed at evaluating their fear and anxiety, to then be sacrificed at 224 days of age by cervical dislocation. Prolonged exposure to elevated levels of stress can impact the overall welfare of the animals, so the greatest precautions have been taken to minimize disturbance and distress of the subjects during implantation. Moreover, the concentration of the corticosterone slow-release pellets was tarred to minimize the harmful consequences of elevated stress hormones. The sacrifice of individuals was warranted to gain access to their brains, and cervical dislocation was used as it involves quickly and humanely breaking the neck of the bird, resulting in immediate loss of consciousness and rapid death. Furthermore, this method ensures the integrity of the brain tissue collected. Overall, the procedure was conducted under ethical permit DNR#50-13. This study allowed to improve knowledge of prenatal maternal stress effects, a phenomenon which shows how the compromised well-being of an individual can lead to perpetuating consequences in their offspring, having long-term consequences on their welfare. Chickens are as of today the most carbo-neutral source of food produced on a large scale. This warrants attention and underscores the need to prioritize the welfare of chicken populations, especially considering the projected growth of the industry. When it comes to the United Nations Sustainable Development Goals, this project can help fulfill the “Good health and Well-being” aspect as we are gaining insight into some neurodevelopmental aspects related to prenatal maternal stress and affective pathology that are also mirrored in humans.

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10 SUPPLEMENTARY MATERIAL

10.1 Absolute Brain Mass

10.1.1 Linear Regression Analysis - Absolute Brain Mass

A linear regression analysis was conducted to examine the influence of body weight, sex, and treatment on total brain mass, telencephalon mass and cerebellum mass.

	ESTIMATE	STD ERROR	T-VALUE	P-VALUE
(INTERCEPT)	2.249	0.213	10.540	<0.001
`BODY_MASS(G)`	<0.001	<0.001	2.220	0.031
SEXM	0.209	0.098	2.133	0.038
SEXM:TREATMENTCONTROL	-0.136	0.093	-1.457	0.151
TREATMENTC	0.103	0.067	1.525	1.336

Supplementary Table 1. Estimated regression parameters, standard errors, t-values and P-values for the linear regression model investigating brain mass.

	ESTIMATE	STD ERROR	T-VALUE	P-VALUE
(INTERCEPT)	1.344	0.121	11.133	5.0557e-15
`BODY_MASS(G)`	0.0001	7.419e-05	1.585	0.1194
SEXM	0.0401	0.00395	1.0171	0.314
TREATMENTCORT	-0.0804	0.00392	-2.0496	0.04577
SEXM:TREATMENTCORT	0.0867	0.00548	1.583	0.1197

Supplementary Table 2. Estimated regression parameters, standard errors, t-values and P-values for the linear regression model investigating Telencephalon mass.

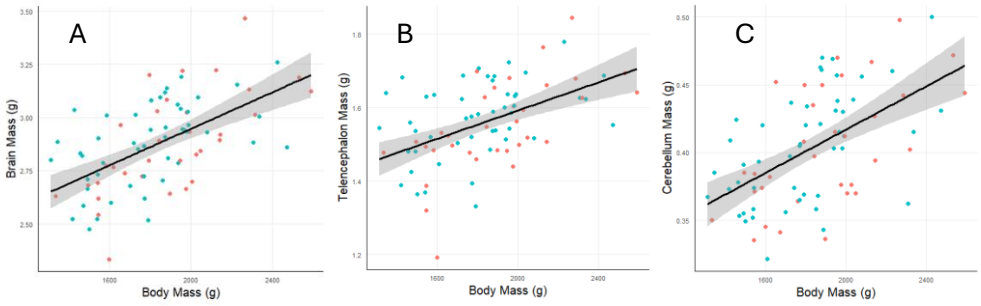
	ESTIMATE	STD ERROR	T-VALUE	P-VALUE
(INTERCEPT)	0.294	0.0441	6.666	2.178e-08
`BODY_MASS(G)`	5.852e-05	2.713e-05	2.156	0.0359
SEXM	0.00106	0.0144	0.733	0.466

TREATMENTCORT	-0.02009	1.434e-02	-1.400	0.168
SEXM:TREATMENTCORT	0.0272	0.02002	1.359	0.180

Supplementary Table 3. Estimated regression parameters, standard errors, t-values and P-values for the linear regression model investigating Cerebellum mass.

