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RESEARCH ARTICLE

Diet reconstruction using next-generation sequencing increases the known ecosystem usage by a shorebird

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ABSTRACT

Molecular scatology and next-generation sequencing identified previously unknown linkages among ecosystems in the diet of Semipalmated Sandpipers (*Calidris pusilla*) in the Bay of Fundy, Canada. During their annual migratory stopover, the birds consumed a wider range of prey items than previously reported, which suggests that they are not selecting for the amphipod *Corophium volutator* and are acting as generalist foragers. Our analysis identified several novel prey items—arachnids, crabs, bivalves, several terrestrial and freshwater insect species, ctenophores, cnidarians, and fish (likely eggs or juveniles)—indicating that Semipalmated Sandpipers consume prey from marine, freshwater, and terrestrial ecosystems. Connections between Semipalmated Sandpipers and freshwater and terrestrial ecosystems were previously unknown in the Bay of Fundy. Current conservation efforts for this species are focused on beach and intertidal habitats; however, we may also need to consider the surrounding freshwater and terrestrial habitat.

Keywords: 454 pyrosequencing, Bay of Fundy, conservation, diet, molecular scatology, next-generation sequencing, Semipalmated Sandpiper, shorebirds

La reconstrucción de la dieta usando secuenciación de última generación aumenta el conocimiento del uso de ecosistemas por parte de un ave playera

RESUMEN

La escatología molecular y la secuenciación de última generación identificaron vinculaciones previamente desconocidas entre ecosistemas en la dieta de *Calidris pusilla* en la Bahía de Fundy, Canadá. Durante sus paradas migratorias anuales, los chorlos consumieron un rango más amplio de presas de lo anteriormente supuesto, sugiriendo que no están seleccionando el anfípodo *Corophium volutator,* y que están comportándose como forrajeros generalistas. Nuestro análisis identificó varias presas nuevas—arácnidos, cangrejos, bivalvos, varias especies de insectos terrestres y de agua dulce, tenóforos, nidarios y peces (probablemente huevos o juveniles)—indicando que los chorlos consumen presas de ecosistemas marinos, de agua dulce y terrestres. Las vinculaciones entre los chorlos y los ecosistemas de agua dulce al igual que los terrestres eran previamente desconocidas en la Bahía de Fundy. Los esfuerzos actuales de conservación dirigidos a los chorlos se enfocan en los hábitats playeros e intermareales; sin embargo, también es posible que necesitemos considerar los alrededores de los hábitats de agua dulce y terrestre.

Palabras clave: 454 pirosecuenciación, aves playeras, Bahía de Fundy, *Calidris pusilla*, conservación, dieta, escatología molecular, secuenciación de última generación

INTRODUCTION

Most migratory shorebirds exhibit strong dietary flexibility (Hicklin and Smith 1979, Pienkowski et al. 1984, Skagen and Oman 1996, Skagen 2006). This is not surprising given the variety of prey items that long-distance migrants encounter. Food quality at migratory stopover sites is important because birds use these areas to accumulate fat prior to the next leg of their migration (Skagen 2006, Niles et al. 2009, Smith et al. 2012). When birds specialize on particular prey items at these stopover areas, they become

vulnerable to changes in the food base (Skagen 2006, Niles et al. 2009). For example, Red Knots (*Calidris canutus*) that forage on horseshoe crab eggs (*Limulus polyphemus*; Tsipoura and Burger 1999, Karpanty et al. 2006) have experienced substantial population declines as a result of human overexploitation of horseshoe crabs (Niles et al. 2009).

The Semipalmated Sandpiper (*C. pusilla*) is an Arctic-breeding shorebird that undergoes a long-distance fall migration to wintering grounds in South America. Much of the population is thought to complete this migration

with a single stop in the Bay of Fundy, Canada (Gratto and Cooke 1987, Hicklin 1987, Gratto-Trevor et al. 2012). While in the region, these birds feed extensively on infaunal prey found on intertidal mudflats (Hicklin 1987, Hamilton et al. 2006, Gratto-Trevor et al. 2012). Historical studies using stomach content analysis suggested that while in the Bay of Fundy, Semipalmated Sandpipers foraged preferentially on the amphipod *Corophium volutator* (Hicklin and Smith 1979, 1984). Thus, like Red Knots, Semipalmated Sandpipers might be vulnerable to changes in their prey base. However, recent work using stable isotope analysis and behavioral observation has revealed that Semipalmated Sandpipers consume a broader range of intertidal prey items than previously reported (MacDonald et al. 2012, Quinn and Hamilton 2012). The results of these studies suggest a diet composed of a variety of polychaete worms, biofilm, *C. volutator*, and ostracods, which matches what we know about the foraging ecology of Semipalmated Sandpipers in general. These shorebirds exhibit a broad diet on their breeding grounds in the Arctic (Holmes and Pitelka 1968, Baker 1977) and, over the course of their annual cycle, consume prey items from at least 25 orders and 37 families of invertebrates (Skagen and Oman 1996, Skagen 2006). Although differing methodology makes it impossible to directly compare historical and current diets, perceived changes over time in Semipalmated Sandpipers' diet in the Bay of Fundy match a general decline in abundance of *C. volutator* at some sites (while other sites increased or remained stable), with concurrent increases in other prey such as polychaete worms (see data in and compare the following: Yeo 1977, Gratto 1979, Peer et al. 1986, Wilson 1988, Shepherd et al. 1995, Barbeau et al. 2009, Gerwing et al. 2015). The observed temporal shift in their diet suggests that Semipalmated Sandpipers are probably feeding as generalists according to prey availability while in the Bay of Fundy, as they do elsewhere during their annual cycle.

Semipalmated Sandpipers in the Bay of Fundy typically feed when the mudflat is exposed, and rest on shore during high tide. Until now, we have assumed that their diet is fully intertidal-based; however, given their opportunism and broad diet, there is no inherent reason why they would not take prey of other origins if given the opportunity. Diet opportunism is common in other shorebirds, such as the Western Sandpiper (*C. mauri*), Least Sandpiper (*C. minutilla*), Pectoral Sandpiper (*C. melanotos*), Lesser Yellowlegs (*Tringa flavipes*), and Killdeer (*Charadrius vociferus*) (Davis and Smith 2001, Smith et al. 2012, Jardine et al. 2015), as well as in birds generally (Poulin et al. 1994). Thus, opportunistic foraging by Semipalmated Sandpipers could generate links between this species and multiple ecosystems.

