# UTILIZATION OF BLOW FLIES (*PHORMIA REGINA*) AS VERTEBRATE RESOURCE DIVERSITY INDICATORS

by

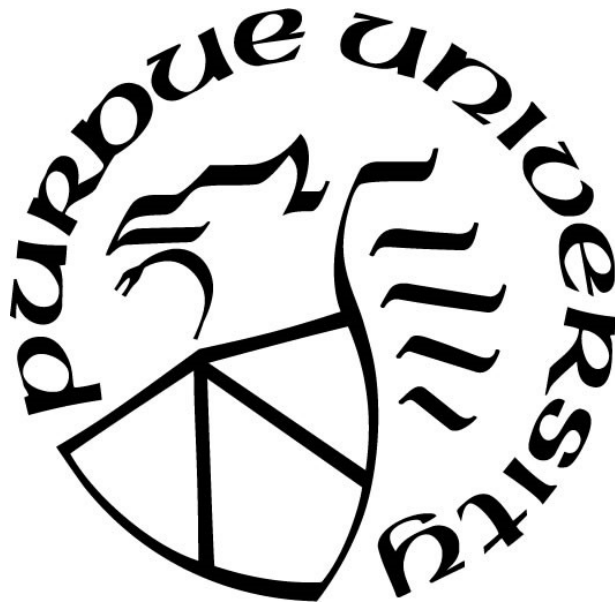**Ashton Brooke Jones**


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*Dedicated to my parents, for their constant love and support. Thank you!*

# ACKNOWLEDGMENTS

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# ABSTRACT

Blow flies are often utilized in the field of forensic science due to their ability to aid in the estimation of time since death. Currently, estimations of postmortem interval require assumptions to be made and are prone to a margin of error, prompting research that may contribute to more accurate postmortem interval estimations and help to fill in the gaps of unknown information. Blow flies are necrophagous, feeding on feces and carrion, and therefore, are constantly sampling the environment. This behavior can be exploited in order to monitor the biodiversity in an environment. Through analysis of DNA isolated from the guts of blow flies, information can be obtained regarding what animals have died in an environment, what animals are still living in that environment, and the abundance and diversity of the animals present in a specific environment. Using fly-derived ingested DNA is a viable method for vertebrate resource identification and biodiversity monitoring. Over the course of a two-summer sampling period, in and around two national parks, a total of 162 blow fly (*Phormia regina*) samples returned a positive vertebrate DNA identification, with 33 species identified from five animal orders. Of the total number of flies collected and analyzed, 23.58% returned a positive vertebrate species identification. The method detected both abundant and common species based on National Park surveys, as well as some uncommon or unknown to the park species. In the SE region, 9 individuals belonging to the Rodentia order, 12 individuals belonging to the Artiodactyla order, 21 individuals belonging to the Carnivora order, 1 individual belonging to the Cingulata order, and 3 individuals belonging to the Lagomorph order were detected. In the SE region, 63% of the individuals detected belonged to the common category, 14% of the individuals detected belonged to the uncommon category, and 23% of the individuals detected belonged to the not in park/unknown category. In the NW region, 42 individuals belonging to the Rodentia order, 46 individuals belonging to the Artiodactyla order, and 28 individuals belonging to the Carnivora order were detected. In the NW region, 52% of the individuals detected belonged to the abundant category, 36% of the individuals detected belonged to the common category, and 12% of the individuals detected belonged to the uncommon category. The relative biodiversity of the sampled environment can be inferred. In the SE region, the Shannon Biodiversity Index was calculated to be 2.28 with an evenness of 0.844, while in the NW region, the Shannon Biodiversity Index was calculated to be 2.79 with an evenness of 0.855. Unsurprisingly, there was greater biodiversity in the Northwest Park samples than in the Southeast

Park samples. Additionally, the ideal weather conditions for blow fly collection were determined be at a temperature of between 60- and 80-degrees Fahrenheit, a relative humidity between 50% and 60%, no precipitation, and a wind speed between 2 and 8 miles per hour. This information has further implications in the field of forensic science, specifically dealing with wildlife forensics, pathogen distributions, and can help to improve accuracy in regards to postmortem interval (PMI) estimations.

# CHAPTER 1.    INTRODUCTION

The first recorded use of blow flies (Diptera: Calliphoridae) in a criminal case occurred in China in the 13th- century and was documented in *The Washing Away of Wrongs* by Sung Tz'u [1]. In this case, a farmer was found in a field murdered by a sharp object; the weapon was suspected to be a sickle, a tool commonly owned by farmers. All suspects were subsequently asked to place their sickles on the ground. After some time, blow flies were attracted to trace amounts of flesh and blood, and only drawn to one sickle. This resulted in the confession by the murderer [1]. Today, in the field of forensic science, blow flies are frequently used for the estimation of postmortem interval (PMI), or the time between death and the discovery of remains [2].

## 1.1    Forensic Entomology

Forensic entomology involves the study of insects and the application of this knowledge to the law. Forensic entomology can be divided into three categories: urban, stored-product, and medicolegal [3, 4]. Urban forensic entomology encompasses civil law and involves insects as house and garden pests. For example, this category may include insects related to structural damage, such as termite damage [2]. Also included in this category is litigations involving the misuse of pesticides [3, 4]. Stored-product forensic entomology involves contamination or infestation of commercial products, such as insects in food products. Medicolegal forensic entomology, the most prevalent aspect of forensic entomology, involves the use of insects and their involvement in violent and non-violent crimes, abuse, and/or contraband trafficking [4]. Investigations involving medicolegal forensic entomology often seeks to establish a timeline of events related to death. Information that can be obtained via forensic entomology may include time since death and the determination of whether the body has been moved [5]. A common insect analyzed in forensic entomology is the blow fly [6]. Blow flies are ubiquitous, existing in a variety of habitats and locations, and are known to feed on vertebrate carrion, as well as feces and organic matter [3, 7].

## 1.2  Blow Fly Life Cycle

Insects belonging to the Calliphoridae family, particularly blow flies, are typically a necrophagous species, depending on carrion and feces for protein, ovary maturation, an oviposition medium, and larval development [8]. Adult calliphorids are typically between 6 and 14 mm in length and are characteristically shiny in their appearance, with a blue, green, or black metallic coloring and are similar in size or larger than the typical house fly [7]. Due to the need for their offspring to develop on decomposing tissue, blow flies are known to quickly locate and colonize their food source using a combination of visual and olfactory cues. Blow flies have been known to arrive on carrion within minutes following death and to oviposit (deposit offspring) within the first few hours following death [4, 9]. The pattern of insect colonization and larval development makes blow flies ideal for the estimation of postmortem interval (PMI) in the field of forensic science [6, 8, 10, 11].

The research presented here involves *Phormia regina* (Diptera: Calliphoridae*),* known as the black blow fly, one of the most common and widely studied blow flies in North America [12]. Due to its ability to thrive in a wide range of temperatures, *P. regina* is commonly found in forensic investigations all throughout the North America and Europe [12, 13]. In addition to carrion, *P. regina* has been known to be attracted to the feces of humans and other animals [12]. Only female *Phormia regina* were utilized for this project. Female blow flies were utilized in this project as they require a protein source for sexual organs maturation as well as a medium for oviposition. Thus, female blow flies must "taste" (or sample) the carrion before they can oviposit.

Adult blow flies have a few dietary needs in order to survive and reproduce. Water is needed to sustain life functions, carbohydrates are necessary for the energy for flight, and protein is necessary for the maturation of the ovaries [10]. In order to obtain these resources, blow flies will visit carrion and feces. Gravid adults, or flies that are carrying eggs, are attracted to carrion for their ability to sustain eggs [14]. Therefore, it is a necessity of blow flies to have excellent odor detection skills. Gravid females in search of suitable carrion are in search of carrion that is decomposed, but not too old, and is not too crowded in order to ensure the survival of their offspring. Presumably, gravid females assess the quality of carrion as soon as they arrive by "tasting" the carrion [15]. Because blow flies are attracted to carrion even from unknown and potentially long distances, they perceive olfactory cues first, with visual cues playing a limited role in initial attraction to carrion [11]. Blow flies sense and are attracted to odors produced by volatile

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organic compounds (VOC's) that are released as a result of the decomposition process [8, 11, 16]. When death occurs, cells begin to die and are digested by various enzymes during the process of autolysis. As decomposition begins, bacteria found in the gastrointestinal tract begin to destroy the soft tissue. This process produces volatile molecules and gases, which attract various insects, especially blow flies [9, 16]. Some of the VOCs known to be produced during decomposition include putrescine, cadaverine, butan-1-ol, butanoic acid, indole, phenol, and sulfur compounds such as dimethyl disulfide (DMDS) [11]. Female blow flies are especially sensitive to these compounds due to their need to find a suitable oviposition medium to ensure the survival of their offspring [11]. Specifically, it has been observed that phenol encourages oviposition in blow flies as the presence of this compound helps in the assessment of carrion quality and availability. Additionally, indole is known to increase in concentration as decomposition proceeds. Therefore, the detection of this compound by gravid blow flies may indicate that the carrion is not a fresh source and may not be suitable for oviposition [17].

Due to the unpredictability of vertebrate populations and gaps in current biodiversity monitoring techniques, there is a lack of knowledge in estimating carrion availability in a given environment. Carrion availability in an environment may be affected by weather extremes, disease, and biodiversity shifts. Weather extremes may include events such as drought, natural disasters, and temperature changes. Vertebrates are susceptible to death caused by these weather extremes, which would increase the proportion of carrion in an environment. Death from disease may also produce a short-term increase in carrion in an environment. Biodiversity shifts may also affect carrion availability in a particular environment and refers to situations in which the biodiversity differs from what is typically observed. For example, if predator populations increase, carrion proportions may also increase. However, if predator populations decrease, a decrease in carrion proportions may also occur. Conversely, a decrease in predator populations could be indicative of a decline in resources, meaning more animals are competing for the same resources. This scenario may cause an increase in carrion, as there are not enough resources to support the current vertebrates in the environment.

Carrion availability in an environment is also known to be influenced and determined by both carcass production and scavenging. Greater carcass production adds to the carrion availability in the environment. However, what is often overlooked is scavenging [18]. Scavenging, and often predation, subtracts from the carrion availability to blow flies in the environment. The presence of

scavenging can be illustrated by the fact that entomologists must use cages in research where carrion is being exposed in order to ensure that insects have the ability to interact with the carrion and the carrion is not being affected by scavengers. Changes in carrion availability have meaningful effects on the scavenger and decomposer populations in an environment [18, 19]. Blow flies depend on carrion for their survival, especially for the survival of eggs. Without a steady supply of carrion, it is unlikely that an environment could support a large blow fly population. If the blow fly population cannot be supported, the blow flies will commence searching for an environment that can support the population and will disperse further until they encounter the required resources. [20].

Once the adult fly has located its food source, blow flies will ingest the carrion either as a source of protein or to assess the suitability of the carrion for oviposition. Blow flies have sponging mouthparts, meaning they suck up a liquid diet via the extension of the proboscis (Figure 1) [10, 21]. The food is liquified using regurgitated saliva and/or digestive enzymes (most commonly seen with larvae and rarely seen in adult feeding) [10]. Once the blow fly has ingested their selected food source, the food source will pass through both the midgut and will be collected in the crop in the center of the abdomen (Figure 1) [10]. The crop is a specialized area of food storage. When the midgut becomes full, any remaining food will pass into the crop. No digestive enzymes are secreted in the crop, but slight digestion does occur here as a result of saliva and enzymes from the regurgitated contents of the midgut [10, 22]. The minimal digestion observed in the crop makes it ideal for the research in this project as vertebrate DNA may be retained for a longer period of time than if it was stored in the midgut.

Blow flies begin their life cycle as eggs. An adult female blow fly typically lays approximately 150-200 eggs per batch [7]. Once the blow fly has emerged from its egg, it goes through three larval stages, or instars, until it has reached a critical weight necessary to ensure the energy needed to complete metamorphosis [23]. Each instar is denoted by the larval size, with length and width increasing as the larvae progress through the three stages. The stages are also denoted by the shape of spiracles, or the breathing holes on the larvae. Once the larvae have reached their full size, feeding stops and wandering begins. During the wandering stage, the larvae are in search of a dry and dark environment so that pupariation can begin. The outer skin of the larvae will then shrink and hardens, forming a rigid and protective encasement, known as the puparium, and metamorphosis will commence [23]. Once pupariation and metamorphosis is

complete, adult eclosion occurs and the blow fly will emerge from its pupal casing as an adult blow fly. [7, 9, 23]. Once the fly has emerged as an adult fly, it must begin looking for a protein source in order for the ovaries to mature. Once the adult blow fly has located its food source and has fed, the ovaries will mature and mating can occur [24]. The gravid blow fly will detect a suitable carrion resource and oviposit its eggs. The life cycle will then be repeated (Figure 3). Laboratory studies have indicated that adult blow flies typically have a mean survival of approximately three or four weeks [24].
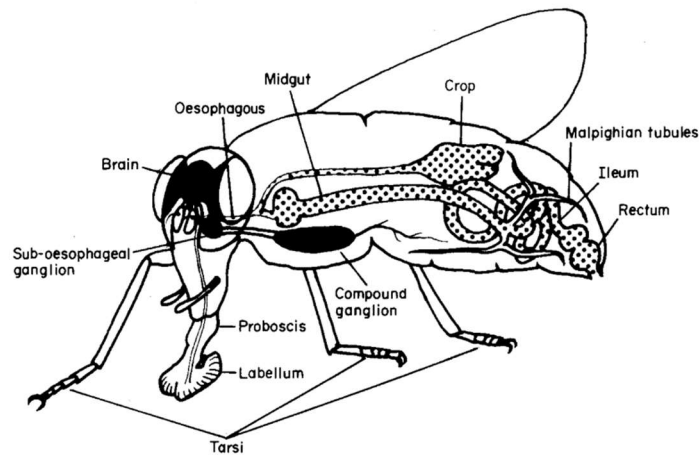


