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Comparisons of the socio-behavioral differences between chimpanzees (Pan troglodytes) and bonobos (Pan paniscus), investigating the impact of a naturally occurring polymorphic microsatellite deletion in the 5' flanking region of arginine vasopressin receptor 1a (AVPR1a) on gene expression and sociality within the Pan genus

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Abstract

Among the great apes, chimpanzees are unique in having a polymorphic deletion of a ~350bp microsatellite containing region (DupB) in the 5' flanking region of the arginine vasopressin receptor 1a (AVPR1a) gene. This results in three genotypes (DupB+/+, DupB+/- and DupB-/-) of AVPR1a in chimpanzees. Variations in the length of microsatellites 5' of AVPR1a have been associated with social behaviors (pair-bonding, paternal care, degree of social interest) and differential levels of expression of AVPR1a in the brains of voles. The polymorphic DupB microsatellite in chimpanzees allows the investigation of microsatellite variation 5' of AVPR1a in higher order primates. We hypothesized that chimpanzees lacking the DupB microsatellite would spend more time alone (in the absence of conspecific social partners) compared to chimpanzees that retain the ancestral genotype. Additionally we collected identical behavioral data on bonobos, who are not polymorphic for the deletion of this microsatellite in order to make comparisons within the Pan genus on sociality and the impact of a polymorphic deletion of the DupB microsatellite. Finally we conduced luciferase reporter assays in order to investigate the impact of the DupB microsatellite on gene expression. These data indicate that bonobos spend more time close to conspecifics and spend more time grooming, compared to chimpanzees. With respect to the DupB deletion and sociality, these data indicate that chimpanzees with a complete deletion of DupB are more likely to spend time "alone" than DupB+ individuals or bonobos.

Chapter 1: Introduction and background

1.1 Chimpanzees and bonobos

The evolutionary significance of sociality can be seen in a variety of species, where variable patterns of social behaviors have been selected for their ability to improve fitness. The adaptive value of increased sociality can be observed among colonial invertebrates, social insects, mammals, non-human primates and even our own species (Wilson 2000). Although living in social groups often requires considerable input of energy to maintain relationships within the group, the benefits of avoiding predation through group defense and aggregation along with collaborative group foraging and access to mates seem to outweigh the costs associated with increased group size. Adaptations for living in increasingly large social groups have been selected for as species evolve patterns of sociality that best match their available resources, the abundance of those resources, the competition for those resources, and predation while acquiring those resources- in short, their habitat-specific ecological conditions (Boyd & Richerson, 2009). Among humans and non-human primates, the ability to maintain social relationships among conspecifics to facilitate the existence of large social groups has evolved in many different forms. Human's closest relatives, chimpanzees and bonobos, are an excellent example of the ability of natural selection to favor differential socio-behavioral patterns between closely related, reproductively isolated species. Chimpanzees and bonobos are members of the genus *Pan*, sharing a common ancestor with humans roughly 6 million years ago (MYA). Chimpanzees and bonobos subsequently diverged from a common ancestor as recently as 1 MYA (Becquet *et al.* 2007; Hey 2010). This

relatively short period of time from divergence is reflected in the physiological similarities between the two species along with comparable components of social behavior (Prüfer *et al.*, 2012). In regard to physical appearance, chimpanzees and bonobos are so similar that they were not even identified as individual species until the early 1930's (de Waal, 1988).

Although they share many aspects of appearance and there is considerable overlap, bonobos are generally smaller and more gracile than their larger more robust chimpanzee counterparts (Goodall, 1986). Despite striking similarities in anatomy and physiology, in terms of social interactions and general aspects of behavior chimpanzees and bonobos have less in common. Strong male-male bonds characterize chimpanzee societies, with males usually holding a higher status in the group than females. In sharp contrast, bonobo societies are matriarchal, with closely bonded, often-related females holding higher hierarchal status than males (Boesch *et al*., 2002). The two *Pan* species also differ in their levels of violence; chimpanzees use aggressive interactions to reinforce the hierarchy among each other, and male chimpanzees will often form parties to patrol the boundaries of their territory (Goodall, 1986). During these patrols they may attack members from other groups along with hunting small monkeys and other mammals (de Waal, 1988). Intergroup aggression has rarely been observed in bonobos, and although they are omnivorous and will consume animal tissue, hunting is much less frequent among bonobos (Surbeck *et al*., 2009). Bonobos and chimpanzees both participate in grooming of conspecifics to reinforce social bonds, but among bonobos sexual interactions are common and occur between sexes indiscriminately. These frequent sexual

interactions are hypothesized to reduce stress and ease conflict within the group (Boesch *et al*., 2002).

The dramatic social differences between members of the *Pan* genus are hypothesized to be explained, at least in part, by the different habitats occupied by chimpanzees and bonobos. Both species are native to sub-Saharan Africa, but while chimpanzees live in a variety of habitats across Africa, bonobos are restricted in their range to a region of jungle on the south bank of the Congo River. Bonobos live allopatrically from chimpanzees and gorillas in this relatively small, densely forested area within the Democratic Republic of Congo (DRC) (Wrangham *et al*., 1996; Yamagiwa, 1999; Yamakoshi, 2004). Within this confined range, there are swampy habitats accessible to bonobos, but the majority of their time is spent in the drier primary and secondary forest (Hashimoto *et al*., 1998). In contrast to the restricted range of bonobos, chimpanzees have been able to occupy a diverse array of habitat types extending from dry, grassy savannah in the Western extremes of their range to dense rainforest near the equator (Goodall, 1986). In addition to the variety of habitats chimpanzees occupy compared to bonobos, they also live sympatrically with gorillas in large portions of their habitat (Stanford and Nkurunungi, 2003; Head *et al.*, 2011). The separation of chimpanzee and bonobo ranges by the Congo River along with bonobos existence allopatric to any of the great apes species are hypothesized as potential driving factors in the differential evolutionary trajectories between chimpanzees and bonobos (Tutin *et al.,* 1991; Malenky & Wrangham, 1994). The isolation of bonobos in a relatively homogenous habitat allopatric from any other great ape species may explain the lack of

specialization observed in bonobos compared to chimpanzees and gorillas. Chimpanzees and bonobos both rely on ripe fruit as a staple of their diet. Gorillas, however, consume large amounts of terrestrial herbaceous vegetation (THV) to support their large body size (Wrangham & Rubenstein, 1986). Although ripe fruit is a major part of bonobos' diets, THV consumption appears to constitute a greater proportion of their total diet (33%) compared to chimpanzees' use of THV (7%) (Wrangham & Rubenstein, 1986). Chimpanzees mostly rely on THV during times of fruit scarcity, but bonobos have been found to consume THV at a relatively constant rate throughout the year (Chapman *et al*., 1994). The law of competitive exclusion states that two species competing for the same limiting resource cannot coexist, and this may explain the dietary specialization observed in chimpanzees and gorillas and lack of specialization for either THV or ripe fruit by bonobos. In short, chimpanzees have faced selective pressures to specialize their diet whereas bonobos have not. The selective pressures to coexist with gorillas and adapt to a greater variety of habitats faced by chimpanzees compared to bonobos have likely also selected for the differential socio-behavioral patterns between the two species.

The ability to make direct comparisons between two so closely related, but socially distinct species provides an excellent opportunity to investigate the ecological and genetic factors responsible for species-specific socio-behavioral patterns. These two species, in particular, provide a unique opportunity to not only investigate the evolutionary origins of sociality, but also the evolution of social behavior in our own species. Unlike other animal models that are often used to investigate the genetic underpinnings of social behavior, such as rodents, the shared

phylogenetic history of chimpanzees, bonobos, and humans make them particularly relevant to understanding the evolutionary history of sociality in humans.

Although comparative research on chimpanzees and bonobos has only been conducted for a relatively short period of time and the nuances of the differences in social behaviors between the two are not yet fully understood, it is widely accepted that there are divergent social strategies between the two species (Goodall, 1986; de Waal, 1988; Tutin *et al.,* 1991; Malenky & Wrangham, 1994). The existence of two distinct patterns of social interactions between closely related, recently diverged, socially complex apes begs several questions. For example, what ecological factors led to this divergence, and how do these different social strategies reflect the ecological challenges faced by individuals? How did these differential behavioral phenotypes evolve over such short evolutionary time? What can we learn about our own ancestors from studying these different adaptations seen in our closest living relatives?

1.2 Genes to behavior

Prior to the genetic revolution during the early 21st century, observational data on behavior had been the primary tool for comparing social behaviors between species. With the increasing availability of genetic tools, however, we are now able to combine observational data on behavior with genetic analysis of individual subjects. The integration of these two disciplines presents the opportunity to tease out the genetic underpinnings of behavior. The molecular basis of behavioral

genetics has primarily been examined through the use of model organisms such as fruit flies and rodents. While valuable insights have been gained through genetic manipulations and knock-out/knock-in experiments in typical model organisms, there is a less-invasive side of behavioral-genetics. The ability to extract DNA using minimally invasive procedures now allows for the integration of behavioral observations with individual genetic analysis in an attempt to better understand the genetic foundations of complex behaviors.

Social cognition refers to the neurocognitive ability to perceive, process and interpret social information (Henry *et al*., 2016). Therefore, social cognition is the functional ability of an individual to react with the appropriate socio-behavioral response when confronted with a social stimulus.

