Identifying spawning events in fish by observing a spike in environmental DNA concentration after spawning

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Abstract
An understanding of the reproductive biology of aquatic organisms is crucial for the efficient conservation and management of species and/or populations. Nevertheless, conventional spawning surveys such as visual- and capture-based monitoring generally require laborious, time-consuming work and are subject to monitoring biases such as observer bias, as well as miscounts due to false spawning. In addition, direct capture often damages eggs or individuals. Thus, an efficient noninvasive approach for monitoring spawning events on aquatic species would be a valuable tool to understand their reproductive biology and conserving biodiversity. Here, we proposed an environmental DNA (eDNA)-based approach for monitoring and understanding spawning events by observing spikes in eDNA concentration after spawning events. We found in hybridization experiment using two medaka species (Oryzias latipes and Oryzias sakaizumii, 1:1 individual per tank) that a spike in eDNA concentration occurred in male species after spawning. Besides, the magnitude of the spike in eDNA concentration was dependent on the number of spawning activities with egg and sperm release. In the field survey during the reproductive season, eDNA concentrations after spawning were 3–25 times higher than before the expected time for spawning. Additionally, there was no spike in eDNA concentration during the non-reproductive season. Therefore, our results demonstrated that spike in eDNA concentration is mainly caused by the released sperm during spawning events, and it can be used as evidence of spawning in the field survey. The presented approach could be a practical tool for studying reproductive biology and provides an opportunity to design effective conservation and environmental management actions.

KEYWORDS
concentration, environmental DNA, medaka, reproduction, spawning, sperm, spike

1 | INTRODUCTION
Reproductive events are important parts of annual life history in organisms. In aquatic species, especially for rare species and important fishery target species, understanding the timing and location of spawning are critical for efficient conservation and management of species and/or populations (Danylchuk et al., 2011; Spear et al., 2015). Nevertheless, conventional spawning surveys (by
direct observation of individuals or eggs) generally require laborious, time-consuming methods and are subject to monitoring biases such as observer bias, as well as geographical constraints and miscounts due to false spawning (mating without egg and sperm release) (Bylemans et al., 2017; Caswell et al., 2004; Diana et al., 2015; Ko et al., 2013; Koster et al., 2014; Miller et al., 2012). Besides, particularly for rare and endangered species, traditional capture survey methods may threaten the persistence of species or populations because of additional mortality among the spawning population and/or eggs (Engstedt et al., 2014; Tsukamoto, 2006; Wei et al., 2009). Thus, an efficient, noninvasive method of monitoring spawning events would be a valuable tool to increase our understanding of species’ reproductive biology and conserving aquatic biodiversity.

Environmental DNA (eDNA) is a generic term used to refer to the DNA that has been shed by organisms into the surrounding environment, and it is thought to derive from skin, feces, mucus, and reproductive materials (e.g., oocytes, ovarian fluid and sperm) (Barnes & Turner., 2016; Ficetola et al., 2008; Merkes et al., 2014; Takeuchi et al., 2019). Thus, in the case of externally fertilizing species, an increase of eDNA concentration in the reproductive season would be expected. In fact, recent works have shown a relatively higher eDNA concentration in water during the reproductive season (Bayer et al., 2019; Bracken et al., 2019; Spear et al., 2015; Takahashi et al., 2018; Thalinger et al., 2019; Tillotson et al., 2018). Furthermore, eDNA analysis could, potentially, avoid the restrictions associated with conventional spawning observations imposed by time, labor, monitoring biases, and invasiveness because eDNA analysis requires only water sampling at the study site. Therefore, the monitoring for eDNA concentration changes would provide promising opportunities to efficiently and noninvasively monitor spawning events in the area.

