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COCCIDIOSIS IN GREEN TURTLES (*CHELONIA MYDAS*) IN AUSTRALIA: PATHOGENESIS, SPATIAL AND TEMPORAL DISTRIBUTION, AND CLIMATE-RELATED DETERMINANTS OF DISEASE OUTBREAKS

Silvia Ban de Gouvea Pedroso,[1,9,12] David N. Phalen,[1,14] Michael Terkildsen,[2] David Blyde,[3] Duane T. March,[4] Anita N. Gordon,[5] Phoebe A. Chapman,[6] Paul C. Mills,[6] Helen Owen,[6] Amber Gillett,[7] Hannah B. Lloyd,[8] Geoffrey A. Ross,[8,13] Jane Hall,[9] Jennifer Scott,[10] Ellen Ariel,[10] Rongchang Yang,[11] and Karrie A. Rose[9,10]

[1] Sydney School of Veterinary Science, The University of Sydney, 415 Werombi Road, Camden, New South Wales, 2570, Australia
[2] Australian Bureau of Meteorology, 300 Elizabeth Street, Sydney, New South Wales, 2000, Australia
[3] Village Roadshow Theme Parks, Sea World, Seaworld Drive, Main Beach, Gold Coast, 4217, Queensland, Australia
[4] Dolphin Marine Magic, 65 Orlando Street, Coffs Harbour, 2450, New South Wales, Australia
[5] Biosecurity Sciences Laboratory, Queensland Department of Agriculture and Fisheries, 39 Kessels Road, Cooper Plains, 4108, Queensland, Australia
[6] School of Veterinary Science, The University of Queensland, Gatton, 4343, Queensland, Australia
[7] Australia Zoo Wildlife Hospital, 1638 Steve Irwin Way, Beerwah, 4519, Queensland, Australia
[8] Office of the Environment and Heritage, NSW National Parks and Wildlife Service, PO Box 1967, Hurstville, 1481, New South Wales, Australia
[9] Australian Registry of Wildlife Health, Taronga Conservation Society Australia, Mosman, 2088, New South Wales, Australia
[10] College of Public Health, Medical and Veterinary Sciences, James Cook University, 1 James Cook Drive, Townsville, 4811, Queensland, Australia
[11] School of Veterinary and Life Sciences, Murdoch University, 90 South Street, Murdoch, 6150, Western Australia, Australia
[12] Current address: Wildlife Health Australia, Suite E, 34 Suakin Drive, Mosman, New South Wales, 2088, Australia
[13] Current address: NSW Environment Protection Authority, PO Box A290, Sydney South, 1232, New South Wales, Australia
[14] Corresponding author (email: david.phalen@sydney.edu.au)

ABSTRACT: An epizootic of coccidiosis in free-ranging green turtles (*Chelonia mydas*) occurred in Australia in 1991 and the parasites were thought to be *Caryospora cheloniae*. Recurring outbreaks over an increased geographic range followed. We used medical records and temporal and spatial data of turtles diagnosed with coccidiosis between 1991 and 2014 to characterize the disease and factors associated with outbreaks. Most affected animals were subadults or older. Neurologic signs with intralesional cerebral coccidia were observed. Coccidia associated with inflammation and necrosis were predominantly found in the intestine, brain, kidney, and thyroid. Cases occurred in the spring and summer. Three major outbreaks (1991, 2002, and 2014) were concentrated in Port Stephens, New South Wales (NSW) and Moreton Bay, Queensland, but cases occurred as far south as Sydney, NSW. Coccidiosis cases were more likely during, or 1 mo prior to, El Niño–like events. Molecular characterization of the 18S rRNA locus of coccidia from tissues of 10 green turtles collected in 2002 and 2004 in Port Stevens and Sydney imply that they were *Schellackia*-like organisms. Two genotypes were identified. The Genotype 3 sequence was most common (in eight of 10 turtles), with 98.8% similarity to the 18S sequence of *Schellackia orientalis*. The Genotype 4 sequence was less common (in two of 10 turtles) with 99.7% similarity to the 18S sequence of the most common genotype (Genotype 1) detected in turtles from the 2014 Moreton Bay outbreak. Our study will help with the identification and management of future outbreaks and provide tools for identification of additional disease patterns in green turtles.

Key words: *Caryospora*, *Chelonia mydas*, coccidiosis, green turtle, pathogenesis, *Schellackia*, spatial distribution, temporal distribution.

INTRODUCTION

A pathogen of the endangered green turtle (*Chelonia mydas*; Seminoff 2004) is a protozoan originally characterized as *Caryospora cheloniae* (Gordon et al. 1993). The parasite was first described in captive green turtle hatchlings in the Cayman Islands, where it caused significant

TABLE 1. Sources of green turtle (*Chelonia mydas*) stranding data including collaborator, collaborator location, and data period in years from New South Wales and Queensland, Australia. Data were from medical and necropsy reports, except for stranding-only data (Office of Environment and Heritage and the Department of Environment and Heritage Protection, Aquatic Threatened Species, StrandNet), and necropsies and tissue examinations (School of Veterinary Sciences, James Cook University).

Collaborator	Collaborator location[a]	Period
Office of Environment and Heritage	Sydney, NSW	1999–2014
Department of Environment and Heritage Protection, Aquatic Threatened Species, StrandNet	Dutton Park, QLD	1996–2014
Dolphin Marine Magic	Coffs Harbour, NSW	2009–2014
Australian Registry of Wildlife Health	Sydney, NSW	1999–2014
Sea World	Gold Coast, QLD	1991–2014
Australia Zoo Wildlife Hospital	Beerwah, QLD	2005–2014
School of Veterinary Sciences, James Cook University	Townsville, QLD	2009–2014

[a] NSW = New South Wales; QLD = Queensland.

morbidity and mortality (Rebell et al. 1974; Leibovitz et al. 1978). In that outbreak, coccidia were restricted to the hindgut and were associated with severe, diffuse, hemorrhagic enteritis (Rebell et al. 1974; Leibovitz et al. 1978). The origin of the outbreak was uncertain; however, inadequate hygiene was presumed to be a factor (Rebell et al. 1974).

