SOCIAL AND PSYCHOBIOLOGICAL REGULATION OF THE HUMAN CHILD's HYPOTHALAMUS-PITUITARY-AXIS: AN ONTOGENETIC PERSPECTIVE

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by

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The undersigned, appointed by the dean of the Graduate School, have examined the dissertation entitled

SOCIAL AND PSYCHOBIOLOGICAL REGULATION OF THE HUMAN CHILD'S HYPOTHALAMUS-PITUITARY-AXIS: AN ONTOGENETIC PERSPECTIVE

presented by Davide Ponzi,

a candidate for the degree of Doctor of Philosophy

and hereby certify that, in their opinion, it is worthy of acceptance.

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Volevo dirti qualcosa adesso che e' notte
e le parole se ne vanno…
dirti che ti voglio bene, figlio,
che il mio sogno sei tu.
Osvaldo Soriano, *L'ora senz'ombra*

Questo lavoro e' dedicato alla memoria di mio padre e a mia madre, le cui vite fatte di sacrifici e sudore hanno reso, ai miei occhi, le loro piccole e quotidiane vittorie uno straordinario viaggio che non e' ancora finito.

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ABSTRACT

On the basis of life history theory, the delayed reproductive maturity represented by an extended period of childhood and juvenility in humans is predicted to be important for learning cultural, social, and ecological skills that help prepare the child for the adult socio-competitive environment. The human child is extremely sensitive to the social milieu and it has been proposed that the type of early social and biological environment shapes life history strategies. During this developmental period, boys and girls show behavioral sex differences in play and social interactions. The hypothalamic-pituitary-adrenocortical (HPA) axis, with its products cortisol and dehydroepiandrosterone (DHEA), is expected to play a pivotal role mediating the relationships between the social environment and an individual's life history strategies. Yet the processes that underlie the biological embedding of social information remain unclear in humans. Drawing from this background, in this dissertation I report results of a 4 months long research conducted during a longitudinal naturalistic study aimed to investigate the family and peer influences on the child's HPA and socio-cognitive development. Using a multidisciplinary approach spanning from human biology, cultural and cognitive anthropology to human ethology, this work illustrates that the early mother-child relationship plays an important role in the development of the child's baseline and reactive HPA activity. Specifically, a strong mother-child bond predicted lower levels of daily DHEA, while a low mother-child bond correlated with high cortisol before a video-recorded interview. Low maternal investment measured as time spent breast-feeding the child predicted lower

cortisol and DHEA concentrations in boys and girls, while more maternal investment was associated with higher cortisol only in girls.

This dissertation also reports data on the potential physiological mechanisms of sex differences in peer-network during mid-childhood. It was demonstrated that in the population under study children' social network is sex segregated, boys tend to have higher clustered friendship ties than girls, but that DHEA is not associated with these behavioral outcomes. Finally, the study reports the effects of coalitional competition on the HPA and hypothalamic-pituitary-gonadal (HPG) axis activity in children and teenagers.

Chapter 1

General Introduction

1.1 Introduction

Several characteristics of *Homo sapiens* appear unusual or even unique to our species. These include: a big brain (Holloway 1996; Lee and Wolpoff, 2003) with complex intellect, large scale coalitionary behaviors and high levels of cooperation and competition between and within groups, concealed ovulation, menopause, absence of hair, up-right posture (Alexander 1990), and a long juvenile period characterized by a "physically helpless but mentally precocial baby" (Alexander 1990). In my dissertation I focus on this last unusual human trait, the long pre-reproductive period of human development.

Paraphrasing C.M. Lessels (2008), as a neurobiologist attempting to study the evolutionary underpinnings of human behavior, my goal is to unravel the chain of causation from the perception of the social environment, via the workings of the neuroendocrine system, to the production of a particular psychological and behavioral phenotype.

This dissertation is based on data collected from a small, rural village on the east coast of Dominica, during approximately 4 months of field work spanning from summers 2008 to 2010. With these data, I will be testing two main hypotheses: i) the stress system during mid-childhood is sensitive to the social environment with the mother-child relationship playing a very important role in its modulation; ii) there will be an activational effect of dehydroepiandrosterone in the expression of sex differences in socio-cognitive

behavior during mid-childhood. The last part of the dissertation is a preliminary study that aims to bring attention and shed light to the hormonal mechanisms that may regulate in-group versus out-group competition during childhood.

1.2 The organization of the dissertation

In this dissertation I investigate the hormonal mechanisms that are associated with the development of human social behavior. My research focuses on monitoring levels of the salivary biomarkers testosterone, dehydroepiandrosterone (DHEA), cortisol and androstenedione and their relationship with child behavior, cognition and early sources of social stress.

The work is organized into 5 main chapters. In chapter 2 I examine the day-to-day stability of cortisol and DHEA across several days of salivary sample collection. Although we have good knowledge of the circadian rhythm of cortisol, we lack accurate, longitudinal analysis on DHEA daily and yearly variation. Moreover, there are few studies looking at cross-population differences and similarities in relation to the hypothalamic-pituitary-axis (HPA) activity. Therefore, I compare how age and sex influence the daily output of cortisol and DHEA with industrialized population from Europe and North-America.

In chapter 3 and 4 I investigate potential sources of early stress, focusing on how maternal investment and the mother-child bond, affect the basal and reactive hormonal status of the child and the nature of their association with child social competence. These two chapters complement the already known effects of early stressful experiences on child development. Chapter 3 also shows that the HPA of young children is sensitive to novel situations involving social challenges, raising doubt about the idea of a period

of hypo-responsiveness of the HPA during childhood (Gunnar and Quevedo, 2007). Chapter 4 calls for a socio-ecological and evolutionary driven approach in studying children sex differences in stress sensitivity.

It is known that beginning with horticulturalist and agriculturalist societies, the peer environment where children grow shows sex segregation. This pattern is different from the one of hunter-gatherers, mostly because of demographic characteristics involving fertility rate among women and the amount of women giving birth during the same year (Konner 2010). Nonetheless, the peer network in these societies are similar in the sense that children play in multi-aged groups of siblings, rising the argument that " same-age peer relations in human infancy and childhood are to a large degree an artifact of laboratory studies and of child care conditions in advanced industrial states" (Konner 2010, pp. 499). In chapter 5 I present an analysis of sex differences in peer social network and their possible biological basis. My working hypothesis is that DHEA has activational effects on the expression of behaviors related to peer sex segregation. I also report results on the relationship between friendship and age, sex and kinship.

The ecological-dominance hypothesis considers humans as evolved in an environment where between-group competition acted as a selective pressure shaping cognition and behaviors that facilitate between-group aggression and within-group cooperation. In collaboration with M. Flinn and D. Geary, I found that the neuroendocrine system is sensitive to within group and between group competition (Oxford et al. 2009; Flinn et al. 2011). An important question that needs to be addressed is how the neuroendocrine system underlying this in-group psychology develops, focusing on what and when

hormones are associated with competition, aggression and cooperation during childhood.

In chapter 6 I empirically approach this question examining the association between hormones and competition in a coalitionary setting, with the aim of understanding if the hormonal reactivity to within-group competition differs with age in male children and adolescents.

1.3 Theoretical background: Evolution of the human child

From an evolutionary standpoint, a long juvenile stage is a puzzle. The delay of sexual maturity increases the probability of pre-reproductive death, resulting in a high fitness cost. In primates the costs of an extended juvenile period include the risk of starvation and predation (Janson and Van Shaik, 1993; Joffe 1997); therefore the benefit of a long developmental phase must be high. One trait that is associated with the length of juvenile development is increased encephalization (Barrickam et al., 2007) and an expanded neocortex (Joffe 1997). A bigger brain, although metabolically costly, may provide benefits from learning ecological and social skills that outweigh the cost of delayed reproduction.

Although these life history trends are present in primates, humans are unique in the extent of altriciality and further extension of the pre-reproductive life stage, both requiring extensive parental and alloparental care (Muehlenbein and Flinn, 2011). The evolution of juvenility is related to high juvenile mortality risk (Janson and Van Shaik, 1993) and maturational constrains to grow a larger brain for learning processes of foraging and social competencies (Alexander 1990; Deaner et al., 2003; Flinn et al., 2007; Kaplan et al., 2000; Joffe, 1997).

Kaplan et al. (2000) posit that a long period of juvenile dependence in humans has the advantage of increasing the time spent in learning skills important for exploitation of the ecological niche. Foraging and hunting skills would increase the adult productivity rate (more calories produced- which for humans is higher compared to chimpanzees) that would feed-forward on the development of a big brain. Although learning ecological information is undoubtedly important, there are some caveats: (1) the brain structures serving physical and biological folk knowledge do not seem to have undergone big changes relative to other primates (Geary, 2005), and (2) physical skills for subsistence activities such as hunting and digging roots do not require a long time to be mastered (Blurton-Jones et al., 2002). These results could be extended to other primates, where although a juvenile may suffer from foraging competition with adults, juveniles are no less efficient in collecting calories (Joffe 1997).

An alternative hypothesis for the extended juvenile period in humans posits that the development of a big brain is the result of selective pressures on mastering social relationships -- the social brain hypothesis (Alexander 1989, Dunbar 1989, Flinn et al. 2005). This hypothesis proposes that as human ancestors became increasingly ecologically dominant (Alexander 1990), the major selective pressure for a larger brain and extended juvenile period was social competition among conspecifics; a process of runaway social selection (Flinn and Alexander 2007). The key point here is that humans have been selected to understand, memorize, and predict behaviors of other individuals (both in one-on-one and group relationships). This social context is not unique to humans. There is a general relation between

expansion of the neocortex and level of group living, wherein size of the social group is considered as an index of social complexity (Dunbar 1989). Nonetheless, humans are the unique species where the complexity of the social system, supported by the evolution of culture, resulted in large scale between-group competition and within- and between- group cooperation.

The presence of psychological and socio-cognitive characteristics such as language, theory of mind, mental time travel and self-awareness that occurred with the extension of the human prefrontal cortex compared to other Great Apes, is supportive of the hypothesis that mastering one-on-one and coalitionary social relationships was an important need during human evolution.

1.4 The social neuro-endocrinology of the human child: an ontogenetic perspective

Human childhood ranges from 3 to 7 years of age and is characterized by a slow rate of growth, immature dentition and motor control, and dependency on older people, generally relatives, in feeding behaviors. During this period the brain has its major development in terms of weight. Important maturational changes in the brain happen during pre-puberty and extend beyond the second decade of life. Synaptic density in the prefrontal cortex is completely developed at around 3 years and a half of age, reaching a plateau that is maintained until late childhood, when a decline in the number of synapses begins (Huttenlocher 1997). An interesting parallel to this pattern of brain maturation is that the cerebral metabolic cost, measured by positron emission tomography (PET), increases rapidly during infancy, it is maintained at a high level during childhood and decrease during adolescence (Huttenlocher, 1997;

Chugani et al., 1987), in a complementary fashion with the somatic growth rate.

From a socio-behavioral point of view, by the age of five children start to interact with peers showing the development of social hierarchies in which low status children affiliate mostly with dominants (Strayer and Trudel, 1984). Furthermore this life stage seems to be very sensitive to the experience of social relationship, setting future social behaviors (Kohlber et al., 1972). As an example, social hierarchies during mid-childhood correlate with social hierarchies during adolescence (Weisfeld, 1999).

Toward the end of childhood, the so called "five-to-seven year shift" (Sameroff and Haight, 1996), children experience an improvement in terms of learning social and behavioral skills and become more independent from parents, assuming the ability to walk and perform some complex behavior as adults do (Locke and Bogin, 2006). This is the transitory period characterized by the onset of adrenarche. Throughout the childhood and mid-childhood period sex differences in coalitional organization strengthen becoming increasingly evident, with boys playing in larger groups than girls. This is then related to a "more integrated social networks" where friends of a boy will become friends of everyone. Conversely, girls spend more time in dyadic interaction than boys at 4-6 years (Geary 2010; Rose and Rudolph, 2006).

Chapter 2

Day-to-day and year-to-year stability of cortisol and DHEA

2.1 Introduction

Cross sectional and longitudinal studies have been widely employed to investigate the relationship between the hypothalamic-pituitary-adrenal cortex axis (HPAA) and pathological conditions. Unfortunately the majority of these studies are limited to westernized-industrial society and there are virtually no longitudinal analyses of salivary adrenal steroids in children from developing countries (Flinn 2009; Nyberg 2011). Here I attempt to fill this gap by presenting a longitudinal analysis of salivary cortisol and DHEA form a population of young Dominican children.

Saliva is an excellent matrix for the measurement of steroid hormones and it is characterized by an easy and un-stressful collection procedure (Riad-Fahmy et al., 1982; Vining et al., 1983). The reliable measurement of steroid in saliva is allowed by the use of high-sensitive radioimmunoassay (RIA) and enzymatic immunoassays (EIA) that results in high correlations between salivary-blood steroids and low cross-reactivity with other steroids (Granger et al., 1999; Riad-Fahmy et al., 1982; Vining et al., 1983).

Cortisol and dehydroepiandrosterone (DHEA) are two adrenal steroids found in many vertebrates that can be easily detected in saliva where they are highly correlated with total blood levels and represent the free hormone concentration in plasma. The presence of high quantities of these two steroids, representing approximately 5% of the total hormone in blood (Goodyear et al., 1996), and their high correlation with plasma concentration is

related to their high solubility through the lipid-rich cell membrane of salivary glands without being affected by salivary flow rate (Vining et al., 1983).

Salivary cortisol represents approximately two thirds of total free hormone in plasma although this relationship seems to be not linear as a consequence of 1) concentration and saturation level of cortisol binding globulines (CBG), 2) the conversion of salivary cortisol to salivary cortisone (Hellhammer et al., 2009; Vining et al., 1983). The link between salivary cortisol and plasma cortisol is also reflected by the similar circadian variation and by the quick appearance of salivary cortisol after an increase of plasma cortisol, generally within few minutes (Vining et al., 1983).

In plasma, DHEA is strongly bound to sex hormone binding globulines (SHBG) and albumin (Dunn et al., 1981). As for cortisol, salivary DHEA represents almost 100% of the free hormone in plasma and is sensitive to dexamethasone treatment and follows the circadian rhythm (Swinkels et al., 1990; Granger et al., 1999).

While the physiological functions of cortisol and glucocorticoids are well known, from the regulation of the catabolic metabolism to the modulation of the stress system (Flinn and England 2003), the actual functions of DHEA remain a major question in endocrinology. DHEA is the most abundant circulating androgen in the human body, and although it has a very low androgenic activity relative to testosterone (4%), it can be converted to testosterone (T), dyhydrotestosterone (DHT) and estrogen in peripheral tissues (Labrie, 2004). DHEA is also produced in the gonads and it is synthesized de-novo in the brain, where it can act as a neurosteroid (Conley and Bird 1997; Corpechot et al., 1981; Majewska, 1995).

Cortisol and DHEA are secreted by two different zones of the adrenal cortex, the zona fasciculata (ZF) and the zona reticularis (ZR), respectively. The cells of these two regions have the same origin and potentially they can produce both steroids. The fate of the biosynthetic pathway that brings either toward the production of DHEA or cortisol is regulated by the different zone-dependent expression of the key enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD), which irreversibly converts Δ^5 steroids (such as pregnenolone) into Δ^4 steroids (such as progesterone).

The secretion of DHEA follows the diurnal pattern of cortisol (Auchus, 2004; Havelock et al., 2004; Ibanez et al, 2000; Rosenfeld et al., 1971) with higher levels during the morning than in the afternoon. A major difference in the secretory pattern between the two adrenal steroids is that DHEA seems to lack the "awakening" effect and is more stable than cortisol during the day (Granger et al., 1999; Hucklebridge at al., 2005). These studies show that the secretion of DHEA is dependent on the adrenocorticotropic hormone (ACTH), the main glucocorticoid stimulating hormone. In support of this view, individuals with impairments in the physiology of ACTH do not show the typical increase of the sulfated conjugate of DHEA, DHEAS, during mid-childhood (Weber et al., 1997).

Nonetheless, several observations and experiments demonstrated that ACTH is not the only player in the regulation of DHEA secretion (Ibanez et al., 2000; Nishida et al., 1977; Parker, 1979; Pintor et al., 1980). Two main differences in the secretion of cortisol and DHEA led to assume a partially different regulatory system for these two hormones. First, while cortisol secretion across the life span follows the ACTH pattern, DHEA increases from

mid-childhood to reach a peak in the mid twenties and then decrease with aging (Havelock et al., 2004). Second, while a feedback mechanism regulating the glucocorticoids secretion is related to the corticotropin releasing hormones (CRH) and ACTH, there is no knowledge of such a regulatory loop for DHEA. Surprisingly, we still lack of knowledge on how DHEA synthesis and secretion are regulated (Hornsby, 2004).

From an ontogenetic point of view the secretion of DHEA from the adrenal cortex is species specific, and in humans it follows six stages: 1) high production of DHEA (and DHEAS) during fetal life from the fetal zone (FZ), which act as a substrate for estrogen synthesis in the placenta; 2) involution of the FZ right after birth and consequent decline of adrenal androgens; 3) the formation of focal parts of zona reticularis (ZR) at three years of age; 4) the development in thickness of a scattered ZR to form a continuous ZR by the age of 6 years, which parallels with an increase in adrenal steroid synthesis and secretion that by 6- 8 years of age will mark the adrenarche; 5) a steep increase in DHEA levels that will peak, with the maturation of ZR, in the mid 20s early 30s, followed by 6) a slow decrease during aging, seemingly with the reduction in thickness of ZR (Auchus et al., 2004; Havelock 2004; Nguyen and Conley 2008).

Cortisol has a completely different pattern of secretion during the life span. Cortisol fetal production rises during the last weeks before parturition and then decreases a few days after delivery. Thereafter the cortisol production rate seems to show stable but continuous increases during development and aging (Cauter et al.,1996; Styne et al., 2008).

Adrenarche is defined as a concentration of DHEAS in plasma equal to 50 µg/dL (Ibanez et al., 2000). Because DHEAS in saliva may not be a reliable measure of the hormone measured in plasma this raises the problem of how we can define adrenarche as salivary biomarker. In a recent study, Ellis and Essex (2007) defined children with salivary levels of DHEA below 16pg/ml as preadrenarcheal. Conversely, if at least one of the multiple samples collected by these authors was equal to or higher than 16pg/ml the child was considered as being in adrenarche. This approach has the limitations of being clearly a subjective definition that apparently does not have any substantial evidence from literature. Alternatively, knowing that the concentration of DHEAS used to define adrenarche in plasma is 50 µg/dL, adrenarche in saliva could be extrapolated using mathematical transformations. For example, DHEAS has been shown to represent 0.2% of the total plasma concentration and that in saliva, DHEA represents 15% of salivary DHEAS (Goodyear et al., 1996). Following this approach, adrenarche in saliva should be equal to approximately to 150 pg/ml of DHEA. Another alternative method is to use the plasma concentration of DHEA reported by studies that found it to significantly rise during specific ages. For example, Parker et al. (1978) found that DHEA was significantly higher in children of ages 7-8 compared to earlier ages. Then, knowing that salivary DHEA levels are approximately 5% of total plasma levels (Goodyear et al., 1996), adrenarche in saliva could be defined as any concentration above 35pg/ml.

Ethnic differences have been shown in adrenal production during prepuberty. Usually African-American children and specifically African-American and Caribbean-Hispanic girls show higher levels of serum and

urinary adrenal androgens (Girgis et al., 2000; Havelock et al., 2004; Pratt et al., 1990). Since the community under study presents a mixed genetic pool of African, Carib and European and the nutritional status of children is quite high, it could be expected that the production rate of DHEA will be close to the pattern of African-American and Hispanic children. No clear prediction could be made for cortisol, since no evidences of ethnic differences on basal and circadian output can be found in the literature.

The aim of this chapter was to analyze and characterize the circadian and year-to-year cortisol and DHEA output in a population of children and adolescents from a horticultural village of Dominica. I also investigated the onset of adrenarche and its reliability as a physiological marker in saliva.

This is one of few studies to use a cross-population and longitudinal approach to understanding DHEA production and regulation.

2.2 Methods

2.2.1 Study site, subjects and saliva collection

Data were collected from a small rural village in the east coast of the island of Dominica. About 500 residents of mixed African, Carib and European descent live in 5 different hamlets. The average annual income is $1900US and many villagers spend months or years doing seasonal work in other islands of the Caribbean, in the USA or in Canada. Despite poverty, children have growth rate comparable to US standards (Flinn et al., 1999). Fifty nine subjects (26 girls and 33 boys) with ages ranging from 5 to 11 years were considered for this analysis. 45 children gave multiple samples during the two summers of collection (2008 and 2009) while the remainder gave several samples only during summer 2008.

A maximum of 12 samples per child were collected on four separate days during the summers of 2008 and 2009. The collection procedure requires the researcher and assistants to walk set routes from house to house three times a day for two days (Flinn et al., 1995). During July 2008, salivary samples were collected once in the early morning (6:00 am-9:00 am), once in the late morning-early afternoon (10:00 am-1:00 pm) and once in the mid-afternoon (2:00 pm-5:00 pm). During August 2009, salivary samples were collected once in the early morning (8:00 am-10:00 am), once in the late morning (10:00 am-12:00 pm) and once in the early-afternoon (12:00 pm-2:00 pm). Saliva was collected by passive drooling through a straw into a polypropylene tube after stimulation of saliva with spearmint gums. During each sample collection, the researcher and assistants recorded information on date and time of collection, wake up time, current health condition, activities and food/drinks, all variables that can affect cortisol secretion (Gibson 1999; Slag 1981). Salivary hormones were measured by means of enzymatic immune assay following the company's procedures (Salimetrics LLC). Intra-assay coefficients of variation (CV) were less than 5% for cortisol (CORT) and less than 8% for DHEA. Inter-assay CV was 16.4% for CORT and less than 10% for DHEA.

2.2.2 Statistical analysis

Growth models with three levels where samples were nested within day and day within subject (Hruschka et al., 2005; Singer 1998) where used to investigate the effect of time of collection relative to time since wake up and the effects of age and sex.

At first a conditional growth model (Model 1) was tested, which helped to partition the variation between and within days and individuals in rate of change. In this model, time since wake-up (in minutes-TSAWK) was entered as a level 1 parameter. Time since wake-up was centered at its grand mean.

This model allowed me to evaluate the relative amount of within versus between individual variation by means of the intraclass correlation coefficient (ICC), once I controlled for time since wake up (Hruschka et al., 2005). The two ICCs of level 2 and 3 are given by equations 2 and 3 (see appendix).

Nested models were compared using the Maximum Likelihood (ML) deviance tests, which simultaneously tests for fixed and random effects (Singer and Willet, 2001). Briefly, this approach requires to perform the subtraction of the -2 Log likelihood (-2LL) between two competing models (a full and a restricted model). The deviance is distributed as a χ^2 with degrees of freedom (*df*) equal to the difference in parameters between the two models. Models that fitted better at $p<0.10$ were retained.

To test if cortisol and DHEA variation was influenced by age, sex and their interaction, three consecutive models were tested adding to each one as fixed effect (age followed by sex followed by the interaction).

Since age was associated with hormonal change, it was important to obtain average values of each hormone across ages, to allow for cross-population comparison. Therefore one additional model was run with age entered as a classification variable and lsmeans was used to obtain the model based estimates.

Cortisol was distributed with a skewness= 11.11 and a kurtosis= 146.42; Kolmogorov-Smirnov $p<0.01$. DHEA was distributed with skewness=

4.79 and kurtosis= 33.14; Kolmogorov-Smirnov $p<0.01$. In order to normally distribute the values, CORT and DHEA were fifth root transformed.

The transformed CORT and DHEA were rescaled multiplying CORT by 10^4 and DHEA by 10^6. The reason for this procedure was that the variance component of an outcome's small values will be small, potentially causing non-convergence of the statistical algorithm (Singer and Willet, 2002). Scaling variables have only "aesthetic effects without changing results of the statistical tests" (Singer and Willet, 2002).

There is no definition of adrenarche in saliva, therefore studies willing to dichotomize children as being post-adrenarche using saliva need to use different approaches based on mathematical transformations of known concentrations of blood DHEA and DHEAS or subjective definitions. In order to evaluate if these different approaches are reliable in identifying children considered post-adrenarche two alternative methods were used. Method 1 uses the definition from Ellis and Essex study (2007), that is, children with a concentration of DHEA below 16 pg/mL were considered as pre-adrenarcheal. In methods two I opted for using Parker et al. results (1978) and I chose a plasma concentration 0.72 ng/ml, the central value obtained from 95^{th} confidence interval of ages 7-8 in Parker's study. Then knowing that salivary DHEA levels are approximately 5% of total plasma levels (Goodyear et al., 1996), adrenarche in saliva was defined as any concentration above 35pg/ml. To test the percentage of children in adrenarche across the ages I used Fisher exact test.

2.3 Results

2.3.1 CORT daily and yearly change and stability

As it is shown in table 1, model 2 demonstrated that CORT, once controlled for time since wake-up, significantly decreases throughout the day (fig 1). A cubic term of time since wake-up showed a better fit than model 2. At this point, I explored the amount of variance that could be explained by 2nd level (day-level) and 3d level (person level) fixed effects and CORT stability by means of the intra-class correlation coefficient (ICC). The ICC for level 2 showed that the correlation between CORT measurements collected within the same day (after controlling for time of day) was 0.22, while the correlation between all samples collected across days was 0.13.

Age showed a trend toward statistical significance showing that cortisol slightly increases throughout childhood (fig 5). The two models with sex did not fit better that the model with only time and age and therefore were discarded.

2.3.2 Differences between averaged mean values of cortisol across ages

No clear differences were found for cortisol between ages, although the growth model showed that cortisol increases throughout childhood (fig. 3). Cortisol values from the population under study are in the range of values obtained from industrialized and developing populations, although the morning samples were somewhat low (see table 3).

2.3.3 DHEA daily and yearly change and stability

Model 2 showed that DHEA output did not change throughout the day (figure 2, table 2). Therefore, the amount of variance that could be explained

by 2nd level (day-level) and 3d level (person level) fixed effects and DHEA stability was explored by means of the ICC. The ICC for level 2 showed that the correlation between DHEA measurements collected within the same day (after controlling for time of day although not significant) was 0.40, while the correlation between all samples collected within the same individual was 0.17.

Age was a significant time varying predictor showing that DHEA strongly increases throughout childhood (fig 4). Because DHEA is known to sharply and then steadily increase at the beginning of midchildhood, a model with a quadratic term for age was used. The model did not significantly fit better than the model with only the linear term of age. The model with sex fit significantly better than the model with only age, with sex showing a trend to statistical significance and indicating that girls had higher levels of DHEA. The model with the interaction term of age and sex was not significant and therefore it was dropped.

2.3.4 Differences between averaged mean values of DHEA across ages

In a similar way to cortisol, if and how DHEA output systematically changes across ages was tested by means of lsmeans multiple comparisons. The results showed that at 9 years of age DHEA increases significantly compared to earlier ages (figure 4). Overall, salivary DHEA levels for this population seems to be lower but comparable to the one found by others (see table 3) .

Defining adrenarche in saliva

The Ellis and Essex method (cut-off at 16 pg/mL of salivary DHEA) resulted in 62.50 % of the children between 6-8 in adrenarche, while 29% were

found in the same age range using method 2 (tables 5 and 6). By the ages of 9-11 more than 80% of the children were adrenarcheal and post-adrenarcheal by Ellis and Essex, while only 47% by method 2. Neither method produced significant results (Fisher exact test, see tables 5 and 6).

2.4 Discussion

As expected based on the scientific literature cortisol and DHEA were measurable in saliva. Cortisol showed a diurnal decrease with higher concentrations in the morning and lower in the late afternoon. It is well documented that cortisol shows an awakening response (CAR) wherein the hormone peaks at around 30 minutes after waking up and then decreases thereafter to reach a nadir in the evening (Clow et al., 2010; Hucklebridge et al., 2005). For DHEA the awakening response is not so strong with a flatter diurnal slope (Hucklebridge et al., 2005).

Cortisol samples collected within the same day were moderately correlated and this correlation was higher than the one between different days. Hruschka et al. (2005) observes that the magnitude of the correlation between samples from the same day should reflect the half-life of cortisol, which is between 70 and 120 minutes (Stewart 2008). Therefore, if samples are collected with a lag of 20 minutes, the correlation between them would be higher than the correlation between samples collected farther apart. The results obtained from the children of this Dominican population are similar to the Hruschka et al.(2005) work on Mongolian children, where four samples were collected in the morning, early and late afternoon and evening.

