# MEDIATORS OF FINE-SCALE POPULATION GENETIC STRUCTURE IN THE BLACK BLOW FLY, *PHORMIA REGINA* (MEIGEN) (DIPTERA: CALLIPHORIDAE)

by

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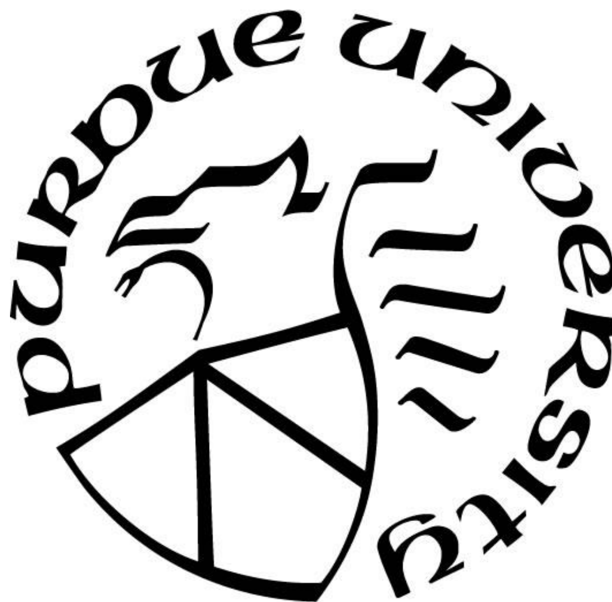


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*This dissertation is dedicated to Mims and Granny.*
*Thank you both for being bad-ass grandmothers.*

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# ABSTRACT

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Population genetic structure is difficult to assess in blow flies (Diptera: Calliphoridae) due to high connectivity and genetic diversity of subpopulations. Previous studies revealed high relatedness among individuals within wild samples of blow fly populations, however broad geographic structure was absent. The aim of this research was to determine if blow fly genetic structure exists at a fine spatiotemporal resolution and, if so, to elucidate the influence of environmental factors and resource availability on fly genetics. Specifically, blow fly population genetic patterns were tested against a null hypothesis that flies adhere to a patchy population model with high genetic diversity (i.e. no structure) and high resource availability. Samples of the black blow fly, *Phormia regina* Meigen (Diptera: Calliphoridae), were collected at six urban parks in Indiana, USA (=urban) in 2016 and 2017 (N = 14 and 16 timepoints, respectively). Additional sampling in different ecoregions was performed to determine if trends observed at a high-resolution scale were also present at a broad geographic scale. Therefore, *P. regina* were also collected at four sites within two national parks (the Great Smoky Mountains and Yellowstone National Parks) over a three-day period. Randomly selected females (N = 10) from each sample underwent the following analyses: 1) gut DNA extraction, 2) molecular analysis at 6 microsatellite loci, 3) vertebrate-specific 12S and 16S rRNA sequencing, and, 4) vertebrate fecal metabolite screening. Flies from the national parks and a comparable subset of urban data also underwent stable isotope analysis (SIA) to determine larval food source. Overall, strong seasonal population genetic structure was

observed over both years in the urban environment (2016 $F'_{ST} = 0.47$, 2017 $F'_{ST}$ 0.34), however spatial structure was lacking, as seen in previous studies (2016 $F'_{ST} = 0.04$, 2017 $F'_{ST}$ 0.03). Weather conditions prior to and on the day of blow fly collections, interspecific competition, and resource availability greatly impacted the genetic diversity and kinship of *P. regina.* A total of 17 and 19 vertebrate species were detected by flies in 2016 and 2017, respectively, and many flies tested positive for vertebrate feces, suggesting that many varied resources are important for maintaining high gene flow among geographic locations. Genetic diversity was non-existent in flies collected from the Smokies ($F'_{ST} = 0.00$), while very slight spatial structure existed in the Yellowstone populations ($F'_{ST} = 0.07$). Environmental factors such as temperature, humidity, and wind speed were all statistically relevant in maximizing fly collections with vertebrate resources. In 720 min of total sampling time in the national parks and a subset of urban data, 28 vertebrate species were identified, and fecal resources appeared to be the most abundant in Yellowstone. Stable isotope analysis revealed a majority of larval resources in the national parks were herbivores, with a more even distribution of carnivore and herbivore carcasses present in the urban environment, which likely explains the high genetic diversity of adult flies in these regions. Overall, the null hypothesis that *P. regina* adheres to a patchy population model could not be rejected for the Smokies populations. However, the urban and Yellowstone populations appear to adhere to a Levins metapopulation model in which variable availability in resources leads to random bottleneck events in the local populations. Overall, environmental conditions, competition, and resource availability are all important factors influencing *P. regina* population genetic structure in different environments.

# CHAPTER 1. INTRODUCTION

Blow flies (Diptera: Calliphoridae) are typically necrophagous flies that are found nearly everywhere on Earth [1]. Given their propensity for locating and colonizing corpses, their life cycles are often utilized in medicolegal investigations [2]. However, population level data for these insects is still lacking, as well a fundamental understanding of their spatiotemporal dispersal and the key environmental factors driving their movement. Furthermore, accurate quantification of important resources available to blow flies has not been investigated, even though these resources are critical for the survival of blow fly populations in any environment. This research involves developing a multidisciplinary model of blow fly population genetic structure that also informs us about wildlife, which can be difficult to survey using traditional (and potentially invasive) methods.

## 1.1 Blow Fly Biology

Although most blow flies are necrophagous, some species have evolved to be both facultative and even obligate parasites [3]. Larvae of necrophagous species depend solely on carrion, whereas adults may visit both feces and carcasses [4], as well as flowers [5]. Given the blow fly's inextricable dependence on vertebrates, they, in their quest for food, mates, and oviposition sites, capture snapshots of vertebrate communities in a natural, non-invasive way that rivals traditional animal survey methods [6, 7]. Adult flies disperse away from their larval resources, sometimes by tens or hundreds of kilometers, in order to search for vital resources [8, 9]. Thus, blow fly dispersal may be mediated largely by vertebrate communities (both alive and dead), ultimately affecting population dynamics and allele frequency changes of these flies over space-time. Finally, given the blow fly's poikilothermic nature, abiotic factors such as temperature

[10-12], humidity [13-15], and local weather in general [16, 17] can have significant effects on development, oviposition, and overall survivorship of fly populations. Biotic factors such as interspecific competition can also radically alter behaviors of flies and potentially lead to local population extinctions if they are driven out or killed by competitors [18-20]. All of these factors together likely impact the effective population size of blow flies in a given area or environment, and these must be understood in order to make inferences using blow flies in medicolegal contexts.

## 1.2 Carrion Ecology

Vertebrate carcasses represent an ephemeral resource that is unpredictable across space and time [21]. Though fleeting, the quality nutrient-dense tissue makes carrion a highly coveted resource among organisms ranging from bacteria to vertebrate scavengers [22]. Patchy distributions, however, require rapid detection and location from organisms vying for an opportunity to utilize the carcass. Carrion-breeding insects fill this niche, as mainly flies and beetles possess the ability to identify postmortem volatile organic compounds (VOC's) released from the carcass bacteria [23, 24]. Blow flies can locate a carcass soon after its deposition into an environment (3 to 9 h) [25] and are usually the first insect to colonize. As each female may lay 100 – 300 eggs [26], relatively few females may be responsible for contributing to a large maggot population on a carcass. This leads to significant and rapid mass loss of a carcass when compared to control carcasses not exposed to blow fly activity [27]. Thus, blow flies are a critical component to any ecosystem as they remove and recycle dead animals of all sizes.

One of the main tenets of this research relies on the ability to quantify vertebrate resource diversity in an ecosystem, which has only been attempted a handful of times, and usually in areas where the carcass biomass is large and obvious (e.g. salmon runs) [28]. Other studies have estimated carcass abundance as a means to study effects of climate change on apex-predator

systems through computer simulations [29], or by using conventional distance sampling of carcasses to determine abundance and density of animal populations [30]. The method outlined in this research relies on an integrated and multidisciplinary set of techniques to determine the vertebrate resource diversity of an area and relate it back to the population genetic dynamics occurring within spatiotemporal blow fly populations.

### 1.3 Identifying Vertebrate Resources

Determining the diet and foraging decisions of any organism is a vital aspect of their ecology as it can reveal patterns of energy flow throughout food webs in an ecosystem [31]. Molecular diet analysis works by using taxon-specific or universal primers to amplify DNA from a substrate. Environmental DNA (eDNA) [32] can be applied to everything from detecting the DNA traces of spider and prey from a spider web [33], to identifying the prey DNA in predator feces [34, 35], to surveying fish diversity from DNA in water samples [36]. eDNA sequencing has also been used frequently in studies of hematophagous disease vectoring insects, such as mosquitoes [37, 38] and ticks [39] in order to determine their host breadth. Mitochondrial genes have been shown to provide accurate species resolution and works well for degraded/partially digested samples (compared to nuclear DNA). Though a few previous endeavors have looked at identifying corpse DNA in maggot crops [40], only recently have researchers used this approach for adult blow flies, mainly for conservation efforts [6, 7]. By using this simple method, it is possible to survey the diversity of carcasses visited by wild flies in the days leading up to their capture, thus determining recent carrion availability.

However, a main limitation of vertebrate DNA sequencing is that the host DNA being amplified may not necessarily originate from a carcass. Some blow fly species (*Chrysomya megacephala* and *Chrysomya putoria*) are known to feed on human and livestock feces and are

capable of spreading fecal pathogens [41]. Feces may play a pivotal role in the female blow fly's life as she requires a protein source to fully develop her ovaries after mating [42]. Stoffolano et al. (1990) demonstrated that non-gravid female *Phormia regina* Meigen (Diptera: Calliphoridae) tend to frequent cat feces more often than gravid females, but that the reverse was true for carcasses [43]. Similarly, Mohr and Tomberlin (2014) demonstrated that carcass attendance by female blow flies is dictated by ovarian status of the female and the age of a carcass, with gravid females making up 40 – 100% of visitors up to 40 h postmortem, and non-gravid females dominating (20 – 100%) after this time period [25]. Coupling this with the fact that vertebrate host DNA can be amplified from feces [44, 45], it may be possible for a fly (especially a female) to ingest host or even prey DNA (if feeding on carnivore scat) [46] from feces without directly feeding on host tissue. For this reason, it was important to develop a method for this study that was capable of differentiating between tissue and feces that did not rely on the host DNA, but instead implements a quick chemical assay that screens for vertebrate fecal metabolites in fly guts.

The above tests can reveal diversity and abundance of vertebrate resources within a few days of fly collections. However, another method exists that could potentially extend resource identification back weeks in time to the larval stage of the flies collected: stable isotope analysis (SIA). The mantra in trophic studies utilizing SIA is "You are what you eat", and for good reason. SIA can be a very useful tool for ecologists as animals incorporate isotopes from food into their own tissues [31, 47, 48]. Since blow flies require carrion for their entire larval development, SIA represents a logical method to determine the kind of carcass an adult fly developed on as a larva [49, 50], which cannot be accomplished with DNA methods (carcass DNA is destroyed during metamorphosis). Since material in the adult fly digestive system is ephemeral, SIA provides a way to detect long-term signatures of diet when molecular analysis can only provide a snapshot into

recent food intake. Overall, stable isotopes were used in this research to determine what kinds of vertebrate carcasses were available to blow flies in an environment prior to sampling.

## 1.4 Population Genetics

Populations genetics is useful for investigating genetic variation within and among target populations [51]. Though many studies have set out to quantify genetic structure of blow fly populations, little evidence of spatial structure exists thus far [52, 53]. Large-scale geographic studies (i.e. spanning the entire United States) have previously revealed that the blow flies lack geographic structure (even among flies collected across the country from each other), but that relatedness within samples was higher than expected by chance [54, 55], indicating that these flies may have exhibited some genetic structure over time. However, no blow fly population genetic study to date has also measured abiotic and biotic factors within the environment from which the blow flies were sampled in order to determine the most important factors affecting population genetics. In this study, modern molecular markers were used to genotype wild individuals of a common blow fly species from a high-resolution spatiotemporal collection scheme spanning two years. To date, no other population genetics study on this type of organism has a similarly intensive sampling design. Additional fly collections were made in two national parks (the Great Smoky Mountains and Yellowstone) in order to compare population trends between different environment types. Though these additional collections were not high-resolution, they served as a validation to the intensive urban sampling. Genetic data from all regions was combined with molecular, chemical, and biogeochemical diet data to produce a comprehensive view of blow fly population genetics.

### 1.5 Research Organism

The black blow fly, *Phormia* regina, is a ubiquitous blow fly found in all regions of North America and Europe [56]. It is the primary colonizer in the northern United States in warmer months and can be found in the southern states in fall and winter. This fly has been the focus of numerous development studies [57-60] due to its prevalence on carcasses and human remains. Because of our previous knowledge of this fly, in addition to its pervasive nature and the ease of collecting it at virtually any time in warm months, *P. regina* serves as the research organism in this study.

### 1.6 Conclusion

The overarching goal of this study was to quantify genetic variation in wild populations of *P. regina*. Previous studies have been limited in the resolution at which populations were sampled and therefore may not fully represent both spatial and temporal genetic patterns. The ecological diversity and microsatellite datasets generated from this work represent some of the most extensive and high-resolution blow fly population data to date. The biogeochemical (SIA) data represents a brand new step in the field of blow fly and decomposition ecology and is the first of its kind for this species. Finally, a more thorough understanding of the drivers of blow fly population dynamics contribute to general population theory and carrion ecology and may aid in conservation efforts using blow flies as vertebrate survey surrogates.

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# CHAPTER 2. CHEMICAL ASSAY FOR THE DETECTION OF VERTEBRATE FECAL METABOLITES IN ADULT BLOW FLIES (DIPTERA: CALLIPHORIDAE)

## 2.1 Introduction

Filth flies have long been implicated in pathogen transmission routes due to their association with unsanitary conditions [1]. The tendency for numerous families of adult filth flies to associate with feces from humans [2-4] and animals [5-7] represents the basis for mechanical transmission theories in which viral and microbial pathogens are assumed to be acquired by the flies directly from infected feces [8, 9]. Visitation to feces may be opportunistic (i.e. the fly may haphazardly alight on animal waste) or it may represent a required nutrient resource for the fly. For example, female blow flies (Diptera: Calliphoridae) can achieve partial or complete follicle development by feeding on carnivore (i.e. dog, cat) or herbivore (i.e. cow, pig, sheep) feces [10-12]. Furthermore, proteins from feces are also important for male blow fly reproductive organ development and increases the probability of successful insemination [11]. It has been shown that adult *Phormia regina* Meigen (Diptera: Calliphoridae) acquire more *Escherichia coli* O157:H7 cells from manure compared to the house fly*, Musca domestica* Linnaeus (Diptera: Muscidae) and distribute these bacteria to food products [13]. Additionally, viral pathogens may be acquired and transmitted in the same manner. For example, the blow fly *Calliphora nigribarbis* Vollenhoven (Diptera: Calliphoridae) was suspected to have played a major role in the spread of H5N1 bird flu in Japan as the H5 influenza A virus gene detected and isolated from the guts of flies captured near infected poultry facilities was identical to the strain found in infected chickens (*Gallus gallus domesticaus* Linnaeus, Galliformes: Phasianidae) and crows (*Corvus macrorhynchos* Wagler, Passeriformes: Corvidae [14]. However, the acquisition route could not be resolved, as flies may

have picked up viral DNA from infected feces, carcasses, living tissue, or even contaminated feed. In tropical areas such as Manila, flies such as *Chrysomya megacephala* Fabricius (Diptera: Calliphoridae) and *M. domestica* even have the potential to disseminate disease-causing parasites as both species have been found to harbor eggs of parasitic worms on the outside of their bodies, presumably acquired from waste [15]. However, in many real-world applications, it is almost impossible to distinguish between potential acquisition modes in a fly-pathogen association, as it may occur as the result of: 1) visitation to feces, resulting in ingestion of pathogens or attachment of pathogens to the outer surface of the fly (i.e. mouthparts, legs [16]); 2) possible acquisition of the pathogen from another contaminated source (e.g. garbage, carrion, offal [17]); and/or 3) an intrinsic association independent of fecal visitation by the adults (e.g. carryover of bacteria from immature stages of *M. domestica* to adulthood, reviewed in [18]).

Pathogenic bacteria associated with filth flies are typically extracted for analysis via microbiological culturing methods [19], molecular methods [14, 20, 21], or by using an integrated approach of culturing and DNA sequencing [13, 22]. Controlled laboratory experiments in which flies are experimentally exposed to vertebrate feces and analyzed for pathogens have given more support to the mechanical transmission theory. Such studies have shown that house flies and blow flies can acquire *E. coli* O157:H7 and *Salmonella enterica* from cow manure and successfully transfer these bacteria to spinach leaves [13, 23]. Female house flies are also capable of acquiring more bacterial colony forming units (CFUs) than males soon after exposure to manure [19]. However, in applied settings in which wild-caught flies are of primary interest, without physically observing flies ingesting or touching infected waste and linking those flies to targeted pathogens also found in the feces, there is no qualitative method for confirming that the source of the pathogen originated from feces.

The purpose of this project was to qualitatively identify vertebrate fecal biomarkers that can be detected in the alimentary canals of blow flies. Screening of these biomarkers in wild flies can then be used to test hypotheses that pathogen acquisition originates from feces. In the clinical/medical field, metabolomic studies have investigated biomarkers associated with human feces to assist in disease detection and to elucidate metabolite pathways [24, 25]. Numerous fecal compounds have been investigated for profiling human and animal metabolites, including steroids [26, 27], various bile acids [28-30], and sterols [31]. Other metabolites of interest include bilirubin and its derivatives, urobilin and stercobilin, as well as the oxidized form of urobilin, urobilinogen, as these metabolites are associated with vertebrate urine and feces [32]. These urobilinoids are formed from the breakdown of bilirubin by gut microflora [33-35] and represent a complex mixture of similar chemical structures which include the colorless urobilinogens, the yellow urobilins, and the brown stercobilins. Urobilin especially has been used as a target compound in environmental wastewater contamination studies using high performance liquid chromatography electrospray mass spectrometry (HPLC-ES-MS) [36]. This compound has also been detected in human fecal samples using ultra performance liquid chromatography quadrupole time of flight mass spectrometry (UPLC/Q-TOF MS) [25], and pressurized liquid extraction turbulent flow chromatography liquid chromatography tandem mass spectrometry (PLE-TFC-LC-MS/MS) [37, 38]. Given the suite of fecal biomarkers characterized by modern analytical techniques in metabolomics, we chose to investigate these compounds using modified liquid chromatography mass spectrometry methods.

In wild-caught flies, evidence that pathogens have been acquired from animal feces would consist of 1) a confirmation that the fly in question is infected with viral or microbial pathogens, and 2) a confirmation that the fly has recently contacted or ingested animal feces. Here, we describe a method that addresses the latter consideration: we implement HPLC MS/MS to detect

compounds associated with vertebrate feces in the alimentary canal of the black blow fly, *Phormia regina*. This fly represents one of the most common blow flies in North America, it has importance in human and veterinary medicine as a secondary myiasis producer [39], and it has forensic utility as a primary colonizer of corpses [40].

## 2.2 Methods

### 2.2.1 Feeding Experiment

In order to determine whether fecal metabolites could be detected from flies, controlled feeding experiments were implemented in which flies were exposed to vertebrate feces, beef liver, or a control (i.e. flies were unfed). A laboratory colony ($G_3$; originally generated from wild-caught *P. regina* collected from Military Park, Indianapolis, IN, USA, housed in 29.85 x 29.85 x 29.85 cm insect rearing cages (BugDorm, MegaView Science Co., Ltd., Taichung, Taiwan)) was maintained at ambient conditions (~22˚C, 50%RH) in the "fly room" at IUPUI, Indianapolis, Indiana, USA and given water and table sugar *ad libitum.* For the feeding experiment, ~100 colony-reared pupae were placed inside of a bleach-cleaned 21 x 12 cm mosquito breeder (BioQuip®, Rancho Dominguez, CA, USA) filled with ~2.5 cm pine shavings to ensure no access to any vertebrate tissue. Upon eclosion, experimental adult flies were transferred to bleach-cleaned rearing cages with non-sterilized water and sugar for 4 d.

Sixty adults were randomly assigned to one of three treatments: dog feces (*Canis lupus familiaris* Linnaeus, Carnivora: Canidae), bovine (*Bos Taurus* Linnaeus, Artiodactyla: Bovidae) liver, or negative control (i.e. unfed). Flies were individually exposed to their respective resources to eliminate competition and give each fly ample opportunity to "taste" the resource. To do this, first a damp, folded Kimwipe™ (Kimberley-Clark™, Dallas, TX, USA) was placed in a. 29.57 mL plastic condiment cup (Diamond™ Multi-Purpose Mini Cups, Jarden Home Brands, Fishers,

IN, USA) and ~1 g of dog feces or beef liver was placed on top (negative control flies were only exposed to the damp Kimwipe™). The purpose of the Kimwipe™ was to prevent desiccation of the resource. Once flies were anesthetized via refrigeration, they were quickly placed inside a feeding cup and sealed with a breathable lid (N = 10 males, 10 females/treatment; N = 60 total flies). Feeding cups were placed inside of a Percival I-36VL incubator (Percival Scientific Inc., Perry, IA, USA) at 28˚C and 65% RH for 4 h and then freeze-killed at -80˚C.

Several additional controls were also analyzed to confirm the presence of urobilinoids in fecal samples derived from animals occupying different trophic positions. Confirmatory samples of dog (*Canis lupus familiaris* Linnaeus, Carnivora: Canidae), lion (*Panthera leo* Linnaeus, Carnivora: Felidae), Grant's Zebra (*Equus quagga boehmi* Matschie, Perissodactyla: Equidae), and Guinea Baboon (*Papio papio* Desmarest, Primates: Cercopithecidae) feces (N = 5 per animal) were analyzed to verify urobilinoids would be present in a sample regardless of the host diet. All exotic animal fecal samples were collected with permission from the Indianapolis Zoo, Indianapolis, IN, USA in the spring and fall of 2016. Exotic animal fecal samples were collected from overnight holding areas in the zoo after animals had been moved to their day-time enclosures, but before the areas were cleaned by staff. Maximum age of fecal samples was approximately 10 h. These samples were collected with a sterilized tongue depressor and placed in sterile 50 mL falcon tubes, which were immediately stored at -20˚C. All dog fecal samples were collected in the same manner shortly after defecation and then stored at -20˚C until needed. Beef liver tissue (N = 5) was also tested as a negative control in order to confirm that urobilinoids would only be present in feces-related samples. All previously frozen fecal material and beef liver tissue was thawed at room temperature for approximately 1 h prior to the beginning the experiment.

### 2.2.2 Wild Blow Fly Collections

*Phormia regina* were sampled from six geographic locations in Central Indiana (Table 1) from March to June 2016. Each sampling site was comprised of a forested park set within, adjacent to, or nearby an urban area. Collections were made with an aerial sweep net at a decayed chicken liver bait (aged 1 – 2 weeks at ambient temperature), which was protected from flies alighting on the bait, over a period of 20 min at each site. Flies were killed in 70% ethanol immediately after this time period and stored at -20˚C until needed for laboratory analyses. For the current study, N = 216 wild-caught flies underwent dissection and DNA extraction as described below.

Table 1. Proportion of *Phormia regina* that tested positive for urobilinoid signals collected in Central Indiana from March 2016 – June 2016. Positive urobilinoid signals were seen in 13% of the flies tested (i.e. 29 out of 216). Values are given as proportions of positive flies from all flies tested per geographic site. Exact location of each park is given by geographic coordinates in degrees, minutes, and seconds (dms).

| Park | City, State | Latitude | Longitude | Proportion Positive Flies |
|---|---|---|---|---|
| Military Park | Indianapolis, IN | 39°46'13.99" | -86°10'06.99" | 5/60 |
| Broad Ripple Park | Indianapolis, IN | 39°52'17.99" | -86°07'49.00" | 5/36 |
| Skiles-Test Nature Park | Indianapolis, IN | 39°52'21.00" | -86°29'49.99" | 6/32 |
| Northwest Park | Greenwood, IN | 39°37'42.99" | -86°08'36.99" | 8/43 |
| University Park | Greenwood, IN | 39°36'39.99" | -86°30'20.00" | 2/24 |
| Province Park | Franklin, IN | 39°28'37.99" | -86°06'39.99" | 3/21 |

### 2.2.3 Gut Dissections and DNA Extractions

The mid- and hindguts of each fly was dissected using flame sterilized forceps for standard organic DNA extractions (DNA is being used for a parallel study in which vertebrate DNA within the gut contents is being sequenced, unpublished). The typically discarded phenol:chloroform:isoamyl alcohol layer from these extractions was then used for chemical analysis. In greater detail, the guts of individual flies were placed inside a sterile 1.5 mL microcentrifuge tube. Digestion was performed by adding 200 uL ChargeSwitch® lysis buffer (Invitrogen™, Carlsbad, CA, USA) and 10 uL 20 mg/mL proteinase K (Invitrogen™) to each

sample and incubated for 4 h at 60°C. 100 uL phenol:chloroform:isoamyl alcohol (PCI, 25:24:1) (Thermo Fisher™, Waltham, MA, USA) was added to the lysate and centrifuged at 5,000 rpm for 5 min, separating the extraction into an organic "waste" layer and an aqueous DNA layer. The aqueous layer was transferred into a new tube to continue the DNA extraction procedure, while the organic layer was refrigerated until use in chemical analysis.

### 2.2.4 Preliminary Presumptive Fecal Test

The Edelman's presumptive test for urobilinoids in feces, which is commonly used in forensic laboratories, detects urobilinogen following a chemical reaction with zinc chloride, mercuric chloride, and isoamyl alcohol [41]. This test was found to be unsuitable for the current application, as the mid- and hind-guts of control flies (i.e. not exposed to any fecal or tissue sources) exhibited background fluorescence (data not shown).

### 2.2.5 Qualitative LC MS/MS Analyses

A 50 µL aliquot of the PCI layer from the DNA extraction was evaporated under a stream of nitrogen at ambient temperature, re-suspended in 50 µL of a 1:1 methanol:water solution, and vortexed for at least 10 min. The samples were separated using an Agilent 1100 HPLC system (Agilent Technologies™, Santa Clara, CA, USA) using reversed phase chromatography on a 100 x 2.1 mm C18 column at a flow rate of 200 µL/min and identified using a LTQ XL™ Linear Ion Trap Quadrupole (Thermo Fisher™) mass spectrometer. The solvents were 0.1% formic acid in water (solvent A) and 0.1% formic acid in 70:30 acetonitrile:methanol (solvent B) over 15 min. The separation began with an initial 1 min hold at 30% B followed by a 9 min linear gradient to 95% B followed by a 4 min at 400 µL/min re-equilibration of the initial mobile phase composition. Mass spectra data were acquired in the positive ion mode. Mass spectral scans consisted of a full

MS followed by directed MS/MS scans at m/z 591, 593, 595, and 597 to screen for various tetrapyrrole urobilinoids. Urobilinogen standards (Thermo Fisher™ and Santa Cruz Biotechnology, Inc., Dallas, TX, USA) were also tested, each consisting of a mixture of isomers with molecular weights ranging from 590.7 to 598.8 g/mol.

### 2.2.6 Statistical Analyses

Experimental and wild flies were compared statistically. The non-parametric Kruskal-Wallis rank sum [42] test was implemented (as peak area data were not normally distributed and could not be transformed), followed by post-hoc paired comparisons using Dunn's test [43]. All statistics were performed in R [44] using the packages stats (native) and dunn.test [45].

## 2.3 Results

Direct extraction of feces as well as gut extractions from feces-fed flies showed several peaks with a m/z of 591 that had MS/MS spectra consistent with tetrapyrroles urobilinoids. Known urobilinoids with m/z 591 include d-urobilinogen and i-urobilin. Two partially resolved HPLC peaks with retention times of 6.3 and 6.6 minutes were obtained (Figure 1A). Both peaks had indistinguishable MS/MS spectra containing m/z 343 and 466 in similar ratios (Figure 1C), both of which were similar to the MS/MS spectrum reported for i-urobilin by [46]. Feces fed fly controls also contained a third prominent peak with m/z 591 eluting at approximately 8.2 minutes, which gave only one prominent fragment ion at m/z 466 (Figure 1B, D). This fragment ion is also suggestive of a urobilinoid, as the neutral loss of 125 mass units is consistent with the loss of a pyrrole unit. We analyzed commercial urobilinoid standards, and peaks with identical retention times and tandem mass spectra were obtained. The commercial standards were complex mixtures of numerous similar isomers, however, so we could not make exact molecular identifications of

the urobilinoids detected in the feces fed flies. Quinn et al (2012) reported on the MS/MS spectra of urobilin and stercobilin, but these spectra were obtained from direct infusion of a standard composed of a mixture of isomers as well.

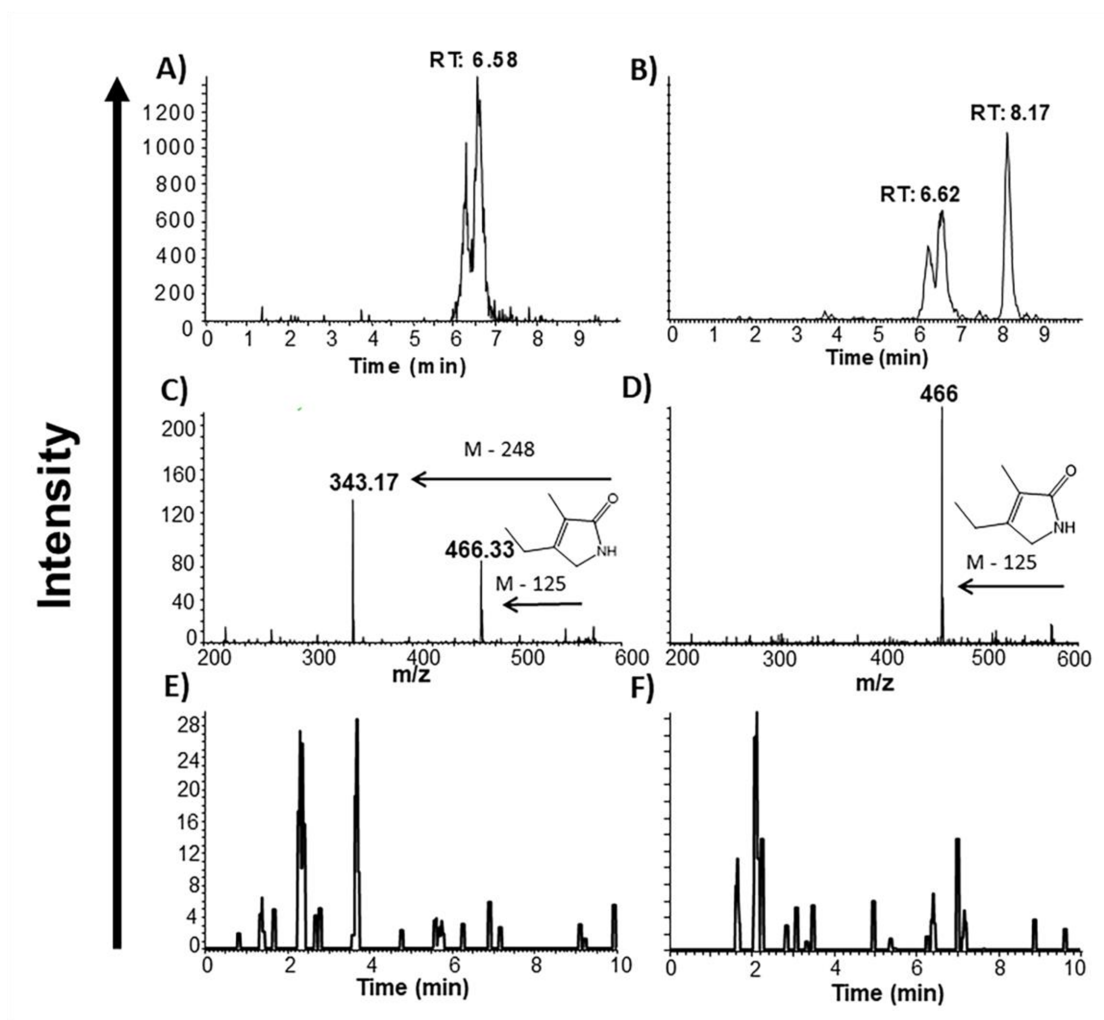


Figure 1. Extracted ion MS/MS chromatograms (XIC) and MS/MS spectra for fly samples (absolute intensities). A) Extracted ion MS/MS chromatogram for m/z 591 → 343 for a feces-fed fly. B) Extracted ion MS/MS chromatogram for m/z 591 → 466 for a feces-fed fly. C) MS/MS spectrum of m/z 591 at 6.5 min. "M" indicates the mass of the primary molecule (591), with 248 and 125 representing neutral loss fragments, e.g. "M – 248" indicates the loss of the 248 molecule to give the 343.17 daughter ion. D) MS/MS spectrum of m/z 591 at 8.2 min. "M" indicates the mass of the primary molecule (591), with 125 representing the neutral loss fragment, i.e. "M – 125" indicates the loss of the 125 molecule to give the 466 daughter ion. E and F) Representative XIC for m/z 591 → 343 (E) and m/z 591 → 466 (F) for a liver fed flies (i.e. no peaks are present at 6 or 8 min).

