JOURNAL OF THE AMERICAN WATER RESOURCES ASSOCIATION
Vol. 50, No. 2 AMERICAN WATER RESOURCES ASSOCIATION April 2014

ASSESSING THE POTENTIAL EFFECTS OF FUNGICIDES ON NONTARGET GUT FUNGI (TRICHOMYCETES) AND THEIR ASSOCIATED LARVAL BLACK FLY HOSTS[1]

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ABSTRACT: Fungicides are moderately hydrophobic and have been detected in water and sediment, particularly in agricultural watersheds, but typically are not included in routine water quality monitoring efforts. This is despite their widespread use and frequent application to combat fungal pathogens. Although the efficacy of these compounds on fungal pathogens is well documented, little is known about their effects on nontarget fungi. This pilot study, a field survey in southwestern Idaho from April to December 2010 on four streams with varying pesticide inputs (two agricultural and two reference sites), was conducted to assess nontarget impact of fungicides on gut fungi, or trichomycetes. Tissues of larval black flies (Diptera: Simuliidae), hosts of gut fungi, were analyzed for pesticide accumulation. Fungicides were detected in hosts from streams within agricultural watersheds but were not detected in hosts from reference streams. Gut fungi from agricultural sites exhibited decreased percent infestation, density and sporulation within the gut, and black fly tissues had elevated pesticide concentrations. Differences observed between the sites demonstrate a potential effect on this symbiotic system. Future research is needed to parse out the details of the complex biotic and abiotic relationships; however, these preliminary results indicate that impacts to nontarget organisms could have far-reaching consequences within aquatic ecosystems.

(KEY TERMS: agriculture; aquatic fungi; black fly; fungicides; nontarget.)

Wilson, Emma R., Kelly L. Smalling, Timothy J. Reilly, Elmer Gray, Laura Bond, Lance Steele, Prasanna Kandel, Alison Chamberlin, Justin Gause, Nicole Reynolds, Ian Robertson, Stephen Novak, Kevin Feris, and Merlin M. White, 2014. Assessing the Potential Effects of Fungicides on Nontarget Gut Fungi (Trichomycetes) and Their Associated Larval Black Fly Hosts. *Journal of the American Water Resources Association* (JAWRA) 50(2): 420-433. DOI: 10.1111/jawr.12166

INTRODUCTION

Modern agricultural practices throughout the world rely on the use of pesticides to combat the effects of an array of pathogens on economically important crops. Inevitably, some of these pesticides are transported to nearby streams via drift, runoff, and infiltration into the groundwater (Relyea and Hoverman, 2006; Rasmussen *et al.*, 2012; Reilly *et al.*, 2012a). In addition, many agricultural areas require field side streams for irrigation purposes,

[1]Paper No. JAWRA-13-0056-P of the *Journal of the American Water Resources Association* (JAWRA). Received March 3, 2013; accepted November 26, 2013. **Discussions are open until six months from print publication**.

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making them susceptible to contamination by pesticides, which are often found at high concentrations (Gilliom, 2007). Indeed, pesticide application to agricultural fields and resulting runoff may be one of the greatest stressors to aquatic ecosystems, with entire communities inadvertently exposed to pesticides (Kolpin *et al.*, 2002; Relyea and Hoverman, 2006; Rasmussen *et al.*, 2012).

Pesticides, at both acute and chronic levels, can impact nontarget organisms, including arthropods. Stream macroinvertebrates can be negatively affected by decreased population densities (Hurd *et al.*, 1996) when exposed to nonpoint source pesticide pollution. Sorption and accumulation of pesticides have been documented in macroinvertebrates, including in the silk of black fly larvae (Brereton *et al.*, 1999). Effects of pesticides vary depending on whether they are acting singularly or interactively (Elskus, 2012). For instance, in the presence of a pyrethroid insecticide, fungicide toxicity to the aquatic crustacean *Daphnia magna* is significantly increased (Norgaard and Cedergreen, 2010).

Fungicides, a group of pesticides that target fungal pathogens, are of particular concern because of the increased tendency for their reapplication during the growing season — in some cases, as much as 10 times per season (Reilly *et al.*, 2012a). In agricultural areas in the United States (U.S.) and Europe, fungicides have been detected in surface and groundwater, sediments, air, and rainfall (Geissen *et al.*, 2010; Schummer *et al.*, 2010; Battaglin *et al.*, 2011; Smalling and Orlando, 2011; Smalling *et al.*, 2013) at concentrations that have the potential to cause adverse effects on a range of aquatic organisms (Battaglin and Fairchild, 2002; Gilliom, 2007; Deb *et al.*, 2010). The modes of action of fungicides are varied and may be detrimental to nontarget organisms, such as macroinvertebrates (Gustafsson *et al.*, 2010; Elskus, 2012). Thus, there has been an increased awareness, interest, and concern about how fungicides may be affecting nonpathogenic fungi (Maltby *et al.*, 2009; Dijksterhuis *et al.*, 2011). Although several studies have focused on aquatic leaf decomposing fungi (Cuppen *et al.*, 2000; Maltby *et al.*, 2009; Bundschuh *et al.*, 2011; Dijksterhuis *et al.*, 2011; Zubrod *et al.*, 2011; Rasmussen *et al.*, 2012), trichomycetes, or gut fungi, are a widespread group of fungi that have not yet been studied in this regard.

Gut fungi are a cosmopolitan group of symbiotic arthropod associates. As endosymbionts, they live in the digestive tracts of many aquatic macroinvertebrates, including immature stages of aquatic insects such as mayflies (Ephemeroptera), stoneflies (Plecoptera), and black flies (Diptera). The relationship between gut fungus and host is thought to shift depending on environmental conditions (McCreadie *et al.*, 2011). Studies have shown their mutualistic potential (Horn and Lichtwardt, 1981) and demonstrated parasitism (Sweeney, 1981), although the fungi are more generally regarded as commensalistic (Lichtwardt, 1986). Whether their role is positive, negative, or neutral, the relationship between the gut fungi and their arthropod host reflects the adaptive responses of the symbiotic partners, both on short- and long-term evolutionary time scales (White, 2006; Hibbett *et al.*, 2007; McCreadie *et al.*, 2011). Beyond being excellent hosts of gut fungi, black fly larvae play important roles in stream ecosystems. They are ecosystem engineers that not only turn over resources in food webs but also serve as a dominant food source for fish and other predators (Wallace and Webster, 1996).

The primary goal of this first assessment was to evaluate the seasonal impact of exposure to broad-spectrum fungicides and other pesticides on gut fungi and their aquatic insect hosts (black fly larvae). The specific objectives of the study were to (1) survey surface waters known to contain fungicides to determine percent infestation and density of gut fungi of black fly larvae residing in those sites and to assess dissolved concentrations of fungicides in the water column, (2) measure fungicide and other pesticide concentrations in black fly host tissue, and (3) compare these metrics with those collected from reference streams. Larval black flies from agriculturally dominated streams containing fungicides were predicted to have a lower percent infestation and density of gut fungi compared to hosts from reference streams.