Salivary DHEA showed higher stability within days and a slight one between days despite the fact that the half lives of both steroids is similar (1 to

3 hrs for DHEA - Kroboth et al., 1999). Hucklebridge et al (2005) showed that samples of salivary DHEA from the same individual were highly correlated within and between days. The result reported here shows that there is a clear difference in the day-to-day stability between DHEA and cortisol, replicating data collected in industrialized countries. One addition to Hucklebridge study is that the correlation between samples is already observable in children.

Age was a positive predictor for the change of DHEA. This result is consistent with the many others showing that basal plasma and salivary cortisol slightly change throughout childhood and adolescence, with some studies showing a correlation with pubertal status (Gunnar and Quevedo 2006; Kiess et al., 1995). Stronger, instead, is the effect of age on DHEA production (Ducharme et al., 1976; Parker et al., 1983; de Peretti et al., 1976).

DHEA begins to be secreted by the adrenal gland starting at around age 3 (Remer et al., 2005) but its levels in plasma are low or undetectable. Beginning around the age of 6 to 8, the adrenal glands produce high levels of DHEA and of its sulfated-conjugate DHEAS (Korth-Schultz et al., 1976).

In my study, the salivary DHEA concentration for children aged from 5 to 8 was significantly lower than children aged 9 and older. Ducharme et al. (1976) found that girls show a marked, significant increase of plasma free DHEA by 6-8 years of age and for boys a little bit later, between ages 8-10. Similarly, dePeretti et al. (1976) found a significant increase in DHEA plasma levels in girls beginning between ages 6 and 7 while boys were gradually but consistently showing increasing concentrations. Finally Hopper and Yen (1975) showed that girls sharply increase DHEA levels between ages 11 and 12, while boys showed a continuous and steady increase throughout late

childhood and puberty. My data demonstrated a slight delay in the increase of DHEA output which was unexpected, once the ethnic composition of the population under study is taken into account.

More complicated is instead the discussion relative to the definition of the onset of adrenarche in saliva and therefore its usefulness as a marker of child endocrinological state. Adrenarche is defined as a concentration of DHEAS in plasma equal to 50μg/dL (Ibanez et al., 2000; Wierman et al., 1986). Since DHEAS in saliva may not be a reliable measure of its plasma levels because of its dependency on saliva flow rate, it follows that it may not be a useful salivary marker for investigating the onset of adrenarche. Similarly, using DHEA as a potential measure of adrenarche may not be desirable, or at least it would require an experimental design comparing salivary and plasma levels of DHEA for pre-adrenarcheal and post-adrenarcheal children or using pubertal stage as a phenotypic marker to be associated with salivary DHEA levels.

In conclusion it was demonstrated that in the present study, cortisol and DHEA show a pattern of secretion and stability in saliva similar to other populations. Reference concentrations of the two steroids for different ages were presented. Although the proportion of children in adrenarche by age 6-8 and the interval of concentrations across all ages were similar to a USA population of same age kids, overall, DHEA from the population of children studied appear to be lower and with the well known sharp increase starting at a later age.

Table 1 Three level multilevel growth model estimates of cortisol

Parameters	Model 1	Model 2	Model 3	Model 4	Model 5
			Fixed effects		
Intercept	3.5362 (0.04275)**	3.4300 (0.04928)**	3.4293 (0.04816)**	3.3742 (0.06023)**	3.3764(0.06026)**
TSAWK	-0.00168 (0.000148)**	-0.0557 (0.0173)**	-0.0569(0.0173)**	-0.0569(0.0173)**	-0.0569(0.0173)**
$TSAWK^2$		0.0149 (0.0032)**	0.0149 (0.0032)**	0.0149 (0.0032)**	0.0149 (0.0032)**
$TSAEWK^3$		-0.0029 (0.0008)**	-0.0029 (0.0008)**	-0.0029 (0.0008)**	-0.0029 (0.0008)**
AGE			-0.03786 (0.02125)†	-0.03967 (0.02104) †	-0.03967 (0.02104) †
SEX				0.1208 (0.08066)	0.1208 (0.08066)
AGExSEX					-0.03251(0.04202)
			Random effects (variance components)		
Level 1 Residual (Within person) σ^2	0.3028(0.02480)**	0.2864 (0.02340)**	0.2862 (0.02336)**	0.286(0.02338)**	0.2863(0.02337)**
Level 2 Intercept (τ_{00})	0.03204(0.02221)†	0.03423(0.02135)†	0.03518 (0.02146)†	0.03397 (0.02115)†	0.03341(0.02103) †
Level 3 Intercept (τ_{000})	0.05433 (0.02326)**	0.05174 (0.02242)*	0.04529(0.02141)*	0.2863(0.02338)*	0.04348 (0.02029)*
			Model fit		
-2 log-likelihood	862.1	840.1	837.0	834.7	834.1

Table 1 shows the models used to test the effect of time of day relative to time since wake-up (Models 1 and 2; TSAWK= time since wake-up), age (Model 3), sex (Model 4) and the interaction between sex and age (Model 5). Model 3 had a better fit and was retained. Models were compared using the Deviance statistics that uses a X^2 statistics based on the difference between the -2 log-likelihood of 2 nested models and with degrees of freedom equal to the differences of parameters between the two nested models. **p<0.01; * p<0.05; †p<0.10

Table 2 Three level multilevel growth model estimate of DHEA

Parameters	Model 1	Model 2	Model 3	Model 4	Model 5
Intercept	26.2479 (0.5221)**	26.2201(0.5064)**	26.5898(0.6999)**	25.4085 (0.6822)**	25.4162 (0.6761)**
TSAWK	-0.00178 (0.001604)				
AGE		0.7153 (0.2663)**	0.7542 (0.2706)**	0.6874 (0.2607)**	0.9862(0.3764)*
AGE^2			-0.1074 (0.1408)		
SEX				1.7284(0.9873)†	1.7717 (0.9789)†
AGExSEX					-0.5694 (0.5181)
			Random effects (variance components)		
Level 1 Residual (Within person) σ^2	25.9193 (2.4952)**	25.7931(2.4734)**	25.7915 (2.4729)**	25.8138(2.4773)**	25.8224 (2.4786)**
Level 2 Intercept (τ_{00})	9.7931 (2.9466)**	9.5627(2.8941)**	9.4974(2.8865)**	9.5230(2.8911)*	9.4661(2.8777)*
Level 3 Intercept (τ_{000})	7.4004(2.8515)**	6.5872(2.6969)**	6.5474(2.6861)**	5.8529(2.5592)**	5.6289(2.5012)**
			Model fit		
-2 log-likelihood	2372.6	2366.7	2366.1	2363.7	2362.5

Table 2 shows the models used to test the effect of time of day relative to time since wake-up (Models 1; TSAWK= time since wake-up), age (Model 2 and 3), sex (Model 4) and the interaction between sex and age (Model 5). Model 4 had a better fit and was retained. Models were compared using the Deviance statistics that uses a X^2 statistics based on the difference between the -2 log-likelihood of 2 nested models and with degrees of freedom equal to the differences of parameters between the two nested models. **p<0.01; * p<0.05; †p<0.10

Table 3 Comparisons with other populations for CORTISOL

Author	Matrix	Methods	Age groups	Morning (nmol/L)	N	Evening (nmol/L)	N
Kiess et al.1995 (Europe)	saliva	DELFIA*	<=5	9.8	23	2.6	23
			6-8	10.9	23	2.7	23
			9-18	10.9	82	3.1	82
Nyberg (2011) (Tsimane)	saliva	DELFIA	1-15	5.27	22	1.94	22
			16-82	6.39	81	2.22	81
Salimetrics Inc. (USA)	saliva	EIA	<5	0.9-17.81	112	1.46-16.76	112
			8-11	2.32-23.17	285	nd-5.93	285
			12-18	0.58-24.39	403	nd- 7.15	403
Gustaffson et al. (2010; Europe)	saliva	EIA	9-16	6.0	130	<2.0	130
Rosmalen et al. (2005;Europe)	saliva	DELFIA	10-12	11.52	1768	1.95	1768
Ponzi et al., (this study ;Dominica)	saliva	EIA	5-7	4.0(±0.51) 0.69-16.29	30	2.79 (±0.55) 0.80-25.16	43
			8-9	3.53(±0.50) 0.91-11.16	23	2.07(±0.16) 0.69-5.33	32
			10-11	4.97(±2.51) 0.96-34.80	13	2.87(±1.21) 0.24-24.97	19

Table 3 represents the comparison between Cortisol values collected from the study in Dominica and other population from Westernized countries. *Time-resolved fluorescent immunoassay

Table 4 Comparisons with other populations for DHEA

Author	Matrix	Methods	Age groups	Morning		Evening		Mean		N
				boys	girls	boys	girls	boys	girls	
Granger et al. (1999;USA)	Saliva	RIA	8	99.1(±19.9)	119.5(±29.7)	29.6(±6.8)	62.9(±11.4)			8
			10	173.3(±42.0)	278.1(±43.0)	72.7(±17.6)	106.8(±20.2)			8
			12	330.4(±69.5)	242.2(±61.1)	129.2(±16.3)	104.1(±16.6)			8
			14	204.6(±33.5)	534.7(±132.4)	94.1(±16.4)	176.2(±22.4)			8
			16	479.4(±87.5)	421.4(±99.5)	238.4(±35.8)	157.8(±35.5)			8
Dorn et al., (2009[a] ; USA)	Saliva	EIA	6-11					51.7 (±39.6)	44.2 (±26.3)	57B,12G
Ponzi et al., (this study[b];Dominica)	Saliva	EIA	5-7	14.65(5.30) <5-54.39	17.69(3.91) <5-46.88	9.47 (3.01) <5-24.62	19.20(5.43) <5-59.83			
			8-9	23.19(6.37) <5-81.22	49.80(25.35) <5-219.40	15.98(3.03) <5-36.45	19.00(10.51) <5-69.71			
			10-11	14.26(6.58) 6.15-27.2 5	37.07(11.16) 7.81-91.80	43.08(7.13) 35.95-50.21	13.71(1.82) 6.70-16.90			

[a]Represents values from Dorn et al., 2009. Salivary values based on a population of 69 North-American healthy children (6 -11 years old) averaged across morning, noon and evening collections. B=boys, G=girls

[b]values are given as mean (±SE) and min and max. N is given as total number of boys and girls in morning (M, 2 hrs since wake-up) and evening (E, 9 hrs since wake-up) samples.

Table 5. % of children in adrenarche based on Ellis and Essex method

Age		**<5**	**6-8**	**9-11**
PA	*f*	4	18	6
		40%	37.50%	17.65%
A	*f*	6	30	28
		60%	62.50%	82.35%

Based on Ellis and Essex method (2007) the cut-off for adrenarche in saliva was 16pg/ml; PA= pre-adrenarche; A= adrenarche; *f*= frequency; X^2 =4.18 df=2 ;Table of probability (P) =0.0064; p= 0.12

Table 6. % of children in adrenarche based on method 2

Age	**<5**	**6-8**	**9-11**
PA	8	34	18
	80%	70.83%	52.94%
A	2	14	16
	20%	29.17%	47.06%

Based on Parker et al (1978) the cut-off for adrenarche in saliva was 35 pg/ml; PA= pre-adrenarche; A= adrenarche; *f*= frequency; X^2 =3.88 df=2 ;Table of probability (P) =0.004; p= 0.13

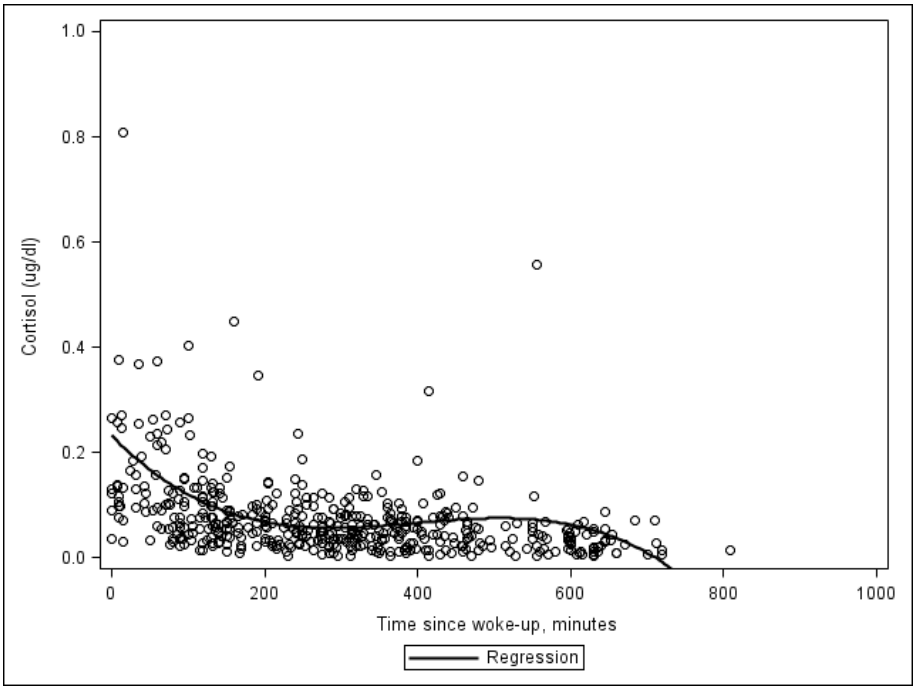


Figure 1. Daily change of salivary cortisol.Salivary cortisol concentration (µg/dL) in relation to time of sample collection. Data represent all the samples collected from subjects aged 5-11 years old during summer 2008 and 2009.

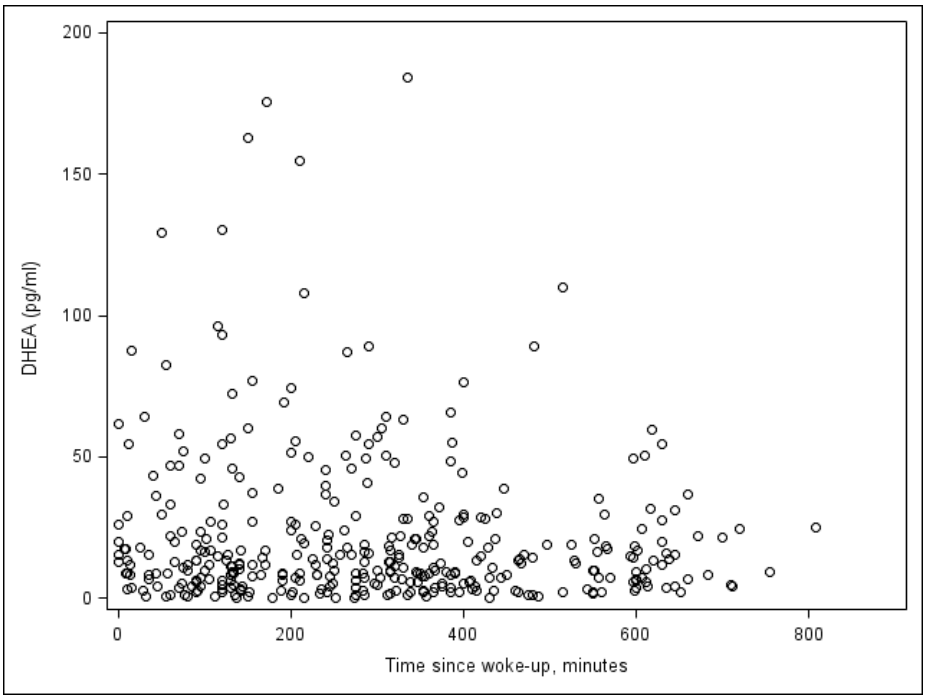


Figure 2. Daily change of salivary DHEA. Salivary DHEA concentration (pg/mL) in relation to time of sample collection. Data represent all the samples collected from subjects aged 5-22 years during summer 2008 and 2009.

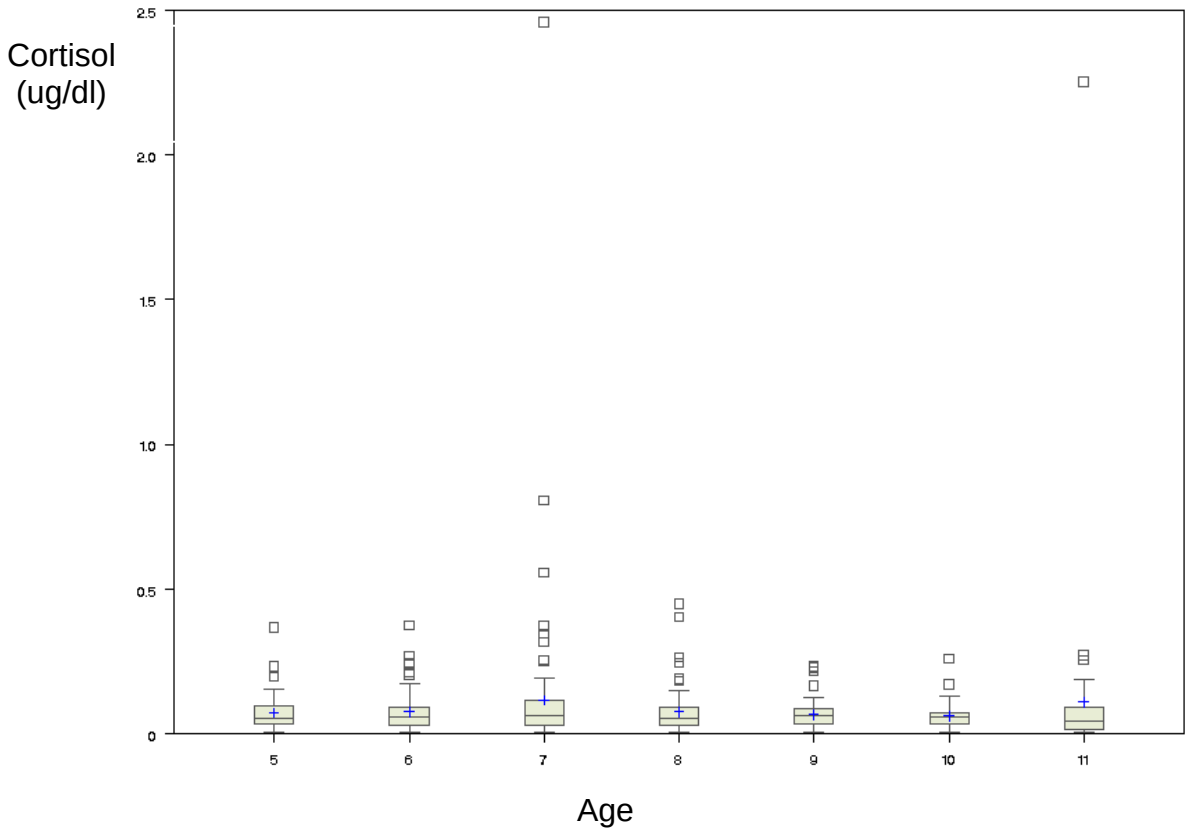


Figure 3. Barplots showing the distribution of salivary cortisol across ages. The upper and lower part of the boxes represent the 75th and 25th percentile, the bar within the box represents the median, the blue cross represents the mean while the two whiskers represent the maximum (95 th percentile) and minimum (5 th percentile) observations. Empty squares represent outliers.

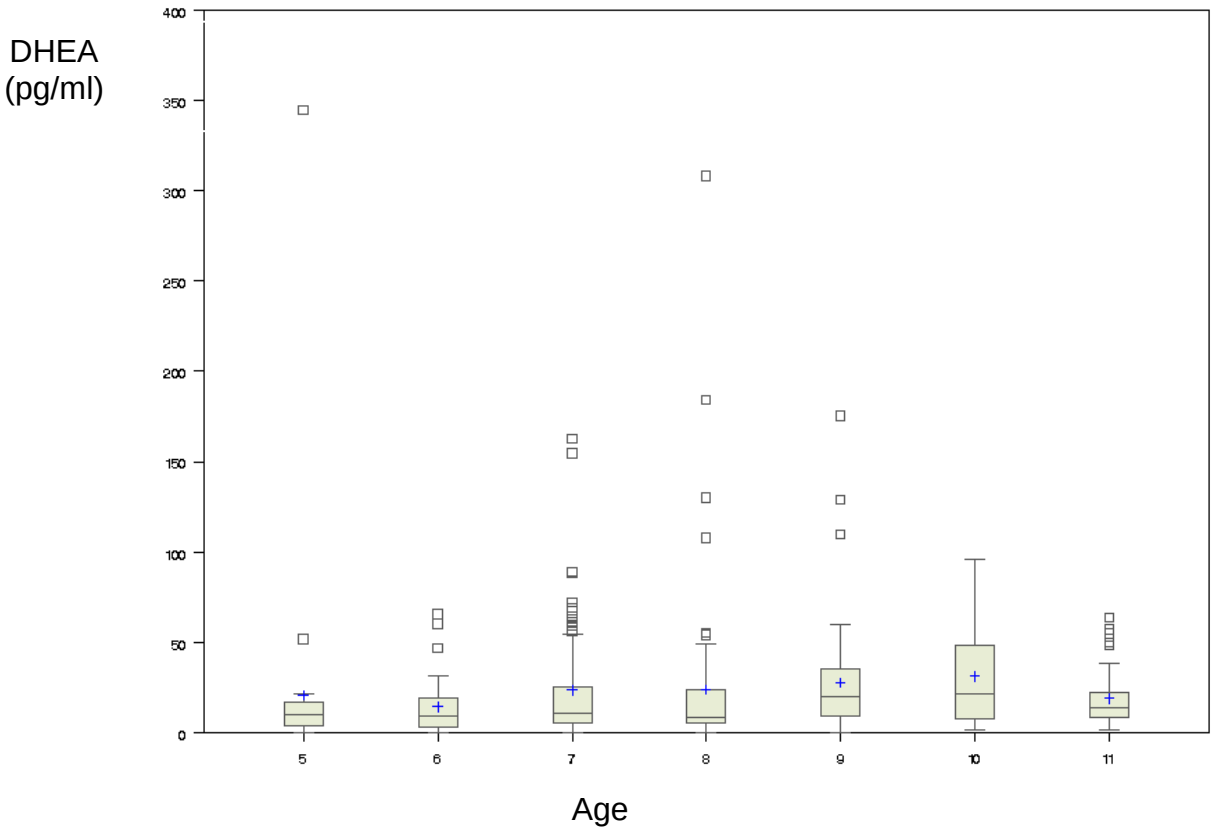


Figure 4. Boxplots representing the distribution of salivary DHEA across ages. The upper and lower part of the boxes represent the 75th and 25th percentile, the bar within the box represents the median, the blue cross represents the mean while the two whiskers represent the maximum (95th percentile) and minimum (5th percentile) observations. Empty squares represent outliers.

Chapter 3

Mother-child bond, stress response and social competence in a rural Dominican community

3.1 Introduction

Early exposure to chronic stress, including parental neglect, is known to affect the hypothalamic-pituitary-adrenocortical (HPA) axis and the autonomic nervous system (ANS) in rodents, non-human primates, and humans (Meaney, 2001; Flinn, 2009; Flinn et al., 2011; Parker and Maestripieri, 2011).

The HPA axis reacts to acute and chronic stressful situations by modulating the circadian release of the glucocorticoid cortisol (corticosterone in rodents; CORT) and the steroids dehydroepiandrosterone (DHEA) and dehydroepiandrosterone-sulphate (DHEA-S) from the adrenal cortex. The activation of the sympathetic-adrenomeduallary system (SAM) brings about the release of the catecholamines epinephrine (Epi), from the adrenal medulla, and norepinephrine (NE) at sympathetic nerve endings.

Cortisol levels are modulated by social stressors, with acute psychosocial challenges leading to a fast increase of salivary cortisol (Foley and Kirschbaum, 2010), while HPA axis hyper-activity followed by hypocortisolism is often observed during chronic stress (Fries et al., 2005; Miller et al., 2007; Jankord and Herman, 2008). Several scholars agree that during infancy and childhood the HPA axis goes under a hyporesponsive period, similar to the stress hyporesponsive period (SHRP) found in rodents (Gunnar and Quevedo 2007). This idea derives from the low number of

studies that have been able to elicit a stressful cortisol response in pre-adolescents children (Gunnar et al, 2009).

Little is known about DHEA and DHEA-S levels in conditions of acute and chronic psychological stress. DHEA is released during acute psychosocial stress concomitantly with CORT (Izawa et al., 2008) and DHEA-S levels are increased by acute stressors (Maninger et al., 2010).

In developmental psychobiology early stressors are often conceptualized as trauma and neglect. Trauma is often related to sexual and/or physical abuse or loss of a parent, whereas neglect is associated with parenting style (e.g. maternal rejection) (Loman & Gunnar, 2010). Children exposed to these experiences show altered HPA axis activity and stress reactivity, resulting in potential long lasting effects (Carpenter et al., 2011; Tyrka et al., 2008). For example, exposure to physical abuse or physical neglect is related to hypocortisolism (Bruce et al., 2009), while hypercortisolism is shown in children with early emotional neglect (Bruce et al., 2009; Flinn, 1996, 2006; Flinn and England, 2003; Gunnar et al., 2001; Mills-Koonce et al., 2011; Nepomaschy and Flinn, 2009). In adult subjects with post-traumatic stress disorder a history of early trauma is related to higher levels of DHEA and DHEA-S (Kellner et al., 2010). In general, low maternal sensitivity is associated with a hyper-reactive stress system in the infant (Hane and Fox, 2006; Tarullo and Gunnar, 2006) and early stressful events, especially in the form of low maternal caring, appear to have consequences for the development of child maladjustment and social competence (Barzman et al., 2010; El-Sheikh et al., 2009; O'Neal et al., 2010; Veenema, 2009).

There is a large variability in how children react to stressful conditions (Cicchetti and Rogosh, 2007) and active coping has been suggested to play an important role in developing resilient behavior after chronic stress (Haglund et al., 2007). Indeed, individual differences in coping styles appear to be relevant for the development of stress-related pathologies (Bartolomucci et al., 2005; Cicchetti and Blender, 2006; Koolhaas et al. 2010). Following the suggestion of Troisi (2002), in this study I used an ethological approach to analyze individual differences in coping behaviors. Ethograms built on nonverbal patterns of behavior that are observed during potentially stressful conditions have been successfully used in the study of psychiatric disorders (Troisi et al., 1996, 1999; Shreve et al., 1999) and stress responsiveness (Pico-alfonso et al., 2008; Sgoifo et al., 2003).

The present study was carried out in a horticultural, matrifocal community in the east side of the island of Dominica. By “matrifocal” I mean that a household is identified by the villagers with the name of the woman that leads it in the domestic and economic domains. These households are usually composed of a woman and her daughters who contribute to child care and house chores. In this matrifocal social system mothers generally invest more in daughters than in sons (Quinlan, 2006) and the importance of this complex family system for the development of the child’s HPA axis has been extensively described by Flinn and collaborators (Flinn et al., 1996, 2011; Flinn and England, 2003; Flinn and Leone, 2006).

The aim of this study is two-fold: (i) to show how children react behaviorally and physiologically to a mild stressor (a videorecorded interview) in their natural environment; and (ii) to verify whether CORT and DHEA are

associated with mother-child bond, i.e. on potential early stress experiences, and social competence.

3.2 Methods

3.2.1 The village

Mwa Bawego is a village situated on the east coast of the island of Dominica. It includes about 500 residents of mixed African, Carib and European descent that live in 160 households. The average annual income is $1900US and many villagers spend months or years doing seasonal work on other islands of the Caribbean, in the USA or in Canada. Despite poverty, children have growth rate comparable to US standards (Flinn et al., 1999).

3.2.2 Participants and general description of the study

Participants were fifty nine subjects (26 girls and 33 boys) with ages ranging from 5 to 11 years living in Bwa Mawego and participating in a longitudinal study on early traumatic experiences and social, psychological, and biological development (Flinn et al., 1996, 2011; Flinn and England, 2003; Flinn and Leone, 2006). As part of this longitudinal study, the subjects provided multiple samples of saliva over different days and years. Of the fifty nine children, thirty-nine (16 girls and 23 boys; mean age=7.89,SD=1.69) participated in a video-interview. The principal objective of the interview was to collect information on the children's social network, while monitoring their physiological and behavioral reactions. The questions were administered by a researcher from outside the village with the assistance of a village preschool teacher. Interviews lasted in average 21.40 minutes, ranging from 10 to 36 min, and often relatives (e.g., siblings, mothers) were present. Children were

asked to sit in front of the camera so that the face and the arms could be visible in the video.

