

Infection dynamics of *Kudoa yasunagai* (Myxozoa: Multivalvulida) infecting brain of cultured yellowtail *Seriola quinqueradiata* in Japan

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ABSTRACT: We monitored infection by a brain-infecting myxozoan *Kudoa yasunagai* in hatchery-reared juvenile yellowtail *Seriola quinqueradiata* at a culturing site in Japan. Infection was detected by PCR and microscopic observation once every 1 to 4 wk during 2010 and 2011. In both years, we detected first infection in mid-July by PCR. Prevalence increased rapidly after the onset of infection, peaking at 100% within 4 wk. Parasites required less than 10 d to reach the brain after invasion. Development of plasmodia and formation of cysts took 4 to 8 wk. Infection did not reach a plateau and number of cysts tended to decline over time, suggesting possible recovery from the infection. A drastic decline in infection prevalence was observed during the season of highest water temperature (>30°C) in 2010. To understand this phenomenon, we conducted a laboratory experiment to compare infection prevalence and cyst formation in fish kept at 25°C and 30°C. However, we could not detect obvious differences between the treatment groups during the 4 wk of the experiment. There was no apparent pathology associated with the infection. These results suggest that pathological effects of *K. yasunagai* may differ between fish species or that other factors are important in the development of infectious signs.

KEY WORDS: Myxozoan · Seasonality · Cyst development · Water temperature

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INTRODUCTION

Kudoa yasunagai (Hsieh et Chen, 1984), originally described as *Septemcapsula yasunagai*, is a multivalvulid myxozoan infecting brains of various marine fish. Since the original finding from Japanese seabass *Lateolabrax japonicus* and striped knifejaw *Oplegnathus fasciatus* (Yasunaga et al. 1981), the parasite has been reported from several important aquaculture fishes in Japan, including red sea bream *Pagrus major*, olive flounder *Paralichthys olivaceus*, tiger puffer *Takifugu rubripes*, yellowtail *Seriola quinqueradiata* and Pacific bluefin tuna *Thunnus orientalis* (Egusa 1986a,b, Whipps et al. 2004, Zhang et al. 2010). It has also been reported from several wild fish in other parts of the world, such as coral catfish *Plotosus lineatus* in the Philippines (Cheung & Nigrelli 1990), whittings *Sillago* spp., and monocle bream *Scolopsis monogramma* from Australia

(Burger & Adlard 2010). Such low host specificity and wide geographical distribution make this parasite a potential threat to marine aquaculture and wild fish resources.

Typical clinical signs of *Kudoa yasunagai* infection in the fish host are abnormal behavior and skeletal deformation. Spinal curvature and/or abnormal swimming in several commercially important fish, including Japanese sea bass, yellowtail, and chub mackerel *Scomber japonicus*, are thought to be due to *K. yasunagai* infection (Yasunaga et al. 1981, Egusa 1986a, Yamamoto 2007). In 2011, we experienced large mortalities of juvenile kingfish *Seriola lalandi* in our culturing facilities in Wakayama, Japan. The hatchery-reared fish started to show signs of infection at about 2 mo after being transferred to net cages. A considerable proportion (exact number not counted, but over 50%) of fish developed S-shaped spinal curvature (scoliosis) and abnormal

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swimming such as whirling or lying on the bottom of the cages. The symptomatic fish had difficulty feeding and suffered from damaged skin due to swimming into the cage net, causing them to die eventually. The infection prevalence of *K. yasunagai* among those fish was over 80%, and we suspected this parasite to be the cause of the problem.

To date, there is no effective control method for *Kudoa yasunagai*, or any other *Kudoa* sp., in aquaculture. One possible way to reduce the parasite damage in a fish farm is to manage the production schedule so fish are not in the open-water cages during the season when infectious parasites are most abundant. However, information about the seasonality of *K. yasunagai* is very scarce, and its infection dynamics are not understood. We conducted a long-term monitoring study and a laboratory experiment aiming to determine (1) the onset and progression of *K. yasunagai* infection in a fish farm and (2) the effect of temperature on parasite development. The purpose of the present study is to obtain basic information about infection dynamics of *K. yasunagai* in order to develop a management strategy to minimize its impact on net cage aquaculture.