METHODS

Site Descriptions and Sample Collection

The four selected study sites in Idaho (Figure 1, Table 1) included two agricultural streams (Sand Run Gulch and Wanstad Ditch) and two reference streams (Cottonwood Creek and Dry Creek). The former were selected because they drain high use agricultural areas (Table 1) and fungicides have been detected in each stream throughout the growing season (Reilly *et al.*, 2012a, b). The latter were selected because they are known to contain insect larvae with robust and well-characterized populations of gut fungi (Bench and White, 2012; Kandel and White, 2012). The reference locations were also chosen based on proximity, accessibility, and, as much as possible, similarity to each other in terms of riparian vegetation and streambed substrate. The agricultural streams were also sampled prior to the start of the survey to ensure there were appropriate hosts for gut fungi.

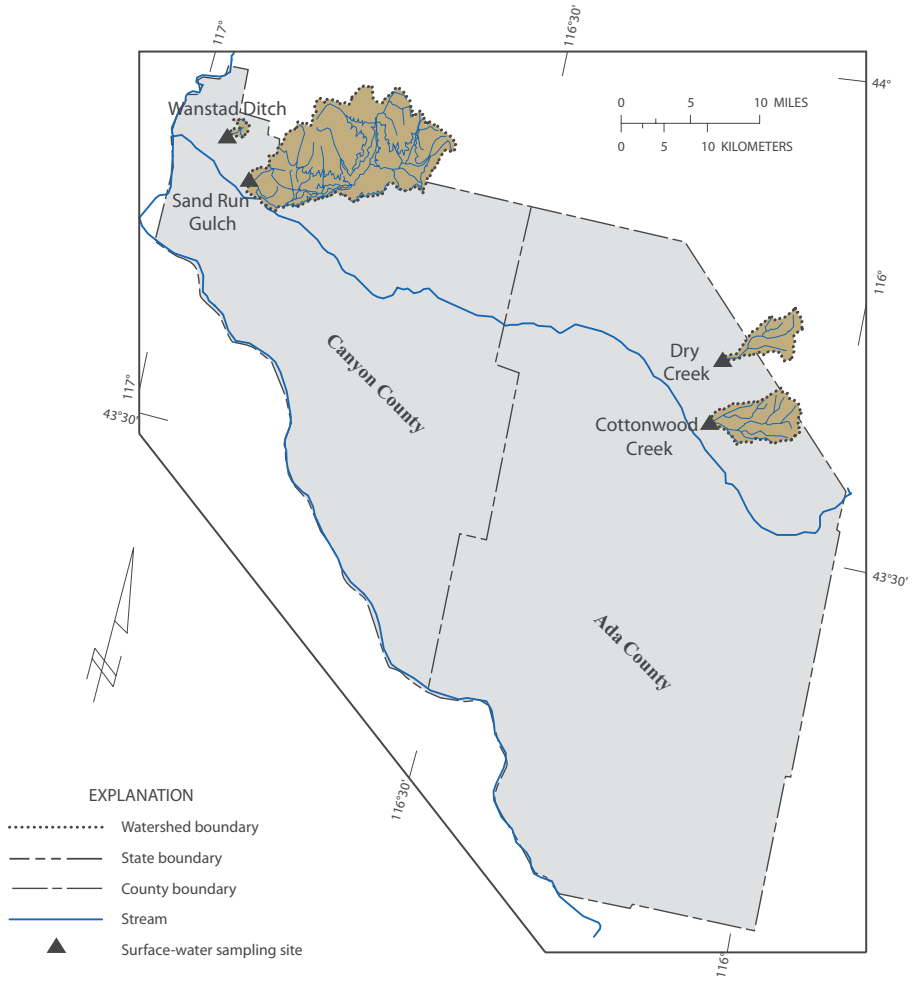

FIGURE 1. Map of Study Area Depicting the Four Surface Water Sampling Sites. Reference sites: Cottonwood Creek and Dry Creek. Agricultural sites: Sand Run Gulch and Wanstad Ditch. For watershed characteristics refer to Table 1 (Reilly *et al.*, 2012b).

TABLE 1. Description of the Four Surface Water Sampling Sites and Associated Watershed Characteristics. Reference sites: Cottonwood Creek and Dry Creek. Agricultural sites: Sand Run Gulch and Wanstad Ditch (Reilly *et al.*, 2012b).

Sampling Site	Latitude	Longitude	Watershed Area (acres)	Percent of Watershed in Agriculture	Dominant Crops (percentage of agricultural acreage)
Cottonwood Creek	43.61972	−116.18525	10,613	0.1	NA
Dry Creek	43.66830	−116.18906	6,171	<0.1	NA
Sand Run Gulch	43.76405	−116.91210	50,877	37.1	Alfalfa (25.0), corn (15.9), winter wheat (14.3)
Wanstad Ditch	43.80303	−116.95663	760	90.5	Winter wheat (27.5), hay (18.1), corn (17.7)

Note: NA, not applicable.

To assess seasonal differences in pesticide concentrations and the density of gut fungi over time, 12 water samples were collected from each of the agricultural streams, and 10 and 11 samples were collected from Dry Creek and Cottonwood Creek, respectively, between April and December of 2010. Samples were collected approximately biweekly starting in April for the two agricultural streams and Cottonwood Creek and starting in June for Dry Creek. This spans the typical growing season in Idaho (Reilly *et al.*, 2012a, b). Sample collection did not target runoff events or other specific hydrologic conditions.

Water quality sampling followed the same procedures at all sites. Samples were collected by immersing precleaned, amber glass bottles (1 l for pesticides and 125 ml for dissolved organic carbon) once at each site to a depth of not less than 0.1 m below the water surface. A 1 l polyurethane bottle was rinsed three

times with stream water, and then filled for determination of basic water quality characteristics (specific conductance in microseimens per centimeter at 25°C [μS/cm], pH in standard units, and turbidity in nephelometric turbidity units). Samples were then packed on ice and shipped overnight from Idaho to the U.S. Geological Survey Organic Chemistry Laboratory in Sacramento, California, for extraction and analysis. Site descriptions, watershed characteristics, and water sampling techniques are further detailed in Reilly *et al.* (2012b).

Host Sampling and Dissection

During each sampling event, a 10-20 m long stream section was sampled for immature stages of black flies using kick nets and/or methanol-cleaned forceps to pick specimens from dangling vegetation and rocks within riffle zones. A minimum of 15-20 black fly larvae were targeted for assessment of gut fungi and tissue pesticide analysis. These were transported in stream water held on ice to Boise State University and stored at 4°C for up to 48 h prior to dissection. If 15-20 hosts were not available within 90 min of searching, all collected insects were used exclusively for fungal metrics. When greater than 20 individuals were recovered in the field, additional subsamples were placed in glass vials using methanol-cleaned forceps and stored at −20°C prior to extraction and analysis of tissue for currently used pesticides.

