

Article

Population-Specific Differences in Respiratory Metabolism and Key Metabolic Enzyme Activities in Manila Clam (*Ruditapes philippinarum*)

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Abstract

Respiratory metabolism reflects energy demand and physiological variation in bivalves, but interpretation of among-population differences requires careful consideration of developmental stage, diet, breeding background, and previous rearing history. In this study, oxygen consumption rate (OR), ammonia excretion rate (NR), O:N ratio, and key metabolic enzyme activities were compared among three Manila clam (*Ruditapes philippinarum*) populations: Laizhou wild-origin stock (LZ), Fujian-origin stock cultured in Liaoning (FJ), and Zebra shell-color selected line (ZS). These populations were selected to represent three common germplasm categories used in Manila clam production: a wild-origin stock, a translocated aquaculture stock, and a Zebra shell-color selected line. Clams with nominal shell lengths of 10, 15, and 20 mm were used for respiratory measurements, and enzyme activities were determined in 15 mm individuals. Two-way ANOVA revealed significant effects of population on OR, NR, and O:N ratio ($p < 0.05$), whereas shell length significantly affected NR and O:N ratio ($p < 0.05$). OR decreased with increasing shell length in LZ and FJ, while ZS showed a different pattern. NR generally declined with shell length across all populations. The O:N ratio increased markedly in LZ but remained relatively low in FJ and ZS. Among enzyme activities, SDH was significantly higher in LZ ($p < 0.01$), whereas AKP activity was highest in ZS ($p < 0.05$). LDH activity did not differ significantly among populations. These results demonstrate that Manila clam populations with different source backgrounds exhibit distinct physiological profiles under standardized laboratory conditions. The observed variation represents population-associated physiological characteristics rather than direct evidence of genetic differentiation, providing baseline information for future germplasm characterization integrating physiological, genetic, and environmental approaches.



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Keywords: *Ruditapes philippinarum*; population; oxygen consumption rate; ammonia excretion rate; O:N ratio; metabolic enzyme activity; metabolic phenotype

1. Introduction

The Manila clam (*Ruditapes philippinarum*) is an important bivalve species along the coast of China because of its wide distribution, strong environmental adaptability, rapid

growth, and suitability for large-scale aquaculture. It plays a major role in marine shellfish aquaculture and coastal food production [1,2]. However, the expansion and intensification of clam farming have been accompanied by increasing challenges, including habitat alteration, sediment degradation, environmental fluctuations, and disease occurrence, which may affect the stability of clam production.

Respiratory metabolism is central to energy conversion and physiological maintenance in aquatic organisms and can provide useful information on their responses to environmental and biological variation [3]. Oxygen consumption rate (OR) and ammonia excretion rate (NR) are routine indicators of respiratory metabolism in bivalves, reflecting aerobic metabolic intensity and nitrogenous waste excretion, respectively [4,5]. The O:N ratio further indicates the relative contribution of proteins, lipids, and carbohydrates to energy metabolism when OR and NR are converted to atomic equivalents [6]. Metabolic enzyme activities also serve as biomarkers of physiological status. Lactate dehydrogenase (LDH) is associated with anaerobic glycolysis, succinate dehydrogenase (SDH) links the tricarboxylic acid cycle and mitochondrial electron transport, and alkaline phosphatase (AKP) is involved in nutrient metabolism and physiological regulation [7,8]. In this study, the term 'physiological profile' or 'physiological strategy' refers only to the observable combination of OR, NR, O:N ratio, and enzyme activity under the specified experimental conditions.

Previous studies have compared Manila clam populations, geographic populations, and color strains in terms of genetic structure, stress responses, oxygen consumption, ammonia excretion, and biochemical traits. Nevertheless, most available studies have either focused on natural geographic populations or examined selected strains under specific stress conditions, whereas fewer studies have integrated whole-animal respiratory indicators with metabolic enzyme activities across shell-length classes while simultaneously considering wild-origin, translocated aquaculture, and shell-color selected germplasm categories. The three populations used in the present study were therefore selected to cover these representative germplasm backgrounds: LZ as a wild-origin stock from Laizhou Bay, FJ as a Fujian-origin stock that had undergone cross-regional culture in Liaoning, and ZS as a Zebra shell-color selected line. This phenotype-level dataset provides a comparative physiological baseline for Manila clam population characterization, not to prove fixed genetic divergence without environmental controls. These three populations were selected to represent three germplasm backgrounds commonly encountered in Manila clam aquaculture: wild-origin, translocated aquaculture, and shell-color selected germplasm.

Previous studies have demonstrated that physiological traits in bivalves can vary among populations with different geographic origins, culture histories, and breeding backgrounds. However, whether populations representing different germplasm backgrounds maintain distinguishable physiological profiles under standardized environmental conditions remains unclear. Therefore, we hypothesized that Manila clam populations with different source backgrounds would exhibit population-associated variation in respiratory metabolism and metabolic enzyme activities when evaluated under common laboratory conditions. Specifically, this study tested whether population background and shell-length category influenced oxygen consumption rate (OR), ammonia excretion rate (NR), O:N ratio, and selected metabolic enzyme activities.

The objective of this study was not to determine genetic differentiation among populations, but rather to characterize phenotype-level physiological variation associated with different germplasm backgrounds. By integrating respiratory metabolic indicators and enzyme activity measurements, this study aimed to establish a comparative physiological baseline for future investigations combining genetic, environmental, and functional analyses in Manila clam germplasm evaluation.

2. Materials and Methods

2.1. Materials

2.1.1. Experimental Populations

Three populations of Manila clam, *Ruditapes philippinarum*, were used in this study, including the Laizhou wild-origin stock (LZ), Fujian-origin stock cultured in Liaoning (FJ), and Zebra shell-color selected line (ZS). The LZ population was collected in March 2025 from the intertidal zone of Laizhou Bay, Shandong Province, China. This population was considered a wild-origin stock based on collection history; however, genetic verification and long-term environmental monitoring data were unavailable. The FJ population originated from Fujian Province and had been cultured in a bottom-seeding aquaculture area in Dandong, Liaoning Province, China before transfer to the laboratory in March 2025. Therefore, this population represented a translocated aquaculture stock with a mixed geographic and culture history. The ZS population was obtained from a zebra shell-color-selected line maintained in pond culture in Panjin, Liaoning Province, China. According to available breeding records, this population has undergone shell-color selection; however, the exact number of selective generations and detailed source background were unavailable. The three populations were selected because they represent three germplasm categories commonly encountered in Manila clam aquaculture: a wild-origin stock (LZ), a translocated aquaculture stock with cross-regional rearing history (FJ), and a zebra shell-color selected line (ZS). This selection enables a practical comparison of metabolic phenotypes among germplasm sources, but it does not by itself separate population background from environmental carry-over or culture-history effects.

All individuals used in the experiment were healthy, with intact shells and no visible external damage. Based on shell length, clams from each population were divided into three nominal size categories: 10, 15, and 20 mm. These size classes were selected to represent juvenile or juvenile-sized stages commonly used in nursery and early grow-out studies and to test size-related metabolic variation within a manageable experimental range. No reproductive-stage selection was performed before the experiment. Histological gonadal staging was not conducted; therefore, reproductive status-related variation cannot be completely excluded. Morphometric characteristics are presented in Table 1, and representative individuals are shown in Figure 1.

