Detection of Fruit Meals Within Laboratory-Raised and Field-Trapped Adult *Drosophila suzukii* (Diptera: Drosophilidae) Guts

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The feeding habits of adult Brachycera are understudied and may provide important context for understanding invasive pest biology, as with the polyphagous small fruit pest *Drosophila suzukii*. We developed molecular methods to study adult *D. suzukii* gut content in order to understand its feeding habits. We designed and verified two primer pairs specific for either blueberries or blackberries and used a qPCR melt curve analysis to determine whether we can detect the presence or absence of berry feeding by adult flies. In a laboratory assay, the blueberry fly meal DNA can be detected for longer periods than the blackberry meal DNA. Generally, female gut contents are less variable than male gut contents. We also tested recently emerged flies that were not fed as adults but developed as larvae in either blueberries or blackberries. Some adult flies from each fruit had detectable fruit DNA in their gut, which could be due to pupal meconium feeding after emergence. Next, we aimed to test the primers in the field to develop techniques to track fruit feeding by *D. suzukii* in its natural field environment. First, to identify the most appropriate collection method, we determined how long we could detect fruit DNA, using previously developed primers within *D. suzukii* gut preserved in four types of trap fluid in the laboratory. The likelihood of detecting blackberry DNA differed by day, trap fluid, and between sexes. For the blueberry primer, the possibility of detecting blueberry DNA differed by trap fluid only. Based on those results, we used RV antifreeze with a Scentry SWD lure in field trials at two research station locations, one containing blackberries and one with blueberries. We established transects away from each fruit planting and collected up to 120 total flies at each point along transects. There were no significant differences in the number of flies containing berry DNA among collection points along the transect in both locations. These results suggest that adult flies move between crop and non-crop habitats and may not be highly dependent on fruit food resources.

Keywords: spotted wing *Drosophila*, integrated pest management, invasive species, gut content, small fruit IPM

INTRODUCTION

Food resource use by adult, non-animal feeding higher flies (Brachycera) has been understudied because it is often considered secondary to adult oviposition in and larval damage of crops (Clarke et al., 2011; Walsh et al., 2011). In contrast, larval resource use, specifically that of invasive polyphagous pest species such as *Drosophila suzukii* (Matsumura) has been well studied (Parrella, 1983; Headrick and Goeden, 1990; Burrack et al., 2009; Ekesi et al., 2009; Emiljanowicz et al., 2014; Jaramillo et al., 2014; Nash and Chapman, 2014; Hardin et al., 2015; Hamby et al., 2016; Silva-Soares et al., 2017; Lewis and Hamby, 2019). However, chemical and cultural control tactics primarily target adult flies (Mahmoud and Shoeib, 2008; Ur Rehman et al., 2009; Van Timmeren and Isaacs, 2013; Erland et al., 2015; Ghabbari et al., 2018; Siddique et al., 2018; Mawtham et al., 2019).

Higher fly morphology determines and restricts feeding (Yeates and Wiegmann, 1999). While blood-feeding flies in the family Muscidae have prestomal teeth that cause the characteristic "bite" felt when they puncture skin, most Brachycera have "lapping" mouthparts (Elzinga and Broce, 1986; Yeates and Wiegmann, 1999). The "lapping" mouthparts evolved 100 million years before flowering plants and limited flies to liquid resources, such as extrafloral nectaries, hemipteran honeydew, flower nectar, decaying materials, fecal matter, yeast, fungi, and other similar substrates (Elzinga and Broce, 1986; Yeates and Wiegmann, 1999). Drosophilidae in particular may be capable of making minute scratches to soft materials to release fluids for feeding but "particulates released would have to be very narrow to pass through the very narrow pseudotracheae found in this family" (Elzinga and Broce, 1986).

In female Brachycera flies, protein appears important to egg production. At the same time, carbohydrate intake promotes adult fly longevity, while diet may have a more species-dependent impact on male reproductive success and survivorship. *Anastrepha* (Diptera: Tephritidae) males of some species had more copulatory success when they fed on higher protein diets while the copulatory success of males in other, closely related species were unaffected by diet (Aluja et al., 2001). Female diet resource has also been linked to reproductive success and longevity. Protein appears to be an important resource for females in developing eggs. In one study, the number of eggs produced was three times higher in *Hermetia illucens* (L.) adult females given a milk-powder-based protein source (Bertinetti et al., 2019). Female *Anastrepha* flies given sucrose and protein diet had a higher egg load than those given sucrose alone (Jacome et al., 1995; Aluja et al., 2001). In developing a fly diet for colony care, researchers found that removing key diet components (amino acid cocktail, cholesterol, etc.) reduced the egg load of *Ceratitis capitata* (Wiedemann) (Chang et al., 2001). Similarly, *Drosophila melanogaster* (Meigen) egg load was suppressed when fed on a diet lacking protein (Lee et al., 2011). Additionally, *D. melanogaster* egg cells may perceive environmental, nutritional status and determine whether they develop or undergo cell death (Terashima and Bownes, 2004). Perhaps unsurprisingly then, the closely related *D. suzukii* have also been shown to increase yeast feeding following mating

(Mori et al., 2017), and egg maturation in *D. suzukii* has shown to be low when fed fruit juice alone (Plantamp et al., 2017). While low protein to carbohydrate levels improves *D. suzukii* fecundity, a high ratio of protein to carbohydrates can slow fecundity within the species (Rendon et al., 2019).