Hosts were dissected and gut fungi prepared per Bench and White (2012). Larval black fly midguts were placed on fresh slides for fungal identification and enumeration. If hindgut fungi were present, they were similarly prepared on a fresh slide and, in either case, fixed and stained in lactophenol cotton blue to later identify the gut fungi to genus. Percent infestation (number of hosts containing gut fungi divided by the number in the sample [Beard and Adler, 2002; Nelder *et al.*, 2005]) was calculated for each sampling occasion.

Fungal Density and Sporulation in Larval Black Fly Midguts

Digital images of dissected, slide-mounted larval black fly peritrophic matrices (the chitinous lining of the midgut, referred to hereafter as PMs) were used to assess the density of thalli (individual fungal hyphae) and trichospores (asexual spores) of *Harpella* spp. (Figure S1). Before fixation in lactophenol cotton blue, the PM, freshly mounted in distilled water, was viewed and digital images were captured according to Bench and White (2012). Thalli and trichospores were counted directly. Previous studies (Beard and Adler, 2002; McCreadie and Beard, 2003) have used grids and ratios to estimate the amount of fungi within the gut. In this case, an exact count was possible by focusing on and enumerating the holdfasts (the structure that attaches the fungus to the PM), even when unbranched but overlapping thalli were present as dense masses in some of the PMs. The density of thalli and spores in the PM was normalized for gut size by counting the number of thalli and spores within the gut and dividing it by the PM area to give the number of thalli or spores per μm^2.

Extraction and Analysis of Surface Water and Tissue for Pesticides

Surface water samples (1 l) were filtered using 0.7 μm glass fiber filters, extracted onto Oasis hydrophilic-lipophilic balance (HLB) solid phase extraction cartridges, dried, eluted with ethyl acetate, reduced, and analyzed for a suite of 90 pesticides by gas chromatography-mass spectrometry operating in electron ionization mode (GC-EIMS) (Hladik *et al.*, 2008; Reilly *et al.*, 2012b). Information on GC-EIMS settings, quality assurance (QA) parameters, and method detection limits are outlined in the Supporting Information (SI).

Thawed composite black fly larvae samples (0.12-2.5 g), containing between 70 and 1,500 individuals from each site, were analyzed for a suite of 12 current-use pesticides. In total, 17 composite larval tissue samples were analyzed based on the compounds historically detected in the water column (Reilly *et al.*, 2012b). Prior to extraction, tissue samples were spiked with trifluralin-d_{10}, ring-^{13}C-*p,p′*-DDE and phenoxy-^{13}C-*cis*-permethrin (Cambridge Isotopes, Andover, Massachusetts) as recovery surrogates. All samples were homogenized with Na_2SO_4 and extracted two times with dichloromethane using a sonic water bath at 30°C for 25 min. Ten percent by volume of each raw extract was allowed to evaporate to a constant weight in a fume hood for gravimetric lipid determination to the nearest 0.001 g using a microbalance. Due to the small sample mass and nondetectable amounts of lipid, no cleanup was necessary. Samples were exchanged to ethyl acetate and further reduced to 200 μl, and an internal standard was added to each sample prior to analysis.

Chromatographic analyses were performed using an Agilent 7890 gas chromatograph coupled to an Agilent 7000 triple quadrupole mass spectrometer (Agilent Technologies, Folsom, California) operating in multiple reaction monitoring mode (MRM). Details of MRM transition, collision energy, and limits of detection are described in Table S1 (see SI), where a complete description of the method, along with more

information on QA parameters and body burden calculations, are also outlined.

Statistical Analysis

Percent infestation was calculated as the number of black flies whose guts contained thalli relative to the total number of black flies in each sample, by site and collection date. To compare thalli and spore density at each site, a mixed linear model on the log transformed data (observed density plus an insubstantial number to allow a log transform when no thalli or spores were found) was employed. These models were used to compare paired sampling dates between Sand Run Gulch and each of the reference sites, and to compare change over sampling dates within each location. Each set of comparisons (for thalli and spore densities) was adjusted for multiple corrections using a Bonferroni correction. The final model included fixed effects for site, date, and their interaction, and separate residuals for each site/date combination to accommodate unequal variance in the observations. Initially, more complex covariance structures were considered to account for within-site correlation and within-sampling date correlation, but these models either did not converge or, if they did, would not allow the specific comparisons needed. In addition, any models considered with these estimated correlations had higher Akaike's Information Criterion (adjusted for small sample size, Burnham and Anderson, 2002) and poor fit based on residual plots; hence, the simpler model was used. Wanstad Ditch was not included in either model due to insufficient or empty (devoid of gut fungi) black fly larvae recovered for density calculations. The spore density model did not converge with the addition of Dry Creek data from June 22 as none of the black flies sampled had any gut fungi present; therefore, this site/date combination was not included in that model.

Sets of comparisons that were corrected for multiple testing included pairwise comparisons of dates within each stream (three sets) and all date comparisons that had a suitable match (i.e., that fell within seven days) (one set). All analyses were done in SAS version 9.3 (SAS Institute Inc., Cary, North Carolina) using PROC MIXED.

RESULTS

Percent Infestation of Gut Fungi

Black fly larvae were present in sufficient numbers ($n \geq 15$) at both reference sites and for 11 of the 12 sampling events at the agricultural site, Sand Run Gulch. In contrast, hosts were absent for 6 of the 12 sampling events at the second agricultural site, Wanstad Ditch, even though the habitat appeared suitable for black flies (Adler *et al.*, 2004). During three of the six sampling events at Wanstad Ditch where black fly larvae were present, only one to seven individuals were collected. Percent infestation of black fly larvae with gut fungi was almost always higher in the reference sites compared to the agricultural sites (Figure 2). At the reference sites, Cottonwood Creek had 100% infestation rates for all sampling events (Figure 2a), and Dry Creek had an average 93% infestation rate (Figure 2b). At the agricultural sites, Sand Run Gulch had an average infestation rate of 54% (Figure 2c) and Wanstad Ditch had an average 33% infestation rate (Figure 2d). The highest percent infestation rate at Sand Run Gulch was observed from July through September (100% for all dates) (Figure 2c). While these rates are comparable to the reference sites, this does not take into account the actual number of fungi within the gut. Therefore, the densities of thalli and spores in the gut were enumerated.

