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Infection dynamics of *Kudoa inornata* (Cnidaria: Myxosporea) in spotted seatrout *Cynoscion nebulosus* (Teleostei: Sciaenidae)

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ABSTRACT: *Kudoa inornata* is a myxosporean parasite that develops in the somatic muscle of spotted seatrout *Cynoscion nebulosus*, an economically and ecologically important fish in estuaries and harbors in southeastern North America. In South Carolina (SC), USA, over 90% of wild adult spotted seatrout are infected. To inform potential mitigation strategies, we conducted 3 experiments using naïve sentinel seatrout and infectious stages of *K. inornata* naturally present in raw water from Charleston Harbor, SC, to determine (1) if *K. inornata* infection follows a seasonal pattern, and (2) how long it takes for myxospores to develop in fish muscle. Infection by *K. inornata* was determined by visual detection of myxospores in fish muscle squashes, and any visually negative samples were then assayed for *K. inornata* ribosomal DNA using novel parasite-specific PCR primers. We observed that *K. inornata* infection in seatrout followed a seasonal pattern, with high prevalence when water temperature was highest (27–31°C; July–September) and infections that were either covert (at ~13–15°C) or not detected (<13°C) at the lowest water temperatures in January–February. Myxospore development occurred within 476 degree-days, i.e. 2 wk in a typical SC summer. Infection was dependent on fish density, which limited presumptive actinospore dose. Our findings suggest that the life cycle of the parasite may be disrupted by preventing spore-rich seatrout carcasses (e.g. at angler cleaning stations) being thrown back into harbors and estuaries throughout the year.

KEY WORDS: Myxosporean · Myxospores · *Kudoa* · Muscle · PCR · Degree-day · Water temperature · Actinospores

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INTRODUCTION

Kudoa Meglitsch, 1947 is a speciose genus of marine myxosporean parasites, described mostly from teleost fishes, including many of economic and ecological value. Parasite myxospores are described from fish tissues including gills, heart, mesenteries, and intestinal muscle, with most developing in the somatic musculature (reviews by Moran et al. 1999a, Eiras et al. 2014). Species including *K. thyrsites* (Gilchrist, 1924) and *K. paniformis* Kabata & Whitaker, 1981 have received much attention because they induce postmortem myoliquefaction ('soft flesh syndrome'), a condition where the fish muscle loses cohesion in response to proteolytic compounds released from the parasite, lowering marketability of the fish and having consequent economic impacts on aquaculture and wild fisheries (Moran et al. 1999a, Funk et al. 2007, Henning et al. 2013, Kasai et al. 2016, Marshall et al. 2016). Further, several species (such as *K. septempunctata* Matsukane, Sato, Tanaka, Kamata, Sugita-Konishi, 2010) have been linked with

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human gastrointestinal illnesses (reviewed by Hallett et al. 2015).

Kudoa inornata Dyková, de Buron, Fiala, and Roumillat, 2009 was described from somatic muscle of a sciaenid fish, spotted (speckled) seatrout *Cynoscion nebulosus* (Cuvier), from estuaries in South Carolina (SC), USA, where over 90% of these fish, when adult, are infected (Dyková et al. 2009, Ware et al. 2014, Arnott et al. 2017). The spotted seatrout (referred to as 'seatrout' hereafter) is an economically and ecologically important fish in the southeastern USA and has been studied extensively (e.g. Bortone 2003, Dippold et al. 2016, Odell et al. 2017). Whereas typical fish hosts of *Kudoa* species are marine and localized offshore, the seatrout is mostly estuary-bound (Smith et al. 2008, Odell et al. 2017). Seatrout is a declared gamefish in most states where it ranges (Bortone 2003). In SC, the health of its population is monitored closely by the Department of Natural Resources, which stocks the fish in Charleston Harbor and routinely educates anglers about its natural history and ecological importance (Wenner & Archambault 1996, Roumillat & Brouwer 2004, Anweiler et al. 2014, ASMFC 2016, O'Donnell et al. 2014, 2016). Although *K. inornata* is not known to cause human gastrointestinal illnesses, the meat of infected fish rapidly softens and loses its quality postmortem (Sundquist et al. 2014). The problem of soft flesh in seatrout is well-recognized by anglers who are advised to quickly consume the fish after it is caught (e.g. www.flmnh.ufl.edu/fish/discover/species-profiles/cynoscion-nebulosus/). The rapid loss of quality of the fish implicates this parasite as a direct negative driver of the state economy.

Although there are practical limits on mitigating parasites in wild fish populations, it is possible to limit certain fishing practices that may elevate parasite prevalence. As such, one particularity of seatrout being a major gamefish is that anglers often fillet fish at cleaning stations located at marinas or on piers, and throw the carcass remains back into the water, particularly the seatrout belly flaps, which have the highest number of *K. inornata* plasmodia (Ware et al. 2014). As infected tissues degrade in the water, they release myxospores that can then infect the presumptive next host in the parasite's life cycle. No *Kudoa* life cycle is known; direct, natural fish-to-fish transmission has not been demonstrated (e.g. Moran et al. 1999b, Yokoyama et al. 2015), and thus it is assumed that *K. inornata* requires an alternate, annelid worm host, as is the case for all marine myxosporeans whose life cycles are known (e.g. *Ellipsomyxa gobii* Køie, 2003, *Gadimyxa atlantica* Køie, Karlsbakk & Nylund, 2007; reviewed by Eszterbauer et al. 2015). Within the annelid host, myxospores undergo complex multiplication and development to ultimately produce actinospores, which then infect fish to complete the cycle. We suspect that in Charleston Harbor and many other seatrout habitats, parasite proliferation is enhanced significantly by the practice of returning myxospore-laden carcasses to the environment proximal to their presumed annelid host.

