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Environmental DNA detects a possible Japanese eel spawning event near a video-recorded anguillid eel in the open ocean

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ABSTRACT: Facultative catadromous eels migrate back to the sea to reproduce, but their spawning behavior and locations have remained elusive. Using environmental DNA (eDNA), we identified a likely spawning site location and time of spawning of the Japanese eel. We detected Japanese eel eDNA at 400 and 600 m and recorded a likely sighting of this species at about 220 m using a deep-tow camera system 6 d before the new moon. A strong eDNA signal was obtained at 400 m from the apparent spawning event the morning after the estimated peak of eel spawning, 3 d before the new moon. These findings indicate that Japanese eels were already within the area where they were going to spawn at least 6 d before the new moon and then may have spawned near the strong eDNA station 3 d before the new moon. We concluded that the eDNA analysis is useful in searching for spawning sites and determining the timing of spawning of aquatic organisms with external fertilization that causes a temporary surge in eDNA, although prior knowledge of likely spawning sites is needed.

KEY WORDS: *Anguilla japonica* · eDNA · Eels · Spawning ecology · Species-specific detection · Underwater camera systems

1. INTRODUCTION

The reproductive ecology of anguillid eels has long fascinated biologists. Even Aristotle, about 2400 yr ago, was said to have noted that gravid eels were never found in rivers (Schmidt 1922). Early in the 20th century, the Danish scientist Johannes Schmidt first discovered the spawning areas of the European eel *Anguilla anguilla* and American eel *A. rostrata* in the Sargasso Sea based on collecting small larvae called leptocephali by net sampling (Schmidt 1922, 1925). Anguillid eels are facultative catadromous, spending most of their lives in rivers, lakes and estuaries before migrating to the sea to reproduce (Schmidt 1922, 1925, Tsukamoto et al. 1998b, 2011, Tsukamoto 2009). Temperate *Anguilla* species migrate long distances of about 3000 to 7000 km to their spawning areas that are located in specific regions of the ocean (Aoyama 2009, Tsukamoto 2009, Béguer-Pon et al. 2015, Righton et al. 2016). However, oceanic spawning events of anguillid eels have never been witnessed, so questions about when and where

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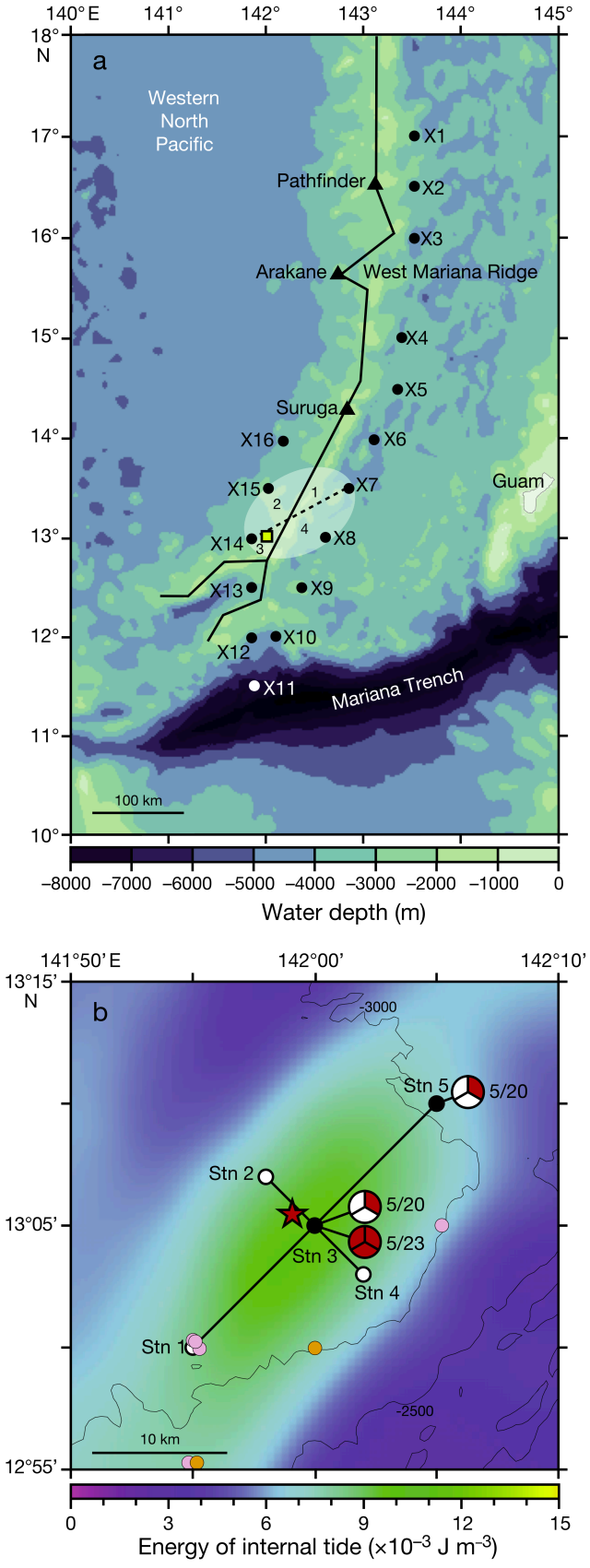
they aggregate and spawn have remained, even after the spawning areas of some other anguillid species have been determined (Miller & Tsukamoto 2017, Kuroki et al. 2020). Recently, telemetry technologies have been used to investigate the spawning migration routes of temperate (Manabe et al. 2011, Béguer-Pon et al. 2015, Chow et al. 2015, Righton et al. 2016, Higuchi et al. 2018, 2021) and tropical (Schabetsberger et al. 2013, 2016) anguillid eels, and although those studies demonstrated that anguillid eels perform clear diel vertical migrations while migrating back to their spawning areas, most studies did not provide much information about spawning locations.

