

Effect of intertidal elevation at Tsuyazaki Cove, Fukuoka, Japan on survival rate of horseshoe crab eggs

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<https://hdl.handle.net/2324/4794751>

出版情報 : Journal of Ocean University of China. 21 (3), pp.601-610, 2022-06. Springer
バージョン :
権利関係 :



1 **Effect of intertidal elevation at Tsuyazaki Cove, Fukuoka, Japan on survival**
2 **rate of horseshoe crab eggs**

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1 **Abstract**

2 Physical factors affecting the survival of *Tachypleus tridentatus* eggs were investigated by
3 translocating their eggs between the high intertidal zone and the low intertidal zone of a known
4 spawning site. The mean egg survival rates per day were highest in the mid intertidal zone ($45.1 \pm$
5 25.4%) and lowest in the low intertidal zone ($13.3 \pm 27.6\%$). Differences in the elevation, air
6 exposure time, and water content of the spawning ground were significant factors determining the
7 egg survival rates. Excessive or insufficient air exposure time resulted in inadequate water content
8 at higher and lower intertidal zones reducing egg survival. On the other hand, moderate saturation
9 and dehydration were repeated with each tidal movement in the mid intertidal zone. This dynamic is
10 considered one of the crucial factors for the survival of eggs and is considered optimal for spawning.
11 Therefore, the protection of the mid intertidal zone is imperative for maximizing the egg survival
12 rate in Tsuyazaki Cove where almost all suitable nesting sites have disappeared due to coastal
13 development. By protecting these optimal sites for spawning and recovering other optimal sites on
14 suitable beaches a positive contribution could be made to future management and conservation.
15 The study also suggests the possibility of translocating eggs from marginal to optimal spawning
16 sites as a recovery strategy for this globally endangered species.

17

18 **Key words** air exposure time; globally endangered species; intertidal elevation; intertidal zone;
19 optimal spawning site; *Tachypleus tridentatus*; translocated horseshoe crab eggs; Tsuyazaki Cove.

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1. Introduction

Horseshoe crabs are a group of chelicerate arthropods known as "living fossils" because their morphological characteristics have hardly changed for about 200 million years. They are important marine organisms in discussing the history of biological evolution (Rudkin and Young 2009). Four species of horseshoe crabs exist in the world: namely, the tri-spine horseshoe crab (*Tachypleus tridentatus*), the Atlantic horseshoe crab (*Limulus polyphemus*), the coastal horseshoe crab (*Tachypleus gigas*), and the mangrove horseshoe crab (*Carcinoscorpius rotundicauda*). Horseshoe crab eggs play an important role in coastal ecosystems as food resources for migratory birds (Botton and Shuster 2003; Botton 2009). In addition, horseshoe crab blood is the only natural resource that can rapidly detect bacterial endotoxins and therefore has been attracting attention in recent developments of new coronavirus vaccines (Wittenberg 2021). Thus, horseshoe crabs are not only biologically valuable but also medically and commercially significant. However, many horseshoe crabs are currently declining worldwide due to overfishing and coastal development (Akbar John et al. 2018; Laurie et al. 2019).

There is only the one species of the horseshoe crab, the tri-spine horseshoe crab (*Tachypleus tridentatus*), in Japan, and Japan is the northern limit of the distribution of this species (Laurie et al. 2019). The species has various habitat types throughout its life history in coastal areas, such as sandy mudflats for juveniles and sandy beaches in calm semi-enclosed bays for spawning (Sekiguchi 1999). Therefore, *T. tridentatus* is recognized as an indicator of a healthy coastal environment (Hsieh and Chen 2009).

Tachypleus tridentatus was once widely distributed in the Seto inland sea and the northern part of Kyushu. However, almost all populations have disappeared due to anthropogenic activities such as landfill projects, and presently the species inhabit only limited areas in Japan (Sekiguchi 1999). *T.*

1 *tridentatus* is designated as critically endangered in Japan (Ito 2014). It has also been listed as a
2 critically endangered species on the Red List of the International Union for Conservation of Nature
3 (Laurie et al. 2019). Thus, *T. tridentatus* is facing extinction both in Japan and the world, and it is
4 imperative to undertake strategic research and take practical measures regarding the conservation of
5 this species.

6 Conservation and restoration of the sandy beach areas of the spawning grounds of *T.*
7 *tridentatus* are one of the most important issues for the survival of this species, whose population is
8 declining significantly (Hsieh and Chen 2009; Wada et al. 2010; Itaya et al. 2019). *T. tridentatus* lay
9 their eggs only on the calm sandy beaches of inner bays. The species spawns in the intertidal zone
10 between the extreme high tide line (EHTL) and the mean high tide line (MHTL) (Sekiguchi 1999).
11 However, the species that have lost suitable spawning sites due to coastal development may lay
12 eggs at lower elevations than the MHTL (Sekiguchi 1999). In our study site, Tsuyazaki Cove, Japan,
13 the number of *T. tridentatus* breeding pairs has decreased remarkably. One of the causes is the
14 decrease in sandy areas suitable for spawning due to coastal development (Itaya et al. 2019).
15 Nesting at lower elevations than the MHTL was also found in our study site (Itaya et al. 2020).
16 Some studies point out that the survival rate of eggs in such an unfavourable environment is low
17 (Penn and Brockman 1994; Vasquez et al. 2015). On the other hand, there are no field-based
18 experiments on the effects of spawning at different elevations from high to low tide levels on *T.*
19 *tridentatus* eggs. Therefore, there is a lack of quantitative data on the causal relationships between
20 egg survival and coastal development impacts for the restoration and conservation of *T.*
21 *tridentatus* spawning grounds.

22 In this study, we translocated *T. tridentatus* eggs at different intertidal elevations. Then, the
23 relationship between the egg survival rate and environmental characteristics was identified to find
24 out the most suitable nesting area within the intertidal zone. The conservation measures for the

1 species which have lost most of the optimal spawning grounds due to coastal development was
2 suggested based on the findings.

3

4 **2. Methods and Materials**

5 **2.1 Study site**

6 This study was conducted at Tsuyazaki Cove (33 ° 47'N, 130 ° 27'E) in Fukuoka, Japan (Fig. 1a).
7 Tsuyazaki Cove was once a vast inner bay and salt marsh, but the landfilling for salt farming began
8 in the Edo period, and most of it was reclaimed (Hirowatari and Shimoyama 1999). Today, the
9 remnants of the salt farms and agricultural areas form the eastern hinterland of the cove, and the
10 tiny mountainous coast located on the west side of the cove. The Tsuyazaki cove is a lagoon-like
11 inland sea. On the north side of the cove, a mudflat, which is a habitat for *T. tridentatus* juveniles,
12 has formed. There are no large rivers flowing into this cove, and therefore it is considered that the
13 cove is unique as a *T. tridentatus* habitat and that it is hardly affected by freshwater (Itaya et al.
14 2019). Spawning of *T. tridentatus* has been found in small sandy areas along Tsuyazaki Cove, of
15 which about 70% of spawning was concentrated around Tsuyazaki Bridge (Wada et al. 2010). In
16 Tsuyazaki Cove, the spawning ground has disappeared significantly due to coastal development
17 after World War II, which is one of the factors for the rapid decrease in the number of breeding pairs.
18 This intensive development has removed almost all optimal sandy intertidal spawning grounds in
19 Tsuyazaki Cove (Itaya et al. 2019).

