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REPORT

Black-spot syndrome in Caribbean fishes linked to trematode parasite infection (*Scaphanocephalus expansus*)

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Abstract Despite the evidence that diseases have increased in marine taxa, parasites remain underrepresented in studies of marine ecology. Recently, observations of black-spot syndrome (BSS) in Caribbean fishes, especially ocean surgeonfishes, *Acanthurus tractus* (Poey, 1860), have been reported, although its cause(s) has remained conjectural. We investigated the etiology of BSS and whether the pathology was functionally and consistently associated with particular infections. By examining the patterns of BSS among reef fishes in Bonaire, we show that observed dermal spots are the encysted stages (metacercariae) of the heterophyid trematode *Scaphanocephalus expansus* (Creplin, 1842). Metacercariae were detected within ten species of fishes, with loads ranging from 1–315 per fish. These represent the first published accounts of the genus *Scaphanocephalus* in the Caribbean and are all new host records. Molecular analysis of sequences identified the infection as a heterophyid, while morphological examinations and comparisons with archival specimens confirmed the species identity as *S. expansus*. Field-based estimates of BSS by scuba divers and subsequent video analysis correlated positively with parasite counts from necropsy. No such correlation was observed between BSS and metacercariae of a second trematode genus, *Bucephalus*. The consistent link between host burdens of *S. expansus* and BSS highlights the potential for low-cost and low-impact assessments in fish populations, including the use of images from citizen scientists and public databases. Taken together, these results help establish the foundation for future investigations into the pathology, geographic distribution, life cycle, and ecological consequences of BSS.

Keywords Emerging infection · Fish pathology · Bonaire · Non-lethal assessment · Black-spot syndrome · *Scaphanocephalus expansus*

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Introduction

Growing evidence highlights the importance of marine pathogens in affecting host population dynamics and even structuring entire ecological communities (Harvell et al. 1999; Groner et al. 2015; Lafferty et al. 2015; Lafferty and Hofmann 2016). Recently, emergence of sea star wasting syndrome, linked to infection by a densovirus (Hewson et al. 2014), has caused mass mortality in multiple species of sea stars and catalyzed subsequent shifts in subtidal communities (Eckert et al. 2000; Menge et al. 2016; Montecino-Latorre et al. 2016; Schultz et al. 2016). Similarly, a widespread mortality of the long-spined urchin

(*Diadema antillarum*) in the 1980s—which fundamentally reshaped coral reef communities across the Caribbean Sea—is believed to have been caused by a bacterial pathogen (reviewed in Lessios 2016). Despite the evidence that infections and disease syndromes have increased in marine taxa (e.g., Ward and Lafferty 2004), parasites remain underrepresented in the marine ecology literature (Timi and Mackenzie 2015; Lafferty and Hofmann 2016; Schultz et al. 2016), and our understanding of their role within such ecosystems is often limited to specific parasite–host systems or case studies.

Many marine parasites have complex life cycles involving sequential infection of multiple host species, often involving host taxa distributed across multiple trophic levels. Parasites are intimately tied to changes in ecological communities, sometimes driving dramatic ecological changes (Sousa 1992; Marcogliese 2002; Wood et al. 2007; Lafferty et al. 2008; Mouritsen and Poulin 2009; Poulin et al. 2016). Because transmission between host species often depends on predation (i.e., trophic transmission), parasite-induced alterations in host behavior or appearance that increase predation risk may be selectively favored (Lafferty 2013; Poulin and Maure 2015; Bernal et al. 2016; Hafer and Milinski 2016; Poulin 2017; Herbison et al. 2018). Some of the most well-documented cases of parasite manipulation occur when fishes are intermediate hosts for larval trematodes (Poulin and Maure 2015; Gopko et al. 2017; Talarico et al. 2017). For example, fishes infected with larval trematodes alter their behavior in such a way as to be more conspicuous to avian piscivores, which are common definitive hosts (Crowden and Broom 1980; Lafferty and Morris 1996; Seppälä et al. 2004, 2005). Such parasite-induced changes emphasize the importance of parasite–host dynamics not just for individual hosts, but also for food web structure, energy flow, and species composition (Wood and Johnson 2015).

In 2012, researchers at the Council on International Educational Exchange Research Station Bonaire noted a large proportion of near-shore coral reef fishes exhibited 'black-spot syndrome' (BSS), a condition characterized by conspicuous black spots on the fins and body (Elmer et al. 2019). Ocean surgeonfish, *Acanthurus tractus* (Poey, 1860; formerly *A. bahianus*; Bernal and Rocha 2011), were most frequently observed with BSS (68.7% of all individuals observed in 2017), particularly in shallow waters. Bernal et al. (2016) also reported a high density of reef fishes displaying symptoms similar to BSS on the nearby island of Curaçao (0.23 infected fish per 10 m^2) and over two-thirds of infected fishes were acanthurids. Of note, the presence of blemishes was not correlated with host functional traits, including diet, mobility, schooling behavior, and position in water column (Bernal et al. 2016), whereas depth was a key predictor in Bonaire (Elmer et al. 2019). Digital analysis of publicly available image databases indicated that symptoms consistent with BSS have been present around Bonaire as far back as 1985 and are widespread throughout the western Atlantic in *A. tractus* (Elmer et al. 2019). Bernal et al. (2016) speculated that such blemishes could be associated with dermal parasites, including digenetic trematodes, turbellarians, or protozoans. Thus far, however, the lack of an identified etiological agent has limited opportunities to understand the mechanism(s) of BSS pathology or inform managers about its potential effects on local fish populations.

The first goal of this study was to investigate the etiology of BSS and determine whether the visible signs were consistently associated with specific forms of parasitic infection. To this end, we comprehensively examined the patterns of dermal anomalies among 44 fishes from Bonaire with a focus on *A. tractus*. Specifically, we evaluated whether black spots aligned with the presence and abundance of macroparasitic infections upon necropsy. Our results indicated that such spots were cysts of a heterophyid trematode, *Scaphanocephalus expansus* (Creplin 1842), for which we provide a detailed morphological and genetic description alongside an in-depth literature review of the genus. We also confirmed the presence of infection in nine additional reef fishes.

The second goal was to compare visual estimates of BSS in *A. tractus* at field sites in Bonaire—using both in situ scuba diver assessments and post hoc analysis of video footage—to parasite counts from necropsy on a subset of specimens, with the aim of determining whether field-based assessment of BSS accurately reflects the presence and degree of infection. These findings highlight the potential use of non-lethal techniques, including analysis of image and video footage obtained by citizen scientists, in the further study of BSS and its implications for reef communities, particularly following the confirmation of *S. expansus* infection in local fish populations.