Seventy years after the famous discovery of anguillid eel spawning areas in the Atlantic Ocean by Schmidt (1922, 1925), in the Pacific Ocean, a big catch of 827 Japanese eel (*A. japonica*) leptocephali in 1991 revealed that its spawning area was in the waters west of the Mariana Islands in the western North Pacific (Tsukamoto 1992). This was followed by the discovery of newly hatched Japanese eel larvae (preleptocephali) in 2005 (Tsukamoto 2006), post-spawning adults in 2008 (Chow et al. 2009, Kurogi et al. 2011, Tsukamoto et al. 2011) and fertilized eggs in 2009 (Tsukamoto et al. 2011) along the southern part of the West Mariana Ridge. Eggs and preleptocephali were also collected in similar areas in subsequent years (Aoyama et al. 2014, Takeuchi et al. 2021). The spawning sites of the Japanese eel are, therefore, the most precisely identified compared to other anguillid eels, whose spawning areas are only known from catches of various sizes of leptocephali (Miller et al. 2015, Miller & Tsukamoto 2017, Kuroki et al. 2020).

The intensive research efforts to understand the spawning ecology of the Japanese eel up to 2015, which totaled 63 cruises, 1335 d and 3675 tows at 1908 stations since 1934 (Shinoda et al. 2011, Takeuchi et al. 2021), have enabled rough estimates of when and where adults spawn. The spawning area appears to be defined latitudinally by the location of a salinity front and longitudinally by the seamount chain of the West Mariana Ridge (Tsukamoto et al. 2011, Aoyama et al. 2014, Takeuchi et al. 2021). The southwestern region of the crossing point where the salinity front intersects the West Mariana Ridge has been referred to as the third quadrant region (Third Quadrant Hypothesis, Fig. 1a), and all the eggs that were collected previously have been obtained from that region (Aoyama et al. 2014, Takeuchi et al. 2021). Previous studies using the otoliths of collected Japanese eel larvae showed evidence of synchronized spawning near the new moon during each month of the breeding season from April to August (Tsukamoto et al. 1998a, 2003, Shinoda et al. 2011). Eggs were collected at a depth of 150 to 180 m, 0 to 3 d before the new moon (Ishikawa et al. 2001, Tsukamoto et al. 2003, 2011, Aoyama et al. 2014), with peak spawning estimated to have occurred between 20:20 and 22:30 h, 3 d prior to the new moon (Higuchi et al. 2020).

Attempts to observe the spawning events of the Japanese eel with submersibles and underwater cameras have failed probably because their deployments were slightly or considerably outside of true spawning sites (Fukuba et al. 2015, Takeuchi et al. 2021), although the head and anterior body of an eel-like species was recorded during a 2012 cruise (Tsukamoto et al. 2013). Because the location of the salinity front that forms within the westward-flowing North Equatorial Current shifts dynamically over time, causing latitudinal changes in the spawning sites (Kimura & Tsukamoto 2006, Aoyama et al. 2014, Takeuchi et al. 2021), to attempt to observe spawning events, it is necessary to first accurately determine the location where the spawning aggregations of this species are likely to occur within the third quadrant region. To achieve this, we previously analyzed environmental DNA (eDNA) — the ubiquitous genetic material in the water originating from sources such as feces, skin and gametes shed by organisms (Ficetola et al. 2008, Taberlet et al. 2012, Thomsen & Willerslev 2015) — within the third quadrant while on board a research ship in 2015 (Takeuchi et al. 2019a). Because little was known about the relationship between eDNA and Japanese eel ecology, we also conducted laboratory experiments using reared Japanese eels. The experiments revealed that older and bigger eels shed more eDNA, the amount of eDNA correlated positively with the number of individuals, and the eDNA concentration after spawning increased 10 to 200 times (Takeuchi et al. 2019b). These results suggested that the Japanese eel spawning events would produce higher eDNA concentrations in the ocean than would the mere presence of individuals of the species.

To observe the spawning behavior of Japanese eels, the objective of this study was to identify the location and timing of spawning using onboard eDNA analysis during the YK17-10 research cruise in 2017. Here, we report the detection of eDNA from a probable spawning event of the Japanese eel in the ocean for the first time and the first clear observation of the whole body of an anguillid eel within its spawning area, although no eel spawning behavior was observed.



2. MATERIALS AND METHODS

2.1. Cruise objectives, survey area and ocean currents

The YK17-10 cruise was conducted on 13 to 27 May 2017 by the RV ‘Yokosuka’ of the Japan Agency for Marine-Earth Science and Technology (JAMSTEC). The cruise aimed to observe the spawning events of the Japanese eel *Anguilla japonica* using underwater observation systems and eDNA detection to further understand the reproductive ecology of this species. Upon reaching the southern part of the West Mariana Ridge, we measured the salinity and water temperature at 16 stations from 11.5° to 17° N along the eastern side (X1–11) and from 12° to 14° N along the western side (X12–16) of the West Mariana Ridge seamount chain from 16 to 18 May 2017 (Fig. 1a). The environmental data were recorded from the surface to 1000 m water depths at each station using expendable conductivity, temperature and depth profiler probes (XCTD; Tsurumi-Seiki) to examine salinity structures along the seamount chain. Consequently, the vertical (Fig. S1 in Supplement 1 at www.int-res.com/articles/suppl/m689p095_supp/) and horizontal (Fig. S2) salinity structure indicated an apparent salinity front between 13.5° N in the east and 13° N in the west along the West Mariana Ridge. The southwestern area was regarded as the third quadrant region along the West Mariana Ridge. This was done to narrow down the area of possible spawning sites using the Third Quadrant Hypothesis (Fig. 1a; Japanese eel eggs were always distributed in the third quadrant region, south of the salinity front and west of the West Mariana Ridge), where spawning can oc-

