Variation of Japanese eel eDNA in sequentially changing conditions and in different sample volume

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Abstract

eDNA surveys have been conducted to evaluate the distribution and abundance of Japanese eels. However, various environmental and biological factors may influence eDNA concentrations. An experiment was conducted using three water sample-replicates (50, 100, and 200 mL) of the same group of eels in a tank that were exposed to sequential non-feeding/feeding and low/high temperature conditions. Slightly higher concentrations occurred at higher temperature (22-23°C) with non-feeding, and the highest concentrations occurred when feeding started even though it was in the lower temperature (16-17°C) condition, but sample volume had no effect.

Key words: environmental DNA, feeding condition, water temperature, Japanese eel, sample volume

Environmental DNA (eDNA) sampling and analysis is a convenient tool for monitoring aquatic animals, especially for rare and endangered species because it only requires water samples instead of catching and possibly injuring the animals (e.g. Pfleger et al., 2016; Thomsen et al., 2012; Weltz et al., 2017). Studies have also been conducted that attempt to estimate the abundance and/or biomass of fishes using eDNA as summarized by Yates et al. (2019).

eDNA surveys have been used with the Japanese eel (*Anguilla japonica*), because it has been listed as an endangered species by both the IUCN red list (https://www.iucnredlist.org) and the Ministry of the Environment of Japan (www.env.go.jp). Itakura et al. (2019) reported that eDNA is an effective method to determine the spatial distribution and abundance of Japanese eels and found a weak positive correlation with eDNA concentrations and electrofishing sampling in 10 rivers in Japan. However, the Japanese eel has a unique catadromous life history and a wide size range of individuals from the elver stage to the early silver eel stage can be present at the same time, and each stage may release different amounts of eDNA (Takeuchi et al., 2019).

Other factors need to be considered when evaluating survey results, because eDNA is unstable as soon as it is excreted by an animal in the water, and its detection depends on the initial concentration, the degradation rate (Sassoubre et al., 2016), distance of eDNA transport (Nukazawa et al., 2018) and other environmental conditions such as temperature (Barnes et al., 2014; Jo et al. 2019). Therefore, biological and environmental factors should be considered before using eDNA for abundance estimations.

There have been reports on positive relationships between biomass and eDNA concentration (Horiuchi et al., 2019; Itakura et al., 2019; Takahara et al., 2012), but for some species it is likely that biological and environmental factors should be considered

when using eDNA for biomass estimation. For example, the Japanese eel is a temperate anguillid species that likely has reduced feeding and movement activity during the winter months when temperatures are lower, which could affect the release of eDNA. Therefore, eDNA variation in response to those conditions should be examined before applying eDNA techniques in natural condition for biomass estimations.

The variation of eDNA release by Japanese eels under different conditions of feeding and temperature does not appear to have been examined, so the aim of this study was to evaluate if eDNA concentrations released by eels can be affected by changing in water temperature and feeding condition using the same individual eels that sequentially experienced four different rearing conditions. The effect of water sample volumes on the eDNA concentration was also evaluated with combination of different water volume, since this is an important methodological consideration during field survey using eDNA.

Twenty cultured eels (yellow eel stage, total length: 496.3±19.1 mm, body weight: 158.1±9.7 g) were purchased from an aquaculture farm and 3 eels were randomly selected for the experiment after checking their haplotype. The haplotype of the eels was tested by real time PCR with specific primer. The 3 eels were reared together in the same 120 L tank filled with approximately 40 L of freshwater for the duration of the experiment. Aeration was set for both water quality and keeping the DNA concentration circulated in the tank.

As hypothesized that fish release more DNA under higher metabolic activities, the experiment was designed for the different treatments and water sampling to occur in the order of ‘Non-feeding & Low temperature’, ‘Non-feeding & High temperature’, ‘Feeding & Low temperature’, and ‘Feeding & High temperature’ so that the former water would have a lower effect on the concentration of the latter sample. Eels were

fasted for one month before the first sampling (25 June 2019). Low and high temperatures were 16-17°C and 22-23°C, respectively, considering the water temperature of natural habitats (excluding extreme conditions) of the Japanese eel. The low temperature was adjusted by room temperature of the rearing chamber (set on 15°C), and the high temperature was controlled by a heater (set on 25°C). About 3 g of frozen non-biting midges (*Chironomus plumosus*) were fed to the eels each feeding time and it was confirmed that eels completely consumed them on the next day of feeding. Eels were reared with three PVC pipes in the tank for shelter and under a 12L: 12D light cycle.

