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Impact of early maternal environment and sex on behaviour and metabolism in two KO mouse models

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Abstract - English

Interactions between genes and environment are recognized to play a critical role in modulating the development of the organism. More in details early maternal environment seems to be a key factor in shaping neuro-behavioural development; the mother-infant bond is the strongest and more common social relationship in mammal species and phylogenetically highly conserved. Experimental evidence demonstrated that maternal behaviour may act on the regulation of genetic expression; in adult life, the offspring that had received high levels of maternal cares showed lower anxiety, less ACTH and Corticosterone production, less aging effect [A1]on hippocampus and more immune activity in response to stress. In the present experiments we investigated the interaction between early maternal environment and specific genetic background, in relation to sex on metabolism and behaviour. We examined two different genetically modified mouse models; the first one is a mouse model characterized by a conditional knock-out of the Y1 receptor in the excitatory neurons of the forebrain; the second one is a knocking mouse model of selective loss of the VGF peptide TLQP-21. To test the hypothesis that the effects of gene deletion may vary in relation to early maternal environment, in the first experiment male and female, Npy Ko and control mice were fostered at birth to dams that displayed high or low levels of maternal care (CD1, FVB – C57, Balb respectively). As adult mice were tested for emotional, aggressive and social behaviour and metabolic changes in response to standard or high fat diets. In the second experiment we performed a detailed analysis of the effect of early maternal environment on control animals of the Npy Y1r KO model. In the third experiment we observed maternal behaviour and reproductive success in ΔTLQP-21 heterozygotes or homozygotes breeders and characterized their offspring by an analysis of emotional and depression-like behaviours in adult males and females. Overall the results of the first study showed that males, but not females, had an increase in anxiety-like behaviour and weighted less than their controls, but only when reared by foster mothers displaying high levels of maternal care. In the second experiment, we demonstrated that early maternal environment affected offspring development but its impact cannot be ascribed entirely to the variation in maternal cares; in fact also maternal milk quality/quantity and nutritional factors could be important variables influencing behaviour and metabolism in adult's life. Lastly, results in the third study indicated that the mutation in TLQP21 gene influence maternal behaviour, with mutant mice showing higher level of maternal care when compared to wild type or heterozygotes breeders (defined by the time spent in the arched back position) [A2]. Such a difference affected the offspring behavioural phenotype as adults, particularly emotional behaviour. Further study will be necessary to investigate the role of nutrition and maternal milk in metabolic and behavioural development of offspring in both NPY KO and WT animals, and to better understand the role of early maternal environment in ΔTLQP-21 mouse. The present findings, however, highlight the importance to take into consideration and control for the complex interaction between genes, early maternal environment and sex-related effects when working with GM mouse models to uncover specific gene functions.

Abstract - Italian

Le interazioni tra geni e ambiente giocano un ruolo chiave nella regolazione dello sviluppo di tutto l'organismo. In particolare, l'ambiente materno potrebbe essere un fattore chiave, soprattutto nella modulazione neuro comportamentale; infatti il legame tra la madre e il figlio è considerato la relazione sociale piu' comune e maggiormente conservata tra le diverse specie di mammiferi. Evidenze sperimentali hanno dimostrato che l'ambiente materno agisce sulla regolazione dell'espressione genetica: da adulti, piccoli che hanno ricevuto alti livelli di cure materne mostrano meno ansia, meno ACTH e corticosterone plasmatici, minore invecchiamento a livello ippocampale e una maggiore attività immunitaria in risposta allo stress. In questo studio abbiamo analizzato l'interazione tra ambiente materno precoce e uno specifico background genetico, in relazione al sesso, e l'effetto metabolico e comportamentale derivato. Abbiamo esaminato due diversi modelli murini geneticamente modificati: il primo è un modello KO condizionale per il recettore Y1 nei neuroni eccitatori del prosencefalo; il secondo è un modello KO selettivo per il peptide TLQP21, che deriva dal gene VGF. Per testare l'ipotesi che l'effetto della delezione genica varia in relazione all'ambiente materno precoce, nel primo esperimento maschi e femmine controllo e KO per il recettore Y1 di NPY, sono stati dati in adozione alla nascita a madri che mostrano diversi livelli di cura materna. In età adulta gli animali sono stati sottoposti a test metabolici (risposta a dieta standard o grassa) e comportamentali, per verificare la presenza di comportamenti simil ansiosi o di aggressività. Nel secondo esperimento invece abbiamo condotto un'analisi dettagliata dell'effetto dell'ambiente materno precoce sugli animali controllo del modello Npy Y1r KO. Infine, nel terzo esperimento, che è uno studio pilota, abbiamo osservato il comportamento materno e il successo riproduttivo in animali ΔTLQP-21 eterozigoti o omozigoti, e cercato di caratterizzare il comportamento dei piccoli analizzandone il comportamento emozionale in età adulta. In generale i risultati del primo studio hanno mostrato che i maschi, ma non le femmine, hanno un aumento del comportamento ansioso, e pesano meno dei loro controlli. Questo però si verifica solo quando gli animali sono allevati da madri che mostrano un alto grado di cure materne. Nel secondo esperimento abbiamo invece dimostrato che l'ambiente materno precoce influenza lo sviluppo dei piccoli, ma non possiamo ascrivere i cambiamenti solo alla variazione delle cure materne; infatti ci sono altri fattori da tenere in considerazione, come la quantità o la qualità del latte materno e dei fattori nutrizionali, che possono influenzare lo sviluppo metabolico e comportamentale in età adulta. Infine, i risultati ottenuti nel terzo studio indicano che la mutazione nel gene TLQP21 influenza il comportamento materno: infatti i mutanti sembrano effettuare una maggiore quantità di cure materne rispetto agli eterozigoti o agli animali wild type. Questa differenza nel livello di cure materne si manifesta in parte nel fenotipo comportamentale dell'adulto, in particolare per quanto riguarda il comportamento emozionale. Saranno sicuramente necessari studi approfonditi per comprendere meglio il ruolo della nutrizione materna nello sviluppo comportamentale e metabolico sia negli animali KO condizionali per NPY, sia negli animali wild type, e per campire meglio quale sia il ruolo dell'ambiente materno precoce nel modello murino ΔTLQP-21. I risultati ottenuti, ad ogni modo, sottolineano quanto sia importante considerare la complessa interazione tra geni, ambiente materno precoce e differenze sessuali, soprattutto nell'utilizzo di modelli murini GM, per la scoperta di funzioni specifiche di diversi geni.

Chapter 1

1 General Introduction

1.1 Gene – environment interaction

In the past few decades there has been an increase in the understanding of the genetic influences on many human diseases. This acknowledge lead us to discover that for many diseases there is a deep complexity, not only due to genetic influences: in fact, both genetic and environmental factors may contribute to susceptibility, and it is not really clear how these factors interact. “Genetic or environmental mechanisms may differ between families or clinically defined subsets of the disease. Moreover, even within a single family, the effect of a susceptibility genotype might be variable because of the modifying effects of other genes or environmental factors” (Ottman, 1996). Several conditions as high blood pressure, diabetes, obesity, or height, are knowing to be the result of several and different variable: in this cases, we know that dietary and behavioural factors play a role, as the heritability does, to create the phenotypes. We need to focus on how heritability can be split in several subcategories, as in its calculation “h2 = genetic components (G) + gene–environment interactions (G × E)”, gene–environment interactions are defined as part of the heritable fraction of variation (Vogel and Motulsky, 1997). In simple words, G × E interaction can be defined as a relationship between a genetic variant that occurs inside a population and an environmental factor to which this population is or has been exposed throughout years (Simon *et al*., 2016). We observe that , at certain levels of exposure, the interaction among genes and environment has different effects in different populations or ethnic groups, or also in people with a difference in phenotype that are genetically dependent. One significant example is the exposure to sunlight that is known to enhance the risk of melanoma much more in fair-skinned than in dark-skinned people: many studies observed a strong interaction between ultraviolet light and skin pigmentation (Dempfle *et al*., 2008). As genes and GxE interaction seem to control the individual’s sensitivity to environmental exposures (Assary *et al*., 2017) this might be considered as a possible explanation to different reactions towards the same stress by different individuals. (Normann and Buttenschøn, 2019). A recent study of Kim J.W. *et al* in 2019 demonstrated that the interaction of two risk factors in the study of Autism spectrum disorders does not always aggravate ASD symptoms but can also alleviate them, which may be key to understanding individual differences in behavioural phenotypes and symptom intensity. In fact, although gene-environment interactions may explain the heterogeneous aetiology of ASD, it is still largely unknown how the gene-environment interaction affects behavioural symptoms and pathophysiology. Interaction among gene and environment results in “phenotypic plasticity”, that is defined as the variation of a phenotype, without changing in genotype. Phenotypic plasticity is very common in nature, but it is still very complicated to understand the evolutionary forces that shape genetic variation for plasticity: the variation of phenotypic plasticity is the results of genotype-by-environment interactions (*G* × *E*) forces that drives the development of the organism. To understand

the interaction among genes and environment is very important because it is the interaction itself that let to maintain the genetic variation: that's why *G* × *E* is quite common within natural populations and species (Des Marais *et al*., 2013). However, we can also think of *G* × *E* as environmental variation for the genetic contribution to phenotype (Josephs, 2018).

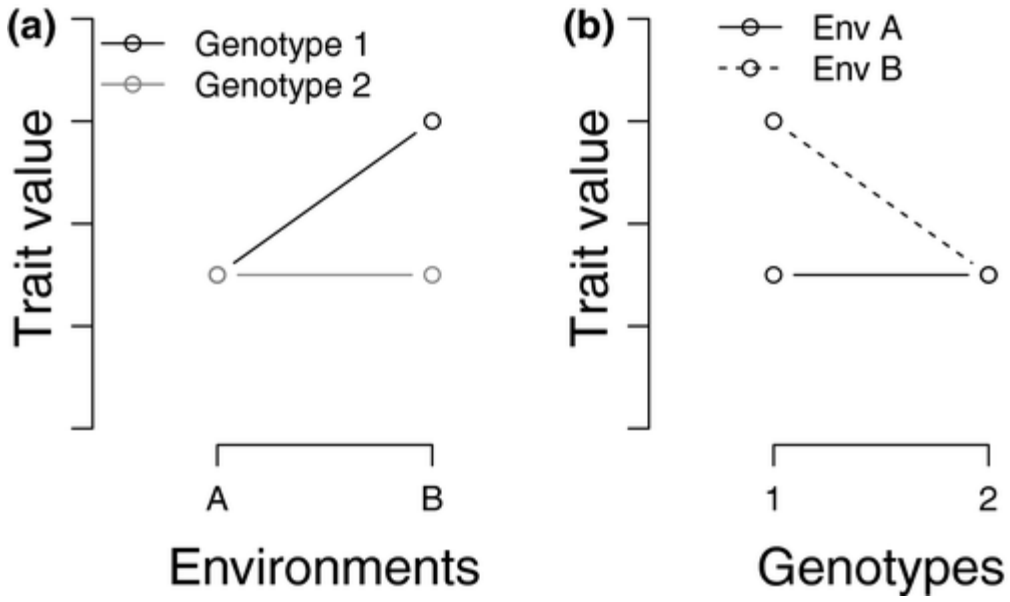

Fig. 1 Visualization of *G* × *E*. (**a**) In this case, the two genotypes, represented by the two lines, differ in response to environments A and B. (**b**) When we observed the same data presented from the perspective of *G* × *E* as variation for environmental effects of genotype, it is clear that environment B elicits a difference between genotypes, whereas environment A does not. (Josephs, 2018)

The importance of G × E interactions might be better understand if we consider the capacity of one environmental factor to enhanced a deleterious genetic variant in a population that bear the mutation. It is important to note that the word "environmental factor" refers to several external features, i.e. environmental pressure or stressors, that might affect an individual that shows the genetic variant. We have to consider that the number of environmental features that might directly or indirectly act on the expression of a genotype is undefined: for this reason the G x E interaction hugely increases the complexity of understanding a phenomenon.

The role of genetic influences like gene-environment (G × E) interactions on many different fields and diseases has been deeply investigated in the last decades. In fact, G x E interaction plays a role in the onset of cardiovascular diseases (Elosua et al., 2018; Elosua, 2018; Bauersachs and Heyman, 2018), heart development (Moreau et al., 2019), asthma (Sam et al., 2018; Sugier at al., 2019), body fat distribution and BMI (Li and Qi, 2019, Garske et al, 2019), over-nutrition and obesity related phenotypes (Tessier et al, 2019), and influences drug metabolism and liver diseases (Broekema et al, 2019). Genetic and environmental factors, i.e. adverse life events, early stressors, might

contribute not only to disease development, but also shape the risk and resilience of an organism. Several environmental factors such as parental monitoring, peer pressure, or socioeconomic status play an important role in the onset of many human diseases: the balance among genes and environmental factors may hold the key to understanding the individual differences in substance use, abuse, and addictive behaviour, and investigate environmental risk factors for addictive behaviour more extensively could be very useful to better understand the interaction between nature and nurture (Vink, 2016). The heterogeneity of a complex traits opens to researchers several paths: one disease, in fact, can hide the genes of another one and recent studies have focused on the possibility to perform a phenotypic deconstruction of comorbid traits that might enable the identification of novel genetic loci (Simon *et al*., 2016).To understanding diseases of incredible complexity, mostly in the field of Psychology and psychiatry, researchers referred to the study of GxE interaction. In fact, if we consider stress and anxiety disorders, it is well known that genetic variation may predispose individuals to resilience or susceptibility to environmental stressors, which may result at the end in the development of psychiatric disorders. Furthermore, without exposure to those environmental stressors, it is possible that the negative outcome does not occur. The hypothesis is that the interaction between genes and environment results critical for the expression of the phenotype of interest (Sharma *et al*., 2016). Genetic variation and environmental influences may not be independent entities: for example, an individual predisposed to high levels of anxiety may find himself engaged in drug addiction in order to alleviate anxiety symptoms. In this case the environment is deeply correlated with the genotype and they cannot be considered independent variables. “Such correlations are deeply embedded biases that have to be tolerated in human studies of G × E interaction” (Dick, 2011). Moreover, it has been recently demonstrated that the environment, particularly early life severe stress or trauma, might induce molecular changes in the form of epigenetic modifications that can drive the organism towards the path of health or disease. In fact, epigenetic modifications might shape and store the molecular response of a cell to its environment as a function of genetic predisposition. Epigenetic modifications provide a robust mechanism for gene-environment interactions: it has been shown that even stable chemical modifications, such as DNA methylation, may affect and force highly dynamic regulation. This potential reversibility makes these mechanisms suitable for encoding the long-term impact of the environment also in post-mitotic tissue such as neurons. The field of epigenetics may provide a possible molecular framework of how genetic and environmental factors interact and shape the risk for psychiatric disorders.

1.2 Early maternal environment and maternal behaviour

Development is a process that derives from recurring accumulation of small changes or events, and it leads to long term modifications in adulthood. In some case the experiences occurring during early postnatal life may affect population biology and break the behavioural reproductive barriers between species (Cirulli *et al*., 2003). The emotional connections among people, regardless the role of individuals (relations between parents and offspring, partners in love, grandparents and grandchildren, or close friends) provide resources to understanding, empathy and love. Attachment is a process that concerns the emotional bond that connects two individuals among space and time, and give to individuals a sense of security that derives from physical and psychological closeness with the attachment figure (Ainsworth, 1973; Bowlby, 1982; Gunnar and Quevedo, 2007).The exchange of information among the subjects that tie the bond gradually modifies them: the effects of mother–infant interaction last throughout life and it has a fundamental role in development (Lucion and Bortolini ,2014). It is important to highlight that, for mammalian infants, the primary caregiver, that usually is the mother, represent the most important source and modulator of stimulation (Hofer,1984). Mother–offspring interactions during the early postnatal period play a key role in neuronal development and formation of adult behaviour (Cirulli *et al*., 2003). In fact, infant organisms are biologically predisposed to develop attachment at their caregiver and its own behavioural systems: this bond is very important because it allows them to increase their survival chances in adverse environments (Bowlby, 1982). As mother infant bond plays a key role in the correct development of individuals, we are not surprised that disruptions in this kind of interaction caused both by physical separation of pups from their mothers and by stress experienced by the mothers during and before lactation can lead to changes in the behaviour of their adult offspring (Babb *et al.,* 2014; Johnson *et al.,* 2011; Kosten *et al.,* 2012; Steenwyk *et al*., 2018). Moreover, a vast body of deleterious psychopathologies and behavioural phenotypes has been associated with negative early life experiences that involve the attachment figure (Anda *et al*., 2006; Cirulli *et al*.,2009; D'Amato *et al.,* 2011). All the data presented in these works suggest that a perturbation in infant attachment may induce neurobiological changes and increase the risk of developing psychopathology during future life (Rincòn- Cortés and Sullivan, 2014). In rodents and humans, mother–pup interaction involves two essential types of actors: the mother and the infant, or several infants at the same time. Many other elements may be involved and/or affect this interaction: the father, the environment, siblings and predators (Mogi *et al.,* 2011). Maternal behaviour is defined as the collection of behaviours by the mother that can increase offspring survival (Krasnegor and Bridges, 1990; Numan and Insel, 2003). Similar nurturing behaviour as maternal behaviours, called as parental behaviour by fathers, and alloparental behaviour by older conspecifics, are widely seen in mammals, and collectively we can refer to them as "parental behaviour" (Kuroda *et al.,*2011). Animal models have been developed to analyse the effects of maternal deprivation on the

development of offspring: Attempts to replace a missing mother or to repair an affection poor childhood (Loman and Gunnar, 2009) are rather difficult. This difficulty probably depends on the fact that there are at least two organisms that are necessarily changed by the interaction (Lucion and Bortolini, 2014). A mother–infant interaction is unique and depends on many factors: for instance, even in large litters, as in rats or mice, mothers show different maternal behaviours toward male and female offspring (Oomen et al.,2009). In rodents, females construct a nest before giving birth, and they exhibit feeding and grooming of the new-borns. They also retrieve the infants back into the nest when they stray and protect them from predators and other dangers. Female rats or mice that have not given birth usually withdraw from or avoid pups, and it has been demonstrated that hormonal changes surrounding the birthing process and pup caring alter those responses to pups (Koch and Ehret, 1989). It has been intensively studied how hormonal changes at parturition enhance the neural mechanisms responsible for maternal behaviour, such as the medial preoptic area or dopamine neural systems (Numan and Stolzenberg, 2009). Disruption of mother–infant bonding in the early lactating period, when the bond is being established, has been well characterized and is known to have a great effect on the developing infant, particularly in altricial species. Moreover, a vast body of deleterious psychopathologies and behavioural phenotypes has been associated with negative early life experiences that involve the attachment figure (Anda *et al*., 2006; Cicchetti and Toth, 1995; Cirulli *et al.*, 2009; D'Amato *et al*., 2011; Gershon *et al*., 2013; O'Connor and Rutter, 2000; Shields *et al*.,1994; Ventura *et al*., 2013). All the data reported suggest that perturbations in infant attachment program the infant's emotional and cognitive resources to adapt to later-life challenges and increase the risk of developing psychopathologies during future life. The changes in quality and quantity of mother–pup interactions, were found to influence adult behaviour and brain morphology.

1.3 Sex differences in behavioural and metabolic development

Sex can be considered an environmental variable because of its ability to condition physiological environment of a gene by changing hormonal pathways. It is simple to measure, distributed in equal parts among the population and it is determined at conception and birth. (Gilks, 2015). It is well-known that differences among males and females in all organisms lead to a very different physiological and behavioural phenotype. Sexual differences may be divided into three subcategories: absolute sexual dimorphism, in which an endpoint can be present only in two exclusive forms, (i.e. traits present in one sex and fully absent in the other, like male bird courtship singing, territory defense, postpartum aggression, etc.). The second one includes all traits in which average among sexes is different but there could be various degrees of overlap, like stress and anxiety responses, memory, aggressiveness and social behaviour. The third category finally, involves situation in which sexes either start from the same point but diverge in response to changes, or they start from different point but lastly converge to a same endpoint (McCarthy et al.,2012). In simple terms sex differences may be considered a difference among males and females in a population for the value of a particular trait that might include anything, from anatomical measurements to expression level of a gene . Sex-related differences in emotional behavior (Johnston and File, 1991; Archer,1975; Gray, 1971) and social relation (Parmigiani et al., 1999, Cox and Rissman, 2011; McPhie-Lalmansingh et al.,2008; Van den Berg et al., 2015) have been well documented in a variety of mammals, included rodents and humans. A study of Palanza et al., 2001, underlined gender bias in social behaviour, demonstrating that male mice significantly differs from females in social context. Male mice appeared to be more aggressive, territorial, an when group-housed they develop a hierarchy head by one single individual, while females showed no aggression towards each other and they only develop a social structure during reproductive periods. Several studies in last decade focused on understanding the role of sex differences in various physical, neurological and psychiatric diseases that usually exhibit gender bias in symptoms, magnitude, illness outcome and response to treatment. Among these, particular attention has been given to neuropsychiatric disorders, that seem to be strictly correlated with sex differences. For instance, females appear to suffer from several neurological and psychiatric diseases such major depression, dementia, Alzheimer's disease, eating disorders and Post Traumatic Stress Disorder (PTSD) (Kessler, 2003; Bao and Swaab, 2011; Bekker and van Mens-Verhulst, 2007); while males are more prone to develop disorders related to development, i.e. Tourette's syndrome, autism, ADHD (Fombonne, 2005; Bao and Swaab, 2011;Werling and Geschwind, 2013). Despite major depression, that is one of the most prevalent disorders worldwide according to WHO statistics in 2015, is presented twice in women than in men the majority of animal studies related to this disorder are carried out in male models only. In fact use of males is tough to be cheaper, easier and less variable than females, for which it is impossible to not evaluate several hormonal factors due to estrus cycle.

Furthermore female models show a manifest struggling to be affected by stressors and metabolic challenges, and results from studies on stressed females usually appear anomalous or in contrast with those obtained in male models (Palanza and Parmigiani, 2017). To figure out all mechanisms underpinning sex differences in disease might help to find the presence of natural disease modifiers, since patient's sex might affect the risk for vulnerability or the development of the sickness. Furthermore, there are sex-related differences in pharmacokinetics and pharmacodynamics, and this should be a crucial clue for investigator in the study of gender differences in drug efficacy (Golden and Voshkul, 2017) also because females occurred to be more sensitive to the effects of various drugs, i.e. psychostimulant, and this lead faster to drug addiction as compared with males (Anker and Carroll 2011; Becker and Hu, 2008). In addition to behavioural and neuropsychiatric studies, many evidences of how sex differences might also shape metabolism are growing in last years. It is well established that gonadal hormones play a major role in definition of sex differences but variation in body size and composition appears to be evident previously to organism exposure to gonadocorticoids, thus probably due to the effect of sexual chromosomes (Zore et al., 2018). These two factors (sexual steroids and sexual chromosomes) anyway are not mutually exclusive and do not act in isolation, since there are several factors (**Fig.1**), including X chromosome imprinting and inactivation, environmental variables, gut microbiome that could synergistically act and lead to changes in gene expression and signalling pathways (Link and Reue, 2017).

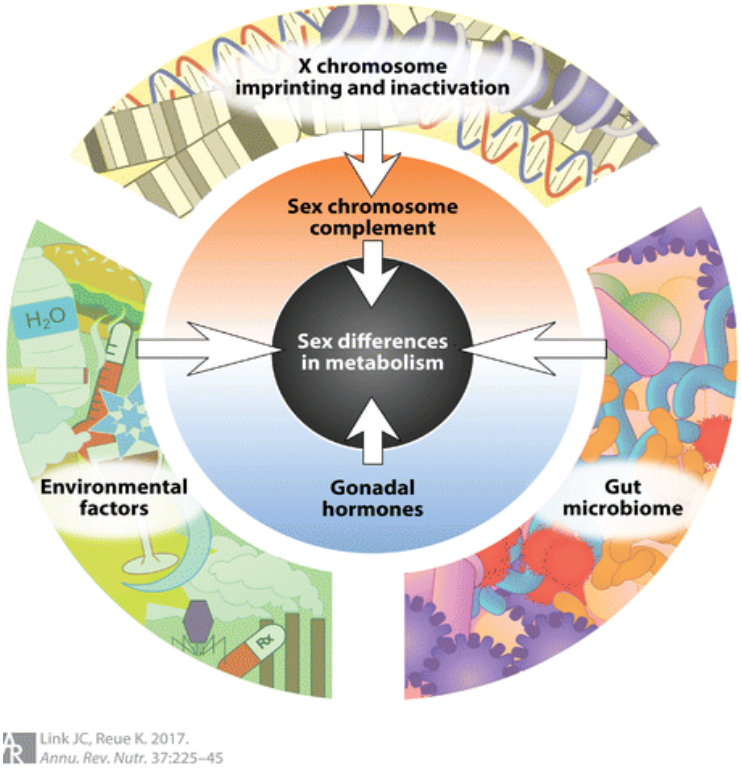

Fig. 1. Several factors influence sex differences more than sex chromosome complement and sexual steroids, for instance gut microbiome, environmental factors (diet, pollution, smoke) (Link and Reue, 2017).

Experimental evidences fully demonstrated that naturally occurring variations in sexual differentiation and development provide a further overview for understanding of the basic differences and the similarities between and within the sexes. To marginally solve the problem of lack of “female” animal models, NIH recently developed policies that “require applicants to report their plans for the balance of male and female cells and animals in preclinical studies in all future applications, unless sex-specific inclusion is unwarranted, based on rigorously defined exceptions” (Clayton and Collins, 2014). Sex is a fundamental biological characteristic that influences many aspects of an organism’s phenotype, including neurobiological functions and behavior as a result of species-specific evolutionary pressures. Sex differences have strong implications for vulnerability to disease and susceptibility to environmental perturbations. (Palanza and Parmigiani, 2017). In a review just submitted for publication, we stressed out that whenever developmental factors are investigated, it is crucial to take into account sexually dimorphic consequences in order to understand sex differences in vulnerability to environmentally-driven diseases for the development of adequate strategies of prevention, detection and treatment. (Palanza et al., submitted)

1.4 Animal models

Since the project has been developed in two different laboratories (Laboratory of Behavioural biology, Unit of Neuroscience, Department of Medicine, University of Parma, Italy ; Bartolomucci's Lab, Department of Integrative biology and physiology, University of Twin cities, Minnesota, USA) I have used different mice model, in order to investigate the complex interaction between genes and early maternal environment. In the laboratory of Behavioural biology in Parma, the experimental mice model I used were the NPY KO conditional ($Npy1r^{rfb)}$ and its respective control model ($Npy1r^{2lox}$). During visiting period in Minnesota the animal model I used for the pilot study was a new murine model engineered by Bartolomucci and Salton, showed and outlined in Chapter 5.

1.4.1 NPY Y1r conditional KO mice

The first animal model we used in the early part of this work (Chapter 3) is characterized by a conditional knock-out of Y1 receptor in the excitatory neurons of the forebrain. The animal model was first developed at the Max Planck Institute of Heidelberg by Rolf Sprengel group and fully described in Bertocchi *et al*. (2011).

Briefly, to generate a modified Npy1r allele, LoxP sites were inserted around exons 2-3, which code for the entire region of the Npy1r, using standard gene targeting techniques (**Fig. 2**). LoxP sites are sensitive to Cre, which catalyzes site-specific recombination inactivating the Npy1r gene in any cell expressing the ricombinase (Sauer, 1998).

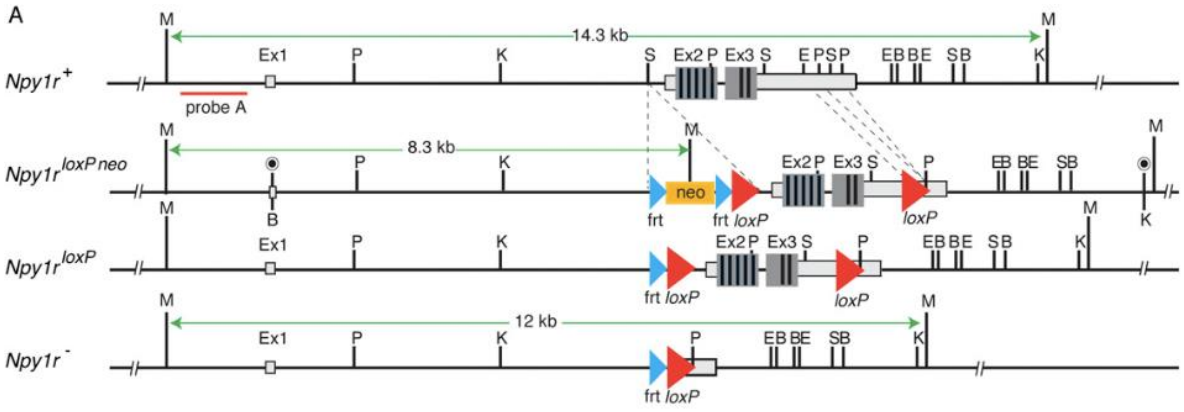

Fig. 2. Generation of a modified Npy1r allele. To generate a *loxP* tagged *Npy1r* allele, *loxP* sites around exons 2−3, which cover the entire Npy1r coding region, were inserted using standard gene targeting techniques in embryonic stem (ES) cells. Light grey boxes represent exons. Dark grey boxes are the coding regions with, in black, the trans-membrane domains. **B**: *BglII*, **E**: *EcoRV*, **K**: *KpnI*, **M**: *MscI*, **P**: *PstI*, **S**: *SphI*. Frt and *loxP* sited are in blue and red triangles, respectively. Circles indicate the **B** and **K** restriction sites; borders of the targeting vector. Arrows indicate the size of genomic fragments generated on Southern blotting by cleavage with *MscI.*

A mouse line carrying a floxed Npy1r allele (Npy1r$^{+/loxP}$ mice) was generated by homologous recombination in embrionic stem cells (ES). Genomic DNA of the positive clones was digested with *MscI* and hybridized with a 1Kb probe, probe A, sitting outside at the 5" end of the targeting vector and obtained by PCR. In the Southern blot, wild-type allele was identified by the presence of a 14.3 Kb fragment, whereas recombinant allele by an additional 8.3 Kb fragment. ES cells of clone 441, positive for the recombination (Npy1r$^{+/loxP\text{-neo}}$), were expanded for injection into C57BL/6J blastocysts that were then implanted into pseudo-pregnant ICR female recipients. PCR and Southern blot analysis of tail DNA revealed that 50% of the pups contained the modified allele. To verify the efficiency of the Cre/*loxP* system, ubiquitous Cre-mediated deletion of the floxed exons was induced by using Cre-*deleter* transgenic mice, in which Cre is expressed in all tissues, including germ cells (Schwenk et al., 1995). Heterozygous mice Npy1r$^{+/-/CREdel}$, were analysedby PCR and Southern blot and then crossed to obtain the homozygous mouse. The ubiquitous Cre-mediated deletion of Npy1r was verified by *in situ* hybridization. The neo cassette was removed *in vivo* by crossing Npy1r$^{+/loxP\text{-}neo}$ mice to flp transgenic mice, carrying the Flippase (flp) recombinase, which recognizes and cuts at Flippase Recognition Target (frt) sites (**Fig. 3A**). Npy1r mRNA expression was observed to extend along the rostral-caudal axis in the brain of Npy1r$^{+/loxP}$ mice and is reduced of about 50% in Npy1r$^{+/-}$ mice and completely absent in Npy1r$^{-/-}$ mice (**Fig. 3B**).

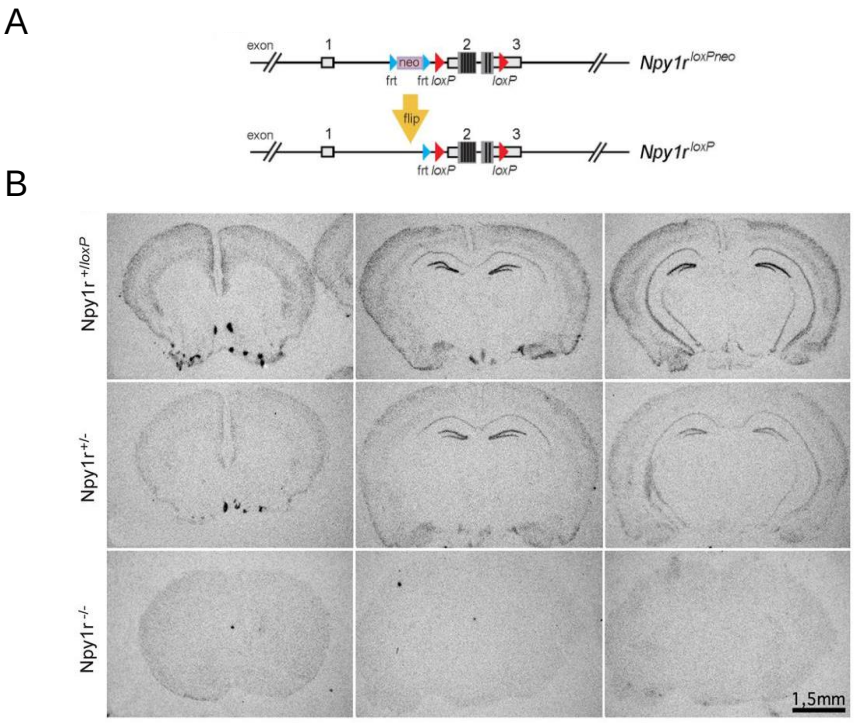

Fig. 3. Verification of the efficiency of the Cre/*loxP* system. (**A**) The *neo* cassette was removed *in vivo* by crossing Npy1r+/*loxPneo* mice. (**B**) *in situ* hybridization on coronal brain sections from Npy1r$^{+/loxP}$, Npy1r$^{+/-}$ and Npy1r$^{-/-}$ germinal knockout mice confirmed the functionality of the LoxP sites at the targeted Npy1r^{loxP} locus

Hereafter, in order to obtain the deletion of the Npy1r gene only in several brain regions, three different mouse lines were generated: 1. Npy1r^{2lox} expressing the LoxP sites flacking the Npy1r gene, 2. Npy1r$^{2lox/Tg\alpha\text{-}CamKII\text{-}tTA}$ mice line, expressing the tTA in a region- and cell type-specific manner under the control of the α-CamKII promoter (Mayford *et al*., 1996) and 3. Npy1r$^{2lox/TgLC1}$ mice line, bearing a transgene containing the tTA-responsive promoter for Cre expression (Schönig *et al*., 2002). In this genetic paradigm, region-specific and postnatal inactivation of Npy1r can be achieved by crossing Npy1r$^{2lox/Tg\alpha\text{-}CamKII\text{-}tTA}$ with Npy1r$^{2lox/TgLC1}$ mice and the extent of Cre expression could be restricted by Doxycycline (Dox) (Chiu *et al*., 2008; Krestel *et al*., 2004). In the resulting animals, chronic treatment with Dox from conception prevents Npy1r inactivation by suppression of tTA-dependent Cre expression. Dox withdrawal, by switching litters at birth to Dox-free foster mothers, induces forebrain specific Npy1r knockout (Npy1r^{rfb} mice) that is fully achieved around postnatal day P40.

To assess temporal and spatial regulation of Cre activity *in vivo*, the pattern of Cre expression in the brain of Tg$^{\alpha\text{-}CamKII\text{-}tTA/LC1}$ mice was assessed by means of immunohistochemistry. Cre immunostaining of coronal brain sections showed a specific, intense labeling of the Tg$^{\alpha\text{-}CamKII\text{-}tTA/LC1}$ mouse anterior forebrain, including the hippocampal formation, the cortex and the amygdaloid nuclei, indicating an α-CaMKII-driven Cre expression in these selected brain regions. Cre-like immunoreactivity was absent in the hypothalamus (**Fig. 4A**). When Dox was applied from conception, consistently with previous results (Chiu *et al*., 2008; Krestel *et al*., 2004), no Cre activity was detectable in the brain. When Dox was applied from conception until birth and pups were shifted at PND0 to Dox-free foster mothers, the Cre specific immunosignal increased over time in the cortex, the hippocampus and the amygdaloid nuclei, reflecting postnatal increase in tTA from the transgenic α-CaMKII promoter (Chiu *et al*., 2008; Krestel *et al*., 2004) (**Fig. 4A**). In order to increse the sensitivity of this analysis, Tg$^{\alpha\text{-}CamKII\text{-}tTA/LC1}$ mice were crossed to the RosaR26 Cre reporter mouse line, harboring a *lacZ* gene silenced with a floxed transcriptional terminator sequence (Soriano, 1999). Through an X-Gal staining, it was demonstrated that *lacZ* gene expression was similar to the regional distribution of Cre immunoreactivity (**Fig. 4B**).

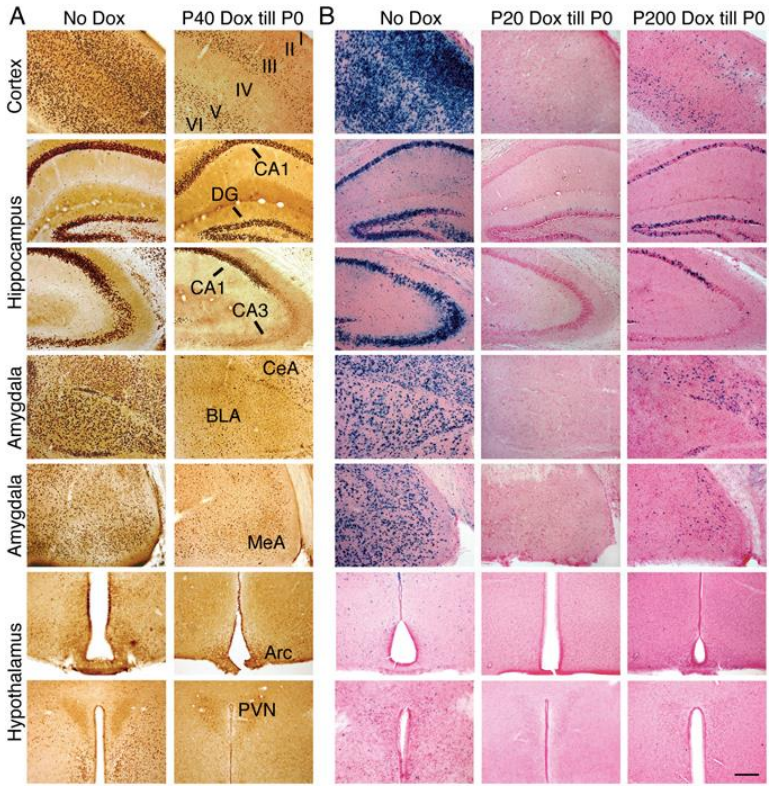

Figure 4. Temporal and spatial regulation of Cre activity. (A) Cre-immunolabeled and (B) corresponding X-Gal–stained (lacZ) coronal brain sections of Dox-naive (No Dox), and prenatally Dox-treated $Tg^{\alpha CaMKII\text{-}tTA/LC1}$ (A) and $Tg^{\alpha CaMKII\text{-}tTA/LC1/Rosa/R26R}$ (B) mice shifted at P0 to Dox-free (Dox till P0). All sections were taken at P20, P40, and P200. The lacZ gene of Rosa/R26R permitted the visualization of Cre activity by X-Gal staining. In Dox naïve mice, the Cre immunoreactivity of coronal brain sections showed a specific, intense labeling in the anterior forebrain of the $Tg^{\alpha CamKII\text{-}tTA/LC1}$ mouse, including the hippocampal formation, the cortex, and the amygdaloid nuclei. In contrast, anti-Cre staining was absent in the hypothalamus. When Dox was applied from conception until birth, the Cre-specific immunosignal was absent at P0 and increased over time in the cortex, the hippocampus, and the amygdaloid nuclei, reflecting postnatal increase in tTA from the transgenic αCaMKII promoter. Cre-induced lacZ expression was similar to the regional distribution of Cre immunoreactivity; Please note the absence of Cre-induced lacZ activity at P20. CA1, CA1 pyramidal cell layer; CA3, CA3 pyramidal cell layer; DG, dentate gyrus granule cell layer; BLA, basolateral amygdala; CeA, central amygdala; MeA, medial amygdala. (Scale bar: 200 μm)

1.4.2 NPY Y1r control mice

As Npy1r^{2lox} didn’t show recombination of the Npy1r allele (Crusio et al., 2009), I have used them as control littermates in all the behavioural and metabolic experiments described in Chapter 3. I also used them as mouse model in Chapter 4, where I evaluated the effect of maternal environment on metabolism, emotional and aggressive behavior in control animals. Briefly, for maintaining the targeted gene and the transgenic lines animals were backcrossed to C57BL/6J mice. The genetic background of the investigated population is very heterogeneous suggesting that observed phenotype is extremely independent from the genetic background (Silva et al., 1997).

1.4.3 ΔTLQP-21 mice

The second animal model I used in this work is a new murine model (background strain: C57BL/6N) engineered by Bartolomucci and Salton in last few years, based on the selective loss of the VGF-derived peptide TLQP21. Details are discussed in Chapter 5 - "TLQP21 mutant mice vs Wild type: the amount of postnatal maternal care and parental genotype influence adults behaviour".

Chapter 2

2 Rational and purpose

2.1 Experimental approach and outline of the thesis

Interactions between genes and environment are recognized to play a critical role in modulating the development of the organism, indeed the result of GxE interaction is well known as «phenotypic plasticity» , a variation in phenotype emerging from a common genotype as a function of variation in environmental conditions. Whether environment influence the phenotypic expression, variations in environmental conditions may lead to individual differences both in behaviour and endocrine responses of an organism. More in details early maternal environment seems to be a key point on the development of behaviour in adult life. Animals, especially mammals and birds, are very sensitive to early life experiences and their future emotional life seems to depend from the formation, conservation, break and renewal of the mother infant bonding. The mother baby bond is the strongest and more common social relationship, widespread between mammalian species and it is phylogenetically conserved among species. Mother exposure to different environmental adversities shapes the mother child interaction and leads to a change in behaviour and endocrine response to stress in offspring. As outcome pups show differences that seem to be and adaptation of their organism to adversities. Handling studies revealed that short separation from dams during sensitive early life periods lead to major parental care when pups are reintroduced in the litters. In adult life, offspring which have received remarkable levels of care showed less ACTH and Corticosterone production, less aging effect on hyppocampus and more immune activity in response to stress. Meaney et al. in 2005 hypothesized that different levels of maternal cares might explain behavioural differences in adults. These analysis were based on the number of licking and grooming of the dams, and time spent in arched back, a position in which mothers crouching over the pups and simultaneously give heat and nutrition to the entire litter at the same time. These behaviours are closely related to each other when the analysis are performed on mother of the same group (High maternal care or Low maternal care groups). As the total amount of interaction with pups didn't decrease in the two groups but the effect of High maternal care were evident (Reduced plasma ACTH and corticosterone, increased hippocampal glucocorticoid receptor mRNA expression, enhanced glucocorticoid negative feedback sensitivity and decreased hypothalamic CRH mRNA levels: in simple terms a modest response to stress, as compared with pups of low maternal cares mothers. Meaney and his team suggested that different amounts of maternal care might shape the adult's behaviour. Previous studies carried out in our laboratory on the Npy1r^{rfb} mouse model aimed to investigate the effects of limbic Npy1r in regulation of metabolism and response to a metabolic challenge in male and female mice reared by mothers that displayed different amounts of maternal care. The results obtained by monitoring body weight growth from weaning to adulthood and by subsequently feeding animals a high fat diet for 4 weeks confirmed the role of limbic Npy1r in

regulating metabolism. Interestingly, the effects of limbic Npy1r inactivation were observed only in male mice and only when reared by foster mothers that displayed an high amount of maternal care (FVB). Indeed, limbic Npy1r deletion did not induce any effects in males reared by foster mothers presenting a low level of maternal care (C57) nor in females reared by both high and low maternal care foster mothers.

All these results pointed to a very interesting question: what is the role of maternal environment? Within all the variables that are connected and implicated in shaping of the behaviour, might the quality of maternal care be the heart of the matter? Are sex differences related with the early maternal environment? How about genes: maternal behaviour may act on genetic expression?

To adress this questions we tried to pursue different goals:

1- The first aim of the research presented in this thesis was to examine the effects of conditional Npy1r inactivation in male and female mice reared by foster FVB and C57 dams on anxiety-like and aggressive behaviour. In a second phase we tried to identify metabolic and neuroendocrine alterations, obesity, pre-diabetic markers, in relation to the diet and early maternal environment, and to determine altered functional processes (Chapter 3).
2- The second aim of this study was to examine the effects of early maternal environment in wild type mice to determine at what extent different levels of maternal care during the first postnatal days might affect anxiety and aggressive behaviour in male and female mice. The second phase of the experiment focused on the examination of the role of dams behaviour in regulating metabolism and more specifically, the susceptibility to metabolic disorders in response to hypercaloric diet (Chapter 4).
3- The third aim is to evaluate if the selective loss of a peptide (TLQP21) could affect the amount of maternal care, and if the imbalance of maternal behaviour could act on the development of different behavioural phenotypes in adulthood (Chapter 5).