Table 1. Morphometric characteristics and nominal size categories of the three Manila clam (*Ruditapes philippinarum*) populations.

Population	Size Group/ Category (mm)	Shell Length (mm)	Shell Width (mm)	Shell Height (mm)	Wet Weight (g)
Laizhou wild-origin stock (LZ)	10	10.00 ± 0.74	3.81 ± 0.24	6.82 ± 0.56	0.18 ± 0.02
	15	14.88 ± 0.43	5.97 ± 0.33	10.34 ± 0.49	0.60 ± 0.07
	20	20.28 ± 0.86	8.45 ± 0.62	13.61 ± 0.79	1.58 ± 0.21
Fujian-origin stock cultured in Liaoning (FJ)	10	10.14 ± 0.62	4.01 ± 0.29	7.16 ± 0.47	0.16 ± 0.04
	15	14.82 ± 1.02	5.81 ± 0.59	10.46 ± 0.85	0.50 ± 0.11
	20	20.52 ± 0.91	9.18 ± 0.73	13.99 ± 1.00	1.73 ± 0.31
Zebra shell-color selected line (ZS)	10	10.36 ± 0.54	4.25 ± 0.28	7.16 ± 0.42	0.19 ± 0.03
	15	15.21 ± 0.82	6.02 ± 0.44	10.25 ± 0.53	0.62 ± 0.10
	20	20.03 ± 0.95	9.38 ± 0.57	15.05 ± 0.81	1.94 ± 0.24

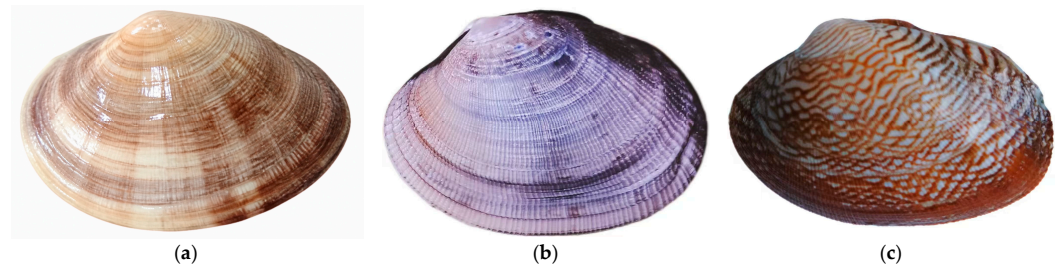


Figure 1. Representative individuals from the three Manila clam (*Ruditapes philippinarum*) populations. The panels are displayed from left to right as (a), (b), and (c). (a) Laizhou wild-origin stock (LZ); (b) Fujian-origin stock cultured in Liaoning (FJ); (c) Zebra shell-color selected line (ZS).

The initial number of clams used in the study was explicitly defined for each assay. For respiratory measurements, 10 clams were placed in each replicate bottle, with three replicate bottles for each population $\times$ size-category combination, resulting in 30 clams per population $\times$ size category, 90 clams per population across the three size categories, and 270 clams in total. For enzyme assays, 60 clams from the same three original populations were cultured in each of three replicate tanks per population, and six individuals were sampled from each tank at the end of the culture period. Thus, the enzyme culture phase included 180 clams per population and 540 clams in total, and enzyme activity was measured in 18 individuals per population and 54 individuals in total. These sample-size definitions clarify the biological experimental units used for statistical analysis.

Before the respiratory experiment, all clams were acclimated in the laboratory for 7 days under continuous aeration, with the water temperature maintained at 20 ± 1 °C and salinity stabilized at 30 ± 1 . The 7-day acclimation period was intended to minimize capture, transportation, and handling stress while avoiding prolonged acclimation that could obscure short-term metabolic signatures associated with source environment and pre-experimental rearing history. During acclimation, all populations were fed the same diet consisting of *Chlorella vulgaris* and spirulina powder, and half of the seawater was renewed daily before feeding. This standardized diet was used to reduce short-term dietary differences among populations during laboratory acclimation. Nevertheless, the diet used during laboratory acclimation may not have been identical to the natural food spectrum or routine aquaculture diet experienced by each population before collection; therefore, potential diet-shift effects were considered in the Discussion. The experimental seawater was collected from the Heishijiao coastal area in Dalian, Liaoning, China, and subjected to sedimentation and filtration before use. To reduce postprandial metabolic effects, all clams were starved for 24 h before respiratory measurements, and no food was supplied during the 24 h respiratory experiment.

Environmental background information for the source populations was limited to source type and culture history. LZ was collected from an intertidal habitat in Laizhou Bay, FJ was a Fujian-origin stock that had been cultured in a Dandong bottom-seeding area, and ZS was maintained in pond culture in Panjin. Because complete source-site environmental datasets were not available for all three populations, including precise geographic coordinates, quarterly seasonal temperature and salinity ranges, dissolved oxygen, pH, natural food abundance, sediment grain size, organic matter content and other sediment chemical properties, as well as pollution indicators, these environmental variables were regarded as potential confounding factors instead of controllable explanatory variables in the present study. The lack of complete source-site environmental data was addressed by standardizing the short-term laboratory conditions and by interpreting the results cautiously.

2.1.2. Substrate and Reagents

The sediment used in this study was collected from the intertidal zone of Heishijiao, Dalian, China. After sieving with 5–100 mesh screens, sediment with a particle size of 251–425 μm was selected as the experimental substrate. This grain-size range represents medium to coarse sand and was selected to provide a standardized burrowing substrate that allowed Manila clams to bury normally while reducing fine-particle suspension during sampling. Because the LZ, FJ, and ZS populations originated from different coastal or culture environments, this standardized substrate was used to minimize short-term substrate variation during the experiment rather than to exactly reproduce the natural habitat of each population. The collected sediment was disinfected with 5% sodium hypochlorite for 24 h and thoroughly rinsed with freshwater to eliminate residual disinfectant before use. All analytical reagents applied for the determination of dissolved oxygen and ammonia nitrogen, including manganese chloride, potassium iodide, sodium thiosulfate, starch indicator, potassium iodate, sodium hydroxide, ammonium chloride, bromate-bromide solution, sulfanilamide, and N-(1-naphthyl)ethylenediamine, were of analytical grade and prepared in accordance with standard analytical protocols. In addition, commercial assay kits for the determination of LDH, AKP, and SDH activities were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) and operated strictly following the manufacturer's instructions.

2.2. Experimental Procedures

2.2.1. Measurement of Oxygen Consumption Rate and Ammonia Excretion Rate

OR and NR were measured using a closed static respirometry method modified from established bivalve physiological energetics and respirometry protocols [5]. For each population and size population, experimental and blank control treatments were established, with three replicate bottles for each treatment. The respiratory experiment included 270 clams in total: three populations, three size populations, three replicate bottles per population $\times$ size category, and ten clams per bottle. The experiment was conducted in 5 L wide-mouth respirometry bottles. A 7 cm layer of sterilized sand substrate, with a particle size of 251–425 μm , was placed at the bottom of each bottle. Ten clams were introduced into each experimental bottle, whereas only sand substrate and seawater were placed in blank control bottles. Each bottle was treated as one independent experimental unit. After adding substrate and clams, filtered seawater was added carefully, and the effective water volume (V) in each bottle was recorded and used in the calculation rather than using only the nominal bottle capacity.

