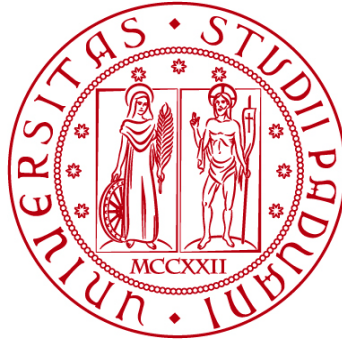


UNIVERSITÀ DEGLI STUDI DI PADOVA

DIPARTIMENTO DI BIOLOGIA

Corso di Laurea magistrale in Marine Biology



TESI DI LAUREA

**Coping with Variability: Physiological Performances and
Plasticity of the Invasive Blue Crab *Callinectes sapidus*
under Fluctuating and Constant Conditions**

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ANNO ACCADEMICO 2024/2025

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1 INTRODUCTION

1.1 Coastal Ecosystems under Climate Change

1.1.1 Climate change: the biggest challenge of the 21st century

Climate change is widely recognized as one of the most serious and pressing threats to coastal environments. Anthropogenic activities and the consequent increase in greenhouse gases emissions are disrupting marine ecosystems (Azra et al., 2020).

A comprehensive meta-analysis has documented the widespread impact of global climate change on marine ecosystems showing that more than 80% of biological responses in marine organisms, such as shifts in distribution and physiological performance, are influenced by ocean warming, providing significant evidence of climate-driven ecological change at a global scale (Poloczanska et al., 2013).

Climate change is not only characterized by a shift in atmospheric and ocean temperatures but also encompasses a variety of other stressors that have a substantial impact on marine life (Poloczanska et al., 2013).

One of the main threats correlated with anthropic activities is ocean acidification. Though oceans can absorb around one-third of human CO₂ emissions, the ocean's natural ability to function as a buffer against greenhouse gas emissions causes major chemical changes in marine ecosystems (Raven and Falkowski, 1999). Lower pH, due to lower carbonate ion concentration, and decreased saturation of CaCO₃ minerals are directly caused by human activity and emissions. Ocean pH, on average, has decreased by 0.1 since preindustrial times, and it could diminish up to 0.4 by 2100 and 0.77 by 2300, which would be the fastest shift over millions of years. If unmitigated, these conditions pose a serious threat to calcifying organisms like corals, mollusks and plankton potentially driving large-scale biodiversity loss and even mass extinctions (Findlay and Turley, 2021).

Sea level rise represents one of the most significant consequences of global climate change. Human societies are also compromised by climate change with sea-level rise driving the displacement of populations of islands and coastal zones (Wetzel et al., 2012). Satellite data show that the global mean in sea level has increased by around 1.7 mm each year in the 20th century, and in recent decades, this has accelerated to over 3 mm per year (Cazenave and Remy, 2011; Mimura, 2013). This is primarily driven by thermal expansion of seawater and melting of permanent glaciers and ice sheets in polar regions, particularly from Greenland and West Antarctica, with big loss of glacial habitats and impacts on specialized species that live on glaciers. Future projections indicate an increase of a total of 18 to 59 cm by the year 2100, recent studies are suggesting that this amount could exceed one meter (Cazenave and Remy, 2011; Mimura, 2013). These variations threaten human settlements as well as coastal ecosystems, endangering the lives of hundreds of millions of people and marine and terrestrial species.

The melting of Greenland's ice sheets is projected to weaken the Atlantic Meridial Overturning Circulation, a key element to global climate and ecosystems which moves considerable amounts of heat from the equator to the poles. This will slow down climate warming, but it will also make regional climates more divergent, making species

extinctions and ecosystem collapse grow in probability, generating trophic effects in marine species networks (Ureta et al., 2022). Melting of Greenland ice also creates substantial alteration on climate zones, with considerable impact on world biodiversity, particularly affecting South American megadiverse countries. Climate patterns will be affected with increase in droughts and extreme precipitation trends, altering crop productivity and pushing humans to expand agricultural areas with an increase in deforestation and disruption of natural habitats (Ureta et al., 2022). Ecosystems in these areas can also become more susceptible to wild fires, with disruption of nursery areas and refuge zones for tropical species (Ureta et al., 2022). Moreover, coastal wetlands such as saltmarshes and mangroves will decline due to sea level rising when the rate of sediment accumulation cannot keep up with rising waters (Nicholls and Cazenave, 2010). Ice glaciers melting also influence biodiversity in glacial habitats with loss of hunting, resting and breeding areas strictly dependent from the ice, with effects in population declines and behavioural changes. The melting alters the sensibility of species of polar bears, seals, narwhals and other marine mammals (Laidre et al., 2008). Finally, sea level rise causes a particular effect called coastal squeeze. This event is defined as a loss of coastal habitats in front of anthropic infrastructure such as sea defenses. Sea level rise brings water closer to human settlements in coastal habitats, causing the narrowing of many intertidal ecosystems and constricting habitats such as beaches, sand dunes, saltmarshes and mangroves to narrow their extension (Pontee, 2013). The loss of these environments will be critical for animals that depend on them for nursery, feeding and shelter, increasing extinction risks for seagrasses, crustacea, fishes and mollusks (Reed et al., 2009). Losing intertidal habitats severely disrupts benthic invertebrate, planktonic and nekton populations and communities. This will alter trophic networks and diminish the stability and productivity of coastal food webs (Colombano et al., 2021).

In addition to sea-level rise and ocean acidification, marine environments are subjected to another critical but less visible consequence of ocean warming: the progressive decline in dissolved oxygen concentrations (Breitburg et al., 2018). Warmer waters tend to hold less oxygen and also enhance metabolic oxygen demand (Pörtner, 2010), resulting in reduced oxygen availability for all marine organisms (Breitburg et al., 2018). In this contest, climate change is exacerbating the formation and expansion of oceanic dead zones, where dissolved oxygen reaches concentrations not suitable to most marine organisms (often under 2 mg/L), via a multitude of interacting mechanisms (Diaz and Rosenberg, 2008). The occurrence of oceanic dead zones has profound consequences on marine biodiversity. Oxygen depletion generates water column stratification with consequent collapse of benthic communities, while alteration of oceanic currents causes mass migrations and death of marine species. Species that can survive under hypoxic conditions can experience loss in growth, reproduction and feeding of species compromising overall fitness (Altieri and Gedan, 2015; Brush et al., 2020; Diaz and Rosenberg, 2008).

Rising temperature, altered precipitation phenomena, ocean acidification and extreme events frequency are contributing to reducing the dissolved oxygen availability and altering ecosystem responses and habitat adaptations (Altieri and Gedan, 2015; Diaz and

Rosenberg, 2008).

Extreme climatic events are rapidly increasing in frequency, intensity and duration with heatwaves representing the most robust signal of human-induced climate change (Brown, 2020). Heatwaves are expected to become longer, more frequent, and more intense, while frost days, on the contrary, are projected to decrease and growing seasons lengthen (Planton et al., 2008). Extreme precipitation events are projected to change, with strong events becoming more common in some regions. Drought trends are showing regional variability, with the Mediterranean region, the terrestrial areas surrounding the Mediterranean sea, showing longer and more persistent events (Planton et al., 2008; Rajczak et al., 2013). Moreover, wind-related extremes are regionally dependent, with potential increase in intensity and strongest storms expected with a lower expected total storm numbers (Planton et al., 2008).

As marine heatwaves are starting to become more frequent and more intense, their combination with declining oxygen levels generates multiple physiological stressors that are particularly impactful for marine ectotherms, whose metabolisms and thermal tolerance directly depend from ambient conditions, undermining the aerobic oxygen consumption of these species (Pörtner, 2010).

Studying how temperature increases affect oxygen consumption and tolerance thresholds becomes essential to predict how organisms will adapt to future oceanic conditions and to act with conservation measures (Pörtner, 2010; Pörtner and Knust, 2007). The thermal limit and metabolic performances of marine organisms of species under changing conditions of gradual and/or extreme warming can be explored more thoroughly by using temperature-dependent mortality curves (thermal ramp) (Li et al., 2015; Pörtner, 2021). These stressors tend to amplify the effects of human-induced eutrophication phenomena, generating conditions of hypoxia in the ocean (Altieri and Gedan, 2015; Diaz and Rosenberg, 2008). Coastal systems are exhibiting increasing sensitivity suggesting that nutrient reduction efforts should be more stringent than in the past, in fact previous eutrophication thresholds may no longer be sufficient to prevent hypoxia zones (Altieri and Gedan, 2015).

Climate change also acts as a driver for biological invasions, generating species redistribution with range shifts as animals try to adapt to changing thermal conditions (Pyšek et al., 2020). The retreat of the glacier and general water warming are opening new areas that are now suitable for colonization, with species adapted to warmer conditions moving towards previously cooler environments (Mancinelli et al., 2017; Pyšek et al., 2020; Rato et al., 2021). In addition, if invasive species exhibit greater physiological plasticity than native ones, they may be able to exploit new environmental conditions, particularly during their establishment phase, having a competitive advantage over native species and consequently altering community structure (Rato et al., 2021).

1.1.3 Coastal Areas: Transitional Ecosystems

Ocean acidification and deoxygenation are exclusively typical to aquatic ecosystems, making coastal areas especially sensitive to particular effects of climate change (Bindoff et al., 2022).

In coastal ecosystems, species richness is particularly vulnerable, and a wide range of stressors (e.g. temperature increase, ocean acidification, oxygen depletion), driven by climate change have a significant impact on biodiversity (Hewitt et al., 2016; Santojanni et al., 2023). Because of this high sensitivity, changes in species composition and biodiversity can actually provide insight into climate-driven alteration in coastal habitats, serving as valuable indicators for detecting them (Hewitt et al., 2016; Santojanni et al., 2023). Intact and diverse communities may be more resilient in terms of survival and reproduction but the ones already close to their ecological tolerance being more vulnerable (Hewitt et al., 2016). Ecological shifts are expected and occur rapidly rather than gradually due to nonlinear and threshold responses (Hewitt et al., 2016).

Temperature represents the main driver of stress on ectotherms. However variability associated with large scale oscillations such as the El Niño–Southern Oscillation (ENSO), a periodic climate phenomenon involving changes in tropical Pacific Ocean temperatures and atmospheric circulation (Battisti and Sarachik, 1995), together with wave exposure also plays a relevant role, further intensifying disturbance at sea level. Also nutrient cycling, sediment dynamics and primary productivity variations that can change the abundance of key-species, ultimately leading to ecosystem shifts (Hewitt et al., 2016). For instance, eutrophication causes algal blooms and consequent oxygen depletion, favouring benthic species that exhibit opportunistic behaviour (Anthony et al., 2009), while loss of sediment dwelling invertebrates disrupt nitrogen cycling, reinforcing oxygen depletion (Altieri and Gedan, 2015). Lastly, loss in primary productivity environments such as seagrass meadows, coral reefs and kelp forests, reduces habitat stability and brings ecosystems towards new irreversible states (ecosystems shifts) (Bindoff et al., 2022).

Moreover, climate change has a major effect on coastal habitats, impacting the composition and functionality of key habitats, such as mangroves, saltmarshes and beach dunes (Bijak et al., 2023; Hoegh-Guldberg et al., 2007; Reed et al., 2022). Ocean acidification and marine heatwaves degrade coral reefs, inducing coral bleaching and biodiversity loss (Hoegh-Guldberg et al., 2007). Under warming and extreme events, seagrass meadows and kelp forests deteriorate, diminishing carbon storage and productivity (Bijak et al., 2023; Reed et al., 2022). Changes in salinity, sediment dynamics, and sea level rise all impact salt marshes and mangroves. Globally, these processes pose a danger to the resilience and stability of coastal ecosystems (Reed et al., 2022).

Transitional environments are habitats such as lagoons, estuaries and coastal wetlands; they are dynamic, highly variable interface systems that lie between marine, terrestrial and river environments (Pérez-Ruzafa et al., 2011). These ecosystems represent a shifting boundary between continental habitats and marine environments. These habitats share features like predominantly shallow waters (with differences in depth), dynamic environment with strong physical gradients and high spatial heterogeneity (Pérez-Ruzafa et al., 2011).

Transitional environments present broad salinity, temperature and oxygen availability variations (Pérez-Ruzafa et al., 2011). The species inhabiting these ecosystems present tolerance to salinity variability and have behavioural adaptations to these constantly changing environments (Pérez-Ruzafa et al., 2011). For instance, fishes actively move to

microhabitats with optimal dissolved oxygen or temperature conditions, in order to maintain their physiological performances towards preferred tolerance range (Kroeker et al., 2020). In general, most mobile marine species tend to move to shallow waters for predatory advantages and to avoid hypoxia conditions (Bell and Eggleston, 2005). Crustacean species, such as *Callinectes sapidus* (Rathbun, 1896) have an adapted reproductive cycles with females migrating towards high salinity areas during reproduction while males typically remain in transitional habitats (Marchessaux et al., 2024; Molina et al., 2021)

As a result of global warming, lagoon and estuary systems are most impacted. Temperature fluctuates quickly there on short spatial and temporal scales (daily and seasonal) (Brito et al., 2012).

Furthermore, dissolved oxygen dynamics are a valuable insight into shallow water ecosystems such as lagoons. Short- and long- term fluctuations reflect the interactions between biotic and abiotic elements: temperature, photosynthetic activity, community level respiration, and atmospheric conditions influence this parameter. High frequency measurements revealed periodic oscillations in dissolved oxygen occurring more times during a day, particularly around a 6-8 hour cycles (D'Autilia et al., 2004). These variations are probably influenced by diurnal metabolic processes and variability in hydrodynamism (D'Autilia et al., 2004).

1.2 Environmental Fluctuations and Ecological Plasticity

1.2.1 Environmental Fluctuations

Marine ecosystems are fundamentally dynamic, with environmental drivers such as temperature, dissolved oxygen, salinity and pH exhibiting natural diel and seasonal fluctuations that deeply impact animal physiological processes and behavioral responses (Bernhardt et al., 2020; Booth et al., 2021; Fusi et al., 2024, 2023). For example, supersaturation conditions during the day can enhance mollusks', like *Mytilus edulis*, and crustacean', such as *Necora puber*, thermal tolerance (Giomi et al., 2019). Moreover, marine ectotherms exposed to elevated temperatures synthesize heat shock proteins to mitigate cellular damage and physiological disturbance (Fusi et al., 2024).

In most aquatic environments, seasonal fluctuations are occurring naturally in major environmental drivers such as temperature, pH, seawater carbonate chemistry, and dissolved oxygen (Kroeker et al., 2020). These natural fluctuations establish the foundation for species' physiological and behavioral responses, which will be addressed in regard to coastal and transitional environments later on.

Organisms living in fluctuating environments characterized by regular diel and seasonal cycles can exploit the predictability of these variations to anticipate upcoming changes, exhibiting a physiological or behavioral changes before the environmental shift actually occurs. This proactive response is known as a feedforward mechanism (Bernhardt et al., 2020; Fusi et al., 2024; Marshall and Burgess, 2015).

