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Long-term effects of prenatal exposure to high-fat diet in an animal model of reduced oxidative stress

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ABSTRACT

Obesity is a world-wide health problem. The increasing incidence of this pathology among women of reproductive age and children highlights the main question of the role played by maternal obesity in setting up a state of individual susceptibility to metabolic disorders in the adult offspring. A growing body of evidence suggests that maternal obesity and maternal adiposity, *per se*, are associated with adverse acute maternal and neonatal outcomes. It has been stated that maternal obesity and, more in general, maternal over-nutrition, such as maternal feeding a diet rich in fats (high-fat diet - HFD), are strongly associated with a raised offspring susceptibility to cardiovascular disease (CVD) and metabolic impairment, such as hyperglycaemia and hyperinsulinemia, type 2 diabetes (T2D) and obesity at adulthood. Obesity is a pathophysiological condition characterized by a low-grade chronic inflammation which contributes to the mechanism for obesity-related metabolic syndrome, insulin resistance (IR) and diabetes. Increased physiological levels of oxidative stress (OS) may be causatively linked to the development and progression of complications associated to obesity and over-nutritional state.

Clinical as well as experimental studies support the theory of the "developmental origins of health and disease" according to which the prenatal environmental adversities may exert lifelong consequences by programming the offspring's development. However, a comprehensive and multi-levels characterization of the long-term impact of maternal HFD is still lacking. In addition, very few studies have paid attention to the long-term effects of exposure to a maternal HFD during the *in utero* life only. The **general aim** of the work presented in this thesis was to evaluate the impact of maternal HFD both on mother and offspring in a long-term perspective and to study the effects of metabolic disturbances occurring in a sensitive time window of the individual life (i.e. fetal development and pregnancy) on the stress response, as well as on the metabolic and emotional profiles. We also aimed at investigating the interaction between an early metabolic stimulus (maternal HFD) and a physiological condition of reduced OS.

In particular, in **Chapter 2**, we report about the long-term effects of prenatal exposure to maternal HFD assessing the metabolic, neuroendocrine as well as the behavioral profile of young adult offspring in a transgenic mouse model characterized by reduced oxidative stress, the $p66^{Shc-/-}$.

In **Chapter 3** we report the effects of a prenatal exposure to N-acetyl-cysteine, a drug characterized by remarkable antioxidant properties, to thwart the physiological response to maternal HFD *in utero*. These studies relied on the assessment of a physiological (Chapter 2) or pharmacological (Chapter 3) condition of reduced OS able to represent a protective condition toward the maternal HFD.

Chapter 4 describes the effects of HFD feeding before and during pregnancy on the neuroendocrine and behavioral profile of dams in the perinatal

period. In addition, in this chapter we propose to investigate the potential role of HFD as a metabolic stressor for the mother with a negative impact for the developing fetus.

Results are discussed considering the impact of HFD feeding during pregnancy on the dam and the possible consequences on the offspring at multiple levels (behavioral, metabolic and endocrine). Particular attention has been paid to a physiological or pharmacological condition of reduced OS as a potential protective factor. Results obtained in this thesis might drive the attention on selected markers useful to detect potential unhealthy states and to develop future preventive strategies as well as pharmacological therapies to limit the effects associated to maternal HFD.

CHAPTER 1

1. GENERAL INTRODUCTION

1.1. RELEVANCE OF OBESITY IN MODERN SOCIETY

Obesity is one of the major health care issues of the 21st century reaching epidemic levels (Ezzati et al., 2005; Hurt et al., 2010). It has been described as a metabolically inflexible state defined as impaired regulatory response to metabolic challenges such as fasting, exercise and overfeeding (Storlien et al., 2004; Phielix and Mensink, 2008; Corpeleijn et al., 2009). Obesity is associated with a markedly elevated risk of type 2 diabetes (T2D) (Pirkola et al., 2010a; Pirkola et al., 2010b), metabolic impairment, such as hyperglycaemia, hyperinsulinaemia and hyperleptinaemia (Weissgerber et al., 2006), in addition to raised blood pressure and impaired cardiovascular function (Khan et al., 2003; Khan et al., 2005), thus resulting in increased risk for cardiovascular disease (CVD). Besides representing a problem in developed countries, overweight and obesity are dramatically increasing in the population of low- and middle income, especially in urban areas (Han et al., 2010; Nelson et al., 2010) and are expected to almost double in the next decade (Kim et al., 2007). In addition to the general incidence, obesity is a major public health concern for women of reproductive age, for which has been registered an increasing global prevalence (WHO, 2000). Currently, 13% of 21- to 30-year-old women and 22% of 31- to 40-year-old women in the UK are obese, with figures estimated to rise to 30 and 47% respectively by 2050 (FORESIGHT, 2001; Mitchell and Shaw, 2014).

1.1.1. Maternal obesity is a predisposing factor for the incidence of obesity

The overall increase in overweight and obesity naturally leads to a disturbing increase in the incidence of weight and metabolic problems in pregnant women. Maternal obesity is indeed associated with adverse acute maternal and neonatal outcomes and the long-term consequences of maternal obesity in pregnancy have large implications both for the mother and her offspring (Martorell et al., 2001; Boney et al., 2005; Catalano and Ehrenberg, 2006; Wardle et al., 2008; Pirkola et al., 2010a; Pirkola et al., 2010b; Dabelea and Crume, 2011). Actually, a particularly alarming increasing rate of obesity has also been registered among children and adolescents, thus driving particular attention on the understanding of the underlying causes. A central dogma to obesity development is that the individual environment is crucial and determines lifestyle choice resulting in an energy imbalance so that caloric intake exceeds caloric expenditure. In this perspective, a sedentary lifestyle and wrong dietary habits can worsen the situation, especially in individuals who already have a tendency to obesity (Hill and Peters, 1998; Swinburn et al., 2004). In addition, despite the relevance of lifestyle, the identification of familial risk factors for obesity (Lee et al., 1997) is suggestive of genetic factors contributing to obesity development (Wardle et

al., 2008). Notwithstanding the recognized role played by genetic factors in the development of obesity, these alone cannot account for the tripling in the prevalence of overweight and obesity over the past 3 decades (Ogden et al., 2006). It is more likely that environment and genes are not acting independently and that gene-environment interactions, also through epigenetic modifications of the genome, are driving the rapid increased prevalence of obesity. To this regard, it has been suggested that epigenetic mechanisms acquired in early life have phenotypic consequences later in development through their role on transcriptional regulation with relevance to the developmental origins of diseases, including obesity (Gluckman and Hanson, 2008; Relton et al., 2012). Changes both in epigenomic patterns and expression of genes involved in the regulation of energy homeostasis are indeed recognized as key mechanisms resulting in phenotypic long-lasting individual differences and thus in metabolic imprinting of permanent alterations.

1.1.2. *In utero* environment programs metabolic development during life

Epidemiological and clinical studies as well as experimental animal work have introduced the concept of "developmental origins of health and disease". This theory supports the hypothesis that prenatal environmental adversities may exert lifelong consequences by programming the offspring's cells, tissues, organs, their structure and function.

Maternal obesity is associated with 2-fold higher incidence of heavier newborns than in non-obese women (Castro and Avina, 2002) and, in turn, fetuses of obese mothers have higher percentage of body fat than fetuses of lean mothers. This could results from imprinting of maternal genes that influence energy balance, but a more widely accepted possibility is that the predisposition is mediated by the *in utero* environment a developing fetus of an obese woman is exposed to (Wardle et al., 2008; Drake and Reynolds, 2010; Nelson et al., 2010). Studies suggest that changes in the intrauterine environment of obese women can cause metabolic adaptations in the fetuses, with immediate or late consequences (King, 2006; Nelson et al., 2010).

Maternal obesity has no major influence on body weight of the offspring, similar to the effect of high-fat diet during pregnancy, suggesting that maternal adiposity *per se* is necessary for the programming effect that predisposes the offspring to obesity (White et al., 2009). This is also supported by further evidence suggesting that maternal body composition *per se* is a driving factor in offspring adiposity (Sacks et al., 2006). Evidence from animal studies supports the hypothesis that the core features underlying obesity result from the obesogenic environment experienced *in utero* rather than pure genetic factors. However, the precise obesogenic environmental factors and the underlying mechanisms that mediate these effects are largely unknown. Several mechanisms may explain the associations between exposure to obesity *in utero*

and long-term metabolic outcomes in the offspring, i.e., genetic and lifestyle factors as well as specific intrauterine effects.

One developmental pathway to obesity is the fetal over-nutrition pathway (Yajnik, 2009), reflecting the effects of hyperinsulinaemia and other factors during fetal life. This creates conditions for later pathophysiological effects of an early obesogenic environment. Beyond increased risk of metabolic disturbances, evidence shows adverse effects of maternal HFD on offspring's brain development, with consequences on affective behavior and cognition (Sullivan et al., 2011). Specifically, animal studies have identified alterations in hippocampal and hypothalamic regions as well as in serotonergic, dopaminergic and opioid neurotransmitter systems, resulting in changes of cognitive and affective behaviors in offspring born to obese mothers (Naef et al., 2008; Walker et al., 2008; Bilbo and Tsang, 2010; Sullivan et al., 2010; Tozuka et al., 2010; Vucetic et al., 2010; Naef et al., 2011; Wright et al., 2011).

1.2. DERANGEMENTS ASSOCIATED TO MATERNAL OBESITY

Alterations in maternal metabolism may contribute to the over-nutrition pathway (Schaefer-Graf et al., 2008). Lipids play a key role in adipocytes differentiation, thus, increased transfer of free fatty acids might affect fetal adiposity by influencing the number, size or activity of fetal adipocytes.

1.2.1. Metabolic regulation and fetal metabolic programming

Adipose tissue is recognized as a major endocrine and secretory organ, releasing a wide range of protein factors and signals, such as leptin, that is directly involved in the regulation of energy balance by a direct action on the hypothalamus (Friedman and Halaas, 1998; Minokoshi et al., 2004), in addition to peripheral signaling (Muoio et al., 1997; Minokoshi et al., 2002). In the hypothalamus leptin stimulates the activation of specific pathways resulting in increased energy expenditure and decreased body weight and food intake (Gao and Horvath, 2007). In obese humans there is a failure of this hormone to exert the appropriate effects, reflective of a leptin resistant state (Liuzzi et al., 1999; Enriori et al., 2007), a key component of obesity. Progression to this state has been shown to involve firstly a gain in adiposity while still retaining responses to peripherally administered leptin, followed by a loss of peripheral leptin responsiveness and, finally, a complete resistance to both peripherally and centrally administered leptin (El-Haschimi et al., 2000). During obesity, there is an acquired and reversible reduction in the transport of leptin across the blood brain barrier with intraperitoneal leptin administration (Banks and Farrell, 2003). There is also a failure within hypothalamic leptin signalling pathways, with a loss of effectiveness of centrally administered leptin on the inhibition of food intake and stimulation of energy expenditure (El-Haschimi et al., 2000; Munzberg and Myers, 2005). However, a preservation of the cardiovascular

effects of leptin is seen, such that obesity and its associated hyperleptinemic state induces further increases in blood pressure, suggesting that 'selective leptin resistance' is an important component of the metabolic syndrome (Correia et al., 2002; Rahmouni et al., 2005).

In addition to the role that leptin plays in adult life, its fundamental action has been also recognized in the establishment of hypothalamic circuitry during the earliest stage of life (Bouret and Simerly, 2006). Prior and colleagues found that offspring from HFD-fed dams exhibits an altered hypothalamic sensitivity to leptin, suggesting that metabolic alterations occurring in a susceptible time window for the developing organism impair central mechanisms regulating leptin secretion at adulthood (Prior et al., 2013).

Obesity is typically associated with insulin resistance (IR). Insulin is a peptide hormone that regulates the metabolism of carbohydrates and fats by promoting the absorption of glucose from the blood to skeletal muscles and fat tissue. In healthy conditions, insulin is provided within the body in a constant proportion to remove excess glucose from the blood, which otherwise would be toxic. In obese subjects, the IR state leads to hyperinsulinemia. In women, this trait seems to confer, in addition to an increased risk for hypertension, central fat accumulation and inflammation, as well as adverse pregnancy outcomes (Catalano, 2010). In addition, it is important to note that IR in the mother predicts the size (Das et al., 2010) and the degree of IR in the fetus (Catalano et al., 2009). Maternal hyperinsulinemia is associated with increased gestational weight gain and weight retention postpartum (Scholl and Chen, 2002). During pregnancy it is usually reported an increase in the mass of visceral fat both in lean and, to a greater extent, in obese women. In the latter case, an increase in fat mass is commonly associated with metabolic-related diseases. Animal studies have shown that the offspring of female mice fed a diet-induced obesity develop IR, hypertension (Samuelsson et al., 2008), adiposity and hyperphagia. Actually, Murabayashi and co-workers (Murabayashi et al., 2013) demonstrated that maternal HFD in mice causes inflammatory changes in the adipose tissue of the offspring. Studies in sheep, fed an obesogenic diet, documented tissue-specific defects in the insulin-signaling cascade in the fetal myocardium and an impaired response to workload stress in the heart (Wang et al., 2010).

1.2.2. Hypothalamic-pituitary-adrenal axis activation

Stress is commonly defined as a state of threatened homeostasis (Chrousos, 1998) by either endogenous or exogenous factors which induce specific response mechanisms, most prominently the sympathetic nervous system and the hypothalamic-pituitary-adrenal (HPA) axis, which result in a series of neural and endocrine adaptations collectively known as the stress response. While the adaptive response to acute stressors is advantageous for survival, periods of chronic stress or stressful life events may be maladaptive and have been associated with a variety of pathological conditions (Charney and Manji, 2004;

Rosengren et al., 2004; de Kloet et al., 2005; Abraham et al., 2007; Dallman, 2010).

A large body of epidemiological evidence indicates a strong link between chronic stress, especially social stress, and metabolic disturbances (Tamashiro, 2011). Specifically, chronic stress represents a major risk factor for the development of abdominal obesity and the metabolic syndrome, i.e. a cluster of metabolic factors that increase the risk to develop CVD and T2D (Rosengren et al., 2004; Chandola et al., 2006; Branth et al., 2007). Indeed, Bartolomucci and colleagues demonstrated how chronic psychosocial stress represents a model of stress induced weight gain and vulnerability to obesity (Bartolomucci et al., 2009). Animal models provide further evidence for an interaction between the stress response and energy balance, suggesting that exposure to stress affects the feeding behavior by regulating caloric intake and body weight alterations (Rosengren et al., 2004; Chandola et al., 2006; Branth et al., 2007; Foster et al., 2009; Tamashiro, 2011).

Strong evidences report the link between stress or glucocorticoids (GCs) exposure to later development features of the metabolic syndrome (Lindsay et al., 1996; Nyirenda et al., 1998). During stress, chow intake decreases but intake of lard and sucrose does not. In addition, Dallman and colleagues suggest that palatability signals and neural signals from fat stores act on the brain to reduce activity in the central stress response system (Dallman et al., 2007). A wide literature demonstrates the involvement of the same neural pathways both in the regulation of the stress responses and in maintaining of the metabolic homeostasis. However, to date relatively little research has focused on the long-term effects of maternal HFD on the offspring's stress response (Nieuwenhuizen and Rutters, 2008) and the molecular basis underlying this relationship remain largely unknown. Nevertheless, the effects of maternal nutritional status, during gestation and lactation, to increase the response to stress is recognized both in mother and, more interestingly, in the offspring. Several data have been reported to this regard demonstrating alterations in HPA axis responsiveness after an acute stressor in neonates and pre-pubertal rats exposed to maternal HFD (Trottier et al., 1998). Moreover, it has been shown that the neonatal HPA axis is sensitive to metabolic signals, including glucose and ghrelin (Schmidt et al., 2006), suggesting that changes in the level of metabolic signals resulting from changes in maternal diet may influence the development of the neonate's HPA axis response. The close communication between metabolic signals and neuroendocrine response is further supported by studies reporting that prenatal stress or GCs exposure in rodents lead to features associated with the metabolic syndrome, such as hypertension and hyperglycaemia (Lindsay et al., 1996; Nyirenda et al., 1998; Mueller and Bale, 2006; Tamashiro et al., 2009). Moreover, the hormonal mediators of the stress response (GCs and catecholamines) are important modulators of metabolism and cardiocirculatory function (Mastorci et al., 2009).

1.2.2.1. Glucocorticoids as mediators of fetal programming effects

GCs are steroid hormones with diverse roles including the regulation of stress responses, glucose homeostasis and immune function, also exerting multiple effects during fetal development, especially in the promotion of tissue maturation and function.

Briefly, GCs, also defined "stress hormones", are produced by adrenal cortex as the final result of the HPA axis activation in response to internal or external stressful stimuli. The response to stress starts after stimulation of paraventricular nucleus in the hypothalamus and the consequent release of corticotropin releasing hormone (CRH), a key mediator of many aspects related to stress (Koob, 1999). CRH stimulate the anterior pituitary gland-release of adrenocorticotropic hormone (ACTH), which in turn lastly stimulate the adrenal glands.

Alterations in the central and peripheral sensitivity to GCs have been described in both humans and animal models of obesity. Increased secretion of GCs by adrenals glands in response to specific stimuli, due either to a centrally hyperactivity of the HPA axis or reduced negative feedback response, altered tissue levels of GCs receptors (GRs) and changes in peripheral GCs metabolism have all been shown to contribute to hypertension, hyperglycemia and impaired insulin sensitivity (Jessop et al., 2001).

The developing fetus is maintained in a low GCs environment to protect it against the potent effects of GCs that accelerate fetal organ maturation in a tradeoff against overall growth. During *in utero* development, the activities of specific placental enzymes provide a barrier that regulates the physiological maternal GCs levels functional to fetal tissue maturation (Benediktsson et al., 1997). More in detail, two steroid enzymes are involved in the maintenance of the GCs gradient between mother and fetus: the 11β-hydroxysteroid dehydrogenase (11β-HSD) type 1 and type 2. Briefly, the 11β-HSD type 1 enzyme activates the maternal circulating GCs to the fetus, while the reverse reaction is driven by the 11β-HSD type 2 isoform, that rapidly converts physiologically active GCs to inert forms (Seckl et al., 2004; Grino, 2005). Human and rodent fetuses show a developmental switch from 11β-HSD type 2 in early and mid-gestation (Murphy, 1978; Brown et al., 1996; Condon et al., 1998) to 11 β-HSD type 1 activity in late gestation (Murphy, 1978; Thompson et al., 2004). The timing of this "switch" is functional to the right fetal development. In accordance with the largely antiproliferative and pro-differentiation effects of GCs and their maturational role in preparation for birth (Fowden et al., 1998), 11β-HSD type 2 is expressed in immature cells, protecting tissues from inappropriate GC exposure during development (Holmes et al., 2006; Wyrwoll et al., 2010), whereas 11β-HSD type 1 is expressed only from late development, apparently amplifying GC maturational effects on fetal tissues.

It has been hypothesized that variations in the expression or in the activity of the fetus-placental 11β-HSD type 2 enzyme may underlie prenatal GCs programming (Edwards et al., 1993). The observation that placental 11β-HSD type 2 enzyme activity correlates directly with birth weight in rodent (Benediktsson et al., 1993) and in most human studies (Stewart et al., 1995; Murphy et al., 2002; Kajantie et al., 2003; Aufdenblatten et al., 2009), suggests that normal variation in fetal exposure to maternal GCs has an impact on fetal growth. More interestingly, preclinical and clinical studies suggest that maternal-mediated metabolic intervention during fetal life affects the enzymatic activity of 11β-HSD type 2 with subsequent alteration of fetal exposure to maternal GCs. In particular, in rat models of maternal malnutrition, the feeding of a calorie restricted or low-protein diet reduces placental levels of 11β-HSD type 2 enzyme and offspring are born of a lower birth weight and present hypertension in later life (Bandyopadhyay and Medrano, 2003; Zeugmann et al., 2010). As well as in clinical studies, protein-restricted diet throughout gestation caused an increase in maternal and fetal GC levels (Lesage et al., 2001; Guzman et al., 2006), together with a decrease in placental 11β-HSD type 2 enzymatic activity (Langley-Evans et al., 1996; Bertram et al., 2001; Stocker et al., 2004). These results suggest that there are common mechanisms underpinning both nutritional and GCs developmental programming.

1.2.3. Offsprings' emotional phenotype

Despite the programming effect of maternal HFD on metabolism and metabolic disorders, little is known thus far about its possible consequences on the psychological/behavioral development of the offspring. Actually, relatively little attention has been paid so far to the impact of overfeeding during pregnancy and the effect it might have on the emotional behavior, such as anxious behavior, depression and, more in general, altered emotional responses and cognitive deficits of the offspring later in life. Indeed, anxiety and depression are amongst the most commonly found comorbid disorders of obesity in humans following diabetes and CVD and thus represent a major concern in today's healthcare system (Luppino et al., 2010). It is unclear whether the natural history of stress-induced effects on behaviors related to mood and anxiety are coincident with alterations observed in food intake. Despite behavior being a key readout for stress induced changes in animal models (Cryan and Holmes, 2005; Lutter et al., 2008; Chuang et al., 2010; Vidal et al., 2011), there is a paucity of behavioral studies on the feedback effects induced by ingestion of highly caloric diets or diet-induced obesity on the stress response (Pecoraro et al., 2004).

1.2.4. Oxidative stress

Oxidative stress (OS) is a state characterizing biological systems in aerobic conditions derived from an imbalance between pro-oxidative and anti-oxidative

molecules where the oxidants override defensive systems. Oxidative molecules, namely Reactive Oxygen Species (ROS), are produced primarily by the physiological metabolism of oxygen (O_2) in cells (Kakkar and Singh, 2007) but also environmental stimuli, such as cytokines, ultraviolet radiation, chemotherapeutic agents, hyperthermia and even growth factors, might contribute to their generation shifting the normal cellular red-ox balance into a state of OS potentially leading to diabetes, ischemia/reperfusion, to mention only a few (Dhalla et al., 2000; Finkel and Holbrook, 2000; Sayre et al., 2001; Jenner, 2003; Dalle-Donne et al., 2006).

Elevated OS, in addition to be involved in a variety of pathological conditions, such as inflammation, atherosclerosis, cancer and several age-related neurodegenerative disorders (Finch and Marchalonis, 1996; O'Banion and Finch, 1996; Hensley et al., 1999; Finkel and Holbrook, 2000; Zhu et al., 2001a; Zhu et al., 2001b), is also a contributing factor to obesity (Itabe, 2012). As already previously mentioned, a pathophysiological feature of obesity is the low-grade chronic inflammation in adipose tissue that secretes a number of inflammatory markers, some of which implicated in pathologies associated with obesity (Pouliot et al., 1994; Foundation, 2005; Trayhurn, 2005). In obese patients, subclinical inflammation has been found to correlate with markers of OS in adipose tissue and this may be the mechanism for obesity-related metabolic syndrome, IR and diabetes mellitus. Furthermore, both OS and low-grade inflammation may be causatively linked to the development, progression and complications of diabetes in obese patients.

1.3. RELEVANCE OF ANIMAL STUDIES

Although there are obvious limitations to the extrapolation of data resulting from research conducted in animal studies to human health, the use of animal models allows for a comprehensive analysis of the offspring and to dissect out discrete periods of offspring development for a mechanistic understanding. In addition, basic research conducted in rodents can allow differentiating between genetic and environmental factors involved in the development of obesity, in addition to studying the interaction between the two factors. Animal models have proven invaluable in interrogation of associations between maternal diet and offspring phenotype (Levin, 2006; Schmidt et al., 2006), providing the ability to study the long-term consequences of maternal diet or prenatal stress on offspring while allowing to control variables that human studies do not afford. These studies support the hypothesis that a diet rich in fat during pregnancy can transmit a propensity for adiposity, glucose intolerance and cardiovascular dysfunction to the offspring. Actually, reported results are sometimes quite controversial. In rodents, some studies have shown that prenatal obesity has been associated with impaired mammary gland development, thus compromising lactation and pups' growth (Rolls et al., 1980;

Rolls et al., 1986; Rasmussen et al., 1998; Flint et al., 2005), while others have shown increased pup growth (Fisher et al. 1992; Gorski et al., 2006).

It should be noted that these results are more commonly reported in male offspring, although many studies observe comparative effects in female littermates (Bayol et al., 2007). Most of these studies suggest that sex differences may be of interest for future investigations in which disturbed glucose homeostasis is a primary outcome.

Rodent models have also been extensively used to study differential susceptibility between individuals to diet-induced obesity (DIO). These models have provided information on the rate of body weight changes induced by feeding to such a diet and have been also able to reveal differences in terms of susceptibility to DIO, both between different animal strains (Prpic et al., 2003) and even within the same species (Levin and Sullivan, 1989; Archer et al., 2003; Enriori et al., 2007). The C57BL/6J mouse strain is an inbred line, although being genetically homogeneous, it exhibits a heterogeneous response to high-fat feeding, with some animals developing obesity and some remaining lean (Burcelin et al., 2002; Collins et al., 2004). This variability in the response to metabolic stimuli is likely to be a function of epigenetic events caused by environmental, biological and social influences imposed on the dams or the offspring themselves during their development. All these factors resemble very closely the human situation, making these animal models of great use in the identification of pathways contributing to obesity development.

1.3.1. The $p66^{Shc-/-}$ mouse model

The $p66^{Shc}$ gene has emerged as a novel gerontogene able to affect lifespan by controlling OS and metabolism (Trinei et al., 2009). $P66^{Shc}$ is peculiar protein acting specifically in the mitochondrion as a red-ox enzyme that generates H_2O_2 to trigger mitochondrial swelling and apoptosis (Giorgio et al., 2005). In the last decade, studies performed on $p66^{Shc}$ knock-out (KO) mice have indicated that this gene is a crucial regulator of ROS levels and that it is involved in age-related dysfunctions. Notably, among ROS, H_2O_2 is the only species that is generated by several specific enzymes in the cell suggesting that its intracellular concentration is tightly regulated and may serve specific cellular functions. The H_2O_2 generated by $p66^{Shc}$ accounts for ~30% of the total pool of intracellular H_2O_2 and is biologically relevant, as shown by the finding that cells and tissues that are derived from $p66^{Shc}$ KO mice accumulate significantly less OS and because $p66^{Shc}$ can induce a mitochondrial apoptotic response (Trinei et al., 2002; Giorgio et al., 2005). $p66^{Shc-/-}$ mice indeed show a healthy phenotype characterized by greater brain and behavioral plasticity, in addition to reduced OS, fat accumulation and incidence of metabolic as well as cardiovascular pathologies (Migliaccio et al., 1999; Berry et al., 2007; Berniakovich et al., 2008; Berry et al., 2008; Berry et al., 2010; Berry et al., 2012; Giorgio et al., 2012).

Studies performed in a semi-naturalistic setting, involving exposure to low temperatures and food shortage, have indicated that $p66^{Shc}$ has been conserved through evolution because of its role as a 'thrifty gene' in energy metabolism. This feature, which increases the probability for survival in harsh natural conditions, can be deleterious when food is constantly available, as in westernized lifestyles, leading to fat accumulation and thus predisposing to metabolic disease, CVD and accelerating brain ageing (Floyd and Hensley, 2002).

