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RESEARCH ARTICLE

Histological observations of the blood cockle, *Tegillarca granosa* (L.), after a mass mortality event in Welu estuary, Thailand

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Abstract. A mass mortality event involving cultured blood cockles (*Tegillarca granosa)* occurred in the Welu estuary in Chanthaburi Province, Thailand, during July and early August of 2013. We collected surviving blood cockles (n = 30) shortly after the mortality event in mid-August and evaluated the physiological conditions of the specimens by performing histological observations of various organs. We found that most specimens of both sexes were at the peak of spawning. Histological observations revealed an absence of food in their digestive ducts and flattened epithelial cells in their midgut glands. The specimens had been experiencing low food availability and nutrient uptake. In addition, rainfall data for Chanthaburi Province revealed that the rainfall in July 2013 was more than twice the average amount received in previous years, and surface salinity data from around the aquaculture site revealed that salinity was < 10 Practical Salinity Unit for longer than a month. Therefore, we concluded that prolonged low-salinity conditions were a major cause of this mass mortality event.

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Introduction

The blood cockle, *Tegillarca granosa* (L.), is a saltwater clam that possesses red-colored bodily fluids owing to the presence of hemoglobin (Broom 1985, Eapen and Patel 1989, Bao et al. 2011, Rattikansukha et al. 2019). The species has been extensively farmed as an aquaculture commodity, especially around estuarine mud flats in Southeast Asian countries; in particular, Malaysia and Thailand are very popular for sowing aquaculture (Pathansali and Song 1958, Broom 1985, Watanabe 2009, Yurimoto et al. 2014a, 2014b, Pahri et al. 2016, Ratchatapattanakul et al. 2017, Chaweepak et al. 2019, Rattikansukha et al. 2019). In Malaysia, domestic production of cockles has flourished on the coast of Selangor since 2007 and a record of approximately 40,000 t was produced in 2010 (Lee et al. 2013, Ramli et al. 2013, Harith et al. 2016, Pahri et al. 2016). However, production has decreased significantly over recent years because of frequent mass mortality events (MMEs) related to water quality deterioration in coastal areas during the aquaculture process (Ramli et al. 2013, 2014, Yurimoto et al.

2014a, Pahri et al. 2016, Shimoda et al. 2016, Teoh et al. 2016). One MME occurred after heavy rainfall in February 2012 and it is suspected that this die-off was related to an associated flood (Yurimoto et al. 2014a). More recently, another MME occurred at an aquaculture site located at the mouth of the Welu River in Chanthaburi Province, Thailand, from July to early August in 2013. Therefore, it is important to identify measures that can be used to improve the survival rate of cultured blood cockles grown in the region. To determine the cause of the die-off, we collected surviving blood cockles shortly after the MME in the Welu estuary (Fig. 1) and conducted histological observations of their organs to evaluate their physiological condition.

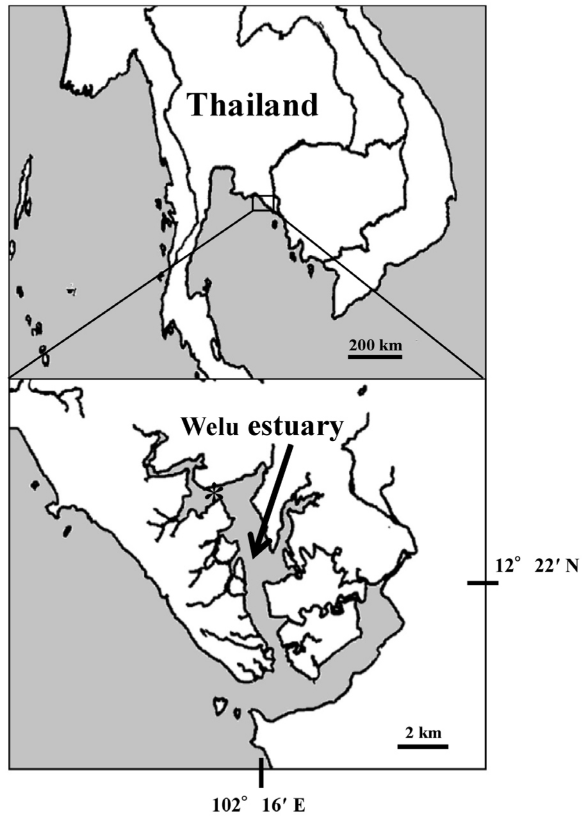

Figure 1. Location of the mass mortality event (*) that occurred in the Welu estuary from July to early-August in 2013.

