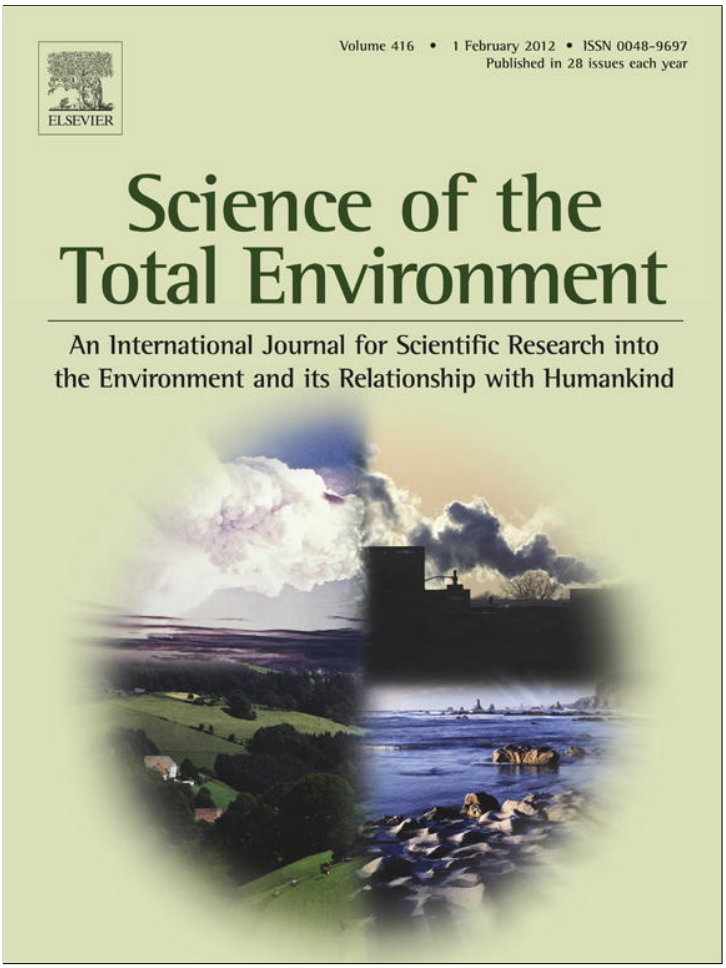

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Using group-specific PCR to detect predation of mayflies (Ephemeroptera) by wolf spiders (Lycosidae) at a mercury-contaminated site

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ABSTRACT

Bioaccumulation of contaminants can occur across ecosystem boundaries via transport by emergent aquatic insects. In the South River, Virginia, USA, aquatic mercury has contaminated songbirds nesting in adjacent riparian forests. Spiders contribute the majority of mercury to these songbirds' diets. We tested the hypothesis that massive annual mayfly emergences provide a vector for mercury from river sediments to the Lycosid spiders most frequently eaten by contaminated songbirds. We designed mayfly-specific PCR primers that amplified mtDNA from 76% of adult mayflies collected at this site. By combining this approach with an Agilent 2100 electrophoresis system, we created a highly sensitive test for mayfly predation by Lycosids, commonly known as wolf spiders. In laboratory spider feeding trials, mayfly DNA could be detected up to 192 h post-ingestion; however, we detected no mayfly predation in a sample of 110 wolf spiders collected at the site during mayfly emergence. We suggest that mayfly predation is not an important mechanism for dietary transfer of mercury to wolf spiders and their avian predators at the South River. Instead, floodplain soil should be considered as a potential proximate source for mercury in the terrestrial food web.

1. Introduction

Mercury is a ubiquitous environmental contaminant with the potential for devastating effects in biological organisms. Since the industrial revolution, humans have increased the atmospheric deposition rate of mercury fourfold, and numerous industrial point sources have raised mercury concentrations in soil, sediment, and water (Wang et al., 2004). Once inorganic mercury is released into aquatic ecosystems, microbes initiate a complex metabolic cascade whereby the mercury becomes methylated. This organic form of mercury is of most concern as a biohazard, because it is readily incorporated into the tissues of eukaryotic organisms and is not easily metabolized or excreted (Clarkson and Magos, 2006).

Organisms at the base of food webs can internally concentrate methylmercury (MeHg), often bioaccumulating it faster than it can be excreted. Because consumers preying on contaminated producers may not be able to excrete MeHg efficiently, they too can become contaminated, retaining much of the mercury burden from their prey and potentially moving it long distances across the landscape and ecosystem boundaries (Blais et al., 2005). At the trophic level of a top predator such as a large fish, MeHg can be biomagnified up the food chain to concentrations millions of times greater than levels in the abiotic environment (Driscoll et al., 2007). Understanding the movement of MeHg through the food web is critical for identifying sites of concern and assessing risk to wildlife and humans.

