

**Research Article****Bile from reproductively mature male largemouth bass *Micropterus salmoides* attracts conspecific females and offers a practical application to control populations**Yasufumi Fujimoto<sup>1,\*</sup>, Hidenobu Yambe<sup>2</sup>, Kiyotaka Takahashi<sup>3</sup> and Shigeru Sato<sup>4</sup><sup>1</sup>The Miyagi Prefectural Izunuma-Uchinuma Environmental Foundation, 17-2 Shikimi, Wakayanagi, Kurihara, Miyagi 989-5504, Japan<sup>2</sup>Tokyo University of Agriculture, Faculty of Bioindustry, Department of Aquatic Bioscience, Abashiri, Hokkaido 099-2493, Japan<sup>3</sup>Society for Shinaimotsugo Conservation, 504-1 Koyachi, Kimazuka, Kashimadai, Osaki, Miyagi 989-4102, Japan<sup>4</sup>School of Marine Biosciences, Kitasato University, 1-15-1 Kitasato, Minamiku, Sagamihara, Kanagawa 252-0373, JapanAuthor e-mails: [fjimo@hotmail.com](mailto:fjimo@hotmail.com) (YF), [h3yambe@nodai.ac.jp](mailto:h3yambe@nodai.ac.jp) (HY), [kiyotaka-toto@ktf.biglobe.ne.jp](mailto:kiyotaka-toto@ktf.biglobe.ne.jp) (KT), [shigeru@kitasato-u.ac.jp](mailto:shigeru@kitasato-u.ac.jp) (SS)*\*Corresponding author*

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**OPEN ACCESS****Abstract**

The largemouth bass *Micropterus salmoides* (Lacépède, 1802) is a highly invasive freshwater species, and has a considerable impact on the diversity of indigenous aquatic organisms. We hypothesized that mature males released sex pheromones to attract mature females to their nests. To develop an effective method for controlling the population in a lake of northern Japan, we studied the efficacy of using bile collected from mature males with the bioactive function to attract mature females. A trap was devised to evaluate the attraction of sample solutions from males and to test practical uses of the pheromone. In the spawning season, when bile from reproductive males (RB) was introduced into the traps, female catches in the trap were significantly higher than when bile from non-reproductive males (NRB) or controls were used. In the non-spawning season, the female largemouth bass did not show any preference for RB. Other fish species did not show any preference for RB in both the spawning and non-spawning season. This study shows that RB contains a sex pheromone that attracts mature females, suggesting that pheromone traps could possibly be used to control largemouth bass populations.

**Key words:** black bass, chemical control, invasive species, pheromone, releaser effect

**Introduction**

Biodiversity has been compromised by various factors such as climate change, disorderly land development, exposure to chemical substances, and species interactions. One of these phenomena is the local extinction of native species caused by invasive foreign species (Schaffelke and Hewitt 2007; Colvin et al. 2005; Yamada and Sugimura 2004). In aquatic animals, the North American signal crayfish *Pacifastacus leniusculus* (Dana, 1852) preyed on macrophytes and other invertebrates, which changed the ecosystem (Dunn 2012). The American bullfrog *Rana catesbeiana* (Shaw, 1802) caused decreases in native frogs in many countries (D'Amore 2012).

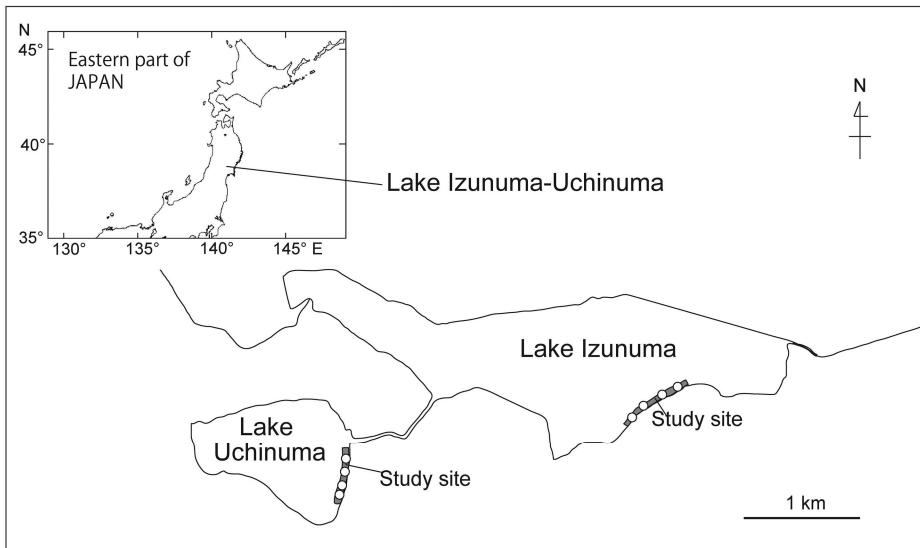
The largemouth bass *Micropterus salmoides* (Lacépède, 1802) is a piscivorous fish species native to North America. This species has been introduced into more than 50 countries (Welcomme 1992) and has caused

