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About this Issue

Statement of Purpose

The Rhodes Journal of Biological Science is a student-edited publication that recognizes the scientific achievements of Rhodes students. Volume XXX marks the ninth year since Mark Stratton and Dr. David Kesler brought the journal back into regular publication in 2006. Founded as a scholarly forum for student research and scientific ideas, the journal aims to maintain and stimulate the tradition of independent study among Rhodes College students. We hope that in reading the journal, other students will be encouraged to pursue scientific investigations and research.

Acknowledgements

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Image Credits

The front cover for this year's edition of the *Rhodes Journal of Biological Sciences* was created by Lexi Perkins, an art major from Murfreesboro, TN. This image illustrates the subject for one of the featured articles in this year's edition which researched cownose stingray.

Editorial Staff

Anna Stachura '15 (**Senior Editor**) is a Biochemistry & Molecular Biology major from Charlotte, North Carolina. Last semester, she participated in the European Studies program where she studied Medieval art and architecture. Anna has completed three years of research under the Snyder lab in the Department of Radiological Sciences at St. Jude Children's Research Hospital through the St. Jude Summer Plus Program. She has also enjoyed coloring with patients at the Grizzlies House and delivering toys to children on the Happy Cart at St. Jude. During her college experience she has volunteered in the Burn Unit at the Regional Medical Center, interned at Baptist Hospice, and served as the Assistant Philanthropy chairperson for her sorority, Chi Omega. She is a member of the Beta Beta Beta, Gamma Sigma Epsilon, and Phi Beta Kappa honor societies. Additionally, she has enjoyed being a teaching assistant for Organic Lab. After graduating this spring, Anna will move to Washington D.C. where she will attend George Washington Medical School and pursue a career as a physician.

Kathryn Roys '16 (Junior Editor) is a Biology major with a double minor in Music and Psychology from New Bern, NC. She is a General Chemistry Lab teacher's assistant and has regularly volunteered at Baptist Memorial Hospital in the emergency department. The summer after her freshman year, she had the opportunity to start an internship in the genetics department at St. Jude Children's Research Hospital investigating the progression of Pancreatic Ductal Adenocarcinoma. She is a member of Beta Beta Beta, Gamma Sigma Epsilon, Delta Epsilon Iota, and Mortar Board honor societies where she has various leadership roles. She is also heavily involved in Reformed University Fellowship, sings with Rhodes Mastersingers Chorale and, during her free time, plays ultimate Frisbee with the Rhodekill club team. This summer she will begin working as a medical scribe in the emergency department and after graduation intends to pursue a career as a Physician Assistant.

Matthew Cannavo '15 is a Biochemistry & Molecular Biology major from Orlando, Florida. Matt participated in the St. Jude Summer Plus International program where he researched with Dr. James Hoffman in the Department of Pharmaceutical Sciences on clinical pharmacogenomics implementation. In addition, Matt has been working with Dr. Loretta Jackson-Hayes investigating fungal cell wall integrity. Outside of the lab, Matt is very passionate about community engagement, serving as a Kinney Coordinator for Healthcare and Wellness, a Bonner Scholar, and a volunteer at Tri Delta Place at St. Jude Children's Research Hospital. He is a member of Mortar Board, Omicron Delta Kappa, Beta Beta Beta, and Gamma Sigma Epsilon honor societies. After graduation, Matt will be heading to Miami, FL to begin medical school at the Herbert Wertheim College of Medicine at Florida International University.

Megan Denny '16 is a Biochemistry and Molecular Biology major and a minor in Urban Studies and Community Health from Hastings, Michigan. On campus she is the Director of Outreach of GlobeMed and the Healthcare and Wellness Coordinator for the Kinney Program. She has also served as the Treasurer for Rhodes United for St. Jude, the National Communications Chair of the American Chemical Society, and the Chapter Leader of the Institute for Healthcare Improvement. Megan is a member of the Beta Beta Beta, Gamma Sigma Epsilon, and the Delta Epsilon Iota honors societies. Off-campus Megan volunteers at LeBonheur Children's Hospital as a Baby Hugger and the Humane Society of Memphis and Shelby County as a Dog Walker. During the school year, Megan works as a Teaching Assistant to Dr. Muesse in the Chemistry Department and she also is an Emergency Department Scribe with the Methodist Lebonheur Healthcare System. Previously, she has held an internship at The Urban Child Institute Department of Research and Education and been the student manager at the Middle Ground Coffee Shop. In previous summers, she has worked as an Animal Care Intern at the Binder Park Zoo. This past summer Megan worked at Genesis Genetics where she worked with the PGD and PGS techniques to track genetic disorders through family pedigrees prior to jetting off to Europe for a semester-long study abroad program. After graduation, Megan intends to receive her M.D. degree with a focus in rural population health and pursue a career in rural clinical health.

Yoonjee Kim '15 is a Neuroscience major from Shreveport, LA. She is a Safe Zone, a Peer Mentor in the multicultural association Serving Our Students program, and Vice President of Active Minds, an organization dedicated to dispelling the stigma surrounding mental illness and providing a judgment-free atmosphere on college campuses. She is a member of the Nu Rho Psi, Beta Beta Beta, and Delta Epsilon Iota Honor Societies and volunteers at St. Jude and the MED, both with children and head trauma victims. Yoonjee was also a clinic intern on the referral team at the Church Health Center, a faith-based nonprofit organization dedicated to providing care to low-income Memphis residents. During the summer of 2013, she performed biochemistry research in a neurogenetics lab, where she spent hours learning to dissect the brains of fruit flies. This is now the most meticulous and distinctive skill she possesses. The summer of 2014, she was certified as a Nurse Assistant and will begin work post-graduation to get valuable experience for her future. After this experience, she hopes to attend a Physician Assistant program.

Gene Lamanilao '16 is a Biochemistry & Molecular Biology major from Memphis, TN. He is currently conducting bio-organic chemistry research in the lab of Dr. Peterson and also regularly volunteers as a Clarence Day Scholar at Compassion Neighborhood Health Clinic, a free health clinic that serves individuals in the Binghampton community. He is the President of Rhodes Rebuilds, an alternative fall and spring break trip that assists in the reconstruction of hurricane-damaged homes in New Orleans, and hosts his own indie music-themed radio show on the student-run Rhodes Radio station. Upon graduation, he plans to attend medical school to pursue a career that combines primary health care and basic research. During his free time, he enjoys studying European Medieval and Renaissance art, collecting vinyl records of British pop bands, Fried Chicken Friday from the Rat, and an occasional jog through Overton Park.

Rachel Nelson '16 is a Chemistry major from Ripley, Tennessee. As a St. Jude Summer Plus Fellow, she is currently doing computational research in the Shelat lab in the department of Chemical Biology and Therapeutics at St. Jude Children's Research Hospital. Her current project focuses on using molecular fields as a way to compare drug similarities. Aside from her research, Rachel is a weekly volunteer in the TICU at Regional One Medical Center and at Lynx Club: a Special Olympics of Memphis Program. She is also a member of Chi Omega fraternity and the national and local chapter of the American Chemistry Society. After graduation she hopes to pursue a career in medicine.

Radhika Puri '16 is a Biology major and Business minor from Germantown, TN. She is a regulatory and public affairs communications intern at FedEx Corporation. She is working with Le Bonheur Children's Hospital and the Memphis Greenline on an injury prevention project as a result of her Clarence Day Scholarship fellowship grant. She loves representing her college as a Rhodes College diplomat and a peer assistant leader for incoming classes. She has been an active volunteer at the St. Jude's Children Research Hospital and Baptist Rehabilitation Hospital in the past years. She hopes to combine her love for the sciences and her desire to help the community with a career in healthcare policy and hospital management.

Fei-Lin Scruggs '16 is a Chemistry major from Durham, NC. She is excited to be back in Memphis this spring after studying abroad in Western Europe last fall through European Studies. She is currently the Chapter President for Alpha Omicron Pi fraternity (Kappa Omicron Chapter), a Ministry Team member for Reformed University Fellowship, a Rhodes College Diplomat, and a member of the American Chemical Society. She has previously served as the Philanthropy Chair for Alpha Omicron Pi, a senator on Rhodes Student Government, and served on Rhodes College Common Table in addition to volunteering at St. Jude Children's Research Hospital and First Evangelical Church. As a St. Jude Summer Plus Fellow, she is currently doing research in the Hendershot lab in the department of Tumor Cell Biology at St. Jude Children's Research Hospital. Her current project is called "Serendipity Leads to Novel Insights into Rules for Glycosylating Proteins." After graduation she plans on attending medical school and pursue a career in pediatrics.

Dispelling the Rumor: Lions do not Bolster Vulture Populations

Alison Hanson

*Many think that vultures use lions as guides both to find carcasses and to eat anything the lions left untouched on their kills. Tembe National Elephant Park, a small game reserve in South Africa, provides an opportunity to examine this assumption as lions (*Panthera leo*) were re-introduced to the park in 2004. Anecdotal evidence supports the claim that the two vulture species in Tembe, the White-backed Vulture (*Pseudogups africanus*) and the Palmnut Vulture (*Gypohierax angolensis*), increased in abundance after the re-introduction of lions. This paper surveys literature on the dietary needs of both vultures and lions, and documented interactions between these two groups. Due to the examples of vultures not appearing at lion kills and the diet of Palmnut Vultures, it appears unlikely that the re-introduction of lions positively influenced the population of both vulture species. Therefore, conservation techniques, both in Africa and abroad, involving endangered species of vultures should not seek to utilize lions or equivalent predators as guides to sources of carrion needed by obligate scavengers. Other conservation techniques, such as vulture restaurants in reserves and minimizing the danger outside of protected areas, will more effectively raise vulture populations.*

Introduction

Scavengers are organisms that forage on dead animals, called carrion, for food (DeVault et al., 2003). Because predation by carnivores only accounts for an estimated 30% of the deaths seen in ungulate species, around 64% of animals die from other causes, such as disease, starvation, dehydration, or old age (Houston, 1995). These deaths produce tons of meat every year, and scavengers are able to utilize this meat in their diets (Houston, 1995). The majority of vertebrate scavengers are mammalian facultative scavengers, foraging on carrion in low quantities, and instead primarily using predation to acquire their food (Houston, 1995).

The minority of vertebrates that are obligate scavengers are avian species, including vultures. Avian scavengers have an advantage in scavenging for food through flight, with which they can cover large areas in less time than mammalian scavengers take to search for carcasses from the ground (Houston, 1995). From the air, avian scavengers can also use other scavengers as guides to carcasses (Houston, 1995). Flying also confers an adaptive advantage by allowing the bird to move over great distances with less energy compared to mammalian scavengers (Houston, 1995). It is hypothesized that the evolution of soaring, a necessary method of flight during the search for carrion, coevolved with the loss of the ability to have enough dexterity to be a successful predator (Houston, 1995).

Vultures are avian scavengers, and they provide a wide array of ecosystem services, and if they are removed from the ecosystem then there is a cascade of negative effects as reviewed by Ogada et al. (2012a). Sometimes when animals are shot by hunters, their carcasses are left in the ecosystem. The Egyptian Vulture (*Neophron percnopterus)* has been found to have increased levels of lead in their blood because they eat the carcasses left behind from these hunting activities and can ingest the bullet by accident (Gangoso et al., 2009). The presence of lead leads to a decrease in calcium mineralization, which could be linked to bone fragility and lower rates of survival among adults (Gangoso et al., 2009). Additionally, the decreased calcium mineralization can be vertically transmitted from adult females to their offspring, causing eggshell thinning and therefore lower reproductive rates (Gangoso et al., 2009). The cumulative impact of lead negatively impacting this species is an example of the threat that bioaccumulation, the process of a chemical or substance accumulating in an organism, poses to multiple species. Another example of bioaccumulation is the chemical dchlorodiphenyltrichloro-ethane (DDT), which breaks down into Dichlorodiphenyldichloroethylene (DDE) in avian and mammalian species (Lundholm, 1997). The DDE present in a portion of some avian species' food supply significantly reduces the calcium content of eggshells once ingested by the females (Lundholm, 1997). Similar to the bullets in Egyptian Vultures, the calcium loss in the egg shells makes the shells more fragile, increasing the likelihood that the weight of the parents will be enough to crush the eggs accidentally, leading to lower reproductive rates (Grier, 1982). Luckily, since the banning of DDT and the resulting DDE in December 1972 there has been an increase in reproductive rates in many species in the United States (Grier, 1982; US EPA).

In Asia, bioaccumulation of the drug diclofenac has caused a large spike in mortality rates in White-rumped Vultures (*Gyps bengalensis*), previously known as Oriental White-backed Vultures (Gilbert et al., 2006). Diclofenac is a drug given to livestock as an anti-inflammatory agent (Oaks et al., 2004). When a cow treated with diclofenac dies, vultures ingest its carcass and the diclofenac causes visceral gout and renal failure in the vulture (Gilbert et al., 2006). The fatal poisoning of White-rumped Vultures is also linked with low reproductive rates, which could signal a future population collapse (Gilbert et al., 2006).

The loss of vultures would severely decrease the health of a biological community, due to the services they provide. For instance, in areas of India there has been a steep decline in vultures that is associated with an increase in feral dogs that survive on the carcasses formerly consumed by vultures (Walker, 2008). With the rise of feral dogs, there has also been an increased prevalence of rabies, leading to a predicted 47,300 human deaths in India (Walker, 2008).

Vultures are important in controlling many other aspects of the ecosystem. The presence of vultures decreases both the species diversity and the total number of animals scavenging at carcasses (Ogada et al., 2012b). In Kenya, the absence of vultures is related to an increase of Spotted Hyenas (*Crocuta crocuta*), Striped Hyenas (*Hyaena hyaena*), and Black-backed Jackals (*Canis mesomelas*) at carcasses (Ogada et al., 2012b). These other scavengers are facultative, and are likely competing for carcasses with the vultures (Ogada et al., 2012b). Additionally, vulture absence increases the chance of these mammals meeting other members of their species at the carcass, which may or may not be members of their social clans (Ogada et al., 2012b). The carcass takes longer to decompose in the absence of vultures, which means the other scavengers will congregate there for longer periods of time (Ogada et al., 2012b). The increases both in time and in number of individuals present increase the chance of the carcass becoming a site of transmission for diseases between the mammalian scavengers (Ogada et al., 2012b). Vultures are therefore important in decreasing the transmission of diseases among mammalian scavengers in African ecosystems (Ogada et al., 2012b). Vultures also increase the rate of nutrient cycling within the environment through their scavenging (Sekercioglu et al., 2004).

Over the past thirty years, there has been a severe decline in scavenging bird populations in Africa (Virani et al., 2011). The decline in population depends upon the species, ranging from a 28% reduction of the Tawny Eagle (*Aquila rapax*) population to 62% of the Hooded Vulture (*Necrosyrtes monachus*), and out of eight species studied, only one, the Bateleur Vulture (*Terathopius ecaudatus*), increased in population size (Virani et al., 2011). The decline in the genus *Gyps* has been linked to the land-use changes in the areas surrounding protected reserves, as humans modify landscapes to increase agriculture (Virani et al., 2011). The other major cause of decline in vulture populations is the purposeful poisoning of carcasses by humans in an attempt to kill mammalian scavengers however, vultures are accidental casualties (Virani et al., 2012). It is estimated that the poisoning of vultures causes 33% of vulture fatalities per year in the Masai Mara National Reserve, Kenya (Kendall et al., 2012b). While it makes intuitive sense that vultures would move into available territories in nature reserves due to the surrounding land-use changes, this phenomenon has not been observed. Many species of *Gyps* cannot live on the reserve alone, because the ungulate species are migratory and therefore are not a stable food source within the reserves (Virani et al., 2012).

With the reduction in habitat outside of nature reserves, African vultures face another threat to their long-run survival, similar to the threat North American vultures currently face: extinction debt. Extinction debt is the lag time between the destruction of a species' habitat and the extinction of the species, which can range from 50 to 400 years in duration (Tilman et al., 1994). This lag time is most commonly observed in species that dominate their competitors, because their efficient use of the available resources after the destruction of the habitat will ensure their survival beyond the species that go extinct immediately afterwards (Tilman et al., 1994). In the Americas, there was once a large diversity of condor species, but many species have since become extinct leaving only three species extant (Emslie, 1998). Ensuring the survival of vulture species beyond extinction debt is critical as the services they provide are invaluable.

The ungulate species migration forces vultures to extend beyond protected areas in search of food whenever the herds are not present (Virani et al., 2012) and coincides with the observation that vultures can have expansive territories, some up to 38,000km^2 (Kendall et al., 2012b). It is possible that the data showing the population declines of vultures within reserves underrepresents the decline in vulture populations as a whole, due to biased reporting of sightings, as only 14 of 78 birds (~18%) were re-sighted (Kendall et al., 2012b). The conservation of vultures focuses on two different ways to help increase vulture populations; limiting the dangers outside of reserves, and increasing utilization of reserves throughout the entire year, instead of limiting their use of it to when the ungulate species are present. There are many efforts looking into the former suggestion, and this paper seeks to speculate about the effectiveness of the latter option, using Tembe National Elephant Park as a study site.

Methods

Tembe National Elephant Park, henceforth called “Tembe”, is a public nature reserve in Kwazulu-Natal, South Africa, and measures 300km^2 (Wright, 2014). Tembe became a park in the 1970’s when many megafauna fled south from Mozambique during the civil war (Wright, 2014). Local members of the Tembe tribe donated the land to the central government to fence in the megafauna, protecting the people from elephant and ungulate damages on crops and structures (Wright, 2014). In the early 2000’s, a small population of lions (*Panthera leo*) was introduced and lions are now are a top predator in the Tembe ecosystem (Wright, 2014).

The lions at the Karongwe Nature Reserve will serve as approximate natural experiment with which we can examine the impact they have on the ecological community’s interaction web at Tembe. Karongwe is another South African park, measuring only 85 km^2, however this park will represent a scaled down version of Tembe, which has not extensively published data on their lion populations. In Karongwe, the population of lions grew 25% each year during a period of six years (Lehmann et al., 2008a). Additionally, the vast majority of cubs (88%) survived beyond their first year of life (Lehmann et al., 2008a). The lion population of Tembe has likewise grown quickly to over 30 members, from an original group size under 10, with several male and female lions being removed from the population due to overcrowding (Wright, 2014).

The lions at Karongwe consume an average of 5.41% of the potential prey species biomass each year (Lehmann et al., 2008b). On average, there was more biomass consumed as the number of lions increased from the first pride of four (in the first year) to a coalition and a subadult group (during the next five years) (Lehmann et al., 2008b). The composition of the lions’ diet included 21 different prey species, with the wildebeest *(Connochaetes taurinus),* warthog *(Phacochoerus africanus)*, waterbuck *(Kobus ellipsiprymnus)*, impala *(Aepyceros melampus)*, and zebra *(Equus burchellii)* representing the largest percentages in number of kills (Lehmann et al., 2008b). All of these species are present in Tembe and therefore the Tembe lions likely have a similar diet, causing a similar impact on the Tembe ungulate populations. The success of lion introduction to Tembe provides an opportunity to examine how lion populations affect vulture populations.

There are only two species of vultures present in Tembe, the White-backed Vulture (*Pseudogups africanus*) and the Palmnut Vulture *(Gypohierax angolensis)*, and they inhabit different niches within the community. The Palmnut Vultures live primarily in forested areas with tall trees, preferring low elevations and a stable source of water (Mundy, 1993). They are distributed throughout Africa, primarily in the central rainforests (Mundy, 1993), and rarely along the east coast of southern Africa (Newman, 2010). There has much speculation as to the validity of calling the Palmnut Vulture a “vulture,” as it exhibits many of the ecological characteristics of snake-eagles (Mundy, 1993). As of 2005, the Palmnut Vulture has been classified phylogenetically as an “Old World Vulture” like the White-backed Vulture. However these two species represent two subfamily clades with different ecological characteristics (Lerner and Mindell, 2005). The Palmnut Vulture is a facultative scavenger and over 30% of its diet is comprised of fruit, including the palmnut fruit from which it receives its name (Mundy, 1993). Though another 30% of its diet comprises vertebrates, the Palmnut Vulture is infrequently observed at carcasses and has been observed killing some of their prey (Mundy, 1993). When Palmnut Vultures do eat carcasses, they primarily eat those of rodents, snakes, other birds, and large mammals (Mundy, 1993).

Contrastingly, the White-backed Vulture preferentially lives in habitats that are dry, open savannas (Mundy, 1993). Their diet primarily consists of the organs and inner meat of mid to large ungulate species, ignoring the skin, and they have been documented as entering a lion enclosure to eat the leftovers of the carnivores (Mundy, 1993). In their natural habitat, White-backed Vultures locate their food through flying and scouring the land for carcasses, other scavengers, and mammalian carnivores (Mundy, 1993). Interestingly, White-backed Vultures are often seen outnumbering other vultures by two or three factors (Mundy, 1993). In Zululand, where Tembe is located, there is an average density of 0.3 White-backed Vultures/km^2 (Mundy, 1993).

The interactions between these two vultures appear to be nearly non-competitive. Many of the food sources for the Palmnut Vulture do not overlap with the food sources of the White-backed Vulture, especially as large ungulates comprise only a small portion of the diet of the Palmnut Vulture. Additionally, the Palmnut Vulture is rarely aggressive with competing species at the same food source, so during the few times they want to utilize the same resource it is likely that there would be no aggression between the two species. There is likely little interactions between the two species, and when they do interact it is probably an amicable amensal relationship where the Palmnut Vultures choose not to compete with the White-backed Vultures for carrion (Mundy, 1993). These two species do not seem to exhibit any substantial relationship, as there is little habitat and diet overlap between the two groups, prohibiting them from either competing for the same resources or for facilitating each other in locating food sources.

There are anecdotal reports of an increase in vulture populations since the introduction of the lions (Hanekom, 2014). Since there is no hard evidence currently available, this paper will discuss the reasons why or why not the presence of lions would increase the habitat suitability for vulture species at Tembe. Additionally, this paper will speculate on reasons that Tembe could or could not be a good model for vulture conservation outside of Africa.

Discussion

Many factors can influence the presence or absence of vultures at carcasses on a population-level basis, and this paper seeks to investigate those factors and compare them to similar situations in Tembe. At the Polish-Belarussian border, Selva et al. (2005) found that the Common Buzzard (*Buteo buteo*) preferentially visited carcasses in open areas, compared to woodlands. Interestingly, they also found that the Common Buzzard visited carcasses of all six origin types, but visited carcasses of harvested ungulate species the most by percentage (Selva et al., 2005). Carcasses killed by Gray Wolves (*Cannis lupus*) and Eurasian Lynx (*Lynx Lynx*) were visited at a frequency of nearly half that of harvested ungulate species (Selva et al., 2005). If this pattern carries over to African vultures, then it can be assumed that the carcasses killed by mammalian predators such as lions, leopards (*Panthera pardus*), or either species of hyenas will be visited proportionally less than the carcasses harvested during culling by humans. However, there is no commercial hunting at Tembe, so there are no extra carcasses available (About: The Ivory Route, n.d.). In addition, many of the carcasses culled by humans in South Africa do not remain in the natural environment, and instead are taken to local communities.

Large, facultative avian scavengers have been found to consume carcasses over a larger extent of time, such as a few days, compared to aggregations of smaller facultative avian scavengers, which consume a carcass over a few hours (Blázquez et al., 2009). In Spain, Common Ravens (*Corvus corax*) form large feeding groups during the winter when food sources are scarcer, and they can outcompete Golden eagles (*Aquila chrysaetos*) if they consume the carcass quickly enough (Blázquez et al., 2009). During the summer, when there is more food available, ravens will feed in small groups or alone, so they do not have the chance to consume large carcasses (Blázquez et al., 2009). The Palmnut Vulture is similar to the Common Raven in this case, as it is a facultative avian scavenger that most likely would not compete with larger scavengers without great numbers, especially since the Palmnut Vulture is non-aggressive at carcasses. During months with greater food availability, which occurs during the dry season in July to October, the Palmnut Vulture possibly has a better chance to consume the large ungulate carcasses like the Common Raven does (Kendall et al., 2014). In months with less food availability, the Palmnut Vulture will probably need to rely on other components of their diets, such as the small mammals and the Palmnut fruit (Mundy, 1993). Similarly, the White-back Vulture draws close parallels to the Golden Eagle, which is outcompeted when other species arrive and quickly consume the carcass. White-backed Vultures of Tembe would need to arrive faster than aggregates of smaller scavengers, in addition to carnivorous mammal species that are eating carcasses as well. Both the Common Raven and the Golden Eagle utilize the resources depending upon food availability, which varies seasonally.

The spatial/temporal variation seen between the two facultative avian species in Spain is similar to the spatial/temporal variation seen in Kenya's vultures at the Masai Mara National Reserve. White-backed Vultures, Ruppell's Vultures (*Gyps rueppellii*), and Lappet-faced Vultures (*Torgos tracheliotos*) were tracked throughout the seasons, and were found to all closely follow the ungulate herds during the dry season, preferentially following the herds due to the higher rate of ungulate mortality during this time (Kendall et al., 2014). Vultures preferred areas with vegetation during this season, compared to areas without vegetation, likely because the herds would stay in areas with enough plant life to support their large herd size. During the rainy season, only White-backed Vultures were found in areas with high amounts of vegetation, and it is speculated that they congregate around areas with high prey abundance, as opposed to prey mortality, which in turn drives the habitat selection of the other two species (Kendall et al., 2014).

The White-backed Vultures at Tembe most likely follow a similar pattern to their northeastern counterparts, and would preferentially forage in the areas of Tembe that have high vegetation and prey abundance. White-baked Vulture's prefer dry, open savannas (Mundy, 1993), and Kendall (2014) found that within this habitat type, these vultures prefer areas with high precipitation and vegetation throughout the year. Tembe is comprised of open and closed woodlands, sand forests, and wetlands. The open woodlands of Tembe are similar to the savannas due to the open woodland's scrub and scattered trees (personal observation), and the wetlands and sand forest probably have enough vegetation to qualify as a "vegetated area" with high precipitation like the vegetated areas in Kendall's (2014) study site. Therefore, White-backed Vultures likely have suitable habitat within Tembe. Both of the studies on avian scavengers' spatial and temporal variations in habitat use and distribution (Blázquez et al., 2009; Kendall et al., 2014) have not addressed competitive or mutualistic relationships with mammalian carnivores, though they have both pointed out other significant impacts on the type of habitats in which vultures choose to forage. If the presence of other avian scavengers at a carcass deters some species from using that resource, could the presence of their own species at carcasses impact individual vultures' decisions to feed there as well? Perhaps with many members of their own species present at a carcass there will be crowding out, causing the later-arriving vultures will seek out alternate sources.

The communal roosting of Turkey Vultures (*Cathartes aura*) and Black Vultures (*Coragups atratus*) increases the foraging ability for each individual, as they can search a larger area more quickly and use each other as guides to the carcasses (Buckley, 1996). This phenomenon is likely seen in Tembe's White-backed Vultures, because they tend to form groups for nesting, where the entire group will spread out over a small patch of habitat forming a "loose colony" (Mundy, 1993). Within these loose colonies, each breeding pair will have their own nest and tree about 50-200m from their nearest neighbor (Mundy, 1993). The presence of loose nesting associations leads to an integrated scavenging system, where the vultures can use each other as cues to quickly locate enough food to feed some members in the colony. This concept of sharing information agrees with the "information center hypothesis," stating that individuals in a colony can utilize each other as resources to locate carrion (Heeb and Richner, 1994) Palmnut Vultures, on the other hand, do not utilize any kind of formal nesting groups, and the average distance between neighboring nests exceeds a kilometer (1.16 km) (Mundy, 1993). The advantage of communal roosting is therefore lost on these vultures, as the costs of living close to one another outweigh the potential benefits of communal roosting and information sharing.

