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Stability of environmental DNA methylation and its utility in tracing spawning in fish

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ABSTRACT

1. The use of environmental DNA (eDNA) is becoming prevalent as a novel method of ecological monitoring. Although eDNA can provide critical information on the distribution and biomass of particular taxa, the DNA sequences of an organism remain unaltered throughout its existence, which complicates the accurate identification of crucial events, including spawning. Therefore, we examined DNA methylation as a

novel source of information from eDNA, considering that the methylation patterns in eggs and sperm released during spawning differ from those of somatic tissues.

2. Despite its potential applications, little is known about eDNA methylation, including its stability and methods for detection and quantification. Therefore, we conducted tank experiments and performed methylation analysis targeting 18S rDNA through bisulfite amplicon sequencing.

3. In the target region, eDNA methylation was not affected by degradation and was equivalent to the methylation rate of genomic DNA from somatic tissues. Unmethylated DNA, abundant in the ovaries, was detected in the eDNA released during fish spawning.

4. These results indicate that eDNA methylation is a stable signal reflecting targeted gene methylation and further demonstrate that germ cell-specific methylation patterns can be used as markers for detecting fish spawning.

1 INTRODUCTION

Reproduction is one of the most significant events in the life cycle of aquatic organisms; therefore, monitoring reproductive activities is necessary for preventing population decline due to environmental changes at local or global scales and for predicting biomass fluctuations for sustainable fisheries management (Erisman et al., 2017; Koenig et al., 2000). The conservation of rare and valuable fishery species depends on a thorough understanding of their breeding activities vis-a-vis breeding grounds and respective time durations to establish appropriate measures (Chollett et al., 2020; Arthington et al., 2016); however, it is difficult to investigate spawning in species that are elusive, occur in small populations or migrate extensively. Even for common species, extensive and high-frequency monitoring of reproductive status requires considerable effort. In recent years, wildlife monitoring methods have moved beyond traditional observations and catch surveys to high-throughput analyses of genetic material. The environmental DNA (eDNA) approach has mainly been used to determine species distribution (Franklin et al., 2019; Tingley et al., 2017); however, it is also effective for monitoring the reproductive activities of individual species. eDNA concentrations show seasonal fluctuations, increasing during the breeding season and spiking during the most active breeding hours of the day (Bracken et al., 2019; Wu et al., 2023a; Tsuji & Shibata, 2021). Furthermore, the increased ratio of nuclear to mitochondrial DNA concentration caused by the low abundance of mitochondria in sperm cells has been used to estimate fish spawning (Bylemans et al., 2017; Wu et al., 2022; 2023b; Saito et al., 2023).

Current analyses of eDNA and interpretation of biological signals from eDNA are based on the detection/non-detection of specific sequences or their concentration; however,

57 eDNA detection and quantification are affected by DNA release and degradation rates, as
58 well as DNA dispersion. Biological factors (e.g. growth stage, body size, population
59 density, and changes in activity) influence the rate of DNA release (Takeuchi et al., 2019;
60 Jo et al., 2019; Yates et al., 2021; Rourke et al., 2022), and environmental factors (e.g.
61 water flow, UV, pH, and temperature) affect DNA degradation and dispersion (Jo et al.,
62 2022; Shogren et al., 2018; Strickler et al., 2015). At present, it is difficult to evaluate the
63 contributions of individual factors in the field; therefore, extracting biological or
64 ecological information based on eDNA concentrations involves inherent uncertainties.
65 Furthermore, reproductive patterns assessed from eDNA are based on quantitative
66 differences among samples, which are not necessarily qualitative and provide indirect
67 evidence. Clear and direct evidence of spawning is required to conserve native species or
68 prevent the colonisation of exotic species (Comizzoli & Holt, 2019; Gutowsky et al.,
69 2020). Therefore, to produce more robust results from eDNA, it is necessary to develop
70 direct indicators of the biological information that is unaffected by such factors.

71 In this study, we examined DNA methylation as a new source of biological information
72 from eDNA. DNA methylation is an epigenetic regulation of gene expression without
73 altering the DNA sequence, and in eukaryotes, methylation of the bi-nucleotide sequence
74 5′-CpG-3′ contributes to the modification and maintenance of gene expression (Law &
75 Jacobsen, 2010). In addition, gene expression states altered by methylation patterns can
76 be passed on across cellular generations (Attwood et al., 2002). Methylation patterns can
77 change in response to cellular differentiation, environmental effects, or pathological
78 processes (Kamstra et al., 2015); therefore, specific methylation has been used in fish as
79 a biomarker indicating sex, age, and genotoxicity (Valdivieso et al., 2023; Mayne et al.,

2020; Head, 2014). Methylation analysis using noninvasive biological residues has gained attention in recent years. Yagi et al. (2023) demonstrated the utility of the noninvasive technique for epigenetic age estimation using dolphin faecal DNA, and this approach appears to be one of the forerunners of the approach involving the methylation analysis of biological residues in water (eDNA in a broad sense). Zhao et al. (2023) reported that the methylation status of freshwater snail eDNA varies with age in tank experiments.

Although eDNA released into water by vertebrates, including fish, is a crucial subject in eDNA studies, little is known regarding its methylation. In this study, methylation analyses of eDNA and genomic DNA (gDNA) were performed in zebrafish (*Danio rerio*). Zebrafish are easy to maintain and breed, and they are frequently used in epigenetic studies to investigate mechanisms of DNA methylation in vertebrates. First, to examine the stability of eDNA methylation, we monitored eDNA methylation stability in eDNA released in fish tanks as it degraded over time (Tank Experiment 1). Second, to determine whether the methylation state of eDNA was equivalent to that of the gDNA, the methylation of gDNA in four tissue types, namely, skin, brain, testis, and ovary, was examined and compared with that of eDNA. Third, to observe ovary-specific hypomethylation in the target region and explore the possibility of using unmethylated DNA as a marker for germ cells, we traced fish spawning in tanks where diurnal reproduction occurred (Tank Experiment 2).

