Estimating the spawning activities of fish species using environmental DNA

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Estimating the spawning activities of fish species using environmental DNA

（環境 DNA による魚類繁殖活動の推定）

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Chapter 1 General introduction

1.1 Fish diversity

Biodiversity loss is widely considered to be one of the most serious ecological challenges facing humanity (Secretariat of the Convention of Biological Diversity, 2006). From 1970 to 2000, average species abundance declined by about 40%; inland water species declined by about 50%, while marine and terrestrial species both declined by about 30% (WWF, 2004). Freshwater fish, which account for a quarter of the world's vertebrates and are a major component of freshwater ecosystems, are facing a crisis of declining diversity (Su et al., 2021). According to the International Union for Conservation of Nature (IUCN) Red List (2020), 80 species of freshwater fish are already extinct and more than 2,400 are critically endangered, including migratory freshwater fish, which have decreased by 76% since 1970, and giant fish, which has decreased by 94% (World Wildlife Fund, 2021). Major threats to fish biodiversity include climate change, invasive alien species, and human activities such as overfishing and dam construction.

1.1.1 Effects of climate change on fish diversity

Climate change affect fish growth and spawning through habitat disturbance (e.g., severe storms, and changes in temperature or salinity), resulting in changes in fish distribution and abundance (Cheal et al., 2002; Wooldridge et al., 2005). In freshwater basins, the biodiversity of fish varies significantly in different climatic conditions (Comte et al., 2013). Arid climate reduces water supply and causes water levels to fall, which hinders depth-dependent fish spawning, and thus threatens inland fisheries (Muneepeerakul et al., 2008). Changes in water

temperature caused by climate change affect the growth rate of fish and interfere with the spawning distribution (Brander, 2007).

1.1.2 Effects of invasive species on fish diversity

Invasive species introduce parasites and pathogens (Smit et al., 2017; Calhoun et al., 2018), increase predation pressure (Britton and Orsi, 2012), decrease of native fish reproduction success due to predation on eggs and offspring (Grabowska et al., 2010). Hybridization of invasive and native fish leads to the loss of unique genetic diversity (Todesco et al., 2016). In 20th century, 68% of fish extinctions in North America are associated with invasive species (Miller et al., 1989). The spread of invasive species poses a strong threat to native fish population structure, and the abundance of invasive species is most important factor in habitat degradation (Hermoso et al., 2011). The establishment and expansion of invasive fish populations depends on spawning adaptations to habitats (Deacon et al., 2011). Therefore, investigating the spawning activities of invasive fish can help to effectively monitor invasive fish populations in the early stage of their establishment and assess their threat to native fish biodiversity.

1.1.3 Effects of human activities on fish diversity

Overfishing significantly reduces the age, size, and geographic diversity of populations and the biodiversity, making fish populations more sensitive to additional stresses such as climate change and invasive species (Brander, 2007). Therefore, sufficient ecological information is needed to guide the planning of closed fishing periods and areas or establishing fish protection area to reduce the impact of overfishing on fish diversity.

Dam construction cause river impoundment and significantly altered ecological change in freshwaters (Poff and Zimmerman, 2010). Dams can hinder fish migration (Hall et al., 2011), affect upstream migration of adult fish and interrupted the downstream migration of their larvae (Lima et al., 2016). Dams alter water flow, water depth, and affect fish habitat ecology, which can impair growth and reproduction of obligatory and facultative riverine fish (Kruk and Penczak, 2003), and cause biohomogenization (Rahel 2000; Poff et al. 2007). Fish taxa such as lampreys (*Lampetra* spp.), eels (*Anguilla* spp.), and shads (*Alosa* spp.) are at particular risk of species loss due to the habitat disturbance by dams (Liermann et al., 2012). Long-term ecological surveys of the dam and its surrounding waters are of great significance to the conservation of fish biodiversity.

Some measures, such as fish stocking (using native and non-native species), the construction of transposition mechanisms and fishery control have been adopted to protect fish biodiversity. However, the use of such measures in the absence of a reasonable monitoring program may have a negative impact on fish spawning (Agostinho et al., 2007). Planned surveys and studies on fish spawning activities can help predict changes in fish population structure and spawning distribution (Kouamélan et al., 2003; Wilson et al., 2009), and provide a reference for the correct implementation of management measures.

1.1.4 The importance of monitoring fish spawning activity

Spawning activity is the basis for the establishment and development of populations, and many factors lead to the loss of fish diversity through interference with spawning activity (Scott et al., 2006). For example, changes in water temperature caused by climate change interfere with

the spatial and temporal distribution of fish spawning; overfishing reduces the reproductive output of populations (Brander, 2007); the dam construction has caused serious habitat disturbance (Kruk and Penczak, 2003). Monitoring the timing and location of spawning activity increases understanding of the fish ecology, helps assess threats to regional biodiversity from external factors; assesses the reproductive output of populations; tracks the establishment and expansion of invasive fish populations; providing important information for conservation and management of fish species to avoid ineffective protective measures（Harada et al., 2015）.

1.2 Investigating fish spawning activities by traditional survey

Changes in the timing of spawning and fecundity of fish were associated primarily with changes in temperature, food, sunshine, and water level, it is difficult to rely on a single factor to predict fish spawning activities (Brander, 2007; Crozier and Hutchings, 2014; Gosch et al., 2006; Matsuzaki et al., 2019). Traditional monitoring methods for fish spawning include may or may not involve destructive or invasive actions on ecosystems or individuals. Destructive or invasive methods include otolith micro-chemistry, gonad maturation, etc. (Ntiba and Jaccarini, 1990; Milton et al., 1997). Otoliths can be used to estimate the time of fish spawning by calculating the age of juvenile fish, and gonad maturation can be used to estimate fish growth and egg formation. These methods require sacrificing all or a subset of organisms and lead to imposing extra mortality rate which makes them undesirable for monitoring spawning activities in rare and endangered fish (Petursdottir et al., 2006). Such methods are often accompanied by fishing activities such as drift nets and electrofishing (Wei et al., 2009). These fishing activities

are time-consuming and labor-intensive, and also cause injury to fish and hindering their natural spawning activity.

Non-destructive or non-invasive methods include acoustic surveys, visual surveys, etc. In acoustic surveys, spawning was identified by large aggregation sounds, so direct evidence of spawning is often not available (Walters et al., 2009), and visual surveys are time-consuming and inefficient (Bracken et al., 2019). Both acoustic surveys and visual surveys are sensitive to observer biases and taxonomic misidentification (Miller et al., 2012). In order to reduce this kind of biases, it is often necessary to combine multiple monitoring methods to jointly estimate fish spawning activities, but this increases the labor and time of field investigation and adds extra cost.

As such, destructive or invasive methods interfere with the natural activities of fish and are not suitable for monitoring the spawning activity of endangered fish. Non-destructive or non-invasive methods have the disadvantages of large observation deviation and low efficiency. Therefore, we need a non-destructive, non-invasive method that can efficiently monitor the fish spawning activities, and it would be a valuable tool for the conservation or management of aquatic biodiversity.

1.3 Environmental DNA (eDNA) technology

eDNA is defined as encompassing the DNA of all organisms present in environmental samples, including microbial, meiofaunal and macrobial taxa (Rodriguez-Ezpeleta et al., 2021). By collecting and detecting eDNA in water, we can determine which organisms live in or around the water body. This technology is called eDNA technology. Currently, eDNA technology has

received attention as a non-destructive, non-invasive survey method (Minamoto et al., 2012). Because this method relies on simply collecting water samples for analysis, it can be used to investigate biological population structures in water bodies without damaging ecosystems or organisms and with a greatly decreased expenditure of time and labor. Using DNA to identify species alleviates the requirement for researchers to have specialized knowledge of species morphology, reduces observer bias, and enables the detection of multiple species from a single eDNA sample, increasing work efficiency (Bohmann et al., 2014).

This technique has been widely used to investigate a wide range of organisms including: vertebrate: e.g., fish (Jerde et al., 2011; Thomsen et al., 2012), amphibians (Ficetola et al., 2008; Goldberg et al., 2011), reptiles (Hunter et al., 2015; Davy et al., 2015), birds (Ushio et al., 2018), mammals (Foote et al., 2012; Ushio et al., 2017); invertebrates: e.g., crustaceans (Tréguier et al., 2014; Wu et al., 2019), insecta (Valentin et al., 2020; Uchida et al., 2020), cnidaria (Minamoto et al., 2017; Takasu et al., 2019), echinodermata (Madduppa et al., 2021), trematoda (Sato et al., 2018; Alzaylaee et al., 2020) and foraminifera (Pawlowski et al., 2014; Cordier et al., 2017); aquatic plants (Scriver et al., 2015; Fujiwara et al., 2016); microorganism: e.g., bacteria (Zhang et al., 2020; Nuñez et al., 2021), fungi (Yan et al., 2018; Heine et al., 2021), virus (Hall et al., 2016; Kaganer et al., 2021).

This technique has been widely used in various ecological surveys. It is frequently be used to estimate the distribution of invasive species, infer the invasion route and assess the threat level of invasive species in different areas (Adrian-Kalchhauser and Burkhardt-Holm, 2016; Dougherty et al., 2016). Through repeated sampling surveys, it has the potential to detect invasive species with very few individuals (Furlan et al., 2019). It is also be used to investigate

community structure and biodiversity, such as assess the relative abundance of different species at different sampling sites by metabarcoding approach using universal primers (Bista et al., 2017; West et al., 2020; Milhau et al., 2021). It can also be used to assess habitat selection for different species by investigating changes in eDNA concentration and population structure with environmental variables (Marshall and Stepien, 2020; Vimercati et al., 2020; Xie et al., 2021). The species migration of aquatic organisms can also be estimated from seasonal changes in eDNA concentration at different regions (Wu et al., 2019; Easson et al., 2020). Some current researches are trying to use eDNA as a non-invasive and efficient method for detecting fish spawning activities (Bylemans et al., 2017; Tsuji et al., 2021).

1.4 Investigating fish spawning activities by eDNA survey

Many aquatic organisms exhibit external fertilization and release a large number of sperm and eggs into their environment during spawning activity (Coward et al., 2002), causing the eDNA concentration to rise during spawning period (Spear et al., 2015; Buxton et al., 2017). Fertilized eggs remain in the water until hatch, but Erickson et al. (2016) found there is no relationship between eDNA concentration and drifting eggs. Takeuchi et al. (2019), Takeshita et al., (2020) and Ostberg et al. (2022) found that fertilized eggs do not seem to produce eDNA or produce low concentrations of eDNA by tank experiments. Although studies have shown that eDNA concentrations are positively correlated with the number of eggs collected during fish spawning period (Hayer et al., 2020), but it could not prove that the high eDNA concentrations were from fertilized eggs. Therefore, it can be considered that the fertilized eggs remaining in the water body after spawning activities will not release a large amount of eDNA, and will not affect the

accuracy of estimating spawning activities by eDNA concentration. Tsuji et al. (2021) proved through the tank experiment that the increase of eDNA concentration during spawning activity mainly depends on the release of sperm, while pseudo spawning activity, which is a reproductive behavior without releasing sperm or egg, has little effect on eDNA concentration. Therefore, it can be considered that the interference of pseudo spawning activity can be excluded by estimating spawning activity through eDNA.

Due to the positive correlation between eDNA concentrations and fish biomass (Takahara et al. 2012), increased eDNA concentration during spawning period may be indistinguishable from eDNA concentration resulting from spawning aggregates. To address this issue, Bylemans et al. (2017) demonstrated by adding fish sperm to a tank, and showed that the release of sperm leads to an increase in the nuclear eDNA/mitochondrial eDNA (nuDNA/mtDNA) ratio in the environment. Using nuDNA/mtDNA ratios to estimate spawning activity can be freed from the effects of fish biomass. However, Saito et al. (2022) found in field investigation that eDNA concentration (nuDNA or mtDNA) could indicate spawning activity, while nuDNA/mtDNA ratio could not accurately indicate spawning activity. The results of the water tank experiment are inconsistent with the actual survey results, and the reasons for this inconsistency have not been scientifically answered.

Some researchers have also conducted ecological surveys related to fish spawning. Tillotson et al. (2018) and Thalinger et al. (2019) monitored the migratory pathways of migratory fish during the spawning season through changes in eDNA concentrations. Antognazza et al. (2019) and Homel et al. (2021) described the fish spawning distribution over a large spatial extent. However, such studies are all conducted on the assumption that "spawning

activity has occurred", so they cannot explain the relationship between changes in eDNA concentration and spawning activity.

In addition to studies on single species, Ip et al. (2022) and Di Muri et al. (2022) attempted to use metabarcoding to detect fish eDNA relative abundance to estimate fish spawning activity. However, relative abundance was affected by the combined effects of eDNA released by all detected species. The changes in local population structure and the simultaneous spawning of multiple species have resulted in complex changes in relative abundance. Existing studies cannot demonstrate the validity and reliability of the method for estimating spawning activity from relative abundance under such complex conditions.

eDNA continues to diffuse and degrade in water. However, almost all prior studies on spawning activity have ignored the effect of this phenomenon on estimating fish spawning activity. Although other studies have shown that the degradation rate of eDNA is affected by factors such as biomass and water temperature (Jo et al., 2020), the duration and diffusion distance of the high concentrations of eDNA produced by spawning activities in water bodies are still unknown. Without this kind of basic knowledge, it is impossible to make accurate and effective survey plans to monitor spawning activities, or to scientifically explain what information about the spawning activity is contained in the peaks of eDNA concentration or ratio.

Basic research on the relationship between eDNA and environmental variables is still lacking. For example, water temperature significantly affects fish spawning activities, but few studies have investigated whether the changes in fish eDNA concentrations and ratios that

accompany changes in water temperature are consistent with fish life history as determined through field work.

All previous studies have only pointed to the phenomenon of increased eDNA concentration or ratio caused by fish spawning activity, but did not establish a specific method to use this phenomenon to estimate fish spawning activity. The previous research limited to the tank experiment could not prove the reproducibility of the experimental results in the field environment, and the limited field investigation research lacked the comparison with the traditional method to illustrate the effectiveness of the eDNA method. Some previous studies have attempted to use metabarcoding to investigate the spawning activity of multiple fish species, but relationship between relative abundance and spawning activity is still unknown. A method to estimate the spawning activity of multiple fish species simultaneously is still lacking.

1.5 Purpose of this study

The purpose of this study was to establish a method for estimating fish spawning activity based on eDNA surveys. For this purpose, a series of studies were conducted as follows. Firstly, artificially induced fish spawning experiments were conducted to investigate spatiotemporal changes in eDNA concentrations during fish spawning, and tried to design an eDNA sampling plan to monitor fish spawning activities. Secondly, a two-years eDNA survey was conducted in a reservoir to develop a method for estimating fish spawning activities, and verify the effectiveness of the method by comparing it with the traditional method. The response of fish eDNA to environmental variables was analyzed to estimate the water temperature conditions required for different fish spawning activity. Thirdly, the quantitative eDNA metabarcoding

approach was performed to simultaneously estimate the spawning activities of multiple fish. Finally, the results of this study and previous study were combined to establish an eDNA-based survey framework for monitoring fish spawning activity.

Chapter 2 Spatiotemporal changes in environmental DNA concentrations caused by fish spawning activity

2.1 Introduction

Monitoring the spawning activity of aquatic organisms is important for the conservation and management of species and populations (Koenig et al., 2000; Merz and Setka, 2004) because spawning activity directly affects population reproduction and future population growth (Scott et al., 2006). The information on the exact timing and location of spawning activities can serve as a basis for establishing closed fishing seasons and/or areas to reduce the interference on the spawning activities of rare species or useful fishery species (Arendse et al., 2007). It can also provide a time reference for the control and management of invasive species (Raghavan et al., 2008). Spawning activity depends on the combined effects of many factors, such as water temperature, food, sunlight, and water level, on aquatic organisms, including fish (Gosch et al., 2006; Matsuzaki et al., 2019). Traditional survey methods for estimating the spawning period of fish include collecting fish eggs or catching fish and examining gonads and otoliths (Allman et al., 2002; Smith and Walker, 2004; Harada et al., 2015). However, these techniques are time-consuming and labor-intensive, and they also cause injury to fish and hindering their natural spawning activity. Monitoring the spawning activities of aquatic organisms using noninvasive methods remains a challenge.

Currently, eDNA analysis has attracted attention as an environmentally friendly and noninvasive survey method (Minamoto et al., 2012; Rees et al., 2014). eDNA represents all DNA present in water derived from biological tissue fragments and excrement (Ficetola et al., 2008). By investigating eDNA in water bodies, it is possible to investigate the distribution of invasive species (Adrian-Kalchhauser & Burkhardt-Holm, 2016; Dougherty et al., 2016; Furlan et al., 2019), community structure and biodiversity (Bista et al., 2017; West et al., 2020; Milhau

et al., 2021), habitat selection preferences of different species (Marshall & Stepien, 2020; Vimercati et al., 2020; Xie et al., 2021), and species migration (Wu et al., 2019; Easson et al., 2020) without interfering with their survival and life. Moreover, species identification using DNA does not require any morphological knowledge. This method has been widely used in ecological surveys of crustaceans (Wu et al., 2018), fish (Takahara et al., 2013, Levi et al., 2019), amphibians (Fukumoto et al., 2015; Evans et al., 2016), reptiles (Piaggio et al., 2014; Nishizawa et al., 2022), and mammals (Franklin et al. 2019).

Many aquatic organisms exhibit external fertilization and release a large number of sperm and eggs into their environment during spawning activity (Coward et al., 2002), causing the eDNA concentration to rise sharply in a short period of time (Buxton et al., 2017; Tsuji & Shibata, 2021). A large number of sperm with low mitochondrial content are released into the water body, which increases the nuDNA/mtDNA ratio (Bylemans et al. 2017).

eDNA continues to diffuse and degrade in water. Although studies have shown that the degradation rate of eDNA is affected by factors such as biomass and water temperature (Jo et al., 2020), the duration and diffusion distance of the high concentrations of eDNA produced by spawning activities in water bodies are still unknown. This lack of basic knowledge prevents peoples from making accurate and effective survey plans to monitor spawning activities. In other words, designing a survey to accurately monitor spawning activities is still challenging.

In the current study, natural fish spawning was simulated through artificially induced fish spawning. The main experimental aims were as follows: 1) to investigate spatiotemporal changes in eDNA concentrations during fish spawning and 2) to design an eDNA sampling plan to monitor fish spawning activities. Common carp (*Cyprinus carpio*) was used as the target

species. Common carp is among the world's worst invasive alien species (Global Invasive Species Database: http://www.iucngisd.org/gisd/). Foreign carp have been released in various places in Japan since the Meiji era (from 1868 to 1912, invasive species of Japan: http://www.nies.go.jp/biodiversity/invasive/index_en.html). Carp spawn in shallow water areas with aquatic plants, and violent mating movements are observed during spawning (Fernández-Delgado, 1990), which can help to visually check the spawning activities of carp.

2.2 Materials and methods

2.2.1 Artificial spawning and water collection

The artificial spawning experiment of carp was conducted five times from March 30 to May 20, 2021. The experimental season was consistent with the spawning period of the carp. The experiments were carried out in a field pond (34°47′23.3″ N, 135°37′00.2″ E) at the Biodiversity Research Center, Research Institute of Environmental, Agriculture and Fisheries, Osaka Prefecture, Japan. The pond shape was approximately rectangular (35 m long, 30 m wide, and 50 m diagonally), and the water flow was almost static. Before the experiment, no carp were present in the pond.

A 2 × 2 m net cage was set up in a corner of the pond, and artificial aquatic plants were placed as spawning media. One male and one female carp were selected from another tank dedicated to carp, injected with 500 or 1000 μL (2500 or 5000 IU) chorionic gonadotrophin according to body size, and transferred to the net cage. The carp were anesthetized throughout the procedure. An infrared camera (HCO-SG560K, ScoutGuard, Australia) was set up on the shore of the net cage to record the possible spawning activities of carp by taking pictures or

videos every 5 min. The following morning, when carp spawning activity was confirmed, water samples were collected. The sampling times were 9:00, 11:00, 13:00, 15:00, 9:00 (24 h), and 9:00 (48 h). Sampling was performed at different distances from the edge of the cage (1, 2, 4, 8, 16, 32, and 47 m [maximum distance]), and samples were collected from far to near. A small bucket with a long handle was used to collect the water sample, and the bucket was washed with a bleach solution (household bleach product containing ~5 % sodium hypochlorite) and pure water between samples. Once a day, pure water was poured into a cleaned bucket and recovered as a blank control.

Forty-two water samples and three blank controls were collected from a single experimental series. Water samples were immediately filtered in a laboratory near the pond using 47-mm glass-fiber filters with 0.7-μm pores (GF/F; GE Healthcare Japan, Tokyo, Japan). Two GF/F filters were used for each water sample to filter 600 mL per sample. The filters were stored at -25°C until DNA extraction. After one experiment, the carp and net cage with eggs were recovered, a new net cage was set up, and different carp individuals were selected for the next experiment, with an interval of 3–4 days between each experiment. In total, 210 water samples and 15 blank controls were collected.

2.2.2 eDNA extraction and quantitative polymerase chain reaction (qPCR)

For each sample, two filters were combined, and the total DNA from both filters was extracted using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the method recommended by the eDNA Society (Minamoto et al., 2021). Briefly, each filter was placed in a Salivette tube (Sarstedt, Nümbrecht, Germany), and 440 μL lysis solution, composed of 400

μL Buffer AL and 40 μL Proteinase K, was added to the filters. The tubes were then incubated at 56°C for 30 min. After incubation, the tubes were centrifuged at 5,000 × g for 3 min. TE buffer (220 μL) was added to the filters, and the tubes were recentrifuged at 5,000 × g for 1 min to increase the DNA yield. The DNA was purified according to the manufacturer's instructions. The total DNA was eluted in 100 μL AE buffer and stored at -25°C until subsequent qPCR analysis.

The concentrations of nuDNA and mtDNA in all samples and blank controls were determined using three qPCR replicates per sample for the target fragments of nuclear internal transcribed spacer 1 (*ITS1*) and mitochondrial cytochrome b (*CytB*). The primers and probes used were as follows: *ITS1*-F, 5′-TTCAAAGACCCCCCGTAAC-3′; *ITS1*-R, 5′-GCCATGCCGCACACA-3′; *ITS1*-probe, 5′-TCACGACCCCCCTTATTTTTTCCAAAACC-3′ (Minamoto et al., 2017); *CytB*-F, 5′-GGTGGGTTCTCAGTAGACAATGC-3′; *CytB*-R, 5′-GGCGGCAATAACAAATGGTAGT-3′; *CytB*-probe, 5′-CACTAACACGATTCTTCGCATTCCACTTCC-3′ (Takahara et al., 2012). Each 20-μL PCR mixture contained 2 μL template DNA, 900 nM of each primer, 125 nM TaqMan probe, 10 μL of 1× Environmental Master Mix 2.0 (Life Technologies, Foster City, CA, USA), and 0.1 μL AmpErase Uracil N-Glycosylase (Thermo Fisher Scientific, Waltham, MA, USA). PCR was performed using a StepOnePlus Real-Time PCR System (Thermo Fisher Scientific) as follows: 2 min at 50°C, 10 min at 95°C, and 55 cycles of 15 s at 95°C and 1 min at 60°C. For each PCR run, prepared triplicate negative controls were prepared and standards diluted to 30000, 3000, 300, and 30 copies/well.

2.2.3 Data analysis

Using the recording results from an infrared camera, the time points at which all carp spawning behaviors may occur were recorded by judging the ripples on the water surface. The median of the time points of the confirmed spawning behaviors for each group of the five artificial spawning experiments was set as 0 h, and the sampling time of each group of experiments was recalculated.

The triplicate PCR results were averaged and used as concentration of each sample, and results with fewer than one copy were removed (four samples were removed), after which the *ITS1*/*CytB* ratio was calculated. Then, logarithmic transformation was performed for the eDNA concentration and *ITS1*/*CytB* ratio with a base of e. The third set of experiments may not represent typical spawning activity because the male and female carp did not cooperate with the spawning activity, and the results were removed from subsequent analyses. The detailed reasons are given in the Appendix 1-1.

The effects of time and distance on changes in eDNA concentrations and ratios were analyzed using generalized additive mixed models (GAMMs). Three GAMMs with a Gaussian distribution were used to evaluate the relationships between log-transformed *ITS1* concentrations, log-transformed *CytB* concentrations, log-transformed *ITS1*/*CytB* ratios (response variables), time, distance, and interactions between time and distance (explanatory variables). Random effects in the four experimental groups were also considered. The degradation rates of eDNA concentrations and ratios after the peak were analyzed using a linear model, and the interaction effect between time and distance was also considered.

2.2.4 Data simulation

Two data simulations were performed to show the probability of the successful detection of a carp spawning event when sampled at random times and distances, given that carp spawning activity occurs in a wider field. Simulation I was performed to estimate how the success rate of spawning activity monitoring varied with sampling time and distance intervals, and Simulation II was performed to estimate the success rate of spawning activity monitoring for different sampling plans.

2.2.4.1 Data preparation

The data from the final sampling in each group of experiments were used as the baseline for the eDNA concentration. Because the final sampling was more than 48 h away after the spawning activity, the eDNA concentration at this time was assumed not affected by the spawning activity. To simulate complete spawning activity, the eDNA concentration was assumed that started to rise beginning at -1 h. Because no -1 h data, the baseline data (data for the final sampling in each group of experiments) were extrapolated as the data for -1 h. Assuming that when the pond became n times larger, the eDNA concentration became 1/n and the baseline of the eDNA concentration remained unchanged, then the simulation data were calculated as follows: (raw data – baseline) / n + baseline. The *ITS1/CytB* ratio was calculated using the simulation data to build new GAMMs based on the simulated concentration and ratio data.

