ParasiteBlitz: Adaptation of the BioBlitz concept to parasitology

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Abstract

A BioBlitz is a rapid and intensive survey of a specific geographic area that brings together experts and often lay participants to assess biodiversity, typically of macrobiota that are easily observed and identifiable on-site. This concept has become popular across taxonomic fields, attracting interest globally to increase knowledge of local biodiversity. Inspired by the success of the approach, we undertook a 'ParasiteBlitz' at an unexplored locality (Stono Preserve, Charleston, South Carolina, USA) to determine its feasibility for parasites, whose assessment of diversity is largely neglected worldwide. We assembled a team of parasitologists with complementary expertise. Over 12 days (3 days in each habitat) in April 2023, we intensively screened fishes and aquatic invertebrates for parasites, and sampled sediment and water for environmental DNA (eDNA) metabarcoding from four aquatic habitats: wetland, freshwater pond, brackish impoundment, and tidal creek. We incorporated assistance from non-parasitologists and students. Details on methodologies and results are provided in individual papers in this Special Collection. Traditional methods revealed the presence of ca. 100 species of seven major metazoan parasite taxa, and the eDNA survey yielded over 1,000 amplicon sequence variants identified as parasites, most with sequences unmatched in GenBank, and resulting in only a few species identified as named species in the one-year post-Blitz timeframe we imposed upon ourselves for identification. Limitations and challenges of the ParasiteBlitz are discussed, and our results support that this approach can be effective for rapid discovery of the dimensions of parasite assemblages in an understudied environment and contribute to parasitology knowledge.

Introduction

Parasites remain typically ignored in biodiversity surveys, despite their vital role in ecosystem dynamics (Dougherty *et al.* 2016; Lafferty *et al.* 2006; Timi and Poulin 2020; Wood 2007) and as significant drivers of evolution (Brunner *et al.* 2017; Combes 1996; Kuris *et al.* 2008; Møller 2005; Timi and Poulin 2020). The well-established, urgent need to curb global biodiversity loss (Chan *et al.* 2022a) nevertheless seems to not be recognized for parasites. Largely, this neglect has been blamed on the fact that parasites are often not visible to the untrained eye, that necropsies are tedious and lengthy, and more significantly, that uncommon and highly specialized taxonomic expertise is needed for discovery and identification.

In addition, the ubiquity of parasites and their associations with their free-living hosts means that they can serve as indicators of overall biodiversity in ecosystems (Moore *et al.* 2024). Yet parasites are still largely disregarded as integral biodiversity components due to the perception of them as disease agents, with their presence being considered 'abnormal' and a target for control or elimination. Such a flawed view has been debunked over the past ~20 years as evidence has increasingly shown that parasites are essential players in ecosystem function and evolution. In short, parasites regulate and structure host populations (Møller 2005) and are integral to food webs (Lafferty *et al.* 2006; 2008; Moore *et al.* 2024), having been shown to outweigh the biomass of predators in certain ecosystems (Kuris *et al.* 2008). Just as with free-living species, parasite diversity and community composition are impacted by biotic and abiotic environmental changes. However, parasites are additionally impacted because of their dependence on hosts (Cable *et al.* 2017; Marcogliese 2023). Cases of zoonotic spillovers attract scientific attention (García-Moreno 2023; Miller *et al.* 2020; Patz *et al.* 2000; Thompson 2013), but more focus is needed specifically on the long-lasting effects of introduced parasites on native, non-human animal populations.

Consequently, true diversity and perceived functions of introduced and native parasites are often understated or erroneous despite their recognized evolutionary and ecological impacts on ecosystems (Goedknegt *et al.* 2016). In the same vein, whereas habitat modifications' effects on parasite distribution are well documented (Bitters *et al.* 2022; Bordes *et al.* 2015) and have at times been used in disease management (e.g., Bjork and Bartholomew 2008; Cable *et al.* 2017), the effects of climate change on parasites are not well understood: some species may face extinction (Carlson *et al.* 2017; Cizauskas *et al.* 2017), while others may experience range expansion (Carlson *et al.* 2017; Okamura *et al.* 2011). The ecological consequences of these parasite species redistributions are largely unknown (Carlson *et al.* 2017; Marcogliese 2016).

The extent of parasite diversity has been a question of interest to numerous parasitologists (e.g., Dobson *et al.* 2008; Poulin and Morand 2004), yet remains difficult and unanswered. As with free-living organisms, gleaning the magnitude of the parasite biosphere is rendered even more intractable by rapid environmental change. Parasites may account for 50–80% of Earth's biodiversity (Dobson *et al.* 2008; Poulin 2014), with 85–95% of parasite species estimated to yet be discovered (Carlson *et al.* 2020; Okamura *et al.* 2018). In parallel, reduced taxonomic expertise in new generations of parasitologists (Poulin and Presswell 2022) hinders species discovery. It is estimated that it would take over 500 years to describe global helminth diversity alone (Carlson *et al.* 2020).

Increases in biodiversity awareness have occurred in a large part thanks to BioBlitzes, which are intense, short-term surveys involving teams of experts who collect, process, and identify specimens of various phyla, and thereby generate valuable datasets both at local and global scales (Meeus *et al.* 2023; Pollock *et al.* 2015; Postles *et al.* 2018; Ruch *et al.* 2010). Since 1996 when the first BioBlitz in the USA was done in Kenilworth Park and Aquatic Gardens in Washington, DC, hundreds have been carried out over the world by a variety of groups including scientific associations or societies (e.g., https://www.esa.org/seeds/2024-national-seeds-bioblitz/), national parks (e.g., https://www.nps.gov/subjects/biodiversity/national-parks-bioblitz.htm), or municipalities (e.g., https://www.keepdurhambeautiful.org/durham-bioblitz-1). BioBlitzes foster community involvement, as they can include non-scientist volunteers and students, and they create learning opportunities with a 'heightened sense of stewardship for all participants' (Gass *et al.* 2021). All of these BioBlitzes, however, have focused on diversity of free-living, macro-organisms (e.g., plants, insects, fish, birds), and to our knowledge, no comprehensive *parasite* BioBlitz *per se* has been carried out.

Reasons for the heretofore taxonomic oversight of parasites in BioBlitzes are likely multifactorial, including the need for expertise when collecting parasites from hosts. This stands in contrast to other taxonomic groups such as plants, many of which can now be 'identified' by non-scientists *via* smartphone applications such as iNaturalist (https://www.inaturalist.org/) that are often used by Bio-Blitz participants (https://libguides.lib.msu.edu/BioBlitz/apps; https://www.seacoastsciencecenter.org/2023/09/12/prepare-to-blitz-using-inaturalist-app-on-the-day-of-bioblitz/). In the few cases when parasites were included in a BioBlitz, expert parasitologists were present (https://today.uconn.edu/2016/06/bioblitz-species-count-set-new-us-record/#). While exhaustively surveying a locality for parasites is practically impossible, we propose that the BioBlitz concept can be adopted as a 'moonshot'-like endeavor (Carlson *et al.* 2020) to examine parasite diversity if the appropriate team of experts is involved. Such a collaboration has the other advantage of spreading taxonomic

effort geographically (Poulin and Jorge 2019) as parasitologists have their skills brought to bear on new field localities and host assemblages. We aimed to test this approach by organizing a team of parasitologists with complementary and methodologically aligned expertise to conduct a *parasite* BioBlitz (hereafter ParasiteBlitz) at Stono Preserve, a coastal locality in South Carolina, USA. Adoption of the BioBlitz concept to the study of parasites, however, inevitably led to the need for adaptations to the typical BioBlitz procedure, which meant allocating more time overall for specimen collection and species identification. The hosts being themselves individual ecosystems, a ParasiteBlitz inherently requires more time given the secondary collection of parasites following the acquisition of hosts, and the lengthy morphological and molecular analyses required to identify parasite species. An additional hinderance but integral dimension for a ParasiteBlitz is the collection and identification of parasites' free-living stages, to complement detections of host-borne parasite stages and to compensate for temporal absences in some hosts.