In the spring of 1991, an epizootic of coccidiosis in free-ranging green turtles occurred in Moreton Bay, Queensland (QLD), Australia and surrounding waters (Gordon et al. 1993). Affected turtles were weak, depressed, and some showed neurologic signs (Gordon et al. 1993). In contrast to the Cayman Island outbreak, lesions occurred throughout the intestine, sparing only the duodenum, and many turtles had extraintestinal lesions containing organisms, particularly in the brain, and the morphology of the oocytes was not identical to those described in the Cayman Island outbreak (Gordon et al. 1993). Genetic characterization of the parasite associated with a Moreton Bay outbreak in 2014 revealed that they were predominately a novel coccidia (Genotype 1) most closely related to several species of *Schellackia* (Chapman et al. 2016). A coinfection with a second coccidia (Genotype 2), associated with lesions in the kidney and thyroid, was also found. A parasite genetically similar to Genotype 2 was identified as an incidental finding in adrenal glands of leatherback turtles (*Dermochelys coriacea*) in North America (Ferguson et al. 2016). The parasite causing disease in the Cayman Island turtles was not molecularly characterized and its identity remains uncertain.

The number and severity of disease outbreaks in marine turtles may be influenced by underlying ecosystem disruptions (Aguirre and Lutz 2004). Coccidiosis cases in green turtles have occurred on the eastern coast of Australia, predominantly involving subadult turtles in El Niño years during drought (Rose et al. 2003; Chapman et al. 2016). We reviewed all known cases of coccidiosis in green turtles in Australia, described the disease, defined host and environmental factors associated with outbreaks, and genetically characterized coccidia from the southern extent of the outbreaks.

MATERIALS AND METHODS

Data collection

We investigated green turtle stranding events between 1991 and 2014. Stranding data and medical records were searched for turtles diagnosed with coccidiosis (Table 1). Analyzed data included cases in which parasite infection was identified by either fecal wet preparations, fecal flotation, necropsy with mucosal scrapings, histopathology, PCR and sequencing (Chapman et al. 2016), or a combination of these methods. Limited data from coccidiosis findings from 32 of 108 stranded turtles in 1990, 1991, 1992, and 1996 were available (Gordon 2005).

Temporal, spatial, clinical, necropsy, and histopathologic data of stranded turtles were summarized. Duplicated cases were merged. Cases where final diagnosis and identification of parasite occurred by histopathology (41) or by PCR with subsequent histopathology (2; Chapman et al. 2016) were subdivided into 'gastrointestinal' if the parasites were found only in the intestine, the intestinal lumen, or stomach, and as 'systemic' if present in any organ other than the gastrointestinal tract (GIT). Cases diagnosed only by mucosal scrapings (4) were classified as gastrointestinal-systemic status unknown. The remaining cases (16) were not classified.

Age, sex, and distribution of infected turtles

The sex of the turtles was inconsistently reported; therefore, it was not used in the analyses. Age was based on curved carapace length (CCL) and divided into juvenile (CCL <65 cm), subadult (CCL $65<90$ cm), and large subadults, pubescent, and adults (CCL >90 cm; Limpus and Read 1985). The GPS coordinates were used to map turtle stranding locations, and the Gaussian blur algorithm was used to render map colors (ArcGIS 2015). When GPS coordinates were not provided, latitude and longitude were estimated based on location and postcode.

Correlation of spatial and temporal data with environmental variables

Correlations with environmental variables were based on the spatial data and stranding or admissions date of turtles diagnosed with coccidiosis. Environmental data were obtained from the Australian Bureau of Meteorology (BOM). The effect of large-scale climatic variability was represented by the monthly Southern Oscillation Index (SOI; BOM 2015). The SOI was defined by the difference in mean sea level pressure between Darwin and Tahiti, reported against a climatologic baseline (1933–1992 for BOM SOI). The impact of ocean temperature was investigated using the average sea surface temperature anomaly data for the Tasman region (BOM 2015).

Atmospheric temperature (daily minimum and maximum) and rainfall (daily) data were obtained (BOM 2015). Specific rainfall and temperature monitoring sites within the latitude range 25°S to 36°S were selected based on temporal coverage and location, closest to the center of each 1°-latitude band. Australian Climate Observations Reference Network–Surface Air Temperature (BOM 2015) sites (Trewin 2013) were preferentially selected for their stability and continuity of temperature observations. Freshwater discharge rate and stream level from selected streams within each river basin were also obtained (BOM 2015). All environmental predictors lagged by zero, one, three, six, and 12 mo to assess the role of the recent history of environmental parameters on the presence of coccidiosis. A lagged predictor was calculated as the median of the daily observations over the lag period prior to the date of the turtle observation. Thus, a lag of 1 mo, for example, referred to the current and previous months.

Molecular analysis

We stored 72 tissue samples collected from 12 green and three hawksbill turtles (*Eretmochelys imbricata*) from NSW either in 70% ethanol or at –20 C prior to molecular analysis. Ten of the green turtles had histologic evidence of coccidia infection. Animals with infection were collected in 2002 (7) and in 2004 (3). Nine of these green turtles were from Port Stephens, NSW and one was from Sydney, NSW. Two green and all hawksbill turtles with no evidence of infection acted as negative controls.

We extracted DNA using the DNeasy Blood and Tissue Kit (Qiagen, Chadstone, Victoria, Australia). We amplified the partial 18S rRNA gene sequence (1,526 base pairs) using the primers BT-F1 (5′-GGT TGA TCC TGC CAG TAG T-3′) and hep1600R (5′-AAA GGG CAG GGA CGT AAT CGG-3′; Megía-Palma et al. 2014).

