**SPECIAL FEATURE: ORIGINAL PAPER**

**Environmental DNA as a Practical Tool for Aquatic Conservation and Restoration**

# Diurnal detection of environmental DNA of the semi-aquatic water shrew *Chimarrogale platycephala* using 25-h water sampling in streams

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## Abstract

Whether environmental DNA (eDNA) testing methods are effective for semi-aquatic organisms that spend time both in rivers and on land remains unclear. We designed a species-specific primer–probe set for the semi-aquatic Japanese water shrew, *Chimarrogale platycephala*, an endangered top predator species for which limited ecological information is available. The designed primer–probe set was used to determine the effective sampling time for detecting eDNA from this species in running water. Hourly water sampling was conducted for 25 h in the autumn of 2020. Water sampling and camera trapping were conducted in the two streams in which feces had been observed on stream rocks. No *C. platycephala* activity was captured by the camera traps at any sampling point during the investigation. However, eDNA was mainly detected at night in both streams and twice during the daytime in one stream; the eDNA detection periods differed between streams (21:00–23:00 vs. 1:00–8:00 and daytime). These results demonstrate that eDNA testing methods are effective for detecting small semi-aquatic mammals. However, the sampling time affected the results, and eDNA was mostly detected at night, possibly because of the nocturnal activity of the species. Therefore, the sampling time should be adjusted to match the activity period of a species to improve the efficiency of eDNA testing for semi-aquatic organisms.

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## Introduction

As indicated by Sustainable Development Goal #15 of the United Nations, conserving freshwater biodiversity is a top global priority (Flitcroft et al. 2019). In recent years, assessment of environmental DNA (eDNA), defined as genetic material obtained directly from environmental samples without capturing the organisms (Ficetola et al. 2008), has been used to determine the presence, abundance, and biomass of organisms (Rees et al. 2014; Goldberg et al. 2015). The eDNA detection in studies of aquatic organisms, such as fish (Miya et al. 2015), crustaceans (Thomsen et al. 2012), amphibians (Fukumoto et al. 2015), and insects (Doi et al. 2017) has been reported in flowing water bodies (Katano et al. 2017; Doi et al. 2021), lakes, and ponds (Bista et al. 2017; Kakuda et al. 2019; Togaki et al. 2020). However, the effectiveness of methods for detecting eDNA from semi-aquatic organisms that spend time both in rivers and on land is unclear.

Semi-aquatic organisms include many small mammals such as the endangered water shrew (Soricidae, Nectogalini), which plays an important role as a top predator in the river food web (Abe 2011). Most small mammals exhibit nocturnal behavior and move quickly (Buchler 1976), making it difficult to study their ecological behavior and distribution using traditional methods such as camera traps and direct capture. These methods are also labor-intensive and costly (e.g., Harper et al. 2019; Lyet et al. 2021). These limitations may be overcome using eDNA detection methods, which are more non-invasive, sensitive, and cost-effective than the traditional survey methods (Thomsen and Willerslev 2015). Yonezawa et al. (2020) determined the distribution of the semi-aquatic Japanese freshwater shrew, *Chimarrogale platycephala* (Soricidae, Nectogalini)*,* in a watershed using eDNA methods with universal primers to analyze the entire mammal community (MiMammal) (Ushio et al. 2017). However, false-negative results have been reported, such as the non-detection of eDNA at stations where the species live, likely because of the low sensitivity of metabarcoding. Quantitative polymerase chain reaction (qPCR) detection using species-specific primer probes may be more sensitive than metabarcoding (e.g., Harper et al. 2018; Wood et al. 2019). For the shrew species, a species-specific primer–probe set was preliminarily developed by one of the authors (Nakamura, personal communication). However, eDNA surveys led to false-negative results when a single water sample was collected during the daytime, even in rivers where inhabitation by the species was visually confirmed (Nakamura, personal communication). We hypothesized that the mismatch between the organism's nocturnal activity and eDNA sampling time could explain this result. In headwater streams with rapid water exchange, water sample collection should be consistent with the activity times of the organisms compared to that in lentic waters (e.g., Ushio et al. 2017). Therefore, in the headwater stream, the sampling timing should be correlated with the nocturnal activity of the organism; however, such testing has not been performed yet.