2.2.4.2 Simulation I

Random sampling was performed at different time intervals and different distance intervals after spawning activity occurred to estimate the probability of successfully monitoring spawning activity. The time intervals were set to -1–11, 11–23, 23–35, and 35–47 h; the distance intervals were set to 0–25 and 25–50 m; and the size of the pond was set to 1–10 times. Random sampling was performed 100 times (10 times at the set time intervals × 10 times at the set distance intervals). For each random sampling, the mean and variance through GAMMs of the simulated data were calculated, and a random point from the normal distribution formed by the mean and variance was considered the sampling result. The upper limit of the 95% prediction interval of the eDNA concentration and ratio of each group of experiments using the raw data from the last sampling (no spawning activity interference) at 1 m (carp very close to the sampling point) was used as the threshold value. When the result of random sampling was higher than the threshold, the spawning activity of the carp was considered to have been successfully monitored. The success rate was calculated as the number of successful monitoring events within 100 random sampling activities during the set time intervals and with the set distance intervals. The simulation was repeated 100 times, and the results were analyzed using linear mixed models (LMMs).

2.2.4.3 Simulation II

To estimate the probability of successful monitoring of spawning activity with different sampling plans (time intervals and distances between sampling sites in larger ponds), a second simulation was performed based on data from -1–47 h and 0–50 m. The pond size was set to 1–10 times larger than that used in this study. The time interval was set to sample every 12 h (four

time points), 24 h (two time points), or 48 h (one time point). Because the maximum distance between the spawning site and the sampling site was half the distance between the two sampling sites, the distance interval was set to sample every 50 m (two sites) or 100 m (one site). Sampling plans were performed 100 times to calculate the success rate (10 groups of random time points × 10 groups of random sites). The simulation was repeated 100 times. Calculation of the sampling results and thresholds was performed as described for simulation I. For multiple samples in a sampling plan, at least one sample above the threshold was considered a successful monitoring of spawning activity. The GAMMs were run using the *mgcv* package (Wood, 2001). The LMMs were run using the *nlme* package. All analyses were performed using *R* version 4.0.3.

2.3 Results

2.3.1 Changes in eDNA concentrations

None of the blank or negative controls showed any amplification. Among 1,260 PCR wells (210 samples [containing the third set of experiments] × two target DNAs × three replicates), 21 wells were not successfully amplified. Among the 210 samples, 208 samples had more than one copy in the PCR results for *CytB* and *ITS1*. Changes in eDNA concentrations and ratios over time and distance were estimated using GAMMs for carp-spawning activity. The results of the GAMMs showed that time, distance, and the interaction of time and distance significantly affected the changes in *CytB* and *ITS1* concentrations; for the *ITS1*/*CytB* ratio, only time exhibited a significant effect (Table 2-1). The eDNA concentration and ratio peaked at approximately 7 h and decreased thereafter (Figure 2-1). As the distance increased, the times at

which the eDNA concentration and ratio peaked were slightly delayed. Compared with that at 1 m, the time at which the eDNA concentration and ratio peaked at 47 m was delayed by approximately 2 h (Figure 2-1), suggesting that carp spawning activity could significantly affect eDNA concentrations at a distance of 50 m, even in still waters. The closer to the net cage, the higher the concentration of basal eDNA released by carp. As a result, the eDNA concentrations showed varying decline processes at different distances. For example, closer to the net cage, the eDNA concentration showed a gentle downward trend earlier (Figure 2-1). Although the GAMM results showed that the interaction between time and distance was not significant, a trend similar to that observed for the concentration was observed (Figure 2-1).

The high eDNA concentration produced by carp spawning activities showed a linear downward trend over a short period of time after reaching the peak. Therefore, a linear model was used to analyze eDNA data for 8–20 h. The results are presented in Table 2-2. The distance and interaction of time and distance did not have a significant effect on the eDNA concentration (Table 2-2), consistent with the results of GAMMs (Fig. 2-1, 8–20 h). The time-dependent coefficients of *CytB* and *ITS1* concentrations showed that as the time increased by 0.1 h, the *CytB* concentration decreased by approximately 5.22%, and the *ITS1* concentration decreased by approximately 7.78% (Table 2-2); thus, the speed at which the *ITS1* concentration decreased was significantly faster than that of the *CytB* concentration. Similarly, the rate of decrease in the *ITS1/CytB* ratio was approximately 2.56% / 0.1 h (Table 2-2), reflecting the difference between the rates of at which *ITS1* and *CytB* decreased.

2.3.2 Results of simulation I

Figure 2-2 shows the random sampling results for different time intervals and different distance intervals after spawning activity occurred. When estimating the spawning activities of carp based solely on the *CytB* concentration, even if the pond was at the original size (1 time) and sampling was performed within 12 h after spawning activity, there was only approximately a 50% probability of successfully monitoring spawning activity. The success rate decreased significantly as pond size increased (Figure 2-2a). When the *ITS1* concentration or the ratio of *ITS1/CytB* was used to estimate the spawning activity of carp, a trend similar to that of the change in pond size was observed (Figure 2-2b, c). Additionally, the success rate decreased significantly as the pond size increased during 11–23 h, and the success rate tended toward 0% after 23 h. The results for the 11–23 h interval showed that the success rate for the 25–50 m distance interval was approximately 6.2% and 12.9% higher than those for the 0–25 m distance interval for the *ITS1* concentration and the *ITS1/CytB* ratio, respectively ($p < 0.001$; Figure 2-2b, c).

2.3.3 Results of simulation II

Figure 2-3 shows the probability of the successful monitoring of carp spawning activity under different sampling plans. As the size of the pond increased, the success rate gradually decreased but had less effect on the results of *ITS1* and the *ITS1/CytB* ratio sampled every 12 h (Figure 2-3b, c). When the pond size became 10 times larger, the success rate was close to 0% for *CytB*, regardless of the sampling plan. For *ITS1*, the success rate of sampling every 100 m was approximately 7.4% lower than that of sampling every 50 m ($p < 0.001$), and the success rates of sampling every 24 and 48 h were approximately 39.0% and 65.4% lower than that of

sampling every 12 h, respectively ($p < 0.001$). For the *ITS1/CytB* ratio, the success rate of sampling every 100 m was approximately 5.72% lower than that of sampling every 50 m ($p < 0.001$), and the success rates of sampling every 24 and 48 h were approximately 35.6% and 65.6% lower than that of sampling every 12 h, respectively ($p < 0.001$). In summary, the sampling frequency over time was more important than that over distance.

2.4 Discussion

Through artificial spawning experiments in carp, trends in the changes of eDNA concentrations and nuDNA/mtDNA ratios produced by spawning activities under the interactions of time and distance were showed. Reductions in eDNA concentrations following peaking after spawning activity were estimated, and differences in the reduction rates between nuDNA and mtDNA are shown. Through data simulation, differences in the results of sampling at different time and distance intervals were estimated, and the probability of successful monitoring of carp spawning activity under different sampling plans was estimated. The results showed that the high eDNA concentrations and nuDNA/mtDNA ratios produced by spawning activities returned to baseline within approximately 24 h. When carp spawning activity occurred, the spawning activity could be successfully monitored by measuring nuDNA concentrations or the nuDNA/mtDNA ratio with a probability of approximately 50–75% based on a sampling plan of collecting samples every 100 m and every 24 h.

The results of changes in eDNA concentrations over time and distance showed that the high concentrations of eDNA released by spawning activity tended to be evenly distributed after peaking in the ponds; this effect was believed to relate to the size of the experimental pond.

The pond with a diagonal of approximately 50 m showed limited diffusion of eDNA, whereas in larger water bodies, eDNA could diffuse unrestricted. In this study, 8–20 h data was used to determine the speed at which the eDNA concentration decreased after peaking. The results showed that distance did not significantly affect the eDNA concentration, which indicated that the eDNA concentration had reached a uniform distribution in the pond. Due to the absence of persistent diffusion, the rate of decrease in the eDNA concentration may have been underestimated. To compensate for this, data simulations were run to simulate experiments with random sampling of larger bodies of water. The results showed that the monitoring of spawning activity solely based on mtDNA concentration was greatly affected by the size of the water body. This is because the concentration of mtDNA released during spawning is much lower than that of nuDNA; therefore, the mtDNA concentration is more susceptible to dilution. In the data simulation, assumed that when the size of the pool was n, the eDNA concentration would become 1/n. In reality, the eDNA concentration would be higher than 1/n owing to factors such as the propagation speed of eDNA and the terrain of the field.

After the eDNA concentration and nuDNA/mtDNA ratio produced by spawning activities peaked, as the time increased by 0.1 h, the mtDNA and nuDNA concentrations decreased by approximately 5.22% and 7.78%, respectively, and the nuDNA/mtDNA ratio decreased by approximately 2.56%. The nuDNA concentration decreased significantly faster than the mtDNA concentration, consistent with the results of a previous study (Jo et al., 2020). At this degradation rate, when the peak of eDNA concentration was 100-fold higher, the time to decrease to the baseline concentration was extended by approximately 9 h (mtDNA) and 6 h (nuDNA). These findings suggested that in the wild environment, even if a large number of

carp spawn simultaneously, the high concentration of eDNA produced by these carp will not persist. The results of simulation I showed that the probability of successful monitoring of spawning activities was close to 0% through sampling after 23 h. Therefore, extremely high eDNA concentrations during the spawning period for specific types of fish may suggest the occurrence of spawning activity within 24 h before sample collection. Moreover, increases in the peak eDNA concentration may prolong the time until the eDNA concentration decreases to the baseline value, but does not prolong the time for the ratio to decrease to baseline. The time required for the ratio to decrease to the baseline value depends on the peak value of the ratio, that is, the ratio of sperm-derived eDNA to all eDNA released during spawning activity. Because the concentration and ratio of eDNA will decrease over time, only whether or not spawning activity has occurred can be estimated, but cannot estimate the scale of spawning activity, such as the biomass involved in spawning activity or the number of eggs released.

A previous study proposed using the nuDNA/mtDNA ratio to estimate fish spawning activity (Bylemans et al., 2017). In this experiment, the maximum value of the *ITS1*/*CytB* ratio of carp was 1227.6, whereas the maximum ratios for *Macquaria australasica* were 309 (sperm only) and 31.5 (field survey) (Bylemans et al., 2017), those for *Micropterus salmoides* and *Lepomis macrochirus* were 3.4 and 35.5 (field survey), respectively (Wu et al., 2022), and that of *Trachurus japonicus* was 178.5 (sperm only) (Tsuji et al., 2022). This indicates that the ratio can vary widely among species. Although field survey data may not reflect the actual maximum nuDNA/mtDNA ratio, this difference should still be considered. When the peak value of the eDNA concentration is high but the peak value of the ratio is not high, the ratio may even decrease because of the difference in the rate of decrease of nuDNA and mtDNA. Therefore,

estimating spawning activity by the ratio alone may be difficult for species with low peak nuDNA/mtDNA ratios.

Through Simulation I, the probability of successful monitoring of spawning activity was estimated when randomly sampled at different time intervals and at different distance intervals after spawning activity. The results showed that the success rate tended to 0% after 23 h, which indicates that the spawning activity monitored by eDNA occurred within 24 h before sampling. The results of the 11–23 h interval showed that the success rate of the 25–50 m distance interval was higher than that of the 0–25 m distance interval for *ITS1* concentration and the *ITS1*/*CytB* ratio, which was contrary to intuition. Can be considered that the uneven distribution of carp in the water bodies caused this result. The net cage was set at the corner of the pond. The location closer to the net cage had a higher baseline eDNA concentration. After spawning activity, the continuous release of fresh eDNA due to the presence of carp accelerates the reduction in the ratio, and the influence decreases with increasing distance. This also shows that the time required for the nuDNA/mtDNA ratio to decrease to the baseline value is also affected by the baseline eDNA concentration, and in water bodies with higher carp densities, the reduction speed of the ratio will be faster. For the *ITS1* concentration, the reason for the above phenomenon may be the accumulation of eDNA in the corner of the pond, and the sampling points of 32 m and 47 m were located in the corner of the pond.

Through Simulation II, the probability of successful monitoring of spawning activity under different sampling plans was estimated. Because different sampling plans for distance had less effect on the success rate, sampling every 100 m was recommend to reduce the workload. Although sampling every 12 h had the highest success rate, sampling every 24 h was still

recommend. Because this experiment simulated the occurrence of a single spawning activity, and the spawning activity of fish is a group behavior, when multiple spawning activities occur at the same sampling point at the same time, a higher peak eDNA concentration will be generated, and when multiple spawning activities occur at different times on the same day, there may be multiple peaks in the eDNA concentration and ratio, which will help improve the success rate of monitoring. Therefore, sampling every 24 h and 100 m is recommended as a general sampling plan. The actual sampling plan should be designed based on the purpose of the survey, workload, biomass, terrain of the field, and life history of the target species.

2.5 Conclusions

Taking the artificial spawning experiment of common carp as an example, the change in eDNA concentration and nuDNA/mtDNA ratio caused by spawning activities over time and distance was showed, estimated the degradation rate of the eDNA concentration, and proposed a sampling plan to monitor fish spawning activities using eDNA analysis. eDNA analysis may have the potential to estimate spawning activities in units of days, and a sampling plan with sampling every 24 h and every 100 m is an appropriate approach to monitor the spawning activities of common carp. Notably, the ratio peaks may differ for different species. This ratio may not be a valid indicator of spawning activity for species with low peak nuDNA/mtDNA ratios. Therefore, combining eDNA concentrations and ratios to monitor spawning activities was recommended.

2.6 Tables

Table 2-1. Results of GAMMs used to fit changes in eDNA concentrations and nuDNA/mtDNA ratios with time and distance

	CytB		*ITS1*		*ITS1/CytB*	
	edf	*P* value	edf	*P* value	edf	*P* value
ti(Time)	3.888	< 0.001 ***	3.880	< 0.001 ***	3.559	< 0.001 ***
ti(Distance)	2.503	< 0.001 ***	2.413	< 0.001 ***	1.582	0.616
ti(Time, Distance)	2.733	0.043 *	2.599	0.028 *	2.170	0.062
random effect	2.944	< 0.001 ***	2.913	< 0.001 ***	2.890	< 0.001 ***

*** < 0.001; ** < 0.01; * < 0.05

“ti()” refers to a smooth term, and “edf” refers to effective degrees of freedom.

Table 2-2. Variations in eDNA concentrations and ratio with time and distance after peak

		Estimate	Std. Error	*P* value
log(*CytB*)	(Intercept)	13.33	0.66	< 0.001 ***
	Time	-0.522	0.055	< 0.001 ***
	Distance	-0.044	0.029	0.144
	Time × Distance	0.003	0.002	0.19
log(*ITS1*)	(Intercept)	21.717	0.897	< 0.001 ***
	Time	-0.778	0.075	< 0.001 ***
	Distance	-0.013	0.039	0.735
	Time × Distance	0.001	0.003	0.702
log(*ITS1/CytB*)	(Intercept)	8.387	0.457	< 0.001 ***
	Time	-0.256	0.038	< 0.001 ***
	Distance	0.03	0.02	0.145
	Time × Distance	-0.002	0.002	0.246

*** < 0.001; ** < 0.01; * < 0.05

2.7 Figures

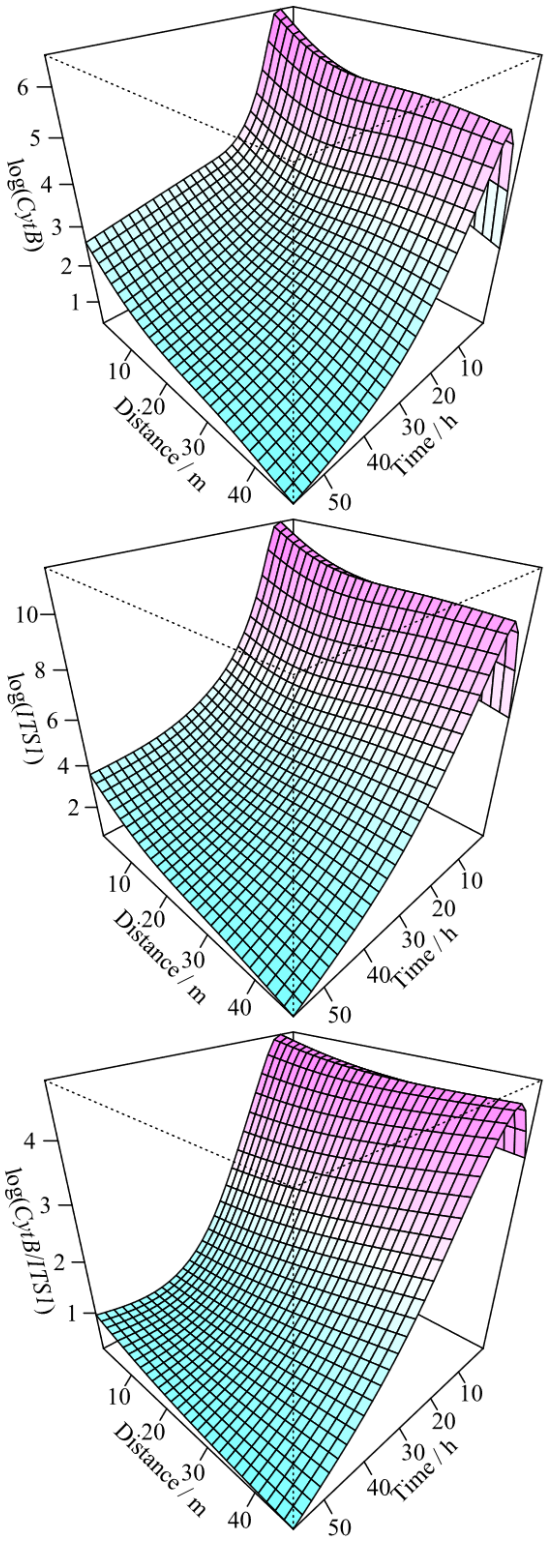

Figure 2-1. Changes in eDNA concentrations and *ITS1/CytB* ratios with time and distance

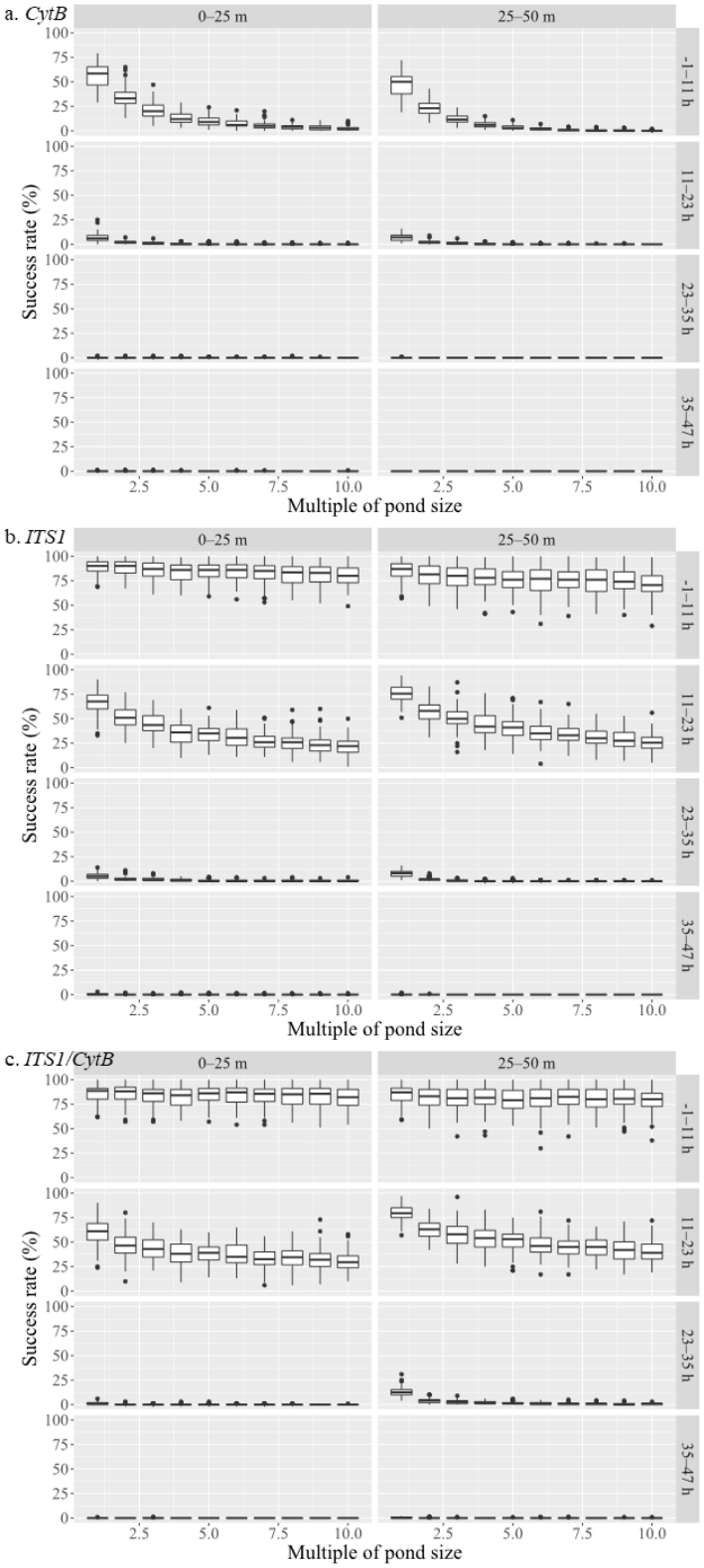

Figure 2-2. Probability of successful monitoring of spawning activity after randomly sampling at different time and distance intervals

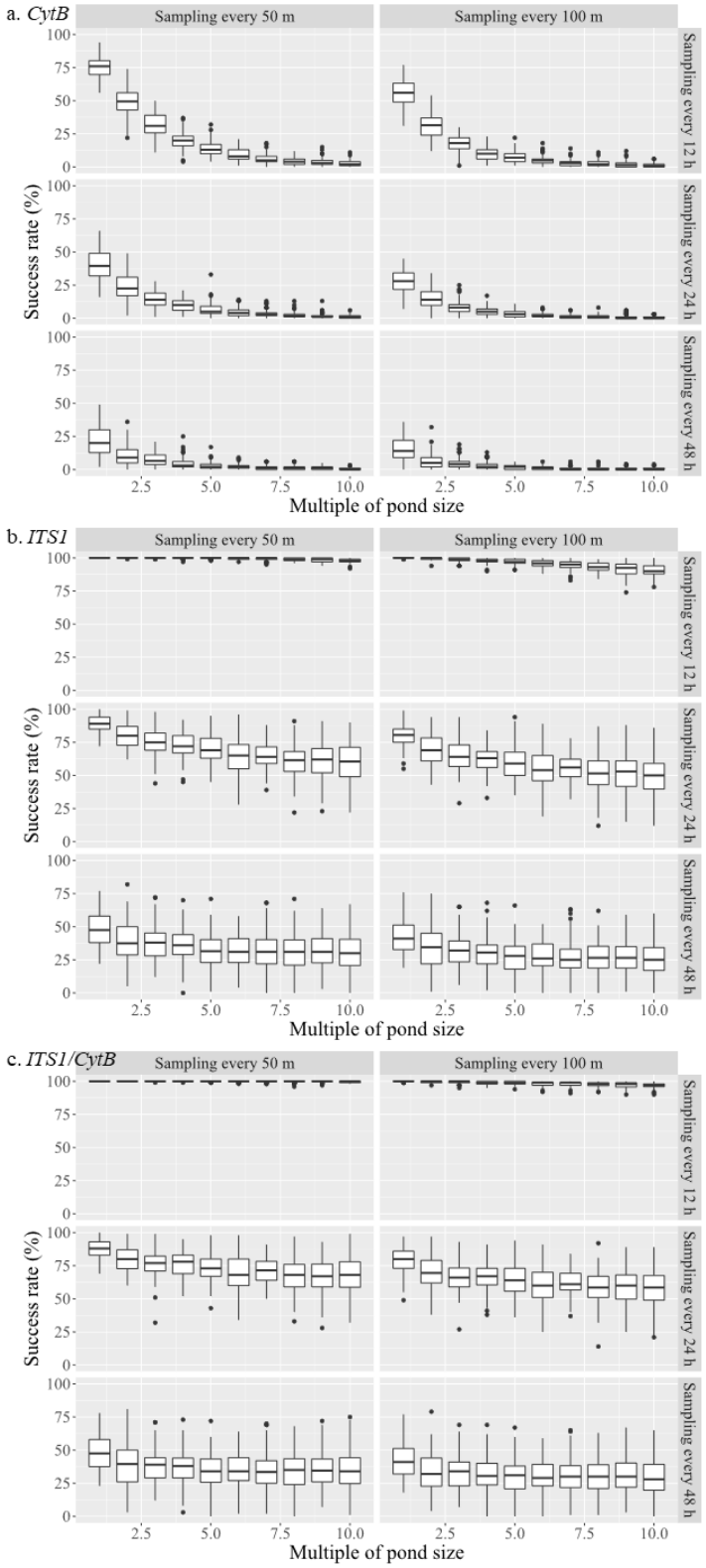

Figure 2-3. Probability of successful monitoring of spawning activity after using different sampling plans

Chapter 3 Estimating the spawning activity of fish species using nuclear and mitochondrial environmental DNA concentrations and their ratios

3.1 Introduction

Fish spawning activity directly affects the reproductive output of a population and future population growth (Scott et al., 2006). Seasonal conditions can have a large impact on juveniles because first-winter mortality is size-dependent and is directly determined by lipid reserves accumulated during the fall. Early spawning allows time for an increase in body size by the time fall is reached, thereby improving overwinter survival and conveying a distinct advantage (Trebitz, 1991; Ludsin and DeVries, 1997). However, some studies have pointed out that early-hatched larvae are subject to variable environmental factors that may reduce survival (Garvey et al., 2002). Monitoring the time and location of fish reproduction can deepen understanding of fish biology and provide important information for effective fish management (Heyman et al., 2019). The spawning activity of fish depends on the combined effects of many factors, such as water temperature, food, sunshine, and water level (Gosch et al., 2006; Matsuzaki et al., 2019), and it is difficult to rely on a single factor to predict fish spawning activities. A common method is to catch adults and juveniles using nets, electric shocks, etc., and then examine their gonads and otoliths to determine the spawning period (Allman et al., 2002; Smith & Walker, 2004), or to collect eggs as direct evidence of spawning (Harada et al., 2015). However, these techniques are time-consuming and labor-intensive, irreversibly damage fish, and hinder their natural reproduction. Therefore, this study aimed to find an accurate and noninvasive method for estimating fish spawning activity.

