# RJBS

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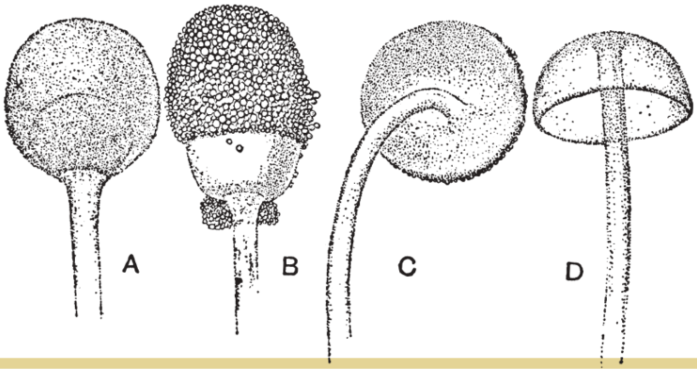
# From the Editorial Team

The Rhodes Journal of Biological Science is a student-edited publication that recognizes the scientific achievements of Rhodes students. Volume XXXIV marks the thirteenth 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 out journal, other students will be encouraged to pursue scientific investigations and research.



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# Activity budget and spatial location of captive Western lowland gorillas (*Gorilla gorilla*)

Amanda Cheang and Gary Wigman

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*Factors which affect the behavior of captive western lowland gorillas (*Gorilla gorilla*) has been a subject of concern. There have been attempts at making the exhibit of captive gorillas more naturalistic, but zoos still differ from the natural environment with regards to space restriction and structures available. This study investigated the effect of exhibit structure type and the gorillas' movement and interaction on the activity budget and spatial location of the four captive western lowland gorillas in the Memphis Zoo. Scan sampling was conducted to record the behavior and locations of the gorillas in the exhibit. Any social interaction, initiation of movement and following, and aggression were recorded using behavioral sampling. All four gorillas spent a larger percentage of scans out in the exhibit than in the indoor enclosure. The most common behavior for the four gorillas was resting. The females differed in the percent of initiation of movement (from 13.9% to 47.2%, not including the male). Two female gorillas were found to have a difference in the frequency of time spent in the open area versus in areas close to walls, structures and trees, spending more time in the latter. When looking at the location used for resting, two gorillas spent a larger percentage of scans resting in areas close to walls, structures and trees than in the open. The other two gorillas had no difference in the location used for resting. The results of this study provide insight into the effect of exhibit structures on the spatial location and activity of captive gorillas in an exhibit. Future research can be done to investigate the specific characteristics of the walls, structures and trees which influence the gorillas' behavior and spatial location in an enclosed space.*

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## Introduction

Western lowland gorillas (*Gorilla gorilla*) are critically endangered and their population size is decreasing (Walsh et al., 2007). Zoos can be a huge resource for ex-situ conservation efforts to increase the gorilla population size through captive breeding for reintroduction into the wild (Conde et al., 2011). It is important to understand the behavior of captive primate animals in zoos for several reasons: animal welfare, the zoo visitor experience, and for behavioral research (Hosey, 2005). The primates' behavior in captivity might shed light on information which cannot be easily observed in the wild (Hosey, 2005). Information known from primates in captivity can be used in conjunction with information known from primates in the wild to evaluate the well-being of the animals (Hosey, 2005).

As much as zoos try to make the exhibits of captive primates naturalistic, zoos still differ from the primates' natural environments in terms of space restrictions and the influx of unfamiliar human visitors (Hosey, 2005). Carder (2008) found evidence for the effect of visitor numbers on the anxiety levels of the gorillas at certain sites, as indicated by the increased scratching and increased visual monitoring of visitors. Captive primates are also being monitored in many aspects such as their diet and health which does not happen in the natural setting (Hosey, 2005). As a result, behaviors not commonly observed in the wild occur in captivity, such as regurgitation and reingestion in captive western lowland gorillas, which might be due to the feeding motivation related to captivity (Lukas, 1999). Captive gorillas' diets are low in fiber and small in size, which may prevent the suppression of hunger, motivating the gorillas to feed more (Lukas, 1999). Therefore, there is a need to study the behaviors of captive gorillas more extensively to see how their behavior in captivity is affected. Using the information from captivity, the captive gorillas can then be compared to wild gorillas, ensuring that the captive animals' needs are being met in zoo environments (Hosey, 2005).

There have been several studies looking at how captive environment can influence the behavior of gorillas. Meder (1992) found that the structure of the exhibit plays a more important role in influencing the behavior of the captive gorillas than the size of the exhibit. Studies have shown that gorillas avoid open spaces (Ross and Lukas, 2005; Stoinski et al., 2002) and prefer building structures over non-building structures (such as rocks and trees) (Stoinski et al., 2002). It is important for captive gorillas to have structures that they can sit near or against, such as walls, trees, rocks and inlets (Meder, 1992). Such structures provide cover, mimicking that which would be found in their natural habitat (Meder, 1992).

Primate social systems are built upon behavioral interactions between individuals (Schaik, 1996). One type of behavioral interaction that exists in gorillas is female to female interaction (Schaik 1996). In the wild, females of a troop of gorillas form hierarchical structures due to competition for food and other resources (Schaik, 1996). Such hierarchies also consider direct familial relation, in which direct relatives are more closely bonded together (Schaik, 1996). Female associations and relationships are linked to ecological factors (Schaik, 1996). Thus, the study of interactions between captive female gorillas can provide insight into the behavior of these captive animals and how they are faring in the captive environment.

We investigated what factors in the exhibit affect the activity budget and spatial location of captive western lowland gorillas. Two hypotheses were tested in this study. We hypothesized that the females in the exhibit impact each other's behavior and spatial location. Thus, we predicted that one female will initiate movements and receive follows more than the other females. We also hypothesized that the exhibit structure affects the activity budget and spatial location of the gorillas. We predicted that the gorillas will spend a larger proportion of time at areas close to walls, structures and trees. We also predicted that gorillas will spend a larger proportion of time resting at areas close to walls, structures and trees than in the open.

In order to effectively provide captive environments which are suitable for the gorillas, it is important to study the behavior of gorillas in captivity and compare to the behaviors in non-captive environments (Hosey, 2005). Understanding the behavior of captive gorillas in their captive environment can highlight if their welfare is taken care of, and also provides suggestions on improving the animal husbandry practices (Hosey, 2005). Once the needs of the gorillas are met, a self-sustaining captive population can be established (Meder, 1992), contributing to successful ex-situ conservation research efforts. Therefore, it is important to study the factors which affect the behavior and spatial location of captive gorillas.

## Methods

### *Study Site and Study Subjects*

The study was conducted on four western lowland gorillas at the Primate Canyon in the Memphis Zoo. The gorillas live in an outdoor exhibit and have access to two indoor enclosures which are out of sight from public view. The exhibit has two double-layered glass viewing windows at the sides of the exhibit (Figure 1). There are three climbing structures in the middle of the exhibit and a stream which provides water for the gorillas (Figure 1). The edges of the exhibit are made up of stone walls. The visitors and the exhibit are separated by a deep moat and a tall wall.

All observations occurred in the outdoor exhibit.

The group of gorillas comprises a silverback male and three females (Table 1). Kebara is the daughter of Penny and is the dominant female (M Peterson, pers. comm). Penny is the only female on birth control (S Shoemaker, pers. comm).

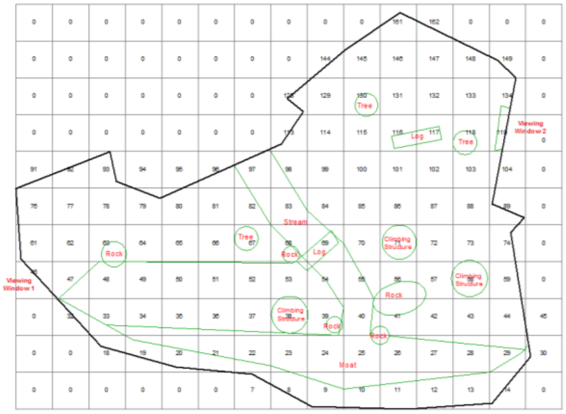

**Figure 1.** The map of the exhibit used when recording the location of the gorillas. The black boundary line represents the boundary of the visitor fencing. Visitors were allowed outside the black boundary line. The grid cells within the exhibit are numbered and they were used in the recording of the gorillas' location. Cells labelled '0' are not within the boundary.

### *Data Collection*

The gorillas were observed for two hours once a week over a period of five weeks. Both scan sampling and behavioral sampling (Martin and Bateson, 2007) were conducted simultaneously. Scan sampling was conducted every two minutes to record the location of each gorilla using Figure 1, and their activity, as listed in the ethogram (Table 2). A map of the exhibit was created using ArcGIS 10.7 using an image from Google Earth (Figure 1). A fishnet was laid on the map and the exhibit was divided into equal grid cells. The grid cells were numbered, and the grid cell number was recorded for the location of the gorillas in the scan sampling. In the case that the gorilla appeared to be in multiple cells, the cell where the gorilla's head was in was recorded. The gorillas were marked as out of site when they went into the indoor enclosure, off-exhibit. The number of visitors was also recorded every two minutes during the scan sampling as the presence of visitors is a confounding variable.

For the behavioral sampling, all subjects were studied, and every social interaction, aggressive behavior and uncommon behavior were recorded. A distinction was made between social interactions and aggressive behaviors (Table 2). Aggression included running, hitting other subjects or objects, pouncing on a subject, throwing objects, and beating of the chest. These actions could also be accompanied by vocalizations. Social interaction referred to any contact or interaction between the gorillas that was not a display of aggression. Any initiating and following of movement, where one individual moved across the exhibit to a new location and was followed by one or more other individuals, was recorded.

### *Data Analyses*

Activity budgets were calculated from the scan sampling data. Behaviors that were used for the scan sampling (Table 2) were reclassified into rest, move, eat, forage and others. Descriptive statistics (mean and standard error) were calculated for every behavior for all the gorillas individually based on the sum of the five weeks of activity budget data. The percentage of scans spent doing each behavior during the study period was then calculated for each gorilla.

The initiation of movement by female gorillas which received one or more follows were calculated from the behavioral sampling data. The number of times a female gorilla moved to a different location in the exhibit and was followed by one or more gorilla was summed up and the frequency total of initiation was used for chi-square analysis to test if the female gorillas differ in the frequency of initiation of movement to a different location.

The location data of the gorillas recorded in the scan sampling data were analyzed to determine the percentage of scans each gorilla spent in open areas as compared to areas close to the walls, structures and trees. The grids along the perimeter of the exhibit as well as grids in which the climbing structures and trees fall in, were counted as areas close to the walls, structures and trees (Figure 1). The structure types were treated as equal. The other grids were considered as open areas. The frequencies of activity budget scans the gorillas spent in the open and at areas close to the walls, structures and trees were used for chi-square analysis.

The data on the resting behaviors and the locations at which the resting behaviors occurred were obtained from the scan sampling data. A calculation was performed to determine the percentage of scans the gorillas spent resting close to walls, structures and trees and in the open. A chi-square analysis was conducted with the frequency data of the location at which the resting behaviors occurred.

## Results

### *Activity Budget*

All four gorillas spent a larger percentage of their scans in the exhibit than out of site in the indoor enclosure (Table 3). Mwelu's combined activity budget was: resting (71.98% ± 6.66), eating (18.51% ± 4.48), other (3.93% ± 2.27), moving (3.57% ± 0.62) and foraging (2.01% ± 1.07) (Figure 2). Kwizera's combined activity budget was: resting (53.56% ± 10.81), eating (24.56% ± 9.82), moving (13.97% ± 2.86), foraging (6.24% ± 1.89) and others (1.67% ± 0.76) (Figure 2). Penny's combined activity budget was: resting (60.15% ± 10.40), eating (20.34% ± 7.22), moving (8.21% ± 2.41), foraging (8.10% ± 3.92) and other (3.20% ± 0.84) (Figure 2). Kebara's combined activity budget was: resting (51.80% ± 4.84), eating (19.07% ± 6.07), foraging (13.06% ± 3.09), moving (12.49% ± 1.57) and other (3.58% ± 2.21) (Figure 2).

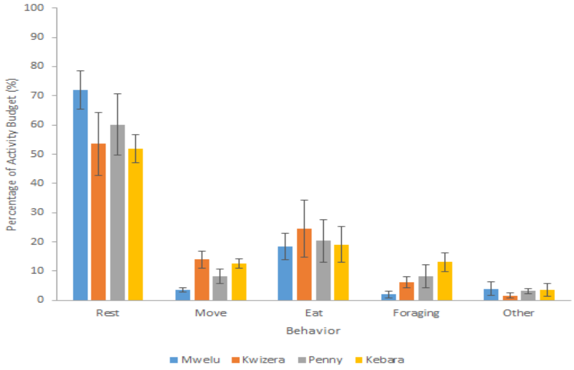

**Figure 2.** Activity budget of the four gorillas (mean ± standard error). All four gorillas spent most of their time resting during the observation period.

### *Initiation of Movement*

There was a total of 36 movements initiated by the female gorillas which received a follow from either one or two other gorillas (Kebara = 47.2%, Kwizera = 38.9%, Penny = 13.9%; Figure 3). There is a difference in the frequency of initiation behavior among the three female gorillas ($X^2_2 = 6.5$, p = 0.0388), between Kebara and Penny ($X^2_1 = 6.545$, p = 0.0105) and between Kwizera and Penny ($X^2_1 = 4.263$, p = 0.039). Kebara and Kwizera initiated more movements which received one or more follows than Penny. There is no difference in the frequency of initiation behavior between Kebara and Kwizera ($X^2_1 = 0.290$, p = 0.5902). Out of the seventeen initiations by Kebara which received a follow, 64.7% were by Kwizera and 35.3% were by Penny ($X^2_1 = 1.471$, p = 0.2252). Out of the fourteen initiations by Kwizera which received a follow, 64.3% of the follows were by Kebara and 35.7% were by Penny ($X^2_1 = 1.143$, p = 0.285). Out of the five initiations by Penny which received a follow, 80% of the follows were by Kebara and 20% were by Mwelu ($X^2_1 = 1.8$, p = 0.1797). Several initiations incurred a chain of response (multiple follows by several gorillas), but the results above show only the first individual which followed the initiator.

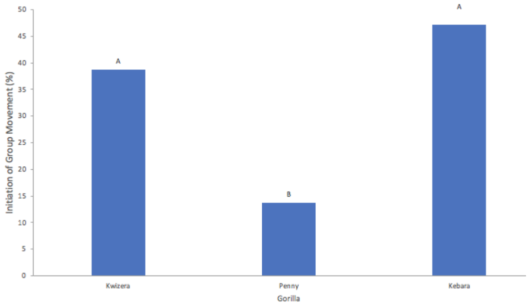

**Figure 3**. Initiation of movement by female gorillas which received one or more follows from the other gorillas. Kebara initiated movement and received follows 17 times; Kwizera initiated movement and received follows 14 times; Penny initiated movement and received follows 5 times. There is a difference in initiation between the three females, between Kwizera and Penny, and Kebara and Penny, as indicated by the letters above the bars.

### *Exhibit Structure*

For the scans which did not include out of site data, Mwelu spent 50.19% of scans in open areas and 49.81% of scans in areas close to walls, trees and structures. Kwizera spent 19.66% of scans in open areas and 80.34% of scans in areas close to walls, trees and structures. Penny spent 48.83% of scans in open areas and 51.17% of scans in areas close to walls, trees and structures. Kebara spent 38.36% of scans in open areas and 61.64% of scans in areas close to walls, trees and structures. Exhibit structure impacted the spatial location of two of the four gorillas observed. Kwizera ($X^2_1 = 65.53$, $p < 0.001$) and Kebara ($X^2_1 = 8.61$, $p = 0.0033$) spent a greater percentage of scans in proximity to walls, trees, and structures (Figure 4). Mwelu ($X^2_1 = 0.0038$, $p = 0.95$) and Penny ($X^2_1 = 0.016$, $p = 0.9006$) had no difference in the percentage of scans spent in the open and at areas close to trees, walls and structures. All three females climbed up the structure several times throughout the study. Mwelu stayed low on the ground the whole time and was not observed climbing up structures.

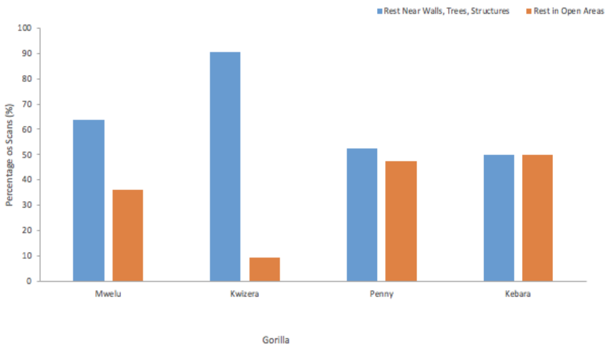

**Figure 4.** Exhibit use of each gorilla, comparing percentage of scans spent in close proximity to walls, trees and structures to time spent in open areas. Kwizera and Kebara were observed have a difference in the exhibit use location, spending more time in close proximity to walls, trees and structures than in open areas, displaying a 60.68% and a 23.28% difference respectively.

All four gorillas spent the largest percentage of their activity budget scans resting (Figure 2). For Mwelu ($X^2_1 = 14.23$, $p = 0.0002$) and Kwizera ($X^2_1 = 62.41$, $p < 0.001$), there was a difference in the location used for resting (in the open or in areas in close proximity to walls, structures and trees). Both Mwelu and Kwizera spent a greater percentage of their resting scans in close proximity to walls, trees, and structures (Figure 5). Penny ($X^2_1 = 0.41$, $p = 0.52$) and Kebara ($X^2_1 = 0.00$, $p = 1$) displayed no difference.

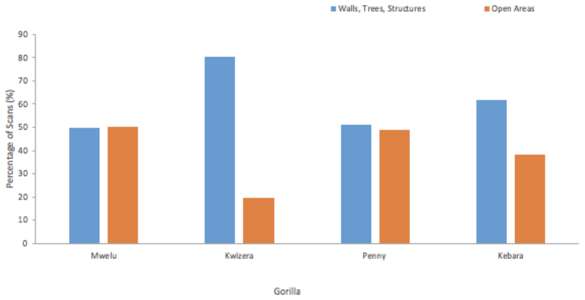

**Figure 5.** Resting location of each gorilla comparing percentage of scans spent resting near walls, trees, and structures versus resting in open. Mwelu and Kwizera rested a greater percentage of time near walls, trees, and structures, displaying a 27.36% and 81.06% difference respectively. Penny and Kebara displayed no discernible difference.

*Aggression and Other Behaviors*

Seven instances of aggression were observed throughout the study period: Mwelu (42.9%), Penny (42.9%) and Kebara (14.3%). Mwelu displayed three instances of aggression during the keeper chat and feeding time, of which two were directed towards Kebara when she reached out for food. The third instance was general aggression not directed at any individual when cabbage given by the zookeeper fell into the moat.

Penny displayed aggression thrice throughout the study period. She ran towards Kwizera and pounded on her chest once. She vocalized at Kebara once when Kebara was reaching out for food during the keeper chat and feeding time. Penny also pounced at the viewing window 1 (Figure 1) once during the last week of observation when the observer turned her back around.

Kebara displayed aggression once throughout the study period by beating her chest. It did not appear to be directed at any individual. Kwizera did not display any aggressive behavior at all throughout the study. There was no difference between the aggressive behavior displayed by the four gorillas ($X^2_3 = 3.827$, p = 0.2773).