IR and abdominal obesity, often observed in older adults, are main components of the metabolic syndrome, a pathological condition characterized by multi-organ morbidity (Folsom et al., 1993; Morley, 2008). However, a growing body of evidence shows that the incidence of obesity, and the often associated metabolic syndrome, is increasing in the young population, especially in western countries (Haffner and Taegtmeyer, 2003). These morbid conditions are accompanied by a whole metabolic unbalance with high levels of OS and inflammation in addition to shorter telomeres (that correlate with increasing BMI), overall resembling a form of precocious ageing (Fadini et al., 2011). Furthermore, patients with metabolic syndrome are often characterized by a higher incidence of mood and cognitive dysfunctions than the general age-matched population that, in turn, emerge as significant risk factors for aggravation of metabolic syndrome and the related health outcomes, particularly CVD and T2D (Engum, 2007; Goldbacher et al., 2009; Muller et al., 2010; van Reedt Dortland et al., 2010; Zeugmann et al., 2010; Raffaitin et al., 2011). Thus, overall, alterations in metabolism and body fat distribution certainly play a role in a vicious cycle that can precipitate even the ageing process, in addition to the onset of diseases (Barzilai et al., 2012).

1.4. RATIONALE AND OBJECTIVES

1.4.1. Current state of the art and statement of the problem

Obesity and related co-morbidities epidemically impact modern societies, both in developed and in developing countries. The increasing incidence of this pathology among women of reproductive age and children highlight the main question of the role played by maternal obesity in setting up a state of individual susceptibility to metabolic disorders at adulthood. The literature reviewed so far, both in clinical and in research field, supports a raised susceptibility in the offspring of obese mothers to CVD and metabolic impairment, such as hyperglycaemia and hyperinsulinemia, T2D and obesity at adulthood. However, a comprehensive behavioral and endocrine characterization of the long-term impact of maternal HFD, combined with metabolic and stressful challenging conditions, is still lacking. Because of the large and complex interplay among the mechanisms underpinning this predisposition, we are still far from a

complete understanding of the mechanisms, let alone putting in place potential countermeasures.

The **general aim** of the work presented in this thesis was to evaluate the impact of maternal HFD both on the mother and the offspring in a long-term perspective and to study the interplay of metabolic disturbances occurring in a sensitive time window (i.e. fetal development and pregnancy) and the stress system under basal conditions, as well as following an adverse challenge. We also aimed at investigating the interaction between an early metabolic stimulus (maternal HFD) and a physiological condition of reduced OS. Our hypothesis was that HFD feeding represents a metabolic stressful stimulus during pregnancy, with relevant effects on the phenotype of the mother and, consequently, on the phenotype of the offspring during the whole life course. Moreover, we hypothesize that reduced OS might result in a more efficient homeostatic control and better abilities to cope with changes in the internal milieu resulting by the maternal HFD. We propose that this analysis may provide information on how a reduction in the oxidative state can affect the physiological perception of an altered metabolic state and the molecular arrangements occurring during pregnancy and parturition in response to such a stimulus.

1.4.2. Rationale of the studies

I. Investigating the long-lasting effects of maternal high-fat feeding on the neuroendocrine, metabolic and behavioral phenotype of the adult offspring in a mouse model of reduced OS, the $p66^{Shc}$ knock-out mouse, paying particular attention to gender-specific outcomes. In addition, we aimed at identifying individual metabolic and/or neuroendocrine markers of vulnerability to metabolic and emotional disorders. The main hypothesis of the work was that the lack of the $p66^{Shc}$ gene might exert a protective role against the detrimental effects of maternal exposure to HFD.
II. Develop a pharmacological model of reduced OS by means of prenatal administration of an antioxidant drug in the attempt to induce, in adult subjects, the ability to cope with metabolic challenges, represented by a maternal HFD, occurring during early life. Identify a time window in the life of the individual during which a pharmacological intervention might exert a preventive efficacy counteracting the consequences associated to maternal HFD.
III. Investigate the effects of high-fat feeding before and during pregnancy on the behavioral and neuroendocrine profile of dams in the perinatal period, both before and after delivery, with a special focus on the effects of HFD on maternal behavior and on the neuronal plasticity which contributes to its establishment, in addition to the molecular adaptation associated with parturition.

1.4.3. Experimental approach and outline of the thesis

In **Chapter 2** and **Chapter 3** are reported two studies aimed at assessing long-term multi-levels effects associated to prenatal exposure to maternal HFD.

Animal models of maternal over-nutrition typically involve the feeding of a HFD to pregnant dams. In both these studies a metabolically altered *in utero* environment has been induced by means of feeding dams a diet rich in fats since 10 weeks before pregnancy, and not limited to the pregnancy period only. It has been widely reported by several preclinical studies the hypothesis that a diet rich in fat during pregnancy can transmit a wide range of metabolic impairments to the offspring. However, while in some instances this was evident in offspring that had been exposed to high-fat feeding *in utero* only, in other cases the exposure was extended to the lactation period (Guo and Jen, 1995; Buckley et al., 2005; Cerf et al., 2005). Thus, it seems likely that more than one critical time window during individual development may play a determinant role in programming offspring disease risks (Alfaradhi and Ozanne, 2011). Animal studies are thus necessary to separate the wide range of confounding exposures and factors during the different developmental phases, which can influence offspring health outcome. Various animal models have already been used to address the transgenerational impact of DIO by fetal exposures throughout pregnancy and also the lactation period (Alfaradhi and Ozanne, 2011), which represents a critical time window for programming in rodents (Purcell et al., 2011; Sun et al., 2012). However, a greater paucity of animal studies have specifically investigated the transgenerational effects of maternal pre-gravid and gestational obesity (Shankar et al., 2008; Shankar et al., 2010), although an impact on early embryo development has been suggested (Igosheva et al., 2010; Jungheim et al., 2010).

So far, the outcomes of maternal obesity have mostly been determined in male offspring (Aiken and Ozanne, 2013) while only few studies have compared the effects on the two sexes (Khan et al., 2003; Han et al., 2005; Elahi et al., 2009; Nivoit et al., 2009; Bayol et al., 2010; Gallou-Kabani et al., 2010). These studies are highly relavant also given the reported sex-dependent association between parental BMI and children's weight and body fat, an observation not being explained as yet (Whitaker et al., 2000; Danielzik et al., 2002). Developmental components to sex-specific differences in human disease risk have also been reported for several common disorders (Ober et al., 2008), such as in diabetes (Mingrone et al., 2008).

Chapter 2 describes a study aimed at assessing the metabolic, neuroendocrine as well as the behavioral profile of young adult offspring in a transgenic mouse model characterized by reduced oxidative stress, the $p66^{Shc-/-}$ mouse. Data were analyzed in order to highlight if the reduced oxidative stress is a physiological condition that might exert a preventive role able to counteract, or at least limit, the effects resulting by prenatal exposure to a metabolic challenge represented by the maternal HFD. Since previous studies have already

demonstrated that the maternal environment experienced early during development affects fetal programming, setting the growth trajectory of the fetus through a different endocrine regulation in the two genders (Maccari et al., 2003; Seckl and Holmes, 2007; Reynolds et al., 2013; Spencer, 2013), gender effects on physiological responses of the adult offspring were also assessed. More in general, all these effects were evaluated in the transgenic mouse model of reduced oxidative stress.

The results obtained by the first study reveal, among other interesting results, a "protective" role exerted by the reduced oxidative stress toward the negative outcomes resulting by the maternal diet. These findings have lead to the study described in **Chapter 3,** in which are reported the effects resulting by the prenatal exposure to N-acetyl-cysteine(NAC), a drug characterized by remarkable antioxidant properties (De Flora et al., 1995a; De Flora et al., 1995b). The time window of exposure to the NAC drug overlapped with maternal HFD exposure. This methodological choice was based upon the widely reported data related to the relevance of the prenatal programming on the physiological shaping of the adult individual (Barker, 1995), rendering such a period relevant for a preventive intervention. In this Chapter a main emphasis was placed on the ability of the NAC drug to counteract the effects of the HFD effect of the adult offspring.

In **Chapters 2 and 3** have been also described the effects of HFD exposure before and during pregnancy on the dams. Results indicate an HFD-induced aberrant maternal behavior toward to the offspring, leading us to hypothesize that HFD feeding might represent a metabolic stressful challenge during pregnancy which might impair the establishment of maternal behavior with detrimental effects on the offspring.

Thus, the studies described in **Chapter 4** were mainly focused on investigating the effects of HFD feeding before and during pregnancy on the neuroendocrine and behavioral profile of dams in the perinatal period. The literature reviewed so far is mainly focused on the effects of maternal obesity or maternal high-fat feeding on the offspring and very few studies have paid attention to the effect of HFD on the mothers' physiology during pregnancy and parturition. In Chapter 4 we proposed a model of HFD feeding before and during parturition in order to investigate its potential role as a metabolic stressor. The comprehension of the mechanisms underpinning the physiological adaptation of the mothers in response to HFD might lead to the identification of neuroendocrine and molecular markers predictive of a general physiological and behavioral alteration.

All data are discussed in **Chapter 5**.

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CHAPTER 2

2. GENDER-DEPENDENT RESILIENCY TO STRESSFUL AND METABOLIC CHALLENGES FOLLOWING PRENATAL EXPOSURE TO HIGH-FAT DIET IN THE P66$^{SHC-/-}$ MOUSE

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ABSTRACT

Metabolic stressful challenges during susceptible time windows, such as fetal life, can have important implications for health throughout life. Deletion of the $p66^{Shc}$ gene in mice leads to reduced oxidative stress (OS), resulting in a healthy and lean phenotype characterized by increased metabolic rate, resistance to high-fat diet (HFD)-induced obesity and reduced emotionality at adulthood. Here we hypothesize that $p66^{Shc-/-}$ (KO) adult offspring might be protected from the detrimental effects induced by maternal HFD administered before and during pregnancy. To test such hypothesis, we fed $p66^{Shc+/+}$ (WT) and KO females with HFD for 13 weeks starting on 5 weeks of age until delivery and tested adult male and female offspring for their metabolic, neuroendocrine, and emotional profile. Prenatal diet affected stress responses and metabolic features in a gender-dependent fashion. In particular, prenatal HFD increased plasma leptin levels and decreased anxiety-like behavior in females, while increasing body weight, particularly in KO subjects. KO mice were overall characterized by metabolic resiliency, showing a blunted change in glycemia levels in response to glucose or insulin challenges. However, in $p66^{Shc-/-}$ mice, prenatal HFD affected glucose tolerance response in an opposite manner in the two genders, overriding the resilience in males and exacerbating it in females. Finally, KO females were protected from the disrupting effect of prenatal HFD on neuroendocrine response. These findings indicate that prenatal HFD alters the emotional profile and metabolic functionality of the adult individual in a gender-dependent fashion and suggest that exposure to high-caloric food during fetal life is a stressful condition interfering with the developmental programming of the adult phenotype. Deletion of the $p66^{Shc}$ gene attenuates such effects, acting as a protective factor.

Keywords: maternal obesity, $p66^{Shc}$ gene, oxidative stress, biomarkers, adipokines, gender, emotionality, animal models.

2.1. INTRODUCTION

The environment experienced early during development is not only crucial for setting the growth trajectory of the fetus but represents also a key factor contributing to overall disease susceptibility in later life (Barker, 1995). In this context, developmental plasticity is a fundamental mechanism matching the growing organism to the environment it will face after birth (Barker, 2003). Early life stress can deeply influence developmental programming. In modern society maternal obesity - often associated with low socio-economic status - is an example of a physiological stressor experienced by women during pregnancy (Reynolds et al., 2013). Preclinical evidence, recently confirmed by clinical studies, suggest that such metabolic condition produces an adverse *in utero* environment for the offspring with long-term detrimental effects (Drake and Reynolds, 2010; Li et al., 2011). In particular, maternal obesity induces a chronic mild inflammatory state and high levels of oxidative stress (OS), resulting in frailty for psychiatric disorders and metabolic complications linked to an increased risk of insulin resistance (IR) and type 2 diabetes (T2D), in addition to cardiovascular disease and other metabolic disorders associated with obesity (Kahn and Flier, 2000; Wang et al., 2004; Fantuzzi, 2005; Taylor et al., 2006; Eriksson et al., 2014). Likewise, high-fat feeding during pregnancy, independently from maternal obesity,causes *per se* placental dysfunction and metabolic impairment in the offspring (McCurdy et al., 2009; Frias et al., 2011). Preclinical studies have shown that extreme changes in maternal diet influence maternal stress responses and, likewise, affect offspring outcome, including adverse changes in behavior and memory (Weinstock, 2001), in cardiovascular responses to stress (Igosheva et al., 2007), in glucose tolerance (Lesage et al., 2004), as well as sexual dimorphisms of brain regions associated with mood and hypothalamic-pituitary-adrenal (HPA) axis functions (Handa et al., 1994; Majdic and Tobet, 2011). Analyzing further these mechanisms could help identifying protective factors and lead to a better understanding of sex differences in the risk for metabolic and mood disorders reported in the adult population. These are important public health issues, given the abundance of dietary fats in western diets.

The $p66^{Shc}$ gene is a mammalian gerontogene involved in metabolism and OS (Trinei et al., 2009) and plays a major role in the aging process. $p66^{Shc}$ is highly expressed within the adipose tissue and is involved in adipogenesis as it contributes to the intracellular insulin-mediated signaling pathway regulating fat accumulation (Berniakovich et al., 2008; Tomilov et al., 2011). In addition to resistance to OS, the lack of $p66^{Shc}$ gene leads to reduced trygliceride accumulation in the adipocytes, reduced fat mass, increased metabolic rate and resistance to diet-induced obesity in the mouse (Berniakovich et al., 2008; Tomilov et al., 2011). Furthermore, the $p66^{Shc}$ mutants show reduced emotionality, both in social and non-social contexts (Berry et al., 2007; Berry et al., 2008; Berry and Cirulli, 2013), associated to a mild hyperdrive of the HPA

axis (Berry et al., 2010). These physiological features of the $p66^{Shc-/-}$ mouse make it a powerful tool to investigate the mechanisms underlying the long-term consequences of maternal exposure to an obesogenic diet on metabolism and emotionality of the adult offspring.

The current study was aimed at investigating the long-lasting effects of maternal high-fat feeding on the neuroendocrine, metabolic and behavioral phenotype of the adult offspring, paying particular attention to gender-specific outcomes. In addition, we aimed at identifying individual metabolic and/or neuroendocrine markers of vulnerability to metabolic and emotional disorders.

Given the role played by the $p66^{Shc}$ gene in cellular metabolism, by mediating the insulin signaling, we hypothesized that the lack of this gene might exerts a protective role against the detrimental effects of maternal exposure to high-fat diet (HFD).To test such hypothesis, $p66^{Shc+/+}$ (WT) and $p66^{Shc-/-}$ (KO) female mice were fed with HFD or control diet (CD) for 13 weeks: from 5 weeks of age until right before delivery. Indeed, while several studies on the effects of maternal high- fat feeding have been performed by extending HFD exposure through pregnancy and/or lactation up until weaning, in this study we wanted to focus mainly on prenatal effects. The offspring was subsequently phenotyped for metabolic, neuroendocrine and behavioral responses.

2.2. MATERIALSANDMETHODS

2.2.1. Animals

Experimental subjects were 5 weeks-old knock-out (KO - $p66^{Shc-/-}$, *n*= 71) and wild-type (WT - $p66^{Shc+/+}$, *n*= 77) female mice on a C57BL/6J background. Animals were housed 2/cage in transparent Plexiglas cages (37×21×19 cm) provided by Tecniplast, in an air conditioned room (temperature 21±1°C, relative humidity 60±10%) under a reversed 12/12h light/dark cycle with lights off from 07:00 a.m. to 07:00 p.m. Fresh tap-water and standard chow (standard diet - SD - energy 3.3 kcal/g, fat 17%, carbohydrate 60% and protein 23%, provided by Altromin-R, Rieper, Italy) were continuously available until 5 weeks of age. Thereafter females were fed *ad libitum* either with HFD (energy 5.56 kcal/g, fat 58%, carbohydrate 25.5% and protein 16.4%; *n*= 40 WT; 37 KO) or control diet (CD - energy 4.07 kcal/g, fat 10.5%, carbohydrate 73.1% and protein 16.4%; *n*= 37 WT; 34 KO) for 10 weeks, i.e. until 15 weeks of age. Females of both genotypes were randomly assigned to HFD or CD groups avoiding difference in the average of body weight between groups. HFD (D12331) and CD (D12328) were provided by Research Diets, Inc., New Brunswick, NJ, USA. After 10 weeks on the diet, all females were mated with males of the same genotype. Body weight was monitored once a week to assess pregnancy. Pregnant females (*n*= 24 WT-CD; 19 KO-CD; 34 WT-HFD; 27 KO-HFD) were kept with either HFD or CD throughout gestation until 3 days before the expected delivery date, i.e. at gestational day 16 (G16). Thereafter all

of them were switched to SD during lactation until pups weaning, occurring at post-natal day 30 (P30). Daily food consumption (24 h) and body weight gain of females maintained on HFD or CD were monitored once a week. The success of birth and the maternal behavior immediately after the birth of pups were also registered. In addition to nest dimension, pups' body weight was also monitored during development at P3, P30 and at 3-months-old (P90). At weaning all pups were weaned onto SD and the onset of puberty markers were checked daily. At 3 months of age, both males and females offspring were tested to assess the metabolic, neuroendocrine and emotional profiles resulting from prenatal exposure to a hypercaloric diet. At this age the Body Mass Index (BMI) of all subjects was also calculated as the ratio between body weight (g) and the square of the anal-nasal length (cm).

A schematic design of the experimental plan is reported in **Figure 1**.

Animal handling and experimental procedures were performed in accordance with the EC guidelines (EC Council Directive 86/609 1987) and with the Italian legislation on animal experimentation (Decreto L.vo 116/92).

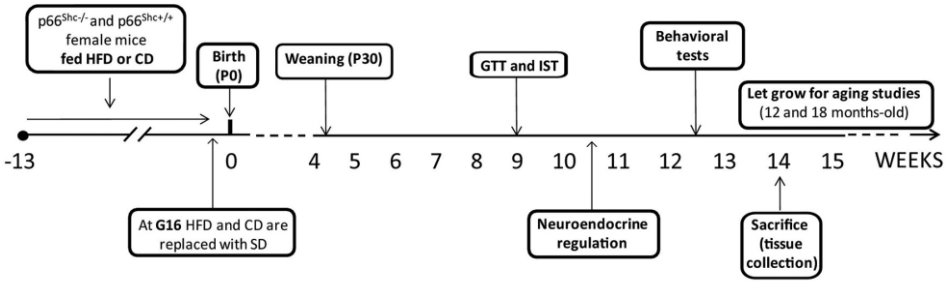

Figure 1. Schematic design of the experimental plan.

2.2.2. Experimental procedure

2.2.2.1. Onset of puberty

The onset of puberty was assessed in all male and female pups, starting from the day of weaning (P30). Vaginal opening (VO) and balano-preputial separation (BPS) were chosen as puber- tal markers (Korenbrot et al., 1977; Rodriguez et al., 1997). Beginning on the day of weaning, the dates of VO for females and BPS for males were recorded. In female mice, VO was deter- mined by daily inspection and recorded as the day on which the vaginal orifice transitioned from tightly closed to patent (Nelson et al., 1990; Azooz et al., 2001; Zhou et al., 2007; Brill and Moenter, 2009). The opening of the vaginal cavity to the skin is an event occurring around the fifth week of life that can be induced in sexually immature mice by beta-estradiol injections (Rodriguez et al., 1997).

The separation of the prepuce from the gland penis (balanus) has been shown to be androgen depen- dent and to occur around the time of puberty (Korenbrot et al., 1977).

2.2.2.2. Metabolic regulation

• Glucose tolerance test (GTT). Intra-peritoneal GTT was performed after a 15 h overnight fasting that took place from 06:30 p.m. until 09:30 a.m. Animals were intra-peritoneally (IP) loaded with 2 g/kg body weight D-glucose (10% D glucose solution; Sigma, St. Louis, MO, USA) (Satapathy et al., 2011). Blood was collected from the tail vein at 0 (baseline), 30, 60, 120 and 180 min (Ranieri et al., 2010) following IP injection and glycemia (blood glucose concentration) was measured using a commercial glucometer (StatStrip Xpress-i, nova biomedical, A. Menarini diagnostic) (Titta et al., 2010).

• Insulin sensitivity test (IST). The test was performed on animals starved for 5 h that took place from 09:30 a.m. until 02:30 p.m. Glycemia was measured using a commercial glucometer (StatStrip Xpress-i, nova biomedical, A. Menarini diagnostic) immediately before (0) and 15, 30, 60, 120 min after IP injection of a 0.4 U/kg body weight (Titta et al., 2010) solution of human recombinant insulin (Humulin, Eli-Lilly, 100 U/mL) (Ranieri et al., 2010).

• Metabolic hormones assessment. Plasma levels of leptin and adiponectin were assessed under starving condition. Blood samples were collected at 08:30 a.m., 12 h after removal of the food, from the tail vein in potassium EDTA coated tubes (1.6 mg EDTA/ml blood; Sarstedt, Germany). After centrifugation, plasma samples were used for the determination of leptin levels, by a Mouse Leptin Elisa kit (Crystal Chem Inc., Downers Grove, IL) (Berniakovich et al., 2008), in addition to adiponectin levels by an Elisa kit (B-Bridge International, Inc.) (Giorgio et al., 2012). The levels of adiponectin were also assessed in the epididymal/periovaric and mesenteric adipose tissues by immunoblot analysis. Epididymal/periovaric and mesenteric adipose tissue pads (100mg) were homogenized with Politron homogenizerin NaCl 150 mM, Tris 25 mM pH 6.8, EDTA 1 mM, supplemented with protease and phosphatase inhibitors, and centrifugated 1000 g for 10 min at 4°C, the fat pad fraction was discarded. A Bradford protein assay (Bio-Rad) was then conducted on the samples to determine the protein concentration for each sample. Proteins (20 mg) were separated by SDS-PAGE (10%) and transferred to nitrocellulose membranes. Membranes were blocked in 5% non-fat dry milk for 1 h and incubated with primary antibody for 1 h. Membranes were washed with TBS/0.1% Tween-20 three times and incubated with secondary antibody for 1 h, then washed with TBS/0.1% Tween-20 three times and reac- tivity was detected by the enhanced chemiluminescence kit (Pierce). Blots were developed using a FluorChem™ R System. Mouse monoclonal adiponectin and actin antibodies were from Abcam, Inc. (Cambridge, MA).

2.2.2.3. Neuroendocrine activation

The activation of the HPA axis was assessed in response to a psychophysical stressful challenge. All subjects underwent an acute restraint stress (30 min) and blood samples were col- lected by a tail nick at different time points, i.e. soon before (0 min) and following (30, 180 and 240 min) the exposure to stress, in order to measure plasma levels of corticosterone (CORT) (MP Biomedicals, LLC, Germany GmbH). Exposure to stress took place at 02:30 p.m., when the levels of free CORT were far from the circadian peak (Kitchener et al., 2004).

2.2.2.4. Behavioral phenotype

After 10 days of washout from the acute restraint stress, all sub- jects underwent behavioral testing to assess spontaneous behavior in the Open Field test, in addition to emotionality and general anxiety in the Elevated Plus Maze test (Pellow et al., 1985; File, 1993). These two tests were performed on different days.

• Open field (OF). Each subject was individually placed in the center of a cubic arena (open field box 40×40×40 cm) made of Plexiglas and allowed to freely explore for a single session lasting 15 min. The OF box was ideally divided into 25 squares and ideally partitioned into a central portion (24×24 cm) and a peripheral one, identified as the remaining part of the arena. When data were analyzed, the session was subdivided in three time blocks (tb), lasting 5 min, and the time spent in each portion of the arena was measured. Furthermore, the duration of locomotion (*crossings* of squares limits with all paws) was scored as index of exploratory behavior.

• Elevated plus maze (EPM). The EPM is made of two open arms(30×5×0 cm) and two closed arms (30×5×15 cm) that extend from a common central platform (5×5 cm). The apparatus, made of Plexiglas (gray floor, clear walls), is elevated to a height of 60 cm above the floor. Mice were individually placed on the central platform facing an open arm and allowed to freely explore the maze for 5 min. Behavioral parameters observed were: % open entries [(open/total)×100] and time spent in the open and closed arms of the maze (File, 2001). Furthermore, the behavioral parameters taken into account were the total amount of time spent in *immobility* and *self-grooming*, a self-directed behavior providing a reliable marker of anxiety (Kalueff and Tuohimaa, 2005).

In all behavioral tests, after each trial/session, the apparatus was cleaned with 50% ethanol to reduce olfactory cues.

At 9 weeks of age mice began the experimental procedure. All subjects underwent the GTT as first metabolic test and after 3 days, in which mice were left undisturbed, all of them were tested for the IST. This time interval was necessary to recover from the fasting and the handling procedures. Five days after the IST the neuroendocrine activation was assessed. After 10 days from the end of the stress procedure subjects underwent the behavioral tests.

2.2.2.5. Tissue collection

At 3 months of age all subjects were sacrificed and the epididymal/periovaric and mesenteric adipose tissue depots dissected out and immediately stored at -80°C for further analysis.

2.2.3. Statistical analysis

Data were analyzed using parametric analysis of variance (ANOVA) with diet (HFD *vs.* CD), genotype (WT *vs.* KO) and gender (females *vs.* males) as between-subjects factors and minutes, zones (EPM: "center" *vs.* "closed arms" *vs.* "open arms"; OF: "center" *vs.* "periphery") and time blocks (0-5 *vs.* 5-10 *vs.* 10-15 min) as within-subject repeated measures, when appropriate (GTT, IST, CORT measurement, EPM and OF tests).

Post-hoc comparisons have been performed using the Tukey's test. A linear regression model was used to assess the effect of body weight on the onset of puberty in the offspring. Fisher's exact probability test was used to compare genotypes and diets for reproductive success of the colony (i.e. number of pregnant females died during the perinatal period and number of litters in which cannibalistic episodes took place: two-by-two contingency table). Statistics were performed with Statview II (Abacus Concepts, CA, USA).

2.3. RESULTS

2.3.1. Dams' body weight and food consumption

Regardless of genotype, dams fed HFD registered a lower daily food consumption than controls (main effect of diet: $F_{(1,59)}$= 14.023, p= 0.0004, **Figure 2A**). Nevertheless, regardless of genotype, HFD determined an increase in body weight during the 10 weeks on diet (main effect of diet: $F_{(1,144)}$= 22.348, p< 0.0001, **Figure 2B**).

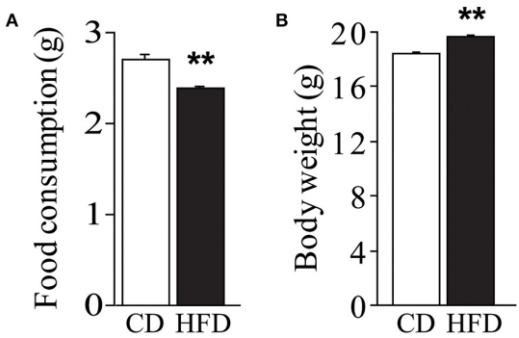

Figure 2. Effect of high-fat feeding on dams. Food consumption **(A)** and body weight **(B)** of dams feed HFD. Data are shown as + s.e.m. *Post-hoc* comparisons, ∗∗p< 0.01(hfd *vs.* cd). Experimental subjects, cd-wt/ko, n= 37/34; hfd-wt/ko, n= 40/37.

2.3.2. Success of birth, cannibalistic behavior and litter size

Feeding HFD results in an increased dams' mortality associated to pregnancy (Fisher's exact probability test: p= 0.0266, **Table 1A**) and increased frequency of cannibalistic episodes (Fisher's exact probability test: p= 0.0308, **Table 1B**) only in WT subjects; interestingly such phenomena were strongly reduced in the $p66^{Shc-/-}$ dams. Regardless of genotype, the HFD affected the littersize reducing the number of pups (main effect of maternal diet: $F_{(1,64)}$= 9.134, p= 0.0036, **Table 1C**).

