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Seasonal monitoring of *Kudoa yasunagai* from sea water and aquaculture water using quantitative PCR

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ABSTRACT: Kudoid myxozoans pose serious chronic problems in marine fisheries by causing pathological damage to host fish, reducing the market value of infected fish and potentially threatening public health. *Kudoa yasunagai* is a cosmopolitan parasite that infects the brains of various marine fishes, including important aquaculture species. We developed a quantitative PCR assay to detect *K. yasunagai* in sea water, and we used it to monitor abundance of the parasite in the environment and in culture through spring and winter. Quantitative PCR detected *K. yasunagai* DNA from sea water, with the lowest reliable threshold of 162 copies 28S rDNA l^{-1}. Parasite DNA was detected sporadically in sea water throughout the study period of May through December 2012. The highest level of detected DNA occurred in mid-December (winter), at 117 180 copies — equivalent to an estimate of over 200 myxospores l^{-1}. Parasite DNA was generally not detected in August or September, the period with the highest water temperature. The reason for this observation is unknown, but the timing of parasite development may play a role. The amount of detected DNA was not different between unfiltered culture water and water filtered through a high-speed fiber filtration system. This result and the past incidence of high infection rate of fish reared in filtered water indicate that the mechanical removal of *K. yasunagai* from culture water is difficult. Detecting the precise onset and time window of infection in host fish will be an important step in the development of measures to control this economically important parasite.

KEY WORDS: Myxozoa · Quantitative PCR · qPCR · Actinospore · Sea water · Seasonality

INTRODUCTION

Kudoid myxozoans (Myxosporea, Multivalvulida) are microscopic parasites of marine fishes. Over 80 species have been described worldwide from a wide variety of fish hosts (Egusa 1986b, Moran et al. 1999, Burger & Adlard 2010a, Meng et al. 2011, Miller & Adlard 2012, Yokoyama et al. 2012b). Infections by *Kudoa* spp. are a chronic problem in finfish mariculture, even though the pathology of *Kudoa* is not generally considered a significant threat to fish survival (but see Cobcroft & Battaglene 2013). Nevertheless, unsightly cyst formations in the flesh (e.g. *K. amamiensis*) or postmortem myoliquefaction (e.g. *K. thyrsites*) render the fish unmarketable and cause economic loss to fish farmers. Furthermore, the effects of *Kudoa* on public health have recently become a serious concern (Martínez de Velasco et al. 2007). Using epidemiological analysis and animal testing, researchers have identified *K. septempunctata*, which infects the somatic muscle of the olive flounder *Paralichthys olivaceus,* as a causative agent of food poisoning. (Kawai et al. 2012, Iwashita et al. 2013). This finding has led to increased interest and concern over *Kudoa* infection in commercially important fish.

Kudoa yasunagai is a brain-infecting kudoid myxozoan found in a wide range of cultured and wild marine fish (Burger & Adlard 2010b, Miller & Adlard 2012). Nearly 20 fish species from various taxonomic groups have been recognized as hosts of *K. yasunagai,* including the red sea bream *Pagrus major*, the

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olive flounder, the tiger puffer *Takifugu rubripes*, the Pacific bluefin tuna *Thunnus orientalis*, the coral catfish *Plotosus lineatus*, and the whiting *Sillago* spp. from Japan, Southeast Asia and Australia (Egusa 1986a,b, Cheung & Nigrelli 1990, Whipps et al. 2004, Burger et al. 2007, Burger & Adlard 2010b, Zhang et al. 2010). Although *K. yasunagai* does not likely affect human health, its low host specificity and wide geographical distribution makes this parasite a potential cosmopolitan pathogen in fish farms. Typical clinical signs of infected fish are abnormal behavior and skeletal deformation, but lethal effects on heavily infected fish have also been suggested (Yasunaga et al. 1981, Egusa 1986a, Yamamoto 2007, H. Yokoyama pers. comm.). At a fish culture site in Tanabe bay, Wakayama prefecture, Japan, *K. yasunagai* infection occurs every year, and the associated skeletal deformities in juvenile kingfish *Seriola lalandi* are a serious problem (Shirakashi et al. 2012). High infection prevalence (>80%) has been detected among juvenile kingfish kept in land-based tanks; therefore, the infection occurs both in the hatchery and after fish are transferred to the open water net cages (S. Shirakashi unpubl. data). In a previous study, we monitored the progression of *K. yasunagai* infection among yellowtail *Seriola quinqueradiata* kept in open water cages in Tanabe Bay. We showed that *K. yasunagai* becomes detectable by PCR in the fish brain around mid-July and the cysts become visible several weeks later, in August (Shirakashi et al. 2012). Therefore, we suspect that the infection to the fish in this region starts sometime around May or June. However, the precise onset of infection and the duration of the invasive season remain unknown.