Chapter 3

3 Behavioural and metabolic effects of early maternal environment on NPY Y1r conditional KO mice

3.1 Introduction

Neuropeptide Y (NPY) is a mammalian neuropeptide of 36 amino acids widely distributed both in the central nervous system and in the periphery. NPY is highly conserved through many species and it forms the family of pancreatic polypeptide together with peptide YY (PYY) and pancreatic polypeptide (PP) (Tatemoto *et al*., 1982). In the CNS, NPY is involved in the regulation of several biological functions including energy balance, emotional and stress reactions, and cognition (Eva *et al*., 2006; Hokfelt *et al*., 1998). For example, injection of NPY into either the cerebral ventricles or the hypothalamus stimulates food intake even in satiated rats, eventually leading to obesity (Clark *et al*., 1984; Levine and Morley, 1984). Furthermore, humans affected by depression and anxiety disorders show abnormally low level of NPY in plasma and cerebrospinal fluid (CSF) (Heilig *et al*., 2004). In rodents, injection of NPY in the third ventricle or in the limbic system reduces both stress-response and anxiety behaviour (Broqua *et al*., 1995; Heilig *et al*., 1993; Sajdyk *et al.,* 2008); conversely, mice lacking NPY showed increased anxiety behaviour (Bannon *et al*., 2000; Heilig, 2004). NPY exerts its action in the CNS through the interaction with a family of G-protein coupled receptors that includes the Y1 (Y1R), Y2 (Y2R), Y4 (Y4R), Y5 (Y5R) e Y6 (Y6R). Within these receptors, the most important is Y1R, which is involved in regulating the main functions of NPY. Pharmacologic and genetic studies suggest that NPY induces anxiolytic effects via activation of the Y1Rs in the amygdala, hippocampus and locus coeruleus (Bertocchi *et al*., 2011; Kask *et al*., 2000; Lin *et al*., 2010; Sajdyk *et al*., 1999). Recently, Bertocchi *et al*. (2011) generated conditional knockout mice in which the inactivation of the Npy1r gene was restricted to excitatory neurons of the forebrain, starting from juvenile stages (Npy1rrfb). Npy1rrfb mice exhibited reduced body weight, less adipose tissue, and lower serum leptin levels. Npy1rrfb mutants also displayed higher peripheral corticosterone and higher density of NPY immunoreactive fibers and corticotropin releasing hormone (CRH) immunoreactive cell bodies in the PVN suggesting that conditional inactivation of limbic Y1R might decrease body weight gain by activation of the HPA axis. Importantly, cross-fostering experiments demonstrated that differences in phenotype between Npy1rrfb and control mice (Npy1r2lox) only became apparent when both genotypes were raised by FVB dams, exhibiting high levels of arched back nursing (HABN), an established index of high maternal care. When conditional mutants and their control littermates were fostered to C57 dams, which showed lower maternal cares towards their adopted pups (LABN), Npy1r mRNA levels were similarly low and both mouse cohorts tested showed no significant phenotypic differences, suggesting that high levels of maternal care increase the expression of limbic Npy1r expression. These phenotypic differences reflect differences in the expression of Npy1r mRNA in the limbic system; only Npy1rrfb male mice reared by HM dams,

but not Npy1rrfb mice reared by low maternal care dams, showed a reduction of the expression in the CA1, CA3 and DG of the hippocampus compared to controls, suggesting that limbic Npy1r expression might be regulated by early maternal environment through epigenetic mechanism (Bertocchi *et al*., 2011).

The conditional Npy1r KO mouse model thus provided the first experimental genetic evidence that limbic Y1Rs are required for regulation of body weight and are key targets of maternal care-induced programming of energy homeostasis (Bertocchi *et al*., 2011). Dysfunctions of NPY system have been implicated in human diseases such as obesity, type II diabetes and metabolic syndrome, raising the possibility that targeting this system may provide therapeutic benefits for these diseases. One variant causing an amino acid change from leucine to proline at codon 7 in the signal peptide of NPY is associated with increased body mass index (Ding *et al*., 2005), impaired glucose tolerance and type II diabetes (Nordman *et al.,* 2005; Ukkola and Kesaniemi, 2007), higher serum levels of total and low- density lipoprotein cholesterol (Karvonen *et al*., 1998) and predicts myocardial infarction and stroke in hypertensive patients (Wallerstedt et al., 2004). Furthermore, another study shows that the Leu7/Pro polymorphism of the NPY gene alters the packaging and secretion of NPY, leading to increased peptide synthesis and release in endocrine cells and CNS neurons (Mitchell *et al.,* 2008). This is consistent with the elevated plasma levels of NPY observed in subjects carrying the NPY Leu7/Pro polymorphism during exercise and elevated sympathetic activity (Kallio *et al*., 2001). Taken together these findings suggest that increased NPYergic tone leading to an enhanced NPY production may be an etiological factor in obesity. From early on, the Y1 receptor has been a focus in obesity research due to its initial recognition as a “feeding receptor” that mediates NPY-induced hyperphagia (Antal-Zimanyi *et al*., 2008; Haynes *et al.,* 1998; Henry *et al*., 2005; Kanatani *et al*., 1999; Kanatani *et al*., 2001; Mullins *et al*., 2001). Later studies revealed that, in addition to feeding, the Y1 receptor plays important roles in mediating other metabolic effects of NPY related to energy homeostasis, such as energy expenditure, lipid metabolism and insulin secretion (Herzog, 2003; Huda *et al.,* 2006; Renshaw and Batterham, 2005). Recently, Bertocchi and coworkers demonstrated that reduced expression of Npy1r in the limbic system induced increased anxiety-like behaviour, higher hypothalamus-pituitaryadrenocortical (HPA) axis reactivity and decreased body weight growth from weaning to adulthood in conditional Npy1r knock-out (Npy1rrfb) mice (Bertocchi *et al*., 2011). Intriguingly, differences in phenotype between Npy1rrfb and control mice (Npy1r2lox) were observed only in male mice and only when reared by high maternal (HABN) cares dams. NPY and Y1Rs have been also implicated in regulating other behaviours, such as aggressive behaviour (Karl *et al*., 2004). Indeed, NPY also is known to alter the expression and secretion of several neurotransmitters such as norepinephrine (Ekblad *et al*., 1984) and serotonin (Shimizu and Bray, 1989), which have been implicated in aggressive behaviour (Kask *et al*., 2002; Olivier *et al.,* 1995). In this study we examined the effects of conditional Npy1r inactivation in male and female mice reared by foster mothers displaying high maternal care (FVB dams) or low maternal care (C57 dams)

on anxiety-like behaviour through the Elevated Plus Maze (EPM) test and the Novelty Induced Suppression of Feeding (NISF) test. Furthermore, considering the role of Y1Rs in modulating aggressive behaviour, we also examined male and female behaviour towards same sex conspecifics in the resident/intruder test. The second aim is to examine the role of Y1R in limbic areas in regulating metabolism and more specifically, the susceptibility to metabolic disorders in response to hyper caloric diet. Our hypothesis is that low limbic Y1R expression induced by conditional gene inactivation, might affect susceptibility to metabolic challenges in adulthood and vulnerability to obesity, metabolic syndrome and associated disorders such as type II diabetes. To this purpose, we exposed mice with low expression of limbic Y1R to a hyper caloric or standard diet to identify metabolic and neuroendocrine alterations, obesity, pre-diabetic markers, in relation to the diet and early maternal environment, and to determine altered functional processes.

3.2 General material and methods

Animals and housing

All the animals used in this work were born and reared in the Laboratory of Behavioural Biology at the University of Parma. All experiments were conducted in accordance with the European Community Council Directive of 24 November 1986 86/609/EEC and 6106/10/EU and approved by the University of Parma Ethical Committee for animal research and by the Italian Ministry of Health (Aut. N 1143/2016 – PR (Protocol 2712C.2 of 13/07/2016). The generation of Npy1r^{2lox} mice and Npy1r^{rfb} was achieved as described in the next paragraph and in Bertocchi et al. (Bertocchi et al., 2011). At the same time, female mice of two different strains FVB/J and C57BL/6J (named FVB and C57) used as foster mothers based on the levels of maternal care displayed were paired with same-strain males. Females were kept with their respective males for 7-10 days. Pregnant females were initially housed in groups of two in a temperature – (22 ± 1 °C) and humidity- controlled room on a 12-hours light/dark cycle (11:00 AM – 11:00 PM). Few days after birth mice were taken and placed in separated cages until delivery. Within 12 hours after birth (Post Natal Day 0) Npy1r^{2lox} mice and Npy1r^{rfb} pups were weighed, sexed and fostered randomly to a lactating female of FVB or C57 group. Maternal behaviour was observed from PND1 to PND7, for two hours a day. At PND27 pups were weaning and housed in groups of siblings of the same sex.

Generation of Npy1r^{rfb} conditional knock-out mice

The generation of Npy1r^{2lox} mice and Npy1r^{rfb} (background strain: C57BL/6J) was achieved as described in Bertocchi *et al.*, 2011. Briefly, two different GM mouse lines were paired: 1) Npy1r^{2lox}/Tg$^{\alpha CamKII\text{-}tTA}$ expressing tTA in a cell type-specific manner under the control of the α-Ca^{2+}/calmodulin kinase II (α−CamKII) promoter (αCaMKII-tTA) and 2) Npy1r^{2lox}/Tg^{LC1} encoding the tTA-responsive Cre transgene LC1 (**Fig. 1**). Breeding pairs were housed in a separate colony room at the University of Parma on a 12-hours light/dark cycle (lights on: 11AM-11PM) and exposed to chronic Dox treatment from conception to birth (50 mg/L in drinking water, 1% sucrose. Dox solution was prepared and changed every other day). Pups with different genotypes were generated in a Mendelian ratio and gene inactivation was achieved in Npy1r^{2lox}/Tg$^{\alpha CamKII\text{-}tTA/+LC1}$ mice (named Npy1r^{rfb}). Littermates comprising Npy1r^{2lox}/Tg$^{\alpha CamKII\text{-}tTA}$, Npy1r^{2lox}/Tg^{LC1} *and* Npy1r^{2lox} were used as controls (named Npy1r^{2lox}) (Bertocchi *et al*., 2011).

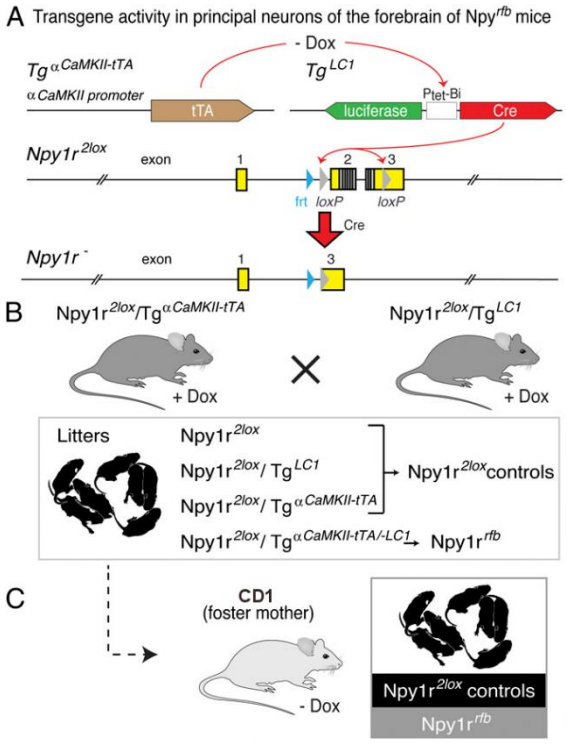

Fig. 1. Generation of Npy1r^{rfb} mutants and Npy1r^{2lox} control cohorts. (A) Diagram depicting the interaction of the different genetic components: After Dox removal, the αCamKII promoter-driven tTA activates transcription of the transgene TgLC1, thereby inducing Cre expression in excitatory neurons of the forebrain. The Cre recombinase interacts with loxP sites in the gene-targeted Npy1r2lox alleles and removes the Npy1r2lox coding region leading to the inactivation of the Npy1r gene (Npy1r^{-}). Frt and loxP sites are in blue and gray triangles, respectively; exons in open boxes, coding regions in gray boxes; black boxes, transmembrane spanning codons. (B) By mating the compound transgenic mice Npy1r^{2lox}/Tg$^{αCamKII-tTA}$ and Npy1r^{2lox}/Tg^{LC1} under Dox treatment, pups with four different genotypes were generated and found in a Mendelian ratio. (C) At the day of birth [postnatal day (PND)0], the litters were transferred to Swiss CD1 Dox naive foster mothers to induce the Cre-mediated Npy1r gene inactivation in the forebrain of Npy1r^{2lox}/Tg$^{αCamKII-tTA/LC1}$ mice (named herein Npy1r^{rfb}). Littermates comprising Npy1r^{2lox}/Tg$^{αCamKII-tTA}$, Npy1r^{2lox}/Tg^{LC1}, and Npy1r^{2lox} genotypes were used as controls (named herein Npy1r^{2lox} controls). (modified from Bertocchi et al. 2011)

Fostering and Genotyping

Within 12 hours after birth (PND 0), mice were weighed, sexed and fostered to Dox-free FVB and C57 lactating females. Every biological mother provided approximately the same number of experimental animals to both Npy1r^{2lox} and Npy1r^{rfb} groups, and each foster mother received 5-6 pups from the same litter. If biological mother gave birth to a lower number of pups, the litter size was increased by adding an adequate number of pups from another litter born on the same day. Receiving foster dams were mated with males of their same strain one or two days before the breeding of biological mothers. For this reason, at fostering (PND0) lactating Dox naïve foster females were prepared to rear Npy1r^{2lox} and Npy1r^{rfb} pups. Genotyping was performed on tail DNA at PND 27. Npy1r^{2lox} mice and Npy1r^{rfb} were identified by using the tTA gene as a marker gene for conditional knock-out. Thus, animals who resulted positive for the tTA were considered as knock-out (Npy1r^{rfb}) whereas the others were controls (Npy1r^{2lox}). Briefly, DNA extraction was performed using the Wizard Genomic DNA purification kit (Promega, Madison, WI, USA). PCR was performed

by using as a marker gene the tTA (primer: tTA_1(GTGATTAACAGCGCATTAGAGC), tTA_4(GAAGGCTGGCTCTGCACCTTGGTG), Eurofins Genomics, Ebersberg) that was amplified in a 20ul reaction mix composed by: 1U of GoTaq Dna polymerase (Promega, Madison, WI, USA), dNTPS 0.2 mM, 8 pmol of each primer in 1X Reaction Buffer. The reaction was carried out at 95°C for 5 min (first denaturation step) followed by 34 cycles of 95°C for 40 sec, annealing at 56°C for 40 sec and elongation at 72°C for 1 min. At the end of the 34 cycles, the reaction was stopped through an elongation step at 72°C for 10 min. The presence of the PCR product (tTA) was evaluated through electrophoresis on 2.5% agarose gel. Mice were then housed in same-sex groups of siblings (2-5 per cage) in Plexiglas cages (45cmx25cmx20cm).

Maternal behaviour observation

Maternal behaviour displayed by foster mothers was observed from PND 1 to 7, by analysing fostered dams in their home cages. Mice are most active during the dark phase of light/dark cycle (Latham and Mason, 2004). Therefore, the observation period (120 min) started at 09 AM hr and was conducted entirely during the dark phase with the aid of 25-W red lights, until 11 AM. Each lactating female was observed once every four minutes, for a total amount of 30 observations in 2 hours. During each 4min observation, the experimenter revealed which behaviour is displayed at the moment of the observation.

Dams behaviour was identified according to behavioural categories , as in Palanza et al. (2002), namely: *Arched back nursing*, the lactating female was giving to all the pups nourishment and heath simultaneously with her body arched over them; In nest: the female was anywhere inside the nest, regardless of the behaviour being exhibited at the moment of observation; *Nursing*, the dam was allowing the offspring to suckle (it is not implying that the whole litter was nursing); *Licking*, the female was licking or grooming pups; *Nest building*, the lactating female was occupied in some activity regarding the nest building, either inside or outside the nest itself; *Grooming*; the lactating female was cleaning herself; *Active*: the female was moving on the cage; *Resting*: the female was lying outside the nest, not displaying any other behaviour, without pups suckling or attached to the nipples, motionless. *Out of nest*; the dam was anywhere in the cage but the nest, regardless of the behaviour being exhibited at the moment of the observation.

Body weight growth until PND 90

Offspring body weight was monitored from birth to adulthood by a digital balance accurate to 0.01g (Sartorius, Germany) with the animal weighing functions (animal's weight is automatically calculated as the average of a defined number of individual weighing operations). From PND 34 to adulthood (PND 90), body weight was measured once a week from 10 AM to 11 AM in order to monitor body weight growth.

Statistical analysis

A 3-way ANOVA (genotype, adoption and time) for repeated measures was used to analyse body weight, food intake and glucose tolerance test data. A 2-way ANOVA (adoption and genotype) was run to analyse abdominal fat pads, emotional, aggressive and social behaviour. A Tukey's test for post hoc comparisons followed the ANOVAs. Npy1r mRNA expression was analysed with Student's "t" tests. Data were analysed with Statistica 10.0 software (Stat-Soft, Tulsa, OK, USA). Significance was determined when $p<0.05$. Data are presented as mean ± standard error of the mean.

3.2.1 Experiment 1 – NPY1r inactivation affects emotional behaviour

Emotional behaviour

Recent studies have demonstrated that reduced expression of Npy1r in the limbic system induced increased anxiety-like behaviour, higher hypothalamus-pituitaryadrenocortical (HPA) axis reactivity and decreased body weight growth from weaning to adulthood in conditional Npy1r knock-out (Npy1rrfb) male mice (Bertocchi *et al*., 2011). These effects seem to be remarkable only when males were reared by mothers that displaying an high amount of maternal care in early postnatal life. For this reason we decided to analyse emotional behaviour in $Npy1r^{2lox}$ and $Npy1r^{rfb}$ mice using the Elevated Plus Maze (EPM) test, and the Novelty induced suppression of feeding test (NISF), both useful to assess stress-induced anxiety. Behavioural tests were carried out on adult male and female $Npy1r^{2lox}$ and $Npy1r^{rfb}$ reared by FVB and C57 dams between PND 90 and PND 110 from 3 PM to 7 PM. At adulthood (PND 90) animals were isolated in Plexiglass cages (45cm*25cm*20cm) and, after a habituation period of 1 week, they were tested for behaviour. All mice were transported to the testing room 1 hour prior the behavioural test for habituation.

Elevated Plus Maze (EPM) test

$Npy1r^{2lox}$ and $Npy1r^{rfb}$ mice reared by FVB and C57 dams were tested in the EPM in a half-lit room in order to simulate the dark phase that is known to be the more active phase of rodents. The method used in this study was based on Gioiosa *et al*. (2007). Briefly, the EPM apparatus consists of a plus-cross shaped (30*5cm) originating from a central platform (5*5cm) elevated 40 cm above the floor. Two arms, opposing each other, are enclosed by Plexiglas walls (20 cm) and the leftover arms are open. Each animal was placed on the centre platform facing one of the open arms and was allowed to explore the maze for 5 minutes. The frequency of entries into, and time spent in, the open arms and closed arms of the maze were recorded with a video camera placed 2 m above the apparatus. To reduce any persistent olfactory cues, the maze was cleaned with clean damp cloth and a solution of 2% ethylic alcohol between successive trials. Behaviours were analysed and scored by an observer, previously trained, using specific software (The Observer, Noldus, NL) to record the latency, duration and frequency of transitions between different arms. In addition we selected 9 behaviours to analyse: • *Walking*, mouse walks; • *Immobility*, mouse is stationary without moving; • *Rearing*, mouse stays vertical, with rear paws on the floor and forward paws on the wall; • *Sniffing*: mouse is stationary and sniffs the air; • *Head-dipping*: mouse move the head towards the open arm; • *Self-Grooming*: mouse cleans the fur; • *Risk Assessment*: from closed arm to centre , or from centre to open arm mouse stretches repeatedly with one or both paws fixed; • *Stretching*: mouse stretches with forward paws from centre to closed arm, or into closed/open arm ; • *Other behaviours*.

Novelty Induced Suppression of Feeding (NISF) test

To measure the latency of an animal to approach and eat a familiar food in a novel environment and assess the animal anxiety-like state NISF test is very effective (Dadomo *et al*., 2011). FVB and C57 fostered male / female offspring underwent the NISF test. For four consecutive days, individual experimental animals were given at 9AM a Petri dish containing a little piece of peanut placed in a corner of their home cage. On the fourth day, the peanut was presented in a novel cage with fresh bedding (Merali *et al*., 2003). The latency the mice presented before starting to consume the peanut was recorded on all days with a cut-off time of 600s. In case of maximum latency, the peanut was left in the cage of the animal. A reduced latency to begin consumption of the palatable snack in the novel cage indicate a reduction of the anxiety

3.2.2 Experiment 2 - Effects of NPY1r inactivation on aggressive behaviour

Aggressive and social behaviour

NPY and his receptor Y1 have been also implicated in the regulation of other behaviours than anxiety and depressive-like behaviour, such as aggressive behaviour (Karl *et al*., 2004). Indeed, NPY is also known to significant alter the regular expression and secretion of several neurotransmitters such as serotonin (Shimizu and Bray, 1989), and norepinephrine (Ekblad *et al*., 1984) implicated in aggressive behaviour (Kask *et al*., 2002; Olivier *et al*., 1995). For this reason we decided to better investigate the role of NPY in aggressive and social behaviour using the Resident / intruder paradigm, a standardized method to measure offensive aggression and defensive behaviour in a setting that retrace the natural surroundings.

Resident / Intruder test

Aggressive and social behaviour was measured both in male and female Npy1r2lox and Npy1rrfb fostered at birth to FVB or C57 mothers. The animals were individually housed for a week. Resident mice were confronted in the Resident/Intruder paradigm with standard same-sex intruder adult male and female Balb mice, which are known to show low level of aggression.Experimental animals were observed and video-recorded for 2 min in their home cage, then an intruder of the same sex was placed in the resident's cage and the encounter was videotaped for 10 min with a camera placed 2 meters above the cages. Latency to the first attack by the resident against the intruder was recorded and the test was terminated if clear signs of distress in the intruder transpired. Behaviour was scored by the software The Observer (Noldus, NL). Specific behaviours analysed were: numbers and

duration of attack, tail rattling (rapid vibration of the tail, typically occurs shortly before and during an attack flurry), circling and chasing (the resident chases rapidly the intruder shortly before an attack occurrs), upright posture (following being defeated, mice rear up on their hindlegs and assume a defensive upright posture), immobility with contact (resident mice are in a freezing posture with contact to the intruder),startle response (defensive response to threatening stimuli), social exploration (resident explores the intruder, it includes ano-genital exploration), immobility without contact (resident mice are in freezing posture within the cage), rearing (mice rear up on their hindlegs), digging (mice dig bedding), environment exploration and walking. In order to promote a clear representation of the data, specific behavioural elements were grouped into the following broad behavioural categories: offensive behaviour (attack, tail rattling, circling and chasing) and defensive behaviour (upright posture, immobility with contact and startle response).

3.2.3 Experiment 3 - Effects of NPY1r inactivation on metabolism

Metabolic challenge

NPY is involved in the regulation of several biological functions including energy balance, and dysfunctions of its system have been implicated in human diseases such as obesity, type II diabetes and metabolic syndrome, raising the possibility that targeting the NPY system may provide therapeutic benefits for these diseases. In particular Y1 receptor has been a focus in obesity research due to its initial recognition as a “feeding receptor” that mediates NPY-induced hyperphagia (Antal-Zimanyi *et al*., 2008; Haynes *et al*., 1998; Henry *et al*., 2005; Kanatani *et al*., 1999; Kanatani *et al*., 2001; Mullins *et al*., 2001). Moreover, further studies revealed that Y1 receptor plays important roles in mediating other metabolic effects of NPY related to energy homeostasis, such as energy expenditure, lipid metabolism and insulin secretion (Herzog, 2003; Huda *et al*., 2006; Renshaw and Batterham, 2005). Accordingly with these results we decided to investigate the role of NPY limbic inactivation on the susceptibility to metabolic challenges in adulthood.

Body weight in STD and HFD

On PND 120 male and female Npy1r^{2lox} and Npy1r^{rfb} mice reared by FVB or C57 dams were weighed and individually housed in Plexiglas cages (45cm*25cm*20cm) with wood shaving bedding changed weekly. After the first week on a standard diet, to obtain baseline measurement, Npy1r^{2lox} and Npy1r^{rfb} FVB and C57 offspring were randomly assigned to two experimental groups: one fed standard diet (STD - 6.55% kcal from fat and 3.9 kcal/g; 4RF21, Mucedola, Italy) and the other fed a custom pelleted high fat diet (HFD - 45% kcal from fat and 5.2 kcal/g manufactured by Mucedola) obtained by a modification of the STD formula. Throughout the study, food and water were available

ad libitum to all experimental animals. Body weight and food intake were determined from 10 AM to 11 AM every two days for 3 weeks (**Fig. 2**). Pre-weighed food was carefully placed in the U-shaped metallic feed hopper of each cage and then reweighed on a per-cage basis. Food crumbles with a diameter greater than 1 mm were recovered from the cage and weighed as well.

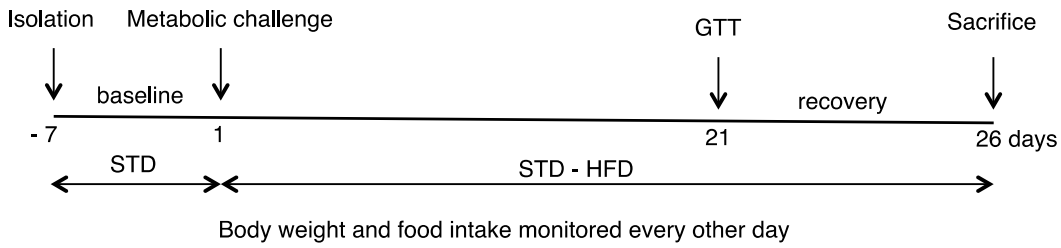

Fig.2. Experimental timeline

Glucose Tolerance test (GTT)

On the 21st day of the diet, Glucose Tolerance Test was performed following overnight fasting (12h). Blood glucose levels obtained from tail bleeding were monitored at 0, 30, 60 and 120 min after i.p. injection of d-glucose (Carlo Erba Reagent S.r.L., Milan, Italy) at 1g/Kg. Blood concentrations were all determined through an Accucheck Aviva (Roche Diagnostics, USA) blood glucose meter during the light phase between 9:00 AM and 11:00 AM. Mice were then returned to their diet regimen for 5 days to allow body weight recovery.

Tissue collection and plasma analysis.

On day 26 of diet exposure, following a 12 hrs fasting, mice were euthanized by decapitation after a brief CO2 exposure. Trunk blood was collected in heparinized tubes (Sarsted, Germany) and centrifuged at 4000 RPM, at 4°C for 10 min, then placed at -20°C until analysis. Plasma triglycerides and LDL, HDL and total cholesterol were measured on a clinical chemistry analyser (HITACHI 911, Roche Diagnostic, USA) using specific Sentinel KIT. Abdominal white adipose tissue (perigonadal, visceral, retroperitoneal fat pads) was collected and weighed, then frozen in liquid nitrogen for later analysis. Brains were removed, immediately frozen in liquid nitrogen, then stored at -80°C until analysis.

3.3 Results

Maternal behaviour observation

The direct observation of mother's behaviour with the pups allows us to calculate the mean percentage of time that FVB (n= 37) and C57 (n=20) dams spent in maternal behaviour (**Fig.3A**). A one-way ANOVA (foster mother strain) and a Tuckey post-hoc test were performed on weekly percentage of time spent in several behaviours (previously described). As we expected, FVB foster dams spent most of their time engaged in pup-related behaviours by crouching over the pups in the arched-back nursing (ABN), compared to the lying posture of nursing pups (main effect of foster mother: $F_{(1,55)}$ =39.4478; p <.001), when compared to C57 foster dams. (**Fig.3B**). Arched back posture is an active form of nursing: the greater time spent in arched back rather than in normal nursing suggests that FVB foster dams are characterized by higher level of maternal care than dams of the C57 strain (effect of foster mother: $F_{(1,55)}$ =36.4928; p <.001). When we analysed time spent out of the nest we observed a significant effect of foster mother strain ($F_{(1,55)}$ =30.9013; p<.001), as FVB dams presented a lower percentage of weekly time spent out of the nest on other activities as compared to C57 foster mothers. Moreover the percentage of time spent in active behaviours, namely all the behaviours that are not included in other categories, underlined a significant difference: in fact, FVB mothers showed a lower percentage of active behaviour as compared with C57 dams (main effect of foster mother strain: $F_{(1,55)}$ =76.0512; p <.001).

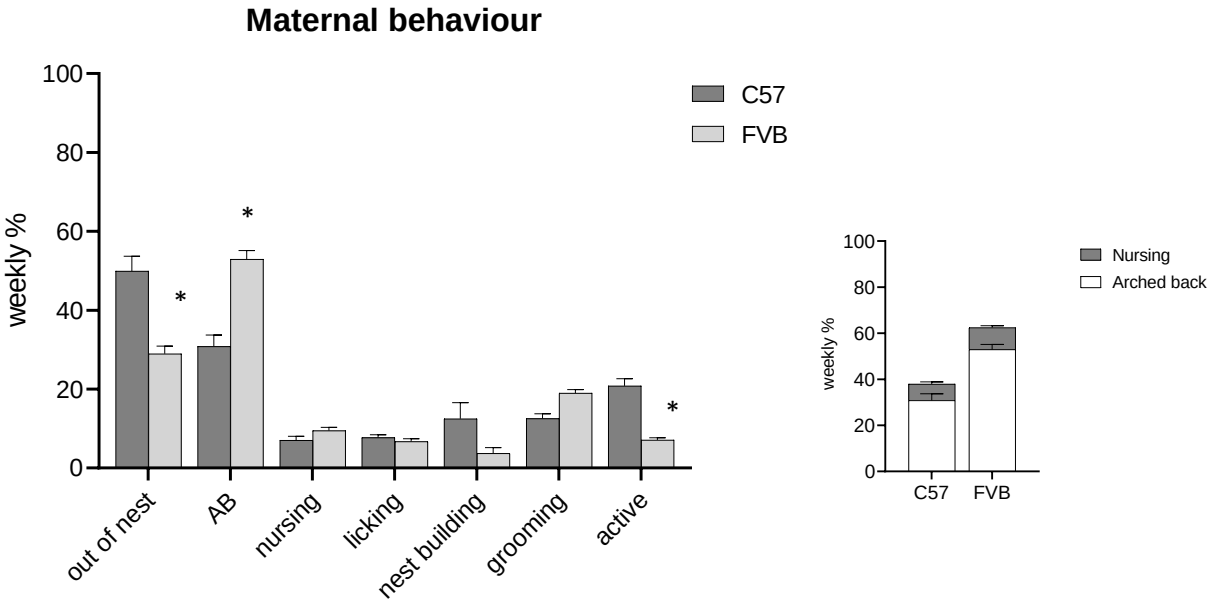

Fig. 3. (A) Average percentage of weekly time spent in spontaneous maternal behaviour by C57 (n=20) and FVB (n=37) foster dams during PND1-7 of observation. **(B)** Total nursing (Arched back + nursing behaviour) of C57 and FVB foster dams during postnatal day PND1-7. Data are presented as mean ± SEM, * indicates a significant difference with p<.05.

Body weight growth

Statistical analysis conducted on all the animals showed that there was a significant interaction among days and sex, as females' body weight was lower than males from PND 34 to PND 90 (days x sex interaction: $F_{(8,1008)} = 76.0512$; $p < .001$). When individually analysed females of both genotype and foster mothers' groups ($Npy1r^{2lox}$ and $Npy1r^{rfb}$ reared by FVB foster mothers, n=15 and n=16, respectively; $Npy1r^{2lox}$ and $Npy1r^{rfb}$ reared by C57 foster mothers, n=22 and n=16 respectively) showed no differences in body weight growth. Indeed in males, $Npy1r^{rfb}$ mice only when reared by FVB foster dams (n= 16) were characterized by a slower body weight growth starting from PND 48 (day of total inactivation of Npy1r gene) than their control male mice ($Npy1r^{2lox}$ reared by FVB foster mothers, n=16) (interaction among days and genotype: $F_{(8,240)} = 4.051$; $p < .001$). On the contrary, the difference in body weight among control animals ($Npy1r^{2lox}$ reared by C57 foster mothers, n= 18) and $Npy1r^{rfb}$ mice (n=16) was not evident in C57 foster mothers' offspring (days x genotype interaction: $F_{(8,256)} = 0.996$; $p = .4392$). (**Fig. 4**)

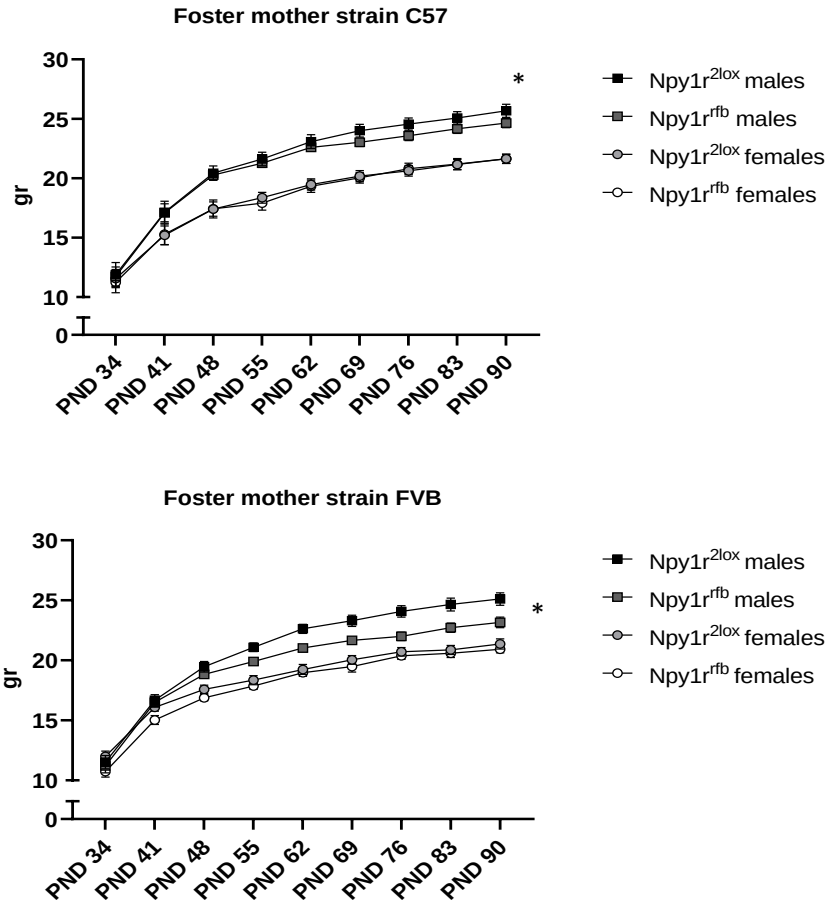

Fig. 4. Body weight growth of $Npy1r^{2lox}$ and $Npy1r^{rfb}$ male mice reared by C57 (n=22, n=16 respectively) and FVB (n=15, n=16 respectively) foster dams, measured from PND 34 to PND 90. Starting from PND 48, $Npy1r^{rfb}$ male mice reared by FVB showed a lower growth rate as compared to $py1r^{2lox}$, while in mice reared by C57 foster dams there was no difference among genotypes. In females no differences due to genotype or foster mother were observed. Values are presented as mean ± SEM, * indicates a significant difference with $p<.05$.

3.3.1 Experiment 1 - NPY1r inactivation affects emotional behaviour

Elevated Plus maze

Data were analysed using parametric analysis of variance (ANOVA) with strain of foster mother (C57 vs FVB), genotype (WT vs KO) and sex (males vs females) as between-subject factors, and total duration, total frequency and zones of the EPM as within-subjects repeated measures. All the animals were analysed for total frequency of entrance and total time spent in the three zones of EPM (closed arms, open arms, centre). Overall, regardless of sex, all subjects spent more time in the closed arms of the apparatus. When we analysed total duration in the close arms of the maze we observed that regardless of sex Npy1r^{rfb} animals reared by FVB foster dams (males n=26; females n=25) spent less time in the close arm of the apparatus than their controls (genotype x adoption interaction: $F_{(1,169)} = 4.1215$; $p<.05$).

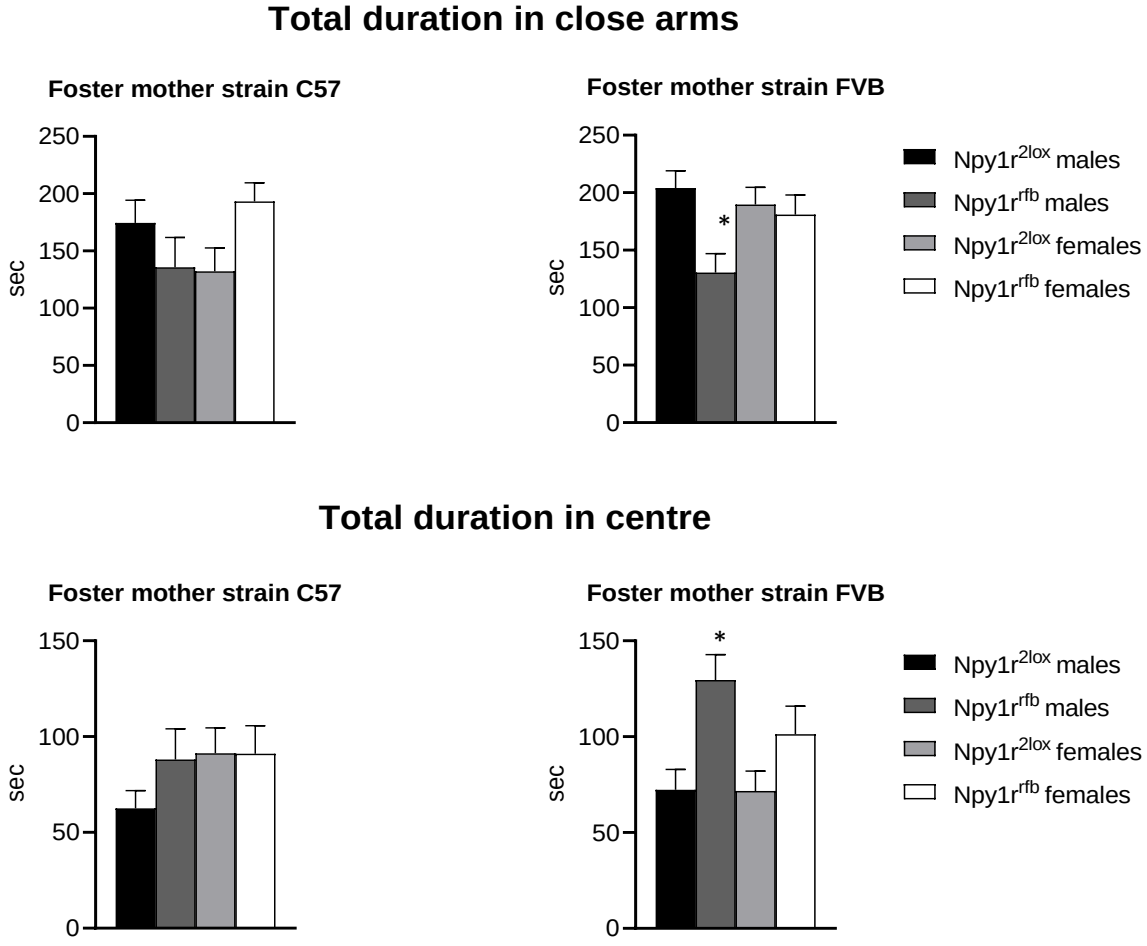

Fig.5. Time spent in the centre and close arm of EPM by males (Npy1r^{2lox} n=20, Npy1r^{rfb} n=26 reared by FVB foster dams; Npy1r^{2lox} n=18, Npy1r^{rfb} n=14 reared by C57 foster dams) and females (Npy1r^{2lox} n=29, Npy1r^{rfb} n=25 reared by FVB foster dams; Npy1r^{2lox} n=22, Npy1r^{rfb} n=20 reared by C57 foster dams). Interaction of genotype x adoption, and sex x genotype were observed in total duration spent in closed arms of the maze, with Npy1r^{rfb} males that spent less time than their controls and respective females in close space. Main effect of genotype detected in time spent in the centre of the apparatus, as Npy1r^{rfb} stood more time in the centre than their controls. Values are presented as mean ± SEM, * indicates a significant difference with $p<.05$.

Moreover Npy1r^{rfb} males of both groups of adoption (Npy1r^{rfb} reared by C57,n=14;) spent less time in close zone than their respective controls (Npy1r^{2lox} reared by C57,n=18; Npy1r^{2lox} reared by FVB, n=20) and also compared with Npy1r^{rfb} females (sex x genotype interaction: $F_{(1,169)}$ =10.2514; p<.005). Npy1r^{rfb} mice, regardless of sex and strain of foster mother differs from their controls in time spent exploring the centre of the maze (main effect of genotype: $F_{(1,169)}$ =8.9994; p<.005). **(Fig.5).** Overall Npy1r^{rfb} males spent also more time in the open arms of the EPM than their respective females, while in control animals there were no significant difference among male and female animals (sex x genotype interaction: $F_{(1,169)}$ =7.03536; p<.01). Furthermore mice reared by C57 foster mothers spent significant more time in the open arms than mice reared by FVB foster dams (adoption: $F_{(1,169)}$ =7.07938; p<.001) (**Fig.6**).

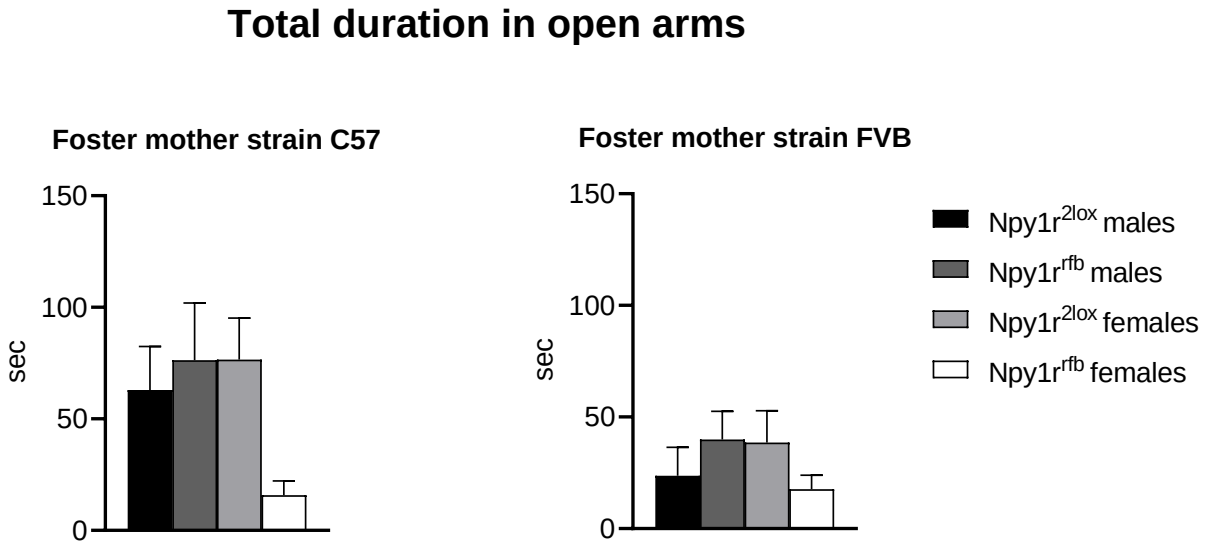

Fig.6. Time spent in the open arm of EPM by males (Npy1r^{2lox} n=20, Npy1r^{rfb} n=26 reared by FVB foster dams; Npy1r^{2lox} n=18, Npy1r^{rfb} n=14 reared by C57 foster dams) and females (Npy1r^{2lox} n=29, Npy1r^{rfb} n=25 reared by FVB foster dams; Npy1r^{2lox} n=22, Npy1r^{rfb} n=20 reared by C57 foster dams). Interaction of sex x genotype in open arms, Npy1r^{rfb} males spent more time in open space than their females, while in control animals there was no difference. Effect of the adoption in time spent in open arms, as animals reared by C57 foster dams lingered more in open spaces than mice reared by FVB foster mothers. Values are presented as mean ± SEM

Statistical analysis conducted on total numbers of entries in centre space of the apparatus underlying the significant effect of genotype ($F_{(1,169)}$ =17.5591; p<.001). Npy1r^{rfb} animals, regardless of sex and foster mother's strain entered less than their controls in the centre of the EPM. Similarly we observed a significant effect of genotype in locomotor activity (number of entries in the close arms), as Npy1r^{rfb} mice had a lower frequency of entrance in close space of the maze ($F_{(1,169)}$ =16.3216; p<.001;)(**Fig.7**). Moreover, accordingly to results of total duration, Npy1r^{rfb} mice reared by C57 dams showed a lower frequency of entrance in open arms as compared to their controls, whereas there's no

difference among genotypes in mice reared by FVB foster mothers (genotype x adoption interaction: $F_{(1,169)} = 6.7810$; $p<.05$)(**Fig.8**).

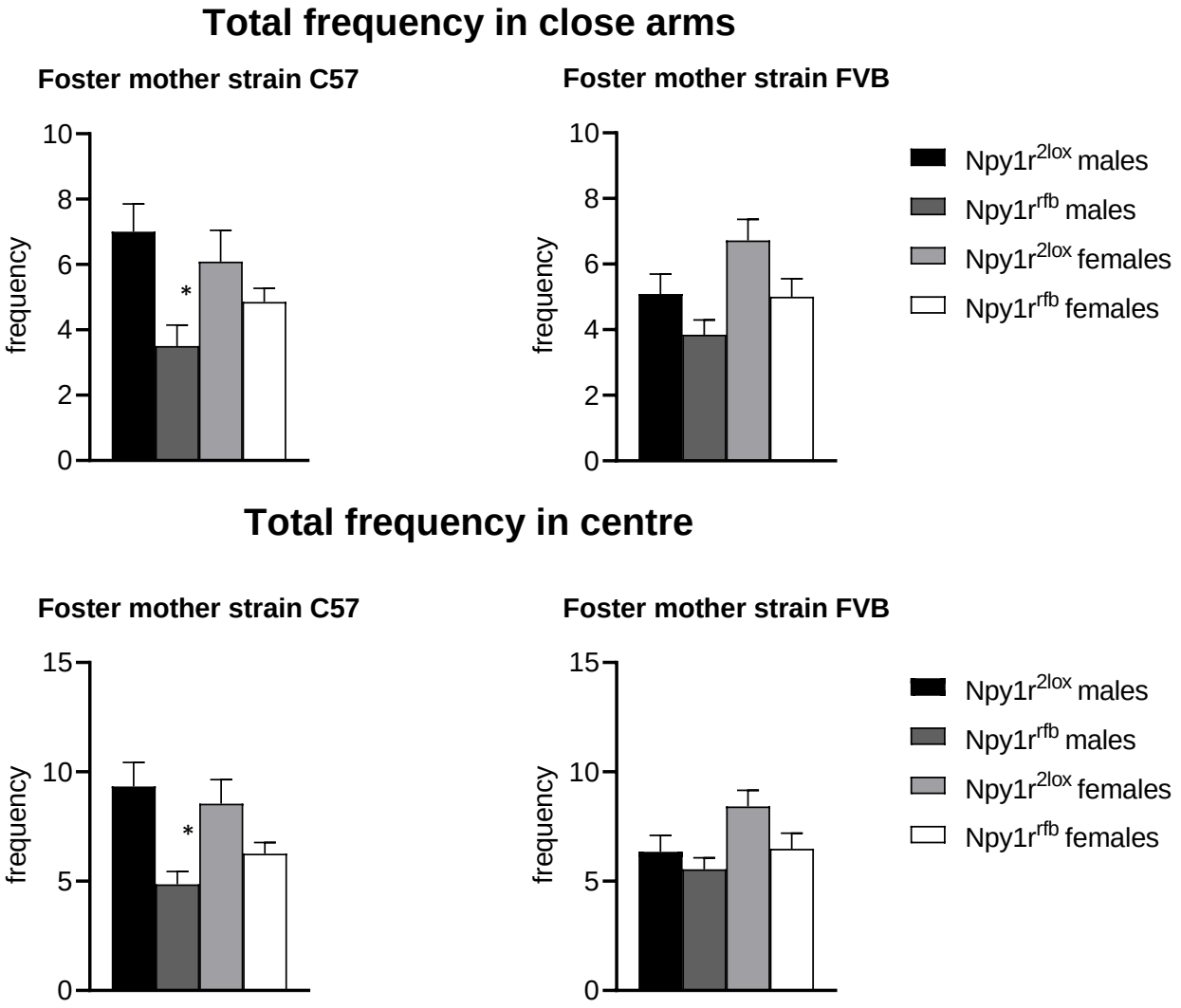

Fig.7. Frequency of entries in the centre and close arm of EPM by males ($Npy1r^{2lox}$ n=20, $Npy1r^{rfb}$ n=26 reared by FVB foster dams; $Npy1r^{2lox}$ n=18, $Npy1r^{rfb}$ n=14 reared by C57 foster dams) and females ($Npy1r^{2lox}$ n=29, $Npy1r^{rfb}$ n=25 reared by FVB foster dams; $Npy1r^{2lox}$ n=22, $Npy1r^{rfb}$ n=20 reared by C57 foster dams). Main effect of genotype detected in frequency of entrance in the centre and close arms of the apparatus. $Npy1r^{rfb}$ entered less times in the centre than their controls. Interaction among adoption and genotype in frequency of entrance in the open arms; $Npy1r^{rfb}$ mice reared by C57 foster dams entered less times in open space than their controls, while in animals reared by FVB foster mothers there was no difference. Values are presented as mean ± SEM, * indicates a significant difference with $p<.05$.

