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Standardizing Olfactory Tests for the Common Marmoset (*Callithrix jacchus*) Aging Model

Evelyn Bartling- John

A departmental senior thesis submitted to the department of Neuroscience at Trinity University in partial fulfillment of the requirements for graduation with departmental honors.

April 30, 2020

Kimberley A. Phillips

Thesis Advisor & Department Chair

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Preface

Overall, my lab experience in Dr. Phillips' lab has sought to understand how animals perceive the world through a sensation and perception lens. However, sensation and perception can be studied through multiple scientific facets. This thesis includes two of those facets within the umbrella of sensation and perception and seeks to understand how nonhuman primates interact with their environment. Within the first facet, hair cortisol concentration (HCC) and hormonal assays, I studied hypothalamic-pituitary-adrenal (HPA) axis activity -a complex neural, negative feedback system. In doing so, I learned how the hormone, cortisol, is regulated by the HPA axis in response to environmental stimuli. In turn, this provided one method to understand how animals perceive their environment. After successfully running through hair cortisol processing and assay protocols, we published one article on field common marmoset (*Callithrix jacchus*) HCC (Chapter 1) and another article on body location influencing HCC in captive common marmosets (Chapter 2). In addition to their link to sensation and perception, these articles exemplify my understanding of experimental design and animal testing. With this experience, I was more equipped to tackle the latter portion of this thesis -my pilot study. In Chapters 3 and 4, I explore animal sensation and perception through olfaction. The literature review in Chapter 3 explains general olfaction, age-related olfaction, compares human and marmoset olfaction, and segues into the my pilot study (in Chapter 4). The pilot study aimed to standardize an olfactory test for the common marmoset to understand age-related olfactory decline. By applying olfaction to the context of age in a nonhuman primate model, I aimed to further understand marmoset olfaction, human olfaction, and the relationship between cognition and olfaction.

Final author submission to *PeerJ*.

Life in a Harsh Environment: The Effects of Age, Sex, Reproductive Condition, and Season on Hair Cortisol Concentration in a Wild Non-human Primate

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Abstract

Hair cortisol concentration (HCC) provides a long-term retrospective measure of hypothalamic–pituitary–adrenal axis activity, and is increasingly used to assess the life history, health and ecology of wild mammals. Given that sex, age, season and pregnancy influence HCC, and that it may indicate ongoing stress, we examined HCC in common marmosets (Callithrix jacchus) naturally inhabiting a hot and dry semi-desert like habitat, Caatinga, in northeastern Brazil. We trapped, measured, weighed, marked and collected shaved hair from the back of the neck of 61 wild marmosets during the wet and dry seasons. Using enzyme immunoassay, we found that HCC was higher in the dry season compared with the wet season among all age/sex classes. Females had significantly higher HCC than males, juveniles had higher HCC than adults, and reproductively active adult females and non-pregnant/non lactating adult females did not differ in HCC. There were no interaction effects of sex, age, group, or season on HCC. The magnitude of the effect of this extremely hot and dry environment (average yearly rainfall was only 271 mm) on HCC in common marmosets is difficult to ascertain as these animals are also experiencing a variety of other stressors. However, the elevated HCC seen in common marmosets during the 5–8 month dry season, suggests these primates face an extended period of heat, water and possibly nutritional stress, which appears to result in a high rate of juvenile mortality.

Subjects: Animal Behavior, Anthropology, Zoology

Keywords: Common marmosets, Callithrix jacchus, Environmental stress, Cortisol, Caatinga

Introduction

Glucocorticoid hormones, of which cortisol is the most prominent in primates, are involved in multiple physiological processes, including the conversion of sugar, fat and protein stores into usable energy, and as an anti-inflammatory preventing tissue and nerve damage (Katsu & Iguchi, 2016). Cortisol is regulated by the hypothalamic–pituitary–adrenal (HPA) axis and its production is increased during periods of stress and elevated blood glucose levels. Environmental stressors such as habitat fragmentation (Seltmann et al., 2017), climate extremes (Bechshoft et al., 2013; Fardi et al., 2018), reduced food availability and anthropogenic disturbances (Carlitz et al., 2016; Strasser & Heath, 2013) impact HPA-axis activity. The implications of such prolonged stress may include decreased fertility (Strasser & Heath, 2013), a weakened immune system (Khansari, Murgo & Faith, 1990; Padgett & Glaser, 2003) and decreased body condition (McEwen, 1998), which can lead to increased mortality.

Hair cortisol concentration (HCC) is increasingly used as a noninvasive measure to assess retrospective HPA axis activity in wild animal populations (Heimburge, Kanitz & Otten, 2019). While the exact mechanism by which cortisol is deposited into hair is unknown, free cortisol is believed to be incorporated into hair via diffusion from follicular capillaries into the medulla of the hair shaft during growth (Meyer & Novak, 2012; Russell et al., 2012). HCC reflects HPA axis activity over a period of weeks to months, depending on the rate of hair growth and the length of hair sampled. However, due to wash out effects, HCC is best used to evaluate ongoing stress, rather than a specific event that occurred in the distant past (Kalliokoski, Jellestad & Murison, 2019). That is, if individuals encounter environmental, nutritional, or social stressors that persist for an extended period, HCC can provide useful information about physiological response and physiological condition.

Environmental stressors including reduced food availability, drought, and exposure to excessive heat and water deprivation have been correlated with elevated cortisol levels in several animal species (Bruener, Delehanty & Boonstra, 2013; Cavigelli, 1999; Fardi et al., 2018; Parker et al., 2003; Weingrill et al., 2004). For example, a decrease in ripe fruit production was the best predictor of increased fecal cortisol levels in wild red-bellied lemurs (*Eulemur rubriventer*; Tecot, 2013). In the case of brown bears (*Ursus arctos*; Cattet et al., 2014) inhabiting Coastal British Columbia, an increase in salmon availability and consumption resulted in a decrease in HCC (Bryan et al., 2013). And, in Merino sheep (*Ovis aries*), elevated plasma cortisol levels were associated with a "loss of body water in excess of that associated with a loss of electrolytes", diuresis and dehydration (Parker et al., 2003). This also was supported in a study of corridale sheep (*Ovis aries*) exposed to alternative regimes of heat stress and water restriction. Ewes exposed to a temperature–humidity index of 27.9 (mild stress), and water restriction for the first 3 h after feeding, exhibited significantly higher wool cortisol levels than ewes with unrestricted or less restricted (2 h post feeding) access to water (Ghassemi Nejad et al., 2014). Long-term exposure to heat, water and nutritional stress can result in the impairment of ovarian function, a decrease in rates of conception, reduced growth, dehydration, increased susceptibility to disease and death (Sanin, Zuluaga Cabrera & Tarazona Morales, 2015; Takahashi, 2012).

HCC is reported to display an age-dependent decline from young to adult ages in captive and domesticated species, including dairy cattle (*Bos taurus*), rhesus monkeys (*Macaca mulatta*), and vervet monkeys (*Chlorocebus aethiops*) (Dettmer et al., 2014; Fourie & Bernstein, 2011; Gonzalez-De-la-Vara et al., 2011; Laudenschlager, Jorgensen & Fairbanks, 2012). This same effect has been documented in wild primates such as vervets and baboons (*Papio* spp; Fourie et al., 2015a, 2015b). Similarly, blood glucocorticoids were reported to be higher in captive infant

and juvenile pig-tailed macaques (*Macaca nemestrina*) compared with adults. This may result from infants and juveniles having lower corticosteroid binding globulin concentration, leading to higher plasma concentrations of free cortisol, which could be deposited into hair during growth (Grant et al., 2017).

There are inconsistent results concerning whether HCC varies by sex in nonhuman primates. Phillips et al. (2018) did not detect significant sex differences among captive adult common marmosets (*C. jacchus*). Similarly, researchers did not find sex differences in HCC in captive chimpanzees (*Pan troglodytes*) and orangutans (*Pongo* spp.) (Carlitz et al., 2014; Yamanashi et al., 2013). However, females were reported to have higher HCC compared to males in captive vervet monkeys (Laudenschlager, Jorgensen & Fairbanks, 2012). This sex difference emerged at puberty and continued into adulthood (Laudenschlager, Jorgensen & Fairbanks, 2012). Finally, wild adult female and male lion tamarins (*Leontopithecus rosalia*) showed similar values of fecal cortisol, except during the final trimester of pregnancy, when values for breeding females increased significantly (Bales et al., 2005, 2006).

In this study we assess the effects of age, sex, reproductive condition and season on HCC in a wild New World primate, the common marmoset (*C. jacchus*, Callitrichinae). Common marmosets are small monkeys (adult body mass = 265−325 g; Garber et al., 2019) endemic to forested habitats of two highly distinct biomes in northeastern Brazil:the Caatinga (CAT) and the Atlantic Forest (AF) (Garber et al., 2019; Rylands, Coimbra-Filho & Mittermeier, 2009). The CAT is a semi-desert biome characterized by a hot (daytime temperatures in the dry season commonly exceed 33 °C) and extended (5–8-month-long) dry season, limited rainfall (250–1,200 mm per year), drought-resistant plant species, and reduced productivity compared with the AF, which is a region of high biodiversity, higher annual rainfall (1,400–2,000 mm per year) and high

primary productivity (Araújo, Castro & Alburquerque, 2007). Based on biogeographical and genetic evidence, it appears that ancestral common marmosets first invaded a Caatinga-like biome some 800,000 years ago (Buckner et al., 2015).

Marmosets (and their close relatives the tamarins) exhibit a highly derived set of reproductive traits that distinguish them from other primate taxa (Garber et al., 2016; Schiel & Souto, 2017). These traits include the production of dizygotic twin offspring, the ability to produce two sets of twin litters per year, and a system of cooperative infant caregiving provided principally by adult males (Garber et al., 2016). Adult males carry, provision and guard the group's infants (Garber, 1997). Studies of several tamarin and marmoset species indicate a significant positive relationship between the number of adult male helpers per group and infant survivorship (Garber, 1997; Koenig, 1995).

Common marmosets live in small multimale–multifemale social groups composed of 5–16 individuals (Garber et al., 2019; Yamamoto et al., 2009). Wild groups contain from one to six adult females and one to five adult males. Regardless of the number of adult females per group, generally only a single female in each group breeds (Garber et al., 2019; Schiel & Souto, 2017). Reproductive competition among resident females for the sovereign or primary breeding position is high (Garber, 1997), and the breeding female is socially dominant to all other group members (De la Fuente et al., 2019). In both the AF and the CAT, a breeding female can produce two litters or a total of four offspring per year (Garber et al., 2019).

Given that the CAT represents an extreme environment characterized by high heat and water stress and reduced plant productivity, especially during the extended dry season, we examined and compared HCC, an indicator of overall health and HPA axis activity, among breeding female, non-breeding adult female, adult male and juvenile CAT common marmosets.

Based on the existing literature, we hypothesized that juveniles would have higher HCC than adults (age effect) and that marmosets, regardless of age or sex, would show higher HCC during the dry season compared to the wet season. Given the joint role that mothers and helpers play in infant care giving, we did not expect differences in HCC between adult males and females; however, given the high nutritional costs of producing twin infants, we expected breeding females to experience increased HCC compared to non-breeding females. Finally, assuming that juvenile marmosets are more susceptible to environmental stressors (climate, access to resources, predation) than adults, we expected high rates of juvenile mortality in our study population.

Method

From February 2015 to July 2018, we trapped, measured, weighed, marked and collected shaved hair from the back of the neck of 61 common marmosets belonging to ten groups inhabiting the Baracuhy Biological Field Station (7°31′42″S, 36°17′50″W) in the state of Paraíba in northeastern Brazil. The field site is a 400-ha thorn scrub Caatinga forest averaging 337 mm of rainfall per year (based on 85 years of data collected by the Instituto Nacional de Meteorologia-INMET). During the 3 years of our study, rainfall averaged 271 mm (SD ± 124 mm) and temperatures reached or exceeded 33 °C (91 °F) on 169 days (± 38) per year (based on an average of 330 days of data collection per year, INMET), making this one of the driest and hottest field sites inhabited by any species of nonhuman primate.

The monkeys were trapped using the Peruvian Capture Method, which involved habituating each marmoset group to a single large trap containing 10 separate compartments, each with its own individually operated door (Garber et al., 2019, for additional information). The traps were baited with bananas and during each capture session we were able to trap all or most group members. Once the group was trapped, we removed one marmoset at a time, injected

the individual with ketamine HCL (50 mg/mL, dosage for juveniles = 0.02 mg and dosage for subadults and adults = 0.04 mg), collected biomedical information, and obtained a shaved hair sample from the back of the animal's neck. The hair was immediately placed in a paper envelope, labeled with the marmoset's identification number, age, sex and date of capture, and stored in a dry container at the field site. Marmoset hair samples were collected in the dry season (February/March) and in the wet season (July/August). Given that HCC is an indicator of stress occurring over a period of weeks or months, our trapping procedure is not expected to have a direct effect on our results.

In order to avoid resampling hair from the same individual during the same trapping season, all trapped individuals were implanted subcutaneously with an RFID microchip for permanent identification (Biomark HPT8 pit tags). During retrapping, we confirmed the identify of each individual using a microchip reader (Biomark 601 Handheld Reader). In addition, adults were fitted with a uniquely color-coded collar for field identification. Juveniles were too young to collar and instead a segment of their tail was shaved for purposes of field identification. Thus, we are confident that we did not collect duplicate samples from the same individual during the same trapping season of the same year. We sampled a total of 61 marmosets: 40 in the wet season and 26 in the dry season. Duplicate samples were obtained from three individuals. One marmoset was sampled twice (wet and dry season), and two individuals were sampled three times (twice in two distinct dry seasons, once in the wet season). We treated these samples as independent for analysis because they were collected between 4 and 24 months apart. Based on the length of the shaved hair samples (12–14 mm) used for analysis, and the fact that hair growth in captive marmosets is approximately .5 cm per month (Phillips et al., 2018), we estimate that the HCC represents marmoset physiology during the preceding 8–12 weeks.

Individuals were categorized as infant (≤4 months of age), juvenile (>4–11 months of age, subadult (12–15 months of age) or adult (>15 months of age) based on body mass, limb, body and genital measurements, and patterns of deciduous and permanent dental eruption (Hershkovitz, 1977). Adults were defined as reproductively mature individuals with all permanent dentition fully erupted (mean weight of adult males and females was 280 g; Garber et al., 2019). Subadults were individuals who had not attained full adult body mass (mean weight of subadults (n = 4) was 234 g) and without canines fully erupted. Juveniles were defined as individuals with a mixture of deciduous and permanent teeth, first permanent molar fully erupted and permanent canine had not yet erupted (Hershkovitz, 1977). Mean juvenile male and female body mass was 162.5 g, n = 21). Infants were not used in this analysis. Although marmoset groups in our population contained from one to four adult females, only one female in each group produced offspring. Adult females were classified as either pregnant/lactating or nonpregnant/nonlactating at the time of capture.

In addition, we conducted field censuses every 2–3 months via behavioral observations and occasional retrapping of 6 focal study groups in order to assess changes in their size, age and sex composition due to births, immigration/emigration, and disappearances. We used this information to obtain estimates of infant mortality (these results were published in Garber et al., 2019) and presumed juvenile mortality (i.e., the absence of juveniles previously present in the group). Given that it was not possible to obtain complete counts of all groups during each census, our data do not permit us to determine the degree to which season had an affect on juvenile mortality.

Permission to trap and collect biological samples from the marmosets was provided by the Federal Rural University of Pernambuco, Brazil (CEUA license number 135/2014 and

SISBIO license number 46770-1). Permission to collect behavioral data on the marmosets was approved by the The University of Illinois (IACUC Protocol #14263).