The 18S sequences obtained from the turtles were deposited into GenBank (accession nos. KY046254 and KY046255). Sequences were aligned with 38 representative sequences from major coccidian groups, including two sequences (KT361639 and KT361640) obtained from green turtles with coccidiosis in QLD (Chapman et al. 2016) and a sequence from North American leatherback turtles (KT956976). The alignment was performed using ClustalW (Phylogeny.fr 2015). Phylogenies were constructed using MEGA 7 (Kumar et al. 2016). Maximum likelihood, neighbor-joining, and maximum parsimony analyses were conducted with Tamura-Nei based on the most appropriate model selection using Model Test in MEGA 7. Bootstrap analyses were conducted using 1,000 replicates.

Statistical analysis

Single-factor differences in the occurrence of coccidiosis for each environmental parameter were assessed using analysis of variance at $P<0.05$. A statistical model was then fitted to the data to assess the relative and combined impact of environmental factors on the occurrence of coccidiosis. Turtle observational data were grouped into spatio-temporal bins of one degree of latitude by 1 mo duration. A model for the probability of turtles with coccidiosis in each bin, as a function of the environmental parame-

TABLE 2. Number and percentage of green turtles (*Chelonia mydas*) in eastern Australia with coccidiosis (gastrointestinal [GI], GI systemic status unknown, systemic, or not classified) as a function of green turtle age class, determined by curved carapace length (CCL).

Age class (Limpus and Read 1985)	CCL range in cm	Coccidiosis classification, no. (%)				Total, no. (%)
		GI	GI systemic status unknown	Systemic	Not classified[a]	
Large subadult, pubescent, and adult	>90		2 (50)	14 (38.9)	6 (37.5)	22 (34.9)
Subadult	65<90	1 (14.3)	2 (50)	14 (38.9)	7 (43.8)	24 (38.1)
Juvenile	<65	5 (71.4)		7 (19.4)	2 (12.5)	14 (22.2)
Unknown age		1 (14.3)		1 (2.8)	1 (6.2)	3 (4.8)
Total		7 (11.2)	4 (6.3)	36 (57.1)	16 (25.4)	63 (100)

[a] Not classified: there was no information of necropsy, histopathology, or PCR for categorization.

ters over time, was fitted to the data. The response variable was the presence or absence of confirmed cases in each month or latitude bin. Mass outbreaks were represented in the model identically to single turtle events, hence the model does not account for the severity of a given outbreak.

A generalized additive mixed modelling (GAMM) approach was used for the binary data. This allowed the relaxation of the a priori assumption of linearity between the predictors and the response variable through the inclusion of smoothing terms. It also accounted for inhomogeneity in the spatio-temporal distribution of turtles and sampling effort through the inclusion of random effects or smoothing terms for location and time. Temporal autocorrelation and partial autocorrelation plots (ACF-PACF) were used to assess seasonal autocorrelation. A cubic-spline function of numerical month, within the model, accounted for seasonal autocorrelation. Model residuals were subsequently checked using ACF-PACF plots to confirm no residual autocorrelation remained. Latitude bin was included as a random effect in the GAMM to account for spatial variability in sampling effort and turtle distribution. Model fitting was achieved using step-wise factor selection of environmental predictors (forwards and backwards), with model impact assessment based on Aikake's information criteria. All statistical analyses were conducted with R3.2.1 (R Core Team 2014) using the R libraries *gamlss* (Rigby and Stasinopoulos 2005) and *gamm4* (Wood and Scheipl 2014).

RESULTS

Signalment and signs

A total of 63 green turtles with coccidiosis between 1991 and 2014 were identified. Four previously reported cases from 1991 (Gordon 2005) were included in the dataset. Coccidiosis was not detected in 120 stranded turtles between Cardwell and Bowen, QLD from 2009 to 2014. Table 2 shows the number and percentage of turtles per age class and disease type. Most turtles (54/63) were euthanized or died. Six turtles were released. The amount of time in care prior to release was 52, 62, 63, 83, 85, and 155 days. Another three turtles were still in care after 88, 127, and 128 d at the time of data collection. Twenty-one of 63 turtles had body condition information: 11 had abundant or good fat deposits, 12 had good hydration, and 15 had good to excellent muscle mass. Clinical signs were observed in 61 turtles. Thirty-one turtles were moderately depressed or moribund. Other signs included floating (24), emaciation (6), diarrhea (2), unilateral head tilt (17), and circling (10). All systemic cases were presented alive. The majority of turtles showed more than one sign. All but one animal with neurologic signs died or were euthanized (17), and 15 of those had histologic evidence of cerebral parasites. Intramonocytic parasites resembling coccidial zoites were found in the blood film from one turtle.

Coccidia distribution and lesions

We identified 35 gross pathology and 43 histopathology reports. Gross lesions were not found in 13 of 35 turtles. Distribution of the microscopic lesions is shown in Figure 1.