The water temperature was maintained at 20 ± 1 °C throughout the respiratory experiment, and salinity was maintained at 30 ± 1 . Dissolved oxygen was measured at the beginning and end of the 24 h incubation to ensure that oxygen depletion was not severe enough to invalidate the closed respirometry measurements. Initial dissolved oxygen and ammonia nitrogen concentrations were measured using the iodometric method and hypobromite oxidation method, respectively. After 24 h of incubation, water samples were collected from the bottom of each bottle using a siphon, and the final dissolved oxygen and ammonia nitrogen concentrations were determined. Blank bottles containing sand and seawater were used to correct for background oxygen consumption and ammonia variation. At the end of the experiment, clams were dried at 60 °C to constant weight, and the total dry weight (G) of clams in each bottle was recorded. The main monitored water-quality variables in the respiratory trial were temperature, salinity, dissolved oxygen, and ammonia nitrogen. Continuous pH, dissolved-oxygen saturation, and full real-time salinity fluctuation records were not available, and this limitation was added to the Discussion.

The oxygen consumption rate and ammonia excretion rate were calculated as follows:

$$OR = V \times [(DO_0 - DO_T) - (DO_1 - DO_2)] / (G \times T), \quad (1)$$

$$NR = V \times [(NR_T - NR_0) - (NR_2 - NR_1)] / (G \times T) \quad (2)$$

where V is the effective water volume in the respirometry bottle after addition of substrate and clams (L); DO₀ and DO_T are the initial and final dissolved oxygen concentrations in the experimental population (mg L⁻¹), respectively; DO₁ and DO₂ are the initial and final dissolved oxygen concentrations in the blank control population (mg L⁻¹), respectively; NR₀ and NR_T are the initial and final ammonia nitrogen concentrations in the experimental population (mg L⁻¹), respectively; NR₁ and NR₂ are the initial and final ammonia nitrogen concentrations in the blank control population (mg L⁻¹), respectively; G is the total clam dry weight (g); and T is the experimental duration (h). OR and NR were expressed as µg O₂ g⁻¹ h⁻¹ and µg NH₄⁺-N g⁻¹ h⁻¹, respectively. The NR formula was based on the increase in ammonia nitrogen concentration during incubation after correction for the blank control.

The O:N ratio was calculated by converting OR and NR into their corresponding atomic equivalents of oxygen and nitrogen according to the method described by Mayzaud and Conover (1988) [9]. Specifically, OR expressed as µg O₂ g⁻¹ h⁻¹ was converted to µmol O atoms g⁻¹ h⁻¹ by dividing by 16, and NR expressed as µg NH₄⁺-N g⁻¹ h⁻¹ was converted to µmol N atoms g⁻¹ h⁻¹ by dividing by 14. The O:N ratio was then calculated as the ratio of atomic oxygen consumption to atomic nitrogen excretion. This ratio was used to characterize relative metabolic substrate utilization. A schematic diagram of the closed static respirometry setup is shown in Figure 2.

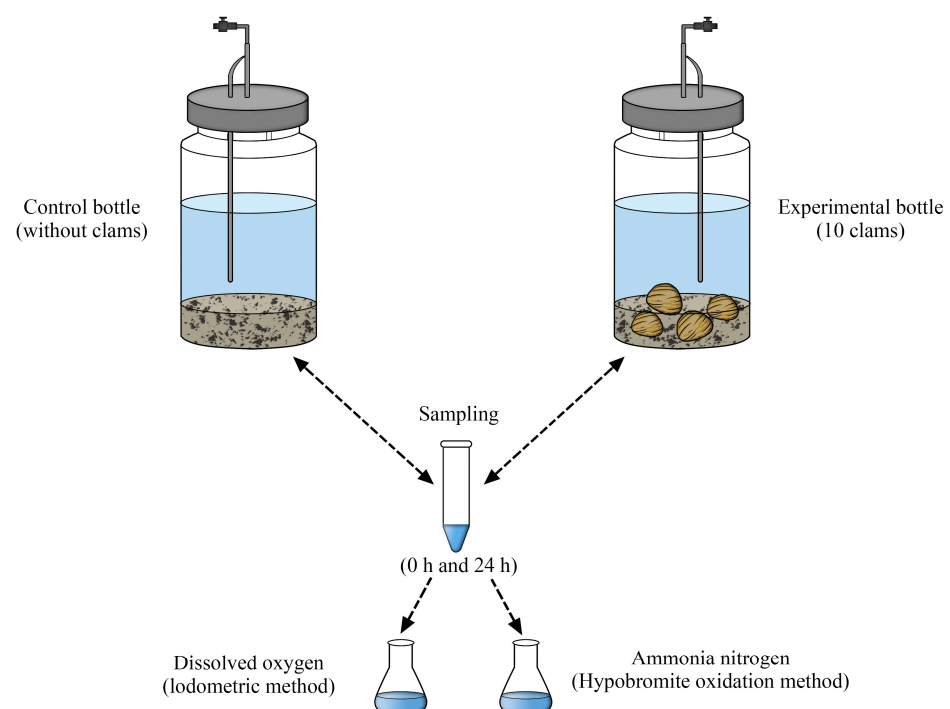


Figure 2. Schematic diagram of the closed static respirometry system used for respiratory metabolism measurements in *Ruditapes philippinarum*. Experimental bottles contained clams, sterilized sand substrate, and filtered seawater, whereas blank control bottles contained only substrate and seawater. Dissolved oxygen and ammonia nitrogen concentrations were measured at 0 and 24 h, and the data were used to calculate oxygen consumption rate (OR), ammonia excretion rate (NR), and O:N ratio. Dashed arrows illustrate the sampling flow for water collection from bottles for subsequent dissolved oxygen and ammonia-nitrogen analysis.

2.2.2. Measurement of Enzyme Activity

Enzyme activities were measured using clams with a shell length of approximately 15 mm. The enzyme-activity experiment used individuals from the same three original populations as the respiratory experiment but applied a different standardized culture regime before enzyme sampling. The experiment was conducted in plastic tanks measuring 30 cm × 21 cm × 12 cm. For each population, three independent replicate tanks were established, with 60 clams in each tank; therefore, 540 clams were initially used in the 45-day enzyme culture phase. A 7 cm layer of sand substrate, with a particle size of 251–425 µm, was placed at the bottom of each tank. The same substrate grain-size range was used as in the respiratory experiment to standardize the culture environment. The effective water volume in each tank after substrate addition was kept consistent among replicate tanks.

During the 45-day culture period before enzyme sampling, the water temperature was maintained at 15 ± 1 °C, and salinity was maintained at 30 ± 1 . Half of the seawater was renewed daily, and all clams were fed the same spirulina powder diet once per day. The 45-day standardized culture period was designed to reduce short-term nutritional and rearing differences before enzyme assays and to obtain a more stable biochemical state. However, it may also have introduced laboratory acclimation effects and partially reduced the original environmental signals from the source populations. In addition, spirulina powder may not have matched the natural or routine diets of all source populations. These trade-offs were addressed explicitly in the Discussion, and the enzyme results were interpreted as complementary indicators rather than as direct mechanistic explanations for all respiratory patterns. Morphometric characteristics of clams used for enzyme activity assays are presented in Table 2.