20

21 **2.2 Egg translocation experiment and analysis**

22 We translocated *T. tridentatus* eggs to test the hypothesis that the egg survival rate is affected by

1 the physical parameters at the different elevations of the intertidal zone within the spawning ground.
2 The high tide and the low tide zones in the translocation site were decided as follows. First, the site's
3 elevation and latitude/longitude data were obtained using the Real-Time Kinematic-Global
4 Navigation Satellite System (RTK-GNSS: Trimble R4 GNSS, Nikon-Trimble Co., Ltd.). Then these
5 data were taken into ArcGIS (ArcMap version 10.8, esri Japan Co., Ltd.) to create a digital
6 elevation map of the site. Next, the EHTL and the MHTL were calculated from the local tide
7 measured using a portable water depth meter (COMPACT-TD ATD-HR, JFE Advantech Co., Ltd.).
8 Finally, the position of the high intertidal zone to the low intertidal zone within the translocation site
9 was determined by overlaying EHTL/MHTL and the digital map. In addition, to confirm the
10 consistency of the elevation map, aerial photographs of the EHTL and the MHTL at the
11 translocation site were taken using a drone (Spark, Dji Co., Ltd.).

12 *T. tridentatus* spawning activities take place at high tide during summer. A female crab digs holes
13 in a sandy beach and lays eggs in the holes. Then a male crab releases sperm there to fertilize
14 (Sekiguchi 1999). The eggs laid by the same breeding pair on 3 July 2019 on the same beach in our
15 study site were collected for translocation on 4 July 2019. Since *T. tridentatus* has external
16 fertilization, we assumed that fertilized eggs were distributed homogeneously in all translocated
17 eggs. Two transect lines, transect A (A1 to A4) and transect B (B1 to B4), were placed on the sandy
18 area of the translocation site. Four translocation points (A1 to A4, B1 to B4) were set on each
19 transect line. The translocation points were located in the high intertidal zone (near the EHTL),
20 upper-mid intertidal zone (between the EHTL and MHTL), lower mid intertidal zone (near the
21 MHTL), and low intertidal zone (below the MHTL). 100 eggs were translocated at each point to a
22 total of 8 locations (Fig. 1b). For translocating eggs, we used a plastic hydroponic mesh pot (600
23 mL) with a nylon net (mesh size 1 mm) spread out inside the pot to prevent egg loss and human
24 disturbance as there are houses nearby, and many people use the site. The translocated eggs were

1 placed in the pots together with the sand collected from the study site. The eggs were slightly
2 separated from each other to prevent infection within the pot (Fig 2). Then, the hydroponic pots
3 were buried 15 cm below the surface of each translocation point on the transects. The translocated
4 eggs were buried at a depth at which horseshoe crabs lay eggs in the wild, as Maeda et al. (2000)
5 and Chen et al. (2004) described.

6 *T. tridentatus* eggs become rotating eggs as they approach hatching (Sekiguchi 1999). After the
7 stage of rotating eggs, the dispersion of the hatched larvae occurs, making it difficult to count the
8 surviving eggs accurately. Therefore, we observed the eggs during the period from eggs (stage 1) to
9 late embryos or rotating eggs (stage 21) based on the classification of Sekiguchi (1990). There are
10 some detailed reports on the development of *T. tridentatus* eggs in captivity (Sugita and Sekiguchi
11 1981; Sugita et al. 1985; Sekiguchi 1999). However, unlike in captivity, it was impossible to
12 observe the detailed developmental stages of horseshoe crab eggs in the field. Therefore, we did not
13 categorize them; instead, we determined whether the eggs were alive or dead. Infected, decayed,
14 and broken eggs were judged as dead eggs. During the inspection of eggs, the eggs at each point
15 were quickly transferred to a plastic tray full of seawater to determine how many eggs were dead or
16 alive. After counting the surviving eggs, we took their photographs and buried them in their original
17 locations. Of the two transect lines, we inspected transect B at about two weeks intervals until
18 August 21, 2019 to observe the survival status of the eggs. For transect A, we counted the final
19 surviving eggs without digging until August 21, 2019.

20 The survival rate of the translocated eggs was calculated as "egg survival rate (%) = the number
21 of eggs survived /number of eggs translocated $\times 100$ ". Since each hydroponic pot contained 100
22 eggs, the survival rate of eggs was equal to the number of surviving eggs. The survival rate of eggs
23 at each translocation site during the experimental period was analyzed using only transect B. For the
24 comparison of the egg survival rates at each translocation point from B1 to B4, the mean survival

rate per day was calculated after interpolating the data using a linear regression approximation formula. A significant test of the mean survival of eggs per day between the translocation points was performed by one-way ANOVA. If a significant difference was evident, then the Tukey-Kramer procedure was used to examine where the significant difference lay.

2.3 Measurement and analysis of physical parameters

To help understand the relationship between egg viability and the physical characteristics of each of the translocation points, salinity, nest temperature, air exposure time, water content, and sediment types were measured and analyzed. We measured seawater near the translocation site for salinity every 30 minutes over the study period (n=2001 for each point) with an underwater electrical conductivity measurement data logger (HOBO U24, Onset Co., Ltd.). A dimensionless PSU (Practical Salinity Unit) was used as the salinity unit. The nest temperature was measured every 30 minutes over the study period (n=2065 for each point) by burying an underwater temperature measurement data logger (HOBO U24, Onset Co., Ltd.) 15 cm below the ground surface at each translocation point. The air exposure time of the translocation points was estimated from the differences in the elevation of each point and the tide levels for all the time zones of the study period (n=42 for each point). For the water content, we selected the flood tide day from August 17 to 18, 2019, and collected about 100 g of soil 15 cm below the ground surface at each translocation point every hour. Then, the water content ratio in the soil was calculated as the difference between wet weight and dry weight ($W = W_w / W_s \times 100$, where W= water content, W_w = weight of water contained in soil, W_s = weight of dried soil). Soil samplings were not possible when the sand was immersed about 10 cm or more below the surface water because the soil mixed with seawater. Therefore, the soil samples for the water content were collected during

low tides (n=25 at B1, n=21 at B2, n=13 at B3, n=7 at B4 respectively). About 100 g of soil 15 cm below the ground surface was collected at each translocation point for the grain size analysis. We used seven types of sieves for the grain size analysis with a mesh size of 0.064 mm to 4 mm. Dried soil samples were passed through each sieve, and the grain size composition was determined from the weight of the particles remaining on each sieve and the ratio of the total weight. In addition, as representative values of the sediment at each translocation point, the median diameter and the grain size-sorting were calculated from the grain size distribution, and the silt-clay content was calculated from the ratio of silt-clay to the total weight. The display of physical data corresponding to each translocation point is described as B1 to B4 in this paper (Fig.1).