To begin to unravel the genetic underpinnings of social behavior and social cognition, we must first direct our attention to the potential genes that could exert an influence over the development of social cognition. The molecular basis of social cognition in animal models has been associated with two neuropeptides: arginine vasopressin (AVP) and oxytocin (OT). Oxytocin is well known for its central role in maternal attachment and lactation in females. Vasopressin's peripheral role in the renal control of water balance has been well documented, but much less is known about vasopressin's central role in the regulation of social behaviors (Skuse & Gallagher, 2011). Recently there has been a growing interest in vasopressin and its relation to behavior from its role in the etiology of autism and altruistic tendencies to its role in dancing ability or male pair bonding abilities (Tansey *et al*., 2007; Ebstein *et al*., 2012; Bachner-Melman *et al*., 2005; Walum *et al*., 2008)

Given the conserved role of OT and AVP in regulating specific aspects of social behavior, they have been targeted by behavioral geneticists seeking to identify and implicate genetic factors associated with these neuropeptides to differential behavioral phenotypes. Particular emphasis has been given to identifying genetic factors that modify the expression of genes coding for receptors of these neuropeptides. It has been proposed that mutations to microsatellites could alter the expression level of specific neuropeptide receptors. These mutations may serve as an evolutionary "control knob" whereby mutations can tweak the expression level and patterns of neuropeptide receptors. These tweaks of neuropeptide receptors consequently modify patterns of social behaviors (King *et al*., 1997). Microsatellites in the non-coding region of these genes are of particular interest as they are much more susceptible to mutations that may modify expression of genes without the deleterious effects of modifications to the actual protein coding sequence of genes (Young & Hammock, 2007). Thus, natural polymorphisms in the non-coding region of genes involved in social cognition may create some degree of plasticity in behavioral phenotypes, especially when experiencing variations in ecological conditions that may select for different patterns of sociality.

Genetic tools such as the polymerase chain reaction (PCR) allow us to amplify specific regions of an individual's genome and identify polymorphic elements among genes involved in social cognition. *In vivo* experiments in rodents have subsequently been used to demonstrate that specific polymorphisms can

modify behavioral phenotypes through differential expression neuropeptide receptors (Hammock *et al*., 2005).

1.3 AVPR1a polymorphisms: From monogamy in voles; to sociality in chimpanzees; to developmental disorders in humans

An example of the utility of rodent research is the identification of a polymorphism in the non-coding region of the gene that codes for an arginine vasopressin (AVP) receptor in the *Microtus* rodent genus of voles. This polymorphism was implicated as having a direct impact on social behaviors in prairie voles (*Microtus ochrogaster*) (Hammock & Young, 2004; Hammock & Young, 2005; Hammock *et al*., 2005). The polymorphism they identified in the 5' flanking region of arginine vasopressin receptor 1a (AVPR1a), was shown to affect the sociability of voles with respect to their level of parental care, mate fidelity and degree of social interest, with an especially pronounced impact on male vole behavior (Hammock & Young, 2004; Hammock & Young, 2005). Young and colleagues have also shown, through *in vivo* experiments, that this regulatory polymorphism is associated with individual differences in patterns of central vasopressin receptor distribution (Hammock *et al*., 2005).

The identification of this polymorphism's ability to modify social behaviors in rodents sparked interest in identifying polymorphisms 5' of AVPR1a in humans to determine if there were similar effects on behavioral phenotypes. Subsequently, it has been shown that specific allele lengths of the RS3 allele in the DupB

microsatellite are associated with altruism, male pair bonding behaviors, incidence of autism, and amygdala activation in response to facial recognition task (Knafo *et al*., 2008; Walum *et al*., 2008; Meyer-Lindenberg *et al*., 2009; Tansey *et al*., 2011). This polymorphism of the RS3 allele length and its behavioral correlations in humans is similar to the polymorphism observed in voles. However, the polymorphism in voles is located at a different part of the 5' flanking region of AVPR1a and involves a complete deletion of a polymorphic microsatellite. Furthermore, the polymorphic microsatellite implicated in prairie vole sociality and the allele length differences shown to impact human sociality are not homologous at all. The microsatellite that has been implicated as having an effect on human behavior is a complex dinucleotide repeat called RS3 and is located ~3600bp from the transcription start site (TSS) of AVPR1a. The polymorphism in voles that modifies social behaviors is a tetranucleotide repeat and is located 553bp from the TSS of AVPR1a (Rosso *et al*., 2008). This is an example of the difficulties involved with the transition of rodent behavioral genetics to human and non-human primate behavioral genetics. Although it has been shown that microsatellite polymorphisms 5' of AVPR1a contribute to differential behavioral phenotypes in humans and voles, these microsatellites are not evolutionarily related. This is not surprising given the phylogenetic separation between humans and voles, and the highly mutable nature of microsatellites outside the coding region of genes (Young and Hammock, 2007). It should be noted that although there are significant differences in the 5' microsatellites of AVPR1a between humans and voles, the sequence of the actual

coding region of AVPR1a is highly conserved between not only humans and voles but also among most mammals (Hammock & Young, 2004).

The vasopressin neuropeptide itself has a conserved role in regulating social behaviors in mammals, and there are indications that microsatellites in the 5' non-coding region may alter individual or species level expression patterns of the receptor for this neuropeptide (Donaldson *et al*., 2005; Tansley *et al*., 2011). Thus, inter-species behavioral patterns may be modulated by the modified expression of vasopressin receptors across the brains of different species. While investigating the phylogenetic history of microsatellites 5' of AVPR1a, Donaldson identified a polymorphic deletion of a ~360bp in the 5' region of chimpanzees named as the DupB microsatellite. The DupB microsatellite contains the RS3 allele that was shown in humans to have an impact on sociality. Furthermore it was shown that chimpanzees are the only great apes that are polymorphic for a complete deletion of this microsatellite (Donaldson *et al*., 2008). It was also determined that among chimpanzee subspecies the DupB deletion is more common in chimpanzees of West African origin. Among captive chimpanzees, of West African origin ~70% have a complete deletion of the DupB microsatellite (DupB-/-) while genotyping of wild Eastern African chimpanzees indicates that ~62% retain the DupB+ microsatellite (Anestis *et al*., 2014). It is interesting to note that the habitat variation experienced by chimpanzees can often be associated with differences in behavior (Boesch, 1994). One behavior in particular that has been shown to vary between chimpanzees inhabiting different habitats is the likelihood of sharing meat or participating in cooperative hunting, which has been shown to be much more

common among forest chimpanzees compared to savanna-woodland chimpanzees (Boesch & Boesch, 1989). To this date the specific differences in behavior between chimpanzee subspecies are still largely speculative but the variation in behavior between chimpanzees inhabiting different habitats is likely related to the different ecological conditions present in those habitats. If the presence of the DupB microsatellite deletion is more common in some chimpanzee subspecies and there are observed behavioral differences between those species, modifications to the non-coding *cis* portion of genes involved in social cognition may have provided a substrate for rapid evolution of behavioral phenotypes to fit variations in ecological conditions (King *et al*., 1997; Young and Hammock, 2007).

In relation to the polymorphic deletion of AVPR1a there have been several attempts using a variety of methods to extrapolate if the polymorphic deletion of this microsatellite has an associated behavioral phenotype. The majority of these studies have relied on quantifying "personality styles" either through handler questionnaires or observational data (Anestis *et al*., 2012, Hopkins *et al*., 2012). Anestis *et al*. demonstrated an association between DupB+ genotype and a "smart" personality type, which was determined by those individuals as often using coalitions in aggressive encounters, receiving more grooming than they give, and being likely to initiate play successfully with peers (Anestis *et al*., 2014). Hopkins *et al*. was able to demonstrate a sex based difference in personality by showing that males with the DupB+ genotype had higher scores of "dominance" and lower scores of "conscientiousness". However, there was no difference in dominance and contentiousness found between males and females with the Dupb- genotype

(Hopkins *et al*., 2012). Staes *et al*. (2015) recently found that males with the homozygous DupB+/+ genotype were more "sociable" as determined by a personality analysis despite a small sample size of three DupB+/+ males (Staes *et al*., 2015). This adds support to the hypothesis that variation at this allele results in differential social behavior (Staes *et al*., 2014). In summary, the current literature suggests that this microsatellite likely exerts some control on socio-behavioral phenotypes, and it appears that this is a sexually dimorphic effect with male socio-behavioral phenotypes being more affected than females.

The most closely related evolutionary relatives of chimpanzees, humans and bonobos, are not polymorphic for the deletion of the DupB locus (Donaldson *et al*., 2008; Staes *et al*. 2014). The social behaviors of humans vary widely and are not easily comparable to those of chimpanzees, but chimpanzees and bonobos shared a common ancestor only 1-2 million years ago and share components of many social behaviors that can be compared directly (Prüfer *et al*., 2012). For this reason chimpanzees and bonobos present an excellent model to study the ability of microsatellites in the non-coding region of genes to alter socio-behavioral phenotypes. Comparisons between closely related, behaviorally similar species with divergent social behaviors will allow us to understand how variations in non-coding genetic elements can contribute to the rise of specific social behaviors. Additionally, the close evolutionary relationship chimpanzees and bonobos share with humans makes this research directly applicable to understanding the genetic factors underlying differential socio-behavioral phenotypes in humans.

