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Impacts of multiple stressors on reproductive performance and gamete metabolism of the blue mussels *Mytilus edulis:* Physiological mechanisms and ecological implications

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Erklärung

Hiermit versichere ich an Eides statt, dass ich die vorliegende Arbeit selbständig angefertigt und ohne fremde Hilfe verfasst habe. Dazu habe ich keine außer den von mir angegebenen Hilfsmitteln und Quellen verwendet. Die aus den benutzten Werken inhaltlich und wörtlich entnommenen Stellen sind als solche kenntlich gemacht.

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I was born on a small island named Gouqi, located in the East China Sea, where many people make a living from mussel aquaculture. Therefore, I got to know the blue mussels when I was a young boy. And until today, 13.07.2025, I completed my PhD thesis with the blue mussels. That's an amazing journey.

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To prepare this work, I used ChatGPT-4o and DeepL to improve the readability and check the grammar of the manuscript. No prompt-based AI-generated content was included in the manuscript. After using the services of ChatGPT and DeepL, I have thoroughly reviewed and edited the content as needed and take full responsibility for the thesis contents.

Abstract

Marine coastal environments in the Anthropocene are increasingly influenced by abiotic stressors such as seawater warming, hypoxia, and salinity fluctuations, driven by both natural variability and climate change, and posing significant challenges to marine ecosystems. Blue mussels (*Mytilus edulis*), as widely distributed ecosystem engineers along temperate coasts, are directly exposed to environmental changes in seawater. As broadcast spawners, mussels release their gametes into seawater for fertilization during the reproductive season. Sperm-egg collision is a necessary step for fertilization and requires the motile sperm to find the eggs in the natural seawater environment. Mitochondria are the essential organelles in spermatozoa that carry out highly efficient ATP production through aerobic oxidative phosphorylation (OXPHOS). Understanding how the seawater environmental changes impact the reproductive capability of *Mytilus* spp. is crucial for assessing the sustainability and adaptation of their populations. This study aimed to explore the metabolic underpinnings and environmental determinants of reproductive performance in mussels by identifying the primary pathway of ATP production in sperm (OXPHOS vs. glycolysis), assessing changes in sperm motility and mitochondrial function following parental exposure to hypoxia and anoxia, and evaluating the effects of salinity and temperature on sperm motility, mitochondrial function, fertilization success, and their interrelationships. Our results demonstrated that mitochondrial OXPHOS is the primary metabolic pathway for ATP generation in *M. edulis* sperm, directly supporting sperm motility. Parental exposure to 72h of anoxia decreased the ATP synthesis capacity and coupling efficiency of sperm mitochondria, while both hypoxia and anoxia increased the production of reactive oxygen species (ROS) in sperm mitochondria. High temperature decreased the respiratory efficiency of mitochondria in sperm and enhanced ROS efflux, whereas osmolarity was not a crucial factor. However, the mitochondria in sperm are resilient and provide stable ATP production even under the adverse effects of environmental stressors, such as temperature and salinity variations. Our study provided new mechanistic insights into energy metabolism in mussel sperm and showed that, while hypoxia and anoxia can impair sperm performance, the effects of temperature and salinity variations on mussel reproduction are unlikely to stem from disruptions in sperm metabolism. Instead, they are more likely driven by factors such as osmotic and temperature impacts on gamete integrity and interactions, or embryonic development.

Kurzfassung

Meeres-Küstengebiete im Anthropozän werden zunehmend durch abiotische Stressfaktoren wie Erwärmung des Meerwassers, Hypoxie und Schwankungen des Salzgehalts beeinflusst, die sowohl durch natürliche Schwankungen als auch durch den Klimawandel verursacht werden und erhebliche Herausforderungen für marine Ökosysteme darstellen. Miesmuscheln (*Mytilus edulis*) sind als weit verbreitete Ökosystemingenieure entlang der gemäßigten Küsten direkt den Umweltveränderungen im Meerwasser ausgesetzt. Als Freisetzungslaicher geben Miesmuscheln während der Fortpflanzungszeit ihre Gameten zur Befruchtung ins Meerwasser ab. Die Kollision von Spermium und Eizelle ist ein notwendiger Schritt für die Befruchtung und erfordert, dass die beweglichen Spermien die Eizellen in der natürlichen Meeresumgebung finden. Mitochondrien sind die essenziellen Organellen in Spermien, die durch aerobe oxidative Phosphorylierung (OXPHOS) eine hocheffiziente ATP-Produktion durchführen. Das Verständnis, wie sich die Veränderungen der Meeresumwelt auf die Fortpflanzungsfähigkeit von *Mytilus* spp. auswirken, ist entscheidend für die Bewertung der Nachhaltigkeit und Anpassungsfähigkeit ihrer Populationen. Ziel dieser Studie war es, die metabolischen Grundlagen und Umweltfaktoren der Fortpflanzungsleistung von Muscheln zu untersuchen, indem der primäre Weg der ATP-Produktion in Spermien (OXPHOS vs. Glykolyse) identifiziert, Veränderungen der Spermienbeweglichkeit und der Mitochondrienfunktion nach Exposition der Eltern gegenüber Hypoxie und Anoxie bewertet und die Auswirkungen von Salzgehalt und Temperatur auf die Spermienbeweglichkeit, die Mitochondrienfunktion, den Befruchtungserfolg und deren Wechselbeziehungen untersucht wurden. Unsere Ergebnisse zeigten, dass die mitochondriale OXPHOS der primäre Stoffwechselweg für die ATP-Produktion in *M. edulis*-Spermien ist und die Spermienmotilität direkt unterstützt. Die Exposition der Eltern gegenüber 72 Stunden Anoxie verringerte die ATP-Synthesekapazität und die Kopplungseffizienz der Spermienmitochondrien, während sowohl Hypoxie als auch Anoxie die Produktion reaktiver Sauerstoffspezies (ROS) in den Spermienmitochondrien erhöhten. Hohe Temperaturen verringerten die Atmungseffizienz der Mitochondrien in den Spermien und verstärkten den ROS-Ausfluss, während die Osmolarität kein entscheidender Faktor war. Die Mitochondrien in den Spermien sind jedoch widerstandsfähig und sorgen auch unter den nachteiligen Auswirkungen von Umweltstressoren wie Temperatur- und Salzgehaltsschwankungen für eine stabile ATP-Produktion. Unsere Studie lieferte neue mechanistische Einblicke in den Energiestoffwechsel in Muschel-Spermien und zeigte, dass Hypoxie und Anoxie zwar die Spermienleistung beeinträchtigen können, die Auswirkungen von Temperatur- und Salzgehaltsschwankungen auf die Fortpflanzung von Muscheln jedoch wahrscheinlich nicht auf Störungen des Spermienstoffwechsels zurückzuführen sind. Stattdessen werden sie eher durch Faktoren wie osmotische und temperaturempfindliche Auswirkungen auf die Integrität und Interaktion der Gameten oder die Embryonalentwicklung verursacht.

List of abbreviations

OXPHOS – Oxidative phosphorylation
ETS – Electron transport system
ROS – Reactive oxygen species
C I – Complex I, NADH-ubiquinone oxidoreductase
C II – Complex II, Succinate-ubiquinone oxidoreductase
C III – Complex III, Ubiquinol-cytochrome c oxidoreductase
C IV – Complex IV, Cytochrome c oxidase
C V – Complex V, ATP synthase
NADH – Nicotinamide adenine dinucleotide
$FADH_2$ – Flavin adenine dinucleotide
COX – Cytochrome C oxidase
FEL – Fractional electron leak
RCR – Respiratory control ratio
ATP – Adenosine triphosphate
2-DG – 2-Deoxy-D-glucose
CASA – Computer-assisted sperm analysis
VCL – Curvilinear velocity
VAP – Average path velocity
FSR – Fertilization success rate
DO – Dissolved oxygen
EEI – Earth energy imbalance
OHC – Ocean heat content
MHWs – Marine heatwaves
LDH – lactate dehydrogenase
BS – the Baltic Sea
NS – the North Sea

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1. General Introduction

1.1 Introduction

1.1.1 Coastal habitats under multiple abiotic stressors

Coastal zones and estuaries are important ecosystems that offer essential services including nutrient cycling, habitats for diverse marine species, and buffering against environmental shocks [1-3]. These regions are subjected to multiple abiotic stressors such as deoxygenation, salinity fluctuations, and seawater warming, which are amplified by the effects of climate change and human activities [4-6].

Since the industrial revolution, the anthropogenic greenhouse gas emissions have increased, resulting in a positive Earth energy imbalance (EEI). The oceans hold a vast heat capacity, absorbing approximately 93% of the excess heat generated by greenhouse gas-driven global warming. This energy uptake by the ocean surface has resulted in a continuous increase in ocean heat content (OHC) [7, 8]. Specifically, at 0 – 700 m depth, the average OHC increased by 191.1 ± 10.7 ZJ ($1\ ZJ = 10^{21}\ J$) globally from 1960 to 2018, according to the Global Climate Observing System [9]. In addition, from the 1960s to the 2010s, the rate of seawater warming has increased from less than 5 to around 10 ZJ per year. Owing to the large thermal inertia of the ocean, seawater warming is predicted to be irreversible in this century [7].

Climate change not only causes the gradual warming of the surface seawater, but also increases the frequencies of extreme events, such as marine heatwaves (MHWs). MHWs are defined as the periods when sea surface temperatures exceed the 90th percentile of the local climatology for at least 5 consecutive days [10, 11]. Ecologically, as a potential consequence of ocean warming, MHWs have wide-ranging impacts on the marine ecosystems. In recent years, MHWs events have been reported frequently, which induce mass mortalities of benthic invertebrates and alter food webs and species interactions [10, 12, 13]. Additionally, the increasing temperature in the sea surface intensifies stratification of the upper ocean, decreases vertical mixing, and therefore disrupts the transport of oxygen from the surface to the deep waters [14]. This reduced vertical exchange, alongside the decreased oxygen solubility and the changed circulation, has led to a measurable deoxygenation in the oceans since the 1960s [4, 15].

Dissolved oxygen (DO) in seawater is the basis for most marine life, and the habitat boundaries of marine organisms are often determined by DO levels, making it one of the most important factors for marine ecosystems [16, 17]. In natural aquatic systems, DO input is primarily derived from the air-sea gas exchange and photosynthesis in the upper water layers. Elevated temperatures reduce the oxygen solubility and result in lower oxygen concentrations in the seawater [18]. Furthermore, coastal eutrophication, the nutrient enrichment primarily driven by human activities, exacerbates hypoxia by fueling algal blooms. When these algae die, they are decomposed by aerobic bacteria, which is a process that consumes large amounts of DO and contributes to oxygen depletion in the

bottom waters and sediments [19, 20]. Hypoxia is commonly defined as the DO concentration less than 2 mg O_2 L^{-1} (< 62.5 μM O_2 or < 30% oxygen saturation) [17]. As a result of these combined stressors, including ocean warming, excessive nutrients, and stratification, coastal hypoxia has emerged as a widespread and intensifying global environmental issue, with far-reaching implications for marine biodiversity, ecosystem functioning, and fisheries.

1.1.2 The Baltic Sea and the North Sea: model systems for environmental change

As one of the largest brackish water bodies in the world, the Baltic Sea spans a surface area of 412,560 km^2, and contains approximately 21,631 km^3 of water [21]. It comprises six sub-regions from west to northeast, including the Danish Straits, the Baltic Proper, the Gulf of Riga, the Archipelago Sea, the Gulf of Finland, and the Gulf of Bothnia [22]. Geographically, the Baltic Sea is a semi-enclosed basin connected to the North Sea only through the narrow Danish Straits. Before the 1980s, frequent flows of dense, saline bottom water from the North Sea entered the Baltic Sea via the shallow and narrow Danish Straits, helping to displace and renew the anoxic deep waters. However, such major inflow events have become increasingly rare in recent decades [23]. The Baltic Sea receives freshwater input from over 200 rivers, resulting in a distinct west-to-northeast salinity gradient [22, 24]. Specifically, the salinity decreases from near-marine conditions in the Kattegat (the transitional zone with the North Sea) to almost freshwater conditions in the Gulf of Finland and the Gulf of Bothnia. Along the Swedish West Coast, salinity spans from 17 to 25; in the Baltic proper, it is from 5.5 to 7.3; ~5 in the Bothnian Sea, as well as 2 to 4 in the Bothnian Bay [25]. A previous survey reported the highest number of marine macrozoobenthic species in the Kattegat (1,154 species), whereas the Gulf of Finland hosted the highest number of freshwater species (410 species), clearly reflecting the influence of salinity on biodiversity distribution [26]. This pattern aligns with the "species minimum" concept developed by Remane in 1934, which describes a hypothetical distribution of benthic invertebrate diversity along a marine–freshwater salinity gradient, the lowest biodiversity occurs at salinity 5 to 8 [27]. Although the validity of Remane's theory has been debated in subsequent studies, including by Whitfield et al. [28], it remains applicable to macrozoobenthic diversity patterns in the Baltic Sea. In addition, the study revealed that the salinity fluctuations in tidal estuaries are the major drivers of species distribution [29]. However, the Baltic Sea, characterized by the lack of tides, exhibits stable temporal salinity. Therefore, the spatial salinity gradients remain the dominant factor structuring benthic biodiversity in the Baltic Sea.

As one of the major sea areas in the European Union, the Baltic Sea is economically and socially important for the 85 million people living in its catchment area [22]. However, the intensive anthropogenic force also has negative impacts on the coastal environments, including agricultural runoff and urban wastewater discharge, which have collectively contributed to severe eutrophication over recent decades [33-35]. The excess amount of nutrients input promotes the growth of microalgae and phytoplankton, as well as the accumulation of organic matter in the ecosystem. The subsequent processes of microbial respiration and

degradation consume DO, thus resulting in seasonal and spatial hypoxia or anoxia in bottom waters [36]. In addition, due to its limited seawater exchange with the broader ocean, the bathygraphic peculiarities, and the permanent halocline produced by estuarine circulation hamper aeration of deep water layers, the Baltic Sea is prone to hypoxia [37]. According to reports, the hypoxic area has expanded from less than 10000 km^2 before 1950 to over 60000 km^2 since 2000 in the Baltic Sea [38]. While anoxia, which is an absolute lack of oxygen, occurs mainly in the deep central part of the Baltic Sea and it has area varies from 10000 km^2 to 50000 km^2 from 2000 to 2017 [39]. Additionally, depending on climate change scenarios and nutrient loads, hypoxic and anoxic zones in the Baltic Sea may change significantly [40].

In contrast to the brackish and stratified Baltic Sea, the North Sea represents a typical temperate marine ecosystem characterized by higher salinity levels (approximately 30–35), strong tidal mixing, and greater DO availability throughout the water body [30]. In terms of the overall structure physiography of the North Sea and the Baltic Sea, the openness of the North Sea contrasts with the closeness of the Baltic, which is land-locked. Its open connection to the Atlantic Ocean through the English Channel and the Norwegian Sea ensures regular water exchange and a relatively stable ecosystem [30]. While the two seas are connected via the narrow Danish Straits, the Baltic Sea receives significant freshwater input and has limited deep-water renewal, making it more susceptible to hypoxia and environmental stress [22, 24, 31]. Despite their proximity and partial connectivity, these seas support distinct environmental regimes [30, 31]. Many marine species, including blue mussels *Mytilus edulis*, occur in both regions [32]. This natural contrast provides an excellent setting for investigating population-specific physiological responses and local adaptation across environmental gradients.

Climate change amplifies many stressors experienced by organisms in the coastal marine ecosystems. Climate change is leading to a steady rise in sea surface temperatures in the North Sea, with warming especially noticeable in southern and shallow areas [205, 206]. At the same time, salinity patterns are changing due to increased river runoff, precipitation, and altered ocean circulation, leading to regional freshening in some parts [207]. These shifts affect water column stratification, reducing vertical mixing and impacting oxygen levels, nutrient transport, and marine ecosystems [208]. The Baltic Sea is warming about 3 to 4 times faster than the global ocean average, with sea surface temperatures rising by approximately 0.5°C per decade [209, 210]. This rapid warming is due to its shallow depth, limited water exchange, and sensitivity to atmospheric conditions. The rising temperature of the Baltic Sea, driven by global warming, enhances the stratification of the water column, thus decreasing DO mixing between surface and deep waters [41]. The microbial respiration and nutrient cycling are also accelerated in warmer seawater, exacerbating the severity and persistence of hypoxia zones. Elevated temperatures induce faster rates of microbial decomposition of organic matter, which increases DO consumption in bottom waters [42]. Moreover, hypoxia alters nutrient cycling by enhancing the release of phosphorus from sediments and shifting nitrogen pathways away from denitrification toward dissimilatory nitrate reduction to ammonium. These potential consequences, particularly the regeneration of bioavailable nutrients

under low oxygen conditions, promote further phytoplankton blooms and organic matter accumulation, thereby increasing the demand for DO and prolonging hypoxic conditions [43]. Therefore, the eutrophication-induced hypoxia and climate-induced seawater warming represent a severe threat to the ecological stability and reproductive functioning of benthic invertebrates in the Baltic Sea.

1.1.3 Mussels *M. edulis*: an ecophysiological model for coastal invertebrates

The blue mussel of the genus *Mytilus*, is the reef-building foundation species that is widely distributed along the North Atlantic and adjacent sea areas. As an important coastal ecosystem engineer, mussels create complex 3D structures (mussel beds) that serve as habitat and nursery grounds for various species and contribute to local community structure, biodiversity and nutrient cycling [44-47]. The blue mussel populations also play a key role in top-down control of algae in coastal and estuarine environments [48]. They enhance water clarity by actively filtering suspended particles such as phytoplankton, detritus, and fine sediments, as well as removing pollutants like excess nutrients and organic matter from the water column [49]. This filtration improves light penetration, supports the growth of submerged aquatic vegetation, and contributes to overall ecosystem stability and water quality [50-52]. In addition, the mussels serve a vital ecological role as the primary food for many animals, including fish, crabs, sea stars, sea birds, sea otters, and seals [44]. Beyond its ecological importance, *M. edulis* is considered to be of great socioeconomic significance. Thousands of years ago, the mussels were already regarded as an important food item, since their shells were discovered in archaeological excavations of kitchen middens from the Mesolithic period [53-55]. Nowadays, mussel aquaculture benefits the coastal countries and people living in the catchment area all over the world [56, 57]. Besides, *Mytilus* is commonly utilized as a sentinel organism for environmental monitoring and environmental quality assessments [58-60].

The blue mussels of the genus *Mytilus* have a broad range of tolerance to abiotic stressors, including temperature, salinity, and DO availability, largely due to their ectothermic physiology and high plasticity [61-63]. Adult mussels can survive within a thermal window of 0–29°C, with optimal performance of 15-20°C [63-65]. They are also osmoconformers and exhibit population-specific tolerance to salinity ranging from ~5 to 35 [66]. Both the Baltic Sea and the North Sea are the natural habitats for the blue mussels, reflecting their broad salinity tolerance [67, 68]. Notably, the two populations of *Mytilus* spp. possess a different genetic background. Specifically, the Warnemünde population (Baltic Sea) originates from a stable hybrid zone between *M. edulis* and *Mytilus trossulus*, with ~ 70% of nuclear markers and 100% of mitochondrial markers of *M. edulis* [69], and is thus classified as natural hybrids of *M. edulis*. In contrast, the Sylt population (North Sea) is genetically pure *M. edulis*, according to prior population genetic assessments [70]. In addition to their thermal and salinity tolerance, adult mussels also exhibit adaptations to low oxygen environments. In hypoxic environments, they initiate metabolic rate depression, with the ATP demand decreased to ≤10% of normoxic levels by downregulating energy-intensive processes (e.g., via phosphorylation-mediated inhibition of glycolytic enzymes and ion channel arrest). Meanwhile, they shift to facultative anaerobic metabolism, which is mainly

rely on the succinate-propionate pathway, sustaining basal cellular functions [71]. These physiological strategies enhance their survival in coastal habitats with the fluctuating environmental conditions. However, it is also important to note that such resilience is largely characteristic of adult individuals, while the early life stages of blue mussel, including larvae and gametes, tend to exhibit lower tolerance to these environmental challenges.

Despite these physiological adaptations, environmental stressors such as reduced salinity and oxygen deficiency can still severely impair mussel fitness. For instance, the local population structure of mussels and growth rates of individuals are limited by low salinity, especially in the Baltic Sea, where the local mussels have thinner shells and smaller sizes than the North Sea population, indicating the suppressed growth under low salinity environments [72, 73]. This growth limitation may result from osmotic stress that increases energy demands for osmoregulation, with fewer energy resources for somatic growth and reproduction. In addition, reduced shell thickness could increase vulnerability of individuals to predators and environmental disturbances, further compromising survival [74, 75]. Furthermore, short-term exposure to hypoxia may lead to compensatory responses such as increased ventilation and metabolic rate; however, prolonged hypoxia results in anaerobic metabolism, which is less efficient and can suppress physiological processes such as immunity, digestion, and growth, and ultimately increasing mortality risks [76-80]. Therefore, the mussel populations and its related ecosystems are threatened by the rising hypoxia and low salinity in the Baltic Sea, and further investigations on population sustainability under these environmental stressors are necessary to strengthen the knowledge in this field.

The life cycle of mussels begins with embryonic development and metamorphosis, gradually transitioning from a planktonic to a benthic lifestyle (Figure 1). The planktonic larval stages of marine mussels are important for population connectivity, facilitating both the colonization of new habitats and maintaining gene flow across spatially separated populations [81-83]. However, these planktonic stages also represent a population survival bottleneck, since larvae exhibit relatively high sensitivity to environmental stressors, including MHWs, salinity fluctuations, and hypoxia [84-86]. Given this vulnerability, understanding the reproductive responses of mussels to environmental change is crucial. As a well-established model organism, mussels provide valuable insights into gamete physiology and reproductive biology under environmental stress [87, 88]. Despite their physiological plasticity, the effects of environmental variations on reproductive traits, particularly on sperm performance, are still insufficiently understood. Investigating these processes is essential for understanding population resilience under ongoing climate change and coastal environmental stressors.

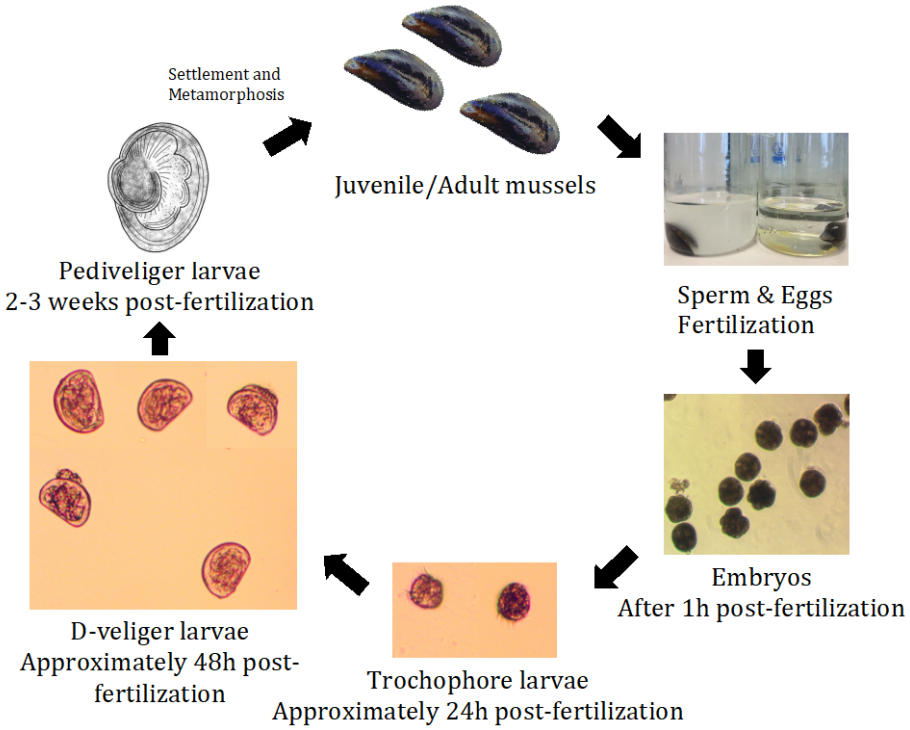

Figure 1. Diagram of the lifecycle of the blue mussel *M. edulis*, showing the development from fertilization to metamorphosis. All larval stages generally last 3-4 weeks before settlement.

1.1.4 Reproductive ecology of marine invertebrates

Broadcast spawning is an evolutionary ancient reproductive strategy commonly found in marine invertebrates. It is characterized by the free release of gametes into the surrounding seawater for external fertilization, with many small and often oligolecithal eggs, followed by the embryonic development in the water column until the planktotrophic larvae [89]. Among weakly mobile or sessile species t, broadcast spawning is a prevalent reproductive strategy [90]. However, this mode of reproduction also faces important limitations, particularly in the form of sperm competition and sperm limitation [91]. In dense populations, sperm released by multiple males may compete to fertilize the same eggs, leading to intensive sperm competition [92]. Conversely, in sparse populations or under strong water movement, sperm may become highly diluted, resulting in low local sperm concentrations and reduced fertilization success, a phenomenon referred to as sperm limitation [93, 94].

External fertilization is a complex process that depends both on the biological traits of gametes and the physical characteristics of seawater environments. It involves several important, highly conserved steps , including (i) gamete release, followed by the sperm activation and sperm chemotaxis in the direction of the egg; (ii) induction of the acrosomal reaction by egg surface components; (iii) sperm-egg binding, where the sperm binds to the egg coat; (iv) acrosome reaction that

involves sperm penetration through the egg coat; (v) sperm-egg fusion; and (vi) egg activation to initiate the developmental program [95]. For the species with external fertilization, the scale and timing of spawning events directly shape the quantity and timing of larval production [96, 97]. Successful fertilization is the first critical step in the organisms' life cycles, establishing the upper limit for population recruitment, regardless of subsequent dispersal by ocean currents. Therefore, while broadcast spawning determines gamete availability, environmental and biological factors jointly constrain fertilization success and thus have important impacts on population dynamics.

Previous research revealed that fertilization success in many species is affected by egg size, sperm motility, and egg-sperm interactions, including the chemotaxis of sperm towards the eggs [98-101]. The trade-off between egg size and quantity is particularly interesting and important, since the eggs provide primary energy for postzygotic fitness [99]. Evolutionarily, both animals and their gametes are subject to natural selection in terms of reproductive strategies to achieve the optimal fertilization success. For instance, larger egg size could increase the likelihood of sperm-egg collision [102]. However, this hypothesis should be approached with caution, as the chemoattractants released by eggs can guide sperm toward them, significantly enhancing fertilization success by over 50-fold in some cases [103]. This suggests that chemoattraction may provide a more efficient and evolutionarily favorable alternative [104]. Nonetheless, this finding does not dismiss the significance of the egg size-quantity trade-off in shaping reproductive strategies of mussels. Not only may the energetic constraints lead to the trade-off, but also the simple space constraints, since the brood capacity of females to hold eggs is limited, especially when a mother produces more offspring, the smaller-sized eggs are preferred so that they can fit within the maternal reproductive structure [105]. Despite these well-established theories, the energy allocation trade-offs in mussels under environmental stressors, particularly hypoxia-induced metabolic challenges, remain poorly understood. It's still necessary to investigate how DO deficiency modulates reproductive investment strategies of the blue mussels.

Environmental conditions such as temperature, salinity, and oxygen levels are particularly crucial for the reproductive performance of marine broadcast spawners, as they directly influence gamete viability, fertilization success, and the timing of spawning events, all of which are essential for successful reproduction and population sustainability. For example, energy re-allocation induced by marine heat waves (MHWs) may cause gonadal resorption in three bivalves, including *Ruditapes decussatus*, *Venerupis corrugata*, and *Cerastoderma edule* [106]. Heat stress induced by warming seawater leads to cellular damage and oxidative stress in bivalves [107], which may impair the gamete quality. In addition, failed spawning events were reported in many invertebrate species under the adverse impacts of MHWs [108]. Furthermore, hypoxia is also a potential limiting factor for reproduction. Sperm motility, velocity, and fertilization success in the sea urchin *Paracentrotus lividus* were negatively affected in hypoxic environments [109]. Larval development and mortality of the eastern oyster *Crassostrea virginica* was detrimentally affected by the low oxygen conditions, especially when they lasted over 24 h [110]. Salinity fluctuations,

commonly found in estuaries, may also jeopardize fertilization of broadcast spawners. Thus, reduced salinity has been shown to decrease sperm motility [111], impair sperm survival [112], and reduce fertilization success [113] in several oyster species. These environmental stressors may lead to reproductive impairments in marine invertebrate species and therefore affect the recruitment and sustainability of their populations.

1.1.5 Sperm performance and mitochondrial function

Sperm performance, including motility and velocity, is crucial to fertilization success [98, 114]. Sperm movement is powered by the flagellum, which is a highly specialized structure that generates rapid, oscillatory motions. The breakdown of adenosine triphosphate (ATP) provides the energy for the movement of flagellum, catalyzed by the dynein-ATPase located in the axoneme of the flagellum [115]. Two main metabolic pathways in sperm are involved in ATP synthesis - anaerobic glycolysis, and aerobic oxidative phosphorylation (OXPHOS). Glycolysis occurs in the sperm head and the principal piece of the flagellum under anaerobic conditions, producing two ATP molecules per glucose molecule metabolized [116]. Conversely, OXPHOS takes place in the mitochondria located in the midpiece of sperm and requires oxygen. It involves a multistep electron transport chain, comprising Complexes I, II, III, and IV, that transfer electrons from energy-rich substrates (NADH and $FADH_2$) onto oxygen pumping protons across the inner membrane of mitochondria in the process. This creates an electrochemical gradient, which is also called the mitochondrial membrane potential (MMP), which is utilized by Complex V, the ATP synthase, to generate ATP [117, 118]. ATP yield per mole of glucose is significantly higher with OXPHOS (30-32 ATP) compared to anaerobic glycolysis (only 2 ATP). Moreover, OXPHOS enables the utilization of a wide range of fuel sources—including lipids, carbohydrates, and amino acids—whereas glycolysis relies exclusively on carbohydrates.

The contribution of glycolysis and OXPHOS to ATP production in sperm varies among animal species. In some mammals, for example, humans and mice, glycolysis has been identified as the major metabolic pathway to produce ATP and support sperm motility [119, 120]. This preference was partially attributed to the limitation on sperm structure, the ATP generated by OXPHOS within the mitochondria is unable to diffuse to the distal area of the flagellum, suggesting the key role of glycolysis [121-123]. However, for the sperm motility in ram, glycolysis and OXPHOS were considered of equal importance [124]. Although glycolysis provides a rapid energy supply under low oxygen conditions, the superior efficiency of OXPHOS in sperm ATP generation cannot be ignored (15-16 fold higher in the case of glucose) [125], emphasizing the importance of mitochondrial function in sperm motility. Given the diversity in metabolic strategies among animal species, further studies are warranted to explore the sperm physiology of marine organisms with different reproductive strategies, especially the broadcast spawners.

Despite the significance of mitochondrial function in ATP synthesis, it is also a major source of reactive oxygen species (ROS), which is the natural by-product of OXPHOS. ROS is a diverse group of reactive molecules, including the radicals such

as hydroxyl ion, superoxide, nitric oxide, and peroxyl; the non-radicals, for example, ozone, single oxygen, lipid peroxides, and hydrogen peroxide; as well as the oxygen derivatives [126]. Generally, at physiological concentrations, ROS play regulatory roles in many physiological reproductive processes, including sperm capacitation, acrosome reaction, and gamete fusion, where redox signaling is essential [127, 128]. However, excessive ROS generation may lead to the imbalance between ROS and antioxidant mechanisms, and thus, oxidative stress occurs. Previous studies have proved that sperm owns a low antioxidant capacity and are particularly sensitive to oxidative stress [129, 130]. Furthermore, the sperm plasma membrane is rich in polyunsaturated fatty acids, which are highly prone to lipid peroxidation. The effects of oxidative stress on membrane lipids are highly detrimental to membrane integrity and fluidity, thereby impairing sperm motility and fertilization capacity [130-132].

In marine invertebrates, sperm mitochondria from different species exhibit variable physiological adaptations to environmental stressors. For instance, MMP in sperm of the mussels *Mytilus galloprovincialis* collapsed under the thermal stress [133]. However, for sea urchin *Heliocidaris tuberculata*, thermal stress had no effect on the mitochondrial activity of sperm, and the thermal-induced fertilization failure was not correlated with sperm mitochondrial activity [134]. While the recent climate change research has investigated sperm motility, viability, and fertilization success of marine organisms, the impacts of environmental stressors on sperm mitochondrial function, which is the essential driver of motility, remain poorly understood.

1.2 Experimental design and research objectives

The aim of the present study was to explore the impacts of multiple environmental stressors, including seawater warming, salinity gradients, and deoxygenation on the reproductive performance and gamete metabolism of the keystone marine bivalve, the blue mussel *M. edulis* spp. from the Baltic Sea and the North Sea, providing the physiological mechanisms and ecological implications. To achieve this objective, we examined the crucial traits of reproduction, such as the motility, ATP production, and mitochondrial function of sperm, and fertilization success in the following specific aims:

- To determine the primary metabolic pathway for ATP generation in sperm of the blue mussel *M. edulis* (glycolysis vs. OXPHOS) (Publication 1).
- To evaluate the impacts of parental exposure to hypoxia and anoxia on the spawning rate, sperm performance, ATP production, mitochondrial function, egg size-number trade off, and fertilization success of the blue mussel *M. edulis* from the Baltic Sea (Publication 2).
- To assess and compare the effects of temperature and salinity gradients on sperm motility, ATP production, and fertilization success of the blue mussel *M. edulis* from the Baltic Sea and the North Sea (Unpublished manuscript 3).
- To investigate the combined effects of temperature and osmolarity on the

sperm mitochondrial function of the blue mussel *M. edulis* from the Baltic Sea (Unpublished manuscript 3).

