

## A Preliminary Study on Sinking Disease in Koi Carp

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## Research article

# A Preliminary Study on Sinking Disease in Koi Carp

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**ABSTRACT**—“Sinking disease” (Sensui-byo in Japanese) has become a major issue in the field of koi carp breeding. Although many diseased fish did not exhibit any significant changes in their appearance, some had ulcers and erosions on the abdomen and at the base of the fins due to physical scratching caused by being at the bottom of a pond for a prolonged period. At necropsy, the swim bladder of the fish was filled with fluid (swim bladder fluid, SBF) and very little gas. Histopathologically, the fish had hyperplastic connective tissue in the tunica intima of the swim bladder, and some were accompanied by inflammation. Furthermore, one to three species of bacteria were isolated from six out of fourteen SBF samples of diseased fish. To reproduce this disease, two experiments were performed using the three major isolates (live or formalin-killed bacteria) from SBF as well as SBF itself. Immobility and the accumulation of SBF occurred in the live bacteria-injected groups. Based on these results, we concluded that the major cause of sinking disease is the accumulation of SBF due to bacterial invasion in the swim bladder and resulting loss of buoyancy.

**Key words:** koi carp, swim bladder, sinking disease, sensui-byo, *Streptococcus*, *Pseudomonas*, *Mycobacterium*

Koi carp (fancy carp; *Cyprinus carpio*) is internationally popular as an ornamental fish. Product value is influenced by a number of factors, including size, shape, color, and variety, and has recently been increasing. Many types of diseases, including Koi herpesvirus (CyHV-3), have been reported in koi carp, and idiopathic diseases are known among farmers and hobbyists (Yasumoto, 2018).

“Sinking disease” (Sensui-byo in Japanese) has become a major issue in the field of koi carp breeding. Diseased fish sink, become immobile, and stay at the bottom of a pond or tank with lethargy, but have no external signs. Their swim bladder is filled with liquid (swim bladder fluid, SBF). This disease occurs in all seasons at an annual incidence of less than 5%, and is more likely to develop in bigger fish that consume large amounts of food. Despite its low incidence, sinking disease has an economic impact because it mainly affects large and expensive koi carp that are typically shown at competitions. Due to a lack of research, limited information is currently available on the cause and prevention of this disease.

To obtain a more detailed understanding of sinking disease in koi carp, we herein examined the gross and histological features of diseased fish from farms.

Bacteria were isolated from the SBF of diseased fish. To reproduce the disease, experimental infections using isolated bacteria were also attempted.

## Materials and Methods

### Observation of diseased fish

Live diseased fish (n = 15) transported from farms were kept in a tank and observed for between 2 wk and 6 mo prior to the initiation of experiments. Fish were then euthanized with quinaldine (2-methylquinoline; final concentration, 20 ppm) and examined.

### Analysis of SBF

The color and cloudiness of SBF were visually assessed and protein concentrations in SBF were measured using the handheld refractometer MASTER-SUR/Jα (ATAGO, Japan). SBF smears were also stained with Löffler’s methylene blue.

### Isolation and detection of pathogens

The inner surface of the swim bladder, SBF (100 μL), the spleen, and kidney were subjected to bacterial isolation on nutrient agar, brain heart infusion, Cytophaga agar, and 1% Ogawa media at 25°C. The identification\* of isolates was performed by BEX Co., Ltd. (Japan). The 16S rRNA gene of isolates were sequenced, and the sequenced data (about 300

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nucleotides) were compared with the BLAST database. Higher ranked species are judged as identified species or closely related species.

The detection of CyHV-3, carp edema virus (CEV), and *Sphaerospora* spp. in the swim bladder, SBF, kidney, and gills was attempted with PCR. DNA extraction from these tissues (10 to 20 mg) and SBF (200  $\mu$ L) was performed using DNAzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Specific primers for CyHV-3 (Yuasa *et al.*, 2005) and CEV (Oyamatsu *et al.*, 1997) were used for detection. To detect sphaerospora, which cause swim bladder inflammation (SBI) in koi carp, SphF and SphR were utilized as primers for the 18S rDNA of *Sphaerospora* spp. (Holzer *et al.*, 2013; Liu *et al.*, 2016).

#### Histopathology

Pieces of the swim bladder, gill, heart, hepatopancreas, spleen, kidney, intestine, lateral muscle, brain, and ulcer sites of diseased fish were fixed in 10% formalin solution and processed for histopathological examinations. Paraffin-embedded tissue sections (4  $\mu$ m) were stained with Mayer's hematoxylin and eosin (HE), azan stain, Berlin blue, and May-Grunwald Giemsa.