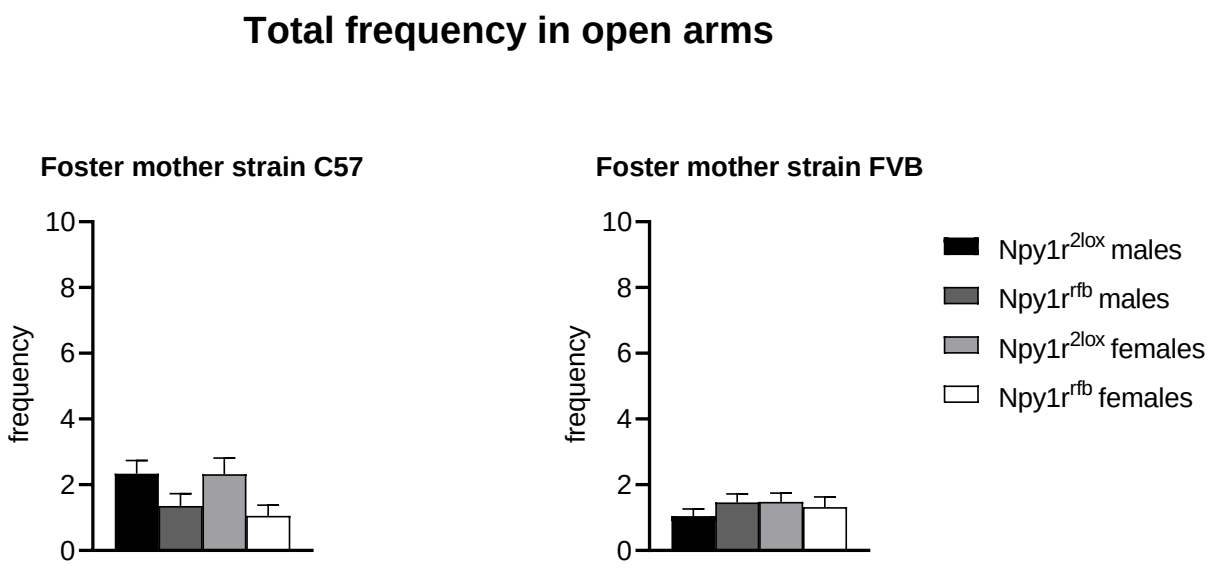

Fig.8. Frequency of entries in the open arm of EPM by males (Npy1r^{2lox} n=20, Npy1r^{rfb} n=26 reared by FVB foster dams; Npy1r^{2lox} n=18, Npy1r^{rfb} n=14 reared by C57 foster dams) and females (Npy1r^{2lox} n=29, Npy1r^{rfb} n=25 reared by FVB foster dams; Npy1r^{2lox} n=22, Npy1r^{rfb} n=20 reared by C57 foster dams). Interaction among adoption and genotype in frequency of entrance in the open arms; Npy1r^{rfb} mice reared by C57 foster dams entered less times in open space than their controls, while in animals reared by FVB foster mothers there was no difference. Values are presented as mean ± SEM

Novelty induced suppression of feeding – NISF

NISF test provides the results on latency to initiate consumption of a palatable snack over habituation and during the test in a novel environment. Npy1r^{rfb} and Npy1r^{2lox} mice reared by FVB and C57 foster mothers have been tested for NISF among 4 days, 3 days of habituation in their home cage plus 1 day of test in a new cage. Statistical analysis conducted on the habituation days showed that females Npy1r^{2lox} (n=17, n=26; number of control females reared by C57 and FVB foster dams, respectively) presented an higher latency of consumption during day 1, as compared to their respective males (n=16, n=20; number of control males reared by C57 and FVB foster dams, respectively), whereas in Npy1r^{rfb} mice no differences due to sex were detected (days x sex x genotype interaction: $F_{(2,300)}$ =3.1181; p<.05) Regardless of sex, there is a significant decrease in the latency to eat a palatable food during the habituation trials (main effect of days: $F_{(2,510)}$ =127.4647; p<.001), but no effect of fostering, genotype or sex was detected. During the test (day 4) we observed a significant main effect of the genotype ($F_{(1,150)}$ =7.9838; p<.01). In fact, regardless sex or strain of adoption, Npy1r^{rfb} mice (n=19, n=23; number of KO females reared by C57 and FVB foster dams, respectively; n=12, n=25; number of KO males reared by C57 and FVB foster dams, respectively) showed lower latencies to eat the novel food as compared with their controls (**Fig.9**).

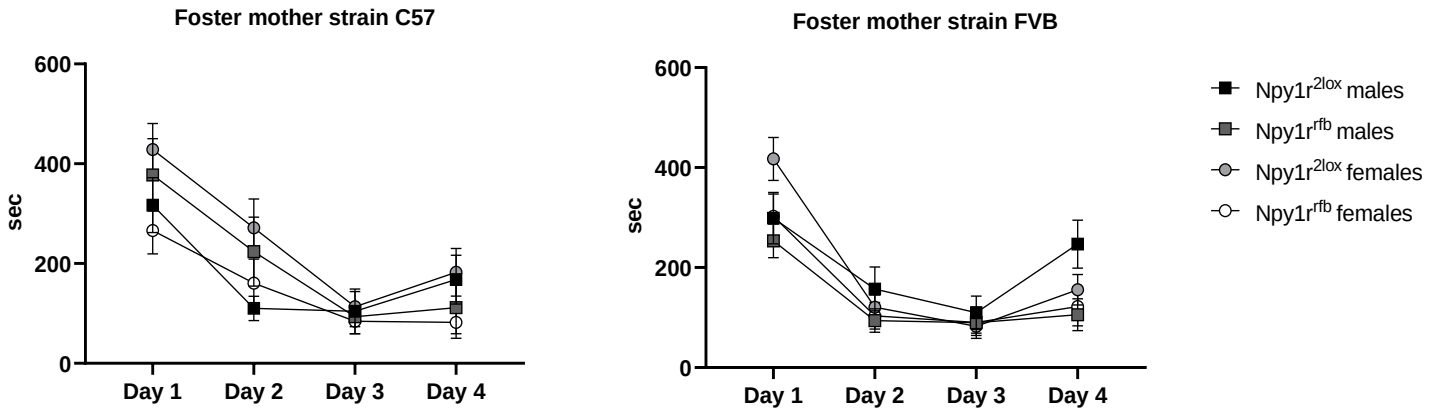

Fig. 9. Novelty induced suppression of feeding; latency to eat the peanuts (sec) of males (Npy1r^{2lox} n=20, Npy1r^{rfb} n=25 reared by FVB foster dams; Npy1r^{2lox} n=16, Npy1r^{rfb} n=12 reared by C57 foster dams) and females (Npy1r^{2lox} n=26, Npy1r^{rfb} n=23 reared by FVB foster dams; Npy1r^{2lox} n=17, Npy1r^{rfb} n=19 reared by C57 foster dams). Day 1-3 represent the habituation, day 4 represent the test in a novel environment. Latency decreased in all groups during the habituation. At day 4 Npy1r^{rfb} lingered less than Npy1r^{2lox} animals to eat the palatable snack. Values are presented as mean ± SEM

3.3.2 Experiment 2 - Effects of NPY1r inactivation on aggressive behaviour

Aggressive and social behaviour

Aggressive behaviour was analysed in Npy1r^{rfb} and Npy1r^{2lox} males reared by FVB and C57 foster mothers through the Resident-Intruder paradigm. Among males reared by C57 foster dams 16 out of 18 control mice attacked the intruder, and interestingly the same number of conditional knockout mice (12 out of 14) displayed aggressive behaviour. Interestingly between males reared by FVB foster mothers, 15 out of 23 Npy1r^{2lox} male attacked and only 9 out of 26 Npy1r^{rfb} displayed aggressive behaviour. In line with this finding, males reared by FVB foster mothers, regardless genotype, were characterized by significantly higher attack latency, as compared with males reared by C57 foster dams (adoption: $F_{(1,77)}$ =9.5805; p<.005) (**Fig. 10 C**). Consistently, males adopted by FVB foster mothers spent significantly less time engaged in offensive behaviour (comprising biting attack, circling, chasing, tail rattling), as compared with male mice reared by C57 foster dams (effect of adoption: $F_{(1,61)}$ =9.05859; p<.005) (**Fig. 10 A**).

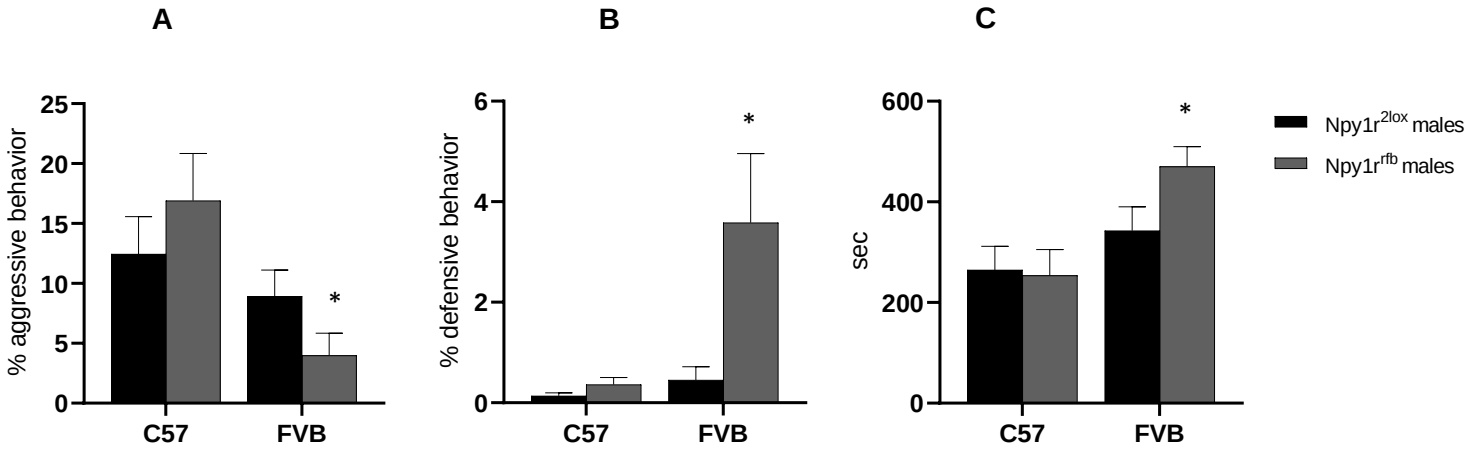

Fig. 10. Resident intruder paradigm to test aggressive behaviour in Npy1r^{2lox} (n= 18, n=23 reared by C57 and FVB foster dams) and Npy1r^{rfb} (n= 14, n=26 reared by C57 and FVB foster dams) male mice; **(A)**Time spent in agonistic behaviour (comprising biting attack, circling, chasing, tail rattling). Males of both genotypes, when reared by FVB foster dams showed a decrease in aggressive behaviour. **(B)** Time spent in defensive behaviour (upright posture, immobility with contact). Interaction among adoption and genotype; KO males reared by FVB dams presented the highest percentage of defensive behaviour. **(C)** Attack latency. In line with the aggressive behaviour displayed, only offspring of FVB foster dams showed an increase in latency of attack. Values are presented as mean ± SEM, * indicates a significant difference with p<.05.

In line with previous results, Npy1r^{rfb} males spent more time performing defensive behaviours (upright posture, immobility with contact) when compared with control males (genotype: $F_{(1,61)}$ =4.024829; p<.05). The foster mother's strain play a significant role too, as offspring of FVB foster

dams displayed more defensive behaviours than males reared by C57 foster mothers (adoption: $F_{(1,61)}$ =4.467568; p<.05) (**Fig. 10 B**).

To test social behaviour we analysed the percentage of time spent in social investigation towards the intruder. As expected, we did not observe any differences due to genotype or adoption in males. Intriguingly, in females, NPY conditional KO displayed a lower amount of social behaviour when reared by FVB foster dams (n=8), as compared with their respective controls (females Npy1r^{2lox} reared by FVB foster mothers n=11) (**Fig. 11**). On the contrary Npy1r^{rfb} females adopted by C57 foster dams (n=12) showed an higher amount of social investigation when compared to their controls (females Npy1r^{2lox} reared by C57 foster mothers n=17) (genotype x adoption interaction: $F_{(1,44)}$ =7.2744; p<.01).

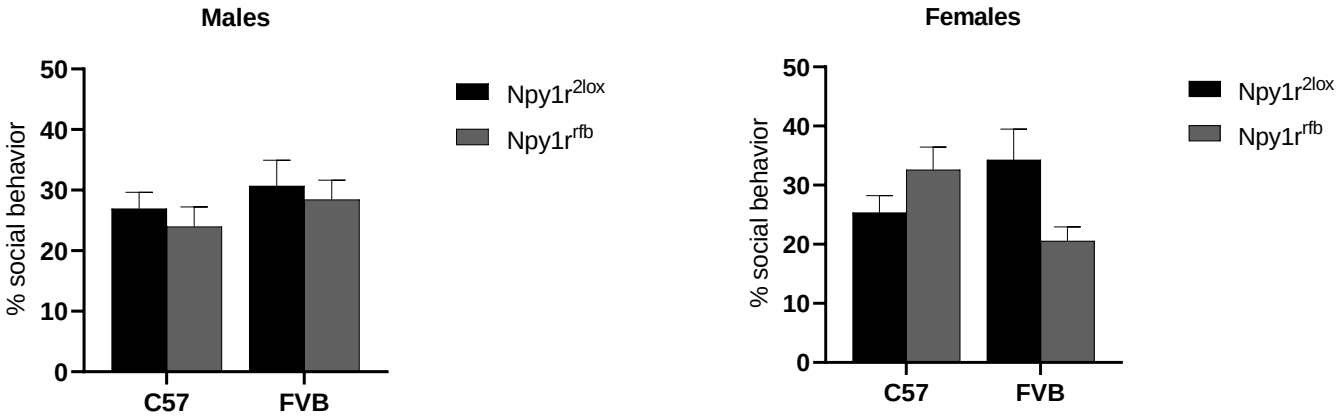

Fig. 11. Resident intruder paradigm to test social behaviour; Percentage of time spent in social interaction by Npy1r^{2lox} (n= 18, n=23 reared by C57 and FVB foster dams) and Npy1r^{rfb} (n= 14, n=26 reared by C57 and FVB foster dams) males and females (Npy1r^{2lox} n= 17, n=11 and Npy1r^{rfb} n=12, n=8 female mice reared by C57 and FVB foster dams). We observed higher amount of social behaviours in females. Npy1r^{rfb} females reared by FVB foster mothers presented a lower amount of social investigation as compared with their controls. On the contrary when reared by C57 dams Npy1r^{rfb} females showed more social behaviours as compared with Npy1r^{2lox} . Values are presented as mean ± SEM

3.3.3 Experiment 3 - Effects of NPY1r inactivation on metabolism

Metabolic challenge

As adults, Npy1r^{2lox} and Npy1r^{rfb} mice were exposed to standard (STD)(males Npy1r^{2lox} n= 5, Npy1r^{rfb} n=3 and females Npy1r^{2lox} n= 9, Npy1r^{rfb} n=5 reared by C57 dams; males Npy1r^{2lox} n=12, Npy1r^{rfb} n=14 and females Npy1r^{2lox} n=8 , Npy1r^{rfb} n=8 reared by FVB dams) or high fat (HFD) diet (males Npy1r^{2lox} n=11, Npy1r^{rfb} n=12 and females Npy1r^{2lox} n=10, Npy1r^{rfb} n=11 reared by C57 dams; males Npy1r^{2lox} n=8, Npy1r^{rfb} n=7 and females Npy1r^{2lox} n=7, Npy1r^{rfb} n=8 reared by FVB dams). Statistical analysis conducted on all the animals first showed a significant effect of diet ($F_{(1,122)}$ =103.92; p<.001) with animals fed with HFD diet gaining more weight than animals fed with STD diet. Males of both genotypes and regardless of the adoption, weighted more than females in both STD and HFD diets (sex: $F_{(1,122)}$ =315.43; p<.001). When individually analysed mice fed with STD diet showed a significant difference due to sex effect ($F_{(1,56)}$ =110.44; p<.001) as males regardless of genotype and adoption gained more weight than females, but no other differences were detected (**Fig.12**).

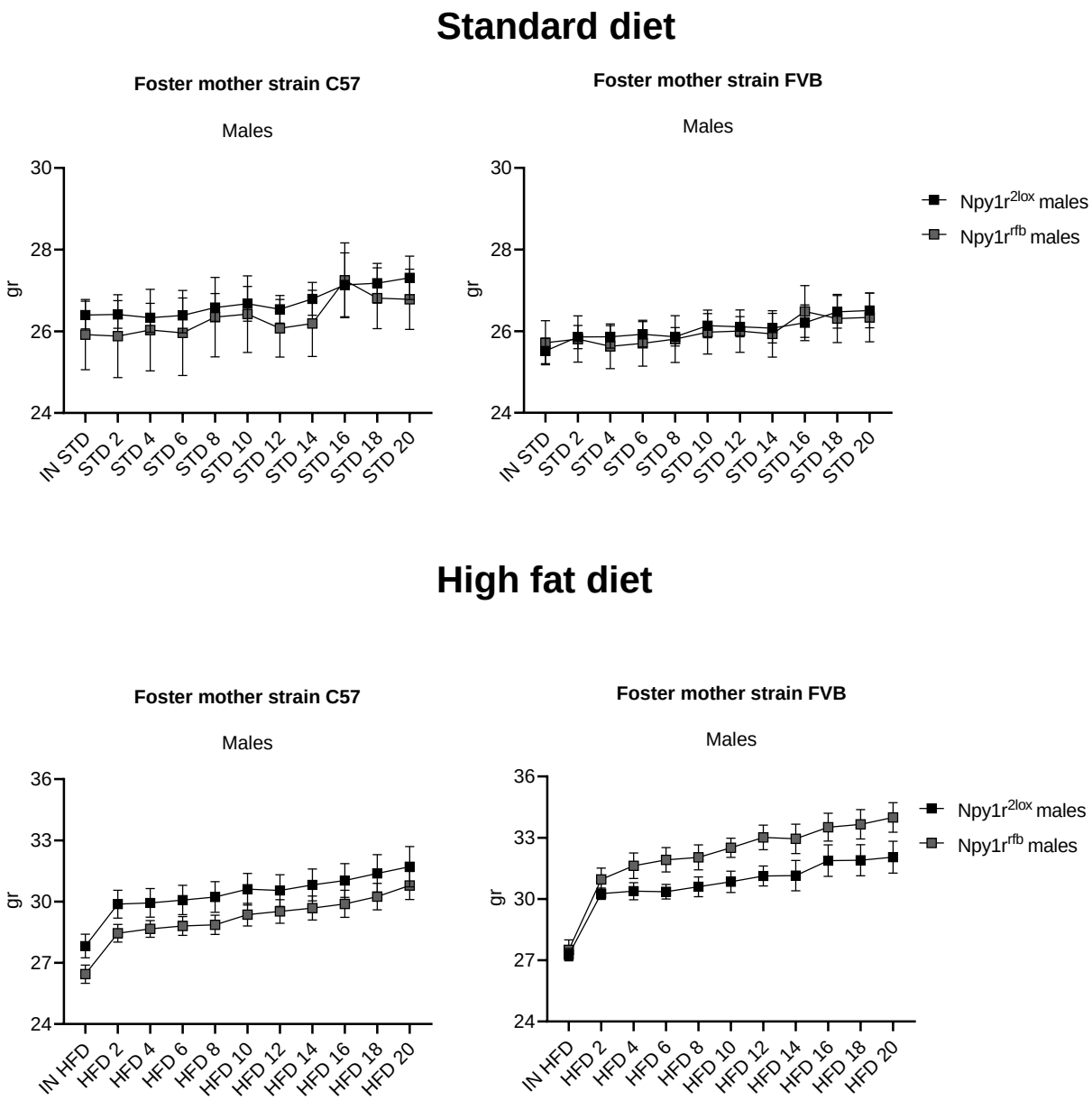

Fig. 12. Body weight during the metabolic challenge. Metabolic consequences of STD in Npy1r^{2lox} (n=5; n=12 males and n=9 , n=8 females of C57 and FVB foster dams respectively) and Npy1r^{rfb} (n=3; n=14 males and n=5 , n=8 females of C57 and FVB foster dams respectively) mice. Regardless of genotype and adoption, male mice on STD showed a greater body weight as compared to females. Values are presented as mean ± SEM.

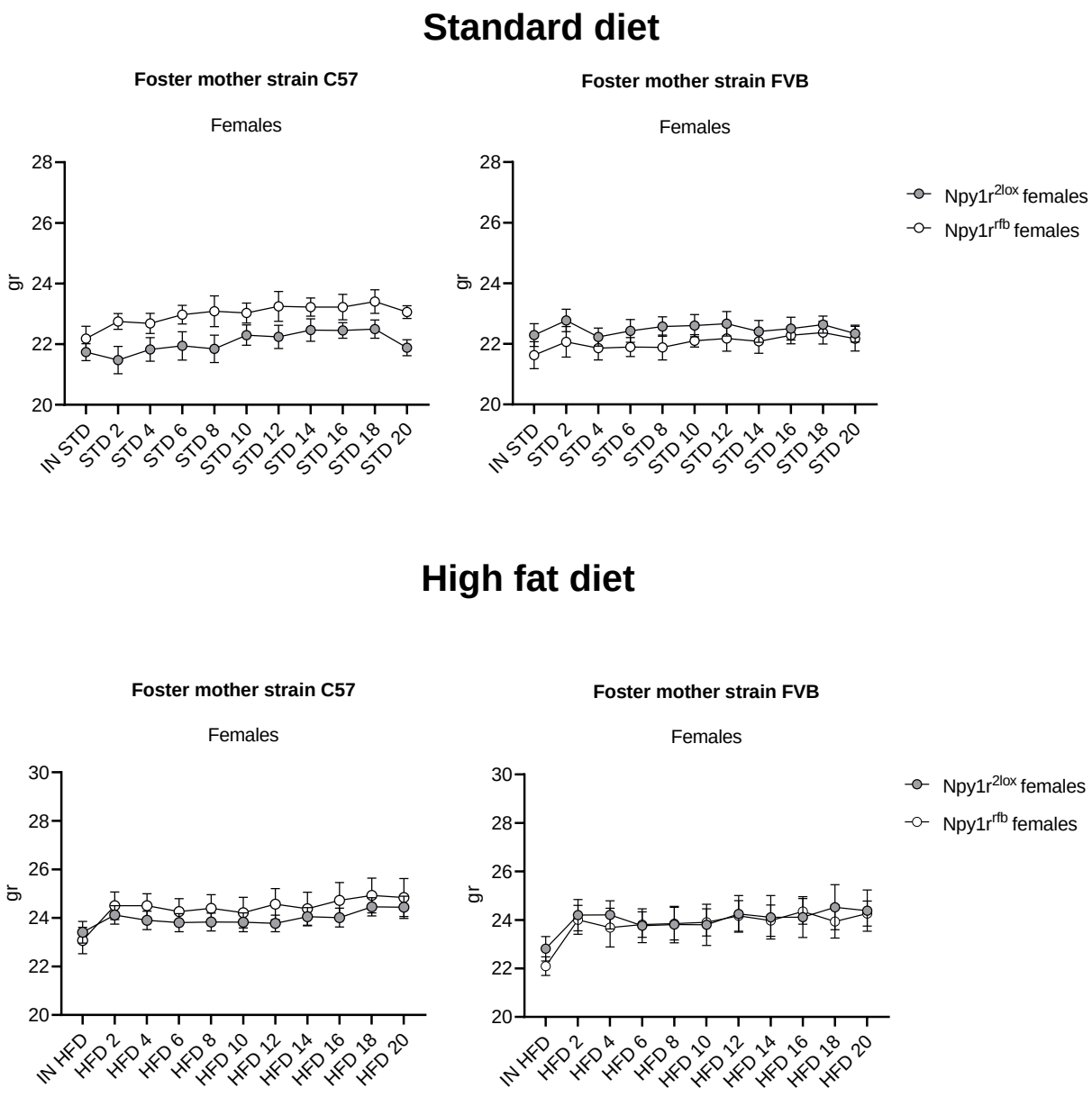

Fig.13. Body weight during the metabolic challenge. Metabolic consequences of HFD in Npy1r^{2lox} (n=11; n=8 males and n=10 , n=7 females of C57 and FVB foster dams respectively) and Npy1r^{rfb} (n=12; n=7 males and n=11 , n=8 females of C57 and FVB foster dams respectively) mice. Male mice on HFD showed a greater body weight as compared to females, and there is an interaction among sex and adoption, as male mice reared by FVB, regardless of genotype weighted more than males reared by C57 foster dams. Moreover, there is a tendency of the interaction among sex, genotype and adoption: Npy1r^{rfb} males reared by FVB dams showed an increase of body weight when compared to their controls, while in males reared by C57 dams KO weighted less than controls. Values are presented as mean ± SEM

When fed with HFD males reared by FVB foster dams, regardless of genotype, gained more weight than male mice reared by C57 foster mothers, whereas in females differences due to adoption were not detected (sex x adoption interaction: $F_{(1,66)}$ =5.14; p<.05). Moreover, there seemed to be a tendency of the interaction of sex x adoption x genotype ($F_{(1,66)}$ =3.62; p=.06). In fact, Npy1r^{rfb} males

reared by FVB foster dams weighted more than their respective controls; on the contrary in C57 foster dams' males we observed that Npy1r conditional KO mice gained less weight as compared with their controls (**Fig.13**).

After 3-week exposure to High fat or Standard diet we performed the Glucose tolerance test (GTT) on all $Npy1r^{2lox}$ (males reared by C57 foster dams and fed with STD n=10 and HFD n=11; males reared by FVB foster dams and fed with STD n=5 and HFD n=7 ; females reared by C57 foster dams and fed with STD n=9 and HFD n=10; females reared by FVB foster dams and fed with STD n=7 and HFD n=8) and $Npy1r^{rfb}$ (males reared by C57 foster dams and fed with STD n=16 and HFD n=12; males reared by FVB foster dams and fed with STD n=7 and HFD n=6 ; females and reared by C57 foster dams and fed with STD n=7 and HFD n=8; females reared by FVB foster dams and fed with STD n=5 and HFD n=8) mice (**Fig.14**). Statistical analysis of metabolic changes on all animals showed that in males $Npy1r^{rfb}$ mice presented higher levels of glycemia as compared to $Npy1r^{2lox}$ whereas in females we did not observed any differences related to genotype (genotype x sex interaction: $F_{(1,110)}=5.524$, $p<.05$). There is also a significant interaction among genotype and diet, as $Npy1r^{rfb}$ mice fed with HFD showed hyperglycemia as compared with their controls, while in STD animals we did not observed any difference. Moreover, we observed an effect of the diet ($F_{(1,110)}=60.840$, $p<.001$), as mice of both sexes, genotypes and adoptions, presented an increase in glycemia levels when fed with HFD as compared with STD animals, and an effect of sex ($F_{(1,110)}=139.177$, $p<.001$), as males regardless genotype, adoption and diet, showed higher levels of blood glucose compared with females. Individual analysis of glycemia levels in standard diet showed that after 30, 60 and 120 minutes from the injection females' blood glucose was significant lower than males' values, whereas at time 0 levels were similar (interaction among time and sex: $F_{(3,144)}=10.917$, $p<.001$), but no effects of genotype or adoption were found. The same result was observed when we analysed mice fed with HFD (interaction among time and sex: $F_{(3,186)}=16.918$, $p<.001$). Interestingly, statistical analysis based on sex, underlined that HFD promoted glucose intolerance in $Npy1r^{rfb}$ compared to $Npy1r^{2lox}$ males, regardless the adoption, as indicated by the higher blood glucose level observed in conditional mutant following the ip glucose injection (diet x genotype interaction: $F_{(1,56)}=4.276$, $p<.05$). Furthermore in females we did not observed any difference but the diet ($F_{(1,54)}=24.954$, $p<.001$).

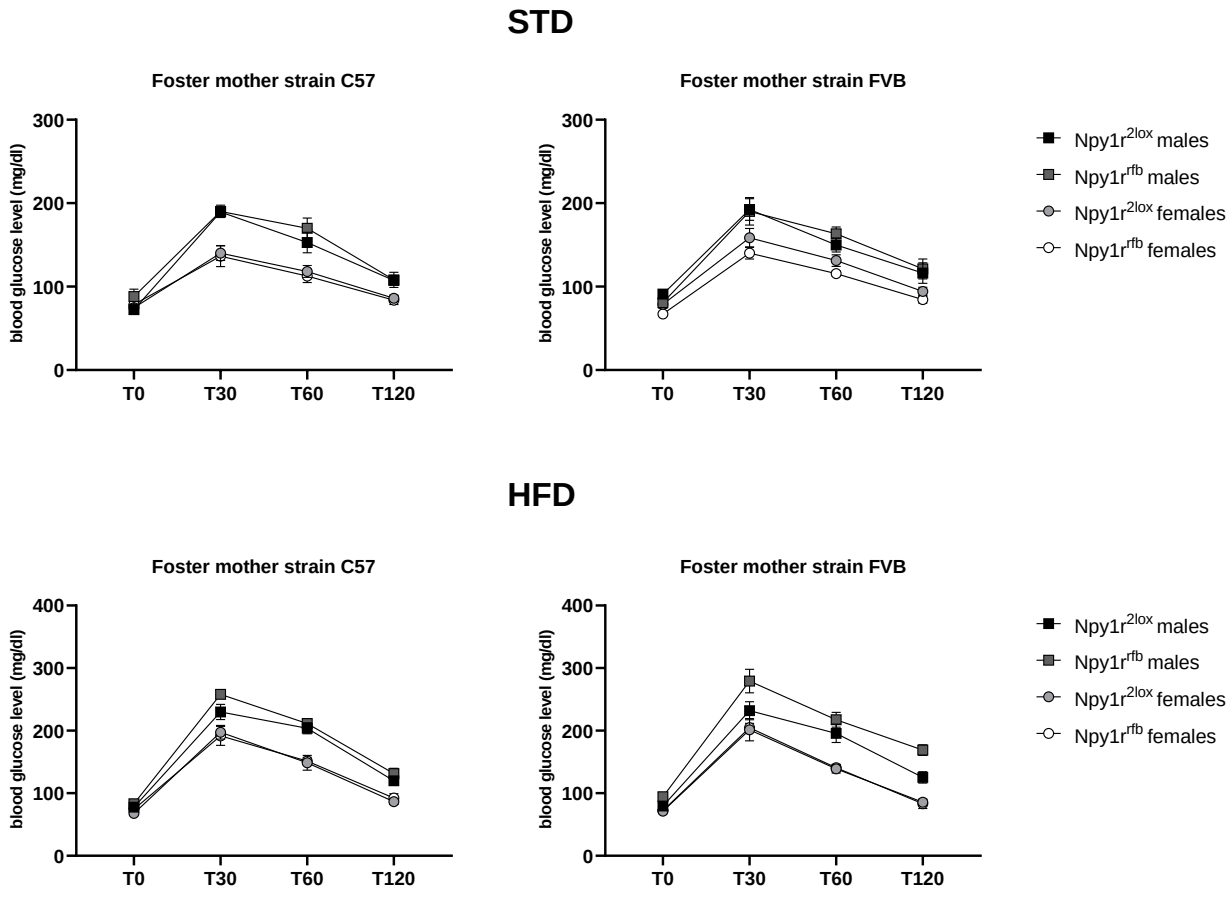

Fig. 14. Glucose tolerance test (GTT). Glucose levels during STD (males Npy1r^{2lox} n= 10, n=5 and Npy1r^{rfb} n=16, n=7 reared by C57 and FVB foster dams; females Npy1r^{2lox} n=9, n=7 and Npy1r^{rfb} n=7,n=5 reared by FVB and C57 foster dams) and HFD (males Npy1r^{2lox} n= 11, n=7 and Npy1r^{rfb} n=12, n=6 reared by C57 and FVB foster dams; females Npy1r^{2lox} n=10, n=8 and Npy1r^{rfb} n=8, n=8 reared by FVB and C57 foster dams). Male mice on HFD, regardless genotype and adoption, showed a higher level of glycemia as compared to females. Npy1r^{rfb} as compared to Npy1r^{2lox} males presented hyperglycemia only when fed with HFD (interaction among diet x genotype). No differences but the diet (STD mice showed less blood glucose than HFD mice) in females. Values are presented as mean ± SEM.

At sacrifice we analysed the total amount of white abdominal fat. As expected males mice fed with HFD (Npy1r^{2lox} n=8, Npy1r^{rfb} n=12 reared by C57 dams; Npy1r^{2lox} n=8, Npy1r^{rfb} n=7 reared by FVB dams), regardless of genotype and adoption, had an higher level of abdominal fat as compared with females fed with high fat diet (Npy1r^{2lox} n=8, Npy1r^{rfb} n=7 reared by C57 dams; Npy1r^{2lox} n=7, Npy1r^{rfb} n=8 reared by FVB dams), while in STD the difference was not significant (sex x diet interaction: $F_{(1,104)}$=63.6587, p<.001). When individually analysed mice fed with STD (males Npy1r^{2lox} n=9, Npy1r^{rfb} n=7 reared by C57 dams; males Npy1r^{2lox} n=5, Npy1r^{rfb} n=7 reared by FVB dams; females Npy1r^{2lox} n=8, Npy1r^{rfb} n=7 reared by C57 dams; females Npy1r^{2lox} n=7, Npy1r^{rfb} n=5 reared by FVB dams) did not showed any difference due to genotype, sex or adoption (**Fig.15**). Indeed, statistical analysis conducted on mice fed with HFD provided data in line with the results of body weight during

high fat diet regimen. In fact male Npy1r^{rfb} mice reared by FVB foster dams presented an higher amount of abdominal fat than their respective controls, whereas on the contrary Npy1r^{rf} males reared by C57 foster mothers showed less white adipose tissue than their controls (sex x genotype x adoption interaction: $F_{(1,57)}$=4.8199, p<.05). Moreover we observed that in females fed with HFD, regardless of genotype, the amount of WAT was higher in female mice reared by C57 foster mothers, as compared to females reared by FVB foster dams (diet x adoption interaction: $F_{(1,49)}$=5.26913, p<.05) (**Fig.15**).

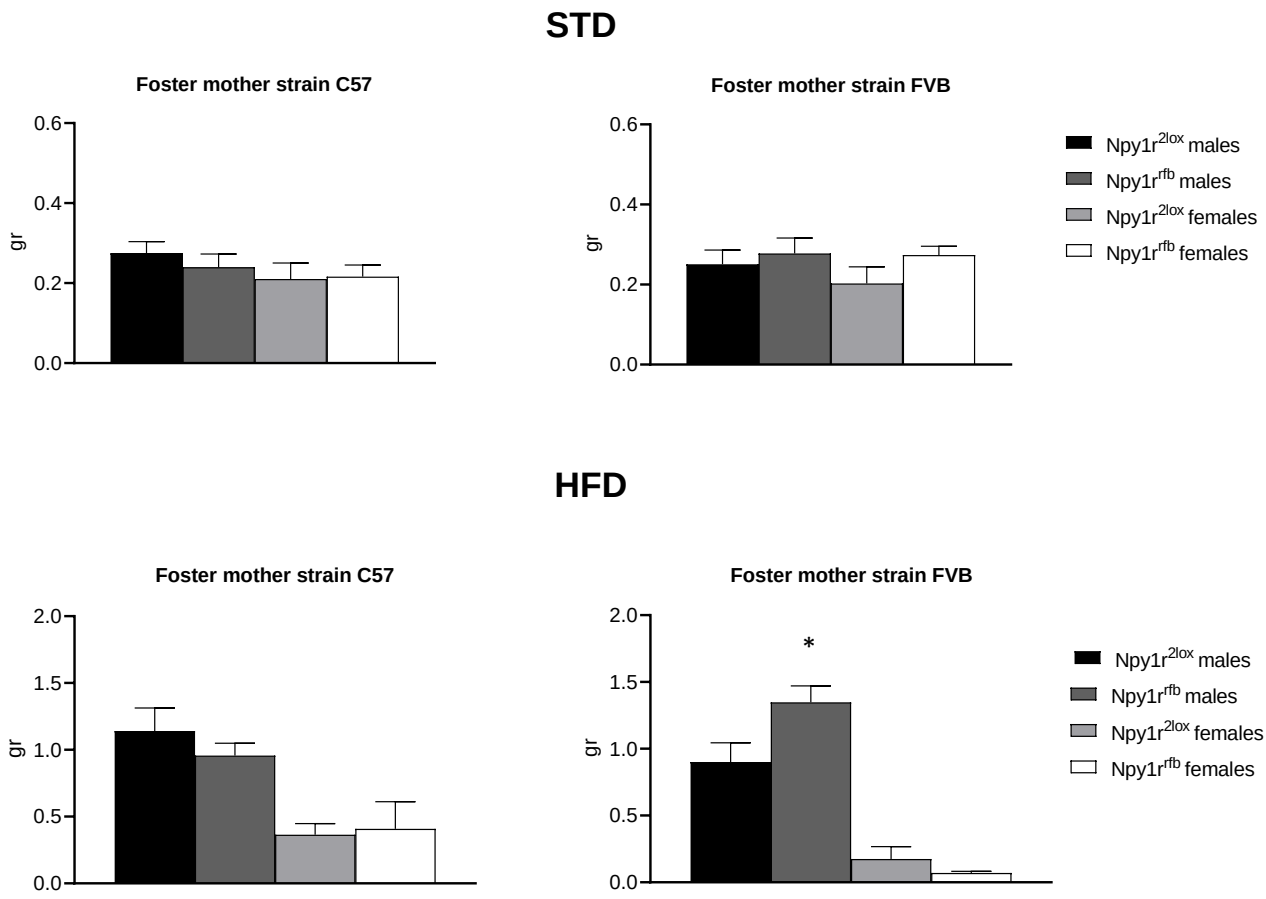

Fig.15 White adipose tissue harvested at sacrifice. Amount of WAT in standard diet STD (males Npy1r^{2lox} n= 9, n=5 and Npy1r^{rfb} n=7, n=7 reared by C57 and FVB foster dams; females Npy1r^{2lox} n=8, n=7 and Npy1r^{rfb} n=7,n=5 reared by FVB and C57 foster dams) and HFD (males Npy1r^{2lox} n= 8, n=8 and Npy1r^{rfb} n=12, n=7 reared by C57 and FVB foster dams; females Npy1r^{2lox} n=8, n=7 and Npy1r^{rfb} n=7, n=8 reared by FVB and C57 foster dams). Overall Npy1r^{rfb} males reared by FVB dams presented an increase in WAT as compared with their controls, on the contrary Npy1r^{rfb} males reared by C57 showed a lower amount of abdominal fat as compared with Npy1r^{2lox}. All values are presented as mean ± SEM, * indicates a significant difference with p<.05

3.4 Discussion

In this study we assessed the effects of conditional Npy1r knockout in brain limbic areas on anxiety-like, aggressive and social behavior, and metabolism of male and female mice that have received different levels of maternal care. To this aim we examined adult male and female control $Npy1r^{2lox}$ and KO $Npy1r^{rfb}$ mice reared by FVB and C57 foster mothers, which displayed high and low maternal care, respectively.

First of all, we analysed maternal behaviour displayed by FVB and C57 foster mothers. FVB dams showed a high maternal care profile, with a significant amount of maternal care directed to the pups (Arched back and nursing behaviour) and low amount of time out of the nest or in other activities as compared with C57 foster mothers. Such a high proportion of time spent by FVB dams in the ABN posture was consistent during the first postnatal week: these results confirm that FVB mothers display high maternal cares (HM), while C57 females display low maternal care (LM), as previously described by Bertocchi and coworkers (2011).

$Npy1r^{rfb}$ and $Npy1r^{2lox}$ fostered to Dox-naïve FVB and C57 dams were then examined for body growth since weaning (postnatal day 27-28); results indicated that male mice characterized by low limbic Npy1r expression induced by conditional gene inactivation showed reduced body weight growth, starting after postnatal day 48, when gene deletion was achieved. In line with previous study (Bertocchi *et al*. 2011), the effects of conditional limbic Npy1r deletion on metabolism are shown in male mice reared by high maternal care dams (FVB dams). $Npy1r^{rfb}$ males showed lower body weight growth starting from approximately PND 48, which coincides with the maximal level of Npy1r gene Cre-mediated inactivatio.

NPY1r effects on emotional behaviour

Anxiety-like behaviour was assessed by means of the Elevated Plus maze (EPM) test and the Novelty Induced Suppression of Feeding (NISF) test. Contrary to what expected, reduced Npy1r mRNA expression in limbic areas did not affect the traditional measure of anxiety in the EPM, open-arms exploration. Overall both $Npy1r^{2lox}$ and $Npy1r^{rfb}$ males and females reared by high or low maternal care dams spent most of the time in the close arms of the EPM and rarely explored the open arms, suggesting that, regardless of sex, genotype and adoption, they are characterized by an anxious-like phenotype and high trait-anxiety. Foster mother strain seems to affect the time spent in open arms, as overall mice reared by C57 foster dams spent more time in open spaces than mice reared by FVB foster mothers.

Interestingly, genotype affected locomotor activity in a sex biased way; conditional inactivation of NPY1r decreased locomotor activity in the EPM in both males and females. These results are

partially in accordance with what was previously reported (Bertocchi *et al*., 2011), as our previous work showed that Npy1r^{rfb} male mice reared by HABN dams (FVB) showed higher level of anxiety than controls both in the EPM and in the Open Field tests. In fact, here we observed only the effect of adoption on time spent in open arms, which is the main index of the level of anxiety; however, genotype-dependent decrease in locomotor activity (number of entries in close arms) can be considered an index of higher anxiety as well.

In the NISF test, anxiety-like behaviour is evaluated through the latency to eat a highly palatable food in a novel environment. This test is based on the principle that rodents show a clear preference for lipid-rich foods, such as peanuts (e.g. Gaillard *et al*., 2008) and that the latency to close in and consume a familiar snack in a novel environment is markedly increased in a novel cage with clean bedding (Merali *et al*., 2003). In this test, regardless of the fostering strain, both male and female Npy1r^{2lox} and Npy1r^{rfb} mice showed a regular daily reduction in the latency to consume the peanut, but the latency only increased in Npy1r^{2lox} male mice when the peanut was presented in a clean bedding-filled cage on day 4. Thus suggesting that Npy1r^{rfb} males showed lower anxiety-like behaviour as compared with their controls: these results are in contrast to what we observed in the EPM, where both control and conditional KO mice displayed a behavioural profile indicative of high anxiety. Remarkably, Npy1r^{rfb} male mice reared by FVB dams showed significantly shorter latency to eat the peanut on all trials as compared to their controls, while Npy1r^{rfb} and Npy1r^{2lox} males reared by C57 showed a similar trend. No differences due to genotype or adoption were observed between Npy1r^{2lox} and Npy1r^{rfb} female mice.

These data indicate that, regardless the fostering strain, male and female Npy1r^{2lox} and Npy1r^{rfb} mice are not so sensitive to novelty-induced inhibition of feeding, as they did not show the expected environment-induced avoidance in approaching the highly palatable food. Since these effects are more pronounced in males and especially in Npy1r^{rfb} males reared by mothers that display a high amount of maternal care, this suggests that a reduction of hippocampal Npy1r may affect brain paths involved in the processing of reward-related behavioural responses. Even if these findings seem to be in apparent contrast with the high level of anxiety observed in EPM it is possible to hypothesize that more than a lower level of anxiety, the behaviour displayed by our subjects in the NISF test might be an indicator of an enhanced reward-related response and further studies are needed to test this hypothesis. An alternative explanation could be related to the display of an autistic-like response: in fact the high anxiety displayed in the EPM associated by insensitivity to a novel environment shown in the NISF test might be factors related to autism spectrum disorder (Crawley, 2007). The absence of the inhibition of palatable food intake in novel environment might be interpreted as a resistance to change and response to the changes in routine, characteristics of autism specter disorders (Crawley, 2007).

NPY1r affects aggressive behaviour

Our results showed that conditional Npy1r inactivation induces lower aggression only in males reared by high maternal care dams. In the Resident/Intruder paradigm, $Npy1r^{rfb}$ male mice were characterized by higher attack latency when reared by high maternal care dams and consequently, they spent less time than control mice engaged in agonistic behaviour. These results are in apparent contrast to what was described by Karl et al. (2004) who showed that germinal ablation of the Y1 receptor gene led to a pronounced increase in territorial aggressive behaviour by affecting the serotoninergic system. Moreover, a critical role of Y1R and 5-HT-1A receptor in integrating behavioural pathways to enabling aggression has been demonstrated (Karl *et al*., 2004). In NPY1r KO mouse model, however, reduced Y1R expression restricted in the CA1, CA3 and DG areas of the hippocampus induced the opposite response and decreased territorial aggression. Consistently, $Npy1r^{2lox}$ mice reared by FVB dams and characterized by higher expression of Npy1r in these hippocampal areas, showed higher aggressive behaviour than KO mice. However, when reared by C57 dams the percentage of time spent by $Npy1r^{rfb}$ male mice in agonistic behaviour increases, suggesting that levels of maternal care might play a role in the regulation of male aggressive behavior at adulthood. It is known that aggressive behaviour is influenced by many environmental and experimental variables and further studies are required in order to clarify the networks involved in this behaviour.