10.1.2 Correlational Analysis - Absolute Brain Mass

Correlational analysis indicated a significant positive correlation between brain mass and body weight (Supplementary Figure 1A, slope=0.585, p<0.001), indicating that individuals with higher body weight tend to have larger brain mass. The same pattern can be found across various brain regions, including the telencephalon (Supplementary Figure 1B, slope=0.491, p<0.01) and cerebellum (Supplementary Figure 1C, slope=0.55, p<0.01).



Supplementary Figure 1. Scatterplots of brain masses (g) of (A) Whole Brain (B) Telencephalon (C) Cerebellum against the mass (g) of the body. Prenatally stressed individuals are shown in orange, and controls in cyan.

10.2 Relative Brain Mass

10.2.1 Linear Regression Analysis - Relative Brain Mass

A linear regression analysis was conducted to examine the influence of body weight, sex, and treatment on relative brain mass of the total brain, telencephalon and cerebellum.

	ESTIMATE	STD ERROR	T-VALUE	P-VALUE
(INTERCEPT)	28.630	1.303	21.975	5.0250e-27
WEIGHT.224_G	-0.00684	0.000800	-8.554	2.747e-11
TREATMENTCORT	-0.839	0.423	-1.984	5.287e-02
SEXM	0.0358	0.426	0.0841	9.332e-01
TREATMENTCORT:SEXM	1.178	0.590	1.995	5.161e-02

Supplementary Table 4. Estimated regression parameters, standard errors, t-values and P-values for the linear regression model investigating brain proportions.

	ESTIMATE	STD ERROR	T-VALUE	P-VALUE
(INTERCEPT)	5.6492e-01	1.776e-02	31.795	2.263e-34
WEIGHT.224_G	-1.093e-05	1.091e-05	-1.002	3.212e-01
SEXM	-3.104e-05	5.810e-03	-0.00534	9.957e-01
TREATMENTCORT	-9.160e-03	5.772e-03	-1.587	1.189e-01
SEXM:TREATMENTCORT	5.398e-03	8.057e-03	0.669	5.060e-01

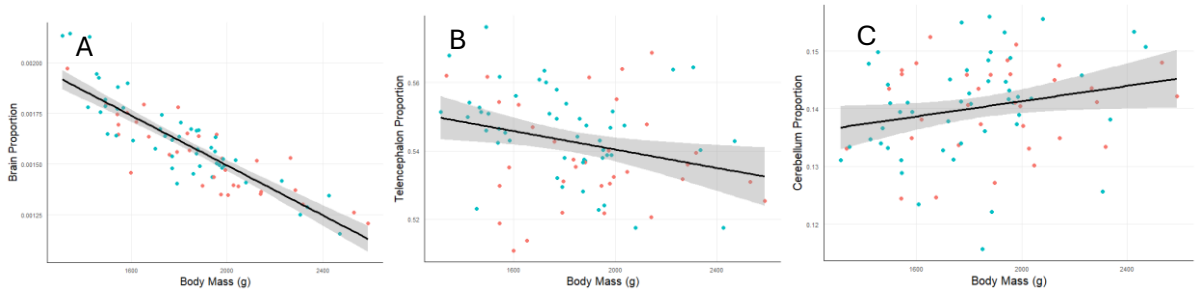
Supplementary Table 5. Estimated regression parameters, standard errors, t-values and P-values for the linear regression model investigating Telencephalon proportions.

	ESTIMATE	STD ERROR	T-VALUE	P-VALUE
(INTERCEPT)	1.295e-01	1.120e-02	11.560	1.317e-15
WEIGHT.224_G	5.695e-06	6.886e-06	0.826	4.122e-01
SEXM	3.662e-04	3.665e-03	0.0999	9.208e-01
TREATMENTCORT	-2.106e-03	3.641e-03	-0.578	5.655e-01
SEXM:TREATMENTCORT	2.954e-03	5.082e-03	0.581	5.637e-01

Supplementary Table 6. Estimated regression parameters, standard errors, t-values and P-values for the linear regression model investigating Cerebellum proportions.

10.2.2 Correlational Analysis - Relative Brain Mass

Correlational analysis revealed a significant negative correlation between relative total brain mass and body mass (Supplementary Figure 2A, slope = -0.864, $p < 0.01$), suggesting that individuals with higher body weight tend to have smaller brain proportions. When looking in detail at the subdivided brain regions, correlational analysis revealed that relative telencephalon mass correlated negatively with body mass (Supplementary Figure 2B slope = -0.272, $p < 0.05$), whereas relative cerebellum mass showed a positive correlation (Supplementary Figure 2C, slope = 0.230, $p < 0.05$). This suggests that with increasing body mass, telencephalon proportional size tends to decrease while cerebellum proportional size rises.



