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Effect of caregiving behaviors and genetic predispositions on human and non-human primates development

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To Ian

Contents

Abstract

Parental sensitivity towards infants' needs influences both the way caregiver-infant interactions unfold and individuals' own development throughout lifetime. The pivotal role of this early interaction with caregivers is especially highlighted by the fact that when the interaction is non-adaptive, infants' development may be severely hindered in various domains, such as cognitive, social, and emotional. Moreover, the quality of the early interaction with caregivers has long-lasting effects since it constitutes a lens through which individuals interpret the social world throughout lifetime. Caregivers' influence on individuals' subsequent behavior is also moderated by their own genetic predispositions. However the way behavioral, physiological and genetic mechanisms dynamically interact over time in shaping the development of caregiver-infant bonding and the long term effects on individuals remains largely unknown. The present project aimed to investigate behavioral and physiological mechanisms underling caregiver-infant interactions and their long-term effects applying a multilevel approach including behavioral, physiological and genetic measurements as well as a comparative approach between human and non-human primates. Specifically, in a first study focused on human adults we investigated the effect of the interaction between early parental care and individuals' own genetic predispositions in moderating adults' subsequent peripheral physiological responses to distressing social stimuli. Next, a second study on a primate model, the marmosets (*Callithrix Jacchus*), focused directly on caregiver-infant dyads applying micro-behavioral analysis during infants' first month of life. Overall findings highlighted a differential importance of environmental and genetic factors in moderating caregiver-infant dyads vs individuals' long-term development. The leading role of environmental factors, namely parental behav-

iors, in determining infants' responses to specific caregivers within caregiver-infant interactions seems to be, on the contrary, smoothed out by individuals' own genetic predispositions when focusing on the long-term effects. Indeed, genetic characteristics determine individuals' sensitivity to environment, either weakening or strengthening the effect of environmental contribution in shaping individuals' physiological mechanisms. Also, interesting similarities between marmosets' and humans' caregiver-infant interactions' structure have been found, making way for future studies investigating the brain mechanisms underlying the development of parent-infant bonding.

Chapter 1

Caregiver-Infant Interactions: Effects on Development

1.1 Longitudinal Impact of Caregiver-Infant Interactions

Altricial mammals - not able to provide for themselves in early life - during infancy need virtually constant care from one or both of their caregivers, completely relying on them for nurture, safety and, overall, survival (Bowlby, 1973). Since in humans the infancy period is so long, the adaptiveness of parental responses to infants' signals and needs plays a pivotal role in infants' cognitive, emotional, and social development and has broad long-lasting effects which influence individuals' development even up to adulthood (Rilling & Young, 2014; Sroufe, 2005; Dalsant *et al.* , 2015). Indeed, previous researches have shown that prompt and adequate parental responses to infants' needs foster a better and more stable emotional development in their offsprings, who will be able to cope with stressful events in an adaptive way and to build functioning, stable and secure relationships with peers and intimate partners (Fraley, 2002; Simpson, 1990). By contrast, children who experience poor parenting tend to develop poorer emotional pathways, reacting to distress with maladaptive responses and more likely developing less functioning social relationships in adulthood (Feeney & Noller, 1990; Sbarra & Hazan, 2008). For example, Apter-Levy *et al.* (2013) found that infants of mothers with chronic post-partum depression were more likely to develop psychopathologies and to show lower social engagement and empathy at 6 years of age compared to infants of mothers not diagnosed with chronic post-partum depression. Although it is undeniable that significant social interactions, subsequent to the one experienced with parents, will contribute in influencing individuals' development and in moderating the effects of parental behaviors, what we learn within the early interaction with our primary caregivers remains a lens through which we will interpret and understand our surrounding social and physical world throughout lifetime (Allen *et al.* , 2002; Cutrona *et al.* , 1994; DiTommaso *et al.* , 2003).

The role played by caregivers' attitude towards infants' psychological needs was first highlighted by Bowlby (1973) in the theorization of the attachment theory. Infants are biologically programmed to perform behaviors, called attachment behaviors which trigger caregivers' care favoring their proximity to the infant. Examples of attachment behaviors are crying, smiling, clinging, or following. However, caregivers' response to infants' attachment behaviors differs between individuals. The mode and the promptness of caregivers' reactions determine the characteristics of the attachment relationship through the formation of internal working models which allow the infants to try to predict how a social interaction will unfold based on his/her own previous experiences. More prompt and adaptive responses will foster a secure attachment by favoring the development of a model representing a safe social relationship within which the infant feels secure in expecting the proximity of caregivers and the fulfillment of his/her needs. On the contrary, delayed or not-attuned caregivers' responses will favor the formation of models representing social interactions as unsafe or unreliable since infants cannot be sure about caregivers' care and responsiveness. In Bowlby's theory, the attachment model developed in early infancy within the relationship with primary caregivers will then be a fixed key through which all social relationships will be interpreted throughout lifetime, even up to adulthood. Subsequent studies pointed out how, actually, the models derived from the attachment relationship with caregivers are malleable and may be influenced and re-shaped by other significant social relationships later in life. However, since we born in a world of which our brains know nothing about, the very first interactions we experienced play a pivotal role in paving the way through which the external world will be interpreted thereafter. Recently, researchers also started to investigate the long-term effects of early caregiving on individuals' physiological development. Children and adolescents who report to be more likely to approach their parents in distressing situations show a higher activation of the left dorsal amygdala in response to their own mother's face compared to the face of a stranger, while in prior institutionalized children a higher connectivity between the amygdala and the medial-PreFrontal Cortex (mPFC) compared to non-prior institutionalized children was found (Tottenham *et al.* , 2012). Higher amygdala-mPFC connectivity is usually related to a more mature brain and, indeed, the connectivity in prior institutionalized children matched the ones in adolescents, both prior institutionalized and not (Gee *et al.* , 2013). Consistently, previously institutionalized children did not show the increase in oxytocin concentration after being touched by their mothers as the one experienced by not-previously institutionalized children (Fries *et al.* , 2005), and smaller white matter volume together with poorer cognitive performances were found in 9 to 14 years old adopted children who experienced early neglect compared to control children from families matched for socio-economic status to the adopted ones (Hanson *et al.* , 2013).

On the other hand the environment is not the only factor that contributes in shaping individuals' development. We do not meet the world completely pristine and unarmed, rather we carry in the world our genetic programming which defines the initial metabolic pathways of our physiology, influencing individuals' development as well as individuals' responses to environmental stimuli. Thus, the contribution of infants' own genetic predispositions in moderating the effects that parenting behaviors have on individuals' development needs also to be taken into account. Between others, genes responsible for the programming of the oxytocinergic system, such as the oxytocin receptor (OXTR) gene, are known to be involved in the emergence of social bonds and affective behaviors, since an optimal functionality of the oxytocinergic system contribute to an adaptive development of these traits (Bradley *et al.* , 2013; Ebstein *et al.* , 2012). This happens because several regions of the OXTR gene, called Single Nucleotide Polymorphisms (SNPs), may exist in the population in different forms, called alleles. Since the form of the gene determines the proteic structure of oxytocin receptors and taken into account that different a morphology may hinder or enhance receptors' functionality, different alleles in the OXTR gene may modify the function of the oxytocinergic system. For example individuals carrying the G allele in the region rs53576 of the oxytocin receptor (OXTR) gene when they have experienced a good family environment in childhood show higher positive affect and more resilient coping in adulthood compared to AA homozygotes

(Bradley *et al.* , 2013), while GG homozygotes in the same region, when they experienced maltreatments in childhood, show more depressive symptoms in adulthood (McQuaid *et al.* , 2013). However, knowledge about how the behavioral experiences, physiological mechanisms and genetic characteristics interact in shaping long-term development is still scarce and it usually does not take into account more than two levels of analysis, either behavioral and genetic or behavioral and physiological. The studies proposed here aim to apply complementary methodologies in order to start shading light on how these three levels interact with each other in mediating individuals' social development.

1.2 Evaluation of Caregiver-Infant Interactions

In collaboration with Bowlby, Mary Ainsworth ran observational studies aiming to describe and operationalize the constructs of secure and insecure attachment relationships in order to classify caregiver-infant interactional patterns (Ainsworth, 1978). Indeed, findings from her observations showed that infants with distinct attachment styles display different but specific behavioral patterns (Ainsworth *et al.* , 2015). To evaluate caregiver-infant interactions Ainsworth and colleagues developed a paradigm called Strange Situation Procedure (SSP), which usually applies to toddlers of 9 to 12 months of age and aims to investigate infants' reactions to a mild distressing situation. The SSP lasts for about 20 minutes and foresees eight different episodes: (i) the mother and the toddler play alone in a room, (ii) an experimenter, stranger to the toddler, enters the room and (iii) joins the play, (iv) the mother leaves the room, (v) the mother re-enters the room while the stranger leaves, (vi) the mother exits the room again leaving the toddler alone, (vii) the stranger returns, (viii) the mother returns. Toddlers with secure attachment explore more willingly and with more interest the environment when the mother is in the room and they are more easily soothed when reunited with her after a separation has occurred. On the other hand, insecure toddlers may display one of two different behavioral patterns. Toddlers displaying insecure avoidant attachment interact less with the mother when she is in the room and look less for proximity with her during the reunion. Toddlers displaying insecure ambivalent attachment, on the other hand, show anger towards the mother during the reunion phase while at the same time they look for her proximity (Ainsworth & Bell, 1970). Lastly, in case of maltreatments, abuse, or severe social deprivation, toddlers may develop the so-called disorganized attachment style which is heavily maladaptive and includes features of both the previous insecure styles without following a recognizable behavioral pattern or using coherent and organized coping strategies (Main *et al.* , 1990). Following studies re-confirmed the presence of different behavioral patters and researchers started to look into the physiological and molecular underpinnings of the attachment styles, finding mixed results. The investigation on physiological responses to the SSP mainly focused on cortisol or salivary-Alpha-Amylase (sAA) concentration, or on Heart Rate variations. Cortisol is a steroid hormone whose secretion is controlled by the Hypothalamic-Pituitary-Adrenocortical (HPA) system, whereas the sAA is an enzyme secreted by the sympathetic-medullary axis. Both the HPA and the sympathetic-medullary axis are controlled by the Autonomic Nervous System (ANS) and, as such, their activity is an index of the internal body state (Kirschbaum & Hellhammer, 1994; Rohleder *et al.* , 2004). Indeed, higher activation of the parasympathetic branch of the ANS would indicate a calmer state whereas the activation of the sympathetic branch is an index of distress or promptness to take action in response to an external stimuli. The heart muscle is also under control of the ANS and therefore variations in the heart rate are also indicators of the activation of the parasympathetic and sympathetic branches (Berntson *et al.* , 1997). Indeed the activation of the vagus nerve triggered by the parasympathetic response induces a decrease in the heart rate, whereas the sympathetic response increases it. Respiratory-Sinus-Arrhythmia (RSA), instead, is solely an index of vagal tone which in under parasympathetic control (Berntson *et al.* , 1993). Specifically, RSA is a natural variation in heart rate which occurs during the breathing cycle, which causes the heart rate to decrease during expiration and increase during inspiration. During distressing situations RSA amplitude decreases because of the withdrawal of the vagal stimulation, which is under parasympathetic control, allowing researchers to use this information as an index of the internal body state (Bazhenova *et al.* , 2001). Both Spangler & Grossmann (1993) and Hertsgaard *et al.* (1995) found a higher cortisol response in disorganized infants compared to insecure and secure infants. Spangler & Grossmann (1993), by measuring cortisol levels 30 minutes after the end of the procedure, also found it to be higher in insecure compared to secure infants. In contrast, in the work by Hertsgaard *et al.* (1995) where the cortisol response measured 30 minutes after the beginning of the SSP was found to be higher in disorganized infants compared to both secure and insecure infants, no difference in cortisol reactivity was found between secure and insecure infants. In line with the results found by Spangler & Grossmann (1993), a higher stress-related physiological response in insecure infants was also found by Hill-Soderlund *et al.* (2008). In their studying Hill-Soderlund and colleagues measured salivary-Alpha Amylase (sAA) concentration as well as Respiratory-Sinus-Arrhythmia and they found the former, sAA, to be higher in insecure compared to secure infants, while the latter, RSA, to be lower in insecure compared to secure infants. These findings both underlie an enhanced stress-related physiological response in insecure infants. Differences in results may be due to the presence of moderators. For example, Gunnar *et al.* (1996) found cortisol levels to increase in insecure infants in response to SSP only when they showed high levels of social fear as measured by the Toddler Behavior Assessment Scale (Goldsmith, 1988) but not otherwise. Also, findings from a recent research by Beijers *et al.* (2017) showed that cortisol increase was higher in insecure infants when they displayed less compared to more fussing behaviors, whereas it was higher in secure infants when they displayed more compared to less self-soothing behaviors while separated from the mother. Another possible explanation for the inconsistent findings may be that the contribution of individuals' genetic predispositions in moderating physiological and behavioral responses.

1.3 Genetic correlates of infants' attachment

The genetic code is the foundation of living beings since it programs and defines the metabolic pathways influencing individuals' development as well as individuals' responses to environmental stimuli. Therefore, researchers investigated which role genetic predispositions play in moderating infants' physiological and behavioral responses to SSP in order to assess the contribution of the genetic programming in the emergence of attachment. The genes mostly considered to be involved in the moderation of attachment relationship were the promoter region of the serotonin transporter gene (5-HTTLPR), the oxytocin receptor gene (OXTR), the catechol-O-methyltransferase gene (COMT), and two regions of the D4 dopamine receptor gene (DRD4 and DRD4/-521).

The serotoninergic system is involved in emotional, affective and mating processes (Cheon *et al.* , 2013; Caspers *et al.* , 2009; Kong, 2015; Fisher, 2000). The structure of the 5-HTTLPR region, which may be long or short, contribute in determining its level of functionality. Indeed the 5-HTTLPR polymorphism is located in the promoter region of the SLC6A4 gene, which encodes for a membrane protein that transports the serotonin from the synaptic space back into the presynaptic neuron, thus terminating its action on the postsynaptic neuron (Iurescia *et al.* , 2016; Weizmann Institute of Science, 2018c). However, for the SLC6A4 gene to be correctly expressed the functionality of the promoter region is essential. This is why different forms of the 5-HTTLPR region, by moderating the expression of the SLC6A4 gene, moderates the efficacy of the serotonin reuptake from the synaptic gap influencing, in turn, the activity of the serotoninergic system as a whole (Canli & Lesch, 2007). The oxytocinergic system is involved in the formation of social bonds (Ebstein *et al.* , 2012; Kendrick *et al.* , 1987; Ross & Young, 2009) and several regions of the OXTR gene are Single Nucleotide Polymorphisms (SNPs), that is in the population it is possible to find two different forms of a specific DNA region and the two forms, the so-called alleles, only differ for one base pair. The form of the gene determines the morphology of the expressed proteins and, thus, modifies their functionality. In the case of the OXTR gene, different alleles will therefore lead to different protein structure in the oxytocin receptors which will then differentially affect the functionality of the oxytocinergic system (Weizmann Institute of Science, 2018b). D4 and COMT genes code for proteins related to the regulation of the dopaminergic system, which is involved in motivation and reward systems (Wittmann *et al.* , 2005). Specifically, D4 gene code for a type of dopamine receptors, the D4 receptors (Weizmann Institute of Science, 2018a). Therefore, as for the OXTR gene, different alleles in the D4 gene affect the morphology and, in turn, the functionality of the D4 receptors. Whereas the COMT gene codes for a protein colled catechol-O-methyltransferase, which helps breaking down dopamine and norepinephrine maintaining appropriate levels of these neurotransmitters, especially in the prefrontal areas of the brain (U.S National Library of Medicine, 2007). Again, different forms of the COMT gene will affect the morphology and the functionality of the catechol-O-methyltransferase and, in turn, will influence the levels of dopamine and norepinephrine present in the brain. Frigerio *et al.* (2009) found that infants carrying the T allele in the region DRD4/-521 of the gene D4 were more likely to show insecure avoidant attachment whereas infants carrying the C allele were more likely to show insecure resistant attachment. Also, insecure infants carrying a short allele of the region 5-HTTLPR had a steeper sAA concentration increase in response to the SSP compared to insecure infants carrying the long allele (Frigerio *et al.* , 2009). However both relations did not survive Bonferroni correction. Lakatos *et al.* (2000) found a link between the presence of 7-repeats in the DRD4 region of the D4 dopamine receptor gene and disorganized attachment in both males and females, whereas Chen *et al.* (2011) showed an association between SNPs in the OXTR gene and the development of secure attachment. However, studies carried on larger cohorts of participants failed to replicate the results, and found only one significant but weak association between genetic characteristics of the COMT gene and the development of disorganized attachment (Roisman *et al.* , 2013). The weak and minimal association between genetic characteristics and the attachment phenotype is likely due to both the broad nature of the attachment construct itself and to the complexity of the interaction between genetic and environmental factors. Indeed, several models of how genes and environment may interact in moderating individuals' development has been proposed. In the next section these different models will be discussed.