3.2.3 Internal and externalizing behavior

These were assessed by means of the short-form assessment for children (SAC, Glisson et al., 2002). The SAC includes 24 items for internalizing behavior and 24 items for externalizing behavior. Each item can be rated as not true (scored as 0), sometimes true (scored as 1) and very true (scored as 2) (Glisson et al., 2002). “Is sad, unhappy or feels down” and “Feels tired a lot” are examples of items aimed to describe internalizing behavior. “Is irritable or stubborn” and “fights a lot” are examples of items measuring externalizing behavior.

The questionnaire was completed by two of the village pre-school teachers who knew all of the children. The subscales were highly consistent (Cronbach alpha =0.95 and 0.99 for externalizing and internalizing behaviors respectively). Agreement between the two raters was acceptable for externalizing (r=0.64) but low for internalizing (r=0.32). Because of the low reliability for internalizing behaviors, only externalizing behaviors were considered. For each child, results of externalizing behavior were obtained as the average of the untransformed (raw) scores relative to each subscale (see Glisson et al., 2002, for a description of transformed-scores).

3.2.4 Social network

The social peer network information collected during the interview was used to determine the network density of the children (Parker and Seal, 1996). Each time a pair of children were identified as friends by other children (not self identified), a value of 1 was added to the total strength of this bond. This process could create redundancy of ties if, for example, child A says that B is friend with C and that C is friend with B. In these situations the strength of the relationship was counted as one and not double. A symmetric co-nomination matrix was built using Ucinet 6.0 (Borgatti et al., 2002). Each cell represented the number of times two children were named as friends by others, and thus represented the association strength between the dyads. Association strengths of one, indicating only one of the 42 other children identified them as friends, were eliminated through the “filtering” process (Croft et al., 2008). Filtering was followed by the elimination of isolated nodes (James et al., 2009). Ucinet 6.0 was then used to obtain a measure of network centrality, Bonacich power (Bonacich 1987).

3.2.5 Mother-child bond

Measures of mother-child bonds were collected using three items: 1) level of attentiveness of the mother towards the child; 2) level of attachment of the child to the mother; 3) level of interaction between mother and child. Three female raters from the village were interviewed and rated each item from 1 (not at all) to 10 (very much). For the first item, inter-rater agreement was high ($r=0.7$, $p<0.01$) for two raters, but not the third, and thus only these two raters were used. For the other two items, the average correlation between the two raters was 0.68. A unique value for each item was obtained

averaging the raters' scores, but the summary scores were highly correlated and thus were averaged to a single mother-child bond score (Cronbach alpha =0.95).

3.2.6 Behavioral assessment

Nonverbal behavior of each child during the interview was measured by means of the Ethological Coding System for Interviews (ECSI; Troisi, 1999). The ECSI is an ethogram that comprises 37 different behavioral patterns related to facial expressions and hand/arm movements. These 37 patterns are grouped in 8 specific behavioral categories, i.e. eye contact, affiliation, prosocial behavior, submission, displacement, flight, assertion, relaxation. Although the audio was recorded during the interview, it was turned off during the behavioral coding. A trained observer unaware of the subject's identity and interview content scored the behavior of the subject in the first 5 minutes of the interview, using a one-zero sampling mode. One-zero scores have been shown to represent accurately the amount of behavioral pattern expression (Troisi, 1999). The 5-min scoring period was divided in twenty 15-s time intervals. The observer recorded whether a certain behavioral pattern occurred or not in each sampling interval. The score of each behavioral pattern was quantified as the proportion of sample intervals during which that pattern occurred.

The 37 behavioral patterns were grouped into 8 categories; specifically, 1) eye contact (i.e. gazing at another's face, that serves to express attention and involvement); 2) affiliation (i.e. series of patterns expressing friendliness and reflecting positive attitude); 3) submission, i.e. a set of behaviors that inhibit hostile reactions and represent a submissive attitude; 4) flight, i.e. the subject disengages from any form of social interaction; 5) assertion, i.e. hand movements and facial expressions that communicate low level aggression and hostility; 6) displacement, i.e. movements that focus on one's own body, are correlated with subjective experience of anxiety and negative states; 7) relaxation, i.e. patterns indicating a low level of emotional arousal and thus a low output of non verbal behavioral patterns that correspond to emotional arousal, and 8) gesture. An additional category, prosocial behavior, was obtained by adding submission and affiliation (Troisi, 1999). Because of the nature of the study, we focused our attention on three specific categories: relaxation, conflict (or displacement) and flight.

3.2.7 Saliva sampling, handling and preparation

Saliva was collected by passive drooling after stimulation with a sugar free spearmint gum. Saliva was then collected in a 5 mL polypropylene centrifuge tube free of NaAz through the help of a straw.

3.2.7.1 Saliva collection during regular days (no-interview)

A maximum of 12 samples per child were collected on four separate days during the summers of 2008 and 2009. The collection procedure requires the researcher and assistants to walk set routes from house to house three times a day for two days (Flinn et al., 1995). During July 2008, salivary

samples were collected once in the early morning (6:00 am-9:00 am), once in the late morning-early afternoon (10:00 am-1:00 pm) and once in the mid-afternoon (2:00 pm-5:00 pm). During August 2009, salivary samples were collected once in the early morning (8:00 am-10:00 am), once in the late morning (10:00 am-12:00 pm) and once in the early-afternoon (12:00 pm-2:00 pm).

3.2.7.2 Saliva collection during the interview

The interview was recorded during summer 2009 on a different day from the saliva sampling for the circadian study. The day of the interview three saliva samples were collected from each child before (T0), right at the end (T1) and 15 minutes after the end (T2) of the interview. Samples were kept in a thermic box with dry ice throughout the sampling procedure. Samples were stored at -20 °C for all the period of the field work and then brought to the department of Biological Sciences of the University of Missouri and stored at -80 °C. On the day of testing all samples were centrifuged at 3000 rpm for 15 minutes to remove mucins. The three samples from each participant were run on the same batch.

3.2.8 Cortisol and DHEA

Cortisol and DHEA were quantified using an enzymatic immunoassay (Salimetrics cat. # 1-3002 and 1-1202) following the manufacturer's company directions. Specifically, the cortisol assay uses 25 µl of saliva and has a sensitivity <0.003 µg/dL. Coefficients of intra- and inter-assay variation were less than 5% and 16.2% respectively. The DHEA assay uses 50 uL of saliva and has a sensitivity of 5 pg/mL. Coefficients of intra- and inter-assay variation were less than 8% and 10 % respectively.

3.3 Statistical Analysis

Growth models with three levels where samples were nested within day and day within subject (Hruschka et al., 2005; Singer 1998) where used to investigate the effects of the interview controlling for the effect of time since wake up, age and sex.

Nested models were compared using the Maximum Likelihood (ML) deviance tests, which allowed simultaneously to test for fixed and random effects (Singer and Willet, 2001). Briefly, this approach requires to perform the subtraction of the -2 Log likelihood (-2LL) between two competing models (a full and a restricted model). The deviance is distributed as a χ^2 with degrees of freedom (*df*) equal to the difference in parameters between the two models. To test if cortisol and DHEA variation was influenced by age, sex and their interaction, three consecutive models were tested adding in each one a fixed effect (age followed by sex followed by the interaction).

To test if children reacted to the interview with an increase in CORT and DHEA, a time-varying dummy variable was coded as 0 if the sample was collected during a regular day and 1 if the sample was collected during the day of the interview. In the case where the interview had a significant effect, the residuals for the samples collected during the interview were used for further analysis. To test if the levels of CORT and DHEA changed throughout the interview, the residuals for the sample collected before the interview (T0) were compared with the samples at T1 and T2 using a Student t-test for dependent samples.

The effects of mother-child bond, externalizing and Bonacich power on the hormonal measures controlling for the effect of the interview were tested

running two additional models for each of these variables, testing the effect of each predictor and its interaction with the baseline (samples collected during non-interview day) and interview hormonal values. For this analysis I report in the tables only the models with significant results.

I could not obtain information on mother-child bonding for four children, on ethological measures for one child and on the SAC and Bonacich power for four children. This will account for differences in sample size between different analyses. This may create problems when performing model comparisons in the multilevel regressions. Therefore, in the analysis testing the effects of these four predictors the AIC measure was used.

Correlation analyses were used to investigate the relations among the residuals of hormonal levels, nonverbal behaviors and questionnaire items.

Cortisol was distributed with a skewness= 11.11 and a kurtosis= 146.42; Kolmogorov-Smirnov $p<0.01$. DHEA was distributed with skewness= 4.79 and kurtosis= 33.14; Kolmogorov-Smirnov $p<0.01$. In order to normally distribute the values, CORT and DHEA were fifth root transformed (Eck et al., 1996).

The transformed CORT and DHEA were rescaled multiplying CORT by 10^4 and DHEA by 10^6 for reasons explained in the previous chapter. Briefly, scaling variables have only “aesthetic effects without changing results of the statistical tests” and help to avoid non convergence of the algorithm (Singer and Willet, 2002).

3.4 Results

3.4.1 Effect of the interview, comparing basal vs interview samples

The multilevel model showed that CORT decreased throughout the day with a cubic exponential function. Age and sex were not significant parameters. The model showed that the day of the interview CORT levels were significantly higher than in other days ($F_{1,381}$=44.89, p<0.001; see table 7; figures 5 and 6). DHEA output did not change throughout the day (Model 2; figure 7, table 8) but resulted significantly associated with age but sex was not significant ($F_{1,258}$=4.17, p<0.05; Table 8). The model showed a significant effect of the dummy variable for day of the interview (figures 7 and 8), with DHEA levels higher during the interview than during a regular day.

There were no significant differences between the pre interview CORT (T0-T1: $t_{(35)}$=0.09; p>0.05; T0-T2: $t_{(34)}$=-1.10; p>0.05) and DHEA (T0-T1: $t_{(34)}$=1.20; p>0.05; T0-T2: $t_{(36)}$=-0.80; p>0.05) samples and the samples collected at T1 and T2.

3.4.2 Correlation analysis

Results concerning the correlation between several variables are shown in table 9. The residuals obtained from the multivariate model representing the samples from the interview showed a negative relationship between the residuals of CORT before the beginning of the interview and mother-child bond (r=-0.44, p<0.01, N=33). CORT residuals right at the end of the interview were negatively associated with levels of relaxation (r=-0.34; p<0.05; N=38). No correlation was found between the residuals of DHEA and CORT except a positive relationship between the residuals of CORT at the

end of the interview and the DHEA residuals 15 minutes after the end. Externalizing behaviors were not associated with any of the variable tested.

3.4.3 Relationship between basal levels of cortisol and DHEA with mother-child bonds, externalizing behaviors and popularity.

For CORT, mother-child bond and externalizing were not significant parameters, while Bonacich power was negatively associated with cortisol ($F_{1,43.8}$= 3.09, p=0.08; table 7). Higehr DHEA concentration was associated with a closer mother-child bond ($F_{1,46.3}$= 4.64, p=0.03; table 8).

3.5. Discussion

In this chapter, I examined the relations between children's hormonal and behavioral reactivity during a videotaped interview in a natural setting, its association with mother-child bond and social competence.

It was demonstrated that during mid-childhood, children's HPA axis is able to secrete significant amount of cortisol and DHEA in response to a novel but, likely, mild stressors. This result contrasts with the "stress hyporesponsive period" that has been suggested for humans, similarly to rodents (Gunnar and Quevedo 2007). Newborns and infants are able to mount stress reactions, but this ability seems to decrease throughout childhood to arise, then, again around puberty (Gunnar and Quevedo 2007). Data on child's stress reactivity to laboratory paradigms produced conflicting results. Therefore there is a possibility that the hypo-responsiveness of children to stress is an artifact of stressor paradigms that do not work in the lab (Gunnar et al., 2009). The children from the community under study showed significantly higher levels of cortisol and DHEA during a video-recorded interview, compared to regular days. As a result, the interview

elicited the activation of the HPA axis. This result would not have been found if I was analyzing only the three samples collected throughout the interview.

That the HPA axis is activated by the interview is further supported by the fact that DHEA was high during the test. DHEA is considered an antiglucorticoid hormone with lower concentrations of DHEA indicative of higher glucocorticoid potency (McEwen 2003). High levels of DHEA and a high DHEA to cortisol ratio have been found in individual with post-traumatic stress disorders (PTSD) (Maninger et al., 2009), but have also been associated with positive resilient outcome (Haglund et al., 2007; Maninger et al., 2009). In studies of acute physical and psychosocial stress, DHEA has been shown to increase with the increased release of cortisol, as it would be expected by the action of ACTH (Manninger 2010). Based on this view, the results presented in this study confirm what is already known in the scientific literature, but extend it to the childhood stage.

CORT levels measured right at the end of the interview were positively correlated with DHEA levels measured 15 minutes after the end of the videorecorded interview. This may indicate that, in this sample of children, perhaps the two steroids are not released concomitantly.

The results from this chapter are also relevant for the early life stress effects on the development of the HPA stress reactivity. Scholars suggest that individual subjected to traumatic and neglectful experiences early in life will have higher stress reactivity in adulthood (Gunnar and Tarullo 2006). Although with the multilevel analysis I did not find a relationship between CORT and mother-child bond, there was a negative correlation between these two variables during the interview. Low mother-child bond was

associated with higher pre-interview CORT. Interestingly, I found a positive effect of mother-child bond on DHEA output, once we controlled for the effect of age and of the interview. This result is somewhat difficult to put in context. On one hand, the antiglucocorticoid nature of DHEA may be in line with a positive effect of the mother-child relationship. On the other hand, it has been shown that high levels of DHEA can correlate with antisocial behavior (Golubchik et al., 2009; van Goozen et al., 1998, 2000) although, in our sample, externalizing behaviors were notassociated with DHEA. Further analysis will be required to understand which of the two alternatives may be correct. Nonetheless, this result confirms the importance of the early social environment, in this case the relationship between a child and his mom, in modulating the development of the HPA axis.

The great variability of individuals' reactivity to stress may be associated with different strategies of active coping (Bartolomucci et al., 2005; Haglund et al., 2007). Under this perspective, the analysis of behaviors that are phylogenetically related to stress reactivity may be of valuable use to predict which children may be at higher risk. Using a methodology introduced by Troisi, I found that children with high cortisol right at the end of the interview were less relaxed, an expected result. Instead, displacement behaviors, that usually correlate with level of HPA activity in primates and rodents, were not associated with any hormonal measure throughout the interview.

There are some limitations to the study. One is the lack of standardization of the interview that is explained by the nature of the field work. The interview was carried out in a familiar environment for the child and, in many occasions, the children were surrounded by kin. Presumably, this setting should reduce the overall stressful impact of the interview. Alternatively, because during the interview each child was asked to rate and talk about (good and bad as they liked it) their friends, being surrounded by mom and other relatives may have created more discomfort in some children. There are potentially many more possibilities that may help to explain what and to what extent the interview could be perceived as stressful. Second, although I focused the analysis to a group of children ranging from 5 to 11 years of age, I was unable to collect information on pubertal stage, which it is known to affect the HPA axis reactivity (Gunnar et al., 2009).

In conclusion, this study showed that during mid-childhood children are able to raise cortisol and DHEA in response to a novel, likely, mild stressor and that the mother-child bond may play an important role in their modulation. Overall, the results support the idea that salivary cortisol and DHEA are potentially useful biomarkers of stress in children and they call for the need of careful ethological and ethnographic approaches when studying the relationships between stress factors and child behavior.

Table 7 Three level multilevel growth model: effect of the interview on cortisol

Parameters	Model 1	Model 2	Model 3	Model 4	Model5
Intercept	3.5843(0.03860)**	3.4484(0.04594)**	3.6577(0.1577)**	3.8540(0.07606)**	3.8816(0.07802)**
TSAWK	-0.07166 (0.00752)**	-0.00450(0.01620)	-0.00505(0.01621)	-0.03824(0.1650)*	-0.03930 (0.01778)*
$TSAWK^2$		0.01318 (0.0023) **	0.01327 (0.0023)**	0.01002(0.0022)**	0.00939 (0.0024)**
$TSAWK^3$		-0.00342(0.0007)**	-0.00340 (0.0007) **	-0.00282 (0.0007)**	-0.00271(0.0007)**
AGE			-0.02666(0.01941)	-	-
INTERVIEW DAY				-0.4689(0.06988)**	-0.4939(0.07431)**
BONACICH					-0.01213(0.0068)†
			Random effects (variance components)		
Level 1 Residual (Within person) σ^2	0.2817(0.02188)**	0.2647(0.02021)**	0.2646(0.02019)**	0.2517(0.01874)**	0.2330(0.01930)**
Level 2 Intercept (τ_{00})	0.06968(0.02397)**	0.05441(0.02041)**	0.05755(0.02076)**	0.03874(0.01695)	0.04470(0.01789)**
Level 3 Intercept (τ_{000})	0.03700(0.01903)*	0.04066(0.01850)*	0.03289(0.01734)*	0.04468(0.01821)**	0.02734(0.01520)*
			Model fit		
-2 log-likelihood	1064.9	1019.1	1017.3	975.6	751.9
AIC	1074.9	1033.1	1033.3	991.6	769.9

Table 7 represents the models used to estimate the effects of the interview on cortisol levels (Model 4) and network centrality (Model 5). Because model 4 and model 5 are not nested within each other they were compared using the Akaike procedure, that resulted in a better fit for model 5. TSAWK = time since wake up. Estimates represents the rescaled variables: time since wake up was mean centered and divided by 60; Age was mean centered. **p<0.01; *p<0.05; †p<0.10

Table 8 Three level multilevel growth model: effect of the interview on DHEA

Parameters	Model 1	Model 2	Model 3	Model 4	Model 5
Intercept	26.6216(0.4748)**	26.5526(0.4657)**	25.9078(0.6250)**	27.5951(0.6948)**	23.5117 (1.9806)**
TSAWK	-0.00072(0.001316)	-	-	-	-
AGE		0.7341(0.2423)**	0.7055(0.2386)**	0.7038(0.2433)**	0.7646 (0.2417)**
SEX			1.3953(0.9152)	-	-
INTERVIEW DAY				-1.3876(0.6799) **	-1.8099(0.7091)*
BOND					0.5540 (0.2571)*
			Random effects (variance components)		
Level 1 Residual (Within person) σ^2	23.8993(2.0775)**	23.8153(2.0619)**	23.8525(2.0677)**	23.6915(2.0431)**	20.3857 (2.0976)**
Level 2 Intercept (τ_{00})	8.5148(2.2575)**	7.9034(2.1890)**	7.8467(2.1840)**	7.5779(2.1294)*	8.1179(2.3217)* *
Level 3 Intercept (τ_{000})	7.4896(2.4142)**	7.1574(2.3601)**	6.6741(2.2566)**	7.3887(2.3711)**	2.3754 (1.4749)†
			Model fit		
-2 log-likelihood	3062.7	3053.9	3051.6	3049.8	2147.7
AIC	3072.7	3063.9	3063.6	3061.8	2161.7

Table 8 represents the models used to estimate the effects of the interview on DHEA levels (Model 4) and mother child bond (Model 5). Because model 4 and model 5 are not nested within each other they were compared using the Akaike procedure, that resulted in a better fit for model 5. TSAWK = time since wake up. Estimates represents the rescaled variables: time since wake up was mean centered and divided by 60; Age was mean centered. **p<0.01; *p<0.05; †p<0.10

Table 9 Correlation among residuals for cortisol, DHEA, mother-child bond, ECSI and externalizing behaviors and Bonacich Power

	CORT1	CORT2	CORT3	DHEA1	DHEA2	DHEA3	CORT	DHEA
C1	-							
C2	0.48(37)*	-						
C3	0.38(35)*	0.41(37)*	-					
D1	-0.003(37)	-0.09(37)	-0.009(35)	-				
D2	0.08(35)	-0.21(37)	-0.01(37)	0.39(35)*	-			
D3	-0.01(37)	0.42(39)*	0.04(37)	0.18(37)	-0.30(39)†	-		
CORT	0.76(39)*	0.80(39)*	0.78(39)*	-0.05(37)	-0.06(37)	0.18(39)	-	
DHEA	0.01(37)	0.08(39)	0.01(37)	0.83(37)*	0.49(37)*	0.54(39)*	0.03(39)	
Bond	-0.34(33)*	-0.16(35)	0.18(35)	0.11(33)	0.11(33)	0.26(33)	-0.14(35)	0.28(35)†
Conflict	0.19(36)	-0.01(38)	-0.23(36)	-0.001(36)	0.12(36)	0.06(38)	-0.03(38)	0.07(38)
Flight	-0.13(36)	-0.09(38)	-0.05(36)	-0.04(36)	-0.15(36)	0.21(38)	-0.11(38)	0.03(38)
Relax	-0.09(36)	-0.34(38)*	-0.17(36)	0.05(36)	0.26(36)	0.003(38)	-0.26(38)	0.16(38)
Externalizing	-0.01(33)	-0.03(34)	-0.23(32)	-0.13(33)	-0.05(32)	-0.07(34)	-0.10(35)	-0.13(35)
Bonacich	0.01(32)	0.06(33)	-0.20(31)	0.05(32)	0.03(31)	-0.02(33)	-0.03(33)	-0.00(33)

Note: CORT1, CORT2 and CORT3 = cortisol before, right after and 15 minutes after the interview. DHEA1, DHEA2 and DHEA3 = DHEA before, right after and 15 minutes after the interview; CORT=average of cortisol residuals across the three sampling times; DHEA= average of DHEA residuals across the three sampling times.
Bond= Mother-child bond; Number in parenthesis represent the sample size; *$p<0.05$, †$p<0.10$

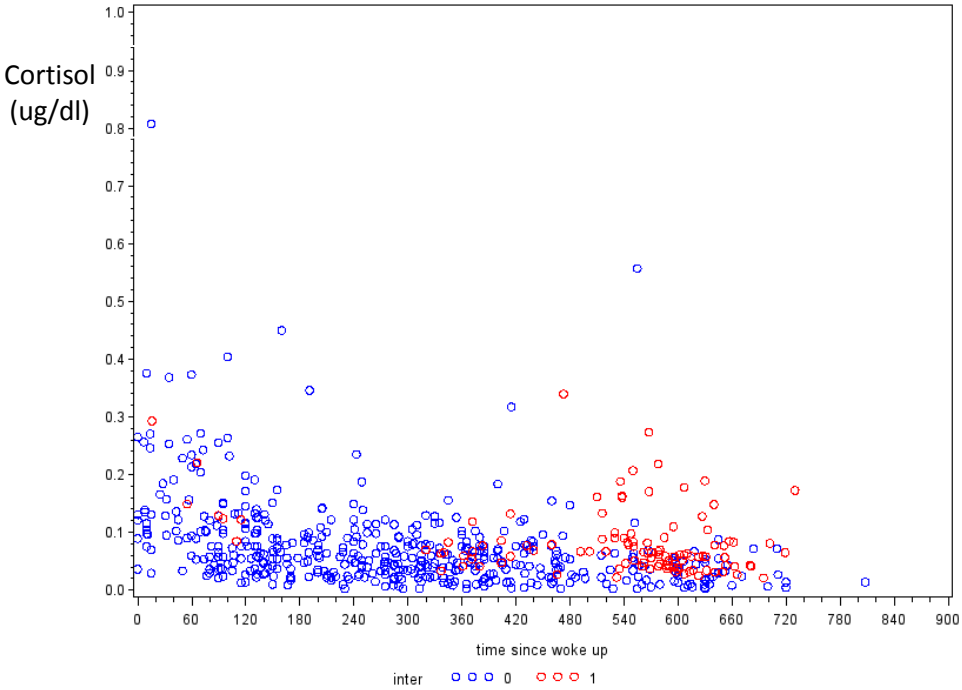


Figure 5. Effect of the interview on the distribution of cortisol values throughout the day. Blue circles represent samples from regular day, while red circles represent samples collected before, during and after the interview.

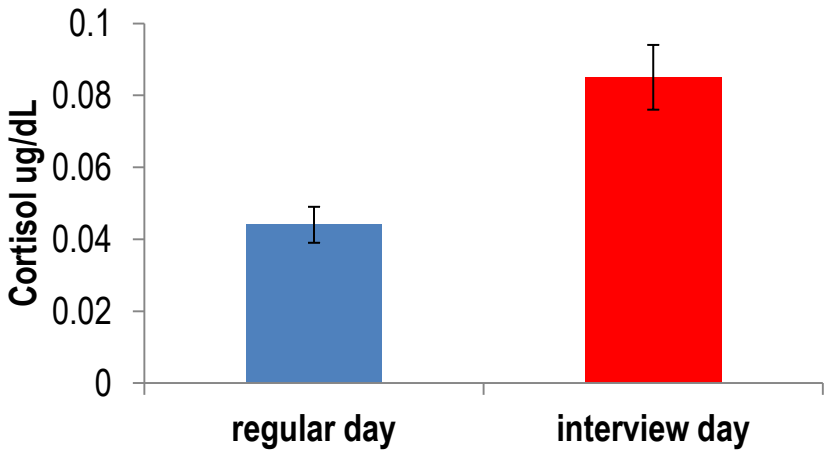


Figure 6. Cortisol levels on the day of the interview. Cortisol values were higher than at the same time in regular days

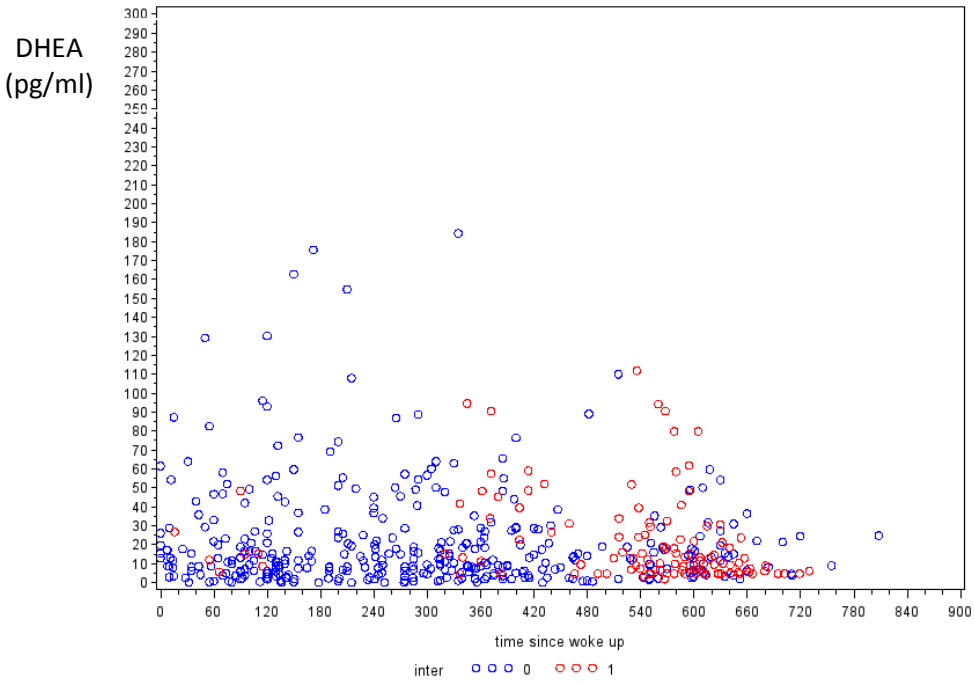


Figure 7. Effect of the interview on the distribution of DHEA values throughout the day. Blue circles represent samples from regular day, while red circles represent samples collected before, during and after the interview.

