Department of Environment Systems
Graduate School of Frontier Sciences
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Master's Thesis

Laboratory Determination of Clams Burrowing Response to Marine Thermal Stress using Acoustic Monitoring

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1. Introduction

1.1 Background

Marine ecosystems are the cornerstone of global ecological balance. They provide vast benefits to both natural systems and human societies, including structural habitat, food and raw materials, nutrient cycling, and climate regulation.[1][2] However, ecosystems are increasingly threatened by more and more frequent climate changes, such as marine warming, acidification, sea level rise and hypoxic dead zones. These changes have become the principal environmental stressors that compromise the stability and biodiversity of marine ecosystems.[3][4] These stressors are usually driven by human activities and often interact with one another. They severely undermine marine foundation species, thereby disrupting the resilience and functioning of marine ecosystems.[5][6][7] Among the marine species affected by anthropogenic stressors, bivalves (class Bivalvia) are considered as one of the most vulnerable groups, due to their crucial ecological and economic significance and high sensitivity to environmental stressors.

As poikilothermic benthic animals, bivalves have important ecological functions and economic value. However, climate change, especially marine thermal stress, is increasingly weakening their survival and behavioral performance. Although numerous studies have focused on their physiological responses under thermal conditions, there is still a lack of systematic quantitative methods and experimental observations on the dynamic variation and regulatory mechanisms of their important physical response, burrowing behavior. This gap limits our further understanding of their ecological adaptability.

Since burrowing is a fundamental behavior that is closely related to predator avoidance, food ingestion, and sediment bioturbation, it may be considered as an effective indicator for assessing ecological adaptability under environmental stress. Therefore, this study primarily focuses on the burrowing behavior of Hamaguri (*Meretrix lusoria*) under thermal stress. By using ultrasound monitoring, we aim to uncover behavioral changes and their ecological implications. To better understand the

ecological importance of this behavior, it is necessary to first explore the role of infaunal clams in benthic ecosystems.

1.1.1 Ecological Importance of Infaunal Clams

Infaunal clams, a subgroup of bivalve mollusks that live beneath the sediments, are recognized as important components of marine benthic ecosystems. They are widely distributed in marine ecosystems and often act as ecosystem engineers since their biological activities physically and chemically modify their surrounding environment.[8][9][10] Their activities can extremely affect sediment structure, water quality, and nutrient cycling.[11] In recent years, with the advancement of marine ecological research, an increasing number of studies have emphasized the irreplaceable role of infaunal clams in ecosystems. A decline in their abundance may lead to substantial reductions in ecosystem stability and resilience.[12][13] Consequently, a comprehensive understanding of the ecological roles of infaunal clams is critical for the effective conservation and sustainable management of marine ecosystems.

One of the most significant influences of infaunal clams is their effect on sediment biogeochemistry. Fig. 1-1 showed biogeochemical transformations associated with infaunal clams.[14] When clams feed and burrow within the seabed, they physically disturb and mix the sediment, facilitating the exchange of solutes such as oxygen, ammonia, nitrate, and silicate between the sediment and overlying water.[15][16][17] These bioturbation activities improve sediment metabolism, increase oxygen availability, and stimulate microbial processes, including coupled nitrification–denitrification, which are critical for nitrogen removal and maintaining sediment health.[18] By regulating redox conditions, infaunal clams also help prevent the excessive accumulation of reduced compounds like hydrogen sulfide and ammonium, which are toxic under anoxic conditions.[19][20] In addition, microbial biofilms on their shells and within their digestive tracts actively participate in biogeochemical transformations, further modulating nutrient cycling and gas fluxes such as methane and nitrous oxide.[21][22] These functions make clams active contributors in maintaining the quality and function of their habitats.

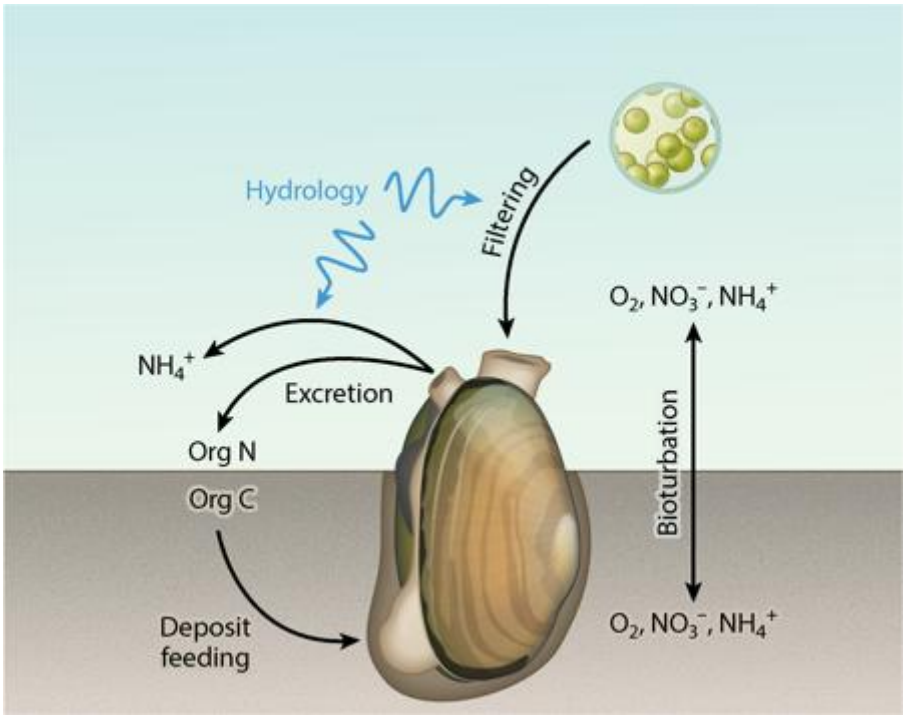


Fig 1-1 Biogeochemical transformations associated with infaunal clams.[14]

Beyond their biogeochemical functions, infaunal clams also contribute significantly to bioremediation and environmental monitoring. Through filtration and deposit feeding, bivalves can take up and bioaccumulate a wide range of contaminants, including heavy metals, nutrients, and pathogens.[23][24] Their capacity to tolerate, sequester, and retain pollutants makes them valuable candidates for bioextraction and sustainable water quality management. For instance, Asian clams (*Corbicula fluminea*) have shown effectiveness in removing metals from acid mine drainage, while hard clams (Mercenaria mercenaria) have been utilized to consume excess nutrients in aquaculture systems.[25][26] These applications not only support water purification but also enable the development of sustainable aquatic resource management strategies.

Moreover, infaunal clams are increasingly recognized as sentinel species for detecting early signs of environmental stress and pollution.[27][28][29] Their sedentary lifestyle and sensitivity to habitat conditions allow researchers to monitor ecological disturbances such as hypoxia, ocean acidification, and thermal anomalies through changes in burrowing behavior and physiological biomarkers.[30][31][32][33] Biomonitoring programs often incorporate clam-based metrics, including population

abundance and health status, into multivariate ecological indices like M-AMBI to assess overall ecosystem quality.[34][35]

Within aquatic ecosystems, infaunal clams are important components of both marine and freshwater food webs. They serve as a key trophic link in benthic–pelagic coupling. By filtering particulate organic matter and phytoplankton from the water column, they transfer this energy to higher trophic levels such as crabs, fish, birds, and mammals.[36] In eutrophic and turbid systems, such filtration activity can enhance water clarity and light penetration. It helps the growth of benthic macrophytes and stimulates secondary production.[37] Furthermore, the biodeposits and excretions of infaunal clams can enrich benthic environments, creating a nutrient pulse that supports benthic food webs.[38] Finally, the decomposition of dead clams supplies organic matter to aquatic and adjacent terrestrial ecosystems, contributing to detrital food chains and nutrient cycling.[39]

Infaunal clams can also be regarded as potential contributors to negative carbon emissions. Their growth helps relieve carbon pressure in the atmosphere. Infaunal clams mainly absorb and utilize carbon in two ways, that is, by carbon input from Dissolved Inorganic Carbon (DIC) uptake and organic carbon through ingestion.[40] As shown in Fig. 1-2, through biocalcification, infaunal clams can fix carbon as calcium carbonate ($CaCO_3$), which contribute to the absorption of atmospheric carbon dioxide (CO_2).[41] According to the related study, each kilogram of farmed Manila clams (*Ruditapes philippinarum*) sequesters about $254\,g\ \ CO_2$ in shells while emitting only $22g$ during cultivation — a net sink of $233\,g\ \ CO_2/kg$.[42] When shells are harvested and removed, the carbon is effectively stored long-term. At the ecosystem scale, especially in systems integrated with macroalgae or seagrass, bivalves further contribute positively to coastal carbon budgets. Thus, infaunal clams serve a dual role: reinforcing sedimentary environments and supporting blue carbon storage.[43]

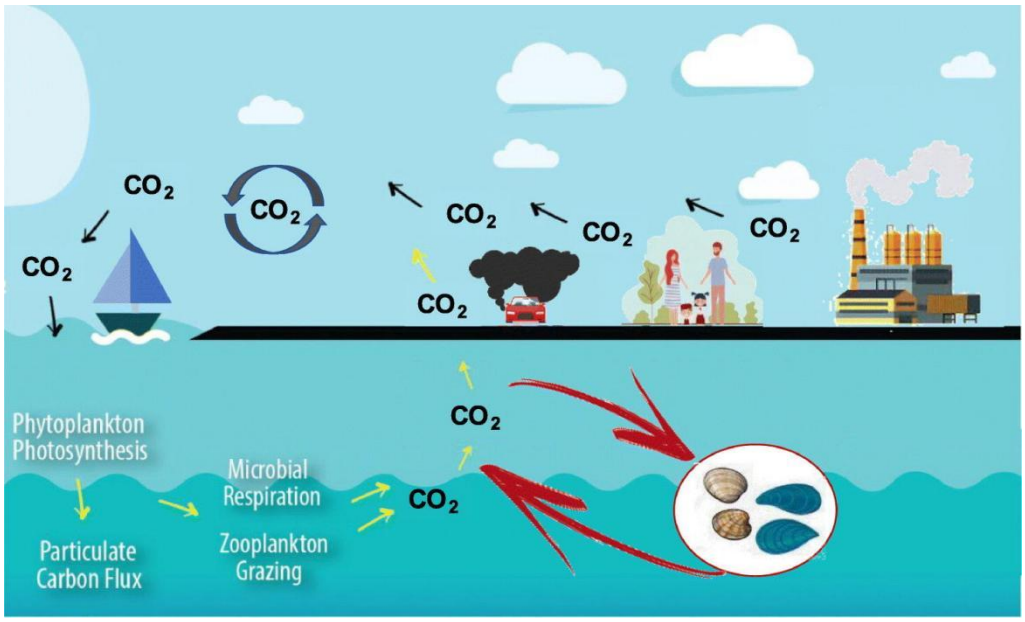


Fig. 1-2 Biogeochemical transformations associated with infaunal clams.[42]

In conclusion, infaunal clams play a foundational role in maintaining the structure and function of benthic ecosystems. Their ecological services, including sediment bioturbation, nutrient cycling, and trophic mediation, render them indispensable components of coastal resilience. As global stressors intensify, incorporating clam biology into monitoring and management frameworks offers a practical pathway toward sustainable marine resource governance.

1.1.2 Impact of Marine Thermal Stress on Infaunal Clams

Over recent decades, increasing greenhouse gas emissions have contributed to more frequent and severe marine thermal issues, resulting in large-scale mortality events and significant shifts in species distributions.[6][44][45]

Fig. 1-3 Dead Manila clams in Washington, following a record heat wave in 2021.

Image source: https://www.eurekalert.org/multimedia/939204

Among the various temperature anomalies, marine heatwaves (MHWs), defined as prolonged high-temperature extreme events in the ocean, have posed serious challenges to marine ecosystems.[46] MHWs now occur more frequently and last longer than they did a century ago.[47] Based on model simulations, the probability and frequency of MHWs will increase further by the end of this century.[44] Meanwhile, their interaction with other environmental stressors such as ocean acidification and hypoxia will lead to further ecological deterioration.[48][49] Although heatwaves represent one form of acute thermal stress, elevated temperatures alone are enough to significantly impair the physiology and behavior of benthic species. Infaunal clams, which lack the mobility to escape thermal shifts, are particularly vulnerable to such stressor.[50] In this sense, understanding how these organisms respond to elevated temperature conditions is critical for assessing their ecological resilience and predicting ecosystem responses under future climate scenarios.

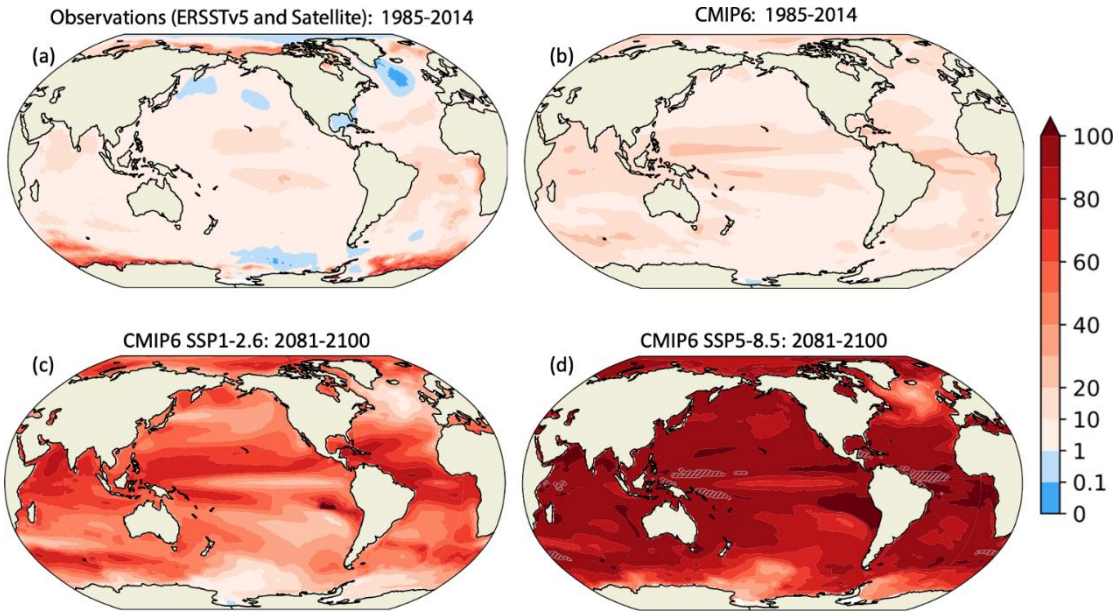


Fig. 1-4 Observed and simulated regional probability ratio of marine heatwaves (MHWs) for the 1985–2014 period and for the end of the 21st century under two different greenhouse gas emissions scenarios. (a) The MHW probability ratio from satellite observations during 1985–2014. (b-d) Coupled Model Intercomparison Project Phase 6 (CMIP6) simulated multi-model mean probability ratio of the 1985–2014 period, and 2081–2100 period. [44]

Temperature is one of the most important factors influencing physiological and behavioral performance of ectothermic animals.[51][52] As poikilothermic organisms, infaunal clams depend on ambient temperatures to regulate metabolic processes and maintain homeostasis.[53] In this sense, they are very vulnerable to temperature fluctuations. Marine thermal stress can therefore trigger a cascade of physiological disruptions in these organisms. Rising temperatures can increase basal metabolic rates, leading to greater oxygen demand in tissues. Under extreme thermal conditions, oxygen demand may exceed the organisms' aerobic scope, causing multiple physiological disruptions. When aerobic capacity becomes insufficient, clams experience metabolic suppression and significantly lowered respiration rates after stress exposure.[31] This oxygen imbalance triggers oxidative stress and lipid peroxidation. Meanwhile, the energy reserves and condition index decline, weakening the organism's physiological status and capacity to recover.[54][55] As a protective mechanism, clams initiate

molecular responses like the upregulation of HSP70, a chaperone protein that stabilizes denatured proteins during thermal stress. However, this upregulation is energetically costly and causes trade-offs that impair immune function, growth, and reproduction.[56] Additionally, studies showed that repeated or prolonged exposure to sublethal heat stress may not cause immediate mortality, but it can lead to cumulative physiological damage, including reduced filtration activity and depressed metabolic efficiency.[57][58]

Except for physiological disruptions, thermal stress also has a negative impact on the burrowing behavior of infaunal clams. As we know, burrowing behavior is essential for clam's feeding, stressor escape and predator avoidance.[59][60] Depending on the extent of temperature increase, burrowing behavior is affected differently. Studies have shown that moderate increases in temperature typically enhance the burrowing activities of clams as a mechanism to avoid thermal stress.[61] For example, experimental research on the Manila clam showed that juveniles had significantly higher burrowing rates than adults when temperatures rose to 30.6℃. However, when the temperature further increased to 44.6℃, nearly all adults died, whereas approximately half of the juveniles survived. Similarly, Zhang et al. also found the clams exhibit faster digging and higher percentage of burrowing rate at temperatures 20℃ and 30℃ as opposed to 10℃ and 34℃.[62] Such findings indicated that under moderate thermal stress, clams mitigate heat stress by increasing their burrowing depth or speed; however, extreme temperatures surpass their physiological limits, resulting in inhibition of burrowing behavior and causing mortality.

In conclusion, marine thermal stress poses significant threats to infaunal clams by impairing their physiological functions and critical behaviors such as burrowing. Although warming may temporarily enhance behavioral adaptations, such as increased burrowing, extreme conditions exceed their physiological tolerance and lead to severe dysfunction or mortality.

These impacts are not just theoretical concerns but have already affected clam populations in many coastal regions. In Japan, several regions have already experienced significant drops in clam yields due to the environmental changes.

1.1.3 Status of Infaunal Clam Production in Hamana Lake

Hamana Lake, located in Shizuoka Prefecture, is one of Japan's important clam production bases. It is famous for its abundant harvests of Asari (*Ruditapes philippinarum*), which play an important role in local fisheries and culinary culture.[63] However, in recent years, as global warming intensifies, clam production in Hamana Lake has sharply declined, raising serious concerns for local fisheries and ecological sustainability.

According to fisheries data provided by Shizuoka Prefectural Fisheries Research Institute, Asari production in Hamana Lake has significantly decreased over the past decade.[64] The annual clam catch in Hamana Lake during the early 2000s ranged between 2,000 to 6,000 tons, but by 2020, it had plummeted to a historical low of just 707 tons.[65] This decline is attributed to a combination of biological, environmental, and anthropogenic stressors. The decline in clam resources is attributed to complex factors, including decrease of broodstock, reduced abundance of planktonic larvae, habitat degradation, and intensified predation pressure.

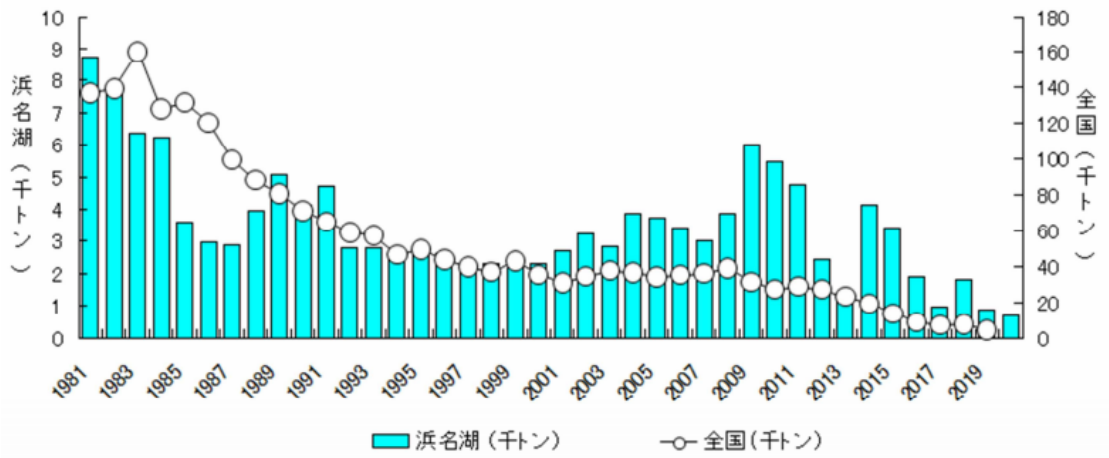


Fig. 1-5 Trends in Asari Clam Harvests in Lake Hamana and Across Japan[64]

Rising temperatures and extreme thermal events have been identified as critical factors affecting the reproduction and survival of clams in Hamana Lake. Especially in summer, hypoxia often occurs near the lake bottom, posing a serious threat to both juvenile and adult clams. As temperatures increase, the thermal layering of the water becomes stronger, making it harder for oxygen to reach the bottom and creating large

areas of unsuitable habitat.

Additionally, increased temperatures also affect the reproductive rhythm of clams. Shown as Fig. 1-6, clams in Hamana Lake usually spawn in spring and autumn, but temperature changes may disrupt the spawning period and reduce the number and quality of planktonic larvae. Studies indicated that the density of planktonic larvae in 2020-2021 was only 1/10 to 1/20 of that in 2004, seriously affecting the source of supplementary individuals.[65]

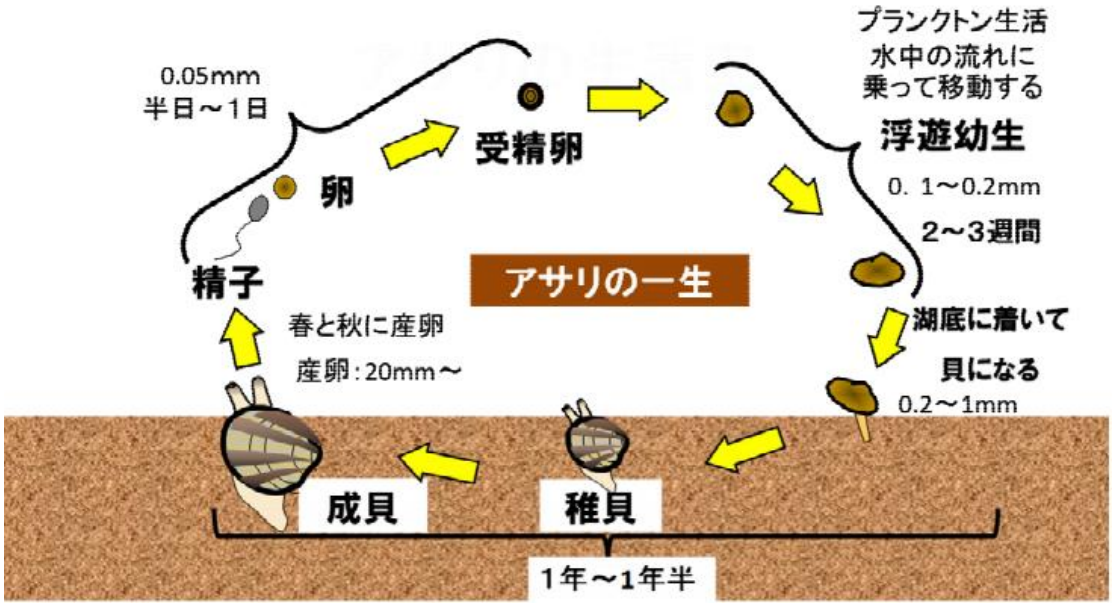


Fig. 1-6 The life progress of clams[64]

The planktonic larvae usually float in the water. During this time, they move with the currents in the lake. Recent observations by local fishers indicated that the water current within Lake Hamana has become faster in recent years, which may prevent planktonic larvae from successfully settling in suitable habitats.[65] Although there is not sufficient evidence in the current report to explain this hydrodynamic change, such shifts may be partially influenced by global warming. Previous studies have shown that global warming enhances thermal stratification and suppresses vertical mixing in lakes, which can alter internal water movement patterns.[66][67] So the increasing temperature may cause “floating failure” of planktonic larvae, leading to the decrease of their amount.