Materials and methods

A blood cockle MME was reported in the Welu estuary during July and early August of 2013 (Fig. 1), and information regarding the event was obtained from local blood cockle farmers, who estimated a mortality rate of > 50%. Accordingly, we conducted a field survey of the aquaculture site on August 15, 2013, to collect still-surviving blood cockles that had a shell length of 24.5–28.3 mm (n = 30). The shell length of each specimen was measured and shells were opened with an oyster knife before immersion in a fixation solution of 10% formalin in seawater. At a later date, various fixed tissues (including the mantle, gills, kidney, midgut gland, and gonads) were dissected from the specimen and, after ethanol dehydration and Lemosol replacement, were embedded in paraffin. Tissue sections with a thickness of 5 μm were prepared from the paraffin blocks with a microtome, and, finally, the tissue sections were stained with hematoxylin and eosin (H&E) to observe the condition of the various tissues under an optical microscope (Eclipse 80i, Nikon, Japan).

From the 30 blood cockles, the tissue sections suitable for detailed histological observation under an optical microscope amounted to 27, 30, 17, 30, and 30 individual specimens for mantle, gill, kidney, midgut gland, and gonad tissues, respectively. Sections of the mantle, gill, and kidney tissues were examined for the presence or absence of tissue damage. In the digestive duct and midgut gland from the midgut, the presence or absence of food and the epithelial cell condition, respectively, were examined. Gonadal tissue was also examined to determine the gender and stage of sexual maturation, according to an established classification scheme used for blood cockles, such as that used for *Scapharca kagoshimensis* (Tokunaga) and *Anadara* (*Tegillarca*) *granosa* (L.) (Yurimoto et al. 2008, 2014b, 2019).

To determine the environmental conditions at the time of the MME, we obtained rainfall data for Chanthaburi Province (latitude: 12°36’ N/longitude: 102°06’ E; altitude: 2 m), from January to December 2013, obtained from the Meteorological Agency of Thailand. Additionally, year-average rainfall data for the same location, which was the 30-year average from 1981 to 2010, were obtained from the Japan

Table 1
Histological observations of mantle, gill, kidney, digestive duct, and midgut gland from blood cockle, *Tegillarca granosa*, specimens collected from the Welu estuary in mid-August 2013 after a mass mortality event

		Mantle			Gill			Kidney			Digestive duct			Midgut gland			
	n	ns	-*	+*	ns	-*	+*	ns	-*	+*	ns	-**	+**	ns	-***	+***	++
Tegillarca granosa	30	3	27	0	0	22	8	13	12	5	0	23	7	0	4	20	6

n: number of specimens collected, ns: tissue section was not suitable for detailed observation, -*: normal tissues in mantle, gill, and kidney, +*: partial necrosis of mantle, gill, and kidney, -**: empty digestive duct, +**: food in digestive duct, -***: normal epithelial cells in midgut gland, +***: partially flattened epithelial cells in midgut gland, ++: extensively flattened epithelial cells in midgut gland.

Meteorological Agency website (http://ds.data.jma.go.jp/gmd/tcc/tcc/products/climate/). In addition, we obtained rainfall statistical data for the region in July, August, and September from 1982 to 2014 from the Japan Meteorological Agency website. Simple regression analyses were performed on these data to clarify the annual rainfall trend for each month. However, rainfall data in the statistical database were not included for July 1982, 1983, 1997, 1999, 2001, 2003, and 2009; August 1985, 1995, 2003, and 2004; and September 1996 and 2003. In addition, *P*-values of these data were calculated by simple regression analysis using Microsoft Excel 365. Furthermore, surface salinity monitoring data from near the blood cockle aquaculture site in the Welu estuary were obtained from the Chanthaburi Coastal Fisheries Research and Development Center, which collected the data from January to December 2013.