However, it is important to note that the seasonal increase of eDNA concentration is not always evidence of spawning; it can be caused by the formation of spawning aggregations (Takeuchi et al., 2019). While still controversial because eDNA persistence and release rates are not constant across different species and environmental conditions, many previous studies have shown a significant positive relationship between abundance/biomass of species and eDNA concentration (Doi et al., 2017; Takahara et al., 2012; Yamamoto et al., 2016). To obtain evidence of spawning events, Bylemans et al. (2017) proposed an approach focused on a change in the ratio of nuclear eDNA fragments to mitochondrial eDNA fragments during spawning. They observed the relative difference in nuclear eDNA concentration and mitochondrial eDNA concentration over approximately 40–60 hr. The increasing of the nuclear eDNA fragment after spawning probably caused by containing the released sperm which has more highly condensed and protected nuclear DNA than somatic cells as the eDNA source. It is inferred from this observation that their approach is suitable for long-term observation of reproductive season, but not for estimating the presence and amount of daily spawning events. Therefore, proposing a novel eDNA approach to monitor daily spawning events would be extremely valuable.

Here, we propose a novel approach to monitoring and understanding spawning events by observing a spike in eDNA concentration of mitochondrial DNA between before and after the expected spawning time. As suggested in previous studies (Bylemans et al., 2017; Takeuchi et al., 2019), when spawning occurs, the released sperm and/or oocytes and ovarian fluid are likely to become major sources of eDNA. These substances are mass released from individuals in a fraction of a second during spawning, and eDNA concentration should be shown a rapid increase. If only the formation of spawning aggregations, a spike in eDNA concentration due to reproductive materials will never be observed between before and after the expected spawning time. First, we examined which of reproductive material (sperm release or oocytes and ovarian fluid release) causes an increase in eDNA concentration during spawning activities using two medaka species which can easily hybridize. Besides, we analyzed the relationship between the magnitude of a spike in eDNA concentration and the number of spawning activities with egg and sperm release in the laboratory. Finally, we examined whether we could detect the spawning events of two medaka species in the field conditions based on the observation of the eDNA concentration spike.

2 | MATERIALS AND METHODS

2.1 | Study species and their spawning activities

The two Japanese medaka species, Oryzias latipes and Oryzias sakaizumii, are small (3–4 cm in standard length) freshwater fish (Iguchi et al., 2018) and listed as “Vulnerable” on the Red List of Threatened Species of Japan (Ministry of the Environment, Japan, 2015). However, many genetically distinct medaka varieties have been created and maintained by fish farmers because they can breed easily in captivity and are very popular as aquarium fish (Nakao & Kitagawa, 2015). While both wild medaka species have black–gray body coloration and are morphologically similar, the most popular cultured medaka, originated from O. latipes, has orange-red body coloration. In the present study, to identify the two medaka species easily, the orange-red variety of O. latipes and the wild type of O. sakaizumii were used for tank experiments. There is no difference in spawning events between the orange-red medaka and wild-type medaka, and they can easily hybridize in both artificial and natural conditions (Kobayashi et al., 2012; Nakao et al., 2017; Sakaizumi et al., 1992).

Spawning events of two medaka species occur when the light condition, water temperature, and adequate nutritional status are at optimum (Kinoshita et al., 2009). If they are kept under appropriate conditions, daily spawning can be induced at any point in the year for up to 3 months of duration. Optimum conditions for daily spawning are as follows: 14 hr of daylight and 10 hr of darkness, the water temperature of 25–28°C, and feeding at least three times a day. Spawning events in medaka species is usually observed within the first hour after the light is turned on (Kinoshita et al., 2009). The main...
steps are as follows (Figure 1): (a) following and positioning (the male approaches the female but keeps near distance); (b) dancing (the male swims in a circle in front of the female); (c) wrapping (the male wraps the female with his dorsal and anal fins); (d) egg release and sperm release; (e) separation (the male leaves the female); and (f) egg stripping (the attachment of the eggs to a substance). However, sometimes the female separates from the male before egg and sperm release in step 4, despite the warm-up steps (steps 1–3) were performed. It is difficult to distinguish true spawning from false spawning when observing from above. In the present study, the spawning event which was observed egg and sperm release was defined as "true spawning," and not observed it was defined as "false spawning."

Under field conditions, spawning events in the medaka species can be observed from approximately May to September (daily mean water temperature >20°C and day length >12 hr). In a wild population, spawning events start from approximately 1 hr before sunrise and continues for approximately 6 hr (Kobayashi et al., 2012). The two medaka species do not migrate to spawn and form spawning aggregations, but the abundance and biomass of each species in their habitat continue to increase during the reproductive season because hatched individuals can grow mature within some (3–4) month.