We screened for urobilinoids with m/z 593, 595, and 597 as well. Stercobilin, with m/z 595, eluted at 6.76 min and yielded two primary fragment ions at m/z 595 → 470 and 345. This peak was identified as stercobilin by comparison with an MS/MS spectra reported in the literature [46]. Though stercobilin is the primary brown pigment in feces, it did not prove to be a reliable marker for feces consumption. Although stercobilin was strongly detected in zebra and baboon feces extracts, it was absent or very low in lion and canine feces extracts (data not shown). This urobilinoid was detected in only a small fraction of feces-fed control flies, and, when present, the intensity was significantly lower than the three m/z 591 urobilinoids. Because of this, stercobilin was eliminated as a targeted compound in this assay. The well-known urobilinoids i-urobilinogen (m/z 593) and i-stercobilinogen (m/z 597) were also not detected in feces-fed control flies.

Data for positive controls (i.e. vertebrate feces) varied considerably among and between species (dog, lion, zebra, baboon); however, all were positive for one or more of the m/z 591 urobilinoids described above (Figure 2). Zebra samples contained the 8.2 min peak, but not the peaks at 6.3 – 6.6 min, whereas lion samples displayed a strong peak at 6.6 min but relatively low intensity peaks for the other two urobilinoids peaks at 6.3 and 8.2 min. However, none of the relevant urobilinoid peaks were present in the beef liver tissue extractions (Figure 2). All feces-related samples contained peaks indicative of urobilinoids that were not present in the other samples tested.

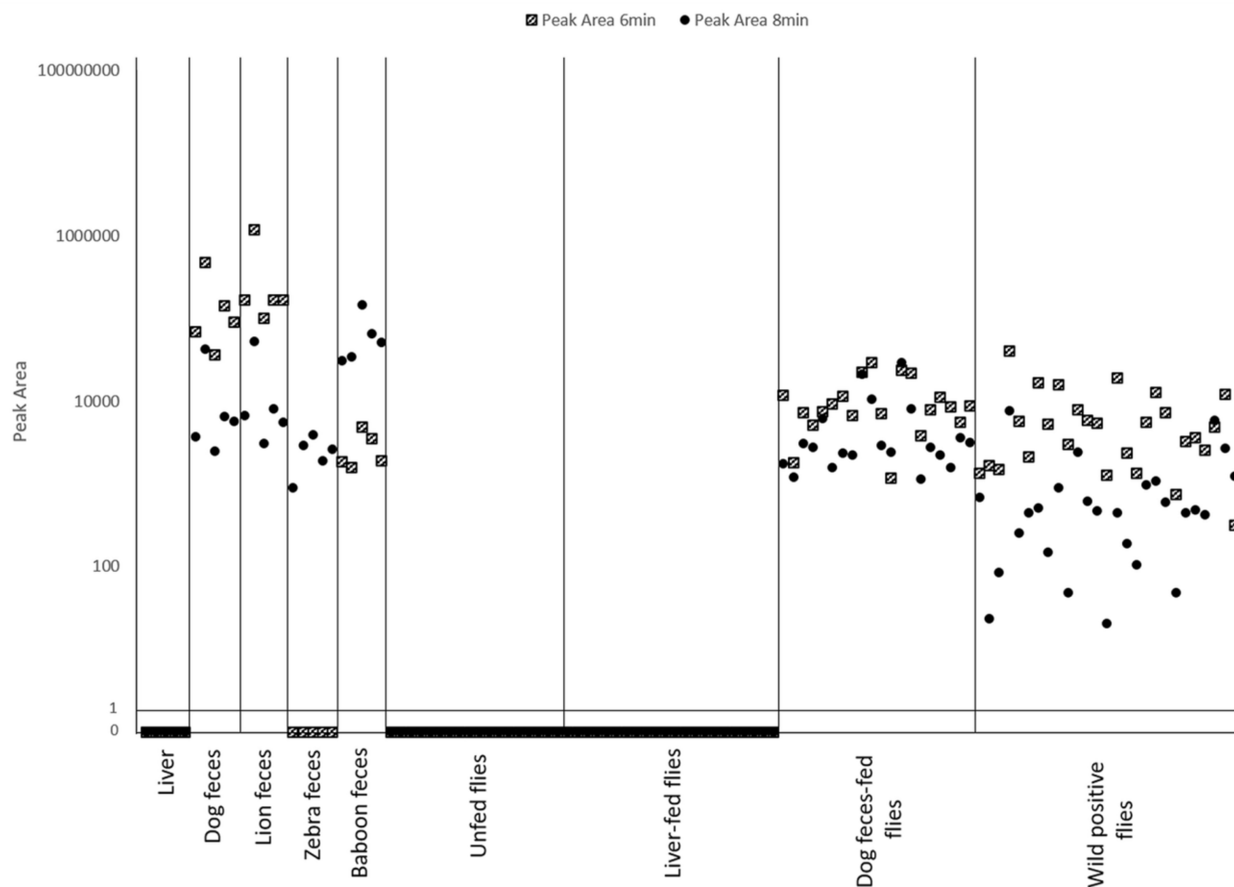


Figure 2. Scatterplot comparison of LC MS/MS 6.3 – 6.6 and 8.2 min peak area data for all tissue and fecal controls, as well as all experimental and wild flies. Controls consisted of beef liver tissue, dog, lion, zebra, and baboon feces (N = 5 per control. Experimental feeding treatments for flies included unfed, beef liver-fed, and dog feces-fed individuals (N = 20 per treatment). Wild flies (N = 29) were collected from March – June 2016 in urban parks in Central Indiana., and samples with positive signals are shown here. Black circles = peak area data at 6 min, striped squares = peak area data at 8 min.

Although peak areas were highly variable across feeding experiment flies as well as wild flies, all feces-fed flies exhibited readily detectable signals for the m/z 591 urobilinoids. None of the unfed or liver-fed flies showed any peaks indicating the presence of urobilinoid compounds (Figures 1, 2). The Kruskal-Wallis test revealed significant effects of treatments at 6 min ($\chi^2$ = 72.8, df = 3, $P < 0.0001$) and 8 min ($\chi^2$ = 78.3, df = 3, $P < 0.0001$), and post-hoc tests revealed significant differences between flies testing positive for urobilinoids and those which tested

negative for these compounds (Table 2). Comparing the peak areas for wild flies vs. experimental flies, there were no statistical differences at 6 min (P = 0.5290), but a significant difference was observed between the two sets at the 8 min peak (P = 0.0205). The urobilinoid signal is greater in feces-fed flies vs. wild flies, though speculative, likely due to the recent feeding event for experimental flies, and an unknown time since feeding for the wild flies. Wild blow fly data showed that 13% (N = 29 of 216) of the *P. regina* collected and tested from urban parks in Indiana, USA were positive for urobilinoids (Figure 2, Table 1). Peak area values (6.3 – 6.6, 8.2 min) of wild flies clustered with all feces control samples (Figure 2). The mean proportion of flies with positive urobilinoid signals at 6.3 – 6.6 and 8.2 min peaks varied slightly by site (mean = 0.14 +/- 0.05).

Table 2. Results for Dunn's test for nonparametric multiple comparisons between experimental (N = 20/treatment) and wild-caught (N = 29) flies for peak areas at 6 and 8 min. Experimental treatments included unfed flies, beef liver-fed flies, and feces-fed flies. An * indicates a statistically significant comparison at P < 0.05.

| | **Peak Area (6 min)** | | **Peak Area (8 min)** | |
|---|---|---|---|---|
| **Paired Comparison** | **z-statistic** | ***P*** | **z-statistic** | ***P*** |
| Feces-fed – Liver-fed | 6.4562 | < 0.0001* | 7.1809 | < 0.0001* |
| Feces-fed – Unfed | 6.4562 | < 0.0001* | 7.1809 | < 0.0001* |
| Feces-fed – Wild | 1.3521 | 0.5290 | 2.7043 | 0.0205* |
| Liver-fed – Unfed | 0.0000 | 1.0000 | 0.0000 | 1.0000 |
| Liver-fed – Wild | -5.5681 | < 0.0001* | -4.9928 | < 0.0001* |
| Unfed – Wild | -5.5681 | < 0.0001* | -4.9928 | < 0.0001* |

## 2.4 Discussion

Surprisingly, few studies have investigated the diet of wild or experimental insects using LC/MS methods, with the exception of detecting sugars in the guts of sand flies (Diptera: Psychodidae) [47, 48] and mosquitoes (Diptera: Culicidae) [49]. Larval, though not adult, blow fly diet has been previously investigated using GC/MS and LC-MS/MS for the purpose of

detecting legal and illicit drugs present in corpse tissues consumed by maggots [50-52]. Our research represents an important first step in qualitatively assessing the diet of an adult filth fly of medical, veterinary, and forensic importance using LC/MS methods. Furthermore, this method was utilized without the aid of microbiological culturing or molecular DNA sequencing, which is common in studies which aim to detect and isolate pathogens/pathogen genes from filth flies. However, the integration of all three methods (microbial culturing and sequencing with urobilinoid analysis) would likely produce an even more precise method for confirming the presence and source of feces ingested by a filth fly, as well as the pathogens associated with the resource.

This study shows that a mixture of the urobilinoids consisting of d-urobilinogen and i-urobilin were definitively and consistently transferred to the guts of *Phormia regina* from ingested feces. Though flies in this study were only exposed to one type of feces (dog), a strong urobilinoid signal observed for all fecal samples (and their absence in tissue samples) indicate that similar results would likely be seen in flies that had ingested feces from various vertebrate species. In addition to experimental verification, this assay also confirmed that wild, randomly sampled blow flies from urban areas consume vertebrate feces. This is not unexpected as several of the collection sites were adjacent to dog parks and residential areas. Sources impacting the variability in signal intensity in positive flies likely include the amount of feces ingested by flies as well as the amount of gut contents subjected to DNA extraction. The liquid-liquid extraction and analytical measurement also contributed to the observed variation. Fluctuations in signal intensity within and between animal fecal samples were expected as the relative amounts of the urobilinoids likely varies depending on the gut microflora [53], or exposure of the samples to air and light, which has been shown to induce a conversion from urobilinogen to urobilin [54]. Furthermore, the amount of urobilinoids present in wild-caught fly guts would likely be significantly affected by variables

such as the amount and type of feces consumed, the time between feeding and collection, and environmental conditions such as temperature, which may affect the speed of fly metabolism. These uncontrollable variables would complicate interpretation of quantitative data. The variability of stercobilin specifically may have arisen from differences listed above, as well as liver functioning of the host animal, which could vary by individual. As such, the observed variability made this compound problematic for our purposes.

The assay described here can aid in testing transmission hypotheses. Specifically, it can confirm that wild flies collected in applied settings (e.g., during routine inspection inside and around farms, during a disease outbreak, as part of ecological surveys, etc.) have ingested animal feces. This method can be applied to several different situations in which determining fecal consumption of flies could be critical, including when the putative, but not confirmed, source of a pathogenic transmission is a filth insect. For example, both *Lucilia sericata* Meigen and *Lucilia cuprina* Wiedemann are thought to exhibit horizontal transmission of bacteria to substrates that they contact [55]. Whole genome shotgun sequencing of both *C. megacephala* and *M. domestica* has revealed that the microbiomes associated with these flies is vast and includes numerous pathogenic species presumably acquired from the environment, as recently-eclosed lab-reared flies had greatly reduced microbial diversity, with a majority of reads coming only from endosymbionts [56]. As blow flies may visit a variety of resources in the environment (i.e. carcasses, feces, and human food, to name a few), it would therefore be valuable to be able to include or exclude feces as a possible bacterial-acquisition source, especially since these flies are often found in human environments. Though most blow flies in the United States do not actually develop in feces, the recent invader *C. megacephala* [57] has been known to breed in human feces in particular [4, 58]. In China, *C. megacephala* and other blow flies (*L. sericata* and *Aldrichina grahami* Aldrich) have

been shown to harbor numerous strains of gram-negative enteric antibiotic-resistant bacteria [59]. Thus, as *C. megacephala* has quickly expanded its distribution from Florida, USA [60] to Indiana, USA [61], and likely is continuing to disperse throughout this region, it will be crucial to investigate its impact on pathogen acquisition from human and animal waste in areas where it is becoming established. Additional filth flies, especially *M. domestica*, are associated with human and animal dwellings and are known to harbor pathogens such as *Coxiella burnetii* [62], numerous strains of *Escherichia coli* [63], *Campylobacter* spp. [22], and *Cryptosporidium parvum* [64], all presumably acquired from feces. The method presented here could easily be implemented with any of the aforementioned filth fly species collected in urban areas to confirm their proclivity for feces in conjunction with determining their pathogen-harboring status, which would further elucidate and solidify their roles in disease transmission routes. Of course, presence of vertebrate fecal metabolites within a fly does not necessarily equate to a pathogen-harboring status of the fly. This assay is simply a qualitative confirmation of ingestion of vertebrate feces.

Though the method presented here is not intended to pinpoint a geographic location of the pathogen source, *per se*, a general knowledge of the recorded dispersal abilities of the species in question would likely benefit those utilizing this method in an applied setting. Greenberg (1973) provides a comprehensive overview of the numerous dispersal distances and rates for several species of filth flies. For *P. regina*, dispersal rates range from 2.25 km to 13.28 km in a 24 hour period, though environment (i.e. urban, rural) was not consistent across studies [9]. However, a comparison of numerous mark and recapture studies of the primary screwworm, *Cochliomyia hominivorax* Coquerel (Diptera: Calliphoridae), showed that a majority of flies recaptured stayed close (< 3 km) to habitats that contained suitable hosts for oviposition, but single females were recorded as traveling up to ~290 km within two weeks of release when environmental conditions

were not ideal [65, 66]. Dispersal patterns of filth flies are important for two reasons: 1) flies may remain in areas where resources are plentiful (e.g. animal facilities rich in feces for protein-feeding), allowing the flies to potentially acquire pathogens); and, 2) flies are also capable of travelling great distances when other necessary resources may be lacking, which could increase the dissemination of pathogens into areas that may be otherwise unaffected by a potential disease outbreak. Therefore, recorded dispersal abilities for blow fly species, known geographic ranges of blow fly populations, and the environmental information contained within each adult fly collected from the wild (e.g. fecal metabolites, bacterial DNA, and vertebrate host DNA) could provide invaluable data when investigating flies potentially involved in pathogen transmission.

Finally, the detection of urobilinoids could prove useful in conservation studies in which filth flies may be used as "environmental drones" to gain ecological information about vertebrate species (targeted or non-specific) in virtually any ecosystem that is conducive for filth flies. Molecular methods have been used previously to determine the species identity of "carrion" in the wild from pooled samples of blow flies [67, 68], though whether the vertebrate DNA obtained in these studies actually originated from carcasses could not be resolved with molecular methods. The method proposed here could be used to improve such studies by differentiating between fecal and tissue resources, which could be critical in conservation studies which are centered on finding evidence that the species of interest is alive. For example, evidence of defecation of an endangered animal would have very different implications to conservation researchers than evidence of the endangered animal's carcass. Utilizing the method provided here in conjunction with vertebrate DNA sequencing tools could lead to a more informed understanding of spatiotemporal distributions of endangered and threatened animals, as well as overall vertebrate diversity of an ecosystem.

## 2.5 Conclusion

Our experimental data show that HPLC MS/MS provides an accurate qualitative test for detection of fecal urobilinoids in *Phormia regina*. Though the intensities of these signals were variable across individuals, chemical signatures associated with feces were evident and strong compared to unfed flies and flies fed liver tissue with no false positives or negatives observed (i.e. urobilinoids were only detected in flies exposed to animal feces), thus supporting the validity of this method in detecting the presence of fecal material as well as providing a strong indication that blow flies feed on animal feces.

The qualitative test presented here may be of great utility to several disciplines, however, there are limitations that should be addressed. Factors that could impact the urobilinoid signal intensity of fly guts include the amount of feces consumed by a fly, as well as the number of meals each fly may take within a given period of time. Currently, the duration of detectable urobilinoid signals in fly guts is unknown (i.e. we cannot determine when a wild fly may have ingested feces in the environment). This is largely due to the variability in peak signals that were observed among individuals in the controlled feeding experiment. Regardless, the sole purpose of this study was simply to illustrate that urobilinoids are only detected in flies exposed to feces, and that these compounds are not associated with flies otherwise. Experimental results provide support that the signals seen from our wild flies were real, indicating that they were in fact feeding on feces in the environment. In the future, this method should be implemented and validated using multiple species of filth flies and other coprophagous insects. This method will be most impactful when combined with microbial culturing and DNA sequencing methods, as well as vertebrate DNA sequencing methods, to precisely identify the source of pathogens mechanically acquired by filth insects from vertebrate feces.

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# CHAPTER 3. FEMALE BLOW FLIES AS VERTEBRATE RESOURCE INDICATORS

## 3.1 Introduction

Biodiversity is integral to ecosystem health and stability, and the loss thereof can have dramatic and cascading consequences on a global scale [1-3]. Given this, it is imperative to implement quick, non-invasive methods to evaluate spatiotemporal changes in animal community compositions [4]. However, traditional surveillance techniques are labor-intensive [5-7], and modern minimal-effort techniques have disadvantages. For example, camera trapping requires sensitive image processing, large data capacities, and may carry a body-size bias [8-10]. Additionally, such methods limit the number of taxa or guilds that can be evaluated simultaneously, requiring implementation of several methods (increasing sampling effort and cost) for total community assessment.

Recent studies suggest utilizing invertebrates, such as blow flies (Diptera: Calliphoridae), to indirectly monitor vertebrates [11-13], with evidence that flies out-perform traditional survey methods in detecting animal richness [14, 15]. Many blow fly species are necrophagous, requiring an animal carcass as a larval developmental substrate [16]. In order to select quality resources to lay eggs, adult female blow flies "taste" carcasses in the wild, effectively sampling and storing host DNA in their bodies. Additionally, female flies also visit animal feces for highly desirable protein, which initiates development of their reproductive organs [17]. As host DNA can also be recovered from epithelial cells found on the exterior surface of feces [18], flies can pick up vertebrate DNA in addition to fecal metabolites when feeding on this type of resource. Given this unique biology of the blow fly, vertebrate DNA (now contained within the fly) can be extracted and sequenced [14, 19] and combined with an analytical assay for fecal metabolites [20],

constituting a powerful approach for remote species identifications in any environment with conditions supporting blow fly activity.

The black blow fly *Phormia regina* (Meigen, Diptera: Calliphoridae) was the primary blow fly species of interest in this study. This species is highly abundant and one of the most forensically important blow flies in the United States [21, 22]. The overarching goals of this work were to enhance current blow fly-based vertebrate DNA methods and illustrate the depth of environmental information gleaned from flies collected from different ecosystems.

## 3.2 Methods

### 3.2.1 Blow Fly Collections

#### Fly Sampling

Blow flies were sampled three times from four urban sites (Indianapolis, IN, USA) and four sites in two national parks: Great Smoky Mountains National Park (=Smokies) and Yellowstone National Park (=Yellowstone; Table 3, (Permits: GRSM-2018-SCI-2039; YELL-2018-SCI-7046). A decayed chicken liver bait inside an aerated container was used to attract flies during each 20 min sampling period (Figure 3). Flies were collected with an aerial sweep net and killed in 70% ethanol on site.

Table 3. Summary of geographic regions and sites used for blow fly collections. Dates given reflect sampling periods. Urban = Indianapolis, IN, USA, Smokies = Great Smoky Mountains national park, Yellowstone = Yellowstone national park. Except for the subsampling experiment (indicated by a #) which examined both sexes of all blow fly species present, only female P. regina were selected for analyses in all other samples. The number of flies analyzed per site, as well as the percentage of flies analyzed out of the entire sample (in parentheses), is given in the last column.

| Region | Site | City, State | Coordinates | Flies Analyzed |
|---|---|---|---|---|
| Urban (March – Oct. 2016) | #Military Park | Indianapolis, IN | 39.770555, -86.168611 | 20 (30.3%); #53 (100%) |
| | Northwest Park | Greenwood, IN | 39.628611, -86.143611 | 30 (30.9%) |
| | #Skiles Test Park | Indianapolis, IN | 39.867882, -86.048541 | 20 (12.3%); #69 (100%) |
| | University Park | Greenwood, IN | 39.611061, -86.050641 | 30 (28.3%) |
| | #Province Park | Franklin, IN | 39.477500, -86.053333 | #141 (100%) |
| Smokies (11 – 13 June 2018) | Site 1 | near Gatlinburg, TN | 35.734722, -83.413333 | 20 (13.6%) |
| | Site 2 | near Gatlinburg, TN | 35.704444, -83.364722 | 20 (13.0%) |
| | Site 3 | near Gatlinburg, TN | 35.663330, -83.526389 | 26 (17.1%) |
| | Site 4 | near Gatlinburg, TN | 35.670833, -83.680000 | 30 (5.2%) |
| Yellowstone (9 – 11 July 2018) | Site 1 | near Gardiner, MT | 44.614170, -110.413600 | 26 (39.4%) |
| | Site 2 | near Gardiner, MT | 44.957780, -110.541700 | 30 (22.6%) |
| | Site 3 | near Gardiner, MT | 44.957780, -110.311900 | 22 (32.4%) |
| | Site 4 | near Gardiner, MT | 44.885560, -110.144400 | 20 (37.7%) |

Figure 3. Holding container for aged chicken liver bait used to attract blow flies. The sides of the container, as well as the lid, have been cut open and covered with mesh. This aerates the container allowing for attractive volatile cues to disseminate into the surrounding environment while preventing the flies from landing on the bait itself.

**Subsampling**

Three urban public parks were investigated at a single timepoint to determine the ideal blow fly species and sample size for vertebrate surveillance (*, Table 3).

### 3.2.2 Molecular and Chemical Analysis

For subsampling experiments, both sexes of all blow fly species collected from each park were analyzed (Military Park: 53 flies; Skiles Test Park: 69 flies; Province Park: 131 flies). For all other sampling, the black blow fly, *Phormia regina* Meigen (Diptera: Calliphoridae), was analyzed

due to its prevalence in the USA. A maximum of 10 female *P. regina* were randomly selected from each spatiotemporal sample for analysis.

**Dissections and DNA Extractions**

Crops and hindguts of each fly were removed using flame-sterilized forceps and placed inside a sterile 1.5 mL microcentrifuge tube. Flies containing mature eggs were deemed gravid. Dissection of guts was followed by digestion in 200uL ChargeSwitch® lysis buffer (Invitrogen™, Carlsbad, CA, USA) and 10uL 20mg/mL proteinase K (Invitrogen™), incubating for 4 h at 60˚C. 100uL phenol chloroform isoamyl alcohol (PCI, 25:24:1) (Thermo Fisher™, Waltham, MA, USA) was added to each lysate and centrifuged at 5,000 rpm for 5 min, separating the extraction into an organic "waste" layer and an aqueous DNA layer. A standard phenol-chloroform DNA extraction was continued [23].

**Chemical Analysis**

Waste layers of each sample underwent vertebrate fecal metabolite analysis according to methods previously outlined [20].

**Molecular Vertebrate Species Identification**

Several vertebrate-specific primer pairs used for amplifying vertebrate DNA in similar studies were tested during preliminary controlled feeding experiments with flies (data not shown). These included two different cytochrome b primer pairs (UNFOR403/UNREV1025 [24] and L14841/H15149 [25]), two 12S rRNA primer pairs (12SV5F/12SV5R [26] and L1085/H1259 [27]), and two 16S rRNA primer pairs (16SMam1/16SMam2 [28] and L2513/H2714 [27]). Both sets of cytochrome b primers resulted in the amplification of fly DNA, which obscured any vertebrate data that may have been obtained. Success rate was variable with the 12SV5 primers as

well as the 16SMam primers. The optimal primers for this study were determined to be the 12S (L1085/H1259; 215 bp amplicon) and 16S (L2513/H2714; 244 bp amplicon) rRNA mitochondrial primers as they consistently produced successful amplification and sequencing of vertebrate DNA without amplifying fly DNA [27]. For each 10uL total volume PCR reaction, each of the following were added: 5uL Promega 2X PCR mastermix (Promega™, Madison, WI, USA), 1uL 5uM forward and reverse primers, 0.5uL 1X bovine serum albumin (BSA; Promega™), and 2.5uL genomic DNA. Amplifications were carried out on a Mastercycler Pro thermocycler (Eppendorf®, Hamburg, Germany) using a 10-step touchdown from 63˚C to 54˚C, then 25 cycles at 54˚C, and a final extension at 7 min.

Samples were purified with 1uL ExoSAP-IT™ (ThermoFisher Scientific, Waltham, MA, USA) following manufacturer's protocols. Amplicon sequencing was performed according to BigDye™ Terminator v3.1 Cycle Sequencing kit (ThermoFisher) protocols. Sequencing products were purified via ethanol precipitation: 1.25uL 125mM EDTA was dissolved into each sample, followed by 20uL 100% ethanol, and incubation for 15 min at room temperature. A second centrifugation was conducted at 2500g for 30 min, followed by removal of ethanol. 20uL 70% ethanol was added and the plate was centrifuged at 2500g for 15 min. After ethanol removal, the plate was inverted and centrifuged at 185g for 1 min. Samples were re-suspended in 10uL HiDi™ formamide (ThermoFisher), vortexed for 15 s, and denatured at 95˚C for 5 min. Sequences were separated and detected on a 3500 genetic analyzer (ThermoFisher) and manually edited and trimmed via Sequence Scanner (ThermoFisher). Only sequences with clearly resolved nucleotides were used (low quality or obvious multiple sequences were discarded). Sequences were then queried using the National Center for Biotechnology Information (NCBI) nucleotide database

using BLASTn®. Only the top hit with a query coverage of >95% and an e-value $<10^{-5}$ was accepted.

### 3.2.2 Vertebrate Diversity Analyses

Reference databases for DNA sequences (like NCBI GenBank) are not complete, which can be a limitation in iDNA studies performed in areas where many native vertebrate species are not represented in the database. Additional assignment methods have been shown to enhance BLAST results (MEGAN) or outperform them altogether (PROTAX) [14]. As the environments sampled in this study had well-characterized vertebrate taxa that were represented in GenBank at the loci used for amplification, the top BLAST result was used with confidence as the true species identification. However, in the event that an appropriate match or identification was not made, a phylogenetic analysis was performed to infer the lowest-possible taxonomic level of samples that could not be resolved to species (i.e. the phylogenetic analyses were used to resolve to either genus or family level). Sequences of multiple genera based on animal distributions were downloaded from NCBI, sequence alignments were done in MEGA-X v10.0.4 [29], and a Tamura-Nei distance tree (500 bootstrap replicates) was generated. Animal body sizes (kg) were obtained from Quaardvark [30] and placed into three subjective categories: small (<5 kg), medium (5-55 kg), and large (>55 kg). Vertebrate species richness (S) and the extrapolation of richness from rarefaction curves were obtained using the R packages *vegan* [31] and *iNEXT* [32], respectively.

### 3.2.3 Statistical Analyses

For subsampling experiments, a one-way ANOVA with a post-hoc Tukey's honest squared differences was performed with native R packages [33] to elucidate differences in richness among female *P. regina*, male *P. regina*, and all other blow flies analyzed. To determine the ideal fly

sample size needed to maximize richness, subsamples of N = 10, 15, and 20 flies were randomly generated from the three subsampling datasets and analyzed via Kruskal-Wallis test with post-hoc Dunn's test using *dunn.test* [34].

An Integrated Nested Laplace Approximation (INLA) algorithm [35] specifically implementing the Besag, York, and Mollie (BYM) spatial autoregression [36] using Hierarchical Bayesian Inference (HBI) was used to determine the most important predictors of vertebrate resource availability. Conditional autoregressive (CAR, random walk) Bayesian Hierarchical Model captures the hierarchical nature of space (both correlated and uncorrelated spatial heterogeneity) and time (both time trend and space-time diffusion) and incorporates abiotic factors as predictors. These predictors included mean temperature (˚C), mean humidity (%RH), mean wind speed (m/s), abundance of *P. regina*, and abundance of gravid females per sample. Response variables included abundance of vertebrate DNA-positive flies, vertebrate richness, and abundance of feces-positive flies per sample. A chi-squared test for independence was also conducted to assess whether the detection of vertebrate DNA and fecal metabolites were independent of each other.

## 3.3 Results

### 3.3.1 Effect of Subsampling

Entire samples (i.e. both sexes of all species) of blow flies were collected at three urban parks (Military Park, Skiles Test Park, Province Park; Table 3) in and around Indianapolis, IN, USA and analyzed to determine the ideal fly species, sex, and sample size for recovering vertebrate diversity. ANOVA revealed a significant difference among the sexes of flies sampled for vertebrate DNA ($P$ = 0.043), with a post-hoc test showing that female *P. regina* detected significantly more vertebrate species than males ($P$ = 0.038, Figure 4). 33% of female *P. regina*

tested positive for vertebrate DNA, compared to 21% and 14% in male *P. regina* and blow flies of other species, respectively. No statistically significant differences were detected between either female or male *P. regina* and blow flies of other species. There was a significant difference in richness when sample sizes increased from 10 to 15 or 20 flies ($P < 0.001$), though there was no difference between 15 and 20 flies. Therefore, up to 15 flies per sample should recover maximum vertebrate richness.

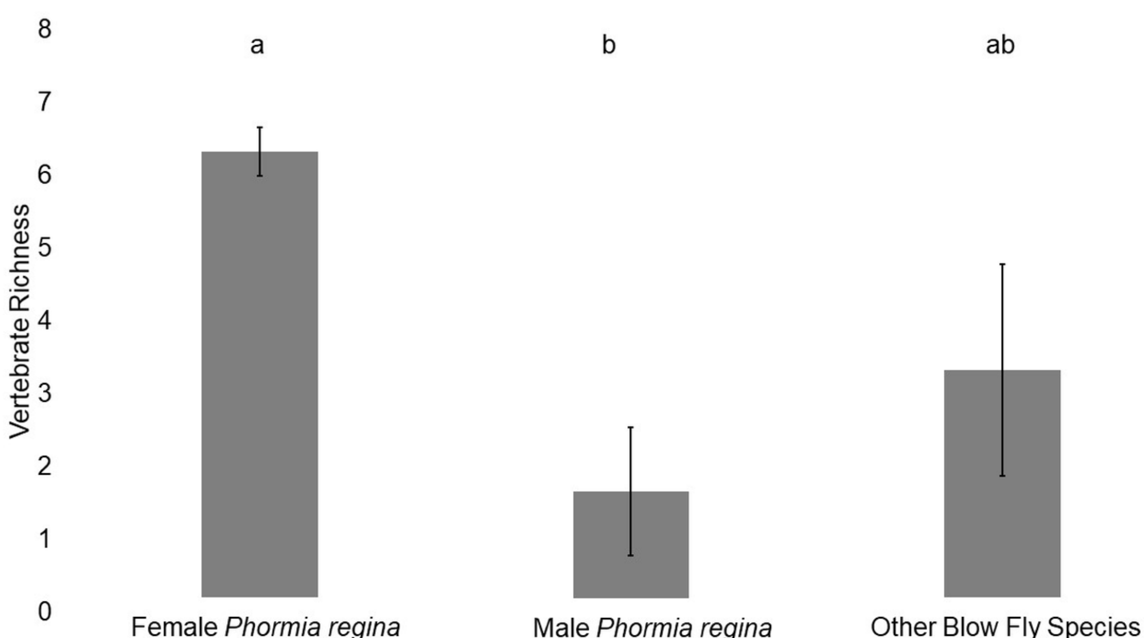


Figure 4. Bar plots summarizing the mean vertebrate richness (with mean SE bars) detected from blow flies from the subsampling experiment. Statistically different values are represented with different letters (P < 0.05).