## Discussion

The results showed support for the hypothesis that females in the exhibit impact each other's behavior and spatial location, as there was a difference in the frequency of initiation behavior among the three female gorillas. There is a difference in the frequency of initiation behavior between Kebara and Penny, as well as Kwizera and Penny, indicating that Kebara and Kwizera both initiate movements which received follows more than Penny. Direct relatives are more closely bonded together (Schaik, 1996), but there was no difference in Kebara and Penny's followers. Penny, Kebara's mother, was not observed to be following Kebara more than Kwizera. Though there was no difference calculated in Penny's followers, it was noted that only Kebara and Mwelu were observed to follow Penny. Kwizera was not observed to follow Penny at all. Female gorillas tend to form hierarchy (Schaik, 1996); the results could potentially provide some information on the female gorillas' hierarchy. Further studies can be done to investigate the hierarchical relationship among the female gorillas.

The results showed partial support for the hypothesis that exhibit structure affects the activity and spatial location of the gorillas. Two gorillas, Kwizera and Kebara, were found to have a difference in the amount of time they spent in the open as compared to areas close to walls, structures and trees, spending more time in close proximity to walls, structures and trees. In addition, two gorillas, Mwelu and Kwizera were found to rest more in areas that were close to walls, structures, and trees than areas in the open. These findings are in congruence with what Meder (1992) found expressing that use of walls, trees and structures as a preferred area to rest and feel secure. There could be other factors influencing the gorillas' location in the exhibit and not just the exhibit structures, therefore the other two gorillas did not have a difference. This study potentially builds upon what Stoinski et al. (2002) found in their study. They found that gorillas prefer building structures over non-building structures such as rocks and trees (Stoinski et al., 2002). In this study, there were no building structures available for the gorillas. There were just non-building structures (walls, trees, structures) and areas in the open, and half of the gorillas were shown to prefer the former. Therefore, there could potentially be a hierarchy of location preference of captive gorillas in captive environment, or it could be individual variation, which can be explored more in future studies.

Aggression was recorded in this study as Stokes (2004) found that agonistic behavior by male gorillas directed at female gorillas is fairly common. Stokes (2004) found that when aggression is displayed by a male silverback gorilla in the wild, females almost always respond by retreating and were never observed to show agonistic behavior back to the male aggressor. Few aggressive behaviors were noted in this study. The only agonistic behavior by Mwelu directed at the females – specifically Kebara – were during the keeper's chat and feeding session, over food. Future studies can be conducted to look further into the situations which male gorillas' direct agonistic behavior toward female gorillas.

Although the two hypotheses were not fully supported, our study still provided insights into learning more about the behavior of captive western lowland gorillas. In contrary to what Meder (1992) found in her study, the captive gorillas in this study spent more time in the outdoor exhibit area visible to the public than in the indoor enclosure out of the public's view. More studies can be done to investigate the potential contributing factors to this variation in results. Similar to what Ross and Lukas (2005) found in their study, the male gorilla stayed on the ground the entire study period and was not observed to climb up any structures. It was the females who were mostly up on the structures. It could be that the females were spacing themselves out around the male (Ross and Lukas, 2005), as it appeared that the females were often up on the structures at the same time that Mwelu was on a rock in the middle at the front of the exhibit (the structures were around the rock). However, there could be other confounding variables at play, such as the keeper's feeding sessions. The keepers often stood at the front of the exhibit in the middle and threw food in. The gorillas would often eat in the same area in the middle of the exhibit where the structures are. Meder (1992) found that staying above ground is a way of withdrawal for captive gorillas. Future studies can investigate and compare several zoos to see the functionality of structures which allow above-ground access for the gorillas as well as study the behaviors of the gorillas that stay above ground. Cross-comparison studies can be done to investigate the relationship between the behavior and spatial location of gorillas within the exhibit.

There are several limitations to this study. The sample size was small, and the duration of the study was short. A small sample size could be contributing to the large p-values. Also, only 10 hours of data was collected over a period of five weeks, therefore the data collected might not have been sufficient to detect trends or patterns in behaviors of the gorillas. Future studies could also potentially take this study one step further to quantify the actual proximity in distance the gorillas are to structures. More long-term studies can also be done to see if the weather, seasonality, tree cover and sunlight exposure affects the spatial location and behavior of the gorillas in the exhibit.

## Conclusions

1. Exhibit structure impacted the spatial location of half of the study subjects. Exhibit structure may have significance on the behavior and spatial location of captive western lowland gorillas and should be taken into careful consideration when these exhibits are designed or refurbished.
2. There was a difference amongst the female gorillas in the frequency of initiation of movement to another location which received follows. The mother and daughter pair were not observed to follow each other more often than the unrelated female.

**Acknowledgements**

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**Table 1.** Information of the gorillas - their name, sex, date of birth, relatedness to each other and the date which they arrived at the Memphis Zoo (S Shoemaker, pers. comm).

| Gorilla | Sex | Date of Birth | Relatedness | Arrival at Memphis Zoo |
|---|---|---|---|---|
| Mwelu | Male | July 16, 1986 | Unrelated | November 15, 2007 |
| Kwizera | Female | March 11, 1988 | Unrelated | December 11, 2009 |
| Penny | Female | May 3, 1989 | Mother of Kebara | May 9, 2008 |
| Kebara | Female | January 18, 1997 | Daughter of Penny | May 9, 2008 |

**Table 2.** The ethogram used in the study to record the behaviors of the western lowland gorillas.

| Activity | Code | Definition |
|---|---|---|
| Eating | EA | Placing solid food in the mouth or the action of chewing. Can be done sitting or standing. |
| Foraging | FO | Searching for food. Can occur simultaneously while eating and/or walking. |
| Locomotion | LM | Movement on the ground at a relaxed pace. |
| Resting Sitting | RS | Sitting in one location. |
| Resting Lying | RL | Lying down in one location. |
| Resting Standing | RST | Standing stationary at a location on the limbs. |
| Regurgitation and Reingestion | RR | Expulsion of material from the mouth and consumption of the expelled material. |
| Climbing | CL | Vertical movement along structures. |
| Vocalization | VO | Sound production. |
| Touching Objects | TO | Contact with any object which can occur while sitting, lying or walking. |
| Urinating/Defecating | UD | Expulsion of waste material. |
| Aggression | AG | Includes running, hitting other subjects or objects, pouncing on subject, throwing objects, beating of the chest. These actions can be accompanied by vocalizations. |
| Drinking | DR | Consumption of water. |
| Interpersonal Contact | IC | Any non-aggressive contact between the gorillas. |
| Copulation | CO | Attempted mounting on another subject. |
| Retreat | RT | Backward movement from a subject. |
| Out of sight | OOS | Not visible in the exhibit. |
| Others | OT | Any behavior which does not fall under any behaviors in the list. |

**Table 3.** The percentage of scans each gorilla spent in the exhibit and out of site.

| Gorilla | Percentage of scans in the exhibit (%) | Percentage of scans out of site (%) |
|---|---|---|
| Mwelu | 84.84 | 15.16 |
| Kwizera | 57.42 | 42.58 |
| Penny | 82.58 | 17.42 |
| Kebara | 51.29 | 48.71 |

# The impact of environmental temperature and body size on the behavior of captive Nile crocodiles (*Crocodylus niloticus*)

Samuel Cloyd and Ryan Palermo

*In ectothermic animal species, environmental temperature plays a role in driving specific patterns of heat-seeking and heat-avoiding behaviors. With an increase in average global temperatures, it is important to understand how susceptible species will respond to the changing environment. Ectothermic behaviors have been linked to specific body temperatures and body size. In our study of captive Nile crocodiles (*Crocodylus niloticus*) at the Memphis Zoo, we identified and measured the effects of environmental temperature on patterns of behavioral thermoregulation. The types of behavior analyzed include energy-demanding behaviors, basking behaviors, and substrate preference. Additionally, we measured the effects of body size on basking behavior by comparing daily behavioral patterns across individuals. Results suggest that there is not a link between temperature and basking behavior, energy-demanding behavior, or substrate preference, as there were significant variations in the individual activity budgets of our study subjects. However, we found that individual body size was negatively correlated with time spent basking. The minimal variation in body size among our study subjects and a relatively small sample size may have yielded results that are not reflected in the general population, so future work on these hypotheses could give further insight into this species. With the results of this study and information learned from future studies, more generalizable claims can be made regarding how this species and related ectotherms might respond to climate change.*

## Introduction

Ectothermic species rely on external sources of heat to regulate their internal body temperatures, and these animals often achieve thermal homeostasis by engaging in characteristic behavioral patterns (Bogert, 1949). Although ectotherm body temperatures tend to fall within species-specific ranges, the body temperature of estuarine crocodiles (*Crocodylus porosus*) has been observed to vary over the course of each day in response to patterns of behavioral thermoregulation, such as heat-seeking and heat-avoidance (Glanville and Seebacher, 2006). These daily variations in body temperature are accompanied by compensatory physiological changes, including altered metabolic rates, which allow for optimized behavioral performance (Glanville and Seebacher, 2006). Taken together, these data suggest that ectotherms utilize a host of behavioral and physiological adaptations to maintain optimal body temperatures (Bogert, 1949; Glanville and Seebacher, 2006).

Members of the class Reptilia are considered ectothermic, including the Nile crocodile (*Crocodylus niloticus*) (Bogert, 1949). These crocodiles inhabit freshwater wetlands along the southern part of the African Nile River and Madagascar (Mazzotti and Dunson, 1989). For freshwater crocodiles, thermoregulation is most commonly mediated via behavioral mechanisms, such as moving between land and water (Seebacher and Grigg, 1997). Crocodilian body temperature has been shown to exhibit regular temporal patterns, with body temperature rising in the morning until it reaches a certain range, which is maintained behaviorally throughout the day, followed by a decrease to eventual equilibrium with water temperatures during the night (Seebacher and Grigg, 1997). Downs et al. (2008) reported that the average body temperature of a Nile crocodile ranges from a daily minimum of 18.8-19.6°C to a daily maximum of 26.929.2°C. Furthermore, Downs et al. (2008) found that basking coincided with rapid increases in body temperature, suggesting the ability of Nile crocodiles to exercise behavioral control over body temperature.

In this study, we seek to identify which behaviors are impacted by environmental temperature and body weight in captive Nile crocodiles. Prior research has helped define certain thermoregulatory behaviors common to ectotherms, including basking [exposing the body to direct sunlight] and gaping [resting with the mouth ajar] (Bogert, 1949; Grigg et al., 1998). Smith (1979) investigated the effects of water and air temperature on patterns of thermoregulation in the American alligator (*Alligator mississippiensis*). When air temperature exceeded the water temperature, alligators were more likely to engage in basking behaviors to increase their body temperature as environmental temperature cooled (Smith, 1979). A similar study by Downs et al. (2008) investigated the effects of environmental factors on the thermoregulatory behaviors of Nile crocodiles inhabiting St. Lucia Lake in southern Africa. Among the factors that were identified, environmental temperature was observed to affect the frequency at which thermoregulatory behaviors were performed (Downs et al., 2008). Therefore, we hypothesize that ambient environmental temperature alters the thermoregulatory behavior of Nile crocodiles. We predict that a decrease in environmental temperature will correlate to increased basking behavior in the activity budgets of our study subjects.

In addition to thermoregulatory behaviors, we are interested in assessing how temperature affects crocodile engagement in energy-demanding behaviors. In a study correlating aggressive crocodile behavior to seasonality, Pooley et al. (2019) found that crocodiles were most likely to attack during seasons when the average temperature was higher. Therefore, we hypothesize that ambient environmental temperature alters Nile crocodile engagement in energy-demanding activities. We predict that a decrease in environmental temperature will correlate to decreased engagement in energy-demanding activities.

Finally, this study explores how body weight influences thermoregulatory behaviors in Nile crocodile activity budgets. Seebacher et al. (1998) investigated the relationship between body weight and thermoregulation in saltwater crocodiles (*Crocodylus porosus*) and found that fluctuations in body temperature became more stable as body weight increased. Furthermore, Seebacher et al. (1998) reported that larger crocodiles had higher average body temperatures. A study of the same species by Grigg et al. (1998) corroborated the finding that increased body mass is correlated to higher body temperatures. Grigg et al. (1998) determined that large saltwater crocodiles spent less time basking than small crocodiles. Therefore, we hypothesize that body weight has an effect on the thermoregulatory behavior of Nile crocodiles. We predict that an increase in body mass will be associated with a decrease in time devoted to basking.

The findings of this study will likely be of particular relevance to Nile crocodile conservation. In a study of the aquatic ecosystem in South Africa’s Olifants River, Ashton (2010) reported that the Nile crocodile population was in a continual state of decline, which will likely be exacerbated by the adverse effects of climate change. Within the present century, it is predicted that the southern African region will experience increasingly severe droughts and significant loss of water supply to local rivers due to projected temperature increases and decreased rainfall (De Wit and Stankiewicz, 2006).

**Table 1.** Ethogram for the Nile Crocodile (*Crocodylus niloticus*)

| Behavior | Description |
|---|---|
| *Thermoregulatory behaviors* | |
| Fully submerged resting | Remaining motionless with the entire body submerged underwater and not exposed to direct sunlight |
| Top water resting | Remaining motionless, near/on the top surface of the water, with a minority of the dorsal side above water level |
| Land basking | Lying motionless, completely out of the water, while exposed to direct sunlight |
| Land basking - gaping | Land basking with mouth slightly open and jaws in a fixed position |
| Water basking | Lying motionless while only partially submerged in the water. The majority of the dorsal side is above water and exposed to direct sunlight |
| Water basking - gaping | Water basking with mouth slightly open and jaws in a fixed position |
| *Energy-demanding behaviors* | |
| Aggression in water | Striking or biting another crocodile within the water |
| Aggression on land | Striking or biting another crocodile on land |
| Feeding | Consuming food items |
| Locomotion in water | Walking or crawling along the bottom of the pool |
| Locomotion on land | Walking or crawling on land |
| Swimming | Actively moving through the water without the contact of feet on the bottom of the pool |

**Figure 1.** Satellite image of the Nile crocodile enclosure at the Memphis Zoo (Google Earth, 2019). Central grassy island surrounded by a pool of water and a sandpit along the perimeter.

Given this projected rise in environmental temperature within regions inhabited by Nile crocodiles, climate change is likely to have far-reaching impacts on the conservation status of the species. It is our hope that our study will provide greater insight into the possible implications of climate change on Nile crocodile behavior and thermoregulation.

## Methods

### *Study subjects and location*

The subjects of this study consisted of one male and three female adult Nile crocodiles, all of whom were estimated to be approximately 35 years of age (S. Reichling and D. Dembiec, pers. comm.). Although the exact body weights of the four subjects were unknown, each crocodile was classified by relative size. The male subject, Skabenga, was the largest of the group (S. Reichling and D. Dembiec, pers. comm.). Two of the females, Makhula and Squaba, were visually similar in body size and were deemed medium-sized, relative to the group (S. Reichling and D. Dembiec, pers. comm.). The smallest of the four subjects was a female named N'Tombi (S. Reichling and D.Dembiec, pers. comm.). Study subjects resided in captivity at the Memphis Zoo, located in Memphis, TN. The four crocodiles are on rotation within the exhibit and, on any given day, there were anywhere from one to three subjects present and available for observation. Within the Nile crocodile exhibit, animals could move freely between four primary substrates: rock, sand, water of varying depths, and grassy land with foliage providing areas of partial shade (**Figure 1**).

### *Data collection*

Specific behaviors were defined within two broad categories: energy-demanding and thermoregulatory behaviors (**Table 1**). In order to maximize the data obtained from behavioral observation, two researchers performed continuous focal animal sampling (Martin and Bateson, 2007). Each researcher monitored the behavior of one to two study subjects, recording the type and duration of every behavior that occurred during the two-hour observation period. Data were collected every Wednesday from 1:30 to 3:30 pm for five consecutive weeks. Additionally, daily environmental temperatures were recorded at the start of each observation period. Outdoor temperature data were obtained from the National Weather Service website (forecast.weather.gov), according to the current temperature reports for Memphis, TN.

It is important to note that our sample size was relatively small for generalizing results to the total population of Nile crocodiles. However, by collecting data via continuous focal animal sampling, all behaviors and changes to behaviors were accounted for in each individual. This form of sampling reduced the likelihood that any trends would be found by chance.

### *Data analysis*

To construct each animal's activity budget, the total amount of time devoted to a specific behavior was divided by the total amount of time that the animal was observed. This calculation yielded the daily proportion of time devoted to each type of behavior.

Descriptive statistics, including averages and standard error, were calculated for each individual based on daily activity budgets.

Due to the small sample size of four individuals, non-parametric statistics tests were utilized for data analysis. For all comparisons, we used Spearman's Correlation tests to determine whether relationships existed between variables.

Using data from each individual crocodile, the first set of variables that we tested was daily temperature versus proportion of time spent basking. We also compared daily temperature to the proportion of time devoted to energy demanding behaviors. Furthermore, we used a Spearman's Correlation test to determine whether daily temperature was related to the proportion of time that individuals spent in the water. A final Spearman's Correlation test was used to discern whether body size was related to average time spent basking. The data for relative body size were operationalized as follows: the one large male was assigned a rank of 3, the two medium females were assigned a rank of 2, and the smallest female of the group was assigned a rank of 1.

## **Results**

During the observational study, environmental temperature ranged from 16°C to 34°C over the course of five days of observation. Skabenga, the male crocodile, was present every day of data collection. However, the three female crocodiles rotated shifts on exhibit, each present on only two out of the five days. Therefore, to reduce the likelihood that the results would be biased toward the activities of the male, the data set from Skabenga was used in isolation. We observed variations in basking and energy-demanding behavior within individual crocodiles over the entirety of the study (**Figure 2** and **Figure 3**).

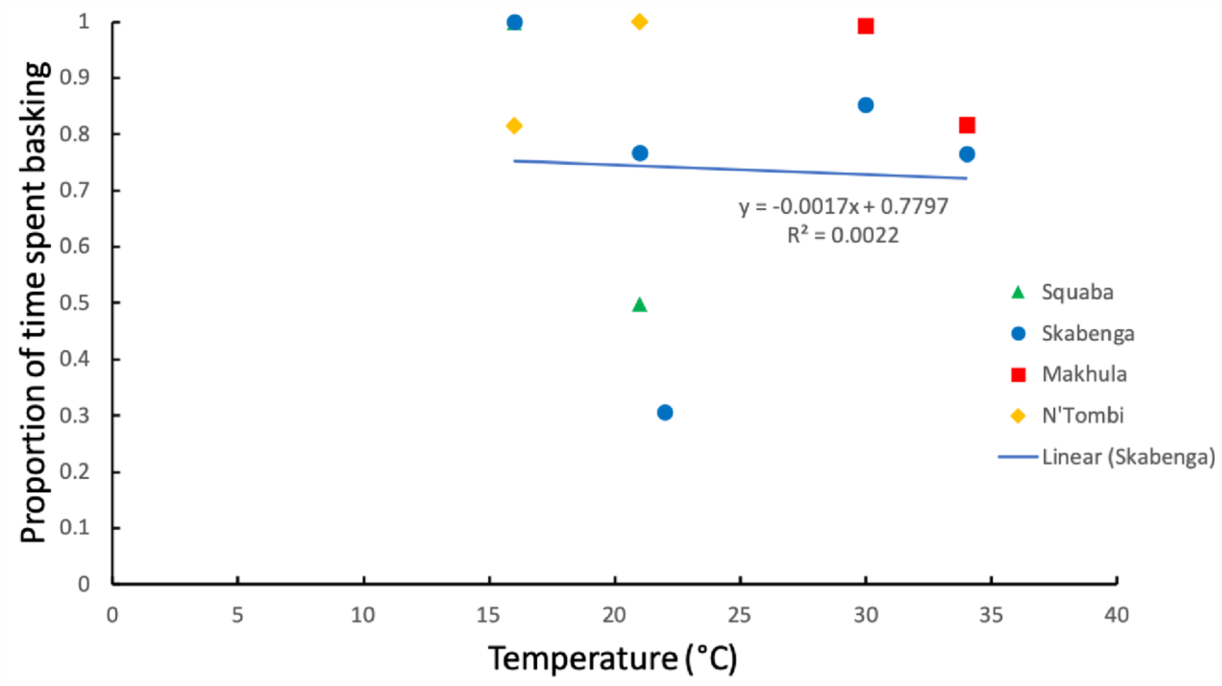

**Figure 2**. The effects of temperature on the proportion of time spent basking for each subject. A trendline was added based on the data for the male crocodile, Skabenga.