Although local authorities have implemented several restoration measures, such as the designation of no-fishing zones, artificial seeding, and protective enclosures, these human-led efforts still face challenges under long-term heat stress. For now, our understanding of behavioral performance and ecological processes in infaunal clam species is still insufficient. Thus, the decline of clam resources in Hamana Lake reflects not only a management issue but also a broader symptom of climate-induced disruption in coastal ecosystems. Future restoration and sustainable fishery management must incorporate climate adaptation strategies and promote interdisciplinary, long-term ecological monitoring to cope with the growing uncertainties imposed by a warming world.

1.1.4 Significance of Burrowing Behavior and Advances in Measurement Techniques

Burrowing behavior is essential for clam survival. One major advantage is predator avoidance. Many clam species rely on burrowing into the sediment as their primary defense mechanism, rather than depending solely on their calcified exoskeletons. This behavioral strategy not only helps to reduce the likelihood of detection by predators but also increases the time and effort required for successful predation.[68][69][70] In fact, infaunal clams will be more active to dig themselves deeper when they detect predators around them. For example, soft-shell clams (*Mya arenaria*) can sense crab predators by chemical and mechanical signals and respond by burrowing deeper to avoid attack.[60] Studies have demonstrated that individuals which maintain greater burial depths would have higher survival rates than those remaining near the surface.[71]

Burrowing behavior also helps infaunal clams to resist thermal stress and desiccation, especially in the intertidal environment. In the intertidal zone, the area would experience low and high tides. So, it would exhibit greater temperature variation than open water areas, during high tides, clams will extend their siphon to the surface for feeding and breathing. While during low tides, they will burrow to greater depths to avoid extreme heat.[72] The sediment's moisture and its lower temperature can provide a stable microhabitat, preventing overheating and water loss.

In addition, burrowing behavior protects clams from physical disturbances and hydrodynamic forces. The sediment plays the role as a barrier against strong currents, anchoring the clam in given position.[72] Hydrodynamic experiments indicated this benefit: nearly 95% of buried clams remained in position under unidirectional currents up to $60\ cm/s$, whereas unburied clams or those in highly turbulent conditions were much more vulnerable to being carried off.[73] By burrowing, clams effectively reduce the impact of water movement and sediment disturbance on their bodies. This protection from mechanical stress not only prevents injury but also conserves energy that would otherwise be spent re-anchoring or burrowing anew after a disturbance.

Overall, burrowing behavior serves multiple survival functions for infaunal clams. Rather than being a simple movement response, burrowing behavior reflects the clam's dynamic interaction with its surrounding environment. Since it closely relates to environmental stress and ecological resilience, burrowing behavior can be considered as an important indicator for assessing individual fitness and ecosystem health under changing conditions.

To investigate such ecologically significant behavior, reliable and non-invasive methods for burrowing observation are essential. Over the past decades, several techniques have been used to monitor clam activity, ranging from traditional observation to imaging techniques.

In most studies, researchers visually observe and record the time it takes for clams to transition from resting on the sediment surface to becoming fully buried, as well as the success rate of this initial burrowing process.[74][75] This approach is easy to implement but cannot track the clam's behavior beneath the sediment. Therefore, some studies have employed transparent containers to directly observe sediment profiles from the side, allowing for visualization of the clam's vertical movement within the sediment.[59] Based on this approach, other studies, such as Román et al., have adopted destructive methods at the end of experiments, such as longitudinally slicing sediment cores to directly measure the final position of clams.[76] Although this method is destructive, conducting it at the end of the experiment allows for accurate measurement of terminal

burial position. Additionally, some studies have used non-contact techniques to monitor the vertical movement of clams, such as attaching a thread to the shell and recording its vertical displacement over time.[77] While this method provides continuous data on burial dynamics, it has limitations: the thread may disturb the clam, and the technique lacks sufficient resolution and accuracy in detecting lateral movements.

In contrast, recent studies have increasingly focused on the bioturbation caused by clam burrowing behavior. Several studies introduced fluorescent particles into the sediment as tracers to track the extent and depth of sediment disturbance.[76][78][79] At the end of the experiments, the sediment cores were sliced vertically and imaged under ultraviolet light, allowing researchers to analyze the depth distribution of the particles and quantify sediment reworking. Compared to traditional measurements such as burial time or final depth, the tracer-based bioturbation analysis provides multidimensional behavioral metrics, helping people to understand clams' burrowing abilities from a sediment perspective. However, this method cannot capture continuous behavioral changes over time and is limited in detecting the precise position or movement trajectory of individual clams.

Despite the advancements in monitoring burrowing behavior, current approaches still face limitations in terms of temporal resolution and spatial accuracy, especially in tracking continuous behavioral changes and precise individual positions within the sediment. Moreover, our understanding of how burrowing behavior dynamically responds to environmental stressors, especially under marine thermal stress, remains incomplete.

1.1.5 Research Motivation

As marine thermal stress intensifies, infaunal clams are facing increasing threats to their survival. However, our understanding of their behavioral responses remains limited. Additionally, conventional behavioral recording methods are often restricted to initial burial time or final depth, making it difficult to capture the complete burrowing process. To address this limitation, the present study employs a high-resolution, non-invasive ultrasound monitoring technique to continuously track changes in burrowing

behavior and to develop more refined behavioral indicators. This approach aims to bridge existing research gaps and offer new tools for understanding the ecological adaptation mechanisms of benthic organisms under environmental stress.

1.2 Research Objectives and Significance

1.2.1 Research Objectives

The primary objective of this study is to explore how infaunal clams behaviorally respond to thermal stress in controlled laboratory conditions. With the utilization of acoustic technology, the research manages to overcome the limitations of conventional behavioral assessment and provide a more comprehensive understanding of clam burrowing dynamics under warming conditions. The specific goals include:

1. Design and build an experimental aquarium system that allows the control of temperature. Using ultrasound probes and optical cameras, the study will obtain raw burrowing behavior data of clams under 22℃ and 30℃.
2. Develop refined behavioral indicators for burrowing activity, such as burrowing time, burrowing depth, displacement and behavioral stability. Aiming to fill the data gap regarding clam responses to thermal stress.
3. Conduct statistical analyses of burrowing behavior to examine how temperature influences specific behavioral indicators and to explore potential mechanistic links between heat stress and behavioral change.

1.2.2 Research Significance

The specific significance is as follows:

1. This study provides new insights into monitoring benthic organisms. The application of ultrasound technique with optical recording offers a non-invasive, real-time monitoring approach. This method can be applied to other benthic species and extends to long-term field observations.
2. By supplying high-resolution burrowing data and refined behavioral indicators, this study fills a critical gap on how infaunal clams react to heat stress, laying the

foundation of behavioral and ecological modelling.

3. The temperature-behavior relationship would help our understanding of thermal impacts on infaunal clams. It also supports practical measures for predicting climate-related risks and conserving habitats in regions like Hamana Lake.

2. Methodology

2.1 Principle of Ultrasound Imaging for Monitoring Clams' Burrowing Behavior

2.1.1 Fundamentals of Ultrasound Imaging

Ultrasound is a kind of mechanical wave with frequency greater than 20 kHz. It propagates through a medium as alternating compressions and rarefactions of local pressure.[80] Since these vibrations can penetrate opaque materials, ultrasound is widely applied in fields such as medical diagnosis, structural flaw detection, and marine surveying to visualize internal structures.

As shown in Fig. 2-1, high-frequency sound pulses are generated by piezoelectric crystals inside the transducer and transmitted into the medium. When these pulses encounter a boundary between materials with different acoustic impedances, a part of the waves is reflected and generates echoes. The transducer then receives the echo and records its arrival time and amplitude. Based on the known speed of sound in the medium, the depth of the reflector can be calculated from the two-way travel time using the following formula:

$$d = \frac{ct}{2}$$

where d is the distance between the transducer and object, c is the speed of sound in the medium, t is the total travel time of the ultrasound pulse.

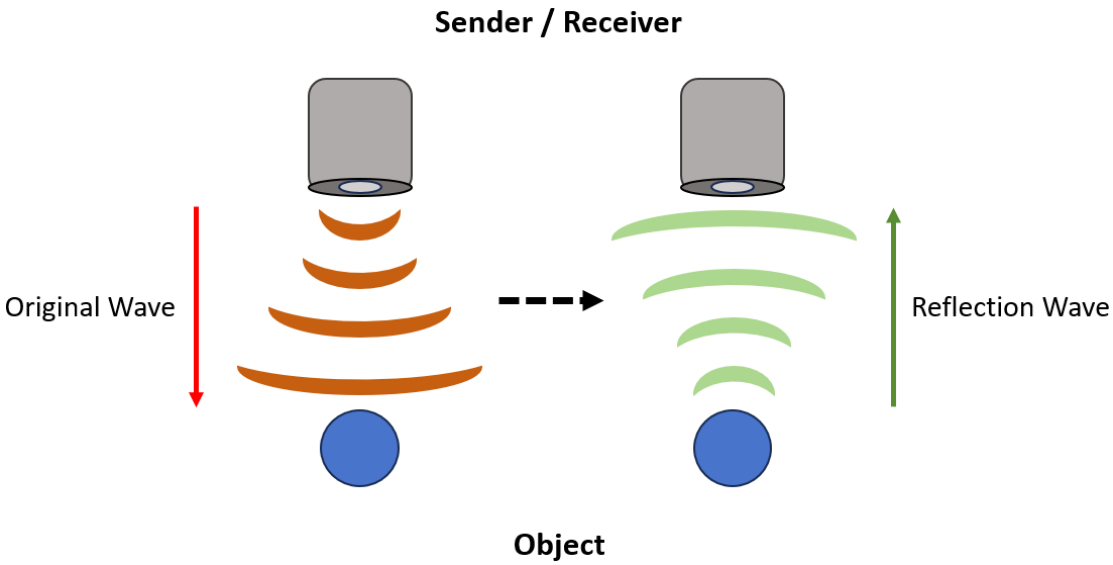


Fig. 2-1 The Basic Principle of Acoustic Transmission and Reflection

In this study, an ultrasound probe was used for both pulse emission and echo reception. As shown in Fig. 2-2, when the probe scans a single point, it generates one-dimensional data along the z-axis (depth). A motorized platform was used to move the transducer across a predefined two-dimensional (XY Plane) scanning area. This mechanical scanning enabled echo acquisition at multiple points. By combining the reflection signals from all scanned points, a three-dimensional acoustic data cube is constructed. The 3D ultrasound data can be further processed for target extraction.

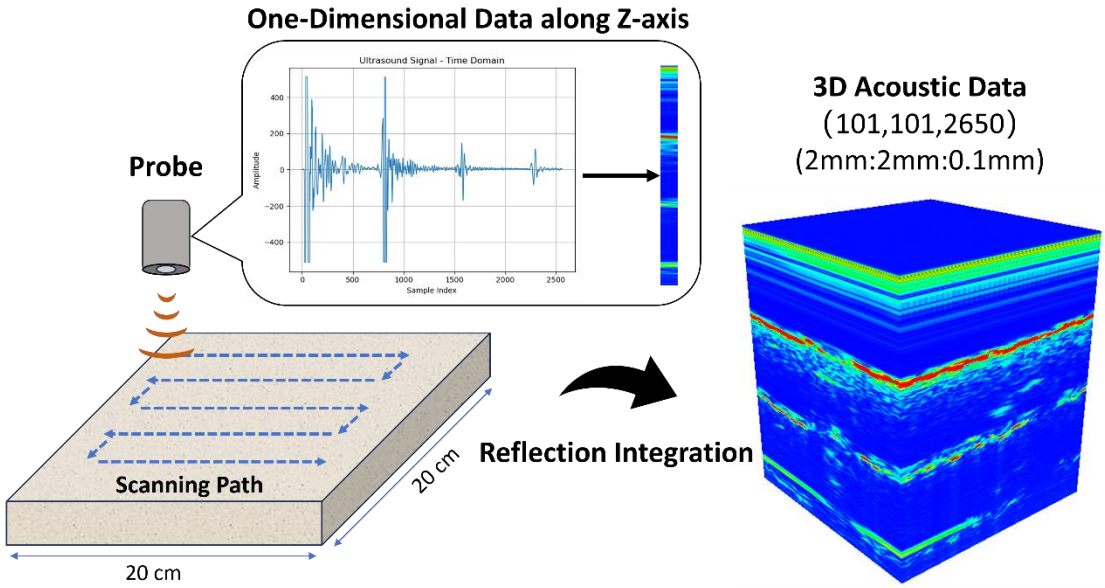


Fig. 2-2 The Principle of Ultrasound Imaging

Compared to electronic phased-array systems, mechanical scanning is structurally

simpler and well suited for high-fidelity data acquisition in laboratory environments. Although the scanning speed is slower, this method allows for higher spatial resolution and the data processing is simpler. This scanning principle lays the foundation for our ultrasound monitoring system used in the research.

2.1.2 Feasibility of Ultrasound Imaging for Monitoring Clams

In ultrasound imaging, when a sound wave travels through one medium and encounters the boundary of another, part of the wave is reflected. The strength of this reflection depends on the difference in acoustic impedance Z between the two media. Acoustic impedance is defined as:

$$Z = \rho \times c$$

where ρ is the density of the medium and c is the speed of sound in the medium.

When a clam is buried beneath the sediment surface, its calcareous shell has a much higher acoustic impedance than the soft tissue, due to their different densities. This difference causes strong echoes at the shell boundaries, which helps to locate and outline the clam. Similarly, the water–sediment interface also creates a clear reflection due to the impedance gap, allowing us to detect the height of the sediment surface. By identifying these reflected interfaces, we can estimate the burial depth of the clam and observe any changes in its posture.

Selection of ultrasound frequency

In natural environments, ultrasound waves often experience significant energy loss during propagation. When traveling through water-saturated granular media, the waves are attenuated mainly by two mechanisms: absorption and scattering. In order to get high-quality ultrasound data, we should carefully choose the ultrasonic frequency used for the measurement. So, it is essential to understand the physical basis and frequency dependence of these mechanisms.

Absorption refers to the gradual conversion of sound energy into heat, which reduces the amplitude of the signal. This process is mainly caused by viscous losses in the pore water and internal friction within the sediment frame. The frequency response of absorption is often described using the dimensionless parameter kd, which is the

product of wave number k and grain diameter d. When kd is less than 1, which means the wavelength is much larger than the grain size, the absorption tends to dominate.

Kimura et al. used the Biot–Stoll poroelastic model to calculate absorption characteristics in water-saturated sediments.[81] The results showed that in the frequency range of 1-100 kHz, the absorption coefficient increased with the square of the frequency ($\propto f^2$). At higher frequencies, the relationship shifted toward a square root dependence ($\propto f^{1/2}$). This indicated that even without scattering, absorption alone can limit the penetration depth of ultrasound imaging.

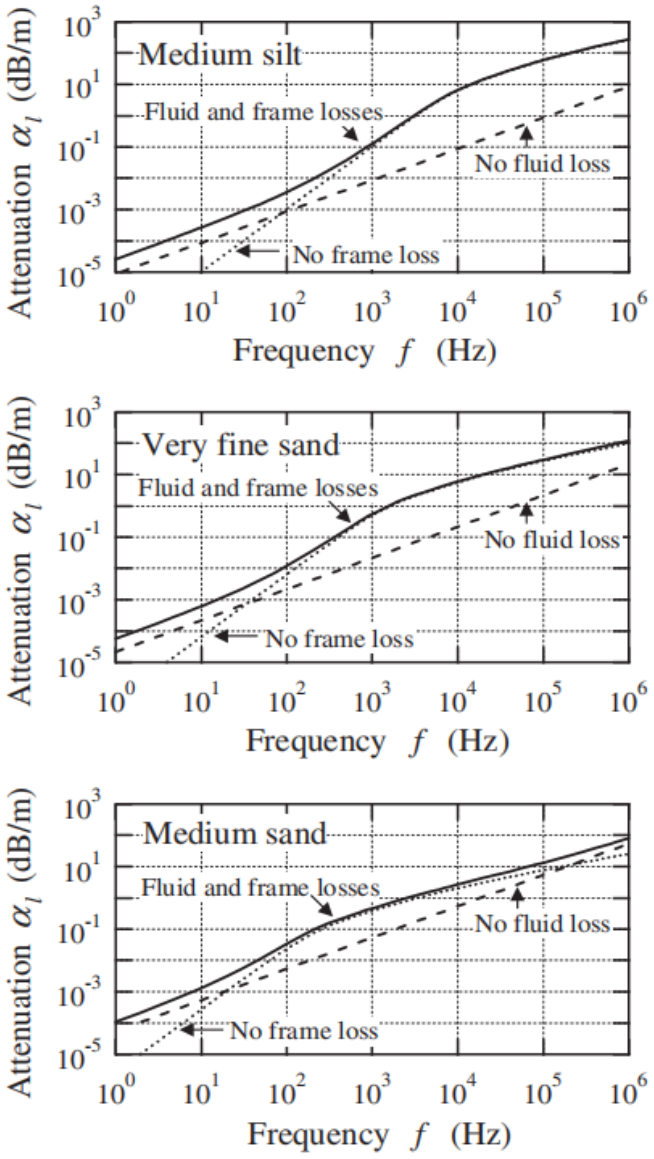


Fig. 2-3 Frequency-Dependent Acoustic Attenuation in Water-Saturated Sediments[81]

Compared to absorption, scattering attenuation is mainly caused by the structural heterogeneity of the sediment medium. This effect becomes more significant when the acoustic wavelength is similar to the size of sediment particles. When the wave encounters interfaces such as grains, pores, or shell fragments, it is deflected or reflected, resulting in scattering. Mizuno et al. showed that, as kd exceeds 1, scattering becomes the dominant attenuation mechanism.[82] It may lead to multiple scattering events, which distort the signal waveform, broaden the signal path, and cause a loss of high-frequency components.

In summary, selecting the ultrasound frequency requires a balance between spatial resolution and penetration depth. The spatial resolution of ultrasound is determined by its wavelength, which is inversely proportional to frequency. The effective spatial resolution is generally considered to be approximately half the wavelength. In this study, natural sediments with particle sizes ranging from 0.1 to 1 mm were used as the burrowing medium. The target organisms were live clams with shell lengths of 3-5 cm, and the required imaging depth was around 10 cm. Considering the clam's size and attenuation in sediments, we selected a frequency of 500 kHz for ultrasound imaging. Based on an approximate calculation, the wavelength in water-saturated sediment is 3.41 mm, $kd \approx 0.98$ and the total attenuation is about 67.29 dB/m ($\alpha_{absorption} = 50.06\ dB/m, \alpha_{scattering} = 17.23\ dB/m$). This frequency is sufficient to detect clams under the sediment. It provides a reasonable compromise between penetration and image clarity, and is suitable for non-invasive, real-time monitoring of infaunal bivalves under laboratory conditions.

Distance from ultrasonic probe to sediment surface

In this study, we used an immersion-type ultrasound probe (model: 0.5K40I–PF70, Japan Probe) with a central frequency of 0.5 MHz and an element diameter of 40 mm. The transducer is composed of a composite piezoelectric element and an epoxy matching layer, enclosed in a stainless-steel housing.

The surface of lens is spherically focused, with a curvature radius of 70 mm, designed to concentrate the acoustic beam at an optimal focal point in the water. This design

enhances signal intensity and spatial resolution at the target depth. The simulation of acoustic beam is shown in Fig. 2-4.

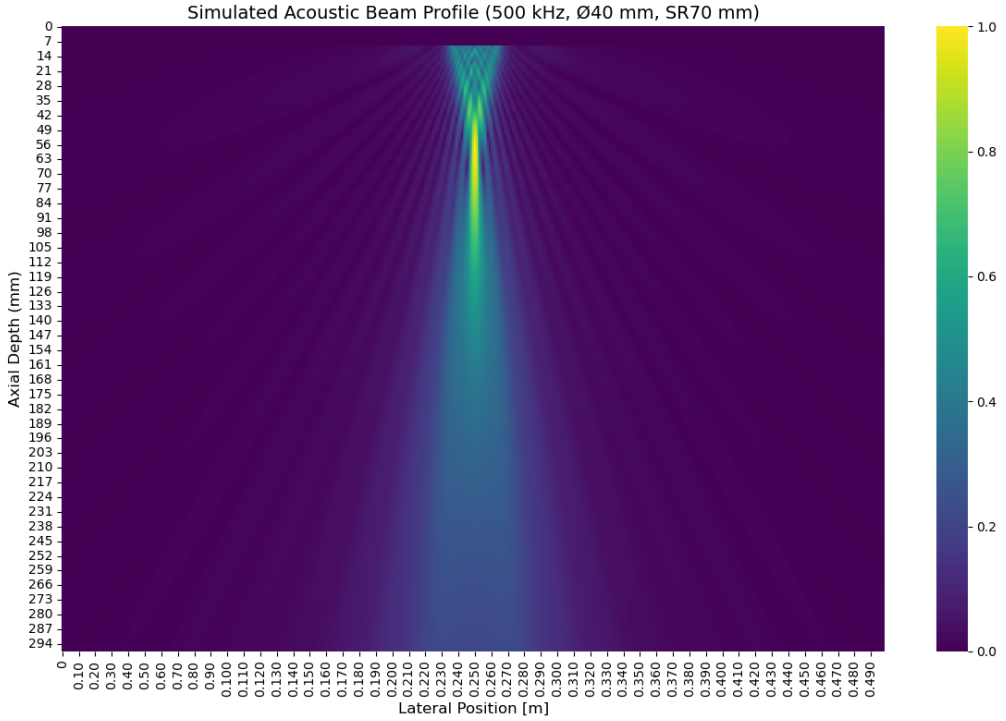


Fig. 2-4 Simulation of ultrasound probe beam

Based on the simulation, the transducer's focal length was estimated to be around $5\ cm - 7\ cm$. Therefore, in our experiments we mounted the probe approximately $5\ cm$ above the sediment surface to align with the focal area and ensure better imaging resolution and penetration.[83]

Initial Testing for Clam Detection

To evaluate the feasibility of detecting buried clams under experimental conditions, we conducted a preliminary imaging test using a live individual. A clam was placed on the sediment surface and allowed to burrow naturally without interference. After the clam was fully buried, we performed ultrasound scans to image the target area. The initial result is shown in Fig. 2-4.