#### Challenge test

To reproduce this disease, two experimental infections (Ex. 1 and Ex. 2) were performed with three major isolates (*Streptococcus* sp. NFUSS17, *Pseudomonas aeruginosa* NFUPA17, and *Mycobacterium* sp. NFUMS18) from the SBF of fish.

Bacteria were cultured in BHI agar at 25°C for 48 h, except for *Mycobacterium* sp. (five days), and suspended ( $1.0 \times 10^9$  cfu/mL) in autoclaved saline (0.85% NaCl). To prepare formalin-killed (FK) bacteria, formalin was added to the suspension at a final concentration of 0.5% (v/v) and allowed to stand at 25°C for 24 h. FK bacteria were washed by centrifugation ( $3,000 \times g$  at 4°C for 15 min, 3 times), resuspended in saline, and adjusted to the same turbidity of live bacteria.

Experimental koi carp (variety: kohaku; body weight  $87.7 \pm 15.3$  g; total length  $18.9 \pm 3.3$  cm) were offspring of the same parental fish, and domesticated at the National Fisheries University (Shimonoseki, Japan). Fish were held in tanks with a water circulation system and fed pelleted diets before the initiation of experiments.

In Ex.1, fifty four fish were divided into nine groups (G-1 to G-9; n = 6), each of which was held in a 150-L tank with an air lift filter at 24°C. A live or FK bacteria suspension (*Streptococcus* sp., *Pseudomonas aeruginosa*, and *Mycobacterium* sp.; each 100  $\mu$ L/fish) was

injected into the anterior chamber of the swim bladder using a needle (21 gauge, 1.5 inch; NIPRO, Japan), in six groups (G-1, live *Streptococcus* sp.; G-2, FK *Streptococcus* sp.; G-3, live *P. aeruginosa*; G-4, FK *P. aeruginosa*; G-5, live *Mycobacterium* sp.; G-6, FK *Mycobacterium* sp.). SBF, autoclaved distilled water (DW), or saline (0.85% NaCl) was injected in the same manner (G-7, SBF; G-8, DW; G-9, saline). SBF (bacteria were not isolated) was obtained from diseased fish from farms by needle aspiration, filtrated (0.45  $\mu$ m), and immediately used for this experiment.

In Ex. 2, ten groups consisted of one hundred twenty fish (groups: S-1 to S-3, P-1 to P-3, M-1 to M-3, C; n = 12). Live bacteria at different concentrations ( $-1$  to  $-3$ ,  $1.0 \times 10^6$  to  $1.0 \times 10^8$  cfu/100  $\mu$ L/fish) were injected into the swim bladder of fish, similar to the first experiment. As a control, saline was injected (group C).

Injected fish were held at 24°C and observed daily for 90 days (Ex. 1) and 30 days (Ex. 2). After experiments, gross and histological features were examined, and bacterial isolation from the swim bladder or SBF was performed same as diseased fish from farms. Identification of re-isolated bacteria was judged by shape and color of colony.

## Results

#### Gross and SBF features of diseased fish

Diseased fish from farms were received in all seasons (Table 1). All fish (n = 15) were motionless at the bottom of a tank but sometime swimming to eat (Fig. 1A). There were no deaths or recovery before sampling. Although nine fish had no external signs, six had ulcers and/or erosions on the ventral abdomen and base of the pelvic and butt fins (Fig. 1B). Three of these fish exhibited exophthalmos, edema of the lateral musculature, and slight abdominal enlargement accompanied by severe fullness of the swim bladder chambers (Fig. 1C).

At necropsy, the majority of fish (14/15) showed the same signs, namely, fullness of SBF in the swim bladder (Fig. 2A) with hyperplasia of the tunica intima (gas was hardly observed; Fig. 2B). Although only three fish had adhesion of the internal organs to each other, other internal organs were normal in the other fish. However, some fish with exophthalmos, edema, and slight abdominal enlargement had deformed internal organs because of compression caused by severe fullness of the swim bladder (Fig. 2C). Only one fish had a normal anterior chamber of the swim bladder and atrophied posterior chamber without SBF (Fig. 2D).

#### Analysis of SBF

SBF was colorless (8/14) or pale yellow (6/14), and some samples of pale yellow SBF (4/6) were also slightly cloudy (Table 1). Protein concentrations in SBF were lower than 2.0 g/100 mL. Slight cellular debris was

\* The Japanese Pharmacopoeia 15th edition, Rapid Identification of Microorganisms Based on Molecular Biological Method, p1741-1742 (March 31, 2006 The MHLW Ministerial Notification No.285)

observed in SBF smear preparations.