In this context, productive aquatic environments, such as the north Adriatic sea and the Venice Lagoon, show abundant primary producers presence. This generates diel

fluctuations of dissolved oxygen, driven by photosynthesis and community respiration (Fusi et al., 2023). The significant diel and seasonal dynamics of aquatic ecosystems are highlighted by periodic shifts (diel and seasonal) in temperature, pH, and dissolved oxygen (Bernhardt et al., 2020; Wylie and Jones, 1987). For instance, habitats like estuaries and coastal lagoons, such as the Venice Lagoon and coastal euphotic zones, are dynamic habitats with highly fluctuating dissolved oxygen (DO), frequently displaying daily cycles from hypoxia (low DO) to hyperoxia (oxygen supersaturation). These oxygen fluctuations are always followed by temperature fluctuations with higher temperatures at peaks of oxygen saturation and lower temperatures during hypoxia conditions (Guadagnin G., 2021; Ministero delle Infrastrutture e dei Trasporti, 2020).

In coastal ecosystems, such as the Venice Lagoon, salinity is a significant contributor to temporal environmental variation in addition to temperature and oxygen dynamics (Zirino et al., 2014). Complex salinity patterns that can change at diel, seasonal, and interannual periods are produced in lagoonal systems by tidal mixing, river inputs, and meteorological conditions. This variability affects ecosystem resilience to anthropogenic and climatic stressors, controls species interactions, and shapes habitat structure (Zirino et al., 2014), since species must adapt their physiological processes to maintain homeostasis in constantly fluctuating conditions (Guerin and Stickle, 1992). Salinity gradients, for example, controls plant zonation in salt marshes by altering their habitats and influencing distribution of species such as *Limonium narbonense*, *Salicornia veneta*, *Salicornia fruticosa* due to the different salinity tolerances (D'Alpaos and D'Alpaos, 2021).

Since environmental variability significantly influences physiological performance and behavioral patterns, examining how species adapt to these natural variations offers important insights into their capability for adaptation as well as the general resilience of coastal ecosystems under climate change. These environmental fluctuations are directly linked to external physical and biological processes that operate on diel and seasonal scales (Bernhardt et al., 2020; Fusi et al., 2024).

Light and temperature cycles produce daily patterns of oxygen fluctuation in the euphotic zones: oxygen supersaturation occurrences during the day, caused by active photosynthesis, and oxygen decreasing conditions during the night, due to absent photosynthetic activity and the prevalence of the heterotrophic respiration (Fusi et al., 2024). Photosynthetic activity produces an abundance of oxygen during the day, potentially reaching supersaturation levels, whereas animal and microbial respiration takes over at night, pushing the system toward undersaturation or even hypoxia (Booth et al., 2021). The availability or the absence of light, which promotes or inhibits photosynthesis, and temperature, which influences metabolism and physiological activity, are intimately related to this oxygen production. The normal amplitude of dissolved oxygen oscillations is amplified by higher temperatures as they raise metabolic needs while decreasing oxygen solubility (Booth et al., 2021; Guadagnin G., 2021).

Shallow water ecosystems, such as lakes, estuarine and coastal habitats, are influenced by solar radiation that drives temperature fluctuations. Solar radiation directly impacts the temperature and stratification patterns of the water, increasing surface warming

during the day and affecting the system's pH fluctuation, oxygen solubility, and metabolic rates (Piccioni et al., 2021).

Although these fluctuations of temperature and dissolved oxygen are natural, climate change is exacerbating their magnitude and frequency: anthropogenic activities are altering key aquatic parameters (temperature, PH, dissolved oxygen, salinity), generating extreme events that exceed the normal range of environmental variability (Brown, 2020; Diaz and Rosenberg, 2008; Pörtner, 2010; Raven and Falkowski, 1999).

Transitional ecosystems like coastal lagoons are biodiversity hotspot and they are especially vulnerable to extreme events and climatic change. They are important ecosystems for evaluating the biological effects of climate change because of their high vulnerability to temperature, salinity, and hydrological variability (Lucena-Moya et al., 2012).

The stability of biological communities is disrupted by the alteration of these environments, which affects floral and faunal assemblages and eventually undermines the resilience and ecological balance of Mediterranean coastal lagoons (Ligorini et al., 2023).

1.2.2 Behavioural and Physiological Adaptations

Behavioral regulation is essential for aquatic organisms to adapt to changing conditions in transitional environments, where environmental factors like temperature, oxygen, and salinity show significant diel and seasonal change. Individuals can mitigate environmental stress and sustain functional performance under fluctuating oxygen and temperature regimes by movement, activity regulation, and microhabitat selection (Claireaux and Chabot, 2016).

Within transitional and coastal ecosystems, habitat heterogeneity offers natural refugia that can protect creatures from oxygen and heat stress in addition to individual behavioral changes. Macroalgae, seagrass beds, mangroves, and coral reefs serve as climatic refuges that can lessen the impact of rising temperatures on marine life (Gunderson and Stillman, 2015).

Environmentally driven changes in fitness are controlled by direct effects on physiological processes and behaviour, which are closely linked. Elevated temperatures and CO₂, either alone or in combination, have been shown to alter behavior in sea hares, including longer time to foraging, poor foraging decisions, slower locomotion, and an increase in metabolic rate (Horwitz et al., 2020).

Ectotherms usually experience an increase in metabolic rate when the ambient temperature rises, which reflects the rate-enhancing effects of temperature on biochemical reactions (Horwitz et al., 2020).

Thermal stress is frequently exacerbated in transitional conditions by the simultaneous action of several stressors. Ambient hypoxia and elevated CO₂ (ocean acidification) aggravate oxygen- and capacity-limited thermal tolerance (OCLTT) by increasing hypoxaemia, low O₂ concentration in blood, constricting the thermal performance window, and diminishing the organism's ability for thermal acclimation. The combined effect of elevated temperature, hypoxia, and CO₂ has been described as the "deadly trio" due to its major effects on physiology and survival (Pörtner, 2021, 2010).

Individuals with behavioral plasticity can react quickly to environmental variety, mitigating short-term effects on fitness. Recurrent exposure to such changes may promote evolutionary adaptation over a longer time scale, leading to populations that are more resilient to stress from heat, hypoxia, or salinity. Therefore, adaptation and acclimatization are complementary processes that allow survival in changing transitional situations (Bernhardt et al., 2020).

Through adaptive behavioural plasticity, individuals may be able to buffer the impact of heterogeneous environments, consequently diminishing intense directional selection pressures and encouraging adaptation in altered or new habitats (Robinson and Dukas, 1999). Many mobile fishes are able to detect large, hypoxic areas and exhibit behaviour to track favourable ranges of temperature and oxygen in order to move there (Kroeker et al., 2020). Moreover, intertidal sea stars are able to fill their body with seawater to resist extreme temperatures (Kroeker et al., 2020).

1.2.3 The concept of Ecological Plasticity

The phenotype of an organism results from a continuous interaction between genetic information and environmental influences (Pelster and Burggren, 2018).

For a species to thrive in a fluctuating environment, a certain level of phenotypic plasticity is necessary (Pelster and Burggren, 2018).

Phenotypic plasticity is the capacity of organisms to modify their physiology and behavior in response to changing environmental conditions. This adaptability allows animals to survive in dynamic environments, but it may come at the expense of energy or produce suboptimal phenotypes in situations that change quickly (Pelster and Burggren, 2018).

In order to improve fitness, organisms that experience regular and predictable cyclical environmental variations, such as diel cycles of temperature and oxygen in coastal habitats, can rely on feedforward mechanisms, which use environmental cues (e.g. light-dark cycles) to predict these changes and anticipate upcoming metabolic demands, subsequently modifying their physiological and behavioral traits accordingly (Bernhardt et al., 2020; Fusi et al., 2024). The more consistently these fluctuations repeat in regular time, the more efficiently these organisms can exploit the environment to optimize their performances, both physiological and behavioural (Bernhardt et al., 2020; Fusi et al., 2024).

One important aspect influencing the evolution of phenotypic plasticity is environmental predictability: plastic responses are favored when environmental cues reliably indicate future conditions; on the other hand, large plasticity may have fitness costs or become maladaptive in extremely unpredictable systems (Reed et al., 2010).

The concept of environmental predictability describes how organisms are able to anticipate changes and modify their physiological processes and life-history strategies when environmental variability is high yet predictable, as through regular seasonal or diel cycles. This predictability improves ecological performance and shapes adaptive trajectories during evolutionary time by facilitating the synchronization of biological responses with recurring environmental variables (Marshall and Burgess, 2015).

Halali et al. (2021) reveal that populations that live in habitats with high periodicity and consistent cues exhibit more phenotypic plasticity, while populations that live in unpredictable, seasonal conditions typically exhibit less plastic responses. Environments with fluctuating conditions that repeat in cycles, are characterized by populations that tend to evolve greater plasticity, enabling them to cope with a broader range of environmental gradients and enhancing their ecological resilience (Sekajova et al., 2025). This ecological plasticity is a strong advantage for animals living in fluctuating environments, while organisms from stable environments frequently develop more specialized but less flexible traits (Sekajova et al., 2025).

The rate of adaptive phenotypic plasticity is thought to determine an individual's fitness cost in response to environmental change. Analyses on ectotherms show significant variation in this trait's plasticity rates among species, with half-times, the amount of time needed for a species to achieve 50% of its overall phenotypic change, ranging from 3.7 to 770.2 h, with amphibians and reptiles having higher rates than fish and crustaceans, while insects exhibit intermediate rates (Einum and Burton, 2023).

According to Davidson et al. (2011), invasive species generally exhibit higher degrees of phenotypic plasticity than co-occurring non-invasives, This finding supports the hypothesis that increased ecological plasticity enhances a species' ability to adapt to new or changing habitats. Because of their adaptability, they can change their morphological, physiological, and behavioral characteristics under a wider variety of circumstances, which makes it easier for them to colonize and survive in disturbed or transitional habitats (Davidson et al., 2011). The wider phenotypic variety of invasive species promotes resistance to environmental change and extreme events, whereas non-invasive species may retain greater fitness homeostasis under resource limitation. Therefore, ecological flexibility serves as a resilience mechanism as well as a major factor in invasiveness, enabling species to outcompete more specialized taxa and take advantage of unexpected circumstances (Davidson et al., 2011).

1.3 The Venice Lagoon as a model system

The Venice Lagoon is a complex and heterogeneous system which is subject to multiple intense natural and anthropogenic stressors that have altered its morphology, biological communities and also bio geochemistry (Solidoro and Bandelj, 2010). Industrial and agricultural activities since the 1940s have caused pollution, sediment contamination, and eutrophication leading to macroalgal blooms (such as *Ulva sp.*) and recurring hypoxic crisis during the 1980s (Solidoro and Bandelj, 2010). Ecological shifts were triggered by these events, bringing loss of biodiversity and dominance of tolerant species (Solidoro and Bandelj, 2010). Mitigation measures such as phosphorus reduction and harvesting of macroalgae, promoted recovery and oligotrophication in the 1990s. In addition, intense fishing of the commercial species of *Ruditapes philippinarum* strongly disturbed sediments, changing benthic structures due to highly destructive gears employed like dredging (Marchini et al., 2015; Solidoro and Bandelj, 2010). More recently, this environment has been characterized by declining inputs of nutrients, with reduced primary productivity as a consequence (Solidoro and Bandelj, 2010). Climate driven

hydrological changes and sea level rise may furtherly reshape the habitat distributions and their trophic dynamics (Solidoro and Bandelj, 2010).

Additionally, the Venice Lagoon environment is highly characterized by a multitude of ecosystem services (Rova et al., 2022). This area is a complex socio-ecological system that requires an integrated and sustainable management to preserve it (D'Alpaos and D'Alpaos, 2021; Rova et al., 2022). Experts have evaluated the ecosystem services, indirect or direct benefits for humans deriving from natural environments, present in this area to prioritize the ones essential for lagoon preservation (D'Alpaos and D'Alpaos, 2021). "Biodiversity and landscape" was defined as the most important ecosystem service, followed by "nursery habitat for fisheries" and "carbon storage" (D'Alpaos and D'Alpaos, 2021). This emphasizes the ecological and economical relevance of the salt marshes systems (D'Alpaos and D'Alpaos, 2021).

The Venice lagoon is a transitional environment characterized by intermediate conditions between freshwater habitats (continental) and marine habitats. It is influenced by a combination of natural and anthropogenic factors: solar radiation, meteorological events, freshwater inputs, exchanges with the sea, and internal physical and biological processes (Ministero delle Infrastrutture e dei Trasporti, 2020; Ramieri, 2000; Varrani and Nones, 2018). This multitude of interactions naturally create a strong spatial and temporal variability in the abiotic properties of this environment. Seasonal patterns are evident in temperature, dissolved oxygen, pH and chlorophyll-a, mainly related to solar radiation variations and biological activity (Solidoro et al., 2004). Salinity is mainly influenced by rainfall and water inflow so it shows a weaker pattern in seasonality (Solidoro et al., 2004). Data coming from the SAMANET network highlighted clear differences between the stations, with stations more affected by freshwater inputs while the other closer to the sea inlets show stronger marine influence (Ministero delle Infrastrutture e dei Trasporti, 2020). The North Lagoon has a fluvial regime with comparatively steady flows all year round, which leads to more consistent freshwater imports. The South Lagoon, on the other hand, has more erratic flows because its waters are frequently controlled by weirs and dams, which can cause significant temporal variability in freshwater inflow and affect salinity patterns locally (Ministero delle Infrastrutture e dei Trasporti, 2020).

Transitional ecosystems are strongly affected by climate change and anthropic pressures: rising sea levels and increasing temperatures are expected to amplify natural erosion processes and alter ecological parameters, such as salinity, nutrients, and tidal regimes (Ramieri, 2000; Varrani and Nones, 2018). The Lagoon's structural balance is threatened by these changes, they promote its gradual transformation into a marine bay affecting biodiversity compositions, both vegetation and fauna communities. Moreover, anthropogenic modifications, such as urban and industrial development, have further reduced the natural resilience of this habitat and its capacity to adapt to changes (Ramieri, 2000). Climate driven stressors are compromising the stability of this naturally delicate system (Ramieri, 2000).

The Venice lagoon was chosen as a model system because it is a transitional ecosystem characterized by strong seasonal and diel fluctuations as well as marked spatial heterogeneity. This environment is affected by both natural and anthropogenic stressors

and benefits from comprehensive long-term monitoring data. Overall, it constitutes a highly vulnerable yet well-studied and closely observed ecosystem (D'Alpaos and D'Alpaos, 2021; Ministero delle Infrastrutture e dei Trasporti, 2020; Ramieri, 2000; Solidoro et al., 2004; Varrani and Nones, 2018).

1.4 Invasive Species: a focus on *Callinectes sapidus*

1.4.1 What is an invasive species?

Climate change can influence biological invasions by affecting the introduction, the establishment, and spread of alien species across terrestrial, freshwater, and marine environments (Rato et al., 2021). Ectothermic organisms appeared to be the most responsive due to their physiology, dispersal abilities and association with human activities (Hulme, 2017).

