OCEAN ACIDIFICATION EFFECT ON THE BIOLOGICAL AND ECOLOGICAL TRAITS OF BANDED-DYE MUREX Hexaplex trunculus (Linnaeus, 1758) POPULATION FROM MALI STON BAY

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This doctoral thesis was conducted at the University of Dubrovnik, Department of Applied Ecology, under the guidance of Ana Bratoš Cetinić Ph.D., associate professor, and Sam Dupont Ph.D., associate professor, University of Gothenburg, as a part of the inter-university doctoral study of Applied Marine Sciences at the University of Split and University of Dubrovnik.

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Abstract

The biological and ecological response of the predatory marine gastropod species *Hexaplex trunculus* to a range of low pH (pH_T 7.95 – 7.22) relevant in the context of ocean acidification was studied. This is the first study on the effects of ocean acidification conducted on a population of a species from the east coast of the Adriatic Sea and the first study on intracapsular development in the class Gastropoda where the parental generation was exposed to a range of low pH in the long-term. The results show that *H. trunculus* employs a variety of trade-off mechanisms to cope with low pH. Feeding rate and ability to find food were not affected by low pH, although gastropods exposed to low pH took less time to reach food. Acute exposure to low pH had a positive effect on *H. trunculus* growth rate, but during energy-limiting periods, shell growth was negatively impacted. However, prolonged exposure resulted in acclimatization with no discernible differences among pH conditions. The net calcification rate was negatively affected by low pH for the whole duration of the experiment. *H. trunculus* managed to maintain the same rate of total body weight over the period of acute exposure, but during the remainder of the period, total body weight decreased with low pH. Soft tissue body weight remained unaffected until the completion of spawning, suggesting a subsequent negative effect. No sex-specific differences were observed in the response to low pH. Female gastropods initially exhibited reduced metabolic rates at low pH, but the balance was restored over time. The pH had no effect on spawning characteristics, but intracapsular development was impaired under certain low pH conditions ($pH_T < 7.51$), impacting post-veliger developmental stages. Acute exposure of spawns to low pH had a negative effect on intracapsular development. Parental exposure generally had a negative effect on intracapsular development, but variability in the response was observed. Negative effects could be partially reversed, as shown by the improved growth of embryos from parents preconditioned to lower pH when transferred to higher pH. This study enhances the understanding of *H. trunculus* responses to future ocean acidification and emphasizes the complexity of adaptive strategies and the importance of considering individual variability and parental influence while assessing species sensitivity.

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Sanja Grđan

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Sažetak

Radi očekivanih globalnih promjena kiselosti mora istraživan je biološki i ekološki odgovor predatorskog puža *Hexaplex trunculus* na pH vrijednosti u rasponu od 7,95 do 7,22. Ovo je prvo istraživanje o utjecaju nižeg pH provedeno na nekoj istočnojadranskoj populaciji te prvo istraživanje intrakapsularnog razvoja predstavnika razreda Gastropoda u kojem je roditeljska generacija dugotrajno izložena rasponu vrijednosti pH. Rezultati pokazuju da *H. trunculus* koristi različite mehanizme prilagodbe sniženom pH mora. Vrijednosti pH nisu imale utjecaja na tjednu stopu prehrane i sposobnost pronalaska hrane, iako je jedinkama iz okoliša s nižim pH trebalo manje vremena za pronalazak hrane. Akutna izloženost niskom pH utjecala je na brži rast duljine kućice, dok je pri nižim temperaturama i smanjenoj metaboličkoj aktivnosti stopa rasta kućice bila smanjena u sniženom pH. Međutim, dugotrajna izloženost nije rezultirala razlikom u duljini kućice između jedinki iz različitih pH uvjeta. Snižen pH je negativno utjecao na neto stopu kalcifikacije tijekom eksperimenta. Tijekom razdoblja akutne izloženosti stopa rasta ukupne mase *H. trunculus* nije se mijenjala, no tijekom trajanja eksperimenta stopa rasta se proporcionalno smanjivala snižavanjem vrijednosti pH. Stopa rasta mase mekog tkiva nije se mijenjala do završetka mriješćenja, nakon čega se masa mekog tkiva u uvjetima niže pH vrijednosti smanjivala. Nije zabilježena razlika mjerenih značajki između mužjaka i ženki. Opažena je smanjena stopa respiracije ženki za vrijeme akutne izloženosti pri niskom pH, ali nakon dugotrajnije izloženosti nije bilo razlike u stopi respiracije između ženki iz različitih pH uvjeta. Vrijednost pH nije imala utjecaja na mriješćenje i značajke mrijesta, ali je opažena razlika u intrakapsularnom razvoju pri pojedinim vrijednostima pH (pH_T < 7,51), te negativni utjecaj na razvojne faze nakon stadija veliger ličinke. Akutna izloženost mrijesta niskom pH imala je negativan učinak na intrakapsularni razvoj. Izloženost roditelja nižim vrijednostima pH rezultirala je manjim rastom tijekom intrakapsularnog razvoja. Bolji rast ličinki nakon prebacivanja u više vrijednosti pH nakon dugotrajne izloženosti njihovih roditelja nižim vrijednostima, ukazuje na mogućnost smanjivanja negativnih učinaka Rezultati ovog istraživanja doprinose razumijevanju odgovora na očekivanu acidifikaciju mora te ukazuju na složenost strategija prilagodbe.

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1. INTRODUCTION

Over the past 250 years atmospheric carbon dioxide (CO_2) levels have increased by as much as 50% compared to pre-industrial times – from 280 parts per million (ppm) to 417 ppm in 2022 (Friedlingstein et al., 2022). The increase in atmospheric CO_2 concentrations is primarily due to the burning of fossil fuel (rapid urbanization, mechanization of industry, intensive agriculture) and is causing significant changes in the carbonate chemistry of seawater (Doney et al., 2009) as oceans absorb about 27% of the released carbon dioxide (Hurd et al., 2018). This perturbation of seawater carbonate chemistry is referred to as ocean acidification and encompasses simultaneous changes in the concentration of hydrogen ions (H^+), dissolved carbon dioxide (CO_2), bicarbonate ions (HCO_3^-), and carbonate ions (CO_3^{2-}) (Hurd et al., 2018). Over the same period, the average surface ocean pH has decreased by 0.11 (Doney et al., 2009). Depending on variations of future anthropogenic CO_2 emissions, the average partial pressure of carbon dioxide (pCO_2) in the open ocean, which is currently around 40.53 Pa (= 400 µatm – standard atmosphere (atm) is a unit of pressure; 1 atm = 101.325 Pa), could reach values between 60.80 (600 µatm) and 101.33 Pa (1000 µatm) by 2100 (Caldeira & Wickett, 2005). Consequently, this means that open-ocean surface pH is projected to decline by 0.08–0.37 pH units in 2081–2100 relative to 2006–2015, depending on the future carbon dioxide emissions (Cooley et al., 2022).

When carbon dioxide from the atmosphere dissolves in seawater, it reacts with water to form carbonic acid, which is unstable and quickly dissociates into hydrogen and bicarbonate ions. The bicarbonate ions then further dissociate into carbonate ions:

$$CO_{2(atm)} + H_2O \rightleftarrows H_2CO_3 \rightleftarrows H^+ + HCO_3^- \rightleftarrows 2H^+ + CO_3^{2-}.$$

The reactions in seawater are reversible and near equilibrium. At an average surface seawater pH of 8.1 and salinity of 35, 90% of the inorganic carbon consists of bicarbonate ions, 9% of carbonate ions, and only 1% of dissolved carbon dioxide. The relative proportions of these inorganic carbon species regulate the pH of seawater over shorter and longer periods of time. The uptake of additional CO_2 from the atmosphere increases the concentration of the dissolved CO_2, bicarbonate, and hydrogen ions. Consequently, the increase of hydrogen ions lowers the pH ($pH = - \log_{10} [H^+]$) (Orr et al., 2005; Doney et al., 2009). Over longer periods of time, the ability of the ocean to absorb atmospheric CO_2 also depends on the dissolution of calcium carbonate ($CaCO_3$) in the water column or sediment, according to the following equation:

$$CaCO_3 + CO_2 + H_2O \rightleftarrows Ca^{2+} + 2HCO_3^-.$$

Calcium carbonate in the ocean originates from shells and skeletons of marine organisms. In the pelagic environment, carbonates sink through the water column and either dissolve or accumulate in shallow and deep-sea sediments. Solid calcium carbonate, which occurs in the ocean primarily in two polymorphic forms – aragonite and calcite – dissolves in seawater through a reaction with free hydrogen ions and forms calcium and bicarbonate ions. The formation of calcium carbonate and the rate of dissolution vary with saturation state (Ω), which is defined as the ion product of calcium and carbonate ions:

$$\Omega = [Ca^{2+}][CO_3^{2-}]/K'sp,$$

where K'sp is the solubility product constant that depends on temperature, salinity, pressure, and the specific mineral phase; aragonite is 50% more soluble than calcite (Feely et al., 2004). The formation of shells and skeletons in organisms occurs in compartments where $\Omega > 1.0$, and their dissolution begins at $\Omega < 1.0$, unless the shells and skeletons are protected by an organic cover, for example. Saturation values are highest in shallow, warm tropical regions and lowest in colder, higher latitudes and greater depths, reflecting the increasing solubility of calcium carbonate with decreasing temperature and increasing pressure (Feely et al., 2004).

In addition to the absorption of carbon dioxide from the atmosphere, changes in the carbonate chemistry of coastal areas are largely influenced by biological processes such as photosynthesis, respiration or the precipitation and dissolution of calcium carbonate, which can act as a source or sink for carbon dioxide. Freshwater inputs, organic runoff from land and natural processes that bring carbon dioxide-rich water from the middle layer of the ocean to the surface can further exacerbate daily and seasonal fluctuations in pH and *p*CO2 in coastal areas (Hall et al., 2020; Osborne et al., 2020). Understanding how changes in the carbonate chemistry of seawater will affect key species, the ecosystem and the services it provides, is of utmost importance. A wide range of impacts have been observed in organisms at all trophic levels. Assessing the effects of ocean acidification on ecosystems is further complicated by interactions with numerous local and global stressors, from eutrophication to increased sea surface temperature, all of which have a variety range of impacts on marine life (Hurd et al., 2018).

1.1 Research rationale, aim and objectives

To facilitate the comparison of experimental data on the effects of ocean acidification on marine organisms, Riebesell et al. (2011) proposed a three-scenario approach for experiments that would correspond to present-day conditions, and *International Panel for Climate Change* (IPCC) projections for the years 2050 and 2100. However, the IPCC projections refer to open ocean

conditions, whereas most of the studied organisms live in coastal habitats where pH already fluctuates naturally. Therefore, local populations may have some adaptive mechanisms to cope with the pH levels they are already exposed to, which could play an important role in defying the species' sensitivity to changes in seawater pH (Vargas et al., 2017). Previous studies examining the biological and ecological traits of organisms have shown differences in response to low pH depending on the experimental setup and the longevity of exposure. To determine the effects of acidification on individual organisms, it is necessary to know the variation in pH in the habitat of the population under study and design experiments based on these data (Dupont et al., 2013). Many published studies have not considered local variability in their experimental design. To maintain consistency throughout the literature review in this study, the term “low pH” refers to a lower pH in the experiment compared to what was used as a control by the respective authors.

The results of previous short-term studies focused on a single life stage did not account for selection, acclimation, and transgenerational effects between successive life stages (Dupont et al., 2013). Acclimation involves phenotypic responses in physiology, morphology, or behavior that help maintain the fitness of the species in a new, altered environment, while adaptation involves genetic selection that shifts the average phenotype toward better fitness for the species (Kroeker et al., 2017). The adaptation potential depends on population size and generation time, with the highest rates of adaptation expected in the species with large populations and short generation times (Dupont et al., 2013). Acclimation can mitigate the population response to the current effects of acidification and give individuals more time to adapt, which may be particularly important for organisms with long generation times. On the other hand, acclimation may require an energy cost that redirects energy needed for other processes such as gonad development (Sunday et al., 2014). Future research on the effects of acidification on marine organisms should focus on long-term experiments spanning more than one generation (Hurd et al., 2018; Saba et al., 2019). In addition, it is necessary to study the response of species in a pH range to assess the adaptive capacity of organisms through acclimation, phenotypic changes, and eventually genetic adaptation (Kroeker et al., 2017).

Although no single experiment can resolve what will happen to future generations of a species, the collection of empirical data is of utmost importance for making a more general assessment of individual species’ responses (Baumann, 2019). One of the gaps in knowledge is the internal variability of a species' sensitivity, or population variability (Baumann, 2019). While some experiments have shown that populations of the same species inhabiting stable habitats have greater tolerance to acidification than populations inhabiting variable habitats (Osores et al., 2017;

Kurihara et al., 2020), numerous studies suggest that these populations may be more at risk because long-term exposure to acidification can lead to diversion of energy from other life processes, such as reduced calcification (Lagos et al., 2016; Osores et al., 2017).

Taking this into consideration, the main objective of this thesis is to investigate the biological and ecological response of the predatory marine gastropod species, *Hexaplex trunculus* (Linnaeus 1758) to a range of low pH relevant in the context of ocean acidification. This study focuses on the population inhabiting the economically and ecologically important coastal region of Mali Ston Bay in the southeastern Adriatic Sea. The study is designed as a long-term experiment that includes both the naturally occurring pH fluctuations in Mali Ston Bay and the values projected for the near future and beyond.

The research presented here is the first study of the effects of ocean acidification on a population of a species from the eastern Adriatic Sea. In addition, this is the first study of intracapsular development in the class Gastropoda that involves long-term exposure of parental generation to low pH tested under a range of pH conditions. These results will contribute to the understanding of the response of marine gastropods to low pH. Specifically, by assessing the response of *H. trunculus*, one of the key species of benthic communities in the study area, it will be possible to better understand and potentially mitigate the effects of ocean acidification in this ecosystem.

The specific aims of this thesis were to:

- investigate the effects of low pH on the food consumption rate of *H. trunculus*

- assess the ability of *H. trunculus* to locate and reach food after long-term exposure to low pH

- examine if a range of pH affects the shell length growth rate of *H. trunculus*

- compare the net calcification rates of *H. trunculus* across a range of pH using the buoyant weight method

- analyze the soft tissue and total weight growth rate of *H. trunculus* across a range of pH

- determine if there is a sex effect in terms of pH on the shell length growth rate, soft tissue and total weight growth rate, and net calcification rate of *H. trunculus*

- assess the metabolic activity of *H. trunculus* females under a range of pH conditions by measuring oxygen consumption

- determine if low pH affects the number of spawned *H. trunculus* females and the size and quantity of spawned capsules

- examine if there is a difference in the intracapsular development of *H. trunculus* under a range of pH by determining embryonic/larval stages

- determine if there is a carryover effect of parental generation exposure to a range of pH on the intracapsular development of embryos and larvae of *H. trunculus*

1.2 Research hypotheses

Two hypotheses are tested in this study:

i) pH levels within the range observed in Bistrina Bay will not significantly affect the studied characteristics of the species, as they might have developed adaptive mechanisms to these values

ii) long-term exposure of parent generation to low pH during gonadal maturation and spawning will have a positive carryover effect on embryonic development.

2. LITERATURE REVIEW

2.1 Response of marine calcifying invertebrates on ocean acidification

Numerous research papers have demonstrated the effects of ocean acidification on various biological processes in marine organisms, such as development, survival, reproduction, calcification, behavior, feeding, etc. (Cooley et al., 2009; Hendriks et al., 2010; Kroeker et al., 2011; Branch et al., 2013; Bednaršek et al., 2019; Melzner et al., 2020; Wang & Wang 2020; Figuerola et al., 2021). Many marine calcifiers are known to be particularly sensitive to ocean acidification, as the additional energy costs associated with acid-base regulation, calcification and protection against dissolution alter the overall energy budget and fitness (Hendriks et al., 2010; Ross et al., 2011).

However, a growing body of research has shown that some calcifying organisms are able to maintain their calcification rate or even thrive under low pH conditions (Findlay et al., 2009; Byrne & Fitzer, 2019; Barclay et al., 2020). For example, the pteropod *Limacina helicina* (Phipps, 1774) has shown the ability to repair the shell and maintain shell integrity by thickening the inner shell wall, although this may come with a metabolic cost (Peck et al., 2018). Similarly, the polar brachiopod species *Liothyrella uva* (Broderip, 1883) was observed to increase the thickness of its inner secondary layer as a compensatory mechanism against shell dissolution under ocean acidification (Cross et al., 2015). In contrast, the temperate brachiopod species *Calloria inconspicua* (Sowerby, 1846) showed less extensive effects of dissolution, and no compensatory mechanisms were involved indicating habitat-specific differences in sensitivity and response (Cross et al., 2016).

In some species, increased rates of growth and calcification have been observed with short-term exposure to low pH, such as in juveniles of the commercial bivalve species *Chamelea gallina* (Linnaeus, 1758) after 74 days of exposure. However, a negative effect on calcification rates was observed in the long term (Sordo et al., 2021). In contrast, the cold-water coral *Lophelia pertusa* (Linnaeus, 1758) initially experienced a decline in calcification in a short-term study, but was able to acclimate after 6 months, resulting in slightly higher growth rates (Form & Riebsell, 2012).