Materials and methods

Study site

Bonaire is a small (294 km^2) island located in the Leeward Antilles of the southern Caribbean Sea (Fig. 1). Fishing provides an important source of livelihood as well as recreation for local populations (Schep et al. 2012). Bonaire's shallow reefs are considered a biodiversity hotspot (Myers et al. 2000) and have been managed since 1979 as the Bonaire National Marine Park by the Stichting Nationale Parken (STINAPA) Bonaire (Becking and Meesters 2014). Its near-shore waters support at least 362 species of

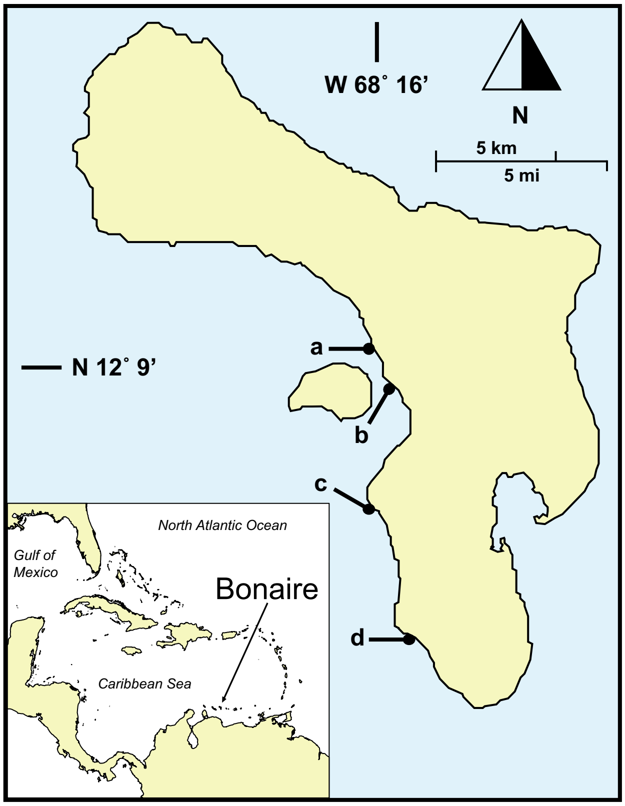

Fig. 1 Island of Bonaire, Caribbean Netherlands, in the southern Caribbean Sea (inset). Collection sites indicated as follows: (a) Cliff (12°10′28″N and 68°17′24″W), (b) Playa Lechi (12°09′37″N and 68°16′55″W), (c) The Lake (12°06′20″N and 68°17′20″W), and (d) Margate Bay (12°03′08″N and 68°16′25″W)

fishes, of which *A. tractus* is a common inhabitant and accounts for a sizeable portion of the shallow-water herbivorous fish population (Lewis and Wainwright 1985; Robertson et al. 2005).

Field sampling and parasite assessment

Fish were collected from the leeward side of Bonaire in the summer and fall of 2017 with approval from the Dienst Ruimtelijke Ordening en Beheer (DROB; Department of Nature; Permit #: 2014002497). The primary species of interest was *A. tractus* (ocean surgeonfish; *n* = 31). Collection was haphazardly designed to acquire a minimum of three individuals of *A. tractus* within each category of BSS severity (stages 0–3, see below; Fig. 2), encompassing a range of levels against which to compare detected infections. We collected seven additional species with visible signs of BSS to confirm that the causative agent was consistent among affected fishes (Table 1). Specimens were collected between 2–10 m of depth at four locations: Cliff, Playa Lechi, The Lake, and Margate Bay (Fig. 1). Fishes were obtained with an Eradicate Lionfish tool (Innovative Scuba Concepts, Colorado, USA), immediately placed on ice, shipped overnight to the University of Colorado, Boulder, and frozen until necropsy. Specimens of two additional species, *Cantherhines pullus* and *Cephalopholis cruentata*, were dissected on Bonaire, for which necropsy was limited to removal and excystment of metacercariae without a quantitative assessment of parasite load or species composition. Host scientific names follow Froese and Paully (2019).

Because the causative agent of BSS is unknown, we comprehensively examined fish hosts (both with and without BSS) for all macroparasitic infections. Total length (mm) of each fish host was measured with digital calipers, and eyes, nares, gills, scales, subcutaneous tissues, and musculature were examined for macroparasites using an Olympus SZX10 stereo dissection microscope (Calhoun et al. 2018). Necropsy included an examination for the larval or adult stages of any trematodes (flukes), nematodes (roundworms), tapeworms (cestodes), spiny-headed worms (acanthocephalans), gill worms (monogeneans), and parasitic arthropods (e.g., isopods, mites, copepods). Isolated parasites were identified following Schell (1985), Grabda (1991), Hoffman (1999), and Williams and Bunkley-Williams (1996). Fresh and recently thawed encysted metacercariae were incubated at 40 °C for 12–24 h in 1.5-ml conical tubes with 1.4 ml of 0.5 w/v % porcine pepsin in distilled water adjusted to pH 2 with 6 N HCl (detailed in Fried and Johnson 2005). Voucher samples were stained with aqueous Meyer’s hematoxylin solution, dehydrated through graded ethanol series, cleared with methyl salicylate, and mounted in Canada balsam to facilitate identification (Calhoun et al. 2018). Molecular vouchers were preserved in 95% ethanol and stored at − 20 °C for genomic analysis.

Morphology analysis

After detecting an association between BSS and the presence of metacercariae of the heterophyid trematode *Scaphanocephalus* sp., we compared our metacercariae (*n* = 9) with previous descriptions from the primary literature, including Jägerskiöld (1904), Yamaguti (1942), Dubois (1960), Pearson and Prévot (1985), Hoffman (1999), Pearson (2008), and Foronda et al. (2009). We also reexamined comparative material from the Harold W. Manter Laboratory (HWML) of the University of Nebraska, Lincoln, consisting of seven adults collected from an osprey from Mexico (HWML 22997) and a single metacercaria collected from a Nassau grouper, *Epinephelus striatus*, from Jamaica (HWML 124300).