Chapter 2: Sociality of chimpanzees and bonobos

2.1 Introduction

We sought to collect behavioral data that would allow us to compare "sociality" within the *Pan* genus. In order to compare the level of sociality between species we chose to collect data on social proximity and grooming. Social proximity was chosen as a measure of sociality because how close an individual chooses to be to his/her conspecifics is a very straightforward measure of how social an individual is with conspecifics (Sibbald *et al*., 2005). The space between individuals has been shown as a method to describe sociality, in which more social individuals are those that spend more time in close social proximity to conspecifics (Hediger, 1950; Heidger 1963). Following Sibbald (2005) we collected data on nearest neighbor distances during our focal follows in order to infer sociality. We created four categories of social proximity so that we could determine how much time is spent at varying social distances (see table 1 for details). Collecting data on the nearest neighbor within four discrete categories of social proximity allowed us to determine if there was a difference between chimpanzee and bonobo preferences for the distance to the closest conspecific. Previous studies have compared proximity of great apes by collecting data on proximity in larger categories (alone vs. close), and the breakdown of social proximity into four categories allowed us to determine if there is a specific distance where chimpanzee and bonobo social tolerance differ (Milne, 2015).

In addition to social proximity, during focal follows we collected data on any grooming in which the focal individual participated. Grooming in *Pan* has been

studied intensely and is considered a major social component in both chimpanzee and bonobo societies (Muroyama & Sugiyama, 1994). Grooming has been associated with reconciliation behaviors and is hypothesized to play a part in maintaining long-term social relationships between individuals (de Waal, 2001). Little data exist, however, making direct comparisons of grooming behaviors between chimpanzees and bonobos. In their 1994 book Chimpanzee Cultures, Muroyama and Sugiyama evaluated grooming rates as they differ between sexes based on the combination of several studies conducted at field sites in Africa. This analysis revealed that male-male grooming interactions are the most common among chimpanzees and that male and female grooming interactions were the most common among bonobos (Muroyama & Sugiyama, 1994). Given the paucity of data comparing captive chimpanzees and bonobos, and scarcity of data comparing grooming in captive apes, our study provides a simple but important step to understanding how grooming activities differ between captive chimpanzees and bonobos.

Quantifying these two components of sociality permit comparisons of the amount of time chimpanzees and bonobos spend at variable social distances and comparisons of the time spent engaged in grooming behaviors. Most comparisons that have been made between the social behaviors of chimpanzees and bonobos have been based on independent studies of the two species in the wild (Muroyama & Sugiyama, 1994). Making comparisons based on data collected on wild apes is crucial to understanding behavioral variation in natural ecological conditions. Captive studies, however, allow data collection without the variability of ecological conditions. In order to directly compare patterns of sociality between chimpanzees

and bonobos, it is necessary to collect data from similar habitats. In captivity the variation among resource availability, group sizes, group composition, and range size can be controlled. The minimal variability in captive ape management between zoos creates the ability to make behavioral comparisons between apes inhabiting similar habitats, removing potentially confounding ecological factors. As discussed previously, chimpanzees and bonobos inhabit a variety of habitats in the wild, thus captive studies are ideal for identifying specific aspects of sociality that differ between chimpanzees and bonobos.

Hypothesis:

Comparing "sociality" of chimpanzees and bonobos as quantified by social proximity and occurrences of grooming will reveal distinctly different socio-behavioral patterns between the two species.

Given the lack of comparative studies between captive chimpanzees and bonobos it is difficult to predict exactly how their patterns of sociality will differ. From observations of these species in the wild, we expect bonobos to be more social based on findings that bonobos maintain larger and more cohesive groups than chimpanzees (Boesch, 2002).

Prediction:

Comparing the relative amount of time spent among four discrete distances of social proximity will reveal that bonobos are more likely to spend more time at a

closer social proximity than chimpanzees. Additionally we predict that the relative amount of time spent engaged in grooming activities will be higher for bonobos than it will be for chimpanzees.

2.2 Methods

Observational data collection

Data were collected at AZA accredited zoos in North America, where we performed 10-minute focal follows with 30-second instantaneous sampling intervals. During each 10-minute focal follow, every 30 seconds a data point was collected on social proximity and grooming for the focal individual. Behavioral data were collected blind to the genotypes of the individuals to avoid biased data collection. Social proximity was recorded as either; close/touching, socially close, solitary, or isolated, and the identity of social partners at the determined social proximity were recorded (see table 1 for definitions of social proximities). This was performed so that only the closest social proximity was recorded, such that if an individual was within 1.5m of the closest conspecific and there was another individual within 5m, only the close/touching social proximity and the partner(s) at that distance were recorded. Additionally if the focal individual was participating in grooming, the directionality of grooming (give, receive, or mutual) and the number and identity of grooming partners were recorded. There was a minimum interval of one hour between observations on each individual, and no more than four focal follows were performed on the same individual in a given day. Prior to collection of

behavioral data, a random list of the individuals present in the group was created so data were collected as evenly across the group as possible. Additionally, the minimum group size that data were collected on was 5 individuals, and data were collected only on individuals that were at least 3 years of age. Any interactions by the focal individual with infants two years of age or younger were not recorded. If the focal individual was out of view (OOV) of the observer this was noted as the social proximity, and if the focal individual was out of view for three consecutive data points (1.5min) then the data from that focal follow were discarded.

Subjects and study sites

Bonobos – Milwaukee County Zoo, Milwaukee, Wisconsin

Milwaukee County Zoo maintains a large breeding colony of bonobos. During the time of our observations there were 23 total individuals, 7 of which were under three years of age. We determined this age represents the minimal age to exhibit sufficient independence from an individual's mother to merit data collection. The remaining individuals ranged between 3 and 49 years old, and we collected data on 4 males and 12 females from this study site.

The bonobo enclosure at Milwaukee County Zoo is composed of two areas that are used seasonally. The primary indoor enclosure is ~10,000 sq ft, and we conducted our observations from glass windows on the ground level of this enclosure. Within their enclosure the bonobos have access to a variety of climbing structures, including a mesh wall at the rear of their enclosure, which extends all the

way to the roof three stories above. They are able to climb this mesh wall, and there are several common locations where the bonobos often congregate to groom or sit close to skylights. They also have large stone climbing structures within this indoor enclosure. The second area to which the bonobos are given access in the summer months is a series of expansive tunnels with varying levels, creating a looped path with visual and auditory access to the majority of the enclosure.

Bonobos – Jacksonville Zoo and Gardens, Jacksonville, Florida

Jacksonville's bonobo colony is made up of 10 individuals between the ages of 2 and 47. Six of those individuals are females and four are males. One of those males was under 3 years old and dependent on his mother, so he was excluded from data collection. The group of bonobos that was observed varied on a daily basis, simulating their fission-fusion social structure. There were 4-6 individuals per group, and on some days that group was given access to the yard for the entire day; on other days the groups were changed at 12:00. Occasionally, individuals from the morning group would be included in the afternoon group on days that groups were changed midday.

The bonobo enclosure at Jacksonville is ~6,940 sq ft surrounded by a ~2ft deep moat filled with water. Within their yard, the bonobos have a two-story waterfall and small pool along with a three story climbing structure. There are ropes and hammocks distributed across the enclosure. Observations of these bonobos were conducted from visitor decks two stories above the yard.

Chimpanzees- North Carolina Zoological Society, Asheboro, North Carolina

North Carolina's chimpanzee group consists of 16 individuals between the ages of 2 and 45. One of the males is under the age of three, so he was excluded from data collection. Of the chimpanzees on which we collected data at North Carolina, there were three males and 12 females. Similar to Jacksonville, these chimpanzees are separated into two groups, but the level of fission-fusion is less among these subjects; the males are never changed between groups, and there are only 3-4 females that are frequently exchanged between groups. This results in frequent groupings of one male and 4-6 females or 2 males and 2-6 females. Access to the outdoor yard is given to one of these groups until 2:00pm when that group is taken inside, food is distributed across the yard, and the other group is given overnight access to the yard until 2:00pm the following day.

Observations on these chimpanzees were conducted from 2 visitor viewing areas, which allowed the viewing of chimpanzees at ground level through glass walls. Although the chimpanzee enclosure at North Carolina is ~45,000 sq ft and chimpanzees have access to a variety of rocks and downed trees on which to climb, they frequently elect to sit by the viewing areas and interact with visitors.

Chimpanzees- Warner Park Zoo, Chattanooga, Tennessee

The chimpanzees at Chattanooga's Warner Park Zoo are relatively recent arrivals to this zoo. In June 2015 they were transferred to their current location following their retirement from research at the Yerkes National Primate Research Center. There are 7 individuals within this group, and they range in age between 24 and 31 years old. Within this group there are two males and five females; they are all given access to a ~5,000 sq ft outdoor enclosure everyday, weather permitting. The chimpanzees at Chattanooga were observed from visitor areas, which allowed us to view the chimpanzees at ground level from behind glass walls. The yard at Chattanooga is surrounded by a ~15 ft wall, with several glass portions where the chimpanzees frequently interact with visitors. There is a waterfall and small pool in addition to several climbing rocks and ropes that provide access to hammocks and further arboreal climbing structures.

2.3 Results

We collected 9,744 total data points for chimpanzees and bonobos: 4,809 on chimpanzees and 4,935 on bonobos. We were able to collect ~10 focal follows per individual. There were three bonobos for which we were unable to collect very much data and one chimpanzee who died during data collection. Removal of these individuals from analyses caused a reduction of 231 data points (Chimpanzee: 42; Bonobo: 189). We identified one bonobo as an outlier for spending time alone; she had recently given birth and was rarely engaged with group members. She was removed from all subsequent analyses.

Statistical analysis

The proportion of time spent at each social proximity (figure 1) was determined by the following formula:

$$\frac{\#\ of\ data\ points\ at\ given\ social\ proximity}{(total\ \#\ of\ data\ points - any\ OOV)}$$

This revealed that the difference in sociality, as inferred by time spent at varying social proximities, lies in the amount of time spent isolated or within the close touching social proximities. As the two intermediate categories of social proximity did not reveal any interspecies differences we reasoned that in order to further determine the differences in sociality between the two species we should compare the amount of time spent within the close/touching social proximity, or "close" and the amount of time spent in every other social proximity, or "alone". The combination of the four social proximities outside of the "close" category to create the "alone" category gives us the best comparison of sociality between the two species, as the tolerance of conspecifics within the "close" proximity indicates a higher degree of sociality (Sibbald *et al*., 2005).