Unfortunately, existing methods of diet assessment such as gut content analysis and stable isotope analysis do not lend themselves to identification of unexpected prey items. Morphological methods suffer from misidentification of similar prey items, overrepresentation of hard-to-digest prey items, and underrepresentation of easily digested prey items (Barrett et al. 2007, Deagle et al. 2007, Pompanon et al. 2011). Stable isotope or fatty acid analyses offer an assessment of diet integrated over an extended period but rely on a priori knowledge of an animal's diet (Deagle et al. 2005, 2007, Casper et al. 2007, Pompanon et al. 2011, Quinn and Hamilton 2012). Therefore, we employed molecular scatology, the use of molecular techniques to identify prey DNA in fecal samples (Barrett et al. 2007, Deagle et al. 2009, Pompanon et al. 2011), as a way of gaining greater insight into the breadth of the Semipalmated Sandpiper diet. Assessing diet via molecular scatology offers a noninvasive and high-resolution snapshot of a predator's diet, even when prey items are degraded (Kohn and Wayne 1997, Deagle et al. 2006, Pompanon et al. 2011).

Populations of most North American shorebirds, including Semipalmated Sandpipers, are declining (Bart et al. 2007, Gratto-Trevor et al. 2012, Galbraith et al. 2014). Because an animal's diet influences every aspect of its biology, understanding diet is essential for conservation and management plans (Fryxell et al. 2014). Full knowledge of Semipalmated Sandpipers' diet on stopover grounds is particularly important because the primary function of these stops is to refuel for the next leg of migration (Davis and Smith 2001, Skagen 2006, Smith et al. 2012). The results of the present study will enhance our understanding of dietary breadth in Semipalmated Sandpipers during their time at this very important stopover region.

METHODS

Study Sites and Sample Collection

We collected fecal samples from Semipalmated Sandpipers in summer 2010 on 3 intertidal mudflats in the upper Bay of Fundy, Canada (Figure 1): Avonport (AV), Grande Anse (GA), and Mary's Point (MP). To assess temporal variability in diet, we sampled at 2 points in the birds' stopover period: August 3–5 and August 22–23. Fecal samples were collected on a receding tide approximately 1–2 hr after the birds ceased roosting and commenced foraging on the mudflat, thus ensuring that we obtained only fresh fecal samples (in that feces dropped before high tide would have been washed away) and potentially allowing us to detect prey obtained during roosting and mudflat foraging. All fecal samples were from the upper intertidal zone, which facilitated clear observation of the birds from shore and quick access to samples as soon as the birds had moved out of the area. Flocks were observed to ensure that fecal samples were collected only from
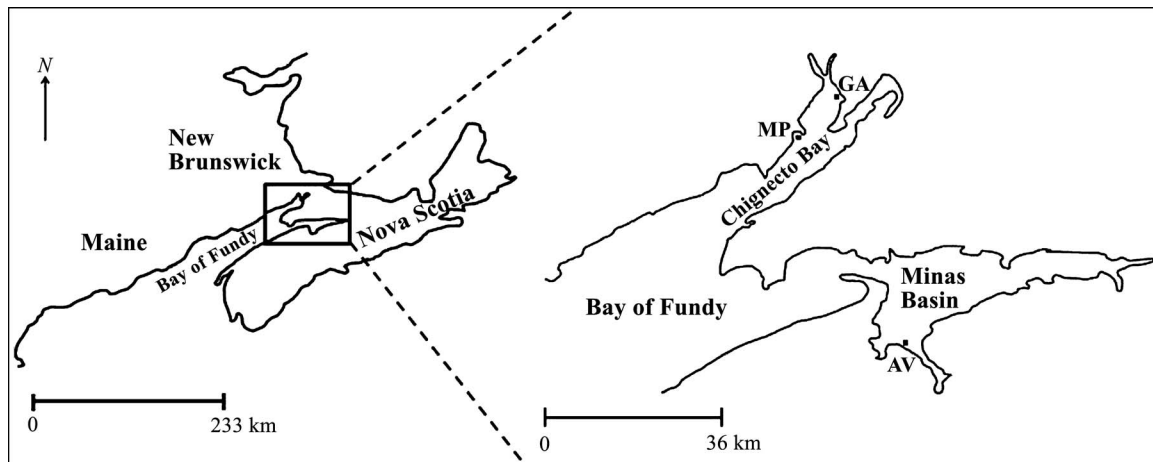
FIGURE 1. Intertidal mudflat sites where fecal samples of Semipalmated Sandpipers were collected during August 2010. Site names: AV = Avonport, GA = Grande Anse, and MP = Mary's Point.

flocks composed primarily of Semipalmated Sandpipers. Although other species of shorebirds were present, Semipalmated Sandpipers typically make up ~95% of shorebirds using the region (Hicklin and Smith 1979, Hicklin 1987). We collected, with sterilized tweezers, 50–75 fresh fecal samples (the brown digested matter at the center and on top of the uric acid), being attentive not to touch the mudflat surface with the tweezers. Each fecal sample was stored in 95% ethanol immediately upon collection and frozen ($-20°C$) within 12 hr. Samples remained frozen until DNA extraction.

Primer Design and DNA Preparation
Given that DNA extracted from feces is often degraded (Kohn and Wayne 1997, Deagle et al. 2006) and that molecular analyses based on a single locus can result in variable coverage of diet composition (Bowser et al. 2013), we used universal primer pairs that targeted short fragments (130–300 base pairs [bp]) of 2 mitochondrial genes: cytochrome *c* oxidase subunit 1 (*COI*; Meusnier et al. 2008) and the 16S ribosomal RNA subunit (*16S*; Deagle et al. 2007). We used a pooled massively parallel sequencing (MPS) approach described by Puritz et al. (2012). To facilitate the recovery and identification of sequences obtained from individual fecal samples, we included a 10-base multiplex identifier (MID) tag between the Lib-L 454 sequencing adapter (26 bp plus a 4 bp signal calibration key) and the universal primer (*16S* or *COI*) in our custom engineered forward and reverse primers. Details of the primer design, including the MID tags used, can be found in Bowser et al. (2013).