2 MATERIALS AND METHODS

2.1 Tank Experiment 1: Monitoring the methylation levels of eDNA undergoing degradation over time

In Tank Experiment 1, to examine the methylation levels of eDNA undergoing degradation over time, water samples were collected immediately after the removal of fish from the breeding tanks for up to 72 h. Three breeding tanks (Tank 1-1, Tank 1-2, and Tank 1-3) containing 9 L of water each and one without fish (a blank tank) were prepared (Figure S1). A water sampling tube was installed in the tanks, and the water inlet was covered with a net. A transparent perforated partition board was mounted in the middle of each breeding tank to prevent fluctuations in the eDNA methylation rate caused by sperm and egg release. Six male and six female fish were placed in separate compartments. All tanks were covered with lids, and boards were placed between the tanks to avoid contamination. Fish were fed once a day, ensuring no food was left over, and water temperature was maintained at 27.5 ± 0.5 °C. The lights were turned on at 10:00 JST and off at 22:00 JST. After 5 days of acclimation and eDNA saturation in the tanks, all fish were quickly removed from each breeding tank. After the removal of the fish, the tank conditions were maintained, except that feeding was stopped. At 0, 1, 3, 12, 24, 48, and 72 h after removing the fish, 500 ml water was sampled from each of the four tanks. Water samples were filtered immediately through GF/F filters (pore size: 0.7 μm) (GE Healthcare Life Science, Little Chalfont, UK) and stored at −25 °C until DNA extraction. A total of 28 samples were collected, with seven samples collected from each of the four tanks. All equipment used for water collection and filtration was bleached in 0.1% sodium hypochlorite before use.

2.2 Tank Experiment 2: Monitoring eDNA methylation during fish spawning

Mature zebrafish can spawn continuously up to 2–3 times per week, and an interval of a few days can be used to optimise mating induction (Nasiadka & Clark, 2012). In Tank Experiment 2, spawning was artificially induced by separating the sexes and bringing them together at the beginning of the light period, and progress was closely monitored using eDNA analysis.

Three breeding tanks (Tank 2-1, Tank 2-2, and Tank 2-3) filled with 9 L of water and a blank tank were used for Experiment 2. The breeding and equipment conditions were the same as those used in Experiment 1. Five males and five females were placed in separate compartments. After 4 days of acclimation, eDNA sampling was performed. The first day of sampling was considered day 1, and water sampling continued until day 4. At 10:00 on day 2, the partitions were removed and fish of both sexes were allowed to mix. The water collection regimes are listed in Table 1. Water was added after each sampling to compensate for the decrease, except for periods of less than 1 hour after light exposure, which may interfere with mating behaviour. The collected 500 ml of tank water was immediately filtered through a GF/F filter, and the filter was stored at -25 °C. Sixteen samples were collected per tank for a total of 64 samples, including the blank tank.

2.3 eDNA extraction

The extraction of eDNA from the filters was performed using a DNeasy Blood & Tissue Kit (Qiagen , Hilden, Germany) following the protocol published by the eDNA Society (Minamoto et al., 2021) with slight modifications, i.e. using buffer ATL instead of buffer AL (Wu & Minamoto, 2023). The concentration of the extracted DNA was determined using a Qubit dsDNA Quantification Assay Kit (Thermo Fisher Scientific, USA) with a Qubit 3.0 Fluorometer (Thermo Fisher Scientific). To prevent cross-contamination, eDNA was extracted in a different laboratory room physically separated from gDNA extraction or PCR.

2.4 gDNA extraction

A DNeasy Blood & Tissue Kit (Qiagen, Germany) was used following the manufacturer's tissue extraction protocol to obtain gDNA from skin, brain, testis, and ovary samples (n = 6) collected from 12 zebrafish (six males and six females). The DNA concentrations were quantified as described above.

2.5 Primer development for qPCR

The nuclear ribosomal internal transcribed spacer (ITS), a nuclear DNA marker with high detection sensitivity due to tandem repeats (Minamoto et al., 2017), was used to quantify the concentration of eDNA in the collected water samples. We downloaded the sequence of the zebrafish rDNA ITS2 region from the National Center for Biotechnology

Information (NCBI) database and searched for a primer set with no intraspecific variation in the sequence covered by the primers, melting temperatures of around 60 °C, T_m difference between primers within 2.0 °C, and an amplificon length of 150 bp or less. Primers with an amplification length of 132 bp were identified, and a probe was designed to hybridise to the respective internal regions (Table 2). The specificity of the primer set was confirmed using Primer Blast (NCBI).

2.6 qPCR

eDNA concentrations were quantified by qPCR using a StepOnePlus Real-Time PCR system (Thermo Fisher Scientific). All runs were performed using triplicates with 20 µL per sample. The reaction mix contained 10 µL 2xTaqMan Environmental Master Mix 2.0 (Thermo Fisher Scientific), 0.1 µL AmpErase Uracil N-glycosylase (Thermo Fisher Scientific), 900 nM of each primer (Dre-ITS2-F1/Dre-ITS2-R1: Table 2), 125 nM probe (Dre-ITS2-P: Table 2), and 2 µL template DNA. The thermocycling conditions were 50 °C for 2 min, 96 °C for 10 sec followed by 55 cycles of 96 °C for 15 sec and 60 °C for 1 min. Standard curves were generated using linear DNA prepared by treating a circular plasmid (pEX-A2J2) containing the target sequence. Standards were prepared through dilution to 3 × 10^1 to 3 × 10^4 copies per reaction. Each PCR run included a negative control using ultrapure water instead of template DNA.

184 **2.7 Primer development for bisulfite amplicon sequencing**

185 We developed a region-specific methylation assay using bisulfite amplicon sequencing
186 (BSAS) (Masser et al., 2015) to measure the methylation rates of the internal regions of
187 18S rDNA. The 18S is arranged in tandem repeats with the ITS region. It exhibits high
188 detection sensitivity, similar to that of the ITS region, and sequence information has been
189 recorded for numerous species, rendering it highly useful as an eDNA marker (Othman
190 et al., 2021). We downloaded the zebrafish 18S rDNA sequence from the NCBI database
191 and used MethPrimer (https://www.urogene.org/methprimer/) to generate two sets of
192 primers that matched the target region after bisulfite base substitution. Two additional sets
193 of primers were designed manually. The following conditions were considered to design
194 the sequencing primers: 20–40 bp per primer an amplicon length of approximately 250
195 bp (allows iSeq100 paired-end sequencing), T_m in the 50–60 °C range, >10 CpG sites in
196 the amplicon, and no CpG sites in the primers (bisulfite can cause TG or CG, biasing the
197 amplification efficiency). Four primer sets were thus generated and were tested *in vitro*.
198 The amplification efficiency and specificity of the primer sets were examined using
199 agarose gel electrophoresis and Sanger sequencing, and one set of primers was selected.
200 Primers with added adapter sequences (Dre_18S_BF2A and Dre_18S_BR2A) were used
201 for BSAS (Table 2). The amplicon of the developed primers was 265 bp long and
202 contained 19 CpG sites.