Currently, environmental DNA (eDNA) analysis has received attention as a noninvasive survey method (Ficetola et al., 2008; Minamoto et al., 2012). Because this method relies on simply collecting DNA fragments that exist in the water for analysis, it can be used to

investigate biological population structures in a body of water without damaging the organisms and with a greatly decreased expenditure of time and labor. This technique has been widely used to investigate fish (Takahara et al., 2012; Thomsen et al., 2012), amphibians (Goldberg et al., 2011; Pilliod et al., 2013), reptiles (Hunter et al., 2015; Davy et al., 2015), crustaceans (Tréguier et al., 2014; Wu et al., 2019) and other organisms. Previous studies have shown that eDNA concentrations increase sharply during fish spawning periods (Spear et al., 2015; Buxton et al., 2017; Tsuji & Shibata, 2021). Furthermore, due to the low proportion of mitochondria in sperm, the ratio of nuclear DNA/mitochondrial DNA (nuDNA/mtDNA) increases during the fertilization activity of fish, thereby allowing the estimation of fish spawning activity (Bylemans et al., 2017).

However, few studies have used eDNA analysis to detect fish spawning activities, and a method to estimate whether fish have spawned based on eDNA concentrations and ratios has been lacking. Water temperature significantly affects fish spawning activities, but few studies have investigated whether the changes in fish eDNA concentrations and ratios that accompany changes in water temperature are consistent with fish life history as determined through field work. In this study, eDNA analysis was used to investigate changes in the eDNA concentration and ratio of common carp (*Cyprinus carpio*), largemouth bass (*Micropterus salmoides*), and bluegill sunfish (*Lepomis macrochirus*) in a small-scale reservoir in Japan. Common carp and largemouth bass are among the top 100 of the world's worst invasive alien species (Global Invasive Species Database: http://www.iucngisd.org/gisd/), and bluegill sunfish is in the top 100 of Japan’s worst invasive alien species (Invasive Species of Japan: http://www.nies.go.jp/biodiversity/invasive/index_en.html). According to the Invasive Species

of Japan database, largemouth bass and bluegill sunfish invaded Japan in 1925 and 1960, respectively, and are now widely distributed throughout Japan. Although there are native carp species in Japan, since the Meiji era (from 1867 to the present), foreign carp have also been released in various places, and hybridization between the native and foreign populations is progressing. These three invasive fishes have had large negative impacts on local ecosystems over a wide range (Fujimoto et al., 2012; Mabuchi and Matsuzaki, 2017). Therefore, these three fishes were used as the target species and attempted to create a method to monitor fish spawning activities using eDNA data to help the management of invasive fishes.

The goals of this study were to develop a method for estimating spawning activity based on eDNA data, to demonstrate that this eDNA data could be used to create high-resolution estimates of spawning activity in terms of both time and space, and to verify the effectiveness of the eDNA method by comparing its results with those from traditional methods. For this purpose, (1) electrofishing was used, and the period of fish spawning activities was estimated from the body length of juveniles; (2) eDNA concentrations and nuDNA/mtDNA ratios were measured based on weekly sampling and investigated their relationship with environmental parameters; (3) models were developed to estimate spawning activity based on the results of weekly sampling, and compared results with those obtained using traditional methods; and (4) new method was applied to samples taken daily, demonstrating the feasibility of using the new method to estimate spawning activity with high resolution.

3.2 Materials and methods

3.2.1 Electrofishing survey

Electrofishing survey was carried out at the Miharu Reservoir in Fukushima Prefecture, Japan (Figure 3-1). The Miharu Reservoir has been in operation since 1998, with a flooded area of 2.9 km^2 and a total water storage capacity of 42,800,000 m^3. Sailed around the bank of the reservoir at a speed of 2–4 km/h in a fiber-reinforced plastic (FRP) boat equipped with an electro-shocker (2.5GPP, Smith Root, USA), to capture largemouth bass and bluegill sunfish. All captured largemouth bass and bluegill sunfish were brought back to the laboratory, and their body lengths were measured. The survey was conducted 48 times from May to October 2019 and 2020. The survey dates are shown in Table S3-3.

3.2.2 Estimating age based on body length and otoliths

Before estimating age based on body length, the relationship between age and body length was examined by analyzing otoliths. The otolith analysis was performed in 2003, 2011, and 2012. Largemouth bass and bluegill sunfish juveniles were captured from the Miharu Reservoir. Body length was measured, and the age of the juveniles was determined using otolith analysis. This information was used to generate linear regression models between the age and body length of juveniles.

The above models were used to estimate the age of largemouth bass and bluegill sunfish captured in electrofishing surveys in 2019 and 2020, performed in parallel with the eDNA surveys below, and estimated the hatching dates of the captured fish. The spawning dates of largemouth bass and bluegill sunfish were estimated by considering the number of days from spawning to hatching, 7 days (Matsuzaki et al., 2019) and 2 days (Akazaki et al., 1970) for largemouth bass and bluegill sunfish, respectively.

3.2.3 eDNA surveys

The survey sites were located in the Hebisawagawa front reservoir at the Miharu Reservoir (Figure 3-1). The front reservoir is an overflow-type reservoir with a capacity of 114,000 m^3 and a retention time of 22 days. The water velocity is close to static. The target species were three species of fish that live in the reservoir: common carp, largemouth bass, and bluegill sunfish. During weekly sampling, 1 L water samples were collected at three sites (MHS-1–MHS-3, Figure 3-1) once a week from March 26 to August 13, 2019, and March 3 to August 25, 2020. Disposable gloves were wearing, and collected water samples from the water surface using new gamma-sterilized plastic bottles. Gloves were changed at different sampling sites to prevent contamination. A total of 135 samples were collected. During daily sampling, 1 L water samples were collected at six sites (MHS-1–MHS-6, Figure 3-1) once a day from June 23 to July 3, 2020. A total of 66 samples were collected.

During the collection of the water samples, the water temperature, pH, and electrical conductivity (EC) were recorded. After the water samples were collected, 1 mL of 10% (mass/volume) benzalkonium chloride solution was added to each sample to prevent DNA degradation (Yamanaka et al., 2017). Water samples were refrigerated, transported back to the laboratory, and filtered within 24 h using 47 mm glass-fiber filters with 0.7 μm pore size (GF/F; GE Healthcare Japan, Tokyo, Japan). All equipment used in filtration were single-use or washed with a bleach solution (diluted household bleach product containing ~0.5 % sodium hypochlorite) and pure water to prevent contamination. For weekly samples, two GF/F filters were used for each water sample to filter 1 L or until clogging. For daily samples, two GF/F filters were used to filter 0.6 L. One liter of ultrapure water was filtered as a negative control

daily, yielding a total of 56 negative controls. The filters were stored at −25 ℃ until DNA extraction.

3.2.4 DNA extraction and qPCR

DNA extraction and PCR preparation were performed in a dedicated room for eDNA analysis, and PCRs were performed in another room. Workspaces were sterilized prior to DNA extraction and PCR preparation using sterile water. For each sample, two filters were combined and the total DNA from both filters was extracted using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the method recommended by the eDNA Society (Minamoto et al., 2021). In brief, each filter was placed in a Salivette tube (Sarstedt, Nümbrecht, Germany), and 440 µl lysis solution, composed of 400 µl of Buffer AL and 40 µl of Proteinase K, was added to the filters. Then the tubes were incubated at 56 ℃ for 30 min. After incubation, the tubes were centrifuged at 5,000 × *g* for 3 min. TE buffer (220 µl) was added to the filters, and the tubes were re-centrifuged at 5,000 × *g* for 1 min to increase the DNA yield. The DNA was purified according to the manufacturer's instructions. The total DNA was eluted in 100 µl AE buffer and stored at -25 ℃ until the polymerase chain reaction (PCR) assay.

The concentrations of nuDNA and mtDNA in all samples were determined by performing three qPCR replicates per sample for the target fragments of nuclear internal transcribed spacer 1 (*ITS1*) and mitochondrial cytochrome b (*CytB*). Each 20 µL PCR mixture contained 2 µL of template DNA, 900 nM of each primer, 125 nM of TaqMan probe, 10 µL of 1x Environmental Master Mix 2.0 (Life Technologies, Foster City, USA), and 0.1 µL AmpErase Uracil N-Glycosylase (Thermo Fisher Scientific, Waltham, MA, USA). The full primer sequences are

shown in Table 3-1. PCR was performed using the StepOnePlus Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) as follows: 2 min at 50 ℃, 10 min at 95 ℃, 55 cycles of 15 s at 95 ℃ and 1 min at 60 ℃.

3.2.5 Data analysis

3.2.5.1 Relationship between eDNA dynamics and environmental variables

The PCR results were kept as integers, and the average value of the triple replicates was calculated and used as the eDNA copy number of the template. The eDNA copy number was used to calculate the *ITS1*/*CytB* ratio. During the calculations, if the denominator value is low enough, a small change can cause a large change in the ratio and distort the results. To avoid this kind of distortion, samples with fewer than four copies were not used to calculate the *ITS1*/*CytB* ratios. Afterward, the copy number of the template was unified to a copy number per liter according to the amount of filtrate and used as the eDNA concentration. Then, a logarithmic transformation was performed with a base of 10. To avoid the problem of a zero value, a value of one was added to all concentration data before log-transformation.

The effects of several environmental variables on changes in eDNA concentration and ratios in the weekly samples were also analyzed. Three generalized additive mixed models (GAMMs) with a Gaussian distribution were used to evaluate the relationship between the log-transformed *ITS1* concentration, the log-transformed *CytB* concentration, and the log-transformed *ITS1*/*CytB* ratio and water temperature, pH, and EC. The random effects on the three sampling sites over the two-year study period were also considered. Linear mixed models

(LMMs) were also fitted to illustrate the necessity of choosing additive models. The details of LMMs are shown in Appendix3-2 (Table S3-4, Figures S3-6, S3-7, S3-8).

3.2.5.2 Modeling spawning probability based on weekly eDNA data

Models were produced for estimating spawning activity based on eDNA data with the following assumptions: in weekly data grouped by site and year, if the *ITS1* concentration, *CytB* concentration, or *ITS1*/*CytB* ratio was greater than the 3rd quartile + 1.5* interquartile range, the fish was considered to have spawned. This estimated spawning activity occurrence was recorded as one, and no spawning activity was recorded as zero. However, even if the above conditions were met, no spawning activity occurrence was recorded if the water temperature exceeded 31 ℃ because the target species do not spawn under such conditions. Based on the binary data obtained, two GAMMs with binomial distributions were generated, one using log-transformed *ITS1* concentration + log-transformed *ITS1*/*CytB* ratio + water temperature and one using log-transformed *CytB* concentration + log-transformed *ITS1*/*CytB* ratio + water temperature. The models were compared and selected according to Akaike's information criterion (AIC). Then the weekly data was used as test data to simply evaluate the selected models and determined the best threshold for judging whether there was spawning activity using the receiver operating characteristic (ROC) curve. If the results from any one of the three sampling sites exceeded the threshold, spawning activity was considered to have occurred that day.

The results of the electrofishing survey were used to determine whether spawning activity occurred in the Miharu reservoir on the days of eDNA sampling. Cohen's kappa was calculated

between the eDNA results and electrofishing results to evaluate the consistency of the two methods.

3.2.5.3 Demonstrating the estimation of spawning probability using daily sampling data

To examine the feasibility of using the method established in this study to estimate spawning probability with high resolution, a model of spawning probability created based on weekly eDNA data was applied to the eDNA data from the daily samples. The *ITS1* concentration, *CytB* concentration, and *ITS1*/*CytB* ratio of eDNA from the largemouth bass and bluegill sunfish were used. The spawning probabilities of both species over 11 consecutive days at the six sites were calculated using the model selected in accordance with the AIC. The confidence interval of the linear predictor was computed using the prediction function in R, and the confidence interval of the spawning probability was then calculated. Among the daily samples, the collection of samples at sites MHS-1, MHS-2, and MHS-3 on June 23 and June 30 overlapped with the collection of weekly samples, and this part of the data did not be excluded from the daily data. The eDNA data from common carp were not used because of the low spawning potential of the common carp during the daily sampling period.

The variance inflation factor (VIF) was used to evaluate collinearity between explanatory variables. GAMMs were run using the *mgcv* package (Wood, 2001), ROC was run using the *pROC* package (Robin et al., 2011), and kappa2 was run using the *irr* package (Fleiss et al., 1969). All analyses were performed using *R*, version 4.0.3.

3.3 Results

3.3.1 Estimating the spawning activity from the body length of juveniles

The linear models of the relationship between body length and age as determined by otolith analysis are shown in Table S3-5 and Figure S3-2. This model was used to estimate the spawning activities of largemouth bass (body length ≤ 70 mm) and bluegill sunfish (body length ≤ 30 mm).

In total, the ages and spawning dates of 1,450 and 1,293 largemouth bass and bluegill sunfish individuals, respectively, were estimated. The results showed that the spawning activity of largemouth bass mainly occurred from April to July, and the spawning activity of bluegill sunfish mainly occurred from July to August (Figure 3-2).

3.3.2 eDNA concentrations and the *ITS1*/*CytB* ratios for the three fish species

All negative controls had zero copies and there was no evidence of cross-contamination. Because partial residual plots showed a possible nonlinear relationship between eDNA concentrations or the ratio and environmental variables (water temperature, pH, and EC) (Figures S3-6, S3-7, S3-8), GAMMs were employed in this study to examine the effects of these variables. The modeling outcomes are shown in Table 3-2. Water temperature had a statistically significant effect on eDNA concentration. For common carp, the eDNA concentration changes with water temperature and reaches a peak at 15 - 18 ℃ (Figure 3-3 a, b), which is consistent with the known life history of the fish (Fernández-Delgado, 1990). The eDNA concentration for largemouth bass and bluegill sunfish increases with increasing water temperature (Figure 3-3 d, e, g, h) but did not show a downward trend. For largemouth bass and bluegill sunfish, pH had a statistically significant effect on eDNA concentration (Table 3-2).

When the pH exceeded 8.5, the eDNA concentrations of the two fishes showed a downward trend, but the effect of pH on the eDNA concentration of the common carp was not statistically significant (Figure 3-4). The EC had a significant effect on the eDNA concentration of largemouth bass; however, the same phenomenon was not observed for the other two fishes (Table 3-2).

For largemouth bass and bluegill sunfish, the water temperature had a statistically significant effect on the *ITS1*/*CytB* ratio (Table 3-2). Although the effect of water temperature on the ratio was not statistically significant for common carp, it showed an inter-year difference. When the two-year data were analyzed separately, the results showed that water temperature had a statistically significant effect on the ratio in 2019 (edf 2.887, $p = 0.023$, Figure S3-5).

3.3.3 Spawning probability modeling based on weekly eDNA data

To estimate spawning probability, two GAMMs with binomial distributions were created and the two models had nearly the same AIC (Table S3-1). The log-transformed *CytB* concentration + log-transformed *ITS1*/*CytB* ratio + water temperature model was selected, and the modeling outcomes are shown in Table S3-2. The spawning probability estimated using this model is shown in Figure 3-5. The ROC curve showed that the optimal thresholds for determining whether the fish spawned were 18.3%, 12.5%, and 9.4% for common carp, largemouth bass, and bluegill sunfish, respectively (Figure S3-3). In accordance with the threshold values, it was determined that the spawning activity of carp mainly occurred from March to May, that of largemouth bass mainly occurred from May to July, and that of bluegill sunfish mainly occurred

from May to August. Cohen's kappa between eDNA results and electrofishing results was 0.3 (p = 0.04) for largemouth bass and 0.423 (p = 0.002) for bluegill sunfish.

3.3.4 Demonstration of the spawning probability model applied to the daily samples

Using the selected GAMM with binomial distribution, the spawning probability of largemouth bass and bluegill sunfish at six sampling sites in the daily samples was estimated (Figure 3-6). Samples showing evidence of higher spawning probability, which was defined as a probability above 90%, included those taken at site MHS-3 on June 23 (confidence interval: 56.2 - 99.4%) for largemouth bass, and at sites MHS-2 and MHS-6 on June 23 (70.5 - 99.2% and 77.1 - 99.7%) and at site MHS-3 on July 2 (93.7 - 100%) for bluegill sunfish.

3.4 Discussion

This study attempted to develop a method for estimating the spawning activity of fish with high resolution from eDNA dynamics data and constructed a framework for estimating spawning probability from eDNA data. The effectiveness of the method was verified by comparing results with those from traditional methods. By applying such a framework, it is possible to estimate the spawning activity of fish with high spatiotemporal resolution. The method was developed using models based on weekly eDNA sampling, and the results showed that the estimated spawning periods coincided with the known fish ecology and the results of traditional methods to a certain extent. By applying this method to daily samples, the feasibility of using this method to estimate spawning probability with high resolution was also examined.

3.4.1 Effect of environmental variables on eDNA concentration

Previous studies have shown that largemouth bass and bluegill sunfish begin to build nests and prepare for spawning when the water temperature exceeds approximately 16 ℃ and 19 ℃, respectively (Kramer and Smith, 1962; Nack et al., 1993; Garvey et al., 2002) and several males repeatedly utilize the same nest site during each of the separate spawning events (Werner and Hall, 1988; Waters and Noble, 2004). The eDNA concentration of largemouth bass and bluegill sunfish increased with increasing water temperature but did not show a downward trend. According to experience, in the Miharu Reservoir, many largemouth bass juveniles will flock to the shore of the lake in August, and the water temperature is relatively high at this time (Figure S3-4). Previous studies have shown that the survival of larval bluegill sunfish is usually highest at temperatures > 23.5 ℃ (Garvey et al., 2002). There is no evidence that large numbers of largemouth bass and bluegill sunfish spawned at water temperatures higher than 31°C. However, as the water temperature rose, the eDNA of largemouth bass and bluegill sunfish did not show a downward trend, which may have been caused by juvenile activity.

3.4.2 Effect of environmental variables on eDNA ratios

For common carp, the eDNA concentration showed a significant peak with water temperature, whereas the *ITS1*/*CytB* ratio only showed a similar peak in 2019. For the largemouth bass, the ratio did not show a significant peak. It can believe that this was caused by a sharp increase in the concentration of mtDNA in the water. When fish spawn, they follow specific spawning behaviors for some time. For the largemouth bass, males and females hover in the nest and touch bellies, quiver, and spawn. Spawning episodes last from 110 min to over 240 min (Isaac et al., 1998). In the process, large amounts of sperm, eggs, and associated mucus are discharged

into the body of water. The copy number of mtDNA in a single cell varies from tens to thousands, depending on individual body condition and cell type (Moraes, 2001; Minamoto et al., 2017). Although the number of mitochondria in sperm is low, other cells discharged into the water body as part of spawning activity do contain large numbers of mitochondria. Therefore, the nuDNA/mtDNA ratio increases depending on the proportion of sperm in the collected sample. In addition, the degradation rate of nuDNA is higher than that of mtDNA (Jo et al., 2020), which may make it more difficult to detect spawning activity solely using the nuDNA/mtDNA ratio.

3.4.3 Effect of time and distance on spawning probability determinations

Based on the weekly eDNA data, a GAMM was generated and applied to the daily data to estimate the spawning probability of the fish. The probability was based on eDNA concentration, ratio, and water temperature; that is, a higher eDNA concentration and ratio provide a higher spawning probability value. However, the eDNA concentration and ratio that increase sharply during spawning activity will degrade over time and be continuously diluted with increasing diffusion distance. Therefore, when spawning activity does occur, the reasons for a decline in spawning probability values can include time and distance. From the daily spawning probability results, the spawning probabilities at different locations were not synchronized, and the data between adjacent time points were not associated with each other. Therefore, considering the average distance (~50m) between sampling locations and sampling interval (once a day), it can conclude that peak eDNA concentration will not diffuse more than 50 m, and the residence time will not exceed 24 h at study sites.

3.4.4 Comparison of the eDNA method and the traditional method

From the results of traditional sampling, the main spawning periods of largemouth bass and bluegill sunfish were estimated to be from April to July and July to August, respectively. The known spawning periods of common carp are from March to April (personal observation by JO). The estimated spawning periods of the three target fish species based on eDNA survey were from March to May (common carp), May to July (largemouth bass), and May to August (bluegill sunfish). Although investigations using traditional methods were not conducted for the common carp, the results of the eDNA method were consistent with commonly recognized carp ecology (Invasive Species of Japan: http://www.nies.go.jp/biodiversity/invasive/index_en.html). For the other two species, investigations were conducted using traditional methods. Cohen's kappa showed that the spawning period estimated by the eDNA method was consistent with those determined using traditional methods for largemouth bass and bluegill sunfish, but the degree of consistency was not high. The traditional method showed that largemouth bass spawned frequently in the Miharu Reservoir from April to May, but the concentration of largemouth bass eDNA concentration was low during this period. The reason for this may be that the low sampling frequency (once a week) did not allow the collection of water samples with high concentrations of eDNA. In addition, the results of the eDNA method showed that the common carp spawned frequently during this period. The active spawning activity of common carp could drive largemouth bass away from sampling sites. From May to June 2020, the eDNA data from some samples suggested that bluegill sunfish had spawning activity during this time, which was inconsistent with the results obtained by the traditional method. This may be related to an increase in eDNA concentration in some areas caused by biological aggregation (Doi et al.,

2017). In summary, it can believe that it is feasible to use the established eDNA-based method to estimate fish spawning activity.

Fish spawning probability was estimated using models based on eDNA concentration, ratio, and water temperature. Due to the rapid decline in eDNA described above, a higher probability means that the sampling time was spatially and temporally close to the spawning time and location of the fish. This means that if sampling occurs at a higher frequency in time and space, the eDNA method has the potential to accurately determine the time and location of spawning activity (e.g., Tsuji and Shibata, 2021).

3.5 Conclusions

This study demonstrated a method for estimating fish spawning activity using eDNA data. A traditional survey was conducted in parallel, and the reliability of the new method was demonstrated by comparing the results of the two methods. For two of the three species, it was able to estimate the spawning period using the method developed in this study, but for one species, still faced some challenges. With this method, fish spawning activities can be estimated without conducting laborious traditional surveys, which can facilitate the management of invasive species and monitoring the reproduction of rare or important fishery species. Although the method is affected by biomass and the diffusion and degradation of eDNA, it has the potential to accurately determine spawning activities on a spatiotemporal scale. Further research on the diffusion distance and degradation time of the eDNA concentration peak caused by fish spawning activity in the water body may help to monitor spawning activity more accurately.

3.6 Tables

Table 3-1. Primers and probes used in this study

Species	Target	Primer and probe	
Common carp	*CytB* (Takahara *et al.*, 2012)	Forward	5'-GGTGGGTTCTCAGTAGACAATGC-3'
		Reverse	5'-GGCGGCAATAACAAATGGTAGT-3'
		Probe	5'-FAM-CACTAACACGATTCTTCGCATTCCACTTCC-TAMRA-3'
	ITS1 (Minamoto *et al.*, 2017)	Forward	5'-TTCAAAGACCCCCCGTAAC-3'
		Reverse	5'-GCCATGCCGCACACA-3'
		Probe	5'-FAM-TCACGACCCCCCTTATTTTTTCCAAAACC-TAMRA-3'
Largemouth bass	*CytB* (Yamanaka *et al.*, 2016)	Forward	5'-GCCCACATTTGTCGTGATGTAA-3'
		Reverse	5'-AGCCCCGGCCGATATG-3'
		Probe	5'-FAM-CTAACGGTGCATCCTTCTTTTTCATCTGCA-TAMRA-3'
	*ITS1**	Forward	5'-GGTACCCAACTCTCCTCCC-3'
		Reverse	5'-GTGGGGTTTGAAAGGGGATGA-3'
		Probe	5'-FAM-CTCCCCCCAGCTCTCGCGG-TAMRA-3'
Bluegill sunfish	*CytB* (Takahara *et al.*, 2013)	Forward	5'-GCCTAGCAACCCAGATTTTAACA-3'
		Reverse	5'-ACGTCCCGGCAGATGTGT-3'
		Probe	5'-FAM-CGACATCGCAACTGCCTTCTCTTCAGT-TAMRA-3'
	*ITS1**	Forward	5'-GGGTACCCAACTCTCCTCTC-3'
		Reverse	5'-TCGGAGGCCACAGTTCAG-3'
		Probe	5'-FAM-AGCTCCCGGCCCGA-MGB-3'

* Designed for this study.