Supplementary Figure 2. Scatterplots of relative brain mass (%) of (A) Whole Brain (B) Telencephalon (C) Cerebellum against the mass (g) of the body. Prenatally stressed individuals are shown in orange, and controls in cyan.

10.3 Telencephalon Cell Counts

10.3.1 Linear Regression Analysis – Telencephalon Cell Counts

A linear regression analysis was conducted to examine the influence of brain mass, sex, and treatment on total, neuronal and non-neuronal cell count in the telencephalon.

	ESTIMATE	STD ERROR	T-VALUE	P-VALUE
(INTERCEPT)	100810710	35036424	2.877312	0.00588
BRAIN_MASS	23000871	12599403	1.825552	0.0739
SEXM	5202778	4955072	1.049991	0.299
TREATMENTCORT	-7341257	4359010	-1.684157	0.0984

Supplementary Table 7. Estimated regression parameters, standard errors, t-values and P-values for the linear regression model investigating total cell counts in the telencephalon.

	ESTIMATE	STD ERROR	T-VALUE	P-VALUE
(INTERCEPT)	80139000	22393000	3.578	0.000790
CEREBRUM_MASS	-9129000	14839000	-0.615	0.541
TREATMENTCORT	2128000	3023000	0.703	0.485
SEXM	8460000	3177000	0.266	0.791
RESEARCHERFELIPE	-10666000	3349000	-3.184	0.00252

Supplementary Table 8. Estimated regression parameters, standard errors, t-values and P-values for the linear regression model investigating Telencephalon neuronal counts.

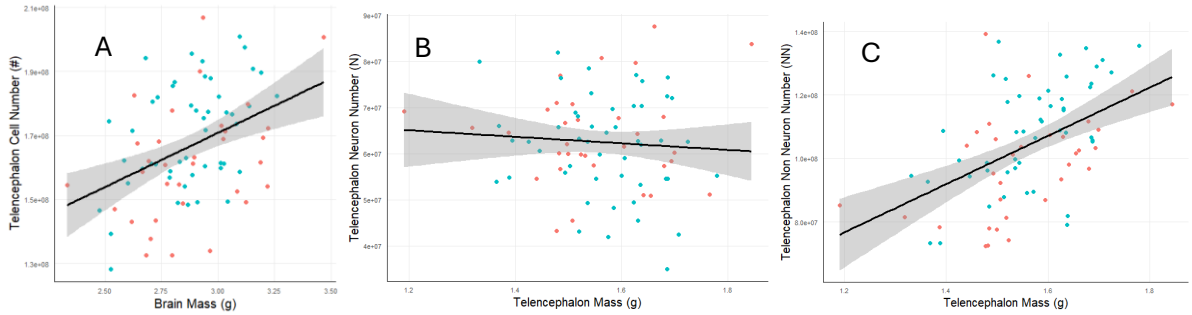
	ESTIMATE	STD ERROR	T-VALUE	P-VALUE
(INTERCEPT)	13633000	26032000	0.523	6.028e-01
CEREBRUM_MASS	54406000	17250000	3.153	2.751e-03
SEXM	4408000	3694000	1.193	2.384e-01
TREATMENTCORT	-6736000	3515000	-1.916	6.116e-02
RESEARCHERFELIPE	17208000	3894000	4.418	5.487e-05

Supplementary Table 9. Estimated regression parameters, standard errors, t-values and P-values for the linear regression model investigating Telencephalon non-neuronal counts.

10.3.2 Correlational Analysis - Telencephalon Cell Counts

Correlational analysis indicated a significant positive correlation between telencephalon cell number and brain mass (Supplementary Figure 3A, slope = 0.406, $p < 0.001$), suggesting that individuals with higher brain weight tend to have higher cell numbers in the telencephalon.

Regarding neuron counts, correlational analysis didn't find a significant correlation between telencephalon neuron number and telencephalon mass (Supplementary Figure 3B, slope = -0.075, $p > 0.05$). However, telencephalon non-neuron number does exhibit a significant positive correlation with telencephalon mass (Supplementary Figure 3C, slope = 0.500, $p < 0.01$). This indicates that individuals with greater telencephalons tend also to have higher non-neuronal numbers in them, but the number of neurons tends to remain constant.