Sucrose concentration has been shown to affect *C. capitata* survival but not egg load (Chang et al., 2001). An intermediate amount of carbohydrates, increases lipid-storage within *D. melanogaster* and increases their longevity (Lee et al., 2011). Similarly, female *D. suzukii* given floral resources had increased carbohydrate reserves, which increased their survival compared to those treatments only given water (Tochen et al., 2016).

Many of the studies discussed so far looked at laboratory-raised colonies of flies in order to understand their biology better. In the research described here, we created a tool to study higher fly molecular gut content at the field-level. For the most part, molecular gut content research has been dominated by studies of predator feeding behavior (Juen and Traugott, 2005; Greenstone et al., 2007; Harwood et al., 2009; Szendrei et al., 2010; Günther et al., 2014; Rondoni et al., 2015; Li et al., 2017; Albertini et al., 2018; Macías-Hernández et al., 2018). We use molecular gut content to understand how a polyphagous, phytophagous fly and major small fruit crop pest, *D. suzukii*, may use cultivated small fruit resources within a field setting. Briem et al. (2018) in Germany recently conducted an assay that evaluated total adult food resource. They captured 40 adult male and female flies within a raspberry field, and sequenced them using high-throughput sequencing on an Illumina MiSeq platform (Briem et al., 2018). The vast majority of their flies had detectable raspberry DNA within their guts, in addition to two other plants (*Urtica dioica* and *Polygonum humifusum*). Half their samples included environmental DNA, or eDNA, from the outside of flies where they did not surface sterilize those samples (Briem et al., 2018). This research provided insight into the total feeding behavior of a small sample of flies. In our experiments, we sought to develop a tool to test large numbers of flies trapped within a field to understand what proportion of *D. suzukii* are using specific fruit resources in a cultivated monoculture resource. This larger scale of testing would likely be more economical for future field-based research.

We built from previous work by our group which tested a putative strawberry primer (see note in section "Materials and Methods") (Diepenbrock et al., 2018). That primer was used to detect fruit feeding using a qPCR melt curve reaction and detected a fruit meal within flies up to 7 days after feeding in a mixed-sex population (Diepenbrock et al., 2018). We further expanded these methods by designing and testing blueberry- and blackberry-specific primers. We then conducted a series of laboratory experiments to inform the interpretation of results from field-collected flies.

MATERIALS AND METHODS

Source Material
All flies from this experiment originated from a colony of *D. suzukii* raised for over 100 generations. These flies stem

from wild flies collected in Laurel Springs, NC in 2010 and have had periodic wild-trapped flies added to maintain genetic diversity. The colony is kept at 20°C 16 L h: 8 D h and fed a cornmeal-based diet as described in Hardin et al. (2015). Adult flies between 3- and 7-days old were used for these experiments.

Primer Design and Selection

We used the *D. suzukii* primer (fwd: AATTGTTACCGCA CATGC; rev: GGAATGCTATATCTGGGTCC) which amplifies a 117-bp region of the COI gene (Dhami and Kumarasinghe, 2014) as a positive control for successful DNA extraction in our samples. All fruit primers were tested on adult flies starved and then fed on fruit. Early assays found that the melt curve temperature profile shifts depending on whether the DNA was extracted straight from the plant or from the *D. suzukii* gut. Throughout this research project, we tested 130 primer pairs. Over time, we built a library of *D. suzukii* fed known varieties of fruit and tested our final primers on multiple varieties of the same fruit type to determine specificity (Please, see the Supplementary Material for more information, including a list of primers tested).

   We successfully found a blackberry-specific primer by searching the literature for published blackberry genes (fwd: CT TCCCCCTATAAATCCCGA/rev: CGTCTCTCTGCAATTCCT CC). The blackberry primer comes from an SSR region (RH_MEa0007aG06, Zurn et al., 2018 from Bassil et al., 2016) that we confirmed was selective for cvs. Von, Ouachita, and wild blackberries fed to adult *D. suzukii* without picking up most of the raspberry accessions grown at Salisbury Research Station (**Supplementary Table 1**).

   The blueberry-specific primer comes from a maturase K gene found within the chloroplast (Accession Number: MH551878) and picks up a large number of blueberry varieties from both highbush (*Vaccinium corymbosum*) and rabbiteye (*Vaccinium virgatum* formerly *V. ashei*) (fwd: GGGCAT TCTATGGGTTTTCA/rev: TGGATCCTTCTTGGTTGAGC). The primer was designed using Primer 3 software (Untergasser et al., 2012).

   Unfortunately, the strawberry primer published in Diepenbrock et al. (2018) was not specific to strawberries and picked up caneberries and potentially other members in the family Rosaceae. A fully annotated blackberry genome was not yet published at the time of these experiments.

Larval Feeding-Adult Gut Content Assay

To determine whether adults contain detectable remains of larval diet, we infested fruit following methods described in Kraft et al. (2020). We held infested blackberries and blueberries in separate containers with mesh bottoms at 20°C for 8 days until pupation. At that time, all pupae were removed from fruit containers and placed on a damp paper towel inside a 60 mm Petri dish until adults emerged. Replicates for each fruit type were processed on the same day. Adult flies were frozen within 12 h of adult emergence and then placed in 96% ethanol in microcentrifuge tubes and held at −20°C until DNA extraction.

Pupal Meconium Extraction Assay

To test whether berry DNA was detectable from pupal meconium, we infested blueberries and blackberries using the same methods described above. When pupae were freshly sclerotized 7 days later, we removed pupae of larvae that had wandered away from the fruit (to reduce potential external contamination). The pupae were rinsed under tap water three times on fresh dish towels to remove external fruit material as in Schnetzer and Tyler (1996). Then, pupae were placed on a damp paper towel inside a 60 mm Petri dish until adults emerged. We extracted DNA from empty pupal cases within 3 h of adult emergence over the course of 2 days. In a follow-up study, after the same rinse protocol, we additionally subjected pupae to UV light within the fume hood for 15 min to further decrease the chances of external fruit DNA presence.