In addition, some species have shown that they can maintain their calcification rates under reduced pH conditions when an abundant food supply is available. For example, the coral species *Porites* sp. maintained the same calcification rate under reduced pH when well fed (Edmunds, 2011). Similarly, the branching coral *Stylophora pistillata* (Esper, 1792) maintained its calcification rates in control and low pH conditions when fed, although other indicators of feeding

performance such as organic nutrient acquisition and dissolved free amino acid uptake rates were reduced, suggesting potential possible negative long-term effects (Houlbrèque et al., 2015). In the cold-water coral *Desmophyllum dianthus* (Esper, 1794) feeding frequency had a significant effect on skeletal growth, with higher feeding frequency resulting in more positive and variable calcification rates (Martínez-Dios et al., 2020). In the case of the Mediterranean mussel *Mytilus galloprovincialis* Lamarck, 1819, optimal feeding regimes led to increased feeding rates in response to low pH, while mussels on suboptimal diets exhibited weak attachment to the substrate and narrow valve opening (Lassoued et al., 2021). Furthermore, Caribbean corals were also able to mitigate the effects of ocean acidification when fed, as they increased their feeding rate and lipid content, while their unfed counterparts did not maintain growth (Towle et al., 2015).

However, it is important to note that the response of feeding performance in marine organisms to ocean acidification does not follow a consistent pattern. The copepod *Centropages tenuiremis* Thompson I.C. & Scott A., 1903, for example, exhibited increased feeding rates with lower pH only after an acclimation period, showing an increase in respiration and feeding rates in response to ocean acidification to balance the energy cost against low pH (Li & Gao, 2012). Blue mussels *Mytilus edulis* Linnaes, 1758, on the other hand, decreased their clearance rates under low pH (Meseck et al., 2020). Furthermore, the effects of pH on feeding habits can also be influenced by the ontogenic stage of the organism. Different responses in consumption and feeding preferences have been observed between adults and juveniles of the amphipod *Orchestoidea tuberculata* Nicolet, 1849 (Benitez et al., 2016). In the case of the California mussel *Mytilus californianus* Conrad 1837, pH had no significant effect on feeding habits such as initiation of feeding, gut fullness, and ingestion rates, but the feeding activity may be negatively impacted (Gray et al., 2017).

Many marine invertebrates exposed to low pH have exhibited metabolic depression, characterized by a reduction in respiration rate (Willson & Burnett, 2000; Michaelidis et al., 2005; Navarro et al., 2013). However, some species have shown no significant impact on their metabolic rate under ocean acidification conditions (Gutowska et al., 2008; Lannig et al., 2010), while others have even increased their metabolic rate (Mardones et al., 2022). For example, a study on three important bivalve species from the southern coast of China revealed different metabolic responses to pH levels. The respiration rate of *Pinctada fucata* (A. Gould, 1850) and *Perna viridis* (Linnaeus, 1758) showed no significant effect under low pH, while the scallop *Chlamys nobilis* (Reeve, 1852) had a significantly lower respiration rate (Liu & He, 2012). Shelled pteropods (Thecosomata) from tropical regions that naturally migrate to oxygen minimum zones, such as *Hyalocylis striata* (Rang,

1828), *Clio pyramidata* Linnaeus, 1767, *Cavolinia longirostris* (Blainville, 1821), and *Creseis virgula* (Rang, 1828), were not affected by low pH. However, *Diacria quadridentata* (Blainville, 1821), which does not migrate to these zones, exhibited reduced oxygen consumption and ammonia excretion at low pH, indicating that the environment of individual species can influence their sensitivity to ocean acidification (Maas et al., 2012). Initially increased oxygen consumption was noted in two species of crabs, *Paralithodes camtschaticus* (Tilesius, 1815) and *Paralithodes platypus* (Brandt, 1851), but after three weeks, there was no observable difference. Their feeding performance did not change, indicating that they likely reduced energy expenditure in other areas, which explains the reduced growth and increased mortality (Long et al., 2019).

Marine invertebrate sexual reproduction reveals the complexity of responses across and between species with some studies revealing significant effects of ocean acidification on reproductive traits (Xu et al., 2016; Conradi et al., 2019; Rossin et al., 2019; Marčeta et al., 2020), and others indicating no significant effects (Reed et al., 2021; Uthicke et al., 2021). As in other traits researched, a longevity of exposure has a crucial role in predicting a species' response. For example, female fecundity of green sea urchin *Strongylocentrotus droebachiensis* (O.F. Müller, 1776) was decreased when acclimated for four months to low pH during reproductive conditioning, but no effect was observed when females were acclimated for 16 months, although preconditioning od adults had a negative carryover effect on subsequent larvae and juveniles performance (Dupont et al., 2013). The sea anemone *Nematostella vectensis* Stephenson, 1935, exposed to low pH for six gametogenic cycles exhibited reduced fecundity but produced larger eggs. After several cycles, fecundity was recovered, but the eggs remained large, indicating long-term acclimation and no effect of parental exposure on larval performance was observed (Glass et al., 2023). Another example that highlights the reallocation of energy is the barnacle *Amphibalanus improvisus* Darwin, 1854, with successful development of gonads after eight months of exposure to low pH, but still failing to produce viable embryos and larvae (Pansch et al., 2018).

Larval stages have demonstrated particular vulnerability to ocean acidification (e.g. Chan et al., 2011; Waldbusser et al., 2015; Bergman et al., 2018; Byrne & Hernández, 2020), as they lack developed compensatory mechanisms for pH homeostasis and require more energy for development and basic life functions compared to adult stages (Stumpp et. al., 2011; Lee et al., 2020). Even in larval stages, a species-specific response has been demonstrated. While most invertebrate species exhibit negative effects on early stages under low pH, some examples show positive effects, such as increased growth rates in larvae of bay scallop *Argopecten irradians* (Lamarck, 1819) (Gobler & Talmage, 2012). Early life stages of the Arctic copepod *Calanus*

glacialis Jaschnov, 1955, were unaffected by increased *p*CO2 after two months of exposure, as measured by developmental rate, dry weight, carbon and nitrogen mass, and oxygen consumption rate (Bailey et al., 2017).

Among other modulating factors, differences in response between sexes has often been overlooked in studying the effect of ocean acidification. Studies conducted so far indicate variation in response between sexes (Ellis et al., 2017; Marčeta et al., 2020; Cui et al., 2022) highlighting the need to consider sex in future studies. Although there is a paucity of studies in the field of ocean acidification that account for sex in the experimental design (Ellis et al., 2017), there are indications that sex can modulate a species' response, especially when it comes to reproduction, gonadal tissue and gamete quality (Ellis et al., 2014; Uthicke et al., 2014; Schram et al., 2016).

Experimental data have shown that species do not respond uniformly to changes in carbonate chemistry, and physiological responses can vary even among closely related species or different populations of the same species. For example, long-term experiments (six months) on adult sea urchins *Paracentrotus lividus* (Lamarck, 1816) from different environments showed differences in the magnitude of response between two populations. Both populations eventually acclimated, but sea urchins from a more variable environment appeared to acclimate faster to low pH (Asnicar et al., 202). A recent study by Vargas et al. (2022) revealed that the impact of experimental ocean acidification scenarios on marine organisms depends on the deviation from the upper pCO_2 level experienced by local populations and highlights the importance of considering the present pCO_2 natural variability for a given population.

2.2 Marine gastropods under ocean acidification

The effects of ocean acidification on marine gastropods (Mollusca: Gastropoda) are less well studied compared to other commercially important species, despite their ecological and economic importance. Herbivorous marine gastropods have shown reduced growth and calcification rates (Noisette et al., 2015; Barclay et al., 2020; Leung et al., 2020), reduced survival at early stages (Li et al., 2018; Wessel et al., 2018), and decreased herbivory (Young et al., 2019; Young & Gobler, 2020) under low pH. However, there is some evidence of no effect on calcification, for example in sea hare *Aplysia punctata* (Cuvier, 1803), although metabolic rates were reduced (Carey et al., 2016). Different gastropod species may respond to ocean acidification differently, such as a decrease in respiration rate in species of the family Littorinidae, *Lacuna vincta* (Montagu, 1803) (Young et al., 2019), whereas *Nassarius conoidalis* (Deshayes, 1833), showed no difference in physiological response (Zhang et al., 2015).

Defense and survival mechanisms following predation may also vary among closely related species inhabiting the same area. For example, two Australian species in the family Trochididae showed differences in their ability to regenerate damaged shells, suggesting that less resilient species are subject to greater predation pressure, leading to potential community changes (Coleman et al., 2014). In addition, differences in sensitivity to ocean acidification have been observed in different populations of the same gastropod species. *Littorina littorea* (Linnaeus, 1758) individuals from populations living at the edge of their natural range in the northeast Atlantic showed greater sensitivity to shell dissolution and a reduced ability to regulate metabolism under lower pH when compared to individuals from populations that inhabit areas with lower pH variability (Calosi et al., 2017).

Feeding habits and their implications for predator-prey interactions have been studied in several gastropod species, revealing complex relationships. The predatory cone snail *Conus marmoreus* Linnaeus, 1758, for instance, exhibited increased activity levels but decreased predation rates under low pH (Watson et al., 2017). In the predatory snail *Reishia clavigera* (Küster, 1860) time required to find prey increased, while movement speed and prey consumption remained unchanged, although the prey handling time decreased significantly (Xu et al., 2017; Li et al., 2020). The intertidal gastropod *L. littorea* displayed thicker shells in the presence of predation cues from crabs, but this process was disrupted under low pH conditions. However, avoidance behavior increased, indicating potential indirect effects on interactions with other organisms (Bibby et al., 2007). Furthermore, the predator-prey interactions between larvae of two interacting mollusk species, the predatory snail *R. clavigera* and the rock oyster *Saccostrea cucullata* (Born, 1778), were investigated under low pH conditions with no significant effect on the mortality, abnormality, or growth of oyster larvae, whereas whelk larvae experienced increased mortality, abnormality, and significantly higher metabolic rates compared to controls, indicating possible shifts in communities (Campanati et al., 2018).

Variations in the response to ocean acidification have also been observed among populations of the economically important species *Concholepas concholepas* (Bruguière, 1789) (Lardies et al., 2014; Vargas et al., 2015). In a study examining *C. concholepas* larvae from three different habitats, it was demonstrated that specimens from areas with a greater influence of freshwater inflow had higher tolerance to low pH conditions when compared to populations inhabiting habitats with lower pH variability (Vargas et al., 2015). Larval stages of *C. concholepas* were particularly vulnerable to low pH, leading to increased mortality and negative effects on shell morphology, size and metabolism were observed (Crim et al., 2011; Manriquez et al., 2014).

Previous research on *C. concholepas* larvae also revealed a negative effect of pH on larval feeding intensity and the selectivity of ingested food, favoring smaller phytoplankton cells (Vargas et al., 2013).

Studies also indicated a negative effect on the reproduction and intracapsular development in several species, for example in gastropod *Haliotis discus hannai* Ino, 1953, fertilization rate and hatching success decreased under low pH (Kimura et al., 2011) while intertidal snail *Littorina obtusata* (Linnaeus, 1758) had longer developmental time and reduced viability of encapsulated embryos exposed to pH 7.6 (Ellis et al., 2009).

Several long-term studies have emphasized the importance of conducting extended experiments to fully understand a species' ability to acclimate. For instance, *Ocenebra erinaceus* (Linnaeus, 1758) from the Muricidae family demonstrated the potential for acclimation to low pH after three months of exposure, with no discernible effects on oxygen consumption and body weight. However, after ten months, it became evident that females failed to invest in reproduction (Mardones et al., 2022).

Studies conducted at volcanic CO_2 vents with natural pH gradients have provided valuable insights into biological responses. For example, no significant effect of low pH was found on the population structure and desiccation tolerance of the intertidal gastropod *Phorcus sauciatus* (F. C. L. Koch, 1845) at a volcanic CO_2 vent off La Palma Island in the Canary Islands where pH fluctuates from 7.0 to 8.2 over tidal cycles). However, significant differences in shell morphology, integrity, and fracture resistance were detected (Viotti et al., 2019). Research at CO_2 vents in the Mediterranean has revealed a significant reduction in the abundance (Hall-Spencer et al., 2008; Kroeker et al., 2011) and proportion of female gastropods (Harvey et al., 2016) exposed to elevated *p*CO2 levels. Additionally, changes in shell shape, size, and thickness in response to changes in carbonate chemistry have been observed in limpets, whelks, top-shells, and nassariid gastropods living near CO_2 vents (Garilli et al., 2015; Duquette et al., 2017; Viotti, 2019).

2.3 Study species, *Hexaplex trunculus*

2.3.1 Biology and ecology

H. trunculus is a widespread gastropod species of the family Muricidae, found along the coasts of the Mediterranean Sea and in parts of the Atlantic Ocean (Houart, 2001). It occurs at depths from the mid-littoral to the infralittoral zone, with a maximum depth of 120 meters (Houart, 2001; Rilov et al., 2004). However, it is most abundant at depths of 0.3 to 10 meters (Rilov et al., 2004). Higher abundances in the Adriatic Sea are found mainly in the areas where the European

flat oyster *Ostrea edulis* Linnaeus, 1758, and the Mediterranean mussel *M. galloprovincialis*, are cultured, reaching densities of up to 120 individuals/m^2 (Zavodnik & Šimunović, 1997).

The spatial distribution of banded-dye murex in the intertidal zone is influenced by sediment type and food availability, as it prefers sandy-muddy and rocky substrates and mostly preys on bivalves (Peharda & Morton, 2006; Elhasni et al., 2017). Populations in the intertidal zone exhibit greater inter-individual variability due to adaptation to unstable environmental conditions, while populations at greater depths exhibit similar growth rates for both shell length and body mass during ontogeny (Elhasni et al., 2018).

H. trunculus is a gonochoristic species with internal fertilization (Matoničkin, 1987). Males can be identified by the presence of a penis behind the right eye tentacle, while females can be identified by the presence of a capsular gland and vaginal opening (Vasconcelos et al., 2008). Gametogenesis and spawning are influenced by environmental factors, particularly sea temperature. Males are reproductively active throughout the year, while females show more intense reproductive activity during certain months, from March to May and from September to February (Vasconcelos et al., 2008). Females can store sperm in a special gland (*receptaculum seminis*) for a longer period of time and fertilize eggs when environmental conditions become favorable (Vasconcelos et al., 2004). Prior to spawning, females significantly reduce their feeding rate and activity (Vasconcelos et al., 2004; Gùler & Lök, 2014). Spawning can last from a few hours to 5 days (Vasconcelos et al., 2004). Females release egg capsules from the pedal gland, which they attach to the substrate and form irregularly shaped clusters. The number of egg capsules per female and per spawning event varies, with an average of 108 to 158 capsules per female (Vasconcelos et al., 2004; Lahbib et al., 2010; Gùler & Lök, 2014). Embryonic and larval development occurs intracapsularly, beginning with the trochophore stage and progressing through veliger and pediveliger stages until hatching. The incubation period lasts about one month and is shorter at lower temperatures. Juveniles range in length from 1.04 to 1.64 mm at hatching (Vasconcelos et al., 2004; Lahbib et al., 2010).

The lifespan of banded-dye murex is estimated to be ten years (Vasconcelos et al., 2006). However, determining the exact age of individuals is difficult as significant differences in growth rate have been found even among individuals from the same spawn (Vasconcelos et al., 2012). The species reaches sexual maturity at two years of age, with a shell length of 35 to 50 mm (Vasconcelos et al., 2006). The ratio of males to females changes with the size of banded-dye murex, with males dominating at smaller sizes, the sex ratio being equal between 40 and 50 mm,

and females dominating at larger sizes (Lahbib et al., 2009; Gharsallah et al., 2010). The behavior and activity of banded-dye murex are strongly influenced by seawater temperature. They can bury themselves shallow in soft sediment to avoid unfavorable conditions such as low winter and high summer temperatures (Rilov et al., 2004).

Both adult and juvenile specimens of banded-dye murex have an important ecological role as predators in benthic communities and can influence the population dynamics of their prey (Menge, 1976; Carriker, 1981; Morton, 2004; Gùler & Lòk, 2018). They consume a wide variety of prey, including various mollusk species, barnacles, and prey on dead fish (Rilov et al., 2004; Vasconcelos et al., 2004; Peharda & Morton, 2006; Morton & Peharda, 2007) and show a tendency to cannibalism when no other food is available (Gùler & Lòk, 2018). Prey consumption rates depend on both individual and group attacks, as well as seawater temperature (Gùler & Lòk, 2016). In addition, banded murex is commercially important for human consumption and as bait for fisheries, and is also considered a potential candidate for aquaculture (Benović, 1997; Peharda & Morton, 2006).