However, myxospore density is only one part of myxozoan infection dynamics. Abiotic factors, particularly temperature, are significant drivers of parasite success (Schmidt-Posthaus & Wahli 2015, Barber et al. 2016). Whereas myxospore and actinospore development and densities of other myxosporeans/*Kudoa* species have demonstrated temperature dependency (El Matbouli et al. 1999, Ray et al. 2012, Alama-Bermejo et al. 2013, Schmidt-Posthaus & Wahli 2015), as have the densities of certain annelid species (Zajac 1991, Gillett et al. 2007), the effect of water temperature on the viability and infectivity of *K. inornata* myxospores or actinospores is not known. Further, whereas prevalence of *K. inornata* infection in adult seatrout is high in SC estuaries (Dyková et al. 2009, Arnott et al. 2017), it is not known whether there are temporal or seasonal patterns of infectionand myxospore production. Although SC is subtropical, estuarine water temperatures vary greatly over the year, typically from ~12°C in winter months (reaching below 5°C during extremely cold winters) to over 31°C during the summer (USGS National Water Information System, http://waterdata.usgs.gov). Since other *Kudoa* species have shown seasonal patterns of infection (Hallett et al. 1997, Moran & Kent 1999, Yokoyama et al. 2015), we predicted that infection by *K. inornata* in spotted seatrout would be seasonal as well.

Given the variation in development time of *Kudoa* spp. myxospores (Moran & Kent 1999, Moran et al. 1999c, Young & Jones 2005, Shirakashi et al. 2012, Marshall et al. 2016), we also wanted to determine the number of degree-days (DD) necessary for development of *K. inornata* myxospores in seatrout. DD are a metric of thermal or physiological time, and represent accumulated daily temperature for a process to occur (Trudgill et al. 2005). Since temperature can vary among studies, the conversion of the average daily temperature (in °C) and time (in days) to DD, allows data to be normalized and compared among experiments. For myxozoan parasites, DD have been used to define the duration of an exposure event (e.g. Jones et al. 2016), and to characterize infection progression (e.g. Marshall et al. 2016), development of

myxospores (Moran & Kent 1999), time to host death (e.g. Ryce et al. 2004, Hallett et al. 2012), and life cycle completion (e.g. Chiaramonte 2013). The purpose of our study was to determine the temporal pattern of infection in seatrout by *K. inornata* and time for development of myxospores, to support our contention that the parasite could be mitigated by limiting the practice of discarding fish remains in estuarine waters. We used 2 methods to detect infection of fish: direct observation of myxospores, and molecular detection of *K. inornata* DNA using novel, specific PCR primers. Whereas PCR detection indicated early stages of infection, only direct observation of myxospores indicated that the parasite had had sufficient time to complete that phase of its life cycle and was ready to infect the alternate host.

MATERIALS AND METHODS

Experimental setup and sources of fish and parasites

All seatrout used in the experiments described below were provided by the Mariculture Section of the SC Department of Natural Resources (DNR). Fish were naïve F1 fish, born and raised in closed circuit, parasite-free seawater (chlorinated and dechlorinated), and fed a diet of pellets and frozen fish (Atlantic mackerel). All fish were kept in these conditions until used in experiments. Given the lack of knowledge of the putative annelid alternate host and fish-infective stage in the *Kudoa inornata* life cycle, lab-based model infections were not possible. Instead, we infected seatrout by exposing them to raw (untreated) water from Charleston Harbor, where *K. inornata* is naturally present. Fish were held in ~2200 l tanks with flow-through raw water. Sediment that accumulated at the bottom of the tanks was removed to limit the deleterious build-up of sulfides. Before water was allowed to fill the tanks again, either no major cleaning was done (Expt 1) or the tanks were totally emptied and scraped of any sessile invertebrates (Expt 2). Fish to be examined were captured haphazardly after most of the water in the tanks was removed. Fish were euthanized one by one with an overdose of MS222 according to the College of Charleston approved ethical procedures (IACUC 2014-002). Fish were processed immediately, and their total length (TL) and weight (W) were recorded. Monthly average water temperatures were collected from the Charleston Customs House by the USGS National Water Information System (http://waterdata.usgs.gov). When data were missing from this system (19 days in November–December 2014), water temperatures were measured directly from the tanks.

Visual and molecular detection of *K. inornata* in fish muscle tissue

Visual detection of parasite myxospores

A muscle punch biopsy (~5 mm in diameter) from the left epaxial musculature close to the dorsal fin was sampled from each fish, as it is known to be a reliable subsample locality to determine infection in fish (Ware et al. 2014). Tissue was squashed between a slide and coverslip, and presence of myxospore-containing plasmodia was determined by scanning the entire squash at 100× total magnification using a compound microscope (Fig. 1). If a squash appeared negative, a second random punch biopsy was taken from the cranial or epaxial muscle and examined. Fish were recorded as negative for myxospores after 2 negative biopsies. All fish were then frozen at −20°C for subsequent DNA extraction.