MATERIALS AND METHODS

Infection monitoring

We investigated the temporal change of *Kudoa yasunagai* infection at the culturing site in 2010 and 2011 using *K. yasunagai*-free juvenile yellowtail *Seriola quinqueradiata* that were raised from eggs in a land-based hatchery. For the experiment, approximately 700 and 400 juvenile fish in 2010 and 2011, respectively (body weight 27.2 ± 5.2 g for 2010, 2.5 ± 0.4 g for 2011) were transferred to a net cage ($3 \times 3 \times 3$ m) in the ocean and were monitored for parasite infection by both microscopy and PCR. We checked 20 individuals prior to the experiment using PCR described below and confirmed that there was no *Kudoa* infection. For 2010 juveniles, infection was monitored for 15 mo between May 16, 2010 and August 15, 2011. Ten fish were sampled every week for the first 5 mo, every second week for the following 5 mo and then at 1 mo intervals for the last 5 mo. For 2011 juveniles, we monitored infection for 1 mo from July 2, to August 3, 2011 by checking 10 individuals every week.

At each sampling, we collected fish from the net cage using a dip net and kept them on ice until dissection. We measured the body size of individual

fish, and their brains were removed. The surface of brain and cranial cavity was checked thoroughly under a dissecting scope for the presence of parasite cysts (plasmodium). The total number of cysts was recorded and the parasite was identified by examining spore morphology in a squashed preparation of randomly selected cysts. In cases where no cysts were visible, PCR diagnosis was performed. DNA was extracted from a portion (approx. 20 mg) of the brain homogenate using the phenol-chloroform method. PCR was performed with primer sets designed to target 18S rDNA of *Kudoa* spp. (610 bp); yassuf (5'-AAC CTA GTG TTT CGG CTT CG-3') and yassur (5'-CTT TTC CCC ACA ACG TCA AG-3'). The PCR reaction mixture contained 0.3 μ M of each primer, 10 μ l of Takara EmeraldAmp PCR Master-MIX (2 \times), and 1 μ l template DNA, and was topped up to 20 μ l with H₂O. Cycling protocol comprised an initial denaturation at 95°C for 3 min, followed by 35 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 30 s followed by a final extension of 72°C for 10 min. PCR products were separated on a 2% agarose gel. To assure the identity of the amplified product with the sequence of *K. yasunagai*, PCR products were sequenced (Meng et al. 2011) from 3 randomly selected isolates obtained in different seasons. Obtained sequences were compared with the data in GenBank.

Throughout the experiment, fish were fed with commercial dry pellets every other day to satiation and checked for mortality. Fresh water-bath treatments were performed on several occasions to control skin fluke *Benedenia seriolae* and *Neobenedenia girellae* infections. Surface water temperature was recorded daily. Any abnormalities in sampled fish, such as spinal curvature or malformation, were recorded with photographs.

Temperature experiment

During the monitoring experiment, we noticed an unexpected sharp decline of infection prevalence during the period of highest water temperature (ca. 30°C). To understand this phenomenon we conducted a laboratory experiment to test whether high water temperature could reduce *Kudoa yasunagai* infection. The laboratory experiment was conducted in August 2011 when the infection prevalence of 2011 juveniles reached 95%. All infections in those fish were detected by the PCR and no cysts were observed at the time. One hundred and forty fish were removed from the net cage and divided into

two 500 l tanks, which were set at 30°C (average $30.4 \pm 0.73^\circ\text{C}$) or 25°C ($24.8 \pm 0.67^\circ\text{C}$) with electric heating and cooling systems. The surface water temperature at the culturing site at the time of transfer was 28.6°C. Fish were kept at the 2 temperature conditions for 4 wk. Twenty fish were sampled from each group at 1, 2 and 4 wk for checking *K. yasunagai* infection using the methods described above. The experimental tanks were provided with flow through water (4.8 l min^{-1}) and the temperature was recorded hourly. Fish were fed every other day with dry pellets ad libitum. Fresh water-bath treatments were performed at the beginning and 2 wk after the experiment was started.