This study focused on the South River, a tributary of the Shenandoah River in Virginia, which was contaminated by the industrial release of mercuric sulfate from 1930 to 1950. Contamination of South River fish was recognized in the 1970s (Carter, 1977), but only recently has it been discovered that birds living along the river also exhibit elevated MeHg, including many insectivorous songbirds with no direct trophic connection to the river (Cristol et al., 2008). Thus, mercury from an ultimately aquatic source can enter a terrestrial food web (Cristol et al., 2008).

Collection of prey items delivered by terrestrial-feeding songbirds to their nestlings in forests along the South River revealed that spiders comprised 25–30% of the biomass consumed, and delivered approximately 75% of the mercury in the birds' diets (Cristol et al., 2008). None of the collected prey was aquatic in origin, and the majority of spiders collected were cursorial (non-web-weaving) and of the family Lycosidae, or wolf spiders (Howie and Cristol, unpublished data). The average total (organic and inorganic) mercury concentration of collected spiders (1.24 ppm dry weight) dwarfed that of moths and caterpillars (0.38 ppm) or crickets and grasshoppers (0.31 ppm), the other major songbird dietary components. In fact, spiders preyed upon by songbirds had average mercury levels higher than prey items in the diet of an obligate fish-eater (0.73 ppm), the belted kingfisher *Ceryle alcyon*, subverting the usual paradigm that fish-eating wildlife are at the greatest risk for mercury exposure (Cristol et al., 2008). This finding raises the question of how the cursorial spiders favored by songbirds obtained such high

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mercury levels. A parsimonious hypothesis is that the spiders obtained mercury by eating the abundant emergent aquatic insects that have accumulated aquatic mercury directly during their life as nymphs (larvae) in the contaminated South River.

Analyses of various stream/terrestrial interfaces have identified the emergent aquatic insect order Ephemeroptera (mayflies) as the dominant contributor to biomass moving out of many rivers into adjacent terrestrial habitats (Burdon and Harding, 2008). Mayflies are eaten by one aerially foraging songbird (tree swallow, *Tachycineta bicolor*) at the South River, but most songbirds forage by picking invertebrates from vegetation or the ground, and the diets of the other three songbird species studied in detail (Carolina wren, *Thryothorus ludovicianus*; house wren, *Troglodytes aedon*; eastern bluebird, *Siala sialis*) contained no recognizable emergent aquatic insects (Brasso and Cristol, 2008; Cristol et al., 2008). At the South River, mayflies are abundant, forming dense clouds that drift far from the river at the height of emergence (pers. obs.), and have elevated mercury levels, making them a potential source of mercury for cursorial spiders living near the river (nymphs: Tom et al., 2010; adults: unpublished data from six adult mayflies collected by A. Condon during the present study indicated total mercury of 0.85 $\pm$ 0.24 μg/g (dry weight) with 93.87 $\pm$ 16.77% in the form of methylmercury). As mayflies molt from their aquatic nymphal form to the flying subimago adult, they often rest on nearby rocks or vegetation before molting again to the imago adult stage (Brittain, 1982). During this period, or when the short-lived imago adults begin to die shortly after mating, they represent a plausible food source for the wolf spiders that constituted the majority of spiders eaten by songbirds near the South River (Howie and Cristol, unpublished data).

Wolf spiders are generalist predators with cryptic and varied predation habits that are difficult to observe directly, e.g., most are nocturnal and do not collect food in webs (Ahrens and Kraus, 2006; Maloney, 2002). Therefore, molecular techniques such as PCR are a favored approach for identifying their prey (Sheppard et al., 2005). The availability of DNA sequence information on Internet databases such as GenBank makes the "barcoding" of invertebrates possible through analysis of mitochondrial DNA (mtDNA) (King et al., 2008). The present study used a group-specific PCR approach to design primers within the Folmer fragment (Folmer et al., 1994) that targeted mayfly mtDNA but excluded that of the spider predator. By coupling this PCR technique with an Agilent 2100 on-chip electrophoresis system, we achieved greater sensitivity for identifying mayfly DNA compared to agarose gels stained with ethidium bromide (Jensen, 2004).

The objective of this study was to determine whether a sample of wolf spiders collected from forests and fields along the South River had been feeding on emerging mayflies. The larger context for this research question was to test the hypothesis that the annual emergences of mayflies provide a vector for the transport of MeHg from the river into the surrounding terrestrial food web that includes spider-eating songbirds. Ultimately, the choice of which environmental remediation methods to pursue at a contaminated site such as this depends on the proximate source and route of exposure to bioavailable mercury.