Detectability Over Time Assay

In this assay, we wanted to determine for how long berry DNA would be detected after a single feeding event. Adult flies aged 3–7 days were held for ∼60 h on a dilute sugar water (5 g sugar in 400 mL of tap water) soaked cotton ball. Flies were then fed a fruit puree made from fresh fruit purchased from the grocery store (blueberries, unknown variety) or from blackberries cv. Von picked at the Piedmont Research Station, Salisbury, NC during summer 2020 with ∼4 g sugar added to increase feeding. Seeds remained present in the puree, but *D. suzukii* mouthparts are a magnitude of size too small to feed on seeds or skin and only fed on juice from the puree. Flies were allowed to feed on the fruit puree for 4–6 h and were checked visually for a purple or lavender stripe in their gut to confirm feeding.

   After fruit feeding, flies were moved to vials of a cornmeal-based diet and placed into two separate growth chambers. For each cohort of the flies that fed on blackberry and the flies that fed on blueberry, one growth chamber held the flies at 20°C with a 16:8 light/dark cycle while the other growth chamber was set to resemble field-typical temperature and light schedule during fruit ripening periods. These field-typical temperatures were selected by taking the mean minimum and maximum temperatures from each Piedmont Research Station, Salisbury, NC (for blackberries) and Castle Hayne Research Station, Castle Hayne, NC (for blueberries) from research station weather data for the last 5 years. For each day by temperature by fruit type treatment, there were a total of 15 male and 15 female flies tested. All treatments by fruit type were set up on the same day with the same cohort of flies.

   A group of control flies was placed in empty vials immediately after feeding, frozen overnight, put into 96% ethanol, and held at −20°C until DNA extraction. Five vials totaling at least 15 live flies of each sex were removed from growth chambers each day for up to 7 days, frozen, and then moved to microcentrifuge tubes with 96% ethanol and held at −20°C until DNA extraction. Therefore, there were a total of 30 fruit by temperature by time treatments, 15 for blackberry, and 15 for blueberry.
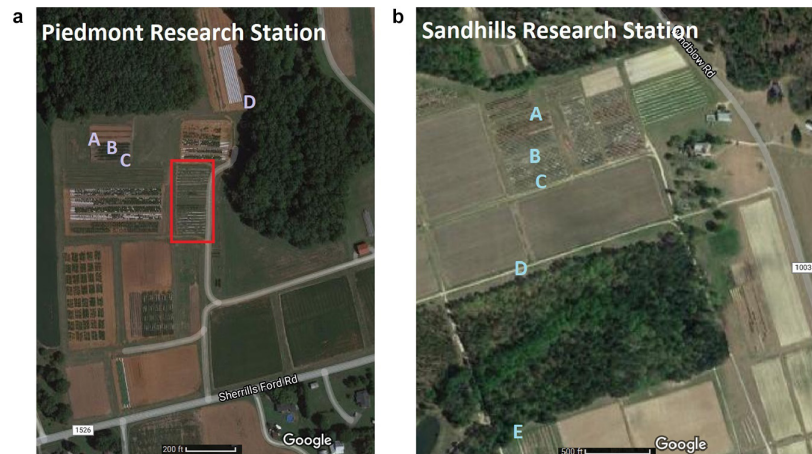
FIGURE 1 | (a) Piedmont Research Station blackberry plots. Traps A, B, and C, are at the edge, center, and edge, respectively, of a Von and Ouachita blackberry plot. Trap D represents the wooded edge. Note that in the outlined square are caneberry breeding plots with blackberry, raspberry, black raspberry, golden raspberry, and other caneberries present at the same time. Coordinates are 35°41′49″N 80°37′45″W (Google, 2021a). **(b)** Sandhills Research Station blueberry plots. Traps A, B, and C were placed in a rabbiteye blueberry germplasm plot. The early-season blueberry plots in the two fields to the right were no longer fruiting during this experiment. Trap D is on a near wooded edge, and E represents a distant wooded edge. Coordinates are 35°11′39″N 79°41′06″W (Google, 2021b).

Trap Fluid Experiment

Our objective for this assay was to determine the appropriate trap fluid to use in the field that would best maintain intact gut berry DNA. We starved adult *D. suzukii* for 72 h on a 5 g sucrose/400 mL water solution. Adult flies were then given a puree of either fresh, store-bought blackberries or blueberries, each with ∼4 g of sucrose, on which to feed for 6 h. To best mimic conditions similar to those in the field, the live adult *D. suzukii* were then immediately placed into small 5 mL plastic condiment containers with 4 mL of trap liquid. One container per trap fluid was set up per day for a total of seven containers; each container had at least 5 male and 5 female flies. The lid was immediately fitted, and the trap fluid was swirled to drown all flies.

We compared four trap fluids, including: (1) molecular grade ethylene glycol, (2) apple cider vinegar, (3) tap water with a drop of liquid detergent added, and (4) propylene glycol (Peak RV & Marine Antifreeze, Northbrooke, IL). Apple cider vinegar and water are commonly used in *D. suzukii* traps along with commercial lures (Lee et al., 2012; Frewin et al., 2017), and ethylene and propylene glycol are used as DNA preservatives in both field and laboratory settings.