There is no effective control measure for *Kudoa yasunagai,* and elimination of the parasite from the fish body is not practical or feasible (but see Jones et al. 2012). Thus, prevention is the only realistic way to reduce infection rates. However, information about the general biology of *K. yasunagai* or *Kudoa* in general is scarce. The typical life cycle of a myxozoan involves 2 alternate hosts: vertebrates (mainly fish) and annelid invertebrates (generally oligochaetes for freshwater species and polychaetes for marine species) (Yokoyama et al. 2012a). Fish become infected upon physical contact with actinospores released from an annelid host. To date, the life cycles of 5 species of marine myxozoans have been described, but neither actinospores nor alternative invertebrate hosts have been found for any *Kudoa* species (Køie et al. 2004, 2007, 2008, Rangel et al. 2009, Karlsbakk & Køie 2012). Such a lack of basic information impedes the development of control measures in aquaculture. Understanding the temporal and geographical distribution patterns of the parasite will be useful for optimizing control measures such as UV or ozone treatments of culture water and for the development of new preventative methods.

In the present study, we developed a quantitative PCR (qPCR) assay to detect *Kudoa yasunagai* in sea water and used this assay to monitor seasonal changes in parasite abundance. Our goal was to develop a reliable method to detect the parasite in sea water which could then be applied to obtain basic information that could be used for the development of control methods against *K. yasunagai*, as well as for other important *Kudoa* species.

MATERIALS AND METHODS

qPCR conditions and primer-probe set

The primers and TaqMan probe for species-specific quantification were designed using GENETYX (GENETYX) against the 28S rDNA of *Kudoa yasunagai*. This region was targeted due to greater polymorphism, which better allows discrimination between closely related species (Meng et al. 2011). The specificity of the primer set was checked initially with a nucleotide BLAST search of the DNA Data Bank of Japan (DDBJ). The primer sequences were as follows: F381, 5′-AGT GAG TGA GTC TGC TCA AGA GCA-3′ and R556, 5′-ACC GAC AAT ACA TCA CCA ACG CTC CTC-3′. The TaqMan probe Kyas-rPCR-FL was 5′ end-labeled with FAM and 3′ end- labeled with TAMRA, and its sequence was as follows: 5′-CAA TGG TCG GCC ATG GCT TTC ACA GCA AAT-3′. Each 25 µl reaction contained 0.2 µM of primers F381 and R556, 0.4 µM of Kyas-rPCR-FL probe, 1 × remix Ex Taq (TaKaRa), and 5 µl of sample DNA. The amplification profile was as follows: initial denaturation at 95°C for 30 s, followed by 50 cycles of repeated denaturation at 95°C for 5 s, annealing and extension at 60°C for 50 s. All qPCR assays were performed using a Smart Cycler II System (Cepheid), except for seasonal monitoring which was performed with Corbett Rotor-Gene 6000 system (Qiagen).

Specificity and sensitivity of qPCR

To confirm specificity, the qPCR amplification was separately applied to stocked DNA extracts of field isolates of 6 *Kudoa* species: *K. yasunagai, K. ama-*