Figure 1. Diagram of a blow fly, including internal and external features important for feeding and digestion. In this research, the crop and gut were dissected and subsequently used for DNA extraction [10].
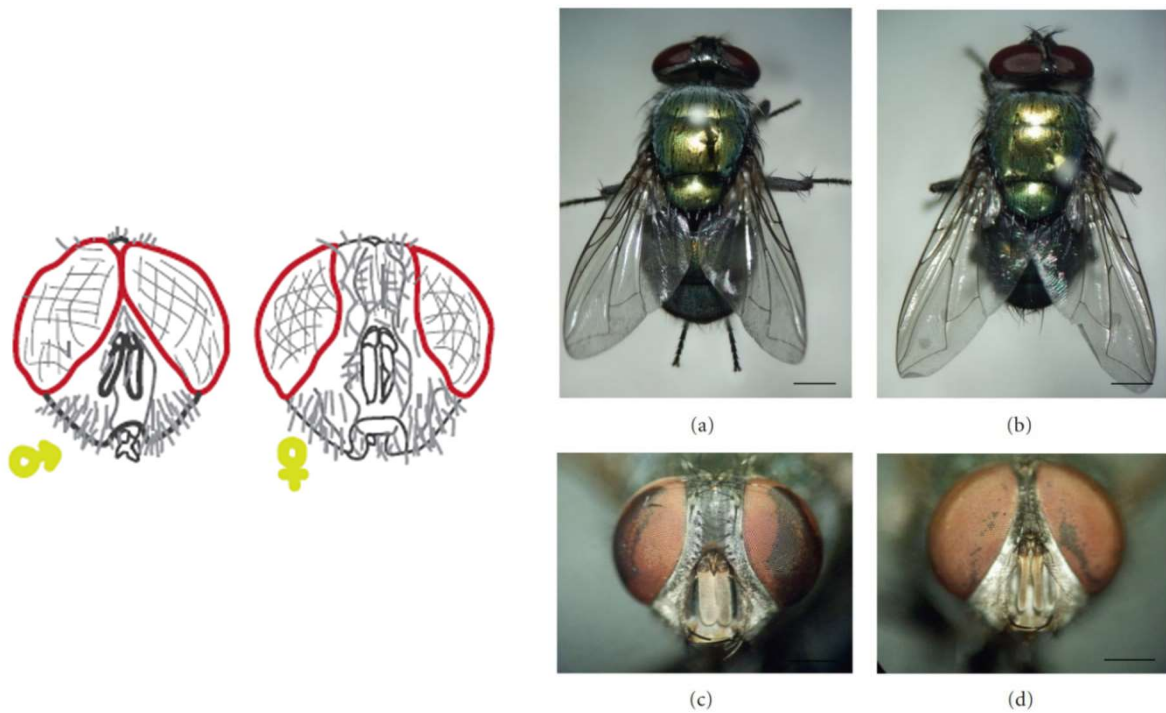
Figure 2. Diagram of male and female blow fly identification. The space between the eyes can be used to determine the sex of the blow fly. Males (B and D) will not have a space between the eyes, while females (A and C) will have a space between the eyes [25].
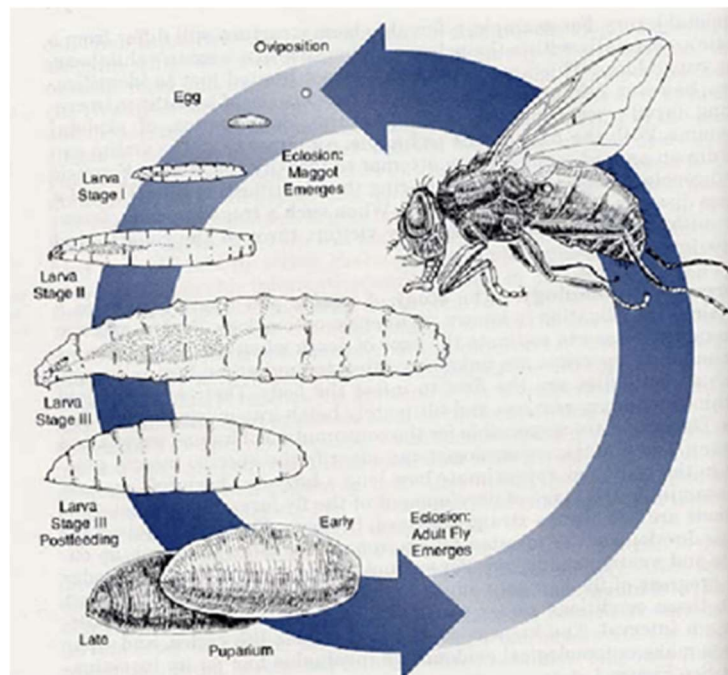


Figure 3. Diagram of the life cycle of a blow fly from egg deposition to emergence as an adult fly [26].

## 1.3    Postmortem Interval and Pre-Appearance Interval

Blow flies are known to quickly visit and colonize decomposing matter, even from long distances. This colonization occurs in a predictable pattern and sequence, known as the entomofaunal succession. Due to the predictability of this sequence, blow flies can be used for postmortem interval (PMI) estimations [11]. When death occurs, blow flies are unable to immediately detect the presence of carrion, or dead and decaying flesh of animals as olfactory cues must be spread before the blow fly can detect a carrion resource [11]. Over time, as carrion decomposes, volatile odors are released. These particular odors are sensed by the blow fly and attract the blow fly to the carrion. Once detected, the blow fly will travel to the carrion to begin colonization. This time between death and the colonization of the carrion by insects is known as the pre-appearance interval (PAI) (Figure 4) [27]. Once blow flies reach the carrion, feeding may begin, as carrion is an exceptional protein source. If the blow flies that are attracted to and arrive on the carrion are gravid and determine the carrion to be a suitable area for oviposition, then the eggs will be oviposited, and colonization occurs. The time beginning at colonization and extending to the discovery of the carrion is known as the period of insect activity (Figure 4) [20, 28].

In order for the estimated minimum PMI to be equal to the PMI, the PAI must be equal to 0, meaning that the blow flies must colonize the carrion immediately after death [28]. Rapid colonization is likely to occur if the environmental conditions (temperature, humidity, wind speed, and precipitation) are perfect and if a large number of gravid females are already present in the environment. The more gravid females that are present in an environment, the quicker oviposition on the carrion resource can occur. In theory, a large number of gravid females already present in an environment requires a large population of male and female blow flies. This only happens when there are enough carrion resources to support this large blow fly population.

There are two methods currently employed by forensic entomologists to estimate PMI [2]. One method, the developmental method, involves estimating the minimum postmortem interval ($PMI_{MIN}$) by estimating the age of the larva(e) using the length of the larva(e) and comparing it to reference development data, taking into consideration the species and environmental conditions. This method is known to estimate the minimum PMI, the shortest amount of time that could have elapsed since death, because it does not take the pre-appearance interval (PAI) into account. The PAI is the amount of time it takes for blow flies to detect, locate, and oviposit on carrion [28, 29]. The only thing that can be estimated via this method is how long the individual insect has been

associated with the carrion (i.e., how old are the larvae?). This method also makes the assumption that the collected and analyzed specimen is representative the entire assemblage present on the corpse, i.e., that the collected specimen is the oldest individual on the carrion, and this may not always be the case [9, 28]. Another problem with this method is that the species of blow flies is difficult to determine at the larval stage. In order to mitigate this problem, entomologists may rear larvae collected from the carrion into adulthood to get a final determination of species. However, even if species is determined, developmental data does not exist for all species.

The second method of PMI estimation involves the observation of the succession of species found on carrion, as well as stage of decomposition of carrion. This method does include the PAI, and can therefore be used to estimate both the minimum PMI and maximum PMI [28, 29]. In order to obtain reference data for insect succession, the forensic entomologist will frequently utilize non-human carrion (typically pigs) in similar environmental conditions. Using this method, a species will be noted as either absent or present. Over time, the reference assemblage of insects will be examined and compared to the collected assemblage in order to estimate the PMI [28]. The succession method of estimating PMI also has its own difficulties. With this method, reference data is scarce and extremely difficult to generate. In order to generate reference data, practical experiments must be performed, requiring many carrion specimens (typically pigs), collections over a great period of time, and replications in various environmental conditions [27, 28, 30]. Due to these difficulties, comprehensive reference data is difficult to obtain.
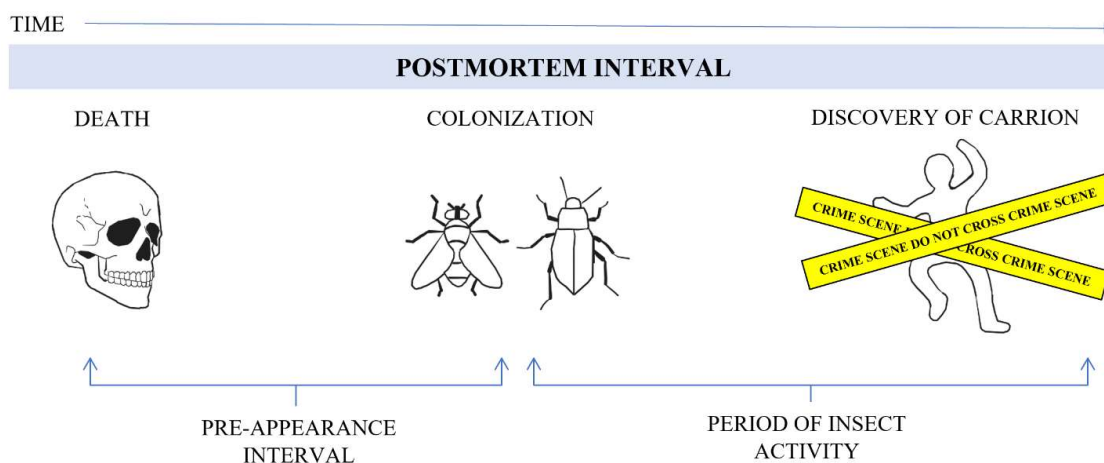


Figure 4. Diagram of postmortem interval (PMI) and the entomological process observed through decomposition.

## 1.4   Effect of Weather Factors on Blow Flies

Colonization of carrion by blow flies can take anywhere from less than an hour to several days [31]. Blow flies are poikilothermic, meaning their body temperature is influenced by the ambient temperature of the local environment [32]. Due to this attribute, various abiotic factors (or environmental conditions) are known to influence the flight activity of blow flies and their attraction to and colonization of carrion, with the potential to influence the PAI and PMI estimations. Of these abiotic factors, temperature is the most widely studied and has been known to have the greatest influence on blow fly activity [31]. A positive relationship has been observed between temperature and blow fly activity; meaning that as temperature increases, blow fly activity also tends to increase. Generally, favorable temperatures for carrion colonization of blow flies is between 12°C and 30°C (between 54°F and 86°F) [32]. Also, blow flies prefer to oviposit in moist conditions, making relative humidity another important factor to consider regarding blow fly flight activity. Relative humidity has been observed to be ideal for blow fly colonization between approximately 40 and 50 percent [31, 32]. However, previous research [31] has observed a negative relationship between relative humidity and blow fly activity, especially when relative humidity was greater than 80%.

Rain and wind speed have also been known to play an important role in blow fly activity. Rain has been known to inhibit the travel of blow flies. The largest effect can be seen when rain lasts for an extended period of time, as flies can continue their travel activity during breaks in the rain [24, 31]. Wind is an especially important environmental factor to consider when it comes to attraction to carrion, as blow flies depend on the wind to spread and deliver olfactory information regarding available vertebrate resources. If the wind does not blow, olfactory cues are not spread, and blow flies who are not in immediate proximity to the carrion cannot locate the resource quickly or may not be able to locate it at all [31]. However, if the wind speed is too great, the ability of the blow fly to travel to the detected resource will be inhibited. It has been reported that blow fly flight activity may decrease when wind speeds exceed 5 miles per hour, and flight is completely inhibited when wind speeds reach 15 miles per hour [24].

Flight activity and colonization is an important factor to keep in mind for this project. If the blow flies are not actively flying and searching for a suitable oviposition medium, they are not traveling to the available vertebrate resources in an environment to feed or oviposit. This is relevant to this research because a lack of flight and a lack of feeding or "tasting" vertebrate resources does

not allow for the blow fly to sample the environment. Without this interaction, vertebrate DNA cannot be detected via blow flies and carrion availability cannot be predicted.

## 1.5    Fly-Derived DNA

Many environments are monitored and evaluated based on the biodiversity of that specific ecosystem. Biodiversity is essential to the health and stability of a given environment [33]. A lack of biodiversity can lead to disastrous effects on the environment and species inhabiting that environment. Blow flies may be especially affected by a loss of biodiversity, as they rely on carrion and feces in the environment to feed and reproduce. If carrion and feces become scarce, a large blow fly population can no longer inhabit an environment [20]. In order to monitor the biodiversity of an environment, researchers may employ various methods such as cameras, traps, tagging, direct observations, and sampling hair, feces, soil, and water. [34-36]. Unfortunately, these methods are labor-intensive, expensive, and only provide small fragments of information; often being biased against the smaller species that frequently comprise a large proportion of the fauna [37].

Blow flies in particular are interesting in the field of biodiversity due to their constant contact with the environment. From a single blow fly, information regarding various environmental aspects can be assessed; such as the animals that have died in that environment, animals that are still living in that environment, and the abundance and diversity of those animals [38]. Using this information, collection of an abundance of these flies can give researchers a detailed and complete picture of composition and biodiversity of a particular environment. As an environment changes, blow flies continue to gather information, allowing scientists the opportunity to monitor these changes [39]. Using this information, the fly can be used to monitor the changing aspects and dynamics of the environment.

By using blow flies to sample a given environment, researchers are provided with much more information by utilizing a species that does all the sampling work for them. Blow flies are known to feed on carrion, open wounds, and feces of other animals. Blow flies are also found in a variety of habitats worldwide and are fairly easy to collect [36, 40]. This project involves DNA that is contained within invertebrates that have fed on vertebrates (invertebrate-derived ingested DNA or iDNA) [34, 35, 41]. iDNA provides the potential to reveal results that are species-specific, site-specific, and offer information relevant to species composition and mammal biodiversity in a given environment [35, 36]. Studies have been performed comparing iDNA and various trapping

methods. These studies have revealed that iDNA has detected more mammal species than traditional camera traps and pitfall trappings [37, 40, 41], suggesting that iDNA detection is a viable method that can be used in current biodiversity monitoring methods.

## 1.6    Research Purpose

Currently, in regards to the developmental method of estimating PMI, the pre-appearance interval (PAI) is unknown and varies based on environmental conditions. These unknowns and variations have the potential to affect PMI estimations, leading to inaccurate estimations and timelines. Inaccuracies in PMI estimations could conceivably cause complications in forensic investigations [27]. The goal of this research is to contribute to the further understanding of the PAI of blow flies. To accomplish this goal, it must first be determined if the methodology of the utilization of fly-derived DNA as a tool for biodiversity monitoring is a viable and effective method of estimating the quantity and diversity of vertebrates in a given environment. Vertebrate abundance and diversity information can then be applied to current PAI knowledge to obtain a more accurate estimate of PAI.  In the scope of this project, it is hypothesized that if there is greater abundance and diversity of carrion available in a given environment, then the blow fly population will also be more abundant. It would also be logical to conclude that in an environment with an increased blow fly population size, the blow flies would be more readily available in the environment, allowing for quicker detection and colonization of carrion, resulting in a shorter pre-appearance interval. Using fly-derived DNA can be used to estimate carrion abundance, and in turn, blow fly abundance. From blow fly abundance, information regarding PAI in unknown environments can be extrapolated.