Table 2. Morphometric characteristics of Manila clams (*Ruditapes philippinarum*) used for enzyme activity assays after the 45-day standardized culture period.

Population	Shell Length (mm)	Shell Width (mm)	Shell Height (mm)	Wet Weight (g)
Laizhou wild-origin stock(LZ)	14.88 ± 0.43	5.97 ± 0.33	10.34 ± 0.49	0.60 ± 0.07
Fujian-origin stock cultured in Liaoning (FJ)	14.82 ± 1.02	5.81 ± 0.59	10.46 ± 0.85	0.50 ± 0.11
Zebra shell-color selected line (ZS)	15.21 ± 0.82	6.02 ± 0.44	10.25 ± 0.53	0.62 ± 0.10

At the end of the culture period, six clams were randomly selected from each replicate tank, giving 18 assayed individuals per population and 54 assayed individuals in total. Soft tissues were dissected and weighed. Each sample was homogenized individually with distilled water at a ratio of 1:5 (*w/v*). The homogenates were then centrifuged at 4000 rpm for 15 min at 4 °C, and the supernatants were collected for enzyme activity determination.

The activities of LDH, AKP, and SDH were measured using commercial assay kits according to the manufacturer's instructions. Protein concentration in the homogenate was determined using the protein assay supplied with the corresponding commercial kit, and enzyme activities were normalized to protein concentration and expressed as U mg^{−1} protein. For statistical analysis, each tank was treated as one biological replicate, and the mean value of the six individually measured clams within each tank was used to avoid pseudoreplication.

2.3. Statistical Analysis

All data are presented as mean ± standard deviation (mean ± SD). Prior to analysis, normality and homogeneity of variance were tested using Shapiro–Wilk and Levene tests, respectively. A two-way analysis of variance (ANOVA) was performed to evaluate the

effects of population, shell length category, and their interaction on OR, NR, and O:N ratio. When significant effects were detected, Duncan's multiple range test was used for post hoc comparisons. Because each population $\times$ shell-length combination included three replicate bottles and each bottle contained ten clams, the bottle rather than the individual clam was treated as the experimental unit for respiratory variables. This resulted in three biological replicates per population $\times$ shell-length category. This design was adequate for detecting major population or shell-length effects but provided limited power for detecting interaction effects. The relatively small number of biological replicates may therefore have reduced statistical power for detecting subtle interaction effects among factors.

Enzyme activities were analyzed using one-way ANOVA among populations, followed by Duncan's multiple range test. Each tank was treated as an independent biological replicate, and the mean value of the six individually measured clams within each tank was used for statistical analysis to avoid pseudoreplication. Statistical significance was set at $p < 0.05$. Because respiratory variables and enzyme activities were measured on different individuals and under different acclimation durations and temperatures, formal individual-level correlation analysis between respiratory rates and enzyme activities was not statistically appropriate. Instead, the parameters were integrated qualitatively in the Discussion.

2.4. Ethical Statement

Manila clams used in this study are invertebrate mollusks. No endangered or protected species were involved. The experimental procedures were conducted in accordance with the institutional guidelines for the care and use of experimental animals at Dalian Ocean University.

2.5. Data Availability and GenAI Statement

The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

Generative AI tools were used only for language refinement and grammar correction. All scientific content, data analysis, interpretation, and conclusions were developed and verified by the authors.

3. Results

3.1. Respiratory Metabolic Indicators Among Clam Populations

Respiratory metabolic indicators differed among the LZ, FJ, and ZS populations of *R. philippinarum*, and the variation pattern varied with shell length. OR, NR, and O:N ratio exhibited population-associated metabolic phenotypic characteristics across the 10, 15, and 20 mm shell-length populations under the standardized respiratory measurement conditions.

3.1.1. Oxygen Consumption Rate

Two-way ANOVA revealed that population had a significant effect on OR ($p < 0.05$), whereas shell length and the interaction effect were not significant ($p > 0.05$).

OR generally decreased with increasing shell length in the Laizhou (LZ) and Fujian (FJ) populations, although slight differences in variation patterns were observed among populations. At 10 and 15 mm shell lengths, the ZS population exhibited significantly lower OR than the LZ and FJ populations ($p < 0.05$). In contrast, at 20 mm shell length, the FJ population showed the lowest OR among the three populations ($p < 0.01$).

For example, at 10 mm shell length, OR values were 13.80 ± 1.90 , 10.12 ± 1.40 , and $2.01 \pm 0.91 \mu\text{g O}_2 \text{ g}^{-1} \text{ h}^{-1}$ in the LZ, FJ, and ZS populations, respectively. At 20 mm shell length, OR values in the FJ population decreased to $1.79 \pm 0.41 \mu\text{g O}_2 \text{ g}^{-1} \text{ h}^{-1}$, which was significantly lower than those of the LZ and ZS populations (Figure 3).

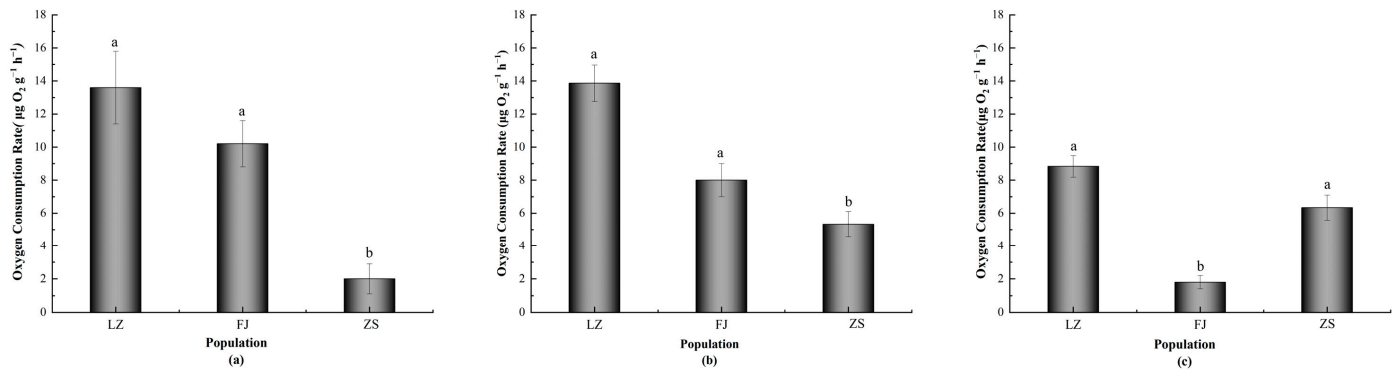


Figure 3. Oxygen consumption rates of three populations at different shell lengths. (a) 10 mm shell length; (b) 15 mm shell length; (c) 20 mm shell length. Values are presented as mean $\pm$ SD ($n = 3$). Different lowercase letters indicate significant differences among populations within the same shell-length populations ($p < 0.05$).

3.1.2. Ammonia Excretion Rate

Two-way ANOVA showed that both population and shell length significantly affected NR ($p < 0.001$), whereas the interaction effect was not significant ($p > 0.05$).