The establishment of non-native species follows a unified framework divided in three phases: Introduction, Establishment, and Diffusion/Impact (Pyšek et al., 2020; Rato et al., 2021). During the first phase, the Introduction, the species are transported out of their natural population range through human vectors (Pyšek et al., 2020). These can be unintentional, such as maritime traffic, ballast waters or artificial waterways, or intentional, such as live seafood trade, ornamental releases, or aquaculture practices (Marchini et al., 2015; Pyšek et al., 2020). The organisms at this phase are referred to as alien species (Pyšek et al., 2020). Later on, the Establishment phase occurs when the introduced species starts to form self-sustaining populations in the colonized environment (Pyšek et al., 2020). This process is largely influenced by phenotypic plasticity and physiological tolerances of the species; they allow better adaptation to local environmental conditions and different stressors (Rato et al., 2021). Lastly, the Diffusion/Impact phase is reached when the individuals starts to exert a measurable ecological or economic impact (oftentimes both) (Pyšek et al., 2020). The expansion can be facilitated by climate change that acts both as driver and passenger of biological invasions, altering species richness, biodiversity and reshaping ecosystems (Pyšek et al., 2020).

Climate change can modify not only which alien species are present and where, but also the ecological consequences of their invasions. If native species become more stressed and weakened due to altered climate conditions, they may become more vulnerable to invasions (Compagnoni and Adler, 2014).

The distribution of species is strongly influenced by global warming of ocean temperature (Pecl et al., 2017). In particular, marine organisms exhibit extensive distributional changes in response to ocean warming: species are moving poleward by 72 km and 18 m into deeper water per decade (Poloczanska et al., 2013).

These latitudinal and bathymetric migrations reflect the influence on species' thermal niches caused by anthropogenic activities and causes the introduction of non-native species into previously unoccupied habitats altering the natural ecological balance of coastal ecosystems (Poloczanska et al., 2013).

Global trade and transport networks are increasing the unintended introduction of exotic species in new regions of the world, with highly connected hubs and frequent movement acting as key pathways for alien species introduction (Banks et al., 2015).

While not all alien species are environmentally problematic, some have the potential to disrupt the local habitats and consequently become invasive, altering native communities, ecosystem functions or outcompete local species (Persson, 2023).

For example, the introduction of non-native the red algae *Gracilaria vermiculophylla* in the Lagoon of Venice has locally reduced eutrophication effects (Marchini et al., 2015).

During the last few decades, the Mediterranean and southern European waters have seen a rapid increase in non-indigenous flora and fauna (Coll et al., 2010).

One of the most disruptive species of the Mediterranean sea is *Caulerpa cylindracea*, a green algae capable of forming dense mats that reduces dissolved oxygen in sediments while suffocating native *Posidonia oceanica* meadows (Piazzi et al., 2016).

Moreover, the puffer fish *Lagocephalus sceleratus*, capable of altering the trophic networks and competing with native species, it is also responsible for many tetrodotoxin poisoning that are currently threatening human health and generating loss in the fishing field (Bentur et al., 2008).

But most importantly, the focal species of this study, the blue crab *Callinectes sapidus* which is currently disrupting the Italian Adriatic coastal trophic networks by eating native crab species and impacting clam aquaculture system, leading to significant losses in both biodiversity and in human economies (Mancinelli et al., 2017).

Firstly recorded in 1949 in the Venice Lagoon (Marchessaux et al., 2024), *C. sapidus* was most probably introduced by ballast waters, as maritime traffic is one of the most plausible sources of introduction in this area (Mancinelli et al., 2017). After this detection until 2015, this species was classified as established but rare, showing limited distribution and restricted to certain habitats in the lagoon (Marchini et al., 2015). However, in the following years, the species rapidly expanded its population across the Adriatic Sea exhibiting clear invasive behaviour (Marchini et al., 2015). This species is considered as highly abundant in the Venice Lagoon, where it acts as a dominant species by altering benthic habitats and massively preying native species (Rifi et al., 2023). It is described as one of the most emblematic events of biological invasions occurring in The Mediterranean Sea (Marchini et al., 2015).

1.4.2 *Callinectes sapidus*: what are the drivers of its success?

The blue crab *Callinectes sapidus*, native to the western Atlantic from Nova Scotia to Argentina, was introduced to European waters during the early 20th century and is now widespread in the Mediterranean and Black Seas (Mancinelli et al., 2017).

In particular, records from the FAO agency have shown the presence of *C. sapidus* from 1947 in the Aegean sea (Serbetis, 1959). In 1949 there were the first records of *C. sapidus* in the Venice Lagoon (Marchessaux et al., 2024).

As an ectotherm animal, *C. sapidus* physiological performance is highly dependent on external water temperature (Marchessaux et al., 2024). This parameter is capable of

affecting population dynamics and spatial distribution by influencing the growth rate and survival of this species (Molina et al., 2021).

This species is characterized by a wide range of tolerances that makes it able to thrive in many environments, especially coastal lagoons and estuarine habitats (Glandon et al., 2019; Rato et al., 2021).



Figure 1. Adult male *Callinectes sapidus* specimen has a PULSE V2 (© 2020 ELECTRICBLUE) sensor applied to its carapace for the purpose of detecting heart rate (BPM), as described later in the Materials and Methods section.

The blue crab is widely categorized as an eurythermal and euryhaline organism, characterized by a wide array of temperature and salinity tolerances that makes it able to colonize a multitude of different habitats. (Herrera et al., 2024; Marchessaux et al., 2024; Rato et al., 2021)

Habitats like estuaries and coastal lagoons, such as the Venice Lagoon, are dynamic habitats with highly fluctuating dissolved oxygen (DO), frequently displaying daily cycles from hypoxia (low DO) to hyperoxia (oxygen supersaturation). These oxygen fluctuations are always followed by temperature fluctuations with higher temperatures at peaks of oxygen saturation and lower temperatures during hypoxia conditions (Guadagnin G., 2021; Ministero delle Infrastrutture e dei Trasporti, 2020).

Such environmental variability demands high physiological flexibility, and the phenotypic plasticity of *C. sapidus* represents one of the key traits enabling its persistence and success in these fluctuating habitats (Azra et al., 2020; Rato et al., 2021).

Regarding its thermal tolerance, *C. sapidus* is able to tolerate temperatures between 0°C and 40°C, meaning that temperature performances are not a limiting factor for its expansion in the Mediterranean sea (Marchessaux et al., 2022).

The optimal temperature for metabolic performance in Mediterranean waters is considered to be 24°C, which closely aligns with the temperature range of 22-23°C where this species reaches its highest global density (Marchessaux et al., 2022).

The critical thermal maximum (CT_{max}) for Mediterranean populations reaches 40°C (Marchessaux et al., 2022), while some tropical populations have shown an even higher upper limit, up to 41.4°C (García-Rueda et al., 2021).

C. sapidus also shows phenotypic plasticity in its thermal limits, showing increased critical temperature with higher acclimation temperature (García-Rueda et al., 2021).

The efficiency of aerobic metabolism in aquatic organisms is characterized by their oxygen consumption rate (MO_2); it serves as a proxy for measuring the physiological response to environmental dissolved oxygen (Pörtner, 2010). Aquatic species are divided into two separate categories depending on how they manage oxygen uptake in response to oxygen partial pressure (PO_2): oxyconformers, which exhibits a proportional decrease in oxygen consumption as water PO_2 decline, and oxyregulators, capable to maintain stable oxygen consumption within a wide range of PO_2 until a critical point (P_{crit}) is reached. P_{crit} divides aerobic from anaerobic metabolism: whenever PO_2 is higher than P_{crit} , aerobic metabolism predominates, when PO_2 drops below P_{crit} species switches to anaerobic metabolism (Pörtner, 2010).

C. sapidus was considered to be strongly susceptible to hypoxia conditions but recent research has categorized it as an oxygen regulator organism: it is able to maintain a constant aerobic metabolic rate until a critical oxygen saturation level is reached (Brill et al., 2015). The P_{crit} of this species, the oxygen saturation percentage under which the animal cannot compensate and uses anaerobic metabolism, is considered to be around 15-20% oxygen saturation for the specimens acclimated at 17-23°C, reaching 36% of oxygen saturation for the organisms acclimated around 28°C (Brill et al., 2015).

The metabolism of tropical *C. sapidus* populations can be at least partially controlled across a fairly broad range of dissolved oxygen levels. However, as would be expected for a tropical species, this capacity is limited to high temperatures (García-Rueda et al., 2021). This species also shows a behavioural response to low dissolved oxygen in waters, particularly at DO equal or lower than 2 mg / L, the animals move to shallower and better oxygenated water (Brill et al., 2015). Other behavioural responses to hypoxia is burrowing: this species exhibits a burrowing behaviour reducing their metabolic activity and energy expenditure and remaining relatively inactive until oxygen levels improve (DeFur et al., 1990).

These traits highlight the generalist behavior of the species, explaining both its invasive potential and its success in colonizing Mediterranean habitats like the Venice Lagoon (Azra et al., 2020; Rato et al., 2021).

Its winter survival rate is believed to be positively impacted by the continuous warming trend in Mediterranean coastal waters (Ulbrich et al., 2013), which fosters greater fitness and subsequent expansion. Furthermore, the invasive potential of this species is strictly

tied with its high phenotypic plasticity that allows them to rapidly adjust to new environmental conditions (Azra et al., 2020; Rato et al., 2021).

2 AIM OF THE STUDY (ABSTRACT)

As the invasive blue crab *Callinectes sapidus* rapidly expands over European coastal habitats, its capacity to adapt to shifting environmental conditions may be essential to its success (Marchessaux et al., 2022). In this study, we compared constant laboratory conditions with fluctuating conditions which reproduce the natural diel and seasonal fluctuations typical of the euphotic zones in the Venice Lagoon (Ministero delle Infrastrutture e dei Trasporti, 2020), in order to assess their potential effects on metabolic performance. Most importantly, the research focused on temperature and dissolved oxygen, considered the main environmental driver of physiological and metabolic processes in marine ectotherms (Booth et al., 2021; Pörtner, 2010; Semsar-kazerouni and Verberk, 2018). Metabolic rates were measured through closed chamber respirometry, while a temperature-dependent mortality curve was used to assess cumulative heat tolerance. Potential feedforward mechanisms linked to environmental predictability were investigated by examining how oxygen consumption rates varied according to diel fluctuations. Our results identify statistical differences between fluctuating (natural) regimes and constant conditions in terms of metabolic responses and mortality, emphasizing the importance of environmental heterogeneity in shaping the environmental plasticity of the blue crab *C. sapidus* and providing insights to its ability to thrive under ongoing climate change. By focusing on naturally occurring fluctuations, the current study demonstrates how realistic fluctuations in the environment can influence metabolic processes and illustrates that constant laboratory settings could fail to accurately represent the physiological responses of organisms. Moreover, this particular approach allows us to evaluate how *C. sapidus* is able to respond to extreme events, providing insights into the resistance and resilience of this invasive species.

3 MATERIALS AND METHODS

All the experiments were conducted at the Stazione Idrobiologica “U D. Ancona” (Chioggia, VE, Italy) of the University of Padova between March and July 2025. Seventy-two adult male *Callinectes sapidus* individuals were tested in both natural fluctuating conditions and controlled laboratory settings.

3.1 Species

The blue crab *Callinectes sapidus* is an invasive species of the Lagoon of Venice. The individuals used in this experiment were male adults in order to avoid variability and uncertainties due to different life stages of the animals, and potential confounding effects due to additional metabolic demands from egg-bearing females.

This species exhibits sex-based spatial segregation: females migrate along salt gradients and frequently head toward locations with higher salinity throughout their reproductive cycle, while males tend to stay in transitional habitats (Molina et al., 2021). In order to avoid sex-related bias associated with female reproductive status (or egg production) and different physiological demands, only male crabs were selected to provide consistent physiological responses.

The experimental crabs were collected in the Lagoon of Venice by local fishermen.

3.2 Acclimation Treatment

To test the influence of environmental fluctuations, the acclimation represented the treatment.

3.2.1 Setting the Acclimation period

The acclimation period was set at 6 days and its duration was selected based on preliminary trials and literature on crustacean acclimation indicating this period was enough for blue crabs to adjust their metabolic and oxygen consumption physiology (Guerin and Stickle, 1992). For species subjected to considerable phenotypic plasticity, such as *Callinectes sapidus* (Azra et al., 2020), this is especially crucial, as their metabolic and respiratory responses can rapidly adjust to environmental shifts, and to ensure the results are due to a stable physiological conditions rather than influenced by transitory stress.

The photoperiod was maintained the same for acclimation Treatments depending on the Season, 12:12 h (light : dark) in March and 16:8 h in July.

Dissolved oxygen and temperature were the two monitored parameters of this acclimation process, and they were continuously recorded using miniDOT (PME Inc © 2021).

During the acclimation treatments, the individuals were regularly monitored to ensure their well-being and their diet consisting of mussels. Crabs were fed only one time on the third day of the acclimation process and food was removed if not consumed in order to not affect metabolism of the animals and microorganism buildup.

Notably, crabs were subjected to a 24 hours fasting period prior to the experiments in order to adjust the metabolic status of the animals (Brill et al., 2015; Glandon et al., 2019). Eventual dead organisms were removed immediately to maintain optimal conditions.

The acclimation was set for organisms to different conditions. Crabs were maintained under either fluctuating, naturally occurring, conditions or constant conditions throughout this phase. These environmental regimes therefore not only served to prepare the animals for subsequent measurements, but also constituted the core of the experimental treatment.

Two different acclimation processes were implemented, namely Fluctuating Treatment and Constant Treatment. These treatments will be detailed in the two following subsections and aimed to test the influence of environmental fluctuations.

Both the fluctuating and constant treatments were replicated in two seasonal periods, spring and summer, in order to factor in potential differences associated with natural seasonal conditions. This design allowed to repeat the experiments under these treatments while considering the environmental context of each season.

3.2.2 Fluctuating Treatment

Organisms were maintained in an external tank (65 x 35 x 400 cm). A continuous flow of brackish water coming from the Lagoon was pumped directly into the tank. Environmental parameters fluctuate naturally due to Lagoon dynamics and seasonal cycles (Fig. 2a). The focus was on temperature and dissolved oxygen.

These fluctuations were not artificially imposed; by using a direct flow from the Lagoon, they replicated the natural daily and seasonal cycles experienced by *Callinectes sapidus* in the wild, including the natural photoperiod.

Further on, to mimic aspects of the natural habitat while minimizing stress from intraspecific interactions among adult males (Taylor and Eggleston, 2000), the external tank was enriched with longitudinally cut PVC pipes and bricks, providing shelter resembling natural crevices. Algae were added to the tank to help maintain natural fluctuations in dissolved oxygen, supporting a more ecologically relevant environment.