#### Isolation and detection of pathogens

Twelve strains of bacteria were isolated from the SBF of six fish (1 to 3 strain species/fish), however no bacteria obtained from the spleen and kidney (Table 1). The obtained strains were identified as *Streptococcus* sp., *S. equi* subsp. *zooepidemicus*, *P. aeruginosa*, *Mycobacterium* sp., *Aeromonas hydrophila*, *A. veroni*, *Plesiomonas shigelloides*, and *P. fluorescens* by partial sequence analysis of 16S rRNA gene. *Streptococcus* sp., *P. aeruginosa*, and *Mycobacterium* sp. were isolated several times from SBF. Although CyHV-3 and sphaerospora were not detected, CEV was detected in the gills of three fish.

#### Histopathological features of diseased fish from farms

The tunica intima of all swim bladders containing SBF showed the hyperplasia of collagen fibers and frequently inflammation (Fig. 3A); however, bacteria were not observed in these lesion sites (date not shown). There were no pathological signs in the tunica externa of the swim bladder. The atrophied posterior swim bladder in diseased fish (Fig. 2D) showed the severe hyperplasia of collagen fibers in the tunica intima without inflammation (Fig. 3B). Only fish with exophthalmos, edema, and slight abdominal enlargement had hemosiderin deposits in the spleen and cloudy hepatocytes in the liver (Fig. 3C and D). The other organs had no pathological signs. Sphaerospora were not detected in any swim bladders and kidneys.

**Table 1.** List of diseased fish from farms

Received date	Farm* <sup>1</sup>	Variety* <sup>2</sup>	Sampling date	TL* <sup>3</sup> (cm)	Sex* <sup>4</sup>	Gross features* <sup>5</sup>	SBF		Isolated bacteria* <sup>8</sup>	
							Color and turbidity	Smear* <sup>6</sup> (mg/100 mL)		
2016/10/31	N	Su	2016/11/22	43.5	F	–	colorless transparent	n	0	–
2016/12/1	H	K	2016/12/19	67.1	F	ul, er, ex, ae, ed, ad	pale yellow slightly cloudy	slight cellular debris	0	–
2016/12/21	N	K	2017/1/20	48.5	M	–	colorless transparent	n	1.0	–
2017/1/27	N	T	2017/2/8	72.0	F	ul, er, ex, ae, ed, ad	pale yellow transparent	n	0	<i>P. aeruginosa</i>
2017/4/7	H	Ss	2017/4/24	53.5	F	–	colorless transparent	n	0.2	–
2017/6/6	Y	K	2017/6/20	43.2	F	–	colorless transparent	n	0	<i>P. aeruginosa</i> <i>P. shigelloides</i> <i>A. hydrophila</i> <i>Streptococcus</i> sp. <i>S. equi</i> subsp. <i>zooepidemicus</i>
2017/7/4	N	K	2017/7/19	51.5	F	ul, er, ex, ae, ed	colorless transparent	n	0	<i>P. aeruginosa</i> * <sup>9</sup> <i>A. veroni</i> <i>P. fluorescens</i>
2017/7/4	N	Ss	2017/7/20	55.4	F	–	pale yellow slightly cloudy	slight cellular debris	2.0	–
2017/7/24	Y	K	2017/8/7	77.5	F	–	colorless transparent	n	0	–
2017/8/7	N	T	2017/8/22	63.2	F	–	pale yellow slightly cloudy	n	0.4	<i>Streptococcus</i> sp.* <sup>10</sup> <i>Mycobacterium</i> sp.
2017/11/15	Y	Kg	2018/4/19	78.0	F	ul, er	–	none	–	–
2018/3/26	T	K	2018/4/20	18.0	U	–	colorless transparent	n	0	–
2018/7/10	N	K	2018/7/26	75.0	F	–	pale yellow transparent	n	0	–
2018/8/31	O	K	2018/9/15	32.8	M	ul, er	pale yellow slightly cloudy	slight cellular debris	0.6	<i>Mycobacterium</i> sp.* <sup>11</sup>
2018/9/18	N	T	2018/10/7	49.5	F	ul, er, ad	colorless transparent	n	0	–

\*<sup>1</sup> H, Hiroshima; N, Niigata; O, Okayama; T, Tokyo; Y, Yamaguchi.

\*<sup>2</sup> K, Kohaku; Kg, Karashigoi; Su, Shiroutsuri; Ss, Showasanshoku; T, Taishosanshoku.

\*<sup>3</sup> Total length.

\*<sup>4</sup> F, female; M, male; U, unknown.

\*<sup>5</sup> ul, ulcer; er, erosion; ex, exophthalmos; ae, abdominal enlargement; ed, edema; ad, adhesion; -, no external sign.

\*<sup>6</sup> n, nothing.