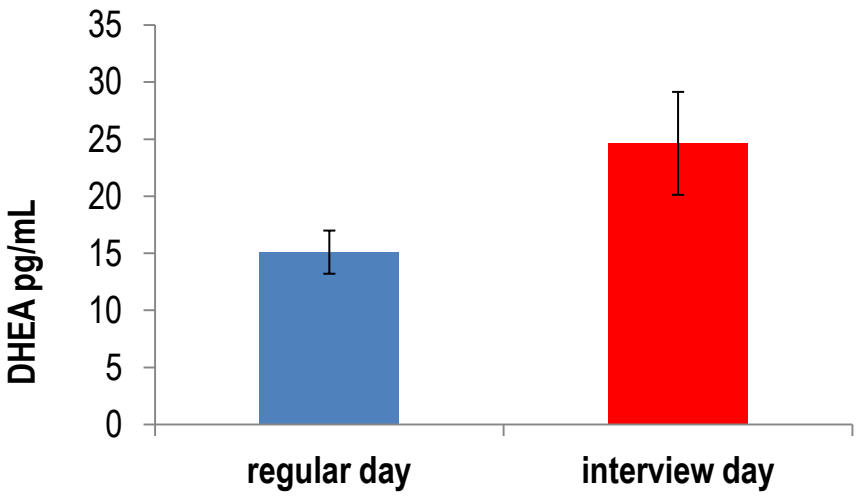


Figure 8. DHEA levels on the day of the interview. DHEA was higher than at the same time in regular days

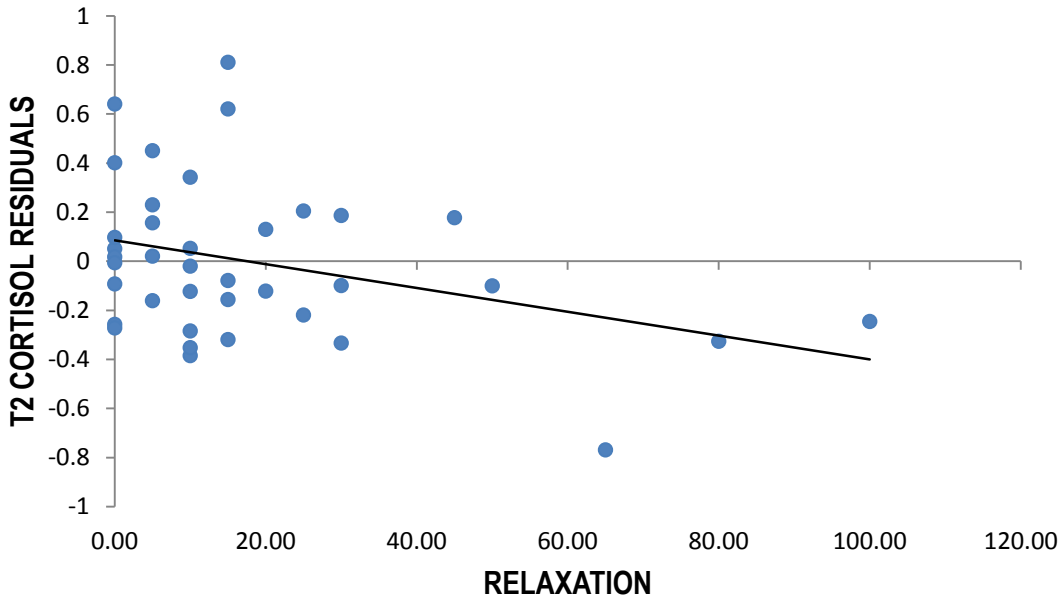


Fig. 9. Cortisol residuals and levels of child's relaxation during the interview. Relationship between level of relaxation during the first five minutes of the interview and the cortisol residuals right at the end of the interview.

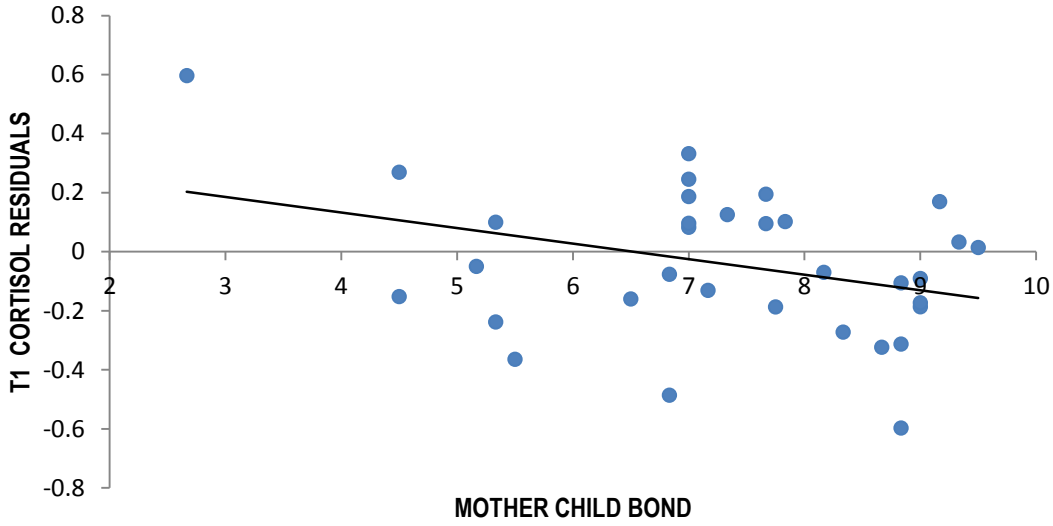


Fig. 10. Cortisol residuals and level of mother-child bond. Cortisol residuals at the beginning of the interview.

Chapter 4

Sex biased maternal investment and sex differences in child' sensitivity to stress

4.1 Introduction

Sex differences in relation to stress reactivity are found consistently across species (Yoshimura et al., 2003; Kajantie et al., 2006) with sex steroids playing a significant role. Although the nature of the stressful experience may influence in different ways the neurophysiology of each sex (Stroud et al., 2002), from a mechanistic point of view, at least in rodents, testosterone is considered to be inhibitory while estrogen excitatory of the HPAA (Yoshimura et al., 2003). For example, male rats show lower basal and stress induced adrenocorticotropic hormone (ACTH) and corticosterone while females show higher corticotropin releasing hormone (CRH) levels, effects that are dependent on the gonads (Viau 2002).

Sex differences in hypothalamus-pituitary-adrenal-axis (HPAA) regulation have been found also in humans with age, menstrual cycle and, likely, the nature of stressful experience playing important roles. Overall, it seems that human males show higher HPAA activity under acute stress than women (Kudielka et al., 2005, 2009; Kajantie et al., 2006), with men showing higher salivary (free) cortisol and plasma ACTH in response to challenges. These results are contrasting with the rodent studies. Within and between species, conflicting results may be explained by species-specific differences in

stress physiology and differences in laboratory protocols, with this last point probably very important for humans.

The observed, and sometime inconsistent, sex differences in stress physiology are usually explained in terms of proximate mechanisms, that is, as organizational vs activational effects of sex steroids on the HPAA axis (Kajantie et al., 2006; Kudielka et al., 2005; Lyons et al., 2000).

In humans, the complex interaction between sex steroids and the stress response becomes critically evident when we look at the sex differences in vulnerability to psychopathologies that are usually observed at the beginning of puberty (McCormick et al., 2007; Romeo 2010; Stroud et al., 2011). The picture becomes even more complicated when considering the long-lasting effects of early trauma (Heim et al., 2010). Parental neglect and maltreatment can have deleterious outcomes on the neurobiological development of children, with different sources of maltreatment resulting in different neurophysiological outcomes during childhood (Cicchetti and Rogosh, 2007; Cicchetti and Valentino, 2006; DeBellis et al., 2003; Tarullo and Gunnar 2006).

An important gap in preclinical and human scientific studies is an adaptationist-evolutionary approach that analyzes the ultimate causes of the observed results. For example, although developmental psychopathologists stress the evolutionary importance of the mother-child relationship and the deleterious effects resulting from the disruption of this form of attachment, few of them have used evolutionary driven theories to test adaptive vs maladaptive outcomes (exceptions are Belsky 1991, Ellis et al., 2006, 2011; Flinn 2006, 2009, 2011; Flinn and Leone, 2006; Del Giudice et al. 2011).

This approach would require the use of sexual selection, parent-offspring conflict (POC) and parental investment (PI), life history theories and marginal values analysis to formulate predictions to be tested by laboratory and epidemiological studies. At the same time, a well-sounded socio-ecological approach, conceptually different from the biosocial model (Granger and Kivlighan 2003), is lacking. The analysis of the species-specific socio-ecology should be integrated with the analysis of proximate mechanisms because it would help to better formulate predictions of biological function derived from evolutionary theory (Macri' et al., 2006).

For example, it could be hypothesized that increases of stress related markers in the offspring during maternal separation are the result of costly signals and thus, unless the mother will fail to provide the offspring with the minimum resources needed to survive and successfully reproduce, it should result in adaptive modification- life history theory- (Lyons et al., 2001; Parker & Mestripieri 2010).

The socio-ecological analysis would help to make different predictions of the neurobiological effects of mother-offspring separation between different species, even in presence of similar physiological outcome. In order to sustain the cost of lactation, mothers of carnivorous and rodent species are forced to leave their pups alone frequently until they are able to follow her. This is different from primates where mothers or other relatives are in continuous contact with their offspring. This is an important consideration for animal paradigms studying the effects of mother-child relationship disruption (Macri' et al., 2006).

Inconsistent results of sex differences in stress physiology that are found across species may be explained by different selective pressures, working on individuals in their species-specific evolutionary history. In the field studying the effects of early stressful experiences, rarely scientist looked for or reported potential sex differences in offspring sensitivity to low parental investment or sources of trauma. For example, in humans there is evidence for sex specific effects on the corpus callosum resulting from different sources of maltreatment, with girls' corpus callosum affected by sexual abuse and boys from neglect (Teicher et al., 2003, 2004). Girls with post-traumatic stress disorders (PTSD) have higher cortisol levels than boys (Carrion et al., 2002). Nevertheless, there are virtually no studies showing a sex specific effect of early trauma on stress response (Gunnar, Frenn et al., 2009; Gunnar and Quevedo 2007).

In this chapter, I aim i) to investigate if children show differences in HPA activity as a consequence of the interaction between their sex and early maternal investment and; ii) to propose that an adaptationist and socio-ecological approach may help to explain some controversial results in preclinical and human studies of early stressful experiences. However, I want to stress that preclinical studies are of tremendous value in order to understand proximate mechanisms, even if they show inconsistencies across species, and that my approach is a preliminary tentative to explain few reported scientific results, therefore it is prone to potential weaknesses.

I will start reviewing concepts of adaptive models of birth sex ratio and parental sex allocation. I will then shortly review few available data on maternal investment and stress reactivity in the offspring in a non-human

primate species. Finally I will present a preliminary, empirical study from a rural community of the island of Dominica.

4.2 Adaptive explanation of sex biased ratio in primate and humans

Since Darwin, the possibility of an adaptive mechanism regulating sex ratio has captured the attention of evolutionary biologists. Fisher (1930) showed that given the equal share of genetic material with each offspring, parents should invest equally in sons and daughters in order to maximize the number of grandchildren, as long as the cost of raising the offspring is the same. Whenever a biased sex ratio happens, a counter-selective process (frequency-dependent) will bring the ratio back to equilibrium, since a mutation that would make a parent to produce the rarer sex would be favored (Fisher 1930). Therefore the equal sex ratio represents an evolutionary equilibrium at the population level. Williams observed that although the sex allocation of a population is at equilibrium, individual parents may use different strategies to produce offspring of different sex, which it may require adaptation at the individual level although the evidence for it is not very strong (Williams 1979).

Several evolutionary biologists proposed different models to explain how a sex biased ratio could be an adaptation at the individual level shaped by natural selection (Clark 1978; Hamilton 1967; Trivers & Willard 1973; Silk 1983). As follow, I will list them as they potentially apply to human and non-human primates.

The Trivers and Willard model (TWM) suggests that when one of the sex gains more from extra parental investment then parents in better

conditions (with more resources) should shift sex allocation toward the sex that has the higher reproductive returns (Trivers and Willard, 1973; Frank, 1990). Therefore mothers in good conditions would favor sons because of their higher reproductive variance, while mothers in poor conditions should produce more females.

Local resource competition (LRC- Clark, 1978; Silk, 1984) is an extension of the local mate competition model proposed by Hamilton (1967). It was first proposed in order to explain sex biased ratios in galagos (Clark, 1978) and then in cercopitechines (Silk, 1984). It suggests that competition for resources among the philopatric sex may be costly for the mother up to the point where maternal sex allocation will be biased toward the dispersing sex. Silk suggested that in cercopitechines, the competition between females would extend to frequent attacks of high status monkeys toward the daughters of low status monkeys.Therefore, low ranking mothers should be forced to produce more sons that daughters. Considering maternal rank as a measure of maternal condition, Silk's model could be an extension of the TWM, but with an opposite outcome.

Local resource enhancement (LRE- Gowaty and Lennartz, 1985; Emlenet al., 1986) is applied to the cooperatively breeding species and predicts the opposite trend suggested by the local resource competition. Here, birth sex ratio will be biased toward the philopatric sex as far as it returns maternal investment (i.e. helper at the nest).

These models of birth sex ratio have been empirically tested in primates, mostly among the family of the cercopitechine- macaques and baboons. These species of Old World monkeys live in matrilineal groups

where daughters inherit maternal rank and where maternal kin are important as helpers and allies (“the advantage daughter” hypothesis- Hrdy, 1987). In these groups females from high ranking mothers are expected to have higher reproductive success than the one from lower ranking females (Altmann 1980).

Empirical studies using maternal rank as a measure of maternal condition in order to test the Trivers and Willard model and to derive specific predictions of the LRC and LRE have been carried out. Interestingly, in primate groups where females are philopatric the TWM seemed to apply but in the opposite direction, namely low ranking females produced more males (Silk 1984; Altmann 1980; Hrdy 1987). Nevertheless, the most comprehensive and latest meta-analysis have shown no support for the regulation of birth sex ratio by maternal rank, thus excluding the Trivers and Willard model as a potential explanation of birth sex ratio in primates when rank is used as a proxy of maternal condition (Brown and Silk, 2002). Instead, models of LRC and LRE appear to have some effect on birth sex ratio (Silk and Brown, 2008).

In humans, data on adaptive secondary sex ratio have not show much support and generally are conflicting (Hrdy 1987; Sieff 1990). Among the many reasons for this result, the potential model for explaining sex bias at birth may need to be modified to consider sexual specialization in parental investment, kin recognition and confidence of paternity, mate exchange systems and kin coalitions (Chagnon et al., 1979; Flinn and Sattenpiel 1990). These variables could all be view as further extensions of “local factors” that may shape parental sex allocation (Cronk 2007; Lazarus 2002), therefore complicating the issue. Moreover there are many theoretical demonstrations of random-non

adaptive explanations of secondary sex ratio in higher vertebrates (Krackow 2002; Williams 1979).

4.3 Adaptive models of sex biased investment after birth

The TWM, LRC and LRE models have also been used to explain sex biased parental investment after the birth of the offspring (Hrdy 1987). Social and ecological constraint could force parents to invest more in the offspring whom sex may have the highest reproductive return (Trivers and Willard 1973), in the sex that would decrease within parent-sibling competition (the migrating sex- Clark 1978; Silk 1983) or in the sex that will return the parental investment (Gowaty and Lennartz, 1985; Emlenet al., 1986). The logic is the same as for the birth sex ratio, but here parents play a direct role, behaviorally, on offspring survival and reproductive quality.

In the most recent review on sex biased investment in primates, Brown concluded that at this time (2001) there is not clear evidence of sex biased maternal investment in offspring, and this is true also for species showing female philopatry. The reason for this conclusion arises from the contrasting results collected by different research groups. Such divergence stands in the difficulty of measuring maternal investment or to unequivocally associate the costs of the investment passed to the offspring (Brown 2001). Examples of measures of maternal investment are gestation length, birth weight, interbirth interval, age at weaning, loss of weight during lactation and mother-offspring relationships (Brown 2001). All these measures are used to indicate which of the two sexes is more costly, an approach that showed opposite points of view (for an example of interbirth interval: Gomendio et al., 1990; Maestripieri 2001; Schino et al., 1999).

In humans there are many studies showing sex biased parental investment. These works have the same methodological problems found for primates in terms of measuring maternal investment (see Cronk 2007). Nonetheless, examples supporting the TWM or the LRE have been found in different populations (TWM: Boone 1986; Bereczkei and Dunbar 1997; Cronk 2000; Dickemann 1979; Voland 1984; LRE: Bereczkei and Dunbar 2002; Margulis et al., 1993; Quinlan et al., 2003, 2005). Interestingly, several of these studies using a LRE model found parental investment to be biased toward daughters.

4.4 Mother-offspring relationships in free ranging and captive social groups of Cercopithecines.

Although mammalian male reproductive success is at higher variance compared to females, it is surprising that in primate species where females are philopatric this may not be the case, or at least, data do not support it. In these female philopatric species, sons migrate incurring high risks of mortality and daughters' reproductive success is related to maternal rank. Therefore, mothers are expected to invest more in daughters (at least high ranking mothers, Altman, 1980; Silk 1981). A partial support of this hypothesis comes from a work on a captive group of bonnet macaques by Silk (1988), where high ranking females left several grand-offspring, while low ranking mothers none. In these matrilineal species mortality before weaning is higher for females, at least in free ranging condition where food availability may be sparse. This result contrasts with the "male frailty hypothesis" and it could be explained by the intense within sex social and food competition supporting the LRC model (Silk 1983; van Shaik and de Visser 1990).

While species-specific differences in social structures are well recognized, the socioecology where old world monkeys are embedded leading to the high female-female competition is presumably shared by guenon, macaques and baboons. In all these species, young and pregnant females are harassed by usually higher ranking females and generally infants are harassed and kidnapped (Bourliere 1970 for guenons; Sackett et al., 1975, Simpson et al., 1981 for macaques; Wasser and Norton 1993 for baboons; see Maestripieri 1994). There is evidence that in different species of the genus *macaca* infant females receive more harassment than males (Dittus 1977, 1979, 1980 cited in Silk 1983; Silk 1981, 1983; Simpson 1983). Therefore, Silk hypothesized that if young daughters receive more aggression, given the importance of having daughters in the matriliny mothers will invest more in protecting them (Silk, 1983).

Based on these socio-ecological pressures, a sex biased mothering style may be expected. Mothers may become more protective or less rejecting and restrain more the offspring that is at risk, daughters. In species where there is high infant harassment mothers invest more time in proximity to their offspring, becoming more protective (Maestripieri 1994a,b,c). Even if nipple access (potentially a measure of maternal investment) for rhesus monkeys has been found to be favored to sons (Simpson 1983; and nipple access may not be a good measure of maternal investment-Brown 2001), mothers restrained more their 8 weeks old daughters and mothers receiving high aggression keep daughters closer (Simpson 1983).

Maternal rejection of sons was found also in a colony of pigtailed macaques (Maestripieri 1998). In conclusion, there seems to be some

evidence that mothers of the genus *macaca* reject more sons and protect more daughters and this trend may be expected in all those species with female philopatry.

Following van Shaik and deVisser (1990) and Hrdy (1987) insights, the opposite pattern may be expected in male bonded groups of primates, where infant males could be at potential risk for infanticide (Nishida 1985). Data on different maternal styles between sons and daughters for chimpanzees were not found in literature.

4.5 Sex differences in infant sensitivity to low maternal care in cercopitechines

Within species variation in mothering style are expected, with genetic influences, temperament, age and previous experiences playing an important role (Stevenson-Hinde 1983; Maestripieri 1994 a,b,c). Moreover, given the importance of maternal care for proper infant development, extreme deviances from the normative pattern may be expected to be deleterious for the offspring.

Nevertheless, based on the socioecological pressures described in the previous paragraphs, sex differences in infant susceptibility to maternal care could be predicted. In cercopithecine, if maternal handling and protection is more important for daughter than for son survival then one could hypothesize that infant males and females macaques have different threshold for sensitivity to low quality of rearing, with females having the lowest one. These differences may manifest themselves in sex differences of distress related behaviors and vocalization upon mother-infant separation. Support for this hypothesis comes from female rhesus macaques using more “coos” upon maternal separation, possibly a sign of more distress (Erwin and Mitchell, 1973; Tomaszycki et al.,

2001; but see Berman et al., 1994 and Kalin and Shelton 1998 for lack of sex differences during distress).

Even if sex differences in distress related behaviors may not be observed during mother-offspring separation, the intensity of the negative experience perceived by the individual could be differently processed by each sex. This and the availability for coping strategies could be reflected in the infant's future ability to cope with stressful events. For example, in Rhesus Berman et al. found that infant did not differ in levels of distress when their mother resumed the mating activity, i.e. when the frequency of maternal separation increases dramatically, but they found that as a consequence of the separation, sons played and daughters groomed significantly more and that daughters tended to stay closer to their mothers (Berman et al., 1994). They explained these results in terms of the potential role of maternal separation in the developmental expression of characteristic behavioral sex differences. Yet, it is tempting to speculate that males and females are using different social coping strategies and that the lack of expressing them may amplify the stressful experience. Clearly, the two views are not in conflict but could complement each other.

Hence, sex differences in stress response to separation can be expected. Specifically for Cercopitechine, upon maternal separation daughters are expected to show a higher HPA reactivity than sons. The opposite should be true for species with male group bonds, such as chimpanzees and spider monkeys, species with high male infant mortality and possibly higher level of adult male attack toward male infants (Hrdy 1988; van Sheick and de Visser, 1990; but see Sackett 1975 for a an opposite point of view). Moreover, if

rearing conditions are expected to have long lasting effects on the stress response, the same predictions could be applied to females from poor rearing condition showing later on higher levels of anxiety-stress than females from good conditions while no or little differences may be expected for males.

4.6 Support from the scientific literature

As outlined in previous sections, rhesus monkeys live in matrilineal societies where mothers bias investment toward females (Maestripieri, 2001). Based on parental investment theory and sexual selection, sex differences in sensitivity to low maternal investment could be predicted in these species. In this social context, male and female youngsters may be expected to receive different maternal treatment because they go under different social selective pressures: females will stay in the group where they are born while males will migrate after puberty, experiencing a high mortality rate (Silk 1983).

In this social context, considering non-pathological familiar conditions and given some trans-generational stability of sex-biased maternal investment, daughters are expected to be more sensitive to maternal care than sons, with the prediction of within and between sex differences in stress related markers after mother-offspring separation.

The following is a tentative review of the studies showing basal and stress reactivity of the HPA system in macaques. Importantly, the predictions suggested earlier have not been tested yet, at least to my knowledge, and because of the paucity of data on the neuroendocrinological effects of maternal rejection in old world monkeys infants, I will focus the attention to paradigms of both mother-infant separation and mothering abusive style and report results whenever sex differences were analyzed.

Looking at the effects of repeated mother-infant separations on infant behavior and HPA activity, Sanchez and coll. found that at 5.5 months of age all infants (both the infants repeatedly separated from their mothers and the ones that never underwent this rearing condition), especially females, showed higher levels of cortisol upon maternal separation. This effect was higher and statistically significant in daughters exposed to the adverse rearing condition but males did not differ across the two conditions (Sanchez et al., 2005). They also found that females reared in the adverse condition showed a flattened diurnal cortisol decrease, a signal of abnormal HPAA activity. In explaining their results, Sanchez and collaborators suggested proximal endocrinological mechanisms (sex steroid organizational effects) and that females may have been sensitized to the separation protocol. No potential (and functional) explanations of why this should not be the case for males were suggested.

In a following but different study, Sanchez et al. (2010) investigated the short and long term effects of abusive mother behavior in infant rhesus macaques using a corticotropin releasing hormones (CRH) test. Overall, they found that abused infants showed higher cortisol levels than non-abused infants across the first 3 years of life. Females also showed the highest levels of cortisol responsiveness irrespective of the abuse experience. Although a non significant interaction between abuse and sex was found, it was evident that abused females had the higher cortisol reactivity to CRH, followed by abused males and control females with comparable values and finally by control males with the lowest reactivity (Sanchez et al., 2010). It is important to stress that in the colony studied by Sanchez and coll., sons and daughters

have the same probability to be abused (Maestripieri and Carroll, 1998; Maestripieri et al., 1997).

It is also note worthy to mention studies with peer-reared monkeys, that are motherless infants raised in nurseries with peers, therefore not socially deprived. These data need to be evaluated with caution, since in natural conditions motherless infant will not survive and therefore any presumably adaptive response of the infant will be hard to infer. Nonetheless, they reveal some interesting pattern. In an experiment testing the acute effects of alcohol, which can act as a physiological stressor by stimulating CRH and arginine vasopressine (AVP) release, adolescents peer reared females had the highest ACTH response and the highest basal levels of cortisol (Barr et al., 2004). Moreover, infant rhesus monkeys carrying the short allele of the serotonin transporter (5HTTLR) show high stress reactivity in form of high levels of ACTH. This effect is increased by the early peer rearing condition (motherless) and it appears to be exacerbated in peer-reared females which also show overall low concentrations of cortisol (Barr et al., 2004).

Unfortunately works investigating the effects of different maternal behaviors on neuroendocrinological measures in infants are few, sometime preliminary and use different paradigms and species (Parker and Maestripieri 2011). Data on variable mothering style on endocrinological and neuronal sex differences in the offspring, taking in account the species-specific socioecology, are virtually absent.

4.7 Application to humans

In humans there is is substantial variability of sex biased parental investment. This variability is cultural dependent but in general it shows a male

preference in pre-industrial societies (Hrdy 1987). This variability is also present within each culture in trends that apparently follows the TWM, with high ranking-rich families- investing more in boys and low ranking families in females, at least where females marry hypergynously (Cronk 2000). Nevertheless, when age at weaning is used as a measure of maternal investment there are many population where females are breast fed for longer (Bereczkei and Dunbar 2002; Margulis et al., 1993; Quinlan et al., 2003, 2005).

In this chapter I use a socio-biological approach to make predictions of HPAA functioning in children from a rural matrifocal population in the island of Dominica. In this population Flinn and collaborators extensively studied the effect of the family environment on children's HPAA development, showing that family composition has sex specific effects on children (Flinn et al., 1996). For example, sons seem to be more sensitive to father presence and daughters to mother absence and these results are age dependent (Flinn et al., 1996). Both sexes are extremely sensitive to the presence of step-fathers, showing unusual cortisol levels.

In this population, Quinlan and Flinn (Quinlan et al., 2003; Quinlan et al., 2005) established that, based on local resource enhancement (LRE), maternal investment is sex biased with girls weaned at later ages and that men are at greater risk for alcoholism and are socio-economically unreliable (Quinlan 2006). However, biased maternal investment may be adaptive to both boys and girls, because it may shape different developmental strategies suited to the socio-sexual ecological demands. For example, mothers appear to be generally less demanding with boys. It is suggested that in a matrifocal society

the mother-son relationship may reflect the social and economic marginality typical of Caribbean men (Quinlan et al., 2003), which in turn may result in more intense male-male competition for resources and social influence (Geary 2010).

If the socio-ecological pressure that led to female biased maternal investment has been stable for enough generations, so that low maternal investment could be “expected” by boys, I suggest that the “neglected” sex should adapt to low maternal investment (up to levels that will not reduce reproductive success), possibly by becoming less sensitive to it, and that girls will be more sensitive to low levels of maternal investment. Therefore, I predict that:

1) Overall, females will be more sensitive to low maternal investment, showing a hyper-responsive HPA to stressful conditions.
2) Females from low maternal investment will show hyper-responsive HPA to stressful conditions compared to females from high investment (within sex differences) and no or small differences between boys
3) If basal levels of adrenal hormones are indicative of early stress experience on the HPAA activity (Gunnar and Vazquez, 2001), i) significant differences in basal levels between low investment daughters and all the other groups ii) significant differences within females, iii) no or small differences within males.

4.8 Methods

4.8.1 Breast feeding

Information about age at weaning was obtained through retrospective health interviews of mothers conducted by a village informant. Data relative to months of breast-feeding were collected for 57 children (28 girls and 29 boys; age min = 3, max = 12) living in Bwa Mawego, a rural village on the east coast of the Commonwealth of Dominica. Of the sample of children, three boys were not breastfed. Although recall of complete cessation of breastfeeding is highly reliable (Quandt 1987; Quinlan et al., 2005), sometimes mothers that claim to breastfeed equally their children show instead sex biased weaning (Quinlan at al., 2005). As it is showed in table 1, in our sample there were several siblings that were reported by mothers to be breastfed for the same length of time. In order to maintain the sample the most possibly reliable, I excluded from the analysis children from family number 3, 11, 13, 14, 16, 28, 30, 33, 35 (table 10). I also excluded children that were not breastfed, two brothers from family 21 and a boy from family 25, and one girl and one boy that were still breastfed during the first collection of saliva in summer 2008. Therefore our final sample size was N=32 (14 girls and 18 boys; age range min=4, max=12).

4.8.2 Salivary sampling

A maximum of 12 samples per child were collected on four separate days during the summers of 2008 and 2009 (see chapter 2 for a description of the collection procedure). Salivary hormones were measured by means of enzymatic immune assay following the company's procedures (Salimetrics LLC). Intra-assay coefficient of variation (CV) were less than 5% for CORT

and less than 8% for DHEA. Inter-assay CV was 16.4% for CORT and less than 10% for DHEA.

