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Short Article

Combined effects of salinity and turbidity on the gaping behavior, clearance rate, survival rate and glycogen content of blood cockle *Tegillarca granosa* (Linnaeus, 1758)

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Abstract: Blood cockle *Tegillarca granosa*, a popular commercial bivalve species in south-east Asia, China and South Korea usually inhabits shallow mudflats in the intertidal zone. Due to the recent unpredictable climate patterns and increase of prolonged heavy rain conditions, bivalves inhabiting mudflats would be exposed to extreme hyposaline and turbid waters which would have an effect on their physiological conditions. The possible influences of such extreme conditions were tested in the laboratory using a 2 x 4 experimental design, where 24 specimens of *T. granosa* were exposed to different salinities (10, 30 psu) and turbidities (0, 100, 200, 300 mg/l) with 3 replicates over a 2-week period. Results indicated that *T. granosa* experienced stress to some extent but was quite tolerable against hyposaline and turbid conditions within the 2-week period. That is, specimens exposed to lower salinity and higher turbidities recorded decrease in clearance rates with delay in the initiation of gaping activity, which were considered as light signs of stress. Despite signs of stress, exposed individuals recorded minimal effect on glycogen content and a high survival rate was obtained. Overall, *T. granosa* is likely to tolerate hyposaline and turbid water sufficiently during the limited 2-week experimental period, showing only a slight stress response.

Key words: *Tegillarca granosa*, salinity, turbidity, clearance rate, glycogen content.

Introduction

Blood cockle *Tegillarca granosa* (Haigai: in Japanese) is known to burrow itself into muddy and sandy sediments near mangrove forests or intertidal zones (Srisunont et al., 2020). In Japan, *T. granosa* is not a commercially utilized species but is consumed locally e.g., in Kashima city, Saga Prefecture (Noma, 2021). Due to its popularity as a shellfish fishery resource and cultured species in southeast Asia, China, and South Korea, we believe *T. granosa* could potentially be an important fishery resource and aquaculture species in Japan.

T. granosa is thought to have been a thriving coastal bivalve species in the past as previous records show their widely distributions across Japan. Specifically, according to fossil records, in Japan about 6000 years ago, *T.*

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granosa was once widely distributed from Kagoshima Bay (southern limit) to Hakodate Bay (northern limit), and its living specimens were recorded in many embayments in central and western Japan until 100 years ago (Sato, 2000).

In recent years, however, due to several causes, mainly anthropogenic coastal developments of estuarine mudflats, bivalves including *T. granosa* have experienced habitat loss (Sato, 2010). The habitats of *T. granosa* have narrowed down to Seto Inland Sea, Ariake Bay and Shiranui Sea (Sato, 2010); see Figure 9 of (Sato, 2010) for specifics on the chronological change of distribution of *T. granosa* in Japan. As an example of coastal development as a cause of habitat loss, in the late 1900s, in one of Japan's most controversial coastal projects, Sato (2001) stated that four months after the Isahaya bay enclosure, mass mortality of *T. granosa* of about 73 individuals/m^2 occurred. There seem to be no recent abundance and distribution data of *T. granosa* in the Kyushu area. The last was in 2008 where studies showed that *T. granosa* distribution had narrowed down to the innermost part of the Sea of Ariake, and additional small habitats in Shiranui and Imari Bay in western Kyushu (Yamashita, 2004; Miyazaki, 2008). At present, *T. granosa* inhabits the mudflats of Tara machi, Saga Prefecture (32°57'30"N, 130°12'52"E), but its abundance is yet to be known. Even with the current habitats present in the inner part of the Sea of Ariake, they seem to be exposed to recent environmental stresses brought about by global warming and climate change.

Furthermore, unpredictable weather patterns caused by global warming and climate change would heighten the influence of environmental stresses on bivalve habitats. According to Japan Meteorological Agency (2023), over the years, due to the effects of global warming on precipitation during the summer months, the long-term frequency of extreme heavy rainfall in Japan shows an increasing tendency. Hirota et al. (2021) reported a similar heavy rain event that occurred in 2020 in the coast of Saga prefecture facing the sea of Ariake which affected environmental factors such as salinity, sea temperature, dissolved oxygen, etc. In a recent article by (Tokunaga et al., 2024), it was reported that during the heavy rainfall in July 2020, Chikugo River reached a daily average flow rate up to 5000 m^3/s causing salinity levels to drop below 10 psu and occurrence of hypoxic water masses in the inner part of the Sea of Ariake. Such events would cause frequent occurrence of low salinity and high turbidity conditions in *T. granosa* habitats in Japan.