203 We also conducted Sanger sequencing of amplicons derived from skin gDNA of males
204 and females and from ovarian gDNA with sequencing primers designed separately to
205 ensure that the primer sequences did not cover sites of intraspecific mutations that would
206 suppress PCR efficiency and to determine reference sequences for use in BSAS.

207

208 **2.8 Bisulfite amplicon sequencing**

209 **2.8.1 Bisulfite treatment**

210 DNA was bisulfited and cleaned up using an EpiTect Fast DNA Bisulfite Kit (Qiagen),
211 according to the manufacturer's instructions. gDNA was diluted and prepared in TE buffer,
212 and 20 µL (400 ng) was processed. For eDNA, 20 µL was processed after ensuring that
213 the amount of eDNA was within the range recommended by the manufacturer (1 ng to 2
214 µg). Finally, 15 µL bisulfite-treated DNA was collected and TE buffer (pH 8.0) was added
215 up to 30 µL. DNA was then stored at -25 °C.

216

217 **2.8.2 Library preparation**

218 The BSAS protocol using next-generation sequencing (NGS) was performed as described
219 by Masser et al. (2015). The first PCR specific to 18S rDNA was performed using
220 bisulfite-treated eDNA or gDNA as the template. TaKaRa EpiTaq HS (Takara Bio, Shiga,
221 Japan) was used as the PCR reagent. The reaction mixture contained 2.5 µL of 10×EpiTaq
222 PCR Buffer, 2.5 µL of 25 mM $MgCl_2$, 3 µL of dNTPs (2.5 mM each), 0.125 µL of EpiTaq
223 HS (5 U/µL), 400 nM each of primers (ER3/ER1: Table 2), 2 µL bisulfite-treated DNA,
224 and ultrapure water up to 25 µL. The thermocycling conditions were 98 °C for 10 min
225 followed by 45 cycles of 98 °C for 30 s, 52 °C for 30 s, and 72 °C for 30 s. Each PCR run
226 included a negative control with ultrapure water instead of template DNA. As a few eDNA
227 samples had significantly reduced DNA content due to degradation, all eDNA samples
228 from Tank Experiment 1 (collected as described in Section 2.3) and gDNA samples

(collected as described in Section 2.4) were subjected to the four reactions of the first PCR, and the products were pooled. The eDNA samples from Tank Experiment 2 were used to produce two replicates of the first PCR, and the products were pooled. PCR products were purified using the SPRI-select Reagent Kit (Beckman Coulter Inc., USA) according to the manufacturer's instructions, diluted to 0.1 ng/μL, and used as a template for the second PCR.

The second PCR was performed by adding Illumina P5/P7 adaptor sequences (Illumina, USA) and 8-bp index sequences at both ends of the amplicon. The reaction mixture for the second PCR contained 6 μL 2×KAPA HiFi HotStart ReadyMix (Kapa Biosystems, South Africa), 300 nM each of F/R primer, 1 μL template, and ultrapure water to a total volume of 12 μL. The thermocycling conditions were 95 °C for 3 min, followed by 12 cycles of 98 °C for 20 s, 72 °C for 20 s, and finally 72 °C for 5 min. Products of the second PCR were pooled and size-selected using E-Gel SizeSelect II agarose gels (2%; Thermo Fisher Scientific). The desired sequence length was verified on an Agilent 2100 BioAnalyzer (Agilent Technologies, USA), and the library was sequenced using an iSeq 100 Sequencing System (Illumina).

2.8.3 Data processing

The NGS data were processed using USEARCH v10 (Edgar, 2010). First, paired-end reads were generated using the 'fastq_mergepairs' command, and reads with more than five alignment mismatches, those with less than 90% alignment match, and those with alignments less than 16 bases were discarded. Next, the primer sequences were truncated

from the paired-end reads using the fastx_truncate command. Quality filtering was performed using the "fastq_filter" command to remove reads with a predicted error rate exceeding 1% and using the "fastx_uniques" command to identify the set of unique sequences.

2.8.4 Identity filtering

Identity filtering was performed on the PCR-amplified sequences to remove sequences with low identity to the zebrafish reference sequence. To roughly remove reads with many mismatches while allowing for mismatches in CpG sites substituted for TGs via bisulfite treatment and to simplify the following process, pairwise alignment to the reference was performed to filter for 80% identity using the 'usearch_global' command in USEARCH v11. When the sequence corresponding to the reference CpG site was TG, it was converted to CG, allowing for variable sequences due to methylation modifications. Subsequently, the sequences were filtered for 95% identity using the 'usearch_global command' again to restrict the sequences to target species. Finally, the data were produced to contain the sequence and number of reads for each unique sequence after identity filtering.

2.8.5 Quantification of methylation rates

The methylation rate in the target region of each sample was calculated as follows: (sum of CGs)/(sum of CpG sites). The quantification limit was set to a read depth of 10. In

general, the ‘methylation rate’ refers to the bulk-level metric of the sample. For example, a sample containing a 1:1 ratio of 100% to 0% methylated cells will exhibit a methylation rate of 50%. To investigate methylation at the single molecule level, we used single-read methylation (read methylation rate) in addition to the ‘methylation rate’ (Kerr et al., 2023). The read methylation rate for each DNA sequence was calculated as follows: number of CGs in the sequence/ number of CpG sites in the sequence = 19. Then, as an indicator of DNA with low methylation, the percentage of unmethylated DNA sequences with a read methylation rate of 0% (CG=0) in each sample was calculated.

2.9 Statistical analyses

To investigate the impact of time on eDNA concentrations, we used a linear mixed model (LMM) included in the lmerTest package of R software version 4.1.2. The DNA concentration (log-transformed +1) was the response variable, elapsed time (log-transformed +1) was the explanatory variable, and the tank number was included as a random effect. We also used a generalised LMM (GLMM) with beta distribution using the glmmTMB package to investigate the impact of time on the methylation rate in the target region of eDNA. The methylation rate was the response variable, elapsed time was the explanatory variable, and tank number was included as a random effect.

In addition, the differences in methylation rates in the target region were analysed by fitting a GLMM with beta distribution among the five groups with regard to gDNA, extracted from four different organ tissues (skin, brain, testis, and ovary), and eDNA, collected immediately after fish removal (after 0 h) in Experiment 1. The methylation

rate was the response variable, the sample group was the explanatory variable, and the number of tanks or individuals was included as a random effect. Multiple comparison tests were performed using Tukey–Kramer post-hoc analysis.

2.10 Ethics approval for conducting animal experiments

Animal experiments were conducted following all relevant laws and guidelines of the countries where the studies were conducted.