This study was conducted to verify the effectiveness of the eDNA detection method for confirming the distribution of the semi-aquatic Japanese freshwater shrew *C. platycephala* using a species-specific primer probe ((Nakamura, personal communication) and to identify effective sampling times for eDNA detection. The effectiveness of diurnal eDNA water sampling and that of camera trap photography were compared in 11 streams inhabited by *C. platycephala*.

## Materials and methods

### Study species

The Japanese freshwater shrew is distributed throughout Japan, except for in the Hokkaido and Shikoku regions, but is ranked in one of the Red List categories in many areas (Yokohata et al. 2008; Ministry of the Environment 2014). This species inhabits mountain streams and feeds on benthic organisms such as aquatic insects, crabs, and fish (Abe 2011). The Japanese freshwater shrew is among the top predators in mountain stream ecosystems and has ecologically important roles (Ichikawa et al. 2005). The breeding season is primarily in spring, with some females also breeding in autumn; their diurnal activity varies depending on the breeding period, rearing period, and other normal periods when the offspring are independent (Ohdachi et al. 2015). Although detailed information on its behavior is lacking, *C. platycephala* is known to nest in terrestrial environments, such as in crevices in rocks in the riparian zone, and dives into the water to forage (Abe 2003). This species defecates on rocks protruding above the stream surface (Fig. 1); *C. platycephala* feces can be identified by its specific shape, size, and location, as described by Sekiya et al. (2017). Occasionally, several feces can be found on the same rock used by many individuals as a fecal site (Yamazaki et al. 2020). This species is active throughout the day but is more active at night (Motoki 2000). The behavior of *C. platycephala* does not change upon the approach of humans (Kitagaki 2016; Saito et al. 2019); therefore, during sampling, we assumed that the presence of humans during water sampling had negligible effects on the shrew's behavior.

### Field sign survey to confirm the presence of *C. platycephala*

Feces surveys were conducted from August to November 2020, which corresponded to 1 or 2 months before the eDNA surveys in the mountain streams in the northern Kii Peninsula. The survey sites included: six tributaries (A–F streams) flowing into the Takami River (Kinokawa River system, Nara Prefecture, Japan) and five tributaries (G–K streams) flowing into the Nabari River (Yodogawa River system, Mie Pref.; Table 1). In 1 month, surveys in each stream were conducted 3–6 times. An approximately 800-m longitudinal section upstream of the confluence of the mainstem was selected as the study area and examined for the presence of *C. platycephala* feces using the method described by Sekiya et al. (2017). When the feces were detected, the samples were photographed to determine their freshness based on the surface appearance such color and wetness (see Fig. 1d), and the number of new feces was counted.

### Field survey for water sampling and camera trap photography

Water sampling surveys and camera trap photography (trophy cam 24MP, Bushnell, Overland Park, KS, USA) were conducted at a station along the two streams where the feces