2.2 | Tank experiments

2.2.1 | Exp. 1: Effect of spawning events on eDNA concentration

The experimental design of tank experiment 1 is shown in Figure 2a. To reveal which of reproductive material (sperm release or oocytes and ovarian fluid release) causes a spike in eDNA concentration after true spawning, we hybridized two medaka species in cross-species pairings. By comparing the increased amount of eDNA between male and female species, we can estimate the main cause of the spike in DNA concentration observed after true spawning. Throughout the present study, we do not consider eggs themselves as a source of eDNA because it is large enough to be visible by the naked eye. The orange-red variety of O. latipes and the wild type of O. sakaizumii were obtained from an aquarium shop and Fukui Prefecture (Japan), respectively. A Pair were placed in tanks in two combinations, (a) male O. latipes (24.6 ± 0.45 mm standard length [SL], mean ± SD) and female O. sakaizumii (24.5 ± 0.55 mm SL) and (b) male of O. sakaizumii (26.46 ± 1.24 mm SL) and female O. latipes (14.0 ± 0.77 mm SL) (three tanks for each combination, dimensions 245 x 225 x 145 mm, 4 L). During the aquarium experiment, an aquarium without a medaka species was also prepared as the experimental control to check for cross-contamination caused by water splash and background DNA. The tanks were kept in the laboratory at 28°C room temperature with a 14:10 hr light/dark cycle for 3–10 days. To maintain optimum nutrition for the experimental fish, formula feed (Hikari medaka no esa for spawning, Kyorin Corporation) was provided four to five times a day.
The water was sampled on the day after true spawning was first observed in each tank. Using a syringe, 50 ml of water was collected from the middle depth at the center of each tank at three time points: (A) 15 min before turning on the light, (B) 15 min after turning on the light, and (C) 45 min after turning on the light. The collected water samples were immediately filtered using glass fiber filters (GF/F; GE Healthcare Japan) and stored at −20°C until DNA extraction. A GF/F filter is the most major filter which is used for the collection of eDNA from a water sample, and its mesh size (0.7 μm, mean) is smaller than the sperm of two medaka species. To avoid contamination, all filtering equipment was dipped in a >10% bleach solution for >10 min, carefully washed with tap water, and finally rinsed with ultrapure water. As a filtration negative control, after all water sampling, 50 ml of water was collected from the middle depth at the center of the experimental control tank and filtered using the same filtering equipment. The eDNA was extracted and quantified according to the methods described in Section 2.4. True spawning events were observed between time points B and C, and the eggs were still attached to the female’s body when water sample was collected from each tank after spawning events (i.e., time point C). In all tanks, all released eggs (approximately 11–23 eggs for one female) were fertilized, and thus, the sperm release from a male during spawning events was confirmed. The eDNA concentrations of the two medaka species were compared at the three time points using the nonparametric Kruskal–Wallis one-way ANOVA, followed by a Tukey honest significant differences (HSD) test on ranks. All statistical analyses in this study were performed using R ver. 3.6.0 software (R Core Team 2019), and the minimum level of significance was set at α = .05.

2.2.2 | Exp. 2: Examining the relationship between the number of spawning events and the concentration of DNA in the water