Flies in trap fluid containers were then placed in an incubator with a temperature ramping program typical of field temperatures during ripening times for each blueberry and blackberry, since trap fluids would be used in the field exposed to fluctuating, high temperatures. The blueberry program started at 21.1°C for 8 h in dark, then gradual increased for 6 h, stayed at 30.9°C for 4 h, and then gradually decreased to 21.1°C for 6 h. The blackberry program followed the same cycle with a minimum of 19.2°C and maximum of 31.2°C. In a preliminary assay, we put raspberry-fed flies outside in July to subject them to typical summer UV and temperatures; we found no difference in feeding based on UV within flies held in the incubator or outside when using a pan-Rosaceae primer (see **Supplementary Material**).

Individual containers were removed daily for up to 7 days. Flies were removed from the liquid, then carefully placed in 1.5 mL microcentrifuge tubes containing 96% ethanol and held at –20°C until DNA extraction and qPCR.

Field Experiment

We sought to test the molecular gut content of wild-trapped *D. suzukii* adults from two different field locations. Field trapping experiments were conducted when fruit load in fields was at a peak from two research stations: Piedmont Research Station, Salisbury, NC for blackberries, and Sandhills Research Station, Jackson Springs, NC for blueberries. At Piedmont Research Station in a blackberry plot containing cvs. Von and Ouachita, adult flies were collected every 3 days from June 1, 2020 to July 27, 2020. At Sandhills Research Station in a blueberry germplasm field (with mostly rabbiteye blueberries ripe during the experiment), samples were collected from July 10, 2020 to July 28, 2020. Traps were created from liter-sized plastic food containers (Lee et al., 2012) filled with ∼250 mL of RV antifreeze with a Scentry *D. suzukii* lure hanging over the trap fluid (Great Lakes IPM, Vestaburg, MI, United States). Traps were hung at the height of 1 m on plastic stakes. To maximize the number of flies trapped, from 3 to 7 traps were placed at each location. Traps were checked every 3 days, and trapped *D. suzukii* were filtered from the fluid. Captured flies were then collected in microcentrifuge tubes with 96% ethanol and stored at –20°C until DNA extraction and qPCR.

We sought to collect flies under field conditions during peak fruit ripening along a transect, including two edges of cultivated fruit plantings, the center of the cultivated fruit plot, and a distant wooded field margin. In the case of Sandhills Research Station, a second wooded margin was also included (**Figures 1a,b**). Between 30 and 120 adult flies were trapped at each trap location.

DNA Extraction

Flies were removed from the –20°C freezer in their solution of 96% ethanol, gently held by the wing and rinsed in a 5% bleach solution, and then twice rinsed in distilled water as described in Diepenbrock et al. (2018). Flies were decapitated before DNA extraction to reduce interference from DNases in the head during DNA extraction. We used a DNeasy blood and tissue kit (catalog no. 69506, Qiagen Inc., Germantown, MD, United States[1]) for all DNA extractions.

DNA Amplification and Comparative C_T

Quantitative polymerase chain reaction (qPCR) reactions were performed in triplicate in 96-well plates (United States Scientific catalog no. 1402-9100) with blackberry, blueberry, or *D. suzukii* primers. Each well contained a total of 5 μl of iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, United States), 1 μl each of 5 μM forward and reverse primer, 2 μl of dd-H2O, and 1 μl of DNA template. Amplification was performed using an Applied Biosystems QuantStudio 6 Flex System in Fast mode preset for SYBR reagents in a comparative C_T experiment. The subsequent PCR products were then subjected to a melt curve analysis to confirm identity. Each plate contained 30 samples, followed by a positive control of a *D. suzukii* starved female fed known fruit and a negative control of dd-H2O instead of DNA template.

Data Analyses

All data was considered binomial based on whether an individual fly was positive or negative for berry DNA for the given primer. Treatments tested were temperature regime, sex, day, and whether the individual fly was positive or negative. No variables were considered random. Data were analyzed using SAS version 9.4 University Edition run on Oracle VM VirtualBox, and all data conformed to the necessary assumptions. The Proc Logistic command was used to run a logistic regression of all treatments except field-trapped flies. "Day" was considered a continuous variable for these analyses.

For the field transects, treatments included the trap location, sex, and whether an individual fly was positive or negative. No variables were considered random. Data were analyzed using a Proc Glimmix command to run a binomial regression of all treatments, adding the "dist = binomial" and "link = logit" to our model statement.
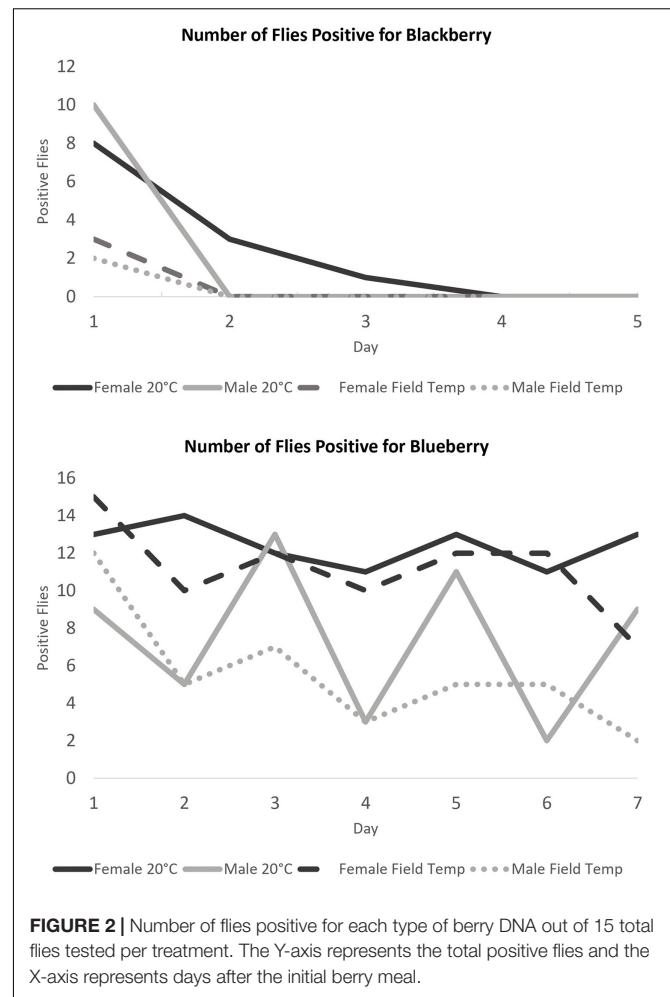
RESULTS