2.3.2 Previous studies of ocean acidification effect on *Hexaplex trunculus*

Despite their ecological and commercial importance, there is a lack of studies on the effects of ocean acidification on *H. trunculus*. To date, only three studies have been conducted, one of which is a laboratory experiment (Chatzinikolaou et al., 2019), while the other two were carried out on individuals from CO_2 volcanic seeps near the Isola Volcano in Italy (Harvey et al., 2016; Duquette et al., 2017). In one long-term study, it was observed that low pH significantly affects the ability of adults and juveniles banded-dye murex to reach their food, although the response time, duration of search, speed of movement and path index remained unchanged. Juveniles hatched and reared at low pH showed better feeding performance than adults, indicating a degree of acclimation (Chatzinikolaou et al., 2019). In volcanic CO_2 seeps, shell properties such as shell length and thickness were affected by low pH (Harvey et al., 2016). Significant shell dissolution and lower shell toughness of inner shell surfaces were also observed at low pH sites, although the mean force required to break the shell and mean shell toughness did not differ significantly between pH (Duquette et al., 2017). In addition, the metabolic activity of banded-dye murex at the low pH sites was significantly higher, although there was no significant difference in the mean metabolic response of individuals reciprocally transplanted between control and low pH sites, indicating the potential for psychological plasticity and acclimation when leaving and entering acidifying areas (Harvey et al., 2016).

3. MATERIALS AND METHODS

3.1 Study area

Mali Ston Bay is located on the east coast of the southern Adriatic Sea and is enclosed by the Pelješac Peninsula and the mainland. It is an important marine habitat with high biodiversity, protected at the national level as a special marine reserve (Official Gazette 124/13). In addition, it is also protected by the European Union ecological framework, Natura 2000 (https://www.bioportal.hr/gis/). Since Roman times, this area has been known for bivalve fisheries (Benović, 1997), with the first archival records of extensive farming of oysters dating back to the 16th century (Tomšić & Lovrić, 2004). The bay is 21 km long, has a maximum width of 2.2 km and a depth between 7 and 28 meters with mostly sandy-muddy bottom, except for the rocky coastal habitats (Benović at al., 2004). The temperature range recorded over the years of monitoring is between 12 and 29 °C. Salinity varies between 25 and 38 due to the inflow of freshwater from both the Neretva River and the underground springs (Pećarević et al., 2020). The only available data on pH are from the 1970s, with an average pH_{NBS} of 8.12 (minimum 7.98, maximum 8.27) (Stjepčević et al., 1981).

3.2 Sample collection and acclimatization to laboratory conditions

Mature adult gastropods were collected in August 2020 in the Bay Bistrina, part of Mali Ston Bay (42°52'19.1 "N 17°42'02.3 "E) (Figure 3.2.1) using plastic trays filled with crushed Mediterranean mussels *M. galloprovincialis*.

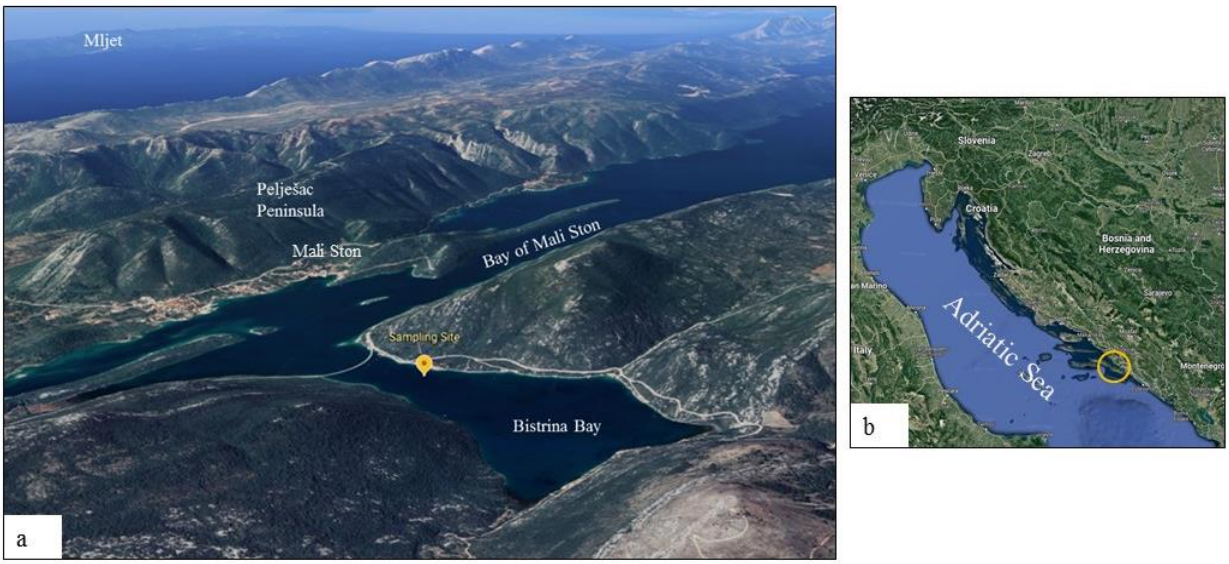

Figure 3.2.1 3D map indicating sampling site (a; yellow pin) in the study area (b; yellow circle), Bistrina Bay, Adriatic Sea (www.googlearth.com)

Trays were placed at a depth of five meters and connected to the dock by a rope. After 24 hours, gastropods were collected from the tray and measured for shell length (Sl) with a digital caliper to the nearest 0.01 mm. Males reach sexual maturity at a shell length of Sl_{50} = 40 mm and females at Sl_{50} =50 mm (Elhasni et al., 2017), therefore specimens with Sl = 49.76 ± 0.86 mm were selected for the experiment. The animals were immediately transferred to the laboratory and carefully cleaned from fouling organisms with a brush. Forty individuals were randomly selected for each experimental pH (total of nine pH treatments, n(individuals) = 360).

For analysis of spawning and embryonic development, 10 females out of 40 gastropods in each tank were marked. Banded-dye murex lacks external sexual dimorphism. Sex can be determined by the presence of a penis or vaginal opening behind the right tentacle on a cephalic region. Still, the strong foot muscle that they use to tightly close the operculum required the use of anesthetic solutions to relax the gastropods. Immersion of gastropods for two hours in an anesthetic solution of magnesium chloride hexahydrate ($MgCl_2$ x 6 H_2O) is an effective and non-invasive method of relaxing gastropods (Gibbs, 1999). For this purpose, 75 grams of $MgCL_2$ x 6 H_20 was dissolved into one liter of seawater. Animals were completely submersed in the solution and left for two hours (Fig. 3.2.2a). At this time, it was possible to carefully pull out the operculum and expose the foot and cephalic region to determine females by the absence of penis (Figure 3.2.2b).

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Figure 3.2.2 Sex determination of *Hexaplex trunculus*: a) anesthetized banded-dye murex, b) penis behind right tentacle indicating male (red circle).

Along with the females, each individual was also marked with a numbered bee tag glued to the shell (Henry & Jarne, 2007). The area of the shell to which the tags were glued with a polyacrylate adhesive was lightly brushed with sandpaper for better adhesion (Figure 3.2.3).

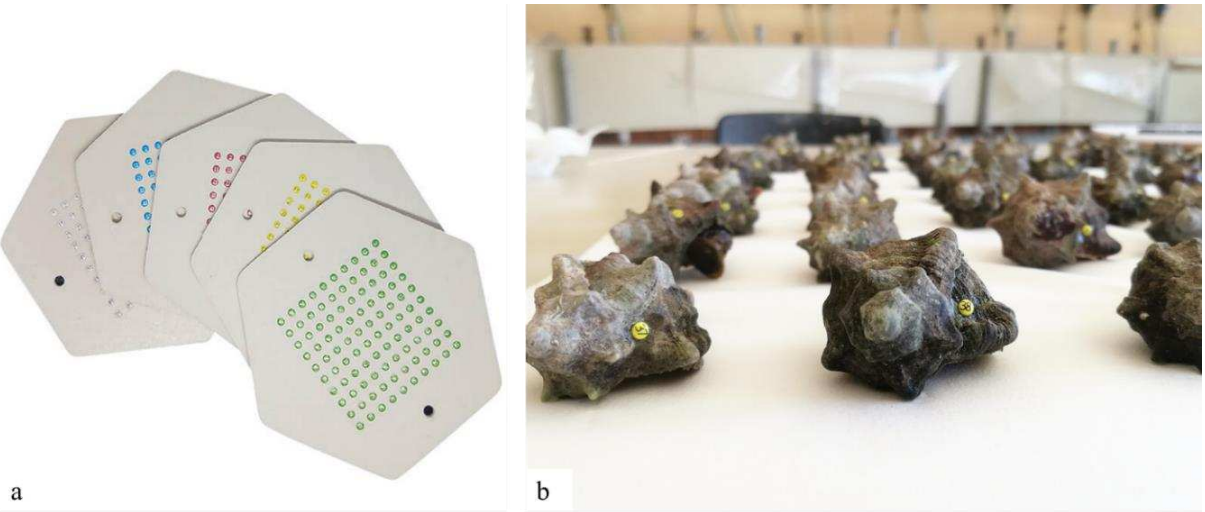


Figure 3.2.3 a) Bee tags used for marking animals, and b) glued on the shells with polyacrylate adhesive.

Before the start of the experiment, the animals were kept in a flow-through system with filtered ambient seawater for two weeks to allow them to acclimate to laboratory conditions. During the acclimation period, they were fed *ad libitum* with Mediterranean mussel. Temperature, salinity and dissolved oxygen concentration were monitored regularly.

3.3 Experiment set-up

The experiment took place in the Laboratory of Mariculture, University of Dubrovnik, located in Bistrina Bay. Nine pH values were selected for this experiment, ranging between an average pH_T 7.95 and 7.22. Experimental treatments were not replicated. pH conditions cover the present values (pH_T 8.07–7.74, this study) and the end-of-the-century range (pH_T 7.7–7.3) of natural variability in Bistrina Bay, and beyond.

Experimental system consisted of nine separate treatment tanks (volume 130L) with a flow-through water system with filtered, UV-sterilized, and aerated ambient seawater pumped from Bistrina Bay adjacent to the laboratory facilities. Randomly selected 40 individuals were placed in each of the nine tanks. The pH in each tank was manipulated separately by bubbling pure CO_2 gas from four CO_2 cylinders (one CO_2 cylinder providing gas for two tanks) and using pH

controllers (Milwaukee MC122) connected to solenoid valves to maintain the desired pH. Lab grade double junction glass electrodes (Milwaukee MA911B/2) were connected to the pH controllers (Fig. 3.3.1). Manual two-point calibration of the pH electrodes was performed twice a month with Milwaukee pH 7.01 and pH 4.01 Calibration Solution Sachets (buffer solution accuracy ± 0.01 pH). In one tank, pH was not manipulated to equilibrate with the atmospheric CO_2 concentration. Schematic representation of the experimental set-up is shown in Figure 3.3.2.

Figure 3.3.1 Equipment used in experimental system: a) CO_2 gas bottles; b) pH controller; c) pH probe; d) experimental tanks

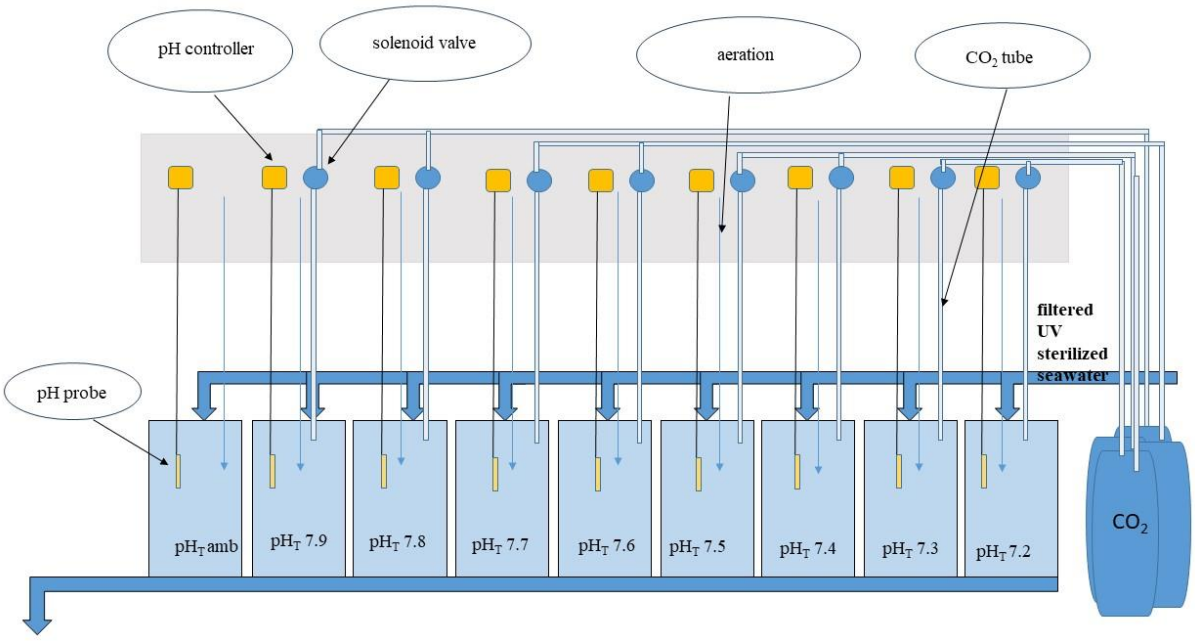


Figure 3.3.2 Schematic representation of experimental set-up.

Temperature (T; ºC), salinity (S; ppt), dissolved oxygen concentration (DO, mgL^{-1}), and pH (on the National Bureau of Standards scale – pH_{NBS}) were measured using multiparametric probes (YSI Pro 30, Oxygen Handy Polaris, and SI Analytics Lab Meter 845, respectively). Salinity and temperature followed the ambient conditions of the incoming seawater and values were confirmed by measuring the salinity and temperature of the seawater at the pump inlet twice a month. Feces and debris were siphoned every other day, and once a week a complete exchange of the seawater and detailed cleaning was done. The animals were fed *ad libitum* once a week Mediterranean mussels *M. galloprovincialis*, obtained from a local shellfish supplier. Mussels were replaced once a week. Adult gastropods were maintained under experimental conditions for 310 days (start: 24 August 2020; end: 30 June 2021).

3.4 Seawater carbonate chemistry

When carbon dioxide dissolves in the seawater, the following reactions take place:

$$CO_2\ (g) \rightarrow CO_2\ (aq)$$

$$CO_2\ (aq) + H_2O \rightarrow H_2CO_3\ (aq)$$

$$H_2CO_3\ (aq) \rightarrow H^+(aq) + HCO_3^-\ (aq)$$

$$HCO_3^-(aq) \rightarrow H^+\ (aq) + CO_3^{2-}\ (aq).$$

The equilibrium relationships between the concentrations of various carbon species in the seawater are as follows:

$$K0=[CO_2] / f(CO_2)$$

$$K1 = [H^+] [HCO_3^-]/[CO_2]$$

$$K2 = [H^+] [CO_3^{2-}]/[HCO_3^-]$$

The expression $f(CO_2)$ represents the fugacity of carbon dioxide in the gaseous state. These equilibrium constants are functions of temperature, pressure, and the salinity of seawater. The concentrations of the carbon dioxide individual species in the seawater cannot be measured directly. There are four parameters that can be evaluated analytically: total dissolved inorganic carbon (DIC), total alkalinity (TA), CO_2 in equilibrium with the seawater sample $f(CO_2)$, and total hydrogen ion concentration (pH). To obtain a description of the experimental seawater carbonate system, two of these parameters should be measured, and together with the equilibrium constants, temperature, pressure, and salinity, it is possible to calculate other parameters (Dickson et al., 2007).

For the purposes of this experiment, pH on a total scale – pH_T and total alkalinity (TA, $\mu mol\ kg^{-1}$) were measured. Other seawater carbonate chemistry parameters (pCO_2, Ω_{Ca}, Ω_{Ar}) were calculated based on known TA and pH_{NBS} for a given salinity using the CO2SYS software, with dissociation constants of Mehrbach (1973) refitted by Dickson and Millero (1987).

3.4.1 pH on a total scale, pH_T

Four different scales are commonly used to measure the pH of seawater. pH_{NBS} (National Bureau of Standards), also known as the IUPAC (International Union of Pure and Applied Chemistry) scale, measures the activity of hydrogen ions, while the pH_{sw} (seawater), pH_F (free hydrogen ions), and pH_T (total hydrogen ions) scales measure hydrogen ion concentrations but differ by measuring different dissociated protons (Dickson, 1984, Dickson, 1993a). The pH_{NBS} scale is optimized for glass membrane electrodes and the use of NBS buffer. This classical method measures free hydrogen ion activity, but the low ionic strength of NBS buffers is not suitable for seawater samples (Dickson, 1993a). The most suitable scale is the total scale, pH_T, as seawater contains high concentrations of sulfate ions and pH_T includes the concentration of free hydrogen ions and protons that dissociate from hydrogen sulfate (HSO_4^-):

$$[H^+] = [H^+]F (1+ ST / KS= \sim [H^+]F + [H_2SO_4^-],$$

where [H]F is the free concentration of hydrogen ions in seawater, ST is the total sulfate concentration ([HSO_4^-]+ [SO_4^{2-}]) and KS is the acid dissociation constant for HSO_4^-. Therefore, it has been recommended to measure and report pH on a total scale in ocean acidification research (Riebesell et al., 2011).