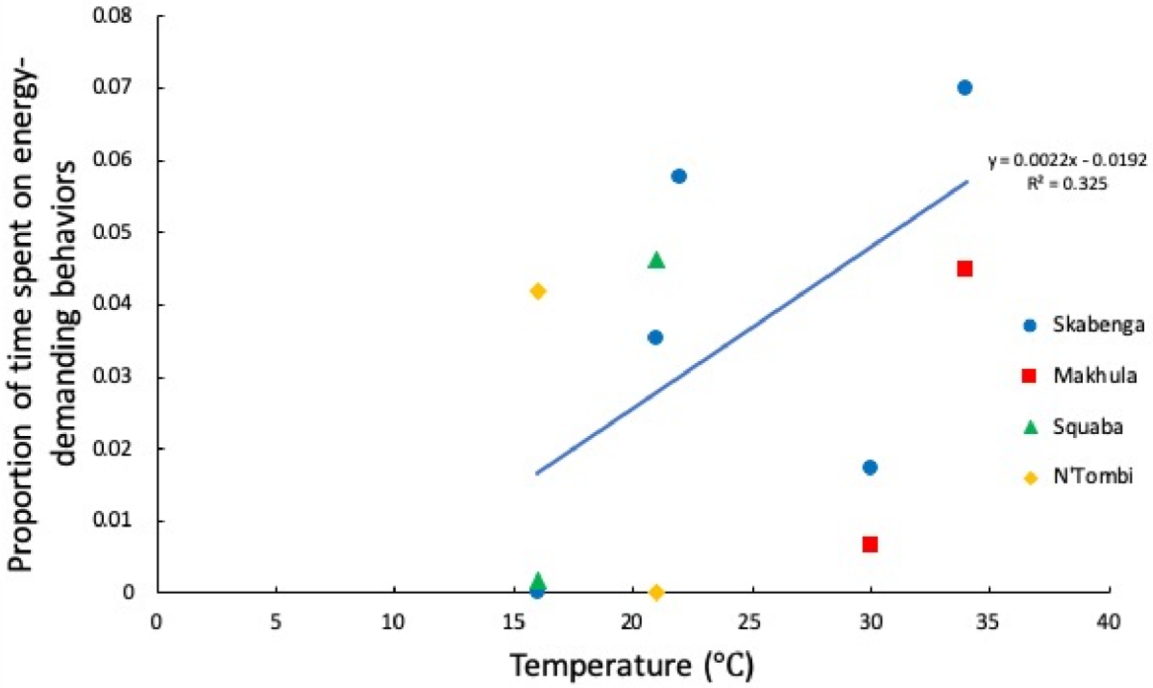

**Figure 3**. The effects of temperature on the proportion of time spent performing energy demanding behaviors. A trendline was added for the data on the male crocodile, Skabenga.

The proportion of time spent basking was 0.7384 ± 0.1160 for Skabenga, 0.9052 ±0.088for Makhula, 0.7481± 0.2503 for Squaba, and 0.9072 ± 0.0928 for N'Tombi (mean ± standard error). The proportion of time devoted to energy demanding behaviors was 0.0353 ± 0.0123 for Skabenga, 0.045 ± 0.0192 for Makhula, 0.0463 ± 0.0223 for Squaba, and 0.0 ± 0.0210 for N'Tombi (mean ± standard error). The proportion of time spent in water was 0.7386 ± 0.1940 for Skabenga, 0.5034 ± 0.4967 for Makhula, 0.5 ± 0.5 for Squaba, and 0.3813 ± 0.3813 for N'Tombi (mean ± standard error).

As noted earlier, all Spearman's Correlation tests were performed using only the data from the male subject due to sample size. For the male, environmental temperature and basking behavior were not correlated ($r_s$ = -0.500, n = 5, p = 0.391). Environmental temperature was not related to time devoted to energy-demanding behaviors ($r_s$ = 0.700, n = 5, p = 0.188).

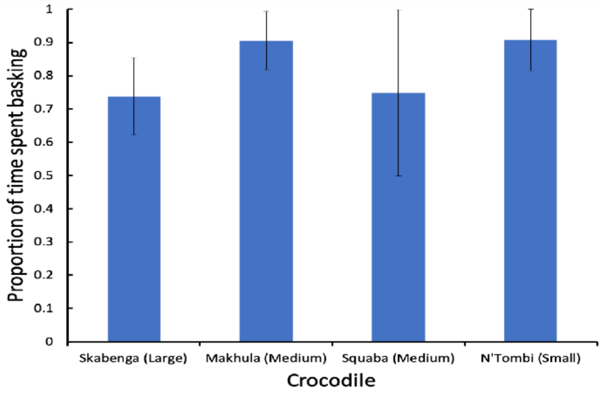

**Figure 4**. Proportion of time spent basking (mean ± standard error) for the crocodiles at the Memphis Zoo. Individuals are ranked based on relative body size: large, medium, and small.

Furthermore, there was no correlation between temperature and substrate preference for the male subject ($r_s$ = 0.671, n = 5, p = 0.215). Although we did not find a correlation between temperature and substrate preference, it is important to note that Skabenga spent all of his time in the water during the two hottest days and all of his time on land during the coldest day recorded. Unlike the prior tests, when all crocodiles were considered, we found that body size and basking behavior were negatively correlated, as larger crocodiles spent less time basking ($r_s$ = -0.949, n = 4, p = 0.051) (**Figure 4**).

## Discussion

The results of this study did not support our hypothesis that environmental temperature alters thermoregulatory behavior nor the hypothesis that temperature alters energy-demanding behavior. Contrary to our predictions, differences in daily temperature were not correlated with changes in the activity budgets of our study subjects. Our observations of the male crocodile at the Memphis Zoo differed from a previous study conducted by Smith (1979), which related changes in air temperature to altered frequencies of thermoregulatory behaviors in Nile crocodiles. These differences could suggest that crocodiles have different behavioral patterns in captivity than in the wild, that activity budgets of individual crocodiles vary greatly over time, or that behavioral patterns are not affected by environmental temperature. The latter suggestion would differ from other observational studies that suggest crocodile attacks or other forms of energy-demanding activities are linked to seasonal temperature (Pooley et al., 2019). It is more likely that one of the former suggestions are true, and further research could give insight into those potential claims. To test whether captive crocodiles differ from wild crocodiles, further studies could include multiple observation sites in the wild and in captivity to increase sample size. To test whether the activity budgets of individual crocodiles vary enough to cause differing results, subsequent research should focus on collecting ample amounts of data from a larger sample of crocodiles.

The results from our study supported our hypothesis that body weight has an effect on thermoregulatory behavior. Our observations matched the prediction that body weight and proportion of time spent basking would be negatively correlated. Our data corroborate results from other studies, suggesting that an increase in body mass is correlated to less time devoted to basking (Grigg et al. 1998). Even though we did not have enough information on the study subjects to determine if the body size was correlated to basking behavior as suggested by the prior research, the large male studied, Skabenga, spent less time basking than the medium-sized females, who also basked less than the smallest female (Seebacher et al. 1998). Our results suggest that, even though body size and basking behavior are negatively correlated, further research could be done using similar methods with a larger sample size and inputting exact weights.

Because we could not obtain the exact body mass of each crocodile, we relied on relative numerical rakings that could have been inherently flawed because of their lack of specificity. By using precise body weight data and a larger sample size, future research could have the potential to produce stronger correlational claims. Since our data showed a negative correlation between body size and basking activity, further study could be undertaken to observe the effects of climate change on body size. If we could identify a trend between average global temperature and average body size of Nile crocodiles, we would be able to predict the population's average proportion of time spent basking. This trend would allow future conservation efforts to focus on maintaining the species by sustaining particular aspects of their habitats. For example, if the effects of climate change are selecting for smaller individuals, the correlation found in this study would suggest that it would be more productive to preserve habitats where basking is likely to occur.

## Conclusions

1. Differences in environmental temperature were not correlated with any specific changes in the amount of time that Nile crocodiles devoted to basking behavior.
2. Changes in environmental temperature did not have an effect on the prevalence of energy-demanding behaviors within the daily activity budgets of our study subjects.
3. Body size was found to be correlated with basking behavior, as smaller crocodiles spent more time basking than larger individuals.

## Acknowledgments

We would like to thank the Memphis Zoo and its staff for the use of their facilities, as well as Dr. Boyle and the Rhodes College Biology Department for their sponsorship of this study. Additionally, we want to thank Dr. Steve Reichling, Director of Conservation and Research for the Memphis Zoo, and Mr. Dan Dembiec, Zoo Curator, for providing background information on the study subjects.

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# Plastic Marsh (The Tenacity): A look back at the varying anthropogenic impacts on estuary systems through microplastic pollution and stable isotopes.

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*As plastic pollution increases, huge amounts of microplastic particles (<5mm diameter) are found in aquatic systems worldwide, posing threats to the biota that inhabit them. Estuarine ecosystems receive plastics from various sources resulting from the variety of human activities that occur in their surrounding land. Little is known about the distribution and abundance of microplastics in these systems. For this study, three estuaries of varying levels of human development in Waquoit Bay were selected. In order to look at the varying anthropogenic impacts on each of these systems, elemental analysis (organic carbon and nitrogen), stable isotopes ($\delta^{13}C$ and $\delta^{15}N$), and abundance of microplastics were examined through time (~30 years) in sediment cores. Results indicate that the distribution and abundance of microplastics are coupled with the intensification of land uses and population density. We observed that plastic pollution occurs in environments ranging from almost pristine natural areas to highly populated estuaries. At all three locations, there was also a higher abundance of fibers found in the sediments in comparison to fragments. This study is the first to survey microplastic contamination in Cape Cod estuaries and to provide insights on the nature and extent of contamination in these habitats.*

## Introduction

Plastic is ubiquitous; it's what you use to wrap your sandwich, it's the material of your phone case is made of, and its scattered along our shorelines. Although the invention of plastic has benefitted our society greatly because of its versatility and desirable properties including durability, light weight and multipurpose use, it has also created a huge problem (Browne 2007). The plastics that have been created can never be fully broken down, they just keep getting smaller. That's the issue we are facing today. Microplastic fragments and fibers of 0.5mm or less get introduced the environment at this size through cosmetic products and clothing. However, it is not the only way the accumulation of these morsels happens, the large macro plastics can also eventually break down into smaller fragments and fibers (Arthur et. al 2009). Microplastics have been observed in the bottom sediments of marine and freshwater environments (Free et. al 2014). Sediments are suggested to be a long-term sink for microplastics. These small unnatural pieces are highly bioavailable to organisms through both direct consumption and indirect trophic transfers

As humans continue to create and dispose of increasing amounts of plastics, the accumulation of microplastics in ecosystems would be potentially linked to increased population densities and intensification of land uses. As other coastal ecosystems, estuarine areas receive plastics from various sources resulting from the variety of human activities that occur in their surrounding land. Because of its influence from the ocean, plastics can arrive with the tides and wash up on the mud flats. But, there is also the influence from human development (residential, surface runoff from roadways, boating etc.) around the watershed that feeds into the estuary system. Our understanding of how, when and where microplastics get into our estuaries is still very limited. As a space that provides many valuable ecosystems services, such as protecting the coastline from erosion and wave action, fixing carbon, and recycling nutrients (REFs), it is essential that we understand how microplastics are accumulating and impacting these systems to better protect them.

To provide answer to some of these questions we located our study area in Waquoit Bay, Cape Cod, Massachusetts. Our study investigates the abundance and distribution of microplastics in intertidal sediments in three estuaries within Waquoit Bay subject to different levels of human development. The differences in population density and anthropogenic inputs across the three sites, allowed us to test a vast range of variability within the same study area. The accumulation of plastics, as well as carbon and nitrogen contents and isotopic signatures were analyzed in intertidal sediment cores, down to depths that allowed us to characterize the impact of anthropogenic activities over the past 30 years. We hypothesize that the presence of microplastics and the impacts on nitrogen content and isotopic signatures will increase, reflecting the effects of increased human development both spatially across sites and temporally along core depth. We also hypothesized that in more recent years we will see higher concentrations of plastic and that sites that are sandier will have a higher abundance of plastics than the ones that are taken from marsh areas. Understanding the abundance and accumulation through time of microplastic in Waquoit Bay can help to identify contributing sources of microplastics as well as inform residents, researchers, and policy makers about their potential hazards.

## Materials and Methods

### *Study Site Description*

Waquoit Bay and its sub-estuaries, located on the south coast of Cape Cod, Massachusetts, are representative of shallow, groundwater-fed estuaries in the northeastern USA (Figures 1 and 2). This well studied system consists of 910 hectares of water.

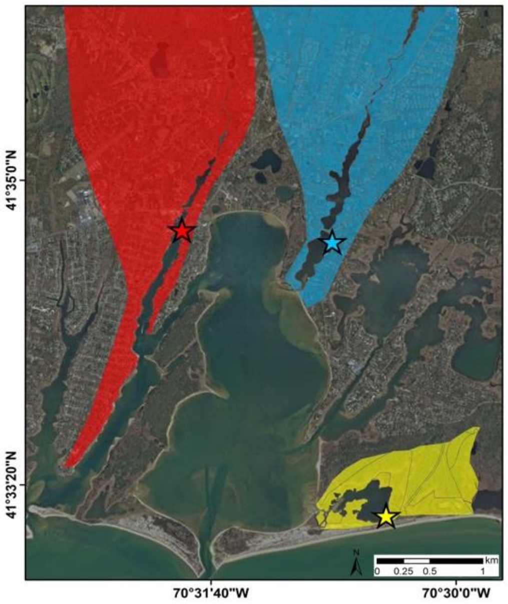


**Figure 1**. Map of the sample locations and watersheds: Childs River in red, Quashnet River in blue, Sage Lot Pond in yellow. The stars are the core sampling locations that were collected in each of the sites.

Within Waquoit Bay there are areas of freshwater, saltwater, brackish water, and different marsh and grasslands (*Waquoit Bay*

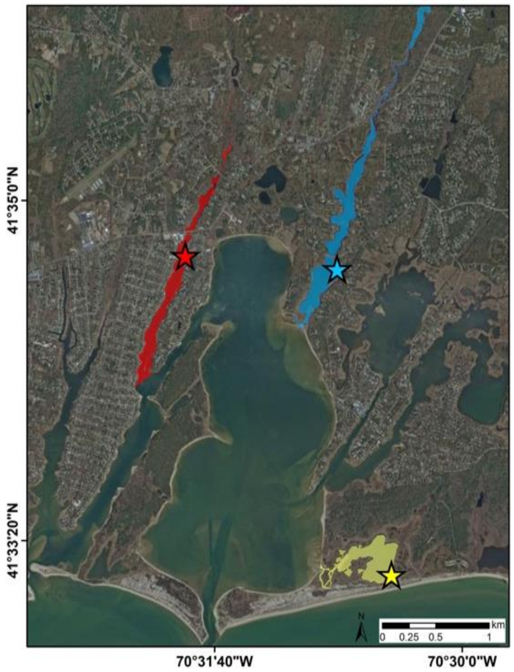


**Figure 2.** Map with the estuaries shaded in. Stars show the GPS location of where the cores were taken from. Red: Childs River, Blue: Quashnet River, Yellow: Sage Lot

*National Estuarine Research Reserve).* Waquoit Bay estuaries receive different N loads due to high diversity in population residency and land uses (Bowen and Valiela 2001) (Valiela et al. 2016) (Figure 3& 4).

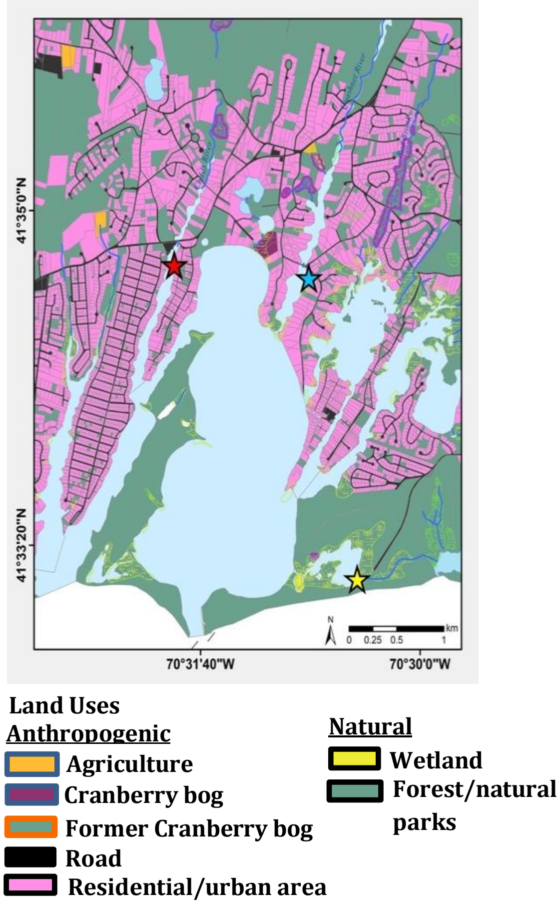


**Figure 3**. Map showing the different land uses that surround the estuary system. The stars represent the location of where the cores were taken. Red: Childs River, Blue: Quashnet River, and Yellow: Sage Lot Pond

Three sub-estuaries within Waquoit Bay were selected: Childs River, Quashnet River, and Sage Lot Pond (Figure 1). The Quashnet River has the largest watershed, 2000 ha, the Childs River is in the middle, 794 ha, and Sage Lot Pond has the smallest, 104 ha. These sites were chosen to represent the varying levels of anthropogenic impact. The Child's River has a high residency level and a large amount of boat traffic. Sage Lot Pond in comparison is a pristine and uninfluenced area of Waquoit Bay. The Quashnet River falls in between there is no heavy boat traffic, but there is a large amount of homes that surround the area. Quashnet also has many current and former cranberry bogs at the top of the river that have influence on the estuary (Figure 2).

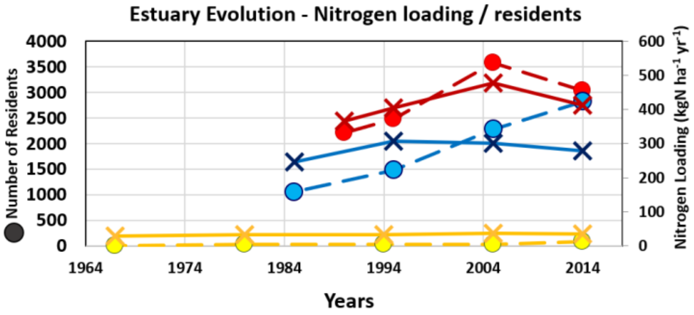


**Figure 4**. Nitrogen Loading denoted by the circle and dashed line and Nitrogen loading denoted by an X and a solid line. The Childs River is in Red the Quashnet River in Blue and Sage lot Pond in Yellow.

Sedimentation rates in Waquoit Bay range from 0.24cm $y^{-1}$ to 0.33 cm $y^{-1}$. The Childs river has the highest accretion rate of 0.33 cm $y^{-1}$ Quashnet falls in the middle with 0.27 cm $y^{-1}$ and Sage Lot Pond is the lowest with 0.24 cm $y^{-1}$. (Kinney 2010).

### *Collection and Preparation of the Sediment Core*

At each site, three to six cores of about 10 cm were taken. At the Childs River and Sage Lot Pond cores were taken from both the mudflats or shoreline as well as from marsh areas with *Spartina alterniflora* to investigate the preferential accumulation of microplastics within the sites. In the Quashnet river only shoreline/mudflat cores were taken.

Using a rubber mallet and piece of wood each core was hammered about 10 cm into the ground. Suction was created using a rubber stopper and then the core was pulled out of the ground, capped, labeled and brought back to the lab. The cores were stored in a fridge set to 4 ° C until they could be processed.