The experimental design of tank experiment 2 is shown in Figure 2b. Six males O. sakaizumii (25.5 ± 1.6 mm SL, mean ± SD) and 10 females O. latipes (22.6 ± 3.6 mm SL, mean ± SD) were placed in a tank (dimensions 450 × 240 × 300 mm) containing 24 L of aged tap water (aerated throughout the experiment). The tank was kept for 18 days under the same conditions as in the tank experiment 1. True spawning was observed every day from day 5; however, fishes did not always perform spawning activities at a specific time and a specific number of times because their conditions are changed every day (ex, physical condition, nutritional condition, and relationships with other individuals etc.). Water sampling was performed on days 5, 9, 16, and 18. Spawning events were also observed on days 6–8, 10–15, and 17. However, we could not perform water sampling because of the fish’s bad condition (temporary weakness in some individuals) and/or the observer’s schedule (S. Tsuji). All spawning events were performed by a single pair, and the sneaking by other male was not observed. Using a syringe, 50 ml of water was collected from the middle depth at the center of the tank at five-time points: 1, at 15 min after turning on the light, and 2–5 at approximately 1 min after the true spawning events (from first to fourth observations). The number and timing of the spawning events with (true spawning) and without (False spawning) egg and sperm release were recorded during water sampling. The collected water samples were stored on ice until filtration and immediately filtered using glass fiber filters (GF/F) after the water sampling at each day. On the final day (day 18), as a filtration negative control, 50 ml of ultrapure water was filtered and treated in the same manner as in the real samples. Filter samples were stored at −20°C until DNA extraction. eDNA was extracted and quantified according to the methods described in Section 2.4. In the eDNA extraction step on day 5 samples, there was a technical error that the first centrifugation to eliminate excess water left in the filters was not performed. However, there should be no difference in extraction efficiency between filters in the sample series of day 5 because extract conditions were the same among filters. Because there is no effect on the relative DNA concentration among filters on day 5, we decided that it would be acceptable to add data of day 5 to analysis. The influence of the number of spawning events on the eDNA concentration was evaluated using a generalized linear mixed-effects model (GLMM; package nlme ver. 3.1–139, Pinheiro et al., 2019) with a random effect of sampling day. In the model, the number of spawning events and log-transformed eDNA copy numbers (the average eDNA concentrations of three qPCR replications per sampling point) was set as explanatory and response variables, respectively. In addition, to examine the influence of false spawning which performed between true spawning events on eDNA concentration, the relationship between the number of false spawning and the difference in eDNA concentration between true spawning (n) and (n + 1) were examined by using GLMM with a random effect of sampling day.

2.3 | Field survey

The field survey was performed in each medaka species’ natural habitat to examine whether the spike in eDNA concentration, caused by spawning events, could be observed under field conditions. The two (L01 and L02; Shiga Prefecture, Japan; O. latipes) and one (S01; Fukui Prefecture; O. sakaizumii) sampling sites were selected for each medaka species. The field type was a small water channel (width <1.0 m; L01 and S01) or a pond (<10 m in diameter; L02). We avoided publishing detailed information of each sampling site because the fish are rare species. Water sampling was performed before and after sunrise in the reproductive season (September 15 or 16, 2019) and non-reproductive season (November 9 or 10, 2019). Detailed information on sampling times, sunrise times, and water temperatures is shown in Table 1 and Table S5. Water qualities were measured using a handy sensor (PCS Tester 35; Advance Instruments & Chemicals). Water flow conditions in each sampling site were not measured; however, all sampling sites had relatively low flow conditions. Additionally, during the reproductive season, a visual survey was performed between sunrise and a second water sampling to confirm spawning.
We collected 0.5 L of surface water from each sampling site at two-time points: approximately 1–2 hr before sunrise and approximately 2–3 hr after sunrise, using a disposable plastic cup (Table 1 and Table S5). The collected water sample was immediately filtered using a glass fiber filter on-site, and each filter was placed in a plastic bag and kept at −20°C until eDNA extraction. As a filtration negative control, the same volume of ultrapure water was filtered at the end of each sampling day and treated in the same manner as the samples. eDNA was extracted and quantified according to the methods described in Section 2.4.

### 2.4 Laboratory analysis

#### 2.4.1 eDNA extraction from filter samples

The eDNA extraction and purification were performed following the procedures described in Tsuji et al. (2020). Briefly, each filter sample was rolled and placed in the spin column (EZ-10; Bio Basic) with the attached silica-gel membrane. The spin column was centrifuged for 1 min at 6,000 × g to remove any excess water remaining in the filter. Then, 310 µl of a mixture, composed of 200 µl of ultrapure water, 100 µl of Buffer AL, and 10 µl of proteinase K, was placed in each of the filter’s spin columns. After incubating the spin column at 56°C for 30 min, the liquid held in the filter was collected by centrifugation for 1 min at 6,000 × g. To increase the eDNA yield, the remaining ultrapure water was filtered at the end of each sampling day and treated in the same manner as the samples. eDNA was extracted and quantified according to the methods described in Section 2.4.

