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Synergistic Impacts of Hypoxia and High Temperature on the Bay Scallop, *Argopecten irradians*: Mortality, Gene Expression, and Hemocyte Responses

Jeong-Hwa Kim[1], Nobuhisa Kajino[1], Jong-Seop Shin[1], Kwang-Sik Choi[1], Hee-Jung Lee[2] and Hyun-Ki Hong[3]

[1]Department of Marine Life Science (BK21 FOUR) and Marine Science Institute, Jeju National University, Jeju 63243, Korea
[2]Southeast Sea Fisheries Research Institute, National Institute of Fisheries Science (NIFS), Tongyeong 53085, Korea
[3]Department of Marine Biology and Aquaculture, Gyeongsang National University, Tongyeong 53064, Korea

ABSTRACT

Hypoxia coupled with high water temperatures during summer poses a significant threat to aquatic animals. This study investigated the combined effect of these stressors on the bay scallop, *Argopecten irradians*, to understand potential causes of mass mortality events. Scallops were exposed to control (19℃, 6.76 mg O_2/L), hypoxia (19℃, 1.01 mg O_2/L), and combined hypoxia and high temperature (28℃, 1.02 mg O_2/L) conditions for 48 hours. Quantitative PCR (qPCR) assessed mRNA expression of hypoxia-inducible factor 1-alpha (HIF1-α) and heat shock protein 90 (HSP90) in the digestive gland. Flow cytometry evaluated cellular responses, including phagocytosis capacity, nitric oxide (NO) production, and reactive oxygen species (ROS) production of hemocytes. Scallops exposed to combined stressors showed 50% mortality within 48 h, starting at 30 h, while hypoxia alone caused only 18% mortality. Gene expression remained unchanged under hypoxia, but HIF1-α and HSP90 were significantly upregulated under combined stressors. Hemocyte parameters did not show statistically significant differences across groups; however, there was a trend of decreased phagocytic capacity and increased ROS and NO production under combined stressors. These results suggest that the synergistic effect of hypoxia and high temperature during summer can exert sublethal to lethal impacts on bay scallops, potentially explaining mortality events in this species. Further research is needed to explore the underlying mechanisms and potential adaptive strategies in scallops facing such environmental challenges.

Keywords: *Argopecten irradians*, Hypoxia, High temperature, Combined stressors, Gene expression, Hemocyte responses

INTRODUCTION

Bay scallops (*Argopecten irradians*) are economically significant bivalve mollusks native to the northwest Atlantic coast of North America (MacKenzie Jr, 2008; Oreska *et al.*, 2017). Historically important since the mid-1600s, particularly in regions like Long Island, New York, they have supported commercial fisheries and local economies (MacKenzie Jr, 2008). However, due to overfishing and habitat degradation, wild stocks have declined significantly since the 1980s (MacKenzie Jr, 2008). In response to declining wild populations and increasing consumer demand, bay scallop aquaculture has expanded globally. This species was introduced to China in 1982, developing into a significant aquaculture industry in northern China (Guo and Luo, 2006, 2016). Similarly, bay scallops

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Corresponding author: Hyun-Ki Hong
Tel: +82 (55) 772-9151, e-mail: hyunki@gnu.ac.kr
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were introduced to Korea from China in 1996, with researchers conducting experiments on artificial spawning and larval development in 1997 (Kang *et al.*, 2019).

In Korea, bay scallop aquaculture has become increasingly important, particularly in the southern coastal areas. Gyeongnam province has emerged as the largest producer, accounting for over 90% of the country's cultivated scallop production. Production has grown rapidly, from 25 tons in 2006 to 11,521 tons in 2023 (http://kosis.kr/). However, the success of bay scallop aquaculture faces challenges from environmental stressors. In recent years, the bays along Korea's southern coast where scallops are cultivated have been experiencing persistent hypoxic conditions along with high water temperatures during summer (Lee *et al.*, 2016, 2018). These combined stressors pose a significant threat to scallop survival and production.