Table 3-2. Results of the generalized additive mixed models (GAMMs)

		CytB Concentration		*ITS1* Concentration		*ITS1*/*CytB* Ratio	
Target Fishes	**Variable**	edf	p-value	edf	p-value	edf	p-value
Common carp	s(Tem)	3.507	< 0.001 ***	3.208	0.003 **	1.000	0.274
	s(pH)	1.818	0.378	2.062	0.248	3.975	0.0756
	s(EC)	1.000	0.064	1.000	0.274	1.000	0.9017
	Random effects	3.695	0.004 **	3.756	0.003 **	4.825	< 0.001 ***
Largemouth bass	s(Tem)	2.225	< 0.001 ***	4.667	< 0.001 ***	1.757	0.027 *
	s(pH)	2.937	< 0.001 ***	2.831	0.013 *	1.000	0.286
	s(EC)	2.303	0.018 *	1.233	0.006 **	1.000	0.391
	Random effects	0.447	0.355	3.789	0.002 **	0.456	0.352
Bluegill sunfish	s(Tem)	4.502	< 0.001 ***	4.188	< 0.001 ***	2.37	0.009 **
	s(pH)	3.466	0.010 **	1.001	0.003 **	1.000	0.171
	s(EC)	2.195	0.376	2.164	0.355	1.000	0.417
	Random effects	4.543	< 0.001 ***	4.369	< 0.001 ***	< 0.001	0.771

Significance codes: '***' <0.001; '**'< 0.01; '*' <0.05

"Tem" refers to water temperature and "EC" refers to electrical conductivity.

"s()" refers to a smooth term, and "edf" refers to effective degrees of freedom.

3.7 Figures

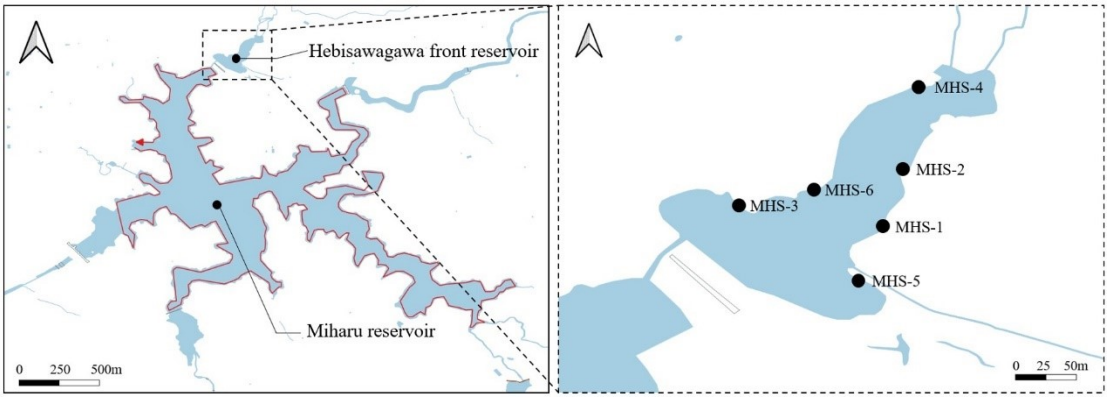

Figure 3-1. Map of electrofishing survey at Miharu reservoir (Fukushima, Japan) and the eDNA survey sites in the Hebisawagawa front reservoir. The red arrow line refers to the approximate path of the electrofishing survey. Weekly eDNA samples were collected at sites MHS-1, -2, and -3 once a week from March 26 to August 13 in 2019 and March 3 to August 25 in 2020. Daily eDNA samples were collected at all six sites once a day from June 23 to July 3 in 2020.

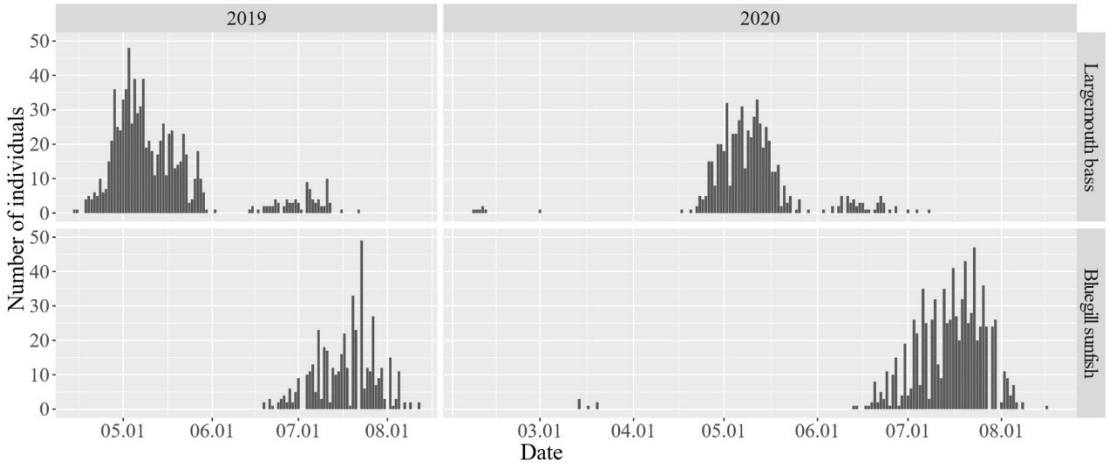

Figure 3-2. Fish spawning periods, as estimated from body lengths of largemouth bass and bluegill sunfish in the Miharu reservoir.

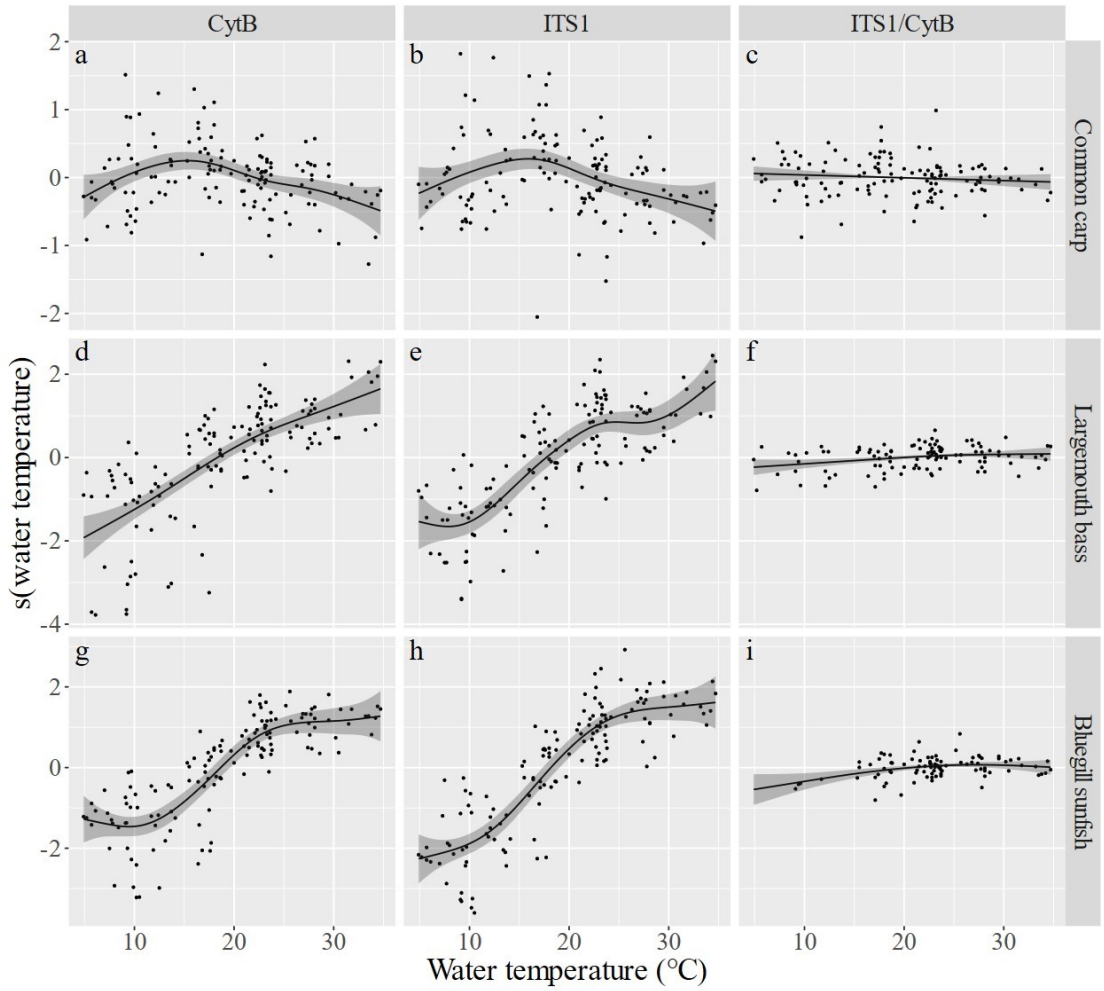

Figure 3-3. Smooth term of water temperature on the eDNA concentration and ratio. Graphs a, d, and g refer to the influence of water temperature on the mitochondrial *CytB* concentration of common carp, largemouth bass, and bluegill sunfish, respectively. Graphs b, e, and h refer to the influence of water temperature on the nuclear *ITS1* concentrations of common carp, largemouth bass, and bluegill sunfish, respectively. Graphs c, f, and i refer to the influence of water temperature on the *ITS1*/*CytB* ratios of common carp, largemouth bass, and bluegill sunfish, respectively.

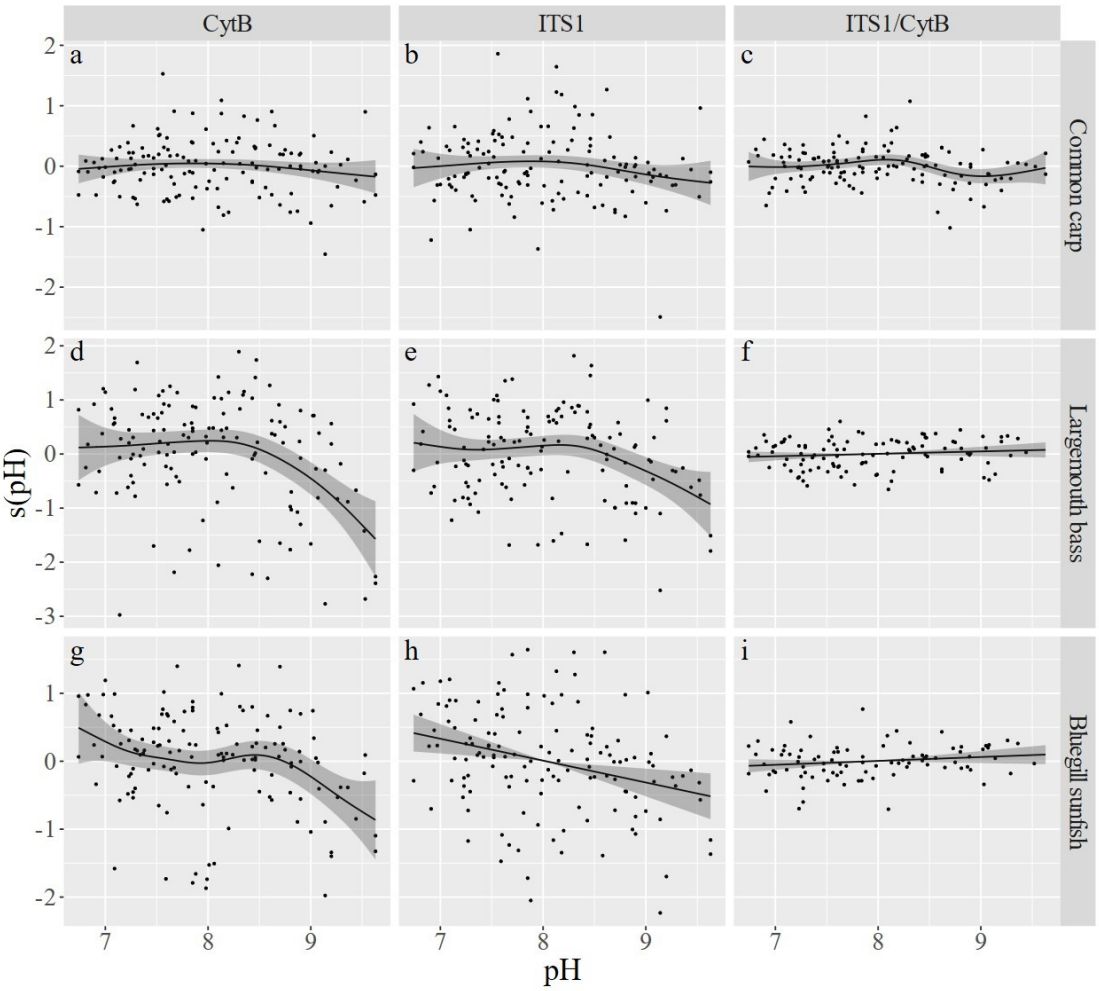

Figure 3-4. Smooth term of pH on the eDNA concentration and ratio. Graphs a, d, and g refer to the influence of pH on the mitochondrial *CytB* concentration of common carp, largemouth bass, and bluegill sunfish, respectively. Graphs b, e, and h refer to the influence of pH on the nuclear *ITS1* concentration of common carp, largemouth bass, and bluegill sunfish, respectively. Graphs c, f, and i refer to the influence of pH on the *ITS1*/*CytB* ratio of common carp, largemouth bass, and bluegill sunfish, respectively.

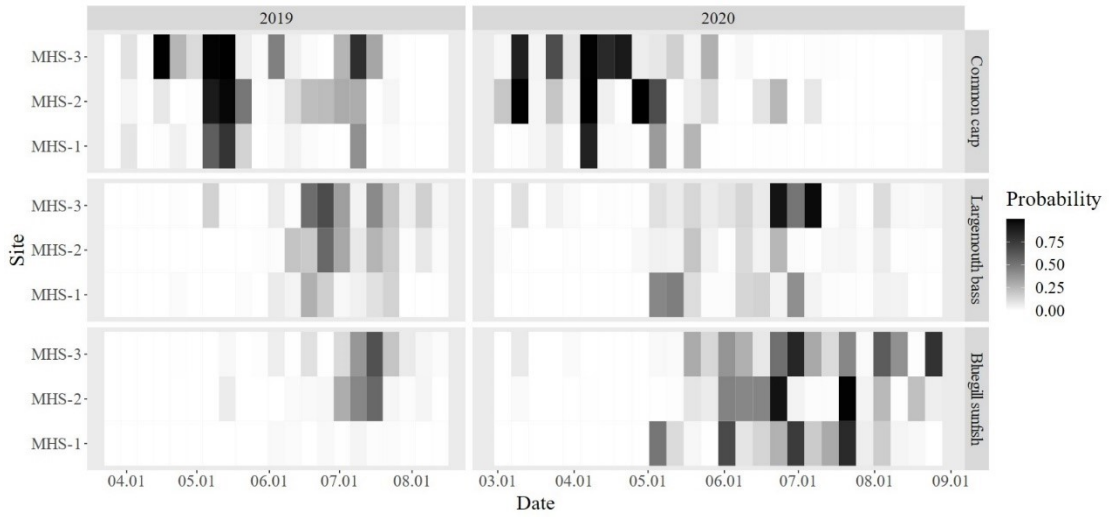

Figure 3-5. Test results of models in weekly samples. Each grid represents a sample, and the color represents the spawning probability calculated by a GAMM with binomial distribution.

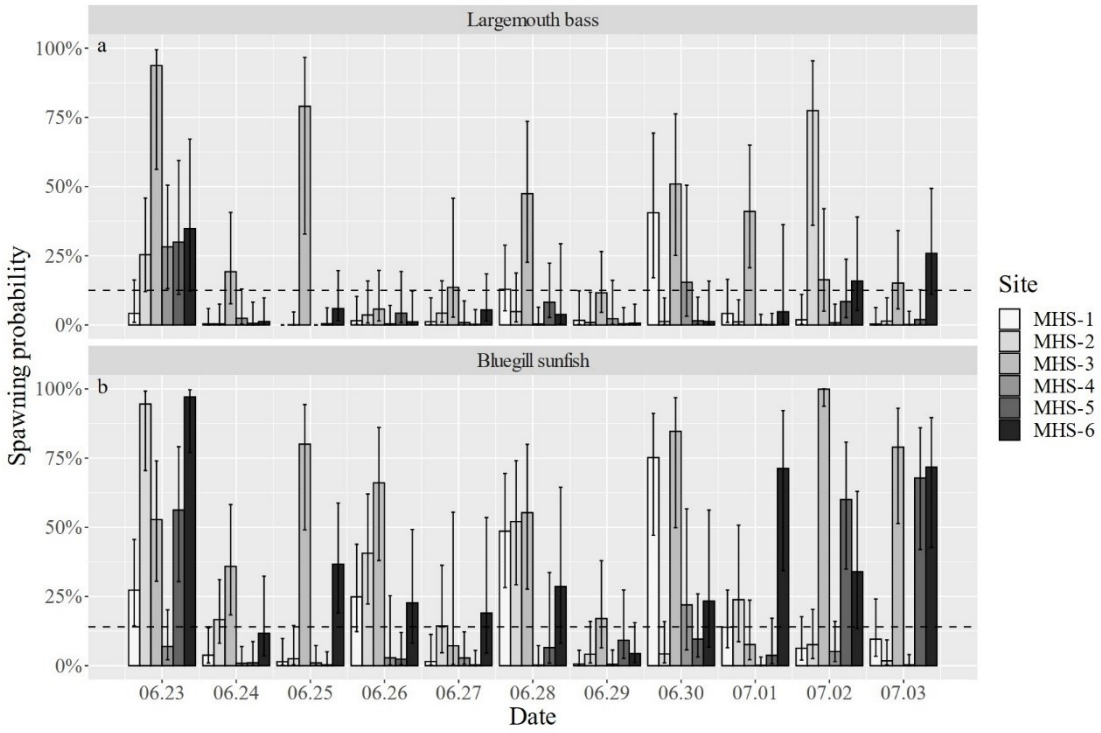

Figure 3-6. Spawning probability of largemouth bass and bluegill sunfish during daily sampling periods. Graphs a and b refer to the spawning probabilities of largemouth bass and bluegill sunfish, respectively. The dotted line represents the threshold determined by ROC analysis.

Chapter 4 Monitoring of multiple fish species by time-series quantitative environmental DNA metabarcoding surveys

4.1 Introduction

Ecological monitoring of fish, such as changes in fish population structure, fish distribution associated with environmental variables, and the time and location of fish spawning activities can provide important information for the protection of endangered fish (Chollett et al., 2020), the sustainable use of fishery resources (King et al., 2009; Erisman et al., 2017), and the management of alien species (Jackson et al., 2004). Traditional methods are used to perform ecological surveys of fish through visual inspection, electrofishing etc. (Copp and Peňáz, 1988). Visual surveys are time-consuming and inefficient (Rowland, 1999), and electrofishing sometimes harm fish and may interfere with their natural spawning activity (Snyder, 2003). A non-invasive method that can efficiently monitor the fish spawning activities would be a valuable tool for the management of aquatic biodiversity.

As a non-invasive method, eDNA technique is being widely used for ecological survey. eDNA technology only needs to collect DNA in water for analysis, and therefore, it has the advantages of greatly reducing the cost and labor of field investigation, and does not damage the environment or interfere with the natural activities of organisms (Thomsen and Willerslev, 2015). eDNA technique has been widely used in the monitoring of fish (Jerde et al., 2011; Takahara et al., 2013), amphibians (Ficetola et al., 2008; Pilliod et al., 2013), crustaceans (Tréguier et al., 2014; Wu et al., 2018), reptiles (Hunter et al., 2015; Davy et al., 2015), birds (Ushio et al., 2018), mammals (Foote et al., 2012; Ushio et al., 2017) and aquatic plants (Scriver et al., 2015, Fujiwara et al., 2016).

eDNA concentration correlates with biomass, therefore previous studies used quantitative PCR (qPCR) to quantify eDNA concentration and then estimated fish biomass (Lacoursière-

Roussel et al., 2016; Doi et al., 2017), and also other studies used metabarcoding to investigate fish β-diversity based on relative abundance (Bagley et al., 2019; Xie et al., 2021). Fish external fertilization will release a large number of sperm and eggs into the water during spawning activity, resulting in a sharp rise in eDNA concentration and nuclear eDNA/mitochondrial eDNA ratio in a short period of time (Tsuji & Shibata, 2021; Bylemans et al., 2017; Wu et al., 2022), which makes the eDNA approach have the potential to accurately monitoring fish spawning activities by day. The previous study tried to estimating fish spawning activities by investigate the extremely high values of the eDNA concentration and ratio quantified by qPCR (Wu et al., 2022). There is also study that use the peak of relative abundance calculated by reads obtained from metabarcoding to estimate fish spawning activities (Di Muri et al., 2022). However, relative abundance was affected by the combined effects of eDNA released by all detected species, the validity of using relative abundance to estimate spawning activity still needs to be verified.

The previous studies used qPCR to quantify DNA concentrations in environmental samples (Doi et al., 2017; Langlois et al., 2021). However, its quantitative performance may be affected by inhibition (Lance and Guan, 2020), and in a single qPCR experiment can only quantify a single target species, and repeated experiments are required if targeting multiple species. For multi-species studies, metabarcoding techniques are widely used (Miya et al., 2015; Nakagawa et al., 2018). PCR was performed using universal primers combined with high-throughput sequencing to detect all target species simultaneously. Compared with qPCR, metabarcoding technology can simultaneously detect multiple target species in a single experiment, but generally does not have the ability of quantification.

Previous study proposed that adding internal standard DNAs to the metabarcoding approach, and converting the sequencing reads into copy numbers, so as to achieve the purpose of quantification (Ushio et al., 2018). Compared with traditional metabarcoding and species-specific qPCR, this method can achieve simultaneous quantification of multiple species with higher efficiency. At present, some studies have adopted this method to measure the copy number of multiple species (Tsuji et al., 2022a; Nakagawa et al., 2022), and showed significant positive relationships between the eDNA concentrations and the abundances among species (Tsuji et al., 2022b), but this approach still lacks the application in long-term time-series field surveys.

In this study, long-term quantitative metabarcoding approach was used to simultaneously quantify the eDNA concentration of multiple fish species in a small reservoir, and combined with the method of previous research to estimating the spawning activities of multiple fish species in the reservoir. The main research objectives are as follows: (1) Using quantitative metabarcoding to monitor the long-term changes in eDNA concentrations of multiple fish species and compare them with the results of traditional surveys to verify the validity of quantitative metabarcoding. (2) Using the quantitative data to investigate the fish composition and the change of fish-specific eDNA concentration with environmental variables. (3) Using long-term quantitative data to estimate the spawning activities of multiple fish species.

4.2 Materials and methods

The survey sites were located in the Hebisawagawa front reservoir at the Miharu Reservoir in Fukushima Prefecture, Japan (Figure 4-1). The total area of the Hebisawagawa front reservoir

was about 44,000 m^2. This front reservoir is connected to the main reservoir by a channel with a width of 5 m and a depth of 5 m. It is isolated during the summer season (June 11th to October 10th) when the water level is low. Both traditional surveys and eDNA surveys were conducted to investigate the individuals and eDNA concentration of fish, respectively. The all eDNA samples are collected in the previous study (Wu et al., 2022), and therefore, the details of sampling sites, measures to prevent cross contamination, and DNA extraction are only briefly described in this paper.

4.2.1 Traditional surveys

Traditional surveys were conducted in Hebisawagawa front reservoir at 2007–2010. A partition net with a mesh size of 10 mm was installed to isolate the front reservoir from main reservoir during periods when the water level was high. The fish were caught using the partition net and the fixed net. Catching with the partition net was carried out using the draw-down operation of the main reservoir when the water level was lowered in preparation for summer floods. Catching with the fixed net was carried out in two stages. In the first stage, the draw-down operation was used to catch fish in the enclosed area when the water level dropped and the shallows dried up. Nets with a mesh size of 10 mm were installed at two locations so as to surround a range of 1.5 m water depth in the shallow area of the reservoir lakeshore. The total enclosed area was about 7,500 m^2. In the second stage, the valve at the bottom of the front reservoir was opened to drain the water, and the water level was lowered by 1.5 m to catch fish with the fixed net. The total enclosed area at the second stage was about 40,000 m^2. In addition

to the above surveys, the 2019 traditional survey data were queried from the River Environmental Database (http://www.nilim.go.jp/lab/fbg/ksnkankyo/dl_82_index.html).

4.2.2 eDNA sampling and extraction

Water samples were collected at three sites (MHS1–MHS3, Figure 1) once a week from March 26 to August 13, 2019, and March 3 to August 25, 2020. Benzalkonium chloride solution (1 mL of 10% mass/volume) was immediately added to the water samples to prevent DNA degradation (Yamanaka et al., 2017). A total of 141 samples were collected. The water temperature (WT), pH, and electrical conductivity (EC) were recorded during the collection of the water samples. Water samples were filtered until clogging (up to 1 L) by using two 47 mm glass-fiber filters with 0.7 μm pore size (GF/F; GE Healthcare Japan, Tokyo, Japan). Ultrapure water (1 L) was filtered as a filtering negative control, yielding a total of 47 filtering negative controls. The filters were stored at −25 ℃ until DNA extraction.

Total DNA was extracted using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the method recommended by the eDNA Society (Minamoto et al., 2021). Briefly, two filters of each sample were combined in a single Salivette tube (Sarstedt, Nümbrecht, Germany). Buffer AL (400 μl) and Proteinase K (40 μl) was mixed well as lysis solution, and then added to the filters. After filters were incubated at 56 ℃ for 30 min, they were centrifuged at 5,000 × g for 3 min to collect eDNA. TE buffer (220 μl) was added to the filters, and re-centrifuged at 5,000 × g for 1 min to increase the DNA yield. Then, the DNA was purified according to the manufacturer's instructions. The total DNA was finally eluted in 100 μl AE buffer and stored at -25 ℃ until paired-end library preparation.