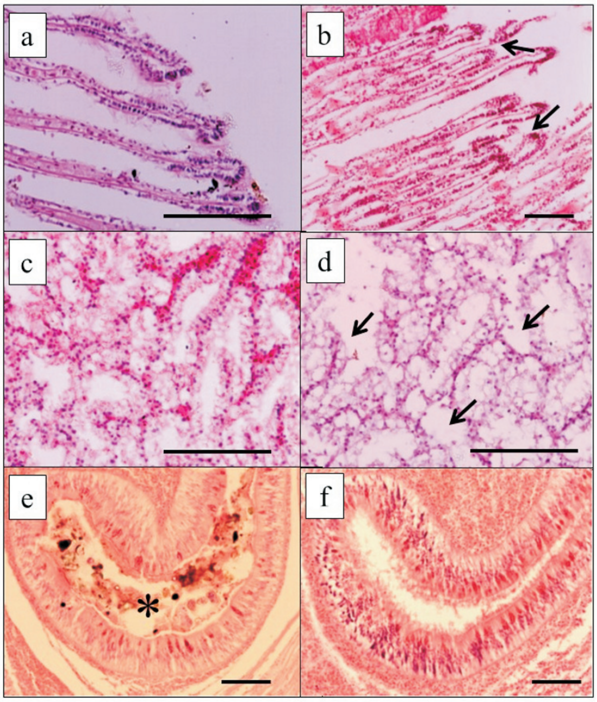

Figure 2. Histological observations of gill, kidney, and digestive duct in blood cockle, *Tegillarca granosa*, specimens collected from the Welu estuary in mid-August 2013 after a mass mortality event. a: normal condition in gill, b: partial necrosis in gill (arrows), c: normal condition in kidney, d: partial necrosis in kidney (arrows), e: food in digestive duct (*), f: empty digestive duct. Scale bars indicate 100 µm.

Results

Histological observations

Samples from some specimens showed partially necrotic gill and kidney tissues, but no damage was observed in the mantle tissue. In damaged gill tissue, necrosis was observed involving the cilia (Fig. 2b), but not in normal gill tissue (Fig. 2a). In damaged kidney tissue, necrosis was observed in renal epithelial cells (Fig. 2d) but not in normal kidney tissues (Fig. 2c). However, the incidence of damage was < 30% in the observed specimens and the extent of this was limited to parts of the organs (Table 1). In contrast, the digestive ducts of some individuals (n = 7) were filled with food (Fig. 2e), but the digestive ducts of most individuals (n = 23) were empty (Fig. 2f). In addition, the conditions of the midgut glands were classified into three categories: I - those with healthy epithelial cells (Fig. 3a), II – partially flattened