In order to obtain accurate PAI estimates for a given environment, further research and extensive experimentation is required. However, environments with a known PAI can also be useful in estimating PAI in environments where PAI is unknown. For example, if PAI is known in one environment (environment A) and unknown in another environment (environment B), using fly-derived DNA, researchers can compare carrion quantity and blow fly abundance of the two environments. If environment A has a greater abundance of carrion and blow flies than environment B, it can be concluded that the PAI of environment B will be greater than environment A. This information can then be used to more accurately estimate PAI in unknown environments. In order to obtain species identity from the DNA extracted from the crops of the blow flies, the

16S mitochondrial rRNA locus was used. This widely used locus was selected because it consists of highly conserved regions interrupted by regions of high variability among species [35, 42]. This stable and specific locus has been shown to allow for differentiation among closely related species as well as species that are not particularly closely related. The 16S region has been sequenced for a multitude of species and is available in the database used for this project, NCBI's GenBank® ([www.ncbi.nlm.nih.gov/genbank/](www.ncbi.nlm.nih.gov/genbank/)) [43]. Based on previous research, the 16S mitochondrial rRNA locus was determined to be the most efficient method for sequencing the iDNA used in this project, as it is more efficient in distinguishing between mammal species as opposed to the 12S mitochondrial region [35]. Also, another previously conducted study [8] concluded that the traditionally employed *cytochrome b* primers resulted in amplification of fly DNA, causing any vertebrate DNA to be obscured.

In summary, the overarching goal of this research is to provide insights to the question: Can PAI be estimated by correlating the abundance of carrion to blow flies, while also taking environmental information into consideration? In order to study this question, this research will seek to determine the type and abundance of carrion present in the environment and to assess the ability of the flies to find these carrion. Weather conditions will also be monitored in order to assess the ideal weather conditions for detecting iDNA. This information can be applied to the PAI to further extrapolate more accurate PMI estimates.

# CHAPTER 2.    BLOW FLIES AS CARRION INDICATORS

## 2.1    Introduction

Biodiversity monitoring is currently a technique used to examine and survey environments [33]. Traditional biodiversity monitoring methods are expensive, invasive, time consuming, and biased toward larger vertebrate species [37]. The method used in this research, iDNA, uses trace amounts of DNA from the environment, specifically derived from vertebrate DNA ingested by blow flies, to detect vertebrate species and monitor the biodiversity of the particular environment [36].

The black blow fly, *Phormia regina*, was the species of interest in this research. *Phormia regina* is one of the most abundant blow flies in North America and one of the most widely studied species of blow fly [3, 12]. Due to the unique biology of blow flies, they are known to rely on carrion for survival. Carrion resources may either be a protein source or an oviposition medium [8]. Regardless of whether the carrion is a food source or oviposition medium, the blow flies are ingesting this resource, effectively sampling and storing DNA from vertebrates present in the environment [36, 37, 40, 44]. The DNA contained within the gut of the blow fly can subsequently be extracted and sequenced to determine the identity of the sampled species in an environment.

The overall goal of this research was to increase knowledge regarding PAI and subsequent PMI estimations. To accomplish this overarching goal, this research explored the determination of carrion identity and quantity derived from iDNA extracted from blow flies. This research also monitored abiotic factors that are known to contribute to blow fly activity and colonization.

## 2.2    Materials and Methods

### 2.2.1    Sample Collections

Flies were collected in the summers of 2018 and 2019 from four national parks; Mammoth Cave National Park and the Great Smoky Mountains National Park in the Southeast (SE) region, and Yellowstone National Park and Grand Teton National Park in the Northwest (NW) region (Figure 5). Furthermore, samples were collected from neighboring National Forests. The SE region included collection sites in Tennessee, North Carolina, and Kentucky, while the NW region

included sites in Wyoming, Montana, and Idaho. Multiple sites were sampled at each park area during these two years. The national parks sites were chosen due to the availability of multi-year mammal survey data of each park. This data is provided and maintained by the National Park Service (NPS) and is accessible online for each park (https://irma.nps.gov/NPSpecies/Search/SpeciesList) [45]. Fly sampling involved an aerated container with decayed chicken liver bait enclosed. The container sides and lid contained considerable air holes in order to allow the attractive VOCs to escape and spread to the surrounding environment, while also preventing the flies from landing on or ingesting the bait contaminating the vertebrate identifications (Figure 6). Once attracted to the bait, adult flies were actively caught with an aerial sweeping net. Once caught, the flies were preserved in 70% ethanol on-site and transported back to the lab [33]. Collections were performed by Drs. Christine Picard and Charity Owings.

Furthermore, the current research includes the addition of supplementary rodent reference samples provided by the National Science Foundation's (NSF) National Ecological Observatory Network (NEON) biorepository. Upon our request, known rodent DNA extracts of 56 species were provided by the NEON biorepository.
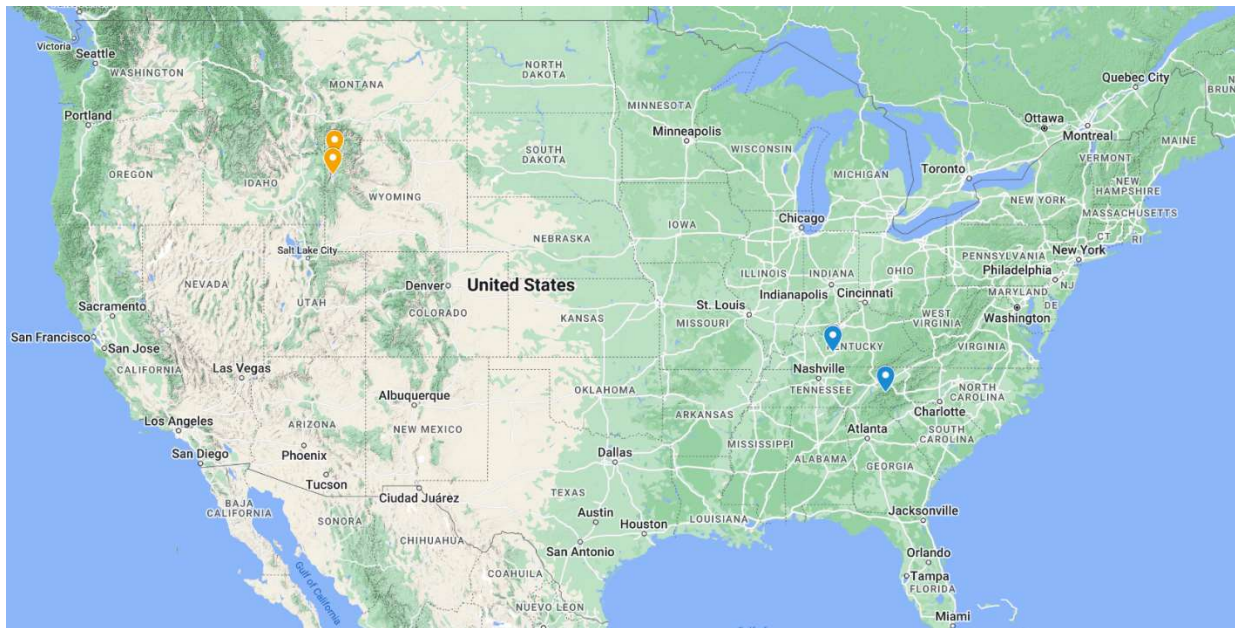
Figure 5. Overall map of areas of collection conducted in 2018 and 2019. Sites included in the SE region can be seen in blue, while sites included in the NW region can be seen in orange.



Figure 6. An example of the set-up used to attract blow flies (aerated vessel containing rotting meat).

### 2.2.2 Abiotic Factors Data Collection

Weather data was also analyzed during this research. Following collection, weather data (including temperature, relative humidity, wind speed, and precipitation) was obtained using a commercial weather service known as Weather Underground. By using the Historical Weather portion of this site, previous weather conditions could be found as recorded by the nearest weather station to the location of collection. Weather data represented consisted of daily averages, as this takes into account the abiotic factors preceding collection of blow flies

### 2.2.3 Gut Dissections and DNA Extractions

Once the flies were collected and killed in 70% ethanol, they were transported back to the lab, still submerged in ethanol, for gut and crop dissections. Before dissections could begin, the collected blow flies were removed from the ethanol and the sex of the blow flies was noted, as only female *P. regina* were utilized for this project (Figure 2). Female blow flies sample the carrion before they oviposit, making them more likely to sample vertebrate DNA than male blow flies [33]. Because the DNA of interest is contained within the gut of the blow fly, the gut must be removed from the remaining portions of the fly for further analysis. Dissection consisted of abdomen excision, then the removal of the crop and midgut (as depicted in Figure 1). Dissections were performed by examining the blow flies under the microscope and removing the abdomen. The abdomen was ripped open with sterile forceps to reveal the crop and midgut (Figure 1). Because of the difficulty of dissecting only the crop from the abdomen, both the crop and midgut were dissected and used for further DNA extraction. The dissected guts were placed a 1.5 mL microcentrifuge tube [33]. A standard organic extraction was then used to extract the DNA from the collected fly guts (performed by Khadija Moctar).

### 2.2.4 Dilution Analysis

DNA extracts were quantified using a Thermo Scientific™ NanoDrop™ 2000 spectrophotometer. Following quantification, extracted DNA samples were diluted in an attempt to dilute PCR inhibitors found in the sample along with the DNA, such as digestive enzymes produced by the fly and/or found in the fly guts. In addition to digestive enzymes, PCR inhibitors

were expected to be present due to the diet of blow flies, as feces and carrion are known to contain PCR inhibitors [46].

In order to determine the optimal concentration for amplification, three separate experiments were performed. In the first experiment, four different DNA extract samples of widely varying concentrations were chosen (627 ng/μL, 469 ng/μL, 110 ng/μL, and 20 ng/μL). In the second experiment, diluted concentrations of the same four DNA extracts, whose concentrations consisted of a narrower range, were chosen (62.7 ng/μL, 46.9 ng/μL , 20 ng/μL , and 11 ng/μL). In the third experiment, a dilution series of a single DNA extract sample was tested (62.7 ng/μL, 12.54 ng/μL, and 6.27 ng/μL). For each experiment, PCR products of these samples were run on a 1% agarose gel with nucleic acids stained with SYBR™ Safe DNA Gel Stain. The concentrations at which a band of the expected size (< 300 bp) were observed, and the optimal range of DNA concentrations was determined based on these results. Samples with quantities greater than 20 ng/μL were diluted to ~15-20 ng/μL prior to PCR amplification.

### 2.2.5   Vertebrate Species Identification

Based on previous research, it was determined that the optimal primers for successful amplification and sequencing of vertebrate DNA (without also amplifying fly DNA) was the 16S primer pair: L2513 (5'-GCCTGTTTACCAAAAACATCAC-3') and H2714 (5'-CTCCATAGGGTCTTCTCGTCTT-3') [8, 47]. The total PCR volume for each reaction was 20 μL. Each PCR reaction was comprised of 10 μL Promega® 2X Master Mix, 1 μL of 5 μM forward and reverse primer, 0.5 μL 1X Bovine Serum Albumin (BSA), 2.5 μL sterile $H_2O$, and 5 μL genomic DNA. Amplification was performed using an Eppendorf™ Mastercycler™ Pro using the following conditions: an initial denaturation at 94°C for 2 minutes, ten touchdown cycles of a 30 second denaturation at 95°C, an annealing step from 63°C to 54°C for 30 seconds, and an extension step at 72°C for 1 minute. This was then followed by 35 cycles of 95°C for 1 minute, 53°C for 1 minute, and 72°C for 1 minute, and a final extension step at 72°C for 3 minutes [8].

To visualize successful PCR amplification, PCR products were run on a 1% agarose gel and nucleic acids stained with SYBR™ Safe DNA Gel Stain. Only samples that exhibited a band at the expected size (< 300 bp) were sequenced and analyzed for further analyses. PCR amplicons (5 μL) were purified using 2 μL ThermoFisher Scientific™ ExoSAP-IT™ following the manufacturer's protocol.

All 2018 samples were sequenced using the ThermoFisher™ BigDye™ Terminator v3.1 Cycle Sequencing Kit manufacturer protocols, purified using BigDye Xterminator™ Purification Kit and then separated and detected on an Applied Biosystems™ 3500 Genetic Analyzer (ThermoFisher™). All purified PCR products from 2019 were sequenced at a commercial facility (Psomagen, Inc. standard sequencing).

Sequences were visualized using CLC Genomics Workbench software. The sequences were manually inspected, and the ends were individually trimmed. To get an initial taxonomic classification, sequences were queried using the National Center for Biotechnology Information (NCBI) nucleotide database (GenBank) using BLASTn®. The top hit was evaluated and recorded.

Due to a gap in the coverage of the database used, the National Center for Biotechnology Information's (NCBI) database, there is a possibility of misidentification of species because the BLASTn® program used identifies the closest match located within the database, not necessarily a true match. Therefore, final species classifications were made based on phylogenetic inferences.

The supplementary NEON rodent reference DNA extracts were amplified and sequenced in the same manner as the DNA extracts from the blow flies. These sequence results from 56 rodent species were then used in conjunction with the reference sequences of 65 species provided by NCBI's GenBank (Table 1) in order to construct phylogenetic trees using MEGA (Molecular Evolutionary Genetics Analysis) software [48, 49] to phylogenetically resolve a number of rodent samples that previously could not be fully resolved.

One at a time, species belonging to taxonomic groups (Artiodactyla, Carnivora, Rodentia, Cingulata, and Lagomorph) were aligned using MUSCLE (Multiple Sequence Comparison by Log-Expectation) multiple sequence alignment tool. Manual trimming of the sequences was performed using BioEdit software, and MEGA software [49] was utilized to build the phylogenetic trees. Using this software, maximum likelihood trees were constructed, and the default settings were applied (bootstrap method test of phylogeny, 100 bootstrap replications, Tamura-Nei model, uniform rates among sites, all sites used, and Nearest-Neighbor-Interchange (NNI) method). Once generated, the tree was rooted on the outgroup and a 50% cut-off value for consensus tree was employed.

The constructed phylogenetic trees show the evolutionary relatedness between organisms [50]. The maximum likelihood tree is one of the most commonly used methods for estimating phylogenetic relationships [50]. This method can be used when sequences are dissimilar from one

another and formulates hypotheses about evolutionary relationships. Final species determinations (Table 2) were made based on constructed phylogenetic trees rather than the BLASTn® results via the GenBank database.

### 2.2.6 Vertebrate Diversity Analysis

Information provided and maintained by the NPS includes what species are known to be present in each National Park and the relative abundance of these species. Species denoted as abundant are seen in relatively large numbers and may be seen daily. Species denoted as common may be seen daily, but not in large numbers or not covering a large portion of the park. Species denoted as uncommon are likely to be seen monthly or sporadically in common habitats. Species denoted as rare are likely to be seen only a few times a year. Species denoted as unknown or not in park are either species where their abundance is unknown to the NPS or are not known to be found in the particular park. Instances of vertebrate DNA where identification could not be resolved to the species level were not included in this particular analysis of abundance. Samples collected near Mammoth Cave National Park were also not included in this analysis of abundance due to the lack of availability of species abundance information.