1.4 Gene-Environment interaction models

[1]The set of genes carried by each individual determines the pool of possible physiological processes and metabolic pathways available during lifetime. However, the developmental path followed is also affected by external environmental conditions. By way of example, let's consider what would happen to an individual whose genetic code favor a high height if he/she grows in environments with different levels of food availability, high vs low availability. In the environment characterized by food scarcity the young individual's genetic predisposition to grow tall will not be expressed, while the opposite would happen in the environment characterized by high food availability. More in general, an individual's resulting physiological and behavioral characteristics, called the individual's phenotype, are the result of a dynamic interplay between genes and environment. This interaction however may take place in different ways: (i) genes can affect the phenotype and the exposure to environmental factors, (ii) the environment can affect genetic expression, or (iii) genetic predispositions and environmental factors can moderate each other's effects. In the first scenario genetic characteristics may play a leading role by affecting individuals' phenotype directly, or by moderating environmental factors. For example in the case of congenital deafness the infant's phenotype is directly affected by the genetic code. Moreover, since congenital deafness is a condition that narrows the environmental stimulation to which

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[1] Section based on:

Onnis, L., Truzzi, A., Ma, X. (2018). Language development and disorders: Possible genes and environment interactions. *Research in developmental disabilities*, 82, 132-146.

an infant might be exposed, in this case the genetic characteristics also moderate the external environmental factors. On the other side of the medal, environmental factors themselves may affect how genes are expressed. The bioecological model (Bronfenbrenner *et al.* , 1994; Rutter *et al.* , 2006) proposes that even though genetic characteristics define a particular phenotype, the phenotype itself might not be revealed if the environmental conditions do not give it the opportunity to be expressed. An example of this model may be the case of the environment characterized by food scarcity proposed above. The genetic programming that allows an individual to grow tall is embedded in the DNA, no matter in which conditions the individual actually grows up. However, the expression of this phenotype depends on the environmental conditions since for the individual to actually grow tall the right amount of food needs to be available. The bioecological model does not stand alone, rather it is part of a series of processes called *epigenetics* that describe mechanisms though which environmental conditions moderates the expression of genes without affecting the structure of the genetic code (Rice, 2012). Research on epigenetic mechanisms is recent and until now it has mostly being focused on animal models. Indeed, in rodents different parental signals within mother-infant interactions may differentially methylate the DNA and therefore hider or enhance the possibility for DNA-expression-related proteins to reach the two strands o the double helix starting genes expression (Kappeler & Meaney, 2010). In some cases these metabolic effects was even found to be stable changes in the genetic expression which also persisted in subsequent generations. In humans, an association between folic acid intake during pregnancy and the risk of severe language delay at three years of age was found (Roth *et al.* , 2011). Folic acid is known to be involved in the regulation of the insulin-like growth factor 2 gene (IGF2) (Steegers-Theunissen *et al.* , 2009) and when pregnant women were given folic acid supplements between 4 to 8 weeks after conception three years later toddlers were less likely to develop a severe language delay. Lastly, it is possible that genetic predispositions and environmental factors mediate or moderate each other. Two models have been proposed to explain this third type of gene-environment interaction: the diathesis-stress model, and the plasticity genes model. The *diathesis-stress model* predicts that some genes, depending on their characteristics and mutations, may be either protective or risk factors towards the external environment (Rende & Plomin, 1992; Rutter *et al.* , 2006). Let's consider two individuals having different alleles on one of these genes. According to the diathesis-stress model, when the two individuals experience a favorable environment they will have the same developmental possibilities and will show similar adaptive phenotypes. On the contrary, when exposed to a disadvantageous environment one of the two individuals will be protected by his/her genetic characteristics and will still display an adaptive phenotype, while the other, carrying the risk genetic factor, will be at risk of developing a maladaptive phenotype. However the interaction between genes and environment may be even more complex, as exemplified by the *plasticity genes model* (Belsky *et al.* , 2009; Belsky & Pluess, 2009b,a). According to this model genes are not protective or risk factors per se, rather they may be either favorable or disadvantageous accordingly to the environment individuals' are exposed to during development. This is because different alleles of a specific gene moderate individuals' sensitivity to external factors. An individual whose genetic characteristics lead to a lower sensitivity to the environment will more or less follow a stable developmental trajectory independent to the environmental factors experienced. On the contrary an individual whose genetic characteristics lead to a higher sensitivity to the environment will likely follow opposite developmental trajectories accordingly to the early experiences. That it, an optimal environment would boost the adaptiveness of the development of high-sensitive individual whereas a negative environment would favor a more maladaptive development compared to less sensitive individuals (Belsky *et al.* , 2009). The plasticity genes model helps to understand how some pools of seemingly disadvantageous genetic mutations have been spared by natural selection. Individually each of this genetic mutations may have favored traits that, although risky in a specific environment, in another context proved to be more useful than other safer alleles (Bishop, 2009).

In the current project it is proposed that the model most fitted to explain the mechanisms underlying the unfolding of parent-infant interactions and infants' development is the plasticity genes model. The respective role of genes and environment will be investigated in adulthood - Chapter 2 - to tackle the long term effects of gene-environment interactions and in early life - Chapter 3. In the second part of the project, presented in Chapter 3, a micro-analysis of caregivers' and infants' behaviors which aims to disassemble the caregiver-infant interaction in its key components is presented. Also, aiming for the investigation of the causal mechanisms underlying these behavioral components, the study was conducted on a primate model, the marmosets (*Callithrix Jacchus*).

1.5 Caregiver-Infant Bonding: Findings from Non-Human Primate Models

The idea that infants' relationship to their mothers is only driven by food provision and physical needs was first challenged by findings from non-human primate models. In the mid 20th century Harlow & Zimmermann (1958) showed that an infant rhesus monkey separated from its own mother if given the choice between two iron-made-cylinder surrogate mothers, one providing food and the other providing no food but covered in warm soft fur, chose to spend the majority of their time attached to the warm furred one, only changing manikin when hungry. Subsequently several other researches corroborated Harlow's findings. Non-human primates have been found to develop an affiliative bond with dogs, and infant titi monkeys primary caregiver could be the father although mothers are the ones that provide nutrition to the infant (Hoffman *et al.* , 1995; Mason & Mendoza, 1998; Mason & Kenney, 1974). The importance of early caregiver-infant interactions in non-human primates and their contribution to infants' feelings of security was reconfirmed in a research by Kostan & Snowdon (2002) whose findings showed that, when a threatening stimuli is present, cotton-top tamarin infants choose proximity with the caregiver that previously fulfilled the infants' own needs the most, that is the one who most carried and shared food with it. Findings from non-human primate models also corroborate the importance of caregivers' responsiveness and sensitivity since chimpanzee infants reared in condition of non-responsive care, where only infants' physical needs were met, showed more disorganized and maladaptive behaviors, such as rocking or clutching to a towel, during a Strange Situation Procedure test and scored lower in cognitive tests compared to infants reared in a situation of responsive care (van Ijzendoorn *et al.* , 2009). Moreover, long-term effects of early social interactions on individuals' development have also been shown in non-human primates. Infant monkeys socially deprived during the first three months of life showed signs of depression when the isolation period ended, so much so that in two out of twelve cases the monkey underwent severe depression, one of them died of starvation while the other starved itself but was force-fed by the experimenter and survived (Griffin & Harlow, 1966). Moreover, early socially deprived monkeys display inappropriate aggression, non-adaptive sexual behaviors, and hindered maternal behaviors. In a study by Ruppenthal *et al.* (1976) infant female monkeys were separated from their mothers within three post-natal days and were either reared in isolation with or without a cloth-surrogate mother or reared together with five age-matched peers but without having access to the mother or other adult females. Females grown up in isolation, when it was their turn to be mothers, showed a higher proportion of indifferent or abusive parenting compared to peer-reared females, failing to nurse their infants or injuring them. However the effect was slightly weakened when females were reared with a cloth surrogate mother, and the proportion of adequate care increased in successive birth, suggesting an important role of experience in moderating caregiving quality, consistently with findings from studies on alloparenting (Stone *et al.* , 2010). Studies on animal models are useful because they allow us to investigate the causal relation underlying physiological systems and output behaviors and this is true also for the mechanisms underlying caregiver-infant interactions. Specifically, the opiate system, given its involvement in reward systems and in the acquisition of preferences, has been one of the most widely studied physiological pathways in relation to the development of caregiver-infant bond. In a study by Kalin *et al.* (1995) twelve infant rhesus monkeys were separated from their mothers for 20 minutes and right prior to reunion the infants or the mothers were injected either saline solution, non-sedating doses of morphine, which binds to opioid receptors in the central nervous system, or naltrexone, which is an antagonist of opioid receptors and as such hinder opioid system's functioning. When infants were injected with naltrexone they spent longer time clinging to the mothers and emitted more calls compared to controls, whereas when infants were injected with morphine they spent less time clinging to the mothers and emitted less calls compared to controls. The same pattern was found when mothers were injected with naltrexone or morphine but only in the first 30 minutes after the start of reunion. These findings highlight the involvement of the opioid system in moderating some affiliative behaviors during reunion and the development of caregiver-infant bonds, however more researches are needed to investigate the involvement of other systems and their relation to other affiliative behaviors.

1.6 Investigating Caregiver-Infant Interactions: A Complementary Approach

The quality of caregiver-infant bond in early life plays a pivotal role in shaping short and long-term individuals' development and this mechanisms are phylogenetically conserved. However, a deep understanding of how the underlying behavioral, physiological and genetic mechanisms dynamically interact with each other in the context of caregiver-infant interactions and their long-term effects is still lacking. The study of caregiver infant interactions, therefore, involves at least two fundamental questions:

(i) How do caregivers' behaviors interact with genetic and physiological mechanisms in moderating individuals' long term development?

(ii) How is adaptive caregiver-infant bonding achieved?

To address these two questions two complementary approaches were applied. At first we focused on adult humans aiming to assess how individuals' genetic predispositions moderated the effects of the experienced parental behaviors on physiological development. We hypothesized that the physiological level works as an interface between genetic fixed characteristics and dynamic environmental factors, because genetic predispositions determines individuals' sensitivity to the environment, that is how much the physiological systems are susceptible to be shaped by external factors. Subsequently we directly focused on caregiver-infant dyads. Micro-analysis of behaviors was applied during a modified strange situation procedure on an animal model, the marmosets (*Callithrix Jacchus*), to investigate caregiver-infant interactions in detail, disassembling them and highlighting their core elements taking into account both sides of the dyads. This analysis had been run on marmosets in order to test the possibility to consider them the leading model in the study of caregiver-infant interactions as well as to pave the way to tackle causal relationships between physiological systems and core behaviors performed within the interaction. In the following chapters these studies will be presented and discussed in detail.

Chapter 2

How do caregivers' behaviors interact with genetic and physiological mechanisms in moderating individuals' long term development?

Chapter based on:

Esposito G., Truzzi A., Setoh P., Putnick D.L., Shinohara K., Bornstein MH. (2017) Genetic predispositions and parental bonding interact to shape adults' physiological responses to social distress. *Behavioral Brain Research*, 325, 156-162, doi: 10.1016/j.bbr.2016.06.042.

Truzzi, A., Bornstein M.H., Senese, V.P., Shinohara, K., Setoh, P., Esposito, G. (2017). Serotonin transporter gene polymorphisms and early parent-infant interactions are related to adult male heart rate response to female crying. *Frontiers in Physiology*, 8(111), doi: 10.3389/fphys.2017.00111.

Truzzi, A., Poquérusse, J., Setoh P., Shinohara, K., Bornstein, M.H., Esposito, G. (2018). Oxytocin receptor gene polymorphisms (rs53576) and early paternal care sensitize males to distressing female vocalizations. *Developmental Psychobiology*, 60(3),

2.1 Introduction

As described in chapter 1, being humans altricial mammals, the caregiving behaviors that we experienced in infancy and childhood strongly affect our subsequent development, influencing our social abilities with effects that last even up to adulthood (Allen *et al.* , 2002; Cutrona *et al.* , 1994; DiTommaso *et al.* , 2003). However, parenting behaviors do not work into an empty framework: each individual has his/her own predispositions defined by the genetic code and some genes which code for proteins important in the control of processes involved in social behaviors and social bonds, are thought to be moderating the long-term consequences of parental behaviors. Two of the most studied genes associated with social behaviors are the oxytocin receptor gene (OXTR) and the serotonin transporter gene (SLC6A4).

Oxytocin Receptor Gene (OXTR) The neuropeptide oxytocin is involved in the modulation of key social behaviors such as bonding and attachment (Kendrick *et al.* , 1987; Ross & Young, 2009; Ebstein *et al.* , 2012) and its involvement in behavioral regulation relies on the functionality of the oxytocin receptors placed on the cells' membrane. Since the functionality of receptors is due to their proteic structure and the proteic structure is defined by the genetic code, genes coding for neurotransmitters and hormones receptors are highly involved in the regulation of behaviors. In the OXTR gene some regions are characterized by the so-called Single Nucleotide Polymorphisms (SNPs). The SNPs are genetic regions of the DNA which exist in different versions in the general populations, different alleles, and the two versions differ for one base pair. It is possible that the two versions of the

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333-339, doi:10.1002/dev.21606.

AT contributed in performing the experiment, analyzing data, interpreting results and writing the manuscript in all three articles.

DNA region do not equally code for optimal functioning proteins. Two of the most studied SNPs of the OXTR gene are in the regions rs2254298 and rs53576. Concerning the region rs2254298, two allelic forms, A and G, have been identified. However, findings about the role which these two allele plays in moderating social development are mixed. In some researches the G allele seems to be a protective factor for an adaptive social development whereas the A allele would be the risk factor, leading to a poorer social development (Furman *et al.* , 2011; Marusak *et al.* , 2015; Thompson *et al.* , 2011; Wu *et al.* , 2012). In a sample of adolescent girls, the ones carrying two copies of the G allele (GG homozygotes) were found to be less sensitive, compared to girls carrying at least one copy of the A allele, to early social adversities, such as having depressed or anxious mothers (Thompson *et al.* , 2011). Also, adults carrying the G allele showed higher empathy (Wu *et al.* , 2012). On the other hand, one research by Costa *et al.* (2009) pointed out that the G allele appears with higher frequency in adults who shows higher separation anxiety levels and in patients affected by depression. Furthermore, previous studies highlighted that also in the other target region of the OXTR gene, the region rs53576, the alleles G and A have a specific and differential influence on the regulation of social behaviors. Individuals homozygous for the G allele have been found to show higher dispositional empathy, pro-social characteristics and physiological arousal in reaction to a social stressor compared to A carrier individuals (A/A or A/G) (Norman *et al.* , 2012; Rodrigues *et al.* , 2009; Smith *et al.* , 2014; Tost *et al.* , 2010). In contrast, the presence of the A allele has been associated to reduced positive affect and empathetic accuracy (Lucht *et al.* , 2009; Rodrigues *et al.* , 2009). On the other hand, other studies found opposite associations or lack of association between the genetic characteristics of the region rs53576 of the OXTR gene and individuals' social traits (Bakermans-Kranenburg & van IJzendoorn, 2008; Li *et al.* , 2015). Indeed, no relation was found between the genetic characteristics in nine different SNPs in the OXTR gene and adults' performances in two economic games with real monetary rewards, the trust game and the dictator game (Apicella *et al.* , 2010). Also, in a recent study, McQuaid *et al.* (2013) found that adults homozygous for the G allele in the region rs53576 of the OXTR gene, when they had experienced severe maltreatment in childhood, were more likely to develop depressive traits compared to A carriers. The mixed findings concerning the OXTR gene may be due to the fact that rather then being absolute risk or protective factor this gene may moderate individuals' sensitivity to environmental factors both for better and for worse (Boyce & Ellis, 2005). Sensitivity to the environment, rather than being positive or negative in itself, may be either a protective or a risk factor depending on the environmental context that individuals experience (Boyce & Ellis, 2005). When genetic characteristics predispose the individual to be less sensitive, a bad or poor environment will have less effects on development but so will a good and rich environment. Whereas highly sensitive individuals will be subjected to greater environmental influences on their development in both directions: in a bad environment they risk to undergo a maladaptive development but the same individuals would benefit more than less sensitive ones from good and rich experiences (Belsky *et al.* , 2007; Pluess, 2017). Indeed, a longitudinal research by Raby *et al.* (2013) showed that G/G homozygotes had higher stability of attanchemt style from childhood to adulthood compared to A carriers, which would be consistent with A carriers being overall more sensitive to environmental factors experienced during lifetime . The exact role of this polymorphism in moderating social behaviors is therefore still to be fully elucidated.

Serotonin Transporter Gene (SLC6A4) Serotonin is involved in reward systems, in social interactions with opposite-sex conspecifics and in mating (Fisher, 2000). When confronting an opposite-sex conspecific individuals have been shown to experience a sudden decrease in serotonin levels. The concentration of serotonin is moderated by the efficacy of serotonin transporters, which are inter-membrane proteins responsible for reuptaking the serotonin into the cell from the synaptic cleft. Therefore the genotype of the serotonin transporter gene (SLC6A4) which codes for the structure of the inter-menbrane transporter and, in turn, for their functionality, is closely related to the levels of serotonin concentration. The promoter region of the serotonin transporter gene includes a polymorphic region (5-HTTLPR) and, as such, it is present in two different forms in the general population: the long (L) and the short (S) alleles. The less transcriptionally efficient form of the gene is the S allele which relates to lower efficiency in setoronin reuptake compared to the L allele (Canli & Lesch, 2007). Different efficiency in serotonin reuptake, in turn, affect individuals' physiological responses and well-being. Indeed, individuals carrying two copies of the S allele have a higher likelihood to develop depression when exposed to stressful and negative life events (Kendler *et al.* , 2005). Moreover, the genotype of the promoter region 5-HTTLPR has been related to differential adaptiveness in social environment and intimate bonds. Individuals homozygotes for the long allele showed reduced intergroup bias and richer bond representations in adulthood, while individuals homozygotes for the short allele showed higher maladaptive behaviors (Caspers *et al.* , 2009; Cheon *et al.* , 2013). At the societal level, Kong (2015) showed that when large societies and higher democracy levels are coupled with a prevalence of the short allele, people tend to rate the society as less trustworthy.