**Fig. 1** Photographs of the study sites and feces of *Chimarrogale platycephala*. **a** G stream in Mitsue, Nara; **b** A stream in Higashi-yoshino, Nara; **c** Feces of *C. platycephala* on a rock protruding from the water surface; **d** enlargement of (**c**). Multiple old and fresh feces were observed
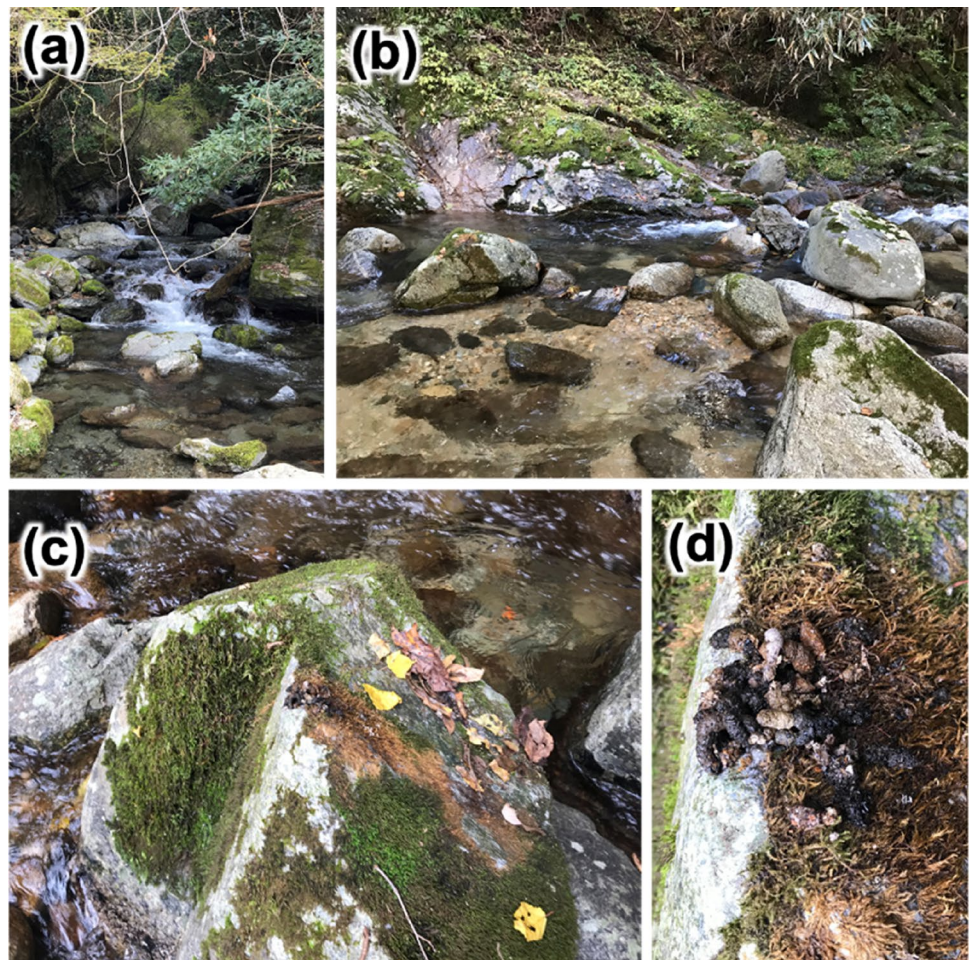


**Table 1** Observed maximum number of new feces per survey day in 11 tributary streams of the Takami and Nabari Rivers from August to November 2020. For each stream, the maximum observed new feces numbers per survey day are shown

| River | Takami river | | | | | | Nabari river | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Tributary | A | B | C | D | E | F | G | H | I | J | K |
| August | 1 | 0 | 0 | 0 | 0 | 0 | – | – | – | – | – |
| September | 1 | 0 | 0 | 0 | 0 | 0 | – | – | – | – | – |
| October | – | – | – | – | – | – | 1 | 0 | 1 | 1 | 0 |
| November | – | – | – | – | – | – | 2–4 | 0 | 0 | 0 | 0 |

–: No survey

were observed in the prior feces survey and where the stream morphology was considered as safe for water sampling, including at night (streams A and G, Table 1). Both water sampling stations along stream A (340 m a.s.l., 3.5 km² watershed area) and stream G (520 m a.s.l., 8.5 km² watershed area) had a width of approximately 2 m and canopy coverage of 60% (Fig. 1a, b). Both sites were 10 m downstream from the rock where the feces was found.