In this experiment, pH was measured on a total scale (pH_T) by the potentiometric method using a glass electrode cell after calibration with a TRIS (2-amino-2-hydroxy-1,3-propanediol) buffer prepared in synthetic seawater to keep activity coefficients similar between the buffer and a sample of seawater (Dickson, 1993b; Millero et al., 1993). The TRIS buffer was prepared according to a simplified method specifically developed for monitoring carbonate chemistry in biological experiments (Paulsen & Dickson, 2020).

3.4.2 TRIS buffer preparation

Simplified volumetric preparation of the TRIS buffer consists of a few steps with the main focus on making sure that the buffer ratio TRIS : H+ is 1 : 1, together with synthetic seawater composition to ensure appropriate activity coefficients consistent with other seawater acid-base constants (Paulsen & Dickson, 2020).

A colometric acid-base titration with methyl red indicator was used to determine the exact molarity content of a ~1M HCl solution. The HCl solution was prepared by diluting 37% reagent grade HCl solution (37% AnalaR NORMAPUR). Approximately one gram of TRIS (99.8 – 100.8 % AnalaR NORMAPUR), recorded to the nearest 0.1 mg, was dissolved in 80 g of deionized water and six drops of 0.1% methyl red indicator were added to the alcoholic solution. The glass beaker is placed on a magnetic stirrer with the yellow TRIS solution titrated by weight with 1 $molkg^{-1}$ HCl using disposable Pasteur pipettes until a distinct pink color is obtained (Figure 3.4.2.1).

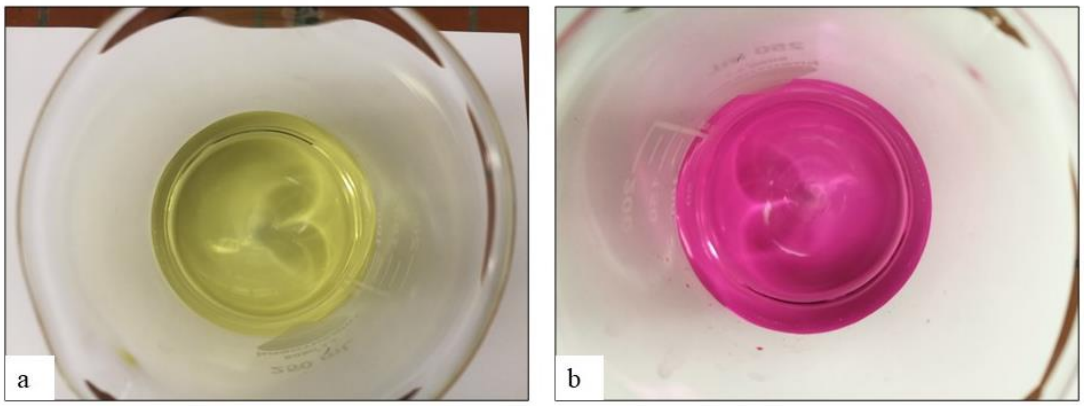


Figure 3.4.2.1 Colorimetric acid-base titration: a) titration of TRIS solution b) pink color indicating the end of titration.

The pipettes were filled with 0.1 mol l^{-1} solution, placed on a small beaker and weighed to 0.1 mg before and after titration to determine the weight of HCl added. Obtained weights (w) were corrected to mass (m) by applying the air buoyancy correction and densities (ρ) of TRIS and HCl, 1.33 g cm^{-3} and 1.02 g cm^{-3}, respectively:

$$m\ (\mathrm{g}) = w\ (\mathrm{g}) \times (1+0.0012 \times (1/\rho - 1/8)).$$

The amount (in mol kg solution-1) of HCl [HCl]titr is calculated based on the mass (m) in g of TRIS and the HCl used in the titration:

$$n(\mathrm{HCl}) = n(\mathrm{TRIS})$$

$$[\mathrm{HCl}] \times m(\mathrm{HCl})_{\mathrm{solution}} = m(\mathrm{TRIS}) / M(\mathrm{TRIS})$$

$$[\mathrm{HCL}]_{\mathrm{titr}}\ (\mathrm{mol\ kg\ solution}^{-1}) = m(\mathrm{TRIS}) / M(\mathrm{TRIS}) \times 1000 / m(\mathrm{HCl})_{\mathrm{solution}}$$

Where n is the amount of substance in moles, mol, M is the molar mass of the substance in $gmol^{-1}$, and square brackets indicate molar concentration in mol per kg $solution^{-1}$.

The calculated amount content of HCl, [HCl]titr was used to determine the weight of HCl needed to prepare the buffer. To prepare one liter of TRIS buffer, HCl was weighed in a volumetric flask to within 0.3 g of the calculated HCl weight. Other buffer component weights (TRIS, NaCl, Na_2SO_4, KCl, $MgCl_2$, $CaCl_2$) were then recalculated and scaled to dispensed HCl to ensure that ratio of moles between all components remains the same:

$$w(\mathrm{component})_{\mathrm{target}} = w(\mathrm{component})_{\mathrm{desired}} \times w(\mathrm{HCl})_{\mathrm{dispensed}} / w(\mathrm{HCL})_{\mathrm{desired}}$$

After re-calculation, other components were carefully weighed and added to the volumetric flask, along with the deionized water and left to stir on the magnetic stirrer (VWR) for a minimum of four hours.

To validate the accuracy of pH_T measurements with the TRIS buffer prepared in the laboratory, it was compared with the TRIS buffer purchased from the Scripps laboratory (Batch T37). The pH probe was calibrated with both buffers (Figure 3.4.2.2) and pH_T was measured. The calculated pH_T after calibration with the TRIS buffer prepared in the laboratory was on average 0.0217 ± 0.0001 pH units larger than after calibration with the purchased TRIS buffer T37.

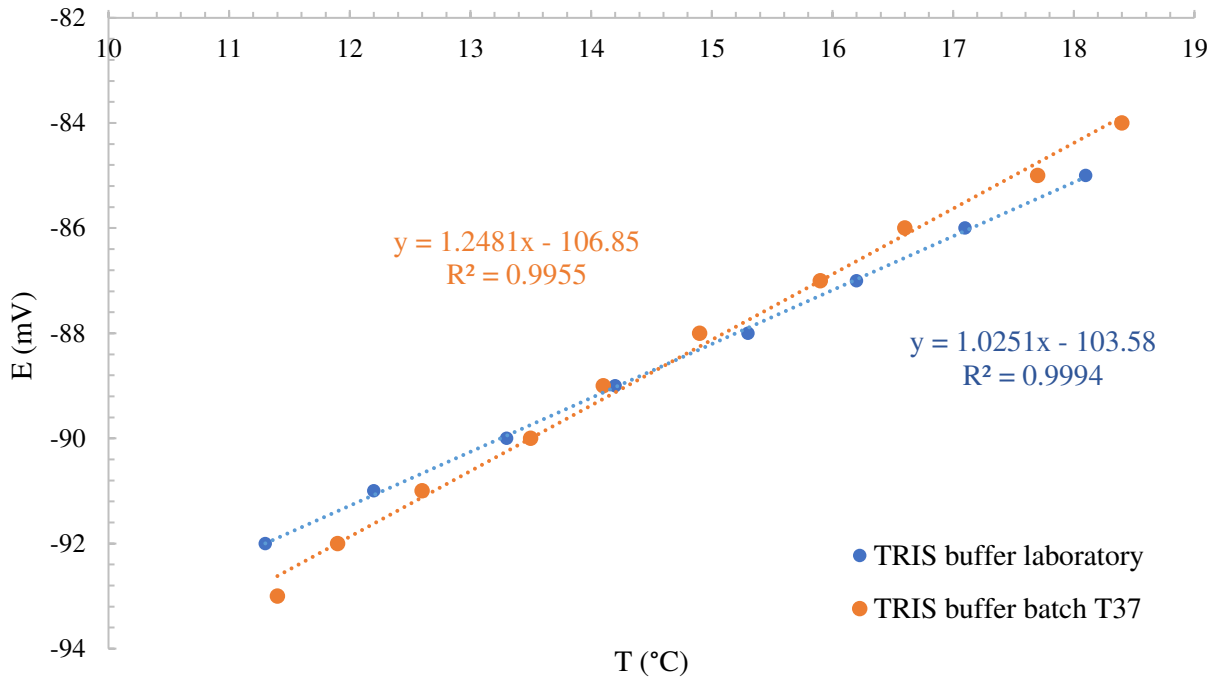


Figure 3.4.2.2 Comparison of pH glass probe calibration with the TRIS buffer prepared in the laboratory (blue circles) and the purchased TRIS buffer batch T37 (orange circles). Electrode potential (E, mV) changes with respect to temperature (T, °C).

3.4.3 Sampling and pH_T calculation

Following the guide to best practices for ocean acidification research (Riebesell et al. 2011) and recommended procedures (Dickson et al. 2007), pH was measured on a total scale (pH_T) every 2 days by the potentiometric method after calibration with the TRIS buffer. The TRIS buffer pH depends on the temperature and salinity, and was calculated as follows:

$$pH_T(TRIS) = (11911.08 - 18.2499 \times S - 0.39336 \times S^2) \times 1/T - 366.27059 + 0.53993607 \times S + 0.00016329 \times S^2 + (64.52243 - 0.084041S) \times \ln(T) - 0.11149858 \times T.$$

Temperature and salinity were measured in experimental units prior to sampling. Seawater samples were taken with a 250 ml glass beaker. Immediately after sampling an electrode potential was measured by immersing the glass electrode into the beaker and gently stirring. pH probe was rinsed with the sample seawater before taking measurement (Figure 3.4.3.1). After taking all the measurements, pH_T was calculated as follows:

$$pH_{T(sample)} = pH_{T\ (TRIS)} + ((E_{TRIS} - E_{sample})/(RT \ln 10/F).$$

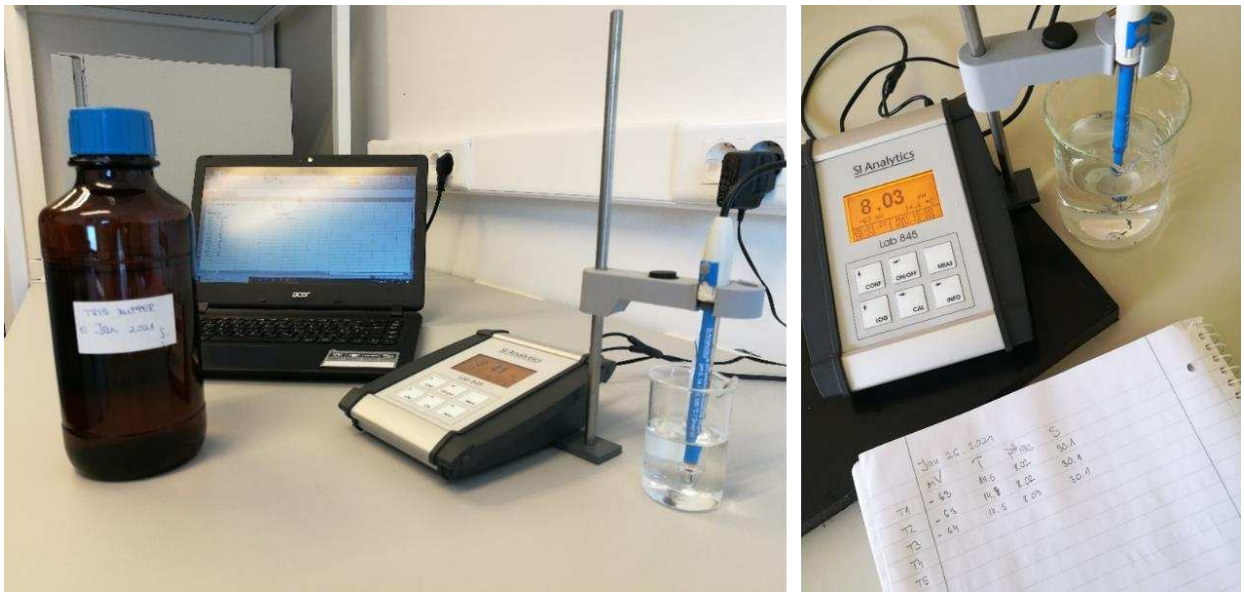


Figure 3.4.3.1 a) pH probe calibration with TRIS buffer), and b) experimental seawater sampling.

pH_T in Bistrina Bay was measured in the course of the experiment, approximately twice a month on the seawater samples taken from the adjacent sea in front of the pump inlet (one-meter depth).

3.4.4 Total alkalinity, TA

The total alkalinity (TA, µmol kg^{-1}) of seawater is defined as the number of moles of hydrogen ion equivalent to the excess of proton acceptors (bases formed from weak acids with a dissociation constant, $K \leq 10^{-4,5}$ at 25 °C and zero ionic strength) over proton donors (acids with $K > 10^{-4,5}$) in 1 kg of the sample (Dickson et al., 2007). Following the guide to best practices for ocean acidification research (Riebesell et al., 2011) and recommended procedures (Dickson et al., 2007), the total alkalinity was determined every two weeks with the potentiometric two-point open-cell titration method where the seawater sample is titrated with 0.1 M hydrochloride acid (HCl) prepared in a 0.7 M sodium chloride (NaCl) solution to approximate the ionic strength of seawater and to maintain approximately constant activity coefficients during the titration (Dickson et al., 2007).

The samples for alkalinity titrations were taken from each pH tank using a Tygon tube and transferred to Winkler bottles to avoid air contact. Both the 0.1 M HCl titrant and the Winkler bottles containing the seawater samples were placed in a water bath to maintain them at the same temperature between 20 and 25 °C. Before titration, the sample bottle was weighed to 0.01 g. The sample was then poured into a 250 ml glass beaker containing a stir bar and placed on a stirrer. The empty bottles were weighed again, and the sample weight was determined by calculating the

difference between the two weights. A pH probe was carefully inserted into the beaker to measure the electrode potential (E, mV). Titration was performed using a manual burette filled with the titrant, and the initial volume was recorded (Figure 3.4.4.1). In the first step of the titration, the seawater sample was titrated with a single aliquot of the 0.1 M HCl titrant to bring the pH slightly above ~ pH 3.5. The dispensed volume of the titrant was recorded. The amount of acid added depends on the approximate alkalinity and sample volume, and was 2 ml for the samples in this study. The acidified sample was then stirred for six minutes to allow for CO_2 degassing. In the second step of the titration, the sample was titrated dropwise (in increments of ~ 0.05 ml) until a final pH of 3.0 was reached (Fig. After each addition, the dispensed volume (ml), E (mV) and sample temperature (°C) were recorded. The recorded data were used to calculate total alkalinity using an Excel spreadsheet template adapted for the open-cell method provided by the Global Observing Ocean Acidification Network (GOA-ON). Initially, the Gran approach was used to estimate alkalinity, followed by the nonlinear least squares method for the final calculation of total alkalinity. The equations used these data to estimate the value of TA based on a linear least squares model. The estimate was further refined by applying the solver function in the second spreadsheet, resulting in a final estimate of TA. Total alkalinity was determined based on the volume of acid required to reach the second endpoint and the acid normality. The inflection point (V2) was determined using a Gran plot (Gran, 1952) that corrected for bisulfate and hydrogen fluoride interference. Sulfate and fluoride concentrations were calculated from salinity and equilibrium constants for bisulfate and hydrogen fluoride (Dickson & Riley, 1979; Dickson, 1981).

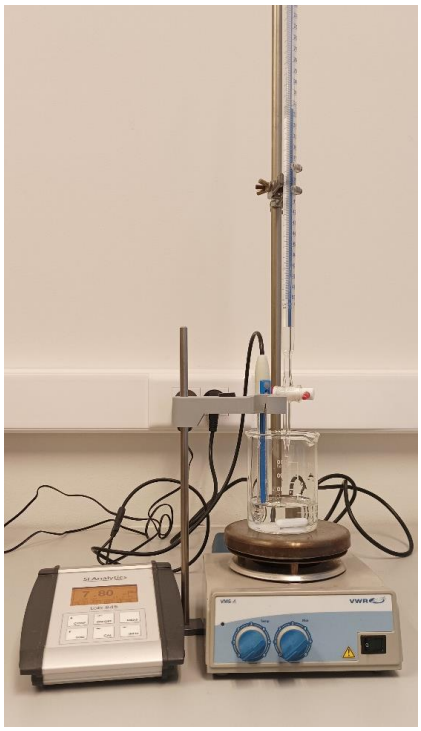


Figure 3.4.4.1 Manual titration set-up for determination of total alkalinity (TA).

3.5 Effect of long-term exposure to different pH on the performance of *H. trunculus*

3.5.1 Feeding

Feeding habits were assessed by weekly food intake and a separate experiment after long-term exposure to low pH. Specimens were fed *ad libitum* for 40 weeks Mediterranean mussels *M. galloprovincialis* (shell length = 67.1 ± 0.83 mm), obtained from a local shellfish supplier (Figure 3.5.1.1a). Mussels were replaced once a week. Empty shells were counted and divided by the number of individuals in each treatment (3.5.1.1.b) The feeding rate (mussel week^{-1}) was calculated as the average number of mussels consumed per week and per individual.