Data herein are presented in log-odds. Log-odds is the logarithm of the odds of success. It is a way of looking at the probability of success over the probability of failure of a binomial dataset.

Larvae Raised in Fruit and Pupal Meconium

No blackberry DNA was present in a total of 30 newly emerged adults who fed on blackberry as larvae. However, out of 30

FIGURE 2 | Number of flies positive for each type of berry DNA out of 15 total flies tested per treatment. The Y-axis represents the total positive flies and the X-axis represents days after the initial berry meal.

adults that fed on blueberry as larvae, 11 flies tested positive with no difference by sex (7 females and 4 males, logistic regression $\chi^2 = 1.2665$, DF = 1, $P = 0.2604$).

After this observation, we set out to determine if pupal meconium was a potential source of the berry DNA within newly emerged fly guts. We found that 25 out of 30 pupal cases from larvae which fed on blackberries were positive for blackberry DNA and 30 out of 30 pupal cases were positive from larvae that fed on blueberries. In the follow-up study where rinsed pupae were subjected to 15 min of UV light to degrade any remaining DNA on the outside of the pupal case, 16 out of 30 pupal cases from larvae fed on blackberries tested positive, while 30 out of 30 pupal cases from larvae that fed on blueberries tested positive.

Blackberry Detectability Over Time

No blackberry DNA was detected after Day 4 in 15 total flies tested for each treatment. In fact, blackberry was detectible in most *D. suzukii* only on Day 1 and not afterward (**Figure 2**). Only females held at 20°C contained detectible blackberry for up to 4 days. We also found there were more males who tested positive for feeding on Day 1 than Day 0 (10 females, 2 males were positive).

All variables (temperature program, sex, and day) were statistically significant via logistic regression. Day (Wald Chi-Square or WCS = 32.9) had a much stronger effect on whether a fly had detectible blackberry in its gut than temperature program (WCS = 6.9) and sex (WCS = 4.9) (**Table 1**).

For a one unit increase in day, the log-odds of having a fly positive for blackberry DNA decreased by a factor of 1.2. The log-odds of a female fly being positive for blackberry DNA increased by a factor of 0.48, and flies held at the cooler 20°C temperature program had increased log-odds of being positive for blackberry DNA by a factor of 0.7 (**Table 2**).

Due to the low number of positive flies in this assay (out of 15 total flies tested), we were unable to make predictions over time at field temperatures, which was our original objective for this assay (χ^2 = 0.2631, DF = 4, P = 0.9921). Despite that, our raw data showed that no flies had detectible DNA after the first day held at field temperature.

Blueberry Detectability Over Time

In stark contrast to the blackberry primer results, blueberry DNA was detectible for a proportion of flies regardless of temperature program or sex for up to 7 days. Detection in males was more variable than in females (**Figure 2**).

In the logistic regression, all variables were statistically significant (temperature program p = 0.0067, sex p < 0.0001, day p < 0.0001). With the blueberry primer, unlike the blackberry primer, the variable resulting in the largest effect size was sex (WCS = 55.3) followed by day (WCS = 18.8) with a smaller effect size due to the temperature program (WCS = 7.4) (**Table 3**).

For each additional day after feeding, a fly decreased its log-odds of being positive by a factor of 0.22. Flies held at the cooler 20°C temperature increased their log-odds of having a detectible blueberry meal by a factor of 0.3. In the case of female flies, the log-odds of having a positive blueberry DNA test increased by a factor of 0.84 (**Table 4**).

For flies held at field temperatures only, the log-odds of a fly being positive on day 1 versus day 7 were increased by a factor of 34.762 (95% CI 7.53-160.407) (χ^2 = 43.2291, DF = 7, P < 0.0001). No other day-to-day comparisons were statistically significant.

Trap Fluid
Blackberry Primers

All variables tested (trap fluid, day, and sex) were statistically significant via logistic regression. Day (Wald Chi-Square or WCS = 15.0) had a more significant effect size on whether a fly had a detectible blackberry meal in its gut than trap fluid (WCS = 10.7) followed by sex (WCS = 7.7; **Table 5**).

A fly fed on blackberry and held in tap water with soap had a log-odds decrease of detectible DNA of 1.1 units compared to a fly held in RV antifreeze. Other comparisons were not statistically significant. Flies tested on day 3 had increased log-odds of being positive by a factor of 1.7. Despite not being consistently significant, there is a small trend toward having increased log-odds of detecting blackberry DNA within a fly within the first 3 days with a slight decrease in the estimate for all later days. The log-odds of a female fly being positive for blackberry DNA increased by a factor of 0.6 (**Table 6**).