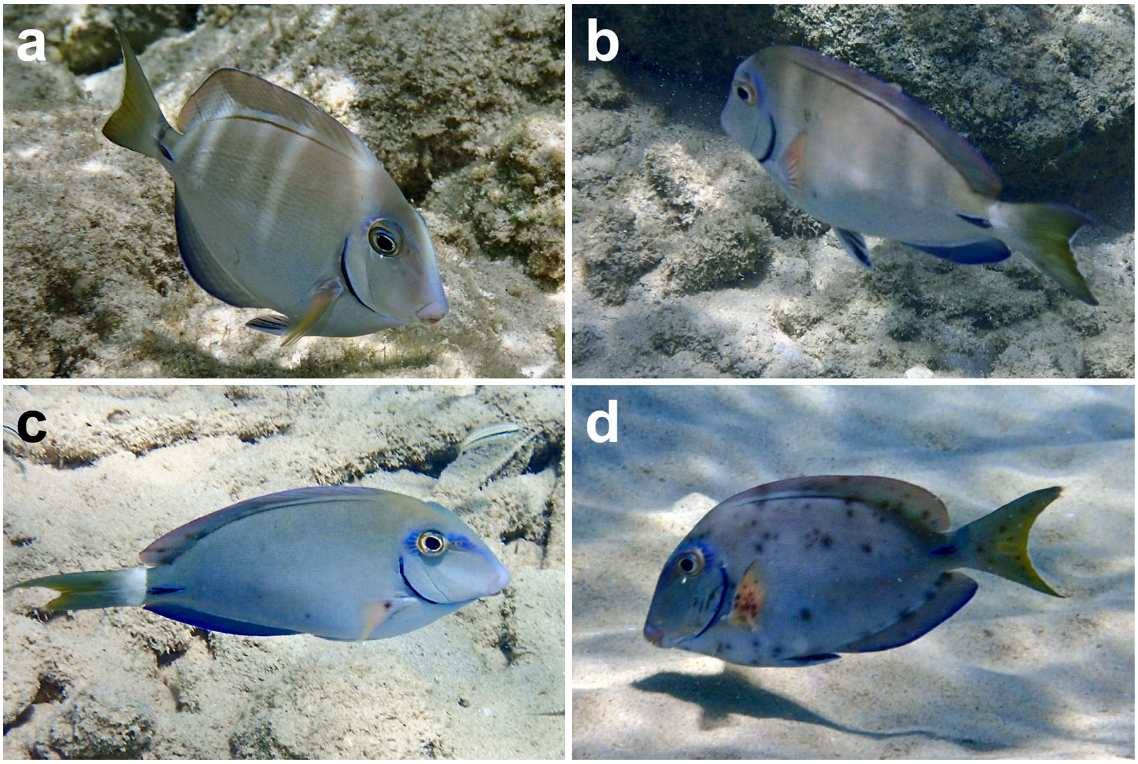

Fig. 2 Stages of black-spot syndrome on *Acanthurus tractus* (ocean surgeonfish) as assessed in the field: **a** stage 0 (no obvious spotting); **b** stage 1 (1–4 spots); **c** stage 2 (5–10 spots); **d** stage 3 (11 or more spots). Only one lateral surface was used for field categorization of specimens

Molecular analysis

Genomic DNA for molecular analysis was isolated from two *Scaphanocephalus* sp. metacercariae obtained from *Sparisoma chrysopterum* following initial morphological identification and excystment. DNA was isolated according to Tkach and Pawlowski (1999). An approximately 1300-bp-long region at the 5′ end of the nuclear 28S rRNA gene (lsrDNA) was amplified using forward primer dig12 (5′–AAG CAT ATC ACT AAG CGG–3′) and reverse primer 1500R (5′–GCT ATC CTG AGG GAA ACT TCG–3′) (Tkach et al. 2003). PCRs were run on a BioRad T-100 (Hercules, California, USA) thermal cycler using OneTaq Master Mix (New England Biolabs, Massachusetts, USA) following the manufacturer's instructions; annealing temperature was 53 °C. PCR products were purified using ExoSAP-IT (Affymetrix, California, USA) and cycle-sequenced directly using BrightDye® Terminator Cycle Sequencing Kit (MCLAB, California, USA). Sequencing reactions were cleaned up using BigDye® Cleaning Beads (MCLAB) and run on an ABI Prism 3130 automated sequencer. Contiguous sequences were assembled using Sequencer^{TM} version 4.2 (GeneCodes Corp., Michigan, USA). Because the sequences were identical, only one was submitted to GenBank® under accession number MK680936.

Newly generated partial 28S rDNA sequences from the *Scaphanocephalus* sp. specimens were aligned with 28 previously published sequences of other trematodes (heterophyids and closely related opisthorchiids) from GenBank®, included in the most recent phylogeny of the group (Kuzmina et al. 2018) using Mega7 software. A cryptogonimid, *Mitotrema anthostomatum* (Manter 1963), was chosen as an out-group based on the results of the phylogenetic analysis by Olson et al. (2003). The alignment was trimmed to the length of the shortest sequence. The final alignment was 877 nucleotides long.

Phylogenetic analyses were conducted using Bayesian inference as implemented in the MrBayes version. 3.2.6 software (Ronquist and Huelsenbeck 2003). The general time-reversible model with estimates of invariant sites and gamma-distributed among-site variation (GTR + I + G) was identified as the best-fitting nucleotide substitution model for both datasets using jModelTest 2 software (Darriba et al. 2012). Bayesian inference analysis was performed using as follows: Markov chain Monte Carlo chains were run for 3,000,000 generations, log-likelihood scores were plotted, and only the final 75% of trees were used to produce the consensus trees. Trees were visualized in FigTree version 1.4 software (Rambaut 2012).

Table 1 Number of *Scaphanocephalus expansus* metacercariae infections as determined by necropsy in fishes collected during SCUBA sessions from near-shore waters of Bonaire, Caribbean Netherlands, from June to October 2017

Host species	*N*	Prevalence (% infected)	Range	Abundance (mean parasites per fish ± SE)	Intensity (mean parasites per infected fish ± SE)
Acanthurus tractus (ocean surgeonfish)	31	90.3	0–132	19.4 ± 9.4	21.5 ± 5.7
Subset used in field and video GLMM analysis	24	87.5	0–78	15.9 ± 4.3	18.2 ± 4.7
Acanthurus chirurgus (doctorfish)	2	50	0–1	0.5 ± 0.5	1
Acanthurus coeruleus (blue tang surgeonfish)	2	100	1–2	1.5 ± 0.5	1.5 ± 0.5
Cantherhines pullus (orangespotted filefish)*	1	–	–	–	–
Caranx ruber (bar jack)	3	100	2–7	3.7 ± 1.7	3.7 ± 1.7
Cephalopholis cruentata (graysby)*	1	–	–	–	–
Paranthias furcifer (creole-fish)	1	100	2	2	2
Mulloidichthys martinicus (yellow goatfish)	1	100	14	14	14
Sparisoma aurofrenatum (redband parrotfish)	3	100	126–315	234.7 ± 56.4	234.7 ± 56.4
Sparisoma chrysopterum (redtail parrotfish)	1	100	17	17	17

Presented are the number of host specimens examined (*N*), the percentage of hosts found infected during necropsy (prevalence), the range in the number of metacercariae per host (range), the average number of parasites per sampled fish (abundance), and the average number of parasites per infected host (intensity). Host species other than *A. tractus* were collected only if black spots were observed in the field. Current host species taxonomy retrieved from Froese and Paully (2019)

* *Scaphanocephalus expansus* infection confirmed, but not quantified in these species

Comparison of SCUBA assessments with necropsy analysis

To evaluate the correspondence between field-based classifications (from scuba surveys) of BSS and quantitative necropsies, we visually categorized apparent infections from 24 *A. tractus* observed at Playa Lechi and The Lake field sites in October 2017. For each fish, a diver categorizes the stage of infection as follows: 0 (no visible spots), 1 (1–4 spots), 2 (5–10 spots), and 3 (more than 10 spots), consistent with previous classifications for one lateral surface of the fish in the field (Elmer et al. 2019). Analysis of video footage (GoPro®) of each animal prior to collection was used to estimate BSS spot counts to compare with field estimates. Captured fish were necropsied to quantify metacercariae in the scales, fins, and dermal tissue, as described above, for comparison with field- and video-based classifications.