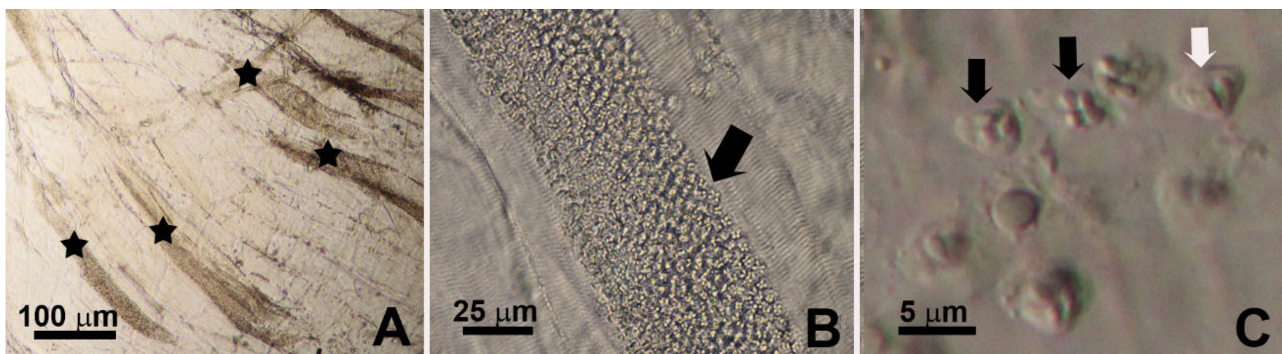

Fig. 1. *Kudoa inornata* in skeletal muscle from spotted seatrout *Cynoscion nebulosus*. (A) Several plasmodia (stars) in a squash of skeletal muscle. (B) Mature plasmodium (arrow) showing developed myxospores. (C) Individual myxospores en face (black arrow) or in profile (white arrow)

Molecular detection of parasite DNA

For each fish that did not have visible myxospores in Expt 2 and controls (described below), we tested muscle tissue for the presence of parasite DNA (presumptive pre-sporogonic parasite stages). Tissue was taken randomly from a minimum of 3 sites in the left fillet of the fish and thoroughly mixed before taking a ~40 mg subsample, from which DNA was extracted using a Qiagen DNeasy Blood and Tissue Kit following the manufacturer's protocol. We developed a *K. inornata*-specific PCR assay, designing primers based on GenBank accession number FJ790311 to amplify a region of small subunit ribosomal DNA (SSU rDNA) that varied when compared to available sequences of other known North American *Kudoa* species (*K. thyrsites*, GU191932; *K. crumena*, KC142196). Primers KINF2 (5′-CAA ATC CCG CAC TTG TGC-3′) and KINR2 (5′-CAT TCC GAT AGT GAA CCA ATT ATG G-3′) generated a 325 base pair (bp) amplicon that we verified by bi-directional Sanger sequencing as identical to the *K. inornata* reference sequence. A *K. inornata*-positive sample (visible myxospores) and a no-template control were included in all assays. The optimized 20 µl total volume PCR contained 1× PCR buffer (Promega), 3.0 mM $MgCl_2$, 0.2 mM dNTPs (Promega), 0.25 mg ml^{-1} bovine serum albumin (BSA, Promega), 1× *Redi*load gel loading buffer (Invitrogen), 2 µM of each primer, 1.25 U Go *Taq* Flexi DNA Polymerase (Promega), and 2 µl template DNA. A 2 min initial denaturation at 95°C was followed by 35 cycles of denaturation at 94°C for 20 s, annealing at 65°C for 20 s, and extension at 72°C for 45 s, and then by a final extension at 72°C for 2 min. Products were electrophoresed on 2% agarose gels (100 V, 30 min), subsequently stained with ethidium bromide or GelRed (Biotium), and visualized under a UV light.

To detect false negatives and verify that DNA was successfully extracted, we assessed the concentration and purity of the genomic DNA of PCR-negative samples using a NanoDrop 2000 UV-Vis spectrophotometer (Thermo-Scientific). Further, to verify that negative results were not due to PCR inhibition, 1 PCR-negative fish from each tank each month was selected randomly, and assayed using universal SSU rDNA primers 16S-A (5′-AAC CTG GTT GAT CCT GCC AGT-3′) and 16S-B (5′-GAT CCT TCC GCA GGT TCA CCT AC-3′; Medlin et al. 1988), to demonstrate that amplifiable DNA was present. The 20 µl total volume reaction contained 1× PCR Buffer (Promega), 1.5 mM $MgCl_2$, 0.2 mM dNTPs (Promega), 0.4 mg ml^{-1} BSA, 0.5× *Redi*load gel loading buffer (Invitrogen), 1 µM of each primer, 0.6 U Go *Taq* Flexi DNA Polymerase (Promega), and 1 µl template DNA. A 10 min initial denaturation at 94°C was followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 45°C for 20 s, and extension at 72°C for 2 min, and then by a final extension at 72°C for 5 min. Products were electrophoresed, stained, and visualized as above.

Experimental infections in sentinel fish

Expt 1: Continuous exposure over 12 months

Naïve spotted seatrout (N = 120) were set in each of 2 duplicate tanks (1A and 1B) and exposed to raw harbor water starting on 10 January 2013 (0 d post-exposure [dpe]). Approximately monthly (27–34 d, due to logistical constraints), 10 fish were subsampled from each tank and assessed for *K. inornata* infection by visualization of myxospores only.

Expt 2: Monthly exposures over 24 months

Naïve seatrout (N = 10) were set in each of 2 duplicate tanks (2A and 2B) and exposed to raw harbor water for periods of 27–34 d (Year 1) or 28 d (Year 2; except for the last exposure, which lasted 36 d due to factors beyond our control) commencing 10 January 2013. Two different stocks of seatrout were used (1 for each year) to avoid potential fish size effects on infection during the second year. At the end of each exposure period, all 10 fish were removed from each tank and assessed for *K. inornata* infection (via visualization of myxospores and PCR), and 10 naïve fish were placed in each tank for the next exposure period.

Expt 3: Myxospore development time

We used controlled-temperature laboratory experiments at 26 and 31°C to more precisely calculate DD to myxospore development. We first established the minimum exposure period needed to infect fish. In mid-June 2014, groups of naïve fish (each N = 15) were exposed to raw harbor water for 2, 3, 4, 5, 6, and 7 d at an average temperature of 26°C, then maintained in parasite-free seawater (Instant Ocean, Doctors Foster & Smith) for up to 4 wk at 26°C. Starting 2 wk post exposure (where Time 0 = Day 1 of exposure), 5 fish wk^{-1} $group^{-1}$ were examined for the

presence of visible myxospores as described above. We determined that a 7 d exposure was necessary for infection to develop in at least 1/5 fish. We then determined DD to myxospore development by exposing 15 naïve fish to raw water at 26°C for 7 d, then held them in parasite-free seawater as before. Starting 15 dpe (i.e. 7 d exposure plus 8 d held), 3 fish were sampled every other day, until all fish had been sampled. We repeated this part of the experiment twice during July 2014 (average water temperature 31°C) during which fish were exposed for 7 d in raw seawater from the harbor and then maintained in parasite-free water at 31°C. Three fish were dissected every day starting at 15 dpe.