2.4 Analysis of the relationship between egg viability and physical parameters

Generalized linear models (GLMs) were applied to find out the relationship between the egg survival rate per day and the physical parameters using R version 3.4.4. In these models, we utilized the elevation, the mean salinity, the mean nest temperature, the mean air exposure time, the mean water content, the median diameter, the grain size-sorting and the silt-clay content as dependent variables (binomial distribution) and the mean egg survival rate per day as a predictor variable. The square value of each physical parameter was also used as a predictor variable (ordinary least squares). The models with a significant p-value ($P < 0.01$) were regarded as robust models, and thus these selected physical parameters were employed to estimate the mean egg survival rate per day by using the following equation:

$$y = 1 / \{1 + \exp[-(a + bx + cx^2)]\} \times 100$$

where: y = mean egg survival rate per day (%), x = dependent variable, a = intercept, b = coefficient

1, c= coefficient 2

3. Results

3.1 Egg survival rate

Rotating eggs were found only in the mid intertidal zone at both transects A and B (Fig. 3). On transect A, the eggs in the mid intertidal zone became rotating eggs 49 days after the translocation on August 21, 2019, but the survival rate was only 1%. On the other hand, 10% of eggs survived as rotating eggs in the mid intertidal zone on transect B. Although rotating eggs were confirmed on transect A, there was not enough data to analyze. Therefore, we used the transect B data only for analysis as described below.

On transect B, rotating eggs were found only in B3, the mid intertidal zone. These rotating eggs were first confirmed 44 days (August 16, 2019) after the translocation. By the 49th day (August 21, 2019), 10% of the B3 rotating eggs were still alive. The lowest survival rate was found in the low intertidal zone (B4), where all eggs died in 13 days (July 16, 2019) after the translocation. In other intertidal elevations, more than 50% of eggs survived until day 13 (July 16, 2019), but in the high intertidal zone (B1) eggs had died out by day 28 (July 31, 2019). In the mid intertidal zone, the survival rate of eggs on the 28th day (July 31, 2019) was 18% for B2 and 40% for B3. However, on the 44th day (August 16, 2019), B2 the eggs all died and only B3 eggs survived (Fig. 4a).

The mean survival rate of eggs per day was highest in the mid intertidal zone and lowest in the low intertidal zone ($27.8 \pm 33.9\%$ at B1, $34.1 \pm 30.8\%$ at B2, $45.1 \pm 25.4\%$ at B3, $13.3 \pm 27.6\%$ at B4). A significant difference in the mean survival rate of eggs per day among the translocation points was demonstrated by one-way ANOVA ($F=9.87$; $df=3, 192$; $p=4.36 \times 10^{-15}$). Multiple

1 comparisons based on the Tukey-Kramer test revealed that the survival rate of eggs was
2 significantly lower in the low tide zone ($P < 0.01$) (Fig. 4b).

3

4 **3.2 Physical parameters**

5 We investigated air exposure time, water content, salinity, nest temperature and sediment to
6 understand the relationship between egg viability and the physical characteristics of the
7 translocation site. The results are described below. The comparison of physical parameters in this
8 study and the previous studies is shown in Table 1.

9 The high intertidal zone (B1) was exposed to the air for almost 24 hours, whereas the low
10 intertidal zone (B4) was submerged for almost 24 hours (Table 1). The mean water content was low
11 at higher elevations and it was high at lower elevations (Table 1). The mean salinity of seawater
12 near the translocation site was 28.5 ± 3.7 (minimum 2.8, maximum 31.5, $n=2001$) (Table 1). The
13 mean nest temperature was highest in the high intertidal zone and lowest in the low intertidal zone
14 (Table 1). The sediment characteristics at the translocation site are shown in Table 1. For the grain
15 size composition, very fine sand accounted for about 90% at B1 and B2. At B3 and B4, medium
16 sand and very fine sand each accounted for about 50%. Thus, all translocation points were composed
17 of sand. The median diameters were 0.17 mm for B1 and B2 and 0.26 mm for B3 and B4,
18 respectively. The grain size sorting was from $\phi = 0.45$ (B1) to 0.76 (B4). The silt-clay content was
19 from 0.09% (B1) to 0.31% (B4).

20

21

Table 1 Comparison of the physical parameters of *T. tridentatus* spawning grounds in this study and previous studies.

Physical parameters		Air exposure time (Hour)	Water content (%)	Salinity (PSU)*2	Nest temperature (°C)	Sediment characteristics				Area		
Studies	*1 Intertidal zone					Grain size classification*3	Median diameter (mm)	Grain size sorting (φ)	Silt-clay content (%)			
This study	B1 High	23.3 ± 0.2	8.8 ± 1.3	28.5 ± 3.7	29.5 ± 3.0	VFS	0.17	0.45	0.09	Tsuyazaki, Japan		
	B2 Mid	18.6 ± 0.3	14.0 ± 2.1	28.5 ± 3.7	28.6 ± 2.8	VFS	0.17	0.50	0.06			
	B3 Mid	11.9 ± 0.1	21.7 ± 1.8	28.5 ± 3.7	27.4 ± 2.5	VFS / MS	0.26	0.70	0.11			
	B4 Low	3.3 ± 0.3	29.0 ± 0.6	28.5 ± 3.7	27.1 ± 2.5	VFS / MS	0.26	0.76	0.31			
Botton et al. 1996				33.0			0.54-0.76	1.06-1.36		Imari, Japan		
Botton et al. 1996							0.33	1.15		Morie, Japan		
Sekiguchi 1999				18.0-33.0	22.0-31.0					Imari, Japan		
Maeda et al. 2000				17.3 ± 6.6	25.7 ± 2.4		0.51			Morie, Japan		
Maeda et al. 2000				26.6 ± 5.5	27.6 ± 1.9		0.40-1.00			Morie, Japan		
Seino et al. 2000						CS	0.42-0.97		Less than3%	Morie, Japan		
Seino et al. 2001						CS	0.52-1.10		1.0-2.0	Nakatsu, Japan		
Chen et al. 2004				9.8-13.7		MS - CS	0.40-1.80			Kinmen, Taiwan		
Ohtsubo et al. 2005				12.0-17.0	5.0-25.0	24.0-29.0	26.2	VFS - CS		negligible	Imazu, Japan	
Hsieh and Chen 2009				3.7-9.3				CS	1.10		Toungshiao / Miaoli, Taiwan	
Wada et al. 2010								VFS - G	0.20-5.00	0.37-1.98	0.0-6.1	Tsuyazaki, Japan
Iida et al. 2017									0.74-4.00			Sone, Japan

Note

* 1. B1 to B4 are the egg translocation points in this study.