adverse impacts on freshwater ecosystems (Whittier and Kincaid 1999; Jackson 2002). Expansion of the largemouth bass population has caused serious damage to freshwater ecosystems. Degradation of fish fauna has been observed after largemouth bass invaded lakes or ponds (Gratwicke and Marshall 2001; Abekura et al. 2004; Kamerath et al. 2008; Ide and Seki 2010) and several fish populations including those of endangered species have become locally extinct (Washitani et al. 2010). The decrease in fish populations has also led to a decrease in the number of fish-eating water birds such as the little grebe *Tachybaptus ruficollis* (Pallas, 1764) and the little egret *Egretta garzetta* (Linnaeus, 1766) (Shimada et al. 2005).

In the late twentieth century, many projects aimed at controlling the population of the largemouth bass have been conducted around the world (Ministry of the Environment 2004; Michalski 2007). Generally, population control efforts depend on the scale of the population's habitat (Grice 2009; Parkes and Panetta 2009). In small habitats like irrigation ponds, eradication of the largemouth bass will be easily achieved by water drawdowns (Tsunoda et al. 2010), because few native species remain to be impacted as well. In large habitats such as swamps and lakes, however, multiple trapping methods that avoid bycatch of native species have been used with the aim to achieve management and control in these habitats (Britton et al. 2011).

Multiple methods that include artificial spawning nests, dip nets, set nets, gillnets, short-length gillnets, and electric fishing boats have so far been used to capture largemouth bass at various stages of their life cycle (Hosoya 2006). However, even though largemouth bass can be captured using these methods, they require considerable effort. The most effective method of controlling invasive species may therefore be the selective capture of gravid females (Ricker 1954; Beverton and Holt 1957). Nonetheless, methods to selectively capture mature female largemouth bass have not yet been established. The development of a practical method of selectively capturing gravid female largemouth bass is urgent and holds promise for controlling populations of this species.

The most effective means of controlling invasive species with due consideration to the environment and native species is a trapping technique based on species-specific ecology (Kolar et al. 2010). In the spawning season (spring), male largemouth bass build a nest in bottom gravel, and conspecific females then approach the nest to spawn with the male (Heidinger 1975). Since some habitats of largemouth bass are eutrophic and have low visibility underwater, we hypothesize that reproductively mature male largemouth bass release sex pheromones to attract gravid females to their nests. In general, the roles of sex pheromones have been categorized into a priming effect that induces physiological changes and a releaser effect that affects behavior (Wilson and Bossert 1963). Many reports have shown that sex pheromones of fishes are contained within various bodily fluids such as semen (Carolsfeld et al. 1997), urine (Yambe



**Figure 1.** Locations of the sampling sites at Lake Izunuma-Uchinuma. Shaded bars on the lakeside indicate spawning locations of largemouth bass. Open circles at the spawning sites indicate the locations of the 8 sampling sites.

et al. 1999; Olsén et al. 2002), bile (Vermeirssen and Scott 2001), or skin mucus (Hara and Macdonald 1976). Our preliminary experiments using these bodily fluids suggest that releaser pheromones of largemouth bass are contained in the bile of nesting males (Fujimoto et al. *unpublished data*).