3 RESULTS

3.1 Collection and verification of analytical data

In the qPCR, no DNA was detected in any of the PCR negative controls or in any blank tank in Tank Experiment 1. In some of the blank tanks in Tank Experiment 2, a very small amount of DNA (less than 0.1% of the DNA concentration in the breeding tanks) was detected; however, this level of contamination did not affect the results. For BSAS, the maximum number of reads from the PCR negative controls after filtering did not exceed three, and all blank tank samples showed no amplification in the 1st PCR, indicating no evidence of cross-contamination affecting the results. A total of 479,877 reads were obtained using the BSAS iSeq100 (Table S1). The number of reads after quality filtering was 117,093 (94.4%) and 87,771 (94.9%) for gDNA and eDNA samples, respectively, in Tank Experiment 1 and 245,412 (93.2%) for eDNA samples in Tank Experiment 2. The final number of reads after identity filtering was 81,941 (70.0%) and 2,547 (2.9%) for

gDNA and eDNA samples, respectively, in Tank Experiment 1 and 79,607 (32.4%) for the eDNA sample in Tank Experiment 2. In Tank Experiment 1, where eDNA degradation was monitored, two tanks at 24 h and one each at 48 and 72 h did not produce the necessary read depths for analysis (Table S2). After excluding these, the samples had a minimum read depth of 12. When methylation analysis was performed without identity filtering to remove reads with a low match to the reference, methylation rates decreased in most eDNA samples, and the decrease rates tended to be higher when the sample contained more reads that were removed by identity filtering (Figure S2).

3.2 Methylation levels of degrading eDNA

In all breeding tanks, DNA concentrations were the highest immediately after fish removal and decreased significantly over time ($p < 0.001$; Figure 1, Table S3). Rapid DNA degradation was observed early in the experiment; however, the degradation rate decelerated over time. In contrast, the methylation rate of the target region remained stable throughout the experiment, and the decrease was marginal but not statistically significant ($p = 0.052$; Figure 1, Table S4).

3.3 Methylation levels of gDNA and eDNA

The methylation rate of the target region in skin, brain, testis, and ovary gDNA was 85.25% ± 3.53%, 87.54% ± 2.15%, 86.76% ± 1.97%, and 14.28% ± 6.20%, respectively, showing pronounced hypomethylation in the ovary (Table S5). The methylation rate of

eDNA was 86.06% ± 5.12% (Table S6), and no significant differences between groups were observed in eDNA and gDNA, except for that in ovary tissue (Figure 2).

In sample types other than ovary tissue, sequences including 19 CpGs (100% read methylation rate) and 18 CpGs (94.7% read methylation rate) were most frequently detected (Figure 3). Sequences with lower read methylation rates were less frequent, and sequences with 0% read methylation rate were hardly detected in skin and brain tissue, with frequencies of 0.20% and 0.05%, respectively, whereas they were more frequent in the testes, at 2.33% (Table S7, Table S8). Notably, 71.39% of the sequences detected in the ovaries were completely unmethylated, indicating that hypomethylation occurred within the target region because the majority of DNA in the tissue was entirely unmethylated.

In addition, most of the detected unmethylated DNA sequences were mismatched with the reference sequences at the same positions. Considering the possibility of bisulfite-induced base substitutions and PCR errors, we sequenced bisulfite-untreated ovary DNA using Sanger sequencing; the results confirmed the mismatches with the reference sequence, and their positions were consistent with those in the ovine rRNA variant (Locati et al., 2017). The minimum identity threshold with the reference was set at 95% to avoid the exclusion of zebrafish rDNA variants from the analysis.

3.4 eDNA methylation levels during fish spawning

After the partition was removed at 10:00 on day 2, at the same time as the light was turned on, courtship behaviour in which males actively pursued females was observed in all

breeding tanks. A rapid increase in DNA concentration was observed in all tanks after removal of the partitions (Figure 4, Table S9). The eDNA concentrations in all tanks peaked within 1 hour. After the peak, the DNA concentrations decreased over time and stabilised at the end of the day at concentrations similar to those before partition removal. In Tank 2-3, a small surge in DNA concentration was also observed on day 3.

Unmethylated DNA was detected in 21 of the 48 samples (Table S8); during the light period, it was detected in 13 of 21 samples within 1 h of light exposure and in 7 of 18 samples after 1 h of light exposure. It was not detected in any of the 9 samples tested during the dark period. The average proportion of unmethylated DNA detected in the detected samples was 2.44% ± 2.39%.

A large amount of unmethylated DNA was detected after removing the partition on day 2 (Figure 5, Table S10). The peak percentages of unmethylated DNA relative to the total DNA were as follows: Tank 2-1 had 2.63%, Tank 2-2 had 3.11%, and Tank 2-3 had 10.87%. The amount of unmethylated DNA decreased, and the number of undetected samples increased over time. On day 3, unmethylated DNA was only detected in Tank 2-3 after light exposure, whereas unmethylated DNA was detected in all tanks after light exposure on day 4. Unmethylated DNA was detected in Tanks 2-1 and 2-3 on the first day of light exposure. Tank 2-3 showed the largest amount of unmethylated DNA and the highest number of water sampling points. The proportion of unmethylated DNA detected in these eDNA samples was higher than the average proportion detected in the somatic tissue samples analysed for methylation (skin: 0.20%, brain: 0.08%), with the exception of three samples.

The methylation rate of the target region in the eDNA samples did not change significantly during the entire experimental period or during the 24 h after removing the partitions (Figure S3, Table S11). The change in the methylation rate differed between tanks, except for a correlated trend of change after midday on day 2, which showed no general diurnal variation. The methylation rates for each tank for the entire experimental period were 82% ± 7.97%, 82.39% ± 4.44%, and 79.41 ± 5.54% for Tanks 2-1, 2-2, and 2-3, respectively (Table S9).

4 DISCUSSION

In this study, we analysed for the first time the methylation state of fish eDNA extracted from underwater. We developed primers to amplify zebrafish 18S rDNA, BSAS was performed, and the methylation of eDNA was shown to be stable in Tank Experiment 1. In addition, methylation analysis of gDNA confirmed the existence of a specific hypomethylation pattern in the 18S rDNA of zebrafish ovaries, and a certain amount of unmethylated DNA was detected in the reproductive organs. In Tank Experiment 2, we detected germline eDNA and found that eDNA methylation levels correlated with zebrafish spawning. These results suggest that spawning can be detected by examining eDNA methylation levels.