While the advent of DNA sequencing has allowed for great strides in the discovery and identification of cryptic species (Heneberg and Ditko 2021; Pérez-Ponce de León and Nadler 2010) and reconstruction of multi-host life cycles (Bartholomew *et al.* 2006; Bennett *et al.* 2023; Hill-Spanik *et al.* 2021; Siegel *et al.* 2018), the recent development of high-throughput sequencing has further expanded DNA barcoding to metabarcoding, which provides large-scale and simultaneous detection of organisms from a single mixed sample (community DNA or eDNA), with millions of sequences being generated in one sequencing run (Hupało *et al.* 2021). However, while this latter approach has been used for numerous biodiversity assessments of bacteria for instance (e.g., McCaig *et al.* 1999; Parfenova *et al.* 2013), parasite metabarcoding studies have been limited in their taxonomic scope. Nevertheless, these studies have proved effective in assessing diversity of free-living parasite stages (e.g., eggs, spores, cercariae) in environmental samples (Davey *et al.* 2021; Hartikainen *et al.* 2016) and uncovering hidden diversity that might have been missed when using classical methodology (Trzebny *et al.* 2020). Hence, to obtain as complete a parasite diversity assessment as possible, and to further inform development of the ParasiteBlitz approach, we used both individual-based DNA barcoding and metabarcoding of environmental samples.

The overarching goal of this study was to implement a ParasiteBlitz to test the feasibility of such an approach for increasing global knowledge of parasite diversity *via* the acquisition of baseline information for a continuum of aquatic environments (Stono Preserve) in coastal South Carolina, USA. Stono Preserve is located about 30 km from Charleston; it is a protected space in a region experiencing rapid development into suburban communities and as such could well be considered into future urban greening of this fast-growing city. It is a natural laboratory with a unique aquatic ecosystem consisting of ephemeral, forested wetland and adjacent freshwater pond and brackish water impoundment, with the latter opening into a tidal marsh. The particular physical attributes of these habitats allowed for representative sampling of free-living and parasitic fauna. We had three objectives: 1) adapt the BioBlitz concept to assess parasite diversity at Stono Preserve using traditional methods (individual-based parasite identification using morphology and DNA sequencing); 2) implement and examine the efficacy of high-throughput eDNA metabarcoding to assess parasite diversity in water and sediment samples and compare results with those using traditional parasitology; and 3) assess whether the data obtained allowed for understanding connectance

among organisms in the four sites studied and whether collecting such data is productive in the framework of a ParasiteBlitz.

Materials and methods

We allocated three days over ~two weeks to investigate each of four ecotypes and imposed a one-year post-Blitz window to identify specimens. In the month leading up to the ParasiteBlitz, field and laboratory equipment were relocated to the Stono Preserve Field Station, accommodations and transportation were arranged, and meals were planned. Below are general descriptions of the team, the locality, and the collection sites as well as hosts, parasites, and water and sediment collection methods. Detailed methods, procedures, and results are provided in respective papers in this Special Collection.

The people

The team was comprised of six parasitologists with expertise in platyhelminthes, acanthocephalans, myxozoans, and eDNA, one ecologist, one ichthyologist, one PhD student, and one Master's student. Several undergraduates were involved in the field for host collections and in the laboratory for necropsies, visualization of parasites, and data recording (Figure 1). Volunteers (i.e., logistics ambassadors) supported the team with tasks including collection of fishes and invertebrates, transport of material around the sites and labs, and daily chores (e.g., meals and cleaning). Essential administrative support was also received (dean, department chair, and academic director of the field station). All are listed in the Acknowledgments.

### The locality and collection sites

The four collection sites at Stono Preserve (32°44'06"N 80°10'48"W) form a continuous ecosystem (Figure 2): a forested freshwater ephemeral wetland, a freshwater pond, a brackish impoundment, and a tidal creek. Salinity and temperature were recorded during each sampling period using a YSI Professional Plus (Yellow Springs, OH, USA).

Host, sediment, and water collection

Fishes were collected via traps, cast nets, hook and line, seine, and electrofishing according to substrate type, water height, and amount of debris present. Fish were euthanized with an overdose of MS222 (IACUC protocol #2023-008). We examined 1–15 individuals for each of the fish species we encountered at each site (with the exception of eastern mosquitofish *Gambusia holbrooki*, which were abundant and the sole species recovered in the two freshwater sites; Table 1). Crustaceans were collected in dipnets, trawls, and traps; bivalves, gastropods, insects, and annelids were collected by hand, dipnets, and opportunistically in the seine. We aimed to examine as many individuals per species as possible as time allowed within 48 h post capture (Table 2). Surface (top 3 mm) sediment samples were collected using 10-cc syringe corers, placed in nuclease-free collection tubes, and kept on ice until storage at -80°C upon return to the laboratory. Four water samples were collected across each habitat in new 0.5-L Nalgene bottles by submerging the bottle while wearing nitrile gloves. Samples were placed on ice and filtered *in situ* using a battery-powered peristaltic pump (Vampire Sampler, Bürkle GmbH) and self-preserving eDNA filter packs (PES membrane, 1.2 µm pore size (Thomas *et al.* 2019)). For the impoundment, tidal creek, and pond, 2 L of water was filtered at each site. In the case