miensis, K. neothunni, K. prunusi, K. septempunctata, and *K. thyrsites.* The sensitivity of the qPCR was determined with a plasmid derived from the 28S rDNA of *K. yasunagai,* constructed as follows: the target region of primers F381 and R556 were amplified by conventional PCR from cyst DNA isolated from the brain of a yellowtail. The amplicon was purified using a QIAquick Gel Extraction Kit (Qiagen), cloned into the pDrive cloning vector (Qiagen) and transformed into *Escherichia coli* XL-1. The correct clone was screened and selected by colony PCR. Plasmid DNA was isolated from cultured cells using a QIAprep Spin Miniprep Kit (Qiagen) and was then stored in small aliquots at −20°C until use. The DNA concentration was determined by spectrophotometry at 260 nm, and the purity was confirmed using the 260/280 ratio. The copy number of plasmid was calculated using the equation: plasmid copies μl^{-1} = [plasmid amount (g μl^{-1}) × (6.02×10^{23})] / [molecular weight of the DNA fragment]. The qPCR was performed using a serial 10-fold dilution of the plasmid ranging from 1×10^6 to 1×10^0 copies per PCR. In addition, myxospores of *K. yasunagai* collected from the brain of a barbel eel *Plotosus japonicus* were counted under a microscope, serially diluted and subjected to qPCR. DNA from the myxospores was extracted using aQIAamp DNA Mini Kit (Qiagen), dissolved in 100 µl of Buffer AE, and prepared as 10-fold dilutions ranging from 1×10^6 to 1×10^1 spores. Each 5 µl portion of the DNA solution was submitted to qPCR, and each reaction contained DNA extracted from the myxospores, ranging from 5×10^{-1} to 5×10^4 spores. qPCR reactions of each concentration of the plasmid and myxospore were run at least in triplicate, and quantification cycle (Cq) values were averaged. For determination of the relationship between the qPCR Cq values and the copy number of plasmid DNA or the number of myxospores, standard curves were generated. The copy number of rDNA contained in a myxospore was calculated from these standard curves.

DNA extraction from sea water

Each 1 l water sample was suction-filtered through a 5 µm pore size nitrocellulose membrane filter (SMWP04700, Millipore), and the filter with residue was folded to fit into a 1.5 ml microcentrifuge tube and preserved at −20°C until DNA extraction. The filter was dissolved (filter was removed from the filtered residue) prior to DNA extraction by acetone dissolution (modified from Hallett et al. 2012). In a preliminary experiment, this process improved the DNA yield estimated by qPCR more than 3 times compared to direct extraction from the filter. To each microcentrifuge tube, 1 ml of acetone was added, and the tube was vortexed until the filter fragmented into fine particles. The tube was then centrifuged and the supernatant was discarded. The process was repeated twice with 1 and 0.5 ml of acetone to ensure elimination of the filter. DNA was extracted from the residue that remained in the tube using a QIAamp DNA Mini Kit (Qiagen) following the manufacturer's protocol, and the DNA was eluted into 100 µl of Buffer AE.

The DNA recovery rate of the extraction procedure was estimated by an additional recovery method. Myxospores of *Kudoa yasunagai* isolated from a yellowtail were added to 1 l of sea water collected from Tanabe Bay. DNA from 5×10^{-1} to 5×10^2 myxospores was extracted and quantified by qPCR using the myxospore standard curve. The recovery rate was calculated as the quotient of the number of detected and added myxospores.

Seasonal monitoring of *Kudoa yasunagai*

The density of *Kudoa yasunagai* rDNA in water samples was estimated using qPCR. Water samples (1 l) were collected every week at 2 sites in Tanabe bay between 25 May and 21 December 2012 (Fig. 1). The 2 sampling sites are approximately 1.5 km apart from each other. Samples consisted of fiber-filtered ('Kemari' system, Daiki Ataka Engineering; specified capture particle size ≥2 µm, suspended substance removal rate <95%, linear velocity <50 m h^{-1}) and unfiltered water from the Fish Nursery Center of Kinki University (Sakata) and surface water from the vicinity of fish cages of the Fisheries Laboratory of Kinki University (Kogaura). The water intake for the Fish Nursery Center is located in an inlet approximately 300 m away from the Center at a depth of 3 m. The species of fish cultured in the sampling area included kelp grouper *Epinephelus bruneus,* red seabream, olive flounder, tiger puffer, yellowtail, and greater amberjack *Seriola dumerili.*