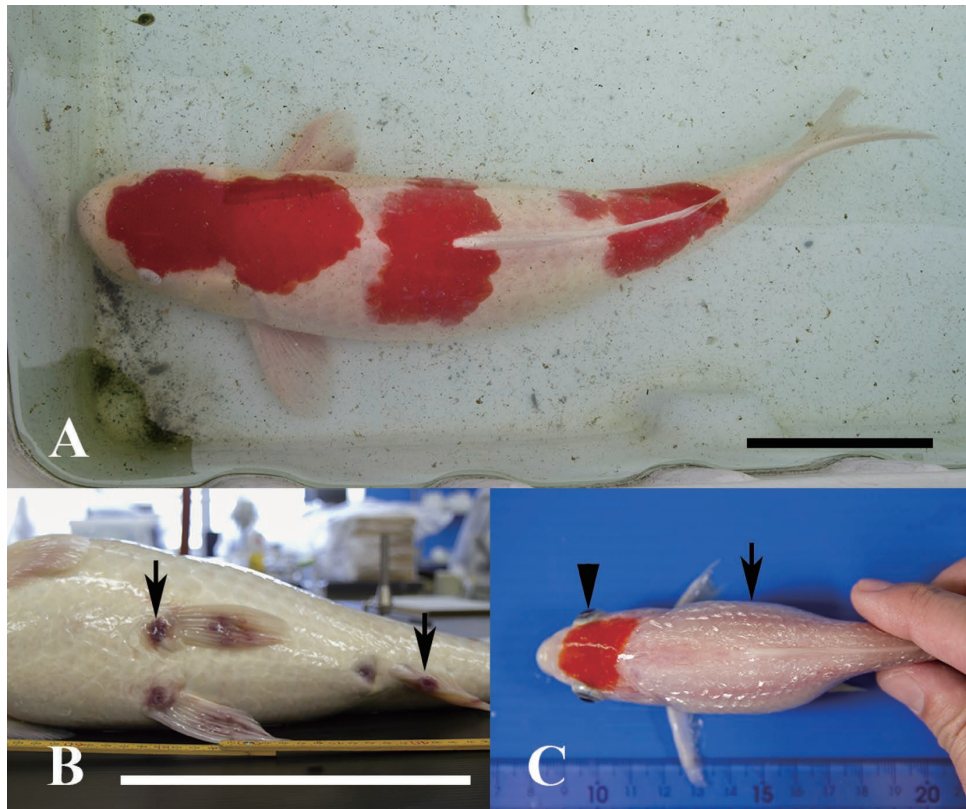
\*<sup>7</sup> Protein concentration.

\*<sup>8</sup> A, *Aeromonas*; M, *Mycobacterium*; P, *Pseudomonas*; S, *Streptococcus*; -, not isolated.

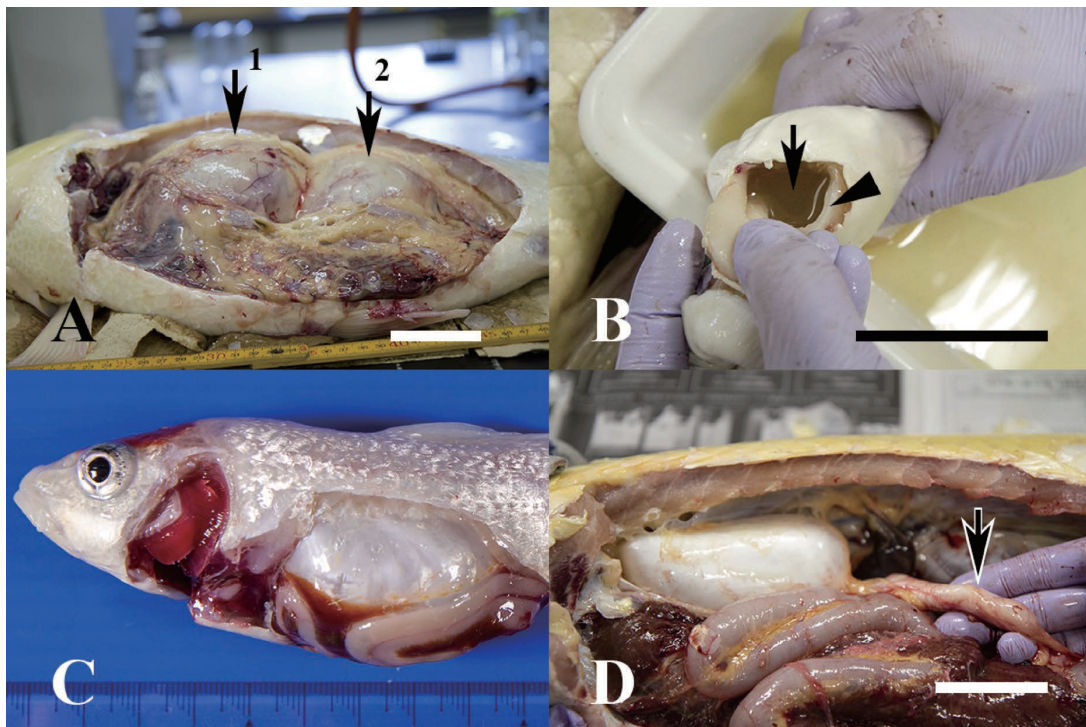
\*<sup>9</sup> NFUPA17.