Hair Cortisol Concentration

Hair samples were stored at ambient temperature out of direct light in opaque paper envelopes until processed. Stored under these conditions, hair cortisol is stable (Greff et al., 2019; Yamanashi et al., 2016).

The protocol for extracting cortisol from hair followed Phillips et al. (2018), which was based on a procedure developed by Meyer et al. (2014). After processing, samples were diluted with PBS 1:40 before being analyzed in duplicate via enzyme immunoassay (EIA) using a commercially available expanded range high sensitivity salivary cortisol kit (#1-3002; Salimetrics, State College, PA, USA). Details of the dilution determination and parallelism tests for validation of the procedure for common marmosets are described in Phillips et al. (2018). We converted the obtained values (μg/dL) to pg/mg for analysis. Inter- and intra-coefficients of variance were 11% and 5%, respectively.

Data analysis

We used ANCOVA to determine if there was a significant effect of (a) sex, age class, or season on HCC controlling for body mass and (b) reproductive condition (reproductively active or not reproductively active) and season on HCC. Adult females who had given birth within 1 month of hair sample collection and lactating or were pregnant as indicated by uterine palpation during the physical exam at the time of sample collection were classified as reproductively active. Analyses were conducted in SPSS 26.0 and α was set at 0.05.

Results

Overall, HCC ranged from 231 to 4295 pg/mg (Table 1). We found that the covariate, body mass, was not significantly related to HCC, F (1, 57) = 0.070, p = 0.792, ηp2 = 0.001. The predicted main effects of age class and season were significant (age class: F (1, 57) = 8.604, p = 0.005, ηp2 = 0.131; season: F (1, 57) = 5.449, p = 0.023, ηp2 = 0.087). Juveniles (M = 2464.71, SE = 196.54) had higher HCC than did subadults and adults (M = 1222.00, SE = 121.23; Fig. 1A). Across all individuals, HCC was higher in the dry season (M = 1816, SE = 164) than in the wet season (M = 1218, SE = 168; Fig. 1B).

Contrary to our prediction, sex was found to influence HCC, F (1, 57) = 4.95, p = 0.03, ηp2 = 0.080, with females showing higher HCC (M = 1823, SE = 197) than males (M = 1379, SE = 151; Fig. 1C). All interactions were non-significant and irrelevant to our hypotheses, all F < 2.357, $p \geq 0.13$, ηp2 ≤ 0.040.

Table 1: Hair cortisol concentration (HCC) in adult and juvenile female and male common marmosets during the wet and dry seasons in the Caatinga forest of northeastern Brazil. Adult females were assigned to pregnant/lactating (P/L) or nonpregnant/nonlactating (NP/NL) states. Superscripts indicate data from three individuals who were sampled repeatedly. * Not applicable.

Season	Age category	Sex	Reproductive state	Body mass (g)	HCC (pg/mg)	Individual ID
Wet	Adult	F	NP/NL	250	794.12	M16
			NP/NL	275	789.23	M17
			P/L	308	535.58	M26
			P/L	320	2,007.78	M32
			NP/NL	290	417.08	M38
			NP/NL	276	677.01	M39
			P/L	297	657.32	M42
			P/L	294	689.13	M44

			NP/NL	279	1,436.53	M511
		M	*	280	328.20	M12
			*	250	366.22	M15
			*	284	1,319.93	M31
			*	245	704.21	M40
			*	244	619.58	M41
			*	282	531.38	M43
			*	288	246.51	M48
			*	264	702.84	M491
	Subadult	M	*	241	1,671.80	M33
	Juvenile	F	*	160	3,491.93	M34
			*	169	2,259.61	M351
			*	162	966.97	M45
		M	*	190	1,181.82	M13
			*	200	1,518.40	M14
			*	120	1,933.15	M18
			*	110	2,084.69	M19
			*	172	1,029.87	M46
			*	119	3,246.44	M47
Dry	Adult	F	P/L	281	817.90	M512
			P/L	357	966.97	M53
			P/L	313	2,741.55	M513
			P/L	315	4,295.46	M63
			P/L	294	2,619.59	M65
			NP/NL	262	997.26	M70
			P/L	297	2,889.62	M72
			NP/NL	294	1,876.08	M73
			NP/NL	247	2,061.67	M81
		M	*	260	933.43	M22

		*	270	231.02	M23
		*	280	970.74	M25
		*	255	631.93	M492
		*	275	686.39	M52
		*	286	835.84	M54
		*	284	466.64	M57
		*	284	1,177.37	M493
		*	284	2,073.61	M64
		*	257	1,021.90	M66
		*	257	585.23	M71
		*	313	1,392.48	M74
		*	271	2,011.95	M76
		*	308	1,671.94	M79
		*	265	2,045.73	M80
Subadult	M	*	215	852.42	M27
		*	233	1,092.49	M28
		*	247	1,959.06	M75
Juvenile	F	*	234	2,113.26	M352
		*	168	3,024.24	M56
		*	162	3,523.09	M58
		*	159	2,793.13	M67
		*	189	1,583.09	M69
		*	183	2,900.54	M82
		*	198	2,808.69	M83
	M	*	172	1,419.66	M24
		*	140	2,725.76	M59
		*	141	2,541.69	M62
		*	139	2,674.82	M77
		*	125	4,088.90	M78

We next evaluated whether there was an effect of reproductive condition and season on HCC in adult females. Body mass was not a significant covariate in this analysis, F (1, 12) = 0.36, p = 0.056, ηp2 = 0.086. Female reproductive condition did not significantly affect HCC, F (1, 12) = 1.260, p = 0.284, ηp2 = 0.179. However, HCC did vary by season in adult females, with HCC higher in the dry season (M = 2107.09, SE = 286.6) than the wet season (M = 933.53, SE = 126.9), F (1, 12) = 4.292, p = 0.061, ηp2 = 0.478 (Fig. 1D).

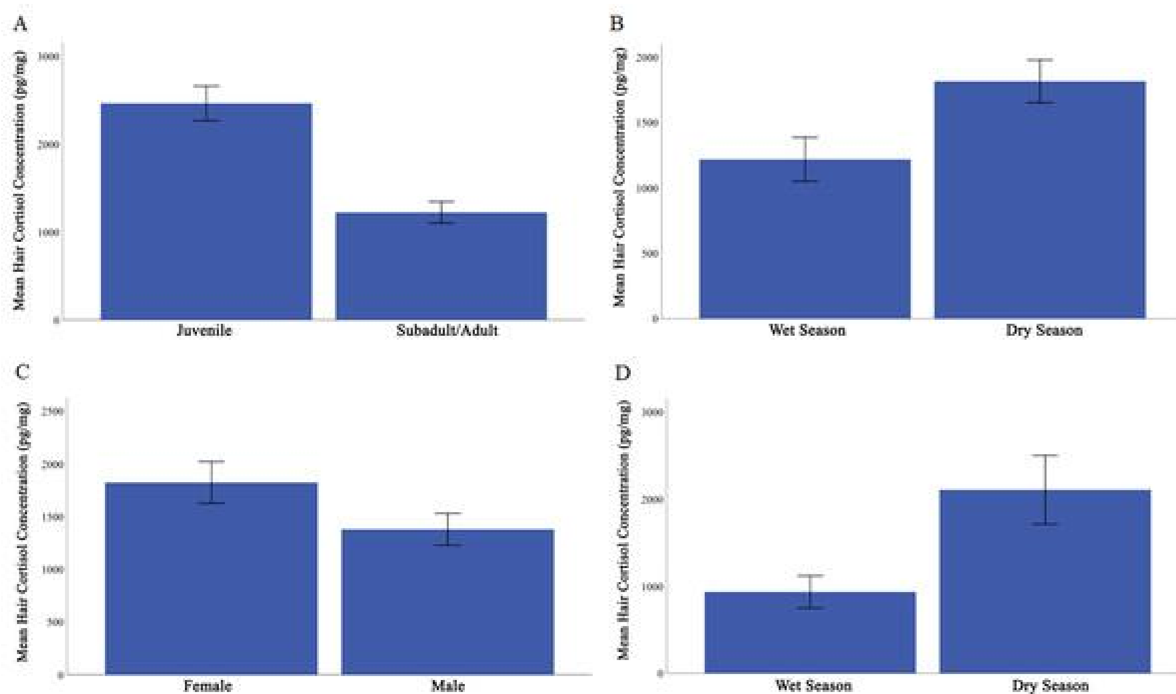

Figure 1: Mean hair cortisol concentration from Caatinga common marmosets. (A) Juvenile and subadult/adult. (B) All individuals during the wet season compared with dry season. (C) Females compared with males. (D) Adult females during the wet and dry seasons. Error bars represent ± 1 standard error.

Finally, during our study we documented 21 birth events involving eight common marmoset females. The number of litters per female ranged from one to five and the number of infants we observed was 39. We do not know whether all births produced twins (total 42 infants) and in three cases one of the twins died shortly after birth (a few days or 1–2 weeks) or whether

three of the 21 births produced singletons. Twenty-nine of the infants survived past weaning (4 months of age), resulting in 25–30% infant mortality. Based on group censuses and retrapping, we found that 15 of the surviving 29 offspring were present in their natal group by 1 year of age (end of juvenile period). Therefore, assuming that juveniles who disappeared from their natal group had died, we estimated that juvenile mortality in this CAT marmoset population was 48%.

Discussion

In this study, we examined HCC in wild adult female, adult male, subadult and juvenile common marmosets naturally inhabiting an extremely hot and dry region of northeastern Brazil. Across the 3 years of our study, rainfall varied from only 177–412 mm per year, with 64–72% of all precipitation occurring during a 3 month period. During the months of October through April, temperatures at our field site reached or exceeded 33 °C on 65–86% of days (Instituto Nacional de Meteorologia-INMET).

Our most important findings were that across all age and sex classes, HCC was higher in the dry season compared with the wet season, female reproductive condition (pregnant and lactating vs. not reproductively active) did not predict HCC, adult females had higher HCC than adult males, and juveniles had higher HCC than adults. Elevated HCC in CAT common marmosets during the dry season appears to represent a physiological response to an extended period of heat, water and, possibly, nutritional stress. The majority of CAT trees are deciduous during the dry season. During this period, fleshy fruits and insects, which account for over 50% of yearly marmoset feeding time, are in limited supply (Amora, Beltrão-Mendes & Ferrari, 2013). CAT marmosets respond to dry season conditions by devoting almost 20% of their total daily activity budget to exploiting exudates (gums and saps, a renewable resource), increasing their dependance on cactus flesh as a reliable source of water and nutrients, resting during the

hottest times of the day, seeking refuge in shaded rock crevices, and licking the dew that collected on leaves in the early morning (Abreu et al., 2016; De la Fuente et al., 2014; Garber et al., 2019). In addition, compared to Atlantic Forest common marmosets, adult Caatinga common marmosets weight 11–20% less, and exhibit a higher surface area relative to body mass, which may play an important role in dissipating body heat in order to maintain thermal homeostasis (Garber et al., 2019). The fact that both males and females exhibited significantly higher HCC in the dry season compared to the wet season, lends support to our hypothesis that during this 5–8 month period of the year, CAT marmosets are characterized by increased levels of ongoing physiological stress.

Effects of Sex and Reproduction on HCC

A second important finding was that, unrelated to season, female reproductive condition (pregnancy/lactation vs. nonpregnant/nonlactating) did not significantly affect HCC in common marmosets. Given their potentially high reproductive output and the energetic costs of reproduction (production of twin offspring, ability to produce two litters per year, combined weight of twins at birth representing 15–20% of maternal weight and increased water intake required to nurse twin infants (Garber & Leigh, 1997), we expected breeding females to have elevated HCC compared with nonbreeding females. However, this was not the case. The fact that adult females, regardless of reproductive condition, were found to have significantly higher HCC compared with adult males suggests that wild female common marmosets face a combination of social and environmental stressors associated with competitive interactions resulting in infertility or subfertility in subordinate adult females (Saltzman et al., 2008) and high reproductive output and reproductive sovereignty in breeding females, that differ from stress loads encountered by males, and contribute to elevated HCC. We note that Phillips et al. (2018) found no evidence of

adult sex-based differences in HCC in captive common marmosets. Her groups, however, were housed in non-currently reproducing family groups containing a single adult male and single adult female. The absence of stressors associated with female reproductive competition and the nutritional costs of producing twin infants in this captive population, may help to explain sex differences in HCC present in our wild marmoset population but absent in captive individuals.

Elevated HCC in Juveniles

Our finding that cortisol decreased with age in common marmosets is consistent with reports from several wild and captive primate populations (Fourie & Bernstein, 2011; Gesquiere et al., 2005; Pryce, Palme & Feldon, 2002). If elevated cortisol in juveniles compared with adults is solely a function of age-specific differences in physiology (i.e., elevated in juveniles in response to lower corticosteroid binding globulin concentration), then it is not a true indicator of increased physiological stress, nutrient and/or water deficiency, or decreased health. However, HCC was found to correlate negatively with survivorship in a population of wild gray mouse lemurs (*Microcebus murinus*; (Rakotoniaina et al., 2017). In this species, survival rates of juveniles during the winter were 19–40% lower compared with those of adults (Kraus, Eberle & Kappeler, 2008). Rakotoniaina et al. (2017) argued that HCC in gray mouse lemurs "may underlie demographic fluctuations of natural populations."

In the case of common marmosets, AF and CAT populations differ in certain critical aspects of demography that may provide an ecological explanation for the consequences of elevated HCC in juveniles. In the CAT, despite the fact that females commonly produce two to four infants per year, group size averages only 6 ± 1 individuals, including three to four adults (based on 13 groups see Table SII in Garber et al., 2019). Our largest CAT group totaled nine individuals. In contrast, although female AF common marmosets also produce two to four

infants per year, group size averages 9 ± 3 individuals (including an average of five adults), with the largest groups containing 16 individuals (Table SII in Garber et al., 2019). Therefore, we asked the question, how can CAT marmosets maintain such small group sizes if the breeding female produces two to four offspring per year.

Two factors that affect group size are infant mortality and juvenile mortality. Infant mortality in both AF and CAT common marmoset populations averages 25–30% (Garber et al., 2019). Although we lack quantitative data on juvenile mortality in AF common marmosets, 48% (14/29) of the juveniles in our CAT population disappeared from their natal groups. Given that each was less than 1 year of age when they disappeared, and that during the 3 years of our study no juvenile was observed to immigrate into any of our six focal groups, we argue that the most plausible explanation is that these immature individuals did not survive the juvenile period. This is supported by demographic data on a close relative of common marmosets, the Amazonian saddle back tamarins (*Leontocebus weddelli*, formerly *Saguinus fuscicollis weddelli*). In Weddell's saddleback tamarin, individuals migrate from their natal groups between 20 and 45 months of age (Goldizen et al., 1996). Mortality to 1.5 years of age in this tamarin population was 14% (9 of 63 natal individuals), which is considerably lower than in our CAT marmoset population.