Additionally, a transparent plastic sheet was fastened over the tank with ropes. This served to limit oxygen re-aeration from the air, ensuring that natural fluctuations in dissolved oxygen were maintained, and to avoid the escape of crabs.

Water parameters (temperature, dissolved oxygen) were continuously monitored using two MiniDOT Loggers (PME Inc © 2021), positioned at the two opposite ends along the longitudinal axis of the external tank. This instrument was adjusted so that the optical sensor did not touch the bottom of the tank while ensuring that measurements were taken at the same depth as the organisms. The sampling interval is set at 1 minute following the instruction on the User Manual (Manual MiniDOT Clear, 2020). This allowed the detection of natural fluctuations while ensuring no extreme values by frequently checking the signal. To take note of the recorded the parameters, the miniDOT Logger was removed from the tank daily. Any temperature and dissolved oxygen values corresponding to the moments when the miniDOT was out of the water were excluded from the dataset, ensuring that only the data coming from actual water measurements were used in the statistical analysis.

By connecting the miniDOT Logger to a computer it is possible to see that three programs are available for data-handling:

- miniDOT Concatenate: employed to concatenate multiple data files coming from different days of sampling into a single uninterrupted data set, enabling easier and comprehensive data analysis;
- miniDOT Plot: utilized for visualization of the data into plots as it is being collected, in particular the fluctuations in temperature and dissolved oxygen;
- miniDOT Control: used to configure the logger settings such as the sampling interval, particularly set at 1 minute.

This arrangement simulates near-natural conditions while protecting the crabs, ensuring the assessment on how natural environmental variability affects physiological responses.

3.2.3 Constant Treatment

The Constant Treatment was designed to replicate the average seasonal conditions derived from the Fluctuating Treatment. The baseline values for temperature and oxygen were established by the seasonal averages recorded by the miniDOT during the Fluctuating Treatment and then applied in the Constant Treatment as fixed (Fig. 2b), non-fluctuating conditions.

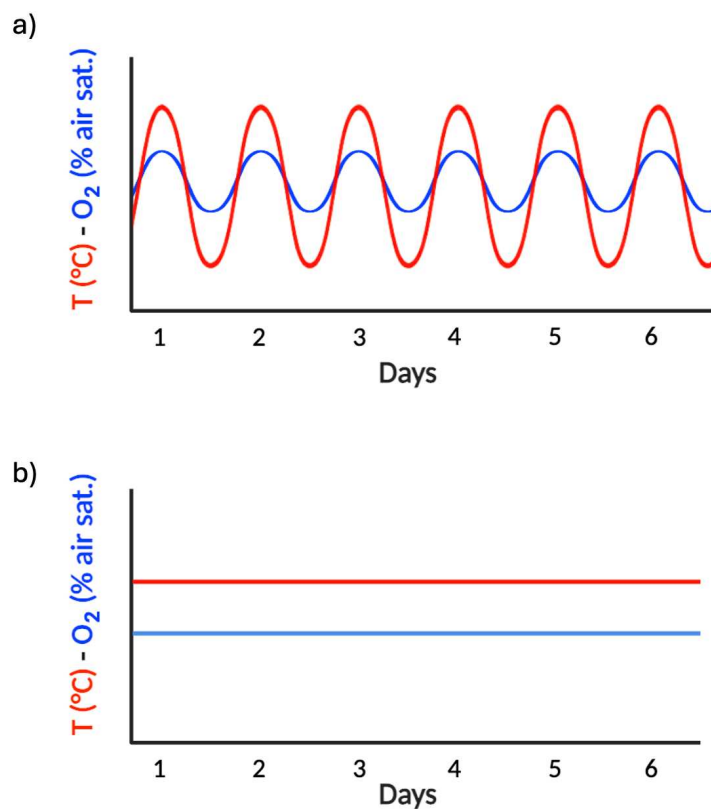


Figure 2. Schematic representation of the Fluctuating (1a) and Constant (1b) Acclimation Treatment. Regarding the Fluctuating Acclimation (Fig. 1a), the acclimation period of 6 days shows the normal fluctuating conditions of oxygen (blue) [%air sat.] and temperature (red) [°C]. On the 7 days from the start of the acclimation treatment, the first experiment is performed: the Temperature-dependent Mortality Curve. Normally, the Respirometry is performed two days after. Regarding the Constant Acclimation (Fig. 1b), organisms underwent a 6 days acclimation period under constant conditions in oxygen (blue) and temperature (red). The average values of temperature recorded during the Fluctuating Acclimation Treatments were used as the baseline values for temperature. On the 7th day the first experiment, the Temperature-dependent Mortality Curve, is performed, followed by the Respirometry experiment two days later.

The Constant Treatment was carried out in four internal tanks (60 x 85 x 40cm). Each tank contained a plastic net mesh that divided the tank into 6 compartments in a 3 x 2 grid (20 x 20cm) (García-Rueda et al., 2021): this prevents intraspecific interaction and aggressive behaviour that would stress the animal and potentially influence the result (Taylor and Eggleston, 2000). The experimental setup was placed into a room controlled by a thermostat connected to a heating/cooling system. The water temperature was kept constant. In addition, each experimental tank had two diffusers installed in order to maintain constant aeration and the oxygen levels were checked daily.

Both dissolved oxygen and temperature were monitored at one-minute intervals using a miniDOT Logger (PME Inc. © 2021) installed in one of the four tanks.

To verify data consistency the HANNA HI 9829 multiparameter probe (<https://hanna.it>) was employed to check whether the temperature and dissolved oxygen levels in the tank constantly monitored by the miniDOT Logger matched the ones in the other three tanks. These measurements were performed daily to ensure that all the experimental tanks maintained identical experimental conditions. As for the fluctuating treatments, the miniDOT Logger was briefly removed daily to monitor the water parameters and the corresponding data points were promptly removed from the statistical analysis dataset to avoid bias.

The feeding schedule and stress reduction practices were the same applied for the fluctuating treatment.

3.3 Temperature Dependent Mortality Curve

To evaluate the thermal sensitivity and tolerance to heatwaves conditions of *Callinectes sapidus* a Thermal Ramp experiment was performed. This progressive heating protocol allows for the assessment of mortality thresholds and physiological measurements, thereby giving a standardized measure of thermal stress under gradually increasing temperature (Giomi et al., 2019; Giomi and Poertner, 2013; Li et al., 2015).

3.3.1 Measured Indicators

Two main physiological and mortality-related indicators were investigated to evaluate the responses of *C. sapidus* under different thermal conditions:

- Lethal Temperature for 50% of individuals - LT_{50} : obtained from the cumulative mortality data using a sigmoidal curve fit. The LT_{50} is a physiological indicator representing the temperature at which half of the individuals within a given population or species perish after exposure for a defined period. It is commonly employed as a parameter of thermal tolerance in individuals (Giomi et al., 2019).
- Heart rate (BPM): continuously monitored in real time using the PULSE V2 logger (© 2020 ELECTRICBLUE), providing sub-second resolution of cardiac activity.
- Temperature coefficient (Q_{10}): indicates the factor by which a biological response (in this case BPM) changes for every 10°C increase in temperature (Zainal and Noorani, 2019). It is calculated using values measured at two different temperatures, as per the following equation (1):

$$Q_{10} = (R_2 / R_1)^{10^\circ C / (T_2 - T_1)} \quad (1)$$

with R_2 and R_1 representing the average of BPM at corresponding temperature T_2 and T_1 (Zainal and Noorani, 2019), collected by PULSE V2 throughout the experiment.

These variables lead to assessing the thermal tolerance and physiological responses of *Callinectes sapidus*.

3.3.2 Thermal ramp

During this experiment, the specimens are subjected to a thermal ramping protocol (Giomi et al., 2019) to evaluate temperature dependent mortality. After each acclimation treatment, 10 crabs were employed, divided in two tanks (60 x 85 x 40cm) with five individuals each. The plastic net mesh was configured as described for the Constant Treatment.

Water was continuously aerated through air stone diffusers (as described for the Constant Treatment), and the HANNA HI 9829 multiparameter probe (<https://hanna.it>) monitors environmental conditions such as temperature and dissolved oxygen for the entire duration of the experiment.

Each one of the ten crabs used in the experimental setup was equipped with an optical sensor from the PULSE V2 heart frequency logger. The device is able to monitor continuously (at sub-second resolution) the cardiac activity of mollusks and crustaceans by recording the heart rate of a maximum of ten organisms simultaneously. The system used non-invasive IR (infrared) sensors that were glued to the exoskeleton directly above the heart. The variations of the IR sensor, calculated in the amount of IR light reflected during each heartbeat, are subsequently converted into electric signals processed independently by each of the ten channels. Data coming from these animals were continuously displayed in a 3,5" LCD screen and immediately stored as CSV files that can be imported in Excel, MATLAB or R Studio for statistical analysis. This method not only constitutes a non-invasive method for heart rate evaluation of mollusks and crustaceans but also real time monitoring of physiological performance without harming the individuals (<https://electricblue.eu/pulse, n.d.>).

The starting temperature for each of the four trials was based on the average seasonal temperature recorded during the Fluctuating Treatment, which represented the natural environmental conditions. For example, during the first spring trial, crabs in the Fluctuating Treatment experienced an average temperature of 12°C, as measured by the miniDOT logger during the acclimation period, while the summer Fluctuating Treatment showed an average temperature of 24°C. This measured seasonal average is not only applied as the baseline for the thermal ramping protocol but also applied as the baseline for the Constant Treatment, ensuring that differences between treatments are solely due to temperature variability rather than differences in mean temperature.

Prior to the start of the thermal ramping protocol, crabs were allowed a 30-minute acclimation period to adjust to the new environment and ensure the correct measure of PULSE V2.

Once the tanks reach the baseline temperature, three electric heaters were inserted into each tank. The temperature was then increased at a constant rate of 1°C per half an hour until 42°C, temperature at which all individuals reach mortality (Giomi et al., 2019).

Individuals' mortality was evaluated by a combination of behavioural and physiological criteria: loss of response to external stimuli, immobilization of the animal, and cessation of cardiac activity as shown by the PULSE V2 sensor. Every time an organism died, morphological measures were acquired: carapace length (cm), carapace width (cm), and wet weight (g) using a precision balance. Furthermore, the exact temperature at which the organisms died was recorded in order to calculate thermal tolerance criteria such as LT_{50} .

Each experiment was conducted across different seasonal treatments, allowing the investigation of both treatment type (Fluctuating vs Constant) and seasonality (spring vs summer) on thermal tolerance.

The resulting data were then analyzed to calculate the LT_{50} values via MATLAB, the temperature at which 50% of the specimens die. The data were later visualized through a sigmoidal curve calculated by R coding, showing cumulative mortality depending on temperature and calculating LT_{50} . Scatterplots were employed to display cardiac activity (BPM) using data coming from the PULSE V2 sensor, enabling the evaluation of physiological responses in thermal stress conditions.

The Q_{10} values were calculated using the average BPM measured at two selected temperatures. To highlight how this coefficient varies across different temperature ranges, the calculation is repeated at intervals of 5°C. Data processing, including the calculation of mean BPM and corresponding Q_{10} values, were obtained using Microsoft Excel. This allows us to assess temperature sensitivity of cardiac output across the thermal gradient applied in the experiments. This calculation was performed in each of the four different experimental setups, accounting for both Treatment (Fluctuating and Constant) and Seasons (Spring and Summer).

2.4 Closed Respirometry system

Respiratory measurements were conducted using a closed-respirometric system, in which each individual was placed in a sealed glass chamber to monitor physiological and metabolic responses under controlled conditions (Laird and Haefner, 1976; Seibel et al., 2021). The temperature of the water within each chamber matched the respective seasonal acclimation temperature, being set at 12°C for spring and 24°C for summer trials.

2.4.1 Measured Indicators

Two key physiological and metabolic indicators were investigated during the closed system respirometry experiments:

- Oxygen consumption rate MO_2 ($mgO_2 h^{-1} g WW^{-1}$): calculated from the decline in dissolved oxygen concentration ($mg O_2 L^{-1}$) over time in each closed respirometry chamber and normalized to individual body mass (g).
- Heart rate BPM ($beat min^{-1}$): continuously monitored with the PULSE V2 logger, providing continuous resolution of cardiac activity.

These indicators provide an integrated assessment of metabolic demand and physiological performance of *Callinectes sapidus* under different experimental conditions (Angilletta et al., 2010; Marchessaux et al., 2022) (Fluctuating vs Constant treatments, morning vs afternoon, spring vs summer).

2.4.2 Experimental Design

Four crabs were used for each trial. Measurements were repeated twice a day: in the morning at 10:30 AM and in the afternoon at 4:30 PM. These times of day were selected because they approximately represent the periods when temperature and dissolved oxygen reach their daily average values within the natural diel fluctuations of the Venice Lagoon and so in order to exclude biases in respiration given by values of these parameters different from their average. Morning measurements were performed at a time when metabolic activity is expected to increase, possibly leading to higher oxygen consumption, whereas the afternoon measurements were conducted when metabolic activity slows down and oxygen consumption may decrease.

Animals were tested after each acclimation treatment in both seasons.

Each crab was placed individually in a closed respirometry chamber with a volume of 3.3 liters. The closed respirometry chambers were cylindrical and made of glass, they were sealed with a wooden lid and a gasket to prevent water leakage. Each respirometry chamber was hermetically sealed, allowing only the passage of heat while preventing any water exchange with the surrounding tank. The four chambers were then placed in an individual plastic tank containing water maintained at 12°C or 24°C, thanks to a chiller. The chiller circulates water through a pump system that can continuously check for the temperature inside the plastic tank filled with freshwater and subsequently heat or cool the water as needed. The constant temperatures maintained were 12°C in spring and 24°C in summer.

A small hole of approximately 1 cm in diameter is cut on top of the wooden lid to allow the insertion of the PULSE V2 sensor. A piece of tape was used to seal the chamber to prevent external air entrance and avoid potential interference with the respirometric measures.

Inside each respirometry chamber, a small magnet stir was placed on the bottom. This magnet rotates in response to an external Vortex, ensuring uniform mixing of the water and homogeneity in oxygen distribution. This is particularly relevant since crabs tend to remain at the bottom of the chamber, and homogeneous mixing ensures that the PyroScience Firesting-O₂ records a representative measure of the entire system by circulating water evenly throughout the respirometry chamber (<https://www.pyroscience.com, n.d.>).

Dissolved Oxygen concentration and cardiac activity were continuously monitored using two instruments:

- PULSE V2
Each crab was equipped with a PULSE V2 heart frequency logger (<https://electricblue.eu/pulse, n.d.>), which records heart rate through non-invasive IR (infrared) sensor attached with glue to the exoskeleton of the

crustacean, directly above the heart. This instrument continuously detects heart rate and provides results about cardiac activity.