NR generally decreased with increasing shell length in all three populations (Figure 4). The ZS population exhibited the highest NR at the 10 mm shell-length category, whereas the LZ population showed the lowest NR at the 20 mm shell-length category. At 15 and 20 mm, the FJ and ZS populations remained higher than the LZ population, and the two did not differ significantly within each shell-length category.

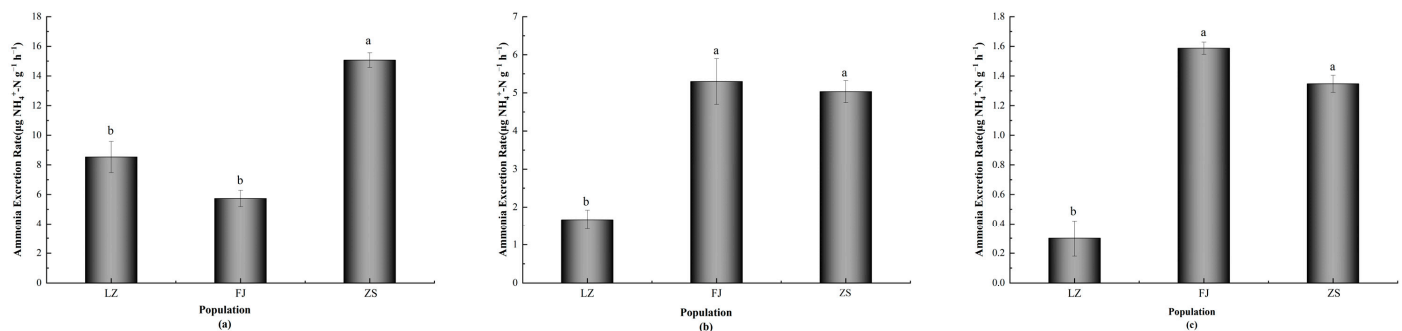


Figure 4. Ammonia excretion rates of three populations at different shell lengths: (a) 10 mm shell length; (b) 15 mm shell length; (c) 20 mm shell length. Values are presented as mean $\pm$ SD ($n = 3$). Different lowercase letters indicate significant differences among populations within the same shell-length population ($p < 0.05$). Note that the y -axis scales differ among shell-length populations to improve data visualization.

At 10 mm shell length, the ZS population exhibited the highest NR ($15.10 \pm 0.49 \mu\text{g NH}_4^+-\text{N g}^{-1} \text{h}^{-1}$), which was significantly higher than that of the FJ and LZ populations ($p < 0.05$). At 20 mm shell length, NR in the LZ population decreased to $0.30 \pm 0.12 \mu\text{g NH}_4^+-\text{N g}^{-1} \text{h}^{-1}$, remaining significantly lower than that of the FJ and ZS populations ($p < 0.01$).

3.1.3. O:N Ratio

Two-way ANOVA revealed significant effects of population and shell length on O:N ratio ($p < 0.001$), whereas the interaction effect was not significant ($p > 0.05$).

The O:N ratio increased markedly with shell length in the LZ population but remained relatively low in the FJ and ZS populations across all shell-length populations (Figure 5).

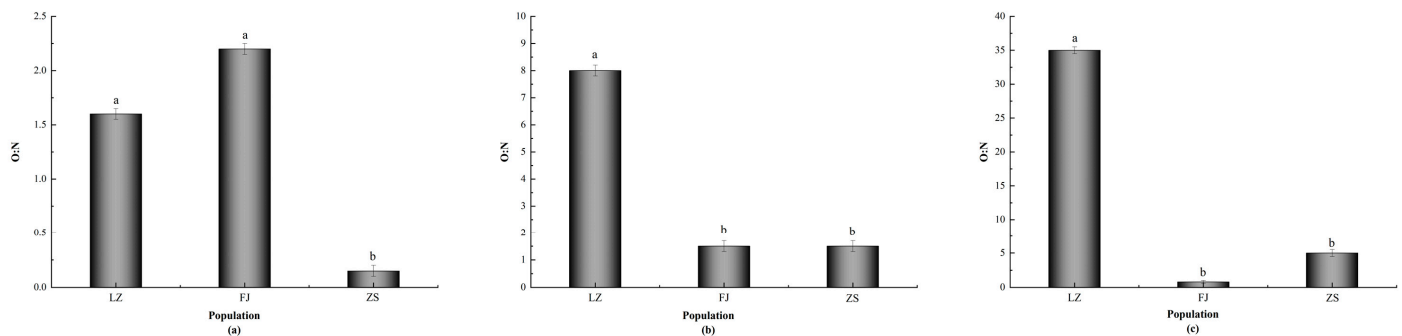


Figure 5. O:N ratios of three populations at different shell lengths: (a) 10 mm shell length; (b) 15 mm shell length; (c) 20 mm shell length. Values are presented as mean $\pm$ SD ($n = 3$). Different lower-case letters indicate significant differences among populations within the same shell-length population ($p < 0.05$). Note that the y -axis scales differ among shell-length populations to improve data visualization.

At 10 mm shell length, the ZS population showed the lowest O:N ratio (0.15 ± 0.04), which was significantly lower than those of the LZ and FJ populations ($p < 0.05$). In contrast, at 20 mm shell length, the O:N ratio of the LZ population increased to 35.12 ± 0.50 , significantly exceeding those of the FJ and ZS populations ($p < 0.01$).

Overall, the marked increase in O:N ratio in the LZ population suggests a potential shift in metabolic substrate utilization during growth, whereas the FJ and ZS populations maintained relatively protein-oriented metabolic characteristics under the present experimental conditions. Because the O:N ratio was not accompanied by measurements of tissue biochemical composition, feeding rate, absorption efficiency, gonadal stage, or complete energy budget, this interpretation should be regarded as an inference based on respiratory and excretory indicators rather than as direct evidence of substrate use.

3.2. Respiratory Metabolic Enzyme Activities Among Clam Populations

The activities of LDH, AKP, and SDH differed among populations, indicating variation in selected biochemical indicators related to anaerobic metabolism, nutrient-related metabolic activity, and aerobic energy metabolism (Figure 6). Because enzyme assays were conducted only in 15 mm individuals and under a different acclimation temperature and duration from the respiratory experiment, the enzyme results were interpreted as complementary evidence rather than as a direct mechanistic explanation for all respiratory patterns.