* 2. Seawater salinity near the survey site.

* 3. The abbreviations for the grain size class are as follows; G for granule, CS for coarse sand, MS for medium sand, VFS for very fine sand.

3.3 Relationship between egg survival rate and physical parameters

The results of analyses of the mean egg survival rate per day and the physical parameters using generalized linear models (GLMs) are shown in Table 2. The following parameters significantly influenced the mean survival rate of the eggs: the elevation, the mean air exposure time, the mean water content, the mean nest temperatures and the grain size-sorting, respectively (Table 2). Of these, the nest temperatures and the grain size-sorting were excluded from further analysis as these parameters were within the known range of *T. tridentatus* spawning grounds (Table 1). Thus, we selected elevation, air exposure time, and water content to predict the egg survival rate.

The change in the predicted values of the mean egg survival rate per day with elevation, air exposure time, and water content are shown in Figure 5. Of all these three physical factors, the estimated egg survival rate per day was the highest in the mid intertidal zone (B2 and B3) and the lowest in the low intertidal zone (B4) (Fig. 5).

Table 2 GML analysis results for the relationship between mean egg survival per day and physical parameters.

Explanatory variable	Estimate	z value	Probability (> z)
Elevation	4.506	4.511	6.44e-06 ***
squared	-3.016		
intercept	-1.994		
Air exposure time	0.342	4.608	4.06e-06 ***
squared	-0.011		
intercept	-2.818		
Water content	0.402	4.024	5.73e-05 ***
squared	-0.011		
intercept	-3.745		
Salinity	NA	NA	NA
squared	NA	NA	NA
intercept	-0.847		
Nest temperature	31.294	2.852	0.00434**
squared	-0.552		
intercept	-443.880		
Median diameter	-1.059	-0.436	0.663
squared	NA	NA	NA
intercept	-0.620	-1.168	0.243
Grain size-sorting	113.323	3.806	0.000141 ***
squared	-95.564		
intercept	-33.090		
Silt-clay content	26.756	2.494	0.01265 *
squared	-78.311		
intercept	-2.391		

Significance codes: ***P<0.001, **P<0.01, *P<0.05

4. Discussion

4.1 Egg survival rate

The eggs translocated at the mid intertidal zone became rotating eggs (stage 21) in 44 days (August 16, 2019) at 27.40 ± 2.52 °C and salinity of 28.5 ± 3.6 (Table 1, Fig. 4a). Our results showed a similar development rate as a laboratory experiment conducted by Sekiguchi (1999). In his experiment, the captive eggs reached stage 21 in 43 days at 30 °C and salinity of 20 to 35

1 (Sekiguchi 1999). It is thought that egg development proceeds at the same rate in the field as in
2 captivity when conditions such as temperature and salinity are met (Maeda et al. 2000). The "wild
3 eggs" that were laid at the mid intertidal zone on the same beach, where our experiment was
4 conducted, also became rotating eggs during the same period (the "wild eggs" were laid on 2 July
5 2019 and became rotating eggs on the 16 August 2019) (Itaya pers. comm.). Thus, our experiment
6 may reflect the average development rate of *T. tridentatus* eggs.

7 The translocated eggs had the highest survival rate in the mid intertidal zone. Survival of eggs
8 decreased at higher and lower elevations, especially 'inferior' in the low intertidal zone (Fig. 3 and
9 4). Ideally speaking, more replications should have been prepared for the experiment. However, we
10 had difficulty preparing enough eggs for the experiment because of the rapid decline of *T.*
11 *tridentatus* and thus a minimal number of breeding pairs coming to our study site (Itaya et al. 2019).
12 Therefore, care should be taken in interpreting the results of the egg survival rate as sufficient
13 statistical analysis could not be performed on the small number of replications (a follow-up
14 experiment with more replications of translocated eggs is necessary to verify the current findings).
15 Nevertheless, we can still conclude that there were differences in egg survival rate at different
16 elevation points (Fig. 4). In addition, the GLM modeling showed that elevation from high tide to
17 low tide was an important factor in determining egg survival rate (Fig. 5a). These differences in egg
18 survival rate because of the elevational difference are noteworthy.

19 The egg survival rate was highest in the mid intertidal zone (B2 and B3). Of these, 10% of B3
20 eggs finally developed to rotating eggs (Fig. 4a). There is almost no field-based research on the
21 survival rate of *T. tridentatus* eggs in the wild. In Taiwan, Hsieh and Chen (2009) found that the
22 hatching rate ranged from 0 to 88.5% (average 33.9%), and a decline in hatching rates occurred in
23 places where the tidal amplitude was small. In this experiment of Hsieh and Chen (2009), there
24 were three spawning areas, A, B, and C on the same beach, but the above survival rate was

1 calculated for only 4 nests in area A where hatching was confirmed (the reason for the exclusion of
2 areas B and C is unknown). However, if we included areas B and C where hatching was not
3 confirmed, the total number of nests would be 12. Then, the average survival rate of 33.9% found
4 by Hsieh and Chen (2009) could be divided by 12 nests instead of 4 nests, and then the survival rate
5 would be about 11%. The egg survival rate in the mid intertidal zone (B3) of this study was 10%
6 (Fig.4a), which is almost the same as their conversion value. In addition, when we consider all of
7 the translocated eggs in transect B, the number of surviving eggs is 10 out of 400 eggs, the survival
8 rate is 2.5%. In summary, the range of egg survival rate in our study is from 2.5% (minimum) to
9 10% (maximum), which is not essentially different from the example in Taiwan reported by Hsieh
10 and Chen (2009). Therefore, it is assumed that the survival rate of *T. tridentatus* eggs obtained in
11 this study may reflect the survival rate in the 'wild'.

12 On the other hand, we found some reddish eggs showing bacterial infection in our translocated
13 eggs (Fig. 3). Sekiguchi (1999) removed infected eggs every time in his laboratory experiment.
14 However, removing the infected eggs in the hydroponic pots was not performed to reduce human
15 influence as much as possible. It is more likely that infection can spread quickly within the closed
16 environment of the hydroponic pots. Thus, this may have led to a decrease in the survival rate of the
17 translocated eggs.