#### 2.4.2 qPCR quantification of eDNA

The number of DNA copies of each medaka species was quantified using the previously developed species-specific primer/probe sets which can detect mitochondrial DNA of each medaka species (Table S1; Tsuji et al., 2018) and the real-time polymerase chain reaction (qPCR) system (QuantStudio 3, Thermo Fisher Scientific). The qPCR was conducted in 15-µl volume with a reaction solution that consisted of 900 nM of each primer (OlaND5-F/R or Osa16S-F/R), 125 nM of TaqMan probe (OlaND5-Pr or Osa16S-Pr), 0.2 µl AmpErase Uracil N-Glycosylase (Thermo Fisher Scientific), and 2.0 µl of DNA template in 1x TaqMan Environmental Master Mix (Thermo Fisher Scientific). For each qPCR run, a four-point standard curve, with triplicate for plasmid DNA of each target region at known copies (3 × 10^1 to 3 × 10^5), was used to estimate the absolute eDNA concentration. Also, a PCR negative control, 2.0 µl of ultrapure water was added and analyzed instead of the DNA template to assess cross-contamination. The qPCR was performed in triplicate for all eDNA samples, standard dilution series, and the PCR negative control, with the following thermal conditions: 2 min at 50°C, 10 min at 95°C, 55 cycles of 15 s at 95°C, and 1 min at 60°C.

### 3 RESULTS

In tank experiment 1, which examined the effect of spawning events on eDNA concentration, the male species’ eDNA concentration increased drastically after true spawning (time point C: 45 min after turning on the light) in all tanks regardless of species (Figure 3a, O. latipes p < 0.001; Figure 3b, O. sakaizumii p < 0.001). The female species’ eDNA concentration increased after true spawning only when the O. sakaizumii was female (Figure 3a, p = 0.05). In all tanks, the increase in eDNA concentration after true spawning was higher in males than in females. After true spawning, male species’ eDNA concentrations were 5.9 (O. latipes) and 16.1 times (O. sakaizumii) higher, compared with those at 15 min, after turning on the light (before spawning). In both species, there were no differences in the eDNA concentrations between time point A (15 min before turning on the light) and B (15 min after turning on the light), regardless of sex. The results of all statistical analyses in tank experiment 1 are summarized in Table S2.

In tank experiment 2, all water sampling was performed within 1 hr and 45 min (Table S3). We found a significant effect on the number of true spawning on eDNA concentration (Figure 4a, p < 0.001). This significant effect was also found when the data of 0 true spawning was omitted. In addition, there was a significant relationship between the number of both true and false spawning and the increase...
in eDNA concentration (Figure 4b, \( p < 0.05 \)). However, there was no significant relationship between the number of false spawning and the increase in eDNA concentration between true spawning (\( n \)) and (\( n + 1 \)) (Figure 4c, \( p = 0.83 \)). On day 18, the first true spawning was observed within 15 min after turning on the light; thus, data on the period before the spawning (i.e., the number of spawning 0) could not be obtained. The results of all statistical analyses in tank experiment 2 are summarized in Table S4.

In the field survey, a spike in eDNA concentrations after sunrise was observed only during the reproductive season (September) in all sites (Figure 5). The eDNA concentrations after sunrise at each site were 25.0, 7.2, and 3.1 times higher compared with samples collected at night. Although a visual survey was performed between sunrise and the second water sampling, spawning event was not observed. In the non-reproductive season (November), there were no notable changes in eDNA concentrations between the points before and after sunrise, regardless of species.

In all experiments, no medaka DNA was detected in the experimental control tank, filtration negative controls, and PCR negative controls, indicating that there was no cross-contamination during experiments. In all qPCR runs, the \( R^2 \) value of the standard curve ranged from 0.984 to 0.991.