Fig. 1. Study area and environmental DNA (eDNA) detection during the YK17-10 research cruise. (a) Positional relationship among the possible location of the salinity front (dashed line), the 3 shallow seamounts (black triangles), the general layout of the West Mariana Ridge (black line) and the survey area (yellow square). The third quadrant region labeled as 3 is defined as being the southwestern area of 4 quadrants numbered anticlockwise from the upper right (see numbers in transparent white oval). Black circles show XCTD stations to examine the salinity structure. (b) Survey area, consisting of 5 stations in the patch of high internal tide energy. Japanese eel eDNA quantitative real-time PCR replicates (n = 3) are in red at positive stations (black circles) on 20 and 23 May 2017. White circles show negative stations with no Japanese eel eDNA detections. Red star represents the location where an apparent Japanese eel was observed. Previous egg collection stations in June 2011 (pink circles; Aoyama et al. 2014) and May 2014 (orange circles; Takeuchi et al. 2021) are also shown

cur at 1 or multiple locations in relation to the salinity front/salinity structure along the seamount ridge (Takeuchi et al. 2021), as this species seems to spawn in shallower depths of 150 to 200 m in the third quadrant region at night a few days before the new moon (Tsukamoto et al. 2011, Aoyama et al. 2014).

Japanese eels might be able to detect a high-energy patch of internal tides as a landmark to aggregate based on factors such as turbulence or the effects of vertical movements of water (T. Higuchi et al. unpubl.), as has been considered for other fish species (Woodson 2018). A high-resolution simulation of oceanic internal tidal energy distribution around the West Mariana Ridge was conducted using detailed bathymetric data (Niwa & Hibiya 2014). This simulation of the internal tide 3 d before the new moon on the dates of the survey, which is the eel spawning peak, showed that high-energy patches occurred in the third quadrant region during the YK17-10 cruise. Based on the distribution of internal tide energy, an elliptical survey area consisting of 5 stations was set in the patch where the spawning events of the Japanese eel were expected to occur (Fig. 1b). The long-axis distance from Stns 1 to 5 in the survey area was about 25.8 km, and the short-axis distance from Stns 2 to 4 was about 10.3 km.

Ocean currents at 200, 400 and 600 m water depths were analyzed to assess how the eDNA may disperse within the survey area using the analysis datasets of the Hybrid Coordinate Ocean Model (HYCOM) data-assimilative system (Chassignet et al. 2009) (version GLBu0.08/expt_91.2, www.hycom.org). The datasets of 21 May were missing in the available HYCOM data, but the analyses were conducted on 19, 20, 22, 23 and 24 May 2017 to evaluate the plausible transport history of eDNA (Fig. S3).

2.2. Onboard eDNA analysis

The eDNA sampling was conducted at 5 stations in the survey area on 20 May and only at Stn 3 from 21 to 24 May. A seawater sample of 10 l was collected from each of 8 water depths (50, 100, 150, 200, 400, 600, 800, 1000 m) at a station using the 16 Niskin bottles attached to the RV 'Yokosuka' deep-tow (YKDT) camera system that was deployed vertically while the ship was stationary (dive nos. 187, 188, 189, 190, 191, 193, 196). The depths for the water collection reflected likely spawning depths (<200 m) and included possible daytime swimming depths of adult eels (400–1000 m). Seawater samples of 10 l were also collected during the observation survey by the YKDT (dive nos. 186, 192, 194, 195, 197; see Table S1 in Supplement 1) while the ship was moving, but all showed negative results. All seawater samples were transferred to 10 l plastic bottles (Sanpo Kasei) once the YKDT system was brought back on board the ship. The seawater samples were immediately filtered using Sterivex filter cartridges (0.45 µm pore size; Millipore). The filtration of large water volumes such as 10 l was done following Miya et al. (2016). As a control, 10 l of ultrapure water was filtered using the same method for each station and prepared as a filtration blank. The plastic bottles were sterilized with 0.5% bleach, thoroughly washed with ultrapure water before reuse and then prewashed with the seawater collected from the depth of each particular sample.

Environmental DNA was extracted from the filter cartridge using a DNeasy Blood and Tissue Kit (Qiagen) following the manufacturer's protocol. Ultrapure water was processed in parallel throughout the eDNA extraction to monitor contamination. We obtained 95 seawater samples and 12 filtration and extraction blanks. Quantitative real-time PCR (qPCR) was carried out on the ship to specifically detect Japanese eel eDNA. qPCR composition, primers, probe and cycling conditions were the same as in a previous study (Takeuchi et al. 2019b). A 5-point standard curve for plasmid DNA at known concentration (3×10^1–3×10^5 copies per reaction) was used to estimate absolute eDNA concentration in seawater samples. Ultrapure water was analyzed as a 2 µl PCR blank instead of seawater samples at each qPCR run. All eDNA extracts were amplified in triplicate qPCR solutions during the cruise. In all qPCR runs, the standard curve slopes ranged from −3.84 to −4.78, y intercepts ranged from 43.4 to 35.3, R^2 values ranged from 1 to 0.92, and PCR efficiencies ranged from 82.3 to 61.9%. All eDNA extracts and qPCR products were preserved in a −20°C freezer on the ship.

We followed all sanitary procedures to avoid contamination in the working room on the ship. Disposable gloves were worn and changed between stations and samples. A working desk, pipettes and equipment were wiped using paper and 0.5% bleach solution, and filter tips were used. Extraction and qPCR setups were conducted on a portable clean bench (Koken). qPCR products were never opened in the working space to prevent DNA dispersing into the air.