- PyroScience Firesting-O₂
Simultaneously, oxygen concentration was measured with the employment of a PyroScience Firesting-O₂ (<https://www.pyroscience.com>, n.d.). This instrument operates by using four fiber-optic cables, each connected to an optical oxygen sensor externally attached to the glass wall of a respirometry chamber. It measures dissolved oxygen concentration (% air sat.) providing results in real time in a PC that needs to be connected to the instrument. Readings were recorded at a 30 second sampling interval. After the experiment, the PC program installed provides a series of data: the data are written in a text file that can be later used by the statistical analysis.

Before the trial starts, crabs were acclimated for a period of 30 minutes in the respirometry chambers. Each measurement continued until the concentration of oxygen reaches 0% in all the four chambers.

Data are then normalized per gram of body mass during the data analysis which encompasses both oxygen consumption and heart rate.

The resulting data allow assessment of oxygen consumption and physiological responses under the two different treatments (Fluctuating vs Constant), time of the day (morning vs afternoon) and seasonal conditions (spring vs summer).

3.6 Statistical Analysis

The statistical analyses were performed using a combination of Microsoft Excel, MATLAB (© 1994–2025 The MathWorks, Inc.), and RStudio (© The R Foundation, 2025). The workflow and methodology are described below.

3.6.1 Temperature Dependent Mortality Curve

The raw data obtained during the Thermal Ramp experiments were first organized and cleaned in Microsoft Excel. Outliers were identified and removed when necessary, and preliminary checks were performed.

Subsequently, cumulative mortality data were imported into RStudio. Scatter plots have been developed to visualize cumulative mortality trends across the combinations of all four treatments (Constant Treatment - Fluctuating Treatment) and seasons (spring - summer). Logistic regression models were fitted to the mortality data using the nlsLM function in R studio. These sigmoidal curves allowed to extrapolate the LT₅₀ (Lethal Temperature for 50% of organisms), corresponding to the inflection point of the fitted logistic function.

Heart rate (BPM) data were extracted from PULSE V2 signals. MATLAB scripts identified peaks using findpeaks function, and one-minute segments were sampled every ten minutes (Zainal and Noorani, 2019). Peaks were counted to derive BPM values for each individual channel.

The MATLAB scripts also included graphical inspections and dataset organization steps, which were not central to the analysis and served as quality control.

Finally, BPM values were examined in R using linear mixed-effects models. Temperature, season, treatment, and their interactions were included as fixed effects. The individual (channel) was considered as a random factor to account for repeated measures (each channel represents an individual). Fixed effects were tested using Type III ANOVA to evaluate the significance of the difference of experimental factors, the “CH” (individual organism) is treated as a random factor. The Satterthwaite’s method was applied to estimate degrees of freedom, as it provides more accurate p-values in unbalanced data (different number of total BPM) and when multiple interaction terms are included.

All MATLAB and R scripts used for this analysis are provided in the Supplementary Appendix (Section 8.1).

3.6.2 Closed System Respirometry

In order to calculate oxygen consumption rates normalized to the organism's wet weight, PO₂ data were first obtained from the PyroScience FireSting-O₂ system, which records oxygen concentration as percentage of air saturation PO₂ (% air sat.) and then elaborated into oxygen consumption MO₂ (mg L⁻¹). The following formulas were adapted from García-Rueda et al., 2021; Laird and Haefner, 1976; Marchessaux et al., 2022.

- $PO_2 \text{ (mg L}^{-1}\text{)} = (PO_2 \text{ (\% air sat.)}/100) * (14,6244 - 0,367134 * T + 0,0044972 * (T^2) - 0,0966 * Sal + 0,00205 * T * Sal) + 0,0002739 * (Sal^2)$
where T is the average temperature (°C) and Sal is salinity (PSU).
- $MO_2 \text{ (mg L}^{-1} \text{ hour}^{-1}\text{)} = (PO_2 (t_1) - PO_2 (t_2)) * 120$
PO₂ (t₁) and PO₂ (t₂) are the oxygen concentrations (mg L⁻¹) at time 1 and time 2 respectively, with a 30 second interval between the two measurements. The multiplication factor 120 accounts for the 30-second sampling interval of the PyroScience system (i.e., 120 intervals per hour).
- $MO_2 \text{ (mg hour}^{-1} \text{ g}^{-1}\text{)} = MO_2 \text{ (mg L}^{-1} \text{ hour}^{-1}\text{)} * V(L) WW(g)^{-1}$
where V is the volume (L) of the closed respirometry chamber and WW is the organism’s wet weight (g).

Oxygen partial pressure (PO₂) data from the PyroScience FireSting-O₂ and the corresponding oxygen consumption (MO₂) of each individual was merged in a dataset and imported into MATLAB.

The effectiveness of aerobic metabolism in aquatic species is reflected in their oxygen consumption (MO₂), which also serves as a crucial indicator of how their bodies react to the availability of oxygen in their surroundings. The way their metabolism manages oxygen consumption divides aquatic animals into two categories: oxyregulators and oxyconformers (Pörtner, 2010). These categories describe how animals control oxygen absorption in response to variations in ambient oxygen partial pressure (PO₂). While oxyconformers consume oxygen proportionally as oxygen partial pressure diminishes, oxyregulators are able maintain a consistent oxygen consumption rate over a broad range of PO₂ values until they hit a critical threshold (P_{crit}), below which anaerobic metabolism starts (Pörtner, 2010). The blue crab *Callinectes sapidus* is considered to be an

oxyregulator (Brill et al., 2015; García-Rueda et al., 2021), which justifies the use of this animal to describe its oxygen consumption dynamics.

This piecewise linear function was used to describe the relationship between oxygen partial pressure (PO_2) and oxygen consumption rate (MO_2) in oxyregulators:

$$(x < a) * (b + c * x) + (x \geq a) * (b + c * a)$$

Two different metabolic regimes are defined by this equation (Ultsch and Regan, 2019). The oxygen-dependent phase (anaerobic) is shown in the linear decrease in oxygen consumption with decreasing MO_2 for PO_2 values below the critical oxygen tension (P_{crit} , $x < a$). MO_2 stays constant for PO_2 values above P_{crit} ($x \geq a$), indicating the plateau phase where oxygen availability no longer limits metabolism (aerobic) (Ultsch and Regan, 2019). The critical oxygen tension (P_{crit}) and the aerobic metabolic rate (MO_2) can both be estimated using this model (Ultsch and Regan, 2019).

The Curve Fitting tool in MATLAB was used to separate aerobic and anaerobic oxygen consumption rates and extract parameters:

- A: critical O_2 tension (P_{crit})
- $B + C \times A$: aerobic metabolic rate (MO_2)

Aerobic MO_2 values were then imported into R, visualized with scatter plots, and analyzed using a PERMANOVA for the general analysis, while Kruskal-wallis was implemented to highlight possible differences between morning and afternoon values in all the combinations of Treatment x Season.

BPM data collected during respirometry were processed similarly to the thermal ramp protocol: signals were divided into consecutive 5-minute windows, peaks were counted, and values were stored in a table including reference time, individual (channel), and BPM. The variable Temperature was included only as a check and has no biological meaning.

Oxygen saturation (PO_2) values were processed using a similar approach: columns were extracted per channel, converted to numeric, resampled every five minutes. This generated a clean dataset suitable for presenting BPM in function of PO_2 in scatter plots. Finally, the BPM response variable was analyzed using a linear mixed model and ANOVA Type III, including treatment, time of the day, and season as fixed effects, and individual (channel) as a random effect. The Satterthwaite's method was applied to estimate degrees of freedom, as it provides more accurate p-values in unbalanced data (different number of total BPM) and when multiple interaction terms are included.

All MATLAB and R scripts used for this analysis are provided in the Supplementary Appendix (Section 8.2).

4 RESULTS

4.1 Acclimation Data

Table 1. During both the Spring and Summer experimental periods, mean (\pm SD) values of dissolved oxygen (DO - %air sat.) and water temperature ($^{\circ}$ C) were recorded under both constant and fluctuating environmental regimes. For each season, the mean temperature recorded under the fluctuating treatment was used as a reference to set the constant condition, in order to ensure comparable thermal regimes between treatments. Minor deviations observed in Summer remained within the standard deviation range and are therefore not considered significant.

Spring				
Treatment	Mean Temp. ($^{\circ}$ C)	SD Temp	Mean DO (% air sat)	SD DO
Fluctuating	11.79	1.59	74.89	9.86
Constant	11.66	0.25	65.17	37.32

Summer				
Treatment	Mean Temp. ($^{\circ}$ C)	SD Temp	Mean DO (% air sat)	SD DO
Fluctuating	23.86	1.69	76.96	9.35
Constant	24.03	0.17	83.75	7.86

MiniDOT Loggers were used to continuously measure the water's temperature ($^{\circ}$ C) and dissolved oxygen concentration (% air sat.). Table 1 reports the mean and standard deviation values for both parameters for each treatment and season. Due to technical issues during the acclimatization period, a significant standard deviation in dissolved oxygen was noted in the Spring Constant treatment. However, this issue did not affect the outcome of the subsequent analyses, as the crabs used for the experiments were not taken from the affected tanks (see Discussion for details).

4.2 Temperature Dependent Mortality Curve

4.2.1 Mortality Curve and LT_{50}

After performing the four different experiments, Fluctuating and Constant Treatment during Spring and Summer, the following results were obtained.

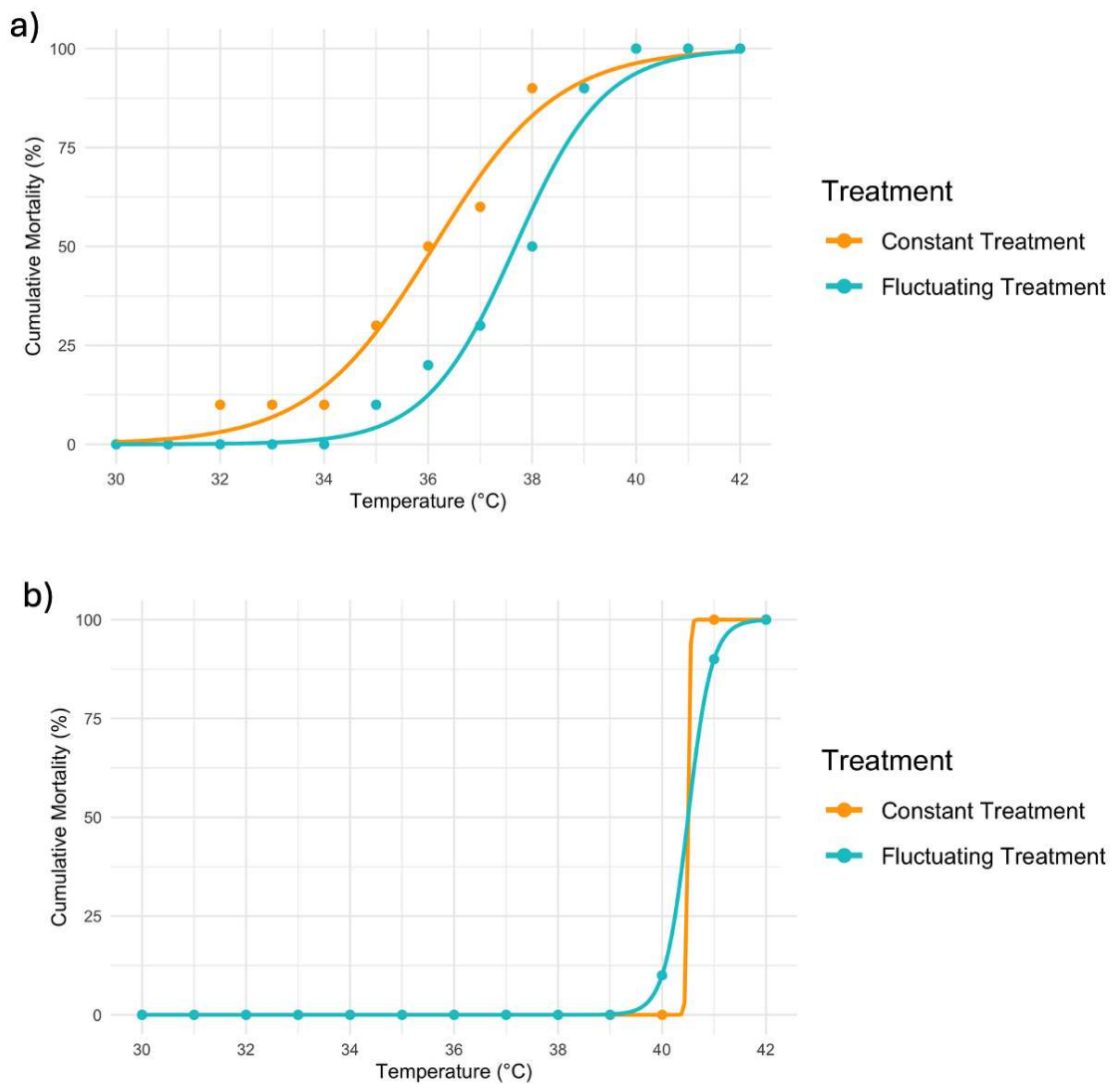


Figure 3. Temperature dependent mortality curves of *Callinectes sapidus* under Constant and Fluctuating Acclimation Treatment. (a) Spring and (b) Summer. The x-axis represents temperature (°C), while the y-axis shows cumulative mortality (%). Each curve displays the sigmoidal trend of cumulative mortality as temperature increases. This highlights the response of crabs to rising thermal conditions in the four experimental settings.

Table 2. LT₅₀ (°C) values of *Callinectes sapidus* under different treatments (Constant vs. Fluctuating) across seasons (Spring and Summer). The table highlights seasonal variations and treatment related differences in thermal tolerance.

	FLUCTUATING	CONSTANT
SPRING	37.7°C	36.1°C
SUMMER	40.5°C	40.5°C

In spring (Fig. 3a) mortality under Constant conditions began at 32°C, while in Fluctuating conditions the first death occurred at 35°C. After both treatments 100% of individuals died at 40°C (Fig. 3a). Table 2 shows that the lethal temperature (LT₅₀) in Spring was 36.1°C under Constant conditions, while individuals exposed to Fluctuating conditions exhibited

a higher LT_{50} of 37.7°C, an increase of 1.6°C. The analysis of thermal tolerance of *C. sapidus* highlighted considerable variations between treatments during the spring period.

Regarding summer conditions (Fig. 3b), the first death occurred at 39°C for Fluctuating conditions, with 8 organisms which died at 40°C and the last one at 42°C. For Constant all the deaths occurred at 41°C. Regarding LT_{50} for summer period, individuals exposed to Fluctuating and Constant treatments resulted in a LT_{50} of 40.5°C (Table 2). In the summer acclimation, the last mortality occurred at 42°C for the Fluctuating treatment and at 41°C for the Constant treatment.

Overall, individuals in spring experienced complete mortality at lower temperatures compared to summer, with the last death occurring at 40°C, whereas in summer mortality occurred at 41 and 42°C.

As seen by Table 2 and Fig. 3, the experiment of the Temperature Dependent Mortality Curve resulted in a seasonal difference, with LT_{50} values being lower in spring compared to summer. Summer also showed no differences in Treatments, having the same LT_{50} for both Constant and Fluctuating conditions.