Role of Physiological Responses Although, genetic factors play a pivotal role in moderating the long-term effects of parental behaviors, they do not directly and univocally determine social behaviors and development. However, little is known about the mechanisms through which genetic and environmental factors interact in shaping individuals' development. Our hypothesis is that genetic and environmental levels meet at the physiological levels which plays the role of an interface between them, since genetic characteristics determine how malleable vs stable the physiological responses will be in front of the environment individuals are introduced to upon birth. Therefore, here we aim to test the effect of the interaction between genetic predispositions in the OXTR and SLC6A4 genes and the parental behaviors experienced in childhood on adults' Autonomic Nervous System (ANS) activations in response to distressing social stimuli (Esposito *et al.* , 2017; Truzzi *et al.* , 2017, 2018). We expect that individuals carrying genetic forms which are associate with higher sensitivity to the environment will show more differential physiological arousal in response to distressing social stimuli accordingly to the experienced parenting compared to individuals carrying less sensitive genotypes.

2.2 Methods and Measurements

2.2.1 Participants

Forty-two non-married and non-parent male adults (M = 24.7 years, SD = 5.05) were recruited through web-announcements and on the University of Trento website. Since only woman adult cries were available and given the evolutionary significance of cry stimuli, to avoid possible confounding effects due to females' specific responses to infant cries (De Pisapia *et al.* , 2013; Messina *et al.* , 2016) only males were included in the present study. All participants were of caucasian ethnicity and had Italian nationality. In order to avoid possibe confounding external factors that could have influence participants' heart rate, they were asked to refrain from smoking, drinking coffee, of perform sports activity for the 24 hours before the experimental session took place. Also, at the time of experiment participants were not involved in agonistic sports. The study was conducted in accord with ethical principles stated in the Declaration of Helsinki and written informed consent was obtained from all participants. The genetic assessment followed the procedures approved by the IRB guidelines of the University of the Nagasaki Graduate School (Japan) and was conducted on anonymized bio-samples at the University of Nagasaki.

2.2.2 Stimuli

Thirty 15-seconds long audio clips of distressing vocalizations were used as stimuli. The audio clips belonged to three different categories, female cries, infant cries and bonobo cries, for a total of 10 clips per category (3 type x 10 clips = 30 stimuli). Cries were used because of their high evolutionary significance. Indeed, cries have been found to elicit in adults specific physiological responses which underlie distress and promptness to action (De Pisapia *et al.* , 2013; Esposito *et al.* , 2012; Messina *et al.* , 2016). Since the present research aimed to assess physiological responses elicited by social distress, and infant and female cries could both have a specific evolutionary salience to male adults, both human infant and adult female cries were included as stimuli. Bonobo cries are non-human vocalizations whose acoustic characteristics bear some similarities with humans' and bonobos' vocal tract is similar to that of humans. Therefore, bonobo cries were used as control stimuli. The mean fundamental frequencies of each cry category were not significantly different (F(2,27) = .28; ns): female cries: f0 M = 480Hz (f0 range = 280Hz-705Hz); infant cries: f0 M = 482Hz (f0 range = 334Hz-736Hz); bonobo cries: f0 M = 384Hz (f0 range = 355Hz-431Hz). Stimuli intensities were normalized (M = 85dB), and the volume was kept constant. Each audio clip was followed by 10 seconds of silence to allow recovery of physiological responses. Three randomized sequences of audio clips were created using the open source software Audacity and each participant was presented with one of the three possible sequences. Overall each participant listened to all the 30 audio clips.

2.2.3 Procedure

The experimental session was divided into three main parts. First, participants filled in an online self-report questionnaire, the Parental Bonding Instrument (PBI) assessing their experiences with their own parents during childhood. Next, in the laboratory, the audio stimuli were presented and participants' heart rate (HR) was recorded all throughout the stimuli presentation. Participants were seated at about 1m from the speakers and the audio was kept constant for the presentation and through participants. Participants' baseline was collected during the 60 seconds before the audio presentation while only a gray screen with a fixation point was presented in a dark and silent room. During baseline collection participants were instructed to only look at the screen and relax. Then, each stimuli was presented for 15 seconds with an inter-stimuli interval of 10 seconds [Figure 2.1]. Finally, for each participant a buccal mucosa sample was collected using a cotton swab. The experimenters that ran the experimental sessions were blind to participants' questionnaire responses and PBI final score.

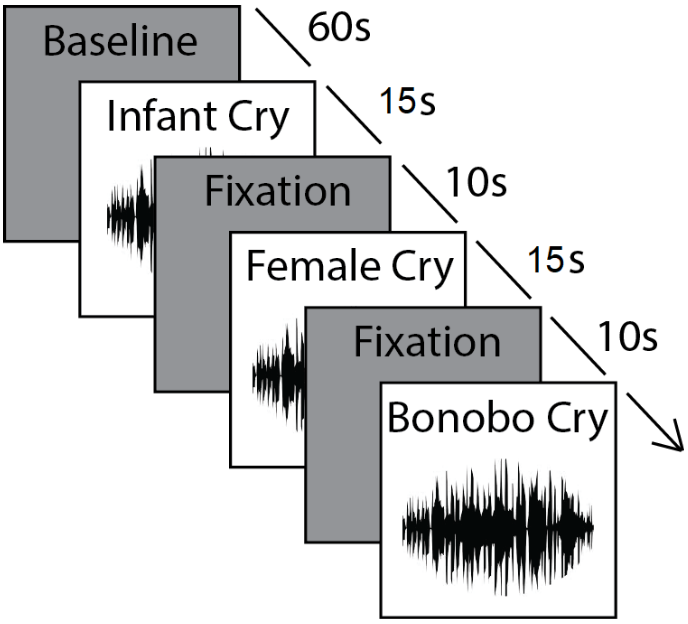

Figure 2.1: Here the flow of stimuli presentation is represented. First, 60 seconds of baseline were recorded, then each audio clip was presented for 15 seconds with an inter-stimulus interval of 10 seconds

2.2.4 Parental Bonding

The Parental Bonding Instrument is a self-report questionnaire organized in 50-item and developed to measure two parental dimensions experienced in childhood: care and overprotection (PBI; (Parker *et al.* , 1979)). These are two of the principal parenting dimensions since they are indexes, respectively, of how much parents payed attention to the child psychological needs and, on the other hand, how much parents were intrusive vs permissive in letting the child exploring the surrounding environment. Therefore high care scores are interpreted as high warmth, parental affection, emotional closeness, and empathy (Arrindell *et al.* , 1998; Dalsant *et al.* , 2015), whereas high overprotection scores represent high parental intrusion which prevents independent behavior and exploration (Arrindell *et al.* , 1998; Rikhye *et al.* , 2008). Participants filled in one form for maternal behaviors and one for paternal behaviors, for a total of two forms. Both dimensions are measured on continuous scales and values range from 0 to 3. In our sample, the Cronbach's alphas were high for both the maternal PBI form (maternal care = 0.73; maternal overprotection = 0.73) and the paternal PBI form (paternal care = 0.65; paternal overprotection = .83), and the averaged PBI Cronbach's alpha for the whole questionnaire was 0.74.

2.2.5 Heart Rate

We measured heart rate (HR) using a pulse oximeter (CONTEC CMS60D) placed on participants' left forefinger. The pulse oximeter is constituted by a LED which emits light in two wavelengths. Light passes through the finger and it is partly absorbed by the tissues. The light not absorbed is then measured by a photodetector on the other side of the finger. The difference between the light emitted by the LED and the light recorded by the detector on the other side is an index of the type of tissues the light passed through. The two specific wavelengths emitted by a pulse oximeter are majorly absorbed by oxygenated hemoglobin and deoxygenated hemoglobin, respectively, allowing us to measure the blood volume in the finger and, consequently participants' heart rate as an increase in blood volume occurs in correspondence to each heartbeat. Measurements were collected at 64 Hz. Then, averaging the recorded values, the device automatically calculated the heartbeat frequency per minute. As such, the sampling frequency of the device's output signal was 1Hz. Heart muscle's contractions are under the control of both branches of the the Autonomic Nervous System, the sympathetic branch and the parasympathetic branch. Therefore HR is an overall index of ANS activation. Specifically, an increase in HR reflects heightened attention, promptness to action or distress, whereas a decreased HR is index of a calming response or a decreased attention in response to external stimulation (Berntson *et al.* , 1997; Bradley, 2009). However, a decrease in HR after being exposed to prolonged stress-eliciting stimuli is an adaptive deactivation of the stress axis and, as such, indicates the presence of an adaptive coping reaction (Pavlov *et al.* , 2015). Indeed, in a recent study Pavlov *et al.* (2015) presented to participants negative-affective eliciting images, and asked them to try reducing their negative feeling towards the pictures. When after being asked to reduce the negative attitude, participants actually gave images less negative ratings they showed physiological indexes of more efficient down-regulation of negative affect, namely lower cardiac blood output and lower total peripheral resistance. On the other hand an increase in HR in response to prolonged stress-eliciting stimuli is index of a failure in the deactivation of the prolonged sympathetic response. On the long run, prolonged sympathetic activations are known to cause maladaptive consequences such as heightened probability of develop a physical or psychological illness.

2.2.6 Genetic Assessment

All participants underwent genotypization for all the three considered polymorphisms. DNA extraction and genotyping were conducted by ACGT, Inc. (Wheeling, IL). DNA was extracted from each kit using the Oragene DNA purification reagent as per manufacturer's instructions. DNA concentrations were evaluated using spectroscopy (NanoDrop Technologies, USA). Each saliva sample was subdivided into three subsamples in order to undergo the three genotypizations. Each sub-sample underwent through a polymerase chain reaction (PCR) amplified for the target region of interest. A PCR reaction of 20 μl consisting of 1.5 μl of genomic DNA from the test sample, PCR buffer, 1 μM each of forward and reverse primers for the target region, 10 μM deoxyribonucleotides, KapaTaq polymerase, and 50 μM of MgCl2 was performed. Cycling conditions included an initial 15 min denaturation at 95°C, and 35 cycles of 94°C (30 s), 60°C (60 s), 72°C (60 s), and a final extension of 72°C for 10 min. PCR reactions were genotyped with an ABI 3730xl Genetic Analyzer (Applied Biosystems Inc.) and normalized with GeneScan 600 LIZ (Applied Biosystems, Inc.) size standards run on each sample. The genotype data were analyzed using GeneMapper ID (Applied Biosystems, Inc.).

Oxytocin Receptor Gene: rs2254298. The rs2254298 target region of the OXTR gene was polymerase chain reaction amplified with the forward primer 5′-TGA AAG CAG AGG TTG TGT GGA CAG G-3′ and the reverse primer 5′-AAC GCC CAC CCC AGT TTC TTC-3′. Here, participants carrying at least one A allele (A/A or G/A) in the rs2254298 region were merged into the A carriers group (Marusak *et al.* , 2015) and they were compared to individuals carrying two copies of the G allele. The distribution of genotypes for this region of the OXTR gene in the general population is 20-30% for A carriers and 70-80% for G/G homozygous. In our sample, all participants considered, the distributions were respectively 29% for A carriers and 71% for G/G homozygous.

Oxytocin Receptor Gene: rs53576. The rs53576 target region of the OXTR gene was polymerase chain reaction amplified with the forward primer 5′-GCC CAC CAT GCT CTC CAC ATC-3’ and reverse primer 5’-GCT GGA CTC AGG AGG AAT AGG GAC-3’. As for the other region on the OXTR gene, individuals carrying at least one copy of the A allele (A/A or G/A) were grouped into the A carriers group and they were compared to individuals carrying two copies of the G allele (G/G). The distribution of genotypes for this region of the OXTR gene in the general population is 50-60% for A carriers and 40-50% for G/G homozygous. In our sample, all participants considered, the distributions were respectively 57% for A carriers and 43% for G/G homozygous.

Promoter Region of the Serotonin Transporter Gene: 5-HTTLPR The target DNA, the 5-HTTLPR repeat region target, was polymerase chain reaction amplified with the forward primer (50-CCA GCA CCT AAC CCC TAAT-30) labeled with 6-FAM (6-carboxyfluorescein), and a reverse primer (50-AGG GAC TGA GCT GGA CAA CCAC-30). Participants carrying at least one long (L) allele (L/L or L/S) were merged into one L carriers group (N=30), while individuals homozygotes for the short allele (S/S) were categorized as a second group (N=12). L carriers were confronted against S/S homozygotes because, being S the less functional allele and considered the limited sample size, we aimed to strength our findings by comparing individuals carrying both less functional alleles with individuals carrying only one or none short allele.

2.2.7 Analysis

An averaged value for HR baseline was calculated for each participant by averaging the HR values of the first 60 seconds of recording. Than a linear model between the HR values recorded during the stimuli presentation and the averaged baseline was computed. The residuals of the resulting model were used as difference from the baseline. One averaged HR value for each condition (female cries, infant cries, bonobo cries) was than calculated for each participant. The responses to the different categories of distress vocalization sounds (female, infant, and bonobo) were kept separate in order to test participants' differential reactions to the three conditions. Further analyses were run starting from the computed averaged values. Outliers, that is values higher or lower than two stardard deviations from the mean of the total distribution were replaced with the mean value of the distribution. The dependent variable, HR changes from baseline, was normally distributed, therefore no transformation was applied to the data (skewness = 0.13). For each genetic polymorphism (rs2254298, rs53576, 5-HTTLPR), one multivariated ANCOVA was performed with the calculated HR average as the dependent variable, the cry type (female, infant, bonobo) as a within-subject factor, the genotype as a between-subject factor, and the four PBI dimensions (maternal care, paternal care, maternal overprotection, paternal overprotection) as continuous covariates. Cohen's d was used to evaluate the magnitude of effects which were found to be significant. Correlations were run as post hoc tests and Pearsons' r coefficients were used to analyze the strength of the effect of the covariate on the dependent variable.

2.3 Results

2.3.1 Oxytocin Receptor Gene: rs2254298

Paternal Care No main effect nor significant interaction effect was found between genotype (rs2254298), vocalization type and paternal care.

Paternal Overprotection A significant 2-way interaction between paternal overprotection and genotype (rs2254298) on HR values emerged ($F(1,41) = 8.54$, $p < .01$, $d = .99$). No significant main effect was found. Paternal overprotection was positively correlated with HR changes from baseline for A carriers, $r = .38$; while it was negatively correlated with HR changes from baseline in G/G homozygous, $r = -.35$. Pearson's r values were strong but

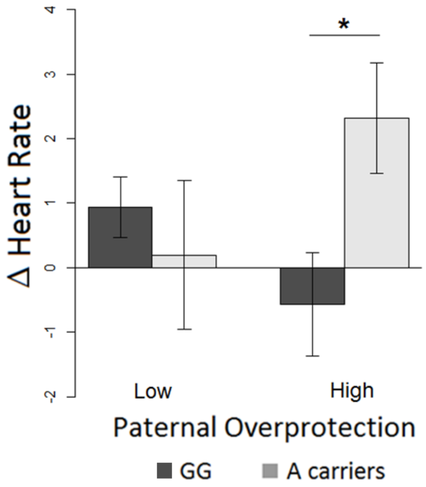

Figure 2.2: Effect of the interaction between paternal overprotection and OXTR rs2254298 genotype on HR changes. Heart Rate responses to distress vocalizations (calculated as difference from baseline) for the following groups are represented: (i) GG homozygotes who experienced low paternal overprotection, (ii) A carriers who experienced low paternal overprotection, (iii) GG homozygotes who experienced high paternal overprotection, and (iv) A carriers who experienced high paternal overprotection. *$p<0.05$, **$p<0.01$, ***$p<0.001$ [From Esposito *et al.* 2017]

not significant. However, the difference between the two slopes of the linear models for for G/G and A carriers, tested with Fisher's z, was statistically significant (z = -2.02, p < .05) [Figure 2.2].

Maternal Care No main effect nor significant interaction effect was found between genotype (rs2254298), vocalization type and maternal care.

Maternal Overprotection No main effect nor significant interaction effect was found between genotype (rs2254298), vocalization type and maternal overprotection.

2.3.2 Oxytocin Receptor Gene: rs53576

Paternal Care A significant 3-way interaction between paternal care, genotype (rs53576) and vocalization type on HR values emerged ($F(1,41) = 4.07$, $p <.05$, $d = .63$). No significant main effect was found. The effect of paternal care was present only in homozygous G/G variants and the effect was particularly strong in response to female crying sounds. The correlation between paternal care and HR changes from baseline was positive. The higher the paternal care, the higher the HR increase ($r = .36$, $p = 0.14$) [Figure 2.3].

