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Introductory Essay

Corticosterone; a teammate in the game of developmental programming in chickens or a misunderstood bench warmer?

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1 Abstract

The developing embryo exhibits a plastic ability to adapt to environmental challenges and these developmental changes in response to the environment may cause permanent consequences in adult life. Mammalian species are constantly susceptible to maternal effects due to their *intra uterine* development and prone to react and respond to the condition of the mother. A detrimental *intra uterine* environment predisposes for an array of epidemiological effects including adult cardiovascular, metabolic and psychological disease but the mechanisms responsible are still mostly unveiled. The chicken serves as a suitable model for ontogenetic and developmental programming investigations. Its *extra uterine* development avoids maternal interactions during embryonic development and the embryo is readily accessible within its egg case for external manipulations. Many studies have been undertaken in order to characterize the mechanisms by which stress shapes the phenotype of the developing chicken. Maternal stress, food enriched with corticosterone as well as corticosterone injections in the egg and female have all been used to investigate maternal effects and fetal programming. However, very low levels of corticosterone have been found in the egg and with the use of high-performance liquid chromatography and the levels found were attributable to cross binding with progesterone. The chicken still serves as a suitable model for developmental programming studies, but not so for corticosterone incorporation by maternal effects. The chick is not resilient to the maternal environment and other steroid hormones might be the pathway of programming. If this is an evolutionary strategy or merely a by-product of maternal constraints still needs to be demonstrated.

2 Developmental Programming

Parental effects, also named less accurately as maternal effects, refer to the phenotypic changes on the offspring directly attributable to the parents' actions or phenotype. Mousseau and Fox also define them as the maternal traits that influence offspring phenotype via non-genetic pathways (Mousseau and Fox 1998). Maternal effects have been reviewed and are shown to be of uttermost importance because of the influences that early developmental experiences have on subsequent life history and ultimately on survival and reproductive success (Metcalf and Monaghan 2001).

Prenatal stress is another widely used term and refers to the challenge an embryo is exposed to prior birth or hatching. A solid definition of the term is, however, missing, and it is used arbitrarily. Here I make an attempt to clarify the subject. The term prenatal stress comprises of stress with different origins and the distinction is seldom addressed. Either the stress is of maternal origin where the embryo is a passive receiver of maternal stress effects (stress hormones, nutrition composition etc.), or it is caused by environmental factors that the embryo on its own is responding to. For example, a challenge cause a stress hormone response, and the hormone can be of either exogenous (maternal) or endogenous (embryonic) origin. In oviparous species the origin of stress hormones is clear cut due to the extra-uterine development but for mammals the distinction is troublesome and in most cases probably not possible.

The developing embryo exhibits a plastic ability to adapt to environmental constraints and these developmental changes in response to the environment may cause permanent consequences in adult life. A series of studies have demonstrated the link between the *in utero* environment and its long term consequences, clearly demonstrating that early life events predispose for adult cardiovascular and metabolic disease in humans and numerous animal models. Low birth weight is strongly predictive to hypertension (Barker, Winter et al. 1989), increased risk and death from coronary heart disease (Barker 1995, Stein, Fall et al. 1996), glucose intolerance (Hales, Barker et al. 1991), non-insulin-dependent diabetes mellitus (McCance, Pettitt et al. 1994) and death from ischemic heart disease (Barker and Osmond 1986, Barker, Winter et al. 1989). These consequences from detrimental uterine environment are independent of classic life-style factors such as sedentary life-style, smoking, obesity, alcohol, social class etc. (Barker, Gluckman et al. 1993).

An early study was conducted on 19-year old men following the Dutch famine in 1944-45. They were exposed to prenatal and early postnatal malnutrition and examined at military induction, and demonstrated that early malnutrition determines subsequent obesity. The consequences of the exposure depend on when during the development it occurs. Fetuses exposed during the last trimester exhibit a decreased risk of obesity in contrast to those exposed early in gestation that exhibit an increased risk. The authors speculate that the early exposure impact the differentiation of the hypothalamic centers regulating growth and food intake and hence program the fetus to accumulation when

nutrition is available. During late gestation the adipose tissue develop and might be reduced when faced to low energy availability during this developmental window (Ravelli, Stein et al. 1976). A more recent study investigating the epigenome of 60 individuals conceived during the famine and compared them with their same sexed siblings now six decades later. They found that the IGF2-gene (insulin-like growth factor 2, a key factor in human growth and development) was hypomethylated as a result of low calorie intake. Individuals exposed late in gestation showed no methylation difference, but instead showed a lowered birth weight compared to controls as well as in comparison to individual exposed early in gestation (Heijmans, Tobi et al. 2008). A series of other genes has now been demonstrated to possess changes in methylation patterns due to famine (Heijmans, Tobi et al. 2009) indicating life-long changes in the epigenome. Further studies following the same famine provides evidence that nutrient restriction during the last trimester predispose for schizophrenia. The relative risk to develop the disorder was found to be 2.16 to 2.54 for women but not for men depending on the class of schizophrenia (Susser and Lin 1992). Two excellent reviews have been published summarizing the prenatal effects from the Dutch famine (Roseboom, Van der Meulen et al. 2001, Painter, Roseboom et al. 2005), but to discuss this further exceeds the scope for this thesis.

Another extensive study investigated the correlation between low birth weight and adult cardiovascular and metabolic diseases in a cohort of 22 000 American men. A low birth weight (lighter than 2.2 kg) was found to strongly predispose for hypertension and type 2 diabetes with a relative risk of 1.26 and 1.75 respectively compared with average birth-weight adult controls (Curhan, Willett et al. 1996).

These correlations were thoroughly investigated by Barker et al (Barker and Osmond 1986, Barker, Winter et al. 1989) and the phenomenon was coined as the Barker hypothesis. This initiated the field of developmental programming and the hypothesis was subsequently further refined to the thrifty phenotype hypothesis (Hales and Barker 1992, Hales and Barker 2001). Their definition is the following: “The thrifty phenotype hypothesis propose that the fetus adapts to an adverse intrauterine milieu by optimizing the use of a reduced nutrient supply to ensure survival, but that favoring the development of some organs over that of others leads to persistent alterations in the growth and function of developing tissues” (Hales and Barker 1992, Simmons 2005).