18 There are several reports on the spawning location of horseshoe crabs within the intertidal zone.
19 97% of *T. tridentatus* spawning was found between the EHTL and the MHTL in Imari, Japan, but
20 most were concentrated near the mid intertidal zone (Sekiguchi 1999). In Malaysia as well, more
21 than 50% of *T. tridentatus* spawning was concentrated in the mid intertidal zone (Mohamad et al.
22 2019). The Atlantic horseshoe crab (*Limulus polyphemus*) laid their eggs between the high intertidal
23 and the mid intertidal zones. Increased egg development was evident in both zones but decreased in
24 the low intertidal zone (Vasquez et al. 2015). Another study pointed out that *L. polyphemus* spawned

in the mid intertidal zone which maximized their egg development rate by avoiding the dry and hot environment in the high intertidal zone and the oxygen-deficient condition in the low intertidal zone (Penn & Brockman 1994). Our results were consistent with these previous studies. The translocated eggs had the highest survival rate in the mid intertidal zone (Fig. 4 and 5). Although no other research was found on *T. tridentatus* investigating egg development at different elevations from high tide to low tide, it is thought that *T. tridentatus* select the mid intertidal elevations of the intertidal gradient for spawning.

4.2 Physical parameters contributing to egg survival rate

Since *T. tridentatus* spawning occurs near the MHTL, the total time the eggs are submerged is relatively short. The air exposure time of eggs at the translocated site was longer at higher elevations of the intertidal zone. Consequently, the water content was lower at higher elevations and higher at lower elevations (Table 1). However, due to the water storage capacity of the sandy beach and the water absorptive function of the egg itself, seawater is retained in the egg even when the beach is exposed to air (Sekiguchi 1999). Penn and Brockman (1994) found that the water content of sand is one of the factors that influences egg development in horseshoe crabs. In this study, the water content of the eggs at each translocated point was found to be determined by the tidal movement and the degree of elevation of the intertidal zone.

In the mid intertidal zone (B2, B3), where egg survival was higher, the mean air exposure time was 11.9 to 18.6 hours per day (Table 1). Although very few studies quantified the air exposure time of spawning grounds, it was 12.0 to 17.0 hours per day in Imazu, Japan (Otsubo et al. 2005), which is almost the same as the results of this study (Table 1). On the other hand, in the high intertidal zone (B1) and the low intertidal zone (B4) where egg survival was relatively poor, B1 was exposed

1 to air for almost 24 hours and B4 was flooded almost all day (Table 1).

2 In this study, the egg survival rate was highest in the mid intertidal zone (B2 and B3) and lowest
3 in the low intertidal zone (B4) (Fig. 4). This trend was also evident in the GLM models, which
4 showed that the air exposure time and water content contributed significantly to the egg survival
5 rate (Table 2, Fig. 5b and c). Otsubo et al. (2005) pointed out that the low intertidal zone is not
6 suitable for spawning due to the amount of dissolved oxygen in the pore water is low in moist sand.
7 A sandy beach takes in abundant oxygen at low tide when exposed to the air. Then, when it
8 immerses in seawater at high tide, the oxygen in the sand dissolves into the seawater and finally is
9 absorbed by the eggs. Therefore, *T. tridentatus* eggs develop better when the seawater is completely
10 removed at a certain frequency than when they are constantly immersed in seawater (Sekiguchi
11 1999). Thus, the low egg survival rate in the low intertidal zone can be explained by the lack of air
12 exposure time, resulting in excessive water content (Table 1), which causes insufficient oxygen for
13 the eggs.

14 The water content in the high intertidal zone (B1) in this study was within the range of other
15 studies (Table 1). However, in the high intertidal zone, the water content temporarily increased
16 during high tides but remained low over the other tidal periods. Furthermore, while other
17 translocated points were submerged into seawater at least once a day, there were three times when
18 the high intertidal zone was not submerged for more than a week during the study period. It
19 accounted for 62% of the total time when the high intertidal zone was not immersed in seawater. In
20 a laboratory experiment with the coastal horseshoe crab (*Tachypleus gigas*), Faizul et al.
21 (2013) showed that eggs in sand develop well when immersed in seawater at a frequency of 1 to 3
22 days. It was suggested that when the eggs are not submerged in seawater for more than a week,
23 desiccation may cause a poor egg development rate (Faizul et al. 2013). Therefore, it is possible that
24 dissolved oxygen did not reach the eggs sufficiently in the high intertidal zone as the immersion

1 time and its frequency were extremely short in this study.

2 Significantly, the mid intertidal zone (B2 and B3) was covered in seawater at a moderate
3 frequency and it was exposed to air for about 30 to 50% each day. This, in turn, would have made it
4 possible for a sufficient supply of oxygen for egg development. Thus, the mid intertidal zone is
5 considered the most suitable spawning zone for *T. tridentatus* with respect to air exposure frequency
6 and the water content.

7

8 **4.3 Impact of coastal development on *T. tridentatus* spawning ground and conservation**

9 The results of this field-based study can be applied to the restoration and conservation of the
10 spawning ground for the critically-endangered horseshoe crab where there is the lack of suitable
11 spawning grounds caused by coastal development. The study revealed that the egg survival rate in
12 the low intertidal zone was meager (Fig. 4 and 5). Some breeding pairs of the area laid eggs in the
13 low intertidal zone suggesting there was probably a shortage of suitable spawning habitat (Itaya et
14 al. 2020). Hence, the egg survival rate of some of the breeding pairs is insufficient to sustain a
15 viable population of horseshoe crabs in Tsuyazaki Cove. It may be necessary to translocate eggs
16 laid in the low intertidal zone to the mid intertidal zone to increase their survival rate. However, as
17 described below, the translocation of the eggs alone may not be sufficient for the recovery of the
18 natural population in Tsuyazaki Cove.

19 The sand supply to suitable sandy spawning grounds has decreased due to the coastal
20 development in Tsuyazaki Cove (Itaya et al. 2019). As a result, the number of breeding pairs has
21 decreased yearly with this habitat deterioration. Over the last decade, more than 90% of breeding
22 pairs have disappeared from our study site due to the development. Currently, the number of

1 spawning pairs coming to the beach every year is only 4 to 10 pairs (Itaya et al. 2019). Thus, the
2 spawning grounds at Tsuyazaki Cove have degraded over time, and natural recovery of the
3 spawning grounds and the population cannot be expected. It is urgent therefore to protect the
4 remaining suitable spawning sites and set about restoring those sites which are degraded. We
5 believe it is urgent to secure the mid intertidal zone for maximizing the egg survival rate in
6 Tsuyazaki Cove by protecting what remains of it and by recovering other suitable sites for spawning.
7 It is even possible to design and build artificial spawning sites. For these practical conservation
8 measures, further research studies will be necessary to estimate the remaining suitable spawning
9 grounds and those degraded in order for conservation policies to give consideration to specific
10 species recovery strategies.