The long-assumed hypothesized association between vultures and lions might not be based in reality. Hunter et al. (2007) found that there was no significant relationship between the presence of lions and medium-sized vultures (including White-backed Vultures), while this same vulture group appeared at carcasses with Jackals and Spotted Hyenas. We can speculate that vultures prefer the kills made by smaller carnivores than lion kills, due to the presence of White-backed Vultures at the kills of Spotted Hyenas and Jackals, which are smaller than lions (Hunter et al., 2007). This finding supports the idea that the presence of lions may not positively impact the presence of vultures in an area because they are not observed sharing carcasses and, by extension, vultures and lions are not in a commensal relationship, where the vultures benefit from unaffected lions.

There is even a concern that the introduction of lions would not only have no effect on vulture species, but also rather negatively affect the vultures. There is evidence that the re-introduction of lions caused a population crash of other species due to the arrival of the predator in an ecological system where the species of prey have not been present for enough time to fully integrate into the system (Miller et al., 2013). Since there is no evidence as to whether Miller's observations carry over to Tembe, it is important to discern if there are aspects from the suggestions at Miller et al.'s study site that can improve the situation at Tembe, regardless of the type of impact on the vultures by Tembe lions. The question therefore becomes how to manage lions better in order to support the entire park, including vultures.

One solution is to prohibit over-productive lions from surpassing the carrying capacity due to the artificial environment of small reserves (Miller et al., 2013). This issue can be solved in two ways: introducing birth control methods to stop overpopulation, and removing adults from the population before they have the chance to breed (Miller et al., 2013). Removing adults from the population can be achieved in several ways; including selling lions to other parks or zoos or allowing commercial trophy hunting (Wright, 2014). While Tembe frequently sends lions to other reserves to keep the lion population in check (Wright, 2014), perhaps they are not keeping the number of lions low enough. By keeping the population of lions small, without sacrificing genetic diversity, each lion may eat less of each carcass and leave remains of the carrion for vultures. This feeding style would be especially helpful in the short term if the park management utilized more harvested ungulates to feed both populations. Alternatively, lions could kill fewer ungulates which would leave less for the vultures to consume.

Another challenged faced by the vultures of Tembe could be the roads both in and around Tembe. Carcasses placed near roads tend to be found more quickly than carcasses farther from roads (Lambertucci et al., 2009). This trend is likely related to the fact that White-back Vultures and Palmnut Vultures prefer open areas for foraging. However, the facilitated location of food comes at a price: larger-bodied birds face significant risks when close to roads, including mortality due to collisions with cars and trucks (Lambertucci et al., 2009). Tembe luckily does not face this issue as much as other places because there are few roads inside the park, and not many cars are allowed inside the park each day (Wright, 2014). The roads of Tembe therefore facilitate the foraging process without many of the risks associated in other locations.

The introduction of lions at Tembe does not serve as an example of a way to bolster the populations of vultures. By extension, the populations of endangered vulture species in the United States, for example, would not benefit from an increased presence of large mammalian carnivores. A close equivalent to Africa's lions could be the Mountain Lion (*Puma concolor*) in North America, and increasing the presence of this mammal could negatively impact the entire ecosystem, as suggested by Miller et al. (2013), in addition to having a negative or neutral impact on North American vultures. In order to improve the conditions for vultures, both in Africa and in the US, there should be an increase in carcass availability during periods of low ungulate mortality that is unrelated to predation by carnivorous mammals.

An easy way to supplement the food source of vultures is the creation and maintenance of "Vulture Restaurants." These restaurants are locations in which harvested or culled ungulate carcasses are compiled in order to provide a stable food source for vultures (Cortés-Avizanda et al., 2010). However, these restaurants are not reflective of actual conditions because the food is concentrated, which facilitates the dominant competitors in outcompeting the other species (Cortés-Avizanda et al., 2010). Therefore to conserve species diversity, park managers need to implement several small restaurants with few carcasses per site to make these resources harder to monopolize (Cortés-Avizanda et al., 2010). The White-backed Vultures of Tembe would be able to utilize these carcasses without monopolizing them, allowing the Palmnut Vultures to use this resource as well. Unfortunately, vulture restaurants will help vulture populations only in the short-term, as they are artificial and create a reliance on human intervention.

There is little overlap between the diets of Palmnut Vultures and lions. White-backed Vultures, on the other hand, survive on a similar diet to the lions, with the major exception being that lions will kill their prey in addition to scavenging, unlike the obligate scavenger White-backed Vulture. With little evidence to suggest that lions and vultures are seen at the same carcasses, there is a serious question as to the validity of the suggested increased sightings of vultures at Tembe after the introduction of lions in 2004. In fact, this review has found no indication that there is a scientific basis for this claim, and it is likely that there was no positive influence by the lions on the vultures at Tembe.

With the assumption of lions positively influencing vulture populations discounted, potential ways to increase vulture presence inside of reserves have one less option. There could be other conservation techniques to keep vultures inside of reservations where they are safe from humans killing them, such as the short-term use of vulture restaurants or the increased presence of smaller mammalian carnivores, such as hyenas or jackals. From the literature surveyed in this paper, however, the most promising technique in vulture conservation will be making the areas surrounding the reserves safer for vultures.

Summary

There is a substantial lack of supporting information for the assumption that the reintroduction of lions led to an increased presence of vultures in Tembe National Elephant Park. Of the two vulture species present, only White-backed Vultures have a large dietary overlap with lions. However, White-backed Vultures are only infrequently observed at lion kills, though it has been documented. While the presence of predators at kills often decreases the presence of vultures, vultures also experience seasonal fluctuations in density due to higher carcass availability in the migratory season (Kendall et al., 2012a).

The most important aspects of promoting the conservation of African Vultures is increasing their use within reserves through the use of vulture restaurants, and making the areas outside of reserves safer. This latter option would include educating local communities on effective ways to reduce mammalian facultative scavengers without harming the vultures, as poisoning carcasses currently harms both of these groups. In terms of conservation, the lack of evidence for a positive interaction between lions and vultures signifies a technique to avoid in the attempts to promote vulture presence. Tembe's introduction of lions does not serve as a positive conservation technique, either for its own vulture populations or for locations outside of Africa.

Further research on the numbers of vultures in Tembe through time is warranted: speculation will not prove anything in terms of the actual events in

Tembe. Other ways to increase the presence of vultures inside reserves demands attentions as well, for if researchers could determine a way to bring about an increase in vulture presence within reserves as “naturally” as possible, then the conservation of these important species would not need to rely solely on the acts of the local community.

References

Blázquez M, Sánchez-Zapata JA, Botella F, Carrete M, and Eguía S. 2009. Spatio-temporal segregation of facultative avian scavengers at ungulate carcasses. *Acta Oecologica* 35: 645–650. doi: 10.1016/j.actao.2009.06.002. Published online before print June 2009. doi: 10.1016/j.actao.2009.06.002 <http://www.sciencedirect.com/science/article/pii/S1146609X09000782>. Accessed 2014 Oct 8.

Buckley NJ. 1996. Food finding and the influence of information, local enhancement, and communal roosting on foraging success of North American Vultures. *The Auk* 113: 473–488. <https://sora.unm.edu/node/25677>. Accessed 2014 Oct 8.

Cortés-Avizanda A, Carrete M, and Donázar JA. 2010. Managing supplementary feeding for avian scavengers: Guidelines for optimal design using ecological criteria. *Biol. Conserv*. 143: 1707–1715. Published online before print May 2010. doi:10.1016/j.biocon.2010.04.016 <http://hdl.handle.net/10261/35432>. Accessed 2014 Oct 8.

DeVault TL, Rhodes J, Olin E, and Shivik JA. 2003. Scavenging by vertebrates: behavioral, ecological, and evolutionary perspectives on an important energy transfer pathway in terrestrial ecosystems. *Oikos* 102: 225–234. Published online before print July 2003. doi:10.1034/j.1600-0706.2003.12378.x <http://onlinelibrary.wiley.com/doi/10.1034/j.1600-0706.2003.12378.x/abstract>. Accessed 2014 Oct 8.

Emslie SD. 1998. An Early Condor-Like Vulture From North America. Auk 105: 529–535. <https://sora.unm.edu/advancedsearch?field_authors_value=&title=An+Early+Condor-Like+Vulture+From+North+America&field_fulltext_value=&field_year_value=>. Accessed 2014 Oct 29.

Gangoso L, Álvarez-Lloret P, Rodríguez-Navarro AAB, Mateo R, Hiraldo F, and Donázar JA. 2009. Long-term effects of lead poisoning on bone mineralization in vultures exposed to ammunition sources. *Environ. Pollut. 157*, 569–574. Published online before print November 2008. doi:10.1016/j.envpol.2008.09.015 <http://www.sciencedirect.com/science/article/pii/S0269749108004478>. Accessed 2014 Oct 8.

Gilbert M, Watson RT, Virani MZ et al. 2006. Rapid population declines and mortality clusters in three Oriental white-backed vulture Gyps bengalensis colonies in Pakistan due to diclofenac poisoning. *Oryx* 40: 388–399. <http://search.proquest.com/docview/294619074?accountid=13503>. Accessed 2014 Oct 27.

Grier JW. 1982. Ban of DDT and subsequent recovery of Reproduction in bald eagles. *Science* 218: 1232–1235.<http://www.sciencemag.org/content/218/4578/1232>. Accessed 2014 Nov 17.

Hanekom C. 2014. Personal Communication.

Heeb P, and Richne H. 1994. Seabird colonies and the appeal of the Information Center Hypothesis. *Trends Ecol. Evol.* 9: 25. <http://www.sciencedirect.com/science/article/pii/0169534794902283>. Accessed 2014 Dec 10.

Houston D. 1995. The Adaptations of Scavengers. In: Sinclair ARE and Norton-Griffiths M, eds. *Serengeti, Dynamics of an Ecosystem*. Chicago: University of Chicago Press. 263–86. <http://books.google.com/books?id=ffLvZbZYpr0C&q=30%25#v=snippet&q=adaptations%20of%20scavengers&f=false>. Accessed 2014 Oct 30.

Hunter JS, Durant SM, and Caro TM. 2007. Patterns of scavenger arrival at cheetah kills in Serengeti National Park Tanzania. *Afr. J. Ecol.* 45: 275–281. Published online before print December 2006. doi:10.1111/j.1365-2028.2006.00702.x <http://onlinelibrary.wiley.com/doi/10.1111/j.1365-2028.2006.00702.x/abstract>. Accessed 2014 Oct 8.

Kendall C, Virani MZ, Kirui P, Thomsett S, and Githiru M, 2012a. Mechanisms of Coexistence in Vultures: Understanding the Patterns of Vulture Abundance at Carcasses in Masai Mara National Reserve, Kenya. *The Condor* 114: 523–531. <http://www.bioone.org/doi/abs/10.1525/cond.2012.100196>. Accessed 2014 Oct 8.

Kendall CJ, Virani MZ, and Bildstein KL. 2012b. Assessing Mortality of African Vultures

Using Wing Tags and GSM-GPS Transmitters. *J. Raptor Res.* 46: 135–140. <http://www.bioone.org/doi/abs/10.3356/JRR-10-87.1>. Accessed 2014 Oct 8.

Kendall CJ, Virani MZ, Hopcraft JGC, Bildstein KL, and Rubenstein DI. 2014. African Vultures Don’t Follow Migratory Herds: Scavenger Habitat Use Is Not Mediated by Prey Abundance. *PLoS ONE* 9: e83470. doi:10.1371/journal.pone.0083470

Lambertucci SA, Speziale KL, Rogers TE, and Morales JM. 2009. How do roads affect the habitat use of an assemblage of scavenging raptors? *Biodivers. Conserv*. 18: 2063–2074. <http://search.proquest.com/docview/21321134?accountid=13503>. Accessed 2014 Oct 8.

Lehmann MB, Funston PJ, Owen CR, and Slotow R. 2008a. Reproductive biology of a pride of lions on Karongwe Game Reserve, South Africa. *Afr. Zool*. 43: 230–236. <http://www.bioone.org/doi/full/10.3377/1562-7020-43.2.230>. Accessed 2014 Oct 8.

Lehmann MB, Funston PJ, Owen CR, and Slotow R. 2008b. Feeding behaviour of lions Panthera leo on a small reserve. *South Afr. J. Wildl. Res.* 38: 66–78. <http://www.bioone.org/doi/full/10.3957/0379-4369-38.1.66>. Accessed 2014 Oct 8.

Lerner HRL, and Mindell DP. 2005. Phylogeny of eagles, Old World vultures, and other Accipitridae based on nuclear and mitochondrial DNA. *Mol. Phylogenet. Evol.* 37: 327–346. Published online before print May 2005. doi: 10.1016/j.ympev.2005.04.010 <http://www.sciencedirect.com/science/article/pii/S1055790305001363>. Accessed 2014 Dec 10.

Lundholm CE. 1997. DDE-induced eggshell thinning in birds: Effects of p,p′-DDE on the calcium and prostaglandin metabolism of the eggshell gland. *Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol.* 118: 113–128. <http://www.sciencedirect.com/science/article/pii/S0742841397001059>. Accessed 2014 Nov 17.

Miller SM, Bissett C, Burger A, Courtenay B, Dickerson T, Druce DJ, et al. 2013. Management of Reintroduced Lions in Small, Fenced Reserves in South Africa: An Assessment and Guidelines. *South Afr. J. Wildl. Res.* 43: 138–154. <http://www.bioone.org/doi/abs/10.3957/056.043.0202>. Accessed 2014 Oct 8.

Mundy P. 1993. The Vultures of Africa Academic Press. 460p.

Newman K. 2010. Newman’s Birds of Southern Africa. Commemorative Edition. Cape Town, South Africa: Struik Nature. 536p.

Oaks JL, Gilbert M, Virani MZ, Watson RT, Meteyer CU, Rideout BA, et al. 2004. Diclofenac residues as the cause of vulture population decline in Pakistan. *Nature* 427: 630–633. Published online before print January 2004. doi:10.1038/nature02317 <http://www.nature.com/nature/journal/v427/n6975/full/nature02317.html>. Accessed 2014 Nov 17.

Ogada DL, Keesing F, and Virani MZ. 2012. Dropping Dead: Causes and Consequences of Vulture Population Declines Worldwide. *Annals of the New York Academy of Sciences* 1249: 57–71. Published online before print December 2012. doi:10.1111/j.1749-6632.2011.06293.x <http://onlinelibrary.wiley.com/doi/10.1111/j.1749-6632.2011.06293.x/abstract>. Accessed 8 Oct. 2014.

Ogada DL, Torchin ME, Kinnaird MF, and Ezenwa VO. 2012b. Effects of Vulture Declines on Facultative Scavengers and Potential Implications for Mammalian Disease Transmission. *Conserv. Biol.* 26: 453–460. Published online before print March 2012. doi:10.1111/j.1523-1739.2012.01827.x <http://onlinelibrary.wiley.com/doi/10.1111/j.1523-1739.2012.01827.x/abstract>. Accessed 2014 Oct 8.

Sekercioglu CH, Daily GC, and Ehrlich PR. 2004. Ecosystem consequences of bird declines. *Proc. Natl. Acad. Sci. U. S. A.* 101: 18042–18047. <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC539768/>. Accessed 2014 Oct 23.

Selva N, Jedrzejewska B, Jedrzejewski W, and Wajrak A. 2005. Factors affecting carcass use by a guild of scavengers in European temperate woodland. *Can. J. Zool*. 83: 1590–1601. <http://search.proquest.com/docview/220491419?pq-origsite=summon>. Accessed 2014 Oct 8.

Tailor Made Safaris [Internet]. n.d. Map of the Park. <http://www.safarisopmaat.nl/uploads/2/0/8/1/20817686/map_tembe_elephant_park.jpg> Accessed 2014 Nov 8.

Tembe National Elephant Park [Internet]. n.d. “About: The Ivory Route.” <http://www.tembe.co.za/about.htm> Accessed 2014 Dec 10.

Tilman D, May RM, Lehman CL, and Newak MA. 1994. Habitat destruction and the extinction debt. *Nature* 371: 65. <http://search.proquest.com/docview/204461498/abstract?accountid=13503>. Accessed 2014 Oct 29.

US EPA [Internet]. 2014 December 4. DDT Ban Takes Effect EPA Press Release-December 31, 1972. <http://www2.epa.gov/aboutepa/ddt-ban-takes-effect> Accessed 2014 Dec 10.

Virani MZ, Kendall C, Njoroge P, and Thomsett S. 2011. Major declines in the abundance of vultures and other scavenging raptors in and around the Masai Mara ecosystem, Kenya. *Biol. Conserv*. 144: 746–752. Published online before print November 2010. doi:10.1016/j.biocon.2010.10.024 <http://www.sciencedirect.com/science/article/pii/S0006320710004726>. Accessed 2014 Oct 8.

Walker M. 2008. Vulture catastrophe becomes human tragedy. *New Sci*. 199: 14.

Wright, C. 2014. Personal Communication.

DFT and MP2 Study of the Binding of Salbutamol within the SULT1A3 Active Site

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*Sulfotransferase 1A3 is important in its role in the regulation of a variety of endogenous substrates and xenobiotics in the body. This study uses the previously validated solvated-relaxed model [Bigler, D.J. et al. Comp. Theor. Chem. 1051, 79 (2014)] to investigate the binding of salbutamol within the SULT1A3 active site. Salbutamol is relevant both as the pharmaceutical medication, albuterol, and as a feed additive. The method M062X was used for the optimization, allowing for implicit solvation, of the ligand and the amino residue R-groups within the SULT1A3 active site. Interaction energies between salbutamol and the active site were then calculated using M062X and MP2 with a 6-311+G*basis set. The results of this study indicate that salbutamol has favorable interaction energies when bound within the SULT1A3 active site and the ligand is predicted to be favorably sulfated.*

Introduction

In recent work, we investigated the binding of ligands to SULT1A3 (Bigler et al. 2014). The electronic interaction energies of dopamine, dopamine analogues, and resveratrol were calculated using three different model chemistries (Bigler et al. 2014). In the current project, we use our previously validated solvated-relaxed model to predict the affinity of SULT1A3 for the pharmaceutically relevant molecule, salbutamol (Fig. 1) (Bigler et al. 2014).

Sulfotransferases catalyze a sulfation reaction that results in the regulation and metabolism of several endogenous ligands and xenobiotics (Gamage et al. 2006). The sulfation of these substrates occurs through abstraction of a proton from a phenolic hydroxyl group by the residue His108 and then attachment of a sulfuryl moiety (SO_3^-) by the cofactor 3’-phosphoadenosine-5’-phosphosulfate (PAPS) (Gamage et al. 2006). The attachment of this group affects water solubility of the ligands and thus regulates movement throughout the body (Gamage et al. 2006). The current study focuses on a cytosolic member of the sulfotransferase family, SULT1A3 (Gamage et al. 2006).

SULT1A3 is shown to play a role in the metabolism of various endogenous ligands such as dopamine; however it also is involved in the metabolism of exogenous ligands such as L-dopa and salbutamol (Bian et al. 2007). SULT1A3 is regulated by glucocorticoids and exclusively metabolizes salbutamol, which makes it a target for additional studies because of its clinical significance (Bian et al. 2007). Knowledge of the specificity of SULT1A3 is especially pertinent to the pharmaceutical understanding of the medication salbutamol, which is used to relieve symptoms of breathlessness associated with asthma and with chronic obstructive pulmonary disease (Jacobson et al. 2015). Salbutamol is structurally similar to dopamine and has a benzylic and phenolic alcohol (Figure 1), which allow it to have the same kinds of interactions within the enzymatic active site (Jacobson et al. 2015).

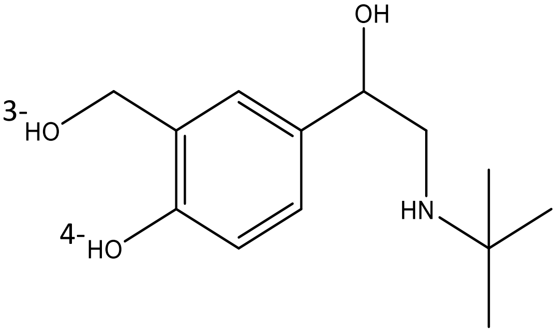


Figure 1. Structure of salbutamol.

In a study by Jacobson, *et al*, the role of SULT1A3 in the metabolism of salbutamol is investigated (Jacobson et al. 2015). Because this enzyme is so important to the metabolism of small phenolic structures similar to dopamine, variation in the specificity of the enzyme could affect the rate of the excretion of xenobiotics from the body (Jacobson et al. 2015). Salbutamol is excreted from the body in urine in both its unchanged form and in the metabolite form (Jacobson et al. 2015). For the metabolism of drugs, hepatic and intestinal SULT1A3 enzymes are of specific interest (Cubitt et al. 2011). However, the capacity of sulfation by SULT enzymes can be constrained by the availability of the cofactor, PAPS (Cubitt et al. 2011).

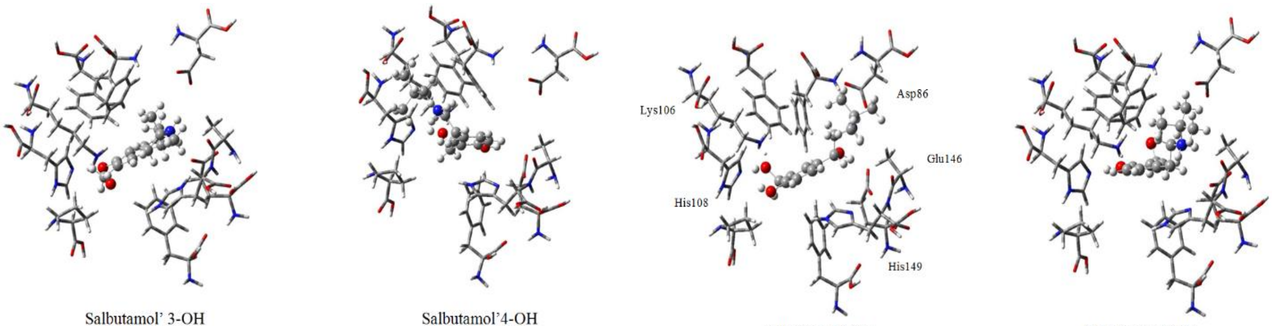
Understanding the process of sulfation of salbutamol is important in the both the food and pharmacological industries (Ko et al. 2012). Salbutamol acts as a β-adrenergic agonist when added to feed for livestock (Ko et al. 2012). Salbutamol is one of several feed additives with the purpose of enhancing the leanness of the meat (Ko et al. 2012). In a study by Ko, *et al*, SULT1A3 was found to be the most prominent sulfotransferase in the metabolism of salbutamol with an activity of about 2.01 mmol/min/mg when exposed to 10μM of salbutamol (Ko et al. 2012). In this study, the kinetic parameters of the sulfation of salbutamol was also investigated and was thought to play a role in the specificity of this substrate towards SULT1A3 (Ko et al. 2012). The highest levels of sulfation of salbutamol was determined to be in the small intestine (Ko et al. 2012). Salbutamol was found to inhibit the sulfation of dopamine in a concentration-dependent manner in the study by Ko, *et al* (Ko et al. 2012). However, affinity of SULT1A3 for dopamine is higher than its affinity for salbutamol (Ko et al. 2012). Therefore, it would require a high concentration of salbutamol to obstruct the homeostasis of dopamine or other endogenous ligands (Ko et al. 2012). Salbutamol only has one phenolic hydroxyl group and thus the major product of sulfation of salbutamol has been found to be salbutamol-4-O-sulfate (Ko et al. 2012).

In this study, the interaction energies between salbutamol and the active site are investigated in four positions. As in previous work, MP2 and M062X with counter-poise corrections were the two methods chosen for calculating the interactive forces, based on their accuracy in determining non-covalent interactions (Bigler et al. 2014; Hohenstein et al. 2008; Tsuzaki et al. 2002; Digiovanni et al. 2013). Optimizations used the Polarizable Continuum Model, allowing for implicit solvation (Tomasi et al. 2005).

Computational Details

Salbutamol, in its four different conformers, was investigated in this computational study. The active site that was used for SULT1A3 was obtained from a crystal structure with bound dopamine and contains ten amino acid residues, as in previous research—Ala148, Asp86, Glu146, His108, His149, Lys106, Phe24, Phe81,, Phe142, and Pro47 (Lu et al. 2005). These residues all contain an atom that is within 3.5 angstroms of the crystal structure of bound dopamine (Bigler et al. 2011). The first orientation of salbutamol favors sulfation at the 4-position; the second favors sulfation at the 4-position, but in a flipped orientation of the ligand (Figure 2). Similarly, in the third and fourth orientations, salbutamol is oriented to allow for deprotonation at the 3-position.

Salbutamol within the active site was optimized using M062X with a basis set of 6-31G. The optimization allowed for the optimization of the R-groups of the amino acid residues as well as implicit solvation using the Polarizable Continuum Model (Tomasi et al. 2005). Salbutamol was optimized in the four previously defined positions and then interaction energies were calculated. Two methods were used for the calculation of the counterpoise-corrected interaction energy, MP2 and M062X, both with a basis set of 6-311+G* (Tomasi et al. 2005). The total interaction energy was calculated by a summation of the individual ligand/active site calculations. All calculations were performed in Gaussian 09 (Frisch et al. 2009).



Salbutamol' 3-OH Salbutamol'4-OH Salbutamol 3-OH Salbutamol 4-OH

Figure 2. Optimized structures (M062x/6-31G) of salbutamol in all four orientations. Optimizations were performed with non-rigid amino acid residues and implicit solvation.

Results

Figure 2 shows all four orientations of salbutamol optimized with the relaxed-solvated model within the SULT1A3 active site (Bigler et al. 2014). In all optimizations, salbutamol retained the protons on the benzylic and phenolic alcohols. To be consistent with previous work, the 3-OH and 4-OH terminology is being used (see figure 1). The protons were thus not abstracted by the amino residue, His108. The positioning of salbutamol in the 3-OH and 4-OH positions is more favorably situated within the active site than salbutamol in the “flipped” orientation (indicated by salbutamol’).

Table 1 shows the interaction energies between salbutamol and five significant amino acid residues within the active site—His108, Lys106, Asp86, Glu146, and His149. In all optimizations of salbutamol, the ligand retains its protons and thus is positively charged. Therefore, there is a strong attractive interaction between salbutamol and the negatively charged residues, Asp86 and Glu146. In this study, negative interaction energies indicate an attractive force between the ligand and the active site and positive interaction energies indicate a repulsive force.