In order to analyze the biodiversity of each region, the Shannon Diversity Index was employed. This index is one of the most commonly applied indices to determine biodiversity [51]. The Shannon Diversity Index was calculated using Equation 1. Also examined was species evenness and species richness. Species evenness examines how evenly species are distributed in a given environment [51]. Species evenness was calculated using Equation 2. Species richness refers to the number of species present in the given environment.

$$H = -\sum [(p_i) \times ln(p_i)]$$

Equation 1. The Shannon Biodiversity Index (H) equation, where $p_i$ is the proportion of each species [51].

$$E = H / \ln(k)$$

Equation 2. Species evenness equation, where H is the Shannon Biodiversity Index and k is the number of species [51].

Species accumulation curves were utilized in this research to illustrate the sufficiency of the conducted survey. In order to demonstrate this, the cumulative number of species detected was plotted as a function of collection sites. A logarithmic curve was then added to this plot to establish the number of unique species that would be expected to be detected if collections were increased. The logarithmic curve was extended until the line approached a plateau, meaning that the number of collections needed to collect the maximum number of species was being approached.

## 2.3    Results and Discussion

### 2.3.1    Reference Data

Table 1. GenBank accession numbers and NEON sample ID numbers for vertebrate reference samples. GenBank accession numbers are depicted in green, while NEON sample ID numbers are depicted in orange.

| Order | Species Name | Common Name | Accession Number or Sample ID |
|---|---|---|---|
| Artiodactyla | *Antilocapra americana* | Antelope/Pronghorn | NC_020679.1:1093-2665, JN632597.1:1093-2665 |
| Artiodactyla | *Bison bison* | Bison | KP668811.1, DQ318383.1 |
| Artiodactyla | *Bos taurus* | Domestic cattle | AB099142.1, AB099144.1 |
| Artiodactyla | *Cervus elaphus* | Elk | KJ870171.1, AB245427.2:1094-2667 |
| Artiodactyla | *Odocoileus hemionus* | Mule deer | KJ870154.1, DQ318369.1 |
| Artiodactyla | *Odocoileus virginianus* | White-tailed deer | KJ870168.1, DQ318370.1 |
| Artiodactyla | *Oreamnos americanus* | Mountain goat | DQ318385.1, FJ207535.1:1091-2664 |
| Carnivora | *Canis latrans* | Coyote | DQ334812.1, MZ042357.1:1092-2670 |

*Table 1 continued*

| | | | |
|---|---|---|---|
| Carnivora | *Canis lupus* | Gray wolf | DQ334813.1, MT796489.1:1091-2670 |
| Carnivora | *Canis lupus familiaris* | Domestic dog | KF799980.1, MW549038.1:1091-2670, KF907307.1:1091-2670, U96639.2:1091-2670, EU740414.1 |
| Carnivora | *Canis rufus* | Red wolf | MZ367921.1:1089-2668, MZ367912.1:1089-2668 |
| Carnivora | *Felis catus* | Domestic cat | LC500148.1 |
| Carnivora | *Felis silvestris* | Wildcat | KJ193070.1 |
| Carnivora | *Lynx rufus* | Bobcat | AY499296.1, AY499295.1 |
| Carnivora | *Martes americana* | Pine marten | MK320897.1:1096-2668, MK320898.1:1096-2668 |
| Carnivora | *Mephitis mephitis* | Striped skunk | NC_020648.1:1095-2667 |
| Carnivora | *Procyon lotor* | Raccoon | AB291073.1:1100-2686, AB462205.1 |
| Carnivora | *Puma concolor* | Cougar | KU884290.1, KC567464.1 |
| Carnivora | *Spilogale putorius* | Eastern spotted skunk | AM711898.1:1094-2670 |
| Carnivora | *Taxidea taxus* | Badger | HM106330.1:1099-2674 |
| Carnivora | *Urocyon cinereoargenteus* | Gray fox | KP129108.1:1095-2677, NC_026723.1:1095-2677 |
| Carnivora | *Ursus americanus* | Black bear | DQ334819.1, AF303109.1:2042-3621 |
| Carnivora | *Ursus arctos* | Brown bear | DQ334820.1, AP012592.1:2177-3755 |
| Carnivora | *Vulpes vulpes* | Red fox | LC500149.1, JN711443.1:1094-2672 |
| Rodentia | *Cavia porcellus* | Guinea Pig | DQ334847.1, NC_000884.1:1078-2642 |
| Rodentia | *Baiomys taylori* | Northern pygmy mouse | CLBJ.20170425.R1709.E, CLBJ.20170428.R1597.E |

*Table 1 continued*

| Rodentia | *Chaetodipus baileyi* | Bailey's pocket mouse | EF156818.1, SRER.20160527.135545.E, SRER.20170324.796345.E |
|---|---|---|---|
| Rodentia | *Chaetodipus californicus* | California pocket mouse | EF156819.1, SJER.20161025.R1962.E, SJER.20170218.R1077.E |
| Rodentia | *Chaetodipus eremicus* | Chihuahuan pocket mouse | EF156820.1, JORN.20170601.796351.E, JORN.20170601.796464.E |
| Rodentia | *Chaetodipus hispidus* | Hispid pocket mouse | EF156822.1, OAES.20160510.L1109.E, STER.20170504.R2833.E |
| Rodentia | *Chaetodipus intermedius* | Rock pocket mouse | EF156823.1, JORN.20160409.188858.E, SRER.20160703.056118.E |
| Rodentia | *Chaetodipus penicillatus* | Desert pocket mouse | SRER.20170325.796354.E, JORN.20170503.796448.E |
| Rodentia | *Dipodomys merriami* | Merriam's kangaroo rat | EF156804.1, JORN.20160613.R1635.E, SRER.20161028.R2003.E |
| Rodentia | *Dipodomys microps* | Chisel-toothed kangaroo rat | DQ422887.2, DQ422888.2, ONAQ.20160511.L1965.E, ONAQ.20161007.R2788.E |
| Rodentia | *Dipodomys ordii* | Ord's kangaroo rat | EF156806.1, CPER.20160825.R2857.E, ONAQ.20160511.R1663.E |
| Rodentia | *Dipodomys spectabilis* | Banner-tailed kangaroo rat | EF156809.1, JORN.20160409.R1217.E, SRER.20161029.R2104.E |
| Rodentia | *Ictidomys tridecemlineatus* | Thirteen-lined ground squirrel | OAES.20160831.L1910.E, CPER.20160603.296137.E |
| Rodentia | *Marmota flaviventris* | Yellow-bellied marmot | NC_042243.1:1111-2672, MH987778.1:1111-2672 |
| Rodentia | *Marmota monax* | Woodchuck | AY227473.1 |
| Rodentia | *Microdipodops megacephalus* | Dark kangaroo mouse | EU861127.1, EU861126.1 |
| Rodentia | *Microdipodops pallidus* | Pale kangaroo mouse | DQ534301.1, DQ534300.1 |
| Rodentia | *Microtus chrotorrhinus* | Rock vole | NC_057557.1:1084-2645, MN058078.1:1084-2645 |
| Rodentia | *Microtus ochrogaster* | Prairie vole | KT166982.1:1132-2651, NC_027945.1:1132-2651 |

*Table 1 continued*

| Rodentia | *Microtus oregoni* | Creeping vole | ABBY.20170421.R0114.E, ABBY.20170621.R0357.E |
|---|---|---|---|
| Rodentia | *Microtus pinetorum* | Woodland vole | KONZ.20160709.R3454.E, SCBI.20170518.R2161.E |
| Rodentia | *Microtus richardsoni* | Water vole | MT381944.1:1091-2651, NC_049220.1:1425-2985 |
| Rodentia | *Microtus xanthognathus* | Taiga vole | BONA.20170731.R2766.E, BONA.20170802.R2438.E |
| Rodentia | *Mus setulosus* | Peter's mouse | GU830868.1, GU830864.1 |
| Rodentia | *Myodes gapperi* | Southern red-backed vole | MLBS.20171012.R2097.E, RMNP.20170816.R1834.E |
| Rodentia | *Myodes rutilus* | Northern red-backed vole | MK482363.1:1088-2603, HEAL.20160601.R0300.E, BONA.20170628.R2032.E |
| Rodentia | *Napaeozapus insignis* | Woodland jumping mouse | BART.20160513.L2361.E, STEI.20160729.R2063.E |
| Rodentia | *Neotoma albigula* | White-throated woodrat | DQ179758.1, DQ179757.1, MOAB.20170621.MR3364.E, JORN.20161104.L2131.E |
| Rodentia | *Neotoma floridana* | Eastern woodrat | DQ179754.1, DQ179721.1, UKFS.20170522.R2076.E, JERC.20161026.R1051.E |
| Rodentia | *Neotoma mexicana* | Mexican woodrat | DQ179747.1, KY707300.1:1596-3104, SRER.20180521.R2772.E, MOAB.20160924.L1955.E |
| Rodentia | *Neotoma micropus* | Southern plains woodrat | DQ179750.1, DQ179740.1, JORN.20170506.R2595.E, CLBJ.20160706.R2100.E |
| Rodentia | *Ochrotomys nuttalli* | Golden mouse | OSBS.20170420.R0414.E, ORNL.20160504.R1576.E |
| Rodentia | *Onychomys arenicola* | Mearn's grasshopper mouse | JORN.20160608.L1797.E, JORN.20160610.R1449.E |
| Rodentia | *Onychomys leucogaster* | Northern grasshopper mouse | NC_029760.1:1172-2739, KU168563.1:1172-2739, STER.20170531.R1541.E, MOAB.20170819.MR3488.E |
| Rodentia | *Onychomys torridus* | Southern grasshopper mouse | JORN.20180508.R2830.E, JORN.20180607.R3062.E |
| Rodentia | *Perognathus flavus* | Silky pocket mouse | EF156826.1, CPER.20161026.120599.E, CPER.20170418.120574.E |

*Table 1 continued*

| | | | |
|---|---|---|---|
| Rodentia | *Perognathus inornatus* | San Joaquin pocket mouse | SJER.20170330.501562.E, SJER.20170427.501633.E |
| Rodentia | *Perognathus parvus* | Great basin pocket mouse | EF156829.1, ONAQ.20160709.355602.E, ONAQ.20170825.128506.E |
| Rodentia | *Peromyscus boylii* | Brush mouse | SJER.20170118.R1959.E, SRER.20160526.R1630.E |
| Rodentia | *Peromyscus crinitus* | Canyon mouse | KY707308.1:1534-3038, NC_035614.1:1534-3038, MOAB.20161027.ML3197.E |
| Rodentia | *Peromyscus eremicus* | Cactus mouse | NC_047188.1:1140-2648, MT078819.1:1140-2648, SRER.20160526.R1713.E, SRER.20170324.R2438.E |
| Rodentia | *Peromyscus gossypinus* | Cotton mouse | DELA.20160630.R1253.E, JERC.20170616.R0042.E |
| Rodentia | *Peromyscus keeni* | Northwestern deer mouse | ABBY.20170420.R0188.E, ABBY.20170716.R0151.E |
| Rodentia | *Peromyscus leucopus* | White-footed mouse | KM225832.1, AF364506.1, GRSM.20160607.R1677.E, BLAN.20160406.R1757.E |
| Rodentia | *Peromyscus maniculatus* | Deer mouse | MH260579.1:1095-2626, NC_039921.1:1095-2626, BLAN.20160607.R1891.E, ABBY.20170420.R0189.E |
| Rodentia | *Peromyscus merriami* | Mesquite mouse | SRER.20160525.R1774.E, SRER.20160701.R1427.E |
| Rodentia | *Peromyscus polionotus* | Oldfield mouse | KY707301.1:1431-2942, NC_035571.1:1431-2942, TALL.20180420.R1791.E, JERC.20161026.R1388.E |
| Rodentia | *Peromyscus truei* | Pinyon mouse | SJER.20170120.R1055.E, MOAB.20160603.R2837.E |
| Rodentia | *Phenacomys intermedius* | Western heather vole | MT381941.1, NIWO.20170628.NR2028.E, NIWO.20170628.NR2029.E |
| Rodentia | *Reithrodontomys fulvescens* | Fulvous harvest mouse | CLBJ.20160802.R1995.E, OAES.20160512.R1950.E |
| Rodentia | *Reithrodontomys humulis* | Eastern harvest mouse | ORNL.20160602.R1381.E, DSNY.20161107.L0132.E |
| Rodentia | *Reithrodontomys megalotis* | Western harvest mouse | SJER.20170218.R1071.E, CPER.20160628.2206654.E |

*Table 1 continued*

| | | | |
|---|---|---|---|
| Rodentia | *Reithrodontomys montanus* | Plains harvest mouse | CLBJ.20170331.R1720.E, CPER.20160602.L2831.E |
| Rodentia | *Sigmodon arizonae* | Arizona cotton rat | SRER.20180521.R3170.E, SRER.20170323.R2479.E |
| Rodentia | *Sigmodon hispidus* | Hispid cotton rat | CLBJ.20160803.R2411.E, ORNL.20160503.R1358.E |
| Rodentia | *Spermophilus armatus* | Uinta ground squirrel | YELL.20180706.R0108.E, YELL.20180707.R0016.E |
| Rodentia | *Sphiggurus insidiosus* | Porcupine | JX312693.1:1097-2669 |
| Rodentia | *Tamias amoenus* | Yellow-pine chipmunk | AF147678.1, KY070171.1:1108-2690, YELL.20180610.R0004.E, YELL.20180706.R0106.E |
| Rodentia | *Tamias minimus* | Least chipmunk | AF147686.1, ONAQ.20160511.R1748.E, RMNP.20170817.R2488.E |
| Rodentia | *Tamias rufus* | Hopi chipmunk | AF147693.1, NC_032371.1:1106-2688, MOAB.20170523.MR2111.E, MOAB.20160925.ML3122.E |
| Rodentia | *Tamias striatus* | Eastern chipmunk | AY227476.1, NC_032375.1:1105-2687, BART.20160512.R2314.E, GRSM.20160512.R1647.E |
| Rodentia | *Tamias townsendii* | Townsend's chipmunk | AF147698.1, ABBY.20170422.R0136.E, ABBY.20170620.844812.E |
| Rodentia | *Tamiasciurus hudsonicus* | Red squirrel | AY227504.1, AF147684.1, TREE.20160712.R3547.E, DEJU.20170727.R2865.E |
| Rodentia | *Zapus hudsonius* | Meadow jumping mouse | BLAN.20170525.R2508.E, WOOD.20160604.R2053.E |
| Rodentia | *Zapus princeps* | Western jumping mouse | WOOD.20170523.R3027.E, NOGP.20160608.R2188.E |
| Rodentia | *Zapus trinotatus* | Pacific jumping mouse | ABBY.20170523.R0145.E, ABBY.20170619.L0369.E |
| Cingulata | *Dasypus novemcinctus* | Nine-banded armadillo | Y11832.1:1105-2682, KF799981.1 |
| Lagomorpha | *Sylvilagus floridanus* | Eastern cottontail | DQ334836.1, AY011158.1 |

## 2.3.2 Dilution Analysis

For this research, it was necessary to determine the optimal concentration of DNA that would result in successful amplification. The first dilution experiment tested four different DNA extract samples with differing concentrations: 627 ng/μL, 469 ng/μL, 110 ng/μL, and 20 ng/μL. When run on a 1% agarose gel, the results of this experiment exhibited a band of the expected size range (< 300 bp) for the sample containing 20 ng/μL of DNA (Figure 7). This indicated that successful amplification occurred with 20 ng/μL of DNA.