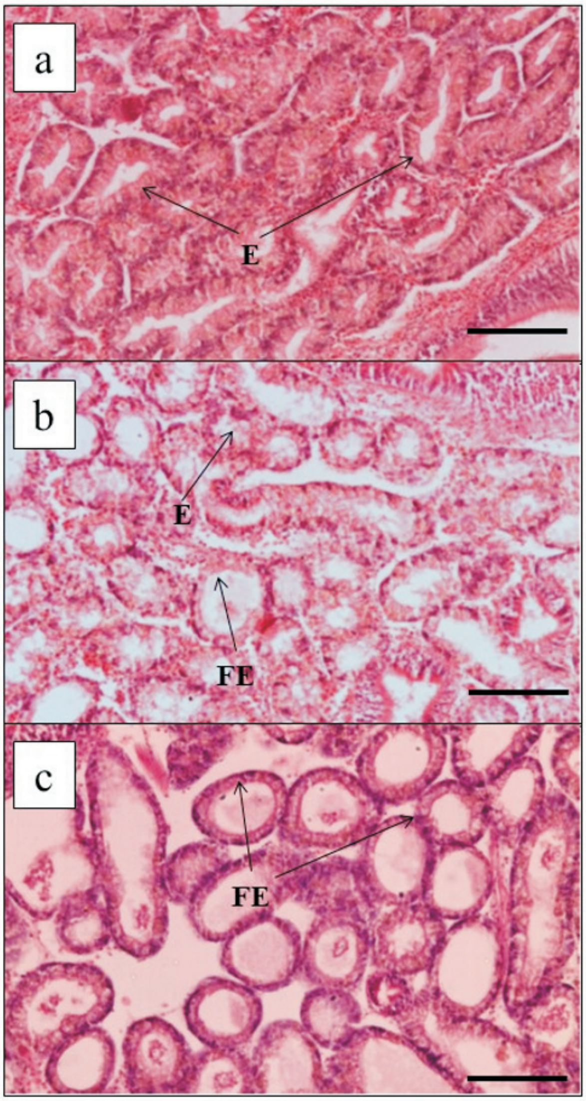

Figure 3. Histological observation of midgut gland in blood cockle, *Tegillarca granosa*, specimens collected from the Welu estuary in mid-August 2013 after a mass mortality event. a: Good condition of epithelial cells, b: Partial flattening of epithelial cells, c: Extensively flattening of epithelial cells. E: normal epithelial cells, FE: flattening epithelial cells. Scale bars indicate 100 µm.

epithelial cells (Fig. 3b), and III – extensively flattened epithelial cells (Fig. 3c), according to the classification proposed by Yurimoto et al. (2014a). Of the 30 specimens examined, epithelial cells were healthy in only four individuals, partially flattened in 20 individuals, and extensively flattened in six individuals (Table 1). Overall, the percentage of individuals with either partially or extensively flattened epithelial cells was > 80%.

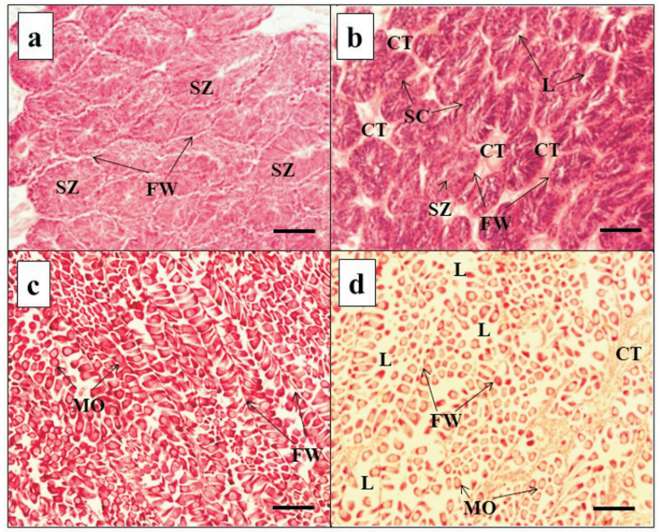

Figure 4. Histological observation of gonad in blood cockle, *Tegillarca granosa*, specimens collected from the Welu estuary in mid-August 2013 after a mass mortality event. a: mature stage in testis, b: partial spawning stage in testis, c: mature stage in ovary, d: partial spawning stage in ovary. CT: connective tissue, FW: follicle wall, L: lumen, MO: mature oocyte, SC: spermatocyte, SZ: spermatozoa. Scale bars indicate 200 µm. Sections in figures b and d were observed some lumens in follicles and some connective tissues in gonads by partial spawning.