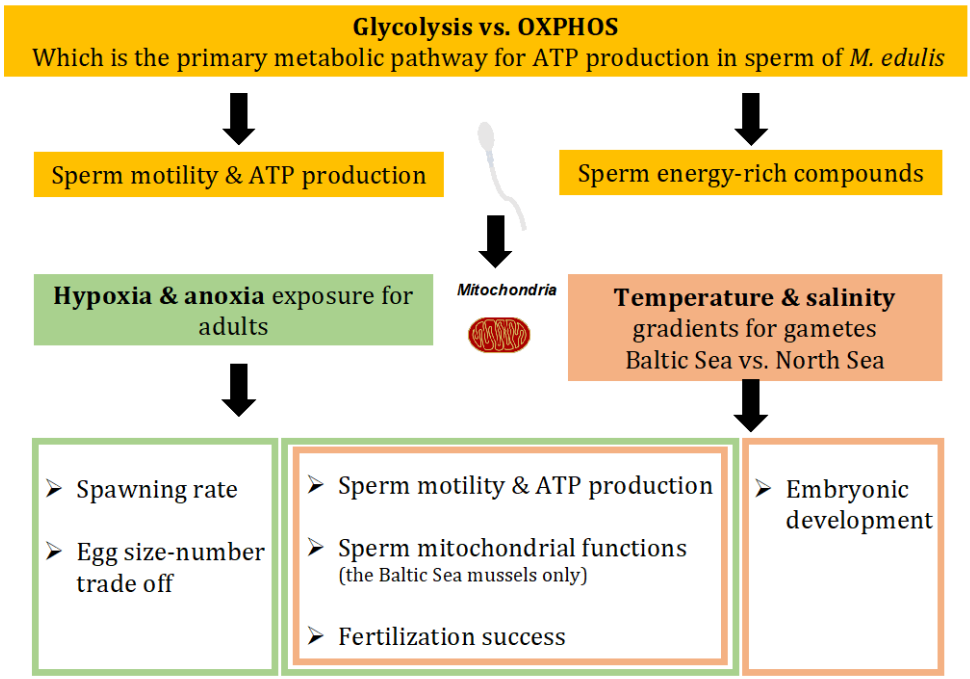

Figure 2. Concept of the experimental design for investigating the reproductive performance and gamete metabolism of blue mussel *M. edulis* spp. from the Baltic Sea and the North Sea under multiple environmental stressors. The yellow boxes represent the sperm ATP energy metabolism experiment; the green boxes represent the hypoxia and anoxia exposure experiment; the orange boxes represent the temperature and salinity exposure experiment.

To address these research aims, three independent experiments have been conducted (Figure 2). In the first experiment, we investigated the metabolic pathway for ATP production in sperm of *M. edulis*, and tested the time effects of different OXPHOS inhibitors on sperm motility (Figure 3). Five treatments were set up to clarify the contributions of glycolysis and OXPHOS, including: (1) control treatment, without inhibitors or solvents; (2) solvent control treatment, that received 0.5% ethanol; (3) 100 µM of a glycolysis inhibitor 2- deoxyglucose (2-DG); (4) 3 µg/ml oligomycin, the inhibitor for F_O, F_1-ATPase in mitochondrial OXPHOS; (5) combined treatment of 100 µM 2-DG + 3 µg/ml oligomycin. To investigate the temporal dynamics of cellular responses to OXPHOS inhibition and assess the specific contribution of mitochondrial Complex I to sperm bioenergetics, we implemented the following treatments: (1) control; 2) oligomycin (3 µg/ml); and (3) rotenone (2 µg/ml), a Complex I inhibitor in the ETS. All inhibitors were applied at concentrations shown to be effective in previous studies on marine mollusks [135–137]. The effects of these treatments were evaluated at multiple exposure durations (Figure 3).

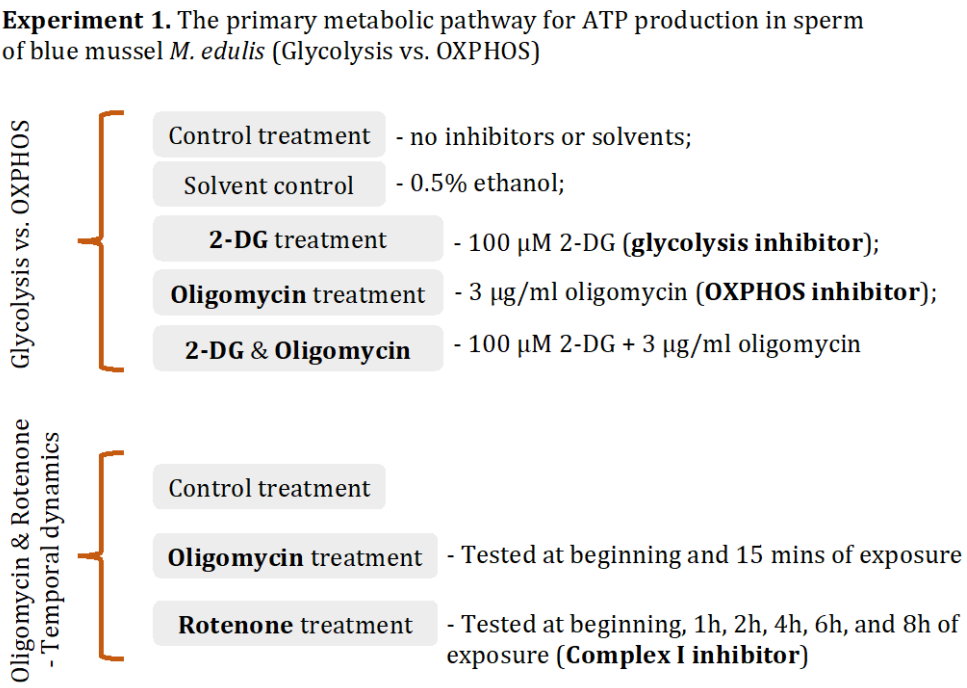

Figure 3. Experimental design to test the primary metabolic pathway for ATP production in mussels' sperm. Fresh sperm suspensions were obtained by inducing spawning through thermal shock and subsequently divided into five or three aliquots for different inhibitor treatments.

The second experiment focused on the impacts of an environmental stressor (deoxygenation) on the reproductive traits of the blue mussel *M. edulis* (Figure 4). We exposed the adult mussels to different DO regimes, including: (1) control treatment, ~ 9 mg O_2/L for 2 weeks; (2) anoxia 24h, 0 mg O_2/L; (3) anoxia 72 h, 0 mg O_2/L; (4) hypoxia 1 week, 2 mg O_2/L; (5) hypoxia 2weeks, 2 mg O_2/L. After the parental exposure, gametes were collected by inducing spawning through thermal shock to test sperm motility, ATP contents and mitochondrial function, egg diameter, as well as fertilization success. Furthermore, the spawning rates of adult mussels from all treatments were recorded.

Experiment 2. The effects of parental exposure to hypoxia and anoxia on the reproductive traits of blue mussel *M. edulis*

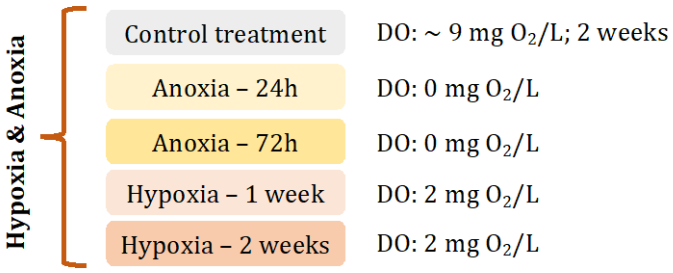

Figure 4. Experimental design for studying the effects of different dissolved oxygen regimes on the reproductive traits of adult mussels. Mussels were exposed to different DO regimes at a temperature of 10 °C and salinity of 15. Gametes were obtained by inducing spawning through thermal shock after the parental exposure and used to evaluate sperm and egg related traits and fertilization success.

In the third experiment, we tested the effects of temperature and salinity variation on sperm performance and fertilization success in the Baltic Sea and the North Sea mussel populations. For temperature experiment, gametes from both mussel populations were exposed to 5 °C, 10 °C, 15 °C, 20 °C, and 25 °C. Due to the different salinity environments of the natural habitats for two populations, the salinity set up are different for two populations. For the Baltic population, the salinity treatments were 5, 9, 13, 17, and 21; whereas for the North Sea mussels, the setting was 9, 17, 21, 30, and 35. We also investigated the combined effects of temperature and osmolarity on the sperm mitochondrial function of blue mussel *M. edulis* from the Baltic Sea. To set up the experimental conditions, five osmolarity levels (150 mOsm, 270 mOsm, 390 mOsm, 510 mOsm, and 630 mOsm) were crossed designed with three temperature levels (15 °C, 20 °C, and 25 °C). Mitochondrial function traits, including oxygen consumption rate ($\dot{M}O_2$), ROS production, and fractional electron leak (FEL) at both OXPHOS and LEAK status, were measured (Figure 5).

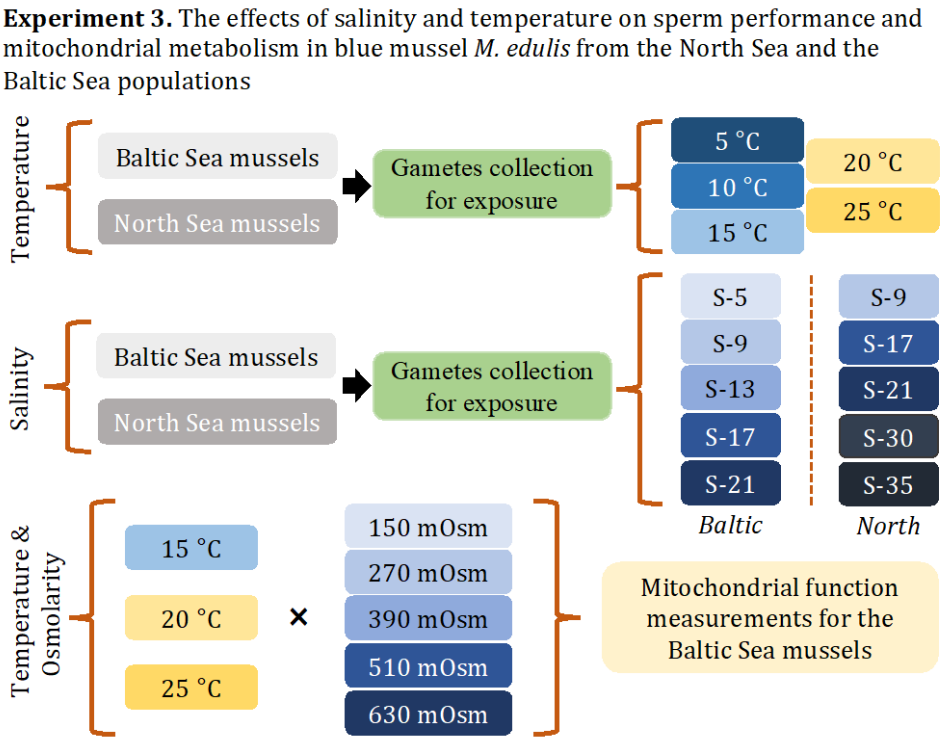

Figure 5. The experimental design of the study examining the effects of temperature and salinity on sperm motility and fertilization success, as well as the combined effects of temperature and osmolarity on the mitochondrial function of the sperm. During acclimation, seawater temperature was maintained at 10 °C for both populations, with control salinities set at 15 and 30 for the Baltic Sea and the North Sea mussels, respectively.

2. Summary and discussion of results

2.1 OXPHOS rather than glycolysis is the primary energy source for sperm motility

The response of sperm motility and ATP production to both glycolysis and OXPHOS inhibitors revealed that oligomycin, the inhibitor of ATP synthase in mitochondria, immediately decreased the motility rate, velocities, and ATP contents of sperm in the blue mussel *M. edulis*, whereas no effect was observed in response to the glycolysis inhibitor 2-DG. Moreover, the inhibitor of CI in OXPHOS, rotenone, also partially impaired the motility rate and velocities of sperm. However, the rotenone-induced suppression of sperm activity was incomplete, indicating that other mitochondrial complexes (most likely the succinate-driven CII) contribute to aerobic ATP production, supporting sperm activity [138, 139]. These results indicate the important role of the mitochondrially-produced ATP in sperm performance of blue mussel *M. edulis*. Furthermore, the ATP levels were strongly correlated with the motility rate and velocities of sperm, indicating that ATP is a direct contributor to sperm movement. This study determined that OXPHOS, rather than glycolysis, is the most important metabolic pathway for ATP

production in mussel sperm, which is consistent with the previous findings [140].

Our temporal dynamics investigation of the impact of oligomycin further supports the key role of OXPHOS in sperm bioenergetics. The response of sperm motility to oligomycin was instantaneous; less than 1% of sperm remained active immediately after the exposure to oligomycin. This result emphasized the crucial importance of the mitochondrial ATP synthesis for the sperm performance High reliance on the OXPHOS was also reported for the sperm of the other broadcast spawners. Regardless of their mitochondrial inheritance strategy—whether strict maternal inheritance or doubly uniparental inheritance—sperm motility of marine bivalves such as scallops, cardiid clams, and mytilid mussels primarily relies on OXPHOS [140]. In contrast to our findings in mussels, a study on the Pacific oyster *Crassostrea gigas* showed that sperm motility rate significantly decreased by the mitochondrial uncoupler CCCP only after two hours of exposure [117]. This result suggested that in oysters, glycolysis or other substrate-level phosphorylation, such as phosphagen breakdown, may contribute to maintaining sperm motility for a few hours post-spawning, potentially supported by internal ATP reserves. However, sustained sperm activity over longer periods also relies on oxidative phosphorylation (OXPHOS) in *C. gigas* [117].

Interestingly, glycolysis did not contribute to the energy supply supporting sperm motility in the blue mussel *M. edulis*, since the glycolysis inhibitor 2-DG did not affect the motility rate, velocities, or ATP production of the sperm. This outcome contrasts with the earlier findings in mammals, where glycolysis is the preferred pathway for ATP production in sperm, particularly under the low-oxygen conditions typical of internal fertilization environments [119, 120]. Interestingly, an earlier study reported moderate suppression of sperm velocities in bivalves, including *M. edulis*, *Ruditapes philippinarum*, *Mercenaria mercenaria*, *Nuttallia obscurata*, and *Placopecten magellanicus*, following treatment with an inhibitor of lactate dehydrogenase (LDH), oxamate [140]. However, due to the limited specificity of oxamate, which can also affect enzymes beyond glycolysis, including those related to mitochondrial OXPHOS [141], these findings should be interpreted with caution. Furthermore, marine mollusks are known to exhibit low baseline LDH activity [142-147], which further supports the view that the observed effects of oxamate on sperm motility could be due to the off-target effects of oxamate, and not be attributable solely to glycolytic inhibition. In comparison, the notable reductions in sperm performance of bivalve species were observed in response to the inhibition of OXPHOS [140]. These results collectively reinforce the conclusion that mitochondrial OXPHOS in sperm is the dominant energy pathway in *M. edulis*, indicating a metabolic strategy well-adapted to the ample DO conditions for external fertilization in marine environments.

In addition, the inhibition of mitochondrial CI by rotenone in this study induced an immediate and substantial reduction (~50%) in the motility rate and curvilinear velocity (VCL) of sperm, which confirms the essential role of mitochondrial OXPHOS in maintaining sperm movement. Residual sperm motility observed despite inhibition of electron transfer at CI suggests that alternative substrates—such as succinate or glycerol-3-phosphate—may partially support mitochondrial ATP production by donating electrons directly to ubiquinone [137,

148]. It is worth noting that rotenone is known to increase mitochondrial ROS production in many cell types [149, 150], raising a possibility that the rotenone-induced decrease in sperm performance may be due to the oxidative stress. However, a previous study on the Pacific oyster *C. gigas* has shown no significant stimulation of mitochondrial ROS production by rotenone [151]. This implies that the observed reduction of sperm motility is likely attributable to impaired ATP synthesis rather than oxidative stress. Our findings highlight that the sperm performance of *M. edulis* is highly dependent on mitochondrially-generated ATP.

Despite the similar length of flagellum between mussels and human sperm (~ 44 μm) and fewer mitochondria quantity (5-6 in mussels vs 50-75 in humans) [152-154], the ATP generated by mitochondria in mussel sperm is likely sufficient to diffuse to the distal end of the flagellum to support sperm movement, since our results indicate that OXPHOS alone sufficiently powers sperm motility in mussels. Furthermore, we observed no measurable depletion in the contents of carbohydrates, lipids, or proteins in sperm over 8 h of activity in seawater, indicating the minimal energetic cost in maintaining sperm motility of *M. edulis*. Interestingly, a slight but significant increase in ATP content was observed between 2 h and 8 h post-activation, which may reflect a reduced energy demand as sperm motility declines, suggesting that ATP synthesis temporarily exceeds ATP consumption at this later time point.

2.2 Effects of hypoxia and anoxia on the reproductive capacity

Hypoxia is a growing issue globally, threatening marine biodiversity [4, 17]. Under hypoxic conditions, mussels reduce their heart rates and shift to anaerobic metabolism to conserve energy [155]. During reproductive events, especially spawning, hypoxia induced to increase the energetic cost (primarily fueled by lipid and protein breakdown), impairs mitochondrial ATP synthesis post-spawning, and hampers energy recovery, increasing the risk of post-spawning mortality in the natural coastal habitats [156]. Our study has demonstrated that the parental exposure of blue mussels *M. edulis* to hypoxia and anoxia on their reproductive capacities, including the spawning rate, function and metabolism of gametes, and fertilization success. Dissolved oxygen in seawater is an essential factor for sperm motility of marine animals [109, 157]. Previous studies in polychaetes and echinoderms have shown that the decline in competitive fertilization success was caused by the reduced swimming speed of sperm under hypoxia [109, 158], which could be attributed to the reduced mitochondrial ATP production under oxygen-limited conditions [159]. In our study, the results show that short-term exposure (24 – 72 h of anoxia or one week of hypoxia) had no lasting adverse effects on mussel sperm motility when evaluated under normoxic conditions. However, the prolonged hypoxia exposure of two weeks led to an impairment of sperm endurance, since we observed a significantly faster decline in sperm motility after 6 h post-spawning compared to the sperm from males maintained in normoxic seawater. These findings indicate that despite the resilience of *M. edulis* sperm to short-term oxygen deficiency in seawater, extended exposure to hypoxia may still compromise male gamete quality. In the natural habitat of mussels, this reduction in sperm endurance, perhaps coupled with the impaired swimming performance [109, 158], may diminish the

reproductive fitness of male individuals in hypoxia-prone coastal areas, especially when spawning events coincide with hypoxic events seasonally or diurnally.

In addition to impairing sperm endurance, prolonged (two weeks) hypoxia exposure significantly decreased the spawning rate of the adult mussels. Although the underlying mechanisms remain unclear, we hypothesize that the suppression observed after two weeks of hypoxia may result from reduced responsiveness to spawning cues, potentially due to gonadal dysfunction, gamete resorption, or endocrine disruption. Similar reproductive impairments have been reported in other marine invertebrates and fish exposed to low DO environments [160, 161]. The combined impacts of decline in spawning rate, impaired sperm endurance, and slower swimming speed may ultimately result in low fecundity and reproductive capacity impairment of mussel populations inhabiting hypoxia-prone coastal areas. Further studies are needed to clarify the effects of DO deficiency on gonadal development in mussels and to gain a more comprehensive understanding of how coastal hypoxia impacts mussel reproduction.

The exposure of males *M. edulis* to low DO conditions has induced altered mitochondrial function in sperm. However, the response of sperm mitochondria to low DO conditions differed from that of overall sperm performance, with significant effects observed after prolonged (72 h) anoxia exposure rather than after two weeks of hypoxia. Specifically, a significant reduction in mitochondrial respiratory control ratio (RCR; indicating the mitochondrial coupling efficiency) of the sperm was observed in 72 h-anoxia treatment, reflecting a decrease in the OXPHOS rate relative to the proton leak rate. Furthermore, a substantial increase in fractional electron leak (FEL) reflective of the ratio of ROS production to O_2 consumption, was also observed in the 72 h-anoxia treatment, suggesting impaired OXPHOS and elevated potential for ROS production. Unexpectedly, our results also showed that sperm ATP content was highest in males after 72 h of anoxia exposure. This may be attributed to reduced ATP breakdown in the sperm, potentially due to anoxia-induced suppression of metabolic activity [162-164]. In anoxia-tolerant species such as bivalves, metabolic rate suppression is a common stress survival strategy that involves the downregulation of energy-intensive cellular functions, including protein biosynthesis and ion pumping [165, 166]. Notably, this metabolic modification occurs without compromising the mitochondrial capacity [167]. With reoxygenation, mitochondrial respiration in marine bivalves generally recovers rapidly and even exceeds baseline levels [151, 168]. When combined with a reduced rate of ATP consumption, this might explain the high levels of ATP observed in the sperm of male individuals pre-exposed to prolonged anoxic conditions.

A decrease in the mitochondrial coupling observed under low DO conditions suggest a potential regulatory mechanism aimed at minimizing oxidative stress in mussel sperm. ROS production in mitochondria is caused by electron leak and increases exponentially with the elevated MMP, so that mild uncoupling of the mitochondrial ETS due to increased proton leak lowers MMP and thereby reduces ROS production [169, 170]. However, this mechanism appears to be of limited effectiveness in mussel sperm, as the fractional electron leak (FEL) in mitochondria from males exposed to 72 hours of anoxia increased by 7.7-fold in

the OXPHOS state and 2.1-fold in the LEAK state, despite a significant decline in mitochondrial coupling efficiency—from 2.91 ± 0.57 to 1.08 ± 0.02. Similarly, in the one-week hypoxia treatment, the mitochondrial FEL in the OXPHOS state increased 4.6-fold, accompanied by a moderate reduction in the respiratory control ratio (RCR) to 1.43 ± 0.16. Interestingly, after the prolonged hypoxia exposure of two weeks, both the mitochondrial RCR and the FEL at OXPHOS state in mussel sperm returned to near-control levels, suggesting that sperm mitochondria in mussel may be capable of functional acclimation. Generally, these findings exhibit the mitochondrial plasticity of mussel sperm under prolonged hypoxia and indicate that sperm metabolism of the blue mussels from the Baltic Sea is resilient to hypoxia.

Egg size and fertilization success are widely recognized as important determinants of reproductive success in marine species [171, 172]. Previous studies have shown that hypoxic conditions could reduce energy allocation to both reproduction and somatic growth in fish such as the common carp (*Cyprinus carpio*) [173], and impair gonadal development [157]. In female *M. edulis*, short-term hypoxia exposure of one week led to a significant increase in egg size, along with a modest but non-significant reduction in egg number. This may indicate an early-stage shift in maternal investment strategy, which produces fewer but larger eggs under hypoxia exposure. However, Spearman's correlation analysis revealed no significant relationship between egg size and fecundity, suggesting that the observed increase in egg size may not decrease the overall reproductive output. Considering that the gametogenesis in female mussels requires an extended period to complete (generally several months in winter) [174], the short-term hypoxia exposure of one to two weeks may be insufficient to fully alter the reproductive allocation. Notably, our results revealed that the fertilization success remained unaffected by parental exposure to low DO conditions, likely due to the normoxic conditions during spawning and fertilization. This outcome contrasts with the findings in other marine invertebrates, such as the sea urchin *P. lividus*, the hypoxia during fertilization decreased fertilization success [109]. These results emphasize that the effects of DO limitation on fertilization are species-specific and highly dependent on the environmental conditions during fertilization process.

2.3 Effects of temperature and salinity on reproductive traits of the blue mussel, *M. edulis*, in the Baltic and North Seas

Understanding how environmental fluctuations affect gamete performance is crucial for evaluating the reproductive success of marine invertebrates, especially for the broadcast spawners, such as mussels, which release gametes directly into seawater for external fertilization. Temperature and salinity fluctuations are key abiotic stressors in coastal environments and can severely influence gamete performance [133, 175-177]. According to previous studies, the effects of gamete quality on fertilization success are well-documented [176, 178, 179]; however, the direct effects of thermal and osmotic stress on sperm performance remain poorly understood. In this study, we demonstrate that both temperature and salinity variation significantly affect the gamete performance in *M. edulis*, with notable population-specific responses between the North Sea (NS) and the Baltic Sea (BS)

mussels. These findings indicate that the two populations have developed distinct reproductive strategies, potentially shaped by long-term adaptation to their differing environmental salinity regimes.

2.3.1 Population-specific effects of salinity on sperm performance and fertilization

In both studied *Mytilus* populations, salinity showed a strong effect on sperm performance and reproductive success, though the patterns differed significantly between the two regions. Our findings indicate that *Mytilus* populations from the maritime North Sea and the brackish Baltic Sea have adapted to distinct salinity regimes, as reflected in their differing reproductive performance across salinity gradients. In the Baltic population, sperm motility and velocity (VCL and VAP) remained high and stable between salinities of 9 and 21, but dropped significantly at salinity 5. Interestingly, this decline in motility was not accompanied by any changes in key metabolic parameters of the sperm mitochondria, including OXPHOS, resting LEAK respiration, mitochondrial coupling, ROS production, or ATP content, all of which remained stable across salinities 5 to 21, measured at a control temperature of 15°C. This suggests that the decrease in sperm performance at low salinity of 5 is not attributed to the disruptions in aerobic metabolism or oxidative stress, and may be mediated by mechanisms unrelated to energy depletion. Similar results were reported in the oyster *C. virginica*, where low salinity of 10 impaired sperm motility without affecting ATP levels [177]. These effects could be linked to the disruptions in ion homeostasis (e.g., K^+, Na^+, Ca^{2+}), the altered osmotic pressure, and the impairments of sperm motility signaling, which is upstream of ATP-dependent motility processes [180, 181]. These factors collectively impair sperm activation and reduce motility. Further investigation is needed to clarify the possible mechanisms underlying the observed salinity-dependent variation in sperm performance unrelated to energy metabolism in the blue mussels.

The BS population also exhibited a relatively wide tolerance range in fertilization success between salinities 9 and 21, though slight declines were observed at both ends of this spectrum. Embryonic development, however, progressed most efficiently within a narrower range of salinities 13–17, with slower development at both salinities 9 and 21. These results suggest the salinity range of 13–17 as the optimal salinity window for successful external fertilization and embryonic development in this brackish population. In contrast, the low salinity of 5 led to extremely low fertilization success, with embryonic development either stalling or being substantially delayed at the first polar body stage, indicating that salinity 5 is a critical threshold at which the mussels' reproduction is severely impaired. Notably, this threshold aligns with the lower salinity boundary for marine macrozoobenthos initially described by Remane, and highlights the reproductive limitations imposed by low-salinity conditions in brackish coastal ecosystems [27].

In contrast to the BS mussels, NS mussels showed optimal sperm function at higher salinities of 30–35, with performance declining gradually at salinities below 30. Sperm motility rate was particularly sensitive, decreasing within the first hour of low-salinity exposure, while velocity (VCL and VAP) declined more

slowly. Fertilization success exhibited the highest level between salinities of 21–30, but dropped significantly at a salinity of 9, where embryonic development also arrested at the first polar body stage, indicating a lower physiological threshold for reproductive capacity in this population.

Across both studied populations, fertilization success was positively correlated with sperm motility and velocity, but motility rate consistently emerged as the strongest predictor of fertilization success, explaining 85% of the variation in the BS population and 66% in the NS population. In contrast, sperm ATP content exhibited no correlation with either motility or fertilization outcomes under varying salinity conditions. This finding supports the hypothesis that salinity-induced disruptions in sperm motility, likely driven by osmotic or ionic stress, rather than energy limitation, underlie the observed variation in sperm performance. Overall, these findings suggest that the salinity-induced limitations on the reproductive success of *M. edulis* are predominantly dependent on sperm motility and its associated performance metrics.

The marked differentiation in salinity tolerance between the Baltic and North Sea *M. edulis* populations aligns with previous evidence of genetic and phenotypic differentiation across these regions, including variation in morphology, growth, metabolism, and genetic composition [72, 182-185]. Sperm motility rate emerged as highly sensitive to low salinities, with the thresholds at the salinity of 5 for the BS population and 9 for the NS population, together with salinity-dependent developmental outcomes, define distinct optimal reproductive salinity windows for the two *M. edulis* populations, which are the salinities of 13–17 and 21–35 for the BS and the NS populations, respectively. These ranges may reflect long-term local adaptation to different salinity regimes, as well as the different genetic backgrounds of the two populations, since the BS population occurs within a hybrid zone (*M. edulis* × *M. trossulus*), carrying ~70% *M. edulis* nuclear markers and 100% *M. edulis* mitochondrial markers [69], whereas the NS population represents a genetically pure *M. edulis* lineage [70]. The Baltic mussels have adapted to low and variable salinities through enhanced osmoregulatory and reproductive plasticity, whereas North Sea mussels, adapted to more stable and saline environments, show reduced tolerance to hyposaline stress. Together, these findings highlight how both environmental adaptation and genetic structure may contribute to population-specific reproductive strategies in *M. edulis*.

2.3.2 Effects of temperature on sperm performance and fertilization

The temperature dependence of sperm performance revealed similar thermal tolerance for *M. edulis* sperm from the BS and NS populations, which may reflect the shared exposure to seasonal temperature fluctuations (5–15°C) during their reproductive period. The experimental temperatures used in this study realistically mimic gradual seasonal warming and occasional MHWs, both of which are relevant thermal stressors under ongoing climate change. Across both populations, sperm motility was maintained between 5°C and 20°C, with a significant decline observed only at 25°C. Notably, the lowest tested temperature of 5°C is below the thermal threshold for spawning induction in field populations, which generally occurs at 9.5–12.5°C [186]. Nevertheless, our results suggest that sperm remained active and functionally comparable to those observed at

temperatures ranging from 10 to 15°C. The mechanisms underlying this cold tolerance remain unclear; however, it may serve as a protective trait to preserve sperm function during occasional cold spells in spring.

Interestingly, despite impaired sperm performance at the elevated temperature of 25°C, fertilization success remained comparable to that at optimal spawning temperatures (10–15°C) and moderate warming (20°C). This may be due to the amount of sperm used in the experiment being sufficient to ensure fertilization despite reduced sperm motility, or to temperature-enhanced fertilization processes that compensated for the lower motility. In contrast, fertilization success at 2 h post-fertilization was significantly decreased at 5°C, even though sperm motility and velocity remained high. This decoupling between sperm activity and fertilization success is supported by the lack of correlation between motility metrics and fertilization success under temperature treatments, unlike the strong relationship observed under salinity variation. These results suggest that, in the context of temperature, the declines in fertilization success are likely attributable to the disruption of fertilization-specific processes such as the induction of the acrosomal reaction, sperm-egg binding and fusion, or egg activation [95], rather than a reduction in sperm quality. Comparable observations in the sea urchin *Anthocidaris crassispina* showed that low temperatures (0°-10°C) significantly impaired sperm binding and acrosome reaction efficiency [187].

Due to reduced metabolism and enzyme activities under low temperature environments, the embryonic development in ectotherms such as blue mussels *M. edulis*, generally slows down. Similar results were observed in other marine invertebrates. For instance, in the basket cockle *Clinocardium nuttallii*, cleavage stages occur significantly later in cold environments [188], and the sea urchin *Arbacia dufresnii* shows a significant delay in early development at 12°C compared to 17°C [189]. These findings support that the apparent arrest of embryonic development in *M. edulis* at 5 °C may reflect a pronounced temperature-dependent delay in the onset of cleavage rather than a complete failure of embryogenesis.

2.3.3 Does mitochondrial impairment contribute to salinity- and temperature-induced decrease in sperm quality?

Mitochondria play an important role in supporting sperm activity, primarily through ATP generation via OXPHOS, which occurs in the inner membrane of mitochondria and is strictly oxygen-dependent [159]. This link between mitochondrial aerobic respiration and ATP production is essential for sustaining sperm motility. Key mitochondrial enzymes, including citrate synthase and succinate dehydrogenase (Complex II), have been linked to sperm performance [190, 191]. Moreover, mitochondrial respiration, measured by oxygen consumption, and respiratory efficiency show a strong correlation with sperm motility [192]. Experimental inhibition of the mitochondrial electron transport system (ETS) with agents such as rotenone and oligomycin significantly reduces mussel sperm motility, further confirming the functional dependence of sperm quality on mitochondrial integrity [159].

Environmental stressors such as temperature fluctuations and osmotic shifts are reported to impair mitochondrial respiration, coupling efficiency, and ATP synthesis in marine ectotherms [167, 193-197], which may contribute to reduced sperm performance. In addition, stress-induced electron leakage from the mitochondrial ETS elevates ROS production, which may damage cellular structures, including sperm membranes, through lipid peroxidation, thus impairing the motility and morphology of sperm [126, 198-201]. Although sperm motility and mitochondrial metabolism have been studied in many marine invertebrates under environmental stress [202-204], the relationship between mitochondrial function and sperm motility remains insufficiently understood. Our results suggest that mitochondrial dysfunction alone does not fully account for the decline in sperm performance observed at high temperatures or low salinities in *M. edulis*. In Baltic mussels, the reduction in sperm performance at low salinity of 5 was not associated with disruptions in mitochondrial metabolism. BS sperm maintained stable OXPHOS rates and low ROS levels across a wide range of salinities, indicating high mitochondrial tolerance to osmotic stress. Although mitochondrial function in NS sperm under different salinities was not directly measured, prior studies on somatic tissues suggest similarly broad osmotic tolerance (160–1200 mOsm) [139]. Consequently, the observed decline in sperm motility at low salinities (5 in BS and 9 in NS mussels) is likely due to the limited osmoregulatory capacity typical of marine osmoconformers, rather than oxidative stress or mitochondrial dysfunction.

Elevated temperature impaired mitochondrial ATP synthesis capacity in *M. edulis* sperm, as evidenced by reduced OXPHOS rates and decreased coupling efficiency at 25°C compared to 15°C. However, this decline was only observed when warming was combined with low salinity (5–9). Despite these mitochondrial disruptions, ATP content remained stable, indicating that energy supply was maintained and that the decline in sperm motility and velocity observed after 4 hours at 25°C is unlikely due to ATP limitation. This implies that mitochondrial capacity was sufficient to sustain ATP production even under suboptimal temperature conditions. In addition, mitochondrial impairment under thermal stress (25°C) was further reflected by increased fractional electron leak (FEL) at salinities of 5–17, resulting in higher relative ROS production compared to O_2 consumption. While excessive ROS production has been proven to cause oxidative damage in spermatozoa through lipid peroxidation, and thus impair sperm function [198-200], our data did not provide direct evidence linking ROS efflux to impaired sperm motility. However, mitochondrial results showed that both sperm mitochondrial ROS efflux and FEL increased with temperature, indicating that elevated temperature enhances electron leakage, potentially pushing it closer to the threshold for oxidative damage in sperm.

Overall, our findings demonstrate that the reductions in sperm motility, velocity, and fertilization success in *M. edulis* under varying salinity and temperature conditions are not primarily attributed to the impaired mitochondrial function in sperm. Although high temperature decreased mitochondrial coupling efficiency and increased ROS efflux, the ATP production in mussel sperm remained stable, indicating that mitochondria in sperm are resilient and able to meet the energetic demands for sperm motility even under the adverse effects of temperature and

salinity fluctuations. These results suggest that alternative mechanisms, beyond energy metabolism, may contribute to the impaired reproductive traits of *M. edulis* under suboptimal environmental conditions. Further studies are necessary to explore the mechanisms of fertilization failure of blue mussels in cold and low-salinity environments. Potential aims include the acrosomal enzyme activity, sperm–egg binding efficiency, and gamete membrane interactions under these conditions. Understanding these mechanisms will be crucial for predicting how marine environmental changes influence reproductive success and population sustainability in marine broadcast spawners.

2.4 Conclusions

The present study investigated the effects of climate change-related environmental stressors, including salinity fluctuation, temperature variation, and DO deficiency, on gamete performance, fertilization success, as well as early embryonic development in the blue mussel *M. edulis*, focusing on the energetic and physiologic mechanisms behind these effects in two geographically distant populations (BS vs. NS). The study revealed novel insights into the reproductive resilience and vulnerability of the marine broadcast spawner *M. edulis* under environmental variations.