Mortality risk during juvenility, a period during which young individuals have attained locomotor and dietary independence but are not yet fully adult-like in body mass, social skills and cognitive development, has been a focus of primate life history theory (Leigh & Blomquist, 2011). And although quantitative data on juvenile risk in wild primates are extremely limited, it is assumed that factors such as predation, disease, and/or reduced competitiveness in obtaining access to high quality resources are the primary drivers of juvenile mortality (Janson & van

Schaik, 1993). Our CAT field site contains at least seven potential marmoset predators including two species of raptors (burrowing owls, Athene cunicularia; Southern crested caracaras, Caracara plancus), two species of predatory snakes (boas, Boa constrictor; rattlesnakes, Crotalus sp.) and three species of carnivores (oncillas, Leopardus triginus; Crab-eating foxes, Cerdocyon thous; Domesticated dogs, Canis familiaris). Each of these predators are present year-round (Cardoso da Silva, Leal & Tabarelli, 2017; Passos Filho et al., 2015). Moreover, we note that compared with other taxa of small New World monkeys (e.g., squirrel monkeys, titi monkeys and night monkeys), immature tamarins and marmosets are characterized by an extended period of dietary dependance and a pattern of delayed brain growth (Garber & Leigh, 1997) during which adult helpers provision the young with fruits and insects well into the juvenile period (Ferrari, 1992; Schiel et al., 2010). What is less clear, however, is the degree to which the transition from provisioning to dietary independence represents a critical period in juvenile survival, especially in a hot, dry and food-limited environment like the CAT. Elevated HCC in juvenile CAT marmosets, therefore, may represent an honest indicator of environmental stress that is consistent with the high level of juvenile mortality in our study population. However, additional data, including quantitative information on changes in food availability and distribution during the wet and dry seasons, mortality rate, and HCC, are necessary to evaluate this hypothesis.

Conclusion

Common marmosets living in the harsh Brazilian Caatinga, which is characterized by an extended hot and dry season and low primary productivity, experienced increased levels of chronic and/or nutritional stress as reflected in HCC. Juveniles had higher HCC than adults; and regardless of age or sex, and individuals experienced higher HCC during the dry season compared with the wet season. Given the set of environmental stressors present in the Caatinga

(heat, water, and food stress, especially during the dry season and year-round predation risk), we suggest the high levels of HCC found in juveniles is an indicator of chronic stress and coincides with high rates of juvenile mortality that characterize this population.

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Competing Interests

- The authors declare that they have no competing interests.

Author Contributions

- Paul A. Garber conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Anna McKenney conceived and designed the experiments, performed the experiments, authored or reviewed drafts of the paper, and approved the final draft.
- Evelyn Bartling-John performed the experiments, authored or reviewed drafts of the paper, and approved the final draft.
- Júlio César Bicca-Marques conceived and designed the experiments, performed the experiments, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- María Fernanda De la Fuente performed the experiments, authored or reviewed drafts of the paper, and approved the final draft.
- Filipa Abreu performed the experiments, authored or reviewed drafts of the paper, and approved the final draft.
- Nicola Schiel conceived and designed the experiments, performed the experiments, authored or reviewed drafts of the paper, and approved the final draft.

- Antonio Souto conceived and designed the experiments, performed the experiments, authored or reviewed drafts of the paper, and approved the final draft.
- Kimberley A Phillips conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.

Animal Ethics

The following information was supplied relating to ethical approvals (i.e., approving body and any reference numbers): Our protocols to trap the marmosets and collect hair samples were approved by the Ethical Committee on Animal Experimentation (CEUA) of the Federal Rural University of Pernambuco, Brazil (CEUA license number 135/2014 and SISBIO license number 46770‑1). The University of Illinois (IACUC Protocol #14263) and SISBIO (license 46770‑1) approved procedures associated with behavioral observations.

Data Availability

The following information was supplied regarding data availability: The hair cortisol concentration for each individual and independent variables sex, age group, and season are available in Table 1.

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The Effect of Body Region on Hair Cortisol Concentration in Common Marmosets (*Callithrix jacchus*)

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Running Title: Effect of Body Region on Hair Cortisol Concentration in Marmosets

Abstract

Glucocorticoid hormones are involved in multiple physiological processes, including the conversion of sugar, fat, and protein stores into usable energy and the inhibition of swelling and inflammation. Glucocorticoid hormones, including cortisol, are regulated by the hypothalamic-pituitary-adrenal (HPA) axis. Quantification of cortisol in hair provides a valid, non-invasive, retrospective biomarker of HPA axis activity and therefore information on animal health, including responses to environmental and social stimuli. Common marmosets (Callithrix jacchus) are a valuable research model in neuroscience and aging research due to their adaptivity, physiological characteristics, and ease of handling. Analysis of long-term HPA activity may be desired; thus, it is essential to obtain valid and reliable measurements. We investigated if hair cortisol concentration varied depending on the body region in marmosets. Hair was collected from the back and tail of nine (9) adult common marmosets during annual health screenings (male n=3; female n=6) and analyzed for cortisol via methanol extraction and enzyme immunoassay. Hair cortisol concentration differed between tail and back regions, with the tail having significantly higher cortisol concentration.

Abbreviations and Acronyms:
HCC: hair cortisol concentration; **HPA:** hypothalamic-pituitary-adrenal

Introduction

Common marmosets (Callithrix jacchus) are small New World Primates endemic to Northeast Brazil. Marmosets are an increasingly important model in behavioral and biomedical research, including studies of models of aging and neurological disease, due to their similarity to humans in physiology, neuroanatomy, cognition, and sociality.[11; 15] The increased utilization of marmosets in biomedical research, particularly studies of aging or development, has resulted in their prominence in longitudinal studies. The ability to frequently and non-invasively assess glucocorticoid hormones as one measure of an animal's response to environmental or social stimuli is vital in longitudinal research.

Glucocorticoids are regulated by the hypothalamic-pituitary-adrenal (HPA) axis, a complex neural negative-feedback system that perceives environmental stimuli and regulates hormones accordingly. Cortisol is a the dominant glucocorticoid hormone found in most mammals[23] and is involved in a number of physiological processes including the conversion of sugar, fat, and protein stores into usable energy and the inhibition of swelling and inflammation.[19] Quantification of cortisol concentration can provide valuable insight as to how an animal is responding to its environment and experimental manipulation, and provide a valid, retrospective biomarker of HPA axis activity and therefore, individual health.[4; 5; 14; 24]

Cortisol concentration can be quantified from blood, saliva, urine, feces, and hair. Each medium provides different information concerning sample type (point, or state, or chronic), time frame, and invasiveness.[20] Point, state, and chronic sample types refer to the time frame from which cortisol is sampled after it has been secreted from the adrenal cortex. Point measures include blood and saliva and reflect a time frame of minutes; state measures, which include urine and feces, reflect a time frame from hours to a day; and finally, chronic samples reflect a time

frame over weeks or months, depending on the length of hair assayed. Retrospective, long-term HPA axis activity can be quantified via hair, as this provides a chronic sample of cortisol concentration over weeks or months, depending upon the length of hair analyzed20 and the growth dynamics of the hair.18 Blood-born substances, such as cortisol, diffuse from blood capillaries into hair follicle cells.[7; 25] Only cortisol molecules that are not bound to proteins diffuse into blood capillaries; therefore, hair cortisol concentration (HCC) represents unbound cortisol molecules.[7; 25] Once cortisol molecules enter the follicle cells, they are deposited into the hair shaft.[7] The cortisol deposition process only occurs at a certain time during hair growth. Cortisol deposition rates into the hair shaft over time are directly proportional to blood cortisol concentration.[7]

The stage of growth in a hair follicle influences the deposition process. All hair has three stages of growth: the anagen, catagen, and telogen. The anagen is the active stage when growth occurs; in the catagen or intermediate stage, old hair shafts break down and newer shafts are produced in preparation for the next round of growth; lastly, the telogen stage is the resting phase of hair growth. Most cut or plucked hair samples are expected to be in the anagen phase while shed hair is likely in the telogen phase.[13] Cortisol deposition occurs during the anagen stage as active hair follicles require nutrients via the bloodstream to grow. This slow rate of deposition provides information of HPA activity over a period of weeks or months.[20]

While hair cortisol concentration is increasingly used to assess retrospective HPA axis activity in captive and wild animals, there is currently no standard body region from which to obtain hair samples. Studies report sampling from a variety of regions, including back of the neck, back, shoulder, chest, base of tail, thigh, deltoid, and inter-scapula.[1; 7-9] In this investigation, we sought to determine if HCC was affected by body region in common marmosets. We

compared HCC from two common and readily accessible locations of sample collection in common marmosets, back and tail.

Method

Subjects

We obtained hair samples from nine (9) juvenile (1 year) and adult (3 – 9 years) common marmosets (Callithrix jacchus; male n=3, female n=6) housed at the Southwest National Primate Research Center, Texas Biomedical Research Institute, San Antonio, Texas, USA. These opportunistic samples were obtained from animals as they underwent health screenings before being assigned to other research protocols at the Center. Juveniles were housed in their family groups at the time of sample collection; adults were non[1]breeding and either pair-housed or single-housed for behavioral reasons. All animals maintained visual, olfactory, and auditory access with other marmosets. Marmosets were maintained in accordance with the Guide for the Care and Use of Laboratory Animals. The facility was AAALAC-accredited and all work was approved by the Texas Biomedical Research Institute's Institutional Animal Care and Use Committee. Room temperatures maintained at 80°F (range 76°F - 84°F) and a 12-hour light-dark cycle. Fresh food was available ad libitum and consisted of a purified diet (Harlan Teklad TD130059 PWD), Mazuri diet (AVP Callitrichid 5LK6), and fresh fruit, seeds, and dairy. Further husbandry specifics followed methods outlined in Layne and Power.[17]

Procedure

Veterinary staff collected hair samples from the back and tail region (~100 g from each location; see Figure 1) of all subjects during a scheduled health screening. All animals were sedated for this exam. An electric shaver was used with care not to nick the skin and contaminate the sample with blood. Hair was collected from the back (between the shoulder blades), then tail

(8 cm from the base). The electric shaver was cleaned with disinfectant solution after each use. After collection, samples were stored out of direct sunlight in 15 mL screw-cap polypropylene centrifuge tubes until processing. The procedure for extracting cortisol from hair followed Phillips et al.,[22] which was based on a procedure detailed in Meyer et al.[21]

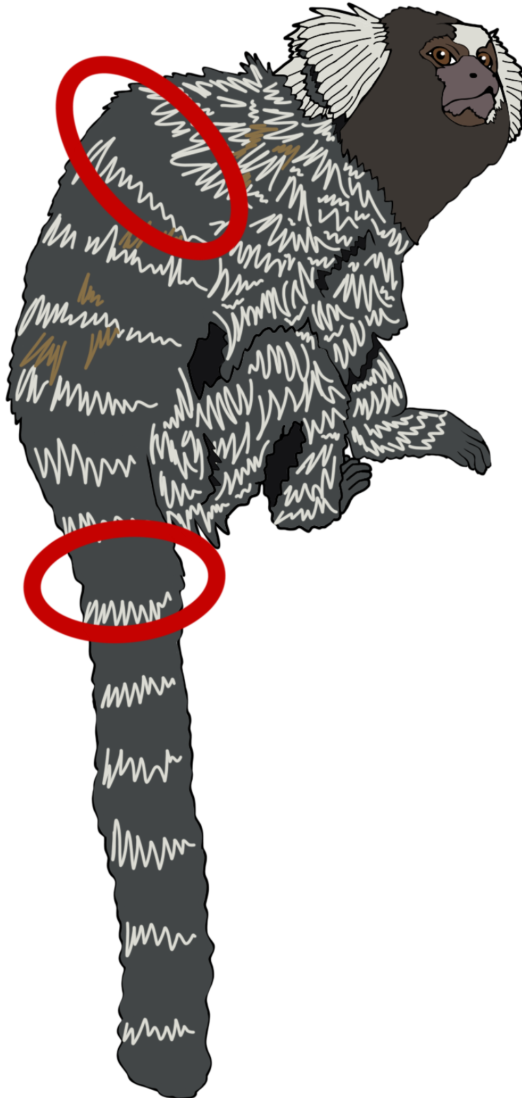

Figure 1. Illustration depicting where the hair was sampled from the back and tail from marmosets.

Processed samples were washed three times in HPLC-grade isopropanol and then air-dried under a hood for 3-5 days. Once dry, samples were ground into a fine powder with a ball mill (MM400; Retsch, Newton, PA). Fifty milligrams of each sample was placed in a 2 mL microcentrifuge tube for methanol extraction. The ground hair incubated while gently rotating in 1.0 mL of HPLC-grade methanol for 24 hours. Samples were immediately centrifuged for two minutes at 14,000 rpm to pellet the powdered hair. Next, we evaporated 600 μL of the sample supernatant for 45 minutes using a nitrogen evaporator. Samples were then reconstituted with 200 μL of assay buffer and diluted 1:40 before being analyzed in duplicate via enzyme immunoassay (EIA) using a commercially available expanded high range sensitivity salivary cortisol EIA kit (#1-3002; Salimetrics; State College, PA). Resulting values (μg/dL) were converted into pg/mg for analysis. Intra-assay coefficient of variation (CV) was 4%, whereas the inter-assay CV was 8.2%. Details of the parallelism tests for validation of the procedure for common marmosets are described in detailed in Phillips et al.[22]

We performed the Shapiro-Wilk test of normality for HCC values obtained from the back and tail before statistical analysis. We conducted a paired-samples t-test, comparing HCC from the back and tail regions in marmosets. We also performed correlational analysis on the HCC values from the back and the tail. Alpha was set at 0.05 and all analyses were performed using RStudio.[26]

Results

The range of obtained HCC in this study is provided in Table 1. All subjects had a cortisol concentration that was lower for hair obtained from the back than from the tail. The Shapiro-Wilk test indicated HCC values for both back and tail were normally distributed (HCC_{BACK} , D(9) =0.958, p = 0.78; HCC_{TAIL} , D(9) =0.962, p = 0.816).

Table 1. Hair cortisol concentration in the back and tail regions, from nine common marmosets.

Sex	Age (yrs)	Back (pg/mg)	Tail (pg/mg)
F	1	3893.7	7937.0
F	1	3227.8	7973.7
F	3	2714.5	7655.2
F	5	5424.6	7288.9
F	5	4303.8	7765.8
F	8	5206.5	8825.6
M	4	3091.9	6772.8
M	4	4718.7	6908.4
M	7	4120.1	6504.6

Visual inspection of the Q-Q plots confirmed normality. A paired-samples t-test confirmed HCC was significantly different between the back and tail regions ($t(8) = 9.43$, $p < 0.001$), with cortisol concentration determined from back hair significantly lower than cortisol concentration obtained from tail hair ($M_{\text{BACK}} = 4077.96 \pm SE_{\text{BACK}} = 314.87$ pg/mg; $M_{\text{TAIL}} = 7514.67 \pm 240.93$ pg/mg). Additionally, there was no overlap between HCC from the back and tail; the highest HCC from the back was still lower than the lowest tail HCC score (Figure 2). HCC values from the back and tail were not significantly correlated ($r(7) = 0.16$, $p = 0.68$).