### 3.3.2 Wild Sampling

Active sampling (240 min/region) yielded a total of 1,784 *P. regina,* of which 293 females were analyzed. Overall, 28 vertebrate species were identified, with 42% of flies containing vertebrate resources: 23% DNA, 5% feces, and 14% both. Not all fecal positive flies resulted in a positive species identification. Resource-seeking behavior is likely influenced by the biology of

the female fly, with gravid females (i.e. females with fully developed eggs) more likely to sample vertebrate resources. This was indeed observed in the data as slightly more than half (53%) of the analyzed flies were gravid, and of those gravid females, 64% contained vertebrate animal information (37% vertebrate DNA, 21% fecal metabolites, and 6% both).

**Urban**

Of the 434 *P. regina* collected in the urban environment, 23% were analyzed. 29% of these flies contained vertebrate resources: 19% DNA, 7% feces, and 3% both (Figure 5A). Most sequenced animal species (>50%) were small to medium (S = 10; Figure 5D, G; Table 4). Rarefaction and extrapolation of species richness and diversity determined that a plateau is approached when sampling ~40 flies (Figure 5G). Unsurprisingly, dogs (*Canis lupus familaris*) were the most common animal detected in the urban environment (making up 37% of all positive species IDs).

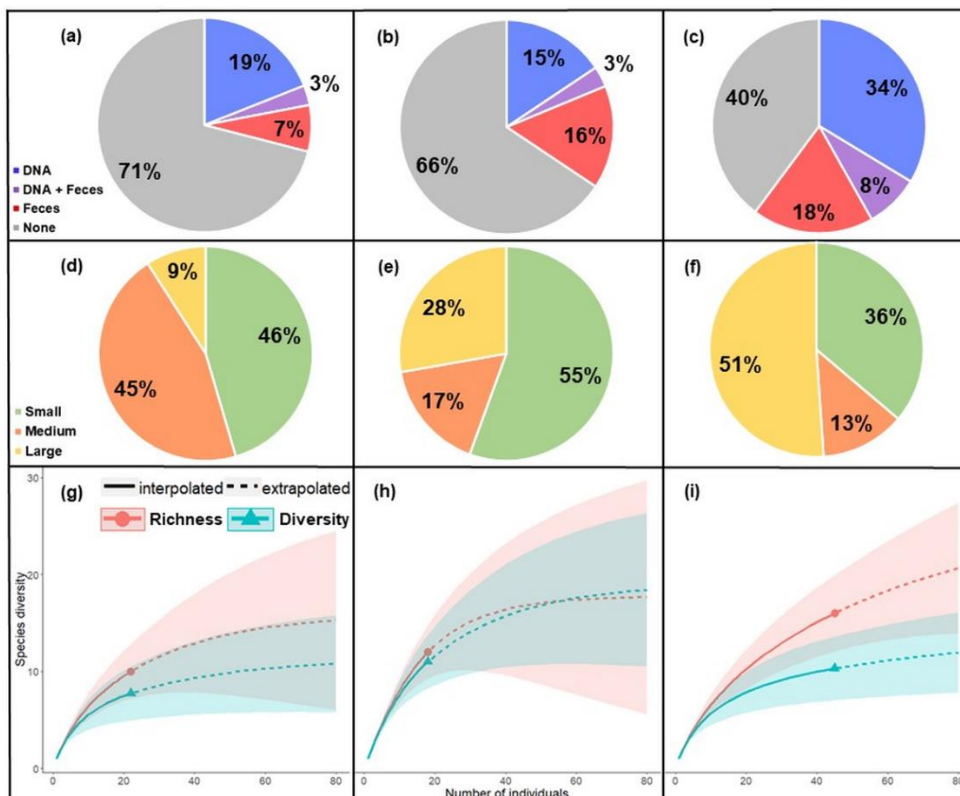


Figure 5. Summary of vertebrate abundance and diversity detected by blow flies from three regions: urban (left column), Smokies (middle column), and Yellowstone (right column). A-C: Pie charts showing the abundance (given as percentages) of flies containing evidence of vertebrate resources (DNA, feces, and flies containing both vertebrate DNA and feces), as well as flies with no vertebrate resources detected. D-F: Pie graphs showing relative body sizes (small, medium, large) of vertebrate species detected by flies. G-I: Rarefaction curves generated from vertebrate data showing both interpolated and extrapolated values for vertebrate richness and diversity (shaded areas represent 95% confidence intervals).

Table 4. Summary of vertebrate species detected by blow flies in three regions: urban, Smokies, and Yellowstone. The lowest taxonomic level resolved by the primers used in this study is given for each animal.

| Species | Urban | Smokies | Yellowstone |
|---|---|---|---|
| *Canis lupus familiaris* | 7 | 2 | 0 |
| *Sylvilagus floridanus* | 3 | 2 | 4 |
| *Felis catus* | 2 | 1 | 1 |
| *Sus scrofa* | 1 | 1 | 0 |
| *Bos taurus* | 1 | 2 | 0 |
| *Marmota monax* | 1 | 0 | 0 |
| *Peromyscus leucopus* | 1 | 0 | 0 |
| *Procyon lotor* | 3 | 0 | 0 |
| *Sciurus niger* | 2 | 1 | 0 |
| *Didelphis virginiana* | 1 | 0 | 0 |
| *Odocoileus sp.* | 0 | 1 | 13 |
| *Sciurus carolinensis* | 0 | 3 | 0 |
| *Ursus americanus* | 0 | 1 | 1 |
| *Canis latrans* | 0 | 1 | 0 |
| *Cavia porcellus* | 0 | 2 | 0 |
| *Neovison vison* | 0 | 1 | 0 |
| *Peromyscus sp.* | 0 | 0 | 1 |
| *Cervus elaphus* | 0 | 0 | 8 |
| *Myodes sp.* | 0 | 0 | 2 |
| *Mus sp.* | 0 | 0 | 1 |
| *Taxidea taxus* | 0 | 0 | 1 |
| *Vulpes vulpes* | 0 | 0 | 1 |
| *Bison bison* | 0 | 0 | 2 |
| *Felis sp.* | 0 | 0 | 1 |
| *Antilocapra americana* | 0 | 0 | 2 |
| *Cynomys sp.* | 0 | 0 | 3 |
| *Urocitellus armatus* | 0 | 0 | 3 |
| *Canis sp.* | 0 | 0 | 1 |

**National Parks**

The abundance of *P. regina* collected from the Smokies and Yellowstone was 1030 (9% analyzed) and 320 (31% analyzed), respectively. Though only 36% of Smokies flies contained vertebrate resources (15% DNA, 16% feces, 3% both; Figure 5B), most (>50%) were small mammals (S = 12, Figure 5E, H, Table 4). In contrast, 68% of Yellowstone flies contained vertebrate resources (34% DNA, 18% feces, 8% both), with >50% being large-bodied animals (S = 15; Figure 5F, I; Table 4). Rarefaction and extrapolation of richness and diversity shows that Yellowstone requires more sampling to reach a plateau compared to the Smokies (Figure 5H, I). 11% of DNA-positive Smokies flies contained dog DNA, whereas only one Yellowstone fly

detected a single occurrence of *Canis lupus*, though whether this was a dog or a wolf could not be resolved given the presence of large wolf packs in Yellowstone and the limitations on domestic dogs in the park. Therefore, in an effort to be conservative, the authors only report the genus of this animal (Table 4). Additionally, the uncommon Pine Marten (*Martes americana*), and a previously unobserved prairie dog genus (*Cynomys* sp.) were detected in Yellowstone (Table 4; [37]). However, as 4 of the 23 rodent species in subfamily Xerinae documented within the park have not been sequenced at the loci used for this study, it is not known whether the *Cynomys* sp. detected here is a true representation of this genus in the park, or whether it is the result of an incomplete molecular database.

### 3.3.3 Parameters Affecting Vertebrate Resource Detection

Hierarchical Bayesian inference using INLA showed no significant results for predicting vertebrate DNA- or fecal-positivity in flies. However, a positive interaction between mean temperature and vertebrate richness was observed (mean = 0.098, sd = 0.048, 0.025Q = 0.004, 0.975Q = 0.192; Figure 6). Other variables (humidity, wind speed, abundance of gravid flies, and total abundance of flies) had no impact on vertebrate detection by flies in the areas and timeframes in which flies were sampled. A chi-square test for independence determined that vertebrate DNA detection and fecal metabolite detection were statistically independent of one another ($\chi^2 = 3.35$, df = 2, $P = 0.187$).

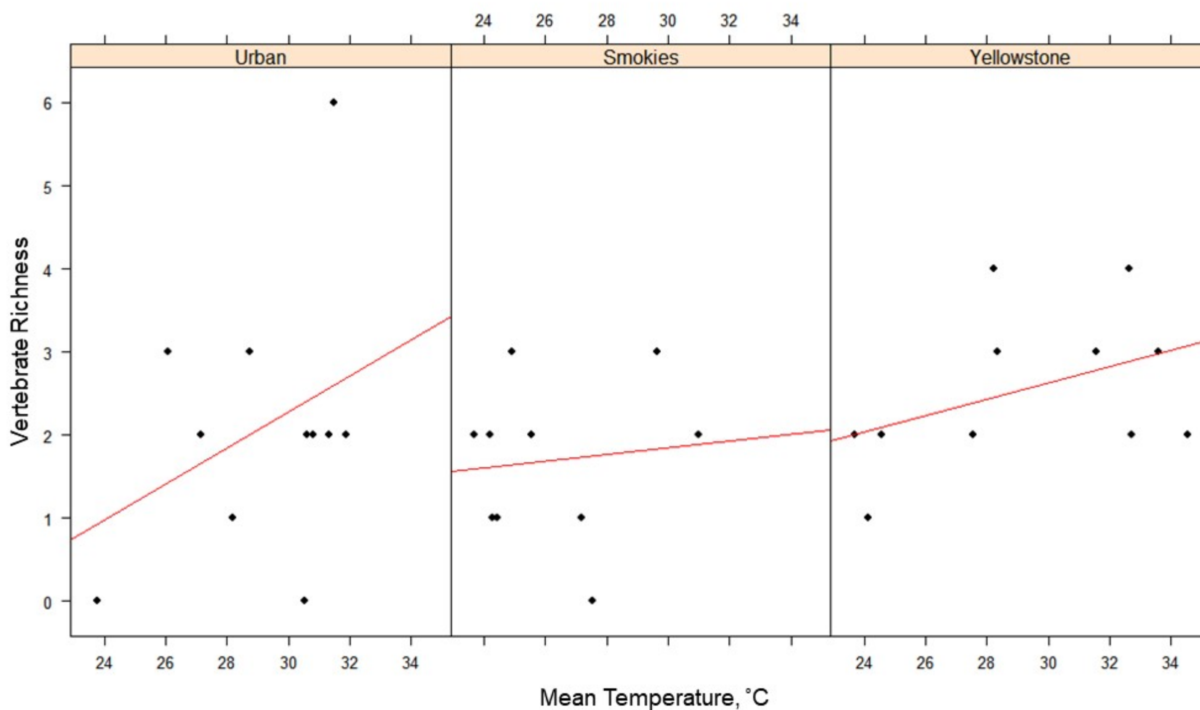


Figure 6. Scatterplots of vertebrate richness by mean temperature for three regions: Urban, Smokies, and Yellowstone. Regression lines are shown in red.

### 3.4 Discussion

Study design is crucial for successfully surveying vertebrate biodiversity with invertebrates. We show that by analyzing a fraction of collected flies (average = 21%) in a limited timeframe (240 min /environment), 26 - 43% of common and abundant mammals (excluding bats) can be detected in three distinct environments [37-39]. Vertebrate DNA detected by flies approximated the mammal richness in the sampled regions. Using vertebrate-specific primers producing short (<250 bp) amplicons, we were able to detect the same number of wild animal species in our urban samples (S = 6, excluding four domestic animal species) as a recent study in Germany, though without the use of blocking primers or next generation sequencing [13]. Similarly, richness values, as well as vertebrate detection rates, from flies captured at the national

parks were comparable to previous studies investigating carrion DNA from flies in pristine tropical habitats [11, 14, 15].

Though it is not surprising that higher temperatures on collections days resulted in more vertebrate species being detected, this is important to point out for non-entomologists wishing to use insects as vertebrate diversity indicators. The poikilothermic nature of insects leads them to be more active during warm weather [40], increasing the speed of population turnover and quickly leading to increases in population abundance. For blow flies, this translates to increased active searching behaviors for required resources, thereby increasing the vertebrate diversity detected by flies. Though the other abiotic variables tested here seemed to have very little impact on vertebrate diversity detected by flies, additional sampling (especially on a temporal scale) would likely reveal more significant microclimatic effects.

Additionally, we have shown that wild female blow flies are better candidates for vertebrate DNA recovery compared to males, likely due to their nutritional requirements for reproduction. While males will visit vertebrate resources such as feces for their own sexual maturation [17], the frequency at which they do so appears to be much less than females. Implementing the use of female blow flies in practice is quite easy to do, even for the non-entomologist. The most obvious sexually dimorphic characteristic to use is the placement of the eyes: the eyes of males are proportionally large and touch in the middle of the head, while the eyes of females are proportionally smaller and separated, usually by an appreciable distance [41]. This simple delineation can be easily used by researchers to enhance the recovery of vertebrate DNA in sampled wild flies.

We have also illustrated the utility of actively capturing blow flies in a limited timeframe and analyzing only female blow flies individually via gut dissection. Active sampling is ideal in

situations where repeated sampling in an area is not possible, as well as because it is known exactly when flies arrive at the bait and when they are killed by the researcher. Furthermore, the measurement of abiotic factors, like wind speed and direction, during active sampling could potentially be important in determining where the fly may be traveling from, as blow flies will fly into the odor plume when attempting to locate the bait. Passive trapping does have its benefits as it may require much less effort on the part of the researcher and potentially result in larger collections over time [14]. However, there is always a chance that baited passive traps can be scavenged or destroyed by other animals. If implemented for iDNA studies, passive traps should be checked frequently to avoid degradation of DNA and potential disturbance by other animals. In terms of preparation of flies, some studies pool samples prior to molecular diet analysis [13, 15]. Though this method may be more cost-efficient when performing next generation sequencing to quickly asses biodiversity of an area, it has been shown that pooling can reduce the number of detectable vertebrate species in a sample [14]. This is likely due to "rare" or low template DNA within individual flies (potentially due to low acquisition or decay of DNA over time) being lost due to PCR bias, resulting in an underestimate of vertebrate diversity. Additionally, valuable individual data, such as whether or not the fly has fed on feces as well as the reproductive status of the fly, is lost if samples are pooled. Overall, the risks and rewards of fly sampling and molecular analysis methods should be weighed carefully against the overall goal of the study.

Our results also show that the process resulting in the presence of vertebrate DNA in flies is potentially independent from the process resulting in the presence of fecal metabolites. This could mean that either 1) the acquisition of these materials occurs separately (i.e. the fly visited both a fecal resource, where it picked up fecal metabolites, and a carcass, where it picked up DNA), or, 2) if both DNA and feces were acquired together, one material must degrade more rapidly than

the other. One previous report found that vertebrate DNA from beef liver tissue was detected in blow fly guts up to 96 h after ingestion by the blow fly *Chrysomya megacephala* (Macquart) [19]. On the other hand, we have found that fecal metabolites can persist in *P. regina* for as long as two weeks post-ingestion (data not shown). The sensitivity of the chemical fecal assay [20] in conjunction with the persistence of the urobilinoid signal in the fly guts points to the high reliability of this assay to detect fecal metabolites if they have been ingested by the fly. Given this finding, it would make more sense that if DNA and fecal metabolites were ingested concurrently, that the fecal signal would outlast the DNA signal over time since feeding. In the context of our wild fly sampling data, this would mean that the flies containing only a vertebrate DNA signal likely obtained the DNA from a carcass or carrion resource. Though feces can serve as an adequate protein source for flies and they seem to visit it frequently [16], the most preferable resource for females is animal carrion. Female blow flies in various stages of vitellogenesis will visit carrion or carcasses [42-44], and protein from this type of resource results in much more rapid egg maturation [17]. As for the flies containing both DNA and feces, it cannot be determined whether DNA was obtained from the fecal resource alone or if two different resources were ingested.

It is important to acknowledge the dispersal potential of blow flies in order to determine if fly-derived data represents the true vertebrate community composition of the area of interest. A comprehensive review of blow fly dispersal outlines the variability in distances traveled by flies, albeit this likely results from a combination of variable abiotic factors (i.e. wind speed, temperature, overall climate) during sampling, as well as varying recovery methods utilized by researchers [16]. Local spatial aggregation is well-known in blow flies, suggesting that though individual flies in a population may disperse somewhat randomly, the population itself persists in an area due to both resource availability and environmental factors [45-47]. However, it has also

been reported that flies can travel up to 13 km in 24 h likely in search of important nutrients or resources which may be lacking in the area [48]. In the context of vertebrate resource sampling, female flies (the flies most important for the method presented in this paper) likely would not disperse far away from areas rich in protein (e.g. vertebrate feces) and oviposition resources (e.g. animal carcasses on which to lay their eggs). Thus, vertebrate DNA detected from female flies should reflect the true vertebrate diversity present in a relatively local area of collection. If sampling in a geographic location that is known to have low vertebrate diversity (e.g. large agricultural areas with little to no animal refugia), then caution should be taken in interpreting vertebrate data detected by flies as the region in which these animals may actually be located likely lies outside of the local area of fly collection.

Overall, blow flies contain vast reservoirs of environmental information waiting to be tapped by scientists. Additional ecological data can also be extracted from flies, such as population genetics of targeted species [12], vertebrate-pathogen associations [49, 50], and even angiosperm diversity in phenological studies (wild blow flies consume nectar [51, 52] and potentially pollen [53]). Furthermore, flies can be used to monitor environmental pollution (e.g. pesticides, agricultural run-off) using similar analytical chemistry methods used to detect fecal metabolites. The possibilities of using blow flies as environmental monitors are nearly endless. With the tools provided here, it is possible to detect taxa spanning multiple trophic levels and spatiotemporal scales with a limited sampling effort, an endeavor unattainable using any other current methods.

### 3.5 Conclusion

Rapid vertebrate diversity evaluation is invaluable for monitoring changing ecosystems worldwide. Here, we have demonstrated the power of blow flies as biodiversity monitors and provide a logical approach to optimize vertebrate diversity measurements from flies that any

researcher can utilize. This method will empower ecologists to test theories previously out of reach due practical challenges associated with traditional sampling.

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# CHAPTER 4. STABLE ISOTOPE ANALYSIS REVEALS LARVAL RESOURCE OF ADULT *PHORMIA REGINA*

## 4.1 Introduction

"You are what you eat" is the mantra for stable isotope analysis in trophic food web ecology. Stable isotope analysis (SIA) is a useful tool for ecologists as animals incorporate isotopes from food into their own tissues, which can provide a record of the animal's diet over time [1]. In other words, the animal's historical diet is reflected in its isotope chemistry. As the short-lived food material in the digestive system only provides a snapshot into recent food intake, SIA is useful over longer periods of time [2]. This makes it possible to track the movement of animals over extended time scales and expansive geographic ranges by their diet history (e.g. plants endemic to specific regions), as in mass migration of terrestrial and marine organisms [3-6].

The foundation for stable isotope analysis lies in the fact that several elements exist for which there are two or more naturally occurring isotopes, which are differentiated by atomic mass [1]. This mass difference leads to environmental variation in relative abundance of each isotope (=fractionation) [7]. Natural fractionation among the heavy and light isotopes of carbon and nitrogen ($^{13}C/^{12}C$; $^{15}N/^{14}N$) makes it possible to quantify their ratios in virtually any type of sample via stable isotope ratio mass spectrometry (IRMS). Carbon and nitrogen stable isotopes (ratios denoted in delta notation: $\delta^{13}C$, $\delta^{15}N$) have been used extensively in food web studies [8]. Dietary carbon can have distinct $\delta^{13}C$ values, and as such, can be used to reveal the source of carbon in an animal's diet. In food webs, carbon usually originates from primary producers (plants), which exhibit differential fractionation of carbon based on the amount of $CO_2$ fixed through different photosynthetic pathways (i.e. $C_3$ plants vs. $C_4$ plants) [7, 9]. The $^{13}C/^{12}C$ ratio becomes incorporated in the tissues of primary (and higher) consumers with little variation, meaning that

carbon isotopic values should be consistent from food to consumer. On the other hand, $\delta^{15}N$ is useful as it can reveal the trophic position of an animal due to preferential incorporation of the heavy isotope into consumer tissues, leading to an enrichment of $^{15}N$ with each trophic level. Several studies have shown that animals consistently exhibit an approximate 3‰ increase of $\delta^{15}N$ relative to their diet [7, 10]. The premise of most trophic studies is that organisms assuming higher trophic levels should have higher $\delta^{15}N$ values relative to lower trophic level organisms, with the highest position being occupied by the organism with the highest $\delta^{15}N$ value.

There have been several studies of stable isotopes in arthropods [11-14], especially in trophic studies of hematophagous disease vectors [15-17]. For example, C/N ratios can distinguish between unfed and bloodfed adult *Aedes albopictus* (Skuse) (Diptera: Culicidae) mosquitoes, and $\delta^{15}N$ in particular can distinguish between their different hosts, even after complete digestion of the bloodmeal [15]. Immature developmental substrates of adults can also be determined via $\delta^{13}C$ and $\delta^{15}N$ in some arthropods. For example, fractionation of these isotopes can resolve larval substrates as being plant or animal detritus in adult *Ochlerotatus triseriatus* (Say) (Diptera: Culicidae) mosquitoes [16]. Differentiation of hosts from blood-fed nymphal ticks (gerbils or rabbits), as well as the age of the tick *Ixodes ricinus* (Linnaeus) (Ixodida: Ixodidae) has also been investigated using this method [17]. This is particularly interesting given the authors were able to resolve between two hosts occupying similar trophic positions, and the fact that ticks became more enriched in both $\delta^{13}C$ and $\delta^{15}N$ over time, potentially due to depletion of the light isotopes during starvation after moulting to adulthood.

Currently, stable isotope data is limited for necrophagous insects. Several species of the carrion beetle genus *Nicrophorus* (Fabricius) (Coleoptera: Silphidae) were shown to have significantly different $\delta^{13}C$ and $\delta^{15}N$ signatures that reflected both size of the beetle as well as size

of the carcass it was reared on [18]. Blow flies specifically have been used to estimate the fate of nutrients and energy entering an ecosystem, as >80% of adult *Calliphora* spp. (Robineau-Desvoidy) (Diptera: Calliphoridae) reared from salmon runs (estimated at 4 – 7 million larvae per watershed) had salmon-based $\delta^{13}C$ and $\delta^{15}N$ values [19]. More recently, Bernhardt et al. (2017) analyzed isotopic enrichment in the blow fly *Lucilia sericata* Meigen (Diptera: Calliphoridae) after rearing larvae on different animal tissues, including human muscle [20]. Though the goal of this particular study was to determine if human isotopic signatures were different enough to distinguish humans from other animals (they were not), it is the most similar study to what is investigated in the current research.

If applied to decomposition ecology and even conservation biology, stable isotope analysis of necrophagous insects can potentially reveal carrion trophic diversity of an ecosystem. However, in order to draw any meaningful conclusions from isotope profiles generated from wild blow flies, it is imperative to develop baseline datasets for comparative purposes [10]. The goal of this study was to determine if the predictable enrichment trends recorded for $\delta^{15}N$ and $\delta^{13}C$ from previous food web studies holds true for *Phormia regina.* Specifically, I attempted to determine if there is a predictable fractionation of $\delta^{15}N$ and $\delta^{13}C$ in the adult fly compared to its larval diet and if this is affected by other variables, such as preservation method and adult feeding. Additionally, we tested the ability of stable isotope analysis of flies to determine the carcass community composition of an area, which currently can only be done with traditional surveying methods and molecular analysis.

## 4.2 Materials and Methods

### 4.2.1 General Procedure for Feeding Experiments

Laboratory colonies (<$G_5$) generated from wild-caught *P. regina* were used for all experiments. When not being utilized for experimental purposes, flies were maintained at ambient conditions (~22˚C, 50%RH) in the "fly room" at IUPUI, Indianapolis, Indiana, USA and given water and table sugar *ad libitum*. Adult flies were allowed to age for 3 to 4 d post-eclosion before being used in any feeding experiment (i.e. they were not given any protein resources during this time). To ensure that each fly had an equal opportunity to land on and taste the resource, flies were always individually placed in sterile, clear medicine cups with 5 g of the respective resource and a kimwipe soaked in distilled water (negative control flies were only exposed to a water-soaked kimwipe). Cups were then randomized and placed inside of a Percival I-36VL incubator (Percival Scientific Inc., Perry, IA, USA) at 28˚C and 65% RH. Exposure time lasted 4 h for all adult feeding experiments to ensure ample opportunities for the fly to taste the resource. Flies were then released from the feeding cups into a sterile container (either a cage or a sterile mosquito breeder; one for each treatment group) with water available until flies were killed.

### 4.2.2 Experiment 1: Effects of Preservation

The first experiment was designed to investigate if the kill/preservation method affects detectable isotopic signatures in the fly tissue. This is relevant as flies that are collected in the field are usually killed in ethanol, while flies used in lab experiments are often frozen. A single colony of *P. regina*, reared on turkey breast to adulthood, was used to investigate this problem. Cohorts of unfed males (N = 10) and females (N = 10) were euthanized by freezing at -20˚C (i.e. "frozen

flies") or by submerging in 95% ethanol (i.e. "ethanol flies"), for a total of N = 20 flies per treatment.

### 4.2.3 Experiment 2: Persistence of Larval Diet Isotopic Signature

The second experiment was designed to determine if larval resources could be differentiated based on the isotopic signature of the adult flies. Three *P. regina* colonies were reared on either beef liver, chicken liver, or salmon filets. Colonies were allowed to go through one generation on the respective resource prior to the current experiment. Flies (N = 5 males, N = 5 females per colony) were not given a protein resource as adults, but were only exposed to water and table sugar, *ad libitum*, and then killed 4 d post-eclosion.

### 4.2.4 Experiment 3: Effect of Single Adult Feeding Event

The third experiment was designed to determine if a larval isotopic signature persists even after feeding as an adult. Two *P. regina* colonies were each reared on an individual beef liver (i.e. different cows used for each colony). Colonies were allowed to complete two successive generations on the livers prior to the experiment. The third generation of each colony was subjected to a feeding experiment in which adult cohorts (N = 3 male, N = 3 female per treatment) were exposed to various tissues: beef liver, chicken breast, salmon filet, and carnivore feces (lion, *Panthero leo* Linnaeus) to test whether different adult resources affect the detection of larval isotopic signatures. A cohort of negative control flies (i.e. unfed as adults) were also tested. Flies were killed 4 h after the end of the feeding period.

### 4.2.5 Wild Sample Validation

In order to test the validity of the experimental isotope results, wild sampling of blow flies was performed in three regions (Indianapolis (=urban), the Great Smoky Mountains national park,

and Yellowstone national park) as described in Chapter 3. All flies that underwent DNA and fecal analysis were also subjected to stable isotope analysis (urban, N = 100; Smokies, N = 96; Yellowstone, N = 98).

### 4.2.6 Isotope Ratio Mass Spectrometry

All flies used for isotopic analysis were decapitated and heads were desiccated in a drying oven set at 50˚C for 24 h. Each fly head was then placed inside of a 9mm x 5mm tin capsule (Thermo Fisher™, Waltham, MA, USA) and weighed on a Micro Balance (Sartorius™ AG, Goettingen, Germany). All samples were combusted in an EAIsoLink elemental analyzer and run under continuous helium flow to a Thermo DeltaV Plus IRMS. Sample values were corrected to the international standards, whose isotopic values bracket the range of the samples. Nitrogen isotope values were expressed relative to international reference materials as follows: USGS-40 L-glutamic acid (U.S. Geological Survey, IAEA, -4.52 ‰), USGS-41 L-glutamic acid (U.S. Geological Survey, IAEA, 47.51 ‰), and Bovine Liver RM 1577c (National Institute of Standards and Technology, 8.14 ‰). Carbon isotope values are expressed in per mil deviations from the international reference material Vienna Pee Dee Belemnite as follows: USGS 40, -26.39 per mil; USGS 41, -37.63 per mil; Bovine Liver RM 1577c, -17.78 per mil. The following equation was used: $\delta^{X}E = [(R_{Sample}/R_{Standard}) - 1]*1000$, where R = $^{15}N/^{14}N$ or $^{13}C/^{12}C$. Analytical precision based on replicate standard analyses was ±0.2 per mil.

### 4.2.7 Carcass Trophic Assignment

In order to make sense of the isotopic signatures observed in wild flies, it was necessary to gather pertinent wild vertebrate data that could potentially correspond to the signatures seen in the flies. Unfortunately, no isotopic repository exists, so a meta-analysis was done in order to obtain

$\delta^{13}C$ and $\delta^{15}N$ values from animals within broad trophic categories: carnivore, omnivore, herbivore (browser), and herbivore (grazer) [20-26]. As there is no standardized method for performing such studies on animals, nor is there a preferred method of reporting data, some values had to be corrected before they were implemented in the current study. For example, many studies report isotopic values for animal feces or hair, but not muscle tissue. Luckily, a correction factor given in [27] made it possible to standardize all values to muscle tissue, which is the most likely animal tissue that larval blow flies will feed on in the wild.

### 4.2.8 Statistical Analysis

An analysis of variance (ANOVA) with post-hoc Tukey's honest squared distance (HSD) was used to determine significant main effects, interaction effects, and paired comparisons for all experimental data. As meta-analysis data were not normally distributed, a Kruskal-Wallis test with post-hoc Dunn's test was performed in order to statistically differentiate between animal trophic positions from the literature. This gave specific categories and ranges of isotopic values for both $\delta^{13}C$ and $\delta^{15}N$ that could be used to determine the actual carcass trophic position derived from the fly isotopic signatures. Experimental enrichment and depletion data averaged from all experiments for female flies specifically were used to transform raw wild fly data to carcass data. Finally, with the trophic categories derived from the literature, the carcass data was placed into putative trophic positions.

## 4.3 Results

### 4.3.1 Experiment 1: Effects of Preservation

No significant main effects or paired comparisons for $\delta^{15}N$ data were observed (Figure 7A, Table 5). Flies killed in ethanol did exhibit a wider range of $\delta^{15}N$ values compared to flies that

were flash-frozen, but this was not significant. Overall, the only significant effect was that of sex, as shown by $\delta^{13}C$ values. This is likely explained by the nearly significant difference between males and females in the ethanol treatment in which females were slightly more depleted in $\delta^{13}C$ than males (*P* = 0.051, Figure 7B). However, based on the available data, the method in which the fly is killed does not impact the nitrogen or carbon stable isotopes of flies, though sex should be considered during isotopic analysis.