Figure 1. ParasiteBlitz setup; a: field laboratory with individual workstations; b: learning moment with student; c, d: undergraduate student involvement; e–g: fish collection using different gear according to habitat; h: setup of passive filter for eDNA; i–n: invertebrate collection.
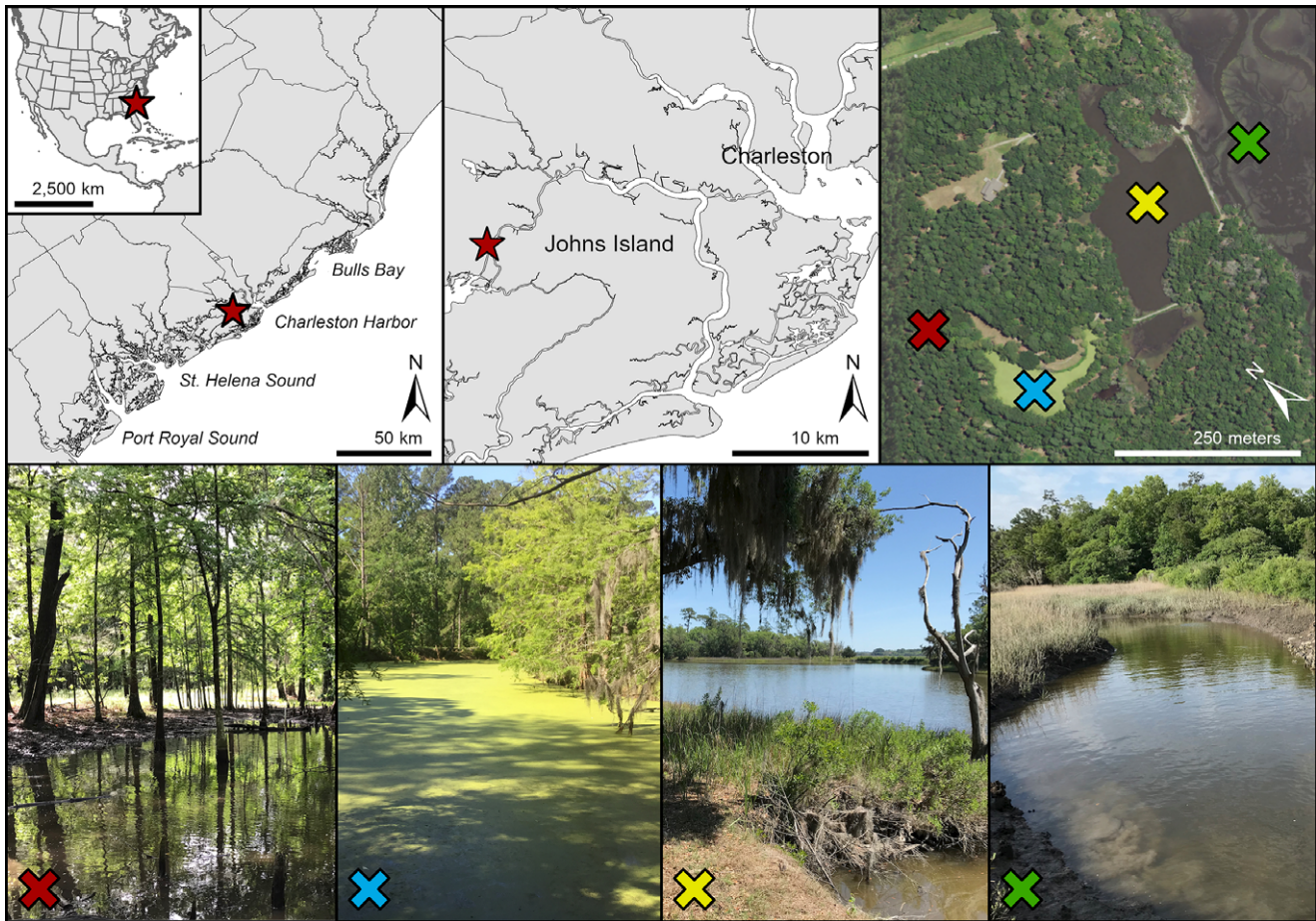
Figure 2. Collection sites at Stono Preserve, Charleston, South Carolina, USA: A forested freshwater ephemeral wetland (red X), a freshwater pond (blue X), a brackish impoundment (yellow X), and a tidal creek (green X).

of the wetland, only 1 L of water was sampled due to its considerably smaller size compared to the other water bodies. One Nalgene bottle filled with 500 mL ultrapure Milli-Q water was also filtered *in situ* at each habitat as a negative control. Passive filters (Whatman cellulose acetate membrane, 0.45 μm pore size) were deployed for 8 h at the same sites as the active samples; membranes were preserved in 100% ethanol (EtOH) after collection. One additional filter per habitat was handled *in situ* as a negative control. Gloves were used, and metal forceps for handling the filters were flamed between samples to avoid contamination.

Parasite collection from hosts

Each host was placed in a tray alongside a ruler, assigned a unique alpha-numeric code, photographed, identified to the lowest taxonomic level morphologically, measured, and tissue-preserved in 100% EtOH for identity verification *via* DNA sequencing as needed. Fishes, insects, and crustaceans were identified by Blitz participants using Carpenter (2002a, b), Merritt *et al.* (2008), and Thorp and Rogers (2015), respectively. Gastropods were identified using Burch (1989) and Wethington (2004) and annelids using Kathman and Brinkhurst (1998). Fish and invertebrates were then processed down the line of specialists: all were first examined for ectoparasites under a stereomicroscope, prior to dissection. For fish, gills were first resected, then the next person dissected the digestive system, then another person made squashes of kidney,

muscle, intestine, brain, and other tissues as time allowed. For invertebrates, insects and crustaceans were examined for parasites in their body cavity and digestive tract, and some muscle tissue was squashed in large individuals; annelids were dissected when large and squashed when small; and snails' shells were crushed, soft tissues removed, and placed in water in individual Petri dishes. Bivalves were shucked, and a portion of gill, mantle, and digestive gland were fixed immediately in 100% EtOH for molecular detection of microparasites. For each parasite taxon encountered, a subset of specimens was examined fresh and photographed; others were fixed in 95% EtOH for molecular studies; others yet were fixed in 80% EtOH, 10% neutral-buffered formalin, or air dried as smears for morphological studies and for preparation of museum vouchers. Data of hosts and gross parasite taxon identities according to each organ examined were recorded by each participant in field notebooks, then digitized into a spreadsheet at the end of the Blitz.

Parasite identification from hosts

After isolation, parasites were taken by each respective expert for further processing, which varied depending on taxa (see taxon-specific ParasiteBlitz papers for details). In short, voucher specimens of helminths were prepared for morphological analysis, including some hologenophores. Where possible, DNA of hologenophores, isogenophores, and paragenophores was extracted, and primers and PCR protocols were selected according to parasite

Table 1. Species and number of individual fish examined at each collection site during the ParasiteBlitz at Stono Preserve, Charleston, SC, in April 2023

Species	Ephemeral wetland	Freshwater pond	Impoundment	Tidal creek
Anchoa mitchilli	0	0	1	5
Fundulus heteroclitus	0	0	7	2
Fundulus majalis	0	0	0	4
Fundulus confluentus	0	0	1	0
Gambusia holbrooki	8	39	5	0
Gobiosoma bosc	0	0	3	2
Lagodon rhomboides	0	0	0	5
Leistomus xanthurus	0	0	3	9
Menidia beryllina	0	0	3	0
Menidia sp.	0	0	0	3
Menidia menidia	0	0	0	4
Mugil curema	0	0	0	8
Mugil cephalus	0	0	1	4
Paralichthys lethostigma	0	0	1	0
Poecilia latipinna	0	0	5	0
Pogonias chromis	0	0	1	0
Pomatomus saltatrix	0	0	0	1
Total: 125	8	39	31	47

taxon. Briefly, cytochrome *c* oxidase I (COI) mitochondrial DNA (digeneans, cestodes, nematodes), 18S small subunit ribosomal RNA (rRNA) (myxozoans, monogeneans, copepods), 28S large subunit rRNA (digeneans, cestodes, monogeneans, copepods) genes, and/or the first internal transcribed spacer region of the rRNA gene (nematodes) were amplified and sequenced. Voucher specimens were deposited at the National Parasite Collection housed at the Smithsonian Institution (Washington DC, USA) and the Museum National d'Histoire Naturelle (Paris, France), as well as in some of our collections at Hasselt University (Diepenbeek, Belgium) and at the Bulgarian Academy of Sciences' Institute of Biodiversity and Ecosystem Research (Sofia, Bulgaria). Representative sequences were deposited in GenBank.

Parasite eDNA metabarcoding from sediment and water

DNA was isolated from homogenized sediment samples and water filters from each habitat. There are no 'universal' parasite markers given their extreme diversity; we targeted microsporidians, myxozoans, nematodes, and platyhelminthes for which taxon-specific primers have been developed (Bhadbury and Austen 2010; Chan *et al.* 2022a, b; Hartikainen *et al.* 2016; Lisnerová *et al.* 2023; Thomas *et al.* 2022; Weigand *et al.* 2016; Zhu *et al.* 1993). We also used universal eukaryote primers (Bråte *et al.* 2010) to capture parasite groups for which no taxon-specific primers have been designed, and tested primers from Littlewood *et al.* (1997) and Lockyer *et al.* (2003) that, to our knowledge, have not yet been used in platyhelminthes' metabarcoding studies. Libraries were sequenced on an Illumina MiSeq, and data were preprocessed and analyzed using various software packages in R version 4.1.2

(R Core Team 2021) including *DADA2* (Callahan *et al.* 2016), *vegan* (Oksanen *et al.* 2022), and *phyloseq* (McMurdie and Holmes 2013).