Female mice confronted with same sex intruders spent most of the time in social investigation: in particular $Npy1r^{2lox}$ females reared by FVB displayed more social behaviour than their respective KO, while $Npy1r^{2lox}$ females reared by C57 spent less time than their respective conditional KO counterparts in social investigation.

Effects of NPY1r inactivation on metabolism

In this study we also analysed the effects of limbic Npy1r on energy balance and vulnerability to high fat diet induced obesity and metabolic syndrome. Our results show that reduction of limbic Npy1r promotes (high fat) diet-induced obesity and partial glucose intolerance only in male mice and only when reared by dams showing high levels of maternal care. These findings are supported by the higher body weight, abdominal adipose tissue and blood glucose level observed in Npy1r knockout male mice fostered at birth to FVB dams. In female mice reduction of limbic Npy1r or fostering at birth did not affect body weight growth on a standard diet and did not induce susceptibility to diet induced obesity.

Male mice characterized by low limbic Npy1r expression induced by conditional gene inactivation showed reduced body weight growth, starting when gene deletion was achieved. When challenged with exposure to a high fat diet, however, $Npy1r^{rfb}$ male mice, were more susceptible to develop

metabolic disorders but only if they had been reared by foster mother that displayed high maternal care. Although both Npy1r^{rfb} and Npy1r^{2lox} male mice fed HFD showed increased body weight growth than STD fed groups, regardless of fostering strain, Npy1r^{rfb} males showed much greater body weight gain than their controls (Npy1r^{2lox}) when on HFD. This greater increase in body weight started from the second day of HFD and persisted throughout the 21 days of the diet regimen. Eventually, Npy1r^{rfb} mice on HFD also showed impaired glucose clearance in the glucose tolerance test. Even though HFD did not induce fasting hyperglycemia in either genotype, Npy1r^{rfb} mice reared by high maternal care mothers on HFD showed higher blood glucose level as compared to their controls 30 minutes after the glucose injection, indicating impairment in glucose clearance. This effect is not present in Npy1r^{rfb} vs Npy1r^{2lox} fostered at birth to C57.

In male mice, HFD induced a greater amount of abdominal adipose tissue in both genotypes than in the STD groups, regardless of the adoption. Remarkably, Npy1r^{rfb} on HFD showed much greater WAT than Npy1r^{2lox} mice on HFD only when reared by high maternal care dams (FVB), indicating that the increase in body weight showed by Npy1r^{rfb} mice fed HFD might be linked to the high level of white adipose tissue.

Contrary to what reported in males, Npy1r^{rfb} female mice did not show lower body weight growth than controls, and when challenged with HFD, they didn't develop diet-induced obesity and glucose intolerance.

Taken together, these results suggest that Npy1r^{rfb} male mice, when reared by mothers that display an high level of maternal behaviour, are characterized by a phenotype that predisposes them to develop metabolic disorders as demonstrated by their high abdominal adipose tissue and glucose clearance impairment both of which are predictive factors for obesity and metabolic syndrome (Björntorp, 1991; Jensen, 2008; Kissebah and Krakower, 1994). According with the results of a previous study (Bertocchi *et al*. 2011), Npy1r^{rfb} males showed lower body weight growth starting from approximately PND 48, which coincides with the maximal level of Npy1r gene Cre-mediated inactivation, but this effect is remarkable only in males reared by dams that displayed high amount of maternal care. Indeed, *in situ* hybridization performed at the end of metabolic challenge on both groups (STD- and HFD-fed mice reared by CD1 foster dams) showed that Npy1r mRNA expression in Npy1r^{rfb} mice on STD was reduced in CA1, CA3 and DG as compared to Npy1r^{2lox} mice (Paterlini *et al*., in preparation). This profile of expression is similar to what was reported by Bertocchi *et al*. (2011), in male mice reared by FVB foster mothers (HABN). After the HFD regimen, however, we observed an upregulation of Npy1r mRNA expression in CA1 and CA3 in Npy1r^{rfb} males fed with HFD; as a consequence, the differences in the expression of Npy1r mRNA between Npy1r^{rfb} and Npy1r^{2lox} disappeared. This finding may suggest that low limbic Npy1r expression might be responsible for the enhanced predisposition to develop diet-induced obesity and glucose intolerance (metabolic syndrome) in response to high fat diet, which in turn may induce increased expression of

Npy1r mRNA in limbic structures due to compensatory mechanisms. HFD *per se* has been reported to influence synaptic plasticity and gene expression (Lennox *et al.*, 2015; Molteni *et al.*, 2002; Stranahan *et al.*, 2008; Underwood and Thompson, 2016).

While Npy1r^{rfb} males reared by FVB mothers and exposed to HFD showed a fast and enduring increase in body weight as compared to controls, Npy1r^{rfb} females seemed to be "shielded" from the effect of a hyper caloric food. In fact, even when challenged with a HFD, both Npy1r^{rfb} and Npy1r^{2lox} female mice, regardless of the adoption, only showed a slight increase in body weight and a small gain of abdominal adipose tissue as compared to the control groups on STD. Interestingly, these effects are found regardless of genotype and adoption, suggesting that neither lower expression of Npy1r in limbic areas or the quality of maternal care are factors affecting metabolic functions and susceptibility to metabolic disorders in female mice. HFD regimen, furthermore, did not promote glucose intolerance in females; Npy1r^{rfb} and Npy1r^{2lox} females on either STD or HFD showed similar blood glucose level curves after glucose administration. Such a weaker effect of high fat diet on females may be due to different factors and/or environmental variables. First of all estrogens have been shown to act like "protectors" against metabolic effects of high fat diet (Litwak *et al*., 2014; Riant *et al*., 2009). In fact, Riant and coworkers (2009) showed that the activation of the estrogen pathway prevents the occurrence and the course of insulin resistance and glucose intolerance induced by high-fat feeding in wild-type mice. For this reason, estrogens might exert a "shield" action in Npy1r^{rfb} and Npy1r^{2lox} female, despite the strain of foster mother and the amount of maternal behaviour displayed in early postnatal life, that resulted in lower HFD-induced metabolic changes than males. Conditional deletion of Npy1r in forebrain excitatory neurons did not affect body weight growth in female mice, although a reduction of Npy1r mRNA expression was observed in CA1, CA3, DG of Npy1r^{rfb} females as compared to controls (Eva, unpublished data). Furthermore, Npy1r mRNA expression was significantly reduced also in the hypothalamic PVN and medial nucleus of the amygdala (MeA): such a reduction in Npy1r expression in PVN and MeA was not observed in Npy1r^{rfb} males (Paterlini S. *et al*, in preparation; Eva, unpublished data). Therefore, the differential pattern of Npy1r inactivation in females and males might be responsible for the sex differences observed in the role of limbic Npy1r in the regulation of body weight.

Based on present and previous studies, we demonstrated here that limbic Npy1r is differently regulated in male and female mice and its actions are strongly dependent upon sex; we also confirmed that early maternal environment affects NPY1r regulation of behavioral and metabolic responses. In this regards, we demonstrated that these differential effects of early maternal environment seem to affect mostly the male.

Chapter 4

4 Impact of Early maternal care on development, metabolism and behaviour of Wild type mice

4.1 Introduction

The experiments discussed in the previous chapter indicated that the early maternal environment influenced the effects of gene inactivation on behavioural development and metabolic functions of the adult offspring in a complex manner. The present series of experiments aimed to clarify the specific effects of different maternal environments on offspring development in relation to sex.

Indeed, mother infant interactions, together with sexual interactions, are the two most common social behaviours in mammals and occur during mating and mother–infant interactions, respectively. The main difference among these social bonds is the duration: in fact, sexual interactions are generally short lasting, while the mother–infant bond is long-term and persisting at least through weaning. The mother–infant bond is the most common enduring social bond in mammals (Numan, and Young, 2016). Survival of infants relies on attachment bond to the caregiver, a process that requires pups to identify, learn, remember and be in contact with the attachment figure (Landers and Sullivan 2012). In most mammals, the endocrine events associated with the end of pregnancy and parturition together with OT release into the brain that results from the vagino-cervical stimulation associated with the birth process, act on the brain to stimulate the immediate onset of maternal behaviour at parturition, but after maternal behaviour becomes established during the early postpartum hours, its maintenance until weaning becomes independent of hormonal control (Numan *et al*., 2006). Mothers of non-primate mammals typically display a stereotyped set of maternal behaviours. In rodents, females construct a nest before giving birth, and they exhibit feeding and grooming of the new-borns. They also retrieve the infants back into the nest when they stray and protect them from predators and other dangers. Female rats or mice that have not given birth usually withdraw from or avoid pups, and it has been demonstrated that hormonal changes surrounding the birthi process and pup caring alter those responses to pups (Koch and Ehret, 1989). It has been intensively studied how hormonal changes at parturition enhance the neural mechanisms responsible for maternal behaviour, such as the medial preoptic area or dopamine neural systems (Numan and Stolzenberg, 2009). OT has been shown to play a key role in this mechanism. In studies using functional magnetic resonance imaging, lactating rat mothers have been shown to exhibit activation of brain areas involved in olfactory, emotional, and reward processing during nursing of pups that corresponded with activation patterns observed following exogenous OT administration (Febo *et al.,* 2005).

The maintenance of maternal behaviours in rodent dams is dependent on and synchronized with the infant postnatal development. In fact, nest building stops on the day when pups can regulate their body temperature, and milk production ends when the pups decrease their suckling times (Leon *et*

al., 1990). Moreover, if mothers are separated from their pups after delivery and not allowed to interact with them at all, their maternal responsiveness declines over the first postpartum week (Orpen and Fleming, 1987). These findings suggest that maternal response to infants is maintained by pup stimuli, that seem to have an intrinsic effect on the mechanisms responsible for maternal behaviour (Mogi *et al*., 2011).

In rodents maternal behavior is variable in quality and quantity and affects offspring development. How mothers mother depends upon genetic and hormonal background, brain neurochemistry, stimuli from the suckling offspring, nutrition state, environmental conditions and social factors (Fleming et al 1999;2002; Bisceglia et al. 2012; Stoltzberg and Champagne 2016). A robust evicence exists in laboratory rodents demonstrating that maternal behavior can vary under stable conditions and can be altered by experimental manipulations (Caldji *et al*. 1998; Champagne *et al*. 2003a; Francis *et al*. 1999). Variations in maternal cares, in turn, affect subsequent offspring physiological and neurobiological development. The series of experiments by the Meaney lab have clearly demonstrated that in rats, levels of specific maternal behaviors (i.e., arched-back posture and licking-grooming) are associated with the long-term development of physiological and behavioral responses to stress in the adult offspring due to epigenetic mechanisms (Francis & Meaney 1999; Francis *et al*. 1999; Liu *et al*. 1997; Meaney 2001; Champagne and Curley 2009). Thus, mother–offspring interactions provide a direct, flexible link between the dam and the pups that shape adult behaviors including social and emotional behavior (Pitizianti et al, 2016; Macri et al 2004), learning (Pryce *et al*. 2003), metabolic functions (Levin, 2000; Monks et al., 2018;) and the development of psychopathology (Cirulli *et al*. 2003). The high plasticity of maternal behavior may thus represent a means by which ecological information could be passed from mother to offspring (McLeod et al 2007;). In rats, also maternal deprivation (180 min/day) during the first postnatal 10 days have been shown to have serious consequences on the offspring as adults later in life, such as increased anxiety (Caldji *et al*., 2000; Plotsky and Meaney, 1993), reduced resistance against cancer (LaBarba and White, 1971), and enhanced neuroendocrine responses to stressors (Francis and Meaney, 1999; Ladd *et al*., 1996; Ogawa *et al*., 1994), associated with epigenetic changes in the central nervous system (Holmes *et al*., 2005; Weaver *et al.,* 2004).

Moreover, several psychiatric and neurobehavioral disorders have been associated with negative early life experiences involving the attachment figure (Anda *et al.,* 2006; Cicchetti and Toth, 1995; Cirulli *et al*., 2009; D'Amato *et al*., 2011; Gershon *et al*., 2013; O'Connor and Rutter, 2000; Shields *et al*.,1994; Ventura *et al*., 2013). All the data reported suggest that perturbations in early maternal environment program the infant's emotional and cognitive resources to adapt to later-life challenges and increase the risk of developing psychopathology during future life. The changes in quality and quantity of mother–infant interactions, were found to influence adult behaviour and brain morphology.

Although these many reports clearly demonstrate the impact of maternal care on shaping the individual's phenotype, its importance is often overlooked in studies using knockout or inbred strains of mice, the most common models to study genetic influences on the development of behavioural phenotypes. For example, in studies using knockout mice, it is not unusual that cross-fostering to a wild-type mother is performed because of low maternal care of the knockout strain. But this procedure can induce unexpected behavioural changes in the adult knockout mice and interfere with conclusions on the role of the targeted gene. Thus, differences found between knockout and wild-type mice that are usually assigned to the gene of interest, might instead reflect differential maternal care received. (Van der Veen, R., *et al*., 2008). Cross-fostering is also widely used with inbred strains of mice to determine the relative contributions of genetic and early environmental factors in shaping phenotypes. In the previous experiment we have demonstrated that maternal behaviour received by foster mothers affects the impact of the NPY1r gene inactivation on behaviour and metabolism in conditional KO mice. Here we aim to assess the specific impact that variable levels of maternal cares exerts on wild type C57BL6J-derived mice. For this reason, we performed an analysis of all the data collected on wild type mice in several experimental cohorts in our laboratory.

Therefore we carried out an extensive analysis of maternal behaviour effects on offspring development focussing on wild type control mice of NPY1r line (as described in the previous experiment - Chapter 3). We combined all the data relative to different experiment replicates and analysed all data derived from $Npy1r^{2lox}$ mice (background strain: C57BL6J – C57) that are considered controls of the $Npy1r^{rfb}$ mice. The protocol of cross fostering and maternal observation provides for random fostering of litters at birth to a lactating female belonging to one of four mouse laboratory strains (FVB, CD1, C57, Balb) that are known for displaying different amount of maternal care. We examined the effects of early maternal environment on wild-type mice to determine at what extent different levels of maternal care during the first postnatal days might affect anxiety and aggressive behaviour in male and female mice. Second, we examined the impact of maternal behaviour on the regulation of offspring metabolism and more specifically, the susceptibility to metabolic disorders in response to hypercaloric diet.

4.2 Material and methods

Animals and housing

All the animals used in this experiment were born and reared in the Laboratory of Behavioural Biology at the University of Parma. All experiments were conducted in accordance with the European Community Council Directive of 24 November 1986- 86/609/EEC and 6106/10/EU and approved by the University of Parma Ethical Committee for animal research and by the Italian Ministry of Health. (Aut. N 1143/2016 – PR (Protocol 2712C.2 of 13/07/2016).

As foster mothers we paired female mice of four different strains (CD1 swiss, FVB/J , C57BL/6J, and Balb/c, hereafter CD1, FVB, C57, Balb) with same strain males for 7-10 days. The pregnant females were initially housed in groups of two in a temperature – (22 ± 1 °C) and humidity-controlled room on a 12-hours light/dark cycle (11:00 AM – 11:00 PM) and few days before birth mice were taken and placed in separated cages until delivery. Simultaneously two different GM mouse lines were paired: 1) $Npy1r^{2lox}/Tg^{\alpha CamKII\text{-}tTA}$ expressing tTA in a cell type-specific manner under the control of the α-Ca^{2+}/calmodulin kinase II (α−CamKII) promoter (αCaMKII-tTA) and 2) $Npy1r^{2lox}/Tg^{LC1}$ encoding the tTA-responsive Cre transgene LC1 (background strain C57) were mated in the way earlier described. Within 12 hours after birth (PND0 0) all pups were weighed, sexed and each litter fostered randomly to a lactating female of CD1, FVB, C57 or Balb strain. Receiving foster dams were mated with males of their same strain one or two days before the breeding of biological mothers so that the lactating phase was approximately the same of biological mothers. At PND27 pups were weaned and genotyped and housed in groups of siblings of the same sex. Only wild-type mice were considered in this study for behavioural tests and metabolic challenge

Maternal Behaviour observations

Maternal behaviour was assessed by observing foster dams in their home cages, from PND 1 to 7. Mice are most active during the dark phase of light/dark cycle (Latham and Mason, 2004). Therefore the observation period (120 min) started at 09 AM hr and was conducted entirely during the dark phase under 25-W red lights, until 11 AM. Each lactating female of any strain (CD1, FVB, C57 or Balb strain) was observed once every four minutes, for a total amount of 30 observations in 2 hours. During each 4min observation, the experimenter recorded which behaviour was displayed at the moment of the observation (instantaneous sampling).

Dams behaviours were identified according to behavioural categories, as described in Palanza *et al*. (2002), namely: *Arched back nursing*, the lactating female was giving to all the pups nourishment

and heath simultaneously with her body arched over them; In nest: the female was anywhere inside the nest, regardless of the behaviour being exhibited at the moment of observation; *Nursing*, the dam was allowing the offspring to suckle but not in AB posture (it is not implying that the whole litter was nursing). *Licking*, the female was licking or grooming pups; *Nest building*, the lactating female was occupied in some activity regarding the nest building, either inside or outside the nest itself; *Eating/drinking*, the dam was crunching some food pellet or drinking from the water bottle. *Grooming*; the lactating female was cleaning herself; *Active*: the female was moving in the cage; *Resting*: the female was lying outside the nest, not displaying any other behaviour, without pups suckling or attached to the nipples, motionless. *Out of nest*; the dam was anywhere in the cage but the nest, regardless of the behaviour being exhibited at the moment of the observation.

Body weight growth

Offspring body weight was monitored from birth to adulthood by a digital balance accurate to 0.01g (Sartorius, Germany) with the animal weighing functions (animal's weight is automatically calculated as the average of a defined number of individual weighing operations). Pups were weighed immediately after birth (PND 0), at PND 10 and at weaning (PND 27). From weaning to adulthood (PND 90), body weight was measured once a week from 10 AM to 11 AM in order to monitor body weight growth.

Behavioural tests

Behavioural tests were carried out on adult male and female mice fostered to CD1, FVB, C57 and Balb dams between PND 90 and PND 110 from 3 PM to 7 PM. At adulthood (PND 90) animals were isolated in Plexiglass cages (45cm*25cm*20cm) and, after a habituation period of 1 week, they were tested for behaviour. All mice were transported to the testing room 1 hour prior the behavioural test for habituation.

1- Emotional behaviour

Elevated Plus Maze (EPM) test: male and female mice fostered to CD1, FVB, C57 and Balb dams were tested in the EPM in a half-lit room in order to simulate the dark phase that is known to be the more active phase of rodents. The method used in this study was based on Gioiosa et al. (Gioiosa et al., 2007). Briefly, the EPM apparatus consists of a plus-cross shaped (30*5cm) originating from a central platform (5*5cm) elevated 40 cm above the floor. Two arms, opposing each other, are enclosed by Plexiglas walls (20 cm) and the leftover arms are open. Each animal was placed on the centre platform facing one of the open arms and was allowed to explore the maze for 5 minutes. The frequency of entries into, and time spent in, the open arms and closed arms of the maze were recorded with a video camera placed 2 m above the apparatus. To reduce any persistent olfactory cues, the maze was cleaned with clean damp cloth and a solution of 2% ethylic alcohol between successive trials. Behaviours were analysed and scored by an observer, previously trained, using specific software (The Observer, Noldus, NL) to record the latency, duration and frequency of transitions between different arms.

Novelty Induced Suppression of Feeding (NISF) **test** measures the latency of an animal to approach and eat a familiar food in a novel environment to assess the animal anxiety-like state (Dadomo *et al*., 2011). Male and female mice reared by CD1, FVB, C57 and Balb foster mothers underwent the NISF test. For four consecutive days, individual experimental animals were given at 9AM a Petri dish containing a little piece of peanut placed in a corner of their home cage. On the fourth day, the peanut was presented in a novel cage with fresh bedding (Merali *et al*., 2003). The latency the mice showed before starting to consume the peanut was recorded on all days with a cut-off time of 600s. In case of maximum latency, the peanut was left in the cage of the animal. A reduced latency to begin consumption of the palatable snack in the novel cage indicate a reduction of the anxiety, using this test.

2- Aggressive and social behaviour

***Resident/Intruder test*:** both male and female mice reared by CD1, FVB, C57 and Balb foster dams were wxamined for aggressive and social behaviour.Mice were previously housed in a single cage for a week. Resident mice were confronted in the Resident/Intruder paradigm with standard same-sex intruder adult male and female Balb mice, which are known to show low level of aggression.

Experimental animals were observed and video-recorded for 2 min in their home cage, then an intruder of the same sex was placed in the resident's cage and the encounter was videotaped for 10 min with a camera placed 2 meters above the cages. Latency to the first attack by the resident against the intruder was recorded and the test was terminated if clear signs of distress in the intruder transpired. Behaviour was scored by the software The Observer (Noldus, NL). Specific behaviours analysed were: numbers and duration of attack, tail rattling (rapid vibration of the tail, typically occurs shortly before and during an attack flurry), circling and chasing (the resident chases rapidly the intruder shortly before an attack occurrs), upright posture (following being defeated, mice rear up on their hindlegs and assume a defensive upright posture), immobility with contact (resident mice are in a freezing posture with contact to the intruder),startle response (defensive response to threatening stimuli), social exploration (resident explores the intruder, it includes ano-genital exploration), immobility without contact (resident mice are in freezing posture within the cage), rearing (mice rear up on their hindlegs), digging (mice dig bedding), environment exploration and walking. In order to promote a clear representation of the data, specific behavioural elements were grouped into the following broad behavioural categories: offensive behaviour (attack, tail rattling, circling and chasing) and defensive behaviour (upright posture, immobility with contact and startle response).

Metabolic challenge

Body Weight and Food Intake. On PND 120 male mice were weighed and individually housed in Plexiglas cages (45cm*25cm*20cm) with wood shaving bedding changed weekly. After the first week on a standard diet, to obtain baseline measurement, mice were randomly assigned to two experimental groups: one fed standard diet (STD - 6.55% kcal from fat and 3.9 kcal/g; 4RF21, Mucedola, Italy) and the other fed a custom pelleted high fat diet (HFD - 45% kcal from fat and 5.2 kcal/g manufactured by Mucedola) obtained by a modification of the STD formula. Thus we obtained eight experimental categories: C57 fostered mice fed with STD (males n=13; females n=12) or HFD (males n=22, females n=14), FVB fostered mice fed with STD (males n=19, females n=8) or HFD (males n=12, females n=18), Balb fostered mice fed with STD (males n=1, females n=1) or HFD (males n=5, females n=5) and CD1 fostered animals either with STD (males n=11, females n=9) or HFD (males n=25, females n=10). Mice reared by Balb foster dams were excluded from statistical analysis run on standard diet data as the number of animals was not sufficient. About HFD, as the number of mice reared by Balb foster mother were lower than other groups we decided to perform two different analysis, one including and the other one omitting values of Balb's offspring. Throughout the study, food and water were available ad libitum to all experimental animals. Body weight and food intake were determined from 10 AM to 11 AM every two days for 3 weeks. Pre-weighed food was carefully placed in the U-shaped metallic feed hopper of each cage and then reweighed on a per-cage basis. Food crumbles with a diameter greater than 1 mm were recovered from the cage and weighed as well. Food intake was quantified by calculating the difference between pre- and post-weighed food for the entire duration of the experiment and averaged over baseline and weekly. Food intake was transformed to kcal and expressed as fold changes vs baseline.

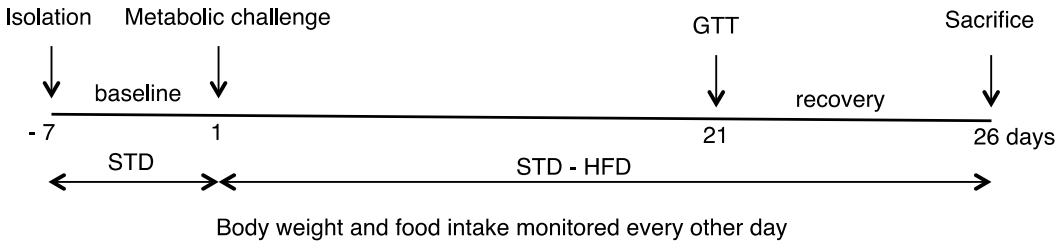

Fig.1. Experimental timeline

Glucose Tolerance test (GTT). On the 21st day of the diet, Glucose Tolerance Test was performed following overnight fasting (12h). Blood glucose levels obtained from tail bleeding were monitored at 0, 30, 60 and 120 min after intra peritoneal. injection of d-glucose (Carlo Erba Reagent S.r.L., Milan, Italy) at 1g/Kg. Blood concentrations were all determined through an Accucheck Aviva (Roche Diagnostics, USA) blood glucose meter during the light phase between 9:00 AM and 11:00 AM. Mice were then returned to their diet regimen for 5 days to allow body weight recovery.

Tissue collection and plasma analysis. On day 26 of diet exposure, following a 12 hrs fasting, mice were euthanized by decapitation after a brief CO2 exposure. Trunk blood was collected in heparinized tubes (Sarsted, Germany) and centrifuged at 4000 RPM, at 4°C for 10 min, then placed at -20°C until analysis. Abdominal white adipose tissue (perigonadal, visceral, retroperitoneal fat pads) was collected and weighed, then frozen in liquid nitrogen for later analysis. Brains were removed, immediately frozen in liquid nitrogen, then stored at -80°C until analysis.

Statistical analysis

A 3-way ANOVA (fostering, sex and time) for repeated measures was used to analyse body weight, food intake and glucose tolerance test data. A 2-way ANOVA (fostering and sex) was run to analyse abdominal fat pads, emotional behaviour. A one-way ANOVA (fostering) was performed on aggressive and social behaviour data. A Tukey's test for post hoc comparisons followed the ANOVAs. Data were analysed with Statistica 10.0 software (Stat-Soft, Tulsa, OK, USA). Significance was determined when $p<0.05$. Data are presented as mean ± standard error of the mean. As the number of Balb animals resulted insufficient we decided to not include animals reared from these foster dams in statistical analysis of metabolic challenge. In behavioural tests the number of mice fostered to Balb mothers resulted sufficient, but lower than N in other experimental groups: due to the asymmetry of data we could not completely evaluatedata provided by animals reared by Balb foster strain.

PCA Analysis: Principal component analysis (PCA) is a useful way to simplify the complexity of a remarkable data-set. The primary purpose of this technique is to reduce a high number of variables (representing all the properties of the studied event) to a limited number of hidden variables that are informative too. The variables of a data-set might present different relevance and it appears to be quite useful to remove the excess of overlapping data. In simple terms PCA is a linear transformation of the variables used in different fields, that help the observer to identify data models based on correlation among different features. In fact, studying a reduced data-set often simplify the recognition of tendencies, models and odd values. To realize a Principal component analysis means

to project the original variables in a new Cartesian system, in which they are organized depending on decreasing value of variance. The variable with the highest variance is projected on the first axis, the second one is projected on the second axis, and so on. PCA technique establishes 5 different steps to obtain the linear transformation: first of all data, represented in a matrix, need to be standardized. The second step impose to calculate the covariance matrix, and at a later time to compute the eigenvectors (directions of the new Cartesian plane) and eigenvalues (numeric values that describe the variance among data). To reduce the dimensionality of the function it is then necessary to project the function itself on a reduced space in which eigenvectors act like new axes and eigenvalues are selected based on their magnitude (the lower values produce less information about data distribution. Eventually there is a calculation of principal components and the obtained values are projected into the new reduced Cartesian system and the reduction of the complexity is reached with the analysis of the principal new variables, depending on the variance.

Principal Component Analysis (PCA Kayser's eigen-value-greater-than-one rule; Keyser-Meyer-Olkin = .851; Bartlett's test: $\times 2 = 3484.4$, $p<.01$) was performed on frequency of behaviours recorded in EPM test (walking, risk assessment, head dipping, sniffing, rearing, self-grooming, stretching, total frequency and duration in close, centre or open arms). The main goal of the work was to gather all the 75 variables (divided by frequency and duration) obtained from EPM analysis with "The Observer" software, into few representative factors of a specific behaviour.

We eventually obtained 8 different factors:

1- Factor 1, named "locomotive activity", included *walking* (total frequency, closed frequency, total duration), *total frequency closed*, *risk assessment* (closed and total frequency), *sniffing* (closed and total frequency), *centre total frequency*.
2- Factor 2, named "active risk assessment", included *head dipping* (centre frequency and duration), *risk assessment* (centre frequency and duration), *sniffing* (centre frequency), *centre total duration*.
3- Factor 3, named "anxious-like behaviours", included *head dipping* (open frequency and duration, total duration and total frequency) and *open total duration*.
4- Factor 4 named "rearing", included *rearing* (total duration and frequency, closed duration and frequency).
5- Factor 5, named "self-grooming", included total duration and frequency of self-grooming activity, and closed duration and frequency of the same behaviour.
6- Factor 6, named "stretching", included *stretching* (total frequency and duration, centre frequency and duration)
7- Factor 7, named "passive risk assessment" included risk assessment (total duration and closed duration) and sniffing (total duration).

8- Factor 8, named “open frequency” included only the entries frequency in the open arms of the maze.

Individual factor scores of each principal factor were subsequently used as independent variables in a two-way analysis of variance (ANOVA) considering foster mother and sex and referring to Npy1r control animals selected among the cohorts. We decided to show graphs of each single factor's frequency in the results section, even if they don’t provide any additional information on the behaviour of animals. When appropriate, post hoc analysis (Tukey's range test) was performed separately in males and females for comparison of the treatment groups.

Factors	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
total_walking_frequency	**0,919**							
walking_close_frequency	**0,902**							
total_walking_duration	**0,894**							
walking_closed_duration	**0,848**							
Close total_number	**0,84**							
ra_close_frequency	**0,818**							
total sniffing frequency	**0,75**							
sniffing_close frequency	**0,742**	-0,32		0,33				
centre_total_number	**0,678**	0,323						-0,49
ra_total_frequency	0,64	**0,625**						
dip_centre_frequency		**0,926**						
ra_centre_frequency		**0,89**						
dip_centre_duration		**0,884**						
ra_centre_duration		**0,827**						
sniffing_centre_frequency		**0,821**						
Centre	-0,36	**0,807**						
sniffing_close_duration		-0,63	-0,43					
Close	0,421	-0,6	-0,48					
sniffing_centre_duration	-0,46	0,517					-0,36	
dip_open_duration			**0,962**					
dip_open_frequency			**0,956**					
Open			**0,898**					
dip_total_duration		0,624	**0,7**					
dip_total_frequency		0,675	**0,681**					
rearing_total_duration				**0,94**				
rearing_close_duration				**0,94**				
rearing_total_frequency	0,369			**0,9**				
rearing_close_frequency	0,368			**0,89**				
sg_totale_duration					**0,9**			
sg_close_duration					**0,88**			
sg_total_frequency					**0,87**			
sg_close_frequency					**0,86**			
sap_total_frequency						**0,86**		
sap_total_duration			0,324			**0,82**		
sap_close_frequency		0,379				**0,82**		
sap_centre_duration		0,326				**0,81**		
ra_total_duration	0,396		-0,36				**0,785**	
ra_close_duration	0,407	-0,3	-0,38				**0,688**	
sniffing_total_duration	-0,55	-0,36	-0,31				**-0,59**	
open_total_number								**0,801**

Fig.2. Results of PCA applied to behaviours of Npy 1r control mice, correlation coefficients of the representative factors .Total variance explained by these factors: 87.969 %.

4.3 Results

Analysis of spontaneous maternal behaviour of foster dams

Spontaneous maternal behaviour of foster dams was observed throughout the seven days of postnatal life. The direct observation of mother's behaviour with the pups allowed us to calculate the mean percentage of time that CD1 (n=33), FVB (n=37), C57 (n=20) and Balb (n=4) foster dams spent in maternal behaviour. A one-way ANOVA (foster mother strain) and a Tuckey post-hoc test were performed on weekly percentage of time spent in several behaviours described in Material and methods. In **Fig.3** we calculated and visualized the mean percentage of time spent in maternal behaviour during postnatal days 1-7. Statistical analysis showed that FVB and CD1 foster dams spent most of their time engaged in pup-related behaviours by crouching over the pups in the active form of nursing known as arched-back (AB) (main effect of foster mother: $F_{(3,90)}= 14.0552$; $p<.001$), compared to the lying posture of nursing pups (effect of fostering: $F_{(3,90)} =4.7261$; $p<.05$) (**Fig.4**). The greater time spent in ABN rather than in normal nursing suggests that CD1 foster dams, and FVB dams, are characterized by higher level of maternal care than dams of C57 and Balb strain. These data are confirmed by the total time spent out of nest, that showed a main effect of foster mother strain ($F_{(3,90)} =13.4873$; $p<.001$). In fact, lactating females of C57 and Balb strains spent most time in other activities completed out of their nest (eating, grooming and active behaviours).

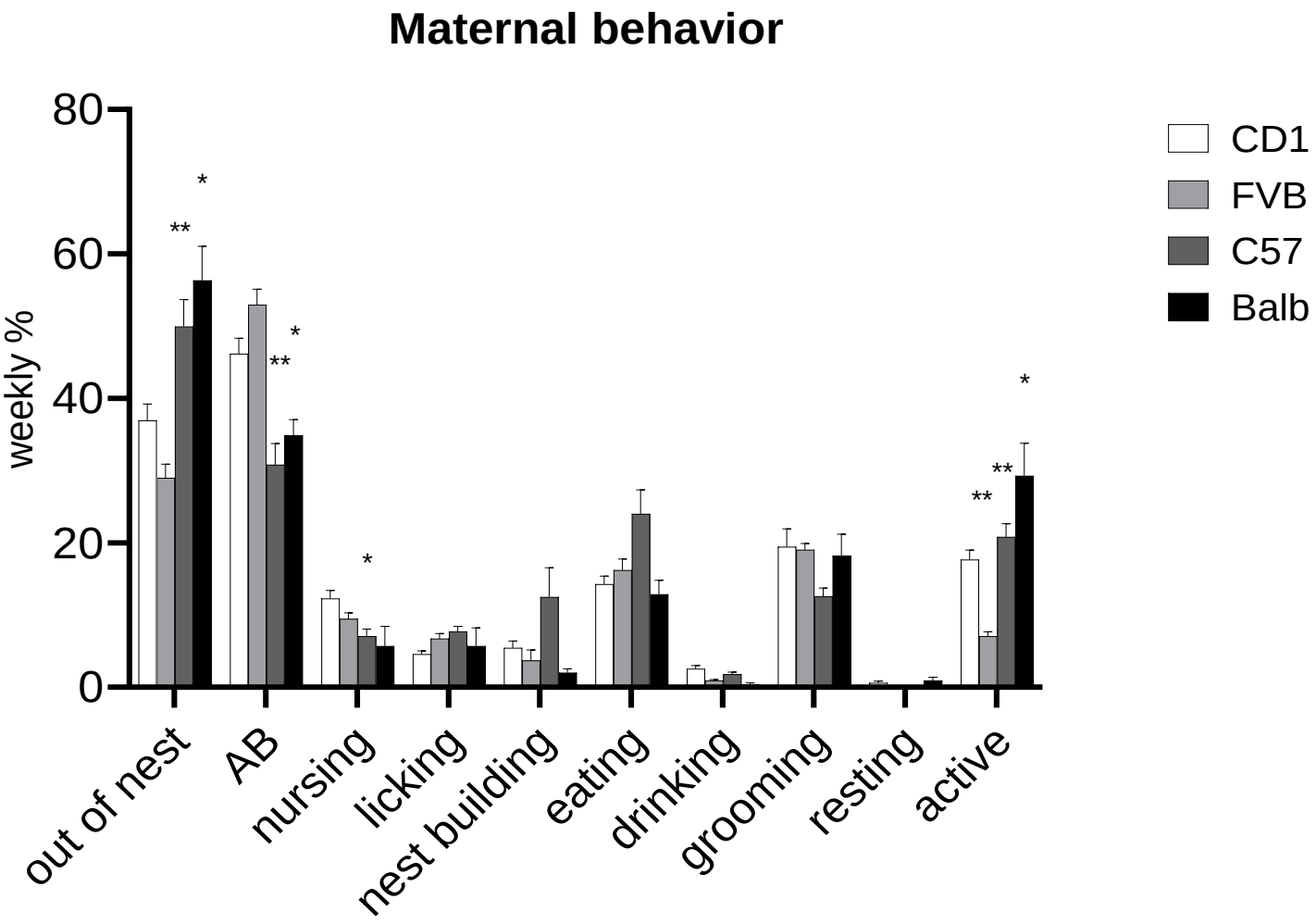

Fig.3. Spontaneous maternal behaviour of CD1 (n=33), FVB (n=37), C57 (n=20) and Balb (n=4) foster dams during postnatal days 1-7, expressed as weekly percentage of time spent in several behaviours (time spent out of the nest, arched back behaviour, nursing, licking pups, nest building, eating and drinking, grooming, resting and active behaviour). As expected C57 and Balb foster dams displayed less behaviours directed to pups'care as compared with FVB and CD1 foster dams. Data are presented as mean ± SEM, * indicates a significant difference with p<.05.** indicates a significant differences with p<.001.

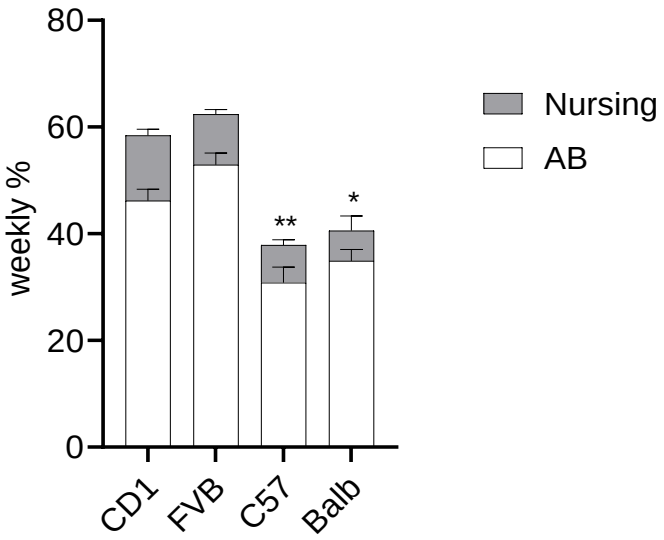

Fig.4. Total nursing (arched back + nursing behaviour) of CD1 (n=33), FVB (n=37), C57 (n=20) and Balb (n=4) foster dams displayed during post-natal day PND1-7. Data are presented as mean ± SEM, * indicates a significant difference with p<.05.** indicates a significant differences with p<.001

Body weight of the offspring

Pups were weighed at birth before cross fostering, then at Post Natal Day - PND 10 and 27, at weaning. No significant differences were found at birth among offspring of the 4 foster groups. On postnatal day 10 all offspring showed an increase in body weight that was more pronounced for litters reared by CD1 and Balb foster mothers.. Statistical analysis showed a significant foster mother effect ($F_{(3,469)}$ =7.083; p=0.00016), and a significant interaction between days and fostering ($F_{(3,469)}$ =149.924; p<.001), with CD1, and partially Balb mice of both sexes presenting a higher increase in body weight from birth to PND10 (**Fig.5**).

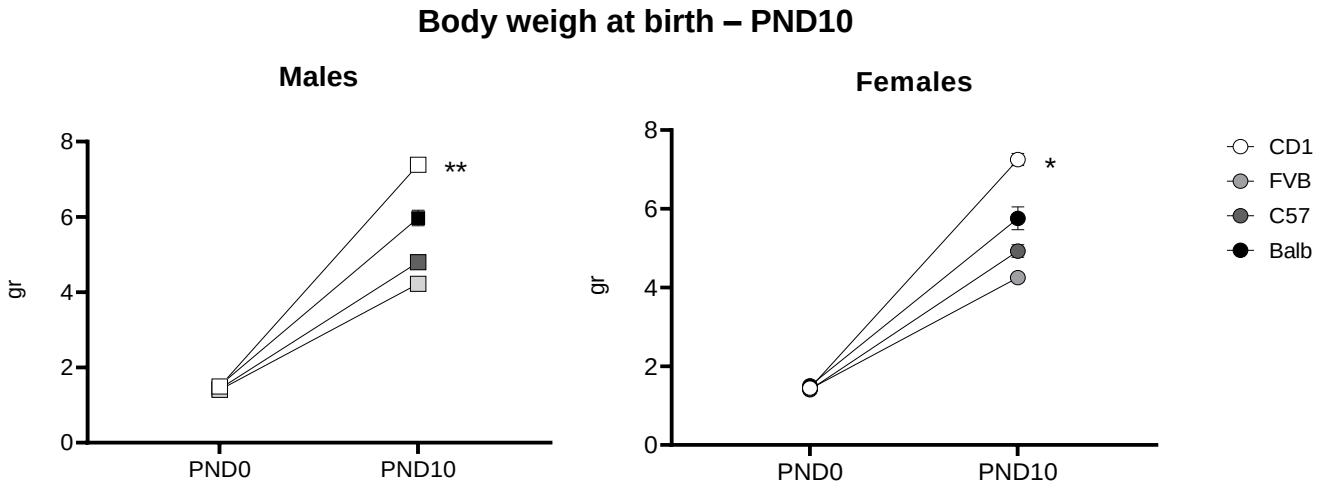

Fig. 5. Body weight growth from PND 0 to PND 10 of males and females reared by CD1(n=78 m, n=93 f), FVB (n=54 m, n=56 f), C57 (n=81 m, n=82 f) and Balb (n=20 m, n=14 f) foster mothers. Animals reared by CD1 and marginally by Balb foster dams showed a higher body mass at PND10. No significant differences were found at birth. Data are presented as mean ± SEM, * indicates a significant difference with p<.05.** indicates a significant differences with p<.001

Regardless sex, the same effect was observed at weaning, with CD1 and Balb pups showing increased body weght compared to FVB and C57, as showed in **fig.6**. (n=81,n=82 males and females, respectively, reared by C57 foster dams; n=20, n=14 males and females, respectively, reared by Balb foster dams; n=54, n=56 males and females, respectively, reared by FVB foster dams; n=78, n=93 males and females, respectively, reared by CD1 foster dams). Statistical analysis at PND27 showed a significant foster mother effect ($F_{(3,260)}$ = 52.250; p< .001) as in both males and females, mice reared by CD1 foster dams had higher body weight compared to other fostering categories, especially those reared by FVB and C57 foster dams.

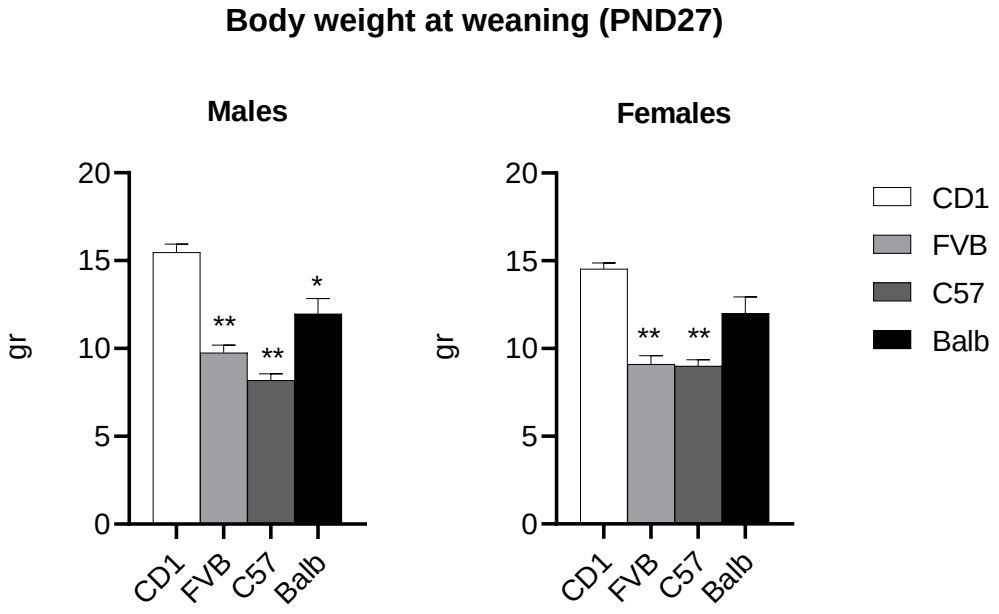

Fig. 6. Body weight of pups reared by CD1 (n=78 m, n=93 f), FVB (n=54 m, n=56 f), C57 (n=81 m, n=82 f) and Balb (n=20 m, n=14 f) foster dams at weaning (PND27). Effect of fostering: pups reared by CD1 foster mothers showed an higher weight than other groups. Data are presented as mean ± SEM, * indicates a significant difference with p<.05.** indicates a significant differences with p<.001

Body weight growth until PND90

After weaning (PND27), animals were weighed once a week until PND 90. A three-way ANOVA (days, sex, fostering) was run on body weight data. Results indicated a significant interaction among days, sex and fostering, as male mice reared by CD1 foster dams had higher body weight ($F_{(27,2385)}$ =2.20; p<.001) compare with the other fostering groups (**Fig.7**). A significant difference between mice reared by the four strains of foster mothers persisted until PND 41 but then disappeared (days x fostering interaction: $F_{(27,2385)}$ =42.20; p<.001) with higher weight for both male and female offspring reared by Balb and CD1 foster dams. This difference was lost after PND 48, and eventually no differences were observed at PND 90. Moreover there was a significant effect of sex ($F_{(1,265)}$ =75.91; p<.001) as males weighed significantly more than females, and of foster strain ($F_{(3,265)}$ =17.12; p<.001) as offspring of CD1 foster dams weighed more than mice reared by the other strains (total mice reared by foster dams: CD1 n=22 m, n=19 f; FVB n=58 m, n=51 f; C57 n=53 m, n=58 f; Balb n=6 m, n=6 f).