Blueberry Primers

Of the three variables tested, only trap fluid was statistically significant via logistic regression for the blueberry primer. The

TABLE 3 | Type 3 analysis of effects for blueberry detectability over time.

Effect	DF	Wald Chi-square	Pr > ChiSq
Temp Program	1	7.3646	0.0067
Sex	1	55.2968	<0.0001
Day	1	18.8052	<0.0001

χ^2 = 82.0530, DF = 9, P < 0.0001.

TABLE 4 | Analysis of maximum likelihood estimates for blueberry detectability over time.

Parameter		DF	Estimate	Standard error	Wald Chi-square	Pr > ChiSq
Intercept		1	1.4726	0.2354	39.1273	<0.0001
Temp Program	20°C	1	0.2953	0.1088	7.3646	0.0067
Sex	f	1	0.8358	0.1124	55.2968	<0.0001
Day		1	−0.2245	0.0518	18.8052	<0.0001

χ^2 = 82.0530, DF = 9, P < 0.0001.

TABLE 1 | Type 3 analysis of effects for blackberry detectability over time.

Effect	DF	Wald Chi-square	Pr > ChiSq
Temp Program	1	6.8909	0.0087
Sex	1	4.9125	0.0267
Day	1	32.9063	<0.0001

χ^2 = 35.8002, DF = 7, P < 0.0001.

TABLE 2 | Analysis of maximum likelihood estimates for blackberry detectability over time.

Parameter		DF	Estimate	Standard error	Wald Chi-square	Pr > ChiSq
Intercept		1	−0.2688	0.3685	0.5322	0.4657
Temp Program	20°C	1	0.7008	0.2670	6.8909	0.0087
Sex	f	1	0.4784	0.2159	4.9125	0.0267
Day		1	−1.1808	0.2058	32.9063	<0.0001

χ^2 = 35.8002, DF = 7, P < 0.0001.

TABLE 5 | Type 3 Analysis of Effects of Blackberry Fed Flies in Trap Fluid.

Effect	DF	Wald Chi-square	Pr > ChiSq
Fluid	3	10.7383	0.0132*
Day	6	15.0356	0.0200*
Sex	1	7.7328	0.0054*

χ^2 = 39.7339, DF = 10, P < 0.0001, * represents statistically significant data at an alpha value of p < 0.05.

effect size for trap fluid was moderate (WCS = 8.3; **Table 7**). A blueberry-fed fly stored in tap water with soap had a decrease in its DNA detectability based on log-odds by a factor of 0.6 compared to RV antifreeze which may be due to bloating. When running the experiment, some of the flies held only in the water and soap solution became so bloated with water that they would pop at the slightest touch. None of the comparisons for day or sex were statistically significant (**Table 8**).

Based on these data, we used RV antifreeze as the trap fluid for the field-experiment and trapped flies every 2–3 days.

Field Tests

There was no statistically significant difference among trap locations nor sex trapped at the Piedmont Research station (**Table 9**). In our testing, between 0 and 11% of flies, by sex, tested positive for blackberry DNA (**Table 10**).

There was no difference between the different trap locations tested nor between fly sex at Sandhills Research Station (**Table 11**). Between 0 and 15% of flies tested by sex were positive for blueberry DNA (**Table 12**).

TABLE 6 | Analysis of maximum likelihood estimates of blackberry fed flies in trap fluid.

Parameter		DF	Estimate	Standard error	Wald Chi-square	Pr > ChiSq
Intercept		1	2.3294	0.2737	72.4416	<0.0001
Fluid	ACV	1	0.0344	0.3511	0.0096	0.9219
Fluid	EG	1	0.4343	0.4481	0.9390	0.3325
Fluid	H2O	1	−1.0806	0.3352	10.3952	0.0013*
Day	1	1	0.9792	0.6770	2.0924	0.1480
Day	2	1	0.5220	0.5813	0.8063	0.3692
Day	3	1	1.7240	0.9040	3.6372	0.0565*
Day	4	1	−0.5347	0.4478	1.4258	0.2325
Day	5	1	−1.2972	0.4596	7.9656	0.0048*
Day	6	1	−0.8926	0.4233	4.4465	0.0350*
Sex	f	1	0.6041	0.2172	7.7328	0.0054*

"ACV" represents apple cider vinegar, "EG" represents ethylene glycol, "H$_2$O" represents the tap water and soap fluid, and "RV" represents the propylene glycol antifreeze.
All fluid types are being compared to RV antifreeze; all days compared to 7; sex compared to male.
*χ^2 = 39.7339, DF = 10, P < 0.0001, * represents statistically significant data at an alpha value of p < 0.05.*

TABLE 7 | Type 3 analysis of effects of blueberry-fed flies in trap fluid.

Effect	DF	Wald Chi-square	Pr > ChiSq
Fluid	3	8.2705	0.0407*
Day	6	9.2968	0.1576
Sex	1	0.5058	0.4770

"ACV" represents apple cider vinegar; "EG" represents ethylene glycol; "H$_2$O" represents the tap water and soap; and "RV" represents the propylene glycol.
All fluid types are being compared to RV fluid; all days compared to 7; sex compared to male.
*χ^2 = 19.8150, DF = 10, P = 0.0311, * represents statistically significant data at an alpha value of p < 0.05.*

TABLE 8 | Analysis of maximum likelihood estimates of blueberry-fed flies in trap fluid.