Controls

We periodically tested spotted seatrout from our naïve stock for the presence of the parasite (via visualization of myxospores in squashes and PCR of muscle tissue): 25 fish at Time 0 (January 2013), 20 fish in September 2013, and 10 fish in January 2014. From our second year stock of naïve fish, 10 control fish were tested at Time 0 (January 2014), and 10 tested every 3 mo thereafter (April, July, October 2014, and January 2015). For Expt 3, naïve sentinel fish (N = 15) were held as controls under similar lab conditions, but without ever being in contact with raw water; these fish were assayed at the end of the experiment.

Statistical tests

Prevalence of infection was recorded for fish from each sample group. In Expt 2, we used a paired *t*-test to examine whether prevalence of infection by myxozoans differed systematically between our 2 experimental tanks over the 24 mo of study. Prior to *t*-tests, monthly percentages of fish infected in each tank (Tank 2A vs. Tank 2B) were arcsine transformed using Anscombe's method (Zar 1996). Since we found no significant difference between tanks (see Results), we pooled data across tanks to examine temporal changes, testing for both seasonal and annual variations in prevalence of infection. A chi-squared test was used to test for seasonal variations. This required data to be pooled into quarterly time periods within each calendar year (January–March, April–June, July–September, October–December) to meet test assumptions. Fisher's exact test was used to test whether overall prevalence of infection differed annually between 2013 and 2014. For this, data were pooled across all months within each calendar year.

RESULTS

Expt 1: continuous exposure

Myxospores were first detected visually in 5% (1/20) of fish subsampled in June 2013 after 5 mo of continuous exposure to raw harbor water. In July 2013, due to an accumulation of sulfides, 40 fish died in Tank 1B, which led to a significant decrease in fish density and increase in fish size in that tank (Table 1). The remaining Tank 1B fish grew >20% larger and heavier than fish in the replicate Tank 1A, where density remained higher (Fig. 2). Data from Tanks 1A and 1B were therefore analyzed independently. In Tank 1B, 100% (7/7) of fish sampled (mean TL = 192 mm, W = 58 g) were infected by November (10 mo continuous exposure), whereas in Tank 1A, the maximum prevalence of infection was 80% (8/10) (mean TL = 176 mm, W = 49 g) in January 2014 after 12 mo of continuous exposure.

Expt 2: monthly exposure

For Expt 2, we verified that there was no tank effect on parasite prevalence (paired *t*-tests: prevalence by visual detection only, $p = 0.78$; prevalence by visual + PCR detection, $p = 0.73$), and data were therefore pooled between replicate tanks (2A and 2B). The percent of fish with visibly detectable myxospores varied significantly between seasons over the full duration of the study (chi-squared test, $p < 0.00001$), as well as among seasons within 2013 ($p < 0.0001$) and within 2014 ($p < 0.01$; Fig. 3). In Year 1 (2013), myxospores were first detected visually in fish exposed in March (collected in April after 32 d exposure; mean TL = 92 mm, W = 10 g). The percent of fish with visible myxospores in each group increased through spring and summer to peak at 50% in fish exposed in August (collected in September after 34 d exposure; TL = 159 mm, W = 37 g). Fish exposed in November and December (examined in December and January after 31 and 30 d exposure, respectively) had no visible myxospores. In Year 2 (2014), visible myxospores were first detected in fish exposed in May (collected in June). Prevalence peaked at 25% in July fish, and no myxospores were seen in fish exposed after October, as also observed in Year 1. *Kudoa inornata* infection was first detected via PCR

Table 1. Expt 1 (continuous exposure): sampling schedule and results of sentinel seatrout *Cynoscion nebulosus* exposed to raw water from Charleston Harbor, South Carolina (USA), over 1 yr starting on 10 January 2013; 120 fish were placed in each of 2 separate tanks (1A and 1B). Dates refer to dates of fish collection and examination. Biometric data of fish at sampling included mean total length (TL) and weight (TW). Number (N) of fish infected in their muscle by myxospores (myxo) was via observation of squashes. Control fish were all negative (infection checked via direct observation of myxospores and PCR)

Date	Exposure time (d)	Mean (min.–max.) water temp. (°C)	Fish sampled (N) Tank 1A|1B	Mean TL (mm) Tank 1A|1B	Mean TW (g) Tank 1A|1B	Myxo-positive fish (N) Tank 1A|1B	Control fish (N)
2013							
10 Jan	0	–	–	63	3	–	25
13 Feb	34	13 (11–16)	10|10	64|69	3|3	0|0	–
15 Mar	64	13 (11–14)	10|10	70|72	3|3	0|0	–
15 Apr	95	15 (12–20)	10|10	82|82	5|5	0|0	–
15 May	125	20 (18–23)	10|10	95|101	6|9	0|0	–
12 Jun	153	25 (21–29)	10|10	102|105	7|8	2|0	–
10 Jul[a]	182	28 (27–30)	10|10	101|109	18|18	0|2	
13 Aug	215	29 (27–31)	10|10	138|163	23|39	3|4	–
13 Sep	246	28 (27–30)	10|10	150|176	28|48	3|5	20
10 Oct	273	26 (23–29)	10|10	167|186	37|57	5|5	–
09 Nov	303	21 (18–24)	10|7	169|192	36|58	4|7	–
09 Dec	334	15 (12–18)	10|0	174|–	46|–	5|–	–
2014							
08 Jan	364	13 (9–16)	10|0	176|–	49|–	8|–	10

[a]A fish kill occurred in Tank 1B, and all fish from 1B were sampled by November and thus, no data were collected for December 2013 or January 2014 for this tank)

in 3/12 visually negative fish exposed in April of Year 1. Note that in a few cases of visually negative fish (1 exposed in February, 5 in April and in May 2013, and 1 in August 2014; Table 2), DNA was not successfully extracted despite repeated attempts, which reduced the number of visually negative fish available for PCR).