We conducted water sampling and camera trap photography surveys on October 20–21, 2020 in stream A and November 24–25, 2020 in stream G. October and November correspond to the second gestation period of some *C.*

*platycephala* females. Most females become pregnant in spring, and only a few females become pregnant in autumn (Ohdachi et al. 2015). Considering the nocturnal behavior of the species, the water sampling survey was conducted for 25 h. At the sampling station, 1 L water sample was directly collected from each of the right- and left-hand sides of the stream center into a DNA-free polypropylene bottle, giving a total of 2 L of water samples hourly from 12:00 noon to 13:00 in the next day. A 0.1% volume of benzalkonium chloride solution was added to the water samples and stirred immediately to prevent DNA degradation prior to analysis (Yamanaka et al. 2017). The water samples were stored in a

cooler box containing 2 L of DNA-free distilled water as a neutral cooler. The water temperature of the stream surface was measured using a stick thermometer at the sampling time.

A field of view for camera trap photography was placed on the rock where the feces was found to obtain photograph of the species; the camera was strapped to a riparian tree. The shutter is released when a moving object enters the field of view.

## Water filtration and DNA extraction

The water samples collected were vacuum-filtered through 47-mm GF/F glass filters (pore size 0.7 μm, GE Healthcare, Little Chalfont, UK). The filter was wrapped in commercial aluminum foil and stored at − 20 °C before eDNA extraction. We incorporated an 'equipment blank' as a negative control (1 L DNA-free distilled water, which was filtered after filtering the samples on each sampling day). Also, a 'cooler blank' was filtered during filtering the samples on each sampling day. DNA from the negative controls was tested along with the sample filters to identify field preparation/transportation, filter equipment, or background contamination. The filters were stored at − 20 °C until DNA extraction.

DNA was extracted from the filters as described by Uchii et al. (2016) and Minamoto et al. (2021). Each filter was incubated in a mixed buffer of 400 μL of Buffer AL (Qiagen, Hilden, Germany) and 40 μL of Proteinase K (Qiagen) using a Salivette tube (Sarstedt, Nümbrecht, Germany) at 56 °C for 30 min. The Salivette tube with the filter was centrifuged at 3500×*g* for 5 min, after which 220 μL of TE buffer (10 mM Tris–HCl and 1 mM EDTA, pH 8.0) was added and the filter was centrifuged at 5000×*g* for 5 min. The dissolved DNA in the eluted solution was purified using a DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's protocol. The extracted sample was eluted in 200 μL of Buffer AE from the DNeasy Blood & Tissue Kit and stored at − 20 °C until qPCR analysis.

## Real-time qPCR

For qPCR, we used Primer3Plus (https://primer3plus.com/) to design species-specific primers to amplify an 81-bp fragment of the 16S ribosomal gene of the mitochondrial DNA of *C. platycephala*. The TaqMan MGB probe was designed using Primer Express version 3 (Thermo Fisher Scientific, Waltham, MA, USA). The primer and probe sequences were as follows: Cpl_16S_F (5′-CCGACACAGGAACGCATT TAA-3′), Cpl_16S_R (5′-TTGGTAAACAGGCGGGGT TTA-3′), and Cpl_16S_probe (5′-[FAM]-TIAAAGAAGTA AAAGGAACTCGG-[MGB]-3′). There is only one species of *Chimarrogale* in Japan; therefore, the specificity of

the primers and probe was evaluated using sequences from *Chimarrogale* species present in Asia. Sequences from the National Center for Biotechnology Information databases (http://www.ncbi.nlm.nih.gov/) for *C. platycephala* and *C. himalayica* were used. *Chimarrogale himalayica* was not detected during in silico specificity screening performed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). We also performed in vivo amplification tests using extracted DNA. DNA was extracted from the species tissue and diluted to 6.5 ng/μL for qPCR. To confirm the specificity of the primers, the PCR amplicons of eDNA from samples collected in a stream in Kanagawa Prefecture were treated with AMPure XP (Beckman Coulter, Brea, CA, USA), and directly sequenced. The sequences were determined using standard protocols on an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Based on the results, we confirmed that the sequence of the PCR amplicon belonged to *C. platycephala*.