4.1 Effect of eDNA degradation on methylation

Although 5-methylcytosine, generated through the methylation of CpG sites, is a stable compound (Wan et al., 2021), it remains unknown how the modified state of DNA is affected *ex vivo*. If demethylation is associated with DNA degradation, the methylation rate of eDNA may decrease over time and no longer match the methylation state of gDNA. Therefore, we first investigated the effects of the gradual degradation of eDNA with time on the methylation rate in Tank Experiment 1. Monitoring of eDNA over 72 h showed that eDNA degraded and decreased over time. On the contrary, the methylation rate in the target region remained stable. The oldest eDNA sample analysed for methylation was obtained 72 h or more after being released by zebrafish into water, which was close to the limit of eDNA detection in our experiments. The effect of time on the methylation rate was marginal. However, the methylation state of eDNA over 72 h was expected to have a negligible impact on the results of methylation analysis because the eDNA concentration at 72 h was less than 1.5% of the saturated state; therefore, even if there was any change, its impact must be negligible. Currently, two demethylation processes (active and passive demethylation) are known. Active demethylation occurs during cellular reprogramming and involves a multistep metabolism mediated by 5-methylcytosine-targeting proteins, whereas passive demethylation occurs when DNA methyltransferases remain inactive during cell division (Bhutani et al., 2011). These processes are unlikely to occur in DNA fragments in water because they require the maintenance of intracellular functions and are controlled by biomolecules. The methylation pattern of individual DNA molecules is considered fixed at the time of release from the body.

While the methylation state of individual DNA molecules is thought to remain unchanged, the possibility that DNA dilution increases stochastic variation in methylation analysis must be considered. In particular, methylation at each CpG site in the sequence yields binary information, i.e., methylated or unmethylated; hence, a low read depth reduces not only the reproducibility of the results but also the resolution of the methylation rate. BSAS, which focuses on a specific target region, requires less data than genome-wide methylation analyses, thereby providing sufficient read depth. In the present study, the minimum read depth set at 10 was considered optimal (Ziller et al., 2015), obtaining accurate quantitative measurement data. Additionally, using rDNA, which is abundant in the genome owing to tandem repeats, as a marker helped detect and examine degraded eDNA at low concentrations.

4.2 Comparison with gDNA

eDNA is considered a mixture of DNA derived from multiple sources (Barnes & Turner, 2016). In the present study, we examined the methylation rates in the target region in tissues belonging to the ectodermal, nervous, and reproductive organ systems, which vary widely in their cellular differentiation. We found that the methylation rates in these tissues were similar, except in the ovary, likely because ribosomes play a housekeeping role within cells. Nuclear rDNA has been found to function as a clock for biological ageing in distant species and has the potential to serve as a universal marker that correlates with age in diverse species, including fish (Wang & Lemos, 2019. In contrast to that in other tissues, ovarian DNA is hypomethylated (see below). Importantly, eDNA methylation

was similar to that of gDNA, with higher methylation rates in skin, brain, and testis DNA. Among these tissues, skin cells are likely a constant eDNA source, suggesting that eDNA methylation reflects that of the source tissue.

The only published study comparing the methylation levels of eDNA and gDNA was conducted on freshwater snails, wherein genome-wide methylation analysis showed that the methylation rate of eDNA is higher than that of gDNA (Zhao et al., 2023), suggesting that methylation enrichment may occur in eDNA. Although methylation enrichment of eDNA was not confirmed in this study, the fact that DNA methylation characteristics differ in many respects between fish and molluscs (Zhang et al., 2022) may account for the discrepancies between the respective results. The differences between the region-specific and genome-wide approaches to methylation analysis in both studies require further investigation.

4.3 Ovary-specific hypomethylation

The methylation rate of the target region in somatic cells was nearly 85%, comparable to that reported in ribosomal gene study (Ortega-Recalde et al., 2019), suggesting that this region is hypermethylated, similar to the average CpG methylation rate of 80% in the adult zebrafish genome (Goll & Halpern, 2011). In contrast, ovarian DNA was markedly hypomethylated (approximately 14%). The ovaries of mature zebrafish contain numerous female germ cells, which likely influence methylation patterns in the ovaries (Wu et al., 2018). Most of the DNA detected in the ovary was unmethylated (0% read methylation), and unmethylated reads were also detected in testis DNA. Because unmethylated DNA is

466 rarely found in somatic tissues, unmethylated DNA or at least partial 18S rDNA can be
467 used as an indicator of germline DNA.

468 Complementary unmethylated rDNA has been identified as an rDNA variant that differs
469 by several base sequences in specific regions expressed in zebrafish ovaries (Locati et al.,
470 2017). This rDNA variant accounts for the majority of rDNA repeats in unfertilised eggs.
471 Amplification and hypomethylation of the rDNA variant occur in the germ line of
472 zebrafish (Ortega-Recalde et al., 2019). This mechanism of hypomethylation differs from
473 the variable modification state of DNA observed in other genes during the regulation of
474 gene expression upon differentiation (Wang & Bhandari., 2019; Potok et al., 2013).
475 Nevertheless, in species such as zebrafish, wherein the composition of gene variants in
476 the genome differs between the germline and somatic cells, the possibility of using
477 germline-specific sequences for detecting spawning using eDNA analysis has also been
478 suggested.

479

480 **4.4 Detecting fish spawning using eDNA methylation analysis**

481 Zebrafish reproduction strongly depends on photoperiod (Blanco-Vives & Sanchez-
482 Vazquez, 2009). In the present study, we ensured that spawning occurs during the 12-h-
483 long light period and confirmed reproductive behaviour, including spawning, in all tanks
484 based on visual observation on day 2. The rapid increase in eDNA concentration and the
485 detection of unmethylated DNA were consistent with the observed spawning and,
486 therefore, are considered indicators of zebrafish spawning. A rapid increase in the eDNA
487 concentration indicates sperm release (Tsuji & Shibata, 2021). However, unmethylated

DNA was suggested to originate from eggs or sperm released from the ovaries or testes. Following the end of visible reproductive activity, eDNA and unmethylated DNA concentrations decreased with time, and at the beginning of the dark period of day 2, eDNA concentrations were approximately the same as those on day 1, and no unmethylated DNA was detected. On day 3, an increase in eDNA and unmethylated DNA were observed only in Tank 2-3, possibly due to the spawning activities of fish that had not participated in mating activities on the previous day. On day 4, unmethylated DNA was detected in all tanks, suggesting that some fish resumed their reproductive activities after undergoing germ cell repair; however, no considerable increase in eDNA concentration was observed on day 4, indicating that monitoring eDNA concentrations alone may not be sufficient to detect spawning consistently and that additional methylation analysis may help detect spawning. Estimating reproductive activity from eDNA concentrations carries the risk of missing small-scale reproductive activity and sensitivity to other factors likely to affect concentration fluctuations (e.g. migration or water flow). In contrast, methylation analysis may provide clear indications of spawning by detecting DNA in unique methylation modification states. Revealing the differences in the timing and extent of spawning among tanks under the same conditions, it also highlighted the importance of testing whether eDNA analysis provides a consistent signal in behavioural experiments with additional replicates.