To test our hypothesis that distinct patterns of sociality are exhibited between chimpanzees and bonobos, we conducted two separate univariate analyses of variance (ANOVA). For the first analysis the four categories of social proximity

were collapsed for use in statistical comparisons such that any proximity other than close/touching became "alone". Collapsing the categories of social proximity allowed us to make a comparison of the time spent alone as opposed to close/touching between the two species. As seen in figure 1, we observed distinct differences between the two species in proportion of time spent either "close/touching" or "isolated" social proximities without any pronounced differences in the time spent between the "socially close" or "solitary" categories of social proximity. Based on this we reasoned that the difference between chimpanzees and bonobos in their tolerance to conspecifics in social proximities lies in the time spent within touching distance (1.5m) or "close/touching". This led us to collapse the categories of social proximity so that comparisons can be made between the amounts of time the two species spend outside of the close/touching social proximity. This proportion was calculated as the time spent "alone" for each individual:

$$\frac{(\#\ of\ data\ points\ SC + \#\ data\ points\ SO + \#\ data\ points\ IS)}{(total\ \#\ data\ points - any\ OOV)}$$

We screened the data for outliers using the boxplot function in SPSS (version 22) using species as the category and percent of time spent alone and the variable of interest. This revealed one outlier that was removed from subsequent analysis.

In order to make statistical comparisons between the grooming activities of each species we combined all the categories of grooming directionality so that we

could create a value for each individual that reflects the proportion of time that the individual was engaged in any grooming activity. We similarly screened the grooming data for outliers using species with proportion of time grooming, which revealed one outlier (not the same as in the first screen) that was removed before proceeding.

Data Analysis

Figure 1 shows the proportion of time spent in each social proximity between chimpanzees and bonobos based on the equation described in *statistical analysis*.

We identified a trend, although not statistically significant, of bonobos spending more time in the close/touching category than chimpanzees and chimpanzees spending more time in the "isolated" category. In order to further compare "sociality" between the two species we followed Sibbald (2005) and reasoned that the amount of time spent in close social proximity is the most descriptive statistic to compare sociality. Thus we collapsed the categories of proximity outside of the close/touching category as described above.

In figure 2 the proportion of time spent "alone" is depicted for both chimpanzees and bonobos. We performed a univariate ANOVA for percentage of time alone, with the fixed factors, sex and species, and group size included as a

covariate. This revealed a main effect for species, no main effect for sex and no interaction between the two factors. Chimpanzees (62.1%) spent significantly more time alone than bonobos (51.59%), $F(1,42)=6.04$, $p=0.019$.

We carried out a second univariate ANOVA to evaluate the proportion of time spent grooming between the two species with the fixed factors, sex and species, and group size included as a covariate. This analysis also revealed a main effect for species, no main effect for sex and no interaction between the two factors. The data indicate that bonobos spend roughly twice as much time grooming (21%), than chimpanzees (11%), $F(1,42)=5.15$, $p=0.029$), (figure 4).

2.4 Discussion

E.O. Wilson (1975) singled out human sociality as one of his four pillars of social evolution, along with colonial invertebrates, social insects and nonhuman mammals. He justified this separation of human sociality from other mammals by the idea that only humans have created societies as complex as social insects and colonial invertebrates. Humans differ from these other "ultra-social" species representing his pillars of social evolution, however, in their lack of shared genetic identity between individuals in the case of colonial invertebrates, or close relatedness in social insects which is fundamental to their society (Richerson & Boyd, 1998; Wilson, 2000). Fossils from pre-human ancestors have allowed the study of the morphological evolution of humans; unfortunately the evolution of sociality cannot be inferred from fossils. In order investigate the evolutionary

origins of sociality we must study the socio-behavioral patterns in extant organisms. Chimpanzees and bonobo's close phylogenetic relationship to humans make them ideal for investigations into the evolutionary origins of sociality. The data presented within this report indicate that chimpanzees and bonobos exhibit different patterns of sociality. As indicated in figure 1, chimpanzees spend more time isolated than bonobos, who seem to spend more time close enough to touch a conspecific. In order to compare these data we collapsed the categories of social proximity. This allowed us to directly compare the proportion of time spent "alone" or "close" between chimpanzees and bonobos. Through this comparison, shown in figure 3, we found a significant difference in the proportion of time spent alone between chimpanzees (62%) and bonobos (51%), $F(1,42)=6.04$, $p=0.019$. Additionally we found that bonobos spend a greater proportion of time engaged in grooming behaviors compared to chimpanzees- 21% and 11% respectively, $F(1,42)=5.15$, $p=0.029$ (Figure 4).

We hypothesized that we would be able to identify distinctly different patterns of social behaviors between chimpanzees and bonobos when comparing social proximity and grooming participation. Based on observations of wild chimpanzees and bonobos we predicted that bonobos would be more social than chimpanzees (Boesch, 2002). Our results suggest distinctly different patterns of behavior that we hypothesized to exist and show statistically significant differences in the patterns of social proximity and involvement in grooming between chimpanzees and bonobos.

Chapter 3:

Genotyping for, and behavioral associations of, a polymorphic microsatellite deletion 5' of AVPR1a

3.1 Introduction

In order to determine the behavioral impact of a deletion of the DupB microsatellite in the *Pan* species we performed observations and collected behavioral data on both chimpanzees and bonobos. Rather than only collecting behavioral data on chimpanzees to investigate the impact of this polymorphism we chose to collect data on both members of the *Pan* genus. This enabled us to not only compare differences in behavior between the two genotypes of chimpanzees but also to the only other extant member of the *Pan* genus. Collecting data of this nature enabled comparisons the sociality of DupB+ chimpanzees to DupB- chimpanzees and to bonobos.

The polymorphic deletion of the DupB microsatellite is unique to chimpanzees and is not found in their close relatives bonobos and humans (Staes *et al*., 2014: Donaldson *et al*., 2008). Recently it was shown that there is no evidence for a deletion of DupB in bonobos through genotyping a sample that includes 90% of the captive founder population of unrelated bonobos (33 wild caught bonobos), so for the remainder of this study we will treat bonobos as DupB+ (Staes *et al*., 2014). Based on these findings we only genotyped the chimpanzees in this study for the DupB deletion. The DupB microsatellite is ~360 bp in length, so its presence can

easily be visually identified in an electrophoresis gel following amplification of the 5' region of AVPR1a using primers designed to amplify this region.

Since the chimpanzees observed at Chattanooga zoo had been previously housed at the Yerkes National Primate Research Center, we were able to obtain their AVPR1a genotypes from others (Bill Hopkins, personal communication). As such, we did not obtain DNA samples as their genotypes have been confirmed in previous analysis. For the chimpanzees housed at North Carolina Zoo, the zoo was able to provide frozen blood samples that had been stored from routine medical examinations for several individuals. Additionally, they were able to provide serum samples for other individuals. The zoo did not have frozen blood/serum samples from all individuals, so we were able to coordinate the collection of buccal swabs from several subjects and a hair sample from one chimpanzee. After genotyping these chimpanzees we will be able to use the behavioral data described in the previous chapter to compare sociality between DupB+, DupB- chimpanzees and bonobos.

Hypothesis:

Chimpanzees that are homozygous for a complete polymorphic deletion of the DupB microsatellite (DupB-) will have a lower level of sociality, as measured by the proportion of time spent alone, than chimpanzees who retain the ancestral genotype (DupB+)

Given that bonobos are not polymorphic for the deletion of this microsatellite and retain the ancestral genotype (DupB+), we predict that: *If the presence of the DupB allele is associated with greater levels of sociality and DupB+ chimpanzees are shown to be more social than DupB- chimpanzees then we predict that when compared to bonobos DupB+ chimpanzees and bonobos will spend less time socially alone than DupB- chimpanzees.*

3.2 Methods

Genotyping of chimpanzees was performed with polymerase chain reactions (PCR) after extraction of DNA from whole blood, serum, buccal swabs, and hair samples. These samples consisted of whole blood or serum samples collected from chimpanzees during routine medical examinations and stored at -20˚C, buccal cell swabs, or a hair sample, all of which were collected by keepers from voluntary chimpanzees at our request.

Buccal swabs were collected with sterile q-tips from willing chimpanzees, which had been trained to present their mouth for swabs. After collection the swabs were stored in a 1.5ml microcentrifuge tube at -20˚C.

A keeper collected the hair sample by plucking 5-10 hairs, including the follicle, and storing/shipping them in a 15ml conical tube at room temperature.

DNA extraction methods:

We followed the instructions from the manufacturer of the GeneSync DNA extraction kit when performing DNA extractions from whole blood and hair samples. In order to extract DNA from the serum and buccal swab samples we modified the protocols from the GeneSync kit; modifications are outlined in the following sections.

Protocol for extraction of DNA from buccal swabs:

We used the GeneSync DNA extraction kit with some modifications. The buccal swab was removed with sterile tweezers from the tube in which it was shipped and placed into a 1.5ml microcentrifuge tube; 500ul of GST buffer was then added to submerge the swab. Proteinase K (20ul) was added, and the sample was vortexed. The sample was then placed in a 40˚C heat block and incubated overnight, with vortexing performed 5-6 times during this incubation. The following day the sample was incubated at 60˚C for 10min before the swab was removed from the 1.5ml microcentrifuge tube and placed into a spin column and collection tube from the GeneSync kit. The swab in the column was then centrifuged at 13,000 RPM for 2min. Following centrifugation, the flow through was added to the sample tube; the swab, column, and collection tube were discarded. From this point, we followed the provided protocol for collection of DNA from solid tissue.