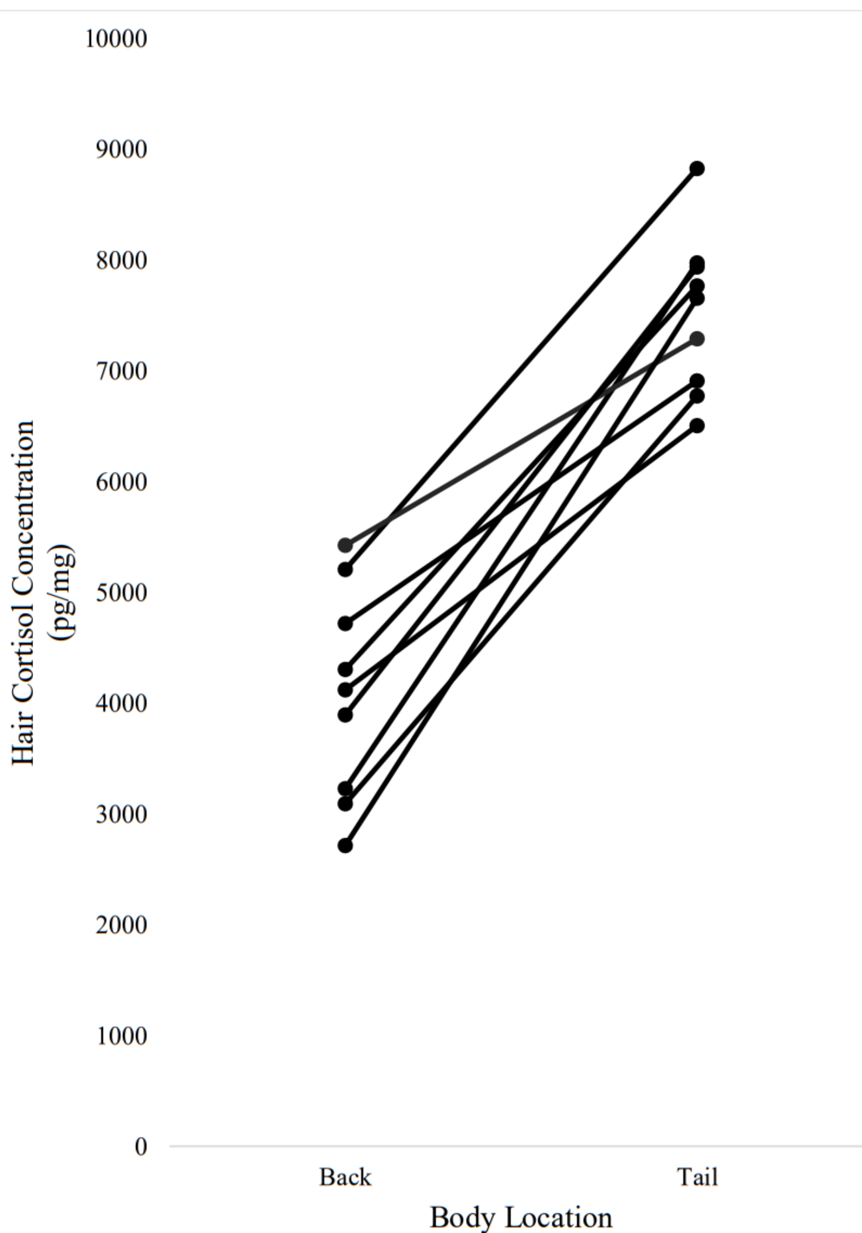

Figure 2. Hair cortisol concentration (HCC) from the back and tail regions in common marmosets. (M_{BACK} = 4077.96 ± 314.87 pg/mg; M_{TAIL} = 7514.67 ± 240.93 pg/mg)

We did not conduct statistical tests to determine if HCC differed in male and female marmosets as a function of body location, as the sample size for males (*n* = 3) was small and thus violated nonparametric testing requirements. However, visual inspection of the data revealed that

overall, females had higher HCC from the tail than males. Additionally, we did not conduct statistical tests to determine if HCC varies as a function of age group (juvenile or adult), as there were only two juveniles in the sample. Visual inspection of these data did not indicate any trend with regards to differences in HCC by age.

Discussion

HCC varied by body region in common marmosets, with samples obtained from the back providing significantly lower values than samples obtained from the tail. These results are consistent with previous research on chimpanzees (Pan troglodytes), yellow baboons (Papio cynocephalus), marmots (Marmota vancouverensis), caribou (Rangifer tarandus granti), reindeer (R. t. tarandus), and grizzly bears (Usrus arctos), which also reported significant differences in HCC as a function of body location.[2; 3; 9; 18; 27]

Why might cortisol vary by body location? Two possible reasons are regional variation in hair growth and regional differences in vascularization. Macbeth et al.[18] considered how timing and patterns of hair growth lead to varying HCC between body regions. In grizzly bears, molting patterns likely played a role in HCC variation by body region, with higher HCC in regions that were preparing to molt. They suggested new hair grew in the neck region before the grizzly bear molted in the neck. Seasonal shedding is not the only determinant of hair growth. While marmosets do not molt like grizzly bears, the molting in grizzly bears highlights that elevated HCC levels are linked to increased hair growth. We therefore suggest these differences found in the present study are due to the timing and pattern of hair growth in marmosets. Hair growth only occurs in the anagen stage, and this is the only hair growth stage where cortisol deposition occurs. Therefore, our data indicate that there are incongruencies in hair stages by body region. Furthermore, we speculate there is a slower rate of hair growth in the tail, thereby providing a

longer period of time for cortisol to be deposited into the hair. Along with hair growth patterns, researchers have theorized that differences in blood flow also contribute to varied HCC by body region.[1; 3] Cortisol is deposited into the hair shaft in proportion to the unbound cortisol in the blood. [7] If blood flow varies by body region, then it is possible that unbound cortisol levels in the blood vary as a result, which in turns affects HCC.

Visual inspection of the data indicated that females had higher HCC from the tail than males. Previous research examining sex differences in HCC in common marmosets, both captive and in the field, has yielded inconsistent results. [12; 22] A field study examining how HCC in common marmosets varied as a function of season, age, and sex, reported significant sex differences, with females having significantly higher HCC than males.[12] However, a study examining HCC in a small cohort of captive marmosets did not report sex differences.[22] Across nonhuman primates, inconsistent results have been reported as to whether HCC varies by sex.[4; 16; 27]

Cortisol levels in New World monkeys are 2 – 10 times higher than Old World monkeys. This difference is also reflected in hair cortisol and believed to be due to the phenomenon of glucocorticoid resistance found in some New World monkeys. Glucocorticoid resistance is the result of species-variation in the glucocorticoid receptor binding affinity and receptor signaling. 6 The HCC values obtained in this study are similar to those reported by Franke et al.10 and Phillips et al.22 These three studies followed the same assay protocol. Phillips et al.22 compared marmoset HCC values across published studies and noted that variation in assay procedure corresponded to differences in obtained values.

These results, combined with previous work[21; 28], highlight the importance of standard protocols and consistent sample collection, both within and across laboratories. Specifically, this

study indicates that intra-individual and inter-individual comparisons of hair cortisol must utilize hair obtained from the same body region. Doing so will allow for greater reliability of comparison of HCC data, During longitudinal investigations or when comparing HCC in marmosets across treatment conditions, hair samples should always be obtained from the same region.

Acknowledgements

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Literature Review

This review covers the general anatomy, signaling, and odorant processing within the olfactory system. As olfaction is reliant upon new and changing stimuli, it is naturally intertwined with adaptation and learning. Upon reviewing the basic anatomy and function of smell, more dynamic components of olfaction -such as adaptation, adult neurogenesis, typical aging, and pathological decline- are discussed to exemplify how smell is inseparable from cognition and aging. As a result, olfaction could contribute to research in cognitive aging. One prominent aging and cognition model is the common marmoset (*Callithrix jacchus*). Marmosets have a different olfactory system from humans, but they have enough physiological similarities to remain valuable research models in olfaction. With a well-developed olfactory system and a proven relevance in cognition and aging, researchers need to establish a standardized olfaction test for marmosets. In doing so, olfactory sensitivity and discrimination could be understood across age groups. Although this thesis mainly sought to understand marmoset olfaction, this work could potentially bridge the gaps in human pathological research and innovate new ways of perceiving well-being through olfaction.

1.0 General Olfaction

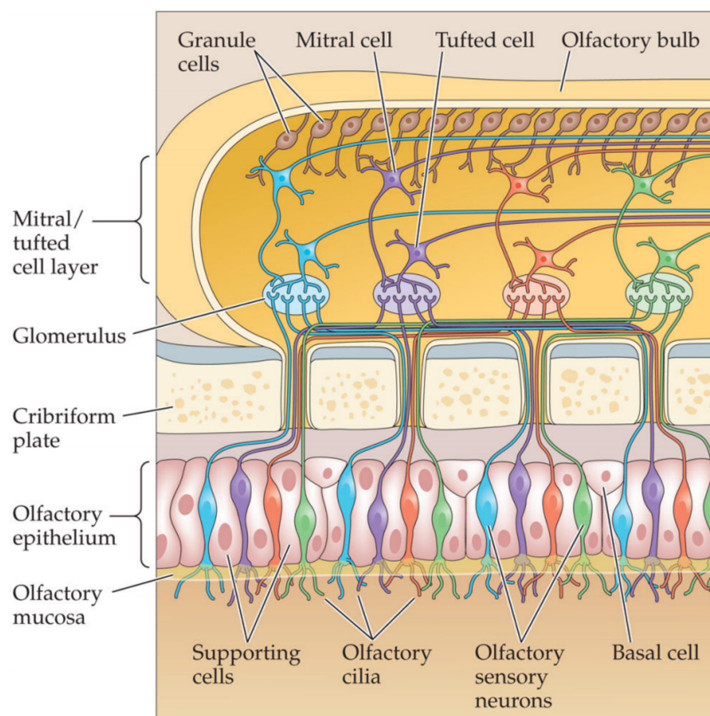
1.1 Anatomy

Each sense is unique in its own right, but olfaction contains functional differences that set it apart from the other sensory systems. Even amongst primates, variations exist in the olfactory system. For instance, all primates have a main olfactory system, but some species (i.e. the common marmoset) also have an accessory olfactory system. Each mammalian olfactory system has evolved to adapt and survive under different conditions (see 3.2 The Olfactory Systems). This work studies marmoset olfaction, which is undeniably distinct from human olfaction.

However, basic olfactory anatomy and function remain similar across all mammals. Thus, a breakdown of general anatomy through human olfaction provides a foundational understanding of smell that will showcase where marmoset olfaction aligns and differs from human olfaction.

Figure 1

Diagram of Olfactory Organization



Note. Depicts the olfactory microanatomy within the olfactory mucus, epithelium, and bulb. From "Chapter 14 Olfaction" by J. Wolfe, K. Kluender, D. Levi, L. Bartoshuk, in R. Herz, R. Klatzy, & D. Merfeld (Eds.), *Sensation and Perception* (5th ed., p. 465), 2017, Oxford University Press. Copyright 2018 by Oxford Publishing Limited. Reproduced with permission of the Licensor through PLSclear.

Before an odorant binds to a receptor site, it must enter the naval cavity from the nostrils or the mouth and be absorbed by the mucus lining the olfactory epithelium. Within the mucus lining and embedded in the olfactory epithelium are olfactory sensory neurons (OSN; Figure 1). The hairlike cilia of the OSN dendrites project through the mucus layer and are the receptor sites

for odorant molecules (Figure 1). These receptor sites are G-coupled protein receptors (Wolfe et al., 2018). The OSNs are the first structures involved in olfactory signal transduction (Wolfe et al., 2018). The olfactory epithelium also contains innervated fibers from the trigeminal nerve (Cranial Nerve V), the nervus terminalis (Cranial Nerve 0), and autonomic fibers from the superior cervical ganglion (Zielinski et al., 1989).

Aside from the receptor cells, the olfactory epithelium contains basal cells, supporting cells, and duct cells from the Bowman's glands (Wolfe et al., 2018). Basal cells act as precursor cells to OSNs (Figure 1; Wolfe et al., 2018). Supporting cells add structural and metabolic support to the epithelium and, among other things, insulate receptor cells from one another (Figure 1; Wolfe et al., 2018). These cells are also involved in the biotransformation of noxious chemicals and in maintaining the ionic environment that surrounds the olfactory receptor cell cilia (Vogalis et al., 2005). When damaged, the olfactory epithelium can be reconstituted from these cells. It is important to note that regeneration declines with age through processes such as telomere shortening (Watabe-Rudolph et al., 2011) and the degree of cumulative damage from previous environmental insults -including those from pollution, viral, and bacterial infections (Harkema et al., 2006). The last structure before the olfactory bulb is the cribriform plate (Figure 1). The cribriform plate is a porous structure that partitions the olfactory epithelium from the olfactory bulb and allows axons to pass from the nose to the brain (Wolfe et al., 2018).

Inside the olfactory bulb are glomeruli, juxtaglomerular neurons, tufted cells, mitral cells, and granular cells (Figure 1; Wolfe et al., 2018). Olfactory sensory organization dictates that each OSN expresses one type of olfactory receptor, which all project to the same type of glomerulus (Figure 1; Wolfe et al., 2018). Glomeruli are spherical conglomerates containing the incoming axons of OSNs (Figure 1; Wolfe et al., 2018). Juxtaglomerular interneurons are around

the glomeruli, producing excitatory or inhibitory signals in response to odorant stimuli (Wolfe et al., 2018). There are multiple types of juxtaglomerular cells that surround a glomerulus, including periglomerular cells (Scott et al., 1993; Shipley & Ennis, 1996). Periglomerular cells have excitatory synapses with olfactory receptor neurons; these synapses convey olfactory information from the olfactory epithelium in the nose (Schoppa et al., 1998; Kasowski et al., 1999).

Beneath the glomeruli layer is a layer of tufted cells followed by a layer of mitral cells (Figure 1; Wolfe et al., 2018). All three cell types respond to odorants, but mitral cells are the deepest and respond to odorants the least (Wolfe et al., 2018). Beyond the mitral/tufted cell layer, are granule cells which synapse with mitral cells (Figure 1; Scott et al., 1993; Shipley & Ennis, 1996; Wolfe et al., 2018). Granule cells make up a network of inhibitory neurons that compile information from the mitral cells to identify specific odorants (Wolfe et al., 2018). In summation, the main olfactory system is structured efficiently and specifically to signal numerous odorants.