Each core was sliced into 1 cm depth intervals. The sedimentation rate of Waquoit Bay was assumed to be on average about 0.3 cm $yr^{-1}$ (Kinney & Valiela 2013). The cores were sectioned for the for the first 10 cm (corresponding to the past ~30 years) (Kinney 2010). Each resolution was placed in a pre-weighed tin, then weighed again to get the wet weight and then dried overnight in an oven set to 65 ° C. Once the samples were dry, they were weighed again to obtain the dry weight of the sample.

Replicate cores from the same site and section depth were combined. The Spartina samples from locations were kept separate due to their unique characteristics. Each combined sample was then homogenized and weighed again, to get a pooled dry weight. Samples from Quashnet River, Childs River and Sage Lot had a small sub sample of 1-3g, from the combined sample, set aside from the 0-1, 3-4, 6-7, 9-10 cm sections for carbon, nitrogen and stable isotopes analyses.

### *TOC, TN and Stable Isotopes*

The subsamples from the sediment sections 0-1, 3-4, 6-7 and 9-10 cm, corresponding about to decade separation, from Childs Quashnet and Sage Lot were analyzed for total organic carbon (TOC), and total nitrogen (TN) concentrations and stable isotopic composition with a Europa 20-20 CF-IRMS interfaced with the Europa ANCA-SL elemental (MBL Stable Isotopes Laboratory). The samples were ground at 20 rpm for 50. Once each sample was in a fine powder, they were each weighed in pre-weighed tin capsules (from 5 to 100 mg, depending on the amount of TOC and TN). were weighed. Prior to analysis, each of the subsamples were pre-treated to remove the carbonates by adding 50 µl of 4% sulfurous acid directly to sampling and evaporating at 55-75°C until no reaction.

### *Organic Material Digestion*

Due to high concentration of organic material in the cores interfering with the microplastics separation using density floatation, the first step of the microplastic isolation process was to oxidize each sample using a 30% aqueous Potassium Hydroxide (KOH) solution. Each sample was mixed with the KOH solution and placed in an oven set to 60 °C overnight. The sample was then sieved through a 63 µm sieve and rinsed to clean the sample of the KOH (Scheurer, 2018).

### *Microplastic Isolation and Quantification*

After the initial organic matter digestion, the sediment was moved into a 1000 ml beaker and added to a solution of Calcium Chloride ($CaCl_2$) with a density of 1.4 $g/cm^2$ was added. The solution was then stirred by hand for five minutes and left to sit for fifteen. After the fifteen minutes, it was stirred again for 30 s and left to sit for at least another 5 min. The floating material was then aspirated of the top of the solution and size fractionated through a series of sieves: 5.6 mm, 1 mm, 250 µm, and a 63µm. The material collected in each sieve was transferred into Falcon tubes, the digestion step using KOH was repeated in the tubes. The material that did not float was set aside for grain size fractionation (see below).

The samples in the tubes were then washed again in with the designated sieve for its size fraction and the one below it just in case any of the plastics were broken down with the KOH. These were then transferred into new falcon tubes and dried completely in the oven set to 65° C.

In the non-floating material from the Childs River Samples it was visually noticed that there were plastics that did not float due to higher density. These samples, both Spartina and Mudflat Childs River samples were refloated using an aqueous solution of $ZnCl_2$ at a density of 1.6 $g/cm^2$. The steps were the same as the $CaCl_2$ floatation. The density of $CaCl_2$ solution allowed the recovery of plastics that were less dense such as polyethylene (PE, 1.2 g/cm$^2$), polystyrene (PS, 1.25 g/cm$^2$), polypropylene (PP, 0.97 g/cm$^{2)}$ low density polyethylene (LDPE, 0.915 g/cm$^2$), high density polyethylene (HDPE, 0.940 g/cm$^2$), and nylon (1.15 g/cm$^2$). Denser plastic polymers such as polyvinyl chloride (PVC, 1.45 g/cm$^2$) and polyethylene terephthalate (PET, 1.50 g/cm$^2$) were not likely to be recovered with the $CaCl_2$ solution, but would be with the $ZnCl_2$ solution (Density of Plastics).

In a final step to separate the plastics from the remaining organic matter 10-15 mL of 65% Nitric acid was added to each of the falcon tubes. They were set in a 45 °C hot water bath for 24hours (Scheurer, 2018). They were then diluted with water and washed. The material went through a final floatation using the same $CaCl_2$ or $ZnCl_2$ solution in the Falcon tube. The floating material was poured off the top and placed in petri dishes for microplastic counting and characterization.

Microplastic particles from each sample, both fractions A (1 to 5.6 mm) and B (250 µm to 1mm), were quantified using a dissecting microscope. Plastics were categorized as fibers, fragments, or pellets. Additionally, within those three categorize they were identified by the material color.

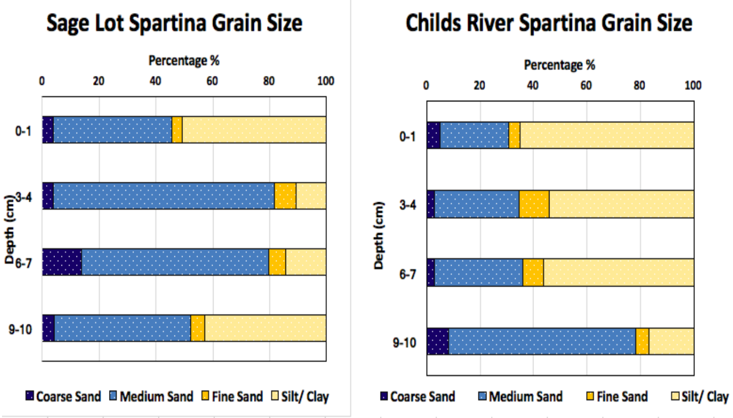
### *Grain Size*

The non-floating material that was set aside earlier was sieved through the same set of sieves (5.6 mm, 1 mm, 250 µm, and 63 µm) and weighed once dry to calculate the relative percentage of each grain size fraction. The 1-5.6 mm, 250 µm-1mm, 63-250 µm and <63 µm grain size fractions correspond to coarse sand, medium sand, fine sand and silt/clay fractions, respectable.

## Results

### *Grain size distribution*

Figure 5 and 6 show the different percentages of grain size that were found in the different sampling sites. The cores from Child's and Quashnet have higher percentages of the coarse sand fraction (1-5.6 mm). The Spartina cores from both Child's and Sage Lot have very high silt/clay fractions, less than 63 µm. The Sage Lot core shows the gradient starting with mostly a sandy layer and ending with a heavily clay/ silt layer.


Sage Lot Spartina Grain Size

Childs River Spartina Grain Size

**Figure 5**. Grain size of the Spartina samples taken from Sage Lot and Childs River. The Coarse sand is 5.6-1mm, Medium sand 1mm-250µm, fine sand from 250µm to 63 µm and the clay fraction was material <63 µm.

### *Elemental and stable isotopic composition*

The organic carbon and nitrogen percentages were the highest in the Childs River and the Quashnet River. The $\delta^{15}$N is the also the highest in the Childs River and follows similar patterns to the Quashnet River. As for the $\delta^{13}$C we found that it was the most depleted in the Childs River. In all four of these analyses the Sage Lot pond stays very consistent and unchanged.

Stable isotope $\delta^{15}$N from the sediment samples positively correlate with the nitrogen loadings in Childs River and Quashnet River. The highest $\delta^{15}$N values and nitrogen loadings are found in most recent layers of

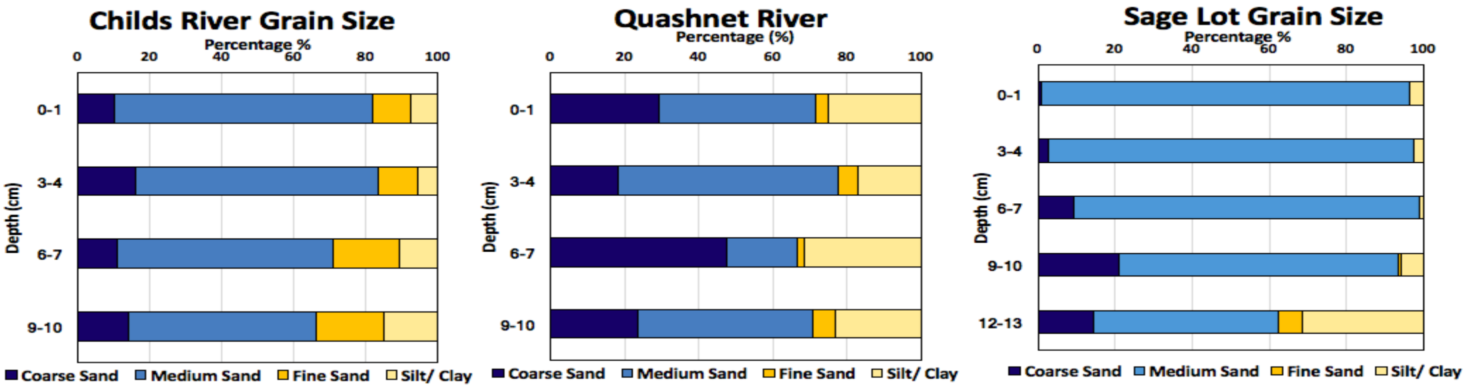


**Figure 6**. Grain size of the samples from the shoreline cores of the Childs River, the Quashnet River and Sage Lot Pond. The Coarse sand is 5.6-1mm Medium sand 1mm-250µm fine sand from 250µm to 63 µm and the clay fraction was material <63 µm.

Childs River, followed by Quashnet River samples. Sage lot there is the lowest nitrogen loading and $\delta^{15}$N.

*Microplastic abundance*

Figure 7 shows the number of plastics per cm$^3$. Taking the number of microplastic particles found in each section was calculated by dividing the number of particles by the volume of the section. The graph on the left shows the number of microplastics that were found in the shoreline cores over the past 40-50 years. The highest concentration of microplastics were found in Childs River in the 1980's and the 1990's. This subestuarine system also overall has the highest abundance of plastic over all the decades. Sage lot and Quashnet have very similar concentrations of plastic and it does not change that significantly over time. In the Spartina marsh cores the levels of plastics are much lower.

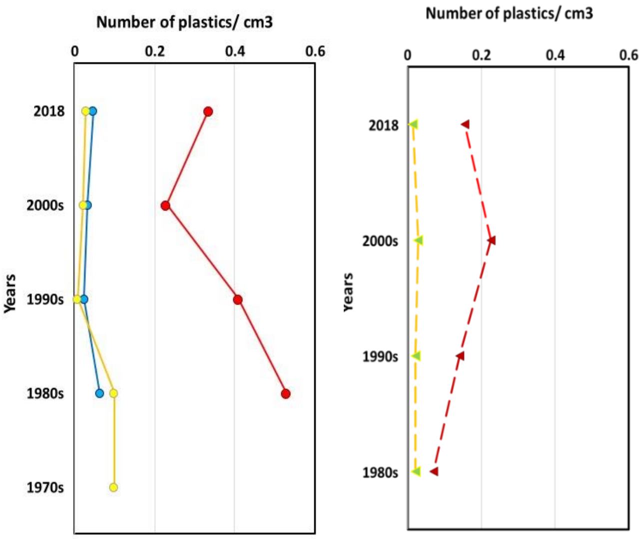


**Figure 7**. Graph showing the number of plastic pieces per cm3 that were found in the shoreline (left, solid lines) and Spartina marsh (right, dashed lines) cores, graphed by the corresponding age of the core section. Childs River (red), Quashnet (blue) and Sage Lot Pond (yellow).

Comparing the abundance of microplastics with the historical nitrogen loading (Figure 4), we observe that, the higher the nitrogen loading to the system the higher the abundance of microplastics that was found in that site. The Childs River was found to have the highest concentrations of plastics per hectare. Sage Lot is found at the lower end of the amount of plastics and nitrogen. However, some of the points are comparable with both Quashnet and Childs river.

Quashnet falls in the middle of the two other sites but, is closer to the range of Sage Lot. There is variability in microplastic particles found, including the presence of both fragments and fibers. Figures 9, 10 and 11 show the number of plastics found in each of the categories in millions per hectare at each of the different sites. It is also broken into size fraction in each resolution. The A fraction represents the pieces that were in the range 1-5.6 mm and B fraction was pieces that were 250 µm-1mm- in size. The Childs River in all the depths had more accumulation of fragments than fibers, with

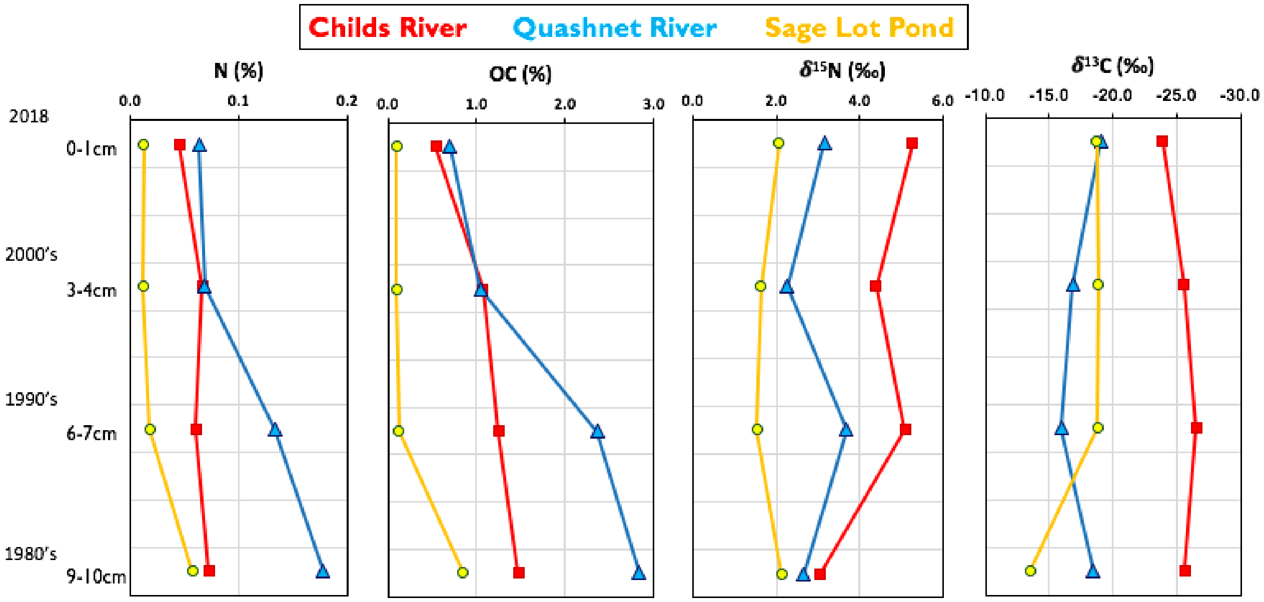


**Figure 8**. Graphs showing the % organic nitrogen, % Organic Carbon, δ 15N and δ 13 C in each of three sites: red with squares s is the Childs River, blue with triangles is the Quashnet river and yellow with circles is Sage Lot Pond. The Axis shows both the depths and the years that correspond with that depth resolution

the highest accumulation in the 9-10 B fraction. In Sage Lot, more fibers were found than fragments in all the depths. The only fraction that had plastic was in the B fraction from depth 12-13 cm... In the Quashnet River there were more fibers than fragments but is much more evenly distributed between the A and B size fractions.

Considering the size of the estuaries for Childs, Quashnet and Sage Lot, (16.9, 32.1, 14.2, respectively) we estimated the number of plastic particles that could be found in each estuary. The Childs River would have the most at 566x10$^6$ then Quashnet with 151x10$^6$ and finally Sage Lot with 42.1 x10$^6$ (Table 2).

## Discussion

The results of this study highlight the evolution anthropogenic impacts on Waquoit Bay subestuaries. The land use data showed that for the first time in history in the Child's River urban uses dominate the landscape (Lloret in prep). Quashnet is also trending in a very similar way to the Child's river. Sage Lot Pond, still maintains extensive areas of natural vegetation. Part of this urbanization is due to the increase of homes being built in the watersheds (Figure 4). This leads to the problem of nitrogen loading that Waquoit Bay faces. The trends between residency and nitrogen loadings (Figure 4) are attributed to the fact that in estuaries like the Child's River, which displays the highest level of nitrogen loading, there are also the highest number of residents. The increased nitrogen comes from human uses on land; as more people move into the estuary's watershed wastewater and fertilizer sources of nitrogen increase in the area (Valiela et al. 2016).

*Elemental analysis and isotopes*

The highest concentrations of organic matter were found both in Childs and Quashnet Rivers. This is because these two rivers are surrounded by forested areas. The annual litter fall contributes to a muddy shoreline. In Sage Lot Pond the shore lines were much sandier. This can be seen in the grainsize distributions shown in figures 5 and 6. The composition of Sage Lot is mostly medium sand with very little to no fine sand or silt clay. While the Childs River and Quashnet river have much higher percentages of the smaller size fractions.

The percent TN varies among the three different experimental sites. For Sage Lot

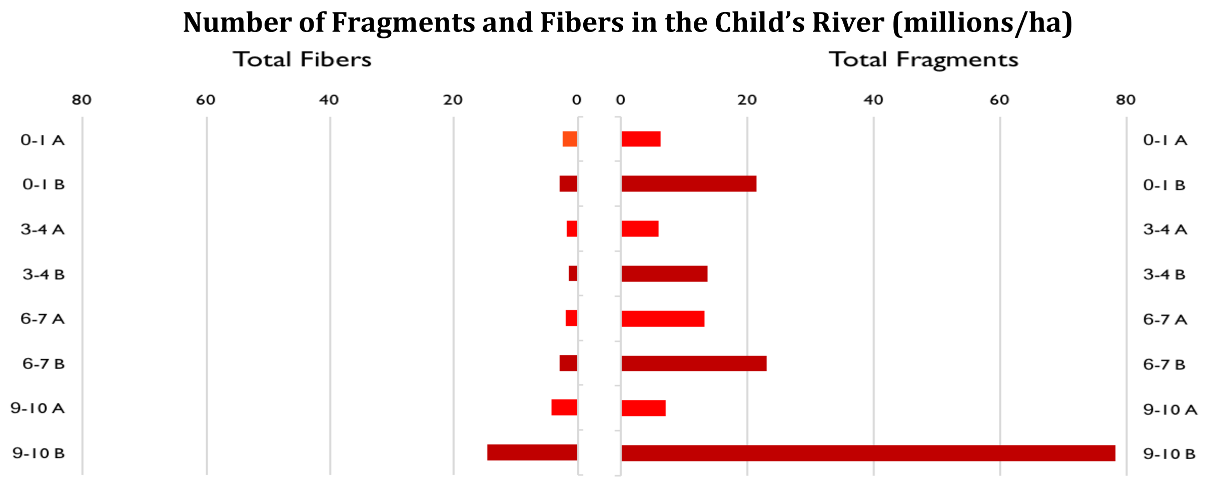


**Figure 9.** Graph showing the number of plastic pieces per hectare that were found in the Childs River cores at each depth and in each size fraction. A is fraction 5.6mm-1mm and B is 1mm to 250µm.