PCR detection of parasite DNA in visually negative fish was over 40% in Year 1, and generally less than 12% in Year 2 when myxospores were observed only

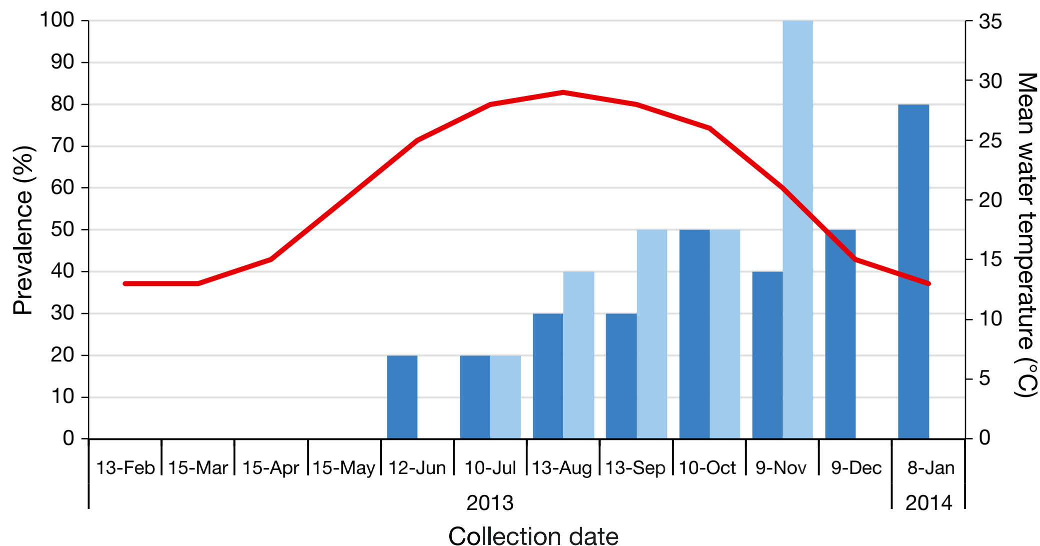

Fig. 2. Expt 1 (continuous exposure): prevalence of infection (%) of sentinel spotted seatrout *Cynoscion nebulosus* by myxospores of *Kudoa inornata* during continuous exposure to raw water from Charleston Harbor, South Carolina (USA). Line indicates average water temperatures. Tank 1A (dark bars) and Tank 1B (light bars) were each stocked with 120 fish on 10 January 2013. Ten fish were removed from each tank at intervals of 27–34 d. A fish kill (40 fish) occurred in July in Tank 1B, which presumably resulted in higher actinospore dose per fish in that tank (as evidenced by higher infection prevalence compared to Tank 1A after July), and no fish remaining to sample in December and January

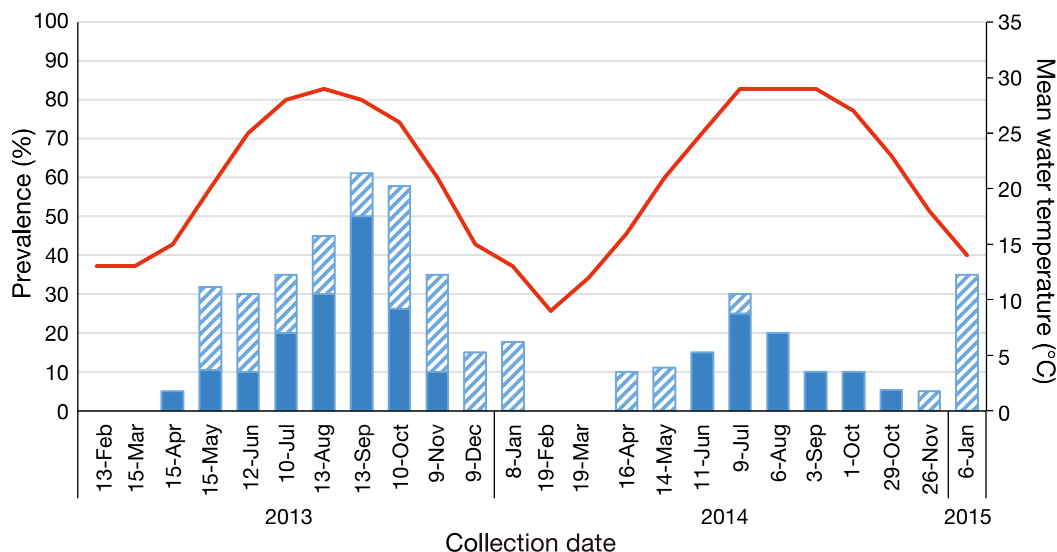

Fig. 3. Expt 2 (monthly exposures): prevalence of *Kudoa inornata* infection (%) in sentinel spotted seatrout *Cynoscion nebulosus* as determined by visible myxospores (solid bars) and by PCR of DNA extracted from muscle tissue (striped bars). Line indicates average water temperatures. Each month, 10 fish in 2 tanks (2A and 2B) were exposed to raw water from Charleston Harbor for 30–32 d during Year 1 (January 2013 – January 2014) and 28 d during Year 2 (January 2014 – January 2015). Data from each tank were pooled for analyses as there was no tank effect

in fish exposed in May through October (collected June through October 2014). No myxospores were seen in fish exposed for 36 d between late November 2014 and early January 2015, but 35% (7/20) of fish were PCR-positive. Neither technique detected *K. inornata* in fish exposed in January and February (examined in February and March, respectively) of either year.