Ethanol was removed from the fecal samples by decanting following 30 min of centrifugation at 4°C. DNA was extracted with QIAamp DNA Stool Mini Kit (Qiagen, Toronto, Ontario, Canada) following the manu-

facturer's protocol. Samples with small amounts of fecal material were eluted with 75–100 μL of buffer AE instead of the recommended 200 μL to increase DNA concentration in the extracted sample. DNA was stored in 2 mL microcentrifuge tubes at $-20°C$ until used for *16S* and *COI* gene amplification. Fecal samples can contain significant amounts of DNA from the host species as a result of the sloughing of cells from the digestive tract (Shehzad et al. 2012). However, we did not attempt to block the amplification of the host DNA during polymerase chain reaction (PCR), because several species of shorebirds forage on the mudflats, and we used the traces of host DNA to exclude samples that did not come from Semipalmated Sandpipers (see below).

454 Library Preparation
Amplification of fecal DNA with *16S* MID-tagged sequencing primers was achieved in 20 μL reactions containing 5 μL undiluted template fecal DNA, 0.2mM dNTP (New England Biolabs, Whitby, Ontario), 1X bovine serum albumin (New England Biolabs), 5 mM $MgSO_4$ (Life Technologies, Burlington, Ontario), 0.5 μM of each primer (Integrated DNA Technologies, Coralville, Iowa, USA), 1X High Fidelity Buffer (Life Technologies), and 1 unit of Platinum *Taq* DNA Polymerase High Fidelity (Life Technologies). The thermocycling protocol for *16S* began at 94°C for 2 min followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 68°C for 45 s, with a final extension of 68°C for 5 min (C-1000 Thermal Cycler, Bio-Rad, Mississauga, Ontario). Amplification with *COI* followed similar component and cycling conditions as the *16S*, but the annealing temperature was dropped to 45°C. Successful amplification of individual PCR samples was verified by electrophoresis in 1.5% agarose and visualized under ultraviolet light using SYBR Safe (Life Technologies).

Amplicons were purified using the QIAEX II Gel Extraction Kit (Qiagen), and the concentration was determined with dsDNA BR Assays on a Qubit 2.0 Fluorometer (Life Technologies). Samples with amplicon concentrations <1 ng μL^{-1} were reamplified using the same PCR conditions. Finally, the pooled library was prepared by combining 20 ng of each of the *16S* and *COI* amplicons and concentrating to 35 ng μL^{-1} in 150 PCR-grade H_2O using the DNA Clean & Concentrator-100 (Zymo Research, Irvine, California, USA). The library was sequenced unidirectionally on half a pico titre plate using the Roche GS-FLX (454) platform at the Genome Quebec Innovation Centre (McGill University, Montreal, Quebec, Canada).

454 Data Sorting and MOTU Identification

Raw reads (DNA sequences) were imported to the CLC Genomics Workbench (Qiagen) for downstream sequence analyses. The library was first sorted by MID tag and then by locus based on the first 5 bases of the primer (GACGA for *16S*; TCCAC for *COI*). Following the trimming of the MID tag and primers, reads were discarded if (1) there were more than 2 ambiguous nucleotides, (2) they had a minimum quality score >0.01 (equivalent to a Phred quality score of 20), and (3) they were <30 nucleotides. Duplicate reads within a sample were removed to increase the representation of unique reads.

The reads were assembled into contigs using the default de novo assembly parameters. Both contigs and singletons were considered "molecular operational taxonomic units" (MOTUs) and were searched in the nucleotide database of the National Center for Biotechnology Information (NCBI; U.S. National Library of Medicine, Bethesda, MD, USA) using the Basic Local Alignment Search Tool for nucleotide sequence alignments (BLASTn) through the CLC Genomics Workbench. We used the following criteria to identify candidate species in the fecal DNA samples: (1) 50-nucleotide minimum length of query sequence, (2) 90% minimum fraction on length consensus between top hit and query sequences, (3) 90% minimum identity between top hit and query sequences, and (4) 60 minimum bit score of top hit sequence. Finally, the identification of each MOTU was accomplished by matching the top-hit *16S* or *COI* MOTU with 69,614 genera and 305,936 species that were registered to the taxonomy database of the NCBI as of July 2014. A list of all animal prey items detected within each fecal sample is presented in Supplemental Material Table S1.

Identification of *Corophium volutator* in Fecal Samples

Although our main goal was to assess the breadth of the Semipalmated Sandpipers' diet, we also wanted to evaluate the prevalence of *Corophium volutator* in their diet. Both empirical and bioinformatics analyses indicated a mis-match between the *COI* primers and the sequence in *C. volutator*. There was an adequate match with the *16S* primers, but the public database did not contain *Corophium* spp. Therefore, *16S* sequences and our BLASTn searches failed to identify *C. volutator* in any fecal sample. To probe our library further, we sequenced the short universal *16S* from 7 different *C. volutator* DNA extractions and constructed our own species-specific reference sequence for this locus. A similar database was not constructed for other infauna (e.g., the polychaetes Nereididae and Nephtyidae; Gerwing et al. 2015) because the public database contained sequences from closely related species, and no mismatches between primers and the available sequences were detected. Using the same settings in CLC Genomics Workbench described above, we aligned all demultiplexed and quality-trimmed *16S* reads and *16S* MOTUs (contigs + singletons) to our *C. volutator* *16S* consensus. As a result, we found 67 of the 1,775 *16S* contigs and 867 of the 6,146 *16S* singletons (11.8% of the 7,896 *16S* MOTUs) matched to the *16S* consensus, positively identifying >100 fecal samples as containing *C. volutator* DNA.

Prey Bins

To ease interpretation and increase confidence in prey identities, DNA sequences were placed into prey bins.

Fecal Samples with and without Semipalmated Sandpiper DNA

Semipalmated Sandpipers always dominated flocks of foraging shorebirds; however, the possibility of collecting feces from other species remained. To assess this, we examined fecal samples for the presence of Semipalmated Sandpiper DNA, as well as that of other shorebirds. Among a total of 389,328 sequences, we detected 16,836 non–Semipalmated Sandpiper avian DNA sequences in 54 fecal samples; therefore, these samples were excluded from all further analysis. To determine whether it would be appropriate to include the samples that contained no DNA of any bird species in our investigation of the Semipalmated Sandpiper diet, we compared prey items found in fecal samples with Semipalmated Sandpiper DNA to those with no bird DNA, using an analysis of similarities (ANOSIM) in the statistical program PRIMER with the PERMANOVA (permutational multivariate analysis of variance) add-on (McArdle and Anderson 2001). A diet matrix was constructed using presence–absence of each prey item (in prey bins) found in feces samples for *COI* and *16S* (individually and combined), using the Bray-Curtis coefficient.