11

12 **4.4 Conclusion**

13 In this study, the relationship between *Tachypleus tridentatus* egg survival and the physical
14 characteristics of the spawning ground was revealed by a first translocation experiment of
15 *Tachypleus tridentatus* eggs in the field. It was found that the difference in elevation from high tide
16 to low tide was a key factor in determining the egg survival. At medium elevation translocation sites,
17 suitable air exposure time, water content and sediment types were met. In that condition, the
18 duration of the beach exposed to air and the water content in the sand caused by the difference in
19 elevation was essential for the eggs to survive, suggesting that the mid intertidal zone is the most
20 suitable for spawning. In higher and lower intertidal zones, excessive or insufficient air exposure
21 time reduced egg survival. In the mid intertidal zone, moderate saturation and dehydration were
22 repeated with each tidal movement and this dynamic is considered one of the crucial factors for the
23 survival of the translocated eggs in Tsuyazaki Cove. We suggest that the protection of mid intertidal

zones as optimal spawning sites would contribute positively to the future management and conservation of this globally endangered species. Also, there is the possibility of translocating eggs to these optimal spawning sites from marginal sites as a recovery strategy for this globally endangered species.

Acknowledgement

We would like to express our gratitude to Dr. John A. Harris (former Head, School of Environmental Science, University of Canberra, Australia) for his suggestions during his visit to our study site and for editing a draft of this paper. We would like to show our gratitude to Dr. Akihiko Koyama at the Fisheries Laboratory of the Graduate School of Kyushu University for his useful advice regarding this paper's structure and data analyses. Mr. Katsuji Motojima of Towa Technology Co., Ltd. gave us many words of encouragement. We would also like to thank Mr. Ayumu Sakurada, Mr. Yu Ono, Mr. Kazuya Maeda, Mr. Daiki Masuda of the Faculty of Engineering, Kyushu University, and Mr. Hiroyuki Koyama of the limited liability company Morisho for their cooperation in the survey of this research.

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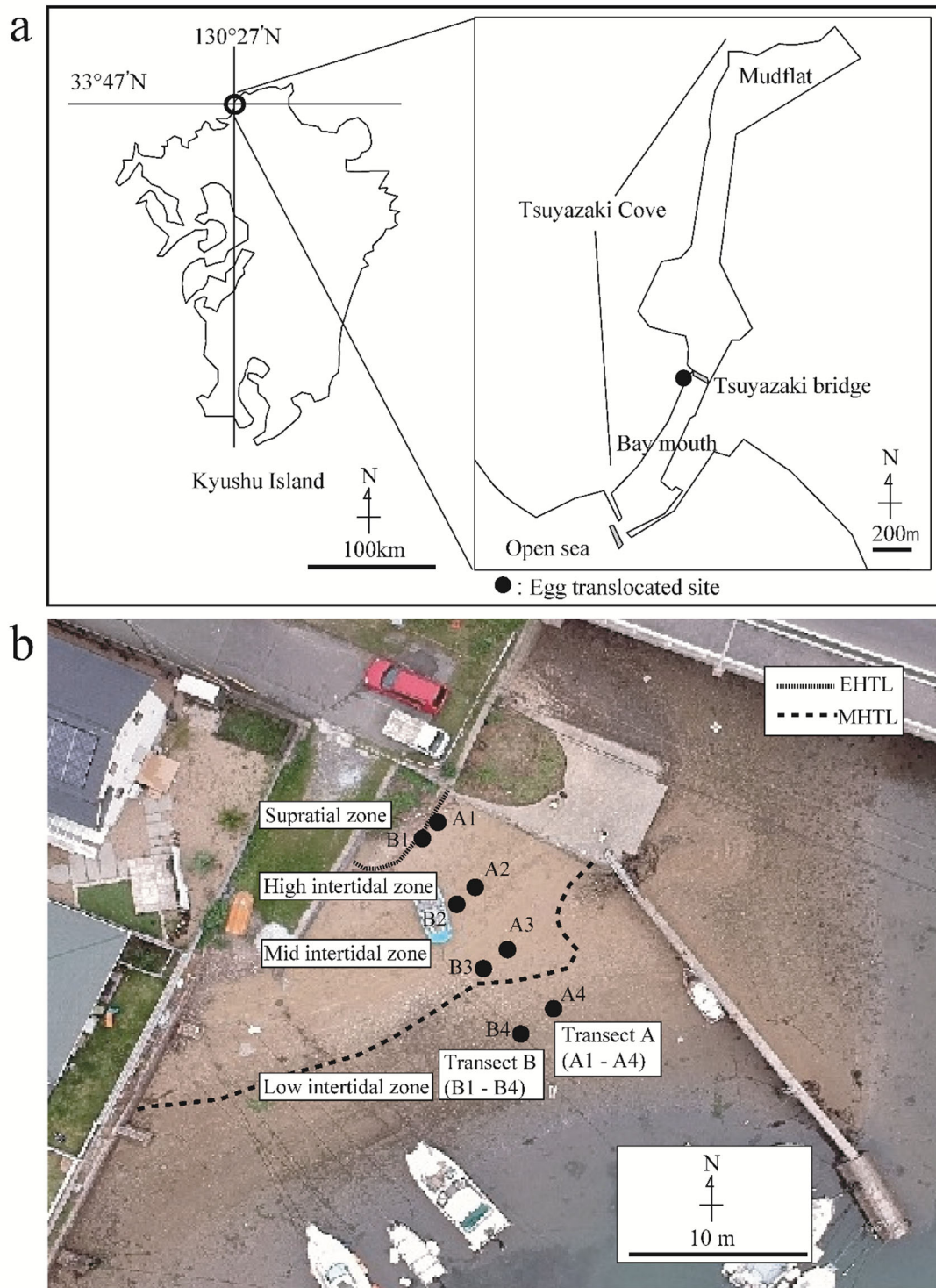


Figure 1 a) Location of the study site in Tsuyazaki, Fukuoka, Japan. The dot indicates the site where *Tachypleus tridentatus* eggs were translocated in this study. b) The translocation site of *Tachypleus tridentatus* eggs in Tsuyazaki, Fukuoka, Japan. The extreme high tide line (EHTL) and the mean high tide line (MHTL) are shown in the broken lines. The dots indicate the translocation points (A1-A4 and B1-B4).