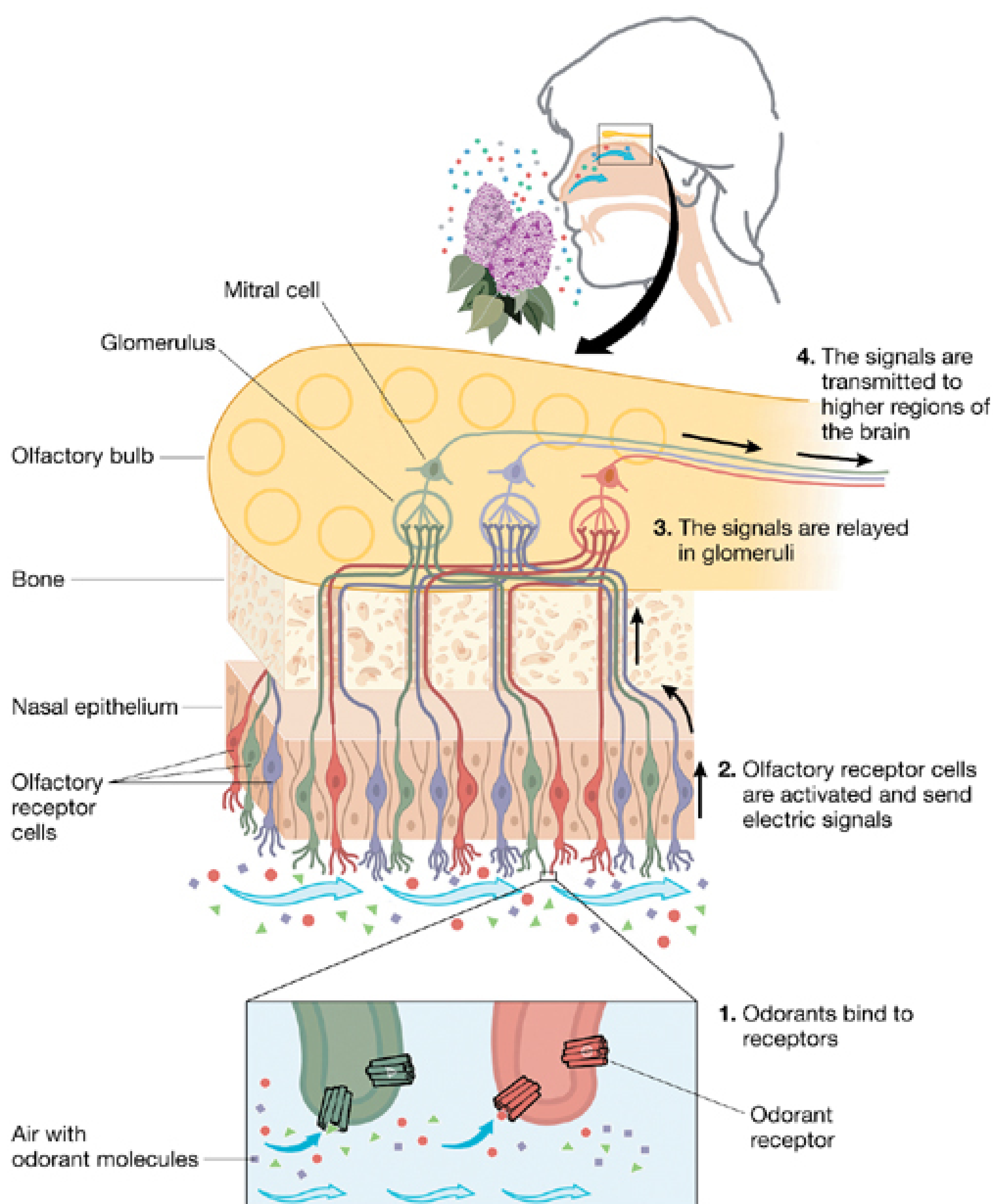
1.2 Signal Pathways and Cortical Processing

The structural organization of the olfactory system provides efficient pathways for signal processing. Olfactory sensation starts when an odorant binds to a receptor in the nose (Figure 2). These receptors are compatible with particular odorants, so many different receptors are in place to allow for optimal olfactory sensation (Wolfe et al., 2018). Olfactory cilia receptors bind to an odorant, triggering an action potential in the OSN (Figure 2). This generated signal travels to the olfactory bulb, where it first reaches the glomeruli; each OSN converges onto two glomeruli (Figure 2; Wolfe et al., 2018). Here, information is processed before being transmitted to mitral and tufted cells. The mitral and tufted cell axons then combine to form the olfactory tract,

carrying the electrical signal out of the olfactory bulb and into the cortex for processing (Wolfe et al., 2018).

Figure 2

Diagram Sequencing the Olfactory Signaling Pathway



Note. Diagram begins with the inception of an olfactory sensory signal and ends with the transmittance of that signal to cortical regions for processing. "The Scent of Life" by A. Rinaldi, 2007, *EMBO reports, 8*(7), 629 (https://doi.org/10.1038/sj.embor.7401029). Copyright 2017 by

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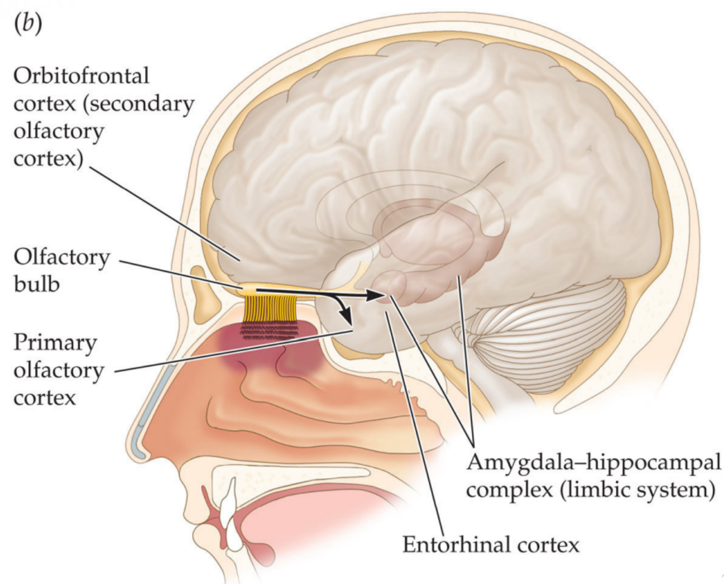
When the olfactory signal leaves the olfactory bulb, it first travels to the primary olfactory cortex -or piriform cortex (Figure 3; Wolfe et al., 2018). The piriform cortex contains the parahippocampal gyrus, amygdala, and interconnected areas called the amygdala-hippocampal complex; the piriform cortex interacts closely with the entorhinal cortex (Figure 3; Wolfe et al., 2018). The entorhinal cortex receives input from other olfactory regions for processing as well. These central structures are part of the limbic system (Wolfe et al., 2018). This connection between olfaction and the limbic system proves unique to other senses and could explain why scents are associated with emotions.

The final step in cortical processing is storage and memory. After the limbic system, olfactory signals travel to the orbitofrontal cortex (Figure 3). The orbitofrontal cortex has been linked to the conscious experience of olfaction, integration of pleasure and displeasure of food, and hedonic judgment (Wolfe et al., 2018). Therefore, this cortex provides the increased emotional associations experienced with scent.

Figure 3

Diagram of Cortical Regions for Olfactory Processing



Note. From "Chapter 14 Olfaction" by J. Wolfe, K. Kluender, D. Levi, L. Bartoshuk, in R. Herz, R. Klatzy, & D. Merfeld (Eds.), *Sensation and Perception* (5th ed., p. 472), 2017, Oxford University Press. Copyright 2018 by Oxford Publishing Limited. Reproduced with permission of the Licensor through PLSclear.

Odor signaling and processing is proven complex and integrates multiple aspects of cognition. For instance, odors can be felt by stimulating the somatosensory system via polymodal nociceptors (Wolfe et al., 2018). As previously mentioned, the olfactory epithelium is innervated with fibers from the trigeminal nerves (V). The trigeminal fibers carry information from somatosensory receptors in the olfactory epithelium and other facial sensors to the thalamus. Then, information is taken to the somatosensory cortex, where odor information is processed as a feeling. This sensory overlay can cumulatively heighten sensory processing and perception, but it also further demonstrates that sensation and cognitive processing are not separate.

In summation, olfaction and cortical processing are integrated to provide animals a grounded perception of their environment. But how does the olfactory system incorporate

change? Impaired olfactory signaling or processing could indicate damage to cortical regions in the brain. However, the olfactory system is built to withstand a fair amount of change -both harmful or not.

1.3 Plasticity, Adaptation, and Adult Neurogenesis

The basic olfactory structures, pathways, and processing regions are all formed early in life. In fact, most of the nervous system develops prenatally, with some neurogenesis occurring postnatally (Altman & Das, 1966). However, sensory systems are dynamic and undergo constant modifications to adapt to their environment. Therefore, most neuroplasticity in the nervous system arises from expanding upon what already exists. Nevertheless, two brain regions exhibit sustained neurogenesis in adulthood. In this section, plasticity, adaptation, and neurogenesis will build off basic olfactory biology to provide a realistic depiction of the working, dynamic olfactory system.

The sense of smell is essentially a change detector, so it is inherently adaptive. The olfactory system likely gains this adaptive learning from neurogenesis. Although some postnatal neurogenesis occurs, most mammalian neurogenesis occurs embryonically (Altman & Das, 1966; Altman, 1969). Even fewer regions of the brain contain neurogenesis in adulthood (Altman & Das, 1966; Altman, 1969; Luskin, 1993; Lois & Alvarez-Buylla, 1994; Kornack & Rakic, 2001; Zhao et al., 2008). As far as we know, adult neurogenesis in mammals only occurs in two regions of the brain: the subventricular zone of the lateral ventricles and the subgranular side of the dentate gyrus in the hippocampus. Neurons generated in the subventricular zone become granule and periglomerular neurons in the olfactory bulb (Zhao et al., 2008).

Granule and periglomerular cells are associated with primary projections in the olfactory bulb (1.1 Anatomy; Scott et al., 1993; Shipley & Ennis, 1996; Isaacson & Vitten, 2003).

Newborn neurons migrate to the olfactory bulb through chain migrations (Zhao et al., 2008). The cells morph and develop before integrating into the granule cell layer (for granule cells) and glomerular cell layer (for periglomerular cells; Zhao et al., 2008). Neurons generated in the subgranular zone become dentate granule cells -which seem to contribute to new episodic memory formation, the spontaneous exploration of novel environments, and other functions (Zhao et al., 2008). Moreover, new granule cells in the adult hippocampus convey ion channel properties that enable enhanced synaptic plasticity (Schmidt-Hieber et al., 2004). This enhanced plasticity suggests that the generation of both interneurons boosts fine olfactory discrimination (Enwere et al., 2004).

In short, new granule and periglomerular cells play a vital role in olfactory function (Enwere et al., 2004). However, this adaptation to environmental change is not simply a by-product of olfactory stimulation but evidence that olfaction depends upon change. For instance, receptor adaptation is defined as the biochemical phenomenon that occurs after continuous exposure to an odorant, whereby the receptors stop responding to the odorant and detection ceases (Wolfe et al., 2018). Ergo, a smell only remains salient when it is dynamic or unfamiliar. Additionally, exposure to novel scents increases the chances that new olfactory interneurons survive (Petreanu & Alvarez-Buylla, 2002; Rochefort et al., 2002) and become active within the olfactory bulb (Belluzzi et al., 2003; Carleton et al., 2003).

As this neurogenesis is continuous, the amount of olfactory interneurons increases with age (Mirich et al., 2002). Adult neurogenesis is regulated by internal and environmental activity. What's more, these new cells prove necessary for some forms of brain function within the olfactory bulb and hippocampus -which are important for learning and memory (Zhao et al., 2008). Continuous adult neurogenesis demonstrates that an animal actively is learning. Ergo,

adult neurogenesis strengthens the argument that olfaction is intertwined with cognition and learning.

The neural mechanisms behind olfaction have proven complex and dynamic. Behaviorally, animals rely on an adaptive sense of smell. Olfaction provides new information about food, mates, predators, and many other environmental dynamics essential for survival. It is often critical to identify novel and familiar scents via olfactory discrimination. Olfaction is unique to other senses because it contains neurogenesis. For this reason, olfactory tests are valuable for measuring various adaptive and learning functions. However, even neurogenesis cannot prevent the progressive, age-related decline in smell. Previous research on mice has suggested that olfactory decline with age is due to an increased number of olfactory interneurons (Mirich et al., 2002).

To reiterate, mammals do share basic olfactory functions, but a mouse's olfactory system is different from a human or marmoset olfactory system. Adult neurogenesis is significantly different between mice and marmosets. Indeed, marmosets showed similar baseline and age-related function to adult neurogenesis in humans (Bunk et al., 2011). According to Bunk et al. (2011), marmosets demonstrated adult neurogenesis in the dentate gyrus in the hippocampus, subventricular zone of the lateral ventricle; as a result, newformed periglomerular and granule cells were found in the olfactory bulb. Although these commonalities advocate for the common marmoset research model, marmoset olfaction still differs from human olfaction. Specifically, marmoset and mouse olfaction both have something that humans do not: an accessory olfactory system (3.2 The Olfactory Systems).

2.0 Aging and Olfaction

2.1 Typical Human Olfactory Declines with Age

Considering olfaction is the only sensory system that integrates new neurons in adulthood, it represents change and adaptation throughout the lifespan. Admittedly, humans are not as scent-driven as the common marmoset. All the same, olfaction provides plentiful information about adaptation, learning, and well-being. Olfaction is completely reliant upon change and adaptation, so the ultimate antagonist to that plasticity is the rigidity of age.

Changes in olfactory sensitivity and discrimination occur as a typical function of age in humans. Studies in the US show that half of people between the ages of 65-80 demonstrate smell loss (Doty et al., 1984; Duffy et al., 1995; Murphy et al., 2002). Unfortunately, olfactory dysfunction makes life more challenging. In lesser extremes, it can cause dissatisfaction with food flavor (Schiffman & Zervakis, 2002). An odor identification assessment involving recall, identification, and recognition of smells, elderly adults scored low on recall and recognition (Murphy et al., 1997). Reasonably, people assumed that structural changes in the nose cause olfactory decline with age. Indeed, as the olfactory bulb ages, neurogenesis persists, leading to an impaired olfactory system (Doty & Kamath, 2014; Enwere et al., 2004, Rochefort et al., 2002; Mirich et al., 2002; Murphy et al., 1997; Wilson, Schneider, et al., 2007). Beyond the olfactory bulb, areas such as the nose, olfactory epithelium, and higher brain structures also deteriorate with age (Doty & Kamath, 2014).

In the nose, shifting airflow patterns influence nasal engorgement (swelling) and mucus secretions. These pattern shifts are often from air temperature, humidity, exercise, stress, and aversive environmental agents (e.i. toxins or allergens; Frye, 2003). The autonomic nervous system mediates much of the nasal engorgement. Ergo, the sympathetic/parasympathetic

dynamics influence the overall swelling of the nasal capillary bodies. With age comes more balance between the sympathetic and parasympathetic nervous systems. The suprachiasmatic nucleus, a brain center involved in controlling several biological rhythms, changes with age and is suggested to mediate this autonomic nervous system effect (Farajnia et al., 2014).

Moreover, the nasal epithelium undergoes atrophy with age, decreasing in mucosal blood flow and elasticity (Bende, 1983). Another significant age-related change is the decline in the size and number of patent foramina (or “pores”) of the porous cribriform plate (Kalmey et al., 1998). A reduction in size can lead to a pinching off or elimination of olfactory receptor cell axons that enter into the brain from the olfactory epithelium (Kalmey et al., 1998).

Additionally, histological studies of the human olfactory epithelium found age-related decline in the number of olfactory receptors, the epithelium, and alterations in the cellular patterns and zonal distributions of the nuclei of the olfactory receptor and sustentacular cells (Nakashima et al., 1984; Paik et al., 1992). The intermingling of supporting and receptor cell nuclei is common because the respiratory epithelium is replacing olfactory epithelium (Nakashima et al., 1984; Paik et al., 1992). Additional findings showed that a declining olfactory epithelium indirectly affects the olfactory bulb and a number of its laminae -most notably the glomerular layer (Bhatnagar et al., 1987). Some of this decline is due to loss of neuronal components, generalized atrophy, and increased numbers of astroglia; however, most of the olfactory bulb decline seems secondary to a damaged olfactory epithelium from nasal infections, chronic rhinitis, lack of airflow, and exposures to xenobiotics (Meurman, 1950; Liss & Gomez, 1958). All of these progressive, structural, and functional changes within the olfactory system are typical with age. In this next section, olfactory decline is presented as a precursor for pathological disease.

2.2 Pathologies Related to Olfaction in Humans

Rapid olfactory decline has been labeled a precursor of rapid cognitive decline associated with pathological dysfunction (Devanand et al., 2000; Enwere et al., 2004; Wilson, Arnold, et al., 2007). Interestingly, olfactory dysfunction showed some specificity to common neurologic and psychiatric disorders (Martzke et al., 1997). For example, impaired olfactory sensitivity was not seen consistently across patients with Huntington's disease or depression (Doty et al., 1991; Warner et al., 1990). However, impaired olfaction can be found in Alzheimer's and Parkinson's disease (Del Tredici et al., 2002; Devanand et al., 2000; Wilson, Arnold, et al., 2007).