The optimized salbutamol 3-OH and salbutamol’ 3-OH were oriented in a way to favor deprotonation at the benzylic alcohol on the ligand. In these orientations, the tail of salbutamol is extended in such a way to allow for favorable interactions with Asp86 within the active site. The interaction energy between salbutamol 3-OH and Asp86 is -104 (kcal/mol), a more favorable interaction than the energy between salbutamol 4-OH and Asp86 is -55.7 (kcal/mol). Similarly, there are more favorable interaction energies between salbutamol in the 3-OH position and Glu146. The interaction energy between salbutamol 3-OH and Glu146 is –93.9 (kcal/mol), a more favorable interaction than the energy between salbutamol 4-OH and Glu146 is -74.4 (kcal/mol). The interaction energies of His108 did not show a notable difference between salbutamol 3-OH or salbutamol’ 3-OH.

The individual interaction energies between the positively charged amino acid residues with salbutamol in all orientations exhibit a repulsive force. In the case of salbutamol 3-OH, the interaction with Lys106 is 22.4 (kcal/mol) and the interaction with His149 is 42.6 (kcal/mol). Salbutamol’ 3-OH exhibits the most repulsive force with an interaction with Lys106 of 27.2 (kcal/mol) and with His149, 54.6 (kcal/mol). In the other three orientations of salbutamol there is a similar trend of repulsive interactions with these positively charged residues.

When salbutamol is positioned in a way that favors deprotonation at the 4-OH position, there is a favorable interaction energy, although not as strongly attractive as when the molecule is positioned to favor the 3-OH position. Again, in the case of salbutamol 4-OH, there is an attractive force between the ligand and the SULT1A3 active site of -96.9 (kcal/mol). The “flipped” orientation also shows a favorable interaction of -66.5 (kcal/mol) but is the least suitable position based on the calculated interaction energies. Again, His108 showed only a minor decrease in interaction energy for salbutamol’ 4-OH (-7.97 kcal/mol) when compared to salbutamol 4-OH (-16.2 kcal/mol).

Table 1. Counterpoise-corrected MP2 and M062X interaction energies of salbutamol in each orientation optimized in the solvated-relaxed model.[1] Salbutamol’ refers to the ligand in a flipped orientation. Energies in kcal/mol.

		Histidine108	Lysine106	Aspartate86	Glutamate146	Histidine149	Total
Salbutamol 3-OH	MP2	-15.7	22.4	-104	-93.9	42.6	-168
	M062X	-17.6	19.4	-109	-96.7	42.9	-181
Salbutamol 4-OH	MP2	-16.2	13.9	-55.7	-74.4	53.3	-96.9
	M062X	-18.9	10.7	-55.4	-74.5	53.2	-107
Salbutamol’ 3-OH	MP2	-15.9	27.2	-60.8	-102	54.6	-113
	M062X	-18.5	24.7	-60.3	-105	53.8	-125
Salbutamol’ 4-OH	MP2	-7.97	18.6	-38.2	-50.5	39.5	-66.5
	M062X	-9.83	15.3	-38.0	-50.8	39.8	-73.1

In previously published work, we investigated the interaction energy between SULT1A3 and the endogenous ligand, dopamine. The total interaction energy for dopamine in the active site was calculated to be -178 (kcal/mol). Thus, salbutamol shows a favorable interaction energy, but calculations suggest that it has less of an affinity for the SULT1A3 active site than dopamine. This trend agrees with experimental data in the study by Ko, *et al* (Ko et al. 2012).

All interaction energy calculations showed a favorable interaction between salbutamol and the SULT1A3 active site.

Conclusions

Calculated energies for salbutamol indicate that this molecule has favorable interactions with the residues in the SULT1A3 active site. The strongest interaction energy of -168 kcal/mol suggests that salbutamol at the 3-OH position is favored for deprotonation.

In line with other work, these findings suggest that the 3-OH would be the more favorable product for sulfation, although experimental work suggests that the sulfation of the 4-OH is more likely (Ko et al. 2012). This contrast would suggest that the binding energy between the ligand and the active site is not the major factor in the selectivity of sulfation of salbutamol, catalyzed by SULT1A3.

Salbutamol in all orientations shows favorable interactions with SULT1A3, although less strong than the attractive forces between the endogenous compound, dopamine, and the active site.

References

Bian HS, Ngo SY, Tan W, Wong CH, Boelsteril UA, Tan TM. 2007. Induction of human sulfotransferase 1A3 (SULT 1A3) by glucocorticoids. Life Sciences. 81(25-26): 1659-1667.

Bigler DJ, Peterson LW, Cafiero M. 2014. Effects of implicit solvent and relaxed amino acid side chains on the MP2 and DFT calculations of ligand-protein structure and electronic interaction energies of dopaminergic ligands in the SULT1A3 enzyme active site. Computational and Theoretical Chemistry. 1051 (1): 79-92.

Cubitt HE, Houston JB, Galetin A. 2011. Prediction of Human Drug Clearance by Multiple Metabolic Pathways: Integration of Hepatic and Intestinal Microsomal and Cytosolic Data. Drug Metabolism and Disposition 39 (5): 864-873.

DiGiovanni KM, Hatstat AK, Rote J, Cafiero M. 2013. MP2/DFT calculations of interaction energies between acetaminophen and acetaminophen analogues and the aryl sulfotransferase active site. Comput. Theo. Chem.1007 (1): 41-47

Gamage N, Barnett A, Hempel N. 2006. Human sulfotransferases and their tole in chemical metabolism, Toxicol. Sci. 90 (1):5-22.

Gaussian 09, Revision C.01, Frisch MJ, Trucks GW, Schlegel HB, Scuseria GE, Robb GA, Cheeseman JR, Scalmani G, Barone V, Mennucci B, Petersson GA, Nakatsuji H, Caricato M, Li X, Hratchian HP, Izmaylov AF, Bloino J, Zheng G, Sonnenberg JL, Hada M, Ehara M, Toyota K, Fukuda R, Hasegawa J, Ishida M, Nakajima T, Honda Y, Kitao O, Nakai H, Vreven T, Montgomery JA, Peralta JE, Ogliaro F, Bearpark M, Heyd JJ, Brothers E, Kudin KN, Staroverov VN, Kobayashi R, Normand J, Raghavachari K, Rendell A, Burant JC, Iyengar SS, Tomasi J, Cossi M, Rega N, Millam JM, Klene M, Knox JE, Cross JB, Bakken V, Adamo C, Jaramillo J, Gomperts R, Stratmann RE, Yazyev O, Austin AJ, Cammi R, Pomelli C, Ochterski JW, Martin RL, Morokuma K, Zakrzewski VG, Voth GA, Salvador P, Dannenberg JJ, Dapprich S, Daniels SD, Farkas O, Foresman JB, Ortiz JV, Cioslowski J, and Fox DJ. 2009. Gaussian, Inc., Wallingford CT.

Hohenstein EG, Chill ST, Sherrill CD. 2008. Assessment of the Performance of the M05-2X and M06-2X Exchange-Correlation Functionals for Noncovalent Interactions in Biomolecules. Journal of Chemical Theory and Computation. 4(12):1996-2000.

Jacobson GA, Yee KC, Wood-Baker R, Walters EH. 2015. SULT1A3 single-nucleotide polymorphism and the single dose pharmacokinetics of inhaled salbutamol enantiomers: Are some athletes at risk of higher urine levels? Drug Testing and Analysis. 7 (2):109-113

Ko KA, Kurogi K, Davidson G, Liu MY, Sakakibara Y, Suiko M, Liu MC. 2012. Sulfation of ractopamine and salbutamol by the human cytosolic sulfotransferases. The Journal of Biochemistry. 152 (3): 275-283.

Lu JH, Li HT, Liu MC, Zhang JP, Li M, An XM, Chang WR. 2005. Crystal structure of human sulfotransferase SULT1A3 in complex with dopamine and 3’-phosphoadenosine 5’-phosphate.

Biochemical and Biophysical Research Communications. 335 (2):417-423.

Tsuzaki S, Honda K, Uchimaru T, Mikami M, Tanabe K. 2002. Origin of attraction and directionality of the π/π interaction: model chemistry calculations of benzene dimer interaction. J. Am. Chem. Soc. 124 (1):104-112.

Tomasi J, Mennucci B, Cammi R, 2005. Quantum mechanical continuum solvation models, Chem. Rev. 105 (1):2999-3093.

The Oral Microbiome: its Role in Oral Cancer and Detection

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The oral microbiome is an environment conducive to many types of bacteria. In recent years, researchers have conducted both saliva and tissue sampling studies to compare the oral microbiome of healthy individuals to those with oral carcinoma. A consensus among researchers reveals that there is a distinct fluctuation between the oral microbiota associated with cancerous versus non-cancerous tissues. Inconsistent techniques and variations in sample location and size in these studies make it difficult to conclude that certain species level bacteria have a direct correlation with oral cancer. Additionally, early detection methods for oral cancer have also not yet been adequately developed or studied. Despite these setbacks, a few studies have identified species that could be linked to oral cancer: Streptococcus anginosus *and* Porphyromonas gingivalis. *These findings demonstrate the potential and importance of identifying species level bacteria for prediction and detection of oral cancer.*

Introduction

The oral microbiome serves as a good site for detecting pathogenic bacteria because of the mouth's well-known microbial make-up and stability in individuals over time. The oral microbiome specifically consists of all of the microorganisms found in or on the mouth, and all of its parts leading up to the distal esophagus (Dewhirst et al. 2010). These surfaces include the teeth, gingiva, tongue, cheeks, hard and soft palates, tonsils, pharynx, esophagus, eustachian tube, middle ear, trachea, lungs, nasal passages, and sinuses (Dewhirst et al. 2010). These structures all house a wide variety of bacteria, and different bacterial species have been found on these surfaces. The oral cavity has a much richer core microbiome than other sites like the nose, stool, skin, or vagina (Huse et al. 2012). The most prominent bacterial communities found in at least 95% of samples taken in a study of a healthy human mouth include Streptococcus, Fusobacteria, Gemella, and Veillonella (Huse et al. 2012). More specifically the core microbiome of most healthy mouths consist of bacteria from five phyla: Firmicutes, Proteobacteria, Actinobacteria, Bacteroidetes, and Fusobacteria (Zaura et al. 2009). These same core bacterial phyla also remain stable in the oral cavity over time (Lazarevic et al. 2009). Lazarevic and colleagues sampled saliva from five healthy individuals and found that the proportion of the core phyla does not significantly differ for each individual (Lazarevic et al. 2010). These phyla not only remain constant between individuals but also do not fluctuate within individuals over time (Lazarevic et al. 2010). These stable characteristics of the oral microbiome make it a predictable site in terms of collecting bacteria and using these bacteria as indicators for cancer. It is important to note that periodontal disease is defined as the progression of gingivitis associated with the microbial shifts from gram-positive organisms to gram-negative organisms (Socransky 2005). While many of the bacteria isolated in these studies are also correlated with periodontal disease, the bacteria in this review will be examined in terms of their relation to cancer, independent of periodontitis.

Oral Cancer Overview

According to the Centers for Disease Control and Prevention (CDC), oral cancer affects more than 30,000 individuals each year, and it has a 5-year survival rate of about 50% (CDC 2013). Unfortunately, this survival rate has not yet improved over the last forty years (CDC 2013). The most common type of oral cancer is oral squamous cell carcinoma (OSCC), and it affects about 90% of those who are diagnosed with oral cancer (Schmitt 2014). More people die from oral cancer than from either melanoma, cervical, or ovarian cancer separately in the United States (Schmitt 2014). High-risk behaviors for developing oral carcinoma include excessive drinking and smoking (CDC 2013). HPV is also a causative agent of oral cancer (CDC 2013). While OSCC is a cancer mainly found in middle-aged men with frequent exposure to alcohol and tobacco, oral cancer is becoming increasingly more common in women and children worldwide (Schmitt et al. 2014; Fan et al. 2014). Oral cancer is traditionally diagnosed through visual means in the office, including community-based visual screens and other additional tools for diagnosis (Patton 2003). However, these methods often do not help significantly decrease morbidity or mortality associated with oral cancer (Patton 2003). Since oral cancer is among the top ten most common cancers and disease rates have not improved in recent years, a scientifically accurate early detection method is needed (Elashoff et al. 2012). Examining the changes associated with the oral microbiome in OSCC patients can help to increase scientists' understandings of the causative agents of oral cancer and help to find earlier detection mechanisms.

Comparing Healthy versus Cancerous Microbiomes

Several experiments have demonstrated a variation in the oral microbiota of healthy patients versus those with oral carcinoma. In the following studies, bacteria were non-invasively isolated from OSCC saliva samples and compared to saliva samples from OSCC-free patients (Henrich et al. 2014; Mager et al. 2005; Pushalkar et al. 2011). An experiment from Mager et al. in 2005 is one of the most cited salivary microbiota studies. This experiment was one of the first modern experiments to isolate specific bacterial species potentially responsible for OSCC. They tested the forty most common oral bacteria in all subjects and found that three species of bacteria were more elevated in patients with OSCC. These three species include *Capnocytophaga gingivalis*, *Prevotella melaninogenica*, and *Streptococcus mitis*. These all come from the Bacteroidetes and Firmicutes phyla. They also found that these three species were able to predict 80% of OSCC cancer cases in their study (Mager et al. 2005). A similar study by Pushalkar et al. in 2005 also discovered bacteria from these same phyla. However, the more specific genera of bacteria found in their subsequent studies include *Streptococcus, Gemella, Rothia, Peptostreptococcus, Lactobacillus, and Porphyromonas (*Pushalkar et al. 2011). There are several explanations for the lack of similarities in the genera of these bacteria samples between the experiments of Mager et al. and Pushalkar et al. The first study sampled a much larger cohort of patients including forty-five patients with OSCC and 299 OSCC-free all matched by age, gender, and smoking history (Mager et al. 2005). The second study represents a smaller portion of the first study. It only sampled oral saliva from five patients in total: three OSCC and two OSCC-free, all male and over the age of fifty with histories of both smoking and drinking (Pushalkar et al. 2011). These methodical differences in sampling may contribute to the lack of genera similarity between their findings. An additional case study found a specific pathogen responsible for oral carcinoma using similar non-invasive saliva methods as the two previous experiments (Henrich et al. 2014). In this study, the subjects were much different than previous saliva sample studies; they included a control group of five healthy patients along with two patients with Faconi anemia (FA) and two with oral tumors. The patients with FA also had oral leukoplakia—a precursor to oral cancer. The saliva samples from these groups of patients help to represent the stages of oral cancer progression: healthy to precancerous to cancerous. This study was able to specifically isolate the presence of *Mycoplasma salivarium* as a major colonizer in those with oral carcinoma. Bacterial loads of *M. salivarium* were detected in all three-subject groups, but the bacterial loads increased with the group's likelihood of having oral cancer. The oral tumor group had the highest quantity of this bacterium, while those with FA also had slightly higher counts than the control group. This study suggests that *M. salivaria* could potentially be used as a bioindicator for oral cancer. Another study by Mizuki and colleagues also found this bacterium to be prevalent in those with oral leukoplakia (Mizuki et al. 2014). Like mentioned earlier, oral leukoplakia is often characterized as a premalignant lesion and presents as a type of white plaque on the tongue and gingiva that cannot be rubbed off. Mizuki and colleagues were able to specifically isolate the presence of this bacterium on the epithelial cells of those with oral leukoplakia (Mizuki et al. 2014). Overall, research from these two groups suggests that *M. salivarium* and its relation to oral cancer must be further studied. Additionally, testing for bacterial loads of *M. salivarium* could help dentists non-invasively diagnosis and assess the severity of precancerous conditions like oral leukoplakia in addition to early stages of oral cancer.

Using a similar concept of comparing cancerous with non-cancerous microbial environments in the mouth, several other studies utilized tissue samples to isolate bacteria instead of saliva samples. In these studies, cancerous versus non-cancerous tissue was sampled via invasive or non-invasive techniques in the same subject, and bacterial DNA was extracted and then sequenced to detect the variety of taxa and species (Hooper et al. 2007; Pushalkar et al. 2012; Schmidt et al. 2014). In 2007 Hooper and colleagues found that the major phyla in the tumor sites were mostly Actinobacteria, Fusobacterium, and Proteobacteria. More specifically the species *Clavibacter michiganensis* , *Fusobacterium naviforme,* and *Ralstonia insidiosa* were all detected in more than 30% of the tumorous samples (Hooper et al. 2007). *Streptococcus anginosus* from the Firmicutes phylum was also present in six patients from this study in both cancerous and non-cancerous sites, suggesting it may play a role in oral carcinogenesis (Hooper et al. 2007). Surprisingly, *Porphyromonas gingivalis,* and *Streptococcus mitis/oralis* were more prominent in control samples, which is contrary to what the saliva sample from Mager et al. found in 2005 (Hooper et al. 2007; Mager et al. 2005), see Table 1. However, a later tumor sample study done by Pushalkar et al. in 2012 found the most prevalent phyla at tumor sites to be Firmicutes, which agrees with all of the studies thus far discussed. Specifically they isolated *Streptococcus sp.*, *Peptostreptococcus stomatis,* *Streptococcus salivarius*, *Streptococcus gordonii*, *Gemella haemolysans*, *Gemella morbillorum*, *Johnsonella ignava*, and *Streptococcus parasanguinis* in their samples of OSCC (Pushalkar et al. 2012). The most recent of these tumor sample studies done by Schmidt et al. in 2014 found extreme deviations from the rest of the research articles discussed in this review (Table 1). They found that the Firmicutes phylum, especially Streptococcus and Actinobacteria, were decreased in the oral cancer regions when compared to the control regions (Schmidt et al. 2014). The finding that the Firmicutes phylum was decreased in oral cancer patients is the opposite of what all the previous studies evaluated. However, Schmidt and colleagues did find that the phylum Fusobacteria was elevated in the cancerous regions, which agrees with the tumor study from Hooper in 2007 (Schmidt et al. 2014; Hooper 2007). Though Schmidt and colleagues did not mirror similar findings to previous studies, they did suggest that changes in the abundance of the Firmicutes might help identify oral pre-cancers (Schmidt et al. 2014). Whether or not the Firmicutes phylum is more or less prevalent in oral cancers, all studies have highlighted this phylum, and its role in oral carcinomas or pre-carcinomas should be further evaluated. Amongst the tumor studies, the sampling methods differed slightly in terms of distance from the cancerous oral site in each of these tissue studies. While the studies from Hooper et al. in 2007 and Pushalkar et al. in 2012 sampled at least 5cm away from the tumor area, the more recent study by Schmidt et al. noted that it was important to sample as far as 7cm from the tumor site to find clinically normal tissue. To ensure that healthy tissue samples were far enough away from cancerous tissue samples, Schmidt and colleagues also sampled matching regions on the other side of the mouth (Schmidt et al. 2014). Their study also differs from the two earlier studies in 2007 and 2012 because it sampled the tissue non-invasively, while the other studies made invasive oral incisions. These three tumor studies were all done with about ten patients, which could be too small of a sample size to accurately study the cancerous oral microbiota. Some of these methodical differences are depicted in Table 2.

Overall, both salvia and tissue detection methods have successfully found a difference between parts of the mouth with oral cancer and those without. However all of these experiments have divergent species level findings (Table 1). The main differences in their findings include those from the Firmicutes phylum. Likewise, only one main difference was found between saliva and tissue studies. Hooper et al. found *Streptococcus mitis/oralis* were more often found in non-cancerous tissue, while the main saliva sample from Mager et al. determined that *Streptococcus mitis* was more prominent in patients with oral cancer. Overall, there are many phylum level correlations between all of these recent oral microbiome studies, but there do not seem to be any consistent genera or species level observations. All of the differences seen in Table 1 are likely due to the methodical differences shown in Table 2.

Table 1. The most prevalent bacterial phylum found in OSCC patients. * The last study identifies the most prevalent bacteria found in the control groups.

Studies	Phyla	Genera or Species
Saliva: Mager et al. 2005	Bacteroidetes/ Firmicutes	*Capnocytophaga gingivalis*, *Prevotella melaninogenica*, *Streptococcus mitis*
Saliva: Pushalkar et al. 2011	Bacteroidetes/ Firmicutes	*Johnsonella ignava, Peptostreptococcus stomatis, Streptococcus salivarius, Streptococcus gordonii, Streptococcus parasanguinis, Gemella haemolysans, Gemella morbillorum*
Saliva: Henrich et al. 2005	Bacteroidetes/ Firmicutes	*Mycoplasma salivarium*
Tissue: Hooper et al. 2007	Actinobacteria/ Firmicutes/ Fusobacteria/ Proteobacteria	*Streptococcus anginosus, Clavibacter michiganensis, Fusobacterium naviforme, Ralstonia insidiosa*
Tissue: Pushalkar et al. 2012	Bacteroidetes/ Firmicutes	*Streptococcus sp.*, *Peptostreptococcus stomatis*, *Streptococcus salivarius*, *Streptococcus gordonii*, *Gemella haemolysans*, *Gemella morbillorum*, and *Streptococcus parasanguinis, Johnsonella ignava*
*Tissue: Schmidt et al. 2014	Actinobacteria/ Firmicutes	*Streptococcus*, *Rothia*

Table 2. The consolidated methods used throughout both saliva and tissue sampling studies. Samples were either conducted invasively or non-invasively and compared to samples from the same individual or compared to others in the study.

Studies	Invasive/non-invasive	Same/different individual	Total number of subjects
Saliva: Mager et al. 2005	non-invasive	different	344
Saliva: Pushalkar et al. 2011	non-invasive	different	5
Saliva: Henrich et al. 2005	non-invasive	different	9
Tissue: Hooper et al. 2007	invasive	same	10
Tissue: Pushalkar et al. 2012	invasive	same	10
Tissue: Schmidt et al. 2014	invasive	same	11

The methodical differences include divergent bacterial sampling methods and extreme variations between the type and number of patients studied. Looking at Table 2, it is evident that one main difference between these two types of studies was that the salvia samples all used different individuals whereas the tissue sample studies all directly sampled from the same patients. This could be a problem if the entire microbiota of the mouth is different in a person with cancer and in one without oral cancer. The salvia detection studies are more feasible in terms of using these practices in a clinical setting than the tissue detection methods because they are less invasive and can sample the microbiota without having to anesthetize the patient. Due to these largely inconsistent findings overall, more specific studies or studies done with similar techniques must be conducted.

Specific Bacteria Associated with Oral Cancer

Despite the inconsistent observations on the species level findings of potentially carcinogenic oral bacteria, some studies take a more specific approach and look at known oral pathogens to determine their link to oral cancer. This method may be a more conclusive way to move forward with the idea that there are different colonizers on the surfaces of oral carcinomas and healthy mouth tissues. *Streptococcus anginosus* and *Porphyromonas gingivalis*, both known pathogens in the oral cavity, are two of the specific organisms that have been isolated and studied most often in relation to oral cancer. *S. anginosus* is both found in a healthy mouth and in areas of decay with dental plaque (Morital et al. 2004). A study from Morita and colleagues found that the amounts of *S. anginosus* increase in individuals as they age (Morita et al. 2004). This study suggests that because *S. anginosus* and oral cancer risk both increase with age, this bacteria and its possible associations with oral cancer should be monitored (Morital et al. 2004). Moving forward with the idea that *S. anginosus* could be linked to oral cancer, Sasaki et al. conducted a specific tissue study in 2005 looking for the frequency of this bacteria's DNA in OSCC saliva and dental plaque. They found *S. anginosus* DNA fragments in almost half of the OSCC samples. This suggests a positive correlation between *S. anginosus* and oral carcinoma. It was also found exclusively in dental plaque, which suggests that plaque is the main habitat for *S. anginosus* (Sasaki et al. 2005).

Porphyromonas gingivalis is one of the bacterial species isolated by Mager et al. in 2005 (see Table 1). A recent study done in 2014 by Inaba et al. specifically studied how this species causes oral cancer. While previous studies were unclear of the exact role that *P. gingivalis* can play in oral cancer, this study found a new molecular mechanism in which this pathogen promotes the invasion of cancer cells (Inaba et al. 2014). Another study of this pathogen by Ahn and colleagues looked at the *P. gingivalis* IgG levels in the blood of those with orodigestive cancer (Ahn et al. 2012). The term orodigestive cancer includes oral cancer and cancers of the digestive system like pancreatic and colorectal cancer (Ansai et al. 2012). Their findings suggest that greater serum levels of IgG correspond to patients with orodigestive cancer (Ahn et al. 2012). Overall these two studies suggest that *P. gingivalis* could be used as a potential biomarker for the risk of death from orodigestive cancer (Inaba et al. 2014; Ahn et al. 2012).

Both of these pathogen-specific studies yielded positive correlations with oral cancer. The blood tests sampling IgG levels may be an easier, more clinically relevant, and quantitative approach towards testing patients for various types of oral cancer and precancerous legions. While salvia and tissue samples allow for testing a broader range of pathogens, perhaps more specific studies may be the next step in trying to more explicitly define the link between oral cancer and the oral microflora.

Non-oral Cancers and Associated Oral Bacteria

While oral pathogens are closely associated and important to study in relation to oral carcinomas, they are also relevant in non-oral diseases (Scannapieco 2013). For instance *Porphyromonas gingivalis* and *Fuscobacterium nucleatum*, both known oral pathogens, can be linked to numerous health problems including atherosclerotic disease, adverse pregnancy outcomes, rheumatoid arthritis, inflammatory bowel disease, colorectal cancer, respiratory tract infections, and other organ inflammation and abscesses (Sessa et al. 2014; Han et al. 2013). More specifically, these two bacteria have been directly related to orodigestive cancers. The orodigestive cancers included in this review are pancreatic and colorectal cancers.

Many studies have looked at the potential correlation between oral pathogens and pancreatic cancer. A general study conducted in 2012 by Farrell et al. found there to be a distinct difference between the oral microbiota in healthy patients and those with pancreatic cancer. Six different strains of oral bacteria were isolated in the salivary microbiota. However, *Neisseria elongate* and *Streptococcus mitis* showed the most significant differences between the subjects and controls. Logistic regression was used to investigate the use of these two bacterial biomarkers to distinguish pancreatic cancer versus healthy controls, pancreatic cancer versus chronic pancreatitis, and pancreatic cancer versus cancer-free controls. The levels of both of these bacterial markers were decreased in patients with pancreatic cancer. They found that in distinguishing pancreatic cancer patients from healthy controls, these tests yielded 96.4% sensitivity and 82.1% specificity and similarly high levels for the rest of the groups as shown in Table 3. This report provides proof that the salivary microbiota can be used as a non-invasive biomarker for disease detection, and the researchers of this study state that the role the oral microbiota plays in the progression and detection of a disease is very important in terms of diagnostic tests (Farrell et al. 2012). Further supporting the correlation of oral bacteria to pancreatic cancer, a large study done in 2013 revealed that individuals with antibodies against *P. gingivalis* had a two-fold higher likelihood of developing pancreatic cancer than those with lower levels of these antibodies (Michaud et al. 2013). The IgG study revealed the need for potential *P. gingivalis* antibody testing in patients who may be in higher risk groups for developing these types of cancers.

In addition to pancreatic cancer, a few other types of cancer like colorectal cancer may also have links with oral bacteria. *Fuscobacterium nucleatum* is an opportunistic commensal bacterium in the oral microbiome that is associated with periodontal disease (Castellarin et al. 2012). This oral bacterium is often found causing extra-oral infections (Rubinstein et al. 2013). A study in 2013 by Rubinstein and colleagues showed that *F. nucleatum* was also found to promote the carcinogenesis in colorectal cancer (Rubinstein et al. 2013).