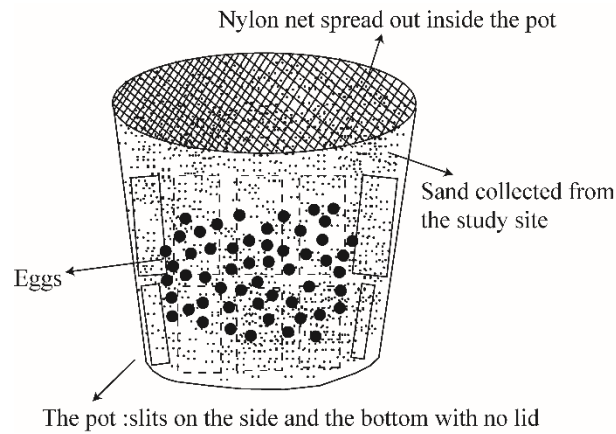


Figure 2 A diagram of a hydroponic mesh pot (600 mL) used for translocating the eggs. A nylon net (mesh size 1 mm) was placed inside the pot to prevent egg loss. 100 horseshoe crab eggs were placed in each pot together with local sand and buried 15 cm below the ground surface, which is the depth at which the horseshoe crab spawns in the wild.

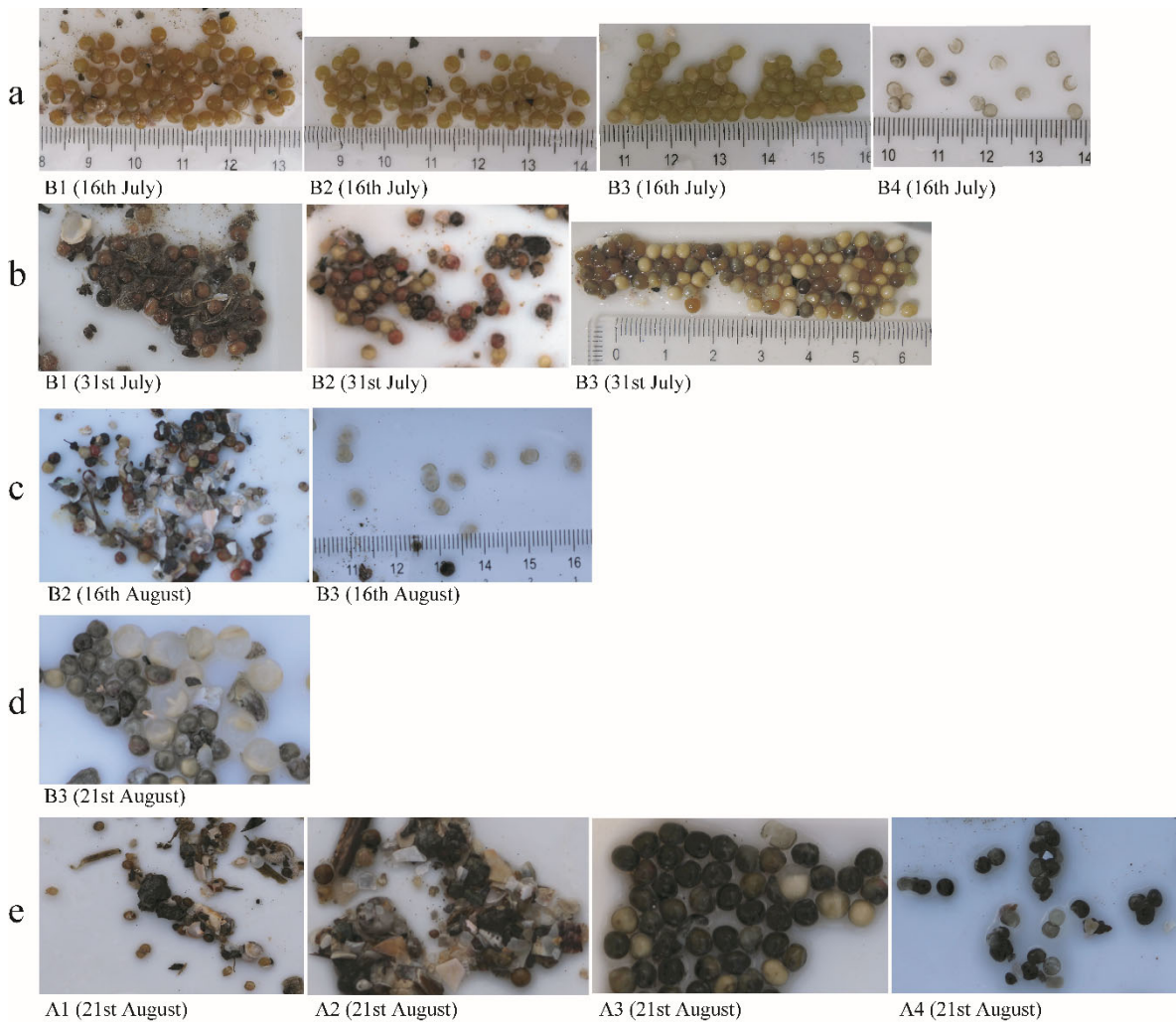
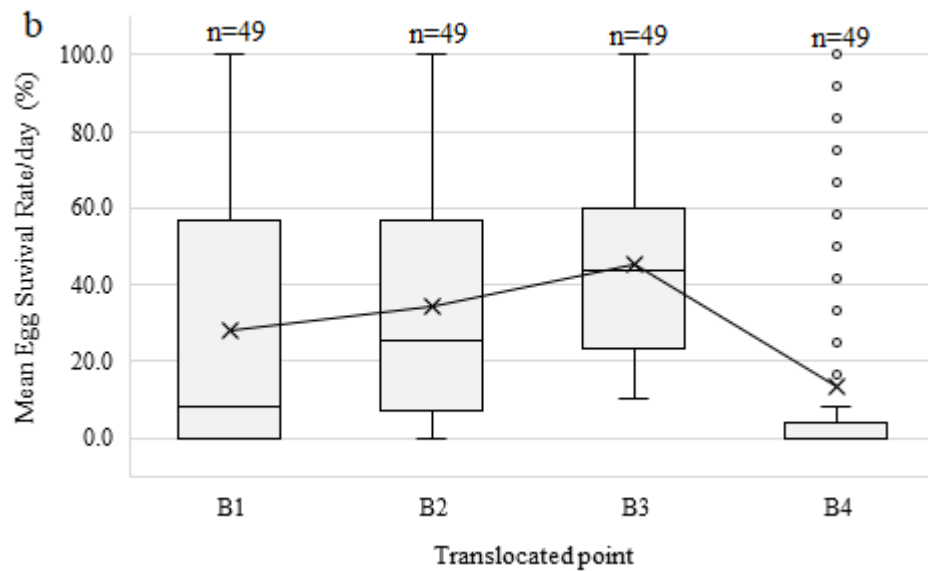
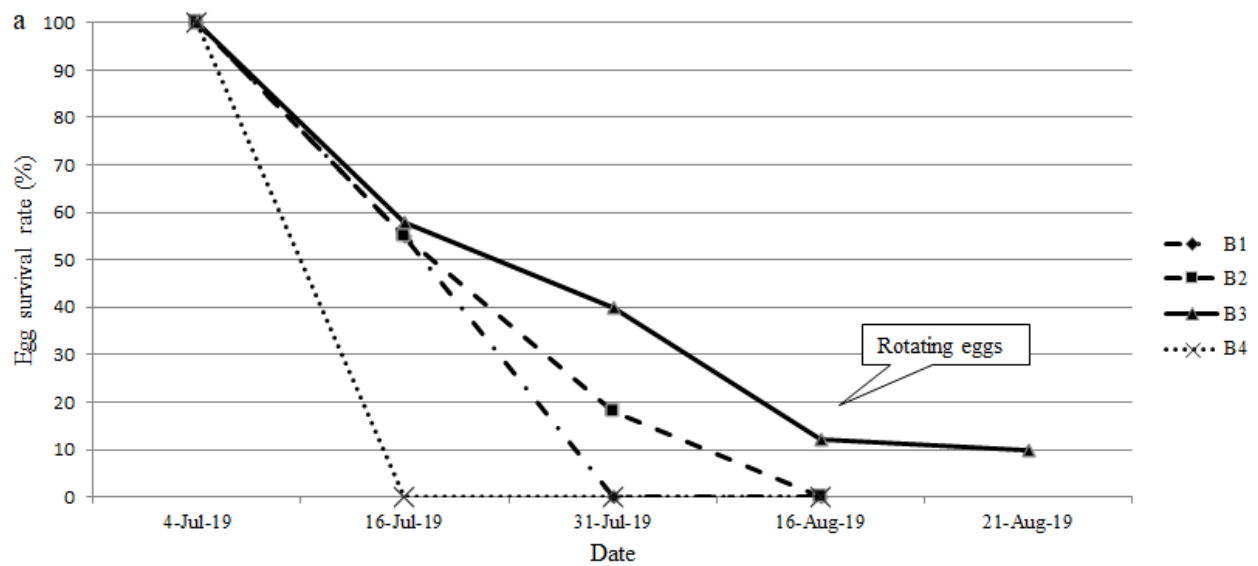