Hales and Barker are focusing on the consequences of malnutrition during development but are not viewing these consequences in the light of adaptations. Gluckman and Hanson further expanded the hypothesis by suggesting that these alterations have adaptive functions and that the fetus programming is an evolutionary strategy to ensure offspring survival. They coin the developmental origin of health and disease and the concept of predicted adaptive response (Parsons) (Gluckman and Hanson 2004, Gluckman, Hanson et al. 2005, Hanson and Gluckman 2008).

The biological “purpose” behind developmental programming is not understood. It is discussed that prenatal plasticity of physiological systems allows environmental factors to alter the set-points, or hardwire the differentiated functions of an organ or tissue system

to prepare the prenatal animal optimally for the environmental conditions *ex utero* (Welberg and Seckl 2001). If the environmental circumstances in later life are not as anticipated, such prenatal programming might produce maladaptive physiology and ultimately predispose to disease (Gluckman and Hanson 2004).

This discussion goes accordingly with the PARs concept stating that the fetus is programmed *in utero* for the environment it will be facing postnatally. PARs is the concept of phenotypic plasticity forecasting the environmental demands based on the intrauterine (or *in ovo*) milieu. When the environment does not match the anticipation, a state of mismatch will occur and the individual will get prone to disease caused by over or under compensations to the environment.

This concept is strong in its belief that the developmental concept urges an adaptive explanation. The consequences might just well be a response to stimuli without a higher cause or a byproduct spawning from constraints the fetus needs to endure during development. The problems mothers-to-be are facing, such as over or under nutrition, sedentary life styles, adiposity, sugar-rich diet and stress are mostly concerns of the modern society of the western world. There simply does not need to be an evolutionary story to these events, it might be just another just-so story.

There is however support for PARs. In insects, females facing the temperature, photoperiod or host availability has been demonstrated affect the probability of diapause in her offspring (Mousseau and Fox 1998). When days get shorter, temperature decreases or hosts become scarce, these are indicators of suboptimal environmental conditions; hence the offspring are entering diapause to endure the upcoming winter. Environmental induced maternal effects have been demonstrated in more than 70 species of insects and similar induced effects on plants have been reported (Mousseau and Fox 1998).

These reports are indicating a short term PARs. The offspring must adapt to the changing environmental demands to survive. However, these organisms have short life spans and no long term developmental programming effects have (or can) been demonstrated. The relevance of these observations in respect to species with long life spans is troublesome and to me it is not convincing that maternal effects as a general rule program the offspring for increased fitness. In insects the plasticity of physiological adaptations to the environment at hatching is crucial and ultimately provide a natural selection pressure of perish or prevail. Due to the short life span, the environmental condition during embryonic development is going to last a substantial period of the insects' life, hence the forecast provide a beneficial trait for survival. Picture an elephant, a blue whale or a leatherback turtle. The embryonic environment only represents a fraction of the total life span and an adaptation to insure those environmental conditions would probably be more detrimental than beneficial in the long run.

2.1 Developmental programming – what do we know in mammals?

An increasing body of knowledge on phenotypic plasticity with deterministic long term consequences has emerged over the last years. Early work focused on finding correlations between neonatal body mass and prenatal constraints and adult disease in massive cohort

studies. Later, more attention has been focused on pinpointing the mechanisms underlying the programming effects, where steroid hormones appears so be of significant importance.

Prenatal stress in rats increases fearfulness and susceptibility to stress (Weinstock 1997) and alter cognitive and motor ability (Braastad 1998). Maternal stress during pregnancy in rats has been shown to feminize male offspring (Ward 1972) and decreases the fertility and fecundity of female offspring (Herrenkohl 1979).

Furthermore, a consequence of maternal stress in rats is an increased anxiety behavior in adult offspring of both sexes (Fride et al., 1986) and reduced learning ability (Vallée, Mayo et al. 1997).

Michael Meaney and co-workers have investigated the effect of maternal nurture quality on stress-coping behavior and hormonal stress response in the offspring. Female rats grooming behavior exhibit a considerable variation seen in pup licking/grooming behavior and arched back nursing. They found that pups provided high nursing quality are less fearful and have a more modest stress response as adults compared to litters from females with poor nursing ability. The hypothalamic-pituitary-adrenal axis (HPA-axis) is altered on many levels including a reduced glucocorticoid plasma concentration in response to acute stress, increased glucocorticoid receptor (GR) messenger RNA expression in the hippocampus, an increased sensitivity in HPA-axis feedback control and decreased levels of corticotropin-releasing hormone (CRH) messenger RNA in the hypothalamus (Liu, Diorio et al. 1997). Furthermore, responses to environmental stressors include cessation of appetite and exploration and increased motivation to escape (Kappeler and Meaney 2010).

Prenatally stressed rhesus monkeys have decreased birth weight, poorer coordination, slower response speed, delayed self-feeding, and more distractible than controls (Schneider 1992) and squirrel monkeys have poorer motor abilities and impaired balance reactions (Schneider and Coe 1993).

Detrimental effects of prenatal stress in humans have been demonstrated by several authors (Stott 1973, Barker 1995, Linnet, Dalsgaard et al. 2003) and fetuses exposed to excessive maternal glucocorticoids might lead to glucose intolerance (Lindsay, Lindsay et al. 1996) and hypertension (Edwards, Benediktsson et al. 1993) .

Synthetic glucocorticoids are administrated to women at risk of preterm delivery and provide an avenue for investigating programming effects of stress hormones. There is a correlation between the administration and attention deficit-hyperactivity disorder (ADHD)-like syndromes. This phenotype seem to be coupled to dopamine signaling suggesting a link between glucocorticoids and the dopamine system, and that the later can be permanently altered by prenatal glucocorticoid administration (Kapoor, Petropoulos et al. 2008).

2.2 Developmental programming – what do we know in birds?

In birds the extra uterine development protects the embryo from detrimental endocrine maternal effect during incubation. Endocrine maternal effects are limited to the egg

formation phase suggesting that the environment during this window might have long term effects depending on the amount of steroid hormones that are incorporated in the egg.

In addition, poor foraging behavior or insufficient foraging time during egg formation can cause eggs of poor quality. This causes the embryo to experience prenatal malnutrition when no compensatory nutrient contribution is possible. The problem arises in species that have an extended egg stage as for precocial species where nutritional limitations are especially acute (Metcalf and Monaghan 2001).