Data Analysis of Binned Prey Items Determining Semipalmated Sandpiper Diet

Data from both *16S* and *COI* loci were combined to maximize prey identification (not all samples were

TABLE 1. Summary of the number of successful amplifications of prey DNA in fecal samples of Semipalmated Sandpipers collected in August 2010, for *16S* and *COI* and the 2 loci combined, excluding all samples that contained non–Semipalmated Sandpiper avian DNA. For site names, see Figure 1.

| | Sample date | | Sample size | | | | | |
| | | | *16S* | | *COI* | | Combined | |
Site	Early	Late	Early	Late	Early	Late	Early	Late
AV	August 3	August 22	29	20	23	20	30	26
GA	August 2	August 23	26	8	23	5	28	24
MP	August 5	August 23	29	24	21	13	33	23

amplified for both loci; Table 1). Frequency of occurrence (FOO; number of fecal samples that a prey item appeared in, divided by total number of samples) was calculated for each prey bin.

PRIMER was also used to examine temporal and spatial variability in the Semipalmated Sandpiper diet. A PERMANOVA was conducted with site and time as random factors. The resemblance matrix was constructed from prey item presence–absence data using the *16S* and *COI* loci as described above for the ANOSIM. We set $\alpha = 0.05$ a priori to assess statistical significance. Following a significant site $\times$ time interaction (see below), we ran a one-way PERMANOVA with all site–time combinations as levels of a single independent variable. Finally, variance components (Searle et al. 1992, Anderson et al. 2008) were calculated to determine how much of the observed variation in the Semipalmated Sandpiper diet was due to spatiotemporal factors.

RESULTS

The ANOSIM detected no differences in diet between fecal samples with Semipalmated Sandpiper DNA and without any bird DNA (*16S*: Semipalmated Sandpiper DNA, $n = 47$; no bird DNA, $n = 101$; $R = 0.04$, $P = 0.18$. *COI*: Semipalmated Sandpiper DNA, $n = 109$; no bird DNA, $n = 162$; $R = -0.008$, $P = 0.65$. Both loci: Semipalmated Sandpiper DNA, $n = 110$; no bird DNA, $n = 173$; $R = 0.001$, $P = 0.87$). Therefore, we included in subsequent analyses all samples that contained either Semipalmated Sandpiper DNA or no bird DNA.

The pooled library generated 389,328 total reads, of which 118,253 unique high-quality sequences were used for analysis after samples containing non–Semipalmated Sandpiper avian DNA and low-quality DNA reads were removed (Table 2). We assembled 7,921 and 26,189 unique MOTUs from the *16S* and *COI* data, respectively. BLASTn analysis of these data identified 132 unique prey taxa in the Semipalmated Sandpiper diet (Table 3), not only from marine (intertidal and pelagic) systems, but also from freshwater and terrestrial ecosystems (to compare FOO of main prey items by locus, see Supplemental Material Table S2). The diet varied over time and space (Table 4); however, the majority of variation was observed between fecal samples (residual in the second analysis: 80%), whereas variation in diet between sites and times accounted for 20% of the observed variation.

Diatoms were present in every sample, and the second most common prey item was *C. volutator*, which was present in >65% of samples from all except one mudflat–time combination (MP; August 5, 2010). Arachnida, Ostracoda, Nemertea, and Nematoda had consistently low FOO, and the remaining prey items generally had higher but variable FOO. The FOO and number of prey items varied over time and space, though there were no systematic temporal trends evident across locations. The most notable variability was at MP during the early sampling period, where Semipalmated Sandpipers appeared to have a substantially different diet than at any other location or time: Relatively few individuals consumed *C. volutator*; use of copepods was much higher than anywhere else; use of various insects increased; and one-

TABLE 2. Total numbers of reads (with percentage of total in parentheses) and numbers of molecular operational taxonomic units (MOTUs) identified by the analysis of 389,328 raw reads sequenced from the pooled library for prey items in feces of Semipalmated Sandpipers collected from the Bay of Fundy in August 2010.

| | Reads remaining after filtering | | De novo assembly | | |
Locus	Demultiplexing (MID tags)	Quality control, duplicate removal	Contigs	Singletons	Total MOTUs
16S	138,418	32,280	1,775	6,146	7,921
COI	235,675	85,973	9,095	17,094	26,189
Total	374,093 (96.1%)	118,253 (30.4%)			

TABLE 3. Frequency of occurrence (%) of prey items in feces of Semipalmated Sandpipers, identified by the combination of 2 DNA loci (the mitochondrial *COI* and *16S*; Table 1) in the Bay of Fundy in August 2010. Total represents the frequency of occurrence pooled over sites and times.

Prey bin	Total	Avonport		Grande Anse		Mary's Point	
		August 3	August 22	August 2	August 23	August 5	August 23
Diatoms	100	100	100	100	100	100	100
Insecta: Diptera	16.5	10.0	15.4	3.7	0	33.3	4.3
Insecta: Caddisflies, mayflies, and damselflies	10.4	16.7	19.2	14.8	0	54.5	0
Insecta: Other terrestrial	13.4	10.0	15.4	14.8	0	9.1	17.4
Insecta: Other marine	7.3	3.3	0	0	0	21.2	0
Arachnida	6.7	0	11.5	7.4	8.3	0.0	13.0
Crustacea: Amphipoda: *Corophium volutator*	85.4	83.3	65.4	96.3	95.8	18.2	91.3
Crustacea: Amphipoda: Other	6.7	3.3	3.8	0	4.2	33.3	21.7
Crustacea: Ostracoda	7.9	3.3	0	0	0	12.1	4.3
Crustacea: Copepoda	7.3	13.3	3.8	7.4	0	84.8	4.3
Crustacea: Other	12.2	3.3	15.4	11.1	8.3	9.1	26.1
Annelida	18.9	13.3	11.5	14.8	8.3	6.1	21.7
Bivalvia	8.5	36.7	7.7	0	0	3.0	0
Gastropoda	31.1	73.3	46.2	3.7	0	9.1	21.7
Cnidaria and Ctenophora	9.8	13.3	11.5	3.7	0	3.0	8.7
Fish	16.5	26.7	30.8	7.4	8.3	12.1	17.4
Nemertea	1.8	0	0	0	0	6.1	4.3
Nematoda	1.2	0	0	0	0	33.3	0

third of the Semipalmated Sandpipers consumed nematodes, which were never present in the diet elsewhere or at other times (Table 3). The number of different prey items (binned) that were consumed was relatively consistent across sites and times, though there was a trend at GA for use of previously unrecognized prey items to be less frequent, especially in the late sampling period, when the diet appeared to be more restricted. The diet at AV exhibited a higher FOO of bivalves, gastropods, and fish, during both periods, compared with MP and GA. Overall,

we observed several prey items not previously identified for this species in the Bay of Fundy: arachnids, crabs, bivalves, several terrestrial and freshwater insect species, fish (likely eggs or juveniles), and cnidarians–ctenophores.