4.8.3 Statistical analysis

Based on previous data showing that female were weaned later than boys, one-tale Wilcoxon-Mann-Whitney test was used to test if weaning was sex biased. To test the hypotheses that early and late weaning affects boys' and girls' cortisol and DHEA secretion in different ways, a multilevel linear modeling using SAS Proc MIXED was implemented (Singer, 1998). The model used is a "growth model" with three levels, where samples were nested within day and day within subject (Hruschka et al., 2005).

Age (in years, AGE) was entered as level 1 time-covariate. After having tested the models with time of day and age as factors, several additional models were implemented with sex (SEX) of the child, age at weaning (in months-WEAN) and the second level interactions between SEX and WEAN entered as predictors. I also entered a covariate (Why-Wean) useful to control the reasons why the mother weaned the child and its interaction with WEAN, thus controlling for the reasons for stopping breast-feeding and the relationship between length of breast feeding and reasons to stop it. Why-Wean was dummy variable coded as 1 if referring to the mother stopping breast-feeding on purpose (examples of possible causes are the mother needed to work and/or the mother left the child with her relatives and she moved away); if the mother had to stop to breast feed because she was sick or the child quit on his own, Why-Wean was coded as 0. The variables time of day, AGE and WEAN were grand-mean centered, therefore, where otherwise specified, the reported

results refers to a 7.71 year old child's CORT or DHEA levels at 5 hours post awake and weaned at 15.28 months.

Nested models were compared using the Maximum Likelihood (ML) deviance tests, which allowed simultaneously to test for fixed and random effects (Singer and Willet, 2001; see chapter 2 for a description). Models that fitted better at $p<0.10$ were retained.

CORT and DHEA were changed in nmol/l. Raw CORT showed skeweness and kurtosis respectively of 9.82 and 107.55. Similarly, DHEA showed skeweness and kurtosis equal to 4.45 and 26.80. In order to normally distribute the values, CORT and DHEA were logarithm and fifth root transformed and then the two transformations were compared. While the logarithm transformation resulted in a skweness = -0.16, kurtosis = 1.13 and Kolmogorov-Smirnov $p<0.01$, the fifth root transformation resulted in a better skweness and kurtosis, 1.04 and 4.72 respectively. For DHEA, the logarithm transformation resulted in skewness = 0.60 and kurtosis = 1.69 and Kolmogorov-Smirnov $p<0.01$. After the fifth root transformation, values where normally distributed with skeweness and kurtosis equal to 0.30 and 0.94 respectively but the Kolmogorov-Smirnov $p>0.05$. Therefore we analyzed the data transformed by means of the fifth root.

The transformed CORT and DHEA were rescaled multiplying CORT by 10^4 and DHEA by 10^6. The reason for this procedure was that the variance component of an outcome's small values will be small potentially causing non-convergence of the statistical algorithm (Singer and Willet, 2002). For similar reasons, I rescaled the time variable by dividing it by 60 (TSAWK / 60) with the result of increasing the growth's rate magnitude. Scaling variables have only

“aesthetic effects without changing results of the statistical tests” (Singer and Willet, 2002; see chapter 2).

4.2.5 Results

4.2.5.1 Sex differences at weaning age

Wilcoxon pair-wise test showed that in this sample of children there were no sex differences in age at weaning (S=215; N females=14; N males=18; p=0.56).

4.2.5.2 Effects of maternal investment on CORT: within and between sex differences

After having controlled for the effects of time of day and age, in model 6 I entered the two main effects SEX (coded as female=1) and WEAN and their interaction. Although this model fitted better than model 5 ($\chi^2_{(3)}$ =6.5 , p<0.10), of the parameters entered in the equation only sex showed a statistically significant trend (p<0.10). Therefore I added two covariates to control for the reasons why the mother interrupted breast-feeding (WHY-WEAN) and the interaction between WHY-WEAN and WEAN. This model performed significantly better than model 5 and 6 (model 5 vs 7, $\chi^2_{(5)}$ =16.1, p<0.01; model 6 vs 7, $\chi^2_{(2)}$ =9.6, p<0.01; see appendix) showing a significant interaction between SEX and WEAN ($F_{1,98}$=4.87, p<0.05; Table 12).

To better understand the interaction, I plotted age at weaning and the child’s predicted CORT concentration by sex (Fig 4.1b). As it can be observed by the plot, CORT is not affected by breast feeding duration in boys while girls showed increased CORT at higher maternal investment. A non-parametric analysis was performed to test sex differences at high and low maternal

investment after having calculated each child's average predicted CORT (across all the child' samples). Wilcoxon exact test showed that at high maternal investment girls have significantly higher concentration of CORT than boys (S =75.0, N girls= 6, N boys=9; $p<0.001$; fig 11), while no differences were evidenced at low maternal investment (S =27.0, N girls= 5, N boys =6; $p=0.66$).

4.2.5.3 Effects of maternal investment on DHEA: within and between sex differences

After having controlled for the effects of time of day and age, in model 6 (see table 4.3) I entered two main effects, SEX (coded as female=1) and WEAN, and their interaction. The model showed to fit statistically better than model 5 ($\chi^2_{(3)} = 9.9$, $p<0.05$). This model showed that the average DHEA concentration of an approximately 7 years old girls at 5 hours post-waking where higher than for a boy of same age at the same time of the day ($F_{1,32.9}=3.01$, $p<0.10$). A significant interaction was also present ($F_{1,38.2}=7.52$,$p<0.01$). Since the random intercept at level 3 became significant at $p<0.10$, I wanted to test if this effect was necessary. I tested a restricted model without the random intercept (see table 12) and found that the variance of this random effect was significantly different from zero ($\chi^2_{(1)} = 3.7$, $p<0.10$) and thus model 6 was retained. In the last model (model 7) I added two covariates, a variable controlling for the reason why mothers interrupted breast-feeding (WHY-WEAN) and the interaction of this variable with breast-feeding duration (WEAN X WHY-WEAN).

The effects of age, sex as well as the interaction between sex and breast feeding duration were still statistically significant after controlling for the

two covariates, although the first two parameters were significant at $p<0.10$. Since the fit of model 7 was not significantly different from model 6 ($\chi^2_{(1)} = 0.9$, $p>0.10$) model 6 was retained as the best model.

As for CORT, to better understand the interaction, age at weaning was plotted against the child's predicted DHEA concentration by sex (Fig 13 and 14). As it can be observed by the plot, DHEA was not affected by breast feeding duration in boys while girls showed increased average DHEA at higher maternal investment. A non-parametric analysis was performed to test sex differences at high and low maternal investment after having calculated each child average predicted DHEA (across all the child's samples). Wilcoxon exact test showed that at high maternal investment girls have significantly higher concentration of DHEA than boys ($S =66.0$, N girls= 6, N boys=9; $p<0.05$), while no differences were evident at low maternal investment ($S =29.0$, N girls= 5, N boys =6; $p =0.93$).

4.2.6 Discussion

First, I was unable to support previous findings of sex biased weaning in the population under study (Quinlan et al., 2003, 2005). But the basal activity of the HPAA axis was related to the level of maternal investment in a sex specific way. For both CORT and DHEA maternal investment seemed to affect more the girls than the boys. The concentrations of these two hormones were different in girls from early and late weaning while no differences were observed for boys. Specifically daughters receiving lower maternal investment showed lower levels of DHEA and CORT.

Sex differences in DHEA levels during childhood can be found in the scientific literature. These results usually show girls entering adrenarche at an

earlier age than boys. Contrary, virtually no sex differences in CORT are found at baseline or under chronic and acute stressful conditions in prepuberal children (Kajantie et al., 2006; Kudielka et al., 2005). An exception seems to be the study of Carrion et coll. (2002) showing that girls with PTSD had higher CORT levels than boys, a result that at first conflicts with ours. Davis and Emory (1995) showed that boys had higher cortisol during the Brazelton test. Few other studies that found sex differences in cortisol in infant during physical examinations or at day-care, glided over them as chance results (Gunnar et al., 1996; Watamura et al., 2002), which of course cannot be excluded.

In this paper I used an adaptationist-evolutionary approach to test predictions of within and between sex differences of the HPA axis during childhood. The directionality of the finding did not support the hypothesis, but the overall results showed the potential usefulness of the method. I tested the hypothesis that different levels of maternal investment in a matrifocal society would results in within and between sex differences of the HPAA activity. Based on the costs of breast-feeding for current and future maternal reproduction, I used as a proxy of maternal investment age at weaning.

Previous research in this population showed that mothers bias the duration of breast-feeding such that daughters are weaned later (Quinlan et al., 2003, 2005). The biased investment was explained by the resource enhancement theory, that is, in a matrifocal society often daughters are more valuable because they return the maternal investment by helping in house-works and child-care (Quinlan et al., 2003, 2005). If age at weaning is a good proxy for early life maternal investment and, low levels of maternal investment early in life have long term consequences on the development of the stress

response, then in this population girls should be more sensitive to lower maternal investment than boys. Therefore, within and between HPAA sex differences could be expected.

As already mentioned, one of the expected result was to observe girls from low maternal investment to be significantly different from any other child. Indeed, if high basal CORT and DHEA levels are indicative of stress load, we would have expected to observe daughters of low investing mothers with the highest CORT and DHEA levels. Support for this hypothesis for cortisol comes from the fact that a hyper-reactive HPAA and high basal cortisol levels have been reported in children from neglectful and/or dysfunctional families and across species (De Bellis 2005; Flinn, 1996, 2006; Nepomaschy and Flinn, 2009; Flinn and England, 2003; Gunnar et al., 2001; Heim et al., 2010). For DHEA, it is known to have antiglucocorticoid effects (Maninger et al., 2009).

The results from the present research seems to support works showing that children from low responsive parents and undergoing chronic stress present high cortisol reactivity that may then translate in later basal hypocortisolism (Bruce et al., 2009; Flinn et al., 1996; Gunnar and Vazquez, 2001). This is of course a speculation that creates the problem of explaining why boys that received high investment show hypocortisolism.

Weaknesses of the study

Undoubtedly one of the most evident weaknesses of this work is the inability to test links between the physiological results and personality, familiar and peer aspects of the child environment, which are important for a full analysis of individual differences in the development of the HPA axis in relation to early stressful experiences.

Another limitation is that the measure of maternal investment that I used could not entirely reflect the aspects of the mother-child relationship during development, while measures of maternal protectiveness/rejection could have been more sounded, based on the theoretical parallelism with the cercopitechine model outlined in the introduction.

Moreover I was unable to gather important information such as patterns of child growth, level of exposure to prenatal sex hormones (which could have been obtained by the 2D:4D finger digit ratio), pubertal status, measures of maternal stress, father presence or absence during infancy and number of living grand-parents or relatives that were taking care of the baby during early development, number of older dependent siblings during infancy. All these variables are very important because they can affect the child HPAA development and the levels of maternal investment. Therefore, they should have been used as control covariates in the statistical analysis. Hence, the results need to be taken as preliminary and warrant further investigation. The sample size could be a weak part of the study as well.

Another potential aspect of critique is the theoretical framework presented and the applicability of it to human studies. First of all, the model requires that species-specific patterns of maternal behavior vary depending on the sex of the offspring. As a consequence of the fact that offspring cannot be considered passive receivers of parental care (Trivers 1974), offspring receiving low parental care may become less sensitive to it if such adjustment will provide future advantages.

Although some results in non-human primates seem to support the view of consistent maternal biases in sex allocation, data are conflicting, probably

reflecting the fact that strategies of maternal investment can change rapidly (are flexible) depending on the mother's present and socio-ecological conditions.

Assuming that this level of flexibility is present within a population and that flexibility of behavior will help the mother to maximize her life-long reproductive success, then a mother is expected to switch investment between males and females across generation. Taking as an example the Trivers and Willard model where maternal condition influences sex allocation, hypothetically the condition could change throughout the life of the mother.

Therefore, it is hard to see how the offspring could have some "expectation" on the level of maternal investment, unless differential sex allocation is a phylogenetic trait evolved under specific socio-ecological pressures. This is even more problematic for humans in consideration of their high flexible behavioral repertoire that is evidenced by how culture changes quickly. Clearly some species-specific differences in maternal caring, present even within the same genus, as a result of different social pressures, shows that there is some strong relationship between mother-offspring relationship and the socio-ecology where they are embedded. But it may not be enough to support the theoretical framework proposed.

Therefore the possibility that mothers switch strategy of sex allocation during their lifetime seems to go against the proposed hypothesis of sex differences in offspring sensitivity to maternal investment.

Someone could argue that, in theories based on pure potential reproductive success, in polygynous species mothers should invest more in males and, following the logic of my framework, males should be more

sensitive to low levels of maternal investment while females should be expected to be more resistant. But this may not always be the case.

Alternatively, sex differences in sensitivity to cues of parental investment do not exist, but sons and daughters are equally affected by low levels of parental care. The resulting sex differences observed in outcomes affected by low parental investment represent genetic and hormonal constraints that may be used by the individual as an internal socio-ecological thermostat for the development of life history strategies (Del Giudice et al., 2011).

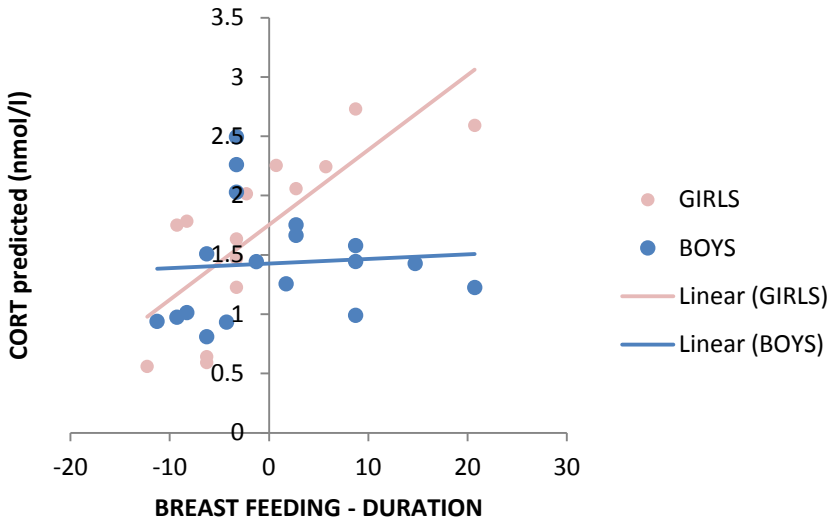
Concluding remarks

Apparent sex differences in sensitivity to early stressful experiences are observed in humans and non-human primates, probably reflecting a complex interaction between genetic, hormonal and environmental factors. In humans, gender differences in the stress response in relation to early trauma or neglect may be related to the different kind of maltreatment that girls and boys usually receive (Heim et al., 2010). Nonetheless, when boys and girls that underwent the same kind of maltreatment were studied, boys showed to be more affected in specific brain areas than girls (De Bellis et al., 2003; Teicher et al., 2003, 2004). Contrary, boys carrying a specific polymorphism for the CRH receptor seem to be protected from depression after trauma experience (Heim et al., 2010).

In macaques, females subject to early traumatic events show an HPAA reactivity completely different from males and non traumatized animals. Moreover a specific polymorphism on the 5HTT increases this sensitivity in females.

Generally these results are either not explained or justify with sex differences in hormonal milieu that organize and activate in a sexually dimorphic way specific brain regions. Interestingly, at least for macaques, some of these sex differences are manifested before puberty, thus excluding the effect of sex steroids in the modulation of the HPAA.

Overall, what is lacking is to answer the question of “why” these sex differences are present or what function, if any, they have. In this study an attempt to answer this last question was made using a socio-ecological approach and, despite potential theoretical flaws, some of the predictions were confirmed.



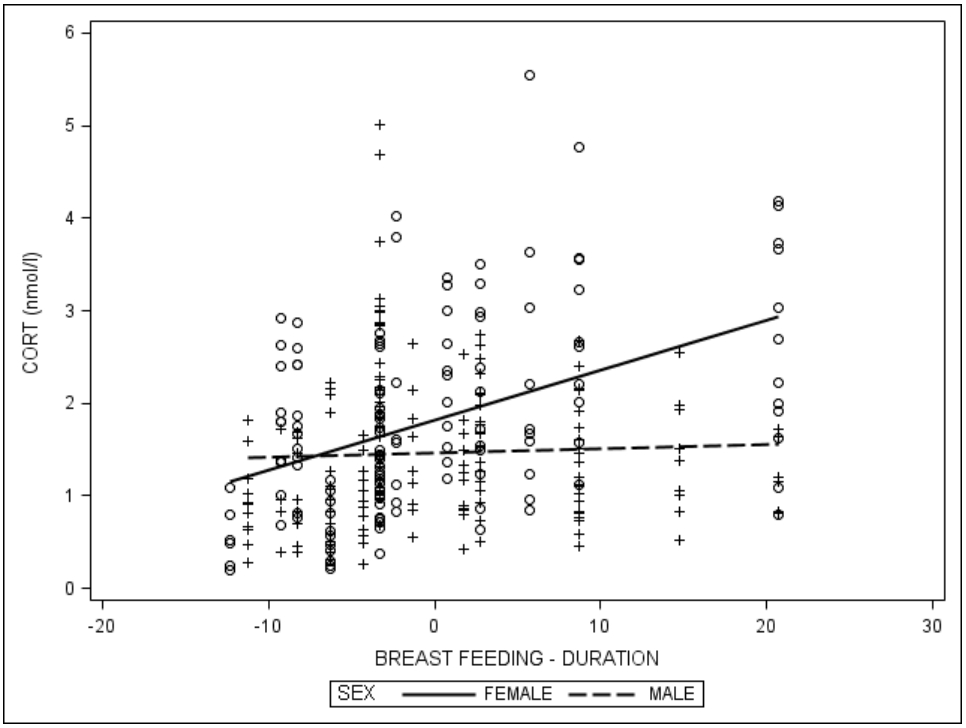
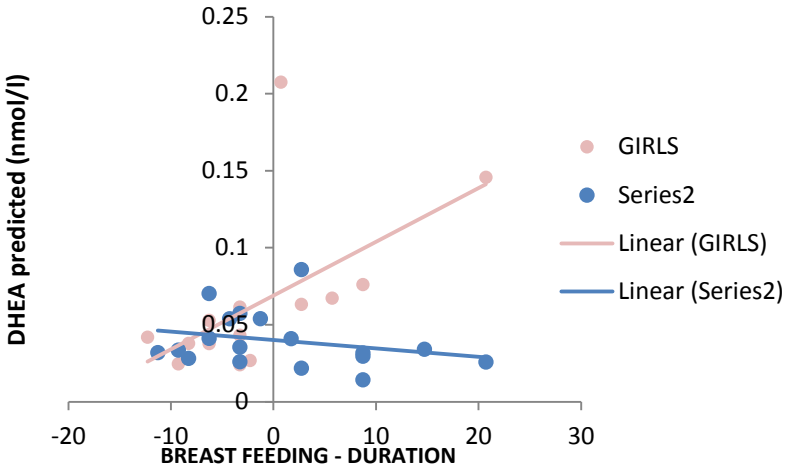


Fig 11 and 12. Effect of high and low investment on yearly average CORT(nmol/l) (A) and single sample (B) predicted levels in boys and girls. High and low investment represent late and early weaning (median split). Weaning time was mean centered (0=15 months). The interaction resulted statistically significant at $p<0.05$.



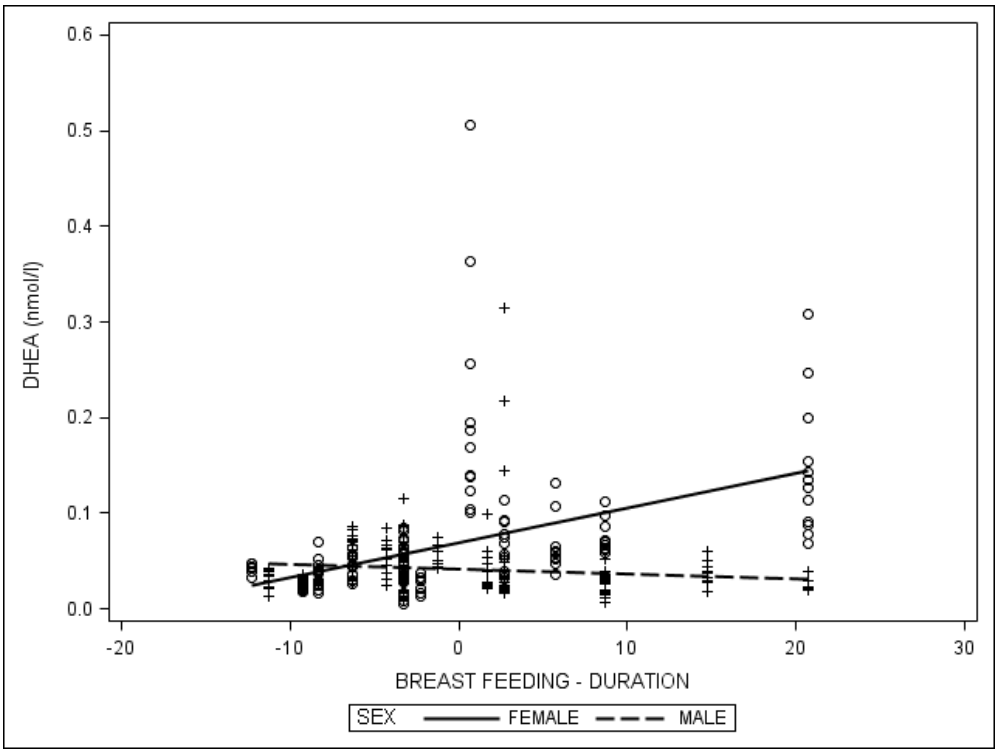


Fig 13 and 14. Effect of high and low investment on yearly average CORT(nmol/l) (A) and single sample (B) predicted levels in boys and girls. High and low investment represent late and early weaning (median split). Weaning time was mean centered (0=15 months). The interaction resulted statistically significant at $p<0.05$.

Table 10. List of children with information for breast feeding

Family	sex	BF	Why-Wean	Explanation	Age
1	female	13	1	work	7
2	male	7	0	--	7
2	male	9	0	--	4
3	female	12	1	not specified	4
3	female	12	1	not specified	9
4	male	12	1	not specified	7
5	male	24	1	not specified	6
5	female	48	1	not specified	3
6	female	9	1	work	11
7	female	7	1	left	9
8	female	16	1	left	7
9	female	12	1	left	7
10	male	36	1	school	4
11	male	12	1	not specified	4
11	female	12	1	not specified	6
12	male	17	0	--	9
13	female	60	1	school	6
13	female	60	1	school	11
14	male	9	0	mom sick	7
14	female	9	0	mom sick	9
15	male	18	0	--	8
16	female	19	1	not specified	11
18	male	24	1	left	5
19	female	9	0	--	11
20	male	12	0	--	6
20	male	30	1	school	4
20	male	11	0		10
21	male	0	.	Not BF	5
21	male	0	.	Not BF	8
22	male	6	0	--	8
23	male	12	1	not specified	6
24	female	21	1	not specified	11
24	male	18	1	not specified	7
25	male	0	.	mom sick	8
26	male	9	0	--	9
27	female	12	1	not specified	9
28	female	12	1	not specified	4
28	female	12	1	not specified	6
28	male	12	1	not specified	9
29	male	4	0	--	6
30	male	24	1	school	8
30	female	24	1	not specified	6
31	female	18	1	not specified	8
31	female	36	1	school	6
32	female	12	0	--	6
33	female	9	1	not specified	3
33	male	9	1	not specified	8
33	female	9	1	not specified	10
34	female	24	1	work	5
35	male	12	1	mom travels	8
35	female	12	1	mom travels	7
36	male	24	0	--	2
37	male	14	1	not specified	9
38	male	24	1	left	8

Notes:BF=breast-feeding duration in months; Work=mother need to go to work; left: mother left the child with grand parents;

Table 11. Three level multilevel growth model estimate of cortisol

Parameters	Model 1	Model 2	Model 3	Model 4	Model 5	Model 6	Model 7
				Fixed effects			
Intercept	10683 (199.43)**	10688(168.40)**	10713(142.29)**	10710(141.70)**	11816(519.28)**	10487(183.85)**	10490(331.84)**
TSAWK	-318.63(36.32)**	-325.97(36.17)**	-323.95(36.05)**	-325.73(36.07)**	-323.30(36.15)**	-320.36(35.95)**	
AGE					-136.24(71.22)†	-146.87(75.01)*	-200.87(77.45)*
SEX						518.52(277.01)†	425.10(279.80)
WEAN						-11.14(24.10)	40.26(48.48)
WHY-WEAN							385.05(391.31)
SEX x WEAN						52.65(33.14)	71.13(32.24)*
WHY-WEANX WEAN							-104.79(54.18) †
				Random effects (variance components)			
Level 1 Residual (Within person) σ^2	4379648(353656)**	3265193(339878)**	3251854(338001)**	3264775(340505)**	3248806(337017)**	3268969(339963)**	3268637(339478)**
Level 2 Intercept (τ_{00})	254667(360955)	635653(345773)*	980396(330527)**	956011(328673)**	913208(319994)**	760557(289793)**	594537(281237)*
Level 3 Intercept (τ_{000})	687552 (353656)*	338900(265053)	-	-	-	-	-
				Model fit			
-2 log-likelihood	5426.2	5358.9	5361.1	5360.5	5357.5	5351.0	5341.4

Table 11 shows the models estimating the effect of maternal investment of cortisol output in boys and girls. TSAWK=time since woke up, in hrs; Whe-Wean= dummy variable identifying the reasons of weaning (see text). **p<0.01; *p<0.05; † p<0.10

Table 12. Three level multilevel growth model estimate of DHEA

Parameters	Model 1	Model 2	Model 3	Model 4	Model 5	Model6	Model7	Model8
Intercept	531274(13823)**	531187(13884)**	531221(13661)**	521230(13187)**	529838(12732)**	515278(14996)**	514933(13098)**	524809(27307)**
TSAWK		-3176.63 (2321.77)	-2267.98(2496.87)	-2495.80(2388.48)	-2541.57(2397.27)	-2039.88(2394.17)	-1744.37(2367.89)	-2029.19(2387.62)
AGE					11687(6045.63)†	10845(5982.14)†	10576(5271.60)*	11202(6256.47)†
SEX						39077(22532)†	41573(19311)*	45357(23705)†
WEAN						-1838.33(1900.85)	-1819.76(1696.12)	-1491.42(3865.89)
WHY-WEAN								-21527(32324)
SEX x WEAN						7003.71(2662.90)*	7301.96(2331.52)**	6985.68(2668.92)*
WEAN x WHY-WEAN								664.85(4253.83)
				Random effects (variance components)				
Level 1								
Residual								
(Within person) σ^2	1.00E10(1.20E9)**	9.99E9(1.19E9)**	9.58E10(1.34E9)**	9.63E10(1.36E9)**	9.43E9(1.32E9)**	9.36E9(1.28E9)**	9.43E9(1.28E9)**	9.29E9(1.26E9)**
Level 2								
Intercept (τ_{00})	3.85E9(1.41E9)**	3.76E9(1.39E9)**	3.15E9(1.32E9)**	3.38E9(1.35E9)**	3.37E9 (1.34E9)**	3.43E9(1.37E9)**	5.11E9(1.39E9)**	3.52E9(1.39E9)**
Slope (τ_{11})			65759131(1.01E8)	6.49E7(9.23E7)	8.36E7(9.29E7)	8.61E7(8.94E7)	7.39E7(8.69E7)	8.78E7(8.82E7)
Covariance (τ_{10})			-5.43E8(2.39E8)*	-6.18E8(2.35E8)**	-6.68E8(2.40E8)**	-6.95E8(2.41E8)**	-7.39E8(2.54E8)**	-7.02E8(2.42E8)**
Level								
Intercept (τ_{000})	3.23E9(1.46E9)*	3.33E9 (1.48E9)*	3.26E9(1.43E9)*	2.86E9(1.27E9)*	2.51E9(1.16E9)*	1.36E9(8.62E8)†	-	1.28E9(8.42E8)†
Slope (τ_{100})			6505964(5.78E7)	-	-	-	-	-
Covariance (τ_{111})			-1.58E8(1.95E8)	-	-	-	-	-
					Model fit			
-2 log-likelihood	6089.5	6087.7	6078.4	6079.1	6075.3	6065.4	6069.1	6064.5

Table 12 represents models estimating the effect of weaning on DHEA output in children. TSAWK=time since woke up (mean centered); Why-wean=dummy variable that represents why the mother weaned; standard error are in parenthesis ; **p<0.01; *p<0.5; †p<0.10

Chapter 5

Proximate causes of sex differences in peer-network characteristics during mid-childhood: the role of DHEA

5.1 The development of sex differences

During child development boys and girls show behavioral sex differences in play and social interactions (Geary & Flinn, 2002; Geary 2010; Geary et al., 2003; Quinlan et al, 2003). From ethnographic studies, these sex differences in peer network organization seem to be observed in agricultural societies but not in hunter-gatherers (Konner 2010). Despite this difference, across many pre industrial societies the peer groups where children play are often multi-aged and kin based (Konner 2010). This last consideration brings to evidence the strikingly difference in peer play group organization between modern and pre-industrial societies.