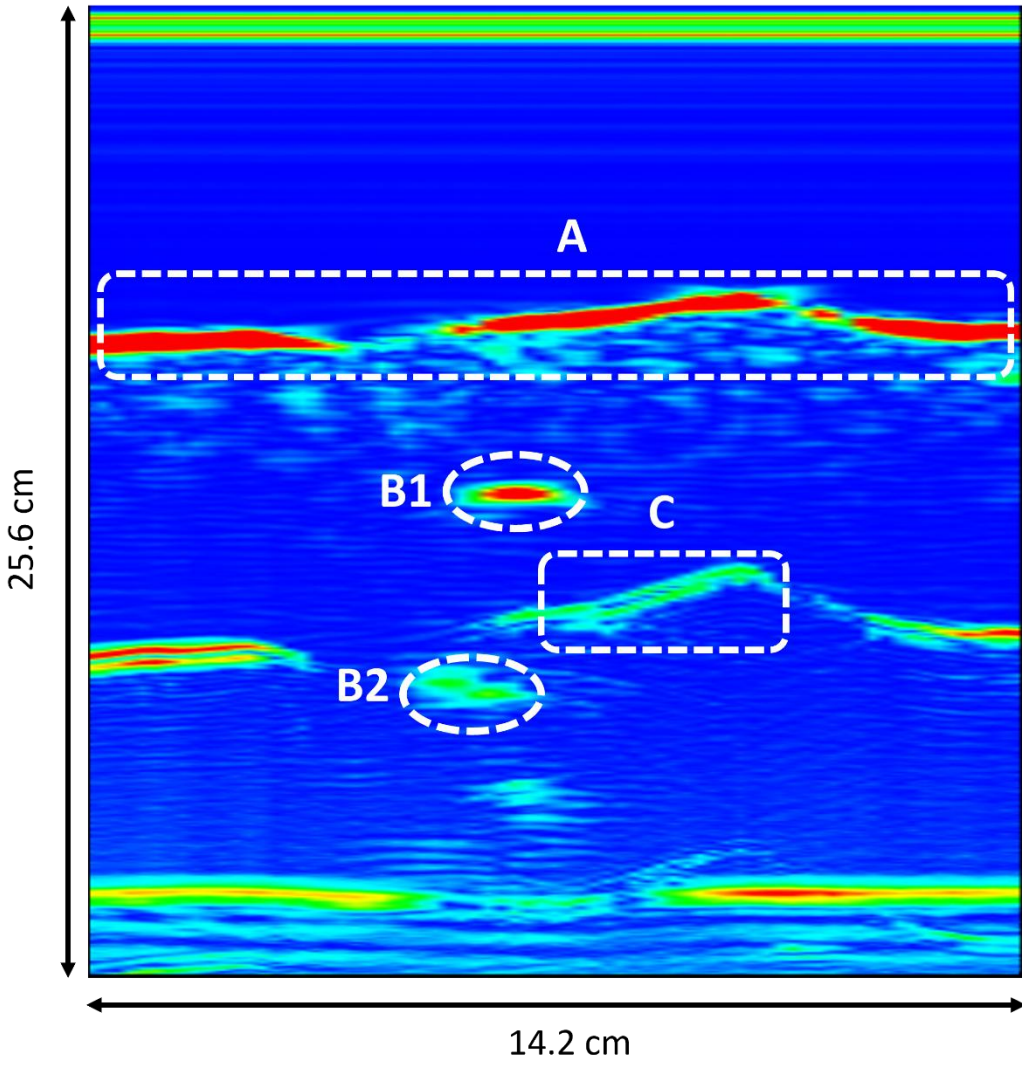


Fig. 2-4 Side slice of ultrasound scanning test

In the acoustic slice, region A shows a strong reflective layer corresponding to the water-sediment interface. Below the interface, elliptical marked reflections labeled B1 and B2 are observed. They represent top and bottom reflections of clam. Region C appears as a slanted, duplicated structure located below the primary targets. It is likely caused by multiple reflections, where part of the acoustic wave bounces between high-contrast interfaces, producing delayed and mirrored signals.

In conclusion, the result suggests that the selected imaging parameters are able to capture the reflection features of clam. The buried targets show sufficient contrast and clarity, supporting the feasibility of using ultrasound to detect clams.

2.2 Experimental Equipment

2.2.1 A-Core-30

In the research, we deployed the acoustic “coring” system, named A-core-30, to obtain the backscatters from the sediment and benthic organisms. The system mainly consists of two parts: a two-dimensional stage controlling unit and an acoustic unit, as shown in Fig. 2-5.

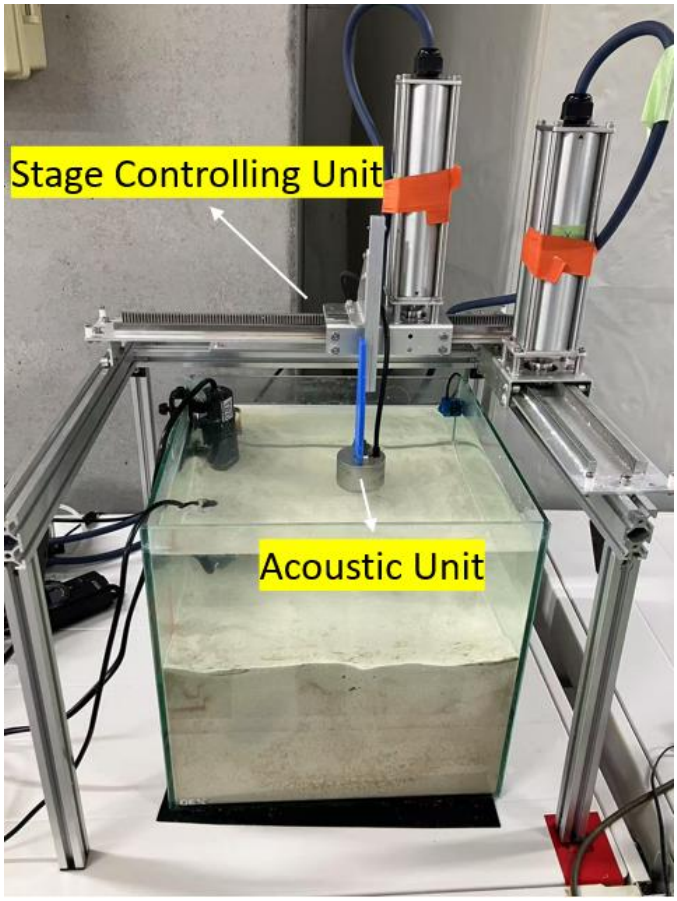


Fig. 2-5 A-core-30

The two-dimensional stage is constructed using aluminum extrusions and linear guide rails, allowing the probe to move precisely along the horizontal X and Y axes. A pair of stepper motors drive the stage with high positional repeatability. The maximum scanning area is defined as $300\,\text{mm} \times 300\,\text{mm}$, which is large enough to accommodate probe scanning.

2.2.2 Aquatic System

In order to maintain stable environmental conditions for the clams during the

experiment, a customized aquatic system was designed, as shown in Fig. 2-6. The system integrates automated feeding, temperature control, and water level regulation to simulate a controlled and semi-natural environment.

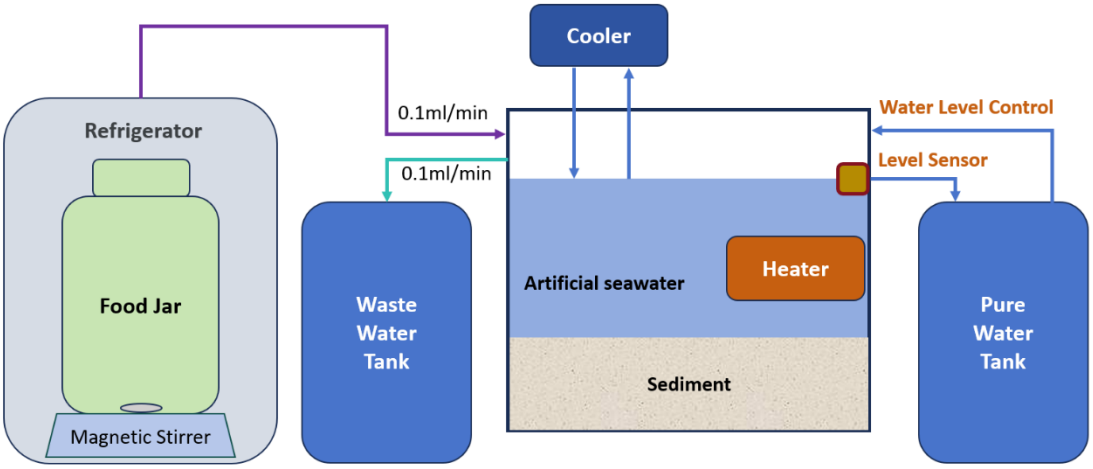


Fig. 2-6 Design of aquatic system

The size of the aquatic tank was $300 \times 300 \times 300\,mm$. It was large enough to accommodate sediment and live clams during observation.

A refrigerator was used to store clam's food in a sealed food jar, which was continuously stirred using a magnetic stirrer to maintain homogeneity. The food solution was delivered into the tank at a constant flow rate of $0.1\,ml/min$ using a precision pump. Simultaneously, an equal amount of water ($0.1\,ml/min$) was drained into a waste water tank to ensure stable salinity and avoid water overflow. This controlled inflow–outflow design also helps maintain water quality and prevents nutrient accumulation.

Temperature regulation was essential since our study had to evaluate clam responses under different temperature conditions. In this study, we used a cooler and a heater for temperature regulation. These two devices worked in coordination to reach and maintain the experimental temperature.

In addition, a water level sensor was installed on the sidewall of the water tank to detect any drop in water level, which might occur due to evaporation or transpiration. When the sensor detects a decrease in water level, it automatically triggers the addition of water from a pure water tank, thereby maintaining a constant water level throughout the experimental period.

Overall, each module in the aquatic system was designed to mimic natural conditions while ensuring experimental consistency and minimizing manual intervention.

2.3 Collection and Maintenance of Experimental Animals

In the research, we used Hamaguri as the experimental animal. Hamaguri is a kind of infaunal clam that is commercially and ecologically important in Japan. They have strong burrowing behavior and are sensitive to environmental changes. Therefore, it is suitable to use for studying behavioral responses under thermal stress.

For animal collection, a total of 22 live clams were collected from Hamana Lake, Shizuoka Prefecture, Japan. Shown in Fig. 2-7. The average length of the collected specimens was $40.42 \pm 0.02\ mm$, the width $33.87 \pm 0.02\ mm$, the height $20.76 \pm 0.02\ mm$, and the wet weight $18.20\ g$.

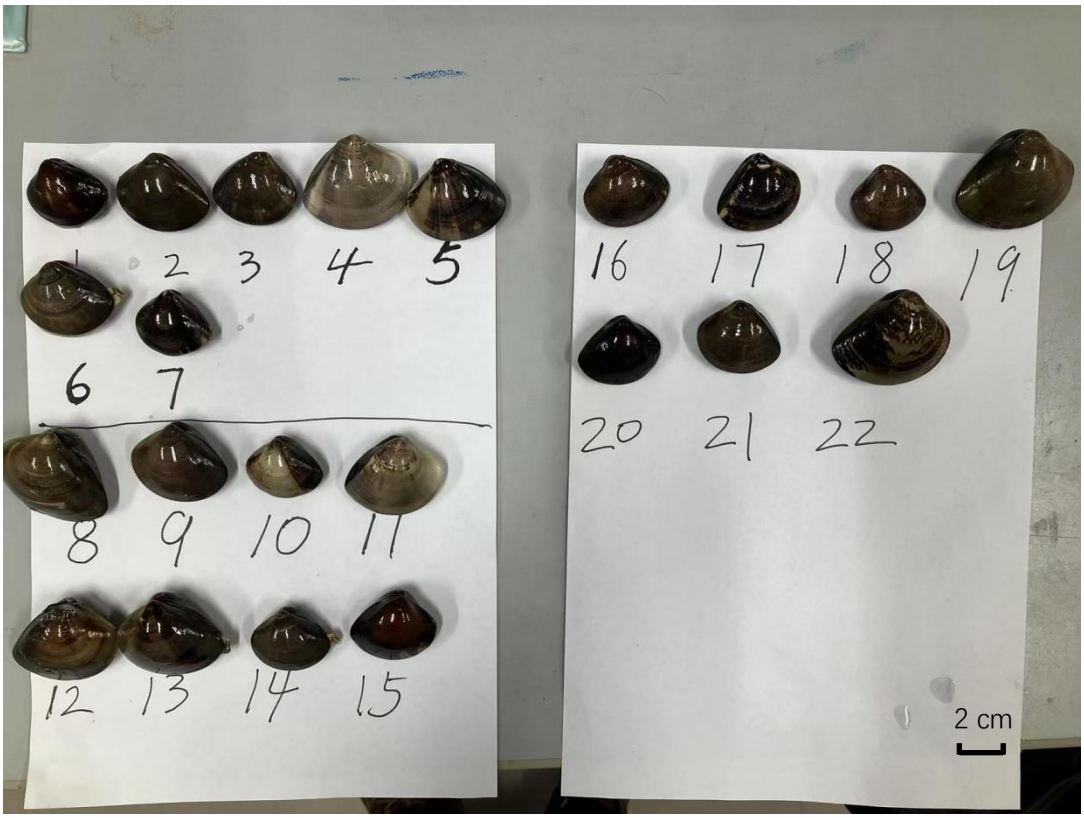


Fig. 2-7 Samples of Hamaguri

During the sampling and transportation process, water parameters such as temperature, pH, dissolved oxygen (DO), and salinity were recorded. After arrival at the laboratory, the clams were transferred to water tanks under controlled conditions that closely

resembled their natural habitat. The maintenance period ensured physiological stabilization before formal experiments. Related environmental parameters were shown as Table 2-1.

Table 2-1 Environmental conditions during sampling, transportation, and laboratory maintenance of Hamaguri.

Phase	Temperature (℃)	pH	DO (mg/L)	Salinity (ppt)
Sampling & Transportation	15.7	7.8	6.28	30
Laboratory	16.0 ± 0.2	8.0 ± 0.2	> 7.0	30

To maintain suitable conditions during the acclimatization and experimental period, the clams were placed in three aquatic systems, with each system containing seven individuals. The food solution was continuously delivered into each tank at a flow rate of $0.1\,ml/min$ using a peristaltic pump. The food was prepared by mixing $2\,L$ of artificial seawater with $144\,ml$ of microalgal concentrate (Tetraselmis gracilis, $1.0 \times 10^8\,cells/ml$; YANMAR). Artificial seawater was prepared by mixing purified water obtained from a reverse osmosis (RO) system (AQUA GEEK) with commercial marine salt. The salinity was adjusted to $30\,ppt$ using a calibrated refractometer.

Fig. 2-8 RO system

To ensure long-term water quality and food freshness, both the artificial seawater and food solution were completely replaced every two weeks. Additionally, a 12:12 hours light-dark photoperiod was maintained using LED lighting and a timer to simulate natural environmental rhythms.

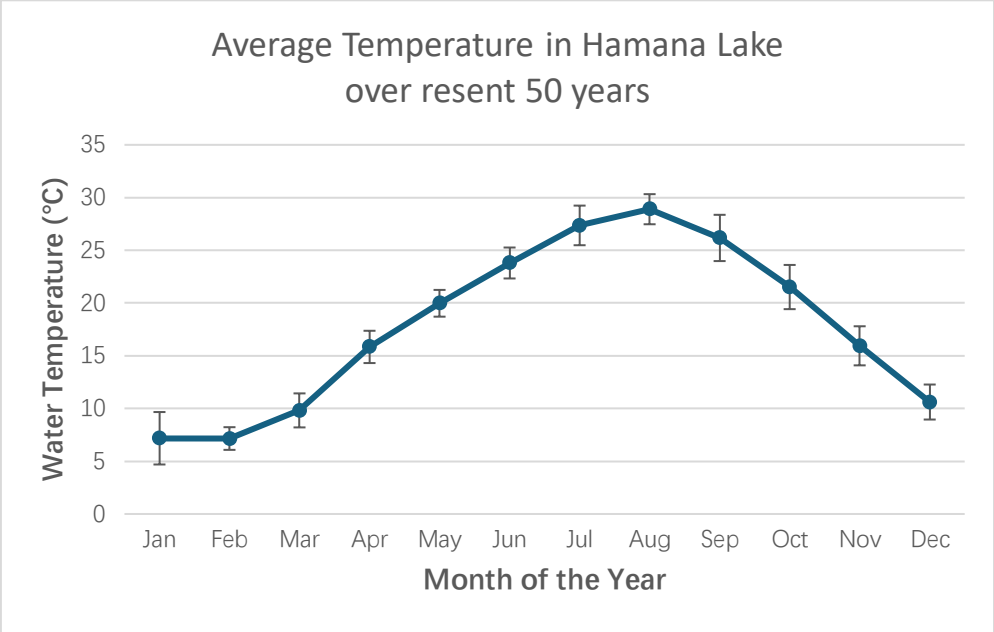
2.4 Experiment Design

2.4.1 Grouping Strategy

A total of 22 Hamaguri clams were temporarily held under laboratory maintenance conditions. Among them, 18 individuals were selected for the experiment and divided into two thermal treatment groups: 22℃ and 30℃. Each group contained 9 clams, consisting of three size classes—large, medium, and small—with three individuals per class. The average shell lengths for each class were $45.51 \pm 3.24\,mm$ (large), $38.98 \pm 1.38\,mm$ (medium), and $34.63 \pm 1.86\,mm$ (small), respectively.

The two temperature levels, 22℃ and 30℃, were selected based on historical environmental data from Hamana Lake. The water temperatures typically reach around 22℃ during early summer and autumn, which are considered optimal for clam growth and physiological stability. In contrast, temperatures in July and August frequently rise to approximately 30℃, approaching the upper threshold of the species' thermal tolerance. In addition, clam mortality usually occurs during summer. Therefore, 30℃ was chosen as a high-stress condition to simulate peak summer warming, while avoiding excessive thermal exposure that could cause acute mortality during the experiment. This selection allowed us to assess behavioral responses under both favorable and thermally stressful yet sublethal conditions.

Fig. 2-9 Average Temperature in Hamana Lake over recent 50 years



2.4.2 Acclimation of Experimental Animals

Before initiating the main experiment, all clams experienced an acclimation phase to reduce potential stress caused by abrupt environmental changes. Acclimation is an essential process in experimental animal studies, particularly when environmental parameters such as temperature are manipulated. It allows organisms to gradually adjust their physiological and behavioral responses to the new conditions, thereby minimizing confounding effects that could interfere with data interpretation.

At the beginning of acclimation, the selected clams were fed in two aquarium systems. They were initially kept at a baseline temperature of 16 ℃, which reflects the ambient condition of their original habitat during the time of collection. After that, the water temperature gradually increased at a rate of 1 ℃ per day until reaching the target experimental temperatures of either 22℃ or 30℃. Once the target temperature was reached, clams were maintained at that condition for one additional week to ensure physiological and behavioral stabilization prior to measurements.

Due to the schedule of the ultrasound measurement, each group needed to be scanned independently to avoid instrument conflict. Therefore, the 22℃ group was scheduled first, followed by the 30℃ group. To guarantee that each group experienced at least

one week of stable thermal conditions prior to measurement, the start dates for acclimation were staggered. As a result, the acclimation periods differed slightly between groups, but both followed the same acclimation measurement and stabilization duration.

During the acclimation period, environmental parameters were maintained within stable ranges consistent with the conditions listed in Table 2-2.

Table 2-2 Environmental Parameters under 22℃ and 30℃

Temperature (℃)	pH	DO (mg/L)	Salinity (ppt)
22 ± 0.2 ℃	8.0 ± 0.2	> 7.0	30
30 ± 0.2 ℃	8.0 ± 0.2	> 6.0	30

In conclusion, this acclimation measurement ensured all clams were physiologically stabilized under their conditions, providing a consistent baseline for subsequent behavioral observations.

2.4.3 Experimental Setup

Behavioral measurements were not conducted in the same tanks used for acclimation. Instead, a physically separate experimental tank was prepared, which maintained the same structural design and environmental controls as the acclimation tanks. This setup ensured consistency in test conditions while facilitating standardized instrument placement and observation.

After the acclimation period, behavioral monitoring was conducted in a series of controlled experiments. As shown in Fig. 2-10, the entire experiment was divided into six monitoring rounds - three for each thermal condition.

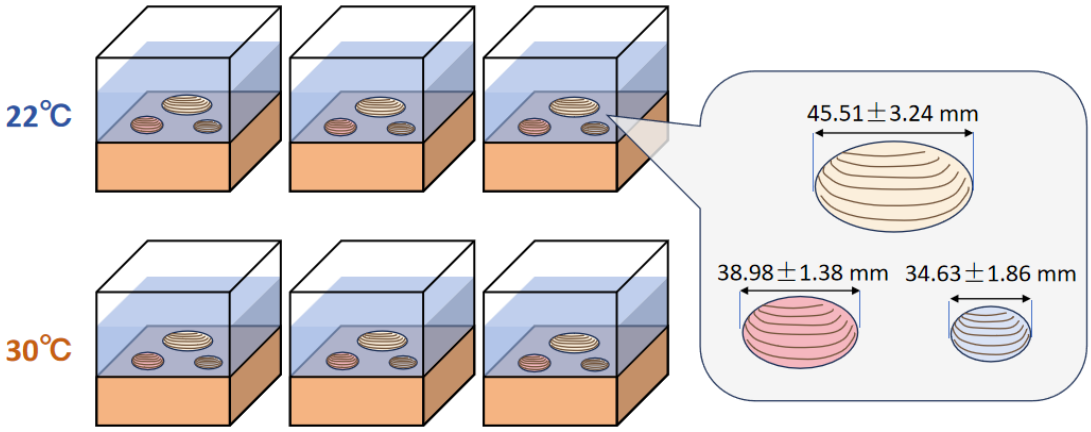


Fig. 2-10 Experimental Design and Size Distribution of Clams

For each monitoring round, one individual from each size class (large, medium, and small) was carefully selected from the acclimation tank and transferred to the experimental tank. Their positions within the sediment were assigned as follows: the large clam was placed in the upper area of the tank, the medium-sized clam in the lower-left, and the small clam in the lower-right. To prevent behavioral interference, clams were spaced at least 5 cm apart from each other during placement.

Each round of behavioral observation lasted 24 hours. The three rounds under 22 ℃ were conducted first, followed by a complete replacement of the artificial seawater and an adjustment day to raise the tank temperature to 30 ℃. The remaining three rounds under 30 ℃ were then conducted following the same protocol.

2.4.4 Data Acquisition

During each 24-hour behavioral monitoring, an ultrasound probe and an interval camera were used to non-invasively observe the burrowing behavior of clams within the sediment. The overall schematic diagram is shown in Fig. 2-11.

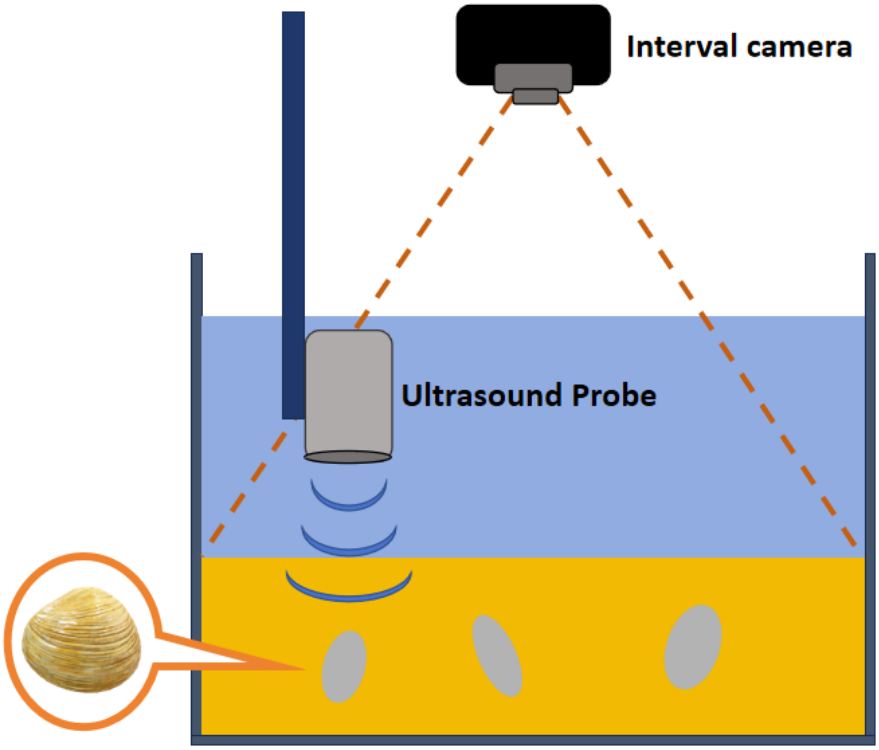


Fig. 2-11 Non-Invasive Monitoring of Burrowing Behavior

The ultrasound probe was used to observe the behavior of clams under sediment. As shown in Fig. 2-12. It scanned a specific area of the sediment ($20 \times 20\ cm$) at regular intervals of 20 minutes. The probe was installed on a motorized scanning platform, and the scanning path followed a raster pattern from left to right, ensuring full coverage of the targeted area. The sediment depth in the experimental tank was maintained at about $15\ cm$, which was sufficient for clams burrowing in the sediment.