The counts of *Scaphanocephalus* sp. metacercariae from each dissected *A. tractus* that matched with a field or video estimate of BSS (24 of 31 sampled) were analyzed using generalized linear mixed models (GLMMs) implemented using the `glmer` function within the `lme4` package in R (Bates et al. 2014; R Core Team 2018). Because counts of parasites per fish were overdispersed, with a mean of 15.9 (range 0–78) and a sample variance of 2994, an overdispersed Poisson model with a log-link function was used to approximate the distribution (Elston et al. 2001; Harrison 2014). The response variable was metacercariae per host (as determined from necropsy) with a fixed effect for the field-based classification (0, 1, 2, or 3) and an observation-level random effect (i.e., the individual host). Numerical predictor variables were centered and scaled prior to inclusion in the model. We calculated approximate *P* values using the `lmerTest` package in R (Kuznetsova et al. 2017). We conducted this analysis twice: once with field classification of SCUBA divers and again using video counts of black spots as a predictor of actual parasite counts from necropsy. As a negative control, we compared the number of black spots assessed in the field with necropsy-based counts of a second trematode taxon, *Bucephalus* sp., found in the fins of and scales of examined fishes but not directly associated with black spots. All means are reported as mean ± 1 SE.

Results

Parasite quantification and within-host distribution

Parasitic infection was assessed by necropsy in fishes collected on Bonaire. Black spots, present in 40 of 44 specimens representing eight new host species, were identified as encysted metacercariae of a digenetic trematode in the genus *Scaphanocephalus* (Fig. 3; Table 1). Metacercariae were contained within thick-walled cysts commonly located within the integument and fin rays of the host. Tissue immediately surrounding the cysts appeared darkly pigmented upon gross observation, leading to the characteristic spotting of BSS (Fig. 3a, b). Among infected fishes, the average intensity (± 1 SE) was 33.9 ± 10.6 metacercariae per fish (range 1–315). The greatest infection loads were observed in *Sparisoma aurofrenatum*, for which the three individuals examined supported loads of 126, 263, and 315 metacercariae (Table 1). Ad hoc necropsies conducted on Bonaire identified *Scaphanocephalus* sp. infection in two additional host species, *Cantherhines pullus* ($n = 1$) and *Cephalopholis cruentata* ($n = 1$; Table 1).

Cysts were especially conspicuous on light-colored fishes, such as *A. tractus*, and were less obvious on multicolored or darkly colored hosts, such as *S. aurofrenatum* or *S. chrysopterum*. However, all metacercariae were readily detectable using microscopy. Parasites often appeared milky white to clear after freezing, although this was not observed among the small number of fishes dissected without freezing in Bonaire (Fig. 3c). Metacercariae of *Scaphanocephalus* sp. were found in the fins, subcutaneous tissues, operculum, and within and around the eyes of infected fishes ($n = 1354$ metacercariae from 40 fishes). The majority of metacercariae were detected on the fins (61.5%), especially the pectoral fins (25.1%). The caudal, pelvic, dorsal, and anal fins supported 12.1%, 9.4%, 8.4%, and 6.4% of total detected cysts, respectively. The remaining 38.5% of infections were found beneath the scales in the epidermis or dermis. Interestingly, in three cases metacercariae were observed in the aqueous humor of one or both eyes. Based on the measurements of 10 individuals, the average size of an encysted metacercaria was 723.0 ± 42.1 µm in length (range 590–950 µm) by 669.0 ± 28.0 µm in width (range 540–775 µm). There was no significant effect of host body size on the number of metacercariae in *A. tractus* (Poisson GLMM with random intercept of host identity: *scale*(*fish length*) = 0.377 ± 0.0249, $P = 0.13$; $n = 31$).

In addition to *Scaphanocephalus* sp., 24 fishes (54.5%) were infected with metacercariae of a second trematode (*Bucephalus* sp.), which was associated with small, white spots visible just under the scales: 20 of 31 *A. tractus*, two of three *S. aurofrenatum*, one of two *Acanthurus coeruleus*,

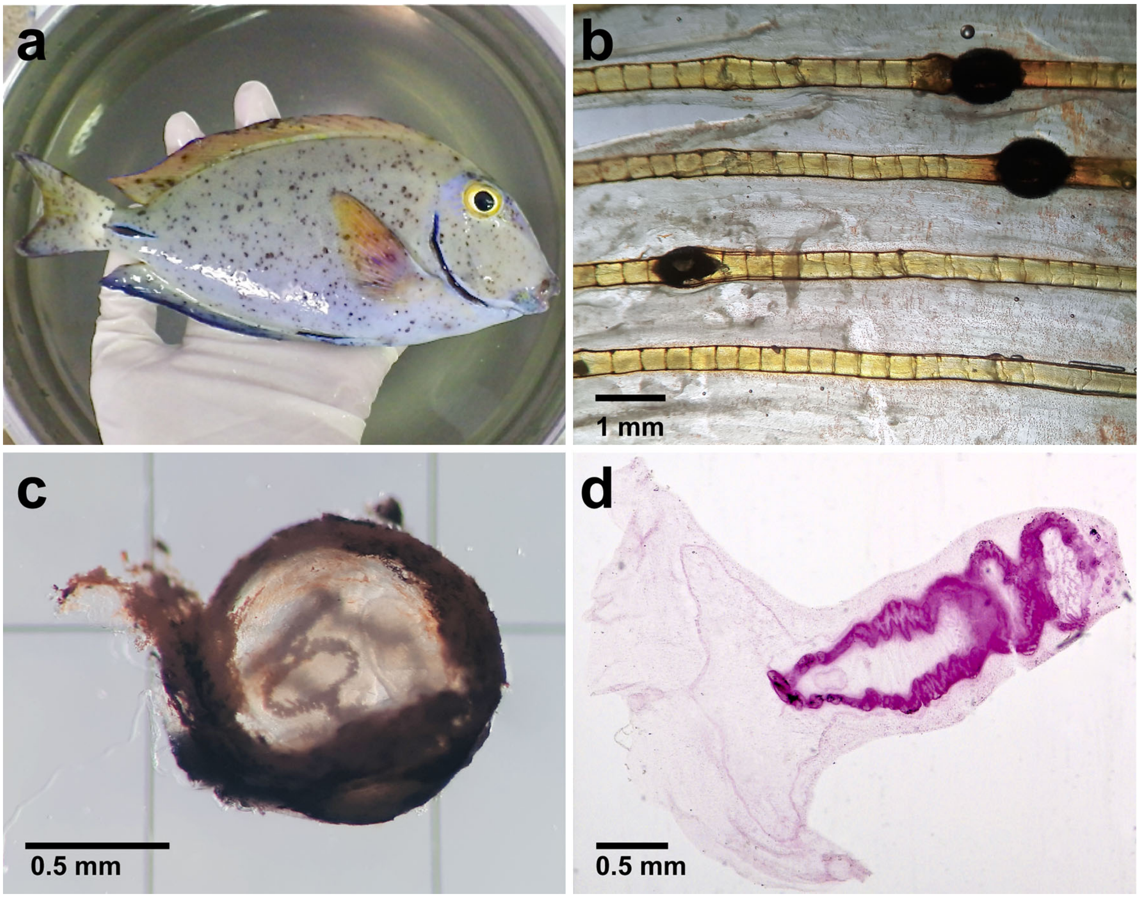

Fig. 3 **a** Black-spot syndrome affected *Acanthurus tractus* (ocean surgeonfish) infected with *Scaphanocephalus expansus*. Metacercariae of *S. expansus* associated with BSS in **b** fin rays and **c** excised from *Cantherhines pullus* (orangespotted filefish). **d** Stained and mounted *S. expansus* metacercaria with characteristic wing-like anterior extensions (excysted from *A. tractus*). All specimens were collected in Bonaire, Caribbean Netherlands

