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Pathophysiology of Cultured Tiger Puffer *Takifugu rubripes* Suffering from the Myxosporean Emaciation Disease

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ABSTRACT—The present study suggests that the myxosporean emaciation disease by enteric infection of *Enteromyxum leei* disrupts intestinal water uptake of tiger puffer *Takifugu rubripes*. This idea is based on a significant negative correlation between plasma chloride concentration and condition factor in diseased fish, and significantly higher osmolarity of plasma and major ion concentrations of the intestinal fluid in infected fish than in healthy control fish. Additionally, surgical ligation of the junction between the stomach and the intestine of healthy fish resulted in a 24% drop of body weight within 50 h, and significantly lower water content of the white muscle (operated fish 74 ± 0.5%; sham-operated fish 82 ± 1%). However, *in vitro* water uptake by isolated intestine sacs was not significantly different between the control and infected fish. Meanwhile, hepatic function appeared to be impaired as evidenced by the significantly lower hepato-somatic index (control fish 8.7 ± 0.8%; infected fish 2.7 ± 0.8%). Plasma activities of LDH, AST and ALT were all significantly lower in the infected fish. We propose that rapid loss of body weight of infected tiger puffer is mainly due to osmoregulatory failure but probably malnutrition is also involved in the pathogenesis.

Key words: myxosporean emaciation disease, *Enteromyxum leei*, *Takifugu rubripes,* pathophysiology, osmoregulation, liver function, tiger puffer

Since mid-1990's, tiger puffer culture in Japan has been plagued by the myxosporean emaciation disease, the causative agents of which have been postulated to be two species of enteric histozoic myxosporeans, *Enteromyxum leei* (formerly *Myxidium* sp. TP) and *Leptotheca fugu*, the latter often hyperparasitized by microsporeans. The other myxosporean, *E. fugu*, also found in the intestine of the diseased fish appears not to be pathogenic (Ogawa and Yokoyama, 2001; Tin Tun *et al*., 2002; Yanagida *et al*., 2004). Clinical symptoms of the diseased fish include a often rapid, heavy loss of body weight, as evidenced by depression of the tissue over the eye socket, loss of musculature over the skull (resulting in protrusion of the skull ridges) and the lower temporal (cheek) region, and tapering of the trunk. Effective treatments or drugs are yet to be developed, and this is likely a cause for the recent reductions of tiger puffer production in Japan.

It has been hypothesized that the enteric myxosporeans impair absorption of nutrients and/or osmoregulation through damaging the intestinal epithelia, which may lead to the characteristic clinical symptoms of this disease as described above (Ogawa and Yokoyama, 2001; Tin Tun *et al*., 2002). Branson *et al*. (1999) pointed out an apparent similarity between morphological disease signs of the farmed turbot *Scophthalmus maximus* infected by a myxosporean (later identified as *E. scophthalmi* by Palenzuela *et al*. (2002)) and those of cachexic and/or dehydrated fish, i.e., anorexia, sunken eyes and a prominent bony ridge on the skull, implying chronic malnutrition and/or osmoregulatory failure as main physiological disorders caused by the disease. On the other hand, Le Breton and Marques (1995) speculated that hepatic dysfunction is the major physiological disorder for two marine cultured

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fish *Puntazzo puntazzo* and *Pagrus major* infected by *E. leei* on the basis of pathological lesion of the liver. Sameshima (2001) reported that tiger puffers suffering from the disease could be emaciated within a few days, which makes it unlikely that nutritional disturbance is the sole cause of the pathogenesis of the disease. However, these assumptions on the pathogenesis of the myxosporean emaciation disease are based on histopathological investigations, and no pathophysiological examination has been conducted yet. Therefore, we aimed at examining influences of the myxosporean emaciation disease on osmoregulation of tiger puffers by analysis of body fluid ion concentrations, determinations of water absorption through isolated intestine, and evaluating effects of surgical occlusion of the intestine on body weight and muscle water content. We also determined basic parameters of hepatic function.

Materials and Methods

Determination of plasma chloride concentration of infected farm fish

Five tiger puffers (0 or 1 year old) were randomly sampled from each of three aquaculture farms that were designated as infected farms by the Kumamoto Prefectural Fisheries Research Center (KPFRC), and used as infected individuals. For comparison, apparently healthy fish (control, N = 4) were selected from tiger puffer stocks reared for 7–19 mo in KPFRC. The control fish were selected on the basis of the following criteria: (1) All fish in stock tanks showed no visual symptom of diseases, and fed and swam normally, (2) No enteric myxozoans were detected in fish randomly sampled from stock tanks, (3) No fish in stock tanks had any record of bacterial diseases, and (4) No fish showed any sign of anemia from natural infection by the monogenean *Heterobothrium okamotoi*. Body weight, body length and condition factor calculated as (body weight/body length^3) $\times$ 1000 were 339.1–891.0 g, 21.3–28.4 cm and 35.1–40.1, respectively, for the control fish (N = 4), and 108.7–844.3 g, 14.7–28.5 cm and 23.4–36.5 for the infected farm fish (N = 14).