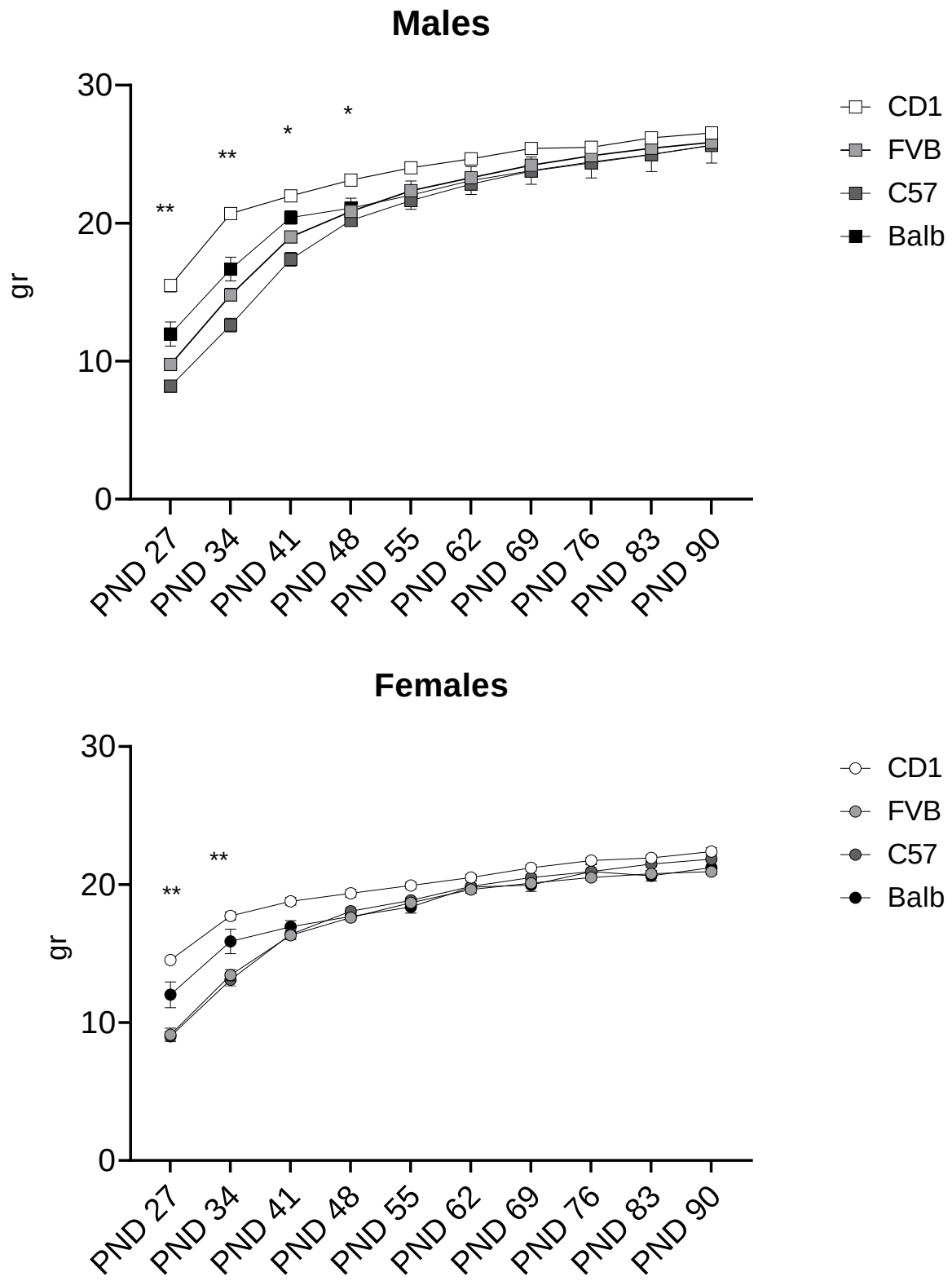

Fig. 7. Body weight growth of male and female mice reared by CD1(n=22 m, n=19 f), FVB (n=58 m, n=51 f), C57 (n=53 m, n=58 f) and Balb (n=6 m, n=6 f) foster dams. Until PND 41, both males and females reared by Balb and CD1 foster dams showed an higher body weight. Starting from PND 48 differences registered in body weight vanished and all the animals reached around the same weight. Values are presented as mean ± SEM, * indicates a significant difference with p<.05.** indicates a significant differences with p<.001

Emotional behaviour

Elevated Plus Maze

Data were analysed using parametric analysis of variance (ANOVA) with strain of foster mother (CD1-FVB-C57-Balb), and sex (females vs males) as between-subject factors, and total duration, total frequency and zones of the EPM as within-subjects repeated measures. All the animals were analysed for total frequency of entries and total time spent in the three zones of EPM (closed arms, open arms, centre), locomotor activity (total duration of walking), total time spent in rearing activity. Total number of mice analysed in Elevated Plus maze paradigm was n=20 males, n=16 females reared by CD1 dams; n=26 males, n=32 females reared by FVB dams, n=19 male, n=24 females reared by C57 dams and n=6 males, n=6 females reared by Balb foster dams.

Overall, all subjects spent high amount time in the closed arms of the apparatus as shown in **fig.8 B–D**, and this might be an index of an anxiety-like phenotype. male mice reared by Balb foster dams and female mice reared by CD1 foster dams spent more time in the closed arms than subjects of the other experimental groups (interaction among sex and fostering: $F_{(3,141)}=3.8078$; $p<0.05$). Both males and females reared by C57 foster mothers spent less time than the other groups in the closed arms of the maze (main effect of fostering: $F_{(3,141)}=6.5435$; $p<.001$). Moreover, no difference was registered in total time spent in the centre of the maze for males (**Fig.8A**), whereas females reared by CD1 spent less time in the centre than the other groups (sex and fostering interaction: $F_{(3,141)}=2.5859$; $p=.055$) as shown in **fig.8C**. Interestingly, female mice reared by C57 foster dams spent more time of the other groups in the open arms of the maze (fostering effect: $F_{(3,141)}=2.84049$; $p<.05$) while males reared by Balb foster dams had a lower frequency and a lower duration in open arms than other groups. A similar trend was found for the numbers of total entries in open arms, thus suggesting a lower level of anxiety in offspring reared by fostered C57 mothers and an higher level of anxiety in Balb's offspring (**Fig.9 A-B**).

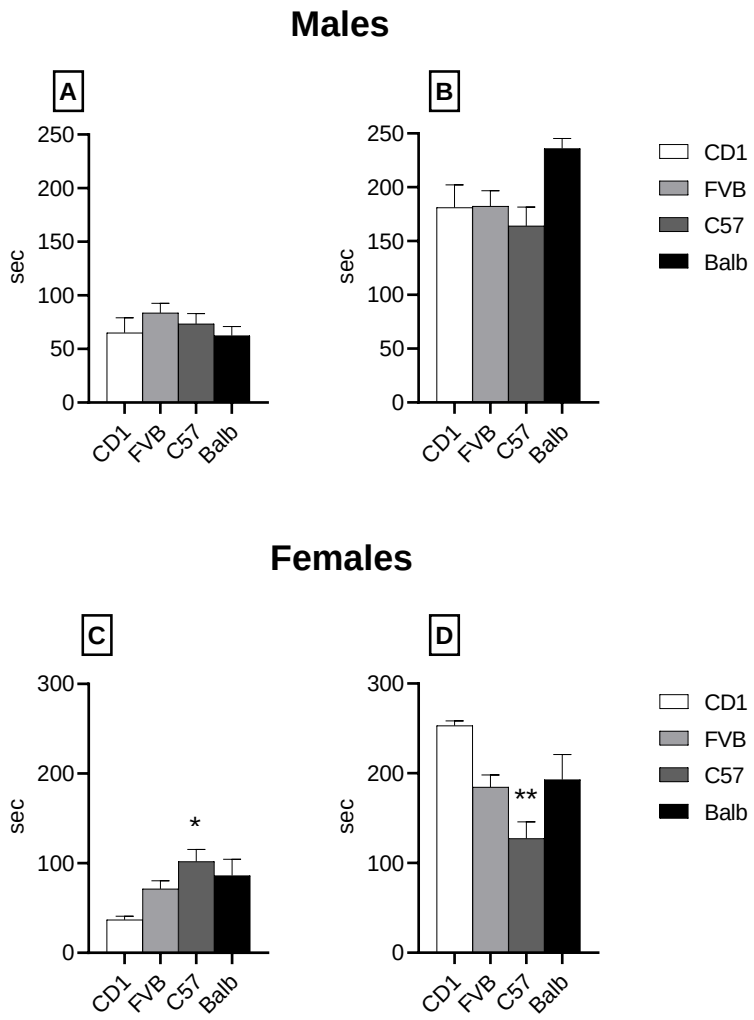

Fig.8. Elevated Plus Maze; **A)** Time spent in centre of the maze by males and **(C)** females reared by CD1 (n=20 m, n=16 f), FVB (n=26 m, n=32 f), C57 (n=19 m, n=24 f) and Balb (n=6 m, n=6 f) foster dams. No significant differences, but females reared by CD1 foster dams spent less time in this zone. **B)** Total time spent in closed arms of EPM by males and **(D)** females reared by CD1, FVB, C57 and Balb foster mothers. All mice, regardless sex and fostering spent most of the time in close arms of the maze. Values are presented as mean ± SEM, * indicates a significant difference with p<.05.** indicates a significant differences with p<.001

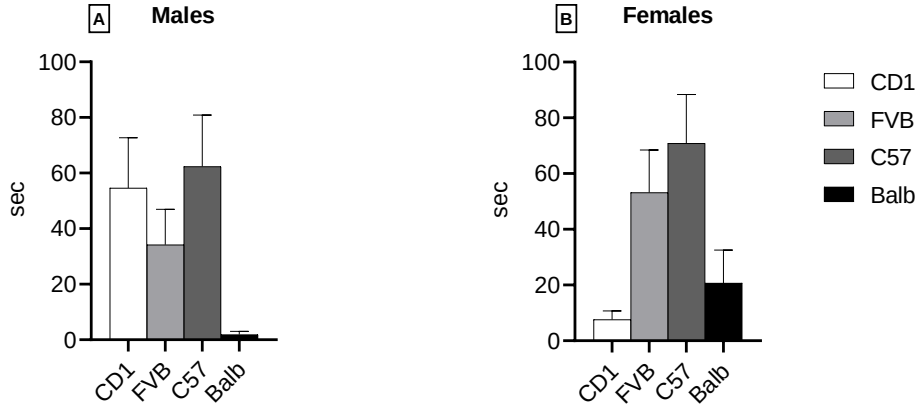

Fig.9. Elevated Plus Maze; **A)** Time spent in open arms of the maze by males and **(B)** females reared by CD1 n=20 m, n=16 f), FVB (n=26 m, n=32 f), C57 (n=19 m, n=24 f) and Balb (n=6 m, n=6 f) foster dams. Especially females reared by C57 spent more time in the open arm as compared to mice reared by the other strains. Values are presented as mean ± SEM

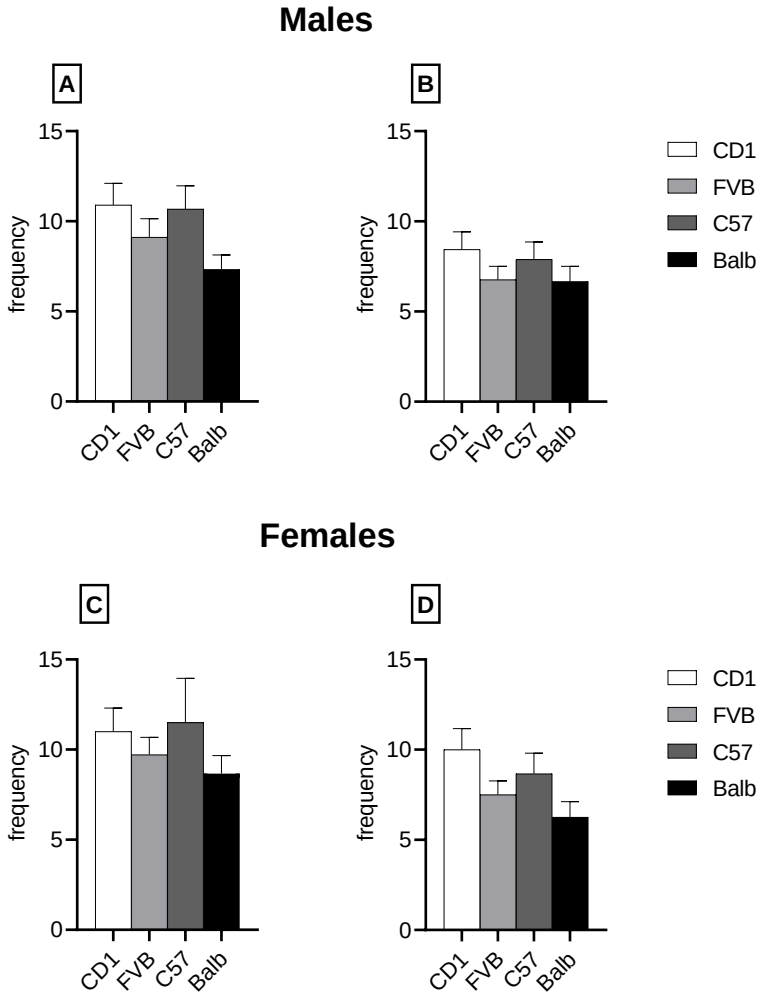

Fig.10. EPM, Total frequencies of entries in the centre space of the maze by males **(A)** and **(C)** females reared by CD1 CD1 n=20 m, n=16 f), FVB (n=26 m, n=32 f), C57 (n=19 m, n=24 f) and Balb (n=6 m, n=6 f) foster dams foster dams. Total frequencies of entries in closed arms of the maze by males **(B)** and females **(D)** reared by CD1, FVB, C57 and Balb foster mothers. No effect of sex or fostering. Data are presented as mean ± SEM

Despite these results in total duration, statistical analysis run on total number of entries in all the space of the EPM did not show any significant effect of sex or fostering (**Fig.10-11**).

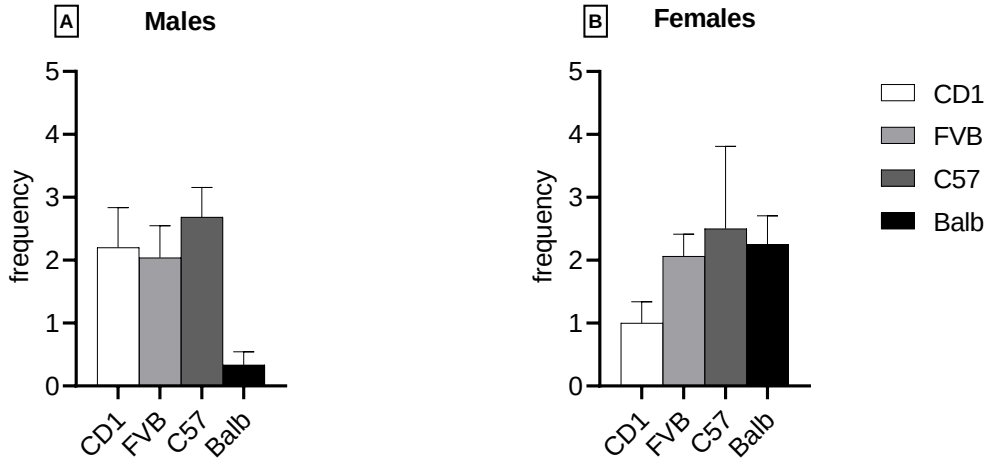

Fig.11. EPM, Total frequencies of entries in the open arms of the maze by males **(A)** and **(B)** females reared by CD1 CD1 n=20 m, n=16 f), FVB (n=26 m, n=32 f), C57 (n=19 m, n=24 f) and Balb (n=6 m, n=6 f) foster dams foster dams. No effect of sex or fostering. Data are presented as mean ± SEM

Locomotor activity

About locomotor activity, comprising total walking in centre, closed and open arms of the maze, we observed a main interaction of fostering and sex ($F_{(3,135)}$ =9.129; p<.005); in fact females reared by CD1 foster dams were more active than those reared by other strains. In particular, females reared by C57 foster mothers showed significant lower locomotor activity than females reared by CD1 lactating mothers. In males we did not observe any significant effect of fostering (**Fig.12**).

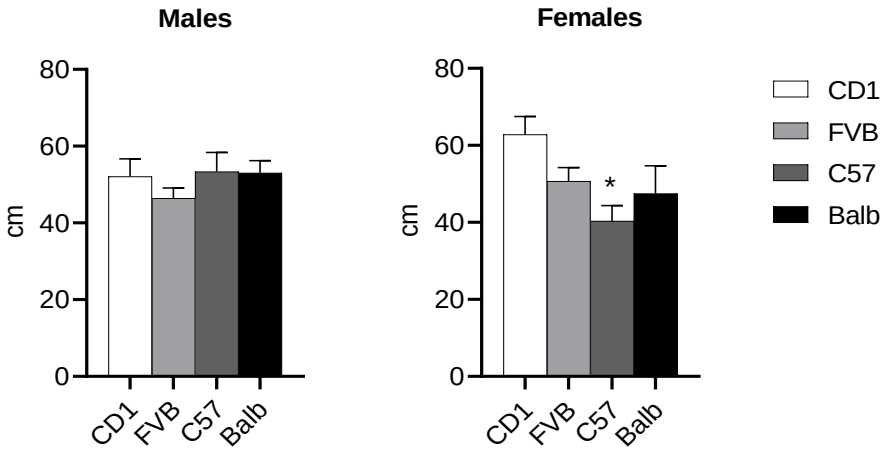

Fig.12. Total distance walked (represented by sum of distance walked in centre, close and open arms of the EPM) in both males and females reared by CD1(n=18 m, n=13 f), FVB (n=26 m, n=31 f), C57 (n=16 m, n=27 f) and Balb (n=6 m, n=6 f). Females reared by CD1 foster dams were more active than other groups, especially those reared by C57 foster mothers. Data are presented as mean ± SEM, * indicates a significant difference with p<.05.

Principal component analysis

Frequency values obtained for each factor are represented **fig.13.** A 2-way ANOVA (fostering and sex) was performed on the 8 factors obtained with PCA using male and female mice to identify how sex and early environment may affect the development of anxiety. Total number of mice analysed with PCA was n=18 males, n=13 females reared by CD1 foster dams; n=26 males, n=31 females reared by FVB foster dams; n=16 males, n=27 females reared by C57 foster mothers and n=6 males, n=6 females reared by Balb foster mothers.

There was a significant interaction among sex and fostering ($F_{(3,135)}$ =2.92692; p<.05) when we analysed factor 1 (locomotor activity): females reared by CD1 dams showed an increased activity when compared with their respective males. Moreover males reared by C57 foster dams showed increased walking as compared with their respective females. No differences were detected in mice reared by FVB or Balb foster dams. For factor 4 (rearing) male and female mice reared by CD1 dams spent more time in rearing activity than mice of both sex reared by other strains of foster dams (main effect of fostering: $F_{(3,135)}$ =19.45781; p<.001), and males showed higher levels of rearing than females (effect of sex: $F_{(3,135)}$ =4.21829; p<.05). In addition females reared by CD1 foster dams spent more time in rearing activity than other females, especially those reared by C57 lactating mothers. This trend is also observed in males, as mice reared by CD1 foster dams performed more rearing behaviour than males of other groups, in particular those reared by C57 and FVB foster dams. In relation to factor 3 (anxious-like behaviour) we observed a significant effect of fostering ($F_{(3,135)}$ =3.951467; p<.05): mice of both sexes reared by C57 dams performed an higher amount of head dipping than mice of other foster groups. About factor 6 (stretching), regardless of sex, mice reared by C57 dams spent more time in stretch attending posture as compared with mice reared by other foster dams (effect of fostering: $F_{(3,135)}$ =3.709257; p<.05). When we analysed factor 8 (open frequency) we found a main effect of fostering ($F_{(3,135)}$ =16.51125; p<.001), as animals of both sexes reared by CD1, and marginally by C57, entered less time in the open arm than offspring fostered to FVB and Balb mothers. More in detail, females reared by Balb foster dams showed an higher frequency of entries in open arms especially when compared with females reared by C57 and CD1 foster dams. In males we observed the same trend, as mice reared by Balb foster mothers entered more times than male animals reared by C57 and CD1 foster dams. We didn't find any significant effect on factor 2, 7 and 5 (Active and passive risk assessment, self-grooming respectively). All factor graphs are represented in **fig. 14** for males and **fig.15** for females.

	CD1		FVB		C57		Balb	
	♂♂	♀♀	♂♂	♀♀	♂♂	♀♀	♂♂	♀♀
Locomotor activity	0,268 ± 0,204	1,013 ± 0,294	0,046 ± 0,174	0,419 ± 0,201	0,782 ± 0,286	0,174 ± 0,223	0,263 ± 0,182	0,642 ± 0,356
Active risk assessment	-0,132 ± 0,185	-0,444 ± 0,112	-0,225 ± 0,195	-0,224 ± 0,172	-0,069 ± 0,259	0,044 ± 0,211	-1,124 ± 0,126	0,066 ± 0,459
Anxious-like behaviour	-0,037 ± 0,187	-0,097 ± 0,207	0,141 ± 0,278	0,314 ± 0,235	0,954 ± 0,401	0,593 ± 0,307	-0,644 ± 0,033	-0,263 ± 0,341
Rearing	1,242 ± 0,296	0,894 ± 0,349	-0,053 ± 0,151	-0,167 ± 0,152	-0,505 ± 0,213	-0,523 ± 0,098	0,495 ± 0,3	-0,472 ± 0,312
Self grooming	-0,384 ± 0,149	-0,507 ± 0,190	-0,082 ± 0,226	-0,378 ± 0,138	0,226 ± 0,173	-0,138 ± 0,241	-0,506 ± 0,210	-0,650 ± 0,347
Stretching	-0,064 ± 0,232	0,078 ± 0,238	-0,066 ± 0,175	0,061 ± 0,167	0,639 ± 0,247	0,537 ± 0,241	-0,295 ± 0,167	-0,467 ± 0,288
Passive risk assessment	-0,438 ± 0,115	-0,414 ± 0,253	0.011 ± 0,225	0,098 ± 0,183	-0,034 ± 0,185	-0,303 ± 0,221	-0,536 ± 0,175	0,543 ± 0,431
Open frequency	-0,419 ± 0,119	-0,550 ± 0,180	0,338 ± 0,224	0,765 ± 0,275	-0,357 ± 0,111	-0,068 ± 0,208	1,562 ± 0,387	2,065 ± 0,609

Fig.13. PCA analysis, frequency values for each factor in mice reared by CD1 (n=18 males, n=13 females), FVB (n=26 males, n=31 females), C57 (n=16 males, n=27 females) and Balb (n=6 males, n=6 females) foster mothers. Data are presented as mean ± SE.

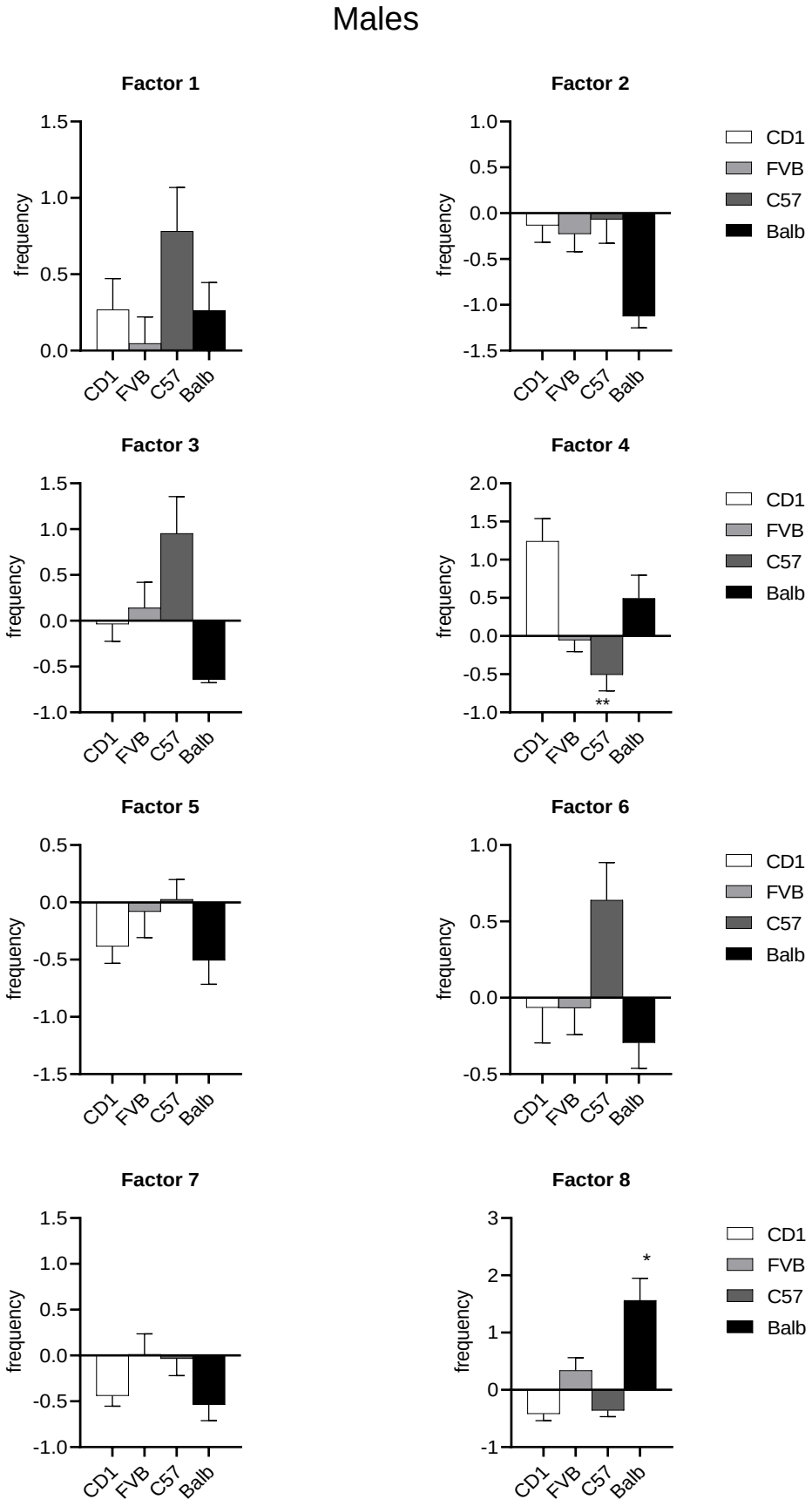

Fig.14. Elevated plus maze, PCA. Graphs of factor 1 (locomotor activity), 2 (active risk assessment), 3 (anxious-like behaviour), 4 (rearing), 5 (self-grooming), 6 (stretching), 7 (active risk assessment), 8 (open frequency) are calculated on the frequencies of major behavioral components (mean ± SEM) in males reared by CD1 (n=18), FVB (n=26), C57 (n=16) and Balb (n=6) foster dams. * indicates a significant difference with p<.05.** indicates a significant differences with p<.001

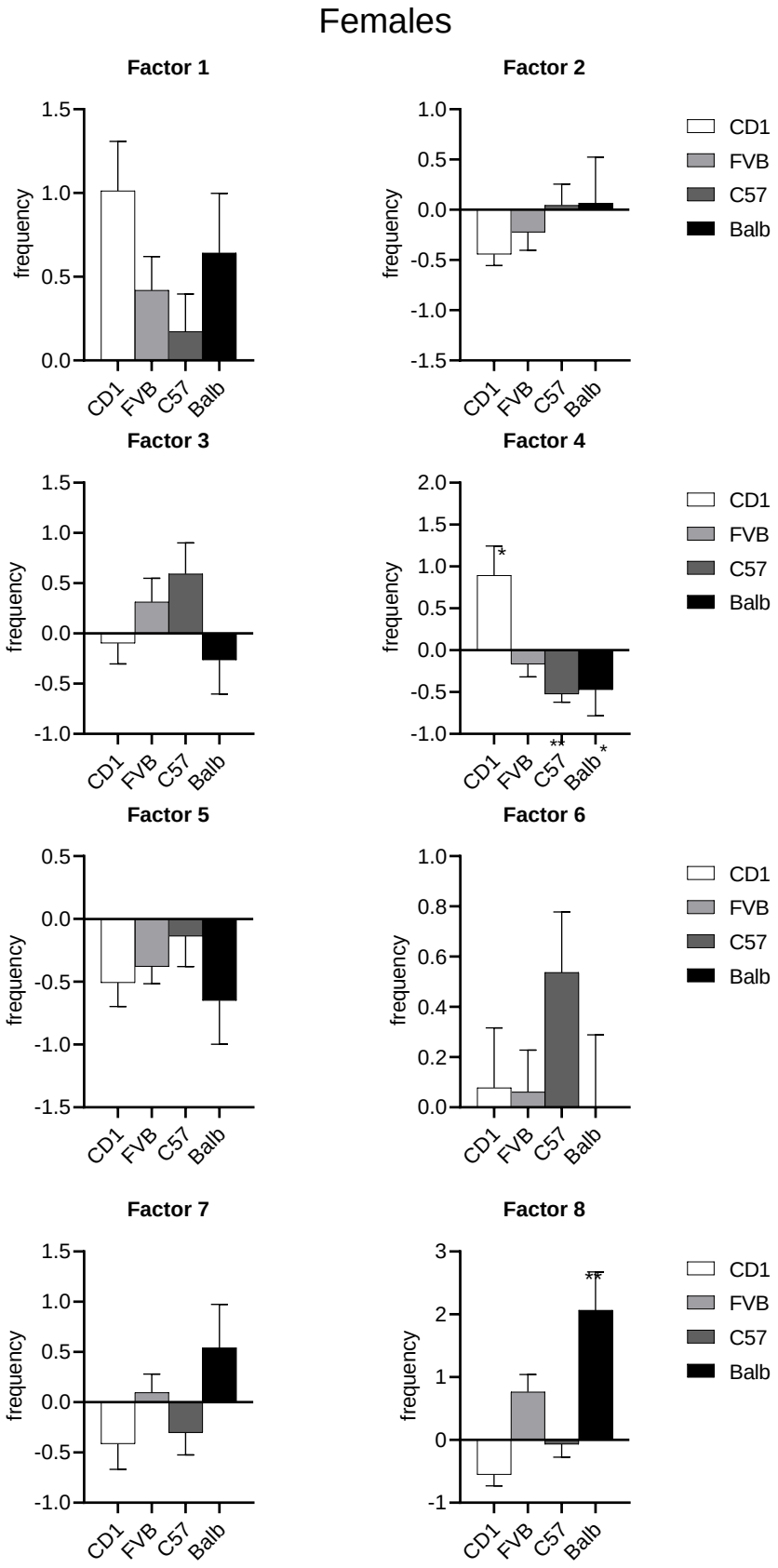

Fig. 15. Elevated plus maze, PCA. Graphs of factor 1 (locomotor activity), 2 (active risk assessment), 3 (anxious-like behaviour), 4 (rearing), 5 (self-grooming), 6 (stretching), 7 (active risk assessment), 8 (open frequency) are calculated on the frequencies of major behavioural components (mean ± SE) in females reared by CD1 (n=13), FVB (n=31), C57 (n=27) and Balb (n=6) foster dams. * indicates a significant difference with p<.05.** indicates a significant differences with p<.001

Novelty induced suppression of feeding

Mice of both sexes reared by C57 (males n=14, females n=17), Balb (males n=6, females n=6), FVB (males n=15, females n=22) and CD1(males n=14, females n=30) foster dams were tested for approach to novel environment with the NISF test. In **Fig.16** are shown results on latency to initiate consumption of a palatable snack over habituation days (1–3) and on day of test (4) in the novel cage of males and females fostered to different strain dams. Regardless of sex, there is a significant decrease in the latency to eat a palatable food during the habituation (main effect of the days: $F_{(3,232)}$ =89.4277; p<.001) but there is no effect of fostering ($F_{(3,116)}$ =1.1061; p=.349616).

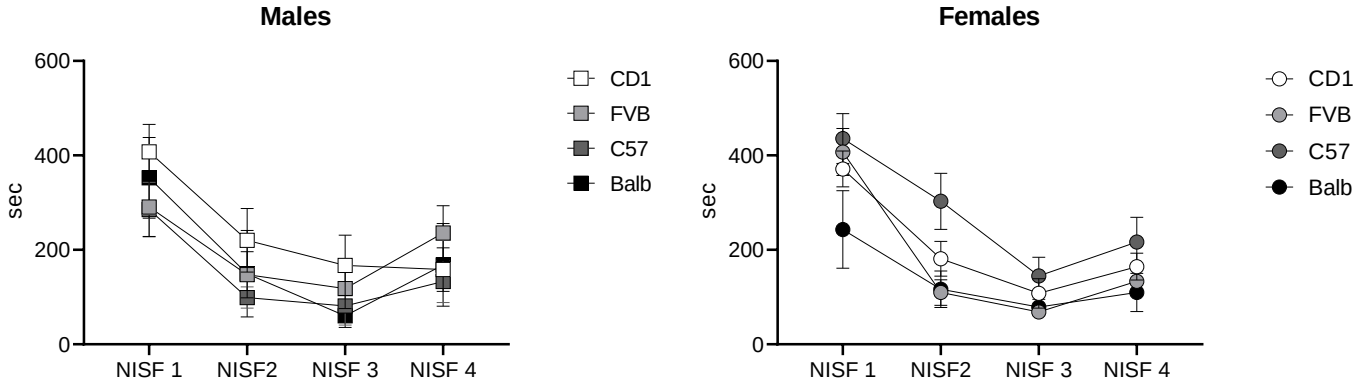

Fig.16. Novelty induced suppression of feeding (NISF) test performed on male and female mice reared by CD1 (n=14 m, n=30 f), FVB (n=15 m, n=22 f), C57 (n=14 m, n=17 f) and Balb (n=6 m, n=6 f) foster dams. Day 1-3 represent the habituation, day 4 represents the test in a novel environment. Latency decreased during the habituation. At day 4 no effect of fostering or sex was detected. Values are presented as mean ± SEM.

Aggressive and social behaviour

Resident/intruder test

Aggressive behaviour was analysed in males reared by C57, Balb, FVB and CD1 foster mothers through the Resident-Intruder paradigm. Among males reared by C57 foster dams 11 out of 14 mice attacked the intruder, and interestingly around the same number (12 out of 17) of males reared by FVB foster dams displayed aggressive behaviour. Among males reared by CD1 foster mothers, 9 out of 10 mice attacked the intruder while only 2 out of 6 males reared by Balb foster dams displayed aggressive behaviour. Consistently, males reared by C57 and FVB foster mothers displayed similar amount of aggressive behaviour (**Fig.17A**), and similar the attack latency differed. In addition, male mice reared by Balb foster dams seemed to display a lower level of aggressive behaviour, in line with the high latency of attack (**Fig.17B**). Despite these results, statistical analysis did not find any effect of fostering on percentage of aggressive behaviour. Males fostered to CD1 dams presented a significant lower latency of attack as compared with other groups, especially compared with males reared by Balb foster mothers ($F_{(3,39)}$ =4.53211; p<.01).

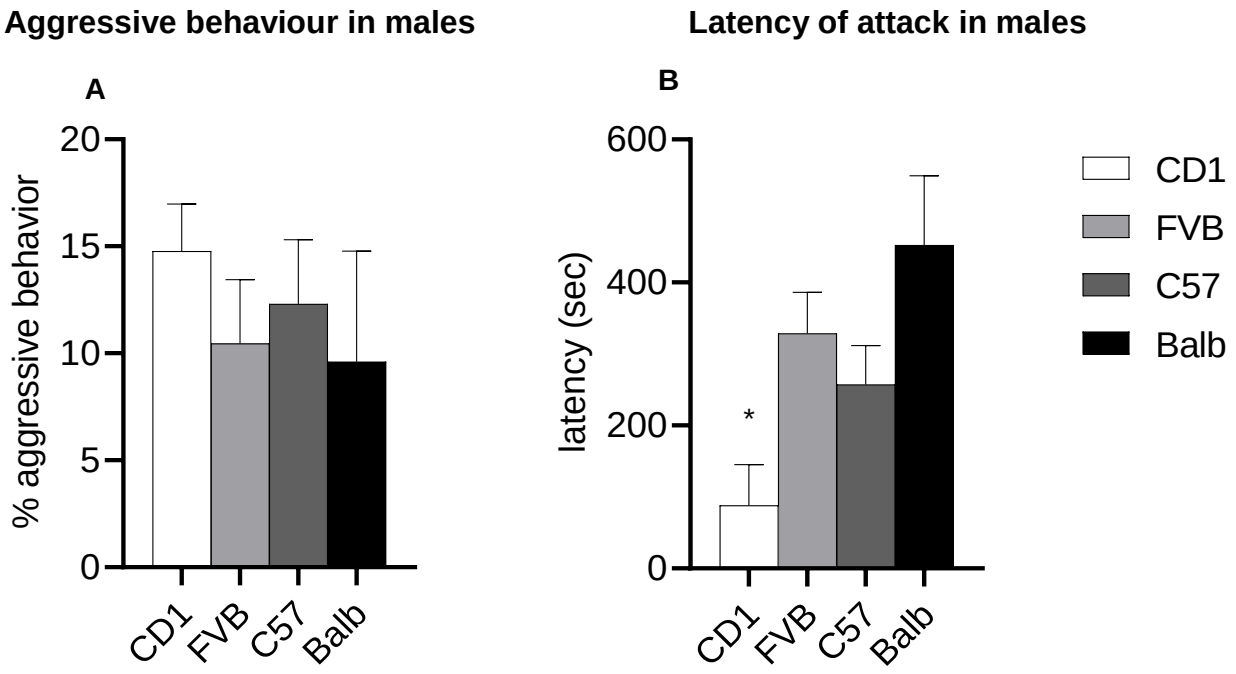

Fig.17. Resident intruder paradigm to test aggressive and social behaviour; (**A**) Time spent by males reared by CD1 (n=10), FVB (n=17), C57 (n=14) and Balb (n=6) foster dams in aggressive behaviour (comprising biting attack, circling, chasing, tail rattling). No significant effect of fostering. (**B**) Latency of first attack in males. We observed an increase of latency in males when reared by Balb dams, while males fostered to CD1 dams showed a lower latency than other groups. Values are presented as mean ± SEM, * indicates a significant difference with p<.05

We also investigated social behaviour with the same resident intruder paradigm in both males and females mice reared by the four strain of adoption (CD1 n=10 males, n=14 females; FVB n=17 males, n=23 females; C57 n=14 males, n=13 females; Balb n=6 males, n=4 females).

Interestingly, we found a significant interaction among sex and fostering ($F_{(3,92)}$ =2.9868; p<.05) as male mice but not females reared by CD1 foster dams displayed less social behaviours than other groups (**Fig.18**). Furthermore females spent more time in social investigation than males (effect of sex: $F_{(1,92)}$ =5.6335; p<.05).

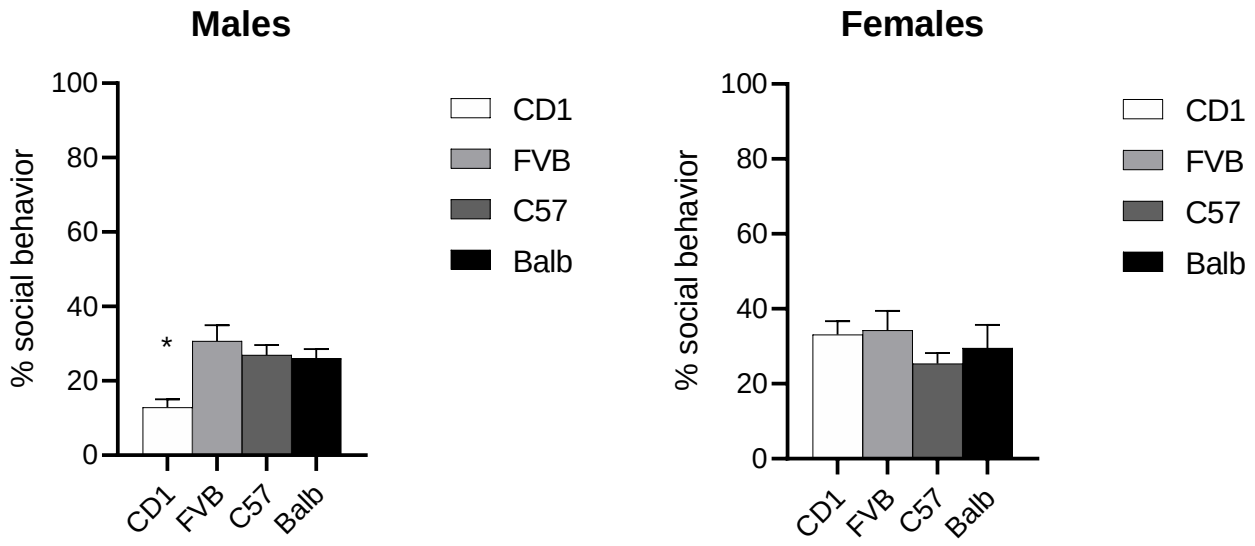

Fig.18. Resident intruder paradigm to test social behaviour; Percentage of time spent in social interaction by males and females reared by CD1 (n=10 m, n=14 f),FVB (n=17 m, n=23 f), C57 (n=14 m, n=13 f) and Balb (n=6 m, n=4 f) foster dams. Statistical analysis underlined sex and fostering interaction, as males reared by CD1 foster dams, but not females, showed a lower level of social investigation directed toward the intruder. We also observed higher amount of social behaviours in females. Values are presented as mean ± SEM, * indicates a significant difference with p<.05.

Metabolic challenge

As adult, C57-derived offspring reared by different strains of foster mothers were exposed to standard (STD) or high fat (HFD) diet. Thus, we obtained eight experimental groups: C57 fostered mice fed with STD (males n=13; females n=12) or HFD (males n=22, females n=14), FVB fostered mice fed with STD (males n=19, females n=8) or HFD (males n=12, females n=18), Balb fostered mice fed with STD (males n=1, females n=1) or HFD (males n=5, females n=5) and CD1 fostered animals either with STD (males n=11, females n=9) or HFD (males n=25, females n=10). Mice reared by Balb foster dams were excluded from statistical analysis run on STD data as the number of animals was not sufficient. About HFD, as number of mice reared by Balb foster mother was lower than other groups we decided to perform two different analysis, one including and other one omitting values of Balb's offspring.

Metabolic analysis on STD – HFD data

When we analysed all data recorded on the animals reared by CD1, FVB and C57 foster dams we observed that mice reared by CD1 foster mothers gained significantly more weight than animals of other groups when fed HFD; while in STD animals we did not observe any significant changes in body mass during days (days x fostering x diet interaction: $F_{(18,1449)}=2.06$; $p<.01$). Regardless of fostering and sex, all the animals increased their body weight when fed with HFD (diet: $F_{(1,161)}=10.55$; $p<.001$). specific analysis based on the diet underlined that, across days, CD1- reared mice fed STD increased their body weight more than other groups (days x fostering interaction: $F_{(18,\ 594)}=2.6$; $p<.001$), and that females reared by CD1 foster dams presented a higher increase in body mass than their respective males (sex x fostering interaction: $F_{(2,66)}=3.4$; $p<.05$) (Fig.**19A-C**). All males increased their body weight during HFD whereas in females there is no significant difference between the first and the last day of diet (days x sex interaction: $F_{(9,\ 855)}=16.18$; $p<.001$). Animals reared by CD1 foster dams presented a higher increase in body weight across days when fed with HFD than mice reared by FVB and C57 foster dams, thus suggesting higher vulnerability to diet-induced obesity (interaction between days and fostering: $F_{(18,855)}=2.72$; $p<.001$) (**Fig.19B-D**).

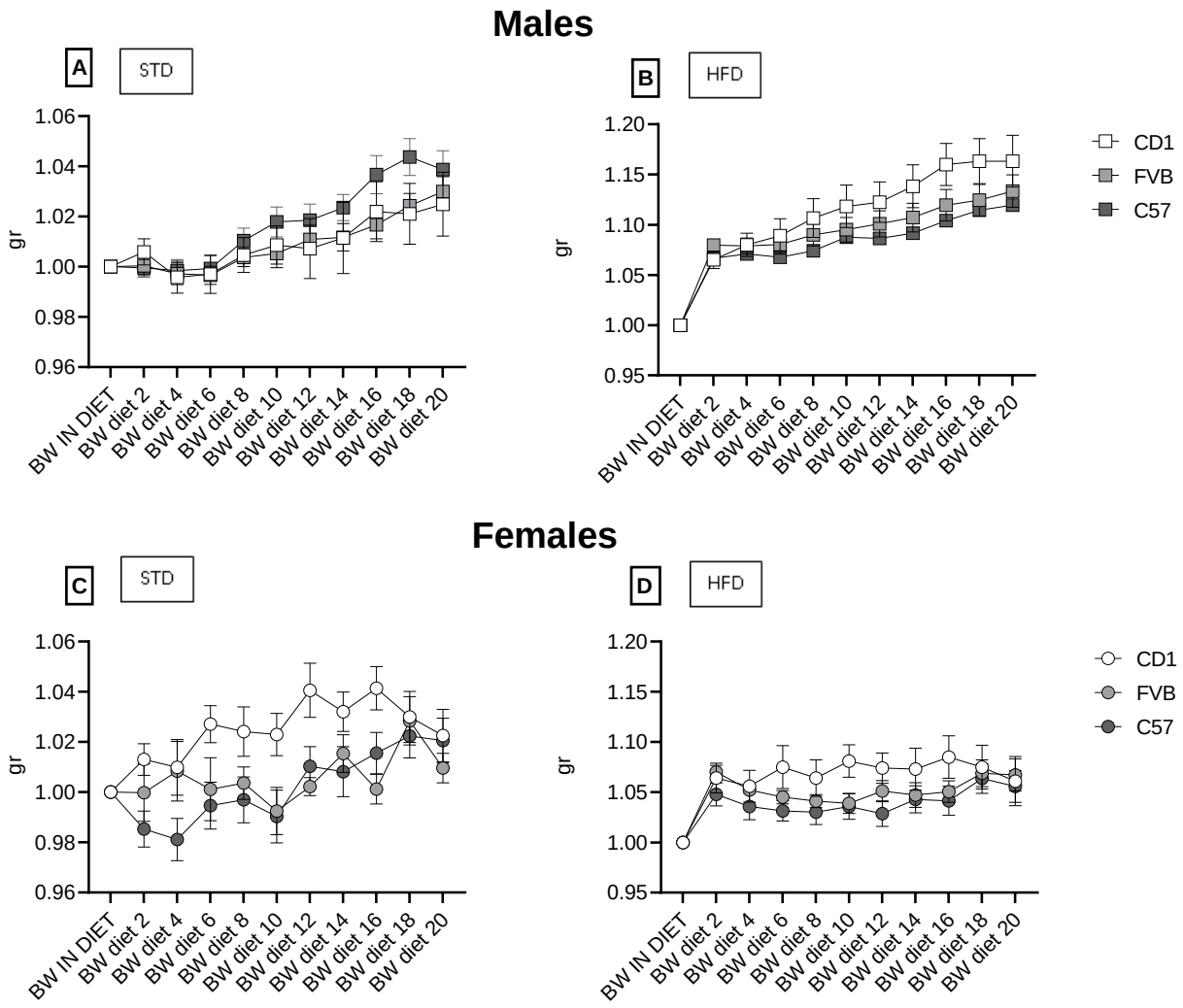

Fig.19. Body weight gain during the metabolic challenge. Metabolic consequences of STD in male **(A)** and female **(C)** mice reared by CD1 (n=11 m, n=9 f), FVB (n=19 m, n=8 f) and C57 (n=13 m, n=12 f) foster mothers. Mice of both sex on STD showed no significant differences in BW gain during the STD regimen, but males reared by CD1mothers presented a major increase in body weight gain than other foster groups. **B**) Metabolic consequences of HFD in males **(B)** and females **(D)** reared by CD1 (n=25 m, n=10 f), FVB (n=12 m, n=18 f) and C57 (n=22 m, n=14 f) foster mothers All males in HFD regimen increased their body weight gain, while females did not show significant increase in body weight. Moreover, males reared by CD1 foster dams presented an higher increase in body weight during days than males in other groups. Values are presented as mean ± SEM.