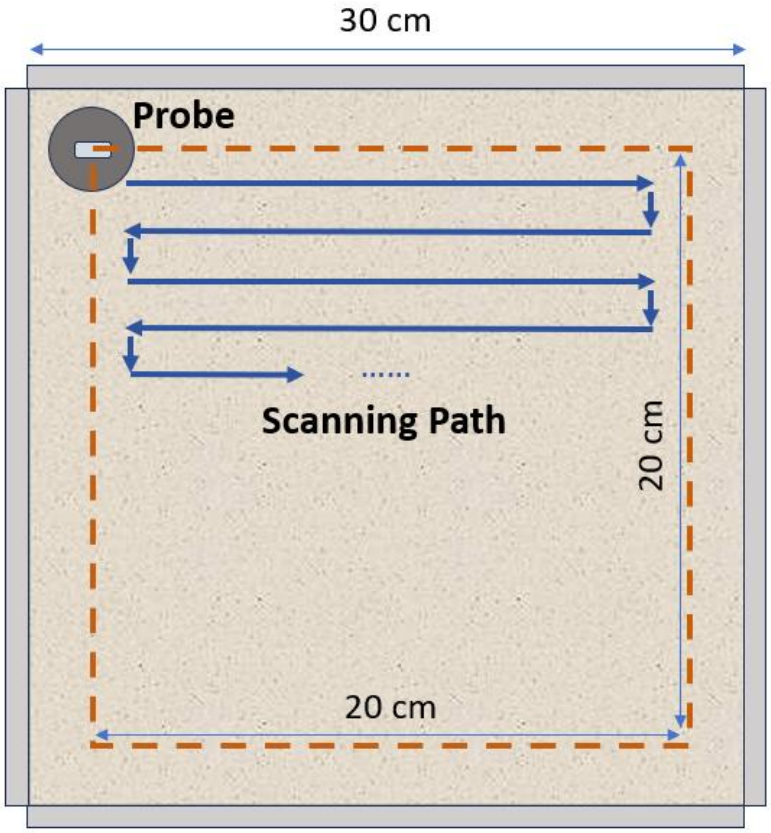


Fig. 2-12 Ultrasound scanning range and path

Meanwhile, an interval camera was positioned above the tank to capture top-view optical images of the sediment surface. The interval camera operated in a time-lapse mode with a temporal resolution of 5 seconds. It can record clear clam movements. The surface trails left by clams provided useful visual cues for approximating their subsurface positions. These cues enhanced the accuracy of subsurface localization during ultrasound image analysis.

After each scanning session, ultrasound data were stored in .csw format and later reconstructed into 3D ultrasound volumes. These data were used for the analysis of burrowing depth, posture, and displacement. The time-lapse videos recorded by the interval camera were saved in .AVI format and then used to analyze clam burrowing timing.

2.5 Quantification of Burrowing Behavior

2.5.1 Data Processing

In this study, each 24-hour monitoring generated 72 ultrasound cubes and one group of videos. The acoustic data contained the features of both the clams and sediment surface. To make the data useful for behavioral analysis, we applied basic processing to extract the feature and prepare for later quantification of burrowing activity. In contrast, the optical data were mainly used to observe the initial burrowing on the sediment surface and thus required no further processing.

An overview of the acoustic data processing workflow is shown in Fig. 2-13, which includes preprocessing and feature extraction.

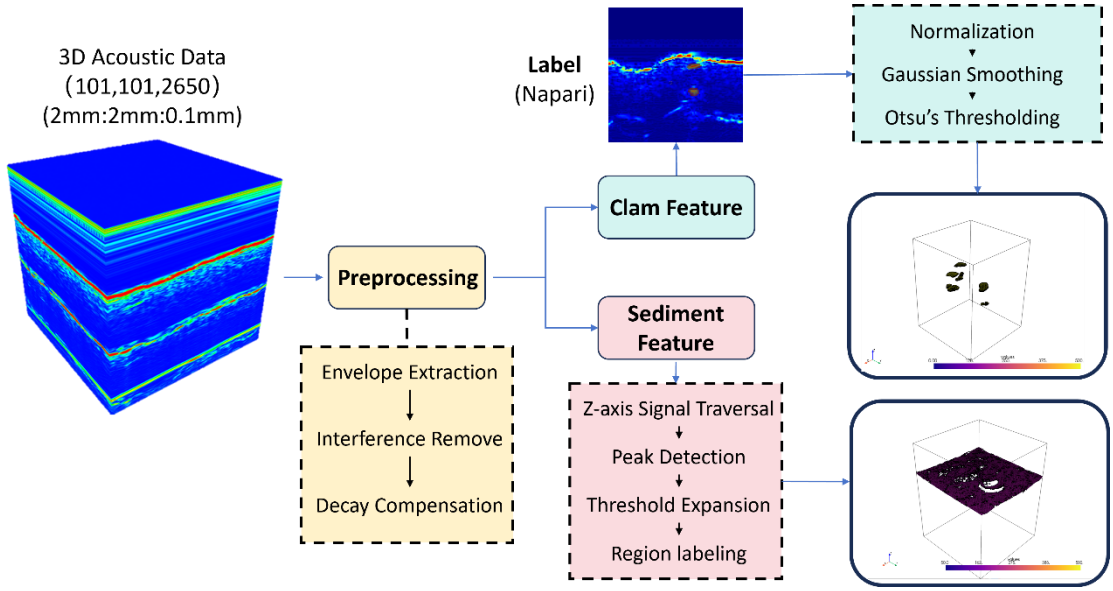


Fig. 2-13 Preprocessing of acoustic data

1. Preprocessing

The original 3D ultrasound dataset comprised $101 \times 101 \times 2650$ voxels, with a spatial resolution of $2\,mm \times 2\,mm$ in the horizontal plane and $0.1\,mm$ along the vertical axis. To ensure the quality and interpretability of the raw acoustic data, preprocessing was implemented prior to feature extraction. Three key procedures were included: envelope extraction, interference removal and attenuation compensation.

First, envelope extraction was applied to smooth the raw acoustic signal and highlight the amplitude profile of reflection intensities. It used the peak interpolation method to identify local peak values along the Z-axis signal and generated a continuous envelope

curve. This measurement was used to improve the detectability of the features.

Next, interference removal was applied to eliminate the reflections from the transducer lens and the bottom of the tank. Shown in Fig. 2-14, reflections from the lens typically appear near the top of the signal profile. Similarly, strong acoustic reflections from the tank bottom also produced strong signals, which were unrelated to the clam features. By setting two height thresholds along the z-axis, we can separate the regions of lens reflections and bottom echoes from the whole data and set their intensity values to zero. This method eliminates the interference of irrelevant reflections and reduces the possibility of false feature detection during segmentation.

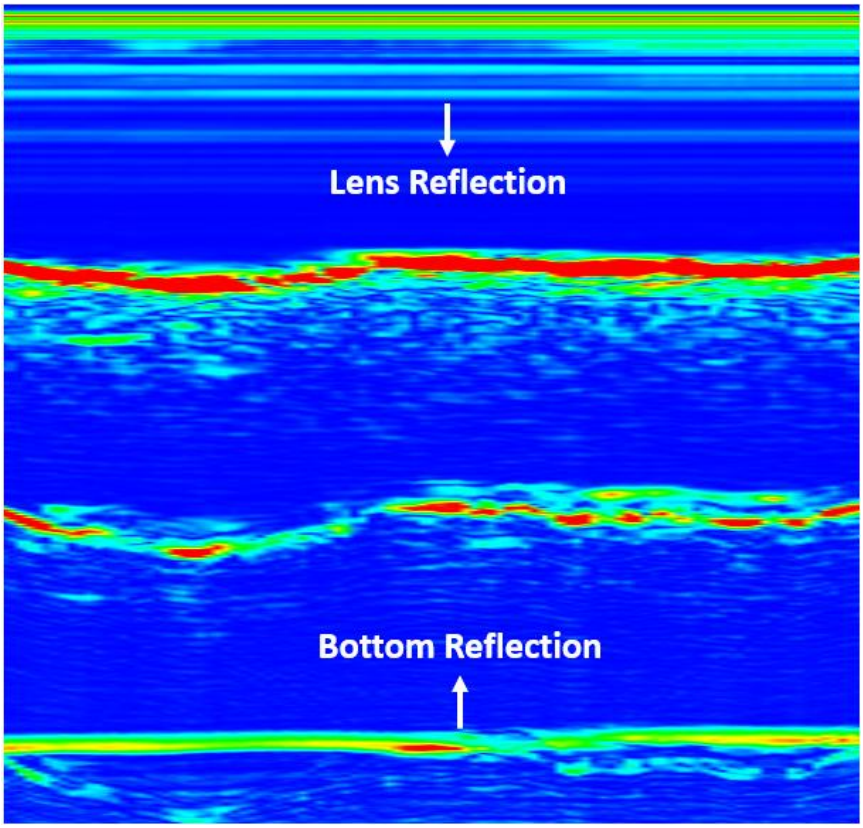


Fig. 2-14 Position of lens and bottom reflection

Additionally, attenuation compensation was performed to correct signal attenuation along z axis. Because acoustic energy decays exponentially with increasing propagation distance, deeper echoes tend to exhibit lower amplitude values. To deal with this issue, we applied an exponential compensation factor to amplify deeper signals, thereby restoring intensity contrast across the full depth range. The equation of compensated amplitude $A_{corr(z)}$ is shown as follows:

$$A_{corr(z)} = A_{(z)} \cdot e^{\propto z}$$

where:

$A_{(z)}$ is the original amplitude at depth z,

α is the attenuation coefficient.

This method enabled consistent visualization of deeper regions and improved the accuracy of feature quantification, particularly for clams that burrowed at deeper depths.

2. Feature Extraction

Feature extraction consists of two parts: clam feature and sediment feature.

Clam Feature Extraction

To extract clam features, we first manually labeled the clam regions by inspecting both vertical and lateral slices of the ultrasound volume. Labeling was based on high-intensity echo zones, typically around a value of 900, which are associated with the clam body. The labeled volumes were saved in .lsm format.

Next, the extracted clam volume matrix was preprocessed to enhance the clarity and separability of clam structures. Specifically, the matrix was first normalized by dividing all voxel values by the maximum intensity. A Gaussian filter was then applied to reduce local noise.

After that, high-intensity regions were separated using K-means clustering method. This method can automatically group voxels into two clusters based on the spatial coordinates of connected high-signal regions. In this sense, it can effectively recognize the top and bottom reflection of the clam body.

For each cluster, Otsu's thresholding method was applied to determine an optimal cutoff for binary segmentation. This method automatically selects the threshold that maximizes the variance between the foreground and background, allowing adaptive separation of signal from noise. As a result, high-intensity clam structures were retained, while low-intensity background and artifacts were effectively excluded. The final segmented volumes were used for subsequent quantitative analysis.

Sediment Feature Extraction

To extract sediment features, clam regions were first removed from the acoustic

volume by masking out all segmented clam voxels. This ensured that sediment signals could be analyzed independently of clam interference.

Then, a custom sediment algorithm was applied to identify the sediment layer. For each (x, y) coordinate, the acoustic signal along the Z-axis was analyzed to detect the first significant peak. This peak represents the upper boundary of the sediment, typically characterized by a sudden increase in reflection intensity due to acoustic impedance changes, though occasionally masked by local signal variations or noise.

For peak detection, we applied a relative threshold of 25% of the maximum signal intensity to exclude weak background fluctuations. After the peak was detected, the sediment region was extended vertically using a continuation threshold of 85% of the peak amplitude, allowing up to three consecutive low-intensity voxels to accommodate minor signal irregularities.

These threshold values were empirically chosen based on visual inspection and prior experience with similar acoustic data. Although not systematically optimized, they provided consistent results in our datasets. Future studies may adjust these parameters to suit different imaging conditions or signal characteristics.

In the end, the output was a 3D binary mask including the sediment region. The voxels within the detected sediment layer were assigned a value of 1 and others set to 0. This mask was then traversed along vertical directions to extract the center position of the sediment layer. The center position is the coordinate of the sediment surface.

2.5.2 Definition of Behavioral Indicators

In this study, we designed a series of indicators to quantify clam burrowing behavior. There are six types of indicators, ranging from initial reaction to long-term sediment disturbance. The indicators were defined as follows:

1. Burrowing Time (s)

Burrowing time is the duration from the start of the monitoring period until the clam's upper surface was fully submerged beneath the sediment. This indicator reflects the initial behavioral response of the clam.

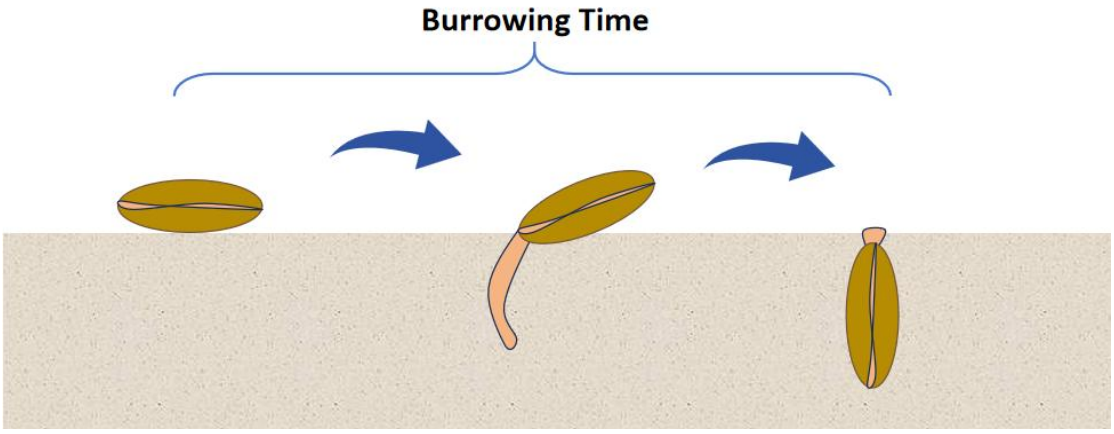


Fig. 2-15 Definition of burrowing Time

2. Burrowing Depth (mm)

The burrowing depth was defined as the shortest three-dimensional Euclidean distance from the top of the clam body to the sediment surface. Shown as Fig. 2-16, to ensure consistency and eliminate the influence of clam-induced sediment disturbance, the sediment surface was used only from the initial scanning before clams placing on the sediment.

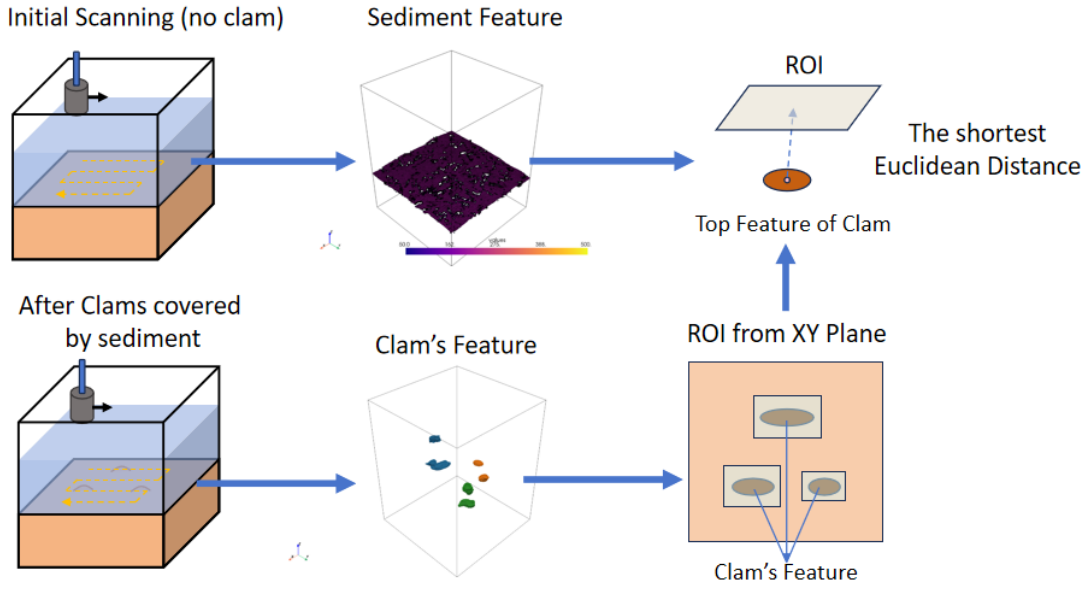


Fig. 2-16 Workflow for defining burrowing depth. The sediment feature acquired prior to the formal measurements was used as a reference. A region of interest (ROI) was defined on the XY plane, within which the shortest Euclidean distance from the centroid of the clam's top feature to the corresponding portion of the sediment surface was calculated.

At each time point, the top surface of the clam was first extracted from its segmented volume. Based on the XY extent of this surface, the minimum and maximum X and Y coordinates were determined to form a bounding box. This box was then expanded by ± 10 pixels in both directions to define a region of interest (ROI) that captured the local surroundings of the clam. Within this ROI, the corresponding portion of the fixed sediment reference surface was retrieved for depth calculation.

Next, the center point of the clam's top surface was identified using the centroid of the segmented top region. The 3D Euclidean distances between this point and all surface voxels within the sediment ROI were computed. The shortest of these distances was taken as the burrowing depth. To distinguish buried from unburied states, the value was recorded as positive if the clam was located below the sediment surface, and negative otherwise.

This method ensured a consistent and precise depth estimation by referencing a fixed sediment surface acquired from the initial undisturbed state, thereby avoiding bias from local bioturbation effects in later time points.

3. Posture (°)

The posture was defined as the angle between the vertical axis and the vector connecting the centroids of the top and bottom segmented feature of the clam. The formula is as follows:

$$tilt = \cos^{-1}\left(\frac{v_z}{\|\vec{v}\|}\right) \times \frac{180}{\pi}$$

where

$\vec{v}$ is the 3D vector connecting the centroids of the top and bottom segmented feature of the clam,

v_z is the Z-component of $\vec{v}$.

A smaller angle indicates a more upright position, whereas a larger angle suggests a tilted posture during burrowing.

4. Displacement (mm)

The displacement of the clam was defined as the Euclidean distance between its positions across consecutive scanning frames. Shown in Fig. 2-17, the clam's position

was calculated as the midpoint of the line segment connecting the centroids of the top and bottom segmented regions. The formula is as follows:

$$d^{(t)} = \frac{p_{top}^{(t)} + p_{bottom}^{(t)}}{2} \qquad p_{top}, p_{bottom} \in R^3$$

$$\Delta d^{(t)} = \left\| d^{(t)} - d^{(t-1)} \right\|$$

where

$p_{top}^{(t)}$ is 3D coordinate of the centroid of clam's top feature at time frame t,

$p_{bottom}^{(t)}$ is 3D coordinate of the centroid of clam's bottom feature at time frame t,

$d^{(t)}$ is the position of the clam at time frame t,

$\Delta d^{(t)}$ is the displacement of clam between time frame $t-1$ and t.

This indicator was used to quantify the overall movement of the clam during the burrowing process. It also served as a basis for the subsequent assessment of behavioral stability, such as identifying periods of active versus stationary behavior.

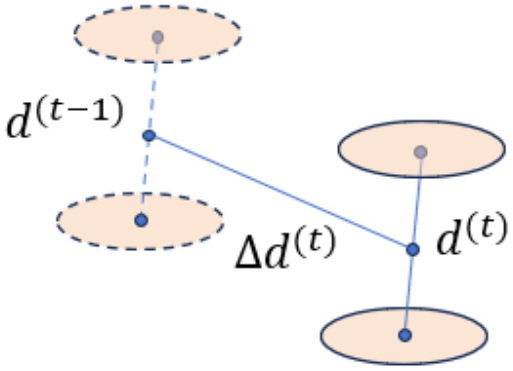


Fig. 2-17 Definition of displacement

5. Behavioral Stability

To quantify the behavioral activity of clams during burrowing, we defined two behavioral states: **"Active"** and **"Stable"**, based on their frame-to-frame displacement. We set up two displacement thresholds—one for horizontal displacement and one for vertical displacement.

At each time frame:

- If the clam's displacement exceeded the threshold, it was classified as **Active**.
- If the displacement was below or equal to the threshold, it was classified as

Stable.

Based on state segmentation, three behavioral stability indicators were computed to quantify the behavioral Stability:

(1) Stable Ratio:

The proportion of time frames classified as "Stable" over the total monitoring period. The equation is shown as below:

$$Stable\ Ratio = \frac{N_s}{N_s + N_A}$$

where

N_S is the number of "Stable" states.

N_A is the number of "Active" states.

(2) Number of Stable Segments (S_n)

The number of continuous "Stable" periods, representing how fragmented the stable behavior is.

(3) Mean Stable Duration

The average duration of stable segments reflecting the persistence of inactivity. The equation is as follows:

$$Mean\ Stable\ Duration = \frac{N_s}{S_n}$$

These indicators were calculated separately for horizontal and vertical displacement to capture direction-specific differences in burrowing dynamics.

6. Disturbed Volume

The disturbed volume is used to describe the intensity of clam's bioturbation. It is defined as the accumulated volume of sediment surface changes over time, calculated only for areas where the vertical displacement between two consecutive frames exceeds a specified threshold (2mm). By using this method, we can calculate the total disturbance volume of the three clams under each monitoring group. The equation is shown as below:

$$Disturbed\ Volume = \sum_{t=2}^{n} \sum_{(x,y):|\Delta Z_t(x,y)| \geq T} |\Delta Z_t(x,y)| \cdot dx \cdot dy \cdot dz$$

where

t is the index of scanning time point,

n is the total number of scanning time points,

$\Delta Z_t(x, y)$ is the vertical voxels difference of sediment surface between frames t and t-1 at 2D coordinates (x, y).

In addition, we estimated the disturbed volume for each individual clam based on a ROI strategy, shown in Fig. 2-18.

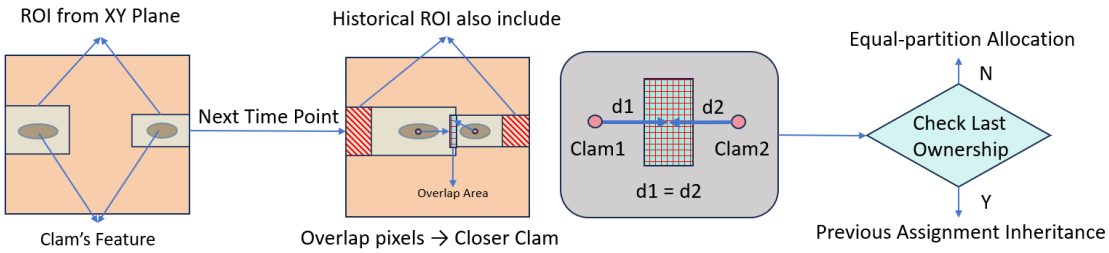


Fig. 2-18 Estimation of disturbed volume for each clam

Similar to the computation of the total disturbance volume, we first calculated the vertical displacement of the sediment surface within the full field of view. Then, for each individual clam, we extracted the corresponding portion of this displacement map using the clam-specific ROI.

Next, for each time point, the XY projection of the clam's body was first determined. The ROI was then defined by expanding the bounding box of the clam's segmented region by a fixed margin (± 20 pixels). To ensure spatial continuity, the ROI also included historical boundaries accumulated over time.

To assign disturbed regions to individual clams, the overlapping pixels between ΔZ and each clam's ROI were evaluated. If a disturbed pixel fell into multiple ROIs, it was assigned to the nearest clam based on Euclidean distance. In cases of distance tie, the algorithm checked the previous frame's assignment. If the same clam owned the pixel previously, the assignment was inherited. Otherwise, the disturbed value was equally divided among all candidate clams.

This dynamic assignment approach ensured that disturbance volumes were reasonably attributed to each clam even in close-proximity or overlapping scenarios.