Supplementary Figure 3. Scatterplots of telencephalon counting of (A) total cells (B) neurons, (C) non-neurons against the mass (g) of the telencephalon. Prenatally stressed individuals are shown in orange, and controls in cyan.

10.4 Cerebellum Cell Counts

10.4.1 Linear Regression Analysis - Cerebellum Cell Counts

A linear regression analysis was conducted to examine the influence of brain mass, sex, and treatment on total cell, neuronal and non-neuronal counts in the cerebellum.

	ESTIMATE	STD ERROR	T-VALUE	P-VALUE
(INTERCEPT)	-81223449	70394833	-1.1538269	0.254
BRAIN_MASS	106923403	25314596	4.2237847	0.000101
SEXM	7719715	9955681	0.7754080	0.441
TREATMENTCORT	-4901754	8758079	-0.5596836	0.578

Supplementary Table 10. Estimated regression parameters, standard errors, t-values and P-values for the linear regression model investigating Cerebellum all cells counts.

	ESTIMATE	STD ERROR	T-VALUE	P-VALUE
(INTERCEPT)	8580000	43036000	0.199	0.842
CEREBELLUM_MASS	393512000	109628000	3.589	0.000765
SEXM	7099000	9467000	0.750	0.457
TREATMENTCORT	-6393000	11670000	-0.548	0.586
SEXM:TREATMENTCORT	6280000	16236000	0.387	0.701

Supplementary Table 11. Estimated regression parameters, standard errors, t-values and P-values for the linear regression model investigating Cerebellum neuronal counts.

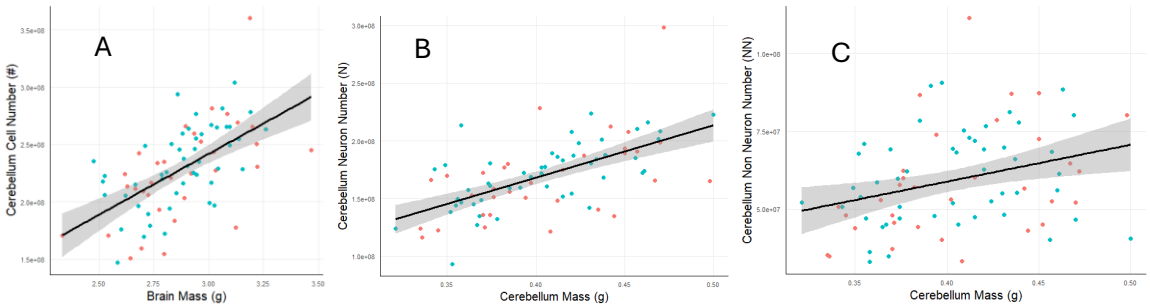
	ESTIMATE	STD ERROR	T-VALUE	P-VALUE
(INTERCEPT)	37216000	22881000	1.626	0.110
CEREBELLUM_MASS	60498000	58287000	1.038	0.304
TREATMENTCORT	-12486000	6204000	-2.012	0.0496
SEXM	-2515000	5033000	-0.500	0.619
TREATMENTCORT:SEXM	19509000	8632000	2.260	0.0283

Supplementary Table 12. Estimated regression parameters, standard errors, t-values and P-values for the linear regression model investigating Cerebellum non-neuronal counts.

10.4.2 Correlational Analysis - Cerebellum Cell Counts

Correlational analysis indicated a significant positive correlation between cerebellum total cell number and brain mass (Supplementary Figure 4A, slope = 0.588, $p < 0.001$). suggesting that individuals with higher brain weight tend to have higher cell numbers in the cerebellum.

Furthermore, correlational analysis indicated a significant correlation between cerebellum neuronal and non-neuronal numbers and cerebellum mass (neuron: Supplementary Figure 4B, slope = 0.606, $p < 0.05$; non-neuron: Supplementary Figure 4C, slope = 0.310, $p < 0.05$;). This indicates that individuals with greater cerebellum tend also to have higher numbers of neurons and non-neurons.



Supplementary Figure 4. Scatterplots of cerebellum counting of (A) total cells (B) neurons, (C) non-neurons against the mass (g) of the cerebellum. Prenatally stressed individuals are shown in orange, and controls in cyan.