Table 1. Success of birth, cannibalistic behavior and pups per litter

A. Dams' mortality

Diet	Genotype	Dead	Alive
CD	$p66^{Shc+/+}$	8	16
CD	$p66^{Shc-/-}$	3	16
HFD	$p66^{Shc+/+}$	11	23
HFD	$p66^{Shc-/-}$	2	25

B. Dams' cannibalistic behavior

Diet	Genotype	Cannibalism	No cannibalism
CD	$p66^{Shc+/+}$	5	16
CD	$p66^{Shc-/-}$	3	16
HFD	$p66^{Shc+/+}$	8	23
HFD	$p66^{Shc-/-}$	1	25

C. Number of pups per litter

Diet	Genotype	Mean
CD	$p66^{Shc+/+}$	5.3 ± 2.4
CD	$p66^{Shc-/-}$	4.5 ± 2.1
HFD	$p66^{Shc+/+}$	3.3 ± 2.2
HFD	$p66^{Shc-/-}$	3.3 ± 1.8

Reduced mortality associated to pregnancy **(A)** and reduced frequency of cannibalistic episodes **(B)** in KO females fed HFD. Reduced size of litters on HFD **(C)**. In the section **(A,B)**, the table reports the number of dams on diet; in the section **(C)** the table reports the average number of pups per litter ± s.e.m.

2.3.3. Offspring body weight and BMI

Overall, exposure to maternal HFD resulted in a decreased body weight at P3 (main effect of maternal diet: $F_{(1,58)}$= 3.835, p= 0.05, data not shown), in the p66$^{Shc-/-}$ offspring (interaction between maternal diet and genotype: $F_{(1,58)}$= 12.120, p= 0.0010, **Figure 3A**). This effect was reverted during growth (P30) (main effect of maternal diet: $F_{(1,85)}$= 15.225, p= 0.0002, data not shown), particularly in males (interaction between maternal diet and gender: $F_{(1,85)}$= 4.099, p= 0.0461, data not shown). In addition, at this age, a specific HFD-induced increase in body weight was found in the p66$^{Shc-/-}$ offspring (interaction between maternal diet and genotype: $F_{(1,85)}$= 6.252, p= 0.0143, **Figure 3B)**. Thus, prenatal HFD was able to override the p66$^{Shc-/-}$ lean phenotype (**Figure 3B**). Overall, the fatting effect of the HFD was maintained until adulthood (P90), when HFD offspring, regardless of genotype and gender, showed a higher body weight than CD subjects (main effect of maternal diet: $F_{(1,85)}$= 4.930, p= 0.0291, data not shown). The interaction between maternal diet and gender, observed at P30, was overturned during growth (P90), when higher body weight was observed only in HFD females (interaction between maternal diet and gender: $F_{(1,85)}$= 6.018, p= 0.0162, data not shown). As for P30, while no difference was observed in WT subjects, KO-HFD offspring maintained a higher body weight compared to KO-CD until 3 months of age (P90) (interaction between maternal diet and genotype: $F_{(1,85)}$= 8.236, p= 0.0052, **Figure 3C**), particularly in males (interaction among maternal diet, genotype and gender: $F_{(1,85)}$= 8.447, p= 0.0047, data not shown).

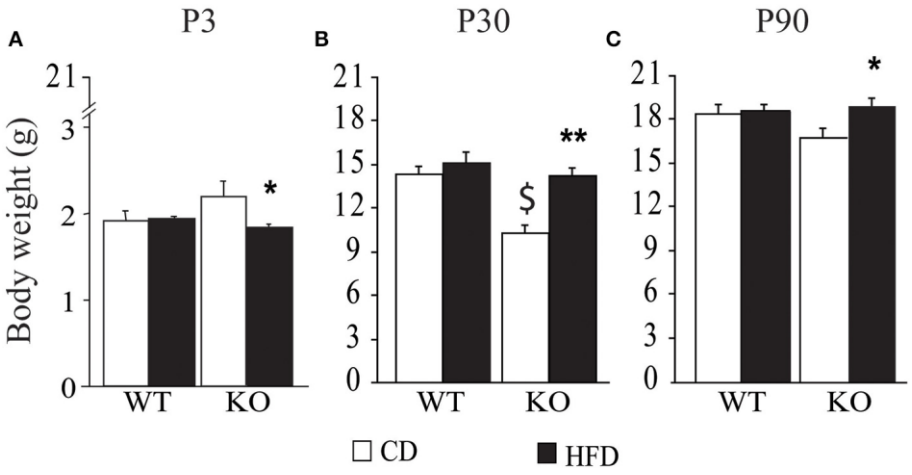

Figure 3. Body weight of the offspring after birth, P3(A), at weaning, P30 (B), and at 3 months of age, P90 (C). Data are shown as + s.e.m. *Post-hoc* comparisons, $*p<$ 0.05 (P3 and P90: hfd-ko *vs.* cd-ko); $ $p<$ 0.01 (P30: cd-ko *vs.* cd-wt); $**$ $p<$ 0.01 (P30: hfd-ko *vs.* cd-ko). Experimental subjects, P3: cd-wt, n= 16; cd-ko, n = 15; hfd-wt, n= 15;hfd-ko, n= 20; P30 and P90: cd-wt-f/m n= 15/10; cd-ko-f/m n= 9/14; hfd-wt-f/m n= 11/9; hfd-ko-f/m n= 11/14.

Regardless of genotype, maternal HFD reduced the difference in BMI usually observed between genders, increasing the BMI of female mice and rendering them more similar to males (interaction between maternal diet and gender: $F_{(1,60)}$= 3.820; p= 0.05, **Figure 4)**.

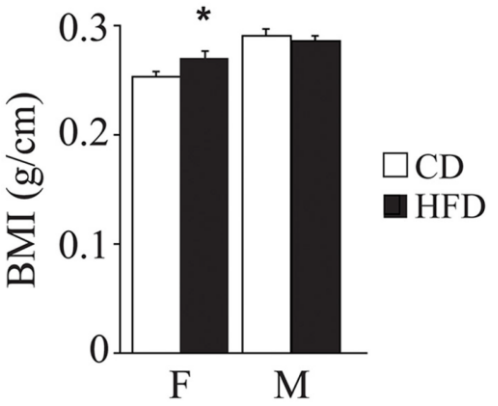

Figure 4. Body mass index. Data are shown +s.e.m. *Post-hoc* comparisons, $*p< 0.05$ (hfd-f *vs.* cd-f). Experimental subjects, cd-wt-f/m n = 8/9; cd-ko-f/m n = 8/9; hfd-wt-f/m n = 8/9; hfd-ko-f/m n = 7/10.

2.3.4. Onset of puberty

Offspring of HFD- and CD-fed dams did not differ as for their onset of puberty (main effect of maternal diet: $F_{(1,130)}$= 0.750; p= 0.3881; interaction among maternal diet, genotype and gender: $F_{(1,130)}$= 0.790; p= 0.3757, data not shown). However, and most intriguingly, while in CD subjects this parameter was associated to changes in body weight ($F_{(1,14)(1,9)(1,9)(1,10)}$= 6.213; 12.808; 14.582; 6.526, p= 0.027; 0.0072; 0.0051; 0.0339, $R2$= 0.323; 0.616; 0.646; 0.449, respectively for females WT-CD and KO-CD; males WT-CD and KO-CD, **Figure 5B)**, this did not occur in HFD offspring ($F_{(1,19)(1,10)(1,19)(1,13)}$= 0.041; 1.496; 0.006; 0.923, p= 0.8945; 0.8444; 0.9419; 0.3557, $R2$= 0.005; 0.143; 0.001; 0.071, respectively for females WT-HFD and KO-HFD; males WT-HFD and KO-HFD, **Figure 5A)**, suggesting that the exposure to HFD during fetal programming might have affected the surge of gonadal hormones, resulting in a disorganized pubertal development. Regardless of diet, lack of $p66^{Shc}$ delayed the time of puberty (main effect of genotype: $F_{(1,130)}$= 6.987, p= 0.0092, data not shown) probably by the lower body weight characterizing the transgenic model and that is known to be relevant in the onset of puberty. This latter point could also justify the delayed onset of puberty observed in female mice (main effect of gender: $F_{(1,130)}$= 20.935, $p< 0.0001$, data not shown).

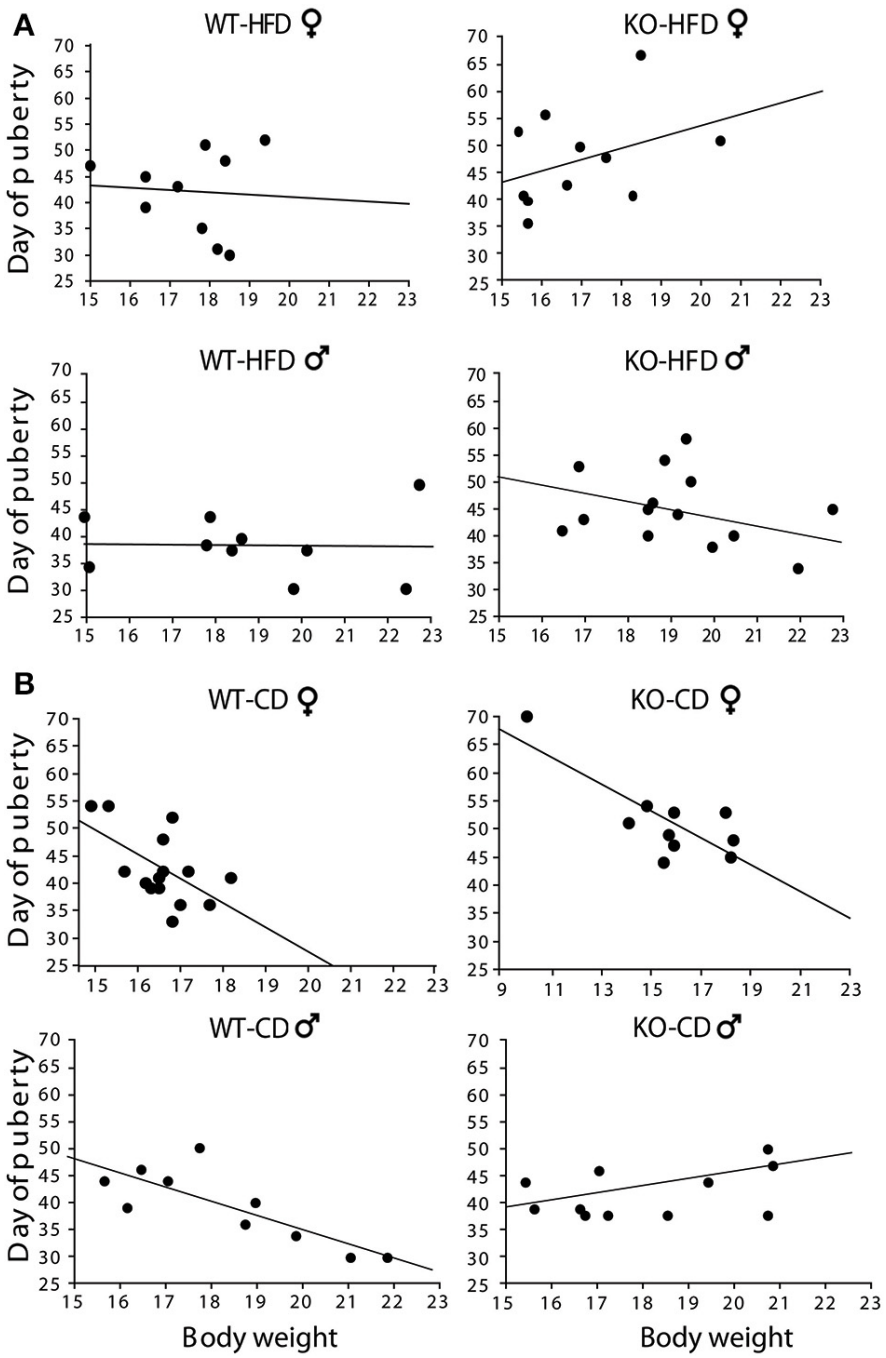

Figure 5. Onset of puberty. Correlation between offspring's body weight and timing of puberty in male and female HFD **(A)** and CD **(B)** offspring. Experimental subjects: cd-wt-f/m, *n*= 15/10; cd-ko-f/m, *n*= 10/11 hfd-wt-f/m, *n*= 10; hfd-ko-f/m, *n*= 11/14.

2.3.5. Metabolic regulation

• Glucose tolerance test (GTT). In both genders and regardless of prenatal diet, $p66^{Shc-/-}$ mice were resistant to an increase in blood glycemia upon glucose injection (main effect of genotype: $F_{(1,77)(1,66)}$= 11, 817; 9.018, p= 0.0009; 0.0038, respectively for male and female subjects, inserts **Figures 6A, B)**. In addition, the HFD affected the metabolic response to GTT in an opposite manner in the two genders, overriding the $p66^{Shc-/-}$ resilience, rendering the glycemic response of $p66^{Shc-/-}$ male mice comparable to that observed in control subjects (interaction among maternal diet, genotype and time course: $F_{(4,308)}$= 1.078; p= 0.3676, **Figure 6A**) and, on the contrary, exacerbating the metabolic resilience of $p66^{Shc-/-}$ females (interaction among maternal diet, genotype and time course: $F_{(4,264)}$= 4.240; p= 0.0024, **Figure 6B**).

• Insulin sensitivity test (IST). Regardless of genotype, prenatal exposure to HFD led to insulin insensitivity later in life (3-months-old) as demonstrated by the higher glycemia observed in young adults (main effect of maternal diet: $F_{(1,77)(1,66)}$= 3.702; 15.504, p= 0.05; 0.0002, respectively for male and female subjects, inserts **Figures 6C, D)**. As already observed in the GTT, overall, KO mice were characterized by a resilient metabolic profile showing higher blood glucose levels in response to a stimulus of insulin (main effect of genotype: $F_{(1,77)(1,66)}$= 21.473; 6.635, p< 0.0001; 0.0122, respectively for male and female subjects, inserts **Figures 6C, D**).

• Metabolic hormones**.** Overall, exposure to maternal HFD did not affect plasma levels of adiponectin in adul toffspring (main effect of maternal diet: $F_{(1,62)}$= 2.392; p= 0.1271, data not shown). In addition, females were characterized by higher peripheral levels of adiponectin than males (main effect of gender: $F_{(1,62)}$= 5.497; p= 0.0223, data not shown). While no differences were observed among male mice regarding both diet and genotype, a greater metabolic variability was found in females. KO female mice prenatally exposed to CD were characterized by higher plasma levels of adiponectin than the WT females and KO males exposed to the same maternal diet (interaction among maternal diet, genotype and gender: $F_{(1,61)}$= 12.691; p= 0.0007, **Figure 7A**). Females were characterized by higher levels of adiponectin also in the adipose tissue (main effect of gender: $F_{(1,32)}$= 6.814; p= 0.0136, data not shown), in particular in the mesenteric fat tissue (interaction between gender and adipose tissue: $F_{(1,32)}$= 10.396; p= 0.0029, data not shown). In addition, we found a strong tendency in increased levels of adiponectin in the mesenteric fat tissue in KO females (interaction among genotype, gender and adipose tissue: $F_{(1,32)}$= 3.778; p= 0.0608, data not shown).

Regardless of genotype, prenatal exposure to HFD led to a sig nificantly higher plasma levels of leptin in females, compared to males (interaction between maternal diet and gender: $F_{(1,50)}$= 5.503; p= 0.0230, **Figure 7B)**.

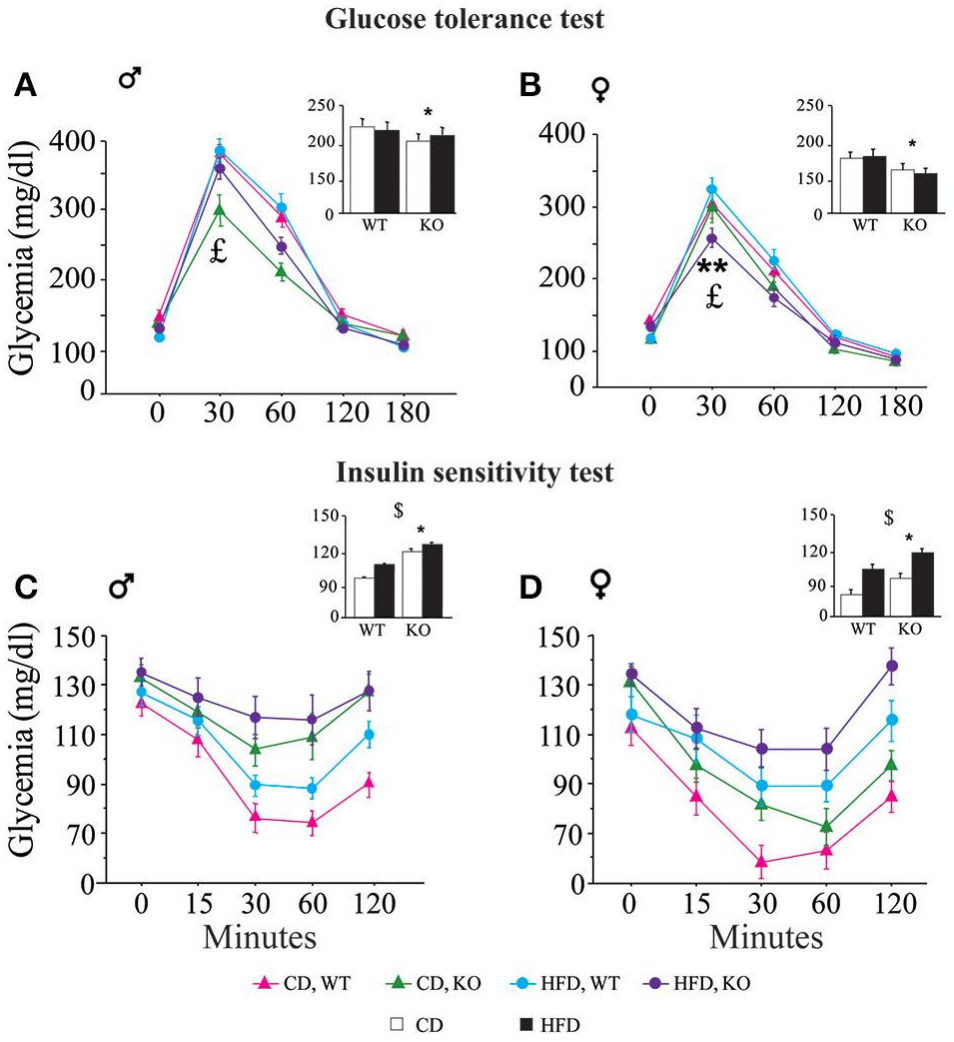

Figure 6. Metabolic regulation. Glucose tolerance assessment in WT and KO male **(A)** and female **(B)** offspring fed CD or HFD. Insulin sensitivity test in WT and KO male **(C)** and female **(D)** offspring fed CD or HFD. Data are shown as ± s.e.m. *Post-hoc* comparisons, $ $p< 0.05$ (cd *vs.* hfd); ∗ $p< 0.05$ (ko *vs.* wt); ∗∗ $p< 0.01$. Experimental subjects: cd-wt-f/m, $n= 17/22$; cd-ko-f/m, $n= 18/19$; hfd-wt-f/m, $n= 18/22$; hfd-ko-f/m, $n= 17/18$.

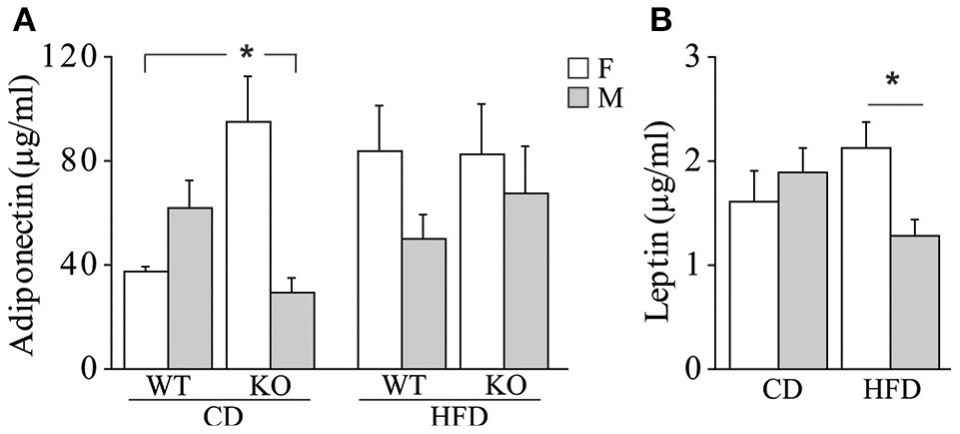

Figure 7. Metabolic hormones. Plasma levels of adiponectin **(A)** and leptin **(B)** in male and female mice prenatally exposed to CD or HFD. Data are shown as + s.e.m. *Post-hoc* comparisons, ∗ $p< 0.05$ (adiponectin: cd-ko-f *vs.* cd-wt-f and cd-ko-m; leptin: hfd-f *vs.* hfd-m). Experimental subjects, adiponectin assessment: cd-wt-f/m, $n= 9$; cd-ko-f/m, $n= 8/9$; hfd-wt-f/m, $n= 8/9$; hfd-ko-f/m, $n= 8/10$; leptin assessment: cd-wt-f/m, $n= 7/9$; cd-ko-f/m, $n= 8/7$; hfd-wt-f/m, $n= 5/7$; hfd-ko-f/m, $n= 7/8$.

2.3.6. Neuroendocrine activation

In female mice maternal HFD exacerbated the HPA axis response to an acute psychophysical stress, increasing the plasma levels of corticosterone (main effect of maternal diet: $F_{(1,32)}= 6.058$, $p= 0.0194$, data not shown). The interaction between maternal diet and genotype was statistically significant ($F_{(1,32)}= 7.395$, $p= 0.0105$, **Figure 8)**. In particular, while the WT females showed a marked increase in corticosterone levels in response to stress when exposed to maternal HFD, KO females were protected from the negative effects of the maternal HFD, showing a neuroendocrine response similar to KO-CD (**Figure 8A**). Moreover, KO females prenatally exposed to HFD showed a prompt feedback of the HPA axis activation displaying a neuroendocrine profile more similar to that of WT-CD subjects (interaction between maternal diet, genotype and time course: $F_{(3,96)}= 2.583$; $p= 0.05$, **Figure 8B)**. No differences in the neuroendocrine activation were observed in male mice in response to the acute restraint stress (interaction among maternal diet, genotype and time course: $F_{(3,87)}= 0.735$; $p= 0.5342$, data not shown).

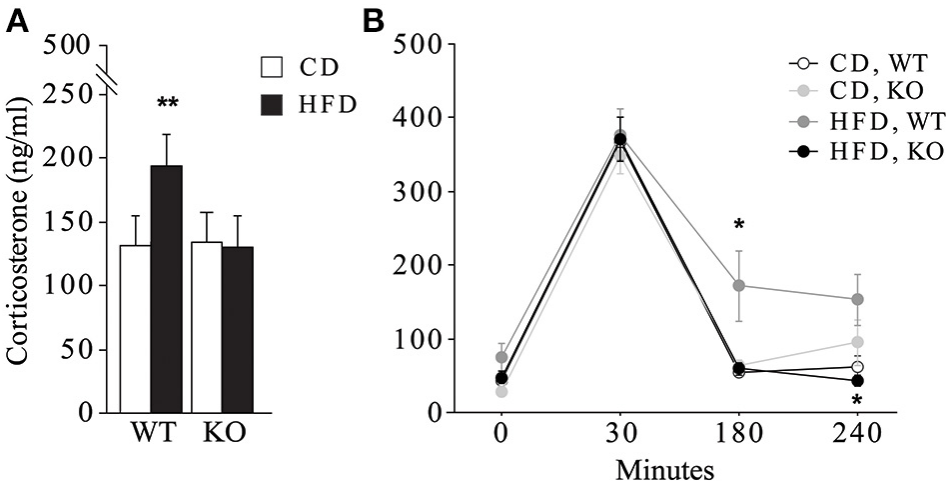

Figure 8. Neuroendocrine activation. Neuroendocrine activation in female mice in response to an acute restraint stress **(A)** and feedback response of the HPA axis activation **(B)**. Data are shown as + s.e.m. *Post-hoc* comparisons, ∗ $p< 0.05$ (hfd-wt *vs.* hfd-ko and wt-cd, at 180 min; hfd-ko *vs.* hfd-wt, at 240 min); ∗∗ $p< 0.01$ (hfd-wt *vs.*cd-wt and hfd-ko). Experimental subjects: cd-wt, $n= 10$; cd-ko, $n= 8$; hfd-wt/ko, $n= 9$.

2.3.7. Behavioral phenotype

• Open field(OF). Overall, regardless of prenatal diet and genotype, all subjects - both males and females – spent significantly less time in the center of the arena, that represents the anxiogenic portion of the apparatus (main effect of zones: $F_{(1,56)}= 5751.755$; $p< 0.0001$, data not shown). Regardless of both genotype and gender, prenatal exposure to HFD gradually reduced locomotion (duration of *crossings*) during the test (interaction between maternal diet and tb: $F_{(2,178)}= 3.910$; $p= 0.0218$, **Figure 9A)**. While no difference emerged between HFD subjects, interestingly, from the assessment of spontaneous behavior emerged that WT subjects performed an uncommon behavior of *jumping* toward the wall of the apparatus and more frequently than KO (Fisher's exact probability test: $p= 0.0361$, data not shown). This behavior indicates an attempt to leave the apparatus and greater risk-taking and aggressive exploration representing an escaping strategy from a novel context. This data suggests that prenatal exposure to HFD reduced activity both in WT and KO mice and that overall KO subjects were less anxious than WT.

• Elevated plusmaze(EPM). Regardless of prenatal diet, genotype and gender, overall, all subjects spent more time in the closed arms of the maze compared to both center and open arms (main effect of zones: $F_{(2,176)}= 29.312$; $p< 0.0001$, data not shown). In addition, prenatal diet, genotype and gender did not affect the percent frequency of entries in the open arms (main effects respectively of prenatal diet, genotype and gender: $F_{(1,58)}= 0.038$; 0.497; 1.150; $p= 0.8463$;

0.4838; 0.2879, data not shown). Regardless of genotype, exposure to maternal HFD *in utero* increased the duration of *immobility*, a reliable index of anxiety-like behavior, in male mice and seemed to reduce that in females (interaction between maternal diet and gender: $F_{(1,88)}$= 3.735; p= 0.05, **Figure 9C)** suggesting a feminization of males exposed to maternal HFD. A similar gender inversion of the behavioral phenotype in response to prenatal exposure to HFD was also related to the time spent in displacement behavior (*grooming*) (interaction between maternal diet and gender: $F_{(1,88)}$= 7.245; p= 0.0085, **Figure 9B**). The inversion in the anxious profile in the subjects prenatally exposed to an obesogenic diet suggests that HFD affected the behavioral profile differently in the two genders, increasing anxiety in male mice and rendering females more uninhibited.