Maternal effects have been studied in birds by multiple authors, which include time to hatch, size at hatching, hatchling growth rate, begging behavior, immune-competence and secondary sexual characteristics (Schwabl 1996, Mousseau and Fox 1998, Mousseau and Fox 1998, Saino, Romano et al. 2005).

Female Barn Swallows produce eggs with elevated corticosterone concentration when exposed to repeated stressor in form of a stuffed cat compared to controls exposed to a stuffed rabbit. Following a physiological infusion of corticosterone in the albumen of freshly laid eggs the eggs had lower hatchability and produced fledglings with decreased body size and slower plumage development than control eggs from the same clutch (Saino, Romano et al. 2005).

Different incubation temperatures affect the development of several characters such as growth rate and corticosterone baseline and stress-induced levels. The negative correlation between body size and plasma corticosterone levels indicates that incubation temperature can influence the HPA-axis (Durant, Hepp et al. 2010).

Prenatal exposures of stress hormones have detrimental effects on growth rate, for example (Metcalf and Monaghan 2001, Eriksen, Haug et al. 2003, Hayward and Wingfield 2004, Durant, Hepp et al. 2010), although some contradictory results suggest that the impact on growth is limited (Janczak, Braastad et al. 2006). Janczak et al did not demonstrate any convincing weight or growth rate difference at hatch or for 1 and 4 week old chicks between control, vehicle and corticosterone treated animals. There was a significant difference between vehicle infused and corticosterone treated animals at 1 week of age (but not compared to control). Otherwise no significant difference was demonstrated (Janczak, Braastad et al. 2006).

Another long term maternal effect is the developmental programming of sex ratio of the progeny. In Collared Flycatchers, *Ficedula albicollis*, the size of the white forehead patch on the mated male correlates to the sex in the progeny. A large patch produces males and a small patch produces females. The size of the forehead patch is a signal of higher quality and hence more attractive to females. The interpretation of these findings suggests that when mated to males with large patches, the female produce more males to increase their female competition ability. When the patch is small it is more beneficial to produce females that due to female choice probably will be mated anyway (Mousseau and Fox 1998).

Pre-incubation injection of corticosterone in chicken eggs caused a significant increase in fearfulness against humans tested by avoidance tests, reduced competitive ability and decreased willingness of crossing a barrier to access a food resource (Janczak, Braastad et

al. 2006). The relevance of the last test is not clear because the mechanisms behind the behavior shift are not determined.

The adaptive purpose of maternally deposited steroid hormones is often assumed, and further discussion often starts with this statement as a general truth. However, the embryo is often view as a passive receiver of the hormones which in turn implies that female plasticity is shaping its progeny for challenges to come (Schwabl 1993). von Engelhardt et al. argue on the evolutionary benefits of maternal steroid effects with the assumption that steroids do have an adaptive function and is not just an epiphenomenon consequence of maternally circulating hormones (von Engelhardt, Henriksen et al. 2009). This assumption further leads to the question if the hormones are adaptive for the mother or for the offspring. The mother benefit from an even distribution of resources among her offspring while in turn the offspring benefit from receiving a larger portion of maternal investment than its siblings. The hormones in itself are not viewed as a resource but their action on development, growth, begging behavior and sibling competition are treated as the adaptive trait. These different strategies cause a mother-offspring conflict but place the mother in the upper hand position in the conflict previously suggested (Schwabl 1993). von Engelhardt et al. implies that the embryo is able to fine-tune the impact of maternal hormones (von Engelhardt, Henriksen et al. 2009).

I question these assumptions. This hypothesis is based on an assumption with no attempt to prove its significance. The key question is if maternal hormones possess an adaptive role or if they are just an epiphenomenon of egg formation. This main question is surprisingly pushed aside for more elaborate stories without a proper consideration. It is beyond doubt that maternal steroids affect embryonic development, but to give these consequences adaptive attribution is jumping too quickly to conclusions.

3 Steroid hormones

3.1 Glucocorticoid functions and effects

Glucocorticoids have two major physiological roles during adult life; an immunological and a metabolic function. As already indicated steroid hormones also exhibit important properties during embryonic development and cognition.

3.1.1 Immunological effects

Glucocorticoids act as an immune response inhibitor by up-regulating the expression of anti-inflammatory proteins and down-regulating the expression of pro-inflammatory proteins. These effects include promoting eosinophil, monocyte as well as T-cell apoptosis, inducing an inhibition of receptor expression and production of cytokines and growth factors (Newton 2000). Glucocorticoids also have an effect on development and homeostasis of T-cells as shown by transgenic mice with either an increased or decreased glucocorticoid sensitivity (Pazirandeh, Xue et al. 2002). Furthermore, glucocorticoids exhibit an inhibitory role in Ca^{2+} independent NO vascular release acting on gene level blocking the expression of the enzyme inducing the vasodilating effect (Ross, Marshall-Jones et al. 1966, Radomski, Palmer et al. 1990). The multifaceted immune response

inhibition marks glucocorticoids to be one of the most potent agents to battle autoimmune diseases.

3.1.2 Metabolic effects

As the name imply, glucocorticoids have a key function in elevating plasma glucose levels (Ross, Marshall-Jones et al. 1966). During hypoglycemic episodes the glucose concentration in the blood stream is increased to ensure homeostasis and several key intermediates in the process is under glucocorticoid control.

Glucocorticoids trigger both the release and increase the availability of metabolic fuels and to convert these metabolites into glucose by gluconeogenesis. Glucocorticoids promote the release of amino acids by striated muscle breakdown and stimulate adipose tissue breakdown to release glycerol from triglycerides, both which are used predominantly in the liver as metabolic fuels (Silverthorn et al. 2009).

Gluconeogenesis involves numerous intermediates produced by enzymatic activity. A well-known glucocorticoid action is the enhancing of the expression of these enzymes. The result in an increased glucose concentration in the plasma restoring the low blood sugar values to normal. Phosphoenolpyruvate carboxykinase , a key enzyme in the gluconeogenesis, is under glucocorticoid regulation (Friedman, Yun et al. 1993) pointing out the crucial role of glucocorticoids. One example of its activity is for CRH deficient mice, who develop hypoglucemia after 24 h of prolonged fasting when controls are able to maintain constant glucose levels throughout the fast (Muglia, Jacobson et al. 1995).