4.2.3 Paired-end library preparation

Five different internal standard DNAs were designed and prepared according to the previous study (Ushio et al., 2022). MiFish-U primer set (forward: 5′-ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT NNN NNN GTC GGT AAA ACT CGT GCC AGC-3′, and reverse: 5′-GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATC TNN NNN NCA TAG TGG GGT ATC TAA TCC CAG TTT G-3′) was used to amplify the hypervariable region of the fish mitochondrial 12S rRNA gene (Miya et al., 2015). Two PCRs were performed to amplify the DNA of the target region and add sequencing primers respectively.

In the first-round PCR (1st PCR), each 12 μl PCR mixture contained 6.0 μl 2 × KAPA HiFi HotStart ReadyMix (Roche, Basel, Switzerland), 0.36 μl each of primers with 300 nM final concentration, 1.0 μl template DNA, 1.0 μl internal standard DNA mix containing 40, 20, 10, 5, and 1 copies per reaction and 3.28 μl pure water. The thermal cycle profile was as follow: an initial 3 min denaturation at 95 ℃, 40 cycles of 98 ℃ for 20 s, 65 ℃ for 15 s, and 72 ℃ for 15 s, with a final extension at 72 ℃ for 5 min. PCR negative controls with internal standard DNAs was employed for each 1st PCR run to monitor contamination during the experiments. Since this samples have been verified by qPCR for the absence of contamination in the previous study (Wu et al. 2022), only seven filtering negative controls were selected from 47 filtering negative controls for experimentation in this study. Four replications were performed for each sample and negative controls to minimize the PCR dropouts. For each sample, the 1st PCR products of four replicates were pooled and size-selected for 200–400 bp using a SPRIselect (Beckman Coulter, Inc.) according to the manufacturer's instructions. The concentrations of the size-selected amplicons were quantified using Qubit fluorometer 3.0 (Thermo Fisher Scientific Inc.)

with Qubit dsDNA HS assay kit, and then diluted to 0.1 ng/μl with sterilized distilled H_2O. All diluted products were frozen at -25℃ until second-round PCR (2nd PCR).

The 2nd PCR was performed to add iSeq adaptor sequences and 8-bp index sequences to both ends of the amplicons. Each 12 μl PCR mixture contained 6 μl 2 × KAPA HiFi HotStart ReadyMix, 2 μl each primer with 300 nM final concentration, 1 μl diluted 1st PCR product and 1 μl pure water. The thermal cycle profile was as follow: an initial 3 min denaturation at 95 ℃, 12 cycles of 98 ℃ for 20 s and 72 ℃ for 20 s, with a final extension at 72 ℃ for 5 min. Since all 2nd PCR products have different index, every 38 products are mixed into a single tube as library, for a total of 4 libraries. The 4 libraries were purified and size-selected (around 370 bp) using E-Gel SizeSelect 2% (Thermo Fisher Scientific Inc.). The size distribution of the purified libraries was checked by Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). Finally, the 4 libraries were diluted to 1 nM with sterilized distilled H_2O, and were sequenced on the iSeq 100 platform (Illumina, Inc) with an iSeq 100 i1 Reagent v2 (Illumina, Inc) for 2× 150 bp pair-end according to the manufacturer's instructions.

4.2.4 Data preprocessing and taxonomic assignment

The raw iSeq data were preprocessed using USEARCH v11.0.667 (Edgar 2010) to generate zero-radius OTUs (ZOTUs) according to the steps as those described by Sakata et al. (2020) with a few modifications. (1) the “fastq_mergepairs” command was used to merge the paired-end reads; (2) the “fastx_truncate” command was used to remove the primer sequences; (3) the “fastq_filter” command was used to remove low-quality reads with an expected error rate of > 1% (Edgar & Flyvbjerg, 2015) and too short reads of < 140 bp; (4) the “fastx_uniques”

command was used to dereplicate the reads and singletons were removed. (5) the “unoise3” command was used to generate ZOTUs. Chimeras and ZOTUs less than 8 reads were removed; (6) the “otutab” command was used to generate ZOTU table under 97% identity threshold; (7) The “usearch_global” command was used to compare the ZOTUs to the local database to determine the internal standards under the condition that a sequence identity of > 98.5% (two nucleotide differences allowed) with the reference sequences and a query coverage of ≥ 90%. Finally, ZOTUs were compared to the NCBI nr database by using blastn under the same condition to perform taxonomic assignment.

The DNA copy numbers were calculated from sequence reads according to the method described by Ushio et al. (2018). In brief, linear regression with intercept set as zero was performed to examine the relationship between sequence reads and the copy numbers of the internal standard DNAs for each sample. The sequence reads of non-standard fish DNAs were converted to copy numbers by dividing the number of iSeq sequence reads by a sample-specific regression slope. To remove contaminants, the copy numbers in the PCR negative control were subtracted from the corresponding sample. Then the copy number per filter volume (copies/L filter volume) was calculated as eDNA concentration according to the filtration volume of each sample. Due to the lack of research on quantitative eDNA metabarcoding limit of quantification, data less than one copy are denoted as zero copy (approximately equal to discarding the reads less than four). Fish species detected only once in 141 samples were removed.

4.2.5 Data analysis

Linear model was performed to evaluate the relationship between fish eDNA concentration and the number of fish individuals obtained from traditional surveys. A Bray–Curtis based analysis of similarities (ANOSIM) was performed to test for statistical differences in fish eDNA composition between samples from different months with 9,999 permutations. Data of concentration were log-transformed based on natural logarithms before calculating the Bray-Curtis distance, and all data were +1 to avoid 0 values. A Bray-Curtis based two-dimensional Nonmetric Multi-Dimensional Scaling (NMDS) was performed to demonstrate differences in fish eDNA composition between samples. GAMs were fitted to the ordination axis scores for the environmental variables (water temperature, pH, EC) and then the smoothed surfaces were plotted over the NMDS by using the "ordisurf" command.

The Hierarchical generalized additive model (HGAM) was performed to estimate the changes of fish eDNA concentration with environmental variables, and the data of 0 copy were not included in the analysis. The model structure is designed as a global smoother plus fish-level smoothers that have the same wiggliness (Pedersen et al., 2019): log(Copy) ~ s(WT,m=2) + s(WT,Fish,bs='fs',m=2) + s(pH,m=2) + s(pH,Fish,bs='fs',m=2) + s(EC,m=2) + s(EC,Fish,bs='fs',m=2) + s(Site,Year,bs='re'). Restricted maximum likelihood (REML) was used to select smoothing parameter (Wood, 2011).

The spawning activities of fish species were estimated according to the method proposed in the previous study (Wu et al., 2022). In brief, in data grouped by site and year, if the eDNA concentration was greater than the 3rd quartile + 1.5* interquartile range, the fish was considered to have spawned. However, even if the above conditions were met, no spawning activity occurrence was recorded if the water temperature exceeded 30 ℃ because the target

species do not spawn under such conditions. Based on the binary data obtained, another HGAM with binomial distributions were generated as: spawning ~ log(copy) + log(copy)*Fish + s(WT,m=2) + s(WT,Fish,bs='fs',m=2). REML was also used. ANOSIM and NMDS was ran by using the *vegan* package (Oksanen et al., 2022), HGAMs was ran by using the *mgcv* package (Wood, 2006). *ggtree* package (Yu, 2022) and *ggplot2* package (Wickham, 2016) was used to plot phylogenetic tree based on neighbor-joining method and other graphs. All analyses were performed using *R*, version 4.2.1 (R Core Team, 2022).

4.3 Results

4.3.1 iSeq raw data preprocessing

The iSeq paired-end sequencing of the 152 libraries for this study [containing 141 samples (47 times sampling × three sites), 7 filtering negative controls, 4 PCR negative controls] yielded a total of 12,630,140 reads, of which 12,288,756 (97.3%) passed the merging processes, and 10,771,744 (85.3%) passed the quality control processes. Subsequently, 657 ZOTUs were generated after denoising and 12,055,551 (95.5%) reads were matched to the ZOTUs (>97% identity). Finally, 11,558,502 (91.5%) reads of 42 ZOTUs were assigned to the standard DNAs and fish taxa (>98.5% identity). Number of non-standard fish iSeq reads were 4,342,992 out of the 11,558,502 (37.6%), of which 4,334,000 (99.8%) were from fish DNAs of field samples and 8,992 (0.2%) were from fish DNAs of negative controls. The copy number of the four PCR negative controls accounted for about 0.03% of the copy number of all samples and filtering negative controls, and this part of the copy number was removed from corresponding samples and filtering negative controls. The average copy number ratio of the seven filtering negative

controls to the corresponding sample copy number was 0.46%, which could be considered as almost no contamination, therefore no more treatment was done for the copy number in the filtering negative controls.

4.3.2 eDNA survey and traditional survey

A total of 15 fish species were detected in 2019 and 2020 using the quantitative eDNA metabarcoding approach (Figure 4-2). The traditional survey results (a total of 6,706 individuals) in 2007, 2008, 2009, 2010 and 2019 showed the presence of 19 fish species (Figure 4-2). A total of 20 species of fish were detected by the two methods, and the dominant species were *Cyprinidae* and *Centrarchidae*. Traditional surveys detected an average of 13.4 fish species each year, and eDNA surveys detected an average of 14.5 fish species each year, showing the same detection capabilities of the two survey methods. *Ctenopharyngodon idella* was only detected in the eDNA approach, and only one individual of *Tridentiger brevispinis*, *Oncorhynchus masou*, two individuals of *Rhynchocypris steindachneri*, three individuals of *Anguilla japonica* and ten individuals of *Rhodeus ocellatus* was detected respectively in the traditional approach. *Anguilla japonica* was also detected in the eDNA approach, but was removed from the data because it was detected only once in 141 eDNA samples. All fish species except extremely rare ones were detected by quantitative eDNA metabarcoding approach, concluding *Rhinogobius* sp., *Gymnogobius urotaenia*, *Micropterus salmoides*, *Lepomis macrochirus*, *Silurus asotus*, *Misgurnus anguillicaudatus*, *Tribolodon hakonensis*, *Zacco platypus*, *Carassius* sp., *Carassius cuvieri*, *Cyprinus carpio*, *Hemibarbus barbus*, *Gnathopogon elongatus*, *Pseudorasbora parva*. The Bray-Curtis distances between different survey results

based on the relative abundance of fish were shown in Figure S4-3. Linear regression was used to evaluate the relationship between the eDNA concentration and the number of fish individuals investigated by traditional survey. The results of the 2019 eDNA survey and the traditional survey showed a statistically significant relationship between the eDNA copy numbers and fish abundances ($p = 0.025$, adjusted R-Squared: 0.278, Figure 4-3). Multi-year merged data from two years of eDNA surveys and five years of traditional surveys also showed a statistically significant relationship ($p < 0.001$, adjusted R-Squared: 0.627, Figure S4-2).

4.3.3 β-diversity

The results of ANOSIM showed that the fish eDNA concentration composition was significantly different in different months ($R = 0.306$, $p < 0.001$), but the difference between sampling sites ($R = 0.025$, $p = 0.007$) and years ($R = 0.09$, $p < 0.001$) was relatively small. The NMDS graph shows the changing trend of fish eDNA composition in different months and the main fish that drive the changes in fish eDNA composition (Figure 4-4a). The distances between samples were large in March and April, and gradually decreased after May. The main characteristic fish in March and April is *C. carpio*, *C. cuvieri* and other fishes of the *Carassius*, in May is *C. idella*, *S. asotus*, *G. urotaenia*, *G. elongatus* and *M. anguillicaudatus*, in June, July and August is *M. salmoides*, *L. macrochirus*, *P. parva* and *H. barbus* (Figure 4-4a). The GAM results of the NMDS ordination axis and environmental variables showed that the change of the ordination axis mainly explained the change of water temperature (Deviance explained = 53.3%, $p < 0.001$), and also explained a part of pH (Deviance explained = 15%, $p < 0.001$) and EC (Deviance explained = 17%, $p < 0.001$).

4.3.4 eDNA concentration and environmental variables

Changes in fish eDNA concentration with environmental variables were fitted using HGAM. The modeling outcomes are shown in Table 1. Figure 5 shows trend of the fish-specific eDNA concentrations changing with environmental variables. The plots including data points are shown in Figure S4. Water temperature, pH, and EC significantly affected the changes in the fish eDNA concentration. Meanwhile, the change trends of the fish-specific eDNA concentration with water temperature and pH were different, but there was no statistical difference with the EC (Table 4-1). The eDNA concentration of different fish species peaked at different water temperatures, *T. hakonensis*, *C. cuvieri*, *C. carpio*, *G. elongatus* peaked at 10-20 °C, *G. urotaenia*, *L. macrochirus*, *Z. platypus*, *H. barbus* and *P. parva* peaked at 20-30°C (Figure 4-5). The variation of eDNA concentration with pH was slightly different among different fish species, and the main difference was in the pH range 7-8.5. The eDNA concentrations of *S. asotus*, *M. anguillicaudatus*, *T. hakonensis*, *C. idella*, and *G. elongatus* showed an upward trend in this pH range, while that of other fish species tended to be flat, but the eDNA concentrations of all fish species showed a downward trend after pH exceeded 8.5 (Figure 4-5). The eDNA concentrations in all fish species showed a uniform downward trend with increasing EC (Figure 4-5).

4.3.5 Fish spawning activities

By detecting outliers from eDNA data, the spawning activities of 13 fish species were estimated for a total of 15 fish species. *S. asotus* and *C. idella* were only detected 4 times and 19 times respectively in 141 samples, and spawning activities could not be estimated due to the failed

identification of outlier values. The spawning activities of *G. urotaenia* estimated using eDNA concentrations was inconsistent between the two years, with the 2019 results showing that the outlier values were mainly concentrated in June-July, while those in the 2020 were mainly concentrated in March-April. The spawning activities of fish species except *G. urotaenia* were roughly in line with the fish spawning period recorded by the Research Institute of Environment, Agriculture and Fisheries, Osaka Prefecture (http://www.kannousuiken-osaka.or.jp/zukan/) (Figure 4-6 dotted box). According to the results of traditional surveys, *Rhinogobius* sp., *Carassius* sp. are mainly composed of *R. kurodai* and *C. a. langsdorfii*, therefore the spawning period of these two fish species was queried.

4.4 Discussion

A long-term eDNA survey was conducted in three sites of Hebisawagawa front reservoir for two years, and the quantitative Metabarcoding approach was used to quantify the eDNA concentration of 15 fish species in 141 samples. The results of the long-term survey showed that the results of the quantitative eDNA metabarcoding approach were highly consistent with the historical results of the traditional survey. The relationship between the eDNA concentration of different fish species detected by quantitative metabarcoding and the individual numbers of fish recorded by traditional survey is statistically significant, which demonstrates the effectiveness of quantitative metabarcoding approach in long-term eDNA survey. Using the advantages of simultaneous measurement of the eDNA concentration of multiple fishes, differences in fish eDNA composition within different samples were revealed by NMDS. The relationships between eDNA concentration and environmental variables in 15 fish species were

also analyzed by performing HGAM. By detecting outliers from eDNA data, the spawning activities of 13 fish species were estimated. Most of the estimated spawning activities were consistent with the known fish spawning periods, which demonstrates the potential of long-term quantitative eDNA metabarcoding to simultaneously monitoring the spawning activities of multiple fishes.

4.4.1 Quantitative metabarcoding

The median adjusted R-squared for a total of 152 linear regressions was 0.987, and most of the adjusted R-squared was above 0.96. The specific distribution is shown in Figure S4-1. The addition of internal standard DNAs takes up a portion of the sequencing reads and may reduce the species detection ability, therefore an average of about 80,000 reads were assigned to each sample to ensure that enough reads were assigned to none internal standard DNAs. Although the total number of fish species detected by eDNA and traditional surveys is not exactly same, but the difference is mainly caused by very rare fish species, such as *T. brevispinis*, *O. masou*, *R. steindachneri* etc. (Figure 4-2), and the average number of fish species detected by the two methods in a single survey is almost the same. Therefore, it can be considered that the addition of internal standard DNAs in quantitative metabarcoding approach will not reduce the species detection ability under the condition of giving sufficient sequencing reads.

The annual relative abundance calculated from fish eDNA concentration was not completely consistent with the relative abundance calculated from the number of fish individuals in traditional surveys (Figure 4-2). *L. macrochirus* has a high relative abundance in the traditional survey, but a low relative abundance in the eDNA survey, and *C. Cuvieri* has the

opposite trend (Figure 4-2). The rate of eDNA release and the number of mitochondria contained in a single cell may vary among fish species (Charitonidou et al., 2022), leading to differences in the relationship between individual number and eDNA copy number across different fish species. There is a long-time interval between eDNA surveys and traditional surveys, and the natural changes in fish population structure may also be one of the reasons for this change. Electrofishing has been conducted a long time in Miharu reservoir to control the individual numbers of *M. salmoides* and *L. macrochirus*, manual intervention may also be one of the important factors affecting this change. There was a significant positive correlation between eDNA concentrations and fish abundances both for 2019 data (Figure 4-3) and multi-year merged data (Figure S4-2). This indicated the feasibility of using quantitative metabarcoding to estimate the abundance of multiple fish species simultaneously. For 2019 data, the adjusted R-Squared of linear regression was lower than that calculated by multi-year merged data. This may be because multi-year surveys have a lower error and can more accurately reflect the number of fish individuals.

4.4.2 β-diversity, eDNA concentration and environmental variables

The reservoir ecosystem is relatively closed comparing with flowing waters and sea areas, therefore although the result of ANOSIM shows that there are differences in the fish population structure in different months, the differences cannot simply be attributed to changes in fish biomass. The rate of eDNA released by individual fish is not constant. It is generally believed that the more active the fish, the higher eDNA released. Therefore, it can be considered that the differences in the fish population structure in different months calculated by eDNA

concentration indicated the degree of fish activity. Leading to a conclusion that *C. carpio*, *C. cuvieri* and other fishes of the *Carassius* are mainly active in March and April, *T. hakonensis*, *C. idella*, *S. asotus* are mainly active in April and May, *M. salmoides*, *L. macrochirus* are mainly active in June, July and August (Figure 4-4a). The fish population structure in June and July is almost same (Figure 4-4a), indicating that the two months have similar active fishes. The main environmental variable that causes changes in fish population structure is water temperature (Figure 4-4b). When the water temperature is low, most fish are inactive, and the released eDNA decreases, resulting in a decrease in detection efficiency. This may be the reason for the large distance between samples in March and April. Therefore, the fish composition based on eDNA can be interpreted as the activity degree of different fish species under different environmental variables (Figure 4-4b, c, d).

Water temperature affects the distribution of fish (Stefan et al., 1996), and the changes in the eDNA concentration of different fish species with water temperature are completely different (Figure 4-5), reflecting the different preferences of different fish species for water temperature. The eDNA concentration of some fish species such as *M. salmoides* and *Carassius* sp. increased with the increase of water temperature but did not show a downward trend, which may be related to the life history of fish. In the Miharu reservoir, *M. salmoides* juveniles were clearly observed to swarm in large numbers near the lakeshore. This swarming behavior may lead to a sharp increase in the local eDNA concentration. The water samples collected in this study were all located in the lakeshore area. Sampling during periods of high-water temperature may reflect juvenile swarming. pH and EC reflect water quality to a certain extent, and the change of eDNA concentration of different fish species with pH also reflects the pH preference

of different fish. In this study, the fluctuation range of pH is small, so the eDNA concentration of most fish does not show a significant peak with the change of pH (Figure 4-5). EC can reflect the content of nutritive salts. EC significantly affected the fish eDNA concentration, but the change of eDNA concentration with EC was not statistically different among different fish species (Table 4-1), which may indicate that freshwater fish are more adapted to survive in waters with low nutritive salts. Because high nutritive salts may lead to eutrophication, resulting in lower dissolved oxygen, which in turn affects fish distribution.

4.4.3 Fish spawning activities

The spawning activities of 13 fish species were simultaneously estimated using quantitative eDNA metabarcoding approach (Figure 4-6). Most of the fish spawning activities estimated based on eDNA is consistent with the known fish spawning period, demonstrating the ability of quantitative eDNA metabarcoding to effectively and efficiently characterize the spatial and temporal nature of fish spawning in reservoirs. The spawning activities of *Carassius* sp., *C. cuvieri* and *C. carpio* mainly occurred in April-May and around July, forming two spawning periods. This is because the spawning period of *C. cuvieri* and *C. carpio* is longer and may spawning multiple times (Fernández-Delgado, 1990). Some estimated spawning activities of *L. macrochirus*, *M. anguillicaudatus*, *Carassius* sp. and *H. barbus* are later than known spawning period, which may be caused by climatic differences or by the swarming of juveniles after hatching. The estimated spawning activities of *G. urotaenia* varied widely between two years and results of 2019 deviates from the known spawning period. The historical records of traditional surveys show that the individual numbers of *G. urotaenia* in the Hebisawagawa front

reservoir is low. Weekly water sampling schedule may fail to capture eDNA concentrations peak caused by spawning activity, and the eDNA detected in 2019 may be derived from the juveniles of *G. urotaenia*, rather than spawning activities. Therefore, for fish with few individuals, more frequent sampling is required to ensure monitoring of spawning activity.

Previous studies have shown that a large number of sperm released during fish spawning activities will not only cause a sharp increase in eDNA concentration, but also a sharp increase in nuclear DNA/mitochondrial DNA ratio due to the low mitochondrial content in sperm (Bylemans et al., 2017). Since there is no universal primer suitable for fish nuclear DNA, in this study, only mitochondrial DNA concentration data obtained based on the quantitative metabarcoding of fish 12S rRNA gene were used to estimate fish spawning activities, and data on nuclear DNA/mitochondrial DNA ratio were lacking. However, other studies have shown that the degradation rate of nuclear DNA is significantly faster than that of mitochondrial DNA (Jo et al., 2020; Wu et al., 2022). Therefore, the peak of nuclear DNA/mitochondrial DNA ratio caused by fish spawning activity will decline rapidly after the end of spawning activity. Due to the different peak of nuclear DNA/mitochondrial DNA ratio in different fish species, detection of spawning activity by nuclear DNA/mitochondrial DNA ratio may not be necessary for species with low peaks (Wu et al., 2022). The estimated fish spawning activity in this study is roughly consistent with the known fish spawning periods except *G. urotaenia*, therefore it can be considered feasible to estimate fish spawning activity from eDNA concentration data alone. For *G. urotaenia* and other fish species with few individuals that may lead to misconceptions, data on nuclear DNA/mitochondrial DNA ratios are needed to further accurately estimate spawning activity.

4.5 Conclusion

Quantitative metabarcoding was used to simultaneously measure the eDNA concentration of multiple fish species. The results showed that quantitative metabarcoding with internal standard DNAs has species detection capabilities similar to traditional surveys, and has more accurate quantitative performance compared to general metabarcoding. Based on the eDNA concentration calculated by this method, the fish composition within samples; the relationship between the fish-specific eDNA concentration and environmental variables were successfully estimated, which is helpful to deepen the understanding of fish ecology. Using the method of estimating fish spawning activities by using the outliers of eDNA data proposed in the previous study, the spawning activity of 13 fish species was successfully estimated, and the spawning activity of 12 fish species was roughly consistent with the known spawning period of fish. All results show that long-term quantitative eDNA metabarcoding is feasible to be used in conducting ecological investigation in multiple fish species simultaneously.

4.6 Tables

Table 4-1. Results of the hierarchical generalized additive model which performed to estimate the changes of fish eDNA concentration with environmental variables

	edf	p-value
s(WT)	2.799	0.038 *
s(WT,Fish)	32.581	<0.001 ***
s(pH)	2.765	0.031 *
s(pH,Fish)	14.704	<0.001 ***
s(EC)	1.001	<0.001 ***
s(EC,Fish)	0.721	0.392
s(Site,Year)	4.749	<0.001 ***

Significance codes: '***' <0.001; '**'< 0.01; '*' <0.05

"WT" refers to water temperature and "EC" refers to electrical conductivity.

"s()" refers to a smooth term, and "edf" refers to effective degrees of freedom.

4.7 Figures

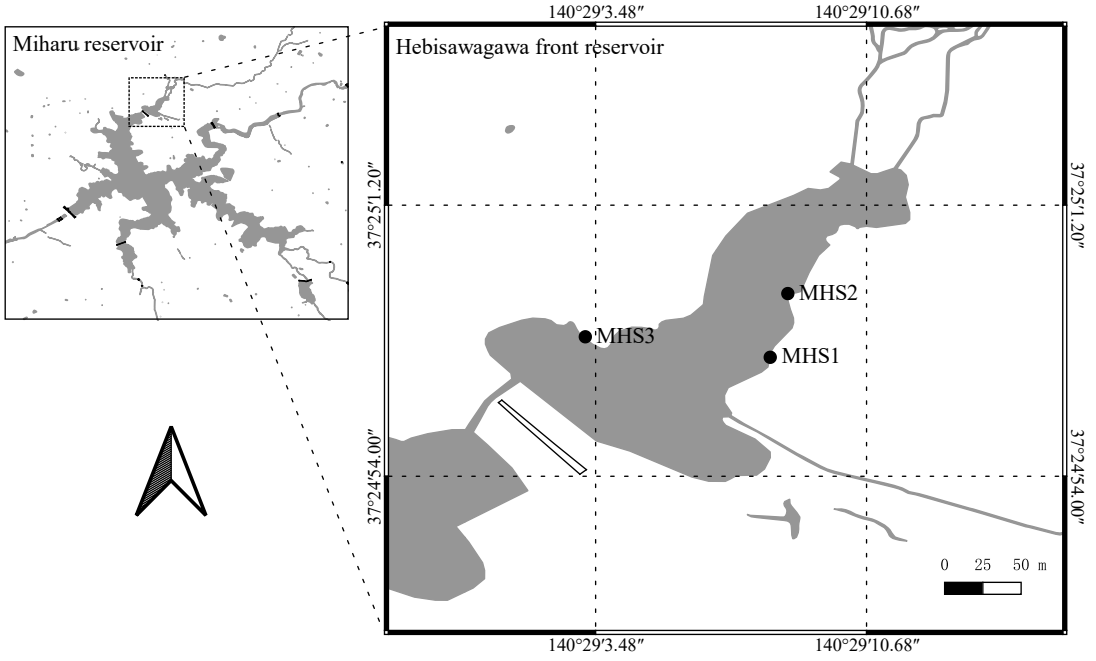

Figure 4-1. Map of the sampling sites within the Hebisawagawa front reservoir.

