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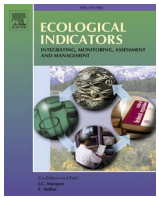

Spatiotemporal changes in environmental DNA concentrations caused by fish spawning activity

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ABSTRACT

Determining the timing and location of spawning is critical for the conservation and management of aquatic species. Environmental DNA (eDNA) analysis can be used to monitor fish spawning activity by detecting peaks in eDNA concentrations and nuclear DNA (nuDNA)/mitochondrial DNA (mtDNA) ratios; however, the duration and diffusion distance of high concentrations of eDNA produced by spawning activities in water bodies are still unknown, preventing us from making accurate and effective survey plans to monitor spawning activities. Using common carp as a target species, we conducted artificial spawning experiments to investigate the spatiotemporal changes in eDNA concentrations during spawning. The results showed that the nuDNA concentration, mtDNA concentration, and nuDNA/mtDNA ratio that were increased by spawning activity decreased at rates of 7.78 %, 5.22 %, and 2.56 %, respectively, per 0.1 h after reaching the peak. Data simulations were performed to estimate the probability of successful monitoring of carp spawning activity during repeated sampling at different times and distance intervals. The results showed that carp spawning activity over the past 24 h could be monitored by eDNA analysis. Additionally, when carp spawning activity occurs once, spawning activity can be successfully monitored by measuring the nuDNA concentration or the nuDNA/mtDNA ratio with a probability of approximately 50–75 % based on a sampling plan of sample collection every 100 m and every 24 h. Thus, the spawning activity of aquatic species can be estimated with high spatial and temporal accuracy using eDNA analysis.

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 and Grimes, 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, environmental DNA (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 et al., 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 and 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

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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 and 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 us 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, we simulated natural fish spawning 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. We used common carp (*Cyprinus carpio*) 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 us visually check the spawning activities of carp.

2. Materials and methods

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. 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, we prepared triplicate negative controls and standards diluted to 30000, 3000, 300, and 30 copies/well.

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 Supplementary Material.

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.

2.4. Data simulation

We performed two data simulations 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.

2.4.1. 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, we assumed that the eDNA concentration at this time was not affected by the spawning activity. To simulate complete spawning activity, we assumed that the eDNA concentration started to rise beginning at −1h. Because we had no −1h data, the baseline data (data for the final sampling in each group of experiments) were extrapolated as the data for −1h. 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.

2.4.2. 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, we considered the spawning activity of the carp 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).

2.4.3. 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), we performed a second simulation 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. We ran the GAMMs using the mgcv package (Wood, 2001). The LMMs were run using the nlme package. All analyses were performed using R version 4.0.3.

3. Results

3.1. Changes in eDNA concentrations

None of the blank or negative controls showed any amplification. Among 1,260 PCR wells (210 samples × 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 1). The eDNA concentration and ratio peaked at approximately 7 h and decreased thereafter (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 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. The distance and interaction of time and distance did not have a significant effect on the eDNA concentration (Table 2), consistent with the results of GAMMs (Fig. 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); 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), reflecting the difference between the rates of at which *ITS1* and *CytB* decreased.