2. Materials and methods

2.1. Collection of spider and mayfly samples

Wolf spider samples were collected along the South River in Augusta and Rockingham counties, Virginia. Collections occurred from 19 to 22 May, 2009 after nightfall (2100–0100) at two riparian sites along the river: Augusta County Forestry Center (2 km of river centered at latitude: 38.17658, longitude: −78.85297), and Grottoes City Park (2 km of river centered at latitude: 38.28572 longitude: −78.83257). Using flashlights held at eye level, spiders were located by their reflective eyes at distances less than 100 m from the riverbank. The majority of spiders were collected < 50 m from the river as they prefer the narrow riparian forest edge habitat that follows the shoreline. Spiders were placed into either type I borosilicate glass vials (29 × 65 mm, Fisher, Pittsburgh PA), or polypropylene scintillation vials (28 × 61 mm, Fisher). Spiders (n = 110) were collected and frozen at −20 °C less than 5 h after collection. A portion of this sample (n = 32) was inadvertently left alive for 25 h after collection, prior to freezing. Within 72 h of collection, the masses of all spiders were measured (wet weight). These 110 spiders were all identified by general morphology and eye configuration as members of the family Lycosidae and were used to determine whether wolf spiders feeding during a mayfly emergence contained mayfly mtDNA in their digestive systems.

To test mayfly-specific primers, adult mayflies were collected with butterfly nets and forceps as they emerged from the river or rested on shoreline vegetation, on the same dates and at the same sites as the spider collections, from 1600 to 2000. Both subimago and imago adults were collected (n = 74) in type I borosilicate glass vials (15 × 45 mm) or polypropylene scintillation vials and frozen within 5 h at –20 °C.

2.2. Dissection and DNA extraction of spider samples

Spiders were processed one-at-a-time, and each individual spider was removed from the freezer and immediately dissected on a new sterile aluminum foil workspace with a sterile scalpel blade and tools. The cephalothorax was bisected from the abdomen, and the abdomen was placed in a sterile polystyrene culture tube (17 × 100 mm). For negative controls, leg segments were removed distal to the femur (n = 7 spiders). Culture tubes were filled with 1.4 ml Buffer ASL (QIAGEN, Valencia CA) and each sample was homogenized using an Omni TH Tissue Homogenizer (Omni International, Kennesaw GA) with a new, sterile Omni hard tissue plastic homogenizer probe for each spider and negative control sample. Parafilm was stretched around the homogenizer probe and the culture tube to form a seal. The contents were then homogenized for 1–2 min at speeds varying between 5000 and 35,000 rpm to minimize bubble formation. Subsequently, the homogenate was incubated at room temperature for 30–60 min, and the contents of the culture tubes were transferred to separate sterile 2.0 ml Eppendorf tubes. DNA extraction was carried out using a QIAGEN DNA Stool Mini Kit according to the manufacturer's specifications for the "Stool Pathogen Detection" procedure, with a 15-min Proteinase K digestion and a 75 °C incubation for increased cell lysis. Extracted DNA was eluted into 200 μl Buffer AE (QIAGEN) and assessed for purity and concentration using a Nanodrop ND-1000 UV–vis spectrophotometer (Thermo Fisher Scientific, Waltham MA). Eluate was then stored at −20 °C.

2.3. Dissection and DNA extraction of mayfly samples

Adult mayfly samples were processed one-at-a-time after legs, wings, and tail filaments were removed with a new sterile scalpel blade. The remaining body was then homogenized and DNA extraction was performed as with spider samples described above. Plastic homogenizer probes were reused during these adult mayfly extractions after being thoroughly washed in detergent, rinsed with distilled water, soaked in 6% w/v hypochlorite bleach for 30 min at room temperature to degrade any residual DNA, and rinsed again with distilled water. DNA eluate was assessed for purity and concentration and stored at −20 °C.

2.4. Design of mayfly-specific PCR primers

Mayfly-specific primers were designed in order to amplify an approximately 206 bp region specific to Ephemeropterans within the

Folmer fragment (Folmer et al., 1994) of the mitochondrial cytochrome *c* oxidase I gene (COI/cox1) (Fig. 1). The forward and reverse primers were designated as (M1WN-F: 5′-AGTATAGTDGAAAGAGGRGCTGG-3′; M1WN-R: 5′-GGAATWCGATCCATWGTTATTCC-3′). Primer sequences are represented here using universal degenerate nucleotide code, as M1WN-F and M1WN-R contain 6-fold and 4-fold degeneracy, respectively. Primers were designed using the freely available Amplicon software package (Jarman, 2004). Clustal DNA sequence alignments were imported into Amplicon, and degeneracy sequences were created for target (mayfly) and excluded (spider) groups. Degeneracy sequences were then aligned and visually inspected for potential primer locations (minimal degeneracy and maximum divergence from the excluded group) (Jarman et al., 2004). After potential primer locations were identified, each location was assessed on the basis of primer length, amplicon length, %GC, secondary structure potential, mononucleotide runs, false priming in the target or excluded groups, and self-compatibility. Primers were selected such that a short amplicon (est. 206 bp) would be generated; retention time of prey DNA after long digestion periods is related to decreasing amplicon length (King et al., 2008; Sheppard and Harwood, 2005).