3.1.3 Arousal and cognition

Together with adrenaline, glucocorticoids are important in the formation of flashbulb memories, i.e. memories created during events associated with both positive and negative strong emotions. Steroids act on the amygdala, hippocampus and frontal lobes and when either glucocorticoids or noradrenalin activity is blocked it results in impaired recall of emotionally relevant information (Cahill and McGaugh 1998). Furthermore, studies conclude that creation of memories during fearful events accompanied by high cortisol levels had better consolidation of this memory, and also enhanced cognitive performance and vigilance. However, the relationship between improved memory capacity and cortisol levels follows a U-shape curve with optimal performance at medium cortisol levels decreasing with either increased or decreased plasma concentration demonstrated by drug administration or adrenalectomy (Lupien, Maheu et al. 2007).

3.1.4 HPA-axis

Hypothalamic paraventricular nucleus (PVN) controls the pituitary-adrenal activity limb of the HPA-axis, and PVN is in turn under negative feedback control from the limbic system, the hippocampus in particular. Parvocellular neurons in the PVN synthesize CRH and arginine vasopressin (AVP) which are secreted into the hypophyseal portal circulation and stimulate the release and synthesis of adrenocorticotropin hormone (ACTH) in the anterior pituitary gland. ACTH then triggers secretion and synthesis of cortisol (or corticosterone) from the adrenal cortex. The cascade release of glucocorticoids is regulated by a feedback system where glucocorticoids via GR and

mineralcorticoid receptors (MR) in the hippocampus and GR at PVN and anterior pituitary inhibit HPA activity (Kapoor, Petropoulos et al. 2008).

3.1.5 Developmental effects

Glucocorticoids are necessary during tissue development. An important example is their role in promoting maturation of the lung and production of the surfactant necessary for extrauterine lung function. Mice with homozygous disruptions in the CRH gene die at birth due to pulmonary immaturity, and feeding the homozygous knock-out female with corticosterone abolish the corticosterone deficiency effect on lung development (Muglia, Jacobson et al. 1995).

Glucocorticoids are also crucial for normal brain development by initiating terminal maturation, remodeling axons and dendrites and affecting cell survival (Lupien, Maheu et al. 2007). Steroids are also involved in the maturation of the HPA-axis demonstrated by perinatal glucocorticoids that permanently affect the HPA-axis response to stress (Meaney, Aitken et al. 1988).

7 % of all pregnant women in North America suffer from preterm delivery, and 75 % of all neonatal deaths in America are accredited to these occurrences (Kapoor, Petropoulos et al. 2008). Glucocorticoids are routinely used to manage these pregnancies to promote lung development, and in rarer cases it is associated with antenatal treatment of fetuses at risk of congenital adrenal hyperplasia (Seckl 2004).

4 Egg properties

The yolk is the nutrient store for the developing embryo and is the major source of vitamins and minerals. It contains all of the egg's fat and cholesterol, and about one-fifth of the protein. The chicken yolk consists of 16 % proteins, 26.5 % fat, 3.5% carbohydrates and 52% water (SDA National Nutrient Database for Standard Reference, Release 23 2010).

The albumen is the clear liquid (with the common name egg white or the glair/glaire) contained within an egg. It is the cytoplasm of the egg, which until fertilization is a single cell (including the yolk). It consists mainly of about 15% proteins dissolved in water. Its primary natural purpose is to protect the egg yolk and provide additional nutrition for the growth of the embryo, as it is rich in proteins (11%) and also of high nutritional value. Unlike the egg yolk, it contains a negligible amount of fat and carbohydrates (0,17% and 0,73% respectively) (SDA National Nutrient Database for Standard Reference, Release 23 2010).

4.1 Maternal deposit of corticosterone in the egg

The primary glucocorticoid in humans and most vertebrates is cortisol. However, in rodents, reptiles and avian species the steroid corresponding to a stress response is corticosterone (deRoos 1961).

In mammals the fetus can be exposed to hormones originating from the maternal circulation. Indeed stress hormones are found in the fetal circulation but maternal

glucocorticoid levels are 5 to 10 times as high as in the fetal circulation. The difference in circulating concentrations has been attributed to 11 β -hydroxysteroid dehydrogenase (11HSD), an enzyme positioned in the placenta that catalyzes maternal glucocorticoids to inert 11-keto products protecting the fetus from high stress hormone levels (refs 20 and 21 in (Nyirenda, Lindsay et al. 1998). By reducing the activity of 11HSD birth weight of the progeny is reduced and subsequently hyperglycemia in adults have been demonstrated (Seckl 1997).

Dexamethasone is a synthetic glucocorticoid with low substrate potential for feto-placental 11HSD type 2. When administered to pregnant rats selectively in the last week of gestation it results in progeny displaying reduced birth weight and in adult offspring fasting hyperglycemia, post glucose hyperglycemia and hyperinsulinemia (Nyirenda, Lindsay et al. 1998). These responses were only visible if the drug was administered during the third week of gestation (Nyirenda, Lindsay et al. 1998) suggesting the developmental window for when the fetus is prone to metabolic developmental programming by glucocorticoids.

In birds some evidence suggests that elevated stress hormone levels in the female circulation might mimic some, but not all, of the effects seen in mammals (Lay Jr and Wilson 2002) but Lay and Wilson only treat the chicken as a model for prenatal stress without regards of any maternal effects. Corticosterone is lipophilic, hence maternally produced corticosterone can be incorporated into developing poultry egg yolk and albumen (Eriksen, Haug et al. 2003, Hayward and Wingfield 2004) with the assumption that carrier proteins are available. Do we then have a maternal effect homologous to mammals?