Although the detection of unmethylated DNA can be a valid indicator of spawning in zebrafish, the methylation rate, considered the average methylation state of the samples, did not vary with light conditions or the time of day. This was due to the small proportion of unmethylated DNA in the total sample. It is possible that a large amount of highly

methylated DNA released through sperm or other behaviours represented most of the DNA input into the library, masking the effect of some unmethylated DNA on the average methylation rate. In the present study, it was not possible to determine whether the temporal changes in methylation rates in Tank Experiment 2 were due to variations among individuals or to other sources of eDNA, such as the digestive tract, which were not targeted; however, the similarity in methylation rate fluctuations among the tanks in the afternoon of day 2 may be associated with diurnal variation due to feeding and other factors. To elucidate this, more biological replicates (tanks and individuals) may be required to allow for individual differences in methylation rate and activity and to analyse multiple regions in the genome with longer-term monitoring.

4.5 Application of BSAS to eDNA samples

Turner et al. (2014) reported that fish eDNA accounted for 0.0004–0.0000004% of total eDNA, and a high population density tank experiment conducted by Hechler et al. (2023) showed that only 0.51% of total nucleic acids were from the target species. Therefore, BSAS, consisting of two screening steps, targeted PCR and identity filtering, was performed in this study for targeted species-focused methylation analysis. It has been suggested that the number of methylated cytosine sites in microbial genomes is lower than that in vertebrates and that the frequency of 5-methylcytosine sites is also lower (Jones & Liang, 2009; Capuano et al., 2014). Therefore, it was expected that if sequences of microbial origin are included in the analysis, the methylation rate will shift towards levels lower than normal. In our experiments, the methylation rates of almost all eDNA

533 samples were recovered after applying identity filtering. As the eDNA samples with
534 relatively more noisy reads, which were removed by filtering, showed an increased shift
535 towards lower methylation rates than normal, it was assumed that these removed
536 sequences were from microorganisms in the aquarium (Figure S1). In tank experiment 1,
537 which monitored the process of eDNA degradation, fewer leads remained after filtering
538 than in tank experiment 2, possibly because of a decrease in fish DNA relative to
539 microbial DNA in the tanks. These results indicate that screening bisulfite-treated DNA
540 using targeted PCR alone remains challenging due to a reason based on the principles of
541 the methodology discussed below.

542 Because bisulfite-treated DNA is AT-rich and primers must be designed to avoid CG
543 sequences, the PCR annealing temperature tends to be lower, resulting in lower target
544 specificity. Additionally, the bisulfite step eliminates the distinction between the thymine
545 converted from unmethylated cytosine and native thymine, potentially increasing
546 nonspecific amplification. Using high-specificity PCR is necessary for BSAS, not only
547 in terms of the detection accuracy of the system but also for resource-efficient sensitivity
548 with low sequencing costs. This issue should be considered for field implementation.

549 Because BSAS quantifies the read proportion of unmethylated sequences, the relative
550 amount of unmethylated DNA can decrease not only when unmethylated DNA is reduced
551 through degradation but also when methylated DNA is increased. This can cause an
552 underestimation of the persistence of unmethylated DNA. Methylation-specific PCR and
553 other methods will be considered to determine the absolute number of DNA copies in a
554 certain methylation state. Since sperm are released in much greater amounts than eggs in

555 fish spawning, the unmethylation that occurs in genetic regions of sperm may be an
556 attractive target (Zhang et al., 2023).

557

558 **5 CONCLUSIONS**

559 In the present study, we developed an assay for bisulfite sequencing of zebrafish 18S
560 rDNA for eDNA methylation analysis and performed two tank experiments. The
561 developed assay was useful for methylation analysis of both eDNA and gDNA. The
562 results of Tank Experiment 1 showed that the quantitative measurement of eDNA
563 methylation was consistent with that of somatic gDNA and that degradation over time did
564 not affect the methylation rate of eDNA. These results indicate that the modified state of
565 eDNA is stable, and that eDNA samples are reliable targets for methylation analysis. In
566 Tank Experiment 2, we used germ cell-specific unmethylated rDNA to specifically detect
567 eDNA derived from germ cells such as unfertilised eggs and sperm and detected
568 unmethylated rDNA during periods of active reproduction, indicating that the presence
569 of germ-cell specific DNA in different methylation states could be used as robust evidence
570 of fish spawning.

571 DNA methylation is a mechanism through which gDNA, whose sequences are static and
572 contain essentially homogeneous genetic information, undergoes dynamic and diverse
573 patterns of gene expression. eDNA has been used to estimate the existence of species and
574 genotypes through sequencing; however, it is expected to be further developed to monitor
575 gene expression, health status, and age through further examination of modification states.
576 In addition, methylation analysis may provide insight into the origin and dynamics of

eDNA. This technology expands the application of eDNA and is expected to play a role in linking epigenetics and ecology more closely in the future.

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CONFLICT OF INTEREST STATEMENT

All authors have no conflict of interest to declare.

BENEFIT SHARING STATEMENT

Benefits Generated: Benefits from this research accrue from the sharing of our data and results on public databases as described above.

DATA ACCESSIBILITY STATEMENT

The data that support the findings of this study are openly available in DRYAD at http://doi.org/10.5061/dryad.8gtht76wg. The data of DNA sequences are openly available in the DDBJ (accession number: LC813236-LC813237).

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AUTHOR CONTRIBUTIONS

ITH and TM conceived the study and designed the experimental setup. ITH conducted the tank experiments, molecular experiments, and data analysis. LW co-developed the workflow for the data analysis. ITH and TM wrote and edited the manuscript. All the authors discussed the results and contributed to the development of the manuscript.