2.6 Statistical Analysis

After calculating the burrowing behavior indicators, it is important to apply statistical tests to evaluate the relationship between the dependent and independent variables. These statistical analyses not only help us quantify group differences but also identify potential behavioral pathways.

2.6.1 Non-parametric comparison of behavioral indicators

First, we used non-parametric statistical methods to assess the effects of temperature and clam size on burrowing behavior. An overview of the statistical questions, applied methods, and corresponding effect size metrics is provided in Table 2-3.

Table 2-3 Summary of statistical methods and effect size metrics for evaluating temperature and size effects

Statistical Question	Statistical methods	Effect Size
Overall temperature effect	Mann–Whitney U test	r (rank-biserial)
Temperature effect within each size class	Mann–Whitney U test	r (rank-biserial)
Overall size effect	Kruskal–Wallis test	η^2 (Kruskal–Wallis eta-squared)
Size effect within each temperature condition	Kruskal–Wallis test + Dunn's test	η^2 (Kruskal–Wallis eta-squared)

1. Mann–Whitney U test

The Mann–Whitney U test is a rank-based non-parametric test used to compare two independent groups.[84] In this study, it was applied to assess whether the distribution of behavioral indicators differed significantly between the two temperature conditions (22 ℃ and 30 ℃), both overall and within each size class. This test is robust to outliers and does not require the assumption of normally distributed data, making it appropriate for our small sample sizes.

The U statistic is defined as:

$$U = n_1 n_2 + \frac{n_1(n_1+1)}{2} - R_1$$

where n_1, n_2 are sample sizes in the two temperature groups, and R_1 is the sum of

ranks in group 1.

To quantify the magnitude of the observed differences, we calculated the rank-biserial effect size r, which provides a standardized measure of how strongly the two distributions differ:

$$r = \frac{Z}{\sqrt{N}}$$

here, Z is the standard score associated with the U statistic, and N is the total number of observations. According to Cohen's guidelines, $|r| = 0.1$ is considered a small effect, 0.3 medium, and ≥ 0.5 a large effect. Reporting effect size allows interpretation of practical significance even when p-values are non-significant.

2. Kruskal–Wallis test

To examine the influence of clam size (large, medium, small) on behavioral indicators, we used the Kruskal–Wallis test—an extension of the Mann–Whitney U test for comparing more than two independent groups.[85] This test was used both across all temperatures (overall size effect) and within each temperature condition (conditional size effect).

The H statistic is given by:

$$H = \frac{12}{N(N+1)} \sum_{i=1}^{k} \frac{R_i^2}{n_i} - 3(N+1)$$

Where k is the number of groups, n_i is sample size of group i, and R_i is its rank sum.

To estimate the strength of the group differences, we also calculated the Kruskal–Wallis eta-squared η^2:

$$\eta^2 = \frac{H - k + 1}{N - k}$$

This statistic represents the proportion of variability in the outcome that can be explained by the grouping factor (size class), helping assess the substantive significance of the result.

3. Dunn's post-hoc test

When comparing more than two groups using non-parametric methods such as the Kruskal-Wallis test, a significant result only indicates that at least one group differs

from the others. However, it does not identify which specific groups are different. Therefore, to identify the source of the overall difference, we applied Dunn's post hoc test for pairwise comparisons among size classes.

In this study, Dunn's test was applied to all pairwise groupings, even when the Kruskal–Wallis test did not reach statistical significance. This approach was taken to fully explore the possible differences among groups, particularly given the small sample size and the exploratory nature of this study.

All p-values were adjusted using Bonferroni correction to control for Type I error inflation. Although this comprehensive post hoc analysis exceeds the typical practice of restricting comparisons to significant omnibus results, we interpreted the outcomes with caution and mainly reported confirmatory findings.

2.6.2 Structural Equation Modeling (SEM)

To further investigate the relationships among behavioral responses under different temperature conditions, we employed piecewise structural equation modeling (piecewise SEM). This method allows the combination of multiple regression models to evaluate both direct and indirect effects within a causal network, even when the data violates assumptions of multivariate normality or independence.

Piecewise SEM is a widely used statistical framework that integrates multiple interrelated equations, enabling researchers to evaluate complex causal hypotheses. As noted by Lefcheck et al., piecewise SEM can effectively resolve complex multivariate relationships among variables, while allowing flexible model structures and response distributions.[86] It has been widely applied in marine and ecological studies to evaluate how environmental factors influence biological processes through multiple interconnected mechanisms.

In this study, we constructed three types of structural models, as shown in Fig. 2-19.

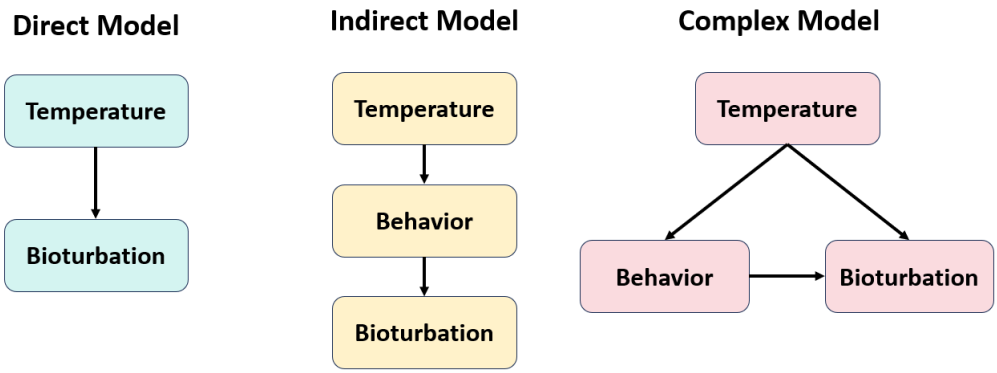


Fig. 2-19 Structural model types used in piecewise SEM analysis

The direct model includes only temperature and size effects on disturbed volume, representing the most simplified structure with no mediating variables.

The indirect model introduces behavioral indicators as mediators between environmental factors and bioturbation outcomes. This model aims to identify potential intermediate processes through which temperature influences sediment disturbance.

The complex model combines both direct and indirect pathways, integrating all measured behavioral traits as potential mediators while retaining direct effects of temperature. The model allows for a more comprehensive evaluation of how thermal stress and individual traits interact to shape bioturbation intensity.

Although the initial analysis of disturbed volume was conducted at the tank level, the SEM was based on individual estimation. This change in analytical unit was necessary to align with the resolution of individual behavioral indicators, such as displacement and stability, which were also measured per individual. While this estimation method introduces some degree of uncertainty, it enables mechanistic interpretation of how specific behavioral patterns contribute to sediment disturbance.

In conclusion, these models were compared to clarify behavioral mechanisms, serving as a complementary approach to the statistical analysis and providing additional insight for clam responses to thermal stress.

3. Result

To comprehensively understand the burrowing responses of clams under thermal stress, we conducted three analytical methods, including descriptive visualization, non-parametric statistical testing, and structural equation modeling.

First, we used boxplots to visualize the distribution patterns of key behavioral indicators across different temperatures and size classes. This initial descriptive analysis helped reveal overall trends and potential group-wise differences.

Subsequently, non-parametric statistical tests were conducted to examine the significance of these differences. By evaluating both statistical significance and effect sizes, we identified which behavioral traits were most sensitive to thermal and size-related factors.

Finally, to uncover the potential behavioral mechanisms underlying sediment disturbance, we constructed SEM. This model allows us to integrate multiple behavioral variables and infer causal relationships, providing insights into how temperature and behavioral stability jointly influence bioturbation intensity.

3.1 Descriptive Trends of Behavioral Indicators

3.1.1 Burrowing Time

As shown in Fig. 3-1, clams exposed to 30℃ exhibited shorter burrowing times compared to those at 22℃. The median burrowing time decreased from 505 seconds at 22℃ to 290 seconds at 30℃, and the data spread was narrower under the higher temperature.

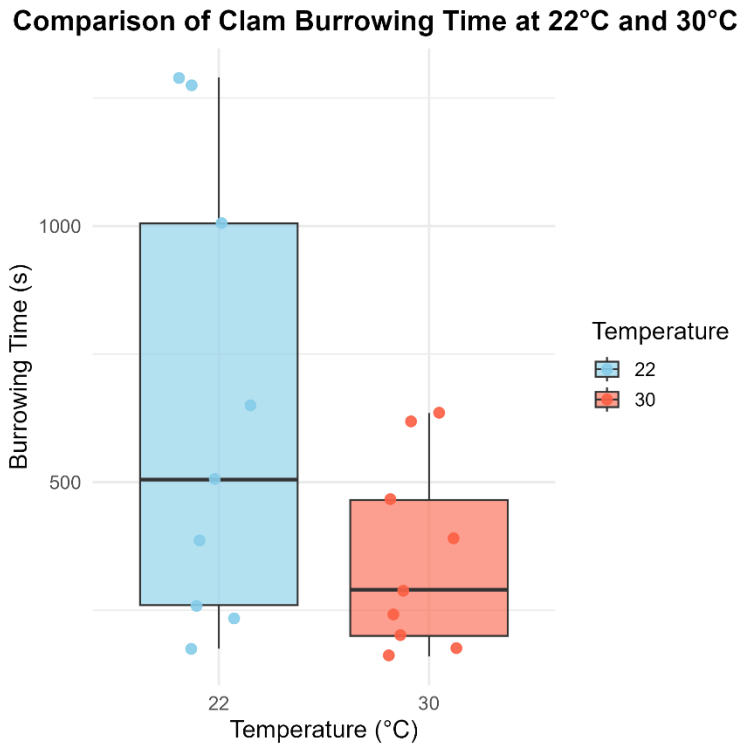


Fig. 3-1 Comparison of Clam Burrowing Time at 22℃ and 30℃

This trend may reflect an acceleration of burrowing behavior under thermal stress, potentially related to a rapid escape response, or increased metabolic activity. However, individual variability remained observable under ambient conditions.

To further examine how temperature effects varied across body sizes, burrowing times were compared across three size classes under each temperature condition, as shown in Fig. 3-2. Although the reduction in burrowing time was consistent across all size classes, the magnitude of change differed: large individuals showed the most pronounced decrease, while medium and small individuals exhibited shorter times at both temperatures.

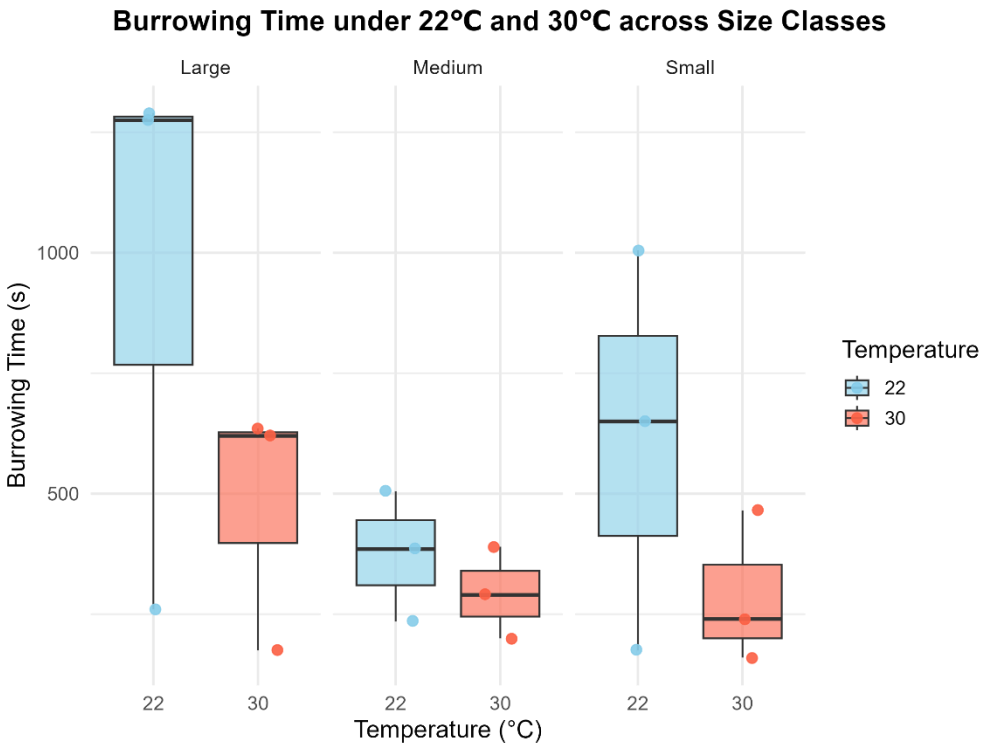


Fig. 3-2 Burrowing Time under 22℃ and 30℃ across Size Classes

3.1.2 Burrowing Depth

To quantify the clams' vertical positioning in the sediment, we calculated the mean burrowing depth for each individual by averaging its depth values across the 24-hour monitoring period. This metric reflects the clam's overall level of burrowing. The higher value indicated deeper burrowing.

As shown in Fig. 3-3, clams in the 22℃ group tend to maintain deeper mean burrowing depths compared to those in the 30℃ group. This trend indicates that elevated temperatures may be associated with reduced burrowing depth, potentially reflecting altered behavioral responses to thermal stress.

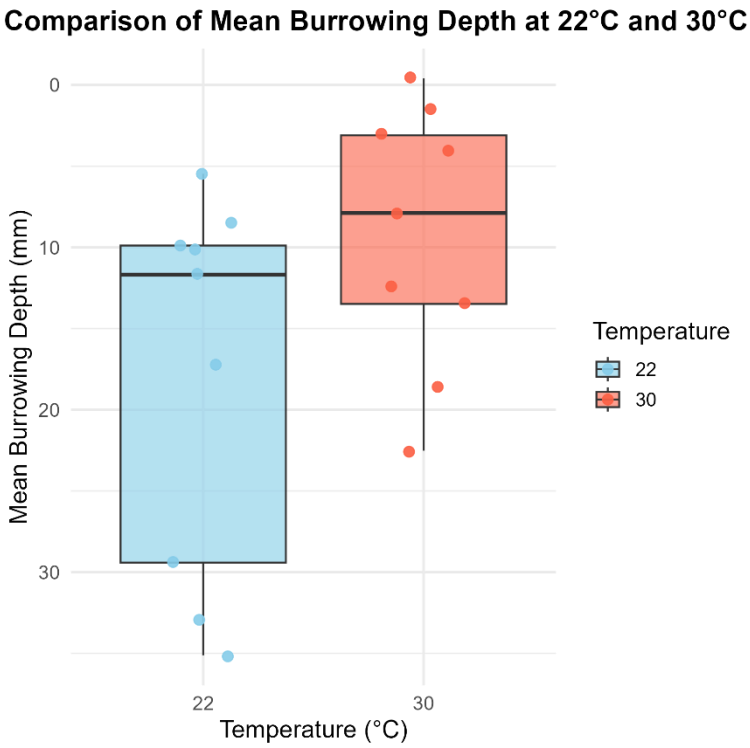


Fig. 3-3 Comparison of Mean Burrowing Depth at 22℃ and 30℃

Next, we further examined burrowing depths across three size classes at both temperatures, shown in Fig. 3-4. Large clams showed observable reduction in burrowing depth under high temperature, while medium and small individuals also exhibited decreasing trends. In addition, small clams appeared to remain at shallower depths under both temperatures, which may reflect size-related tendencies in burrowing depth.

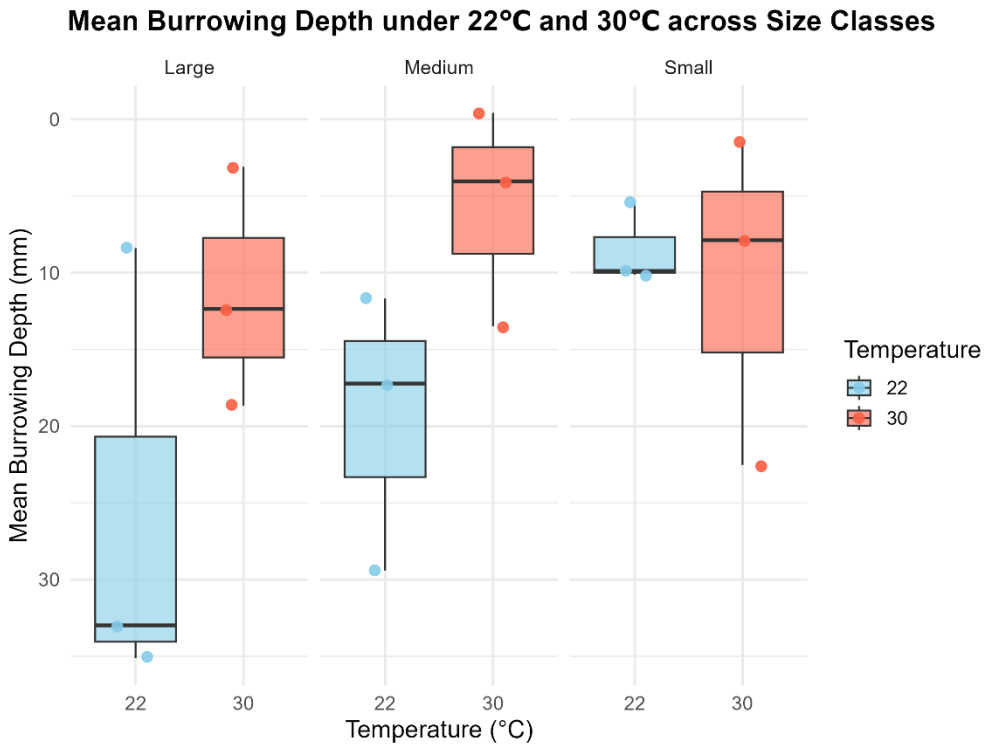


Fig. 3-4 Mean Burrowing Depth under 22℃ and 30℃ across Size Classes

3.1.3 Posture

To evaluate body orientation in the sediment, we calculated the mean tilt angle for each individual clam, defined as the average degree between the clam's body axis and the horizontal plane. A tilt angle close to 180° indicates that the clam remained upright, with its body axis nearly perpendicular to the sediment surface.

As shown in Fig. 3-5, the mean tilt angle remained consistently high in both temperature groups. Although the variation appeared slightly greater at the higher temperature, the overall difference was minimal.

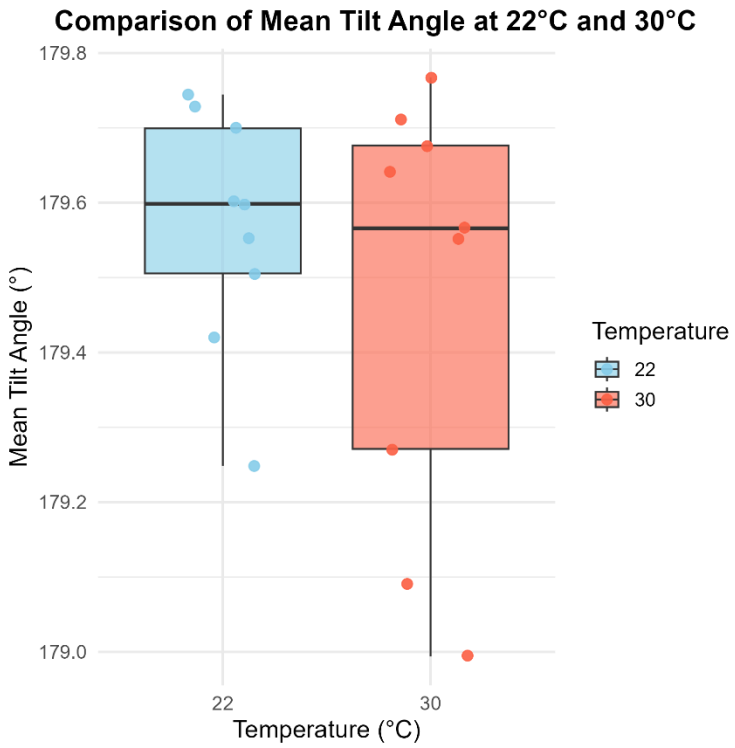


Fig. 3-5 Comparison of Mean Tilt Angle at 22℃ and 30℃

Due to the limitations of ultrasound imaging and scanning intervals, we may not have captured the tilt angles during rapid movements. However, the results indicate that the clams remained in a nearly upright posture for most of the observation period.

3.1.4 Displacement

To evaluate the movement of clams, we tested their cumulative displacement in both the horizontal (XY plane) and vertical (Z axis) directions. Specifically, we used the total XY cumulative displacement and Z cumulative displacement recorded at the final time point of a 24-hour observation period following the completion of initial burrowing. These indicators can reflect the total movement of the clams under the sediment.

As shown in Fig. 3-6 and 3-7, clams in the 30℃ group tend to have greater horizontal displacement compared to those in the 22℃ group. This trend was consistent across all size classes, with bigger differences observed in large and medium individuals. The median displacement and interquartile ranges were increased under higher temperatures, suggesting more intense lateral movement under thermal stress.

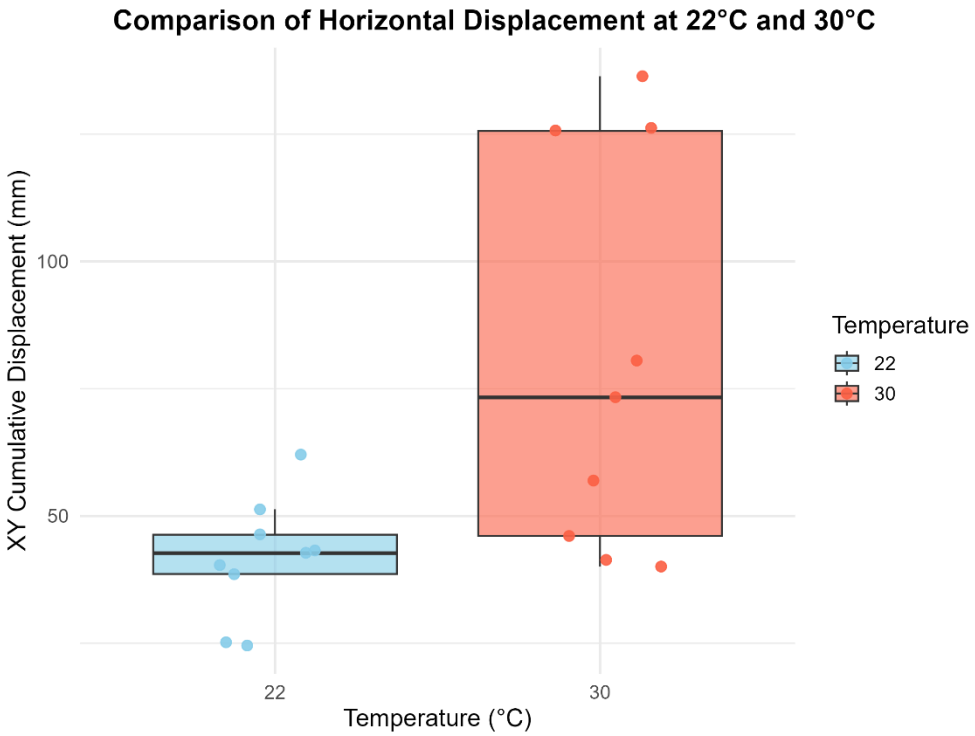


Fig. 3-6 Comparison of Horizontal Displacement at 22℃ and 30℃

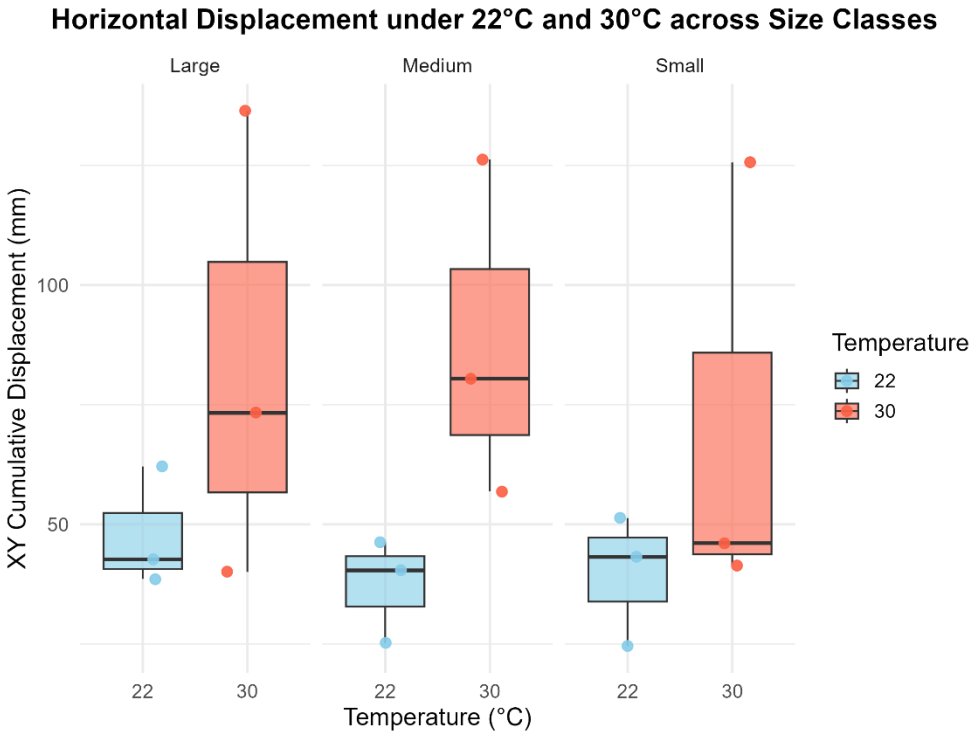


Fig. 3-7 Horizontal Displacement under 22℃ and 30℃ across Size Classes

Shown in Fig. 3-8 and 3-9, different from the horizontal dimension, there is no observable difference in vertical displacement. The median values at 30℃ were

similar to those at 22℃ for most size classes. Only clams in big size showed a little difference to thermal stress.