The levels of steroid hormones in the egg vary both within- and between clutches in relation to a range of factors; such as position in the laying order, food availability, season, quality of the male or density and social interactions and have both short- and long-lasting effects on offspring (Schwabl 1993, Gil 2003, Gil, Heim et al. 2004, Groothuis, Müller et al. 2005, von Engelhardt and Groothuis 2005, Groothuis and Schwabl 2008, Moore and Johnston 2008)

The corticosterone serum concentration in laying hens varies from 0.8 ng/ml during night to maximum of about 1.5 ng/ml in the middle of the light cycle (de Jong, van Voorst et al. 2001). During acute stress, corticosterone concentration in hen plasma varies from 3.4 to 26.8 ng/ml when measured 5 min after exposure to stressor (Eskeland and Blom 1979). Plasma corticosterone concentration in hens peak 10 minutes after a stressor (handling) and are returning to baseline after approximately 45 minutes (Downing and Bryden 2008). But is this circulating corticosterone incorporated in the forming egg?

Indeed, the serum concentration found in adult hens is similar to that found in the yolk and albumen of eggs from unstressed laying hens varying between 1.17 and 1.55 ng/ml in unfertilized eggs (Eriksen, Haug et al. 2003). In cage hens exposed to moderate stress in form of weighing, the plasma concentration was found as high as 1.8 (mean 1.5 ng/ml) in the albumen (ongoing study Janczak et al., personal communication in (Janczak, Braastad et al. 2006))

Hens kept at 30°C produced eggs with significant higher albumen concentrations than birds kept in 18°C. An elevation of albumen corticosterone are obvious up to four days after an insult (in this case moving from single cages to either a cage containing 5 birds, 2 birds or singles not moved as controls) (Downing and Bryden 2008).

The corticosterone levels in blood plasma in the Japanese quails were chronically elevated by an implant increasing levels from 1.28 to 11.68 ng/ml which corresponded to an increase in the eggs 7 days after implantation from 0.92 to 2.06 ng/ml (Hayward and Wingfield 2004). All these findings suggest that maternal corticosterone might be incorporated in the egg and hence influence the development of the offspring.

However, there are a few questions regarding embryo development in response to maternal hormones that needs to be addressed (see also under page 10 in this report). Numerous investigations suggest the presence of maternal corticosterone in the egg but it has been criticized as corticosterone levels in the egg has not been properly investigated or proven and is merely an assumption (Rettenbacher, Möstl et al. 2005). Little is known how stress hormones get incorporated in the egg and according to Groothuis et al. (Groothuis and Schwabl 2008) the only evidence of corticosterone in eggs rely on immunoassays that might detect structurally similar substances other than corticosterone (Rettenbacher, Möstl et al. 2005). More is known about sex hormones but they are secreted and synthesized by the ovary and not by the adrenals as with corticosterone (Rettenbacher, Möstl et al. 2005). Androgens have been thoroughly investigated with mass spectrometry and its presence in the egg been clarified (Schwabl 1993). Also the presence of gestagens has been satisfactory clarified (Möstl, Spendier et al. 2001, Rettenbacher, Möstl et al. 2009). The use of high-performance liquid chromatography (HPLC) showed no traces of corticosterone suggesting that in an untreated egg the hormone is only present in trace amount, if at all (Rettenbacher, Möstl et al. 2005). This study was repeated and once again no traces of corticosterone were found. The earlier publications relying on immunoassays have been biased by cross reactivity with progesterone and its precursors, probably 5 α - and 5 β -pregnanes and pregnenolol (Rettenbacher, Möstl et al. 2009).

The ovaries are the main producer of sexual hormones which provide a possibility of incorporation by the vicinity of production and egg formation. Corticosterone is produced by the adrenal glands and must rely on the blood circulation and carrier mechanisms to reach the forming egg in the ovary.

The three cell hypothesis is suggesting that the steroids produced by the ovary are synthesized by three different tissues. The granulosa cells is the predominant source of gestagens which is converted to androgens by theca interna which in turn is metabolized to estrogens by theca externa (Porter, Hargis et al. 1989). During the follicle maturation the production of the different steroids changes because of the changing production capacities of each synthesizing group of cells. At later stages aromatase activity is decreased and estrogen is no longer produced (Kato, Shimada et al. 1995) and in the large preovulatory follicle cells the enzymatic activity shuts down completely (Etches and Duke). Besides entering the blood stream from where they act as signals of reproductive

status they end up in the developing follicle as well, either by passive diffusion or by unknown mechanisms (Groothuis and Schwabl 2008).

If this temporal and spatial separation of the hormone synthesis is true, the consequences should be seen in the distribution and concentrations of hormones within the yolk. It has been demonstrated that at laying, different yolk layers contain different concentrations of steroids ((Elf and Fivizzani 2002, Hackl, Bromundt et al. 2003, Groothuis, Müller et al. 2005). If the yolk is analyzed in concentric layers gestagens are mostly found in the outer layers, androgens in the middle layers and estrogens in the center of the yolk. Furthermore, the highest concentrations is found of gestagens, the lowest of estrogens and androgens is found in middle amount (Lipar, Ketterson et al. 1999). These findings support the three cell hypothesis as well as suggesting that the incorporations of steroids are due to passive diffusion from producing cells in the oocytes vicinity. This further suggests that corticosterone, produced by the adrenals and hence is depending on the circulatory system to reach the ovary, is not incorporated in the chicken egg (Rettenbacher, Möstl et al. 2009).

Subcutaneous injection of radioactive labeled corticosterone in egg laying hens caused a significant increase of corticosterone in the albumen. The correlation demonstrate that plasma corticosterone concentration are mirrored by the egg albumen, but with a fivefold higher plasma concentration than albumen concentration (Downing and Bryden 2008). However, in this study they measure radioactive decay in blood plasma and egg albumen, but not the hormone itself. They fail to demonstrate that it is not corticosterone metabolites they measure in the egg.

Furthermore, hormones deposited into the egg before embryonic development onset has to be absorbed by the developing embryo. It is unclear how the highly lipophilic steroids are extracted from the yolk by the embryo (Moore and Johnston 2008) and this will also be address later in this report.

Cortisol half-life is reported to be 101.6 minutes in healthy human subjects following a 100 mg intravenous injection (Melby and Spink 1958), and 102 minutes following a 20 mg intravenous injection (Tunn, Mollmann et al. 1992) and around 60 minutes in puberty male subjects (Kerrigan, Veldhuis et al. 1993). Any maternally incorporated glucocorticoids in the egg might therefore be degraded before it has a developmental effect. However, the degradation is probably generated by enzymatic activity and therefore under enzymatic control. Before these enzymes are expressed there is a possibility that these hormones can survive for a long period of time.