2.5. PCR amplification using mayfly-specific primers

Mayfly-specific primers were prepared in equimolar concentrations and purified using HPLC to ensure a large yield of full-length oligonucleotides (Integrated DNA Technologies, Coralville IA). PCR contents were mixed in a separate laboratory area from the DNA extractions within a UV-sterilized Airclean AC600 PCR hood (Raleigh, NC) to prevent DNA cross contamination. Before PCR, 8-reaction master mixes were freshly made and added to 45 ng template DNA in each reaction, diluted into sterile double-deionized water filtered by a Nanopure ultrapure water system (Thermo Fisher Scientific, Waltham MA) to bring the reaction volume to 50 μL. One reaction from each 8-reaction master mix was run with all components except DNA template, to ensure that no DNA carryover occurred and that master mix contents were free of DNA contamination. Reagents were diluted to achieve the following concentrations in each reaction: Herculase II Reaction Buffer (1×) (Agilent, Santa Clara CA); dNTPs (200 μM each) (Promega, San Luis Obispo CA); primers (0.5 μM each) (Integrated DNA Technologies); Herculase II DNA Polymerase (1 μl) (Agilent). Reactions were loaded into a BioRad iCycler thermocycling platform (BioRad, Hercules CA). Cycling times were as follows: initial denaturation at 95 °C for 2 min, 36 cycles of 94 °C for 00:30, 55 °C for 00:30, and 72 °C for 2 min. Final extension was carried out at 72 °C for 8 min. Amplification of the Folmer fragment (1994) was carried out with the same conditions and parameters listed above, except for an annealing temperature of 49 °C and use of the primers LCO1490: 5′-GGTCAACAAATCATAAAGATATTGG-3′; HC02198: 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′. All PCR products were stored at −20 °C.

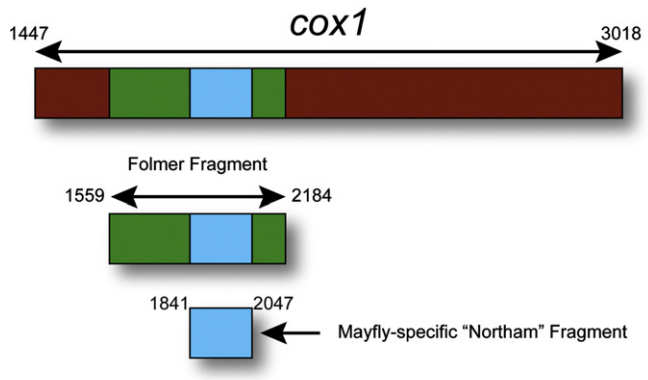

Fig. 1. The cytochrome c oxidase I (COI / cox1) gene (red) is shown above where the Folmer fragment (green) is located. The Folmer fragment was located using a BLAST2seq nucleotide alignment of a sequenced mayfly from the South River and the full mitochondrial genome of a mayfly (Zhang et al., 2008). The nucleotide positions of the Folmer fragment are shown in a linearized form, along with the novel mayfly-specific fragment (blue) amplified by the primers designed in this study.

2.6. Visualization and analysis of PCR products

PCR products were visualized using an Agilent 2100 Bioanalyzer, providing an estimated 25 times greater sensitivity than ethidium bromide staining in an agarose gel (Jensen, 2004). A DNA-1000 on-chip electrophoresis kit was used, with sizing ranging from 25 to 1000 bp (Agilent). One microliter undiluted PCR product was loaded into each of the 12 sample lanes per DNA-1000 chip according to the manufacturer's specifications. Sizing and quantification of PCR products were accomplished using the 2100 Expert software package (Agilent). Default software settings were used for the waveform integrator, except for the sizing threshold, which was set to 5 fluorescence units (FU). Any waveforms below 2 FU in height were considered to be background noise.