The fish from the farms were sacrificed by a sharp blow on the head immediately following capture from fish cages. The control fish were killed in the same manner. The hepatic vein was carefully exposed through a midline incision of the peritoneal cavity, and the blood was withdrawn into a plastic syringe for measurements of plasma chloride concentration.

Laboratory experiments of ion analyses of plasma, intestinal fluid, urine and bile, in vitro *intestinal water uptake measurement, and hepatic functions*

Fish: Control fish (0 year old) were selected from tiger puffer stocks reared for 4 mo according to the criteria described above. Infected fish (0 year old) were obtained from the same farms as above, and reared for 1 mo in KPFRC, and those showing clear symptoms of the emaciation disease were used for the experiment.

Sample collection: Fish were sacrificed by immersing them for 5–10 min in anoxic water that had been vigorously bubbled with N_2 gas to reduce dissolved oxygen concentration. The fish were completely flaccid upon removal from the water. This method gave blood parameters all within the ranges reported for cultured, healthy tiger puffer (Nakauchi *et al*., 1994), and thus was considered acceptable for the present purposes. The intestine was exposed, and the intestinal fluid was collected from the portion immediately anterior to the colon, and analyzed for osmolarity and inorganic ion concentrations. The intestine posterior to the opening of the bile duct was then isolated, and prepared for *in vitro* measurement of water uptake rate as described below. Following the isolation of the intestine, blood was sampled from the hepatic vein, and urine and bile were collected from the urinary and the gall bladder, respectively. Then, viscera, gills and inner operculum were visually inspected, and the number of *H. okamotoi* on the inner operculum was counted. Finally, stamp slides of the remaining intestine were prepared to count enteric myxozoans. Inspection of *H. okamotoi* and myxozoans was done for 4 fish of each group. Liver weight was measured to obtain hepato-somatic index [HSI = (liver weight/body weight) $\times$ 100].

Analysis: Blood was analyzed for hematocrit, osmolarity and plasma inorganic ions, enzyme activities (lactate dehydrogenase LDH, aspartate aminotransferase AST and alanine transaminase ALT), plasma albumin and plasma total protein concentrations. Hematocrit was determined with a microcentrifuge (11,000 rpm, 5 min). The enzyme activities, albumin and total protein concentrations were determined on 1 mL of plasma with an automated dry chemistry analyzer (Kyoto Daiichi Kagaku, SP-4410). The remaining blood, intestinal fluid, urine and bile were centrifuged (11,000 rpm, 5 min), and the obtained supernatants were analyzed for osmolarity with a vapor pressure osmometer (Wescor 5520). The remaining samples were stored at –80°C for later analyses of inorganic ions. Sodium and potassium concentrations were analyzed with a flame photometer (Ciba Corning Diagnostics, Model 480). Magnesium and calcium concentrations were determined with an atomic absorption photometer. Chloride concentration was measured with a chloride analyzer (Jokoh CA 200).

In vitro determination of intestinal water uptake: Isolated intestine was rinsed with the Ringer's solution for pufferfish as prescribed by Katzman *et al*. (1969). Water uptake rate of the intestine was determined following the method of Ando (1975). After rinsing, the intestine was cut into the anterior (length 8.1 $\pm$ 2.1 cm (control, mean $\pm$ SD, N = 7), 6.8 $\pm$ 1.1 (infected, N = 7); surface area 16.7 $\pm$ 3.1 cm^2 (control, N = 7), 12.8 $\pm$ 2.1

(infected, N = 7)) and posterior portions (length 10.5 ± 3.0 (control), 7.9 ± 1.5 (infected); surface area 22.9 ± 6.3 (control), 14.9 ± 3.7 (infected)), and intestinal sac of each was filled with the Ringer's solution. Net water flux was measured gravimetrically and described as positive in case of absorption from mucosa to serosa. Flux measurement lasted for 2 h. Values were expressed as mL per unit intestinal surface area assuming seawater density of 1.02.