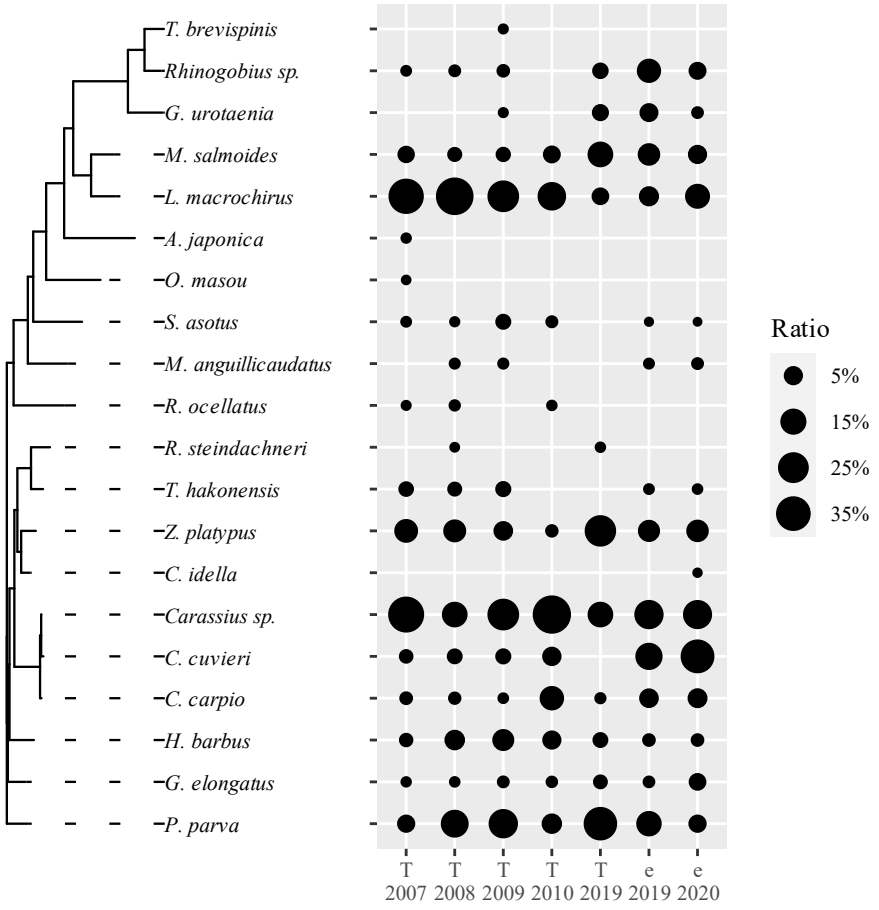

Figure 4-2. Phylogenetic tree based on neighbor-joining method and the relative abundance of 20 fish species. “T” refers to the traditional survey, and “e” refers to the eDNA survey.

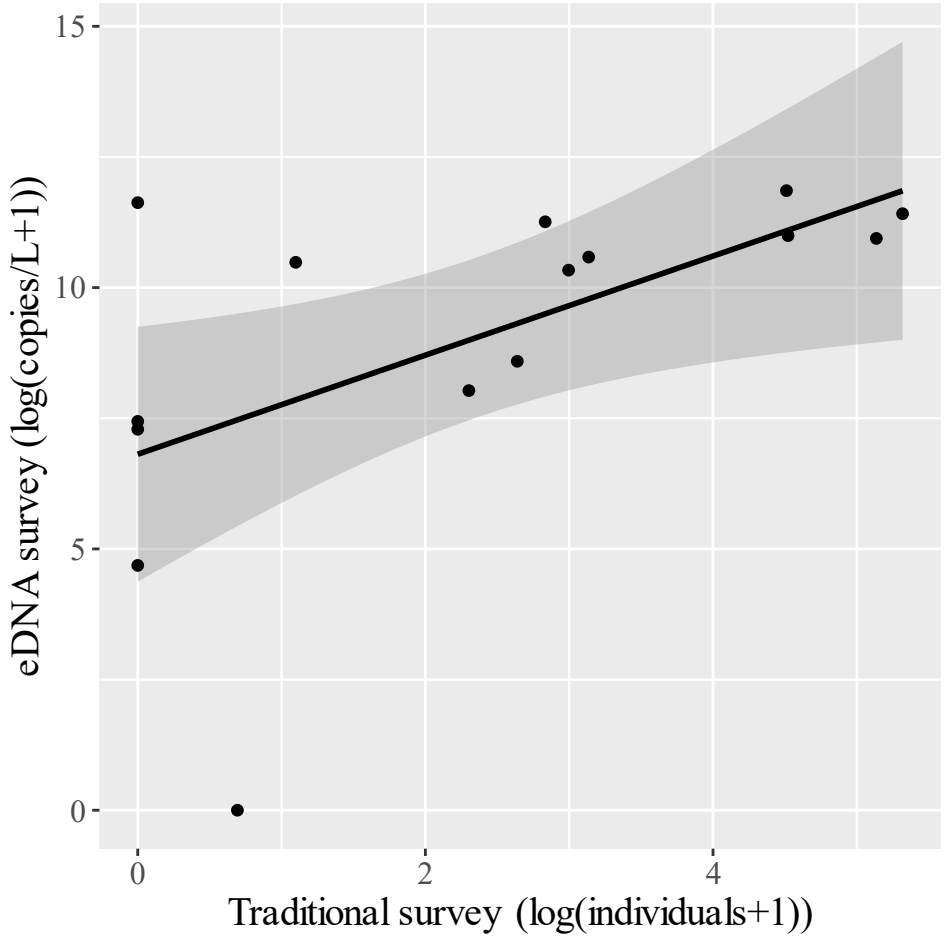

Figure 4-3. Linear regression to test the correlation between the eDNA concentration and the individual numbers of fish from the 2019 eDNA survey and the traditional survey. Shading refers to the 95% confidence interval.

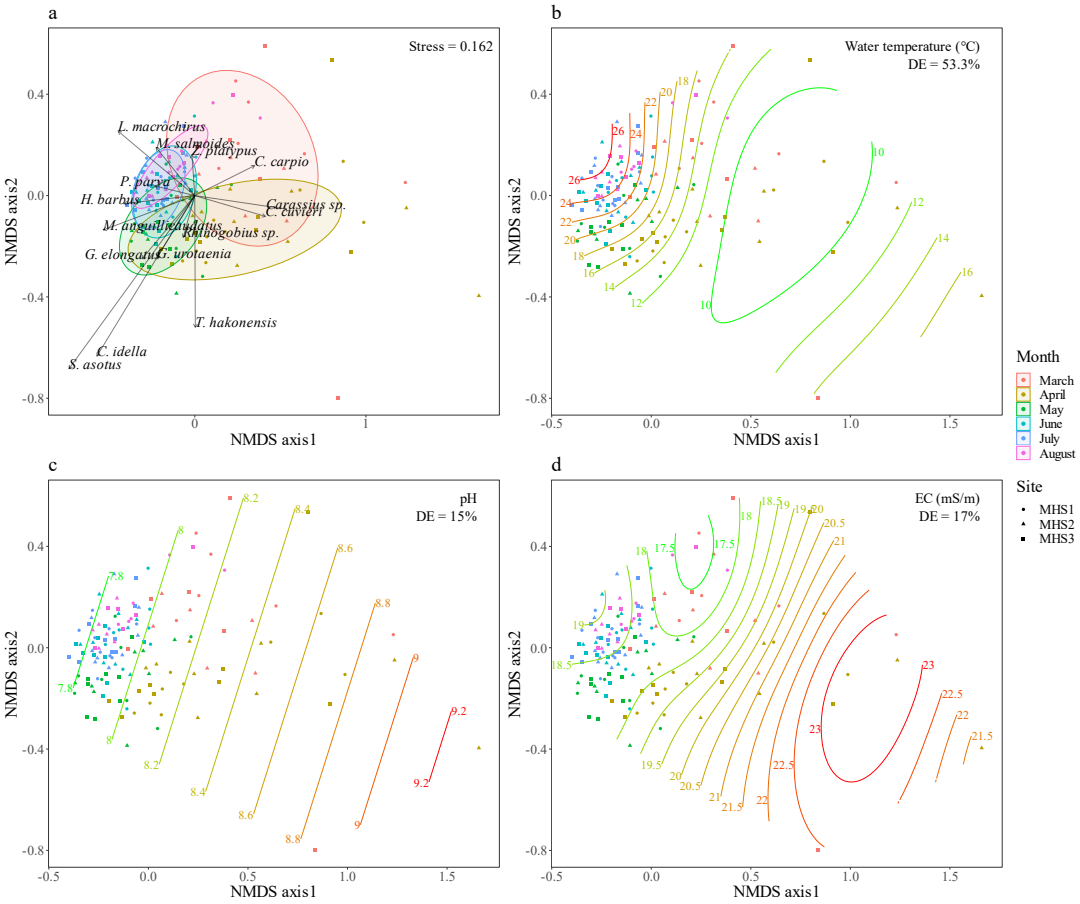

Figure 4-4. NMDS ordination with fitted GAM surfaces overlaid to illustrate the relationships between fish species composition and environmental variables. Shaded refers to the 80% confidence ellipse. “DE” refers to deviance explained.

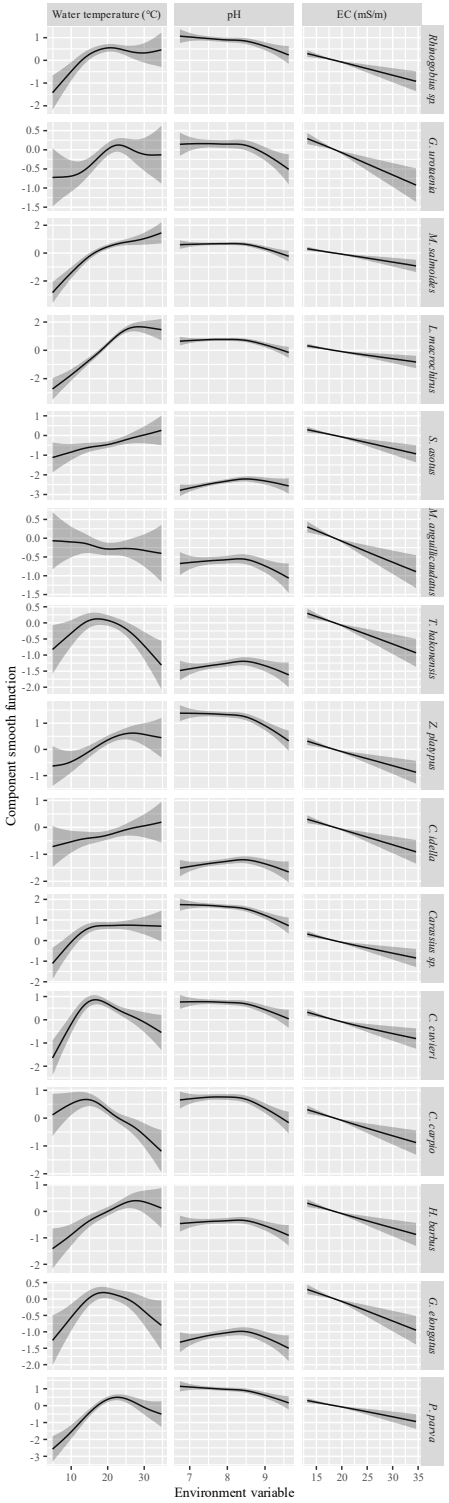

Figure 4-5. Relationships between fish-specific eDNA concentration and environmental variables. The x-axis represents environmental variables. The y-axis represents the linear predictor of component smooth functions.

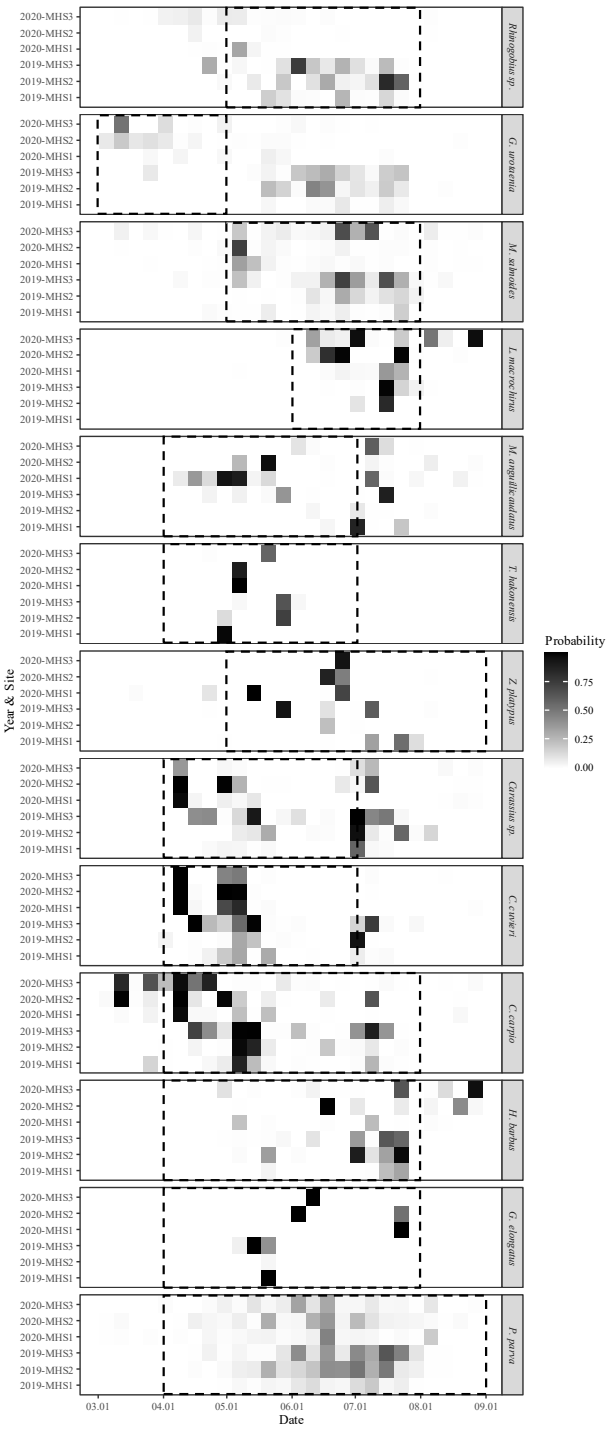

Figure 4-6. Spawning probabilities estimated by eDNA concentration. The x-axis represents sampling time. The y-axis represents years and sites. The dotted box refers to the fish spawning period recorded by the Research Institute of Environment, Agriculture and Fisheries, Osaka Prefecture.

Chapter 5 General discussion

In this study, a series of experiments were carried out around fish spawning activity based on eDNA. A sampling plan was proposed to monitor fish spawning activities using eDNA. A method for estimating fish spawning activity using eDNA data was demonstrated. The feasibility of using quantitative metabarcoding technology to simultaneously estimate the spawning activity of multiple fish in long-term eDNA surveys was validated. It solves the three difficulties that there is currently no feasible sampling plan, no eDNA-based spawning activity estimation method, and no accurate method for detecting the spawning activity of multiple fish species.

In Chapter 2, *Cyprinus carpio* was used as a target species, and artificial spawning experiments were conducted to investigate the spatiotemporal changes in nuclear and mitochondrial eDNA concentrations during spawning. After the eDNA concentration and nuDNA/mtDNA ratio produced by spawning activities peaked, as the time increased by 0.1 h, the mtDNA and nuDNA concentrations decreased by approximately 5.22% and 7.78%, respectively, and the nuDNA/mtDNA ratio decreased by approximately 2.56%. The nuDNA concentration decreased significantly faster than the mtDNA concentration, consistent with the results of a previous study (Jo et al., 2020). These results indicated that the high eDNA concentrations and nuDNA/mtDNA ratios will return to baseline within approximately 24 h, giving eDNA method the potential to estimate spawning activity on a daily basis. Through data simulation, the probability of successful monitoring spawning activity under different sampling plans was estimated, and sampling every 24 h and 100 m was recommended as a general sampling plan.

In Chapter 3, in order to verify the feasibility of estimating fish spawning activities through eDNA technology, traditional surveys and eDNA surveys were performed two years in Miharu reservoir and Hebisawagawa front reservoir. In traditional surveys, spawning activities of *Micropterus salmoides* and *Lepomis macrochirus* was estimated from fish body length and daily rings of otolith. In the eDNA survey, *Cyprinus carpio*, *Micropterus salmoides* and *Lepomis macrochirus* were used as target species, and an eDNA based method was established to estimate the spawning activity of the three fish species using the outlier of the eDNA concentration and the nuDNA/mtDNA ratio. The effectiveness of the method was verified by comparing estimated spawning activities with those from traditional methods and the results showed that the estimated spawning periods coincided with the known fish ecology and the results of traditional methods to a certain extent. By applying such an eDNA based method, it is possible to estimate the spawning activity of fish with high spatiotemporal resolution.

In Chapter 4, to estimate the spawning activity of whole fish assemblages in Hebisawagawa front reservoir, the quantitative metabarcoding approach was applied to simultaneously quantify the eDNA concentration of all fish species. The results showed that the detected fish species of the quantitative eDNA metabarcoding approach were highly consistent with the historical results of the traditional survey. The relationships between eDNA concentration and environmental variables in 15 fish species were also analyzed by performing HGAM. Using the method established in Chapter 3, the spawning activities of 13 fish species were estimated and most of the estimated spawning activities were consistent with the known traditional knowledge on fish spawning periods. These results showed that quantitative eDNA metabarcoding is useful for monitoring spawning activities of multiple fish species.

In Chapter 2, the variation of high concentrations of eDNA produced by fish spawning activities with time and distance was estimated, and a sampling plan of every 100 meters every 24 hours was derived. But in Chapter 3 and Chapter 4, since the purpose of the research is to establish a method for estimating fish spawning activities, it is not necessary to accurately estimate the spawning dates, and therefore, a weekly sampling plan was adopted. In Chapter 2, only *Cyprinus carpio* was used as the target species, but different fishes would have different sperm and eggs releasing regime and different survival times of sperm and eggs, which will lead to different eDNA reduction rates. Therefore, the reduction rate of high concentration eDNA caused by the spawning activities of different fish still needs further study.

In Chapter 3 and Chapter 4, different methods were used to estimate fish spawning activities. In Chapter 3, the eDNA concentrations of *Cyprinus carpio*, *Micropterus salmoides* and *Lepomis macrochirus* were detected by qPCR, and the spawning activities of the three fishes were estimated based on the eDNA concentration and the nuclear DNA/mitochondrial DNA ratio. In Chapter 4, the eDNA concentrations of 15 fish species were measured simultaneously by quantitative metabarcoding technology, and the spawning activities of 13 fish species were estimated only based on the eDNA concentration. Although different methods were used to estimate fish spawning activity, the estimated fish spawning probabilities by these two methods were highly correlated: *Cyprinus carpio* ($r = 0.843$ [*Spearman's rank correlation coefficient*], $p < 0.001$), *Micropterus salmoides* ($r = 0.821$, $p < 0.001$), and *Lepomis macrochirus* ($r = 0.906$, $p < 0.001$). In Chapter 3, eDNA and electrofishing results agreed well (Cohen's kappa [κ] = 0.3, $p = 0.04$ for *Micropterus salmoides*; $\kappa = 0.423$, $p = 0.002$ for *Lepomis macrochirus*). When the quantitative metabarcoding data in Chapter 4 was used to estimated

fish spawning activities and compared with electrofishing results, they were still in good agreement ($\kappa = 0.386$, $p = 0.007$ for *Micropterus salmoides*; $\kappa = 0.327$, $p = 0.014$ for *Lepomis macrochirus*). The method based on qPCR is quantitatively accurate and the experimental procedure is relatively simple, but one experiment can only target one species. The method based on quantitative metabarcoding can target multiple species simultaneously, but the experimental process is more cumbersome, and there is a lack of universal primer for nuclear DNA. Both methods have their own advantages and disadvantages, and the corresponding method should be selected according to the research purposes.

Based on the results of this study and previous studies, an eDNA based survey framework for monitoring fish spawning activity is summarized as Figure 5-1. In the step to determine the sampling plan, a general sampling plan is to sample every 24 h and 100 m along the lakeshore. The actual sampling plan should be designed based on the purpose of the survey, workload, biomass, terrain of the field, and life history of the target species. Depending on the purpose of the survey, if necessary, using qPCR to accurately quantify the nuDNA and mtDNA concentrations of the target fish, and further verify the spawning activities through the nuDNA/mtDNA ratio. This is a complete survey framework for monitoring fish spawning activity based on eDNA, including sample collection, data collection, and data analysis. The fish spawning activities estimated by this framework are in statistically significant consistency with those estimated by traditional electrofishing surveys, and most of the spawning activities are consistent with the known records of fish spawning period. According to the temporal and spatial distribution of high concentrations of eDNA after spawning activities, this survey

framework has the potential to be used to estimate the fish spawning activities within 24 h before water sample collection and within 50 m from the sampling point.

This survey framework is based on eDNA and does not rely on traditional surveys. In contrast to traditional destructive, injurious methods such as otolith micro-chemistry, gonad maturation (Ntiba and Jaccarini, 1990; Milton et al., 1997), this framework does not require the collection of fish tissue samples, therefore it does not cause harm to fish and does not generate an additional fish mortality due to the survey process. The framework only needs to collect a small amount of water samples for a single sampling, does not rely on traditional fishing activities such as drift nets and electrofishing (Wei et al., 2009), therefore it does not interfere with the natural activities of fish, and is an eco-friendly survey method. In contrast to traditional non-invasive methods such as acoustic surveys, visual surveys (Walters et al., 2009; Walters et al., 2009), this survey framework provides direct evidence of fish spawning activity without relying on professional biomorphological knowledge, avoiding observer biases and taxonomic misidentification.

The established eDNA-based survey framework is a non-invasive, highly species-specific method, and suitable for detecting the spawning activities of rare fish. It deepens people's understanding of fish ecology, and provides a reference for the implementation of measures such as the establishment of closed fishing periods and areas. Metabarcoding using universal primers for fish enables rapid response to invasive fish, provides monitoring tools in the early stages of invasive fish population establishment, and provides important intelligence for invasive fish management and native fish diversity conservation. The sampling process of the survey framework does not rely on professional tools, which allows the framework to be applied

to many water areas where traditional survey methods cannot be performed normally due to natural conditions. The sampling method is simple, which greatly reduces the workload of field investigation. Under the same time conditions, this framework can establish more frequent sampling work in time and space than traditional methods. Although the survey framework relies on molecular experiments, which may increase the workload in the laboratory, but molecular experiments can be run with a high degree of automation (Ivanova et al., 2006), which allows the survey framework to be run on large survey programs with heavy workloads. Although this study focused on fish spawning activities, the eDNA-based survey framework established in this study has the potential to be applied to other aquatic species that rely on external fertilization as well as some elusive species such as frogs and salamanders (Vimercati et al., 2020; Takeshita et al., 2020). In external fertilization species often need to excrete large amounts of sperm in order to increase the probability of fertilization (Yund, 2000; Stoltz and Neff, 2006), these high levels eDNA will provides the basis for the realization of this framework.

The eDNA-based survey framework for estimating fish spawning activities established in this study relies on the detection of outliers, which means that long-term eDNA sampling is necessary. Although this study did not discuss the influence of the time span of the samples on the survey results, it can be considered that the time span of the samples must be long enough to cover the fish spawning period and part of the non-spawning period to accurately estimate spawning activities. Because eDNA concentrations decrease with dispersal, dense sampling at spatial scales is required to accurately determine fish spawning sites. This means that a large number of samples need to be taken on both temporal and spatial scales when investigating fish spawning activities in a wide water area. This leads to a large field sampling workload and high

molecular experiment costs. On the other hand, such spatial-temporal surveys can not only be used to estimate fish spawning activities, but also help to deepen the understanding of fish ecology. As done in Chapter 3 and Chapter 4, fish seasonality was explained by the relationship between eDNA concentrations and environmental factors such as water temperature, pH, etc. Mastering this kind of information is helpful to estimate the impact of environment changes on fish abundance and population structure, and provide an important basis for the implementation of fish conservation measures.

The eDNA-based survey framework for estimating fish spawning activities established in this study still has some drawbacks. The detection of eDNA outliers can be affected by the uneven distribution of eDNA. High concentrations of eDNA resulting from fish gathering can potentially be misinterpreted as spawning activity. The nuDNA/mtDNA ratio is not affected by the abundance of fish, but field surveys show that fish spawning activity cannot be estimated only by the nuDNA/mtDNA ratio. Therefore, how to exclude the influence of abundance when estimating fish spawning activities still needs further research. Investigations based on eDNA must consider DNA degradation factors, therefore it is necessary to take proper measures to prevent DNA degradation during the transportation and storage of samples. In addition, changes in water turbidity leading to a decrease in filtration capacity may also affect the measurement of eDNA concentration. The eDNA-based survey relies on fish-specific sequences, and closely related species that cannot be distinguished by DNA will affect the estimated results. The universal primer used in Chapter 4 which target mitochondrial 12S rRNA gene can not distinguish some species belonging to *Carassius*, *Rhinogobius* or else, leading to estimation of

spawning activity based on the genus level. Species level estimation for these species relies on the development of novel primers.