After exposure to Standard or High fat diet we performed the Glucose tolerance test (GTT), to evaluate the level of blood glucose at different time points (n=19 , n=17 males and females reared by C57 foster dams, respectively; n=11 ,n=14 males and females reared by FVB foster dams, respectively; n=12 n=10 males and females reared by CD1 foster dams, respectively). First of all, sex resulted to affect the response to a glucose challenge regardless of the diet, with males characterized by increased glycaemia as compared with females ($F_{(1,138)}$ =84.135; p=.051). Overall, among animals fed with HFD, there was a significant difference between males and females

reared by CD1 foster dams, especially after 30 and 60 minutes from the glucose injection, while ino differences were found in mice reared by other foster strains (time x sex x fostering interaction: $F_{(6,231)}$ =2.124; p=.051). Mice exposed to STD had regular levels of glycaemia, but there was an interaction between time, sex and adoption ($F_{(6,183)}$ =2.847; p<.05) as in males only mice reared by CD1 foster dams increased significantly the amount of blood glucose as compared with other foster mice. Moreover, in females we did not observe any changes among fostering groups (**Fig 20**).

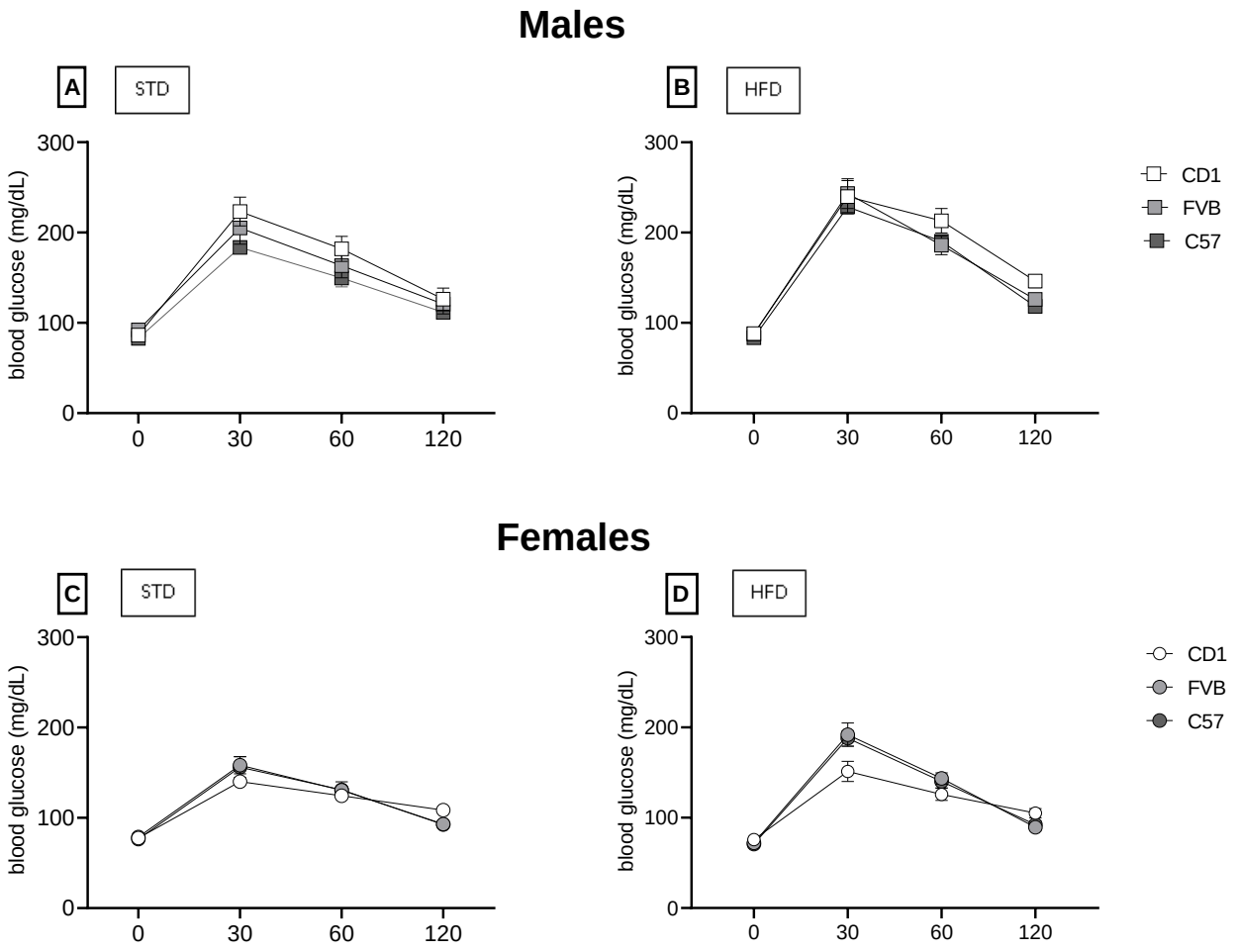

Fig.20. Glucose tolerance test (GTT). Glucose levels during STD in male **(A)** and female mice **(C)** reared by CD1 (n=7 m, n=9 f), FVB (n=11 m, n=8 f) and C57 (n=14 m, n=18 f) foster dams. No differences but the diet (STD mice showed less blood glucose than HFD mice) in females. Glucose levels during HFD in males **(B)** and females **(D)** reared by CD1(n=12 m, n=10 f),FVB (n=11 m, n=14 f) and C57 (n=19 m, n=17 f) foster dams Male mice, regardless genotype and adoption, showed a hyperglycemia only when fed with HFD, and presented an increase in levels of blood glucose as compared with females. Males reared by CD1 foster mothers presented major differences after 30 and 60 minutes with their respective females, while in other groups no significant differences based on time and fostering were detected. Values are presented as mean ± SEM.

Increased body weight in HFD-fed mice reflected an increase in visceral adiposity, with significantly higher abdominal fat weight in male mice fed HFD than in mice fed with STD (sex x diet interaction: $F_{(1,151)}$ =8.7221; p<.005). Overall males showed a higher amount of abdominal white adipose tissue (effect of sex: $F_{(1,151)}$ =13.1756; p<.001) as compared with females, and mice fed with HFD showed a higher amount of abdominal fat (diet: $F_{(1,151)}$ =42.0234; p<.001). Male mice reared by CD1 foster dams and challenged with STD had double the amount of white adipose tissue than the other groups while in females levels of adipose fat were around the same (sex x fostering interaction: $F_{(2,59)}$ =4.2746; p<.05) (**Fig.21A-C**). When analysed individually, HFD mice showed a strong effect of sex ($F_{(1,83)}$ = 22.3969; p<.001), with an increase of abdominal white fat in males compared with females. (**Fig.21B-D**).

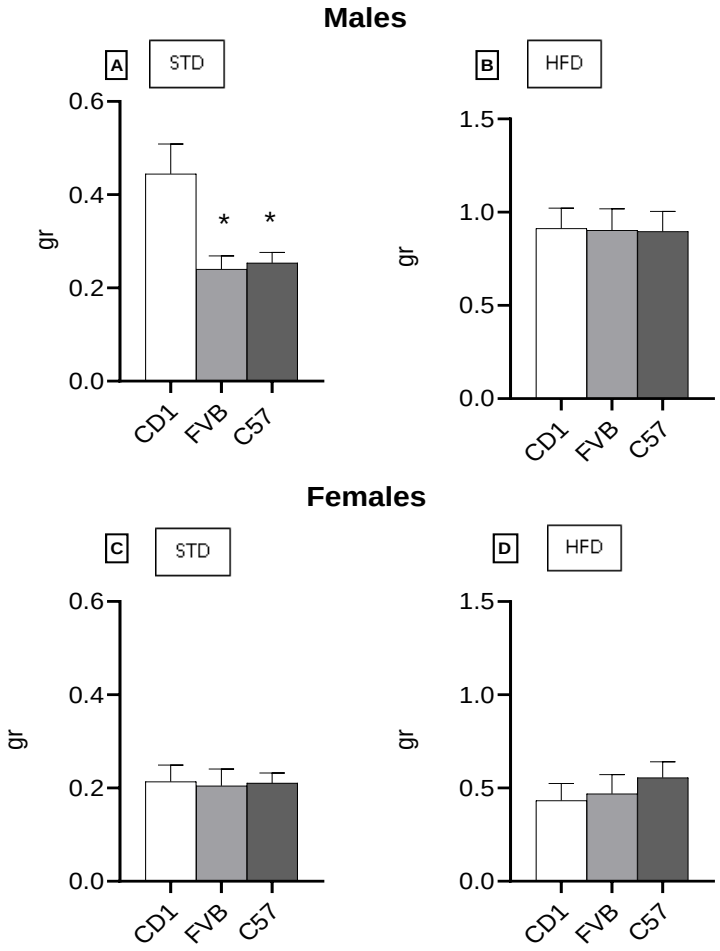

Fig. 21. White adipose tissue harvested at sacrifice. Amount of WAT in male **(A)** and female mice **(C)** reared by CD1 (n=11 m, n=9 f), FVB (n=7 m, n=8 f) and C57 (n=13 m, n=17 f) foster mothers on STD diet. Male mice on STD reared by CD1 dams showed more WAT as compared with males reared by other strains. Amount of WAT in males **(B)** and females **(D)** reared by CD1 (n=12 m, n=10 f), FVB (n=17 m, n=19 f) and C57 (n=16 m, n=15 f) foster dams and fed with HFD diet. Overall males, regardless the adoption, presented an increase in WAT as compared with females. All values are presented as mean ± SEM, * indicates a significant difference with p<.05.

Metabolic analysis on HFD data including mice reared by Balb foster dams

In a second phase we analysed data derived only from HFD. In this analysis we integrated also animals reared by Balb foster mothers (n= 5 males, n=5 females). Body weight gain across days seemed to be affected by foster strain, as mice reared by Balb and CD1 foster dams presented an higher increase in body mass across days as compared with other groups (days x fostering interaction: $F_{(27,927)}$ =2.68; p<.001). Moreover we observed a significant effect of sex difference , with females gaining less weight than males (days x sex: $F_{(9,927)}$ =9.21; p<.001) and a significant effect of foster mothers, as Balb mice presented an higher body weight as compared with other groups (effect of fostering: $F_{(3,103)}$=4.61; p<.005) (**Fig.22**).

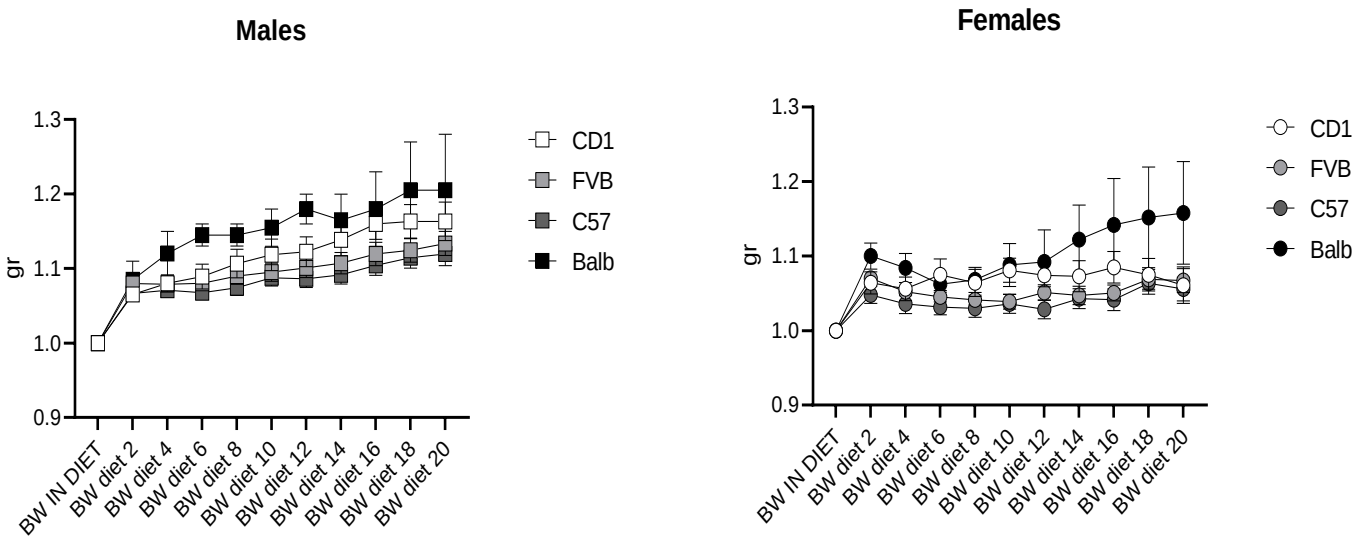

Fig. 22. Body weight gain during the metabolic challenge. Metabolic consequences of HFD in male and female mice reared by CD1 (n=25 m, n=10 f), FVB (n=12 m, n=18 f), C57 (n=22 m, n=14 f) and Balb (n=5 m, n=5 f) foster mothers. Effect of fostering x days interaction, as in both sex animals reared by Balb foster mothers showed a major body weight. Females gained less weight than males (effect of sex: $F_{(1,103)}$=16.96; p<.001). Data are presented as mean ± SEM.

After exposure to High fat diet we performed the Glucose tolerance test (GTT), to evaluate the level of blood glucose at different time points (**Fig.23**). We observed an interaction among time and fostering, as mice reared by Balb foster dams had a major increase in glycaemia levels at different time points as compared with other foster groups (time x fostering interaction: $F_{(9,252)}$=2.486; p<.01). Moreover females, regardless of the strain of foster mothers, showed less blood glucose increase 30, 60 and 120 minutes after the injection as compared with males (interaction among time and sex: $F_{(3,252)}$=18.076; p<.01).

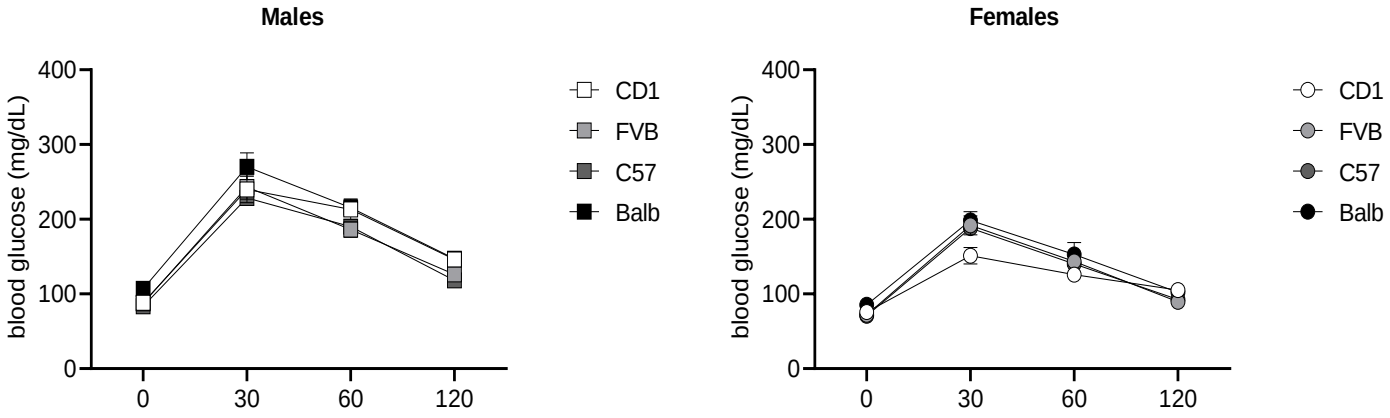

Fig. 23. Glucose tolerance test (GTT) . Glucose levels during HFD in male and female mice reared by CD1 (n=12 m, n=10 f), FVB (n=11 m, n=14 f), C57 (n=19 m, n=17 f) and Balb (n=5 m, n=5 f) foster dams. Male mice, regardless adoption, showed higher levels of glycaemia as compared with females. Mice reared by Balb foster dams presented an increase in glycaemia levels at different time points. Data are presented as mean ± SEM.

Finally, Balb males showed an higher level of white adipose tissue as compared with other groups but no significant difference were found among different fostering groups. Females, indeed, presented a lower amount of WAT as compared with males (sex: $F_{(1,92)}$=30.048; p<.001) (**Fig.24**).

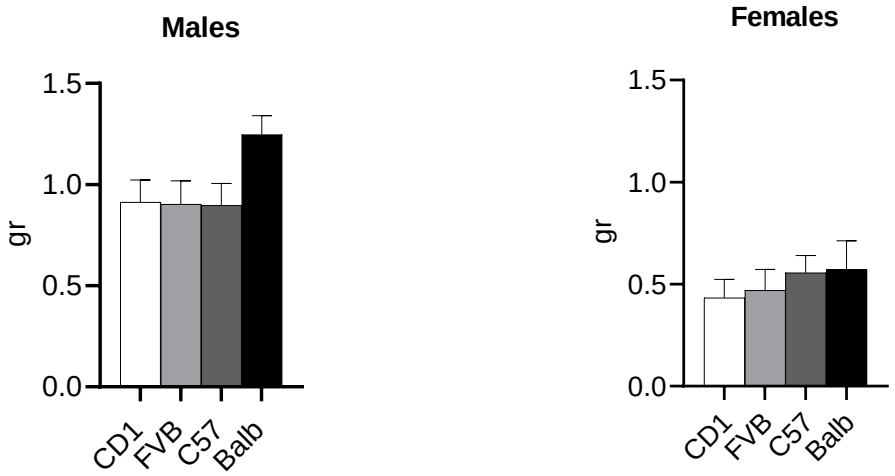

Fig. 24. White adipose tissue harvested at sacrifice. Total amount of WAT in male and female reared by CD1 (n=12 m, n=10 f), FVB (n=17 m, n=19 f), C57 (n=16 m, n=15 f and Balb (n=6 m, n=5 f) mice fed with HFD diet. Overall males, regardless the adoption, presented an increase in WAT as compared with females. All values are presented as mean ± SEM.

4.4 Discussion

In this study we assessed the effects of different levels of maternal care on anxiety-like, aggressive and social behavior, and metabolism of male and female mice that were fostered at birth to mothers belonging to different mouse strains. To this aim we examined adult male and female reared by CD1, FVB, C57 and Balb foster dams. In order to define the quality of maternal care displayed foster mothers, we firstly analysed the amount of maternal behaviour displayed by all the lactating females during the first seven days after delivery. we specifically focused on Maternal behaviours thought to increase pups' survival through provision of an optimal thermoregulation and level of nutrients to pups, namely the arched-back posture of nursing; in fact, when dams crouched over pups in this nursing posture they provide "ventral heat" to offspring as well as access to nipples to allow suckling (Champagne *et al*., 2007). Previous studies from our laboratory, CD1 and FVB dams showed a high maternal care profile, with a significant amount of maternal behaviours directed to pups (Arched back and nursing behaviour) and spent less time out of the nest or in other activities, when compared with C57 or Balb mothers. Such high proportion of time spent by FVB and CD1 dams in the ABN posture was consistent during the first postnatal week; these resultsconfirm that these foster mothers' display high maternal cares(HM), while C57 and Balb females dislpay low maternal care (LM), as the amount of time spent in activities non related to pups was definitely higher than CD1 and FVB dams. These results of C57 and Balb strain are in line with what Ws reported by Shoji and Kato in 2006 and 2009.

Overall, our findings show that early maternal environment, here represented by different strains of foster mothers, plays a major role in modulating anxiety-related behaviours. We examined anxiety like behaviours in the Elevated Plus maze (EPM) test and the Novelty Induced Suppression of Feeding (NISF) test. All the animals spent most of the time in the close arms of the EPM, and the frequency of the open arm exploration was lower than the other spaces, suggesting that, regardless of sex and fostering strain, all the strains of mice are characterized by an anxious-like phenotype. Interestingly, based on time spent in the close and open arm of the maze and frequency of entries, maternal environment appeared to affect both males and females level of anxiety. Mice of both sexes reared by C57 dams, in fact, spent less time in the close arms and more time in the open arms. These results are in line with total time spent in closed arms, with males reared by Balb dams and females reared by CD1 dams spending most of the time in closed space. Moreover, we assessed the total distance walked by the animals in the arena; Female mice reared by CD1 foster mothers were more active in the EPM than mice reared by C57 and BALB dams, thus suggesting a more complex profile of maternal care effects, as higher locomotion is an index of arousal and pro-active behaviour in response to a novel environment. In males we did not observe any significant difference in total distance walked.

Furthermore we performed a PCA on dataset of control mice reared by foster strains. Results obtained from Principal component analysis may help us to understand the effects of maternal environment on offspring emotional behavior.

The subsequent ANOVA ran on the 8 factors, using them as variables (see **Fig.2**) revealed clear effects of early maternal environment. Regardless of sex, mice reared by CD1 foster dams were much more active in the EPM, as they showed significant higher amount of behaviours included in factor 1 and 4, locomotor activity and rearing, respectively, than mice reared by the other strains of foster mothers. Moreover females fostered to CD1 dams resulted more active than their respective males. It is known that cage-climbing activity is also associated with higher anxiety-like behaviour in mice (Pietropaolo et al. 2007) and we observed a significant difference in factor 4 (rearing activity) among male and females reared by CD1 foster dams and other groups. This seems to confirm the results obtained analyzing total time spent in the open arms of the EPM, as males but overall females reared by CD1 foster mothers tended to linger less than other groups in open spaces of the maze. Mice reared by C57 dams showed increased head dipping in open spaces and time spent in open arms (factor 3) as compared with other groups. As the amount of time spent in the open arms is anyway extremely low in all these mice, the slight increase observed in C57-reared mice could be not a strong indication of lower anxiety levels in this mouse strain. However, if we consider head dipping in open space as an index of neophilia (in motivational terms the attraction that an animal displays towards a novel object or a place, in behavioural terms a curiosity-based approach; Brown and Nemes, 2008) the increased head dipping might indicate decreased anxiety-like behavior. Altogether, these results indicate that the levels of maternal care and the early maternal environment might affect the response to a novel environment, particularly locomotor activity and behaviours associated to an anxiety phenotype, in a sex-dependent way. Mice reared by HM mothers, in particular CD1 dams, were indeed much more active and displayed more anxiety-related behaviours. Mice reared by LM mothers, especially C57 dams, were less active and explored more the open areas showing less anxiety-like behaviours as compared with other groups. these results are partially in line with previous studies of Kessler *et al*., 2011, showing that offspring reared by mothers displaying an higher amount of maternal care show an higher level of anxiety in adult life, while offspring reared by dams that performed less nursing and arched back present lower amount of anxiety-like behaviours in adulthood.

In the NISF test, anxiety-like behaviour is evaluated through the latency to eat a highly palatable snack in a novel environment. This test is based on the principle that rodents show a clear preference for lipid-rich foods, such as peanuts (e.g. Gaillard *et al*., 2008) and that the latency to close in and consume a familiar snack in a novel environment is markedly increased in a novel cage with clean bedding (Merali *et a*l., 2003). In this test regardless of sex all mice showed a regular daily reduction in the latency to consume the peanut, but the latency only increased in male mice reared by FVB dams, when the peanut was presented in a clean bedding-filled cage on day 4. Thus suggesting that

males reared by FVB dams present higher anxiety-like response as compared with other groups. These data suggest that, regardless the fostering strain, male and female mice seem to be not so sensitive to novelty-induced inhibition of feeding, as they did not show the expected environment-induced avoidance in approaching the highly palatable food. These effects seem to be more pronounced in males and especially in males reared by mothers that display high amount of maternal care, thus suggesting that an increase of maternal care during early postnatal days might affect the reward system and the related behavioural responses. It is possible to hypothesize that the behaviour displayed by our subjects in the NISF test might be an indicator of an enhanced reward-related response and surely further studies will be needed to test the hypothesis. Another possible explanation to further explore, could be the display of an autistic-like response, as the absence of the inhibition of palatable food intake in novel environment has been interpreted as a sort of resistance to change that is characteristics of autism (Crawley, 2007).

In the Resident/Intruder paradigm male mice were characterized by lower attack latency when reared by high-maternal-care dams (CD1 and FVB) than males reared by Low-maternal-care foster mothers (C57 and Balb). Our results, indeed, indicated that male offspring reared by CD1 dams showed the highest level of aggressive behaviour, with a lower latency of attack and a tendency to an higher percentage of time spent in aggression, and lower time spent in social behaviour; while the lowest aggressive mice were the males reared by Balb dams, which showed low maternal care (LM). In fact, males reared by Balb mothers had the highest latency of attack and the lowest percentage of time spent in aggressive behaviour, Previous studies have suggested that maternal environment can modulate the amount of serotonin and the metabolism of the neurotransmitter in rodents (Shah *et al.*, 2018; Chiavegatto and Randy, 2003; Carola *et al*, 2011) and a major finding seems to be that exposure to both low and high serotonin levels during the perinatal period can lead to behavioural alterations in adulthood (Shah *et al*., 2018). Our results showed that the level of male aggressive behaviour is affected by the quality of maternal care received during the first week of life, even if the specific impact of the amount of care is still unclear. Further studies will be required in order to to understand the interconnection between 5HT, early environment and the development of aggressive behaviour in adult males.

It is well known that levels of aggressive behaviour in non-reproductively active female mice are almost absent, while levels of social behaviour are higher in females: our results, as expected, showed that females presented a high amount of social behaviour as compared with males, with no effect of fostering. Interestingly, among males, we observed a lower level of social behaviour in animals reared by CD1 foster dams, consistent with the tendency to higher aggression displayed by CD1 reared males. further investigations using different paradigms (i.e. three chamber sociability task) are required to better understand how early environment and maternal care affect social behaviour in male and female mice.

In this study we also analysed the effects of fostering and sex on energy balance and vulnerability to high fat diet induced obesity and metabolic syndrome. Our results show that different maternal environment can affect the susceptibility to diet-induced obesity in male but not in female mice. However, there is no clear relation between the amount of maternal care received during early postnatal days and males' propensity to develop diet induced obesity. Indeed, higher body weight gain and higher abdominal fat characterized male mice reared by either CD1 (high amount of maternal care) or Balb (low amount of maternal care) dams and fed with HFD as compared to males reared by FVB (HABN) and C57 (LABN) foster mothers. Thus suggesting a possible role of maternal milk quantity and/or quality.

In female mice, fostering at birth did not affect body weight growth on a standard diet and did not affect susceptibility to diet induced obesity. Sex differences in metabolic disorders such as obesity (Palmer and Clegg, 2015) and atherosclerosis (Wang *et al.,* 2011) are well described and biologically important elements for understanding their aetiologies. However most *in vivo* studies focus on male mice, However, a number of studies have noted that the development of obesity and hyperglycaemia is strain-dependent and sex-dependent (Ingvorsen *et al.,* 2017). The weaker effects of high fat diet on females may be due to several factors and environmental variables, like the action of estrogens, that have been shown to exert a protective action against metabolic effects of high fat diet (Litwak *et al*., 2014; Riant *et al.*, 2009). In fact Riant and coworkers showed that the activation of the estrogen pathway prevents the occurrence and the course of insulin resistance and glucose intolerance induced by high-fat feeding in wild-type mice (Riant *et al*., 2009). In our study we reported a difference in glucose tolerance due to the diet, as the glycaemia increased in animals of both sexes when fed with high fat diet regardless of the mother's strain. Sex however influenced the level of blood glucose, as male mice showed a higher glucose intolerance as compared with females counterparts.

We can speculate that different amounts of maternal care influenced adult metabolism but that other factors, such the nutritional values and the milk composition actively influenced the development of the offspring and the metabolic patterns in adults. In fact previous studies showed that amino-acids concentration like dietary L-serine (Nagamachi *et al.,* 2017), Taurine and β-alanine (Nishigawa, *et al.,*2018), but also epigenetic regulation (Hashimoto and Yoshihiro, 2018) or breeding technology using artificial suckling (Yasuda *et al*., 2016) might modify the early development and the metabolism of the offspring in mice. Indeed, in conclusion we can suggest that the quality of milk and the amount of nutrients could be one relevant variable, in addition to maternal care during postnatal period, that should be considered to underly the complex role of the early environment in the subsequent adult life. In fact, maternal behaviour could not explain by itself the reported alterations in behaviour and metabolism, but more concurrent factors may interact to modulate the offspring developmental trajectories and their behaviour and metabolic functions throughout life.

Chapter 5

5 Selective loss of TLQP-21 acts on postnatal maternal care and parental genotype might influence anxiety behaviour in adulthood (pilot study)

5.1 Introduction

VGF (non-acronymic), that has been first identified as VGF8a, NGF33.1, and a2 on the basis of its rapid induction in PC12 cells treated with the nerve growth factor (NGF), is a neurotrophin-induced gene, namely genes or gene products that are regulated by neurotrophin at a transcriptional, translational, or posttranslational level. Based on recent data, it has been outlined a role for VGF gene in neural plasticity, neuroendocrine changes, and depression. The prohormone convertases (PC), PC1/3 and PC2 are responsible for the process of VGF polypeptide (Trani *et al*., 2002) that it's predominantly synthesized in neuronal and neuroendocrine cells (Levi *et al*., 1985; Salton *et al*.,1991; van den Pol *et al*., 1994; Snyder *et al*., 1998). VGF in rodents encodes a 617 amino acid protein stored in dense core granules and processed to yield several bioactive peptides, whereas in humans the protein is composed by 615 amminoacid but it is believed to execute similar functions. VGF mRNA in the brain is mainly expressed in neurons of the hippocampus, cerebral cortex, amygdala, midbrain and thalamus; the highest concentrations of VGF immunoreactivity is to be found in the medial hypothalamus, particularly in the regions of arcuate nucleus (ARC), paraventricular nucleus (PVN), supraoptic nucleus (SON), and suprachiasmatic nucleus (SCN). VGF is robustly induced by exercise, neurotrophic factor signaling, and synaptic plasticity (Razzoli *et al*., 2012). VGF derived peptides (**Fig.1**) include TLQP-62, TLQP-21, HHPD-41, AQEE-30, AQEE-11, LQEQ-19, and NERP-1 and -2 (neuroendocrine regulatory peptides-1 and -2). All of this VGF-derived-peptides present specific neuronal bioactivities and execute many different functions (Lewis *et al*., 2015). Various studies showed that TLQP-62 and AQEE-30 could increase the synaptic transmission of hippocampal neurons, induce neurogenesis, and present anti-depressive properties (Alder *et al*., 2003; Thakker-Varia and Alder, 2009; Cattaneo *et al*., 2010), while HHPD-41, AQEE-30, AQEE-11, and LQEQ-19 facilitate penile erection in rats and stimulate the sympathoadrenal system (Hahm *et al*., 1999; Succu *et al*., 2004); and finally TLQP-21 and NERP-2 could regulate energy expenditure and the balance between intake of food and output of work, in association with the increase of serum epinephrine (Bartolomucci *et al*., 2006; Toshinai *et al*., 2010). TLQP-21 in addition may regulate gastric contractility and gastric acid secretion, nociception, as it has analgesic properties, may decrease blood pressure and exerts a neuroprotective action, protecting rat cerebellar granule cells subjected to serum and potassium deprivation, modulating the extracellular signal-regulated kinase1/2 and enhancing intracellular Ca^{2+} release (Possenti *et al*., 1999; Severini *et al*., 2008; Fargali *et al*., 2014;). The vast majority of the studies focused on VGF's role in energy balance, nociception modulation, gut contractility and sexual behaviour, but a growing body of data

also supports its role as mediator of antidepressant action. Recent studies suggest an involvement of TLQP21 in pituitary gonadotrope and/or lactotrope function (Ferri *et al*., 1995) : in fact in female rat VGF immunoreactivity seemed to be restricted to gonadotroph and lactotroph cell population in the anterior pituitary, and it has been shown that VGF peptide/s degranulation occurred at estrous pick and it was associated with an increase in Vgf mRNA expression. Moreover, in female sheep, it has been demonstrated that in pituitary cells the VGF immunoreactivity varies also through different reproductive seasons (Brancia et al., 2005). Most of the research activity in last decade focused on the role of VGF-derived peptide TLQP-21 that extends from residue 556 to residue 576 of the precursor sequence. TLQP-21 has been identified as a peptide linked to multiple functions, as regulation of energy balance and body mass, gastric motor function and secretion, nociception, and reproductive function (Razzoli *et al*., 2012).

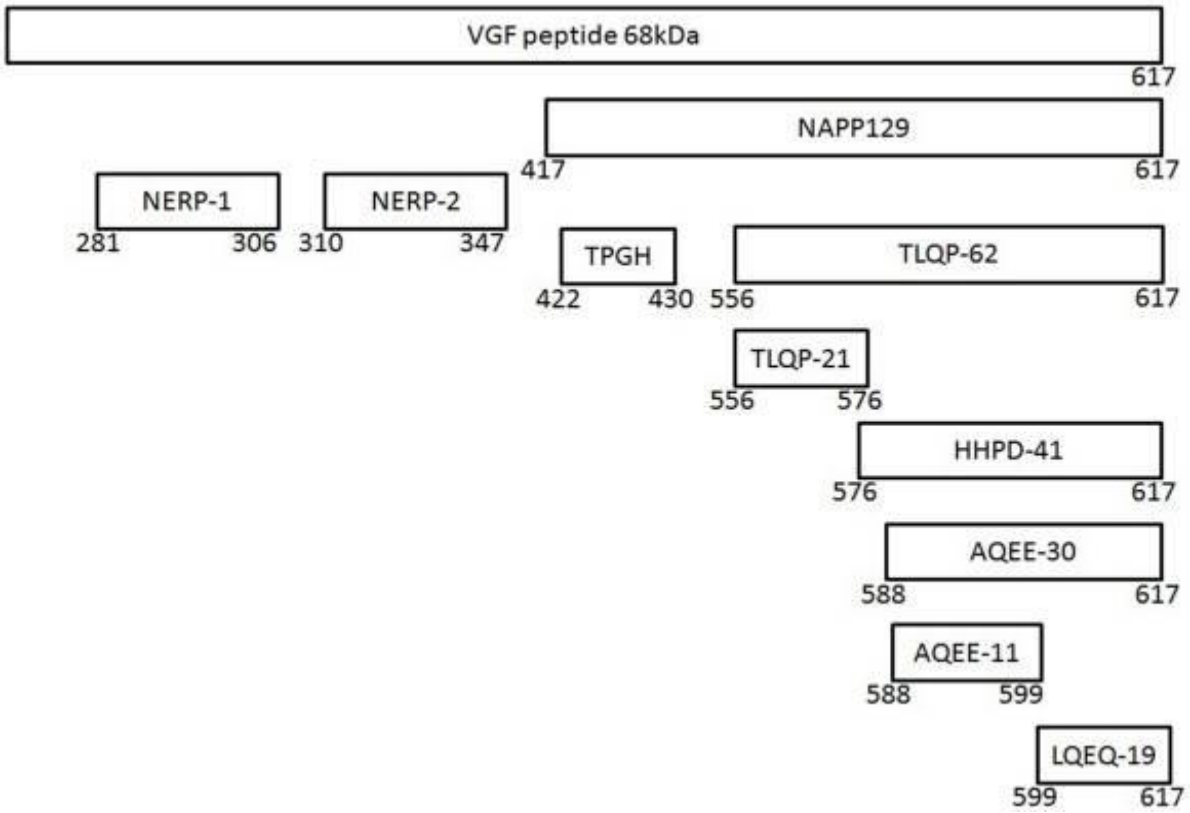

Fig 1. VGF gene and its derived peptides (TLQP-62, TLQP-21, HHPD-41, AQEE-30, AQEE-11, LQEQ-19, and NERP-1 and -2). VGF polypeptide is the precursor of several peptides, that play a role in intercellular communication. The gene itself contains various specific sequences highly conserved among the species and all of them represent potential cleavage sites for the convertases of the proteinase family, namely prohormone convertases-1/3 and -2 (Lewis *et al*., 2015)

TLQP-21, named after its four N-terminal amino acid residues *Thr-Leu-Gln-Pro*, is a 21- aminoacid neuropeptide, derived from the cleavage of the 617-aminoacid VGF pro-petide by prohormone

convertase (PC) 1/3 and PC2. (Guo *et al*., 2018) and encoded by the pro-peptide VGF, that mainly targets the C3aR1 (complement 3a receptor 1, a G-protein coupled receptor protein involved in the complement system) (Hannedouche *et al*., 2012, Cero 2014). Neuropeptide TLQP-21 is expressed in different regions of the central nervous system and peripheral nervous system, as well as in the sympathetic nerve terminal in the adipose tissue. As previously described TLQP-21 regulates energy balance, pain modulation, blood pressure, reproductive behaviour and stress (Bartolomucci *et al*., 2006, 2011; Cero *et al*., 2017; Doolen *et al*., 2017; Fairbanks *et al*., 2014; Fargali *et al*., 2014; Jethwa *et al*., 2007; Petrocchi-Passeri *et al*., 2015; Razzoli *et al*., 2012; Rizzi *et al*., 2008; Severini *et al*., 2009; Trani *et al*., 1995). It has been proved that infusions of TLQP-21 in mice brain produce an increase of energy expenditure and it prevent the onset of obesity. Moreover, Possenti *et al*., in 2012 demonstrated that TLQP- 21 might modulate adrenergic-induced lipolysis and increased sympathetic innervation to the fat pads. Other studies demonstrated that TLQP-21 attenuates the development of type 2 diabetes enhancing the outliving and function of islet b-cell (Stephens et al., 2012), and may normalize hypertension due to obesity (Fargali et al., 2014). Importantly, a recent study has provided the first evidence of a direct effect of TLQP-21 peptide on the reproductive function, demonstrating in pubertal male rats, that central administration of TLQP-21 induced acute gonadotropin responses and stimulated LH secretion directly at the pituitary level (Pinilla *et al*., 2011). Another study of Petrocchi-Passeri *et al*, 2013 suggested the possible role of TLQP-21 as a real mammotrophic peptide: results indicated that TLQP-21 could have a neural modulation effect as hormone when it is released from the hypothalamic area in median eminence or acting as paracrine-autocrine hormone on the pituitary gland.

Although a growing body of evidence suggests that TQLP-21 is the main candidate, among vgf-derived peptide, to carry out a role in obesity, reproduction, stress response and sex behaviour, relatively little is known at this time about the effects of TLQP-21 on behaviour. To address this question we used a new mouse model, engineered by Bartolomucci and Salton to investigate metabolic effect of selective loss of TLQP21. We decided to inquire its behavioural phenotype. For this reason we decided to observe whether this genetic mutation might act on maternal care and how a feasible change in mother's behaviour could affect behavioural development in adulthood. We settled up a preliminary application of a study protocol to verify whether the project could be carried out in a larger scale. Pilot studies are often recommended to address various issues (i.e. effects to expect, reasonable models, difficulties to solve, correct sample size to answer the questions keeping the desired cost/benefit ratio, etc.) as well as to estimate response rate and investigate the feasibility of a study. A pilot study often is a good way to troubleshoot any equipment problems, familiarize the team with the procedures, and see if the experiment design has any potential flaws. First of all, in this pilot study we performed an examination of maternal behaviour in mice parents, that were divided into three experimental groups based on the genotype. The first group included heterozygous parents, the second and the third one included homozygous parent both carrying (ΔTLQP-21 /

ΔTLQP-21) or not carrying (WT / WT) the mutation. Once we observed whether and how TLQP21 acts on maternal care, we analysed the effect of TLQP21 loss on anxiety-like behaviours, performing Elevated Plus Maze test on ΔTLQP-21 and WT offspring reared by ΔTLQP-21, WT and heterozygous parents, rating the effect of paternal line too. Our hypothesis was that selective loss of TLQP21 may affect somehow the amount of maternal care, and this imbalance could lead to the development of a different anxiety phenotype in adulthood.

5.2 Material and methods

Animals and housing

Male and female mice (C57BL/6J;129Sv background backcrossed at least 6 generation to B background) presenting selective loss of TLQP21 (hereafter ΔTLQP-21) were initially housed with same sex littermates and maintained in a 12:12h light-dark cycle at 22 ± 2°C. In a same way, male and female mice (C57BL/6J background) heterozygotes or WT (hereafter WT) were housed in a similar way with same sex individuals. When sexual maturity was achieved, male and female mice with the same genotype (ΔTLQP-21, WT, heterozygous) were mated, obtaining three different groups. Twelve hours after birth we started maternal observation (PND1-14). Pups were weaned at PND21 and we calculated reproductive success based on number of pups alive at weaning. Lastly adult mice were tested for anxiety and depressive like behaviour. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Minnesota.

Generation of ΔTLQP-21 mice

As selective loss of a peptide encoded by the pro-peptide VGF has never been reported thus far, the question on which VGF-derived peptide exert the critical role for energy expenditure and for the balance among food intake and output of work is still unanswered. To reply this question Bartolomucci and Salton engineered a knockin mouse model of selective loss of TLQP-21 (ΔTLQP-21). In this model the endogenous arginine 21 was replaced with an alanine (Arg576Ala mutation in the pro-peptide) thus preventing cleavage by the pro-hormone convertase. In the ΔTLQP-21 the WT codon encoding for R21 (crucial for peptide activity as well as a recognition site for peptidases (Bartolomucci *et al*., 2011) was mutated to encode for A21 (**Fig.2A**). A preliminary set of data were obtained to validate this mouse model: first of all, the mutation was confirmed with Sanger sequencing (**Fig.2B**) and the selective loss of TLQP-21 was confirmed by IHC (**Fig.2C**) and a new targeted MS/MS proteomic assay after in-gel digestion (Jedrychowski *et al*.,2015). Proteomic analysis identified various transitions of the non-natural HFHHALPPA**A**HHPDLEAQARR fragment in the ΔTLQP-21 but not WT mice (**Fig.2D**). Targeted in-gel digestion based MS analysis demonstrated that ΔTLQP-21 mice have imperceptible level of TLQP-21 in the hypothalamus pituitary and adrenals (not shown) while the VGF precursor and other VGF-derived peptides (i.e.TLQP-62) remain unaffected. Preliminary in vivo evidences demonstrate that selective TLQP-21 ablation does not affect early growth, general behaviour or metabolic functions in normal housing conditions thus ruling out a critical role for this peptide in development (unpublished, not shown). However, ΔTLQP-21 are resistant to HFD-induced obesity in presence of normal food intake and increase oxygen consumption, a result which is similar to the metabolic phenotype of VGF KO

mice.(Fig.2E). -Overall, preliminary data suggest that TLQP-21 is the critical VGF encoded peptide regulating adaptive regulation of energy balance in adult mice. Next, Bartolomucci and his team characterized the metabolic phenotype of male and female DTLQP-21 mice (in preparation).

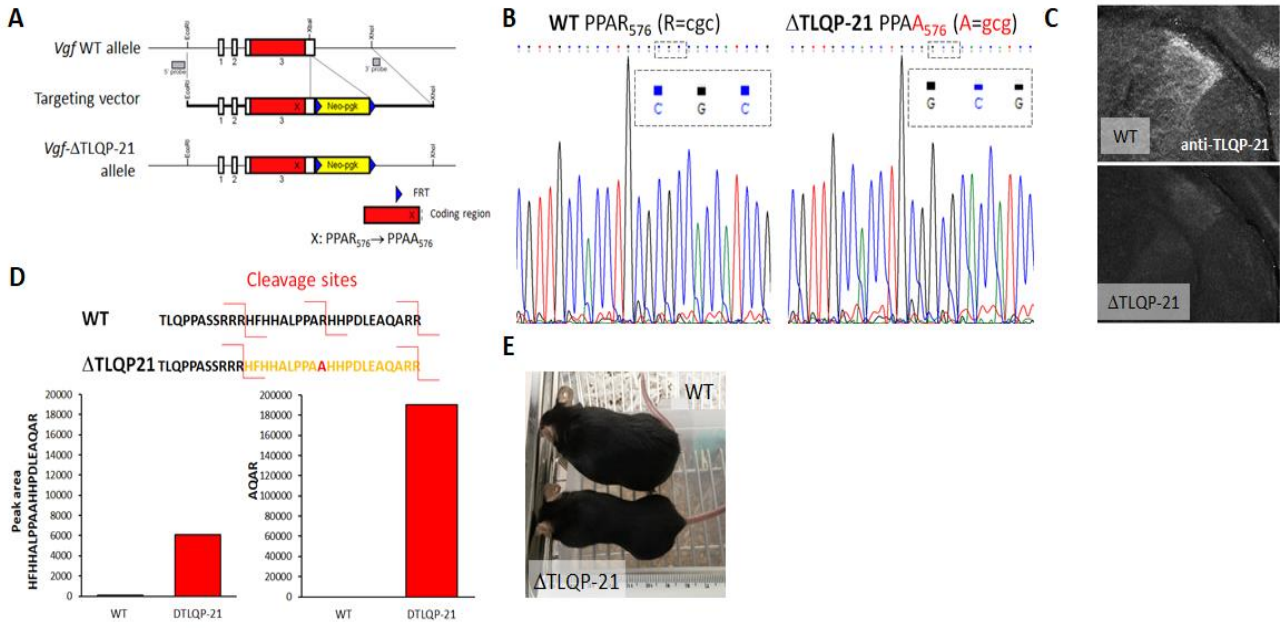

Fig. 2. **A)** Generation of the ΔTLQP-21 strain. **B)** Genomic DNA from WT and ΔTLQP-21. VGF region was PCR-amplified and the resultant amplicon was gel purified and subjected to Sanger sequencing. **C)** IHC of the thalamus of representative WT and ΔTLQP-21 using a specific anti-TLQP-21 antibody[20]. **D)** Targeted MS approach to characterize the misprocessing of TLQP-21. Proteins from adrenals were run in a gel and bands were excised between ~2-5KD and subjected to in-gel tryptic digestion. Peptide digestions were solubilized and run in Applied Biosystem 5500 iontrap. Transitions and parameters predicted from an in silico tryptic digestion using the Skyline program were considered. Unpublished

Maternal observation

Maternal behaviour was assessed by observing ΔTLQP-21 (n=6), WT (n=5) and heterozygous (n=7) mothers in their home cages, from PND 1 to 14. Mice are most active during the dark phase of light/dark cycle (Latham and Mason, 2004). As it was a preliminary study and mice were placed in a common room with other strains, we were not able to change the timing of dark/light cycle. Therefore, based on different works about maternal observations we previously studied and analysed, we decided to start the observation period (120 min) at 06 PM hr, during the last two hours of the light phase, until 8PM. This decision was made to allow the animals to remain in their common environment and stress them as less as possible. Observations were performed by the same operator who was unaware of the mouse genotype. Each lactating female was observed once every four minutes, for a total amount of 30 observations in 2 hours. During each 4min observation, the experimenter revealed which behaviour is displayed at the moment of the observation.