Definition of the degree of emaciation: Fish suffering from the emaciation disease were characterized by the following symptoms; (1) depression of the tissue over the eye socket, (2) protrusion of the skull ridges, and (3) depression of the lower temporal (cheek) region. Fish were qualitatively classified into four categories on the basis of visual inspection of the above symptoms. Degree of emaciation (DE) ±: Fish without any symptoms or slightly showing one of them, DE 1+: Fish clearly showing one of the three symptoms, DE 2+: Fish clearly showing two of the three symptoms, DE 3+: Fish clearly showing all three symptoms.

Microscopic observations of enteric myxozoans: Stamp slides of the intestinal epithelium was stained with Diff-Quik, and density of parasites was qualitatively evaluated for each of the five species of enteric myxosporeans (three species) and hyperparasitic microsporeans (two species) under a microscope.

Effect of pylorus ligation on body weight and muscle water content

Surgical ligation of the intestine: Eighteen healthy tiger puffers reared for 14 mo in KPFRC were used for this experiment. The junction between the stomach and the intestine (pylorus) was occluded surgically for six fish (the operated group). After anesthesia for 2 min in a 400 ppm 2-phenoxyethanol solution, the fish were placed supinely on a surgical table, and the gills were irrigated with a well-aerated 200 ppm 2-phenoxyethanol solution. The anterior intestine was exposed by a ca. 4 cm incision in the midline of the abdomen, and the anteriormost end of the intestine was ligated. The incision was then closed by several interrupted stitches. At the end of the surgery, fish were photographed for individual discrimination on the basis of color pattern, and weighed. The whole procedure took 10–15 min. Sham-operated fish were treated in the same way but the intestinal ligature was cut after once ligating the intestine. Control fish were anesthetized for 2 min, and transferred to a tank (see below) without surgery. All fish recovered from the surgery within 1 min and began swimming when placed in water. Body weight, body length and condition factors were 18.7–22.9 cm, 253.0–318.8 g and 21.3–54.4, respectively, for the control fish (N = 6), 19.5–22.4 cm, 249.3–305.5 g and 23.0–36.8 for the sham-operated fish (N = 6), and 18.3–22.6 cm, 206.9–345.4 g and 23.2–39.6 for the operated fish (N = 6).

Protocol: Six fish from each experimental group (control, sham-operated, and operated) were kept up to 66 h after the surgery in a 5-ton capacity tank without feeding. When found, moribund fish were immediately removed from the tank for the measurement of body weight and tissue sampling. All surviving fish were sampled at the end of the 66-h experimental period. After taking photographs, white muscle samples (ca. 2 g) were excised from each side of the dorso-lateral trunk behind the pectoral fins for the determinations of water content by freeze drying (lowest temperature –40°C, pressure 0.3 torr, duration 24 h, Iwaki Asahi Techno Glass, FRD-51). The abdominal cavity was opened to confirm occlusion of the intestine by the ligature, and to sample the liver to calculate HSI (see above). Enteric myxozoans were observed by stamp slides as described above. Water temperature ranged from 25.0 to 25.6°C during the experiment.

Statistics

Data were analyzed by F-test to compare variances of samples, and then by t-test when variances were not significantly different or otherwise by Mann-Whitney U test. Linear regression analysis was conducted to examine the relationship between condition factor and plasma chloride concentration. Body weight changes in the pylorus ligation experiment were compared by Kruskal-Wallis test. Significant levels were set at $p < 0.05$ and 0.01.

Results

Plasma chloride concentration of infected farm fish

A significant negative correlation ($p = 0.002$) was found between plasma chloride concentration and condition factor (Fig. 1). Fish in one farm (black circles) were considered to be in the process of recovery on the basis of a rapid decline of newly emaciating individuals (see Discussion). Plasma chloride concentration was much lower in these supposedly recovering fish than in the individuals of comparable condition factor from the other farms. Data of these supposedly recovering individuals were not included in the computation of the regression line.

Laboratory experiments of ion analyses of plasma, intestinal fluid, urine and bile, in vitro *intestinal water uptake measurement, and hepatic functions*

Gross disease signs of infected fish

Body shape of the infected fish markedly differed from the healthy, control fish; the head and body of the infected fish were heavily thinned, resulting in protrusion of the skull ridges, depression of the tissues over the eye sockets and the temporals (see Ogawa and Yokoyama, 2001). Both body weight and condition factor of the infected fish were much lower than in the control fish