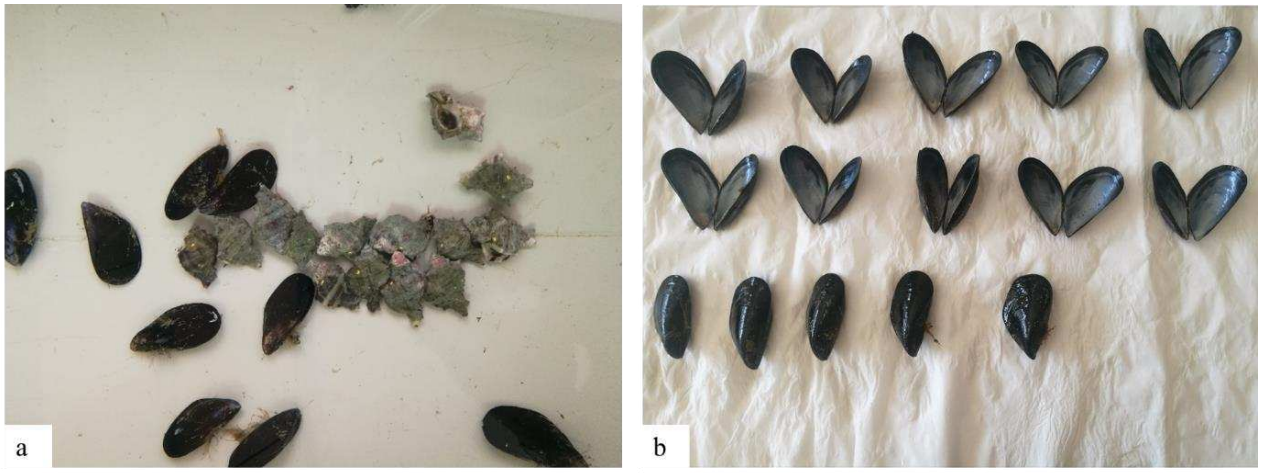


Figure 3.5.1.1. a) *Hexaplex trunculus* feeding on a Mediterranean mussel; b) weekly counting of consumed mussels.

For the second part of the experiment, three pH_T treatments were selected to cover the range of the experimental design – pH_T 7.95, 7.67 and 7.22. The percentage of individuals (%) reaching their food and the time needed to reach it (min) were estimated for gastropods exposed for 60 weeks to each pH condition. All individuals were starved for two weeks prior to the experiment. Plastic trays (52 x 35 x 20 cm) were filled with seawater from each respective pH treatment. Four fresh mussels were manually opened and placed on one side of the tray. Four randomly selected individuals were chosen and placed on the opposite side with the anterior-facing mussels (Figure 3.5.1.2). The time to reach the food (min) was measured with a stopwatch, for a maximum of 60 minutes. The number of individuals that successfully reached their food was noted. For each pH treatment, the experiment was repeated three times.

Figure 3.5.1.2 Experiment set-up to test the pH effect on the percentage of *Hexaplex trunculus* to reach the food and time needed.

3.5.2 Shell length growth rate

Shell length (SL, mm) was measured as the maximum length along the central axis, from the apex of the shell to the end of the siphonal canal (Vasconcelos et al., 2016) (Figure 3.5.2.1). Measurements were performed on the same individuals nine times in total over the course of the experiment. Each marked individual was measured with a digital caliper to the nearest 0.01 mm. Shell length growth rates for each individual per pH (SGR in mm day^{-1}) were calculated from changes in the shell length between successive observation points.

Figure 3.5.2.1 *Hexaplex trunculus* shell length measured as the maximum length from the apex of the shell to the end of the siphonal canal.

3.5.3 Net calcification rate

Ocean acidification may impact organisms' process of calcium carbonate precipitation (gross calcification) and increase the dissolution of calcified structures (Findlay et al., 2009). Net calcification is defined as gross calcification minus dissolution (Smith & Key 1975) and indicates a net change in calcium carbonate in marine shells or skeletal structures (Findlay et al., 2009).

The buoyant weight technique is a method commonly used to measure net calcification or changes the in mineral composition of marine shells (Fitzer et al., 2019). This method is non-invasive (Molina et al., 2005) and is often used to determine net calcification over long periods of time (Herler & Dirnwober, 2011).

Non-destructive technique described by Palmer (1982) was applied to measure shell weight and evaluate net calcification of *H. trunculus* over the course of the experiment.

The weight of the whole individual in seawater (buoyant weight) was determined by placing it on an improvised holder made of copper wire attached directly to the hook at the bottom of the scale (Mettler toledo JL602-G/L). The scale was placed on a stand with a hole in the center, under which was the container of seawater (Figure 3.5.3.1). Immediately before weighing, the gastropods were carefully forced to close the operculum to clear their mantle cavities of possible air. The balance was tared to compensate for the weight of the improvised holder, and the weight of the submerged gastropods was recorded to the nearest 0.01 g. The procedure was repeated seven times during the experiment.

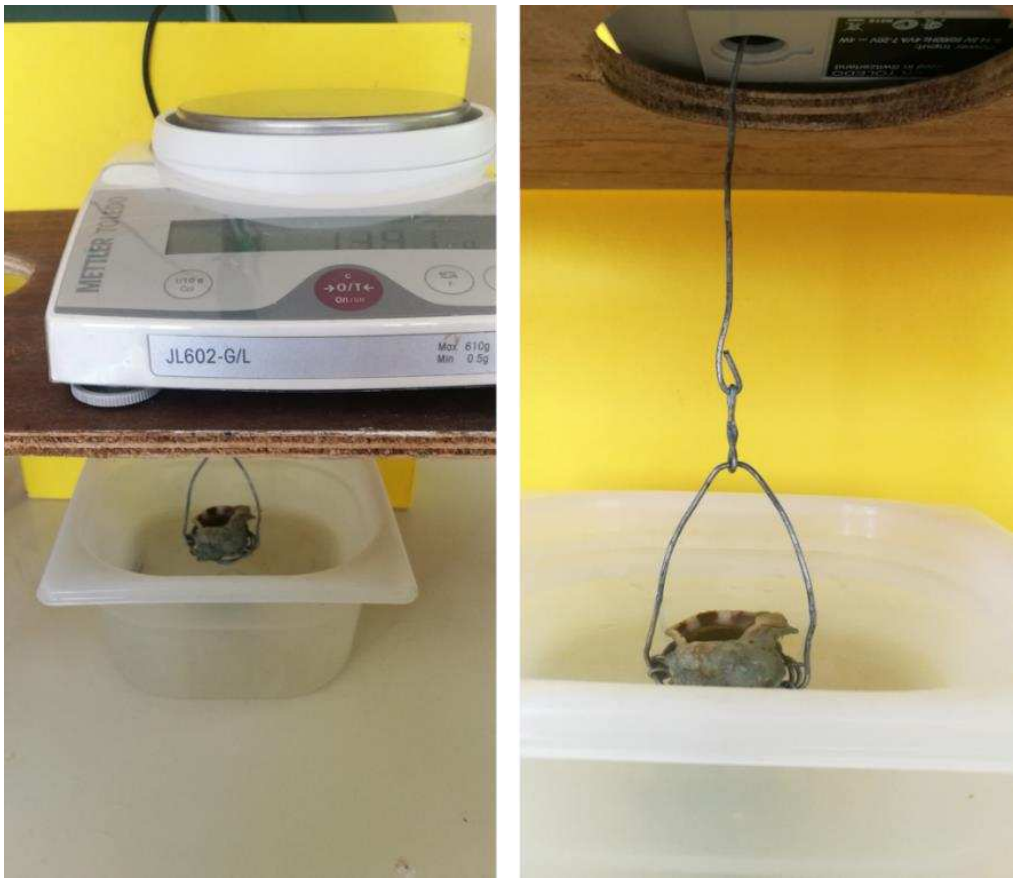


Figure 3.5.3.1 Measuring *Hexaplex trunculus* buoyant weight in the container with seawater placed on an improvised wire holder.

To obtain estimates of shell weight from immersed weight it was necessary to compute a regression of actual shell weight on immersed weight. For this purpose, 20 individuals from the size range of individuals in the experiment were collected from Bistrina Bay and their immersed weight were recorded. The gastropods were then sacrificed and their shells were carefully separated from soft tissue and dried to constant weight at 80 °C. A linear regression between shell dry weight and the immersed weight was calculated (Figure 3.5.3.2). Slope and intercept were used to estimate actual shell weight from the immersed weight of gastropods in the experiment:

$$\text{Shell weight (g)} = 1.4226 \times \text{buoyant weight (g)} - 0.3922$$

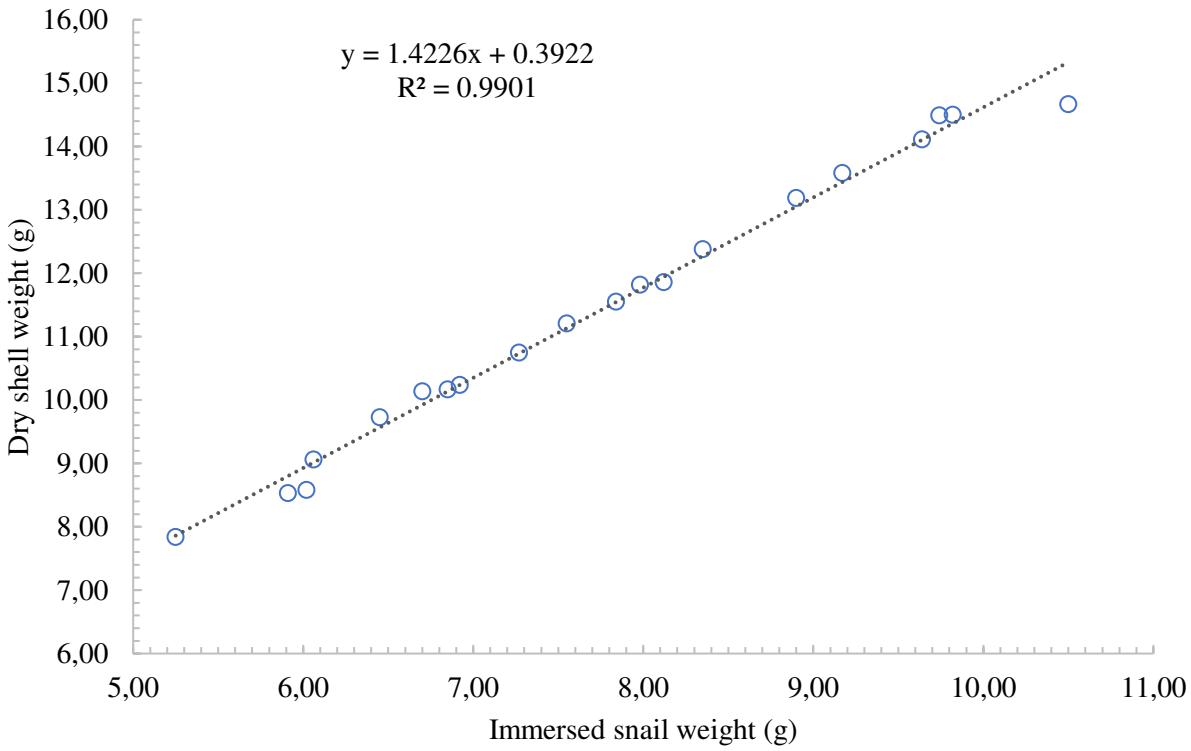


Figure 3.5.3.2 Relationship between dry shell weight (g) and immersed *Hexaplex trunculus* weight indicating that immersed weight can provide an accurate estimate of shell weight (SLR $F(1,19) = 1807.02$, $R^2 = 0.9901$, $p = > 0.001$).

Net calcification rates for each individual per pH (CR in g day^{-1}) were calculated from changes in the shell weight between successive observation points.

3.5.4 Total weight growth rate

Total gastropod weight was measured to the nearest 0.01 g on the top-load scale (Mettler toledo JL602-G/L) for the same individuals nine times in total over the course of the experiment. Prior to weighing, gastropods were placed on absorbent blotting paper for approximately 20 minutes to remove excess water (Figure 3.5.4.1). Total weight growth rates for each individual per pH (TWGR in g day^{-1}) were calculated from changes in the total weight between successive observation points.

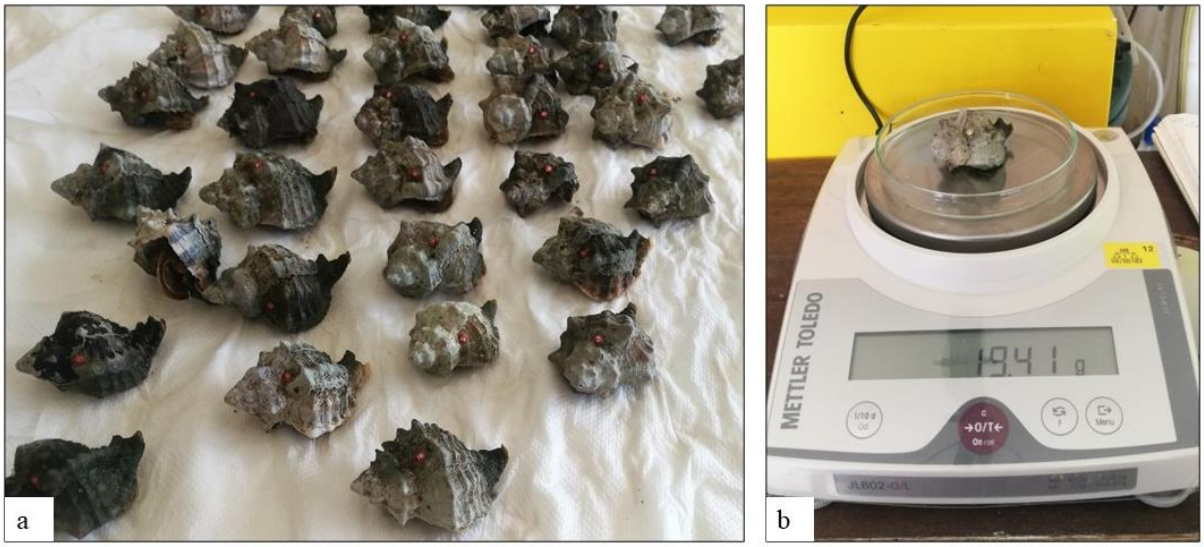


Figure 3.5.4.1 Measurements of total body weight: a) *H. trunculus* left on blotting paper to remove excess water; b) measuring of weight on a top-load scale.

3.5.5. Soft tissue weight growth rate

The total weight of gastropods consists of both shell and soft tissue weight. With previously described measurements of the total and shell gastropod weight, the soft body weight can be calculated as follows:

Soft tissue weight growth rate (g) = total weight growth rate (g) – shell weight growth rate (g).

Soft tissue growth rates for each individual per pH (STWGR in mm day^{-1}) were calculated as the difference in the total weight growth rate and shell weight growth rate between successive observation points.

3.5.6 Sex determination

At the end of the experiment, specimens from all treatments were sampled and frozen until further analysis. For sex determination, shells were cracked with a vice to expose the soft tissue. Males were identified by the presence of a penis behind the right tentacle, and females by the vaginal opening.

In total, there were 162 males and 183 females with the sex ratio of 1:1.08. The percentage of males and females in each pH_T is presented in Figure 3.5.6.1.

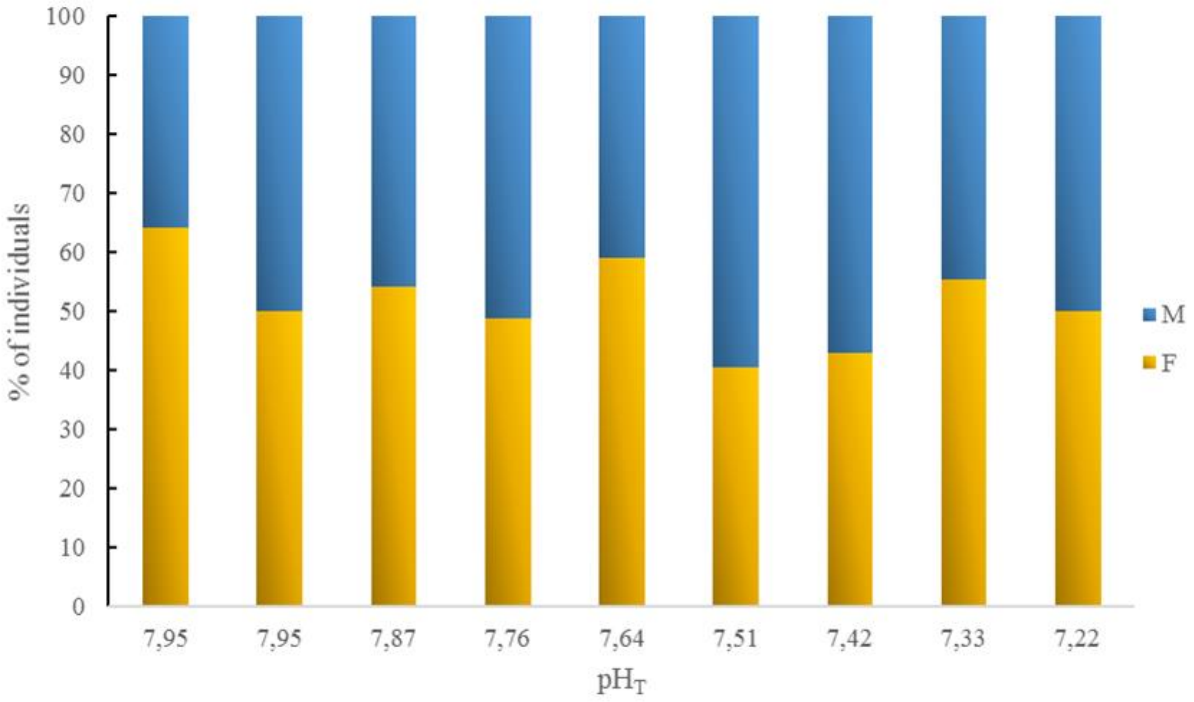


Figure 3.5.6.1 Percentage (%) of *H. trunculus* males (blue) and females (yellow) in the different pH treatments.