4.2.2 BPM Analysis

An increase in heart rate (BPM) with temperature was observed in all treatments.

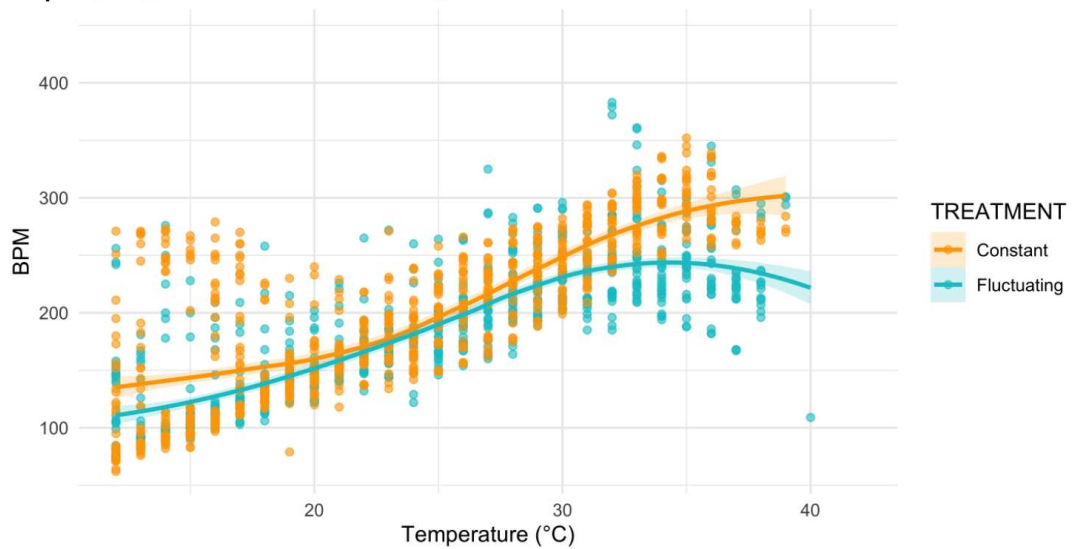
In Spring (Fig. 4a), BPM rose steadily with temperature, with a stronger bell-shaped pattern particularly evident in the Fluctuating treatment but visible also for the Constant conditions. Individuals from the Constant treatment showed higher BPM compared to those from the Fluctuating conditions across all experimental conditions. Individuals from the Constant treatment started at around 140 BPM and peaked at around 300 before mortality occurred, whereas those from the Fluctuating treatment started at around 120 BPM at 12°C, peaked at 250 BPM at 35°C with a slight decrease to 220-230 BPM right before mortality at around 37/38°C. This slight decrease, visible in the Fluctuating treatment, clearly marks the onset of the descending phase of the bell-shaped response. In Spring the upper thermal limit was about 40°C and BPM values tended in a plateau around 36-37°C for the organisms coming from the Constant Treatment, while around 35°C and a slight decrease around 36-37°C for the Fluctuating Treatment organisms', corresponding to the onset of mortality.

In Summer (Fig. 4b), the overall trend was reversed: crabs exposed to Fluctuating conditions exhibited consistently higher BPM values than those in the Constant treatment. Both treatments started at much higher BPM values (around 170-175 BPM at 24°C) compared to Spring ones (120-140 BPM at 12°C). In the Constant group, BPM has a decrease around 32-33°C before increasing steadily up to 280-290 BPM near the upper limit (41°C). Conversely, individuals under Fluctuating conditions exhibited a nearly continuous increase, reaching slightly above 300 BPM before mortality. At higher temperatures (38-41°C), both treatments seem to show a reduced rate of BPM increase, indicating the approach of a plateau.

While Spring data displayed a clear-bell shaped relationship between temperature and BPM, Summer results indicated a less strong approach to a plateau, with an inversion of the relative position of the two treatment curves between seasons.

It is important to mention that, immediately before removing each crab from the tank because of mortality, a visual decrease in BPM was always observed. To prevent including artifacts caused by PULSE V2 sensor noise after death, all BPM data for each organism obtained during the 30-minute period in which death occurred were excluded. The elimination of that time window guarantees that only accurate physiological values are kept because the device continues to generate misleading signals rather than recording a true 0 BPM value.

a) Spring: Constant vs Fluctuating



b) Summer: Constant vs Fluctuating

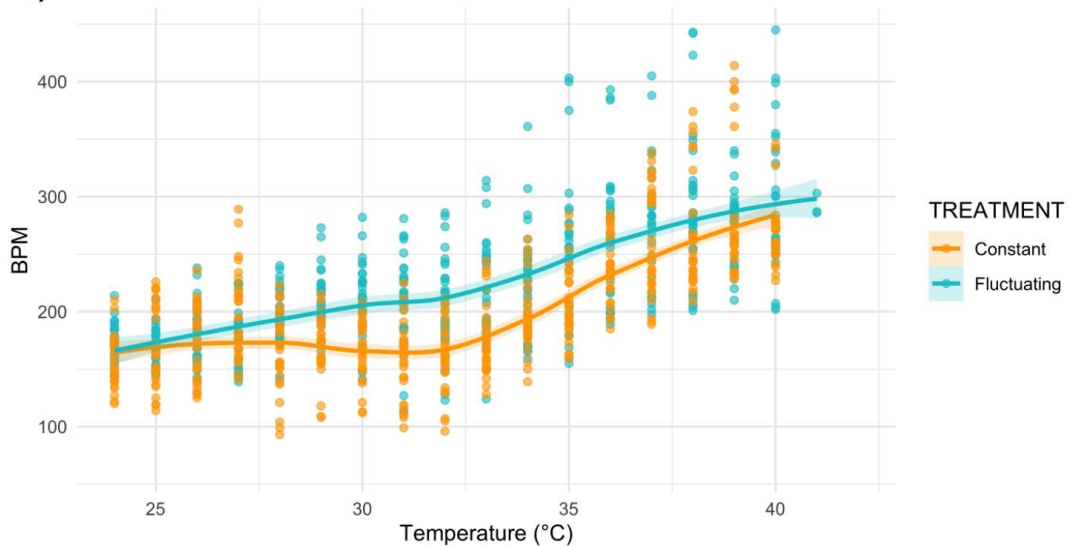


Figure 4. *Callinectes sapidus* heart rate (BPM) under Fluctuating and Constant Acclimation Treatments throughout the Temperature Dependent Mortality Curve trials. (a) Spring and (b) Summer. The x-axis represents temperature (°C) and the y-axis depicting BPM. Curves resume the trend of cardiac activity across increasing temperature levels. The shaded area represents the 95% confidence interval.

Table 3. The table presents the temperature coefficients (Q₁₀) values calculated from BPM measurements across different temperature conditions. Each section corresponds to the four experimental conditions: Fluctuating - Spring, Constant - Spring, Fluctuating - Summer and Constant - Summer. For each section the left column shows the values of temperature (°C) at which BPM was measured, while the right column reports the corresponding Q₁₀ values, reflecting the temperature sensitivity of cardiac activity under specific treatment and seasonal conditions. This provides insight into Q₁₀ trends across Treatments and Seasons.

T (°C)	Q ₁₀ FLUCT SPRING	T (°C)	Q ₁₀ COST SPRING
12-17	1.131	12-17	2.089
17-22	1.715	17-22	1.003
22-27	1.501	22-27	1.498
27-32	1.473	27-32	1.674
32-37	0.816	32-37	1.003
37-40	0.089	37-40	1.018

T (°C)	Q ₁₀ FLUCT SUMMER	T (°C)	Q ₁₀ COST SUMMER
24-27	1.126	24-27	2.184
27-32	1.346	27-32	0.669
32-37	1.817	32-37	2.609
37-41	1.129	37-41	1.077

Table 3 indicates a clear trend in Q₁₀ values (as already seen in Fig. 4), derived from BPM measurements at various Temperature points, across Treatments and Seasons.

Q₁₀ values coming from Spring data show a progressive increase in values from temperature range 12-17°C to 17-22°C. After this initial rise, the rate of increase progressively slows down between 22-27°C after the Fluctuating Treatment, becoming more moderate between 27-32°C after both treatments. Q₁₀ values in these ranges are still above 1, indicating that BPM is increasing along with the increase in temperature. Beyond the threshold of 32°C, that we can consider the inflection point (Fig.4a), Q₁₀ values strongly drop, around 32-37°C and more importantly at 37-40°C, suggesting the reach of the upper thermal limit (40°C).

Constant individuals, instead, have a completely different response with an abrupt and very high initial peak between 12-17°C, and a significant drop in the next interval (17-22°C). After 22°C an increase in values is observable until 27-32°C. Like the Fluctuating organisms, Constant individuals shows an abrupt decrease in Q₁₀ values with values approaching 1 as the upper thermal limit arrives (40°C).

In Summer, the Q₁₀ values are comparable to the Spring ones with some differences. Fluctuating individuals show a similar increase in Q₁₀ at lower temperature intervals, as 24-27°C and 27-32°C, reaching a clear maximum value around the range of 32-37°C. Both

Fluctuating Treatments in either Spring or Summer behave in parallel, but a shift into higher temperature ranges is observable, with 37°C representing the threshold for the decrease in Q_{10} values. In fact, values approach 1 in the last temperature range 37-41°C as the upper thermal limit is neared (42°C). Furthermore, Constant treatments individuals are comparable to the ones in Spring. A sharp initial peak is observable at 24-27°C, followed by a marked drop at 27-32°C. The drop is followed by a secondary rise around 32-37°C, before decreasing again at the last temperature range (37-41°C), as the upper thermal limit is approached (41°C).

Overall, across both Seasons Fluctuating individuals show a much regular response in Q_{10} with rising temperatures. In contrast, Constant individuals exhibit a sharp increase in Q_{10} at the beginning of the thermal ramp, generally being less regular in the increase in Q_{10} values. Moreover, a clear seasonal difference is also observable, with a general shift into higher temperature for all the different thresholds, markedly 32°C in Spring for the starting of the decrease in BPM while 37°C in Summer.

In conclusion, the difference between Treatments is also stronger in Spring rather than Summer.

These results are confirmed after performing the Type III Analysis of Variance (ANOVA) for BPM with Temperature, Season, and Treatment (Table 4) which are all highly significant ($p < 0.001$), as well as all their two- and three-way interactions. This suggests that, in line with the trends seen in Fig. 4, BPM varied considerably and in a complex manner over the temperature range, between Spring and Summer, and between Fluctuating and Constant treatments.

Table 4. Results of the Type III ANOVA performed on the linear model testing the effects of Temperature, Season (Spring, Summer), and Treatment (Constant, Fluctuating) on heart rate (BPM). The table reports the main effects and all interaction terms (Temperature \times Season, Temperature \times Treatment, Season \times Treatment, and Temperature \times Season \times Treatment). All single and interaction effects were found to be statistically significant ($p < 0.001$), indicating a strong combined influence of thermal conditions, seasonality, and treatment on the cardiac response.

Type III Analysis of Variance	Table with Satterthwaite's method			Den DF	F value	pr (> F)	Sign. Code
	Sum Sq	Mean Sq	Num DF				
TEMP	1462611	1462611	1	1679.8	1042176	< 2.2E-16	***
SEASON	58033	58033	1	1679.6	41351	1.654E-10	***
TREATMENT	22403	22403	1	1680.6	15963	6.737E-05	***
TEMP : SEASON	21486	21486	1	1679.8	15310	9.487E-05	***
TEMP: TREATMENT	25345	25345	1	1681.0	18060	2.258E-05	***
SEASON : TREATMENT	22168	22168	1	1680.7	15795	7.356E-05	***
TEMP : SEASON : TREATMENT	47925	47925	1	1681.0	34148	6.120E-09	***

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 1

4.3 Respirometry

4.3.1 Oxygen Consumption Analysis

Regarding Oxygen consumption ($\text{mg O}_2 \text{ h}^{-1} \text{ gWW}^{-1}$), no consistent diel pattern was observed across the two treatments, as shown in Fig. 5, where oxygen consumption remained relatively stable between morning and afternoon in all experimental groups. Only Spring under Constant conditions showed a significant effect ($p < 0.05$) in the Kruskal-

Wallis test (Table 5) on Time of Day across all Season × Treatment combinations, indicating a decline in values from morning to afternoon.

Table 5. Results of the Kruskal–Wallis tests comparing Morning and Afternoon aerobic MO_2 values within each combination of Treatment (Constant, Fluctuating) and Season (Spring, Summer). The table reports p -values for each comparison, with symbols (*) indicating statistically significant differences ($p < 0.05$).

Results Kruskal-Wallis (Morning vs Afternoon):			
SEASON	TREATMENT	p-value	Sign. Code
Spring	Constant	0.0209	* $p < 0.05$
Spring	Fluctuating	1.0000	
Summer	Constant	0.5637	
Summer	Fluctuating	0.7728	

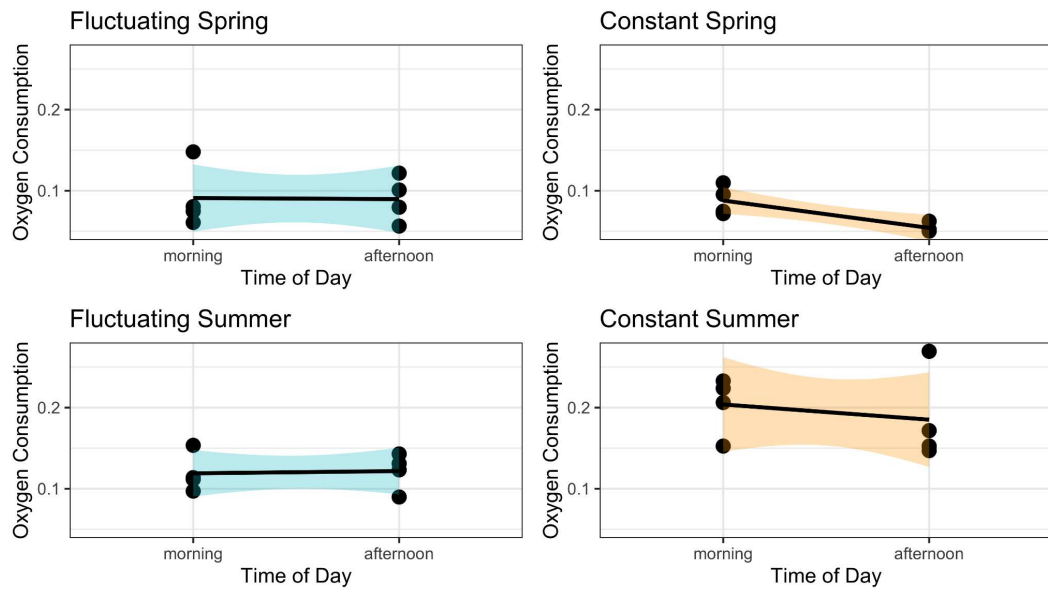


Figure 5. Oxygen consumption (mg O₂ h⁻¹ g⁻¹) of *Callinectes sapidus* under Fluctuating and Constant Acclimation Treatments during Spring and Summer. The x-axis represents the time of day (morning and afternoon), while the y-axis shows oxygen consumption data. Each point represents one individual, while shaded areas represent the 95% confidence interval.