Dams behaviours were identified according to behavioural categories, as in Palanza *et al.* (2002). First of all, we observed the *Arched back nursing*, a behaviour characterized by a kyphosis of the dam's back, that allows the lactating female to control thermoregulation and nourishment of the pups providing them ventral heat as well as access to the nipples. *Nursing* behaviour refers to dam's capability to provide maternal milk to pups; the female is laying on a side, quiescent, and her nipples are exposed to pups. Other behaviours we observed were l*icking,* in which the female licks or grooms her pups not only to keep them clean but to help them urinate/defecate, regulate their body temperature and stimulate their movement to allow them access to nipples (Champagne *et al*., 2007) ; *Nest building*, the lactating female was occupied in some activity regarding the nest building, either inside or outside the nest itself, for example transporting bedding materials towards the nest or manipulating the materials to form the enclosed nest edge (Kuroda *et al*., 2011); *Grooming*; the lactating female was cleaning herself *; Active*: including all the behaviours the female made in or out of the nest, that may not be brought back to any behaviours previously outlined*.* Finally we calculated the time spent o*ut of nest;* considering all the observations during which the dam was anywhere in the cage but the nest, regardless of the behaviour exhibited at the moment of the observation. In addiction we put together all behaviours related to pups (AB, nursing, nest building, licking pups) and we compared it with total of behaviours not related to pups (out of nest, active, grooming). After 21 days pups were weaned and housed in groups of siblings of the same sex. A small tissue sample from the ear of each mouse was collected using an ear puncher during weaning procedure, thus allowing us to establish offspring's genotype using qPCR.

Behavioural test

Behavioural tests were carried out on adult male and female mice between PND 90 and PND 110 from 9 AM to 1 PM. All mice were left in their experimental room during test procedures. We tested mice for anxiety-like behaviour using Elevated plus maze paradigm.

Elevated Plus Maze (EPM) test: The Elevated plus maze is an ethologically-based model of anxiety that let understand better how rodents respond to a novel approach or avoidance situation. The EPM apparatus (**Fig. 3**) consists of a plus-cross shaped (30*5cm) originating from a central platform (5*5cm) elevated 40 cm above the floor. Two arms, opposing each other, are enclosed by Plexiglas walls (20 cm) and the leftover arms are open. WT and ΔTLQP-21 male and female offspring of heterozygotes or homozygotes WT or ΔTLQP-21 parents were tested in the EPM in their experimental room during early morning, to reduce the amount of stress induced by place them in a novel environment. Each animal was placed on the central platform facing one of the open arms and was allowed to explore the maze for 5 minutes from that moment. A video camera placed 2 m above the apparatus recorded the entire test, and the frequency of entries into, and time spent in the open and closed arms of the maze were analysed. To reduce any persistent olfactory cues that could alter the performance, between successive trials the maze was cleaned with clean damp cloth and a solution of 2% ethylic alcohol. Behaviours were analysed and scored by an observer, previously trained, using specific software (The Observer, Noldus, NL) to record the latency, duration and frequency of transitions between different arms.

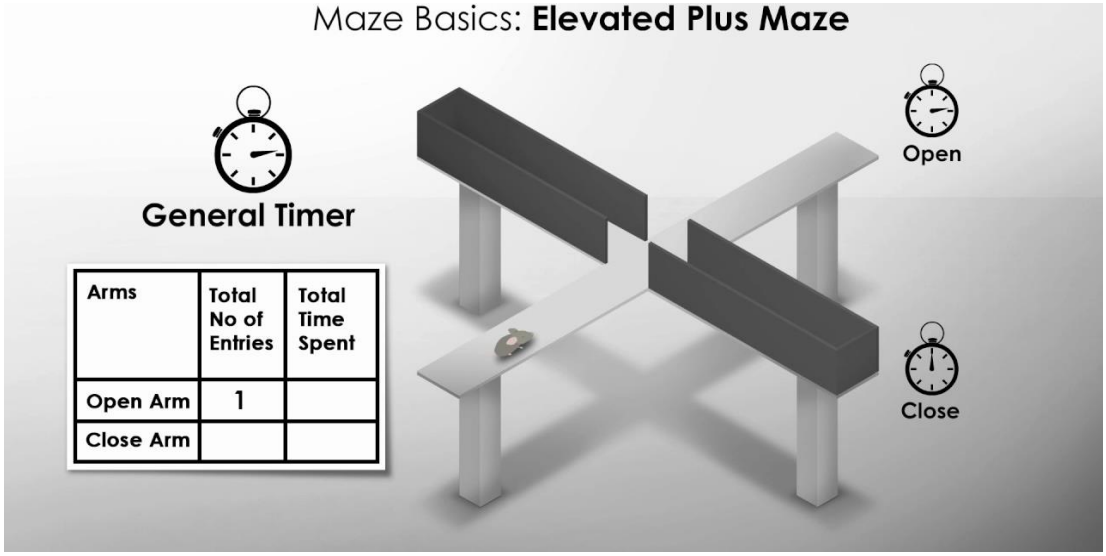

Fig. 3. Elevated Plus Maze apparatus

Statistical analysis

Data were analysed with ANOVA followed by Tuckey`s HSD where appropriate, using Statistica 10. Unless otherwise noted data are presented as average + standard error of the mean. Significance was set at 0.05.

5.3 Results

Analysis of maternal behaviour

Spontaneous maternal behaviour of dams was observed throughout the fourteen days of postnatal life. Data are presented as mean percentage of time spent in different maternal behaviour. Values are obtained by direct observation of ΔTLQP-21 (n=6), WT (n=5) and heterozygous (n=7) mothers with the pups. Observations are divided in two weeks since what we expected was a change in days of the total time spent displaying different behaviours. A two-way ANOVA (genotype, week) was performed to analyse data. We focused on results of behaviours that are considered main components of maternal behaviour, such AB, nursing and licking of pups , and we put aside minor behaviours like allogrooming and nest building. In general, statistical analysis on time spent out of the nest underlined the interaction among genotype and weeks: in fact heterozygous dams during week 2 spent more time out of nest than WT and ΔTLQP-21 mothers, as shown in **fig.4** ($F_{(2,26)}$ = 8,4348; p<.05). [A3]Moreover heterozygous mothers significantly increased time out of nest from the first to the second week ($F_{(1,26)}$ = 7.8680; p<.05).[A4]

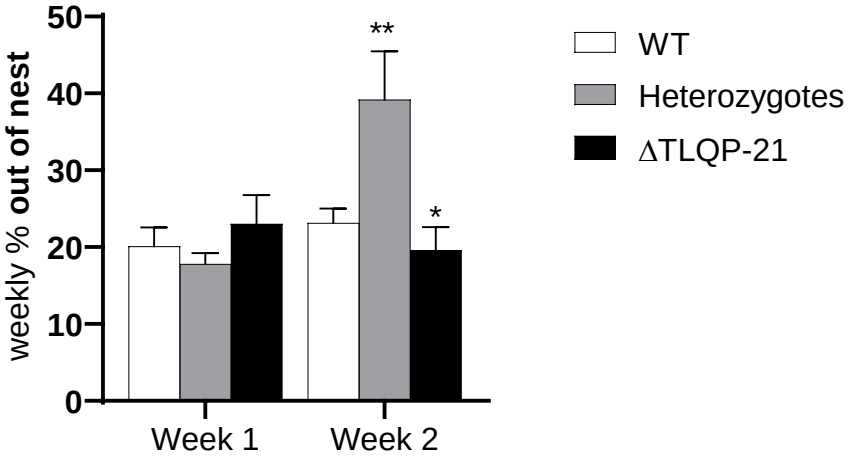

Fig. 4. Weekly percentage of time spent out of nest by ΔTLQP-21 (n=6), WT (n=5) and heterozygous (n=7) mothers. In week 2 Heterozygous mothers spent significant more time out of the nest than other groups. Data are presented as mean ± SEM, * indicates a significant difference with p<.05, between groups in week 2.** indicates a significant differences with p<.001, in heterozygotes, among week 1-2

Overall percentage of time spent in arched back (AB) behaviour decreased in weeks ($F_{(1,26)}$ =69.643; p<.001), especially in heterozygous and ΔTLQP-21 dams as shown in **fig.5A** (effect of genotype: $F_{(2,26)}$ = 5.587; p<.05). Interestingly ΔTLQP-21 mothers significantly increased nursing behaviour in the second week of observation as compared with other groups (interaction among genotype and weeks: $F_{(2,26)}$ = 8,2408; p<.05). Furthermore the value increased in mutant group from week 1 to week 2 ($F_{(1,26)}$ = 23.9429; p<.001) (**Fig. 5B**).

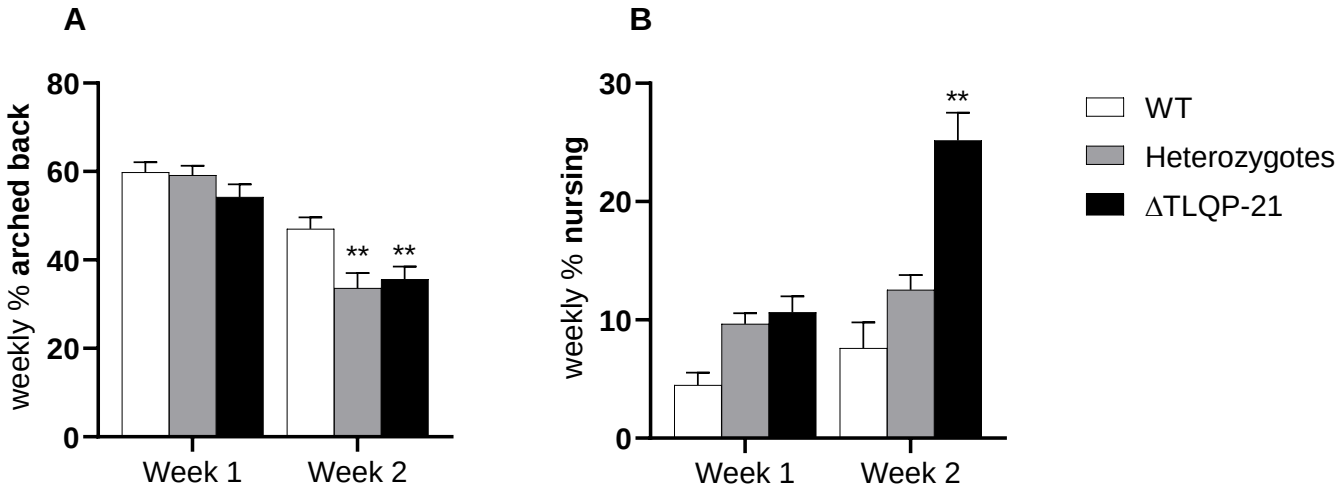

Fig. 5. Weekly percentage of time spent in **(A)** arched back and **(B)** nursing behaviour by ΔTLQP-21 (n=6), WT (n=5) and heterozygous (n=7) mothers. **(A)** All mothers decreased arched back in the second week, especially heterozygous and ΔTLQP-21 dams. (**B)** ΔTLQP-21 mothers significantly increased nursing behaviour in the second week of observation. Data are presented as mean ± SEM,** indicates a significant differences with p<.001, from week 1 to week 2.

Analysis of licking behaviour (**Fig.6**) highlighted an interaction among genotype and weeks ($F_{(2,26)}$ = 5.5810; p<.05) as we observed that heterozygous dams decreased from week 1 to week 2, while on the contrary ΔTLQP-21 slightly increased, following the trend we observed for nursing behaviour.

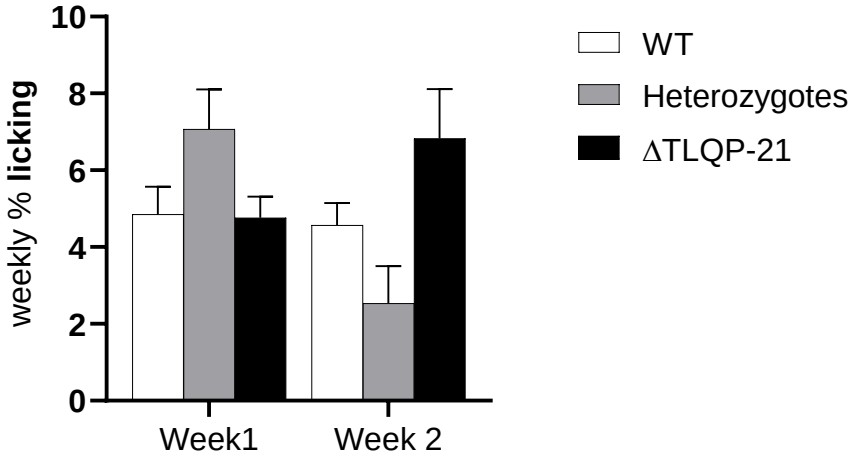

Fig. 6. Weekly percentage of time spent by ΔTLQP-21 (n=6), WT (n=5) and heterozygous (n=7) mothers licking pups. Data are presented as mean ± SEM

Heterozygous mothers showed a typical increase of active behaviour from week 1 to week 2, following the regular tendency of dams to be more active when the pups grow (interaction among genotype and weeks: $F_{(26,130)}$ = 9.8039; p<.001); moreover heterozygous mothers showed a significant difference as compared with mutant dams in week 2 (**Fig.7**). Interestingly when we analysed total time spent in actions related to pups' care, heterozygous dams behaved exactly as we expected, showing a decrease in weeks due to the growth of the offspring. In homozygous WT

this tendency is present but not as strong as in heterozygotes, instead in homozygous ΔTLQP-21 the amount of maternal care seemed to remain the same in all the fourteen days of observation (interaction among days and genotype: $F_{(2,26)} = 7.568$; $p<.01$). This could be explained with the significant increase in ΔTLQP-21's nursing behaviour during week 2 (**Fig.8A**) while the amount of arched back decreased, so total time spent weekly in behaviour related to pups didn't decrease at all. Finally total amount of time spent in behaviours not related with pups' care underlined once more that heterozygous mothers significantly differ from week 1 to week 2 ($F_{(1,26)} = 17.6103$; $p<.001$), and this difference is visible also when heterozygous group is compared with ΔTLQP-21 dams in week 2 (interaction among weeks and genotype: $F_{(1,26)} = 9.6053$; $p<.001$) (**Fig. 8B**).

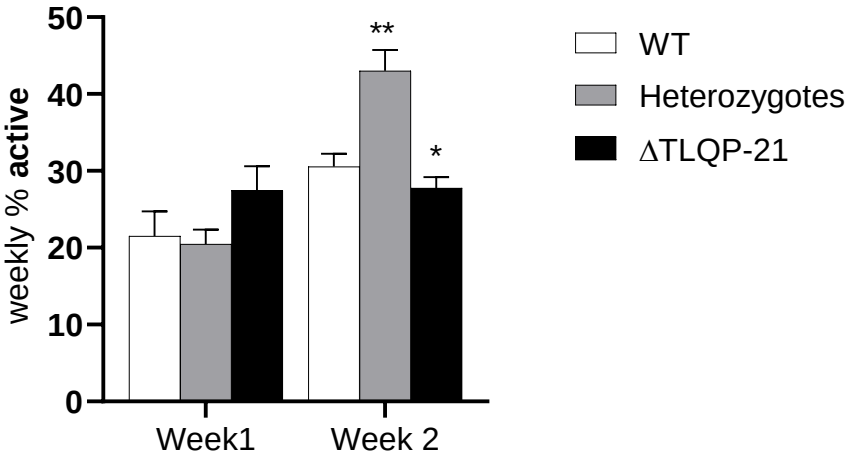

Fig. 7. Weekly percentage of time spent by ΔTLQP-21 (n=6), WT (n=5) and heterozygous (n=7) mothers in active behaviour. Heterozygous mothers increased activity from week 1 to week 2, and during week 2 the difference was significant as compared with ΔTLQP-21 dams. Data are presented as mean ± SEM, * indicates a significant difference with p<.05 of ΔTLQP-21 with heterozygous dams.** indicates a significant differences with p<.001 from week 1 to week 2.

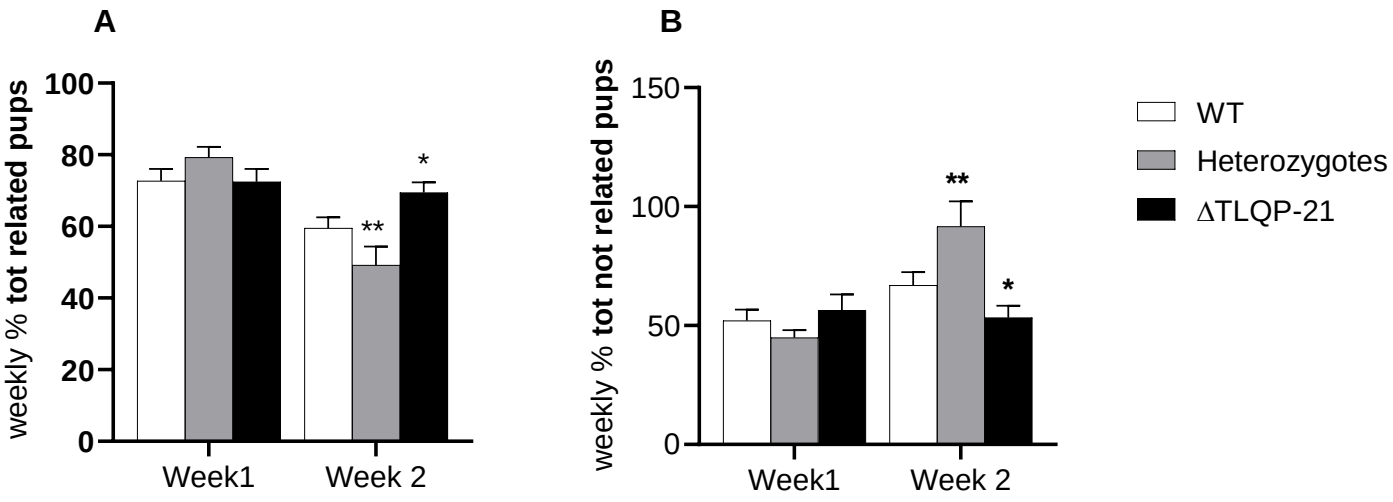

Fig. 8. Weekly percentage of time spent by ΔTLQP-21 (n=6), WT (n=5) and heterozygous (n=7) mothers in **(A)** behaviours related and **(B)** not related to pups. Heterozygous in both cases showed a significant change in second week, and also a difference with mutant group. Data are presented as mean ± SEM, * indicates a significant difference with p<.05, of ΔTLQP-21 with heterozygous dams .** indicates a significant differences with p<.001 from week 1 to week 2.

TLQP-21 does not affect litter size in mice

A one-way ANOVA (genotype) performed on data of the reproductive success of ΔTLQP-21, WT and heterozygous dams showed no significant effect of genotype on birth rate or survival at weaning ($F_{(1,18)} = 3.5$; p=.09) (**Fig.12**).

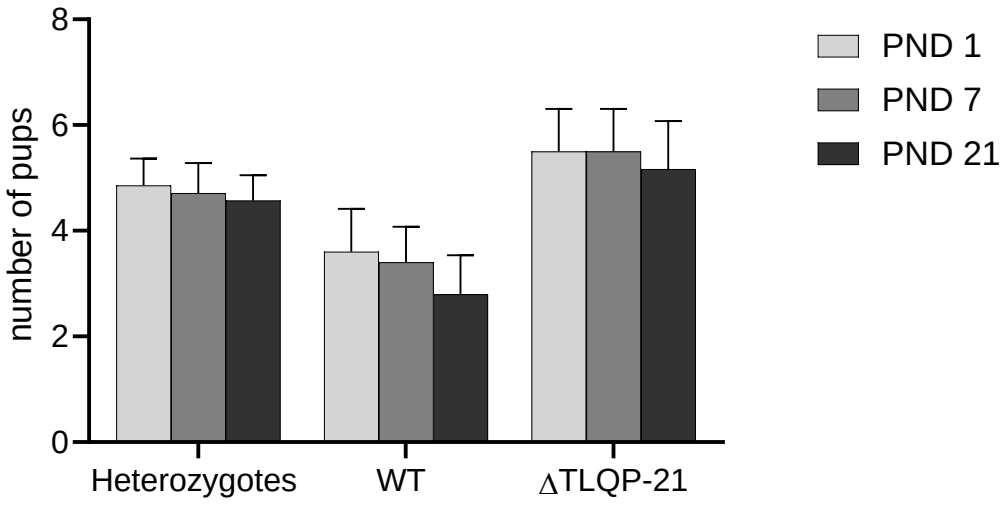

Fig. 12. Spontaneous maternal behaviour of ΔTLQP-21 homozygous ΔTLQP-21 dams. Reproductive success, calculated as number of pups from PND1 to weaning (PND21). Data are presented as mean ± SEM.

Emotional behaviour

Data were examined using parametric analysis of variance (ANOVA) with parents' genotype (homozygotes vs heterozygotes), genotype of the offspring (WT vs ΔTLQP-21) and gender (females vs males) as between-subject factors, and total duration, total frequency and zones of the EPM as within-subjects repeated measures. All animals were analysed for total frequency of entries and total time spent in the three zones of EPM (closed arms, open arms, centre),and locomotor activity (total distance walked). We decided not to include heterozygous offspring to focus on the possible effects of the loss of TLQP21 as compared with WT animals.

Number of animals we achieved was low as compared with a regular study (n=10 males, n= 5 females WT and n=9 males, n=5 females ΔTLQP-21 from heterozygous parents; n=3 males, n=3 females WT and n=10 males, n=9 females ΔTLQP-21 from homozygous parents). Sample size was quite reduced for the group of WT animals from homozygous parents (n=3 males, n=3 females) so statistical analysis may not result as relevant as in other groups.

Locomotor activity

ANOVA of locomotor activity meant as total distance walked in EPM underlined an effect of parents' genotype ($F_{(1,46)} = 24.320$; $p<.001$) in both males and females regardless of their genotype. In fact offspring of homozygous parents walked more than their respective controls reared by heterozygous parents. No effect of sex and genotype, as shown in **fig.13**.

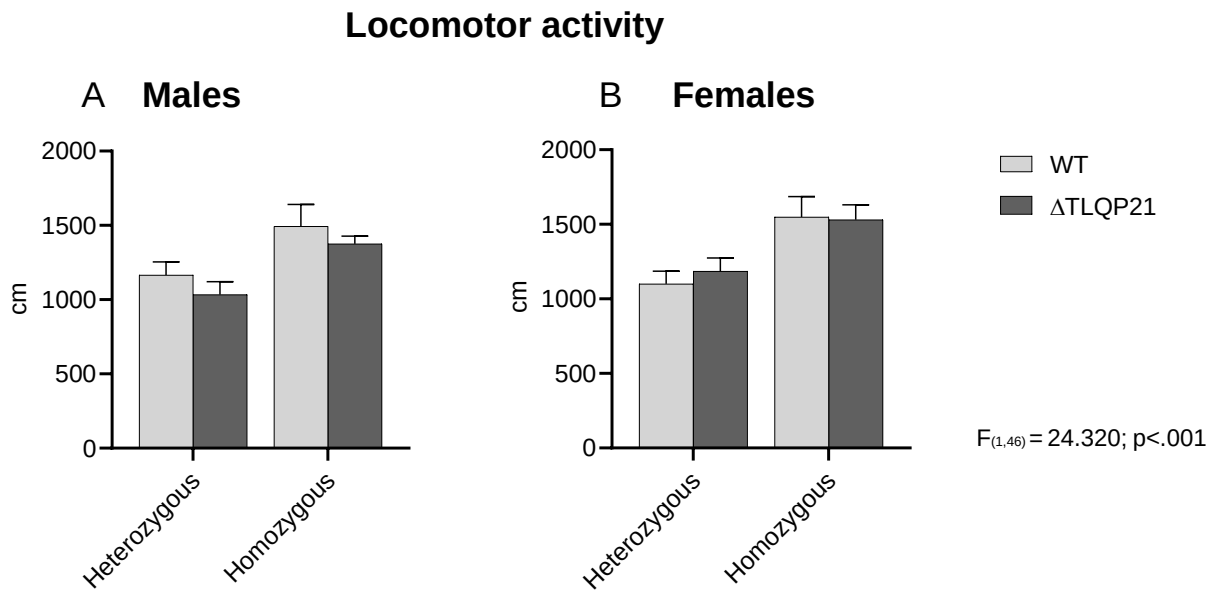

Fig. 13. Total distance walked by male and female WT and ΔTLQP-21 mice from heterozygous (n=10 males, n=5 females WT and n=10 males, n=9 females ΔTLQP-21) or homozygous (n=3 males, n=3 females WT and n=9 males, n=5 females ΔTLQP-21) parents. No significant effect of genotype or sex. Homozygotes' offspring were less active than mice from heterozygous parents, regardless of their genotype. Data are presented as mean ± SEM.

Duration and frequency in EPM zones

All the animals, regardless of parents, sex and genotype, spent most of the time in the closed arm of the apparatus ANOVA run on total time spent in close arms of the apparatus revealed that the main effect is due to parents' genotype ($F_{(1,46)} = 14.309$; $p<.001$): (**Fig.14B-D**). When we analysed total time spent in the centre of the maze, regardless of genotype, in both sexes there was an effect of parents ($F_{(1,46)} = 19.8830$; $p<.001$). Homozygotes' offspring spent more time than heterozygotes' offspring in the centre of the maze. This effect is significant in ΔTLQP-21 males and females of homozygous parents as compared with their counterparts by heterozygous parents (**Fig.14A-C**).

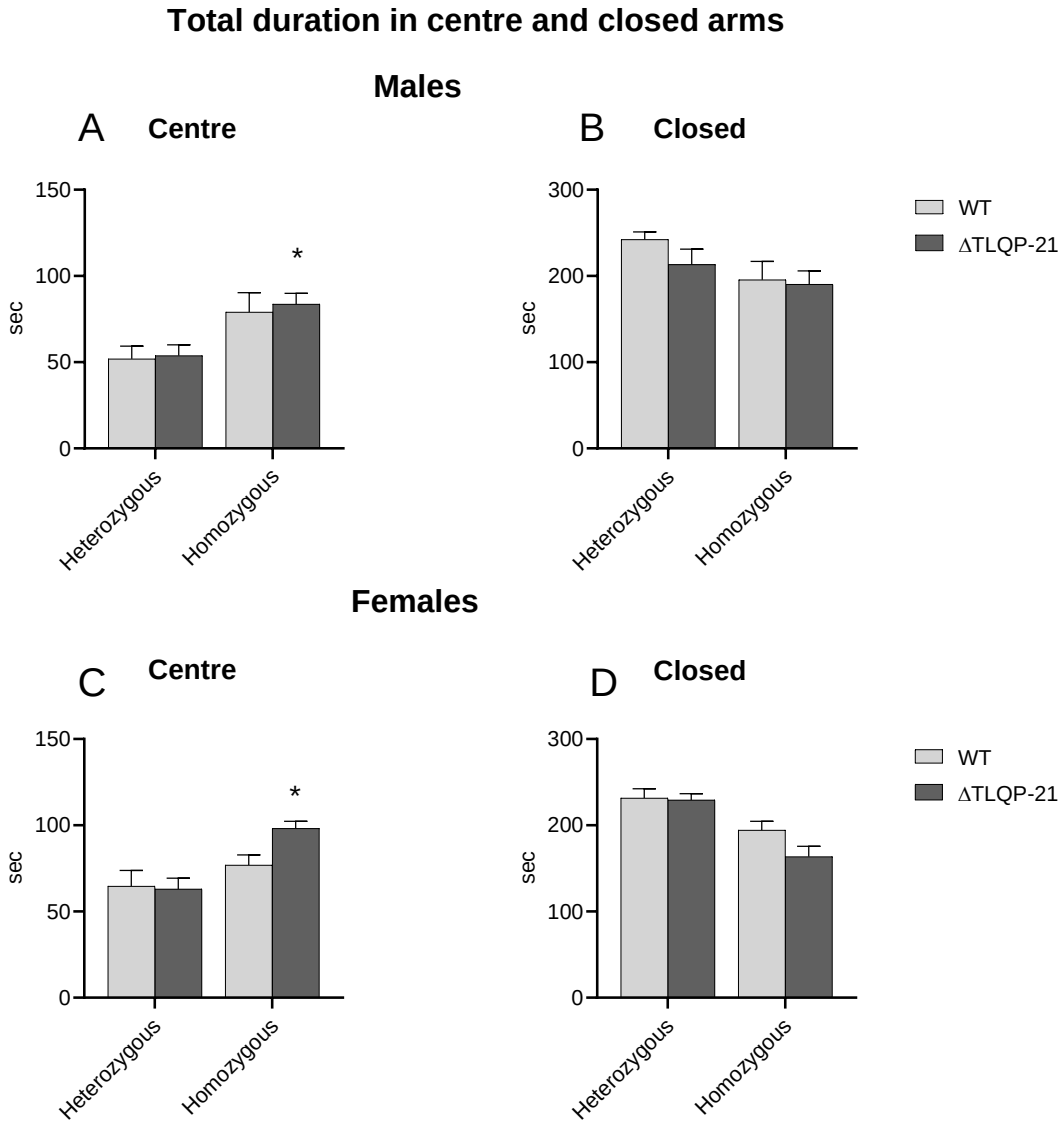

Fig. 14: Total time spent in the centre (**A-C**) and close arms (**B-D**) of the EPM by male and female WT and ΔTLQP-21 mice from heterozygous (n=10 males, n=5 females WT and n=10 males, n=9 females ΔTLQP-21) or homozygous (n=3 males, n=3 females WT and n=9 males, n=5 females ΔTLQP-21) parents. Male (**A**) and female (**C**) offspring of homozygotes spent more time in centre than their counterparts of heterozygotes.. Data are presented as mean ± SEM, * indicates a significant difference with $p<.05$, among heterozygous and homozygous parents.

These trends are confirmed by numbers of entries in centre and closed arms (**Fig.15**). Homozygous' offspring of both sexes and genotypes showed an increased amount of entries in the centre, as compared with offspring of heterozygotes (effect of parents' genotype: $F_{(1,46)} = 17.0689$; $p<.001$) (**Fig.15A-C**). Moreover despite they spent less time in the close arms, there was a higher frequency of entrance in close space as compared with counterparts of heterozygous parents (main effect of parents: $F_{(1,46)} = 8.1555$; $p<.01$) (**Fig.15B-D**).

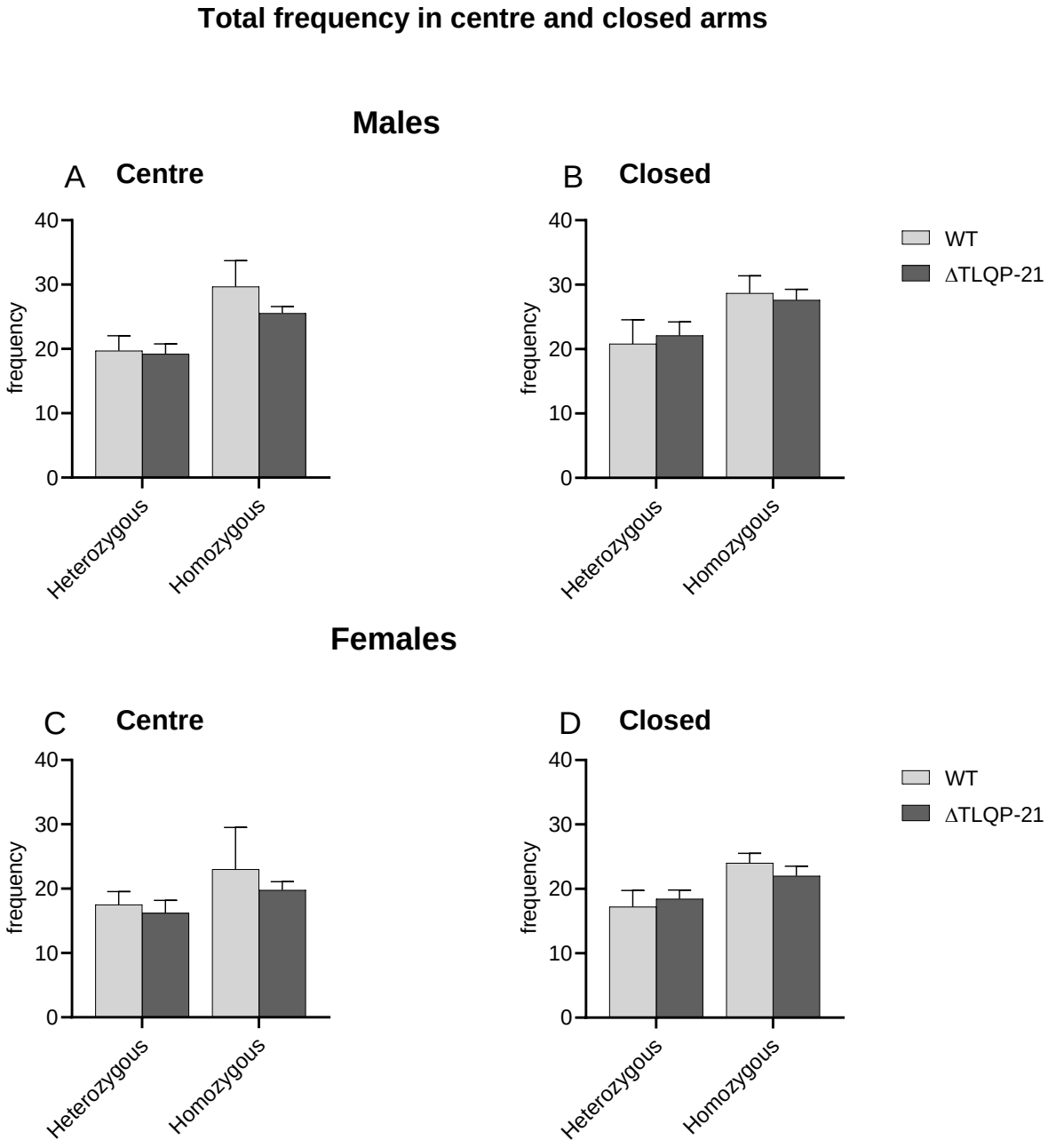

Fig. 15: Total frequency of entries in centre (**A-C**) and close arms (**B-D**) of the EPM by male and female WT and ΔTLQP-21 mice from heterozygous (n=10 males, n=5 females WT and n=10 males, n=9 females ΔTLQP-21) or homozygous (n=3 males, n=3 females WT and n=9 males, n=5 females ΔTLQP-21) parents. Male (**A**) and female (**C**) offspring of homozygotes had a higher frequency of entries in centre and also in close arms (**B**-males; **D**-females). Data are presented as mean ± SEM.

Lastly, we analysed the frequency and the total time spent in the open arms of the maze. First, no significant effect of sex, genotype and parents was detected in analysis of total time in the open arm of the apparatus, as shown in fig.**16A-B**. Nevertheless ANOVA highlighted an effect of parents ($F_{(1,46)}$ = 8.1555; p<.01) in the frequency of entries in open spaces: homozygotes' offspring regardless of genotype and sex showed a higher frequency in open arms as compared to counterparts (**Fig.17A-B**).

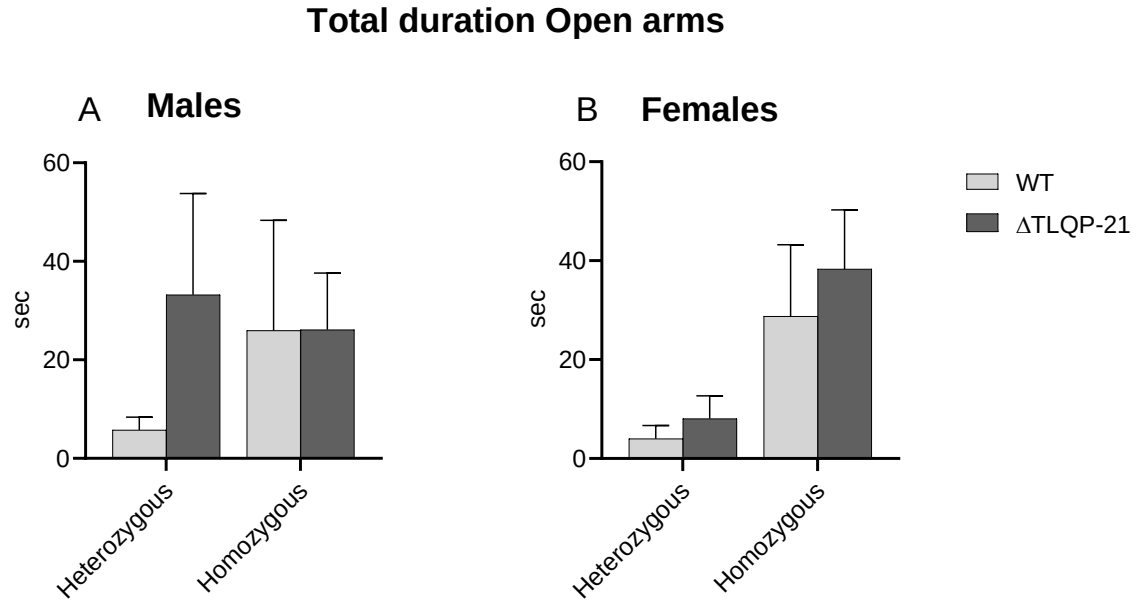

Fig. 16: Total time spent in open arms of the EPM by male (**A)** and female (**B**) WT and ΔTLQP-21 mice from heterozygous (n=10 males, n=5 females WT and n=10 males, n=9 females ΔTLQP-21) or homozygous (n=3 males, n=3 females WT and n=9 males, n=5 females ΔTLQP-21) parents. Data are presented as mean ± SEM

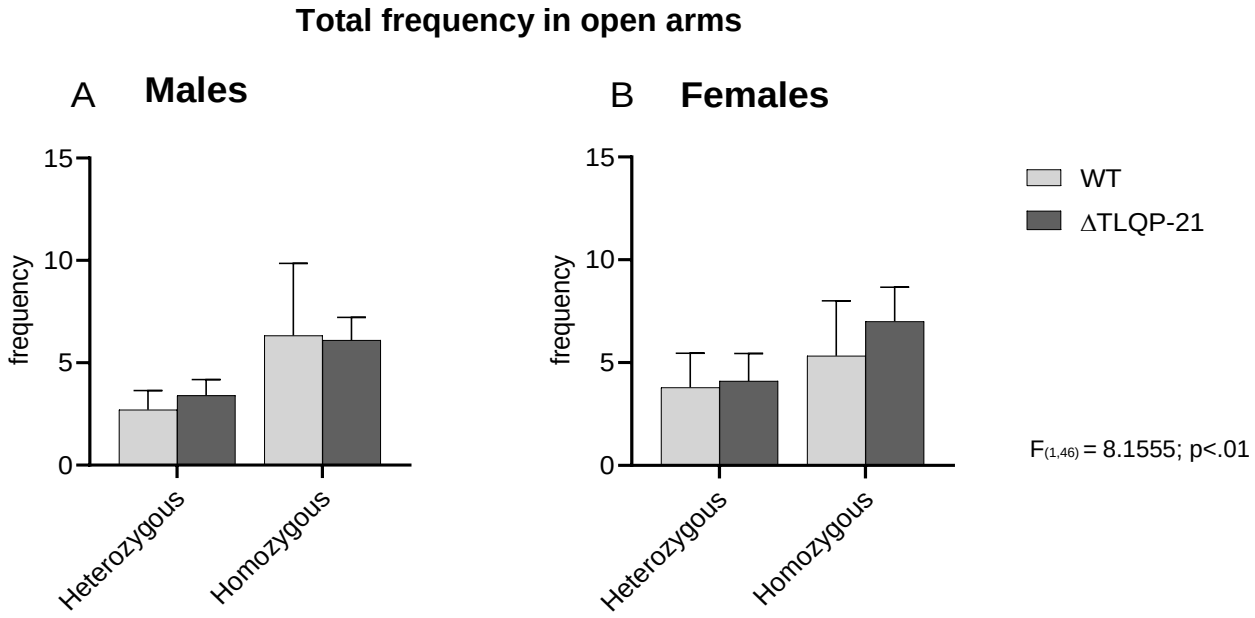

Fig. 17: Total frequency of entries in open arms of the EPM by male (A) and female (B) WT and ΔTLQP-21 mice from heterozygous (n=10 males, n=5 females WT and n=10 males, n=9 females ΔTLQP-21) or homozygous (n=3 males, n=3 females WT and n=9 males, n=5 females ΔTLQP-21) parents. Data are presented as mean ± SEM

5.4 Discussion

In this pilot study we decided to test the hypothesis that selective loss of TLQP-21 peptide could affect maternal behaviour and that this difference in maternal care could lead to different behavioural phenotypes of anxiety in adulthood. TLQP-21 (VGF- derived peptide) exerts multiple functions related to various fields (influence stress response, reproductive behaviour, nociception and pain modulation, energy expenditure and body mass) but nothing has been reported about its effect on maternal care and anxiety. Bartolomucci and Salton recently engineered a new model of selective loss of TLQP21, named ΔTLQP-21 mouse, to better understand several metabolic effects of the VGF-derived peptide. Since the phenotyping of this mouse model is still ongoing, we decided to conduct a pilot behavioural phenotyping by adding information about maternal care and anxiety behaviours Anxiety phenotype might be a consequence of an impairment in several functions, i.e. the coping response to stress and it may be correlated with the amount of maternal care performed by dams in early life (Caldji *et al*., 2000; D'Amato *et al*., 2011; Liu *et al*., 2000b; Plotsky and Meaney, 1993). First of all to understand the role of maternal care in shaping offspring development in mice it's important to figure out the characteristics of variations in maternal behavior, especially for a new model in which most of the behavioural phenotypes are unknown.

Results of maternal observations underlined a main difference among the groups, since Heterozygous , WT and ΔTLQP-21 dams presented different levels of maternal behaviours. In fact, heterozygous dams presented a significant decrease from week 1 to week 2 of observation especially in behaviours related to pups. On the contrary, total amount of time spent in behaviours not related to pups increased from week 1 and week 2, as confirmed by the time spent out of the nest or involved in active behaviours (i.e. eating or drinking). Time weekly spent in arched back behaviour and nursing was expected to decrease, since newborn mice are unable to regulate their body temperature and to eat anything but maternal milk for the first days; that's why mothers usually leave the nest only for brief periods during the first week. Around 10-12 day pups start to eat solid food and maternal milk is only a "complement" to diet (Guénet *et al*., "Genetic of the mouse", 2015). Statistical analysis of WT dams highlighted a similar trend but the difference among behaviours in weeks was less remarkable than in heterozygous mice. Vice-versa homozygous ΔTLQP-21 dams slightly decreased the amount of arched back behaviour, one of the main index of quality of maternal care (Palanza et al., 2002), during week 2 of observation, and significantly increased the percentage of time spent in nursing behaviour from week 1 to week 2. Difference in nursing behavior perhaps, was the most peculiar, since we observed it only in mutant dams. For this reason we hypothesized that this altered maternal behaviour might be due to the selective loss of TLQP-21. This appeared to be in contrast to what observed in Petrocchi-Passeri *et al*., 2013, where TLQP-21 is proved to play a role in stimulation of prolactin expression and secretion. Moreover Salton et al. in 2000 demonstrated that VGF-deficient female mice are unable to nurse their pups, perhaps for an

incomplete development of mammary glands. Nevertheless, since it's known that different transmitter systems act in concert to induce full maternal behavior, it is possible that loss of TLQP-21 might affect the production or the release of another main hormone related to nursing behaviour. However, since this study is exploratory and sample size are extremely reduced, further and deeper studies are necessary to better understand the role of TLQP-21 in maternal and nursing behaviour.

The second phase of the pilot study was aimed to observe how a feasible change in mother's behaviour could affect behavioural development, especially anxiety-like behaviour, in adulthood. Unfortunately sample size of one of the observed groups (WT males and females from heterozygous parents) was lower than others, so data need to be analysed cautiously. Overall observations of the Elevated Plus maze data suggested that the main factor that seems to influence the expression of anxiety could be parents' genotype. Preliminary results indicated that mice of both sexes, regardless of genotype, when reared by heterozygous parents showed less locomotor activity than mice reared by both homozygous ΔTLQP-21 or WT parents. Males and females reared by heterozygous spent also more time in the closed arm of the EPM and less time in centre. On the contrary, offspring of homozygous WT and ΔTLQP-21 mice spent less time in the close arms of the maze as compared with heterozygotes' offspring. No significant effect is detected for time spent in open arms, the main value used in EPM to analyse anxiety behaviors. Data may indicate that the level of maternal care received in early postnatal days, due to parents' genotype might affect the expression of anxiety in adulthood, but reduced sample size of some groups made hard to understand the results. In fact, no effect of sex or offspring's genotype has been detected in any statistical analysis performed on dataset. A recent study of Mizoguchi *et al.*, 2017, demonstrated that the overexpression of VGF and its products might lead to a decreased anxiety. Here we only observed that the absence of VGF derived peptide TLQP-21 doesn't seem to be relevant to the development of anxiety like phenotype in this murine model. By the way, the amount of maternal care received in early life, and perhaps connected to selective loss of TLQP-21 might play a role in development of anxiety behaviours in ΔTLQP-21 mouse model .

Taken together these results offer a starting point for further investigation directed to better understand the behavioural role of TLQP21, its influence on maternal care and whether or not early maternal environment could act on behavioural development in ΔTLQP-21 mice.