DNA extraction from water and qPCR were performed as described above. All qPCR assays of the monitoring samples were performed using a Corbett Rotor-Gene 6000 system (Qiagen). Each sample was run in single. No-template controls (3 to 4) were included in every run to determine the threshold of a negative response. The standard curve for seawater monitoring was generated from the Cq values of DNA

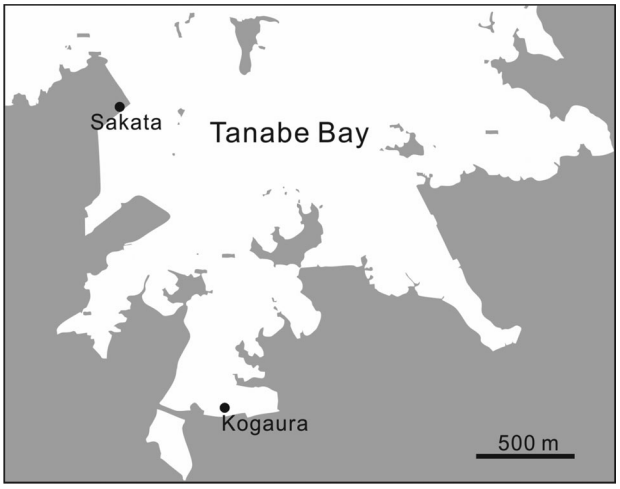

Fig. 1. Tanabe Bay, Japan, indicating sampling sites: the Fisheries Laboratory of Kinki University at Kogaura (33° 40′ 48″ N, 135° 21′ 39″ E), and the Fish Nursery Center of Kinki University at Salkata (33° 41′ 36″ N, 135° 21′ 27″ E). The 2 sites are approximately 1.5 km apart

extracted from serial 100-fold dilutions of the plasmid ranging from 1×10^1 to 1×10^7 target copies using the built-in software of the qPCR system. To distinguish true target negatives from potential PCR inhibition in seawater extracts, TaqMan Exogenous Internal Positive Control (IPC) Reagents (Life Technologies) were added to each reaction of the monitoring samples and negative controls following the manufactures instruction (Hallett et al. 2012). No effect of the IPC with the assay was observed; thus, the amplification of the IPC in each tube was monitored simultaneously with the target reaction and compared between each sample and negative controls.

To confirm species-specific amplification, the amplicons obtained from 9 qPCR-positive reactants, sampled on 25 May, 6 July and 5 October were sequenced. The reactants were separated on a 2% agarose gel. Each band was excised from the gel and purified using a QIAquick Gel Extraction Kit (Qiagen). Dideoxy sequencing was performed by Macrogen Japan.

Data analysis

All standard curves except for those used in field monitoring were generated by Microsoft Excel 2010 software (Microsoft), followed by regression analysis. The amount of detected DNA in each pair of filtered and unfiltered water samples were compared using Student's *t*-test performed with JMP 10 (SAS Institute).

RESULTS

Specificity and sensitivity of qPCR

The primer set amplified a product of the expected size (202 bp) by qPCR from the extracted DNA of *Kudoa yasunagai*. This qPCR assay was specific to the target it was designed for and did not detect the 28S genes of the 5 other *Kudoa* species. Cq values showed a linear relationship to the log number of plasmids in the range of 1×10^2 to 1×10^5 copies. However, the Cq value corresponding to a range of 1×10^0 to 1×10^2 copies of plasmid was approximately 39, which did not correlate to the amount of target. Therefore, the lower limit for accurate quantification of the qPCR from plasmid DNA was approximately 1×10^2 copies. The slope of the standard curve constructed from the range of 1×10^2 to 1×10^6 copies was −3.3287, and the R^2 after linear regression was 0.945. The copy number of 28S rDNA contained in a myxospore of *K. yasunagai*, calculated from the relationships among Cq values and the corresponding numbers of plasmids and myxospores, was 410 to 880. The minimum level of accurate quantification of the plasmid was equivalent to 0.1 to 0.2 myxospores (2 to 4 spores l^{-1} sea water).

DNA extraction from sea water

The DNA recovery rate of myxospores added to sea water was 38%. Introducing an acetone dissolution step for filter removal improved the recovery rate remarkably (data not shown).