\*<sup>10</sup> NFUSS17.

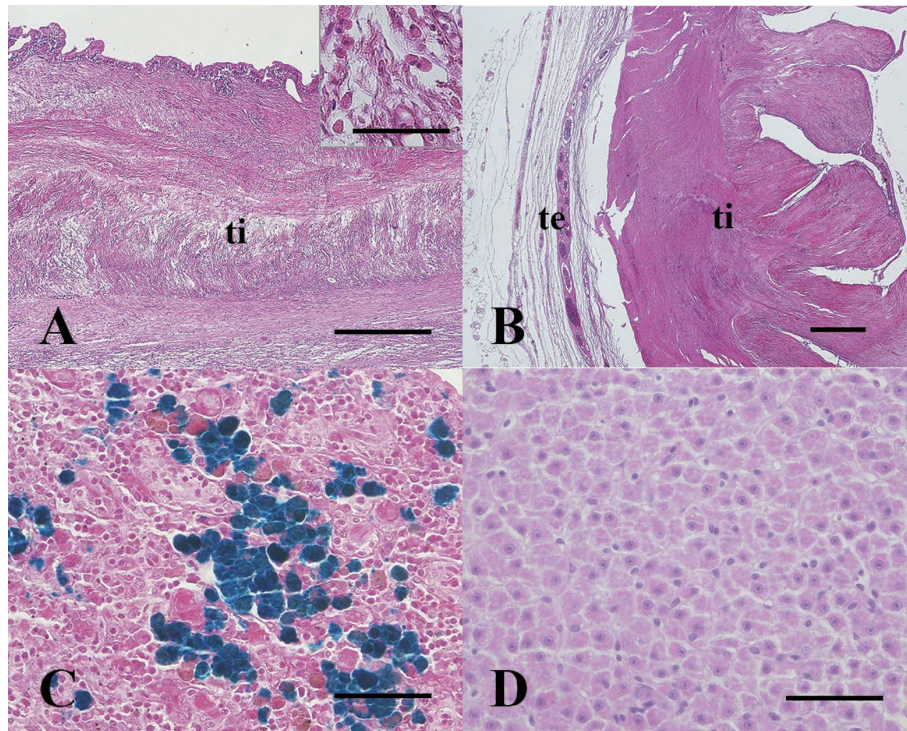
\*<sup>11</sup> NFUMS18.



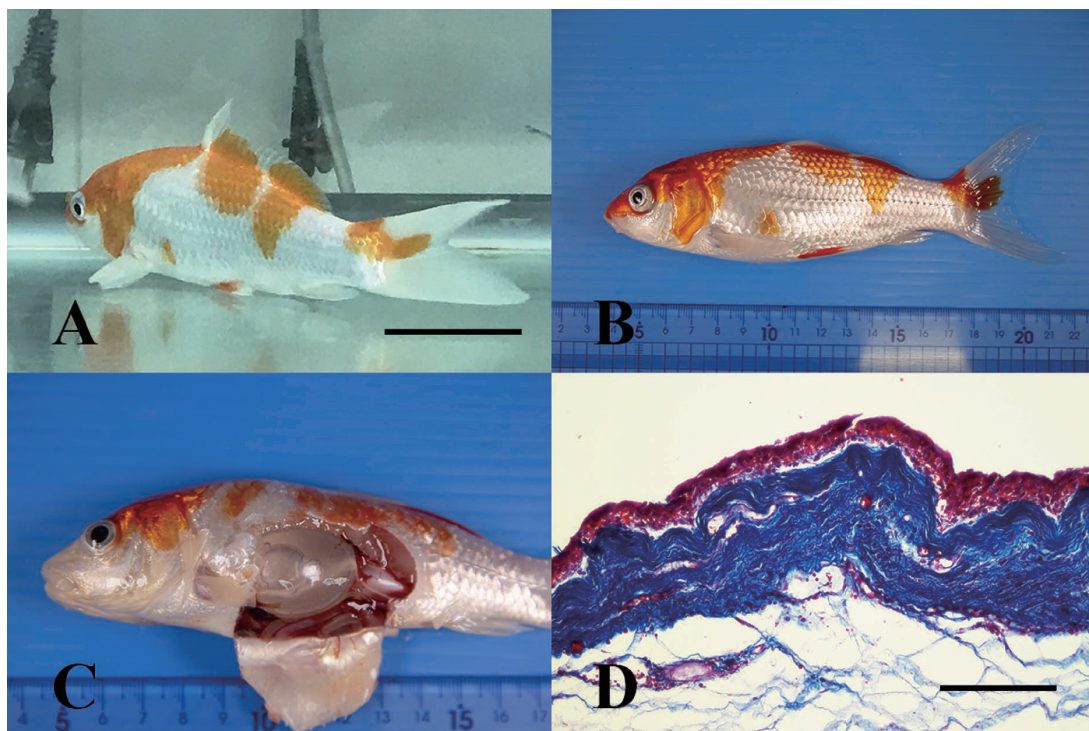
**Fig. 1.** Gross features of diseased fish from a farm. A: Fish became immobile with a spreading pectoral fin at the bottom of the tank. B: Ulcers and erosions on the ventral abdomen and base of pelvic and butt fins (arrows). C: Abdominal enlargement (arrow) due to the severe fullness of liquid in the swim bladder and exophthalmos (arrowhead). Bars: 20 cm.