5 Embryonic endogenous corticosterone production, steroid receptors and steroid metabolism

The embryo must possess the ability to respond to maternal hormones by establishing battery pathways including receptors, second messengers, DNA binding sites etc. Indeed the presence of estrogen and androgen receptors have been demonstrated as early as day 4 of development demonstrated by mRNA detection (Andrews, Smith et al. 1997, Smith,

Andrews et al. 1997) and immunohistochemically using antibodies against progesterone receptors (Andrews, Smith et al. 1997). Endogenous steroid hormones appear after these receptors are present (see (Bruggeman, Van As et al. 2002) which suggest that these receptors are expressed as a physiological adaptation to maternal hormones.

Furthermore, the embryo may produce hormone-metabolizing enzymes that inactivate or modify the activity of maternal hormones. Steroid metabolism by the embryo or the extra-embryonic membranes (yolk sac, allantois, amnion, chorion) has been observed in all species studied so far (fish, amphibians, reptiles, birds and mammals) (see (von Engelhardt, Henriksen et al. 2009). Enzymes or mRNA for enzymes that convert steroids have been found as early as day 2 of incubation (Nomura, Nakabayashi et al. 1999), Woods and Weeks, 1969). Parsons found that testosterone is clearly metabolized by day 2 blastoderm to mainly 5- β -reduced androgens (Parsons 1970). Antila et al. 1984 found in 2 day old blastoderm *in vitro* studies metabolites from an array of steroids suggesting that the early embryo possess steroid metabolizing enzymes with a more general ability of steroid degradation (Antila 1984).

During early development there is a clear conversion from apolar to polar entities of steroid hormones (corticosterone and testosterone) suggesting that the steroids conjugate with each other but the actual substances are not identified (von Engelhardt, Henriksen et al. 2009). The authors suggest that the metabolizing enzymes do not stem from a maternal origin but rather of an endogenous one based on the assumption that hormones of maternal origin would be evenly distributed in the yolk (although as already described the different yolk layers contain different amount of hormones). The highest proportion of polar metabolites was found in the yolk sac tissue and in the sub-embryonic tissue and lesser amounts was found further away from the embryo. Furthermore, the increase of hormones found in the albumen is suggesting that polar metabolites diffuse or transports from the yolk to the albumen (von Engelhardt, Henriksen et al. 2009). A study performed using turtles demonstrated that radioactive estradiol administered to the egg shell at laying was recovered by the albumen and yolk but not to the embryo within a few days of incubation. It was converted to more polar compounds that could be detected in the yolk, extra-embryotic membranes and the embryo but not the albumen (Paitz and Bowden 2008)). It is suggested that the polarity helps the absorption of the molecule by the embryo.

In conclusion, the hormones are metabolized to unknown compounds, but the original hormone remains as well at day 6 of development, especially in the yolk (von Engelhardt, Henriksen et al. 2009).

Cortisol, produced by embryos from about day 9, is converted *in vitro* primarily to 20- β -dihydrocortisol by the chorioallantoic membrane of 6- to 7-day-old embryos (McNatt, Lane et al. 1992). Is cortisol present in the egg then?

With this body of knowledge it is clear that the embryo has a steroid metabolizing machinery operating already from the second day of incubation. Antila et al (1984) suggest that the blastoderm is capable of a general degradation but to my knowledge no one has identified the responsible enzymes. In mammals the 11 β -hydroxysteroids

corticosterone and cortisol degradation are governed by 11HSD which convert these steroids to the hormonally inactive 11-oxosteroids cortisone and 11-dehydrocorticosterone respectively. There are two isoforms described, the 11HSD1 (NADP⁺(H)-dependent) and 11HSD2 (NAD⁺-dependent) which has a Michaelis-Menten constant in the range of micromolars for 11HSD1 and in nanomolars for 11HSD2 (Nyirenda, Lindsay et al. 1998, Kučka, Vagnerová et al. 2006). In non-mammalian species only a few studies have been performed suggesting similar mechanisms in toads and teleost fish. In contrast, birds have been shown to, beside 11HSD activity, also possess 20-hydroxysteroid dehydrogenase (20HSD) that convert glucocorticoids to 20-derivatives (Kučka, Vagnerová et al. 2006). In chickens 20HSD has demonstrated activity in numerous tissues including the uterus, magnum, ovary, testis, brain, liver and predominantly in the intestine and kidney. 11HSD activity is absent in the ovary, testis and brain, modest activity in uterus, magnum, intestine and liver and predominantly in the kidney (Kučka, Vagnerová et al. 2006). We do not know if these hormones also are functional during embryonic development but as there is steroid metabolism one might assume so.

In the chicken the adrenal gland becomes able of independent secretion from day 8 until day 14. At this point the pituitary and hypothalamus begin to influence adrenocortical corticosterone secretion and sympathetic innervation of the adrenal occurs. After 11 days of development negative feedback of adrenal glucocorticoids on pituitary ACTH release becomes functional. Glucocorticoid secretion appears independent of ACTH until 14 days of incubation. Two peaks of glucocorticoids appear during development, one at day 14-16 and one at hatching (Jenkins and Porter 2004).

The timing of HPA-axis development is species dependent and is coupled to the maturity of the progeny at birth. Mammals with highly developed young (guinea pig, primates, pig etc.) have at birth a well-developed brain where the maximal brain growth takes place in utero. In species with immature progeny at delivery (mice, rat, rabbit) a greater proportion of brain development and neuroendocrine maturation occurs in the postnatal period (Kapoor, Petropoulos et al. 2008). With this perspective in mind it is worth noticing the use of altricial and precocial avian species. The chicken is a precocial species and hence more similar to primates and pigs while the rodents and hares resemble the altricial bird species. Therefore, in the light of hatch and birth maturity the chicken model might be more suitable than mice?

As many of the maternal effects by the glucocorticoid pathway in mammals appear late in gestation this further suggests the intriguing but highly speculative idea that corticosterone is not important during early development in chickens, and hence maternally incorporated hormones in the egg is of limited use. To push even further this might suggest that fetal exposure to maternal glucocorticoids is a tradeoff for intrauterine development. As viewed today this trait must be beneficial, but many studies demonstrate detrimental effects. Perhaps hormone transport from mother to fetus is of no evolutionary adaptation value and birds, reptiles etc by their extra uterine development are blissfully protected from detrimental maternal effects?