Results (Tables 1, 2; Figures 3–6)

Traditional sampling led to the examination of 125 fish of 12 genera and 17 species (Table 1). Parasites were found in 16 fish species, and these infections comprised mainly flatworms (cestodes, digeneans, monogeneans), cnidarians (myxosporeans), and to a lesser extent, crustaceans (copepods, branchiurans), nematodes, annelids (leeches), microsporidians, and a variety of 'protists' including myxozoans (apicomplexans, perkinsids), 'ciliates', and 'flagellates' (Figures 3, 4). No acanthocephalan was found in fishes, crustaceans, or insects. Furthermore, we encountered three cases of hyperparasitism and two unknown forms on fish gills (Figures 3, 4). With respect to invertebrates, we examined individuals of 6 species of mollusks (3 of gastropods and 3 of bivalves), 9 species of crustaceans (decapods), oligochaete and polychaete annelids (only some of which we identified to species level), and 4 families of insects (Table 2). Parasites encountered were typical developmental stages in the respective host taxa: digeneans in gastropods, decapods, and annelids; perkinsids and copepods in bivalves; nematodes in insects. The impoundment and the tidal creek (brackish waters) were the most diverse in terms of both hosts and parasites, and showed the highest levels of infection compared to the freshwater pond and forested wetland where we could only sample invertebrates and one species of fish due to difficult terrain for fishing (e.g., vegetation, woody debris, stumps). At the fish species level, a primary coarse assessment uncovered 90 host-parasite combinations, which loosely represents the diversity of fish species/parasite group systems found in the habitats sampled (Figure 5a).

This representation, however, is typically an underestimation of the parasites' diversity, as each parasite group often included several species, and later morphological and molecular analyses allowed finer resolution of diversity (see example in Figure 5b). Flatworms were the most numerous and had the highest diversity of all parasite groups encountered. We found 42 digenean, 8 cestode, and 9 monogenean (8 monopisthocotylean and a single polyopisthocotylean) species, several of which represented new host and/or locality records. Digeneans comprised 14 adult (in fishes), 26 metacercarial (in fishes, decapods, and mollusks), and 2 cercarial stages (in mollusks), and DNA sequence data provided evidence of the transmission pathways for four digenean species. All cestodes were metacestodes in fishes. Myxozoans comprised 22 species as myxospores in fish hosts, 2 species as myxospores hyperparasitic in monogeneans in two fish hosts, and 1 actinospore type in an annelid; this annelid-infecting stage was previously known, and its DNA sequence was matched to one of the hyperparasite stages, and thus represents the first finding of the life cycle of a hyperparasitic myxozoan. In fishes, in addition to flatworms and myxozoans, we found nematodes (not all could be identified to species level), 8 species of copepods, 2 species of leeches, and unidentified microsporidians and 'protists' (ciliates, 'flagellates', myzozoans); we also found several nematodes in insects (not identified to species level), 1 species of cirriped in crabs, 3 species of apicomplexans in oysters, and unidentified gregarines in annelids (Figure 6).

From eDNA samples, six amplicon libraries were produced using metabarcoding primers that targeted platyhelminthes (two

Table 2. Species and number of individual invertebrates collected at each collection site during the ParasiteBlitz at Stono Preserve, Charleston, SC, in April 2023

| Species	Ephemeral wetland	Freshwater pond	Impoundment	Tidal creek
Annelida				
Amphitrite ornata	0	0	0	5
Annelid unidentified	0	0	0	3
Cirratulidae	0	0	0	31
Limnodrilus cf. *hofmeisteri*	0	8	0	0
Lumbriculidae	0	44	0	0
Manayunkia aesturina	0	0	0	104
Naididae sp. 1	0	4	0	0
Naididae sp. 2	0	12	0	0
Naididae sp. 3	0	23	0	0
Naididae sp. 4	0	0	0	15
Naididae sp. 5	3	0	0	0
Nereidae sp.	0	0	3	6
Polychaete (unidentified sp. 1)	0	0	5	9
Polychaete (unidentified sp. 2)	0	0	0	19
Polychaete (unidentified sp. 3)	0	0	0	8
Streblospio sp.	0	0	5	124
Tubificidae	0	0	0	13
Decapoda				
Alpheus heterochaelis	0	0	0	1
Armases cinereum	0	0	2	0
Callinectes sapidus	0	0	0	2
Creaserinus fodiens	0	2	0	0
Minuca pugnax	0	0	3	2
Palaemonetes sp.	0	0	0	1
Panopeus herbstii	0	0	1	3
Procambarus troglodytes	5	4	0	0
Zaops ostreus	0	0	0	1
Insecta				
Dytiscidae sp. 1	5	0	0	0
Dytiscidae sp. 2	7	0	0	0
Gyrinidae sp.	1	0	0	0
Hydrophylidae sp. 1	4	0	0	0
Hydrophylidae sp. 2	3	0	0	0
Libellula sp.	9	0	0	0
Tramea sp.	46	0	0	0
Mollusca				
Crassostrea virginica	0	0	5	10
Geukensia demissa	0	0	0	1
Ilyanassa obsoleta	0	0	17	162
Littoraria irrorata	0	0	72	50
Physa sp.	0	33	0	0
Tagelus plebeius	0	0	0	2
Total: 898	83	130	113	572
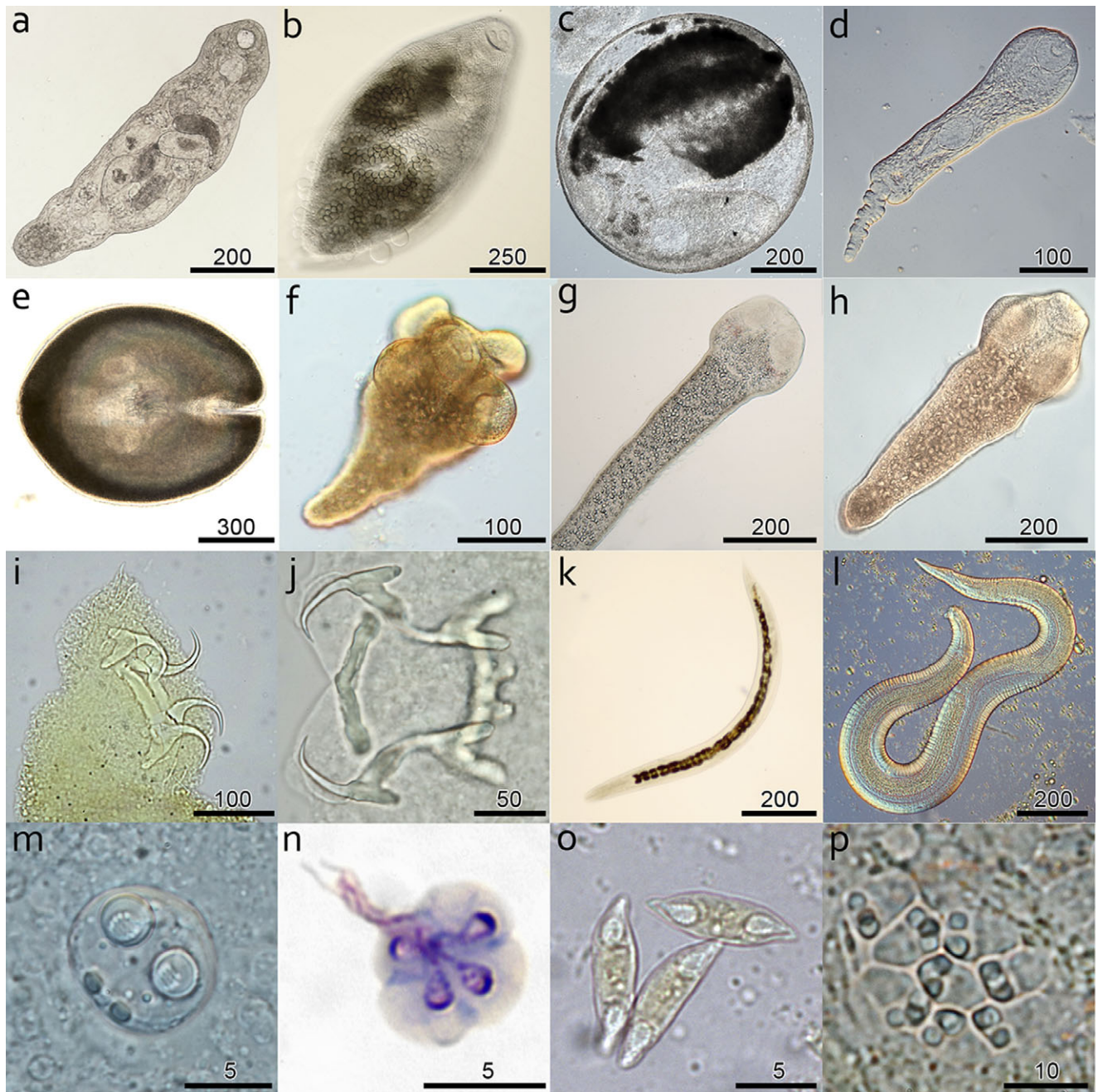
Figure 3. Representative diversity of the most common parasitic metazoans encountered in fish and invertebrates in April 2023 during the ParasiteBlitz at Stono Preserve, South Carolina, USA. Scales are in μm. a–b) adult digeneans; c) metacercaria; d) cercaria; e–h) metacestodes; i–j) monogeneans; k–l) nematodes; m–n) myxospores (myxosporeans); o–p) myxosporean hyperparasitic in monogenean with myxospore (o) and actinospore (p) stages.