Figures and Tables

Table 1. Sampling schedule for Tank Experiment 2

Date (YYYY/M/DD)	Day	Time	Light	Partition	Water added after sampling
2022/9/13	1	11:00	on	+	+
2022/9/13	1	23:00	off	+	+
2022/9/14	2	10:00	on	−	−
2022/9/14	2	10:20	on	−	−
2022/9/14	2	10:40	on	−	−
2022/9/14	2	11:00	on	−	+
2022/9/14	2	12:00	on	−	+
2022/9/14	2	13:00	on	−	+
2022/9/14	2	15:00	on	−	+
2022/9/14	2	17:00	on	−	+
2022/9/14	2	19:00	on	−	+
2022/9/14	2	21:00	on	−	+
2022/9/14	2	23:00	off	−	+
2022/9/15	3	11:00	on	−	+
2022/9/15	3	23:00	off	−	+
2022/9/16	4	11:00	on	−	+

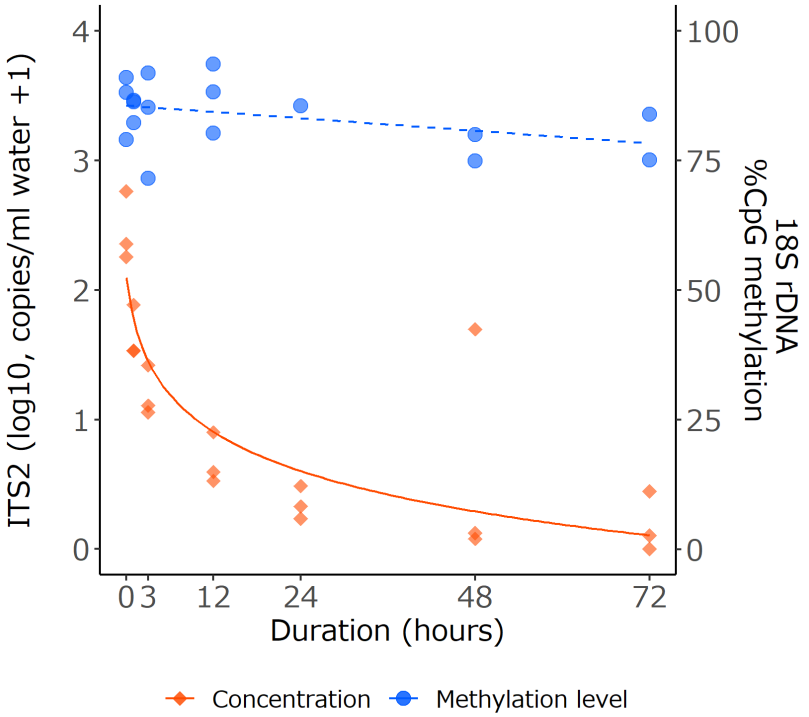
The light was turned on at 10:00 and turned off at 22:00. Partitions separating the sexes in the tanks were removed at 10:00 on Day 2. Water was added to the tanks after each sampling to compensate for this decrease, except for the duration of less than an hour after light exposure, which may interfere with mating behaviour.

856 **Table 2.** Sequences of the developed primers and a probe

Region	Primer/Probe name	Sequence (5′-3′)	Purpose
nuDNA (ITS2)	Dre-ITS2-F1	ACCGTCACGCCACCG	qPCR
	Dre-ITS2-R1	AGCGGTCGGGGTCAG	qPCR
	Dre-ITS2-P	FAM-CGAACCTCCGTCTTCCCCTCGTCA-TAMRA	qPCR
nuDNA (18S)	Dre_18S_BF2A	ACACTCTTTCCCTACACGACGCTCTTCCGATCT NNNNNNTTTAATTTTTTAGAGGGATAAGTGG	Bisulfite sequence
	Dre_18S_BR2A	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT NNNNNNATATATACAAAAAACAAAAACTTAATCAAC	Bisulfite sequence
	EF3	GGAATTGACGGAAGGGCAC	Sanger sequence
	ER1	ACGGGCGGTGTGTACAAAG	Sanger sequence

857

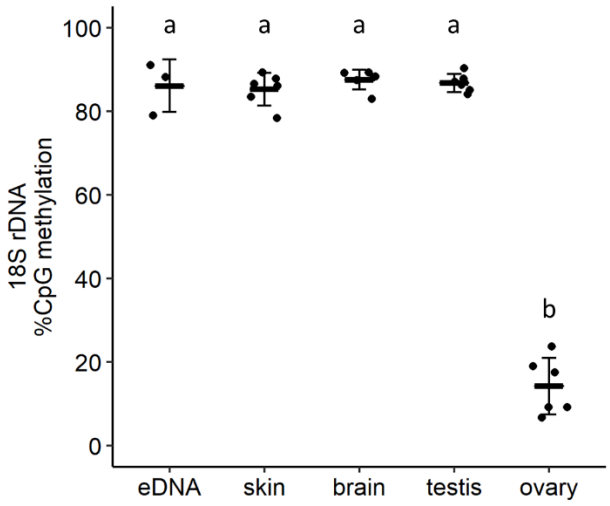
858



859

860 **Figure 1.** eDNA concentrations and methylation rates over time. The vertical axis indicates
861 concentration (log10-transformed +1) of ITS2 copies (left) and methylation rate (right), and the
862 horizontal axis indicates time. DNA concentration and its regression line (concentration: exponential
863 curve, methylation rate: linear line) for each sample were plotted. We used a linear mixed model
864 (LMM) for log-scaled concentration and a generalised LMM (GLMM) with beta distribution using
865 the logit link function. DNA concentrations changed significantly over time ($p < 0.001$), whereas
866 methylation rates changed marginally, but the change was not statistically significant ($p = 0.052$).

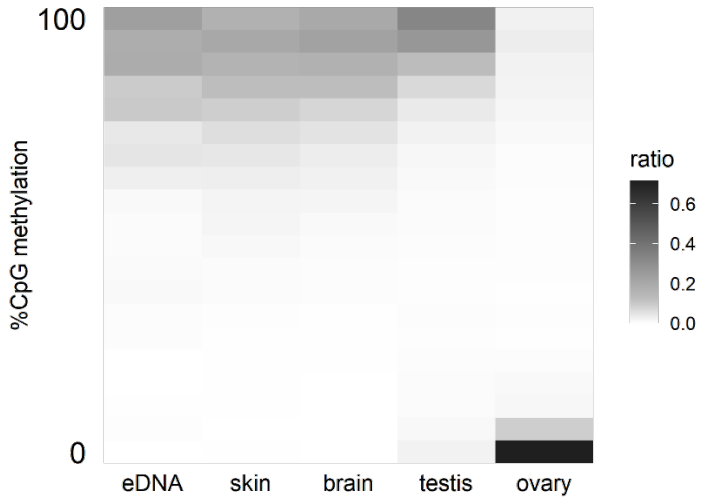
867



868

869 **Figure 2.** Methylation rates of eDNA (0 h after removing fish) and gDNA in various tissues. We
870 used a generalised linear mixed model (GLMM) with beta distribution using the logit link function,
871 and multiple comparison tests were performed using Tukey–Kramer *post-hoc* analysis. Samples with
872 the same letter did not differ significantly ($p > 0.05$). Ovary DNA was significantly hypomethylated.