4 | DISCUSSION

We found spikes in medaka DNA concentration during medaka spawning events. Furthermore, although both sperm from males

![Figure 3](image)

**Figure 3** Comparison of eDNA concentration among three time points in tank experiment 1. Three time points are as follows: A – 15 min before turning on the light; B – 15 min after turning on the light; and C – 45 min after turning on the light. Spawning events were performed between time points B and C in all tanks. Orange squares and blue circles indicate *Oryzias latipes* and *Oryzias sakaizumii*, respectively. The species combination was as follows: The left panel (a) was male *O. latipes* and female *O. sakaizumii*, the right panel (b) was male *O. sakaizumii* and female *O. latipes*.

![Figure 4](image)

**Figure 4** Relationship between the *Oryzias sakaizumii* DNA concentrations and the number of spawning events in the tank experiment 2. The x-axis in each figure represents the number of (a) true spawning only, (b) both of the true and false spawning, (c) false spawning between true spawning events, respectively. The difference in eDNA concentration between true spawning events in panel (c) was calculated by the following equation; true spawning \((n + 1)\) – true spawning \((n)\). Squares, circles, triangles, and asterisk indicate sampling day 5, 9, 16, and 18, respectively.
and secretions from females such as oocytes and ovarian fluid can be a source of eDNA, the significantly higher increase in eDNA on male species suggested that sperm release was the main factor of the spike in eDNA concentration by spawning events (Figure 3). To our knowledge, this is the first study that experimentally identified the main factor for the increase in eDNA concentration caused by spawning events. Our result is supported by the fact that the head length and head width of the sperm of *O. latipes* are 2.6 and 1.7 μm, respectively, and can be trapped by GF/F filter with a mesh size of 0.7 μm (mean) (Iwanatsu, 1999). These facts also suggest that the monitoring of spawning events based on the observation of the spike in DNA concentration can be applied to many other externally fertilizing species.

Our results showed a significant positive relationship between the number of true spawning events and the increase in eDNA concentration, suggesting that the magnitude of the spike in eDNA concentration has the potential to use for comparison of the relative amounts of true spawning among sites. Since decreased data reliability due to the observation of false spawning is a serious limitation for estimating spawning amounts by conventional methods, eDNA-based monitoring which reflects only true spawning events would be useful to assess of spawning events. However, it is probable that the magnitude of the spike in eDNA concentration caused by spawning events is different for each species in relation to the difference in the amount of sperm emission and/or the mating systems. A recent study shows that there is a relationship between DNA copy number and the cell abundance of artificially added a laboratory sperm dilution series (Bayer et al., 2019). In tank experiment 1, we found a difference in the increased amount of eDNA caused by true spawning between *O. latipes* and *O. sakai-zumii*. The difference in the increased amount is most likely due to the difference in the amount of released sperm based on the difference in body size of male species (t test, p = 0.068, close to significance). On day 9 and 16 in experiment 2, a rapid increase of eDNA was observed when the first spawning was performed by comparing with when the second and subsequent times of spawning was performed. This probably resulted from the strongest (i.e., largest body size) individual of male performed the true spawning with multiple females during a day, and the more amount of sperm was released on first true spawning. Other males tried to spawn with females, it stopped short of performing the true spawning due to own alertness and the attack by other males. In addition, we agitated the water during tank experiment 2 by aeration but it is possible that the water containing released sperm was not agitated sufficiently until water sampling. However, the sperm cell count and observation of the amount of sperm emission were not carried out in both tank experiments. In addition, although me-daka species perform spawning activities in monogamous pairs, there are many species which perform spawning activities by many and unspecified individuals (e.g., aggregation spawning) in other species. In this case, it remains to be seen if the relationship between the number of spawning events and the increase in eDNA concentration can be observed. Thus, further insights into these aspects are left future studies.