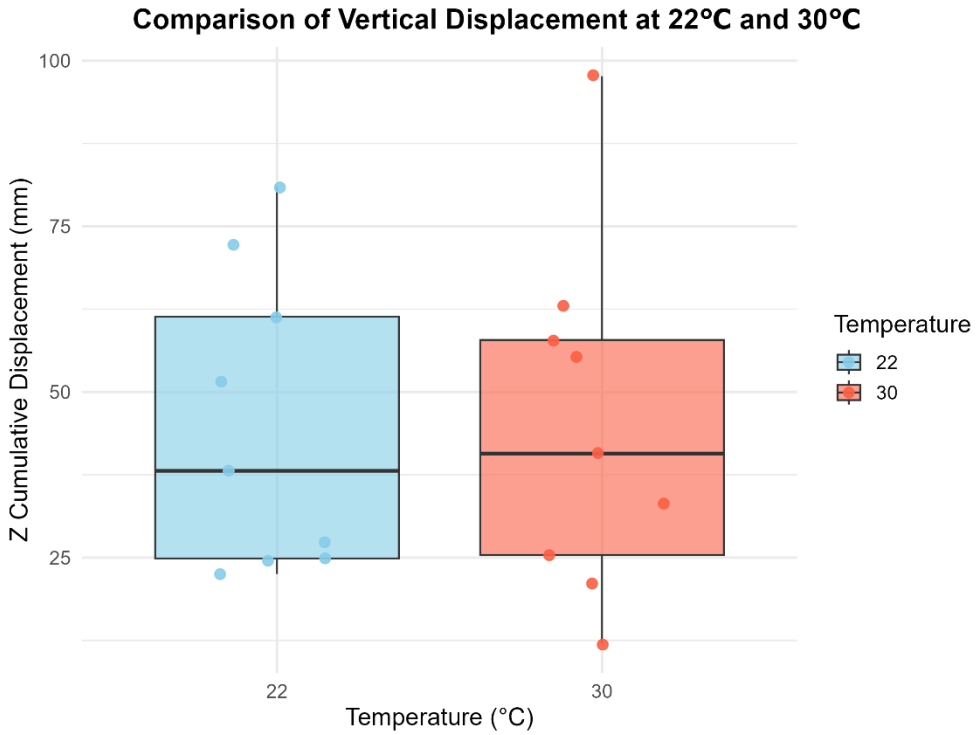


Fig. 3-8 Comparison of Vertical Displacement at 22℃ and 30℃

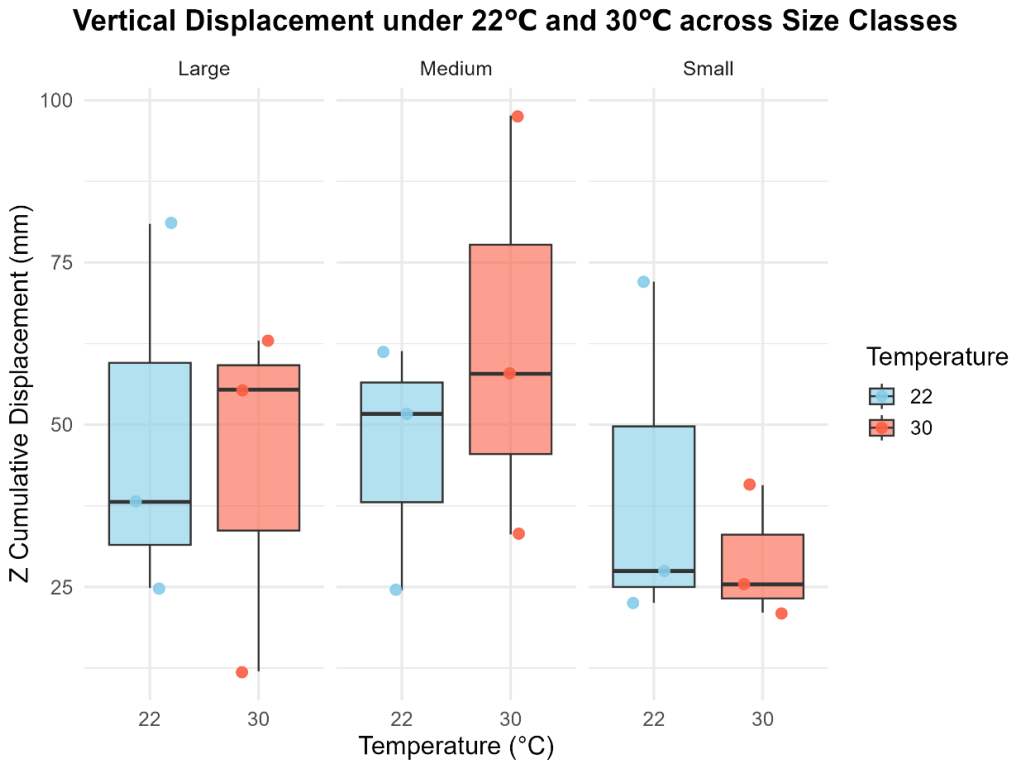


Fig. 3-7 Vertical Displacement under 22℃ and 30℃ across Size Classes

3.1.5 Behavioral Stability

We evaluated behavioral stability separately in the horizontal and vertical directions using the stable ratio.

As shown in Fig. 3-8 and Fig. 3-9, clams exposed to 30℃ tended to exhibit lower stable ratios in the horizontal movement, particularly among large and medium sized individuals.

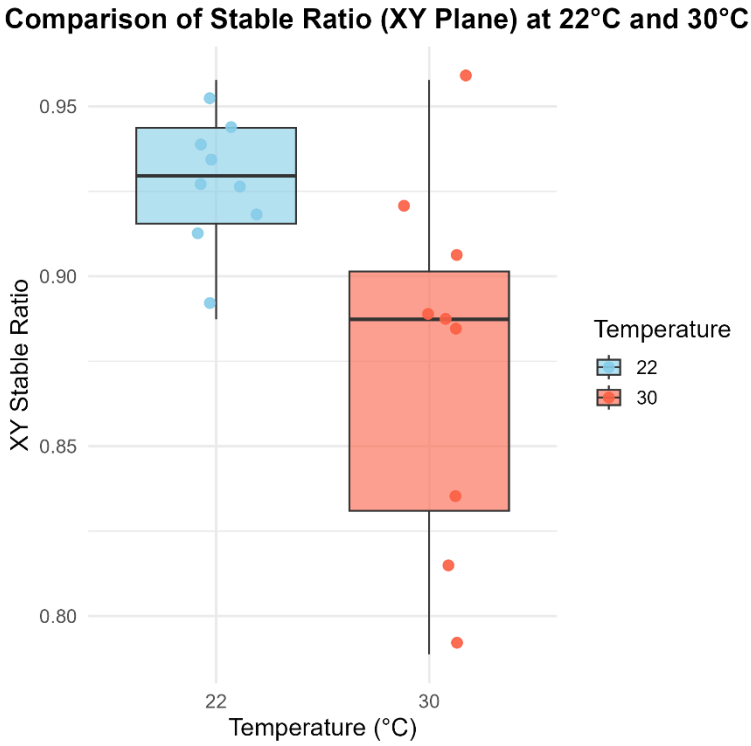


Fig. 3-8 Comparison of Stable Ratio (XY Plane) at 22℃ and 30℃

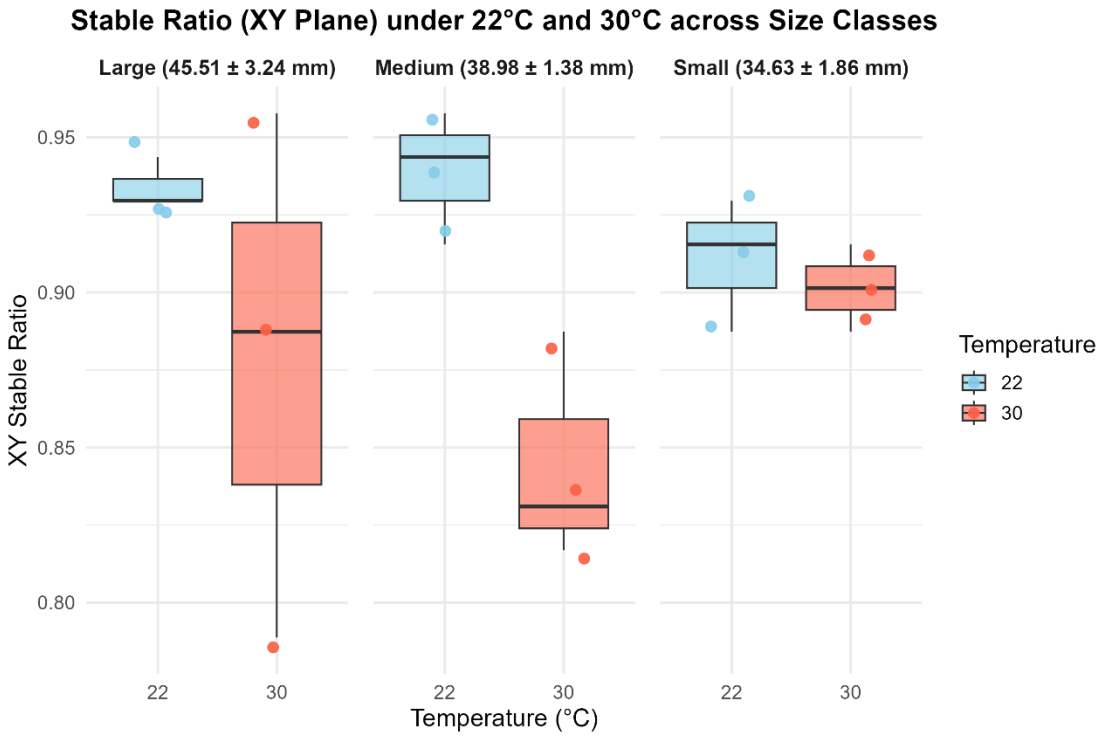


Fig. 3-9 Stable Ratio (XY Plane) under 22℃ and 30℃ across Size Classes

In contrast, differences in vertical movement were not notable, as shown in Fig. 3-10.

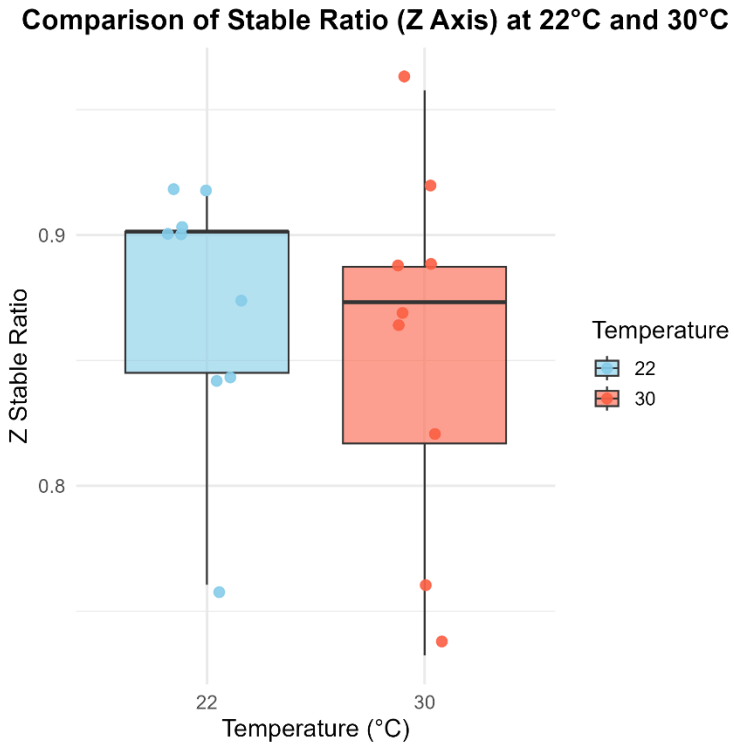


Fig. 3-10 Comparison of Stable Ratio (Z Axis) at 22℃ and 30℃

However, in medium size, the stable ratio of vertical displacement decreased in 30℃ compared to 22℃, which may indicate that the medium size clams are more sensitive to heat stress.

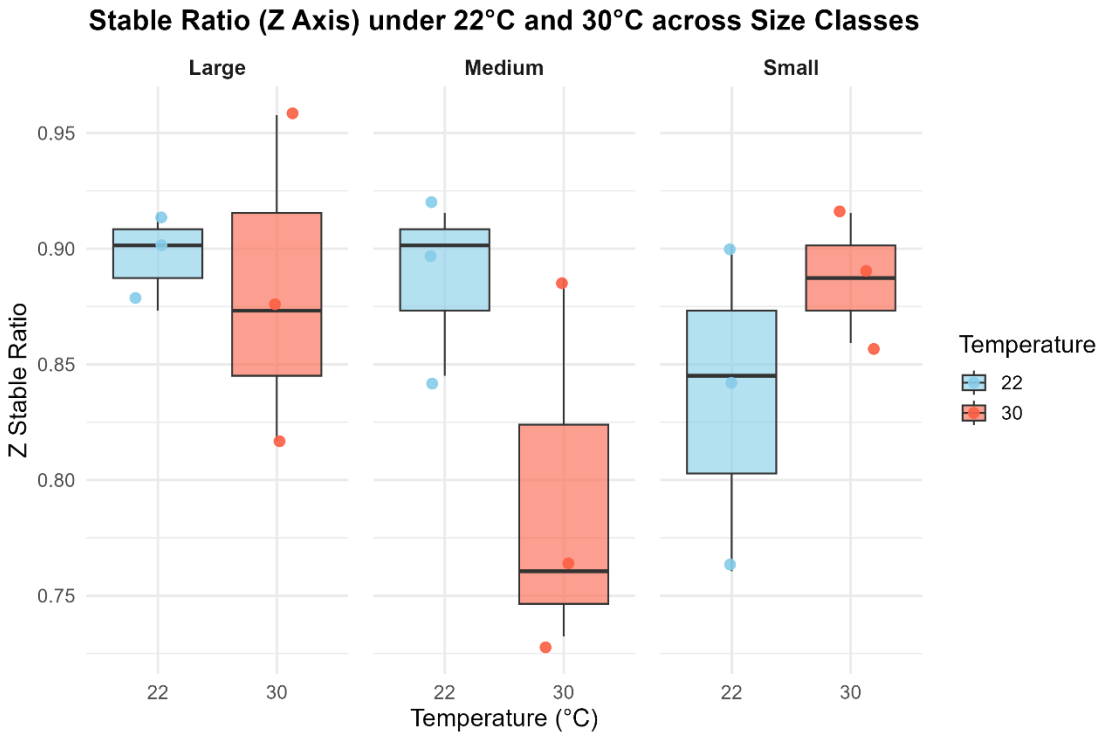


Fig. 3-11 Stable Ratio (Z Axis) under 22℃ and 30℃ across Size Classes

3.1.6 Disturbed Volume

To quantify the bioturbation intensity caused by clam activity, we calculated the disturbed volume within each tank across all scanning frames. For each experimental group, the cumulative disturbed volume over a 24-hour period was summed to represent the total extent of sediment reworking. This value reflects the overall magnitude of burrowing-induced disturbance and was used for our analysis.

As shown in Fig. 3-12, clams at 30℃ generated a larger disturbed volume than those at 22℃. The distribution of values indicates greater inter-individual variability at the higher temperature, with both the median and interquartile range shifted upward. This pattern may indicate increased burrowing activity or sediment disturbance under elevated temperature, though further statistical support is necessary to confirm this interpretation.

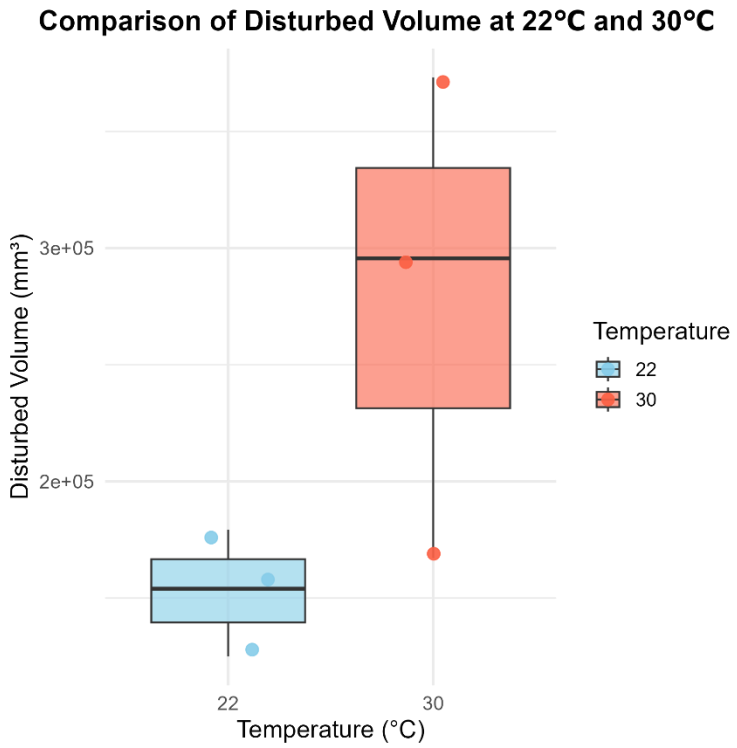


Fig. 3-12 Comparison of Disturbed Volume at 22℃ and 30℃

3.2 Non-Parametric Statistical Analysis of Behavioral Responses

Following the initial descriptive examination, a series of non-parametric statistical tests were applied to determine whether the observed behavioral differences across temperature and size classes were statistically significant. Given the limited sample size and the potential violation of normality assumptions, Mann–Whitney U tests were used for pairwise comparisons between temperature groups, while Kruskal–Wallis tests were employed to assess size-related effects within each temperature condition.

In addition to p-values, we calculated effect sizes to assess the magnitude of group differences. Even when statistical significance was not achieved, effect sizes provided valuable information about the potential biological relevance of the observed patterns. This approach allows for a more comprehensive understanding of the data, especially in cases where small sample sizes may limit statistical power.

3.2.1 Burrowing Time

Assessed by the Mann-Whitney U test, burrowing time exhibited a moderate effect between temperature groups ($r = 0.333$). Although the difference was not statistically significant, the effect size suggests a potentially meaningful biological trend, with clams under different temperature conditions showing variation in burrowing initiation time. When analyzed within size classes, a moderate temperature-related effect ($r = 0.445$) was observed in both large and small individuals. Despite the lack of statistical significance, this may reflect temperature sensitivity in specific size groups.

By contrast, no statistically significant size effects were detected under either temperature condition. Size alone may not strongly influence burrowing time.

Table 3-1 Summary of temperature and size effects on burrowing time

Behavioral Dimension	**Temp. Effect**	**Temp. Effect by Size**	**Size Effect**	**Size Effect by Temp.**
Burrowing Time	No Sig. $r = 0.333$ (Moderate)	No Sig. Size: Large & Small $r = 0.445$ (Moderate)	No Sig.	No Sig.

3.2.2 Burrowing Depth

We evaluated burrowing depth by using mean, minimum, and maximum values, showed varying sensitivity to temperature and size conditions.

Table 3-2 Definitions of burrowing depth metrics used in analysis.

Indicator	**Definition**
Mean burrowing depth	The average burrowing depth during the entire observation period
Minimum burrowing depth	The shallowest depth recorded during the entire observation period.
Maximum burrowing depth	The deepest depth reached by the clam during the entire observation period.

For mean burrowing depth, a moderate effect size was estimated between temperature

groups ($r = 0.364$), although the difference was not statistically significant. When examined within size classes, the effect size was moderate in large individuals ($r = 0.445$) and relatively large in medium-sized individuals ($r = 0.624$). These effect sizes may suggest a potential trend in which medium-sized clams exhibit greater adjustments in average burial depth in response to temperature, though this interpretation remains tentative without statistical support. No main effect of size was detected overall; however, under 22℃, the Kruskal–Wallis test yielded a large effect size ($\eta^2 = 0.215$), which may reflect increased size-related variability at this temperature.

For minimum burrowing depth, a moderate temperature-related effect size was also present ($r = 0.427$), and a particularly large effect was noted in large individuals (r = 0.802). While these observations did not reach statistical significance, they may indicate that large clams responded more strongly in terms of the shallowest depths they occupied. Similar to mean depth, no overall size effect was statistically confirmed, but at 22℃, a large effect size ($\eta^2 = 0.659$) was observed, which could reflect size-associated differences under ambient temperature conditions.

In contrast, maximum burrowing depth showed no significant difference between temperature groups. Nevertheless, a moderate temperature effect size was found in medium-sized clams ($r = 0.445$). Unlike the other two depth metrics, a moderate main effect size of body size was estimated ($\eta^2 = 0.0795$), and this effect persisted at 22℃ ($\eta^2 = 0.126$), possibly indicating some size-related variation in maximum burial capacity, particularly under milder thermal conditions.

While none of these results were statistically significant, some of the estimated effect sizes, especially under 22℃, may reflect biologically relevant trends. These findings remain exploratory and highlight the need for future studies with larger sample sizes to validate these potential behavioral patterns.

Table 3-3 Summary of temperature and size effects on burrowing depth

Behavioral Dimension	Temp. Effect	Temp. Effect by Size	Size Effect	Size Effect by Temp.
Mean burrowing depth	No Sig. $r = 0.364$ (Moderate)	No Sig. Size: Large $r = 0.445$ (Moderate) **Size: Medium** $\boldsymbol{r = 0.624}$ **(Large)**	No Sig.	No Sig. Temp: 22℃ $\eta^2 = 0.215$ (Large)
Minimum burrowing depth	No Sig. $r = 0.427$ (Moderate)	No Sig. **Size: Large** $\boldsymbol{r = 0.802}$ **(Large)**	No Sig.	No Sig. Temp: 22℃ $\eta^2 = 0.658$ (Large)
Maximum burrowing depth	No Sig.	No Sig. Size: Medium $r = 0.445$ (Moderate)	No Sig. $\eta^2 = 0.0795$ (Moderate)	No Sig. Temp: 22℃ $\eta^2 = 0.126$ (Moderate)

3.2.3 Posture

All of the statistical results about clam's posture were not significant, and their effect sizes remained small. This may be due to the relatively small variation in posture across individuals and the limitation in the measurement method.