Gonad condition

According to our gonad observations, all males (n = 14) and females (n = 12) were categorized as mature (males Fig. 4a, females Fig. 4c) or partial spawning (males Fig. 4b, females Fig. 4d). More specifically, in the mature male stage, spermatozoa filled the lumens of follicles in the gonad tissue and sperm were lined up radially toward the center of the lumen (Fig. 4a). In the male partial spawning stage, spermatozoa were still present in the lumens, but some of these were already released, some follicles were collapsed, and connective tissue was conspicuous (Fig. 4b). In the mature female stage, mature oocytes filled the follicles (Fig. 4c) and the follicle walls were thin. In addition, in the female partial spawning stage, mature oocytes were still present in the lumen but some of them were already released and space in the lumen was conspicuous, some follicles had already collapsed, and connective tissue was evident (Fig. 4d). In addition, we also detected a number of hermaphrodite individuals

Table 2
Histological gonad observation of blood cockle, *Tegillarca granosa*, specimens collected from the Welu estuary in mid-August 2013 after a mass mortality event

	n	Male		Female		Hermaphrodite
		Mature	Partial Spawning	Mature	Partial Spawning	
Tegillarca granosa	30	1	13	1	11	4

n: number of samples.

with various germ cells mixed (n = 4) (Fig. 5), which accounted for approximately 13% of the specimens. Additionally, the observed sex ratio (male: female) of specimens from the Welu estuary was 0.54:0.46 (n = 26). Our observations of gonad maturity and stage (i.e. mature) clearly demonstrated that the cockles were well into the peak of the spawning season in mid-August (Table 2), which correlates with the rainy season in the region.

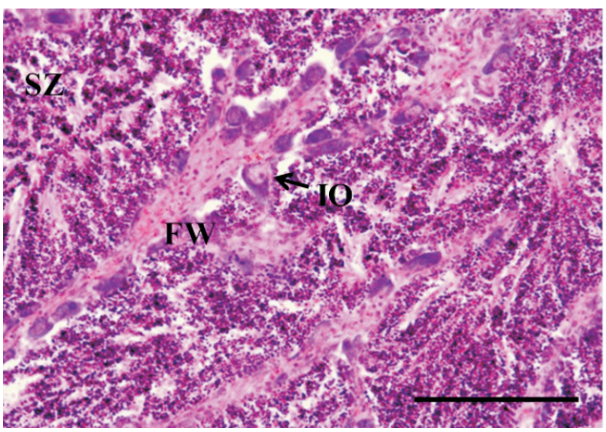

Figure 5. Hermaphrodite gonad in blood cockle, *Tegillarca granosa* (Linnaeus,1758), specimens collected from the Welu estuary in mid-August 2013 after a mass mortality event. FW: follicle wall, IO: immature oocyte, SZ: spermatozoa. Scale bar indicates 200 μm.

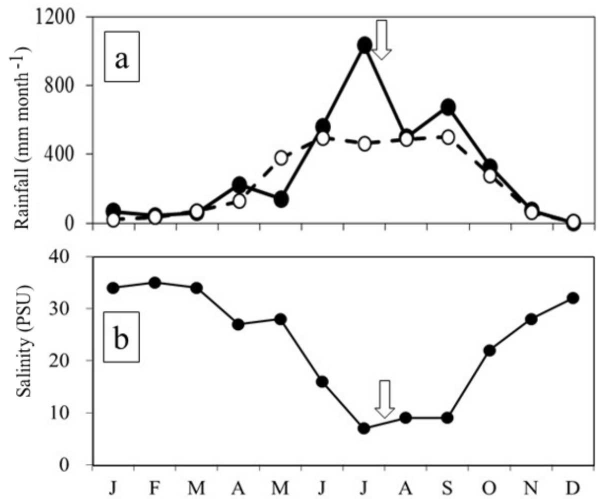

Figure 6. Annual change in rainfall in Chanthaburi Province (a) and salinity near the blood aquaculture site in the Welu estuary (b) from January to December 2013. Arrows indicate the occurrence of the mass mortality event from July to mid-August. The rainfall data for 2013 (a; ●) was obtained from the metrological agency of Thailand and the 30-years average of rainfall amount data (a; ○) was obtained from an internet homepage of the metrological agency of Japan.