Previous studies have shown that marine bivalves, including scallops, exhibit various physiological, biochemical, and behavioral responses to hypoxia and high temperatures. Under hypoxic conditions, bivalves may suppress metabolic rates and activate anaerobic pathways (Anestis *et al.*, 2010; Nokko *et al.*, 2005; Meng *et al.*, 2018; Adzigbli *et al.*, 2024). High temperatures can lead to cellular damage, oxidative stress, and impaired immune function (Monari *et al.*, 2007; Rahman *et al.*, 2019; Zhu *et al.*, 2021). The production of heat shock proteins is often elevated in response to thermal stress, helping maintain cellular integrity (Zhu *et al.*, 2021). When bivalves face the dual challenge of hypoxia and high temperature, the combined effects can exacerbate physiological challenges, as elevated temperatures increase metabolic rates and oxygen demand (Artigaud *et al.*, 2014). When combined, these stressors can have synergistic effects more severe than each alone, potentially leading to mass mortality events (Orr *et al.*, 2020). Despite the importance of understanding these combined effects, there is a scarcity of studies examining multiple stressors simultaneously, particularly in the context of aquaculture. This research gap limits our ability to predict and mitigate the impacts of environmental changes on scallop production.

The study aims to investigate the combined effects of hypoxia and high water temperature on the bay scallop, *Argopecten irradians*, with the objective of understanding the potential causes of mass mortality events during summer conditions. Specifically, the research seeks to assess the molecular and cellular responses of bay scallops to both hypoxia alone and the combined stress of hypoxia and high temperature. It also aims to evaluate the survival rates of bay scallops under different stress conditions. The study is guided by several hypotheses: first, that the combination of hypoxia and high temperature will have a more severe impact on bay scallop survival than hypoxia alone; second, that exposure to combined stressors will induce significant changes in the expression of stress-related genes, such as hypoxia-inducible factor 1-alpha (HIF1-α) and heat shock protein 90 (HSP90), compared to hypoxia alone or control conditions; third, that hemocyte responses, including phagocytic capacity, reactive oxygen species (ROS) production, and nitric oxide (NO) production, will be significantly altered in scallops exposed to combined stressors relative to those exposed to hypoxia alone or control conditions; and finally, that the synergistic effect of hypoxia and high temperature will lead to sublethal or lethal impacts on bay scallops, potentially explaining observed mortality events in natural populations during summer. These objectives and hypotheses aim to elucidate the mechanisms underlying the vulnerability of bay scallops to combined environmental stressors and provide insights into the causes of mass mortality events in this species.

MATERIALS AND METHODS

In August 2021, the bay scallops were obtained from a commercial farm located in Jaran Bay on the southern coast of Korea. The scallops were transported to the laboratory within 3 h under cool conditions and maintained in a tank with aerated