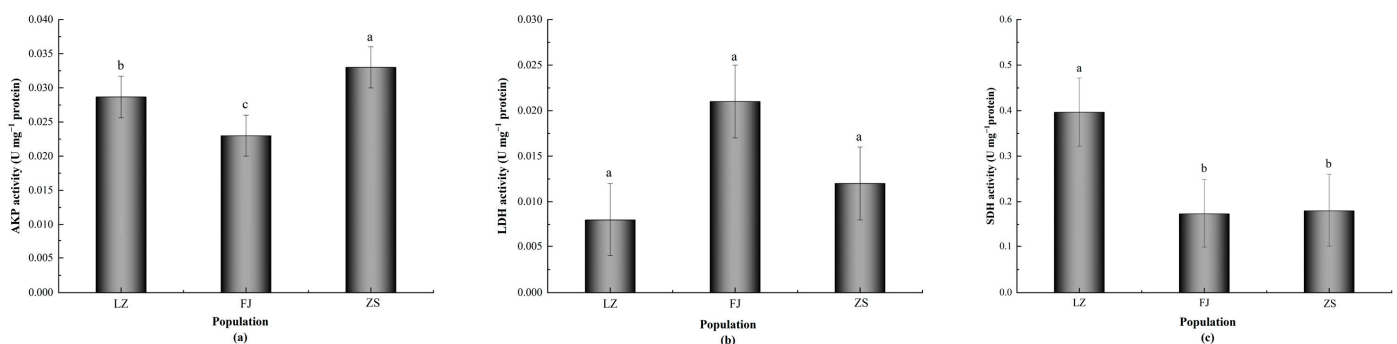


Figure 6. Key metabolic enzyme activities in the populations. (a) Lactate dehydrogenase (LDH) activity; (b) alkaline phosphatase (AKP) activity; (c) succinate dehydrogenase (SDH) activity. Values are presented as mean $\pm$ SD ($n = 3$). Different lowercase letters indicate significant differences among populations ($p < 0.05$). Note that the y -axis scales differ among enzymes.

No significant differences in LDH activity were detected among the three populations ($p > 0.05$), although the FJ population showed the highest numerical value ($0.021 \pm 0.005 \text{ U mg}^{-1} \text{ protein}$), followed by the ZS and LZ populations.

In contrast, AKP activity differed significantly among populations ($p < 0.05$). The ZS population exhibited the highest AKP activity ($0.033 \pm 0.004 \text{ U mg}^{-1} \text{ protein}$), whereas the FJ population showed the lowest value ($0.023 \pm 0.003 \text{ U mg}^{-1} \text{ protein}$).

SDH activity also showed significant metabolic differentiation ($p < 0.01$). The LZ population exhibited significantly higher SDH activity ($0.397 \pm 0.075 \text{ U mg}^{-1} \text{ protein}$) than the FJ and ZS populations, while no significant difference was observed between FJ and ZS ($p > 0.05$).

Combined with the relatively high O:N ratio observed in larger LZ individuals, the elevated SDH activity in LZ may represent variation in aerobic metabolic characteristics under enzyme assay conditions; however, this interpretation cannot be directly linked to respiratory measurements because the two datasets were obtained under different experimental designs.

3.3. Comparison of Respiratory and Enzyme Activity Profiles

The respiratory and enzyme datasets provide complementary physiological information, although they were obtained from independent experimental cohorts.

Independently, respiratory measurements indicated that LZ showed a higher O:N ratio at larger shell sizes, whereas enzyme assays showed higher SDH activity under the separate enzyme-assay conditions.

LZ showed relatively higher OR values in smaller individuals, an increase in the O:N ratio with shell length, and higher SDH activity under the enzyme-assay conditions. ZS combined low OR at small sizes, high NR at 10 mm, and the highest AKP activity after standardized culture. FJ showed reduced OR at 20 mm and relatively low AKP activity but maintained moderate NR. However, a formal correlation matrix among OR, NR, O:N ratio, LDH, AKP, and SDH was not calculated because the respiratory and enzyme variables were measured from different individuals under different acclimation durations and temperatures. This constraint was addressed by qualitative integration in the Discussion rather than by unsupported statistical correlation.

4. Discussion

4.1. Population-Associated Respiratory Physiological Variation

Respiratory metabolism reflects physiological status and energy demand in aquatic organisms. In the present study, the LZ, FJ, and ZS populations of *R. philippinarum* exhibited population-associated differences in OR, NR, and O:N ratio under standardized laboratory measurement conditions.

These differences represent population-associated physiological variation observed under the present experimental conditions. Because the present design did not include common-garden breeding, reciprocal transplantation, or genomic analysis, the relative contributions of source background, environmental history, and their interaction cannot be quantitatively separated. Therefore, the observed differences should be considered as phenotype-level variation among germplasm categories rather than evidence of genetic adaptation.

4.1.1. Interpretation Limitation of Population Variation

A key limitation of this study is that a full common-garden or multi-generational design was not implemented. Therefore, population background effects and environmental history effects cannot be statistically separated in the present dataset. Accordingly,

the observed variation should be considered as an integrated phenotype-level outcome associated with source background, previous culture history and pre-experimental environmental exposure.

Population background and environmental history could not be statistically separated in the present study; therefore, observed differences were interpreted as combined effects of population lineage and rearing history.

4.1.2. Interpretation of Variation in Oxygen Consumption Rate

OR is widely used as an indicator of basal metabolic level and aerobic metabolic characteristics in aquatic organisms [10,11]. In the present study, OR generally declined with increasing shell length in the LZ and FJ populations, which is consistent with the metabolic scaling patterns commonly reported in bivalves [12,13]. Ibarrola et al. [13] reported that mass-specific metabolic rate in *M. galloprovincialis* decreased with increasing body size, reflecting reduced relative energy demand during growth. Similar metabolic allometry has also been described in other marine bivalves [12].

Compared with the LZ and FJ populations, the ZS population exhibited relatively low OR at smaller shell-length classes. Similar physiological differences have been reported among bivalve populations with different culture backgrounds; however, in the present study, no multi-generation selection records or controlled population background were available. Therefore, this difference is interpreted only as a strain-associated phenotypic variation under laboratory conditions. Zhang et al. [14] demonstrated that selectively bred pearl oysters exhibited altered metabolic and feeding characteristics compared with cultured populations, suggesting that breeding background may affect energy utilization patterns.

The relatively high OR observed in the LZ and FJ populations at smaller shell lengths may be associated with size-dependent physiological variation and pre-experimental rearing history. Previous studies have shown that fluctuations in temperature, salinity, dissolved oxygen, food availability, and diet quality can significantly affect respiratory metabolism in marine bivalves [12]. However, because source-site environmental datasets were incomplete, their contributions cannot be quantitatively evaluated in the present study.

Although slight differences in OR variation patterns were observed among populations, the interaction effect between population and shell length was not significant. Therefore, these differences should not be interpreted as evidence of distinct ontogenetic metabolic trajectories. In addition, because the FJ population originated from Fujian but was cultured in Liaoning before the experiment, its metabolic phenotype may reflect both original stock background and subsequent rearing conditions.

At larger shell lengths, the ZS population showed relatively stable OR values. Bordinon et al. [15] reported that juvenile *R. philippinarum* can maintain rapid growth under suitable environmental conditions, implying sustained energy demand during development. However, because growth rate, feeding activity, assimilation efficiency, and energy allocation were not directly measured in the present study, the biological meaning of this pattern remains uncertain.

4.1.3. Interpretation of Variation in Ammonia Excretion Rate

NR is an important physiological indicator reflecting protein catabolism and nitrogenous waste excretion [5]. When interpreted together with OR, NR provides useful information regarding metabolic substrate utilization and physiological energy demand.

In the present study, NR generally decreased with increasing shell length, which is consistent with previous reports showing that excretion rates generally decline with increasing body size in aquatic invertebrates [4,16]. However, population differences

were still observed within the same shell-length categories, although the ranking was not fully consistent across all sizes. Therefore, results are described as population-associated variation rather than a uniform inter-population hierarchy.