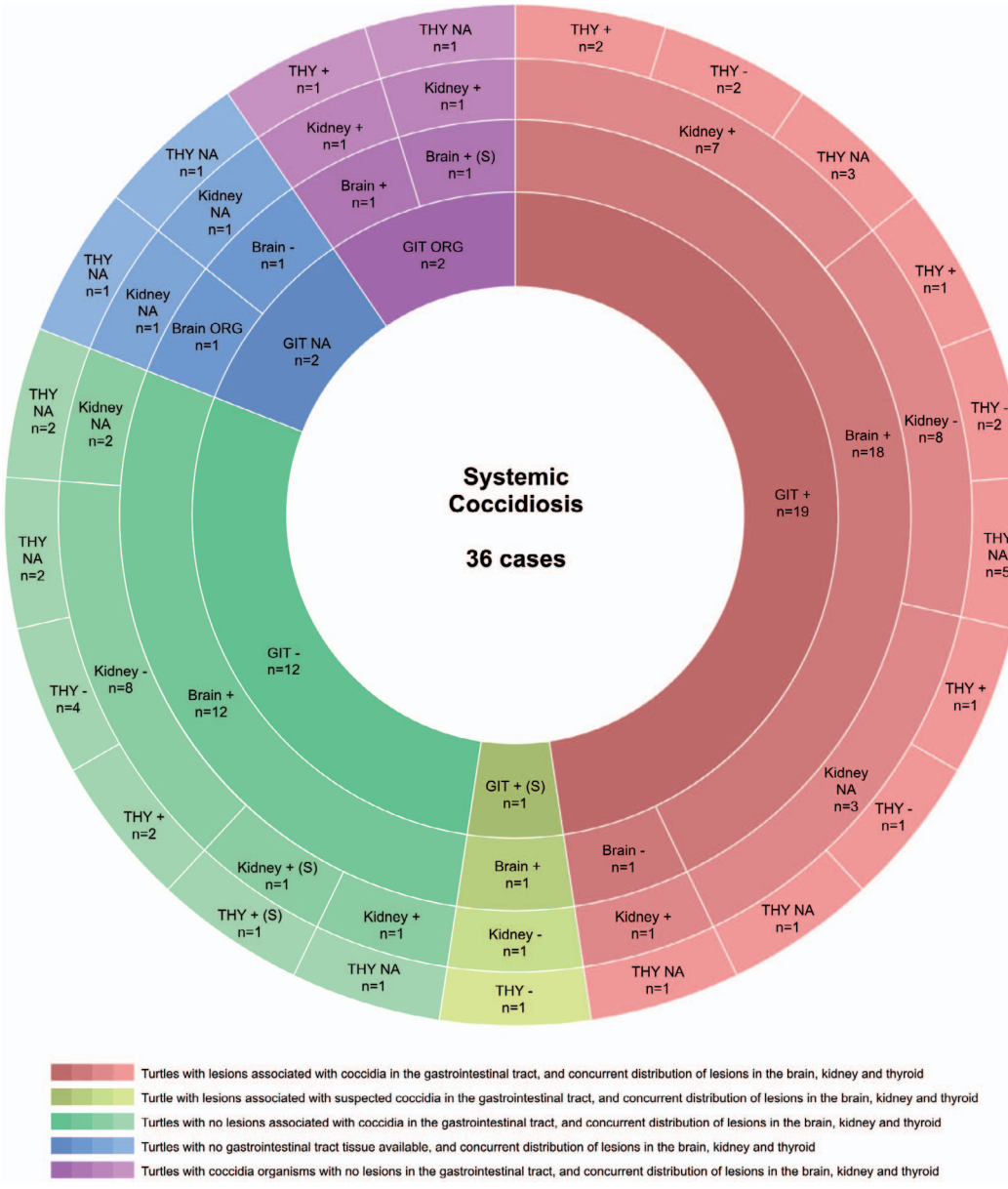

FIGURE 1. Distribution of microscopic lesions and organisms in the gastrointestinal tract (GIT), brain, kidney, and thyroid (THY) associated with coccidiosis in 36 systemic cases in of green turtles (*Chelonia mydas*) in eastern Australia. Tissues were assessed for lesions associated with coccidia (+) or suspected (S) coccidia, no microscopic lesions (–), coccidia organisms only (ORG), or tissues not available (NA) for examination. One turtle with GIT NA, Brain –, Kidney NA, and THY NA was considered a systemic coccidiosis case, given lesions associated with organism were found in the lung. This animal was also PCR positive in the brain and thyroid, but histologic lesions on these organs were not described (Chapman et al. 2016).

Gross lesions in the GIT (14) ranged from mild inflammation with granular appearance of the mucosa (5) to more-severe cases of fibrinous erosive and ulcerative enteritis (9) with pseudomembranous plaque formation (5). In the GIT, histopathologic alterations in 31 turtles included all parts of the intestine, ranging from hypertrophy and hyperplasia of