Seasonal monitoring

The IPC was amplified normally in all samples assayed, and no inhibition of the PCR reaction was observed. Some negative control samples showed a faint increase in the fluorescence value; therefore, the threshold of Cq values between positive and negative signals was set to the minimum Cq value among negative controls. We set the reliable threshold at Cq 43.5, which is equivalent to 0.02 myxospores. *Kudoa yasunagai* genes were detected from sea water collected at both sampling sites. The seasonal change of the target gene density is shown in Fig. 2. Positive signals were detected intermittently throughout the investigation period, but no reliable signal was detected in early August through the end of September, when the water temperature was the

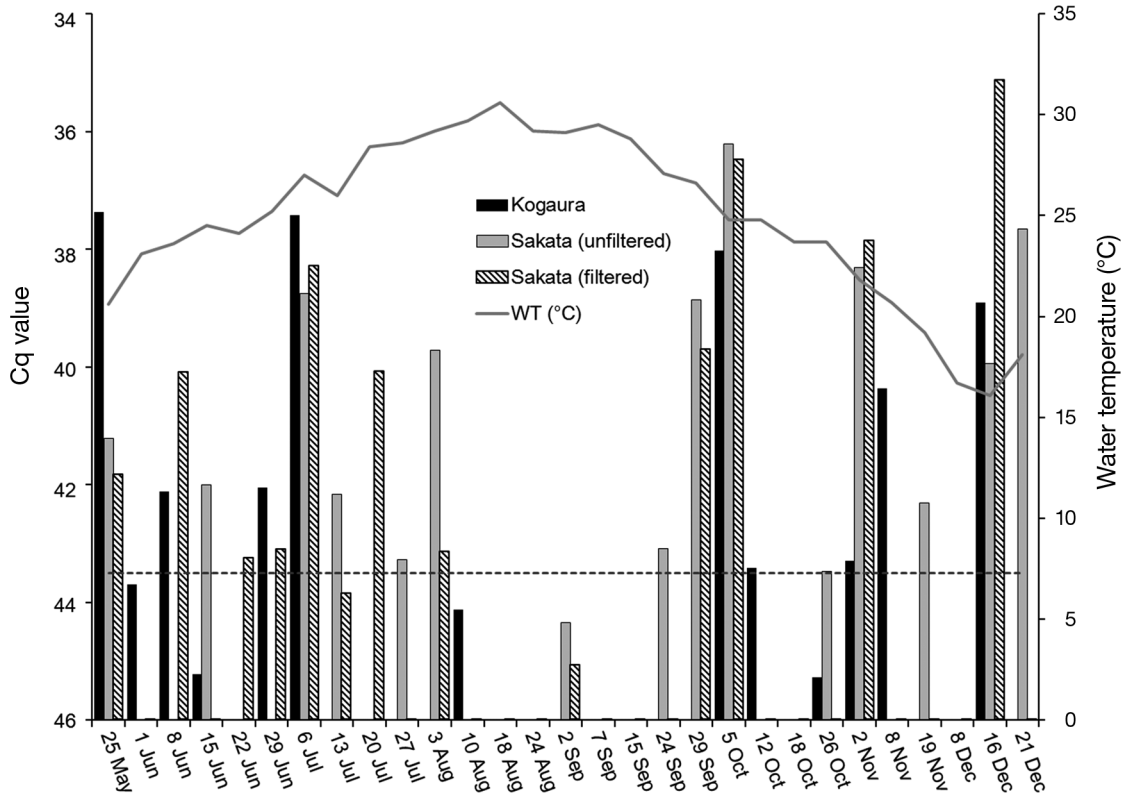

Fig. 2. Seasonal changes in *Kudoa yasunagai* rDNA density at the 2 sites near Wakayama, Japan, from May (spring) to December (winter) 2012. Data are shown as the cycle quantification (Cq) value of quantitative PCR (qPCR). Sea water samples were collected from the surface at Kogaura and the bottom (3 m depth) at Sakata. Dashed line is the threshold of reliable detection. WT: water temperature

highest. At the Fish Nursery Center, there was no difference in the target DNA density between filtered and unfiltered water (*t*-test, p = 0.1576). Sequencing of 9 qPCR products from our environmental monitoring revealed a high degree of similarity (>99%) with known sequences of the corresponding *K. yasunagai* gene deposited in Genbank (e.g. GU808775–GU808777, AY302736, JQ26230–JQ26232).