Pond the values stay consistent and are very low (Figure 8). The land use has not changed greatly over the past 40 years and TN levels are generally low. Sage Lot Pond, as briefly described above, is a sand flat and there is not a lot of organic material that gets deposited in to the system every year. This is also the reason that the TOC is almost zero (Figure 8). However, both elemental analyses showed a peak in the 1980s (9-10 cm). When looking at the Sage Lot Pond core the bottom of it had a muddy *Spartina alterniflora* deposition. At this depth, the grain size composition also changes, the percentage of material in the medium sand size fraction goes down and the silt/clay fraction goes up. In the Childs River, the sediment has a higher percentage of fine sand and silt and clay than Sage Lot Pond. The organic material that falls into the river and gets broken down and increases the percent of carbon and nitrogen in the system. However, over time the percentages have been decreasing. This is potentially due to an increase in urbanization and a decrease in forested/ natural areas (Figure 5). With less forested area there is less material that falls

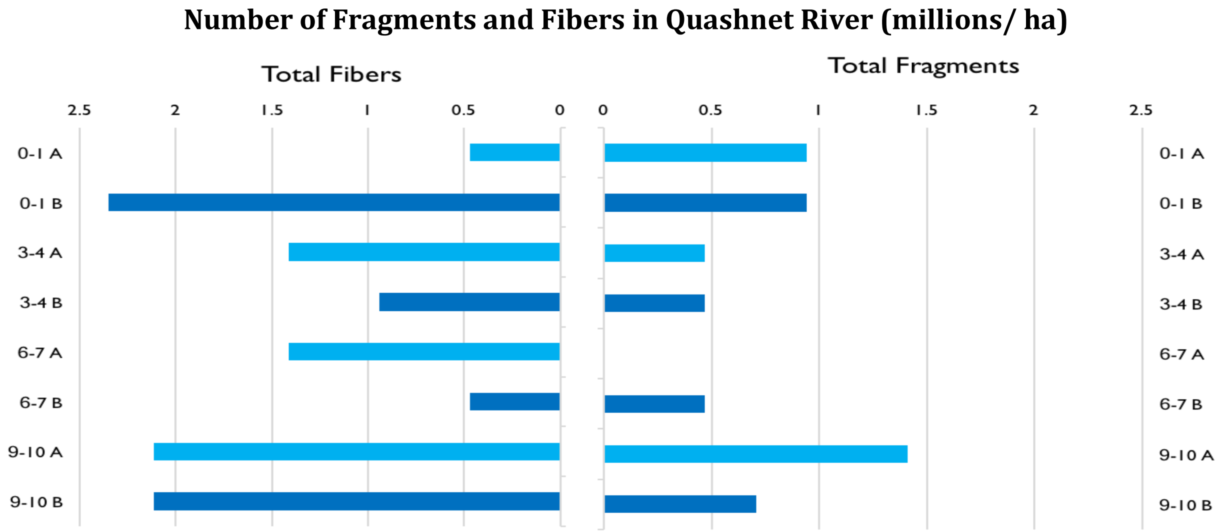


**Figure 10**. Graph showing the number of plastic pieces per hectare that were found in the Quashnet River cores at each depth and in each size fraction. A is fraction 5.6mm-1mm and B is 1mm to 250µm.

into the river every year decreasing the percent organics that accumulate in the sediments. A similar profile of percent carbon and nitrogen is found in the Quashnet River. However, Quashnet River showed much higher nitrogen and carbon in the 1990s and 1980s. Potentially this could be due to the cranberry bogs that are at the top of the Quashnet River. The number of cranberry bogs and agricultural lands were cut in half from 1950 to 1990 and there for much less fertilizer is being used and washed down the river (Bowen et al 2001). Seeing that the 1990s is the last elevated percent carbon and nitrogen it is a good hypothesis that this could be from the decrease in this type of land use. In the current years, their values have nearly matched up with the Childs River. The trends that are depicted in the elemental analysis complements the silt clay fraction percentages. This suggests that there is a correlation between the two. The higher the percentage of the silt clay fraction the higher the total organic carbon and nitrogen value.

The $\delta^{15}$N value can change based on the influence of wastewater in a system. Wastewater sources have very high $\delta^{15}$N values. The Childs River has been increasingly more impacted by humans and it saw an overall change in $\delta^{15}$N 3‰ from the 1980s to now. This change is most likely due to the increased residency producing more wastewater inputs from homes. The Quashnet River also sees a slight increase of about 1‰ the number of homes in the area is increasing so the amount of waste increases the nitrogen inputs however, because the estuary itself is larger the added nitrogen has more room to spread out and be used by the system making the value not as high as the one of the Childs River. Even though currently they both have almost the same number of residents in both of their watersheds. Sage Lot Pond the estuary's $\delta^{15}$N is very low and consistent throughout the decades. With no real change in human impact the $\delta^{15}$N does not change that much over time. (Figure 8).

The next correlation that was looked into was the one between $\delta^{15}$N and nitrogen loading. Because they are both impacted by wastewater and human influence the nitrogen loading values from figure 16 (Valiela et al. 2016) were graphed by the $\delta^{15}$N. From this we can see that there is a linear relationship between these two variables. It is then

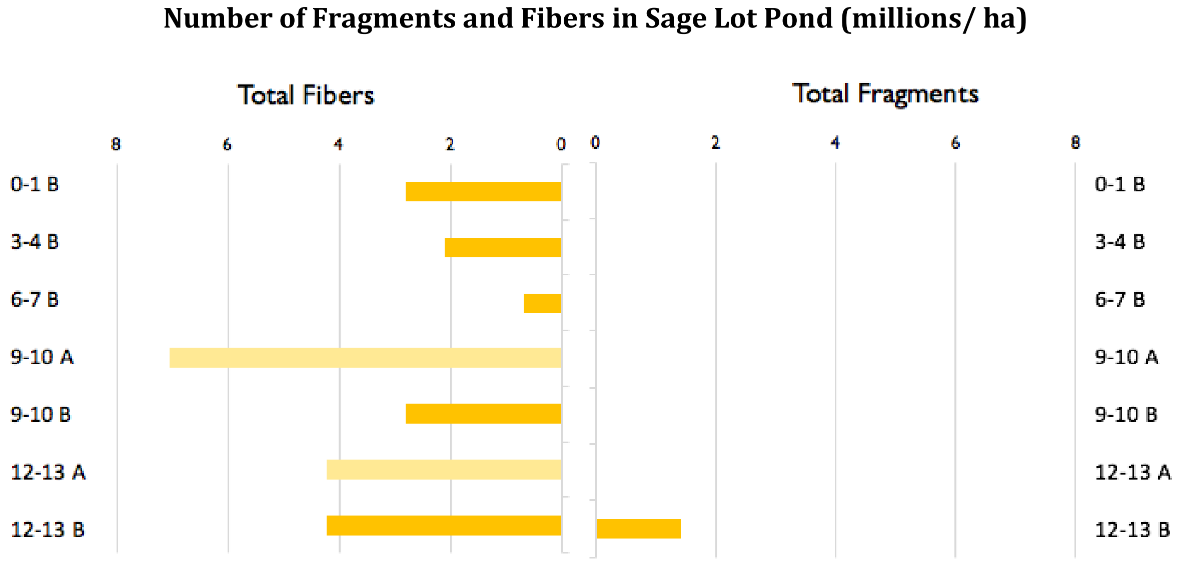


**Figure 11**. Graph showing the number of plastic pieces per hectare that were found in Sage Lot Pond cores at each depth and in each size fraction. A is fraction 5.6mm-1mm and B is 1mm to 250µm.

arguable that the $\delta^{15}$N can be used as an approximation for nitrogen loading in this system.

*Microplastics*

Plastic pollution is highly present in Waquoit Bay. Even in the more pristine environment that was selected for this study, Sage Lot Pond, there are plastic particles (Figure 11). The contrasted concentrations of plastic accumulation in the three subestuaries mirror the land uses and their evolution across recent decades.

We observed preferential accumulation of microplastics in the shoreline cores versus the *Spartina alterniflora* marsh (Figure X). This could be explained by the fact that the *Spartina alterniflora* marsh only gets washed over when the tides are very high which does not happen as frequently as the daily tides that hit the shoreline (Minello 2011). With less frequent waves there is less of a chance for plastic to be carried over and accumulated in the Spartina systems.

The type of microplastic variability (fragments *versus* fibers) through time also reflected the anthropogenic impact level evolution. The Childs River consistently has more plastics in the form of fragments in every depth. This abundance over fibers could be due to the systems main inputs are larger macro-plastics that eventually break down into these small fragments. Along the Childs River there is a high residency level, as well as high boat traffic. Both of these sources are potentially contributing macroplastics directly into the water way (Figure 13). Looking at the Quashnet River the system has a significantly lower abundance of plastic than Childs River. It was also found to have a higher abundance of fibers than fragments (Figure 10). In Quashnet River, unlike the Childs River, there is no boat traffic along the river and a much lower residency right along the river. This potentially impacted the number of fragments because there are less direct sources of plastic to the system. Whereas the higher concentration of fibers could be a result of fibers traveling from densely populated area through the air and runoff from storms (Anderson et. al 2016) In Sage Lot Pond, which was assumed as the pristine environment in this experiment, presented a higher accumulation of fibers to fragments. Similarly, to the Quashnet River there are less direct inputs to Sage Lot. The system has very few homes or developed areas, so most of the plastic would have to travel into the system. The abundance of fibers then can be explained by the trend in the Quashnet River. The fibers are more easily traveled and came to the system by air. The other explanation for the presence of fibers is from clothing, this area is frequented by human foot traffic. The fibers from our degrading clothing could be lost to the system and contribute to the plastic pollution (Carr 2017) (Figure 11).

The fraction 250 µm -1mm has the highest abundance of microplastic in both fibers and fragments in all three subestuaries. This could be due to the types of plastics and how the polymer breaks down. Plastics are broken down in varying ways, most commonly by biodegradation (consumption from living organisms) and, photodegradation (sunlight) (Andrady 2011). Further studying the types of plastics and how they break down in Waquoit Bay could provide insights into why the 250 µm -1mm is predominant in all the systems.

The largest amount of plastic found in the three subestuaries was in the 9-10 cm sediment section which dates these plastics back to about the 1980s. This directly conflicts with the hypothesis that the we would find the highest accumulation in the most recent section. A potential reasoning for this is that in the 1980s there were less regulations and awareness about plastic consumption. The amount that the United States now recycles has nearly doubled since the 1980s (EPA 2018). Local legislation in many states including Massachusetts have changed to limit the number of single use products that are consumed. This could potentially be decreasing the plastic that is available to break down into microplastics, explaining why the deeper fractions have more plastic accumulation.

An estimate for the total accumulation of plastics in the past 40 years could not be made due to the fact that only the tops of the decades were recorded and not the entire core profile. However, the estimation plastic accumulation in the last 3 years shows just a fraction of the pieces that could be found in this estuary, and the magnitude of the problem. These estimates suggest that there could be millions of tiny plastics in just these three small estuaries inside of Waquoit Bay.

## Conclusions

Sediments can be used as a tool to chronologically look back in time. They preserve the different markers that show land use changes through the decades. Elemental analysis and stable isotopes are a useful way to track land use between different locations well as understand the different land use changes over time. Organic carbon and nitrogen analysis can provide an understanding of the type of organic material that is predominant in a system. The coupling of nitrogen loadings in the estuarine watershed, directly related to residency /land use evolution, $\delta\ ^{15}N$ stable isotopes of sediments and microplastic abundance is an effective tool to look for different anthropogenic impact over time in estuarine systems.

This study shows for first time that there are abundant microplastics in Waquoit Bay sediments. Different land uses have an impact not only on the accumulation of plastic but on the type of plastic that is found in each of the sites. Fragments are more prevalent in systems that have higher residency levels and more direct human contact to them, like boating. In more isolated systems plastic is more abundantly found in the form of fibers since they travel easier. Even in the systems that we assume are untouched are facing human influence. Continuing studies surrounding microplastics can help understand how humans are impacting and changing our aquatic systems.

## Acknowledgements

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# The effect of diet on honeybee microbiome

Yufei Zhang

*Honeybees (*Apis mellifera*) are one of the most valuable pollinators for global food production and exert key influences on maintaining plant diversity. However, honeybees have suffered greater overwintering population losses in the past decade. Climate change, loss of habitat, and industrial agriculture actives have been major driving factors in honeybee's population decline. Lethal pathogens have been identified that induce gut microbiome dysbiosis, deformed wing formation and other diseases. Honeybee microbiome diversity and composition, largely influenced by diet and food forage, are crucial to their heath and survival. This review aims to investigate the effect of diet on honeybees' microbiome composition, and to establish an organized overview on this issue that deepens our understanding in honeybees' diet, microbiome, and heath. I have concluded that several dietary factors such as quantity, protein composition, Nosema infection, substitude and diversity alter and influence honeybee health. My conclusion also points out the current lack of understanding of honeybee microbiome strain-level variation, the future potential in studying the effect of diet on honeybee microbiome on a global scale, and the need of further investment in developing novel antibiotic treatments for nosemosis control.*

## Honeybee background and importance

Bees of the genus *Apis* are found to be distributed throughout a diverse range of habitats in the world (Conte and Navajas., 2008). Even though *Apis mellifera* is not endemic to the U.S., it was estimated that over 2.9 million honeybee colonies were identified for pollination purposes in America (Morse and Calderone, 2000). As one of the most economically valuable pollinators in the world, western honeybees are estimated to be responsible for pollination services worth USD 215 billion annually worldwide (Smith et al., 2013).

Honeybees also provide the ecological service of maintaining plant biodiversity in various ecosystems (Conte and Navajas., 2008). Studying honeybee microbiome has wide-reaching benefits such as establishing a tractable model for symbionts studies, reducing possible pathogenic infections, revealing seasonal prevalence novel viruses, and microbiota engineering (Kwong and Moran, 2016; Runckel et al., 2011; Mueller and Sachs, 2015). In addition, because both honeybee and mammalian microbiota systems consist of host-adapted gut microorganisms, a practical model of gut microbial community was established to future study gut microbial communities in a host context (Kwong and Moran, 2016). Further studies on honeybee microbiota are able to deepen our understanding on mammalian microbiota (Kwong and Moran, 2016).

## Honeybee current threats

The continuing honeybee population decline in the past decade is ascribed to several stressors (Potts et al., 2010). Previous studies have showed the negative impact that antibiotics exert on the honeybee gut microbiome, which further alters honeybee health in general (Raymann et al., 2017). Especially considering the abusive usage of antibiotics in modern agriculture for parasite control and infection control, honeybees are inevitably overexposed to antibiotics (Raymann et al., 2017). On an individual level, oxytetracycline, either in the form of syrup or dust, is one of the popular choice of antibiotics for beekeepers in the United States for pathogenic mites control. However, the application of the antibiotics oxytetracycline, tylosin, and lincomycin was found to be toxic to *A. mellifera* larvae with a

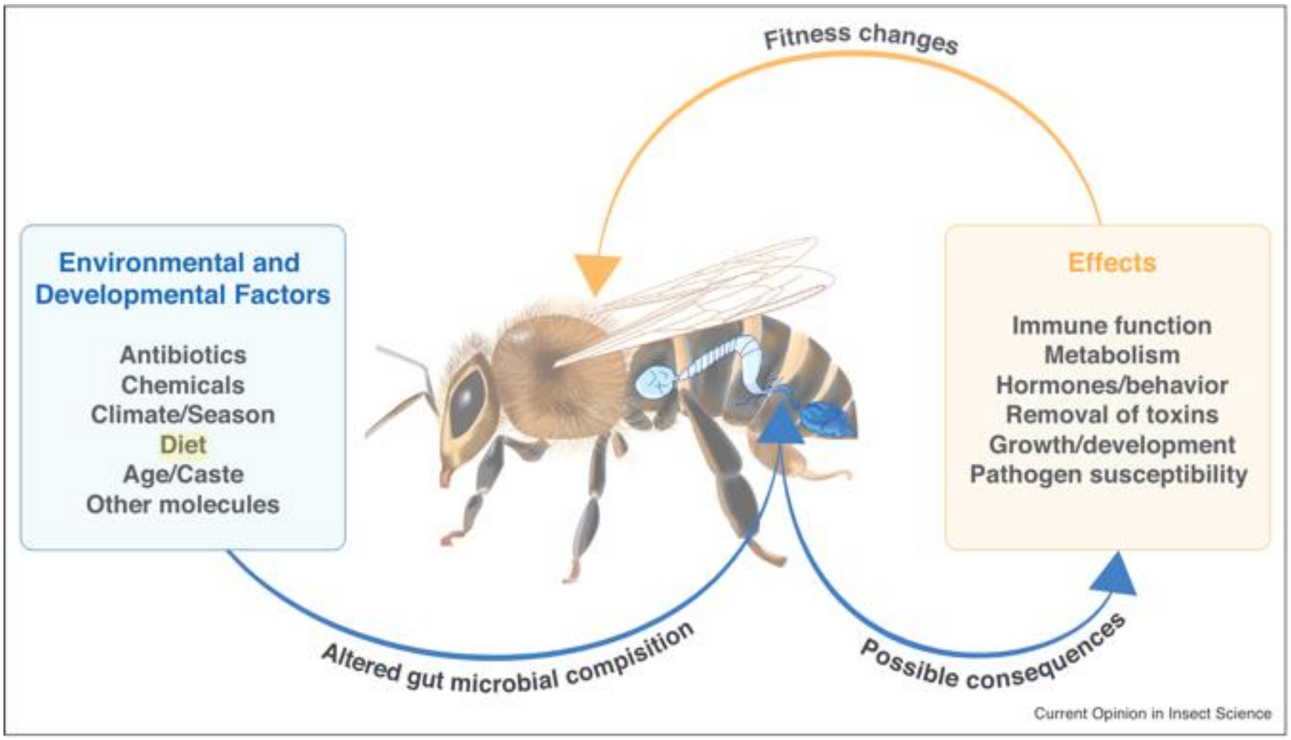

**Figure 1.** From Raymann and Moran (2018)

65% increase in larval mortality (Pettis et al., 2004). Several antibiotic treatments are also capable of inducing gut microbiota dysbiosis to adult worker bees, where an unhealthy shift in honeybee gut microbiome enhances opportunistic pathogenic infections and lowers honeybee survival rates (Raymann et al., 2017). Similar to antibiotic exposure, pesticide products induce lethal toxicity to honeybee populations when honeybees are in contact with insecticide particles in the air (Rortais et al., 2004).

On a regional level, industrial pollutants are partially responsible for altering colony dynamics and decreasing honeybee survival (Hladun et al., 2015). Certain toxic metal contaminations such as lead, zinc, and nickel are able to mix with pollen and nectar of flowers and accumulate within honeybee body systems, leading to honeybee deaths and toxic honey (Silici et al., 2013). Colonies under heavy exposure to industrial metal particles were found to contain more dead pupae, and to have adult worker honeybees with lighter weight (Hladun et al., 2015).

On a global scale, climate change has impacts on honeybees in different aspects (Conte and Navajas, 2008). Honeybees originally were capable of surviving and forming colonies in the Arizona Desert (Conte and Navajas, 2008). However, as the aridity in the Arizona Desert rises due to the pressure of climate change, honeybees are no longer able to extract enough water to raise their offspring and hydrate their hives to establish the ideal humidity, which leads to collapsed colonies (Conte and Navajas, 2008). In addition, the climate caused shift of global floral distribution and diversity limits the food sources and colony development of honeybees (Thuiller et al., 2004). As figure 1 illustrates, multiple environmental stresses are found to be responsible for the acute declines of honeybee population occurring worldwide (Pettis et al., 2010; Becher et al., 2013).

## Honeybee microbiome

Honeybee gut microbial community is structured with complex genomic diversity, extensive microbial species and many strain-level variations (Ellegaard and Engel et al., 2019). The honeybee microbiome is crucial for maintaining health and can be easily targeted by environmental stresses and viruses to trigger diseases (Kevill et al., 2019). As the intestinal microbiota contains a wide collection of microbes, perturbing the balance of gut microbial community gives opportunities for the intrinsic pathogens *Frischella perrara* and *Parasacharibacter apium* to stimulate gut microbiome dysbiosis as they infect the honeybee ileum, mouthparts and hypopharyngeal glands (Brown et al., 2012; Maes et al., 2016). Dysbiosis induced inflammatory infections usually involve defunctionalized mucosal barriers and increased virulence possibilities (Stecher, 2015). A loss of 30-90% of honeybee colonies was reported in 2016 with symptoms associated with colony collapse disorder in the United States (Ellis et al., 2015). Colony collapse disorder is a driving force that partially explains the significant increase in overwintering honeybee population loss in the past decades (vanEngelsdorp et al., 2009).