2.7. PCR cleanup and DNA sequencing

PCR products were purified using a QIAquick PCR purification kit (QIAGEN) according to the manufacturer's specifications, and eluted into 20 μl DNA Elution Buffer (QIAGEN). Purified PCR products were assessed for nucleic acid purity and concentration using a Nanodrop ND-1000 spectrophotometer and stored at −20 °C. PCR products were prepared for sequencing in ultra-thin wall plastic strip tubes with dome caps. Each sequencing reaction was prepared in 12 μl total volume, containing 50 ng purified PCR product, 4 pmol of each primer (LCO1490 and HC02198 for the Folmer fragment or M1WN-F and M1WN-R for the mayfly-specific fragment) and sterile double-deionized water. PCR products were sequenced in the forward and reverse directions at Yale DNA Analysis Facility (New Haven CT) using Big Dye Terminator 3.1 cycle sequencing chemistry (Applied Biosystems, Carlsbad CA) with 1/8 chemistry overall.

2.8. DNA sequence editing and alignment

Post-processed DNA sequence reads were received in ABI (.ab1) format as chromatograms. Sequences were analyzed to screen for base-calling errors and were trimmed manually. FinchTV software (Geospiza, Seattle WA) was used to view and edit chromatograms. Trimmed sequences were then imported into CLC DNA Sequence Viewer or CLC Main Workbench software (CLC Bio, Cambridge MA) and aligned using the following parameters: (gap open cost = 50; gap extension cost = 1). Alignments were formatted as ClustalW files for subsequent analysis including primer design. DNA sequences using Folmer's PCR primers (Accession nos. HQ116459–HQ116493) and the mayfly-specific PCR primers (Accession nos. HQ116494–HQ116522) were deposited in NCBI GenBank.

2.9. Feeding trial experiments

To test the detection time limit for mayfly DNA from wolf spider gut contents, 38 wolf spiders were captured at the site, transported directly to the laboratory without opportunities to feed, fed live mayflies in the laboratory, then sacrificed after varying periods of digestion. Adult mayflies were captured as described above and held at 4 °C until 30 min before feeding trials began, when they were allowed to equilibrate to room temperature. Each spider (captured as described above and at an additional site nearby: Grand Caverns Park, latitude: 38.25567, longitude: −78.83331) was placed into a separate plastic container (approximately 4 l) filled with grass clippings, and

allowed 30 min to adjust to its surroundings before feeding. A mayfly was then introduced to the container and the spider was allowed to attack and eat this single, live prey item. After ingestion was complete, the time was recorded and spiders were kept in the same container on a cycle of 16:8 h L:D with no further food, but water available *ad libitum*. Different digestion times were achieved by freezing the spiders at −40 °C after the following number of hours of digestion: 3–4 (n = 3), 10–12 (n = 4), 24 (n = 5), 48 (n = 4), 72 (n = 4), 96 (n = 3), 120 (n = 3), 144 (n = 3), 168 (n = 3), 192 (n = 3), 216 (n = 2), and one spider that died between 192 and 216 h. Sacrificed spiders were then stored at −20 °C in a clean type I borosilicate glass vial until DNA analysis.

3. Results

3.1. Mayfly-specific primers cover a majority of South River mayflies

A total of 74 adult mayflies were collected from the South River during the same days in which spider collection occurred. Overall, 76% of the collected mayflies demonstrated diagnostic PCR amplification, indicating that the primers covered a majority of mayflies emerging from the river. To investigate the scope of mayfly genetic diversity at the South River, 27 individual samples with either moderate or no amplification were sequenced using Folmer's universal invertebrate primers (Folmer et al., 1994), in addition to eight individual samples from preliminary collecting trips. GenBank queries for these sequences confirmed the degree of genetic diversity among the mtDNA of South River mayflies; samples were grouped into the genera *Ephemerella, Stenacron, Acentrella, Baetis*, *Isonychia,* and *Stenonema*.

3.2. Feeding trials demonstrated high sensitivity and robust detection periods

To estimate the length of digestion time mayfly DNA can survive in the gut of a spider, feeding trials were conducted with various durations of digestion time, and no further feeding, before the spider was frozen. Digestion times ranged from 3 to 216 h, with multiple replicates for each time period. For every digestion time up to 192 h (>1 week), mayfly DNA was detected in at least one sample (Table 1; Fig. 2A). Importantly, amplification strength had high variability and did not correspond closely with digestion time. Mayfly mtDNA that had been digested for 192 h still displayed diagnostic PCR amplification, with fluorescence levels higher than some samples with shorter digestion times (Table 1). Since the genetic diversity of South River mayflies prevented detections of some mayfly species that were fed to spiders, median detection time (T_{50}) was not calculated as has been done for other predator–prey studies (Sheppard and Harwood, 2005). Given sufficient homology with the mayfly-specific primers, it is likely that mayfly mtDNA can be detected within spider gut contents at digestion times up to 192 h under laboratory conditions, and we have not conclusively ruled out that detection is possible after longer time periods. All 22 amplicons from feeding trials were confirmed to be of mayfly origin by DNA sequencing followed by identification using the NCBI GenBank database (Ball et al., 2005).