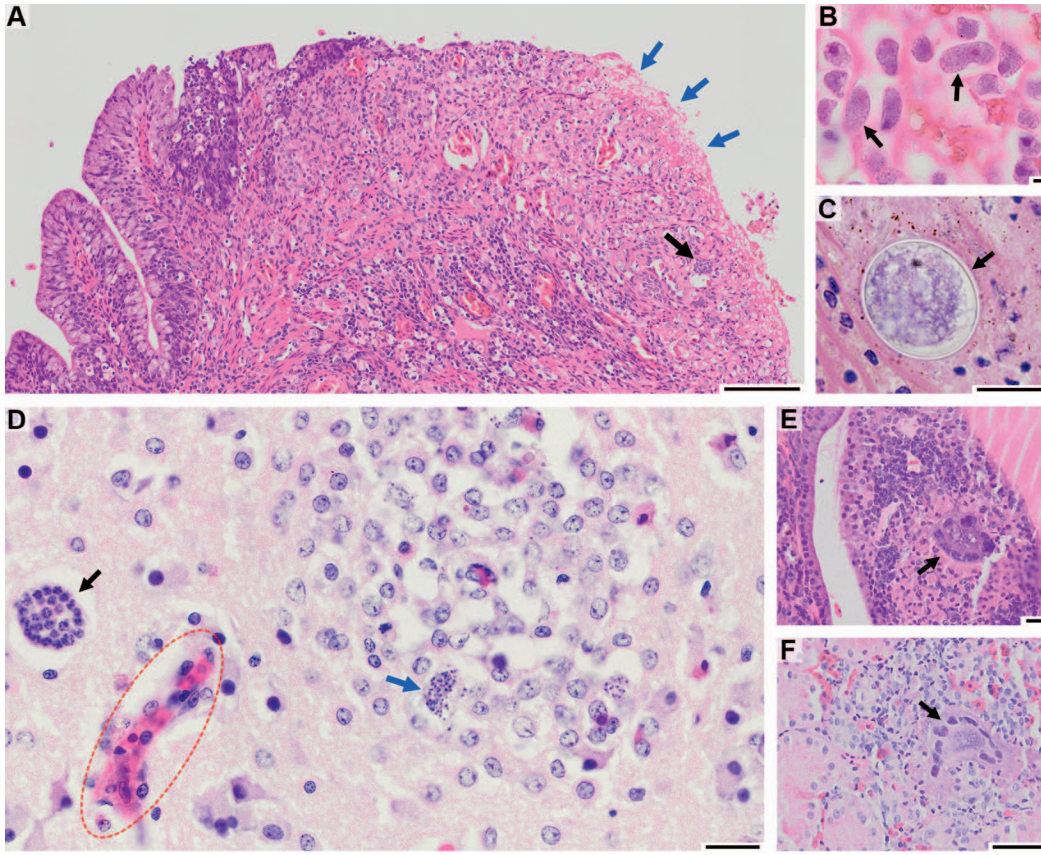

FIGURE 2. Hematoxylin and eosin–stained tissue sections of representative cases of coccidiosis in green turtles (*Chelonia mydas*) in eastern Australia: (A) Erosions in the small intestine (blue arrows) and a schizont in the lamina propria (black arrow). Bar=100 μm. (B) Intestinal luminal elongated oval late gametogenous to early oocytes stages within fibrinoid material from a systemic case. Bar=5 μm. (C) A large, round, luminal oocyte from a gastrointestinal coccidiosis case. Bar=20 μm. (D) An intact schizont in the brain (black arrow) and free merozoites (blue arrow) in an area of spongiotic change in the neuropil with macrophages and granulocytes. Bar=20 μm. (E) A megaloschizont in the thyroid. Bar=20 μm. (F) A megaloschizont in the kidney. Bar=50 μm.

infected mucosal epithelial cells (6) to erosion and ulceration (8), fibrin deposits (3), diphtheritic membrane formation (3), and deep to transmural foci of necrosis (16). These alterations were usually associated with the presence of oocysts in the intestinal lumen, gametogenous coccidial stages in the mucosa, and schizonts, merozoites, and more rarely megaloschizonts in the lamina propria (Fig. 2). Cases of systemic coccidiosis typically contained luminal oocysts that were uniformly elongate and oval whereas some cases of intestinal coccidiosis (3) were associated with round mucosal gametocytes and luminal oocysts. When coccidia were present in the stomach (5), inflammatory cells in the epithelium were found in the presence of gametogenous coccidial stages, luminal oocysts, and schizonts.

Miliary (1–2 mm diameter) white foci were grossly observed in the brain of nine animals with systemic coccidiosis. Microscopically, 33 animals had lesions ranging from mild to severe and multifocally extensive perivascular, nonsuppurative (25) to granulomatous (7) inflammation with foci of malacia throughout the brain and meninges (25). Acute lesions were associated with the presence of free merozoites. Necrosis and granulomatous inflammation ranged from acute foci containing

variable mixtures of gitter cells, multinucleate giant cells, and granulocytes to organized granulomas with caseous centers surrounded by a wall of macrophages, multinucleate giant cells, lymphocytes, and peripherally scattered granulocytes. Schizonts were often clustered adjacent to neural vasculature, unaccompanied by inflammation, with the exception of two cases with extensive encephalomalacia and many schizonts. Megaloschizonts were found in the brain of two turtles. In the spinal cord, Wallerian degeneration in the white matter and schizonts were observed.

Occasionally, miliary white or tan foci were grossly found in the kidney and thyroid gland while schizonts surrounded by lymphocytes, macrophages, and multinucleated cells were observed histologically. Granulomas and megaloschizonts were also observed in both organs (kidney: 4, thyroid: 2). Schizonts were present in the eye of one of nine turtles, and merozoites were seen in the spleen of one of 25 turtles. One gastrointestinal case may have been systemic, as it had suspected coccidia in the brain. Suspected protozoal forms were also found in the stomach (1), bladder (1), thyroid and kidney (1), and lung (1) of turtles with coccidia in the brain. Coccidiosis was attributed to one meningo-encephalitis case with coccidial forms in the kidney and another with splenitis and hepatitis, although organisms were seen only in the brain and thyroid. Representative microscopic images of lesions (Fig. 2) and organism measurements (see Supplementary Material Table S1) are shown.

Other histologic findings

Histologic examination of the GIT in 10 systemic cases did not find coccidia, but GIT necropsy findings included congestion, linear areas of mucosal erythema and erosion, generalized edema, intestinal impaction, ileus, ascites, and fibrinous coelomitis. Histologically, lesions in organs where coccidia were not present but could have been caused by the parasite included nonsuppurative nephritis, pseudomembranous enteritis, nonsuppurative granulomatous meningitis and encephalitis, uveitis, and granulomatous to heterophilic and necrotizing thyroiditis. Lymphoid depletion and fibrinoid vasculitis in the GIT and other tissues may also be associated with the disease.

Temporal and geographic distribution of cases

At least one case of coccidioisis was diagnosed in 14 of the 24 yr studied. Case numbers were noticeably higher in 1991 (24), 2002 (11), and 2014 (24). Cases were found between Sydney and south of Fraser Island and were concentrated in Moreton Bay in 1991 (Gordon et al. 1993) and 2014 and in Port Stephens in 2002 and 2003 (Fig. 3). Cases occurred from September to February. Green turtle stranding data from southern QLD (from Fraser Island to Gold Coast) and from the NSW north coast to greater Sydney were compared to coccidiosis occurrences. The recurrent cases of coccidiosis along the years did not reflect the overall increasing numbers of stranded green turtles in those regions. Most green turtle strandings (928) occurred in October, when there was an increase in the proportion (3.8%) of turtles diagnosed with coccidiosis (see Supplementary Material Fig. S1).