In addition, an external parasitive mite, *Varroa destructor*, is identified as one of the key activators in deformed wing virion multiplication and transfer between honeybees on an individual level (Genersch and Aubert, 2010). With a global distribution, deformed wing virus (DWV) affects *A. mellifera* by concentrating DWV master variants in the head, abdomens, and wings, which results in memory loss, learning impairment, deaths, and colony collapse (Kevill et al., 2019; Iqbal and Mueller, 2007). Other than the evident loss of wings and morphological changes, DW- honeybees also displayed stalled responsiveness to food sources as their associative learning ability is impaired by the virus (Iqbal and Mueller, 2007).

## Diet-related microbial interactions

As generalist consumers, European honeybees feed from a wide variety of sources worldwide including the pollen and nectar from apple orchards, blueberry plants, cranberry bogs, and even fallow fields (Colwell et al., 2017). Due to the huge differences in food sources geographically, pollen qualities from different sites usually result in various protein uptake (Colwell et al., 2017). After examining and quantifying the nutritional content in honeybees' diet and the plant species they forage on, it was found that protein and lipid content differed significantly due to floral species diversity as the proportions of amino acid in their diets revealed different floral sources (Donkersley et al., 2017). As a result, honeybees develop diverse enzymes and provide suitable habitat for different species of microbes in their gastrointestinal system to facilitate metabolism for energy purpose and to retain neuroactive compounds for brain functional support (Romero et al., 2019). Studies have indicated that diet is a major factor of shifting gut microbial environment and compositions, and altered gut microbiota can possibly implement further destructive effects on the overall health of honeybees (Raymann and Moran, 2018).

Comparing fresh pollen and 14 days old pollen reveals that the age of the diet significantly influences honeybee health (Maes et al., 2016). Honeybees fed on aged diets displayed stalled development, decreased survivorship, and shifted gut microbial dysbiosis in comparison to the honeybees fed on fresh diet (Figure. 2a) (Maes et al., 2016). By examining the microbial communities in the ileum, rectum, mouthparts, and hypopharyngeal glands of honeybees, Maes et al. (2016) has discovered that consumption on aged diet facilitates gut microbiome dysbiosis by increasing intrinsic pathogen *Frischella perrara* while reducing a core ileum bacterial commensal *Snodgraseella alvi*.

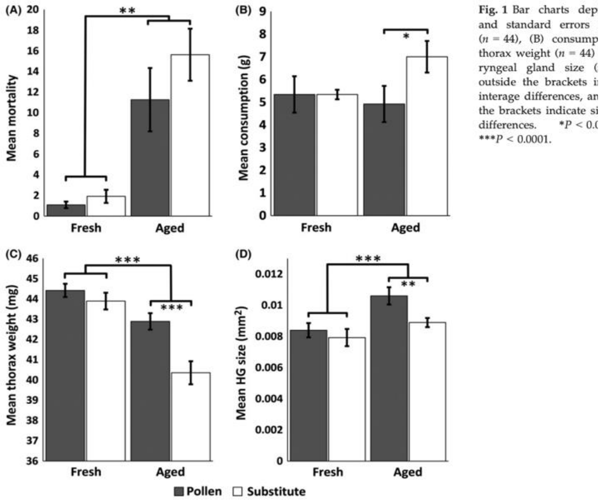

**Figure 2.** From Maes et al. (2016)

Maintaining the honeybee population over winter seasons has been the main obstacle for beekeepers to overcome; therefore, commercial pollen substitutes, including soy flour, brewer's yeast, vitamin C, and dry milk, were invented to overcome such loss (Barker, 1977). In the application of pollen substitutes, toxins derived carbohydrates in certain pollen substitutes such as glucuronic acid, and polygalacturnoic acid were detected (Barker, 1977). Other studies on pollen substitute discovered that honeybees fed on commercial pollen substitute diets experienced higher level of *N. ceranae* infection in comparison to honeybees that fed on wild floral pollen (Flemming et al., 2014).

With diverse dietary components, both monofloral diet and polyfloral diet modify the baseline immunocompetence by regulating honeybee microbiota's susceptibility to diseases (Alaux et al., 2010). However, honeybees fed on mixed- floral-source pollen demonstrated improved immune functions than the honeybees fed on monofloral diet (Alaux et al., 2010).

## Nosemosis alters honeybee microbiome and induces mortality

Additionally, *Nosema ceranae*, spread by hive cleaning behaviors and highly pathogenic to *A. mellifera*, is a microsporidian parasite that causes lethal infections among honeybee populations and sudden colony collapse (Porrini et al., 2011; Higes et al., 2007). As figure 3 shows, a study conducted in Spain successfully tracked the progress of *N. ceranae* sporulation and germination in ventricular cells *in vivo* with electron microscopy and the transmission and escalation of nosemosis illustrated the prevalence and the invasiveness of such disease in *A. mellifera* (Higes et al., 2007).

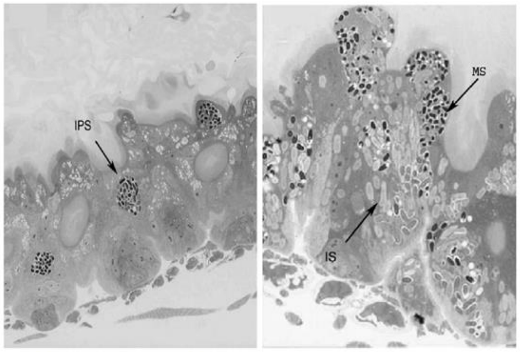

Fig. 1–2. Infection of *Apis mellifera* ventricular (midgut) cells with *Nosema ceranae*. Methylene blue. (Fig. 1) Day 3 p.i. A few epithelial cells at the tips of the folds showing intracellular parasite stages (IPS). 200×. (Fig. 2) Day 6 p.i. Heavily infected tissue with tightly packed parasites. Basophilic mature spores (MS) in apical region of epithelial cells. Larger heterogeneously stained parasitic structures at the bottom of the folds, suggesting different life cycle stages 400× (IS, immature stages).

**Figure 3.** From Higes et al. (2007)

Protein regulation in honeybee diet influences *N. ceranae* infections in the gut microbial community in the same way that higher protein density in floral diet contributes to increased *N. ceranae* intensity, followed by higher mortality rate (Rinderer and Elliott, 1977). A previous study has showed that pollen quantity plays an important role in not only maintaining the microbial balance in honeybee gut microbiota, but also regulating *Nosema ceranae* infections (Figure 4). Increased

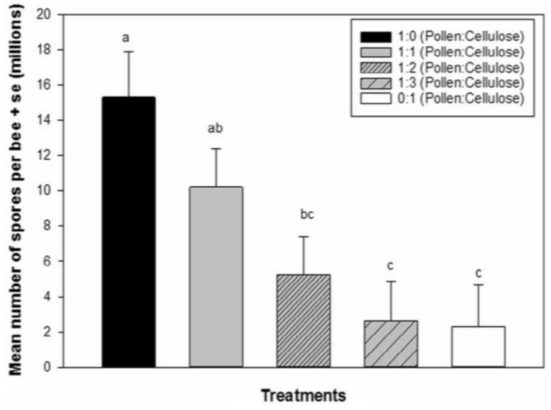

**Fig. 1.** Mean number of *Nosema ceranae* spores per bee (+se) fed different pollen concentrations and sampled 16 days after infection. Means with different letters indicate significant differences among the treatments ($P < 0.0001$).

**Figure 4.** From Jack et al. (2016)

pollen quantity in *A. mellifera* diet contributes to higher survival rates and higher *N. ceranae* intensity at the same time (Figure 5). This counterintuitive experimental conclusion was explained with several hypotheses. One hypothesis is that higher pollen quantity in diet increased fat body content, a part of immunoprotein synthesis, thus increasing the longevity of *A. mellifera*. Another hypothesis is that high pollen quantity might be able to compensate for the loss of energy and nutrients taken by *N. ceranae* (Jack et al., 2016).

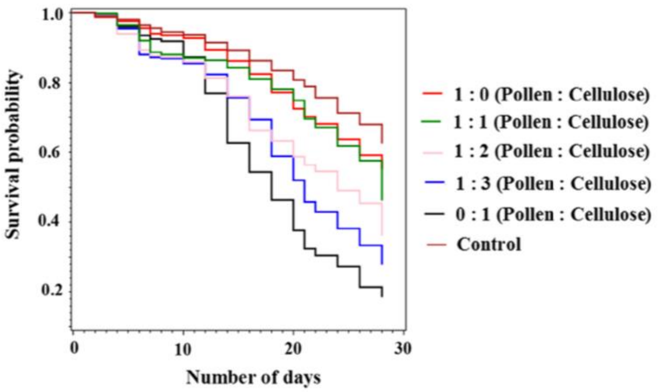

**Figure 5.** From Jack et al. (2016)

Considering the large impact of *Nosema* in honeybee mortality, colony collapse, and loss of economical production, *N. ceranae* infection control has been the subject of ongoing research attention over the past decades (Webster, 1994; Williams et al., 2008; Huang et al., 2013). Fumagillin dicyclohexylammonium, a possible nosemosis treatment, penetrates infected epithelial cells and interferes with *N. ceranae* enzyme methionine aminopeptidase2 and further protein folding, which was supported with high efficacy of *N. ceranae* decline in fumagillin treated cells (Williams et al., 2008). Water soluble Bicyclohexylammonium Fumagillin, Fumagilin B, was formulated successfully to lower *N. ceranae* infection as they are practical and applicable when diluted in sucrose syrup (Webster, 1994). However, due to the fact that fumagillin has been the only effective antibiotic control approved for nosemosis, the extensive usage and dependency on fumagillin has led to *N. ceranae* escaping fumagillin control and continued honeybee microbiome alteration (Huang et al., 2013).

## Concluding remarks

Honeybees are influenced by multiple environmental stressors including climate change, industrial pollution, loss of habitat, and diet. Diet specifically exerts profound influences on honeybee populations on many levels. The regulation of nutrients, differing pollen quantity, replacement of pollen by substitute, protein components, floral sources, and *N. ceranae* intensity alter honeybee microbiome that inevitably impacts honeybee health and change the overall fitness of the population. Honeybee population declines are occurring worldwide, yet much of the research was concentrated regionally. An examination of worldwide honeybee population declines due to altered microbiome would serve as an overview of the current status with a global perspective. Such collection of information would be useful in pointing out where research and conservation efforts is the most needed. Even though a comprehensive collection of microbial identifications within honeybee gut microbiota has been established, many more precise questions remain regarding the strain-level variations of bacterial interaction and composition in the honeybee microbiome. In addition, there is a continued need to advance our understanding of nosemosis control in that novel treatments reduce the current dependency on fumagillin as the sole effective antibiotic treatment and frees the honeybees from possible abusive exposure to fumagillin. Considering the current dependency on fumagillin and observed resistance, novel antibiotic sources are in acute need of discovery, research, and application.

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# Grazing patterns of captive Grant’s gazelles and Bongo antelopes

Alejandra Nawrocki and Natalie Thomas

*Grant’s gazelles* (Nanger granti) *and bongo antelopes* (Tragelaphus eurycerus) *are prevalent ungulate populations within zoos. While ungulates are commonly grouped together in exhibits, previous studies have found that they display different behavioral and grazing patterns. This study focused on the bongo antelopes and Grant’s gazelles at the Memphis Zoo. We test 3 hypotheses: Grant’s gazelles and bongo antelopes exhibit different behavioral patterns, gazelles display group behavior while bongos will tend to be more solitary, and the two species have specific grazing patterns. In this study, we determine whether there were differences in behavioral and grouping tendencies between the gazelles and bongos. Additionally, we identify the areas of the exhibit in which the bongos and gazelles concentrate their feeding behavior. Over the course of our study, we collected behavioral, grouping, and spatial data on the two species. Although we did not find a difference between their behaviors, we found statistical evidence to support the hypothesis that bongos are more likely to graze alone, and gazelles are more likely to graze in larger groups. In addition, we found that gazelles demonstrate grazing preferences in the open area of the exhibit near the front, while bongos tend to concentrate in the shaded back area. Through this research, we hope to aid husbandry practices at the Memphis Zoo, as well as contribute to the literature on captive ungulates that can be used to study the migratory and behavioral patterns of ungulates in the wild.*

## Introduction

Ungulates, such as Grant’s gazelles (*Nanger granti*) and bongos (*Tragelaphus eurycerus*), are prevalent in zoo collections around the world (Huffman, 2013). Grant’s gazelles demonstrate diverse behaviors, making gazelles interesting study subjects both in the wild and in captivity (Huffman, 2013). These behaviors include resting and grazing in large groups of ten or more individuals in order to increase vigilance and awareness of predators (FitzGibbon, 1993). Free-ranging Grant’s gazelles inhabit a wide variety of climates and landscapes throughout Ethiopia and Tanzania (Jarman, 1974; Walther 1972). In the wild, Grant’s gazelles prefer brush and woodland areas, feeding on grasses and browse within a large perimeter (Martin, 2000). Gazelles are intermediate feeders, or species adapted to both grazing and browsing, and focus their eating behavior on legumes and small shrubs (Tieszen et al., 1979). Generally, gazelles consume 24% grasses and 68% browse (Tieszen et al., 1979). In captivity, gazelles benefit from a mixture of hay and access to browse within their exhibit, which provides additional fiber to aid in combatting digestive issues (Huffman, 2013). In order to increase vigilance and awareness of predators, wild Grant’s gazelles rest and graze in large groups of ten or more individuals.

Bongo antelopes (*Tragelaphus eurycerus*) are the largest antelope species by body size yet are one of the least studied (Forthman et al. 1993). Bongos are found throughout the eastern and western regions of Africa’s Lowland Forest Zone and in small pockets of East Africa’s montane forests (Ralls, 1978). There is a considerable lack of information about the behavior of wild bongos compared to other antelopes (Ganslosser and Brunner, 1997). From what has been observed, they are highly selective, concentrate feeders (Wright et al., 2011). Thus, they exhibit flexible grazing patterns that are dependent on food distribution (Wright et al., 2011; Ganslosser and Brunner, 1997). Bongos prefer to consume the leaves of dicotyledonous plants or some seasonal grasses while frequently obtaining minerals from natural salt licks (Wright et al., 2011; Ganslosser and Brunner, 1997). Eating behavior of captive bongos is largely cooperative, with individuals preferring to eat under clumped conditions, resulting in longer feeding times when around other bongos (Ganslosser and Brunner, 1997). As few specifics are known about the eating habits of bongos in the wild, there has been difficulty determining the most ideal diet for captive bongos (Wright et al., 2011). Thus, captive bongos are primarily fed browse and forge consisting of regional grasses and vegetation (Wright et al., 2011). Zoos have been reliant on trial-and-error processes to improve the health of their captive bongos (Ganslosser and Brunner, 1997).

A more expansive knowledge of the feeding habits and behavior of ungulates is vital in aiding conservation efforts (Wright et al., 2011; Huffman 2014). Ungulate populations can be found in almost all geographic regions (Forthman et al., 1993). While methods to observe ungulate behavior in the wild are necessary, data from smaller samples held in captivity are especially valuable for the conservation of these species (Forthman et al., 1993). Research on captive behavior is especially important in the case of bongo antelopes, as the species has become critically endangered in the wild (Wright et al., 2011). Additionally, behavioral studies of captive animals help to maximize the health of the animals being studied and overall captive populations (Wright et al., 2011). In order to continue to improve ungulate husbandry, it is also beneficial to focus on the space provided to captive animals (Browning et al., 2019). Feeding behavior can change depending on the space and graze made available to ungulates, with less-than-ideal conditions leading to more aggression and lower overall health (Ganslosser and Brunner, 1997). These aggressive behaviors could influence the perception that zoo visitors have of the animals (Ganslosser and Brunner, 1997). The ability to make choices allows captive animals to feel a sense of control, which extends to the ability to move according to environmental factors such as rain and sunshine (Browning et al., 2019). Ultimately, an emphasis on behavioral and spatial analysis can bolster ungulate welfare both in captivity and in the wild (Martin, 2000; Browning et al., 2019).

Our research question focuses on the differences between Grant's gazelles and bongos in behavioral, grouping, and grazing patterns. In this experiment, we test three different hypotheses. With regards to behavior, we hypothesize that Grant's gazelles exhibit different behavioral trends than the bongos. We predict that the gazelles will spend more time grazing, while the bongos will spend more of their time resting. For grouping, we hypothesize that gazelles tend to group together, while bongos are more solitary. Here, we predict that gazelles will graze in groups of four or more, and bongos will graze alone. We predict that Grant's gazelles will display more group behavior because of their basic tendency for gregariousness in the wild (Walther 1972). In comparison, free-ranging bongos have been observed grazing longer in groups, but often graze alone when food is not in clumped conditions (Ganslosser and Brunner, 1997). Finally, we hypothesize that the gazelles and bongos will utilize different areas of the exhibit for grazing. Our final prediction is that while gazelles will spend most of their time grazing in the area in front of the watering hole, bongos will prefer to graze behind the watering hole. We expect Grant's gazelles to utilize the larger front portion of the exhibit for grazing due to their large home range in the wild (Martin 2000). Because bongos do not live in open areas like gazelles, we expect them to prefer being in the shade and surrounded by trees or shrubbery (Ralls, 1978).

## Methods:

### *Study Subjects and Location*

Our study was focused on the multispecies African Veldt exhibit at the Memphis Zoo. The study subjects included the five Grant's gazelles and six bongo antelopes contained within the exhibit. The gazelle population in the exhibit consists of two adult females, two juvenile females, and one adult male. All gazelles are offered Mazuri ADF-16 pellets in four bowls that vary in location throughout the exhibit. In addition, the gazelles are offered free-choice alfalfa hay throughout the day in various locations and have access to the ostrich's food. There are six bongos that cohabitate with the gazelles. They are two juvenile males, one adult male, and three adult female bongos. These bongos are offered the same Mazuri ADF-16 pellets in the barn each morning, and any leftovers are placed in one bowl that is left outside of the barn. The bongos are also offered free-choice alfalfa hay throughout the day in various locations around their enclosure. At the beginning of our study, our sample size included five Grant's gazelles and six bongo antelopes. However, halfway through our observation period, the two juvenile bongos were transferred to another zoo, decreasing our bongo sample size from six to four animals.

The multi-species exhibit has a perimeter of around 246 meters and is roughly rectangular in shape (Figure 1). It is a fully outdoor enclosure made of semi-natural material and centered around a large stone fountain and watering hole. There are numerous large trees growing in the exhibit as well as scattered shrubbery. A stone wall forms the back of the enclosure with the doors to the barn set into the rocks. There is a large imitation termite mound in the back-right area of the enclosure. A ditch surrounds the grassy areas and acts as an extra barrier between the animals and the public. The back-left portion of the exhibit contains several cypress trees, has little grass, and is usually quite muddy. There is also a ditch in the back-right corner, with a rocky border placed between the grassy areas and the ditch. A wooden gate reinforced by steel bars surrounds the exhibit on all sides. To the right of the enclosure are the elephants, in front are the rhinos, oryx, and zebras, and left giraffes .

**Figure 1.** Satellite image of exhibit with gridlines and numbered cells (Google Earth).

### *Data Collection*

Data were collected by utilizing a group scan sampling method with two-minute time intervals (Martin and Bateson, 2007). At the two-minute mark, the behavior being demonstrated by the gazelles and bongos was recorded using an ethogram (Table 1), and the numbered grid map was referenced in order to determine animal's location within the exhibit (Figure 1). In addition, the grouping behavior was approximated by whether bongos or gazelles were two body lengths away from members of their own species. One person collected behavioral and spatial data on the Grant's gazelles while the other collected the same type of data on the bongos. Data were collected for two hours every Wednesday from 1:30 to 3:30 in the afternoon for five consecutive weeks.