Firstly, we clarified the ATP generation pathway in the sperm of *M. edulis* and confirmed the essential role of OXPHOS in the energy metabolism of sperm in marine mussels. Our results showed that sperm motility in *M. edulis* can persist for at least 8 hours with stable ATP production by OXPHOS. Also, we observed minimal endogenous fuel consumption, suggesting highly efficient energy utilization by the mussel sperm. The significant correlation between sperm motility rate, velocities (VCL and VAP), and ATP content further supports the potential use of ATP as a reliable biomarker for evaluating sperm performance.

Secondly, our study demonstrates that prolonged hypoxia (up to two weeks) significantly affects reproductive processes in adult *M. edulis* individuals, resulting in a marked decrease in spawning rate and impairments in gamete quality. In addition, both hypoxia and anoxia affected sperm energy metabolism in *M. edulis*, as evidenced by the increased FEL and ROS production in mitochondria, as well as the reduced mitochondrial coupling efficiency and ATP synthesis capacity. However, fertilization success in the gametes from the parental mussels pre-exposed to low DO conditions remained unaffected. Notably, these findings are based on a limited number of biological replicates, and the spawning and fertilization were carried out in normoxic environments, which should be considered when interpreting the results. Taken together, our results indicate that sustained hypoxia could impair spawning and gamete performance in *M. edulis*, potentially influencing the long-term sustainability of mussel populations, whereas anoxia (up to 72 h) appears to have transient and less adverse effects.

Thirdly, we assessed the population-specific responses of mussel sperm, embryos, and fertilization under varying salinity and temperature conditions. BS and NS populations exhibited distinct optimal salinity windows for sperm performance and fertilization, which were 21–35 in NS and 13–17 in BS. Sperm motility and fertilization success were highly sensitive to salinity, with thresholds at 9 and 5

for NS and BS populations, respectively. However, sperm ATP content did not change significantly across salinity treatments, and sperm mitochondrial function was mostly unaffected by osmotic variations, indicating non-energetic mechanisms, including ion homeostasis disruption and osmotic stress, to be responsible for the motility loss. On the other hand, sperm from both populations tolerated the temperature from 5°C to 20°C, while significant impairments in sperm motility were observed at the high temperature of 25°C. A mismatch between sperm motility and fertilization success was reported at 5°C, which may be attributed to the disruption of fertilization-specific processes such as the induction of the acrosomal reaction, sperm-egg binding and fusion, or egg activation. Nevertheless, sperm ATP contents across varying temperatures in both populations were stable, indicating mitochondrial capacity was sufficient to sustain ATP production even under suboptimal temperature conditions.

In conclusion, mitochondrial OXPHOS is the primary metabolic pathway for ATP generation, supporting sperm motility in *M. edulis*. This finding makes mitochondria the target organelle for studying sperm performance under climate change-related environmental variations. Hypoxic and anoxic exposure of adult mussels significantly affects sperm mitochondrial function, specifically evidenced by reduced mitochondrial coupling efficiency and enhanced ROS efflux and FEL. The exposure of gametes from two *M. edulis* populations to different temperatures and salinities significantly affected sperm motility, mitochondrial function, and fertilization success, whereas no impact on sperm ATP contents. This finding suggests the resilience in the mitochondria of mussel sperm, which provide sustained, stable ATP production even under the adverse impacts of temperature and osmotic variations.

3. References

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4. Appendix

Contributions to publications included in this thesis

Publication 1: **Kong, H.**, & Sokolova, I. M. (**2024**). Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 270, 110909.

"Oxidative phosphorylation rather than glycolysis is the primary energy source for sperm motility in the mussels Mytilus edulis."

I contributed approximately 80% to this work through the writing of the original draft, visualization, validation, methodology, investigation, funding acquisition, formal analysis, data curation, and conceptualization.

Publication 2: **Kong, H.**, & Sokolova, I. M. (**2025**). Science of The Total Environment, 972, 179103.

"Effects of hypoxia and anoxia on the reproductive performance of the blue mussel Mytilus edulis."

I was responsible for the majority of this research, including writing the original draft, visualization, validation, methodology, investigation, funding acquisition, formal analysis, data curation, and conceptualization. My overall contribution to this work is approximately 80%.

Manuscript 3: **Kong, H.**, & Sokolova, I. M. Under review.

''Salinity and temperature-driven variations in sperm performance and mitochondrial metabolism in North Sea and Baltic Sea mussels Mytilus spp.''

I contributed approximately 80% to this work through the writing of the original draft, visualization, validation, methodology, investigation, funding acquisition, formal analysis, data curation, and conceptualization.

4.1 Oxidative phosphorylation rather than glycolysis is the primary energy source for sperm motility in the mussels *Mytilus edulis*

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4.2 Effects of hypoxia and anoxia on the reproductive capacity of the blue mussel *Mytilus edulis*

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Oxidative phosphorylation rather than glycolysis is the primary energy source for sperm motility in the mussels *Mytilus edulis*

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ABSTRACT

Broadcast-spawning marine mussels rely on high sperm motility for successful fertilization in the dynamic seawater environment. Mitochondria are typically considered the primary source of ATP generation via oxidative phosphorylation (OXPHOS); however, the ATP generation pathways of mussel sperm have not been fully characterized. To better understand the importance of both OXPHOS and glycolysis for mussel sperm function, we conducted experiments inhibiting these pathways in sperm from *Mytilus edulis*. Our results indicate that oligomycin, an inhibitor of the mitochondrial ATP synthase, immediately decreased sperm motility rate, velocity, and ATP content, while 2-deoxy-D-glucose, a glycolysis inhibitor, had no effect. The OXPHOS inhibitor rotenone also partially reduced sperm motility rate and velocity. Interestingly, no evidence was found for the inhibitors' effects on the content of energy-rich compounds (lipids, carbohydrates, and proteins) in the mussels' sperm, indicating only modest energy demand to fuel sperm motility. Based on these findings, we conclude that OXPHOS is the primary energy source for sperm motility in marine mussels. Our study sheds light on the intricacies of mussel sperm physiology and highlights the importance of understanding the energy requirements for successful fertilization in broadcast-spawning marine invertebrates.

1. Introduction

Spermatozoa, the specialized haploid cells produced in male gonads, play a pivotal role in fertilization by delivering the paternal genome to female gametes, resulting in the formation of a zygote (Gallo et al., 2021). In the marine realm, successful fertilization requires sperm to navigate a complex, three-dimensional seawater environment to locate eggs. Therefore, sperm motility is a crucial determinant of the fertilization success of broadcast spawning marine invertebrates (Boulais et al., 2015; Boulais et al., 2017). This mating strategy primarily relies on the velocity of sperm, as gamete collision occurs when the eggs remain stationary (Shi et al., 2017; Styan, 1998; Vogel et al., 1982).

The flagellum is a specialized structure that drives sperm movement and enables penetration of the egg through high-speed oscillatory movements (Inaba, 2003). The energy required for this movement is provided by the breakdown of adenosine triphosphate (ATP), which is catalyzed by dynein-ATPase located in the flagellum (Cosson, 1996). Sperm can generate ATP through two metabolic pathways: glycolysis and oxidative phosphorylation (OXPHOS). Glycolysis, which occurs in the head and principal piece of the flagellum, produces two ATP molecules per glucose molecule without the involvement of oxygen (du Plessis et al., 2015). In contrast, OXPHOS occurs in mitochondria and requires oxygen. It involves the coordinated activity of the electron transport system (ETS), comprising four membrane-bound enzymatic complexes (Complex I, II, III, and IV) that generate an electrochemical gradient of protons across the inner mitochondrial membrane. The ATP synthase (F_O, F_1-ATPase) then uses the protonmotive force to synthesize ATP (Boulais et al., 2015; Piomboni et al., 2012). OXPHOS exhibits higher efficiency and metabolic flexibility than glycolysis due to the ability of mitochondria to oxidize diverse substrates, such as carbohydrates, fatty acids, and amino acids, and produce considerably more ATP per unit substrate (16–18 fold higher in the case of glucose) (Liemburg-Apers et al., 2011).

Sperm motility relies on both glycolysis and OXPHOS for ATP production, according to previous studies (du Plessis et al., 2015; Storey, 2004). In mice, inhibition of glycolysis by 2-Deoxy-D-glucose (2-DG) reduced sperm motility even in the presence of respiratory substrates, indicating the importance of glycolysis as the preferred energy source for sperm motility (Mukai and Okuno, 2004). Similarly, the glycolytic pathway is the primary energy source for human sperm motility, with

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limited contribution from OXPHOS (Nascimento et al., 2008). However, in ovine sperm motility, both OXPHOS and glycolysis were considered equally important (Losano et al., 2017). The specific structure of spermatozoa may explain this variability in energy production, as the head and principal piece of the flagellum lack sufficient respiratory enzymes and ATP generated by OXPHOS in the sperm midpiece may not effectively spread to the distal area of the flagellum, making sperm dependent on glycolysis (De Kretser, 1997; Turner, 2003). Nevertheless, the superior efficiency of aerobic OXPHOS over glycolysis in ATP production cannot be ignored (du Plessis et al., 2015; John, 2002; Ruiz-Pesini et al., 2007), highlighting the importance of mitochondria in sperm motility. To clarify the relative roles of OXPHOS and glycolysis in sperm metabolism across animal species, further investigation is warranted.

External fertilization in marine broadcast spawning species requires synchronized release of oocytes and spermatozoa to maximize fertilization success. Sperm motility and fertilization in marine organisms depend critically on ATP levels, as they affect the duration of sperm movement. This has been demonstrated in various species, including the European sea bass *Dicentrarchus labrax* (Dreanno et al., 1999a), the turbot *Psetta maxima* (Dreanno et al., 1999b), and the Pacific oyster *Crassostrea gigas* (Boulais et al., 2015; Boulais et al., 2017). The globally distributed mussel *Mytilus edulis* is an economically important bivalve and a typical marine broadcast spawner that can serve as an excellent model organism for studying the gamete physiology of broadcast spawners (Gosling, 1992). In this study, we tested two hypotheses regarding mussel sperm physiology: (1) due to the greater accessibility of dissolved oxygen in seawater compared to the internal environment of mammalian fertilization, mussel sperm will rely more on OXPHOS than glycolysis for efficient ATP production; and (2) mussel sperm can flexibly use internal energy stores, including carbohydrates, lipids, and ATP pools, to support their motility. We measured mussel sperm performance (motility rate and velocity) in the presence and absence of specific inhibitors of glycolysis (2-DG) and OXPHOS (oligomycin and rotenone) and determined the effects of these inhibitors on cellular stores of lipids, carbohydrates, and ATP in mussel sperm.

2. Materials and methods

2.1. Animal collection

Adult mussels (4–6 cm) *M. edulis* were collected from the Baltic Sea near Rostock, Germany (54°10′49.6"N 12°05′21.5″E) in January 2020. Healthy adults were transferred into the recycling aerated seawater system at a density of ~60 individuals per tank with 35 l seawater. Mussels were acclimated at 10 °C and salinity of 15 (practical salinity units) for two weeks. They were fed ad libitum by continual addition of mixed microalgal diet to the maintenance tanks including a mixture of freshly cultured *Nannochloropsis* sp. and *Rhodomonas salina*, and a commercial shellfish diet (DT's Live Marine Phytoplankton, Coralsands, Germany).

2.2. Mussels spawning

Mussels were induced to spawn by a transient thermal shock, increasing the seawater temperature from 10 °C to 20 °C within 30 min on a water bath (Ramesh et al., 2017). Once sperm release was detected, mussels were individually transferred to a beaker containing 250 ml of filtered (0.2 μm) seawater (FSW) and maintained at 20 °C for 1 h until complete sperm release. Sperm concentration was determined in each sample using a hemocytometer (M-NZ-4CH, Kisker Biotech). Briefly, 0.5 ml of sperm suspension was fixed with 0.5 ml of 10% formaldehyde solution, and 10 μl of fixed sperm suspension was used for sperm cell enumeration. Sperm counts were performed in triplicate for each sample, and the mean was used to calculate the sperm concentration. Samples from separate spawning events of different males were used as biological replicates (n = 3–10, depending on the assay).

2.3. Experimental design

2.3.1. Experiment 1: OXPHOS vs. glycolysis in sperm motility

To investigate the role of glycolysis and OXPHOS in mussel sperm motility, we divided sperm samples obtained from separate spawning events into five equal volume aliquots and placed them in 50 ml Falcon tubes (Fisher Scientific, Germany). Two specific inhibitors were used to assess the contribution of glycolysis and OXPHOS in ATP production. 2-Deoxy-D-glucose (2-DG; Carl Roth, Germany), an anti-metabolite of glucose, was used to inhibit the glycolytic pathway (Nascimento et al., 2008). As a D-glucose mimetic, 2-DG is phosphorylated to 2-deoxy-D-glucose-6-phosphate (2-DG-6-P), which cannot be isomerized to fructose-6-P due to the lack of a 2-OH group, resulting the accumulation of 2-DG-6-P inside the cell and the inhibition of glycolysis (Pajak et al., 2019). Oligomycin (Sigma-Aldrich, Germany) was used to inhibit ATP synthesis by inhibiting mitochondrial F_O, F_1-ATPase without inhibiting electron transport system (ETS) activity (Slater, 1967; Adzigbli et al., 2022a). To set up the five treatments, five aliquots of each sperm sample (36 ml) received one of the following treatments: 1) control treatment – no inhibitors or solvents; 2) solvent control (0.5% ethanol); 3) 100 μM 2-DG; 4) 3 μg/ml oligomycin; 5) 100 μM 2-DG + 3 μg/ml oligomycin. The working concentrations of all inhibitors were chosen based on the published studies that report the effective concentrations of 2-DG, oligomycin and rotenone in marine mollusks (Koyama et al., 2020; Cherkasov et al., 2006; Sokolov et al., 2021). Stock solutions of 2-DG were made in distilled water, while oligomycin solutions were made in ethanol. The final ethanol concentration in the oligomycin-containing treatments was the same as in the solvent control (0.5% vol.).

Sperm motility, including the percentage of motile sperm, average path velocity (VAP, which indicates the velocity on a path constructed using a roaming average), and curvilinear velocity (VCL, indicating the total distance traveled per second along the point-to-point trajectory), were evaluated at exposure times of 0, 1.5, 4.5, and 7.5 h. Video recording of the sperm motility took 30 min, after which sperm samples were collected for measurements of the content of energy-rich compounds, Therefore, the content of energy-rich compounds in the sperm was assessed after 0, 2, and 8 h post-spawning. An independent experiment was conducted to measure ATP content of the sperm after 0, 2, and 8 h post-spawning to match the exposure times used for the measurements of other energy-rich compounds. The sample size for motility, velocity, and ATP content measurements was five. Ten biological replicates were used for the assessment of the content of energy-rich macromolecular compounds in the sperm. Each biological replicate represented a sperm sample from a different male.

2.3.2. Experiment 2: The effects of oligomycin and rotenone on sperm motility

In light of the substantial impact of oligomycin on sperm motility observed in the first experimental series, we sought to investigate the temporal dynamics of cells' response to inhibition of ATP synthase and whether inhibition of a Complex I in ETS yields similar effects to oligomycin. To address the first query, we exposed three aliquots of each sperm suspension to one of the following treatments: 1) control treatment (sperm in filtered seawater with no additions); 2) solvent control (0.5% ethanol in filtered seawater); 3) 3 μg/ml oligomycin treatment (with 0.5% ethanol as a solvent). We determined the motility rate, velocity average path (VAP), and velocity curvilinear (VCL) of the sperm immediately after inhibitor and solvent addition (time 0) and after 15 min of incubation at exposure times 0 and 15 min. To investigate the contribution of Complex I of ETS in sperm energy provision, we exposed mussel sperm to rotenone, a specific inhibitor of mitochondrial Complex I. We subjected three aliquots of each sperm suspension to one of the following treatments: 1) control; 2) solvent control (0.5% ethanol); 3) 2 μg/ml rotenone (in 0.5% ethanol). We measured sperm motility immediately (time 0) and after 1, 2, 4, 6, and 8 h. In all treatments, the sample size was three, with each biological replicate corresponding to a

sperm sample from a distinct male.

2.4. Sperm motility

Sperm motility was evaluated using computer-assisted sperm analysis (CASA) software (Shi et al., 2017; Wilson-Leedy and Ingermann, 2007), which involved recording sperm movement videos under a microscope. A 34 μl sperm sample from each treatment was diluted to 1000 μl, and a 17 μl aliquot of the diluted sperm suspension was transferred into a hemocytometer placed under an Olympus microscope (model CH3-TR45) equipped with a UCMOS camera (14000KPA) and the ToupView software. The sperm movement videos were captured at the frame rate of 30 (frames per second) for 60 s, and three replicate videos were recorded for each trial. The motility rate, velocity curvilinear (VCL), and velocity average path (VAP) of the sperm were determined using the CASA plugin developed for ImageJ (https://imagej.net/software/imagej/#publication), and adapted to mussel sperm.

2.5. Sperm ATP content

To determine the ATP content, we collected 5 ml of sperm suspension immediately (time 0) and after 2 and 8 h of various treatments (as described in section 2.3.1). The sperm was subjected to centrifugation at 8000 $\times g$ for 2 min to remove the supernatant, and the pellet was resuspended in 300 μl of phosphate buffer (0.1 M, pH 7.5) (Zhang et al., 2015). Next, the sperm suspension was aliquoted into three wells, incubated at room temperature for 30 min, and 100 μl of CellTiter-Glo® 2.0 Reagent (Promega, Germany) was added to each well and shaken for 2 min. After allowing 10 min for signal stabilization, we recorded the luminescence using the Spectra Max ID3 Multi-Mode Microplate Reader (Molecular Devices, USA).

2.6. Energy-rich compounds in sperm

Marine mussels can utilize lipids, carbohydrates, or proteins as fuels for ATP production, depending on the physiological condition of the mussels and the availability of these compounds (Haider et al., 2018; Wu et al., 2021). Thus, we evaluated the levels of these energy substrates in the mussel sperm exposed to various inhibitor treatments (as described in section 2.3.1). We anticipated that inhibition of the ATP production by inhibitors will decrease energy fuel utilization and thus increase the concentration of the respective energy-rich compound. To test this, we collected sperm suspensions (5 ml per sample) immediately (time 0) and after 2 and 8 h of exposure. The samples were then subjected to centrifugation (8000 $\times g$ for 2 min), and the supernatant was removed. The pellet was then resuspended in 1.5 ml of the supernatant and snap-frozen in liquid nitrogen for storage at −80 °C until further analysis.

To measure the concentrations of the energy-rich compounds, we subjected the sperm samples to sonication (3 × 15 s with 2 min of vortexing between each cycle) to disrupt the sperm cells. Lipid content was quantified using a standard colorimetric assay with chloroform-methanol extraction (Van Handel, 1985; Wu et al., 2021). Carbohydrate content was determined using the phenol-sulfuric acid method (Masuko et al., 2005), while protein content was measured using the Biorad Bradford Protein Assay Kit and bovine serum albumin as a standard, according to the manufacturer's protocol (Bio-Rad, Hercules, CA, USA). The absorbances were measured using the Spectra Max ID3 Multi-Mode Microplate Reader (Molecular Devices, USA). To calculate the total energy content, we converted the lipid, carbohydrate, and protein contents to energy equivalents using established methods (Gnaiger, 1983).

2.7. Statistical analysis

The data analysis was performed using SPSS version 23.0 (IBM Corp., Armonk, NY, USA), and figures were generated using GraphPad Prism version 8.3 (GraphPad Software Inc., La Jolla, CA, USA). Normality of variance was tested using Shapiro-Wilk's test, and homogeneity was tested using Levene's test. Percentage data were transformed using arcsine-square root prior to analysis. Two-way repeated measures ANOVA was used in SPSS with exposure times and inhibitor treatments as independent variables. One-way repeated measures ANOVA followed by the Fisher's least significant difference (LSD) post-hoc test (for normally distributed data) and Friedman's ANOVA (for non-normally distributed data) were used to analyze the effects of exposure times. One-way ANOVA followed by the post-hoc Tukey tests and Student's *t*-test were used to test for significant effects of inhibitor treatments when significant interactions were observed. Kruskal-Wallis test and Mann-Whitney test were performed to analyze significant differences under non-normality and non-homogeneity of variance. Pearson's correlation was used to assess the relationships among the variables. The data are presented as the mean ± standard error of the mean (SEM). Statistical significance was considered at $p < 0.05$.

3. Results

3.1. Effects of inhibitors on sperm motility

During a 1-h spawning period with 10 male mussels in 250 ml filtered seawater (FSW), sperm concentrations ranged from 2.61×10^7 to 8.44×10^7 cells ml^{-1} (Supplemental Table 1). At the start, 28.55 ± 3.55% sperm were motile (Fig. 1A), with an average curvilinear velocity (VCL) of 272.71 ± 71.01 μm/s (Fig. 1B) and average path velocity (VAP) of 91.31 ± 12.08 μm/s (Fig. 1C). The ATP content of spermatozoa at time 0 was 0.28 ± 0.05 nmol/10^7 spermatozoa (Fig. 1D). There was no evidence of a decrease in VAP during the 7.5-h exposure, nor any evidence of a solvent effect (0.5% ethanol) on sperm performance (Fig. 1A, B, C, D). Sperm motility rate and VCL remained stable between 1.5 and 4.5 h, but showed a modest decrease after 7.5 h in the control, control solvent and 2-DG treatments (Fig. 1A, B). Sperm ATP significantly increased from 2 to 8 h of exposure in the control, control solvent, and 2-DG treatments (Fig. 1D). No time effects were detected in the oligomycin treatments and 2-DG & oligomycin treatments (Fig. 1A, B, C, D).

Inhibition of ATP synthase with 3 μg/ml oligomycin significantly decreased the motility rate, as well as the VCL (curvilinear velocity), VAP (average path velocity), and ATP contents of sperm at all time points of exposure (Fig. 1A, B, C, D). Conversely, the inhibitor of glycolysis, 100 μM 2-DG, had no effect. In all experiments, there was a strong correlation between the percentage of sperm motility and both VCL ($R = 0.814$) and VAP ($R = 0.865$) ($p<0.01$; Table 2). Additionally, the velocities demonstrated a significant correlation with each other ($R = 0.743$). Notably, the ATP content showed a significant correlation with both the sperm motility rate ($R = 0.855$), as well as VCL ($R = 0.695$) and VAP ($R = 0.781$), ($p<0.01$, Table 2).

Further investigation using short-term exposures revealed immediate and significant impacts of oligomycin on sperm performance. Sperm motility rate decreased from 24.29 ± 2.23% to 0.57 ± 0.51% and 0.22 ± 0.04% after 0 and 15 min, respectively (Fig. 2A). However, the evidence of this decrease was weak ($p = 0.051$ and 0.066 at 0 and 15 min, respectively), possibly due to the limited sample size ($n = 3$). Weak evidence ($p = 0.066$) was also detected for the impairment of VCL by oligomycin at 15 min (Fig. 2B) and of VAP at both 0 and 15 min (Fig. 2C).

Exposure to a Complex I inhibitor, rotenone, resulted in an approximately 50% decrease in both motility rate and VCL of sperm immediately after the inhibitor addition compared to the control (motility rate: 28.57 ± 3.80% vs 13.45 ± 1.36%; VCL: 272.09 ± 4.91 μm/s vs 135.76 ± 13.68 μm/s) (Fig. 3A, B; Table 1). Rotenone exposure significantly decreased sperm motility rate after 2 h ($p = 0.022$, Fig. 3A) and VAP after 4 h ($p = 0.023$, Fig. 3C). Interestingly, motility rate slightly increased between 0 and 2 h of exposure in the control and control

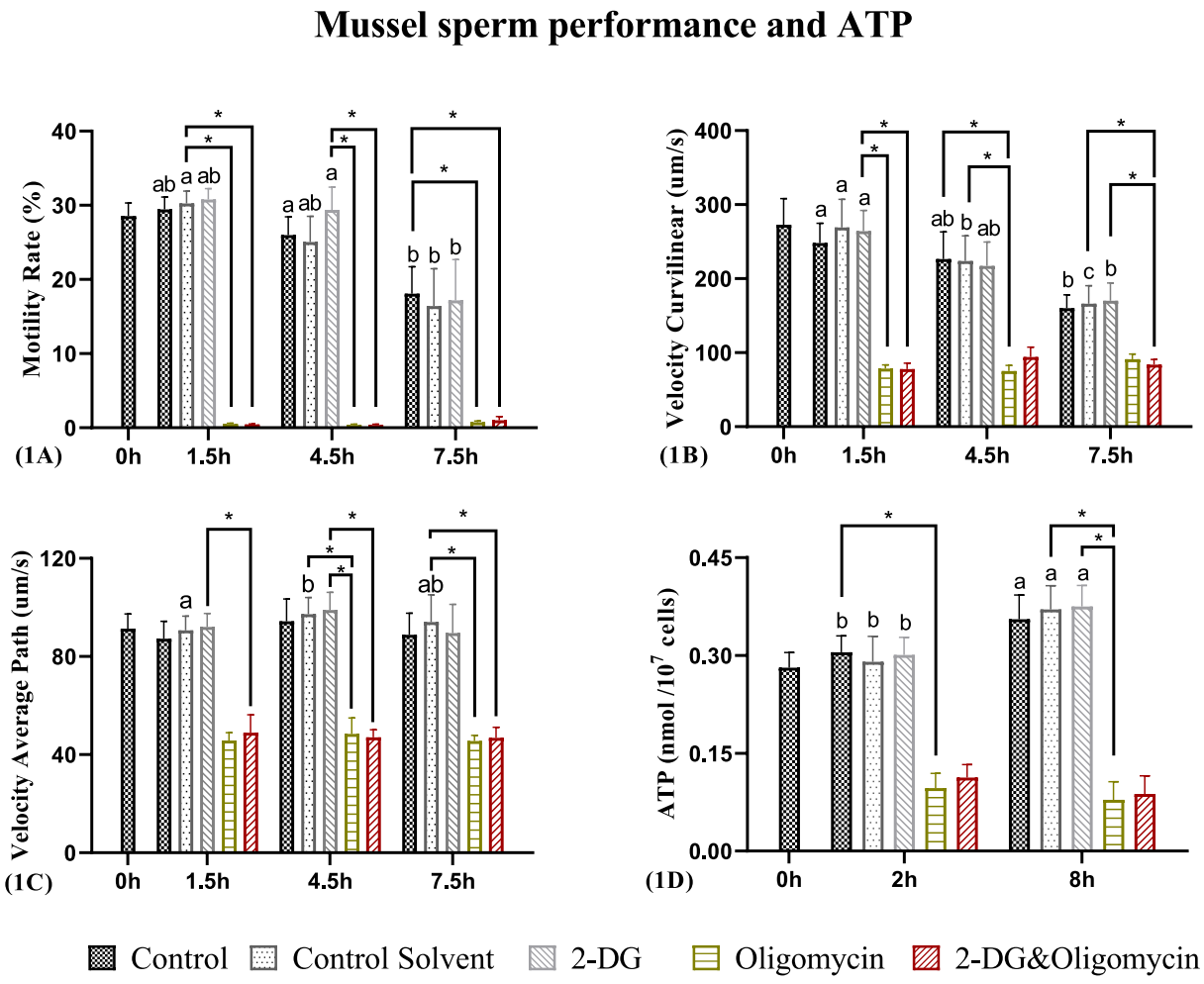

Fig. 1. Motility rate (1A), velocity curvilinear (1B), velocity average path (1C), and ATP contents (1D) of sperm exposed to different inhibitors.
X axis – duration of exposures in the presence or absence of the inhibitors of OXPHOS (oligomycin), glycolysis (2-DG) or their combinations. VCL - velocity curvilinear, VAP - velocity average path. Different letters indicate significant differences among the exposure times within the same treatment group ($p < 0.05$). When the letters are absent, the corresponding values are not significantly different between the different time points ($p > 0.05$). Asterisks indicate significant differences among different inhibitors treatments within the same time group ($p < 0.05$). $n = 5$.

solvent groups and gradually declined afterwards (Fig. 3A). After 8 h of exposure, over a half of viable sperm lost motility in the control and solvent control groups (control: 28.57 ± 3.80% vs 10.23 ± 8.02%; solvent control: 25.73 ± 1.98% vs 11.44 ± 7.35%) (Fig. 3A). Nonetheless, the suppressing effect of rotenone remained evident even at this late exposure time point (Fig. 3A).

3.2. Energy-rich compounds in sperm

The freshly collected sperm displayed a mean lipid content of 23.3 ± 9.9 mg/g (milligram per gram sperm pellet), a mean carbohydrate content of 3.14 ± 1.55 μmol/g, and a mean protein content of 8.76 ± 2.61 mg/g, resulting in a total energy of 1.16 ± 0.43 kJ/g (Fig. 4A, B, C, D). Neither the inhibitor treatments nor the exposure time showed any significant impact on the lipid, carbohydrate, protein, and total energy content of the mussel sperm (Fig. 4A, B, C, D, Table 1). Weak correlations were observed between carbohydrate content and lipid ($R = 0.334$, $p<0.01$) as well as protein contents ($R = 0.251$, $p<0.05$) (Supplemental Table 2).

4. Discussion

Successful fertilization in mussels relies heavily on the maintenance of high sperm motility, which is crucial for navigating the dynamic and complex seawater environment. Thus, adequate ATP supply is paramount in preserving this key feature. We conducted a comprehensive analysis of the relationship between energy supplementation, sperm motility, and energy consumption in the mussel species *M. edulis*. Our findings revealed an extended period of *M. edulis* sperm activity lasting 7.5 h with minimal decline. Previous research on the sperm of a scallop *Pecten maximus* also demonstrated sustained activity and velocity for several hours, although a gradual decline in sperm motility rate and VAP was observed during the 10-h observation period (Suquet et al., 2013). The ability of sperm to sustain their swimming performance for an extended period is especially crucial for marine bivalves that undergo external fertilization. Interestingly, intertidal bivalves such as *M. edulis* (VCL = 272.71 ± 71.01 μm/s; this study) and *Perna canaliculus* (VCL = 177.06 ± 5.91 μm/s) (Smith et al., 2012) exhibit higher sperm swimming speeds than subtidal bivalves such as the Japanese pearl oyster *Pinctada fucata martensii* (99.27 ± 6.54 μm/s) (Basti et al., 2013). This might be an adaptation to a shorter submersion time in the intertidal populations that require faster sperm swimming to ensure reaching the eggs. These adaptations, in conjunction with other mechanisms such as synchronizing gamete release via environmental cues and chemical signals, can enhance the chances of successful fertilization (Lotterhos and Levitan, 2010). This difference in the sperm activity is unlikely to be related to the type of the mitochondrial inheritance as *Perna* and *Pinctada* species have strict maternal inheritance of mtDNA whereas

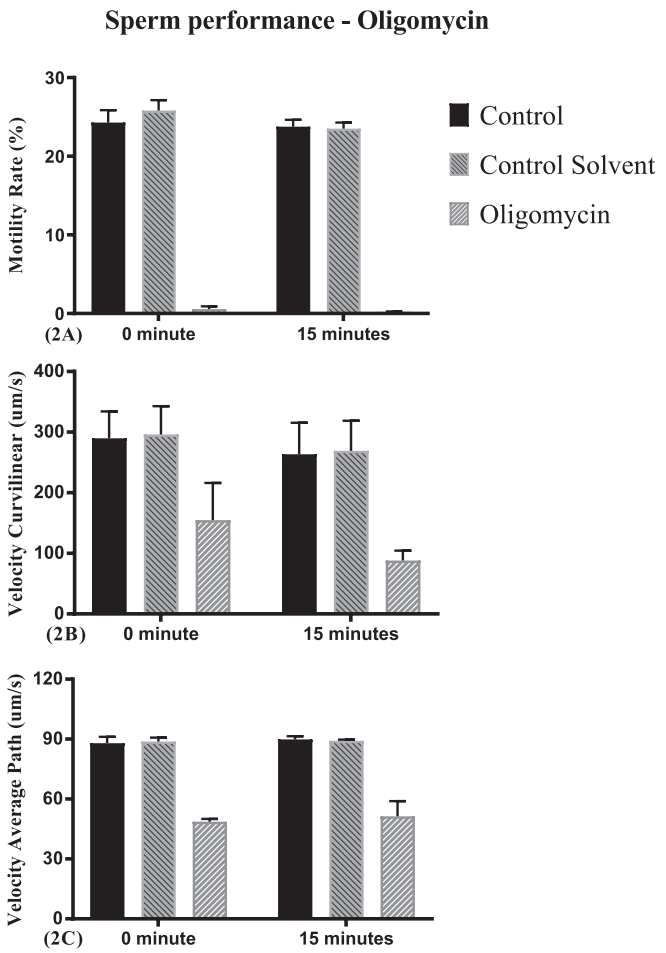

Fig. 2. Motility rate (2A), velocity curvilinear (2B), and velocity average path (2C) of sperm after the short-term exposure to oligomycin (Experiment 2-Oligomycin).
X axis – duration of exposure (0 and 15 min) in the presence or absence of the inhibitor of OXPHOS (oligomycin). VCL - velocity curvilinear, VAP -velocity average path. n = 3.

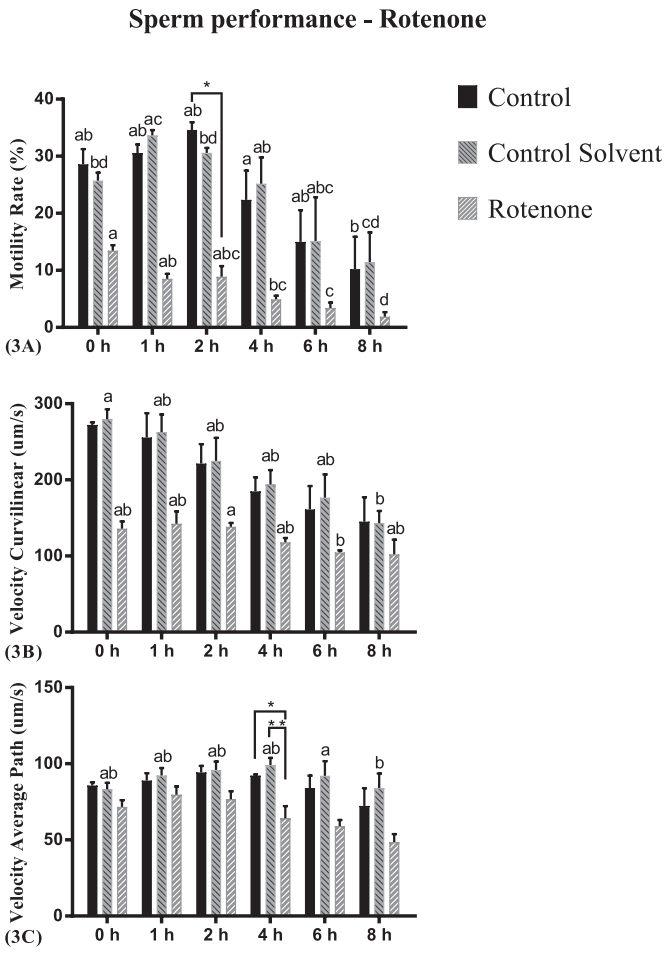

Fig. 3. Motility rate (3A), velocity curvilinear (3B), and velocity average path (3C) of sperm after exposure to rotenone (Experiment 2-Rotenone).
X axis – duration of exposure in the presence or absence of the inhibitor of Complex I of ETS (rotenone). VCL - velocity curvilinear, VAP -velocity average path. Different letters indicate significant differences among the exposure times within the same treatment group ($p < 0.05$). When the letters are absent, the corresponding values are not significantly different between the different time points ($p > 0.05$). Asterisks indicate significant differences among different inhibitors treatments within the same time group (* - $p < 0.05$, ** - $p < 0.01$). $n = 3$.