Monthly average water temperatures were similar in both years except for winter 2014, which was colder (Table 2). The combined prevalence of infection by myxospores (visual + PCR detection) varied significantly between seasons over the duration of the experiment (chi-squared test, $p < 0.00001$). A strong seasonal pattern was evident in 2013 (chi-squared test, $p < 0.00001$), but this pattern was weaker during 2014 ($p = 0.052$), which experienced a generally lower prevalence of infection (2013 vs. 2014; Fisher's exact test, $p = 0.02$). In both years, infection by *K. inornata* first appeared in spring, rose to highest prevalence in summer, decreased in fall, and was not detected during the coldest winter months (Table 2, Fig. 3).

Expt 3: myxospore development time

At 26°C, no fish became infected with myxospores after exposure to raw water for 2, 3, 4, 5, or 6 d. Myxospores appeared in 1/15 fish (mean TL = 130 mm, W = 19 g) that were exposed for 7 d, when examined 21 dpe. Hence, we considered 7 d the minimum exposure time required for *K. inornata* infection to develop in fish at this temperature. In the replicated experiments, after a 7 d exposure, again no fish were found to be infected with myxospores before 21 dpe at 26°C, or before 16 dpe at 31°C. Given the 7 d exposure window, development of myxospores required 17.5 ± 3.5 d at 26°C (= 364–546 DD) and 12.5 ± 3.5 d at 31°C (= 279–496 DD). When combined with temperature data and detection results from Expt 2 (April and December 2013), we refined the minimum time needed for myxospore development to be between 468 and 476 DD.

DISCUSSION

We used 2 methods to detect *Kudoa inornata* in seatrout: a novel species-specific PCR assay, and direct microscopic observation of mature parasite myxospores. Whereas PCR detection indicated cryptic, early stages of infection, direct observation of myxospores indicated that the parasite had completed development within the time frame of exposure at the respective water temperatures.

Presence of *K. inornata* myxospores in seatrout showed a seasonal pattern similar to that of *K. thyrsites* in salmon (Moran & Kent 1999), with mature myxospores first visible in late spring, and prevalence peaking when water temperatures were

Table 2. Expt 2 (monthly exposures): sampling schedule and results of sentinel seatrout exposures to raw water of Charleston Harbor over 2 yr starting on 10 January 2013; 10 fish were placed into each of 2 separate tanks (2A and 2B) and removed after each exposure period (27–36 d). Dates refer to dates of fish collection and examination. Water temperatures were measured during exposure periods. Degree days were calculated using average of daily minimum and maximum water temperatures. Biometric data of fish at collection include total length (TL) and weight (TW). Number (N) of fish infected in their muscle either by myxospores (myxo) via squashes or presumed non-sporulated stages (via PCR) of *Kudoa inornata.* Asterisks (*) indicate sampling with some unsuccessful DNA extraction. Control fish were all negative (infection checked via direct observation of myxospores and PCR)