DISCUSSION

Researchers have previously used qPCR to detect and monitor the abundance of actinospores from freshwater environments. Bartholomew et al. (2007) showed that the abundance of *Parvicapsula minibicornis* and *Ceratomyxa shasta* in river water, determined by qPCR, correlated with infection prevalence in their salmonid hosts. Similarly, the temporal and geographical distribution of *C. shasta* in a river water system has been successfully determined using qPCR (Hallett & Bartholomew 2006, Atkinson & Bartholomew 2010, Hallett et al. 2012). Generally, detection of DNA from the marine environment is more problematic due to a low parasite density and a high diversity and abundance of organic matter (Cox & Goodwin 2013). There has only been 1 multiseasonal study that successfully monitored the density of a myxozoan (*Ceratomyxa puntazzi*) from a marine environment using qPCR (Alama-Bermejo et al. 2013). We successfully detected and quantified *Kudoa yasunagai* DNA from sea water without noticeable inhibition of the PCR assay. DNA sequencing of selected samples confirmed that the detected DNA was indeed from *K. yasunagai*. The amount of target DNA detected from 1 l water samples varied from calculated values of 1960 to 117 180 copies, which was estimated to be the equivalent of approximately 4 to 210 myxospores, respectively. These values are comparable to, or even greater than, the values obtained for freshwater myxozoans (Hallett & Bartholomew 2006, 2009, Griffin et al. 2009). Alama-Bermejo et al. (2013) reported that the density of *C. puntazzi* at the Spanish Mediterranean coast is much lower, a maximum of approximately 1 spore per 8 l of sea water. Although the prevalence of infection in

fish from our study area and from the Spanish Mediterranean were both high (maximum >90%) (Shirakashi et al. 2012, Alama-Bermejo et al. 2013), parasite density at the 2 sites differed markedly with higher abundances of *Kudoa* seen in our study. Differences in parasite density between these sites could be due to parasite and/or site-specific factors, or combinations thereof, including differences in transmission dynamics between parasite species and their hosts, abundance and/or distribution of invertebrate and/or fish hosts, patterns of water circulation and exchange, and physical and chemical characteristics of the water columns.

Our sampling sites are located in a deep part of a small bay with relatively little water movement. In addition, the area contains extremely high densities of cultured and wild fish, as well as annelids that live on the floats, frames and ropes of culture cages. Determination of factors affecting parasite density will be an important step to understanding the infection dynamics and developing control measures.

In the present study, a relatively high amount of *Kudoa yasunagai* DNA was detected throughout the study period, which began in late May. In our previous study, there was a sharp increase of infection prevalence in fish brains during mid-July (from 0 to 90% within 3 wk); thus, we suspected the infection of the fish occurred within a narrow window of time, most likely in June or early July (Shirakashi et al. 2012). However, if the parasites detected during May and June in the present study were actinospores, then the invasion could have started much earlier, and the window of infection may be wider than we originally thought. It is unclear whether the parasite was abundant before May, but it seems likely that it is present in the water throughout the year.

However, the parasite density in the water seemed to be reduced during the summer months when the water temperature was the highest (August and September). Interestingly, we observed a similar trend for the infection prevalence in the fish host. During our monitoring of *Kudoa yasunagai* in yellowtail, we noticed a sharp decline of infection prevalence in mid-August when the water temperature reached its peak (Shirakashi et al. 2012). We hypothesized that this decline in infection rates was due to elimination of the parasite by the host immune response, facilitated by the high water temperature. However, this hypothesis was not supported by our experiment comparing parasite development at 2 temperature conditions of 25° and 30°C (Shirakashi et al. 2012). It is not known why the parasite densities were lower in both sea water and fish brains during the period when water temperature was high. We suspect that the underlying mechanisms for the 2 phenomena are likely different. One possible explanation for the reduced density in the water is the development of the parasite. In our previous study, *K. yasunagai* had reached the brains of yellowtail by early July and took at least 4 wk to develop into cysts (Shirakashi et al. 2012). The period after parasite invasion up to the development of cysts seems to correspond to the period with the lowest parasite abundance in the water. It may be that the rise of water temperature in early summer triggers the release of actinospores, as in other myxozoan systems. This release may be hindered above a threshold temperature of approximately 30°C (El-Matbouli et al. 1999, Stocking et al. 2006, Alama-Bermejo et al. 2013). If this is the case, then infection of fish likely occurs before July, and the parasite starts to develop into myxospores after August with release into the water after September (Shirakashi et al. 2012). In other words, the parasites detected before and after the summer months may be at different developmental stages (actinospores and myxospores, respectively).