Environment

Local rainfall data show that the MME that occurred from July to August was correlated with the peak rainy season (Fig. 6a) and that the monthly rainfall in July 2013 (approximately 1,000 mm) was approximately twice the normal value. In addition, surface salinity data revealed that salinity near the aquaculture site in the Welu estuary during July–August 2013 was lower than 10 Practical Salinity Unit (PSU) (Fig. 6b). For comparison, the surface salinity was > 28 PSU during the 2013 dry seasons (January–May and November–December), < 22 PSU during the 2013 rainy season (June–October), and < 10 PSU during the peak rainy season (July–September) (Fig. 6b). Additionally, annual changes in precipitation were analyzed for July, August, and September from 1982 to 2014 (Fig. 7). The changes in July tended to increase significantly year by year (P < 0.01; Fig. 7a). In contrast, changes in August tended to significantly decrease year by year (P < 0.01; Fig. 7b) and no significant trends were observed for September (P > 0.05; Fig. 7c).

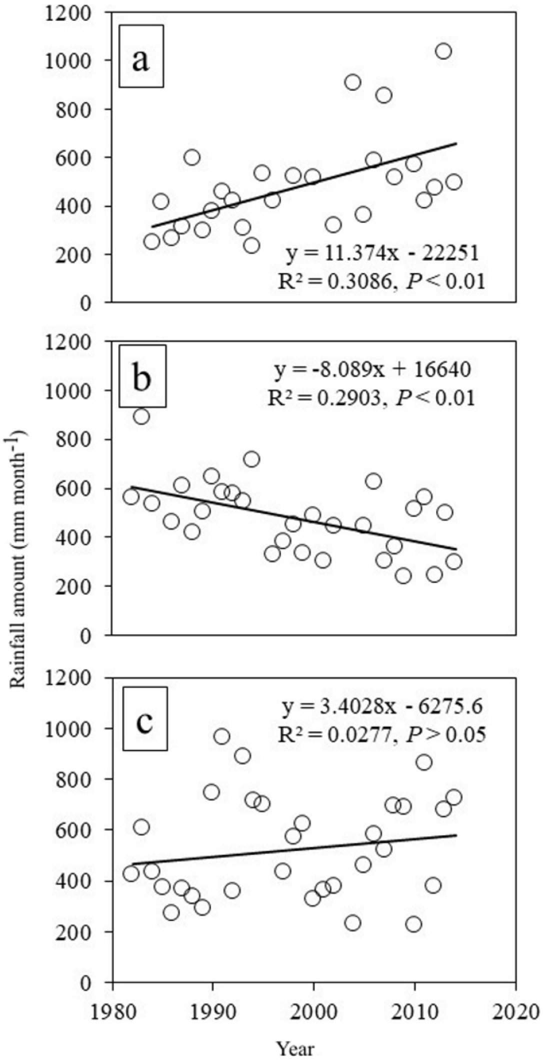

Figure 7. Changes in rainfall in Chanthaburi Province on each month of July, August, and September from 1982 to 2014. a: the changes of July (n = 26), no data in 1982, 1983, 1997, 1999, 2001, 2003 and 2009, b: the changes of August (n = 29), no data in 1985, 1995, 2003 and 2004, c the changes of September (n = 31), no data in1996 and 2003. *P*-values were calculated by simple regression analysis using Microsoft Excel 365. The data was obtained from an internet homepage of the metrological agency of Japan.

Discussion

Histological observations

Some specimens showed partially necrotic gill and kidney tissues. However, the observed incidence of damage involved < 30% of the specimens (Table 1) and the extent of damage was limited to parts of the organs. Therefore, we estimated that such damage had little effect on individual survival. When we determined the feeding status of the specimens, we looked for phytoplankton, such as diatoms, which typically fill the digestive duct of blood cockles that have access to enough food (Yurimoto et al. 2014a, 2014c). We confirmed that the digestive ducts of some individuals (n = 7) were filled with food (Fig. 2e), but we also observed that the digestive ducts of most individuals (n = 23) were empty (Fig. 2f). Therefore, we determined that food availability during the MME was insufficient (Table 1). In addition, we examined the condition of the midgut glands and classified our observations into three categories, according to the classification proposed by Yurimoto et al. (2014a). Of the 30 specimens examined, we found that epithelial cells were healthy in only four individuals, partially flattened in 20 individuals, and extensively flattened in six individuals (Table 1). Overall, the percentage of individuals with either partially or extensively flattened epithelial cells was > 80%. This result suggested that the blood cockles were experiencing low food availability and low nutrient absorption during the MME.