Date	Exposure time (d)	Mean (min.–max.) water temp. (°C)	Mean (min.–max.) degree-days	Fish sampled (N) Tank 2A|2B (total)	Mean TL (mm) Tank 2A|2B	Mean TW (g) Tank 2A|2B	Myxo-positive fish (N) Tank 2A|2B (total)	Myxo-negative fish DNA extracted (N) Tank 2A|2B (total)	DNA extraction successful (N) Tank 2A|2B (total)	Fish muscle PCR+ (N) Tank 2A|2B (total)	Overall infection (N) Tank 2A|2B (total)	Control fish (N)
2013												
10 Jan	0	–	–	–	63	3	0	25	25	0	0	25
13 Feb	34	13 (11–16)	439 (426–452)	10|10 (20)	64|64	3|3	0|0 (0)	10|10 (20)	10|10 (20)	0|0 (0)	0|0 (0)	–
15 Mar	30	13 (11–14)	377 (364–390)	10|10 (20)	84|83	5|5	0|0 (0)	10|10 (20)	9|10 (19)*	0|0 (0)	0|0 (0)	–
15 Apr	32	15 (12–20)	476 (446–487)	10|10 (20)	92|92	7|7	1|0 (1)	9|10 (19)	9|10 (19)	0|0 (0)	1|0 (1)	–
15 May	30	20 (18–23)	605 (584–625)	10|9 (19)	125|121	17|16	2|0 (2)	8|9 (17)	8|4 (12)*	3|0 (3)	5|0 (5)	–
12 Jun	28	25 (21–29)	696 (679–713)	10|10 (20)	130|129	19|19	1|1 (2)	9|9 (18)	4|9 (13)*	0|3 (3)	1|4 (5)	–
10 Jul	29	28 (27–30)	819 (801–837)	10|10 (20)	137|137	23|23	2|2 (4)	8|8 (16)	8|8 (16)	2|1 (3)	4|3 (7)	–
13 Aug	34	29 (27–31)	985 (964–1007)	10|10 (20)	150|150	28|28	3|3 (6)	7|7 (14)	7|7 (14)	2|1 (3)	5|4 (9)	–
13 Sep	31	28 (27–30)	883 (864–901)	8|10 (18)	158|160	33|41	3|6 (9)	5|4 (9)	5|4 (9)	1|1 (2)	4|7 (11)	20
10 Oct	27	26 (23–29)	696 (682–709)	10|9 (19)	162|162	44|42	3|2 (5)	7|7 (14)	7|7 (14)	2|4 (6)	5|6 (11)	–
09 Nov	30	21 (18–24)	645 (633–657)	10|10 (20)	166|166	44|59	1|1 (2)	9|9 (18)	9|9 (18)	2|3 (5)	3|4 (7)	–
09 Dec	31	15 (12–18)	467 (453–482)	10|10 (20)	170|169	46|46	0|0 (0)	10|10 (20)	10|10 (20)	1|2 (3)	1|2 (3)	–
2014												
08 Jan	30	13 (9–16)	394 (381–408)	10|7 (17)	175|173	48|44	0|0 (0)	10|7 (17)	10|7 (17)	3|0 (3)	3|0 (3)	10
22 Jan	0	–	–	–	142	19	0	10	10	0	0	10
19 Feb	28	9 (7–11)	255 (242–267)	10|8 (18)	144|143	20|20	0|0 (0)	10|10 (18)	10|8 (18)	0|0 (0)	0|0 (0)	–
19 Mar	28	12 (10–13)	339 (324–355)	10|10 (20)	152|155	31|32	0|0 (0)	10|10 (20)	10|10 (20)	0|0 (0)	0|0 (0)	–
16 Apr	28	16 (12–21)	444 (425–462)	10|10 (20)	155|162	33|37	0|0 (0)	10|10 (20)	10|10 (20)	2|0 (2)	2|0 (2)	10
14 May	28	21 (16–26)	582 (561–602)	9|9 (18)	166|173	39|42	0|0 (0)	9|9 (18)	9|9 (18)	1|1 (2)	1|2 (3)	–
11 Jun	28	25 (23–28)	709 (695–723)	10|10 (20)	178|176	45|43	1|2 (3)	9|8 (17)	9|8 (17)	0|0 (0)	1|2 (3)	–
09 Jul	28	29 (27–31)	807 (792–823)	10|10 (20)	176|184	44|50	2|3 (5)	8|7 (15)	8|7 (15)	0|1 (1)	2|4 (6)	10
06 Aug	28	29 (28–30)	814 (802–826)	10|10 (20)	175|179	42|45	2|2 (4)	8|8 (16)	8|7 (15)*	0|0 (0)	2|2 (4)	–
03 Sep	28	29 (28–31)	823 (809–837)	10|10 (20)	180|181	42|41	1|1 (2)	9|9 (18)	9|9 (18)	0|0 (0)	1|1 (2)	–
01 Oct	28	27 (23–30)	767 (755–780)	10|10 (20)	186|187	48|50	2|0 (2)	8|10 (18)	8|10 (18)	0|0 (0)	2|0 (2)	10
29 Oct	28	23 (21–26)	659 (646–671)	10| 9 (19)	197|195	59|53	0|1 (1)	10|8 (18)	10|8 (18)	0|0 (0)	0|1 (1)	–
26 Nov	28	18 (10–23)	493 (486–500)	10|10 (20)	194|195	58|61	0|0 (0)	10|10 (20)	10|10 (20)	1|0 (1)	1|0 (1)	–
2015												
06 Jan	36	14 (12–15)	485 (475–495)	10|10 (20)	203|190	70|56	0|0 (0)	10|10 (20)	10|10 (20)	2|5 (7)	2|5 (7)	10

warmest (Table 1, Fig. 2), with only 1 peak of infection, in summer (both years of Expt 2; Table 2, Fig. 3). PCR (used in our second experiment) detected parasite DNA, presumably from cryptic, pre-sporogonic forms, in fish muscle most of the year (Table 2, Fig. 3). The only period when fish were both PCR and myxospore negative was when they were exposed during January and February, the coldest periods during both years, with a minimum of 7°C.

The emergence of myxosporean infections in the spring has been described for multiple species, and correlates with an increase in actinospore density in the water (Hallett et al. 2012). Although the role of actinospore dose cannot be experimentally verified for *K. inornata* for which no annelid alternate host is yet known, recent progress in unravelling marine myxosporean life cycles (Eszterbauer et al. 2015, Rangel et al. 2016) supports the assumption that annelids are involved. An increase in water temperature is considered to be the triggering factor in actinospore release from the annelid hosts (El-Matbouli et al. 1999, Stocking et al. 2006, Alama-Bermejo et al. 2013), and the progressive increase in prevalence of infection, with a peak in the summer, in seatrout maintained in equal density (Expt 2: 10 fish tank^{-1}) supports the hypothesis that actinospore infection of seatrout by *K. inornata* is density-dependent. Although we observed a direct correlation of infection and water temperature, Shirakashi et al. (2012) observed a distinct decrease in prevalence at the highest water temperature, suggestive of an actinospore-viability temperature threshold, which was not evident in our experiments.

We further hypothesize that the decrease in prevalence of infection in late fall and winter was due to a decrease in actinospore density, as has been shown for *Ceratonova shasta* in the Klamath River (Hallett et al. 2012) and *Ceratomyxa puntazzi* in the Mediterranean (Alama-Bermejo et al. 2013) in studies that combined quantification of waterborne stages (through quantitative PCR of filtered water samples) and parallel sentinel fish exposures. Fewer actinospores may result from a seasonal decrease in annelid population (Zajac 1991), an increase in actinospore development time in the worm host (El-Matbouli et al. 1999, Liyanage et al. 2003), or perhaps limitation in actinospore viability known to be temperature- and myxozoan species-dependent (Kallert & El-Matbouli 2008, Bjork & Bartholomew 2009). The common idea that annelid densities may decline in winter because of harsh environmental conditions may not generally be the case, and several species in SC increase in density during winter months (Gillett et al. 2007). Alternatively, the colder 2014 winter may have limited viability of myxospores themselves. Given that the overall water temperatures were similar throughout the 2 yr of the second experiment however, the lower prevalence of infection observed in 2014 may be due to a decrease in density of the presumptive alternate host due to other factors. For example, in 2014, Charleston Harbor was dredged, which potentially disrupted annelid habitat and physically removed worms from the system. The peak of PCR-positive fish in January 2015 (exposed December 2014) may indicate that the annelid population had recovered to the point where infected worms were again present and minimum temperatures were still above a presumptive minimum release/infection threshold.