COI mtDNA libraries), nematodes, myxozoans, microsporidians, and eukaryotes (four 18S rDNA libraries). We recovered ~12,900 amplicon sequence variants (ASVs) from all libraries (~8,200 from 18S, ~4,700 from COI). After filtering data to keep only parasite groups, >1,000 parasite ASVs were identified, not including those of potential myco- and phytoparasites as well as parasites of plants because of our lack of expertise and the general lack of knowledge regarding these groups and their modes of living. We identified ASVs of parasite taxa, including apicomplexans and microsporidians, that were not targeted in the traditional survey. Some parasite groups (e.g., leeches) were not detected despite being found in the traditional survey. ASVs of free-living animals were also identified,

including host taxa that we examined in the traditional survey (e.g., bivalve and gastropod mollusks, fishes, annelids) and potential hosts that fell outside the scope of our collections (e.g., amphibians and mammals). Primer specificity varied: for example, the microsporidian primers exhibited high target fidelity, whereas the nematode primers were less specific.

Discussion

Our findings demonstrate that a ParasiteBlitz is effective at increasing knowledge of parasite diversity and species distribution, and
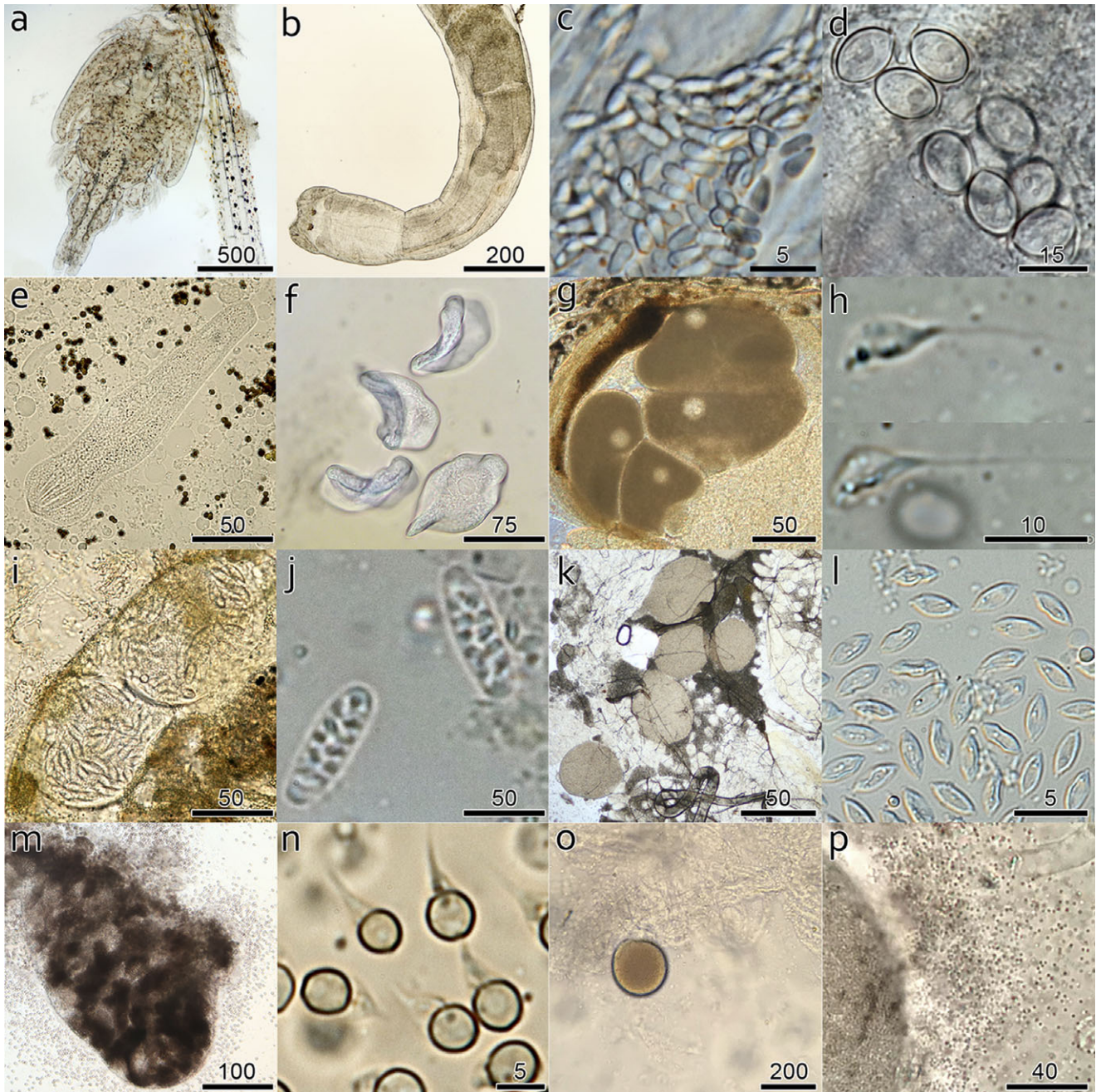
Figure 4. Representative diversity of parasitic organisms encountered in fishes and invertebrates in April 2023 during the ParasiteBlitz at Stono Preserve, SC, USA, and for which we had no taxonomic expertise. Some were identified later by external experts and others remain unidentified. Scales are in μm. a) copepod from gills of mummichog *Fundulus majalis*; b) branchiobdellid leech from external surface of crayfish *Procambarus troglodytes*; c) microsporidia from tegument of sabellid polychaete *Manayunkia aestuarina*; d) coccidia from Eastern oyster *Crassostrea virginica*; e) ciliate from intestine of a freshwater naidid annelid; f) gregarines from intestine of annelids; g) gregarine from intestine of water scavenger beetle (Hydrophilidae); h) composite image of flagellates from gill of Marsh killifish *Fundulus confluentus*; i–j) unknown cyst and spores from coelom of a spionid polychaete; k–l) unknown cyst and spores from haemocoel of water scavenger beetle (Hydrophilidae); m–n) haplosporidian from Grass shrimp *Palaemon* sp.; o–p) unknown pigmented cyst and motile spores from gills of Mosquito fish *Gambusia holbrooki*.