**Table 1.** Ethogram of the recorded behaviors of Grant's gazelles and bongo antelopes

| Behavior | Code | Definition |
|---|---|---|
| Walking | WA | Moderate movement from one part of the exhibit to another. |
| Eating | EA | Grazing and consumption of grass, leaves and feed. |
| Resting | RE | Inactivity for longer than five seconds while laying down or standing. |
| Other | OT | Any other behavior (i.e. climbing, fighting, and drinking). |

### *Analysis*

Behavioral data were analyzed by calculating the mean proportion of time spent exhibiting each type of behavior. Chi-Square tests were used to determine if there was a difference in time spent exhibiting specific behaviors between the Grant's gazelles and bongos. Grouping data was gathered through calculating the mean proportion of time each species spent alone, in pairs, in groups of three, or in groups of four or more. Chi-Square tests were used to establish if there was a difference between the grouping trends of gazelles and bongos. Additionally, Chi-Square tests were also used to compare grouping behavior before and after the juvenile bongos were removed from the exhibit. In order to determine grazing preferences, all areas of the map in which gazelles and bongos demonstrated eating behavior were presented according to the average percentage of scans they spent eating per grid (Figure 4; Figure 5). The areas in which Grant's gazelles and bongos exhibited over five percent of their eating behavior were marked on the original exhibit grid, using different colors to distinguish between the species (Figure 6).

### Results:

There was no difference in the average percent of time spent eating ($X^2_1$= 1.49, p= 0.22), walking ($X^2_1$ = 3.14, p= 0.08) resting ($X^2_1$= 2.39, p= 0.7) or other ($X^2_1$ = 0.14, p= 0.7) between gazelles and bongos (Figure 2).

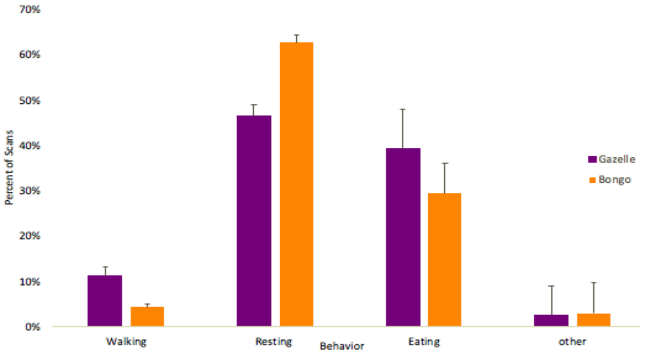

**Figure 2:** Percent of displayed behavior over five days (mean±SE).

There was no difference found between the average percent of pair groups ($X^2_1$= 1.056, p=0.3) and groups of three ($X^2_1$= 0.101, p=0.75) in gazelles and bongos. However, there was significance between individual groups ($X^2_1$= 14.4, p<0.01) and groups of four or more ($X^2_1$= 16.99, p<0.01) (Figure 3).

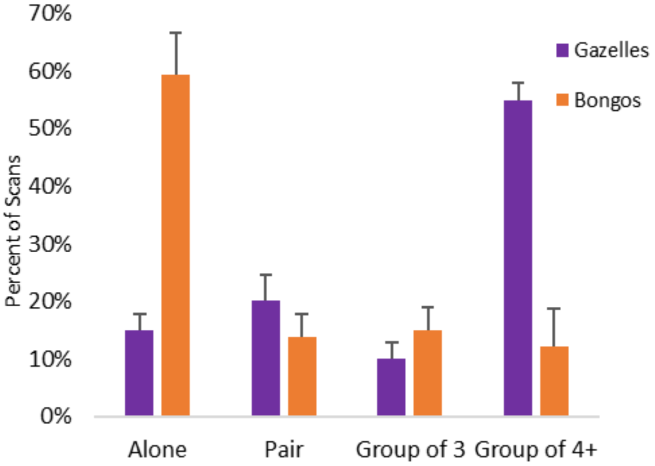

**Figure 3.** Percent of grouping behavior displayed over five days (mean±SE).

Out of the areas in which eating behavior was exhibited, Grant's gazelles demonstrated concentrated their grazing in the front portion of the exhibit in front of the watering hole. Eating behavior of the bongos was most frequent in the area behind the watering hole, closest to the back of the exhibit (Figures 4 and 5).

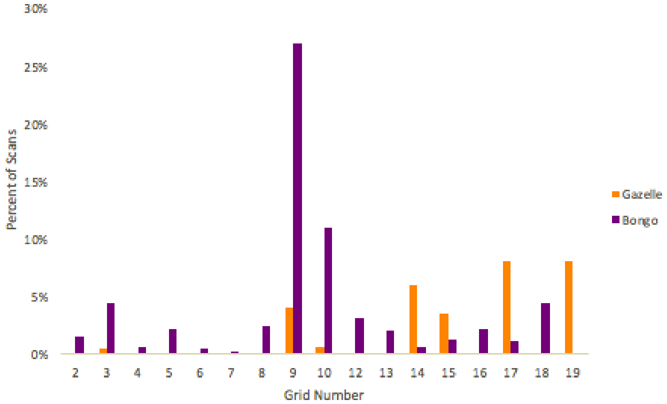

**Figure 4.** Percent of eating behavior exhibited per grid for grids 2-19 for both gazelles and bongos. Does not include grids where no eating behavior was exhibited.

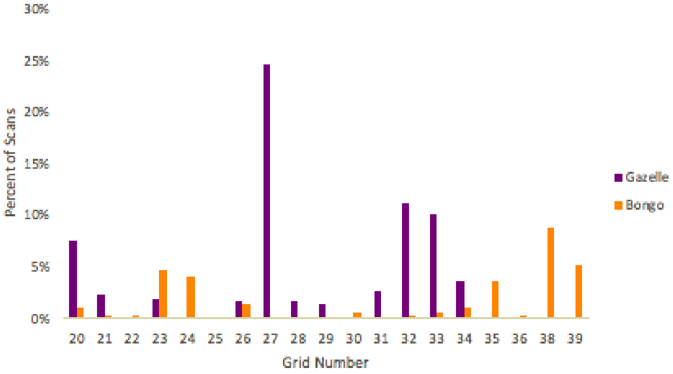

**Figure 5.** Percent of eating behavior exhibited per grid for grids 20-39 for both gazelles and bongos. Does not include grids where no eating behavior was exhibited.

Grouping data between when there were six and four bongos were also compared using Chi-square tests (Figure 7). There were differences between how often the bongos were alone ($X^2_1$=7.69, $p<0.01$) and in groups of four or more ($X^2_1$= 20.61, $p < 0.01$). There was no difference in the time they spent in pairs ($X^2_1$= 0.35, p= 0.55) or in groups of three ($X^2_1$= 2.11, p= 0.15).

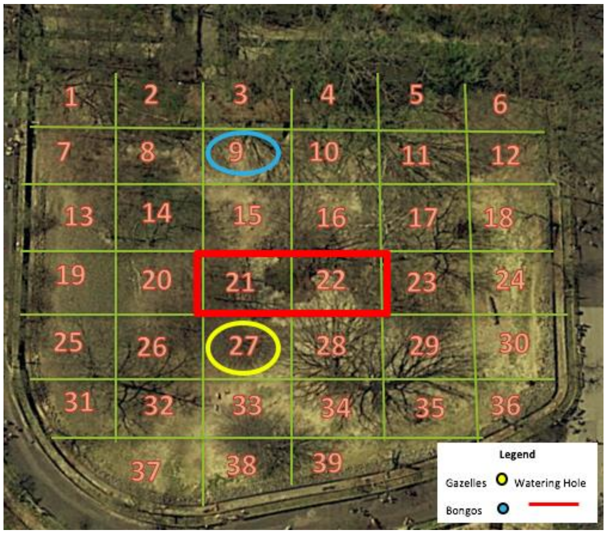

**Figure 6.** Satellite image of exhibit with greatest areas of eating concentration (Google Earth). Colored circles indicate where each species exhibited over 20% of their total grazing behavior.

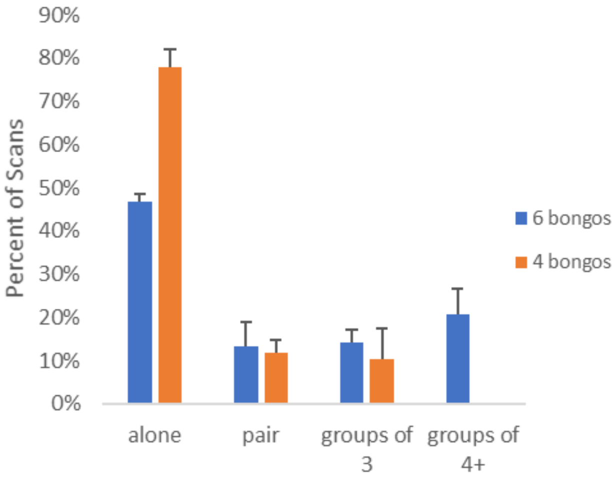

**Figure 7.** Average grouping behavior over the three days with six bongos present and two days with four bongos present (mean±SE).

## Discussion

Contrary to our original prediction that Grant's gazelles and bongo antelopes would display different behavioral trends, our statistical tests show that there were no differences in their exhibited behavioral patterns. However, there were differences in the grouping behavior of Grant's gazelles and bongos in overall percent of scans spent alone or in groups of four or more. These results support our second prediction that Grant's gazelles would display an overall tendency for gregariousness, while bongos would tend to remain solitary. These findings relate to previous studies on both captive and wild populations of bongos and Grant's gazelles. Bongos typically graze in smaller groups or alone if they are large in body size, while smaller bongos prefer larger groups and are rarely alone because they are an easier target for predators (Jarman, 1974). The preference of smaller bongos to graze in groups is evident in the bongos' grouping patterns before and after the two juveniles were removed (Figures 7). Moreover, social group size of wild gazelles is affected by the available forage, and larger groups will be found in more lush environments (Martin 2000). This is supported by the Grant's gazelle's tendency to concentrate in groups of four or more within the well-resourced zoo enclosure. Our spatial analysis supports our prediction that that bongos would concentrate in areas behind the watering hole. The captive bongo's concentration of grazing behavior within the more vegetative, back portion of the exhibit is reflective of their classification as selective grazers, meaning that they move according to where the best food source is (Ganslosser and Brunner, 1997). In contrast, the tendency of the Grant's gazelles to exhibit most of their eating behavior in the more open area at the front of the exhibit could be reflective of the dry regions in which free ranging Grant's gazelles are found and their adaptation to water scarcity (Walther 1972).

While there were originally six bongos in the exhibit, halfway through our observation period, the two juvenile bongos were transferred to another zoo. The last four hours of data were collected with only four of the bongos. This may have altered the results of the research, as the sample size changed. Despite this, we accounted for how the bongos' grouping behavior changed after the removal of the two juveniles, demonstrating that the bongos spent more time alone when there were only four individuals in the exhibit (Figures 6 and 7). However, this could have been attributed to the collection of less grouping data after the juveniles were moved.

Additional factors which could have influenced our results were the weather, zoo visitors, and behavior of other animals. During the first three days of observation, we experienced very high temperatures, while the last two days had lower temperatures. It is possible that the bongos and gazelles altered their behavior depending on the weather conditions. There were not many visitors at the zoo during the very hot hours, but visitor numbers rose drastically when the weather was cooler. An increase in zoo visitors during this time period could have resulted in more stimulation for our subjects. Furthermore, the bongos and gazelles were sharing their exhibit with a female ostrich and three male guinea fowl, who may have had an occasional influence on their behavior. The exhibit is also to the right of the African elephant enclosure, across from the rhino, zebra, and oryx enclosure, and to the left of the giraffe enclosure. There were occasional vocalizations from the animals in these exhibits, particularly the elephants, which could have had an influence on our subjects. There were also loud vocalizations from the primate section of the zoo that could clearly be heard from the African Veldt.

It is important to study grazing patterns of captive ungulates in order to better observe how climatic changes and ecological shifts are affecting these species in the wild. Both Bongos and gazelles have been deeply impacted by humans and will continue to be affected in the future (Martin, 2000; Wright et al. 2011). This is especially true of the bongo, which is an endangered species that has more individuals in captivity than in the wild (Wright et al., 2011). Through better understanding the diet, activity, and general husbandry preferences of captive bongos, scientists can employ more efficient conservation efforts to protect wild bongos and their habitats (Wright et al., 2011). Behavioral studies on captive gazelles can also be beneficial in determining the effects of human development on wild gazelle mobility patterns (Martin, 2000). As humans have settled in gazelle's wild habitats, gazelle populations have been forced to migrate outside of their preferred environments in search of resources. By better understanding gazelles' mobility patterns in captivity, we may be able to better predict how future development will affect migration patterns in certain habitats (Martin, 2000).

## Conclusion

In our study, we found that there were no differences in the behavioral patterns between the Grant's gazelles and bongo antelopes at the Memphis Zoo. There was a difference in their grouping behavior, with gazelles preferring to stick together and the bongos remaining more solitary. However, we did find differences in their grouping behavior, with gazelles preferring to group together in groups of four or more while the bongos were more solitary. Finally, we identified spatial grazing preferences for both the bongos and the gazelles, observing that gazelles portioned most of their grazing behavior in the more exposed, front area of the exhibit, while the bongos concentrated in the more vegetative and shaded back of the exhibit.

## Acknowledgments

We would like to express our gratitude to the Memphis Zoo for allowing us to conduct our research at their facility, Abby Drake for providing us with information on our study subjects, Dr. Sarah Boyle for answering our many questions and allowing us to have this opportunity, Claire McGuire and Madi Holton for helping us with our statistical tests, and the Rhodes College Department of Biology for offering this Animal Behavior class. Finally, we would like to thank the three guinea fowl in the exhibit (Moe, Larry, and Curly) and the ostrich (Olive Oyl) for providing us with constant entertainment and laughs throughout our many hours at the zoo.

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# Bottlenose dolphins in the Mississippi: sound and their preference of trawling vessels

Grace Lovett

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*The cognitive ability of dolphins is a result of their larger brain (Samuels and Tyack, 2000), and because of this, dolphins have demonstrated some of their unique abilities in non-primate studies (Samuels, Tyack, 2000). Dolphins are unique because of their ability to discriminate different frequencies in a way that is almost equivalent to that of a human's abilities (Tyack, 2000). This study demonstrates bottlenose dolphin social behavior and ability to distinguish different vessel frequencies. To determine if the dolphins distinguish different vessel frequencies, the number of individual dolphins, their proximity to the boat, and their social behaviors displayed in 10-minute intervals on each vessel was recorded. Dolphins were shown to appear more frequently and closer to the Miss Peetsy B.*

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## Introduction

The Bottlenose dolphin, *Tursiop truncatus,* exhibit diurnal behavior that is dependent on seasonality (Miller, 2010). In the Mississippi Sound it has been found that the local bottlenose dolphin population is the largest in the United States (Mann, 2000) and numbers are highest in the summer (Miller, 2010). However, anthropogenic disturbances such as watercraft traffic pose a risk to dolphin populations. The elevated background noise that is anthropogenically-made can prevent detection of other sounds that are important to cetaceans such as echolocation (Buckstaff, 2006) Bottle nose dolphins have the ability to filter sounds with as little as 0.2% difference in frequency (Tyack, 2000), and it has been observed that dolphins discriminate between boats based on the sounds of the vessels (Leatherwood, 1975).

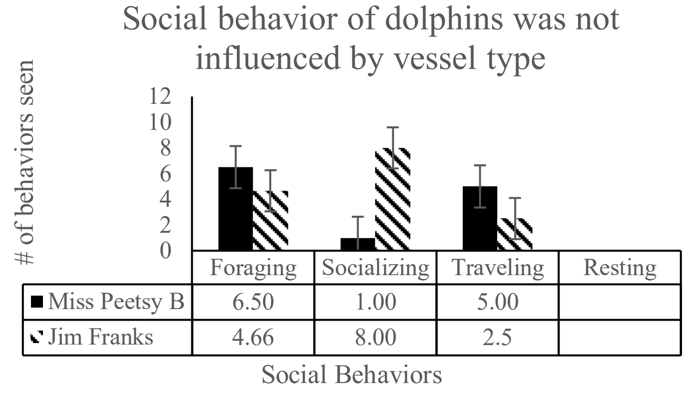
It is hypothesized that the abundance of dolphins and their behaviors will be determined by the sounds of vessels near the dolphins.

## Materials and Methods

This study took place in the costal habitat of the Mississippi Sound in the northern Central Gulf of Mexico. The study was bordered by Pascagoula to the east, south to Horn Island, west to Cat Island, and north to Bay St. Louis. Observations were conducted during five days between 21 May 2019 and 30 May 2019. Two days were non-consecutive (May 21st & 23rd), and three days were consecutive the following week (May 28th - 30th). Two different boats were used to observe dolphins' interactions with various types of boats and to document the activities of these boats. The first research boat, the *R/V Jim Franks*, is 18.3-meter-long, 7.3-meter-wide steel catamaran, that was used four out of the five days. The *Jim Franks* was equipped with a 2.4-meter draft used for pulling a standard size 4.88-meter shrimp trawl behind the vessel. The other one day of observation occurred on the *Miss Peetsy B* which was 10.36 meter-long, 3.32-meter-wide fiberglass-hull vessel. Dolphin proximity to the boat and observed behavior, such as resting, socializing, traveling or foraging, was encoded onto ethograms using continuous sampling in 10-minute segments during trawling. Using SPSS, a t-test was used to determine the significance ($p<0.05$) of two variables; proximity and abundance of dolphins. Social behaviors were tested using a paired t-test that measured socializing, foraging, and traveling for each vessel.

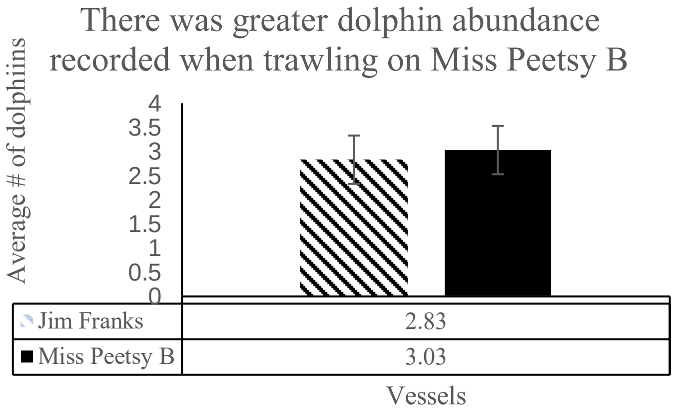
## Results

A significant difference was noted in figure 1. between social behaviors, specifically socialization, and the vessels. Socialization between dolphins appeared to more frequent when aboard the *Jim Franks*, however, it has been tested insignificant (t= .819, p= .499). The results depicted in figure 2**.** tested to be statistically significant in that a greater number of dolphins were observed from the *Miss Peetsy B* than the *Jim Franks* (t=8.9, p<0.001). It was also found in figure 3. that dolphins maintained closer proximity to the *Miss Peetsy B* when trawling than the *Jim Franks* (*t*=14.2, *p*<0.001).