Table 6. Aerobic oxygen consumption rates (mg O₂ h⁻¹ gWW⁻¹) of *Callinectes sapidus* performed at two times of day (morning and afternoon). Experimental individuals were subjected to Fluctuating and Constant Acclimation Treatments during both Spring and Summer. Each different value represents the aerobic oxygen consumption rate of a single crab, with four individuals per experimental condition.

	FLUCTUATING		CONSTANT	
	morning	afternoon	morning	afternoon
SPRING	0.148	0.101	0.072	0.062
	0.080	0.080	0.110	0.050
	0.075	0.056	0.075	0.052
	0.061	0.122	0.095	0.052
SUMMER	0.097	0.143	0.233	0.153
	0.112	0.090	0.224	0.147
	0.153	0.131	0.206	0.172
	0.114	0.123	0.153	0.269

The results coming from the PERMANOVA (Table 7) revealed that Season had a significant effect on oxygen consumption rates ($p < 0.001$), with higher metabolic rates recorded in Summer compared to Spring. In addition, Treatment had a significant main effect ($p < 0.05$). Finally, a significant Season x Treatment interaction ($p < 0.01$) was also detected, indicating that the influence of the acclimation treatment (Fluctuating and Constant) varied between seasons (Spring and Summer).

In conclusion, a clear seasonal variation was observed, with oxygen consumption values being consistently higher in Summer compared to Spring.

Table 7. Results of the PERMANOVA (adonis, 9999 permutations, reduced model) testing the effects of Season (Spring, Summer), Treatment (Constant, Fluctuating), and Time of the day (Morning, Afternoon) on oxygen consumption rate (MO₂). The table reports to p-values for each factor and their interactions, with significance indicated by asterisks (*).

Permutation test for adonis under reduced model (PERMANOVA) - 9999 permutations						
	Df	Sum Of Sqs	R2	F	Pr (> F)	Signif. Codes
SEASON	1	0.047116	0.47845	45.3886	0.0001	***
TREATMENT	1	0.006017	0.06110	5.7961	0.0244	*
TIME	1	0.001299	0.01319	1.2509	0.2827	
SEASON : TREATMENT	1	0.017440	0.17710	16.8004	0.0008	***
SEASON : TIME	1	0.000182	0.00185	0.1757	0.6790	
TREATMENT : TIME	1	0.001451	0.01473	1.3976	0.2530	
SEASON : TREATMENT : TIME	1	0.000060	0.00061	0.0575	0.8129	
Residual	24	0.024913	0.25299			
Total	31	0.098477	1.00000			

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 1

4.3.2 BPM Analysis

Figure 6 shows that the temporal dynamics of cardiac activity differ markedly depending on season, time of the day and whether animals experienced Fluctuating or Constant conditions.

Spring period and Fluctuating conditions: Morning readings started at about 120 bpm, while afternoon readings started at about 140 bpm. The two curves intersected at about 68% oxygen saturation: the morning peaked at about 140 bpm at about 40% PO₂, while the afternoon concurrently reached its lowest point at about 100 bpm. At about 12.5% PO₂, there was a second inversion, and both series converged at about 125 bpm. After this, both curves decreased; by the end of the experiment, the morning curve was slightly below 100 bpm while the afternoon curve was still slightly above 100 bpm.

For Spring period and Constant conditions, Morning started at around 120 bpm and declined, overlapping the Afternoon curve within 25-30% PO₂, showing a clear inversion of their relative positions. The curves crossed again at around 2% PO₂: Morning displayed brief peaks about 140 bpm before settling into a plateau toward the terminal phase, whereas Afternoon, which had started at little under 100 bpm, increased to about 120 bpm.

For Summer period and Fluctuating conditions, morning values were consistently above afternoon across all the experiment. The morning started at about 170 bpm, was mostly constant or slightly higher, and ended with a strong pre-mortem peak that was close to 300 bpm at the lowest PO₂ (~0%). The afternoon began at approximately 140 bpm, dropped to about 100 bpm at about 12.5% PO₂, and then experienced a very steep increase during the last oxygen fall, almost reaching the morning levels (~200–300 bpm) at the terminal point.

For Summer period and Constant conditions: morning curve started at around 150 bpm, dropped slightly to about 110 bpm at around 25% PO₂, and then quickly increased to about 300 bpm as PO₂ approached closer to zero. The afternoon started off slightly higher (~160-170 bpm), dropped to about 150 bpm close to 5% PO₂, and then showed a sharp terminal peak as well to almost 300 BPM.

When analyzed together, these patterns show intricate relationships between diel timing, treatment, and season. While summer animals, especially under fluctuating regimes, show higher cardiac reactivation that peaks abruptly right before pre-mortality, spring individuals exhibit many crossover points and mild terminal responses.

A Type III ANOVA (Table 8) performed on BPM revealed significant main effects of Time and Season ($p < 0.001$) and of Treatment ($p < 0.01$), as well as a significant two-way interaction between Treatment \times Time ($p < 0.001$) and a significant three-way interaction among Treatment \times Time \times Season ($p < 0.001$).

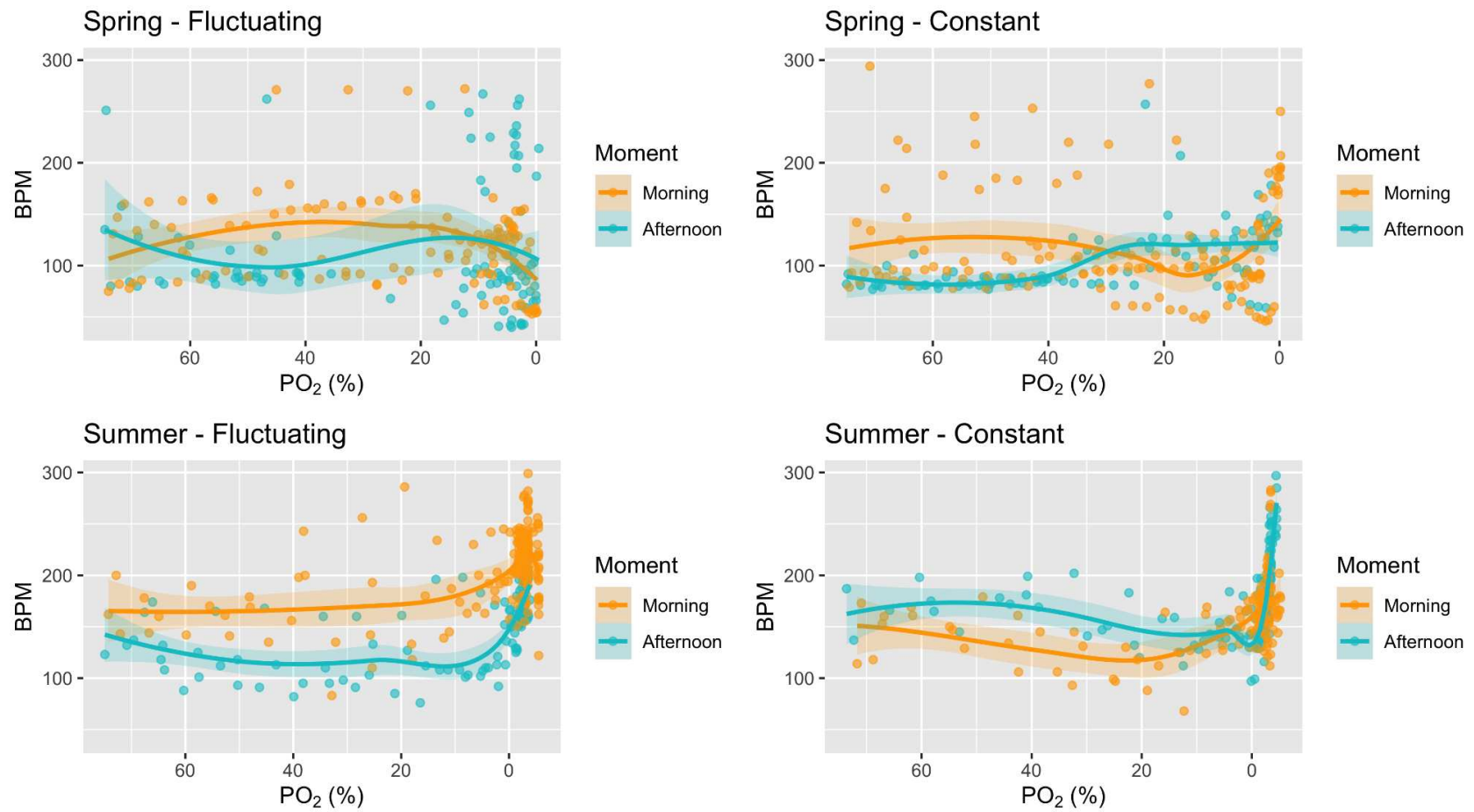


Figure 6. Heart rate (BPM) in *Callinectes sapidus* exposed to various experimental treatments as a function of oxygen partial pressure (PO₂ - %). The spring season is represented by the upper panels, and the summer season is represented by the lower panels. Constant treatments are displayed on the right, whereas fluctuating treatments are presented on the left. Dots show individual heart rate readings taken in the morning (yellow) and afternoon (blue) of the day, while lines show the average heart rate trend. The 95% confidence interval is shown by the shaded regions surrounding the lines.

Table 8. Type III Analysis of Variance (ANOVA) using Satterthwaite's method testing the effects of Treatment (Constant, Fluctuating), Time of the day (Morning, Afternoon), and Season (Spring, Summer) on heart rate (BPM). The table also reports all two-way and three-way interactions among these factors. p-values and corresponding significance levels are indicated, with significance codes represented by asterisks (*).

Type III Analysis of Variance	Table with Satterthwaite's method						
	Sum Sq	Mean Sq	NumDF	DenDF	F value	pr > F	Sign. Codes
TREATMENT	16247	16247	1	1043.4	8.4914	0.003644	**
TIME	65904	65904	1	1043.1	34.4438	5.889e-09	***
SEASON	836050	836050	1	1043.2	436.9524	< 2.2e-16	***
TREATMENT : TIME	69881	69881	1	1043.8	36.5227	2.097e-09	***
TREATMENT : SEASON	138	138	1	1043.4	0.0721	0.788316	
TIME : SEASON	2462	2462	1	1043.1	1.2869	0.256872	
TREATMENT : TIME : SEASON	122580	122580	1	1043.8	64.0651	3.189e-15	***

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 1

5 DISCUSSION

The results supported the claims that natural environmental fluctuation, diel and seasonal, significantly modulates the physiological responses and metabolic performances of *Callinectes sapidus*. Acclimation history strongly influenced both metabolic and cardiac processes, with complex and differentiated mechanisms depending on the metabolic response analysed. We found some evidence of a potential feedforward mechanism under predictable fluctuations to give insights on the role of environmental predictability, able to shape metabolic performances.

5.1 Acclimation

MiniDOT Loggers were used to continually monitor temperature and dissolved oxygen (DO) during the acclimatization period in order to guarantee environmental stability across treatments. With the exception of the Spring Constant condition, which displayed an exceptionally high SD in DO ($\approx 37\%$), mean and standard deviation (SD) values were generally stable throughout seasons and treatments, with higher SD in Fluctuating Treatment due to natural fluctuating conditions. Two of the four constant-treatment tanks, including the one with the MiniDOT logger, were affected by a technical issue with the oxygen supply, which led to this variation. As a result, the DO levels in that tank abruptly decreased from typical levels ($\sim 70\text{-}80\%$) to almost zero, with two total days of anoxic conditions, falsely increasing the variability and slightly lowering the treatment's overall mean DO.

Since the crabs from the impacted tanks were discovered dead and were not utilized in the tests, this incident had no bearing on the physiological investigations that followed. The temperature and oxygen levels in the remaining tanks were unchanged and similar to those seen in the other treatments, according to daily measurements taken with the HANNA HI 9829 multiparameter probe. Therefore, rather than reflecting biological or environmental variability, the increased SD found in the Spring Constant condition should be seen as a technical artifact.

The reliability of the data utilized for the subsequent analysis on thermal tolerance and oxygen consumption is supported by the overall regularity of temperature and oxygen levels in the other tanks, which supports the environmental setup.

5.2 Thermal Sensitivity

Temperature dependent mortality curve findings in thermal tolerance showed a distinct seasonal pattern and, in some instances, a marked impact of treatment. LT_{50} increased by 1.6°C in the spring when crabs accustomed to variable conditions showed a greater LT_{50} (37.7°C) than those kept under constant conditions (36.1°C). This difference should have biological significance and as it implies that when individuals are accustomed to relatively cool settings, environmental fluctuations may increase the LT_{50} of *C. sapidus*. In a number of ectothermic species, such as *Oryzias melastigma*, *Homarus americanus* and the genus *Mytilus*, where fluctuating conditions cause physiological plasticity and encourage heat-hardening responses, production of heat response (either metabolic or with production

of heat shock proteins), similar effects of daily or periodic temperature fluctuations on thermal limits have been previously documented (Fusi et al., 2024; Morash et al., 2018). In summer, however, the difference between variable and continuous treatments is not marked, and both groups obtained the same LT_{50} value (40.5 °C). This convergence probably represents the attenuation of treatment effects at high acclimation temperatures, when performance curves flatten and individuals already function near their maximum thermal limits (Jost et al., 2012; Rato et al., 2021). In fact, regardless of treatment, all individuals died at 41 or 42 °C throughout the summer. In line with the 12 °C difference in mean environmental temperature during the acclimatization phase (12 °C in spring vs. 24 °C in summer), the highest absolute LT_{50} values reported in the summer compared to the spring (up to +4 °C difference) show a considerable seasonal acclimation effect.

Trends in heart rate (BPM) supported these conclusions. In Spring, while Fluctuating crabs attained lower maximum rates (≈ 250 BPM) and showed an early decrease near 36-37 °C, constant-acclimated crabs continuously showed higher BPM values across the temperature range in spring, peaking at about 300 BPM. During the summer, however, the pattern was reversed: constant-acclimated crabs showed lower BPM than fluctuating ones, although the two curves remained overall quite similar. This inversion supports the theory that the physiological response to additional warming is modulated by thermal history, maybe as a result of alterations in energy allocation or the cumulative stress load under prolonged high temperatures (Morash et al., 2018). The separation between in BPM curves is much stronger in Spring rather than Summer, suggesting that animals acclimated to lower temperatures shows more sensitivity to the presence or absence of diel fluctuations. In contrast, in Summer a higher baseline thermal stress is masking and compressing the metabolic response making the effect of fluctuations less detectable (Morash et al., 2018; Brill et al., 2015).