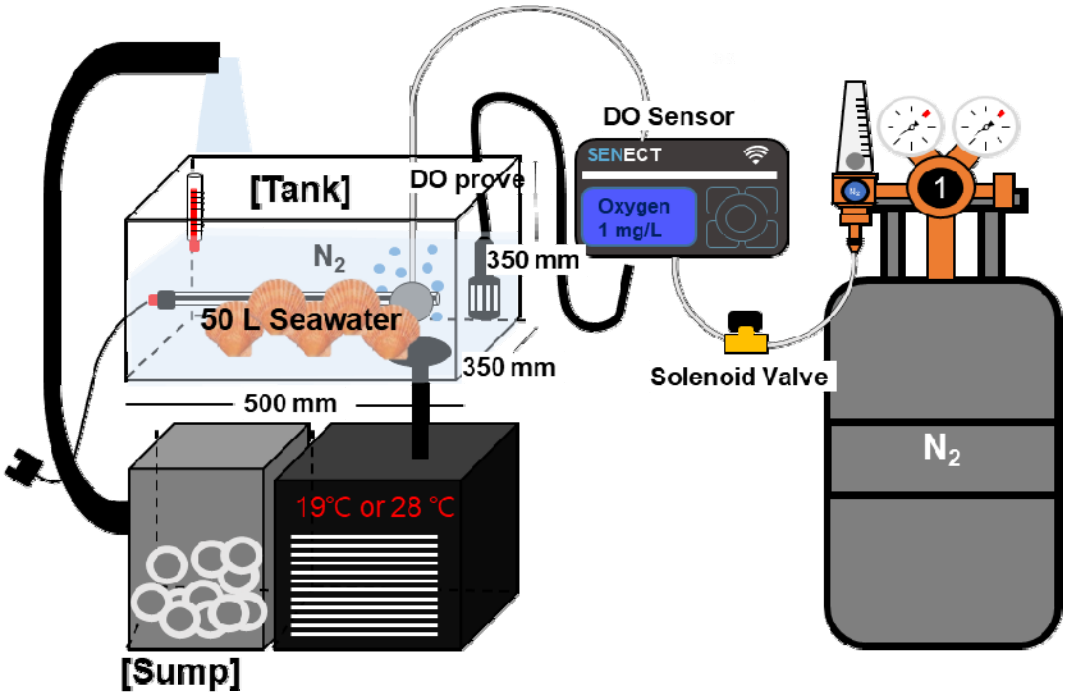

Fig. 1. Experimental setups used in the hypoxia and high-temperature exposure experiments.

seawater (salinity 31; water temperature 19℃) over 48 h to minimize physiological stress induced during transportation. The shell height (i.e., the straight-line distance from the umbo to the outer shell margin) of the scallops used in the experiment ranged from 34.9 to 48.8 mm, with a mean of 42.1 mm. A total of 50 randomly selected scallops were exposed to control (19℃, 6.76 mg O_2/L), hypoxia (19℃, 1.01 mg O_2/L), and combined hypoxia and high temperature (28℃, 1.02 mg O_2/L) conditions for 48 h (Fig. 1). The hypoxia condition was maintained at 1 mg/L using nitrogen gas with SENECT TWO (Senect GmbH, Landu, Germany) dissolved oxygen automatic controller, and the high seawater temperature condition was maintained at 28℃ using heater rod (Thermocontrol 250, EHEIM GmbH & Co. KG, Germany) mechanical thermostat (Fig. 1). During treatments, the scallops were fed once a day with an LPB frozen shellfish diet (Reed Mariculture, USA) at a concentration of 1×10^6 cells/mL. After feeding, the seawater was replaced once a day. The mortality was recorded at 3-hour intervals to calculate the cumulative mortality rate.

To determine immune responses at the end of the experiment, hemolymph was collected from 25 randomly selected individuals under each experiment condition after 48 h. Hemocyte parameters, including phagocytosis capacity, reactive oxygen species (ROS), and nitric oxide (NO) production of individual scallops, were analyzed using a CytoFlex flow cytometer (Beckman Coulter, SA). Approximately 1 mL of hemolymph was extracted from the adductor muscle using a 22G needle-fitted syringe and immediately transferred to ice-cooled microtubes to prevent coagulation. For phagocytosis capacity, 100 µL of hemolymph was mixed with 100 µL of sterilized seawater and 30 µL of 2% diluted fluorescent latex beads (2 µm diameter; Polyscience Inc., SA). The mixture was incubated in the dark at room temperature for 120 minutes, and phagocytic capacity was expressed as the percentage of cells engulfing more than three beads. ROS production was evaluated using 2',7'-dichlorofluorescin diacetate (DCFH-DA; Sigma-Aldrich, USA). In this assay, 100 µL of hemolymph was diluted with an equal volume of sterilized seawater, and 2 µL of DCFH-DA solution

Table 1. Sequence of primers used in this study

Primer Name	Primer Sequence (5' → 3')	Reference
β-actin AF	TAT GCC CTC CCT CAC GCT AT	Song *et al.*, 2006
β-actin AR	GCC AGA CTC GTC GTA TTC CT	Song *et al.*, 2006
HSP90 AF	AGG AAA AGG CAA CCG CAG ACA	Yang *et al.*, 2015
HSP90 AR	ATG TGT TCC AGG CTC CTC CAA TG	Yang *et al.*, 2015
HIF-1α AF	AAC CAG CTG GAC AAA GCA TC	Ferrufino. 2006
HIF-1α AR	TGC AGG TTG TCA ACA TCG TC	Ferrufino. 2006

(final concentration 10 μM) was added. The mixture was incubated in the dark at room temperature for 90 minutes, with ROS production expressed as the level of green fluorescence in arbitrary units (A.U.). For NO production, 100 μL of hemolymph was diluted with an equal volume of sterilized seawater, and 2 μL of DAF-FM Diacetate solution (final concentration 10 μM) was added. After incubating in the dark at room temperature for 60 minutes, NO production was expressed as fluorescence units (A.U.) using the green fluorescence detector.