and one of one *S. chrysopterum*, with no detections in the remaining fish species. Infection intensity for *Bucephalus* sp. averaged 140.8 ± 26.6 metacercariae per infected host (range 2–438). Metacercariae of *Bucephalus* sp. were considerably smaller than those of *Scaphanocephalus* sp., averaging 281.9 µm ± 19.0 µm in length (range 190–340 µm) and 152.4 ± 7.8 µm in width (range 110–190 µm; $n = 10$). *Bucephalus* sp. metacercariae occurred most frequently within the pectoral fins although, relative to *Scaphanocephalus* sp., cysts were embedded more shallowly within the tissue of the fin rays and scales. Twenty-one of the 24 *Bucephalus* sp. infected fishes were also infected with *Scaphanocephalus* sp., yet the differences in metacercaria size, location, and morphology made differentiation straightforward; notably, only metacercariae of *Scaphanocephalus* sp. were clearly associated with black-spot syndrome. No other parasite species were detected under the scales or in close proximity to the black spots. Of the fishes that were comprehensively necropsied, eight other parasite taxa were detected in the gills, intestines, mouth and body cavity (two monogeneans, four digenetic trematodes, one nematode, and one isopod ectoparasite; Table S1).

Morphological identification

Metacercariae excysted from black spots were identified as *Scaphanocephalus expansus* (Creplin, 1842; Trematoda: Heterophyidae) based on the following combination of characteristics: forebody with wing-like lateral expansions; hindbody subcylindrical; ovary multilobate, median, pretesticular; testes tandem, highly ramified, in posterior third of body; genital atrium with gonotyl opening near junction of two body regions. All of these characteristics, originally described in the adult *S. expansus*, were also well developed in the metacercariae (Fig. 3d; Table 2). Specimens examined from Bonaire fishes were consistent with specimens re-examined from museum collections, including seven adult *S. expansus* from an osprey in Mexico (HWML 22997) and a metacercaria collected from a Nassau grouper (*Epinephelus striatus*) in Jamaica (HWML 124300). They were broadly similar to published descriptions and morphologies of metacercariae and adults by Jägerskiöld (1904), Yamaguti (1942), Dubois (1960), and Foronda et al. (2009; Table 2). Our specimens had deeply lobed testes and large eggs, differentiating them from *Scaphanocephalus australis*, which is described as having slightly lobed testes and smaller eggs relative to *S. expansus* (Johnston 1917; Table 3).

Molecular results

The sequences obtained as part of this study are the first for *Scaphanocephalus* in GenBank®. A BLAST search of the GenBank® database clearly demonstrated that our sequences represented a heterophyid digenean, consistent with the identification of *Scaphanocephalus*. Our Bayesian inference phylogenetic analysis produce a tree with overall well-supported topologies (Fig. 4). The sequence of our *S. expansus* samples—alongside a sequence of the trematode *Cryptocotyle lingua*—appeared in a well-supported (100%) clade. This clade belonged to a larger cluster that included genera traditionally placed in the trematode family Heterophyidae (*Euryhelmins*, *Apophallus*) or the

Table 2 Morphological description of *Scaphanocephalus expansus* in the literature and current study

	Source	Current study ($n = 1$)	Manter (HWML 123400; $n = 1$)	Yamaguti (1942; $n = 1$)	Foronda et al. (2009; $n = 43$)
	Location	Bonaire	Jamaica	Japan	Spain
	Host	*Acanthurus tractus*	*Epinephelus striatus*	*Parupeneus multifasciatus*	*Pandion haliaetus*
Measure	Stage	Metacercaria	Metacercaria	Metacercaria	Adult
Total length		3270	2650	2400	3300 ± 400
Anterior end width		2100	1700	2100	1700 ± 500
Posterior end width		940	740	900	900 ± 100
Oral sucker length		103.7	85	90	101 ± 6
Oral sucker width		96.8	135	105	86 ± 3
Anterior testis length		205.7	125	150	375 ± 74
Anterior testis width		629.3	500	600	593 ± 72
Posterior testis length		219.5	145	220	428 ± 89
Posterior testis width		656.4	540	675	634 ± 86
Ovary length		132.2	85	100	171 ± 37
Ovary width		310.2	290	260	298 ± 75

All measurements given in micrometers (µm)

Table 3 Records of *Scaphanocephalus* sp. infections documented in previous literature

Genus species	Host	Life stage	Location	Intensity	Prevalence
*Scaphanocephalus adamsi**	*Bodianus mesothorax* (splitlevel hogfish)[1]	Metacercaria	Manila, Philippines	Present	1/1
Scaphanocephalus australis	*Haliaeetus leucogaster* (white-bellied sea eagle)[2]	Adult	Sydney, Australia	3	1/1
Scaphanocephalus expansus	*Archosargus rhomboidalis* (western Atlantic sea bream)[3]	Metacercaria	Florida, USA	Present	Present
	Eucinostomus argenteus (silver mojarra)[3]	Metacercaria	Florida, USA	Present	Present
	Lutjanus griseus (gray snapper)[4]	Metacercaria	Florida, USA	Present	Present
	Micropogonias undulatus (Atlantic croaker)[3]	Metacercaria	Florida, USA	Present	1/1
	Pandion haliaetus (osprey)[5]	Adult	Europe	Present	Present
	P. haliaetus[6]	Adult	Egypt	Present	Present
	P. haliaetus[7]	Adult	Egypt	Present	Present
	P. haliaetus[8]	Adult	France	Present	Present
	P. haliaetus[9]	Adult	Sonora, Mexico	35	1/1
	P. haliaetus[10]	Adult	Florida, USA	361	2/5
	P. haliaetus[11]	Adult	Canary Islands, Spain	43	1/3
	P. Haliaetus carolinensis (osprey)[12]	Adult	Iowa, USA	30	1/1
	P. Haliaetus haliaetus (osprey)[13]	Adult	Malaysia	Present	Present
	Pristis pectinata (smalltooth sawfish)[14]	Metacercaria	Florida, USA	Present	Present
	Parupeneus multifasciatus (manybar goatfish)[15]	Metacercaria	Naha, Japan	Present	Present
Scaphanocephalus sp.	*Arothron meleagris* (guineafowl puffer)[16]	Metacercaria	Baja California Sur, México	3.67	6/8
	Cephalopholis panamensis (Pacific graysby)[17]	Metacercaria	Baja California Sur, México	Present	2/43
	Hemiramphus brasiliensis (ballyhoo halfbeak)[18]	Metacercaria	Florida, USA	Present	Present
	Pomacentridae fish[16]	Metacercaria	Baja California Sur, México	15	1/1