The second dilution experiment tested the same four DNA extract samples at diluted concentrations: 62.7 ng/μL, 46.9 ng/μL, 20 ng/μL, and 11 ng/μL. When run on a 1% agarose gel, the results of this experiment exhibited a band of the expected size range for the sample containing 20 ng/μL and a possible faint band for the sample containing 62.7 ng/μL of DNA (Figure 8). This indicated that successful amplification occurred with 20 ng/μL and possibly with 62.7 ng/μL of DNA.

The third dilution experiment tested a dilution series of the same DNA extract sample at three DNA concentrations: 62.7 ng/μL, 12.54 ng/μL, and 6.27 ng/μL. Because the results of the previous dilution experiment indicated a possible band of the expected size range for the sample containing 62.7 ng/μL of DNA, this sample was run on the gel again. When run on a 1% agarose gel, the results of this experiment exhibited a band of the expected size range for the samples containing 12.54 ng/μL and 6.27 ng/μL of DNA (Figure 9). This indicated that successful amplification occurred with 12.54 ng/μL and 6.27 ng/μL of DNA. Based on the results of these three experiments, it was determined that amplification was successful with DNA concentrations between approximately 6 ng/μL to 20 ng/μL.
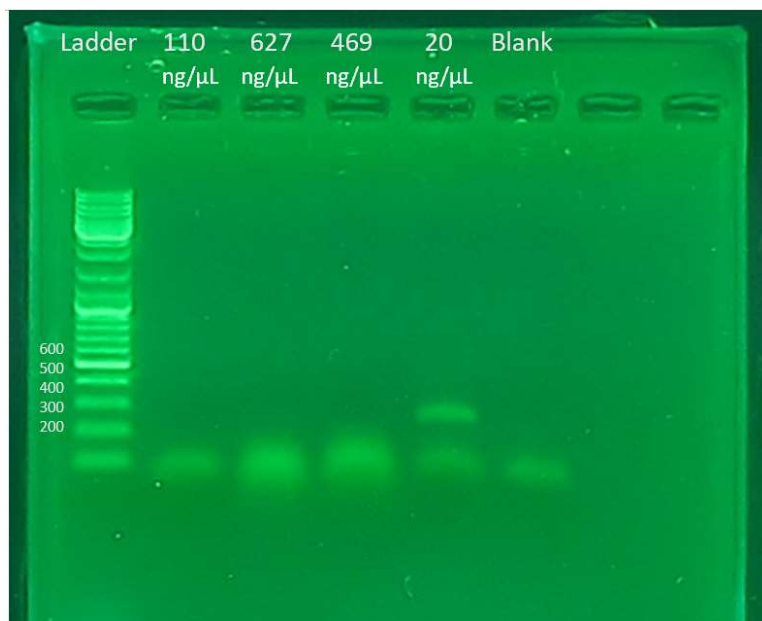
Figure 7. Gel results of the first dilution experiment. The DNA concentrations tested included 627 ng/µL, 469 ng/µL, 110 ng/µL, and 20 ng/µL. A band of the expected size was observed for the sample containing 20 ng/µL of DNA.
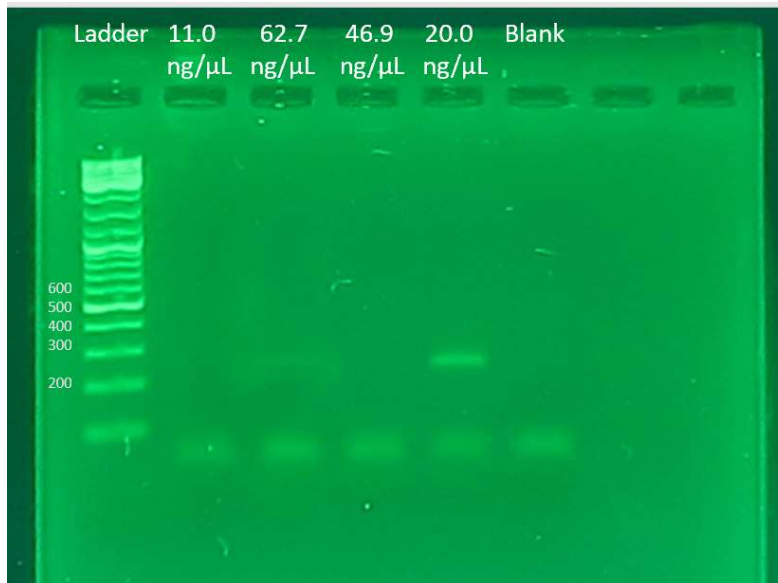


Figure 8. Gel results of the second dilution experiment. The DNA concentrations tested included 62.7 ng/µL, 46.9 ng/µL, 20 ng/µL , and 11 ng/µL. A band of the expected size was observed for the sample containing 20 ng/µL of DNA and a possible band was observed for the sample containing 62.7 ng/µL of DNA.
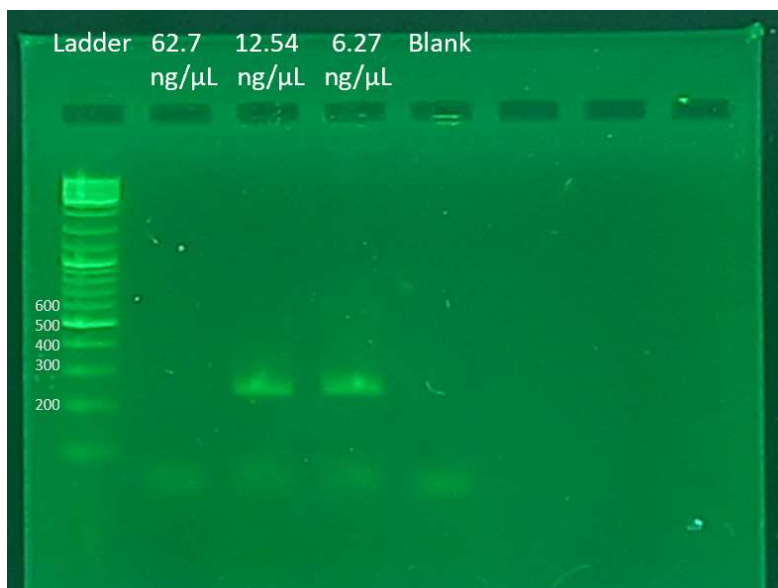
Figure 9. Gel results of the third dilution experiment. The DNA concentrations tested included 62.7 ng/µL, 12.54 ng/µL, and 6.27 ng/µL. A band of the expected size was observed for the sample containing 12.54 ng/µL of DNA and the sample containing 6.27 ng/µL of DNA.

### 2.3.3 Species Identifications and Abundance

A total of 691 female *P. regina* were collected for further testing from both park regions over the course of two summers (297 samples originating from the SE region and 394 samples originating from the NW region). Of these, 304 samples successfully amplified (44%, 111 samples originating from the SE region and 193 samples originating from the NW region). The sequence results of the samples that were successfully amplified were analyzed, and the National Center for Biotechnology Information (NCBI) nucleotide database (GenBank) was searched utilizing the BLASTn® program. A presumptive taxonomic classification was done based on the BLASTn® results.

Of the samples that successfully amplified, approximately 31% (93 samples) were revealed to be sequence mixtures that could not be deconvoluted, meaning flies may have fed on multiple carrion resources. This was evident based on overlapping peaks visible in the electropherograms. Also, due to the fact that this research was focused on vertebrate resources specifically, samples returning human DNA were excluded from further analysis. Of the samples that successfully amplified, approximately 16% (49 samples) were determined to contain human DNA.

When resolutions to the species level were unable to be made, family names were utilized for further analysis. A total of 10 families were unable to be resolved to the species level. These families included deer (*Odocoileus* sp. – 22 instances), bears (*Ursus* sp. – 6 instances), canids (*Canis* sp. – 29 instances), squirrels (*Sciuridae* sp. – 4 instances), voles (*Arvicolinae* sp. – 2 instances), red-backed voles (*Myodes* sp. – 3 instances), meadow voles (*Microtus* sp. – 1 instance), kangaroo rats (*Dipodomys* sp. – 2 instances), pocket mice (*Perognathinae* sp. – 1 instance), and an unknown rat or mouse family (*Neotominae* sp. – 1 instance). The unresolved species identifications of these samples proved to be a limitation observed over the course of this project. This has been especially evident in the rodent species. This may be due to gaps still present in the reference samples found within the database and the NEON rodent samples.

The locations where blow fly collection occurred in the SE region over the course of 2018 and 2019 can be observed in Figure 10. The array of species that were detected at each of these locations over both years can be seen in Figures 11 and 12. Of the vertebrate species detected in 2018 in the SE region, 41.2% were Rodentia (yellow-bellied marmot, southern flying squirrel, guinea pig, and eastern chipmunk), 29.4% were Carnivora (canid, gray fox, and bear), 17.6% were Lagomorpha (eastern cottontail), and 11.8% were Artiodactyla (white-tailed deer and cattle). Of the vertebrate species detected in 2019 in the SE region, 55.2% of the species were Carnivora (canid, raccoon, bear, and red fox), 34.5% of the species were Artiodactyla (white-tailed deer, cattle, and elk), 6.9% were Rodentia (woodchuck, and chipmunk), and 3.4% were Cingulata (nine-banded armadillo). Similarly, Figure 13 illustrates the locations where blow fly collection occurred in the NW region over the course of 2018 and 2019. The array of species that were detected at each of these locations over both years can be seen in Figures 14 and 15. Of the vertebrate species detected in 2018 in the NW region, 57.4% were Artiodactyla (deer, elk, bison, and antelope/pronghorn), 25.5% were Rodentia (Uinta ground squirrel, yellow-bellied marmot, red-backed vole, unknown rat species, and unknown squirrel species), and 17.0% were Carnivora (canid, red fox, bear, and badger). Of the vertebrate species detected in 2019 in the NW region, 43.5% were Rodentia (yellow-bellied marmot, Uinta ground squirrel, red-backed vole, Taiga vole, water vole, southern flying squirrel, red squirrel, porcupine, pocket mouse, kangaroo rat, unknown squirrel species, and unknown vole species), 29.0% were Carnivora (canid, pine marten, bear, badger, red squirrel, and bobcat), and 27.5% were Artiodactyla (elk, deer, cattle, mountain goat, and bison).

A compiled list of common names, species names, relative locations of collection, number of instances of each species detected, and the percentage of the total detected vertebrates can all be found in Table 2. Vertebrate species detected included species belonging to five orders – Artiodactyla, Carnivora, Cingulata, Lagomorpha, and Rodentia. Samples collected from the SE region returned a considerable number of instances of canid species. Unfortunately, the canids could not be resolved to the species level which includes domestic dogs (i.e., brought in by those visiting the park as domestic dogs are not known to be naturally present in the park), coyotes, and/or wolves. Wolves are not known to be present in the SE region park, but other canid species are known to be present [45]. Samples collected from the NW region returned a considerable number of instances of deer species (the species of deer could not be resolved in this instance, but both white-tailed deer and mule deer are known to be present in the park), canid species, and yellow-bellied marmots. When collection locations were combined, a total of 33 vertebrate species were detected. Of the total species identified, 17.90% of the species detected were individuals belonging to the canid family, 13.58% of the species detected were deer, 12.35% of the species detected were elk, 8.02% of the species detected were yellow-bellied marmots, 5.56% of the species detected were domestic cattle (likely present in areas surrounding the national parks as domestic cattle are not known to be present in the parks), and 4.94% of the species detected were Uinta ground squirrels (Table 2). The remaining species detected comprised less than 4% of the total samples detected.
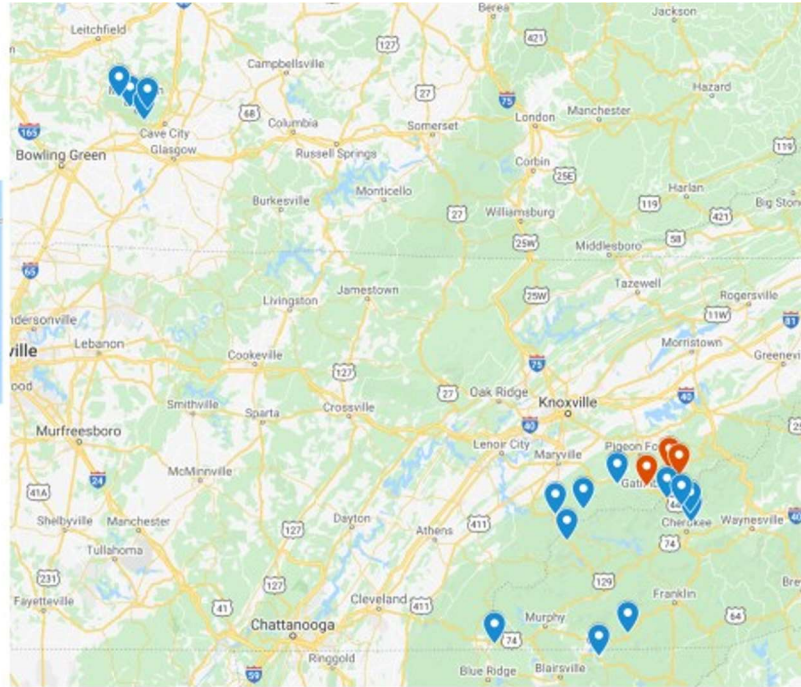
Figure 10. Locations of collections occurring in the SE regions. Markers in red are collections that occurred in 2018 and markers in blue are collections that occurred in 2019.



Figure 11. Map of what vertebrate species and how many were detected in the SE region in 2018. Site 1 is seen in blue, Site 2 is seen in orange, Site 3 is seen in yellow, and Site 4 is seen in purple. Each site was sampled on each of the three days on which collection occurred.

## 2019 SE Park



Canid sp. (2)
White-tailed deer (2)
Cow (3)

Canid sp. (4)
Raccoon (1)
White-tailed deer (1)
Cow (1)
Bear sp. (1)
Nine-banded Armadillo (1)
Eastern Chipmunk (1)

Canid sp. (2)
White-tailed deer (1)
Raccoon (1)

Canid sp. (4)
Cow (1)
Elk (1)
Red fox (1)
Woodchuck (1)

Figure 12. Map of what vertebrate species and how many were detected in the SE region in 2019. Sampling locations on Day 1 are seen in blue, Day 2 are seen in red, Day 3 are seen in purple, and Day 4 are seen in yellow.