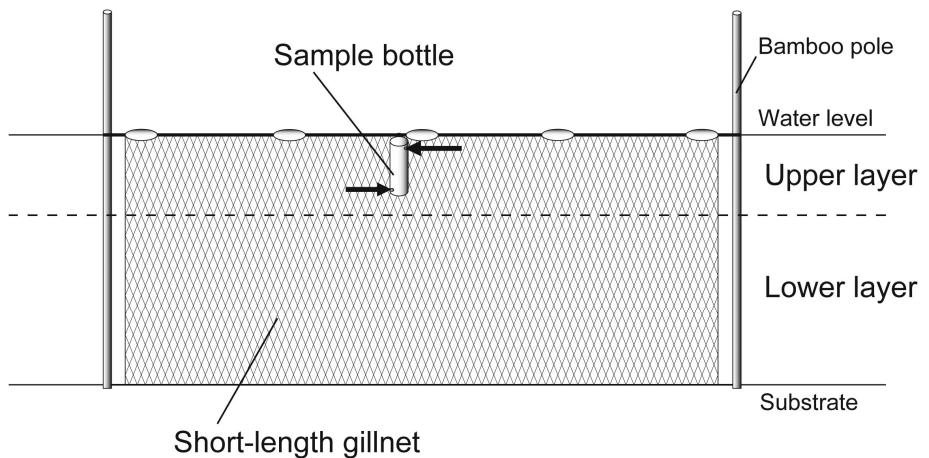
In the present study, field tests to develop the pheromone trap of largemouth bass were performed using the bile of nesting males during the spawning and non-spawning seasons of largemouth bass. Based on these results, we confirmed the seasonal change and the sex- and species-specificity of the pheromonal effect in the bile. We next discuss the practical application of the pheromone trap in controlling populations of largemouth bass.

## Materials and methods

### Study sites

Field experiments were conducted at Lake Izunuma-Uchinuma ( $38^{\circ}43'N$ ;  $141^{\circ}07'E$ ), Miyagi Prefecture, northeastern Japan, from 2008 to 2010 (Figure 1). This lake system consists of two interconnected lakes, Lake Izunuma and Lake Uchinuma. The surface areas of the lakes are 3.69 and  $1.22 \text{ km}^2$  with a mean depth of 0.76 and 0.78 m, respectively. The sea levels of the lakes are equal (6 m), and the lakes are connected by a channel of about 0.8 km in length and 30 m in width. There are no barriers in the channel; fish move freely between both lakes through the channel. The control activities of largemouth bass have therefore been conducted with the two lakes considered as a uniform habitat.

Most of the bottom substrate consists of muddy and partially sandy areas. Previous surveys revealed that the sandy bottom areas were the main spawning sites of largemouth bass (Takahashi 2005; Saito et al. 2007). We therefore assumed that mature largemouth bass aggregate at these sites to



**Figure 2.** Schematic view of the field assay system. A short-length gillnet (2 m long, 0.9 m deep) was tied to bamboo poles. A sample bottle was set at the center of the float line of the gillnet. Arrows pointing to the sample bottle indicate the locations of two small openings (4 mm diameter). The broken line indicates the borderline between the upper and lower layers of the gillnet.

spawn during the spawning season. To evaluate the pheromonal effect of bile on largemouth bass, a cluster of four sampling sites (0.6–0.9 m water depth) was set in each of two spawning locations for a total of eight sampling sites (Figure 1). The shorelines of the spawning sites were populated with the common reed *Phragmites australis* (Cav.) Trin. ex Steud. and willows *Salix* spp. The water clarity of the sites was 0.3–0.5 m. The low visibility was caused by resuspended mud and phytoplankton. The water flow rate at the surface layer of the sites was about 2 cm/s.