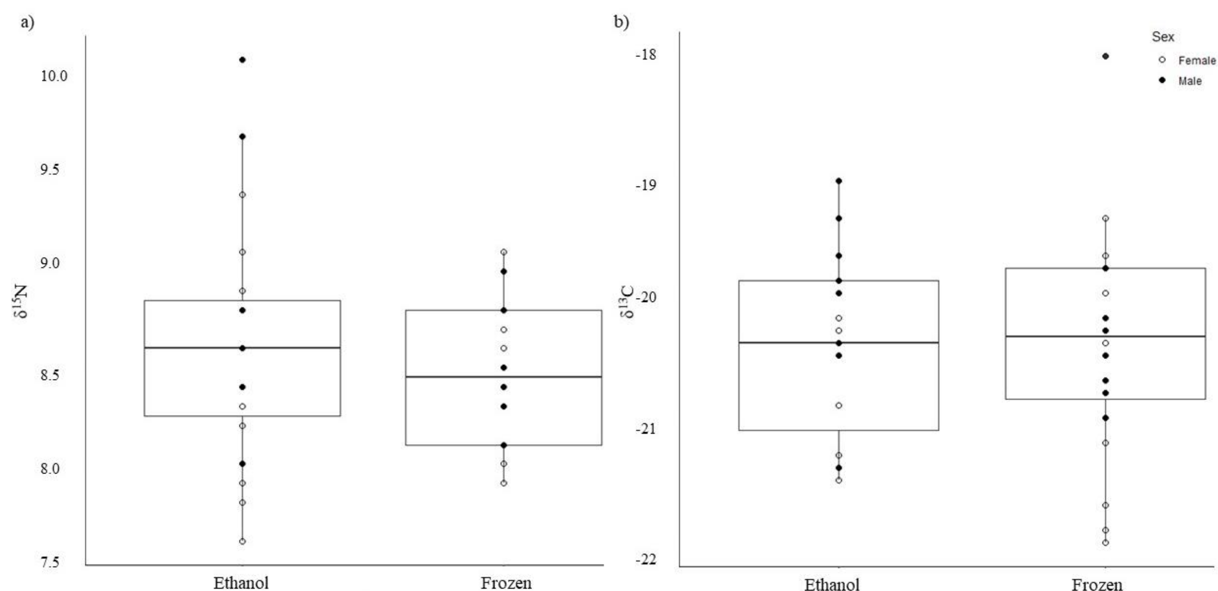


Figure 7. Boxplots summarizing results from experiment 1 (preservation experiment). A) $\delta^{15}N$ values, B) $\delta^{13}C$ values. Ethanol = ethanol killed flies, Frozen = flash-frozen flies. Open points = females, closed points = males.

Table 5. ANOVA results for experiment 1 (preservation experiment). $\delta^{15}N$ = nitrogen stable isotope, $\delta^{13}C$ = carbon stable isotope. Bold = P < 0.05.

| | | df | SS | MS | F | P |
|---|---|---|---|---|---|---|
| **$\delta^{15}N$** | Kill Method | 1 | 0.164 | 0.164 | 0.618 | 0.437 |
| | Sex | 1 | 0.298 | 0.298 | 1.122 | 0.297 |
| | Interaction | 1 | 0.431 | 0.431 | 1.626 | 0.211 |
| | Residuals | 35 | 9.826 | 0.265 | | |
| **$\delta^{13}C$** | Kill Method | 1 | 0.022 | 0.022 | 0.037 | 0.849 |
| | **Sex** | **1** | **2.649** | **2.649** | **4.335** | **0.045** |
| | Interaction | 1 | 1.827 | 1.827 | 2.990 | 0.093 |
| | Residuals | 35 | 21.388 | 0.611 | | |

### 4.3.2 Experiment 2: Persistence of Larval Diet Isotopic Signature

Adult flies exhibited predictable carbon and nitrogen fractionation from their respective larval resources (beef liver, chicken liver, salmon filet). There was a significant difference of treatment ($P < 0.0001$) for both $\delta^{15}N$ and $\delta^{13}C$ (Table 6), which was expected. However, sex was again significant for $\delta^{13}C$ ($P < 0.0001$), which aligns with results obtained from the preservation experiment. Specifically, females had low $\delta^{13}C$ values when compared to males (Table 7). Flies reared on beef liver and chicken liver had similar mean fractionation values for $\delta^{15}N$, however salmon flies exhibited much lower nitrogen fractionation. Beef liver flies were more depleted in $\delta^{13}C$ compared to chicken and salmon flies (Table 7, Figure 8). In all treatments, female *P. regina* exhibited nearly identical $\delta^{15}N$ compared to males, but were always more depleted in $\delta^{13}C$ (Table 7). Overall fractionation of flies compared to their larval diet was 3.9‰ for $\delta^{15}N$ and -2.03‰ for $\delta^{13}C$.

Table 6. Summary of ANOVA for experiment 2 (persistence of larval diet isotopic signature). $\delta^{15}N$ = nitrogen stable isotope, $\delta^{13}C$ = carbon stable isotope. * = significant at 95% confidence.

| **Isotope** | **Effect** | **Df** | **SS** | **MS** | **F** | ***P*** |
|---|---|---|---|---|---|---|
| $\delta^{15}N$ | Treatment | 2 | 135.57 | 67.79 | 726.426 | <0.0001* |
| | Sex | 1 | 0.10 | 0.10 | 1.065 | 0.312 |
| | Interaction | 2 | 0.17 | 0.09 | 0.928 | 0.409 |
| | Residuals | 24 | 2.24 | 0.09 | | |
| $\delta^{13}C$ | Treatment | 2 | 106.89 | 53.45 | 275.700 | <0.0001* |
| | Sex | 1 | 7.66 | 7.66 | 39.500 | <0.0001* |
| | Interaction | 2 | 0.17 | 0.08 | 0.433 | 0.654 |
| | Residuals | 23 | 4.46 | 0.19 | | |

Table 7. Mean isotopic and fractionation values for δ$^{15}$N and δ$^{13}$C of adult flies by treatment (beef liver, chicken liver, or salmon filet) and sex (male, female) for experiment 2. Fractionation = fly – diet, given in ‰ notation.

| | | δ$^{15}$N | | δ$^{13}$C | |
|---|---|---|---|---|---|
| **Treatment** | **Sex** | **Isotopic Value** | **Fractionation Value** | **Isotopic Value** | **Fractionation Value** |
| Beef Liver | Male | 11.3 (0.2) | 4.2 | -23.2 (0.3) | -2.4 |
| | Female | 11.0 (0.3) | 3.9 | -24.2 (0.5) | -3.4 |
| | Overall | 11.2 (0.3) | 4.1 | -23.7 (0.7) | -2.9 |
| Chicken | Male | 8.8 (0.4) | 5.0 | -18.7 (0.7) | -1.2 |
| | Female | 8.7 (0.3) | 4.9 | -19.9 (0.4) | -2.4 |
| | Overall | 8.7 (0.4) | 4.9 | -19.2 (0.8) | -1.8 |
| Salmon | Male | 13.9 (0.3) | 2.8 | -22.6 (0.3) | -1.0 |
| | Female | 14.0 (0.2) | 2.9 | -23.4 (0.4) | -1.9 |
| | Overall | 13.9 (0.2) | 2.8 | -23.0 (0.6) | -1.4 |

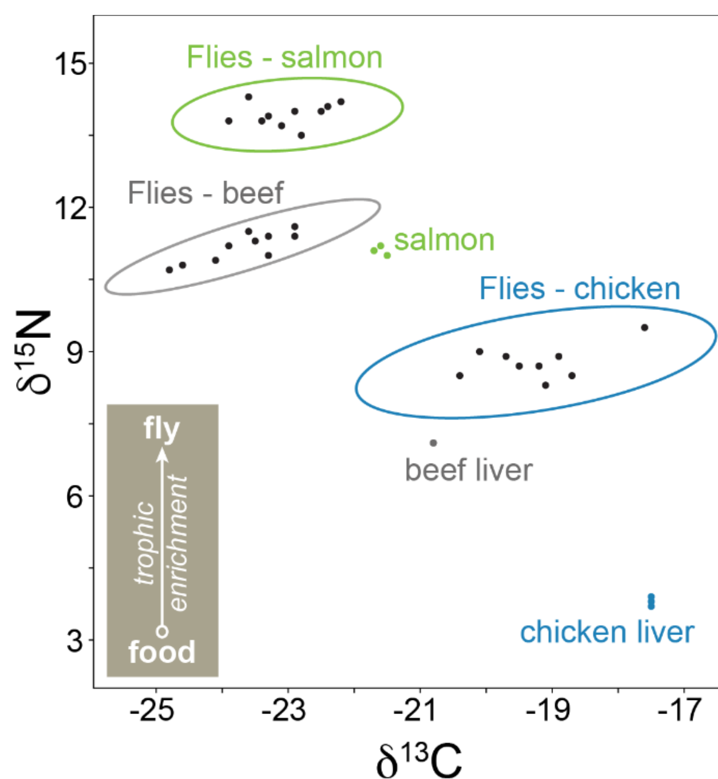


Figure 8. Scatterplot of δ$^{15}$N and δ$^{13}$C isotope values obtained from experiment 2. Circled clusters of data points represent adult flies that were reared on their respective larval diets: chicken liver (blue), beef liver (grey), or salmon filet (green).

### 4.3.3 Experiment 3: Effect of Single Adult Feeding Event

All flies exhibited predictable fractionation relative to their larval resource (beef liver) and there was no effect of adult feeding on carbon or nitrogen isotopic signatures. There were no significant main effects, interaction effects, or post-hoc paired comparisons for $\delta^{15}N$ data (Table 8). Although there is slight variation among treatments, it was not significant and all flies were enriched from their common larval resource (beef liver tissue) by an average fractionation value of 3.8‰ (Figure 9A, Table 9). Sex was the only significant factor for $\delta^{13}C$ data, as observed for the previous experiments (*P* < 0.0001, Table 8). However, $\delta^{13}C$ data show a similar trend as the $\delta^{15}N$ data: despite variation among treatments, most flies are enriched by an average of 1.8‰ relative to their larval food source (Figure 9B, Table 9). Overall, there are no significant differences among adult feeding treatments for $\delta^{15}N$ and $\delta^{13}C$.

Table 8. ANOVA results for experiment 3 (effect of adult diet). $\delta^{15}N$ = nitrogen stable isotopes, $\delta^{13}C$ = carbon stable isotopes. * = significant at 95% confidence.

| **Isotope** | **Effect** | **Df** | **SS** | **MS** | **F** | ***P*** |
|---|---|---|---|---|---|---|
| $\delta^{15}N$ | Treatment | 3 | 1.514 | 0.505 | 0.749 | 0.530 |
| | Sex | 1 | 0.291 | 0.292 | 0.433 | 0.515 |
| | Interaction | 3 | 1.738 | 0.579 | 0.860 | 0.470 |
| | Residuals | 38 | 25.589 | 0.673 | | |
| $\delta^{13}C$ | Treatment | 3 | 0.167 | 0.056 | 0.183 | 0.907 |
| | Sex | 1 | 12.175 | 12.175 | 40.169 | <0.0001* |
| | Interaction | 3 | 0.195 | 0.065 | 0.215 | 0.885 |
| | Residuals | 38 | 11.517 | 0.303 | | |

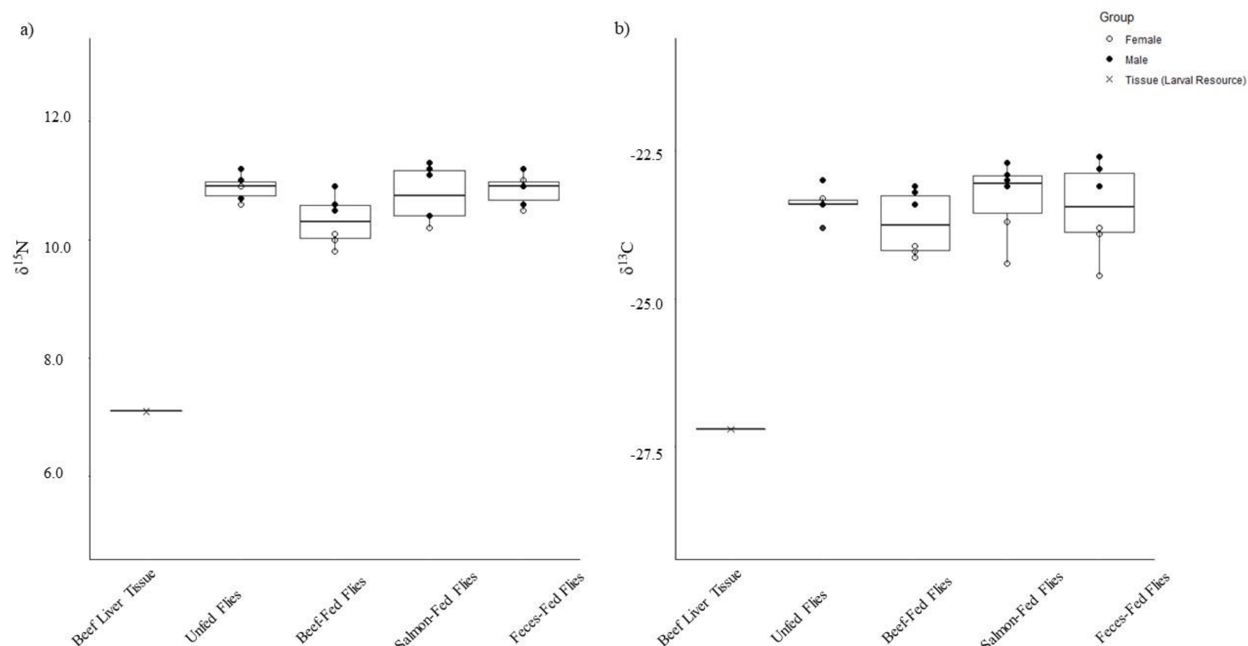


Figure 9. Boxplots summarizing results of experiment 3 (effect of adult diet). A) $\delta^{15}N$ data, B) $\delta^{13}C$ data. Open dots = females, closed dots = males, “X” = larval resource.

Table 9. Mean fractionation values (fly – diet) of flies from various adult feeding treatments (experiment 3) relative to their larval resource (beef liver tissue), given in ‰ notation. Unfed = flies not fed as adults (negative controls), beef-fed = flies exposed to beef liver as adults, salmon-fed = flies exposed to salmon filets as adults, and feces-fed = flies exposed to carnivore feces as adults.

| | **Fractionation Values (‰)** | |
|---|---|---|
| **Adult Feeding Treatment** | **$\delta^{15}N$** | **$\delta^{13}C$** |
| Unfed | 3.94 | 2.12 |
| Beef-fed | 3.53 | 1.49 |
| Salmon-fed | 3.92 | 2.19 |
| Feces-fed | 3.92 | 2.10 |
| ***Overall*** | *3.83* | *1.98* |

### 4.3.4 Wild Fly Validation

Based on the animal isotopic values gathered from the literature (Table 10), four distinct, conservative trophic categories were statistically differentiated based on $\delta^{15}N$ and $\delta^{13}C$ values: fish, carnivores, browsers, and mixed herbivores (Figure 10, Table 11). Though fish do not

technically represent a true trophic position, their isotopic values (particularly $\delta^{15}N$) are different enough from all other vertebrates that they were left as their own category for the purposes of this study. Omnivores were excluded as a definitive category as their isotopic values (both $\delta^{15}N$ and $\delta^{13}C$) overlapped with several other categories and could therefore not be statistically differentiated (Figure 10).

Table 10. Animal isotopic values gathered as part of the meta-analysis to obtain relevant isotopic values with which to compare to wild fly isotopic signatures.

| Study | Trophic Level | Animal Type | Species | $\delta^{15}N$ | $\delta^{13}C$ |
|---|---|---|---|---|---|
| Newsome et al. (2010) | Carnivore | Mammal | Kit fox | 10.6 | -19.8 |
| Newsome et al. (2010) | Carnivore | Mammal | Kit fox | 7.9 | -17.4 |
| Codron et al. (2016) | Carnivore | Mammal | Spotted Hyena | 6.8 | -18.0 |
| Codron et al. (2016) | Carnivore | Mammal | Spotted Hyena | 8.6 | -15.3 |
| Codron et al. (2016) | Carnivore | Mammal | Brown Hyena | 6.8 | -16.5 |
| Codron et al. (2016) | Carnivore | Mammal | Lion feces | 10.9 | -14.6 |
| Bernhardt et al. (2017) | Carnivore | Mammal | Cat | 6.2 | -22.1 |
| Bernhardt et al. (2017) | Carnivore | Mammal | Dog | 8.0 | -22.3 |
| Codron et al. (2016) | Browser | Mammal | Giraffe feces | 7.2 | -24.1 |
| Codron et al. (2016) | Browser | Mammal | Giraffe feces | 5.9 | -24.5 |
| Codron et al. (2016) | Browser | Mammal | Greater Kudu feces | 7.8 | -24.5 |
| Codron et al. (2016) | Browser | Mammal | Greater Kudu feces | 6.8 | -24.3 |
| Codron et al. (2016) | Browser | Mammal | Greater Kudu feces | 7.3 | -23.2 |
| Newsome et al. (2010) | Grazer | Mammal | Gopher hair | 6.1 | -25.4 |
| Ehrich et al. (2015) | Grazer | Mammal | Lemming | 2.5 | -26.1 |
| Ehrich et al. (2015) | Grazer | Mammal | Muskox | 4.4 | -23.7 |
| Ehrich et al. (2015) | Grazer | Mammal | Lemming | 5.9 | -27.3 |
| Ehrich et al. (2015) | Grazer | Mammal | Lemming | 2.5 | -27.1 |
| Ehrich et al. (2015) | Grazer | Mammal | Lemming | 1.3 | -26.3 |
| Ehrich et al. (2015) | Grazer | Mammal | Narrow-headed vole | 4.9 | -26.5 |
| Ehrich et al. (2015) | Grazer | Mammal | Middendorf's vole | 5.8 | -25.5 |
| Ehrich et al. (2015) | Grazer | Mammal | Mountain hare | 2.4 | -25.9 |
| Ehrich et al. (2015) | Grazer | Mammal | Norway lemming | 1.6 | -27.8 |
| Ehrich et al. (2015) | Grazer | Mammal | Root vole | 2.8 | -27.5 |
| Ehrich et al. (2015) | Grazer | Mammal | Grey Red-backed Vole | 1.0 | -26.5 |
| Ehrich et al. (2015) | Grazer | Mammal | Mountain hare | 3.6 | -26.6 |
| Ehrich et al. (2015) | Grazer | Mammal | Arctic hare | 1.8 | -23.4 |
| Ehrich et al. (2015) | Grazer | Mammal | Muskox | 3.1 | -24.2 |
| Bernhardt et al. (2017) | Grazer | Mammal | Sheep | 7.6 | -26.1 |
| Ditmer et al. (2003) | Mixed Herbivore | Mammal | Deer | 5.8 | -27.0 |

Table 10 continued

| | | | | | |
|---|---|---|---|---|---|
| Codron et al. (2016) | Mixed Herbivore | Mammal | Impala feces | **6.9** | **-16.7** |
| Codron et al. (2016) | Mixed Herbivore | Mammal | Impala feces | 7.2 | -17.6 |
| Codron et al. (2016) | Mixed Herbivore | Mammal | Impala feces | 7.1 | -19.6 |
| Codron et al. (2016) | Mixed Herbivore | Mammal | Nyala feces | 8.0 | -24.1 |
| Ehrich et al. (2015) | Mixed Herbivore | Mammal | Reindeer | 5.9 | -23.5 |
| Ehrich et al. (2015) | Mixed Herbivore | Mammal | Reindeer | 3.1 | -23.4 |
| Ehrich et al. (2015) | Mixed Herbivore | Mammal | Reindeer | 2.3 | -25.2 |
| Ehrich et al. (2015) | Mixed Herbivore | Mammal | Reindeer | 4.2 | -25.7 |
| Bernhardt et al. (2017) | Mixed Herbivore | Mammal | Cattle (C3/C4) | 5.9 | -17.9 |
| Bernhardt et al. (2017) | Mixed Herbivore | Mammal | Roe Deer | 1.9 | -27.1 |
| Bernhardt et al. (2017) | Mixed Herbivore | Mammal | Deer | 4.4 | -20.3 |
| Bernhardt et al. (2017) | Omnivore | Mammal | Shrew | 8.3 | -25.4 |
| Bernhardt et al. (2017) | Omnivore | Mammal | Squirrel | 4.4 | -22.5 |
| Newsome et al. (2010) | Omnivore | Mammal | Ground squirrel hair | 6.5 | -21.7 |
| Newsome et al. (2010) | Omnivore | Mammal | Deer Mouse | 6.3 | -19.9 |
| Newsome et al. (2010) | Omnivore | Mammal | Humans hair | 8.8 | -19.1 |
| Newsome et al. (2010) | Omnivore | Bird | Birds feathers | 7.8 | -19.2 |
| Kurle et al. (2014) | Omnivore | Mammal | Rat | 10.8 | -19.0 |
| Koenig et al. (2008) | Omnivore | Bird | Woodpecker | 6.4 | -22.9 |
| Koenig et al. (2008) | Omnivore | Bird | Woodpecker | 5.3 | -23.5 |
| Ditmer et al. (2003) | Omnivore | Mammal | Black Bear hair | 5.8 | -23.4 |
| Ditmer et al. (2003) | Omnivore | Mammal | Black Bear hair | 5.4 | -23.6 |
| Ditmer et al. (2003) | Omnivore | Mammal | Black Bear hair | 5.6 | -22.2 |
| Ditmer et al. (2003) | Omnivore | Mammal | Black Bear hair | 4.9 | -22.9 |
| Newsome et al. (2010) | Omnivore | Mammal | Kangaroo rats hair | 8.5 | -22.0 |
| Bernhardt et al. (2017) | Omnivore | Mammal | Boar (C3/C4) | 6.7 | -19.9 |
| Bernhardt et al. (2017) | Omnivore | Mammal | Human Muscle | 8.7 | -22.7 |
| Bernhardt et al. (2017) | Fast-food | Bird | Chicken | 2.8 | -23.4 |
| Ehrich et al. (2015) | Fish | Fish | Salmon | 11.3 | -19.7 |
| Ehrich et al. (2015) | Fish | Fish | Arctic cisco | 13.1 | -24.5 |
| Ehrich et al. (2015) | Fish | Fish | Burbot | 14.6 | -25.9 |
| Ehrich et al. (2015) | Fish | Fish | Navaga | 13.8 | -20.5 |
| Ehrich et al. (2015) | Fish | Fish | Northern Pike | 11.8 | -26.7 |
| Ehrich et al. (2015) | Fish | Fish | Clupeid fish | 12.6 | -19.8 |
| Ehrich et al. (2015) | Fish | Fish | Arctic char | 14.6 | -21.6 |
| Ehrich et al. (2015) | Fish | Fish | Whitefish | 12.0 | -25.4 |
| Ehrich et al. (2015) | Fish | Fish | Broad whitefish | 11.0 | -22.4 |
| Ehrich et al. (2015) | Fish | Fish | Whitefish | 10.2 | -29.2 |
| Ehrich et al. (2015) | Fish | Fish | Grayling | 11.5 | -27.6 |
| Ehrich et al. (2015) | Fish | Fish | Salmon bone | 12.1 | -19.9 |
| Bernhardt et al. (2017) | Fish | Fish | Fish | 13.6 | -20.9 |

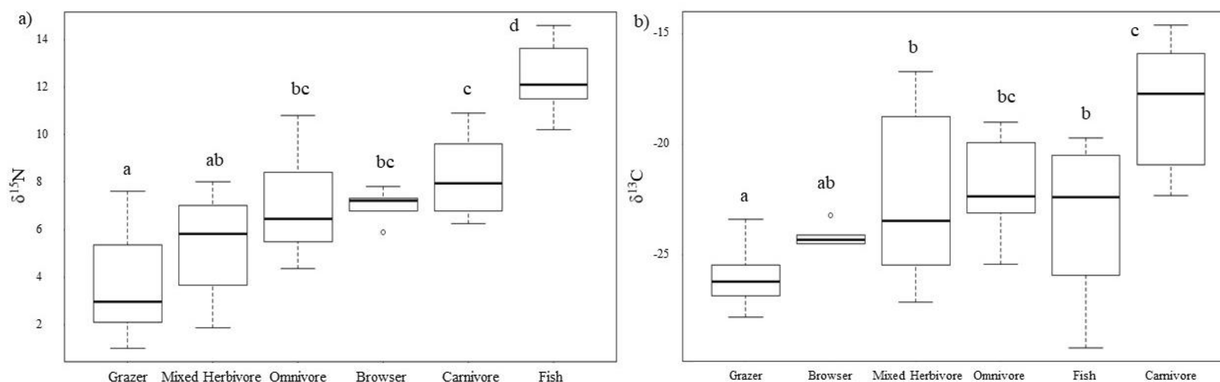


Figure 10. Boxplots summarizing the statistical differences between animal trophic values taken from the literature. $\delta^{15}N$ is given on the left (A) and $\delta^{13}C$ is given on the right (B). Lowercase letters represent statistical significance at P = 0.05, with different letters indicating a statistical difference between paired groups and the same letter indicating no difference between groups.

Table 11. Ranges of $\delta^{15}N$ and $\delta^{13}C$ values for each vertebrate trophic positions obtained from the meta-analysis taken.

| **Trophic Level** | **$\delta^{15}N$** | **$\delta^{13}C$** |
|---|---|---|
| Fish | 9.97 – 13.87 | -26.70 – -20.08 |
| Carnivore | 7.72 – 9.96 | -21.16 – -15.33 |
| Browser | 6.29 – 7.71 | -24.66 – -23.58 |
| Grazer/Mixed Herbivores | 1.00 – 6.41 | -27.60 – -21.26 |

Wild fly $\delta^{15}N$ values ranged from 5.7 to 14.8‰ and $\delta^{13}C$ ranged from -29.2 to -19.2‰. Raw data distributions grouped roughly according to region (urban, Smokies, Yellowstone; Figure 11). Flies collected in the urban environment exhibited significantly higher $\delta^{15}N$ values compared to the other two environments ($P < 0.0001$), with more similar $\delta^{15}N$ values seen between the two national parks. In terms of $\delta^{13}C$, Yellowstone flies were significantly more depleted compared to flies from the urban environment and from the Smokies ($P < 0.0001$).

In order to determine the carcass trophic positions derived from wild fly isotopic signatures, all experimental fractionation data for female flies was averaged for both $\delta^{15}N$ and $\delta^{13}C$, which showed 3.6‰ enrichment in $\delta^{15}N$ and 0.3‰ depletion in $\delta^{13}C$. To obtain carcass

values, 3.6 was subtracted from wild fly $\delta^{15}N$ values and 0.3 was added to $\delta^{13}C$ values (Figure 12). Carcass $\delta^{15}N$ values ranged from 2.1 to 11.2 and $\delta^{13}C$ ranged from -28.9 to -18.9.

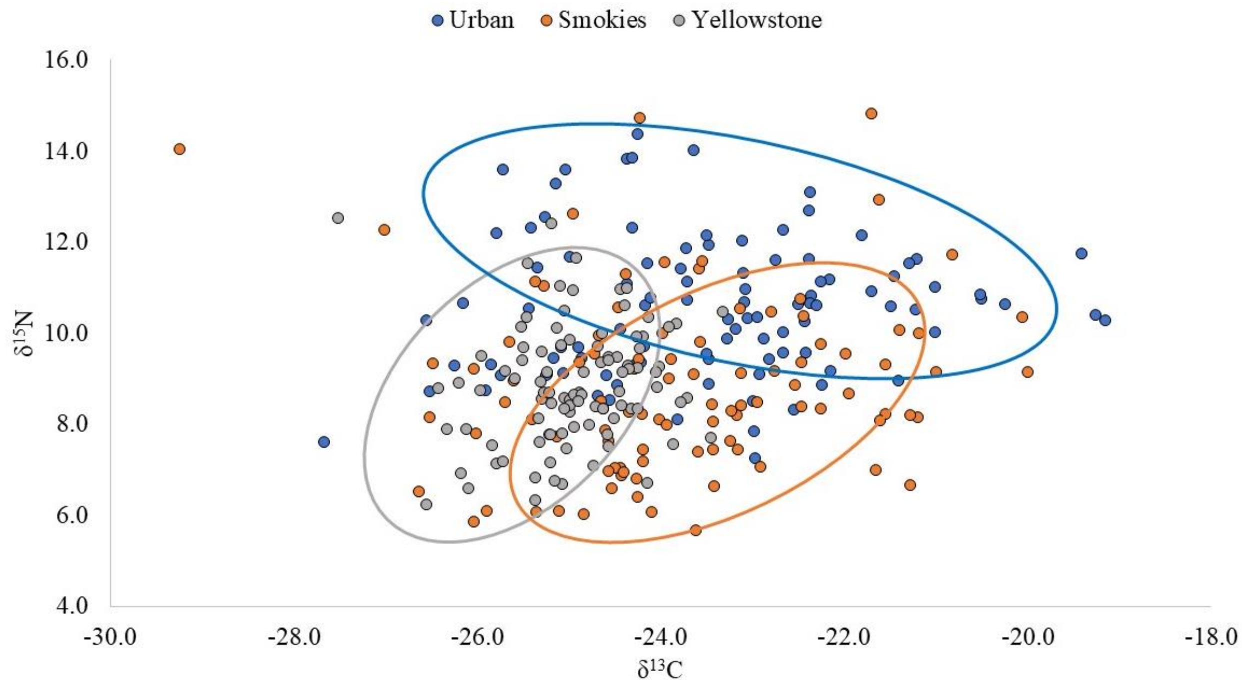


Figure 11. $\delta^{15}N$ and $\delta^{13}C$ isotopic values of wild flies collected in three different regions: urban (=blue), Smokies (=orange), and Yellowstone (=grey). Values are raw and untransformed.

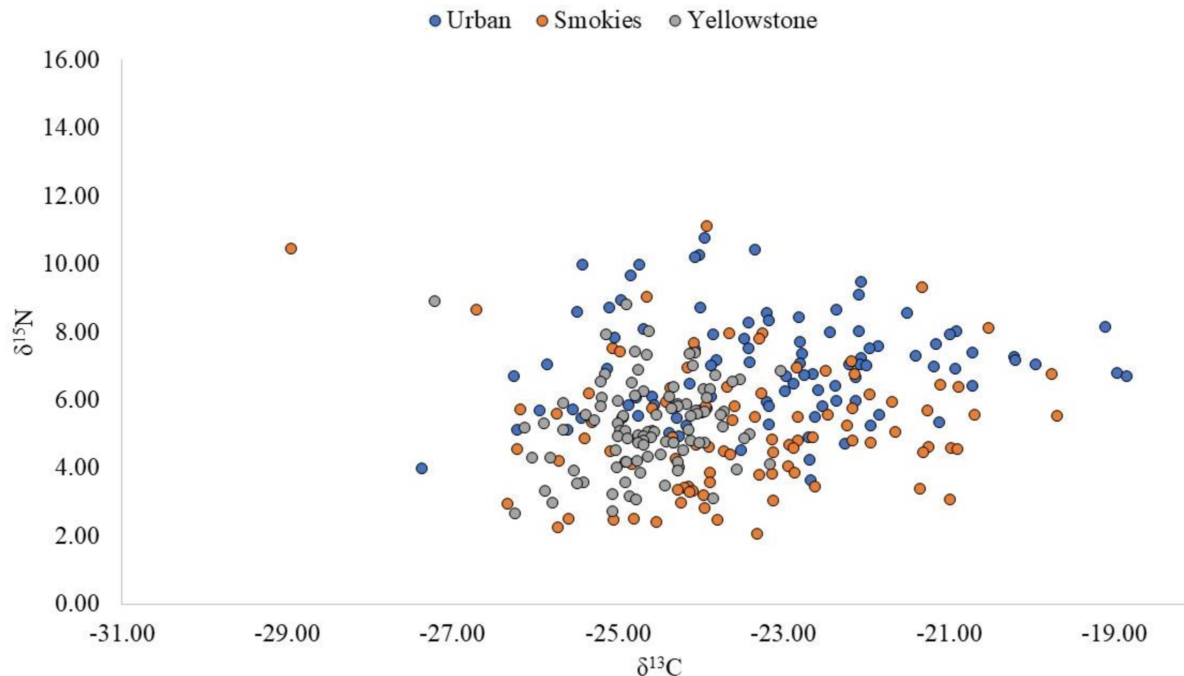


Figure 12. Putative carcass $\delta^{15}N$ and $\delta^{13}C$ isotopic values derived from wild flies from urban (=blue), Smokies (=orange), and Yellowstone (=grey). Wild fly data were transformed using average experimental fractionation values of adult female flies.