Intensity is the average number of parasites among infected hosts (not including uninfected hosts)

Prevalence represents the number of infected hosts relative to the total number examined, if reported. Citations for each record indicated by superscript and listed below. Current host species taxonomy retrieved from Froese and Paully (2019)

**Scaphanocephalus adamsi* may be synonymous with *S. expansus* (Yamaguti 1958)

[1]Tubangui (1933); [2]Johnston (1917); [3]Skinner (1978); [4]Overstreet and Hawkins (2017); [5]Creplin (1842); [6]Jägerskiöld (1904); [7]Gohar (1935); [8]Dubois (1960); [9]Schmidt and Huber (1985); [10]Kinsella et al. (1996); [11]Foronda et al. (2009); [12]Hoffman (1999); [13]Lee (1966); [14]Hutton (1964); [15]Yamaguti (1942); [16]Pérez-Urbiola et al. (1994); [17]Inohuye-Rivera (1995); [18]Bullard and Overstreet (2008)

Opisthorchiidae (*Opisthorchis*, *Clonorchis*, *Amphimerus*). The remaining tree topologies were identical to those in Kuzmina et al. (2018), the most recent phylogeny that included Heterophyidae.

Comparison of field-based classifications and necropsy

The GLMM analysis indicated a strong, positive relationship between diver-based classifications of severity (categorizing the number of black spots into uninfected [0], stage 1 [1], stage 2 [2], stage 3 [3]) and the number of isolated metacercariae of *S. expansus* upon necropsy (Poisson GLMM: *intercept* = 0.67 ± 0.388, $P = 0.08$; *field value coefficient* = 0.855 ± 0.184, $P < 0.0001$; $n = 24$; Fig. 5a). This suggests that, for each one-unit increase in the ordinal diver-based classification of severity, there was a 2.4-fold increase in the total number of metacercariae (based on exponentiation of the coefficient from the Poisson model). Notably, however, the stage 3 category from field assessments was used to classify any fish with > 10 spots; therefore, this classification encompassed a much larger potential range of parasites; for instance, metacercariae per fish in the high [3] category varied from 16–78 (mean 38.7 ± 10.1; $n = 7$), which makes the gradient of this variable decidedly uneven.

The number of spots observed on one lateral surface of a fish using video footage, ranging from 0–35, offered

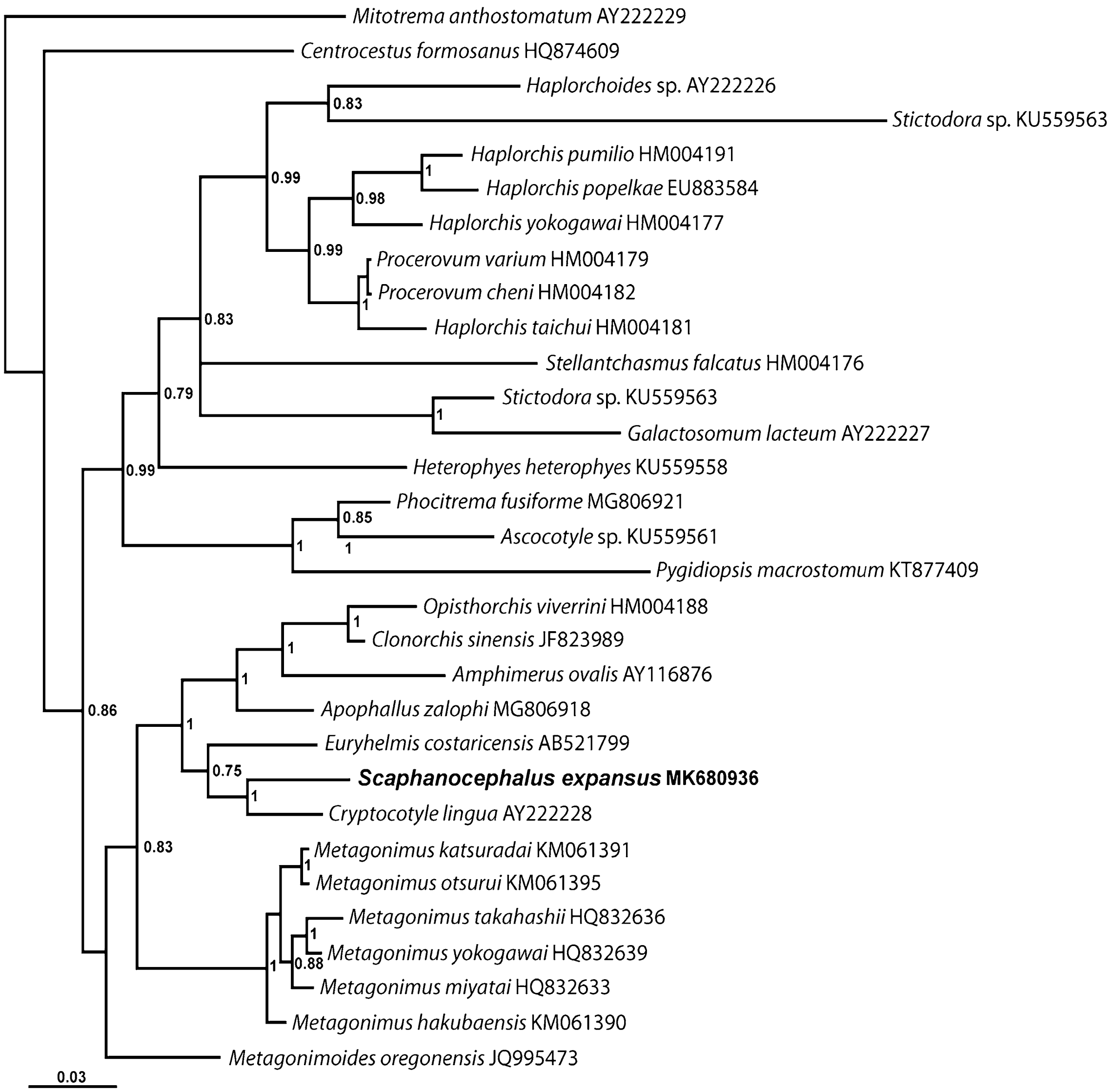

Fig. 4 Phylogenetic tree resulting from Bayesian analysis (3,000,000 generations) of partial sequences of the 28S rRNA gene. The sequence of *Scaphanocephalus expansus* obtained in this study is in bold. Numbers at internodes indicate nodal support as posterior probabilities (only values 0.75 or greater are shown)

comparable explanatory power to the ordinal classification system (ΔAIC = + 2). More specifically, the estimated slope between black spots counted on video and those quantified from the same fish upon dissection was 0.107 ± 0.024 (Poisson GLMM: *intercept* = 1.31 ± 0.287, $P < 0.0001$; *field value coefficient* = 0.106 ± 0.025, $P < 0.0001$; $n = 24$; Fig. 5b). Exponentiating this slope coefficient, for each one-unit increase in the number of spots detected by video there was an ~ 11% increase in the number of metacercariae quantified during necropsy. Once again, however, video-based assessments classified the spots on only a single side of the fish host, whereas necropsies quantified the total number of parasites on both sides, which bears remembering when interpreting such results.