Parameter		DF	Estimate	Standard error	Wald Chi-square	Pr > ChiSq
Intercept		1	1.9761	0.2096	88.9294	<0.0001
Fluid	ACV	1	−0.5003	0.3000	2.7810	0.0954
Fluid	EG	1	0.3535	0.3438	1.0571	0.3039
Fluid	H2O	1	−0.5846	0.2860	4.1786	0.0409*
Day	1	1	0.6695	0.5496	1.4836	0.2232
Day	2	1	−0.3208	0.4103	0.6116	0.4342
Day	3	1	−0.4925	0.3954	1.5520	0.2128
Day	4	1	0.3474	0.4930	0.4966	0.4810
Day	5	1	0.6397	0.6655	0.9239	0.3364
Day	6	1	0.0890	0.4559	0.0381	0.8453
Sex	f	1	0.1272	0.1789	0.5058	0.4770

*χ^2 = 19.8150, DF = 10, P = 0.0311, * represents statistically significant data at an alpha value of p < 0.05.*

TABLE 9 | Type 3 analysis of effects of flies from Piedmont Research Station blackberry plot.

Effect	Num DF	Den DF	F value	Pr > F
Trap	3	411	0.73	0.5371
Sex	1	411	0.00	0.9830
Trap × Sex	3	411	0.40	0.7557

TABLE 10 | Total positive flies out of flies tested at Piedmont Research Station blackberry plot by trap location.

	Positive females/Tested females	Positive males/Tested males
Trap PDT-A	5/60	1/60
Trap PDT-B	5/45	3/45
Trap PDT-C	2/43	2/46
Trap PDT-D	0/60	2/60

TABLE 11 | Type 3 analysis of effects of flies from Sandhills Research Station blueberry plot.

Effect	Num DF	Den DF	F value	Pr > F
Trap	4	437	0.17	0.9554
Sex	1	437	0.00	0.9752
Trap × Sex	4	437	0.19	0.9414

DISCUSSION

Laboratory-Raised Flies

The 20°C treatments for both blackberry and blueberry detectability over time assays had different results. Time had the most substantial effect on whether a fly would test positive for blackberry DNA after a known feeding event. Both sex and temperature also had an effect on the log-odds of testing positive for blackberry DNA but were smaller in effect size. Additionally, there was no detectable blackberry DNA in most flies after Day

TABLE 12 | Total positive flies out of total flies tested at Sandhills Research Station blueberry plot by trap location.

	Positive females/Tested females	Positive males/Tested males
Trap SH-A	3/60	1/60
Trap SH-B	3/20	0/10
Trap SH-C	1/33	1/24
Trap SH-D	2/60	1/60
Trap SH-E	1/60	1/60

1 for both temperature treatments. This could be because the adult flies starved and given blackberry DNA ate a smaller food meal than those for the blueberry experiment. The blackberry puree with ~4 g of added sugar may have been less sweet than the blueberry puree with ~4 g of sugar, since blackberries have fewer sugars than blueberries (Mikulic-Petkovsek et al., 2012; de Souza et al., 2014). It is also possible that a blackberry fruit meal was more easily digestible and was broken down faster than the blueberry fruit meal. In this study, there was no way to test the berry meal volume taken by each fly. Further research feeding individual *D. suzukii* different volumes of fruit could help elucidate answers to this question.

Alternatively, decreased detection of blackberry fruit meal over time compared to blueberry could be due to the nature of the primer region selected to test for blackberry DNA. Because both of the designed primers come from different regions within the whole plant genome, and because they are different lengths with various sequence complexity they could have different melt curve profiles; therefore, they cannot be directly compared. Additionally, since the blueberry primer comes from chloroplast DNA versus the blackberry primer from nuclear DNA, there are more copies of chloroplast DNA in a sample (Daniell et al., 2016). Therefore, there is an increased amount of blueberry DNA present within each sample which is likely adding to its increased detection time, even in non-green tissues like fruit which have low chloroplast protein synthesis but still contain plastids in the chromoplast (Daniell et al., 2016). It is possible that larval nutrition within blackberry was significantly better than within blueberry, resulting in less hungry newly emerged adults who did not need to immediately feed on pupal meconium (Hardin et al., 2015; Young et al., 2018; Olazcuaga et al., 2019). It has been shown that preferred hosts (blackberry) may result in heavier pupal weight, and that adult flies may preferentially choose a preferred host, like blackberry, if their natal host was an inferior host (i.e., pokeweed, *Phytolacca americana* L.) (Diepenbrock et al., 2016); thus, there is evidence that larval host influences adult behavior.

Blueberry DNA was detected for longer durations than blackberry, but detection was more variable within males than females. The dependence of male mating metrics on adult nutrition is species-specific (Aluja et al., 2001). It is possible that males within *D. suzukii* are less dependent on regular adult nutrition than females, perhaps because sperm is less energetically costly than developing eggs. An alternate hypothesis may be that males require less nutrition because they have less locomotor function than females and require less energy for

movement (Ferguson et al., 2015). One study found that adult females of *D. suzukii* are four times more active within a semi-natural field environment than males, which may be specific to females' mated status based on follow-up laboratory experiments (Ferguson et al., 2015). Starved adults of *D. melanogaster* typically ingest a larger than typical food meal than flies fed *ad libitum* (Bowdan and Dethier, 1986; Edgecomb et al., 1994). It is possible that the relatively short feeding period (6 h) may have served to better capture female feeding behavior and that males may take smaller meals more frequently. Together, reduced male activity and differential feeding behavior may impact the predictions made when testing these methods in a field setting.