The sex of each marked individual was recorded to evaluate the effects of sex on shell length growth rate, total weight growth rate, net calcification rate and soft tissue weight growth rate, and to test whether the effect of sex differs across pH levels.

3.5.7 Respiration rate of females

To evaluate the metabolic rate of females, respiration was measured a total of five times during the experiment (days 88, 149, 181, 209, and 240). Individual gastropods were placed in hermetically sealed containers filled with air-saturated seawater taken from each respective pH treatment. The seawater was not filtered. Background oxygen consumption was measured in three blank samples per pH to adjust the final calculations. Dissolved oxygen concentration was measured at the beginning and end of incubation (maximum of 2h) using an oxygen microprobe (UMS Oxyscan 300 Lab). The respiration rate was standardized to the total body weight of the gastropods (TW). Weight measurements were taken immediately after the incubation period. Individuals were left on blotting paper for 15 minutes to remove as much excess water as possible, and the total weight was measured on a top-load scale (Mettler toledo JL602-G/L).

The respiration rate was calculated as follows:

$$R= [((c_2-c_1) – c_0) / t] / TW$$

Where:

R – respiration rate (O_2 mg L^{-1} O_2 min^{-1} g TW^{-1})

c_1 – DO concentration at the beginning of incubation

c_2 – DO concentration at the end of incubation

C_0 – DO concentration in blank samples

t – incubation time, in minutes

TW – total weight, in grams.

3.5.8 Reproduction and intracapsular development

The expected spawning time of *H. trunculus* is late spring (Vasconcelos et al., 2004), when the sea temperature begins to rise. Females tend to aggregate together for spawning, making communal spawns by attaching their capsules to each other. Therefore, to obtain individual spawns, marked females were separated into individual containers at the beginning of May 2021. Each female was transferred to a 5-litre plastic canister cut open at the top, filled with water at the treatment's pH, and covered with a net to prevent escapes (Figure 3.5.8.1). In addition, longitudinal openings were cut three centimeters from the bottom of the tank on opposite sides to allow water to flow through the container. Until the start of spawning, females were fed Mediterranean mussel, *M. galloprovincialis*.

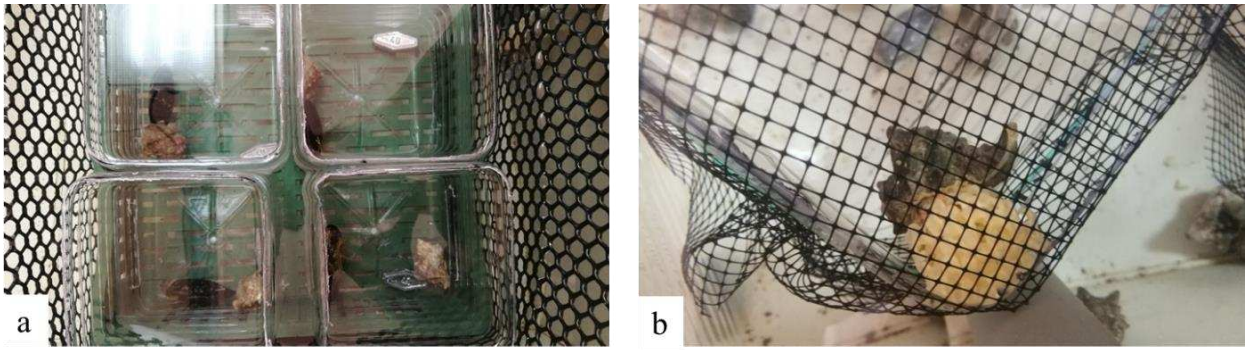


Figure 3.5.8.1 a) females separated into plastic tanks; b) female spawning.

3.5.8.1 Spawning

The start and duration of spawning were recorded for each female. After the completion of spawning, females were taken out from the containers and placed back in the tank with the other individuals. Females were separated into individual containers for approximately 45 days. Spawn was left in the container. The number of females spawning was recorded for each pH treatment. Immediately after spawning, ten randomly selected capsules from each spawn were measured with

a digital caliper (precision 0.01 mm) for length (cl, mm), width (cw, mm) and thickness (ct, mm), with the length being the greatest distance between the basal membrane and the apex, width the greatest distance between lateral edges at right angles to length, and thickness the greatest distance from convex side to concave side at right angles to length and width (D'Asaro 1970, 1986). Five capsules were carefully opened with a scalpel; eggs were emptied onto a microscope slide and counted under a stereo microscope (Olympus SZ40).

3.5.8.2 Intracapsular development

The method for monitoring intracapsular development was modified following previous studies by Vasconcelos et al. (2004), Lahbib et al. (2010), and Gűller and Lok (2014). Four days after spawning, a minimum of two capsules were carefully removed from each spawn. Fertilized eggs were emptied onto a microscope slide and photographed with a microscope digital camera (Olympus DP72) under a light microscope (Olympus BX51). The diameter of a minimum of 50 eggs from each capsule was measured with the software Fiji.

To determine the stage of intracapsular embryonic development, random capsules were sampled four times until hatching at minimum. The capsules were preserved in 4% solution of formaldehyde in seawater for further analysis. Each capsule was carefully opened with a scalpel and emptied onto a slide. The embryos were photographed under a light microscope and the length was measured using the Fiji software. The developmental stage was determined based on the characteristic structures. The first larval stage, the trochophore, was determined by the fine cilia on the anterior side (Lahbib et al., 2010). The first characteristic structure of the veliger stage is the development of a short, bi-lobed velum, eyes, and visceral mass, indicating an early veliger (Lahbib et al. 2010, Gűller and Lok 2014). Further veliger stage was determined by shell formation and more pronounced velar lobes (Vasconcelos et al., 2004). The development of the foot and the large four-lobed velum indicated pediveliger larvae. At the end of intracapsular development, the velum began to degenerate and the shell became pigmented yellow-brown. The hatchlings pierced the fine membrane covering the capsular opening and crawled outside (Vasconcelos et al., 2004; Lahbib et al., 2010) (Figure 3.5.8.2.1).

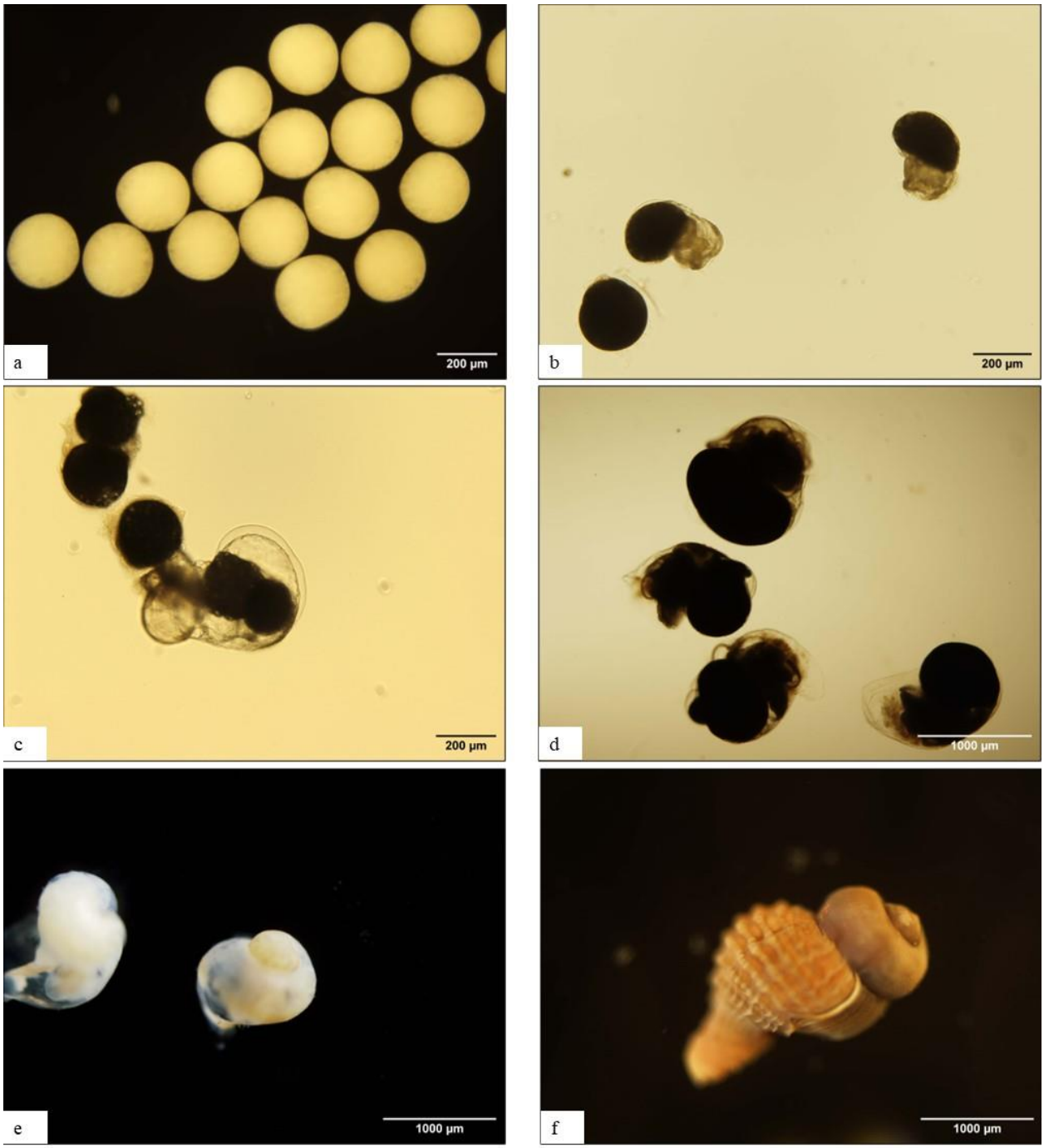


Figure 3.5.8.2.1. Stages of *Hexaplex trunculus* intracapsular development: a) fertilized eggs, b) trochophore, c) early veliger, d) veliger, e) pediveliger, f) hatchling.

3.5.8.3 Carryover effect

To evaluate the carryover effect of parental exposure to the embryos sensitivity to pH, spawn were transferred to different pHs (Figure 3.4.8.3.1). Spawns were selected based on their size and accessibility in the container. After females completed the spawning, the selected spawns were carefully separated with a scalpel and cut in half (Figure 3.4.8.3.2). One-half of the spawn

was returned to the pH_T from which it had been removed, and the other half was placed in the designated pH_T. At minimum four times over the course of the intracapsular development, two capsules were carefully removed from transplants and placed in 4% formaldehyde for further analysis (measurement of embryo size and developmental stages).

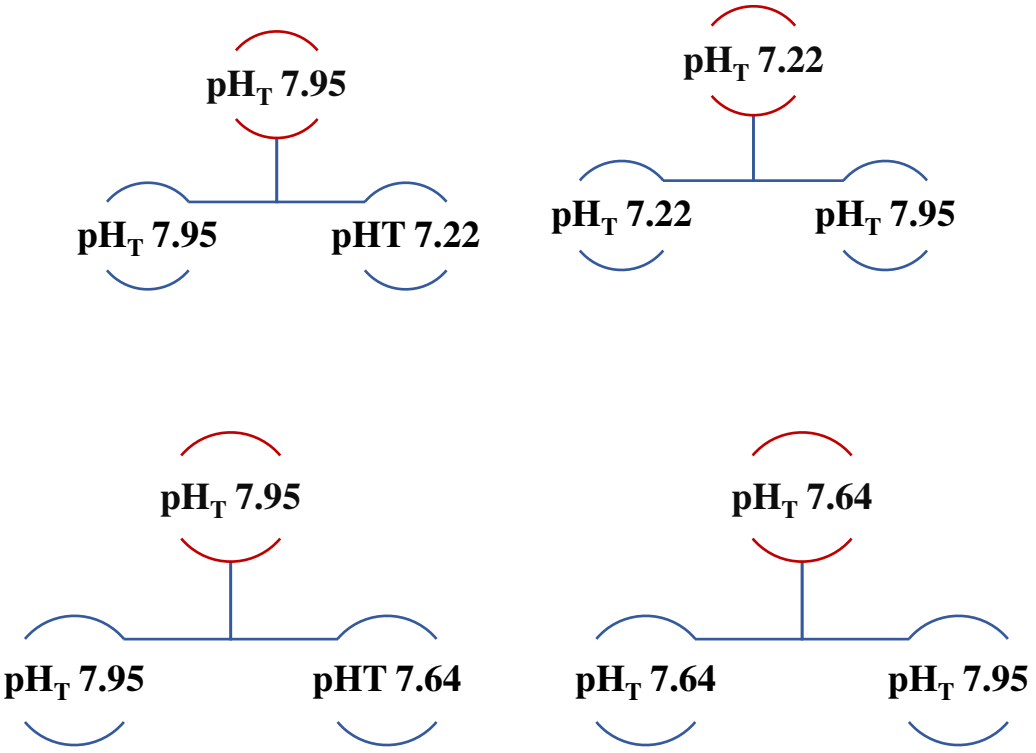


Figure 3.5.8.3.1 Schematic representation of *H. trunculus* spawns cross-transplants between pH_T. Red half-circles indicate pH_T of the parental exposure, blue half-circles indicate pH_T of embryo exposure. Each transplantation was repeated in triplicates.

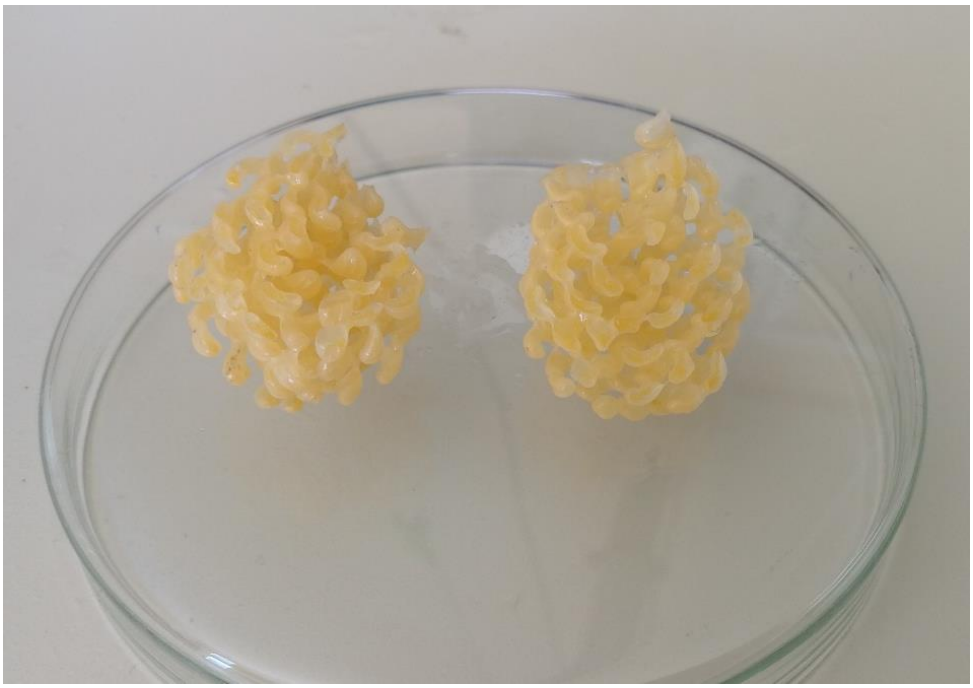


Figure 3.5.8.3.2 *Hexaplex trunculus* spawn cut in half for cross transplantation.