**Fig. 2.** Anatomies of diseased fish from a farm. A: An enlarged swim bladder (arrows) due to SBF accumulation (1, anterior; 2, posterior). The internal organs were adhering each other. B: A swim bladder filled with SBF (arrow), the tunica intima of which shows hyperplasia (arrowhead). C: Deformation of internal organs due to a severely enlarged swim bladder. D: The atrophied posterior chamber of the swim bladder. Bars: 5 cm.



**Fig. 3.** Histopathological features of diseased fish from a farm (A, B, & D, HE; C, Berlin blue). A: Hyperplasia of collagen fibers and severe inflammation in the tunica intima (ti) of the swim bladder with SBF accumulation. Inset: Details of several types of inflammatory cells in the tunica intima. B: The atrophied posterior chamber of the swim bladder shown in Fig. 2D. Note hyperplasia of collagen fibers in the tunica intima [ti; No pathological signs in the tunica externa (te)]. C & D: Deposition of hemosiderin in the spleen (C) and cloudy hepatocytes in the liver (D) of fish with exophthalmos, edema, and slight abdominal enlargement. Bars: A, 500  $\mu\text{m}$ ; inset in A, 50  $\mu\text{m}$ ; B, 500  $\mu\text{m}$ ; C, 50  $\mu\text{m}$ ; D, 50  $\mu\text{m}$ .



**Fig. 4.** Gross and histopathological features of diseased fish from the reproduction test. A & B: Fish were immobile with a spreading pectoral fin at the bottom of a tank, but did not have ulcers or erosions on the ventral abdomen or base of the fins. C: The fullness of SBF in both chambers of the swim bladder. D: Diseased fish showed the weak hyperplasia of collagen fibers in the tunica intima (azan). Bars: A, 5 cm; D, 100  $\mu\text{m}$ .

**Table 2.** Results of reproduction tests in which fish were injected with bacterial species isolated from swim bladder fluid (SBF)

Experiment <sup>*1</sup>	Group	Injection <sup>*2, *3, *4</sup>	Number of diseased fish <sup>*5</sup>	Re-isolation of injected bacteria <sup>*6, *7</sup>
Ex. 1	G-1	<i>Streptococcus</i> sp.	3	1
	G-2	FK <i>Streptococcus</i> sp.	0	nb
	G-3	<i>P. aeruginosa</i>	2	1
	G-4	FK <i>P. aeruginosa</i>	0	0
	G-5	<i>Mycobacterium</i> sp.	1	0
	G-6	FK <i>Mycobacterium</i> sp.	0	0
	G-7	Filtrated SBF	0	nb
	G-8	Sterilized distilled water	0	nb
	G-9	Saline (sterilized 0.85% NaCl)	0	nb
Ex. 2	S-1	live <i>S.</i> sp. ( $1.0 \times 10^6$ cells/100 $\mu$ L/fish)	2	0
	S-2	live <i>S.</i> sp. ( $1.0 \times 10^7$ cells/100 $\mu$ L/fish)	2	1
	S-3	live <i>S.</i> sp. ( $1.0 \times 10^8$ cells/100 $\mu$ L/fish)	5	3
	P-1	live <i>P. aeruginosa</i> ( $1.0 \times 10^6$ cells/100 $\mu$ L/fish)	1	0
	P-2	live <i>P. aeruginosa</i> ( $1.0 \times 10^7$ cells/100 $\mu$ L/fish)	3	1
	P-3	live <i>P. aeruginosa</i> ( $1.0 \times 10^8$ cells/100 $\mu$ L/fish)	3	0
	M-1	live <i>M.</i> sp. ( $1.0 \times 10^6$ cells/100 $\mu$ L/fish)	1	0
	M-2	live <i>M.</i> sp. ( $1.0 \times 10^7$ cells/100 $\mu$ L/fish)	0	0
	M-3	live <i>M.</i> sp. ( $1.0 \times 10^8$ cells/100 $\mu$ L/fish)	2	1
	C	Saline (sterilized 0.85% NaCl)	0	0

\*1 Ex. 1, n = 6 in each group; in Ex. 2, n = 12 in each group.