DISCUSSION

Diet Breadth and Variability
Fecal samples from Semipalmated Sandpipers staging in the Bay of Fundy contained a wide variety of prey items

TABLE 4. Results of PERMANOVA investigating whether the diet of Semipalmated Sandpipers varied over time and space at 3 intertidal mudflats (sites AV, GA, and MP; see Figure 1) in the Bay of Fundy, Canada. Diet was assessed during 2 periods in 2010 at each site: "early" (August 2–5) and "late" (August 22–23). Prey items were identified via presence–absence in Semipalmated Sandpiper feces, using the *16S* and *COI* mitochondrial loci. In the first analysis (A), site and time are random factors. In the second analysis (B), the random factor "site–time" combines site and time in a single factor with 6 levels (AV$_{Early}$, AV$_{Late}$, GA$_{Early}$, GA$_{Late}$, MP$_{Early}$, MP$_{Late}$); 999 permutations were conducted for this second analysis. Significant and interpretable sources of variation are in bold ($\alpha = 0.05$).

(A)

Source	df	MS	Pseudo-*F*	Permutations	*P*	
Site	2	24,428	5.39	329	0.132	
Time	1	6,557	1.45	338	0.355	
Site × time	2	4,530	2.60	999	**0.010**	
Residual	158	1,741				
Total	163					

(B)

Source	df	MS	Pseudo-*F*	*P*	Variance component estimate	Variance components (%)
Site–time	5	13,382	7.69	**0.001**	**20.7**	**19.7**
Residual	158	1,741			**41.7**	**80.3**
Total	163					

and varied over time and space (Tables 3 and 4). In the past, it was thought that Semipalmated Sandpipers foraged preferentially on *C. volutator* in this region (Hicklin and Smith 1979, 1984), and we did observe *C. volutator* in the majority of fecal samples. However, this does not suggest selection or preference, because *C. volutator*, while perhaps not as abundant as it once was (Yeo 1977, Gratto 1979), is still the most abundant infaunal resident of these mudflats (Gerwing et al. 2015). A high FOO of the most common prey item would be expected if Semipalmated Sandpipers are generalist foragers. Further, the low FOO of *C. volutator* in Semipalmated Sandpiper feces at MP on August 5 (see below) supports our view that Semipalmated Sandpipers are not dependent on this intertidal amphipod. The generalist foraging detected in the present study is consistent with results of recent studies of the Semipalmated Sandpiper diet in the Bay of Fundy (MacDonald et al. 2012, Quinn and Hamilton 2012), with the species' diet while breeding in the Arctic (Holmes and Pitelka 1968, Baker 1977), and with the breadth of prey items consumed over the species' annual cycle (Skagen and Oman 1996, Skagen 2006).

The fact that Semipalmated Sandpipers appear to be generalist foragers in the Bay of Fundy is encouraging in a conservation context. Populations of most North American shorebirds, including Semipalmated Sandpipers, are declining. These declines are partially a product of anthropogenic alteration of key shorebird habitat (Bart et al. 2007, Gratto-Trevor et al. 2012, Galbraith et al. 2014), especially migratory stopover sites (Davis and Smith 2001, Skagen 2006, Smith et al. 2012). The broad diet we documented may suggest that these birds will be relatively resilient to changes in their environments, given that diet generalists are more resistant to changing environmental conditions than specialists (Colles et al. 2009).

The observed variation in the Semipalmated Sandpiper diet over space and time (Tables 3 and 4) was not surprising. Jardine et al. (2015) observed that the diet of Western Sandpipers varied between foraging sites located within 100 km of each other along the west coast of Canada. Similarly, Davis and Smith (2001) reported that diets of several shorebirds varied over time within the same migratory stopover point. In our study, although there was substantial spatiotemporal diet variability, the majority of it was between individuals. Some of the observed variability in the Semipalmated Sandpiper diet is likely linked to variation among sites in prey abundance (see data in Gerwing et al. 2015) and suitability. For example, the Semipalmated Sandpiper diet at AV frequently included gastropods, but these were relatively rare prey items at MP or GA. The mudsnail *Nassarius obsoletus*, although present at all the sites, would not normally be considered a potential prey item for Semipalmated Sandpipers because of its size and protec-

tive shell. However, during August 2010 there was an unusually high density of very small juvenile snails in the upper intertidal of AV (T. G. Gerwing personal observation), which could easily be consumed by foraging Semipalmated Sandpipers; this may explain the higher FOO of Gastropoda at AV during August.

Conversely, other observed foraging patterns appear to be unrelated to prey availability. At MP on August 5, 2010, the FOO values of *C. volutator* and of copepods were very low and high, respectively, compared with other sites and with that site later in the season. This was not matched by the availability of these prey items, given that *C. volutator* was very abundant ($\sim$80,000 individuals m^{-2}) and copepods were also readily available ($\sim$20,000 individuals m^{-2}) at that time (Gerwing et al. 2015). Thus, availability of prey did not drive this observed variation in the Semipalmated Sandpiper diet. It is possible that the sampling in early August followed an unusual foraging bout for Semipalmated Sandpipers, in which they fed in a localized area that was not representative of the prey availability on the broader mudflat. More detailed prey sampling in specific areas where birds were observed feeding would be required to clarify this point. However, our findings do highlight the fact that the species' diet can vary widely within a relatively short period, again pointing to foraging flexibility.