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

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

*** < 0.001; ** < 0.01; * < 0.05.
"ti()" refers to a smooth term, and "edf" refers to effective degrees of freedom.

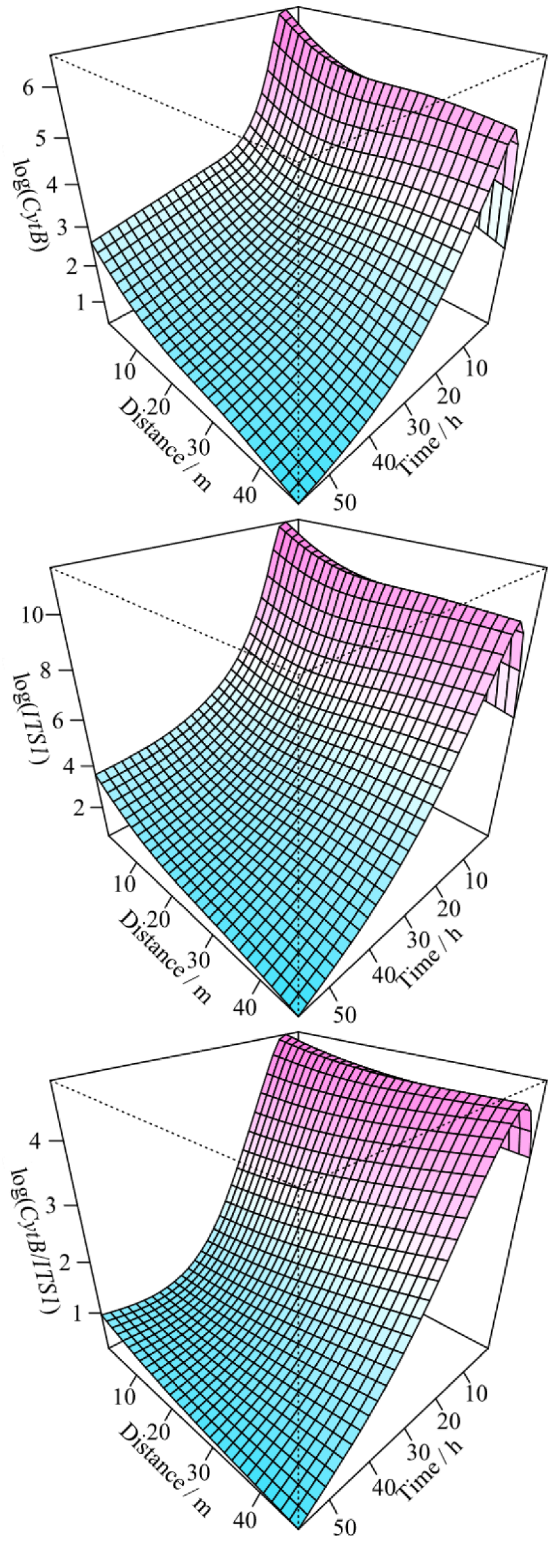

Fig. 1. Changes in eDNA concentrations and *ITS1/CytB* ratios with time and distance.

3.2. Results of Simulation I

Fig. 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 (Fig. 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 (Fig. 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 ($p < 0.001$; Fig. 2b, c).

Table 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.

3.3. Results of Simulation II

Fig. 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 (Fig. 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.

4. Discussion

Through artificial spawning experiments in carp, we showed trends in the changes of eDNA concentrations and nuDNA/mtDNA ratios produced by spawning activities under the interactions of time and distance. 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

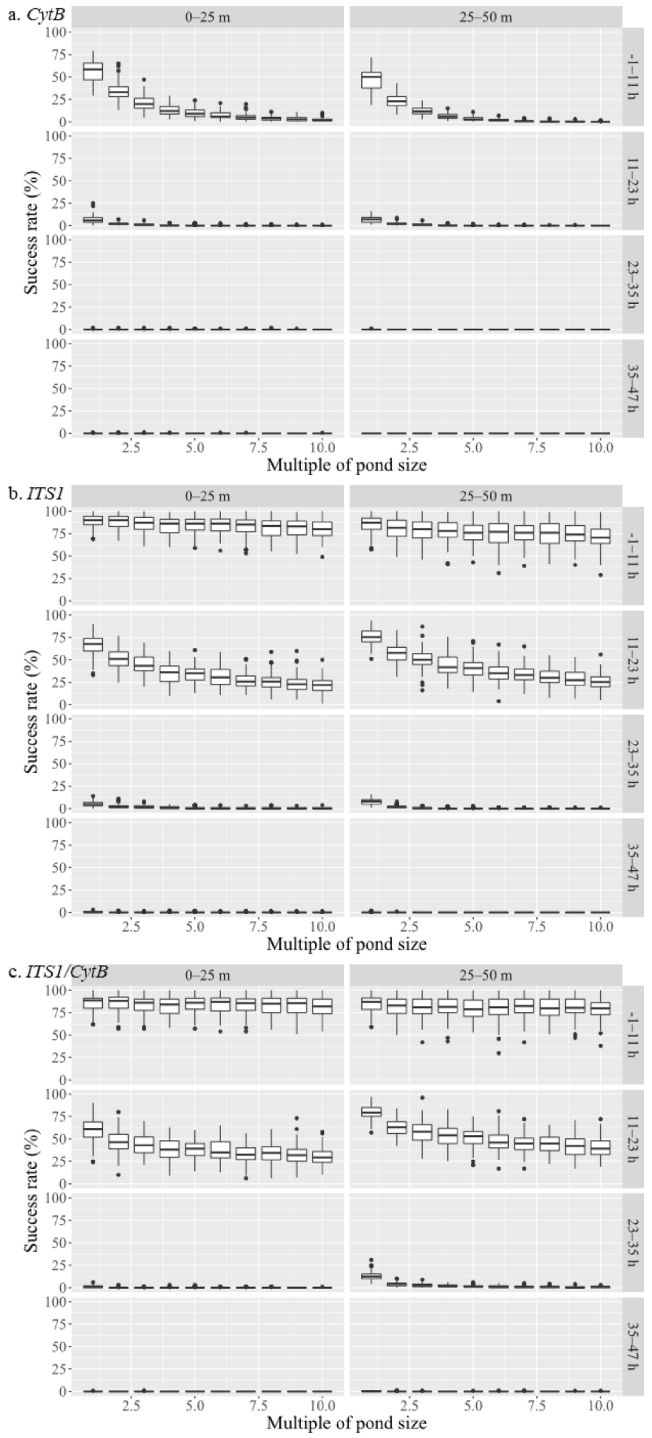

Fig. 2. Probability of successful monitoring of spawning activity after random sampling at different time and distance intervals.