M. edulis shows double uniparental mtDNA inheritance (DUI) (Weber et al., 2009; Doucet-Beaupré et al., 2010).

Our study provides evidence for the critical role of the mitochondrially-generated ATP in supporting sperm motility in *M. edulis*. Specifically, we found that ATP levels are strongly correlated with both sperm motility rate and velocities, highlighting the direct contribution of ATP as an energy source for sperm movement. Across all experimental treatments, variation in the sperm ATP content explained ~48% of the variation in the VCL, 61% of the variation in VAP, and 73% of the variation in the motility rate. These findings support the notion that ATP content is an important factor in determining the sperm motility. Furthermore, we determined that oxidative phosphorylation (OXPHOS) - rather than glycolysis - is essential to provide ATP and maintain sperm motility in this species, which is consistent with the findings of Bettinazzi et al. (2020). Our investigation of ATP depletion further supports these findings. Specifically, oligomycin, an inhibitor of mitochondrial ATP synthesis, immediately halted sperm motility, with <1% of sperm remaining motile after 15 min of exposure. This result suggests that without functional mitochondrial ATP synthesis, the mussel sperm do not have sufficient ATP to maintain movement. High reliance on OXPHOS was also demonstrated in other broadcast-spawning marine bivalves regardless of whether they showed the strict maternal inheritance (like scallops or cardiid clams) or the doubly uniparental inheritance (like mytilid mussels) of mitochondria (Bettinazzi et al., 2020). In contrast, the mitochondrial uncoupler CCCP significantly reduced sperm motility rate in the Pacific oyster *Crassostrea gigas* only after two hours of exposure (Boulais et al., 2015), indicating that during the initial hours after spawning the oyster sperm can maintain motility on internal ATP stores or by substrate-level phosphorylation (e.g. glycolysis or breakdown of phosphagens).

Interestingly, our study found that glycolysis does not appear to play a role in supporting sperm movement in *M. edulis*, as treatment with the glycolytic inhibitor 2-deoxyglucose (2-DG) had no effect on either sperm motility or ATP generation. This result contrasts with previous studies in mammals, where glycolysis is considered the preferred energy source for sperm motility in the low-oxygen environment of the female reproductive tract (Mukai and Okuno, 2004; Nascimento et al., 2008). Moreover, an earlier study by Bettinazzi et al. (2020) using an inhibitor of lactate dehydrogenase (LDH), oxamate, showed a slight but significant decrease in the motility and velocity of the sperm of bivalves, including mussels. These results apparently contradict the conclusion that glycolysis is not involved in ATP production of the mussel sperm. However, it is worth noting that oxamate is a less selective inhibitor and can suppress multiple glycolytic enzymes as well as OXPHOS pathways (Moreno-Sánchez et al., 2017). Additionally, marine mollusks including mussels exhibit

Table 1
Two-way repeated measures ANOVA of studied bioenergetics and activity parameters of *M. edulis* sperm exposed to different inhibitors treatments for 8 h.

	VCL in Experiment 2-Oligomycin			Motility rate in Experiment 2-Rotenone			ATP		
	df	F	P	df	F	P	df	F	P
Time	1	6.192	**0.047**	1.41	19.373	**0.001**	1	25.625	**< 0.001**
Time * IT	2	0.69	0.538	2.82	0.939	0.458	4	12.503	**< 0.001**
	Lipid			Carbohydrate			Protein		
	df	F	P	df	F	P	df	F	P
Time	1	0.17	0.682	1	1.8	0.188	1	2.156	0.149
Time * IT	4	0.726	0.58	4	0.583	0.677	4	1.261	0.3
	Available Energy								
	df	F	P						
Time	1	0.072	0.79						
Time * IT	4	0.774	0.55						

Only the variables that showed normal distribution are included. VCL - velocity curvilinear, IT - inhibitor treatments. Significant effects ($p < 0.05$) are highlighted in bold.

Table 2
Overall correlations among VCL, VAP, motility rate, and ATP of sperm in mussel *M. edulis* under different inhibitors treatments in all experiments.

Correlations	VCL	VAP	Motility Rate	ATP
VCL	1			
VAP	0.743**	1		
Motility Rate	0.814**	0.865**	1	
ATP	0.695**	0.781**	0.855**	1

$n = 11$ for VCL, VAP, and motility rate; $n = 5$ for ATP. Values represent correlation coefficient, ** indicates significant correlations ($p < 0.01$).

low baseline activity of LDH (Dando et al., 1981; Sato et al., 1993; Ohmori et al., 1997; Hammen, 1969; Gäde and Ellington, 1983; Santini et al., 2001), supporting the notion that oxamate-induced suppression of the mussel sperm activity might be due to the off-target effects of oxamate. Regardless of the oxamate specificity towards glycolysis in mussels, its effects on the sperm motility of bivalves were considerably weaker than those of OXPHOS inhibitors (Bettinazzi et al., 2020), consistent with the conclusion of the predominant reliance on OXPHOS for ATP generation in the bivalve sperm. Taken together, these findings suggest that the reliance on OXPHOS as an energy source for sperm motility is a well-suited metabolic strategy for marine broadcast spawners that have access to ample dissolved oxygen in seawater. Further studies are needed to determine whether this metabolic pattern is found in other taxonomic groups with broadcast spawning and external fertilization.

Sperm morphology may influence the energetic provision and activity of spermatozoa. The sperm head of *M. edulis* measures 3.5 μm in length and features a barrel-shaped nucleus (1.3 × 1.6 μm) surmounted by a 2 μm-long conical acrosome and followed by a ring consisting of 5–6 mitochondria with 0.6–0.7 μm in diameter (Hodgson and Bernard, 1986). In human sperm, for instance, the diffusion potential of ATP from the mitochondria-rich midpiece to the distal end of the flagellum is believed insufficient to sustain motility (du Plessis et al., 2015; Ford, 2006). However, our investigation reveals that mitochondrial oxidative phosphorylation (OXPHOS) is essential and adequate to maintain sperm activity in mussels, despite their similar sperm dimensions to humans (~44 μm flagellum length) and possessing notably fewer mitochondria (5 in mussels vs. 50–75 in humans) (Ankel-Simons and Cummins, 1996; Cao et al., 2004; Mossman et al., 2013). This conclusion is supported by the sharp decline in mussels' sperm motility and ATP content following the inhibition of F_O, F_1-ATPase by oligomycin, and the immediate and strong reduction (by ~50%) in VCL and motility rate caused by the mitochondrial Complex I inhibitor, rotenone. The greater decrease in sperm motility and speed caused by oligomycin compared to rotenone suggests that mitochondrial OXPHOS in mussel sperm can be fueled by alternative substrates (like succinate or glycerol-3-phosphate) that donate electrons to ubiquinone bypassing Complex I (Sokolov et al., 2021; Sukhotin et al., 2020). Potentially, rotenone might impair sperm motility not only by the inhibition of Complex I, but also by the generation of mitochondrial reactive oxygen species (ROS) (Gibb et al., 2014). However, earlier studies in bivalves showed that rotenone addition does not stimulate ROS efflux in the bivalve mitochondria (Adzigbli et al., 2022b), indicating that oxidative stress is an unlikely mechanism of rotenone-induced inhibition of the mussel sperm motility. Taken together, our findings indicate that the mussel mitochondria are highly dependent on the mitochondrial ATP generation and that there are no restrictions on the diffusion of ATP from the mitochondria-containing sperm midpiece to the flagellum in mussels.

Notably, we observed no reduction in the overall amount of potential energy fuels (namely, carbohydrates, lipids, and proteins) in the sperm of mussels following 8 h of activity in seawater. In contrast, prior studies on the sea urchin *Hemicentrotus pulcherrimus* revealed a discernible decrease in phospholipid and glycogen levels in sperm just 15–60 min post-activation (Mita and Yasumasu, 1983). Phosphatidylcholine was identified as the preferred substrate for sperm motility in sea urchins (Masatoshi and Nobuo, 1988). However, our findings suggest that the consumption of energy resources by moving mussels' sperm may be too small to register as a decline in the bulk mass of the respective fuels. This aligns with the mussel sperm's dependence on highly efficient aerobic pathways of ATP generation to support motility. Interestingly, we found a small but statistically significant increase in ATP content of the untreated mussel sperm between 2 and 8 h of exposure. This might indicate that at this late time point, ATP synthesis exceeds ATP usage, possibly due to the time-related decline in the sperm motility. We now included this statement in the discussion.

5. Conclusion

Our study elucidates the ATP generation pathway for sperm motility in the mussel *M. edulis*, demonstrating the crucial role of oxidative phosphorylation (OXPHOS) in the energy metabolism of marine mussel spermatozoa. We find that sperm motility in *M. edulis* can persist for at least 8 h but requires continuous ATP generation through OXPHOS. Our results also suggest that the consumption of the energy-rich macromolecular compounds of the sperm during the 8-h activity period is minimal, highlighting the efficient energy fuel use by the mussel sperm. Furthermore, our study identified strong correlations between sperm motility rate, velocities, and ATP content, indicating the potential use of the latter as a reliable marker for assessing sperm performance. Our findings provide valuable insights into the metabolic adaptations of these broadcast spawners to sustain prolonged sperm motility in dynamic marine environments. They also suggest that the reproductive success of these organisms is highly sensitive to seawater oxygenation levels and highlights the importance of oxidative phosphorylation in sustaining prolonged sperm motility in challenging marine environments.

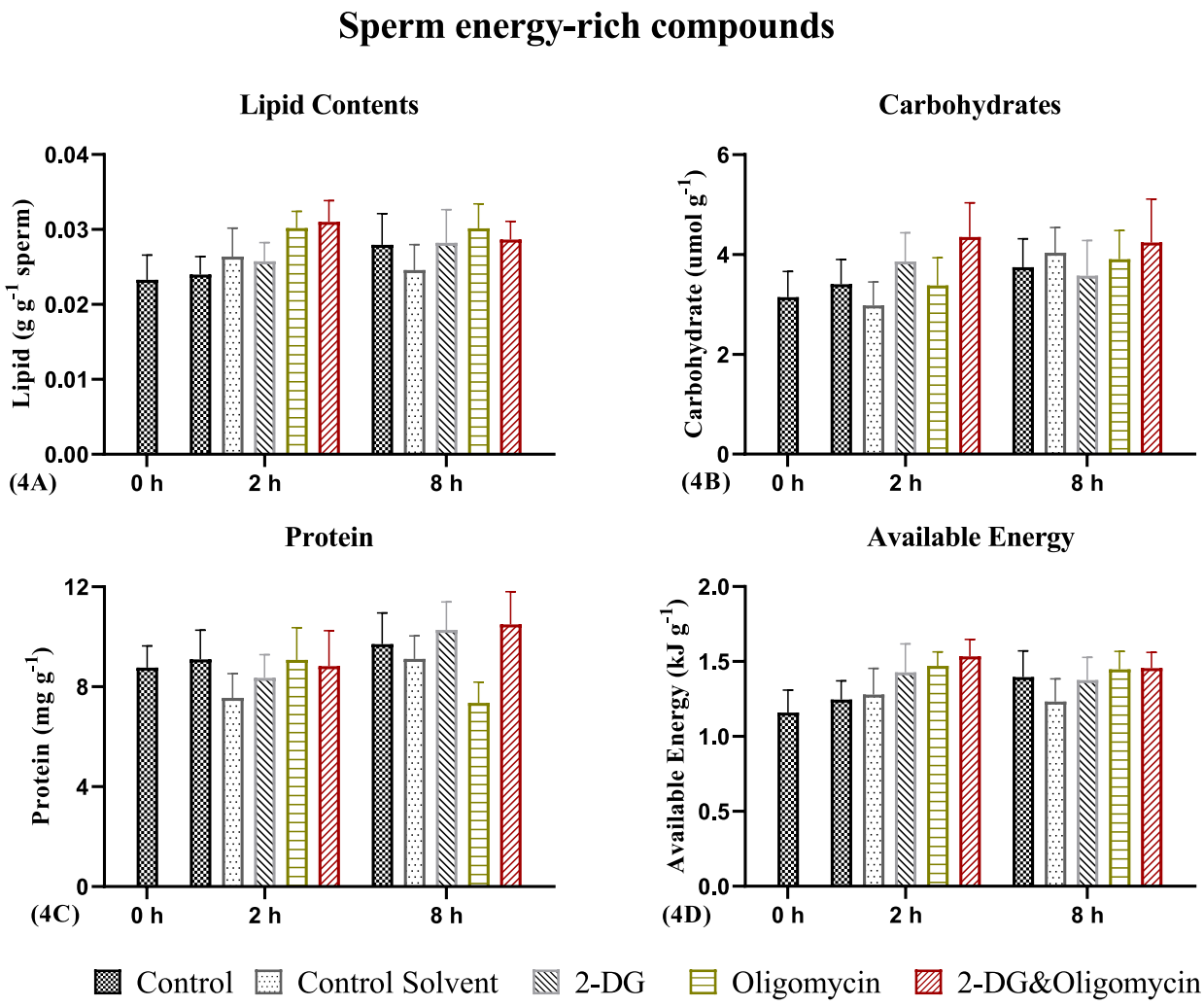

Fig. 4. Lipid (4A), carbohydrate (4B), protein contents (4C), and available energy (4D) in *M. edulis* sperm samples exposed to different inhibitors. The contents of the respective energy-rich compounds were measured after exposure for 8 h in the presence or absence of the inhibitors of OXPHOS (oligomycin), glycolysis (2-DG) or their combinations. $n = 10$ for lipid, carbohydrate, and protein. For available energy, $n = 9$.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpb.2023.110909.

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Effects of hypoxia and anoxia on the reproductive performance of the blue mussel *Mytilus edulis*

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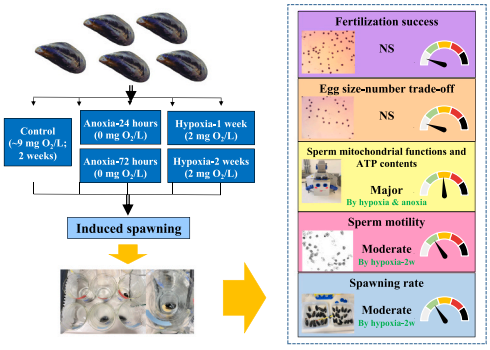
HIGHLIGHTS

- Spawning rate decreases under prolonged hypoxia.
- Long-term hypoxia (2 weeks) impairs sperm velocity and mitochondrial function.
- Short-term anoxia (up to 72 h) has less damaging effects.
- Fertilization success remains unaffected by parental exposure to hypoxia or anoxia.
- Mussels are resilient to short-term hypoxia, but prolonged exposure impairs reproduction.

GRAPHICAL ABSTRACT



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ABSTRACT

Coastal hypoxia poses a growing threat to marine biodiversity, significantly impacting the survival and reproductive success of sessile benthic organisms such as mussels. While much research has focused on the physiological responses of adult mussels to hypoxia, the effects on their reproductive processes remain underexplored. This study investigates how hypoxia (2 mg O_2 l^{-1}, with one-week and two-week exposures) and short-term anoxia (<0.1 mg O_2 l^{-1}, with 24-h and 72-h exposures) affect the reproductive parameters (including sperm motility and metabolism, egg size and number, and fertilization success) of the Baltic Sea blue mussel, *Mytilus edulis*. Our findings show that prolonged (two-week) hypoxia significantly decreases the spawning response rate of adult mussels and leads to a reduction in sperm velocity by ~50 % six hours post-spawning. Short-term (24–72 h) anoxia had no effect on the spawning rate or the sperm velocity. However, sperm mitochondrial ATP synthesis capacity and coupling efficiency diminished after 72 h of anoxic exposure, while hypoxia exposure had no effect on these traits. Increased reactive oxygen species production in sperm mitochondria was noted under both hypoxic and anoxic conditions. Maternal investment strategies, as indicated by the relationship between egg size and number, did not show significant changes following exposure to hypoxia and anoxia. However, one week of hypoxia exposure was associated with the release of larger eggs by female mussels. Despite the negative impact of low oxygen conditions on spawning rates and sperm quality, no decrease in fertilization success was observed, possibly due to fertilization being conducted under normoxic conditions. Our data suggest that a reduced spawning rate from prolonged hypoxia could disrupt spawning synchronization, challenging natural

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reproduction. While sperm from males pre-exposed to hypoxia and anoxia maintained their ability to fertilize eggs under normoxia, impaired sperm quality may affect fertilization success under low oxygen conditions, warranting further investigation.

1. Introduction

Hypoxia, commonly defined as dissolved oxygen (DO) concentrations below 2 mg O_2 L^{-1}, is an increasingly prevalent global issue with significant ecological consequences and a major driver of biodiversity loss in marine environments (Breitburg et al., 2018; Vaquer-Sunyer and Duarte, 2008). Coastal oxygen levels are declining at a particularly high rate, outpacing the reduction of surface-layer oxygen concentrations in the open ocean (Gilbert et al., 2010). Since the 1960s, the expansion of dead zones in coastal oceans has intensified, with over 500 hypoxic regions documented globally across estuaries, coastal seas, and continental shelves (Diaz and Rosenberg, 2008; Breitburg et al., 2018). These deteriorating oxygen conditions are primarily driven by two anthropogenic factors: nutrient overloading, leading to eutrophication, and anthropogenic climate change (Diaz and Rosenberg, 2008; Breitburg et al., 2018). The Baltic Sea, one of the world's largest brackish water bodies, is characterized by significant oxygen and salinity gradients and is a hotspot for hypoxia (Griffiths et al., 2017; Snoeijs-Leijonmalm et al., 2017). Between 1955 and 2009, 115 sites within the Baltic Sea experienced hypoxia, accounting for over 20 % of the coastal hypoxic sites globally (Conley et al., 2011; Carstensen et al., 2014). The coastal zone of the Baltic Sea exhibits a concerning trend, with hypoxia steadily increasing since the 1950s, disrupting nutrient biogeochemical processes, ecosystem services, and coastal habitats (Conley et al., 2011; Carstensen et al., 2014). Benthic organisms, such as the *Mytilus* species, are particularly vulnerable in this region and serve as valuable models for studying the effects of hypoxia.

Coastal "dead zones" with prolonged and severe oxygen depletion cause mass mortalities among benthic organisms, including invertebrates and fish. However, even moderate hypoxia can have detrimental effects on the physiology, growth, and reproduction of marine organisms including such ecologically and economically important groups as bivalves, crustaceans and fish (Diaz and Rosenberg, 2008). Physiologically, the primary impact of hypoxia stems from its adverse effects on aerobic energy metabolism (Sokolova et al., 2019). This disruption leads to energy deficiency, inducing energetically demanding behavioral, physiological, or morphological adjustments to enhance oxygen uptake and delivery. These adaptations can include increased ventilation rates, enhanced circulation, or alterations in metabolic pathways to mitigate the effects of reduced oxygen availability (Earhart et al., 2022; Mandic et al., 2009; Pollock et al., 2007; Song et al., 2024). Under extreme oxygen deficiency, an organism's primary adaptive strategy is to suppress its metabolic rate by significantly reducing both energy supply and demand (Boutilier and St-Pierre, 2000; Storey, 1998; Storey and Storey, 2004). These compensatory mechanisms place physiological stress on the organism, potentially affecting overall health, growth, and reproductive success. Bioenergetic models suggest that the negative impacts of hypoxia and anoxia on an organism's energy balance result in a trade-off between survival mechanisms and the production of biomass, including gonads and gametes, thereby impairing reproduction. While empirical studies have primarily focused on fish, documenting the negative effects of hypoxia on gonadal development, gamete production, fecundity, and endocrine regulation of reproduction (Saha et al., 2022; Thomas et al., 2006; Wang et al., 2023; Wu et al., 2003), there is still a need for research on how these impacts extend to other marine organisms including bivalves (Song et al., 2024).

Blue mussels (*Mytilus* spp.) are key habitat-forming species in temperate coastal ecosystems, contributing to habitat formation, community structure, and nutrient cycling (Kotta et al., 2020; Rönnbäck et al., 2007). In the Baltic Sea, they are prominent on hard substrates and in soft-bottom habitats (Kautsky, 1981). The mussels improve seawater quality by filtering out pollutants and suspended particles, thus improving water clarity and reducing contaminant levels. Furthermore, they support diverse marine life by creating complex habitats that provide shelter and breeding place for various species (Beyer et al., 2017; Vaughn and Hoellein, 2018; Arribas et al., 2014). However, hypoxia reduces their growth rates and increases mortality (Altieri and Witman, 2006). Initially, mussels respond to low oxygen by increasing respiration, heart rate, and ventilation. If hypoxia persists, they shift to anaerobic respiration, leading to reduced immune function, digestion, growth, and survival (Hall et al., 2023; Khan et al., 2020; Nogueira et al., 2017; Wang et al., 2011). Thus, rising hypoxia in the Baltic Sea threatens *Mytilus* species and the ecosystems that depend on them, warranting further investigation of the ecological tolerance of these important ecosystem engineers to oxygen variability.

Reproduction and early development in marine invertebrates, including mussels, are highly sensitive to environmental stressors (Byrne and Przeslawski, 2013; Pandori and Sorte, 2019; Przeslawski et al., 2015). External fertilization in the marine environment requires sperm to navigate a complex three-dimensional seawater matrix to successfully reach and fuse with eggs (Shi et al., 2017). For broadcast-spawning bivalves like mussels, fertilization success is closely linked to sperm motility, which is enhanced by higher sperm velocity (Boulais et al., 2015; Boulais et al., 2017; Vogel et al., 1982). Sperm motility relies on aerobic ATP production via oxidative phosphorylation (OXPHOS), which is oxygen-dependent (Kong and Sokolova, 2024). Thus, hypoxia can impair sperm motility and, consequently, fertilization success. Additionally, hypoxia may influence maternal investment strategies, affecting the trade-off between offspring size and fecundity (Fleming and Gross, 1990; Guisande et al., 1996; Marshall and Steinberg, 2014). Energy limitations often constrain females to producing either fewer, larger eggs or more, smaller ones (Kasimatis and Riginos, 2016). Increasing fecundity at the expense of egg size can be advantageous in harsh conditions with size-independent larval mortality (Morrongiello et al., 2012). Oxygen availability affects reproductive energy expenditure and allocation in mussels (Mredul et al., 2024; Thomas et al., 2019; Wang and Widdows, 1991). However, the impact of hypoxia on egg size-fecundity trade-offs and gamete performance in mussels is not well understood and requires further research.

This study aimed to evaluate the impact of oxygen deficiency (hypoxia and anoxia) on the reproductive performance of Baltic Sea blue mussels (*M. edulis*), focusing on gamete performance and fertilization success. We hypothesized that exposure to hypoxia or anoxia would impair sperm motility and fertilization success by suppressing mitochondrial respiration and ATP synthesis capacity while increasing oxidative stress due to elevated reactive oxygen species (ROS) production. Furthermore, we predicted that prolonged hypoxia would alter maternal investment, resulting in the production of fewer but larger eggs to enhance embryonic survival under low-oxygen conditions (Einum et al., 2002). To test these hypotheses, we assessed sperm performance by evaluating motility (including the percentage of motile sperm and sperm velocity) using computer-assisted sperm analysis (CASA). Additionally, we measured sperm ATP content and mitochondrial function, including respiration rates, ROS production, ATP synthesis capacity, and mitochondrial coupling efficiency. Female gamete quality was determined based on egg size and quantity. Finally, we quantified fertilization success using sperm and eggs from adults exposed to different oxygen regimes. This study aims to clarify the reproductive challenges faced by blue mussels in hypoxic Baltic Sea environments and assess the broader implications of oxygen variations for mussel population

sustainability and marine ecosystem health.

2. Methods and materials

2.1. Animal collection and maintenance

Mussels *M. edulis* were collected from the Baltic Sea in Warnemünde (54°10′49.6"N 12°05′21.5″E), Germany in February 2022. The average water temperature in the Warnemünde area was 4.33 ± 0.02 °C, salinity was 16.22 ± 0.13, and dissolved oxygen (DO) concentration was 11.51 ± 0.05 mg L^{-1} during February–March 2022 (TFO5 site, https://odin2.io-warnemuende.de/). Over the past 10 years (2015–2025), with over 1500 DO recordings, only one hypoxic event (DO <2 mg L^{-1}) has been recorded, lasting for just one minute and likely representing an instrumental artefact. Therefore, the mussel population used in this study is not pre-adapted to hypoxia.

Healthy adult mussels (shell length 4–6 cm) were transported to the laboratory at the University of Rostock within 2 h. The epibionts were removed from the mussels' shells, the mussels were rinsed with cold seawater and transferred to a closed, recycling, aerated seawater system with 12 flow-through tanks containing 570 L seawater at 10 °C and salinity of 15 (practical salinity units). The housing density was approximately one mussel in 1.5 L seawater. The seawater (salinity 15) was prepared by mixing 20 % of natural seawater collected from Warnemünde with 80 % of artificial sea water (Tropic Marin® Pro-Reef, Tropic Marin AG, Switzerland) and aerated for three days before use. Dissolved oxygen, NH_4^+, nitrate, and nitrite concentrations were routinely monitored throughout the preliminary acclimation and experimental exposures to ensure good water quality. The mussels were fed by continual addition of a mixture of freshly cultured *Nannochloropsis* sp. and *Rhodomonas* sp., and a commercial shellfish diet (DT's Live Marine Phytoplankton, Coralsands, Germany). Chlorophyll a concentration in seawater was routinely monitored to ensure sufficient food for mussels. Prior to the experimental exposures, mussels were acclimated in the seawater system for three weeks under control conditions, which were maintained at a temperature of 10°C and a salinity of 15.

2.2. Experimental design

To investigate the effects of hypoxia and anoxia on the reproductive capacity of *M. edulis*, we tested sperm motility, ATP contents and mitochondrial function, egg diameter, as well as fertilization success in five experimental treatments. Parental mussels were exposed to five treatments: 1) control (normoxic) treatment; 2) anoxia 24 h; 3) anoxia 72 h; 4) hypoxia 1 week; 5) hypoxia 2 weeks (Table 1). All treatments were conducted at 10 °C and salinity of 15. Hypoxia is defined as a concentration of DO in seawater of 2 mg L^{-1} and anoxia (near zero DO in this study) represents the "dead zones" in coastal oceans (Diaz and Rosenberg, 2008). Hypoxia was achieved by continually bubbling a predefined mixture of nitrogen and oxygen into the seawater of the experimental tanks (two replicate tanks per treatment, each containing 80 L of seawater). Each tank housed approximately 50 mussels during the hypoxia exposure. The gas flow was regulated using the Qcal Gas Mixing Machine Unit (GMS_3_CH) connected with the GMS_3_Channel Version 5.0 software (QCAL Messtechnik GmbH, Oberostendorf, Germany). DO and temperature were continuously monitored with an oxygen and temperature sensor (LDO101, Hach, Colorado, USA) connected with the portable multimeter HQ40d, and additionally manually recorded at 9 am, 1 pm, and 5 pm every day. Salinity was measured twice a week using a salinity sensor (CDC40103, Hach, Colorado, USA) and adjusted as needed. For the control treatment, approximately 50 mussels were exposed to normoxic seawater for 2 weeks in two replicate 80 L tanks, each housing 50 mussels. Anoxia was achieved by continually bubbling nitrogen into the 2-L air-tight jars filled with seawater until DO of ~0 mg L^{-1} was recorded by a portable multimeter HQ40d (Hach, Colorado, USA) connected with an oxygen sensor (LDO101, Hach, Colorado, USA). The jars were then sealed to prevent gas exchange. Four replicate jars were placed in the water bath at 10 °C, each containing 5 to 6 mussels. Because the jars remained sealed for the duration of anoxic exposure, measurements of seawater temperature, salinity, and dissolved oxygen (DO) were not possible in the anoxia treatments; only the temperature of the external water bath was recorded (Table 1).

After exposure to different oxygen regimes, mussels were transferred to a spawning tank for gametes collection. Spawning was conducted in normoxic seawater for all experimental treatments for approximately 1.5 h in total, including 30 minutes for spawning induction and 1 h for completion. Parental mussels were then removed, and measurements started 1 h post-spawning.

Table 1
Temperature (T) and dissolved oxygen concentration (DO) monitoring in experimental exposures of *M. edulis*. Data are from March to May 2022. For temperature and DO, N is the same as the exposure monitoring days. For salinity, $N = 6$ in control treatment, N = 4 in hypoxia-1w treatment, and $N = 9$ in hypoxia-2w treatment. The DO (~0 mg l^{-1}) and salinity (15) was set in the jars used in the anoxic exposures prior to their sealing, and was not determined (ND) during the 24–72 h of exposure.

	Temperature (°C)	DO (mg O_2 L^{-1})	Salinity	Exposure monitoring days
Control	10.07 ± 0.08	9.13 ± 0.13	15.09 ± 0.03	22
Anoxia-24 h	10.07 ± 0.04	ND	ND	6
Anoxia-72 h	10.08 ± 0.02	ND	ND	8
Hypoxia-1w	9.94 ± 0.03	1.99 ± 0.02	15.13 ± 0.04	14
Hypoxia-2w	9.97 ± 0.02	2.02 ± 0.01	15.09 ± 0.02	31

2.3. Mussels spawning and fertilization

Mussels were induced to spawn by the thermal shocking method (Kong and Sokolova, 2024; Ramesh et al., 2017). Briefly, seawater temperature was increased from 10 °C to 20 °C within 20 min on a water bath. Once gametes' release was detected, mussels were individually transferred into a beaker with 200 mL of 0.2 μm filtered seawater (FSW). The seawater (salinity 15) was prepared as described above (section 2.1 „Animal collection and maintenance"), and filtered through a 0.2 μm filter before use. Mussels were kept in the beakers at 20 °C for 1 h to complete gametes release. Sperm concentration was determined in each sample using a hemocytometer (M-NZ-4CH, Kisker Biotech, Germany). Briefly, 0.1 mL of 4 % formaldehyde solution was mixed with 0.1 mL of sperm suspension to fix the sperm, after that, 10 μL of fixed sperm suspension was pipetted into the chamber in the hemocytometer for sperm cell enumeration. To determine the egg concentration, 50 μL of a seawater stock sample containing eggs was diluted to 1000 μL. After thorough mixing, 50 μL of the diluted sample was placed on a concave slide, and the number of eggs was counted under a microscope to calculate the concentration. Sperm concentration from each male was adjusted to 10^7 cells mL^{-1} to obtain a 100:1 sperm: egg ratio for fertilization (Bierne et al., 2002). For fertilization, sperm suspension from one male and eggs from one female were gently mixed and left undisturbed for 2 h (Bechmann et al., 2011; Pechenik et al., 1990). Fertilization success was assessed by cell cleavage and the release of polar bodies (Sprung and Bayne, 1984; Ventura et al., 2016). Three sperm samples and three egg samples from different mussels exposed to each of the five oxygen regimes were used to assess fertilization success, creating three mussel families with different dams and sires for each treatment (sperm sample 1 × egg sample 1; sperm sample 2 × egg sample 2; sperm sample 3 × egg sample 3).

2.4. Sperm motility and eggs diameter

Sperm motility of mussels were measured by CASA as described earlier (Kong and Sokolova, 2024; Shi et al., 2017; Wilson-Leedy and Ingermann, 2007). Sperm suspension at the concentration of 10^7 cells mL^{-1} was transferred to a hemocytometer after five-fold dilution. Sperm movement videos were recorded under the microscope (Primo Star from Carl Zeiss) equipped with a UCMOS camera (14000KPA) and the ToupView software, and then processed using the CASA plugin via Image J. For each trial, three replicate videos of sperm movement were recorded at a frame rate of 30 frames s^{-1} for 60 s. The motility rate, curvilinear velocity (VCL) and average path velocity (VAP) of the sperm were measured using a CASA plugin developed for Image J (https://imagej.net/software/imagej/#publication) and adapted to mussel sperm (Fig. 1). The parameters used in this analyses were as follows: minimum sperm size 5 pixels; maximum sperm size 120 pixels; minimum track length 10 frames; maximum sperm velocity between frames 26 pixels. The VCL indicates the total distance traveled per second along the point-to-point trajectory, and the VAP measures the velocity on a path constructed using a roaming average (Kong and Sokolova, 2024). Sperm VCL and VAP measurements were based on the motile sperm recognized by CASA.

Five aliquots of egg samples from each female were photographed under the microscope. For each aliquot, a single photograph was taken, and in each photograph, 10 eggs were randomly selected for diameter measurements by ToupView software. Transverse and longitudinal diameters were measured for each egg, and the average of the two diameters was used to represent the diameter of that egg. Eggs from three female mussels were used as three biological replicates in each DO treatment ($N = 3$).

2.5. Sperm ATP content

Sperm ATP content was determined by the luminescence measurements with the Spectra Max ID3 Multi-Mode Microplate Reader (Molecular Devices, USA) as described elsewhere (Kong and Sokolova, 2024). Briefly, 10 mL of sperm suspension from each male was collected by centrifugation at 8000 $\times g$ for 2 min and resuspended in 300 μL of phosphate buffer (0.1 M, pH 7.5). The sperm suspension from each sample was aliquoted (100 μL) into three wells and incubated for 30 min at ambient temperature. Next, 100 μL of CellTiter-Glo® 2.0 Reagent (Promega, Germany) was added to each well and shaken for 2 min. The final luminescence was recorded using the Spectra Max ID3 Multi-Mode Microplate Reader after allowing 10 min for signal stabilization. For each set of measurements, ATP standard samples were prepared according to the technical manual of CellTiter-Glo® 2.0 Assay, at concentrations of 100 nM, 1 μM, and 10 μM to construct a standard curve. Luminescence from both the standards and experimental samples was measured concurrently. The ATP concentrations of experimental samples were then calculated from this standard curve. Final ATP content of experimental samples were calculated based on the ATP concentrations and sperm cell count used in the assay.