Because the initial water sample size affects the final eDNA concentration that is calculated as copies μL^{-1} or copies L^{-1} after PCR analysis, the relationship between sample volume and eDNA concentration was also examined. A total of nine filter samples were collected for each experimental condition, which consisted of triplicate filter samples obtained from the three different water volumes (50, 100, and 200 mL; see supporting information Figure S1, S2 for methods details). Samples were collected after two weeks of acclimation for the next experimental condition. The rearing tank was cleaned by exchanging half of the water volume once per week before the first water sampling and twice per week during the experiment, with feeding occurring twice per week.

DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), in a final volume of 110 μL according to the manufacturer's protocol described by Miya et al. (2016). Quantification was immediately performed after extraction with a specially designed primer and probe following Kasai et al. (2020). Details of the quantitative real time PCR are summarized in the supporting information (Table S1, S2).

126 To examine the effect of rearing condition and water sample volume on the
127 eDNA concentration, general linear models (GLMs) were applied. The eDNA
128 concentration in each experimental condition was used as a response variable. To avoid
129 the overdispersion, the concentration was logarithmically transformed. Feeding
130 condition, water temperature, and sample volume were explanatory variables. Model
131 selection among GLMs, used the *dredge* function in the *MuMIn* package (Bartoń,
132 2017). The best model was selected using Akaike's information criterion (AIC), having
133 the lowest AIC value. Following Burnham & Anderson (2002), models with $\Delta AIC < 2$
134 were assumed to be reasonable alternatives to the best model and thus were retained
135 (Table 1).
136 Among the GLMs for evaluating the effect of rearing condition and sample
137 volume on the eDNA concentration, two models with $\Delta AIC < 2$ were obtained (Table
138 1). Both models included 'Feeding' and 'Temperature', whereby the eDNA
139 concentrations were significantly affected by the feeding condition and water
140 temperature ($P < 0.001$). There was also an interaction between 'Feeding' and
141 'Temperature' ($P < 0.001$). Sample volume was included in the 1st model, but it had no
142 significant effect ($P > 0.1$).
143 Obtained results indicated that the volume of sample did not affect the eDNA
144 concentration. It was mentioned that the high concentration of eDNA can be detected
145 due to large fragment of DNA (Itakura et al., 2019). Also, it was discussed that the huge
146 difference of eDNA concentration among the eels in same stage can be the individual
147 variability or there was bias in eDNA distribution inside the tank (Takeuchi et al. 2019),
148 which is good accordance with present study. Therefore, concentration might depend
149 rather by size of DNA fragment which randomly drifted in the water than sample
150 volume.

151 The eDNA concentrations were lowest in the 'Non-feeding & Low
152 temperature' treatment at the start of the experiment (Figure 1) despite the variability
153 among the 3 filter replicates of the 3 water sampling volumes for this and the other
154 treatments (Supporting information Table S3). The concentrations increased after the
155 water temperature was changed from 16-17°C (1.26±0.4 copies μL^{-1}) to 22-23°C
156 (2.74±1.0 copies μL^{-1}). The eDNA concentrations then became almost 30 times higher
157 at maximum under the 'Feeding' condition (10.62±7.0 copies μL^{-1}) when the water
158 temperature was reduced and feeding had occurred (4 feeding times). The
159 concentrations then dropped back down in the higher temperature feeding condition
160 (4.33±1.1 copies μL^{-1}), but not as low as the 'Non-feeding' condition when the
161 temperature was increased.
162 Considering that metabolic rate increases at higher temperatures and while
163 feeding, it was expected the eDNA concentration would increase with each
164 experimental condition so that the 'Feeding & High temperature' treatment would have
165 the highest value. Feeding is expected to have an obvious effect on eDNA release due to
166 the excretion of waste products. However, the concentration was the highest in the
167 'Feeding & Low temperature' treatment. This might be explained by feeding after the
168 long-term fasting period causing a steep increase of eDNA emission. It has been
169 reported that starvation causes an increase of nutrient carrier (peptide transporter 1) and
170 digestive enzyme (trypsinogen) in Japanese eels (Ahn et al., 2013), which might result
171 in more efficient digestion and excretion of more waste at the beginning of the feeding
172 period. Another possibility is that there was a faster rate of decay of the eDNA at the
173 higher temperature as has been found in other studies (Barnes et al., 2014; Jo et al.,
174 2019; Kasai et al., 2020), with that effect being less important during the non-feeding
175 conditions.