The eDNA concentration was measured using a StepOne qPCR system (Applied Biosystems). Each PCR mixture contained 900 nM of each primer (F, R), 125 nM TaqMan probe in 1× PCR master mix (TaqPath GC; Life Technologies, Carlsbad, CA, USA), and 2 μL of DNA solution. The total volume of each reaction mixture was 20 μL. The PCR conditions were as follows: 2 min at 95 °C and 55 cycles of 15 s at 95 °C and 60 s at 60 °C. Each sample was evaluated in triplicate; a positive value for any of the replicates was considered to indicate the presence of *C. platycephala* DNA. Each real-time PCR assay included three no-template controls, which showed no amplification. Real-time PCR measurement included three replicates, as described in a previous eDNA study of aquatic species detection (Takahara et al. 2013), and was performed according to the MIQE checklist (Bustin et al. 2009). The PCR setup and real-time PCR were performed in two separate rooms to avoid contamination.

The qPCR results were analyzed using StepOne software ver. 2.3 (Applied Biosystems). A standard curve for the target gene was constructed using a dilution series of 10,000, 1000, 100, and 10 copies per PCR sample. For the standard curve, we used the standard DNA isolated from the cloned into a plasmid. The $R^2$ values of the standard curves were 0.995–0.998 and PCR efficiencies were 78.45–95.65%. The DNA concentration in the collected water (DNA copies $L^{-1}$) was calculated from the volume of the filtered water (2 L). Mean DNA copy numbers were evaluated from the triplicates, including a negative detection of zero. The limit of detection of qPCR for the three replicates was one copy per reaction.

## Statistical analysis

All statistical analyses were performed using the R software (version 4.1.1) (R Core Team 2021). We performed

Student's *t* test to compare eDNA concentrations between the two streams to detect differences in eDNA concentrations for different amounts of feces. We also used general additive models with the "mgcv" package ver. 1.8–37 to confirm the diurnal dynamics of the eDNA concentrations using a Gaussian distribution as the error distribution. We set the criteria of the significance as 0.05 ($\alpha = 0.05$).

## Results

Feces from *C. platycephala* were observed in the A, G, I, and J streams but only detected continuously for the 2 months of evaluation in the A and G streams (Table 1). Although only one fecal sample was observed at each time point in stream A, several fecal samples were observed at several time points in stream G. Based on these results and the safety of sampling at night, we performed a 25-h sampling survey in the A and G streams.

On the eDNA sampling date, sunset and sunrise times were 17:16 and 6:07 at stream A and 16:48 and 6:40 at stream G, respectively. The water temperatures in streams A and G were 11.5–12.9 °C and 8.1–9.3 °C, respectively (Table 2). Although the camera traps captured no activity of the species during the 25-h survey at either sampling point, eDNA was detected in the water samples (Table 2). The diurnal dynamics of the eDNA concentration showed that eDNA was detected mainly at night in both streams (Fig. 2). All positive results were detected continuously at night in stream A (21:00–23:00), whereas in stream G, positive results were obtained three times per night (01:00, 04:00, and 06:00) and twice in the daytime (13:00 and 08:00), although the detected eDNA concentration at 13:00 was very low (0.023 copies $L^{-1}$). The eDNA concentration did not significantly differ between the two streams ($p = 0.76$, $t = -0.33$, *t* test, Fig. 3).