Table 3. Consolidated information from interactive dot diagram analysis and receiver operating characteristic (ROC) curve analysis for the predictive power of combined salivary bacterial biomarkers in terms of sensitivity and specificity for the three groups (data originally published by Farrell et al. 2012).

Groups	Sensitivity (%)	Specificity (%)
Pancreatic vs. healthy control	96.4	82.1
Pancreatic cancer vs. chronic pancreatitis	85.7	55.6
Pancreatic cancer vs. non-cancer	85.7	52.7

Conclusions

Oral bacterial pathogens have not only been associated with oral carcinoma, but also with non-oral cancers. All of these current research articles help to demonstrate the importance of identifying and detecting disease causing oral bacteria for future studies. Recent studies about oral cancer detection have shown that overall survival and disease-free survival rates are significantly higher in patients with early stage cancers than advanced cancers (Fan et al. 2014). Therefore, the need for early prediction and treatment of oral malignancies is necessary to decrease mortality and morbidity rates. As examined in this review, there is a detectable difference between the microflora of cancerous oral surfaces both in the same and in different individuals. The studies examined often use divergent sampling techniques and look at elderly population groups with relatively few individuals. Sequencing large populations with consistent sampling methods may yield better and more consistent genera level results. The species-specific studies yielded more applicable findings to support the idea of early detection mechanisms, but much still remains to be done in terms of identifying the roles that bacteria play in the development of oral carcinoma. A good direction for future research might be to conduct more antibody studies that looking for responses to known periodontal pathogens. Detection through blood samples also leaves less room for bacterial contamination and can serve as an accurate and easily replicable mechanism to test for oral cancers in any clinical setting. While broader studies have shown that there is a difference between the microbial composition in healthy and diseased individuals, additional targeted research will likely identify more applicable results to disease comprehension and prevention.

References

Ahn J, Segers S, Hayes RB. 2012. Periodontal disease, Porphyromonas gingivalis serum antibody levels and orodigestive cancer mortality. Carcinogenesis. 33:1055–1058.

Ansai T, Takata Y, Yoshida A, Soh I, Awano S, Hamasaki T, Sogame A, Shimada N. 2013. Association between tooth loss and orodigestive cancer mortality in an 80-year-old community-dwelling Japanese population: a 12-year prospective study. BMC Public Health. 13:814.

Castellarin M, Warren RL, Freeman JD, Dreolini L, Krzywinski M, Strauss J, Barnes R, Watson P, Allen-Vercoe E, Moore RA, Holt RA. 2012. Fusobacterium nucleatum infection is prevalent in human colorectal carcinoma. Genome Res. 22:299–306.

CDC - Oral Cancer - Division of Oral Health. [cited 2014 Nov 22]. Available from: http://www.cdc.gov/oralhealth/oral_cancer/index.htm

Dewhirst FE, Chen T, Izard J, Paster BJ, Tanner ACR, Yu W-H, Lakshmanan A, Wade WG. 2010. The human oral microbiome. J Bacteriol. 192:5002–5017.

Elashoff D, Zhou H, Reiss J, Wang J, Xiao H, Henson B, Hu S, Arellano M, Sinha U, Le A, et al. 2012. Prevalidation of salivary biomarkers for oral cancer detection. Cancer Epidemiol Biomarkers Prev. 21:664–672.

Fan Y, Zheng L, Mao M-H, Huang M-W, Liu S-M, Zhang J, Li S-L, Zheng L, Zhang J-G. 2014. Survival analysis of oral squamous cell carcinoma in a subgroup of young patients. Asian Pac J Cancer Prev. 15:8887–8891.

Farrell JJ, Zhang L, Zhou H, Chia D, Elashoff D, Akin D, Paster BJ, Joshipura K, Wong DTW. 2012. Variations of oral microbiota are associated with pancreatic diseases including pancreatic cancer. Gut. 61:582–588.

Han YW, Wang X. 2013. Mobile Microbiome. J Dent Res. 92:485–491.

Henrich B, Rumming M, Sczyrba A, Velleuer E, Dietrich R, Gerlach W, Gombert M, Rahn S, Stoye J, Borkhardt A, Fischer U. 2014. Mycoplasma salivarium as a Dominant Coloniser of Fanconi Anaemia Associated Oral Carcinoma. PLoS One [Internet]. [cited 2014 Nov 22]; 9. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3958540/

Hooper SJ, Crean S-J, Fardy MJ, Lewis MAO, Spratt DA, Wade WG, Wilson MJ. 2007. A molecular analysis of the bacteria present within oral squamous cell carcinoma. J Med Microbiol. 56:1651–1659.

Huse SM, Ye Y, Zhou Y, Fodor AA. 2012. A Core Human Microbiome as Viewed through 16S rRNA Sequence Clusters. PLoS ONE. 7:e34242.

Inaba H, Sugita H, Kuboniwa M, Iwai S, Hamada M, Noda T, Morisaki I, Lamont RJ, Amano A. 2014. Porphyromonas gingivalis promotes invasion of oral squamous cell carcinoma through induction of proMMP9 and its activation. Cell Microbiol. 16:131–145.

Lazarevic V, Whiteson K, Hernandez D, François P, Schrenzel J. 2010. Study of inter- and intra-individual variations in the salivary microbiota. BMC Genomics. 11:523.

Mager D, Haffajee A, Devlin P, Norris C, Posner M, Goodson J. 2005. The salivary microbiota as a diagnostic indicator of oral cancer: A descriptive, non-randomized study of cancer-free and oral squamous cell carcinoma subjects. J Transl Med. 3:27.

Michaud DS, Izard J, Wilhelm-Benartzi CS, You D-H, Grote VA, Tjønneland A, Dahm CC, Overvad K, Jenab M, Fedirko V, et al. 2013. Plasma antibodies to oral bacteria and risk of pancreatic cancer in a large European prospective cohort study. Gut. 62:1764–1770.

Mizuki H, Kawamura T, Nagasawa D. 2014. In situ immunohistochemical detection of intracellular Mycoplasma salivarium in the epithelial cells of oral leukoplakia. J Oral Pathol Med.

Morita E, Narikiyo M, Nishimura E, Yano A, Tanabe C, Sasaki H, Hanada N. 2004. Molecular analysis of age-related changes of Streptococcus anginosus group and Streptococcus mitis in saliva. Oral Microbiol Immunol. 19:386–389.

Patton LL. 2003. The effectiveness of community-based visual screening and utility of adjunctive diagnostic aids in the early detection of oral cancer. Oral Oncol. 39:708–723.

Pushalkar S, Ji X, Li Y, Estilo C, Yegnanarayana R, Singh B, Li X, Saxena D. 2012. Comparison of oral microbiota in tumor and non-tumor tissues of patients with oral squamous cell carcinoma. BMC Microbiol. 12:144.

Pushalkar S, Mane SP, Ji X, Li Y, Evans C, Crasta OR, Morse D, Meagher R, Singh A, Saxena D. 2011. Microbial Diversity in Saliva of Oral Squamous Cell Carcinoma. FEMS Immunol Med Microbiol. 61:269–277.

Rubinstein MR, Wang X, Liu W, Hao Y, Cai G, Han YW. 2013. Fusobacterium nucleatum promotes colorectal carcinogenesis by modulating E-cadherin/β-catenin signaling via its FadA adhesin. Cell Host Microbe. 14:195–206.

Sasaki M, Yamaura C, Ohara-Nemoto Y, Tajika S, Kodama Y, Ohya T, Harada R, Kimura S. 2005. Streptococcus anginosus infection in oral cancer and its infection route. Oral Dis. 11:151–156.

Scannapieco FA. 2013. The oral microbiome: Its role in health and in oral and systemic infections. Clinical Microbiology Newsletter. 35:163–169.

Schmidt BL, Kuczynski J, Bhattacharya A, Huey B, Corby PM, Queiroz ELS, Nightingale K, Kerr AR, DeLacure MD, Veeramachaneni R, et al. 2014. Changes in Abundance of Oral Microbiota Associated with Oral Cancer. PLoS One [Internet]. [cited 2014 Nov 22]; 9. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4041887/

Sessa R, Pietro MD, Filardo S, Turriziani O. 2014. Infectious burden and atherosclerosis: A clinical issue. World J Clin Cases. 2:240–249.

Socransky SS, Haffajee AD. 2005. Periodontal microbial ecology. Periodontology 2000. 38:135–187.

Zaura E, Keijser BJ, Huse SM, Crielaard W. 2009. Defining the healthy. BMC Microbiology. 9:259.

Cownose Stingrays (*Rhinoptera bonasus*) Enrichment Preference and Spatial Distribution Patterns Before, During, and After Enrichment

Morgan Fuller and Mitchel Trychta

*Understanding the spatial distribution and enrichment preference of captive populations of cownose stingrays (*Rhioptera bonasus*) informs future fish husbandry practices. We investigated the preference that cownose stingrays had for tactile and food enrichment and the location of the rays before, during, and after enrichment was in the pool. We quantified the proportion of interactions that cownose stingrays had with the food and tactile enrichment and found that the stingrays exhibited a preference for food enrichment over tactile enrichment during the first eight minutes of the observational period. Neither, however, was significantly preferred afterwards. When enrichment was placed in the pool, the cownose stingrays shifted their spatial distribution towards the enrichment objects located in the inside area of the tank. Upon removal of the enrichment objects the cownose stingrays shifted their spatial distribution to primarily occupy the outside area of the tank. The purpose of this study was to ascertain the effectiveness of the current enrichment programs for the captive population of cownose stingrays. Since enrichment aims to improve the welfare of the captive individuals, utilizing enrichment that the captive species does not interact with or that does not offer a high difficulty level can result in the presentation of stereotypic behaviors and decreased health of the animal.*

Introduction

Known for their ability to leap from the water when disturbed, cownose stingrays (*Rhinoptera bonasus*) are members of the eagle ray family. The cownose stingray has an expansive range including the Chesapeake Bay, the Western Atlantic, and throughout the Gulf of Mexico (Bedore et al. 2013). As large active swimmers with laterally positioned eyes, the cownose stingray has an extensive field of vision used to survey broad foraging areas (Bedore et al. 2013). Cownose stingrays capture slow moving mollusks and crustaceans by rapidly opening and closing their mouth to create a suction which pulls prey and sand into the oral cavity; the prey is then crushed between fortified interlocking dental plates, while the sand is filtered out using the adapted gills (Sasko et al. 2005). Cownose stingrays are group foragers, coordinating the use of their powerful wings to stir up substrate and disturb prey (Ajemian and Powers, 2011). Cownose stingrays frequently forage in brackish eelgrass habitats, where the extensive plant growth limits prey visibility (Ajemian and Powers, 2012). Electro-sensitive pores clustered along the ventral surface of the stingray are used to locate prey by detecting cryptic bioelectric signals emitted by prey hidden in the sand (Bedore et al. 2013). The cownose stingray is capable of decimating commercial populations of mollusks and crustaceans, which poses economic concerns and a popular tendency to view these stingrays as a pest species; however, they preferentially feed on noncommercial bivalves (Ajemian and Powers, 2011). The cownose stingray is a social stingray, aggregating into massive schools composed of thousands in the Mid-Atlantic during the migration northward in the spring and southbound in the fall towards the Gulf of Mexico (Neer and Thompson, 2005).

Cownose stingrays are often misconceived as being limited to instinctual responses and simplistic behavior, however it becomes increasingly evident that cownose stingrays are capable of learning and performing complex behavioral repertoires (Corwin, 2012). Stingrays can even learn from each other in order to solve problems (Kuba et al., 2010). For instance, when presented with a simple puzzle in order to obtain food stingrays will use a combination of trial-and-error and observational learning to receive the reward (Kuba et al., 2010). Thus progress in fish husbandry practices such as enrichment should continue to be developed as a means to further improve the overall welfare of captive animals (Brydges and Braithwaite, 2009). Enrichment practices aim to promote natural behaviors and improve captive animal welfare (Newberry, 1995); consequently captive cownose stingrays are capable of benefiting from environmental enrichment which fulfills complex psychological needs by providing autonomy of action (Ben-Ari, 2001).

Effective enrichment programs strive to assess the many components that may influence the wellbeing of the captive animals (Mellen and MacPhee, 2001).The science of environmental enrichment assists in determining what enrichment practices promote favorable behavior and activity patterns, what behaviors validate a cumulative assessment of health, and how to effectively plan, implement, execute, and document attempted enrichment programs (Mellen and MacPhee, 2001).

Our study contains two primary research questions. The first examines cownose stingray behavioral data to determine what type of enrichment cownose stingrays prefer, while the second primary research question assesses how cownose stingray distribution within the pool changes during and after enrichment. The following hypotheses were tested. (1) There is no preference in enrichment type among cownose stingrays. Therefore, we expected that there would be no significant difference between the number of interactions between food and tactile enrichment. (2) Cownose stingrays prefer tactile enrichment. Thus, we predicted that there would be a significantly higher number of interactions with the tactile enrichment object than with the food enrichment object. (3) Cownose stingrays prefer food enrichment; hence, we anticipated that cownose stingrays would demonstrate a significantly higher number of interactions with food enrichment objects than with tactile enrichment objects. (4) Cownose stingrays do not change their spatial distribution during enrichment activities. We predicted that there would be no significant difference between stingray spatial distribution when comparing before and during enrichment. Likewise we expected no significant difference between spatial distribution during and after enrichment. Alternatively, we hypothesized (5) cownose stingrays do alter their spatial location during the presence of an enrichment object. Therefore, we expected a significant difference between stingray spatial distribution before and during enrichment, and/or a significant difference between spatial distribution during and after enrichment.

The factors of enrichment that merit the greatest derived benefit are unknown (Brydges and Braithwaite, 2009). Commercial fishing practices have illustrated that captive behavior can be positively modified by incorporating aspects of environmental enrichment, resulting in lower stress and higher behavioral flexibility of exposed specimens (Brydges and Braithwaite, 2009). Fortunately, the accessibility granted to researchers in this study permits the coordinated scientific behavioral observation of cownose stingray enrichment object preference. This accessibility allowed researchers to determine if specific types of enrichment ellicit a higher proportion of interactions. Developing an understanding of preferred enrichment objects allows zoos and aquariums to confidently allocate resources towards current and future enrichment programs, while also increasing opportunities for natural behavior in their captive stingrays by providing a proper rotating array of stimuli. Monitoring of pool location preference of cownose stingrays in response to enrichment placement broadly informs fish husbandry practices used to care for captive fish populations within zoos, aquariums, and the commercial fishing industry. Furthermore, the overall impact of imploring the systematic observation of captive cownose stingrays enlightens caretaking programs for captive fish within zoos and laboratory settings across the scientific community.

Methods

Study subjects and location

The cownose stingrays are held in a pool approximately three-feet deep with a waterfall feature at the south end (Figure 1). The waterfall is part of the filtration and circulation system that helps maintain a balanced environment. There is a small nursery pool constructed from PVC pipe lined with wire fencing that has a radius of approximately four feet that keeps the cownose pups enclosed. The nursery pool is also kept in the pool. There are between 20-50 cownose stingrays held in the pool at one time. They are allowed to swim freely and are able to interact with the public. Visitors to the exhibit can touch and feed the stingrays. Along with the cownose stingrays, southern stingrays (*Dasyatis americana*) and spotted-bamboo sharks (*Chiloscyllium plagiosum)* are held in the exhibit.

We controlled for confounding variables. Weather at the stingray exhibit was not a concern as the exhibit is covered with a tent. The water is kept at a constant temperature and the lighting level is always constant. We attempted to have the same individuals in the pool during each data collection period, but only two days had the same group of individuals.

Moreover, sample size was a concern as there were more than 20 cownose stingrays in the exhibit and individuals were not easily distinguished, consequently, we recorded group behavior instead of identifying individuals or sex. Individual identification would have reflected completely different results.

Data collection

Before beginning data collection, we waded around in the pool (Figure 1) for 20 minutes to allow the cownose stingrays to acclimate to our presence. We had to allow the rays time to recognize that we did not have food and to prevent them from treating us as an enrichment activity ourselves. This allowed minimal interference from the rays while we were observing them when enrichment was placed in the pool. We also moved the cownose stingray pup nursery to the edge of the pool such that it was directly opposite the waterfall and would not obstruct our view of the rays. After the allotted 20 minutes had passed, we stationed ourselves at the predetermined observations points (Figure1). These observation points were based on the ability to clearly see the cownose stingrays the glare across the water caused by the overhead lights. Observation points were moved directly outward to the side of the pool during one collection period when the zookeeper had to leave as we were not allowed in the pool while the keeper was not present. We also stood still in our positions without bending towards the water during the allotted data collection time to prevent attracting the stingrays and, falsely interpreting the stance as a food signal.

We began by observing the spatial distribution of the cownose stingrays in our respective areas for 30 minutes before we placed the enrichment in the pool. We recorded the locations (inside or outside area of the pool) of the cownose stingrays by counting the number of rays within the inside and outside regions of the pool (Figure 1). This measurement was taken in two minute intervals for a total of 15 sample data points, gathered over 30 minutes of observation. We also began each count on the left side of pool and then scanned to the right.

After the first 30 minute interval ended we immediately placed one food and one tactile enrichment activity in the pool. We placed the activities on opposite sides of the pool directly across from each other (Figure 1). One researcher faced the food-focused activity and the other watched the tactile activity. The groupings were as follow: food ball and small sea grass; chains and target training; and food ball and large sea grass (Table 1). For recording the frequency of interactions, we used the scan sampling technique to scan the entire population of cownose stingrays for interaction with the enrichment in addition to an adjusted method of focal continuous recording (Martin and Bateson, 2007). Specifically, focal continuous recording measured the total number of interactions occurring over a specified interval of two minutes; therefore, a total of 15 sample data points was gathered over 30-minutes of observation. We defined interactions as touching any part of the enrichment or following any part of the activity.

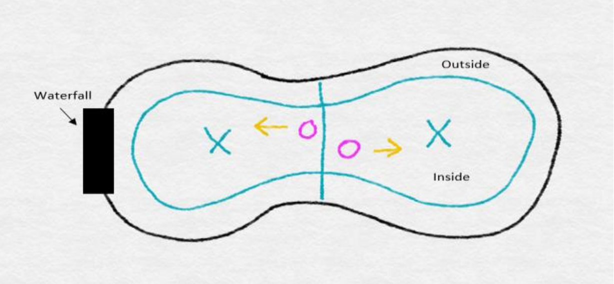
While we were recording interaction with enrichment, we were also recording the spatial distribution of the rays in the same manner as before enrichment was placed in the pool.

After the second 30 minute interval was over, we immediately removed the enrichment from the pool and began another 30 minute interval of spatial distribution tracking.

Table 1. Description of enrichment items used categorized as either food or tactile enrichment.

Enrichment Type	Specific Activity	Description
Food	Food Ball	Food is placed inside a ball with holes and attached to a tether, rays are able to suck out food
	Target Training	Rays touch a red target on the end of a rod, individuals that touch the target are given food
Tactile	Chains	A triangle structure made of pipe has yellow plastic chains attached, the chains float up when placed in water which rays can swim through
	Small sea grass	Wire grid (2”x2”) with green ribbon attached stimulate sea grass which rays can swim through
	Large sea grass	Wire grid (4”x2”) with green ribbon attached stimulate sea grass which rays can swim through

Figure 1. Stingray Bay Description of the pool. The pink circle represents the researchers. The arrows shows which direction the researchers look. The blue X’s signify enrichment placement. The inner blue circle separates the pool into two regions, inside and outside.



Analysis

After data was converted to depict a proportion, enrichment preference data across all three trials was examined by calculating the mean of the number of interactions per time point grouped by enrichment type. A figure was then created based on the mean number of proportional interactions for an enrichment object during the corresponding time period. We used a Mann-Whitney U test to find if a statistical difference existed between the mean proportion of interactions for the food and tactile enrichment activities.

We also calculated the mean proportion of individuals per time point in the outside and inside regions of the pool before, during and after enrichment based on the three collection days. We ran seven paired t-tests to find a statistical difference (Table 2).

Comparison Groupings
Outside Before vs Outside During
Outside During vs Outside After
Inside Before vs Inside During
Inside During vs Inside After
Outside Before vs Inside Before
Outside During vs Inside During
Outside After vs Inside After

Table 2. Outline of the comparison groupings that we used to run the various paired t-tests in order to establish if a statistical difference existed in our data of the spatial distribution of cownose stingrays before, during, and after enrichment.

Results

When there was no enrichment in the pool, the cownose stingrays were found more often in the outside region, but when enrichment was present, the rays were located in the inside region (i.e. the region with the enrichment activity) (Figure 2). Within the outside area, there were more rays before the enrichment (0.137 ± 0.507%) than during enrichment (0.108 ± 0.582%) ($t_{14} = 148.13$, $p < 0.001$). There were also more stingrays in the outside of the pool after (0.130 ± 0.342%) enrichment than during (0.108 ± 0.582%) ($t_{14} = 3.47$, $p = 0.004$). For the inside area, a difference was found before and during the activities ($t_{14} = 105.54$, $p < 0.001$), with more rays being on the inside of the pool during enrichment (0.139 ± 0.788%) than before (0.114 ± 0.475%). Another difference was found during and after the enrichment ($t_{14} = -2.83$, $p = 0.013$), as more cownose rays were in the inside of the pool during (0.139 ± 0.788%) the enrichment than after (0.111 ± 0.323%). Between the outside and inside region before enrichment, we found a difference ($t_{14} = 3.06$, $p = 0.008$) due to a higher proportion of rays present in the outside region (0.138 ± 0.507%) than the inside (0.114 ± 0.475%). There was, again, a statistical difference demonstrated between the two areas during enrichment ($t_{14} = -2.86$, $p = 0.012$). More stingrays were found in the inside (0.139 ± 0.788%) than in the outside area (0.107 ± 0.582%). The final difference was uncovered between both regions after enrichment ($t_{14} = 3.45$, $p = 0.004$) with a higher proportion of cownose stingrays swimming in the outside of the pool (0.130 ± 0.342%) than inside (0.111 ± 0.323%).

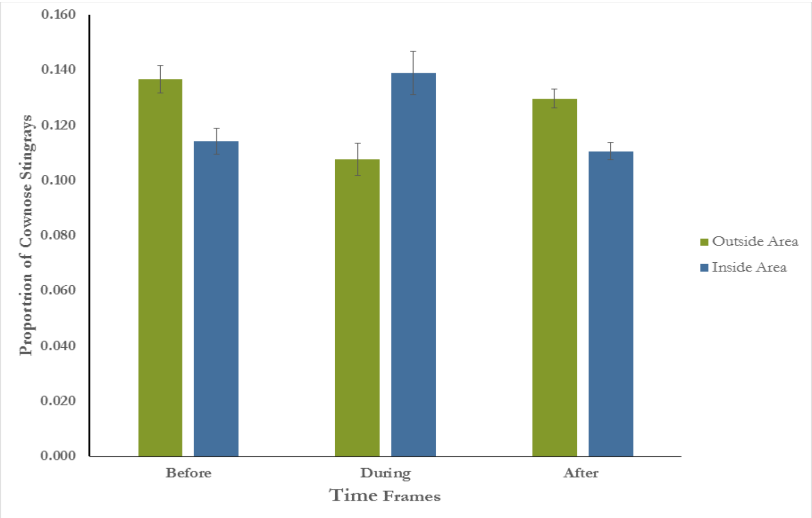


Figure 2. Location of Cownose Stingrays Before, After, and During Enrichment. Relationship of pool location to time period and proportion (mean ± standard error) of stingrays in a specific area before, during, and after enrichment.

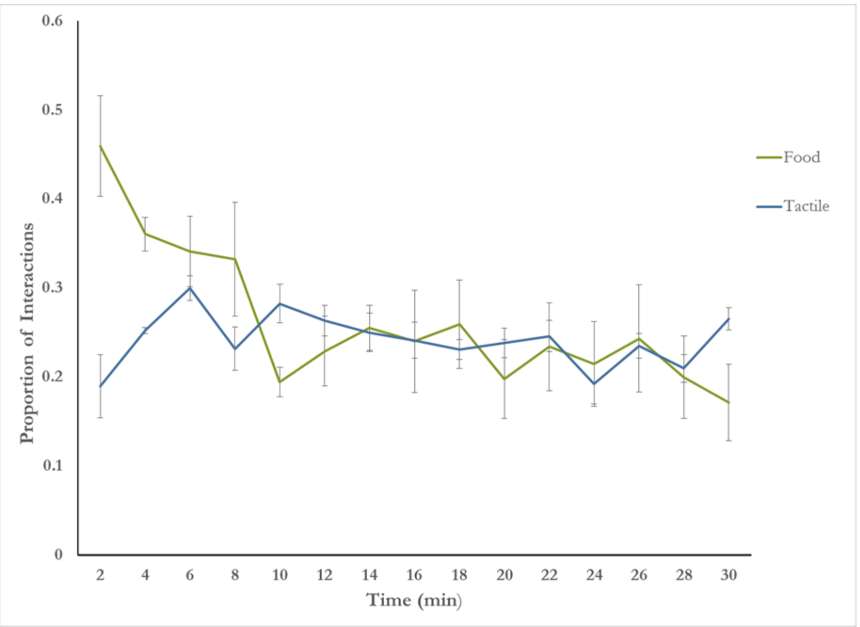


Figure 3. Interaction with Food and Tactile Enrichment over Time. Relationship between proportion (mean ± standard error) of interactions and time as measures of enrichment preference.

There was no difference in the proportion (mean ± standard error) of interaction the cownose stingrays had with the food (0.262 ± 0.0202) and tactile enrichment (0.242 ± 0.0078) (U = 111, $N_1 = N_2 = 15$, p = 0.967). There was, however, a difference in the proportion of interaction that the rays had with food and tactile enrichment during the first eight minutes (U = 0.0, $N_1 = N_2 = 15$, p = 0.029). The stingrays had a higher proportion of interaction with the food enrichment (0.051 ± 0.025) than the tactile enrichment (0.040 ± 0.020) in the first eight minutes (Figure 3).

Discussion

Our results showed that cownose stingrays changed their spatial distribution when presented with an enrichment activity, supporting hypothesis 5. As naturally schooling mesopredators, cownose stingrays often forage in a large group following the movement of the school at large (Ajemian and Powers, 2012). Therefore the presence of food enrichment may have stimulated communal foraging behavior causing the rays to collectively adjust their spatial distribution towards the epicenter of the 'prey' or food enrichment object. In the future, if the keepers need the cownose rays to use more of the exhibit or to be closer to the edge of the pool so that the public can interact with them, they should place the enrichment in the specific region that the keepers want the rays to frequent.

Overall the cownose stingrays did not display a preference for enrichment type which provides support for hypothesis 1. By looking at the first eight minutes of the enrichment being placed in the pool, we did, however, find that the stingrays had a preference for the food enrichment which technically does not support our other hypothesis because our methods established a 30 minute data collection period instead of an 8 minute period. This significant difference between enrichment types during the first 8 minutes is important to address as the food enrichment seemed to be treated as tactile enrichment after 8 minutes had passed. We noted that this was the approximate marker when the rays ate all of the food in the food ball. The comparable values for the food enrichment object, although devoid of food, and the tactile enrichment object indicates that the food enrichment object did not lose its entire value for behaviorally stimulating the stingrays. Instead, the food enrichment object may have taken on a new nature as a novel tactile enrichment object. This result also suggests that a more complex food enrichment activity needs to be developed. The difficulty level could be increased by creating an activity that would elicit natural foraging behaviors, such as those mentioned by Sasko et al. (2006).