2.3. Sequencing of eDNA by a cloning method

All the samples were transported to the laboratory in ice. To ensure the accuracy of eDNA detection,

qPCR products were thawed and sequenced using a cloning method. The amplified qPCR products were separated using a 2% agarose gel and stained with SYBR® safe DNA gel stain (Invitrogen). A target band (154 bp) was cut from the gel under a LED imager (MaestroGen) to remove redundant nucleotides and primers and then purified using the Wizard® SV Gel and PCR Clean-Up System (Promega). The purified DNA was cloned into a pGEM-T Easy Vector (Promega). Transformation was done using *Escherichia coli* CJ236 (Takara) because a master mix for the qPCR included deoxyuridine triphosphate instead of deoxythymidine triphosphate. A colony-direct PCR was performed using the same primer as for the qPCR. Three positive colonies (including the target sequence) were selected, and each colony was propagated using SOC liquid medium. Plasmid DNA was purified using the Wizard® Plus SV Minipreps DNA Purification System (Promega), and sequences were determined by the Sanger method (Sanger et al. 1977) using primers T7 (5′-TAA TAC GAC TCA CTA TAG GG-3′) and SP6 (5′-TAT TTA GGT GAC ACT ATA G-3′). The sequences obtained were compared to those of the morphologically well-identified yellow eels, *A. japonica*, *A. marmorata* and *A. bicolor pacifica* (accession nos. AB038556, AP007242 and AP007237, respectively, from Minegishi et al. 2005), because larvae of the 3 *Anguilla* species were previously collected in the spawning area of the Japanese eel (Kuroki et al. 2014). The eDNA detection was considered positive if the sequences obtained were identical to those of the Japanese eel.

2.4. Videography using a towed system

To observe the spawning aggregations and behavior of the Japanese eels, nighttime observations using the YKDT camera system were conducted from approximately 19:00 h until approximately 05:45 h from 19 to 24 May 2017, for a total of 51 h 31 min. The YKDT was towed from Stn 1 to Stn 3 (dive no. 186 on 19–20 May), from Stn 2 to Stn 4 (dive nos. 192 on 20–21 May and 197 on 23–24 May) and from Stn 5 to Stn 3 (dive nos. 194 on 21–22 May and 195 on 22–23 May) at a ship speed of 1 to 2 knots (Fig. 1b). The YKDT was deployed from a cable using a trawl winch to about 200 m, where the Japanese eels were expected to spawn (Tsukamoto et al. 2011, Aoyama et al. 2014), and then towed horizontally, with its depth moving up and down between a depth range of about 200 to 250 m. The camera system consisted of a high-definition video camera, a black-and-white video camera, a compact camera and a still camera. The YKDT system has an electronic cable enabling real-time observation of the video recording (www.jamstec.go.jp/j/about/equipment/ships/deeptow.html). One camera was forward facing, another faced sideways, and the still camera faced downward, with each camera being equipped with a light. Scientists watched the live video feed and made notes about marine organisms that were observed during the cruise, and the entire recording was viewed after the cruise. A note was made about a fish that was briefly seen during the no. 186 to no. 197 YKDT deployment, which was then checked on board and confirmed as probably a Japanese eel. The Shinkai 6500 submersible of JAMSTEC was also deployed from 19 to 24 May to survey for eels at deeper daytime depths, but no eels were observed, so those survey dives are not included in the present study (dive nos. 1492–1496).

3. RESULTS

3.1. Determination of a survey area

The hydrographic stations along the eastern side of the West Mariana Ridge and the shorter line of stations in the southern area on the western side of the ridge (Fig. 1a) showed that the vertical salinity structure (Fig. S1) consisted of the lowest salinity (34 psu), extending deeper to about 50 m at 13.5° to 13° N on the eastern side and at 13° to 12.5° N on the western side of the West Mariana Ridge. The water mass with relatively low salinity (≤34.5 psu) was detected on the eastern side of the West Mariana Ridge, which extended up to 16.5° N. The horizontal salinity structure (Fig. S2) also showed a salinity gradient and a depression of the lowest salinity to deeper than 50 m at 13.5° to 13° N. We designated the southwestern area of the crossing point where the salinity front (13.5°–13° N) intersected the West Mariana Ridge as the third quadrant region, where the Japanese eel is most likely to spawn, and set 5 stations to be sampled (Fig. 1b). Using a hypothesis that Japanese eels might spawn within patches of high internal tidal energy (T. Higuchi et al. unpubl.), we centered the survey area on Stn 3, which had the highest internal tide energy among the 5 sampling stations in the third quadrant region (Fig. 1b), based on a global internal tide analysis (Niwa & Hibiya 2014). The water current within the survey area was generally flowing in a southwesterly direction during the eDNA and video observation survey (Fig. S3).

3.2. eDNA detection of Japanese eels

Of 3 replicates of qPCR for each sample, eDNA concentrations of 0.99 and 14.5 copies per reaction were detected in only 1 replicate from a 600 m depth (Stn 3; 13° 05′ N, 142° 00′ E) at 08:42 h and 1 from 400 m (Stn 5; 13° 10′ N, 142° 05′ E) at 13:33 h on 20 May, respectively (Fig. 1b, Table 1). Subsequently, the eDNA survey was made at only Stn 3 from 21 to 24 May using the same methods. All 3 qPCR replicates from 400 m at Stn 3 at 07:54 h on 23 May detected similar concentrations of Japanese eel eDNA of an average of 55.3 copies per reaction (Fig. 1b, Table 1), which was a considerably higher concentration than that detected on 20 May. Of 95 seawater samples, Japanese eel eDNA was successfully amplified from 3 samples, and the rest of the 92 samples showed non-detection of eDNA while on the ship (Table S1). After the cruise, all qPCR products from each of the 3 eDNA detection stations were sequenced by a cloning method, and they corresponded with sequences of the Japanese eel. All blanks produced negative results, suggesting no contamination had occurred during the eDNA analysis.