Gonad condition

We categorized all male (n = 14) and female (n = 12) cockles as mature (males Fig. 4a, females Fig. 4c) or spawning (males Fig. 4b, females Fig. 4d). In addition, we detected some hermaphrodite individuals (n = 4) (Fig. 5), which accounted for approximately 13% of the specimens. The percentage of hermaphrodites was much higher than that previously observed (< 1%) at other sites in Malaysia (Yurimoto et al. 2014b, 2014d). Thus, we suggest that the environmental conditions of the Welu estuary may have some unknown factor influencing hermaphroditism occurring in blood cockles. In contrast, the observed sex ratio (male:female) of specimens from the estuary was 0.54:0.46 (n = 26), which is not significantly different from the ratios observed at other sites in

Malaysia. Yurimoto et al. (2014b, 2014d) reported that the sex ratio of blood cockles in the Matang mangrove estuary and in Selangor is 0.57:0.43 (n = 150) and 0.47:0.53 (n = 92), respectively. Our observations of gonad maturity and stage (i.e., spawning) clearly demonstrated that the cockles were well into the peak of the spawning season in mid-August (Table 2), which correlates with peak rainfall in the region. Thus, these results showed that a similar situation exists with blood cockles in the Welu estuary and those at two other sites in Malaysia, in that their peak spawning occurs in the rainy season (Yurimoto et al. 2014b, 2014d).

Environment

The rainfall showed that the MME that occurred from July to early August was correlated with the peak rainy season (Fig. 6a) and that the monthly rainfall in July 2013 (approximately 1,000 mm) was approximately twice the average amount in previous years. In addition, surface salinity near the aquaculture site in the Welu estuary during the peak rainy season was < 10 PSU (Fig. 6b). The blood cockles were cultivated using the sowing method on the seabed. However, the site is a shallow area approximately 2 m in depth. Therefore, salinity can also be at the same low level even on the seabed. Thus, we considered the possibility that excessive rainfall during the peak rainy season could have caused low-salinity conditions, which began in July but could have persisted for up to three months. Davenport and Wong (1986) reported the effects of low salinity (16 PSU) and high water temperature (28–30°C) on blood cockles, which responded by hardly closing their shells upon exposure, and this response only continued for three–five days. Davenport and Wong (1986) also examined the survival rate of blood cockles at even lower salinities (6.4 and 9.6 PSU), which are closer to the values experienced by the cockles in the Welu estuary. In their study, there was no difference in terms of survival in response to the two different salinities and the observed rates of mortality were 7% at 72 h, 87% at 120 h, and 100% at 144 h. Thus, if blood cockles are exposed to salinity conditions of < 10 PSU for > six days, catastrophic physiological effects are likely to result. In this MME, salinity conditions < 10 PSU were observed for the Welu estuary from July to mid-August (Fig. 6b) and blood cockles in shallow water were likely exposed to such conditions for more than a month. In addition, Moon and Shin (2010) conducted a similar low-salinity tolerance experiment involving blood cockles. They found that adult individuals (shell length 36.0 ± 1.5 mm) had greater low-salinity tolerance than juvenile individuals (shell length 19.5 ± 1.3 mm) and high temperature (25°C) conditions reduced this low-salinity tolerance more than low temperature (10°C) conditions. These authors reported that the 11-day LS_{50} (median lethal salinity) at 25°C was 16.8 PSU (reliability limit 12.9-21.2 PSU) and 22.4 PSU (reliability limit 20.5-24.7 PSU) for adult and juvenile individuals, respectively. Furthermore, the cockles were in peak spawning season at that time (Table 2), so it is highly possible that mature adults were less resistant to environmental stresses as a result of spawning stress. In addition, results of digestive duct observations suggested that the blood cockles were experiencing low food availability at that time and epithelial cells in midgut glands were flattened, indicating low nutrient absorption (Fig. 3, Table 1). Thus, it is possible that the absence of recent feeding was a result of: (1) low food availability, (2) the cockles suspending feeding activity to close their shells in response to low-salinity conditions, and/or (3) abnormal feeding behavior caused by spawning and/or low-salinity conditions. As such, we concluded that a combination of these factors contributed to the MME.