Paternal Overprotection A significant 3-way interaction between paternal overprotection, genotype (rs53576) and vocalization type on HR emerged ($F(1,41) = 3.60$, $p <.05$, $d = .59$). No significant main effect was found. The effect of paternal overprotection was present only in A carriers and the effect was particularly strong in response to female crying sounds. The correlation between paternal overprotection and HR changes from baseline was negative. The higher the paternal overprotection, the higher the HR decrease ($r = -.28$, $p = 0.19$). No significant main effect of paternal overprotection was found [Figure 2.4].

Maternal Care No main effect nor significant interaction effect was found between genotype (rs53576), vocalization type and maternal care.

Maternal Overprotection No main effect nor significant interaction effect was found between genotype (rs53576), vocalization type and maternal overprotection.

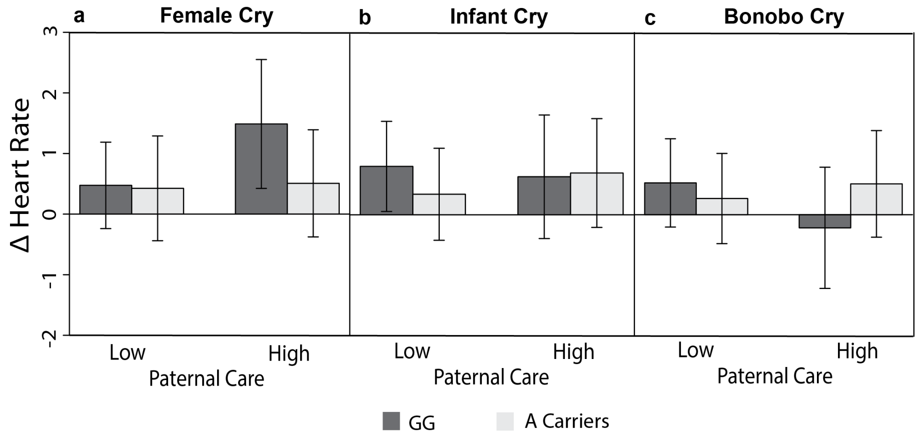

Figure 2.3: Effect of the interaction between paternal care and OXTR rs53576 genotype on HR changes. (a) Heart Rate responses to female cry (calculated as difference from baseline) for the following groups are represented: (i) GG homozygotes who experienced low paternal care, (ii) A carriers who experienced low paternal care, (iii) GG homozygotes who experienced high paternal care, and (iv) A carriers who experienced high paternal care. (b) HR responses to infant cry. (c) HR responses to bonobo cry. *$p<0.05$, **$p<0.01$, ***$p<0.001$ [For the original figure see Truzzi *et al.* 2018]

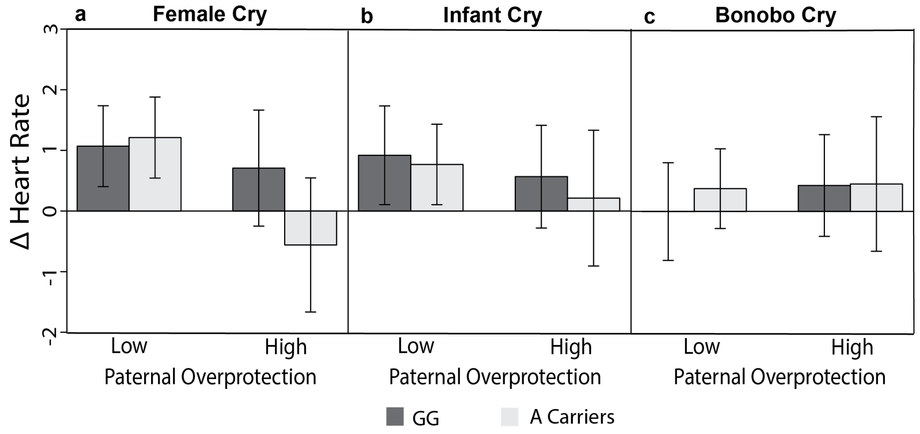

Figure 2.4: Effect of the interaction between paternal overprotection and OXTR rs53576 genotype on HR changes. (a) Heart Rate responses to female cry (calculated as difference from baseline) in the following groups are represented: (i) GG homozygotes who experienced low paternal overprotection, (ii) A carriers who experienced low paternal overprotection, (iii) GG homozygotes who experienced high paternal overprotection, and (iv) A carriers who experienced high paternal overprotection. (b) HR responses to infant cry. (c) HR responses to bonobo cry. *$p<0.05$, **$p<0.01$, ***$p<0.001$ [For the original figure see Truzzi *et al.* 2018]

2.3.3 Promoter Region of the Serotonin Transporter Gene: 5-HTTLPR

Paternal Care No main effect nor significant interaction effect was found between genotype (5-HTTLPR), vocalization type and paternal care.

Paternal Overprotection No main effect nor significant interaction effect was found between genotype (5-HTTLPR), vocalization type and paternal overprotection.

Maternal Care No main effect nor significant interaction effect was found between genotype (5-HTTLPR), vocalization type and maternal care.

Maternal Overprotection A significant 3-way interaction of maternal overprotection, genotype (5-HTTLPR), and cry type on HR emerged (F(1,41) = 6.37, p < .01, d = .84). No significant main effect was found. L carriers showed an opposite pattern of activation compared to S/S homozygotes, although the linear regression slopes did not significantly differ between the two groups. Specifically, the maternal overprotection reported by L carriers positively correlated with HR changes from baseline in response to female cry sounds, r = .19, p = .33; wherease the maternal overprotection reported by S/S homozygotes negatively correlated with HR changes from baseline in response to female cry sounds, r = -.27, p = .39. In both cases the effect sizes were small. [Figure 2.5].

2.4 Discussion

As expected, genetic and early environmental factors interact in moderating individuals development. Specifically, individuals with distinct genetic char-

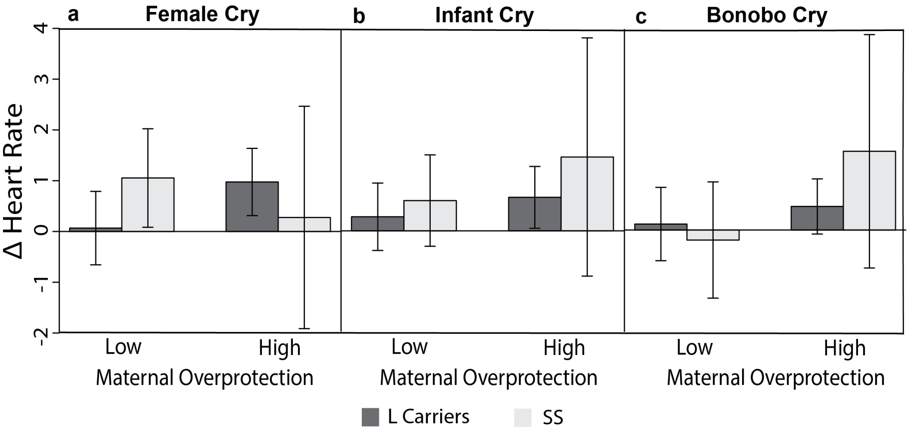

Figure 2.5: Effect of the interaction between maternal overprotection and 5-HTTLPR genotype on HR changes. (a) Heart Rate responses to female cry (calculated as difference from baseline) for the following groups are represented: (i) L carriers who experienced low maternal overprotection, (ii) SS homozygotes who experienced low maternal overprotection, (iii) L carriers who experienced high maternal overprotection, and (iv) SS homozygotes who experienced high maternal overprotection. (b) HR responses to infant cry. (c) HR responses to bonobo cry. *$p<0.05$, **$p<0.01$, ***$p<0.001$ [For the original figure see Truzzi *et al.* 2017]

acteristics did show differential sensitivity to the environment experienced in early childhood and this sensitivity translated in a differential shaping of physiological activations in response to distressing stimuli. Individuals carrying the A allele in the region rs2254298 of the OXTR gene showed opposite physiological responses compared to G/G homozygotes accordingly to the level of paternal overprotection experienced in childhood and these activations were not specific for the type of distressing stimuli since no effect of cry category was found. For A carriers there was a positive association between the experienced paternal overprotection and HR, so that the higher the paternal overprotection the greater the HR increase in response to distressing stimuli. For G/G homozygotes the patter was reversed: the higher the paternal overprotection the greater the HR decrease in response to distressing stimuli. Therefore both genotypes in this region of the OXTR gene seem to be sensitive to paternal overprotection but in opposite directions. It may be that individuals carrying different genotype may be more prone to interpret paternal overprotection in contrasting ways such as, for example, G/G homozygotes who experienced higher levels of protection in childhood learnt not to activate in response to distressing stimuli since someone else will deal with them and therefore would be less aroused by distressing stimuli. In contrast A carriers could interpret high paternal overprotection as an intrusion which increases their physiological arousal in response to distressing events. However, this interpretation need to be specifically investigated in future researches by asking participants explicit judgments and feeling towards the experienced parenting behaviors, rating it as a positive or negative experience. Concerning the second OXTR target region, rs53576, the two possible genotypes were differentially sensitive to paternal overprotection and paternal care. Specifically, individuals' carrying the A allele showed more sensitivity than G/G homozygotes to paternal overprotection. In contrast G/G homozygotes were found to be more sensitive to paternal care, whilst A carriers were not sensitive to paternal care at all. In both cases the effect of the interaction between genotype and paternal behavior was stronger on individuals' responses to female cries compared to activations in response to other cry types. A carriers who experienced higher paternal overprotection showed a higher HR decrease in response to female or infant cries compared to the one displayed by G/G homozygotes, whereas G/G homozygotes who experienced higher paternal care showed a HR increase in response to female or infant cries which was absent in A carriers. In the end, the two distinct genotypes in the promoter region of the serotonin transporter gene were differentially sensitive to the level of maternal overprotection experienced in childhood but the effect was specific for physiological changes in response to female cries.

Individuals' carrying two copies of the short allele, when they experienced higher levels of maternal overprotection, showed a HR decrease in response to female cries whereas individuals' carrying at least one copy of the long allele who experienced the same maternal overprotection levels showed a HR increase in response to female cries. Taking into account all the results together it is interesting to notice that the genotype in the OXTR gene seems to be affecting more individuals' sensitivity towards paternal behaviors whereas the genotype in the serotonin transporter gene seems to be regulating more individuals' sensitivity towards maternal behaviors. This difference may be due to the distinct systems in which oxytocin and serotonin are involved. Oxytocin is more involved in bond creation and affiliative behaviors whereas serotonin is more highly involved in reward system ans mating behaviors (Ebstein *et al.* , 2012; Fisher, 2000). Since only males were tested it makes sense that the genotype regulating affiliative behaviors may greatly affect the bond with the father compared to the mother whereas the genotype involved in mating would regulate more the relation with the mother since adults' social interactions with intimate partners were found to be influenced by the parenting behavior showed by the opposite-sex parent during childhood (Apostolidou, 2006). Also, it seems that genetic predispositions tend to moderate sensitivity towards overprotective behaviors with more frequency than sensitivity towards caring behaviors. A possible interpretation of these result could be that overprotection occurs in the context of possible dangerous situations or neutral situation judged as dangerous by the parents. Therefore the sensitivity of the sympathetic system may be more involved with parenting overprotection levels. The limitations of the present study points to future directions to pursue. First the small sample size decreases the power of the performed analysis and future studies will be needed to replicate the findings and generalize them including larger and different populations such as, for example, women in order to investigate their own pattern of sensitivity towards maternal vs paternal behaviors. Also, a larger sample would allow to consider all the possible combinations of alleles in the two considered genes at the same time in order to obtain a wider and more complete perspective on how genetic and environmental factors shape individuals' physiological responses towards social stimuli. Nevertheless, the distribution of the alleles in the tested sample was coherent with the distribution found in the general population and, as such, we considered the results reliable and worth to be reported. Moreover, the nature of the questionnaire used to measure the quality of child-parent bond represents a second limitation to the present study. Indeed the child-parent bond was measured by asking adults about their opinion and their memories of their parents' behaviors during childhood. And self-reported information may not be accurate and in line with the attachment style (Borelli *et al.* , 2013). However, even though the PBI may not be as objective as a direct observation of parent-infant interactions what the adults retain of the interaction with the parents is still informative about his/her internal perceptions and since the PBI asks questions about specific behaviors related to the two dimensions of care and overprotection avoiding direct questions about the participants' explicit evaluation of the target relationship the bias due to the self-report are minimized as much as possible. Also, Manassis *et al.* (1999) found that although the PBI may be unreliable in clinical sample, for the typical population the attachment information obtained from the PBI and another more structured and thorough measure of adult attachment - the Adult Attachment Interview (AAI) - are comparable. Nevertheless future researches should directly address and solve this issue by performing a longitudinal study which would enable researchers to directly observe parent-infant interactions and individuals' development thoughout lifetime. Concerning the physiological measurements, the heart pace is under the control of the sympathetic and the parasympathetic nervous system and, as such, its rate is the result of the balance between the activity of the two systems. Since in the present study the focus was on the differential physiological activations between individuals with distinct genetic predispositions and childhood experiences the heart rate was a suitable measure to obtain an overall view of the ANS responses. However, it would be interesting in future studies to tackle the two branches individually in order to more precisely characterize individuals' peripheral nervous system activations. Two measures suited to address the parasympathetic activations would be the Respiratory Sinus Arrhythmia (RSA) and the Positive Expiratory Pressure (PEP). Since respiration rate is related to the activation of the vagus nerve which is under the control of the parasympathetic nervous system and, as such both the RSA and the PEP are index for the parasympathetic activity. On the other hand the Galvanic Skin Response (GSR), which occurs in response to a threatening stimuli or distress situation, is part of the fight-or-flight response and, as such, it is a good index of the sympathetic nervous system activity. Other than the RSA, PEP, and GSR it would also be interesting to measure the responses of the central nervous system to investigate the effects of gene-environment interaction also on higher cognitive functions. Lastly, to improve and generalize our understanding, individuals' responses to different stimuli, for example calming social stimuli or visual stimuli, should be tested.

Chapter 3

How is adaptive caregiver-infant bonding achieved?

3.1 Introduction

The mechanisms through which parenting styles begin to affect early development are still largely unknown. To start investigating them, we applied behavioral micro-analysis on non-human primate caregiver-infant dyads to better comprehend the core events and behaviors underlying the interactions. Also, an animal model was chosen bearing in mind the goal to investigate the causal relations between neurophysiological activations and behaviors. Non-human primates are likely to be more suitable than other animals to model social behaviors given their phylogenetic closeness to humans (Miller *et al.* , 2016). Also, by separating non-human primate infants from their parents researchers found similarities between human and non-human primates in the role covered by caregivers' behaviors in determining infants' developmental adaptiveness. Indeed, depriving non-human primates infants of the interaction with caregiver or providing them with unresponsive care caused the individuals to develop disorganized attachment and hindered social behaviors, as well as depressive symptoms (Griffin & Harlow, 1966; Ruppenthal *et al.* , 1976; van Ijzendoorn *et al.* , 2009). Furthermore, in presence of a threatening stimuli infant cotton-top tamarins were found to choose proximity to the caregiver which best fulfilled their needs, which may not have corresponded with the mother (Kostan & Snowdon, 2002). Within non-human primates we focused on marmosets since marmoset' colonies are organized in families in which newborns are cooperatively bred, resembling human familial structure. Fathers and mothers create stable mating couples and the load of newborns' nurturing is divided between them and the older siblings which are still part of the family. This characteristic makes the marmoset very well suited to model the development of caregiver-infant interactions and to understand the underlying causal mechanisms. However, in order to be able to rely on marmosets as animal models to study caregiver-infant bonding, it is first necessary to describe caregiver-infant interactions in marmosets highlighting similarities and differences in order to see to what extent they are comparable to humans'. During the first month of life pups are carried 24hours long by family members and fathers are the ones that carry the pups most (Kazutaka et al., *in preparation*). In the perspective of a cooperative breeding this task division is highly adaptive since while fathers use more energy to carry the pups, mothers can feed more and therefore increase their lactation's nutritional power and, in turn, boost pups' development. During the first month of life being alone, separated from any caregiver, is an unusual situation for a pup and when it happens, infants emit distress calls. Figure 3.1 represents the spectrogram of an audio signal recorded while the infant is separated from and then reunited (green vertical line) with the caregiver. It shows how the pup emits distress calls with high time-frequency during separation

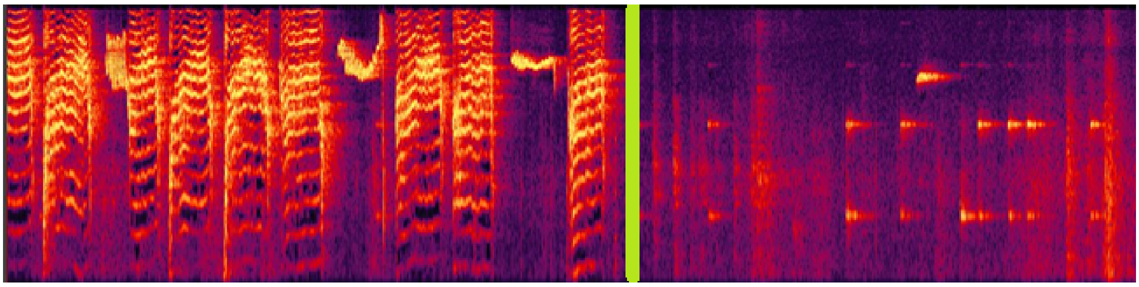

Figure 3.1: Spectrogram of infant distress calls emitted when separated froma nd then reunited with the caregiver. The moment of reunion is marked with a green vertical line.

and than, as soon as it is reunited with the caregiver distress calls cease completely.