Among the scallops used to collect hemolymph, 10 scallops were used in mRNA expression of hypoxia-inducible factor 1-alpha (HIF1-α) and heat shock protein 90 (HSP90) in the digestive gland using quantitative PCR (qPCR). Total RNA was isolated from the digestive gland of scallops using TRIzol reagent (Invitrogen, USA) according to the manufacture's instructions. RNA concentration and purity were assessed by measuring absorbance at 260 nm and 260/280 nm ratio, respectively, using a NanoDrop spectrophotometer. The expression of HIF1-α and HSP90 was measured using quantitative RT-PCR (qRT-PCR). Reactions were performed using the Thermal Cycler Dice® Real Time System III (Takara, Japan) with the One Step TB Green® PLUS RT-PCR Kit (Perfect Real Time, Takara, Japan). The β-actin gene was used as an internal control for normalizing the expression of target genes. Primers for the real-time PCR analysis are listed in Table 1. The qRT-PCR amplifications were conducted in a final reaction volume of 25 μL, containing 2 μL of total RNA, 12.5 μL of 2× One Step TB Green RT-PCR Buffer 4, 1.5 μL of TaKaRa EX Taq HS Mix, 0.5 μL of PrimeScript PLUS RTase Mix, 1 μL each of forward and reverse primers (10 μM), and 6.5 μL of RNase-free dH_2O. The cDNA synthesis program (Stage 1) was performed at 42℃ for 5 minutes, followed by 95℃ for 10 seconds. The RT-PCR program (Stage 2) conditions were as follows: For β-actin: 94℃ for 5 minutes, followed by 22 cycles of 94℃ for 1 minute, 58℃ for 1 minute, and 72℃ for 1 minute (Song *et al.*, 2006). For HSP90: 94℃ for 5 minutes, followed by 30 cycles of 94℃ for 1 minute, 58℃ for 1 minute, and 72℃ for 1 minute (Yang *et al.*, 2015). For HIF-1α: 95℃ for 10 minutes, followed by 40 cycles of 95℃ for 15 seconds, 60℃ for 1 minute, and 72℃ for 30 seconds (Ferrufino, 2006). The relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method, where ΔCt = Ct (Target gene) – Ct (Housekeeping gene), ΔΔCt = ΔCt (sample) – Average ΔCt (negative control), and target amount = $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001).

Differences in cellular and molecular parameters were assessed using one-way ANOVA followed by Tukey's HSD post hoc test at a 95% confidence level. Pearson correlation analyses were performed for each parameter, and relationships were deemed significant at $P < 0.05$. Statistical analyses were conducted using SPSS software (version 25.0; IBM, Armonk, NY, USA).

RESULTS AND DISCUSSION

During the 48-h experiment, no mortality was observed in the control tank, indicating optimal conditions for scallop survival (Fig. 2). Under hypoxic

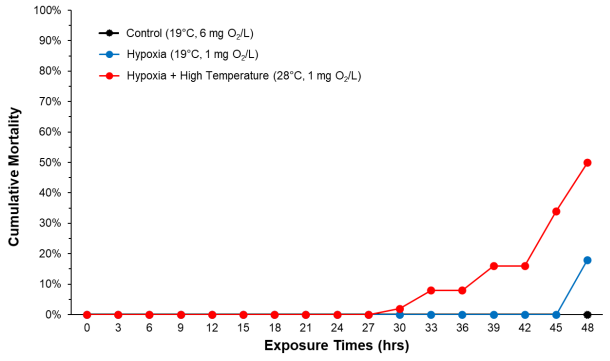

Fig. 2. Cumulative mortality of *Argopectin irradians*. Mortality was monitored at 3-hour intervals.

conditions, mortality was first noted at 48 h with a rate of 18% (Fig. 2). In the tank exposed to combined stressors, mortality began at 30 h with 2% and reached 50% by the end of the experiment (Fig. 2). The rapid mortality increase under combined stressors suggests a synergistic effect, highlighting scallops' vulnerability to multiple simultaneous stressors. In contrast, the delayed mortality under hypoxia indicates some initial resilience to low oxygen levels, although unsustainable over time.