All samples were collected in still waters such as ponds and dam lakes with gentle flow, so the survey framework established in this study may not be suitable for flowing waters. Although the results of the pond experiments in Chapter 2 showed that even in still waters, high concentrations of eDNA produced by spawning activities could be detected at a distance of 50 m from the spawning site, the current in flowing waters would further accelerate the diffusion of eDNA, making the estimated spawning site deviate from the actual site. The additional diffusion caused by the water flow will cause the eDNA to fall back to the normal level more quickly, which will shorten the duration of the high concentration of eDNA, reduce the probability of successful detection of spawning activity, and increase the sampling labor. Further research is needed on the investigation methods of fish spawning activities in flowing waters.

For fish resource conservation, determining the magnitude of spawning events is important to monitor changes in the effective population size. Although prior studies have suggested a potential relationship between eDNA concentration and the magnitude of spawning events (Bracken et al., 2019), the rapid decline in eDNA concentration over time and distance makes it nearly impossible to measure eDNA concentrations released by different spawning activities under the same condition in field surveys. Therefore, it can be considered that the framework established in this study can only detect the occurrence of spawning activities, but cannot detect the magnitude of spawning events.

Fish reproduction is generally a group behavior. The larger spawning scale, the higher concentration of eDNA released, and the longer it takes for the eDNA to fall back to the normal level, which is beneficial to estimate the spawning activity through the eDNA concentration. A previous study proposed using the nuDNA/mtDNA ratio to estimate fish spawning activity (Bylemans et al., 2017). The theoretical maximum peak of the nuDNA/mtDNA ratio occurs in pure semen, and the actual nuDNA/mtDNA ratio regardless of spawning size, and the peak only depend on the proportion of eDNA provided by sperm to the total eDNA, resulting the nuDNA/mtDNA ratio less affected by spawning size. Compared with the spawning activities of multiple males to one female, the proportion of eDNA derived from sperm may be lower in the spawning activities of one male to one female fish, resulting in a lower nuDNA/mtDNA ratio. When the peak value of the eDNA concentration is high but the peak value of the ratio is not high, the ratio may even decrease because of the difference in the decreasing rate of nuDNA and mtDNA. The nuDNA/mtDNA ratio can vary widely among species (Bylemans et al., 2017; Tsuji et al., 2022), therefore estimating spawning activity by the ratio alone may be difficult for species with low peak nuDNA/mtDNA ratios. This explains the reason why some previous studies could not rely on nuDNA/mtDNA ratios to correctly estimate fish spawning activities (Saito et al., 2022). The results of quantitative metabarcoding based on 12S rRNA gene in Chapter 4 also showed that fish spawning activities can be correctly predicted without using nuDNA/mtDNA ratios, which may imply that nuDNA/mtDNA ratios are not a mandatory parameter for estimating spawning activity, but nuDNA/mtDNA ratios are still an excellent parameter to exclude biomass interference. The results in Chapter 2 show that the use of nuDNA has a higher probability to detect fish spawning activities than mtDNA, and there is currently

no universal primer for fish based on nuDNA, so in Chapter 4, the 12S rRNA gene belonging to mtDNA was selected for quantitative metabarcoding. In the future, the development of nuDNA-based universal primers for fish will be more conducive to the application of the fish spawning activity detection framework proposed in this study.

After a series of studies, an eDNA-based fish spawning activity detection framework was finally established. The establishment of this framework complements the current gap in the detection of fish spawning activities by eDNA, helps people deepen the understanding of fish ecology, and provides assistance for the protection of fish biodiversity.

5.1 Figure

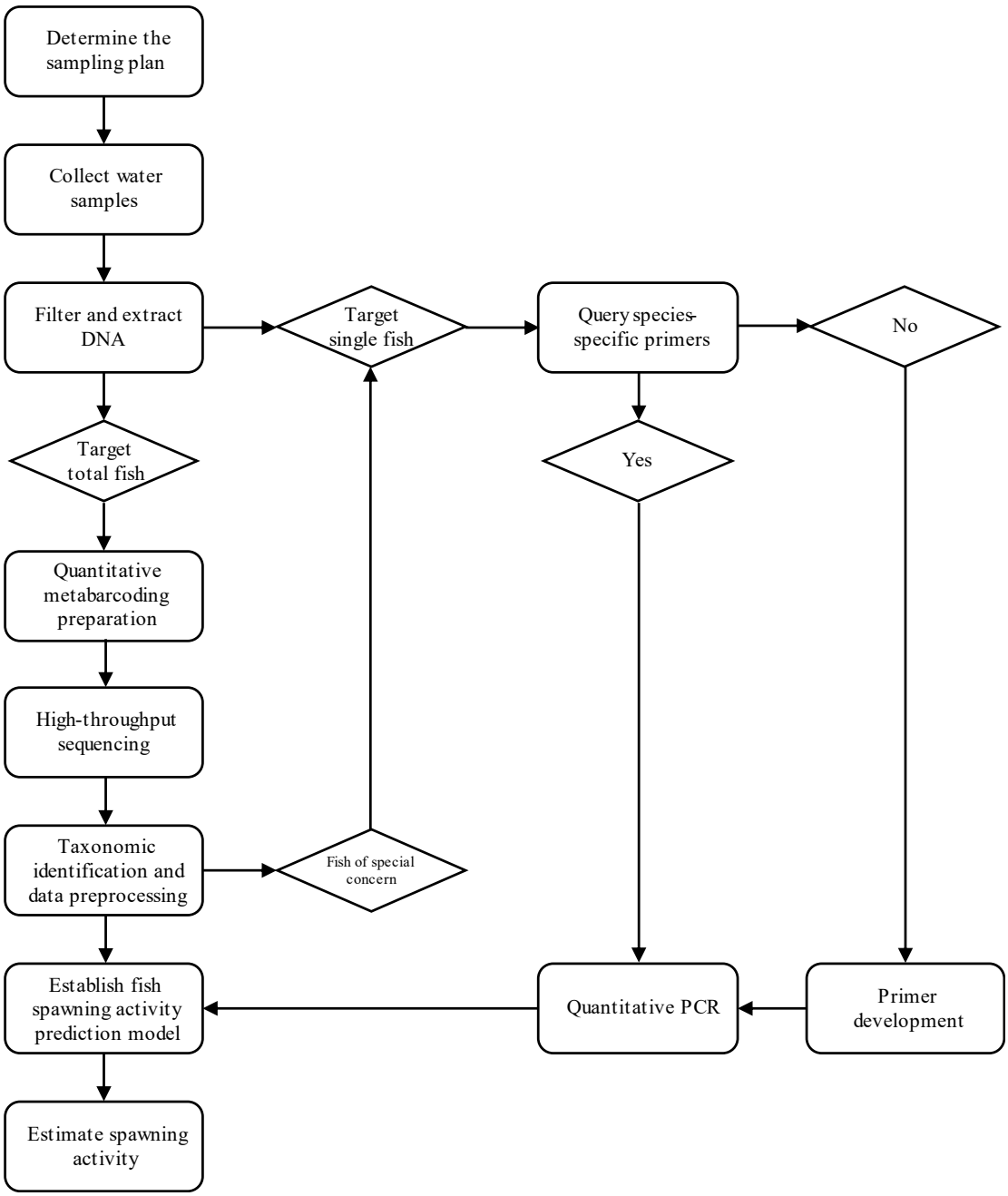

Figure 5-1 eDNA based survey framework for monitoring fish spawning activity.

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Appendix

Appendix 2-1

Carp in the third group of experiments were injected with chorionic gonadotrophin on the first day of the experiment, spawning activity was observed on the morning of the third day, and egg production was confirmed. Sampling was performed afterwards; however, the experimental results showed that the *ITS1/CytB* ratio in the third group of experiments was significantly lower than that in the other experimental groups (Figure S2-1). By examining the camera recording data, it can be found that there was continuous possible spawning activity from the first day to the early morning of the second day of the experiment, although no egg production was observed the next morning. It can be considered that the male carp may have released a lot of sperm in the early morning of the second day, but the female carp did not cooperate with the spawning activity; on the third morning, the female carp released eggs, but not enough sperm were released owing to the spawning activity from the previous days, resulting in a decrease in the *ITS1/CytB* ratio on day 3. Thus, the third set of experiments may not represent typical spawning activity, and these data was excluded from the data analysis in the main text.

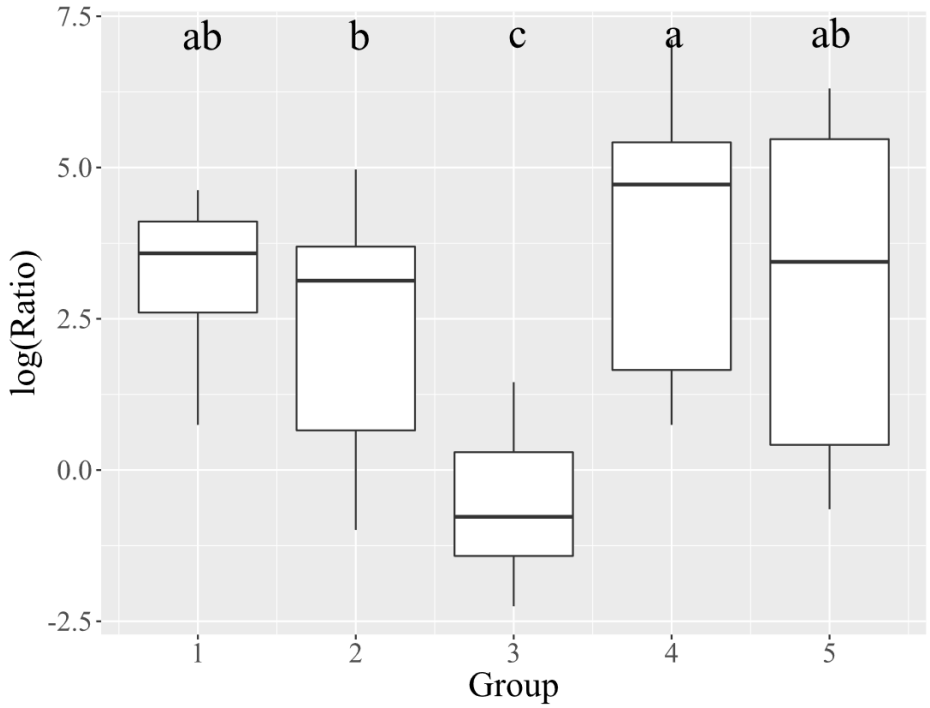

Figure S2-1. The *ITS1/CytB* ratios in different experimental groups. The letters are from the Tukey tests after analysis of variance. The same letter indicates that there was no significant difference.

Appendix 3-1 Designing the primers for the *ITS1* region

To design primers for the *ITS1* region of the largemouth bass and bluegill sunfish, the *ITS1* region of the target and a related fish species, smallmouth bass (*Micropterus dolomieu*) were sequenced. DNA was extracted from the fins and scales of the three fish using the dNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. 25 μL Polymerase chain reaction (PCR) mixtures contained 0.5 μL of KOD -Plus- Neo (Toyobo, Osaka, Japan), 2.5 μL of 10xKOD Buffer, 2.5 μL of 2mM dNTPs, 1.5 μL of 25mM $MgSO_4$, 900 nM of each primer, and 2 μL template DNA. Universal primers: forward 5′-TGGTGCATGGCCGTTCTTAGT -3′ and reverse 5′- GTCTCGTCTGATCTGAGGTC -3′ were available from Bylemans *et al.* (2017). PCR was performed using the StepOnePlus Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) as follows: 2 min at 94 ℃, 35 cycles of 10 s at 98 ℃, 30 s at 53 ℃, 2 min at 68 ℃, and 10 min at 68 ℃. The products were Sanger sequenced and specific primers and probes were designed based on the sequencing results. Next, 20 ng of tissue DNA samples from the three fish were used to verify the specificity of the developed primers and probes. The PCR conditions were the same as those described in the Materials and Methods section. The results showed that, for the primers and probes for bluegill sunfish, the target tissue DNA of bluegill sunfish was successfully amplified, and the amplification curves of largemouth bass and smallmouth bass did not increase. For the primers and probes for largemouth bass, the target tissue DNA of largemouth bass and smallmouth bass was amplified, and the amplification curve of the bluegill sunfish did not increase. Because the sequences of largemouth bass and smallmouth bass are very similar, the primers and probes designed cannot be used to distinguish the two bass species, but this did not affect subsequent

experiments because smallmouth bass do not live in the study area, the Hebisawagawa front reservoir.

Appendix 3-2 Linear mixed models

Linear mixed models (LMMs) were established to estimate the changes of the eDNA concentration and ratio of the three target fishes with environmental factors. The preprocessing of the data used in LMMs is consistent with GAMMs. The results of LMMs are shown in Table S4. Partial residual plots showed possible nonlinear relationships between eDNA concentrations or ratio and environmental variables (Figure S6, S7, S8). For example, in the change of the eDNA concentrations or ratio of carp with water temperature, a plot of the partial residuals showed a peak in the interval of 10 ~ 20℃ (Figure S6). These results are biologically reasonable. Because there should be a certain suitable range of environmental factors for the spawning activity, the relationship between environmental factors and spawning behavior is expected to be non-linear rather than linear. Therefore, choosing GAMMs is more suitable for this study.

Table S3-1. The AIC of two GAMMs with binomial distribution used to estimate fish spawning activity.

	AIC	
Target Fishes	*CytB* + Ratio + s(Tem)	*ITS1* + Ratio + s(Tem)
Common carp	55.04	55.05
Largemouth bass	58.30	58.30
Bluegill sunfish	52.08	52.09

"Tem" refers to water temperature.

Table S3-2. Results of the GAMMs with binomial distribution used to estimate fish spawning activity.

Target Fishes	Linear				Smooth term		
	Variable	Estimate	Std. Error	P	Variable	edf	P
Common carp	Intercept	-29.977	6.799	<0.001***			
	CytB	5.784	1.375	<0.001***	s(Tem)	1.069	0.086
	Ratio	3.801	1.210	0.002**			
Largemouth bass	Intercept	-14.053	4.275	0.001**			
	CytB	2.865	0.998	0.004**	s(Tem)	2.162	0.137
	Ratio	4.717	2.060	0.022*			
Bluegill sunfish	Intercept	-19.372	5.055	0.001**			
	CytB	3.800	1.138	<0.001***	s(Tem)	1	0.022 *
	Ratio	6.167	2.248	0.006**			

Significance codes: '***' <0.001; '**'< 0.01; '*' <0.05

"Tem" refers to water temperature.

Table S3-3. Electric fishing survey time

2019 year	2020 year
5/30	5/7
5/31	5/8
6/6	5/28
6/7	5/29
6/13	6/4
6/14	6/5
6/17	6/11
6/18	6/12
6/27	6/17
6/28	6/18
7/8	7/2
7/9	7/3
7/23	7/13
7/24	7/15
9/11	7/20
9/12	7/21
9/13	9/9
9/17	9/10
9/18	9/11
9/24	9/17
9/25	9/18
10/1	9/26
10/2	9/27
	9/30
	10/1

Table S3-4. Results of the linear mixed models (LMMs)

		***CytB* concentration**			***ITS1* concentration**			***ITS1*/*CytB* ratio**		
Targrt fishes	**Variables**	Estimate	Std	p	Estimate	Std	p	Estimate	Std	p
Common carp	Intercept	4.338	0.51	<0.001***	7.169	0.644	<0.001***	2.812	0.444	0.002**
	Tem	-0.014	0.006	0.016*	-0.017	0.007	0.015*	-0.006	0.004	0.105
	pH	-0.086	0.063	0.17	-0.148	0.077	0.057	-0.049	0.04	0.226
	EC	-0.022	0.015	0.152	-0.02	0.019	0.292	0.001	0.01	0.921
Largemouth bass	Intercept	5.623	0.988	<0.001***	5.207	0.878	<0.001***	-0.802	0.315	0.012*
	Tem	0.118	0.011	<0.001***	0.131	0.01	<0.001***	0.011	0.004	0.01*
	pH	-0.474	0.124	<0.001***	-0.417	0.108	<0.001***	0.033	0.041	0.419
	EC	-0.043	0.029	0.149	-0.065	0.026	0.014*	0.01	0.011	0.358
Bluegill sunfish	Intercept	2.993	0.922	0.004**	3.807	0.947	<0.001***	-0.078	0.309	0.801
	Tem	0.129	0.009	<0.001***	0.17	0.01	<0.001***	0.014	0.006	0.015*
	pH	-0.394	0.101	<0.001***	-0.49	0.109	<0.001***	0.033	0.041	0.421
	EC	0.024	0.025	0.324	-0.008	0.026	0.758	-0.006	0.011	0.55

Significance codes: '***' <0.001; '**'< 0.01; '*' <0.05

"Tem" refers to water temperature and "EC" refers to electrical conductivity.

Table S3-5. Results of the linear model between body length and age

Fish		Estimate	Std. Error	P
Largemouth bass	Intercept	-7.58	1.431	< 0.001 ***
	Age	0.948	0.035	< 0.001 ***
Bluegill sunfish	Intercept	3.827	1.925	0.056
	Age	0.302	0.036	< 0.001 ***

Significance codes: '***' <0.001; '**'< 0.01; '*' <0.05

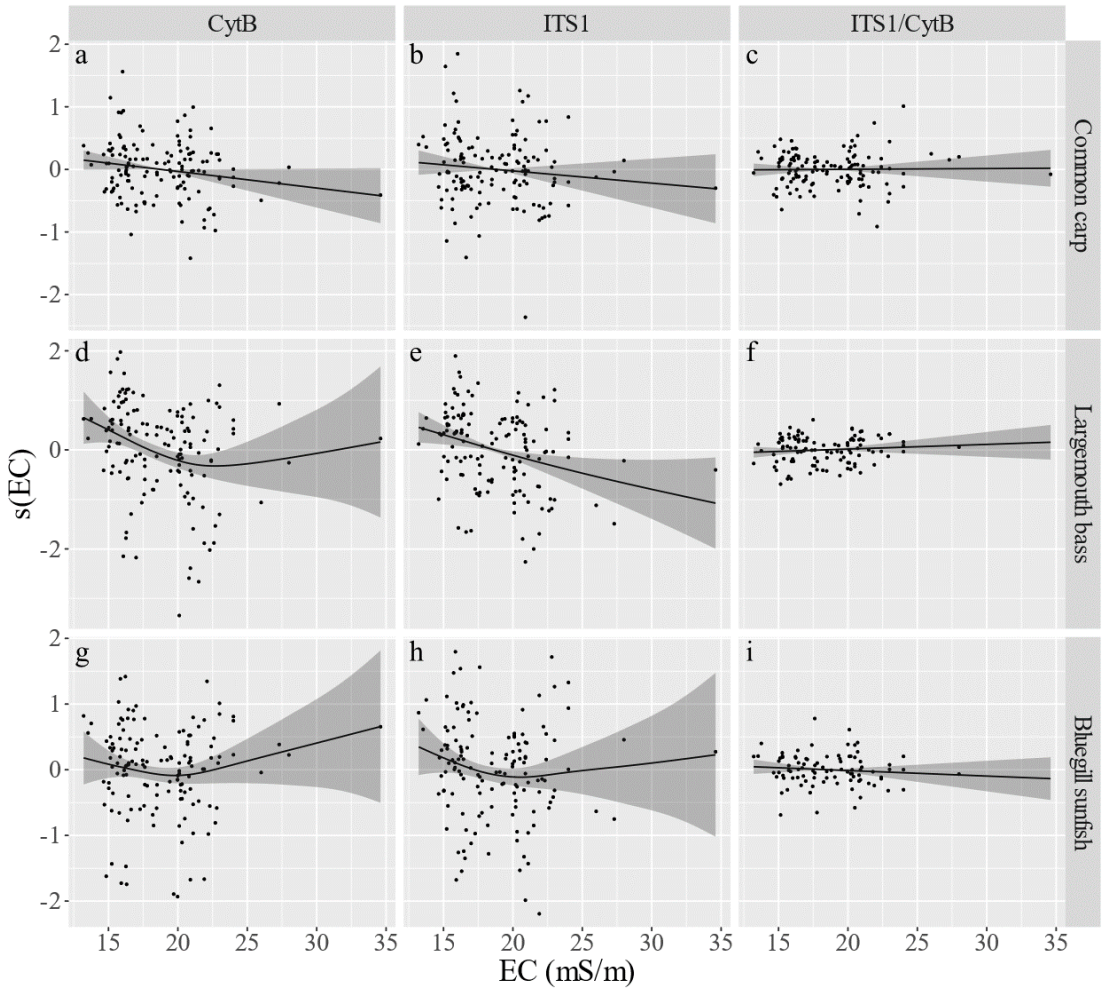

Figure S3-1. Smooth term of EC on the eDNA concentration and ratio. Graphs a, d, and g show the influence of EC on the mitochondrial *CytB* concentration from common carp, largemouth bass, and bluegill sunfish, respectively. Graphs b, e, and h show the influence of EC on the nuclear *ITS1* concentration from common carp, largemouth bass, and bluegill sunfish, respectively. Graphs c, f, and i show the influence of EC on the *ITS1*/*CytB* ratio of common carp, largemouth bass, and bluegill sunfish, respectively.

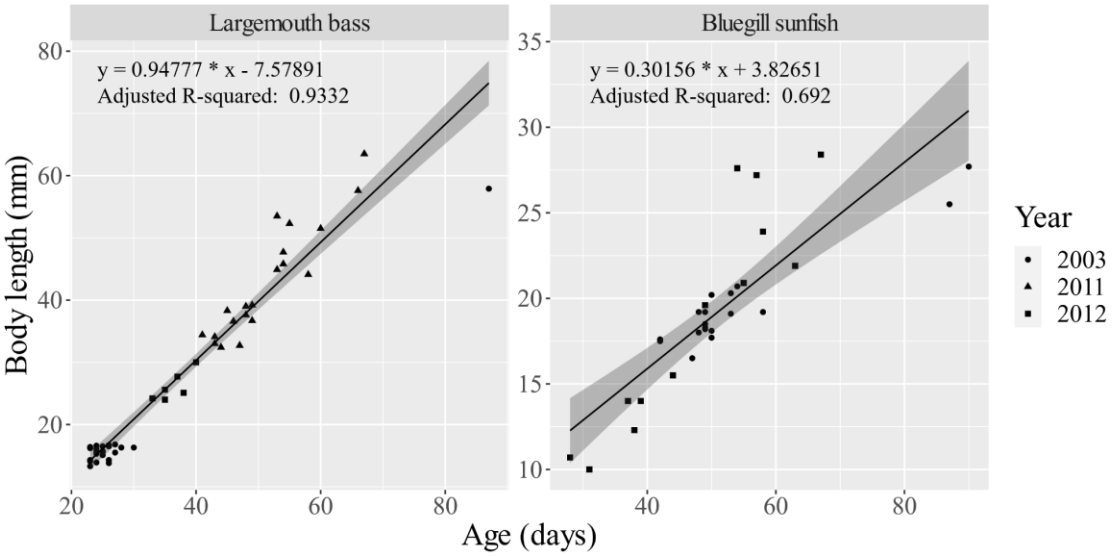

Figure S3-2. Linear model between age and body length. The y refers to age and the x refers to body length.

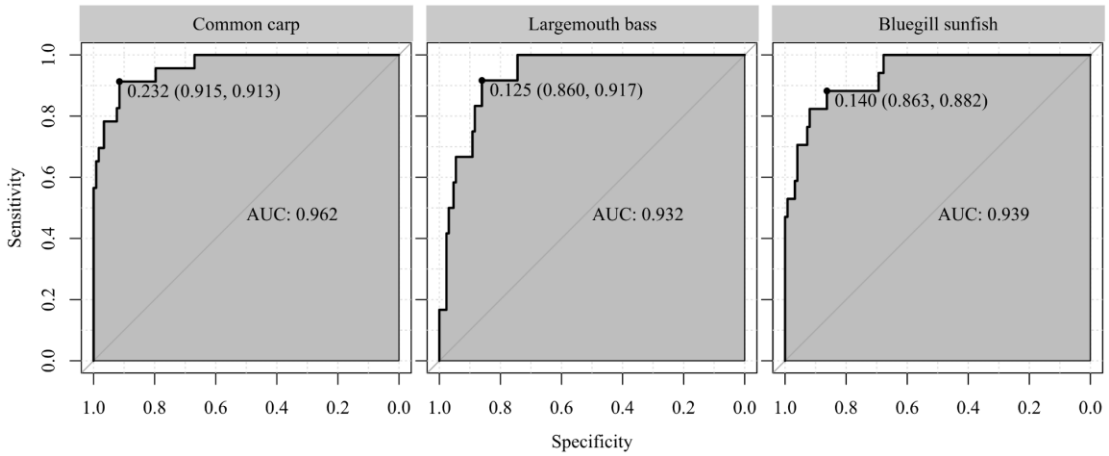

Figure S3-3. The ROC curves of the GAMM results used to estimate fish spawning activity.