5.1 Manipulation of corticosterone concentrations in the egg

Injection of corticosterone requires a lipid vehicle because of the lipophilic character of the hormone. Such vehicle is usually sesame oil. An injection in the middle of the egg will not stay stationary but migrate due to the lower density of sesame oil upwards along the yolk and cause a droplet close to the developing blastoderm (von Engelhardt, Henriksen et al. 2009). This raises concerns that calculated injected concentration might be an overestimate because the oil will not evenly distribute in the egg. The infused vehicle of the hormone will instead travel to the developing embryo facing it with a high dose of the hormone.

Von Engelhardt et al demonstrate some interesting things in their study of 2009. First, the injected hormone does not diffuse evenly in the egg, albumen or yolk. By day three the hormone is mostly present in the upper yolk, and modest amount in the yolk sac and embryo. By day 6 the hormone was distributed in the whole egg with highest concentrations in the yolk sac followed by the upper yolk. Some was found in the albumen, especially the upper part but also in the allantois, amnion and embryo. Second they found that infused labeled hormones were present in the 3 day old embryo suggesting (for the first time) that an early embryo does take up maternal hormones.

Rodricks et al describes an alternative rout of drug administration suggesting that a single dose of corticosterone delivered onto the chorioallantoic membrane seem to be a good way of administration (Rodricks, Miller et al. 2006). Rettenbacher et al. used several avenues of corticosterone administration to quantify the amount of maternal hormones subsequently incorporated in the egg. They infused tritium-labeled corticosterone into the vena cutanea ulnaris, ACTH infusion to stimulate adrenocortical activity and fed hens with one dose corticosterone (Rettenbacher, Möstl et al. 2005).

Less than one percent of the radioactivity-labeled hormone entered the egg (around 0.42 % in the yolk and 0.25 in the albumen) with maximum amount of albumin corticosterone immediately after administration slowly moving inwards through the layers in the yolk over the subsequent 10 days. The same amount was recovered after radiometabolism studies suggesting that the maternal transfer of corticosterone is very low (Rettenbacher, Möstl et al. 2005). Similar pattern of distribution following an intramuscular injection of radioactive labeled steroids was previously found by Hackl et al (Hackl, Bromundt et al. 2003).

Administration of ACTH had no effect on yolk corticosterone concentration, and orally administrated corticosterone resulted in an increase in the yolk, though of minor quantity (Rettenbacher, Möstl et al. 2005).

These findings imply several interesting aspects of corticosterone induced developmental programming. First, offspring from the same clutch from the same female differ in aspect of corticosterone exposure and therefore the environment *in ovo* is based on laying order from a stressful insult. If corticosterone is administrated the formation stage of the egg decides where the stress hormone will be incorporated. If the egg is close to ovulation the hormone will be found mostly in the albumen, and if injection takes place 3 days prior ovulation the concentration will be higher in the yolk. Second, the findings suggesting that less than 1 percent of corticosterone administrated to the hen (these levels were

pharmaceutical and not physiologically relevant) is transferred to the egg and no changes of corticosterone concentration followed an ACTH injection. Finally no corticosterone was found in control eggs with the use of HPLC. Combined, these observations strongly question the relevance of corticosterone in a natural, not pharmacological, situation for the development of the chicken embryo.

There are as I see it three ways to decrease the artificial impact of corticosterone manipulation in the egg. First, as described in von Engelhardt et al 2009 the injected droplets will end up on top of the yolk in the same area as the embryonic disc. If the egg is positioned with the air cell down during the first days of development the supraphysiological exposure of corticosterone to the developing embryo is minimized. Second, injection of corticosterone is performed in the eggs in extensive time before put in the incubator. The authors have injected the radioactive labeled steroids just prior to incubation onset and therefore not allowing the hormones to disperse by passive diffusion. Third, the authors have injected the steroid-containing vehicle directly in the albumen. This is an avenue for infection (although described as limited), and also provides a “point” distribution of the steroid. If the vehicle is applied on top of the air cell, or as described by Rodricks et al 2006 on the chorioallantoic membrane, the concentration gradient and the high concentration close to the developing embryo will be avoided. The problem with this technique is the absorption of the steroid by the embryo. During the first days of development the embryo will not be exposed to the steroid because of diffusion time and lack of, or premature chorioallantoic membrane.

6 Critical windows of development: where and when do things happen?

The appearance of the different parts of the HPA-axis machinery is important to pin-point when insults during ontogeny have crucial effects. First the embryo must be susceptible to circulating glucocorticoids. In mammals glucocorticoids can be of either endogenous or exogenous origin and maternal transfer is possible by the time the placenta is developed. In birds the absence of maternal corticosteroid in the egg limit the embryo to steroids only of endogenous origin.

GR are expressed as early as 9.5 days of development in mouse, and gene disruption by homologues recombinant in the mouse stem cell impaired development of lungs, liver and adrenal functions, and the majority died after birth (Cole, Blendy et al. 1995) Many vertebrate species demonstrate a short burst of androgens around birth. This secretion permanently program a set of phenotypes including steroid metabolizing enzymes in the liver, the size, connection and neurochemistry of specific hypothalamic nuclei, and some sexual behavior (Seckl 2004) . The hormones are not limited to androgens, also estrogen has organizational programming effects on the central nervous system (Seckl 2004)

This hormonal control of tissue can only be exerted during specific perinatal periods but the effects last during the whole life span regardless of subsequent sex steroid manipulation (Seckl 2004). For example in rat, the sexually dimorphic nucleus of the preoptic hypothalamic area is governed by testosterone. This area is larger in males, and

during postnatal day 6 to 10 testosterone inhibit cell apoptosis selectively for this locus producing this phenotypic difference (Seckl 2004).

Growth hormone-secreting cells in the pituitary have been demonstrated to be under corticosterone control in chicken embryos. By embryonic day 11 an increase in corticosterone achieved by injection increased the number of growth hormone-secreting cells two days later. If the administration was performed prior to day 11 of development no changes could be seen on cell numbers illustrating the critical window for corticosterone-induced proliferation of pituitary cells (Bossis and Porter 2000).