Environmental factors such as water temperature and salinity were measured by sensors on the YKDT camera system while we collected seawater samples for the eDNA analysis. As shown in Fig. 2, water temperature and salinity structure were almost identical among the 5 stations. The 150 m depth layer was within the middle of the thermocline (mostly between 100 and 200 m), and salinity reached a maximum value (about 35 psu) near that depth. Temperature and salinity became more stable deeper than about 300 m. Therefore, the depths at which eDNA was detected did not seem to have specific temperature (6.43–9.22°C) or salinity (34.40–34.43 psu) characteristics that could

Table 1. Summary of environmental DNA detection of the Japanese eel *Anguilla japonica* along the West Mariana Ridge in May 2017. Temp: temperature; Conc: concentration; qPCR: quantitative real-time PCR; Ct: threshold cycle; ND: not detected

Stn	Sampling date	Days before new moon	Sampling time (h)	Water depth (m)	Temp. (°C)	Salinity (psu)	qPCR result					
							Replicate 1		Replicate 2		Replicate 3	
							Ct value	Conc. (copies per reaction)	Ct value	Conc. (copies per reaction)	Ct value	Conc. (copies per reaction)
3	20 May	6	08:42	600	6.43	34.45	38.00	0.99	ND	0	ND	0
5	20 May	6	13:33	400	9.22	34.40	37.03	14.52	ND	0	ND	0
3	23 May	3	07:54	400	8.56	34.43	28.74	54.52	28.73	55.07	28.68	56.44

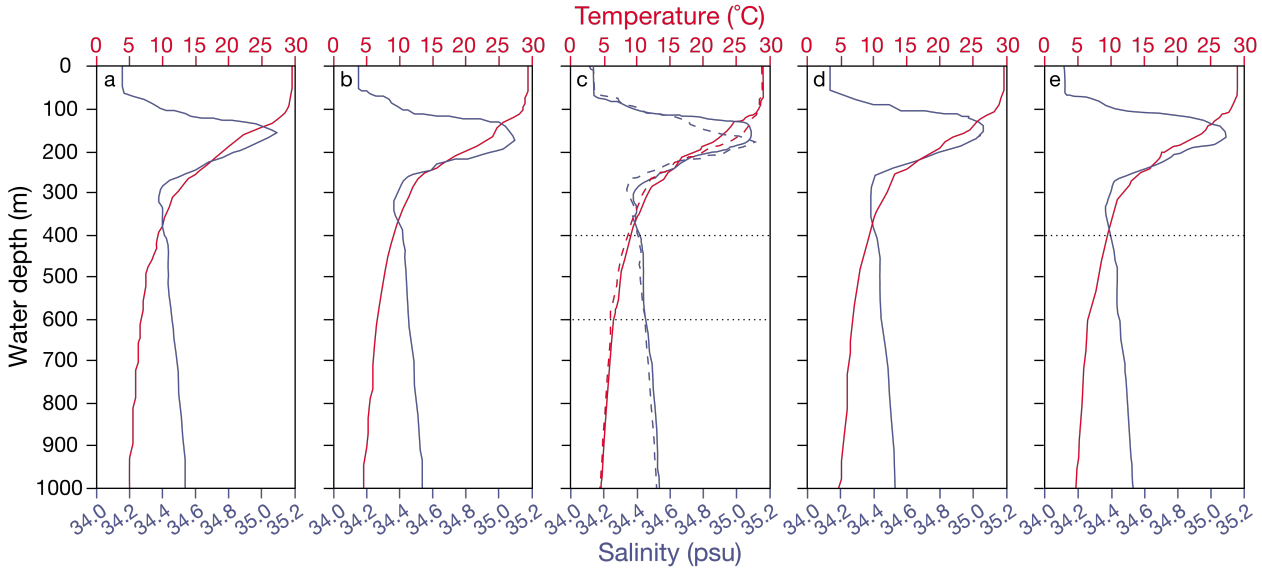


Fig. 2. Temperature (red) and salinity (blue) measured during RV 'Yokosuka' deep-tow casts for environmental DNA (eDNA) water collections at Stns (a) 1, (b) 2, (c) 3, (d) 4 and (e) 5 on 20 May (solid lines) and at Stn 3 on 23 May (in c; dashed lines). Dotted horizontal lines represent the depth where Japanese eel eDNA was detected

serve as landmarks for eel aggregation formation (Fig. 2, Table 1).

3.3. Video observation of a possible Japanese eel

The YKDT was obliquely towed up and down through the bottom of the thermocline layer at around about 200 m, where spawning is thought to occur (Fig. 2, Fig. S4). During one of the deployments, we recorded an apparent sighting of a Japanese eel using the YKDT camera system (dive no. 192) (Fig. S5 in Supplement 1 and Video S1 in Supplement 2 at www.int-res.com/articles/suppl/m689p095_supp/). The recording was made at an area 2.59 km away from Stn 3 at 21:42 h on 20 May (Fig. 1b). The individual swam just below the depth of the camera and lights system that was at a depth of 222 m, which was near the bottom of the thermocline, where the water temperature and salinity were 16.7°C and 34.7 psu, respectively (Fig. S4).

In the 7 s video, the eel's head appears to remain in a fixed posture, while its lateral body and tail showed undulations (Video S1), which has been observed in other anguilliform fish when they swim in a forward direction (D'Août & Aerts 1999, Herrel et al. 2011). The head shape of the individual seems similar to that of the Japanese eel, and it clearly differed from other types of eels of the Anguilliformes such as the Nemichthyidae, Serivomeridae, Eurypharynigidae and Cyematidae that live in the mesopelagic or deeper zones, because they have pointed or unusual jaws (Tsukamoto et al. 2013, Poulsen et al. 2018). Although it could not be identified with certainty (either *Anguilla japonica* [Tsukamoto et al. 2011] or *A. marmorata* [Miller et al. 2002, Kuroki et al. 2009] that also spawn in the area, but *A. marmorata* typically has a thicker body), the observed individual appears to be a Japanese eel based on the eDNA detected on the day of the recording, the pattern of swimming and the shape of its head and body.