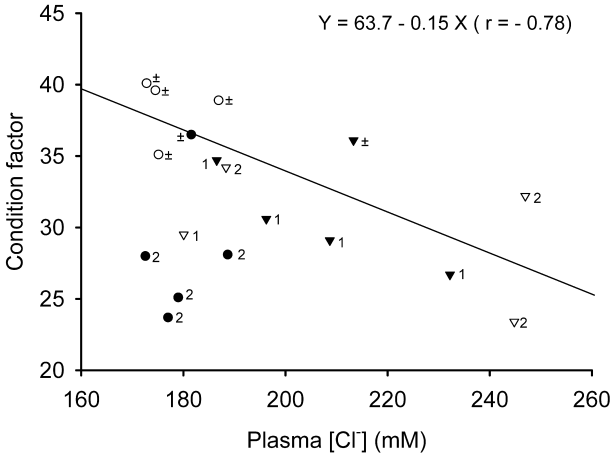

Fig. 1. Relationship between plasma chloride concentration and condition factor of cultured tiger puffer *Takifugu rubripes* sampled from two farms where the myxosporean emaciation disease was proliferating (black and white triangles), and one farm where the disease was subsiding (black circles). Data from laboratory-reared apparently healthy fish were also plotted for comparison (white circles). The regression line was calculated for the pooled data from the two infected farms and laboratory-reared fish, not including the data of the recovering fish (black circles). The ± signs and the numbers beside each symbol represent degree of emaciation as defined in Materials and Methods.

Table 1. Ranges of body weight, body length and condition factor of control and infected tiger puffer used for laboratory experiment

	Control fish (N = 7)	Infected fish (N = 7)
Body weight (g)	162.0–253.0 (203)	58.7–79.2 (67.8)
Body length (cm)	15.4–18.6 (16.8)	13.6–16.4 (14.5)
Condition factor[a]	35.9–51.3 (43.1)	17.2–27.1 (22.2)

[a] Calculated as (body weight/body length^3) × 1000. The values in parenthesis represent averages.

(Table 1). Body length of the infected fish was smaller than in the control fish, though the difference was small (Table 1). The spleen of the control fish slightly hypertrophied, but infection by *H. okamatoi* was either undetected (2 fish) or low (2 in one fish and 9 in the other) among 4 fish examined. No fish showed any sign of anemia. The seven infected fish represented different degrees of emaciation; two was classified as DE 3+, three DE 2+, and the remaining two individuals each were DE 1+ and DE ±. The number of *H. okamotoi* was slightly higher, ranging from 0 to 17 among 4 infected fish examined in this respect.

Myxosporean infection

No enteric myxozoans were found for the 4 control fish examined. In contrast, all infected fish (N = 4) had more than 6 individuals of *E. leei* in the intestine in a 200 × microscopic field. Similarly, a large number of *E. fugu* were found in the intestine of the infected fish, although the latter species is not pathogenic (Ogawa and Yokoyama, 2001; Tin Tun *et al*., 2002; Yanagida *et al*., 2004). No *Leptotheca fugu* was found in any fish.

Osmolarity and ion concentrations of plasma, intestinal fluid, urine and bile

Hematocrit was not significantly different between the groups (Table 2). Plasma osmolarity and chloride concentration were significantly higher in the infected fish than in the control fish, whereas the reverse was true for plasma calcium (Table 2). Chloride, potassium and calcium concentrations of the intestinal fluid were higher in the infected fish than in the control, but osmolarity was not significantly different between them (Table 3). Magnesium was lower than in the control. Urine osmolarity was significantly higher (infected 341.6 ± 23.0 mOsm/kgH_2O, N =7; control 316.0 ± 5.0, N = 6, p < 0.05), but calcium was lower in the infected fish (infected 8.1 ± 4.7 mmol/L, N = 7; control 16.4 ± 4.4, N = 6, p < 0.01). Magnesium concentration of the bile of the infected fish (12.8 ± 5.3 mmol/L, N = 7) was four times higher than in the control (3.3 ± 0.4, N = 7, p < 0.05), while sodium concentration was significantly lower in the infected fish (infected 240.0 ± 41.0, N = 7; control 311.7 ± 5.5, N = 7, p < 0.01). Plasma total protein concentration was significantly lower in the infected fish (2.5 ± 0.2 g/100 mL, N = 4) than in the control fish (4.0 ± 0.1, N = 4,

Table 2. Comparison of hematocrit, plasma osmolarity and ion concentrations of control and infected tiger puffer

	Control fish	Infected fish
Hematocrit (%)	19.0 ± 2.0 (7)	14.7 ± 6.7 (7)
Plasma osmolarity (mOsm/kgH_2O)	330.9 ± 6.8 (7)	355.4 ± 25.2 (7)*
Plasma [Cl^-] (mmol/L)	153.6 ± 3.5 (7)	171.1 ± 10.5 (7)**
Plasma [Na^+] (mmol/L)	183.3 ± 4.5 (6)	193.1 ± 11.8 (7)
Plasma [K^+] (mmol/L)	3.6 ± 1.6 (6)	4.6 ± 1.6 (7)
Plasma [Ca^{2+}] (mmol/L)	3.4 ± 0.3 (7)	2.8 ± 0.6 (7)*
Plasma [Mg^{2+}] (mmol/L)	0.67 ± 0.56 (7)	1.55 ± 1.27 (7)

*, ** Significantly different from the corresponding control values (* p < 0.05, ** p < 0.01). The numbers in parenthesis indicate number of samples. Mean ± SD.