Sex differences increase and strengthen with age to become highly identifiable by early childhood (Rose and Rudolph, 2006). Consistent with an evolutionary history of coalitional competition, boys shows more dense social networks, tend to invest more time in organizing groups of peers, among which they form hierarchies, and compete with other groups (Geary et al., 2003; Rose and Rudolph, 2006).

Throughout child development, boys spend significantly more time playing highly energetic games, such as rough and tumble, and they seems to correlate with the establishment of dominance hierarchies (Pellegrini, 2004), and by being more physically aggressive (Del Giudice et al., 2009). Sexual

differences are also observed in different kind of play, such as object-related play, probably adapted to the development of hunter-gather skills (Pellegrini and Bjorklund, 2004). Many of these sex differences appear early during childhood and become evident around the mid-childhood transition (Geary 2010). During this transition, children experience an improvement in terms of learning social and behavioral skills and become more independent from parents, assuming the ability to walk and perform some complex adult-like behavior (Lancy and Grove, 2011; Locke and Bogin, 2006).

The competitive-behavioral activities that boys show during the juvenile transition require low emotional investment (Benenson and Christakos, 2003; Geary et al., 2003) and, probably, a shift of energies toward monitoring resource distribution on a large scale (Charlesworth, 1994; Benenson 2001, 2010). For boys dealing in group of same sex peers, cognitive mechanisms useful for weighting the costs and benefits for aggression and triadic awareness (Patton 2000) are fundamental in dealing within and between coalitions. These cognitive mechanisms as well as an efficient accuracy in recognizing the strength of other peers social relationships are expected to develop during mid-childhood, between 5 and 7 years of age. Indeed children of 3-6 years are unable to predict with accuracy the social relationships of others, while this is possible in kids between 8-10 years (cited in Grammer, 1992). Nevertheless it is not clear if these abilities differ between boys and girls.

In the context of an ontogenetic perspective of human coalitionary behavior, vigorous play and peers social interactions (i.e aggression), that would result in the formation of social hierarchies and resource control, seem

to correlate with levels of prenatal and pre-puberal exposure to androgens (Archer, 2005; Cohen-Bendahan et al., 2005). On the other hand, boys' prosocial behavior is critical because of the importance of negotiating relationships to form and maintain a coalition (Geary et al., 2003).

The extent to which these behaviors are expressed by each individual throughout childhood certainly depends on the neurobiological and socio-cognitive development of the child and, likely, it is a consequence of each child's life history strategies (Archer, 2005; Del Giudice et al., 2009; Pellegrini, 2004). Indeed, the behavior of a child could be adaptive in one stage of development and not in another, creating therefore what are called "ontogenetic adaptations" (Bjorklund, 1997). An example is the case of moving from aggressive to controlling behavior in peer dominance (Charlesworth, 1994; Del Giudice et al., 2009) and source monitoring and metacognition (Bjorklund, 1997).

5.2 Adrenarche and adrenal androgens: Activational effects for mid-childhood sex differences?

The transition from childhood to mid-childhood is characterized by the onset of an adrenarche. Adrenarche is a physiological event characterized by a sharp increase of adrenal androgens production (DHEA and DHEAS) beginning at mid-childhood and followed by a steady increase that peaks in humans in their mid-twenties, by then steadily declining throughout senescence (Auchus, 2004; Havelock, 2004). Adrenal androgen secretion and the event of adrenarche are unique physiological aspects of primates and few other species, with adrenarche being present only in Humans, Chimpanzees and probably Gorillas (Campbel, 2006; Copeland et al., 1985; Cutler et al.,

1978; Hornsby, 2004; Labrie, 2004; Smail et al., 1982). Although adrenarche seems to be shared with chimpanzees, its timing and the relative androgenic production trajectory differ between the two species (fig 4.1; Campbel, 2006; Copeland et al., 1985).

DHEA and DHEAS – i.e. DHEA(S)- are weak androgens that can be converted in target tissues into more powerful androgens such as androstenedione and testosterone (Labrie 2004). The neurobiological effects of DHEA(S) seem to be related to their functions as neurosteroids in the brain, where their action covers neuroprotection and, by interacting with the serotonin and dopamine systems, neuromodulation, (Baulieu and Robel, 1998; Compagnone et a., 1998; Corpechot et al., 1981; Do Rego et al., 2009; Majewska, 1995; Maninger et al., 2009; Perez-Neri et al., 2008). Based on their anti-glucocorticoid effects, DHEA(S) confers neuroprotection and immunomodulation in humans (Hazeldine et al., 2010; Maninger et al., 2009; McEwen, 2003).

Alternatively, DHEA(S) could also have a more direct behavioral effect modulating aggressive behaviors (Soma et al., 2001; Wingfield and Soma, 2001). Likely because of their conversion to androstenedione and testosterone, DHEA(S) have been positively associated with some levels of aggressive and/or antisocial behavior in pre-puberal children, affecting mostly boys (Archer, 2005; Azurmendi et al. 2006; Dorn et al., 1999; van Goozen et al., 1998; Sanchez-Martin et al., 2010).

The rise of DHEA(S) during midchildhood and their neuroprotective and neuromodulatory functions suggest that adrenarche plays a very important role during a period of life when children are potentially exposed to high levels

of psychosocial stress related to solve “social puzzles” (Campbel, 2006, 2011; Flinn et al., 2011). Moreover, as described in the previous section, during mid-childhood behavioral sex differences become more evident. Therefore, the onset of adrenarche and its age dependent and specie-specific pattern of steroids secretion assume the aspect of an important node for human life history and for the human juvenile transition (Del Giudice et al., 2009).

Hence, DHEA(S) and other androgens of adrenal derivation can have multiple effects on the development of pre pubertal children. Behavioral and potentially cognitive sex differences promoting, during mid-childhood, a sex dependent divergence in relation to coalitionary behaviors are hypothesized to be mediated by the different effects that adrenal androgens may play on the brain of boys and girls.

5.3 Aim of the study

In this chapter I first investigated if boys and girls in the population of children under study segregate in different groups based on sex. This was done by means of social network analysis. Then I proceeded further investigating if children’ peer network characteristics and cognitive aspects of triadic awareness were age and sex dependent. Finally I investigated if adrenarche and DHEA predicted children social network parameters such as number of same sex friends, network density and cognitive abilities to detect others’ relationships.

5.4 Methods

5.4.1 Sociometrics

Data were collected through two separate interviews held during summers 2008 and 2009. The sociometric information and the social network were investigated with a methodology inspired by the socio cognitive maps (SCM) introduced by Cairns et al (1995).

Briefly, each child was asked to nominate up to 5 best friends. For each friend listed, children were then asked to list up to five best friend's friends. The age range of the children interviewed during the two summers varied from 5 to 11 years. Many of these children were going to school outside the village, increasing their range of acquaintances with other children and therefore increasing the potential for having friends outside of the community. Thus children were free to nominate friends from any place (neighborhood, school).

5.4.2 Pile sort

A pile sort technique was used during summer 2008 to better study child's perception of friendships ties in the community with a top down (successive) pile sort (Boster 1996).

A total of 54 pictures of children from age 5 to 10 have been shown to a total of 37 children ranging from 5 to 10 years of age. Specifically, the 54 items were presented shuffled in a random fashion on a table. Each child was asked to observe all the pictures and to move out the children he/she did not recognize. Before eliminating the unknown item, some attempts were made in order to help the child to remember the specific item (indeed in same case the quality of the picture might have confounded the children). The child was also asked to eliminate from the total items his own picture, in order to avoid any

focal point during the performance of the exercise. Once the child was ready, he was asked to put together in piles children that he thought were friends. He was free to do all the piles he wanted. The number of piles was recorded. Then the child was asked if he thought that some of the piles could have been put together following the same logic of friendship ties. This process was repeated until only two piles were left. Then returning to the original piles, the child was asked to remove from the piles children that he thought were notvery close friends until he felt he couldn't separate anybody else. This technique is different from an unconstrained, simple pile sort since it allows to control for the effect of splitters/lumpers (individuals that make many piles vs individuals that make few of them), therefore allowing comparisons of individuals (Weller and Romney 1988).

Of the 37 children interviewed, 8 were notable to perform the successive pile sort, but either did it wrong or did only a simple pile sorting. They were eliminated from the analysis. Consequently the total number of children used in the analysis was 29.

5.4.3 Perception of others' relationships: pile sort accuracy

An individual proximity matrix was built following the methods described in Anthropac (Analytic Technologies, USA) for multiple sorts (Anthropac 4, Reference manual). Each cell of an individual proximity matrix represents the number of times two of the 54 children were placed in the same pile divided by the number of total piles the rater performed. This number represents the rater's perceived strength of friendship between two individuals.

From each proximity matrix an aggregate matrix was built making the simple average of the 29 individual proximity matrices. Therefore each cell of

the aggregate proximity matrix represents the average (from the 29 raters) perceived strength of friendship between two of the 54 children. A correlation between each individual proximity matrix and the aggregate matrix was performed and each correlational value indicated the level of agreement between each child perception of others' friendship and the population overall perceived pattern of friendship. These procedures were performed using Ucinet 6.0 (Borgatti et al., 2002).

5.4.4 Social network

The peer social networks for summer 2008 and 2009 were constructed using the friendship nominations collected for the sociometric study. During 2008, 31 children were interviewed (13 girls and 18 boys) while during 2009, 42 children were interviewed (20 girls and 22 boys). The procedure required that each time a pair of children were identified as friends by other children (not self identified), a tie value of 1 was added to the total strength of this bond. This process could create redundancy of ties if, for example, child A says that B is friend with C and that C is friend with B. In these situations the strength of the relationship was counted as one and not as two.

A symmetric co-nomination matrix was built using Ucinet 6.0 (Borgatti et al., 2002). Each cell represented the number of times two children were named as friends by others, and thus represented the association strength between the dyads. Association strengths of one, indicating that only one of the interviewed children identified two other kids as friends, were eliminated through the "filtering" process (Croft et al., 2008). Filtering was followed by the elimination of isolated nodes (James et al., 2009).

The two resulting networks were different and therefore not comparable. The differences were mainly due to the fact that they did not have the same subjects (in 2008 there were 39 nodes while in 2009 the nodes were 56). Therefore separate results will be presented for each network.

5.5 Statistical analysis

5.5.1 Sex differences in peer nominations

Sex differences in the number of best friends nominated were investigated for 2008 and 2009. In case of heterogeneity of variance in the distribution of the nominations, Wilcoxon-Mann-Whitney test was used. Otherwise a t-test was selected.

5.5.1 Sex segregation and friendship predictors in the social network

Sex segregation within the network was analyzed by means of the algorithm “Joint-Count” (Ucinet 6.0). Joint-Count performs a randomization test of autocorrelation on the symmetric adjacency matrix to compare if the density of ties within and between two groups differs from a random distribution (Hanneman and Riddle). To test if boys have a higher density of ties than girls, a T-test (calculated through permutations with Ucinet 6.0) was run based on the clustering coefficient. The clustering coefficient is a measure of how dense are the ties between the actors of ego’s neighborhood. Moreover, by means of a QAP multiple regression (MRQAP) via double-dekker semi-partialling approach (Ucinet VI; Dekker et al., 2007) it was investigated how age, sex and children relatedness (level of kinship) were related to the distribution of friendship. Level of kinship was calculated using the software Descent 0.2 (E.

Hagen) from genealogical information collected by Mark Flinn and his collaborator Robert Quinlan.

5.5.2 Accuracy

To test the hypothesis that accuracy in recognizing other's friendship ties depends on the sex of the child, an ANCOVA was performed on data from the pile sort using PROC GLM in SAS. Accuracy was entered as the dependent variable and sex as the predictor variable. Age was entered as a covariate. Furthermore what drive children consensus in identify others' relationship was investigated with matrix statistics. First a consensus analysis was run to see if there were subpopulations (or subcultures) within the group of children performing the pile sort. The presence of consensus or subculture was then tested using the eigenvalues obtained by consensus analysis (Borgatti and Halgin, forthcoming). Then, once the presence of "one culture" (Borgatti 1998) was showed, a MRQAP via double-dekker semi-partialling approach was used to test if age, sex and level of kinship were associated with level of agreement between the kids.

5.5.3 Association between accuracy and DHEA

To explore if DHEA was associated with the ability of perceiving accurately others' friendships, a mixed linear model was implemented in SAS using the hormonal data collected during summer 2008 from 25 of the 29 children that participated to the pile sort. The hierarchical model had 2 levels (subject and day of collection). Since day of collection did not show to be a significant random effect and SAS was returning the warning message "G matrix not positively defined" it was dropped from the model. DHEA was entered as the dependent variable.

An unconditional model was tested. Since it was significant, I proceeded testing an unconditional growth model entering time since wake up. Then in the following up models I entered age (mean centered), sex and accuracy and the second level interaction between age and accuracy and sex and accuracy. These interactions were meant to test if sex and/or age mediate the association between DHEA and accuracy.

Deviance statistics were used to test the stochastic part of the model by means of REML statistics. Fixed effects were dropped if not significant at $p<0.10$.

5.5.4 Effect of DHEA on the child's sex segregation and network centrality

To tests the hypothesis that sex segregation and child's network centrality (popularity) were related to DHEA, for each peer network I run one analysis for repeated measures using a multilevel linear modeling with proc Mixed. Since I was interested in a possible association between the two variables I choose to enter DHEA as the dependent variable. The model was then tested with an unconditional model. REML statistics was used to test the need for day and within-person levels and random effects. Because of the small sample size, fixed effects were dropped if not significant at $p<0.10$.

In both multilevel analysis, DHEA was rescaled as explained in previous chapters.

5.5 Results

5.5.1 Peer network: Sex segregation

Results showed that the 2008 peer network was sex segregated (see table 13 and fig 16). In table 5.1 the first column shows the density of ties within girls (1-1), between groups (1-2) and within boys (2-2). If boys and girls were randomly tied and there was no sex segregation, then for the network under study a density value of 44.25 was expected. The observed value of 12.00 resulted statistically significant (the probability of randomly finding a result as small as 12.00 was $P<0.001$). Boys appeared to have a higher density of ties (49.00 compared to 27.00 for girls). A t-test was executed in Ucinet to test if boys had a greater clustering coefficient than girls. A trend toward a higher clustering within boys friendship was found (table 15; $p=0.07$)

The same procedure was run with the peer social network from 2009. As for the 2008 network boys and girls were segregated within same sex groups. The observed density of ties between the two groups was 13.00 compared to the expected 52.36 based on random distribution of ties (table 14 and fig 17). The probability to find a result as small as 13.00 was very small ($p<0.001$) supporting the statistical significance of the finding. Sex differences in ego clustering coefficient were tested with a t-test in Ucinet. A trend toward a higher clustering coefficient for boys than for girls was found (table 16; clustering coefficient of boys > girls- one tail-, $p=0.059$).

5.5.2 Peer network: demographic predictors of friendship

If age, sex and level of relatedness were associated with presence or absence of a friendship tie was investigated. A QAP semi-partialling multiple

regression analysis was run on two different dependent matrices (tables 17 and 18).

Sex and age were significantly related to the distribution of friendship while relatedness between two children was not as strong of a predictor. As an example, table 17 represents the regression analysis of the 2008 network. The overall fit is significant (r^2=0.084, p<0.001). A friendship relationship between two kids is stronger if the two kids have the same age (b= 0.14, p<0.01), same sex (b=0.10, p<0.01) and are close kin (b=0.29, p=0.06). Similar results were found for the 2009 network with the difference that kinship was a significant positive predictor of friendship (b=0.30, p<0.05).

5.5.3 Peer network: effects of DHEA

The association between sex segregation and DHEA was tested. Sex segregation was represented as the percentage of friends in ego's network having the same sex of ego. Two multilevel linear models were run, one for each year of network data.

The model for 2008 showed that age was a significant fixed effect (b= 141930, t=2.00, p=0.05). Sex was not significant. DHEA was not associated with sex segregation (b=-96058, t=-1.15, p>0.10).

A similar procedure was used for the data collected on 2009. Age and sex were not significant fixed effect. The variable measuring sex-segregation was not associated with DHEA (b= -106028, t= -0.45, p>0.10).

5.5.4 Sociometrics: summer 2008

During July 2008, 31 children were interviewed (13 girls and 18 boys). In average each child nominated 4.77 best friends (SE 0.10). The friendship nominations were not normally distributed (skeweness= -2.48, Kurtosis= 5.33

and Kolmogorov-Smirnoff $p<0.05$). Moreover the variances of the two groups were heteroscedastic as it was shown by the parametric test of Levene ($p=0.01$). Therefore the exact Wilcoxon-Mann-Whitney (Wilcoxon rank sum test) test was used to investigate if boys and girls differ in the number of friend nominated. The result showed a trend toward significance ($S=177.50$; $p=0.10$) with boys having a higher sum of scores than girls.

During July 2009, 41 children were interviewed using the same methodology of 2008 (20 girls and 21 boys). In average each child nominated 3.59 best friends (SE 0.17). The friendship nominations were not normally distributed (skeweness= -0.02, Kurtosis= -0.042 and Kolmogorov-Smirnoff $p<0.01$). Anyway, the sample was homoscedastic ($p=0.11$). Therefore a Student t-test for independent samples was run to investigate sex differences in friendship nominations. Data showed that boys nominated in average more bestfriends than girls ($t=2.32$; $df=40$; boys= 3.95 (0.19); girls= 3.2 (0.26); $p<0.05$).

5.5.5 Pile sort

Accuracy was distributed with a skewness = 0.09 and kurtosis = -1.69. Kolmogorov-Smirnov $p <0.01$. Therefore accuracy was not normally distributed. Levene test showed that the variance was homogeneous ($p = 0.56$). Although not necessary, accuracy was squared root transformed. Results showed that the model was significant ($F_{(2,26)}= 5.84$; $p<0.01$). Accuracy was not affected by the sex of the child, but age was a significant covariate ($p<0.01$). Correlation analysis showed that age was positively associated with accuracy (fig 18; $r = 0.53$, $p<0.01$).

To better understand what may drive consensus between the children in the knowledge of the friendship ties within the community beside age, a consensus analysis (Romney, Weller and Batchelder 1986) was run. Once the consensus matrix representing the agreement between each of the 29 children was obtained, correlations between the consensus matrix and other matrices representing shared characters between the 29 children were implemented in Ucinet with a quadratic assignment procedure (QAP). The QAP procedure is composed of two steps. In the first step, it computes Pearson's correlation coefficient between corresponding cells of the two matrices. In the second step, it randomly permutes rows and columns (synchronously) of one matrix and re-computes the correlation.

Results showed that sex and age were not affecting significantly the level of consensus between the 29 children. Since in the village under study each neighborhood is mainly formed by kin related families, I extended the analysis using MRQAP. The dependent matrix was obtained by the consensus analysis and represented the agreement between each child about the distribution of friendship ties. Three matrices were entered as independent predictors: age, sex and relatedness. Only the children for who I had relatedness measures were used, therefore each matrix was composed of 21 children (21 X 21) instead of the 29 for whom I collected the information of accuracy. The results clearly showed that sex, age and relatedness were not associated with level of agreement between the children (table 20).

5.5.6 Association between accuracy and DHEA

The multilevel model implemented to study the presence of an association between the 2008 DHEA levels and child accuracy in detecting

others' relationships showed that day of collection as a level of analysis was not associated with significant variance (moreover the SAS output was reporting that the variance was negative –"G-matrix not positive defined"; To fix this sort of problems a random effect is usually dropped resulting in a more parsimonious model, Singer and Willet 2003). Time since wake up was not a significant parameter (b= 385.82, t= 0.11, p>0.10) showing that for this subsample of children DHEA was not varying during the day. Therefore time since wake up was dropped from the model. Next, accuracy and age of the child and their interaction were entered.

Accuracy was not significant as a main effect (b= -2104.11, t=-0.02, p>0.10). Age showed to be a significant main factor (72.012, t=2.31, p=0.03). The interaction between age and accuracy showed a trend toward significance (b= -210298, t= -2.05, p=0.05). A visual inspection of this interaction (fig 19) showed that age mediated the association between accuracy and DHEA so that for kids younger than 7, high levels of DHEA where positively associated with accuracy while the opposite was true for kids older than 7. Sex (b= 26239, t= 0.32, p>0.10) and the interaction between sex and accuracy (b= -106785, t= -0.43, p>0.10) were not significant and therefore dropped from the model.

5.6 Discussion

In this study I first examined if the children's peer network of a small rural community of the island of Dominica showed sexual segregation and if there were sex differences in peer nominations and density of friendship ties. In order to test the hypothesis that DHEA has activational effects in determining sex differences during childhood, I investigated the possible relationships between the child's social network characteristics and the levels

of DHEA. Moreover, the ability of a child to recognize others' relationships as a function of his age, sex and DHEA levels was explored. In the following sections I will discuss in detail and offer some potential explanation for the results.

The two peer networks obtained during the two summers of field work were showing a clear sexual segregation and in both networks boys showed higher clustering coefficient. In this context the clustering coefficient represents the level of friendships between one child's friends. Therefore it could be assumed as a measure of coalition strength. Moreover, boys were observed to nominate slightly more friends than girls in both years.

These results are a cross-cultural corroboration of studies from the social sciences showing that boys' and girls' social structures of friendship are different (Baines and Blatchford 2009; Benenson et al., 1998; Rose and Rudolph, 2006).

Somehow surprisingly, the distribution of friendship ties was similar to the same age peer playgroups usually found in industrialized society, with age and sex predicting the presence of a friendship tie. This is somehow conflicting with the multi-age play groups found in hunter gatherer, agricultural and horticultural societies (Konner, 2010). Relatedness had a slightly minor effect in the 2008 network while friendships were clearly distributed based on level of kinship in the 2009 network. Overall it could be concluded that kinship plays an important role in how children consider friendship in this village. This finding is corroborated by my personal observation that in the village under study, children play and spend more time within their hamlet where it is more likely for them to have a relative as playmate.

The hypothesis that DHEA was associated with the level of segregation in a child's network was tested. If DHEA acts through its weak androgenic activity or by being metabolized to androstenedione or testosterone in target tissues, then one might expect an activational effect of these hormones during mid-childhood, a period when DHEA is produced at higher rates. As a consequence, this activational effect would result in sex differences in several behavioral and cognitive domains. The results reject this view. Within the two social networks obtained during 2008 and 2009 there was no relationship between DHEA and level of sex segregation. The lack of relationship between the endocrine titer and sex segregation could have been caused by the low sample size. Although a possibility, it seems unlikely.

To explain this point it occurs to consider that sex segregation begins at early ages, at around 3 years, and becomes highly evident during midchildhood (Geary 2010; Konner 2010; Rose and Rudolph, 2006). It may follow that DHEA is not the right hormone to investigate when looking for sex differences in peer network. This concept could be extended to all the sex differences observed prior to puberty. Sex differences during pre-puberty have been found in several species of primates (chimpanzees - Pusey, 1990; macaca fascicularis- Van Noordwiijk et al., 1993; gorillas - Watts and Pusey, 1993) and usually explained in terms of the organizational exposure to prenatal sex hormones or by processes of socialization and adrenarche had been excluded as a potential mechanism (Nadler 1987). To target other characters that are sexually dimorphic during mid-childhood would be an alternative approach. For example, parallel to an increase of sexual segregation, during midchildhood there is also an increase in aggression for

boys and in nurturing behaviors in girls (Konner 2010). It may be interesting to study if DHEA is associated with these types of behaviors.

Cognitive mechanisms supporting the ability to accurately recognize the strength of other peer social relationships are expected to be highly relevant in a species where coalitionary behaviors and exogamy have been considered an important selective pressure (Alexander 1990; Alexander and Flinn 2009; Chapais 2008). Boys and girls behaviors during midchildhood and adolescence seem to reflect this evolutionary history of homo sapiens (Benenson and Heath 2006; Geary 2003, 2010). As supporting this view, it has been described that children of 3-6 years are unable to predict with accuracy the social relationships of others, while this is possible in kids between 8-10 years (cited in Grammer, 1992). Nevertheless it is not clear if these abilities differ between boys and girls.

Using a technique from cognitive anthropology (Boster 1994) I was able to compare the ability of children to recognize others' relationship and to look if DHEA was associated with it, after controlling for sex and age of the child. The results show that age and accuracy were positively correlated. Once again, DHEA was not associated with accuracy, therefore not supporting my hypothesis. Specifically, younger children with high DHEA had higher accuracy than younger children with low DHEA while the opposite was true for the older kids. If DHEA have an activational effect on the aforementioned cognitive skills, the expectation was to find sex differences with boys outperforming girls (see Markowitz and Benenson 2010) and that DHEA was positively associated with cognitive maturation. The finding that high DHEA were predicting better cognitive performance in young children seems to go against the many finding

of deleterious psychological effects of children showing premature adrenarche (Dorn 1999; 2009; vanGoozen 1998).

Concluding remarks

In this chapter sex segregation in the social network of children from the community under study was found. Boys showed a higher clustering of friendship ties and to name more friends than girls. The effect of DHEA on this sex difference was not supported. Moreover, there were no sex differences in the ability to understand others' social relationships, but age was a positive and significant predictor.

Table 13. 2008 sex segregation: analysis with joint-count

	Expected	Observed	Diff	P >= Diff	P <= Diff
1-1	16.594	27.000	10.406	0.007	0.996
1-2	44.251	12.000	-32.251	1.000	0.000
2-2	27.154	49.000	21.846	0.000	1.000

Social network 2008; Number of iterations = 10000; 1= GIRLS; 2= BOYS
This table is showing that girls and boys are segregated in different peer groups. The number of observed ties between boys and girls was significantly lower than the one that would have been expected by chance.

Table 14. 2009 sex segregation: analysis with joint-count

	Expected	Observed	Diff	P >= Diff	P <= Diff
1-1	27.155	37.000	9.845	0.028	0.982
1-2	52.369	16.000	-36.369	1.000	0.000
2-2	23.476	50.000	26.524	0.000	1.000

Social network 2009; Number of iterations = 10000; 1= GIRLS; 2= BOYS
Description as for the previous table.

Table 15. Ttest for clustering coefficient 2008: Basic statistics on each group.

	Girls	Boys
Mean	0.280	0.403
Std Dev	0.305	0.297
Sum	4.486	9.262
Variance	0.093	0.088
SSQ	2.742	5.755
MCSSQ	1.484	2.025
Euc Norm	1.656	2.399
Min	0.000	0.000
Max	1.000	1.000
N of Obs	16.000	23.000

Difference in Means	One-Tailed Tests		Two-Tailed
	Group F > M	Group M > F	
-0.122	0.928	0.072	0.2242

Notes: F=females; M=males

Table 16. Ttest clustering coefficient 2009 Basic statistics on each group.