Statistical analyses

At each sampling, encysting prevalence and infection prevalence were calculated. Encysting prevalence was determined as the proportion of fish with apparent cysts on the brain surface. Infection prevalence was determined as the proportion of individuals with cysts plus those identified positive by the PCR. Average number of cysts was calculated from the fish possessing cysts (excluding the fish without cysts). Difference in prevalence between each sampling time point was tested using a Pearson chi-square test. Spearman's correlation test was used to understand the temporal change in the number of cysts. Numbers of cysts between the 2 experimental groups were compared using a Wilcoxon rank-sum test. All analyses were performed using JMP8 (SAS Institute).

RESULTS

Infection monitoring

The spores we observed during the study possessed 6 to 7 polar capsules, and their morphology resembled the description of *Kudoa yasunagai* (Hsieh & Chen 1984, Burger & Adlard 2010). The DNA sequences of randomly sampled cysts were all identical with a *K. yasunagai* isolate in Genbank (Accession No. AY302741).

In 2010, we detected the first *Kudoa yasunagai* infection by PCR on July 19, which was 9 wk after the fish had been transferred into the net cage. The

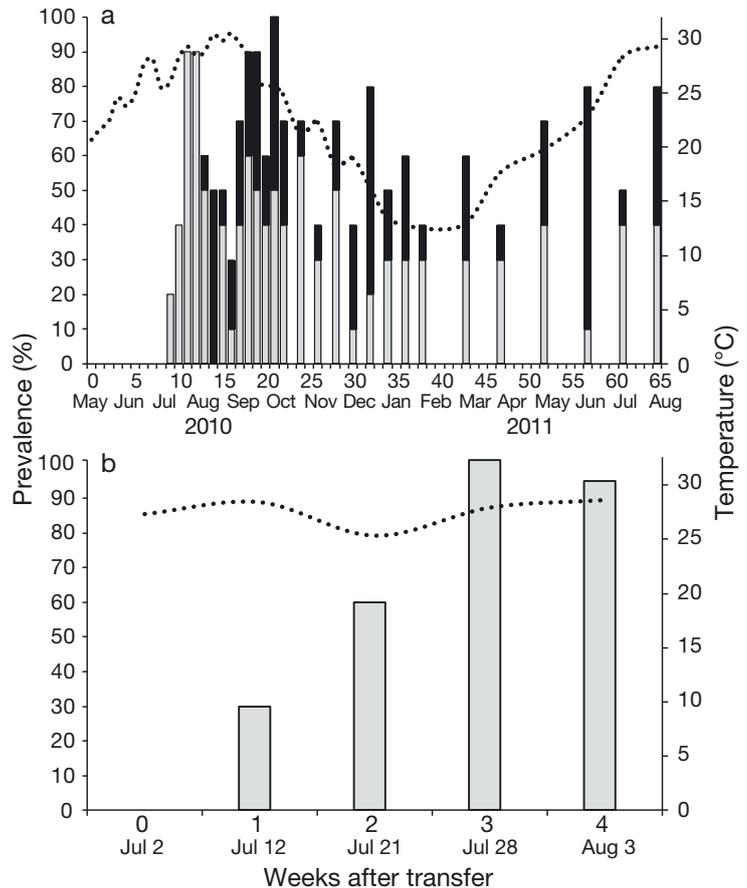


Fig. 1. *Kudoa yasunagai* infecting *Seriola quinqueradiata*. Seasonal change of infection prevalence in juvenile yellowtail after transfer from hatchery to net cages in open water as detected by PCR (grey bar) and visual determination (black bar) of the presence of cysts on the brain surface, during (a) 2010, when the date of transfer was May 16, and (b) 2011, when the date of transfer was July 2. Dotted line: surface water temperature

infection prevalence drastically increased thereafter from 20 to 90% in the following 3 wk (Fig. 1). After reaching the first peak in early August, the prevalence declined to as low as 30% in the beginning of September. This sudden decline of the infection appeared to correspond to the high water temperature, which reached a peak of over 30°C at the time (Fig. 1). After the decline, the infection reached a second peak of 100% prevalence in mid-October and stayed within the range of 40 to 80% thereafter. In 2011 juveniles, the onset of infection occurred at around the same time as in 2010, even though the 2011 fish were transferred to the net cages 1.5 mo later compared to 2010. Also, the progression of infection in 2011 was similar to 2010. We first detected infection on July 12, 2011, a week after the fish had been placed in the net cage (Fig. 1). Infection then increased rapidly, reaching 100% within 3 wk.