Table 3. Comparison of intestinal fluid osmolarity and ion concentrations of control and infected tiger puffer

	Control fish	Infected fish
Osmolarity (mOsm/kgH_2O)	330.8 ± 8.9 (3)	326.5 ± 21.4 (7)
[Cl^-] (mmol/L)	106.9 ± 7.9 (3)	150.8 ± 18.2 (7)**
[Na^+] (mmol/L)	41.0 ± 10.4 (3)	76.7 ± 29.0 (7)
[K^+] (mmol/L)	2.2 ± 0.1 (3)	4.8 ± 1.7 (7)*
[Ca^{2+}] (mmol/L)	9.8 ± 3.2 (3)	18.4 ± 4.5 (7)*
[Mg^{2+}] (mmol/L)	20.6 ± 0.2 (3)	19.3 ± 0.2 (7)**

*, ** Significantly different from the corresponding control values (* p < 0.05, ** p < 0.01). The numbers in parenthesis indicate number of samples. Mean ± SD.

$p < 0.05$). Plasma albumin concentration was lower than detection limits (1.0 g/100 mL) in both groups.

In vitro *determination of intestinal water uptake*

There was no significant difference in the water uptake rate between the infected and control fish, as determined for the anterior (Fig. 2A) or posterior intestine (Fig. 2B). Anterior intestine of the infected fish tended to show lower rates but the differences were not significant ($p = 0.08$ for 1 h, 0.307 for 2 h). There was no apparent relationship between *in vitro* water uptake rate and the DE of the test fish.

Indices for liver function

Table 4 summarizes values of selected indices for liver function. The LDH, AST and ALT values were all lower in the infected fish than in the control fish. No apparent relationship was found for any of these values and the degree of emaciation. The liver of all the infected fish was atrophied (discoloration was obvious in some), and the bile duct was obstructed in two of the seven fish inspected. The HSI value was significantly lower in the infected fish than in the control (Table 4).

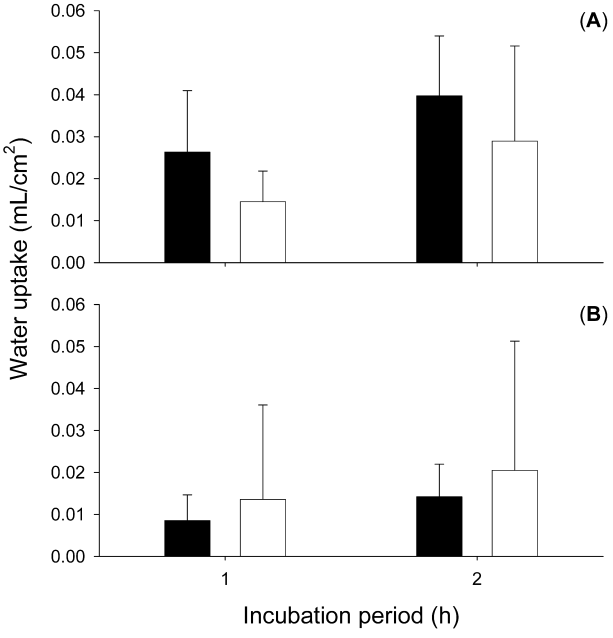

Fig. 2. *In vitro* water uptake rate across the isolated intestinal sac prepared from the anterior (A) and posterior (B) intestines of control, uninfected tiger puffer *Takifugu rubripes* (black bars) and those infected by *Enteromyxum leei* (and *E. fugu*, white bars). Data are obtained after 1 and 2 h of the incubation. Mean + SD. N = 7.

Table 4. Comparison of indices of liver function between control and infected tiger puffer

	Control fish (N = 4)	Infected fish (N = 4)
HSI (%)[a]	8.7 ± 0.8	2.7 ±10.8**
LDH (IU/L)	24.3 ± 11.2	n.d. (< 15)
AST (IU/L)	22.0 ± 14.6	3.3 ± 3.8*
ALT (IU/L)	61.8 ± 16.2	16.3 ± 8.3**

[a] Hepato–somatic index = (liver weight/body weight) × 100, n.d.: not detectable. *,** Significantly different from the corresponding control value (* $p < 0.05$, ** $p < 0.01$). Mean ± SD.