#### *Preparations of traps and bile*

At each sampling site, three traps during spawning season and two traps during non-spawning season were installed to evaluate the response of female largemouth bass to the bile from the males (Figure 2). The trap was configured of a short-length gillnet (2 m long, 0.9 m deep, with 105 mm or 120 mm mesh) for catching fish and a sample bottle (200 mm long, 65 mm diameter; 500 ml volume) made of transparent polypropylene for diffusing the sample solutions. Two small openings (4 mm diameter) were bored at the lateral sides of the bottle in order to diffuse the sample solution. The bottle was set at the center of the float line of the gillnet. It is possible that fish attracted to the sample solution defused from the bottle were caught in the upper layer of the gillnet. On the other hand, fish usually swim in the middle and lower layers of the water, because Osprey *Pandion haliaetus*, a piscivorous bird of prey, hunts fish in the lake and fish generally avoid the upper layer. Therefore, to evaluate the attraction of largemouth bass to the sample solutions, the range of the upper layer was determined to be one-third the height of the net, and the other two-thirds of the net was called the lower layer. The three traps during spawning season and the two traps during non-spawning season were set at 20 m intervals at each sampling site. The locations of the traps were 0.8 m in depth (about 15 m off the

shoreline), and when necessary, were shifted to accommodate fluctuations in water levels.

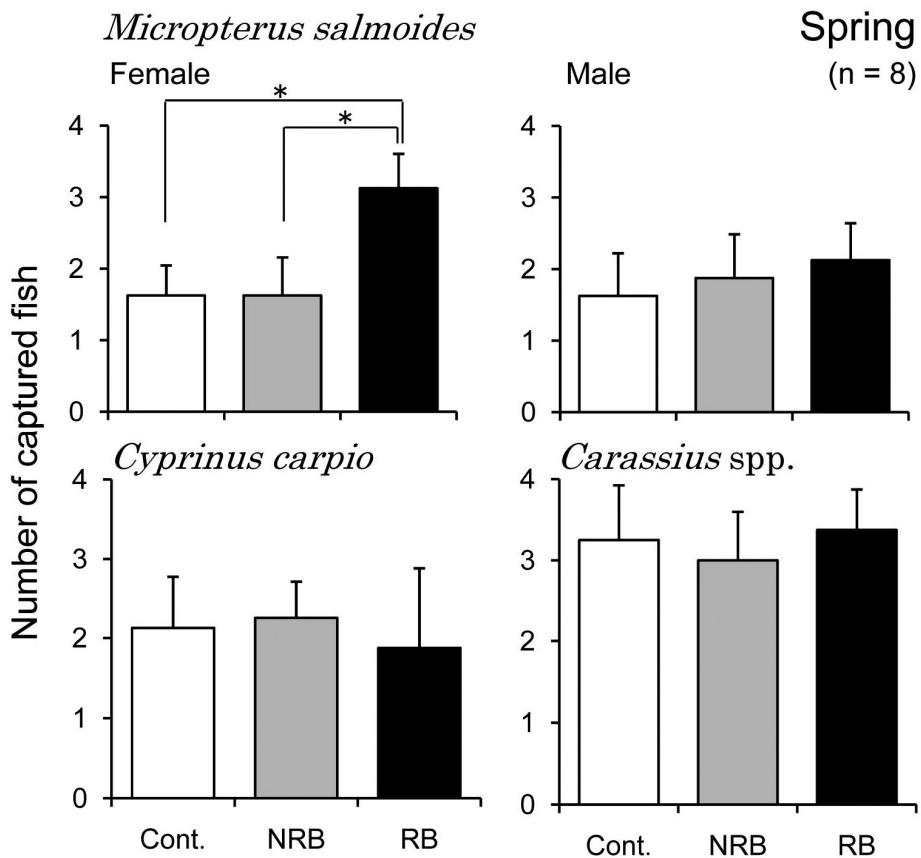
Male largemouth bass for samples were captured using gillnets during the spawning season (May to June in 2007, 2008, and 2009) and the non-spawning season (November to December in 2007 and 2008). We did not use NRB in the non-spawning season because NRB did not show any effect in the test during the spawning season (described below). Since most of the males captured during the spawning season had expressible milts, we determined that the males were reproductive. In both seasons, most of the captured largemouth bass were alive when we checked the gillnet. Captured males were sacrificed immediately to collect test samples of bile which were collected directly from the gall bladder. The mean volume of bile collected from each male largemouth bass was about 600 µL. Bile samples collected from 10 individuals were pooled, and then divided into 300 µL portions each. These sample portions were stored at -18 °C until the field tests. Prior to the tests, each sample was diluted in 500 mL of lake water.