3.2.4 Displacement and Behavioral Stability

We evaluated movement and behavioral stability in both the horizontal and vertical directions using four key metrics: displacement, stability ratio, number of stable segments, and mean stable duration. These indicators provide complementary perspectives on both the intensity and consistency of burrowing activity under different thermal and size conditions.

Table 3-4 Definitions of displacement and behavioral stability metrics used in analysis.

Indicator	Definition
Displacement	The total cumulative movement recorded over 24 hours.
Stability Ratio	The proportion of time spent in stable states relative to total time.
Stable Segment	The number of distinct continuous stable periods observed during the monitoring period.
Stable Duration	The average length of time for each stable period.

XY displacement exhibited a statistically significant difference between temperature groups (p = 0.0244), accompanied by a large effect size ($r = 0.531$), indicating that horizontal movement increased under thermal stress. When analyzed within size classes, medium-sized individuals showed a particularly strong response ($r = 0.802$), while large individuals showed a moderate effect ($r = 0.445$). No significant main effect of size or temperature–size interaction was detected.

For the XY stability ratio, a significant temperature effect was also observed ($p = 0.016, r = 0.578$), suggesting reduced horizontal stability under elevated temperature. Medium-sized clams again showed a large effect ($r = 0.802$). While no main size effect was present, a large size-related effect was detected at 22℃ ($\eta^2 = 0.17$), indicating increased inter-individual variability in stability at ambient temperature.

Regarding the number of stable segments in the horizontal plane, temperature had a significant impact ($p = 0.0426, r = 0.489$). Medium-sized individuals exhibited a large effect ($r = 0.734$), and large individuals showed a moderate effect ($r = 0.367$). Similarly, mean stable duration in the XY plane also showed a significant temperature effect ($p = 0.0378, r = 0.500$), with a strong effect observed in medium-sized clams ($r = 0.802$). No significant size effects were found in either metric.

Table 3-5 Summary of temperature and size effects on horizontal movement

Behavioral Dimension	**Temp. Effect**	**Temp. Effect by Size**	**Size Effect**	**Size Effect by Temp.**
XY Displacement	**$p < 0.05$ (Significant) $r = 0.531$ (Large)**	No Sig. Size: Large $r = 0.445$ (Moderate) **Size: Medium $r = 0.802$ (Large)**	No Sig.	No Sig.
XY Stability Ratio	**$p < 0.05$ (Significant) $r = 0.578$ (Large)**	No Sig. **Size: Medium $r = 0.802$ (Large)**	No Sig.	No Sig. Temp: 22℃ $\eta^2 = 0.17$ (Large)
XY Stable Segment	**$p < 0.05$ (Significant) $r = 0.489$ (Large)**	No Sig. Size: Large $r = 0.367$ (Moderate) **Size: Medium $r = 0.734$ (Large)**	No Sig.	No Sig.
XY Stable Duration	**$p < 0.05$ (Significant) $r = 0.500$ (Large)**	No Sig. **Size: Medium $r = 0.802$ (Large)**	No Sig.	No Sig.

In contrast, Z displacement showed no significant differences across any factor. For Z-axis stability, no overall temperature effect was found, but within-group analysis revealed strong trends. The stability ratio showed a large effect size in medium-sized ($r = 0.624$) and moderate in small-sized ($r = 0.445$) clams, suggesting possible

variation in vertical movement regulation across body sizes. Both the number of stable segments and mean stable duration in the Z direction showed moderate overall temperature effects ($r = 0.336\ and\ r = 0.333$), with large effects again observed in medium-sized individuals ($r = 0.734, r = 0.802$). Notably, a moderate size effect was present under 30℃ for Z stable duration ($\eta^2 = 0.0815$), suggesting that the influence of body size on vertical stability may become more pronounced under heat stress.

Table 3-5 Summary of temperature and size effects on vertical movement

Behavioral Dimension	**Temp. Effect**	**Temp. Effect by Size**	**Size Effect**	**Size Effect by Temp.**
Z Displacement	No Sig.	No Sig.	No Sig.	No Sig.
Z Stability Ratio	No Sig.	No Sig. **Size: Medium** **$r = 0.624$** **(Large)** Size: Small $r = 0.445$ (Moderate)	No Sig.	No Sig.
Z Stable Segment	No Sig. $r = 0.336$ (Moderate)	No Sig. **Size: Medium** **$r = 0.734$** **(Large)**	No Sig.	No Sig.
Z Stable Duration	$r = 0.333$ (Moderate)	No Sig. **Size: Medium** **$r = 0.802$** **(Large)**	No Sig.	No Sig. Temp: 30℃ $\eta^2 = 0.0815$ (Moderate)

3.2.5 Disturbed Volume

The analysis was based on the total cumulative volume of sediment disturbance

recorded during each 24-hour monitoring session under different temperature conditions. From the result, a large effect size was observed between 22℃ and 30 ℃ conditions ($r = 0.642$), with higher disturbed volumes recorded under elevated temperature. The size effect may reflect enhanced sediment disturbance under thermal stress.

3.3 Structural Equation Modeling of Behavioral Mechanisms

In this study, we designed three SEMs to find the potential behavioral mechanisms underlying sediment disturbance in clams exposed to thermal stress. The three types of models were: Direct, Indirect, and Complex. The Direct model explored the straightforward effect of temperature on disturbed volume. The Indirect model investigated whether temperature influenced disturbed volume indirectly via behavioral variables. The Complex model combined both direct and indirect pathways. In the analyses, behavioral variables included displacement and stable ratio of XY and Z directions. For disturbed volume, we estimated the cumulative disturbance volume for each clam. The results are shown below.

For direct model, as shown in Fig. 3-13, temperature positively influenced disturbed volume (0.51, $p > 0.1$). However, the path is not significant.

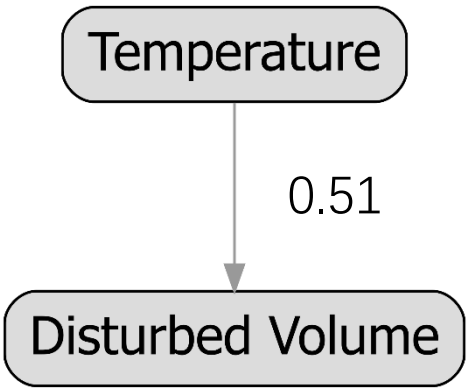


Fig. 3-13 Direct Model

In the indirect models, each model offered different hypotheses about the behavioral

mechanisms linking temperature and sediment disturbance. For horizontal displacement model, temperature exhibited a marginally negative effect on XY stability ($-0.58, p < 0.1$), which in turn strongly decreased XY displacement ($-0.76, p < 0.001$). XY displacement showed a strong positive effect on disturbed volume ($0.80, p < 0.001$), suggesting that temperature-induced instability leads to increased horizontal activity and sediment disturbance. For vertical displacement model, it revealed a similar path. Although the effect of temperature on Z stability was weak and non-significant ($-0.16, p > 0.1$), the path from vertical stability to Z displacement ($-0.48, p < 0.05$) and then to disturbed volume ($0.75, p < 0.001$) remained significant. For combined path model, both horizontal and vertical behavioral pathways were included. While the individual links remained statistically consistent, the final path coefficients from XY displacement ($0.39, p < 0.1$) and Z displacement ($0.43, p < 0.1$) to disturbed volume were weaker and only marginally significant. This reduction may reflect shared explanatory variance and possible collinearity between displacement dimensions, indicating that when modeled together, their individual contributions to sediment disturbance become less distinct.

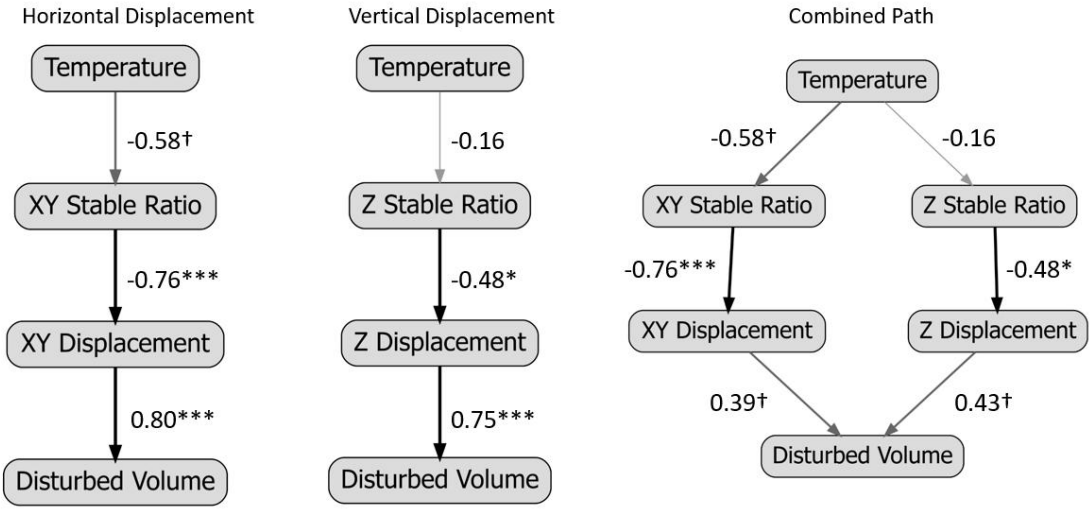


Fig. 3-14 Indirect Model

In the complex models, both direct and indirect effects of temperature were evaluated. For the horizontal displacement model, the direct path from temperature to disturbed volume decreased substantially ($0.07, p > 0.1$), while the indirect pathway through XY stable ratio and XY displacement remained significant ($-0.76, p <$

$0.001;\ 0.75, p < 0.01$). This shows the dominant role of horizontal activity in influencing disturbed volume. For the vertical displacement model, the direct temperature effect on disturbed volume persisted ($0.51, p > 0.1$), while the indirect pathway through Z displacement remained statistically significant ($-0.48, p < 0.05;\ 0.73, p < 0.001$). This suggests that vertical displacement contributed little to mediating the temperature effect, as the magnitude of the direct path remained largely unchanged, indicating limited mediating influence of vertical displacement on the temperature-disturbance relationship.

In the combined model, only Z displacement retained a significant and positive effect on disturbed volume ($0.57, p < 0.05$), whereas the effect of XY displacement became negative and non-significant ($-0.21, p > 0.1$). This reversal and attenuation may result from multicollinearity or shared variance between horizontal and vertical behavioral components. Additionally, it may reflect potential model specification issues, such as overlapping pathways or insufficient sample size for complex path structures, which can affect path stability and interpretability.

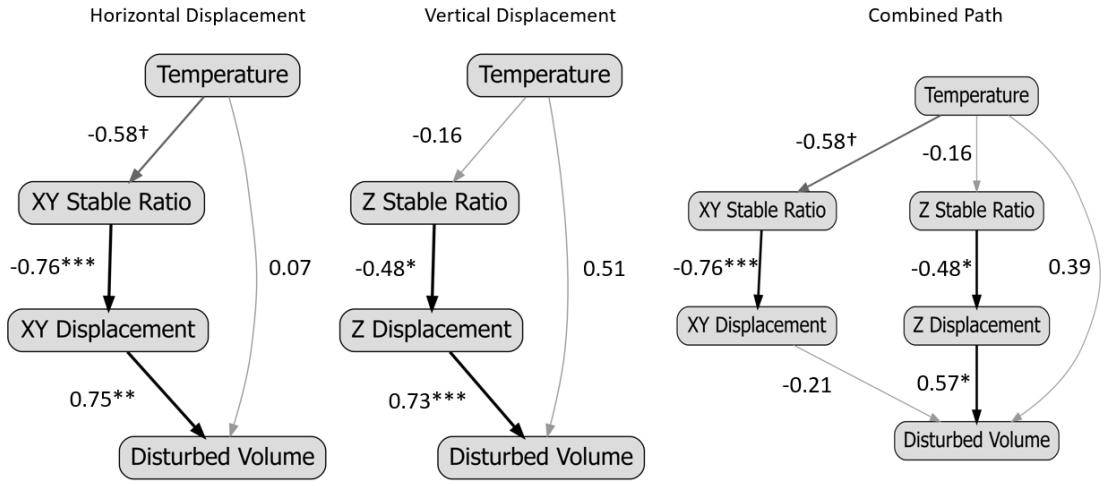


Fig. 3-15 Complex Model

To evaluate and compare the performance of different SEMs, we considered several key indicators:

Akaike Information Criterion (AIC) measures model quality by balancing goodness of fit and model complexity, with lower values indicating better performance.

R^2 for Disturbed Volume represents the proportion of variance in disturbed volume

explained by the model, reflecting explanatory power.

Fisher's C statistic is used to assess overall model fit based on d-separation principles; a non-significant p-value (> 0.05) indicates acceptable fit.

P-value associated with Fisher's C quantifies the likelihood that the observed lack of fit could occur by chance; lower values suggest poor model fit or missing pathways.

In the end, we summarized the model performance for SEM, shown in table 3-6.

Table 3-6 Model performance for SEM

Model		AIC	Disturbed_Volume R^2	Fisher's C	P-value
Direct		393.791	0.409	\	\
XY	Indirect	498.543	0.624	3.999	0.677
	Complex	483.725	0.617	3.341	0.503
Z	Indirect	510.728	0.851	7.079	0.314
	Complex	492.384	0.854	3.203	0.524
Joint	Indirect	609.743	0.758	32.332	0.02
	Complex	592.404	0.821	29.486	0.021

For XY models, both the indirect and complex models exhibited moderate explanatory power ($R^2 = 0.624, 0.617$), with acceptable model fit ($\text{Indirect: Fisher's C} = 3.999, \text{p} = 0.677; \text{Complex: C} = 3.341, \text{p} = 0.503$). Although the indirect XY model showed a slightly higher R^2, the Complex XY model had a lower AIC, indicating a better balance between model fit and parsimony.

Among Z models, both the indirect and complex models achieved high explanatory power ($R^2 = 0.851, 0.854$) and maintained a good fit. The complex Z model performed better than the Indirect model in terms of AIC and R^2, making it better for the explanation of vertical behavior.

For joint models, which integrated both XY and Z behavioral indicators, showed relatively high explanatory power ($R^2 = 0.785, 0.821$). However, both exhibited poor model fit ($p = 0.02, 0.021$), indicating potential structural misspecification or over-

complexity.

In conclusion, based on their acceptable model fit and explanatory power, both complex XY model and complex Z model are considered suitable for further interpretation of the behavioral mechanisms underlying sediment disturbance.

4. Discussion

This study investigated the burrowing behavioral responses of Hamaguri clams under thermal stress using high-resolution acoustic monitoring. By integrating descriptive statistics, non-parametric testing, and SEM, we preliminarily evaluated the influence of temperature and body size on behavioral indicators, including burrowing time, depth, posture, displacement, stability, and sediment disturbance. Although further research is needed, the results indicate that thermal stress may alter burrowing dynamics in clams, with possible consequences for sediment disturbance processes.

4.1 Behavioral Acceleration

One of the important findings is the reduction in burrowing time under rising temperatures. As summarized in Fig. 3-16, although the result of burrowing time was not statistically significant, a moderate effect size ($r = 0.333$) suggests that clams exposed to 30℃ initiated burrowing more rapidly than those at 22℃. Meanwhile, this behavioral acceleration also varied by body size. At 22℃, both large and small clams originally required more time to initiate burrowing. However, the elevated temperature shortened their burrowing time. This effect in large size is more obvious.

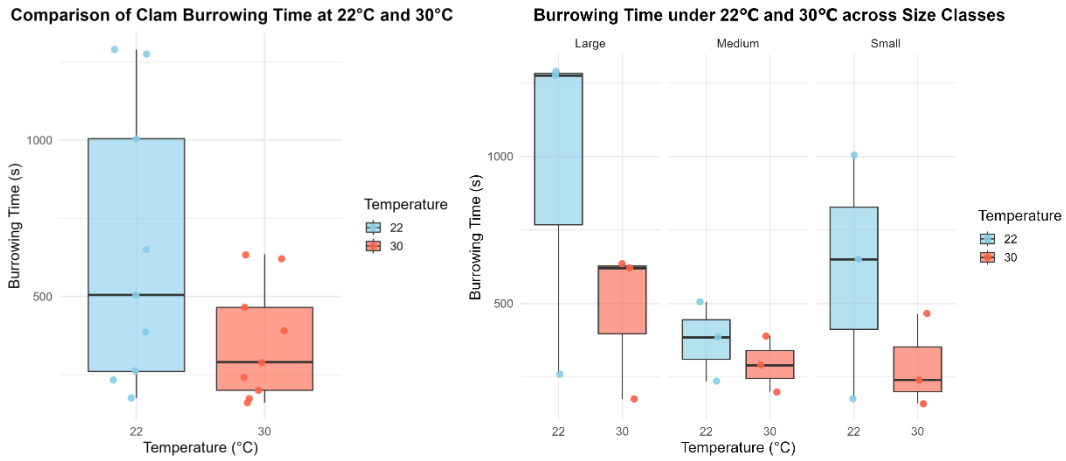


Fig. 3-16 Boxplot summary of burrowing time

For poikilothermic animals, this behavioral acceleration may reflect a thermal escape response. To further examine the thermal conditions faced by the clams, we measured the sediment temperatures at various depths and positions (four corners and the center)

of the experimental tanks. In the 30℃ group, the sediment temperature gradually decreased with depth. From the sediment surface to approximately 9 cm deep, the temperature dropped by about 3℃. Although this temperature gradient was modest, it may have been sufficient to partially alleviate the thermal stress experienced by the clams. Correspondingly, thermal stress can induce foot activity, driving the clams to actively bury themselves into the sediment to avoid excessive heat.[87] Interestingly, the effect appeared stronger in large and small individuals, suggesting that body size may modulate the behavioral sensitivity to temperature during initial burrowing.

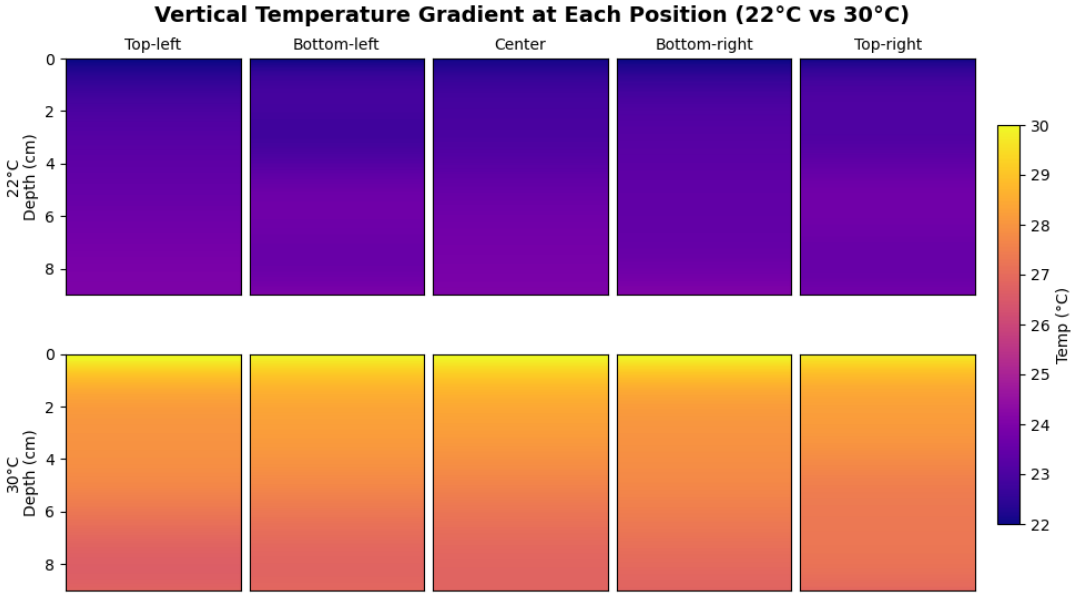


Fig. 3-17 Vertical temperature distribution in sediment at each tank position

4.2 Depth Regulation

Through the experimental results, we observed not only the regulatory effect of temperature on burrowing behavior, but also the influence of body size. Here, we further examined the maximum burrowing depth of each clam in addition to the average depth, and summarized the data using boxplots, as shown in Fig. 3-18.

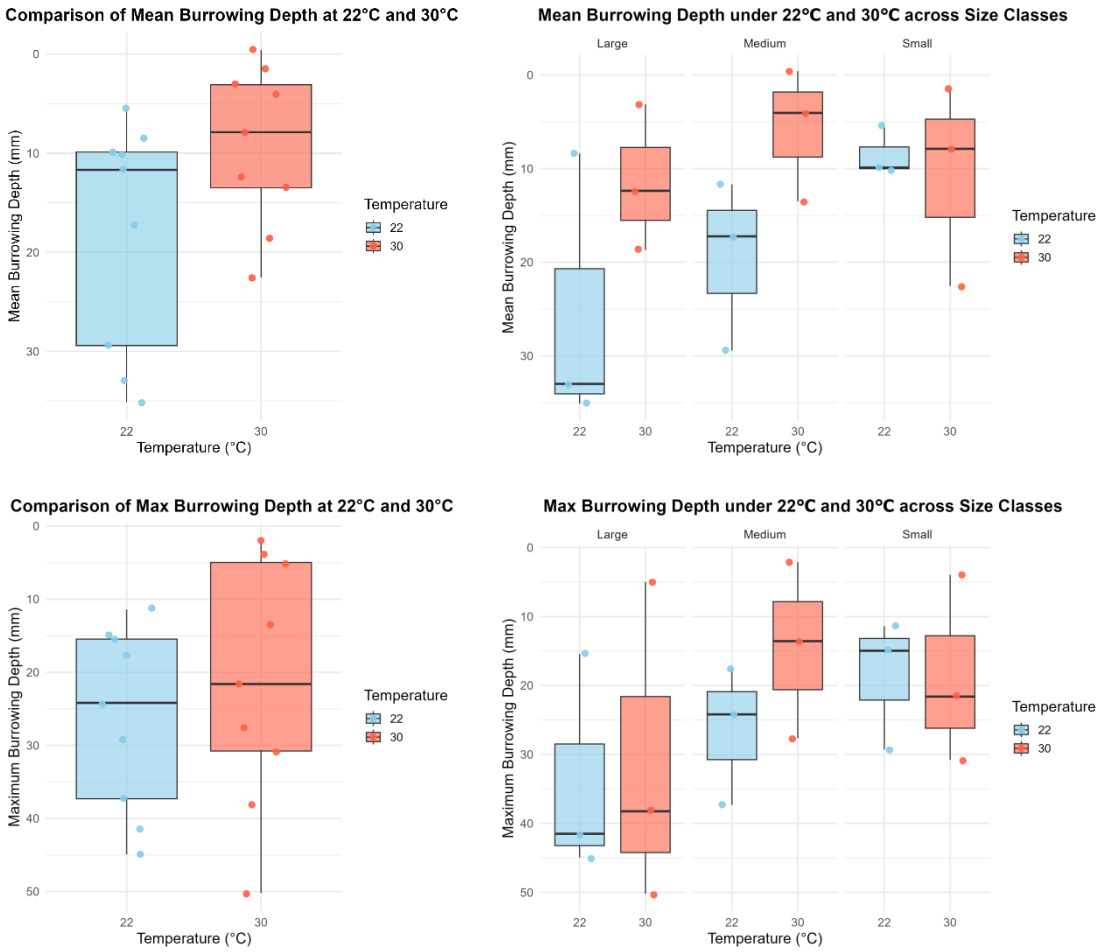


Fig. 3-18 Boxplot Summary of mean and max burrowing depth

Based on the boxplots, elevated temperature tended to slightly reduce the burrowing depth of clams, with the extent of this response varying by body size. Medium-sized individuals exhibited the greatest decrease in average burrowing depth ($r = 0.624$), whereas large individuals showed the most pronounced shallow burial response in terms of minimum depth ($r = 0.802$). In addition, body size itself also influenced burrowing behavior: under 22℃, larger clams generally burrowed deeper than smaller ones.