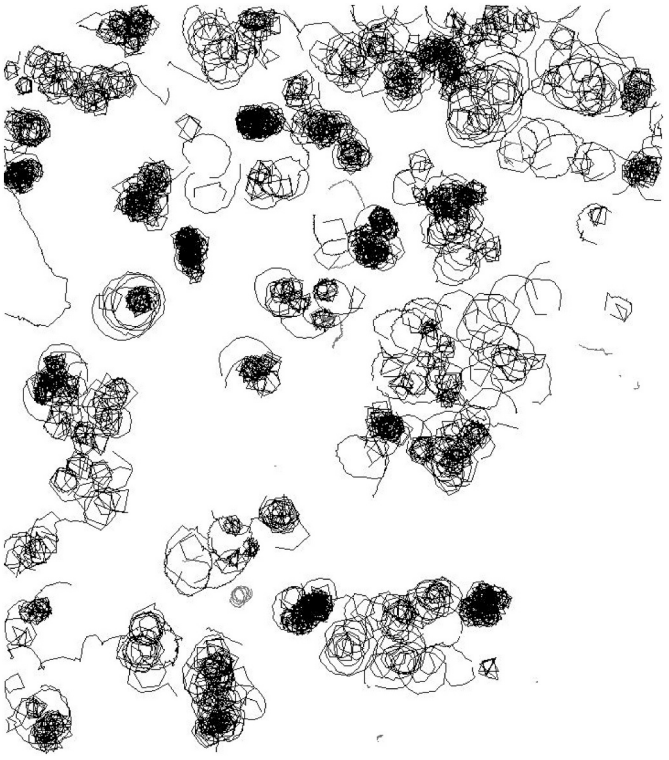

Fig. 1. Sperm movement path analysis using the CASA plugin in ImageJ. The video was randomly selected from the second technical replicate of the first biological replicate in the Anoxia-72 h group, recorded at 3 h post-spawning.

2.6. Sperm mitochondrial functions

To assess the sperm mitochondrial functions of mussels from different DO treatments, the sperm suspension from each male was transferred into a 50 mL centrifuge tube and centrifuged at 8000 *g* for 2 mins at 4 °C. The supernatant was removed and more sperm suspension from the same male was added, repeating the centrifugation and addition steps 2–3 times to obtain sufficient sperm cells. The sperm pellet was resuspended in 500 μL ice-cold homogenization buffer containing 30 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) pH 7.5, 100 mM sucrose, 200 mM KCl, 100 mM NaCl, 8 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), and 30 mM taurine. Protease inhibitors (1 mM phenylmethanesulfonyl fluoride and 2 μg mL^{-1} aprotinin) were freshly added to the homogenization buffer.

We used a high-resolution respirometer (Oxygraph 2-k, Oroboros, Innsbruck, Austria) with integrated software (DatLab 6, Oroboros Instruments) to measure the oxygen consumption and reactive oxygen species (ROS) efflux of sperm mitochondria at 15 °C. Sperm suspension (n = 4–14 per treatment) was transferred into the temperature-controlled chamber containing assay buffer (30 mM HEPES pH 7.2, 185 mM sucrose, 130 mM KCl, 10 mM NaCl, 10 mM glucose, 1 mM $MgCl_2$, 10 mM KH_2PO_4, and 1 % bovine serum albumin). The oxygen and ROS sensors were calibrated as described elsewhere (Ouillon et al., 2021; Steffen et al., 2023). ROS efflux was measured by the rate of hydrogen peroxide (H_2O_2) release using the Fluorescence-Sensor Green (525 nm) connected with Oxygraph 2-k, in the assay buffer supplemented with 10 μmol l^{-1} Amplex™ UltraRed as a reporter, 1 U mL^{-1} horseradish peroxidase (to catalyse the H_2O_2-dependent conversion of Amplex Red to its fluorescent form), and 5 U mL^{-1} superoxide dismutase (convert superoxide radicals to detectable H_2O_2) (Steffen et al., 2023). 0.2 μmol l^{-1} H_2O_2 was used to calibrate the fluorometric sensors before every measurement. After the calibration, 200 μL concentrated sperm suspension was injected into the chamber. Digitonin (10 μg mL^{-1}) was added to permeabilize the sperm membrane, and after 10 min of incubation, measurements of oxygen consumption and ROS efflux started.

The following substrate–uncoupler–inhibitor titration (SUIT) protocol was used for the measurement of sperm mitochondrial respiration and ROS efflux: (1) 5 mM pyruvate and 1 mM malate to stimulate Complex I (LEAK I, NADH-linked, state 2) respiration; (2) 10 mM succinate to stimulate electron flux through Complex II (LEAK I + II, state 2); (3) 3.75 mM ADP to achieve maximum ATP synthesis and OXPHOS activity (OXPHOS respiration, state 3); (4) 5 μM of oligomycin to inhibit mitochondrial F_O,F_1-ATPase (LEAK I + II; state 4); (5) 1 μM rotenone to inhibit electron flux through Complex I; (6) 10 μM antimycin A to inhibit Complex III; (7) 0.5 mM *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD) and 2 mM ascorbate to stimulate the activity of Complex IV

(COX); (8) 50 mM sodium azide to measure non-mitochondrial oxygen consumption. Respiratory control ratio (RCR) calculated as a ratio of OXPHOS to LEAK I + II respiration (state 3 to state 4) was used to assess the mitochondrial coupling (Estabrook, 1967; Ouillon et al., 2021; Steffen et al., 2023). LEAK state respiration (state 2 or state 4) indicates the oxygen demand of idling mitochondria that are not synthesizing ATP. It reflects the maintenance cost of the mitochondrial membrane potential, compensating for all futile proton and cation cycles not coupled to ATP synthesis (Jastroch et al., 2010; Sokolova, 2023). Pilot studies showed that the average differences between the LEAK respiration in state 2 and state 4 in mussel sperm do not exceed 15 % (data not shown). Therefore, we only report the LEAK MO_2 and ROS efflux after oligomycin addition as LEAK I + II state. State 3 (OXPHOS) respiration was used as a proxy for the maximum ATP synthesis capacity of the mitochondria. The fractional electron leak (FEL) rate was calculated as the ratio of the H_2O_2 efflux rate to the mitochondrial oxygen consumption rate ($\dot{M}O_2$) in the LEAK and OXPHOS mitochondrial states.

2.7. Statistical analysis

Data analysis was conducted with SPSS version 23.0 (IBM Corp., Armonk, NY, USA), and all figures were produced by GraphPad Prism version 8.3 (GraphPad Software Inc., La Jolla, CA, USA). Shapiro-Wilk's test was used to test the normality of data distribution, and Levene's test was used to test the homogeneity of variance, both based on the raw data. Percentage data were transformed using the arcsine-square root before analysis. One-way ANOVA followed by the Turkey's post hoc test (for normally distributed data) and Kruskal Wallis test followed by the pairwise comparisons across all groups (for non-normally distributed data or non-homogeneity of variance) were used to analyze the effects of DO regime on the studied response variables. For traits that showed no significant effects of exposure time (including spawning rate, and ROS and FEL of the sperm mitochondria), we conducted an additional analysis by pooling data from different time points within each oxygen condition. This approach increased the sample size and enhanced the power of the statistical analysis. Student's test and Mann-Whitney *U* test were used to analyze the difference between the sperm velocities at 3 h and 6 h post-spawning. Spearman's correlation analysis was used to assess the relationships between egg diameter and number.

The data are presented as the mean ± standard error of the mean (SEM). Statistical significance was considered at $p < 0.05$. For sperm motility, egg number and diameter, and fertilization success, $N = 3$ per treatment, with 3 technical replicates averaged for each biological replicate. For sperm mitochondrial OXPHOS respiration, LEAK I + II respiration, RCR, and ROS efflux, $N = 7$ in control, $N = 4$ in anoxia-24 h, $N = 8$ in anoxia-72 h, $N = 14$ in hypoxia-1 week, and N = 8 in hypoxia-2 weeks. For FEL, N = 7 in control, $N = 5$ in anoxia-24 h, N = 8 in anoxia-72 h, N = 14 in hypoxia-1 week, and N = 8 in hypoxia-2 week. For sperm ATP content, N = 3 in all treatments. For determination of the spawning rate: $N = 6$ in control treatment, N = 5 in anoxia-24 h treatment, N = 6 in anoxia-72 h treatment, N = 6 in hypoxia-1w treatment, and N = 7 in hypoxia-2w treatment, where N indicates the number of recorded independent spawning events.

3. Results

3.1. Seawater temperature, salinity, and DO monitoring

Seawater temperature in the control, one week of hypoxia (hypoxia-1w), and two weeks of hypoxia (hypoxia-2w) treatments was 10.07 ± 0.08 °C, 9.94 ± 0.03 °C, and 9.97 ± 0.02 °C, respectively. For anoxia treatments, temperature was monitored in the water bath outside the jars, and was 10.07 ± 0.04 °C and 10.08 ± 0.02 °C in anoxia-24 h and 72 h treatments, respectively. The DO in the control treatment was 9.13 ± 0.13 mg O_2 L^{-1}. The DO was 1.99 ± 0.02 mg O_2 L^{-1} and 2.02 ± 0.01 mg O_2 L^{-1} for hypoxia-1w and 2w treatments, respectively. Seawater salinity in the control, the hypoxia-1w, and the hypoxia-2w treatments was 15.09 ± 0.03, 15.13 ± 0.04 and 15.09 ± 0.02, respectively (Table 1). In addition, final pH values, measured once per treatment at the end of the experimental exposures, were 7.75, 7.94, and 7.98 for the control, hypoxia-1w, and hypoxia-2w treatments, respectively.

3.2. Effects of hypoxia and anoxia on spawning, gamete quality and fertilization

The spawning rate (the fraction of mussels that spawned within 1 h of warming to 20 °C) of control mussels was 71.3 ± 6.2 % (Fig. 2). The spawning rate was not affected by 24 h of anoxia exposure compared to control treatment. There was a mild and not statistically significant decline in the spawning rate in mussels exposed to 72 h of anoxia or 1 week of hypoxia to 49.7 % ± 4.6 % and 45.1 % ± 5.8 %, respectively. Two weeks hypoxia exposure significantly decreased the spawning rate of mussels to 33.4 % ± 8.0 % (Fig. 2). The spawning rate in the two hypoxia treatments combined (1-week and 2-week exposure) were significantly lower than the control treatment or the two anoxia treatments (24 h and 72 h) combined (Fig. 2).

Parental exposure to hypoxia and anoxia had no significant effect on mussel sperm motility percentage at 3 h and 6 h post-spawning ($p > 0.05$). At 3 h post-spawning, motility percentages were comparable across the five treatments, ranging from 89.4 ± 1.5 % to 95.6 ± 0.6 %. By 6 h post-spawning, a significant decline in motility percentage was observed in all treatments compared to 3 h, with statistically significant reductions in the control group (93.6 ± 0.7 % to 83.3 ± 1.0 %), anoxia-72 h treatment (95.6 ± 0.6 % to 79.4 ± 7.0 %), and hypoxia-2-week treatment (89.8 ± 2.9 % to 53.4 ± 2.2 %) (Fig. 3A).

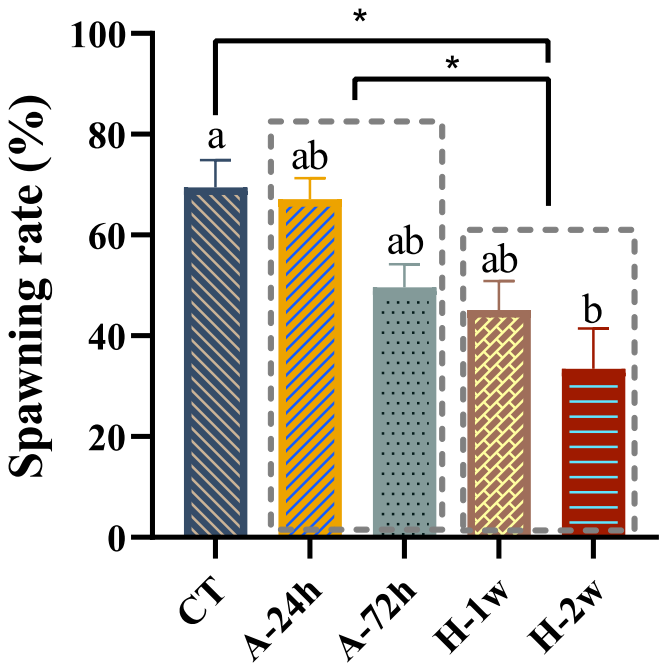

Fig. 2. Spawning rates of parental mussels from five oxygen regime exposures. Different lowercase letters indicate the significant difference among five treatment groups including control (CT), anoxia 24 h (A-24 h), anoxia 72 h (A-72 h), hypoxia 1 week (H-1w) and hypoxia 2 weeks (H-2w) ($p < 0.05$). * represents the significant difference among the control group, the combined anoxia exposure group (A-24 h and A-72 h combined, the gray dashed box) and the combined hypoxia exposure group (H-1w and H-2w combined, the gray dashed box) ($p < 0.05$). N = 6 in control treatment, $N = 5$ in anoxia-24 h treatment, N = 6 in anoxia-72 h treatment, N = 6 in hypoxia-1w treatment, and $N = 7$ in hypoxia-2w treatment. The number of replicates (N) indicates the number of recorded independent spawning events, and the number of mussels for each spawning event varies from 6 to 32.

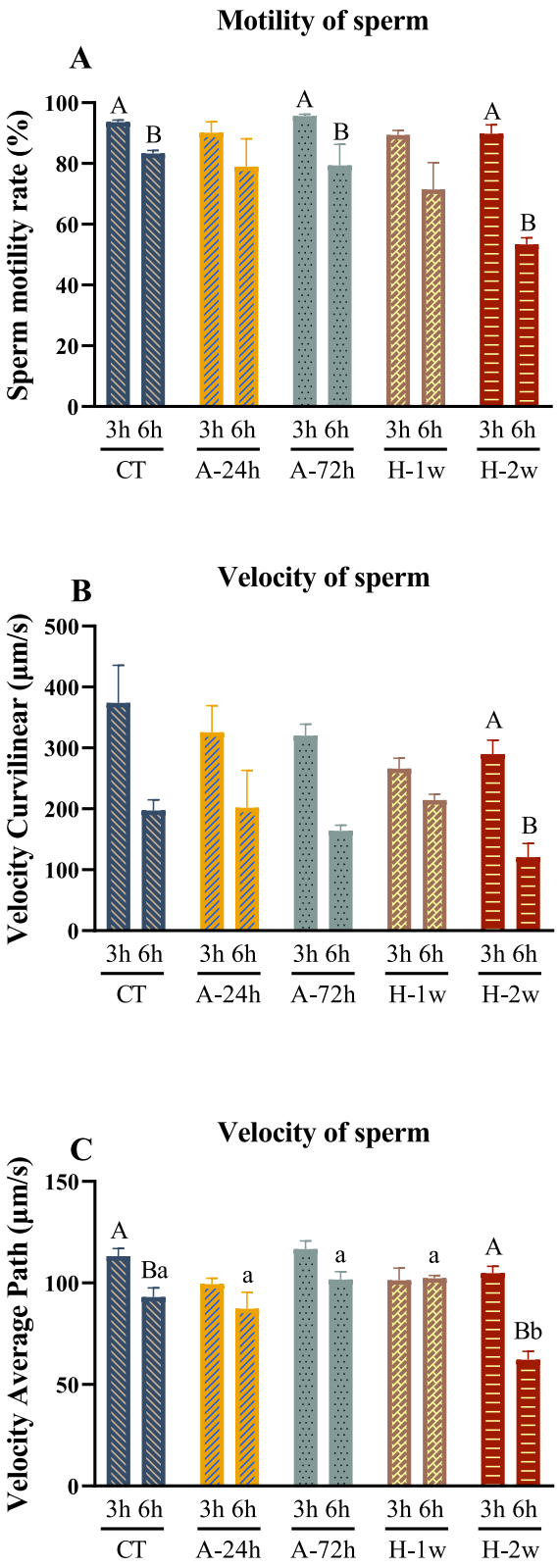

Fig. 3. Motility percentage (A), curvilinear velocity (B) and average path velocity (C) of sperm at 3 h and 6 h post-spawning from *M. edulis* exposed to different DO regimes.

Different capital letters represent the significant differences between 3 h and 6 h time points ($p < 0.05$). Different lowercase letters indicate the significant difference among five DO treatment groups within the same time point ($p < 0.05$). Abbreviations for the treatment groups are as shown in Fig. 2. $N = 3$, with three technical replicates averaged for each biological replicate.

No significant differences in sperm VCL were observed among the five treatments at 3 and 6 h post-spawning ($p > 0.05$). At 3 h post-spawning, sperm VCL was highest in the control group (373.8 ± 61.8 μm s^{-1}) and showed varying degrees of reduction in the experimental treatments, ranging from 244.6 ± 72.3 μm s^{-1} in the 24 h anoxia group to 191.7 ± 40.1 μm s^{-1} in the 1-week hypoxia group. By 6 h post-spawning, sperm VCL declined across all treatments. In the control group, VCL decreased by 47.2 % (to 163.2 ± 21.2 μm s^{-1}). The most pronounced and statistically significant reduction was observed in the 2-week hypoxia group, where VCL decreased by 58.3 % (to 61.7 ± 3.4 μm s^{-1}). In the remaining experimental groups, the reduction in VCL between 3 and 6 h post-spawning was comparable to that of the control, with sperm VCL at 6 h post-spawning recorded as 158.7 ± 66.6 μm s^{-1} (24 h anoxia), 136.0 ± 91.0 μm s^{-1} (72 h anoxia), and 159.2 ± 94.5 μm/s (1-week hypoxia) (Fig. 3B).

Sperm VAP at 3 h post-spawning ranged from 99.5 ± 2.8 μm s^{-1} to 116.6 ± 4.1 μm s^{-1}, with no significant differences among the five treatments ($p > 0.05$). By 6 h post-spawning, a significant reduction in VAP was observed in the control group (17.8 % decrease) and the 2-week hypoxia group (40.7 % decrease) compared to their respective values at 3 h post-spawning. At 6 h post-spawning, sperm VAP in the 2-week hypoxia group (62.2 ± 4.1 μm s^{-1}) was significantly lower than in all other treatments, where values remained between 87.3 ± 8.2 μm s^{-1} (24 h anoxia) and 102.3 ± 1.4 μm s^{-1} (1-week hypoxia) (Fig. 3C).

One week of hypoxia exposure significantly increased egg diameter of female mussels compared to the control treatment (Fig. 4A). The number of eggs released by a single female in a single spawning event ranged from 500,000 to 4,667,000, and there was no significant difference detected among the five treatments (Fig. 4B). Spearman's correlation analysis showed no significant correlation between egg diameter and number with the coefficient − 0.379 ($N = 15$, $p > 0.05$). Fertilization success was not affected by anoxia or hypoxia exposure of parental mussels (Fig. 5).

3.3. *Effects of hypoxia and anoxia exposure on the sperm mitochondrial functions*

OXPHOS respiration of the sperm mitochondria from the control group was 1.17 ± 0.34 nmol O_2 min^{-1} 10^{-7} cells (Fig. 6A). The lowest OXPHOS rate was observed in sperm from males exposed to 72 h of anoxia (0.37 ± 0.05 nmol O_2 min^{-1} 10^{-7} cells), while the highest was in sperm from males exposed to 24 h of anoxia (1.48 ± 0.67 nmol O_2 min^{-1} 10^{-7} cells). However, neither of these values differed significantly from the control (Fig. 6A). No significant differences were observed in sperm LEAK I + II respiration across the five treatments, although the 1-week hypoxia group showed a 45.2 % increase in mean respiration (0.64 ± 0.13 nmol O_2 min^{-1} 10^{-7} cells) compared to the control group (0.44 ± 0.09 nmol O_2 min^{-1} 10^{-7} cells) (Fig. 6B).

The mitochondrial coupling efficiency assessed by RCR was significantly lower in the sperm of the males exposed to 72 h of anoxia (1.08 ± 0.02), compared to the control group (2.91 ± 0.57). RCR in the sperm of the mussels exposed to 1 week of hypoxia was 50.8 % lower than in the control treatment. However, after 2 weeks of hypoxia exposure, sperm mitochondrial RCR significantly increased (2.86 ± 0.22) compared to the 1 week of hypoxia treatment (1.43 ± 0.16) reaching a level similar to the control group (Fig. 6C).

Sperm ATP exhibited the highest level after the males were exposed to anoxia for 72 h (1.76 ± 0.09 nmol 10^{-7} cells vs 0.85 ± 0.05 nmol 10^{-7} cells in the control treatment, $p < 0.05$). When combined across all anoxia-exposed males, sperm ATP contents (1.50 ± 0.15 nmol 10^{-7} cells) was also higher compared to the control. However, no significant effect was observed in the two hypoxia exposure treatments relative to the normoxic control (Fig. 6D). Anaerobic respiration (glycolysis) did not contribute to ATP generation in mussel spermatozoa after the exposure of the males to anoxia for 24 h and 72 h, with OXPHOS remaining the primary pathway for ATP production (Supplementary

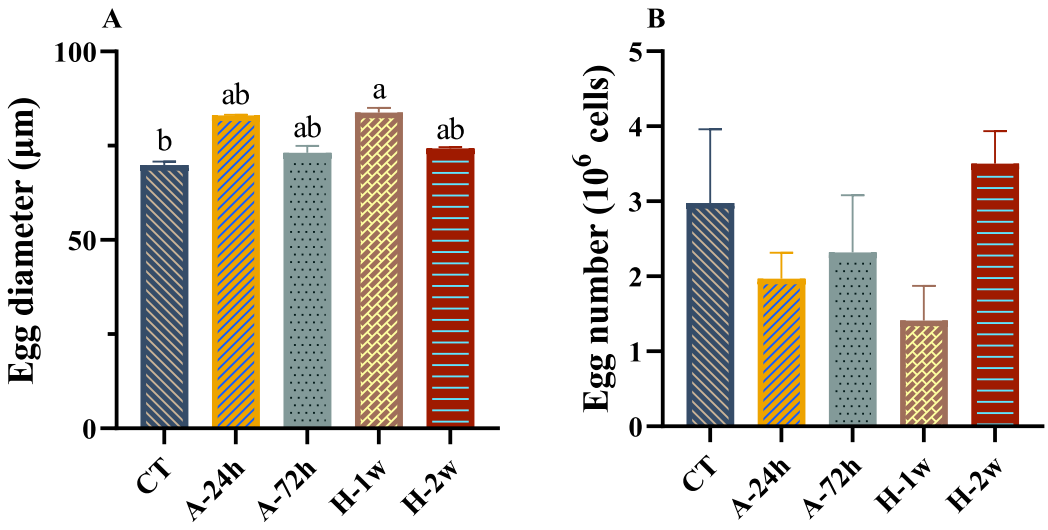

Fig. 4. Egg diameter (A) and number (B) from three females exposed to different DO regimes.
Different lowercase letters indicate the significant difference among five groups ($p < 0.05$). Abbreviations for the treatment groups are as shown in Fig. 2. $N = 3$.

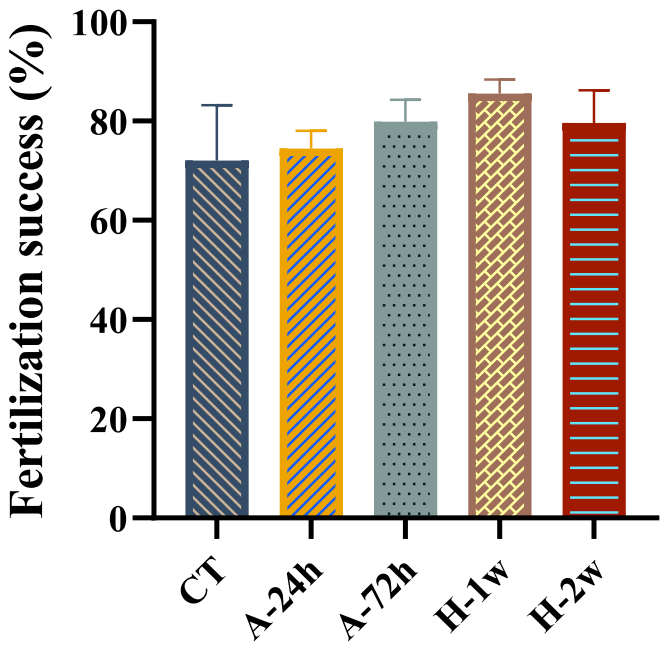

Fig. 5. Effects of parental exposure to different DO regimes on the fertilization success of mussels *M. edulis*.
No significant differences between the DO treatments were found. Abbreviations for the treatment groups are as shown in Fig. 2. N = 3.

Fig. 1S).

The ROS efflux of the sperm mitochondria in the OXPHOS state showed a non-significant increasing trend in 72 h of anoxia and both hypoxia treatments (Fig. 6E). A similar trend was found in the ROS efflux of the LEAK state mitochondria, reaching significance in the combined hypoxia groups relative to the control treatment (Fig. 6F).

The fractional electron leak (FEL) in the OXPHOS state was similar after 24 h of anoxia (0.16 ± 0.03 %) and the control treatment (0.16 ± 0.04 %). The FEL of sperm mitochondria in the OXPHOS state significantly increased in the males exposed to 72 h of anoxia (1.35 ± 0.50 %). In the hypoxia-exposed group (combining 1 and 2-week treatments), sperm mitochondria showed a significantly elevated FEL in the OXPHOS state (0.77 ± 0.16 %) compared to the control treatment (Fig. 6G). In the LEAK state, the sperm mitochondrial FEL was elevated in the males exposed to 72 h of anoxia (1.66 ± 0.56 %), and a significantly elevated FEL was observed between the control group and the hypoxia-exposed group (combining 1 and 2 week treatments) (0.53 ± 0.22 % vs 1.24 ± 0.23 %) (Fig. 6H).

4. Discussion

DO fluctuations are common in coastal marine ecosystems, with hypoxic periods lasting from a few hours (such as during the tidal emersion leading to shell closure or diurnal fluctuations leading to near-bottom hypoxia at night) to several days or weeks in the eutrophicated areas and seasonal "dead zones" (Cooper et al., 2002; Nilsson and Östlund-Nilsson, 2004). Severity of hypoxia can also vary from mild deoxygenation to complete anoxia, resulting in variable physiological and behavioral responses of marine benthic organisms (Diaz and Rosenberg, 1995; Low and Micheli, 2018; McCormick and Levin, 2017). Studies show that DO is an important factor for sperm motility of marine organisms (Graham et al., 2016; Wu et al., 2003). In polychaetes and echinoderms, hypoxia impaired sperm swimming speed resulting in lower competitive fertilization success (Graham et al., 2016; Shin et al., 2014). Suppressed motility was observed when the sperm was directly exposed to hypoxic water (Graham et al., 2016; Shin et al., 2014) and might be attributed to lower sperm ATP production due to oxygen limitation of mitochondrial OXPHOS (Kong and Sokolova, 2024). Our present study, based on the results of 3 and 6 h post spawning, shows that parental exposure to short-term (24–72 h) anoxia or one week of hypoxia does not have lasting negative effects on the sperm motility, when assessed in normoxic seawater. However, two weeks exposure of males to hypoxia led to lower endurance of their sperm as shown by a much faster loss of sperm motility after 6 h in this group compared with the sperm from the males maintained under normoxia. This indicates that even though the mussel sperm is resilient to short-term oxygen deficiency, prolonged hypoxia may compromise the male gamete quality in *M. edulis*. Low sperm endurance combined with lower swimming speed of sperm (Graham et al., 2016; Shin et al., 2014) might therefore reduce male fitness in hypoxia-prone coastal environments, especially if spawning coincides with a seasonal or diurnal hypoxic event.

Furthermore, exposure to prolonged (two weeks) hypoxia suppressed the spawning rate in both sexes of *M. edulis*. Our study does not permit determining whether this suppression reflects gonadal dysfunction, gamete resorption or endocrine disturbance leading to loss of responsiveness to spawning cues. Negative effects of hypoxia on gonad growth has been reported in marine invertebrates like sea urchins

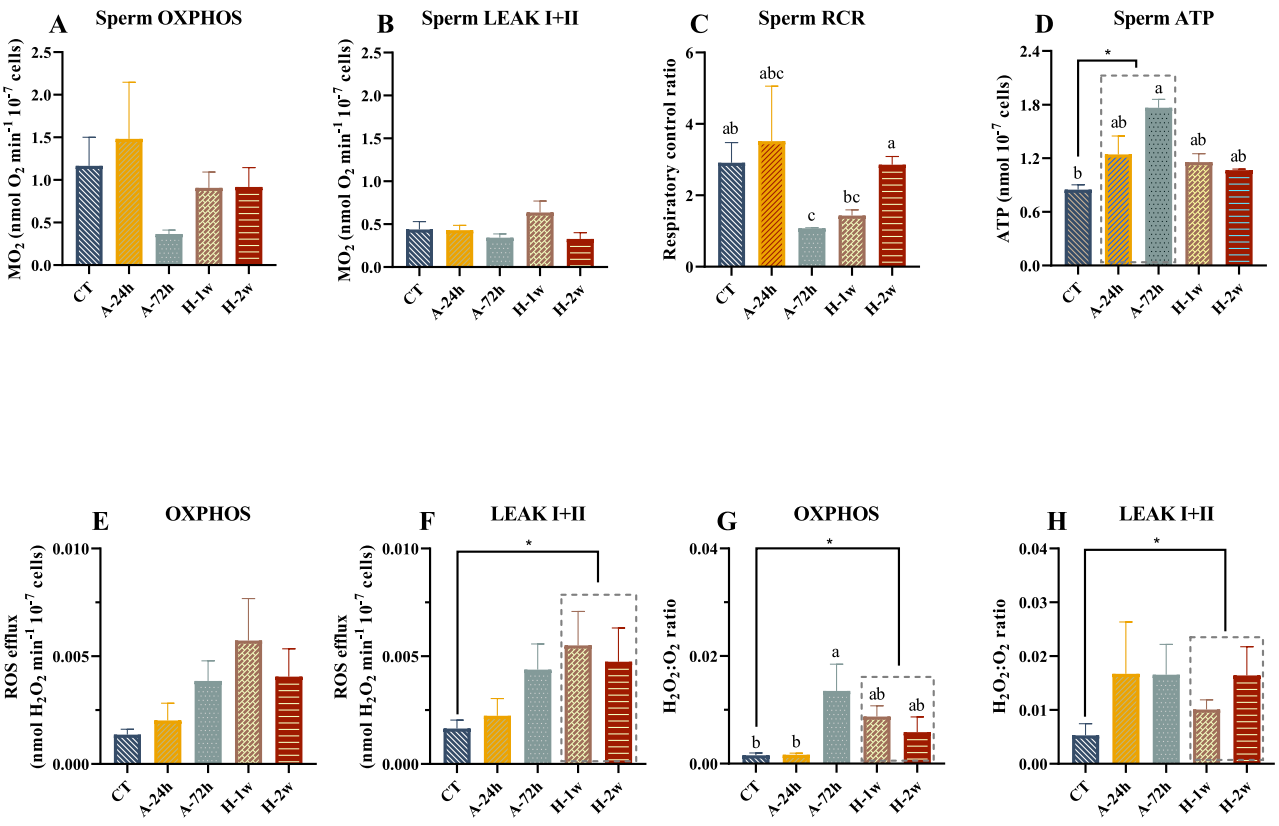

Fig. 6. Effects of parental exposure to oxygen regimes on the sperm mitochondrial functions and ATP contents of *M. edulis*.
A: OXPHOS respiration (nmol O_2 min^{-1} 10^{-7} cells) of the sperm mitochondria. B: LEAK I + II respiration (nmol O_2 min^{-1} 10^{-7} cells) of the sperm mitochondria. C: Respiratory control ratio (RCR) of the sperm mitochondria. D: Sperm ATP contents. E: ROS efflux (nmol H_2O_2 min^{-1} 10^{-7} cells) at OXPHOS state. F: ROS efflux (nmol H_2O_2 min^{-1} 10^{-7} cells) at LEAK I + II state. G: Fractional electron leak (FEL; H_2O_2:O_2 ratio) of the sperm mitochondria at OXPHOS state. H: FEL at LEAK I + II state. Different lowercase letters indicate the significant difference among five DO treatment groups ($p < 0.05$). Abbreviations for the treatment groups are as shown in Fig. 2. * represents the significant difference among control group, combined anoxia exposure groups (A-24 h and A-72 h, with the gray dashed box) and combined hypoxia exposure groups (H-1w and H-2w, with the gray dashed box) ($p < 0.05$). For sperm OXPHOS respiration, LEAK I + II respiration, RCR, and ROS efflux, $N = 7$ in CT, $N = 4$ in A-24 h, $N = 8$ in A-72 h, $N = 14$ in H-1w, and N = 8 in H-2w. For sperm ATP, $N = 3$. For FEL, N = 7 in CT, $N = 5$ in A-24 h, N = 8 in A-72 h, N = 14 in H-1w, and N = 8 in H-2w.

Strongylocentrotus droebachiensis (Siikavuopio et al., 2007) and in fish (Saha et al., 2022). Further studies are needed to determine whether prolonged hypoxia has similar effects on the gonad growth and development in *M. edulis*. However, regardless of the underlying physiological mechanisms, the combination of suppressed spawning rate, lower sperm endurance and slower swimming speed may lead to the loss of fecundity and impair the reproductive capacity of mussel populations in hypoxic coastal areas.

Exposure of males of *M. edulis* to anoxia and hypoxia had impact on the mitochondrial traits of the sperm. Unlike the sperm swimming endurance that was impaired by long-term hypoxia, the mitochondria coupling efficiency and fractional electron leak (reflective of the ratio of ROS production to O_2 consumption) showed the strongest effect of prolonged (72 h) anoxia exposure. The mitochondrial coupling efficiency (RCR) decreased in the sperm of males exposed to 72 h of anoxia, reflecting a decrease in the OXPHOS rate relative to the proton leak rate. Interestingly, despite a decline in OXPHOS rate and significantly lower coupling efficiency, the sperm ATP was the highest in the males exposed to 72 h of anoxia. This may indicate lower ATP breakdown rates in the sperm, possibly due to the metabolic rate suppression induced by prolonged anoxia (Cox and Gillis, 2020; Hochachka, 1986; Ivanina et al., 2010). Metabolic rate suppression in anoxia-tolerant organisms including bivalves involves downregulation of energy-demanding processes like protein synthesis and ion pumping (Sokolova, 2021; Sokolova et al., 2012), but is not associated with the loss of the mitochondrial capacity (Sokolova, 2018). Mitochondrial respiration quickly returns to normal levels (or even responds with an overshoot) during reoxygenation in marine bivalves (Adzigbli et al., 2024; Adzigbli et al., 2022), which, combined with the lower rates of ATP consumption, might explain high levels of ATP in the sperm of mussels pre-exposed to anoxia.