Because newly emerged adults that fed on blueberry as larvae have detectable fruit DNA within their guts, it will be important to incorporate that understanding of fly biology into the interpretation of field data. Because pupal meconium may be a source of this berry DNA, this study provides evidence that adult flies are feeding on pupal meconium following eclosion, which has long been suspected as a means of acquiring symbiotic bacteria (Leach, 1934; Moll et al., 2001; Broderick and Lemaitre, 2012).

Our study of *D. suzukii* molecular gut content detectability over time provides important evidence that adult resource use within *D. suzukii* is species-specific and potentially substrate-specific even within fruit types. Additionally, it provides the first evidence that adult *D. suzukii* use pupal meconium as a nutritional resource after eclosion.

Field-Trapped Flies

The difference in detectable DNA by sex seen in the blackberry primers may be an artifact of variability in fly feeding behavior, particularly because it is only seen in the blackberry primers and not the blueberry primers, as discussed in the laboratory assay above. Because they were held at different temperatures, the trap fluid samples from each fruit type cannot be directly compared to each other statistically. Based on previous research by our group, the blackberry primers detect a fruit meal for flies that fed on blackberry from 1 to 4 days after feeding, with detection dropping precipitously after the first 24 h. The blueberry primers were able to detect a blueberry meal for 7 or more days. Our hypothesis that there would be a higher proportion of flies that had recently fed on either berry type within the crop was rejected. Similar proportions of flies having fed on fruit were found at in-crop, edge-of-crop, and woodline locations. This adds to the body of evidence that within a crop field and its surrounding suitable habitat, adult *D. suzukii* are likely moving short distances and are evenly dispersed (Klick et al., 2016; Leach et al., 2019).

Our trapping method may have selected for flies that had not been fruit fed. Previous studies have showed that traps such as those we used attract more female flies who have not yet found ovipositional substrate, more unmated flies than mated, and—importantly for our study—more starved flies than fed (Swoboda-Bhattarai et al., 2017; Wong et al., 2018).

We found a low proportion of adult feeding in individuals assessed with either set of primers. For the blackberry primers, the range of proportion of feeding was 0 to 11% of all flies. Briem et al. (2018) found that 38% of their trapped flies had fed

on raspberry from their raspberry crop, though half of their samples were not surface-sterilized. Alternatively, the NGS method used by their group may be more sensitive and detect lower DNA levels than the qPCR methods used here (Briem et al., 2018, Chapter 2).

For flies feeding on blueberries, the proportions may have been low because blueberries are a less preferred ovipositional substrate compared to caneberries (Lee et al., 2011; Burrack et al., 2013; Kinjo et al., 2013). Blueberries may also be a less attractive adult food source for related reasons. The thick skin and firmer fruit of the blueberry require greater penetration force, which is why it is less appealing for oviposition (Lee et al., 2011; Burrack et al., 2013; Kinjo et al., 2013). This may result in fewer opportunities for adults to feed due to fewer ovipositional wounds. That said, the blueberry bushes in the breeding plots at Sandhills Research Station that were evaluated in this study had many fruit clusters with large feeding wounds created by paper wasps (*Polistes* sp.), honeybees (*Apis mellifera*), and/or Japanese beetles (*Popilia japonica*). There were also opportunities for *D. suzukii* to feed on punctured blueberries on the ground.

It is impossible to truly compare the blackberry-feeding flies to the blueberry-feeding flies because they were separated geographically, living under different weather conditions, and tested using different primers. It stands to reason that the blueberry-feeding flies, if all things were the same, would have detectible blueberry in their guts at least twice as often as blackberry-feeding flies simply because the blueberry primers can be detected for more than 7 days compared to less than 4 days in blackberry. Furthermore, since female flies are up to four times more active than male flies (Ferguson et al., 2015), we would have expected to see a difference in fruit feeding by sex, yet we did not. Additionally, *D. suzukii* that fed on blossoms, fruit, or laboratory-based diet fly longer and farther than starved flies (Wong et al., 2018). Altogether, the low proportion of fruit feeding, inconsistent with past data on feeding behavior, may instead point to a different narrative. Perhaps adult *D. suzukii* are more dependent on a different nutritional resource than berry juice in the field or are less dependent on nutritional resources as adults than we hypothesized. Judging by the high energetic cost of mating and finding ovipositional substrate, as well as the clues from this study and others that flies are moving up to 120 m within a few days (Klick et al., 2016; Leach et al., 2019), fruit may be a less-important nutritional resource for adults than some other substances. Mushrooms and bird manure have both been suggested as alternative host resources for oviposition

(Stockton et al., 2019). Clearly, fruit feeding provides a small but meaningful proportion of *D. suzukii* with nutritional resources, but they may depend more heavily on other nutritional resources than fruit juice alone.

The results of this research support past findings that *D. suzukii* are regularly moving between crop and edge of crop locations at regular time intervals. Adult *D. suzukii* may depend more heavily on other nutritional resources than berry juice. This research contributes to a growing body of evidence that elucidating adult resource use in higher flies is crucial to understanding the biology of these and other polyphagous invasive pest species. Studying Brachycera feeding behavior in the wild has been difficult due to the high motility of adult flies and a lack of funding, so using pest species to further develop our knowledge of Dipteran biology can fill in gaps in our understanding of this diverse group of insects.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI (accession: FF683693.1 and MH551878).

AUTHOR CONTRIBUTIONS

LK conceived the research, conducted the experiments and statistical analyses, analyzed the data, and wrote the manuscript. LD, TS, and HB assisted with experimental design and research. LK, RA, HA, and GF contributed material. HB secured funding. All authors reviewed, read, and approved the manuscript.

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