**Table 2** Results of water temperature (°C), camera observation, and eDNA detection ratios of *Chimarrogale platycephalus* eDNA obtained from three replicates

| Time | A stream | | | G stream | | |
|---|---|---|---|---|---|---|
| | Water temperature | Camera observation | eDNA | Water temperature | Camera observation | eDNA |
| 12:00 | 12.7 | 0 | 0/3 | 9.0 | 0 | 0/3 |
| 13:00 | 12.6 | 0 | 0/3 | 9.3 | 0 | **1/3** |
| 14:00 | 12.7 | 0 | 0/3 | 9.3 | 0 | 0/3 |
| 15:00 | 12.8 | 0 | 0/3 | 9.3 | 0 | 0/3 |
| 16:00 | 12.8 | 0 | 0/3 | 9.3 | 0 | 0/3 |
| 17:00 | 12.9 | 0 | 0/3 | 9.2 | 0 | 0/3 |
| 18:00 | 12.9 | 0 | 0/3 | 9.1 | 0 | 0/3 |
| 19:00 | 12.7 | 0 | 0/3 | 9.0 | 0 | 0/3 |
| 20:00 | 12.5 | 0 | 0/3 | 8.8 | 0 | 0/3 |
| 21:00 | 12.3 | 0 | **1/3** | 8.7 | 0 | 0/3 |
| 22:00 | 12.1 | 0 | **1/3** | 8.6 | 0 | 0/3 |
| 23:00 | 12.0 | 0 | **1/3** | 8.5 | 0 | 0/3 |
| 0:00 | 11.8 | 0 | 0/3 | 8.4 | 0 | 0/3 |
| 1:00 | 11.7 | 0 | 0/3 | 8.2 | 0 | **1/3** |
| 2:00 | 11.6 | 0 | 0/3 | 8.1 | 0 | 0/3 |
| 3:00 | 11.6 | 0 | 0/3 | 8.1 | 0 | 0/3 |
| 4:00 | 11.6 | 0 | 0/3 | 7.9 | 0 | **1/3** |
| 5:00 | 11.6 | 0 | 0/3 | 7.8 | 0 | 0/3 |
| 6:00 | 11.5 | 0 | 0/3 | 7.7 | 0 | **1/3** |
| 7:00 | 11.5 | 0 | 0/3 | 7.6 | 0 | 0/3 |
| 8:00 | 11.6 | 0 | 0/3 | 7.7 | 0 | **1/3** |
| 9:00 | 11.8 | 0 | 0/3 | 7.7 | 0 | 0/3 |
| 10:00 | 12.2 | 0 | 0/3 | 7.8 | 0 | 0/3 |
| 11:00 | 12.6 | 0 | 0/3 | 8.4 | 0 | 0/3 |
| 12:00 | 12.7 | 0 | 0/3 | 8.6 | 0 | 0/3 |
| 13:00 | 12.8 | 0 | 0/3 | 9.0 | 0 | 0/3 |