The mean phagocytosis capacity of hemocytes in control scallops was 15.4 ± 1.5% (mean ± standard error, SE) (Fig. 3A). Although no statistically significant differences were observed between experimental groups, a trend of decreasing phagocytosis capacity was noted (Fig. 3A). Compared to the control, phagocytosis capacity showed a non-significant reduction under hypoxia (13.5 ± 1.6%) and a further decrease under combined stressors (8.3 ± 1.4%) (Fig. 3A). This trend suggests potential immune impairment due to environmental stressors. The phagocytosis capacity of hemocytes serves as a crucial indicator of the immune function in scallops (Zhou *et al.*, 2013; Song *et al.*, 2015). The reduction under stress conditions aligns with observations in other bivalve species, such as decreased phagocytosis capacity in *Perna viridis* under hypoxia (Wang *et al.*, 2012) and in *Mytilus edulis* under elevated temperatures (Mackenzie *et al.*, 2014). This trend, although not statistically significant, is consistent with the concept of

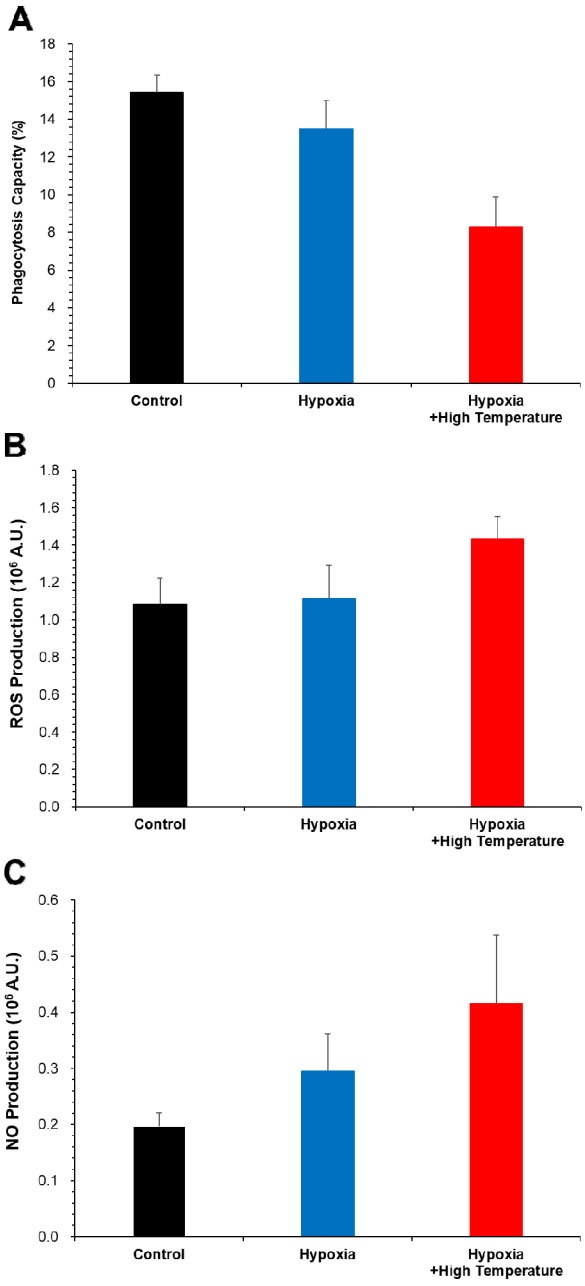

Fig. 3. (**A**) Phagocytosis capacity, (**B**) reactive oxygen species (ROS) production, and (**C**) nitric oxide (NO) production in hemocytes of *Argopectin irradians* determined by flow cytometry. Values are presented as the mean ± standard error.

energy-limited stress tolerance (Sokolova *et al.*, 2012), suggesting energy reallocation from immune responses to critical life-supporting processes under stress.