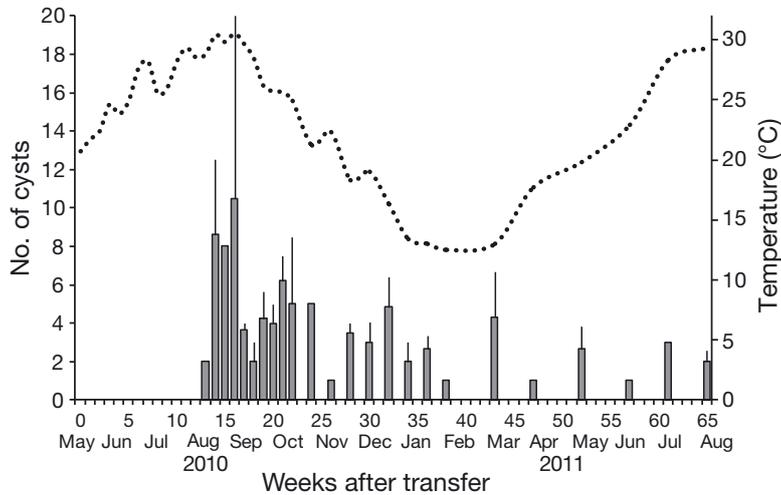


Fig. 2. *Kudoa yasunagai* infecting *Seriola quinqueradiata*. Seasonal changes in the average (+SE) number of cysts. Dotted line: surface water temperature

These fish were subsequently used for the laboratory experiment.

We observed the first cysts in 2010 fish on August 16, 4 wk after our initial detection by the PCR diagnosis (Fig. 1). The encysting prevalence was variable between the samples and did not show an obvious increase as we expected. It remained between 10 and 60% throughout the experiment except for one sampling in August where cysts were detected in all infected fish (Fig. 1). Fig. 2 shows the change in the average number of cysts during the monitoring period. After the first detection, cyst number rapidly increased for several weeks, and then gradually declined during the rest of the experimental period. Overall, there was a significant negative correlation between the number of cysts and the monitoring weeks (Fig. 2; Spearman's $\rho = -0.44$, $p = 0.0002$). No cysts were observed during our 5 wk monitoring of 2011 fish.

During the 2 yr monitoring, a total of only 6 mortalities were noted. All deaths were of 2010 juveniles and occurred mostly during the summer when skin fluke infections were severe. We noticed occasional abnormal swimming behaviors such as flashing and sudden erratic movement, but they were most likely associated with skin fluke infections. We did not see any individuals showing whirling or lying on the cage bottom as seen for the infected kingfish. Out of nearly 500 sampled fish, 6 showed apparent spinal deformities. Their heads were bent upward showing a V-shaped spinal curvature (lordosis) and not an S-shaped curvature (scoliosis) which was commonly observed for the infected kingfish. Infection preva-

lence of *Kudoa yasunagai* among these deformed fish was 33% (2 out of 6 fish). We did not notice any obvious inflammation or pathological signs on the brain surface.

Temperature experiment

Temporal change of the infection prevalence for the 2 temperature treatments is shown in Fig. 3. The prevalence increased and reached 100% by 4 and 2 wk in the 25°C and 30°C treatments, respectively. For 30°C groups, infection prevalence then appeared to decline at the end of the experiment. However, the difference in prevalence was not significant between sampling weeks within either group. Infection prevalence between the treatments did not differ at 1 wk, but the difference became significant at 2 and 4 wk ($\chi^2_1 = 21.20$, $p < 0.0001$, $\chi^2_1 = 22.01$, $p < 0.0001$, respectively). The encysting prevalence increased over time and reached over 60% in both groups at the end of the experiment (Fig. 3). There was no statistical difference in the encysting prevalence between the temperatures at any week.