The ZS population showed relatively higher NR at smaller shell lengths. Similar patterns have been reported in juvenile bivalves; however, this observation should be interpreted only as a higher nitrogen excretion level under laboratory conditions, without inferring specific physiological mechanisms.

Previous studies have shown that increased NR variation may be associated with multiple biological processes [16,17]. Pan et al. [17] reported higher nitrogen turnover in rapidly growing juvenile bivalves; however, such associations cannot be directly inferred in the present study because growth rate, feeding activity, and tissue biochemical composition were not measured. Therefore, differences in NR among populations may reflect a combination of population background and short-term physiological condition under experimental conditions.

For populations with different source backgrounds and rearing histories, variation in NR may also be associated with differences in shell-length-associated physiological regulation, diet history and energy allocation. However, because growth rate, biochemical composition, feeding rate, and assimilation efficiency were not directly measured in the present study, the physiological mechanisms underlying these differences remain uncertain.

At larger shell lengths, the FJ population exhibited relatively low OR together with moderate NR values.

Some previous studies have linked combined changes in respiratory and excretory metabolism to reproductive processes in marine bivalves [18,19]. Hasan Mredul et al. [18] reported that reproductive activity can significantly influence metabolic demand in marine broadcast spawners, whereas Li et al. [19] demonstrated that glycogen metabolism changes substantially during gametogenesis in oysters. Such metabolic characteristics may be influenced by developmental stage, reproductive condition, or diet-related energy allocation. However, in the present study, no histological or reproductive indicators were measured, and all individuals were treated as juvenile-sized clams; therefore, reproductive interpretation remains speculative.

4.1.4. Interpretation of Variation in O:N Ratio

The O:N ratio is widely used to evaluate metabolic substrate utilization in aquatic animals. Generally, low O:N ratios indicate greater reliance on protein metabolism, whereas higher values suggest increased contributions of lipids and carbohydrates [7].

In the present study, all three populations exhibited relatively low O:N ratios at smaller shell lengths, suggesting that protein metabolism played an important role during early growth. Similar metabolic characteristics have been reported in rapidly growing juvenile bivalves, in which tissue formation and protein synthesis require substantial nitrogenous compounds [17]. Pan et al. [17] reported that high protein turnover is a characteristic feature of early developmental stages in marine bivalves.

As shell length increased, the O:N ratio of the LZ population increased markedly and became significantly higher than those of the FJ and ZS populations. This pattern suggests a potential shift toward broader use of metabolic substrates during growth. Similar metabolic shifts have previously been associated with changes in physiological demand, energy allocation, and reproductive preparation in marine invertebrates [18,19]. However, the exact threshold at which metabolism shifts from protein-dominated to more lipid- or carbohydrate-supported metabolism cannot be defined from O:N ratio alone because biochemical composition, diet assimilation, and energy-budget variables were not measured.

Previous studies have also demonstrated that geographic origin and environmental background can influence physiological metabolism in *R. philippinarum* populations [20]. Tan et al. [20] reported population-associated transcriptional responses in Manila clams under temperature stress, suggesting that long-term environmental history may contribute to physiological differentiation among populations. The present study could not quantify this contribution because source-site temperature, salinity, dissolved oxygen, pH, natural food abundance, sediment chemistry, and pollution indicators were not fully available.

The FJ and ZS populations maintained relatively stable O:N values across size classes, indicating consistent metabolic balance under experimental conditions. However, this statement is limited to descriptive observation and does not imply specific metabolic substrate dominance.

Previous studies have shown that salinity stress can induce significant physiological and metabolic adjustments in aquatic invertebrates, affecting osmotic regulation and overall energy metabolism [21].

However, interpretation of O:N ratio should be approached cautiously because this parameter can be affected by multiple physiological and environmental factors. Additional measurements of biochemical composition, reproductive status, and energy allocation would be necessary to further clarify the metabolic significance of the observed patterns.

4.2. Complementary Biochemical Characterization

Metabolic enzyme activities provide additional insight into selected biochemical differences among *R. philippinarum* populations. In the present study, SDH and AKP activities differed significantly among populations, whereas LDH activity remained relatively stable. These results suggest that aerobic metabolic processes and nutrient-related physiological processes may vary among populations under the enzyme-assay conditions, whereas basal anaerobic metabolic characteristics appear comparatively conserved. However, because enzyme assays were performed after 45 days of standardized laboratory culture at 15 ± 1 °C, the results may reflect both original population differences and laboratory acclimation effects.

4.2.1. Succinate Dehydrogenase Activity

SDH is a key enzyme involved in both the tricarboxylic acid cycle and mitochondrial electron transport chain and is commonly used to evaluate aerobic metabolic characteristics and mitochondrial function [22,23]. In the present study, the LZ population exhibited significantly higher SDH activity than the FJ and ZS populations, suggesting relatively stronger aerobic metabolic potential under the experimental conditions.

This result is generally consistent with the respiratory physiological profiles observed in the present study. The LZ population also exhibited relatively high OR values and markedly elevated O:N ratios at larger shell lengths, indicating possible differences in aerobic energy metabolism and substrate-utilization indicators compared with the other populations. However, because the respiratory and enzyme datasets were generated under different acclimation conditions, the apparent consistency between SDH activity and respiratory traits should be treated as supportive but not conclusive evidence.

Previous studies have demonstrated that geographic differentiation exists among *R. philippinarum* populations and that environmental heterogeneity may contribute to variation in physiological and metabolic traits [24,25]. Tan et al. [25] reported that population structure and migration dynamics differed among Manila clam populations along the Chinese coast, suggesting potential ecological differentiation among geographic populations. In addition, northern populations often experience relatively large seasonal fluctuations in temperature and environmental conditions, which may influence respiratory metabolism and transcriptional regulation [26].

However, differences in SDH activity should not be interpreted solely as evidence of inherent geographic metabolic superiority. Physiological metabolism in *R. philippinarum* can also be affected by dietary conditions, developmental stage, reproductive status, environmental exposure, and selective breeding background [27,28]. The standardized spirulina diet used before enzyme sampling reduced short-term diet variability among populations, but it may also have differed from their routine or natural diets, potentially influencing enzyme activity.

Therefore, the elevated SDH activity observed in the LZ population was likely influenced by multiple interacting factors, including population background, environmental history, physiological status, and laboratory diet/acclimation effects. Because all populations were not reared under identical long-term environmental conditions before the experiment, the relative contributions of genetic and environmental factors could not be fully separated. A common-garden design over multiple generations would be required to test whether these differences represent heritable population effects.

4.2.2. Alkaline Phosphatase Activity

AKP is involved in dephosphorylation processes and is associated with nutrient absorption, transmembrane transport, tissue growth, and physiological regulation [28,29]. In bivalves, AKP activity is commonly regarded as an indicator of nutritional metabolism and physiological status.

In the present study, the ZS population exhibited the highest AKP activity among the three populations, suggesting relatively active phosphate-related metabolic regulation under the experimental conditions. Previous transcriptomic studies have shown that immune and stress-related pathways (e.g., HIF and NF- κ B signaling) are involved in physiological responses of *Ruditapes philippinarum* under hypoxic stress, indicating that metabolic enzymes may be indirectly linked to environmental stress response processes. Jia and Nie [30] further demonstrated that AKP activity may participate in immune-related and stress-responsive physiological regulation in Manila clams.