can provide fundamental data for illuminating the role of parasites in local ecosystems. While we encountered challenges that limited the productivity of the ParasiteBlitz (see below), improvements could be made by streamlining the overall organization and procedures. Herein, we share our reflections on what was achieved and the limitations of this approach. Guidelines and recommended dos and don'ts to further develop the ParasiteBlitz approach and enable and inspire other parasitologists to pursue this endeavor for

biodiversity discovery are provided in a separate publication in this Special Collection.

Appropriateness of site

Site selection strongly influences the productivity of any diversity survey. In our case, sampling four habitats yielded large amounts of host-parasite data that can be used in an ecological analysis. The inclusion of four different ecotypes in our study was governed
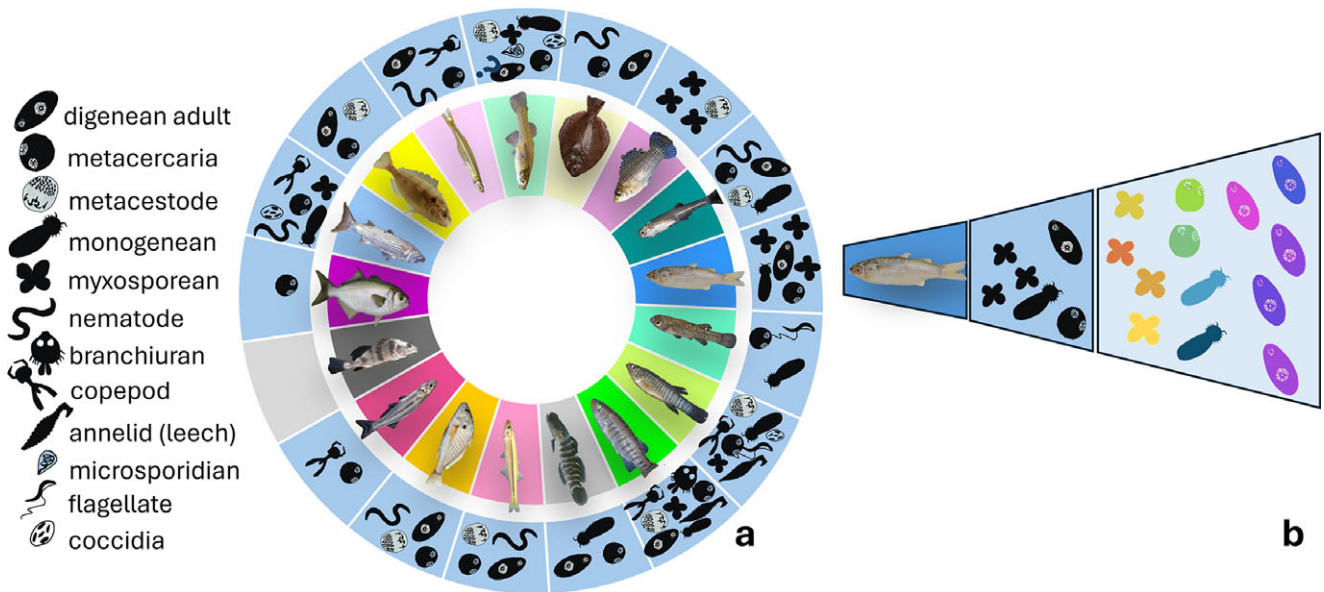
Figure 5. Fish host-parasite encountered at Stono Preserve during ParasiteBlitz. a) 90 host-parasite combinations in 16/17 fish species examined as determined from raw observations immediately after the ParasiteBlitz. Parasites were only identified coarsely as belonging to a given group (black silhouettes in outer ring); grey box shows no infection in only one fish species; b) Example of refined resolution of host-parasite associations after identification of parasites to species level one year post-Blitz (colored silhouettes) showing that combination at the host species level (black silhouettes) is often an underestimation of parasite diversity. Here, for fish species *Mugil curema*, six initial combinations (three myxosporeans, one monogenean, one adult digenean, and one metacercaria) was refined to 13 species (four myxosporeans, two monogeneans, and seven digeneans, including five adults and two metacercariae). Photo credit: Wikimedia.
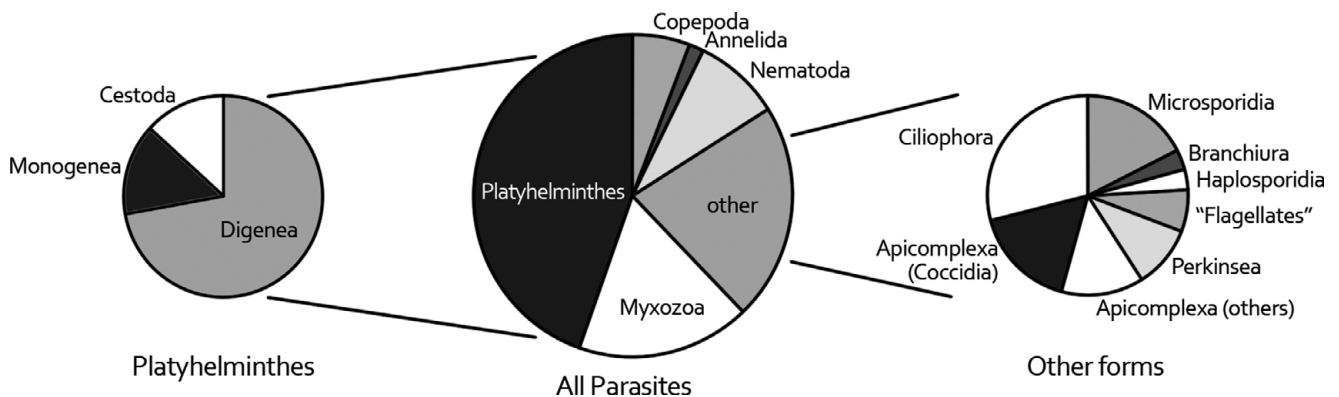
Figure 6. Most parasitic species found were members of Platyhelminthes, which were mostly digeneans. Myxozoans were second most diverse. The 'other forms' group refers to unidentified parasites (mostly ciliates, myzozoans (apicomplexans and perkinsids), and microsporidians).

largely by funding requirements. However, this broad scope proved too ambitious as it impeded the outcome in terms of species identification; simply because of lack of time we could dedicate to any one site both during and post-Blitz. We suggest that future ParasiteBlitzes limit their sampling target to limited geographical areas (e.g., a pond *vs*. a lake; a creek *vs*. a harbor) to improve the depth and resolution of host-parasite data and to ensure Blitz success.