4. DISCUSSION

4.1. Possible Japanese eel spawning event inferred from eDNA

In this study, the eDNA-based method was used as a new tool to detect the presence/absence and spawning sites of the Japanese eel at specific locations or depths in real time on the ship. The survey area was arranged around 13° 05′ N and 142° 00′ E based on the salinity front, seamount ridge, new moon, third quadrant region and internal tide (Fig. 1). In the area, we detected concentrations of Japanese eel eDNA, including a relatively high signal that could have been a spawning event of this species in the vast ocean for the first time. Based on other studies, detection of eDNA points to the distribution of a given species but can also be an indicator of spawning events of the species because a temporal surge in eDNA concentration occurs at spawning (Bylemans et al. 2017, Tillotson et al. 2018, Takeuchi et al. 2019b, Tsuji & Shibata 2021). The eDNA concentrations detected on 20 May (6 d before the new moon) were 0.99 and 14.5 copies per reaction, and those on 23 May (3 d before the new moon) ranged from 54.52 to 56.44 copies per reaction (Table 1). The latter detection had eDNA concentrations about 3.8 to 50 times higher than the other detections and was found at 07:54 h on the morning after the estimated spawning peak between 22 and 23 May, estimated using measurements of the ascending speed of reared Japanese eel eggs and previously obtained environmental data (Higuchi et al. 2020) (Fig. 3a,b). This higher concentration of eDNA was detected at the expected timing and place for it to have come from a spawning event, but there are other possible by-chance explanations such as many eels being in that area or an unusually large amount of sloughed-off DNA entering the water sample. However, the eDNA concentration at a spawning site appears to increase with the congregation of large-bodied adults and surges greatly from the release of sperm, sloughed skin cells and other DNA-containing material during spawning events (Bylemans et al. 2017, Tillotson et al. 2018, Takeuchi et al. 2019b, Tsuji & Shibata 2021). After spawning, eDNA in the marine environment diffuses exponentially with distance from a release point to below the limit of detection (Thomsen et al. 2012, Hansen et al. 2018, Takeuchi et al. 2019a), as illustrated in Fig. 3c.

In the case of the Japanese eel and other anguillid eels, the migrating eels use a wide range of depths because of their diel vertical migration behavior (Manabe et al. 2011, Schabetsberger et al. 2013, 2016, Béguer-Pon et al. 2015, Chow et al. 2015, Righton et al. 2016, Higuchi et al. 2018, 2021). Previous tracking studies have demonstrated that adult Japanese eels in or near their spawning area region swim at deeper depths of about 800 m during the day and at shallower ~200 m depths at night during their diel vertical migrations (Higuchi et al. 2018, 2021). These eel swimming depths directly overlap with the water depths where the eDNA was detected during the day (400–600 m) and where the eel was recorded at night