Overall, trophic composition varied greatly between the urban region and the national parks (Figure 13). A more even trophic position distribution was seen in the urban region, where mixed herbivores, browsers, and carnivores were seen in roughly the same proportions, with fish being the least detected carcass. Mixed herbivores made up a majority of the trophic positions detected by flies in the Smokies (79%) and Yellowstone (81%). No fish were observed in Yellowstone, and the fewest number of flies with carnivore isotopic signatures were detected in this region as well (4% vs. 7% and 23% in the Smokies and urban environments, respectively).

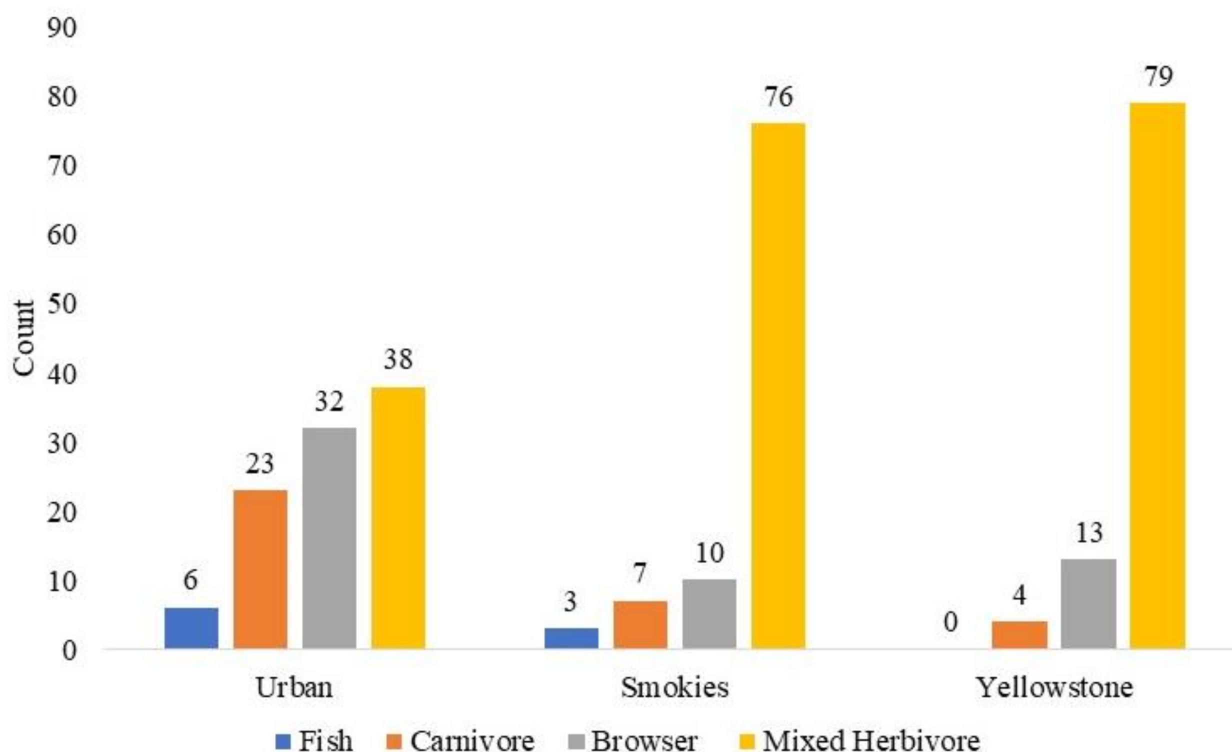


Figure 13. Bar plot summarizing number of wild flies from Urban, Smokies, and Yellowstone regions with isotopic signatures indicative of four trophic positions: fish (=blue), carnivore (=orange), browser (=grey), and mixed herbivore (=yellow).

## 4.4 Discussion

This research has shown that there is predictable fractionation of $\delta^{15}N$ in adult flies compared to their larval diet, though females tend to be more depleted in $\delta^{13}C$ compared to their diets than males. This could be explained by quicker turnover rates that might be occurring in female flies throughout their lifetimes as compared to males. In vertebrates, it is known that $\delta^{13}C$ turnover can vary between different tissues of the same individual due to physiological processes like lipid synthesis [28]. Larvae of other fly species, like *Chironomus acerbiphilus* (Diptera: Chironomidae), can exhibit increased $\delta^{13}C$ during periods of starvation due to the preferential breakdown of the heavy isotope, $^{13}C$, when dietary carbon is unavailable [29]. Thus, the differences in female versus male $\delta^{13}C$ in *P. regina* in this study could be due to the breakdown of

already assimilated $^{12}C$ from the larval diet in the females, perhaps due in part to reproductive needs of the maturing female flies. Conversely, males may break down the heavy isotope ($\delta^{13}C$) quicker than females, leading to the differences observed (males with higher enrichment in carbon). However, as the heavily sclerotized head of the fly was used for analysis in this study, and not any part of the body containing fat or organs, it is still unknown why this process would be occurring in these flies. Regardless of the exact mechanism, it will be important for future studies to take sex of the fly into consideration when deriving meaningful interpretations from carbon isotopic data derived from flies (i.e. larval diet trophic positions).

Our data also show that the integrity of the carcass isotopic signature in the adult fly remains intact even after adult feeding. This means that the isotopic signatures obtained from flies can be reliably attributed to the fly's larval resource and not environmental or physiological conditions experienced by the adult fly itself. However, as a temporal component was not implemented in this study, it is not known whether the turnover of $\delta^{13}C$ and $\delta^{15}N$ in the fly tissues change with the age of the fly. It is unlikely that this is the case, at least in the head of the fly (used for analysis in this study). Once the cuticle of the exoskeleton is laid down and sclerotized (i.e. hardened) after eclosion from the puparium, it is unlikely to change throughout the rest of the blow fly's life. Adult blow flies do not molt, so once they emerge as adults, the sclerotized parts of their bodies (head, thorax, legs) do not physically change. Therefore, the age of the fly likely does not change the isotopic signature within highly sclerotized regions of the body, like the head.

The overall isotopic fractionation of wild female blow flies aligns with similar values compiled from the extensive body of animal isotopic literature [30], lending more confidence to deriving carcass trophic diversity metrics from these flies. The trophic positions of carcasses given here align with much of the molecular and chemical data from the same flies presented in Chapter

3. Vertebrate DNA data from flies in the urban environment showed a greater proportion of medium sized carnivores (e.g. dogs and raccoons) visited by adult flies compared to flies collected in the national parks. However, even though herbivores (e.g. white-tailed deer, groundhogs, eastern cottontails) made up a much smaller proportion of animals detected from molecular analysis of urban flies, isotope data suggests that these animals are important larval resources for flies central Indiana. On the other hand, vertebrate DNA from flies in the Smokies and in Yellowstone aligned well with the stable isotope data, suggesting that herbivore carcasses are not only important protein sources for adults, but that they serve as the prominent larval resources in these areas.

Overall, higher $\delta^{15}N$ values were observed in flies from the urban environment compared to the other regions. This is likely due to a higher number of carnivore carcasses available due to road mortality. Indianapolis is the 13$^{th}$ largest city in the United States [31] with 20,000 km of roadways [32]. Subsampling of roadkill deaths in Indiana from 2005 to 2006 showed 563 mortality events in 9 locales, with 457 of those being mammals, 179 specifically being the Virginia opossum, and 144 being raccoon [33]. This aligns with both molecular and isotopic data from this study, as both opossums and raccoons make up a large portion of vertebrate DNA derived from flies in the urban environment, and they may have potentially served as a portion of the 23% of carnivores utilized by flies for larval development. Therefore, road mortality is a likely explanation for at least some of the animals that were detected by blow flies in this area as they were used as larval developmental substrates in the warmer months of the year. Additionally, close proximity of a roadkill carcass to the road often deters vertebrate scavengers, which makes the carcass more available to insect colonizers.

Higher $\delta^{13}C$ values were observed more in flies from the urban and Smokies environments compared to flies from Yellowstone. This could likely be an influence of corn-based foods being more highly available in this environment. Domestic or agricultural animals being fed or supplemented with corn-based diets make up a portion of the animals detected by DNA in these areas (e.g. cows, pigs, chickens), therefore their muscle tissues may be more enriched with $C_4$, leading to higher $\delta^{13}C$ values. Additionally, opportunistic vertebrate scavengers (e.g. raccoons, opossums, black bears) may also have access to human junk food, fast food (often deep-fried in corn oil, [21]), and corn-based food waste that could have the same isotopic effect on their tissues. If flies develop on the tissues of any of these animals, their $\delta^{13}C$ isotopic signatures would more likely resemble those of corn-fed animals. Conversely, flies collected in Yellowstone had much more depleted $\delta^{13}C$ values compared to the other regions, which indicates that the herbivores they fed upon were consuming more $C_3$ plants, like native grasses. Given all of the molecular and chemical data, as well as species occurrence and abundance lists from these areas[34-36], the stable isotope data gleaned from these flies aligns with all previous observations of vertebrates in these regions.

## 4.5 Conclusion

When used in conjunction with other analytical methods, it is clear that stable isotope analysis can add another rich dimension to community assessment survey methods using carrion insects Today's changing climate requires special tools to assess community diversity that can be quickly implemented and be as minimally invasive as possible to maintain the integrity of fragile ecosystems. The use of stable isotope analysis of necrophagous insects, like blow flies, allows researchers to gain a snapshot of the overall vertebrate community composition of an ecosystem without disturbance. Simultaneous use of isotopic, molecular, and chemical data derived from flies

in these areas can give a quick, comprehensive survey of vertebrate communities that rivals any traditional survey method currently being used. Additionally, stable isotope analysis of blow flies allows researchers to assess the dead vertebrate community in a way that is difficult to accomplish with molecular techniques. As the blow larvae are limited to developing on dead animals, isotopes only reveal the available carcasses in an environment, with no additional potential resources (e.g. feces or garbage) confounding interpretations. Overall, there are many applications of this method in ecology, forensics, and even conservation biology.

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# CHAPTER 5. ABIOTIC AND BIOTIC MEDIATORS OF SPATIOTEMPORAL *PHORMIA REGINA* POPULATION GENETIC STRUCTURE IN INDIANA

## 5.1 Introduction

### 5.1.1 Population Genetics and Theoretical Models

Populations are finite groups of interacting individuals limited spatially and temporally to some extent [1]. These groups tend to form naturally as species abundance increases and thus distributions expand, which may lead to fragmentation into smaller subsets. When populations divide, and if they become geographically isolated, reproductive isolation can occur [2] which can lead to eventual accumulations of genetic changes within each divergent subpopulation. Theoretical models predict a range of connectivity (i.e. gene flow) among subpopulations in order to explain population dynamics and genetics. These models describe a range populations from fully panmictic to completely isolated, with many models falling somewhere in the middle of the spectrum. Panmictic populations may arise due to the presence of highly connected, spatiotemporally variable patches, or areas with high resource availability. Due to high dispersal, and thus increased migration among patches, subpopulations are effectively unified into a single large population (patchy model, Figure 14A) [3]. Metapopulations represent groups of local populations connected by migration events (Figure 14B). In contrast to the patchy model, moderate gene flow can occur even though genetic differentiation among subpopulations may be observed in these populations [4]. Originally formulated to target pest populations, the classic metapopulation model proposed by Levins (1969) assumes that distinct local populations persist due to regional recolonization of homogeneous (e.g. size and quality) patches after a local extinction event [5]. Thus, there is constant balance and little variability between subpopulations,

with movements occurring over generations. The source-sink metapopulation model is differentiated from the Levins metapopulation model by its focus on quality, rather than size, of the available patches [1] (Figure 14C). Lastly, on the opposite end of the population connectivity spectrum from the patchy model lies the isolated population model (Figure 14D). Species experiencing limited spatial dispersal have a greater degree of genetic differentiation among their subpopulations as the geographic distance increases, due to reduced gene flow and increased effects of random genetic drift [2, 6]. This can be seen in naturally and deliberately isolated populations of cutthroat trout (*Oncorhynchus clarkii*) in the Rocky Mountains, which due to spatial isolation, have experienced dramatic genetic diversity loss that could potentially endanger their future survival in the wild [7].

At its core, the field of population genetics provides useful tools for investigating heritable biological variation within and among populations [8]. Specifically, allele frequencies are used to expose evolutionary forces that have made a historical impact on the genetic makeup of a species, such as natural selection, random genetic drift, gene flow, and mutation. With these tools, it is possible to explore not only a species' evolutionary past, but to also predict its future population dynamics across space and time. Thus, population genetics tools (specifically microsatellite markers) will be used to investigate populations of the black blow fly *Phormia regina* (Meigen) (Diptera: Calliphoridae) and determine if any of the proposed population models best explains the observed population genetic patterns.

The patchy population model represents the null hypothesis for this research. Failure to reject this model would be evidenced by: 1) adherence to Hardy-Weinberg equilibrium principles (HWE); 2) high genetic diversity and low substructure ($F_{ST} < 0.01$) among populations; 3) large

numbers of migrants per population and generation ($N_M$); 4) low kinship within populations (R ~ 0); and, 5) consistent and abundant resource availability.

The metapopulation model (specifically Levins) is proposed as the first alternative hypothesis to explain blow fly population structure. Failure to reject this model would be evidenced by: 1) a population not adhering to HWE; 2) moderate genetic differentiation ($F_{ST}$) among populations; 3) moderate migration ($N_M$) among samples; 4) high relatedness within populations ($R > 0.10$); and, 5) variability in carrion and fecal abundance and diversity among population**.** Additional support for a metapopulation model would be given if there is evidence of subpopulation structure adhering to a source-sink type model. If a source-sink model were true for blow flies, high gene flow would be evident and low $F_{ST}$ would be observed between one site and all other populations, and reduced gene flow between the other populations.

The final alternative hypothesis to explain blow fly population structure is that *P. regina* forms isolated subpopulations. Failure to reject this model would include: 1) a population not adhering to HWE; 2) significant genetic differentiation among populations (high $F_{ST}$); 3) negligible migration between populations; 4) high relatedness within populations ($R > 0.10$), and 5) high variability of carrion and feces abundance/diversity among subpopulations.

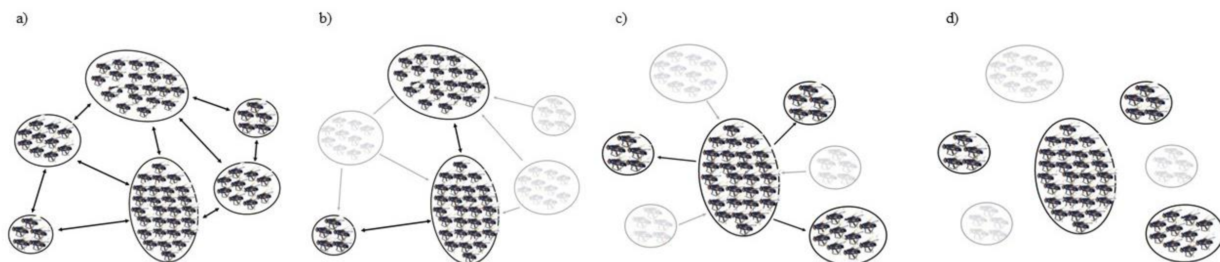


Figure 14. Potential population models explaining blow fly population structure. Decreasing connectivity between subpopulations occurs from left to right. Arrows indicate migration (i.e. gene flow), and grey subpopulation represent extinction events. A) patchy population model, B) Levin's metapopulation model, C) source-sink population model, D) isolated population model.

### 5.1.2 Potential Mediators of Blow Fly Population Structure

Several attempts have been made to quantify genetic differentiation among blow fly populations, though there has been little evidence of spatial structure thus far. Population genetic studies of the blow fly, *Lucilia sericata*, in Australia have indicated that spatial genetic structure may exist in this species when using the mitochondrial COI gene [9]. However, global surveys using the same gene have not been able to correlate genetic data of blow flies to their geographic origins [10]. A survey spanning 25 locations throughout the United States again showed that *P. regina* and *L. sericata* exhibited little spatial structure (even among flies collected thousands of miles apart), but that flies collected at the same time were highly related to each other [11, 12], which may indicate signs of local temporal structure. These results were echoed in Indiana *P. regina* [13] as well as in a Florida survey focusing on the invasive blow fly species *Chrysomya megacephala* (Macquart) [14]. Thus far, genetic diversity of blow flies has been assessed via many molecular means, including RAPD analysis [15-17], AFLP [11, 12, 14], mtDNA COI [18] and COII [19] gene sequencing, and microsatellites [20-24]. Microsatellites (or short tandem repeats, STRs) are composed of sections of adjacent two, three, four, or five nucleotide repeats bounded by unique flanking regions. Microsatellites represent a powerful tool in assessing genetic variation among populations given they are codominant markers (allowing for scoring of heterozygotes), widely abundant throughout the genome, possess a high number of alleles per locus, have a high polymorphism rate, and have high heterozygosity. Thus, microsatellites are the marker of choice for this research.

It is possible that blow flies, due to the unpredictable nature of carrion [25] and the remarkable ability of the flies to locate it [26], may exist as a large panmictic population. However, they may also exist in a metapopulation state, fluctuating between colonization and extinction as

carrion may be unavailable for extended periods of time. Lack of carrion in an area could span one to several blow fly generations, leading to rapid local extinction in those areas. Given the blow fly's dependence on this ephemeral resource, carrion availability likely prominently impacts blow fly population dynamics and, in turn, their genetics. Additionally, vertebrate feces is a vital protein resource for female blow flies to develop their reproductive organs [27, 28], and it is also potentially used as a mating site. Since feces may be a more predictable protein resource than carrion, its near constant presence in some areas (e.g. dog parks) could drastically influence the genetic structure of local populations.

Numerous abiotic factors elicit strong physiological responses in insects as they are physiologically dependent on their external environment, including local weather [29, 30]. Temperature may be the most well-documented of these abiotic factors, as it has a predominant impact on development, distribution, and survival due to the poikilothermic nature of insects [31-33]. Humidity can also have a significant impact on the average number of eggs oviposited by female insects [34-36], and photoperiod can affect oviposition, diapause timing, and development rate [37-39]. Precipitation has been shown to have variable effects on insect community diversity depending on the habitat (e.g. arid vs. tropical), potentially due to complex interactions occurring among organisms in the ecosystem (e.g. multitrophic interactions) [40, 41]. Additionally, landscape features, like elevation gradients and mountain slopes, represent potential physical barriers that prevent gene flow and may thus facilitate eventual isolation, substructuring, and even speciation of populations [42, 43]. Biotic factors can also greatly impact insect populations. Interspecific competition in blow flies is well documented and previous data suggests that intense competition in some species can radically alter behaviors of flies [44-46]. Thus, the presence and ensuing competition with other blow fly species could impact the ability of flies to successfully

colonize carcasses in a patch, which could potentially lead to local extinctions. Food availability (as well as limitation) may be one of the biggest drivers of population dynamics and genetics for most animal species [47-49]. As such, the availability of ephemeral resources like carcasses should have a large impact on blow flies due to their inherent carrion-breeding nature. Though all of the aforementioned factors are known to have large impacts on insect populations, they have currently not been investigated in conjunction with blow fly population genetics.

The overarching goal of this research was to determine how abiotic factors (e.g. temperature, humidity, wind speed, precipitation) and biotic factors (e.g. vertebrate resource availability, interspecific competition) significantly impacted *P. regina* population genetic structure. Though previous studies of this family found no strong evidence of genetic structure on a spatial scale, those results may have been a consequence of limited sampling. It was my goal to test multiple population model hypotheses regarding blow fly population structure.

## 5.2 Methods

### 5.2.1 Blow Fly Collections

Spatiotemporal collections (6 geographic sites, 14 time periods for 2016, 16 time periods for 2017) of *P. regina* were made from March to October 2016 and 2017 in Central Indiana, USA. Collection sites consisted of 2 replicate regions (i.e. counties) containing three urban parks each (Figure 15, Table 12). Fly collections were made using an aged chicken liver bait (inside a "cage" to prevent flies from landing on the bait without hindering the ability to detect its chemical cues) and an aerial sweep net. Fly sampling lasted 20 min starting at the time of bait exposure.

Figure 15. Map of blow fly collection sites in central Indiana. Numbers 1 – 3 indicate Marion County sites: Military Park, Broad Ripple Park, and Skiles Test Park, respectively. Numbers 4 – 6 indicate Johnson County sites: Northwest Park, University Park, and Province Park, respectively. Distances (in km) are given between sites in each county.

Table 12. Summary of blow fly collection sites in central Indiana during 2016 and 2017.

| Site | City, State | County | Coordinates |
|---|---|---|---|
| Broad Ripple Park (BR) | Indianapolis, IN | Marion | 39.871666, -86.130278 |
| Military Park (MP) | Indianapolis, IN | Marion | 39.770555, -86.168611 |
| Northwest Park (NW) | Greenwood, IN | Johnson | 39.628611, -86.143611 |
| Skiles Test Park (SP) | Indianapolis, IN | Marion | 39.867882, -86.048541 |
| University Park (UP) | Greenwood, IN | Johnson | 39.611061, -86.050641 |
| Province Park (PP) | Franklin, IN | Johnson | 39.477500, -86.053333 |

### 5.2.2 Abiotic Factor Data Collection

Temperature and relative humidity were measured throughout the collections using a Datalogging RH/Temperature Pen (SPER Scientific, Scottsdale, Arizona, USA) elevated ~1 m above the ground. Wind speed and direction were also measured at three arbitrary times during each collection using a Digi-Sense® Mini Vane Anemometer (Cole-Parmer, Vernon Hills, Illinois,

USA). After the 20 min collection window, flies were immediately killed in 70% ethanol for transportation to the lab and stored at -20˚C.

### 5.2.3 Dissections and DNA Extractions

A maximum of ten female *P. regina* were randomly selected from each spatiotemporal sample for molecular and chemical analysis. Gut dissections and organic DNA extractions underwent the same protocol outlined in Chapter 2 [50].

### 5.2.4 Microsatellite Amplification and Kinship Analysis

Microsatellite markers specific to the *P. regina* were developed by scanning the *P. regina* genome for di-, tri-, and tetra-nucleotide repeats [13]. Six microsatellite loci with tetra-nucleotide motifs were used to assess the genetic relationships of *P. regina* (Table 13). These loci were are polymorphic, with a range of 11 to 86 (mean = 56.7, SE ± 27.5) alleles per locus, and they are known to be stable over time [13]. PCR amplification of each *P. regina* DNA extraction was carried out in two separate multiplex reactions each containing three primer pairs. Both reactions consisted of 5.0 µL 2x PCR MasterMix (Promega), 0.5 µL 1x Bovine Serum Albumin (BSA), and 0.5 µL nuclease-free water, and 1 µL of each primer according to Table 13. PCR conditions consisted of an initial 2 min denaturation step at 95˚C, nine touchdown cycles of a 15 s denaturation step at 95˚C, an annealing step from 63˚C to 55˚C for 30 s, and an extension step at 72˚C for 2 min. This was followed by 22 cycles of 95˚C for 15 s, 55˚C for 30 s, and 72˚C for 2 min, with a final extension step at 72˚C for 7 min (Mastercycler Pro thermocycler, Eppendorf®, Hamburg, Germany). Following amplification, 1 µL of each PCR product was added to 9.7 µL HiDi formamide and 0.3 µL LIZ internal size standard in a 96-well optical plate. Fragment analysis was

performed on a 3500 genetic analyzer (Applied Biosystems™) and alleles were individually analyzed using GeneMarker® software (SoftGenetics®, State College, PA, USA).

Table 13. Primer sequences, motif, and size range for the six microsatellite loci used in the evaluation of the genetic diversity of P. regina in Indiana.

| Locus | Primer Sequence (5'- 3') | Tm (°C) | Repeat Motif | Observed Fragment Sizes (bp) | Label |
|---|---|---|---|---|---|
| L3 | F: TGTATGACTTGTTGTATTCCTTTGC | 59 | ACAT | 280-490 | VIC |
| | R: ACAGTACCGCTATTTAGGCAC | 59 | | | |
| L8 | F: ACAATCAGCGCCCATTTCC | 59 | ACAT | 328-529 | 6-FAM |
| | R: GGATCCACTTTGACGGATGG | 59 | | | |
| L9 | F: ACCACTGTGCAACGTCAAAC | 60 | ACAT | 201-249 | 6-FAM |
| | R: ACGCTGAATTATAGCCGTTCATC | 60 | | | |
| L12 | F: TGGACTGGGTACTGGTTAGC | 59 | ACAT | 404-620 | 6-FAM |
| | R: AGGCCTACCTCCCAATGAC | 59 | | | |
| L13 | F: TGAAGTATTCCAGTGTTTCAGCG | 60 | ACAT | 257-469 | PET |
| | R: ACGAACGCAACGTCTAAGTG | 60 | | | |
| L14 | F: GTGAATATTTGCAGTTTGGGAGAC | 59 | AGAT | 232-508 | NED |
| | R: TGTTTAGAGGCTAATCCTTGTCG | 59 | | | |

### 5.2.5 Vertebrate Amplification and Sequencing

Methods followed those outlined in Chapter 3.

### 5.2.6 Fecal Metabolite Analysis

Methods followed those outlined in Chapter 2 [50].

### 5.2.7 Statistical Analyses

An analysis of molecular variance (AMOVA) was used to analyze the genetic and geographic differentiation between spatiotemporal populations partitioned by either spatial or temporal units using GenAlEx 6.5 [51]. Several F statistics were obtained from the AMOVAs,

including $F_{IS}$, which measures the local within-population level deficit of heterozygotes; $F_{ST}$, which measures the Wahlund effect (reduction in heterozygosity due to substructure); and $F_{IT}$, which measures global deficit of heterozygotes. Additionally, the $F'_{ST}$ statistic, which standardizes the $F_{ST}$ value by its maximum value given the within-population observed heterozygosity, was specifically used to assess population substructure, and the number of migrants per population and generation ($N_M$) was used to estimate gene flow. Latent genetic clustering was performed in STRUCTURE 2.3.4 [52] using k = 2 – 10 with three repetitions and a burn-in of 10,000 and 100,000 iterations. Putative number of populations was determined using the Evanno method in STRUCTURE HARVESTER [53]. Relatedness coefficients (*r*) were determined using Spatial Pattern Analysis of Genetic Diversity (SPAGeDi v1.5) [54]. The program FSTAT was used to generate pairwise $F_{ST}$ values between all spatial, temporal, and spatiotemporal populations to determine the source geographic population [55].

As no geographic structure was observed (see Results), all statistical analyses were performed with data grouped according to collection date. Specifically, geographic sites were pooled into temporal populations, weather data from regional archived records was used, and vertebrate diversity data, blow fly diversity data and population genetics were calculated from pooled spatial data. Additionally, data from both 2016 and 2017 were combined and analyzed as a single dataset for the statistical analyses. The genetic variables used as responses in statistical analyses were mean kinship and mean $F_{ST}$ within temporal populations. Abiotic predictors included temperature, humidity, wind speed, precipitation, blow fly diversity (richness and evenness), and vertebrate diversity (richness and evenness). Each weather data variable was taken from five different datasets: 1) on-site during each fly collection (excluding precipitation data), representing the local microclimate; 2) data collected from the local airport weather station

archived for each collection date, representing the regional weather; 3) archived regional data for the day before each collection was made; 4) archived regional data for the week prior to each collection; and 5) archived regional data from the entire week of collections. Archived regional month-to-date precipitation data was also obtained for each collection date.

A combination of the Integrated Nested Laplace Approximation technique (INLA) and boosted regression trees (BRT) was used to determine the most significant factors contributing to blow fly population structure. INLA modeling is a form of conditional autoregressive (CAR) Bayesian hierarchical modelling that takes into account both correlated and uncorrelated spatial heterogeneity, as well as time [56]. Numerous other variables (such as the abiotic and biotic factors investigated in this research) can also be used to indicate space-time dependence on important predictors. For INLA, the Besag, York, and Mollie spatial autocorrelation model was used [57]. As p-values were not generated by the R-INLA program, statistical significance was determined with the mean and standard deviation of each coefficient generated by the regression analysis. An analysis was determined to be significant if the mean was at least twice as large as the standard deviation. BRT analysis is a machine learning-based statistical method used to generate the best fit of models and to rank variables that contribute the most to trends seen in the data [58]. Thousands of decision trees (i.e. different models) made from recursive binary splits were fitted to the data, and combining numerous simple models enhances predictive power. For BRT analysis with the data in this research, learning rates (lr) varied from 0.05 to 0.0001 in order to obtain an optimal number of trees (>5000) for each run. Bag fraction was kept at 0.5, and tree complexity was kept at 5. The Gaussian family of distributions was used for all runs. Importance values of each variable were calculated out of 100, therefore the cutoff for the most important variables was calculated by dividing 100 by the total number of variables and then rounding up to the nearest

whole integer. Partial dependency plots, which average out the effects of all but one variable to plot the average fitted value, were generated for all important predictors.

## 5.3 Results

### 5.3.1 Population Structure

A total of 2,335 and 1,559 *P. regina* were collected in 2016 and 2017, respectively, with a proportion (24.5% and 27.4%) analyzed for this study (24.5%). Extensive migration among sites and moderate to high gene flow resulted in negligible geographic structure. However, temporal genetic structure was detected in both 2016 and 2017 samples. Significant departures from HWE were observed for spatial and temporal populations over both years, as observed genetic diversity was significantly lower than expected, likely influenced by the high inbreeding measured among individuals (Table 14).

Table 14. Summary of population genetic measures of P. regina collected in Indiana 2016 and 2017. Data was partitioned into spatial, temporal, and spatiotemporal components. N, $N_A$, $N_E$, $H_O$, and $H_E$ are all presented as mean per population standard error). N = number of individuals, $N_A$ = number of alleles, $N_E$ = number effective alleles, $H_O$ = observed heterozygosity, $H_E$ = expected heterozygosity, $F_{ST}$ = genetic structure of the subpopulation relative to the total population, $F_{IS}$ = genetic structure of the individual relative to the subpopulation, $F_{IT}$ = genetic structure of the individual relative to the total population, $F'_{ST}$ = genetic structure of the subpopulation relative to the total population standardized by the $F_{ST}$ Max, $F_{ST}$ Max = maximum value $F_{ST}$ can attain, $N_M$ = number of migrants (migration rate).

| Year | Data Partition | N | $N_A$ | $N_E$ | $H_O$ | $H_E$ | $F_{ST}$ | $F_{IS}$ | $F_{IT}$ | $F'_{ST}$ | $N_M$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **2016** | Space | 84.08 (3.12) | 59.61 (3.67) | 38.73 (3.08) | 0.52 (0.03) | 0.95 (0.01) | 0.00 | 0.48 | 0.48 | 0.04 | 103.61 |
| | Time | 36.04 (1.45) | 31.00 (1.62) | 21.79 (1.35) | 0.52 (0.02) | 0.91 (0.01) | 0.03 | 0.47 | 0.48 | 0.47 | 7.28 |
| **2017** | Space | 55.47 (3.70) | 45.58 (3.53) | 30.31 (2.80) | 0.48 (0.04) | 0.93 (0.02) | 0.00 | 0.52 | 0.53 | 0.03 | 122.35 |
| | Time | 23.77 (1.97) | 21.43 (1.82) | 15.56 (1.36) | 0.48 (0.03) | 0.85 (0.02) | 0.03 | 0.51 | 0.53 | 0.34 | 8.38 |

When testing the hypothesis that there might be a source population, one location (Skiles Test Park) exhibited limited gene flow relative to the other sampling locations in 2016 (Table 15),

however, no evidence of substructuring was observed when examining all other pairwise AMOVAs between 2016 sites. Analysis of 2017 flies also revealed negligible structure and remarkably high gene flow among all sites. Given the data from two years of sampling, there likely was not a single source population responsible for supplying migrants in central Indiana in the years sampled for this study.