### *Field experiments*

During the spawning season (May to June 2008), we evaluated the behavioral response of reproductively mature largemouth bass to bile collected from reproductive (RB) and non-reproductive (NRB) males. Three gillnets were set at each of the eight sampling sites. We set three bottles containing sample solutions (control [distilled water], NRB, and RB) at each gillnet at around 6 a.m., and then recorded the number and species of captured fish in each trap after 24 h. The traps were set for 22 days during the spawning season. After each test, the positions of the sample solutions in each bottle were rotated at each sampling site to avoid location bias so that all gillnets were almost equally conditioned by each test solution.

To evaluate which layer—upper or lower—attracted females, we recorded the position of the fish captured in the nets (Figure 2). In addition, the sex and the developmental stage of captured largemouth bass were determined by macroscopic observation of the gonads. According to Chiu et al. (1991), the ovaries of captured females were categorized into the vitellogenic or the periovulatory stage based on the most-developed oocytes in the individual. In the ovary of the vitellogenic stage, oocytes were opaque and less than 1 mm in diameter. The ovary in the periovulatory stage (final maturation) included transparent oocytes (about 1.2 mm in diameter) or ovulated oocytes.

During the non-spawning season (October to November 2009), we evaluated the behavioral response of largemouth bass to bile collected from reproductively mature males (RB). Testing was conducted at eight sampling sites, and a pair of control and RB traps was set at each sampling site for 16 days. The procedure of this test followed that of the test in the spawning season.



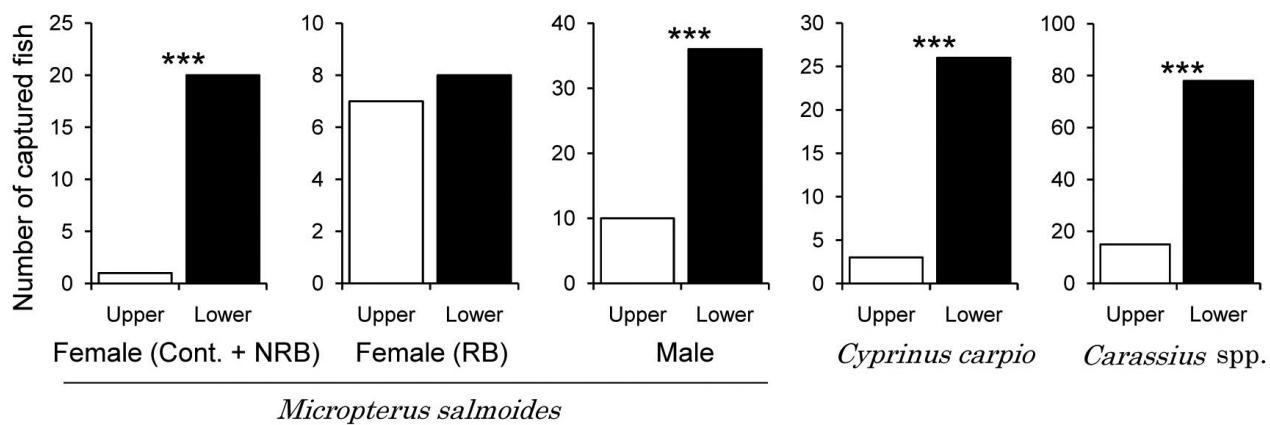
**Figure 3.** Number of fish captured by control (Cont.), non-reproductive bile (NRB) and reproductive bile (RB) in the spawning season of largemouth bass. Bars on the columns indicate standard errors of the mean. \*  $p < 0.05$ .

### Data Analysis

In the test of the spawning season, the mean number of captured fish per sampling site for 22 days was compared among the three types of traps using Friedman's test followed by Scheffe's multiple comparison test (Excel Statistics 2008). We used non-parametric tests because the number of samples was small and the data obtained were not normally distributed. The catches in the upper and lower layers of the traps were compared using the Wald chi-square test. The frequency of the two stages of ovarian development in the types of traps was also compared using the Wald chi-square test. In the test of the non-spawning season, the mean number of fish captured in the control and RB traps at each sampling site for 16 days was compared using the Wilcoxon paired rank test in the same software. All the significance levels were set at  $p = 0.05$ .