Density and Spore Production of Gut Fungi

Fungal density and spore production in larval black fly PMs varied over time in all of the sampled sites. Of interest at the reference sites, thalli densities from the June and early July sampling dates were statistically lower than sampling dates during August through November (with the exception of October 4 at Cottonwood Creek and September 15 at Dry Creek) (Figure 3). Spore densities at the reference sites were more consistent over the sampling period showing spikes on October 26 at Cottonwood Creek and October 4 and November 22 at Dry Creek (Figure 4). At Sand Run Gulch, thalli and spore density both were significantly higher on August 16 than sampling dates between June and July (Figures 3 and 4). Due to insufficient numbers of black flies and *Harpella* thalli recovered, no statistical analysis was conducted for the agricultural site Wanstad Ditch.

Densities were compared between Sand Run Gulch and each of the reference sites at six paired sampling dates. Thallial densities at Dry Creek were statistically significantly higher on five of the six dates, with the magnitude of the difference increasing throughout the sampling period (Table 2). At Cottonwood Creek, densities were significantly higher on three of the sampling dates in mid June, late August, and early September (Table 2). Spore density was not significantly different from Sand Run Gulch at either reference site in June, early July, or August. However, spore density

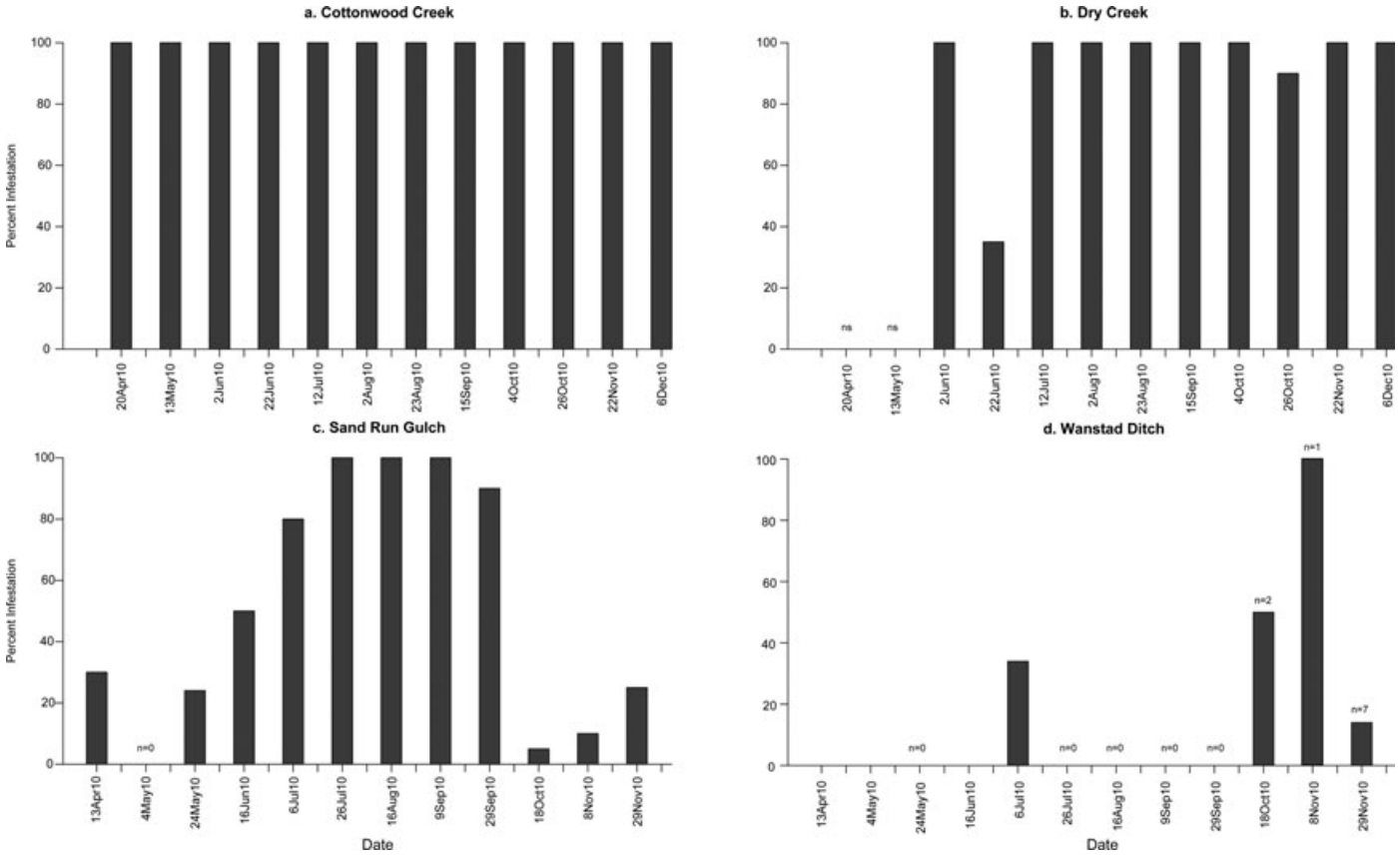

FIGURE 2. Percent Infestation of Gut Fungi in Black Fly Larvae from Reference Sites (a) Cottonwood Creek, (b) Dry Creek, and Agricultural Sites (c) Sand Run Gulch, (d) Wanstad Ditch in Idaho. All samples are $n \geq 15$ unless otherwise noted; ns, not sampled.

was significantly higher at both reference streams in the two September comparisons (Table 2).

For reference sites, the maximum number of thalli/μm^2 was 2.9×10^{-4} and 2.6×10^{-4} at Cottonwood Creek and Dry Creek, respectively (Figure 3). Cottonwood Creek had a maximum number of spores/μm^2 of 3.6×10^{-4} while Dry Creek had 3.2×10^{-4} spores/μm^2 (Figure 4). At agricultural sites, the maximum number of thalli/μm^2 was 0.8×10^{-4} and 0.04×10^{-4} at Sand Run Gulch and Wanstad Ditch, respectively (Figure 3). The maximum number of spores/μm^2 at Sand Run Gulch was 0.4×10^{-4}. Wanstad Ditch had even fewer with 0.08×10^{-4} spores/μm^2 (Figure 4).

Pesticides in Surface Water and Black Fly Tissue

Fungicides were not detected in any surface water samples collected from the reference sites (Table S2). Only two pesticides were detected in samples from these reference sites during the course of this study. In surface water samples from the agricultural sites, 22 pesticides (eight fungicides, ten herbicides, two insecticides, and two degradates) were detected (Table S2). The fungicides azoxystrobin and boscalid were detected in 11 of the 12 (92%) water samples collected from Wanstad Ditch and in 8 of the 12 (67%) sampling events at Sand Run Gulch.