Our analysis identified diatoms in all fecal samples, and there is other evidence that Semipalmated Sandpipers consume diatoms in this system (MacDonald et al. 2012, Quinn and Hamilton 2012). Similarly, biofilm has been identified as a major diet component in numerous other small-bodied sandpipers (Kuwae et al. 2012, Jardine et al. 2015). Although we were careful not to contact the sediment when collecting feces, we cannot exclude the possibility that diatom contamination occurred when feces contacted the sediment. Therefore, although our results are consistent with those of other studies with respect to diatom consumption, they should not be viewed as an independent confirmation.

Use of Multiple Ecosystems

Notably, we detected a variety of terrestrial, freshwater, and pelagic taxa in the diet of Semipalmated Sandpipers. Although links to nonmarine systems have not previously been identified for Semipalmated Sandpipers while in the Bay of Fundy, they may not be new. Hicklin and Smith (1979) observed unknown insects in 7.1–66.6% of Semipalmated Sandpiper gut contents in the Bay of Fundy, but these insects accounted for only 0.1–1.4% of Semipalmated Sandpiper gut content volume. Gut content analyses can miss rare prey and can skew results, depending on time of collection, digestibility, and the presence of identifiable parts that resist digestion (Deagle et al. 2005, Barrett et al. 2007, Pompanon et al. 2011). On the basis of our findings,

we suggest that nontraditional prey items, including those from other ecosystems, make up a variable but nontrivial component of the Semipalmated Sandpiper diet and merit further investigation.

Freshwater prey items (mayflies, caddisflies, and damselflies) may have washed onto the mudflats through small streams that discharge into the Bay of Fundy. Semipalmated Sandpipers also frequently forage near these streams in the upper intertidal area, potentially picking up such items before reaching the mudflats. Variability in FOO (0–54%; average = 16.5%) suggests that freshwater insects are taken opportunistically and may be more accessible at some sites or times than at others, possibly depending on insect life stage. Terrestrial prey items, on the other hand, are probably consumed opportunistically during roosting (T. G. Gerwing personal observation) or as birds are moving off the beach toward mudflats as the tide starts to recede. The FOO values for terrestrial items were usually low, all <20% except for Diptera on one occasion (Table 3). Therefore, terrestrial prey items appear to be minor but consistent components of the Semipalmated Sandpiper diet, present in at least some birds at all sites and times. Pelagic prey items (Atlantic herring, flounder, unknown fish, cnidarians–ctenophores) likely consist of eggs or the smallest juveniles, along with detrital remains deposited on the mudflats as the tide recedes and then consumed opportunistically.

Novel Prey Items

Using molecular scatology, we observed several dietary items (arachnids, crabs, bivalves, several terrestrial and freshwater insect species, fish, and cnidarians–ctenophores) not previously identified as prey. Although Hicklin and Smith (1979) observed the gastropod *Hydrobia totteni* in Semipalmated Sandpiper gut contents, we found DNA from several gastropod taxa (*Littorina* spp., *Hydrobia* spp., *Nassarius obsoletus*). In some cases, snails were relatively common in the Semipalmated Sandpiper diet (FOO: 0–73.3%; average = 26.4%), which suggests that snails may occasionally comprise the bulk of prey consumed during a foraging bout. Their consumption probably depends on the availability of appropriately sized individuals, because the bill of the Semipalmated Sandpiper is too small to consume any but the smallest gastropods. Other novel prey were less common; for instance, crab DNA appeared in only 5 fecal samples, 3 with invasive green crab (*Carcinus maenas*) and 2 with native *Cancer* spp. It is likely that birds consumed crab eggs or pieces of crab in detritus. Bivalves, another novel prey item, were consumed sporadically, mostly at AV. As with snails, the availability of very small bivalves probably determined consumption; however, Semipalmated Sandpipers also ingest bivalves as detritus, or in potentially nonlethal consumption of bivalve siphons (Skilleter and Peterson 1994, Maire et al. 2010).

Finally, our study was the first to detect fish (Atlantic herring, flounder, and unknown fish) and cnidarians–ctenophores in Semipalmated Sandpiper diets, albeit at low frequencies at all sites. It is likely that fish eggs or the smallest juveniles, along with detrital remains of both groups of animals, are consumed by Semipalmated Sandpipers.

Methodological Limitations

Molecular scatology can often detect the prey items of prey items, a phenomenon referred to as "secondary consumption" (Deagle et al. 2005, 2007, Barrett et al. 2007, Pompanon et al. 2011). It is therefore possible that some prey items may have been detected indirectly; however, we suggest that secondary consumption did not greatly influence our results. Prey at low trophic levels (diatoms and the invertebrates that consume them, such as *C. volutator*) would be more likely to be detected through secondary consumption because they are food for higher consumers such as annelids. However, these low-trophic-level taxa are already well established in the diet of Semipalmated Sandpipers (Baker 1977, Hicklin and Smith, 1979, 1984, Quinn and Hamilton 2012). Further, the FOO of lower-level consumers was much higher than that of the most likely sources of secondary consumption. Finally, some of the more unusual prey items (e.g., insects, bivalves, snails) are unlikely to have been consumed, except perhaps as detritus, by animals that Semipalmated Sandpipers commonly eat.

Molecular scatology is also limited by the presence–absence data it produces, and by the reference databank available for comparison. Bay of Fundy intertidal infauna were underrepresented in the NCBI database, which forced grouping into higher taxonomic levels to ensure confidence in identification. This introduced complications for taxa like nematodes, which may have been either free-living species consumed by birds or parasites of prey items or of the birds themselves. A more complete reference library of DNA sequences in this region, especially for infaunal prey items, is required to clarify the dietary relationships reported here.

Finally, we collected feces shortly after birds completed roosting near shore. This maximized our ability to detect prey of all origins, but the overall frequency of these novel prey in the Semipalmated Sandpiper diet throughout the feeding cycle is unknown. The timing of collection may have resulted in the low FOO of annelids (polychaetes), which have been found to contribute substantially to the diet of Semipalmated Sandpipers in other studies (MacDonald et al. 2012, Quinn and Hamilton 2012). It also may have inflated the importance of some novel prey items that are most likely to be detected following periods when birds were near shore. Collecting fecal samples from throughout the tidal cycle would help clarify the relative importance of

both newly observed prey items and previously recognized prey.