## NW Park Collection Locations
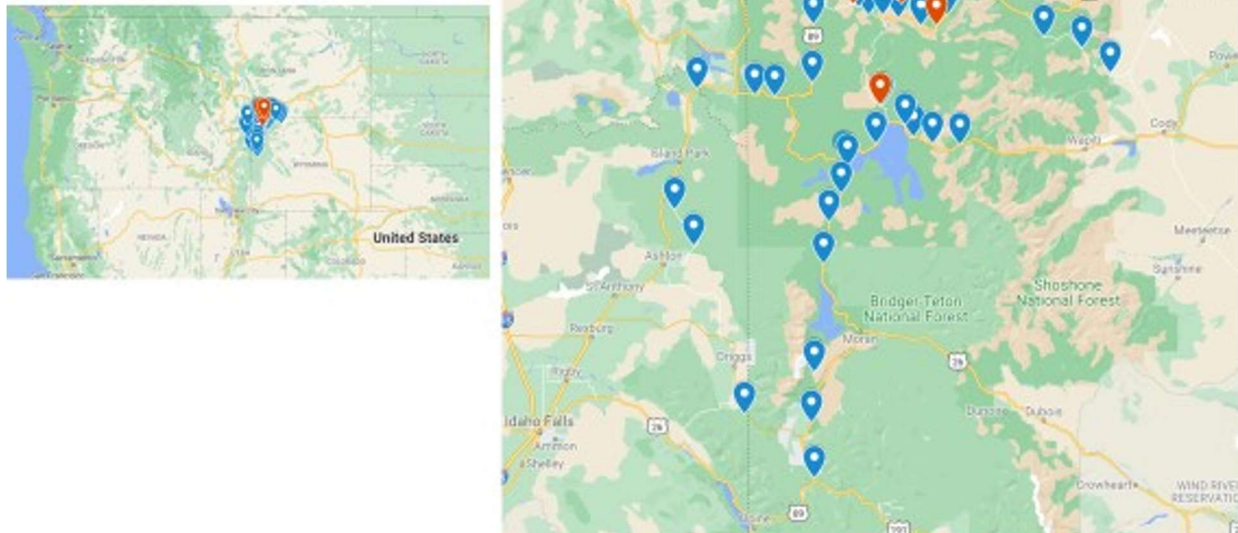


Figure 13. Locations of collections occurring in the NW regions. Markers in red are collections that occurred in 2018 and markers in blue are collections that occurred in 2019.

Figure 14. Map of what vertebrate species and how many were detected in the NW region in 2018. Site 1 is seen in blue, Site 2 is seen in orange, Site 3 is seen in yellow, and Site 4 is seen in purple. Each site was sampled on each of the three days on which collection occurred.

Figure 15. Map of what vertebrate species and how many were detected in the NW region in 2019. Sampling locations for Day 1 are seen in dark blue, Day 2 are seen in purple, Day 3 are seen in gray, Day 4 are seen in green, Day 5 are seen in orange, Day 6 are seen in blue, and Day 7 are seen in yellow.

Table 2. Summary of the vertebrate species detected via invertebrate-derived ingested DNA (iDNA). Species are separated based on mammal order (Artiodactyla, Carnivora, Rodentia, Cingulata, and Lagomorpha) and information regarding the common name, scientific name, location and number of instances of each species, and the percent of the total amount of species detected can be found.

**Mammal Order:** Artiodactyla    Carnivora    Rodentia    Cingulata    Lagomorpha

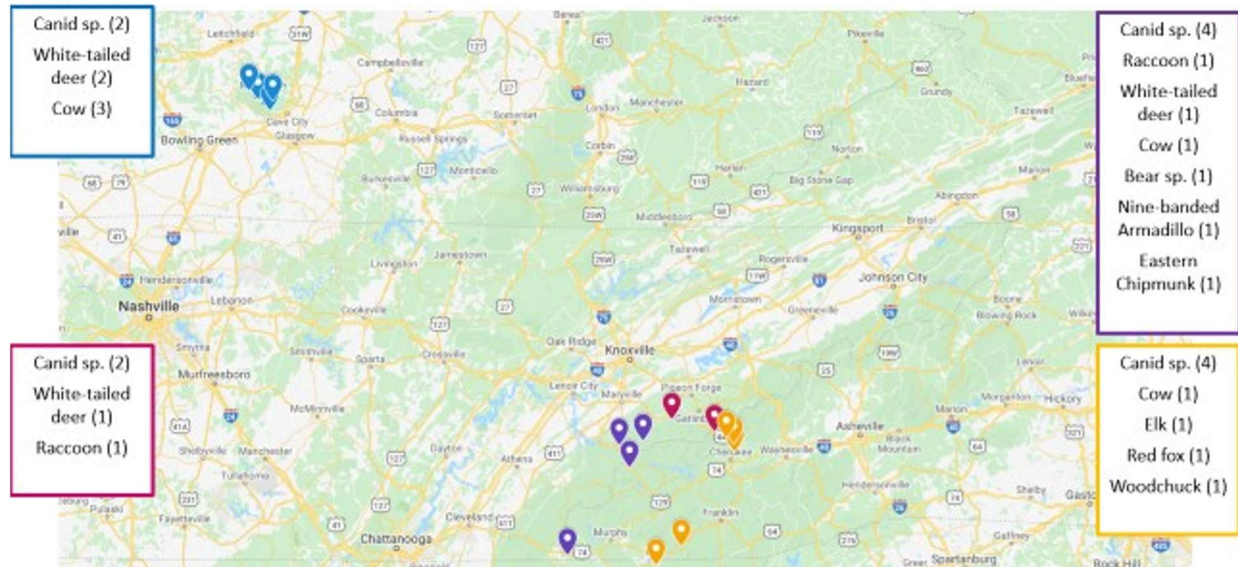| Common Name | Species | SE Park | NW Park | Total | % of samples |
|---|---|---|---|---|---|
| Cow | *Bos taurus* | 6 | 3 | 9 | 5.56 |
| Elk | *Cervus elaphus* | 1 | 19 | 20 | 12.35 |
| Bison | *Bison bison* | -- | 5 | 5 | 3.09 |
| Mountain Goat | *Oreamnos americanus* | -- | 1 | 1 | 0.62 |
| Antelope | *Antilocapra americana* | -- | 1 | 1 | 0.62 |
| Deer | *Odocoileus sp.* | 5 | 17 | 22 | 13.58 |
| Canid | *Canis sp.* | 15 | 14 | 29 | 17.90 |
| Bear | *Ursus sp.* | 2 | 4 | 6 | 3.70 |
| Gray Fox | *Urocyon cinereoargenteus* | 1 | -- | 1 | 0.62 |
| Red Fox | *Vulpes vulpes* | 1 | 4 | 5 | 3.09 |
| Bobcat | *Lynx rufus* | -- | 1 | 1 | 0.62 |
| Badger | *Taxidea taxus* | -- | 3 | 3 | 1.85 |
| Raccoon | *Procyon lotor* | 2 | -- | 2 | 1.23 |
| Pine Marten | *Martes americana* | -- | 2 | 2 | 1.23 |
| Nine-Banded Armadillo | *Dasypus novemcinctus* | 1 | -- | 1 | 0.62 |
| Eastern Cottontail | *Sylvilagus floridanus* | 3 | -- | 3 | 1.85 |
| Guinea Pig | *Cavia porcellus* | 2 | -- | 2 | 1.23 |
| Yellow-Bellied Marmot | *Marmota flaviventris* | 2 | 11 | 13 | 8.02 |
| Woodchuck | *Marmota monax* | 1 | -- | 1 | 0.62 |
| Porcupine | *Sphiggurus insidiosus* | -- | 1 | 1 | 0.62 |
| Eastern Chipmunk | *Tamias striatus* | 2 | -- | 2 | 1.23 |
| Southern Flying Squirrel | *Glaucomys Volans* | 2 | 2 | 4 | 2.47 |
| Uinta Ground Squirrel | *Spermophilus armatus* | -- | 8 | 8 | 4.94 |
| Red Squirrel | *Tamiasciurus hudsonicus* | -- | 2 | 2 | 1.23 |
| Squirrel | *Sciuridae sp.* | -- | 4 | 4 | 2.47 |
| Red-Backed Vole | *Myodes sp.* | -- | 3 | 3 | 1.85 |
| Taiga Vole | *Microtus xanthognathus* | -- | 2 | 2 | 1.23 |
| Water Vole | *Microtus richardsoni* | -- | 2 | 2 | 1.23 |
| Meadow Vole | *Microtus sp.* | -- | 1 | 1 | 0.62 |
| Vole | *Arvicolinae sp.* | -- | 2 | 2 | 1.23 |
| Pocket Mouse | *Perognathinae sp.* | -- | 1 | 1 | 0.62 |
| Kangaroo Rat | *Dipodomys sp.* | -- | 2 | 2 | 1.23 |
| Unknown Rat or Mouse | *Neotominae sp.* | -- | 1 | 1 | |
| | | | | | |
| | | | Total Vertebrate ID: | 162 | |

### 2.3.4 Species Separation

Species returning a positive vertebrate identification were separated for analysis based on mammal order [40]. A total of five mammal orders were detected: Artiodactyla (even-toed ungulates), Carnivora (carnivores), Rodentia (rodents), Cingulata (armored mammals), and Lagomorpha (hares and rabbits). Species detected belonging to each order can be seen in Table 2.

The sites of collection were also separated for further analysis. Collection sites in and/or around the Great Smoky Mountains National Park were classified as originating from the Southeast Park (SE Park) (Figures 10-12). Collection sites in and/or around Yellowstone National Park were classified as originating from the Northwest Park (NW Park) (Figures 13-15).

As exemplified in Figure 16, five orders of species were detected in the SE region over the course of the two years of collection. These orders included Rodentia, Artiodactyla, Carnivora, Cingulata, and Lagomorpha. In the SE region over two years, a majority of the vertebrate species detected were of the Carnivora order, with nearly half (48%) of the detected vertebrate DNA belonging to this order. A quarter (25%) of the detected vertebrate DNA belonged to the Artiodactyla order, 19% of the detected vertebrate DNA belonged to the Rodentia order, 6% of the detected vertebrate DNA belonged to the Lagomorpha order, and 2% of the detected vertebrate DNA belonged to the Cingulata order. In the NW region over the two years of collection, only three orders of vertebrate species were detected. These orders included Rodentia, Artiodactyla, and Carnivora, with the most frequently detected species belonging to the Artiodactyla order, comprising 40% of the detected vertebrate DNA. Additionally, 36% of the detected vertebrate DNA belonged to the Rodentia order and 24% of the detected vertebrate DNA belonged to the Carnivora order.

One of the reasons these National Parks were chosen as locations of collection was because of the retained list of species present and their abundance in each park that is maintained by the NPS. In the SE region over the two-year period of collection, the majority of vertebrate species detected were of the common category (Figure 17). In the detected vertebrate DNA, 63% belonged to the common category (species included eastern cottontail, southern flying squirrel, eastern chipmunk, raccoon, gray fox, black bear, and white-tailed deer), 23% belonged to the not in park or unknown category (species included nine-banded armadillo, yellow-bellied marmot, and guinea pig), and 14% belonged to the uncommon category (species included elk, red fox, and woodchuck) (Figure 17). In the NW region over the two-year period of collection, the largest majority of

vertebrate species detected were of the abundant category (Figure 17). In the detected vertebrate DNA, 52% belonged to the abundant category (species included elk, bison, red-backed vole, red squirrel, and Uinta ground squirrel), 36% belonged to the common category (species included badger, bear, antelope/pronghorn, red fox, porcupine, and yellow-bellied marmot), and 12% belonged to the uncommon category (species included pine marten, bobcat, southern flying squirrel, and water vole) (Figure 17).
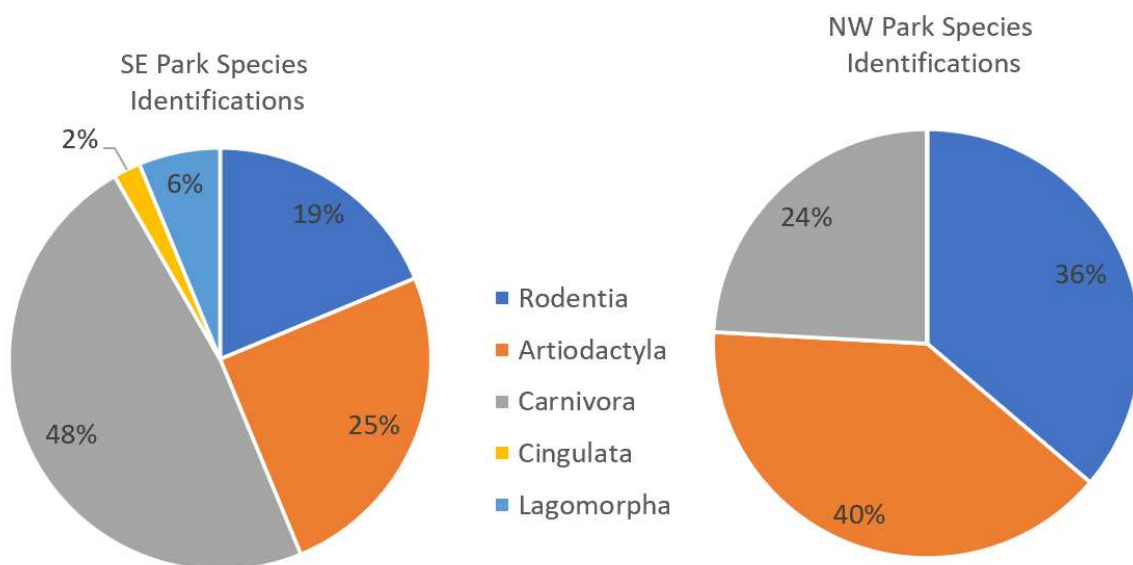


Figure 16. Vertebrate species identifications for each region separated based on mammal order.
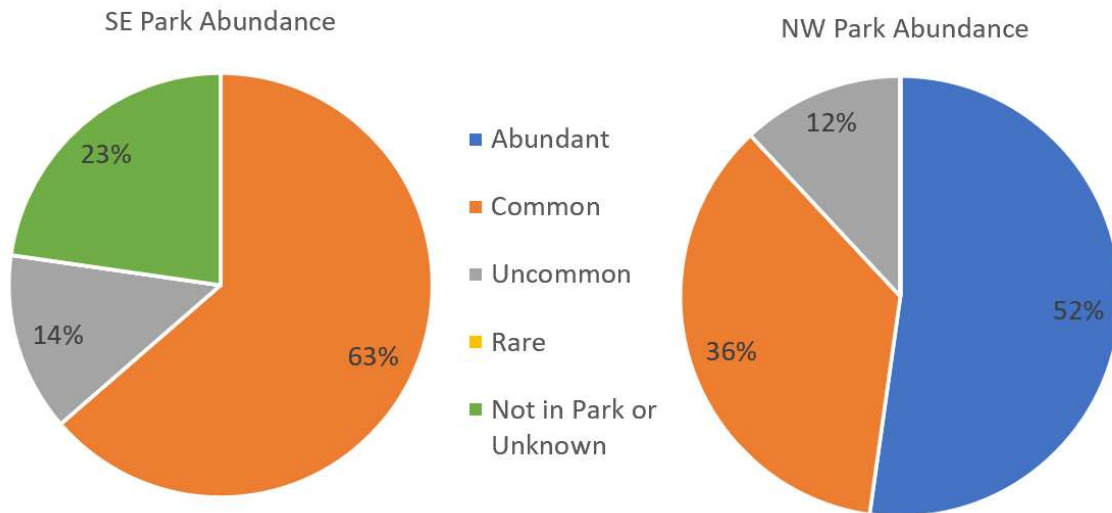
Figure 17. Vertebrate species identifications for each region separated by abundance of species in each park. Abundance information was obtained from the database maintained by the National Park Service (NPS). (Note that samples collected from Mammoth Cave National Park were excluded from this data as no abundance information was available for this park).