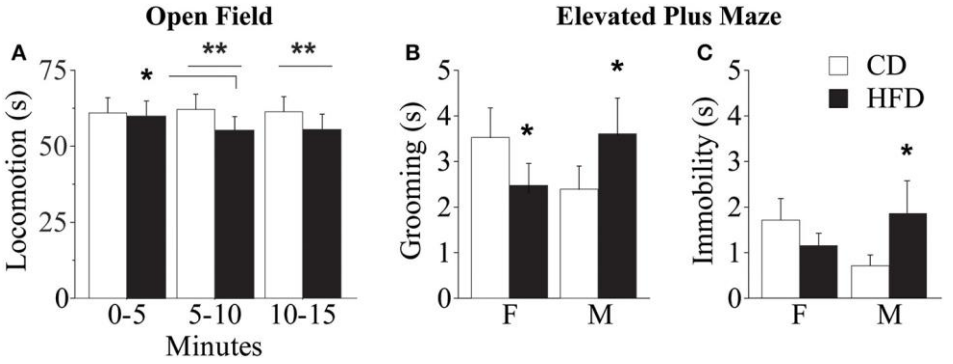

Figure 9. Behavioral phenotype. Duration of locomotor activity in the Open Field test **(A)**. Feminization of males' emotional profile prenatally exposed to HFD measured by the duration of *grooming* **(B)** and *immobility* **(C)** in the Elevated Plus Maze test. Data are shown as + s.e.m. *Post-hoc* comparisons, ∗ p< 0.05 (OF: hfd 0-5 *vs.* 5-10); ∗∗ p< 0.01 (hfd *vs.* cd at 5-10 and 10-15 min); ∗ p< 0.05 (EPM: hfd-f *vs.* cd-f; hfd-m *vs.* cd-m). Experimental subjects, n= 12.

2.4. DISCUSSION

Overall, results of this study reveal that HFD, administered before and during pregnancy, has detrimental effects on both the dam and the offspring. Reproductive success in both WT and KO females is compromised by exposure to HFD, which leads to increased maternal mortality rate during pregnancy and increased frequency of cannibalistic episodes immediately after delivery. The most relevant findings are the long-lasting and gender-dependent effects of maternal HFD feeding on metabolic and neuroendocrine profile of the adult offspring. Maternal HFD produced more marked consequences in the adult female offspring, such as increased BMI and higher leptin levels compared to the CD group. Even greater effects were found in interaction with $p66^{Shc}$

deletion, KO female offspring showing enhanced glucose tolerance and insulin resistance, in addition to being protected from HPA axis hyperactivity induced by HFD.

Our data indicate that high-fat feeding for 13 weeks till delivery results in maternal body weight gain accompanied by a reduced daily HFD consumption, possibly due to the high energetic and caloric intake associated with HFD. In addition, we found an increased mortality rate of pups born from HFD-fed dams, as a result of cannibalistic events and a strong association between HFD and maternal mortality in the days immediately before the expected delivery date, or during parturition. Furthermore, we found that maternal HFD results in a decreased number of pups delivered that may be due to composition of dietary fatty acids which reportedly affect fertility and induce a higher resorption number (Buckley et al., 2005). It is worth noting that similar findings have been previously reported by Flint and co-workers, in mice, and others in a different species, the rat, further corroborating the critical role played by maternal diet in such time window (Siemelink et al., 2002; Buckley et al., 2005; Flint et al., 2005). In this context, the lack of the $p66^{Shc}$ gene plays a protective role, at least for selected parameters such as dam mortality and cannibalistic behavior.

We have observed that pups of HFD-fed dams showed a significantly lower body weight at birth (P3) but an increased weight during adolescence and at adulthood (P30 and P90). Such effect was significantly marked only in the $p66^{Shc-/-}$ mice. These data are consistent with previous studies reporting that poor fetal growth programs pups to quickly catch-up in adult life (Ozanne and Hales, 2004; Bieswal et al., 2006). Also Flint, et al., 2005 found a markedly decreased weight gain in mice pups of HFD-fed dams during the first day of lactation, as a result of an impairment of the mammary gland function, and consequent reduced milk production, induced by the diet. However, in line with our results showing an increased body weight in HFD offspring during adolescence and adulthood, such impairment was recovered in the following days (Flint et al., 2005).

Despite HFD induced differences in body weight, no change has been registered in the BMI. Such discrepancy may be due to the fact that, notwithstanding BMI is considered an adequate index of adiposity, it is poor at discriminating the ratio of fat to lean tissue (Wells et al., 2006), thus resulting not strictly related to body weight. Indeed, in the maternal-HFD exposed offspring, adult females, compared to males, had a lower body weight while showing an increased BMI.

In the present study, we did not find an effect of HFD on the onset of puberty either in males or in females. These results are apparently not in line with some previous studies showing that HFD exposure during pregnancy and/or the early postnatal period advances puberty onset in female rats (Boukouvalas et al., 2008; Chang et al., 2008; Sloboda et al., 2009; Akamine et al., 2010; Boukouvalas et al., 2010; Li et al., 2012). Such discrepancy may be

due to the fact that percent of fat content has been suggested to be critical for the quality of the effects produced by the diet (Chang et al., 2008; Moral et al., 2011; Lie et al., 2013). Indeed, advancement of puberty onset has been found in studies using a diet containing not more than 45% of fat, while the HFD used in the current study, with a 58% fat content, did not advance puberty onset as in other studies using similar diets (Lie et al., 2013).

In addition to diet, body weight affects puberty according to an inverted relation, with increased body weight associated with earlier pubertal onset (Rosenfield et al., 2009). Accordingly, we found such inverted relation in experimental groups exposed to CD. However, in the HFD-KO group, the increased body weight did not go with a change in the mean age of puberty. This may be due to the disrupting effect of HFD on such relationship described here and in previous studies. In particular, perturbations of the timing of female puberty has been found in rats exposed to metabolic stress *in utero*, induced through administration of hypercaloric diet to the mother (Ibanez and de Zegher, 2006b, 2006a; Ibanez et al., 2006). This effect might be mediated by an alteration of gonadal hormones secretion (Mah and Wittert, 2010; Teerds et al., 2011; Pinilla et al., 2012) resulting in a disorganized pubertal development of the offspring. Such effect warrants to be further investigated.

Here we found that $p66^{Shc}$ gene regulates response to metabolic stimuli. In particular, KO mice show a greater resistance toward glucose challenge compared to WT. Such effect was affected by the diet in a gender-dependent fashion. Indeed, in KO females prenatally exposed to HFD, we observed a higher glucose tolerance demonstrating a better ability to metabolize the excess of blood glucose while, in KO males, we found an opposite profile as HFD determined severe glucose intolerance in comparison with their own controls (CD-KO). It is not possible to exclude that measuring glycemia sooner after the metabolic challenge (e.g., after 15 min) might provide additional information.

In the light of these points, it is reasonable to suggest that HFD overrides the effects of $p66^{Shc-/-}$ on metabolism in males, reducing the glucose resilience, and exacerbating it in females. Accordingly, we found that the lack of the $p66^{Shc}$ gene leads to greater resistance in the IST test. Resilience to an insulin challenge was affected not only by the genotype, but also by the maternal diet, since HFD individuals showed greater insulinin sensitivity. In particular, such insulin resistance has been suggested by (Turdi et al., 2013) to result from a severely dampened insulin-signaling-cascade. The metabolic alterations described in KO mice may be due to the reported involvement of $p66^{Shc}$ gene in insulin signaling (Berniakovich et al., 2008), which leads to hypothesize that its deletion results in insulin desensitization with the consequent impairment in the metabolism of glucose.

Leptin is involved in the long-term regulation of body weight and energy balance by acting as a hunger suppressant signal to the brain and has been found to be increased in obese subjects (Hamed et al., 2011). In the present study, maternal HFD results in a difference in leptin plasma levels between

genders in the adult offspring. Prior and colleagues found that offspring from HFD-fed dams exhibits an altered hypothalamic sensitivity to leptin, suggesting that metabolic alteration occurring in a susceptible time window for the developing organism impairs central mechanisms regulating leptin secretion at adulthood (Prior et al., 2013). Previous studies have already demonstrated that the maternal environment affects fetal programming, driving the development of central nervous system circuitries through a different endocrine regulation in the two genders (Maccari et al., 2003; Seckl and Holmes, 2007; Reynolds et al., 2013; Spencer, 2013).

Increased adiponectin levels were found both in plasma and in adipose tissues in KO females. Adiponectin is produced by adipocytes and its expression is inversely related to adipose tissue mass (Yamauchi et al., 2001; Antoniades et al., 2009; Ntaios et al., 2013), a result in line with the reduction of fat tissue found in KO females. In addition, as adiponectin enhances hippocampal neurogenesis (Zhang et al., 2011), the increased levels of this adipokine in KO females fit with our previous data showing increased neurogenesis in aged KO females (Berry et al., 2012).

One of the main findings of this study is the programming effect of HFD on the neuroendocrine fetal development, which leads to greater susceptibility to stressful challenges later in life. In particular, we found that HFD significantly increases the response to an acute restraint stress in adult WT females. This effect may be due to an impairment of the negative feedback of the HPA axis, as suggested by the long lasting increase in corticosterone levels induced by the stressor. Interestingly, despite their exposure to maternal HFD, KO females were protected from such effect, showing an efficient HPA axis feedback, undistinguishable from CD. It is worth noting that the alterations of the neuroendocrine profile induced by maternal HFD overlap with those induced by prenatal stress. For instance, Louvart and colleagues reported that female offspring of rat dams exposed to a chronic restraint stress during the last trimester of pregnancy display at adulthood a higher response to restraint stress (Louvart et al., 2009). The overlapping consequences of maternal HFD and exposure to stressful conditions during the prenatal period leads us to hypothesize that maternal HFD mimics psychophysical stress, as both interfere with the fetal programming of neuroendocrine development, altering the HPA axis activity during the entire lifespan (Maccari et al., 2003; Seckl and Holmes, 2007; Reynolds et al., 2013; Spencer, 2013).

Previous studies have reported increased anxiety-like behavior in adult subjects of both genders exposed to HFD during fetal development (Peleg-Raibstein et al., 2012), although in some cases a gender dependent effect is reported (Bilbo and Tsang, 2010). In line with the latter finding, we show different effects in males and females. In particular, we observed increased *grooming* levels in the EPM, considered an endpoint of anxiety-like behavior, in CD females compared to CD males. Such difference in basal levels of anxiety response between genders is widely described in the literature (Kessler et al.,

1994; Weissman et al., 1994; Kessler et al., 1995; Weissman et al., 1996; Gater et al., 1998). However, such profile was inverted by the exposure to maternal HFD, suggesting a feminization of the emotional profile of male mice. This is especially interesting in light of the results from prenatally stressed subjects, which are characterized by the same feminization effect (Weinstock, 2001). Such finding corroborates the idea that prenatal metabolic alterations acts as a psychophysical stressful stimulus with potential implications for brain function, both at adulthood and during the aging process, confirming the data about the neuroendocrine profile observed in HFD-WT females (Maccari et al., 1995; Maccari et al., 2003; Spencer, 2013).

Overall, results from this study indicate that maternal HFD might represent a detrimental metabolic stimulus for the dam and, in particular, for the offspring. Since our model of HFD feeding did not lead to maternal obesity, the observed effects on the offspring are purely due to alterations of maternal metabolism. In particular, in our study, maternal HFD seems to be a condition sufficient to induce "metabolic misprogramming" causing offspring metabolic and neuroendocrine alterations. Moreover, the gender-dependent effects here described might suggest a potential modulating role played by sex hormones on the long-term consequences of maternal HFD on the offspring. To explore such hypothesis further studies are warranted.

Finally, the overall resilience of the KO in response to metabolic challenges confirms the notion that $p66^{Shc}$ is an important molecular target for future studies investigating pathological states induced by stressful or metabolic factors during early life.

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CHAPTER 3

3. ANTIOXIDANTS COUNTERACT LONG-TERM METABOLIC IMPAIRMENT FOLLOWING PRENATAL EXPOSURE TO HIGH-FAT DIET IN MICE

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ABSTRACT

Previous data have shown that maternal high-fat diet (HFD) throughout gestation has long-term consequences on the offspring's metabolic phenotype. More interestingly, these data showed a reduced incidence of the detrimental long-lasting effect of maternal HFD during fetal development in an animal model of reduced oxidative stress (OS), the $p66^{Shc-/-}$ mouse, thus suggesting a protective role played by this condition in a such susceptible period of the individual life. We hypothesized that a fetal environment characterized by decreased OS might represent a healthy condition able to thwart the effect of maternal HFD on the fetal programming, possibly reducing HFD-induced long-term effects on the offspring both on metabolic and emotional phenotype. To test such hypothesis, female C57BL/6J mice were fed a HFD for 13 weeks, starting on 5 weeks of age until delivery, and were exposed to N-acetyl-cysteine (NAC) drug for 8 weeks (from 10 weeks of age until right before delivery). Adult offspring of both genders were tested at three months of age for their metabolic, neuroendocrine and emotional profile. Overall, results of the study show that prenatal exposure to NAC counteracted some of the negative effects played by HFD on the adult offspring. Despite that, NAC during pregnancy failed to restore reproductive success in HFD female mice. NAC drug reproduced the metabolic effects of the $p66^{Shc}$ gene deletion, conferring glucose tolerance and insulin resistance to young adult male offspring. In addition, maternal HFD and NAC differently affected the metabolic phenotype of the offspring of both genders, suggesting a long-lasting and gender-dependent effect. Moreover, prenatal NAC overturned the effect of maternal HFD on the emotional and behavioral phenotype of the offspring. Overall, NAC mimicked the effects associated to the lack of the $p66^{Shc}$ gene, at least as concerning the metabolic response, confirming the strict relationship between oxidative stress and metabolism.

Keywords: antioxidant, N-acetyl-cysteine, high-fat diet, pregnancy, metabolism, animal models.

3.1. INTRODUCTION

In modern society the consumption of high fat diet (HFD) as well as a sedentary lifestyle has been associated with metabolic derangements and impairment of brain function (Schrauwen and Westerterp, 2000; Unger and Orci, 2001; McGarry, 2002; Molteni et al., 2002; Molteni et al., 2004). While it is generally accepted that HFD-induced obesity *per se* is associated with adverse health outcomes, studies have also shown that maternal obesity is specifically associated with a variety of pregnancy complications, fetal and neonatal death, maternal hypertension and gestational diabetes (Lu et al., 2001; Bhattacharya and Sood, 2007; Thompson, 2008). It is possible that maternal obesity might precipitate adverse outcomes that persist through adulthood in offspring. Indeed, evidence suggests that epigenetic events initiated during the prenatal period can result in persistent adaptations in structure, physiology and metabolism predisposing offspring towards disease and impaired physiology (Barker, 1995; Lucas, 1998). Furthermore, in addition to negatively impacting metabolic regulation, resulting in excess energy intake, increased adiposity and overall impaired several metabolic processes (Woods et al., 2004; Zhang et al., 2009), HFD is also associated with a low grade inflammation and an increased production of inflammatory cytokines (Chandalia and Abate, 2007).

Several studies have shown that administration of high fat or high calorie diets to rodents increases the generation of reactive oxygen species (ROS) (Zhang et al., 2005) and protein oxidation (Souza et al., 2007), thus resulting in increased oxidative damage in the brain (Cecarini et al., 2007; Bruce-Keller et al., 2009; Zhang et al., 2009) and, more in general, in oxidative cell injury (Gutteridge and Halliwell, 2000). This experimental evidence is consistent with the possibility that increased oxidative stress (OS), in turn, mediates the effects of HFD consumption both on brain pathogenesis and cognitive disturbances and exacerbates HFD-related metabolic impairment.

In a previous work (Bellisario et al., 2014) it was found that the detrimental effect of prenatal exposure to a maternal HFD was counteracted by the lack of the $p66^{Shc}$ gene that results, among several features, in reduced OS and resistance to diet-induced obesity. These findings suggested that the reduced OS might represent the key mechanism resulting in increased resistance to impaired physiological adaptation driven by maternal HFD in offspring during adult life. In light of that, this study was aimed at developing a pharmacological model of reduced OS able to cope with metabolic challenges occurring during early life by means of prenatal administration of N-acetyl-cysteine (NAC). This drug is an analogue and precursor of reduced glutathione and its clinical efficacy and safety have been widely established in the lasts decades as a mucolytic drug, in the therapy of respiratory diseases and, more recently, as a remedy towards several acute intoxications (Crystal, 1991). More importantly, NAC works through a variety of coordinated mechanisms, the most important of which depend on its remarkable antioxidant properties, which

warrant possible applications in the prevention of cancer and other pathological conditions associated with damage by free radicals (De Flora et al., 1995a; De Flora et al., 1995b). Given this evidence, we hypothesize that the administration of the antioxidant NAC during prenatal life might counteract with a greater efficacy the negative effects exerted by the maternal HFD on fetus development. To test such hypothesis female mice were fed a HFD or control diet (CD) for 13 weeks (from 5 weeks of age until right before delivery) and were exposed to NAC drug for 8 weeks (from 10 weeks of age until right before delivery). The offspring was subsequently phenotyped for metabolic and behavioral responses at adulthood.

3.2. MATERIALS AND METHODS

3.2.1. Animals

Experimental subjects were the male and female adult offspring (3 months of age) of C57BL/6J female mice fed experimental diets.

All mice were housed 2/cage in transparent Plexiglas cages provided by Tecniplast, in an air-conditioned room (temperature 21±1°C, relative humidity 60±10%), under a reversed 12/12 h light/dark cycle with lights off from 07:00 a.m. to 07:00 p.m. Fresh tap-water and standard chow (standard diet - SD - energy 3.3kcal/g, fat 17%, carbohydrate 60 % and protein 23 % provided by Altromin-R, Rieper, Italy) were continuously available until 5 weeks of age.

3.2.2. Diet administration

At 5 weeks of age, all females (n= 77) were fed *ad libitum* either with a hypercaloric (high-fat diet- HFD - energy 5.56 kcal/g, fat 58%, carbohydrate 25.5% and protein 16.4%; n= 43) or a control diet (CD - energy 4.07 kcal/g, fat 10.5%, carbohydrate 73.1% and protein 16.4%; n= 34) for 13 weeks, i.e. until right before delivery. Females were randomly assigned to HFD or CD groups avoiding difference in the average of body weight between groups. Obesogenic (D12331) and Control (D12328) diets were be provided by Research Diets, Inc., New Brunswick, NJ, USA.

3.2.3. Antioxidant administration

After 5 weeks on the diets, all females underwent antioxidant treatment with NAC, for 5 weeks, until the end of dietary treatment. NAC (Sigma-Aldrich) was daily administered in drinking water (Balansky et al., 1996), in order to minimize stress due to excessive handling procedure by the experimenter, to yield an average dose of 1 g NAC/kg body weight.

3.2.4. Breeding procedure

At 15 weeks of age, after 10 weeks on the diet and 5 weeks of NAC administration, all females were bred. In order to optimize reproductive success

of experimental females, a handful of sawdust from a male's cage was added to the females' cages every morning for 3 days, so that males' pheromones from dirty sawdust (urine) induced the estrus cycle (also known as Whitten effect) (Brockman et al., 1998). On the 4th day, a male was introduced in each cage and females were checked twice a day for the presence of a vaginal plug to tentatively confirm pregnancy. If a vaginal plug was observed, the male was removed from the females' cage. When the vaginal plug was not observed the male were kept in the females' cage for 10 days and the body weight was weekly checked.

After mating, dams were kept with either HFD or CD and NAC throughout gestation until 3 days before the expected delivery date, that is at the gestational day 16 (G16). At this data, the antioxidant treatment (NAC) was stopped and all dams were shifted on SD.

Body weight gain was monitored once a week throughout the 5 weeks of combined exposure to HFD and NAC. Birth success was also registered. Pups birth was considered as post-natal day 0 (P0). At P3 pups' weight was registered, as well as at P30, when all pups were also weaned onto SD. At 3 months of age, offspring of both genders were weighed and tested to assess the metabolic and emotional profiles resulting from prenatal exposure to a hypercaloric diet and the role of antioxidants on these regulations.

A schematic design of the experimental plan is reported in **Figure 1**.

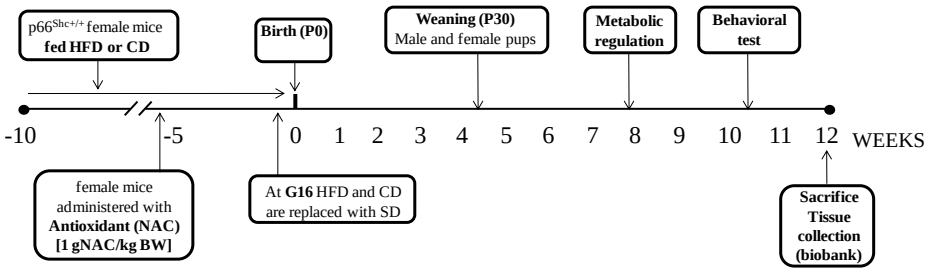

Figure 1. Schematic design of the experimental plan.

Animal handling and experimental procedures were performed in accordance with the EC guidelines (EC Council Directive 2010/63/UE) and with the Italian legislation on animal experimentation (Decreto L.vo 26/2010).

3.3. EXPERIMENTAL PROCEDURE

3.3.1. Metabolic regulation

• Glucose tolerance test (GTT). Intra-peritoneal GTT was performed after a 15 h overnight fasting that took place from 06:30 p.m. until 09:30 a.m. Animals were

intra-peritoneally (IP) loaded with 2 g/kg body weight D-glucose (10% D glucose solution; Sigma, St. Louis, MO, USA) (Satapathy et al., 2011). Blood was collected from the tail vein at 0 (baseline), 30, 60, 120, and 180 min (Ranieri et al., 2010) following IP injection and glycemia (blood glucose concentration) was measured using a commercial glucometer (StatStrip Xpress-i, nova biomedical, A. Menarini diagnostic) (Titta et al., 2010).

• Insulin sensitivity test (IST). The test was performed on animals starved for 5 h that took place from 09:30 a.m. until 02:30 p.m. Glycemia was measured using a commercial glucometer (StatStrip Xpress-i, nova biomedical, A. Menarini diagnostic) immediately before (0) and 15, 30, 60, 120 min after IP injection of a 0.4 U/kg body weight (Titta et al., 2010) solution of human recombinant insulin (Humulin, Eli-Lilly, 100 U/mL) (Ranieri et al., 2010).

3.3.2. Behavioral phenotype

After 5 days of washout from the manipulation required by the assessment of metabolic regulation, all subjects underwent behavioral testing to assess spontaneous behavior in the Open Field test, in addition to emotionality and general anxiety in the Elevated Plus Maze test (Pellow et al., 1985; File, 1993). These two tests were performed on different days.

• Open field (OF). Each subject was individually placed in the center of a cubic arena (open field box 40×40×40 cm) made of Plexiglas and allowed to freely explore for a single session lasting 15 min. The OF box was ideally divided into 25 squares and ideally partitioned into a central portion (24×24 cm) and a peripheral one, identified as the remaining part of the arena. When data were analyzed, the session was subdivided in three time blocks (tb), lasting 5 min, and the time spent in each portion of the arena was measured. Furthermore, the frequency of *sniffing* and *rearing* was scored as index of exploratory behavior and the latency and frequency of *immobility* were considered as index of emotional profile. *Grooming* frequency and the latency to the first event was also taken into account, providing a reliable marker of high or low stress in specific behavioral context (Katz and Roth, 1979; Kalueff and Tuohimaa, 2005).

• Elevated plus maze (EPM). The EPM is made of two open arms (30×5×0 cm) and two closed arms (30×5×15 cm) that extended from a common central platform (5×5 cm). The apparatus, made of Plexiglas (gray floor, clear walls), is elevated to a height of 60 cm above the floor. Mice were individually placed on the central platform facing an open arm and allowed to freely explore the maze for 5 min. Behavioral parameters observed were: % open entries [(open/total) × 100] and time spent in the open and closed arms of the maze (File, 2001). Furthermore, the behavioral parameters taken into account were the frequency of *head-dipping* and of explorative behavior such as *sniffing* and locomotion (*crossings* of squares limits with all paws).

In both behavioral tests, after each session, the apparatus was cleaned with 50% ethanol to reduce olfactory cues.

At 9 weeks of age mice began the experimental procedure. All subjects underwent the GTT as first metabolic test and after 3 days, in which mice were left undisturbed, all of them were tested for the IST. This time interval was necessary to recover from the fasting and the handling procedures. After 5 days from the end of the metabolic measurement subjects underwent the behavioral tests.

3.3.3. Statistical analysis

Data were analyzed using parametric analysis of variance (ANOVA) with treatment (NAC *vs.* WATER), maternal diet (HFD *vs.* CD) and gender (females *vs.* males) as between-subjects factors and minutes, zones (EPM: "center" *vs.* "closed arms" *vs.* "open arms" and OF: "center" *vs.* "periphery") and time blocks (0-5 *vs.* 5-10 *vs.* 10-15 min) as within-subjects repeated measures, when appropriate (GTT, IST, EPM and OF tests).

Post hoc comparisons have been performed using the Tukey's test. In analyzing GTT and IST data, this test was used in the absence of significant ANOVA effects according to the indications given by Wilcox (Wilcox et al., 1987). Fisher's exact probability test was used to compare treatment and diets for reproductive success of the colony (i.e., number of pregnant females able to successfully deliver pups: two-by-two contingency table). Statistics were performed with Statview II (Abacus Concepts, CA, USA).

3.4. RESULTS

3.4.1. Dams' body weight

Overall, feeding a HFD determined an increase in body weight in the dams (main effect of diet: $F_{(1,73)}= 16.080$; $p= 0.0001$). While no main effect of NAC was registered on body weight ($F_{(1,73)}= 0.75$; $p= 0.3893$), an interaction between NAC and diet was found over weeks ($F_{(5,365)}= 4.166$; $p= 0.0011$), with NAC inducing a gaining in body weight of CD females, rendering them more similar to HFD females, especially in the last week of combined exposure to HFD and NAC.

3.4.2. Reproductive success

No main effect of both NAC and HFD was found to affect reproductive success of females (Fisher's exact probability test: $p= 0.5554$ and $p= 0.2368$, for NAC and HFD, respectively). In addition, despite NAC administration, HFD females were characterized by a reduced reproductive success than CD (Fisher's exact probability test: $p= 0.05$).

3.4.3. Body weight of the offspring

Regardless of maternal diet, offspring prenatally exposed to antioxidant treatment were characterized by a higher birth weight than controls (main effect of NAC: $F_{(1,30)}$= 3.955, p= 0.05, **Figure 2A**), an effect also maintained when young adult, at 3 months of age, regardless of diet and gender (main effect of NAC: $F_{(1,121)}$= 22.485, p< 0.0001, data not shown). At this age the interaction among maternal diet, NAC and gender resulted to not affect the body weight of the offspring ($F_{(1,121)}$= 1.429, p= 0.2342, **Figure 2C**). In addition, while maternal HFD did not result in increased offspring's body weight at birth and when young adult (main effect of maternal diet: $F_{(1,30)(1,121)}$= 0.560; 1.730, p= 0.4602; 0.1909, respectively at P3 and at three months of age), prenatal exposure to that increased offspring's body weight at P30 (main effect of maternal diet: $F_{(1,76)}$= 4.391, p= 0.0395, data not shown). At this age an interaction among maternal diet, NAC and gender has been also found with NAC contributing to increased body weight in HFD male mice ($F_{(1,76)}$= 4.473, p= 0.0377, **Figure 2B**).

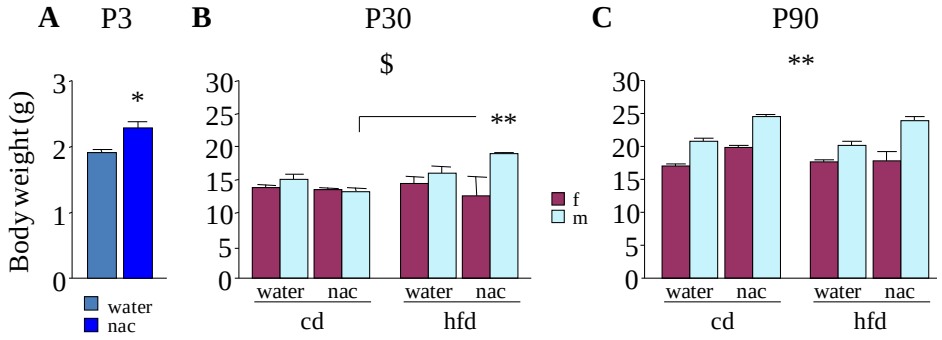

Figure 2. Body weight of the offspring after birth, P3(A), at weaning, P30 (B), and at 3 months of age P90 (C). Data are shown as + s.e.m. *Post-hoc* comparisons, * p< 0.05; ** p< 0.01 (P3 and P90: nac *vs.* water); $ p< 0.05 (P30: hfd *vs.* cd); ∗∗ p< 0.01 (P30: nac-hfd-m *vs.* nac-cd-m). Experimental subjects, P3: cd-water, n= 14; cd-nac, n= 6; hfd-water, n= 13; hfd-nac, n= 1; P30: cd-water-f/m, n= 18/14; cd-nac-f/m, n= 7/19; hfd-water-f/m, n= 11/9; hfd-nac-f/m, n= 2/4; P90: cd-water-f/m, n= 25/26; cd-nac-f/m, n= 7/19; hfd-water-f/m, n= 22/23; hfd-nac-f/m, n= 2/5.