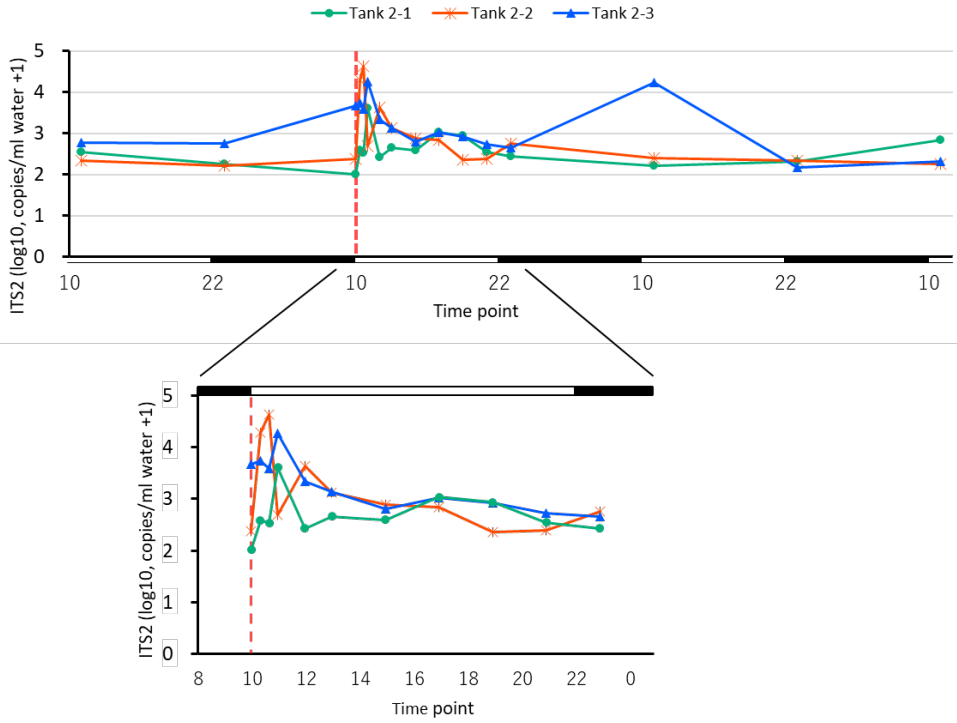
873



874

875 **Figure 3.** Distribution of read methylation rates for sequences detected in each group. A gradient
876 was observed that was more frequently detected in reads with higher methylation rates; however, this
877 trend did not apply to ovary DNA, in which most of the reads had a methylation rate of 0%.

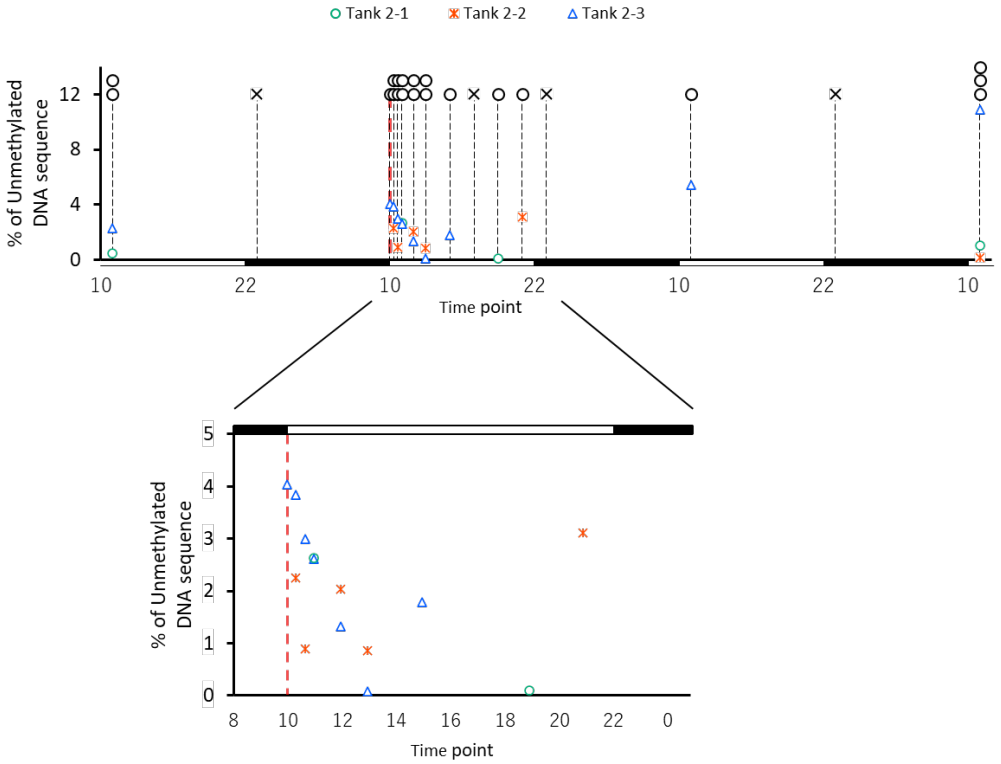
878



879

880 **Figure 4.** Time series of eDNA concentration in Tank Experiment 2. The upper and lower figures
881 show the entire experimental period and 24 hours after the partition was removed on day 2,
882 respectively. The eDNA concentrations at each water sampling point are plotted. The black and
883 white horizontal bars indicate the dark and light periods, respectively. The red dotted vertical line
884 indicates the time when the partition was removed.

885



886

887 **Figure 5.** Time series of the proportions of unmethylated DNA sequences. The upper and lower
888 figures show the entire experimental period and 24 hours after removal of the partition on day 2,
889 respectively. The time points at which unmethylated DNA sequences were detected and the
890 proportions of unmethylated DNA sequences are shown. The black and white horizontal bars
891 indicate the dark and light periods, respectively. The time at which the partition was removed is
892 indicated by the red dotted vertical line. The black vertical dotted line indicates the time of water
893 sampling, and the number of white circles indicates the number of samples detected with
894 unmethylated DNA (up to 3).

895