Table 15. Summary of pairwise AMOVAs between all sites in 2016 and 2017. ** indicates that the number of migrants between populations and generations could not be calculated due to the $F'_{ST} = 0$.

| Year | Pop 1 | Pop 2 | $F_{ST}$ | $F'_{ST}$ | $N_M$ |
|---|---|---|---|---|---|
| 2016 | MP | BR | 0.001 | 0.023 | 257 |
| | MP | SP | 0.004 | 0.043 | 71 |
| | MP | NW | 0.000 | 0.011 | 635 |
| | MP | UP | 0.010 | 0.035 | 191 |
| | MP | PP | 0.040 | 0.096 | 55 |
| | BR | SP | 0.005 | 0.101 | 53 |
| | BR | NW | 0.001 | 0.031 | 198 |
| | BR | UP | 0.001 | 0.024 | 258 |
| | BR | PP | 0.000 | 0.008 | 619 |
| | SP | NW | 0.004 | 0.102 | 58 |
| | SP | UP | 0.002 | 0.052 | 113 |
| | SP | PP | 0.006 | 0.120 | 40 |
| | NW | UP | 0.000 | 0.012 | 559 |
| | NW | PP | 0.004 | 0.086 | 64 |
| | UP | PP | 0.001 | 0.022 | 248 |
| 2017 | MP | BR | 0.002 | 0.026 | 145 |
| | MP | SP | 0.003 | 0.059 | 76 |
| | MP | NW | 0.004 | 0.069 | 62 |
| | MP | UP | 0.001 | 0.023 | 187 |
| | MP | PP | 0.003 | 0.052 | 72 |
| | BR | SP | 0.001 | 0.009 | 411 |
| | BR | NW | 0.000 | 0.000 | ** |
| | BR | UP | 0.000 | 0.000 | ** |
| | BR | PP | 0.000 | 0.000 | ** |
| | SP | NW | 0.005 | 0.081 | 53 |
| | SP | UP | 0.000 | 0.000 | ** |
| | SP | PP | 0.008 | 0.113 | 33 |
| | NW | UP | 0.000 | 0.000 | ** |
| | NW | PP | 0.001 | 0.010 | 362 |
| | UP | PP | 0.002 | 0.032 | 111 |

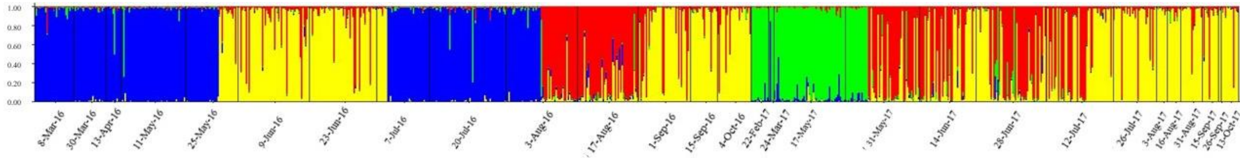


Figure 16. STRUCTURE bar plot summarizing the latent population substructure over both years 2016 and 2017 (K = 4). Collection dates are given on the x-axis and proportion of shared alleles are given on the y-axis. Each bar represents an individual fly.

Temporal population genetic structure aligned approximately with season (Figures 16, 17). When both years are combined, a single population is revealed as being genetically dissimilar from all other populations (Figure 16, in green). There appear to be a few individuals in the last collection date (October 4) of 2016 that share the same genotype as the flies in the early Spring 2017 population. This likely indicates that flies in this unique population diapaused over the winter and constituted the early spring 2017 population. When each year was observed separately, STRUCTURE results revealed four latent temporal populations in 2016 (spring, early summer, late summer, and fall; Figure 17A) and three temporal populations in 2017 (spring, early summer, and late summer/fall; Figure 17B). For 2016, there was evidence of highly structured populations and negligible gene flow from spring to early summer, early summer to late summer, and late summer to fall, with moderate structure between spring and late summer populations (Figure 17A, Table 16). Most flies in 2016 were unrelated, though a few timepoints of relatively high relatedness were observed in each of the early summer, late summer, and fall populations (25 May, 7 July, and 17 August; Figure 18A). The early summer and fall populations experienced increased gene flow resulting in an almost complete absence of population structure.

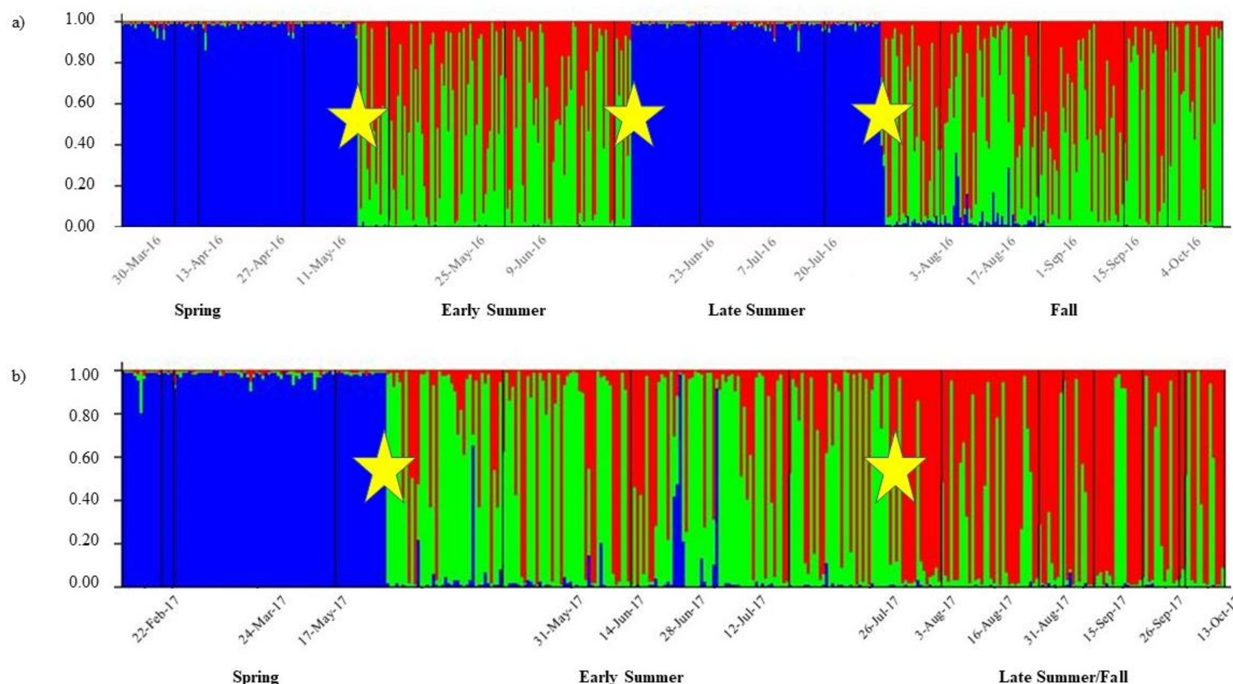


Figure 17. STRUCTURE bar plots by year, 2016 (A) and 2017 (B) (K =3 for both years). Collection dates are given on the x-axis (with corresponding seasons given below) and proportion of shared alleles are given on the y-axis. Each bar represents an individual fly. Yellow stars represent likely population bottlenecks between seasons.

For 2017, highly structured populations existed between spring and early summer, and spring and late summer/fall, with a large increase in genetic diversity from early summer to late summer/fall (Figure 17B, Table 16). Regardless of the several bottlenecks that occurred over both years, considerable gene flow was still observed among temporally non-adjacent populations across years. Specifically, comparing the 2016 early summer and fall populations to the 2017 fall population revealed high genetic similarity. The 2016 early fall and 2017 summer populations were also somewhat genetically similar and exhibited low population structuring. Thus, there seems to be several reproductive events over time that were responsible for the lagged genetic similarity between some populations.

Table 16. Summary of pairwise AMOVAs between seasons within each year, as well as between populations in both years.

| Year | Pop 1 | Pop 2 | F'$_{ST}$ | N$_M$ |
|---|---|---|---|---|
| 2016 | Spring | Early Summer | 0.769 | 5 |
| | Spring | Late Summer | 0.255 | 19 |
| | Spring | Fall | 0.739 | 5 |
| | Early Summer | Late Summer | 0.648 | 5 |
| | Early Summer | Fall | 0.010 | 315 |
| | Late Summer | Fall | 0.605 | 6 |
| 2017 | Spring | Summer | 0.776 | 4 |
| | Spring | Fall | 0.869 | 3 |
| | Summer | Fall | 0.072 | 37 |
| Combined | 2016 Fall | 2017 Spring | 0.883 | 3 |
| | 2016 Early Summer, Late Fall | 2017 Fall | 0.005 | 575 |
| | 2016 Early Fall | 2017 Summer | 0.067 | 44 |

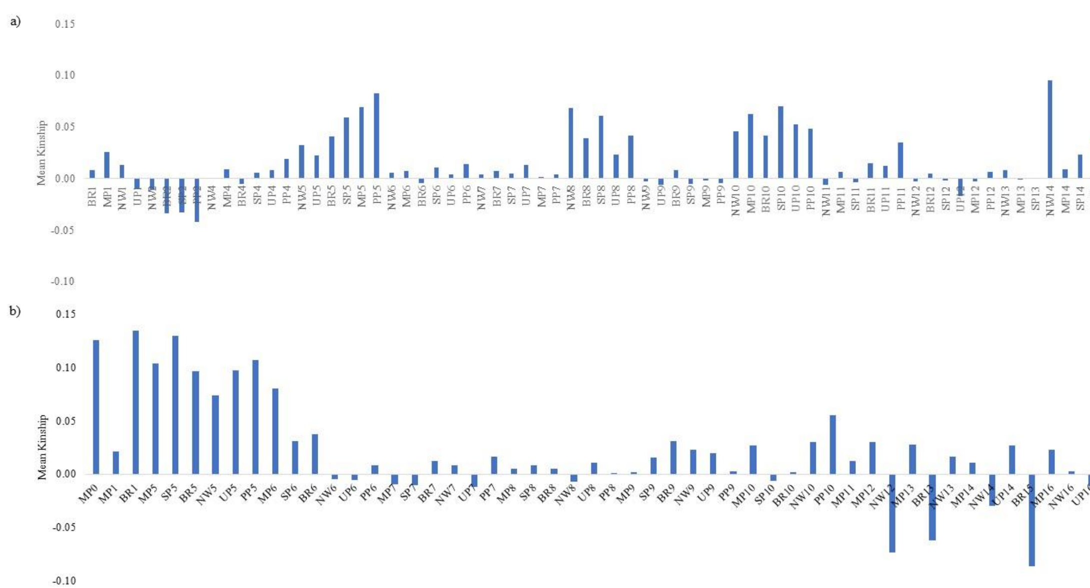


Figure 18. Bar plots summarizing mean kinship within spatiotemporal populations for 2016 (a) and 2017 (b).

In order to further investigate population genetic patterns of *P. regina* in Central Indiana, STRUCTURE plots were also generated for each geographic site that was sampled in both 2016 and 2017 (Figure 19). Overall, the temporal trend seen in the yearly data is obvious for each site, however, there was some variation in the amount of genetic diversity seen by location. For example, Broad Ripple Park and Skiles Test Park (Figure 19C, E) both have very little genetic

variation (K = 3) whereas Province Park (Figure 19F) appears to have a greater amount of admixture over both years (K = 5). Additionally, genetic similarity between at least a few individual flies from the last 2016 population and flies from the early spring 2017 population is evident for Military Park (Figure 19A), Northwest Park (Figure 19B), and Broad Ripple Park (Figure 19C). This indicates that a small founder event (potentially only a handful of individual flies) at or around each geographic location in late 2016 was responsible for the first cohort of flies to appear in early 2017.

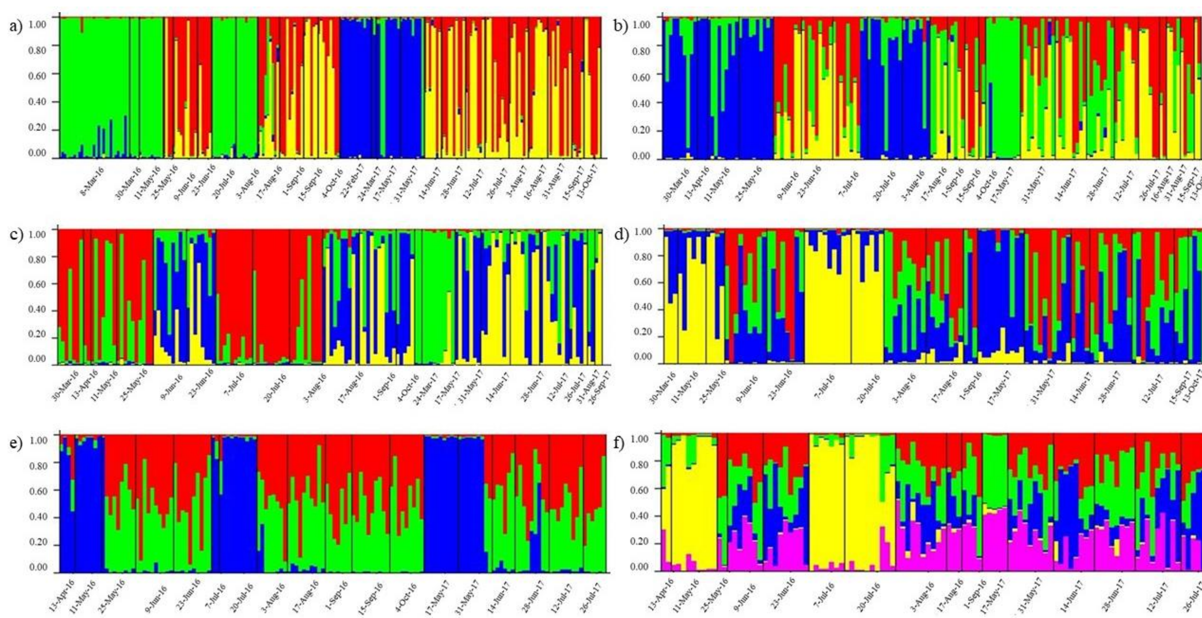


Figure 19. STRUCTURE bar plots summarizing the latent population substructure by geographic site over both 2016 and 2017. Left column: Marion County parks, A) Military Park (K = 4), C) Broad Ripple (K = 3), E) Skiles Test Park (K = 3). Right column: Johnson County parks, B) Northwest Park (K = 4), D) University Park (K = 4), F) Province Park (K = 5). Collection dates are given on the x-axis and proportion of shared alleles are given on the y-axis. Each bar represents an individual fly.

### 5.3.2 Abiotic Factors

Temperature, humidity, and wind speed showed little change over time, however precipitation varied greatly throughout both years. The lowest temperatures recorded during the sampling period for 2016 occurred in the spring (8 March to 13 April) where temperatures averaged approximately 15˚C (Figure 20A). Temperatures began to rise in late April and peaked during the month of August, with decreases occurring beginning in September. Though there was an obvious seasonal change from spring to summer, the majority of sampling in the summer experienced very little deviation in temperatures during this time period. No obvious changes in humidity were noted in 2016 (Figure 20B). On-site relative humidity tended to be lower than regional data in most cases. Wind speed was highly variable between timepoints, as well as between local and regional data (Figure 20C). Local data indicated lower wind speeds than what was recorded at the regional level. This could be due to the proximity of the anemometer to the ground (~1 m for local data, ~10 m for regional data) and because the regional data averages the wind speeds over a 24 h period. However, higher wind speeds at the regional level was common during most of the summer months, likely contributing to the dispersal of flies to various locations around central Indiana. There was a moderate amount of rain during the spring (up to ~10 cm), with a short dry period during the early summer. This dry period corresponded to the new cohort of genetically similar flies during that time period (Figure 20D). The late summer continued to experience more rainfall (up to ~14 cm in July), with the most rain occurring during the timeframe corresponding to the fall population of *P. regina* in central Indiana.

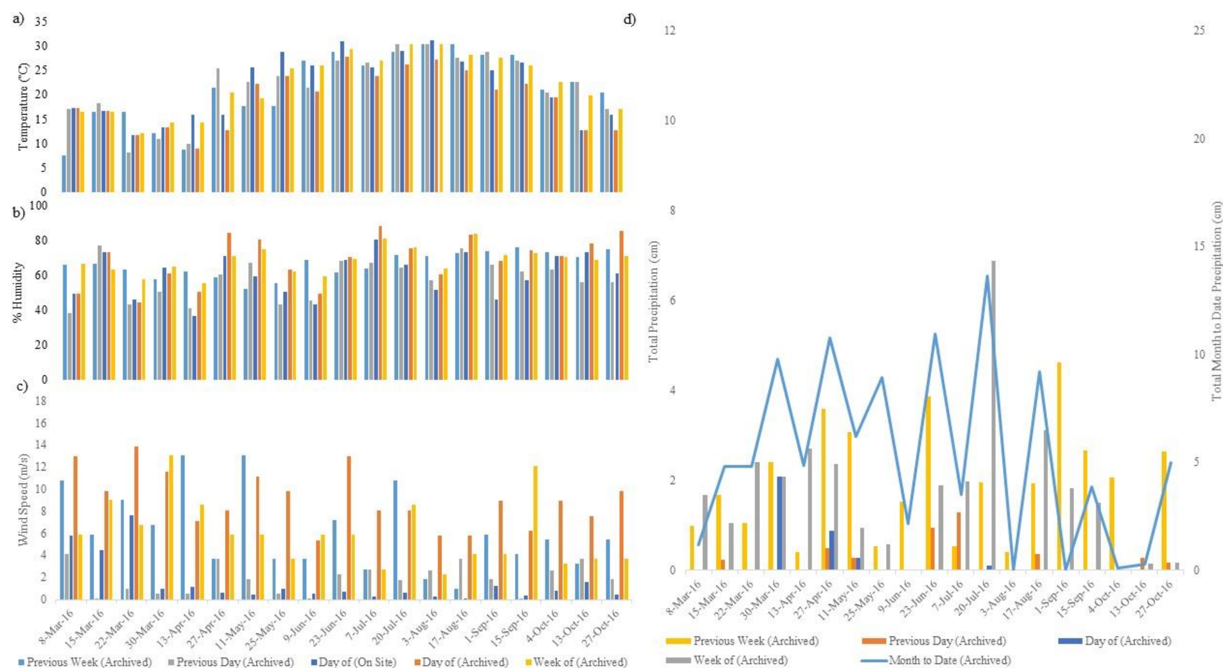


Figure 20. Summary of weather data for 2016. Temperature (A), humidity (B), and wind speed (C) have local data collected on site on the day of collections, regional data from the day of collections (archived from the local weather station), regional data from the day and week before collections, and average regional data for the week of collections. Precipitation data (d) could not be collected on-site, but archived regional data from the day of collections, day before, week before, and weekly averages were obtained. Additionally, total regional month to date precipitation data was also obtained for each collection date.

The lowest temperatures in 2017 occurred at the tail ends of the collecting season (February to March, September to October), with higher, more consistent temperatures during the summer (Figure 21A). On-site temperature data was on average approximately 6˚C higher than regional archived data, but overall displayed the same trends over time. This trend was not observed in 2016. There was little to no variation in humidity over the duration of the sampling period (Figure 21B). Similar to 2016 data, on-site humidity was always lower than regional data collected on the same day. Wind speed was relatively similar across time periods, with the exception of four time periods (24 March, 17 May, 14 June, and 11 July) in which wind speeds reached >12 m/s (Figure 21C). Precipitation did not occur on the day of collections, but often on the previous day, week, or some other time during the same week of collections (Figure 21D). Significant rainfall occurred

during the month of May (21 cm), corresponding to the delineation between the spring and summer *P. regina* populations. Heavy rainfall (9.6 cm) the day before collections on 12 July (as well as the weekly total) marks the delineation between the early summer and late summer/fall populations of *P. regina*. By the end of July, there was a marked decrease in the amount of precipitation recorded in central Indiana.

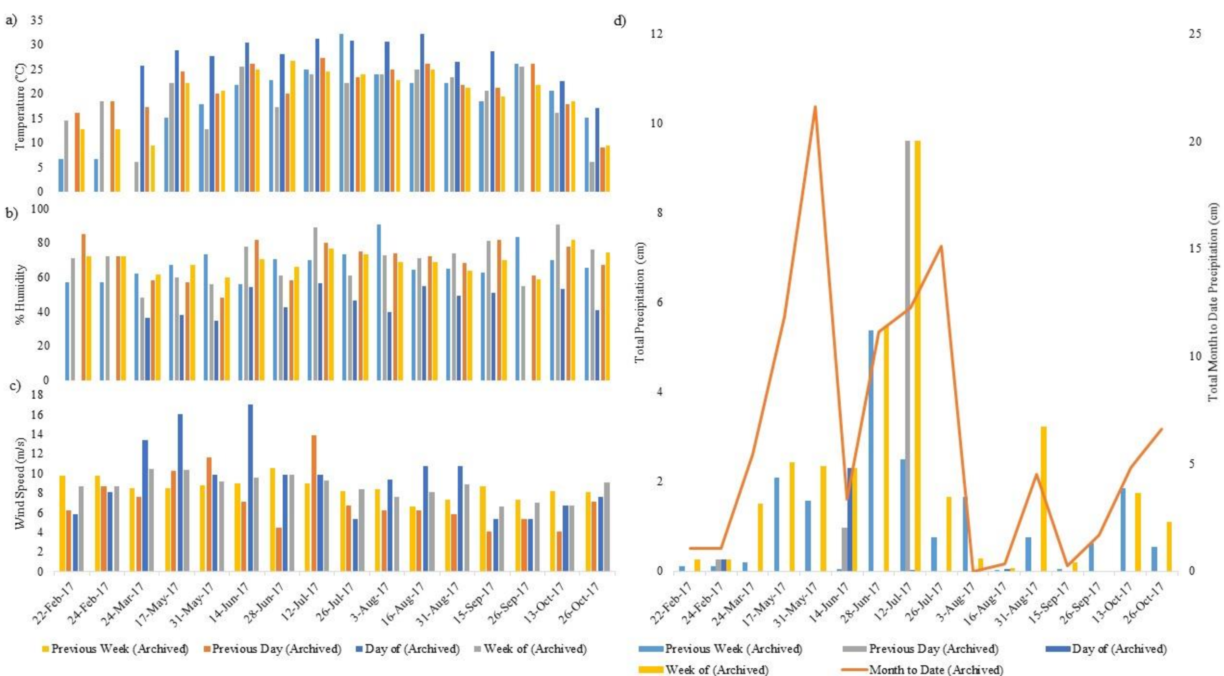


Figure 21. Summary of weather data for 2017. Temperature (A), humidity (B), and wind speed (C) have local data collected on site on the day of collections, regional data from the day of collections (archived from the local weather station), regional data from the day and week before collections, and average regional data for the week of collections. Precipitation data (d) could not be collected on-site, but archived regional data from the day of collections, day before, week before, and weekly averages were obtained. Additionally, total regional month to date precipitation data was also obtained for each collection date.

### 5.3.3 Interspecific Competition

*Phormia regina* abundance was directly impacted via competition, with the lowest abundances noted on days with increased blow fly richness and evenness. In 2016, blow fly

richness was the greatest during 25 May and 7 July collections (S = 10), whereas the greatest evenness was observed during 13 April (J = 0.882), which was also the date with the fewest number of *P. regina* collected (N = 15, Figure 22A). The spike in *P. regina* abundance on the 9 and 23 June sampling dates (N = 393, 357) corresponds with the high genetic diversity seen in the early summer population, with the sudden decrease beginning on 7 July corresponding with the genetically homogenous late summer population. Though 2017 aligned with the same overall trends as 2016, there was only a portion of the year (corresponding roughly with the early summer population) in which *P. regina* was highly abundant (mean N = 243 individuals per sampling time, Figure 22B). At all other times, *P. regina* populations remained low (mean N = 23 collected individuals per sampling time). Blow fly richness was highest on 12 July (S = 10) and evenness was highest on 26 September (J = 0.917) when collected *P. regina* were fewest (N = 2).

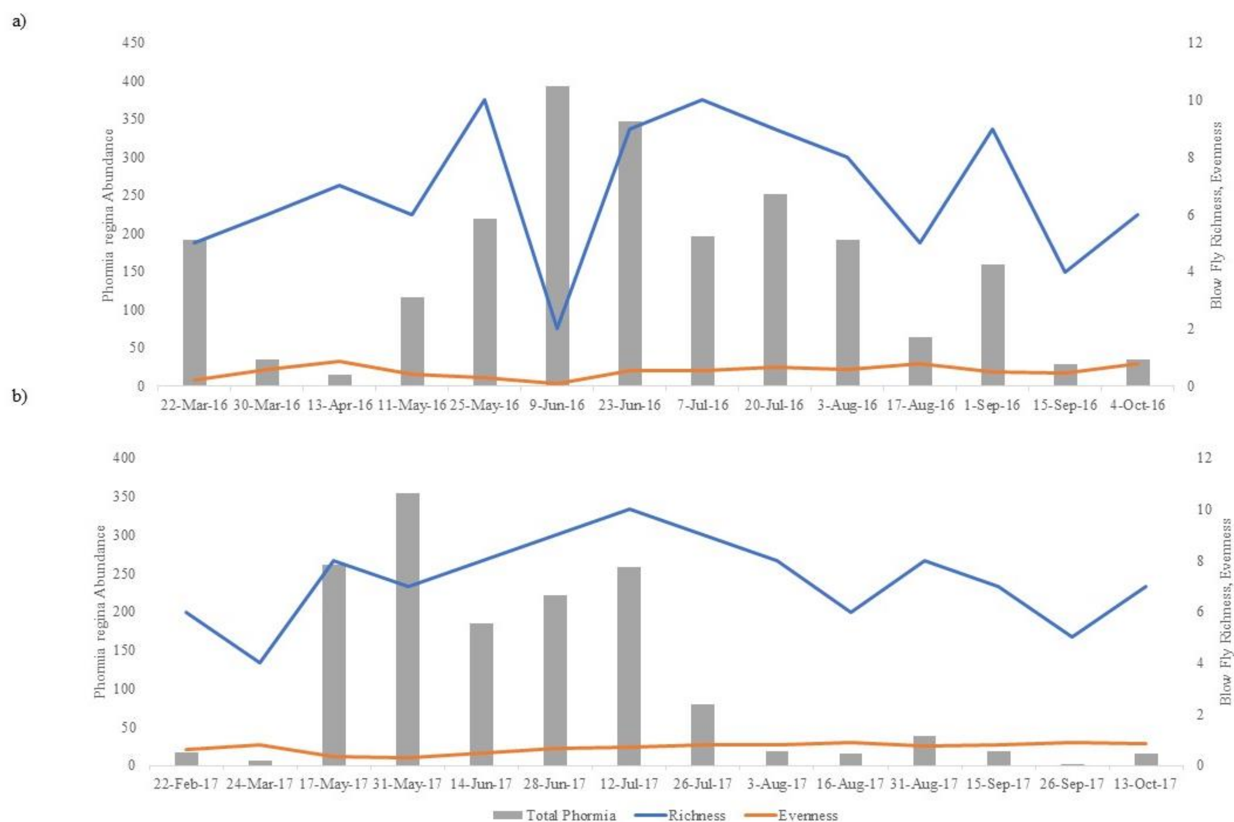


Figure 22. Summary of blow fly diversity measures for 2016 (A) and 2017 (B). Total P. regina abundance for each collection period is given on the primary y-axis, while blow fly richness and evenness is given on the secondary y-axis.

### 5.3.4 Resource Availability

Determining the type of vertebrate resources available to blow flies in their environments was important to establish how these resources mediate blow fly population genetics. When interpreting 2016 data, it appears that the majority of the blow fly diet in spring and early summer was feces (mostly from dogs; Figure 23A). During the spring and late summer, flies containing a greater proportion of detectable vertebrate animal DNA were more abundant than other times of the sampling periods. In 2017, the highest proportion of flies positive for feces (with and without species identification) occurred in the early summer, with the lowest being in the fall (Figure 24A).

In fact, the prevalence of feces-positive flies was almost or even greater than the proportion of flies with only DNA detected, showing that this resource was very important in 2017.

The vertebrate DNA detected from flies revealed a trend in the sizes of animals detected over time for both years. For example, in 2016, medium-bodied animals were dominant in the earliest sampling times, while small-bodied animals were detected more in the summer, and the fall was comprised of a more even distribution of body size categories (Figure 23B). A more consistent distribution of carcass sizes was observed in 2017 as medium-sized animals were the most prominent body size detected throughout the year, followed by small and large animals (Figure 24B).

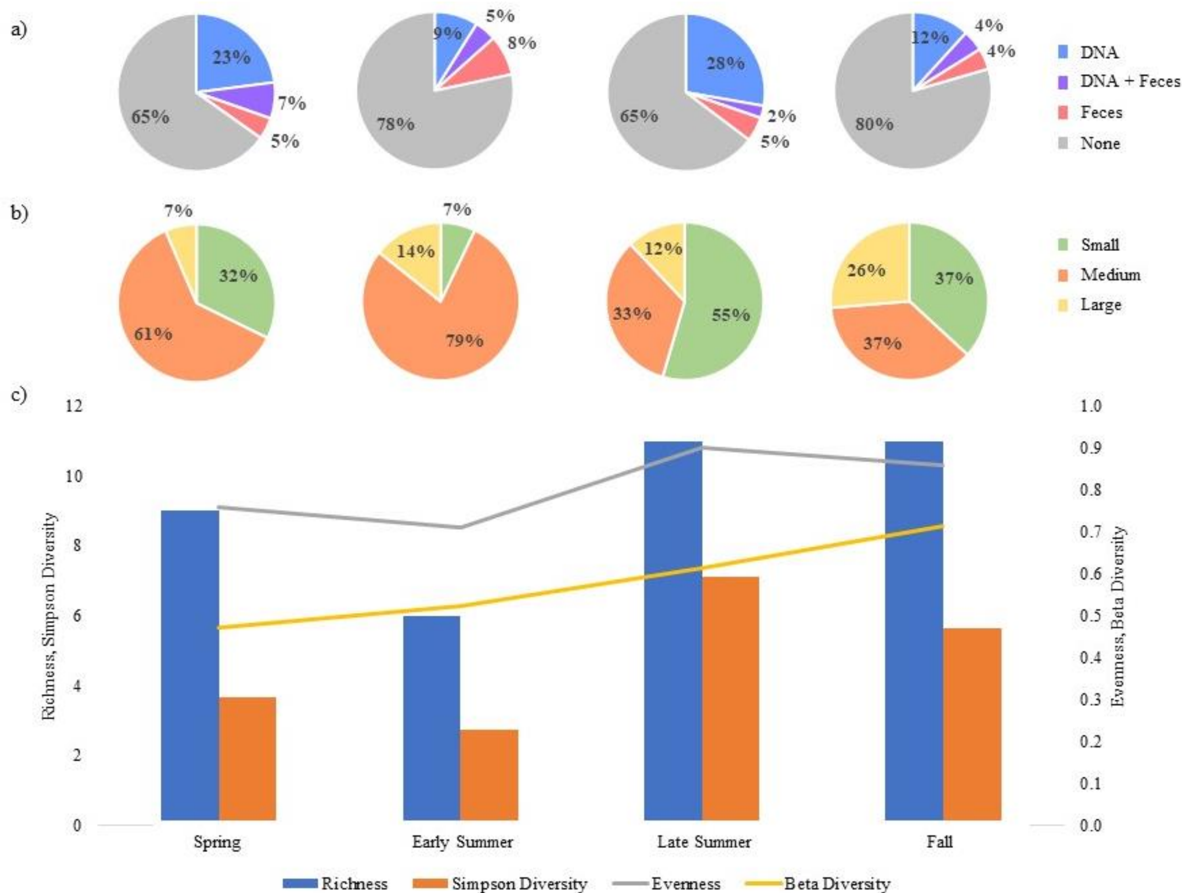


Figure 23. Summary of vertebrate resources detected by flies in each seasonal population of 2016. A) pie graphs indicating the proportion of analyzed flies positive for vertebrate DNA only, vertebrate DNA and fecal metabolites, fecal metabolites only, and flies with no resources detected; B) pie graphs indicating the relative body sizes of vertebrate resources (excluding feces) detected by flies; C) bar and line plot summarizing vertebrate diversity statistics (richness, Simpson diversity, evenness, beta diversity).