3.4.4. Metabolic regulation

• Glucose tolerance test (GTT). Overall, gender resulted to strongly affect the metabolic response to a glucose challenge, with male subjects being characterized by increased glycemia, suggesting reduced glucose tolerance (main effect of gender: $F_{(1,105)}$= 18.487, p< 0.0001, data not shown). Given the significant difference between genders, a finer analysis was performed in order

to better investigate the metabolic response to glucose in male and female subjects independently.

When individually analyzed, overall, prenatal exposure to maternal HFD and NAC independently affected the metabolic response in an opposite manner in the two genders. In detail, maternal HFD led to glucose sensitivity in females (main effect of maternal diet: $F_{(1,25)}$= 4.546, p= 0.0430, data not shown), while did not affected metabolic response in males (main effect of maternal diet: $F_{(1,59)}$= 0.051, p= 0.8218, data not shown). On the contrary, prenatal exposure to NAC induced glucose tolerance in males (main effect of NAC: $F_{(1,59)}$= 6.751, p= 0.0118, data not shown), while no metabolic effects were observed in females (main effect of NAC: $F_{(1,25)}$= 0.007, p= 0.9359, data not shown).

In male mice, despite not being statistically significant (interaction between maternal diet, NAC and minutes: $F_{(4,236)}$= 1.209; p= 0.3078, **Figure 3A**), *post-hoc* comparison confirm that prenatal exposure to NAC led to glucose tolerance in CD males but was not able to counteract the HFD-induced glucose sensitivity (*post-hoc* comparison: NAC-CD *vs.* NAC-HFD and NAC-CD *vs.* WATER-CD, p< 0.01).

In female mice, no effect of the interaction among maternal diet, NAC and minutes was observed in response to metabolic stimulus with glucose in females ($F_{(4,100)}$= 0.990; p= 0.4168, **Figure 3B**).

• Insulin sensitivity test (IST). Unlike to glucose, no difference in the metabolic response has been registered between genders after an insulin challenge (main effect of gender: $F_{(1,102)}$= 2.892, p= 0.0921, data not shown).

As for the GTT, also in the IST an independent statistical analysis was run for the two genders.

In male mice prenatal exposure to HFD led to insulin resistance (main effect of maternal diet: $F_{(1,63)}$= 18.744, p< 0.0001) and, overall, prenatal NAC exacerbated this response (interaction between maternal diet and NAC: $F_{(1,63)}$= 5.279, p= 0.0249). Nonetheless, a main effect of prenatal NAC was not found ($F_{(1,63)}$= 3.008, p= 0.0877). Moreover, despite no statistically significant (interaction among maternal diet, NAC and time course: $F_{(4,252)}$= 0.911, p= 0.4582, **Figure 3C**), *post-hoc* comparison confirm that prenatal exposure to NAC exacerbated insulin resistance characterizing HFD male mice in particular after 15 and 30 minutes after insulin injection (*post-hoc* comparison: NAC-HFD *vs.* NAC-CD and NAC-HFD *vs.* WATER-HFD, p< 0.01).

In females no main effect of both maternal diet and NAC was registered (main effect of maternal diet and NAC: $F_{(1,39)}$= 0.311; 0.962, p= 0.5805; 0.3327, respectively). In addition, no significant difference in the glycemic response to insulin was registered among experimental groups over time (interaction among maternal diet, NAC and minutes: $F_{(4,156)}$= 0.862, p= 0.4884, **Figure 3D**).

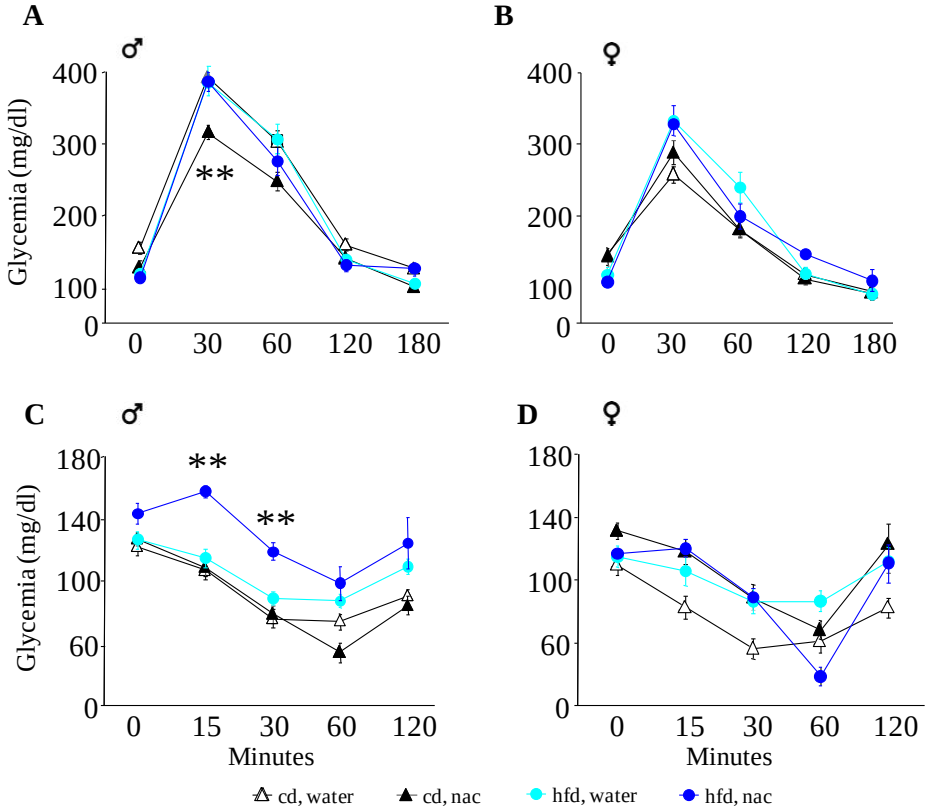

Figure 3. Metabolic regulation. Glucose tolerance assessment in male **(A)** and female **(B)** offspring exposed to maternal CD or HFD and NAC. Insulin sensitivity test male **(C)** and female **(D)** offspring exposed to maternal CD or HFD and NAC. Data are shown as ± s.e.m. *Post-hoc* comparisons, ** $p< 0.01$. Experimental subjects; GTT: cd-water-f/m, $n= 10/19$; cd-nac-f/m, $n= 7/19$; hfd-water-f/m, $n= 10/20$; hfd-nac-f/m, $n= 2/5$; IST: cd-water-f/m, $n= 17/22$; cd-nac-f/m, $n= 6/19$; hfd-water-f/m, $n= 18/22$; hfd-nac-f/m, $n= 2/4$.

3.4.5. Behavioral phenotype

• Open field (OF). Overall, all mice spent significantly less time in the center of the arena (main effect of zone: $F_{(1,63)}= 2604.056$, $p< 0.0001$, data not shown), in particular mice prenatally exposed to maternal HFD (interaction between maternal diet and zone: $F_{(1,63)}= 12.278$, $p= 0.0008$, data not shown). On the contrary, prenatal exposure to NAC resulted in increased time spent in the center of the open field in all subjects (interaction between NAC and zone: $F_{(1,63)}= 64.581$, $p< 0.0001$, data not shown), being also able to counteract the effect of maternal HFD, as demonstrated by the interaction among maternal HFD, NAC and zone ($F_{(1,63)}= 3.742$, $p= 0.05$, **Figure 4A**).

While no difference has been registered as a result of HFD (main effect of maternal diet: $F_{(1,63)}$= 0.108; 0,111, p= 0.7439; 0.7400, respectively for frequency of *sniffing* and *rearing*), prenatal exposure to NAC determined an increased exploratory behavior, as indicated by the increased frequency of environmental *sniffing* and *rearing* (main effect of NAC: $F_{(1,63)}$= 4428.106; 10.390, p< 0.0001; p= 0.0020, respectively for frequency of *sniffing* and *rearing*), in particular in females, being more explorative than males (interaction between NAC and gender: $F_{(1,63)}$= 9.237, p= 0.0035, for frequency of *sniffing*).

Overall, mice prenatally exposed to NAC were characterized by higher latency and reduced frequency of *immobility* than controls (main effect of NAC: $F_{(1,63)}$= 21.422; 20.966, p< 0.0001; p< 0.0001, respectively for latency, **Figure 4B**, and frequency). Interestingly, while no main effect of maternal diet was observed ($F_{(1,63)}$= 0.001, p= 0.9811, data not shown), HFD increased emotional response in a context of novelty in male mice, as indicated by the lower latency to *immobility* than both CD males and HFD females (interaction between maternal diet and gender: $F_{(1,63)}$= 7.428, p= 0.0083, data not shown).

In addition, regardless of maternal diet and gender, prenatal exposure to NAC increased frequency of *grooming* and reduced the latency to the first episode (main effect of NAC: $F_{(1,63)}$= 51.151; 19.246, p< 0.0001; p< 0.0001, respectively for frequency and latency).

• Elevated plus maze (EPM). Overall, all subjects spent more time in the closed arms of the apparatus (main effect of zone: $F_{(1,63)}$= 7.534, p= 0.0079, data not shown).

In addition, no difference was registered in % time spent in the open arms of the apparatus in response to both HFD and NAC independently (main effects: $F_{(1,63)}$= 0.852; 2,020, p= 0.3595; 0,1602, respectively for HFD and NAC). Although not affecting % time spent in open arms, prenatal exposure to NAC resulted in increased emotionality, as indicated by increased latency to first enter in the open arms of the apparatus (main effect of NAC: $F_{(1,63)}$= 6.479, p= 0.0134), particularly in offspring exposed to maternal HFD *in utero* (interaction between maternal diet and NAC: $F_{(1,63)}$= 7.961, p= 0.0064, **Figure 4C**) and by the reduced frequency of *head-dipping* behavior (main effect of NAC: $F_{(1,63)}$= 12.448, p= 0.0008, **Figure 4D**).

Moreover, regardless of both maternal diet and gender, NAC also increased exploratory behavior as indicated by the increased frequency of environmental *sniffing* (main effect of NAC: $F_{(1,63)}$= 14.334, p= 0.0003, data not shown) and by increased locomotion, particularly in female mice (interaction between NAC and gender: $F_{(1,63)}$= 13.575, p= 0.0005, data not shown).

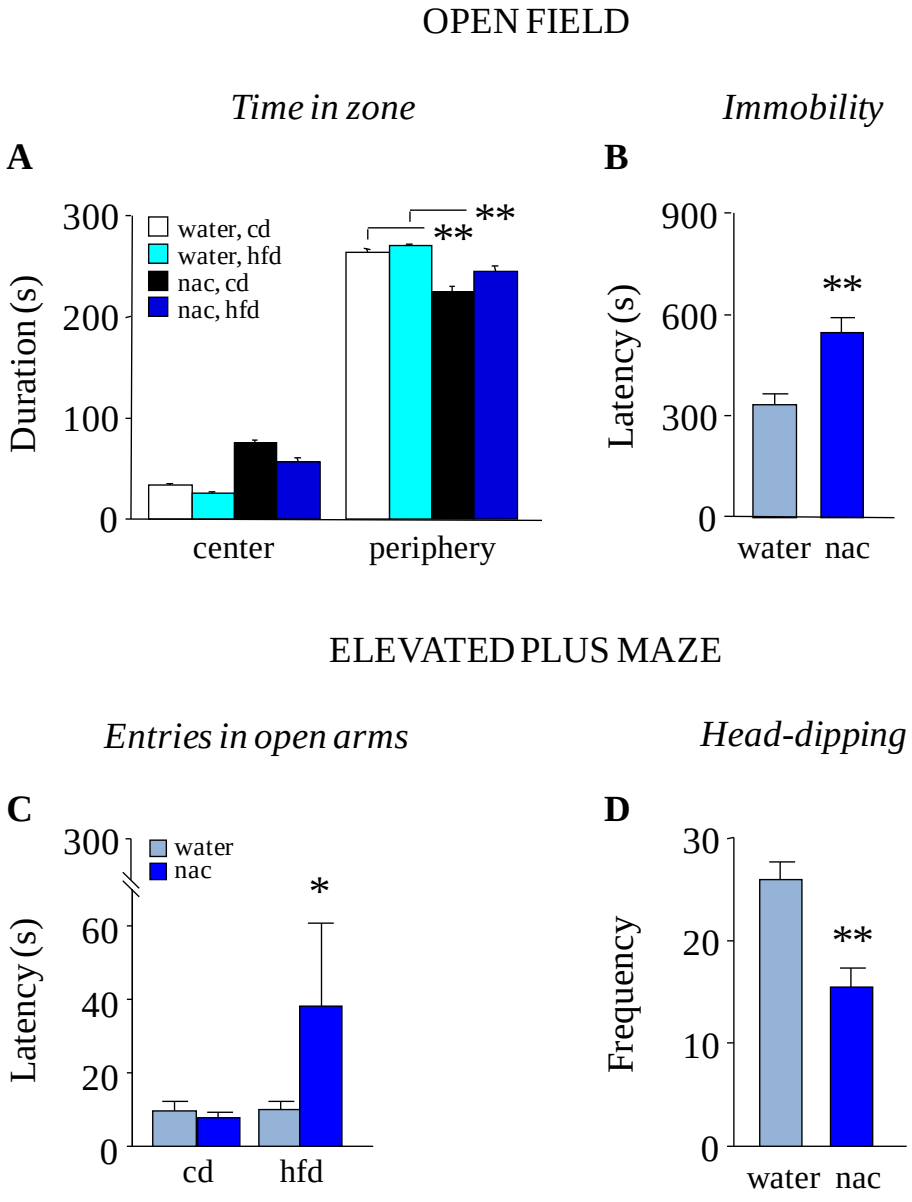

Figure 4. Behavioral phenotype. Time spent in zone of the arena **(A)** and latency to first *immobility* **(B)** in the Open Field test. Latency to first enter in open arms of the maze **(C)** and frequency of *head-dipping* behavior **(D)** in the Elevated Plus Maze test. Data are shown as + s.e.m. *Post-hoc* comparisons, ∗∗ $p< 0.01$ (nac-hfd *vs.* water-hfd; nac-cd *vs.*water-cd; nac *vs.*water); ∗ $p< 0.05$ (hfd-nac *vs.* hfd-water and cd-nac). Experimental subjects: water-cd/hfd-f/m, $n= 12$; nac-cd-f/m, $n= 7/10$; nac-hfd-f/m, $n= 2/4$.

3.5. DISCUSSION

Overall, results from this study support previous data showing that maternal HFD represents a detrimental metabolic stimulus for both the dam and the offspring. This is confirmed by the failure of NAC treatment to restore the reproductive success lacking in HFD female mice. The most relevant finding is the action of the NAC drug, which replicate the metabolic effect of the $p66^{Shc}$ gene deletion, conferring glucose tolerance and insulin resistance to young adult male offspring. Interestingly, maternal HFD and NAC differently affect the metabolic phenotype of the offspring of both genders suggesting a long-lasting and gender-dependent effect. Moreover, prenatal NAC overturned the effect of maternal HFD on the emotional and behavioral phenotype of the offspring.

As previously found, lasting feeding a HFD induced a gaining in body weight in females (Bellisario et al., 2014). In addition, NAC induced a gaining in body weight in controls female mice only, suggesting that the potential effect as a dietary supplementation is particularly evident in standard dietary condition and that this effect could be covered by the stronger effect of a HFD.

Moreover, results from this study indicate that prenatal antioxidant treatment missed to thwart reproductive failure characterizing HFD-fed dams. Several studies have shown delivery failure in addition to fetal resorption during gestation in female rats fed a HFD diet (Niculescu and Lupu, 2009), suggesting a detrimental action to embryo development due to high OS. This hypothesis is further supported by previous findings in a transgenic mouse model of reduced OS, the $p66^{Shc-/-}$ mouse. This phenotypic feature played a protective role in reproductive failure associated to maternal HFD (Bellisario et al., 2014), furthermore suggesting that antioxidant administration (NAC) did not exert a comparable effect with a more effective genetic condition. Indeed, pregnancy is a physiological condition in which body unexpectedly responds to both endogenous and exogenous stimulus and in which hormonal stimulation is potentiated. Taking into account such evidences, prenatal exposure to NAC is not enough to induce the expected protective effect.

Taking into account the offspring, as already observed in the transgenic model of resistance to OS (the transgenic mouse model $p66^{Shc-/-}$), prenatal NAC resulted in increased birth weight of the offspring. This could represent an offset effect toward the reduced birth weight usually associated to prenatal HFD. Interestingly, a comparable profile between the genetic and the pharmacological model of reduced OS used in this study was also found in body weight at P30, when maternal HFD resulted in a greater gain in body weight only in males also prenatally exposed to NAC, as was previously found in the $p66^{Shc-/-}$ mice only. A previous study performed by White and co-workers on rats showed that offspring of maternally obese dams weighed significantly more than the offspring of non-obese dams at weaning (White et al., 2009). The prenatal exposure to an antioxidant seemed not to be able to counteract the effect of maternal HFD while, on the contrary, to potentiate its effect on weight gain in

the offspring at weaning age. This effect might be due to the evidence that the weaning age is a critical period in which several physiological adjustments occur, resulting in significant consequences for the offspring with behavioral and hormonal changes defining the growing individual (Lhuillery et al., 1984; Martin et al., 1984). In this context the prenatal exposure to maternal HFD might major impact the ticklish weaning age. The current study did not found effects in offspring body weight at three months of age as a consequence of maternal consumption of HFD. Interestingly, this feature has been previously reported by Platt and colleagues in a mouse model of maternal HFD (De Flora et al., 1995a). They found that the differences in lard pup weight registered before weaning disappeared after that age, proposing several explanations, among the others a greater content of sucrose in control diet compared to the hypercaloric diet. This variation of sucrose in the diets could have masked potential differences in offspring body weight after weaning in growing offspring (De Flora et al., 1995a). Similarly, the diets used in the present study provided a different calorie intake in fats and carbohydrates, which are respectively 58% fat and 25.5% carbohydrate for the HFD and 10.5% fat and 73.1% carbohydrate for CD. Despite the same kinds of diet were used in a previous study inducing a significant increasing in body weight, both at weaning and at three months of age in HFD offspring, it is possible hypothesize that prenatal exposure to NAC metabolically interfere with the maternal diets thus eclipsing the effect of HFD and resulting in a mild metabolic outcome in the offspring body weight.

Moreover, the effects of prenatal exposure to maternal HFD in combination with prenatal NAC have been tested by measuring the responsiveness to metabolic exogenous stimuli, those are glucose and insulin. Data shows that prenatal exposure to maternal HFD and NAC differently drive the metabolic response to a bolus of glucose in young offspring of both genders. Male mice prenatally exposed to NAC show a greater resistance toward glucose challenge compared to controls. However, such effect was lost in males prenatally exposed to HFD, being characterized by severe glucose intolerance in comparison with their own controls (NAC-CD) and suggesting that prenatal exposure to NAC is not enough able to counteract the negative metabolic effect driven by maternal HFD. The increased resistance to a glucose challenge showed by NAC males is supposedly due to the reduced oxidative stress associated to pharmacological treatment. Previous data showed that the metabolic profile induced by a glucose challenge is strictly similar in NAC subjects and in $p66^{Shc-/-}$ genetic mouse model of reduced OS (Bellisario et al., 2014). Accordingly, when tested in the insulin sensitivity test, prenatal NAC exacerbated the insulin resistance characterizing HFD male mice, as the lack of the $p66^{Shc}$ gene also done. The evidence of a comparable metabolic action between the pharmacological treatment and the genetic condition is given by the glycaemic trend of NAC subjects in response to metabolic stimuli perfectly following that characterizing the genetic model of reduced OS. These features

strongly suggest that the effect of prenatal NAC mimics the lack of $p66^{Shc}$ gene on the metabolic response. It is interesting to note that a completely different metabolic profile has been registered in females, in which prenatal NAC did not mainly affect the metabolic response neither to glucose or insulin. In addition, maternal HFD driven a greater glucose resistance in females, while did not affect males' response, and, on the contrary, induced a greater insulin resistance in males did not affecting females metabolic profile. These data confirm previous data related to a gender-dependent resiliency metabolic challenges following prenatal exposure to high-fat diet (Bellisario et al., 2014).

As concerning the behavioral profile, in the open field test, HFD offspring spent less time in the center of the arena, suggesting an HFD-induced anxiety-like profile. Indeed, the open field test, in addition to reflect deficits in motor behavior, is appropriately used to test partial anxiety (Sharma et al., 2010). Previous studies have reported increased anxiety-like behavior in adult subjects of both genders exposed to HFD during fetal development (Sasaki et al.; Bilbo and Tsang, 2010; Peleg-Raibstein et al., 2012), although in some cases a gender dependent effect is reported (Bilbo and Tsang, 2010). In line with the latter finding, we observed reduced latency to *immobility*, considered an endpoint of anxiety-like behavior, characterizing HFD males compared to CD male and HFD females. More interesting prenatal NAC is able to counteract the anxiogenic effect of maternal HFD increasing the time spent in the center of the arena both in HFD and CD mice and increasing latency and reducing frequency of *immobility*. A similar effect was also found by Chen and colleagues, which found NAC improved social interaction and ameliorated anxiety-like behaviors in the valproate-exposed male offspring of a rat model of autism (Chen et al. 2014). Prenatal exposure to NAC also resulted in increased frequency of *grooming*. Nevertheless some reports *grooming* as an anxiety related behaviors and an increased frequency of these events as a greater level of anxiety (Lister, 1990), others state that *grooming* behavior might just represent a displacement response (Espejo, 1997a, 1997b) that may be essential to restore homeostasis (Kalueff and Tuohimaa, 2005) after exposure for the first time to a novel environment. Also, it has been stated that rodents' *grooming* activity occurs under conditions of extreme arousal, in both high and low stress situations (Katz and Roth, 1979; Kalueff, 2000). Interestingly, prenatal exposure to NAC leads to increased exploratory behavior both in the open field, as suggested by the increased frequency of *sniffing* and *rearing*, and in the elevated plus maze, as suggested by increased frequency of environmental *sniffing* and increased locomotion, in particular in females. Unlike to the reduced emotionality found in the open field, in the elevated plus maze NAC subjects showed increased emotionality as indicated by the reduced frequency of *head-dipping* behavior. It is generally accepted that increased exploratory head dipping behavior in the open arms of the apparatus is an index of decreased levels of anxiety (Blanchard et al., 2001).

Overall, results from this study confirm the negative effect associated to maternal HFD for both the dam and the offspring. In addition, the present work mainly investigated the potential effect of a synthetic antioxidant administered during the prenatal life, the N-acetyl-cysteine, to thwart the effect of maternal-HFD and supposedly associated to the resulting increased oxidative stress. In this regard, results obtained in the present study indicated that prenatal antioxidant administration is able to limit some long-term consequences deriving from HFD exposure during fetal development. It is possible hypothesize that this effect is due to the contemporary exposure to both stimuli, thus rendering the antioxidant action more efficacy because working on the developing fetus in the attempt to reduce the fetal misprogramming. The main effect of prenatal NAC was observed in the metabolic phenotype characterizing the young adult offspring, in particular in males, confirming a gender-dependent metabolic sensitivity maybe driven by hormones. The most important result is that the prenatal exposure to antioxidants mimics some of the effects of the genetic mouse model of reduced oxidative stress, the $p66^{Shc}$ gene knock out, particularly on the metabolic response. This partial effect may be associated to the short time window of exposure that could not be compared with a stable genetic condition. This finding highlights the key role played by oxidative stress in the metabolic response thus representing an important target for future studies investigating potential therapeutic interventions to precociously stop the long-term detrimental effect of the maternal HFD experienced early in life.

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CHAPTER 4

4. HIGH-FAT DIET DURING PREGNANCY ACTS AS A STRESSOR INCREASING MATERNAL GLUCOCORTICOIDS' SIGNALING TO THE FETUS AND DISRUPTING MATERNAL BEHAVIOR THROUGH CHANGES IN NEURONAL ACTIVITY IN THE OLFACTORY BULB IN C57BL/6J MICE

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ABSTRACT

Maternal diet during pregnancy can impact maternal behavior as well as the intrauterine environment, playing a critical role in programming of the offspring's physiology. In a preliminary study we found a strong association between high-fat diet (HFD) during pregnancy and increased cannibalistic episodes and dams' mortality during late pregnancy and parturition. Therefore, we hypothesized that HFD during pregnancy could negatively affect neuroendocrine and metabolic regulations occurring during the final stages of pregnancy, disrupting maternal behavior. To test such hypothesis, female C57BL/6J mice were fed HFD or control diet before and during pregnancy up to delivery. Neuroendocrine and molecular endpoints were assessed at different time points, both in the mothers and in the pups. More in detail, Hypothalamic-Pituitary-Adrenal (HPA) axis activity and the levels of c-Fos in the olfactory bulbs were measured, in addition to peripheral leptin levels. Dam's emotional behavior and social anxiety, in addition to maternal locomotor activity were assessed before parturition. Maternal behavior was also evaluated on post-natal day 1. Placental levels of 11-β-dehydrogenase-2 (11-βHSD2) and 11-β-dehydrogenase-1 (11-βHSD1), regulating fetal exposure to maternal glucocorticoids, were also assessed. HFD led to an increased rate of cannibalism and to an aberrant maternal behavior, with dams showing behavioral uninhibition and locomotor activity, in addition to an overall reduced expression of c-Fos in the olfactory bulbs. HFD-feeding resulted in increased levels of maternal corticosterone and decreased levels of leptin, in addition to reduced enzymatic activity of 11β-HSD2 and reduced levels of the 11β-HSD1 gene expression in the placenta. Overall, these data suggest that HFD acts as a stressful challenge during pregnancy, impairing the neuroendocrine system and the neural activity of brain regions involved in the processing of relevant olfactory stimuli, with aberrant consequences on mother's physiology and maternal behavior.

Keywords: high-fat diet, pregnancy, maternal behavior, anxiety, stress, biomarkers, animal models.

4.1. INTRODUCTION

In modern society, a diet rich in fats (high-fat diet - HFD) is an example of a physiological stressor experienced by women during pregnancy (Reynolds et al., 2013). High-fat feeding during pregnancy causes placental dysfunction (McCurdy et al., 2009; Frias et al., 2011) and plays an important role in fetal development promoting metabolic programming in the offspring with long-term detrimental effects on health during life (Drake and Reynolds, 2010; Li et al., 2011). Maternal HFD during pregnancy "programs" lifelong susceptibility to adult disorders, representing a risk factor for psychiatric disease, such as depression and schizophrenia (Barker, 2004; Eriksson et al., 2004; Huizink et al., 2004), metabolic complications, such as insulin resistance (IR) and type 2 diabetes (T2D), in addition to cardiovascular disease and other metabolic disorders usually associated with obesity (Kahn and Flier, 2000; Wang et al., 2004; Fantuzzi, 2005; Taylor et al., 2006; Eriksson et al., 2014). It is well known that the environment experienced early during development is not only crucial for setting the growth trajectory of the fetus but also represents a key factor contributing to overall disease susceptibility in later life (Barker, 1995). In this context, developmental plasticity is a fundamental mechanism matching the growing organism to the environment it will face after birth (Barker, 2003).