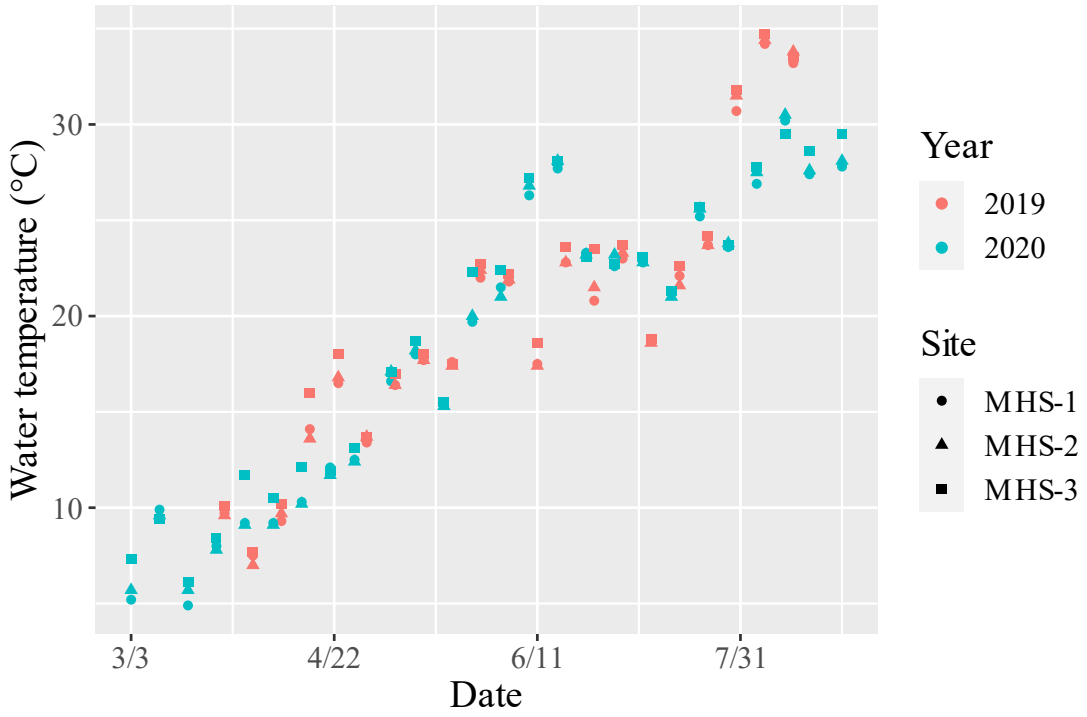

Figure S3-4. Water temperature changes in 2019 and 2020.

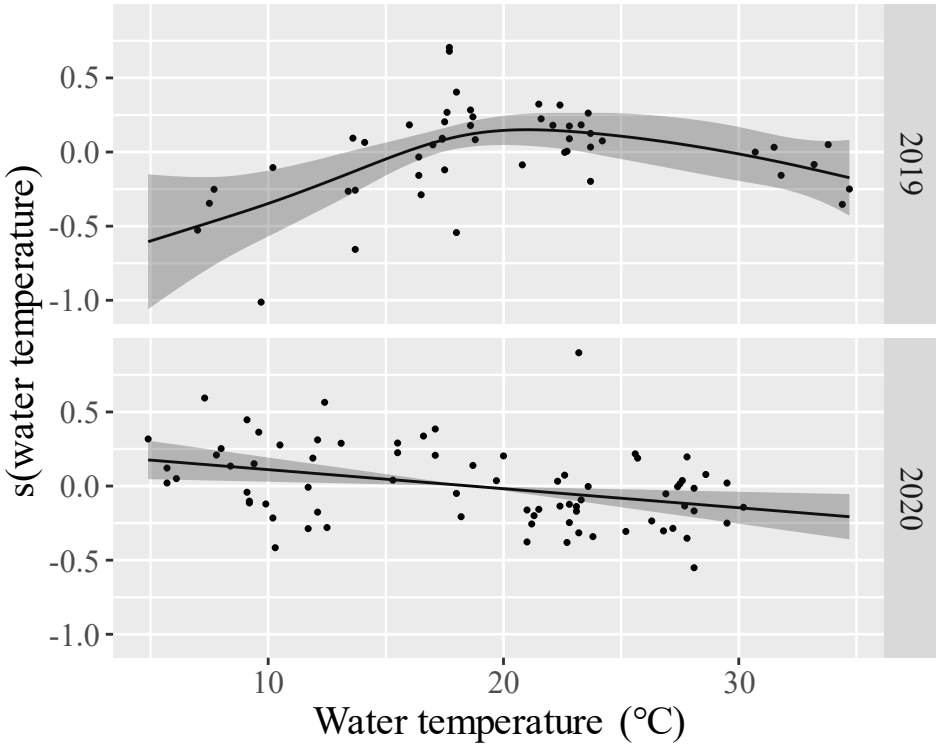

Figure S3-5. Smooth term of water temperature on the *ITS1*/*CytB* ratio for common carp in 2019 and 2020.

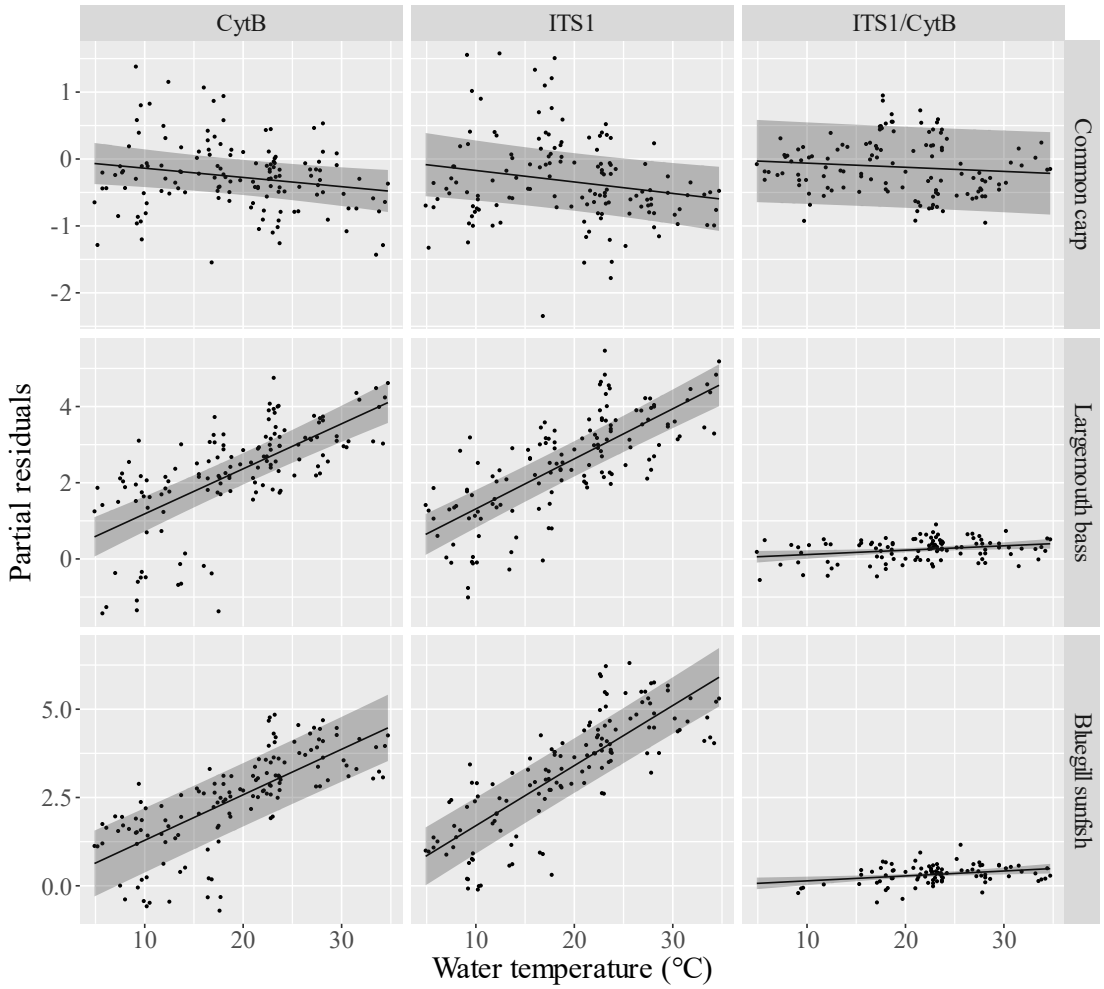

Figure S3-6. Partial residual plots for linear mixed models of water temperature on the eDNA concentration and ratio.

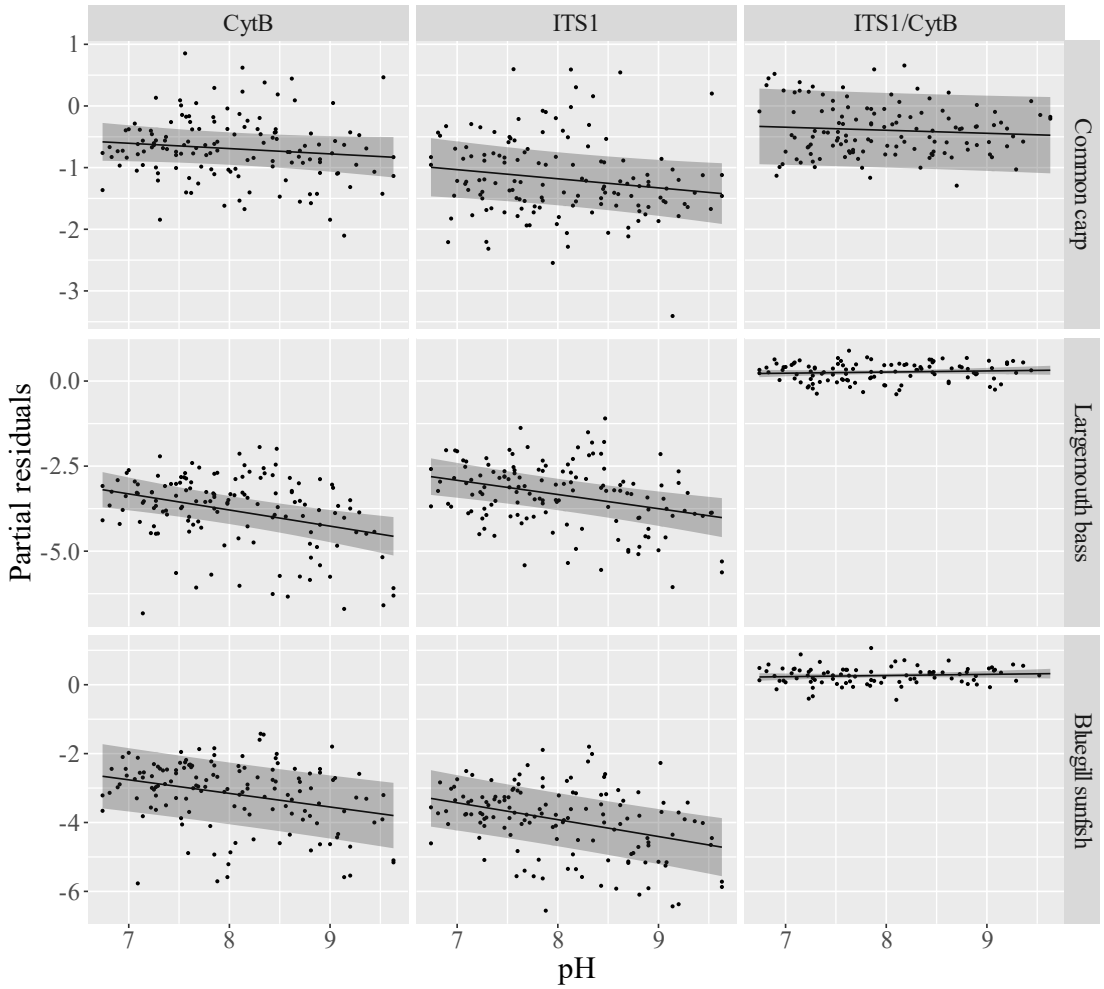

Figure S3-7. Partial residual plots for linear mixed models of pH on the eDNA concentration and ratio.

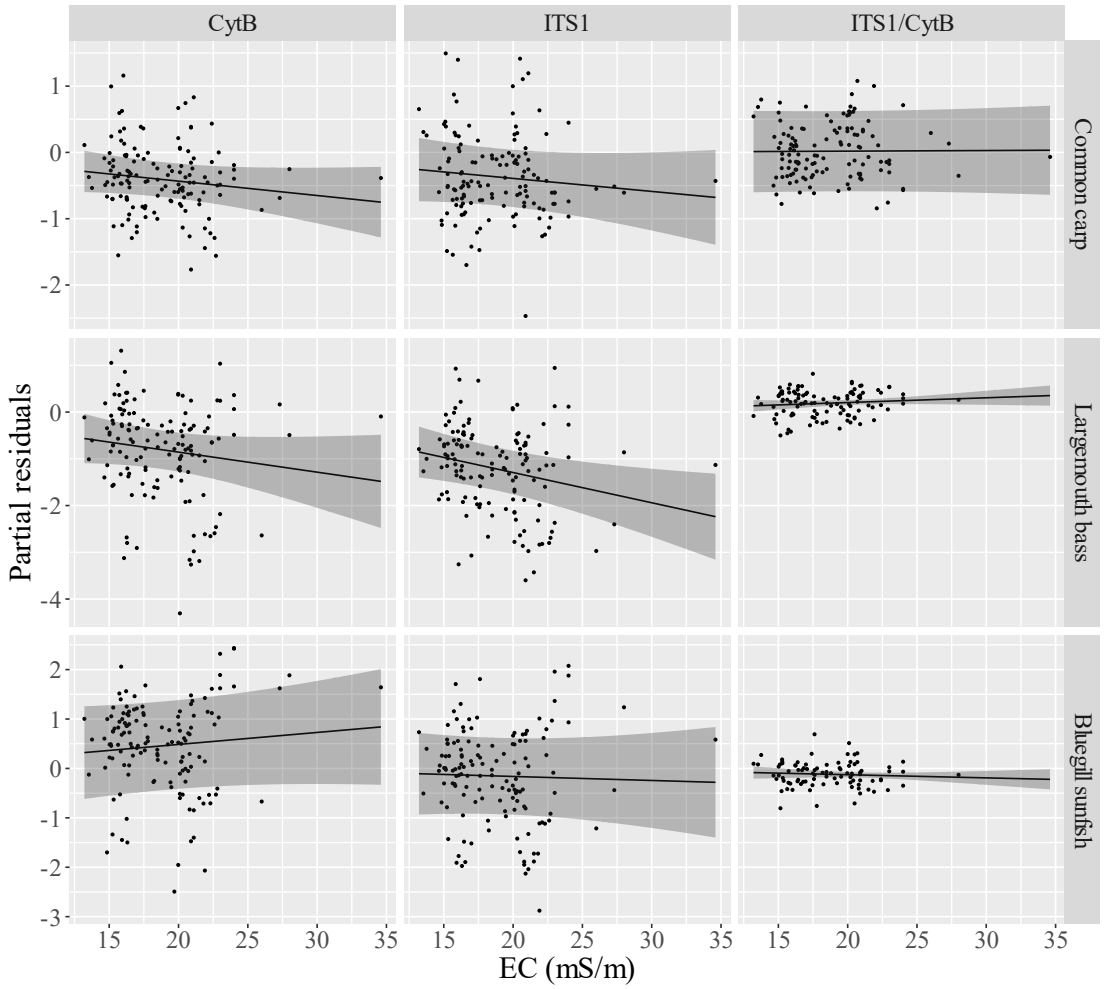

Figure S3-8. Partial residual plots for linear mixed models of EC on the eDNA concentration and ratio

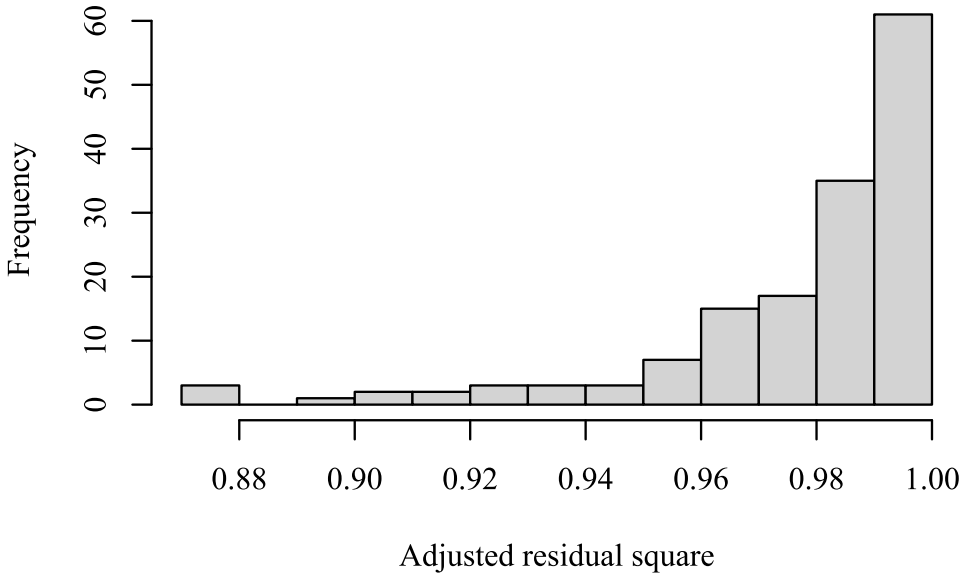

Figure S4-1. Distribution of adjusted R-square of linear regression performed to examine the relationship between sequence reads and the copy numbers of the internal standard DNAs for 152 libraries.

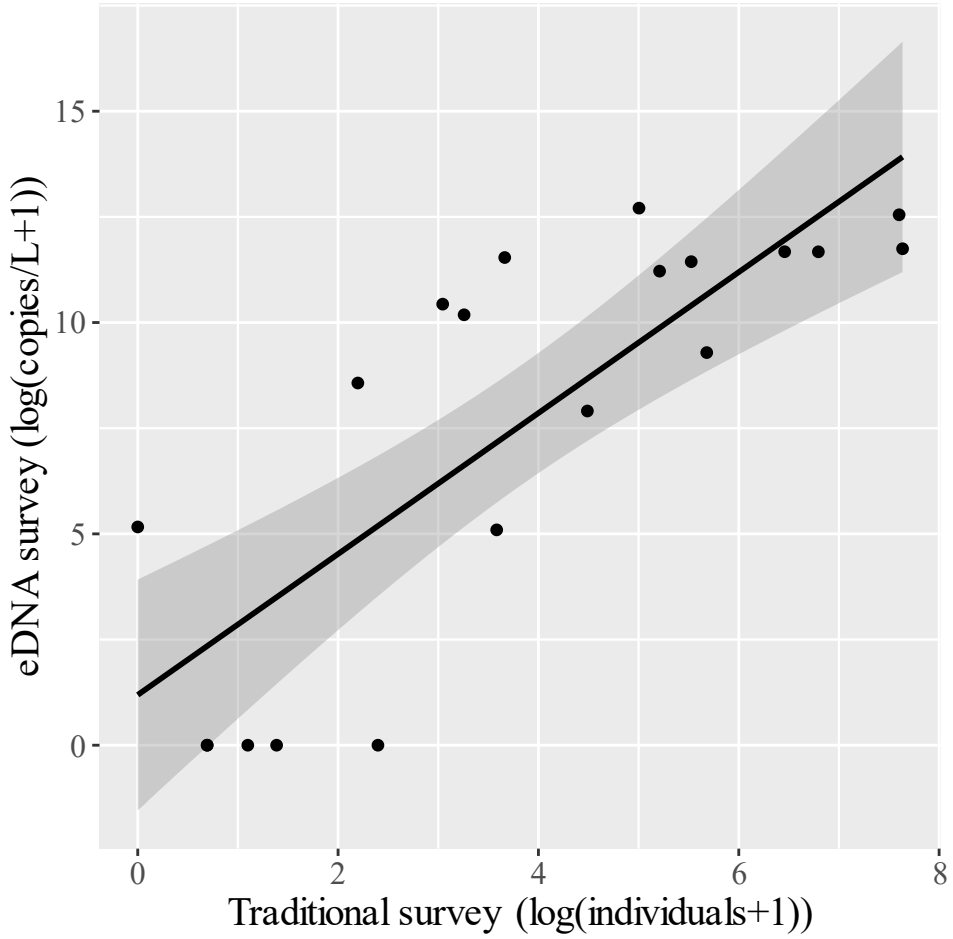

Figure S4-2. Linear regression to test the correlation between the eDNA concentration and the individual numbers of fish from multi-year merged eDNA surveys and traditional surveys. Shading refers to the 95% confidence interval.

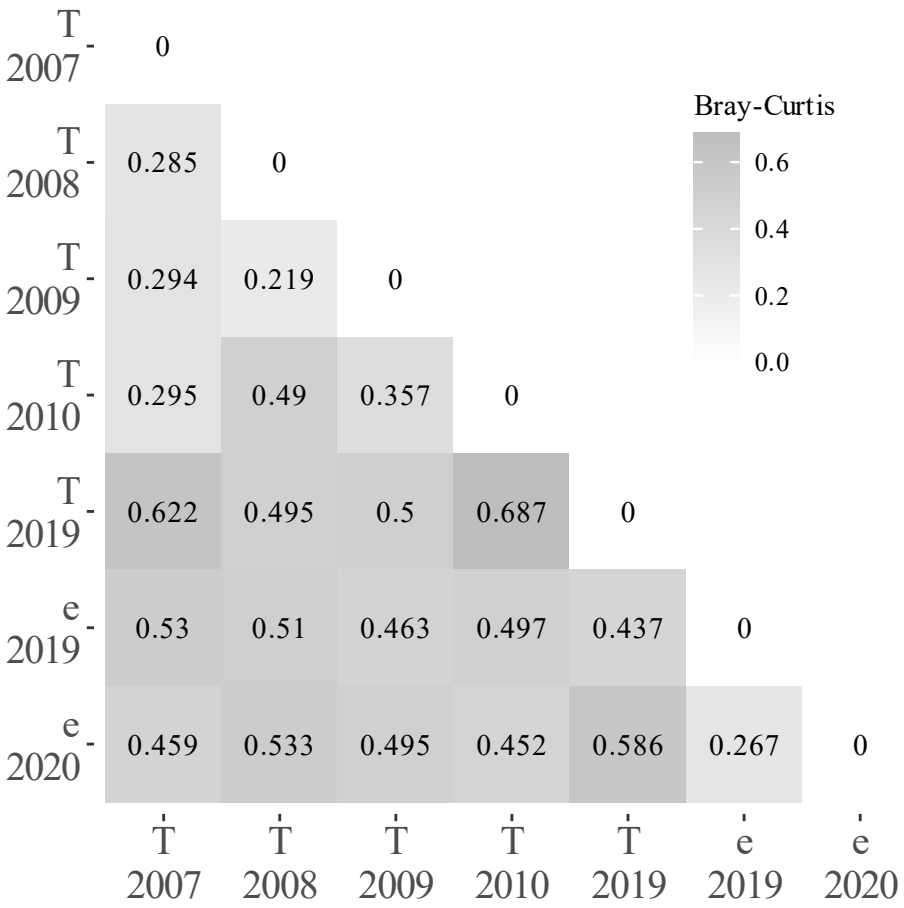

Figure S4-3. The Bray-Curtis distances between different survey results based on the relative abundance of fish.

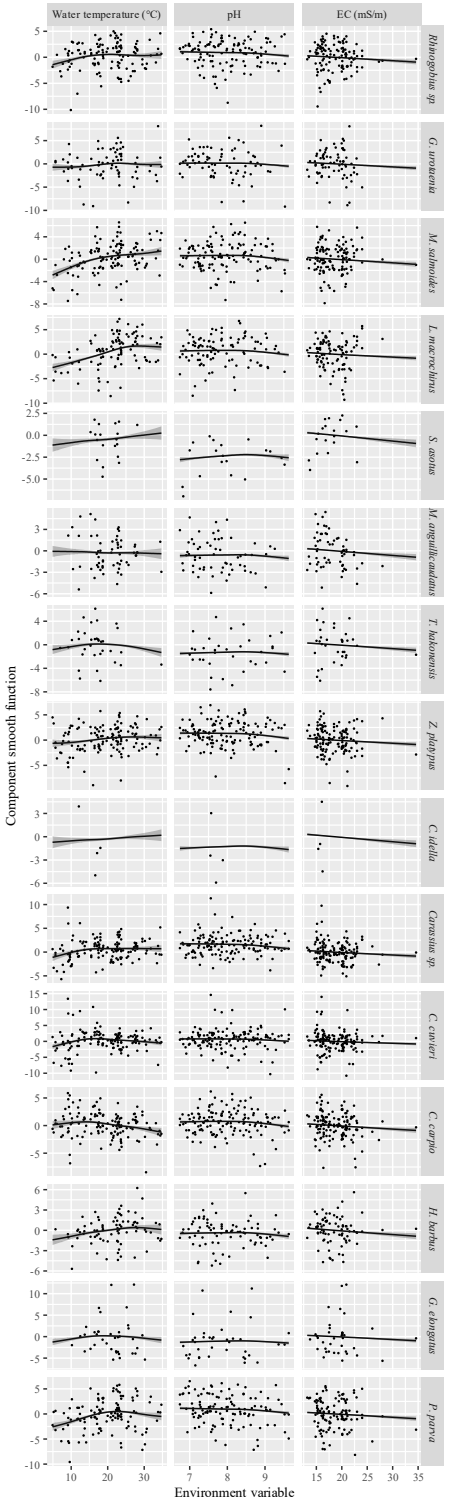

Figure S4-4. Relationships between fish-specific eDNA concentration and environmental variables. The x-axis represents environmental variables. The y-axis represents the linear predictor of component smooth functions. Points represent partial residuals.