Infection appeared during spring in both Expts 1 and 2; however, myxospores appeared later in Expt 1 (June/July) than Expt 2 (April/June), and prevalence of infection (visible myxospores) never reached the same level in Expt 1 fish. All experimental seatrout were exposed to raw seawater under the same conditions, except fish density. Expt 1 fish were maintained at higher stocking density that began at 120 ind. tank^{-1}, versus Expt 2 in which fish density never exceeded 10 ind. tank^{-1}. Additionally, during Expt 1, fish density in Tank 1B dropped following a fish kill in July 2014, and myxospore prevalence thereafter was always equivalent or higher in the remaining 1B fish compared to the 1A fish. Furthermore, prevalence in 1B reached 100%, but 1A only peaked at 80%, where fish density remained higher (Table 1). Host density thus appeared to affect prevalence of infection, with stocking fewer fish in a tank resulting in a higher per-fish dose of presumptive actinospores and consequently higher prevalence of infection. These observations also provide further evidence of an actinospore dose-dependent threshold for infection in the fish host, which is consistent with other myxosporeans, such as *Ceratonova shasta* (see Bjork & Bartholomew 2009, Hallett et al. 2012). Although there was correlation of infection with water temperature, other abiotic factors such as photoperiod or dissolved oxygen content could also have affected actinospore production, release, and/or viability. Quantification of these factors would be greatly aided by discovery of the alternate host of this parasite and an understanding of its natural history.

The DD metric is useful for estimating and comparing time for parasite development under different thermal regimes and for different species. Although wild seatrout could become infected with *K. inornata* (as indicated by PCR detection) during almost any month (except January or February), DD accumula-

tion was sufficient to result in visible myxospores within ~1 mo during summer only; in colder months, the parasite did not have sufficient DD to develop myxospores before the fish were sampled. We determined that the minimum DD to myxospore development in Expt 2 was between 468 (there was no development at 467 DD in December 2013) and 476 (April 2013). Expt 3 used fixed-temperature conditions (26 or 31°C) to confirm these minimum DD estimates (455 ± 91; 388 ± 109, respectively). Practically, these DD estimates demonstrated that mature myxospores can develop in seatrout within 2 wk during a typical SC summer (water temperatures >26°C). This is more than twice as fast as *K. thyrsites* in Atlantic salmon (~1000–2000 DD; Moran & Kent 1999, Marshall et al. 2016). However, the minimum time for detection of presumptive pre-sporogonic stages in muscle is similar for both parasites: *K. inornata* in seatrout (Expt 2, 394 DD); *K. thyrsites* in Atlantic salmon (355 DD in a high-prevalence farm, 509 DD in a low-prevalence farm; Marshall et al. 2016). The implications of having a short myxospore developmental time are manifold for host–parasite dynamics. These effects are amplified by local conditions, as a typical SC summer can extend to over 4 mo. Seatrout that die during this period (or are caught by anglers and their carcasses thrown back into the water) release myxospores that could infect more annelid hosts, which in turn could release actinospores within the same season. Further, while the fact that mature myxospores are observed rarely in wild juvenile seatrout (Arnott et al. 2017) may be due to a partitioning in foraging habitats during ontogeny (Deary & Hilton 2016), we suspect this could also be a result of sampling bias, as moribund fish are rarely captured. Given that the period of high prevalence of infection corresponds with the peak of the spawning season (June–September; Roumillat & Brouwer 2004) and warmest temperatures, and our experimental observations with heavily infected juveniles (data not shown), we hypothesize that juvenile wild seatrout are seriously negatively affected by the immediate, heavy parasite burden, and thus never recruit to the breeding population.

Given the dynamics that we observed for *K. inornata* development, taken together with the well-recognized impacts of climate change on parasites (e.g. Marcogliese 2001, Lafferty 2009), we anticipate that additional developmental cycles will likely occur if the projected warming trends occur. This would only exacerbate the impacts of *K. inornata* on seatrout populations through removal of offspring. Any measures aimed at increasing awareness of this parasite and to reduce the release of myxospores (and actinospores) in the environment could limit prevalence and intensity of *K. inornata* infections. While regular dredging might disrupt potential annelid host populations, it is neither an ecologically nor economically sound solution. Angler education to change the way fish remains are discarded would be an easily applicable, cost-effective, long-term measure to reduce myxospore input back into the harbor. Given the observed infection dynamics of *K. inornata*, however, it would need to occur throughout the year to be most effective.

CONCLUSIONS

We have gleaned insight into the dynamics of infection of spotted seatrout *Cynoscion nebulosus* by the marine myxosporean *Kudoa inornata.* Infection appears to have a distinct seasonal pattern with a single peak of high prevalence in summer, indicating that actinospores may be released more intensely during this period, and with almost continual release through the rest of the year (as shown by presumptive covert infections through early winter). Seatrout appear to have an actinospore dose-dependent threshold for infection, which would be exceeded rapidly in the hottest months. Myxospores of *K. inornata* develop rapidly, compared with other myotropic *Kudoa* species, and unlike, for example, *K. thyrsites* infection in salmon (Moran & Kent 1999), *K. inornata*-infected seatrout appear to retain myxospores throughout their life as no signs of resistance or inflammation are typically observed (Dyková et al. 2009). Therefore, once infected, seatrout remain a source of infectious stages for the next host in the cycle. Given this dynamic, we anticipate that the life cycle of the parasite may be disrupted by preventing spore-rich seatrout carcasses being thrown back into harbors and estuaries throughout the year.

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