Chicken fetuses experiencing hypoxia get impaired memory ability and the timing of the insult is of importance. Embryos exposed to 14% hypoxia on day 10 of development get significantly affected short-term memory and subsequently long-term memory, while embryos exposed on day 14 of development possess an intact short-term memory ability and only affected short-term memory (Rodricks, Rose et al. 2004). These results were mimicked by an *in ovo* corticosterone injection at day 10 and day 14 of development, and corticosterone concentration elevation in the circulation was demonstrated showing that corticosterone is responsible for the deviant memory in prenatal hypoxic chickens (Rodricks, Miller et al. 2006). Hypoxic or corticosterone injection insults before day 9 or after day 15, or day 11 or 12 of development did not affect memory (Rodricks, Rose et al. 2004, Rodricks, Miller et al. 2006).

A similar study demonstrated no memory effects when administrated on day 17 or day 18, but when administrated at day 19 or 20 memory was enhanced rather than reduced (Sui, Sandi et al. 1997)

In the chicken the adrenal gland become able of independent secretion from day 8 until day 14, at which point the pituitary and hypothalamus begin to influence adrenocortical corticosterone secretion and sympathetic innervations of the adrenal occurs. After 11 days of development negative feedback of adrenal glucocorticoids on pituitary ACTH release becomes functional. Glucocorticoid secretion appears independent of ACTH until 14 days of incubation. Two peaks of glucocorticoids appears during development, one at day 14-16 and one at hatching (Jenkins and Porter 2004).

6.1 Corticosterone function in embryonic development and developmental programming

Fetal cortisol levels are elevated in human intrauterine growth retardation (Goland, Jozak et al. 1993) and administration of glucocorticoids during pregnancy reduces birth weight in mammals and humans (Reinisch, Simon et al. 1978).

The impact of maternal care on HPA-axis control have been thoroughly investigated in rats and briefly discussed earlier in this thesis. Pups nursed by females exhibiting high licking/grooming behavior express an increased stress coping ability manifested by an enhanced negative feedback-loop control of corticosterone secretion. High maternal grooming quality increase GR expression in the hippocampus which enables a stronger feedback response. The GR gene is under epigenetic modulation. Several cytosine residues are methylated modulating the transcription of the gene, and it seems that the

behavior from the mother, by a still unknown enzymatic pathway, strip the promoter region of the GR gene from its methyl group. These effects have been demonstrated being chronic, and cross-fostering of pups from high licking/grooming mothers reared by low licking/grooming have clearly shown that these effects have a maternal and not a genetic origin (Kappeler and Meaney 2010).

CRH is critical in lung development. Progeny from CRH-deficient homozygous knockout mice died within 12 hours postnatally by lung dysplasia, whereas heterozygous control survived. Homozygous progeny from heterozygous parents survived as well indicating that maternal corticosterone pass over the placenta and compensate for deficient endogenous corticosterone production. This was also demonstrated by feeding the homozygous knockout female during gestation with corticosterone abolishing the corticosterone deficiency effects on lung development (Muglia, Jacobson et al. 1995). Also, adult knockout mice survived suggesting that CRH is important during fetal development but not during adult life (Muglia, Jacobson et al. 1995).

To me this is saying that it is crucial for survival during fetal development but not during adult life. Saying that CRH is lacking importance seems like an over statement because it also implies that a HPA-axis response is of no use.

Corticosterone baseline levels in Magellanic Penguin's chicks were investigated from an undisturbed and a tourist visited area. Incubation disturbance and exposure of young chicks from habituated tourist-visited birds are less frequent than in birds breeding remote from human disturbances. Interestingly the newly hatched chicks from tourist-accustomed birds demonstrate a significant lower corticosterone baseline than from undisturbed areas suggesting that intermittent incubation increases corticosterone levels in the chicks (Walker, Dee Boersma et al. 2005).

Starling chicks demonstrate a significant higher level of stress-induced free corticosterone by parentals exposed to stressors during incubation in comparison to control parental incubation (Cyr and Michael Romero 2007). The chronic stress stretched over 8 days during incubation where a rotating scheme of four stressor daily spaced by 1-2 h causing the brooding female to abandon the nest and exposing the eggs for up to 30 minutes.

At the beginning of the egg laying period, barn swallows were subjected to a stressor (a stuffed cat) for 2 hours in the afternoon (egg white takes approximately 10-16 hours to form around the yolk prior laying, and laying is performed during morning hours) and corticosterone was measured in the albumen. Corticosterone concentration was significantly higher in eggs laid by stressed females than controls relative the concentration in the first laid egg (prior to the stressor). Body mass was significantly lower in stressed offspring and significantly different in laying order (Saino, Romano et al. 2005). The data however seem a bit obscure because in figures no significant differences are pointed out, and by ocular inspection the results indicate no differences. The whole story is weak in the light of the critique against maternally incorporated stress

hormones in the egg and without stronger support once again birds seems resilient to maternal stress.

7 Transgenerational effects

Transgenerational effects of synthetic glucocorticoid administration have been demonstrated in mice. F1 females whose mothers were treated with dexamethasone during the last week of gestation were paired with male experienced with the same treatment produced offspring with reduced birth weight in comparison to controls. The hepatic phosphoenolpyruvate carboxylase, the rate limiting enzyme in the gluconeogenesis, was significantly increased in F2 males, and consistently, glucose levels was significantly elevated in F2 males (Kapoor, Petropoulos et al. 2008).

For these findings, two explanations are presented. The first involves altered maternal endocrine cardiovascular adaptation to pregnancy in the F1 offspring and this would in turn lead to altered endocrine regulation. The second involves epigenetic mechanism as the explanatory foundation.

The later get supported by the participation of paternal transmission of transgenerational effects of antenatal synthetic glucocorticoids (Kapoor, Petropoulos et al. 2008).

Also it had been demonstrated that epigenetic markers are not completely erased during gametogenesis, opening an avenue of phenotypic variability not explained by either genetics or environment (Drake, Tang et al. 2007, Kapoor, Petropoulos et al. 2008).

A beautifully designed study demonstrated the linkage between maternal environment and developmental programming by epigenetic patterns. Pregnant rats were exposed to protein restriction which initiated a methylation reduction of the hepatic GR promoter and a corresponding increase hepatic GR expression in the offspring (Lillicrop, Phillips et al. 2005).

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