Distress calls usually stop as soon as the pups are retrieved and carried again by one of the caregiver, however we noticed that sometimes, pups did not completely sooth when reunited with the caregivers and this differential behaviors seemed to be related to caregivers' own behaviors towards pups. Here we aimed (i) to apply behavioral micro-analysis in order to understand how and which caregivers behaviors moderated infants' soothing levels during reunion and (ii) to investigate the suitability of the marmoset as the reference animal model for the study of caregiver-infant interactions and bonding in humans. Specifically we hypothesized that (i) infant behaviors within the interaction with a specific caregiver, especially the soothing behaviors displayed after a separation period during the reunion with the caregiver, are driven by the caregiver's own behavioral pattern showed towards the target infant and therefore that (ii) pups' responses are specific for each caregiver. Furthermore, (iii) we hypothesized a certain level of similarity between the caregiver-infant bonding behavioral displays find in marmoset infants and the ones measured in human infants while performing the Strange Situation Procedure. The presence of such similarities would be consistent with the use of marmosets as a reference model for the study of caregiver-infant bonding in humans.

3.2 Methods and Measurements

3.2.1 Retrieving Test

Each pups' own home cage was used as experimental setting. Home cages are constituted of three main blocks. All family members but for the target pup and the target caregiver involved in the experimental sessions were confined in the left section of the home cage. The experimental setting, the test cage, was therefore formed by the central and right parts of the whole home cage. The measures of the test cage were the following: W43cm * D60cm * H66cm.The target caregiver was confined in the central section of the home cage while the pup was separated from the caregiver and moved inside a cylindrical basket (H15cm * dia.15cm), too high for it to escape by itself, placed on the right section of the home cage. The central and right sections were connected via an external corridor placed in front of the test cage (W75cm * D21cm * H30.5cm). The connection between the corridor and the right section of the home cage was kept opened while the entrance to the corridor from the central section of the home cage, where the caregiver was confined, was regulated with a sliding door maneuvered from the outside by the experimenter. The session started when the sliding door was opened and the caregiver was therefore allowed to reach and retrieve the infant [Figure 3.2].

From the moment of retrieving, the session lasted for 10 minutes. All through the session, both caregiver's and pup's behaviors as well as vocalizations were recorded using two video cameras and one directional microphone,

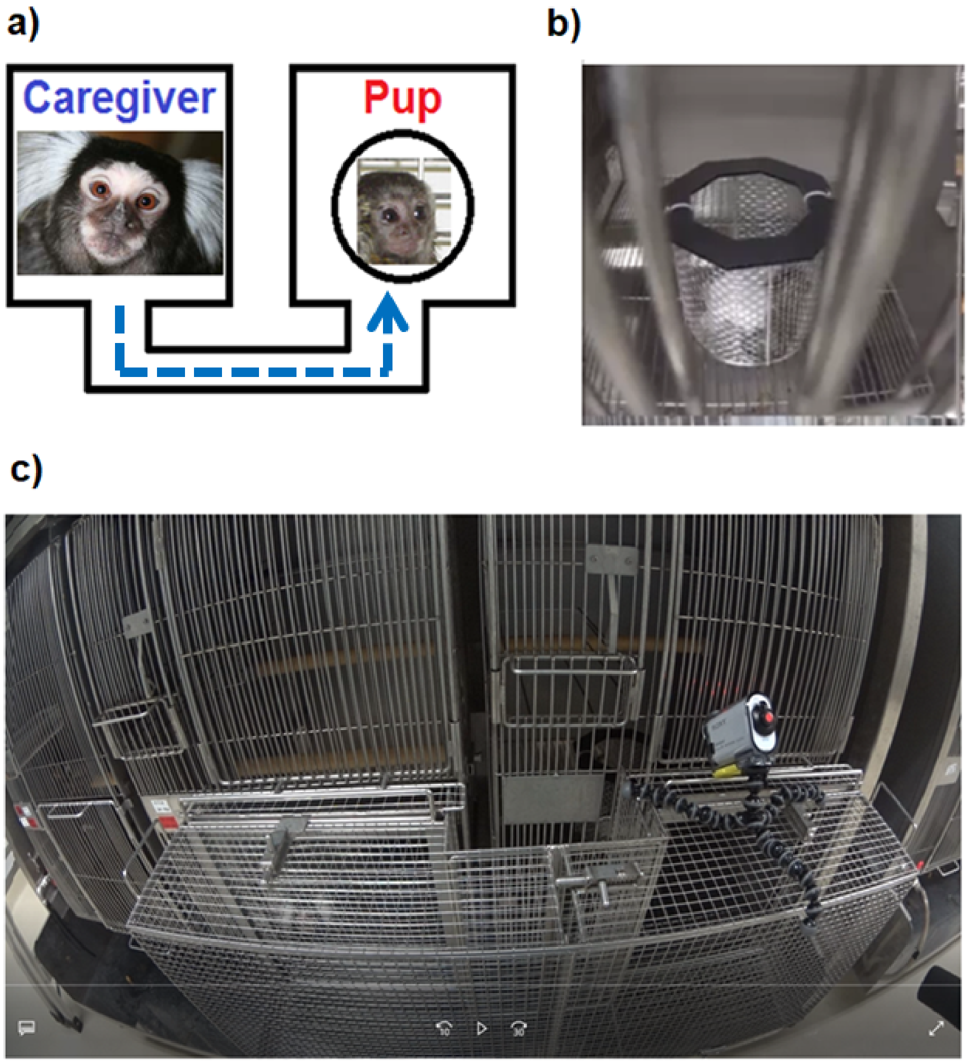

Figure 3.2: Here the experimental setting - central and right parts of the home cage - is depicted. a) Schematic representation of the experimental setting from top view. When the slide blocking the entrance to the corridor connecting the caregiver's cage with the pup's basket was opened, the caregiver walked down the corridor th reach the pup in the basket (blue arrow). b) Close-up view of the pup's basket - frontal view. c) Frontal view of the experimental setting. On the left, the caregiver's starting cage, on the right the part of the home cage where the pup's basket was placed, in front the corridor connecting the caregiver with the pup's basket. On top on the corridor, in front of the right part of the cage, the close-up camera focused on the pup's basket is visible.

respectively. Videos and audio recordings were then coded by a researcher in order to calculate the frequency per minute for each variable of interest. Furthermore, the researcher performing the experiment carried out an online coding of behaviors at low time-resolution – 30sec-long time windows – on a standardized coding sheet. Repeated measures were collected since for each dyad multiple retrieving tests were carried out in subsequent post-natal days. Therefore, the total number of sessions included in the study was N = 261.

3.2.2 Recordings

Video Recordings

One video camera (HDR-AS100V Sony) was placed on top of the corridor connecting the two section of the cages in order for it to be focused on the cylindrical basket where the pup was placed when separated from the caregiver. The distance between the camera and the basket was 30cm. The second camera (HDR-AS100V Sony) was placed at first right on top of the cage, in contact with the cage, in order to obtain a bird view over both of the sections of interest. This camera setting was used for 58 sessions. In a second moment, for the remaining 203 sessions, the second camera was placed in front of the cage, in order to obtain a frontal view of both the two sections of interest and of the corridor connecting them. This change of setting was chosen in order to improve the visibility of marmosets' behaviors. The distance between the front panel of the test cage and the second camera was 40cm. The two cameras were started together using a single controller (DR-60DMKII (TASCAM), recorded with 24bit, 96kHz) and, as such, usually the two videos were perfectly synchronized. However, the synchronization between each couple of video was checked and where a mismatching was present the videos were synchronized by cutting them using the Movie Maker software (2012).

Audio Recordings

In the first 116 sessions the vocalizations were coded starting from the audio track extracted directly from the video cameras, whereas in the remaining 145 sessions the vocalizations produced by the target marmosets were recorded using a directional microphone (MKH 416 SENNHEISER) placed at a distance of 50cm from the pup's basket. The focus of the microphone was chosen to be directed towards the cylindrical basket where the pup was placed during separation. The change of microphone setting was applied to improve the quality of audio recording and, as a consequence to improve the discrimination and the duration measurement of pups' and caregivers' calls.

3.2.3 Marmosets' Families

Six families, for a total of 14 caregivers and 21 pups, were involved in the study [Table 3.1]. Each family was reared in a different cage and each pup's own home cage was used as experimental setting. Three couple of parents were tested for more than one birth. One father, Tochan, was part of two different families because of the death of its first partner, Kachan. Two couples of parents have already had parenting experience previous to the beginning of the retrieving tests. Siblings were only involved in retrieving tests of one generation of twins, the one subsequent to them, which was consider birth number one for the siblings, being their first retrieving experience. One mother, Ribbon, had previous experience in performing the retrieving test, as a sister. In Ribbon's case we considered the retrieving tests performed while she was a sister as birth number 1 for sisterhood, whereas retrieving tests performed when she was a mother were again considered birth number, 1 albeit for motherhood. This approach was adopted because a huge change in caregiving behaviors was detected together with the caregiver status change. Indeed, Ribbon showed less adaptive behaviors as a mother compared to when she participated in the retrieving test as a sister.

3.2.4 Measurements

Behavioral Coding

Online Coding While performing the retrieving tests the experimenter carried out a pen and paper online coding of caregivers' behaviors. Starting from the moment of retrieving, the following relevant behaviors were recorded on the paper sheet: carrying, rejection, lactation, ano-genital licking, stereotypic behavior, self-scratching, feeding behavior, substrate marking, playing with object in the cage, adults' vocalizations. The online coding sheet included a 20-rows table. Each row represented a 30 seconds time bin, for a total of 600 seconds. Starting from the moment of retrieving, a pre-setted timer alarm rang every 30 seconds until the end of the experimental session, which lasted up to 10 minutes after the occurrence of retrieving. When the alarm rang the experimenter was required to code caregivers' behaviors in the successive row, correspondent to the right time-bin.

Video Coding First the synchronization between the two videos recorded within a same session was checked. In case the videos were not perfectly synchronized, they were cut accordingly using as time reference the moment of a highly recognizable caregiver's behavior (i.e. the retrieving time, or the first time the caregiver touched the pup's basket). Caregivers' and infants' behaviors were then coded at 5Hz (0.2 seconds-long time bins) using a coding

Table 3.1: List and composition of marmosets' families. In the "Pups" column black horizontal lines divide pups born from different pregnancies

Family	Caregivers	Pups
Family 1	Hime(Mother) Oji(Father)	Kate(F) Bill(M)
		Charlotte(F) George(M)
		Diana(F) Henry(M)
		Eugenie(M)
Family 2	Fastner(Mother) Chuck(Father)	Hook(M)
		Wendy(F)
		John(M)
		Cubby(M)
Family 3	Lame(Mother) Mogol(Father) Ribbon(Sister) Beads(Brother)	Pearl(F) Stone(M)
Family 4	Kachan(Mother) Tochan(Father) Maru(Brother)	Yu(F) Pon(M)
		Gaku(M) Saku(M)
Family 5	Mama(Mother) Tochan(Father)	Ataro(M) Setaro(M)
Family 6	Ribbon(Mother) Kabu(Father)	Shirayury(F) Suisen(F)

software, Solomon Coder [for the complete list of behaviors see Table 2]. At each time point, the presence (1) or absence (0) of each behavior was numerically coded. Behaviors which occurred in less than 20% percent of the sessions were excluded from further analysis. Lactation was also excluded since it was both difficult to spot from video recordings and it was only present in females while the project aimed to test all caregivers. 5 session were double coded from two different observers in order to calculate inter-rater reliability. Inter-rater reliability, calculated using Cohen's Kappa, was high (Mean = 0.81; Median = 0.84; Range = [[0.55,1.00]).

Vocal Coding

Vocalizations were coded using the PRAAT software ver. 6.0.14. Every call from the target dyads in the audio tracks was labeled. The coded infant vocalizations were the following: vhee, phee, trill, twitter, tsik [Figure 3.3] (Bezerra & Souto, 2008; Pistorio *et al.* , 2006; Primate Society of Great Britain, 2018).

Vhee calls are broadband mid-length calls specific to infants. They are of particular interest because of their acoustic characteristics that bear some similarities with human infant cries. Phee calls are high-pitch long calls know to be used as long-distance communication even between adults. Phees were of particular interest because in a recent paper Takahashi *et al.* (2015) proposed that during marmosets' development, from infancy to adulthood, these long distance calls would substitute the short and broadband Vhee calls, underlying the presence of a complex vocal development. Trills are mid to long-length calls characterized by a vibrato – a repetitive fluctuation of pitch. Twitters are short calls characterized by a raising pitch followed by a decrease. There could be a single twit emission or a series of twitters in a

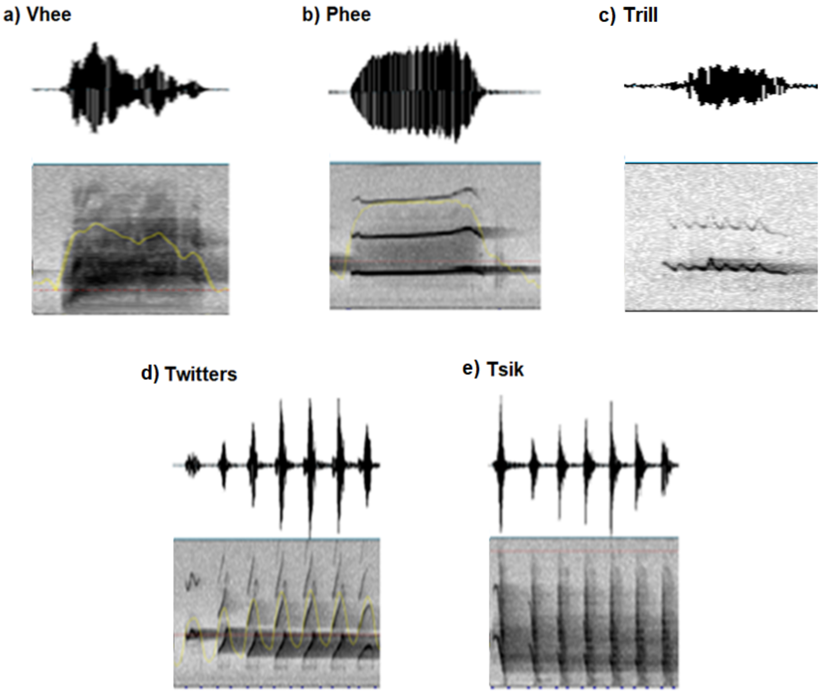

Figure 3.3: Spectrograms and waveforms of infants' calls.

row. A series of twitters, characterized by intervals between twitters shorter than 0.2 seconds, counts as a single call. Tsiks are, again, short calls, however tsiks differentiate from twitters because they lack the beginning pitch raising only showing a pitch falling. In the case of tsiks also there could be a single tsik emission or a series of tsiks in a row. A series of tsiks, characterized by intervals between twitters shorter than 0.2 seconds, counts as a single call. Given that in the first audio setting – audio obtained directly from the camera microphone – it was not easy to call twitters and tsiks apart and considered their similar characteristics, in this study twitters and tsiks were combined together. Concerning caregivers, the adults' coded vocalizations were: phee, twitter, ek, chatter [Figure 3.4].

As for infants, adult's phees are high pitch long calls meant for long distance communications and adult's twitters show the same acoustical characteristics

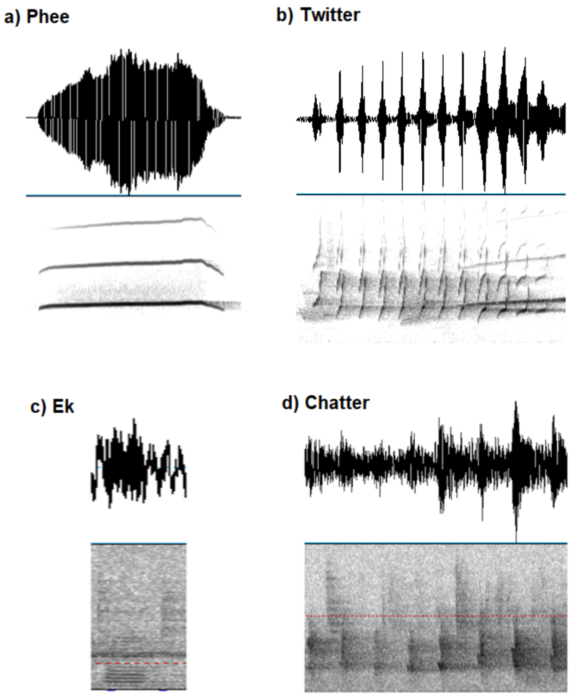

Figure 3.4: Spectrograms and waveforms of caregivers' calls.

as infant's ones. Since the form of phees and twitters were similar between adults and infants, to clarify which member of the dyad emitted the call the online coding sheets, where adults' calls had been recorded during the session, were consulted. Eks are short broadband calls emitted by adults which seems to indicate mild anxiety. Chatters are highly emotive sounds characterized by repeated low-pitch harsh staccato calls emitted in response to threatening aggressive situations. Chatters had very few occurrences within our sample, however we included them in the analysis given the remarkable social importance of these calls [Table 3.2].