3.6 Statistical analyses

Statistical analyses were performed using SPSS Statistics v.26. Mean differences in temperature and salinity among pH treatments were tested with one-way Analysis of Variance (one-way ANOVA). The relationship between pH_T and feeding rate was tested with simple linear regression (SLR), whilst the mean difference in time to reach the food between treatments was tested with one-way ANOVA. The effect of pH on the shell growth rate, calcification rate, total weight and soft tissue weight was tested with the linear mixed model (LMM) with pH and sex as fixed variables and individual gastropod ID as a random variable to account for the repeated measurements on the same individuals over time. Measurements closer in time are expected to be more correlated than measurements further away, therefore an auto-regressive (AR-1) covariance structure was applied. Main effects of the fixed factors (pH and sex) on the dependent variable were tested, and whether the effect of sex on the dependent variable differs across pH was analyzed with an interaction term between sex and pH included in the model (sex*pH). The relationship between the respiration rate and pH_T was tested with simple linear regression. The binary logistic regression model was applied to determine if pH had a significant effect on the likelihood of spawning and on the likelihood of reaching a developmental stage (when applicable), with the regression coefficient estimated (β) interpreted as a predicted change in log odds for every single unit increase of pH. The relationships between pH_T and capsule length, width and thickness, average number of eggs per capsule, number of spawned capsules and egg diameter per spawn

were tested with simple linear regression. The mean difference in the average developmental stage length among pH_T was tested with one-way ANOVA, and the relationship between the day post spawning when a respective developmental stage was reached and pH_T was tested with simple linear regression (SLR). Average intracapsular growth rate for each pH_T was calculated from the log-linear relationship between developmental stage length and developmental time (µm log day^{-1}). The growth rate was then plotted against pH_T to test for relationship. Intracapsular growth rate for each transplanted spawn was calculated from the log-linear relationship between developmental stage length and developmental time (µm log day^{-1}). After log linearization of the data, embryo length between transplants was compared with ANCOVA with developmental time as a covariate. Prior to analysis, the data were tested for normality of residuals with a Q-Q plot or Shapiro-Wilk's test, and for the equality of variances with Levene's test. All data met the assumptions. The threshold for significance was set at $p < 0.05$. When a significant effect was observed, a post-hoc Tukey pairwise comparison was applied with Bonferroni correction for multiple comparisons. Estimated marginal means (EMMs ± SE) obtained from the model were used to further investigate the trend of the relationship between pH_T and the dependent variable.

4. RESULTS

4.1 Seawater parameters

Temperature and salinity varied with time, between 8.4 and 26.6 °C for temperature and 22.6 and 35.3 for salinity (Figure 4.1.1). The tested pH had no effect on temperature and salinity (one-way ANOVA: $F(8, 855) = .02$, $p = 1.00$), $F(8, 882) = .00$, $p = 1.00$, respectively). The data were therefore combined for further analysis.

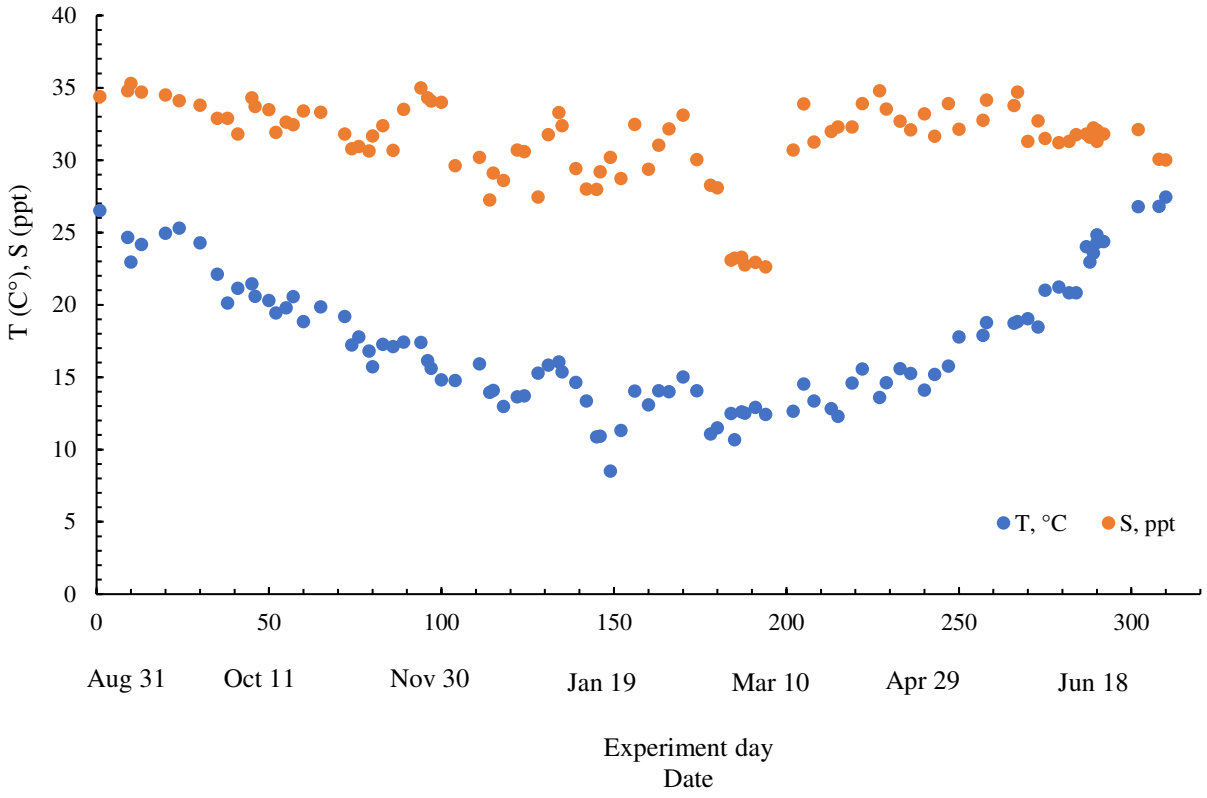


Figure 4.1.1 Temperature (T, °C) and salinity (S, ppt) fluctuations showing seasonal variations

The dissolved oxygen concentration never fell below 6.28 mg L^{-1} O_2. The pH_T of seawater in the unmanipulated pH treatment varied between 7.75 and 8.05 during the experiment, corresponding to the nearshore pH variability in Mali Ston Bay (Figure 4.1.2).

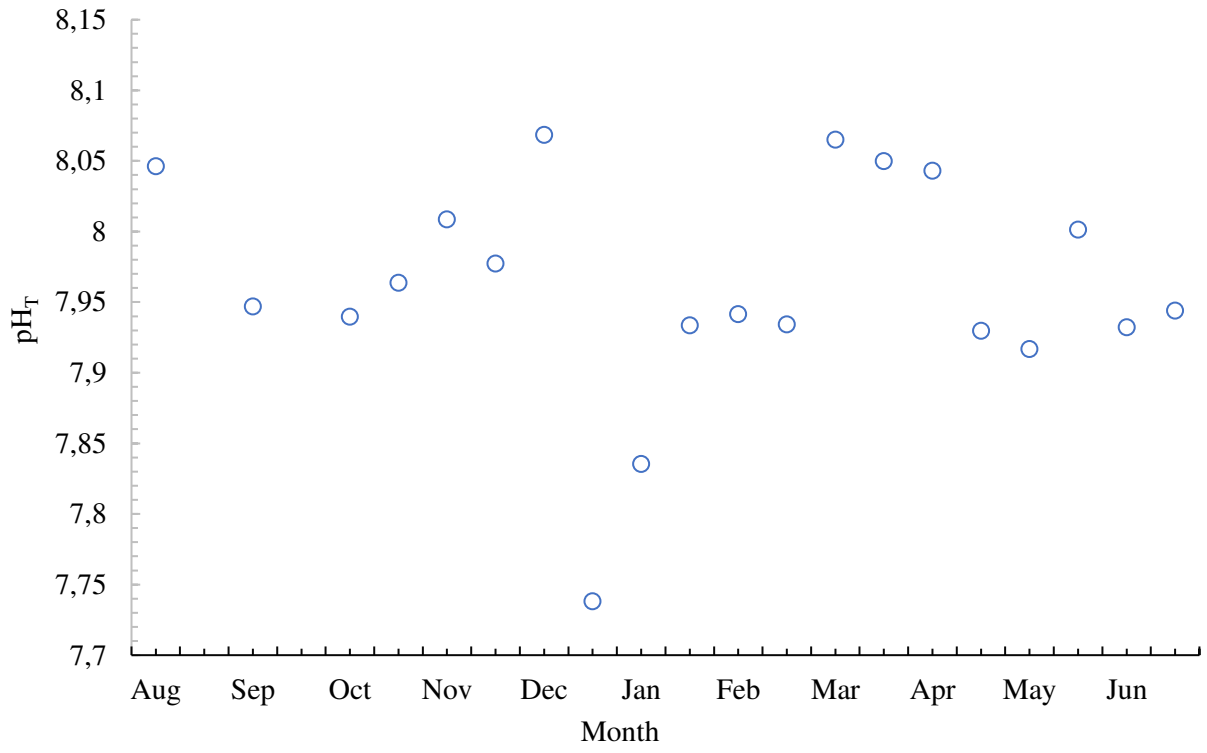


Figure 4.1.2 Measured pH_T in Bistrina Bay over the course of the experiment.

Seawater was undersaturated with respect to calcite only at pH_T 7.22 (Ω Ca = 0.9 ± 0.6), whereas undersaturation in respect to aragonite occurred at pH_T 7.42, 7.33 and 7.22 (Ω Ar = 0.9 ± 0.1, 0.7 ± 01 and 0.6 ± 0.1, respectively). The measured and calculated carbonate chemistry parameters are listed in Table 4.1.1.

Table 4.1.1 Seawater carbonate chemistry parameters presented as mean ± SD. Measured: Seawater pH on a NBS scale (pH_{NBS}), total scale (pH_T), salinity (S; ppt), temperature (T; ºC) and total alkalinity (TA; mmol kg^{-1}). Calculated: CO_2 partial pressure (pCO_2; µatm), calcite and aragonite saturation states (Ω_{Ca} and Ω_{Ar}, respectively).

	Measured				Calculated			
Target pH_{NBS}	pH_{NBS}	pH_T	S (ppt)	T (ºC)	TA (mmol kg^{-1})	pCO_2 (µatm)	Ω_{Ca}	Ω_{Ar}
Control	8.08 ± 0.07	7.95 ± 0.07	31.3 ± 2.8	17.4 ± 4.6	2976 ± 216	692 ± 18	3.9 ± 0.8	2.5 ± 0.6
8.1	8.07 ± 0.07	7.95 ± 0.08	31.3 ± 2.8	17.4 ± 4.6	2950 ± 176	698 ± 19	3.9 ± 0.9	2.5 ± 0.6
8.0	8.00 ± 0.05	7.87 ± 0.08	31.4 ± 2.8	17.4 ± 4.6	2940 ± 190	809 ± 77	3.3 ± 0.4	2.1 ± 0.3

7.9	7.90 ± 0.07	7.76 ± 0.07	31.3 ± 2.8	17.4 ± 4.5	2955 ± 201	1064 ± 99	2.7 ± 0.4	1.7 ± 0.3
7.8	7.81 ± 0.05	7.64 ± 0.07	31.3 ± 2.9	17.5 ± 4.6	2935 ± 207	1335 ± 12	2.2 ± 0.3	1.4 ± 0.2
7.7	7.69 ± 0.05	7.51 ± 0.07	31.3 ± 2.9	17.5 ± 4.6	2917 ± 208	1759 ± 17	1.7 ± 0.2	1.1 ± 0.2
7.6	7.60 ± 0.05	7.42 ± 0.07	31.3 ± 2.8	17.4 ± 4.5	2946 ± 179	2187 ± 18	1.4 ± 0.9	0.9 ± 0.1
7.5	7.52 ± 0.04	7.33 ± 0.06	31.3 ± 2.9	17.4 ± 4.6	2891 ± 207	2601 ± 24	1.2 ± 0.2	0.7 ± 01
7.4	7.43 ± 0.05	7.22 ± 0.07	31.4 ± 2.8	17.4 ± 4.6	2851 ± 184	3221 ± 25	0.9 ± 0.6	0.6 ± 0.1

4.2 Effect of long-term exposure to different pHs on the performance of *H. trunculus*

4.2.1 Feeding

The amount of food consumed varied throughout the experiment. These variations seem to be correlated with changes in temperature, i.e. consumption rates were lower during the colder period from December to March (Figure 4.2.1.1). The average amount of consumed mussels per individual over the 40 weeks was 3.5 ± 0.17 mussels.

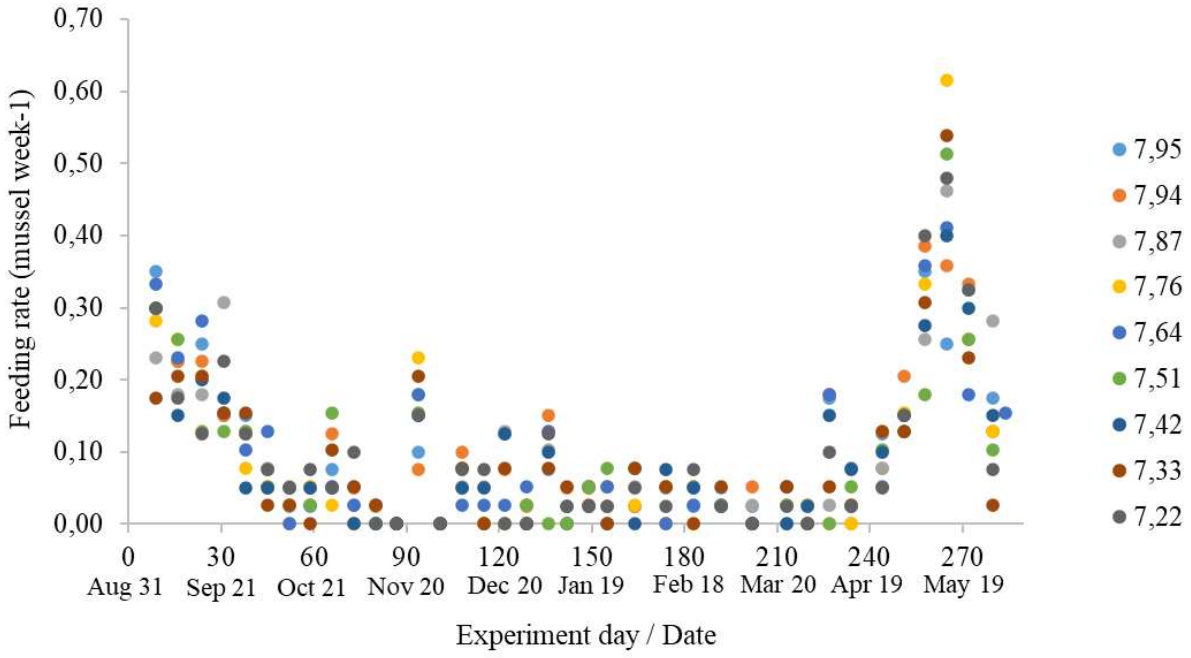


Figure 4.2.1.1 Average weekly feeding rate (mussel week^{-1}) over the course of the experiment (Experiment day/Date) indicating seasonal variations.

A notable increase in consumption in all treatments was observed between 7 May and 21 May (258 – 272 days of experiment) (Figure 4.2.1.2).

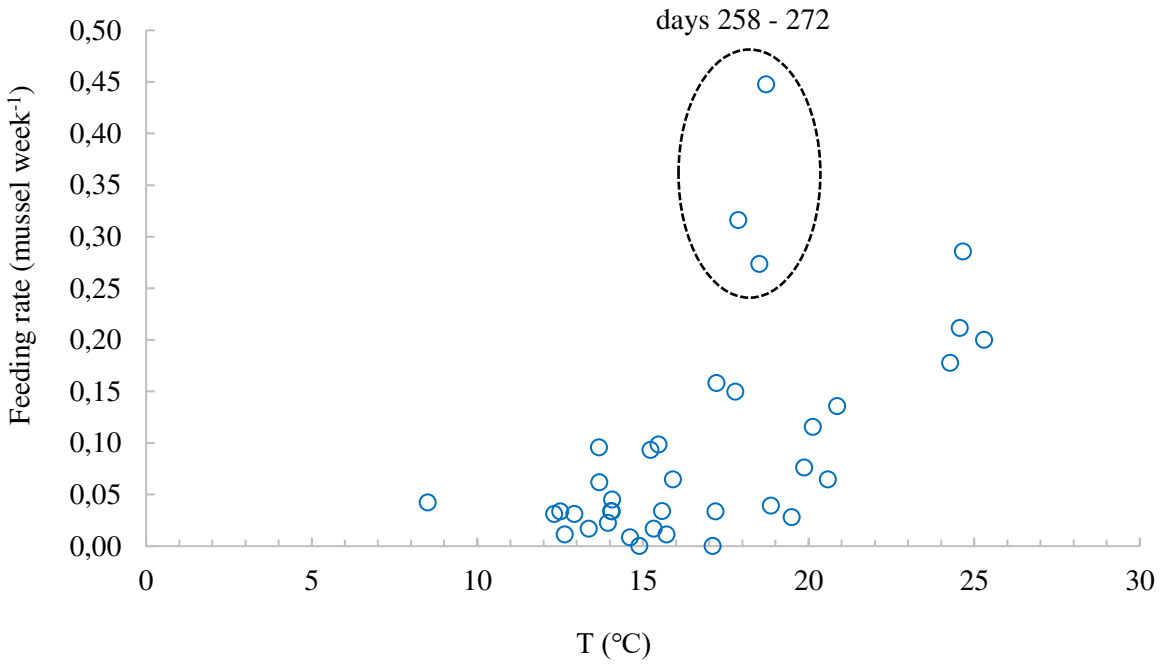


Figure 4.2.1.2. Relationship between the temperature (T, °C) in the seawater and the average weekly feeding rate (mussel week^{-1}) across pH treatments. Weeks 36–38 are highlighted.