Preclinical studies have shown that extreme changes in maternal diet influence maternal stress responses in rats (Weinstock, 2001). In particular, the administration of a HFD causes the sensitization of the hypothalamic-pituitary-adrenal (HPA) axis (Legendre and Harris, 2006) resulting in increased basal levels of maternal glucocorticoids associated with increased emotionality (Tannenbaum et al., 1997; Boukouvalas et al., 2008). The activity of the maternal HPA axis undergoes physiological adaptations in order to achieve regular development of the fetus avoiding adverse effects on both mother and offspring (Duthie and Reynolds, 2013). In turn, the fetus is protected from high glucocorticoids levels by the activity of specific placental enzymes, 11β-hydroxysteroid dehydrogenase (11β-HSD) type 1 and 2, acting as a placental barrier, which regulates the transfer of glucocorticoids from mother to fetus (Benediktsson et al., 1997; Holmes et al., 2006).

In a previous study we found that maternal HFD-feeding during pregnancy has detrimental effects both on the dam, increasing cannibalistic episodes and dam mortality rate in the perinatal period, and on the offspring, with long-lasting and gender-dependent effects on metabolic and neuroendocrine profile at adult age (Bellisario et al., 2014). Clinical studies and animal models have reported altered placental growth, gene expression and nutrient transport following HFD feeding during pregnancy (Jones et al., 2009; Farley et al., 2010; Gallou-Kabani et al., 2010; Gabory et al., 2012). Nevertheless, data concerning the mechanistic effects of HFD on the physiological adaptations occurring during the perinatal period are still lacking.

Understanding the maternal physiological alterations driven by HFD during pregnancy is of major public health importance, and could provide predictive factors of susceptibility for maternal HFD-related metabolic and mood disorders reported in the adult population. Given the abundance of dietary fats in western diets, these are important public health issues.

The current study aimed at investigating the effects of high-fat feeding before and during pregnancy on the neuroendocrine, metabolic and behavioral profile of dams in the perinatal period, both before and after delivery, with a special focus on the effects of HFD on the molecular adaptation associated with parturition, delivery and on the onset of maternal behavior. We hypothesized that HFD-feeding could represent a metabolic, stressful challenge during pregnancy able to negatively affect physiological adaptation occurring during perinatal period and the maternal behavior. To test such a hypothesis, C57BL/6J female mice were fed with HFD or control diet (CD) for 11 weeks: from 5 weeks of age until the onset of delivery. Neuroendocrine activation and levels of c-Fos in the olfactory bulbs were measured both before and after delivery. The behavioral profile of dams and maternal behavior towards the pups was characterized, in addition to leptin levels in adipose tissue. Furthermore, the modulation of fetal exposure to glucocorticoids was also determined.

4.2. MATERIALS AND METHODS

4.2.1. Animals

Experimental subjects were 5 week-old C57BL/6J females (*n*= 100) and same strain experienced male mice (*n*= 34). Females were housed 3/cage and males were individually housed in transparent Plexiglas cages (37×21×19cm) provided by Tecniplast, in an air conditioned room (temperature 21±1°C, relative humidity 60±10%) under a reversed 12/12hrs light/dark cycle with lights off from 07:00 a.m. to 07:00 p.m. Fresh tap-water and standard chow (standard diet -SD- energy 3.3kcal/g, fat 17%, carbohydrate 60% and protein 23%, provided by Altromin-R, Rieper, Italy) were continuously available during the 7 days of habituation before the experimental procedure took place. Thereafter females were fed *ad libitum* either with high-fat diet (HFD - energy 5.56 kcal/g, fat 58%, carbohydrate 25.5% and protein 16.4%; *n*= 50) or control diet (CD - energy 4.07 kcal/g, fat 10.5%, carbohydrate 73.1% and protein 16.4%; *n*= 50) for 8 weeks. Females were randomly assigned to HFD or CD groups avoiding difference in the average of body weight between groups. HFD (D12331) and CD (D12328) were provided by Research Diets, Inc., New Brunswick, NJ, USA. During the 8 weeks on diet food consumption (daily) and body weight gain (weekly) of females was monitored and thereafter all females were bred (for the breeding procedure see paragraph 4.2.2.1). Pregnant females were kept on either HFD or CD throughout gestation until 3 days before the

expected delivery date, i.e. at gestational day 16 (G16), when all of them were switched to SD.

Animals were handled consistently by the same personnel and manipulations were performed under red light. Noise and disturbances to the animals were kept to a minimum during the mating protocol, except for husbandry requirements and the procedures detailed below.

Animal handling and experimental procedures were performed in accordance with the EC guidelines (EC Council Directive 86/609 1987) and with the Italian legislation on animal experimentation (Decreto L.vo 116/92).

4.2.2. Experimental procedure

4.2.2.1. Breeding procedure

After 8 weeks on diet, a handful of sawdust from a male's cage was added to a female's cage every morning for 3 days, so that males' pheromones from dirty sawdust (urine) induced the estrus cycle (Whitten, 1973). On the third day, one male was introduced into each female's cage for 24 hours. Thereafter all females were checked twice daily to assess the presence of a vaginal plug (after 8 hours at 06:00 p.m. and 24 hours at 10:00 a.m.) to tentatively confirm pregnancy. Body weight (BW) was monitored every 2 days to assess pregnancy. Females were individually housed once pregnancy was confirmed.

The HFD feeding procedure and the breeding protocol previously described was also used in two different laboratories (the Centre for Cardiovascular Science, University of Edinburgh, UK and the Max Planck Institute of Psychiatry, Munich, Germany). In the present work data related to cannibalism also collected in these laboratories will be reported. No further data are available from these labs, due to the difficulties associated with breeding and parturition with the high-fat diet chosen for the study.

4.2.2.2. Experimental groups

Pregnant females (*n*= 73) were randomly assigned to four experimental groups for assessment of maternal levels of corticosterone (CORT) both before and after delivery, for *in situ* hybridization (ISH) determination on dams' brain and for genetic, cellular and structural analysis on placentas, in addition to behavioral phenotyping of dams. The success of birth was also registered, in addition to the body weight of both fetuses and pups.

- Group A (*n*= 8, for both CD and HFD) was designed to assess the prenatal levels of maternal CORT, at gestational day 16 (G16) (see paragraph 4.2.2.3). Thereafter all dams underwent a caesarean section procedure and whole brains were harvested and immediately frozen for gene expression analysis (see paragraph 4.2.2.5.). Moreover, two placentas, one for each side of the horn of uterus, were also collected and immediately frozen in order to assess the levels of the 11β-HSD2 enzyme (see paragraph 4.2.2.4.).

• Group B (*n*= 7, for both CD and HFD) was designed to assess the prenatal levels of maternal CORT, on G18 (see paragraph 4.2.2.3). Thereafter all dams underwent a caesarean section procedure. Two placentas, one for each side of the horn of uterus, were also collected, weighed and immediately frozen for the assessment of mRNA levels of 11β-hydroxysteroid dehydrogenase (HSD)-1 placental gene (see paragraph 4.2.2.5.).

At this age a pool of fetal blood was collected for CORT determination and the placental and fetal weight was registered. The success of birth was also registered.

• Group C (*n*= 8, for both CD and HFD) was designed for the assessment of emotionality and social anxiety associated to pregnancy. In more detail, before delivery females underwent behavioral tests aimed at assessing emotionality (Elevated Plus Maze - EPM), at G16, and social anxiety (Social Avoidance Test - SAT), at G17 (see paragraph 4.2.2.6). In addition, after parturition, on post-natal day 1 (P1), all dams underwent to a Retrieval test assessing maternal behavior and body weight of pups was also registered. After 24 hrs, on P2, dams were sacrificed and whole brains and periovaric adipose tissue were dissected out and immediately frozen for further analysis.

• Group D was designed to assess general activity associated to parturition (*n*= 8, for CD and *n*= 7 for HFD) and postnatal (P1) levels of maternal CORT (*n*= 8, for CD and *n*= 4 for HFD) (fsee paragraph 4.2.2.3). Thereafter, dams were sacrificed and underwent to the same procedure also described for the group A. Moreover, the abdominal fat tissue of mothers was collected for leptin detection (see paragraph 4.2.2.3). The lower number of mice used in CORT assessment was due to a technical hitch.

A schematic design of the experimental plan is reported in **Figure 1**.

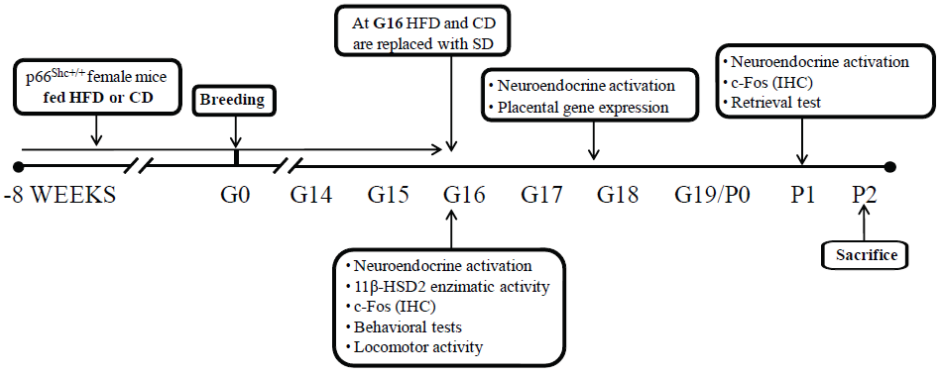

Figure 1. Schematic design of the experimental plan.

4.2.2.3. Hormonal regulation

• Metabolic response. The levels of leptin were assessed in the periovaric adipose tissue by immunoblot analysis. Adipose tissue pads (100 mg) were homogenized with Politron homogenizer in NaCl 150mM, Tris 25 mM pH 6.8, EDTA 1 mM, supplemented with protease and phosphatase inhibitors, and centrifugated 1000g for 10 min at 4°C, the fat pad fraction was discarded. A Bradford protein assay (Bio-Rad) was then conducted on the samples to determine the protein concentration for each sample. Proteins (20 mg) were separated by SDS-PAGE (10%) and transferred to nitrocellulose membrane. Membrane was blocked in 5% non-fat dry milk for one hour and incubated with primary antibody for one hour. Membrane was washed with TBS/0.1% Tween-20 three times and incubated with secondary antibody for 1 hour, then washed with TBS/0.1% Tween-20 three times and reactivity was detected by the enhanced chemiluminescence kit (Pierce). Blots were developed using a FluorChem™ R System. Mouse monoclonal adiponectin and actin antibodies were from Abcam, Inc. (Cambridge, MA).

• Neuroendocrine activation. The basal activity of the HPA axis of dams was assessed both before (G16 and G18) and after parturition (P1). In particular, CD and HFD dams underwent a blood samples collection by a tail nick at 08:30 a.m., when the levels of circulating CORT were far from the circadian peak (Kitchener et al., 2004), in order to measure basal plasma levels of CORT (MP Biomedicals, LLC, Germany GmbH). The neuroendocrine activity of fetuses was measured as well. In detail, after the cesarean section procedure occurring at the 18th day of embryonic development (E18), trunk blood of all fetuses was pooled in order to determine the circulating levels of CORT.

4.2.2.4. Enzyme activity

11-β-HSD2 activity was measured in intact transfected cells after they were seeded in poly-D-lysine coated 96-well plates (25,000 cells/well) 24 h before the assay. The medium was removed, followed by addition of 40 l of fresh medium and 10 l that contained 10 nCi of radiolabeled cortisol or corticosterone and unlabeled corticosterone (20 to 500 nM) or cortisol (50 to 2000 nM). Reactions were incubated for 2 to 12 h at 37°C under 5% CO_2. Kinetic analyses were performed by nonlinear regression using Data Analysis Toolbox (Elsevier MDL, San Ramon, CA), assuming first-order rate kinetics.

4.2.2.5. Gene expression

• rtPCR. Total RNA was extracted from tissue samples using TRIZOL reagent (Life Technologies Inc., Paisley, UK). Extracted RNA was treated with deoxyribonuclease I (Invitrogen, Paisley, UK) to remove contaminating genomic DNA. RNA (1 µg) was then reverse transcribed at 55 °C for 50 min using Moloney murine leukemia virus reverse transcriptase (Promega, Southampton, UK) according to the manufacturer's instructions. Real-time PCR (rtPCR) was performed using the TaqMan ABI Prism 7900 sequence detector

(Applied Biosystems, Chester, UK). Expression levels were quantified using Lightcycler 480 Probes Master (Roche Diagnostics, Burgess Hill, UK) with primer probe sets (Applied Biosystems) for the 11β-HSD-1 gene. Data acquisition was performed using Sequence Detector 1.6.3. software (Applied Biosystems). A standard curve for each primer probe set was generated in triplicate by serial dilution of pooled cDNA. Each sample was run in triplicate, and the mean values of the triplicates were used to calculate transcript level from the standard curve. The results are expressed as a ratio to 18S rRNA to normalize the transcript levels. Reverse transcriptase negative controls and intron spanning primers were used where possible to prevent genomic DNA amplification.

• In situ hybridization (ISH). Frozen brains were sectioned at -20°C in a cryostat microtome at 18 µm, thaw mounted on Super Frost Plus slides, dried and stored at -80°C. In-situ hybridization using a 35S UTP labeled ribonucleotide probes for c-Fos was performed as described previously (Schmidt et al., 2007). Briefly, sections were fixed in 4% paraformaldehyde and acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine/ HCl. Subsequently, brain sections were dehydrated in increasing concentrations of ethanol. The antisense cRNA probes were transcribed from a linearized plasmid. Tissue sections were saturated with 100 ml of hybridization buffer containing approximately 1.5×10^6 cpm 35S labeled riboprobe. Brain sections were coverslipped and incubated overnight at 55 °C. The following day, the sections were rinsed in 2 × SSC (standard saline citrate), treated with RNAse A (20 mg/l) and washed in increasingly stringent SSC solutions at room temperature. Finally, sections were washed in 0.1×SSC for 1 h at 65 °C and dehydrated through increasing concentrations of ethanol. The slides were exposed to Kodak Biomax MR films (Eastman Kodak Co., Rochester, NY) and developed. Autoradiographs were digitized, and expression was determined by optical densitometry utilizing the freely available NIH ImageJ software. The mean of four measurements of two different brain slices was calculated for each animal. The data were analyzed blindly, always subtracting the background signal of a nearby structure showing no specific c-Fos expression from the measurements.

4.2.2.6. Maternal behavioral phenotype

• Elevated plus maze (EPM). Three days before the expected delivery date (G16), dams of the group B underwent to EPM test. The apparatus is made of two open arms (30×5×0 cm) and two closed arms (30×5×15 cm) that extended from a common central platform (5×5 cm). The apparatus, made of Plexiglas (grey floor, clear walls), is elevated to a height of 60 cm above the floor. Mice were individually placed on the central platform facing an open arm were and allowed to freely explore the maze for 5 minutes. Behavioral parameters observed were: % open entries [(open/total) × 100] and % time spent in open and closed arms of the maze (File, 2001). Furthermore, the frequency of *rearing* was taken into account as a behavioral index of exploratory activity but that

may also be determined by emotionality (Gironi Carnevale et al., 1990; Thiel et al., 1998), providing a marker of anxiety.

• Social avoidance test (SAT). Two days before expected delivery date (G17) dams underwent a SAT (Berton et al., 2006). Pregnant females were introduced in a plastic open field arena (42×42 cm), made of grey Plexiglas, for two consecutive sessions, 2.5 min each (a test duration chosen for its optimal sensitivity/throughput ratio (Berton et al., 2006)). During session I ("no target"), the open field contained an empty wire mesh cage (diameter= 10 cm, h= 10 cm) located in the middle of one side of the arena. During session II ("social target"), a social target (an unfamiliar CD-1 male mouse) was introduced into the wire mesh cage. Between the two sessions, the pregnant dam was placed back into its home cage for 1 min. The time spent in the "interaction zone" (8 cm wide corridor surrounding the cage) *vs.* the "corners" (opposite to the location of the cage, 8×8 cm each) was scored as a reliable measure of social avoidance. The percent time spent in the interaction zone was considered as a reliable indicator of social avoidance.

In all behavioral tests, both before and after each trial/session, the apparatus was cleaned with 50% ethanol to reduce olfactory cues which might compromise the result of the test.

• Retrieval test. CD and HFD underwent a Retrieval test to assess maternal behavior. Dam and pups were removed from their home cage for 15 min and placed in two different clean holding cages. Pups were placed in an incubator in order to maintain an approximate body temperature of 30±1°C i.e. comparable to that of the nest (Cirulli et al., 2003; Capone et al., 2005). Thereafter, 4 pups, 2 males and 2 females, were placed back in their home cage, evenly distributed between the four corners, while the mother was introduced in the center of the cage. The latency to first *sniffing a pup* and to *retrieve each pup* was scored. The cut-off time was 20 min. Only pups brought into the nest were counted as retrieved.

4.2.2.7. Locomotor activity

A group of dams was housed in a special rack provided with motion sensors (*n*= 8, for CD and *n*= 7 for HFD) and monitored for spontaneous locomotion over the three days preceeding the expected delivery date. Locomotor activity was monitored through an automatic device using small infrared sensors positioned on the top of each cage (ACTIVISCOPEsystem, NewBehaviour Inc., Zurich, Switzerland (Dell'Omo et al., 2002; Macri et al., 2013)). The sensors detected any movement of mice (except when mice were asleep or inactive) with a frequency of 20 events per second (20 Hz). Average scores obtained during 60 min intervals, expressed as counts per hour, were recorded by an IBM computer with dedicated software. We averaged the data obtained during the three days before the expected delivery date (G16-G19) of continuous registration to obtain a circadian profile (23 points, 1h each) of locomotor activity.

4.2.3. Statistical analysis

Data were analyzed using parametric analysis of variance (ANOVA) with diet (HFD *vs.* CD) as between-subjects factors and zones ("centre" *vs.* "closed arms" *vs.* "open arms") and sessions ("no target" *vs.* "social target") as within-subject repeated measures, where appropriate (EPM and SAT tests).

Post hoc comparisons have been performed using the Tukey's test. In analyzing SAT data, this test was used in the absence of significant ANOVA effects according to the indications given by Wilcox (Wilcox, 1987). The statistical 2-tailed T-test was used to assess variations by diets in genes' expression (11-β-HSD-1 and c-Fos) and in enzymatic activity (11-β-HSD-2). Fisher's exact probability test was used to compare diets for reproductive success of the colony (i.e. dam mortality rate during the perinatal period, number of litters in which cannibalistic episodes took place, pup mortality rate in the two days after delivery and number of dam retrieval rate in the Retrieval test: two-by-two contingency table). Statistics were performed with Statview II (Abacus Concepts, CA, USA).

4.3. RESULTS

4.3.1. Dams' body weight and food consumption

Female mice fed a HFD gained more body weight than CD (main effect of diet: $F_{(1,98)}$= 129.883; p< 0.0001, insert **Figure 2**) through the 8 weeks of exposure (interaction between diet and weeks: $F_{(8,784)}$= 94.851, p< 0.0001, **Figure 2**). Nevertheless, no difference in food consumption was not registered (main effect of diet: $F_{(1,32)}$= 0.069, p= 0.7945, data not shown).

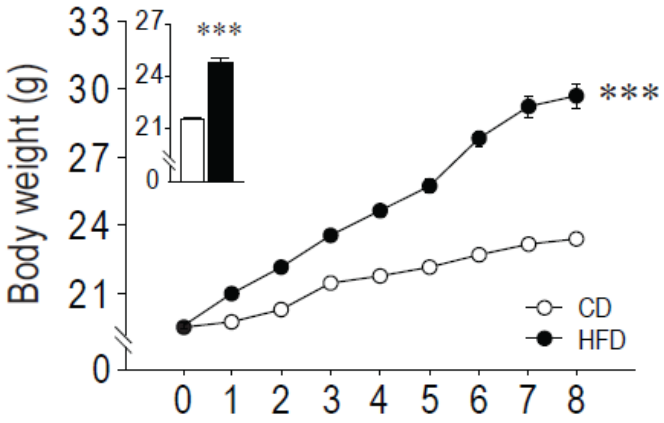

Figure 2.Dams' body weight. Body weight of dams during the 8 weeks of diet exposure and average of body weight gain (insert). Data are shown as +S.E.M. *Post-hoc* comparisons: ***p< 0.001 (HFD *vs.* CD). Experimental subjects, CD/HFD, n= 50.

4.3.2. Pregnancy duration, cannibalistic behavior and pups' mortality

Female mice fed a HFD before and during pregnancy resulted in a prolonged pregnancy compared to CD (main effect of diet: $F_{(1,15)}$= 14.412, p= 0.0018, **Table 1A**).

Feeding HFD resulted in an increased frequency of cannibalistic episodes (Fisher's exact probability test: p= 0.0351, **Table 1B**), which was not observed in CD mothers. An increased pups' mortality associated to HFD was also registered (Fisher's exact probability test: p< 0.0001, **Table 1C$_1$**). These data were reinforced by further results obtained in a separate laboratory adopting the same protocol of diet feeding, the Centre for Cardiovascular Science at the University of Edinburgh (Fisher's exact probability test: p= 0.0001, **Table 1C$_2$**). Data reported in table's sections C_1 and C_2 refer to the number of pups found dead. Similar results were also found in the laboratory of the Max Planck Institute of Psychiatry, where an increased mortality in the HFD nests was registered (nests' mortality: CD= 2, HFD= 15; nests' surviving: CD= 11, HFD= 2) (Fisher's exact probability test: p= 0.0001, data not shown).

Table 1.

A. Days of pregnancy

Diet	Mean
CD	19.5 ± 0.5
HFD	20.7 ± 0.7

B. Dams' cannibalistic behavior

Diet	Cannibalism	No cannibalism
CD	0	11
HFD	5	6

C. Pups' mortality

	Diet	Dead	Alive
C_1.	CD	16	54
	HFD	36	19
C_2.	CD	14	62
	HFD	29	13

Table 1. Duration of pregnancy, pups' mortality and cannibalistic behavior. Increased duration of pregnancy in females fed a HFD **(A)**. High frequency of cannibalistic episodes in HFD dams **(B)** and an increased pup mortality rate in HFD nests **(C)** in two different laboratories involved in the study, at Istituto Superiore di Sanità, Rome, Italy **(C_1)** and at Centre for Cardiovascular Science, University of Edinburgh, UK **(C_2)**. In the section A the table reports the average number of days of pregnancy ±S.E.M., in the sections B the table reports the number of dams on diet, in the sections C_1 and C_2 the table reports the number of pups.

4.3.3. Offspring body weight

Overall, exposure to maternal HFD resulted in a decreased placental weight at the 18th day of embryonic development (E18) (main effect of diet: $F_{(1,54)}$= 6.131, p= 0.0165, **Figure 3A**), in addition to a reduced fetal weight (main effect of diet: $F_{(1,54)}$= 35.826, p< 0.0001, **Figure 3B**). Regardless of gender, this effect was lost after birth (P1), when no difference was registered in body weight of CD and HFD pups (main effect of diet: $F_{(1,80)}$= 0.817, p= 0.3686). However, at this time, gender affected body weight, with females weighing less than males (main effect of gender: $F_{(1,80)}$= 7.523, p= 0.0075, **Figure 3C**).

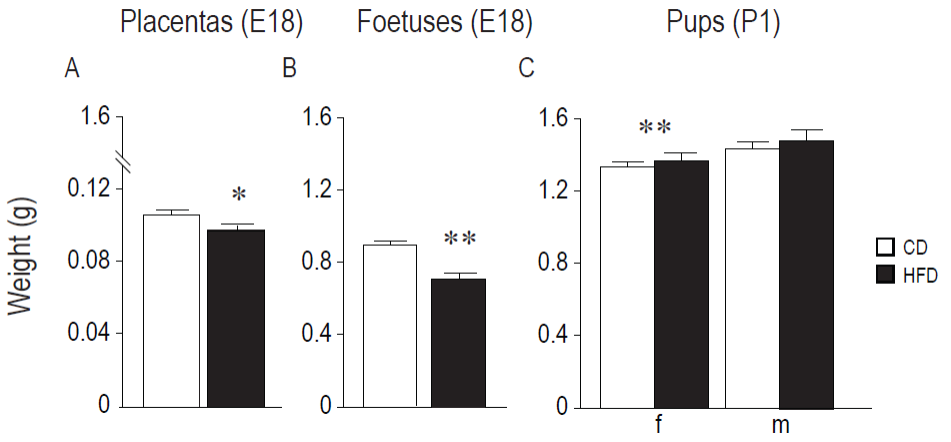

Figure 3. Weight of placentas (A) and fetuses (B) at E18 and birth weight of pups (C). Data are shown as +S.E.M. *Post-hoc* comparisons: *p< 0.05 (HFD *vs.* CD); **p< 0.01 (fetus: HFD *vs.* CD; pups: HFD-f *vs.* CD-f). Experimental subjects: Placentas: CD/HFD, n= 28; Fetus: CD/HFD, n= 28; Pups: CD-f/m, n= 28/32, HFD-f/m, n= 14/10.

4.3.4. Hormonal regulation

• Metabolic response. Overall, reduced levels of leptin were found in the periovaric adipose tissue of females fed a HFD (main effect of diet: $F_{(1,10)}$= 5.029; p= 0.0488, **Figure 4**).

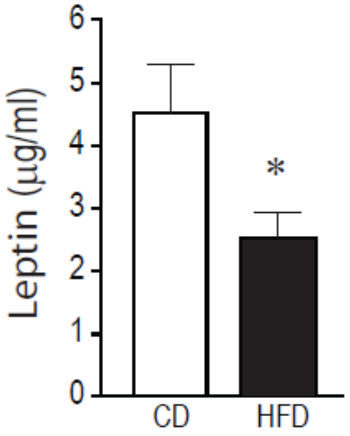

Figure 4. Levels of leptin in periovaric adipose tissue. Data are shown +S.E.M. *Post-hoc* comparisons: *p< 0.05 (HFD *vs.* CD). Experimental subjects: CD/HFD, n= 6.

• Neuroendocrine activation. A HFD strongly enhanced HPA axis activation, overall increasing circulating levels of CORT in basal conditions (main effect of diet: $F_{(1,41)}$= 5.795; p= 0.0207, insert **Figure 5**). Regardless of diet, the more stressful period of pregnancy was found at G16, when the plasma levels of CORT were seven fold higher than G18 and P1 (main effect of day: $F_{(2,41)}$= 389.157; p< 0.0001, data not shown), particularly in females fed a HFD (interaction between diet and day: $F_{(2,41)}$= 4.664; p= 0.0150, **Figure 5**).

4.3.5. Enzyme activity and gene expression

Feeding with HFD before and during pregnancy resulted in down-regulation of the 11ß-HSD2 enzymatic activity in the placentas at G16 (main effect of diet: T_{14}= 3.85; p= 0.0018, **Figure 6A**), in addition to a reduced expression of the placental gene 11ß-HSD1 at G18 (main effect of diet: T_{11}= 5.416; p= 0.0002, **Figure 6B**).