The combined effect of salinity and turbidity conditions due to prolonged heavy rain on the Japanese population of *T. granosa* has not been studied. However, there are several reports of studies carried out in southeast Asia. For example, in Kongkong Laut estuaries, Malaysia, a 4-month growth performance monitoring study of *T. granosa* was performed and authors concluded that salinity level (26.92 ± 4.79 ppt) recorded the highest cockle growth increment and salinity around 17.65 ± 5.73 ppt recorded the lowest growth (Joni et al., 2019). Joni et al. (2019) findings further indicated that prolonged high turbidity levels led to higher mortality rates. In another study carried out in Welu estuary, Chanthaburi Province, Thailand, rainfall in July 2013 was more than twice the average usual amount in previous years, causing salinities around *T. granosa* aquaculture sites to drop below 10 psu for more than a month, resulting in mass mortality (Yurimoto et al., 2021).

Therefore, as mentioned earlier, with the recent unpredictable weather patterns and increase in prolonged heavy rainfall in Japan, there is a need to investigate how these extreme conditions of low salinity and high turbidity influence the physiological behavior of *T. granosa* in Japan. Reasons as to why these two factors were chosen is because both are likely to fluctuate the most during heavy rainfall. Furthermore, instead of a single factor design, the combination of multiple stressors is important as they may induce both additive, synergistic, or antagonistic responses (Kim et al., 2013, 2018; Przeslawski et al., 2015).

Materials and methods

Sample Collection & Experimental Design

T. granosa is known to be distributed along the coasts of east and south-east Asian countries (Ni et al., 2012; Lai et al., 2020). Blood cockles *T. granosa* were obtained from a local fisherman near Kashima, Saga Prefecture and were transported to the Nagasaki Fisheries Technology Institute in Nagasaki Prefecture where experiments were carried out. The blood cockles were acclimatized in a 300 l tank with free-flowing seawater of salinity 30-34 psu and fed on a daily basis with *Chaetoceros gracilis* in abundance for about a week. Feeding was stopped 24 hours before the experimental period began.

A 2 x 4 experimental design with three replicates were set up for each treatment. Each replicate contained a huge tank as a water bath (water temperature 25 ± 1°C) and inside each water bath, eight 30 l tanks were immersed halfway in to create uniformity in water temperature across all tanks. Each 30 l tank was filled with 25 l of seawater with different levels of salinity (10, 30 psu) and turbidity (0, 100, 200, 300 mg/l) and stocked with 1 individual/tank. Salinity and turbidity levels were adjusted by adding freshwater and bentonite, respectively. Turbidity levels were maintained by fixing an underwater fan-like pump in each tank to induce mixing. Experimental organisms were fed with *C. gracilis* at a density of 30,000 cells/ml once a day and water exchange was carried out on a daily basis throughout the experimental period.

Measurements

Clearance rate (CR) measurements were carried out on Day 1, 7 and 13 for all treatments. As mentioned earlier, each 30 l tank containing 25 l of seawater stocked with one *T. granosa* specimen was fed 30,000 cells/ml of *C. gracilis*. Using a fluorometer, Aquafluor (Turner Designs Co.), readings of fluorescence (0^{th}, 1^{st}, 2^{nd}, 3^{rd}) with two-hour intervals spread over a 6-hour period were taken. From the aqua fluor data obtained, CR was calculated using an equation by (Fox et al., 1937) of the form:

$$m = M\frac{b-a}{n\,.\,t}$$

where '*m*' is the CR (RFU ml/min), '*M*' is the volume of seawater (ml), '*b*' is the logarithmic concentration of the original suspension ($\log_e conc_{(o)}$), '*a*' is the logarithmic concentration of the suspension ($\log_e conc_{(t)}$) after time '*t*' and '*n*' is the number of individuals per tank. As for the CR control, the above steps were repeated for tanks with *T. granosa* individuals absent (blank tanks) and CR was calculated. CR values with *T. granosa* present was then subtracted with CR values from blank tanks to obtain real CR values.

Gaping behavior (valves open or shut) of each individual was observed and recorded on a daily basis, before water exchange was carried out in the morning. For individuals that showed signs of valve opening, the number zero '0' was recorded, and the number one '1' for individuals that had their valves closed. For this experiment, since the test organism had no siphon, the assumption of valve open (filtering) and valve closed (not filtering) was used as good and bad conditions, respectively. Data collected on each day were averaged for each treatment and then plotted against each other as valve open: close ratio.

Survival rate was also recorded throughout the experimental period. The experiment was carried out for a period of 2-weeks. After the experimental period, all specimens were sacrificed, and the foot of each specimen was removed and stored in a freezer at −30°C.