Mild uncoupling observed in the sperm mitochondria of the males exposed to hypoxia and anoxia might be a mechanism to counteract the electron leak in the mitochondrial ETS. Mitochondrial electron leak and consequently ROS production exponentially increase with increasing membrane potential and mild uncoupling caused by proton leak therefore mitigates ROS production (Divakaruni and Brand, 2011; Jastroch et al., 2010). Thus, mitochondria from the sperm of the males exposed to 72 h of anoxia showed a 7.7-fold increase in the OXPHOS state FEL and a 2.1-fold increase in the LEAK state FEL along with a strong decrease in the mitochondrial respiratory coupling (from 2.91 ± 0.57 to 1.08 ± 0.02). A 4.6-fold increase in the FEL of the OXPHOS state mitochondria in the males exposed to one week of hypoxia was associated with a moderate decrease of RCR down to 1.43 ± 0.16. The FEL of the LEAK state mitochondria of the sperm was generally higher than in the OXPHOS state and less responsive to hypoxia and anoxia exposure of the males. Interestingly, after two weeks of hypoxia exposure, the RCR and the OXPHOS state FEL of the sperm mitochondria returned to the near-control levels indicating acclimation of the sperm mitochondrial function after prolonged hypoxia exposure. These findings indicate that that the sperm metabolism of the Baltic Sea blue mussels is resilient to the hypoxia (~2 mg O_2 L^{-1}).

Egg size and fertilization success are critical determinants of

reproductive success in marine organisms (Arcand-Hoy and Benson, 1998; Fåhræus-Van Ree and Payne, 1997). Hypoxia has been shown to reduce the energy allocated to reproductive function and somatic growth in the common carp (*Cyprinus carpio*) (Zhou et al., 2001) as well as gonad size and development (Wu et al., 2003). In our study, one-week exposure to hypoxia resulted in a significant increase in egg size in female mussels, accompanied by a modest non-significant decrease in egg number. This suggests a potential shift in maternal investment strategy, with the production of fewer, larger eggs under hypoxic conditions. However, the correlation between egg size and fecundity was not statistically significant, as indicated by Spearman's correlation analysis. Since gametogenesis in female mussels typically begins in the autumn and winter and requires an extended period to complete (Fearman and Moltschaniwskyj, 2010), the short-term hypoxia exposure (one to two weeks) may not have been long enough to significantly alter the egg size-fecundity relationship. Furthermore, our findings indicate that parental exposure to hypoxia and anoxia did not affect fertilization success, likely due to the normoxic conditions during spawning and fertilization. This contrasts with findings from a study on the sea urchin *Paracentrotus lividus*, which reported decreased fertilization success under hypoxic conditions (Graham et al., 2016). These contrasting results highlight that the effects of hypoxia on fertilization success in marine invertebrates may be species-specific and dependent on the conditions during spawning and fertilization.

5. Conclusions, limitations and outlook

Our findings demonstrate that prolonged hypoxia (up to two weeks) significantly impacts reproductive processes in adult mussels, leading to notable reductions in spawning rate and alterations in gamete quality. Specifically, prolonged hypoxia slowed sperm velocity six hours post-spawning in males, while in females, it increased egg size at the expense of fecundity. Additionally, hypoxia exposure delayed the spawning response, reducing spawning rate and potentially affecting the synchronization of gamete release in mussels. Both hypoxia and anoxia altered sperm metabolism, increasing fractional electron leak and ROS production in sperm mitochondria. While severe anoxia significantly impaired sperm mitochondrial ATP synthesis capacity and coupling efficiency, the effects of hypoxia were mild and transient. Despite these changes, fertilization success in the gametes of mussels pre-exposed to hypoxia and anoxia remained unaffected. It is important to note that these results are based on a limited number of biological replicates, which should be taken into account when interpreting the findings. Furthermore, since fertilization trials were conducted under normoxic conditions, recovery of hypoxia- and anoxia-exposed gametes is possible.

Taken together, our study suggests that sustained hypoxia may impair spawning and gamete quality in mussels, potentially affecting the long-term viability of their populations, while short-term anoxia (up to 72 h) appears to have transient and less damaging effects. This study highlights the need for further research into the effects of long-term hypoxia on mussel reproduction, as well as the combined effects of other abiotic factors, such as warming, salinity fluctuations, and ocean acidification, which often coincide with hypoxia or anoxia in the Baltic Sea. Additionally, hypoxia exposure during critical periods of gametogenesis (autumn and winter) and its potential impact on regulatory processes, such as endocrine control of gonadal development and gametogenesis, require further investigation. Another promising area for research is the determination of the effects of chronic hypoxia, spanning from gametogenesis through fertilization and embryonic development, to better assess the long-term consequences of chronic hypoxia on the sustainability of mussel populations in the hypoxia-prone Baltic Sea environment.

CRediT authorship contribution statement

Hui Kong: Writing – original draft, Visualization, Validation, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Inna M. Sokolova:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2025.179103.

Data availability

Data will be made available on request.

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Salinity and temperature-driven variations in sperm performance and mitochondrial metabolism in North Sea and Baltic Sea mussels *Mytilus* spp.

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Running title: Salinity-temperature impact on *Mytilus* sperm

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Abstract: Climate change, including seawater warming and salinity fluctuations, is increasingly affecting marine ecosystems worldwide. The blue mussel *Mytilus edulis*, widely distributed along the temperate coasts of the Northern Hemisphere, thrives in environments characterized by temperature fluctuations and salinity gradients. In particular, populations in the Baltic and North Seas are exposed to significant variations in these factors, which can affect the reproductive capacity of blue mussels essential for sustainability of their populations. This study assessed the effects of varying temperatures and salinities on the reproductive performance of blue mussels from the Baltic and North Seas, focusing on sperm motility, ATP content, and fertilization success. Additionally, sperm mitochondrial function in Baltic Sea mussels was examined under different temperature and osmolarity conditions. Our results showed that mussels from both populations tolerated seawater warming, but were sensitive to cold and low salinity, with sperm motility and fertilization success significantly impaired under these conditions. The salinity window for sperm motility and fertilization was population-specific, with the North Sea mussels unable to reproduce at the ambient salinity of the Baltic Sea. High temperature reduced mitochondrial respiratory efficiency and increased reactive oxygen species (ROS) production, while osmolarity did not appear to be a key factor. These findings highlight population-specific reproductive traits in *M. edulis* and demonstrate the relationship between sperm motility, fertilization success, and mitochondrial function under varying environmental conditions providing new mechanistic insights into how coastal environmental changes affect the adaptation of benthic species.

Keywords: *Mytilus edulis*, sperm motility, mitochondrial function, ROS production, osmolarity, fertilization

1. Introduction

Reproduction is a fundamental biological process that ensures the continuity of species through the production of offspring (Fusco and Minelli, 2019). In marine bivalves, such as mussels, environmental factors like temperature and salinity play a crucial role in reproduction (Boroda et al., 2020; Lymbery et al., 2021; Tackett et al., 2024). As broadcast spawners, marine bivalves release their gametes directly into the seawater for external fertilization, making them particularly vulnerable to environmental stressors (Boni et al., 2016; Leite et al., 2024; Parker et al., 2009).

Climate change poses significant challenges to marine ecosystems, with rising seawater temperatures and increased frequency of marine heatwaves (Fernández et al., 2024; Frölicher et al., 2018; Hobday et al., 2016). In addition, extreme weather events lead to changes in seawater salinity, particularly in semi-enclosed seas like the Baltic Sea (Lehmann et al., 2022; Meier et al., 2022; Rutgersson et al., 2022). These temperature and salinity fluctuations have been shown to negatively affect the reproductive capacity of marine bivalves such as mussels and oysters (Eads et al., 2016; Gregory et al., 2023). For instance, increased temperature and reduced salinity have been linked to reduced sperm survival in *Crassostrea gigas* (Cheng et al., 2024), as well as decreased fertilization success in *C. gigas* exposed to hyposaline waters (Mooney, 2016). In mussels, *Mytilus galloprovincialis*, thermal stress has been associated with reduced sperm concentration, mitochondrial membrane potential collapse, increased lipid peroxidation, DNA fragmentation, and morphological abnormalities (Boni et al., 2016). Consequently, warming seawater and fluctuating salinity may disrupt the reproductive success of coastal bivalves, potentially leading to shifts in population dynamics and marine ecosystem structure.

The blue mussel *Mytilus edulis*, which is widely distributed along the northern Atlantic coasts, demonstrates significant physiological plasticity reflecting adaptations to variable environmental conditions (Larsson et al., 2017). The thermal tolerance window of adult *M. edulis* spans from 0 to 29°C with an optimum temperature around 20°C (Larsson et al., 2017; Seuront et al., 2019; Sorte et al., 2019), and both the North Sea

and Baltic Sea serve as natural habitats for the species, suggesting broad salinity tolerance (Kautsky, 1982; Sprung, 1983). However, low salinity limits local population structure and growth rates, particularly in the Baltic Sea, where populations exhibit smaller sizes, thinner shells, and slower growth compared to their North Sea counterparts (Kautsky et al., 1990; Westerbom et al., 2002). Despite these known physiological adaptations, little is understood about the reproductive strategies of these populations, particularly in relation to sperm performance and fertilization under varying salinity and temperature conditions.

In marine bivalves, oxidative phosphorylation (OXPHOS) in sperm provides the energy necessary for motility via aerobic ATP production in the mitochondria, a process that is highly oxygen-dependent (Kong and Sokolova, 2024). However, mitochondrial responses to environmental stressors, such as thermal stress, vary among male gametes of marine invertebrate species. For example, thermal stress in *M. galloprovincialis* induces a collapse in mitochondrial membrane potential of the sperm (Boni et al., 2016), while no such effect has been observed in the sea urchin *Heliocidaris tuberculata* that showed no correlation between sperm mitochondrial activity and fertilization success (Binet and Doyle, 2013). While climate change studies on sperm performance have largely focused on motility, viability, and fertilization success, little is known about how environmental stressors affect mitochondrial function in sperm and the relationship between mitochondrial activity and sperm performance.

This study examined how temperature and salinity affect the reproductive performance of *M. edulis*, comparing sperm motility, ATP content, fertilization success, and embryonic development between North Sea and Baltic Sea populations. We also assessed mitochondrial function in sperm from Baltic Sea mussels under different temperature and salinity conditions, measuring oxygen consumption rate (MO_2), reactive oxygen species (ROS) production, and fractional electron leak in active ATP synthesis (OXPHOS) and resting (LEAK) states. We hypothesize that warming and low salinity will reduce mitochondrial efficiency and increase ROS production in mussel sperm. Additionally, we expect sperm from Baltic Sea mussels to perform better in low salinity than those from the North Sea, as they are more adapted to brackish

conditions. Lastly, we propose that temperature and salinity fluctuations will significantly affect sperm performance, fertilization success, and embryonic development in both populations. This study aims to clarify how environmental variability impacts blue mussel reproduction, linking sperm metabolism, motility, and fertilization success to broader population resilience under climate change.

2. Materials and Methods

2.1 Animal collection and maintenance

Mussels *M. edulis* of the Baltic Sea population were collected from Warnemünde (54°10'49.6"N 12°05'21.5"E), Rostock, Germany in February 2023 and 2024. Mussels collection in 2023 corresponded to the temperature and salinity exposure for the North Sea mussels and the temperature exposure for the Baltic Sea mussels; while the mussel collection in 2024 was only based on the Baltic Sea mussels and corresponded to the salinity exposure and sperm mitochondrial experiments. The North Sea population mussels were collected from Sylt, Germany in March 2023. Adult mussels (4-6 cm) were immediately transported to laboratory at the University of Rostock. The mussels were kept at 10 °C for at least 2 weeks in two independent recycling aerated systems with a salinity of 30 (practical salinity units) for the North Sea mussels and a salinity of 15 for the Baltic Sea mussels. Each system had 12 flow-through tanks with a total volume of 570 L of seawater. To prepare the seawater, 20% natural seawater from Warnemünde was mixed with 80% artificial seawater (Tropic Marin® Pro-Reef, Tropic Marin AG, Switzerland) to achieve different salinity levels. The mixture was aerated for three days before use. A feeding device (Reefdoser EVO 4, Aqua Medic) continuously added microalgae mixture to the maintenance systems at a rate of 20 ml h^{-1}. The mixture consisted of live *Nannochloropsis* sp., live *Rhodomonas salina*, and a commercial shellfish diet (DT's Live Marine Phytoplankton, Coralsands, Germany) at a ratio of 1:1:1. Chlorophyll a concentration in seawater was routinely monitored to ensure sufficient food for mussels.

The two studied populations of *Mytilus* spp. possess a different genetic background. Specifically, the Warnemünde population (Baltic Sea) originates from a stable hybrid zone between *M. edulis* and *Mytilus trossulus*, with ~ 70% of nuclear markers and 100% of mitochondrial markers of *M. edulis* (Stuckas et al., 2017), and is thus classified as

natural hybrids of *M. edulis*. In contrast, the Sylt population (North Sea) is genetically pure *M. edulis*, according to prior population genetic assessments (Knöbel et al., 2020). It is important to note that all fertilization experiments in the present study were conducted using eggs and sperm of the mussels belonging to the same population.

2.2 Mussel spawning and fertilization

A thermal shock method was used to induce the spawning of mussels as previously described (Kong and Sokolova, 2024; Ramesh et al., 2017). Briefly, the adult mussels were placed individually in beakers filled with 250 ml of 0.2 μm filtered artificial seawater (FSW). Salinity of the FSW was 30 and 15 for the North Sea mussels and the Baltic Sea mussels, respectively. Temperature was gradually increased from 10 °C to 23 °C within 20 min on a water bath. Eggs and sperm were collected one hour after the start of spawning to ensure a complete release of gametes. The sperm concentration was determined with a hemocytometer (M-NZ-4CH, Kisker Biotech). Egg concentration was measured by first removing the adult mussels from the beaker containing 250 mL of seawater, where eggs had settled. The seawater was gently swirled to evenly distribute the eggs without causing mechanical stress, which could compromise egg quality. A 50 μL sample of this homogenized suspension was transferred into 950 μL of water in a 2 mL Eppendorf tube, achieving a 20-fold dilution. After mixing the diluted sample well, a 50 μL subsample was placed on a concave slide for microscopic examination (Olympus, CH3-TR45). Egg counting was performed thrice (n=3), with the concentration calculated as the average count multiplied by 400 (eggs/mL). The final egg concentration for sperm-egg ratio calculation in fertilization was the mean of these three measurements. For fertilization, active sperm and eggs were gently mixed at a 200:1 of the sperm: egg ratio and left undisturbed for 2 h (Bechmann et al., 2011; Pechenik et al., 1990; Tidau et al., 2023). Fertilization was conducted at approximately 2.5 h post-exposure. In the salinity experiment, fertilization for all treatments was carried out at 15°C. In contrast, in the temperature experiment, fertilization was performed at the respective exposure temperature of each treatment.

For each fertilization trial, eggs and sperm were collected from a single female and a single male, respectively, from the corresponding population (North Sea or Baltic Sea), to minimize inter-individual variability in gamete quality. The first polar body release and the zygote cleavage were used as markers of fertilization success (Sprung and

Bayne, 1984; Ventura et al., 2016). The fertilization success rate (FSR) was calculated as the percentage of fertilized eggs out of the total number of eggs used in the fertilization trials.

2.3 Experimental design

2.3.1 Experiment 1: Effects of temperature and salinity on the reproductive traits of the BS vs NS mussels

To examine the effects of temperature and salinity on reproductive performance, we assessed sperm motility and ATP content, fertilization success, and embryonic development in blue mussels *M. edulis* from the North Sea (NS) and Baltic Sea (BS). Fresh sperm and egg suspensions were collected one hour after spawning, induced by thermal shock, and divided into five aliquots (40 ml for sperm and 30 ml for eggs) before exposures to prepare the five temperature or salinity treatments. The spawning medium salinity was set to 15 for BS mussels and 30 for NS mussels. Temperature and salinity exposures were carried out in separate experimental runs.

In the salinity exposure experiment, sperm and eggs were subjected to one of five salinities spanning a habitat-specific salinity range. Salinity in each of the five aliquots of sperm or egg suspension was adjusted by gradually adding distilled water or sea salt (Tropic Marin® Pro-Reef, Tropic Marin AG, Switzerland) under constant gentle mixing, with five biological replicates for all salinity treatments (n=5 per sex and population). Gametes from the BS mussels were exposed to salinities of 5, 9, 13, 17, and 21, while those from the NS mussels were exposed to salinities of 9, 17, 21, 30, and 35 (Supplemental Figure. 2). The test salinity ranges were selected to reflect typical habitat salinities (9–15 for BS and 28–35 for NS populations) and laboratory acclimation salinities (15 for BS and 30 for NS mussels). To enable cross-population comparisons, three common salinities (9, 17, and 21) were included in the experimental design. Fertilization experiments were initiated 2.5 h after the target salinities were achieved. Sperm suspension was gently added to 30 ml of FSW containing eggs to achieve a sperm-to-egg ratio of 200:1, followed by thorough mixing. Fertilization success was assessed by counting zygotes, embryos, and unfertilized eggs in photographs taken under a microscope (Olympus, CH3-TR45) 2 h post-fertilization. The sample size for the fertilization experiments was 5, with each family derived from

gametes of different males and females. The final salinity of each experimental sample, including gamete suspensions and fertilization media, was measured using a multimeter (Hach, HQ40d) after exposure.

To evaluate the effect of salinity on sperm performance, sperm suspensions exposed to different salinities were maintained for 4 h post-spawning at 15 °C. Sperm performance metrics, including motility percentage, velocity curvilinear (VCL), and velocity average path (VAP), were assessed at the spawning salinity (15 and 30 for BS and NS populations, respectively) immediately prior to salinity adjustment (time 0) and after 1 and 4 h of salinity exposure. Sperm movement videos were recorded using a microscope (Olympus CH3-TR45) equipped with a camera connected to the software ToupView on a computer. Videos were captured at 30 frames per second for 60 seconds, with three replicate videos taken for each sperm suspension sample. Video analysis was performed using the computer-assisted sperm analysis (CASA) plugin for ImageJ (https://imagej.net/software/imagej/#publication), adapted for mussel sperm (Kong and Sokolova, 2024; Shi et al., 2017; Wilson-Leedy and Ingermann, 2007). Additionally, sperm ATP content was measured 3 hours post-exposure using the CellTiter-Glo® 2.0 cell viability assay kit (Promega, Germany) with a SpectraMax ID3 Multi-Mode Microplate Reader (Molecular Devices, USA), following the protocol previously described by Kong and Sokolova, 2024. Sperm samples from 5 males in each population were analyzed (n=5 for all treatments).

In the temperature exposure experiment, gametes (sperm or eggs) were collected from five individual mussels per sex for each population (n=5 per sex and population), except for the sperm samples from the Baltic Sea mussels (n=6). Each gamete sample was evenly divided into five aliquots, which were then exposed to five temperature conditions (5 °C, 10 °C, 15 °C, 20 °C, and 25 °C) in a water bath (Supplemental Figure. 2). Salinity was maintained at 15 for BS gametes and 30 for NS gametes across all temperature treatments. The temperature range was selected to represent environmental conditions from early spring, when occasional spawning might occur at low temperatures (around 5 °C), through the main reproductive season (April–June, 10–15 °C), and including elevated temperatures (20–25 °C) associated with spring and summer heat waves. In the Baltic Sea, seawater salinity generally remains low and

stable throughout the year, while temperatures increase gradually from early spring (~ 5 °C) to late spring/early summer (~ 15°C), with occasional heatwaves reaching up to ~ 25 °C (Naumann et al., 2018; Rutgersson et al., 2022). In contrast, the North Sea shows higher baseline salinities with distinct seasonal temperature dynamics and occasional heatwaves. These temporal dynamics were considered when selecting the experimental temperature range to reflect both gradual seasonal changes and episodic extreme events.

After 4 h of exposure to the respective temperatures, the same endpoints—sperm motility, ATP content, fertilization success, and embryonic development—were measured as in the salinity exposure experiment. The detailed timing and sequence of these measurements are illustrated in Supplemental Figure 1. The temperature of each treatment, for both sperm suspension and fertilization samples, was controlled using automated water baths (Thermo Haake K10, Thermo Haake SC100, Thermo Haake D1, Biometra KH-3, and Fisherbrand FBC620). Temperatures were recorded with a digital thermometer (EBI 20-TE1) before and after the exposure to ensure accuracy.

The temperature and salinity conditions for all experimental exposures are shown in Table 1.

2.3.2 Experiment 2: Combined effects of temperature and osmolarity on the sperm mitochondrial function

Mitochondrial functions in sperm from Baltic Sea males were assessed using a high-resolution respirometer (Oxygraph 2-k, Oroboros Instruments, Innsbruck, Austria) equipped with integrated software (DatLab 6). Sperm suspension samples (50 ml) were collected from individual males one hour post-spawning and centrifuged at 8000 × g for 2 min. The resulting sperm pellet was resuspended in 500 µl of ice-cold homogenization buffer (30 mM HEPES pH 7.5, 100 mM sucrose, 200 mM KCl, 100 mM NaCl, 8 mM EGTA, 30 mM taurine) supplemented with freshly added protease inhibitors (1 mM phenylmethanesulfonyl fluoride and 2 µg ml^{-1} aprotinin). The sperm suspension was injected (200 µl per chamber) into the temperature-controlled chambers of the Oxygraph 2-k for measurements. Sperm concentrations of both raw and resuspended samples were determined using a hemocytometer (Supplemental Table 1).

Sperm mitochondrial function was assessed across three temperatures (15 °C, 20 °C, and 25 °C) and five osmolarities (630, 510, 390, 270, and 150 mOsm) that mimic seawater osmolarities at salinities of 21, 17, 13, 9, and 5, respectively (Santos et al., 2013; Thurman et al., 2013). A fully crossed temperature × osmolarity design was used (n=5−8 per treatment; Supplemental Figure. 2), with assay buffer compositions adjusted for each osmolarity (Supplemental Table 2).

Calibrations for oxygen and ROS sensors were conducted following established protocols (Ouillon et al., 2021; Steffen et al., 2023). ROS efflux was measured using the Fluorescence-Sensor Green (525 nm) integrated with the Oxygraph 2-k. Hydrogen peroxide (H_2O_2) production was quantified using 10 µmol l^{-1} Amplex™ UltraRed (AMR) as a reporter, 1 U ml^{-1} horseradish peroxidase to catalyze H_2O_2-dependent AMR conversion, and 5 U ml^{-1} superoxide dismutase to convert superoxide radicals to H_2O_2. Fluorometric sensors were calibrated with 0.2 µmol l^{-1} H_2O_2 prior to measurements.

For each measurement, 200 µl of sperm suspension was injected into the 2 ml chambers following calibration. Digitonin (10 µg ml^{-1}) was added to permeabilize sperm membranes, and real-time monitoring of oxygen consumption and ROS efflux was performed. Mitochondrial respiration and ROS efflux were evaluated in permeabilized sperm by measuring these parameters in the LEAK state (state 2) after adding 5 mM pyruvate, 1 mM malate, and 10 mM succinate to stimulate electron flux through Complexes I and II, and in the OXPHOS state (state 3) following the addition of 3.75 mM ADP to activate F_o,F_1-ATP synthase. Respiration rate in the LEAK state indicate the oxygen consumption required to maintain mitochondrial membrane potential without ATP production, reflecting the cost of compensating for futile proton and cation cycling (Jastroch et al., 2010; Sokolova, 2023). OXPHOS state (state 3) respiration served as a proxy for the maximum ATP synthesis capacity of mitochondria (Sokolova, 2023). The respiratory control ratio (RCR), a measure of mitochondrial coupling efficiency, was calculated as the ratio of OXPHOS to LEAK respiration (state 3 to state 2) (Estabrook, 1967). Fractional electron leak (FEL) was determined as the ratio of H_2O_2 efflux to oxygen consumption ($\dot{M}O_2$) during both LEAK and OXPHOS states, based on simultaneous measurements of mitochondrial oxygen consumption and ROS efflux.

2.4 Statistical analysis

SPSS version 23.0 (IBM Corp., Armonk, NY, USA) was used for statistical analysis, and GraphPad Prism version 8.3 (GraphPad Software Inc., La Jolla, CA, USA) was used to generate figures. Shapiro-Wilk's test was applied to assess the normality of data distribution, and Levene's test was used to evaluate homogeneity of variance. Percentage data were arcsine square-root transformed to meet assumptions of normality and homogeneity where required. For Experiment 1, two-way repeated measures ANOVA was conducted to examine interactions between temperature or salinity and exposure time on response variables, including sperm motility rate, VCL, and VAP. The effects of temperature or salinity were further analyzed using one-way ANOVA followed by Tukey's post hoc test for normally distributed data, or the Kruskal-Wallis test for non-normally distributed data or datasets violating homogeneity of variance. The effects of exposure time were analyzed using one-way repeated measures ANOVA for normally distributed data or Friedman's ANOVA for non-normally distributed data.

For Experiment 2, one-way ANOVA followed by Tukey's post hoc test was used for normally distributed data, while the Kruskal-Wallis test was applied for non-normally distributed data. Welch's ANOVA followed by Games-Howell post hoc test was used for normally distributed data with unequal variances. Two-way ANOVA was not performed due to non-normal distribution in parts of the dataset. Pearson's and Spearman's correlation were conducted to evaluate relationships among sperm motility, ATP, fertilization success, and mitochondrial functions, based on normality of the variables. All data are presented as mean ± standard error of the mean (SEM). Statistical significance was set at $p<0.05$.

3. Results

3.1 Effects of temperature or salinity on the reproduction of mussels, the Baltic Sea vs the North Sea

3.1.1 Sperm motility

The percentage motility of sperm from the BS population remained high and stable in relatively high-salinity seawater environments (salinity 13, 17, and 21; approximately

80% motile sperm), compared to low-salinity seawater environments (salinity 5, with only 26.5 ± 6.7% and 23.5 ± 2.7% motile sperm at 1 h and 4 h post-exposure, respectively, significantly lower than in the other treatments). At salinity 9 (close to the average habitat salinity of the BS population), 74.1 ± 3.9% of BS sperm were motile. A significant decrease of sperm motility was observed in all salinity treatments, except for salinity 5, after 4 h post-exposure compared to 1 h (Fig. 1A).

The sperm motility percentage in the NS population was slightly lower than in the BS population immediately after spawning (time 0), with 82.6 ± 3.1% motile sperm in NS and 89.2 ± 3.3% in BS (Fig. 1A, 1B). Sperm from the NS population showed much less tolerance to low-salinity environments. Only 11.2 ± 1.1% and 11.2 ± 1.9% of sperm were motile at 1 h and 4 h post-exposure to salinity 9, respectively. At salinity 17, 40.7 ± 13.1% of NS sperm were active after 1 h exposure, compared with 83.1 ± 4.6% in the BS population. Additionally, 64.1 ± 8.5% of NS sperm were motile at 1 h post-exposure in salinity 21, but this decreased significantly to 42.1 ± 12.4% after 4 h. The optimal salinity for NS mussel sperm motility was found at 30, where 78.4 ± 5.0% of sperm were motile after 1 h, and no significant reduction was observed after 4 h. In contrast, at salinity 35, the sperm motility significantly decreased after 4 h, with 77.1 ± 2.4% motile at 1 h post-exposure dropping to 54.0 ± 10.7% after 4 h (Fig. 1B).

Sperm from both populations showed tolerance to temperatures ranging from 5°C to 20°C, maintaining high motility percentages during the first hour after spawning. However, at 25°C, a decrease in sperm motility of the BS population was observed at this time point (Fig. 1C). After 4 h of exposure, sperm motility percentages declined in all temperature conditions, except for the NS population at 5°C (Fig. 1C, D). The BS sperm appeared to be less tolerant to extreme temperatures compared to that of the NS population. In the BS population, sperm motility decreased after 4 h at 5°C, while it remained stable in the NS population. After 4 h at 25°C, sperm motility was reduced to 18.1 ± 4.2% in the NS population and 14.9 ± 1.2% in the BS population (Fig. 1C, D). Besides, significant interactions between exposure time and temperature were observed in the motility rate of sperm from both BS and NS populations (Supplemental Table 4).

3.1.2 Sperm velocities and ATP content

The sperm VCL of BS mussels remained relatively stable after 1 h in the salinity range of 9-21, with a significant decline observed at salinity 5. The highest sperm VCL values (316-337 μm/s) were recorded at salinities 13 and 17 in the BS population. In contrast, the average VCL in other treatments remained below 300 μm/s, with the lowest values observed at salinity 5 (151.2 ± 16.8 μm/s). Furthermore, after 4 h, VCL in BS sperm decreased at salinities greater than 5 (with significant reductions at salinities 9, 13, and 21) compared to 1 h post-exposure (Fig. 2A). At salinity 5, sperm VCL remained at low levels, similar to 1 h post-exposure. In NS mussels, sperm VCL remained robust across all salinity treatments (9-35) after 1 h of exposure. However, after 4 h, VCL significantly decreased in the salinities 9, 17, and 35 treatments, with VCL in the salinity 30 treatment being significantly higher than in both the 9 and 17 treatments (Fig. 2B).

Sperm VCL in both the BS and NS populations was resilient to seawater temperature variations. Only after 4 h at 25°C did VCL of the BS sperm significantly decrease compared to the 10°C treatment. Over time, VCL in all five temperature treatments slowed in the NS population, while in the BS population, significant effects of exposure duration were observed at 10, 15, and 25°C (Fig. 2C, D). However, no significant interaction was observed between exposure time and salinity/temperature on sperm VCL in both BS and NS populations (Supplemental Table 4).

Sperm VAP in the BS population decreased at salinity 5 at both time points (1 h and 4 h), but remained relatively stable at salinities 9-21, with a modest but statistically significant decline observed between 1 h and 4 h of exposure (Fig. 3A). In NS mussels, VAP of the sperm decreased only at salinity 9 after 4 h post-exposure, while remaining stable across all other salinities after 1 h and across salinities 17-35 after 4 h (Fig. 3B).

In both BS and NS mussels, sperm VAP showed no significant effect of temperature after 1 h of exposure. In BS sperm, VAP decreased after 4 h of exposure to 25°C compared with 5-20°C (Fig. 3C). In NS mussels, no effect of temperature on sperm VAP was found after 4 h (Fig. 3D). No significant interaction was observed between exposure time and salinity/temperature on sperm VAP (Supplemental Table 4).

No significant difference was observed in the ATP content of sperm across different salinity or temperature treatments for both the BS and NS populations (Fig. 4).

3.1.3 Fertilization success and embryonic development

The optimal salinity range for fertilization in the BS mussel population spanned from salinity 13 to salinity 17, with fertilization success rates (FSR) of 84.7 ± 3.0% and 83.3 ± 3.2%, respectively. Outside this optimal range, FSR decreased as seawater salinity increased or decreased. Notably, exposure to salinity 21 and salinity 5 resulted in a sharp decline in FSR, with values of 64.8 ± 6.6% and 6.2 ± 1.6%, respectively (Fig. 5A). In contrast, the optimal salinity range for fertilization in the NS mussel population was between salinity 21 and 30, yielding FSR values of 94.0 ± 1.8% and 95.9 ± 1.4%, respectively. These values were significantly higher than the FSR observed at salinity 9 (2.5 ± 1.6%) (Fig. 5B). This contrasts sharply with the FSR in the BS population, where FSR was 68.8 ± 4.4% at salinity 9 (Fig. 5A).

Fertilization in both mussel populations showed resilience to elevated temperatures but was notably reduced in cold seawater conditions. FSR remained above 80% across the temperature range of 15-25°C, with a sharp decline to 24-30% at 10°C (Fig. 5C, D). The lowest FSR, falling below 10% in the BS population and below 20% in the NS population, was observed at 5°C (Fig. 5C, D).

The fertilization success in the temperature experiments for both mussel populations was not correlated with sperm motility traits, including motility rate, VCL, VAP, and ATP content. However, in the salinity experiments for both populations, fertilization success was significantly correlated with sperm motility rate and VCL (also including VAP for BS population; $p<0.05$ for all), although no correlation was observed with ATP content (Table 2).

Compared to salinity 5, where most eggs remained unfertilized, high percentage of embryos reached the four-cell and eight-cell stage after 2 h at salinities 13-17 in the BS mussel population, reaching 35.6 ± 7.9% and 10.0 ± 3.7% in the salinity 13, and 33.5 ± 7.1% and 10.9 ± 4.5% in the salinity 17 (Fig. 6A, Supplemental Table 3). Embryonic development was slightly slower at salinities 9 and 21 compared to 13-17, though the

differences were not statistically significant. In contrast, for the NS mussel population, the percentage of embryos in the four-cell and eight-cell stages was highest at salinities 30-35, significantly exceeding those at salinity 9 (Fig. 6B, Supplemental Table 3).

Unlike the BS population, where fertilization and embryonic development were stalled at 5°C, in the NS population, a small fraction of fertilized eggs successfully progressed to the two- and four-cell stages (8.5 ± 6.9% and 1.0 ± 1.0%, respectively) after 2 h post-fertilization. A significant and abrupt increase in the percentage of eight-cell stage embryos was observed in the BS mussel population as seawater temperature rose from 15°C to 20°C, increasing from 4.02 ± 1.95% to 39.01 ± 6.01%. Concurrently, the percentage of four-cell stage embryos decreased from 65.20 ± 5.06% to 27.56 ± 4.34%, indicating that more than 50% of the four-cell stage embryos successfully progressed to the eight-cell stage. Additionally, the percentage of eight-cell stage embryos in the 20°C and 25°C treatments was significantly higher than in the 5°C and 10°C treatments (Fig. 6C, Supplemental Table 3). Similarly, in the NS population, embryos displayed faster development under warmer seawater conditions. The percentage of eight-cell stage embryos in the 25°C treatment was significantly higher than in the 5°C treatment (21.55 ± 5.46% vs 0). The highest percentage of four-cell stage embryos was observed in the 15°C treatment, at 46.33 ± 9.95%, compared with 1.03 ± 1.03% in the 5°C treatment (Fig. 6D, Supplemental Table 3). In both populations, embryos generally showed optimal development in warmer seawater temperatures (15°C - 25°C).

3.2 Combined effects of temperature and osmolarity on sperm mitochondrial function of mussels from the Baltic Sea

Sperm OXPHOS respiration was significantly reduced at 25°C when combined with low osmolarity (0.158 ± 0.036 and 0.182 ± 0.049 nmol O2 min^{-1} 10^{-7} cells at 270 mOsm and 150 mOsm, corresponding to salinity 9 and 5) relative to the values measured at 15°C (0.505 ± 0.080 and 0.854 ± 0.129 nmol O2 min^{-1} 10^{-7} cells at 270 mOsm and 150 mOsm, respectively). Notably, significant effects of osmolarity on sperm OXPHOS respiration were observed only at 25°C, where respiration in the 630 mOsm treatment was significantly higher than in the 270 mOsm and 150 mOsm treatments (Fig. 7A). In contrast, no significant differences in LEAK state respiration was found in the BS

sperm mitochondrial across temperature and osmolarity treatments (Fig. 7B). The respiratory control ratio (RCR) of sperm mitochondria showed a general decline with increasing temperature (Fig. 7C). While osmolarity had minimal impact on RCR at 15°C and 20°C, it became a significant determinant at 25°C. The combined effects of elevated temperature (25°C) and low osmolarity (270 mOsm and 150 mOsm) produced RCR values close to 1, indicative of a loss of mitochondrial coupling (Fig. 7C).