Selected black fly larvae were analyzed directly for a suite of 12 pesticides (four fungicides and eight herbicides) based on the detection frequency and maximum concentrations observed in surface water samples during the study. A total of 17 larval tissue samples were analyzed, 5 from Cottonwood Creek, 4 from Dry Creek, 1 from Wanstad Ditch, and 7 from Sand Run Gulch. No fungicides were detected in any larval tissue samples from the reference sites. The herbicides atrazine, simazine, and trifluralin were detected in 22, 67, and 67%, respectively, of the larval tissues from reference sites with composite concentrations ranging from 5 to 234 μg/kg wet weight (Table S3). A total of 11 pesticides (four fungicides and seven herbicides) were detected in larval tissue samples from agricultural sites with detection frequencies ranging from 50 to 88% and composite concentrations ranging from 21 to 840 μg/kg wet weight (Table 3). Azoxystrobin, one of the most frequently detected fungicides in the U.S. (Battaglin *et al.*, 2011), and boscalid, the most frequently detected fungicide in the study areas (Reilly *et al.*, 2012a), were detected in 85% of the tissue samples and 71% of the water samples, respectively.

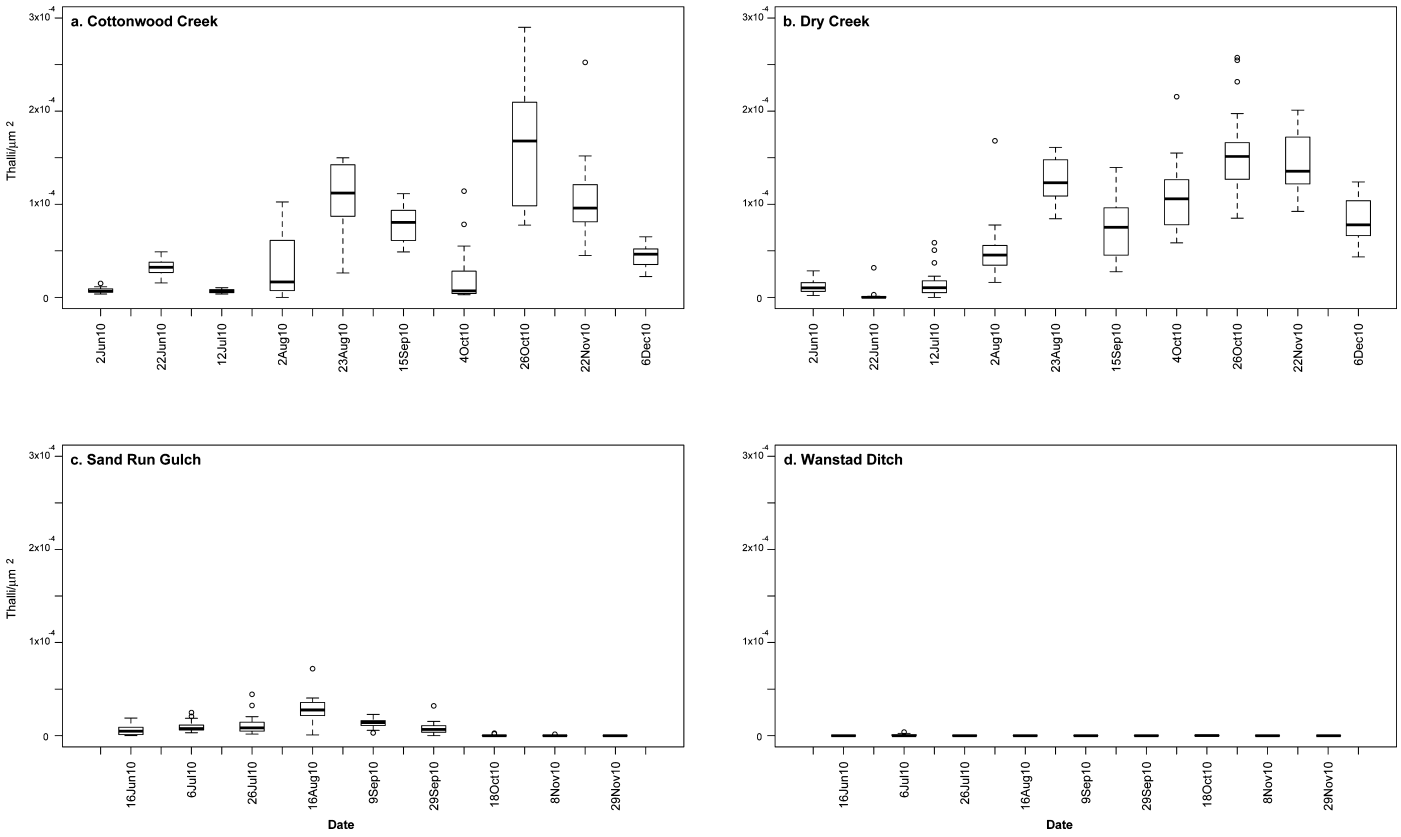

FIGURE 3. Number of Thalli Per μm^2 of Peritrophic Matrix in Black Fly Larvae from Reference Sites (a) Cottonwood Creek, (b) Dry Creek, and Agricultural Sites (c) Sand Run Gulch, (d) Wanstad Ditch. Boxes on plots represent median (thick bar) and interquartile range (25-75%). Whiskers represent adjacent values: upper = largest observation that is ≤ upper quartile plus 1.5 times length of interquartile range; lower = smallest observation that is ≥ lower quartile less 1.5 times length of interquartile range (Adler, 2010).

Within the agricultural sites, fungicides were detected frequently in surface water and larval tissue from Sand Run Gulch throughout the sampling period (Table 3). However, Wanstad Ditch only yielded enough larval black flies for one tissue sample (Tables S3, S4). All four of the fungicides analyzed were detected in larval black fly tissue at Sand Run Gulch. Of those detected, only azoxystrobin and boscalid were detected in the surface water samples that corresponded with the tissue samples; indeed, pyraclostrobin had the highest composite tissue concentration of any of the fungicides (Table 3). Composite tissue concentrations of all fungicides detected were highest in the July and early September samples, which also corresponded to the highest surface water concentrations (Table 3).

DISCUSSION

This is the first exploration of fungicide effects on gut fungi and these results contribute to a better understanding of pesticide impacts across trophic levels in aquatic systems. Current-use fungicides have the potential to effect nontarget organisms, and current test organisms may not be adequate in predicting the effects of fungicides on nontarget fungi (Maltby *et al.*, 2009). The organisms selected for this study are nontarget fungi that exist as endosymbionts of stream invertebrates, thus giving a potential glimpse into the trophic consequences that may be impacted from fungicide exposure.