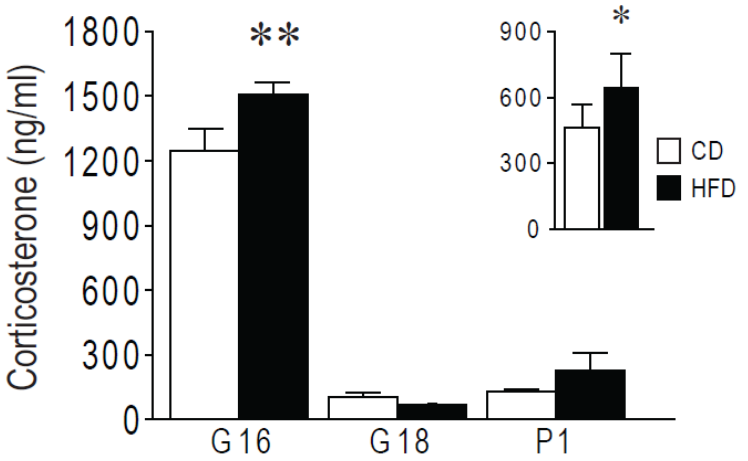

Figure 5. Neuroendocrine activation. Basal neuroendocrine activation in dams measured both before (G16 and G18) and after (P1) delivery and average of basal CORT levels (insert). Data are shown as +S.E.M. *Post-hoc* comparisons: *p< 0.05 (HFD *vs.* CD); **p< 0.01 (G16: HFD *vs.* CD). Experimental subjects: CD-G16/G18/P1, n= 8/10/8; HFD-G16/G18/P1, n= 8/9/4.

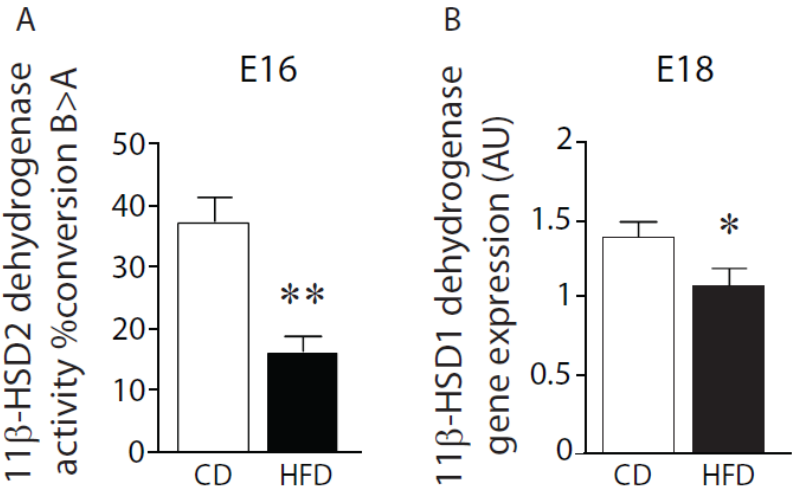

Figure 6. Enzymatic activity and gene expression. Activity of 11β-HSD-2 placental enzyme at E16 **(A)** and expression of 11β-HSD-1 gene in placentas at E18 **(B)**. Data are shown as +S.E.M. *Post-hoc* comparisons: *p< 0.05; **p< 0.01 (HFD *vs.* CD). Experimental subjects: CD-E16/E18, n= 8/7; HFD-E16/E18, n= 6/8.

4.3.6. Locomotor activity

A HFD significantly reduced the locomotor activity of pregnant females during the three days before delivery, and in particular during the central hours of the dark phase and soon after the onset of the light phase (interaction between diet and hours: $F_{(23,299)}$= 2.957; p< 0.0001, **Figure 7**).

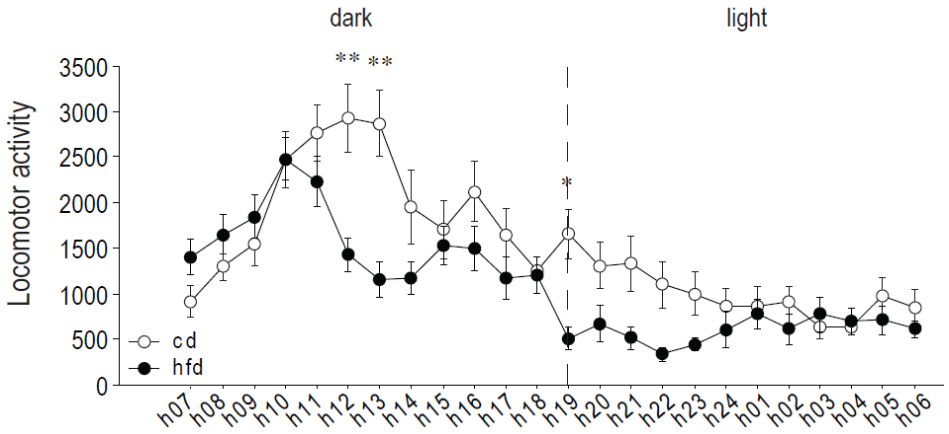

Figure 7. Locomotor activity. Locomotor activity during the three day before delivery. Data are shown as ± S.E.M. *Post-hoc* comparisons: *p< 0.05 (h19: HFD *vs.* CD); **p< 0.01 (h12 and h13: HFD *vs.* CD). Experimental subjects: CD, n= 8; HFD, n= 7.

4.3.7. Maternal behavioral phenotype

• Elevated Plus Maze (EPM). HFD feeding did not affect the time spent in open versus closed arms (interaction between diet and zone: $F_{(1,14)}$= 1.695; p= 0.2140, data not shown). Also, no effect of HFD was found in the percentage of entries into the open arms of the maze (main effect of diet: $F_{(1,14)}$= 1.111; p= 0.3098, data not shown). Moreover, HFD females showed a reduced frequency of *rearing* in the closed arms of the apparatus (interaction between diet and zone: $F_{(2,28)}$= 3.508; p= 0.0437, data not shown).

• Social Avoidance Test (SAT). Regardless of diet, all pregnant dams spent more time in the interaction zone of the arena during both sessions (interaction between stimulus and zone: $F_{(1,14)}$= 6.112; p= 0.0269, data not shown). Despite not being statistically significant (interaction between diet and zone: $F_{(1,14)}$= 2.078; p= 0.1714, **Figure 8A**), *post-hoc* comparison showed that pregnant females fed a HFD spent more time than CD in the interaction zone when the social stimulus was introduced in the wire mesh cage (*post-hoc* comparison for the % time spent in interaction zone HFD-"social stimulus" *vs.* HFD-"no stimulus", p< 0.05).

• Retrieval test. While no difference was registered in the latency to *retrieve* the first pup in the nest between CD and HFD (Fisher's exact probability test: p= 1.000), the latter group was characterized by an increased latency to first *sniffing* a pup (main effect of diet: $F_{(1,14)}$= 10.429; p= 0.0061, **Figure 9A**). In addition, females fed a HFD spent significantly more time than CD *sniffing pups* (main effect of diet: $F_{(1,14)}$= 14.350; p= 0.0020, **Figure 8B**) and in *grooming* behavior (main effect of diet: $F_{(1,14)}$= 6.133; p= 0.0266, **Figure 8C**).

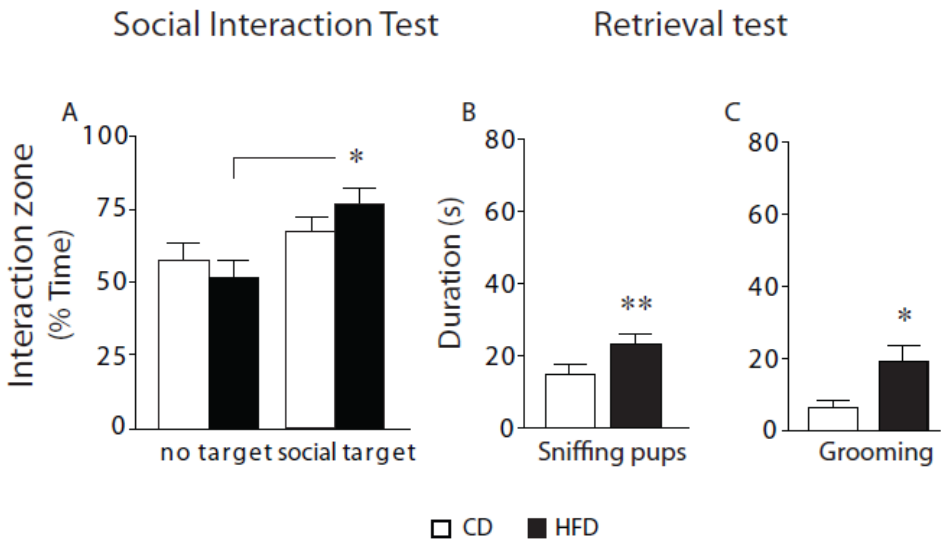

Figure 8. Maternal behavioral phenotype. Time (%) spent in the social interaction zone in the Social Avoidance test (A) and maternal behavior in the Retrieving test (B). Data are shown as +S.E.M. *Post-hoc* comparisons: *p< 0.05 (SAT: HFD-social target *vs.* HFD-no target), (*grooming* duration: HFD *vs.* CD); **p< 0.01 (*sniffing pups*: HFD *vs.* CD). Experimental subjects: CD/HFD, n= 8.

4.3.8. In Situ Hybridization

C-Fos gene expression was used as a marker of neuronal activity to assess neuronal activity within the sensory cortex, olfactory bulb, caudate putamen and paraventricular nucleus (PVN) of the hypothalamus at G16 and P1 (**Table 2**). Exposure to maternal HFD reduced c-Fos expression in the olfactory bulbs, which reached significance at G16 (main effect of diet: T_{14}= 2.684; p= 0.018, **Figure 9B**). Likewise, a HFD reduced c-Fos expression in the PVN of dams assessed at P1 (main effect of diet: T_6= 3.320; p= 0.016, **Figure 9C**).

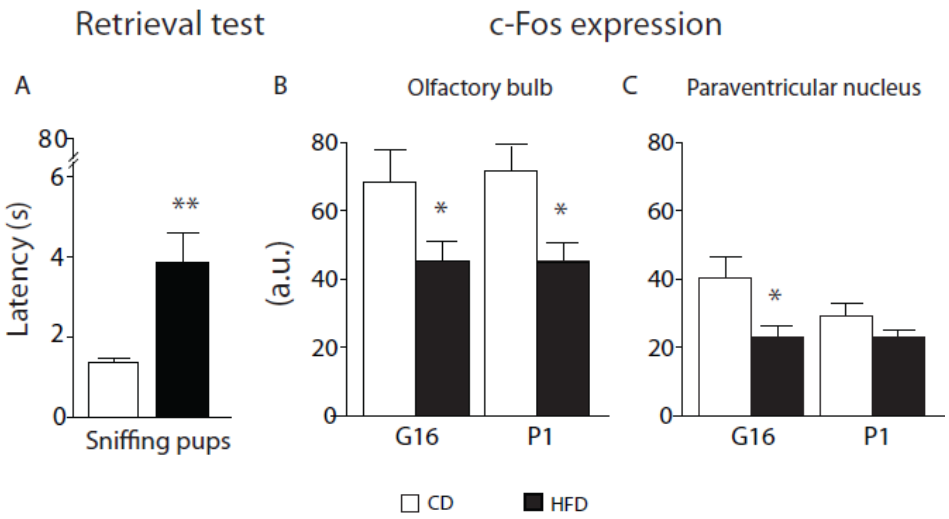

Figure 9. Maternal behavior and olfactory bulb activity. Latency to first *sniffing pups* **(A)** and c-Fos expression in the olfactory bulb **(B)** and in the paraventricular nucleus of the hypothalamus **(C)** of dams both before (G16) and after (P1) delivery. Data are shown as +S.E.M. *Post-hoc* comparisons: *p< 0.05; **p< 0.01 (HFD *vs.* CD). Experimental subjects: CD/HFD, n= 8.

Table 2.

			Sensory Cortex	Olfactory Bulb	Caudate Putamen	PVN
G16	Chow	Mean	48.12	71.42	9.03	29.61
		S.E.M.	7.60	8.58	1.04	2.12
	HFD	Mean	35.01	44.37	7.47	24.71
		S.E.M.	3.73	5.29	0.56	2.38
	Diet p-value		**0.13**	**0.018***	**0.20**	**0.15**
P1	Chow	Mean	34.51	68.43	6.58	40.39
		S.E.M.	4.81	9.10	0.44	3.16
	HFD	Mean	28.87	45.61	7.02	24.96
		S.E.M.	4.95	5.71	0.57	2.70
	Diet p-value		**0.51**	**0.14**	**0.57**	**0.016***

Table 2. C-Fos gene expression. Assessment of neuronal activity within the sensory cortex, olfactory bulb, caudate putamen and paraventricular nucleus (PVN) of the hypothalamus of dams fed a HFD or CD at G16 and P1.

4.4. DISCUSSION

Overall HFD-feeding resulted in increased levels of maternal stress hormones (CORT), associated to reduced 11β-HSD2 enzymatic activity and expression of the 11β-HSD1 gene in the placenta in addition to decreased levels of leptin in the adipose tissue. From a behavioral point of view, HFD dams were characterized by reduced exploratory activity during the active (dark) phase and disrupted social and maternal behavior at parturition. A highly relevant finding concerns the reduced neural activity in brain regions involved in olfactory processing and social recognition, which could underlie the inappropriate behavioral patterns and the cannibalism characterizing HFD-fed dams.

Our data indicate that, despite no difference in daily food consumption, HFD administration for 8 weeks induced a significant gain in body weight of females as previously shown (Bellisario et al., 2014). Moreover, HFD dams were characterized by cannibalistic behavior, an event not registered in CD, and by an increased pup mortality rate. Interestingly, a similar increase in pups' mortality rate has been registered in two separate laboratories involved in a collaborative study, in addition to what has been previously reported by our own laboratory (Bellisario et al., 2014), thus making this a highly replicable finding, although often under-represented in the literature.

In this study we found that the weight of placentas collected at E18 was lower in HFD dams, compared to controls. This result is in line with previous reports from Liang and colleagues, who observed a trend of reduced placental weight at E19 in C57 females fed a HFD for 1 month before the breeding procedure, a result that these authors attributed to elevated oxidative stress due to the HFD (Liang et al., 2010). It is possible that a longer period of diet exposure, like the one used in the present study, may result in greater effects compared to previous reports (Liang et al., 2010). At the same stage of prenatal development (E18) we registered a parallel reduction in HFD fetuses' weight. Several studies report reduced fetal weight with maternal obesity or overnutrition (Akyol et al., 2009; Hayes et al., 2012). Further, Jansson and colleagues demonstrated that changes in placental transport could cause altered fetal growth (Jansson et al., 2006). A reduction in the surface of the transporting epithelium in the HFD placentas might result in a decrease in the nutrient transport, thus reducing fetus weight.

Interestingly, females fed a HFD were also characterized by a significantly extended pregnancy duration compared to CD. So far, no data are available about the effect of a hypercaloric diet on the duration of pregnancy. Nevertheless, it is possible to hypothesize that the extended pregnancy period is an attempt to compensate for the reduced fetal weight registered at E18, allowing for further fetal growth. Indeed, a strong association between maternal HFD-feeding before and during gestation and a lower pup birth weight was previously reported (Flint et al., 2005; Bellisario et al., 2014; Voltolini and

Petraglia, 2014). In the present study no such association was registered between HFD and CD pups' weight at P1.

In the current study we found an increased neuroendocrine response in female mice fed a HFD, particularly at G16, when HPA axis activation was 7-fold higher than at G18 and P1. During pregnancy, the maternal brain triggers fundamentally adaptive mechanisms, allowing normal fetal development and protection for both mother and fetus against adverse programming during parturition (Voltolini and Petraglia, 2014), particularly in the days preceding delivery, when hormonal adaptations occur in preparation for delivery (Sanchez-Muniz et al., 2013). Notably, plasma CORT levels were markedly elevated in HFD dams at term, indicating that the effects of the diet represent a significant stress factor (Connor et al., 2012). An excessive level of stress hormones can result in a number of complications, such as intrauterine growth restriction, which leads to pups with reduced body weight, a condition also found in our study, suggesting an impairment in placental adaptive responses (Jackson, 2000; Sanchez-Muniz et al., 2013). These results are particularly interesting when considering the reduced activation of the 11 β-HSD2 enzyme found in the HFD placentas at G16. This enzyme is highly expressed within the placenta at the maternal-fetal interface and regulates fetal exposure to maternal glucocorticoids (Holmes et al., 2006; Cottrell et al., 2013). In placental mammals, cortisol/corticosterone levels in the maternal circulation are 5- to 10-fold higher than in the fetus (Beitins et al., 1973; Dalle et al., 1978; Montano et al., 1991), a gradient thought to be maintained by high placental 11β-HSD2 (Brown et al., 1993; Brown et al., 1996). This enzyme catalyses the inactivation of maternal glucocorticoids before they are transferred to the fetus (Benediktsson et al., 1997; Chapman et al., 2013; Cottrell et al., 2013). Down-regulation of placental 11β-HSD2, in addition to being correlated with a significant reduction in fetal weight both in humans and rodents (Stewart et al., 1995; Lindsay et al., 1996; Murphy et al., 2002), increases fetus vulnerability to the detrimental effects of maternal HFD. Indeed, in a previous study we found that maternal HFD programs the neuroendocrine fetal development, leading to greater susceptibility to stressful challenges later in life (Bellisario et al., 2014).

Analysis of gene expression in the placenta revealed a reduced HFD-induced expression of the 11β-HSD1 gene at E18. It is worth noticing that the 11β-HSD1 is minimally or not expressed in fetal life until late in gestation when levels are greatest in organs where glucocorticoid activity is required for late maturation prior to birth (Staab et al., 2011). It is possible to speculate that the reduced expression of the 11β-HSD1 gene at that time could be associated to a HFD-induced impaired fetal development, further supported by the reduced fetal weight registered at the same time (G18). Thus, it is reasonable to state that maternal HFD mediates an imbalance in the expression of the genes that regulate the time window of fetal exposure to maternal glucocorticoids (Chapman et al., 2013).

In addition to the above mentioned results, HFD feeding strongly affected the maternal behavioral profile, resulting in a less explorative profile of pregnant females fed a HFD, as indicated by the reduced locomotor activity measured during the three days before delivery and the reduced frequency of *rearing* observed in the EPM on G16. Actually, it has been suggested that while spontaneous locomotor activity largely reflects exploratory behavior, *rearing* may also be an expression of emotionality (Gironi Carnevale et al., 1990; Thiel et al., 1998), suggesting that a HFD reduced emotionality of pregnant females resulting in a disinhibited behavior profile. Such a response has been also observed in the SAT. In this test, HFD pregnant females displayed reduced social anxiety spending more time in close proximity of the unknown CD-1 mouse, suggesting reduced ability to discriminate an unfamiliar social stimulus. A similar response was also observed in the maternal behavior measured in the retrieving test, in which HFD dams spent more time in social investigation of pups (*sniffing pups*). These data could indicate an HFD-induced arousal towards social stimuli rather than increased maternal behavior. Indeed, although no difference in *pups retrieving* was registered between CD and HFD dams, the latter showed a greater latency to approach the pups and spent more time performing *grooming*, a displacement behavior related to negative emotions, such as pain and anxiety (Vos et al., 1994; Kalueff and Tuohimaa, 2005; Xu et al., 2008; Yao and Sessle, 2008), suggesting a lack of adaptation in such a context.

One potential explanation for such changes in the behavior of the dams comes from our findings of reduced c-Fos expression in the olfactory bulbs of HFD dams both before (G16) and after delivery (P1), indicating reduced neuronal activation in these brain regions involved in the processing of relevant olfactory information (Sharp et al., 1993; Spencer and Houpt, 2001).

Previous data have suggested that HFD, regardless of the degree of adiposity, can impact the neuronal structure of the olfactory system resulting in reduction in olfactory discrimination (Thiebaud et al., 2014). Thus in our case, a reduction in neural activity following HFD feeding before and during pregnancy might underlie significant changes in maternal behavior, suggesting that the cannibalistic behavior characterizing HFD dams could depend upon an inability to discriminate the olfactory stimuli provided by the newborn pups.

Results also indicate reduced levels of leptin in the periovaric adipose tissues in HFD dams, an effect possibly associated to HFD-induced leptin resistance. Preclinical and clinical studies have reported HFD-induced hypothalamic inflammation (Cai and Liu, 2012), resulting in leptin resistance through activation of the negative regulators of leptin signaling (Cai and Liu, 2012; Milanski et al., 2012). Interestingly, in the past decade, several metabolic markers of a nutritional status, such as leptin, have been recognized as direct modulators of the peripheral and central neuronal pathways involved in olfactory functions (Palouzier-Paulignan et al., 2012; Thiebaud et al., 2014).

Thus, reduced peripheral levels of leptin found in HFD-fed dams might also be reflected in a reduced neuronal activity of the olfactory bulb.

It is worth noticing that the olfactory system drives behavioral decisions about food choice and consumption (Thiebaud et al., 2014) and the odors associated with food are essential to control food intake (Le Magnen, 1959). Therefore, a change in the activity of the olfactory system could also affect food choices, reinforcing inappropriate behaviors, such as the choice of poor quality food (Fadool et al., 2000; Lacroix et al., 2008; Tucker et al., 2013).

Overall, results from this study indicate that HFD is a stressful challenge during pregnancy with relevant consequences on fetal development. Maternal HFD has been widely hypothesized to be one of the causes leading to the metabolic alteration of the offspring during life. The present study provides a thorough investigation on the mechanisms underlying such detrimental effects, suggesting that HFD can be interpreted as a stressful, metabolic stimulus, reinforcing neuroendocrine activation linked to parturition and delivery.

More importantly, HFD-feeding also impairs the emotional profile of pregnant dams and the neural activity of brain regions involved in the development of maternal behavior with aberrant consequences on the offspring (Cirulli et al., 2003).

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CHAPTER 5

5. GENERAL DISCUSSION

Maternal obesity and HFD consumption during pregnancy have profound effects on offspring health, ranging from metabolic to behavioral disorders in later life, although a comprehensive characterization of the long-term impact of the maternal-mediated metabolic stimulus is still lacking. **The objectives** of the studies described in this thesis were 1) to explore the impact of prenatal exposure to maternal HFD on the behavioral, endocrine and metabolic phenotype of the adult offspring and 2) to investigate if HFD feeding during pregnancy might represent a metabolic stressful stimulus for the mother. **To this aim** the impact of exposure to HFD was evaluated both on fetal development - in a long-term perspective- as well as on maternal behavior and physiology in a mouse model of reduced OS (the $p66^{Shc-/-}$ mouse). In this context a main emphasis was given to the interaction between this metabolic stressful stimulus (maternal HFD) and a physiological condition of reduced OS in the offspring in both genders.

5.1. MAIN FINDINGS

5.1.1. Maternal HFD acts as a psychophysical stress affecting offspring physiology in a lifelong, gender-dependent fashion

Early life experiences, including the prenatal fetal development, are an important programming factor on a number of aspects of the offspring's physiology, representing a risk factor possibly impairing a proper metabolic and neuroendocrine function and also affecting the emotional and behavioral profile of the individual in a lifelong perspective (Spencer, 2013). In this regard, early life stress, as well as extreme metabolic conditions, such as prenatal exposure to a maternal diet highly rich in fats, can deeply influence developmental programming. In fact, preclinical studies have shown that changes in maternal diet also influence the response to stress with relevant consequences on the offspring, including adverse behavioral changes (Weinstock, 2001), cardiovascular responses to stress (Igosheva et al., 2007), glucose tolerance (Lesage et al., 2004), as well as sexual dimorphism of brain regions. This latter point suggests that there might be gender-dependent differences in the risk for metabolic and mood disorders associated to maternal HFD *in utero* as shown in the adult population.

Previous preclinical studies have reported increased anxiety-like behavior in adult subjects of both genders exposed to HFD during fetal development (Peleg-Raibstein et al., 2012), although in some cases a gender dependent effect is reported (Bilbo and Tsang, 2010). Results described in **Chapter 2** are in line with the latter finding, reporting that female subjects exposed to maternal HFD during prenatal life show a reduced emotional profile compared to male mice. In addition, and more interestingly, prenatal HFD

drives an inversion in the emotional profile if compared to that observed in CD offspring. More in detail, prenatal HFD reduces the anxiety-like behavior in female while increasing it in males, suggesting a feminization of the emotional profile of male mice. This is particularly interesting in light of the results obtained by Weinstock and colleagues in a rat model of prenatal stress that observed a similarfeminization effect in male subjects (Weinstock, 2001), suggesting that prenatal metabolic alterations act as psychophysical stressful stimuli with potential implications for brain function. This latter point is further corroborated by results related to the HPA-axis activation of the adult offspring after an acute psychophysical stress. In particular, while no difference in the neuroendocrine activity was observed in male mice, females prenatally exposed to HFD showed an overall significantly increased response to an acute restraint stress, as a result of an impairment of the negative feedback of the HPA axis. It is worth noticing that the alterations of the neuroendocrine profile induced by maternal HFD largly overlap with those observed in animal models of prenatal stress. For instance, Louvart and colleagues reported that female offspring of rat dams exposed to a chronic restraint stress during the last trimester of pregnancy display, at adulthood, a stronger response to restraint stress (Louvart et al., 2009). In this context it is possible to hypothesize that a metabolic challenge (maternal HFD) could mimic a psychophysical stress, as both interfere with the fetal programming of neuroendocrine development, altering the HPA axis activity during the entire life span (Maccari et al., 2003; Seckl and Holmes, 2007; Reynolds et al., 2013; Spencer, 2013). One potential explanation for how stressful and nutritional challenges occurring during the prenatal life can influence the neuroendocrine mechanisms in a long-term perspective is that they can permanently alter the sensitivity of the HPA axis and thus the overall levels of GC the animal is exposed to (Spencer, 2013).

A gender-dependent effect of maternal diet was found in response to metabolic stimuli. Several data have been reported on differences between genders in the perception of metabolic signals (Lonnqvist et al., 1997). This point deserves further investigation since it could contribute to understanding the observed differences in the prevalence rates of eating disorders and obesity between genders (Kaye, 2008). The study described in **Chapter 2** refers to an overall increase in glucose tolerance in females prenatally exposed to HFD, suggesting a better ability to metabolize the excess of blood glucose, while an opposite profile was found in males, in which HFD determined greater glucose intolerance.

Moreover, the studies presented in **Chapter 2** provide new insights into the critical role that early life environment plays in programming the subsequent responses to stress and feeding behavior. In particular, it is worth noticing that high-fat feeding during pregnancy, independently from maternal obesity, causes *per se* placental dysfunctions in the mother as well as metabolic impairments in the offspring (McCurdy et al., 2009; Frias et al., 2011). In addition, since the early life environment has also a specific influence on brain development and

circuitry, stress and the early life nutritional environment can acutely affect the connectivity within the hypothalamic region necessary for regulating feeding leading to a long-term abnormal foraging behavior.

Overall, these results strongly point to the prenatal environment as a main developmental sensitive period. This time window appears to be equally, if not more, important than other sensitive developmental steps, such as lactation and adolescence, in programming the individual vulnerability/responsivity to stress, nutritional needs and feeding behavior and, more importantly, how stress and metabolic needs might influence one another. Therefore early life environment may at least in part account for these inter-individual differences.

5.1.2. Reduced oxidative stress characterizing the $p66^{Shc-/-}$ mouse thwarts the effects of prenatal exposure to maternal HFD

Previous findings reported that functionality loss of the $p66^{Shc}$ gene in mice protects from the negative effects induced by adult exposure to HFD - including adiposity and IR - through multiple mechanisms, also comprising reduction of systemic OS (Frankel and Rogina, 2012).

Chapter 2 describes the multi-level protective role played by $p66^{Shc-/-}$ gene after maternal exposure to HFD. Such a role is observed both in the mother during the last phases of pregnancy and during parturition as well as in the adult offspring, particularly as concerns the metabolic and the neuroendocrine profile.