A spike in eDNA concentration after sunrise was observed in each field only during the reproductive season (September), suggesting that we can use the spike in eDNA concentration as evidence of spawning event in field surveys. As a spawning occurred every day at each sampling site in the reproductive season, it seems reasonable to consider that the spike in eDNA concentration occurred every day and exhibited a diurnal change pattern. This was supported by the fact that the lowest eDNA concentration was observed before spawning on days 9 and 16 in tank experiment 2, despite spawning events occurring every day. Additionally, in the reproductive season, we found that eDNA concentration after sunrise was 3.1- to 25.0-fold higher than that observed before sunrise, and the increase rate was higher in the sampling site with higher water temperature. This observation could indicate that the size of the spike in eDNA concentration reflects the magnitude of spawning events in each sampling site. Especially, the water temperature was kept at optimal (i.e., approximately 25°C) in L01, the expectation was that spawning would occur most actively across the three sites. However, spawning events were not observed by the visual survey. Observing spawning events under field conditions is challenging because the fish are small and can easily hide in the waterweeds. In our visual survey, juveniles and females with eggs were frequently observed, but not spawning events. Our results suggest that eDNA is likely to be a sensitive tool for monitoring spawning events and has greater success than visual surveys.

We propose that spikes in eDNA concentration between before and after the expected time for spawning events may be used to monitor and understand of spawning events. When the spawning...
timing is unclear, monitoring the seasonal change and/or diurnal change of eDNA concentration is an effective means of estimating spawning timing. An estimate of spawning site and/or timing is difficult using conventional methods such as visual survey and drift nets because these methods are strongly limited by field conditions (e.g., visibility, terrain) and the characteristics of the species (e.g., body size, infauna) and the labor-intensiveness of the tasks (Takeuchi et al., 2019). There is only one study which observed medaka species spawning events under natural conditions (Kobayashi et al., 2012) because of the difficulty of the observation. In fact, in the field survey in the present study, we tried to observe the spawning events by a visual survey, but it was never observed because they are small and hide under waterweeds. We can narrow down the spawning season and time without these limitations more efficiently by observing spikes in eDNA concentration because eDNA analysis requires only water sampling at the study site. In addition, we may avoid the survey restrictions on conventional spawning surveys imposed by time, labor, monitoring biases, and invasiveness, for the same reason. Therefore, monitoring for eDNA concentration changes would provide promising opportunities to efficiently and noninvasively monitor spawning events. We can also estimate the preferred spawning site by comparing the amount and/or magnitude of the spikes in eDNA concentration among sites. Understanding preferred environments for spawning is critical for efficient species and/or population conservation and management. Furthermore, comparing the size of spikes in eDNA concentration can also be used to evaluate the effectiveness of artificial spawning grounds and the fish ladder as a pathway to the spawning site.

The present study also has implications for the sampling designs of future studies. When detecting rare species and/or invasive species at the initial stage of invasion, false-negative results are typically caused by low DNA concentrations (Carim et al., 2019; Jerde et al., 2013). Thus, because eDNA concentration temporarily increases, we expect to increase the detection rate with water sampling during the reproductive season and spawning hours. On the other hand, when estimating biomass by quantifying eDNA concentration, we recommend avoiding surveys during the reproductive season. The eDNA concentration will change easily and widely depending on the frequency of spawning events, and this will lead to confusion when interpreting the results. In addition, in metabarcoding using universal primers, the particular species DNA that increased by spawning events is likely to inhibit PCR amplification and sequence determination of minor species’ DNA because of the consumption PCR enzyme and sequence reads. Therefore, it is necessary to design a research plan that considers whether the sampling season and hours are appropriate for the detection methods and the study purpose.

In conclusion, the spikes in medaka eDNA concentration we observed in our studies were the result of spawning events. Observing a spike in eDNA concentration from spawning provides the opportunity to monitor and understand spawning timing and site with less labor, time, and invasiveness than conventional methods. Knowing and understanding reproductive biology is critical and useful for efficient conservation and management of species and/or populations. Although the presented approach shows many applications as a practical tool for reproductive biology, additional studies examining the degradation and transport of released sperm would provide better insights.

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CONFLICT OF INTEREST
None declared.

AUTHOR CONTRIBUTION
S.T. conceived and designed research. S.T. and N.S. performed sampling and experiments. S.T. and N.S. performed data analysis. S.T. wrote the early draft and completed it with significant inputs from N.S.

DATA AVAILABILITY STATEMENT
Full details of the samples, qPCR results, and statistic results for each experiment of the present study are available in the supporting information (Table S1–S4).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.