The metrics used for assessing effects on insect hosts were abundance of black fly larvae and estimations of body burden of select pesticides in their tissue. Potential habitats for black flies are quite varied and include impacted areas such as irrigation channels and drainage ditches (Crosskey, 1990) similar to those sampled at the agricultural sites. However, black fly larvae can have a decreased abundance and species composition in agricultural streams (Pramual and Kuvangkadilok, 2009). Black fly larvae were absent for approximately half of the sampling events at the agricultural site Wanstad Ditch (Figure 2), which may have been due to the presence of contaminants, life history, or environmental conditions

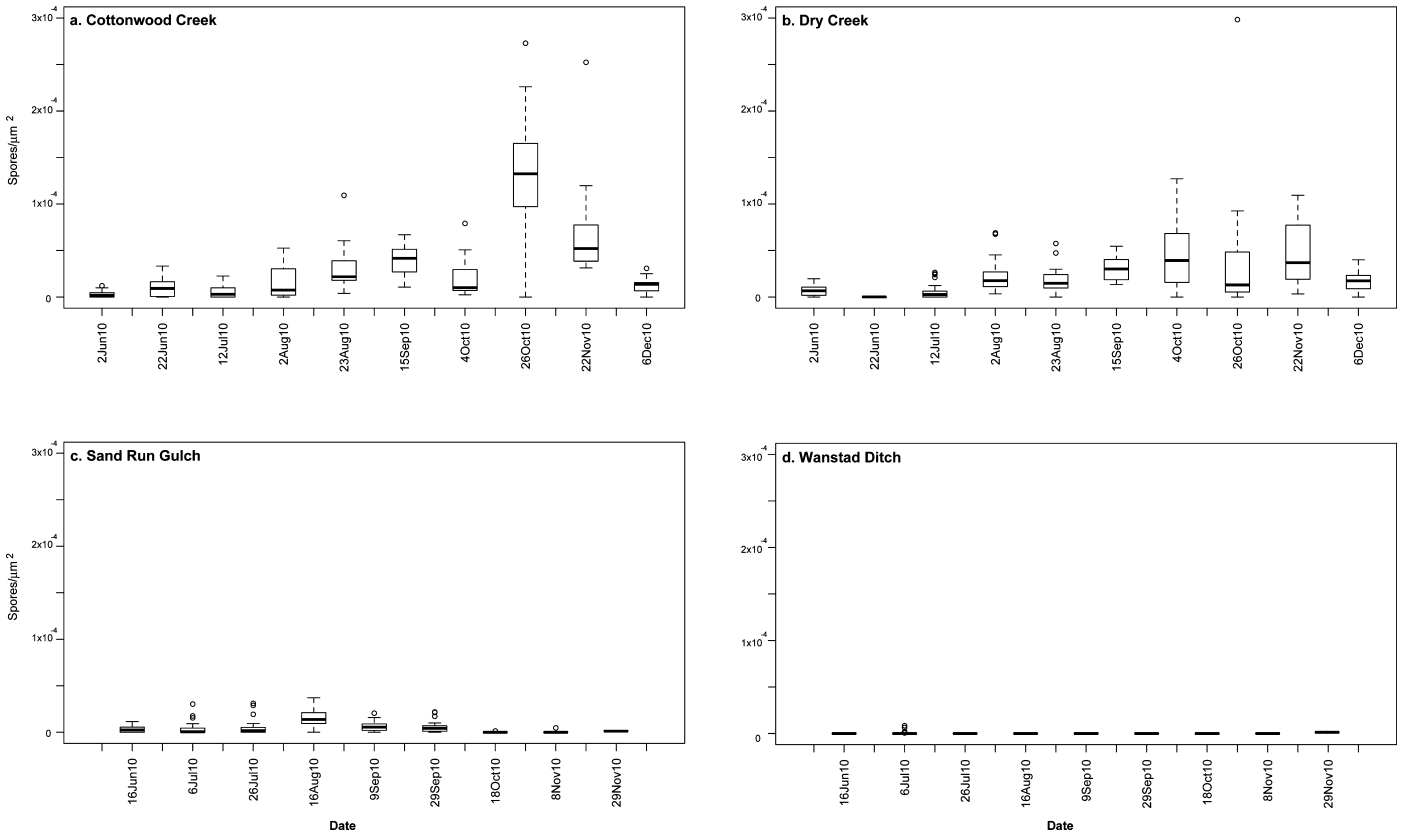

FIGURE 4. Number of Spores Per μm^2 of Peritrophic Matrix in Black Fly Larvae from Reference Sites (a) Cottonwood Creek, (b) Dry Creek, and Agricultural Sites (c) Sand Run Gulch, (d) Wanstad Ditch. Boxes on plots represent median (thick bar) and interquartile range (25-75%). Whiskers represent adjacent values: upper = largest observation that is ≤ upper quartile plus 1.5 times length of interquartile range; lower = smallest observation that is ≥ lower quartile less 1.5 times length of interquartile range (Adler, 2010).

(Beard *et al.*, 2003). Pesticides have been detected in up to 97% of surface water near agricultural land and often at least two pesticides are present per water sample (Gilliom, 2007). In this study, fungicides were detected in nearly 80% of the samples collected from the agricultural streams, which is likely related to repeated field application throughout the growing season (Reilly *et al.*, 2012a). With this high frequency of detection, the organisms living in these waterways are potentially receiving chronic exposure to a mixture of pesticides. In this instance, fungicides were detected at very high concentrations in their tissue: orders of magnitude higher than were detected in the surrounding water column (Table 3).

Fungal percent infestation, density, and spore production in sampled black fly midguts were used to assess the health and fitness of gut fungi. On average, black fly larvae from the reference sites were nearly 100% infested compared to 33-54% at agricultural sites (Figure 2). These lower rates are not unlike those found by others when surveying black fly larvae for gut fungi (Beard *et al.*, 2003; Nelder *et al.*, 2006). However, the actual density of fungi in the gut is even more rarely taken into account in other studies. Certain gut fungus colonization rates can differ between species, although PM-dwelling *Harpella* spp. typically have the highest colonization rates regardless of host taxa (Lichtwardt and Williams, 1988; Beard and Adler, 2002). In this study, various larval *Simulium* spp. were collected (Table S5), but it was assumed that all species had an equal probability of being colonized by *Harpella* spp. Therefore, the low number of gut fungi in agricultural sites is more likely due to an effect of stream conditions, including fungicide exposure, than differences in host species.

The reference sites had at least an order of magnitude higher density and spore production in larval black fly PMs compared to those in agricultural sites, but there was a great deal of variation in both number of thalli and spores over time (Figures 3 and 4). Seasonality has been documented in several other studies on gut fungi where fungal infestation and species composition can change over time and across environmental gradients (Beard and Adler, 2002; Beard *et al.*, 2003; Nelder *et al.*, 2006; Bench, 2009). However, prior to this study, the influence of chronic nonpoint source pesticide exposure in a natural

TABLE 2. Model-Based Means of Thalli and Spores Per μm^2 (±95% confidence intervals). *p*-Value tests null hypotheses that mean number of thalli and spores, respectively, are equivalent between reference sites and Sand Run Gulch, the agricultural site where there were enough fungi for comparisons. Due to insufficient numbers of black flies Wanstad Ditch was omitted from the model. Dates are staggered to represent alternation of sampling weeks. *p*-Values have been adjusted for multiple testing with Bonferroni correction. See Table S6 for mixed model results.