We did not detect cysts at the beginning of the experiment, but as many as 20 cysts were found in some fish only after a week (Fig. 4). Cysts numbers then increased rapidly, reaching an average of about 40 (maximum 120 cysts on a brain) at 4 wk. There was no statistical difference in cyst numbers between the groups at any sampling point. No fish died during the experiment, and we did not notice any obvious abnormality or deformity.

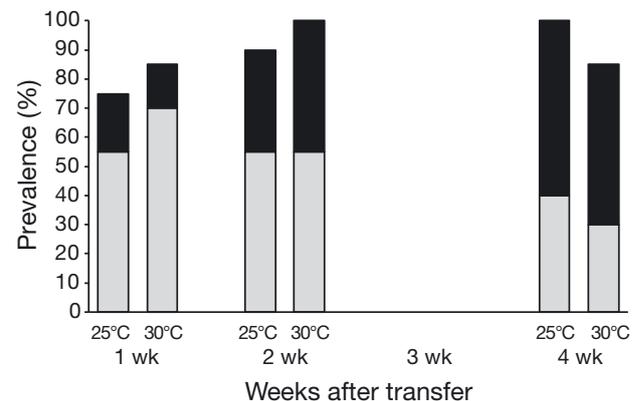


Fig. 3. *Kudoa yasunagai* infecting *Seriola quinqueradiata*. Infection prevalence detected by PCR (grey bars) and visual detection (black bars) of the presence of cysts for fish kept at 25°C and 30°C over the first 4 wk after transfer

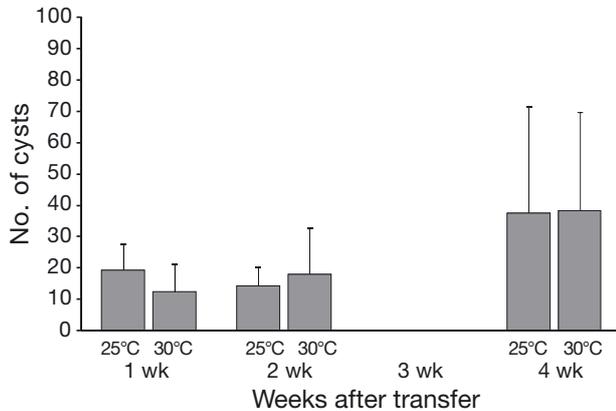


Fig. 4. *Kudoa yasunagai* infecting *Seriola quinqueradiata*. Average (+SE) number of cysts found in fish kept at 25°C and 30°C over the first 4 wk after transfer

DISCUSSION

Infection of *Kudoa yasunagai* in the monitored farming site started around mid July. Since we did not detect any signs of infection during land-based rearing, we can assume that the fish acquired the parasite after they had been transferred to the net cages. The first infection was detected at a similar time in both 2010 (July 12) and 2011 (July 19) despite the time difference in transferring fish to the net cages (May or July). This is consistent with a previous report that *K. yasunagai* infection in cultured tiger puffer in Nagasaki prefecture, Japan, starts in mid-July (H. Yokoyama pers. comm.). In the present study, we observed drastic increases of initial infection prevalence in both 2010 and 2011, 0 to 90% and 0 to 100% within 3 wk, respectively. Certain environmental and biological cues that are characteristics of this time of the year likely trigger the release of *K. yasunagai* actinospores from an unidentified invertebrate, resulting in a high abundance of the infective parasite stages in the water column. Such sharp increase of prevalence is commonly observed in myxozoans showing seasonal infection. Moran et al. (1999b) reported that infection prevalence of *K. thyr-sites* in Atlantic salmon *Salmo salar* increased from 0 to 80% within 3 wk at the end of June. Similarly, infection of *Tetracapsuloides bryosalmonae*, *Chloromyxum schurovi*, and *Myxobolus* sp. in brown trout *S. trutta* fry increased from 0 to >80% within a 1 mo period (Holzer et al. 2006). Many myxozoan infections occur in the spring and early summer when naïve young fish hosts are abundant (Markiw 1991, 1992, Kent et al. 1994, Moran et al. 1999c, Holzer & Schachner 2001). Actinospores of *M. cerebralis* are released at temperatures of 15 to 16°C, when suscep-

tible rainbow trout fry are abundant (Markiw 1991, 1992, Kent et al. 2001). *K. yasunagai* has low host specificity and possibly utilizes multiple fish species as a host. Spring through early summer is the breeding seasons for many marine fishes, and the parasite's strategy may be to release the infective stage during this period when high densities of juvenile hosts are available.