Table 17. Summary of vertebrate species detected by flies in 2016 and 2017. SP = spring, ES = Early Summer, LS = Late Summer, FA = Fall, LS/FA = Late Summer/Fall.

| Species | Common Name | 2016 | | | | 2017 | | |
|---|---|---|---|---|---|---|---|---|
| | | *SP* | *ES* | *LS* | *FA* | *SP* | *ES* | *LS/FA* |
| *Canis lupus familiaris* | Dog | 19 | 11 | 8 | 9 | 4 | 30 | 6 |
| *Sylvilagus floridanus* | Eastern Cottontail | 4 | 1 | 3 | 2 | 1 | 2 | 4 |
| *Felis catus* | Domestic Cat | 2 | 1 | 2 | 1 | 1 | 4 | 0 |
| *Sus scrofa* | Pig | 0 | 1 | 1 | 1 | 0 | 2 | 0 |
| *Bos Taurus* | Cow | 0 | 0 | 3 | 4 | 0 | 4 | 1 |
| *Marmota monax* | Groundhog | 0 | 0 | 2 | 0 | 0 | 0 | 1 |
| *Peromyscus leucopus* | White-Footed Mouse | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| *Ondatra zibethicus* | Muskrat | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| *Procyon lotor* | Raccoon | 8 | 6 | 6 | 3 | 0 | 1 | 0 |
| *Sciurus niger* | Fox Squirrel | 2 | 0 | 7 | 2 | 3 | 2 | 2 |
| *Didelphis virginiana* | Virginia Opossum | 2 | 0 | 2 | 0 | 0 | 2 | 2 |
| *Odocoileus virginianus* | White-Tailed Deer | 2 | 1 | 0 | 1 | 0 | 0 | 1 |
| *Microtus ochragaster* | Prairie Vole | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| *Panthera tigris* | Tiger | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| *Carassius auratus* | Goldfish | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Castor canadensis* | American Beaver | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Tamiasciurus hudsonicus* | American Red Squirrel | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| *Panthera leo* | Lion | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| *Blarina brevicauda* | Northern Short-Tailed Shrew | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| *Gallus gallus* | Chicken | 0 | 0 | 0 | 0 | 0 | 2 | 0 |
| *Micropterus salmoides* | Large-Mouth Bass | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| *Scalopus aquaticus* | Eastern Mole | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| *Tamias striatus* | Eastern Chipmunk | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| *Vulpes vulpes* | Red Fox | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| **Vertebrate Diversity Measures** | *Richness, S* | 9 | 6 | 11 | 11 | 5 | 14 | 11 |
| | *Simpson Diversity, 1/D* | 3.66 | 2.74 | 7.12 | 5.63 | 3.57 | 3.91 | 3.86 |
| | *Evenness, J* | 0.76 | 0.71 | 0.90 | 0.86 | 0.88 | 0.70 | 0.75 |
| | *Beta Diversity, β* | 0.47 | 0.52 | 0.61 | 0.71 | 0.43 | 0.58 | 0.45 |

A total of 17 vertebrate species were detected by flies during 2016, five of which were unique to that year (Figure 23C, Table 17), whereas 19 vertebrate species were detected in 2017, seven of which were unique to that year (Figure 24C, Table 17). In 2016, vertebrate richness, diversity, and evenness peaked in the late summer. However, the highest beta diversity occurred in the fall, indicating that although vertebrate species were evenly distributed in each sampling location, there were some major differences in species composition among sites. In 2017, the

highest vertebrate richness, Simpson diversity, and Beta diversity occurred in the early summer and the highest evenness was observed in the spring when vertebrate diversity measures were the lowest. Seven unique species were detected in the early summer compared to 5 unique species detected in the late summer/fall population, which explains why Beta diversity was high for both of these time periods. The most prominent resources detected from flies in the 2016 spring, early summer, late summer, and fall populations were dog, feces (no species ID), and fox squirrel, respectively (Table 17). The most prominent vertebrate resource of 2017 was feces (no species ID), though this was tied with raccoon DNA in the late summer/fall population.

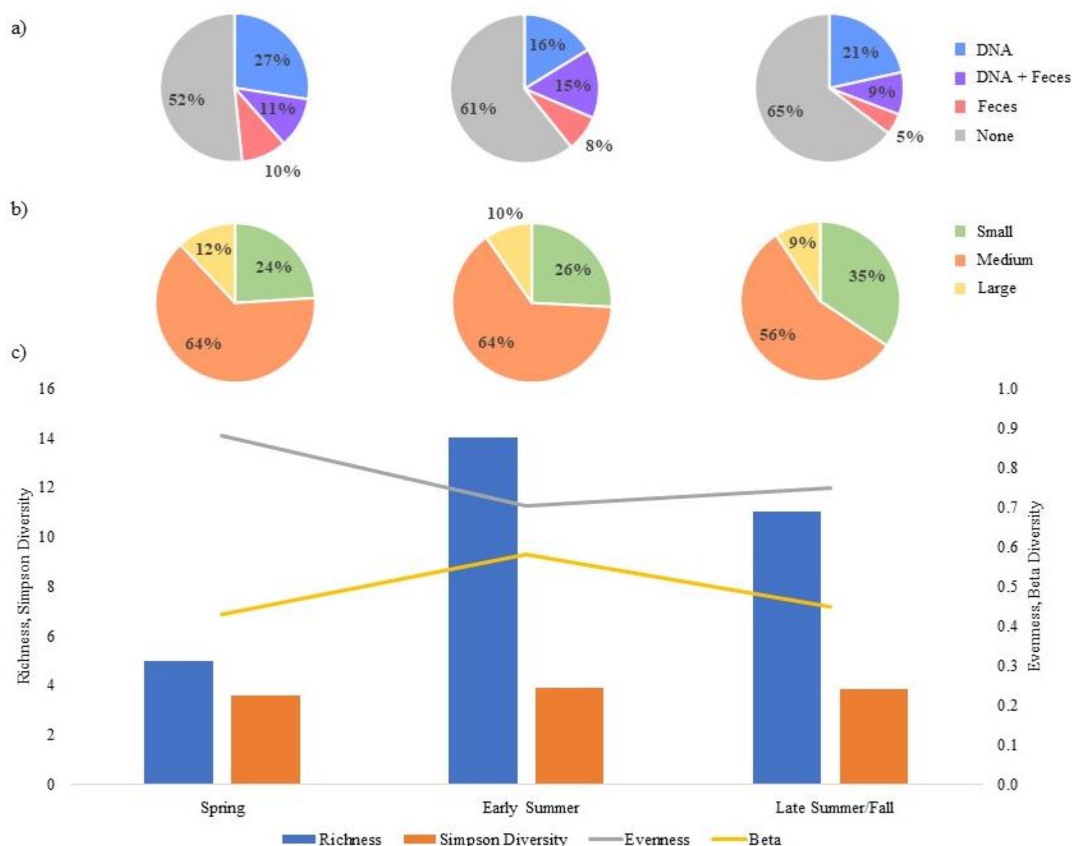


Figure 24. Summary of vertebrate resources detected by flies in each seasonal population of 2017. A) pie graphs indicating the proportion of analyzed flies positive for vertebrate DNA only, vertebrate DNA and fecal metabolites, fecal metabolites only, and flies with no resources detected; B) pie graphs indicating the relative body sizes of vertebrate resources (excluding feces) detected by flies; C) bar and line plot summarizing vertebrate diversity statistics (richness, Simpson diversity, evenness, beta diversity).

### 5.3.5 Significant Mediators of Population Structure

Two different statistical models (INLA and BRT) were used to understand how biotic and abiotic factors influence fly population structure. When interested in whether a particular sample contained a large number of related individuals (=small effective population size), the INLA model determined eight abiotic factors were important (Table 18). Regional temperatures, humidity, wind speed, and fly richness and evenness were all positive predictors (i.e. increase in kinship as predictor increases), whereas, precipitation and blow fly richness negatively influence fly

population structure (i.e. these factors contribute to an increase in genetic diversity, Table 19). The evenness of blow fly species collected on sampling days was the most important predictor of *P. regina* kinship using the INLA analysis.

The BRT model revealed temperature and humidity, as well as wind speed and vertebrate richness, were important predictors of samples with reduced genetic diversity (increased kinship). In fact, when the previous weeks' temperatures were < 15˚C, samples of flies were much more proportionally related than when temperatures increased (Figure 25A). A minimal negative relationship was observed between fly kinship and wind speed from the previous day, contradicting results from the INLA model. Interestingly, when vertebrate richness increased (> 9 species), fly kinship values also increased. Gradual increases in humidity appear to lead to higher kinship values observed in collected flies.

Table 18. Summary of INLA and BRT statistics on combined years temporal data. A "✓" indicates that the test was significant (P < 0.05) for INLA or highly important (>100/number of variables) for BRT.

| ABIOTIC PREDICTORS | | GENETIC RESPONSE | | | |
|---|---|---|---|---|---|
| | | *Kinship* | | $F_{ST}$ | |
| | | INLA | BRT | INLA | BRT |
| **Temperature** | Collection Day (local) | | | | ✓ |
| | Collection Day (regional) | ✓ | | | ✓ |
| | Previous Day | | | | |
| | Previous Week | | ✓ | | ✓ |
| | Collection Week | | | | |
| **Humidity** | Collection Day (local) | | | | |
| | Collection Day (regional) | ✓ | | | |
| | Previous Day | | | | |
| | Previous Week | | ✓ | | |
| | Collection Week | | | | |
| **Wind Speed** | Collection Day (local) | | | | ✓ |
| | Collection Day (regional) | | | | |
| | Previous Day | ✓ | ✓ | | ✓ |
| | Previous Week | | | | |
| | Collection Week | ✓ | | | |
| **Precipitation** | Collection Day (regional) | ✓ | | | |
| | Previous Day | | | | |
| | Previous Week | | | | |
| | Collection Week | | | | |
| | Month to Date | ✓ | | | |
| **Blow Fly Diversity** | Richness | ✓ | | | ✓ |
| | Evenness | ✓ | | | - |
| **Vertebrate Diversity** | Richness | | ✓ | | ✓ |
| | Evenness | | | | ✓ |

Table 19. Summary of means and standard deviations of significant INLA predictors of P. regina kinship.

| Significant Predictors of Kinship | Mean | s.d. |
|---|---|---|
| Temperature Collection Day (regional) | 5.127 | 2.221 |
| Humidity Collection Day (regional) | 3.784 | 1.431 |
| Wind Speed Previous Day | 2.896 | 1.281 |
| Wind Speed Collection Week | 2.701 | 0.858 |
| Precipitation Collection Day (regional) | -13.941 | 6.992 |
| Precipitation Month to Date | -1.620 | 0.653 |
| Blow Fly Richness | -1.943 | 0.804 |
| Blow Fly Evenness | 29.941 | 9.217 |

Kinship is a measure of an individual sample's genetic diversity, while $F_{ST}$ is a measure of how related samples are to one another. When modeling the data using INLA, no abiotic variables were found to be significant predictors of $F_{ST}$ (Table 18). However, eight variables (temperatures, wind speeds, blow fly richness, and vertebrate richness and evenness) were predictive of overall population structure. Unsurprisingly, as wind speeds increased, population substructuring decreased (the wind was physically facilitating the dispersal flies via odor dispersal, Figure 25B). Though local temperature showed a gradual negative relationship with $F_{ST}$ (as temperatures increased $F_{ST}$ decreased), regional and previous week temperatures illustrate a more drastic negative relationship in which low $F_{ST}$ values were seen more often at temperatures >15˚C. Vertebrate evenness had a slight positive relationship with $F_{ST}$, wherein evenness >0.80 resulted in higher $F_{ST}$ values. Vertebrate richness above nine species resulted in higher $F_{ST}$ values. However, an increase in interspecific competition negatively affected $F_{ST}$ of *P. regina*, with more than six species present resulting in slightly lower $F_{ST}$ values.

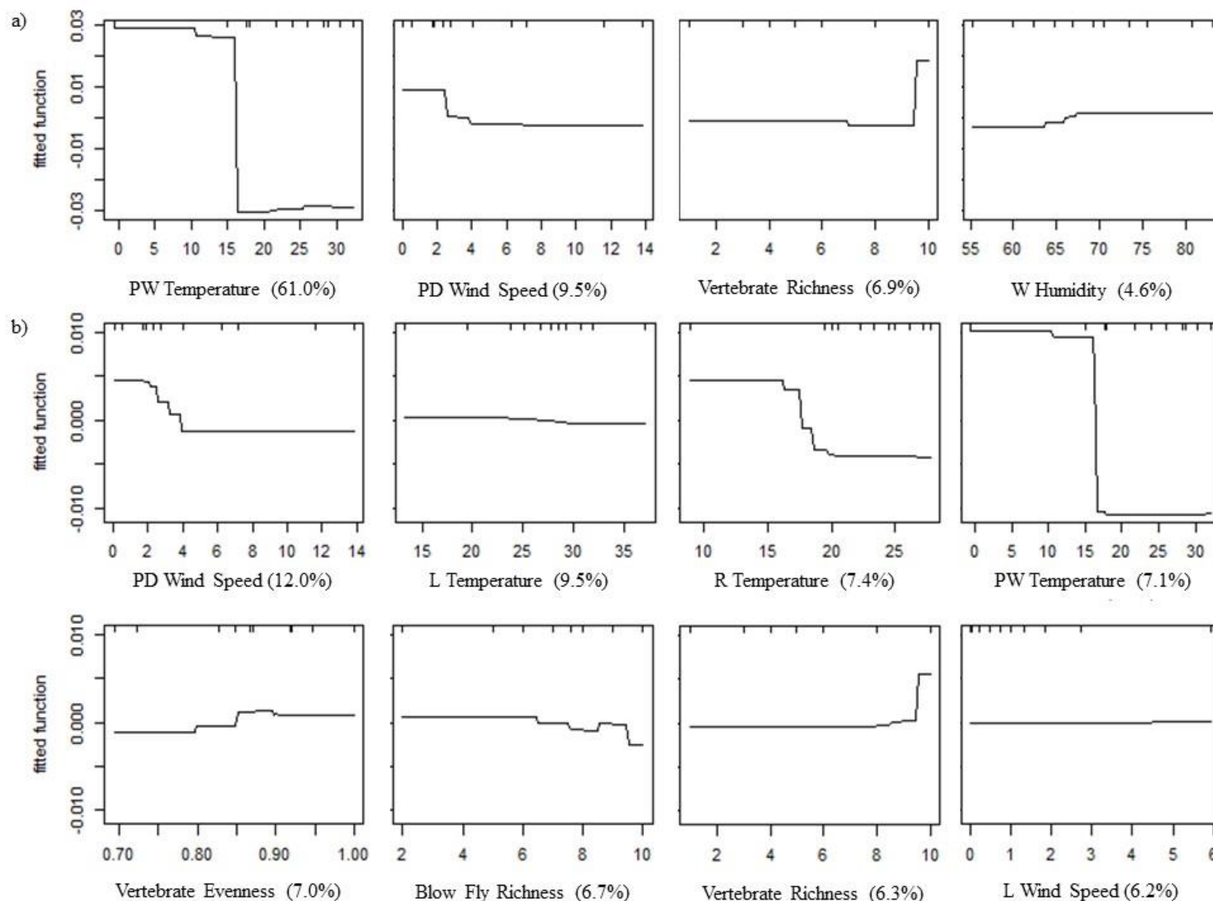


Figure 25. Partial dependency plots of kinship (A) and $F_{ST}$ (B) from boosted regression analysis for the most important predictors. Importance values were calculated by dividing 100 by the number of variables (N = 24) and rounding up to the next highest integer (cutoff = 5). PW = previous week, PD = previous day, W = week of collections, L = local data take on-site, R = regional archived data.

## 5.4 Discussion

It is clear from these results that abiotic and biotic factors do in fact mediate *P. regina* population genetic structure. Local and regional weather patterns, as well as vertebrate resource availability and competition with other blow fly species, impacted genetic diversity of *P. regina* by either facilitating migration between sites or creating conditions in which population bottlenecks were inevitable. Though no evidence was found to support the theory of geographic

substructure in *P. regina,* there was abundant evidence that these populations displayed strong temporal structure that appears to align with seasonal shifts.

The weather itself, both directly and indirectly, had a large impact on *P. regina*. For example, dry periods (like the one observed in late summer/fall 2017) corresponded with bottlenecks in fly populations, which greatly impacted their genetic diversity and created substructure from the recent heterozygote deficit. However, these dry spells may have also provided more vertebrate resources for the flies as heat and drought may result in higher vertebrate mortality [59]. This may explain why the proportion of flies with detectable vertebrate resources only decreases slightly from the early summer to late summer/fall 2017 populations.

The temperature of the week prior to collection appeared to be one of the most important predictors of *P. regina* relatedness, showing a definite temperature threshold above which relatedness was essentially reduced to zero. However, temperatures on and near the day of collections showed a slight increase in the chance of sampling more highly related flies on warmer days. These seemingly contradicting results were likely due to highly related flies being sampled in the cooler early spring of both years when fly population numbers and genetic diversity were low. Above the 15˚C temperature threshold, fly kinship remained low, though there were slight variations throughout the summer and into the fall. These variations tended to be influenced by the immediate local weather conditions (i.e. increases in temperature lead to slight increases in kinship and $F_{ST}$) in the warmer times of the year. This same trend was also seen with wind speed. Similarly, more humid collection days and weeks appeared to slightly increase the likelihood of sampling more highly related flies. Precipitation, however, was related to an overall decrease in fly relatedness, which, when examining the month-to-date data, could be explained by low survivorship of the flies, leading to small bottlenecks during periods of heavy rain. On collection

days where precipitation was recorded, low relatedness among flies could simply be due to limited sampling of the available population. Flies may have taken refuge even in a light mist, which could have greatly reduced the pool of local flies to sample from, artificially reducing genetic diversity of flies in a sample.

Interspecific competition had a marked effect on *P. regina* genetic diversity. Specifically, when blow fly evenness was high, or when samples were very uneven but *P. regina* was not the most dominant species, *P. regina* typically was collected in small abundance and samples were usually more highly related. Blow fly evenness was one of the most predictive variables impacting *P. regina* kinship, and it likely has to do with the arrival of species such as *Lucilia sericata*, *Cochliomyia macellaria*, and *Chrysomya rufifacies* and the ensuing competition for larval and adult resources. Once a species such as *L. sericata* becomes established by colonizing local carcasses, its abundance increases and soon adults begin searching for the same adult resources (feces, nectar, carrion) as *P. regina*. However, *P. regina* appears to have outcompeted these other species as it continued to be the most prominent fly collected into the cooler months of the year. The availability of many different types of vertebrate resources in approximately equal frequency led to higher relatedness among flies in both years of sampling. This could potentially mean that cohorts of highly related flies utilized similar resources because they traveled together, which is a phenomenon observed mainly in vertebrates [60, 61]. The most likely scenario for something like this to happen in blow flies likely occurs soon after adult emergence from the puparium. If many highly related flies emerged around the same time, and local resources were available in the immediate vicinity, many of the flies may have visited these resources before dispersing randomly into the environment. If enough resources were available (e.g. water, carbohydrates, protein, mates, oviposition sites), the flies may not have dispersed out of this environment at all.

In 2016, there appears to be high variability in the abundance of available resources, however this could be dependent on the size of the fly populations throughout the year. For example, almost twice as many flies were collected in early summer vs. spring 2016, though 22% of flies in the early summer contained vertebrate resources, while 35% of flies in the spring population were positive for resources. However, the only way to maintain such a large population of flies in the early summer is to also maintain a large number of resources. Therefore, it is likely that resource availability remained high throughout the summer, but increased fly abundance in the early summer led to intense intraspecific competition for resources and proportionally fewer flies containing evidence of vertebrate resources. The same trend can again be seen in the early summer 2017 flies, where almost twice as many flies were collected compared to the spring, yet proportionally fewer contained resources.

It has also been shown that blow flies will frequent flowers for their nectar, which serves as a carbohydrate resource to maintain the fly on its day-to-day activities [62, 63]. However, recent work shows that blow flies may also utilize pollen as a protein source in the wild [64]. In laboratory experiments, the blow fly *Lucilia sericata* responded positively to floral cues and was able to develop mature oocytes when fed adequate amounts of pollen [64]. Therefore, both angiosperms and gymnosperms may be a vital, yet hitherto under-investigated, resource important to the biology, ecology, and population genetics of blow flies like *P. regina.* Specifically, the phenology of certain plants may play a large role in the seasonal substructuring of *P. regina* that was observed in this study.

It is interesting to note that the spring 2017 *P. regina* population was genetically distinct from all other fly populations collected over the two-year period in this study. Originally, we hypothesized that the earliest spring collections in 2017 would reveal flies that were genetically

similar to the last cohort of flies collected in fall 2016, as *P. regina* is known to diapause during the winter [65]. Our data support this hypothesis, however the founder population seemed to be much smaller than originally expected. It is likely that this population consisted of migrants from an area outside of central Indiana that made their way into this region during the late fall of 2016 and managed to diapause. This unique cohort of flies was highly related, but a combination of the cool, dry weather, as well as the lack of resource diversity, likely led to their population bottleneck before summer 2017.

Though seasonally adjacent populations (e.g. 2016 spring and early summer) showed very little gene flow and high genetic structure, seasonally non-adjacent populations (e.g. 2016 spring and late summer) showed the opposite trend. This was seen in both 2016 and 2017, with some gene flow occurring among seasonal populations spanning both years (e.g. 2016 early summer, 2016 late fall, and 2017 fall). This trend is likely due to generational effects in which adults from an earlier generation in the year (e.g. spring) reproduced, and the offspring, after completing development and metamorphosis, constituted the later temporally non-adjacent population (e.g. late summer). Such reproductive temporal structuring has also been observed for numerous fish species (e.g. perch, roach, salmon), though they tended to also be spatially limited to some extent [66-68]. This could be indicative of sweepstakes reproductive events, whereby a few adults "in the right place at the right time" for mating opportunities, resource availability, and resource colonization are allowed the rare chance to contribute genetic material to the next generation, resulting in low spatial structure and high temporal genetic structure [69]. The difference in genetics between temporally adjacent populations in *P. regina*, however, was likely due to a combination of a bottleneck effect that occurred at the end of one season with simultaneous migration of flies from another area into central Indiana. A continuation of reproductive events

throughout the year thus led to a hopscotch-like effect of each genetically similar populations over time.

As most population models are rooted in spatial ecology, the temporal dimension of this research requires a more complex explanation of what factors impact fly populations in the wild. The combination of high spatial gene flow, lack of substructure, and low kinship suggests that *P. regina* exists in spatially patchy populations. However, the data do not provide evidence to suggest that a source population exists in central Indiana, thus eliminating the source-sink model as a possible explanation. Furthermore, there is no evidence to support an isolated population model due to the spatial genetic heterogeneity. Given the high spatiotemporal genetic variation as well as variability in resources, the Levins metapopulation model cannot be rejected as an explanation for *P. regina* population structuring in Indiana over the time periods sampled. The Levins metapopulation model is quite commonly used to explain insect populations, particularly for flying insects. For example, the Glanville Fritillary butterfly (*Melitaea cinxia*) has maintained a consistent metapopulation for over 30 years even though hundreds of local extinction events have been documented [70]. Highly dispersive biting flies (and disease vectors) such as the yellow fever mosquito (*Aedes aegypti*) and several species of phantom midges (*Chaoborus* spp.) have also been documented to adhere to a metapopulation model, depending largely on urbanization of natural environments by humans [71, 72]. Temporally explicit metapopulation models have also been characterized for marine populations, such as limpets, in which the animal's complex life cycle and increased dispersal patterns contribute to a highly chaotic metapopulation structure [73, 74]. Overall, high dispersal abilities of flies combined with the variability of vertebrate resource availability contributes largely to the increased connectivity between sampled geographic sites. Sudden decreases in resources along with limited reproductive events and the time it takes to

complete the blow fly life cycle all contribute to the metapopulation structuring observed in these flies.

### 5.5 Conclusions

This work adds another dimension to blow fly population investigations by using an interdisciplinary method to determine the most important mediators of genetic structure. Specifically, by integrating local and regional weather data, molecular screening of vertebrate DNA, and chemical assays to determine vertebrate fecal metabolites in flies, an invaluable, more comprehensive picture of wild flies was captured. This method can potentially be used to track range distributions of flies that are forensically, medically, or ecologically important, as well as track vertebrate populations.

### 5.6 References


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# CHAPTER 6. USING BLOW FLY KINSHIP AND STABLE ISOTOPE ANALYSIS TO DETERMINE CARCASS ABUNDANCE

## 6.1 Introduction

Animal carcasses and carrion represent the single most important resource at the individual and population level for all necrophagous blow fly species [1-3]. Without available carcasses, local populations are likely to dwindle to inevitable extinction. However, as female blow flies can lay between 100 – 300 eggs at a time [4], relatively few individuals may prevent local populations from crashing [5]. In theory, the proportion of highly related larvae should be higher on smaller carcasses (e.g. a mouse) compared to large carcasses (e.g. a deer) given the limited oviposition opportunity and the intense larval competition on small carcasses. An area with many small carcasses available would likely show the presence of many "families", or pockets of related individuals, even though the overall population would appear more genetically diverse when considering genotypes of all individuals from all families. Conversely, as large carcasses are capable of supporting larvae from tens or hundreds of females (leading to thousands or hundreds of thousands of larvae [6]), the likelihood of sampling any two highly related individuals from an area with many large carcasses is very low. The genetic similarity of the local blow fly population immediately after adult emergence from the puparium, but before random dispersal into the environment, should be very high if those flies developed on a small carcass, and low if they developed on a large carcass (proportionally). If many large carcasses are available in an area, especially over an extended period of time, then it is expected that genetic diversity will be very high in the local population (i.e. substructure will be low). Overall, the genetic diversity of blow flies sampled from wild populations should reveal important information about the type and

prevalence of carcasses in the environment. However, this is where the problem lies: quantifying the abundance and diversity of carrion (=carcasses) is very difficult to do.

Coincidentally, we can use blow flies to determine carrion diversity and abundances. Using eDNA and analytical techniques (see Chapters 2 and 3), it is possible to survey the vertebrate diversity of an environment using blow flies. However, these methods only reveal a snapshot of the resources available to the adult flies at the time of sampling. The inclusion of stable isotope analyses (SIA, Chapter 4) further enhances the ability to differentiate between the adult and larval resources (because a larval resource is unlikely to be present for the adults that developed on that carcass). Specifically, when used in conjunction with kinship analysis of flies [7, 8], SIA can help to answer questions about how carcass type affects the relatedness of individual flies and how these flies might be dispersing throughout the environment. Additionally, it has been hypothesized that cohorts of related flies may seek out resources together as adults soon after eclosion as they are all physiologically responding to the same chemical cues, which would explain why baited collections have consistently resulted in samples of highly related flies [9-11]. Therefore, kinship analysis of flies sharing similar isotopic signatures was performed and their adult resources compared. In this chapter, we use SIA and kinship analysis to determine if flies with similar isotopic signatures are proportionally more highly related than if they exhibited different carbon and nitrogen values.

## 6.2 Methods

### 6.2.1 Full Sibling Generation

Newly emerged adult *P. regina* (< 1 week old) from laboratory colonies (< $G_{10}$) were used for the generation of full-sibling flies. Flies were maintained under ambient conditions with water and sugar *ad libitum* until needed for the experiment. Female flies were presented with chicken

blood as a protein source to develop their eggs for several days leading up to the experiment. Once female flies appeared gravid, N = 3 random females were individually isolated and presented with chicken liver to induce oviposition. Eggs of each female were placed on approximately 50 g chicken liver (each representing a replicate) and allowed to hatch and develop to third instar larvae. N = 10 random full sibling larvae from each replicate were killed and DNA was extracted according to the procedure outlined in Chapter 3. Microsatellite amplification, fragment analysis, and kinship analysis was performed as outlined in Chapter 5. Genotypes of unrelated flies were used from samples that were taken in different years and in different geographic regions than the lab colony flies, therefore they were guaranteed to be unrelated. Likelihood ratios were calculated by dividing the probability of relatedness between two individuals by the probability of no relatedness between those two individuals. Likelihood ratios derived from forensic paternity indices and used previously for blow fly kinship analysis were used to create thresholds for relatedness among individuals. Methods for probability distribution generations and threshold choice followed those outlined in Picard and Wells (2012) [7].

### 6.2.2 Wild Fly Validation

Blow flies were collected from three regions (urban, Smokies, and Yellowstone) as outlined in Chapter 3. DNA extraction and vertebrate DNA amplification and sequencing protocols follow those outlined in Chapter 3. *Phormia regina* microsatellite amplification, fragment analysis, and population genetics analyses follow procedures outlined in Chapter 5. Vertebrate fecal metabolite screening was carried out via methods outlined in Chapter 2 [12]. Stable isotope analysis and carcass trophic position assignment was performed as described in Chapter 4.

## 6.3 Results

### 6.3.1 Full Sibling Generation

Unrelated flies exhibited a narrow distribution of kinship values with a mean near 0 (R = 0.05, Figure 26), while a wider distribution of relatedness values was observed for full siblings (R > 0.250). Arbitrary thresholds for likelihood ratios were used (LR>1, >20 and >1000) which resulted in kinship thresholds of R = 0.22, R = 0.38 and R = 0.55, respectively. Overall, 59, 25, and 7% of full sibling flies met likelihood ratio thresholds of R = 0.22, 0.38, and 0.55, respectively (Figure 27).

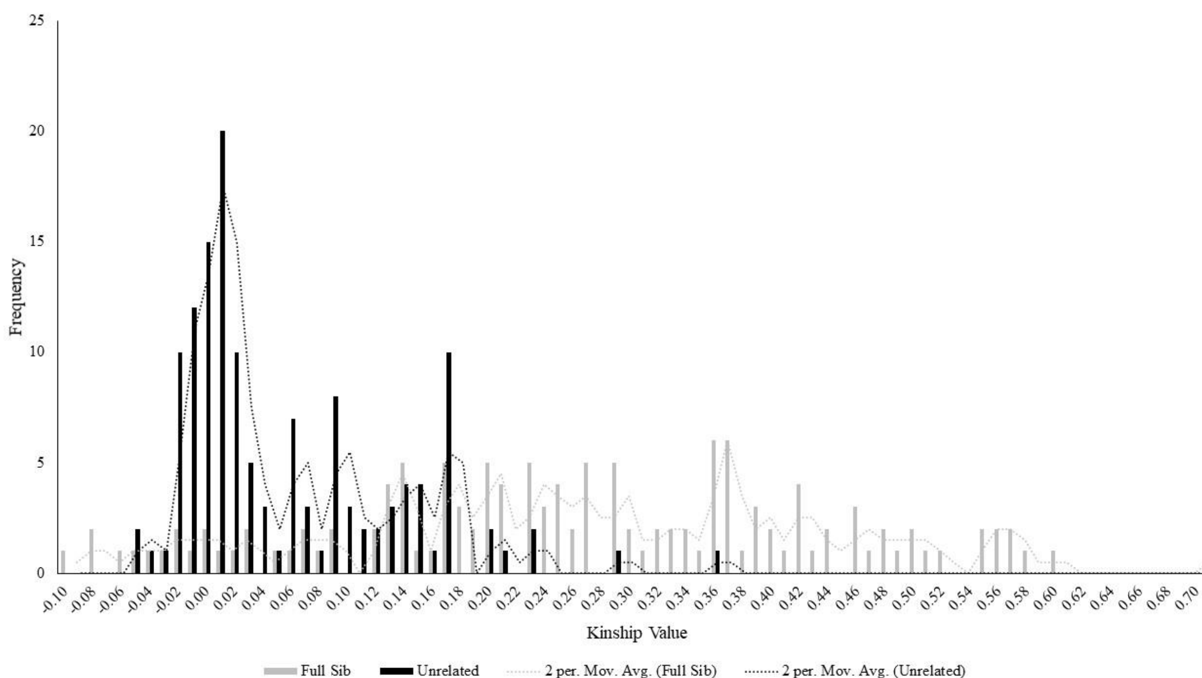


Figure 26. Distribution of kinship values for unrelated flies (black) and full sibling flies (grey). Kinship values represent the proportion of shared alleles between any pair of individual flies from a single sample. Likelihood ratios (corresponding to R = 0.22 – 0.37, >0.38) derived from forensic paternity indices are given in order provide a threshold of determining high relatedness between pairs of flies.