Table 1
The percentages of positive mayfly ID across different feeding trial digestion times.

Digestion time (h)	Spider sample size	Positive mayfly ID	% Positive	Average fluorescence (FU)
3–4	3	2	67	166 ± 208
10–12	4	3	75	233 ± 328
24	5	5	100	136 ± 131
48	4	3	75	160 ± 181
72	4	1	25	88.9
96	3	2	67	3.9 ± 0.35
120	3	1	33	3.5
144	3	1	33	5.8
168	3	2	67	74.9 ± 102
192	3	2	67	26.1 ± 18
216	2	0	0	0

Feeding trials resulted in at least one positive identification of mayfly mtDNA for each digestion time up to 192 h. The percentage of spider samples with positive identifications ranged from 33 to 100% for each time period, except for 216 h, when no mayfly mtDNA was detected. The ability to amplify mayfly mtDNA with group-specific primers is likely influenced by mayfly genotype in addition to digestion time, since positive identifications did not correspond with time. The average fluorescence values from the mayfly-specific amplicons are shown with SD to indicate the high variability in amplification as a likely consequence of differing mayfly genotypes combined with duration of digestion.

3.3. Wolf spiders had not fed on mayflies at the South River

No amplification of mayfly mtDNA was observed in any of the 110 wolf spiders collected near the South River during mayfly emergence (Fig. 2B). Using an Agilent 2100 Bioanalyzer that detects nucleic acids based on laser-induced fluorescence, no fluorescence was recorded above background levels (>2 FU), indicating that no amplification of the mayfly-specific mtDNA fragment had occurred. Spider mass averaged 0.118 g ± 0.148 (SD) (range: 0.022–0.808 g, n = 110), and all spiders were as large or larger than the spiders in the feeding trials that successfully preyed upon live, adult mayflies. To ensure that PCR results were interpreted correctly, a series of negative and positive controls were performed. Distal leg samples from 16 of the feeding trial spiders were used as negative controls, since spider gut contents do not extend into the distal leg segments (Foelix, 1996). None of the 16 negative controls exhibited amplification of the mayfly-specific fragment, indicating that the primers remained mayfly-specific despite the abundance of spider mtDNA, and that no mayfly DNA carryover occurred during DNA extraction. To assess the possibility of DNA contamination in the PCR master mixes, each 8-reaction master mix was used to run one negative control reaction with no DNA template; all were negative. Positive controls consistently demonstrated the efficacy of the mayfly-specific PCR, using mayflies directly (see Fig. S1) and spiders that had been experimentally fed mayflies (Fig. 2A).

4. Discussion

By designing mayfly-specific primers and testing them on adult mayflies, abdomens of spiders that had been experimentally fed live mayflies, and distal leg samples from the same spiders, we have demonstrated high sensitivity for mayfly mtDNA detection, even among proportionally overwhelming amounts of spider DNA. Furthermore, false priming was never observed to generate the approximately 206 bp amplicon; DNA sequencing confirmed that amplification from these primers remained mayfly-specific. Through this approach, any potential cross-amplification with other prey items would be identified, and all amplicons would be subject to molecular identification via NCBI GenBank. A survey of adult mayflies emerging during the same days as spider collection revealed that the primers allowed mtDNA to be amplified from the majority of collected samples (76%), despite a diversity of mayflies.

To assess whether mayfly mtDNA could be detected over extended periods of digestion time, feeding trials were conducted, showing that such detection was possible even after 192 h of digestion in live spiders. It should be noted that our feeding trials were conducted under conditions that may have prolonged retention of gut contents —food deprivation during captivity in an air-conditioned laboratory —so we have not established how long mayfly DNA would be retained in the guts of spiders feeding actively in hot temperatures under natural conditions. The objective of the feeding trials was as a proof-of-concept, and they clearly succeeded in demonstrating that our