The ROS efflux of sperm mitochondria in the OXPHOS state showed an increasing trend with rising temperatures in higher osmolarity treatments (390–630 mOsm). In contrast, at lower osmolarities (150–270 mOsm), ROS efflux peaked at 20°C and showed a decreasing trend at 25°C, although the difference was not statistically significant (Fig. 8A). Similarly, in the OXPHOS state, FEL remained consistently low in the 630 mOsm treatment across all tested temperatures. A significant increase in FEL with warming was observed at 150 and 510 mOsm, whereas no significant temperature-dependent changes were found at 270 and 390 mOsm (Fig. 8C). In the LEAK state, ROS efflux remained stable in mitochondria exposed to 630 mOsm, and at this osmolarity level, FEL showed a decreasing trend with increasing temperature, although this change was not statistically significant (Fig. 8B, D). In the LEAK state, mitochondria exposed to 510 mOsm showed a significant increase in ROS efflux with warming (Fig. 8B), whereas at 270 mOsm, ROS efflux peaked at 20°C and declined significantly at 25°C. FEL in the LEAK state showed a similar pattern at lower osmolarities (150–510 mOsm), increasing with temperature and peaking at either 20°C (270, 510 mOsm) or 25°C (150, 390 mOsm).

Correlational analysis revealed that sperm mitochondrial respiration at the OXPHOS and LEAK states were strongly correlated ($p < 0.01$), while RCR showed a significant correlation with mitochondrial respiration at the OXPHOS state but not the LEAK state. FEL at the OXPHOS state was positively correlated with ROS efflux and FEL-LEAK, while negatively correlated with mitochondrial respiration and RCR. FEL at the LEAK state exhibited similar significant correlations with FEL-OXPHOS, except for RCR. ROS efflux at the OXPHOS and LEAK states were significantly correlated with each other but showed no significant correlations with other traits (Table 3).

4. Discussion

The performance of gametes under varying environmental conditions is crucial for understanding the reproductive success of marine invertebrates, particularly broadcast spawners like mussels, whose gametes are directly exposed to seawater during external fertilization. Climate change introduces multiple stressors, such as temperature and salinity variations, which can significantly influence gamete performance. These factors are particularly relevant in light of gametes' sensitivity to environmental conditions (Boni et al., 2016; Cuccaro et al., 2022; Gallo et al., 2020; Tackett et al., 2024). Although previous research has highlighted the critical role of gamete quality in fertilization success (Budhwar et al., 2017; Gallo et al., 2020; Lewis and Ford, 2012), the specific effects of abiotic stressors on male fertility, including sperm metabolism and performance, remained underexplored. Our study demonstrated that temperature and salinity variations significantly influence the gamete performance of *M. edulis* populations from the North Sea and the Baltic Sea. Additionally, population-specific differences in tolerance to these environmental factors were observed, highlighting the adaptations of these populations to the differing salinity conditions in their respective habitats.

4.1 Population-specific effects of salinity on sperm performance and fertilization

Our study demonstrated that adaptation to distinct salinity regimes in the maritime North Sea population and the brackish Baltic Sea population is reflected in differing optimal salinity ranges for sperm performance, fertilization success, and embryonic and larval development in *M. edulis*. *M. edulis* sperm from the brackish Baltic Sea (BS) population was characterized by a broad salinity window for optimal motility and velocity parameters (VCL and VAP), which remain stable between salinities of 9 and 21, with a significant decline observed at salinity 5. Despite this decline in motility, key metabolic parameters, including oxidative phosphorylation (OXPHOS), resting LEAK respiration, and mitochondrial coupling, remained stable across salinities 5–21 at a control temperature of 15°C. Additionally, reactive oxygen species (ROS) levels and fractional electron leak were unaffected, indicating that the decrease in sperm performance at salinity 5 is not attributable to disruptions in aerobic metabolism or oxidative stress. Furthermore, ATP content in BS sperm was stable across all salinities

tested, suggesting that the observed decline in motility at salinity 5 is likely mediated by mechanisms unrelated to energy depletion. Similar results were observed in the Eastern oyster (*Crassostrea virginica*) sperm motility, the low salinity of 10 significantly reduced sperm motility and progressive movement, while ATP levels remained unaffected (Tackett et al., 2024). This could be explained by the impairment of sperm motility signaling under low salinity, which is upstream of ATP-dependent sperm motility (Boulais et al., 2019). Additionally, low salinity inhibited sperm motility of *C. virginica* by disrupting ion homeostasis (K^+, Ca^{2+}, Na^+) and altering osmotic pressure (Nichols et al., 2021), while Na^+ and Ca^{2+} were involved in the regulation of [pH]i and activation of axonemal proteins, respectively (Alavi et al., 2014; Boulais et al., 2018). These factors collectively impair sperm activation and reduce motility efficiency. Further investigation is needed to determine the possible mechanisms underlying the observed salinity-dependent variation in sperm performance unrelated to energy metabolism.

The fertilization success rate of the Baltic Sea *M. edulis* population also showed a relatively broad tolerance range between salinities 9 and 21, though a declining trend was observed at the extremes of this range. Embryonic development progressed most rapidly at salinities 13–17, while development at salinities 9 and 21 was notably slower. These findings suggest that the optimal salinity window for external fertilization and embryonic development in this brackish population is 13–17. In contrast, salinity 5 led to extremely low fertilization success, with embryonic development either stalling or being substantially delayed at the first polar body stage, identifying salinity 5 as a critical threshold at which reproduction is severely impaired. This threshold aligns with the critical salinity for marine macrozoobenthos initially proposed by Remane and highlights the physiological constraints imposed by low salinity environments on the reproductive success of *M. edulis* populations in brackish ecosystems (Telesh et al., 2011).

The sperm of the North Sea (NS) *M. edulis* population showed an optimal performance window shifted toward higher salinities (30–35) compared to the BS population, with a gradual decline in sperm performance observed between salinities 21 and 9. Among the measured parameters, sperm motility percentage showed the strongest salinity-

dependent variation, gradually declining below salinity 30 within 1 h of exposure. In contrast, declines in velocity parameters (VCL and VAP) were only observed after 4 h of exposure to salinities below 21. Fertilization success in the NS population was high within the salinity range of 17–35, with modest reductions at the extremes (17 and 35) compared to the intermediate range (21–30). However, embryonic development at salinity 17 was delayed, indicating that reproductive success diminishes outside the optimal salinity range of 21–35. At salinity 9, fertilization success was severely disrupted, with only 2.48 ± 1.62% of eggs fertilized, and development stalled at the first polar body stage. These results suggest that salinity 9 falls below the reproductive adaptation threshold for the NS population, highlighting significant differences in salinity tolerance and reproductive performance between the NS and BS populations.

In both studied populations, salinity-dependent variation in fertilization success was strongly and positively correlated with sperm motility percentage and velocity parameters (VCL and VAP). While sperm motility percentage and velocity were also strongly correlated with each other, sperm motility percentage emerged as the strongest predictor of fertilization success, explaining 85% of the variation in fertilization rates in the BS population and 66% in the NS population. Notably, sperm ATP content showed no correlation with either sperm motility or fertilization success across different salinity exposures in both populations. This finding supports the hypothesis that salinity-induced disruptions in sperm motility, likely driven by osmotic or ionic stress, rather than energy deficiency, underlie the observed variation in sperm performance. These results also show that the salinity-induced limitations on the reproductive success of *M. edulis* are predominantly dependent on sperm motility and its associated performance metrics.

The pronounced differentiation in sperm and embryo salinity tolerance between the studied *M. edulis* populations is consistent with previous reports of genetic and phenotypic differentiation between the mussel populations from the Baltic and North Seas, encompassing variations in morphology, growth, metabolism, and genetic composition (Johannesson et al., 1990; Kautsky et al., 1990; Koehn and Gaffney, 1984; Rodhouse et al., 1986; Varvio et al., 1988). However, these differences may not only reflect local adaptation but also genetic background differences, as the Baltic

555 population is part of a known hybrid zone (*M. edulis* × *M. trossulus*) with the mussels
556 showing ~70% of nuclear markers and 100% of mitochondrial markers of *M. edulis*
557 (Stuckas et al., 2017), while the North Sea population is genetically pure *M. edulis*
558 (Knöbel et al., 2020). Sperm motility percentage emerged as highly sensitive to low
559 salinities, with critical thresholds identified at salinity 5 for the BS population and
560 salinity 9 for the NS population, directly influencing fertilization success. Embryonic
561 development of *M. edulis* was also salinity-dependent, and integrating the sperm
562 performance and embryonic development parameters indicates optimal salinity ranges
563 for reproduction of 13–17 in the BS population and 21–35 in the NS population. The
564 observed differences in optimal salinity ranges between the two populations may also
565 reflect long-term local adaptation to distinct environmental salinity regimes. Baltic Sea
566 mussels experience consistently low and fluctuating salinity, leading to enhanced
567 osmoregulatory capacities and reproductive performance at lower salinities. The North
568 Sea mussels are adapted to stable, high-salinity conditions and thus show reduced
569 performance under hyposaline stress.

570 **4.2 Effects of temperature on sperm performance and fertilization**

571 The temperature dependence of sperm performance revealed similar temperature
572 tolerances for *M. edulis* sperm from the Baltic and North Sea populations, which may
573 be due to the shared seasonal temperature fluctuations (5–15°C) they experience during
574 their reproductive season. The selected experimental temperatures thus realistically
575 represent both gradual seasonal warming and occasional heatwaves, which may exert
576 comparable thermal selection pressures on both populations. Sperm from both
577 populations showed resilience to seawater temperature variations within the range of
578 5–20°C, while the sperm performance was significantly impaired at an elevated
579 temperature of 25°C. The lowest tested temperature (5°C) is below the threshold for
580 spawning induction in field populations of *M. edulis*. The natural spawning events
581 typically occur in late spring, when seawater temperature reaches from 9.5°C to 12.5°C
582 (Chipperfield, 1953). Nevertheless, our results indicate that at 5°C the mussel sperm
583 maintain activity levels comparable to those observed within the optimal spawning
584 temperature range (10–15°C) for this species. The mechanisms underlying this cold

tolerance remain unclear; however, it may serve as a protective trait to preserve sperm function during occasional cold spells in spring.

The delay in warming-induced sperm impairment may account for the absence of a significant effect of elevated temperature (25°C) on fertilization success when compared to the typical spawning temperature range (10–15°C) or moderate warming (20°C). Conversely, fertilization success at 2 h post-fertilization was markedly reduced at 5°C, despite high sperm motility and velocity under these conditions. This apparent mismatch between temperature effects on sperm activity and fertilization success is further reflected in the lack of a significant correlation between sperm motility rate and fertilization success, in contrast to the clear relationship observed in salinity variation treatments. These findings suggest that, in the context of temperature, the reduction in fertilization success is likely attributable to the disruption of fertilization-specific processes such as the induction of the acrosomal reaction, sperm-egg binding and fusion, or egg activation (Hirohashi et al., 2008), rather than a decline in sperm quality. Similar results were reported in the sea urchin *Anthocidaris crassispina*, where low temperatures (0°-10°C) significantly reduced the number of sperm bound to the egg coat and the rate of the acrosome reaction (Mita et al., 1984). Additionally, it should be considered that in ectotherms such as mussels, low temperature can slow down fertilization and embryonic development. Thus, the apparent arrest of development observed at 5°C after the 2-hour fertilization period could reflect a temperature-dependent decrease in the cell division rate rather than complete inhibition. Nevertheless, it is worth noting that the Baltic Sea population was more sensitive to the cold-induced delay in the onset of cell division than the North Sea one.

Notably, the embryonic development generally slows under low temperature conditions due to the reduced metabolism and enzyme activity, which delays cell divisions. For instance, in the basket cockle *Clinocardium nuttallii*, the early embryogenesis was significantly delayed under low temperature conditions, with cleavage stages reached much later than at optimal temperatures (Liu et al., 2008). Similarly, the sea urchin *Arbacia dufresnii* shows markedly delayed cleavage and development under low temperature (12°C vs 17°C) (Fernández et al., 2021). These findings align with our observations in *M. edulis* mussels and support the view that the apparent arrest of

embryonic development at 5 °C may reflect a pronounced temperature-dependent delay in early cleavage rather than a true developmental inhibition.

4.3 Does mitochondrial impairment contribute to salinity- and temperature-induced decrease in sperm quality?

Mitochondria play a key role in sperm activity, primarily through ATP generation via oxidative phosphorylation (OXPHOS), which is strictly oxygen-dependent (Kong and Sokolova, 2024). This link between mitochondrial aerobic respiration and ATP production is crucial for sperm function. Activity of key mitochondrial enzymes, such as citrate synthase and succinate dehydrogenase (Complex II), have been linked to sperm performance (Ruiz-Pesini et al., 1998; Ruiz-Pesini et al., 2000). Furthermore, mitochondrial respiration, measured by oxygen consumption, and respiratory efficiency are strongly correlated with sperm motility (Stendardi et al., 2011). Inhibition of the electron transport system (ETS) with agents such as rotenone and oligomycin has been shown to negatively affect mussel sperm motility (Kong and Sokolova, 2024).

Environmental stressors such as temperature and osmotic changes can impair mitochondrial respiration, coupling efficiency, and ATP synthesis in marine ectotherms (Bal et al., 2021; Ballantyne and Moyes, 1987; Heise et al., 2003; Ivanina et al., 2012; Poertner et al., 1999; Sokolova, 2018), which may contribute to reduced sperm performance. Stress can also elevate reactive oxygen species (ROS) production, as electron leakage from the ETS generates ROS. Excessive ROS can lead to oxidative damage, such as lipid peroxidation, impairing sperm function (Aitken, 2017; Dutta et al., 2020; Fernández et al., 2024). Moreover, ROS production has been shown to negatively correlate with sperm morphology and motility (Aziz et al., 2004; Gallo et al., 2021). While sperm motility and mitochondrial metabolism have been studied in various marine invertebrates under stress (Binet et al., 2014; Gallo et al., 2018; Schlegel et al., 2015), the relationship between mitochondrial function and sperm motility remains underexplored. Our investigation of the combined effects of temperature and osmolarity on mitochondrial function in *M. edulis* sperm suggests that mitochondrial dysfunction alone does not fully account for the decline in sperm performance observed at elevated temperatures or reduced salinities. In Baltic mussels (BS), the decrease in sperm performance at low salinities (5) was not linked to disruptions in mitochondrial

metabolism. BS sperm mitochondria displayed high osmotic tolerance, maintaining stable OXPHOS rates and low ROS production across a wide range of salinities. While mitochondrial performance in NS sperm under different salinities was not assessed, studies of somatic tissues in *M. edulis* indicate that mitochondria in NS mussels also exhibit broad osmotic tolerance (160–1200 mOsm) (Wiesenthal et al., 2025). Consequently, the observed decline in sperm motility at low salinities (5 in BS and 9 in NS mussels) is likely due to the limited osmoregulatory capacity typical of marine osmoconformers, rather than oxidative stress or mitochondrial dysfunction.

Elevated temperature reduced mitochondrial ATP synthesis capacity in mussel sperm, as indicated by lower OXPHOS rates at 25°C compared to 15°C, along with a decline in coupling efficiency. However, this decline was only observed when warming was combined with reduced salinity (5–9). Despite these mitochondrial disruptions, sperm ATP levels remained unaffected, suggesting that the observed decline in motility and velocity after 4 h at 25°C was not due to an energy deficit. This implies that mitochondrial capacity was sufficient to sustain ATP production even under suboptimal temperature conditions. At 25°C, mitochondrial impairment was further indicated by increased fractional electron leak at salinities of 5–17, which led to higher relative ROS production compared to O_2 consumption. While excessive ROS production has been shown to cause oxidative damage in spermatozoa (e.g., lipid peroxidation), impairing sperm function (Aitken, 2017; Dutta et al., 2020; Fernández et al., 2024), our data did not provide direct evidence linking ROS efflux to impaired sperm motility. However, mitochondrial results showed that both sperm mitochondrial ROS efflux and fractional electron leak increased with temperature, suggesting that elevated temperature enhances electron leakage, potentially pushing it closer to the threshold for oxidative damage in sperm.

Overall, our findings demonstrate that the observed reductions in sperm motility, velocity, and fertilization success of the blue mussels from the Baltic and North Sea under varying salinity or temperature conditions are not primarily driven by impaired mitochondrial functions. Although high temperature decreased the mitochondrial coupling efficiency and increased the ROS production and FEL, these changes did not

significantly affect ATP production. Thus, the mitochondrial tolerance appears sufficient for sperm energy demands, suggesting that other mechanisms may contribute to impaired reproduction under distinctive temperature and salinity conditions, especially for fertilization failure.

As fertilization is a complex process and requires gametes well coupled, future studies should focus on exploring the mechanisms of fertilization failure under cold and low-salinity seawater environments, such as the potential loss of acrosomal enzyme activity required for successful fertilization, particularly under cold stress. Additionally, investigating how these factors interact with sperm-egg recognition and early embryonic development will be essential for understanding the full impact of environmental fluctuations on mussel reproduction.

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Table 1. Temperature and salinity conditions in experimental exposures of gamete suspensions (Gametes) and fertilization media (Fertilization) of *M. edulis* from the Baltic Sea (BS) and the North Sea (NS) population. Salinities are in practical salinity units, n - the number of biological replicates.

BS: Salinity	Gametes	n	Fertilization	n
5	5.11 ± 0.02	5	5.12 ± 0.02	5
9	9.03 ± 0.05	5	9.10 ± 0.01	5
13	12.93 ± 0.02	5	13.12 ± 0.02	5
17	16.98 ± 0.03	5	17.03 ± 0.05	5
21	20.87 ± 0.02	5	20.91 ± 0.02	5
NS: Salinity	Gametes	n	Fertilization	n
9	9.05 ± 0.07	5	9.02 ± 0.08	4
17	17.50 ± 0.22	5	17.04 ± 0.11	4
21	20.63 ± 0.12	5	21.15 ± 0.24	4
30	30.12 ± 0.15	5	30.20 ± 0.32	4
35	34.76 ± 0.21	5	34.68 ± 0.28	4

BS: Temperature	Gametes and fertilization media		
	Beginning (°C)	End (°C)	n
5 °C	4.93 ± 0.02	4.97 ± 0.02	6
10 °C	9.97 ± 0.02	9.93 ± 0.05	6
15 °C	15.03 ± 0.02	15.05 ± 0.02	6
20 °C	20 ± 0	20 ± 0	6
25 °C	24.90 ± 0.03	24.92 ± 0.03	6

NS: Temperature	Gametes and fertilization media		
	Beginning (°C)	End (°C)	n
5 °C	5.04 ± 0.02	5.02 ± 0.02	5
10 °C	10 ± 0.07	9.94 ± 0.04	5
15 °C	15.02 ± 0.04	15.02 ± 0.02	5
20 °C	20.02 ± 0.02	20 ± 0	5
25 °C	24.96 ± 0.02	24.98 ± 0.02	5

Table 2. Correlations between sperm performance traits and fertilization success rates under various salinity or temperature conditions for the Baltic Sea (BS) and the North Sea (NS) mussels.

BS-Salinity exposure				
Correlations	% Motility	VCL	VAP	Fertilization
% Motility	**N= 25**	**25**	**25**	**25**
VCL	0.850**			
VAP	0.729**	0.759**		
Fertilization	0.845**	0.832**	0.531**	
ATP	-0.322	-0.423*	-0.119	-0.359
BS-Temperature exposure				
Correlations	%Motility	VCL	VAP	Fertilization
%Motility	**N=30**	**30**	**30**	**25**
VCL	0.888**			
VAP	0.786**	0.878**		
Fertilization	0.278	0.188	0.082	
ATP	-0.354	-0.335	-0.079	-0.148
NS-Salinity exposure				
Correlations	%Motility	VCL	VAP	Fertilization
%Motility	**N=25**	**25**	**25**	**25**
VCL	0.873**			
VAP	0.669**	0.860**		
Fertilization	0.700**	0.479*	0.308	
ATP	-0.363	-0.392	-0.149	-0.261
NS-Temperature exposure				
Correlations	%Motility	VCL	VAP	Fertilization
%Motility	**N=25**	**25**	**25**	**25**
VCL	0.853**			
VAP	0.643**	0.459*		
Fertilization	-0.207	-0.146	-0.204	
ATP	-0.661**	-0.531**	-0.518**	-0.051

705 Bold numbers in the first row show the sample size (N) used to calculate the correlation
706 coefficients. Values represent correlation coefficient, * and ** indicate the significant
707 correlations at 0.05 and 0.01 levels, respectively (2-tailed).
708

709 Table 3. Correlations among all sperm mitochondrial traits under various osmolarity and temperature conditions for the blue mussel *M. edulis*
710 from the Baltic Sea.

Correlations	MO_2-LEAK	MO_2-OXPHOS	RCR	ROS-LEAK	ROS-OXPHOS	FEL-LEAK	FEL-OXPHOS
MO_2-LEAK	1						
MO_2-OXPHOS	0.804**	1					
RCR	0.028	0.574**	1				
ROS-LEAK	0.026	0.01	-0.042	1			
ROS-OXPHOS	0.14	0.041	-0.14	0.872**	1		
FEL-LEAK	-0.636**	-0.525**	-0.021	0.710**	0.539**	1	
FEL-OXPHOS	-0.500**	-0.699**	-0.482**	0.578**	0.634**	0.789**	1

711 n=92 for MO_2-LEAK, MO_2-OXPHOS, and RCR; n=89 for ROS-LEAK, ROS-OXPHOS, FEL-LEAK, and FEL-OXPHOS. Values represent
712 correlation coefficient, ** indicate the significant correlations at 0.01 level (2-tailed), and "-" indicates the negative correlation.

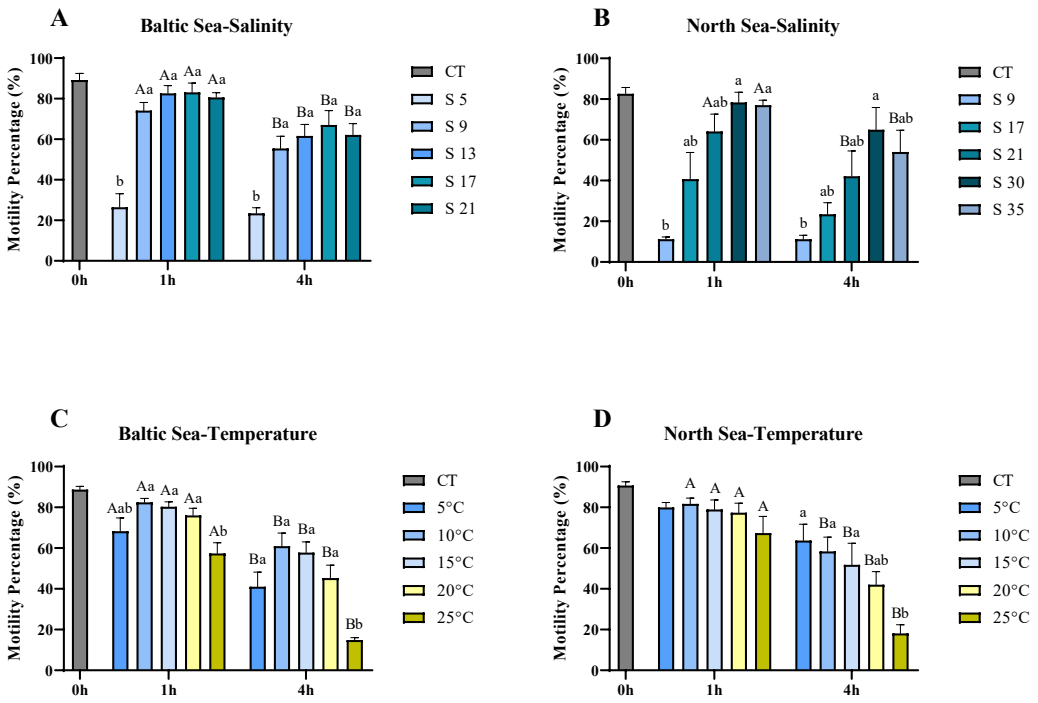
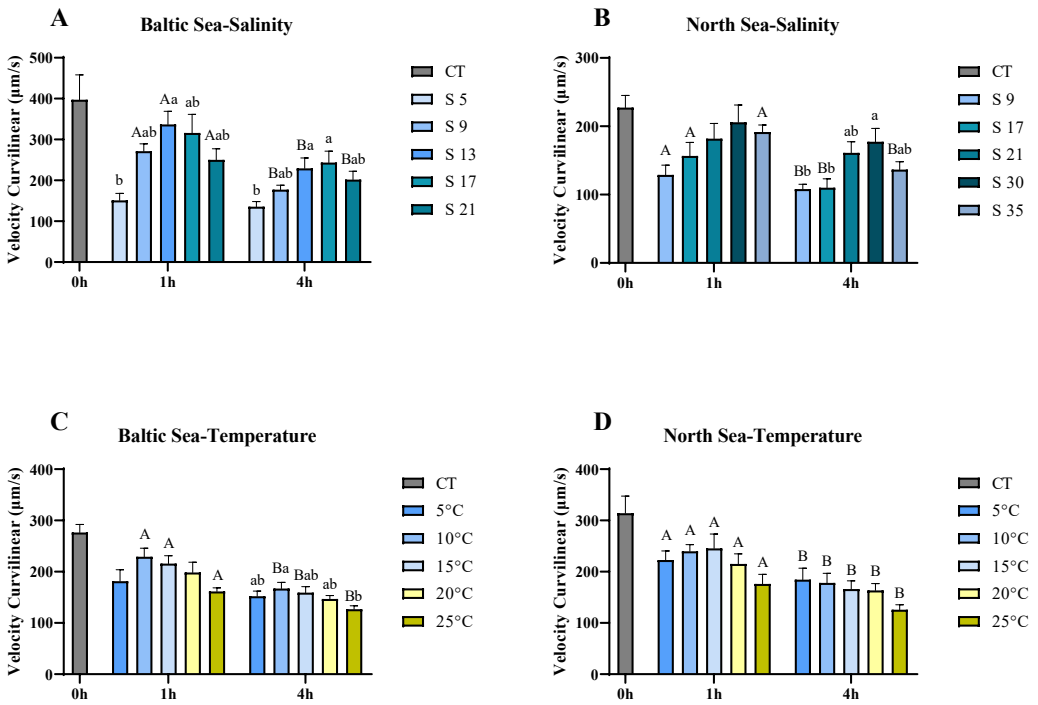


Figure 1. Motility percentage of sperm exposed to different temperature or salinity conditions after 1h and 4h post-exposure in blue mussels *M. edulis* from the Baltic Sea and North Sea.

A: Sperm motility of the BS mussels in salinity exposures, n=5; B: Sperm motility of the NS mussels in salinity exposures, n=5; C: Sperm motility of the BS mussels in temperature exposures, n=6; D: Sperm motility rate of the NS mussels in temperature exposures, n=5. The 0 h exposure in all graphs represents the sperm measurements immediately prior to the salinity and temperature exposures (time 0), taken at the acclimation conditions (15°C, salinity 15 for the BS mussels and salinity 30 for the NS mussels). Different capital letters represent the significant differences between 1h and 4h time points (p<0.05). Different lowercase letters indicate the significant difference among five temperature/salinity treatment groups within the same time point (p<0.05).



729

730 **Figure 2.** Velocity curvilinear of sperm exposed to distinct temperature or salinity
731 conditions at time of beginning (0h), 1h, and 4h post-exposure in blue mussels *M. edulis*
732 from both the Baltic Sea and North Sea.

733 A: VCL of sperm in mussels from the Baltic Sea in salinity experiments, n=5; B: VCL
734 of sperm in mussels from the North Sea in salinity experiments, n=5; C: VCL of sperm
735 in mussels from the Baltic Sea in temperature experiments, n=6; D: VCL of sperm in
736 mussels from the North Sea in temperature experiments, n=5. Different capital letters
737 represent the significant differences between 1h and 4h time points (p<0.05). Different
738 lowercase letters indicate the significant difference among five temperature/salinity
739 treatment groups within the same time point (p<0.05).

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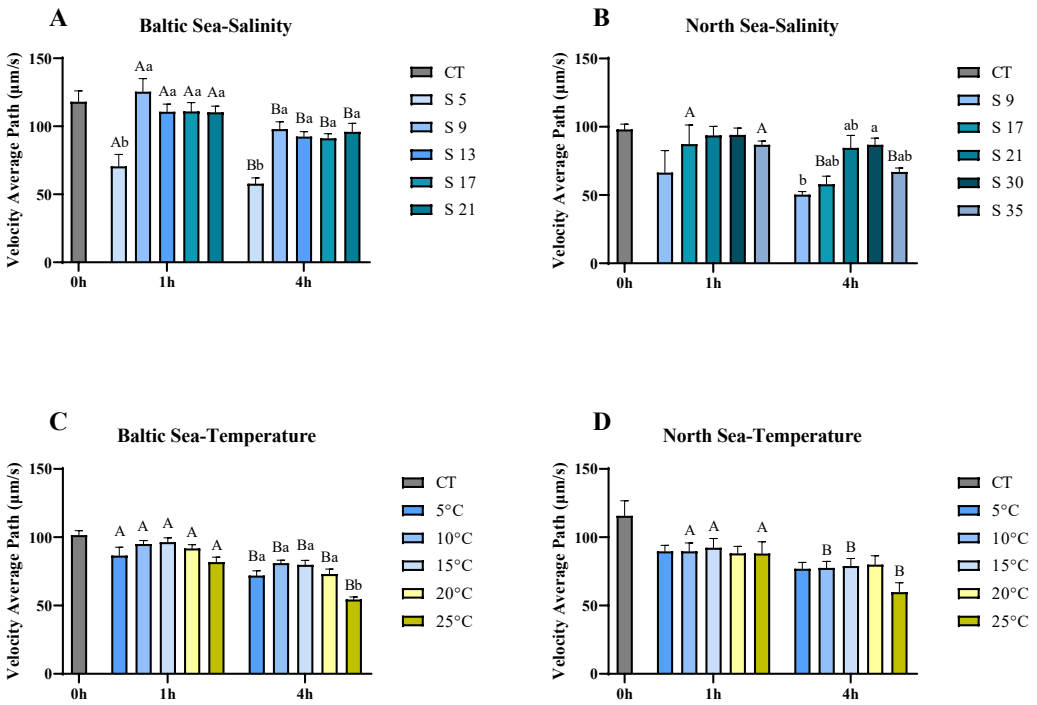

742

743 **Figure 3.** Velocity average path (VAP) of sperm exposed to distinct temperature or
744 salinity conditions at time of beginning (0h), 1h, and 4h post-exposure in blue mussels
745 *M. edulis* from both the Baltic Sea and North Sea.

746 A: VAP of sperm in mussels from the Baltic Sea in salinity experiments, n=5; B: VAP
747 of sperm in mussels from the North Sea in salinity experiments, n=5; C: VAP of sperm
748 in mussels from the Baltic Sea in temperature experiments, n=6; D: VAP of sperm in
749 mussels from the North Sea in temperature experiments, n=5. Different capital letters
750 represent the significant differences between 1h and 4h time points (p<0.05). Different
751 lowercase letters indicate the significant difference among five temperature/salinity
752 treatment groups within the same time point (p<0.05).

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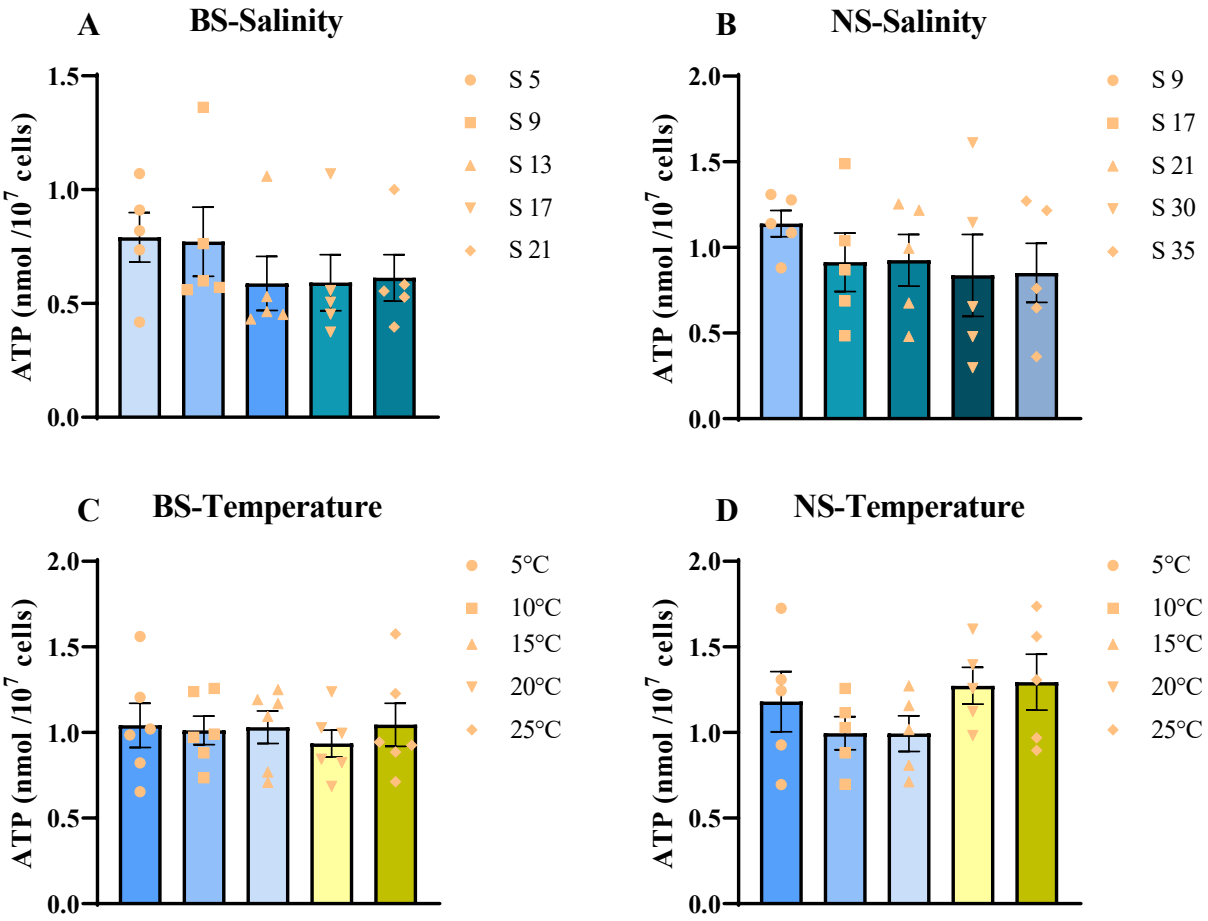
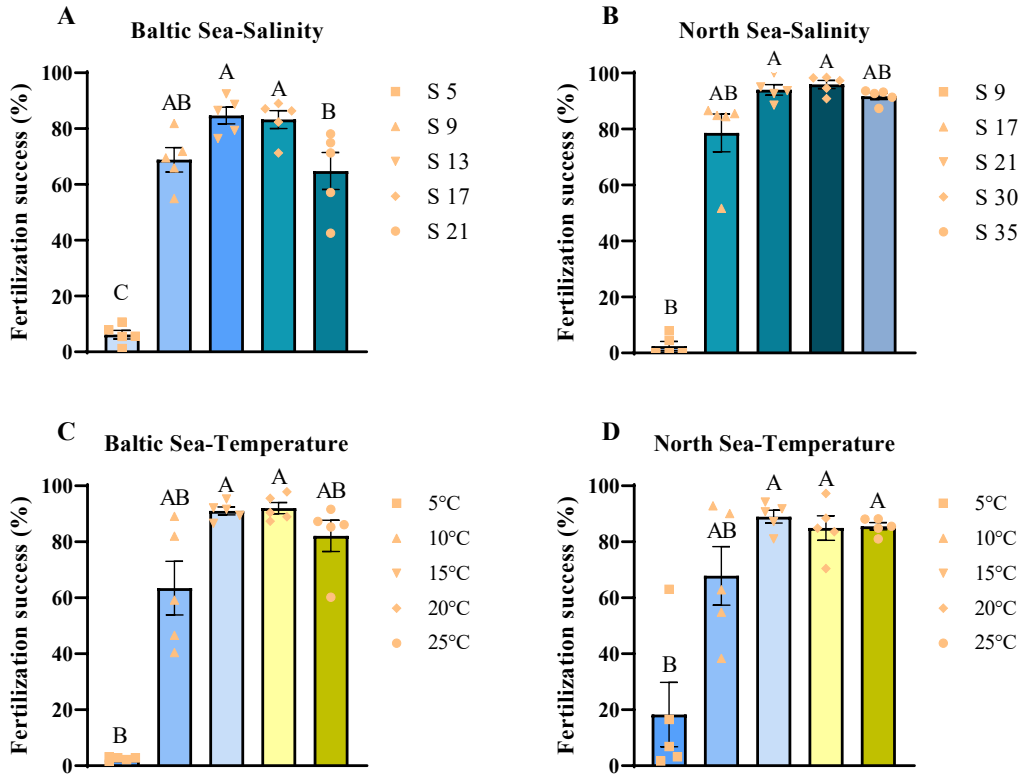


Figure 4. ATP contents of sperm exposed to distinct temperature or salinity conditions at 3h post-exposure in blue mussels *M. edulis* from both the Baltic Sea and North Sea. Color shading indicates salinity (top panels, light to dark blue) or temperature (bottom panels, dark blue to yellow) levels. Data points represent individual biological replicates.