Glycogen content of each individual's foot was measured using the Anthrone method (Kamada and Hamada, 1985). Bivalves stores most of its excessive energy produced in the form of glycogen and this stored energy could be influenced by factors such as growth, sexual maturation, food availability and other surrounding environmental factors (Uzaki et al., 2003, Yurimoto, 2015, Marmita et al., 2022). Therefore, as an indicator of change in physiological state exposed to low salinity and high turbidity over a period of time, glycogen content was measured in this study.

Statistical Analysis

Multiple linear regression (MLR) was performed to test whether salinity, turbidity, or their interaction affect the clearance rate (Day 1, 7, and 13) and glycogen content of foot of *T. granosa* for Day 13 significantly ($p < 0.05$). This MLR was done with the function "lm" of R ver. 4. 3. 2 (R Core Team, 2023).

Results

Clearance Rate (CR)

On Day 7 & 13 significant effects of salinity, turbidity, and their interaction ($p < 0.05$) were recorded. As for overall trends, it was noticed that for individuals exposed to 30 psu, CR decreased as turbidity levels increased throughout Day 1, 7 and 13 measurements (Fig. 1). For individuals exposed to 10 psu and 100 to 300 mg/l, CR slightly increased from Day 1 to Day 13, and this was related to the fact that valve opening started from Day 7 to Day 9 which then initiated filtration, hence the slight increase in CR on Day 13.

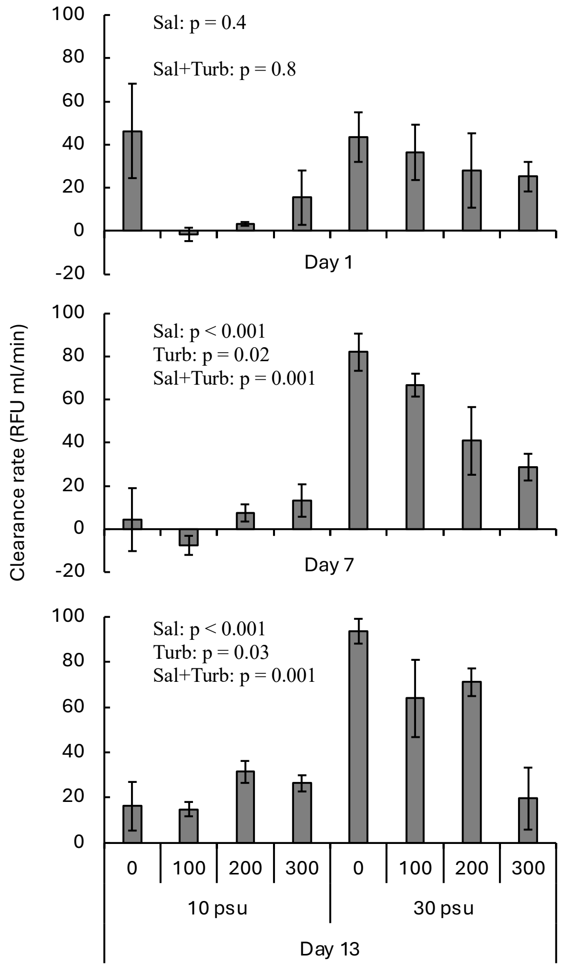

Fig. 1 Clearance rate of *T.granosa* individuals on day 1, 7 & 13 exposed to salinity levels (10, 30 psu) and turbidity levels (0, 100, 200, 300 mg/l) over a 2-week period. Significant effects were recorded for factors that showed values of $p < 0.05$. Vertical bars indicate standard error. Sal: salinity; Turb: turbidity; Sal+Turb: salinity & turbidity interaction.

Gaping Activity

Gaping behavior data showed that for individuals exposed to 30 psu, irrespective of turbidity, had their valves open from the start till the end of the experiment (Fig. 2). On the other hand, individuals exposed to 10 psu, 0 mg/l recorded valve opening from Day 7 at the earliest and as turbidity levels increased from 100-300 mg/l, time taken for valves to show signs of opening delayed by a few days.

Survival Rate

Survival rate for all treatments throughout this study was 100%, except for the combination of 10 psu and 0 mg/l which recorded one dead individual on Day 9 resulting in a 67% survival rate.

Glycogen content

Glycogen content of foot measured after 2-weeks of exposure to low salinity and high turbidity levels ranged from 9.35-12.85 mg/g of wet tissue on average (Fig. 3). There were no significant main or interaction effect of salinity and turbidity on the glycogen content of *T. granosa* foot ($p > 0.05$).