While recording data, we also noticed what appeared to be stingrays learning by observing other individuals. Only a few stingrays showed any interest in the floating bucket of food at the beginning of the tests, but towards the end of the enrichment period, the bucket became the primary focus of the stingrays as evidenced by consistent bumping of the bucket instead of the red target ball and empty food ball. We perceived this to be a type of observational learning. In light of the work done by Thonhauser et al. (2012), inexperienced stingrays may have actually learned how to effectively manipulate the food enrichment from the experienced stingrays. Studies on fish, such as Thonhauser et al. (2012), have further demonstrated the capacity of higher fish to learn what to eat and what not to eat from their subordinates.

The cownose stingrays continuously interacted with all of the enrichment activities over the 30 minutes that the activities were in the pool, but we would be interested to investigate their level of cognitive functioning and extent to which the rays can solve problems. In another study, South American fresh water stingrays (*Potamotrygon castexi*), which are a relative of the cownose stingrays, showed they were capable of learning and using water as a tool (Kuba et al., 2010). The area of cognition in elasmobranches is waiting to be explored as Kuba et al. (2010) elucidates new questions, such as to what extent can stingrays learn from each other and what other problems can they solve?

We were limited in a few ways in our ability to control for all confounding variables. The exhibit, Stingray Bay, was scheduled for closure earlier than anticipated so we were forced to have two consecutive days of data collection. We were, as a result, unable to control the time of day that the enrichment was placed in the pool. Because a zoo event was taking place at the time of the last two data collections, crowd influx increased. We are curious to understand the effects of a large crowd on the cownose stingrays and their proportion of interaction with enrichment. In future studies of enrichment and cownose stingrays, it will be important to control for time of day and crowd flow. Also, we had predetermined observation points, but when the keeper left for a meeting during one data collection period we were no longer allowed to be in the pool. Determining unchanging observation points will be important to establish in future studies. One final area of improvement would be observing the same individuals during the entire study. We were only able to keep the same group of rays for two out of three data collection periods. Expanding the number of behavioral analysis trials to a more suitable number such as ten would assist in ascertaining more accurate baseline behaviors and mean values for interacting with enrichment objects. Additionally, trained researchers would allow for more specific spatial distribution analysis, and higher observer reliability. Finally, the ability to proactively acquire age and sex information would allow for behavioral comparisons between age and sex groups, assuming a method such a video recording is implored for accurately identify individuals during testing.

Despite the fact that the captive population at the Memphis Zoo will not be reintroduced into the wild (Emily Burford, pers. comm.), being able to sustain a captive population is necessary in order to understand behaviors and preferences of wild cownose stingrays (Baldassin et al., 2008). Predatory fishing and habitat destruction currently threaten wild cownose stingrays and by providing a suitable complex habitat for captive populations keepers can encourage high rates of reproduction which will allow researchers to repopulate the species if extinction becomes an immediate threat (Baldassin et al., 2008).

Acknowledgements

We would like to acknowledge the Memphis Zoo for allowing us access to Stingray Bay. Thank you to the zookeepers Emily, Kori, and Forrest for assisting us in our study and answering our numerous questions about cownose stingrays. We are grateful to the staff of Stingray Bay for showing us how to feed the rays and their helpful attitudes. Much appreciation to Professor Sarah Boyle of Rhodes College for her continual guidance and support in our endeavor.

References

Ajemian MJ, Powers SP. 2011. Foraging effects of cownose rays (*Rhinoptera bonasus*) along barrier islands of the northern Gulf of Mexico. Journal of Experimental Marine Biology and Ecology 439: 119-128.

Ajemian MJ, Powers SP. 2012. Habitat-specific feeding by cownose rays (*Rhinoptera bonasus*) of the northern Gulf of Mexico. Environ Biology of Fish 95: 79-97.

Baldassin P, Gallo H, Azevedo V. 2008. Reprodution of the Cownose ray, *Rhinoptera bonasus* Mitchell, 1815 (Elasmobranchii, Rhinopteridae), in captivity and newborn care. Brazilian Journal of Biology 68: 905-906.

Bedore CN, Harris LL, Kajiura SM. 2013. Behavioral response of batoid elasmobranchs to prey-simulating electric fields are correlated to peripheral sensory morphology and ecology. Zoology 117: 95-103.

Ben-Ari ET. 2001. What's new at the zoo? Zoo biologists are taking a scientific approach to improving the quality of life for captive animals. Bio Science 51: 172 -177.

Brydges NM, Braithwaite VA. 2009. Does environmental enrichment affect the behavior of fish commonly used in laboratory work. Applied Animal Behavior Science 118: 137-143.

Corwin AL. 2012. Training Fish and Aquatic Invertebrates for Husbandry and Medical behaviors. The Veterinary Clinics of North America, Exotic Animal Practice: 455-467.

Kuba M, Byrne R, Burghardt, G. 2010. A new method for studying problem solving and tool use in stingrays (*Potamotrgon castexi*). Animal Cognition 13: 507-513.

Martin F, Bateson P. 2007. Measuring behavior: An introductory guide. 3rd edition. Cambridge: Cambridge University Press. 176.

Mellen J, MacPhee MS. 2001. Philosophy of Environmental Enrichment: Past, Present, and Future. Zoo Biology 20: 211-226.

Neer JA, Thompson BA. 2005. Life history of the cownose ray, *Rhioptera bonasus*, in the northern Gulf of Mexico, with commonts on geographic variability in life history traits. Environmental Biology of Fishes 73: 321-331.

Newberry, R. 1995. Environmental enrichment: Increasing the biological relevance of captive environments. Applied Animal Beahviour Science 44: 299-243.

Sasko D, Dean M, Motta P, Hueter R. 2006. Prey capture behavior and kinematics of the Atlantic cownose ray *Rhinoptera bonasus*. Zoology 109: 171-181.

Thonhauser KE, Gutnick T, Byrne RA, Kral K, Burghardt GM, Kuba MJ. 2013. Social Learn Behavior in Cartilaginous fish (stingrays *Potamotrygon falkneri*). Animal Cognition 16: 927-932.

The Role of Solvation in the binding of Morphine, Met-Enkephalin, and Other Ligands in the µ-Opioid Receptor and Acetylcholine Binding Protein

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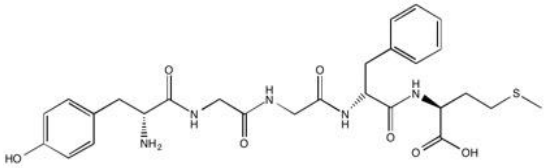
Neurochemical receptors are important targets for drug design. Two important aspects of ligand binding in these receptors include the attraction of the ligand to the active site and the effect of displacing the waters of solvation that surround the ligand and active site. In order to investigate the effect of solvation on hydrophobic active sites, we have studied ligand binding and water displacement in two hydrophobic active sites, those of the acetylcholine binding protein and the µ- opioid receptor. Active site models were obtained from crystal structures of the proteins, catalogued in the Protein Data Bank. Morphine, cocaine, and met-enkephalin were docked in the active-sites, and the ligand-protein complexes were then optimized using M062X/6-31g. Interaction energies between the ligands and the proteins were calculated using M062X and MP2 with the 6-311+G basis set. The role of water was explored by placing explicit waters of solvation both in the empty active-sites and around the ligands. The energy required to displace the waters upon ligand binding was calculated using the same methods mentioned above. Preliminary results suggest that solvation plays a larger role than ligand-active-site binding.*

Introduction

The chemical behavior of neurologically active molecules depends upon interactions with function-specific enzymes and proteins found in the central nervous system. On a biochemical level, protein-ligand interactions occur in the protein active site, a small pocket of amino acids located in an area of the protein that is accessible to potential ligands, which can be either exogenous xenobiotics or endogenous molecules. In biological systems, proteins and ligands interact not only with each other, but also with the water molecules present in the body's aqueous solutions of blood, cytosol, etc. Thus, aqueous solvation affects the behavior of protein active sites and ligands during association. In this investigation, we will look at two neurologically active proteins, the µ-opioid receptor and the acetylcholine binding protein, and their interactions with respective ligands. Specifically, we will analyze the role of solvation in protein-ligand interactions in order to determine its role in protein-ligand binding behaviors. The µ-opioid receptor (MOR), one of three pharmacologically active opioid receptors in the brain, is found throughout the brain and spinal cord. It is most heavily concentrated in the caudate putamen and thalamus but is also present in several other nervous system regions (Arvidsson et al. 1995). Binding of the µ-opioid receptor results in sensorimotor sensations such as pain modulation and analgesia (Manglik et al. 2012). Ligands responsible for eliciting analgesic effects when bound to the µ-opioid receptor include morphine and enkephalin, a pentapeptide, as exogenous and endogenous substrates, respectively (Figure 1). The affinity of each ligand can be determined computationally, providing a comparison of relative strength of binding of the ligand to the active site.

(a) Met-Enkephalin



(b) Morphine (c) Cocaine

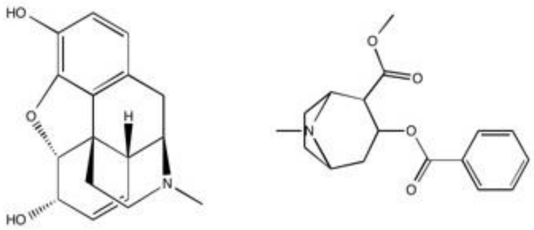


Figure 1. The chemical structures of ligands used in the analysis of interaction energies in the acetylcholine binding protein and the µ-opiod receptor. Ligands (a) and (c) were analyzed in complexed with MOR and ACHBP, respectively, while ligand (b) was investigated as a ligand of both active sites.

The acetylcholine binding protein (ACHBP) is a member of the family of nicotinic receptors, and it is involved in interneuronal synaptic transmission. It has been shown, specifically, to suppress cholinergic transmission through a positive feedback mechanism (Smit et al. 2001). When more acetylcholine is present, the release of acetylcholine binding protein is increased, thus decreasing the net effect of acetylcholine. It interacts with other nicotinic ligands as well. Its pharmacological activity occurs in an active site characterized by a number of aromatic amino acids, creating an aromatic box (Smit et al. 2001).

Like in the μ-opioid receptor, binding behavior of ligands in this aromatic active site can be determined via *in silico* models, providing computational representation of relative affinities. In this investigation, cocaine and morphine will be the ligands of interest when studying the ACHBP (figure 1).

Computational analyses of active site-ligand complexes with the MOR and ACHBP active sites and their respective ligands are performed in an assumed gas phase model. In order to more accurately model the biological behavior of enzymatic active site binding, however, the crystal structure of the protein-ligand complex must be optimized using a model of solvation. Solvation can be modeled implicitly through the Polarizable Continuum Model (PCM) designed by Tomasi et al (Tomasi et al. 2005). Optimization with the PCM yields more accurate charge distributions and dipole moments occurring on the ligand and in the amino acid residues of the active site. This stabilization of charge in the crystal structure model allows for a better determination of charge-dependent intermolecular forces occurring in ligand-active site association (Cramer and Trublar 1999). Though implicit solvation optimization has yielded consistent results between MP2 and M06L, a density functional theory method (Bigler et al. 2015), it does not account for the effects of desolvation.

Desolvation occurs during ligand binding when waters of solvation surrounding both the ligand and the active site residues are displaced. Desolvation, therefore, has contributions to the free energy of the system. This contribution is not accounted for, however, in the polarizable continuum model of solvation. In order to accurately determine the contribution of desolvation on ligand binding, the waters of solvation can be analyzed in various stages. During the desolvation process, both the ligand and active site start with explicit waters of solvation. These water molecules form hydrogen bonds on hydrogen bond receptor sites on both the ligand and the active site residues. When ligands are similar in size and chemical nature, they should be associated with a similar number of water molecules. Thus, the dissociation of water from the ligand during active site binding will result in relatively equal contribution of free waters to the bulk solution regardless of ligand. The desolvation of the ligand and active site is favorable when the molecule and residues have hydrophobic qualities, removing unfavorable hydration from non-polar, uncharged regions. The hydrophobic nature of desolvation can conversely be unfavorable because the removal of water, which was interacting with charged, polar groups, results in the destabilization of hydrophilic regions. This unfavorable aspect of desolvation is resolved by the resultant exposure of polar or charged areas on both the active site and the ligand, allowing for those areas to interact and stabilize the complex (Friesner et al 2006).

In order to account for the contribution of desolvation to free energy, which is not included in the implicit solvation model, this investigation will require a multi-step explicit solvation analysis. Using explicit solvation, we will determine: 1) the amount of energy released upon the dissociation of solvating waters and the ligand, 2) the energy released upon the desolvation of the active site, 3) the energy gained when newly released, free waters associate with each other in bulk, and 4) the energy of the desolvated, complexed active site and ligand. The energies of the different active site-ligand complex conditions are cyclically related, as seen in Figure 2. The cyclic nature of these conditions allows for the determination of desolvation's energetic contribution as a driving force in ligand binding in enzymatic active site, as described by the following equations:

$$\Delta E_1 + \Delta E_3 \leftrightarrow \Delta E_2 + \Delta E_4$$
$$\boldsymbol{\Delta E_4 = \Delta E_1 + \Delta E_3 - \Delta E_2}$$

By solving for ΔE_4, the energy resulting from displacement of waters during ligand binding can be determined. The determination of ligand behavior in the active site and of energy of solvent displacement will be the principle topics of this investigation.

Methods

Computational Details: Optimization and ligand Interaction energy

The enzymatic active sites were chosen because of their neurological properties and biological applicability in a solvation study. The ligands chosen compliment the active sites because they are common exogenous and endogenous substrates of the enzymes. In order to prepare for computational analysis, crystal structures of both the acetylcholine binding protein and μ-opiod receptor were retrieved form the protein data bank. The active sites were isolated from the greater protein crystal structures, containing eleven and thirteen amino acids for ACHBP and MOR, respectively. Models of the ligands were constructed using GaussView software.

Ligands were docked in the active site crystal structures using ArgusLab software and were further optimized with the density functional theory method M062X/6-31g using the Gaussian09 platform. Optimizations were performed with non-flexible active site residues and an assumed gas phase condition.

Interaction Energies were determined as a summation of individual interaction energies between each amino acid and ligand. Energies were calculated using both the M062X and MP2 methods, both with 6-311+g* basis set. Two methods were used to verify the accuracy of each method and to ensure consistent results when comparing energies of different ligand/active site complex.

Computational Details: Explicit Solvation

Explicit solvation structures, were optimized using M062X/6-31g. The crystal structure active site/ligand optimization for the explicit solvation calculations is the same optimization used to determine the interaction energy of morphine with acetylcholine binding protein. Total energies were determined from the optimization calculation. Five waters of solvation were strategically placed around both the ligand and the active site to ensure that the proportion of dissociation energy would be equal for both parts of the investigation. Five water molecules were designated as the number of solvating agents because there are 5 regions on morphine that are likely to hydrogen bond.

Results

Optimizations and Interaction Energies

Images of the optimized crystal structures for the acetylcholine binding protein and the µ-opioid receptor, with each of their respective ligands, are show in in Figure 3(a-b). The optimization of each ligand in its respective active site, which was performed using the M062X/6-31g method with assumed gas phase and non-flexible residues, computationally determined the most energetically stable conformation of the ligand in the active site.

By optimizing the ligand models in complex with the active site residues, the counterpoise corrected interaction energy of each amino acid are determined with both MP2 and M06L methods. The interaction energies between the ligands and each individual residue are listed in Table 1. In the acetylcholine binding protein, morphine yielded a total interaction energy of -17.1 kcal/mol in both methods, while cocaine had an interaction energy of -39.4 and -39.6 kcal/mol with M062X and MP2 respectively. Overall, the magnitude of cocaine's interaction energy is roughly twice that of morphine, showing that cocaine has roughly twice the affinity when acting as a substrate of the acetylcholine binding protein. In the µ-opioid receptor, the interaction energy of morphine was found to be -31.0 kcal/mol while met-enkephalin had a total interaction energy of -63.8 kcal/mol, both with M062X method. Therefore, with an interaction energy double that of morphine, met-enkephalin has twice the affinity as a ligand of the µ-opioid receptor. This result confirms the intuitive conclusion that met-enkephalin would be a more favorable ligand than morphine in the µ-opioid receptor because met-enkephalin is a naturally occurring, endogenous substrate whereas morphine is foreign to the body.

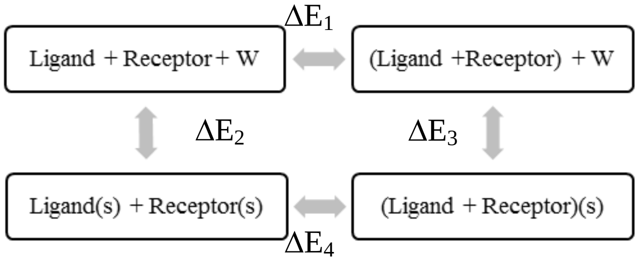


Figure 2. A cyclic depiction of the stages of relationship between ligand, receptor, and waters of solvation. This cycle will be used to determine the energy contribution of water displacement during binding.

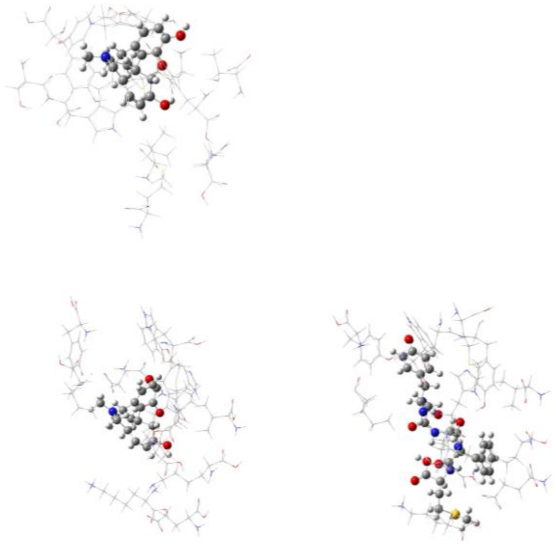


Figure 3. (a) Crystal Structure of the optimized ligands in the active site of the acetylcholine binding protein for morphine (left) and cocaine (right). (b) Crystal Structure of the optimized ligands in the active site of the µ-opioid receptor for morphine (left) and Met-Enkephalin (right).

Acetylcholine Binding Protein

		Trp	Tyr	Tyr	Tyr	Tyr	Cys	Met	Ile	Ser	Gln	Thr	TOTAL
Morphine	**M062X**	-4.71	-0.694	-0.754	-3.67	0.0830	-3.24	-0.00500	-1.41	-0.472	-0.866	-1.95	**-17.7**
	MP2	-6.20	0.0680	0.382	-4.67	0.255	-3.67	-0.0810	-0.0810	-0.543	-0.970	-2.21	**-17.7**
Cocaine	**M062X**	-5.78	-2.42	-9.43	-3.67	-3.53	-4.99	-1.03	-4.02	-0.713	-3.56	-0.581	**-39.4**
	MP2	-5.52	-2.31	-8.57	-4.01	-3.61	-5.24	-1.33	-4.25	-1.03	-2.79	-0.913	**-39.6**

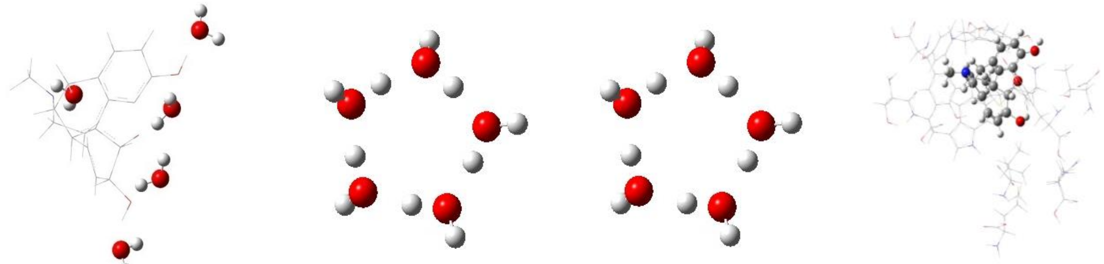
μ-Opioid Receptor

		Glu	Ile	Tyr	Lys	Lys	Met	Trp	Tyr/Asp	Val	Val	His/Ile	TOTAL
Morphine	**M062X**	-4.51	-0.475	-0.676	6.59	3.33	-11.5	0.472	-20.7	-1.21	-1.48	-0.694	**-31.0**
	MP2	-4.33	0.929	-0.971	7.45	2.67	-7.56	1.424	-18.604	-1.69	-1.38	--	--
Met-Enkephalin	**M062X**	-22.3	-1.99	3.25	6.48	-6.95	-1.51	-2.151	-31.0	-0.196	-2.822	-4.65	**-63.8**
	MP2	-21.1	-2.46	4.07	9.37	-6.76	-2.25	--	--	-0.421	0.080	--	--

Table 1. Counterpoise corrected interaction energies for morphine, cocaine and met-enkephalin in their respective ligands, using M062X Optimization. Both M062X and MP2 methods were used for the determination of interaction energies. *Values listed as "—"correspond to calculations that were unable to be completed due to convergence failure as a result of limited computational capacity.

Explicit Solvation

Explicit solvation calculations were completed using morphine docked in the acetylcholine binding protein to determine a model for the effect of desolvation on ligand binding. Explicit solvation analysis required energy determinations of optimized, solvated structures. The five sets of conditions are shown in Figure 4. The net energy of solvation was determined to be the difference between the magnitude of energy gained and of energy lost. Energy is gained from waters of solvation, from both morphine and the active site, associating in bulk and from morphine and binding site associating. Energy is lost from waters dissociating from morphine and from the active site. Thus, based on the values in Table 2, the net energy of solvation is determined to be 28.3 kcal/mol. Because the total energy gained was -123.1 kcal/mol and the total energy lost was -151.4 kcal/mol, the net energy of solvation has more contribution from energy of dissociation than from energy of association. Therefore, ligand binding in the active site is energetically driven more strongly by the displacement of waters of solvation than by the association of the ligand and active site or by the association of displaced waters with the bulk solution.

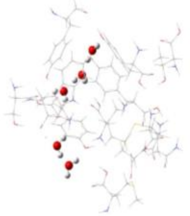


(a) Energy of waters and Morphine Dissociating

(a) Energy of waters from Morphine associating

(a) Energy of waters from active site associating

(a) Energy of morphine and active site associating



(a) Energy of waters from active site dissociating

Figure 4. *In silico* models used in the explicit solvation analysis of the effect of solvation on protein-ligand binding. The ligand in these models is morphine and the active site is taken from the crystal structure of the acetylcholine binding protein. All structures were optimized using M062X/6-31g.

Type of Energy Change	Molecules Involved	Calculated Value (using M062X, in kcal/mol)
Gained	Water (from active site) associating	-52.7
	Water (from morphine) associating	-52.7
	Morphine and active site associating	-17.7
	Total Energy Gained	**-123.1**
Lost	Active site and water dissociating	-81.6
	Morphine and water dissociating	-69.8
	Total Energy Lost	**-151.4**
	Net Energy of Solvation	**28.3**

Table 2. Energies lost and gained from the solvation process. Energy is lost when favorable interactions are dissociated and gained when new interactions are formed.

Conclusions

Through *in silico* modeling of the acetylcholine binding protein, it was determined that cocaine is a more viable ligand than morphine. It was also shown that met-enkephalin binds more favorably than morphine in the active site of the µ-opioid receptor. The higher binding affinity of met-enkephalin follows logical conclusions because met-enkephalin is an endogenous compound and thus should behave more favorably as compared to an exogenous substrate. The study of binding affinity in enzymatic active sites provided a confirmation of computational method consistencies, with little difference in the results between the calculations performed with M062X versus MP2. The similarity in results confirms that both methods provide the same accuracy when analyzing *in silico* models.

The investigation of explicit solvation analyzed the energetic contributions of water displacement and molecular interactions in ligand-active site binding. It was shown that the displacement of waters of solvation from both the ligand and the active site contributes more strongly to ligand binding than the actual energetic interaction of the ligand and the active site. Thus, the displacement of water, which contributes to the favorable entropy of the system, is the main driving force in ligand-active site binding.

References

Arviddson U, Riedl M, Chakrabarti S et al. 1995. Distrubution and targeting of µ-opioid receptor (MOR1) in brain and spinal cord. J Neurosci. 15(5):3328-3341.

Bigler D, Peterson L, Cafiero M. 2015. Effects of implicit solvent and relaxed amino acid side chains on the MP2 and DFT calculations of ligand-protein structure and electron interaction energies of dopaminergic ligands in the SULT1A3 enzyme active site. Comput Theoret Chem. 1051:79-92.

Cramer CJ, Truhlar DG. 1999. Implicit Solvation Models: equilibria, structure, spectra and dynamics. Chem Rev. 99:2161-2200.

Friesner R, Murphy R, Repasky M et al. 2006. Extra precision glide: docking and scoring incorporating a model of hydrophobic enclosure for protein-ligand complexes. J Med Chem. 49(21):6177-6619.

Manglik A, Kruse AC, Kobilka TS et al. 2012. Crystal structure of the µ-opioid receptor bound to a morphinian antagonist. Nature. 485(7398):321-326.

Smit AB, Syed NI, Schaap D et al. 2001. A glia-derived acetylcholine binding protein that modulates synaptic transmission. Nature. 411(6835):261-268.

Tomasi J, Mennucci B, Cammi R. 2005. Quantum mechanical continuum solvation models, Chem. Rev. 105:2999-3093.

Asthmatic Disease: A Complex Interaction of Environmental, Medical, and Age Factors in Affecting the Microbiome

Breanna Durbin

Asthma is a common condition characterized by the narrowing and swelling of the airways. In 2009, it affected one in eight or twenty-five million Americans and continues to grow year by year (American Academy of Allergy Asthma and Immunology). As a disease that affects so many people, there are many studies focusing on how asthma arises and what can be done to treat or prevent it. There is a range of topics being researched including: what characterizes the microbiome of the airway, the hygiene hypothesis concerning the exposure of bacteria to children, the interactions of drugs with the human microbiota, and the relationship of asthma and the microbiome in adults. In this paper, these topics will be explored to create a more cohesive idea of the interaction of the human microbiome and asthmatic disease.