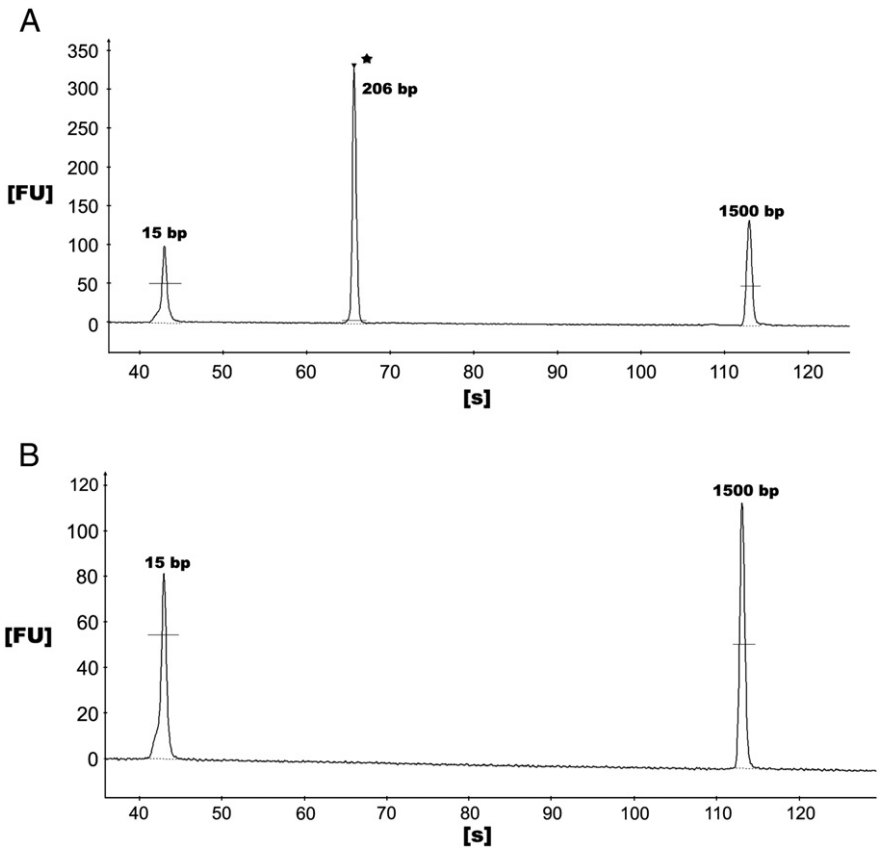

Fig. 2. (A) A representative positive control of a spider experimentally fed a mayfly with 24 h of digestion. The peak at 206 ± 5 bp is mayfly mtDNA amplified using the mayfly-specific primers. (B) Representative results for PCR amplification of sampled terrestrial spiders. As in all 110 collected spiders, no amplification is seen in this sample. Electrophoresis time (i.e. DNA fragment size) is plotted against fluorescence intensity (fluorescence units, FU). Peaks corresponding to 15 and 1500 bp are sizing markers.

technique allows detection of mayfly prey for a reasonable period of time after consumption. Our primary objective, however, was to test for the presence of mayfly mtDNA in the abdomens of the type of spider most responsible for delivering mercury to songbirds foraging on a contaminated river's floodplain.

We detected no mayfly prey in 110 sampled wolf spiders collected during mayfly emergence, suggesting that these spiders living in the floodplain of the mercury-contaminated South River were not feeding on mayflies during the days prior to collection. We therefore have failed to find evidence that emerging mayflies are an important source of dietary mercury for the spiders most commonly fed upon by songbirds at this site. Given the huge volume of mayflies emerging annually at this site, the high levels of mercury in both mayflies and wolf spiders, and the predominance of spider-derived mercury in the diets of songbirds here, rejecting this parsimonious hypothesis for mercury transport from river sediment to birds is an important step forward in understanding the movement of mercury in food webs.

The diversity of mayflies emerging from the South River created difficulty in designing primers that could account for their collective genetic variations. That our primers did not cover 24% of the collected mayflies is unsurprising considering that at least six different genera were present. However, a group-specific approach such as that presented here is useful with this level of mayfly diversity, since an overwhelming array of species-specific primers must be used in multiplex to achieve a similar result. Additionally, knowledge of the exact species and genotype of the target is not required for group-specific primers; any DNA that can be amplified can subsequently be sequenced to determine its genetic identity with taxonomically meaningful information (Ball et al., 2005). In this way, the effort required to map out the genetic diversity of mayfly populations, or to detect predation, is markedly decreased when compared to designing PCR primers or monoclonal antibodies for each species of interest (Sheppard and Harwood, 2005).