Scope of hosts examined

As for all surveys, the scope of host species we captured depended on gear, experience, effort, knowledge of habitat, and season, as well as content and limitations of collection permits and institutional ethics protocol authorizations (e.g., IACUC). Based on these factors, we were most productive with collection of fish (17 species),

even though we had intended to dedicate one out of the three days per site for invertebrate collections and examinations. Fish necropsies took longer than planned and tended to stall because of the diversity of parasites found, which resulted in necropsies not being as exhaustive as intended, and overall, less effort was dedicated to examination of invertebrates. This led to too shallow of an invertebrate survey and consequently incomplete data for the connectance analysis. The effort needed for invertebrate hosts is proportionally much higher and productivity much lower, as prevalence of infection in invertebrates is often much lower than in vertebrate hosts, and because it monopolizes one person's time to examine a single annelid, snail, or oyster for finding few parasite taxa. Thus, within the time limitations of a ParasiteBlitz, the trade-off is that necropsies of fish (or vertebrates in general) are more productive in terms of parasite diversity information acquisition despite the rare gems to be discovered in invertebrate hosts. Therefore, arguably, vertebrate hosts

are better targets for a ParasiteBlitz. The trade-off here is that detection of invertebrate infections can be extremely informative in revealing the presence of parasites that otherwise would not be discoverable, such as those infecting protected species, animals dangerous to capture, or simply those whose vertebrate stages are seasonally absent during the ParasiteBlitz.

Traditional parasite characterization

The traditional parasite characterization component of this ParasiteBlitz yielded new hosts and locality reports, and novel parasite species that are described formally in dedicated manuscripts in this Special Collection. As expected, given the gaps in our current knowledge, the diversity of parasites encountered underlined the importance of having as broad a panel of taxonomists as possible to make the use of hosts ethical and efficient, by exhaustively collecting and identifying all parasites whenever possible. Significantly, DNA sequences for ~80% of species encountered using traditional methods did not provide species-level identification, meaning that these species are either novel or lack a GenBank/BOLD reference sequence. Thus, one significant accomplishment of this ParasiteBlitz is the population of reference databases with more parasite sequences associated with accurate species identifications and voucher specimen depositions in curated collections.

Digeneans and myxozoans were the most diverse parasite groups that we encountered. Our observations at Stono Preserve echo global findings of these groups: the magnitude of their diversity remains vastly unknown due to their relatively small size, the morphological homogeneity of closely related species, and the lack of information about their developmental stages and non-fish hosts for most described species. An obvious consequence of such high myxozoan and digenean diversity is that the number of combinations of host/parasites that we found were significantly less than the actual number of parasite species encountered, and thus, the ParasiteBlitz can produce a rapid, albeit coarse, first assessment of parasite species richness of one or more target host species. Our initial number of host-parasite combinations had its value for informing modifications to the ParasiteBlitz procedures (e.g., to optimize the number of taxon-specific experts needed to balance out necropsies that slowed because of the high diversity of particular parasite groups). Monogeneans were found in ~25% of fishes, but cestodes (all larval), copepods, and leeches were neither abundant nor highly diverse. This may translate in terms of practicality and optimization of ParasiteBlitz organization as taxonomic expertise for these latter groups to possibly be considered secondary, and thus, specimens could be provided to external taxonomists; alternatively, at a minimum, none of these groups appear to need duplication, contrarily to what we concluded regarding digeneans and myxozoans in particular (see below). Of course, this may vary depending on the locality explored, and this is when prior knowledge of surrounding areas or repeated ParasiteBlitzes would allow customization and optimization of events. To further illustrate this point, in our case, the abundance and apparent diversity of gregarines, flagellates, and coccidia indicate the necessity to integrate multiple (and diverse) protistologists in future ParasiteBlitzes if the aim is to be as holistic and exhaustive as possible. We neglected to include protistologists, even though protists are widely understudied (Skovgaard 2014) and may be considered the neglected of the neglected parasites. This oversight could have been better anticipated if we had baseline information for surrounding areas. Thus, we can argue that a first ParasiteBlitz dataset will be such a baseline.

The lesson here is that when in doubt, bring on board protistologists and multiple experts for each major metazoan taxon.

Metabarcoding

Together with our traditional parasitology approaches, we successfully incorporated metabarcoding of environmental DNA (eDNA) extracted from sediment and water, the latter of which was sampled both actively and passively. Metabarcoding revealed ~10 times more parasite diversity than what we found using traditional isolation of specimens from hosts. This was not surprising as metabarcoding applied to parasitology allows detection of parasite DNA (either genomic/intracellular or extracellular) from free-living stages (Bass *et al.* 2015, 2023). We generated and submitted ~12,900 eDNA sequences to the Sequence Read Archive (Leinonen *et al.* 2010), and although they are not associated with voucher specimens, they will serve as a baseline for parasite diversity at Stono Preserve, which as mentioned above is associated with the College of Charleston, largely an undergraduate institution, and could be the impetus to begin long-term surveys of this locality for teaching purposes.

The most challenging aspect of including eDNA metabarcoding as part of the ParasiteBlitz was keeping true to the timeline post-Blitz. In brief, library preparation was slow due to troubleshooting PCR protocols, which we expect will not be as cumbersome as more specific and novel parasite metabarcoding primers are developed. Involvement of more students in the library preparation workflow would have been a win-win in terms of creating more learning opportunities and expediting this process. The bioinformatic analysis also was bogged down due to challenges with regard to assigning taxonomy to our sequences and computer memory requirements. Collaborating with a more experienced bioinformatician from the beginning would have helped, but ultimately, the development of parasite reference sequence databases will likely have the most impact on increasing efficiency.

Roles for participation of non-parasitologists

A core component of traditional BioBlitzes is participation by non-experts: community members, students, and other volunteers. As such, we incorporated non-parasitologists into our ParasiteBlitz, which is a shift from traditional parasite surveys carried out by one or a few parasitologists used to working together, who benefit from familiarity, mutual respect, and trust. Bringing in non-parasitologists has the potential to lead to error, as we must rely on those volunteers despite their being on a steep learning curve over a very short time. All community-science based data collection must deal with errors, some of which can be found and corrected by regular data curation, or otherwise somewhat diluted in the large amount of data generated. We consider that the issue can be mitigated simply by engaging non-scientists in most parts of the ParasiteBlitz aside from actual parasite sampling – turning a potential disadvantage (participation of non-experts) into Blitz success. For instance, just like in a regular BioBlitz, roles existed for non-experts to participate in typical field-support roles (e.g., transporting supplies to and from the field, notetaking, cleaning). Furthermore, the collection of invertebrate hosts was suited for non-expert participants, as this does not require institutional animal use protocol permits and can be done with virtually no training. These important support roles, which we underappreciated in the planning phase, revealed themselves as the backbone of our ParasiteBlitz and essential to efficient and effective operation on-site. Furthermore, despite the minute size of many parasites, and hence

the difficulty in collection and recognition by non-experts, increasing the potential for mistakes, ParasiteBlitzes do offer opportunities for training students prior to or during the event on specialized tasks depending on their interests. The close collaboration of diverse experts with engaged students is a rich opportunity created by the ParasiteBlitz and represents a practical pathway to enhance interest in taxonomy. Students can be effectively 'apprentice parasitologists' and be trained to then take a lead role in describing specimens as an undergraduate or graduate research project.