Influence of Environment on the Microbiome and Asthmatic Risk

A major topic of asthma research focuses on its causes. Several studies have been conducted to look at the effect that the environment has in altering the microbiota, particularly of children. This idea comes from the hygiene hypothesis, which refers to the increase in the occurrence of allergy and asthma due to a reduced exposure of children at a young age to bacteria, viruses, parasites, and other factors causing infectious burdens (Okada et al. 2010). Several researchers have looked into how different environments shape the human microbiome and affect the potential for asthmatic disease. In one such study by Hugg et al. (2008), the researchers looked into the differences in the risk of asthma to children in both Finland and Russia who were exposed to different environments growing up. The children in Finland were exposed more often to dogs, which resulted in a protective effect to asthma, as opposed to the Russian children who were exposed more often to cats and experienced increased risk of asthma. Both groups of children, age 7-16, experienced an increased risk of asthma with exposure to farm animals. The study was questionnaire-based and looked at both prenatal and postnatal exposure to these animals. The results support the fact that there is susceptibility of children in early-life to acquire asthmatic disease as influenced by the environment and the bacteria in the environment surrounding them (Hugg et al. 2008). Taking the idea of the different environment exposure even farther, Douwes et al. (2008) explored the rate of asthmatic disease in New Zealand children exposed to dairy, sheep, beef, and horticulture farms prenatally, early in life, and currently. Once again, this was designed as a questionnaire-based study. The researchers found that increased exposure to farm animals, hay, grain, and bacteria led to a decreased risk of asthma. Particularly the children who were exposed in utero by their pregnant mothers and children in contact with farm animals in the first two years of life experienced symptoms of asthma less often than those children who were only exposed to farms within the last twelve months or lived in rural areas but not on farms. Furthermore, children who were exposed to a farming environment in utero as well as were currently exposed exhibited the highest reduced risk of asthma (Douwes et al. 2008).

Another environmental factor under research includes not only exposure to pets, but also siblings. In a study by Azad et al. (2013), the researchers focused on how microbiota richness and diversity were affected by the surrounding environment in children involved in the Canadian Healthy Infant Longitudinal Development birth cohort. Again, information was collected from mothers through questionnaires. The results showed increased microbiota richness and diversity of children living in homes with pets, while children with older siblings experienced a decrease. With further research, it was discovered that specific bacteria were over and underrepresented in children with differing environments, suggesting that both pets and siblings alter the microbiota of children, yet both result in protection from asthma and other atopic diseases (Azad et al. 2013).

To elaborate on decreased risk of childhood asthma based upon exposure to microorganisms, Ege et al. (2011) brings in an alternative way of collecting data other than through questionnaires. His research centered upon testing samples of mattress dust from children's beds. The groups of children either lived on a farm or were in a reference group, not residing on a farm. Ege et al. conducted studies characterizing the presence of bacteria and fungi on the mattresses. Once again the children growing up on farms were found to have a decreased prevalence of asthma than the children in the reference group because they had been exposed to more bacteria, as found in their mattress collections. They proposed an inverse relationship between having asthma and being raised in a farm environment (Ege et al. 2011). This method of sampling different microbe sites was again used in a study by Delfino et al. They focused on the prevalence of endotoxin in monitors issued to research participants of age nine to eighteen in both Riverside and Whittier, California. Endotoxin makes up the cell walls of Gram-negative bacteria and was measured through the monitors at indoor home sites, outdoor home sites, and at ambient location. This study provides an alternative to what the previously mentioned studies claim, in that endotoxin exposure at home may not be representative of daily personal exposure. Thus, the hygiene hypothesis of environment influencing microbiota and therefore susceptibility to disease is challenged due to a recognition of the multiple different living environments in which children travel daily. However, this study did support the fact that living with a dog, as well as living with a cat, increased personal endotoxin levels. The environment shapes the microbiota present in humans, but as seen here, it may not be representative of all smaller environments to which they are exposed (Delfino et al. 2011).

In summary, these studies provide evidence that the environment takes a role in shaping the communities of bacteria in the human microbiome; and in general, children who are exposed to more pathogens, animals, and bacteria have a decreased risk of asthmatic disease, suggesting a role of microbiota somehow regulating atopic disease responses in humans.

The Role of Antibiotics in Affecting the Microbiome and Asthmatic Disease

To continue the concept of the hygiene hypothesis in children, researchers have studied the exposure of children to antibiotics and how disease susceptibility and the microbiome changes. In a particular study using a murine model, Russell et al. (2012) discovered that the antibiotic, vancomycin, reduced bacterial diversity in the murine gut microbiome and increased the severity of asthmatic disease. The disease severity was characterized by increases in inflammatory cells, eosinophils, airway hyperresponsiveness, IgE levels, and histopathology evaluation scores. The researchers saw an altered microbial community as well as a decrease in regulatory immune cells, particularly in the colon. The loss of these cells led to increased asthmatic disease severity by way of the gut-lung axis, a newly characterized interactive pathway, although the mechanisms of action are not yet known (Russell et al. 2012). In an addendum to their work, Russell et al., continued to build upon the idea of how exposure to vancomycin in early life exacerbates asthma symptoms. They focus more in this article on the increased IgE levels as well as the decreased regulatory T cells. They characterize the time period from birth to 3 weeks of life to be most influenced by vancomycin. The greatest shifts in the microbial community occur during this time period. This exemplifies the importance of limiting exposure of antibiotics to youth very early in life to ensure minimal alteration to the microbiota and robust immune response throughout a lifetime (Russell et al. 2013). In 2014, Metsala et al. investigated the effects of prenatal exposure of children to antibiotics as compared to postnatal exposure. This group studied children in Finland born between the years of 1996 and 2004 who had been diagnosed with asthmatic disease after the age of three. The researchers evaluated the use of antibiotics by the mothers during pregnancy and also the children's use of antibiotics before the diagnosis of asthma was obtained. The results indicated that both prenatal and postnatal exposure to antibiotics produced an increased risk of asthma in the children in a dose-dependent relationship, based on the number of antibiotic purchases made by both mother and child. These researchers propose that antibiotics alter the microbiota of both the mother and the child and affect their ability to regulate the immune response, which leads to asthmatic disease (Metsala et al. 2014).

The Metsala et al. (2014) and the Russell et al. (2012) studies found similar conclusions that the altered gut microbiota of infants has a role in regulating immune response. More research on this subject of study is necessary to determine the mechanism of action and to find preventative treatments for asthmatic disease. The results of these studies presented so far come together to form a larger picture of the hygiene hypothesis, in which exposure of children to bacteria through different environments is beneficial in decreasing their risk for asthma, and antibiotic exposure should be avoided in order to maintain a rich bacterial diversity, and reducing susceptibility to asthma.

Causes and Risk Factors of Adult Asthma

Research into asthmatic disease in adults has yielded more contrasting results from children. Instead of heightened exposure to bacteria lowering the risk of disease or disease severity, it leads to increased responsiveness of asthma in adults. This may be because adults are no longer in the critical period where exposure is beneficial to them. Looking back at the research by Russell et al. (2012), a completely different gut bacterial community is seen in neonatal mice treated with vancomycin versus adult mice treated with the same antibiotic (Russell et al. 2012). Neonates' guts were largely populated by *Lactobacillaceae*, while adults were populated by *Paenibacillaceae.* The microbiomes of adults compared to children are drastically different and most likely have an effect on disease occurrence. Not only do the microbiomes of adults differ from children, but they also differ in the presence of asthmatic disease. In a study by Huang et al. (2011), the researchers characterized the composition of the microbial community in the airways of patients with asthma. They found that the diversity of the bacteria populating the airways was higher among patients with asthma compared to healthy control subjects. The presence of *Comamonadaceae*, *Sphingomonadaceae*, and *Oxalobacteraceae* were associated with increased airway hyperresponsiveness in patients (Huang et al. 2011). The adult response to exposure to bacteria is further explored in the research done by Thorne et al (2005). They found, through vacuum sampling of homes across the nation, a correlation between the amount of endotoxin and the prevalence of asthma, wheezing, or asthma medication usage. The researchers claim that the largest effect of endotoxin exposure is airway inflammation rather than allergy (Thorne et al. 2005). In another study by Marri et al. (2013), researchers again found that changes in the prevalence of specific bacterial phyla were associated with the presence of asthma in patients. These researchers collected sputum samples from the lower respiratory tracts of both asthmatic and non-asthmatic sample groups and found a deviation in phyla present and bacterial diversity present. The greater presence of Proteobacteria was associated with asthmatic patients. The characterization of the lung microbiome is important as previous knowledge claimed that the respiratory system was sterile. Now that it is evident that bacteria do reside in the airways, it is possible to study adults' environments and bacterial interactions with drugs. These researchers conclude that the diversity of the respiratory tract is not due to asthma medication, or corticosteroid, usage, but rather is a hallmark of both mild and severe asthmatic disease. (Marri et al. 2013). With growing knowledge on what bacteria phyla characterize both diseased and healthy lung microbiomes, researchers are able to look deeper into the role of bacteria, such as *Chlamydophila pneumoniae*.

Two such studies focus on this bacterium as an infectious agent that exacerbates asthma. Cosentini et al. (2008) compared how *C. pneumoniae* and *Mycoplasma pneumoniae* infections resulted in disease. This study focused on determining the nature of emergency room visits in Milan, Italy for acute asthma inflammation in 58 subjects. Almost half of the patients had an acute atypical infection with one of the two bacteria mentioned above. The researchers concluded that patients with these infections had a more severe exacerbation of asthma, with *C. pneumoniae* infection being the most prevalent (Cosentini et al. 2008). This conclusion is supported by the research of Hahn et al. (2012) who focused specifically on the presence of *C. pneumoniae* IgE in patients with asthma. As shown previously, the patients who tested positive for IgE levels specific to these bacteria tended to have more severe cases of asthma. This supports, but does not prove, a causal function of *C. pneumoniae* resulting in severe asthma (Hahn et al. 2012).

After characterization of prospective associative factors of asthma exacerbation, researchers began to look further into treatments and their efficacy of eradication of bacteria. In a study by Johnston et al. (2006), it was found that the use of antibiotics in adults results in different outcomes than in children. Two hundred and seventy eight adults diagnosed with asthma were either given the antibiotic telithromycin or a placebo. The researchers found that telithromycin was beneficial in curbing acute asthmatic disease in adults. The studies mentioned earlier by Russell et al. (2012) and Metsala et al. (2014) supported a negative effect of antibiotics on asthma in children, while the research by Johnston et al. (2006) supports a beneficial effect of antibiotics in adult asthma (Johnston et al. 2006). Other researchers who studied the interaction of medications with bacteria and the microbiome include Goleva et al. (2013), who attempted to determine how bacteria altered the cellular response to corticosteroids in people with resistant versus sensitive asthma. The findings indicated some corticosteroid-resistant patients had greater prevalence of Gram-negative bacteria, which triggers the TAK1/MAPK pathway to activate and prompt corticosteroid resistance. These Gram-negative bacteria tended to be Proteobacteria, which increased the prevalence of asthmatic disease in the previously mentioned paper by Marri et al. (2013). One of the specific Proteobacteria that was upregulated in the airways of resistant patients, characterized by Goleva et al., (2013) was found to be *Haemophilus parainfluenzae*, which was associated with increased IL-8, mitogen-activated kinase phosphatase 1 (MKP-1), and MAPK expression after macrophage activation. This potential mechanism for activation of a resistance pathway will be useful for developing an alternative method for treating asthma without ensuing resistance (Goleva et al. 2013). In determining other pathways for adult activation of asthma, studies have looked deeper into the role of the human immune response in disease exacerbation. One such study by

Koh et al. (2010) found that Natural Killer (NK) T cells, known to bridge the innate and the adaptive immune systems, are not required for the activation of allergen-induced features of chronic asthma. It is suggested that NK T cells may be involved in viral induction of chronic asthma (Koh et al. 2010). This paper brings to light the complexity of the causes of both acute and chronic asthma. The interaction between the human immune system and the microbiota should be an important topic in which to continue research in order to develop a way to treat those who are affected by asthmatic disease.

The complexity of asthmatic disease triggers a need to continue research into the alterations that occur due to changing environments, drug usage, and age. So far, research has demonstrated that young children should be exposed to different environments to ensure a diverse microbiota and healthy immune response, while antibiotics in children should be avoided. Research into adult asthma has led to opposite conclusions, where adults should use antibiotics to curb asthmatic disease while limiting bacterial exposure. The difference in guidelines for children compared to adults concerning asthma treatment creates a complicated situation for people trying to maintain health. Difficulty may arise in families consisting of both children and adults requiring differing amounts of exposure to bacteria for non-asthmatic outcomes. Further study of the interactions between the human microbiomes and the human immune system must be conducted to better characterize differences in asthmatic disease. This research is important in controlling the more than 25 million cases of asthmatic disease afflicting Americans each year.

References

American Academy of Allergy Asthma and Immunology. Asthma Statistics. [Internet]. [cited 2014 Nov 26]. Available from http://www.aaaai.org/about-the-aaaai/newsroom/asthma-statistics.aspx.

Azad MB, Konya T, Maughan H, Guttman DS, Field CJ, Sears MR, Becker AB, Scott JA, Kozyrskyj AL. 2013. Infant gut microbiota and the hygiene hypothesis of allergic disease: impact of household pets and siblings on microbiota composition and diversity. Allergy Asthma Clin Immunol. [Internet]. [cited 2014 Nov 23]; 9(1):15.

Cosentini R, Tarsia P, Canetta C, Graziadei G, Brambilla AM, Aliberti S, Pappalettera M, Tantardini F, Blasi F. 2008. Severe asthma exacerbation: role of acute Chlamydophila pneumoniae and Mycoplasma pneumoniae infection. Respir Res. [Internet]. [cited 2014 Nov 23]; 9(1):48.

Delfino RJ, Staimer N, Tjoa T. 2011. Personal endotoxin exposure in a panel study of school children with asthma. Environ Health. [Internet]. [cited 2014 Nov 23]; 10(1):69.

Douwes J, Cheng S, Travier N, Cohet C, Niesink A, McKenzie J, Cunningham C, Gros GL, Mutius E von, Pearce N. 2008. Farm exposure in utero may protect against asthma, hay fever and eczema. Eur Respir J. [Internet]. [cited 2014 Nov 23]; 32(3):603–611.

Ege MJ, Mayer M, Normand A-C, Genuneit J, Cookson WOCM, Braun-Fahrländer C, Heederik D, Piarroux R, von Mutius E. 2011. Exposure to Environmental Microorganisms and Childhood Asthma. N Engl J Med. [Internet]. [cited 2014 Nov 23]; 364(8):701–709.

Goleva E, Jackson LP, Harris JK, Robertson CE, Sutherland ER, Hall CF, Good JT, Gelfand EW, Martin RJ, Leung DYM. 2013. The Effects of Airway Microbiome on Corticosteroid Responsiveness in Asthma. Am J Respir Crit Care Med. [Internet]. [cited 2014 Nov 23]; 188(10):1193–1201

Hahn DL, Schure A, Patel K, Childs T, Drizik E, Webley W. 2012. Chlamydia pneumoniae-Specific IgE Is Prevalent in Asthma and Is Associated with Disease Severity. PLoS One. [Internet]. [cited 2014 Nov 23]; 7(4).

Huang YJ, Nelson CE, Brodie EL, DeSantis TZ, Baek MS, Liu J, Woyke T, Allgaier M, Bristow J, Wiener-Kronish JP, et al. 2011. Airway microbiota and bronchial hyperresponsiveness in patients with suboptimally controlled asthma. J Allergy Clin Immunol. [Internet]. [cited 2014 Nov 23]; 127(2):372–381.e3.

Hugg TT, Jaakkola MS, Ruotsalainen R, Pushkarev V, Jaakkola JJ. 2008. Exposure to animals and the risk of allergic asthma: a population-based cross-sectional study in Finnish and Russian children. Environ Health. [Internet]. [cited 2014 Nov 23]; 7(1):28.

Johnston SL, Blasi F, Black PN, Martin RJ, Farrell DJ, Nieman RB. 2006. The Effect of Telithromycin in Acute Exacerbations of Asthma. N Engl J Med. [Internet]. [cited 2014 Dec 3]; 354(15):1589–1600.

Koh Y-I, Shim J-U, Lee J-H, Chung I-J, Min J-J, Rhee JH, Lee HC, Chung DH, Wi J-O. 2010. Natural killer T cells are dispensable in the development of allergen-induced airway hyperresponsiveness, inflammation and remodelling in a mouse model of chronic asthma. Clin Exp Allergy. [Internet]. [cited 2014 Nov 23]; 161(1):159–170.

Marri PR, Stern DA, Wright AL, Billheimer D, Martinez FD. 2013. Asthma-associated differences in microbial composition of induced sputum. J Allergy Clin Immunol. [Internet]. [cited 2014 Nov 23]; 31(2):346–352.e3.

Metsälä J, Lundqvist A, Virta LJ, Kaila M, Gissler M, Virtanen SM. 2014. Prenatal and Postnatal Exposure to Antibiotics and Risk of Asthma in Childhood. Clin Exp Allergy. [Internet]. [cited 2014 Dec 3]; 45(1):137–145.

Okada H, Kuhn C, Feillet H, Bach J-F. 2010. The “hygiene hypothesis” for autoimmune and allergic diseases: an update. Clin Exp Immunol. [Internet]. [cited 2014 Nov 26]; 160(1):1–9.

Russell SL, Gold MJ, Hartmann M, Willing BP, Thorson L, Wlodarska M, Gill N, Blanchet M-R, Mohn WW, McNagny KM, et al. 2012. Early life antibiotic-driven changes in microbiota enhance susceptibility to allergic asthma. EMBO Rep. [Internet]. [cited 2014 Nov 23]; 13(5):440–447.

Russell SL, Gold MJ, Willing BP, Thorson L, McNagny KM, Finlay BB. 2013. Perinatal antibiotic treatment affects murine microbiota, immune responses and allergic asthma. Gut Microbes. [Internet]. [cited 2014 Nov 23]; 4(2):158–164.

Thorne PS, Kulhánková K, Yin M, Cohn R, Arbes SJ, Zeldin DC. 2005. Endotoxin Exposure Is a Risk Factor for Asthma. Am J Respir Crit Care Med. [Internet]. [cited 2014 Nov 23]; 172(11):1371–1377.

Rhino Conservation in Chitwan National Park, Nepal

Maddie Carwile

Chitwan National Park, Nepal, is home to 500 one-horned rhinos, a species endangered due to habitat loss, poaching, and human-wildlife conflict. The area around Chitwan National Park is highly impoverished, and relies on tourism for a substantial portion of income. My research trip to Chitwan focused on identifying the main threats to rhino conservation and investigating strategies that local people, conservationists, and government agencies employ to counter these threats. I found that while poaching and human-wildlife conflict have been significant threats in the past, the largest threat is the invasive plant species, Mikenia micrantha, *which covers the native grass species consumed by the rhinos. Another significant threat is the possible road and railway system proposed to cut through the National Park.*

Introduction

In August of 2014, I took a research trip to Chitwan National Park, Nepal, to study rhino conservation. Nepal is a small, landlocked country about the size and shape of Tennessee. It is bordered by China to the north and India to the south. The geography of the country is divided into three distinct bands. In the north of Nepal are the Himalayas, including Mount Everest. The large, middle section of the country is covered by the "hills," and the south of Nepal is the Terai, or lowland jungle. While the north of the country can certainly be cold, in the summer temperatures in Nepal can be above 100ºF. A major event in recent history was a civil war between Maoist insurgents and the government fought from 1996 to 2006 (Whelpton 2). This civil war not only impacted Nepal politically, it also had a profound effect on the rhino population. Park rangers and soldiers were unable to police the park due to the conflict, and poaching numbers drastically increased (Pokheral, pers. Comm.; Poudyal et al., 2009).

My research took place in Chitwan National Park in the lowland jungles. Chitwan is home to many species of animals, including elephants, tigers, crocodiles, deer, wild boar, and, of course, rhinos. The one-horned rhino (*Rhinoceros unicornis*), also known as the Indian rhinoceros, is a species endangered due to poaching and habitat loss. One-horned rhinos are solitary animals that live in the grasslands and forests in India and Nepal (Poudyal et al., 2009). Chitwan National Park has around 500 rhinos, around a quarter of all those left in the wild (Chiran Pokheral, pers. Comm.).

Nepal is a very impoverished country. The per capita income is US$700 per year, and a quarter of the population lives below the International Poverty Line of $1.25 a day (USAID/World Bank, 2014). Around 80% of the population works in agriculture, and as the country becomes more urbanized and developed, habitat loss is a real concern for native species (USAID, 2014). While the effect of current population growth and agricultural impacts is unknown, past increases in agricultural development have had significant impacts on wildlife populations. For instance, the Nepalese government's Integrated Agriculture Program increased agricultural production in Nepal, but also caused large-scale clearing of forests and a significant decrease in the rhino population (Poudyal et al., 2009).

My research focused on the relationship between extreme poverty and wildlife conservation. How can local and national governments and local and international organizations help impoverished people make a living while at the same time caring for the welfare of animals? How can wildlife conservation be promoted in areas with high poverty rates? I looked at threats to rhino conservation in Nepal, and how community members, wildlife agencies, and government officials are combatting these threats.

It is interesting to note, that despite Nepal's poverty, the country has a strong track record when it comes to rhino conservation. In the 1950s, the population numbered around 800, but poaching brought the population under to less than 100 by 1968 (Martin and Vigne, 1996). Current estimates using ID-based rhino monitoring place the population at over 500 individuals, largely due to a decrease in poaching and more effective monitoring (Chiran Pokheral, pers. Comm.). March 2014 marked an entire year without incidents of rhino poaching, and there has only been one incident in the last year, and two rhinos in the past four years (Republica). On the other hand, countries such as South Africa have upwards of 1000 rhinos poached every year. Conservationists consider the drastic increase in rhino population in Chitwan National Park an essential success story (Martin and Vigne, 1996). My research focused on reasons for Nepal's success and conservation efforts, and I wanted to study whether these strategies could be applied to other countries.

Methods

I met with conservationists both in Chitwan National Park and in Kathmandu, the capital city of

Nepal. I spent most of the summer working for the United States Agency for International Development in their Social, Environment, and Economic Development (SEED) Office. At USAID, I learned about their Hariyo Ban environmental program. Hariyo Ban means "Green Forests," in Nepali, and focuses on mitigating the effects of climate change and conserving native species. One major component of the Hariyo Ban Program is that it emphasizes community-based natural resource management and focuses on helping poor and marginalized groups build resiliency.

After preliminary research and interviews in Kathmandu, I travelled south to Chitwan. The area around the National Park is home to the indigenous Tharu people. Around 6.7 million people live in the Chitwan region, with 4.5 million cattle (Abdul Ansari, pers. Comm.). This part of Nepal is more impoverished than the country as a whole, with 50% of people living below the poverty level and an average income per capita of only US$90 a year (Abdul Ansari, pers. Comm.).

In Chitwan, I interviewed members of the Community Forestry User Groups and Anti-Poaching Groups. I also visited the World Wildlife Fund Office and met with the director of the Terai Arc Landscape program, Mr. Abdul Ansari. I stayed at the Biodiversity Conservation Center (BCC) for the National Trust for Nature Conservation (NTNC), and participated in ID-based rhino monitoring. The NTNC is one of the foremost wildlife conservation agencies in Nepal, and it was a privilege to stay at their center. One highlight was a baby rhino that roamed free on the compound. He enjoyed sleeping under the stairs to my dorm, and liked to be pet as though he were a dog. When I sat on my stairs, he would stick his snout through the railing and suck on my fingers. The young rhino had claw marks on his face and leg, and was likely attacked by a tiger. The conservationists at the NTNC believe that his mother chased the tiger away, and was separated from her son in the process. As of January 2015, the rhino still lives at the BCC, and due to the improbability of succeeding in the wild he will likely move to the Central Zoo in Kathmandu. Since my research trip, the BCC has rescued another juvenile who was attacked and severely injured by a tiger. Tiger attacks are a major cause of death for rhino calves in Nepal (Martin and Vigne, 1996). The NTNC's rehabilitation of injured and orphaned rhino calves is a ground-level approach to preventing rhino deaths that works in conjunction with larger-scale conservation programs.

The most enjoyable part of my research was participating in ID-based rhino monitoring. Each rhino has a distinguishing feature, such as a spot or a ridge in their armor, and my guide was able to recognize different rhinos and tell me their ages, sex, and names. We went out on elephant back- it's the best way to traverse the jungle and provides an elevated point for searching (Figure 1).

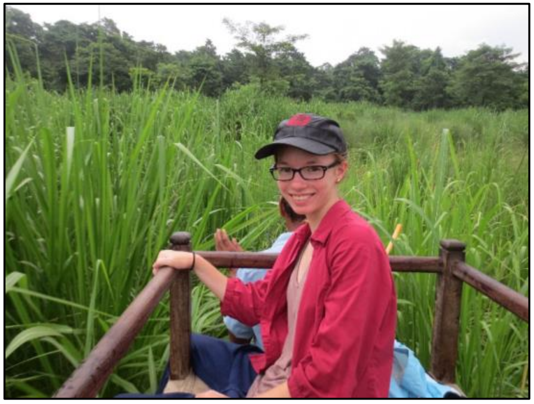


Figure 1. Rhino observations made using elephant transportation.

The BCC keeps their own elephants for monitoring and field work. ID-based rhino monitoring is a way for park officials to monitor the number of rhinos in the park, and helps determine if poaching is occurring (Chiran Pokheral, pers. Comm.).

I was lucky enough to see many rhinos while in Chitwan (Figure 2). However, spotting them can often be difficult due to the dense foliage and high grasses in the summer months. These problems were exacerbated by the fact that my research took place in August, which is monsoon season in Nepal. The rhinos are not skittish and once observed, can be approached easily.

Figure 2. Rhino spotted from elephant back. Photo by author.

Results

Tourism and Human-Wildlife Conflict

In order to address problems surrounding wildlife conservation, it is first necessary to understand the close relationship that the people in the Chitwan area have with the wild animals. The buffer zones that border the National Park have many small villages. It's not uncommon to have wild animals, particularly deer and boar, come into the villages and eat or trample crops. Elephants, also native to the region, have been domesticated and are used in logging, farming and for tourism (Figure 3). At the breeding center, government officials breed and train elephants for use in patrolling the park (Figure 4). Elephants are also used for elephant safaris for tourists (Figure 5).

Initially, I found three main threats to rhino conservation: poaching, habitat loss, and retaliatory killings. These fall into the category known as human-wildlife conflict. Rhinos are most often poached for their horns, which are used in Chinese medicine to treat ailments ranging from typhoid to arthritis to vomiting (Bronwyn Llewellyn, pers. Comm.; Ellis 121). One horn can cost more than $8,000 on the black market, a huge source of income for people who may make less than $100 in an entire year (Ellis 114). Habitat loss occurs with higher frequency as people cut down trees for farmland and pasture land for their animals. Retaliatory killings of wildlife occur when animals eat crops, kill livestock, or damage property. Human-wildlife conflict is increasing as the population grows (Martin and Vigne, 1996). For rhinos specifically, human-wildlife conflict includes crop damage, injury, and even death (Sitidevi Community Forestry User Group; Gyaneshwor Community Forestry User Group, pers. Comm.).

One USAID project that seeks to mitigate human-wildlife conflict is power fencing. One kilometer of fencing costs 100,000 rupees (US$1,000), but can save millions in crop damage or livestock loss (Hariyo Ban Annual Report). The fencing is supposedly effective against rhinos, wild boar, and spotted deer, and has led to a significant decrease in human-wildlife conflict and retaliatory killings (Hariyo Ban Annual Report). I had the opportunity to speak with villagers in Sitidevi and Gyaneshwor Community Forests and ask them their opinions on power fencing. The community members in Gyaneshwor said that the fencing is 75% effective in keeping out animals, while the villagers in Sitidevi said that it works well for rhinos but does not always prevent deer and boar from entering the village. One problem is that Nepal does not have enough electricity for the whole country, which means that the government employs load-shedding. People are without power for up to 18 hours a day. The villagers would like continuous current for the electric fences, as well as compensation for crop and livestock damage.