Correlation of environmental and temporal data

Relationships between environmental data and the occurrence of coccidiosis were not observed in discharge rate and stream flow in preliminary models, and considerable variability in these parameters among streams was noted. In addition to spatial and temporal correlation, the final model included the fixed effect of SOI lagged by 1 mo. Atmospheric temperature, sea surface temperature, and rainfall did not improve the fit of the GAMM and were not included in the final model (median SOI over previous and current months, coefficient 0.092, SE 0.027, $P<0.01$).

Coccidiosis was preferentially associated with El Niño–like conditions of negative SOI in the current or preceding month. The statistical modeling indicated that after adjustment for seasonal effects and spatial inhomogeneity in sampling effort and turtle

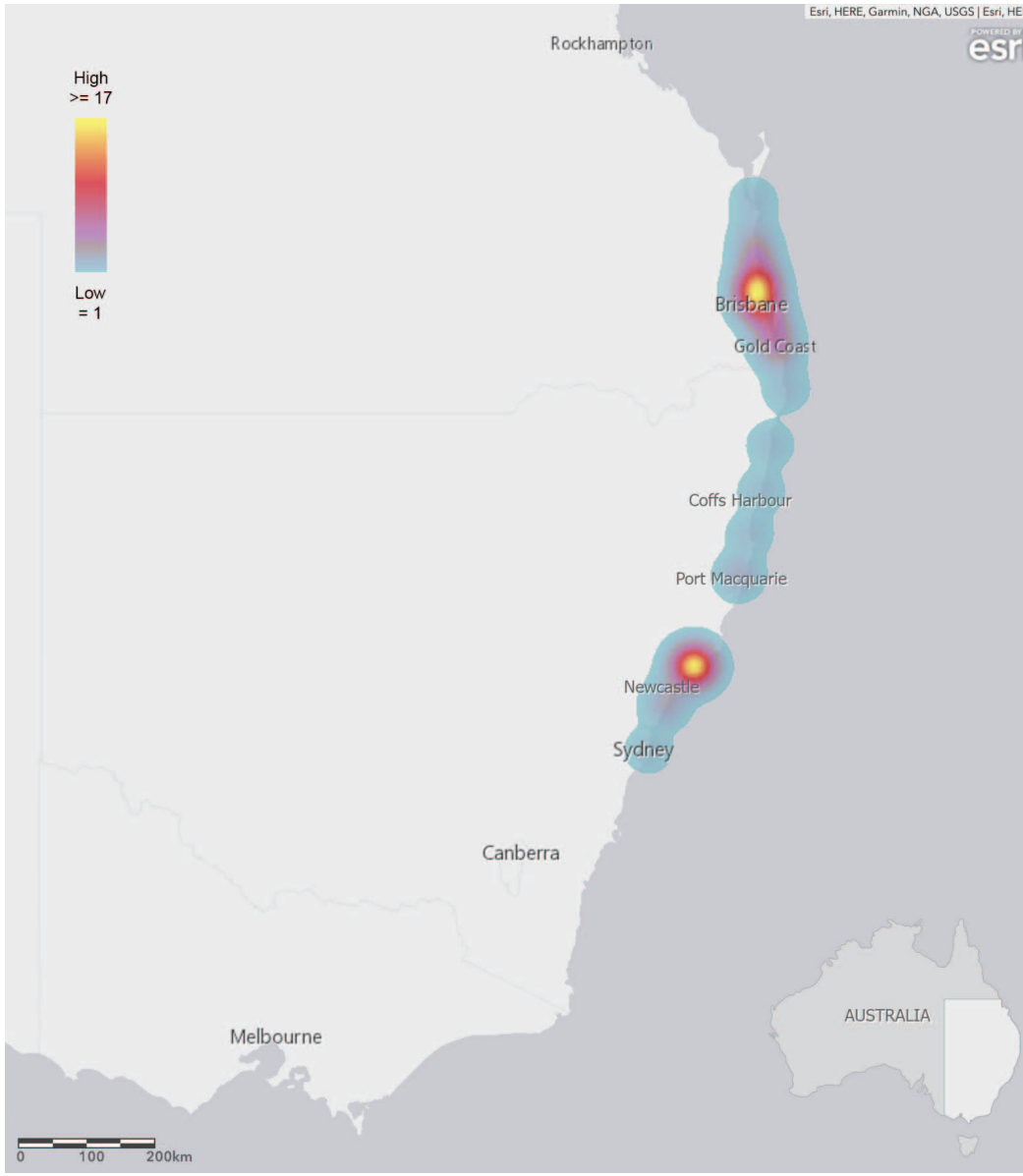

FIGURE 3. Map showing concentrations of green turtle (*Chelonia mydas*) strandings in eastern Australia in 1991 to 2014, diagnosed with coccidiosis in the present study, between the southern end of Fraser Island (latitude 25°48′0′′S, longitude 153°3′36′′E) in Queensland (QLD) and Bondi beach (33°53′24′′S, 151°16′48′′E) in Sydney, New South Wales (NSW), Australia. Seventeen or more cases were concentrated in each Moreton Bay region (between 27°27′36′′S and 27°2′24′′S and 153°3′0′′E and 153°15′0′′E) in QLD and the Port Stephens area (between 32°43′48′′S and 32°39′36′′S and 151°58′48′′E and 152°9′36′′E) in NSW. This figure was produced on the basis of Esri Maps (Redlands, California, USA).

distribution, a decrease in median SOI of 10 units increased the odds of coccidiosis by 150% ($P<0.01$) on average across the latitude range.