Conclusion

Notwithstanding the limitations discussed above, our results reveal a generalist diet and links between Semipalmated Sandpipers and nontraditional environments. While encouraging with respect to this species' ability to adapt to a changing prey base, use of these novel prey items also raises new ecological questions and conservation considerations. Disturbances or contamination of prey from freshwater (Kraus et al. 2014), terrestrial (Hallmann et al. 2014), or marine systems (Simmons et al. 2014) could affect Semipalmated Sandpipers. Therefore, the magnitude of the relationships observed here requires additional investigation. Conservation of stopover points is crucial for migratory shorebirds (Davis and Smith 2001, Skagen 2006, Smith et al. 2012), and current conservation plans for Semipalmated Sandpipers in the Bay of Fundy revolve around protecting beach and intertidal habitat. Our results, however, suggest that this strategy may not protect all resources used by Semipalmated Sandpipers. Further, the results of our study suggest that it may be useful to consider use of nontraditional prey and habitats by other shorebirds with similar ecologies.

LITERATURE CITED

Anderson, M. J., R. N. Gorley, and R. K. Clarke (2008). Permanova+ for Primer: Guide to Software and Statistical Methods. PRIMER-E, Plymouth, UK.

Baker, M. C. (1977). Shorebird food habits in the eastern Canadian Arctic. The Condor 79:56–62.

Barbeau, M. A., L. A. Grecian, E. E. Arnold, D. C. Sheahan, and D. J. Hamilton (2009). Spatial and temporal variation in the population dynamics of the intertidal amphipod *Corophium volutator* in the upper Bay of Fundy, Canada. Journal of Crustacean Biology 29:491–506.

Barrett, R. T., K. Camphuysen, T. Anker-Nilssen, J. W. Chardine, R. W. Furness, S. Garthe, O. Hüppop, M. F. Leopold, W. A. Montevecchi, and R. R. Veit (2007). Diet studies of seabirds: A review and recommendations. ICES Journal of Marine Science 64:1675–1691.

Bart, J., S. Brown, B. Harrington, and R. I. G. Morrison (2007). Survey trends of North American shorebirds: Population declines or shifting distributions? Journal of Avian Biology 38: 73–82.

Bowser, A. K., A. W. Diamond, and J. A. Addison (2013). From puffins to plankton: A DNA-based analysis of a seabird food chain in the northern Gulf of Maine. PLOS One 8:e83152.

Casper, R. M., S. N. Jarman, B. E. Deagle, N. J. Gales, and M. A. Hindell (2007). Detecting prey from DNA in predator scats: A comparison with morphological analysis, using *Arctocephalus* seals fed a known diet. Journal of Experimental Marine Biology and Ecology 347:144–154.

Colles, A., L. H. Liow, and A. Prinzing (2009). Are specialists at risk under environmental change? Neoecological, paleoecological and phylogenetic approaches. Ecology Letters 12:849–863.

Davis, C. A., and L. M. Smith (2001). Foraging strategies and niche dynamics of coexisting shorebirds at stopover sites in the southern Great Plains. The Auk 118:484–495.

Deagle, B. E., J. P. Eveson, and S. N. Jarman (2006). Quantification of damage in DNA recovered from highly degraded samples—a case study on DNA in faeces. Frontiers in Zoology 3:11.

Deagle, B. E., N. J. Gales, K. Evans, S. N. Jarman, S. Robinson, R. Trebilco, and M. Hindell (2007). Studying seabird diet through genetic analysis of faeces: A case study on Macaroni Penguins (*Eudyptes chrysolophus*). PLOS One 2:e831.

Deagle, B. E., R. Kirkwood, and S. N. Jarman (2009). Analysis of Australian fur seal diet by pyrosequencing prey DNA in faeces. Molecular Ecology 18:2022–2038.

Deagle, B. E., D. J. Tollit, S. N. Jarman, M. A. Hindell, A. W. Trites, and N. J. Gales (2005). Molecular scatology as a tool to study diet: Analysis of prey DNA in scats from captive Steller Sea Lions. Molecular Ecology 14:1831–1842.

Fryxell, J. M., A. R. E. Sinclair, and G. Caughley (2014). Wildlife Ecology, Conservation, and Management, third edition. Wiley, Chichester, UK.

Galbraith, H., D. W. DesRochers, S. Brown, and J. M. Reed (2014). Predicting vulnerabilities of North American shorebirds to climate change. PLOS One 9:e108899.

Gerwing, T. G., A. M. Allen Gerwing, D. Drolet, M. A. Barbeau, and D. J. Hamilton (2015). Spatiotemporal variation in biotic and abiotic features of eight intertidal mudflats in the upper Bay of Fundy, Canada. Northeastern Naturalist 22:1–44.

Gratto, C. L., and F. Cooke (1987). Geographic variation in the breeding biology of the Semipalmated Sandpiper. Ornis Scandinavica 18:233–235.

Gratto, G. (1979). Further faunal and ecological surveys of the intertidal invertebrates of Scots Bay and the western Minas Basin. Final Report of Job Corps Project 16-01-0055.

Gratto-Trevor, C., R. I. G. Morrison, D. Mizrahi, D. B. Lank, P. Hicklin, and A. L. Spaans (2012). Migratory connectivity of Semipalmated Sandpipers: Winter distribution and migration routes of breeding populations. Waterbirds 35:83–95.

Hallmann, C. A., R. P. B. Foppen, C. A. M. van Turnhout, H. de Kroon, and E. Jongejans (2014). Declines in insectivorous birds are associated with high neonicotinoid concentrations. Nature 511:341–343.

Hamilton, D. J., A. W. Diamond, and P. G. Wells (2006). Shorebirds, snails, and the amphipod (Corophium volutator) in the upper Bay of Fundy: Top-down vs. bottom-up factors, and the influence of compensatory interactions on mudflat ecology. Hydrobiologia 567:285–306.

Hicklin, P. W. (1987). The migration of shorebirds in the Bay of Fundy. The Wilson Bulletin 99:540–570.

Hicklin, P. W., and P. C. Smith (1979). The diets of five species of migrant shorebirds in the Bay of Fundy. Proceedings of the Nova Scotian Institute of Science 29:483–488.

Hicklin, P. W., and P. C. Smith (1984). Selection of foraging sites and invertebrate prey by migrant Semipalmated Sandpipers, Calidris pusilla (Pallas), in Minas Basin, Bay of Fundy. Canadian Journal of Zoology 62:2201–2210.

Holmes, R. T., and F. A. Pitelka (1968). Food overlap among coexisting sandpipers on northern Alaskan tundra. Systematic Zoology 17:305–318.