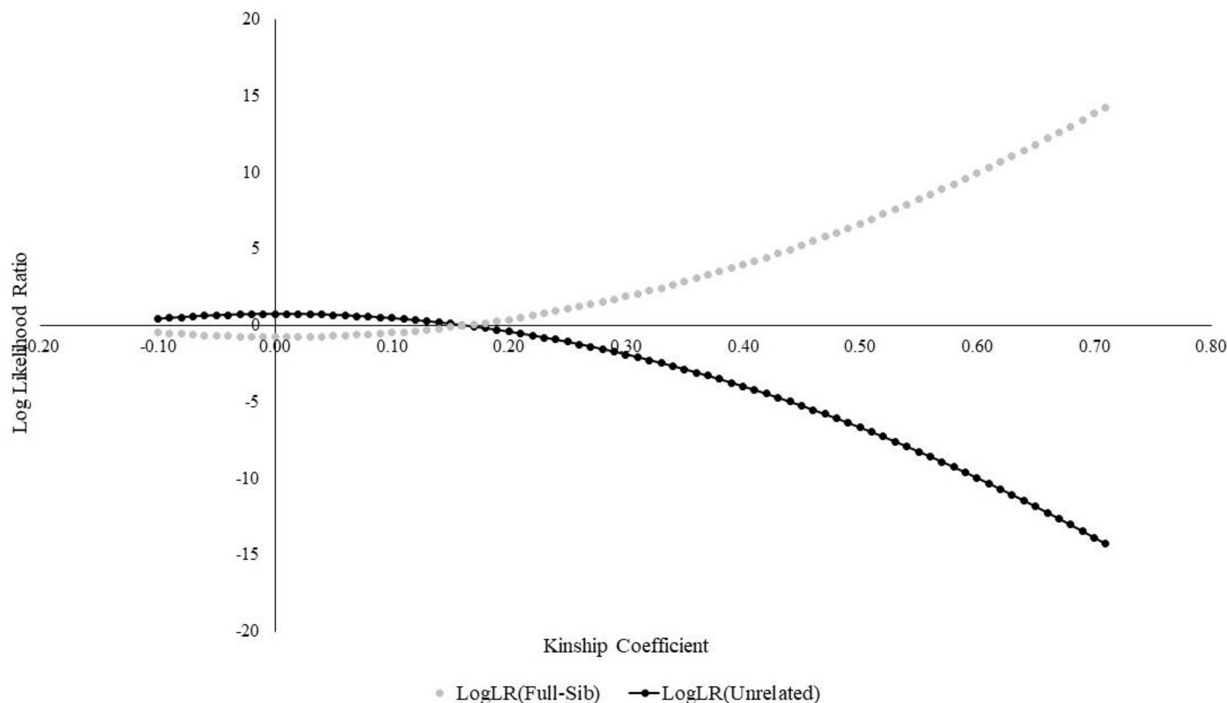


Figure 27. Log likelihood ratios (LR) for unrelated (black) and full sibling P. regina (grey). The first relatedness threshold, LR > 1, occurs at R = 0.22, the second threshold, LR > 20, occurs at R = 0.38, and the final threshold, LR > 1000, occurs at r = 0.55.

### 6.3.2 Wild Fly Genetics

Populations of *P. regina* in all three regions (urban, Smokies, Yellowstone) had significantly less genetic diversity than predicted by HWE (Table 20). The urban environment exhibited strong substructuring between the early and late summer populations, as discussed in Chapter 5 (Figure 28). No evidence of population structure was observed for the Smokies, likely due to a large amount of gene flow occurring on the spatial level (estimated number of migrants per population and generation > 1000). Yellowstone flies exhibited low but measurable gene flow and structure. Genetic similarity was observed between the urban and Smokies environments as 2% of paired kinship comparisons between individuals in these areas were related (R = 0.20 – 0.50) even though the samples were taken ~2 years apart (Table 21). This could indicate long term gene flow due to connectedness of these areas by major roadways and interstates (human mediated

travel or by “roadkill highways”). Aside from the potential gene flow between these two regions, there was no evidence that much gene flow occurred at a large spatial scale (i.e. between national parks), which is further supported by high $F'_{ST}$ values.

Table 20. Summary of population genetics statistics generated for three regions (urban, Smokies, and Yellowstone).

| Region | N | $N_A$ | $N_E$ | $H_O$ | $H_E$ | $F_{ST}$ | $F_{IS}$ | $F_{IT}$ | $F'_{ST}$ | $N_M$ |
|---|---|---|---|---|---|---|---|---|---|---|
| Urban | 21.75 (0.99) | 24.50 (1.83) | 18.91 (1.62) | 0.54 (0.01) | 0.93 (0.01) | 0.04 | 0.45 | 0.47 | 0.57 | 6 |
| Smokies | 22.21 (0.77) | 22.88 (1.55) | 17.02 (1.48) | 0.50 (0.05) | 0.91 (0.02) | 0.00 | 0.49 | 0.49 | 0.00 | 1484 |
| Yellowstone | 21.46 (0.93) | 20.92 (1.69) | 15.37 (1.54) | 0.47 (0.04) | 0.87 (0.03) | 0.01 | 0.51 | 0.52 | 0.07 | 39 |

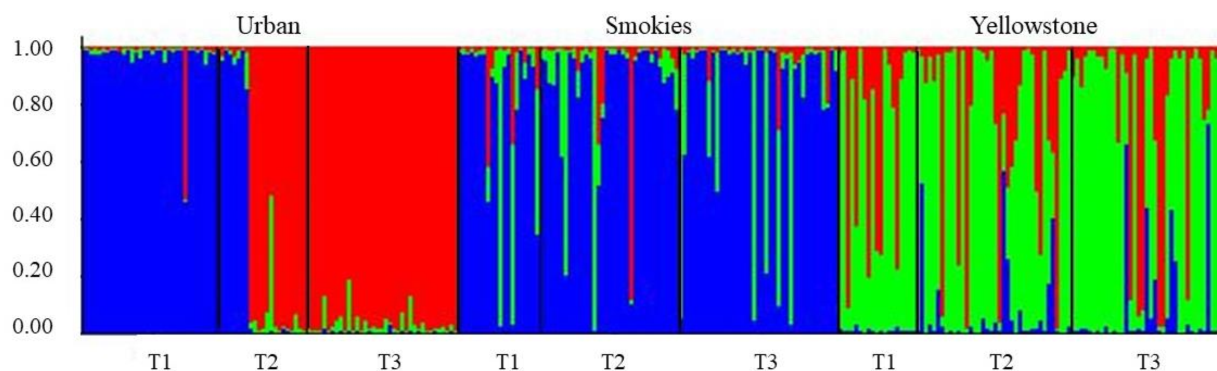


Figure 28. STRUCTURE bar plot summarizing proportion of shared alleles among individuals from three regions (urban, Smokies, Yellowstone). T1 – 3 represents the three temporal sampling periods for each region.

Table 21. Summary of pairwise AMOVAs between populations: Urban (Early Summer, ES), Urban (Late Summer, LS), Smokies, and Yellowstone.

| Pop 1 | Pop 2 | $F'_{ST}$ | $N_M$ |
|---|---|---|---|
| Urban (ES) | Urban (LS) | 0.119 | 46 |
| Urban (ES) | Smokies | 0.134 | 34 |
| Urban (ES) | Yellowstone | 0.800 | 4 |
| Urban (LS) | Smokies | 0.503 | 9 |
| Urban (LS) | Yellowstone | 0.692 | 5 |
| Smokies | Yellowstone | 0.608 | 5 |

### 6.3.3 Evaluating Influence of Larval Diet on Fly Kinship

Mean kinship was highest for flies in the carnivore, fish, and browser carcass categories in the urban, Smokies, and Yellowstone environments, respectively (Table 22). The highest proportion of flies meeting the LR > 1 threshold (R = 0.22) occurred in the browser (urban) and carnivore (Smokies and Yellowstone) categories. Most pairwise comparisons did not meet the LR > 20 threshold (R = 0.38; Figure 29A,C-D, Figure 30, Figure 31C), however 1.3% of pairs in the urban browsers exceeded this threshold (LR > 1000, Figure 29B), indicating a high likelihood that these pairs are made up of full siblings. Though Yellowstone flies did not reach kinship values quite that high, 0.3 and 1.8% of pairs in the mixed herbivore and browser categories exceeded the LR > 20 threshold (Figure 31A, B). Overall, though there were some related pairs of individuals sharing similar larval diets, a majority of flies in each of these categories were mostly unrelated to each other.

Table 22. Summary of fly kinship by region (urban, Smokies, Yellowstone) and trophic category (mixed herbivore, browser, carnivore, and fish). Kinship is given as the mean across all pairwise comparisons within each group, with the standard deviation given in parentheses. The total number of individuals within each trophic category is given, as well as the total number of pairwise comparisons. Finally, the proportion of pairwise comparisons above the relatedness thresholds of R = 0.22 and 0.38 are given.

| Region | Trophic Category | Mean Kinship (s.d.) | Total Individuals | Total Pairwise Comparisons | % R > 0.22 | % R > 0.38 |
|---|---|---|---|---|---|---|
| Urban | Mixed Herbivore | 0.015 (0.069) | 38 | 630 | 1.7 | 0.0 |
| | Browser | 0.007 (0.138) | 33 | 525 | 6.3 | 1.3 |
| | Carnivore | 0.028 (0.080) | 22 | 210 | 2.9 | 0.0 |
| | Fish | 0.010 (0.063) | 6 | 15 | 0.0 | 0.0 |
| Smokies | Mixed Herbivore | 0.019 (0.078) | 72 | 2556 | 2.3 | 0.0 |
| | Browser | 0.012 (0.080) | 10 | 45 | 2.2 | 0.0 |
| | Carnivore | 0.030 (0.096) | 7 | 21 | 4.8 | 0.0 |
| | Fish | 0.046 (0.122) | 3 | 3 | 0.0 | 0.0 |
| Yellowstone | Mixed Herbivore | 0.043 (0.082) | 71 | 2485 | 2.5 | 0.3 |
| | Browsers | 0.050 (0.092) | 11 | 55 | 5.5 | 1.8 |
| | Carnivore | 0.032 (0.144) | 4 | 6 | 16.7 | 0.0 |

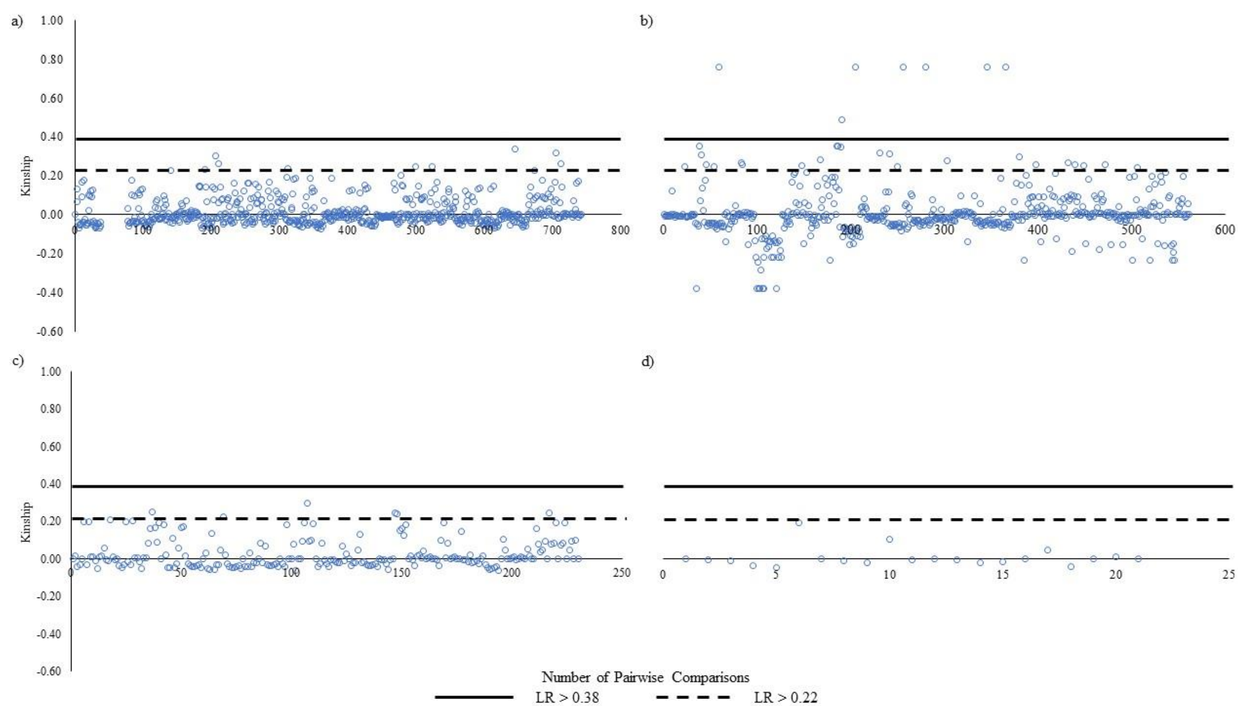


Figure 29. Scatterplots of pairwise kinship comparisons for the Urban environment for each trophic category: A) Mixed Herbivore, B) Browser, C) Carnivore, and D) Fish. The solid black line represents the threshold for kinship values above R = 0.38, indicating high likelihood of relatedness. The dashed black line represents the lower threshold for kinship values (R = 0.22), indicating a moderate degree of relatedness.

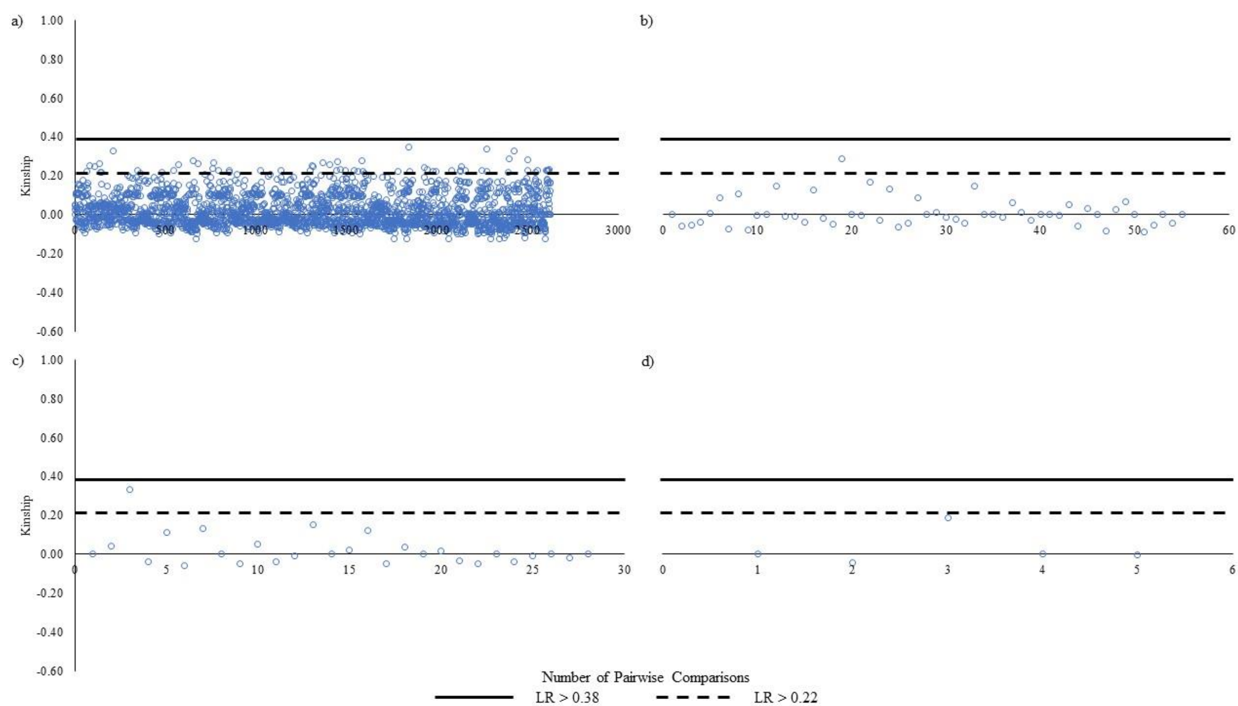


Figure 30. Scatterplots of pairwise kinship comparisons for the Smokies for each trophic category: A) Mixed Herbivore, B) Browser, C) Carnivore, and D) Fish. The solid black line represents the threshold for kinship values above R = 0.38, indicating high likelihood of relatedness. The dashed black line represents the lower threshold for kinship values (R = 0.22), indicating a moderate degree of relatedness.

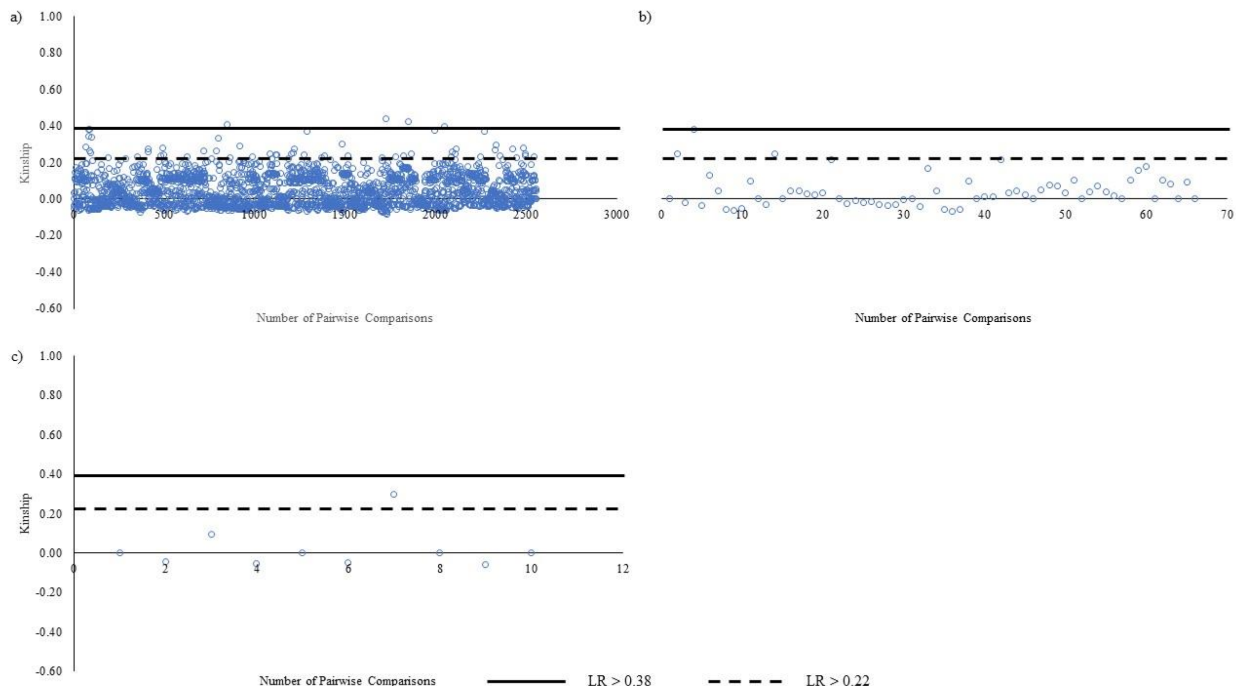


Figure 31. Scatterplots of pairwise kinship comparisons for Yellowstone for each trophic category: A) Mixed Herbivore, B) Browser, and C) Carnivore. The solid black line represents the threshold for kinship values above R = 0.38, indicating high likelihood of relatedness. The dashed black line represents the lower threshold for kinship values (R = 0.22), indicating a moderate degree of relatedness.

Though related flies likely shared the same or similar larval resources, it does not appear that these flies shared the same adult resources (Table 23). Most flies sampled did not test positive for vertebrate resources, indicating that the resources could not be detected by the investigator or that these flies had not fed on a vertebrate resource. Only two pairs of related individuals shared similar isotopic values and adult resources. However, since three of four flies consumed feces from an unknown source, it cannot be known whether the feces they consumed was from the same animal.

Table 23. Summary of pairwise kinship values greater than 0.38. Ind. A refers to the first individual in the pairwise comparison, whereas, Ind. B refers to the second individual in the comparison. Comparisons are broken down by region (urban, Smokies, and Yellowstone) as well as trophic level (mixed herbivore, browser). The adult resource is given for each individual. ND = none detected. Comparisons in boldface indicate related individuals who have consumed the same adult resource.

| Region | Trophic Level | Ind. A | Ind. A Adult Resource | Ind. B | Ind. B Adult Resource | Kinship |
|---|---|---|---|---|---|---|
| Urban | Browser | Ind26 | ND | Ind75 | ND | 0.761 |
| | | Ind26 | ND | Ind38 | ND | 0.488 |
| | | Ind32 | ND | Ind75 | ND | 0.761 |
| | | Ind33 | ND | Ind75 | ND | 0.761 |
| | | Ind37 | ND | Ind75 | ND | 0.761 |
| | | Ind38 | ND | Ind75 | ND | 0.761 |
| | | Ind4 | ND | Ind75 | ND | 0.761 |
| Yellowstone | Mixed Herbivore | **Ind193** | **Feces (no ID)** | **Ind209** | **Feces (no ID)** | **0.381** |
| | | Ind193 | Feces (no ID) | Ind212 | ND | 0.381 |
| | | Ind209 | Feces (no ID) | Ind212 | ND | 0.408 |
| | | Ind239 | Cat | Ind240 | ND | 0.376 |
| | | Ind240 | ND | Ind262 | Rocky Mountain Elk | 0.395 |
| | | **Ind230** | **Feces (no ID)** | **Ind268** | **Rocky Mountain Elk feces** | **0.439** |
| | | Ind233 | Feces (no ID) | Ind273 | ND | 0.425 |
| | Browser | Ind198 | ND | Ind207 | ND | 0.381 |

## 6.4 Discussion

There were likely many carcasses available in the regions sampled as most flies were unrelated (i.e. kinship was very low). This also explains why genetic diversity was so high (i.e. low structure) at the spatial scale for all sampled environments. Molecular and chemical data (Chapter 3) also suggest that there was no scarcity of resources. However, within each region, a small proportion of individuals that shared isotopic signatures were also found to be more related than expected by random chance. Some of these related individuals were very likely full siblings (e.g. the six pairs of flies with R ~ 0.80 in the urban browsers category), which indicates that they shared the same larval resource (carcass). Female flies will lay their full complement of eggs during oviposition, rather than ovipositing some eggs on one carcass and "saving" the others for another carcass (there is no guarantee they will be able to find another carcass). Given this, finding two full sibling flies that were reared on different carcasses would be extremely unlikely.

Therefore, the fact that these several pairs of individuals share a large proportion of their genetic makeup and have similar isotopic signatures are good indicators that they are actually siblings that developed on the same carcass. This is relevant as it can potentially be used to estimate the actual abundance of carcasses in an area.

Furthermore, pairs of related individuals seemed to be randomly distributed in each region, as “families” were not aggregated spatially. For example, one fly collected in Military Park in the urban environment was highly related to a fly collected in Province Park, instead of both flies being sampled at a single site. This is likely due to the dispersal and migration of flies after eclosion into new areas that may contain the required resources. The fact that related flies do not seem to be traveling together is also supported by vertebrate DNA data: out of the related flies that have actually taken an adult meal, only two pairs potentially consumed the same resource (feces). The high geographic migration rate of these flies (as indicated by molecular data, Chapter 5) is likely due to flies searching for new resources, which explains why highly related individuals were collected in different places.

## 6.5 Conclusion

Overall, this research enhances current methods for determining the mediators of blow fly population structure, especially kinship of flies. The carcass is the quintessential resource that sustains most blow fly species at the individual and population level. Therefore, it makes sense that similar carcass signatures are observed in flies with high relatedness values. However, the absence of relatedness in most flies is an indicator that carcasses are abundant in a region, which is a vital piece of information when modeling the movement and dynamics of highly dispersive insects like blow flies.

## 6.6 References

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# CHAPTER 7. CONCLUSION

The overall results of this research seem glaringly obvious: food and environmental conditions have a large impact on blow flies at the individual and population level. However, until now, no research has been implemented to quantify vertebrate resources utilized by flies, as well as their impact on blow fly dispersal and population genetics. To date, weather and climate data has never been implemented in any other studies of blow fly population genetics, nor has a long-term temporal sampling scheme. We now have a multidisciplinary method that can inform us about fly genetics, the types of food and oviposition resources available to flies around the time of sampling, the changes in availability of those resources in different geographic regions and time scales, and the type of carcasses in the environment in the recent past. The type of information generated from this research is crucial to understanding the mechanisms of resource location by flies, range expansion into previously uninhabited areas, and even speciation when considered on an extended temporal scale.

High-resolution spatiotemporal sampling over a two-year period in the urban environment revealed that *P. regina* showed the same trend as every other blow fly population genetics study previously: there was no spatial structure. However, strong genetic structuring by season was observed for the first time ever with these insects, indicating that there are in fact limits to gene flow and some form of isolation of reproductive groups. This high-resolution sampling also revealed highly variable abundance of *P. regina* seasonally due to a combination of local weather, interspecific and intraspecific competition, and availability of vertebrate carcasses and feces. In the urban environment, we found that many medium-sized vertebrate carcasses (mainly dogs, Chapters 3 and 5) were available to adult flies, and that there was a mostly even distribution of carnivore and herbivore carcasses available for blow fly colonization (Chapter 4). The availability

of multiple types of carcasses, as well as plentiful fecal resources for adult protein consumption, may explain the spatial heterogeneity in the fly genetic data. However, seasonal variation in the availability of resources along with abiotic factors like precipitation potentially produced population bottlenecks (Chapter 5), explaining the strong temporal structure seen in this region. Therefore, data collected in the urban environment over an extended period of time and spanning several geographic locations support the null hypothesis (Chapter 5) that blow flies adhere to a Levins metapopulation model.

In order to determine if the trends observed in the extensive urban collections were valid in other ecoregions, *P. regina* was also sampled in two national parks: the Great Smoky Mountains and Yellowstone. Though these regions were not sampled intensively over time (i.e. over a three-day period), the results from these collections are extremely important for understanding the drivers of *P. regina* population genetics and dynamics in different environments. A large number of genetically diverse flies (i.e. no spatial structure) were collected in a short period of time in the Smokies. These flies revealed that a moderate amount of mostly small herbivores resources (carcasses and feces) were present in the park around the time of sampling. However, isotopic signatures indicated that most sampled flies developed on herbivore carcasses. This means that this region had many small herbivore carcasses (squirrels, rabbits) available to flies or potentially a few large carcasses (e.g. deer). Due to the sheer abundance of flies captured, as well as their extremely high genetic diversity, it is likely that large herbivore carcasses contributed proportionally more individual flies to the local population than small carcasses, which would have produced a much smaller number of more highly related flies. Therefore, high resource availability in the Smokies facilitated increased migration and gene flow over space, maintaining a large, genetically diverse blow fly population that aligns with the spatially patchy population model.

A slightly different story can be told of Yellowstone, however. A higher proportion of related individuals were measured in Yellowstone compared to the other regions, and populations in this region show a slight degree of spatial structure. Yellowstone also happens to be the region in which the fewest number of *P. regina* were collected overall and the highest proportion of flies containing vertebrate resources, especially feces, occurred (Chapter 3). This likely indicates that the overall *P. regina* population size in Yellowstone is much lower than in the other regions sampled. However, despite the higher prevalence of related individuals comparatively, a majority of flies were still unrelated, which points to there being no severe lack in carcass availability in this environment. Yellowstone represents a unique area to study blow fly population dynamics due to the prevalence of large vertebrate predators and facultative scavengers (grizzly bears, wolves, coyotes, bald eagles, and ravens, to name a few) which likely compete with the local necrophagous insect community for the rights to carrion [1, 2]. Carcasses in Yellowstone can come from a variety of sources, including natural deaths, roadkill events, predation events, and to some extent hunter-induced kills immediately outside of the park. Blow flies would likely be more successful at locating and successfully colonizing roadkill and animals that have died naturally, as they would likely be incapable of colonizing abandoned predator (e.g. wolf) kills, in which avian scavengers almost always lay claim to the remains immediately after wolves leave [3]. Competition with vertebrate predators and scavengers means that flies lose out on an invaluable opportunity to contribute genetic material to the next generation if they fail to colonize a carcass immediately [1]. This intense competition likely limits the size of blow fly populations in this area, explaining why a larger proportion of sampled flies show some degree of relatedness. Given the present data, Yellowstone would appear to adhere to one of the metapopulation models as populations likely experience many colonization and extinction events throughout the park due to the presence of

vertebrate scavengers. However, it is difficult to determine if a Levins or source-sink metapopulation model is occurring without more high-resolution temporal sampling in this region. What is clear is that the current data do not support adherence to the patchy or isolated population models for flies in Yellowstone.

Given the isotopic data for all three regions, it seems that even though some related flies likely utilized the same carcasses for larval development, they did not visit similar resources as adults. Therefore, it is unlikely that related flies are traveling together in cohorts to find resources. It is possible that if the flies had actually attended the same resources, our DNA or fecal screening methods were unable to detect traces of these molecules and chemicals, possibly due to degradation. Excluding this possibility, there may have been intense inter- or intraspecific competition for resources, or a lack of resources altogether, making it difficult for flies to attend the same, if any, vertebrate resources as adults. Regardless, it seems as though individual perception of resource or environmental cues, and not familial bonds, likely influence a fly's decision to disperse into the environment.

Ultimately, this research shows that all of the information needed to ascertain the life history of a fly is contained within the fly itself. This research reveals the importance of both vertebrate resources and other biotic and abiotic factors contributing to the population genetics of *P. regina*, both at a fine spatiotemporal scale, and at a broad geographic scale. Taking these factors into account is crucial for understanding how insects behave at a population level, which is the unit at which evolution acts. This research can be applied to numerous endeavors involving blow flies, including estimating a minimum postmortem interval in forensic entomology, to using blow flies as drones to survey vertebrate populations, and even to estimate the dispersal of pathogens from ground-zero disease outbreaks into human-inhabited areas. Beyond blow flies, this

multidisciplinary method of studying population genetics can be applied to nearly any type of insect, whether they are necrophagous, predacious, or herbivorous, and may be especially impactful for studies of disease vectors and crop pests. Overall, we now have a better understanding of why previous population genetics trends in blow flies (i.e. lack of spatial structure) were observed, and highlight the importance of high-resolution spatiotemporal sampling, as information gleaned from only one sampling dimension (spatial or temporal) results in only part of the story being told.

## 7.1 References

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