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

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ABSTRACT

Filth flies are commonly implicated in pathogen transmission routes due to their affinity for vertebrate waste and their synanthropic associations. However, solidifying the link between flies and infected feces in the wild can be difficult, as microbial methods to reach this goal may produce misleading results. We present an analytical assay using liquid chromatography mass spectrometry-mass spectrometry (LC MS/MS) to detect vertebrate fecal metabolites (urobilinoids) in adult blow fly guts. Proof of concept experiments consisted of controlled feeding in which flies were grouped into three treatments (unfed, exposure to beef liver tissue, exposure to canine feces; N = 20/treatment) using the black blow fly *Phormia regina* Meigen (Diptera: Calliphoridae). It was revealed that only feces-related samples exhibited peaks with a m/z of 591 and MS/MS spectra consistent with urobilinoids. These peaks were not seen for beef liver tissue, flies exposed to beef liver tissue, or unfed flies. Samples taken directly from beef liver tissue and from feces of several animals were also tested. To test this assay in wild flies, 216 flies were additionally analyzed to determine if they had ingested vertebrate feces. 13% of the wild flies exhibited these same peaks, providing a baseline measure of blow flies collected in urban and residential areas consuming feces from the environment. Overall, this assay can be used for *P. regina* collected in an applied setting and its integration with microbial culturing and sequencing methods will help to improve its use.

KEYWORDS

Feces, filth flies, urobilinoids, liquid chromatography

INTRODUCTION

Filth flies have long been implicated in pathogen transmission routes due to their association with unsanitary conditions (Greenberg 1971). The tendency for numerous families of adult filth flies to associate with feces from humans (Bohart and Gressitt 1951, Liu et al. 1957, Steyskal 1957) and animals (Fatchurochim et al. 1989, Axtell and Arends 1990, Conn et al. 2007) 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 (Hall 1948, Greenberg 1973). Visitation to feces may be opportunistic (i.e. the fly may haphazardly light 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 (Linhares and Avancini 1989, Stoffolano et al. 1990, Stoffolano et al. 1995). Furthermore, proteins from feces are also important for male blow fly reproductive organ development and increases the probability of successful insemination (Stoffolano et al. 1995). 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 (Pace et al. 2017). 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 (Sawabe et al. 2006). 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 (Monzon et al. 1991). 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 (Barro et al. 2006)); 2) possible acquisition of the pathogen from another contaminated source (e.g. garbage, carrion, offal (Fischer et al. 2001)); 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 (Nayduch and Burrus 2017)).

Pathogenic bacteria associated with filth flies are typically extracted for analysis via microbiological culturing methods (Thomson et al. 2017), molecular methods (Sawabe et al. 2006, Brazil et al. 2007, Scully et al. 2017), or by using an integrated approach of culturing and DNA sequencing (Szalanski et al. 2004, Pace et al. 2017). 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 (Talley et al. 2009, Pace et al. 2017). Female house flies are also capable of acquiring more bacterial colony forming units (CFUs) than males soon after exposure to manure (Thomson et al. 2017). 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 for the purpose of testing hypotheses that pathogen acquisition may originate 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 (Cao et al. 2011, Buzatto et al. 2014). Numerous fecal compounds have been investigated for profiling human and animal metabolites, including steroids (Eneroth et al. 1964, Ziegler and Wittwer 2005), various bile acids (Grundy et al. 1965, Eneroth et al. 1966, Perwaiz et al. 2001), and sterols (Isobe et al. 2002). 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 (McMaster and Elman 1925). These urobilinoids are formed from the breakdown of bilirubin by gut microflora (Pullman and Perault 1959, Moscowitz et al. 1970, Fahmy et al. 1972). The urobilinoids are 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) (Jones-Lepp 2006). 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) (Cao et al. 2011), and pressurized liquid extraction turbulent flow chromatography liquid chromatography tandem mass spectrometry (PLE-TFC-LC-MS/MS) (Song et al. 2016b, a). Given the suite of fecal biomarkers characterized by modern analytical techniques in metabolomics, we decided 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 (James 1947), and it has forensic utility as a primary colonizer of corpses (Byrd and Allen 2001).

METHODS

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 were approximately 10 hours old. 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 hour prior to the beginning the experiment.

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.

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.

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 (Li 2015). 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).

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, , respectively) were also tested, each consisting of a mixture of isomers with molecular weights ranging from 590.7 to 598.8 g/mol.

Statistical analyses. Experimental and wild flies were compared statistically. The non-parametric Kruskal-Wallis rank sum (Kruskal and Wallis 1952) 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 (Dunn 1964). All statistics were performed in R (R Core Team 2017) using the packages stats (native) and dunn.test (Dinno 2017).

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 (Quinn et al. 2012). 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 and 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.

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 (Quinn et al. 2012). 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.

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 (χ^2 = 72.8, df = 3, P < 0.0001) and 8 min (χ^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).

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) (Moore et al. 1987, MacVicker et al. 1990) and mosquitos (Diptera: Culicidae) (Burkett et al. 1999). 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 (Campobasso et al. 2004, Pien et al. 2004, Bushby et al. 2012). 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.

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. Flies in this study were only exposed to one type of feces (dog), though 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 (Vítek et al. 2006), or exposure of the samples to air and light, which has been shown to induce a conversion from urobilinogen to urobilin (Wilbur and Addis 1914). 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 that affect the speed of the fly metabolism. These uncontrollable variables would complicate interpretation of quantitative data. The variability of stercobilin could also arise from differences listed above, as well as liver functioning of the host animal, which could vary by individual, and as such, this 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 (Singh et al. 2015), which has implications for other substrates they may visit in the wild (i.e. carcasses or feces to human food). Therefore, it would be pertinent to be able to differentiate whether the bacteria that these blow fly species are carrying originate from carcasses or feces, especially since these flies are often found in human environments. Though most blow flies in the United States do not develop in feces, the recent invader *C. megacephala* (Wells 1991) has been known to breed in human feces in particular (Liu et al. 1957, Goff et al. 1991). 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 (Liu et al. 2013). Thus, as *C. megacephala* has quickly expanded its distribution from Florida, USA (Gruner et al. 2007) to Indiana, USA (Picard 2013), and likely is continuing to disperse throughout this region, it will be crucial to investigate its impact on pathogen acquisition and transmission 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* (Hucko 1984), numerous strains of *Escherichia coli*

(Förster et al. 2007), *Campylobacter* spp. (Szalanski et al. 2004), and *Cryptosporidium parvum* (Graczyk et al. 1999), 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 (Greenberg 1973). 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 (Hightower et al. 1965, Mayer and Atzeni 1993). Dispersal patterns of filth flies are important for two reasons: 1) flies may remain in areas where resources are plentiful (e.g. animal facilities that provide feces for protein-feeding), allowing the flies to potentially acquire pathogens); and, 2) flies are also capable of travelling great distances, which could increase the dissemination of pathogens in areas that may be otherwise unaffected by a potential disease outbreak.

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 (Calvignac-Spencer et al. 2013, Schubert et al. 2015), 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.

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 sequencing methods, as well as vertebrate sequencing methods, to precisely identify the source of pathogens mechanically acquired by filth insects from vertebrate feces.

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FIGURES

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).

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.