We suspected that a high density of infective parasite in mid-July caused the sudden increase of infection level. However, we checked infection only in the brain, and how long before infection the fish acquired the parasite is not clear. Myxozoans invade a fish body via skin, fins, buccal cavity and/or gills (Yokoyama et al. 2012). The infection route of *Kudoa yasunagai* is still unknown, but migration to the brain may require some time. Dzulinsky et al. (1994) reported that developing plasmodia of *Myxobolus neurophilus*, a brain-infecting myxozoan of yellow perch *Perca flavescens*, became microscopically detectable several weeks after the invasion. In 2011, we detected *K. yasunagai* DNA from the brain 10 d after fish were placed in the net cage. This indicates that *K. yasunagai* may take less than 10 d to reach the brain after invasion into the fish.

In both 2010 and 2011, *Kudoa yasunagai* cysts (plasmodia) appeared on the brain surface 4 wk after the parasite was detected by the PCR. During the subsequent 4 wk, the average number of cysts showed a 400% increase. In the laboratory experiment, no cysts were found in fish at the beginning (95% positive by PCR), but the encysting prevalence reached over 50% within the following 4 wk. These results suggest that the development of cysts in the brain takes at least 4 wk and over 8 wk in some cases. This duration is comparable with other myxozoans. The duration of plasmodium development and spore formation may range from less than 3 wk (at 17.8°C) in *Ceratomyxa shasta* (Yamamoto & Sanders 1979) to nearly 6 mo (average temperature 14°C) in *K. thyr-sites* (Moran et al. 1999a,b,c). Combining the results of our monitoring and laboratory experiments, the infection dynamics of *K. yasunagai* in juvenile *Seriola quinqueradiata* is considered as follows. Actinospores are released from invertebrate hosts and start to invade fish in late June or early July. The parasites reach the brain within a few weeks by mid-July, gradually multiply, and develop into the spores in a few months. By mid-August, they start to form plasmodia and the cysts become visible on the brain surface. It remains unclear whether the infection occurs primarily in mid-July or continues for the rest of the year.

Our study also raised a question about the fate of the parasite. Throughout the experiment, we consistently found a considerable proportion of infected fish without cysts. This seems unusual. Since the majority of experimental fish had become infected in July and August, the proportion of fish with cysts should keep increasing as the parasites develop. However, the encysting prevalence never reached 100%, except for one sampling in August 2010. Moreover, after reaching the initial peak of 90 to 100%, the infection prevalence (PCR and cysts) became variable between the samplings and never reached a plateau. There were only a few mortalities and no new fish were added to the cage. Therefore, the possible explanations for such phenomena include (1) artifacts of experimental procedures, such as non-random sampling caused overestimation of prevalence and/or inaccuracy in parasite detection, (2) the parasite never developed into cysts in some fish, or (3) the parasite developed into cysts but disappeared after a while.

The probability of the first scenario is quite low because we randomly sampled the fish and a single person performed the PCR (with both negative and positive controls) and microscopic observation in which probability of overlooking relatively large *Kudoa yasunagai* cysts (>1 mm in some cases) is low. The second explanation is possible. Although we used hatchery-reared fish with low genetic diversity (S. Shirakashi unpubl. data), individual variation may lead to differences in the development rate of the cysts. However, even if cyst formation was delayed in some fish, the infection should accumulate in the population and the proportion of infected fish without cysts should eventually reach a high plateau. We suggest the third explanation is the most likely. After the formation of the cysts, or even before cyst formation in some cases, some parasites might have disappeared from the brain, possibly by the host immune response. This hypothesis is also supported by the declining tendency of cyst intensity, which otherwise should increase over time. Degeneration or rupture of cysts has been observed in several myxozoan–fish systems. *Myxobolus artus* cysts in the skeletal muscle of common carp *Cyprinus carpio* degenerate and spores are transferred to gill lamellae causing anemia (Ogawa et al. 1992, Yokoyama et al. 1996). Similarly, degeneration of *Henneguya lateolabracis* plasmodia in the bulbus arteriosus of Chinese sea bass *Lateolabrax* sp. causes gill damage by the massive influx of spores. Degeneration of plasmodia has been observed in *Kudoa ciliatae* (Hallett et al. 1997) and *K. thyrsites* (Moran et al. 1999b) as well. However, whether degeneration and elimination of parasite