### 2.3.5 Abiotic Factor Data

Blow fly activity varies depending on environmental conditions, such as temperature, precipitation, wind speed, and relative humidity. At the time of blow fly collection, weather data was monitored and recorded. As can be observed in Figures 18 and 19, the proportion of each mammal order detected with samples collected on each day is displayed. Also contained within these figures is weather data, including wind speed, precipitation, temperature, and relative humidity.

Based on these results, ideal weather conditions for collection can be inferred. In the SE region on 06/11/2018, minimal rain was observed. This day also resulted in the least amount of detected vertebrate DNA in that year. This suggests that the minimal rain observed (0.08 inches) may have negatively impacted the proportion of positive vertebrate identification. It is likely that blow flies are not actively feeding or colonizing carrion (and in turn, are not "tasting" or sampling carrion resources) during precipitation events. In the NW region on 07/09/2019 and 07/10/2019, low temperatures and precipitation were observed. These days also resulted in the lowest

proportions of positive vertebrate DNA identifications observed during this year. This suggests that the low temperatures and precipitation may have negatively impacted the proportion of positive vertebrate identifications, with precipitation likely impacting blow fly flight activity and low temperatures not being ideal for colonization Also, in the NW region on 07/08/2019, a lack of wind may not have allowed for the VOCs and odors to be spread and detected by the blow flies, contributing to the lack of positive vertebrate identifications on this day.

In the SE region on 06/03/2019, the greatest number of instances of positive vertebrate DNA identification was observed. This occurred at a temperature between 70-75 degrees Fahrenheit and a relative humidity of approximately 50%. This suggests that the ideal weather conditions for collection in this region was at a temperature of 70-75 degrees Fahrenheit, a relative humidity of approximately 50%, no precipitation, and a wind speed of less than 8 miles per hour. In the NW region on 07/07/2019, the greatest number of instances of positive vertebrate DNA identification was observed. This occurred at a temperature of approximately 60 degrees Fahrenheit and a relative humidity of approximately 50-60%. This suggests that the ideal weather conditions for collection in this region was at a temperature of approximately 60 degrees Fahrenheit, a relative humidity of approximately 50-60%, no precipitation, and a wind speed of 5 miles per hour or less.
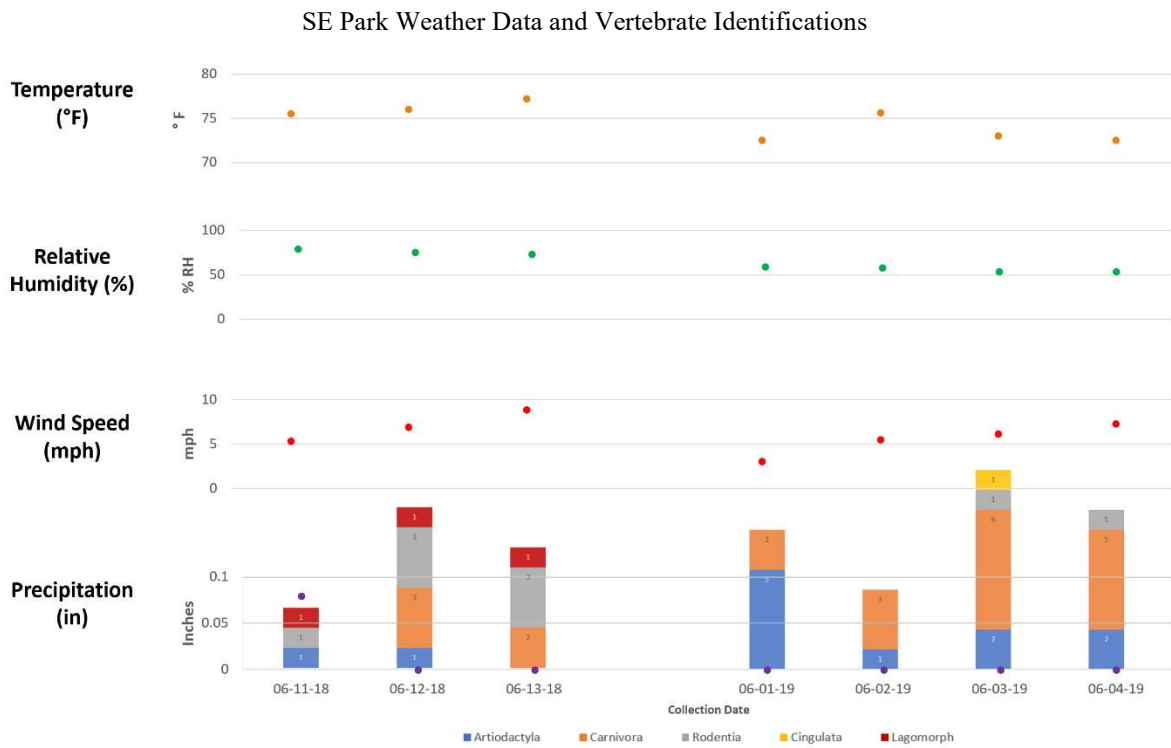
Figure 18. Vertebrate identifications and weather data from the SE region. Vertebrate identifications are separated by day of collection and mammal order (Artiodactyla species in blue, Carnivora species in orange, Rodentia species in gray, Cingulata species in yellow, and Lagomorpha species in red). Daily averaged weather data is also separated by collection day and includes weather data such as temperature (yellow), relative humidity (green), wind speed (red), and precipitation (purple).
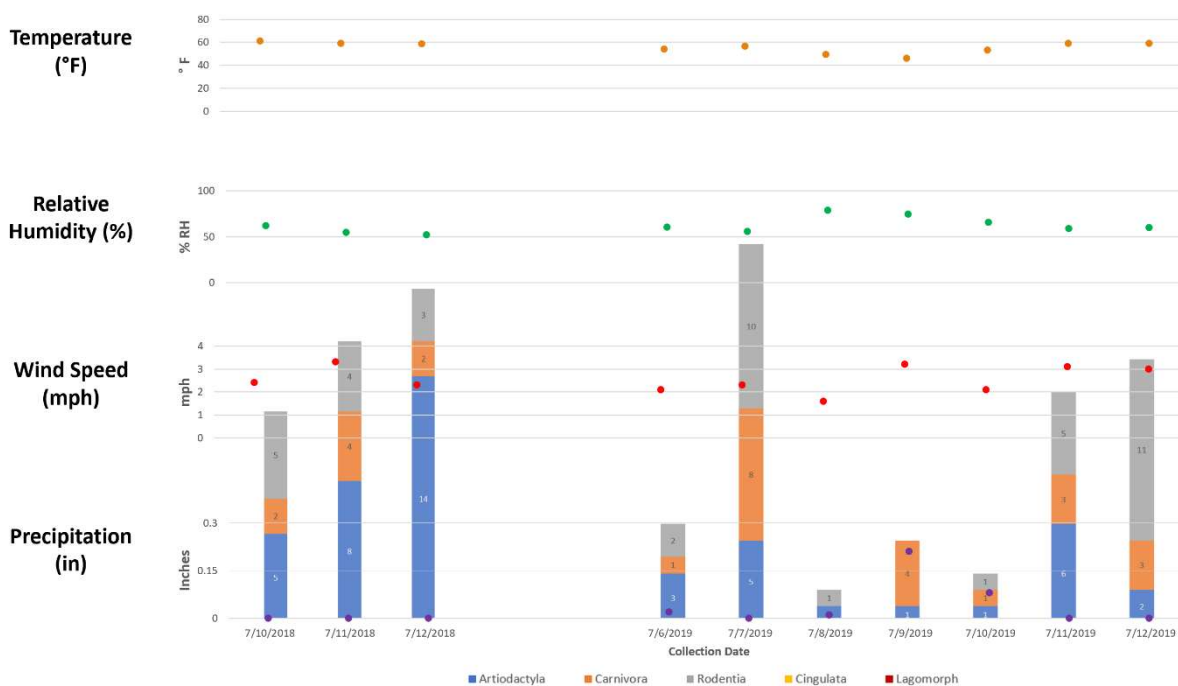
Figure 19. Vertebrate identifications and weather data from the NW region. Vertebrate identifications are separated by day of collection and mammal order (Artiodactyla species in blue, Carnivora species in orange, Rodentia species in gray, Cingulata species in yellow, and Lagomorpha species in red). Daily averaged weather data is also separated by collection day and includes weather data such as temperature (yellow), relative humidity (green), wind speed (red), and precipitation (purple).

### 2.3.6   Biodiversity Estimates

Of the total blow fly samples collected (162 out of 691 samples), vertebrate DNA was detected in 23.6% of the samples, with 15.5% (46 out of 297 samples) of the samples collected from the SE region detecting vertebrate DNA and 29.4% (116 out of 394 samples) of the samples collected from the NW region detecting vertebrate DNA. The Shannon Diversity Index is one of the most commonly used indices to examine diversity in ecological studies [51]. The Shannon Diversity Index is used to measure biodiversity in a given environment when each member belongs to a different group (i.e., species). The Shannon Diversity Index (Equation 1) displayed in Table 3 is greater than 0, meaning that there is some degree of diversity present in both collection locations sampled. The higher the value of the Shannon Diversity Index, the higher the diversity of species present in a particular environment. This value also increases as both the richness and evenness of the community increase. Typically, this value is between 1.5 and 3.5. The calculated Shannon Biodiversity Index was 2.28 for the SE region, 2.79 for the NW region, and 2.89 for both regions combined. Therefore, based on Table 3, the NW park was slightly more diverse than SE park.

Species evenness (Equation 2) refers to the homogeneity of species, or relative distribution of abundance. It is how close the abundance numbers of each species in an environment are to one another. Species evenness increases when the number of each species is more equal. Based on the collected information, the evenness of the SE region was 0.844, the evenness of the NW region was 0.855, and the evenness of both regions combined was 0.827. Therefore, based on this information, the populations of the NW population were more even than the populations of the SE region.

Species richness refers to the number of species present. Based on the information collected in this research, in the SE region, 15 species groups were detected (12 unique species and an additional 3 families were detected as these samples could not be resolved to the species level) out of a total of 46 individuals. In the NW region, 26 species groups were detected (16 unique species and an additional 10 families were detected as these samples could not be resolved to the species level) out of a total of 116 individuals. Therefore, the number of unique species was greater in the NW region than the SE region. In both locations combined, a total of 33 species groups were detected (23 unique species and an additional 10 families were detected as these samples could not be resolved to the species level) out of a total of 162 individuals.

Table 3. Percent of samples returning a positive identification of vertebrate DNA and Shannon Diversity Index calculated for each location (SE Park and NW park) separately and all locations combined.

| Park | % of samples identifying vertebrate species | Shannon Diversity Index | Evenness | Richness | Total # individuals |
|---|---|---|---|---|---|
| SE Park | 15.49 | 2.28 | 0.844 | 15 | 46 |
| NW Park | 29.44 | 2.79 | 0.855 | 26 | 116 |
| Overall | 23.44 | 2.89 | 0.827 | 33 | 162 |

Species accumulation curves are often used to express the sufficiency of the survey being conducted. This is done by recording the cumulative number of species detected as a function of the search. In this instance, the cumulative number of unique species is measured as a function of number of sites of collection sampled. Shown in Figures 20 and 21, a logarithmic trendline was included, describing the expected number of unique species that would be detected if effort was increased (i.e., if more collection sites were sampled). Based on this information the SE region may benefit from increased sampling, as the trendline does not appear to be approaching a clear plateau. The NW region appears to have been sampled sufficiently, as the trendline appears to be reaching a plateau.