\*2 FK, formalin-killed.

\*3 The concentration of injected bacteria in Ex. 1 was  $1.0 \times 10^8$  cfu/100  $\mu$ L/fish.

\*4 *Streptococcus* sp., NFUSS17; *Pseudomonas aeruginosa*, NFUPA17; *Mycobacterium* sp., NFUMS18.

\*5 Diseased fish that exhibited immobility and the accumulation of SBF.

\*6 The number shows diseased fish with injected bacteria re-isolated from SBF.

\*7 nb, not bacteria.

### Challenge tests

The results of Ex. 1 and 2 are shown in Table 2.

In G-1 (live *Streptococcus* sp. NFUSS17 injected group), five fish were in an inactive state around the bottom of the tank until five days post injection (dpi), and two subsequently recovered by 10 dpi. The other three fish gradually sunk and remained immobile at the bottom.

In G-3 (live *P. aeruginosa* NFUPA17 injected group) and G-5 (live *Mycobacterium* sp. NFUMS18 injected group), three fish each were in an inactive state, similar to G-1, until 8 dpi; however, one in G-3 and two in G-5 recovered by 15 dpi. Two and one fish became immobile in G-3 and G-5, respectively. Once fish became immobile at the bottom, they did not recover. In the other groups, diseased fish in an inactive state were not observed during the experimental period. All diseased fish obtained exhibited symptoms of sinking disease; they were immobile with a spreading pectoral fin at the bottom of the tank with no external signs (Fig. 4A and B), fullness of SBF in the swim bladder (Fig. 4C), and the weak hyperplasia of collagen fibers in the tunica intima of the swim bladder (Fig. 4D). SBF was transparent and protein concentrations were lower than 0.4 g/100 mL, and injected bacteria were re-isolated from the SBF of only two diseased fish (33.3%; G-1 and G-3). The other organs of diseased fish had no pathological signs. Asymptomatic fish were normal.

In Ex. 2, all fish in S-1 to S-3 (*Streptococcus* sp. NFUSS17 injected groups) were in an inactive state until 6 dpi, and some gradually became immobile and remained at the bottom of the tank. Two, two and five diseased fish were obtained in S-1 to S-3, respectively. In P-1 to P-3 (*P. aeruginosa* NFUPA17 injected groups) and M-1 to M-3 (*Mycobacterium* sp. NFUMS18 injected groups), approximately 50% of fish were in an inactive state until 8 dpi. One fish in P-1, three in P-2, three in P-3, one in M-1, and three in M-3 eventually exhibited the symptoms of sinking disease. The gross, histopathological, and SBF features of diseased fish were the same as those in Ex. 1 and no significant differences were observed in the experimental groups of Ex. 2. Injected bacteria were re-isolated from six diseased fish (31.6%; S-2, S-3, P-2, M-3).

### Discussion

The present study was performed in order to obtain more detailed information for the control of sinking disease.

Fifteen diseased koi carp, which were provided from farmers, were included in the present study. Although no significant changes occurred in the appearance of many fish, some presented with exophthalmos, edema, and abdominal enlargement as well as ulcers and erosions on the abdomen and at the base of the fins. The

abdomen and base of the fins of diseased fish were directly in contact with the bottom of the pond. Therefore, we suspected that ulcers and erosions were a result of physical scratching caused by being at the bottom of the pond for a prolonged period of time.

At necropsy, the swim bladder was filled with SBF and very little gas in diseased fish, which appeared to result in the loss of buoyancy and, ultimately, immobility. Histopathologically, diseased fish had hyperplastic connective tissue in the tunica intima of the swim bladder, and some were accompanied by inflammation; however, artificially diseased fish exhibited weak hyperplasia without inflammation. None of the artificially diseased fish had external signs. Therefore, we speculated that the pathological changes in diseased fish from farms resulted in the chronic accumulation of SBF. In fish that presented with exophthalmos, edema, and abdominal enlargement, the swim bladders were enlarged due to the severe accumulation of SBF, which compressed internal organs. The deposition of hemosiderin in the spleen and the cloudiness of hepatocytes were also detected. These results indicated that significant enlargement of the swim bladder caused physical pressure to be applied to the internal organs, which caused functional damage and resulted in hypoproteinemia (Yasumoto *et al.*, 2015). In one of the fish that did not have SBF, the posterior chamber of the swim bladder was atrophied without SBF accumulation, and there was a significant amount of hyperplastic collagen fibers in the bladder wall. These results indicated that this particular fish became immobile at the bottom of the pond because it had lost buoyancy as a result of atrophy of the posterior chamber of the swim bladder. However, no diseased fish in Ex. 1 and 2 had atrophied swim bladder without SBF accumulation. Thus, the cause of atrophy and relation with the sinking disease remains unclear.