### Results

In the test of the spawning season, a total of 51 female largemouth bass were captured. The mean number of captured mature female largemouth bass per sampling site over a period of 22 days during the spawning season in spring was 1.92 times higher in the RB trap than in the control or NRB traps (Figure 3; Friedman's test:  $p = 0.019$ ; post-hoc Scheffe's multiple comparison


*Micropterus salmoides*
**Figure 4.** Total catches of fish in the upper and lower layers of gillnets in the spawning season. \* p < 0.001.

**Table 1.** Proportions of developmental stages of oocytes in mature female largemouth bass captured during the spawning season.

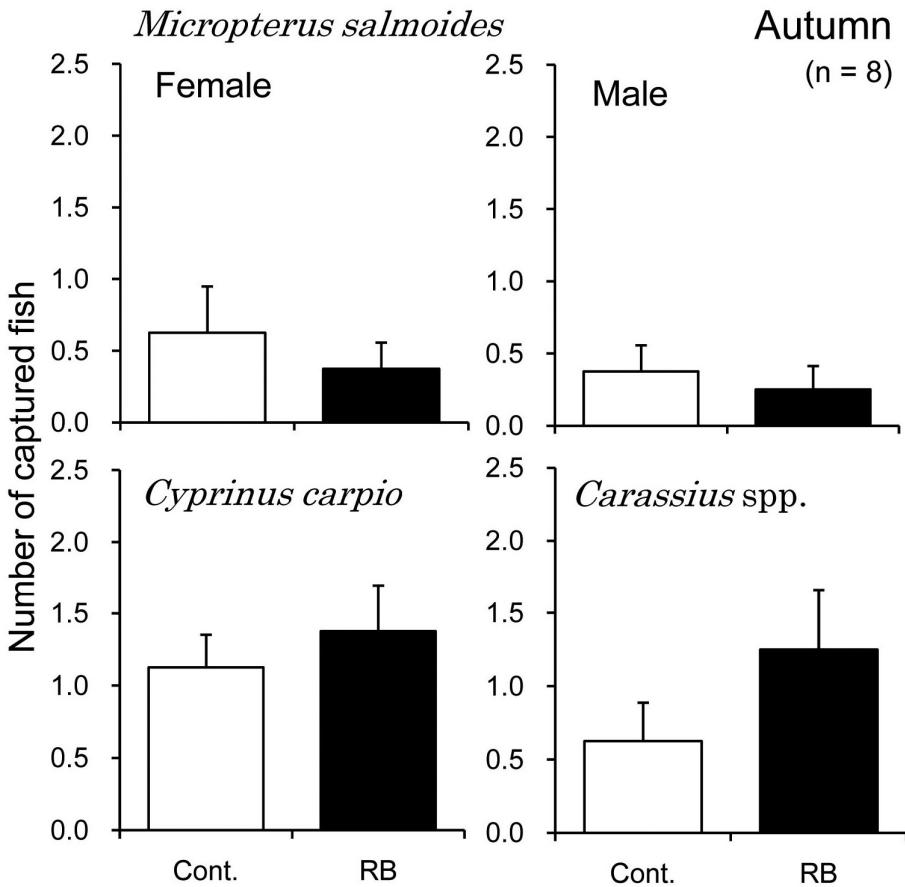
Type of trap	Vitellogenic	Periovulatory
Control	2	11
NRB	3	9
RB	6	15
Total	11 (23.9%)	35 (76.1%)

Values represent numbers of mature female largemouth bass.  
NRB = non-reproductive bile; RB = reproductive bile

test: p = 0.036 or 0.049). For mature male largemouth bass, *Cyprinus carpio* (Linnaeus, 1758), and *Carassius* spp., the mean number of captured individuals showed no difference among the control, NRB, and RB traps (Friedman's test; male largemouth bass: p = 0.661; *Cyprinus carpio*: p = 0.380; *Carassius* spp.: p = 0.417). The mean standard length of captured females was 361 mm (ranging from 273 to 423 mm).