Jardine, C. B., A. L. Bond, P. J. A. Davidson, R. W. Butler, and T. Kuwae (2015). Biofilm consumption and variable diet composition of Western Sandpipers (Calidris mauri) during migratory stopover. PLOS One 10:e0124164.

Karpanty, S. M., J. D. Fraser, J. Berkson, L. J. Niles, A. Dey, and E. P. Smith (2006). Horseshoe crab eggs determine Red Knot distribution in Delaware Bay. Journal of Wildlife Management 70:1704–1710.

Kohn, M. H., and R. K. Wayne (1997). Facts from feces revisited. Trends in Ecology & Evolution 12:223–227.

Kraus, J. M., T. S. Schmidt, D. M. Walters, R. B. Wanty, R. E. Zuellig, and R. E. Wolf (2014). Cross-ecosystem impacts of stream pollution reduce resource and contaminant flux to riparian food webs. Ecological Applications 24:235–243.

Kuwae, T., E. Miyoshi, S. Hosokawa, K. Ichimi, J. Hosoya, T. Amano, T. Moriya, M. Kondoh, R. C. Ydenberg, and R. W. Elner (2012). Variable and complex food web structures revealed by exploring missing trophic links between birds and biofilm. Ecology Letters 15:347–356.

MacDonald, E. C., M. G. Ginn, and D. J. Hamilton (2012). Variability in foraging behavior and implications for diet breadth among Semipalmated Sandpipers staging in the upper Bay of Fundy. The Condor 114:135–144.

Maire, O., J. N. Merchant, M. Bulling, L. R. Teal, A. Grémare, J. C. Duchêne, and M. Solan (2010). Indirect effects of non-lethal predation on bivalve activity and sediment reworking. Journal of Experimental Marine Biology and Ecology 395:30–36.

McArdle, B. H., and M. J. Anderson (2001). Fitting multivariate models to community data: A comment on distance-based redundancy analysis. Ecology 82:290–297.

Meusnier, I., G. A. C. Singer, J.-F. Landry, D. A. Hickey, P. D. N. Hebert, and M. Hajibabaei (2008). A universal DNA mini-barcode for biodiversity analysis. BMC Genomics 9:214.

Niles, L. J., J. Bart, H. P. Sitters, A. D. Dey, K. E. Clark, P. W. Atkinson, A. J. Baker, K. A. Bennett, K. S. Kalasz, N. A. Clark, J. Clark, S. Gillings, et al. (2009). Effects of horseshoe crab harvest in Delaware Bay on Red Knots: Are harvest restrictions working? BioScience 59:153–164.

Peer, D. L., L. E. Linkletter, and P. W. Hicklin (1986). Life history and reproductive biology of Corophium volutator (Crustacea: Amphipoda) and the influence of shorebird predation on population structure in Chignecto Bay, Bay of Fundy, Canada. Netherlands Journal of Sea Research 20:359–373.

Pienkowski, M. W., P. N. Ferns, N. C. Davidson, and D. H. Worrall (1984). Balancing the budget: Measuring the energy intake and requirements of shorebirds in the field. In Coastal Waders and Wildfowl in Winter (P. R. Evans, J. D. Goss-Custard, and W. G. Hale, Editors). Cambridge University Press, Cambridge, UK. pp. 29–56.

Pompanon, F., B. E. Deagle, W. O. C. Symondson, D. S. Brown, S. N. Jarman, and P. Taberlet (2011). Who is eating what: Diet assessment using next generation sequencing. Molecular Ecology 21:1931–1950.

Poulin, B., G. Lefebvre, and R. McNeil (1994). Diets of land birds from northeastern Venezuela. The Condor 96:354–367.

Puritz, J. B., J. A. Addison, and R. J. Toonen (2012). Next-generation phylogeography: A targeted approach for multi-locus sequencing of non-model organisms. PLOS One 7: e34241.

Quinn, J. T., and D. J. Hamilton (2012). Variation in diet of Semipalmated Sandpipers (Calidris pusilla) during stopover in the upper Bay of Fundy, Canada. Canadian Journal of Zoology 90:1181–1190.

Searle, S. R., G. Casella, and C. E. McCulloch (1992). Variance Components. Wiley, New York, NY, USA.

Shehzad, W., T. Riaz, M. A. Nawaz, C. Miquel, C. Poillot, S. A. Shah, F. Pompanon, E. Coissac, and P. Taberlet (2012). Carnivore diet analysis based on next-generation sequencing: Application to the leopard cat (Prionailurus bengalensis) in Pakistan. Molecular Ecology 21:1951–1965.

Shepherd, P. C. F., P. W. Hicklin, and V. A. Partridge (1995). Changes in sediment types and invertebrate fauna in the intertidal mudflats of the Bay of Fundy between 1977 and 1994. Canadian Wildlife Service, Atlantic Region, Environmental Conservation Branch, Ottawa, ON, Canada.

Simmons, D. B. D., M. E. McMaster, E. J. Reiner, L. M. Hewitt, J. L. Parrott, B. J. Park, S. B. Brown, and J. P. Sherry (2014). Wild fish from the Bay of Quinte Area of Concern contain elevated tissue concentrations of PCBs and exhibit evidence of endocrine-related health effects. Environment International 66:124–137.

Skagen, S. K. (2006). Migration stopovers and the conservation of Arctic-breeding calidridine sandpipers. The Auk 123:313–322.

Skagen, S. K., and H. D. Oman (1996). Dietary flexibility of shorebirds in the Western Hemisphere. Canadian Field-Naturalist 110:419–444.

Skilleter, G. A., and C. H. Peterson (1994). Control of foraging behavior of individuals within an ecosystem context: The clam Macoma balthica and interactions between competition and siphon cropping. Oecologia 100:268–278.

Smith, R. V., J. D. Stafford, A. P. Yetter, M. M. Horath, C. S. Hine, and J. P. Hoover (2012). Foraging ecology of fall-migrating shorebirds in the Illinois River valley. PLOS One 7:e45121.

Tsipoura, N., and J. Burger (1999). Shorebird diet during spring migration stopover on Delaware Bay. The Condor 101:635–644.

Wilson, W. H., Jr. (1988). Shifting zones in a Bay of Fundy soft-sediment community: Patterns and processes. Ophelia 29: 227–245.

Yeo, R. K. (1977). Animal-sediment relationships and the ecology of the intertidal mudflat environment, Minas Basin, Nova Scotia. M.Sc. thesis, McMaster University, Hamilton, ON, Canada.