Table 3.2: List and definition of caregivers' and infants' coded behaviors and their respective Cohen's Kappa values for inter-rate reliability

	Name	**Description**	**Cohen's Kappa**
Latencies	Retrieving Latency	Retrieving latency(sec)	-
	Reach Basket Latency	First time the caregiver arrives within arm-distance to the basket (sec)	-
	Touch Basket Latency	First time the caregiver touches the basket with any limb (sec)	-
	Touch Infant Latency	First time the caregiver touches the pup with any limb (sec)	-
Infants' variables	Vhee	Infant Vhee calls (Number of occurrences/min)	0.78
	Trill	Infant Trill calls (Number of occurrences/min)	0.68
	Twit	Infant Twit calls (Number of occurrences/min)	0.84
	Phee	Infant Phee calls (Number of occurrences/min)	0.67
	Infant Movement	The infant is not carried and moves around the cage alone (Frequency/min)	0.84
	Search for Contact	The pup looks for contact with the caregiver	-[a]
Caregivers' variables	Carrying	The caregiver carries the pup on its back or belly (Frequency/min)	0.97
	Rejection	The caregiver rejects the pup by pushing, biting or rolling against the cage (Frequency/min)	0.75
	Contact	The caregiver and the pup touch each other without carrying (Frequency/min)	0.97
	Play	The caregiver plays with any object found in the cage or in the basket (Frequency/min)	0.91
	Self-Scratch	The caregiver scratch itself (Frequency/min)	1
	Locomotion	The caregiver walks, runs or jumps (Frequency/min)	0.65
	Stereotypy	The caregiver runs in vertical circles three times or more (Frequency/min)	0.91
	Adult Phee	Adult Phee calls (Number of occurrences/min)	1
	Adult Twit	Adult Twit calls (Number of occurrences/min)	1
	Adult Ek	Adult Ek calls	-[b]
	Adult Chatter	Adult Chatter calls	-[b]
	Marking Substrate	The caregiver marks its territory	-[b]
	Self Grooming	The caregiver grooms itself	-[b]
	Feeding	The caregiver eats	-[b]
	Yawn	The caregiver opens the mouth and protrudes the tongue	-[b]
	Grooming	The caregiver grooms the infant	-[b]
	Licking	Ano-genital licking on the pup	-[b]
	Breast-Feeding	Mother breast-feeds the pup	-[c]

[a] Few occurrences and excluded because it requires interpretation of pups' intentions, [b] Few occurrences,[c] Only maternal behavior

3.2.5 Preliminary Analysis

Each experimental session was divided into six time windows as follows: (i) Separation (S), (ii) Holding (H), (iii) Transport (T), (iv) Carrying (C), (v) Rejection (R), (vi) Alone (A). "S" included the time from the start of the experimental session to the time of retrieving. "H" included all the time points when the pup was attached to the caregivers' body and at the same time the caregiver was not moving. "T" included all the time points when the pup was attached to the caregiver's body and the caregiver was moving – walking, running, jumping, showing stereotypic behavior. "C" included both H and T time points. "R" referred to all the time points when the caregiver is either biting, pushing, or rolling in order to get the pup leave its body. A referred to the time when the pup was not attached to the caregiver's body after the retrieving had taken place. Behavioral frequencies for each time phase was calculated where appropriate [Table 3.3]. To make sure that the infant calls considered within the H and T phases were not a reaction to a recent rejection, if in the 5 seconds before the onset of the call any rejecting behavior was present the call was excluded. Furthermore, the times necessary for the caregiver to (i) reach the pup's basket (Reach Latency), (ii) touch the pup's basket (Touch Basket Latency), (iii) touch the infant (Touch Infant Latency) and (iv) retrieve the infant (Retrieving Latency) were calculated.

3.2.6 Analytic Plan

Firstly the analysis focus on the parental behaviors. Principal Component Analysis (PCA) coupled with k-means clustering will be applied to investigate the structure of caregivers' behavioral patterns and the relations between different behaviors as well as the presence of different clusters of caregiving

Table 3.3: List of behaviors for which frequency was calculated for each time phase

Time Phase	Caregiver/Infant	Behavior
All Session		Retrieving Latency Reach Basket Latency Touch Basket Latency Touch Infant Latency Carrying Rate
Separation (S)	*Caregivers*	Contact Stereotypy Self-Scratch Play Locomotion AdultPhee AdultTwit
	Infants	Vhee Trill Twit Phee Infant Movement
Carrying (C)	*Caregivers*	Stereotypy Locomotion Mean Carrying Bout Duration
Holding (H)	*Caregivers*	Rejection Self-Scratch Play AdultPhee AdultTwit
	Infants	Vhee Trill Twit Phee
Transport (T)	*Infant*	Vhee Trill Twit Phee
Alone (A)	*Caregivers*	Contact Stereotypy Self-Scratch Play Locomotion AdultPhee AdultTwit
	Infants	Vhee Trill Twit Phee Infant Movement

quality. Next, the same analysis – PCA coupled with k-means clustering – will be applied to pups' behaviors in order to investigate the structure of infants' behavioral patterns and the presence of clusters of infants. In the end, the behaviors of caregivers and pups will be analyzed together moving the focus on the dyads. Correlations between all caregivers and infants behaviors will be investigated and then the caregiving behaviors which mostly correlates to the presence of infants' calls during reunion with the caregiver will be more deeply investigated in order to check the contribution of caregiving behaviors vs infants' own temperamental predispositions in determining the frequency of distress calls when reunited with the caregiver. Hierarchical clustering will then be applied on all behaviors averaged per dyad to categorize caregiver-infant interactions and investigate whether it is possible to evaluate them starting from behavioral frequencies. In the end, a General Linear Model (GLM) will be run to test the presence of possible moderators, such as caregiver status (father, mother, brother, or sister), pups' PND, or birth number, on the emittance of infants' calls during reunion.

3.3 Results

3.3.1 Caregivers

An averaged value of each behavior was computed for each caregiver and birth. That is, when a caregiver participated in the retrieving test by rescuing pups from successive births, a different value was calculated for each birth, since the caregivers' behavior could have even greatly changed from one birth to the next. The Elbow method highlighted the presence of four major clusters of caregiving behavioral patterns [Figure 3.5].

In addition the Principal Component Analysis (PCA) highlighted the multi-

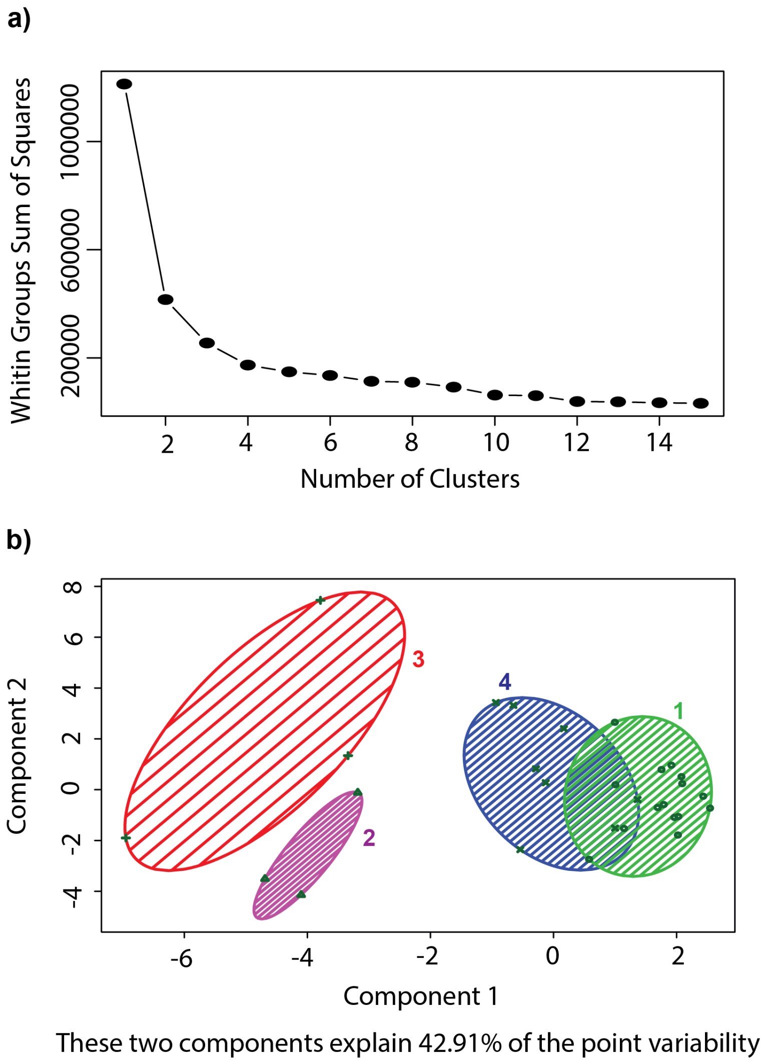

Figure 3.5: Analysis of caregivers' behavioral patterns. a) In the first panel results form the application of the Elbow method are shown. The plot shows the presence of a significant difference between behavioral patterns up to 4 clusters. b) In the second panel, results from the Principal Component Analysis are shown. The first two components explain 42.91% of the variability. The dots represent the caregivers distributed onto the two principal components and categorized in 4 clusters.

dimensional nature of the dataset. Figure 3.5 shows the dataset projected on its first two major components, which explain about 42.91% of the variability, and how the four parental clusters are distributed on these two components. To describe the characteristics of caregiving styles the averaged caregiving behaviors of the four parental clusters were then considered, with a particular focus on the variables which most contributed to the cluster creation. The contribution of each variable to clusters' definition was inferred considering the loading each variable had in determining the first component of the PCA [Table 3.4]. Cluster number 1 included more caring caregivers since they showed higher carrying rate, longer carrying bouts and shorter retrieving latencies coupled with lower rejection frequency and less stress-related behaviors, such as stereotypy, both during Separation and Alone time windows compared to the other clusters. Clusters 2 and 3 were both characterized by low carrying and higher rejection rates. However, caregivers in cluster 2 had shorter carrying bouts and played more with objects during Separation and Alone phases compared to cluster 3 caregivers, whereas in cluster 3 caregivers showed very long retrieving latencies and more frequent distress-related behaviors compared to caregivers in cluster 2. Cluster 4 was collocated mid-way between the more adaptive cluster, cluster 1, and the poorer caregivers, clusters 2 and 3.

Tochan, Chuck, Fastner and Oji in different births belonged to different clusters. This means that their behavioral pattern changed in subsequent births. Specifically, Tochan and Chuck belonged to cluster 2 at the beginning but they subsequently moved to clusters 4 and 1 respectively. Fastner moved from cluster 4 to cluster 1, while Oji belonged to cluster 1 for the first 3 births and it moved to cluster 4 for the 4th birth. Therefore, Tochan, Chuck and Fastner improved the adaptiveness of their caregiving behavior, while

Table 3.4: Caregivers' behaviors averaged for cluster and ordered for PCA loadings of 1st component

Behaviors	**Loadings on 1st PCA component**	**Cluster 1**	**Cluster 2**	**Cluster 3**	**Cluster 4**
Carrying	0.38	54.11	14.15	18.66	41.59
Carrying Bout Duration	0.38	556.38	71.22	175.29	395.32
Rejection	-0.32	0.25	3.96	4.44	1.02
Locomotion(A)	-0.31	1.10	8.25	5.55	2.71
Stereotypy(A)	-0.30	0.15	2.50	3.29	0.14
Retrieving Latency	-0.27	25.21	98.56	189.25	74.99
Play(A)	-0.26	0.29	4.83	2.16	0.63
Touch Infant Latency	-0.21	18.55	18.45	171.28	57.65
Stereotypy(S)	-0.21	0.10	0.21	2.56	0.03
Play(S)	-0.19	0.04	10.56	0.34	0.61
Touch Basket Latency	-0.17	20.69	10.36	113.32	38.44
Play(H)	-0.15	3.06	13.41	3.57	3.18
Self Scratch(S)	-0.15	0.00	0.00	0.24	0.07
Reach Basket Latency	-0.13	7.91	8.30	33.90	14.81
Contact(A)	-0.12	1.27	5.83	1.44	2.74
Locomotion(S)	0.10	19.45	14.18	15.65	18.22
AdultTwit(A)	0.04	0.04	0.13	0.09	0.08
AdultPhee(H)	0.08	0.92	0.03	0.00	0.41
Contact(S)	0.08	17.28	24.77	4.92	12.28
Self Scratch(H)	-0.05	0.21	0.17	0.29	0.37
AdultTwit(S)	0.05	0.18	0.09	0.02	0.72
Stereotypy(C)	-0.05	0.66	0.59	0.72	0.49
AdultTwit(H)	0.04	0.20	0.17	0.19	0.15
AdultPhee(S)	0.04	0.05	0.05	0.00	0.23
Locomotion(C)	0.01	6.21	5.34	5.69	4.49
AdultPhee(A)	-0.01	0.05	0.04	0.00	0.10
Self Scratch(A)	0.01	0.07	0.02	0.09	0.08

Table 3.5: Caregivers' clusters

Cluster	**Caregiver**	**Birth**
1	Chuck (Father)	3-4
	Fastener (Mother)	2-3-4
	Oji (Father)	1-2-3
	Hime (Mother)	1-2-3-4
	Kachan (Mother)	2-3
	Beads (Brother)	1
2	Chuck (Father)	1
	Tochan (Father)	2-3
3	Kabu (Father)	1
	Ribbon (Mother)	1
	Mogol (Father)	2
4	Chuck (Father)	2
	Fastener (Mother)	1
	Lame (Mother)	2
	Ribbon (Sister)	1
	Mama (Mother)	1
	Tochan (Father)	4
	Oji (Father)	4
	Maru (Brother)	1

Oji in the 4th birth showed a poorer parenting style. Ribbon on the other hand changed cluster together with caregiver status, from sister to mother. As a sister Ribbon belonged to cluster 4 while as a mother Ribbon became a poorer caregiver and moved to cluster 3 [Table 3.5].

3.3.2 Infants

An averaged value of each behavior was computed for each infant. The Elbow method highlighted the presence of four major clusters of infants' behavioral patterns [Figure 3.6]. In addition the PCA confirmed the multidimensional nature of the dataset. Figure 3.6 shows the infants projected on the first two major components of the dataset, which explain 41.68% of the variability, and how the four infants' clusters are distributed on these two components. To describe the characteristics of infants' behavioral patterns the averaged infants' behaviors of the four clusters were then considered, with a particular focus on the variables which most contributed to the cluster creation. The contribution of each variable to clusters' definition was inferred considering the loading each variable had in determining the first component of the PCA [Table 3.6]. Cluster 1 was overall characterized by more frequent calls during all the time phases compared to the other clusters but for five cases. Trills during Separation were more frequent in cluster 3, while Twitters during Alone phase, and Vhee, Phee, and Infant Movement during Separation were more frequent in cluster 4 compared to other clusters. Also, the only pup included in cluster 4 emitted fewer calls during Holding, Transport and Alone time windows compared to clusters 2 and 3, whereas it showed a higher calls' frequency during Separation.

Pups from the same parents were usually clustered together but for Eugenie, Kate, Saku, and Wendy. Between the parents of these pups there were the ones more likely to change caregiving behavioral pattern in subsequent births, namely Oji (Eugenie's and Kate's father), Tochan (Saku's father), Chuck (Wendy's father) and Fastner (Wendy's mother). Also, twins retrieved by Ribbon when she was a sister belonged to cluster 2, whereas twins retrieved by her when she was a mother and changed her behavioral pattern were included in cluster 1. [Table 3.7].