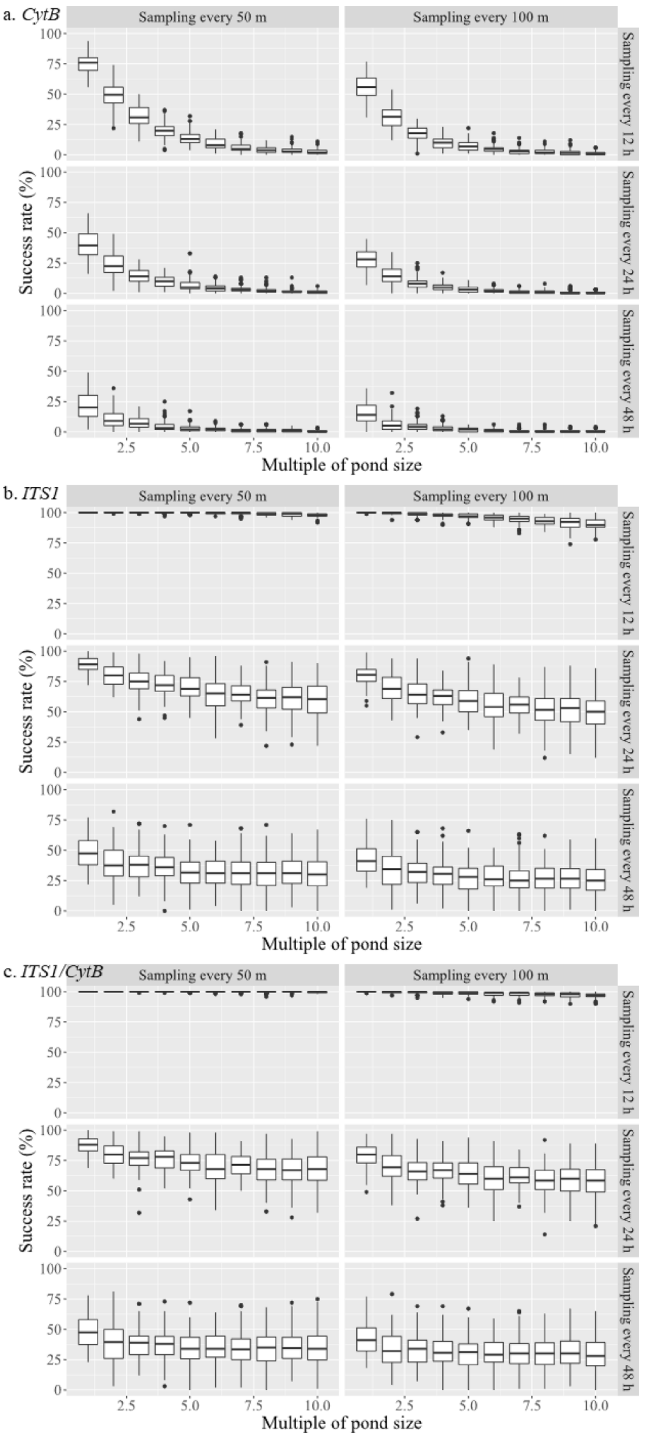

Fig. 3. Probability of successful monitoring of spawning activity after using different sampling plans.

distance showed that the high concentrations of eDNA released by spawning activity tended to be evenly distributed after peaking in the ponds; we believe this effect was related 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, we used 8–20 h data 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, we may have underestimated the rate of decrease in the eDNA concentration. To compensate for this, we ran data simulations 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, we 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, we can only estimate whether or not spawning activity has occurred, 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., under review), 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, we estimated the probability of successful monitoring of spawning activity 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 our intuition. We believe 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, we estimated the probability of successful monitoring of spawning activity under different sampling plans. Because different sampling plans for distance had less effect on the success rate, we recommend sampling every 100 m to reduce the workload. Although sampling every 12 h had the highest success rate, we still recommend sampling every 24 h. 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, our recommended general sampling plan is to sample every 24 h and 100 m. 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.

5. Conclusions

Taking the artificial spawning experiment of common carp as an example, we showed the change in eDNA concentration and nuDNA/mtDNA ratio caused by spawning activities over time and distance, 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, we recommend combining eDNA concentrations and ratios to monitor spawning activities.

Author contributions

LW, YY, and TM conceived of and designed the study. LW, YY, and YS conducted the spawning experiments. LW collected eDNA water samples and performed molecular experiments and statistical analyses of the data. LW and TM wrote and edited the manuscript, respectively. All authors have discussed the results and contributed to the development of the manuscript.

CRediT authorship contribution statement

Luhan Wu: Conceptualization, Formal analysis, Investigation, Writing – original draft, Visualization. **Yoshihiko Yamamoto:** Investigation, Resources, Writing – review & editing. **Shogo Yamaguchi:** Investigation, Resources, Writing – review & editing. **Toshifumi Minamoto:** Conceptualization, Resources, Writing – original draft, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All the raw data used to generate figures and tables can be found in the Supplementary Material.

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Appendix A. Supplementary data

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