The major shortcoming of the present study is that we could not distinguish if the detected DNA came from actinospores released from invertebrate hosts or from myxospores originating from fish. This complication makes it difficult to evaluate the precise time window for infection. To obtain such information, we need to perform further experiments, such as exposing uninfected fish to water collected during various seasons. Additionally, the study should be conducted over multiple years to see if we consistently detect such a seasonal pattern. Other possible reasons for the low parasite abundance in the summer include a higher abundance of plankton, which may ingest the spores as well as possibly affect the DNA extraction process and analysis of samples. It is also possible that fragile actinospores may have reduced survival in sea water at higher summer water temperatures. El-Matbouli et al. (1999) showed that the longevity of *Myxobolus cerebralis* actinospores was significantly reduced at 20°C compared to 15°C. Determining the underlying mechanisms for this phenomenon will help to inform the development of new control measures for this parasite.

Detection of *Kudoa yasunagai* from environmental water by qPCR has many applications. It could be used when choosing the location of net cages and water inlets to use in hatcheries to reduce infection rates. Additionally, qPCR is a highly sensitive and accurate diagnostic method for various myxozoan infections. For instance, qPCR is a more sensitive

method to detect *Parvicapsula minibicornis* infection in the kidneys of Chinook salmon compared to histological examination (True et al. 2009). Similarly, Kelley et al. (2006) showed that qPCR was a highly reliable method of detecting and quantifying *Myxobolus cerebralis* from rainbow trout, among 5 different methods compared. Moreover, qPCR is used as a sensitive and reliable method for the detection of *K. septempunctata* from fish, as well as from fecal samples of food poisoning patients (Harada et al. 2012, Iijima et al. 2012). Thus, the qPCR used in the present study could also be used to check for the presence of *K. yasunagai* in various fish tissues, which could help to determine the onset of infection, the route of invasion and the development of the parasite within the fish body, as well as to identify the invertebrate host.

The amounts of DNA detected from environmental sea water or unfiltered and filtered culture water were comparable. This result confirms the past incidence of high infection levels (>80%) of juvenile kingfish reared in the filtered water in the hatchery and also indicates that the high-speed fiber filtration system used in the hatchery is not capable of physically removing *Kudoa yasunagai* spores. Cobcroft & Battaglene (2013) also reported that mechanical filtration at 25 µm followed by foam fractionation does not prevent *K. neurophila* infection in the striped trumpeter *Latris lineata*. It is possible that the infective stage of *K. yasunagai* is extremely small (<2 µm) and pass through the filtration system of the hatchery. However, it was suggested that a sand filtration system (10 µm) may prevent the infection of *K. septempunctata* in a flounder hatchery (H. Yokoyama pers. comm.). The high-speed fiber filtration system uses floating fiber filter media which capture suspended solids in the water. Such filtration mechanisms may not be feasible to capture actinospores in comparison to conventional filtration systems. Nevertheless, an appropriate filtration system capable of removing actinospores is necessary to prevent *Kudoa* from entering culture water. Thus, treatments that kill or inactivate the fish-infective stage, such as UV irradiation or ozonation of cultured water, are more practical methods to prevent infection (Cobcroft & Battaglene 2013). However, the long-term use of such treatments requires large operational costs and may have side effects on the fish (e.g. skin damage caused by ozonated water). Our next step will be to determine the precise onset and time window of *K. yasunagai* invasion of fish hosts. With such information, we could optimize treatment and production schedules to minimize the impact of infection on culture facilities.

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