Discussion

Results indicated that the Japanese population of *T. granosa* was able to withstand a 2-week exposure to heavy rain induced extreme conditions of low salinity and high turbidity. Survival rate was reflective of this, as throughout the span of the experimental period, only one

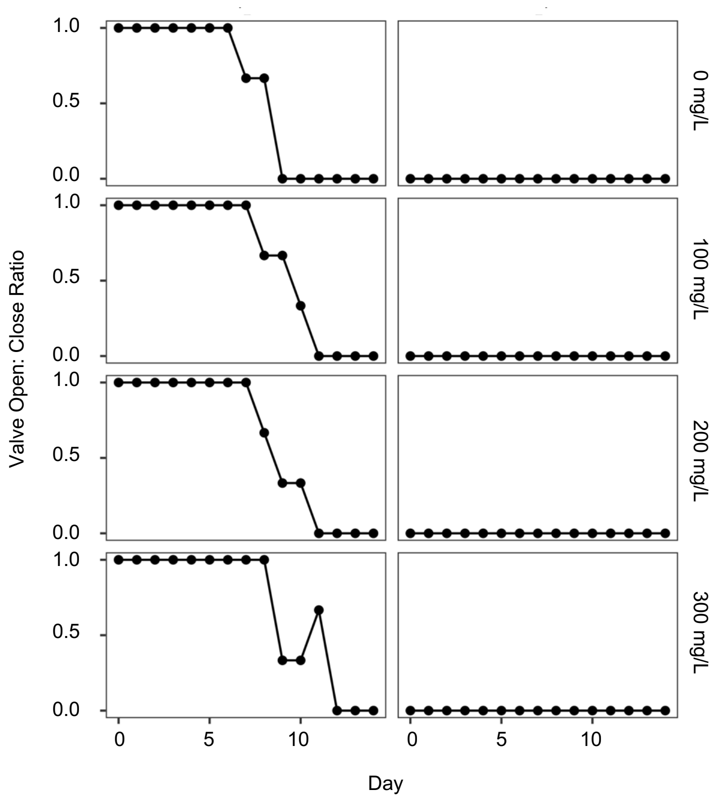

Fig. 2 Gaping activity of *T.granosa* individuals exposed to salinity levels (10, 30 psu) and turbidity levels (0, 100, 200, 300 mg/l) throughout the 2-week experimental period. On the veritcal axis, zero (0) represents valve open and one (1) represents valve close.

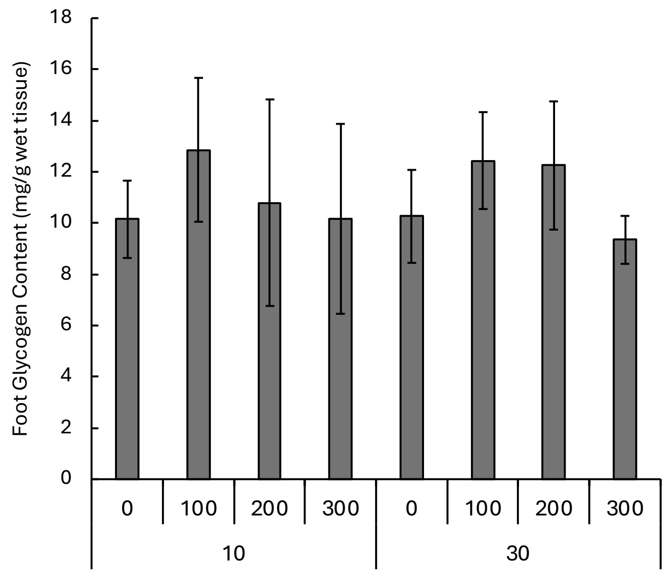

Fig. 3 Foot glycogen content of *T.granosa* individuals exposed to salinity levels (10, 30 psu) and turbidity levels (0, 100, 200, 300 mg/l) after 2-week exposure. Vertical bars indicate standard error.

mortality was recorded. Slight increase in CR after a few days of exposure paired with initiation of gaping activity and appears to indicate adaptation to the low salinity and high turbidity environment. In addition, as shown in Figure 3, glycogen content of *T. granosa* foot showed no significant differences across all treatments indicating minimal influence of the extreme conditions on *T. granosa* energy reserves. Exposed individuals had their valves shut through the first half of the experiment and it was assumed that *T. granosa* would use its energy reserves to cater for the lack of food consumption, however, this was not the case. Andrade et al. (2017) stated that in mussels, when valves are shut due to unfavorable surrounding conditions, they can no longer filter feed and take in oxygenated water, so the total metabolic output decreases and the ability to excrete waste is hindered. This halt in gaping activity maybe correlated with a reduction in metabolic rates of mussels (Andrade et al., 2017), so when exposed to a period of prolonged stress, bivalves may readjust their metabolic profiles and save energy through these mechanisms (Anestis et al., 2007, Riisgård and Larsen, 2015). This might have been the reason for minimum influence on glycogen content in the current study.