Effect of pylorus ligation on body weight and tissue water content

Four fish in the operated group died 41–43 h after the surgery. The remaining two fish were found moribund at 51–52 h. In contrast, no mortality occurred in the sham-operated or the control group. Fish in the operated group lost 23.9 ± 0.01% of body weight, which is highly significantly higher than in the sham-operated (0.3 ± 0.06%, $p < 0.01$) and the control fish (–3.2 ± 0.02%, $p < 0.01$, Kruskal-Wallis test). On the basis of experimental duration and the observed body weight loss, the rates of body weight change during the experiments were estimated to be –5.3 ± 0.73 g/kg body weight/h (operated), 0.1 ± 0.96 (sham-operated) and –0.5 ± 0.25 (control). Muscle water content was significantly lower in the operated fish (73.6 ± 0.5%) than in the sham-operated fish (81.5 ± 1.2%, $p < 0.01$, t-test; no data available for the control fish). External appearance of the moribund or freshly dead fish of the operated group closely resembled tiger puffers suffering from the disease. No enteric myxozoans were found for the fish in the operated and sham-operated groups (no data available for the control fish). HSI calculated as liver weight/initial body weight was ca. 7%, and not significantly different between the groups. All operated fish clearly showed all three symptoms of the emaciation disease (DE 3+).

Discussion

Marine teleosts continually lose water from their body fluids to the surrounding seawater because their body fluid osmolarity is maintained at about one third of seawater (1,000 mOsm/kgH_2O). At the same time, they passively gain ions due to electrochemical gradients that favor movements of ions from seawater into fish body. In order to compensate for this passive water loss, marine teleosts ingest seawater and imbibed seawater is initially desalinated by absorption of NaCl in the esophagus before water is absorbed across the intestinal wall. This intestinal water absorption is driven by active absorption of Na^+ and Cl^- across the intestinal wall, and as a result marine teleosts face with additional ionic loads, which must be counterbalanced to maintain body fluid homeostasis. Thus, marine teleosts extrude ions (predominantly Na^+ and Cl^-) through mitochondria-rich chloride cells of the gills (Marshall and Grosell, 2006). The intestine of marine teleosts is the only site where they can gain water, and, together with the gills

and kidney, plays a pivotal role for osmoregulation of the animals.

In the present study, we hypothesized that the rapid loss of body weight seen in tiger puffer suffering from the myxosporean emaciation disease was caused by disruption of intestinal water absorption. Water drinking rates of marine teleosts range from 1 to 5 mL/kg body weight/h (Marshall and Grosell, 2006). Given that fish is in steady state in terms of water balance, then the drinking rates are expected to be equal to the rates of water loss due to osmotic gradients and urination. Therefore, if intestinal water absorption is disrupted by lesion of the intestinal tissues, as reported as the main histological change of tiger puffer suffering from the myxosporean emaciation disease (Tin Tun *et al*., 2002), the fish are likely to lose body weight rapidly. In fact, the average rate of body weight loss for the pylorus-ligated fish was 5.3 g/kg body weight/h, which agrees with the expected rate of water loss from the body.

However, it seems apparent that water loss alone cannot account for the body weight loss of the infected fish. Assuming that total body water amounts to 700 mL/kg in tiger puffer as in other teleosts (Olson, 1992), that the body weight of the infected fish before infection had been similar to that of the control fish on the basis of the almost identical body length (Table 1), and that the body weight loss was due solely to water loss, then the observed difference in body weight between the two groups of fish (130 g cf. Table 1) would mean that the infected fish had lost 90% of total body water. It is unlikely that tiger puffer can survive dehydration of this extent, since even an extremely dehydration-tolerant animal can hardly survive water loss equivalent to 50% of its body weight (Bentley, 1971). Furthermore, the data on muscle water content of the pyrolus-ligated fish indicate that the observed body weight loss of these fish can not be accounted for by the tissue water loss alone. Assuming that muscle (red + white) constitutes 50% of body weight (Schultz *et al*., 1999) and that changes in water content in red and white muscles are the same, the observed decrease of water content in white muscle (8%) would reduce body weight only by 4%, which is much smaller than the actual body weight loss of 24%. The difference (ca. 20% = 24–4%) may have resulted from changes in fluid volumes in primary (= blood, 30–60 mL/kg; Olson, 1992) and secondary circulatory systems (~50 mL/kg; Steffensen and Lomholt, 1992), and interstitial and transcellular fluid compartments of other tissues and organs. It is worth pointing out that tiger puffer starved for one month was reported to lose body weight only slightly with no sign of the external characteristics of the diseased fish described above (Sameshima, 2001). Although a more rigorous investigation is needed in this respect, this observation seems to dismiss the possibility that the 24% body weight loss within 50 h was due to denied food intake. More importantly, it contradicts the supposition that body weight loss of diseased fish, which often occurs rapidly, can be ascribed to malnutrition.