Reference Sites					Agricultural Sites					*p*-Values*	
Date	Site	No. Samples	Average (±CI) Thalli/μm^2	Average (±CI) Spores/μm^2	Date	Site	No. Samples SRG/WD	SRG Average (±CI) Thalli/μm^2	SRG Average (±CI) Spores/μm^2	Thalli *p*-Value	Spores *p*-Value
June 2, 2010	CC	18	7.4E-06 (6.3E-06, 8.8E-06)	6.4E-07 (1.6E-07, 2.5E-06)							
	DC	23	1.0E-05 (7.8E-06, 1.3E-05)	3.1E-06 (1.3E-06, 7.3E-06)							
					June 16, 2010	SRG/WD	18/18	4.0E-06 (2.2E-06, 7.2E-06)	5.3E-07 (1.2E-07, 2.3E-06)		
June 22, 2010	CC	18	3.2E-05 (2.8E-05, 3.7E-05)	2.0E-06 (4.4E-07, 9.4E-06)						$p < 0.0001$	1.000
	DC	18	9.9E-07 (5.8E-07, 1.7E-06)	0 (all observations)						0.009	nt
					July 6, 2010	SRG/WD	20/29	9.2E-06 (7.3E-06, 1.2E-05)	2.3E-07 (4.9E-08, 1.1E-06)		
July 12, 2010	CC	35	7.0E-06 (6.4E-06, 7.8E-06)	4.1E-07 (1.3E-07, 1.3E-06)						0.412	1.000
	DC	25	9.0E-06 (5.5E-06, 1.5E-05)	9.7E-07 (2.8E-07, 3.4E-06)						1.000	1.000
					July 26, 2010	SRG/WD	20/0	9.1E-06 (6.4E-06, 1.3E-05)	6.5E-07 (1.6E-07, 2.6E-06)		
August 2, 2010	CC	20	1.6E-05 (7.8E-06, 3.4E-05)	3.6E-06 (9.7E-07, 1.4E-05)						1.000	0.754
	DC	19	4.6E-05 (3.6E-05, 5.8E-05)	1.7E-05 (1.2E-05, 2.6E-05)						$p < 0.0001$	0.001
					August 16, 2010	SRG/WD	20/0	2.4E-05 (1.7E-05, 3.5E-05)	1.0E-05 (4.5E-06, 2.3E-05)		
August 23, 2010	CC	20	1.0E-04 (8.6E-05, 1.3E-04)	2.3E-05 (1.7E-05, 3.3E-05)						$p < 0.0001$	0.646
	DC	19	1.2E-04 (1.1E-04, 1.4E-04)	5.8E-06 (1.4E-06, 2.3E-05)						$p < 0.0001$	1.000
					September 9, 2010	SRG/WD	20/0	1.3E-05 (1.0E-05, 1.6E-05)	2.3E-06 (7.4E-07, 7.4E-06)		
September 15, 2010	CC	20	7.8E-05 (7.0E-05, 8.7E-05)	3.6E-05 (2.9E-05, 4.5E-05)						$p < 0.0001$	0.001
	DC	20	6.7E-05 (5.4E-05, 8.3E-05)	2.8E-05 (2.3E-05, 3.4E-05)						$p < 0.0001$	0.003
					September 29, 2010	SRG/WD	18/0	6.2E-06 (3.9E-06, 1.0E-05)	1.3E-06 (3.3E-07, 5.4E-06)		

(continued)

TABLE 2. Continued.

Reference Sites					Agricultural Sites					*p*-Values*	
Date	**Site**	**No. Samples**	**Average (±CI) Thalli/µm²**	**Average (±CI) Spores/µm²**	**Date**	**Site**	**No. Samples SRG/WD**	**SRG Average (±CI) Thalli/µm²**	**SRG Average (±CI) Spores/µm²**	**Thalli *p*-Value**	**Spores *p*-Value**
October 4, 2010	CC	20	1.1E-05 (6.7E-06, 1.9E-05)	1.3E-05 (8.2E-06, 2.1E-05)						1.000	0.027
	DC	20	1.0E-04 (8.8E-05, 1.2E-04)	1.9E-05 (6.8E-06, 5.3E-05)						$p < 0.0001$	0.033
					October 18, 2010	SRG/WD	20/2	All zero except two observations	All zero but one observation		
October 26, 2010	CC	20	1.5E-04 (1.2E-04, 1.8E-04)	7.7E-05 (2.7E-05, 2.2E-04)						nt	nt
	DC	20	1.5E-04 (1.3E-04, 1.7E-04)	6.6E-06 (1.6E-06, 2.7E-05)						nt	nt
					November 8, 2010	SRG/WD	20/1	All zero except one observation	All zero except one observation		
November 22, 2010	CC	15	9.9E-05 (7.8E-05, 1.3E-04)	6.0E-05 (4.4E-05, 8.2E-05)						nt	nt
	DC	20	1.4E-04 (1.3E-04, 1.6E-04)	3.4E-05 (2.2E-05, 5.2E-05)						nt	nt
					November 29, 2010	SRG/WD	20/7	All zero	All zero		
December 6, 2010	CC	20	4.4E-05 (3.9E-05, 5.1E-05)	6.2E-06 (2.2E-06, 1.8E-05)						nt	nt
	DC	19	8.2E-05 (7.1E-05, 9.4E-05)	1.1E-05 (4.4E-06, 2.5E-05)						nt	nt

Notes: *p-Value is Bonferroni-corrected to test null hypothesis that the density of thalli between CC or DC and SRG is equal.
CC, Cottonwood Creek; DC, Dry Creek; SRG, Sand Run Gulch; WD, Wanstad Ditch; nt, not tested.

TABLE 3. Composite Fungicide Concentration (μg/kg wet weight) in Sampled Black Fly Larvae and Surface Water (μg/l) Collected from Sand Run Gulch (agricultural site). Only water data with corresponding tissue data are presented.

	Azoxystrobin		Boscalid		Imazalil		Pyraclostrobin	
Date	Tissue	Water	Tissue	Water	Tissue	Water	Tissue	Water
2010-05-03	56.0	0.00220	93.0	0.01020	181.0	nd	nd	nd
2010-07-06	121.0	0.00629	33.4	0.01076	326.0	nd	nd	nd
2010-07-26	377.0	0.01140	112.0	0.01600	374.0	nd	426.0	nd
2010-09-09	428.0	0.01250	90.0	0.01490	178.0	nd	842.0	nd
2010-09-29	136.0	nd	106.0	nd	21.0	nd	285.0	nd
2010-10-18	35.0	0.00259	29.0	0.00541	66.0	nd	163.0	nd
2010-11-08	nd	nd	nd	nd	nd	nd	nd	nd

Note: nd, not detected.

setting had not been explored. Future investigations modeling variables of season, environment, host species, and other factors (including pesticide concentrations) would offer a window into the forces driving fungal patterns observed in this symbiotic system.