Protocol for extraction of DNA from serum samples:

Since serum samples should contain very few cells, we optimized the protocol from the GENESYNC DNA extraction kit. In order to increase the yield of DNA when extracting DNA from serum, we loaded the column with several preparations of lysed serum samples prior to washing and eluting DNA. Serum samples were lysed as outlined by the manufacturer, and three preparations were loaded to the column before following the manufacturer's instructions for washing and elution of DNA from the column. This method allowed us to extract as much DNA as possible from the serum sample while still keeping the final elution volume low enough that the concentration of DNA was high enough for PCR.

PCR methods:

In order to amplify the region of interest, we used previously published primer sequences that had been used for genotyping this polymorphism. This set of primers corresponds with chimp/human nucleotide differences in order to decrease potential cross-species contamination (Hopkins *et al*., 2014; Donaldson *et al*., 2008). The primers were: forward primer 5'-GCATGGTAGCCTCTCTTTAAT-3' and reverse primer 5'-CATACACATGGAAAGCACCTAA-3'. We used the KaPa HiFi hotstart ready mix for the PCR reactions with an annealing temperature of 65.9˚C.
PCR products were visualized on a 1% agarose gel and stained with ethidium bromide. Staining of the DNA was performed after the PCR products had been separated by gel electrophoresis, by a 15 minute immersion in 1:1000 ethidium:1x TAE buffer solution followed by a 15-min de-staining step in 1% TAE. Gels were

then photographed on the BioRad imager and genotypes visually determined. These genotypes were confirmed with two independent PCR amplifications and subsequent electrophoresis gel visual determination of genotypes.

3.3 Results

We identified 2 DupB+/- individuals out of the 15 chimpanzees that we successfully genotyped from North Carolina zoo. We were unable to extract enough DNA from serum samples of one additional individual see (table 3 for details). Additionally, after behavioral data collection was completed on chimpanzees from Chattanooga zoo it was revealed that two of those chimpanzees are DupB+/-, with the other 5 having the DupB-/- genotype. We collected behavioral data on 21 chimpanzees, 20 of which we were able to genotype for the DupB polymorphism; 16 (80%) of them were found to have a complete deletion of the DupB microsatellite and 4 four were genotyped as DupB+/- (20%).

We used the collapsed categories of social proximity as described in chapter 2 to perform a univariate ANOVA analysis based on the percentage of time spent alone by chimpanzees of each genotype. There were no main effects or interaction. This may be due to the low number of DupB+ chimpanzees present in our data set, an issue that we were not aware of until after behavioral data collection was completed and genotyping performed.

In order to supplement the data collected for this report we were able to acquire a data set from one of our colleagues, which consisted of similar proximity

data from 68 chimpanzees housed between Yerkes National Primate Research Center in Atlanta, GA and M. D. Anderson Cancer Center in Bastrop, TX. This data set only used two categories of proximity- close (within 1.5m) and alone (further than 1.5m) from conspecifics; however, the interval of data collection- 10-min focal follow with a data point taken every 60 seconds - very similar to the behavioral data collected for this project. In the supplementary data set there were 3 DupB+/+ individuals; following the literature precedent, we removed those individuals from our analysis as we lacked sufficient representation of this genotype to make legitimate comparisons (Hopkins *et al*., 2012). After screening for and removing the outliers, as in chapter 2, we performed a univariate ANOVA with the dependent variable, percent of time spent alone and AVPR1a genotype (DupB+, DupB- or bonobos) as the between subject variable. As indicated in Figure 5, there is a statistically significant difference in the proportion of time spent alone between the three genotypes, $F(1,104)=3.224$, $p=0.044$. DupB- chimpanzees appear to spend more time alone (58%) than DupB+ chimpanzees (50%) and bonobos (51%).

3.4 Discussion

Among the chimpanzees for which behavioral data were collected for this report, genotyping revealed that only 4 out of the 21 chimpanzees in our data set were DupB+. Given this relatively small sample size as well as variability among rearing conditions and other factors, and their potential subsequent impact on behavioral patterns, significant associations between time spent alone and AVPR1a

genotype were not observed. Fortunately, we were able to access a data set on the proximity of 68 additional chimpanzees housed at two primate research centers. With the addition of these data, we found that DupB- chimpanzees spend more time "alone", while DupB+ chimpanzees spend more time "close" to conspecifics. These comparisons were made by collapsing the categories of proximity used in the data collection as described in chapter 2, *statistical analysis*. We converted the 4 categories of proximity to match the added data set, either close: within 1.5m or alone: greater than 1.5m to the nearest conspecific. As seen in figure 4, this revealed a statistically significant difference in the proportion of time spent alone between DupB+ (50.92%) and DupB- (58.12%) chimpanzees. Furthermore we were able to show that bonobos percentage of time spent alone (51.59%) is more comparable to the chimpanzees who also retain the DupB microsatellite, which supports our hypothesis that bonobos will have more comparable sociality to DupB+ chimpanzees than to DupB- chimpanzees.

Chapter 4: Gene reporter assays

4.1 Introduction

If the polymorphic deletion of the DupB microsatellite in chimpanzees is able to modify behavioral phenotypes, it must do so by exerting some control over the

expression of the AVPR1a gene. This functionality could be in the form of an enhancer that acts to upregulate the expression of AVPR1a, or the DupB microsatellite could act as a repressor and downregulate the expression of AVPR1a. Additionally the functionality of this microsatellite may exert a differential effect on expression in a cell type dependent manner, acting as a repressor to transcription in some brain regions while enhancing transcription in other regions (Tansey *et al*., 2011). Through either upregulation or downregulation of the expression of the AVPR1a gene, the presence of this microsatellite likely alters expression in some way, consequently modifying the neurological response to stimuli and, in turn, creating differential socio-behavioral phenotypes.

Gene reporter assays have been used to show that in voles the deletion of the polymorphic microsatellite 5' of the vole AVPR1a gene resulted in lower expression of AVPR1a in the brains of these rodents (Hammock & Young, 2004). Similar results were obtained using transgenic mice, by substituting the 5' region of the mouse AVPR1a gene with the corresponding 5' region of AVPR1a from prairie voles. This experiment showed increased expression of AVPR1a in several brain regions compared to wild type mice (Hammock *et al*., 2005). Additionally it has been shown by experiments with human DNA sequences that allele length differences of the RS3 microsatellite, contained in the DupB tandem repeat region, can modify the expression of luciferase in a neural cell line (Tansey *et al*., 2011). However, the impact of a complete deletion of the DupB region has not been investigated through luciferase reporter assays. In 2009, Hong and colleagues attempted to determine if allele length differences of the RS1 microsatellite in chimpanzees modified

expression of luciferase. However, they found no differences in expression based on allele lengths of this microsatellite in chimpanzees (Hong *et al*., 2009). Hong and colleagues examined the impact of the RS1 microsatellite because it is similar in composition and has the same relative location to the TSS of AVPR1a as the polymorphic microsatellite implicated in modifying the social behaviors of voles (Hong *et al*., 2009). However based on recent research it is clear that the polymorphic deletion of the DupB region in chimpanzees is much more likely to modify expression of AVPR1a. Investigating the DupB region is additionally much more applicable to human medicine as human and chimpanzee behavior have been shown to be modified by either length variation or complete deletion of this region (Hopkins *et al*., 2012; Staes *et al*., 2014; Anestis *et al*., 2015). For these reasons we chose to investigate the impact of performing luciferase reporter assays with 2 plasmid constructs; the first containing both the DupA and DupB microsatellites and the second only the DupA microsatellite. This will allow us to measure the impact of removing the DupB microsatellite by quantifying the amount of luciferase produced by each plasmid.

Hypothesis:

Luciferase production will differ between two experimental plasmids, one with, and one without, the DupB microsatellite, indicating that the presence or absence of this microsatellite has an impact on gene expression.

The relationship between the vole microsatellite and expression of AVPR1a, and Tansey et al.'s 2011 luciferase reporter assays investigating human RS3 allele length both indicate that longer alleles/presence of a microsatellite has an upregulatory impact on gene expression (Donaldson *et al*., 2008; Tansey *et al*., 2011).From those findings we predict that:

The DupB+ experimental plasmid will have higher expression of luciferase than the DupB- experimental plasmid.

4.2 Methods

To investigate the role of the DupB microsatellite presence on gene expression, we created luciferase vectors to represent the two genotypes (DupB+ and DupB-). These vectors were created based on the 5' flanking DNA sequence of a chimpanzee named Beleka (Genbank: EU780070.1). This individual's AVPR1a upstream region had been sequenced for a prior study and was available on NCBI (Donaldson *et al*., 2008). This individual was DupB+, so we used the DupA/DupB region for the DupB+ vector and for the DupB- vector we removed the 343bp DupB microsatellite from this sequence and thus created an artificial DupB- individual (plasmid sequences available upon request). This technique was used specifically in place of using the allele of a DupB- individual because we wanted to avoid the issue of SNPs and allele length differences between individuals. By creating this artificial DupB- fragment from the same genome of the DupB+ individual, we compared the

exact same sequence with the only variation between the two vectors being the presence or absence of the 343bp DupB region.