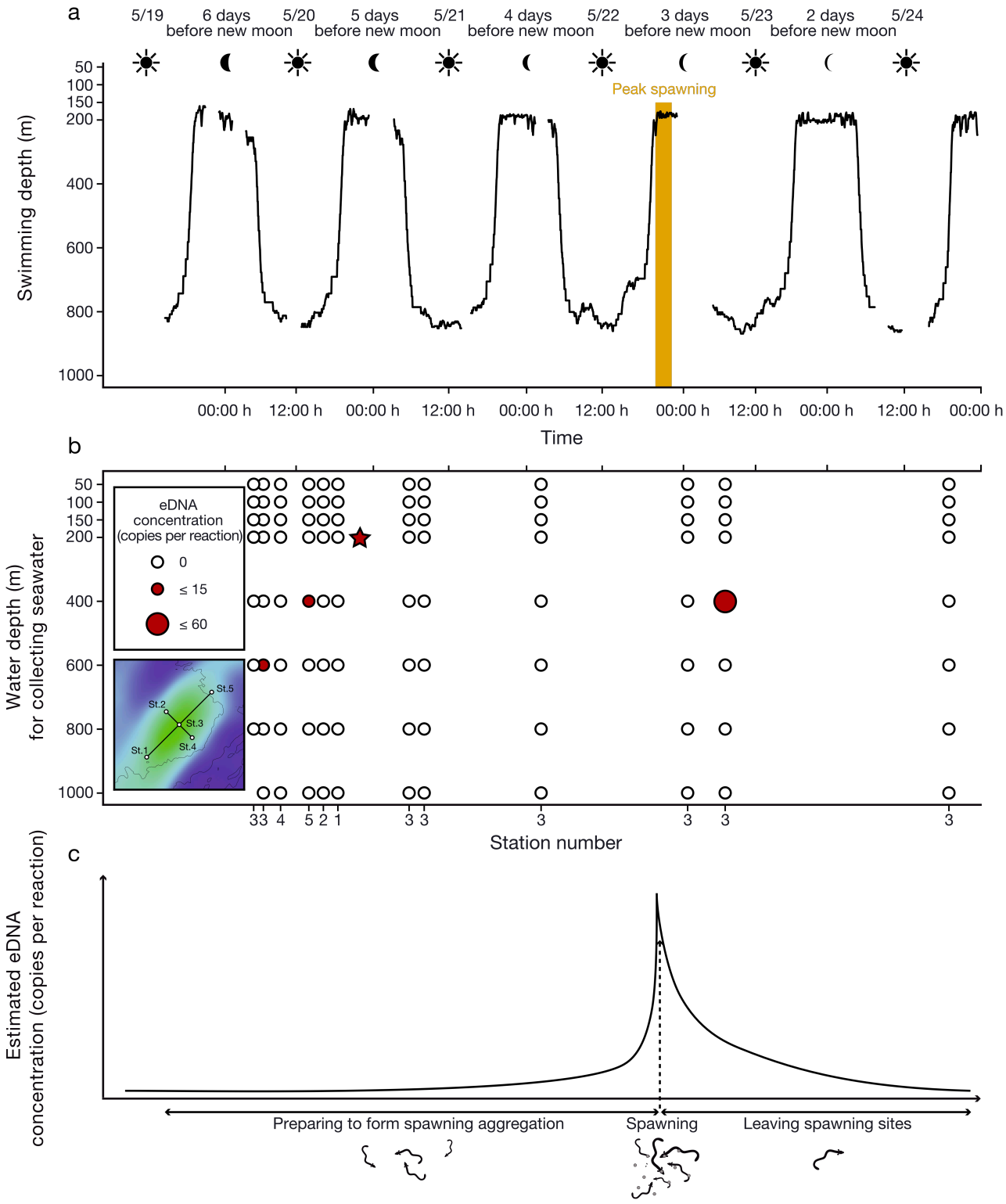


Fig. 3. Spawning ecology of the Japanese eel and environmental DNA (eDNA) concentration. (a) Previous tracking data showing that an adult Japanese eel repeated diel vertical migration near the spawning area prior to the new moon (solid line; blanks indicate missing data; Higuchi et al. 2018). Peak spawning was estimated at 20:20 to 22:30 h, 3 d before the new moon (orange bar; Higuchi et al. 2020). (b) Study results showing 3 detections of eDNA from the Japanese eel (red circles) and potentially recorded eel (red star). Non-detections of eDNA at each depth and station are shown in white circles. (c) Hypothetical schematic phase diagram of eDNA concentration with a series of Japanese eel spawning that illustrates the likely general pattern of eDNA concentration resulting from gamete release during synchronized spawning and then dispersion of the released materials

(>222 m) (Fig. 3a,b, Fig. S5). If these data (eDNA and video) are representative, this suggests that Japanese eels were already within their spawning area at least from 20 May at 6 d before the new moon. Furthermore, our previous laboratory experiments (Takeuchi et al. 2019b) detected relatively high concentrations of Japanese eel eDNA, which suggests that a spawning event had occurred at night near Stn 3 between 22 and 23 May.

Another finding of this and our previous study (Takeuchi et al. 2019a) was that eDNA was only detected in the 250 to 600 m depth range. We previously detected Japanese eel eDNA from 250 and 400 m at a similar 2015 cruise study area (13° 10′–12° 50′ N, 141° 50′–142° 10′ E) (Takeuchi et al. 2019a) and from 400 and 600 m in this 2017 cruise (Figs. 1 & 2). As in Fig. 2, water depths shallower than the 200 m layer showed high temperatures of about 20 to 30°C. The concentration of Japanese eel eDNA degrades by 20 to 60% at 20 to 30°C after about 36 h (Kasai et al. 2020), and higher temperatures make eDNA degradation faster because of the DNA denaturation by increasing enzyme kinetics and microbial metabolism (Barnes et al. 2014, Eichmiller et al. 2016). Spawning events of this species were estimated to occur at shallower layers of 150 to 200 m (Tsukamoto et al. 2011, Aoyama et al. 2014, Takeuchi et al. 2021), so if the eDNA originated from spawning, it was likely to immediately commence degrading thereafter, and eDNA would be expected to be detected at only small spatio-temporal scales (Thomsen et al. 2012, Hansen et al. 2018, Takeuchi et al. 2019a). In fact, the strong eDNA signal presented here was found at Stn 3 at 07:54 h on 23 May (YKDT dive no. 196), but about 37 h later, no eDNA was detected at the same station at about 21:00 h on 24 May (YKDT dive no. 197) (Table S1). During the 37 h, the eDNA was probably transported in a southwesterly direction (Fig. S3) or was degraded and diluted to below the detection limit.

It is also possible that the release of eggs, sperm and any other materials containing DNA associated with spawning aggregations would sink from the spawning depth and be detected at 400 m. If peak spawning occurred at 200 m at 22:00 h, for example, the eDNA collected about 10 h later at 400 m would have had a downward sinking rate of about 480 m d^{-1}. This sinking rate is faster than sinking rate estimates of small particles such as marine snow (~10–117 m d^{-1} from Shanks 2002, Sukigara et al. 2019), and one of these representative *in situ* sinking rates (74 m d^{-1} from Alldredge & Gotschalk 1988) has been used to estimate an eDNA sinking rate, because there is no published information about eDNA sinking speed (Andruszkiewicz et al. 2019). However, eDNA sinking rate might vary depending on water depths because eDNA is released into the water in the form of a variety of tissue materials with different particle sizes (Turner et al. 2014, Thomsen & Willerslev 2015) and is gradually broken down into smaller particles while it sinks to greater depths (Turner et al. 2015).

If our detection of a high concentration of eDNA was from a spawning event as hypothesized, we estimated a spawning site of the Japanese eel more accurately than ever before. In May 2017, the third quadrant region for determining the distribution area of the eggs was a large region up to 36 780 km^2 (Fig. 1a). The elliptical survey area overlapping with the patch of high internal tide energy narrowed a possible spawning area down to about 208.6 km^2 (Fig. 1b). Another factor is that using eDNA allows for greater precision because the target fish eDNA is detectable within 30 to 150 m from the point of release in marine environments (Yamamoto et al. 2016, Murakami et al. 2019). Therefore, eDNA detection likely reflects the presence of Japanese eels or a spawning site within a few hundred meters (Takeuchi et al. 2019a). Similarly, while the collection of larvae using a net is evidence of their presence below the water surface along a sampling line and provides information on their developmental stage or age, the capture depth and location are not clear. In contrast, eDNA analysis can provide specific information on the location and depth of a spawning site of the Japanese eel.

However, there is a risk of meaningless ecological inferences occurring that are caused by false negative (species not detected where present) or false positive (species detected where not present) results when we applied eDNA analysis in the ocean. To find plausible interpretations of the eDNA detections, we considered the relationship between the eDNA detections and the Japanese eel spawning ecology that has already been elucidated: previous egg collections near the survey area (Aoyama et al. 2014, Takeuchi et al. 2021) (Fig. 1b), eel tracking data (Manabe et al. 2011, Chow et al. 2015, Higuchi et al. 2018, 2021), estimated spawning peak (Higuchi et al. 2020) and how to determine a spawning site (Tsukamoto et al. 2003, 2011, Tsukamoto 2009, Aoyama et al. 2014, Takeuchi et al. 2021). This indicated that the Japanese eel was present and likely spawned within our survey area a few days before the new moon (Fig. 3). The eDNA analysis of the present study demonstrates the value of this onboard methodology to examine Japanese eel spawning ecology

when used in conjunction with published research and other conventional methods such as net sampling and video observations.