For instance, Wilson, Schneider, et al. (2007) tested cognition and odor identification in older participants and found that low odor identification scores were associated with a lower cognitive baseline. Soon after, Wilson, Arnold, et al. (2007) concluded in another study that identifying odors might precede cognitive impairment and the transition from mild cognitive impairment to Alzheimer's disease. In Devanand et al. (2000), a clinical group of patients with mild cognitive impairment scored low in their olfactory sensitivity, and got diagnosed with Alzheimer's disease in their follow-up appointment. With an association to diseases such as Alzheimer's disease, olfaction further demonstrates an active role in human well-being.

Structurally, odor identification has been associated with the development of neurofibrillary tangles in the entorhinal cortex and CA1 region of the hippocampus (Wilson, Arnold, et al., 2007) -which are considered the first sights of change in olfaction with Alzheimer's disease (Braak & Braak, 1991). Additionally, brain regions involved in olfaction are early sites of Lewy bodies and have been associated with Parkinson's disease, dementia, and impaired olfaction (Del Tredici et al., 2002; Wilson, Arnold, et al., 2007). In summation, rapid olfactory decline is connected to pathological dysfunction. Thus, if researchers perfect an

olfactory test for typical and atypical decline, olfaction could act as an early biomarker for Parkinson's disease and Alzheimer's disease, giving patients more time before the onset of symptoms. After all, the earlier we can identify the symptoms of a pathology, the earlier we can intervene.

3.0 Olfaction in the Common Marmoset (*Callithrix jacchus*)

As olfaction researchers continue to understand typical and atypical aging patterns in humans, animal researchers are working parallel to bridge the gaps in human function. Various animal models are used to study olfactory decline with age. Rodents are the most common mammalian model in research. As such, they provide relevant information about humans for the betterment of society. For instance, Enwere et al. (2004) found that aged mice had fewer new interneurons in the olfactory bulb than their younger counterparts. However, aged mice had more olfactory interneurons -total- than younger mice. With these neuroanatomical differences, aged mice could easily differentiate distinct odors, but had difficulty distinguishing similar odors. This distinction implies a decline in fine olfactory discrimination with age. These findings are relevant to human olfaction, but rodent physiology does not always translate to human function.

3.1 Why the Common Marmoset?

Nonhuman primates have proven essential to bridging the gap between rodent and human functionality. To further understand human olfaction and cognition with age, researchers have used the common marmoset (*Callithrix jacchus*). The common marmoset is a valuable animal model in behavioral research, utilized as a model of aging and neurological disease; their physiological, neuroanatomical, cognitive, and social similarities to humans make marmosets excellent subjects for this pilot study (French, 2019; Kishi et al., 2014; Ross et al., 2019).

3.1.1 Aging Model.

As an aging model, the common marmoset exhibits phenotypical changes similar to a human. Like humans, marmosets show disruption in their executive functioning (Ross et al., 2019); executive functioning consists of various cognitive processes like task switching, response inhibition, and goal-directed attention (Gunning-Dixon & Raz, 2000; Raz et al., 1998). Similarly, marmosets show an altered cardiac function through mean arterial pressure and diastolic pressure (Ross et al., 2019). Marmoset motor behavior, such as leaping and movement about the cage, also declines with age along with VO2 -which is the maximum amount of oxygen bodies can utilize during exercise (Ross et al., 2019). Lastly, immune function noticeably decreases with age in marmosets (Ross et al., 2019). These physiological and cognitive similarities justify marmosets as a prominent model in human aging and cognition.

3.1.2 Olfactory Model.

Conjointly, marmosets are also an excellent model for assessing olfaction, as they use their olfactory system for many functions. As a species, marmosets have a developed odor communication system. Additionally, marmosets seem cognitively capable of adapting to new scents (Smith, 2006). In summation, the well-developed olfactory system and the complicated social interactions and olfactory-driven dynamics confirm that marmosets are an excellent research model for olfaction. In conclusion, common marmosets are a valuable model for this research because they contain behavioral and physiological traits that effectively formulate, deposit, and receive scent stimuli, producing a developed olfactory system (Epple et al., 1993).

3.2 The Olfactory Systems

Thus far, this review has detailed the similarities between the marmoset olfactory system and human olfactory system. As a New World monkey, marmosets possess an accessory

olfactory system in addition to their main olfactory system (Nakajima et al., 1998; Smith et al., 2004; Taniguchi et al., 1992). At first glance, the presence of an accessory olfactory system may dissuade researchers. However, the marmoset model is more physiologically similar to humans than mice; and while the mouse model has proven valuable in olfaction research, marmosets are evolutionarily closer to humans (French, 2019; Kishi et al., 2014; Ross et al., 2019). Moreover, mice also have an accessory olfactory system (Taniguchi et al., 1992), so the presence of an accessory olfactory system should not discourage research on marmoset olfaction. Ergo, marmosets should be the next research model utilized in understanding human olfaction. In doing so, any disparities between marmoset and human olfaction should be understood.

3.2.1 The Main Olfactory System.

In the main olfactory system, scent stimuli travel to the main olfactory bulb. The olfactory epithelium is the receptor organ of the main olfactory system and receives sensory information about odorants and transduces it to the olfactory bulb (Takayuki et al., 1998). However, most main olfactory information is covered in 1.0 General Olfaction because the main olfactory system of a common marmoset is similar to human olfaction.

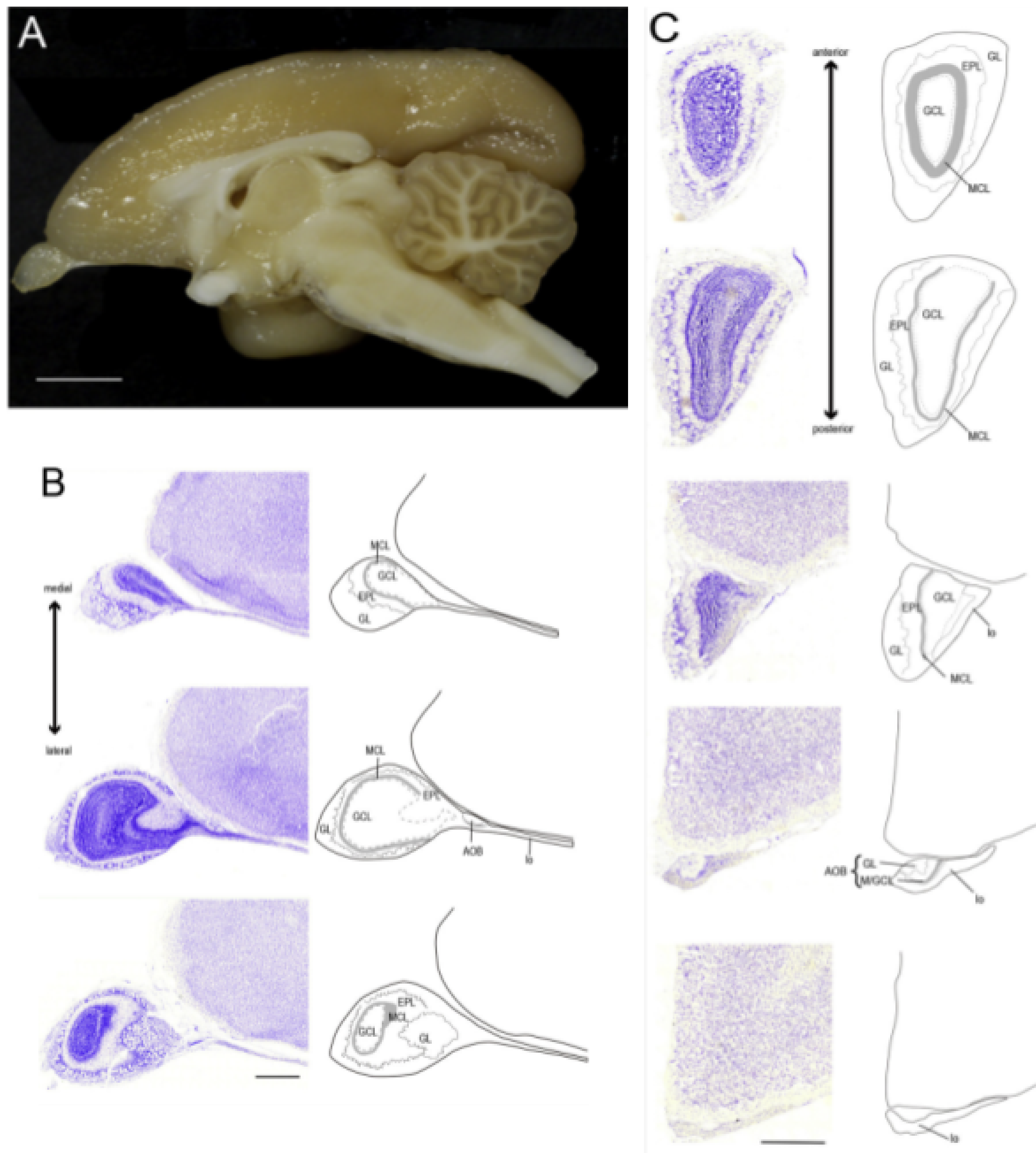
3.2.2 The Accessory Olfactory System.

Dissimilar to humans, marmosets have an accessory olfactory system. The accessory olfactory system functions differently across mammals (Taniguchi et al., 1992). In the accessory system, pheromonal molecules (relating to reproduction and social behavior) are received by the vomeronasal organ and transmitted to the accessory olfactory bulb (Nakajima et al., 1998; Takayuki et al., 1998; Taniguchi et al., 1992). In the common marmoset, the accessory olfactory bulb is located medially to the olfactory tract and posteriorly to the external plexiform layer (Figure 4). The vomeronasal organ interacts with the external world differently across species

(Taniguchi et al., 1992). For instance, some vomeronasal organs detect odorants via the nasal cavity, while other vomeronasal organs activate from odorants in the oral cavity (Halpern, 1987).

Figure 4

Structure of the Marmoset Olfactory Bulb



Note. (A) Midline view of marmoset brain. Scale bar indicates 5 mm. (B) Nissl-stained sections of the olfactory bulb in parasagittal planes and associated schematic drawings. Interval of each panel is 500 m. Scale bar indicates 1 mm. (C) Nissl-stained sections of the olfactory bulb in coronal sections and associated schematic drawings. Interval of each panel is 1000 m. Scale bar indicates 1 mm. AOB, accessory olfactory bulb; EPL, external plexiform layer; GCL, granule cell layer; GL, glomerular layer; lo, lateral olfactory tract; MCL, mitral cell layer; M/GCL, mitral and granule cell layer. From "The Olfactory Bulb and the Number of its Glomeruli in the Common Marmoset (*Callithrix jacchus*)" by K. Moriya-Ito, I. Tanaka, Y. Umitsu, M. Ichikawa, & H. Tokuno, 2015, *Neuroscience Research*, *93*, 160 (https://doi.org/10.1016/j.neures.2014.12.007). Copyright 2015 by Elsevier. Reprinted with permission from Elsevier.

Although humans lack an accessory olfactory bulb, the vomeronasal organ has been found in humans (Johnson et al., 1985). However, the organ itself is no longer very developed and has no sensory epithelium (Johnson et al., 1985). Compared to marmosets, humans did not heavily rely on their sense of smell. As a result, the accessory olfactory system in humans has become useless (Taniguchi et al., 1992). However, Taniguchi et al. (1992) found that the accessory olfactory bulb and vomeronasal system were fully functional in the common marmoset. Therefore, marmosets have a more developed sense of smell because humans have no proven, distinct pheromonal olfactory system. Thus, marmoset olfaction encompasses social (i.e. pheromonal) and non-social odorants. Moreover, an animal like the marmoset, which is heavily reliant upon smell, can be studied to understand how much scent integrates into marmoset well-being.

3.3 Olfactory Behaviors

Like many primates, olfactory cues help common marmosets discriminate between individuals (Smith, 2006). Olfactory discrimination is critical for most social behaviors, such as group relations between kin, parents, or a mate. Phenotypically, marmosets are heavily scent-driven in social contexts and have a distinct scent-mark (Epple, 1986; Epple, 1993; Sutcliffe & Poole, 1978). Scent marking, the act of a marmoset depositing their scent-mark, is intentional and often most concentrated in the feeding areas (Coimbra Filho & Mittermeier, 1978), sleeping sites (Sutcliffe & Poole, 1978), and general home territories (Rylands, 1990). Scent marking appears to influence female intrasexual competition and intersexual communication (Abbott et al., 1998; Smith et al., 2001; Smith, 2006). Despite an indistinguishable chemical composition between scent-marks individual females, each female marmoset correctly distinguished their scent-marked from other female marmosets (Smith et al., 2001). Because of this, Smith et al. (2001) theorized that female marmosets might have a unique ratio of highly volatile chemicals that make their scent-mark distinguishable from other scent-marks.

Marmosets use scent stimuli to adapt and orient themselves within the environment (Smith, 2006). Smith et al. (1997) hypothesized that familiar and unfamiliar scents of a dominant female influence social behavior. Smith et al. (1997) pondered if any odor from a dominant female marmoset would dictate group movement and social behavior. In fact, marmosets remained in their home territory by orienting to the familiar dominant female scent (Smith et al., 1997). Therefore, a familiar odor regulates the frequency of intergroup relations. Fornasieri and Roeder (1993) supported this finding by discovering that marmosets scent mark significantly more in unfamiliar environments. Thus, marmosets must have a functioning olfactory system to

survive complex social dynamics (Smith, 2006). These olfactory-influenced behaviors require adaptive capabilities and are, therefore, a measure of cognition in the common marmoset.

4.0 Olfactory Testing Standards and Considerations for Non-Human Primates

The marmoset scent-mark is comprised of secretions from glands and genital tract, urine, feces, and old scent material (Sutcliffe & Poole, 1978); those individual odorous components represent a marmoset's unique scent (Smith, 2006; Smith et al., 1997). Previous studies frequently use urine to study marmoset olfaction (Smith, 2006; Smith et al., 1997; Sutcliffe & Poole, 1978). Urine contains multiple chemicals that researchers attribute to animal biological and behavioral activity (Karlson & Luscher, 1959; Johnston, 2000). In female mice, active substances within the urine evoke multiple endocrine responses (Jemiolo et al., 1986). In marmosets, urine is a main component of the circumgenital scent-mark (Sutcliffe & Poole, 1978). Similar to mice, urine influences marmoset biological activity (Smith, 2006). Moreover, urine influences observable behavior (Jemiolo et al., 1986; Smith, 2006; Smith et al., 1997). Thus, urine scent provides excellent olfactory stimulation for marmosets for this pilot study.

To structure the protocol of this pilot study, we found a social olfactory test from Stanford's Behavioral and Functional Neuroscience ("Olfactory Test," n.d.) for mice that utilize urine. In trials, researchers used water as a control and urine from other mice (unfamiliar opposite sex and familiar opposite sex) as stimuli. Cotton swabs were dipped in the solution and placed into a microcentrifuge tube with small holes drilled at the base. The tube is secured to the floor on one end of the testing cage, and the mouse is introduced into the cage at the opposite end. Subjects were allowed three minutes in the testing cage. Stimuli was presented in a random order pre-testing session for three trials.

The quality of a testing environment makes the difference between a reliable and unreliable trial. Many studies conduct olfactory discrimination tests within an animal's home cage (Smith et al., 1997; Smith 2006). As marmosets are frequent scent markers, their cage contains many potent olfactory distractions. Although a test apparatus attached to the home cage is salient on its own, researchers must reduce the number of in-trial distractions to provide more reliable results. Additionally, any/all cage mates temporarily removed for testing were still somewhat accessible to the test subject. Although previous studies visually separated, cage mates often remained olfactorally and acoustically present during trials (Smith et al., 1997; Smith, 2006). Therefore, cage mates might provide an additional distraction that hinders a trial run.