Fungicides were detected frequently in the tissue of black flies collected from Sand Run Gulch throughout the sampling period (Table 3). It is difficult to put these data into context as there are few field studies available for the presence of current-use fungicides in aquatic insect tissue, although several laboratory studies have documented the effects of organophosphate insecticides (chlorpyrifos and diazinon) on aquatic insects (Stuijfzand *et al.*, 2000; Buchwalter *et al.*, 2004). It has been suggested that pesticide impacts on larval insects are related to the timing of pesticide occurrence in the stream and the life stage of the organism (Stuijfzand *et al.*, 2000). Several field studies have documented current-use pesticides in other aquatic tissues including crab embryos (Smalling *et al.*, 2010), sand crabs (Dugan *et al.*, 2005; Anderson *et al.*, 2010), and fish (Sapozhnikova *et al.*, 2004; Dugan *et al.*, 2005). The precise effects of fungicides within the host tissue on fungal endosymbionts are unknown and are difficult to separate from the possible effects of dissolved fungicides, whether in the water itself, bound with particulates, or passing through the hosts.

Fungicides constituted 37-81% of the pesticide mass detected in each individual tissue sample. Composite concentrations for individual fungicides ranged from 21 to 840 μg/kg wet weight (Table 3). The median body burdens of the detected fungicides and herbicides were 65.2 and 18.2 μg/g wet weight, respectively. Where pesticides were detected in the water column, pesticides in individual black fly larvae constituted between 0.01 and 0.1% of the mass of an individual larva (Table S3). The estimated body burden on a black fly larva is substantially higher than what has been observed in other studies (Sapozhnikova *et al.*, 2004; Dugan *et al.*, 2005; Smalling *et al.*, 2010). Although, organismal effects, including effects on fungi and exposure pathways in the field, are still unknown, future research is needed to determine not only how the accumulation directly affects the gut fungi within the host but also, more broadly, how this could affect whole aquatic systems.

As it currently stands, the role and responsiveness of symbiotic gut fungi within their arthropod hosts is not completely understood. However, if fungicides in streams are impacting nontarget mutualistic, endosymbiotic fungi, the symbionts may not be available or sensitive to the needs of their host in times of stress (i.e., during exposure to other pesticides and toxicants). Gut fungi are also common in aquatic bioindicator taxa, including but not limited to, Ephemeroptera, Plecoptera, and Trichoptera (Lichtwardt, 1986), but to date there have not been any studies examining the relationship between host sensitivity to pesticides or other stressors and the prevalence of gut fungi in these taxa.

This pilot study shows a significant difference in gut fungus densities in agricultural compared to reference streams while taking into account change over time. Given the nature of this study, direct causation cannot be explicated; however, the lack of hosts and fungi at Wanstad Ditch, the increased detection frequency of fungicides, and the correspondingly high amount of accumulation within the black fly tissue is intriguing and clearly invites further effort. These findings all point toward environmental stressors possibly influencing the symbiotic system. Research is needed that distinguishes between, and ascertains the significance of, natural and varying environmental conditions and the presence of dissolved pesticides as potential stressors affecting gut fungi in nonpristine habitats.

Future studies should continue to explore the mechanisms driving the decreased prevalence of gut fungi in agriculturally impacted streams, with a particular focus on hosts that are beneficial to stream health. There is a need to monitor aquatic ecosystems for fungicides, as demonstrated by the potential impact on nontarget fungi, such as gut fungi and

their black fly hosts. This is yet another example of the potential of pesticides to affect nontarget organisms, but this may represent only a small fraction of impacts among the myriad cascades and associations connecting organisms within watersheds. Such delineations, formed holistically, would pave the way for connecting the interface between host, symbiont, stream health, and environment in this complex web of interactions.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article: detailed information on the methods used to measure pesticides in black fly larval samples. These include the following: instrument parameters, quality assurance, and detection limits of pesticides. Tables S1-S6 and Figure S1 are also included in the supporting information.

Figure S1. Composite image of black fly peritrophic matrix (PM) colonized by *Harpella* spp. from Cottonwood Creek (reference site) slide ID-84-E1. Scale bar = 100 μm.

Table S1. Retention time, MRM conditions, qualifier and qualifier ions, average percent recovery of matrix spikes, and instrumental limits of detection (LOD) for pesticides analyzed in larval black fly tissue.

Table S2. Pesticide concentrations (ng/l) in surface water from the two reference (Cottonwood Creek and Dry Creek) and two agricultural (Wanstad Road Ditch and Sand Run Gulch) sites collected between April and December 2010.

Table S3. Composite pesticide concentration (μg/g wet weight) in sampled black fly larvae collected from reference sites (Cottonwood Creek, Dry Creek) and agricultural sites (Sand Run Gulch, Wanstad Ditch).

Table S4. Estimated pesticide concentrations (μg/g wet weight) of individual black fly larvae collected from reference sites (Cottonwood Creek, Dry Creek) and agricultural sites (Sand Run Gulch, Wanstad Ditch).

Table S5. Identified black fly larvae from 2010 samples. *N* refers to the number of black fly larvae identified from that site over the eight-month sampling period (421 total).

Table S6. Mixed model results for thalli/μm^2 and spores/μm^2. The log of thalli and spores (plus 5E-07) were modeled, with fixed effect of date, site, and date by site interaction. Due to insufficient numbers of black flies, Wanstad Ditch was not included in the model. Separate residuals were modeled for each date by site combination to accommodate unequal variation.

ACKNOWLEDGMENTS

Research was funded by the National Science Foundation DEB 098182 and by generous support from the U.S. Geological Survey Toxic Substances Hydrology Program. The authors thank the following members of the USGS for their support: Kathryn Kuivila, Mark Hardy, William Battaglin, Adria Elskus, Megan McWayne-Holmes, Jim Orlando, and Alexandria Ethridge; graduates and undergraduates at Boise State University: Sarah Oman, Korinne Wade, Donavon Carrie, Yan Wang, Eric Tretter, and Eric Johnson; other personnel at BSU: Julie Heath and Department of Biological Sciences; as well as Rusty Rodriguez (USGS), Mike Thornton and Bill Buhrig (University of Idaho), Eddie Beard and Peter Adler (Clemson University), Bruce Horn (USDA), and Robert Lichtwardt (University of Kansas).

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