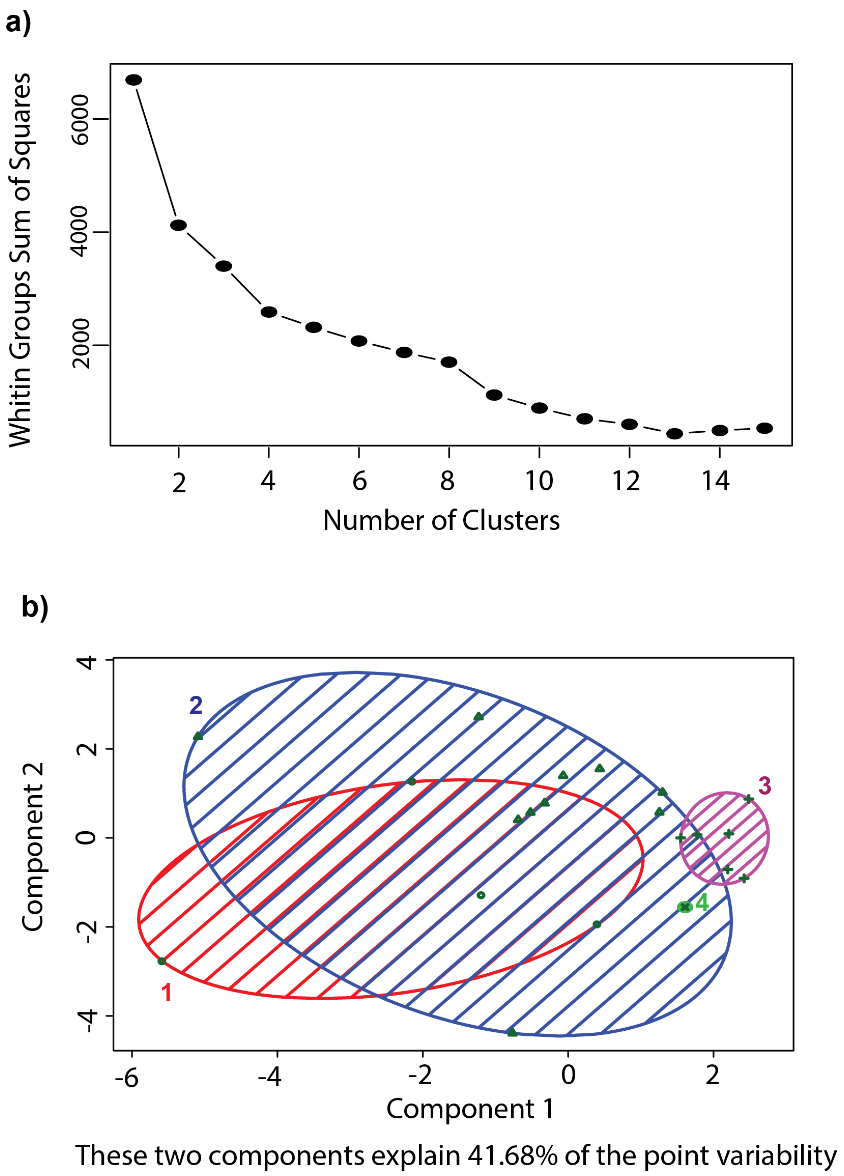

Figure 3.6: Analysis of infants' behavioral patterns. a) In the first panel results form the application of the Elbow method are shown. The plot shows the presence of a significant difference between behavioral patterns up to 4 clusters. b) In the second panel, results from the Principal Component Analysis are shown. The first two components explain 41.68% of the variability. The dots represent the infants distributed onto the two principal components and categorized in 4 clusters.

Table 3.6: Infants' behaviors averaged for cluster and ordered for PCA loadings of 1st component

Behaviors	**Loading on 1st PCA component**	**Cluster 1**	**Cluster 2**	**Cluster 3**	**Cluster 4**
Twit(T)	-0.40	0.61	0.33	0.04	0.00
Vhee(A)	-0.34	12.26	5.01	0.67	1.56
Vhee(T)	-0.34	0.16	0.19	0.01	0.00
Twit(H)	-0.34	0.41	0.27	0.04	0.00
Phee(A)	-0.32	1.61	1.73	0.04	0.46
Phee(T)	-0.28	0.13	0.10	0.01	0.00
Trill(T)	-0.26	0.26	0.14	0.09	0.00
Vhee(H)	-0.23	0.10	0.13	0.00	0.00
Trill(S)	0.19	9.07	7.22	11.69	9.08
Trill(A)	-0.19	4.38	2.75	0.63	0.88
Trill(H)	-0.18	0.19	0.13	0.03	0.00
Phee(H)	-0.17	0.03	0.04	0.01	0.00
Twit(A)	-0.15	12.91	5.74	0.50	31.77
Twit(S)	-0.13	36.20	15.90	22.54	21.99
Infant Movement(A)	-0.12	5.68	1.86	3.08	4.94
Vhee(S)	0.06	17.68	5.17	14.92	31.34
Phee(S)	-0.04	4.82	4.36	3.99	5.53
Infant Movement(S)	-0.02	3.69	2.90	1.41	8.76

Table 3.7: Infants' clusters

Cluster	**Infant**	**Parents**	**Older Siblings**	**Birth**
1	Eugenie	Hime-Oji	-	4
	Shirayury	Ribbon-Kabu	-	1
	Suisen	Ribbon-Kabu	-	1
	Saku	Kachan-Tochan	-	3
2	Ataro	Mama-Tochan	-	Mama:1 - Tochan:4
	Setaro	Mama-Tochan	-	Mama:1 Tochan:4
	Hook	Fastner-Chuck	-	1
	John	Fastner-Chuck	-	3
	Cubby	Fastner-Chuck	-	4
	Pearl	Lame-Mogol	Ribbon,Beads	2
	Stone	Lame-Mogol	Ribbon,Beads	2
	Yu	Kachan-Tochan	Maru	2
	Pon	Kachan-Tochan	Maru	2
	Gaku	Kachan-Tochan	-	3
3	Bill	Hime-Oji	-	1
	Charlotte	Hime-Oji	-	2
	George	Hime-Oji	-	2
	Diana	Hime-Oji	-	3
	Henry	Hime-Oji	-	3
	Wendy	Fastner-Chuck	-	2
4	Kate	Hime-Oji	-	1

3.3.3 Dyads

After having described the different possible patterns of behaviors within caregivers and within infants, the focus of analysis was moved on the interactions by considering caregiver-infant dyads as single units. One averaged value of each caregivers' and infants' variable across sessions was calculated for each caregiver-infant dyad. To investigate which caregivers variables were related to infants behaviors, correlations were computed between all the caregiver and infants variables [Figure 3.7]. Pearson's r were used to evaluate the strength of the associations. Figure 3.7 highlights that six caregivers' variables are strongly correlated to the frequency of infants calls during both H and T phases. Specifically, the carrying rate and the mean duration of the carrying bouts were negatively correlated to infants calls production during Holding and Transport, such as the higher the carrying rate and the longer each carrying bout was, the less infants called during reunion. In contrast, rejection rate and the rates of play, locomotion and stereotypy during Alone phase were positively correlated with infants calls during reunion. That is the more, on average, the infant was rejected, the higher the frequency of calls during reunion, and the more frequently caregivers played with objects, or moved around in the cage displaying stereotypic behaviors, the more an infant cried during reunion. Furthermore, the rate of caregivers' play during Separation was positively correlated with Vhee and Phee calls during both Holding and Transport whereas the rate of caregivers' play during Holding only positively correlated with the frequency of infants' Phee calls during reunion. Specifically, the more caregivers' played with object during S the more the infant emitted distress calls and long distance calls during reunion, whereas the more the caregiver played during Holding the higher the frequency of long distance calls emitted by infants during reunion.

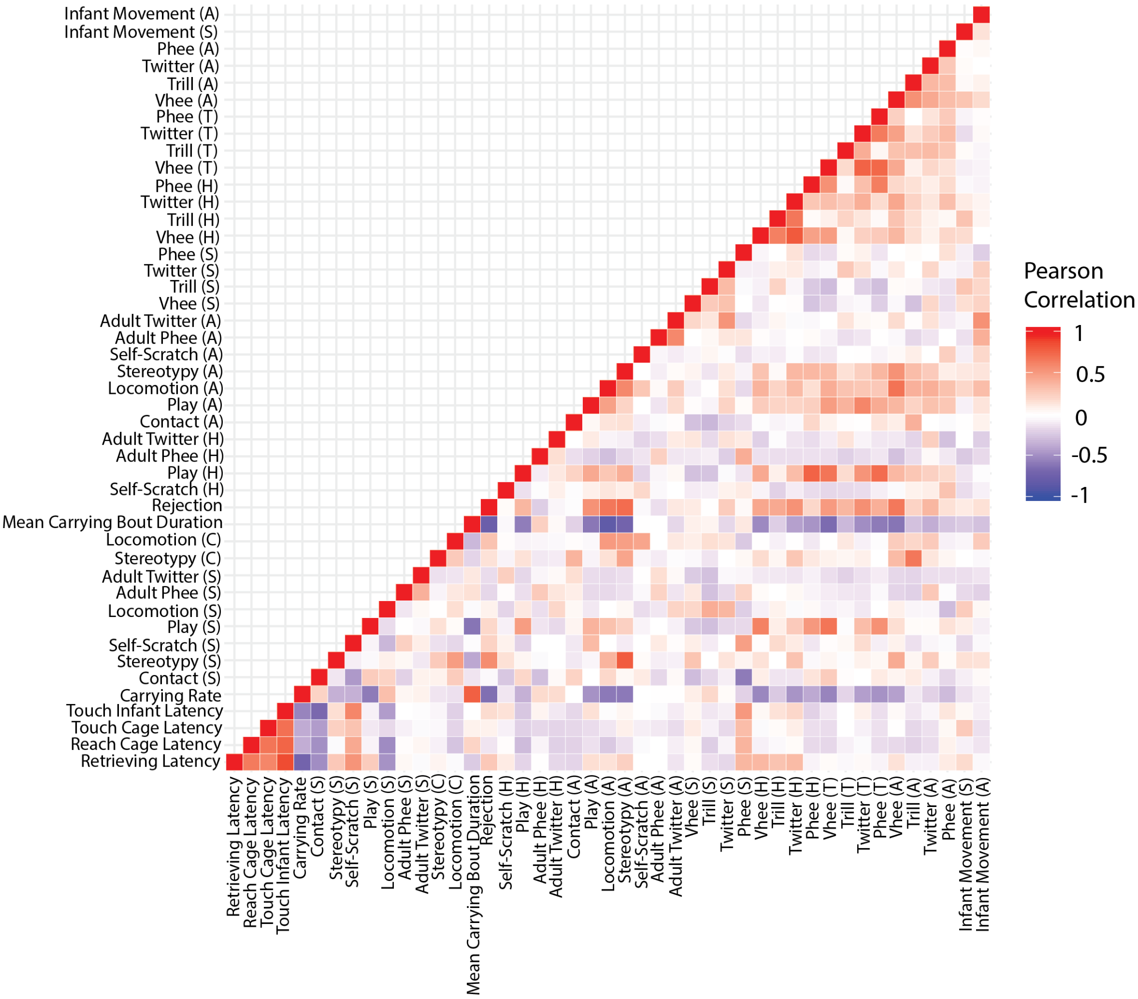

Figure 3.7: Correlation matrix between behaviors within caregiver-infant dyads

Therefore, some caregivers' behaviors co-relate with infants soothing levels during reunion. However, information about the direction of the relation is still missing. Insights about the direction of the relation was found by looking at (i) how a same pup differentially behaved in response to distinct caregivers accordingly to the caregivers' own behavioral pattern and (ii) whether the pups' behaviors changed when the caregivers' own average parenting style modified in successive births. Figure 3.8 shows the first scenario. Here the same pup, Pon, showed a very low frequency of calls during Holding when the rescuer was the mother, Kachan, which never rejected it, had a very high carrying rate, dysplayed few or none play with object in any time window and no locomotion or stereotypy during A. In contrast the same pup showed a high frequency of calls production during Holding phase when the rescuer was the father, Tochan, which often rejected the pup, had a very low carrying rate and displayed a high frequency of play with object, locomotion and stereotypy at any time point.

Figure 3.9, instead, shows the second scenario. Here the behavior of a same father, Oji, in two successive birth is shown as well as the frequency of calls emitted by the pups while held by Oji. Overall this father showed adaptive parenting behavior but, nonetheless, in the last birth, showed on the right side of the panel, Oji decreased the carrying rate and increased the frequency of rejection, play with objects, locomotion, and stereotypy. Accordingly, the first pup, Bill, who experienced a highly adaptive caregiving behavior from Oji did not emit calls during H whereas the last pup, Eugenie, who experienced a less adaptive parenting from Oji showed an increased, although still low, frequency of calls during H when the rescuer was the father itself. This considered, we feel to propose that not only caregivers' behaviors and infant' calls production during reunion are related, but it also seems that infants'

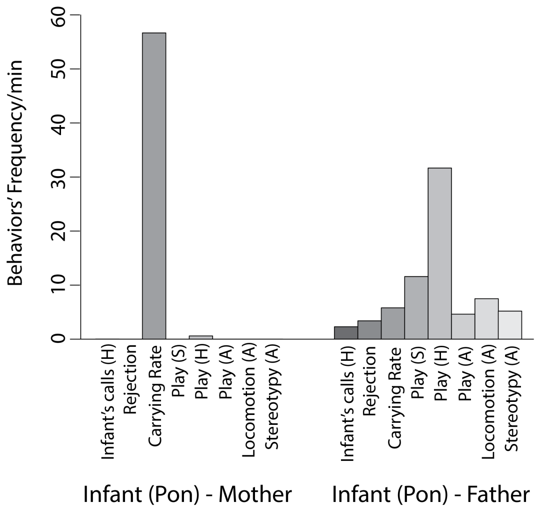

Figure 3.8: Here a same pup showing opposite behavioral patterns accordingly to caregivers' own behaviors is represented. In the left side of the barplot, infants' average call frequency when reunited with the mother as well as the mothers' own average behaviors are shown. In the right side of the barplot, infants' average call frequency when reunited with the father as well as the fathers' own average behaviors are shown

responses, rather than being caused by infants' own internal predispositions, are actually moderated by the caregivers' parenting style.

The Elbow method highlighted the presence of five major clusters of dyadic behavioral patterns [Figure 3.10a]. Applying hierarchical clustering dyads were categorized accordingly to their averaged behavioral frequencies. It was therefore possible to apply data-driven methods to evaluate the quality of caregiver-infant interactions [Figure 3.10b].

Cluster 1 and Cluster 2 include the dyads with neglectful and rejecting caregivers. In these clusters pups emit calls during reunion with higher frequency compared to the other clusters. Cluster 4 include the most adaptive caregivers, careful and less or absolutely none rejecting. In these cluster pups nearly never emit calls during reunion with the caregivers. Cluster 5 collocates mid-way between cluster 4 on one side and clusters 1 and 2 on the

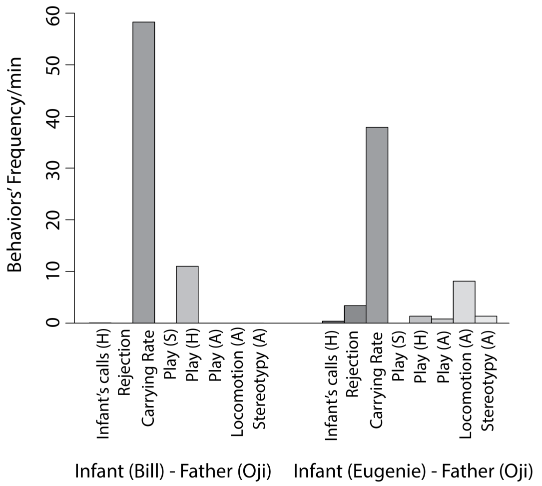

Figure 3.9: Here a same caregiver showing different behavioral patterns and the respective pups' behavioral responses are represented. In the left side of the barplot, one infants' (Bill - 1st birth) average call frequency when reunited with the father as well as the fathers' own average behaviors towards Bill are shown. In the right side of the barplot, another infants' (Eugenie - 4th birth) average call frequency when reunited with the same father as well as the fathers' own average behaviors towards Eugenie are shown.

other. Cluster 3 instead is somewhat atypical. It is constituted by only two dyads, one father, Mogol, with its two only pups, Beads and Pearl. In this dyads the father's rejection rate is zero, however the father is highly neglectful since it carries the pup rarely. On the other hand, when this father does carry the pup the duration of the carrying bout is long because the pup is never rejected. In reaction to this behavioral patter, pups never call during reunion [Table 3.8]. Interestingly for us was that this father, Mogol, was the only artificially reared marmosets in the colony and, as such, this result was consistent with previous literature and findings which showed how early social deprivation hinders the development of social behaviors and parenting abilities. Therefore, the model was able to separately categorize this atypical pattern of interaction. In the end, to rule out the presence of possible moderating factors a General Linear Model was run. As dependent variable we

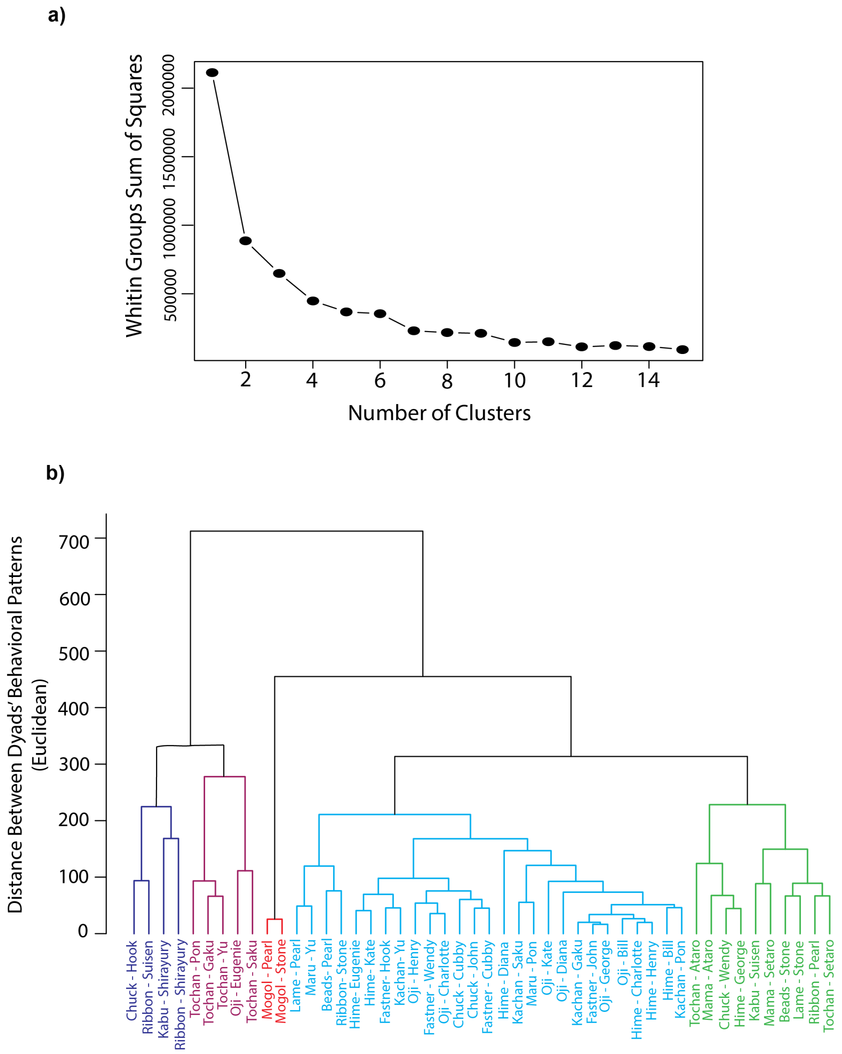

Figure 3.10: Analysis of dyads' behavioral patterns. a) In the first panel results form the application of the Elbow method are shown. The plot shows the presence of a significant difference between behavioral patterns up to 4 clusters. b) Categorization of the dyads in 5 clusters applying the hierarchical method. Here the distance between dyads and clusters is measured by calculating the Euclidean distance and its shown in the dendrogram. Starting from the root level, which include the total population, the sooner two clusters are separated the greater is the distance between them.