This study and a variety of other research indicates that several factors can influence eDNA concentrations, that should be considered when designing surveys on species such as the Japanese eel that attempt to estimate the biomass of the target species. One factor is that eDNA emission varies not only according to metabolism but also by life stage of the Japanese eel (Takeuchi et al. 2019). The eDNA concentration increased in the later life stages, while the concentration per body weight decreased. The same result was found by Maruyama et al. (2014) with the bluegill sunfish (*Leponis macrochirus*), with the release rate of eDNA by adults being 3-4 times higher than that of juveniles, even though the release rate per body weight was higher in juveniles. Maruyama et al. (2014) emphasized the importance of careful interpretation when analyzing quantitative eDNA data to avoid over- or underestimation.

In addition to eDNA emission by fish, the concentration changes according to other factors. Research on eDNA degradation tried to simulate the decay rate with time and distance (Nukazawa et al., 2018; Sassoubre et al., 2016). In addition, the abiotic (e.g. light, oxygen, pH, salinity, water temperature, sediment) and biotic (e.g. microbial community, extracellular enzymes) environments are possible factors that influence eDNA degradation (Barnes et al., 2014; Tsuji et al., 2017). The characteristics of DNA itself (e.g. conformation, membrane-bound, length) also affects the degradation rate (Barnes et al., 2014). Humic acid from plants should also be considered since it can inhibit polymerase chain reactions and lead to false negative results (Thomsen and Willerslev, 2015).

Considering all these possible factors, estimation of biomass of the Japanese eel requires careful consideration when using eDNA. Understanding of physiology of target species, environmental features of their habitats and water chemistry is needed

especially comparing biomass across the region or season. Since eDNA concentration fluctuate even in the same conditions, taking multiple samples is also important.

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Supporting Information

Figure S1. Samples for each experimental condition.

Figure S2. Schedule of rearing and sampling.

Table S1. Specific primers and probe for identifying the Japanese eels.

Table S2. Summary of all quantitative PCR analysis.

Table S3. Replicate data of eDNA concentration calculated by quantitative PCR.

Contribution

H.A. designed, conducted experiment and wrote the manuscript.