In addition, the bell-shaped trend between temperature and BPM is indicative of a standard Thermal Performance Curve, in which metabolic and physiological responses increase along with temperature until an optimum, after which the organisms exhibit a decline in performances while they approach the upper thermal limit and mortality (Morash et al., 2018; Sinclair et al., 2016). A typical Thermal Performance Curve (TPC) has the best performance at the optimum temperature, and lower performance at other temperatures (Morash et al., 2018; Sinclair et al., 2016). This trend suggests that different treatments and seasons indicate different thermal sensitivity and potential acclimation effects (Sinclair et al., 2016). The bell-shaped trend was visually evident mainly in the Spring fluctuating group.

A clear seasonal difference in initial BPM values was also observed. In Spring, when experiments started at around 12°C, individuals exhibited baseline heart rates between 120–140 BPM. Conversely, during Summer, with an initial temperature of approximately 24°C, both treatments started from much higher values, around 175 BPM. This strong seasonal difference reflects the influence of acclimation temperature on baseline metabolic activity and highlights the substantial physiological adjustment of *C. sapidus* to warmer environmental conditions (Brill et al., 2015; Zainal and Noorani, 2019).

These interpretations were supported by the Q_{10} analysis, which showed that season and treatment had an impact on the heat sensitivity of heart activity. Across all treatments and seasons, Q_{10} resulted in a typical Thermal Performance Curve trend (Morash et al., 2018; Sinclair et al., 2016). Initially Q_{10} rose above 1 as temperature increased, after a while, reaching the optimum temperature, the values reached their highest values, starting to decrease right after corresponding to enhanced stress right before mortality (Morash et al., 2018; Sinclair et al., 2016). Higher Q_{10} were observed between 27 and 32 °C in the spring, which coincided with the start of heat stress and lowering of BPM. The peak moved to higher temperatures (32-37 °C) during the summer, demonstrating that individuals who were acclimated to the summer maintained effective physiological performance across a larger temperature range. This pattern reflects a different seasonal acclimation of metabolic processes due to higher temperature, consistent with the thermal acclimation in ectotherms and their ability to adapt (Pörtner, 2010; Pörtner and Knust, 2007). Additionally, Q_{10} levels tended to be higher in Constant treatments than in Fluctuating ones in both Spring and Summer. This suggests a steeper increase in metabolic rate and probably higher energetic expenditures under constant settings, differently from natural fluctuating ones. Similar trends have been noted in other fish and crustacean species, such as *Salmo salar* and *Gammarus fossarum* (Booth et al., 2021; Pörtner, 2010; Semsar-kazerouni and Verberk, 2018), where constant conditions result in less adaptable metabolic responses and a decreased capacity to withstand high temperatures (Morash et al., 2018; Pörtner, 2021). Such differences might be explained by a priming effect, as previous exposure to fluctuating thermal conditions can precondition the organisms, improving their resilience to future temperature challenges (Pörtner, 2021).

Lastly, the ANOVA's significant three-way interactions on BPM analysis (Temperature × Season × Treatment), together with the significance of all main factors and two-way interactions, confirm that *C. sapidus*'s thermal performance is influenced by both the type of environmental variability and the acclimation temperature. Daily oscillations during acclimatization seem to increase resistance in colder season, but this beneficial effect fades at higher acclimation temperatures as the organism gets closer to their upper thermal limits. Overall, these findings shed light on the relationship between seasonal acclimation and environmental fluctuations, and they imply that fluctuating environments may improve thermal tolerance and ecological plasticity, though these benefits may vanish as organisms get closer to their upper thermal limits.

5.3 Oxygen Consumption

During the respirometry trials, oxygen consumption (MO_2) was measured at two fixed times of day (10:30 and 16:30), which approximately corresponded to the average temperature and dissolved oxygen levels of the daily fluctuation cycles. According to the environmental predictability hypothesis, it was expected that oxygen consumption would be lower in the afternoon as both variables begin to decrease toward their minimum and greater in the morning as temperature and oxygen start increasing toward their daily peaks (Fusi et al., 2024). Contrary to expectations, the analysis did not show any consistent diel patterns between morning and afternoon observations, since the

temperature and oxygen values at the experimental time (10:30 and 16:30 – Table 9) did not match average values of these two parameters across the six days of acclimation (Table 1).

By examining the environmental data recorded by the miniDOT Loggers, both the temperature and dissolved oxygen in each measurement day at 10:30 and 16:30, it became evident that the chosen time points did not always match the overall means across the six days of acclimation. While in the Spring Fluctuating treatment the morning temperature was slightly below (-0,5°C) and the afternoon temperature was almost +1°C above the daily average (Table 1 and Table 9), in the Summer Fluctuating treatment the temperatures at both time points were substantially above the mean values, roughly +1°C in the morning and +2°C in the afternoon, with correspondingly elevated levels of dissolved oxygen often exceeding 100% saturation, distant from the average of 77% (Table 1 and Table 9). Therefore, rather than the lack of a feedforward mechanism proposed for oxygen consumption by the environmental predictability hypothesis, the deviation of the sampling periods from the real mean of temperature and oxygen conditions is a good explanation for the lack of a consistent diel trend in *C. sapidus*' oxygen consumption.

Interestingly, under Constant Spring conditions, where temperature was maintained around 12°C, a significant diel difference was observed, with higher oxygen consumption in the morning (Kruskal–Wallis, $p < 0.05$). In contrast to the Summer Constant, where the much closer proximity to the species' upper temperature threshold (~24°C) probably limits metabolic flexibility, this shows that the feedforward may be prominent under cooler, less constraining settings (Bernhardt et al., 2020; Fusi et al., 2024; Morash et al., 2018; Pörtner, 2010). The results of the thermal tolerance tests, which showed that Spring people showed enhanced ecological plasticity in LT_{50} than Summer individuals, are also compatible with this hypothesis.

Furthermore, the results demonstrate that the seasonal context, specifically the acclimatization temperature, has a significant impact on oxygen consumption. Considering that *Callinectes sapidus* is an ectothermic species, its metabolism is temperature-dependent, resulting in much higher oxygen consumption rates in the summer than in the spring in both treatments (Glandon et al., 2019; Li et al., 2015). This seasonal variation represents the metabolic flexibility characteristic of ectotherms, whose physiological function is strongly influenced by the average ambient temperature and the conditions of acclimatization prior to testing (Glandon et al., 2019; Li et al., 2015; Marchessaux et al., 2022). In this case, warmer acclimatization during Summer probably increases metabolic activity, which raises the oxygen demand in comparison to the colder Spring settings.

The PERMANOVA showed a significant main effect of Treatment ($P < 0.05$) in addition to the large seasonal effect ($P < 0.001$), suggesting that the thermal regime to which crabs were exposed also had an impact on the oxygen consumption rate (MO_2). Nevertheless, there was no consistent pattern between fluctuating and constant settings across the seasons. In particular, during the Spring, oxygen consumption tended to be slightly higher in Fluctuating treatments than in Constant ones; in the summer, however, the opposite

pattern was seen, with higher levels under Constant settings. The strong interaction between Season and Treatment further supports this trend, indicating that the seasonal context affects how the presence of natural fluctuations affects metabolic rate (Morash et al., 2018; Pörtner, 2010).

Heart rate (BPM) analysis, on the other hand, showed a broader variation in responsiveness: significant effects were found for Season, Treatment ($p < 0.01$), and Time of the day, as well as for the Treatment \times Time and Treatment \times Time \times Season interactions (all $p < 0.001$). This suggests that diel fluctuations, environmental variability, and seasonal acclimation all have an impact on cardiac activity (Bernhardt et al., 2020; Zainal and Noorani, 2019) which is complex. Therefore, heart rate dynamics revealed a combined effect of both environmental predictability and acclimation history, while oxygen consumption remained more stable, with only minimal diel modulation, representing a more conservative strategy.

Together, these two different but complimentary strategies allow the organisms to withstand to stressful environmental conditions.

Table 9 provides the temperature (°C) and dissolved oxygen (% air saturation) measured at the precise times that correspond to the respirometry trials (10:30 and 16:30). These data represent the environmental conditions recorded during the six days preceding the first temperature-dependent mortality curve experiment. The table includes only the two fluctuating treatments, with the upper panel referring to Spring and the lower one to Summer. The temperature and dissolved oxygen data recorded at these particular hours differ from the mean daily averages calculated over the full six-day period (see Table 1).

days	time	T (°C)	DO (%)
16-mar	10:30	13.25	71.43
16-mar	16:30	15.43	69.09
17-mar	10:30	11.99	76.20
17-mar	16:30	12.87	77.47
18-mar	10:30	10.18	76.07
18-mar	16:30	11.12	80.80
19-mar	10:30	9.84	99.24
19-mar	16:30	13.32	69.95
20-mar	10:30	10.84	75.77
20-mar	16:30	11.42	93.28
21-mar	10:30	11.50	89.65
21-mar	16:30	12.19	97.96

days	time	T (°C)	DO (%)
03-jun	10:30	24.92	98.88
03-jun	16:30	25.64	120.15
04-jun	10:30	24.21	98.28
04-jun	16:30	25.46	125.80
05-jun	10:30	25.09	98.35
05-jun	16:30	26.16	142.14
06-jun	10:30	25.67	95.75
06-jun	16:30	26.00	112.64
07-jun	10:30	24.57	93.36
07-jun	16:30	26.00	117.57
08-jun	10:30	24.73	94.63
08-jun	16:30	27.68	130.23

7 CONCLUSIONS

The invasive species *Callinectes sapidus* is strongly affecting human activities and indigenous communities, posing not only significant socio-economic obstacles but also relevant ecological challenges in the Venice Lagoon (Marchessaux et al., 2024; Marchini et al., 2015). Its capacity to flourish in changing environments is strongly associated with its significant phenotypic plasticity, a trait particularly found in invasive species, offering a competitive edge over indigenous species, especially in the context of temperature rising and oxygen depletion due to climate change (Azra et al., 2020; Rato et al., 2021).

This study primarily aimed to evaluate the effects of constant and natural fluctuating environmental conditions in a laboratory setting on the metabolic and physiological responses of *C. sapidus*, while investigating the possible existence of feedforward mechanisms linked to the environmental predictability hypothesis.

Our findings predominantly support the original hypotheses: mortality under heat waves, metabolism performances and heart rate (BPM) showed both seasonal and treatment-related differences, indicating that the presence or absence of natural fluctuations influences mortality during extreme events and overall physiological function.

Thermal tolerance, indicated by LT_{50} , was greater in crabs adapted to variable conditions in spring, whereas in summer, the LT_{50} measurements aligned between treatments because of the elevated acclimation temperatures, closer to the upper thermal limit. Temperature coefficients (Q_{10}) somehow reflected BPM trends, exhibiting a gradual metabolic rise until reaching a plateau, subsequently followed by a slowdown or decrease, more pronounced in spring compared to summer, aligning with upper thermal thresholds and standard Thermal Performance Curve behaviors.

Oxygen consumption (MO_2) was affected by both season and treatment, showing no distinct diel pattern.

Overall, these results suggest that thermal history and seasonal acclimation may influence physiological and metabolic reactions, while exposure to natural environmental changes improves phenotypic plasticity and heat resistance, especially at cooler temperatures. The inconsistency in diel trends for oxygen consumption seems primarily linked to the sampling times and daily mean values, indicating that increased temporal resolution could uncover further dynamics, similar to those possibly observed in spring constant treatments. Future studies should pay attention to the selection of experimental times to actually reflect diel fluctuations and their mean values in temperature and dissolved oxygen, as this fact may be crucial to uncover potential feedforward mechanisms in different conditions.

Ecologically, these findings show that *C. sapidus* displays significant resilience and phenotypic flexibility, validating its success as an invasive species and its ability to endure extreme heat events and different environmental conditions. This underscores its competitive edge in the temperate waters of the Venice Lagoon, a situation expected to become more pronounced with continuing warming trends (Hulme, 2017).

From a methodological standpoint, our results highlight the significance of simulating natural environmental conditions in lab experiments. Steady conditions, most often employed in ectotherm research, might either underestimate or overestimate

physiological reactions, possibly biasing interpretations of metabolic efficiency and performance.

In summary, this research verifies that the season and acclimation process significantly affect the metabolic reactions of *C. sapidus* and offers perspectives on possible feedforward mechanisms associated with environmental predictability. These findings enhance our comprehension of ectotherm adaptability in variable environments and provide insights for upcoming research in authentic, natural settings.

6 BIBLIOGRAPHY

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8 APPENDIX

8.1 Temperature Dependent Mortality Curve Code

The workflow used to estimate the LT_{50} from thermal ramp experiments is reported below.

Raw data were cleaned in Excel, imported into RStudio, and analyzed using logistic regression models (nlsLM function).

The following MATLAB and RStudio scripts summarize the analytical steps:

```
# Logistic regression to estimate LT50
logistic_model <- function(data) {
  nlsLM(mortalita ~ 100 / (1 + exp(-k * (temperatura - x0))),
        data = data,
        start = list(k = 1, x0 = median(data$temperatura)))
}
model1 <- logistic_model(data1)
model2 <- logistic_model(data2)
LT50_1 <- coef(model1) ["x0"]
LT50_2 <- coef(model2) ["x0"]

% Heart rate (BPM) extraction from PULSE V2 signals
num_rows = 1498; % rows per minute
step = num_rows * 10;
time_sampled = [];
channels_sampled = cell(1,10);
for i = 1:step:length(time_12gradi)-num_rows
  time_sampled = [time_sampled; time_12gradi(i:i+num_rows-1)];
  for ch = 1:10
    channels_sampled{ch} = [channels_sampled{ch}; ...
      eval(sprintf('ch%d_12gradi(i:i+num_rows-1)',
ch))];
  end
end
```

Further analysis was then conducted in RStudio using a linear mixed model:

```
mod1 <- lmer(BPM ~ TEMP * SEASON * TREATMENT + (1 | CH), data
= dati)
summary(mod1)
anova(mod1)
```

8.2 Closed-System Respirometry Code

The oxygen consumption rate (MO_2) was derived from the PyroScience FireSting- O_2 recordings and normalized to wet weight following García-Rueda et al. (2021).

The MATLAB code used for BPM processing during respirometry sessions is shown below:

```
% 5-minute windows BPM extraction
num_rows = 1498; spacingMinutes = 5;
spacingRows = spacingMinutes*num_rows;
nWindows = floor((length(ch1_24gradi) -
num_rows)/spacingRows)+1;
BPM_matrix = zeros(nWindows,4);
channels = {ch1_24gradi, ch2_24gradi, ch3_24gradi,
ch4_24gradi};
for w = 1:nWindows
    s = start_idx(w); e = end_idx(w);
    for ch = 1:4
        data = channels{ch}(s:e);
        [~, locs] = findpeaks(data);
        BPM_matrix(w,ch) = numel(locs);
    end
end
end
```

The resulting BPM and PO_2 datasets were analyzed in R through mixed-effect models:

```
lmm_model_log <- lmer(log(BPM + 1) ~ TREATMENT * TIME * SEASON
+ (1|Canale), data = data)
anova(lmm_model_log)
```