Figure 20. Species accumulation curve for the SE region. Actual detected species are denoted by the blue solid line. The logarithmic trendline of this data is denoted by the blue dotted line.
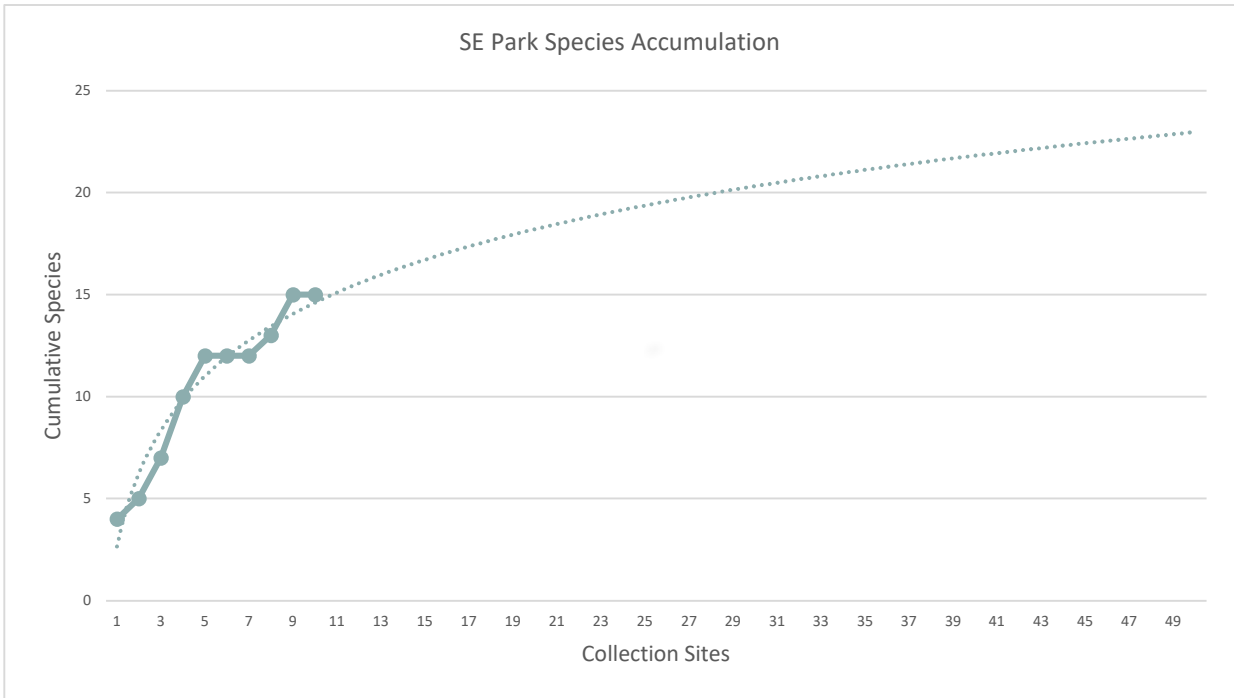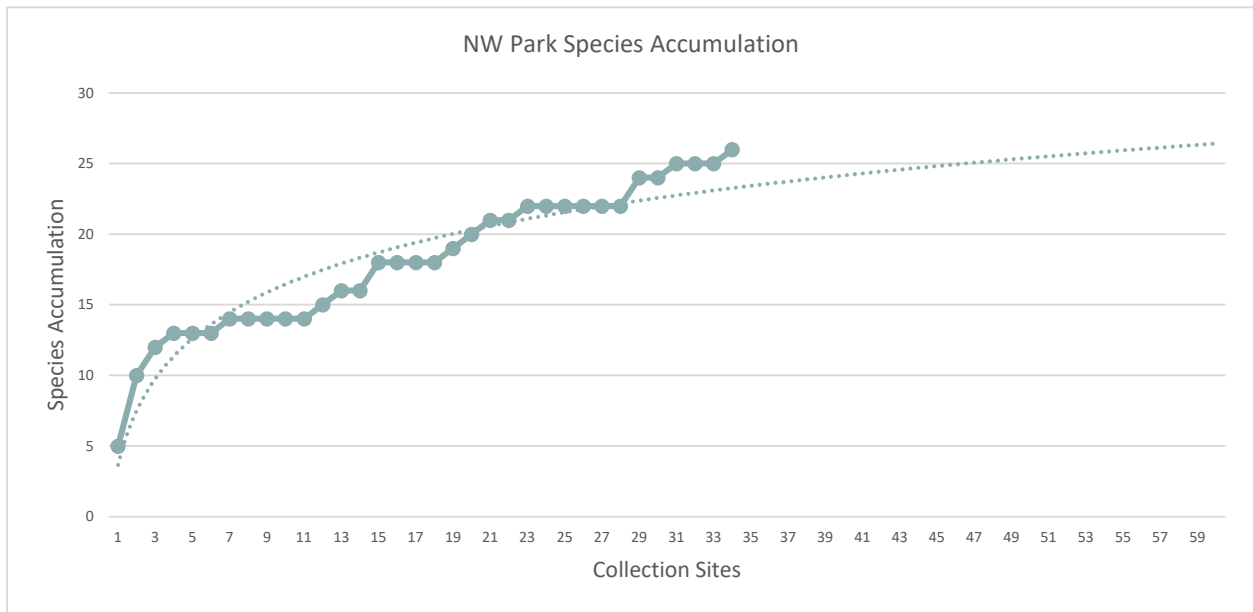


Figure 21. Species accumulation curve for the NW region. Actual detected species are denoted by the blue solid line. The logarithmic trendline of this data is denoted by the blue dotted line.

### 2.3.7 Discussion

Blow flies utilized in this project were collected over the summers of 2018 and 2019 from four National Parks (Mammoth Cave National Park and the Great Smoky Mountains National Park in the SE region, and Yellowstone National Park and Grand Teton National Park in the NW region) and neighboring National Forests. A total of 691 female *P. regina* were collected, with 297 of these blow flies originating from the SE region and 394 originating from the NW region. Out of the total blow flies collected, vertebrate DNA was detected in 23.4% (162 samples). This is most likely due to the collection of blow flies that had not recently fed or "tasted" the carrion source prior to oviposition. If a blow fly has not recently ingested carrion or feces, vertebrate DNA will not be detected via this method.

Of the blow flies collected, 44% (304 samples) successfully amplified. Of the samples that successfully amplified, 31% (93 samples) were revealed to be sequence mixtures and 16% (49 samples) were determined to contain human DNA. The sequence mixtures are likely due to blow flies that have fed on multiple vertebrate resources and both sources of vertebrate DNA were present in the blow fly guts analyzed. The human DNA detected is likely from human feces, rather than human carrion, as human feces is commonly encountered in the national parks. The remaining 53% of the successfully amplified samples (162 samples) were revealed to contain vertebrate DNA that yielded a positive identification.

Abiotic factors such as temperature, relative humidity, precipitation, and wind speed were observed in order to determine if these factors affected blow fly collection and the ability of vertebrate DNA to be detected from the collected blow flies. Based on the weather data collected, the overall ideal conditions for collected and positive vertebrate identifications were determined to be a temperature of between 60 and 80 degrees Fahrenheit and a relative humidity between 50% and 60%. This is unsurprising, as it has been previously documented that blow flies are reliant on the temperature of the local environment, with an increased temperature typically leading to an increase in blow fly activity, as well as the preference of oviposition in moist environments (i.e., a higher relative humidity) [32]. Also based on the weather data collected during this research, the overall ideal weather conditions were determined to be in environments where no precipitation is occurring, and wind speeds are less than 8 miles per hour but greater than 2 miles per hour. Again, this is unsurprising as it has been previously documented that if the wind does not blow, olfactory cues are not spread [31]. Though this weather information is not surprising, it is important to

confirm this information for those who may want to use blow flies to detect vertebrate DNA. However, more research is needed in regards to the effect of abiotic factors on blow fly activity and colonization. For example, more research would be beneficial to determine if and how activity and colonization in certain environmental conditions varies based on species.

Biodiversity is often used as a measurement of the health of an environment. In general, a large number of species would increase diversity, especially if each individual detected belongs to a different species [36]. The vertebrate DNA detected by blow flies was used to approximate the diversity of the sampled environments. The biodiversity index utilized in this research was the Shannon Diversity Index. The diversity index was calculated to be 2.28 in the SE region, 2.79 in the NW region, and 2.89 in all collections conducted through this research. The species evenness was calculated to be 0.844 in the SE region, 0.855 in the NW region, and 0.827 in all collections conducted through this research. This revealed that the diversity was greater in the NW region than the SE region. There could be a few plausible explanations for this. One explanation may be that more female *P. regina* were collected in the NW region. A total of 394 samples were collected from the NW region, as opposed to the 297 samples collected from the SE region. Another explanation may be that more sampling locations were visited in the NW region. In 2018, a total of three days of collection were conducted in both the SE and NW regions. However, in 2019, four days of collection were conducted in the SE region, while a total of 7 collection days were conducted in the NW region. An analysis of the species richness revealed that 15 species groups (12 unique species and an additional 3 families) were detected in the SE region, 26 species groups (16 unique species and an additional 10 families) were detected in the NW region, and 33 species groups (23 unique species and an additional 10 families) were detected in both regions combined. This data revealed that more vertebrate species were detected from the NW region than the SE region. This was unexpected as, according to the databases maintained by the NPS, 89 mammal species are known to be present in the SE region parks, while 69 mammal species are known to be present in the NW region parks [45]. However, again, this may to the fact that more female *P. regina* were collected in the NW region.

Vertebrate species detected belonged to five orders of species - Rodentia, Artiodactyla, Carnivora, Cingulata, and Lagomorpha. Unsurprisingly, a majority of the vertebrate species detected in both regions were known to be abundant or common in their respective parks, while the smallest proportion of detected vertebrate species were known to be uncommon in their

respective park. While this is not new information, it does align with the information collected by the NPS [45]. Interestingly, guinea pigs were detected in the SE region. However, guinea pigs are not known to be present in the park. The source of this DNA is likely to be guinea pigs present in areas surrounding the national parks or brought in by visitors to the parks.

Based on species richness curves (Figures 20 and 21), the trendline of the SE does not appear to be approaching a clear plateau, indicating that this region may benefit from increased sampling. The NW region trendline appears to be reaching a plateau, indicating the region has been sampled sufficiently. However, it is important to note that more sampling would be beneficial in any study in order to increase the accuracy and confidence of results.

### 2.3.8   Conclusion

Throughout the research conducted for this project, fly-derived iDNA was evaluated as a way to monitor biodiversity in a given environment. The results of this research indicated that fly-derived iDNA is indeed a viable method for biodiversity monitoring and vertebrate species detection. From DNA extracted from the guts of blow flies, species present in an environment were detected and a biodiversity index was able to be calculated for the environments sampled. The ideal weather conditions for blow fly collection were also determined, along with various abiotic factors that may affect blow fly collection.

Information collected involving biodiversity of a particular environment also has further implications for PMI estimations. Specifically, there is a gap in knowledge regarding the PAI. Carrion quantity and biodiversity monitoring can be used to increase knowledge regarding PMI estimations and PAI estimations. Environments that do not have readily available carrion resources, are unable to support a large blow fly population. A larger blow fly population will likely decrease the PAI, as more blow flies are likely present in that environment. A smaller blow fly population will likely increase the PAI, as less blow flies are likely present in that environment. Biodiversity monitoring of a given environment can provide researchers with information regarding the health of an environment. Environmental health is likely to affect carrion availability, and in turn affect blow fly populations.

# CHAPTER 3.    CONCLUSION

## 3.1    Forensic Importance

This research shows that information contained within a single fly can allow researchers to understand a fly's life history. This research can reveal how various environmental factors, biotic and abiotic, can affect insect behavior. The conclusions drawn from this research can then be applied to the estimation of PMI using blow flies, surveying vertebrate communities using blow flies as environmental drones, and the estimation of pathogen dispersal patterns [8]. These conclusions can also be applied to a variety of insects, giving researchers a full picture of a particular environment.

Currently, blow flies can be used in forensic science to estimate postmortem interval using two methods. Using a developmental method, a minimum postmortem interval (PMI), also known as the minimum time since death can be estimated [2, 28]. As blow flies are usually the first insect to locate and colonize carrion, entomologists can use the developmental stages of the blow flies that are present to estimate the age of the species present on the carrion [52]. Using a succession method, minimum and maximum postmortem interval can be estimated. As a variety of insects are attracted to carrion in a predictable pattern, entomologists can use the assemblage of insects present on a corpse to estimate how long the carrion has been dead and exposed to the environment [2, 28]. Theoretically, researchers can also use the size of the blow fly population to help estimate a more accurate postmortem interval by estimating the pre-appearance interval. If a smaller fly population is observed, it is hypothesized that the pre-appearance time may increase. However, if a larger fly population is observed, or it is known that the environment can support a large blow fly population, then it can be expected for the pre-appearance time to decrease. While the exact pre-appearance interval is still unknown, this research can provide additional information that can aid in inferences regarding the pre-appearance interval.

However, blow flies have additional applications to the discipline of forensic science. Specifically, in the field of wildlife forensics, determining the biodiversity, monitoring changes in biodiversity, and tracking vertebrate populations of the environment can alert researchers to potentially problematic changes seen in a specific environment [42]. This is especially helpful when monitoring environments that are susceptible to various wildlife crimes such as poaching

and/or trafficking [53-55]. When unexpected and/or drastic changes to vertebrate diversity are seen, wildlife forensic scientists can be alerted to environments that may need to be investigated more closely, as they have the potential to be influenced by various external factors. With blow flies, and the DNA derived from the guts of blow flies, this information can be obtained without invasive trapping methods or expensive camera methods.

In addition, due to a diet that includes feces and carrion, blow flies are capable of spreading fecal pathogens as well as any pathogens found on carrion. Blow flies may transmit pathogens via the sticky pad of their feet, body or leg hairs, mouthparts, and/or vomit [7]. By analyzing blow flies, researchers are able to monitor and test hypotheses regarding the origin of a particular pathogen as well as disease detection [8]. Based on this data, associations can be made between the vertebrates themselves and pathogens. It is even possible to estimate the dispersal of disease outbreaks and/or pathogen distribution into human-inhabited areas [8].

## 3.2    Conclusion and Discussion

This study investigated the use of blow fly ingested DNA as a way to identify vertebrate species present in the environment and monitor biodiversity. Because blow flies feed on carrion and feces, they can be considered environmental drones that are able to constantly sample particular environments. This is accomplished by blow flies feeding on the animals that have died in the environment (carrion) and/or feeding on the feces of the animals that are currently living in the environment. Due to gaps in coverage in the database of vertebrate sequence data used (NCBI's GenBank [43]), phylogenetic methods were employed in order to more confidently resolve vertebrate species.

The results of this research indicated that the use of blow fly ingested DNA to identify and monitor vertebrate resources is a viable method of biodiversity monitoring. From this research, blow flies were collected from the SE region and NW region over the course of two summers (2018 and 2019). A total of 162 samples revealed a positive identification of vertebrate DNA, with a total of 33 unique species being detected. The detected vertebrate species ranged in species abundance from abundant (seen daily) to uncommon (seen sporadically) in their particular environment. The NW region was shown to be slightly more diverse than the SE region. With regards to both regions, of the total 691 female *Phormia regina* collected, 23.6% allowed for the

positive identification of vertebrate DNA. The Shannon Biodiversity Index for both regions combined was calculated to be 2.89 and the species evenness was calculated to be 0.827.

Collection and the proportion of positive vertebrate identifications was affected by environmental conditions such as precipitation, wind speed, temperature, and relative humidity. The overall ideal conditions for collected and positive vertebrate identifications were determined to be a temperature of between 60 and 80 degrees Fahrenheit, a relative humidity between 50% and 60%, no precipitation, and wind speeds less than 8 miles per hour but greater than 2 miles per hour.

## 3.3   Future Studies

A future step in this research involves the use of alternative technology, such as the Oxford Nanopore Technologies MinION or any massively parallel sequencing technology; the goal of which would be to resolve mixed electropherogram readings to distinguish different species present in samples identified as mixtures. Samples with overlapping peaks seen during sequence analysis have been classified as mixed samples and many of these samples have not been able to be resolved at the species level with traditional sequencing. Previous sequencing methods were inadequate at resolving mixed samples but with MinION metabarcoding technology, real-time long read sequencing is capable of distinguishing organismal groups within a mixed sample [42].

Another future step in this project includes the addition of population genetics methods. The goal of this would be to use DNA extracted from the blow flies themselves. Microsatellites could then be used to determine the relatedness of the blow flies sampled from the national parks [8]. This information would be of interest because based on the results already obtained, multiple instances of the same species were found at the same sites. Population genetics methods can be used to help determine if multiple flies were sampling the same animal or if these were different individuals of the same species, as full siblings would likely share the same food source. If related flies are emerging at the same time, and local resources are available in the area, flies will visit these resources before dispersing into environment (if they even disperse at all). From relatedness information, it can be determined if the sampled vertebrates were the same individual or different individuals of the same species. Using this information, the population structure of the vertebrates of the environment in question can be analyzed and predicted. Information regarding the blow fly population itself can also be obtained. For example, the genetic structure and relatedness of blow

fly populations can be studied, as smaller populations are more likely to be inbred than larger populations.

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