HFD-feeding is associated to increased mortality rate of pups, due to impaired maternal behavior. Very intriguingly, an increased mortality rate was also observed in HFD dams during the days immediately before the expected delivery date or during parturition. Similar findings were previously reported by Flint and co-workers, further corroborating the critical role played by maternal diet in such time window (Siemelink et al., 2002; Buckley et al., 2005; Flint et al., 2005). However, in this context, the lack of the $p66^{Shc}$ gene plays a protective role, at least for selected parameters such as dams' mortality and aberrant maternal behavior, which, in turn, results in cannibalistic behavior.

Moreover, in **Chapter 2** it is well described the protective action of reduced OS characterizing the $p66^{Shc-/-}$ mice on the metabolic and neuroendocrine negative effects of the early exposure to maternal HFD. Also in this case, as previously briefly mentioned a strong gender-dependent effect was found as a result of gene deletion.

As far as the metabolic challenges are concerned, the lack of the $p66^{Shc}$ gene was able to modulate the response to metabolic stimuli, resulting in a greater resistance towards a glucose challenge, compared to WTs. Such effect is affected by the diet in a gender-dependent fashion. Indeed, in KO females, prenatal exposure to HFD drove a higher glucose tolerance resulting in a better ability to metabolize an excess of blood glucose, while in KO males an opposite

profile was found, with HFD determining severe glucose intolerance in comparison to controls (CD-KO). In the light of these points, it is reasonable to hypothesize that HFD overrides the effects of the lack of p66 gene on metabolism in males, reducing the glucose resistance, and exacerbating it in females.

Lack of the $p66^{Shc}$ gene leads to greater resistance in the IST test. Resilience to an insulin challenge is affected not only by the genotype, but also by the maternal diet, since HFD individuals showed greater insulin insensitivity. In particular, Turdi and co-workers suggested that such IR could result from a severely dampened insulin-signaling-cascade (Turdi et al., 2013). Thus, the metabolic alterations described in KO mice might be due to the reported involvement of $p66^{Shc}$ gene in the insulin signaling (Berniakovich et al., 2008), which leads to hypothesize that its deletion results in insulin desensitization with the consequent impairment in glucose metabolism.

Another main finding, which highlights the protective role exerted by the lack of the p66 gene, is related to the programming effect of HFD on the neuroendocrine development and responsiveness later in life. More in detail, in female mice, HFD significantly increases the response to an acute restraint stress, however KO subjects are protected from such effect, showing an efficient HPA axis feedback that is comparable/specular to thatobserved in controls (**Chapter 2**). There is a great body of evidence suggesting that HFD consumption is strongly associated to an increased condition of general inflammation (Contestabile, 2001) It is important to note that *in vitro* studies have shown that increased ROS generated upon inflammatory challenges, , may attenuate the GC negative feedback suggesting that neuroendocrine function may be finely modulated upon OS/inflammatory signaling pathways (Okamoto et al., 1999; Asaba et al., 2004).

5.1.3. Antioxidants mimic the metabolic response of the $p66^{Shc-/-}$ gene following prenatal exposure to HFD

Increasingly experimental and clinical data suggest that antioxidants have a beneficial role in the prevention of metabolic disorders, such as IR (Masha et al., 2009; Novelli et al., 2009). N-acetyl-cysteine (NAC) is an interesting target because of its antioxidant and anti-inflammatory effects. Indeed, NAC has been recently shown to exert a protective effect against some metabolic disorders in rats (Haber et al., 2003; Song et al., 2005; Diniz et al., 2006; Novelli et al., 2009). In these studies, it has been found that NAC supplementation is able to prevent hyperglycemia and glucose intolerance associated with the preservation of glucose-induced insulin secretion and improved insulin sensitivity. It is relevant to note that these data are also confirmed by a very recent study conducted by Lasram and colleagues (Lasram et al., 2014).

Interestingly, in line with literature data, results obtained in **Chapter 3** describe that male mice prenatally exposed to NAC show a greater resistance

toward a glucose challenge compared to untreated controls. However, such effect is lost in males prenatally exposed to maternal HFD, showing a severe glucose intolerance in comparison with their own controls (NAC-CD), suggesting that prenatal exposure to NAC is not completely able to counteract the negative metabolic effect driven by maternal HFD.

However, results related to the effects of NAC on the metabolic response to insulin, reported in Chapter 3, do not agree with literature data. Indeed, while several studies reported greater NAC-induced insulin sensitivity, we found that prenatal NAC exacerbated insulin resistance characterizing HFD male mice.

The mechanism by which NAC affects the metabolic response to glucose and insulin is not yet completely understood. NAC promotes the synthesis of growth stimulating hormone (GSH) (Song et al., 2005), which has been found to have cellular functions unrelated to its antioxidant activity, such as acting on several signaling pathways. The few studies performed so far lead to hypothesize that the effects of NAC on glucose homeostasis are mediated by the increase of hepatic GSH output, which in turn positively affects body red-ox status and early events of the insulin signaling pathway, mainly by a reduction of OS.

The described mechanism of action of NAC drug appears supported by the evidence that the metabolic profile induced by a glucose challenge is strictly similar in NAC subjects and in $p66^{Shc-/-}$ mice, in particular in male mice (see **Chapter 2,** paragraph 3.5 and **Chapter 3,** paragraph 5.4).

Similarly, in the insulin sensitivity test a comparable action between the NAC drug and the lack of the $p66^{Shc}$ gene has been registered. This is shown by the glycemic trend of NAC subjects in response to the insulin stimulus that perfectly follows that characterizing the genetic model of reduced OS. This evidence strongly suggests that the effect of prenatal NAC mimics the lack of $p66^{Shc}$ gene on the metabolic profile. It is possible to hypothesize that this effect is due to the parallel exposure of the developing fetus to both stimuli, i.e. maternal HFD and NAC, which might have rendered the antioxidant action more efficient in an attempt to reduce the HFD-induced fetal misprogramming.

In addition, Goodson and co-workers assessed NAC effectiveness as a chemotherapic agent, finally suggesting that NAC may be more useful as a chemopreventive agent in the context of acute UV exposure (Goodson et al., 2009). The preventive action against the UV-induced oxidative stress, demonstrated by Goodson and co-workers, is ascribable to the potent antioxidant capacity of NAC (Goodson et al., 2009). Similarly, from the study described in Chapter 3 emerges that the effects of NAC on the metabolic response are mainly ascribable to the precocious time window of exposure, allowing for a preventive pharmacological action of the drug.

5.1.4. HFD during pregnancy is a stressful challenge resulting in aberrant maternal behavior and impaired neuronal activity

The mechanisms by which prenatal stress alters later life metabolic outcome remain unknown. Stress during pregnancy alters maternal hormones, including circulating GCs levels. Normal physiological levels of GCs during fetal development are essential for tissue growth and maturation, however, when they exceed, GCs have been shown to affect negatively maturation of tissues and organs (Bian et al., 1992; Fowden, 1995). Elevated maternal GCs can cross the placenta (Cottrell et al., 2012) and this has been shown to affect growth and function of brain and peripheral tissues during fetal development (Duthie and Reynolds, 2013). Exposure to increased level of GCs either through administration of dexamethasone during late gestation or via stress exposure (malnutrition, adverse environment exposures) reduces birth weight and impacts maturation of major organs (Lesage et al., 2001; Seckl, 2004). Dams exposed to prenatal variable stress had heavier adrenal mass and lower fecal CORT secretion *vs.* non-stressed controls (Paternain et al., 2012). This is suggestive of a reduced CORT clearance in these stressed dams during gestation.

In line with literature data, results in **Chapter 4** describe an increased neuroendocrine activity characterizing female mice fed a HFD, particularly at G16, when HPA axis activation is 7-fold higher than at G18 and P1. During pregnancy, maternal brain triggers adaptive mechanisms allowing normal fetal development and protection for both mother and fetus against adverse programming (Voltolini and Petraglia, 2014). This particularly happens in the days preceding delivery, when hormonal adaptations occur in preparation for delivery (Sanchez-Muniz et al., 2013). Notably, plasma CORT levels are markedly elevated in HFD dams at term, indicating that the effects of the diet represent a significant stress factor (Connor et al., 2012). An excessive level of stress hormones can result in a number of complications, such as intrauterine growth restriction, which leads to pups with reduced body weight, a condition also found in our study, possibly suggesting an impairment in placental adaptive responses (Jackson, 2000; Sanchez-Muniz et al., 2013).

These results are particularly interesting when considering the reduced HFD-induced activity of the 11β-HSD2 enzyme at E16, in addition to the reduced expression of the 11β-HSD1 gene at E18. These specific placental enzymes protect fetus from high GCs levels, acting as a placental barrier, which regulates the transfer of GCs from mother to fetus (Benediktsson et al., 1997; Holmes et al., 2006). Stocker and colleagues reported that likely, as with maternal stress, maternal nutrition regulates placental 11β-HSD2 levels, in turn, influencing fetal exposure to GCs (Stocker et al., 2004; Stocker et al., 2005). Thus, results of the study might suggest that HFD compromises the timing of the switch from 11β-HSD type 2, in early and mid-gestation (Murphy, 1978; Brown et al., 1996; Condon et al., 1998), to 11 β-HSD type 1 activity, in late gestation (Murphy, 1978; Thompson et al., 2004), functional to a correct fetal

development. In addition, it is possible speculate that the reduced expression of the 11β-HSD1 gene at E18 could be associated to a HFD-induced impaired fetal development, an hypothesis further supported by the reduced fetal weight registered at the same time. An HFD-mediated imbalance in the expression of the genes regulating the time window of fetal exposure to maternal GCs (Chapman et al., 2013) has been reported to have a number of effects, interfering with synaptic pruning during brain development in regions that are important for the control of the HPA axis, the prefrontal cortex and hippocampus (Jacobson and Sapolsky, 1991; Spencer et al., 2005).

A relevant result reported both in **Chapters 2 and 3** is the increased mortality rate of pups from HFD-fed dams as a result of cannibalistic events. This observation was not previously reported in literature. These evidences lead us to hypothesize that this event might be associated to an HFD-induced impairment in maternal behavior.

In the attempt to find a neurobiological support to the behavioral observation, in the study described in **Chapter 4**, in addition to maternal behavior, the c-Fos expression in several brain areas was also measured. It is usually assumed that c-Fos expression provides a reliable marker of neuronal activation (Sharp et al., 1993; Spencer and Houpt, 2001). Particular attention has been paid to the olfactory bulbs, a brain region involved in the identification of olfactory stimuli and particularly relevant in a macrosmatic species, like the mouse. Results reported in Chapter 4 do not register differences in *pups retrieving* between CD and HFD dams; however the latter group showed a greater latency to approach the pups, another reliable index of reduced maternal behavior. This data seems to confirm the hypothesis that HFD during pregnancy might impair the development of maternal behavior.

In addition to maternal behavior, HFD strongly affected the explorative and emotional profile of pregnant females. HFD feeding resulted in a less explorative profile, as indicated by the reduced locomotor activity measured during the three days before delivery and the reduced frequency of explorative behavior (*rearing*) observed in the EPM on G16. Previous studies suggested that *rearing* may also be a behavioral index of emotionality (Gironi Carnevale et al., 1990; Thiel et al., 1998), thus suggesting that HFD reduced emotionality of pregnant females resulting in a disinhibited behavior profile. This is further confirmed by the behavioral profile observed in the SAT, in which HFD pregnant females displayed reduced social anxiety spending more time in close proximity of the unknown CD-1 mouse, but also suggesting a reduced ability to discriminate an unfamiliar social stimulus.

A potential explanation for such changes in the behavior of the dams comes from our findings of reduced c-Fos expression in the olfactory bulbs of HFD dams both before (G16) and after delivery (P1), indicating reduced neuronal activity in those brain regions involved in the processing of relevant olfactory information (Sharp et al., 1993; Spencer and Houpt, 2001). Taken together, these results demonstrate complex interactions between the nutritional

status of an animal and its behavioral and neuronal olfactory bulb responses to odor stimuli. Our results also indicate a modulating role of nutritional status, that is HFD, on c-Fos olfactory bulb responses to olfactory stimuli, as previously suggested (Thiebaud et al., 2014).

5.2. IMPACT AND PERSPECTIVES

Due to the epidemic proportion of obesity and overweight (Hurt et al., 2010), and the related healthy consequences, and taking into account its increasing incidence among children and pregnant woman, in addition to the adult population, (Mitchell and Shaw, 2014) the evaluation of physiological adaptation to diets rich in fats, ranging from behavioral to hormonal and molecular endpoints, has stimulated the scientific interest in several fields.

Growing evidence suggests that maternal obesity increases the risk of childhood obesity and subsequent adult disease. Many studies have reported higher incidences of obesity and several metabolic disturbances, such as hyperglycemia as well as IR and T2D as a result of prolonged high-fat feeding (Oakes et al., 1997; Tschop and Heiman, 2001; Buettner et al., 2007). More importantly, such phenomenon not only directly affects the HFD-consuming individuals but also predisposes subsequent generations to similar health concerns. There is therefore a real and immediate need for interventions that target the development of offspring obesity.

Human studies have identified maternal risk factors associated with disturbed offspring health, such as gestational weight gain, elevated adiposity, hyperglycaemia and IR. These outcomes provide key targets for interventions that seek to improve offspring health. Recent evidence suggests that altering maternal carbohydrate and/or physical activity levels can improve maternal pregnancy outcomes. Indeed, pregnant women are an extremely susceptible population, given their higher nutritional demands both in quantity and in quality terms. Inappropriate nutrient supply during this critical period of time inevitably results in an early onset of chronic diseases in the future generations. Therefore, a better understanding of the physiological origin of HFD-related effects will not only benefit the success of conception but also protect the progeny against unfavorable environmental challenges.

Alhtough longitudinal studies adequately describing the long-term impact of these early metabolic interventions on offspring health are lacking,, basic research provides us highly valuable information that might allow translational applicability in the clinical field. Increasing evidence supported by a substantial amount of animal studies has indeed demonstrated that pre- and peri-natal exposure to HFD profoundly alters the intrauterine environment leading to permanent phenotypic alterations with adverse outcomes that persist through the adulthood of the progeny. Compromised nutritional environment during early development stages targets both immediate health problems in newborns and continuing consequences in adulthood. These outcomes manifest

at certain physiological levels including metabolism, emotional disturbances and learning ability, endocrine alterations and susceptibility to chronic diseases (Napoli et al., 2002; Srinivasan et al., 2006; Elahi et al., 2009).

It is relevant to note that human studies are limited mainly by the restrictions to invasive methodologies in offspring. Animal models therefore provide an alternative in which interventions that seek to protect offspring from a conferred obesity risk may be investigated. Animal studies reported in this thesis provide a great clinical value, allowing early prevention and development of therapeutic treatment.

This thesis was mainly focused on a maternal model of HFD, in which the diet was given to the dams both before and during gestation in order to investigate the transgenerational impact of maternal feeding patterns on fetal outcomes. Fat overconsumption prior to or during pregnancy results in an unfriendly intrauterine environment, on which the concentrations of energy, hormones and blood supply are less optimized and, not surprisingly, leads to abnormal fetal development and growth (Srinivasan et al., 2006; Chen and Scholl, 2008; Samuelsson et al., 2008; McCurdy et al., 2009; Bilbo and Tsang, 2010).

Taken together, data presented in this thesis are consistent with the hypothesis of an early obesogenic nutritional "hit" and a developmental origin of sex-dependent susceptibilities for the HFD exposure-related consequences. The placenta might play a crucial role in mediating such effects to the fetus and offspring. Previous studies report that feeding C57BL/6J mice a HFD during pregnancy leads to modified expression of imprinted genes and local or genome-wide DNA methylation in placenta and that these effects are sex-specific (Gallou-Kabani et al., 2010; Mao et al., 2010). It is possible to speculate that the sex-dimorphic differences we observed in the adult offspring may represent the consequence and transgenerational fixation of detrimental dietary exposures on the placenta during the early prenatal period. In humans, this would correspond to the commonly encountered gestational conditions of overweight or milder forms of obesity. Due to the lack of evidence on long-term health outcomes, those conditions have not yet been included in recent clinical guidelines for preventive action to ameliorate obesity in adults or specifically in women of fertile age (Moyer, 2012). Nonetheless, at least in our rodent model, such a nutritional environment during the earliest phases of development is sufficient to induce long-term adverse effects in offspring phenotypic outcomes. This indicates varying developmental vulnerabilities between sexes towards overnutrition.

More importantly, data reported in this thesis indicate that not only obesity, but also the consumption of diet rich in fats and sugars per se has relevant consequences for the health outcomes of both mother and offspring. The negative effects of wrong dietary habits, which might result in unhealthy metabolic condition such as IR and diabetes, often are not accompained with a gain in body weight or any relevant symptom. The metabolic disturbancies,

which might firstly develop through an asymptomatic phase, might thereafter become evident when conclamate pathologies. In this prospective a preventive intervention is the only way to walk. A sensitization to the multi-levels health consequences deriving from wrong dietary habits and, more in general, wrong lifestyle should be promoted in order to guide people toward an informed nutritional behavior.

Several studies have reported a causative link between HFD feeding and nervous system dysfunction and perturbed neuronal plasticity (Park et al., 2010; Pierce and Xu, 2010; McNay et al., 2012; Yi et al., 2012). As also reported in **Chapter 4,** HFD-feeding has been found induce lower neuronal activity in several brain area and particularly in the olfactory bulb, the main brain system driving behavioral decisions about food choice and consumption (Thiebaud et al., 2014). The odors associated with food are essential to control food intake (Le Magnen, 1959). Therefore, HFD-induced changes in the activity of the olfactory system might affect food choices thus reinforcing inappropriate behaviors, such as the choice of poor quality food (Fadool et al., 2000; Lacroix et al., 2008; Tucker et al., 2013). The trigger of this vicious cycle might be relevant as well as detrimental implication for health.

One of the most relevant finding of this thesis is that HFD experienced during pregnancy represents a potential physiological stressor for dams. This effect is demonstrated by the increased HPA-activity characterizing HFD pregnant dams and is further supported by the aberrant maternal behavior observed after delivery. Despite the paucity of literature data regarding the effect of HFD on both HPA-axis activity during pregnancy and maternal behavior, data obtained in this thesis concordantly support this hypothesis.

Importantly, our results acquire a major relevance taking into account the several clinical studies supporting the stressful role played by diets rich in fats in women during pregnancy (McCurdy et al., 2009; Frias et al., 2011; Reynolds et al., 2013).

This observation drives a raising attention on the negative role played by HFD, and more in general by unhealthy diets, not only on metabolism and on related-pathologies both in the mother and in the offspring, but in a more wide prospective on the psycho-physiological outcome and on the behavioral and emotional profile of mothers which is directly reflected on the offspring.

Thus, in addition to maternal interventions, focus can be placed on offspring outcomes associated with obesity development, metabolic-related pathologies and psychological disorders. To date, there is a paucity of studies investigating potential intervention strategies in either over-nourished mothers or in their offspring that aim to attenuate offspring predisposition to these disorders. Addressing such possibilities is therefore an important research need. These should include targeting maternal parameters associated with excess nutrition and/or obesity (hyperglycaemia, hyperlipidaemia, hyperinsulinaemia and increased stress and inflammatory markers), as well as the phenotypic consequences in the offspring (hyperphagia and reduced physical activity).

Results may then help to combat the cycle of maternal obesity and offspring obesity.

5.2.1. Intervention strategies

Results obtained and discussed in **Chapter 3** describe the potential effect of a synthetic antioxidant administered during the prenatal life, that is NAC, to limit some of the long-term consequences deriving from prenatal exposure to maternal-HFD. However, prenatal exposure to NAC is not completely able to counteract the negative metabolic effect driven by maternal HFD. This finding suggests that a long-lasting rather than a short-term pharmacological intervention could be resolutive to prevent and thwart metabolic disturbances.

However, given the expense and potential long-term risks of pharmacological therapies, lifestyle interventions have also to be seriously considered as a viable alternative. Lifestyle, such as healthy diet and physical exercise, can be useful in the prevention and treatment of obesity and metabolic disorders.

Prevention strategies rather than treatment have gained newfound interest, particularly with the increasing incidence of obesity and risk of attendant complications in developing countries. Having obese women lose weight and achieve a normal BMI prior to conception would be the ideal goal, but realistically quite difficult to achieve, given the known problems of achieving optimal glucose control in women planning to become pregnant. Treatment modalities such as minimization of weight gained during pregnancy, combined with the institution of healthy eating and exercise regimens, may represent an ideal option for these interventions.

This kind of intervention should also have an evident positive economic impact not only for the individual, but in a more wide perspective, for the Public Health System allowing a great cost reduction.

5.2.2. Impact and relevance of the $p66^{Shc}$ gene

The $p66^{Shc}$ gene appears to be one of the converging points linking OS, metabolism and the genetics of ageing, its lack of functionality leading to a plethora of positive effects, accounting for retarded aging, in addition to a more general healthier status (Berry et al., 2007; Berry et al., 2008; Berry et al., 2010). Overall the "thrifty function" of $p66^{Shc}$ appears desirable when food is scarce and resources need to be stored. However, fat accumulation promoted by $p66^{Shc}$ can be detrimental when food is constantly available - a condition characterizing westernized lifestyle - and may lead to increased vulnerability to develop degenerative diseases such as diabetes, CVD and cancer, accelerating the aging process (Neel, 1962; Neel et al., 1998). Being at the crossroad of signaling pathways involved in both central and peripheral stress responses and in the regulation of energy homeostasis, $p66^{Shc}$ is a good candidate molecule to address the mechanisms underlying healthy phenotype and to be targeted for the

development of novel pharmacological tools for the prevention or cure of metabolism-related pathologies.

Future studies should address the issue of the cause-effect involvement of $p66^{Shc}$ in metabolic-related disorders. In addition, the assessment of changes in the $p66^{Shc}$ expression early in life might represent a sensitive marker allowing the detection of early changes in the metabolic/health status in humans. Therefore, $p66^{Shc}$ should be potentially used in clinical practice as specific peripheral biomarkers of a pathological metabolic state indicating a potential risk not only for metabolic disorders but, given the pivotal role played in the red-ox cellular processes, even and importantly for age-related pathologies and the associated disorders.

Finally, data discussed in this thesis set the conditions for future studies aimed at better understanding the role of $p66^{Shc}$ in the adaptive response to metabolic stressors in order to develop novel diagnostic and therapeutic tools aimed at promoting healthy phenotype mainly in an aging perspective.

5.3. CONCLUSIONS

There has been a significant increase in the prevalence of obesity in the past decades both in developed and in developing countries. This increased prevalence is not confined to adults only, but is beginning to affect even youth, having been reported with increasing frequency in adolescents and children as young as 2 years of age. Although there is significant morbidity and mortality associated with obesity in and of itself, there are additional risks associated with the metabolic syndrome, which is a component of and may in itself be a consequence of obesity.

The early nutritional environment is thought to be a major determinant to the development of obesity in the young and adult offspring. Moreover, the exposure to particular diets during critical early periods, e.g., during pregnancy, might result in subjects that are exceptionally vulnerable to environmental factors that contribute to obesity (Delisle, 2005).

It is clear that maternal HFD has a profound influence on the response of the offspring, in both the periphery and the brain, affecting not only physiological phenotype (metabolic and endocrine), but also the behavioral profile. These effects can have enduring consequences for brain functioning and the onset of neurodegenerative disorders, affecting both males and females, often with different outcomes. The perinatal period is a crucial time when adverse experiences have a major impact on developing systems and neuronal and HPA axis activity are critical in mediating the influence of the environment on the individual. In an ontogenetic perspective, a novel concept to be explored is how the quality and quantity of different early experiences affect neural, endocrine, metabolic and behavioral domains relevant across diverse disorders, ranging from mental health to neurodegeneration and obesity. Such wide range

of effects may explain the co-morbidity often found between different pathologies, which can greatly affect quality and even longevity of life.

Knowledge on specific measures capable to alleviate fetal overgrowth in obese pregnant women can allow the development of early intervention strategies that could contribute to a decrease in the prevalence of obesity and diabetes, and more in general of metabolic disorders, in future generations. Additional studies are needed to establish the critical time window during perinatal development when HFD exposure influences the behavioral and neural responses observed in this study. Determining how maternal HFD specifically affects brain signaling pathways in offspring will allow a more time-selective targeting of interventions to counteract the consequences of overnutrition on developmental programming.

Another relevant contribution of these studies, which should inform future research, is the knowledge that metabolic stimuli can act as stressors, potentially affecting the neuroendocrine system, with greater effects if experienced during critical developmental periods. This point is worth taking into account, given the emergency of obesity and the world widespread consumption of high fat food, independently from obesity.

Several studies have shown that administration of high fat or high calorie diets to rodents increases the cellular oxidative state (Zhang et al., 2005; Souza et al., 2007), thus also resulting in increased oxidative damage in the brain (Cecarini et al., 2007; Bruce-Keller et al., 2009; Zhang et al., 2009). In turn, increased OS mediates the effects of HFD consumption both on brain pathogenesis and cognitive disturbances and exacerbates HFD-related metabolic impairment.

The $p66^{Shc-/-}$ mouse is a valid model of reduced OS (Trinei et al., 2009) interestingly characterized, among several other features, by reduced trygliceride accumulation, increased metabolic rate, decreased fat mass and resistance to diet-induced obesity (Berniakovich et al., 2008; Tomilov et al., 2011). This cluster of combined characteristics represents a stimulus for the growing interest in the study of the relationship between maternal HFD and OS and its effect on the offspring.

Finally, all these processes, which also interact with the genetic background of the individual, can lead to epigenetic changes participating to long-term effects of early life experiences and thus offer a platform for environmental and/or pharmacological intervention.

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LIST OF PUBLICATIONS (2012-2014)

Publications within the Ph.D. Research Project

BellisarioV., Panetta P., Berry A., Nathan O., Capoccia S., Holmes M., Seckl J., Schmidt M., Balsevich G, Baumann V., Raggi C. and Cirulli F. "High-fat diet during pregnancy acts as a stressor increasing maternal glucocorticoids' signaling to the fetus and disrupting maternal behavior through changes in neuronal activity in the olfactory bulb in C57BL/6J mice". *Frontiers in Endocrinology.* [Submitted].

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Berry A., Amrein I., Nötzli S., Lazic S.E., **Bellisario V.,** Giorgio M, Pelicci P.G., Alleva E., Lipp H.P. and Cirulli F. "Sustained hippocampal neurogenesis in females is amplified in P66 (Shc-/-) mice: An animal model of healthy aging". *Hippocampus*. 2012; 22:2249-2259.

Other publications

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exposed to prenatal stress: preventive effect of lurasidone treatment during adolescence". *European Journal of Neuropsychopharmacology*. 2014; 24(6):986-995.

*Capoccia S., *Berry A., **Bellisario V.,** Vacirca D., Ortona E., Alleva E., Pelicci P.G., Giorgio M. and Cirulli F. "Quality and Timing of Stressors Differentially Impact on Brain plasticity and Neuroendocrine-Immune Function in Mice". *Neural Plasticity*. 2013;Epub 2013 Mar 31.

Berry A., Vacirca D., Capoccia S., **Bellisario V.,** Malorni W., Ortona E. and Cirulli F. "Anti-ATP synthase autoantibodies induce neuronal death by apoptosis and impair cognitive performance in C57BL/6 mice". *Journal of Alzheimer's Disease.* 2013; 33:317-321.

Berry A., **Bellisario V.,** Capoccia S., Tirassa P., Calza A., Alleva E. and Cirulli F. "Social deprivation stress is a triggering factor for the emergence of anxiety- and depression-like behaviours and leads to reduced brain BDNF levels in C57BL/6J mice". *Psychoneuroendocrinology*. 2012; 37:762-772.

APPENDIX:
THESIS PUBLICATIONS