On the other hand, during the 2-week experimental period, results also indicated that *T. granosa* individuals experienced some forms of stress due to exposure to hyposaline and turbid waters. Specifically, signs of stress were seen in the delay in the initiation of gaping activity and the low or decrease in CR. The following paragraphs outline the stresses and possible signs of adaptation that salinity and turbidity had on the physiological behavior of *T. granosa*.

Firstly, we will discuss the signs of stress and adaptation of salinity on exposed individuals. As clearly shown in Figure 1 and 2, as salinity levels dropped from 30 psu to 10 psu, CR plunged, and gaping activity ceased. According to previous studies, sudden changes in the external environment, in this case, salinity, has been confirmed to have effects on the cell volume regulation of bivalves, hence, the closure of valves at the start of exposure (Pierce, 1971; Shumway et al., 1977; Shumway and Youngson, 1979). For blood cockle *Anadara granosa* (now known as *Tegillarca granosa*), Davenport and Wong (1986) mentioned that they were osmoconformers that respond to low salinity levels by tight and effective closure of their valves triggered below 19 psu. Valve closure often leads to a halt in filtration, resulting in a drop in CR which was the case in the current study, and such cessation of valve closure and decrease in CR at low salinities have been reported for many other bivalves (McFarland et al., 2013; Kurihara, 2017; Casas et al., 2018). On the other hand, for 10 psu individuals, there was a slight increase in CR from Day 1 to Day 13 (Fig. 1) and this might have been due to the initiation of gaping activity data (Fig. 2) from Day 7 onwards, which showed signs of adaptation, and recommencement of filtration activity. Bartberger and Pierce (1976) stated that during the acclimation of mussels (*Modiolus demissus*) to low salinities, free amino acids are released intact from the cells into the hemolymph and are then subsequently degraded elsewhere, probably by specific body tissues. Davenport and Wong (1986) stated that this behavior of valve closure provides protection (from unfavorable surrounding environmental conditions) for relatively short periods of time, especially for cockles that are vulnerable to low environmental salinities which persists beyond about 3 days. However, in the current study, *T. granosa* closed its valves to protect itself from unfavorable low salinity conditions up to 7 days which was way longer than the cockles used in Davenport and Wong's study. Valve closure periods would vary depending on each individual's tolerance ability and also the type of experimental set up or conditions they are exposed to.

Secondly, indication of turbidity stress on CR and gaping activity were distinct in the current study with barely any sign of adaptation. With regards to turbidity influence on CR, throughout the experimental period, individuals exposed to 30 psu showed decrease in CR as turbidity levels increased. As turbidity levels increase, especially the particulate inorganic matter (PIM) proportion, PIM tend to directly affect suspension feeding bivalves by clogging feeding structures like gills and labial palps interfering with particle selection (Thrush et al., 2004), hence, the decrease in CR in this study. This was in total agreement with previous studies, where increase in PIM had significant negative effects on CR of various bivalves (Bricelj and Malouf, 1984; Iglesias et al., 1996; Navarro and Widdows, 1997). Therefore, individuals exposed to salinity 30 psu showed no signs of recovery and this might have been mainly due to the disruption in filtration caused by the increase in PIM proportion. On the other hand, as shown in Figure 2, with increase in turbidity at salinity 10 psu, valve opening slightly delayed. There might be a possibility that individuals exposed to salinity 10 psu, were stressed out to a point where they were quite sensitive to the increase in turbidity levels during their adaptation period, hence, the delay in gaping activity. However, to what extent is turbidity influential on gaping activity is questionable because individuals exposed to 30 psu with increase in turbidity levels showed no influence on gaping behavior as valve openings were recorded from the start till the end of the experimental period. It was difficult to point out signs of recovery to high turbidity in this study due to salinity having a more prominent effect on individuals exposed to low salinity.

The above paragraphs states that there were some forms of stress induced on *T. granosa* individuals during the 2-week period and could the effects of this stress be terminal during recovery remains unclear. Therefore, in future studies, to better understand these carry-over effects that exposed individuals might experience, it would be best to expose individuals to extreme environmental conditions in the laboratory and return them to the field for recovery.

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