ROS production in hemocytes of control scallops was 1.1 x 10^6 ± 0.2 x 10^6 A.U. (mean ± standard error, SE) (Fig. 3B). Under hypoxic conditions, ROS level remained similar to control (1.1 x 10^6 ± 0.1 x 10^6 A.U.) (Fig. 3B). Although not statistically significant, scallops exposed to combined stressors showed elevated ROS production (1.4 x 10^6 ± 0.2 x 10^6 A.U.) (Fig. 3B). ROS production is a marker of oxidative stress and cellular response to environmental challenges (Lesser, 2006; Lushchak, 2011). The similar ROS levels in control and hypoxic conditions suggest effective antioxidant mechanisms for coping with short-term hypoxia, consistent with findings in Pacific oysters *Crassostrea gigas* (Sussarellu *et al.*, 2010). The higher ROS levels under combined stressors indicate elevated oxidative stress, aligning with the concept that multiple stressors can overwhelm antioxidant defenses (Sokolova, 2018). The trend implies increased metabolic activity or stress under adverse conditions, potentially leading to cellular damage if sustained.

NO production in hemocytes of control scallops was 2.0 x 10^5 ± 0.7 x 10^5 A.U. (mean ± standard error, SE) (Fig. 3C). Although not statistically significant across groups, NO levels increased under hypoxia (2.9 x 10^5 ± 1.2 x 10^5 A.U.) and further with combined stressors (4.2 x 10^5 ± 1.7 x 10^5 A.U.) (Fig. 3C). The increase in NO production under stress conditions aligns with its role as a key signaling molecule in physiological and pathological processes (Locascio *et al.*, 2023). The more pronounced increased under combined stressors suggests a potential synergistic effect on physiological response. The role of NO in reducing metabolic rates in invertebrates exposed to hypoxia (Kotsyuba and Dyachuk, 2023), may indicate an adaptive mechanism to cope with low oxygen conditions.

Gene expression analysis revealed a 1.6-fold increase in HSP90 expression under hypoxia conditions compared to controls, though not statistically significant (Fig. 4A). Under combined stressors, however, scallops exhibited a significant 5.1-fold increase in HSP90 expression relative to the control group (Fig. 4A). These findings align with

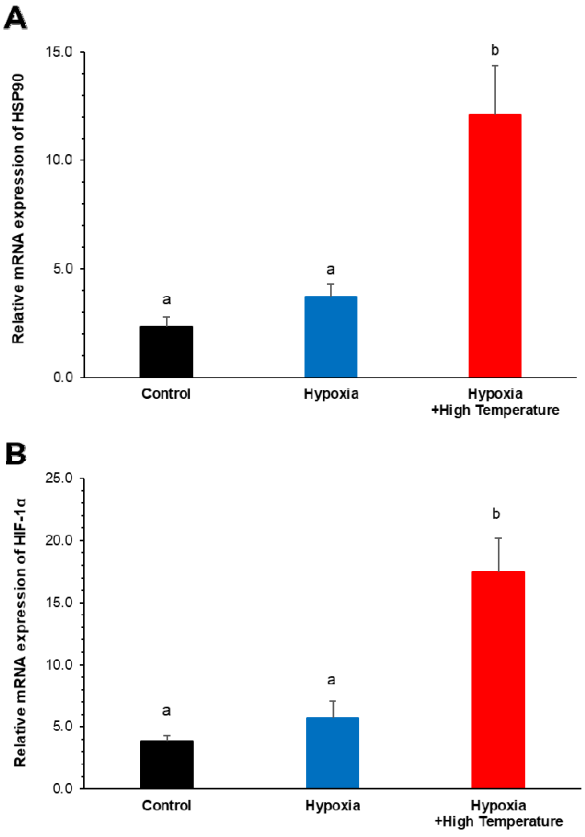

Fig. 4. Relative mRNA levels of (**A**) Heat Shock Protein 90 (HSP90) and (**B**) Hypoxia-Inducible Factor-1α (HIF-1α) in digestive glands of *Argopectin irradians*. Values are presented as the mean ± standard error. Groups with different letters show significant differences ($P < 0.05$).

previous studies of marine bivalves when multiple stressors often elicit more pronounced molecular responses than single stressors. For instance, in the scallop *Chlamys farreri*, exposure to thermal stress resulted in significant upregulation of HSP90 (Cheng *et al.*, 2020; Yu *et al.*, 2021). This response is similar to the heightened HSP90 expression observed in our study under combined stressors. The modest increase in HSP90 expression under hypoxia alone, while not statistically significant, suggests that hypoxia may induce a baseline stress response. This is consistent with findings in other marine bivalves, where moderate hypoxia induced only mild HSP90 upregulation. The lack of statistical significance in the hypoxia group might indicate that this condition alone does not sufficiently challenge the cellular

environment to trigger a robust HSP90 response. The significant 5.1-fold increase in HSP90 expression under combined hypoxia and high temperature stress demonstrates a synergistic effect of multiple stressors. This heightened response likely reflects the scallops' attempt to maintain cellular homeostasis and protect against protein denaturation under more severe environmental conditions. HSP90 is known to interact with various client proteins involved in signal transduction, cell cycle regulation, and stress response pathways (Zhang and Burrows, 2004; Prodromou, 2017). Its upregulation may therefore represent a critical mechanism for scallops to cope with the increased cellular damage and metabolic demands imposed by multiple environmental stressors.