Osmoregulatory failure in the pathogenesis of the myxosporean emaciation disease is also supported by the following three lines of evidence. First, the negative correlation between plasma chloride concentration and condition factor shown in Fig. 1 indicates that ionic and water balances were more adversely affected in those fish showing higher degrees of emaciation. The relatively high plasma chloride concentration of the farm fish shown in Fig. 1 (cf. Table 2) may be partly due to the sampling method employed (Ishimatsu *et al*., 1997), but overall relationship between condition factor and plasma chloride should hold true since blood sampling was conducted in exactly the same way for all the farm fish. Second, the data on plasma osmolarity, and the concentrations of major ions of plasma and intestinal fluid also agree with the hypothesis (Tables 2 and 3). Plasma osmolarity and chloride concentration of the infected fish were significantly higher than those of the control fish, although the sodium concentration was not significantly different (Table 2). However, inspection of the data from individual fish revealed that plasma sodium concentrations were higher (> 200 mmol/L) in those individuals with higher degrees of emaciation (data not shown). Third, the data on intestinal fluid analysis further supported the hypothesis. All but sodium concentrations of the intestinal fluid were significantly higher for the infected group than in the control (Table 3), suggesting disturbed intestinal ion uptake in the infected fish, which offers the driving force for water absorption through intestinal wall (Marshall and Grosell, 2006). On the contrary, the results of *in vitro* intestinal water uptake determinations failed to support the hypothesis in that there was no significant difference between water uptake rate of the infected and control groups (Fig. 2). The fact that the intestinal fluid was nearly iso-osmotic to the plasma (Tables 2 and 3) indicated that the desalination process in the esophagus was unaffected by the disease.

In addition to the data suggesting osmoregulatory failure, the liver of the infected fish showed signs of degeneration, i.e., lower HSI, discoloration, and obstruction of the bile duct. In addition, all the enzyme activities conventionally used as indices for liver inflammation (LDH, AST and ALT) were lower in the infected fish (Table 4). These might be indirect, secondary reactions to intestinal infection, because Tin Tun *et al*. (2002) confirmed no infection of the liver, spleen, kidney, heart, gonad, skin, skeletal muscle, gills and urinary bladder in tiger puffer suffering from the myxosporean emaciation disease. Nonetheless, prolonged intestinal lesion caused by myxosporean infection (Tin Tun *et al*., 2002) must complicate nutritional state of the infected fish through impaired absorption of nutrients.

Recently, Yanagida *et al*. (2006) reported that tiger puffers can be experimentally infected with both *E. leei* and *E. fugu* by feeding gut tissues obtained from donor fish for two or three consecutive days. Investigations using those experimentally infected fish should be most useful for studying the etiology and the pathophysiology of the emaciation disease; time-course analysis needs to be done to follow changes in body weight, osmoregulatory parameters and nutritional parameters to understand how and in what sequence intestinal myxozoans will affect different aspects of the physiology of infected individuals. Also important is to investigate the recovery phase. In this latter aspect, the data shown in Fig. 1 may be worth mentioning. Those individuals with lower plasma chloride and condition factor (shown as black circles in Fig. 1) were obtained from a farm in which we considered the fish were recovering from infection. This was based on the observation that the number of newly emaciating individuals was rapidly declined. Restoration of osmoregulation is likely to precede recovery of nutritional state because of generally rapid recovery of injured intestinal epithelia as shown in mammals (Blikslager *et al*., 2007) and much larger turnover rate of body water.

In conclusion, the balance of evidence suggests that osmoregulatory failure is involved in the pathogenesis of the emaciation disease due to enteric infection by *E. leei*. However, it is unlikely that osmoregulatory failure alone is responsible for the body weight loss of the infected fish. Significant liver lesion suggested malignant nutritional status of the infected fish. We propose that often observed rapid loss of body weight of infected fish (Sameshima, 2001) is mainly due to osmoregulatory failure by histological lesion of intestinal epithelium by *E. leei* infection, but malnutrition from impaired intestinal nutrient absorption also plays some role for the pathogenesis of the disease.