The elevated AKP activity observed in the ZS population may therefore reflect differences in nutrient metabolism, tissue-level physiological activity, or environmental response capacity. Because ZS represents a Zebra shell-color selected line maintained under aquaculture conditions, its physiological characteristics may reflect both selection history and subsequent culture environment.

Previous studies comparing wild and cultivated Manila clam populations under temperature, salinity, hypoxia, and cold stress have similarly demonstrated that breeding background and environmental history can influence physiological responses [31].

Environmental conditions such as temperature fluctuations and sediment-related hypoxia have been shown to trigger cascading environmental effects, including reduced dissolved oxygen and increased hydrogen sulfide accumulation, which can significantly influence physiological processes in *Ruditapes philippinarum* [32].

In contrast, the FJ population exhibited relatively low AKP activity together with reduced OR at larger shell lengths. This pattern may indicate relatively lower metabolic investment in nutrient processing and physiological regulation under the present enzyme-assay conditions. However, because biochemical composition, reproductive development, immune indices other than AKP, and growth performance were not directly evaluated, the physiological significance of AKP variation among populations should be interpreted cautiously.

4.2.3. Lactate Dehydrogenase Activity

LDH is a key enzyme involved in glycolysis and anaerobic metabolism and is commonly used to evaluate physiological responses to hypoxia and environmental stress [33].

In bivalves, LDH activity is often associated with hypoxia tolerance, anaerobic compensation, and short-term environmental fluctuation.

In the present study, LDH activity did not differ significantly among populations, although the numerical values followed the order FJ > ZS > LZ. This result suggests that basal anaerobic metabolic characteristics remained relatively stable among populations under experimental conditions.

Previous studies have shown that differences in anaerobic metabolic enzyme activity are often more pronounced under environmental stress conditions than under resting physiological states [34]. Papadopoulos et al. [34] demonstrated that thermal stress can significantly alter LDH-related metabolic responses in marine bivalves, whereas Meng et al. [35] reported that hypoxia tolerance in oysters is associated with inducible variation in anaerobic energy metabolism.

Compared with SDH and AKP, LDH exhibited relatively limited population differentiation in the present study. This finding may indicate that basal glycolytic metabolism is comparatively conserved among populations in the absence of acute environmental stress. Similar observations have been reported in Manila clams exposed to salinity, hypoxia, and temperature fluctuations, in which LDH responses were more closely associated with stress intensity than with basal population differences [35,36].

Clara et al. [37] further demonstrated that oyster populations from different geographic origins exhibited distinct inducible glycolytic gene expression patterns under hypoxic stress, suggesting that population-level differences in anaerobic metabolism may become more apparent under environmentally challenging conditions.

Therefore, although no significant LDH differences were detected under the present non-stress experimental conditions, future stress-challenge experiments involving hypoxia, salinity fluctuation, temperature stress, and controlled diet treatments may provide further insight into anaerobic metabolic plasticity and physiological adaptation among populations.

4.3. Complementary Interpretation of Respiratory and Enzyme Indicators

Respiratory metabolism and enzyme activities provide complementary physiological information, although these measurements were obtained from independent experimental cohorts under different acclimation regimes. Respiratory measurements reflected whole-animal oxygen consumption and nitrogen excretion patterns under short-term standardized conditions, whereas enzyme assays represented selected biochemical states after a longer laboratory culture period. Therefore, these datasets were not quantitatively integrated but were used to characterize different aspects of population-associated physiological profiles.

Under the present experimental conditions, the three populations exhibited distinct combinations of respiratory and biochemical characteristics. The LZ population showed relatively higher OR values at smaller shell lengths, an increasing O:N ratio with shell length, and higher SDH activity in the enzyme assay. These observations indicate that LZ exhibited a distinct physiological profile involving respiratory and biochemical indicators; however, whether these differences reflect persistent population characteristics or the influence of previous environmental history cannot be determined from the current design.

The ZS population showed relatively lower OR at smaller shell lengths, higher NR at the 10 mm shell-length category, and the highest AKP activity after standardized culture. These patterns represent differences in nitrogen excretion and enzyme activity under the specific experimental conditions but should not be interpreted as evidence of a particular metabolic strategy or functional advantage. The FJ population showed relatively lower OR at larger shell lengths and lower AKP activity, while maintaining moderate NR values. Together, these observations suggest that physiological variation among populations cannot be fully explained by shell length differences alone.

Importantly, the observed respiratory and biochemical patterns should be interpreted as phenotype-level variation associated with different source backgrounds and culture histories rather than as direct evidence of genetic differentiation or adaptation. Multiple factors, including environmental history, developmental condition, reproductive status, dietary background, biochemical composition, and laboratory acclimation, may contribute to the observed variation. Future studies using multi-generation common-garden experiments, reciprocal environmental treatments, and genomic approaches will be required to determine the relative contributions of genetic and environmental factors.

The present study does not aim to redefine metabolic scaling patterns in bivalves, as the decline of mass-specific metabolic and excretory rates with increasing body size is widely recognized. Instead, the contribution of this work is the establishment of a comparative physiological baseline by simultaneously evaluating respiratory indicators, O:N ratio, and selected metabolic enzymes among Manila clam populations representing different source backgrounds: a wild-origin stock, a cross-regionally cultured stock, and a Zebra shell-color selected line. This phenotype-level dataset provides measurable physiological references for future germplasm characterization and generates testable hypotheses regarding whether population-associated physiological differences persist under controlled long-term common-garden conditions.

4.4. Scientific Implications and Future Validation

The main contribution of this study is not to establish causal mechanisms underlying population differences, but to provide a phenotype-level physiological baseline for Manila clam germplasm characterization. Under standardized laboratory conditions, populations representing different source backgrounds exhibited distinct combinations of respiratory and biochemical traits.

These physiological profiles provide testable hypotheses for future studies. For example, whether differences in SDH activity in LZ or AKP activity in ZS represent persistent population characteristics requires validation through multi-generation common-garden experiments, genomic analysis, and controlled environmental manipulation.

Therefore, the present study establishes a comparative physiological framework that links germplasm background with measurable functional traits while recognizing the necessity of integrating genetic and environmental approaches to resolve underlying mechanisms.

5. Conclusions

Overall, this study demonstrated population-associated differences in respiratory metabolism and selected metabolic enzyme activities among LZ, FJ, and ZS populations of *R. philippinarum* under standardized laboratory conditions. OR generally decreased with shell length in LZ and FJ, NR generally declined with shell length across populations, and the O:N ratio increased markedly in LZ but remained relatively low in FJ and ZS.

The enzyme results showed significantly higher SDH activity in LZ and higher AKP activity in ZS, whereas LDH activity did not differ significantly among populations. These results suggest variation in aerobic metabolic potential, nitrogen excretion, and nutrient-related physiological processes, but they do not prove fixed genetic differences because source environment, diet history, developmental status, and laboratory acclimation were not fully separated.

The findings provide baseline physiological information for Manila clam germplasm-associated physiological characterization and aquaculture management. Future studies using multi-site sampling, controlled diets, gonadal staging, complete environmental monitoring, growth and biochemical measurements, and common-garden or multi-generation designs are needed to test the mechanisms underlying the patterns observed here.

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