**Fig. 2** Diurnal pattern of mean environmental DNA (eDNA) concentrations of *Chimarrogale platycephalus* in both **a** A and **b** G streams. The lines show the spline regressions determined using general additive models (GAMs) to show the peaks
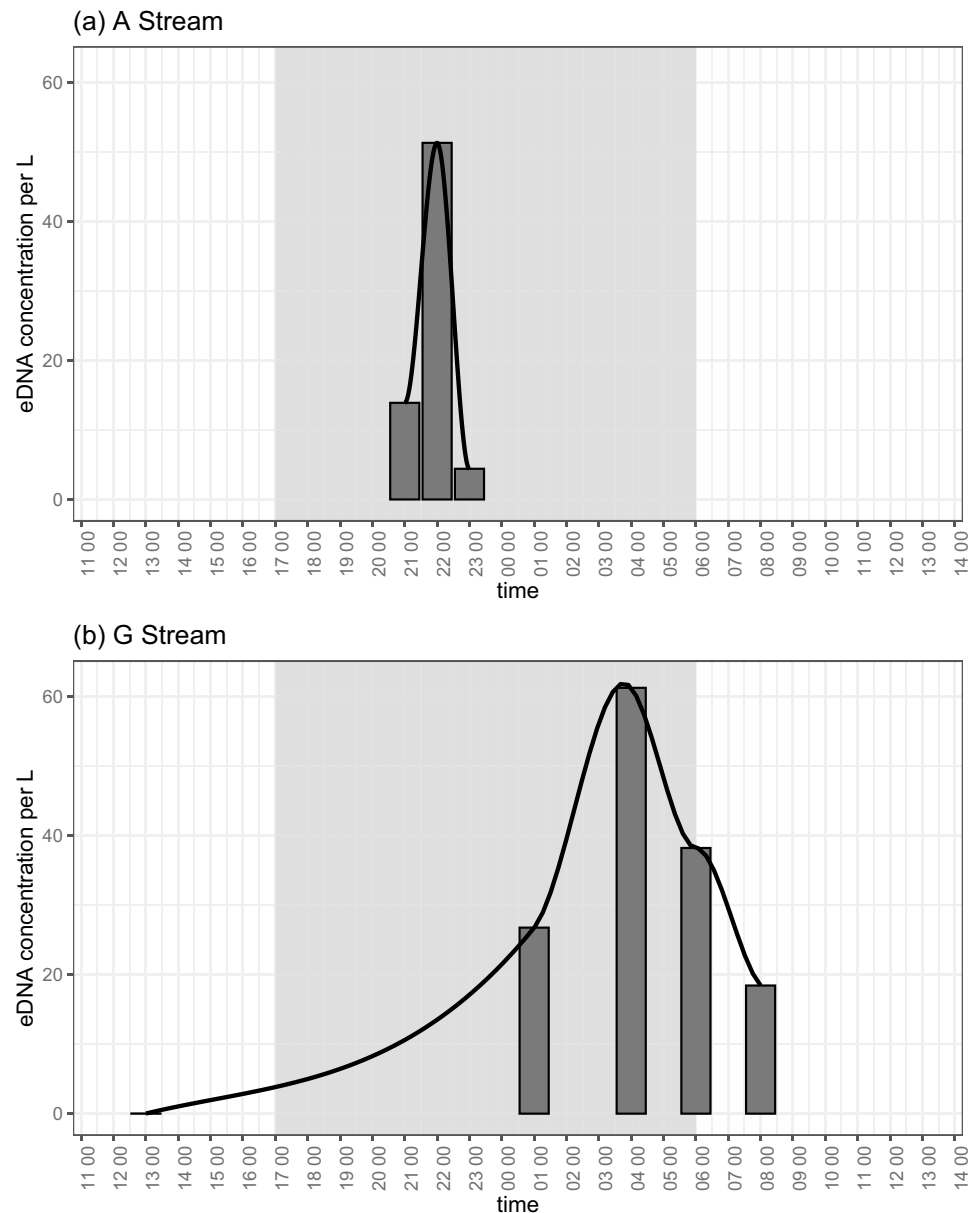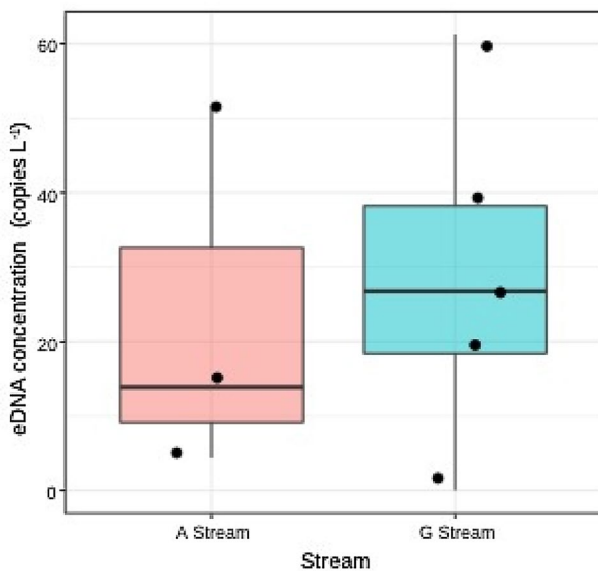