Chapter 6

6 General conclusions

Interactions between genes and environment are recognized to play a critical role in modulating the development of the organism. In mammals, early maternal environment is recognized as a key factor in shaping neuro-behavioural and physiological development of the offspring throughout the lifespan (Fleming et al 1999). However, Individual's sex is a fundamental biological factor, which plays a key role in the regulation of energy homeostasis (Kautzky-Willer et al., 2016), cognition (Andreano and Cahill 2009; Gurvich et al. 2018) and behavior (Palanza and Parmigiani 2017; Choleris et al. 2018). Thus the functional interplay between genetic and environmental factors is likely to differ in males and females. The aim of this thesis was to assess the complex interaction between early maternal environment, genes and sex on metabolic and behavioural development in different mouse models, focussing on three main experiments .

In the first experiment the aim was to assess the impact of early maternal environment and sex in a conditional Knock-out mouse model. We examined the effects of conditional Npy1r inactivation in male and female mice reared by foster FVB and C57 dams on anxiety-like and aggressive behaviour, and metabolic functions. Data confirmed that FVB and C57 dams present different profiles of maternal care and are characterized by high levels (FVB) and low levels (C57) of maternal behaviours addressed to pups. Npy1r^{rfb} mice, in which NPY1r gene was selectively inactivated in excitatory neurons of the brain limbic system as adults, showed a lower body weight growth only when reared by foster dams of the FVB strain (high maternal care), as reported in Bertocchi *et al*., 201. However, we demonstrated that when challenged with HFD, Npy1r^{rfb} mice are more sensitive to diet-induced obesity and glucose intolerance. This is confirmed by the higher body weight and amount of white adipose tissue, as compared with controls. No Differences in body weight and response to HFD are present in Npy1r^{rfb} males reared by C57 foster dam (low maternal behavior), thus indicating that the early maternal environment and genotype both contribute to the expression of vulnerability to diet induced metabolic phenotype in males. On the contrary, Npy1r^{rfb} females did not show a different body weight growth compared to control, regardless of early maternal environment, either when at standard or high fat diet, suggesting a resistance against the effect of hypercaloric regimen, possibly due to the protective actions of estrogens (Litwak *et al*., 2014; Riant *et al*., 2009).

The analysis of emotional behavior reveal an anxious-like phenotype in all mice, regardless genotype, sex and maternal care; however, Npy1r^{rfb} mice present a lower locomotor activity in the EPM and a lower latency to approach new snack in the NISF test. Moreover, mice of both genotypes reared by C57 foster dams, showed slightly lower anxiety in the EPM, thus suggesting a possible effect of maternal care on anxiety responses, in line with Kessler *et al*., 2011. Finally, aggressive and social behaviors appear to be affected by genotype and maternal environment; Npy1r^{rfb} mice

showed a decrease in male aggression and in female social behavior only when reared by high the high maternal care strain (FVB), as compared with their counterparts.

Based on these results, we have demonstrated that limbic Npy1r is differently regulated in males and females and that its actions are strongly dependent upon sex. We also were able to demonstrate the early maternal environment that different effects seem to be linked to early environment and the amount of maternal care received during first postnatal days, especially in males.

In the second study, we aimed to examine the effects of the early maternal environment and sex in control NPYlox mice (wild-type C57BL6J-derived) and to determine at what extent different levels of maternal care during the first postnatal week affect anxiety, aggressive behaviour and metabolism in males and females. To this aim, we performed an analysis on all the data collected from 13 different experimental cohorts of mice, including 4 different strains of foster mothers that were characterised for displaying different levels of maternal cares; CD1 and FVB foster dams presented higher amounts of maternal behaviour compared to C57 and Balb dams. Although pups' body weight didi not different at fostering, both sex offspring reared by CD1 and Balb foster dams showed higher body weight at day 10 and at weaning compared to FVB and C57 reared offspring, perhaps due to the increased body size and milk quantity/quality in these strains. This foster-mother effect on the offspring body weight growth disappear in adult mice on a standard diet, but it reemerges when animals were subjected at the HFD regimen. Male mice reared by CD1 and Balb foster dams seem to be more prone to develop diet-induced obesity, based on their robust body weight growth and higher abdominal fat (WAT). Our results suggest that the sex and early maternal environment affect metabolism functions during adulthood, but factors other than the amount of maternal cares received, such as the nutritional values and the milk composition actively influenced the development of the offspring and metabolic patterns in adults.

Maternal cares appear to also play a key role in shaping anxiety behaviour. In fact, our results on behavioral responses in the EPM and the applied principal component analysis, indicate that mice reared by foster mothers displaying a high level of maternal care (more specifically, CD1 dams) present higher arousal and motor activaction in response to the novel environment associated to high anxiety-like response than their counterparts reared by low maternal care dams (C57). Furthermore in the NISF test, only male mice reared by FVB strain (high maternal care) have a higher latency of approach to novel palatable snack, confirming that early maternal environment and marginally sex, may affects anxiety levels.

On the other side, foster mothers' strain also affetcs aggression and sociability in males, as mice reared by CD1 foster dams (HM) tend to be more aggressive, attack more quickly an intruder and display lower social behaviours. Thus suggesting that different amounts of maternal care might influence emotional and aggressive behaviour, sometimes in a sex-dependent way.

Lastly, we carried out a pilot study to evaluate whether the selective loss of a VGF-derived peptide (TLQP-21) could affect the amount of maternal care, and, in turn, how the imbalance in maternal behaviour could be related to the development of different behavioural phenotypes in WT or mutant mice of the TLQP-21 line. Maternal observation of the three experimental groups indicate that ΔTLQP-21 dams display a higher amount of nursing during the second week of observation, suggesting that loss of the VGF-derived peptide might somehow increase the hormonal pathway connected with maternal care. Even if results are in apparent in contrast with previous studies (Salton, *et al*., 2000; Aguilar *et al*., 2013), we hypothesized that in this new mouse model the lack of TLQP-21 might affect the production or the release of another main hormone related to nursing behaviour.

Analysis of anxiety behaviour using Elevated Plus Maze paradigm doesn't show any difference due to genotype (WT or ΔTLQP-21) or sex. The only weak effect underlined by ANOVA is due to genotype of parents (heterozygous or homozygous), and this may be possibly caused by two different factors. First, as ΔTLQP-21 model currently is not entirely phenotyping from behavioural side, the absence of a clear anxiety outline may suggest that the selective loss of the peptide not induce any behavioural change from the control mouse. Further studies will be required, perhaps using a different paradigm, i.e. open field, and increasing the number of replicates to validate or refute current results. Secondly, since it was a pilot study, number of animals in each group analysed in EPM was significant reduced, in particular male and female mice with WT phenotype and reared by heterozygous dams was extremely lower (n=3 m, n=3 f). Actually, the main purpose of a pilot is to examine the viability of an approach that is tough to be studied in a larger scale, and there are critical limitations to their role and interpretation, as they are not a hypothesis testing study and therefore efficacy are not evaluated (Leon et al., 2011). Our preliminary findings suggest that the selective mutation of VGF gene, directed to the loss of TLQP-21 peptide, could increase the amount of maternal care displayed by mothers, but it doesn't seem to affect anxiety phenotype in adult offspring.

Altogether, present findings suggest that early maternal environment, genes and sex, equally contribute to shaping metabolism and behaviour of individuals. For this reason, their complex interaction needs to be further investigated in many fields, as the relations among these three factors might be a crucial step in understanding phenotypic differences and uncover the factors influencing individuals' susceptibility to neurobehavioral and metabolic disorders

7 References

Ainsworth, M. S. "Infant–mother attachment." *American psychologist* 34.10 (1979): **932**.

Alder, J., et al. "Brain-derived neurotrophic factor-induced gene expression reveals novel actions of VGF in hippocampal synaptic plasticity." *Journal of Neuroscience* **23**.34 (2003): 10800-10808.

Anda, R.F., et al. "The enduring effects of abuse and related adverse experiences in childhood." *European archives of psychiatry and clinical neuroscience* **256.3** (2006): 174-186.

Andreano, J.M., and Cahill, L. "Sex influences on the neurobiology of learning and memory." *Learning & memory* **16**.4 (2009): 248-266.

Anker, J.J., and Carroll, M.E. "Females are more vulnerable to drug abuse than males: evidence from preclinical studies and the role of ovarian hormones." *Biological basis of sex differences in psychopharmacology*. Springer, Berlin, Heidelberg, (2010). 73-96.

Antal-Zimanyi, I., et al. "Pharmacological characterization and appetite suppressive properties of BMS-193885, a novel and selective neuropeptide Y(1) receptor antagonist." *Eur. J. Pharmacol.* **590**, (2008): 224–232.

Archer, J. "Rodent sex differences in emotional and related behavior." *Behavioral biology* **14**.4 (1975): 451-479.

Assary, E., et al. "Gene-environment interaction and psychiatric disorders: Review and future directions." *Seminars in cell & developmental biology*. **Vol. 77**. Academic Press, 2018.

Avgustinovich, D.F., et al. "Response of the serotoninergic brain system to social stress of various duration in male mice C57BL/6J and CBA/Lac." *Rossiiskii fiziologicheskii zhurnal imeni IM Sechenova* 87.4 (2001): 532-542

Babb, J.A., et al. "Transgenerational effects of social stress on social behaviour, corticosterone, oxytocin, and prolactin in rats." *Hormones and behaviour* 65.4 (2014): 386-393.

Bannon, A.W. et al. "Behavioural characterization of neuropeptide Y knockout mice." *Brain Res.* **868**, (2000): 79–87

Bao, A.M, and Swaab, D.F. "Sexual differentiation of the human brain: relation to gender identity, sexual orientation and neuropsychiatric disorders." *Frontiers in neuroendocrinology* **32**.2 (2011): 214-226.

Bartolomucci, A., et al. "The extended granin family: structure, function, and biomedical implications." *Endocrine reviews* **32.6** (2011): 755-797.

Bartolomucci, A., et al. "TLQP-21, a VGF-derived peptide, increases energy expenditure and prevents the early phase of diet-induced obesity." *Proceedings of the National Academy of Sciences* **103**.39 (2006): 14584-14589.

Becker, J.B., and Hu, M. "Sex differences in drug abuse." *Frontiers in neuroendocrinology* **29**.1 (2008): 36-47.

Bekker, M.HJ., and van Mens-Verhulst, J. "Anxiety disorders: sex differences in prevalence, degree, and background, but gender-neutral treatment." *Gender medicine* **4** (2007): S178-S193.

Bertocchi, I., et al. "Regulatory functions of limbic Y1 receptors in body weight and anxiety uncovered by conditional knockout and maternal care." *Proc. Natl. Acad. Sci.* **108**, (2011): 19395–19400.

Bisceglia, R., et al. "Arginine vasopressin 1a receptor gene and maternal behavior: evidence of association and moderation." *Genes, Brain and Behavior* **11**.3 (2012): 262-268.

Björntorp, P. "Metabolic implications of body fat distribution." *Diabetes Care* **14**, (1991): 1132–1143.

Bowlby, J. "Attachment and loss: retrospect and prospect." *American journal of Orthopsychiatry* **52.4** (1982): 664.

Bowlby, J. "Attachment and Loss", vol 1, Attachment, Basic, New York, 1980.

Brancia C., et al. "Differential expression and seasonal modulation of VGF peptides in sheep pituitary." J Endocrinol 2005; **186**:97- 107.

Brodkin, E.S. "Balb mice: low sociability and other phenotypes that may be relevant to autism." *Behavioural brain research* 176.1 (2007): 53-65.

Broqua, P., et al. "Behavioural effects of neuropeptide Y receptor agonists in the elevated plus maze and fear-potentiated startle procedures." *Behav. Pharmacol.* **6**, (1995): 215–222

Brown, G.R., & Nemes, C. "The exploratory behaviour of rats in the hole-board apparatus: is head-dipping a valid measure of neophilia?". *Behavioural processes*, ***78***(3), (2008): 442-448.

Caldji, C., et al. "Variations in maternal care in infancy regulate the development of stress reactivity." *Biological psychiatry* **48.**12 (2000): 1164-1174.

Can, A., et al. "The mouse forced swim test." *Journal of visualized experiments : JoVE* ,**59** e3638. 29 Jan. 2012, doi:10.3791/3638

Carola, V., et al. "Effect of the interaction between the serotonin transporter gene and maternal environment on developing mouse brain." *Behavioural brain research* **217.1** (2011): 188-194.

Carter, M., and Shieh, J. in Guide to Research Techniques in Neuroscience (Second Edition), (2015)

Cattaneo, A., et al. "The expression of VGF is reduced in leukocytes of depressed patients and it is restored by effective antidepressant treatment." *Neuropsychopharmacology* 35.7 (2010): 1423.

Cero, C., et al. "The neuropeptide TLQP-21 opposes obesity via C3aR1-mediated enhancement of adrenergic-induced lipolysis." *Molecular metabolism* **6.1** (2017): 148-158.

Champagne, F.A., and Curley, J.P. "Epigenetic mechanisms mediating the long-term effects of maternal care on development." *Neuroscience & Biobehavioral Reviews* **33**.4 (2009): 593-600.

Champagne, F.A., et al. "Natural variations in postpartum maternal care in inbred and outbred mice." *Physiology & behavior*, ***91***(2-3), (2007): 325-334.

Chiavegatto, S., and Nelson, R.J, "Interaction of nitric oxide and serotonin in aggressive behaviour." *Hormones and Behaviour* **44.3** (2003): 233-241.

Chiu, C., et al. "Developmental Impact of a Familial GABA(A) Receptor Epilepsy Mutation." *Ann. Neurol.* **64**, (2008): 284–293.

Choleris, E., et al. "Sex differences in the brain: Implications for behavioral and biomedical research." *Neuroscience & Biobehavioral Reviews* **85** (2018): 126-145.

Cicchetti, D., et al. "Bowlby's dream comes full circle." *Advances in clinical child psychology*. Springer, Boston, MA, (1995). 1-75.

Cirulli, F., et al. "Early disruption of the mother–infant relationship: effects on brain plasticity and implications for psychopathology." *Neuroscience & Biobehavioural Reviews* **27**.1-2 (2003): 73-82.

Cirulli, F., et al. "Early life stress as a risk factor for mental health: role of neurotrophins from rodents to non-human primates." *Neuroscience & Biobehavioural Reviews* 33.4 (2009): 573-585.

Clark, J.T., et al. “Neuropeptide Y and human pancreatic polypeptide stimulate feeding behaviour in rats.” *Endocrinology* **115**, (1984): 427–429

Clayton, J.A., and Collins, F.S. "Policy: NIH to balance sex in cell and animal studies." *Nature News* 509.7500 (2014): 282.

Cox, K.H., and Rissman, E.F. "Sex differences in juvenile mouse social behavior are influenced by sex chromosomes and social context." *Genes, Brain and Behavior* **10**.4 (2011): 465-472.

Crawley, J.N. “Mouse behavioural assays relevant to the symptoms of autism.” *Brain Pathol.* **17**, (2007): 448–59

Crusio, W.E., et al. "Standards for the publication of mouse mutant studies." *Genes, Brain and Behavior* **8**.1 (2009): 1-4.

Dadomo, H., et al. “Vulnerability to chronic subordination stress-induced depression-like disorders in adult 129SvEv male mice.” *Prog. Neuro-Psychopharmacology Biol. Psychiatry* **35**, (2011): 1461–1471

D'Amato, F.R., et al. "Unstable maternal environment, separation anxiety, and heightened CO2 sensitivity induced by gene-by-environment interplay." *PLoS One* **6.4** (2011): e18637.

Dempfle, A., et al. "Gene–environment interactions for complex traits: definitions, methodological requirements and challenges." *European Journal of Human Genetics* **16**.10 (2008): 1164.

Des Marais, et al. "Genotype-by-environment interaction and plasticity: exploring genomic responses of plants to the abiotic environment." *Annual Review of Ecology, Evolution, and Systematics* **44** (2013): 5-29.

Dick, D.M. "Gene-environment interaction in psychological traits and disorders." *Annual review of clinical psychology* **7** (2011): 383-409.

Ding, B. et al. “Human neuropeptide Y signal peptide gain-of-function polymorphism is associated with increased body mass index: possible mode of function.” *Regul. Pept.* **127**, (2005): 45–53.

Doolen, S., et al. "Complement 3a receptor in dorsal horn microglia mediates pronociceptive neuropeptide signaling." *Glia* **65**.12 (2017): 1976-1989.

Dow, H.C., et al. "Genetic dissection of intermale aggressive behaviour in BalbJ and A/J mice." *Genes, Brain and Behaviour* **10**.1 (2011): 57-68.

Ďureje, L., et al. "No postnatal maternal effect on male aggressiveness in wild-derived strains of house mice." *Aggressive behaviour* **37**.1 (2011): 48-55.

Ekblad, E., et al. “Neuropeptide Y co-exists and co-operates with noradrenaline in perivascular nerve fibers.” *Regul. Pept.* **8**, (1984): 225–235.

Eva, C., et al. “Physiology and gene regulation of the brain NPY Y1 receptor.” *Front. Neuroendocrinol.* **27**, (2006): 308–339.

Fairbanks, C.A., et al. "The VGF-derived peptide TLQP-21 contributes to inflammatory and nerve injury-induced hypersensitivity." *PAIN*® **155.7** (2014): 1229-1237.

Fargali, S, et al. "Role of neurotrophins in the development and function of neural circuits that regulate energy homeostasis." *Journal of Molecular Neuroscience* **48.3** (2012): 654-659.

Fargali, S. et al. “The granin VGF promotes genesis of secretory vesicles, and regulates circulating

catecholamine levels and blood pressure." *FASEB J.* **28**, 2120–33 (2014).

Febo, M., et al. "Oxytocin modulates unconditioned fear response in lactating dams: an fMRI study." *Brain research* 1302 (2009): 183-193.

Ferri, G.L., et al. "The" VGF" protein in rat adenohypophysis: sex differences and changes during the estrous cycle and after gonadectomy." *Endocrinology* **136.5** (1995): 2244-2251.

Fleming, A.S., et al. "Mothering begets mothering: the transmission of behavior and its neurobiology across generations." *Pharmacology Biochemistry and Behavior* **73**.1 (2002): 61-75.

Fombonne, E. "The epidemiology of pervasive developmental disorders." *Recent Developments in Autism Research, MF Casanova, ed.(New York, Nova Science Publishers Inc.)* (2005): 1-25.

Francis, D.D., and Meaney, M.J. "Maternal care and the development of stress responses." *Current opinion in neurobiology* **9**.1 (1999): 128-134.

Gaillard, D., et al. "Molecular mechanisms of fat preference and overeating." *Ann. N. Y. Acad. Sci.* **1141**, (2008): 163–175

Gershon, A., et al. "The long-term impact of early adversity on late-life psychiatric disorders." *Current psychiatry reports* **15**.4 (2013): 352.

Gilks, W.P. "Sex differences in disease genetics." *eLS* (2001): 1-7.

Gioiosa, L., et al. "Developmental exposure to low-dose estrogenic endocrine disruptors alters sex differences in exploration and emotional responses in mice." *Hormones and behaviour* **52**.3 (2007): 307-316.

Golden, L.C., and Voskuhl, R. "The importance of studying sex differences in disease: The example of multiple sclerosis." *Journal of neuroscience research* **95**.1-2 (2017): 633-643.

Gray, J.A. "Sex differences in emotional behaviour in mammals including man: endocrine bases." *Acta psychologica* **35**.1 (1971): 29-46.

Guénet, J. L., et al. "*Genetics of the Mouse*." Berlin: Springer. (2015)

Gunnar, M.R., and Quevedo, K.M. "Early care experiences and HPA axis regulation in children: a mechanism for later trauma vulnerability." *Progress in brain research* **167** (2007): 137-149.

Guo, Z.K, et al. "Clearance kinetics of the VGF-derived neuropeptide TLQP-21." *Neuropeptides* 71 (2018): 97-103.

Gurvich, C., et al. "Sex differences and the influence of sex hormones on cognition through adulthood and the aging process." *Brain sciences* **8**.9 (2018): 163.

Hahm, S., et al. "Targeted deletion of the Vgf gene indicates that the encoded secretory peptide precursor plays a novel role in the regulation of energy balance." *Neuron* 23.3 (1999): 537-548.

Hannedouche, S., et al. "Identification of the C3a receptor (C3AR1) as the target of the VGF-derived peptide TLQP-21 in rodent cells." *Journal of Biological Chemistry* **288**.38 (2013): 27434-27443.

Hashimoto, K., and Ogawa, Y. "Epigenetic switching and neonatal nutritional environment." *Developmental Origins of Health and Disease (DOHaD)*. Springer, Singapore, 2018. 19-25.

Haynes, A.C., et al. "Characterisation of the neuropeptide Y receptor that mediates feeding in the rat: a role for the Y5 receptor?" *Regul. Pept.* **75**–**76**, (1998): 355–361.

Heilig, M., et al. "Corticotropin-releasing factor and neuropeptide Y: Role in emotional integration."

Trends Neurosci. **17**, (1994): 80–85

Heilig, M., et al. “Decreased cerebrospinal fluid neuropeptide Y (NPY) in patients with treatment refractory unipolar major depression: preliminary evidence for association with preproNPY gene polymorphism.” *J. Psychiatr. Res.* **38**, (2004): 113–121

Henriques, T.P., et al. "Stress in neonatal rats with different maternal care backgrounds: monoaminergic and hormonal responses." *Neurochemical research* **39**.12 (2014): 2351-2359.

Henry, M., et al. “Energy metabolic profile of mice after chronic activation of central NPY Y1, Y2, or Y5 receptors.” *Obes. Res.* **13**, (2005): 36–47.

Herzog, H. “Neuropeptide Y and energy homeostasis: Insights from Y receptor knockout models.” In *European Journal of Pharmacology*, (2003): pp. 21–29.

Hofer, M.A. "Relationships as regulators: A psychobiologic perspective on bereavement." *Psychosomatic medicine* 46.3 (1984): 183-197.

Hokfelt, T., et al. “Neuropeptide Y: some viewpoints on a multifaceted peptide in the normal and diseased nervous system.” *Brain Res. Brain Res. Rev.* **26**, (1998): 154–166

Holmes, A., et al. "Early life genetic, epigenetic and environmental factors shaping emotionality in rodents." *Neuroscience & Biobehavioural Reviews* 29.8 (2005): 1335-1346.

Hsieh, L.S., et al. "Outbred CD1 mice are as suitable as inbred C57BL/6J mice in performing social tasks." *Neuroscience letters* 637 (2017): 142-147.

Huda, M. S. B., et al. “Gut peptides and the regulation of appetite.” *Obes. Rev.* **7**, (2006): 163–182.

Ingvorsen, C., et al. “The role of sex and body weight on the metabolic effects of high-fat diet in C57BL/6N mice.” *Nutrition & diabetes* vol. **7,4** e261.(2017)

Jedrychowski, M. P. et al. “Detection and Quantitation of Circulating Human Irisin by Tandem Mass Spectrometry.” *Cell Metab.* **22**, 734–740 (2015).

Jensen, M. D. “Role of body fat distribution and the metabolic complications of obesity.” *J. Clin. Endocrinol. Metab.* **93**, (2008): S57-63

Jethwa, P.H., et al. "VGF-derived peptide, TLQP-21, regulates food intake and body weight in Siberian hamsters." *Endocrinology* **148**.8 (2007): 4044-4055.

Johnson, N. L., et al. "Adolescent opiate exposure in the female rat induces subtle alterations in maternal care and transgenerational effects on play behaviour." *Frontiers in psychiatry* **2** (2011): 29.

Johnston, A.L., and File, S.E. "Sex differences in animal tests of anxiety." *Physiology & behavior* **49**.2 (1991): 245-250.

Josephs, E.B. "Determining the evolutionary forces shaping G× E." *New Phytologist* 219.1 (2018): 31-36.

Kallio, J., et al. “Altered intracellular processing and release of neuropeptide Y due to leucine 7 to proline 7 polymorphism in the signal peptide of preproneuropeptide Y in humans.” *FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol.* **15**, (2001): 1242–1244.

Kanatani, A. et al. “A typical Y1 receptor regulates feeding behaviours: effects of a potent and selective Y1 antagonist, J-115814.” *Mol. Pharmacol.* **59**, (2001): 501–505

Kanatani, A., et al. “The novel neuropeptide Y Y(1) receptor antagonist J-104870: a potent feeding suppressant with oral bioavailability.” *Biochem. Biophys. Res. Commun.* **266**, (1999): 88–91

Karl, T., et al. “Y1 receptors regulate aggressive behaviour by modulating serotonin pathways.” *Proc. Natl. Acad. Sci. United States Am.* **101**, (2004): 12742–12747

Karvonen, et al. “Association of a leucine(7)-to-proline(7) polymorphism in the signal peptide of neuropeptide Y with high serum cholesterol and LDL cholesterol levels.” *Nat. Med.* **4**, (1998): 1434–1437

Kask, A., et al. “Neuropeptide Y attenuates the effect of locus coeruleus denervation by DSP-4 treatment on social behaviour in the rat.” *Neuropeptides* **34**, (2000): 58–61.

Kask, A., et al. “The neurocircuitry and receptor subtypes mediating anxiolytic-like effects of neuropeptide Y.” *Neurosci. Biobehav. Rev.* **26**, (2002): 259–283

Kautzky-Willer, A. et al. "Sex and gender differences in risk, pathophysiology and complications of type 2 diabetes mellitus." *Endocrine reviews* **37**.3 (2016): 278-316.

Kessler, M. S., et al. “Maternal care differs in mice bred for high vs. low trait anxiety: impact of brain vasopressin and cross-fostering.” *Social neuroscience*, ***6***(2), (2011): 156-168.

Kessler, R.C. "Epidemiology of women and depression." *Journal of affective disorders* **74**.1 (2003): 5-13.

Kim, J.W, et al. "Gene-environment interaction counterbalances social impairment in mouse models of autism." *Scientific reports* **9**.1 (2019): 1-10.

Kissebah, A.H. and Krakower, G.R. “Regional adiposity and morbidity.” *Physiol. Rev.* **74**, (1994): 761–811.

Klengel, T., and Binder, E.B., "Epigenetics of stress-related psychiatric disorders and gene× environment interactions." *Neuron* 86.6 (2015): 1343-1357.

Koch, M, and Ehret, **G.,** "Estradiol and parental experience, but not prolactin are necessary for ultrasound recognition and pup-retrieving in the mouse." *Physiology & behaviour* **45**.4 (1989): 771-776.

Kosten, T.A., et al. "Early life manipulations alter learning and memory in rats." *Neuroscience & Biobehavioural Reviews* **36**.9 (2012): 1985-2006.

Krasnegor, N.A., and Bridges, R.S. "Mammalian parenting: Biochemical, neurobiological, and behavioural determinants." *National Institute of Child Health and Human Development Conference on Biological and Behavioural Determinants of Parental Behaviour in Mammals, Sep, 1987, Leesburg, VA, US*. Oxford University Press, 1990.

Krestel, H. E., et al. “A Genetic Switch for Epilepsy in Adult Mice.” *J. Neurosci.* **24**, (2004): 10568 LP-10578.

Kuroda, K.O., et al. "Neuromolecular basis of parental behaviour in laboratory mice and rats: with special emphasis on technical issues of using mouse genetics." *Progress in Neuro-Psychopharmacology and Biological Psychiatry* **35**.5 (2011): 1205-1231.

LaBarba, R.C., and White, J.L. "Litter size variations and emotional reactivity in Balb mice." *Journal of comparative and physiological psychology* **75**.2 (1971): 254.

Ladd, C.O., et al. "Persistent changes in corticotropin-releasing factor neuronal systems induced by maternal deprivation." *Endocrinology* **137**.4 (1996): 1212-1218.

Landers, M.S., and Sullivan, R.M. "The development and neurobiology of infant attachment and fear." *Developmental neuroscience* **34**.2-3 (2012): 101-114.

Latham, N., and Mason, G. "From house mouse to mouse house: the behavioural biology of free-living Mus musculus and its implications in the laboratory." *Applied Animal Behaviour Science* **86**.3-4 (2004): 261-289.

Lennox, R.,et al. “Effects of glucose-dependent insulinotropic polypeptide receptor knockout and a high-fat diet on cognitive function and hippocampal gene expression in mice.” *Mol. Med. Rep.* **12**, (2015): 1544–1548.

Leon, A.C., et al. "The role and interpretation of pilot studies in clinical research." *Journal of psychiatric research* **45**.5 (2011): 626-629.

Levi, A., et al., "Molecular cloning of a gene sequence regulated by nerve growth factor." *Science* 229.4711 (1985): 393-395.

Levin, B.E. "The obesity epidemic: metabolic imprinting on genetically susceptible neural circuits." *Obesity Research* **8**.4 (2000): 342-347.

Levine, A. S. and Morley, J. E. “Neuropeptide Y: a potent inducer of consummatory behaviour in rats.” *Peptides* **5**, (1984): 1025–1029

Lewis, J. E. et al. “Neuroendocrine Role for VGF.” *Frontiers in endocrinology* vol. **6** 3. 2 Feb. 2015, doi:10.3389/fendo.2015.00003

Lin, E.-J. D., et al. “Adult-onset hippocampal-specific neuropeptide Y overexpression confers mild anxiolytic effect in mice.” *Eur. Neuropsychopharmacol.* **20**, (2010): 164–175

Link, J.C., and Reue. K. "Genetic basis for sex differences in obesity and lipid metabolism." *Annual review of nutrition* 37 (2017): 225-245.

Litwak, S.A., et al. “Estradiol prevents fat accumulation and overcomes leptin resistance in female high-fat diet mice.” *Endocrinology* **155**, (2014): 4447–4460

Liu, D., et al. "Maternal care, hippocampal glucocorticoid receptors, and hypothalamic-pituitary-adrenal responses to stress." *Science* **277**.5332 (1997): 1659-1662.

Liu, F., et al. "Disrupted cortical hubs in functional brain networks in social anxiety disorder." *Clinical Neurophysiology* **126**.9 (2015): 1711-1716.

Loman, M.M., and Gunnar, **M.R.** "Early experience and the development of stress reactivity and regulation in children." *Neuroscience & biobehavioural reviews* **34**.6 (2010): 867-876.

Lucion, A.B., and Bortolini, **M.C.** "Mother–pup interactions: rodents and humans." *Frontiers in endocrinology* **5** (2014): 17.

Macrí, S., et al. "Dissociation in the effects of neonatal maternal separations on maternal care and the offspring's HPA and fear responses in rats." *European Journal of Neuroscience* **20**.4 (2004): 1017-1024.

Mayford, M., et al. “Control of memory formation through regulated expression of a CaMKII transgene.” *Science* **274**, (1996): 1678–1683.

McCarthy, M.M., et al. "Sex differences in the brain: the not so inconvenient truth." *Journal of Neuroscience* **32**.7 (2012): 2241-2247.

McPhie-Lalmansingh, A.A., et al. "Sex chromosome complement affects social interactions in mice." *Hormones and behavior* **54**.4 (2008): 565-570.

Meaney, M.J. "Maternal care, gene expression, and the transmission of individual differences in stress reactivity across generations." *Annual review of neuroscience* **24**.1 (2001): 1161-1192.

Meaney, M.J. and Szyf, M. “Maternal care as a model for experience-dependent chromatin plasticity?” *Trends Neurosci.* **28**, (2005): 456–463.

Merali, Z., et al. “Validation of a simple, ethologically relevant paradigm for assessing anxiety in mice.” *Biol. Psychiatry* **54**, (2003). 552–565

Mitchell, G.C., et al. “A common single nucleotide polymorphism alters the synthesis and secretion of neuropeptide Y.” *J. Neurosci.* **28**, (2008): 14428–14434.

Mizoguchi, T., et al. "Behavioural abnormalities with disruption of brain structure in mice overexpressing VGF." *Scientific reports* **7**.1 (2017): 4691.

Mogi, K., Nagasawa, M. and Takefumi K. "Developmental consequences and biological significance of mother–infant bonding." *Progress in Neuro-Psychopharmacology and Biological Psychiatry* 35.5 (2011): 1232-1241.

Molteni, R., et al. “A high-fat, refined sugar diet reduces hippocampal brain-derived neurotrophic factor, neuronal plasticity, and learning.” *Neuroscience* **112**, (2002): 803–814

Monks, J., et al. "Maternal obesity during lactation may protect offspring from high fat diet-induced metabolic dysfunction." *Nutrition & diabetes* **8**.1 (2018): 1-13.

Mullins, D., et al. “Identification of potent and selective neuropeptide Y Y(1) receptor agonists with orexigenic activity in vivo.” *Mol. Pharmacol.* **60**, (2001): 534–540

Nagamachi, S., et al. "Dietary L-serine modifies free amino acid composition of maternal milk and lowers the body weight of the offspring in mice." *Journal of Veterinary Medical Science* (2017): 17-0577.

Nishigawa, T., et al. "Taurine and β-alanine intraperitoneal injection in lactating mice modifies the growth and behaviour of offspring." *Biochemical and biophysical research communications* 495.2 (2018): 2024-2029.

Nordman, S., et al. “Leu7Pro polymorphism in the neuropeptide Y (NPY) gene is associated with impaired glucose tolerance and type 2 diabetes in Swedish men.” *Exp. Clin. Endocrinol. Diabetes* **113**, (2005): 282–287

Normann, C., and Buttenschøn, **H.N.** "Gene–environment interactions between HPA-axis genes and stressful life events in depression: a systematic review." *Acta neuropsychiatrica* (2019): 1-7.

Numan, M. and Stolzenberg, D.S. "Medial preoptic area interactions with dopamine neural systems in the control of the onset and maintenance of maternal behaviour in rats." *Frontiers in neuroendocrinology* 30.1 (2009): 46-64.

Numan, M., and Insel, T.R., "Motivational models of the onset and maintenance of maternal behaviour and maternal aggression." *The neurobiology of parental behaviour* (2003): 69-106.

Numan, M., and Young, L.J. "Neural mechanisms of mother–infant bonding and pair bonding: similarities, differences, and broader implications." *Hormones and behaviour* **77** (2016): 98-112.

O'Connor, T.G., et al. "Attachment disorder behaviour following early severe deprivation: Extension and longitudinal follow-up." *Journal of the American Academy of Child & Adolescent Psychiatry* **39**.6 (2000): 703-712.

Ogawa, T., et al. "Periodic maternal deprivation alters stress response in adult offspring: potentiates the negative feedback regulation of restraint stress-induced adrenocortical response and reduces the frequencies of open field-induced behaviours." *Pharmacology Biochemistry and Behaviour* **49**.4 (1994): 961-967.

Olivier, B., et al. “Serotonin receptors and animal models of aggressive behaviour.” *Pharmacopsychiatry* **28** Suppl 2, (1995): 80–90.

Oomen, C.A., et al. "Opposite effects of early maternal deprivation on neurogenesis in male versus female rats." *PloS one* 4.1 (2009): e3675.

Orpen, B.G., and Fleming A.S. "Experience with pups sustains maternal responding in postpartum rats." *Physiology & Behaviour* **40**.1 (1987): 47-54.

Ottman, R. “Gene-environment interaction: definitions and study designs.” *Preventive medicine* vol. **25**,6 (1996): 764-70.

Palanza, P., and Parmigiani, S. "How does sex matter? Behavior, stress and animal models of neurobehavioral disorders." *Neuroscience & Biobehavioral Reviews* **76** (2017): 134-143.

Palanza, P., et al. "Exposure to a low dose of bisphenol A during fetal life or in adulthood alters maternal behaviour in mice." *Environmental health perspectives* **110**.suppl 3 (2002): 415-422.

Palanza, P., et al. "Social stress in mice: gender differences and effects of estrous cycle and social dominance." *Physiology & behavior* **73**.3 (2001): 411-420.

Palanza, P., et al. ”Sex-Biased Impact of Endocrine Disrupting Chemicals on Behavioral Development and Vulnerability to Disease: Of Mice and Children” **Submitted to** *Neuroscience and biobehavioral reviews* (2020)

Palmer, B.F., and J. Clegg. "The sexual dimorphism of obesity." *Molecular and cellular endocrinology* **402** (2015): 113-119.

Parmigiani, S., et al. "Selection, evolution of behavior and animal models in behavioral neuroscience." *Neuroscience & Biobehavioral Reviews* **23**.7 (1999): 957-970.

Passeri, P.P., et al. “Neuropeptide TLQP-21, a VGF internal fragment, modulates hormonal gene expression and secretion in GH3 cell line.” *Neuroendocrinology*, ***97***(3), (2013): 212-224.

Petrocchi-Passeri, P., et al. "The VGF-derived peptide TLQP-62 modulates insulin secretion and glucose homeostasis." *J Mol Endocrinol* **54**.3 (2015): 227-239.

Pettersson, U.S. et al. “Female mice are protected against high-fat diet induced metabolic syndrome and increase the regulatory T cell population in adipose tissue.” *PloS one* vol. **7**,9 (2012): e46057. doi:10.1371/journal.pone.0046057

Pietropaolo, S. et al. “The behavioral sequela following the prevention of home-cage grid-climbing activity in C57BL/6 mice.” *Behavioral neuroscience*, ***121***(2), (2007): 345.

Pinilla, L., et al. "Characterization of the reproductive effects of the anorexigenic VGF-derived peptide TLQP-21: in vivo and in vitro studies in male rats." *American Journal of Physiology-Endocrinology and Metabolism* **300**.5 (2011): E837-E847.

Pitzianti, M. et al. "Neurological soft signs are associated with attentional dysfunction in children with attention deficit hyperactivity disorder." *Cognitive neuropsychiatry* **21**.6 (2016): 475-493.

Plotsky, P.M., and Meaney, M.J. "Early, postnatal experience alters hypothalamic corticotropin-releasing factor (CRF) mRNA, median eminence CRF content and stress-induced release in adult rats." *Molecular brain research* **18**.3 (1993): 195-200.

Popova, N.K., et al. "Involvement of brain serotonin 5-HT 1A receptors in genetic predisposition to aggressive behaviour." *Neuroscience and behavioural physiology* **37**.6 (2007): 631-635.

Possenti, R., et al. "Expression, processing, and secretion of the neuroendocrine VGF peptides by INS-1 cells." *Endocrinology* **140**.8 (1999): 3727-3735.

Pryce, C.R., et al. "Comparison of the effects of early handling and early deprivation on conditioned stimulus, context, and spatial learning and memory in adult rats." *Behavioral Neuroscience* **117**.5 (2003): 883.

Razzoli, M., et al. "Implication of the VGF-derived peptide TLQP-21 in mouse acute and chronic stress responses." *Behavioural brain research* **229**.2 (2012): 333-339.

Renshaw, D. and Batterham, R.L. (2005). Peptide YY: a potential therapy for obesity. *Curr. Drug Targets* **6**, 171–179

Riant, E., et al. “Estrogens protect against high-fat diet-induced insulin resistance and glucose intolerance in mice.” *Endocrinology* **150**, (2009): 2109–2117.

Rincón-Cortés, M. and Sullivan, R.M. "Early life trauma and attachment: Immediate and enduring effects on neurobehavioural and stress axis development." *Frontiers in endocrinology* **5** (2014): 33.

Rizzi, R., et al. "The VGF-derived peptide TLQP-21: a new modulatory peptide for inflammatory pain." *Neuroscience letters* **441**.1 (2008): 129-133.

Sadahiro, M. et al*.* “Role of VGF-derived carboxy-terminal peptides in energy balance and reproduction: analysis of "humanized" knockin mice expressing full-length or truncated VGF.” *Endocrinology* **156**, (2015): 1724–38

Sajdyk, T.J., et al. “Amygdalar neuropeptide Y Y1 receptors mediate the anxiolytic-like actions of neuropeptide Y in the social interaction test.” *Eur. J. Pharmacol.* **368**, (1999): 143–147.

Sajdyk, T.J., et al. “Neuropeptide Y in the amygdala induces long-term resilience to stress-induced reductions in social responses but not hypothalamic-adrenal-pituitary axis activity or hyperthermia.” *J. Neurosci.* **28**, (2008): 893–903

Sankoorikal, G.M.V., et al. "A mouse model system for genetic analysis of sociability: C57BL/6J versus BalbJ inbred mouse strains." *Biological psychiatry* **59**.5 (2006): 415-423.

Sauer, B. “Inducible gene targeting in mice using the Cre/lox system.” *Methods* **14**, (1998): 381–392.

Schönig, K., et al. “Stringent doxycycline dependent control of CRE recombinase in vivo.” *Nucleic Acids Res.* **30**, **.** (2002): e134–e134

Severini, C., et al. "In vitro and in vivo pharmacological role of TLQP-21, a VGF-derived peptide, in the regulation of rat gastric motor functions." *British journal of pharmacology* **157**.6 (2009): 984-993.

Shah, R., et al. "Abnormal serotonin levels during perinatal development lead to behavioural deficits in adulthood." *Frontiers in behavioural neuroscience* **12** (2018).

Sharma, S., et al. "Gene× environment determinants of stress-and anxiety-related disorders." *Annual review of psychology* **67** (2016): 239-261.

Shimizu, H. and Bray, G.A. “Effects of neuropeptide Y on norepinephrine and serotonin metabolism in rat hypothalamus in vivo.” *Brain Res. Bull.* **22**, (1989): 945–950.

Shoji, H. and Kato, K. "Maternal behavior of primiparous females in inbred strains of mice: a detailed descriptive analysis." *Physiology & behavior* 89.3 (2006): 320-328.

Shoji, H., and Kato, K. “Maternal care affects the development of maternal behavior in inbred mice.” *Developmental Psychobiology: The Journal of the International Society for Developmental Psychobiology*, ***51***(4), (2009): 345-357.

Silva, A.J., et al. "Mutant mice and neuroscience: recommendations concerning genetic background." *Neuron* **19**.4 (1997): 755-759.

Simon, P.HG. et al. “Key Considerations and Methods in the Study of Gene–Environment Interactions”, *American Journal of Hypertension*, Volume **29,** Issue 8, August 2016, Pages 891–899,

Snyder, S.E., and Salton, S.R.J. "Expression of VGF mRNA in the adult rat central nervous system." *Journal of Comparative Neurology* **394**.1 (1998): 91-105.

Soriano, P. “Generalized lacZ expression with the ROSA26 Cre reporter strain.” *Nat. Genet.* **21**, (1999): 70–71.

Stephens, S.B., et al. "A VGF-derived peptide attenuates development of type 2 diabetes via enhancement of islet β-cell survival and function." *Cell metabolism* **16**.1 (2012): 33-43.

Stolzenberg, D.S., and Champagne, F.A. "Hormonal and non-hormonal bases of maternal behavior: the role of experience and epigenetic mechanisms." *Hormones and behavior* **77** (2016): 204-210.

Stranahan, A.M., et al. “Diet-induced insulin resistance impairs hippocampal synaptic plasticity and cognition in middle-aged rats.” *Hippocampus* **18**, (2008): 1085–1088.

Succu, S., et al. "Pro-VGF-derived peptides induce penile erection in male rats: possible involvement of oxytocin." *European Journal of Neuroscience* **20**.11 (2004): 3035-3040.

Tatemoto, K., et al. “Neuropeptide Y--a novel brain peptide with structural similarities to peptide YY and pancreatic polypeptide.” *Nature* **296**, (1982): 659–660.

Thakker-Varia, S., and Alder, J. "Neuropeptides in depression: role of VGF." *Behavioural brain research* 197.2 (2009): 262-278.

Toshinai, K., et al. "Neuroendocrine regulatory peptide-2 regulates feeding behaviour via the orexin system in the hypothalamus." *American Journal of Physiology-Endocrinology and Metabolism* 299.3 (2010): E394-E401.

Trani, E., et al. "Isolation and characterization of VGF peptides in rat brain. Role of PC1/3 and PC2 in the maturation of VGF precursor." *Journal of neurochemistry* **81**.3 (2002): 565-574.

Trani, E., et al. "Tissue-specific processing of the neuroendocrine protein VGF." *Journal of neurochemistry* **65**.6 (1995): 2441-2449.

Ukkola, O. and Kesaniemi, Y.A. “Leu7Pro polymorphism of PreproNPY associated with an increased risk for type II diabetes in middle-aged subjects.” *Eur J Clin Nutr* **61**, (2007): 1102–1105

Underwood, E.L. and Thompson, L.T. “High-fat diet impairs spatial memory and hippocampal intrinsic excitability and sex-dependently alters circulating insulin and hippocampal insulin sensitivity.” *Biol. Sex Differ.* **7**, (2016): 9

Van Den Berg, W.E., et al. "Sex-specific mechanism of social hierarchy in mice." *Neuropsychopharmacology* **40**.6 (2015): 1364-1372.

Van Den Pol, A.N., et al. "VGF expression in the brain." *Journal of Comparative Neurology* 347.3 (1994): 455-469.

Van der Veen, R., et al. "Impact of intra-and interstrain cross-fostering on mouse maternal

care." *Genes, Brain and Behaviour* **7**.2 (2008): 184-192.

van Steenwyk, G., et al. "Transgenerational inheritance of behavioural and metabolic effects of paternal exposure to traumatic stress in early postnatal life: evidence in the 4th generation." *Environmental Epigenetics* **4**.2 (2018): dvy023.

Velez, L., et al. "Differences in aggressive behaviour and DNA copy number variants between BalbJ and BalbByJ substrains." *Behaviour genetics* **40**.2 (2010): 201-210.

Vink, J.M. "Genetics of addiction: future focus on gene× environment interaction?." *Journal of studies on alcohol and drugs* **77**.5 (2016): 684-687.

Vogel, F., and Motulsky. A.G. "Gene action: genetic diseases." *Human Genetics*. Springer, Berlin, Heidelberg, (1997). 257-360.

Wallerstedt, S.M., et al. "Association analysis of the polymorphism T1128C in the signal peptide of neuropeptide Y in a Swedish hypertensive population." *J. Hypertens.* (2004). **22**, 1277–1281

Wang X., et al., "Sex differences in lipid and lipoprotein metabolism: it's not just about sex hormones." J Clin Endocrinol Metab (2011); **96**: 885–893

Weaver, I.C.G., et al. "Epigenetic programming by maternal behaviour." *Nature neuroscience* 7.8 (2004): 847.

Werling, D.M., and Geschwind, D.H. "Understanding sex bias in autism spectrum disorder." *Proceedings of the National Academy of Sciences* **110.**13 (2013): 4868-4869.

Wold, S., et al. "Principal component analysis." *Chemometrics and intelligent laboratory systems* **2**.1-3 (1987): 37-52.

Yasuda, H., et al. "Artificially reared mice exhibit anxiety-like behaviour in adulthood." *Experimental animals* **65**.3 (2016): 267-274.

Zore, T., et al. "Sex differences in obesity, lipid metabolism, and inflammation—A role for the sex chromosomes?." *Molecular metabolism* **15** (2018): 35-44.