Amplification of mayfly DNA even after 192 h of digestion in spider gut was shown to be possible in feeding trials without an obvious correspondence between digestion time and detection ability, which is fortuitous considering the theoretically lowered efficiency of degenerate primers relative to normal oligonucleotides (Jabado et al., 2006). Ours is not the first PCR-based study of spider predation using feeding trials (Greenstone and Shufran, 2003). Others have demonstrated the detection of multiple-copy DNA (mtDNA or rDNA) from large prey (medfly, *Ceratitis capitata*) after digestion times up to 96 h (Monzó et al., 2009) and 120 h from smaller prey (diamondback moth larvae, *Plutella xylostella)* (Ma et al., 2005). Generally, amplified fragment length is significantly correlated with the ability to detect prey DNA; shorter amplicons yield stronger detection at longer digestion times (Agustí et al., 1999; Agustí et al., 2000; Hoogendoorn and Heimpel, 2001; Zaidi et al., 1999). With the efficiency of the mayfly-specific primers, the multiple-copy nature of mtDNA, and the amplified fragment length of approximately 206 bp, it is likely that the detection of mayfly DNA could be extended further than the 192 h demonstrated in the present study. For example, it may be that two mayflies with low homology to our primers were used, by chance, in the 216 h feeding trial, in which case it would mistakenly appear as though no mayfly mtDNA had been present. Detection of prey DNA despite 192 h (8 days) of digestion is consistent with the hypothesis that spiders can depress their metabolic rates during periods of starvation (Walker et al., 1999), from a resting rate that is already lower than many other comparable poikilotherms (Greenstone and Bennett, 1980).

Because the 110 wolf spiders we sampled were all negative for mayfly mtDNA, it is unlikely that, during our early summer sampling

period, emerging mayflies were a major pathway for aquatic mercury into wolf spiders. The mayfly and Lycosid samples used in the feeding trials were specifically collected at the same sites and time periods as the 110 spiders used to answer the question of whether wolf spiders had been feeding on mayflies. Since these feeding trials yielded many positive amplifications, in addition to the positive controls from individual mayflies, it is unlikely that PCR inhibition played a significant role in our negative findings from the 110 wolf spiders. The high sensitivity of the mayfly-specific primers coupled with their ability to identify 76% of emerging mayflies, and the persistence of mayfly DNA in the spider gut, should have led to detection of even a low level of mayfly predation for up to a week prior to spider collection. The finding that mayflies did not contribute to the diets of sampled wolf spiders does not discount the role of mayflies in the biomagnification of MeHg at the South River; their prevalence in the diets of tree swallows provides evidence of their direct transfer of mercury out of the river to at least one species of aerially foraging bird, and presumably other terrestrial vertebrates at this site feed on them as well (Brasso and Cristol, 2008). However, wolf spiders represent the most important source of mercury for at least three species of forest-dwelling songbirds that do not eat mayflies at this site.

Mayflies, because of their extreme abundance and elevated mercury levels, seemed a likely source of mercury for these spiders, but the results of this study do not support that hypothesis. There are several reasons that wolf spiders may not consume many mayflies. Mayflies are larger than some species of wolf spiders and remain airborne or in treetops for much of their short adult lives, so cursorial, ground-dwelling wolf spiders are less likely to prey on mayflies than arboreal web-weaving spiders. However, it is wolf spiders, not arboreal web-weaving spiders, that deliver most of the mercury to songbirds, so our objective was to determine whether wolf spiders acquire aquatic mercury from this abundant source. Other emergent aquatic insects are potential vectors of river sediment-derived mercury to wolf spiders, including caddisflies (Order Trichoptera), damselflies (Order Odonata), and stoneflies (Order Plecoptera). Further study of the diets of Lycosid and other cursorial spiders is necessary, and could utilize "blocking primers" to amplify any of their invertebrate non-spider prey (Vestheim and Jarman, 2008).

5. Conclusions

We demonstrated that emerging mayflies were not a major dietary source for MeHg in sampled Lycosid spiders. Since we did not link contamination of the river sediment to wolf spiders via mayfly emergence, this study should focus attention on the possibility that the river floodplain is the proximate source of MeHg for wolf spiders, and subsequently, songbirds. Floodplain soils are well-characterized sinks for anthropogenic toxicants, including heavy metals, and some hold large deposits of MeHg (Lair et al., 2010). The soils of the South River floodplain have enough mercury that the eroding river banks now serve as a significant non-point source of mercury back into the river (Eggleston, 2009; Flanders et al., 2010). Ruling out the direct transfer of river sediment MeHg from emerging mayflies to wolf spiders is an important step in narrowing the possible sources and routes of exposure of bioavailable mercury to the terrestrial food web. Choosing the optimal method of environmental remediation requires knowledge of the proximate source and exposure route. If wolf spiders were eating emergent aquatic insects contaminated from river bottom sediments, remediation efforts focused on the river channel would reduce exposure of songbirds in the surrounding forest. However, if spiders obtain MeHg from terrestrial floodplain soils via plants and terrestrial invertebrates, river channel remediation will not affect mercury exposure of forest songbirds and other techniques will be required (Wang et al., 2004).

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