Since burrowing is an energy-consuming behavior, clams with different sizes have varying energy reserves and excavation capacities.[88] Under thermal stress, the average burrowing depths of large and medium-sized clams were not as deep as those observed at 22℃, which may reflect an energy trade-off strategy. As shown in Fig. 3-17, although the sediment temperature decreases with depth, the gradient is small. For clams, while digging deeper leads to lower temperatures, it also requires greater energy

expenditure. Because the temperature reduction is limited, burrowing deeper is evidently not cost-effective for clams. Therefore, clams may have made a balance between energy consumption and thermal avoidance, selecting an acceptable point that achieves some degree of heat avoidance while conserving energy.

4.3 Burrowing behavior under sediments

Individual clams exhibited variability in their movement strategies beneath the sediment. Similarly, we summarized the boxplots of horizontal and vertical displacements, as shown in Fig. 3-19.

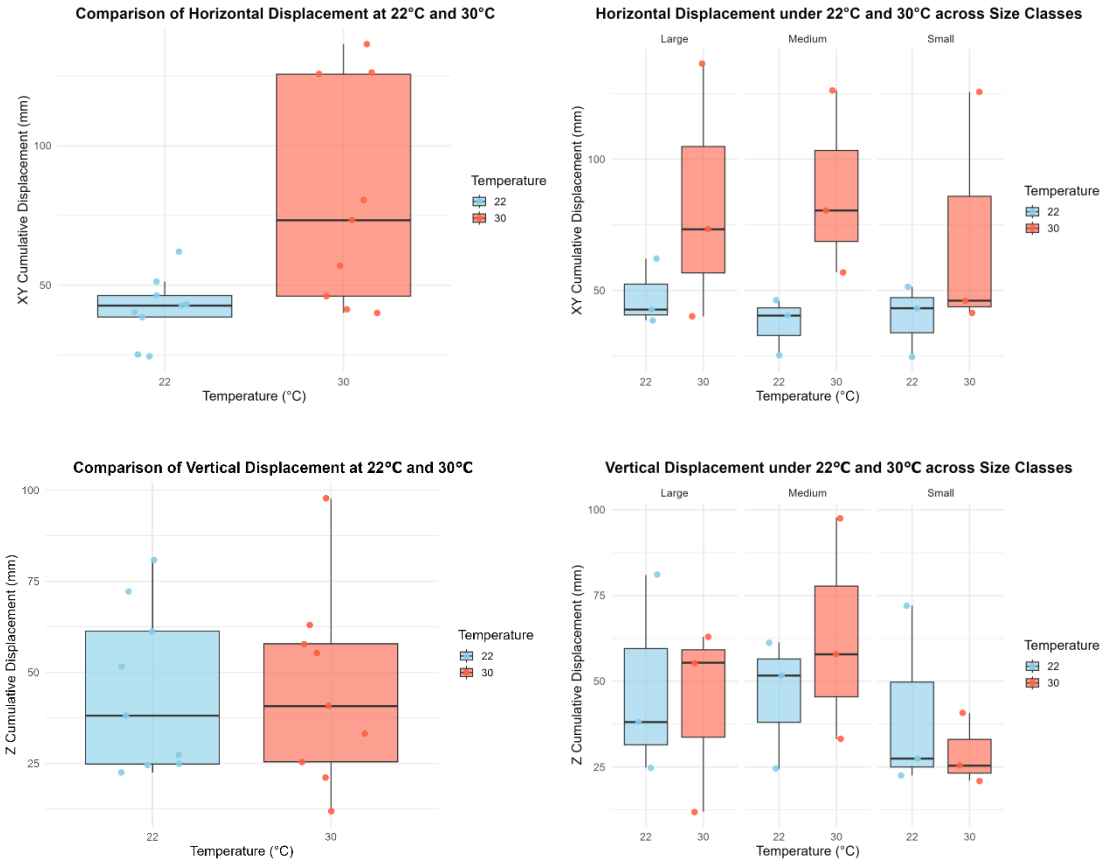


Fig. 3-18 Boxplot Summary of horizontal and vertical displacements

From the boxplots, it can be observed that elevated temperatures increased horizontal displacement in individual clams, while vertical displacement remained unaffected. This pattern is further supported by the results of non-parametric statistical tests, in which the temperature effect on XY displacement was statistically significant ($p = 0.0244$) with a large effect size ($r = 0.531$), whereas no significant effect was found

for Z displacement.

Although separating clam displacement into horizontal and vertical components provides a more refined assessment of burrowing behavior, especially helping us to understand which direction is favored under thermal stress, it does not clarify the underlying movement strategy. It remains unclear whether clams move laterally first and then burrow vertically, or whether they adopt an oblique burrowing path. To address this uncertainty, we further examined movement trajectories by visualizing the positional coordinates used in the displacement calculations.

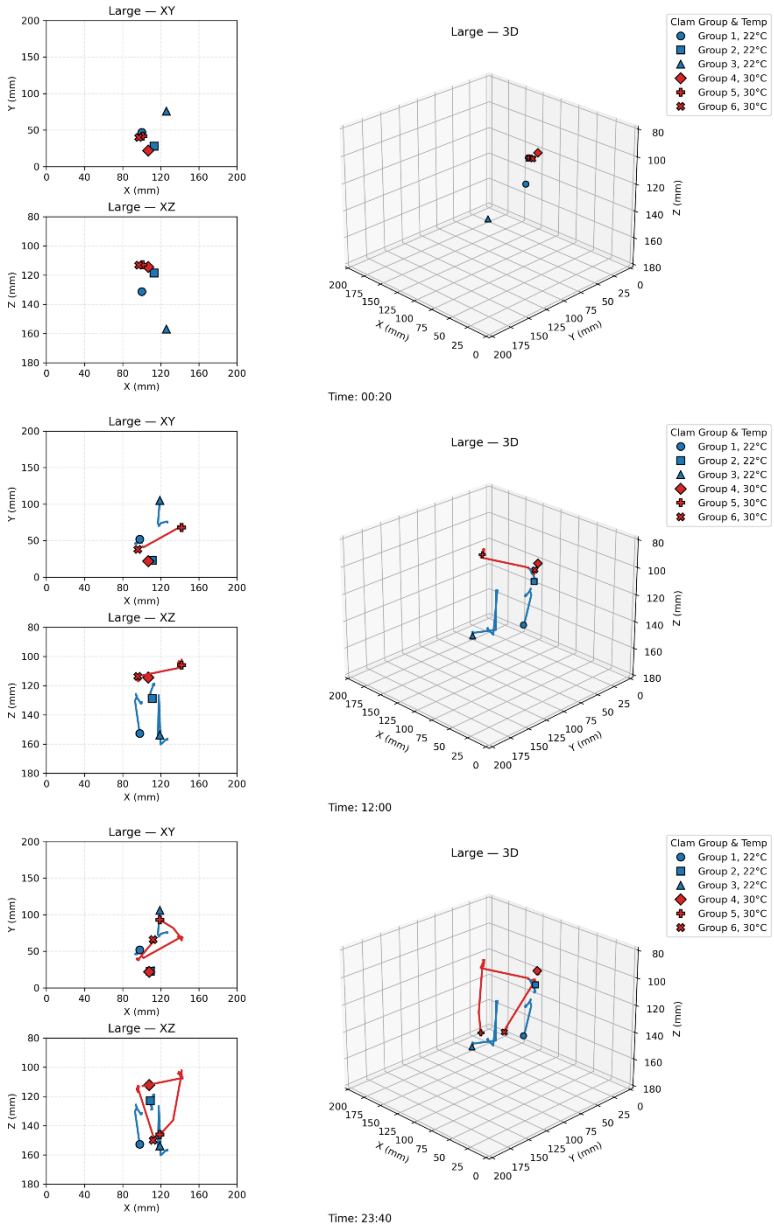


Fig. 3-19 Movement trajectories of large clams at 00:20, 12:00 and 23:40 under 22℃

and 30℃ conditions.

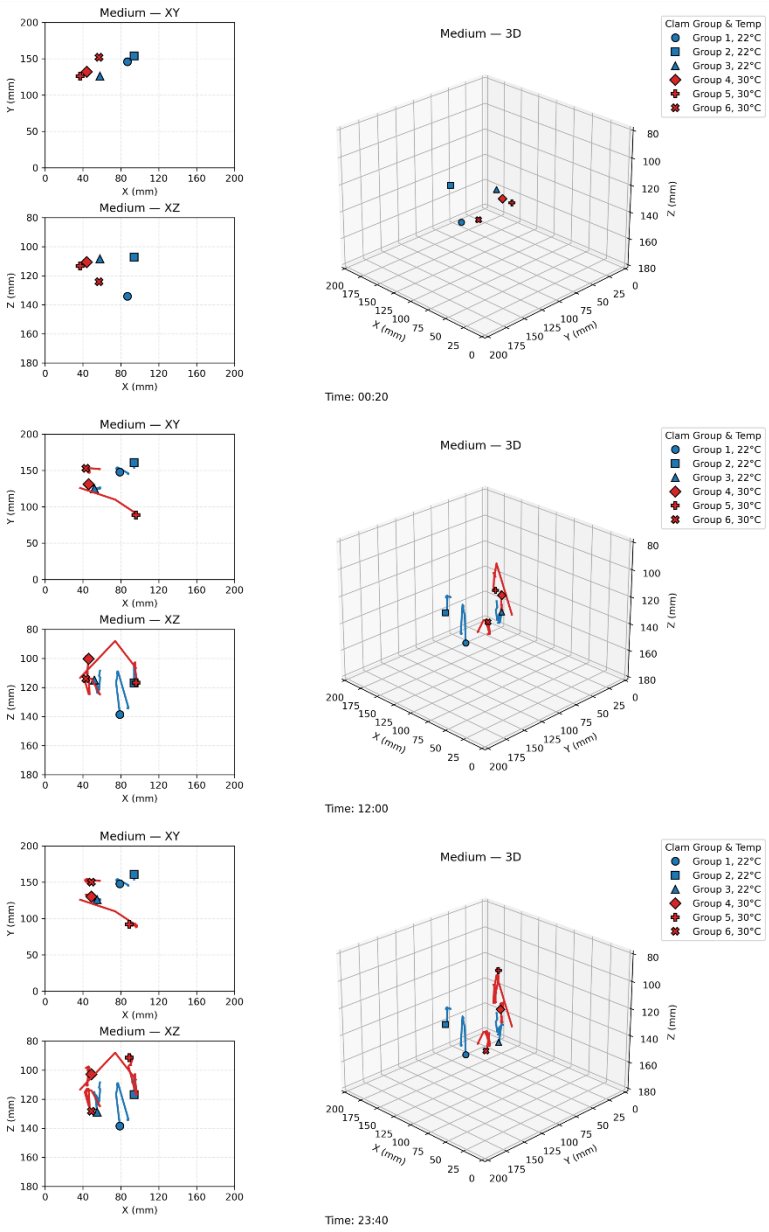


Fig. 3-20 Movement trajectories of medium clams at 00:20, 12:00 and 23:40 under 22℃ and 30℃ conditions.

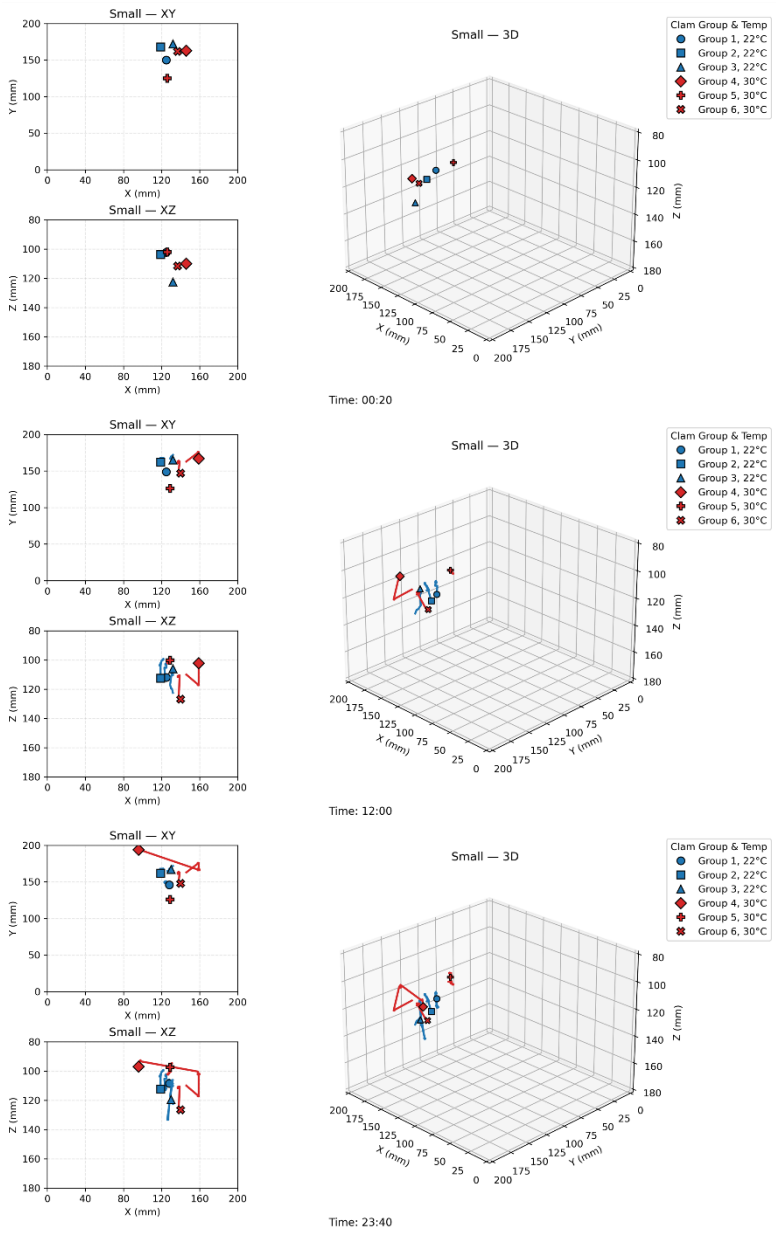


Fig. 3-21 Movement trajectories of small clams at 00:20, 12:00 and 23:40 under 22℃ and 30℃ conditions.

Due to the limited sample size, we did not conduct classification of movement trajectories. However, from the observed trajectories, the clams exhibit various movement paths under thermal stress. At higher temperatures, some individuals moved horizontally under the sediment, while others adopted oblique burrowing paths. This diversity in movement patterns may reflect adaptive behavioral strategies.

For the preference of horizontal movement, clams may be exploring the sediment surface in search of microenvironments with more favorable environmental

conditions.[89][90] The rising temperatures can reduce DO in water and sediments. Clams in such conditions may increase the horizontal movement to locate areas with better oxygen availability, thermal buffering, or sediment characteristics. In this sense, horizontal displacement may serve as a behavioral mechanism for habitat selection under suboptimal thermal and chemical conditions.

In contrast, oblique burrowing may represent an energetically efficient strategy for penetrating the sediment. Shinji Sassa et al. demonstrated that infaunal clams can actively adjust their burrowing angle and adopt oblique burrowing to reduce the energy required for penetration.[59] By decreasing the insertion angle and limiting burial depth, this behavioral strategy helps prevent the energetic cost of burrowing from exceeding their physiological capacity. Therefore, under elevated temperatures, clams may adopt a similar energy-saving strategy: by burrowing obliquely, they can achieve partial thermal avoidance while reducing locomotor cost.

4.4 The behavioral pathways of temperature affecting disturbed volume

In this study, we further dissected the behavioral mechanisms underlying sediment disturbance by using SEM method. Here, we separately modeled displacement in the horizontal and vertical directions. Both models revealed significant indirect pathways linking thermal stress to disturbed volume via behavioral stability and displacement magnitude, the result summary is shown as Fig. 3-22.

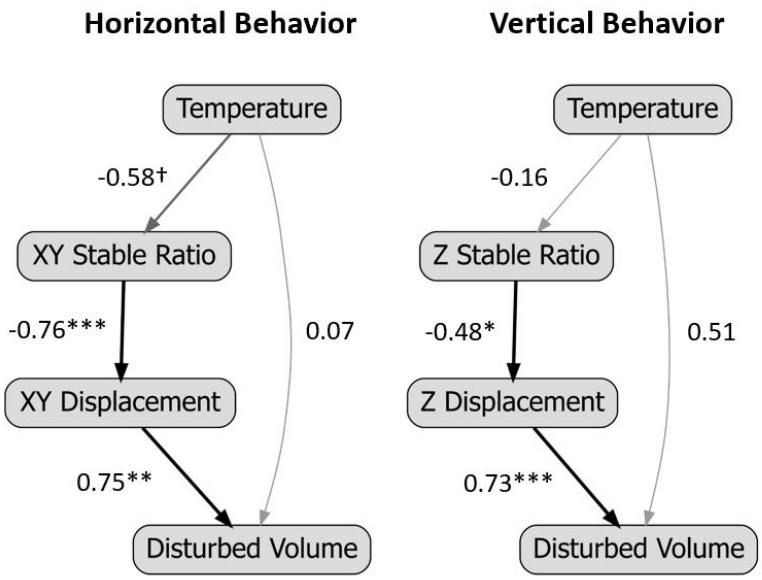


Fig. 3-22 Path models of horizontal and vertical behavior linking temperature to disturbed volume

For the horizontal behavior analysis, increased temperature was associated with reduced horizontal stability, ultimately increasing disturbed volume. This suggests that under thermal stress, clams shift to more frequent or fragmented horizontal movement, contributing to bioturbation activity.

In contrast, the path of vertical behavior showed a weaker link between temperature and vertical stability. However, the downstream path from stability to displacement and from displacement to disturbed volume remained strong. These results suggest that the elevated temperatures may not fragment vertical behavioral stability or increase total vertical displacement. Instead, vertical displacement may serve as a baseline contributor to sediment disturbance, providing a stable effect.

These findings offer a practical perspective for understanding how clams respond to warming conditions. While vertical movement plays a steady role in disturbing the sediment, it is the changes in horizontal behavior. Since these changes are easier to detect and more sensitive to temperature, they could serve as useful early warning signs of rising temperature. In this sense, monitoring horizontal displacement and sediment disturbance may help us better assess the impacts of marine thermal stress and the role of infaunal clams in shaping their habitats.

5. Conclusion

In this study, we used a high-resolution acoustic system to evaluate the burrowing responses of clams under marine thermal stress. Compared to conventional approaches, our method enables non-invasive, continuous monitoring of clam positions beneath the sediment. A spatial resolution of $2\,mm \times 2\,mm \times 0.1\,mm$ in the X, Y, and Z axes is sufficient to capture subtle movements in benthic environments. In addition, we developed a comprehensive set of behavioral indicators to quantify burrowing activity, including burrowing time, depth, displacement, posture, behavioral stability, and disturbed sediment volume. These indicators refined the existing indicators of burrowing behavior, filling the methodological gap of monitoring behavioral dynamics under thermal stress.

From the results, we observed behavioral patterns similar to those reported in previous studies. For instance, elevated temperatures led to shorter burrowing time, faster digging response, and depth regulation.[62][91] In addition, our study revealed several novel findings. Under thermal stress, clams exhibited increased horizontal displacement and adopted not only lateral movement but also oblique burrowing trajectories, indicating energy-saving strategies. Further, the SEM analysis showed that increased temperature tended to reduce horizontal behavioral stability, leading to more fragmented movement, greater cumulative displacement, and ultimately enhanced bioturbation intensity. The findings suggest that the monitoring of individual trajectory and sediment disturbance could serve as potential indicators for detecting early behavioral responses of benthic organisms to thermal stress.

In conclusion, this study provides an experimental foundation for understanding the burrowing responses of infaunal clams to marine thermal stress. As a preliminary investigation, it demonstrates the feasibility of using high-resolution acoustic monitoring to continuously capture behavioral changes beneath the sediment. Compared to conventional approaches, our method offers non-invasive, high-resolution tracking of clam movements. These findings highlight the value of integrating behavioral indicators with environmental data to explore species responses under

climate stress, and they offer a basis for further ecological and physiological research on thermal adaptation in benthic bivalves.

6. Limitations and Future Work

6.1 Research Limitations

Despite the completion of the experiment, this study still has several experimental limitations that should be acknowledged.

First, regarding experimental design, a total of 18 clams were used in this study. While sufficient for initial exploration, this sample number is relatively small for biological experiments. In addition, the clams varied in size and were not uniformly distributed across the size spectrum, which further reduced the effective sample size when categorizing individuals into distinct size classes. This limitation may have weakened the statistical power of our analyses and contributed to the presence of non-significant results despite observable behavioral trends. Moreover, only two temperature conditions were tested, limiting our ability to capture gradual or nonlinear thermal responses across a broader temperature gradient.

Second, in terms of experimental procedure, the placement of clams at the beginning of each trial was not randomized. Individuals of different size classes were consistently positioned in fixed zones within the sediment tank. While this approach ensured visual clarity and convenience during labeling, it may have introduced spatial bias or tank effects. A randomized placement strategy would be more appropriate to eliminate potential confounding factors related to location.

Third, during the feature extraction, some processing thresholds were determined empirically based on prior experience and visual inspection. Although these choices yielded consistent results in the present dataset, they were not optimized or validated systematically, which may limit the generalizability or reproducibility of the method under different conditions or in future studies.

6.2 Future Work

Our understanding of how benthic organisms respond to environmental changes remains limited. To effectively monitor and protect benthic populations, it is crucial to

improve our knowledge of their behavioral and physiological responses under climate stressors.

First, I aim to expand upon the current thermal stress experiment. In natural settings, clams typically inhabit intertidal zones, where they are exposed to periodic tidal fluctuations. These fluctuations, when combined with marine heatwaves, can cause large amounts of mortality. To simulate such environmental conditions in the laboratory, I plan to design an experimental system with multiple sensor-controlled elements to mimic tidal cycles under heatwave scenarios. This system will allow dynamic control of water levels and temperature, enabling precise simulation of stress patterns observed in the field. To achieve a more comprehensive understanding of behavioral responses, I plan to adopt a multi-modal monitoring method. Specifically, ultrasound imaging will be utilized to monitor burrowing activity under sediment, while optical cameras will be applied to record initial burrowing behavior. In addition, infrared imaging may be used to visualize the spatial distribution of temperature across the sediment surface. In the end, miniature sensors such as accelerometers may be attached to the clam shells to measure body inclination angles, helping to quantify posture changes during burrowing. Based on this extended experimental platform, I will also compare behavioral responses across multiple infaunal clam species to identify interspecific variation in thermal tolerance and burrowing strategy. In addition to behavioral data, basic physiological metrics will be incorporated, such as respiration rate, metabolic rate, and condition index, to gain a more holistic view of organismal responses. Field data collection in natural intertidal habitats will be essential. By comparing laboratory findings within situ behavioral and environmental observations, we can better validate experimental results and ensure ecological relevance.

Second, beyond thermal stress, other climate-related stressors such as hypoxia and ocean acidification also have negative impact on infaunal clams. Therefore, it is necessary to develop parallel experimental setups that allow investigation of these stressors, either independently or in combination, to examine how clams respond under multi-stressor scenarios.

Finally, it would also be ecologically meaningful to explore biotic interactions. For example, in habitats where seagrass beds or mangroves are present, it is worth investigating whether vegetated environments can help buffer thermal extremes, offering clams microrefugia and potentially mitigating stress impacts. Understanding these habitat-level interactions may inform habitat restoration and conservation efforts under future climate scenarios.

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