Molecular characterization

Two 18S genotypes were identified (Genotypes 3 and 4) in NSW turtles. Analyses of the Genotype 3 and Genotype 4 sequences using

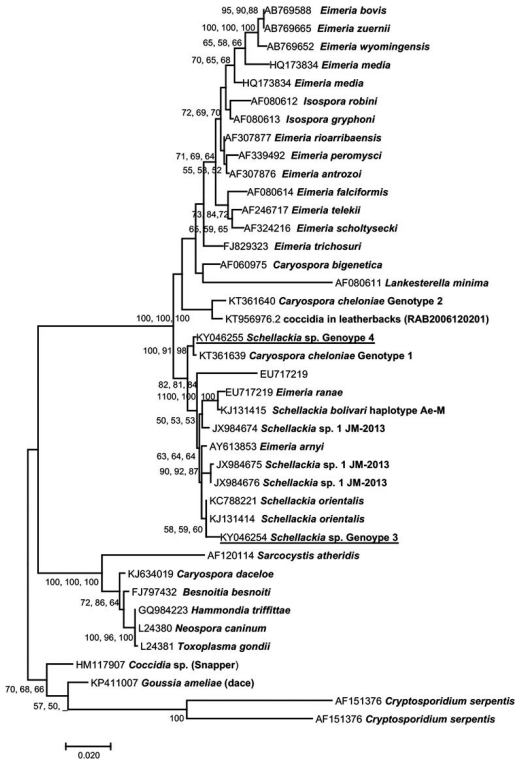

FIGURE 4. Evolutionary relationships of coccidia from the green turtles (*Chelonia mydas*) in eastern Australia inferred by distance analysis of 18S rRNA sequences. Percentage support (>50%) from 1,000 pseudoreplicates from distance, maximum likelihood, and parsimony analysis, respectively, is indicated at the left of the support node ('_'=value was <50%). The KT956977 sequence identified from a North American leatherback turtle fecal sample is closely related to KT956976 and was omitted here.

distance, parsimony, and maximum likelihood analyses produced similar results. Both the Genotype 3 and 4 18S sequences from this study were grouped in a clade with multiple *Schellackia* spp. (Fig. 4.). The Genotype 3 sequence was the most common, infecting eight green turtles; it had a 98.8% similarity of *Schellackia orientalis* (KC788221), a parasite of the Asian grass lizard (*Takydromus sexlineatus*; Telford 1993). The Genotype 4 sequence was only found in two turtles and was almost identical (99.7% similarity) to Genotype 1 (KT361639), the most common genotype found in a previous study of coccidiosis in green turtles from QLD (Chapman et al. 2016; Table 3).

Both Genotypes 3 and 4 had a similar tissue distribution and disease pattern, and there was reasonable correlation between tissues that were histologically positive and the PCR results (see Supplementary Material Table S2). The PCR detected organisms in 11 of 17 histologically positive tissues but not in the histologically positive kidney (4) and intestinal samples (2). Of 32 samples PCR tested and histologically negative, 13 were PCR positive. None of the tissues from the histologically negative turtles were PCR positive.

DISCUSSION

We show that coccidiosis should be suspected in green turtles stranded during the months of September to February, peaking in October, on the Australian eastern coast from Sydney, NSW to Fraser Island, QLD. Our study confirms that the expected signs of coccidiosis in green turtles relate to disease of the neurologic and gastrointestinal systems. Body condition is not useful diagnostically. The prognosis for clinically ill turtles with coccidia is grave.

Our findings illustrate that the parasite can be identified via fecal flotation and wet preparation, GIT mucosal scrapings, histopathology, and PCR. Coccidiosis should be suspected when enteritis or gastritis is grossly present, although absence of coccidia in these lesions does not exclude infection. Intestinal lesions were not always diffuse, so multiple sections of the intestine should be examined grossly and microscopically. Gross lesions in the brain, kidney, and thyroid, consistent with multifocal inflammation, were suggestive of coccidiosis. Microscopically, we found brain lesions that were more severe and extensive with higher numbers of schizonts and more widespread encephalomalacia than previously described, although other histologic findings were largely consistent with other case descriptions (Gordon et al. 1993).

Organism sizes at various life stages varied considerably from those previously studied (Leibovitz et al. 1978; Gordon 2005), possibly due to shrinkage of parasites subsequent to

TABLE 3. Comparison of coccidian genotypes, PCR-positive tissues, stranding locations, and dates of green turtles (*Chelonia mydas*) in eastern Australia.

Coccidia genotype[a]	Turtles PCR-positive (n[b] tested)	PCR-positive tissues[c]	Location[d]	Latitude and longitude coordinates	Season and year
1	10 (11)	Brain, GIT, lung	Between Sandgate in the Moreton Bay region and Teewah Beach in the north of Sunshine Coast, QLD	Between 27°18′43′′S, 153°4′10′′E and 26°16′37′′S, 153°4′59′′E	Spring 2014
2	2 (11)	Kidney, thyroid	Golden Beach and Teewah Beach in Sunshine Coast, QLD	26°48′55′′S, 153°7′59′′E and 26°16′37′′S, 153°4′59′′E	Spring 2014
3	8 (10)	Brain, GIT, lung, spleen, kidney	Port Stephens area, NSW	Between 32°43′48′′S and 32°39′36′′S and 151°58′48′′E and 152°9′36′′E	Spring 2002 and 2004
4	2 (10)	Brain, GIT, lung, spleen	Tahlee in Port Stephens area and Bondi beach in Sydney region, NSW	32°40′12′′S, 152°0′0′′E and 33°53′24′′S, 151°16′48′′E	Spring 2002

[a] Genotypes 1 and 2 identified by Chapman et al. (2016); turtles with Genotype 2 had coinfection of Genotype 1 in the brain; Genotypes 3 and 4 identified in present study.

[b] n=number of turtles with histologic evidence of coccidia infection tested by PCR.

[c] Not all tissues were positive in PCR-positive turtles. See Supplementary Material details in Table S2. GIT = gastrointestinal tract.

[d] QLD = Queensland; NSW = New South Wales.

tissue fixation, although megaloschizonts were notably smaller than those reported in Gordon (2005). Additionally, we provide descriptions of zoites in monocytes, free zoites in tissues, and single and multiloculated schizont sizes in the brain. Schizonts type I were not observed in the GIT. Also, two types of oocysts, elongated oval in systemic cases and rounded in some GI cases, have not been previously described. Finally, we describe sexual stages in the gastric epithelium and ocular coccidiosis.