cysts occurs in the brain of yellowtail is not clear. We did not observe obvious residual or degenerating cysts. Histological studies are currently being undertaken, and hopefully this will reveal the underlying mechanisms behind the observed phenomena. We hypothesized that the sudden sharp decline of prevalence observed in August 2010 is due to elimination of the parasite under high water temperature conditions. If this is the case, it opens up the possibilities of developing a control measure. However, our hypothesis was not supported by the laboratory experiment. There was no difference in the infection prevalence, encysting prevalence and cyst intensity between the fish kept at 25 and 30°C, suggesting similar parasite development at both temperatures. This indicates that the water temperatures in our tested range affect neither development nor survival of *K. yasunagai*. Although the overall prevalence in the 30°C group appeared to decline at the end of the experiment, this difference was not statistically significant. Long-term experiments may provide different results. However, in the monitoring experiment, infection prevalence showed a 60% decline within 4 wk, and such a drastic change was not observed in the laboratory. Therefore, the decline of prevalence observed in the monitoring experiment is likely to have been caused by factors other than temperature, or a combination of various factors. The upper threshold for the *K. yasunagai* could possibly be 30°C, with infection diminishing at higher temperatures. Fish immunity may also be important because low temperatures have an adverse effect on immune response (Le Morvan et al. 1998). High density and stressful laboratory conditions might suppress the fish immune response which otherwise would reduce infection as in the net cage condition. The reduced infection in high temperature season could simply be an artifact of sampling, and we need to continue monitoring to see if a similar phenomenon consistently occurs.

Unexpectedly we did not see obvious pathologies in our experimental fish despite high *Kudoa yasunagai* infection. Mortality was minimal; only 6 fish died in 15 mo (<1%). We observed occasional erratic swimming, but they were likely associated with the skin fluke infections. Several fish possessed abnormal spinal curvature (head bent upward), but their infection prevalence was only 33%, lower than overall average. A V-shaped spinal curvature, similar to the one observed in the present study, is known to be caused by abnormal development of the swim bladder (Kitajima et al. 1994). Considering the low infection prevalence and the type of spinal curvature, we believe that the spinal deformities seen in the ex-

perimental fish were not associated with *Kudoa yasunagai*, but probably the abnormality of hatchery-reared fish. It remains unknown why there were no apparent clinical signs in the infected yellowtail while kingfish, a member of the same genus, showed severe whirling and scoliosis. Differences in pathological effects between fish species have been documented in other brain-infecting myxozoans. For instance, *Myxobolus acanthogobii* causes severe dorso-ventral spinal curvature (kyphosis) in cultured chub mackerel, S-curvature (scoliosis) in yellowtail, and irregular deformities in Japanese bluefish *Scombrops boops*, but appears to cause no skeletal abnormalities in yellowfin goby *Acanthogobius flavimanus* and brown-lined puffer *Canthigaster rivulata* (Yokoyama et al. 2004, 2005). *K. yasunagai* may have less effect on yellowtail than kingfish. However, there have been records of spinal curvature in *K. yasunagai*-infected yellowtail (H. Yokoyama pers. comm.). The combination of various factors such as host growth rate, age of fish at infection, parasite development speed, infection site within the brain, and/or environmental factors may be important in the development of pathological signs.

Our study raised more questions than it provided answers. Our knowledge about general biology, life-cycle, infection dynamics and host-parasite interaction of *Kudoa* spp. is still very limited. We believe accumulation of basic information is important to deepen our understanding of *Kudoa* and develop control measures in aquaculture.

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