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References

Ando, M. (1975): Intestinal water transport and chloride pump in relation to seawater adaptation of the eel, *Anguilla japonica*. *Comp. Biochem. Physiol*., **52A**, 229–233.

Bentley, P. J. (1971): Endocrines and osmoregulation. Springer-Verlag, Berlin, 300 p.

Blikslager, A. T., A. D. Moeser, J. L. Gookin, S. L. Jones and J. Odle (2007): Restoration of barrier function in injured intestinal mucosa. *Physiol. Rev*., **87**, 545–564.

Branson, E., A. Riaza and P. Alvarez-Pellitero (1999): Myxosporean infection causing intestinal disease in farmed turbot, *Scophthalmus maximus* (L.), (Teleostei: Scophthalmidae). *J. Fish Dis*., **22**, 395–399.

Ishimatsu, A., H. Maruta, T. Oda and M. Ozaki (1997): A comparison of physiological responses in yellowtail to fatal environmental hypoxia and exposure to *Chattonella marina*. *Fish. Sci*., **63**, 557–562.

Katzman, R., G. M. Lehrer and C. W. Wilson (1969): Sodium and potassium distribution in puffer fish supramedullary nerve cell bodies. *J. Gen. Physiol*., **54**, 232–249.

Le Breton, A. and A. Marques (1995): Occurrence of a histozoic *Myxidium* infection in two marine cultured species: *Puntazzo puntazzo* C. and *Pagrus major*. *Bull. Eur. Ass. Fish Pathol*., **15**, 210–212.

Marshall, W. S. and M. Grosell (2006): Ion transport, osmoregulation, and acid-base balance. In "The physiology of fishes" (ed. by D. H. Evans and J. B. Claiborne). CRC Press, Boca Raton, pp. 177–230.

Nakauchi, R., M. Matsuura and S. S. Kubota (1994): Studies on blood biochemical analysis in the cultured puffer (*Takifugu rubripes*) – I. Establishment of standard values without changes in age and season. *J. Vet. Med*., **47**, 537–543. (In Japanese)

Ogawa, K. and H. Yokoyama (2001): Emaciation disease of cultured tiger puffer *Takifugu rubripes*. *Bull. Natl. Res. Inst. Aquacult., Suppl*., **5**, 65–70.

Olson, K. R. (1992): Blood and extracellular fluid volume regulation: role of the renin-angiotensin system, kallikrein-kinin system and atrial natriuretic peptides. In "Fish physiology Vol. XIIB, the cardiovascular system (ed. By W. S. Hoar, D. J. Randall and A. P. Farrell)". Academic Press, San Diego, pp. 135–245.

Palenzuela, O., M. J. Redondo and P. Álvarez-Pellitero (2002): Description of *Enteromyxum scophthalmi* gen. nov., sp. nov. (Myxozoa), an intestinal parasite of turbot (*Scophthalmus maximus* L.) using morphological and ribosomal RNA sequence data. *Parasitology*, **124**, 369–379.

Sameshima, M (2001): The latest information on the myxosporean emaciation disease of cultured tiger puffer. *Yoshoku*, **38**, 94–97. (In Japanese)

Schultz, I. R., M. G. Barron, M. C. Newman and A. M. Vick (1999): Blood flow distribution and tissue allometry in channel catfish. *J. Fish Biol*., **54**, 1275–1286.

Steffensen, J. F. and J. P. Lomholt (1992): The secondary vascular system. In "Fish physiology Vol. XIIA, the cardiovascular system (ed. By W. S. Hoar, D. J. Randall and A. P. Farrell)". Academic Press, San Diego, pp. 185–217.

Tin Tun, K. Ogawa and H. Wakabayashi (2002): Pathological changes induced by three myxosporeans in the intestine of cultured tiger puffer, *Takifugu rubripes* (Temminck and Schlegel). *J. Fish Dis*., **25**, 63–72.

Yanagida, T., Y. Nomura, T. Kimura, Y. Fukuda, H. Yokoyama and K. Ogawa (2004): Molecular and morphological redescriptions of enteric myxozoans, *Enteromyxum leei* (formerly *Myxidium* sp. TP) and *Enteromyxum fugu* comb. n. (syn. *Myxidium fugu*) from cultured tiger puffer. *Fish Pathol*., **39**, 137–143.

Yanagida, T., M. Sameshima, H. Nasu, H. Yokoyama and K. Ogawa (2006): Temperature effects on the development of *Enteromyxum* spp. (Myxozoa) in experimentally infected tiger puffer, *Takifugu rubripes* (Temminck and Schlegel). *J. Fish Dis*., **29**, 561–567.