Rainfall amount

We considered whether the high amount of rainfall in July 2013 occurred by chance. Therefore, to understand inter-annual rainfall trends in Chanthaburi Province, we focused on statistical precipitation data for the peak rainy season (July–September) in the region and analysed the annual changes in each month from 1982 to 2014 (Fig. 7). We found that changes in

July tended to increase each year (P < 0.01), changes in August tended to decrease each year (P < 0.01), and no significant trends were observed for September (P > 0.05) during this period. We also found that the peak of annual rainfall occurred in August in previous years, but now occurs mainly in July. As a result of such climactic changes, it is likely that low-salinity conditions would be prolonged and that additional MMEs will occur in the future. Therefore, to stabilize the blood cockle industry in the region, it will be necessary for the aquaculture industry to address low-salinity measures during the peak rainy season, mainly from July to August.

In Thailand, the inflow of freshwater due to heavy rain has had an adverse effect on the survival of farmed blood cockles. This was observed not only in Chanthaburi Province, which was surveyed as this research site, but also in Bandon Bay in Surat Thani Province and Bang-tabun Bay in Phetchaburi Province (Ratchatapattanakul et al. 2017, Srisomwong et al. 2018). Srisomwong et al. (2018) surveyed suitable sites for blood cockle aquaculture, around the estuarine area of Bangtabun Bay in Phetchaburi Province, and found that there was a significant correlation between salinity and chlorophyll *a* concentrations in blood cockle growth. They clarified that the highest growth rate of blood cockles was at the highest levels of both salinity and chlorophyll *a* concentration. This means that aquaculture grounds with abundant phytoplankton and less of the influence of freshwater are suitable for blood cockle aquaculture, and it is important to select good culture grounds based on these indicators. In addition, the zoning of coastal areas is known as an effective method of fisheries management and the method has already been studied on fisheries grounds, including with regards to the blood cockle aquaculture in Bandon Bay (Jarernpornnipat et al. 2003). Therefore, it is important to create a suitable aquaculture ground map for blood cockles based on local salinity levels and the distribution of chlorophyll *a* concentration in the Welu estuary. Moreover, it is expected that differences in distribution maps between the rainy and dry seasons will provide information for more effective aquaculture management of blood cockles.

Conclusions

Our histological observations, which were conducted to determine the cause of a blood cockle MME that occurred at an aquaculture site in Welu estuary, Thailand, revealed that the animals were at the peak of their spawning period at that time. In addition, the cockles were experiencing low nutrient absorption and poor food availability, as indicated by flattened epithelial cells in the midgut gland and the absence of food in the digestive ducts. Moreover, the rainfall in Chanthaburi Province during July 2013 was approximately two times greater than the normal value and surface salinity around the aquaculture site was < 10 PSU for longer than a month. Thus, it was determined that prolonged low-salinity conditions were the main cause of the blood cockle MME. Furthermore, because weather statistical data show that July rainfall in the province is increasing yearly, it is likely that similar MMEs will affect blood cockle aquaculture in the future. Therefore, to stabilize the blood cockle industry in the region, it will be necessary for the aquaculture industry to engage measures to tackle low salinity during the peak rainy season, mainly from July to August.

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Author contributions. T.Y. summarized the histological data, analyzed weather data, and drafted the manuscript. T.C. and K.S. collected the cockle samples, provided environmental data, and organized this study. K.M. suggested on the study design and discussed on the results with T.Y. and revised a draft version of the manuscript. All authors read and approved the final manuscript.

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