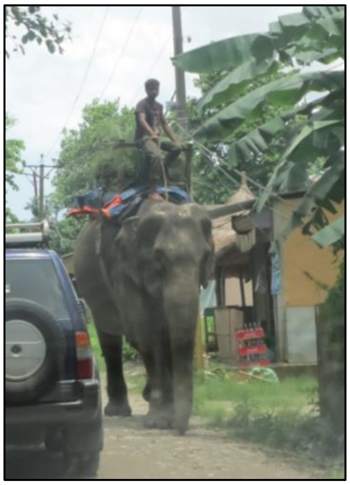


Figure 3. Elephant and driver walking down street. Photo by author.

Figure 4. Mother and young elephant at the breeding center. Photo by author.

Figure 5. Tourists take part in an elephant safari. Photo by author.

Nepal has had a successful history of conservation for several reasons. It's true that in impoverished communities, poaching is a way for people to earn money (Community-Based Anti-Poaching Unit, pers. Comm.). In 1993, a rhino horn cost over US$11,000 per kilogram, a significant amount of money in a region where the average income per capita is only $90 a year (Martin and Vigne, 1996). As prices are likely increasing, poaching is a lucrative opportunity for impoverished Nepalis and Indians alike (Bronwyn Llewellyn, pers. Comm.) However, not only is tourism a significant source of income, it also benefits the whole community rather than a select few individuals. There are dozens of hotels around the National Park that employ locals as cooks, waiters, housekeepers, and guides. Tourism in Chitwan focuses on experiencing the wildlife through elephant safaris and canoe rides. I was even able to participate in an elephant bath, riding an adult female bareback down to the river. These wildlife tourism activities employ locals with indigenous knowledge of the region, and provide money to improve infrastructure, education, and nutrition. People living around Chitwan understand that loss of rhinos and tigers means loss of tourism, so they do not support poachers. To quote Abdul Ansari, the director of the World Wildlife Fund's Terai Arc Landscape (WWF-TAL) program, "without conservation, there is no tourism."

In fact, one of WWF's main projects actually focuses on increasing and improving tourism by promoting eco-tourism. Home stays of visitors benefit the community directly, and with regular tourism, the hotels and resorts employ local guides, as well as cooks, and housekeepers. However, more studies should be conducted on the effects of so many people around Chitwan National Park (Abdul Ansari, pers. Comm.). Each area of the forest only has a limited carrying capacity, or the number of species that an environment can sustainably support, but dozens of elephants and hundreds of people may use the forest every day. In the future, a study should be done to see if there is a lack of engagement in normal behavior for rhinos, such as mating (Bronwyn Llewellyn, pers. Comm.). Certain behaviors may be distorted due to the large numbers of people and elephants using the same areas surrounding the national park.

While the effects of tourism on the local environment is still be studied, tourism has had a significant economic benefit for the community. Park entry fees alone bring 220 million rupees, or around $2 million, a year (Abdul Ansari, pers. Comm.). Approximately 50% of this money returns to local communities (Abdul Ansari, pers. Comm.). Hotels and businesses also promote community involvement for tourists, and many adopt schools or villages.

Invasive Species

While in Chitwan, I met with the director of the program for WWF-Nepal, Abdul Ansari. He says that the largest threat to the rhino population is not poaching or other forms of human-wildlife conflict. Instead, he said that the largest threat is alien invasive species on grasslands.

Mikenia micrantha, a fast-growing plant also known as "mile-a-minute," is becoming a significant threat to the rhino population in Nepal (Abdul Ansari, pers. Comm.). Nepal underwent a civil war from 1998 to 2008, and during this time the army covered their barracks with mile-a-minute to hide them from the Maoist insurgents (Sapana Lodge guide, pers. Comm.). The plant has since spread, and is covering the native grasses that the rhinos usually eat (Figure 6). The rhinos cannot eat the *Mikenia* plant, and park officials are unsure of how to destroy it (Abdul Ansari, pers. Comm.).

Figure 6. Invasive species *Mikenia micrantha* covering grass, the main food source for rhinos. Photo by author.

Combatting Poaching with Community Involvement

Community members in Chitwan have also established Anti-Poaching Groups to prevent poaching of animals such as rhinos and tigers. Their main focus is improving awareness for the poorest communities via songs during festivals, household visits, quiz competitions in schools, and advertisements on local radio. According to the leaders with whom I met, education is the most important aspect of reducing poaching. By teaching children about the importance of wildlife, the anti-poaching members hope that parents and family members will also learn. The anti-poaching groups also have a cadre of informants, make observations while patrolling, and report suspicious behavior to the National Park Authorities.

The Anti-Poaching members are all volunteers. When I asked the five men their motivations, two answered conservation of forests, one said to improve the world for future generations, another responded with protection of animals, and only one person responded that his main motivation was to help the local economy promote tourism. Nepal's success rate cannot just be attributed to the economic benefits of tourism.

Conservationists agree that the best way to promote wildlife protection is to engage and mobilize the community (Wells and Sharma, 1998; Bronwyn Llewellyn, pers. Comm.). When people take ownership of natural resources, they have motives to protect them that go beyond money. The people living around Chitwan National Park have a generally positive attitude towards the animals and see them as a source of pride. All of the villagers that I interviewed said that they liked the rhinos and did not consider them to be a pest. Such positive opinions seem conflicting when looking at the damage that rhinos can cause. In Sitidevi Community Forest, one person was recently killed by a rhino while cutting grass in the forest, and four people were injured since the spring. In Gyaneshwor Community Forest, three people were killed by rhino attacks and two were injured in the past seven years. According to the villagers, rhinos are the most dangerous animals in the jungle, followed by tigers and leopards.

Villagers that live next to the jungle are less accepting of conservation and anti-poaching efforts, due to wild animals eating their crops and livestock. Since the Community-Based Anti-Poaching groups protect these animals, villagers often view them as responsible for their loss of livelihood. People involved in combatting poaching are not always safe. The anti-poaching volunteers said that they feel unsafe during night patrols and told me about a masked man on a motorcycle who would drive behind them and make death threats. And sadly, in October, two National Park employees were shot and one was killed likely by wildlife smugglers (Republica).

When I met with the anti-poaching group, they were all men. While women are involved in planning and organizing events, there are no women patrolling in the field. Anti-poaching leaders say that patrolling is too physically demanding for the women, and claim that women cannot run or climb trees if pursued by an animal. They also worry about women encountering poachers. The main problem, however, is societal and cultural pressure. It would be considered improper to send women into the jungle with men, and there are no patrolling groups that are composed solely of women.

Current Issues and Future Threats

Nepal has some advantages that other countries lack when it comes to promoting conservation efforts and reducing poaching. In part due to its extreme poverty, Nepal often provides soldiers for United Nations Peace-Keeping missions, and these soldiers train by patrolling national parks (Bronwyn Llewellyn, pers. Comm.). There are more than 1,000 soldiers stationed in Chitwan National Park alone, and they have been effective in reducing the amount of poaching (Abdul Ansari, pers. Comm.). When poachers shot and killed a rhino in May, they did so during a change of command in the army (Abdul Ansari, pers. Comm.). The presence of the army is a strong deterrent for poachers.

Chitwan National Park is also a relatively small area at 932km^2 (Martin and Vigne, 1996) It is surrounded by buffer zones, so poachers must pass through populated areas to get in and out of the park. Between the anti-poaching members, soldiers, and villagers, it can be difficult for poachers to remain unseen. This is not the case in many wildlife reserves and parks in Africa, where the vast areas cannot always be adequately patrolled (Bronwyn Llewellyn, pers. Comm).

Other countries may not be able to fully replicate Nepal's success in rhino conservation, which benefits from the strength of the army and the small size of the park. However, other programs can learn from Nepal's focus on community engagement and mobilization. The anti-poaching groups and community forest groups take charge when it comes to conservation and natural resource management. Sense of ownership and self-governance encourages local people to not only participate in conservation, but to become leaders. Any and all conservation practices need to have the full support of the local community and a strong understanding of the culture.

The future of rhino conservation in Nepal looks positive. However, certain challenges remain. Potential problems are a planned railroad and postal roads connecting India and Nepal. The current development plan has the railroad and roads cutting directly through Chitwan National Park (Bronwyn Llewellyn, pers. Comm.; Abdul Ansari, pers. Comm.). While the railway and roads would bring economic benefit to the people living in the area, they would likely have a severe and long-lasting effect on wildlife conservation. If the railway or the postal roads are constructed, conservation agencies and government officials will need to work with community members to ensure that rhinos and other species are protected.

Due to the relatively small size of the rhino population, genetic diversity may also become a threat in the future. There are genetic problems in the tiger population due to its small size of 200 individuals within the park, but no studies have been conducted on the rhinos (Bronwyn Llewellyn, pers. Comm.). A study should be done on genetic diversity, as a bottleneck may have occurred following the reduction in population size during the Maoist insurgency or in years of poaching prior. During the insurgency, at least 29 rhinos were killed in Chitwan as soldiers left their posts at the national Park to fight the Maoists (Ellis 116).

In the future, studies should also be conducted on the carrying capacity of the park and buffer zone area to see the impact of tourism on the environment. Agencies working in Chitwan should promote active involvement and leadership by women, which would not only help with conservation efforts but also in raising the status of women in an area where they have fewer rights and opportunities than men. Since women and men cannot go together into the jungle, all-women anti-poaching teams should be established. The local government should work with the national government and aid agencies to expand solar-powered electric fencing to all communities, to reduce human-wildlife conflict, crop damage, and death. The two biggest challenges for the future will be the effects of the possible roads and railways and the habitat loss caused by *Mikenia micrantha.*

While there are several barriers for Nepal to overcome, the future looks promising. Rhino populations continue to rise as poaching decreases, and the open collaboration between the government and conservation agencies continues to create new solutions for environmental sustainability and rhino protection.

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References

Ansari, Abdul. Personal interview. August 2014.
Ellis, Richard. *Tiger Bone & Rhino Horn*. London: Shearwater Books, 2005.
Llewellyn, Bronwyn. Personal interview. July 2014.
Martin and Vigne. “Nepal’s Rhinos- One of the Greatest Conservation Success Stories.” *Pachyderm*, 21, 1996.
Pokheral, Chiranjibi. Personal interview. August 2014.
Poudyal et al. “Ecological and economic analysis of poaching of the greater one-horned rhinoceros (Rhinoceros unicornis) in Nepal.” *Ecological Applications*, 19 (7) 2009, pp. 1693-1707.
United States Agency for International Development. *Hariyo Ban Annual Report*, 2013. Online.
Wells and Sharma. “Socio-economic and political aspects of biodiversity conservation in Nepal.” *International Journal of Social Economics*. 25.2, 1998, pp.226-243.
Whelpton, John. *A History of Nepal*. Cambridge: Cambridge University Press, 2005.

Lateralized Behavior in Captive African Penguins

Allen Travis and Lucas Grim

Behavioral lateralization occurs when an animal indicates a notable bias toward one side of the body. This asymmetry may take place in social species due to coordinating constraints placed on the individuals within a population (Ghirlanda and Vallortigara 2004). This particular study at the Memphis Zoo, was among the first to assess behavioral lateralization within captive African black-footed penguins. Through utilizing ad libitum behavioral sampling, individuals were examined for an overall side preference, and later tested against known population factors. In total, 336 behavioral samples were collected with the majority of behaviors being exhibited by 9 of the 22 penguins. Overall, one penguin performed only two behaviors, (one left and one right), while another (with 34 behaviors) displayed more lateralization at 22 left versus 11 right. In the analysis of the combined sampled behaviors using a minimum cutoff value (8), it was discovered that a slight (14%) right side preference in male versus female penguins existed; however, this trend did not meet requirements for significance. Additionally, although the largest numerical difference occurred in the preening behavior, no significant difference was found. Likewise, there was no correlation between age and proportion of laterized behaviors displayed for either sex.

Introduction

Behavioral lateralization is characterized as showing a preference for one side of the body in an animal species that displays physical bilateral symmetry (Ghirlanda and Vallortigara 2004). Studies have suggested that the functional purpose of cerebral lateralization is to facilitate parallel processing of information within brain hemispheres, thus allowing individuals to manage simultaneous tasks with optimal efficiency (Rogers et al. 2004). However, currently the full extent of the relationship between the physiological lateralization in the brain and the often seemingly emergent lateralization of behaviors is a subject of ongoing research (Brown and Magat 2011). In initial studies, lateralization was first viewed as a uniquely human attribute (e.g., handedness in primates), and thought to be a resultant property of complex human brain organization (Ghirlanda and Vallortigara 2004). However, in the past few decades, this notion of lateralized behaviors has been identified and studied more extensively across the animal kingdom (Ghirlanda and Vallortigara 2004).

In particular, research seems to draw special interest with regards to lateralization in birds, and moreover, there is a wide range of data to suggest that birds may display some functional dominance between brain hemispheres (Prior 2006). Peluso and Anderson (2014) discovered an observable trend in several Caribbean flamingos living in captivity that could be used to predict age, as well as the frequency and outcome of agonistic bouts. Older and more successful birds preferred to scratch using the right foot (Peluso and Anderson 2014). However, no studies have focused on laterization in African black-footed penguins (*Spheniscus demersus*), a captive endangered species (Crawford et al. 2011). The African penguin is an ideal candidate for such research purposes; these birds live in highly socialized groups and are quite active during the day, exhibiting a wide range of natural behaviors, including diving, aquatic foraging, and preening (Eggleton and Siegfried, 1979). As a semi-aquatic species, black-footed penguins naturally reside in temperate/tropical climates off the southern coast of Africa (Brown et al. 1982). These penguins display almost no sexual dimorphism in African penguins, which grow to be about 2 ft. tall, and weigh between 7-8 lbs. (Brown et al. 1982). In comparison, captive penguins, much like their wild counterparts, display some behaviors that may or may not be influenced by the frequency with which other individuals in the group perform the same behavior (Foerder et al., 2013). The aforementioned phenomenon, known as synchrony, was identified in multiple captive penguin species (Foerder et al. 2013). Perhaps, these findings may suggest possible evidence to support the theory that behavioral lateralization may have developed in an adaptive strategy by asymmetric individuals within a population to achieve some level of coordination with conspecifics (Ghirlanda and Vallortigara 2004).

Although it is neither feasible nor relevant to the purpose of this study to parse out which behaviors are synchronized, attempts to account for this variable will be resolved through broadening the statistical approach. This approach will consider analyses of individual preferences alongside notable population trends. The goals of this study are manifold: 1) to determine whether individual African penguins exhibit strongly lateralized behaviors, and if so 2) to examine within-population trends that are associated with behavior frequencies. It is therefore hypothesized that captive African penguins exhibit a significant degree of lateralization for the sampled behaviors. It is predicted that the penguins will display right-side dominant lateralization (Peluso and Anderson 2014). Additionally, it is hypothesized that the captive African penguin population displays sex-related, and/or age-related lateralization trends. It is also predicted that significant differences in right-sided preferences between male penguins versus females, as well as age-dependent changes in right-side behavioral frequencies (Peluso and Anderson 2014).

Because hemisphere dominance within higher-order species is very quickly becoming the rule, rather than the exception, and because such neurological organization has been shown repeatedly to accompany quantifiable behavioral attributes (e.g., lateralization), this research presents a contribution to the expanding catalogs animal psychology and neurodevelopment (Brown and Magat 2011). There can be no overstatement regarding the place of behavioral research to accommodate emerging fields that will result from a clearer understanding of neurophysiology. Lateralization studies mark an important primary junction for examining the secret lives of African penguins and other endangered species, which unfortunately may not always exist in numbers suitable for population studies.

Methods

Subjects & Study Site

Our study was comprised of 22 African penguins, (10 males, 12 females) at the Memphis Zoo in Tennessee, ranging in age from 1 to 27 years (Table 1). Individual penguins were equipped with color-coded tags to facilitate recognition. Penguins were housed outdoors in a roughly circular island exhibit with tall waterfalls and steep climbing structures intended to simulate rock-strewn cliffs on the interior. Surrounding this interior region was a ring shaped pool of water, several feet deep. There were just a few hiding places for the birds underneath the concrete decks that extended into the water, as well as within the rocky fascia of the island. Enrichment consisted of natural items placed around the habitat (e.g., shells, gravel, deadwood) as well as various daily interactions that took place between the penguins, their zookeepers and staff. Importantly, zookeepers interacted with the penguins, whereas the zoo staff primarily interacted with the exhibit (e.g., performing maintenance). As such, keeper presence both in and around the exhibit was common, although the minor disruptions did not seem to noticeably impact penguin behavior. Group feeding for the birds took place each day at 3 pm, and consisted of small fish.

Table 1. Penguin Distribution and Grouping Data

Name	Sex	Age	Name	Sex	Age
Alyran	Male	5	Windy	Female	7
Hotch	Male	4	Betty	Female	2
Liam	Male	8	Emily	Female	6
LT	Male	2	Genevieve	Female	14
Mundo	Male	27	Julie	Female	16
Neville	Male	1	Maria	Female	9
Opus	Male	14	Millie	Female	3
Peanut	Male	7	Myles	Female	2
Shasa	Male	14	Nymphadora	Female	2
Spencer	Male	6	Penelope	Female	6
Sunny	Female	8	Rosie	Female	1

Observation Protocol

All penguins were simultaneously observed using ad libitum behavioral sampling (Martin and Bateson 2007). The behaviors in our ethogram (Table 2) were the only behaviors sampled during the observation period. Animal observation took place on Mondays (1:30pm-4pm), over four weeks from late September to November. On the last observation day, less than 2 hours of data were collected. For consistency, the afternoon feeding time always coincided with the observation period. Still, because so few behaviors were observed initially during the brief feeding times, these scheduled events were omitted from the record. In total, 336 behavioral samples were collected. Both observers were present during all observation periods. In order to ensure consistency, efficiency, and comprehensive inclusion of as many behaviors as possible, we observed independently when the penguins were in distinguishable groups. When this was the case, the penguins were grouped and divided roughly evenly between the two observers. Other times, when the penguins were together in one large group, we divided our roles by having one observer call out the color of the ID tag and the behavior, while the other observer recorded the dictation. This technique was adapted in order to maximize efficiency, but more importantly, to avoid sampling the same behaviors twice. Chosen behaviors were limited to those that had bilateral equivalents, and likewise recordings were confined to situations where an obvious side preference had occurred. During a typical recording, the observer would note first the ID of the penguin performing the action, then the behavior code, and finally the right or left side designation.

Statistical Analysis

Using the IBM software product SPSS, a series of statistical tests of the aforementioned hypotheses were performed to assess trends and significance of potential lateral biases. Levene's test for equality of variance was conducted to ensure that our data fulfilled the assumptions required to proceed with parametric testing. An independent samples t test was simultaneously carried out to determine whether a difference existed between male and female side preference within the population. Individual penguin data chosen for the t test were required to meet our cutoff value of 8 or more total samplings. Establishing cutoff values for the data helped to reduce skew created by outliers with poor representation. Sampling totals for eligible penguins were then converted to decimal values based on the frequency of left-sided behaviors out of the total number of behaviors, resulting in one value between 0-1 for each of the 17 eligible penguins (8 male, 9 female). A Pearson's-chi squared test was performed using compiled data for just the preening behavior, only when 3 or more samplings had occurred (20 eligibilities). Lastly, two separate Pearson correlations were run (one for males, one for females) to test whether lateral biases varied in proportion with age. Both correlations utilized the same left-sided percentages with cutoff described for the t test as the Y variable (sex specific), and age in years of those same cutoff penguins as the X variable (sex specific).

Results

Of the 336 behavioral samples collected, it was found that the majority of those behaviors were exhibited by 9 of the 22 penguins. The ranges of individual lateralization were highly varied. Genevieve performed only two behaviors, (1 left versus 1 right) while Betty, with 34 behaviors, was the most lateralized (22 left versus 11 right). Variance between tested groups did not differ (F = 0.379, p = 0.547); therefore, parametric tests were appropriately used. There was no clear side preference for any of the five individual behaviors that were sampled (Figure 1).

Table 2. Ethogram for African penguin behaviors.

Behavior	Code	Description
Preening	P	An individual reaches back and plucks at the base of a feather on one side or the other
Waterproofing	W	An individual uses its beak to reach back to get a waterproofing substance from the base of its tail to help waterproof its feathers
Scratching	S	An individual uses the right or left foot to scratch a part of their body
Aquatic Roll	R	An individual, while in the water, rolls left or right, and floats unrelated to another action. The direction of the roll is based on the side that remains in the water.
Head Rub	H	An individual rubs his or her head on one side of the body or the other

There was no significant difference between expected and observed frequencies of lateralized preening in penguins ($X^2 = 0.2$, df = 1, p = 0.655). There was a right-side behavior preference (mean ± standard error) for male penguins (61.62% ± 6.60) versus females (47.56% ± 4.02), but no significant difference between the total proportions of lateralized behaviors for each group (t = -1.869, df = 15, p = 0.081). Finally, there was found to be no correlation between the total proportions of lateralized behaviors and the ages of penguins for either males ($r_s = 0.106$, n = 8, p = 0.802), or females ($r_s = 0.330$, n = 9, p = 0.386, Figure 2).

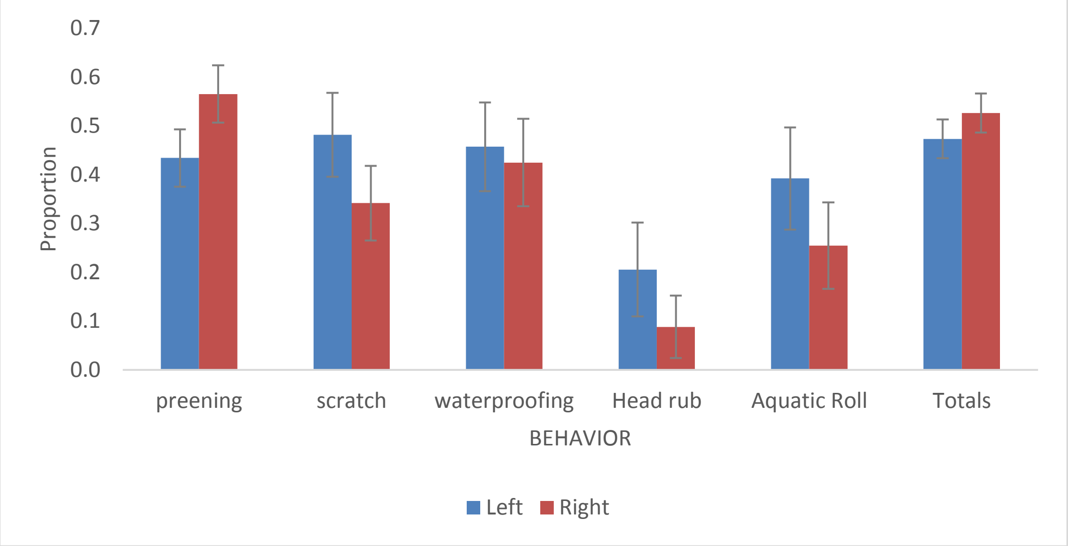

Figure 1. Activity budget (mean ± standard error) of lateralized behaviors exhibited by individuals with 8 or more total samplings.

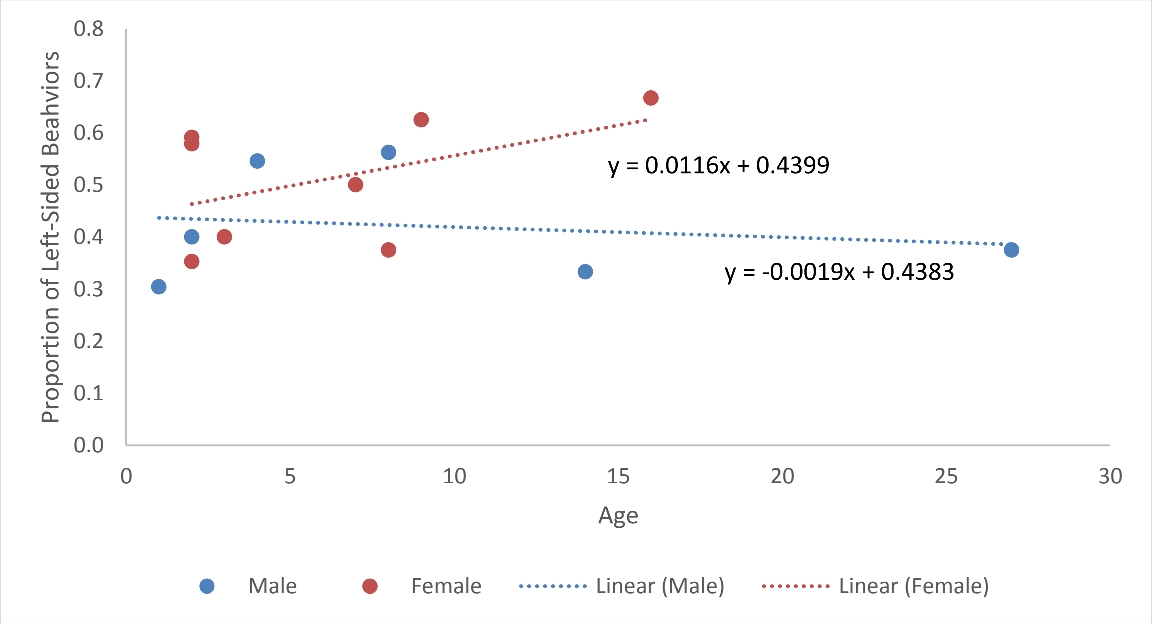

Figure 2. Age vs. proportion of lateralized behaviors shown for both males and females. Only individuals with 8 or more total samplings were included.

Discussion

These results suggested that only the null hypothesis, which stated that that there was no significant lateralization of the sampled behaviors, was statistically supported. However, it did reveal a relationship whereby male penguins were 14% more likely than female penguins to show a right-side preference when analyzing the combined behaviors. Male penguins performed a right-sided behavior nearly 62% of the time. Although this value was not technically significant, is did suggest a possible trend. Interestingly, no age-dependent relationship existed for behavioral lateralization. However, the skew of low ages within the resident penguin population at the Memphis Zoo offers a possible explanation for this finding.

Unlike the flamingo study by Peluso and Anderson (2014), this study's findings did not uncover any noticeable patterns that could have later been studied to provide a more direct link to biological fitness. There are certainly quite a few theories and/or possible explanations that could account for the differences in the expected and observed results. For one, captivity has long been known to have the potential to alter the natural behavior of a wild species over time (Rowell 1967). If we assume, as proposed by Ghirlanda and Vallortigara (2004), that behavioral lateralization is the result of social pressures, then it is totally feasible that a small, captive population in a zoo might be influenced to develop different behavioral strategies, or even demonstrate rapidly shifting population trends.

Overall, we remain conservative and optimistic about our findings and their implications. In total, only 336 behavioral samples were collected. The results of any behavioral study are fully contingent on the process and scale at which the data is compiled; broader characterization of these penguins could be achieved through carrying out the data collection over a much longer period. Future study in this area is undoubtedly warranted to either strengthen or contest this research. Research needs to investigate the question why less than half of the population displayed nearly all of the documented behaviors. Perhaps, the addition of genealogical information and/or medical histories could be used to elucidate more meaningful relationships regarding health and dynamics of the group. Other avenues for possible exploration include examining lateralization within specific foraging patterns. Doing so might yield more promising results, though it would be more difficult to extend the findings of a captive feeding study to wild penguin populations that hunt for live prey over much greater distances (Brown et al. 1982).