To further validate the link between *S. expansus* metacercariae and observations of black spots, we tested for an association between the number of diver-quantified black spots and the necropsy-based counts of metacercariae for the trematode, *Bucephalus* sp. (not noticeably linked to BSS). Among the 24 fishes for which this infection was detected (range 2–386 metacercariae per host), we found no significant relationship between diver-based assess-

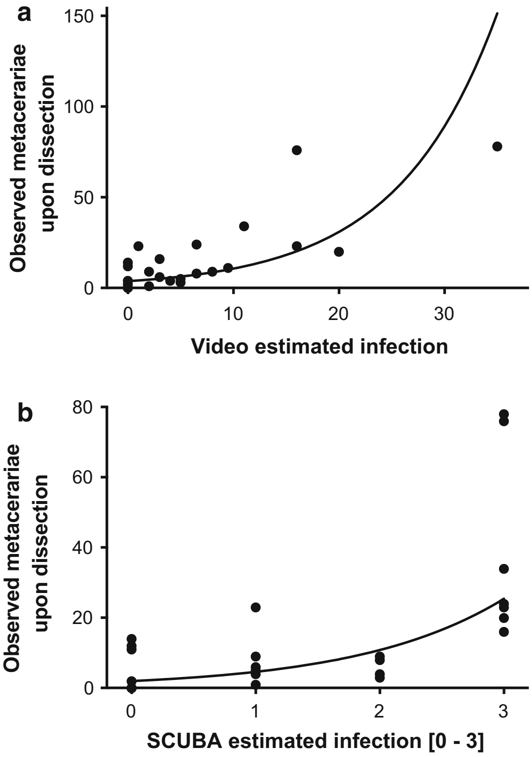

Fig. 5 Relationship between **a** video-estimated infection and **b** field-assessed infection severity of black-spot syndrome (BSS) compared to the number of *Scaphanocephalus expansus* metacercariae observed during necropsies of *Acanthurus tractus* (ocean surgeonfish). Severity was assessed in the field with four stages: stage 0 (no obvious spots); stage 1 (1–4 spots); stage 2 (5–10 spots); and stage 3 (11 or more spots). Video and field estimates were obtained by SCUBA divers with GoPro cameras in near-shore waters of the island of Bonaire, Caribbean Netherlands. Field-based assessments were of a single side of each fish, whereas dissections were comprehensive. Poisson GLMM for **a** video estimates: *intercept* = 0.67 ± 0.388, $P = 0.08$; *field value coefficient* = 0.855 ± 0.184, $P < 0.0001$; $n = 24$. Poisson GLMM for b field estimates: intercept = 1.31 ± 0.287, $P < 0.0001$; field value coefficient = 0.106 ± 0.025, $P < 0.0001$; $n = 24$

ments of black spots and the number of parasites detected at necropsy (Poisson GLMM: *intercept* = 3.75 ± 0.61, $P < 0.0001$; *field value coefficient* = 0.068 ± 0.325, $P = 0.835$). This result indicates two things: even when divers observed no spots on a fish, hosts still supported an average of 42.5 *Bucephalus* sp. metacercariae (i.e., the intercept was significantly greater than zero), and second, increases in the load of *Bucephalus* sp. per host had no significant influence on the change in observed BSS among fishes. Results were comparable when using video-based assessments as the predictor. Thus, field-based observations of BSS—whether measured by divers directly or through video footage—associated only with infection by *S. expansus*, not with other trematode infections.

Discussion

Despite observations of BSS from multiple fish species and islands in the Caribbean and western Atlantic dating as far back as 1985 (Bernal et al. 2016, Elmer et al. 2019), the etiological agent(s) responsible has remained conjectural. Using a combination of morphological and genetic techniques, we provide evidence that BSS in Caribbean fishes is the result of infection by a heterophyid trematode in the genus *Scaphanocephalus*. Upon detailed examination in the laboratory, localized hyperpigmentation in the dermis and fins of affected fishes was closely associated with the encysted metacercariae of *S. expansus*. These metacercariae have a tough, opaquely colored cyst, for which the inner layer of the wall encloses the parasite, often making it difficult to identify without liberation from the cyst (Yamaguti 1942). Once excysted, *S. expansus* have a highly distinctive anterior body shape, which widens to form unique wing-like structures (Yamaguti 1942, 1958; Hoffman et al. 1953; Hoffman 1999; Fig. 3d; Table 2). Based on comparisons of field observations (diver- or video-based assessments) with host necropsies, the presence and severity of BSS on ocean surgeonfish associated closely with the quantified abundance of *S. expansus* metacercariae, whereas no such relationship was detected for metacercariae of a second trematode species (*Bucephalus* sp.) that was not found to form such melanized cysts. These findings offer evidence that infection by *S. expansus* is a necessary and sufficient condition to explain BSS in Caribbean reef fishes, at least within the study region around Bonaire.

Adult and metacercariae of *S. expansus* are distinguished by unique anterior wing-shaped expansions not described in any other trematode taxon (Pearson 2008). Although the exact life cycle is unknown, species in the genus *Scaphanocephalus* have a complex life cycle likely involving marine snails as the first intermediate hosts, fishes as second intermediate hosts, and fish-eating birds as the definitive hosts. The snail intermediate host of *S. expansus* remains unknown, and the physical structure of the free-living cercariae—including whether they possess the distinctive wing-like structures of later stages—remains undescribed. All records of adult *Scaphanocephalus* are from *Pandion haliaetus* (ospreys; Jägerskiöld 1904; Yamaguti 1942; Hoffman et al. 1953; Schmidt and Huber 1985), with the exception of *S. australis* from *Haliaeetus leucogaster* (white-bellied sea eagles; Johnston 1917; Tables 2 and 3). The geographic distribution of *S. expansus* broadly mirrors the global distribution of osprey (Bierregaard et al. 2014; Wiley et al. 2014), including Egypt, Central and North America, and Europe (Table 3).