After creating the two sequences for the DupB+ plasmid and the DupB- plasmid *in silico*, we sent the two sequences along with a sample of the pGL4.23[luc2/minP] plasmid to GeneWiz, who synthesized the representative fragments for each genotype and then cloned those fragments into the pGL4.23[luc2/minP] plasmid at the KpnI and NheI restriction sites.

Upon delivery of the experimental plasmids we performed bacterial transformations of both experimental plasmids, the empty pGL4.23(luc2/minP) plasmid and the pGL4.70(hRluc) plasmid in NEB DH5(a) cells. These transformations were subsequently mini-prepped using the Zyppy™ Plasmid Miniprep Kit, following the manufacturers instructions. To confirm the identity of the transformed plasmids we conducted a restriction digest and visualized the products on a 1% ethidium bromide gel.

Transfections

We performed each assay in a triplicate format, using 24 well plates, but only plating cells in 12 of the wells. In preparation for the assays we thawed Human Embryonic Kidney (HEK-293T) cells from liquid nitrogen storage. These cells were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum, 1mM sodium pyruvate, non-essential amino acids and 1x penicillin/streptomycin. Cells were kept at 37˚C, 5% CO_2, 85% humidity in an

incubator until ~90% confluence at which point we removed a sample and counted the number of cells using a BD-biosciences C6 ACCURIE flow cytometry. We then plated ~19,000 cells in each of the 12-wells and allowed them to grow to ~50% confluence before transfecting the following day. Transfections were performed using Lipofectamine 3000, according to the manufacturers instructions. For each well 1 µg experimental plasmid DNA (DupB+, DupB- or empty pGL4.23, respectively) was co-transfected along with 100ng pGL4.70(hRluc) to allow us to control for transfection efficiency and cell health/density.

After transfecting the cells with their respective plasmids, they were allowed to grow for 2-days before performing the Dual-Glo assay (Promega). The Dual-Glo kit from Promega was designed for optimal use in a 96well format, but we modified the protocols in collaboration with a Promega representative for use in a 12 well format.

The modified protocol that we followed for the Dual-Glo assay is as follows:

1) Media was aspirated from each well, and replaced with 100 µl of fresh media (because the Dual-Glo kit is optimized for use in the presence of media, and is prepared 2x so it works best in 1:1 ratios of reagent to cell culture media)
2) Reagent 1 (cell lysis and luciferase substrate) was added to each well (100 µl) and allowed to incubate in the tissue culture hood for 10min
3) After 10 minute incubation the contents of each well was pipetted into a labeled 1.5ml microcentrifuge tube

4) The GloMax 20/20 luminometer (Promega) was then used to measure the RLUs from each sample (with the caps of the tubes open, in the order that reagent 1 was added)
5) After measuring the luminescence, reagent 2 (stop and glow reagent, prepared 1:100 substrate to buffer) was added to each well (100 µl) and allowed to incubate for 10min before the Renilla RLUs were measured in the GloMax 20/20 luminometer (with the caps of the tubes open, in the order that reagent 2 was added)

4.3 Results

We performed six independent luciferase assays, each in triplicate, containing three wells that were transfected with the pGL4.23(luc2/minP)+DupA/DupB fragment, three wells that were transfected with pGL4.23(luc2/minP)+DupA only fragment, three wells transfected with the empty pGL4.23(luc2/minP) vector containing no experimental insert and three wells that were not transfected.

One of these experiments was not included in our analysis because of inconsistencies in the volume of DNA that was transfected, which rendered normalization impossible; thus, our statistical analysis was limited to five assays. For each independent experiment, we normalized each well for transfection efficiency through the use of the co-transfected *Renilla* plasmid. The pGL4.70(hRluc) plasmid was co-transfected along with the respective pGL4.23 vector in the

experimental and control wells. The presence of this co-transfected plasmid allowed us to divide the RLUs produced by the firefly luciferase by the RLUs produced in the same sample by the Renilla plasmid to normalize for cell health and transfection efficiency in each well. This gives us a ratio of firefly luminescence/Renilla luminescence for each well. From that point we averaged the ratios from the three wells of: pGL4.23(luc2/minP)+DupA/DupB, pGL4.23(luc2/minP)+DupA, Empty pGL4.23(luc2/minP) and the untransfected wells. After averaging the ratios for each assay, the separate triplicate assays are treated as n=1. In order to make comparisons between the expression of the two experimental plasmids, we divided the ratio of firefly/Renilla expression of each experimental plasmid to the ratio of firefly/Renilla expression of the empty pGL4.23(luc2/minP). This normalization allowed us to compare the results from all five assays and perform statistical comparisons.

Our data indicate that there is no difference in the expression between the two experimental plasmids we did identify a trend in the data. Based on our experiments we found that the pGL4.23(luc2/minP)+DupA (DupB-) plasmid had higher expression on average compared to the pGl4.23(luc2/minP)+DupA/DupB (DupB+) plasmid (figure 5).

4.4 Discussion

Despite extreme variability among assays, we were able to identify a trend showing that the DupB- plasmid had higher level of expression of the luciferase gene

than the DupB+ plasmid (figure 5). We expected to identify a trend in the opposite direction, which would indicate that the DupB region acts as a promoter to induce higher expression of the luciferase gene. This would suggest that the DupB region acts as a promoter to enhance expression of the AVPR1a gene. Our data, however, suggest that the DupB region may actually act as a repressor and, in turn, decrease the expression of AVPR1a when it is present. We expected that when the DupB microsatellite was present it would enhance the expression of AVPR1a, leading to greater density of the vasopressin 1a (V1a) receptor in the brains of DupB+ individuals. Our data suggest, however, that DupB- individuals may have greater central expression of the V1a receptor compared to DupB+ individuals. This finding, although contrary to our expectations is still very interesting in the context of gaining a better understanding of the impact elements in the non-coding region of genes have on gene expression and modifications to behavioral phenotypes.

A potential limitation in our design of the plasmid constructs is that by only cloning in the DupA/DupB, or DupA microsatellites we are eliminating ~3300bp of potential promoter elements in the 5' flanking region of the AVPR1a TSS. To correct for this it would be interesting to examine DupA/DupB plus the rest of the 5' flanking region of the AVPR1a gene in luciferase assays.

To expand on the gene reporter assays we performed, it would be of value to repeat these experiments in a different cell line, such as the neuronal cell line (SH-SY5Y) that was used when investigating the effect of human RS3 allele length on luciferase expression (Tansey *et al*., 2014). We used HEK cells because they have been found to endogenously produce the AVPR1a gene, so we considered them a

representative cell line. The drawback to using this cell line, however, is that we are interested in how this polymorphism modifies the central density of the AVPR1a gene, so performing these experiments in a peripheral cell line might not be representative of how expression is modified by this microsatellite in a neural context. Young and colleagues (2004; 2005) showed that microsatellite variation 5' of the AVPR1a gene may modify expression in a cell-type specific manner, and it is has been shown that the density of neural expression of V1aR often shows variation between brain regions (Hammock & Young, 2004; Hammock & Young, 2005). Despite the multitude of variables to consider when investigating the factors that can contribute to differential gene expression, along with potential limitations in our plasmid design, and lack of experiments in multiple cell lines, our results nonetheless suggest that this polymorphism exerts influence on gene expression.

It should not be taken lightly that this polymorphic microsatellite is only one of many putative genetic elements that may contribute to the development of differential behavioral phenotypes. That being said, the identification of such putative genetic elements is the first step to integrating advances in genomics, *in vivo* experimentation, and behavioral observations, to identify the genetic underpinnings of differential behavioral phenotypes.

Summary:

In this report we present data that indicate differences in sociality between captive chimpanzees and bonobos. Given the lack of direct behavioral comparisons

between captive chimpanzees and bonobos, these data are valuable to our understanding of how chimpanzees and bonobos differ in terms of sociality. The ability to make observations and collect data on these apes in captivity, where potentially confounding ecological conditions can be controlled, allows us to make direct comparisons between the two species. To quantify "sociality" we used behavioral data collected on the social proximity and grooming activities of focal individuals. These data indicated that chimpanzees spend more time "alone" (62.1%) (further than 1.5m from a conspecific) than bonobos (51.59%), and that bonobos spend more time engaged in grooming activities (21.28%) than chimpanzees (10.21%).

We also investigated the impact of a polymorphic deletion of the DupB microsatellite in the 5' flanking region of the AVPR1a gene in chimpanzees. These data indicated that there is a difference in the amount of time spent further than 1.5 meters from the nearest conspecific. We defined this social proximity as "alone", as opposed to "close" social proximity, which indicates the presence of a conspecific within 1.5m of the focal subject. Our data indicates that DupB- chimpanzees spend more time "alone" than both DupB+ chimpanzees and bonobos. As bonobos are not polymorphic for the deletion of the DupB microsatellite, we expected to find that DupB+ chimpanzees would have more similar patterns of sociality compared to bonobos than the DupB- chimpanzees, owing to the impact this deletion has on central expression of V1aR and the subsequent impact on behavioral phenotypes from this difference in expression.

We used luciferase reporter assays to determine if there was a molecular explanation for the association between the presence of the DupB microsatellite and observed differences in social behavior. These assays allowed us to measure the impact of the presence or absence of the DupB microsatellite through the relative expression of a luciferase gene contained in the experimental plasmids. After controlling for transfection efficiency and cell health with the internal control and normalizing to background luminescence produced from the "empty" plasmid backbone without an experimental region of interest, we did not find a statistically significant difference in the expression of luciferase between the two experimental plasmids. We did, however, identify a trend with the DupB- plasmid having higher expression of luciferase than the DupB+ plasmid. While this is contrary to our expectations based on similar assays evaluating the vole polymorphism and human RS3 allele length differences, the indication that the presence or absence of the DupB microsatellite has implications for the expression of luciferase still lends support to our hypothesis that this microsatellite exerts influence over the expression of the AVPR1a gene.