5.0 Gaps in Literature

Although many discoveries have been made, furthering our understanding of marmoset olfaction, some questions remain unanswered. At present, we do not know how long a marmoset can retain a familiar scent -or if odor retention changes with age (Laska & Metzker, 1998; Smith, 2006; Smith et al., 1997). Is olfactory sensitivity to a mate -or other significant social experience- more salient or sustained long-term? In a food aversion task, marmosets showed retention of an olfactory cues' significance for up to four weeks (Laska & Metzker, 1998), suggesting that scents can be retained for some time. Is this retention time different for social scents? Furthermore, a "familiar" odor also implies learning (Enwere et al., 2004). Olfactory learning plays a role in the significant behavioral difference between novel and familiar scents. So, by studying behaviors influenced by one scent, we observe the learned behavioral patterns that particular scent elicits. This pilot study will redo previous studies of familiar vs. unfamiliar scent sensitivity and hopefully strengthen current scent retention findings - which are essential to

understand if we are to standardize an assessment of marmoset olfaction (Smith, 2006; Smith et al., 1997; Smith et al., 2001).

Current research on common marmoset olfaction studies social behavior rather than cognition. Cognitive capability is certainly implied through social behavior. However, olfaction is connected to adaptation and learning in or out of a social context (2.3 Plasticity, Adaptation, and Adult Neurogenesis). Marmosets use olfactory cues to adapt to social dynamics, but olfaction provides other environmental information as well. The neural mechanisms behind olfaction have proven complex and dynamic. Olfaction is unique to other senses because it contains neurogenesis. For this reason, olfactory tests are valuable for measuring various adaptive and learning functions. In the aging process, the olfactory system operates differently from other sensory systems, making it a unique correlate with age-related cognition.

In summation, olfaction has been studied in aging and cognitive decline research, as well as marmoset sociality. However, marmoset olfactory studies in social contexts do not overtly factor in cognitive function or age. Despite this, olfactory discrimination plays a role in cognitive functions like learning and memory. The ability to encode, store, and retrieve odors appear to predict odor recall and performance better than recognition and identification (Murphy et al., 1997). In this study pilot, we attempt to assess social influences on cognition and brain aging.

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Pilot Study: Age-Related Changes in Olfactory Sensitivity in a Social Context

Olfaction is vital for animal survival and requires sufficient levels of sensitivity and discrimination. A progressive decline in olfactory sensitivity and discrimination is typical with age. However, olfactory decline has been associated with pathologies, such as Parkinson's and Alzheimer's disease, and, therefore, is used in current research to increase our understanding of human well-being. Researchers have utilized the common marmoset (*Callithrix jacchus*) model to study aging and cognition due to the animal's adaptivity, physiological characteristics, and ease of handling. Five minute trials were performed on seven geriatric and one adult common marmoset. Animals were exposed to urine from a familiar marmoset and an unfamiliar marmoset. Although these data are preliminary, they still indicated that olfactory decline was measured through urine scents. There was also a larger disparity between same-sex urine investigations than opposite-sex investigations. To ensure that marmosets exhibit age-related olfactory decline, future research should run fMRI scans when a subject is exposed to an odorant. In doing so, olfactory impairment can be materialized via functional neuroimaging. In conclusion, these data will guide future studies towards a standard olfactory test for the common marmoset. Upon these olfactory studies, we will develop a more holistic understanding of aging and cognition within such a lucrative animal model, expanding our perception of human functionality.

Keywords: common marmoset, olfaction, aging, urine, olfactory test

Pilot Study: Age-Related Changes in Olfactory Sensitivity in a Social Context

Olfactory sensitivity and discrimination are vital for an animal to survive. Changes in olfactory sensitivity and discrimination occur as a normal function of age in humans. With age comes a progressive decline in the human senses. Smell, or olfaction, is no exception. However, olfactory decline in humans indicates both typical aging and pathological dysfunction (Devanand et al., 2000; Doty et al., 1984; Duffy et al., 1995; Murphy et al., 2002; Wilson, Arnold, et al., 2007; Wilson, Schneider, et al., 2007). The typical olfactory decline with age is subtle. As humans age, they are continually exposed to environmental substances. Some of those damaging substances induce progressive olfactory decline (Meurman, 1950; Liss & Gomez, 1958). Conjointly, olfactory decline has also been associated with pathologies. For instance, decreased olfaction can be an early sign of neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease (Del Tredici et al., 2002; Devanand et al., 2000; Wilson, Arnold, et al., 2007). As a connection to many neurological paradigms, olfaction can aid in our understanding of the brain and human well-being.

To address these cognitive changes in humans, nonhuman models for aging must be further developed. Common marmosets (*Callithrix jacchus*) are a valuable animal model for aging and cognition due to their adaptivity, physiological characteristics, and ease of handling (French, 2019; Kishi et al., 2014; Ross et al., 2019). Additionally, common marmosets have a well-developed olfactory system for social behavior and environmental adaptation (Epple et al., 1993; Smith, 2006). Although marmosets show their importance in aging, researchers do not currently have a standardized technique to assess marmoset olfactory sensitivity and discrimination -let alone a way to study age-related changes in their olfaction.

Olfaction could help further explain marmoset behavior, aging, and cognition. Social behaviors of the common marmoset are highly scent-driven (Epple, 1986; Epple, 1993; Taniguchi et al., 1992). Thus, understanding their age-related changes in olfactory sensitivity and discrimination in a social context could explain a large portion of a marmoset's cognition. As a forager, olfaction plays a crucial role in marmoset survival (Coimbra et al., 1978; Rylands, 1990; Sutcliffe & Poole, 1978). Throughout their life, marmosets must continue to have sufficient olfactory capabilities to survive in the wild (Epple, 1986; Epple, 1993; Smith, 2006). So, if we established typical olfactory sensitivity parameters within the younger marmoset through baseline olfactory tests, we can compare young and old olfaction. In doing so, we can discover any age-related changes in marmoset olfactory sensitivity and discrimination.

To establish baseline olfactory sensitivity across age groups, we looked to previous studies on marmoset olfaction. Marmosets are known to have a distinct scent-mark (Sutcliffe & Poole, 1978). Part of their unique scent can be found in their urine; Smith et al. (2001) theorized that each female marmoset might have a unique ratio of highly volatile chemicals that make their scent-mark distinguishable from other scent-marks. Moreover, Smith et al. (1997) studied whether familiar and unfamiliar scents of a dominant female influence social behavior and found marmosets remained in their home territory by orienting to the familiar dominant female scent. Fornasieri and Roeder (1993) supported this finding by discovering that marmosets scent-mark significantly more in unfamiliar environments. Therefore, familiar and unfamiliar odors are salient to a common marmoset and can likely be used as a baseline to measure olfactory sensitivity and discrimination.

In conclusion, common marmosets are an excellent model for this research because they contain behavioral and physiological traits that effectively formulate, deposit, and receive scent

stimuli, producing a developed olfactory system (Epple et al., 1993). By standardizing olfactory sensitivity and discrimination in the marmoset, we aim to increase our understanding of marmoset olfaction and determine whether marmosets show age-related changes in olfactory sensitivity. We hypothesized geriatric marmosets would demonstrate reduced olfactory discriminatory ability from adult marmosets. Specifically, we believe that younger marmosets will perform better on our olfactory tests than geriatric marmosets.

Method

Subjects

This project originally started with four sets of pair-housed geriatric marmosets and one set of pair-housed adult marmosets. Due to untimely deaths or re-pairing, three subjects were temporarily single-housed and/or re-paired. One marmoset was single-housed during habituation trials but paired with a mate before test trials. Another subject, subject b, became single-housed after care staff paired the subject's mate with another marmoset. Near the end of the test trials, subject b moved cage rooms to get re-paired with a new mate, ending subject b's participation in this trial. The last marmoset, subject c, became single-housed when their mate died of old age. As this also occurred near the end of data collection, subject c was removed from further test trials.

Despite these setbacks, trials were still performed on seven geriatric (> 10 yrs) and one adult common marmosets (5 - 8 years) housed at the Southwest National Primate Research Center, Texas Biomedical Research Institute, San Antonio, Texas, USA were used in this investigation. Rooms were set at 80°F (ranging 76°F - 84°F) and maintained a 12-hour light-dark cycle. Subjects were fed Mazuri diet (AVP Callitrichid 5LK6), purified diet (Harlan Teklad

TD130059 PWD), and fresh fruit, seeds, and dairy. All subjects were not breeding throughout the project; however, not all subjects remained pair-housed for the study's duration.

Procedure

Fresh urine was collected daily before the test trial. Urine was not frozen because temperatures below -20 °C denature major urinary proteins and, therefore, elicit less behavior in subjects (Hoffmann et al., 2009). Urine samples were collected between 7:00 am and 10:00 am to capture the morning urination. To collect the urine, clean, scentless paper or a plastic garbage bag was cut and laid between the wire mesh and the cage tray. Urine was collected with a cotton swab and then stored in a microcentrifuge tube to preserve odorants and sample potency. If any feces, food, water, or fur fell into the urine, the sample would be considered contaminated and unusable.

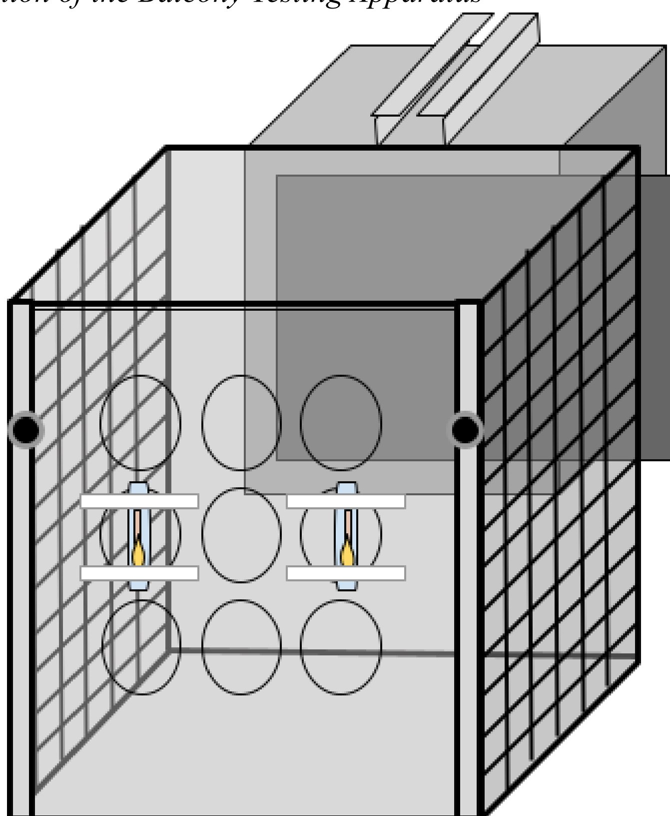
For this pilot study, subjects were exposed to familiar urine (from a marmoset in the same room) and unfamiliar urine (from a marmoset in a different room) to evaluate olfactory discrimination and sensitivity. Both urine samples in a trial were of the same sex. Therefore, if it was unclear who had urinated in a pair-housed cage, that urine was not sampled. Familiar and unfamiliar urine was placed randomly pre-testing session for trials. Familiar urine samples must remain consistent throughout the testing trials, but unfamiliar urine samples must change between trials.

For scent collection, cotton swabs were dipped and saturated in urine and placed into a 2.0 mL microcentrifuge tube with four small holes drilled at the base. Subjects were tested in a balcony unit attached to their home cage (Figure 1). The balcony unit contains two rooms and a sliding metal door that acts as a partition between the cage and the balcony. The tube was secured to a plexiglass wall on one end of the testing apparatus, and the marmoset was

introduced into the cage at the opposite end. This transparent wall provided easy viewing access for recording trials. To prevent an escape, the plexiglass wall was secured with plastic screws. Lastly, the testing apparatus was cleaned thoroughly after each subject to remove the scent from the previous test subject and the cleaning supplies.

Figure 1

Illustration of the Balcony Testing Apparatus



Note. The rear room is inside the home cage and was used to enter the balcony unit once the metal-sliding partition was removed.

Trials started when the metal partition was removed and ended when the partition was reinserted. Subjects were introduced to the testing environment and protocol through at least two habituation trials. For habituation trials, urine from the most easily accessible marmoset was collected and utilized during the habituation trials. Upon testing, animals were allotted five minutes of access to the balcony unit.

Data Scoring and Analysis

All trials were recorded and scored by the frequency of certain investigative behaviors as a measure of olfactory sensitivity and discrimination. The following behaviors were considered investigative: biting, licking, grabbing, and sniffing. Biting was categorized as the head moving towards the tube followed by the subject gripping the tube with their mouth/teeth. Meaning, gnawing is considered one bite. A lick was counted when the tongue touched the tube. Licks and bites can occur simultaneously but should be scored separately. A grab occurred when the subject wrapped a hand around and successfully gripped the tube. Therefore, two hands wrapped around the tube are considered 2 “grabs.” A hand that releases its grip on the tube but remains wrapped around the tube is not considered an additional grab. The hand must unwrap itself from the tube and then re-envelop to be considered an additional grab. However, the arm does not have to lower and then raise to be considered another grab. Pulling at the tube does not change the count. Lastly, any attempts to grab at the tube, whether that be reaching or partially wrapping around the tube, were not considered grabbing. Lastly, a sniff was quantified when the head moved toward the tube, bringing the nose close. Often, marmoset would also indicate a “sniff” by closing their eyes with the head movement.

Each investigative behavior will be scored individually and sorted by which urine type (unfamiliar female, familiar female, unfamiliar male, and familiar male); but, each trial will also sum up every behavior to count the total investigative behavior. These data will then be added up as all test trials to produce a sum of total investigative behaviors for each urine type.

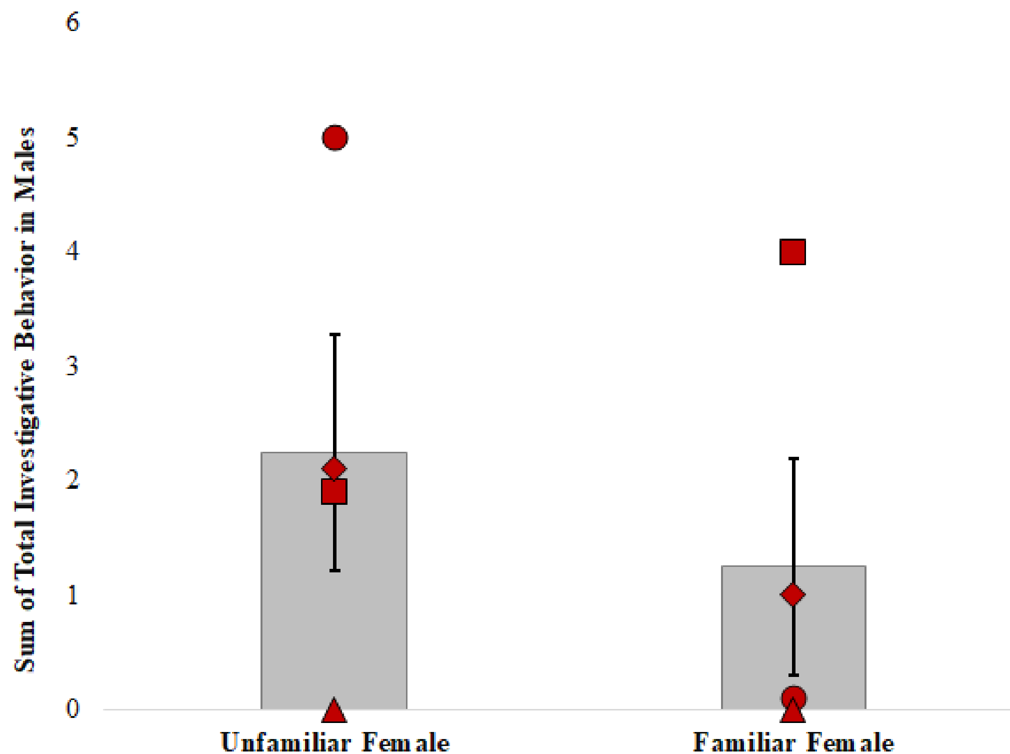
Results

This project aims to determine a reliable measure of olfactory discrimination to understand the olfactory differences between young and geriatric marmosets. In doing so, we

hope to increase our understanding of marmoset olfaction and use that knowledge to improve animal well-being. Although this pilot study sought to differentiate olfactory sensitivity between geriatric and young/adult marmosets, there are too few adult marmosets (n = 2) to reliably identify age-related olfaction traits. Although these data are unexpected, they can still guide age-related olfaction trends. At this stage of the study, only two females successfully interacted with the microcentrifuge tubes. Therefore, it is premature to run statistical analyses on such a small sample size. Despite this, there are still observable trends in these data that can guide future studies onto the next steps.