Our samples of metacercariae are consistent with previous morphological descriptions of *S. expansus*. Yamaguti (1958) recognized only two species: *S. expansus* and *S. australis*. *Scaphanocephalus expansus*, which is far more frequently reported (see Table 3), is longer and narrower in body shape than *S. australis* with more deeply lobed testes and smaller eggs. A third species of *Scaphanocephalus*, *S. adamsi*, was described by Tubangui (1933) from metacercariae encysted in the fins and under the scales of the splitlevel hogfish, *Bodianus mesothorax* (formerly *Lepidaplois mesothorax*), in the Philippines, although Yamaguti (1942) considered it synonymous with *S. expansus*. Correspondingly, the metacercariae observed in this study also aligned closely with the features noted in archived vouchers of adults and a metacercaria of *S. expansus* that were re-examined, including the deeply lobed testes, two unbranched ceca extending into the posterior body, and the large, anterior, wing-like expansion (Fig. 3d; Table 2). Based on the genetic analysis of *S. expansus*, our samples clearly represent a trematode from the family Heterophyidae, consistent with the current classification of this genus (Fig. 4). Because there are no previously reported sequences for *Scaphanocephalus* sp. in GenBank®, we were not able to directly compare our samples with established material; nonetheless, the vouchers and sequences we deposited will offer a valuable foundation for future comparisons.

Our results represent the first records of *S. expansus* from the Caribbean, and all ten fish species are new host records, including four new family records. The use of second intermediate hosts by *S. expansus* appears to be minimally constrained to bony and cartilaginous fishes, with multiple known host species observed across broad regions of the Pacific and Atlantic oceans (see below and Table 3). Metacercariae of *S. expansus* have been reported in six fish species, including one report from the elasmobranch *Pristis pectinata* collected in Florida (Table 3; Hutton 1964). Additionally, specimens of *Scaphanocephalus* sp. have been described from one fish species from the southwest Pacific (possibly *S. expansus*; Tubangui 1933; Yamaguti 1942), three species from the Gulf of California (Pérez-Urbiola et al. 1994; Inohuye-Rivera 1995), and one species from the western Atlantic (Bullard and Overstreet 2008; Table 3). While not confirmed as *S. expansus*, Bernal et al. (2016) also described dermal parasites in Curaçao with pigmentation characteristic of BSS in *A. tractus* (19.7%), *C. pullus* (18.2%), and *C. ruber* (17.7%). Given the similarity of the dermal black spots and the matching predominance in surgeonfish, it is likely that *S. expansus* infection also explains these previous observations of BSS within the Caribbean, however this requires empirical validation. Osório and Godinho (2017) reported similar dermal pigmentation around the Rocas Atoll, which could suggest the infection extends as far south as Brazil, though these individuals were not investigated further. While *S. expansus* has not been reported in fish or osprey from South America, the parasite is likely present given the proximity of the Caribbean Netherlands to the South American mainland (Bierregaard et al. 2014; Fig. 1).

We detected a strong, positive relationship between both diver- and video-based estimates of BSS severity and the number of *S. expansus* metacercariae quantified upon necropsy in ocean surgeonfish (Fig. 5). These findings validate the second goal of the study, which was to evaluate whether non-lethal measurement techniques can approximate *S. expansus* infections in different fishes and locations. Both methods offered similar explanatory power in predicting fish infection load (ΔAIC ≤ 2), although the video-based approach has the dual advantages of allowing (a) the direct counting of black spots (rather than an ordinal classification system) and (b) long-term storage of the footage to be further validated or examined by multiple observers. This provides a potentially low-cost and low-impact monitoring option for management and conservation agencies interested in tracking the epidemiology of BSS, particularly within lightly colored fish, such as ocean surgeonfish, in which BSS is more conspicuous. Following the sacrifice of a subset of individuals to confirm parasite identification—a process simplified by the distinctive wing-like structures of *Scaphanocephalus*—local stakeholders and citizen scientists could participate in surveys to document temporal and spatial changes in BSS prevalence. Using this approach, Elmer et al. (2019) found that BSS in Bonaire was less severe at dive sites adjacent to salt production ponds used by *P. haliaetus* (osprey) as hunting grounds, less severe at greater depths, and that the intensity of spots in surgeonfish has increased over the past five years. Ocean surgeonfish have become increasingly important as ecosystem engineers since the Caribbean-wide die-off of *D. antillarum* during the early 1980s, emphasizing the value of monitoring this group of herbivorous reef fishes into the future, especially using non-lethal techniques.

In coastal habitats, shallow-water marine fishes, including specifically *Acanthurus* spp., represent critical prey species of *P. haliaetus* (Martins et al. 2011; Fig. 6). Osprey populations in the Caribbean are comprised of the non-migratory Caribbean osprey (*P. haliaetus ridgwayi*) as well as seasonally present North American osprey (*P. haliaetus carolinensis*; Bierregaard et al. 2014; Wiley et al. 2014). In Bonaire, osprey are most commonly observed seasonally (May through September), often foraging near-estuarine salt flats (Simal 2009; Smith et al. 2012), or along the shallow regions of the coastal reef. As the sole definitive host genus of *S. expansus*, migratory osprey are likely responsible for the broad global distribution of *S. expansus*,

Fig. 6 *Pandion haliaetus* (osprey) with an *Acanthurus tractus* (ocean surgeonfish) affected with black-spot syndrome (black arrows) on Bonaire, Caribbean Netherlands. Photograph courtesy of Steve Schnoll (www.reeftraveler.com)

whereas non-migratory osprey may be important for sustaining local populations. Osprey populations have been rebounding since the 1970s and 1980s following global reductions in use of the pesticide DDT (reviewed in Bierregaard et al. 2014) and there is intriguing evidence that osprey numbers in the Caribbean have increased between the periods of 1951–1961 and 1977–1989 (Prins et al. 2009). Whether such shifts in osprey populations have similarly altered the range, prevalence, or abundance of *S. expansus* in marine habitats has yet to be systematically investigated.

The consequences of *S. expansus* infections for individual host fishes and reef communities remain unknown. Such infections can be associated with reduced host growth or altered behavior, with potentially important implications for risk of predation by piscivorous birds (Lemly and Esch 1984; Quist et al. 2007). Thus far, records of pathology associated with *S. expansus* are rare, although this is likely because of infrequent study rather than infrequent infection. Black spots have been previously associated with *S. expansus* infection in fishes (Overstreet and Hawkins 2017), and infection by *S. adamsi* was linked to the death of several captive wrasses in the Philippines (Tubangui 1933). Two intriguing lines of inquiry in the continued study of this system are, first, whether the inverse relationship between depth and infection rate is due to shallow-water preference post-infection, or increased infection rates in already shallow preferring individuals (Elmer et al. 2019). Second, whether the large, black spots associated with *S. expansus* metacercariae make infected fishes more conspicuous in shallow marine habitats, potentially increasing their risk of predation by ospreys, and thus the likelihood of the parasite completing its life cycle (Overstreet and Hawkins 2017). Additional study under both natural and experimental conditions will be necessary to elucidate whether and how *S. expansus* infection affects fish behavior, physiology, habitat use, and vulnerability to predation by suitable definitive hosts.

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Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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