Figure 3 Egg survival status over the study period. a) All eggs were dead in the low intertidal zone at transect B on July 16, 2019. b) On July 31, 2019, no live eggs were found in the high intertidal zone at transect B, and reddish eggs were found in the high and mid intertidal zones. c) On August 16, 2019, all eggs died in the upper mid intertidal zones, but the eggs in the lower mid intertidal zones became rotating eggs at transect B. d) At transect B, in which the eggs were inspected regularly, 10% survived as rotating eggs on August 21, 2019. e) On August 21, 2019, rotating eggs were found only in the mid intertidal zone at transect A, which was not inspected regularly. The survival rate in the mid intertidal zone at transect A was 1%.



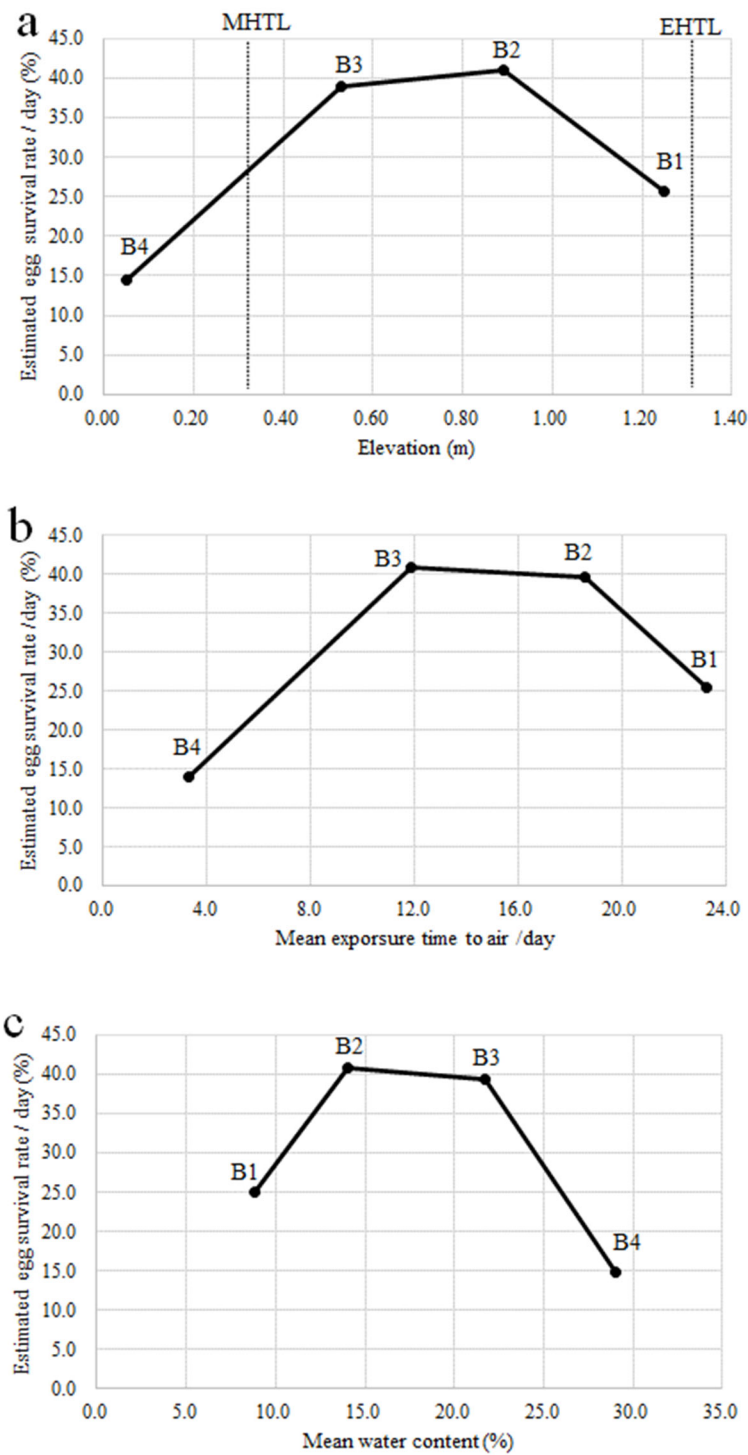


Figure 5 Change in estimated mean egg survival rate per day with significant physical parameters at the translocation site. a) Change in mean egg survival rate per day with elevation, b) Change in mean egg survival rate per day with mean air exposure time per day, c) Change in mean egg survival rate per day with mean water content.