	Girls	Boys
Mean	0.487	0.533
Std Dev	0.366	0.342
Sum	9.748	13.319
Variance	0.134	0.117
SSQ	7.424	10.011
MCSSQ	2.673	2.916
Euc Norm	2.725	3.164
Minimum	0.000	0.000
Maximum	1.000	1.000
N of Obs	20.000	25.000

Difference in Means	One-Tailed Tests Group F > M	Group F > M	Two-Tailed Test
-0.045	0.941	0.059	0.6676

Notes F=females; M=males

Table 17. 2008 QAP regression analysis kin vs presence-absence of friendship

1. MODEL FIT

R-square	Adj R-Sqr	Probability	# of Obs
0.084	0.083	0.000	2450

2. REGRESSION COEFFICIENTS

	Un-stdized Coefficient	Stdized Coefficient	Significance	Proportion As Large	Proportion As Small	Std Err
Intercept	-0.006889	0.000000				
AGE	0.145703	0.200690	0.0005	0.0005	1.0000	0.107195
SEX	0.103302	0.200026	0.0005	0.0005	1.0000	0.111514
KINSHIP	0.298170	0.047647	0.0630	0.0630	0.9375	0.059612

Table 18. 2009 regression QAP analysis kinv vs presence-absence of friendship

1. MODEL FIT

R-square	Adj R-Sqr	Probability	# of Obs
0.060	0.059	0.000	3080

2. REGRESSION COEFFICIENTS

	Un-stdized Coefficient	Stdized Coefficient	Significance	Proportion As Large	Proportion As Small	Std Err
Intercept	0.000512	0.000000				
AGE	0.109935	0.145697	0.0005	0.0005	1.0000	0.109556
SEX	0.091442	0.182991	0.0005	0.0005	1.0000	0.130299
KINSHIP	0.305792	0.056053	0.0225	0.0225	0.9780	0.082988

Table 19. QAP correlations between age, sex and accuracy

	AGE	SEX
ACCURACY	-0.022	-0.024
QAP P-values	0.32	0.29

Table 20. QAP Regression analysis between Accuracy, age, sex and kinship

1. MODEL FIT

R-square	Adj R-Sqr	Probability	# of Obs
0.001	-0.004	0.236	420

2. REGRESSION COEFFICIENTS

	Un-stdized Coefficient	Stdized Coefficient	Significance	Proportion As Large	Proportion As Small	Std Err
Intercept	0.112893	0.000000				
AGE	-0.009487	-0.022282	0.3253	0.6752	0.3253	0.050207
SEX	-0.008607	-0.024430	0.2984	0.7021	0.2984	0.049756
KINSHIP	-0.001100	-0.000301	0.5887	0.4118	0.5887	0.049826

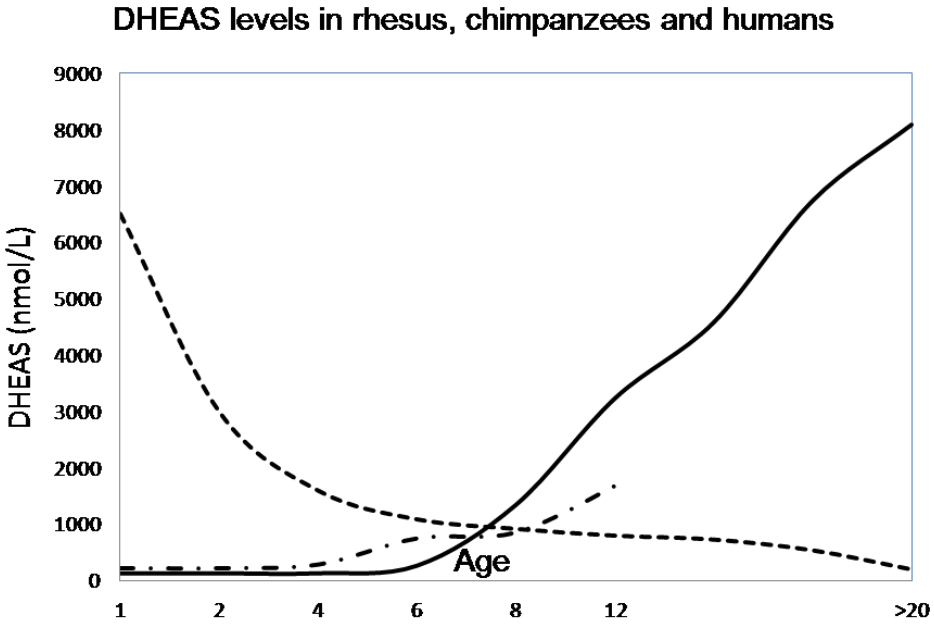


Figure 15. Human DHEAS levels (solid lines) from birth throughout juvenility compared to two different species of primates, rhesus macaques (dashed lines) and chimpanzees (dash-dotted lines). Data have been approximated using values obtained from Copeland et al. (1985), Kemnitz et al. (2000) and Korth-Schutz et al. (1976).

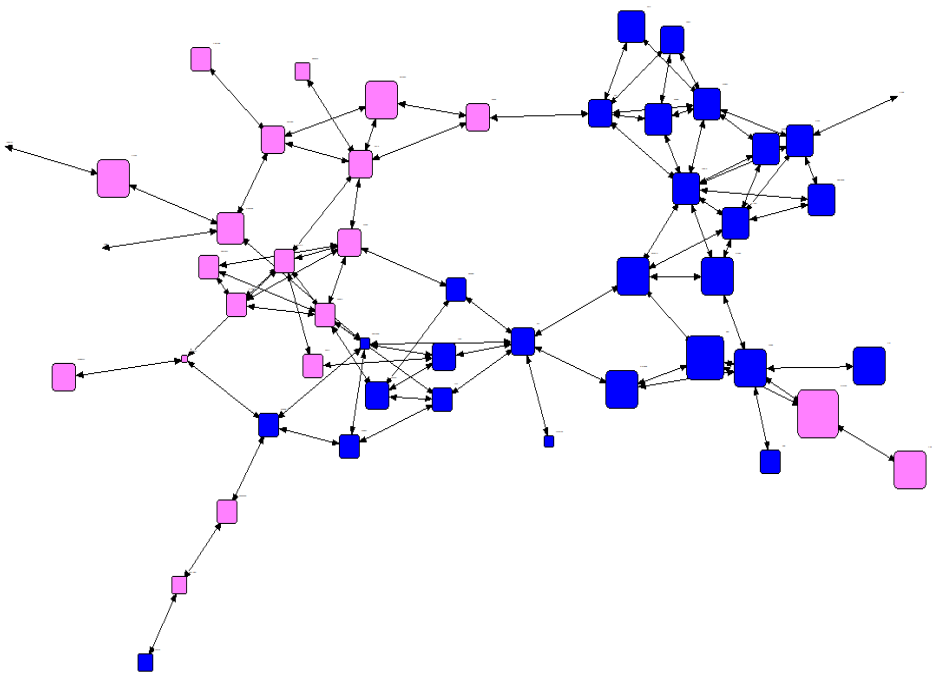


Fig 16: Sex differences in social network, summer 2008. Illustration of the sex segregation. Pink= girls; Blue= boys; size proportional to the age

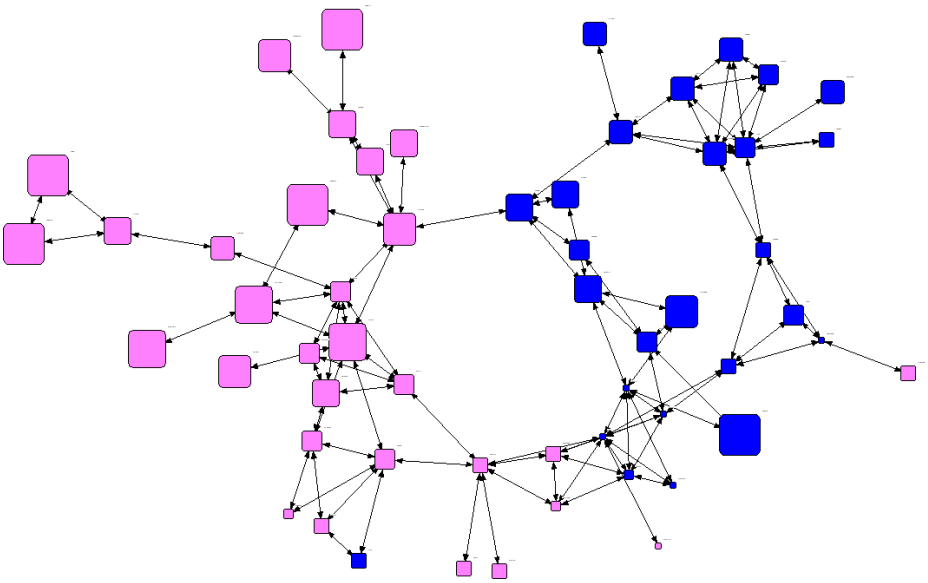


Fig 17: Sex differences in social network, summer 2009. Illustration of the sex segregation. Pink= girls; Blue= boys; size proportional to the age

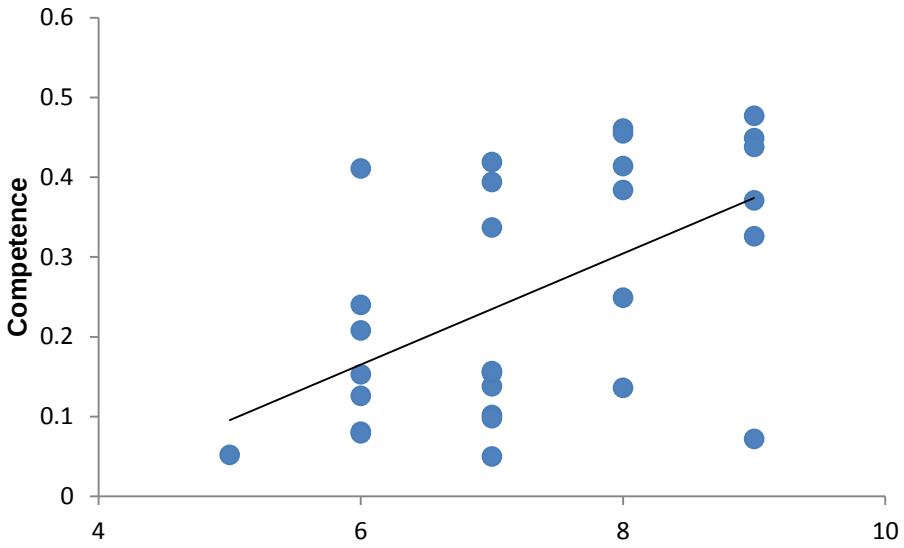


Figure 18. Correlation between accuracy and age. Accuracy represents the ability to recognize other's relationships (X axis: age in years)

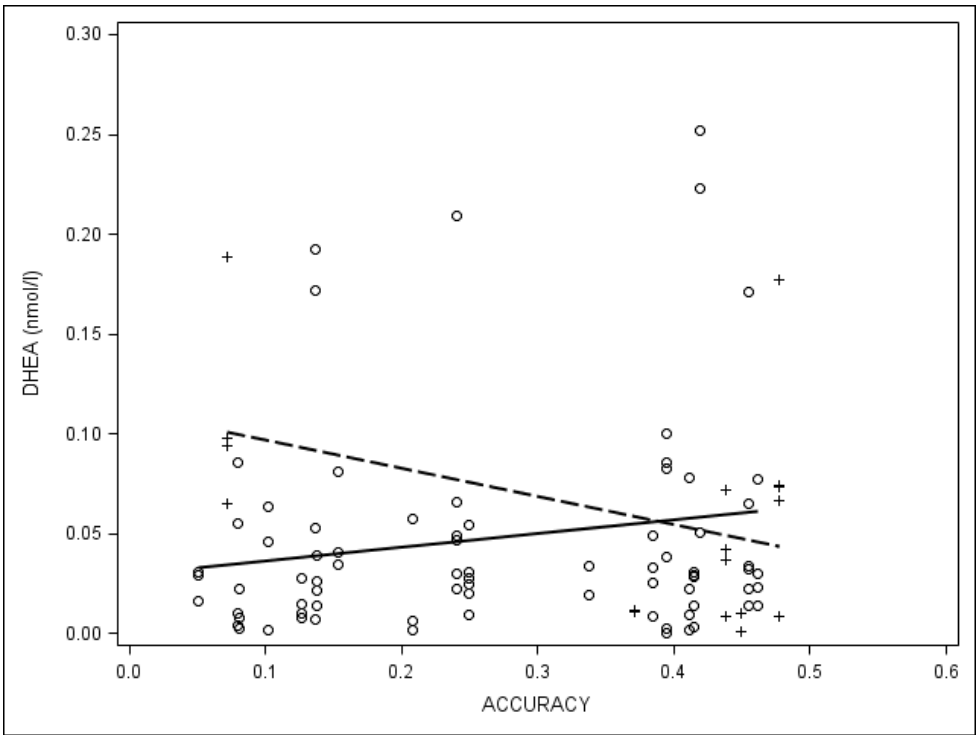


Fig 19. Interaction between accuracy and DHEA levels. Dashed line represents children 8 y.o and older; Solid line represents children 6 y.o. and younger.

Chapter 6

Hormonal mechanisms of coalitional competition during development

6.1 Introduction

Humans, as many other species, compete, sometime fiercely, for ecological and reproductive resources. The forms through which these competitive behaviors are carried out include between individuals and between groups aggressive challenges. The vis-à-vis competition between two opponents is phylogenetically the most ancient form of dispute over different sorts of resources. The group-vs-group competition, instead, appears to be a trait evolved later in few species of mammals, such as dolphins, wolves, chimpanzees and humans (Wrangham 2011). Inter-group conflicts in humans can span from killer raids that usually leaves few dead on the field (Kelly 2005; Wrangham 2011), to complex wars that see thousands of soldiers on the battle field (First World War). The complexity of these conflicts is somehow enhanced by examples where male children (usually at the beginning of mid-childhood) are either trained as warriors (Spartans, Spadolini 2007) or are forced to participate in the war (Elbert et al., 2010).

Chimpanzees are able to conduct coalitional killings that exterminate neighboring communities (Manson and Wrangham et al. 1991; Wrangham 2011). Wrangham (2011) suggest that the rate of intentional killing between communities is comparable between chimpanzees and pre-state societies. He argues that this phenomenon is under the same decision making heuristic, that is, the imbalance of power: a deadly raid will be carried out only if the costs of the aggression are low, meaning that they will happen only when the raiding group outnumbers the individuals of the other community.

On the other hand, the complexity of the human social systems, comprehending genetic and lineage kinship rules, may have selected for cognitive and behavioral characteristics that probably set them apart from chimpanzees (Flinn et al., 2011). Drawing from the ecological dominance hypothesis (Alexander 1990; Flinn et al., 2005), on one hand group vs group competition has probably shaped the cognitive and behavioral traits that enhance within-group cooperation and the reduction of reproductive conflict of interests (socially imposed monogamy). On the other hand, it has favored the expression of between group competition and out-group derogation (Geary 2010). To complicate the picture, within group competition is also expected, resulting in hierarchical organization through social dominance (Geary 2010).

How these cognitive and behavioral traits are regulated by the neuro-endocrine system during development and in adulthood are not known. My guess is that the same hormones and neurotransmitters that are thought to motivate dominance seeking, such as testosterone (T), aggression (serotonin-5HT), prosocial behavior (oxytocin) and the stress system (cortisol), are all intermingled in their action. That is, I dismiss the simplistic view of some hormone as having one specific cognitive function (Flinn et al., 2011; i.e. oxytocin as being “the pro-social” hormone). It is hard to imagine how a soldier that has just killed many enemies would sacrifice his life to save few comrades with the actual theoretical dichotomization of hormonal effects on behaviors. For example, in adults low levels of 5-HT have been associated with higher rate of impulsive aggression, while higher levels seems to increase social dominance and cooperation (Tse and Bond, 2002; Crocket et al., 2010).

Similarly, the trustworthiness stimulated by oxytocin seems to be directed only to in-group members (Dedreu 2011).

In many vertebrates, high levels of testosterone (T) are associated with increased aggressiveness (Wingfield 1990, Gleason et al., 2009). In male rodents, a peak of testosterone after a won encounter seems to have two functions: by activating the rewarding limbic circuit, the mouse is motivated to acquire (or reinforce the ownership of) the territory. Moreover, the peak of T would increase the probability to be successful in next competitive challenges (Fuxjager et al., 2011; Gleason et al., 2009).

In adult humans, T is not associated with aggression, but instead it has a positive relationship with striving for dominance (Archer 2006). It has been suggested that the positive effect of T on dominance is mediated by the HPA axis, specifically by low cortisol levels (Mehta and Josephs 2007). Overall, the scientific literature is clear in showing that during competition, humans may increase T levels if victorious and may show high levels of pre-competition T, which may help to prepare cognitively and physically to the actual or future challenge (Salvador 2005). T has also been observed to decrease in case of defeat in social anxious males, potentially showing the willingness to submit (Maner et al., 2008). Testoterone reactivity during a social challenge do not necessarily requires physical excercise (Mazur and Lamb, 1998, Hasegawa et al., 2008), since the androgenic outcome of a social context are also observed in bystanders (Bernhardt et al., 1998).

Data on children focused mostly on pathological population and aggression. What is know is that levels of adrenal androgens are often correlated with antisocial and aggressive behaviors (Dorn et al., 1999, 2008;

VanGoozen et al., 1998; VanGoozen and Fairchild 2007). In one study looking at the effect of peer victimization in 12-13 years old boys and girls, T was higher in bullied children, therefore conflicting with the prediction that T would decrease with social defeat. It is also known that dominance hierarchies during mid-childhood correlate with adolescent dominance status (Weisfeld 1999), but there are no data on neuroendocrine correlates.

Wagner et al. (2002), Oxford et al., (2009) and Flinn et al. (2011) showed clearly that T output is differently affected depending on the relationship between the competitors. Usually T is not affected if the competitors are from the same group while it rises if winning against an outgroup. In a study of intercollegiate soccer, Edwards et al., (2006) found that, for men, social connectedness with teammates was positively correlated with the increase of T during the game.

Although male children organize themselves in groups and compete and discriminate between ingorup and outgroup (Geary 2011), there are no data on the hormonal developmental processes underlying ingroup vs outgroup competition. Consequently, there are several key questions that need to be answered. If and when children begin to show hormonal correlates of social competition: during childhood T is not released by the testis, with the consequent very low concentration in serum. Therefore, there is the possibility that other androgens, such as DHEA and androstenedione, play the role that T plays in adulthood. Children may not show a different behavioral and hormonal reactivity between ingroup vs outgroup challenges: this is because young children may still be straggling for social dominance. Instead, such group context effect on T may be shown by teenagers that have known each other

for longer time and had many more chances to test their social and physical skills (Weisfeld 1999).

In this chapter I expand the adult empirical results previously cited (FLinn et al., 2011) to the study of teenagers and young children.

6.2 Methods

6.2.1 Teenagers: Cricket game and saliva collection

During summer 2008, a cricket match for teenagers of the village was organized to examine variability of hormonal responses to competition between coalitions of the same village. Sixteen teenagers participated in the match and divided themselves into two teams, each team representing a different hamlet: eight teenagers within a team (Team “S”, where S stands for the initial of the hamlet; age = 12.37, stdev = 1.30) and eight within another team (Team “F’; age= 13.59, stdev = 1.03). Since for one of the subject we did not obtained parental permission, saliva samples from fifteen players were collected immediately before and 10 minutes after the match. Saliva was collected by passive drool (via use of a straw) into a 5ml polypropylene centrifuge tube without sodium azide before and after the game. Free testosterone and cortisol were quantified via an enzyme immunoassay (Salimetrics) according to manufacturer’s instructions. All individual samples were run in duplicates with standards and high and low concentration controls (controls within acceptable ranges). All samples were run in a single assay, with the intra-assay coefficient of variation equal to 3.8% and 2.3% for testosterone and cortisol respectively. One of the players of the team that lost had undetectable levels of T (below the sensitivity of the assay) before the

game. This subject was not considered for further analysis concerning testosterone.

6.2.3 Teenagers: Measures of coalitional support and kinship

During summer 2008, 19 male teenagers from the village were interviewed by means of a top-down successive pile sort technique (Boster, 1994). Each teenager was presented with cards. On each card were written the first and last name of 37 adolescents and young adult villagers (age ranging from 11 to 19). Each subject was asked to pile the cards based on who would support who in case of a fight. From each resulting individual pile sort, an individual proximity matrix was built following the methods described in Anthropac for multiple sorts (Anthropac 4, Reference manual). In short, each cell of an individual proximity matrix represents the number of time two teenagers "survived" the splitting process divided by the number of total splits the subject performed. This number represents the perceived (by the informants) strength of coalitionary support between the two individuals. From each proximity matrix an aggregate matrix was built making the simple average of each individual proximity matrix. All these procedures were made using Ucinet 6.0. Using the aggregate matrix, two measures of coalitionary strength for each kid were obtained. One measure, that represents the average strength of perceived coalitionary support within the 37 teenagers used in the pile sort, was calculated in the following way: the overall strength of coalitionary support for individual *i* was equal to the average coalitionary support between *i* and every other teenager (in matrix terms it represents the average of all the values in the *i*th row). The other measure represents the average strength of perceived coalitionary strength within the cricket team (see

below), then named as “team bonding”, and calculated in a similar way but considering only team mates.

During the same summer a cricket match for teenagers of the same village was organized. Seventeen of the teenagers considered in the pile sorts participated to the match and divided themselves in two teams. The two teams were representing a particular hamlet of the village. Saliva samples from fifteen of the teenagers that played the cricket game were collected before and after the cricket game. Testosterone was analyzed by means of an enzymatic immunoassay (Salimetrics).

Kinship relationships between 31 of the 37 teenagers were obtained using Descent 0.2 (E. Hagen).

6.2.4 Children: Cricket game

During summer 2010 a within-village and a between-villages cricket matches were organized for children. Seventeen children (age ranging from 8 to 11) played the within-village game, while 12 children (ages ranging from 8 to 13) played the between-village game. The games were played in two different days. The within-village game was not terminated because of thunder storms, therefore the two games cannot be compared in terms of competition outcome. Both games were played in the early afternoon (2 pm c.a.).

Two salivary samples were collected by passive drool (via use of a straw) into a 5ml polypropylene centrifuge tube without sodium azide, one sample before and one after the game. Free testosterone (T), androstenedione (A) and cortisol (CORT) were quantified via an enzyme immunoassay (Salimetrics) according to manufacturer’s instructions. For the hormonal analysis, the salivary samples of only 11 players (the one that

played both games) were chose. All individual samples were run in duplicates with standards and high and low concentration controls (controls within acceptable ranges). All samples were run in a single assay, with the intra-assay coefficient less that 5% for all hormones.

6.3 Statistical analysis

6.3.1 Teenagers

For teenagers, testosterone and cortisol were distributed with a skeweness of 1.85 and 1.93 respectively and a kurtosis of 3.81 and 3.50. Since cricket requires short bursts of activity (both for batters-runners and for fielders), and acute exercise typically results in transient increases in testosterone level (Di Luigi et al. 2006; Edwards et al. 2006; Thomas et al. 2009), I examined if T and CORT levels increased after the end of the competition, independently of the outcome, by means of a Wilcoxon signed rank sum test (SAS) using the overall sample. Changes in T and CORT levels across events (pre- and post-competition) were examined within individuals by means of Wilcoxon signed rank sum test. Percent change in testosterone and cortisol levels across events (pre- and post-competition) were examined between winners and losers by means of Wilcoxon sum rank test using proc npar1way in SAS (Wilcoxon exact test). Non parametric correlation (based on permutations using Ucinet VI) was used to study the relationship between coalitionary support and kinship. Spearman ρ was used to study the correlation between the hormonal measures and individual level of team bond.

6.3.2 Children

Nine out of 38 samples (23%) for androstenedione and 4 out 40 (10%) samples for testosterone were below the sensitivity of the assay. Because of the already small sample size, these samples were assigned a value of 4.9 pg/ml for androstenedione and 0.9 pg/ml for testosterone. These values are just below the detection limit of the assay. This method has been used by others (Decker 2006).

For children, androstenedione, testosterone and cortisol were not normally distributed. Distribution skeweness for androstenedione, testosterone and cortisol were 1.02, 3.20 and 0.79 respectively. Kurtosis for androstenedione, testosterone and cortisol were -0.07, 14.60 and -0.29. Wilcoxon signed rank sum test was used to examine the effect of exercise on testosterone and androstenedione changes. To test the effect of the within- vs between- village competition, Friedman test was run using proc freq in SAS. Where the Friedman statistic was significant, multiple comparison analysis with relative pair-wise adjustment was used, following Ipe (1987). Specifically, after having found a statistically significant result with Friedman test, using the ranks for the hormone within each subject, a GLM analysis with LS means was run in SAS. Although Ipe did not adjust for the type 1 error, I used Tukey's adjustment for the multiple comparisons.

6.4 Results

The game did not elicit a significant change in testosterone for teenagers ($S=3.5$, $p>0.10$ for T and $S=24$, $p>0.10$ for CORT). Teenage males did not elevate testosterone after defeating a team from their community, but slightly decreased them ($S=-13$, $n=8$, $p=0.07$; fig. 20). But unlike predicted,

testosterone levels were slightly elevated in males on the defeated team compared to baseline (S=8.5, n=6, p=0.09; Figure 21). This trend was further supported by the fact that losers had a significant percent increase of T compared to winners (S=69, p<0.01; Figure 22). CORT did not changed in the winners (S=3, p>0.10; Figure 23), but was slightly elevated in losers (S=8.5, n=6, p=0.09; Figure 24). Percent change of CORT was not different between winners and losers (S=54, p>0.10; Figure 25).

The level of coalitonary support was positively correlated with kinship (see table 21 and figure 26). Furthermore, pre-match testosterone levels were highly and negatively correlated with team bond (rho=-0.79, N=14, p<0.001; Figure 27) while post-match were not (rho=-0.02, N=14, p>0.10; Figure 28). A trend towards a similar and statistically significant correlation was present for pre-match cortisol levels (rho=-0.43, N=15, p=0.10; Figure 29).

For children, testosterone was the only hormone significantly affected by the cricket games. Friedman test resulted significant (χ^2=8.06, df=3, p<0.05). Post-hoc comparisons showed that testosterone collected before the within-village game was lower than testosterone collected after both games (p=0.08 for the before-after within-village game, p<0.05 for the before within-after between village game; figure 30). Androstenedione and cortisol were not affected by the cricket games (figures 31 and 32).

6.5 Discussion

In this study I show that testosterone and cortisol are sensitive to the social context within which the competition is carried out. This reactivity seems to begin early in development, since young children were showing some slight change in T levels depending on the game played. Moreover, the level of

social support within the team may play an important role in modulating T and cortisol levels during social challenges.

I first describe the weaknesses of the analysis and then, after accounting for them, I will propose a plausible, although speculative, psychobiological explanation of the effects observed. Clearly the sample size is very small, making impossible to analyze the data in detail. Among the many factors that could have affected the hormonal output there are pubertal stage, measures of growth (% of muscles), personality and rating of players' competitive efforts, all variables that have been shown to affect testosterone reactivity in sport context (Di Luigi et al., 2006; Salvador, 2005). Keeping this in mind, the following is an analysis of some potential explanation of the results.

It is well accepted that exercise induces increases of T in children (Di Luigi et al., 2006; Thomas et al., 2009). Based on the fact that cricket is a sport that requires short burst of activity (both for runners and for fielders) a rise of T would be expected if the changes in T levels were reflecting exercise effort. I did not find an overall difference between pre and post game T levels for teenagers, but there was a trend for younger kids. Specifically for the teenagers, were I was able to compare the effect of winning on T, the two teams showed two opposite trajectories that might reflect cognitive reactions (i.e. the effect of winning and losing) to the match outcome. The fact that there were not significant differences in percent change of cortisol between winners and losers seems to support this point, while excluding the physical effort option.

Winners did not show significant changes while losers had significant increases of T levels after the match. The lack of an increase of T in winners could reflect its modualtion by the within group social context. In contrast, the increased T levels of the loser team would not fit the ingroup/outgroup modulation of the neuroendocrine system. One appealing and yet speculative scenario is that during adolescence the striving for dominance within someone's own community may be higher than for adults.

Dominance in adults could be expected as less susceptible to changes because based on the results of socio-competitive events dating back to the young-adulthood period. If this was to be the case, in teenagers a within-group competition could represents a good opportunity to determine someone's dominance and an increase in T levels after a defeat could translate in the willing to compete again (Mehta and Joseph, 2006). Partially supporting this view is the following description of what happened during the day of the game.

The cricket tournament was organized as a robin round league in such a way to have between and within villages games. Game one was played by the two within-village teams. The loser team was then going to play against the outside village team. No matter the outcome of this game, the outside village team was then going to play against the winner of game number one. The winner team of this last game was then becoming the champion. Things changed considerably instead. In game two, the loser team from game one (team F) won against the outsiders, opening a "crisis": they wanted to change the rules so to play now a final game against their co-villagers winners (Team S).

Since the majority of the adults present at the game supported this new point of view, a “revenge” game was played, which it concluded with the losers of the first game winning the final and becoming the actual champions with great disappointment from the other teams… Unfortunately I was unable to collect saliva samples during these events because of many constraints that become evident in naturalistic settings.