Table 3.8: Dyads clusters' description: caregivers' and infants' averaged behaviors

Behaviors	**Cluster 1**	**Cluster 2**	**Cluster 3**	**Cluster 4**	**Cluster 5**
Retrieving Latency	186.77	30.10	247.38	40.23	80.74
Carrying Rate	14.27	20.67	15.48	52.92	39.02
Carrying Bouts Duration	138.46	129.26	619.95	569.32	411.46
Rejection	8.35	5.26	0.00	0.20	1.11
Play(S)	4.43	5.848	0.99	0.09	0.68
Play(H)	4.24	16.84	0.00	2.66	4.30
Phee(A)	5.52	5.85	0.00	0.37	2.30
Locomotion(A)	12.61	10.48	0.00	1.79	6.05
Stereotypy(A)	5.75	3.33	0.00	0.17	0.15
Vhee(H)	0.35	0.22	0.00	0.02	0.12
Trill(H)	0.25	0.09	0.00	0.05	0.21
Twit(H)	0.79	0.35	0.002	0.11	0.27
Phee(H)	0.009	0.17	0.00	0.02	0.02
Vhee(T)	0.45	0.71	0.00	0.05	0.004
Trill(T)	0.17	0.34	0.00	0.11	0.09
Twit(T)	0.85	1.16	0.00	0.18	0.04
Phee(T)	0.08	0.56	0.00	0.02	0.05

Table 3.9: Results of the General Linear Model

	Estimate	**Standard Error**	***t* value**	***p* value**
Intercept	1.17	0.48	2.45	0.02*
PND	0.001	0.01	0.06	ns
Caregiver Status: Father	0.27	0.47	0.58	ns
Caregiver Status: Mother	-0.59	0.45	-1.29	ns
Caregiver Status: Sister	-0.67	0.78	-0.85	ns
Infant Sex: Male	-0.29	-1.10	0.27	ns
Birth Number	-0.08	0.13	-0.59	ns

considered the overall frequency of calls during reunion (Holding + Transport) for each experimental session, while the model included as independent factors infants' PND, infants' sex, caregivers' status (mother, father, brother, sister) and the birth number. No significant effect of any of the factors was found, ruling out the presence of external moderator and corroborating the hypothesis that the emittance of infants' distress calls during reunion is a specific reaction to each caregivers' own parenting style [Table 3.9].

3.4 Discussion

The micro-analysis of behaviors allowed us to disassemble caregiver-infant interactions in core behaviors, quantitatively clusterizing caregivers and infants, as well as caregiver-infant dyads accordingly to their behavioral patters and highlighting which core behaviors play a pivotal role within the interaction. Caregivers' behavioral patters were categorized into four main clusters which underlined the presence of a continuum of caregiving adaptiveness. Indeed, the behaviors mostly involved in cluster definitions were (i) care-related behaviors, such as the amount and the length of carrying, the latencies in reaching and retrieving the pup, and the amount of object play while the pup was separated, (ii) rejecting behaviors, and (iii) stress related behaviors, such as stereotypy and high frequency locomotion. Little or no contribution was given by the presence of adults' calls. Clusters 2 and 3 were the less adaptive but while caregivers in cluster 2 showed overall less rejecting and stress-related behaviors together with more neglectful behaviors, caregivers in cluster 3 were more rejecting and more stressed but somewhat less neglectful, although it took a very long time for them to reach and retrieve the pup. The most adaptive cluster was the first one whereas in cluster 4 caregivers showed care coupled with a fair amount of rejectiveness. In both the adaptive clusters, 1 and 4, stress-related behaviors appeared with very low frequency. Within infants, the most important behaviors in defining clusters were the frequency of calls emitted at the time of reunion with the caregiver, either during Transport or Holding, whereas calls emitted during separation and the amount of movement alone had few or non importance in cluster categorization. Infants in cluster 3 showed more soothing compared to other clusters both during reunion and separation or alone phases, whereas infants in cluster 4 showed high soothing during reunion coupled with higher call frequency during alone and separation phases compared to infants in cluster 3. Cluster 1 infants showed high calls production both during reunion and separation, whereas cluster 2 infants showed less soothing during reunion compared to clusters 3 and 4 but they also produced less calls during separation and alone phases compared to cluster 1 infants. The behaviors performed in the Alone phase, when the pup was left alone after the reunion already occurred proved to be important in both caregivers and infants categorization and we think this is especially because the presence of the Alone phase was not assured in all sessions since the pup could have never left caregiver's fur, therefore the presence of Alone phase is likely part of the definition of categories by itself. Caregiver and infants therefore do show differential behavioral patterns, however we still do not know how they do interact between each other. To test how caregivers' and infants' behaviors intertwined and influence each other we moved the focus onto the dyads considered as whole units. Consistently with previous results, the caregiving behaviors that were found to relate more with infants' calls frequency during reunion with the caregiver were the carrying rate and length, the amount of time taken by the caregiver to play with objects when separated from the infant, the rejection, and the stress-related behaviors. Also, from the distribution of behaviors between dyads it seems that caregivers' behaviors are moderating infants' soothing in response to specific caregivers rather than for caregivers' behaviors to be a response to infants' own predispositions. Indeed, a same pup behaved differently, even showing opposite soothing levels, in response to distinct caregivers accordingly to caregivers' own behavioral pattern. Also, when caregivers changed behavioral pattern in successive birth, infants' soothing during reunion changed accordingly even though siblings share 50% of genetic characteristics. Consistently, the frequency of infants' calls during Separation was of less importance, compared to the calls emitted during reunion, in defining infants' clusters highlighting how overall infants called out similarly when asking for caregivers' rescue and therefore it seems less likely that they affected caregivers' behaviors accordingly to their tendency in calling. Rather, since pups were not aware of the identity of the caregiver involved in each session until it reached the infants' basket, it is more likely that pups changed their behavior when they identified the caregiver's identity, adapting it to the caregiving style they were facing in each specific session. This hypothesis was also corroborated by ruling out the contribution of possible external moderators. Indeed, findings from the GLM showed that the infants' PND and sex as well as caregivers' status (mother, father, sister, brother) had no effect on the frequency of calls emitted during reunion. Furthermore, by categorizing dyads based on dyadic-average behaviors it was possible to evaluate caregiver-infant interactions in the framework of the attachment theory. Indeed, clusters of dyads highlighted a continuum of caregiver-infant interactions' adaptiveness. In dyads where the caregivers were more rejecting, neglectful and stressed, infants were not soothed by reunion (clusters 1 and 2), similarly to human infants displaying ambivalent attachment in response to erratic caregivers. Interactions presented themselves on a continuum: the more caregivers were rejecting or stressed and the less they were careful, the less the infant soothed during reunion. In contrast the more the caregiver showed caring behaviors coupled with a decrease in rejection and stress-related behaviors, the more the infants soothed during reunion consistently with a human-infant secure attachment style (clusters 4 and 5). In the end, the atypical behavior showed by the artificially reared father in cluster 3, characterized by very long retrieving times and low carrying rate but without any sign of rejectiveness caused infants not to call at all when reunited with the caregiver. This behavior in infants marmosets is consistent with avoidant attachment in human infants. Pups knows the caregiver is not going to readily answer to their needs but they also know it is not going to reject and hurt them therefore they may being somewhat withdrawing from the interaction just taking in what the caregiver is providing without spending energies and effort to ask for more. However, this interpretation need to be corroborated in future steps by studying other dyads including artificially reared or socially deprived caregivers. In this experimental framework, findings from Takahashi *et al.* (2015) about vocal development were not corroborated. Takahashi *et al.* (2015) found that during pups development while Vhee calls decrease the Phee calls increase and they interpreted it as Phee calls substituting Vhee calls due to development of individuals' vocal tract. However, although in some social situations the increase in Phee calls may couple with the decrease of Vhee calls, during the retrieving tests, therefore in a distressing social situation, this was not the case. Our interpretation of this discrepancy is that marmosets may have a broad vocal repertoire which they adapt to different social situations. Moreover, a distressing social situations as well as caregiver-infant interactions may have a particular status and may therefore being responded to with specific vocal patters, different form the everyday-life ones. Marmosets are therefore suitable to model caregiver-infant interactions and bonding development. Limitations of the present study point to future directions. First, only correlational measures were collected and this characteristics do not allow us to infer more than hypothesis about the underlying causal mechanisms. However we are nowadays investigating the role of the Medial PreOptic Area (MPOA) in the marmosets' brain in determining caregiving behaviors. Previous studies found this area to be pivotal in the emergence of caregiving behaviors in mice and our results seem to confirm its role in marmosets, although specific for the presence of tolerance-related behaviors, that is accepting to carry the pup and sharing food with infants. MPOA activity seems therefore to be involved in an evolutionary conserved mechanisms that induce parenting in mammals. Second it was not possible to obtain long-term measures of marmosets adult social behavior although this knowledge would allow to test which model of gene-environment interaction would be the most fit to explain marmosets' development and whether this non-human primate's developmental trajectories are similar to humans'. Future researches will need to foresee longitudinal data collection in order to obtain information about both parent-infant interactions in early life and subsequent adults' social behaviors towards peers as well as parenting behaviors towards infants. Lastly, the application of the micro-analytic approach to the evaluation of caregiver-infant interactions to human caregiver-infant dyads will need to be investigated. Being able to evaluate the functionality of the interactions based on core elements would allow us to apply machine learning approaches in order to recognize adaptive vs maladaptive patterns of behaviors, and to detect non-optimally functioning caregiver-infant interactions. This would give us the possibility to intervene faster and earlier to restore, if necessary, an adaptive interaction, providing caregivers with concrete advices about which specific behaviors to improve.

Chapter 4

General Discussion and Future Directions

Caregiving behaviors are once again confirmed to play a pivotal role both in short and long term development. In the short term, parenting behavioral style forges the quality of the caregiver-infant interactions (Ainsworth & Bell, 1970; Ainsworth *et al.* , 2015; Bowlby, 1973) whereas in the long term it affects the development of socially-related responses, even up to adulthood (Rilling & Young, 2014; Sroufe, 2005; Dalsant *et al.* , 2015). However, caregivers' behaviors are not the only factor which shapes development. Individuals' own genetic predispositions are important characteristics which, by influencing individuals' own behaviors, contribute in moderating individuals' responses to external stimuli (Fisher, 2000; Furman *et al.* , 2011; Marusak *et al.* , 2015; Thompson *et al.* , 2011; Wu *et al.* , 2012). Findings from the current project highlighted that the difference between short vs long term effects may reside in a different balance between genetic and environmental contributions. Concerning short term effects, although infants' predispositions always plays a role, the contribution of parenting behaviors is more relevant in determining how caregiver-infant interactions build and unfold over time. Indeed, the frequency of pups' calls during the separation was less important than calls during reunion in evaluating caregiver-infant dyads. Moreover, even when target infants' genetic characteristics were equal or similar, pups showed differential behavior accordingly to the caregivers' style. So that a same pup reacted even with opposite soothing levels to different caregivers accordingly to the level of caring, rejectiveness and stress, or siblings from successive birth reacted in opposite ways to a same parent when it changed parenting style form one birth to another. In contrast, long term effects are determined by the interaction between the experienced parenting behaviors and individuals' genetic predispositions. Here genes play a pivotal role in moderating to what extent parenting behaviors will contribute in shaping long-term development because genetic characteristics play a role in shaping individuals' sensitivity to the external environment (Belsky *et al.* , 2007). Specifically, findings from the present research show how genetic factors modulate the malleability of physiological systems, that is how much physiological systems are fixed vs modifiable by environmental factors. Individuals whose DNA codes for more fixed physiological systems are going to develop the same, less influenced by parenting behaviors, whereas individuals whose DNA codes for more flexible physiological systems, will be highly affected by the experienced parenting behaviors, both for good and for bad. The presence of plasticity genotypes was likely selected through a balancing evolutionary pressure (Pluess, 2017): both the plastic and non-plastic genotypes favor development and survival in two different scenarios. In a good or favorable environment a higher flexibility and greater physiological adaptability would be beneficial since it would hugely boost individuals' development whereas in the presence of bad environmental factors a more stable and fixed physiological system would be more resilient and would help preventing individuals' maladaptive development. To strengthen our findings on long term effects, future researches will need to consider combinations of genotypes increasing the sample size, especially including women, and adding the investigation of central nervous system responses to assess how higher cognitive processes are affected by the early experienced parenting behaviors. Also the dynamics and the causal mechanisms underlying long-term effect will be investigated in the marmoset model. The investigation of these two mechanisms can be achieved by applying two different techniques: lesion studies and transgenic models. Indeed using lesion studies it will be possible to individuate the brain areas responsible for good vs bad parenting behaviors and then to hinder or trigger one of the two behavioral type in order to investigate whether the alteration in parenting causes a switch in infants' responses. We started on this path finding that the Medial PreOptic Area (MPOA) in the marmosets' brain is causally involved in tolerance-related parenting behaviors, that is accepting to carry the pup and sharing food with infants. Future studies will tackle the causal role of these parenting behaviors in altering infants' responses to caregivers. Furthermore, in order to test the causal role of genetic predispositions and the fitness of the plasticity genes model in caregiver-infant interactions in marmosets, the application of transgenic techniques would be of great importance. Transgenic techniques allow us to modify part of the genetic code even in the germline and, thus, to have control over genetic characteristics. In marmosets, this line of research is still young. The first transgenic marmosets was recently presented by Sasaki and colleagues in a study published in 2009 (Sasaki *et al.* , 2009). Nowadays we are proceeding towards marmosets models of some neurodegenerative and neurodevelopmental diseases (Kishi *et al.* , 2014; Tomioka *et al.*

, 2018), even though a fined grain manipulation of marmosets DNA as the one we obtained in mice is still to be fully conquered (Schatten & Mitalipov, 2009; Sasaki, 2015). However, the marmosets are particularly suited for the application of transgenic techniques since they are phylogenetically closer to humans than mice, their metabolism and hormonal mechanisms are closer to humans, they are small animals easy to breed, and they reach sexual maturity in 12-18 months having 40 to 80 offsprings during their lifetime (Sasaki *et al.* , 2009; Sasaki, 2015). Also, the refinement of state of the art genetic tools together with the scientists' huge effort of mapping how genes are express in the neonate marmosets' brain (Shimogori *et al.* , 2018) will allow in future researches to manipulate the target regions of interest in the DNA of the marmosets in order to investigate how these mutations influence pups' sensitivity to parenting behaviors and individuals' social development. On the other hand, concerning the mechanisms underlying the short-term effects of caregiver-infant interactions, a study on human caregiver-infant dyads is now undergoing. The study aims to apply the behavioral mirco-analysis which proved efficient in the animal model to disassemble the interaction in its core elements and to look for the physiological correlates of these key behaviors by measuring infants' heart rate responses to maternal behaviors in a modified strange situation paradigm where the infants and the mothers interact for 2 minutes, are separated for 2 minutes and then reunited for 2 more minutes. Interactions will then be evaluated applying machine learning methods based on the measured behavioral and physiological elements in order to categorize adaptive vs maladaptive caregiver-infant interactions. If a reliable categorization model will be obtained, this model will be applied to evaluate the quality of early caregiver-infant interactions in clinical settings.

Taken together results from marmosets and human studies may pave the way for clinical applications such as more efficient treatments of caregiver-infant interactions in the case of neurodevelopmental disorders (i.e. Autism Spectrum Disorders (ASDs)) or Post-Partum Depression (PPD). Indeed, by being able to reliably evaluate the quality of the interactions by observing them it would be possible to identify the atypical ones which may hinder infants' adaptive development. Also, being able to apply this technique during infancy would allow us to apply earlier, faster and more efficient treatments to restore an adaptive interaction and we would also be able to target specific core behaviors in order to improve their effectiveness and overall to increase both the dyads' and individuals' well-being. As for the mechanisms causing the neurodevelopmental disorders, the application of transgenic techniques and the study of cellular mechanisms in marmosets models will give insights on what actually causes the disorders and maybe on how to treat them at their core rather than trying to smooth their consequences.

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