SBF was colorless or pale yellow, and some pale yellow SBF were also slightly cloudy. Protein concentrations were lower than 2.0 g/100 mL, and microscopic examinations revealed that SBF only contained a slight amount of cellular debris. Based on the criteria published by Light (2007), SBF is categorized as a transudate. Between one and three species of bacteria were isolated (a total of 8 species of bacteria were isolated) from 43% (6 out of 14 diseased fish from farms that had SBF) of SBF samples. Notably, *Streptococcus* sp., *P. aeruginosa*, and *Mycobacterium* sp. were isolated from several samples. *Streptococcus* sp. and *Mycobacterium* sp. both cause disease in various freshwater fish, while *P. aeruginosa* causes infections in humans (Kodama *et al.*, 1974; Goslee and Wolinsky, 1976; Jo, 1982; Okuda, 1982; Collins *et al.*, 1984). These three major species, including five other species, exist in ponds as indigenous microorganisms. Neither the swim bladder nor SBF contained CyHV-3 or CEV, which are known to infect koi carp (Oyamatsu *et al.*, 1997; Yuasa, 2016). CEV was

isolated from the gills of three fish; however, CEV infection did not markedly affect the results obtained because most koi farms have existing surveillance programs with a live virus to prevent CEV infection. Similarly, neither the swim bladder nor SBF contained microspores that cause SBI, which results in significant inflammation of the swim bladder in koi carp (Holzer *et al.*, 2014; Gómez *et al.*, 2015; Chang *et al.*, 2016). This result was also confirmed by histopathology. Therefore, indigenous bacteria that exist in ponds enter the swim bladder, resulting in the accumulation of SBF and lesions in the swim bladder, and we attempted to perform two challenge tests.

In Ex. 1, the swim bladder of experimental fish was exposed to one of the following: live or formalin-killed bacteria, filtered and sterilized SBF, DW, and saline. Fish injected with live species of bacteria (G-1, G-3, and G-5) exhibited the symptoms of sinking disease, and histopathological changes of their swim bladder were similar to those found in diseased fish from farms. The symptoms of sinking disease were not observed in any of the other experimental groups, indicating that the disease is caused by live bacteria rather than foreign materials or viruses. In Ex. 2, varying concentrations of bacteria were injected into the swim bladder. The results obtained demonstrated that while a high concentration of bacteria was the most effective at causing the symptoms of sinking disease. The results of bacterial re-isolation in Ex. 1 and 2 showed that injected bacteria were obtained from below 33.3% diseased fish. One to three species of bacteria were also isolated from 43% of the SBF samples of diseased fish from farms. Foreign materials that are injected into the swim bladder are known to be phagocytosed and subsequently removed from the body by induced white blood cells (Endo *et al.*, 1997; Matsuyama *et al.*, 1999). Therefore, some or all of the bacterial species that were seeded in artificially diseased fish were likely removed by white blood cells. However, it currently remains unclear why the causal bacteria was not isolated from all of the diseased fish in the present study. The present results suggest that the SBF might be the major symptom contributing to the pathogenesis of this condition. It is very possible that "sinking disease" would be characterized by other symptoms, such as swim bladder atrophy, however, we conclude that the SBF could be induced by experimental inoculation of the three bacterial isolates into the anterior chamber of the swim bladder.

The disease that causes abnormalities in the swim bladder is also found in the ayu *Plecoglossus altivelis*, which is a physostomous fish similar to koi carp. In larval ayu, accidental aspiration may introduce foreign materials and pathogens into the swim bladder and cause a disease characterized by severe abdominal enlargement due to the accumulation of SBF, ultimately resulting in death (Ochiai *et al.*, 1977). This mechanism

may also apply to the present study; accidental aspiration may have introduced bacteria into the swim bladder of diseased fish.

Therefore, we concluded that the major cause of sinking disease is the accumulation of SBF due to bacterial invasion in the swim bladder and, as a result, a loss of buoyancy. By being at the bottom of a pond for a prolonged period of time, ulcers and erosion develop on the abdomen and at the base of the fins in chronically diseased fish as they scratch against the bottom of the pond. Exophthalmos, edema, and abdominal distention were also observed as the disease progressed. Future studies are needed to elucidate the mechanisms underlying the accumulation of SBF and develop measures to prevent and treat sinking disease in koi carp.

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