(a) A Stream

(b) G Stream

## Discussion

Using the designed species-specific primer–probe, we detected the eDNA of *C. platycephala* in streams containing feces from this species, even when no individuals were observed in the camera trap. Because the species is very susceptible to stress during trapping (Ichikawa et al. 2005; Fujimoto et al. 2011), previous distribution surveys relied on laborious fecal observations or camera trap methods; however, both methods had some limitations. Feces may be difficult to observe depending on the stream channel topography, and the camera trap does not always record small, fast-moving organisms because of difficulties in adjusting the shutter; in some cases, there were no camera recording from nights when new feces were dropped. We found that

the eDNA method may overcome the false-negative results obtained when using camera-trap surveys alone. In addition, the water sampling time affected eDNA detection, with eDNA detected mostly at night. This result may reflect the nocturnal activity and night foraging behavior of the species. Although eDNA of aquatic organisms that are always in the water can be detected during the day and night (such as for hellbender and eel, Takahashi et al. 2018, 2021), we showed that the detection of eDNA from semi-aquatic organisms can show positive or negative results depending on the diurnal cycle and sampling time.

Such eDNA false-negative results related to the sampling time can occur frequently in headwater streams where water is constantly exchanged, where eDNA is rapidly lost. eDNA constantly present in the water can be collected at any time,

**Fig. 3** Comparison of environmental DNA (eDNA) concentrations in the G and A streams. The horizontal bars in the boxes represent the median values, upper and lower edges of the boxes correspond to inter-quartiles, vertical bars correspond to 1.5×quartiles, and points represent the raw data

even in headwater streams, such as for nocturnal salamanders (Katano et al. 2017). However, we found when performing eDNA sampling for nocturnal semi-aquatic mammals in headwater streams, their diurnal activity cycles must be considered. Daytime sampling is possible for nocturnal mammals present in lentic waters (Ushio et al. 2017).

Based on the abundance of feces, we expected that the estimate of the species' population size in stream A would be lower than that in stream G. Only one fecal specimen was found per survey day in stream A, indicating a population size of one or a few individuals; in contrast, stream G harbored a few individuals or more, as many feces samples were found in each survey. The samples positive for eDNA in the daytime in the G streams may reflect the presence of multiple individuals; there may be competition between individuals, and the outcompeted organism may be active during the daytime alternatively. However, the eDNA concentrations did not significantly differ between the two streams and thus would not be markedly increased by the presence of multiple individuals.

Only two positive daytime results were obtained for stream G, one of which corresponded to a very low concentration (three orders of magnitude smaller than the other positive samples). This species was active even during the daytime, although it mainly exhibited nocturnal behavior (Motoki 2000). Our survey period corresponded to normal life cycles of the species (Ohdachi et al. 2015), during which diurnal activity was observed. In the other life-cycle

phases, such as in the winter when many females become pregnant and the spring when they are in the rearing phase, the behavior, particularly that of female individuals, may shift to increase daytime activity (Ohdachi et al. 2015); however, no field data on these behaviors have been reported. Further eDNA surveys in various life-cycle phases may provide more detailed information on the diurnal activities of *C. platycephala*. Such ongoing diurnal eDNA surveys at various life-cycle phases would reveal the possibility of daytime water sampling in other seasons.

We designed a species-specific primer–probe set for *C. platycephala* and detected eDNA from streams. In the 25-h eDNA survey, nighttime sampling was more effective than daytime sampling for semi-aquatic mammals in streams, as it reflected the nocturnal activity of the species. We confirmed the effect of sampling time on eDNA detection and importance of adjusting the sampling time based on the active periods of animals.

**Author contributions** All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by MN, TS and HI, NS and IK, IK and HD, respectively. The first draft of the manuscript was written by IK and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

## Declarations

**Conflict of interest** The commercial affiliations of the authors [MN and TS] did not alter our adherence to the journal policies on the sharing of data and materials. MN and TS are employed by the company serving environmental assessments. However, none of the authors directly benefit financially from publication of this paper.

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