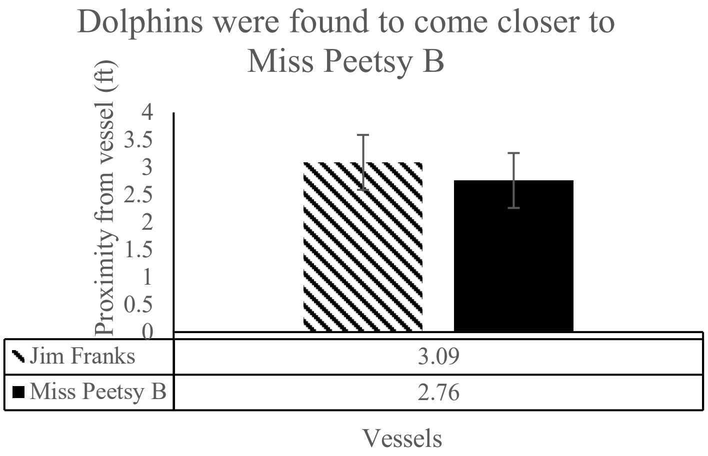
| | Foraging | Socializing | Traveling | Resting |
|---|---|---|---|---|
| ■ Miss Peetsy B | 6.50 | 1.00 | 5.00 | |
| ▨ Jim Franks | 4.66 | 8.00 | 2.5 | |

**Figure 1.** The mean observed social behaviors is insignificant in relation to boat type. Error bars indicate that there is overlap between data sets and that there could be high variance (t= .819, p= .499)



| | |
|---|---|
| ▨ Jim Franks | 2.83 |
| ■ Miss Peetsy B | 3.03 |

**Figure 2.** The number of dolphins is significantly greater when aboard the Miss Peetsy B (t=8.9, p<0.001).



| | |
|---|---|
| ▨ Jim Franks | 3.09 |
| ■ Miss Peetsy B | 2.76 |

**Figure 3.** The proximity of dolphins and the boat is significantly greater when aboard the Miss Peetsy B (t=14.2, p<0.001). The high t-value indicates that there is a significant difference.

## Discussion

The results indicated that the original hypothesis could be rejected because not all three variables tested to be significant. The *Miss Peetsy B* had more dolphins with closer proximity to the boat than the *Jim Franks,* however, it should be noted that the *Miss Peetsy B* was smaller, slower, and more familiar to the dolphins than the *Jim Franks.* This is important to note because it restricted trawling to Ocean Springs, however, social behaviors determined by boat were not significant. Replication of this study should use vessels with the same travel ranges, and more tests could be run in a longer amount of time to reinforce significant data. Insignificant data that was connected to dolphin behavior between boats could be a result of the unequal amount of days spent on each boat. The information gained from this study could possibly help future researchers determine which boats to use when observing dolphins. Additionally, watercraft pollution has caused cetaceans to display avoidance behavior (Buckstaff, 2006). It has also been found that dolphins have established relationships with certain vessels (Buckstaff, 2006). Therefore, by measuring the number of dolphins present at each trawling vessel could help establish a frequency that is more tolerable to local dolphin populations.

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# Clinical, Demographic, and Behavioral Correlates of TMS Cortical Excitability Measure in Focal Motor Epilepsy and Tumor Patients

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*We investigated whether resting motor threshold (rMT), a transcranial magnetic stimulation (TMS)-derived measure of cortical excitability, could index the altered cortical excitability in patients with epilepsy and serve as a marker for the epileptogenic hemisphere (EH). In this clinical cohort, we expected to demonstrate a correlation of rMT with age, previously observed in healthy controls, and significantly differing rMT in the EH, when compared to the non-lesional hemisphere (NH). In a chart review, we identified 114 patients with focal motor seizures due to epilepsy or brain tumor who had undergone rMT determination as part of clinical TMS motor mapping and correlated their clinical, demographic, and behavioral measures with rMT for EH and NH. Age was significantly negatively correlated ($p<0.01$) with rMT in the two hemispheres (EH: $r=0.44$, NH: $r=0.53$). Similar to healthy controls, age and rMT correlation in the patient cohort indicated that a mature motor network of older individuals was more excitable and required a lower TMS intensity to elicit a motor response. We did not observe significant differences in rMT between EH and NH, with the rMTs in the two hemispheres correlating with each other significantly ($r=0.73$, $p<0.01$). These findings are novel as this is the first study in a large pediatric cohort to establish an association between rMT and age. However, rMT did not serve as a marker of excitability in the EH in this cohort. Therefore, future studies should be aimed at identifying TMS parameters that can serve as markers of disease-specific abnormalities in cortical excitability.*

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## Introduction

Transcranial magnetic stimulation (TMS) is a non-invasive brain stimulation technique used to study the integrity of the motor pathways and cortical excitability. TMS applied to the primary motor cortex (M1) in humans elicits a brief spike in baseline activity, called the motor evoked potential (MEP), in the contralateral target muscle, measured by electrodes placed over the muscle belly. The intensity of TMS required to elicit an MEP reflects the overall excitability of the motor cortex. One measure of the overall excitability state of the corticospinal neurons contributing to the MEP response is the resting motor threshold (rMT). The rMT is defined as the TMS intensity that elicits MEPs of at least 50 μV in amplitude in 50% of trials in a non-active muscle (Rossini et al., 2015). When compared to rMT in conditions of normal excitability, the rMT is higher in conditions where cortical excitability is decreased, and is lower in conditions where cortical excitability is increased. Therefore, TMS is well suited to study disorders of cortical excitability such as epilepsy, a neurological disease characterized by repeated seizures caused by abnormal electrical activity and altered cortical excitability in the brain.

Previously, variable patterns of alterations in the motor cortical excitability patterns have been reported in individuals who have epilepsy (Badawy et al., 2013). For instance, when compared to healthy individuals, drug-naïve newly diagnosed patients with temporal lobe epilepsy were found to have higher cortical excitability in the hemisphere with the seizure focus, i.e., the epileptogenic hemisphere (EH) (Badawy et al., 2013). Pharmacological treatment has been shown to reduce this baseline hyperexcitability only in patients who become seizure-free, and not those who continue to have seizures (Badawy et al., 2013; Badawy et al., 2010). These studies have included only small numbers of adolescents (≥ 14 years), and there are no studies that have examined the cortical excitability changes specifically in children with epilepsy. Furthermore, while there are few reports on the age related increases (i.e., developmental changes) in cortical excitability in healthy children (Nezu et al., 1997; Garvey & Gilbert, 2004; Määttä et al., 2017; Säisänen et al., 2018), no studies to date have examined the evolution of cortical excitability in children with epilepsy.

To expound on these findings, we examined the TMS-derived rMT in a clinical cohort of primarily children who were evaluated for epilepsy or brain tumor at our institution. In this clinical cohort, we expected to demonstrate decreasing rMT with age, previously observed in healthy controls (Nezu et al., 1997; Garvey & Gilbert, 2004; Määttä et al., 2017; Säisänen et al., 2018). We also hypothesized that rMT in the EH would be significantly decreased when compared to the non-lesional, or non-epileptogenic, hemisphere (NH) and thereby serve as a marker for identifying the epileptogenic hemisphere (EH).

## Methods

### *Patient Cohort*

Three hundred potentially eligible patients who underwent motor mapping with TMS between January 2013 and May 2019 were identified through a retrospective review of clinical evaluations performed at the Epilepsy Monitoring Unit of the Le Bonheur Comprehensive Epilepsy Program, Le Bonheur Children's Hospital. The institutional review boards at the University of Tennessee Health Science Center and the Le Bonheur Children's Hospital approved the retrospective chart review. The patient inclusion flow chart is detailed in Figure 1.

The patient cohort included in the final analysis consisted of 114 patients having focal motor seizures due to epilepsy or brain tumor. The demographics, clinical history, and TMS-derived MT for the two groups cohort included in the final analysis are listed in Table 1.

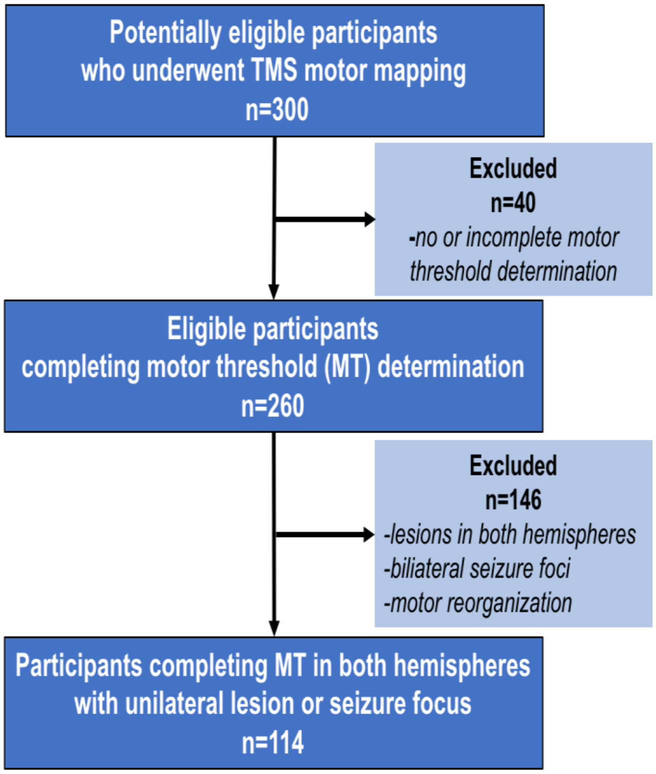

**Figure 1.** Standards for Reporting Diagnostic Accuracy Studies (STARD) flow diagram of patient inclusion in the final analysis. Data extracted from each patient included demographics (age and gender), findings from clinical evaluation (seizure hemisphere and seizure type), and TMS-derived motor threshold.

### *Transcranial Magnetic Stimulation*

In all individuals included in the study, motor mapping was performed using an MRI-navigated TMS system using a figure-8 coil (NBS system 4.0; Nexstim Inc., Atlanta, GA). The coil with current pulse passing through it is placed on the scalp, and the resulting magnetic field passes through the scalp and skull and non-invasively stimulates the underlying brain tissue. The high-

|  | Study Cohort |
|---|---|
| **Total number of patients** | 114 |
| **Children (≤ 18 years)** | 73 |
| **Adults (> 18 years)** | 41 |
| **Females** | 56 |
| **Males** | 58 |
| **Age (years) Average ±SD** | 17.8 ± 8.1 |
| **Age range (years)** | 5.0 - 41.0 |
| **Epileptogenic Hemisphere - Left** | 68 |
| **Epileptogenic Hemisphere - Right** | 46 |
| **Epileptogenic Hemisphere Motor threshold (Volts/m) average ±SD** | 112.1 ± 39.4 |
| **Non-epileptogenic Hemisphere Motor threshold (Volts/m) average ±SD** | 108.6 ± 36.0 |

**Table 1.** The demographics, clinical history, and TMS-derived rMT for the two groups cohort included in the final analysis. SD: standard deviation

resolution T1-weighted MRI of each patient was co-registered to the patient's head using anatomical landmarks and surface matching procedure implemented in the Nexstim NBS system. The MEP elicited by single pulse TMS was recorded by surface electromyography (EMG) from the adductor pollicis brevis muscle in the hand and brachioradialis muscle in the forearm using disposable electrodes (Neuroline 720, Ambu Inc., Maryland, USA) and sampled at 3 kHz and band-pass filtered from 10 Hz to 500 Hz. The motor mapping procedure began with applying TMS to cortex around the central sulcus so that the cortical location where TMS elicited the highest MEP amplitude in the contralateral hand muscle was identified. The rMT was determined at this location as the TMS intensity required to elicit an MEP ≥ 50 µV in 50% of the trials and expressed as the strength of electric field at the cortical location in Volts/m. Single pulses of TMS were then applied sequentially to neighboring cortical areas. The MEP from each site of stimulation was recorded and the spatial extent of the motor cortex, or the 'motor map', was generated and then projected on the MRI. The rMT determination and motor mapping was completed in both hemispheres.

### *Data Analysis*

For each patient included in the final analysis, age, gender, type of seizure, hemisphere of seizure focus, and rMTs in EH and NH were tabulated. We examined the association between rMT in the two hemispheres and the age of the patients in this cohort. We also examined whether the rMT in the EH was different than the rMT in the NH in this study group. The correlations were tested for significance.

## Results

### *Resting MT and age*

We found that the resting motor threshold in both epileptogenic and non-epileptogenic hemispheres decreased with increasing age of the patient. The rMT decreased more steeply in children up to the age of 18 years. In this age group, the correlation of EH and NH with age had correlation coefficients (r) of 0.44 and 0.53, which were found to be significant ($p<0.01$). In the youngest pediatric cohort (first quartile n=19, age 5-10.9 years), the average rMTs in the EH and NH were 143 V/m and 142 V/m respectively, and were significantly higher ($p<0.05$, one-tailed t-test) than the average rMT values in the two hemispheres in the patients in other three quartiles. In the oldest pediatric cohort (fourth quartile n=18, age 16-18 years), the average rMT in the EH was 111 V/m and in the NH was 99 V/m. The rMT in both hemispheres did not significantly ($p>0.05$) change with age in adults (EH: r=0.26, NH: r=0.21) with average rMT of 99 V/m in EH and 100 V/m in NH.

### *Relationship between rMT in EH and NH:*

There was no significant difference between the mean rMTs in the EH and NH in this cohort (paired t-test, p=0.18), with about half the group having higher rMT in EH and the other half having higher rMT in NH. The rMTs in the two hemispheres were found to behave in a similar manner across individual

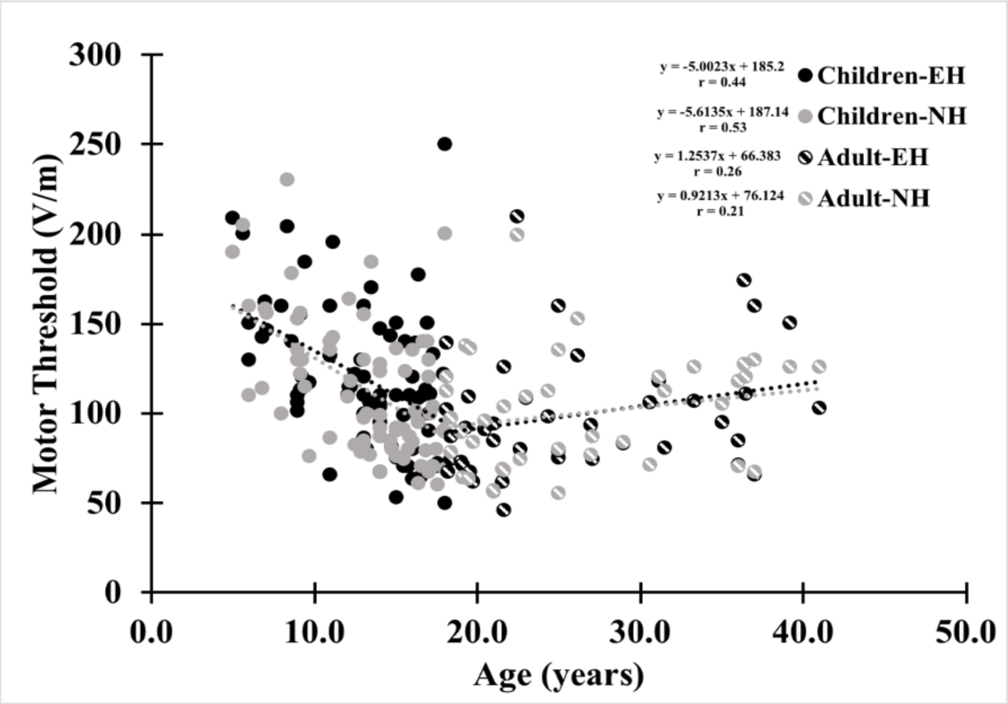

**Figure 2.** Relationship between rMT and age**.** A significant ($p<0.01$) negative correlation between age and rMT (EH: $r=0.44$, NH: $r=0.53$) indicating age-related maturity of the motor system. The rMT in both EH and NH were not significantly ($p>0.05$) correlated with age in adults (EH: $r=0.26$, NH: $r=0.21$).

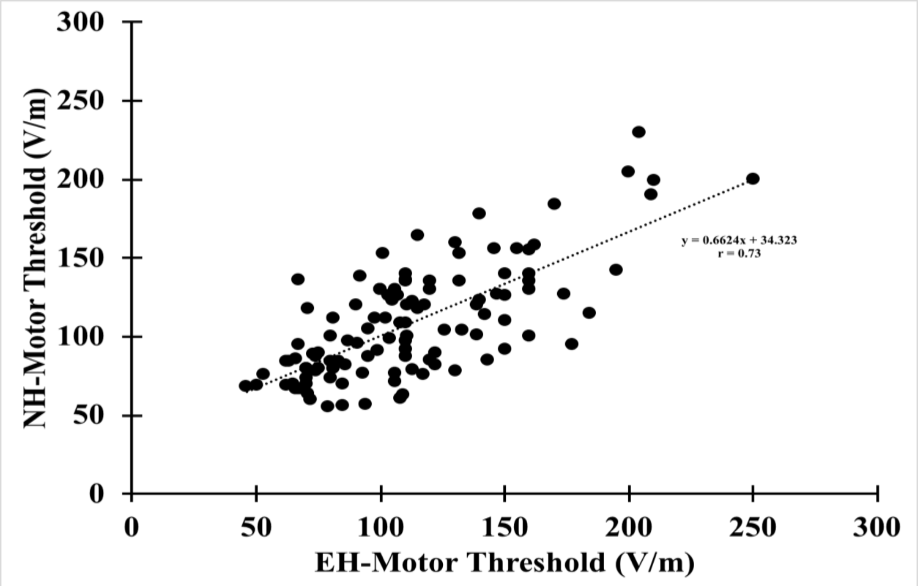

**Figure 3.** Motor thresholds in the two hemispheres. The motor thresholds in EH and NH significantly correlated with each other ($r=0.73$, $p<0.01$).

patients. In individuals where rMT in EH was found to be high, the rMT in NH was also found to be high, and vice versa. Thus, there was a significant correlation ($r=0.73$, $p < 0.01$) between the rMT in the two hemispheres.

## Discussion

This is one of the largest pediatric cohort studies examining the relationship between motor cortex excitability and epilepsy. In this cohort with focal motor seizures due to epilepsy or brain tumor, the rMT demonstrated a significant decrease with age. Age-related decrease in rMT reflects an increased excitability, which in turn represents age-related maturation of the motor system. Previously, decreasing rMT with age in a similar manner has been demonstrated in healthy children (Nezu et al., 1997; Garvey & Gilbert, 2004; Määttä et al., 2017; Säisänen et al., 2018). In our study, the rMT decrease was most notable in children under 16 years of age with the rMT in both hemispheres decreasing by approximately 25%. The rMT in the 16- to 18-year age group and the adults was not significantly different indicating, in this cohort, that motor cortex excitability reached adult levels by 16 years of age.

We did not observe significant differences in the rMT between EH and NH. Instead, we observed a significant positive correlation between the rMTs in the two hemispheres. In our cohort with children and adults with epilepsy or brain tumor having focal motor seizures, rMT was not able to identify altered cortical excitability in the EH. Previous reports of decreased rMT in the EH were in patients with new onset epilepsy or those who had just began pharmacological treatment (Badawy et al., 2013). However, our cohort was diverse and included patients with different epilepsy types and syndromes whose epilepsy was refractory to several classes of anti-epileptic drugs.

## Conclusion

These findings from this study are novel as this is the first study in a large pediatric clinical cohort to confirm an association between motor excitability and chronological age. We also found that epilepsy and/or brain tumor did not alter the overall trajectory of motor development. While motor threshold was useful in indexing global brain developmental patterns, i.e., age-related maturation, it was not effective in identifying epilepsy-induced alterations in cortical excitability. Future studies should be aimed at identifying other TMS parameters that can serve as markers of disease-specific abnormalities in cortical excitability.

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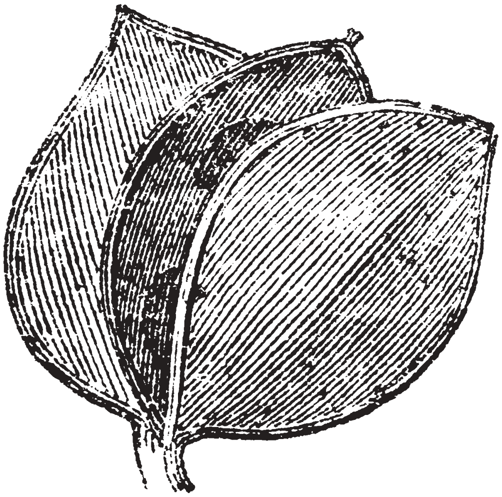
YOUR CO-EDITORS,

MADIE HOLTON & CLAIRE MCGUIRE



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