Together this is a behavioral, genetic, and molecular investigation of behavioral variations between chimpanzees and bonobos with emphasis on the naturally occurring polymorphic deletion of the DupB microsatellite in chimpanzees. We have provided evidence that among captive chimpanzees and bonobos there are distinct patterns of social behavior with respect to social proximity and grooming activities. Although not statistically significant or in line with our predictions, the gene reporter assays we conducted indicate that the

presence of the DupB microsatellite does have some control over genetic expression.

Integration of thesis research:

Over the course of this research we have utilized tools from several distinct disciplines concomitantly. In order to quantify differences in socio-behavioral patterns we collected observational data on social proximity and the grooming activities of chimpanzees and bonobos. This data makes up the behavioral component of our study and is representative of typical observational data collected during investigations of behavioral biology. During the genotyping of chimpanzee DNA samples we utilized several tools commonly used in genetics research such as DNA extraction kits, PCR and gel electrophoresis. Additionally, luciferase reporter assays were incorporated into this study to evaluate the effect of the DupB polymorphism on gene expression. This component of the project required the use of cell culture, bacterial transformations, and transfection of cells along with additional tools of microbiology. In summary the completion of this project required the successful integration of components of behavioral biology, genetics, and microbiology. The integration of these disciplines has enabled us to evaluate social behaviors of great apes from a genetic perspective and begin to provide a molecular explanation for the differential behavioral phenotypes associated with a polymorphic microsatellite deletion in the 5' flanking region of the AVPR1a gene.

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Figures and Tables:

Table 1: Physical Proximity Defined

Proximity	Description
Close/Touching	Focal individual is in physical contact with a conspecific or close enough that it could touch a conspecific without relocating (~≤1.5 meters)
Socially close	Focal individual is ~1.5-3 meters from the nearest conspecific
Alone	Focal individual is ~3-5 meters from the nearest conspecific

Secluded	Individual is >5 meters from the nearest conspecific

Table 2: Grooming Defined

Type of Grooming	Description
Groom give	Focal individual is grooming a conspecific without any receiving any grooming from the conspecific it is grooming
Groom receive	Focal individual is receiving grooming from a conspecific and is not returning any grooming
Mutual Groom	Both the focal individual and the social partner(s) are actively grooming each other

Table 3: DNA concentrations and genotypes from North Carolina Chimpanzees

Name of Individual:	DNA concentration:	Genotype:
Terry	25.3ng/µl	DupB-/-
Maggie	13.5ng/µl	DupB-/-
Ruthie	35.4ng/µl	DupB-/-
Amy	15.5ng/µl	DupB-/-
Tammy	26.7ng/µl	DupB-/-
Ruby	1.6ng/µl	n/a
Jonathan	10.7ng/µl	DupB+/-
Sokoto	3.0ng/µl	DupB-/-
Lance	6.7ng/µl	DupB-/-
Kendall	24.9ng/µl	DupB-/-
Nori	25.6ng/µl	DupB-/-
Ebi	8.6ng/µl	DupB-/-
Gari	3.5ng/µl	DupB+/-
Gerre	3.1ng/µl	DupB-/-
Gigi	6.3ng/µl	DupB-/-
Genie	15.7ng/µl	DupB-/-

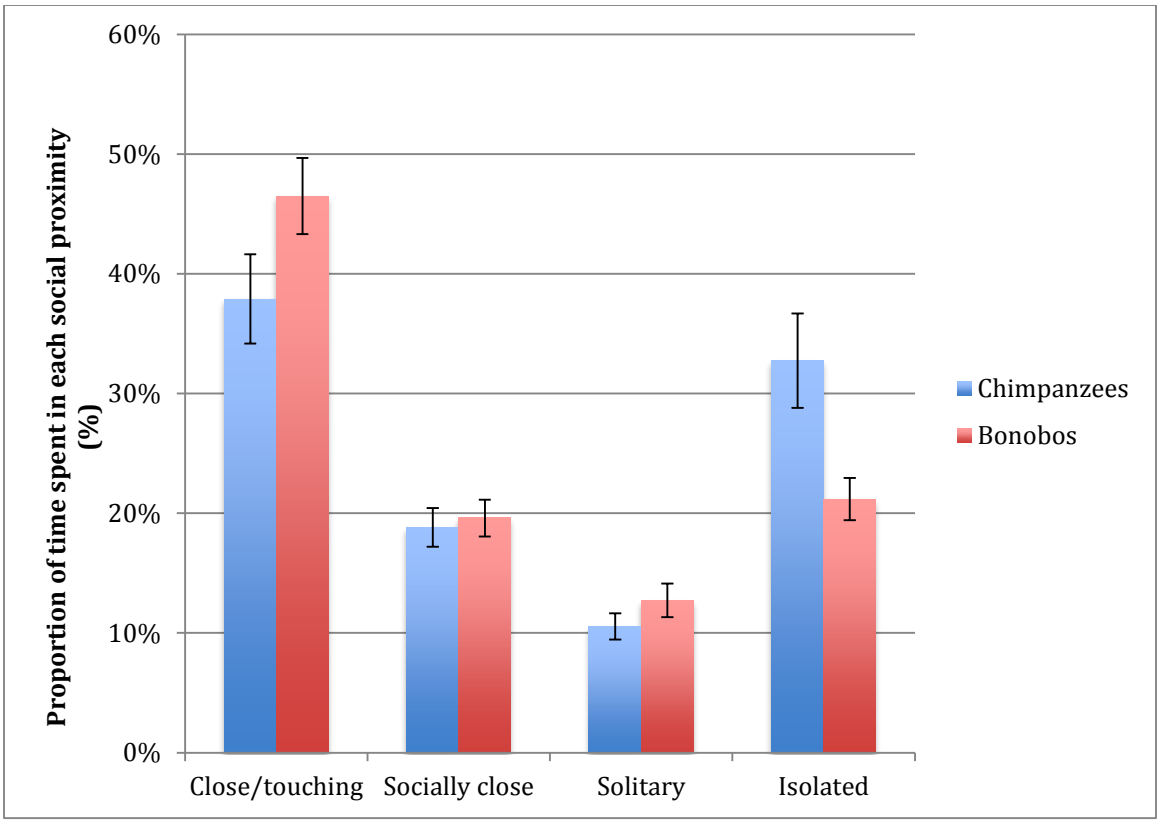

Figure 1: Mean proportion of time spent at each social proximity for chimpanzees and bonobos (error bars indicate standard error)

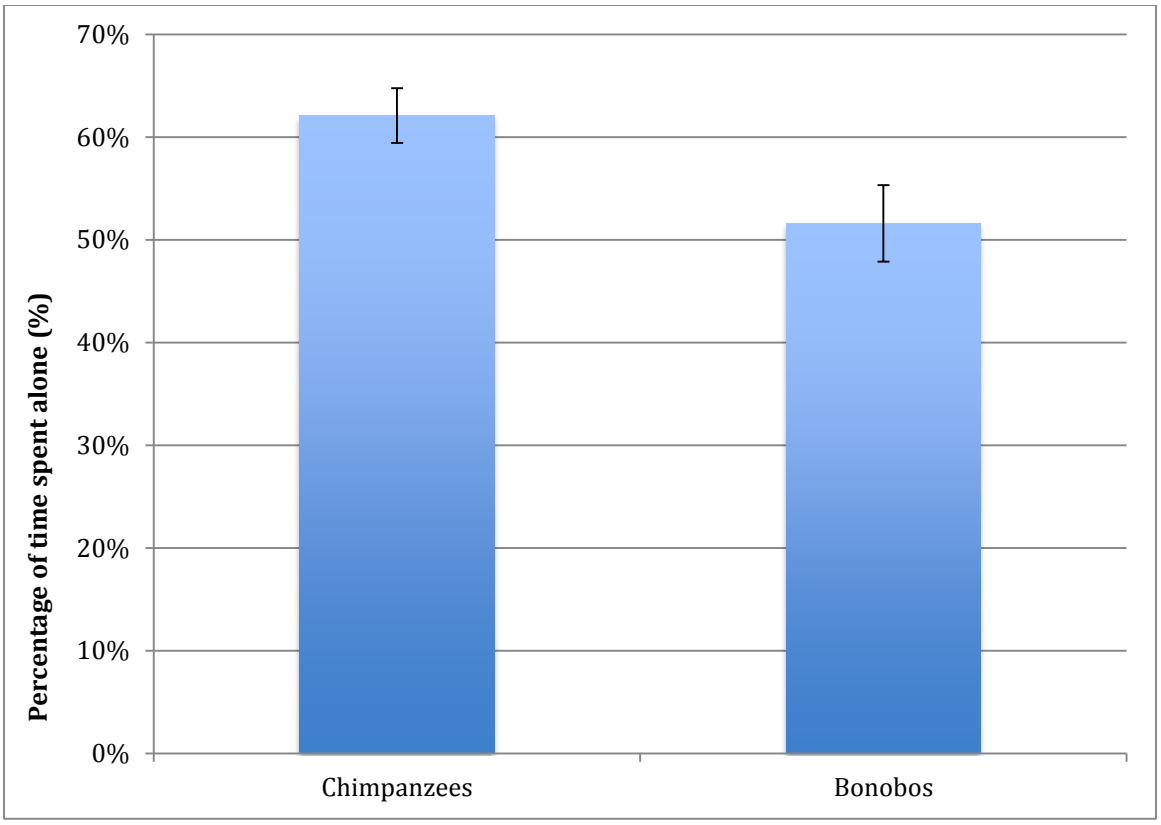

Figure 2: Percentage of time spent "alone", in a social proximity further than 1.5m from nearest conspecific, between chimpanzees and bonobos (error bars indicate standard error)