Figure 2

Dot Plot of Males Investigating Female Urine

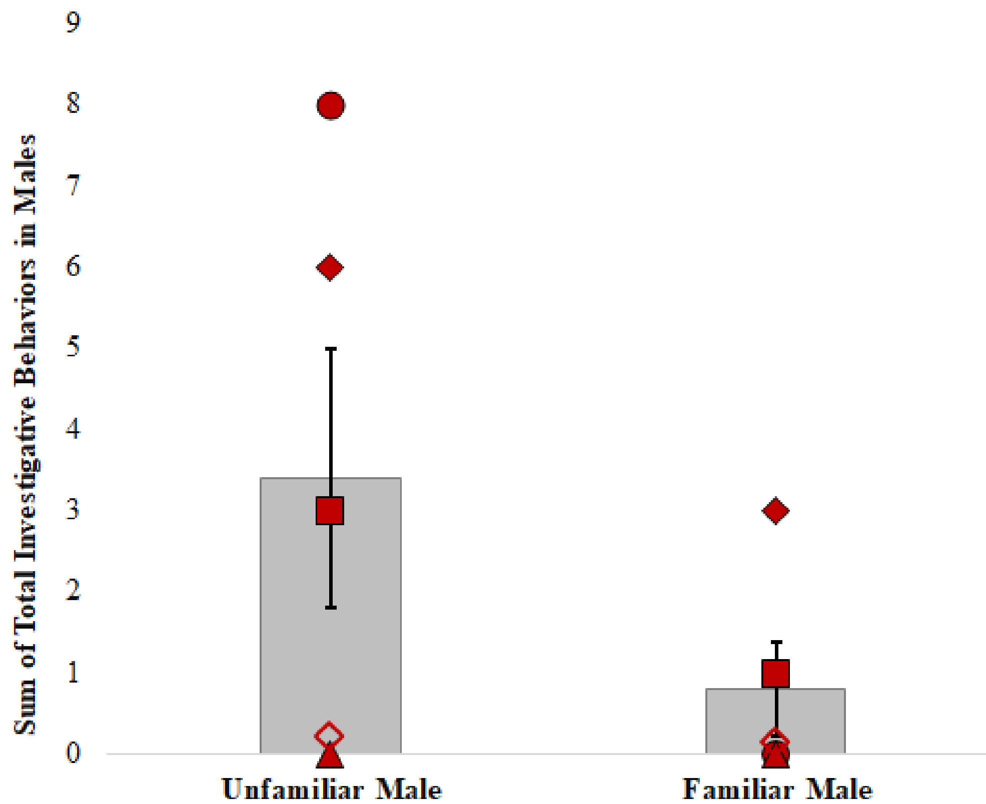


Note. The sum of total investigative behaviors for each female subject has been marked with a unique datum point (▲, ◆, ●, ■) to compare olfactory sensitivity within a subject relative to the mean. The ■ subject is adult among geriatric marmosets. Total investigative behaviors are the combined frequencies of biting, licking, grabbing, and sniffing. The error bars represent SEM.

For instance, male subjects investigated the unfamiliar female (UF) scent slightly more than the familiar female scent (FF; $M_{UF} = 2.3 \pm SE = 1.0$, $M_{FF} = 1.3 \pm 0.9$, $n = 4$; Figure 2). Furthermore, higher levels of investigation towards the UF scent are shown individually in addition to the mean. The ■ and ◆ subjects investigated both UF and F female scent. The ◆ subject investigated UF scents more than FF scents, while the ■ subject showed the opposite preference. The ● male showed the most investigative behavior toward the UF scent and no investigative behavior toward the FF scent. The ▲ subject did not investigate either scent.

Figure 3

Dot Plot of Males Investigating Male Urine

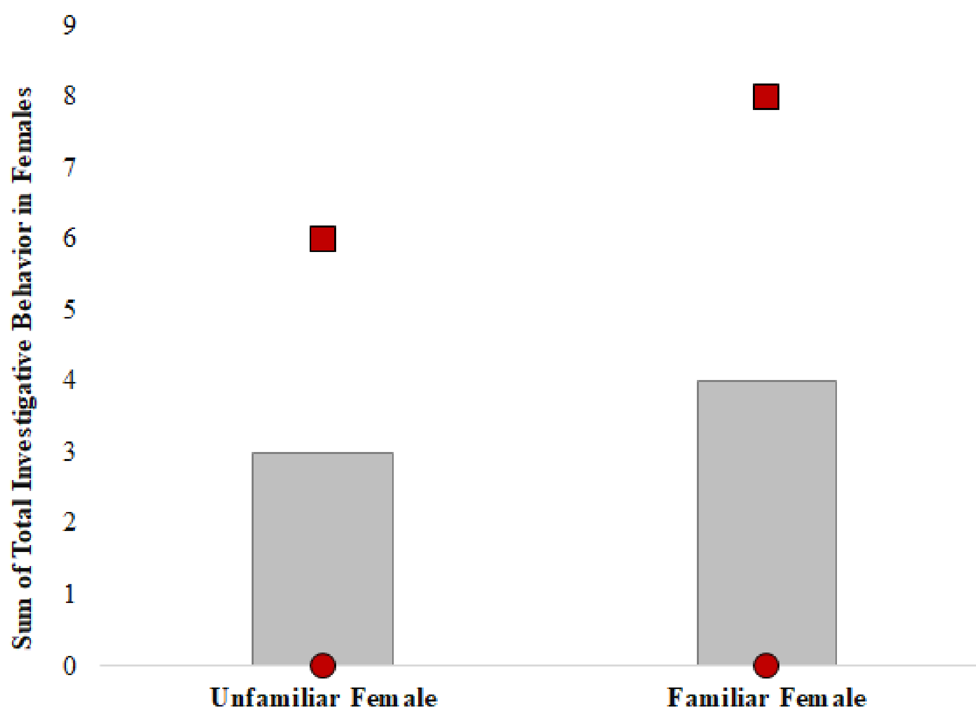


Note. The sum of total investigative behaviors for each male subject has been marked with a unique datum point (▲, ◆, ●, ■, ◇) to compare olfactory sensitivity within a subject relative to the mean. The ■ subject is adult among geriatric marmosets. Total investigative behaviors are the combined frequencies of biting, licking, grabbing, and sniffing. The error bars represent SEM.

Males investigated the unfamiliar male scent (UM) more than the familiar male scent (FM; $M_{UM} = 3.4 \pm 1.6$, $M_{FM} = 0.8 \pm 0.6$, $n = 5$; Figure 3). The ●, ◆, and ■ subjects all showed to investigate the UM scent more. The ▲ and ◇ males did no investigation. Interestingly, males seemed to have a larger disparity between UM and FM than UF and FF ($M\ difference_{UM/FM} = 2.6 \pm 1.0$, $M\ difference_{UF/FF} = 1.0 \pm 0.1$).

Figure 4

Dot Plot of Females Investigating Female Urine

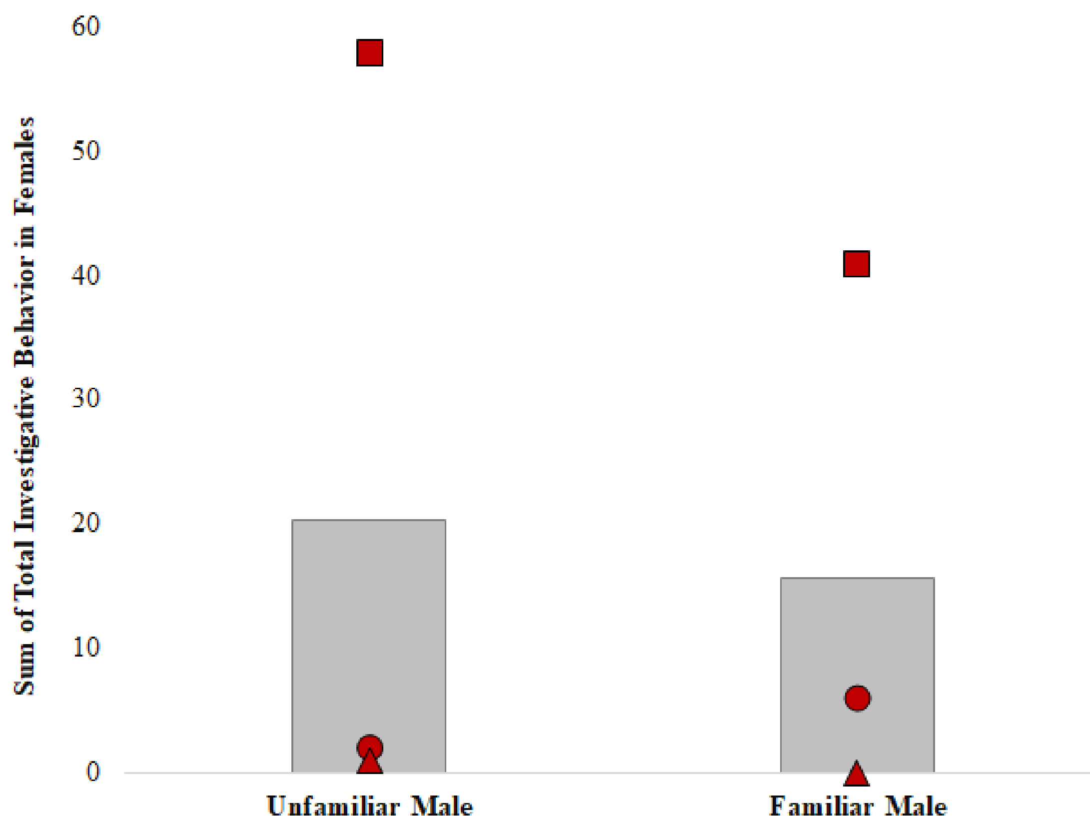


Note. The sum of total investigative behaviors for each female subject has been marked with a unique datum point (●, ■) to compare olfactory sensitivity within a subject relative to the mean. Total investigative behaviors are the combined frequencies of biting, licking, grabbing, and sniffing. Given the smaller sample size, no error bars were used.

As for the female subjects, there is an observational difference in investigative behaviors. Unfortunately, there are only two females in this sample, so we cannot see any clear trends. According to the data, the ● subject did not investigate the tubes, and the ■ subject investigated the FF more than the UF scent ($total_{■UF}$ = 6, $total_{■FF}$ = 8; Figure 4).

Figure 5

Dot Plot of Females Investigating Male Urine



Note. The sum of total investigative behaviors for each female subject has been marked with a unique datum point (▲, ●, ■) to compare olfactory sensitivity within a subject relative to the mean. Total investigative behaviors are the combined frequencies of biting, licking, grabbing, and sniffing. Given the smaller sample size, no error bars were used.

Lastly, female subjects appeared to investigate the UM scent more than the FM scent (M_{UM} = 20.3 ± 18.8, M_{FM} = 15.7 ± 12.8, n = 3; Figure 5). The ■ female investigated both UM and FM urines, investigating the UM 17 times more than the FM urines. The ▲ female did not investigate either scent, and the ● female appeared to investigate the FM scent more than the UM scent. Although both sexes are small sample sizes, they both show a common trend of investigating unfamiliar urine more.

Discussion

To reiterate, this pilot study aims to identify age-related changes in olfactory sensitivity to urine using one adult and seven geriatric marmosets. In studying olfaction by age groups, we also assess if the test design (a social scent, urine) provides a reliable standard for testing future age-related olfaction. Based on previous studies, the geriatric age group was predicted to exhibit measurable olfactory decline. Although these data are preliminary, they still indicated that olfactory decline was measured through urine scents. Some marmosets showed a strong individual pattern of investigating UF urine, indicating that geriatric marmosets are still capable of olfactory discrimination when it comes to urine. However, some marmosets did not investigate the urines at all. The subjects exhibiting no investigative behavior may look discouraging at first, but is it possible those subjects could not detect at all? If so, these findings indicate that we have successfully measured age-related olfactory dysfunction. With more subjects and trials, this protocol could become a standard test to measure marmoset olfaction with age.

In addition to more test trials, these data can be evaluated further for age-related olfactory dysfunction through cognitive and behavioral (i.e. gait) data from these particular animals on another project. In mentioning gait, age-related olfaction can be linked back to Parkinson's disease. As gait is an important measure of motor impairment from Parkinson's disease, this research could close some conceptual gaps between olfaction and aging pathology.

Now, what information does this pilot study provide for future studies? When looking at the disparity by sex of the urine and subject, it seems like subjects showed higher disparities in investigating urine of their same-sex over opposite-sex urine. Smith and colleagues (2001) and Smith (2006) found similar results when studying female marmoset response to urine from other

females (Abbott et al., 1998; Smith et al., 2001; Smith, 2006). Now, these results could guide future studies in two ways. As one researcher found that finer olfaction diminishes more with age (Enwere et al., 2004), it might be more beneficial for future research to study opposite-sex urine because there is less contrast between familiar and unfamiliar scents. Conversely, it might be beneficial to study same-sex urine discrimination so that a smaller, progressive difference can be compared across age groups. In summation, if researchers seek to have a discrete disparity between scents to contrast the investigative behaviors of young and old marmosets, then opposite-sex urine should be used. Inversely, to differentiate between the two scents more, researchers should utilize same-sex urine.

Moreover, urine will stimulate the accessory olfactory system. Although it is crucial to understand every aspect of marmoset olfaction, scents that isolate main olfactory activity will develop a holistic understanding of marmoset olfaction and bridge the conceptual gaps in human olfaction. Marmosets are highly scent-driven in their sociality and rely on other senses for scavenging or hunting (Laska & Metzer, 1998). Mouse models struggle with finer olfactory discrimination with age more than distinct discrimination (Enwere et al., 2004). Food-based olfaction may diminish with age quicker than social-based olfaction. Therefore, food-based olfaction should be evaluated in adult marmosets and geriatric marmosets. As this sample consisted of one adult marmoset, we were limited to doing social scent olfactory tests. Lastly, to test that marmosets are exhibiting age-related olfactory impairment, research should run fMRI scans when a subject is exposed to an odorant. This way, olfactory impairment can be materialized in brain imaging.

In conclusion, these data indicate where future studies should go to standardize a marmoset olfaction test. And upon these olfactory studies, we expand our comprehension of

aging and cognition within such an impactful animal model and pave the way to understanding human functionality.

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