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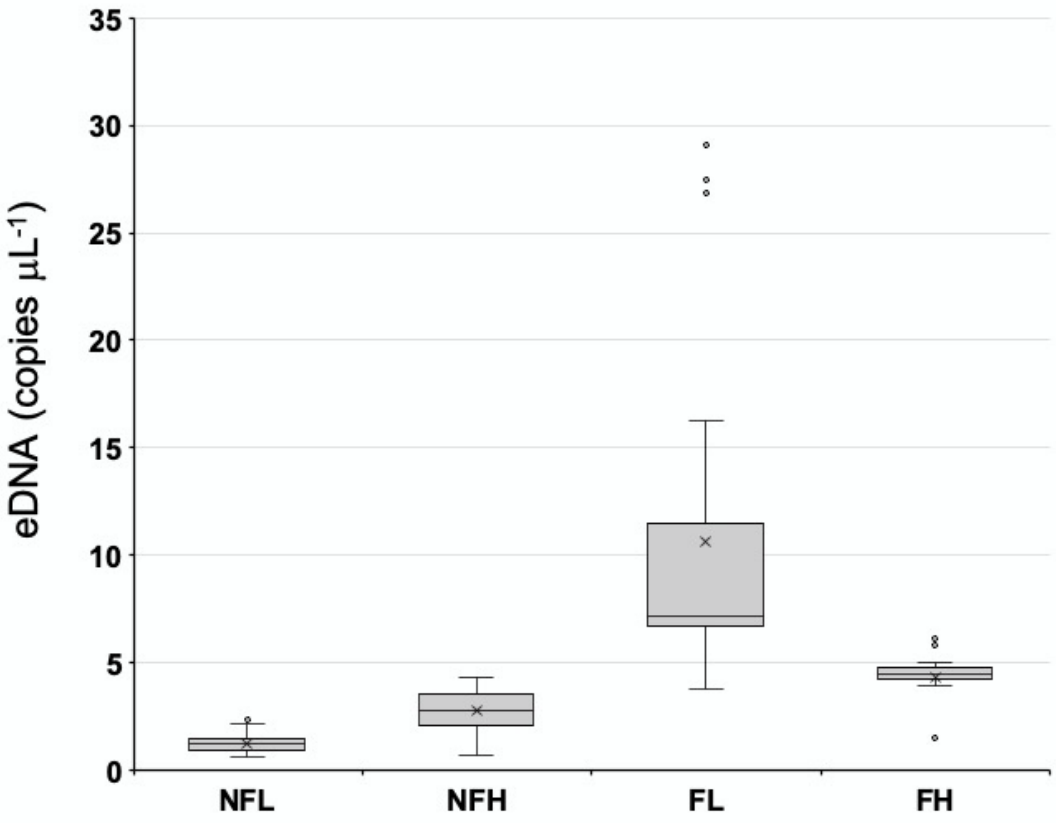
280
281
282 **Table 1.** Summary of models with ΔAIC < 2[†] used to assess the effect of feeding
283 condition, water temperature, and sample volume on the eDNA concentration.

Model	Variable					Weight	df	AIC	ΔAIC
	(Intercept)	Feeding	Temp	Volume	Fed:Temp				
1	13	4.31***	0.074***	-0.001	-0.152***	0.504	6	136.1	0
2	12.89	4.31***	0.074***		-0.152***	0.496	5	136.1	0.03

284
285 [†]Based on comparison of null and full models in general linear model results; β
286 coefficients of predictor variables are shown.
287 Abbreviations: AIC, Akaike's information criterion; Temp, Temperature; Fed:Temp,
288 Interaction of Feeding and Temperature
289 ***$P < 0.001$
290

Figure 1



Supporting Information

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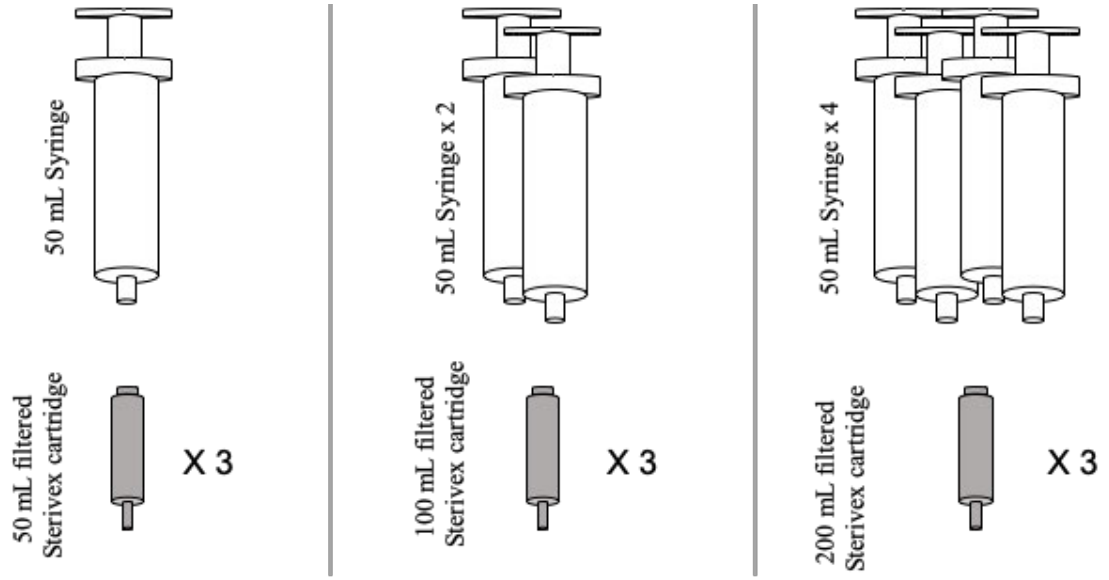