4.2. Improvements and challenges of oceanic eDNA surveys to detect spawning events

Previous studies revealed that eDNA analysis can be used to detect fish spawning events in freshwater areas (Bylemans et al. 2017, Tillotson et al. 2018) and the open ocean (Takeuchi et al. 2019a). In this study, despite the high effort invested in the eDNA survey, only 3 of 95 seawater samples showed positive detections for Japanese eel eDNA (Fig. 3b, Table S1). To increase the chances of species detections in the open ocean, either more water samples can be collected from a station (Ficetola et al. 2008), the volume of water filtered can be increased (Miya et al. 2016), or more PCR replicates can be made (Ficetola et al. 2015). However, these improvements do not consider trade-offs in cost, time and labor. Increasing the number of collected water samples and the volume of seawater filtered would increase costs and time invested and require more researchers compared to creating more PCR replicates. Increasing the number of PCR replicates may provide a better cost-effective way to obtain positive eDNA detections (Furlan et al. 2016) as well as time and labor effectiveness. At least 8 PCR replicates should be performed if detection probability is low (Ficetola et al. 2015), such as in the open ocean with low target eDNA concentrations.

Another requirement that seems to be unique to oceanic surveys searching for fish spawning events is that some physical and chemical signposts (e.g. water temperature, salinity, chlorophyll concentration, seamounts, internal tide and direction of water currents) are needed to narrow down the likely spawning areas in advance for the eDNA survey, as was clearly the case in our study. Within the areas of likely spawning, intensive water sample collections should be conducted vertically and horizontally for the eDNA analysis, which will provide high-resolution information about the presence/absence of the target species before and after spawning and about locations where spawning would occur. In the case of using onboard eDNA analysis, water collection stations can be adjusted if no detections occur in the survey area.

A key technical challenge faced by further use of the eDNA-based method of searching for eel spawning sites is to distinguish between the presence of eels, aggregations and spawning events. Positive relationships have been found between the eDNA concentration and abundance or biomass of other fishes (Doi et al. 2017, Itakura et al. 2019, Rourke et al. 2022). A possible solution has been developed by detecting a change to higher concentrations of nuclear eDNA (nu-eDNA) relative to mitochondrial eDNA (mt-eDNA), which occurs due to a change in the ratio of sperm (mitochondrial genome is relatively low) and somatic cells in a water sample (Bylemans et al. 2017). However, Bylemans et al. (2017) did not consider the dispersal rates of sperm, and the method has not been applied in the open ocean yet. The continuous movements of water in the open ocean would likely rapidly dilute and disperse the sperm, and then the ratio between nu- and mt-eDNA may immediately return to the no spawning indicated case, because the water samples do not contain sperm. Although it might detect very recent spawning, the nu- and mt-eDNA methods may have difficulty detecting spawning events when a seawater sample includes a low amount of sperm. In this study, as mentioned above, the depth and timing of the morning after the estimated peak of eel spawning 3 d before the new moon (Fig. 3a) suggest that the high eDNA concentration may have come from a spawning event of Japanese eels, because just the presence of eels or an aggregation may not be able to produce such a high concentration in one 10 l water sample in the diffusive environment of the open ocean. However, future research should develop a method to amplify specific DNA fragments derived from fish sperm directly from water samples, which will provide evidence that the spawning has occurred using only eDNA.

4.3. Video recording of an anguillid eel

This study succeeded to capture a whole-body image of what appears to be a Japanese eel within an area where spawning was likely occurring and has occurred nearby in previous years (Aoyama et al. 2014, Takeuchi et al. 2021). The previous video observation of part of some type of eel (Tsukamoto et al. 2013) did not appear to be an anguillid eel. The eel that was observed in this study was consistent in body shape with an anguillid eel. It also had a somewhat deteriorated body condition with whitish color areas (Video S1), which is consistent with the highly deteriorated body conditions of Japanese eels caught by large fisheries trawls in this West Mariana Ridge spawning area region (Chow et al. 2009, Kurogi et al. 2011, Tsukamoto et al. 2011). Histological gonad

analyses of those spawning-condition Japanese eels indicated that they may spawn more than once during successive new moons (Tsukamoto et al. 2011, Shimizu et al. 2021). However, it is unclear if the swimming eel we recorded had already spawned once during the previous new moon and was searching for other eels or if it was a more slender male eel, although the eel did not appear to have a highly swollen abdomen that female anguillid eels are known to have just before spawning (Dou et al. 2007, Tsukamoto 2009, Tsukamoto et al. 2011). No other types of eels with that body shape would be swimming in the upper midwater of the ocean, so it seems that this eel was swimming at the bottom of the thermocline, where Japanese eel spawning is thought to occur based on the previous depth of catches of both eggs and preleptocephali (Tsukamoto et al. 2011, Aoyama et al. 2014, Takeuchi et al. 2021).

4.4. Conclusions

This study represents the culmination of spawning area research on the Japanese eel, which resulted in eDNA detection from an apparent spawning event, or at least from spawning-condition eels, being traced to a specific time of night using abiotic landmarks, such as salinity front, seamount chain, new moon and internal tide, to help narrow down spawning sites and times of this species. We also recorded video of an apparent Japanese eel within its spawning area at the estimated depth where spawning has been hypothesized to occur. Another of our recent surveys used the same set of factors to deploy different types of underwater observation systems in combination with net sampling, and although eels were not video recorded, a few eggs and larger numbers of preleptocephali were collected (Takeuchi et al. 2021). Combining all our research results, eDNA analysis was able to complement conventional survey methods and is useful for searching for spawning sites and determining the spawning time of the Japanese eel in the ocean. The method of using eDNA in both horizontal and vertical dimensions in combination with knowledge about the spawning ecology of other marine organisms with external fertilization can likely be used to study their reproductive biology.

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