A: Sperm ATP contents of mussels from the Baltic Sea in salinity experiments, n=5; B: Sperm ATP contents of mussels from the North Sea in salinity experiments, n=5; C: Sperm ATP contents of mussels from the Baltic Sea in temperature experiments, n=6; D: Sperm ATP contents of mussels from the North Sea in temperature experiments, n=5.



768

769 **Figure 5.** Fertilization success rate (FSR) under distinct temperature or salinity
770 conditions at 2h post-fertilization in blue mussels *M. edulis* from both the Baltic Sea
771 and North Sea. Color shading indicates salinity (top panels, light to dark blue) or
772 temperature (bottom panels, dark blue to yellow) levels. Data points represent
773 individual biological replicates.

774 A: FSR of mussels from the Baltic Sea in salinity experiments; B: FSR of mussels from
775 the North Sea in salinity experiments; C: FSR of mussels from the Baltic Sea in
776 temperature experiments; D: FSR of mussels from the North Sea in temperature
777 experiments. Different capital letters indicate the significant difference among five
778 temperature/salinity treatment groups (p<0.05). n=5.

779

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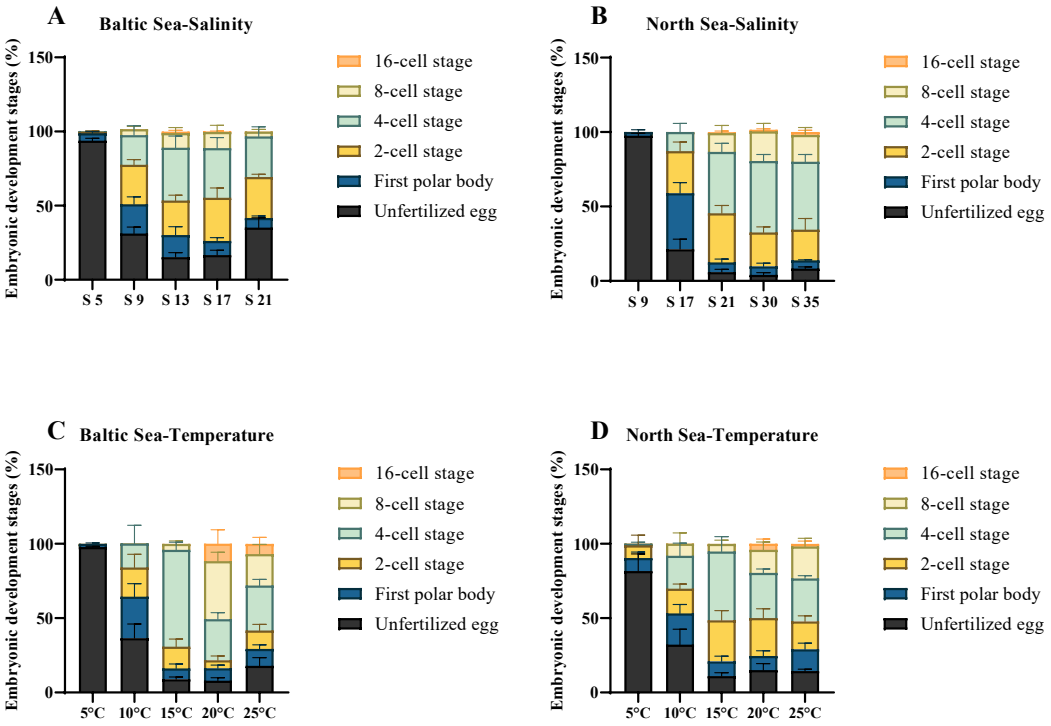
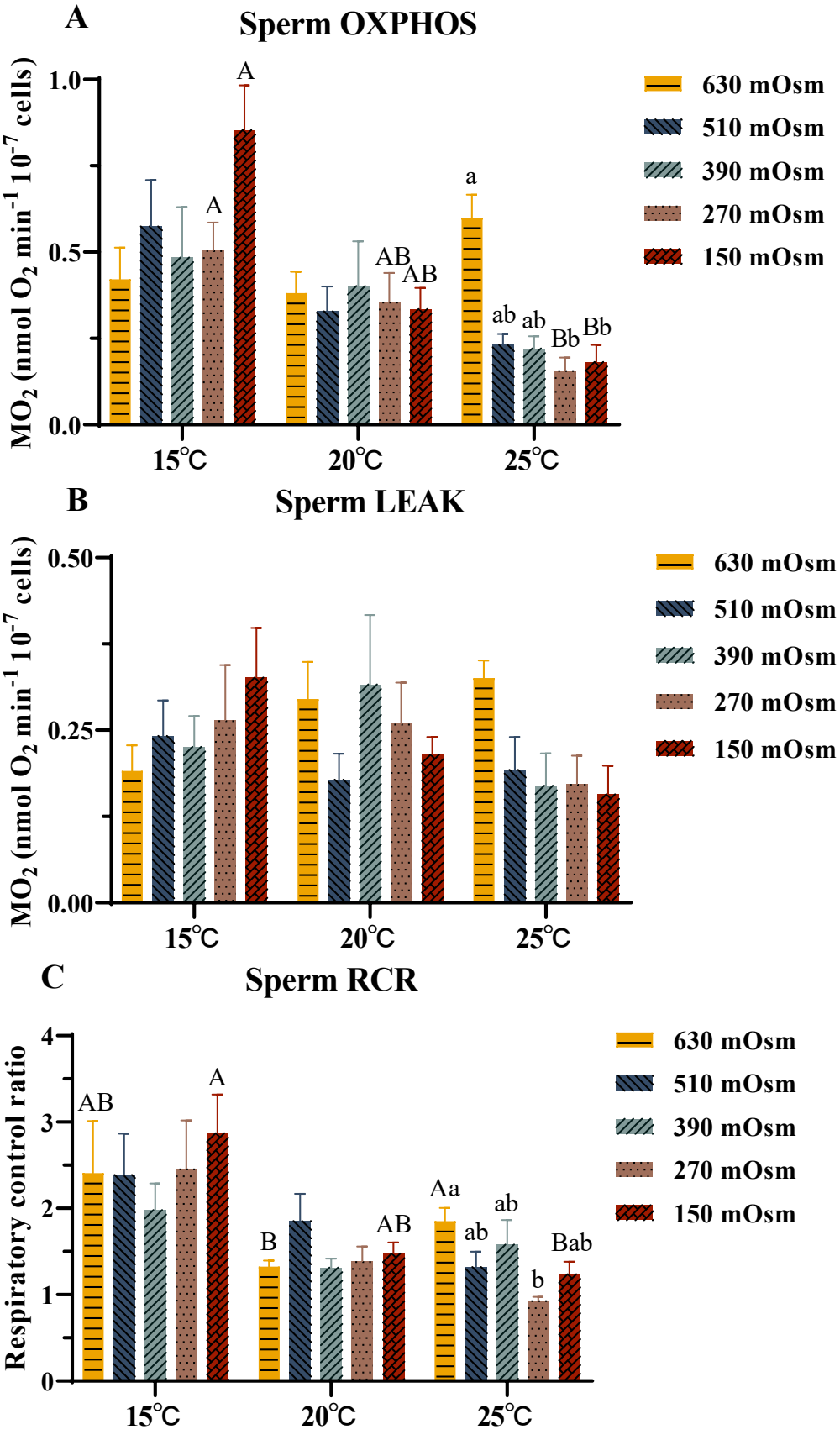


781

782 **Figure 6.** Embryonic developmental stage distribution (EDSD) under distinct
783 temperature or salinity conditions at 2h post-fertilization in blue mussels *M. edulis* from
784 both the Baltic Sea and North Sea.

785 A: EDSD of mussels from the Baltic Sea in salinity experiments; B: EDSD of mussels
786 from the North Sea in salinity experiments; C: EDSD of mussels from the Baltic Sea in
787 temperature experiments; D: EDSD of mussels from the North Sea in temperature
788 experiments. Due to the extensive details, directly displaying the significance on the
789 figures would overly complicate them and affect readability. The significant differences
790 in the outcomes are shown in Supplemental Table 3. n=5.

791


A
Sperm OXPHOS
MO2 (nmol O2 min-1 10-7 cells)
1.0
0.5
0.0
A
A
AB
AB
a
ab
ab
Bb
Bb
15°C
20°C
25°C
630 mOsm
510 mOsm
390 mOsm
270 mOsm
150 mOsm
B
Sperm LEAK
MO2 (nmol O2 min-1 10-7 cells)
0.50
0.25
0.00
15°C
20°C
25°C
630 mOsm
510 mOsm
390 mOsm
270 mOsm
150 mOsm
C
Sperm RCR
Respiratory control ratio
4
3
2
1
0
AB
A
B
AB
Aa
ab
ab
b
Bab
15°C
20°C
25°C
630 mOsm
510 mOsm
390 mOsm
270 mOsm
150 mOsm

792

Figure 7. Combined effects of temperature and osmolarity on the sperm mitochondrial functions of mussels *M. edulis* from the Baltic Sea.

A: Mitochondrial OXPHOS respiration (nmol O_2 min^{-1} 10^{-7} cells) of the sperm exposed to distinct temperature and osmolarity conditions. B: Mitochondrial LEAK I+II respiration (nmol O_2 min^{-1} 10^{-7} cells) of the sperm exposed to distinct temperature and osmolarity conditions. C: Respiratory control ratio (RCR) of the sperm mitochondria under different temperature and osmolarity treatments. Different capital letters represent the significant differences among three temperature treatment groups at the same osmolarity (p<0.05). Different lowercase letters indicate the significant difference among five osmolarity treatment groups at the fixed temperature (p<0.05). n=5-8.

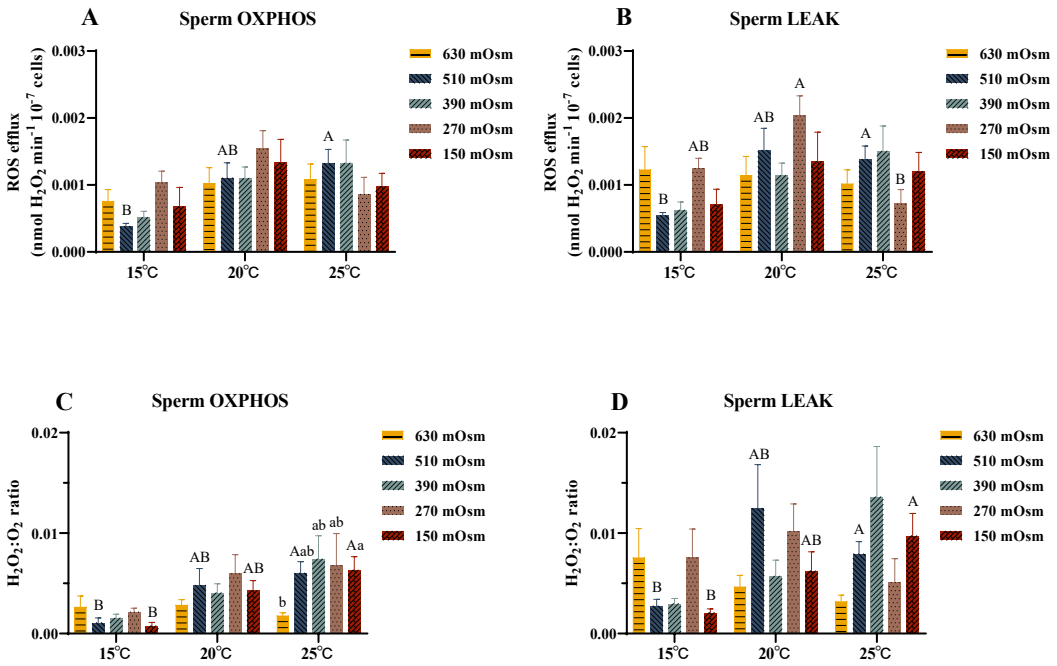


Figure 8. Combined effects of temperature and osmolarity on the sperm mitochondrial functions of mussels *M. edulis* from the Baltic Sea.

A: Reactive oxygen species (ROS) efflux in sperm mitochondria in OXPHOS status under distinct temperature and osmolarity treatments. B: ROS efflux in sperm mitochondria in LEAK I+II status under distinct temperature and osmolarity treatments. C: Fractional electron leak (FEL; expressed as H_2O_2:O_2 ratio) of sperm mitochondria in OXPHOS status under distinct temperature and osmolarity treatments. D: FEL of sperm mitochondria in LEAK I+II status under distinct temperature and osmolarity treatments. Different capital letters represent the significant differences among three temperature treatment groups at the same osmolarity (p<0.05). Different lowercase letters indicate the significant difference among five osmolarity treatment groups at the fixed temperature (p<0.05). n=5-8.

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Supplementary materials for

Salinity and temperature-driven variations in sperm performance and mitochondrial metabolism in North Sea and Baltic Sea mussels *Mytilus* spp.

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Supplemental Table 1 Sperm concentration of male mussel *M. edulis* individuals before and after centrifugation for sperm mitochondrial function measurements. Raw indicates the raw sperm concentration (10^7 cells ml^{-1}) before centrifugation and stock represents the sperm concentration (10^9 cells ml^{-1}) after centrifugation.

Treatments	15 °C×630 mOsm		15 °C×510 mOsm		15 °C×390 mOsm		15 °C×270 mOsm		15 °C×150 mOsm	
	Raw	Stock	Raw	Stock	Raw	Stock	Raw	Stock	Raw	Stock
N1	5	5.23	8.4	3.4	4.8	2.4	5.3	2.2	5.2	1.3
N2	7.5	4.52	11.5	3.8	5.3	2.8	4.4	4.5	6.9	3.9
N3	7.75	5.5	12.3	4.3	14.5	7.2	4	1.6	22.7	3.8
N4	10.1	8.5	23.4	7.3	6.9	4.7	17.9	2.2	5.5	5
N5	7.1	3.1	5.7	2.1	5.5	2.85	10.9	2	14.6	4.8
N6	3.9	2.4			4.9	2.8				
Treatments	20 °C×630 mOsm		20 °C×510 mOsm		20 °C×390 mOsm		20 °C×270 mOsm		20 °C×150 mOsm	
	Raw	Stock	Raw	Stock	Raw	Stock	Raw	Stock	Raw	Stock
N1	10.2	4.4	3.1	1.9	16.8	8.4	9	5.25	12.5	3.85
N2	5.6	2.75	5.1	4.4	12.6	7	7.7	4.05	9.6	4
N3	5.4	3.5	5	3.95	4.5	2.4	6.3	1.9	21.2	6.85
N4	9.5	4.35	6.5	4.1	5.8	4.05	7.3	5.75	8.3	2.6
N5	3.7	2.8	8.3	4.5	23	3.45	7.9	3.2	16	3.3
N6	10.5	3.6	4.5	3.4	19.9	3.3	6.5	2.15	6.8	2.75

N7	7.2	4.5	8.4	4.1			10.1	3.8	5.2	2.45
N8	3.8	2.85							6.5	3.75
Treatments	25 °C×630 mOsm		25 °C×510 mOsm		25 °C×390 mOsm		25 °C×270 mOsm		25 °C×150 mOsm	
	Raw	Stock	Raw	Stock	Raw	Stock	Raw	Stock	Raw	Stock
N1	6.7	3.5	3.4	2.6	1.8	2.05	6.1	3.9	7.3	6.1
N2	5.5	3.3	2	1.5	2.5	1.9	10.9	9.4	6.6	3.55
N3	7	4.55	2.4	2	6.1	2.9	5.8	3.7	3.2	3.9
N4	8.5	6.4	10.2	6.6	2.7	3.2	6.1	3.55	3.3	2.5
N5	19.4	5.8	3.4	2.75	5	3.65	3.7	2.8	3.6	4.55
N6	18.4	6.75			4.2	3.3	4.3	4.7	8.8	6.6

Supplemental Table 2 Chemical compositions in the assay buffer for distinct sperm mitochondrial exposure.

Assay buffers	Salinity of 5	Salinity of 9	Salinity of 13	Salinity of 17	Salinity of 21
Osmolarity	150 mOsm	270 mOsm	390 mOsm	510 mOsm	630 mOsm
Sucrose	0	0	47mM	167mM	287mM
KCL	43.5mM	103.5mM	130mM	130mM	130mM
NaCl	0	0	10mM	10mM	10mM
HEPES	30mM	30mM	30mM	30mM	30mM
Glucose	10mM	10mM	10mM	10mM	10mM
$MgCl_2$	1mM	1mM	1mM	1mM	1mM
KH_2PO_4	10mM	10mM	10mM	10mM	10mM
BSA	1%	1%	1%	1%	1%
pH	7.2	7.2	7.2	7.2	7.2

Supplemental Table 3. Significant difference between the treatments on the embryonic development of both mussel populations under distinctive salinity and temperature conditions. *p* values marked in red were only recorded when the significant difference occurred.

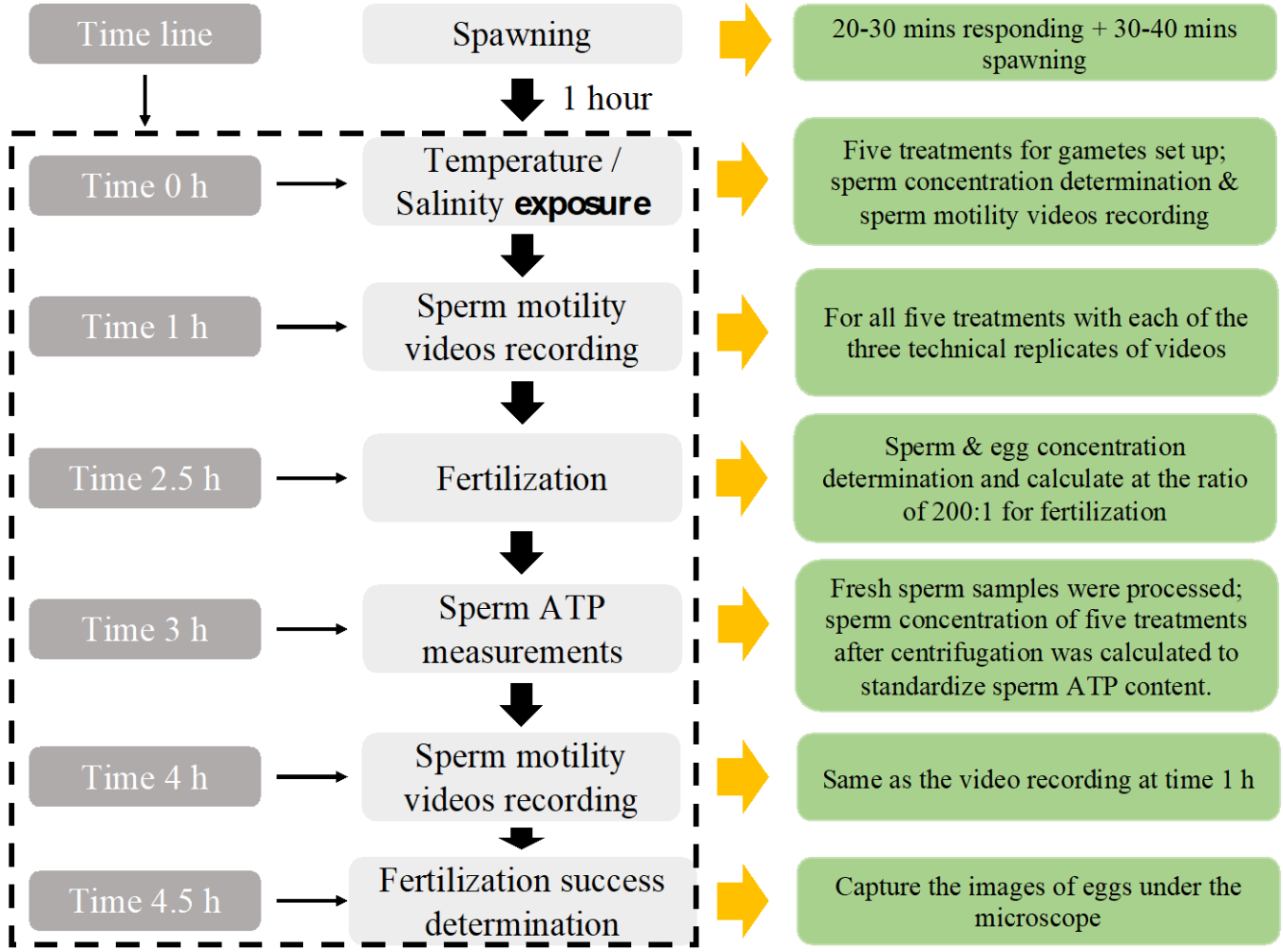
BS-Salinity	Unfertilized egg stage		First polar body stage		Two-cell stage		Four-cell stage		Eight-cell stage	
	Treatments Comparison	*p*	Treatments Comparison	*p*	Treatments Comparison	*p*	Treatments Comparison	*p*	Treatments Comparison	*p*
	5 vs 9	<0.001			5 vs 21	0.019	5 vs 13	0.034	5 vs 13	0.012
	5 vs 13	<0.001					5 vs 17	0.044	5 vs 17	0.015
	5 vs 17	<0.001								
	5 vs 21	<0.001								
	13 vs 21	0.021								
	17 vs 21	0.04								
BS-Temperature	Unfertilized egg stage		First polar body stage		Two-cell stage		Four-cell stage		Eight-cell stage	
	Treatments Comparison	*p*	Treatments Comparison	*p*	Treatments Comparison	*p*	Treatments Comparison	*p*	Treatments Comparison	*p*
	5 vs 15 (°C)	0.004	5 vs 10 (°C)	0.012	5 vs 15 (°C)	0.046	5 vs 15 (°C)	0.001	5 vs 20 (°C)	0.002
	5 vs 20 (°C)	0.003			5 vs 25 (°C)	0.02	10 vs 15 (°C)	0.024	5 vs 25 (°C)	0.025
									10 vs 20 (°C)	0.002
									10 vs 25 (°C)	0.025

									15 vs 20 (°C)	0.003
NS-Salinity	Unfertilized egg stage		First polar body stage		Two-cell stage		Four-cell stage		Eight-cell stage	
	Treatments Comparison	*p*	Treatments Comparison	*p*	Treatments Comparison	*p*	Treatments Comparison	*p*	Treatments Comparison	*p*
	9 vs 21	0.009	9 vs 17	0.004	9 vs 17	0.034	9 vs 21	0.001	9 vs 30	0.025
	9 vs 30	0.002			9 vs 21	0.006	9 vs 30	<0.001	9 vs 35	0.017
							9 vs 35	0.001	17 vs 30	0.025
							17 vs 30	0.046	17 vs 35	0.017
NS-Temperature	Unfertilized egg stage		First polar body stage		Two-cell stage		Four-cell stage		Eight-cell stage	
	Treatments Comparison	*p*	Treatments Comparison	*p*	Treatments Comparison	*p*	Treatments Comparison	*p*	Treatments Comparison	*p*
	5 vs 15 (°C)	0.015					5 vs 15 (°C)	0.003	5 vs 25 (°C)	0.013
	5 vs 20 (°C)	0.014								
	5 vs 25 (°C)	0.022								

Supplemental Table 4. Two-way repeated measures ANOVA of the exposure time and salinity/temperature on sperm motility and velocity of mussels *M. edulis* from the Baltic and North Sea. Only data following the normal distribution are presented.

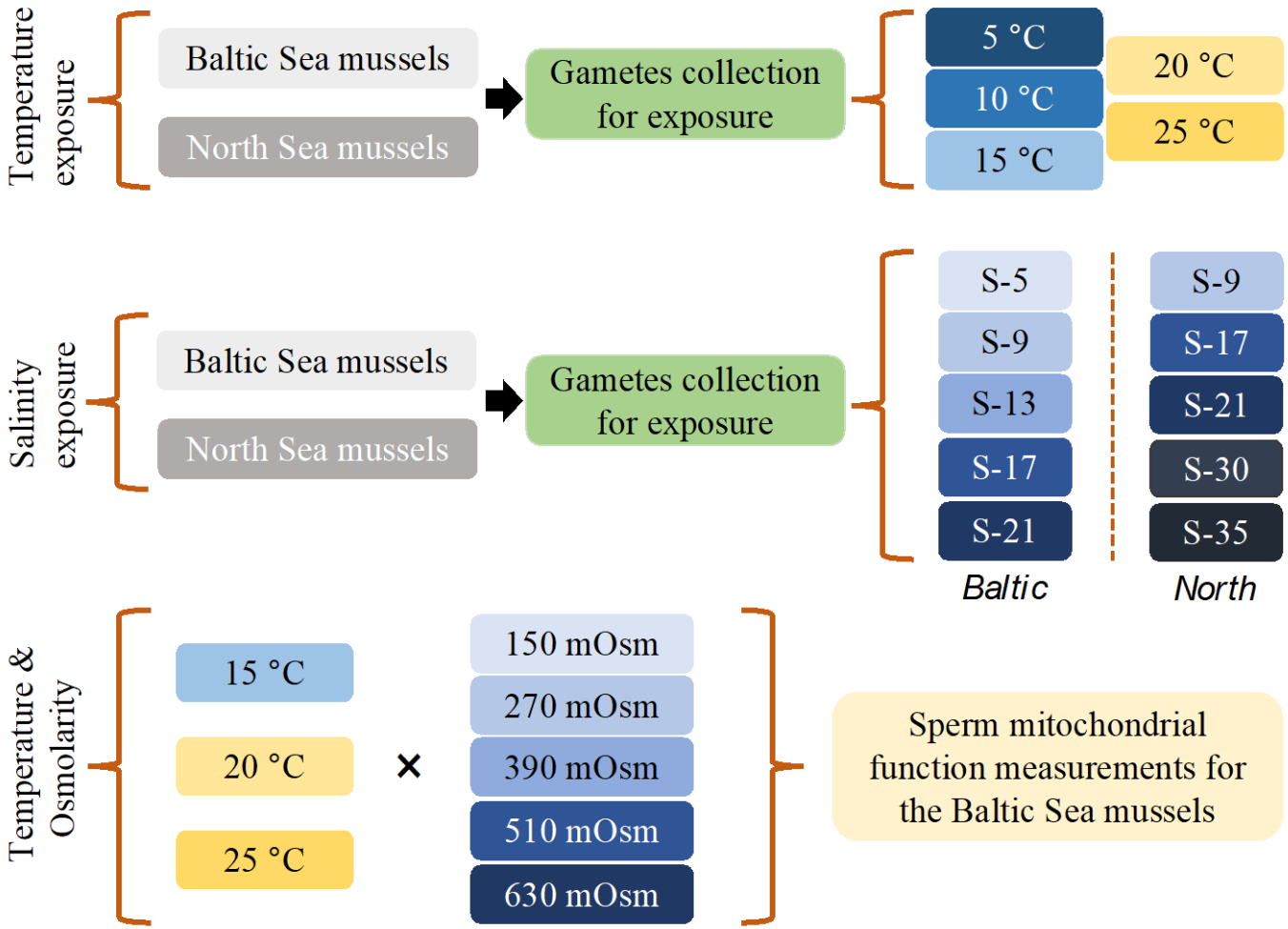
VAP		BS-Salinity	
Source	df	*F*	*p*
Exposure time	1	60.024	<0.001
Exposure time * Salinity	4	1.159	0.358
Motility rate		BS-Temperature	
Source	df	*F*	*p*
Exposure time	1	181.474	<0.001
Exposure time * Temperature	4	3.12	0.033
VCL		BS-Temperature	
Source	df	*F*	*p*
Exposure time	1	58.921	<0.001
Exposure time * Temperature	4	1.068	0.393
VAP		BS-Temperature	
Source	df	*F*	*p*
Exposure time	1	145.351	<0.001
Exposure time * Temperature	4	2.556	0.064

VAP		NS-Salinity	
Source	df	*F*	*p*
Exposure time	1	15.191	0.001
Exposure time * Salinity	4	0.898	0.484
Motility rate		NS-Temperature	
Source	df	*F*	*p*
Exposure time	1	202.061	<0.001
Exposure time * Temperature	4	7.09	0.001
VCL		NS-Temperature	
Source	df	*F*	*p*
Exposure time	1	118.027	<0.001
Exposure time * Temperature	4	1.75	0.179



Exposure of temperature / salinity timeline

Supplemental Figure. 1 Experimental timeline for sperm performance and fertilization assays. Adult mussels were induced to spawn by the thermal shock method over a period of 1 h (including ~20–30 minutes of thermal response and ~30–40 minutes of gamete release). Collected sperm and eggs were then divided into five treatments for temperature or salinity exposure. Sperm motility was recorded at 1 h and 4 h post-exposure, fertilization was performed at time 2.5 h, sperm ATP content was measured at time 3 h, and fertilization success was assessed at time 4.5 h.


Temperature exposure
Baltic Sea mussels
North Sea mussels
Gametes collection for exposure
5 °C
10 °C
15 °C
20 °C
25 °C
Salinity exposure
Baltic Sea mussels
North Sea mussels
Gametes collection for exposure
S-5
S-9
S-13
S-17
S-21
S-9
S-17
S-21
S-30
S-35
Baltic
North
Temperature & Osmolarity
15 °C
20 °C
25 °C
×
150 mOsm
270 mOsm
390 mOsm
510 mOsm
630 mOsm
Sperm mitochondrial function measurements for the Baltic Sea mussels

Supplemental Figure. 2 The effects of salinity/osmolarity and temperature on sperm performance and mitochondrial metabolism in blue mussel *M. edulis* from the North Sea and the Baltic Sea populations.

Curriculum Vitae

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Since 04/2021 **PhD study in Institute of Biological Sciences, University of Rostock**

Topic: “Impacts of multiple stressors on reproductive performance and gamete metabolism of the blue mussels *Mytilus edulis*: Physiological mechanisms and ecological implications”

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09/2017–06/2020 **Master study in College of Fisheries and Life Science, Shanghai Ocean University**

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09/2013–06/2017 **Bachelor study in College of Fisheries and Life Science, Shanghai Ocean University**

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List of Publications (*related to the PhD thesis)

11) *Kong, H., & Sokolova, I. M. (**2025**). Effects of hypoxia and anoxia on the reproductive performance of the blue mussel *Mytilus edulis*. Science of The Total Environment, 972, 179103.

10) *Kong, H., & Sokolova, I. M. (**2024**). Oxidative phosphorylation rather than glycolysis is the primary energy source for sperm motility in the mussels *Mytilus edulis*. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 270, 110909.

9) Wu, F., Kong, H., Xie, L., & Sokolova, I. M. (**2024**). Exposure to nanopollutants (nZnO) enhances the negative effects of hypoxia and delays recovery of the mussels' immune system. Environmental Pollution, 351, 124112.

8) Mredul, M. M. H., Sokolov, E. P., Kong, H., & Sokolova, I. M. (**2024**). Spawning acts as a metabolic stressor enhanced by hypoxia and independent of sex in a broadcast marine spawner. Science of The Total Environment, 909, 168419.

7) Xu, G., Kong, H., Chang, X., Dupont, S., Chen, H., Deng, Y., Hu, M., & Wang, Y. (**2021**). Gonadal antioxidant responses to seawater acidification and hypoxia in the marine mussel Mytilus coruscus. Environmental Science and Pollution Research, 28(38), 53847-53856.

6) Khan, F. U1., Hu, M1., Kong, H., Shang, Y., Wang, T., Wang, X., Xu, R., Lu, W., & Wang, Y. (**2020**). Ocean acidification, hypoxia and warming impair digestive parameters of marine mussels. Chemosphere, 127096.

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3) Kong, H., Clements, J. C., Dupont, S., Wang, T., Huang, X., Shang, Y., Huang W., Chen, J., Hu, M., & Wang, Y. (**2019**). Seawater acidification and temperature modulate anti-predator defenses in two co-existing *Mytilus* species. Marine Pollution Bulletin, 145, 118-125.

2) Kong, H., Jiang, X., Clements, J. C., Wang, T., Huang, X., Shang, Y., Chen, J., Hu, M., & Wang, Y. (**2019**). Transgenerational effects of short-term exposure to acidification and hypoxia on early developmental traits of the mussel *Mytilus edulis*. Marine Environmental Research, 145, 73-80.

1) Shang, Y., Wang, X., Kong, H., Huang, W., Hu, M., & Wang, Y. (**2019**). Nano-ZnO impairs anti-predation capacity of marine mussels under seawater acidification. Journal of Hazardous Materials, 371, 521-528.