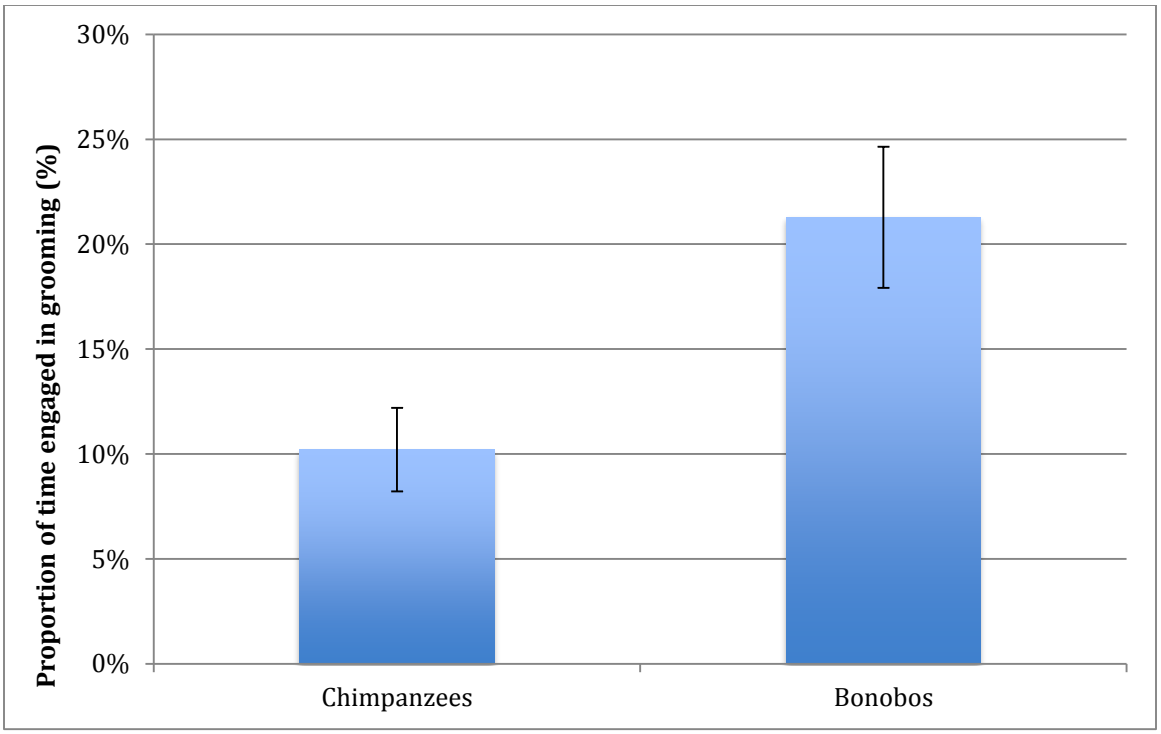

Figure 3: Percentage of time engaged in any grooming activity for chimpanzees and bonobos (error bars indicate standard error)

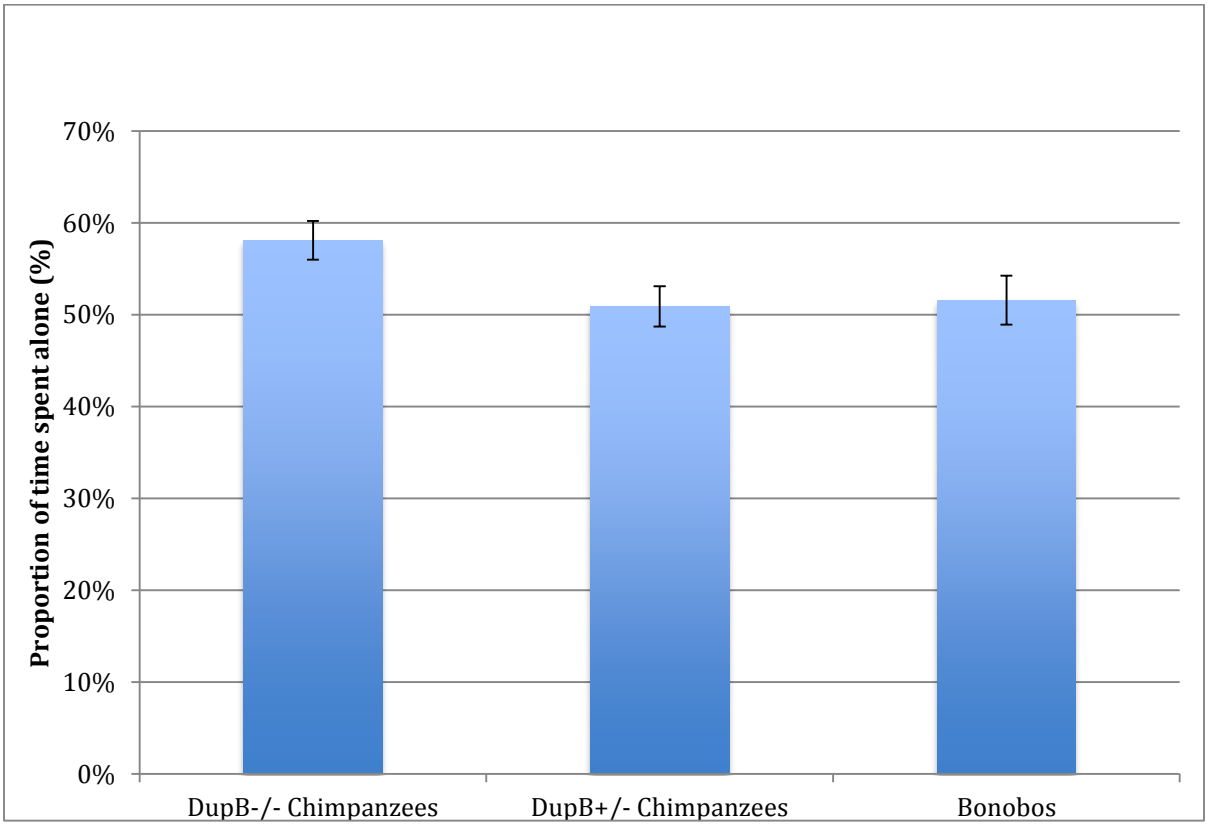

Figure 4: Percentage of time alone, further than 1.5m from nearest conspecific, between chimpanzees with and without the DupB microsatellite and bonobos (error bars indicate standard error)

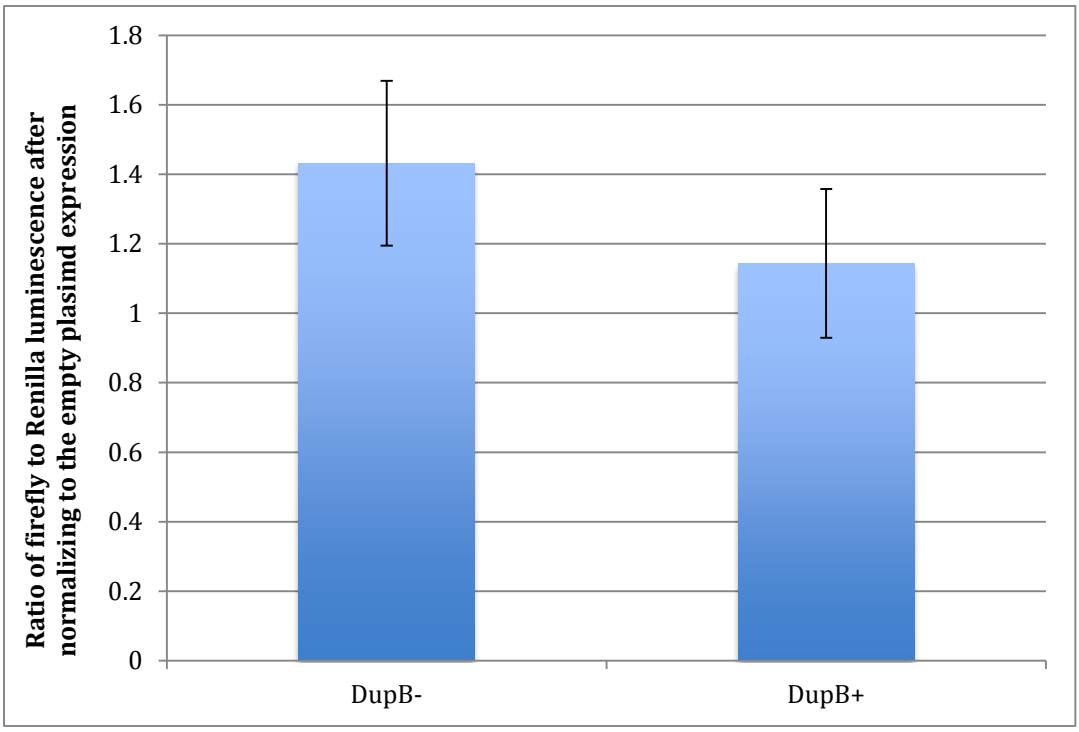

Figure 5: Relative luminescence of the two experimental plasmids (DupB+ & DupB-) after normalizing to the internal control (pGl4.70(hRluc)) and empty plasmid (pGL4.23(luc2/minP) background luminescence (error bars indicate standard error)

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