Figure S1. Samples for each of the four experimental conditions consisted of nine filter samples that were obtained for the 'Non-feeding & Low temperature', 'Non-feeding & High temperature', 'Feeding & Low temperature', and 'Feeding & High temperature' conditions, with triplicate filter samples obtained from the three different water volumes (50, 100, and 200 mL, respectively). Approximately 1200 mL of water was moved to a plastic beaker after mixing the water inside the rearing tank for each experimental condition. A syringe (Terumo Corp, Tokyo, Japan) was used for each water volume sample to transfer the desired amount of water from the beaker into each Sterivex cartridge for immediate filtering (pore size, 0.45 μm; Merck Millipore, Billerica, MA, USA). The filter was then immediately immersed in 1.6 mL RNAlater Stabilization Solution (Thermo Fisher Scientific, Waltham, MA, USA) and stored at -30°C. Disposable equipment was used for sampling and filtering.

Sunday	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
23-Jun	24	25 S	26 C	27	28	29 C
30-Jun	1-Jul	2	3 C	4	5	6 C
7-Jul	8	9 S / F	10 C	11	12 F	13 C
14-Jul	15	16 F	17 C	18	19 F	20 C
21-Jul	22	23 S / F	24 C	25	26 F	27 C
28-Jul	29	30 F	31 C	1-Aug	2 F	3 C
4-Aug	5	6 S / F	7 C	8	9 F	10 C

Figure S2. The schedule of rearing and sampling that shows sampling (S), feeding (F), and tank cleaning (C) dates. As hypothesized that fish release more DNA under higher metabolic activities, the experiment was designed for the different treatments and water sampling to occur in the order of 'Non-feeding & Low temperature' (25 June), 'Non-feeding & High temperature' (9 July), 'Feeding & Low temperature' (23 July), and 'Feeding & High temperature (6 August)' so that the former water would have a lower affect the concentration of the latter sample. Eels were fed 3 hours after water sampling.

Table S1. Specific primers and probe for the Japanese eel designed from D-loop region of the mitochondrial DNA.

Primers and probe	Sequences
Forward primer	5′–TACATTTAATGGAAAACAAGCATAAGCC–3′
Reverse primer	5′–CGTTAACATTACTCTGTCAACTTACCTG–3
Probe	5′–FAM–ACCCATAAACTGATAAATAG–MGB–3′

Table S2. Summary of the quantitative PCR analysis. NFL: Non-feeding & Low temperature, NFH: Non-feeding & High temperature, FL: Feeding & Low temperature, FH: Feeding & High temperature.

qPCR run	Slope	Y intercept	R^2	PCR efficiency
#1 (NFL)	-3.779	42.96	0.999	83.928
#2 (NFH)	-3.799	43.03	1.000	83.327
#3 (FL)	-3.832	44.26	0.999	82.364
#4 (FH)	-3.775	44.04	1.000	84.029

Table S3. All replicate data of number of DNA copies in 2 uL of reaction volume (mean±SD) calculated by quantitative PCR. NFL: Non-feeding & Low temperature, NFH: Non-feeding & High temperature, FL: Feeding & Low temperature, FH: Feeding & High temperature, NTC: Non-template negative control, ND: Not detected. Some of the variability among sampling replicates may have been due to uneven distribution of eDNA within the water.

Experimental condition	eDNA concentration									
	50 mL			100 mL			200 mL			
	replicate 1	replicate 2	replicate 3	replicate 1	replicate 2	replicate 3	replicate 1	replicate 2	replicate 3	NTC
NFL	846±7	1297±22	606±40	2164±61	4181±192	2253±148	3613±158	4208±285	5330±47	ND
NFH	1982±59	3740±194	3276±80	3691±150	6465±130	1366±106	9956±181	10917±370	9898±590	ND
FL	8670±242	10140±264	3551±149	12419±412	28516±1081	50573±2112	24059±366	25662±353	25471±1197	ND
FH	5511±176	3945±229	3883±263	8579±466	8221±151	8906±253	15452±277	5832±431	15660±492	ND