The results for the young children seem to be less clear. Cortisol and androstenedione were not associated with any kind of game, but as already mentioned, T was lower before the beginning of the within village game that compared to the end of the within and between village challenges. If this was caused by an increased physical effort is not clear, although if this were to be the case, cortisol may have followed the same pattern. Cortisol usually increases after acute physical exercise in adolescents, but data on children a lacking (Budde et al., 2010). As for cortisol, data on androstenedione and physical exercise in children were not found. Nevertheless, androstenedione was associated with aggression in preschool children (Azurmendi et al., 2006).

In general, high levels of testosterone are often associated with status seeking, antisocial behavior and decreased generosity (Archer, 2006; Zak et a., 2009) while low levels with affiliative behaviors. It follows that low T levels seems to be advantageous in group compared to individual competition since they would moderate cooperation between team-mates and decrease personal dominance seeking (Mehta et al., 2009). In my opinion, the cognitive aspects that interact with the neuroendocrine mechanisms in response to inter-group coalitional competition and in-group social relationships require a more complex analysis. On one hand, T levels during group competition are

subjected to be cognitively modulated depending on the relationship with the competitors. In these circumstances perhaps T enable physiological and psychological responses for fighting, but also for the comradeship among a victorious band of brothers (Flinn et al., 2011).

Therefore, T likely interacts with other neuroendocrine systems (oxytocinergic, vasopressinergic, serotoninergic) so to modulate the long-term effects of the social interaction beyond, for example, the end of the challenge. This last point seems to be very important since it requires the need to explain how extreme burst of violence during inter-group competition is accompanied by extreme acts of altruism and social bonding within the group.

Scoring high in coalitionary support within someone's own team was highly associated with the levels of T at the beginning of the game, so that who is expected to have higher support from teammates had the lowest levels of pre –match testosterone.

This relationship between coalitional support from team-mates and levels of pre-match testosterone is interesting and it finds support in literature (Kivligan et al., 2005). Our measure of coalitional support may indicate several potential characteristics of the subject: social dominance, social competence, prosocial and cooperative behaviors toward friends, and/or being part of a larger kin network. Indeed, the measure of coalitional support was associated with level of kinship.

High ratings may indicate that an individual is considered an important and valuable key player in his social network. This could provide a sense of security in stressful situations, i.e. competition. A trend toward a similar and statistically significant correlation was present for pre-match cortisol levels

(rho=-0.43, p=0.10). Alternatively, individuals that score higher in dominance may not perceive within group challenges as important, and this could be reflected by the lower anticipatory T levels. Nonetheless, it must be reported that one study looking at a similar relationship between teammates found the opposite relationship between T and level of team social support (Edwards et al., 2006), although the measure of “social connectedness” was based on self-report.

In conclusion, at present, it is not possible to know if the hypothesized neuroendocrinological mechanisms that regulate adult ingroup-outgroup behaviors are at work in youngster. In this work, it seems that T is somewhat regulated by the social nature of the context probably also in young children.

Table 21. QAP Correlation between kinship and coalitionary support

	Obs Value	Signif.	Prop >= Obs	Prop <= Obs
Pearson Correlation	**0.260**	0.000	0.000	1.000

Number of permutation =5000

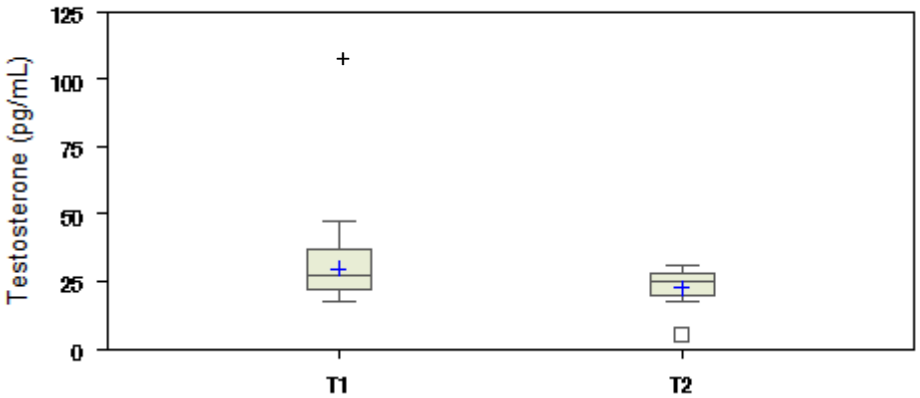


Figure 20. Testosterone levels of teenagers on victorious team. Winning against a team of friends is not associated with increased testosterone levels in young teenagers. Instead a slight decrease in T levels was found after the end of the game (T1 = immediately before the match; T2 = 10 minutes after the match). $^{+}$ $p<0.10$

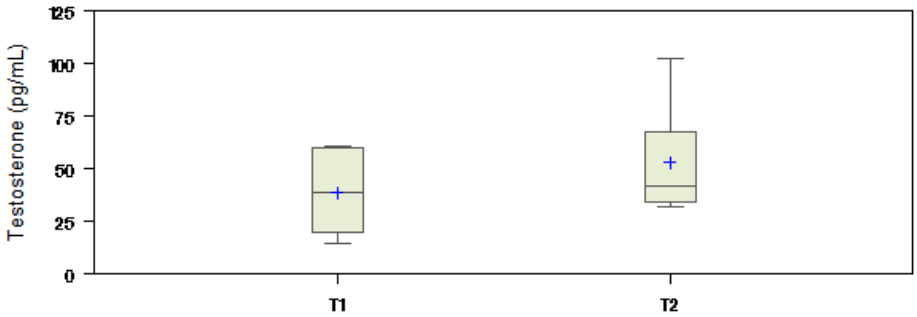


Figure 21. Testosterone levels of teenagers on defeated team.
Losing against a team of friends was associated with increased testosterone levels in young teenagers (S=12, n=6, p=0.09).

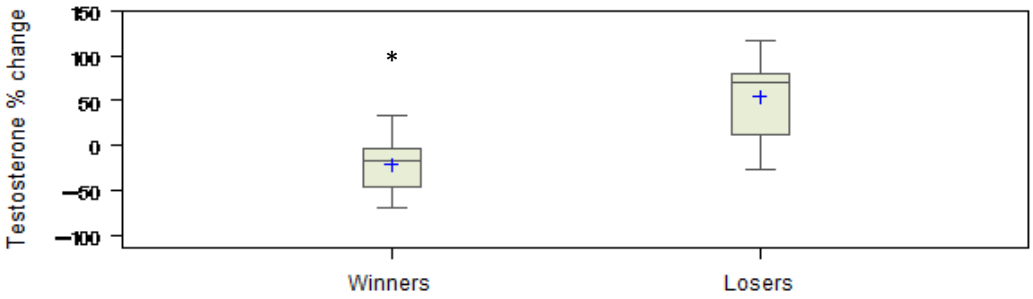


Fig 22. Percent change in testosterone at the end of the teenagers cricket competition. Losers showed a statistically significant increase in T levels compared to winners. * p<0.05

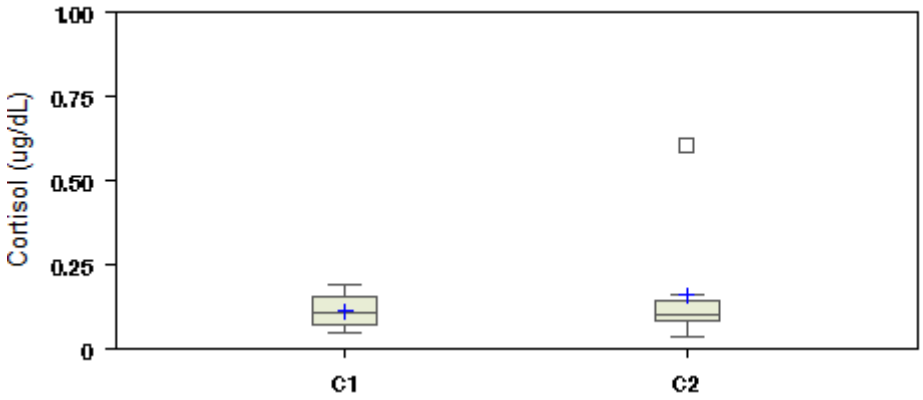


Fig 23. Cortisol levels in teenager winners. Winning against a team of friends is not associated with increased cortisol levels in teenagers (S=3,n=8, p>0.10). C1 = immediately before the match; C2 = 10 minutes after the match.

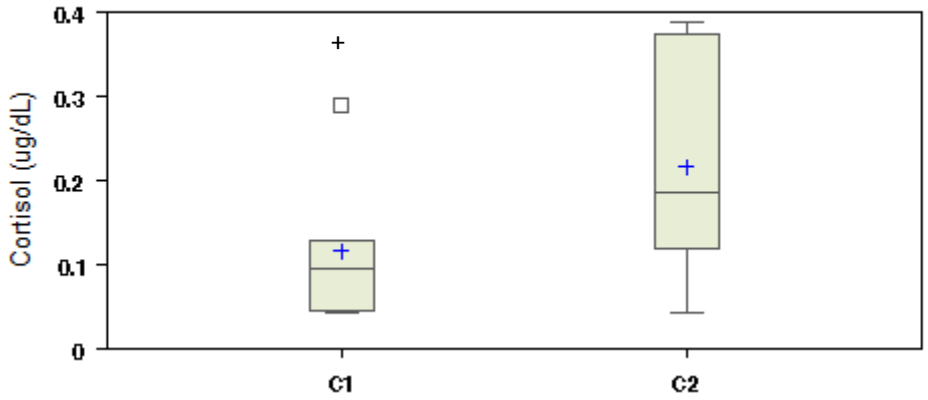


Fig 24.Cortisol levels in teenager losers. Loosing against a team of friends slightly increased cortisol levels in teenagers (C1 = immediately before the match, C2 = 10 minutes after the match). $^{+}$ p<0.10

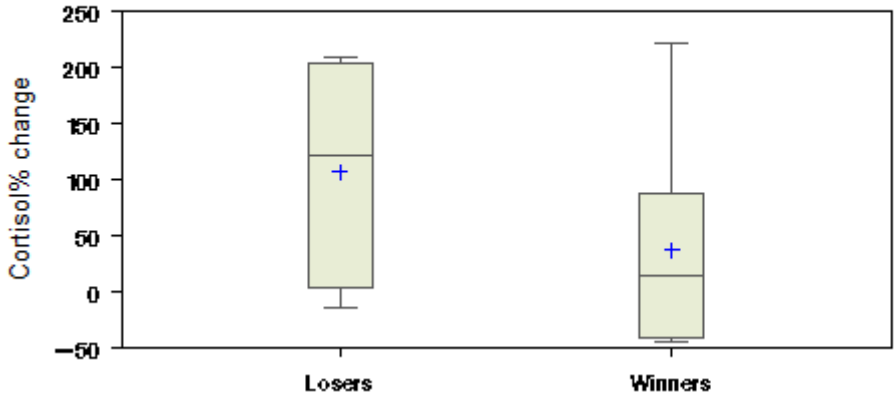


Fig 25. Percent change in teenagers' cortisol. Overall, no differences were found in percent change of cortisol between the two teams.

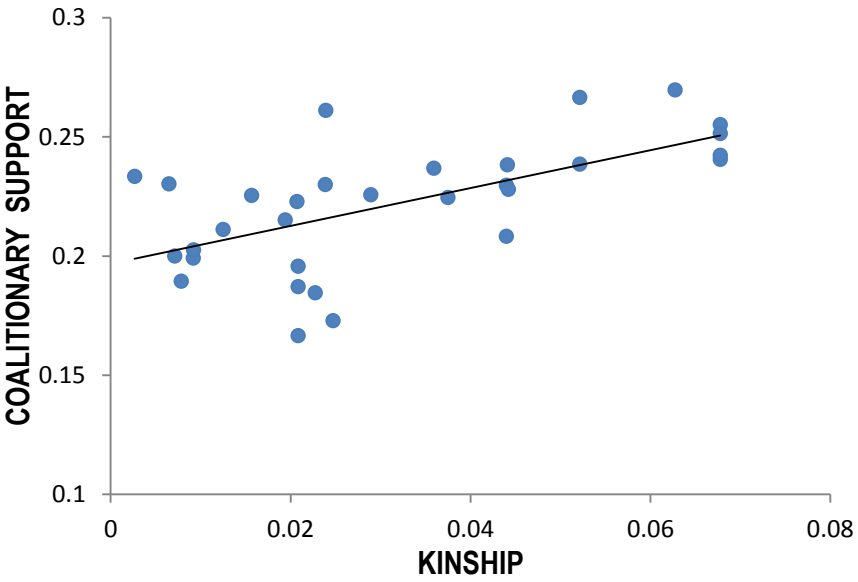


Fig. 26. Relationship between kinship and coalitionary support. The perception (of the informants) of who will support who in case of a fight increases with increasing kinship.

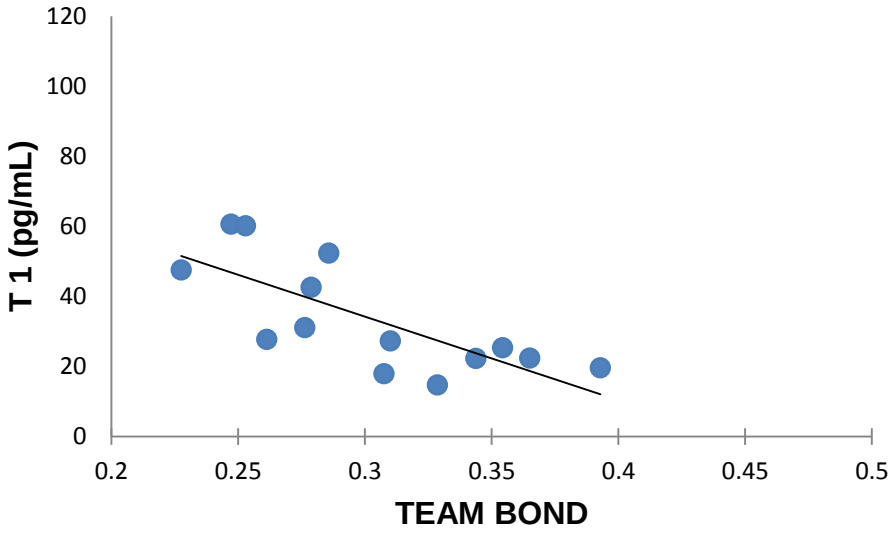


Fig. 27. Relationship between pre-match testosterone levels and coal are associated with perceived levels of coalitionary support within own team (rho=-0.77, N=14, p<0.01).

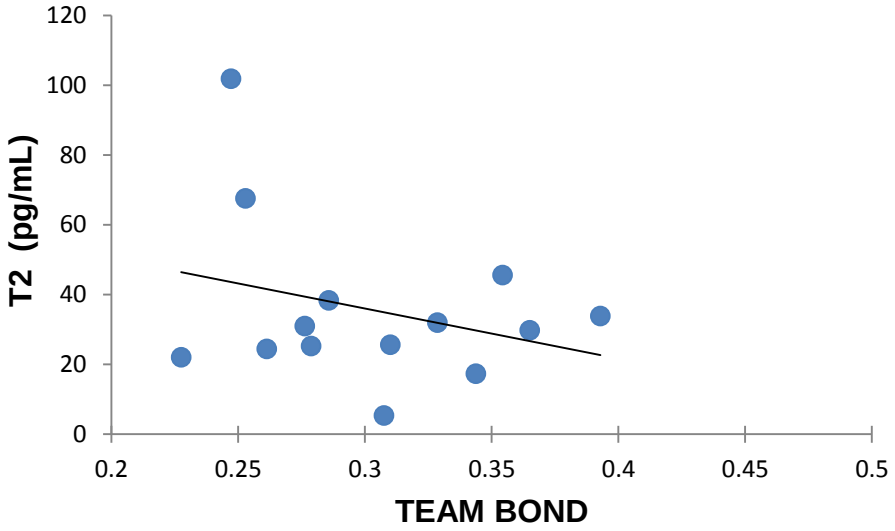


Fig 28. Relationship between post-match testosterone levels and perceived levels of coalitionary support within own team (rho=-0.02, N=14, p>0.10).

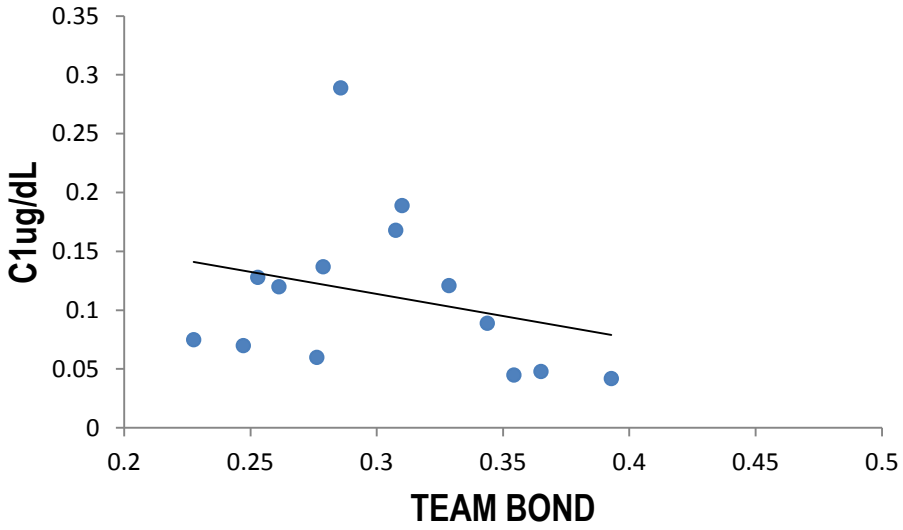


Fig. 29. Relationship between pre-match cortisol levels and perceived levels of coalitionary support within own team (rho=-0.43, N=15, p=0.10)

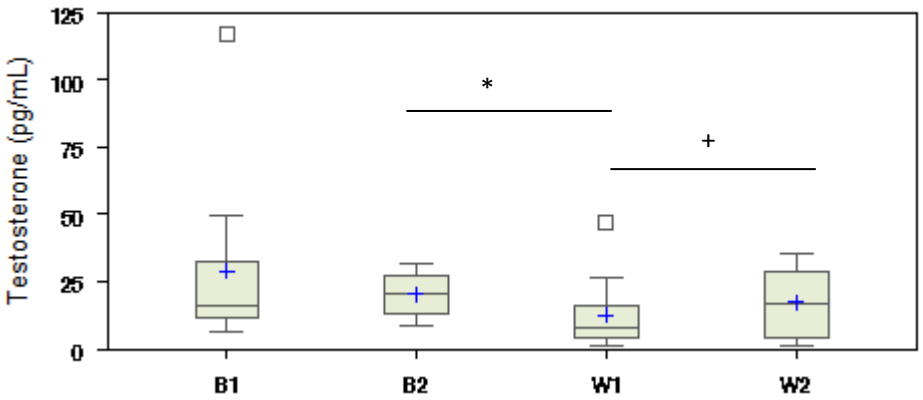


Fig 30. Children's testosterone levels during within village (baseline= W1 and post game = W2) and between village (baseline =B1 and post game = B2) cricket games. * $p<0.05$, $^{+}$ $p< 0.10$

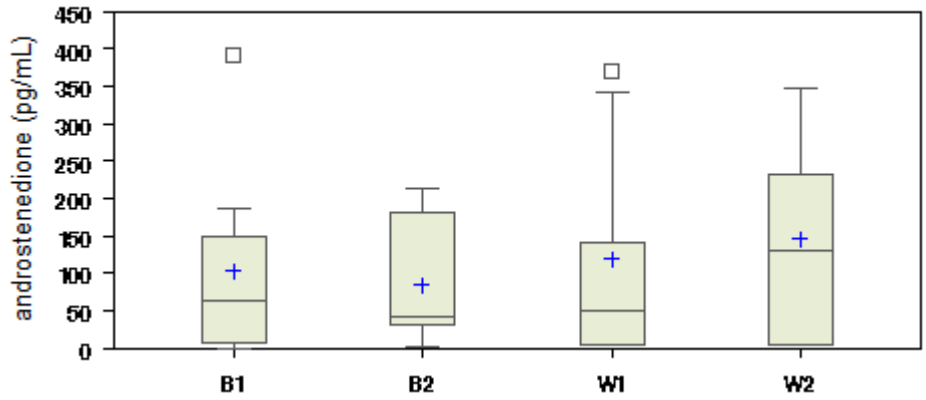


Fig 31. Children's androstenedione levels during within village (baseline= W1 and post game = W2) and between village (baseline =B1 and post game = B2) cricket games.

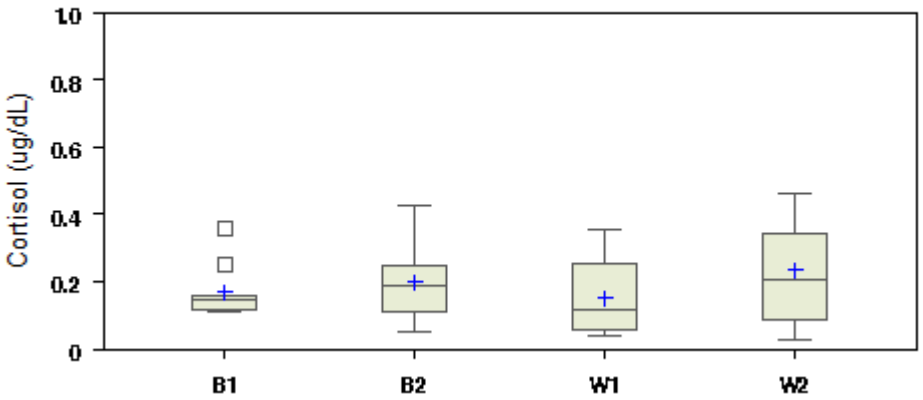


Fig 32. Children's cortisol levels during within village (baseline= W1 and post game = W2) and between village (baseline =B1 and post game = B2) cricket games.

Chapter 7

General discussion

In this dissertation I examined several aspects of the development of children' social behaviors and their relationships with the neuroendocrine system. My goal was to study the biological mechanisms underlying the extreme sensitivity of children to the social environment using an evolutionary framework. In order to fulfill the aim, I tested hypotheses in relation to the regulation of the stress system by age, sex and maternal investment. I also tested the hypothesis that sex differences in behavior and triadic awareness during mid-childhood are associated with levels of adrenal androgens. Finally, I presented some preliminary results on the effects of coalitional competition during childhood and adolescence.

The results demonstrated that pre-pubertal children mount a clear stress reaction to potentially socially stressful events, the children from the village studied showed a sexually segregated peer network and that their distribution of friendship ties is associated with age and kinship. Lastly, the result showed that teenagers and young children may differ in relation to how they react physiologically to group based competitions.

A first glance, the interdisciplinary approach I used for this work may appear for some presumptuous. In my opinion, instead, I shed light on the usefulness and need to unify methodologies from sociology, psychology, cultural and evolutionary anthropology and neuroendocrinology to study the development of human behavior and its related health issues.

Taken as a whole, this report underscores the importance of conducting longitudinal naturalistic studies. And of course, many if not all scientists

working in the field will agree. As it was shown in chapter three, the ability to identify even subtle effects on the HPA axis are amplified by the possibility to collect multiple samples from the same individuals across multiple years. In this way, factors that affect between and within individual variability can be identified and controlled for. Moreover, in a longitudinal setting, the subjects become familiar with the researchers and the methodology. Clearly this is an aspect of extreme importance when studying the stress system.

My opinion is that studies conducted in laboratory have the disadvantage that the "baseline" samples may already be burst up. For example, Gunnar et al. (2009) pointed out that laboratory protocols for the study of human stress reactivity that works for adults are obviously not adapted for children (for example, ethical reasons). They highlight the fact that children may not (or cannot) go over the same, long process of acclimatization to the laboratory procedure as adults do. Some researchers try to overcome this problem having children collecting several samples in their own home, controlling therefore for the novelty of the laboratory setting. This may be an approach that may work, but unfortunately more samples are collected, higher will be the cost the experiment, a problem that is exacerbated in longitudinal naturalistic settings.

A less obvious advantage of a longitudinal study is the one of "getting to know" the culture of the population under study. And here I want to stress the importance of using ethnography and cultural anthropology for this purpose. Once again, this is nothing new, at least in the study of developmental psychology (Pellegrini and Bjorklung 1991), but often overlooked.

The importance of framing studies on the development of the stress response within an evolutionary, socio-ecological context was pointed out in chapter four. In humans, this may require weighting the effects of cultural change on the embedded biology of an individual, focusing on the development of life history strategies, but, evidently, not forgetting phylogenetic constraints. It was shown that this approach may be valuable in order to test predictions of the HPA activity.

Sex differences in relation to behavior and cognition are present in humans (Geary 2010; Hines 2011). Many of these differences appear early in life and become more evident with the process of development (Geary 2010; Rose and Rudolph 2006). Hormonal and cultural factors play an important role in the expression of these differences (Geary 2010; Hines 2011; Lancy and Grove 2011). Prenatal androgens are fundamental in the dimorphic organization of the nervous system (Hines 2011). In this work, the hypothesis that DHEA could activate sex differences in peer network was refused. Knowing that sex differences in group size and sex segregation begin approximately when DHEA levels are low (at around three years of age), it was suggested that a longitudinal approach of hormonal and social network analysis should begin at around the age of three.

Based on my knowledge, the last empirical chapter of this dissertation is the first work that tried to study the development of the hormonal system that could modulates the aspects of social categorization during competitive challenges. Although it can be considered a pilot study that will be extended in the future, it showed that during mid-childhood, kids may have a functional feedback process between the social context within which they compete and

the neuroendocrine system. With no doubt, for teenagers being embedded within a group of kin has evident effects on the HPA and HPG axis during competition. What does it means in terms of behavioral and cognitive processes is a question that will need to be approached soon in the future.

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Appendix

Chapter 2.

Equation *(1)*

Level 1 $CORTsdi = \pi 0di + \varepsilon sdi \quad \varepsilon sdi \sim N(0, \sigma^2)$

Level 2 $\pi 0di = \beta 00i + \mu 0di \quad \mu 0di \sim N(0, \tau_{\mu 0}^2)$

Level 3 $\beta 00i = \gamma 000 + v00i \quad v00i \sim N(0, \tau_{v0}^2)$

where s = sample, d = day and i = subject

Equation (2)

$ICC_{L2} = (\tau_{\mu 0}^2 + \tau_{v0}^2) / (\tau_{\mu 0}^2 + \tau_{v0}^2 + \sigma^2_{\varepsilon})$, this is the intra-class correlation coefficient for the sample nested within day. It expresses how correlated are two samples collected within a day (and within child).

Equation (3)

$ICC_{L3} = (\tau_{v0}^2) / (\tau_{\mu 0}^2 + \tau_{v0}^2 + \sigma^2_{\varepsilon})$, this is the intra-class correlation coefficient for the sample within subject. It expresses the correlation between two samples collected from the same child but, in a different fashion from the previous one, far apart.

Chapter 4

Multilevel linear models comparisons

ML Deviance test-Model comparison : Cortisol

Model	Δ-2LL	Δ#parm	p
1 vs 2	67.3	1	p<0.01
3 vs 2	2.2	1	p>0.10
3 vs 4	0.6	2	p>0.10
3 vs 5	3.6	1	p<0.10
5 vs 6	12.4	4	p<0.05
6 vs 7	3.7	1	p<0.10

Model 7 was chosen

ML Deviance test-Model comparison : DHEA

Model	Δ-2LL	Δ#parm	p
1 vs 2	8.2	1	p<0.01
3 vs 2	19.3	4	p<0.01
2 vs 4	18.6	2	p<0.01
3 vs 4	0.7	2	p>0.10
4 vs 5	3.8	1	p<0.10
5 vs 6	9.9	3	p<0.05
7 vs 6	3.7	1	p<0.10
6 vs 8	0.1	1	p>010

Model 6 was chosen

VITA

Davide Ponzi was born in Parma (Italy) on January 8, 1977 from Pierino Ponzi and Zaira Landini. He has lived in Parma until 2004, when he came to Columbia Missouri for the first time. Since then he has been living in Columbia, where in 2005 he begun to attend graduate school at the University of Missouri. As an undergraduate, Davide studied and graduated in biology and pathophysiology from the University of Parma, Italy. He is married to Jessica Burroughs Garcia.