We also analyzed catches found inside the upper and lower layers of the traps (Figure 4). In both the total catches of female largemouth bass by two traps with NRB and distilled water (DW; control) and the total catches of male largemouth bass, *Cyprinus carpio*, and *Carassius* spp. by three traps with RB, NRB, and DW, a significantly higher number of individuals were captured in the lower layer of the gillnets. In the catches of female largemouth bass by traps with RB, however, there was no difference between the upper and lower layers (Wald chi-square test, p > 0.05). These results show that female largemouth bass are attracted to the upper layer that is proximal to the point where RB was introduced.

A large majority (76.1%) of all captured females were in the periovulatory (i.e., final maturation) stage. The number of female catches in each type of trap showed no statistical difference between the vitellogenic and periovulatory stages (Table 1). In the test of the non-spawning season, conducted over a period of 16 days in autumn during the non-spawning season of largemouth bass, no statistical difference was shown between the control and RB traps in catches of female and male largemouth bass, *Cyprinus carpio*, and *Carassius* spp. (Figure 5; Wilcoxon paired rank test;



**Figure 5.** Number of fish captured by control (Cont.) and reproductive bile (RB) in the non-spawning season of largemouth bass. Bars on the columns indicate standard errors of the mean.

female largemouth bass:  $z = 0.67$ ,  $p = 0.50$ ; male largemouth bass:  $z = 1.00$ ,  $p = 0.32$ ; *Cyprinus carpio*:  $z = 0.40$ ,  $p = 0.69$ ; *Carassius spp.*:  $z = 1.15$ ,  $p = 0.25$ ).

## Discussion

This study showed that the presence of a pheromone to attract conspecific females was contained in the bile of mature males of largemouth bass captured during the spawning season. Further, the field test revealed that the sex pheromone could be practically applied in the development of a pheromone trap and contribute to controlling the biomass of largemouth bass. During the spawning season, the number of captured females in the RB trap was 1.92 times higher than those in the control and NRB traps. In this test, most of the captured females (76.1%) were shown to be in ovulation. These results indicate that the bile of reproductive males (RB) contains a sex attractant, since no significant difference was shown between the numbers of females captured in the control and NRB traps. Bile has been proposed as one of the sources of pheromones in teleost fish (Doving et al. 1980), and bile acids contained in bile have been shown to be a potent odorant for fish olfaction (Fraude et al. 2002). Over three decades, it has been demonstrated that sea lamprey *Petromyzon marinus* utilize migratory or sex pheromones, and more recent studies revealed the chemical compounds in these pheromones (Li et al. 2002; Sorensen et al. 2005).

In the reproductive season (spring), sex-specificity of the pheromone was also shown, since females indicated significant differences among test samples while males indicated no difference, as reported in other species (Yambe et al. 2006; Appelt and Sorensen 2007; Marentette and Corkum 2008). We also showed seasonal differences in female responses to RB between the spawning (spring) and non-spawning (autumn) periods. On the other hand, in the case of *Cyprinus carpio* and *Carassius* spp., no significant difference was found in the number of captured individuals among the control, NRB, and RB traps, suggesting species-specificity for the sex pheromone.

Male largemouth bass nest alone in sheltered sites surrounded by aquatic macrophytes or submerged deadwoods to protect their eggs from predators (Heidinger 1975). Many habitats of largemouth bass are located in water areas with low visibility (Jenkins 1975). It would be difficult for females to detect the nests using sensors other than chemical senses. Females thus seem to detect the nests using their olfactory organs. The present study showed that bile from reproductively mature male largemouth bass contained a releaser pheromone to attract mature females into their spawning areas. In sea lamprey, it has been reported that bile acids containing sex pheromones are released from the gill (Li et al. 2002). In general, however, fish excrete much bile with the feces (Schmidt and Weber 1973). Male largemouth bass probably also excrete feces containing bile, and then the bile pheromone continues to diffuse from the feces dropped around its nest. A sex pheromone in male bile will thus contribute significantly to the reproductive success and fitness of largemouth bass. In addition, the absence of a differential response by female largemouth bass in autumn could be due to two factors: bile from males outside the spawning season (NRB) is qualitatively different from bile collected during the spawning season (RB), or alternatively, females are receptive and attracted to male bile only when they are searching for a male with whom to spawn. Future studies are therefore needed to clarify the physiological mechanisms between male and female largemouth bass.