Our findings suggest that the organisms causing systemic coccidiosis were well established in the coastal waters of eastern Australia. The original reports of coccidiosis in green turtles were from southeast QLD (Gordon et al. 1993; Chapman et al. 2016). Our analysis shows that the disease occurrence now includes a wider, but still discrete, portion of the east coast of Australia from Sydney, NSW to the south end of Fraser Island in QLD. The green turtle is rarely found in south coast waters of NSW (OEH 2014), so the southernmost extent of the parasite distribution may be limited by turtle range. The green turtle's range extends along the entire QLD coast, yet coccidiosis was not detected in stranded turtles from north of Fraser Island. The current spatial boundaries of this disease suggested environmental conditions may define its distribution.

The distribution of cases was not random. Most were concentrated in and around Moreton Bay, QLD and Port Stephens, NSW, which may have been associated with their extensive estuarine ecosystem as well as agricultural, industrial, and urban effluents. Moreton Bay catchments have significantly degraded areas and are subjected to algal blooms (Paerl and Huisman 2008; Healthy Waterways 2014). Exposure to anthropogenic toxins or biotoxins and unfavorable environmental conditions may have made turtles more prone to infection or favored persistence or concentration of the parasite. The concen-

tration of cases may have also reflected green turtle density on those feeding grounds near highly populated urban areas.

The age distribution also suggests that green turtles were exposed to coccidian parasites following recruitment to coastal feeding grounds. In the 1991 Moreton Bay outbreak (Gordon et al. 1993) and in our study, fatal coccidiosis occurred with higher prevalence in large subadult and pubescent green turtles, perhaps reflecting increased exposure or susceptibility to these parasites. Larger green turtles are known to favor different habitats within Moreton Bay (Limpus et al. 1994), and these may have been areas where infection was acquired.

Given that outbreaks were not annual, a possible correlation between cyclic climatic change and outbreak cycles was investigated. Seasonal occurrence in warmer months and concentration of hosts in specific sites were accounted for because the models considered the lack of homogeneity in the spatial-temporal distribution. Although the models did not include outbreak intensity, its concept was similar to past studies of strandings of marine species (Truchon et al. 2013; Meager and Limpus 2014), and the chances of coccidiosis occurrence were enhanced when negative SOI was observed in the preceding and current months of the disease.

Rainfall, atmospheric temperature, and sea surface temperature did not show relevance to the models predicting coccidiosis. Although SOI is known to significantly affect rainfall across eastern Australia (BOM 2015), a recent study indicated there was no correlation between El Niño and rainfall patterns in coastal regions across the eastern seaboard, where rainfall may be influenced more by the impacts of east coast low pressure systems (Pepler et al. 2014). Nevertheless, 50% or more of Australian El Niño events provoked droughts across the eastern seaboard (BOM 2015), suggesting El Niño events may indeed be associated with coccidiosis occurrence, possibly when east coast lows were not present. The reasons why outbreaks of green turtle coccidiosis were associated with weather patterns is not known. However, possible explanations included changes in water temperature or salinity that might allow the shed oocysts to remain infectious for longer in the environment, decreased water flow that might allow increased concentrations of oocysts in the environment, or green turtles eating plants closer to their roots and thus ingesting soil that might contain higher concentrations of oocysts.

Recent findings in QLD turtles suggested that disease was caused by two novel coccidia; the common Genotype 1 that was usually present in the brain and intestine and the less common Genotype 2 that occurred as a coinfection and was only identified in the thyroid and the kidney (Chapman et al. 2016). If these observations are true, coinfections with both of these coccidia are likely to be common, as many of the turtles in the present study had histologic evidence of parasites in the brain and kidney, brain and thyroid, or brain, kidney, and thyroid.

Molecular analysis of the two organisms identified in NSW turtles at the 18S rRNA locus confirmed that these coccidia have a close relationship with the *Schellackia* genus. The Genotype 4 sequence grouped with and was nearly identical to the common Genotype 1 from turtles dying with systemic coccidiosis in QLD (Chapman et al. 2016). In contrast, the Genotype 4 sequence was only found in two of the 10 green turtles from NSW. The Genotype 3 sequence was the most common, was novel, and was most closely related to *Schellackia orientalis*. The less common Genotype 2 sat in the same clade as North American leatherback turtle coccidia RAB2006120201 but was not identified in the NSW turtles. These results suggested that the coccidia causing systemic coccidiosis in green turtles in Australia were genetically heterogeneous.

Possible explanations for why the Genotype 3 was more common than the Genotype 4, and the absence of Genotype 2 in this study, include the possibility of different spatial distributions of these genotypes or changes in prevalence of genotypes in diseased turtles from year to year. Therefore, genotyping more

samples from across the range of this disease, including samples from different years, is recommended.

The absence of PCR-detectable organisms in the control tissues from three hawksbill turtles and two nonaffected green turtles provide additional assurance of a cause and effect relationship between the observed organisms identified by PCR and the associated disease. There were some discrepancies between histologically positive and negative tissues and PCR results. It was not surprising that some histologically negative tissues are PCR positive, as PCR is likely to be more sensitive than is histopathology. Also, given the sensitivity of PCR, tissue contamination at the time of collection could not be ruled out. Six histologically positive samples were PCR negative. The cause of this finding is unknown but may have represented copurification of an inhibitor or DNA degradation during storage.

This retrospective analysis describes the signs and circumstances of coccidiosis outbreaks, characterizing it as an important recurring disease in green turtles in Australia and identifying genetically heterogeneous organisms causing systemic disease. It provides a valuable illustration of the potential interplay between climatic conditions and disease expression.

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SUPPLEMENTARY MATERIAL

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