Limitations of the ParasiteBlitz approach

Compared to a typical survey of parasites, a ParasiteBlitz has pros and cons. Limitations include the need for heavy planning and logistics, constraints of time both on-site and post-Blitz, the need for funding – all of which occur to some extent for all extramural surveys but are amplified with Blitzes in proportion to the number of people involved and the pressure for short turn-around of identifications. Though, perhaps the greatest concern may be that speed can sacrifice thoroughness and lead to mistakes. In this regard, it is important to recognize that one major difference between a BioBlitz and a ParasiteBlitz is that collection and identification of specimens are two separate processes in the latter, and that while collection is rapid, identification is not. Hence, if the terms of BioBlitzes are strictly followed, the productivity of ParasiteBlitzes would be limited to host-parasite combinations, which would be inaccurate, and adjustments are therefore necessary given the current state of the parasitologists' identification toolbox.

Pre-Blitz hurdles include the need for collecting permits, which can be difficult for non-local participants, and for IACUC approved protocols. These obstacles can be alleviated if Blitzers have the support of a governmental or similar fish and wildlife agency that already has staff, permits, and equipment to collect hosts. This coordination can greatly relieve the organizational burden from the ParasiteBlitz team, and such help typically brings knowledge of the locality and host taxonomic expertise. Such symbiotic collaborations can represent a win-win as agencies may have time budget allowance for outreach-type activities. Further logistical limitations include the organization of the panel of experts who must agree to follow the same protocols and put aside their individual habits, however good they are, and therein, human factors are important and must be considered. Success in this gathering of many people at one spot for a limited amount of time requires a good deal of chance weather-wise and sustained collaborative effort from all team members for the gathering not to fall into chaos, which is not something participants in more traditional long-term and often slower surveys must deal with. Furthermore, based on the diversity of parasites we encountered, it would be unrealistic to envisage being able to assemble a panel of parasitologists to cover all taxa. Our panel of six covered the most common parasites that we expected to encounter (helminths and myxozoans) but lacked taxonomic expertise in protists, which we found to be diverse and ubiquitous. This taxonomic knowledge gap could be addressed by adding more experts to the Blitz team, by limiting the scope of the Blitz to particular taxa to match the team, or by recruiting additional specialists post-Blitz (which we did for some taxa – however, not for 'protists' because we were not set up to collect them). The latter case implies that team members must be trained pre-Blitz about appropriate collection of parasites outside their expertise. Although in this regard, a ParasiteBlitz does not differ from a typical survey, in all cases, we recommend that effort should be made to collect all parasites from hosts both for ethical reasons and

to optimize the usage of people and monetary resources, even if specimens cannot be identified within the planned scope and timing of the Blitz.

Time and money

Time is a limited commodity in all BioBlitzes, and its scarcity was particularly amplified in our ParasiteBlitz. The time required to examine hosts is typically longer than the time to collect hosts, requiring dedicated collection management so as not to kill hosts that cannot be given due examination. The problem is compounded as examinations slow down as parasites are discovered and worked up. There is therefore an onus on each expert to have their specimen characterization (or at least preservation) protocols refined to be as efficient as possible. Even with careful host collection management, Blitzers may have to choose between generating qualitative or quantitative data from the available material. However, we suggest that this situation could be minimized if the panel is comprised of duplicated experts for the same taxa – in particular, those known to be typically most abundant (digeneans and myxozoans, in our case). And, as already mentioned, increased participation of students in necropsies and as assistants to the experts can increase dedicated focus of the parasitologists to the parasites per se.

Constraints associated with funding can be a major limitation to organization of Blitz-like events, as funding agencies want more for their buck. When compared to typical BioBlitzes, ParasiteBlitzes are more expensive, as molecular identification of parasites (and less common hosts) is best practice. The funding dynamic of a ParasiteBlitz is also novel, given its short duration and high spend rate compared to longer-term traditional parasite surveys. We had an imposed funding-dependent one-year post-Blitz deadline to submit results to our grant agencies, which did not allow time to identify all species either morphologically or molecularly to species level. The silver lining here is that this reflects the parasite diversity encountered, and thus the numerous species that need description and/or sequencing to populate databases (e.g., GenBank, BOLD), and this diversity further emphasizes the promising productivity of ParasiteBlitzes.

Haste, not waste

Finally, as mentioned above, one major concern about Blitzes is that their inherent speed may lead to sloppiness and mistakes. It is thus important to emphasize that the implied speed of a Blitz translates only to sample collection but does not mean identification is less rigorous. One further difference between a BioBlitz and a ParasiteBlitz to keep in mind is that voucher specimens of parasites are deposited into curated collections so verification of identity will always be possible in the future. Nevertheless, parasite identification is still a lengthy process, and we see it currently at the same point as other taxa were at in the first BioBlitzes; modern BioBlitzes are way more productive with the advent of applications on mobile phones. Because thoroughness in identification must be maintained, faster results will occur only if processing can be accelerated. For parasites, improved online databases, checklists, and access to digitized types from museums will reduce identification time. Even though depositing voucher specimens remains technically optional, their critical importance cannot be stressed enough. As such, the request to deposit vouchers has been made repetitively to parasitologists (Buckner *et al.* 2021; Galbreath *et al.* 2019; Hoberg *et al.* 2015; Thompson *et al.* 2021). The common recommendation to include (and frequently require) a taxonomically informative DNA

sequence as part of parasite identity adds a layer of time requirement beyond a traditional Blitz. Yet technological advancement is at the point of enabling host and parasite DNA sequencing within the several-day time scope of a Blitz. Technologies like the Oxford Nanopore MinION portable long-read sequencing device enable real-time and on-site DNA sequencing, which we envision could be readily coupled with established, robust protocols using parasite-taxon-specific primers to generate daily sequence identities. We propose that a team of two or three additional experts could wrangle on-site PCR and sequencing and take the ParasiteBlitz to its next level of being a rapid, focused, and accurate means of generating parasite diversity data. Recognizing novel species remains the major goal behind the adoption of the Blitz approach, and being able to quickly filter out known species would allow more time for such recognition and descriptions.

Concluding summary

The adoption of the BioBlitz concept to parasitology has overall yielded positive outcomes. In particular, we generated a census of metazoan parasites at a previously unexplored locality, and we added novel host records and discovered unnamed parasite species. Hence, our objective to test the concept as a shot-gun approach to increase global parasite diversity knowledge was fulfilled. We enhanced the BioBlitz concept to apply it to parasitology given both expected and unexpected challenges. In particular, we were unable to assign taxon identity to most of our eDNA sequences, which again, highlights the crucial need for more sequences in reference databases in general, but especially for parasites. However, the sequences generated by using the traditional methods will allow for more accurate and comprehensive reference databases, facilitating taxonomic assignment in future metabarcoding projects. This alone is an incentive to continue ParasiteBlitzes to support better utilization of eDNA and other non-invasive techniques, thereby limiting the need to sacrifice too many hosts to assess parasite diversity. In a nutshell, the positive counterbalances to incomplete databases, and time and funding limitations for a ParasiteBlitz, include potential for speedy discoveries; quick acquisition of baseline information for target localities; active exchange of information among host and parasite taxonomists; active learning from students; enhancement of student engagement; increased awareness of the true role of parasites in ecosystems among helpers, administrators, and funding agencies; and last but not least, opening of future research questions to further the understanding of parasites' natural history and role in function of local ecosystems.

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