Since the control activities were being conducted in the lake since 2004, the population density of largemouth bass in the lake had decreased (*unpublished data*). The size of the largemouth bass population in the lake has been estimated since 2010. The estimated female largemouth bass population was 850 individuals in 2010. The total catch of females by RB, NRB, and Control traps was 25, 13, and 13 individuals, respectively. The total catch of females in the study was 6% of the estimated number in 2010. Therefore, we don't think the total catch by these small gillnets was low.

In general, many fundamental studies on pheromones are conducted in research laboratories. This study, however, was conducted in an aquatic field and we could clearly demonstrate the female attraction toward bile from the male. These results will thus readily contribute to the development

of a pheromone trap that will attract gravid female largemouth bass. An investigation into the relationship between the concentration of pheromone in male bile and the effective range is especially important for developing an effective design that will trap largemouth bass using pheromone. The effective range of the trap with 300 µL of bile, which was set to evaluate the releaser effect of the bile, may thus be inadequate to be of practical value in reducing the numbers of largemouth bass. To enhance the effect of pheromone traps under such conditions, a larger amount of bile should be released from the trap. Furthermore, ambient water at the study sites undoubtedly contained sex pheromones from male largemouth bass, as many nests made by males were found around the sites. It is therefore possible that the effective range of the bile released into the trap was shortened due to acclimation by pheromones already in the water. It is also likely that the effective range of the bile will be greater if population density is decreased by other methods including gillnets.

In Lake Izunuma-Uchinuma, fish catches for species other than largemouth bass had decreased by up to about one-third compared to previous catches before largemouth bass increased there (Takahashi et al. 2001). If we use a non-selective method such as a normal gillnet, catches of native fishes other than target species will increase and the capture rate of largemouth bass will decline. This approach will probably hamper efforts to restore the populations of native fishes. Bycatches of native fishes will reduce the catch per unit effort (CPUE) of the target species and we will experience a decline in working efficiency. In the present results, most of the non-target fish were captured in the lower layer of the net, while half of mature largemouth bass females in the RB trap were captured in the upper layer. These results suggest that the bile released from a gillnet floating in the top third of the water column and open underneath would make it possible to selectively capture female largemouth bass. To reduce the bycatch rate of native species, it is therefore recommended to produce a new pheromone trap with a gillnet that does not touch the lakebed. Also, there is a possibility of adding this attractant to a net type other than a gillnet so that non-target species could be trapped and then released without harm.

Illegal releases of largemouth bass by anglers have spread in Japan and are continuing (Yodo and Iguchi 2004; Tsunoda et al. 2011). The pheromone trap would be one of the most effective methods for monitoring the presence of pest or foreign species (Wyatt 2003). Pheromone-based monitoring may contribute to early detection of illegal introductions of largemouth bass, due to the specific effect of pheromone even under lower densities of the target species. A combination of several methods can be efficiently employed according to the population density of the fish. The CPUE of gillnets, set nets, electric fishing boats, angling and other control implements declines with decreases in the population density of the target fish and makes it difficult to eradicate invasive species (Myers et al. 1998).

Since the potential efficiency of a pheromone trap is dependent on the pheromone of males at their nests and is also inversely proportional to the population density of target species, when densities of largemouth bass are lower, pheromone traps are likely used to capture the target individuals compared to nets, electric shocks, or other methods.

Pest control using insect pheromones has long been practiced to protect agricultural industries (Wyatt 2003). In vertebrates, in order to eradicate invasive jawless fish, the chemical identity of sex and migratory pheromones in the sea lamprey were identified (Li et al. 2002; Sorensen et al. 2005), and pheromone traps using these chemical substances have already been tested (Wagner et al. 2006; Johnson et al. 2009; Meckley et al. 2012). In teleost fishes, however, pest eradication using pheromones is performed at the beginning stage of a development project. We will continue to capture largemouth bass by using pheromone traps with male bile. In tandem with the field work described in this paper, it is necessary to elucidate and synthesize the chemical compounds in the bile that attract reproductive females. Future control programs that employ pheromone traps to reduce the numbers of largemouth bass are expected to contribute to the recovery of native fish populations including *Cyprinus carpio* and *Carassius* spp.

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