Conclusions

1. None of the captive African penguins exhibited a significant degree of lateralization for any of the behaviors sampled.
2. Males within the captive African penguin population displayed a slight right-side bias of the combined behaviors.
3. Relative proportions of total lateralized behaviors were not found to be age dependent in either sex.

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References

Brown C, Magat M. 2011. Cerebral lateralization determines hand preference in Australian parrots. Biology Letters. 7: 496-498.

Brown LH, Urban EK, Newman K. 1982. The Birds of Africa, Vol. I. New York: Academic Press. 314-316.

Crawford RJM, Altwegg R, Barham BJ, et al. 2011. Collapse of South Africa's penguins in the early 21st century. African Journal of Marine Science. 33(1): 139-156.

Eggleton P, Siegfried WR. 1979. Displays of the jackass penguin. Ostrich. 50(3): 139-167.

Foerder P, Chodorow M, Moore DE. 2013. Behavioural synchrony in two species of communally housed captive penguins. Behaviour. 150: 1357-1374.

Ghirlanda S, Vallortigara G. 2004. The evolution of brain lateralization: a game-theoretical analysis of population structure. Proceedings of the Royal Society of London B. 271: 853-857.

Martin P, Bateson P. 2007. Measuring behavior: An introductory guide. 3rd Edition. Cambridge: Cambridge University Press. 176.

Peluso AI, Anderson MJ. 2014. The role of lateralization in feeding behavior and scratching preference in relation to social behavior in captive Caribbean flamingos (*Phoenicopterus ruber*). Animal Behavior and Cognition. 1(1): 51-65.

Prior H. 2006. Lateralization of spatial orientation in birds. In: Y Malashichev, AW Deckel, editors. Behavioral and Morphological Asymmetries in Vertebrates. Washington, DC: Landes-Bioscience. 75-85.

Rogers LJ, Zucca P, Vallortigara G. 2004. Advantages of having a lateralized brain. Proceedings of the Royal Society of London B. 271: S420-S422.

Rowell TE. 1967. A quantitative comparison of the behaviour of a wild and a caged baboon group. Animal Behaviour. 15(4): 499-509.

Determining How Dominance Hierarchy in Captive Pumas (*Puma concolor*) Influences Behavior

Alyssa Tews and Andrew Williams

*For public appeasement and space concerns, zoos often place naturally-solitary creatures together in exhibits. To handle the close proximity of other individuals, species may create dominance hierarchies. Dominance hierarchies allow an individual to exert agonistic behaviors over others in order to establish a social division of the dominant individual and subordinates. In their natural environment, cougars (*Puma concolor*) are solitary felids; therefore, the objective of this study was to determine if a dominance hierarchy existed in the population of cougars at the Memphis Zoo and to what extent this hierarchy affected the cougars' behaviors. A dominant individual (Olympia) and two subordinates (Jane and Sue) were statistically determined and used to test other variables of interest such as amount of active behavior, exhibit use, and average vocalizations. However, no significance was found in the proportion of observed active behaviors (active behaviors $U = 0$, $N1$, $N2 = 2$, $p = 0.66$) and average observed vocalizations (vocalization behaviors $U = 0$, $N1$, $N2 = 2$, $p = 0.66$) between the groupings of dominant versus subordinate cougars. The mapping of exhibit use revealed that the dominance hierarchy did not greatly influence exhibit space differences between the dominant and subordinate individuals (Olympia's total exhibit usage = 41.176%, Jane's total exhibit usage = 31.372%, Sue's total exhibit usage = 28.431%). By understanding how cougars interact and how the hierarchy is assigned, husbandry of captive cougars may be altered to minimize the social stress created by a dominance hierarchy. In addition, this research study could help to predict the effects of impeding habitat destruction by urbanization—as populations in the wild may begin similar hierarchies to cope with the limited space availability.*

Introduction

As solitary carnivores, cougars (*Puma concolor*) define their social interactions through chemical scents, vocalizations, and aggressive behaviors (Hamsen et al., 2010). In the wild, cougars use chemical signaling such as scrape markings to define territories and communicate reproductive status (Hamsen et al., 2010). To maintain these markings, cougars often patrol their territories to keep communication of dominance and status available to others (Hamsen et al., 2010). Interestingly, communication between cougars is unique to their genus as their solid hyoid does not permit cougars to vocalize a roar (Busch, 2004). Instead, cougars vocalize through screams, growls, purrs, chirps, and snarls (Macarrao et al., 2012). These differing sounds can communicate reproductive status of females, calls for potential mates, or imitations of prey (Hamsen et al.; 2010, Macarrao et al., 2012).

Cougars' territories can either be held by an individual for a long period of time or cougars can migrate to follow the migratory patterns of prey such as mule deer (Pierce et al., 1999). As observed by Pierce et al. (1999), under certain circumstances cougars are able to overlap territories for a brief period of time when mating or migrating, but later separate. In the wild, male cougars hold large territories while females have much smaller territories (Hamsen et al. 2009). If these felids approach one another, the encounters tend to result in aggressive displays of hissing, attacking, and fighting until an individual backs down (Hamesen et al., 2009). However, when multiple animals are placed together in captivity, they find means to live with lessened agonistic behaviors through what is believed to be a dominance hierarchy (Vandierendonck et al., 1995). The dominant position is gained by aggressive and agonistic behaviors towards other individuals until they submit or show no aggressive behaviors in return (Chase et al., 2001). Although some dominant-subordinate behaviors are observed in the wild, in captivity, the subordinate cannot run away or leave the enclosure when challenged.

In studies where human-interference and habitat loss are factors influencing cougar behavior, scientists such as Kertson et al. (2013) found that cougar-human interactions were more common in residential areas in Washington. Although a proportion of the cougars adapted to this new setting and continued to hunt, travel, and scent-mark in the residential areas, some cougars avoided the residential areas. This avoidance forced cougars to inhabit a smaller range of territory (Kertson et al. 2013). Therefore, this habitat loss may require cougars to create an environment where members of this species interact and encounter other cougars more often.

Since cougars may encounter similar species in the wild due to habitat loss, and as a result establish a social structure, it is important to understand how numerous individuals in captivity behave when housed together and later generalize these findings to wildlife situations. The purpose of this study is to analyze if a dominance hierarchy changes natural behavior of solitary creatures. When multiple cougars are kept in an enclosure together, how will their behaviors and space usage be influenced by a dominance hierarchy?

Three hypotheses were constructed to determine if a dominance hierarchy influences behavior and spatial usage in captive cougars. (1) To evaluate if the dominance hierarchy influenced one of the cougars to display more active behaviors than the other cougars as means for defining its territory, it was hypothesized the dominant individual would display more active behaviors than subordinate individuals. (2) Since the dominant individual ought to have a larger range of exhibit use of space compared to the subordinate individual, it is hypothesized that having a dominance hierarchy created unequal or limited use of space by the captive cougars. (3) Also, with cougars having many different vocalizations to communicate, it is hypothesized that a dominance hierarchy influenced the dominant individual to vocalize more frequently than the subordinate cougars.

Methods

Study Subjects and Location

The study at Memphis Zoo involves three female cougars: Olympia, Calamity Jane (Jane), and Lakota Sue (Sue). Olympia originally came to the zoo with two of her litter mates from Washington but her siblings are no longer at the zoo (Melanie Lewis, pers. comm). Olympia has neither of her ears cuffed (shortened), a pale-brown coat, and is ten years old (Melanie Lewis, pers. comm). Jane and Sue are both two year-old female litter mates who came from North Dakota (Melanie Lewis, pers. comm). Although they both have one of their ears cuffed, Jane has a darker, broader face than her sister Sue. Therefore, these cougars have distinguishable characteristics for identification during this study.

The cougar exhibit at Memphis Zoo consisted of a dirt and grass floor with rock and wooden beam structures for climbing and resting. Visitors can look down upon the ground-level of the exhibit, but the rock structures allow for cougars to climb higher than eye-level and be above guests. The cougar exhibit is across and adjacent to other animal exhibits, thus permitting indirect interactions between species.

Data Collection

Data collection was split into two types of collection methods. One method involved recording a two-minute interval scan-sampling (Martin and Bateson, 2007) of the behaviors observed from the defined ethogram (Table 1).

Table 1. Cougar Ethogram

Behavior	**Description**
Resting (R)	Cougar is resting on side or belly, but not actively responding to stimuli; make note of asleep or awake and where
Pacing (PA)	Cougar is walking perimeter of exhibit at a constant speed and repetitively
Grooming (G)	Cougar is grooming/licking fur and/or paws
Vocalization (V)	Cougar is making noises to communicate with others; includes: hissing, growling, purring, chuffing, squeaking, screeching
Climbing (CL)	Cougar is climbing trees and/or rocks in exhibit; locomotion without touching ground
Walking (WA)	Cougar is walking around exhibit, on ground
Chasing (CH)	Cougar is chasing another cougar around exhibit
Scratching (S)	Cougar is scratching self
Touching Objects (TO)	Cougar is scratching and/or touching objects
Watching (WT)	Cougar is actively watching another cougar or object; note position of ears (back vs. forward), whether tail is twitching, and where in exhibit
Attacking/Agonistic (AA)	Cougar is pouncing, stalking, pawing, crouching, or aggressively interacting with another cougar or person
Playing (P)	Cougar is non-aggressively playing with, chasing, pouncing on other cougar or object
Stretching (ST)	Cougar is stretching limbs and/ or body
Out of Sight (OOS)	Cougar is out of sight of observer
Other (OTH)	Cougar is displaying behaviors other than ones listed
Interaction with Keeper (IK)	Cougar has immediate change in behavior in presence of Zookeeper; note for how long and which individual

In performing the scan, the location of the three cougars was charted with the gridded maps of the exhibit (Figure 1). In addition, a continuous behavior-sampling (Martin and Bateson, 2007) was conducted in order to record any agonistic behaviors or vocalizations. The duration and frequency of these behaviors were recorded as well. Datum was collected for two hours at a time for two days a week (Mondays and Wednesdays). The total data collection time period spanned over five weeks.

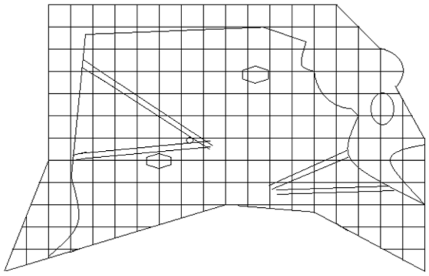

Figure 1. The map of the cougar exhibit at the Memphis Zoo. This map is the total exhibit and not broken into the different levels.

For the purpose of this study, the cougar exhibit at the Memphis Zoo was divided into layers and made into gridded maps with each cell individually numbered. To help determine exhibit use, the exhibit was further divided into three levels (bottom, middle, top). The bottom level was defined as the area in which the cougars are not off the ground. The middle included areas that were at eye level to the observer and any wooden logs off the ground in the exhibit. The top level consisted of the highest-elevated areas accessible to the cougars and included the top pathway that runs L-shaped in the left corner and the top of the highest rock feature. A gridded map was made of each level and each cell was individually numbered. The mapping was performed to denote placement of the cougar in the exhibit and what level the cougar was at.

Analysis

Dominance was defined as the individual who instigated the most aggressive behavior instances towards another individual. Using a Chi-Square test, it was determined if there was a significant difference between the dominant and submissive cougars and the numbers of times they instigated an aggressive behavior. Once a cougar was defined as the dominant individual by the significantly higher instigated behaviors, non-parametric Mann Whitney U tests were performed to account for other variables in the hypotheses. These variables included whether the dominant cougar had significantly higher values for displaying more active behaviors and performed more vocalizations than the other cougars. To determine if was a difference in exhibit use by the individuals, the gridded maps were color-coordinated to calculate the overall difference in percent of time each cougar spent in different grid cells.

Results

It was found that Olympia was dominant over Jane and Sue by interactions involving various vocal and non-vocal behaviors ($\chi^2 = 10.84, p > 0.001$). In total, there were 73 observed agonistic behaviors instigated by Olympia versus the 39 instigated agonistic behaviors seen from Jane and Sue combined. Jane was observed to rest the most (*resting average ± standard error = 0.509 ± 0.074*), Sue the most pacing (*pacing average ± standard error = 0.525 ± 0.105*), and Olympia as the most vocal (*vocalization average ± standard error = 0.3 ± 0.089*) (Figure 2).

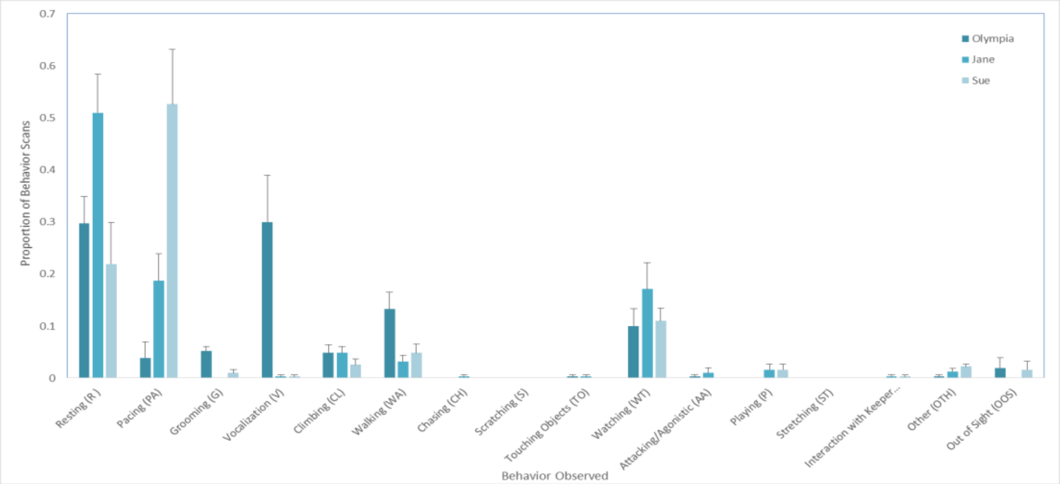

Figure 2. Activity budget of individual cougars and their proportion of behavior scans observed.

Looking in the main categories of behaviors such as resting, active behaviors, and vocalizations, Sue displayed the most active behaviors (*active behavior average* ± *standard error = 0.761* ± *0.113*) and Jane displayed the most resting behaviors (*resting behavior average* ± *standard error = 0.509* ± *0.086*) (Figure 3). There was no significance between any of the broad categories of behavior between cougars (*active behaviors U = 0, N1, N2 = 2, p = 0.66, resting behaviors U = 1, N1, N2 = 2, p = 1.00, vocalization behaviors U = 0, N1, N2 = 2, p = 0.66*) (Figure 3). In regards to stereotypy, the activity budget data revealed Sue to have paced more than half (*pacing average = 0.525*) of the time observed. Sue's high pacing average heavily skewed the data for determining which cougar had the most active behaviors. For exhibit use, the gridded maps showed that Olympia appeared to have the wider area-use for the bottom level despite the fact that Sue was predominantly in the bottom level (*percent time spent in level C = 0.651*) (Figure. 4).

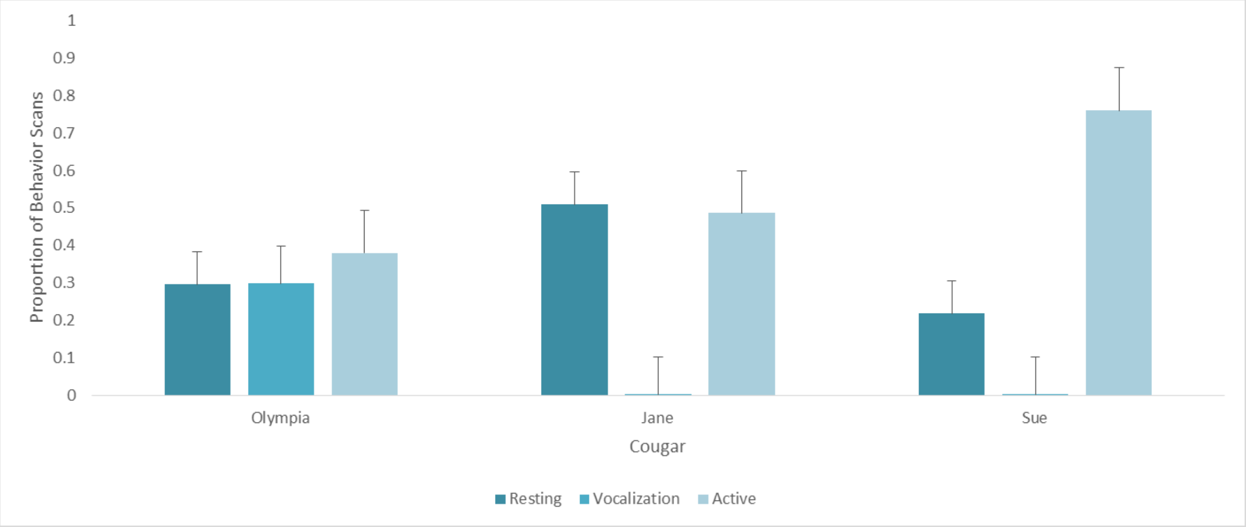

Figure 3. Broad categories of average resting, vocalization, and active behaviors between cougars.

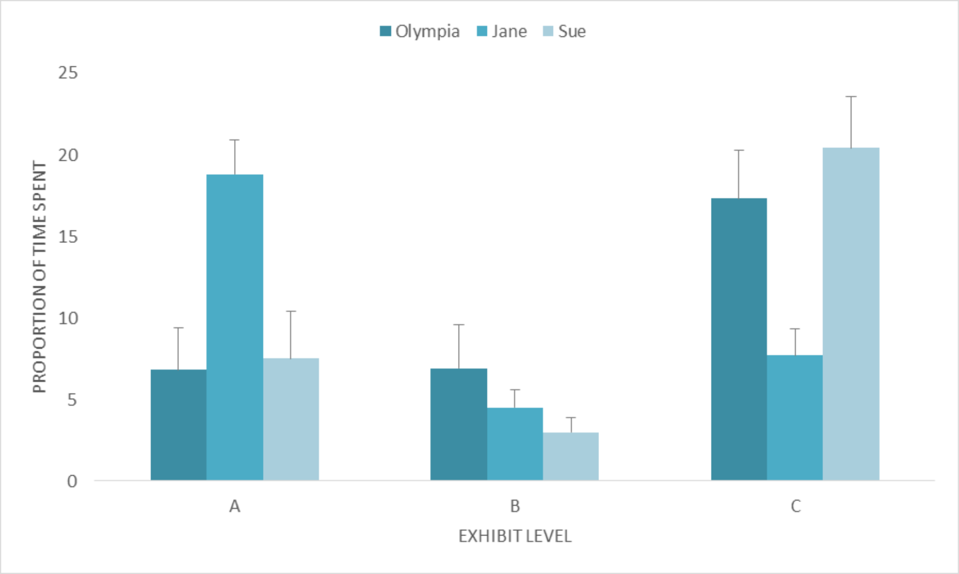

Figure 4. Proportion of time spent on different exhibit level versus individual cougars. As shown, Jane spent most of her time on level A (*proportion of time spent on level A = 18.8).* However, Olympia and Sue both spent the most time on level C (*Olympia proportion of time on C = 17.3, Sue's proportion of time spent on level C = 20.4*).

Jane was observed to occupy the top level more than half the time (*percent time spent in level A = 0.563*). The other maps appeared to show no large differences in exhibit use between cougars (*Olympia's total exhibit usage = 41.176%, Jane's total exhibit usage = 31.372%, Sue's total exhibit usage = 28.431%*) (Figures 5-7).

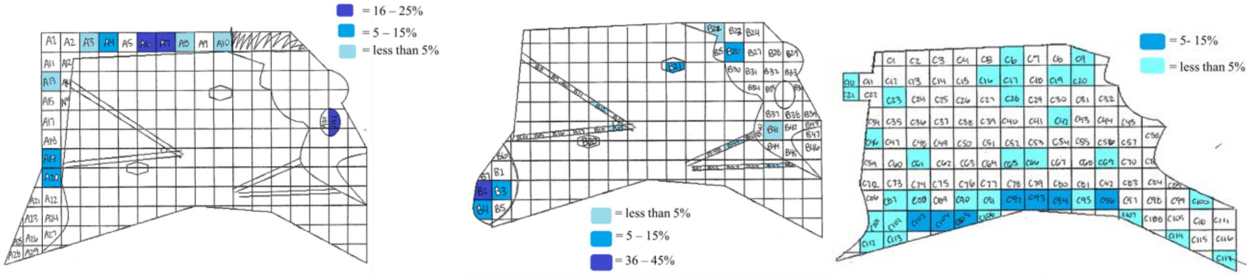

Figure 5. Sue's % exhibit use across the different levels of the exhibit. Different colors represent percent time observed in that grid cell. Total observed exhibit use across all three levels was 28.43% which was lowest of all three cougars.

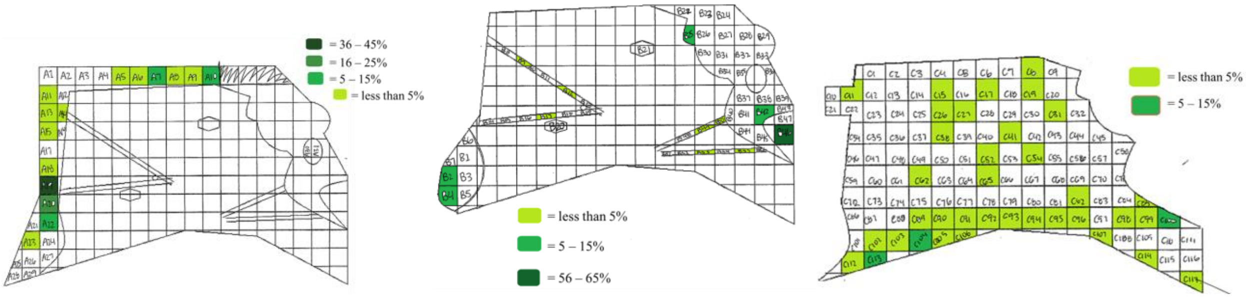

Figure 6. Jane's % exhibit use across the different levels of the exhibit. Different colors represent percent time observed in that grid cell Total observed exhibit use across all three levels was 31. 37%.

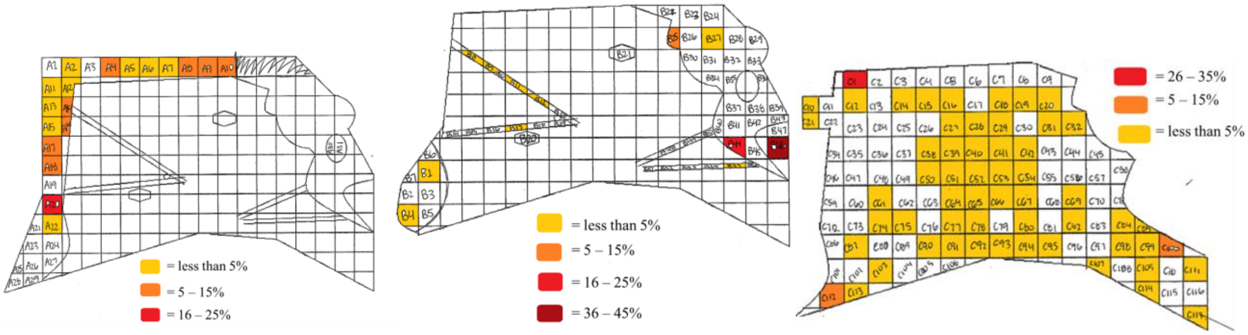

Figure 7. Olympia's % exhibit use across the different levels. Different colors represent percent time spent in that grid cell. Total observed exhibit use across all three levels was 41.17% which was highest of all three cougars.

Discussion

Through data analysis, it was determined that a dominant individual and subordinates existed in the cougar population of Memphis Zoo. Olympia, the dominant cougar had the highest number of instigated agonistic behaviors over Jane and Sue. There was no significance found between active, resting, and vocalizing behaviors in the dominant versus subordinate cougars. This finding demonstrated that the dominance hierarchy has little or no effect on these behaviors. Despite the wider range of areas Olympia was seen to inhabit, there were no major differences between dominant and subordinate use in the exhibit. Although there is an established dominant individual, the cougars' overall behaviors and exhibit usage has no significant difference. These results have no direct support for the initial hypotheses tested; however, they help in determining patterns to be furthered analyzed. As mentioned before, it is important to note Sue's apparent pacing behavior and Jane's resting behavior which was observed in over half of the time spent in the study. These behaviors may be due to the time of day datum was collected or signs of stereotypic behavior in regards to Sue's pacing.

A factor that drastically impacted behavior, vocalizations, and aggression between the individuals was Olympia going into heat during the study period. When Olympia went into heat, it appeared as though she was in closer proximity to Jane, more aggressive to Sue, and vocalizing majority of the study observations. However, before and after measurements of these specific observations were not recorded to draw conclusions on the effect of how a dominant individual going into heat affected a change in behavior frequency. In addition, a sample size of three individuals greatly limited finding significance in the data. Larger sample sizes are needed in further experiments to contribute to determining differences in the mean values of the data.

For further studies, the next step would be to increase the sample size by including different cougar populations from several zoos. By having a diverse population, we could test to see if there is always a dominant individual established and what behaviors are observed across the individuals in the group with regards to proportion of active behaviors, exhibit use, and average vocalizations per individual.

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References

Busch, Robert H. 2004. The cougar almanac: a complete natural history of the mountain lion. Guilford, CT: Lyons Press. 19 p.

Chase ID, Tovey C, Spangler-Martin D, Manfredonia M. 2001. Individual differences versus social dynamics in the formation of animal dominance hierarchies. PNAS 99: 5744-5749.

Harmsen BJ, Foster RJ, Gutierrez SM, Marin SY, Doncaster CP. 2010. Scrape-marking behavior of jaguars (*Panthera onca*) and pumas (*Puma concolor*). Journal of Mammology 91: 1225-1234.

Harmsen BJ, Foster RJ, Silver SC, Ostro LET, Doncaster CP. 2009. Spatial and temporal interactions of sympatric jaguars (*Panthera onca*) and pumas (*Puma concolor*) in a Neotropical forest. Journal of Mammology 90: 612-620.

Kertson BN, Spencer RD, Grue CE. 2013. Demographic influences on cougar residential use and interactions with people in western Washington. Journal of Mammology. 94: 269-281.

Macarrao A, Corbo M, Araujo CB. 2012. Cougar (*Puma concolor*) vocalization and frequency shift as a playback response. Biota Neotrop 12: 134-135.

Martin P, Bateson P. 2007. Measuring behavior: An introductory guide. 3rd edition. Cambridge: Cambridge University Press. 176 p.

Pierce BM, Bliech VC, Wehausen JD, Bowyer RT. 1999. Migratory patterns of mountain lions: implications for social regulation and conservation. Journal of Mammology 80: 986-992.

Vandierendonck MC, Devries H, Schilder MBH. 1995. An analysis of dominance, its behavioural parameters and possible determinants in a herd of Icelandic horses in captivity. Neth J of Zool 45: 362-385.