HIF1-α expression increased by 1.5-fold under hypoxia conditions compared to controls, without statistical significance (Fig. 4B). Under combined stressors, expression rose by 4.5-fold compared to controls, indicating an enhanced hypoxic response likely facilitating metabolic adaptation (Fig. 4B). HIF-1α is a master regulator of cellular responses to hypoxia, playing a crucial role in the transcriptional activation of genes involved in angiogenesis, erythropoiesis, energy metabolism, and cell survival (Fu *et al.*, 2023). The observed baseline increases in HIF-1α expression under hypoxia, albeit not statistically significant, suggest that hypoxia alone may not be sufficient to trigger a robust HIF-1α response in scallops. This is consistent with findings in other marine invertebrates, where moderate hypoxia induced only mild HIF-1α upregulation (Kodama *et al.*, 2011). The significant 4.5-fold increase in HIF-1α expression under combined hypoxia and high temperature stress indicates a synergistic effect of multiple stressors. This aligns with previous studies on marine invertebrates, where combined stressors often elicit more pronounced molecular responses than single stressors (Peng *et al.*, 2023). For instance, in the white shrimp *Litopenaeus vannamei,* HIF-1α expression was significantly upregulated under hypoxia, and this response was further enhanced when combined with thermal stress (Mao *et al.*, 2022). The heightened HIF-1α response under combined stressors likely reflects the scallops' attempt to maintain cellular homeostasis and adapt to the challenging environmental conditions. HIF-1α activation leads to the transcription of numerous genes involved in glycolysis, glucose transport, and angiogenesis, which are crucial for survival under low oxygen conditions (Hao *et al.*, 2022).

Correlation analyses revealed several relationships among the measured parameters. Phagocytosis capacity showed negative correlations with other parameters, though these were not statistically significant. ROS production positively correlated with NO production, HSP90 expression, and HIF-1α expression, with a significant correlation ($P < 0.05$) observed for NO production. NO production also positively correlated with HSP90 and HIF-1α expression. Notably, the strong significant positive correlation ($P < 0.05$) was found between HSP90 and HIF-1£ expression (Table 2).

The study demonstrates that while individual stressors like hypoxia did not significantly alter gene expression or physiological parameters, their

Table 2. Correlation coefficients among the parameters of *Argopecten irradians* exposed to hypoxia and hypoxia combined with high temperature. $P < 0.05$ was regarded as a significant correlation. PHG, phagocytosis capacity; ROS, Reactive oxygen species production; NO, nitric oxide production; HSP90, heat shock protein 90; HIF-1α, Hypoxia-Inducible Factor-1α

Parameters	PHG		ROS		NO		HSP90	
	Coefficient	P	Coefficient	P	Coefficient	P	Coefficient	P
ROS	–0.189	0.125						
NO	–0.207	0.090	0.257	0.036				
HSP90	–0.301	0.119	0.238	0.222	0.221	0.259		
HIF-1α	–0.323	0.101	0.165	0.411	0.070	0.730	0.944	< .0001

combination amplified stress responses in scallops. The rapid mortality under combined stressors, coupled with trends in reduced phagocytic capacity and increased production of ROS and NO, suggest potential immune impairment and elevated oxidative stress. These findings underscore the importance of considering both individual and combined effects of environmental stressors when assessing marine organism health and developing conservation strategies.

Bay scallops *Argopecten irradians* are significantly affected by environmental stressors, particularly when these stressors are combined. The synergistic effects observed highlight the vulnerability of scallops to environments with multiple adverse conditions. Further research is needed to elucidate underlying mechanisms and explore adaptive strategies in scallops facing changing environmental conditions.

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