There was no significant relationship between pH_T and the feeding rate (Figure 4.2.1.3; SLR, $R^2 = 0.24$, $F(1, 8) = 2.20$, $p = .18$).

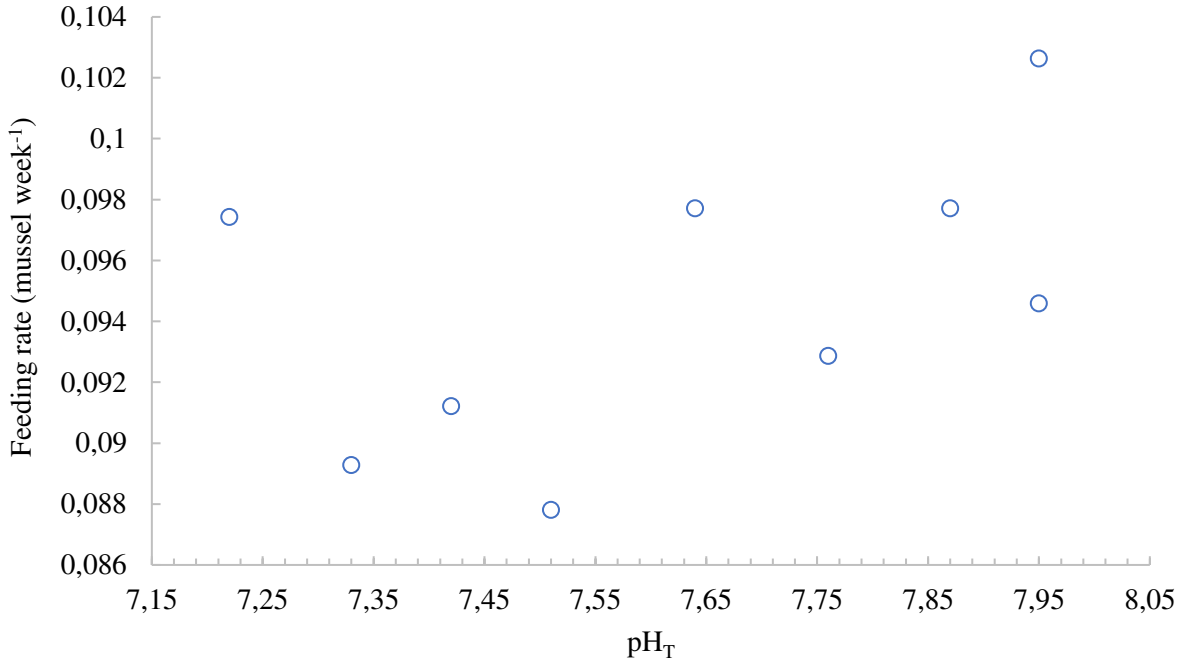


Figure 4.2.1.3 Relationship between average weekly feeding rate (mussel week^{-1}) and pH_T indicating no effect of pH on the feeding rate.

After 60 weeks of exposure, pH had a significant effect on the time needed by the gastropods to reach their food (one-way ANOVA; $F(2, 21) = 3.98$, $p = .034$). A post hoc Tukey's pairwise analysis showed that the individuals in pH_T 7.22 took significantly less time to reach their food than in pH_T 7.95 ($T = -2.78$, $p = .029$), although the number of individuals that successfully reached their food (success, %) had not changed, with a success rate of 66.7% in all treatments (Table 4.2.1.1).

Table 4.2.1.1 Average values (± SD) of time to reach food (duration, min) and success of individuals that reached food (success, %) for a given number of individuals (N). pH_T and temperature in respective treatments (T, °C) at the time of the experiment.

Treatment	N	pH_T	T (°C)	Duration (min)	Success (%)
pH 7.95	12	7.94	26.7	32.88 ± 5.6	66.7 ± 14.4
pH 7.67	12	7.65	26.8	27.75 ± 2.6	66.7 ± 38.2
pH 7.22	12	7.23	26.7	18.13 ± 2.1	66.7 ± 28.9

4.2.2 Shell length growth rate

Shell length growth rates were calculated as the daily increase in shell length between the two successive observation points (SGR, mm day^{-1}). The growth rate followed a similar trend in

the different pH treatments over the course of the experiment, with periods of higher growth at the beginning and end of the experiment and periods of no growth and dissolution in between (Figure 4.2.2.1).

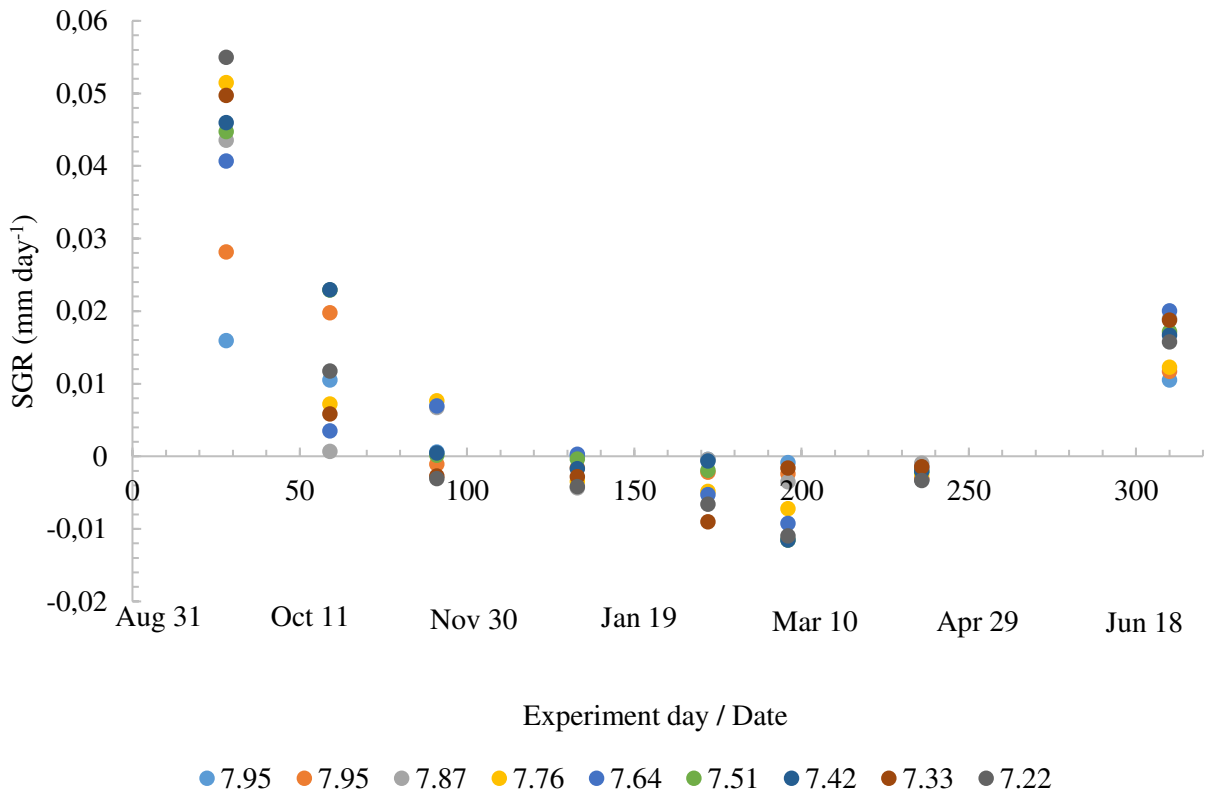


Figure 4.2.2.1 Shell growth rate (SGR, mm day^{-1}) of banded-dye murex, *Hexaplex trunculus*, over the course of the experiment (Experimental day) showing seasonal pattern. Colored dots indicate respective pH_T.

These differences in shell growth rate over time can be explained by temperature. The shell growth rate is highly impacted by temperature and is only positive for temperatures above 20 °C (Figure 4.2.2.2).

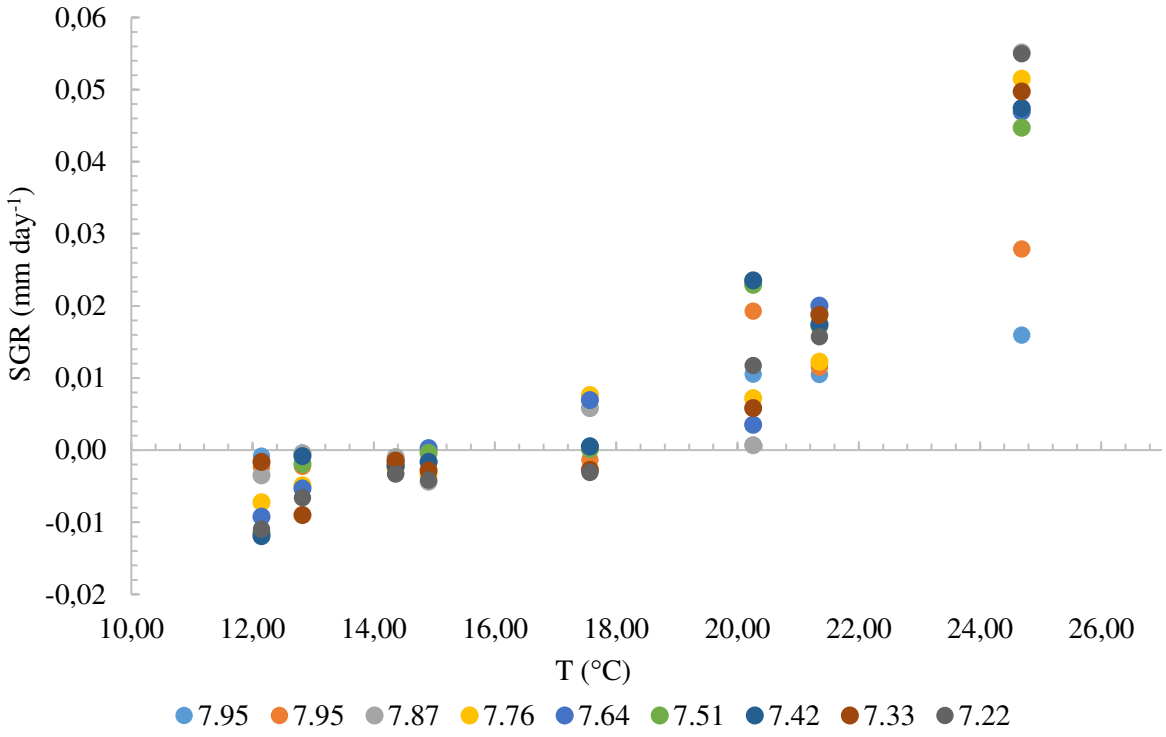
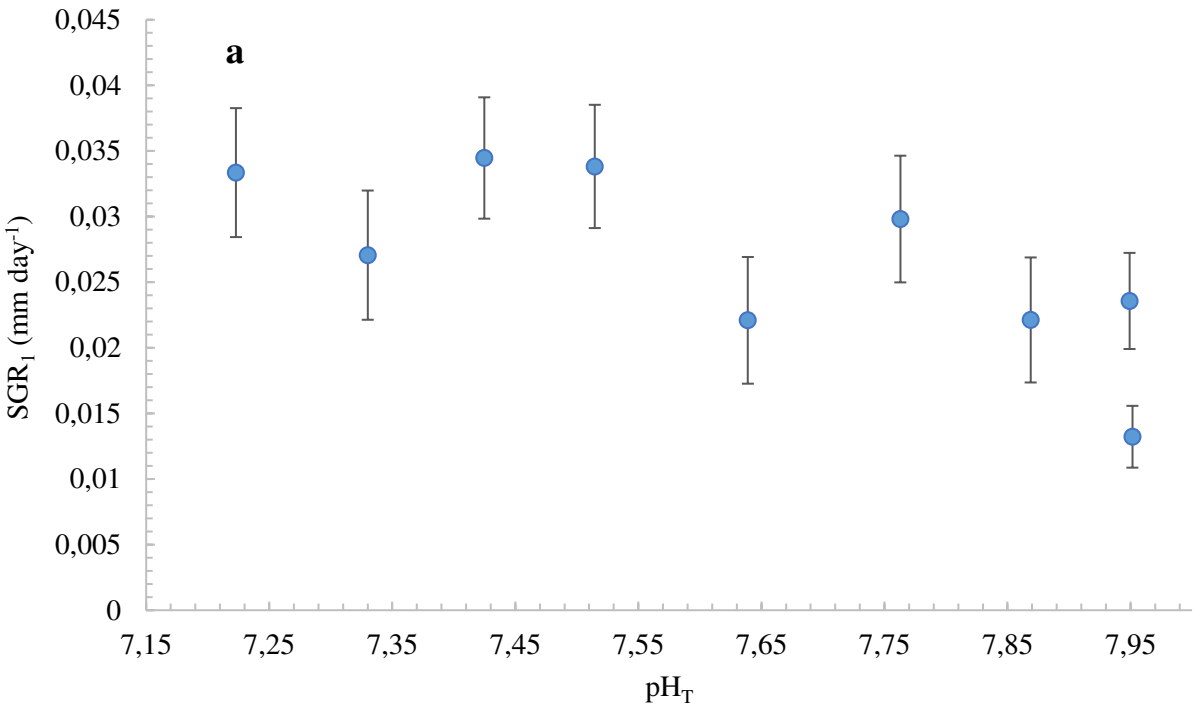
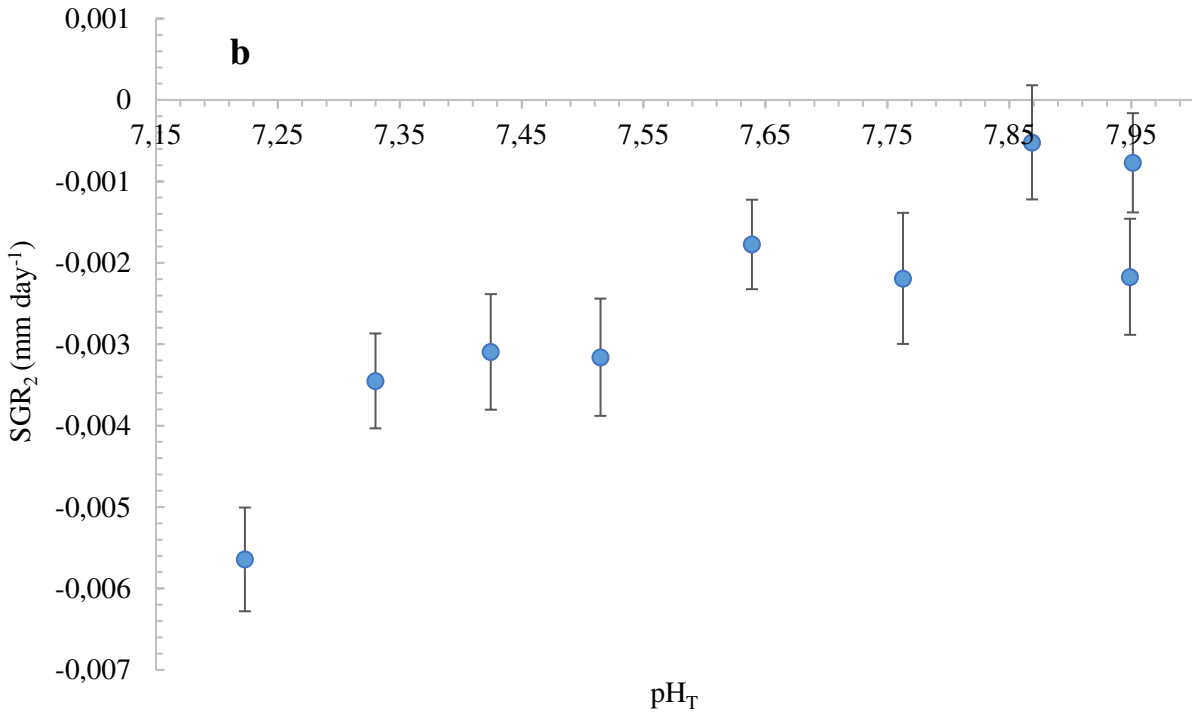


Figure 4.2.2.2 Relationship between shell growth rate (SGR, mm day^{-1}) of banded-dye murex, *Hexaplex trunculus*, in pH treatments (colored dots) and seawater temperature (T, °C).

Analysis of the shell growth rate was therefore divided into three periods to account for the temperature effect: (i) first 59 days of exposure with temperature above 20 °C and positive growth (SGR_1, T = 22.4 ± 2.37 °C), (ii) between 59 and 236 days with temperature below 20 °C (SGR_2, T = 14.4 ± 2.29 °C), and (iii) the last 74 days with temperature above 20°C (SGR_3, T = 22.1 ± 3.9 °C).

For the first 59 days, pH had a significant effect on the shell growth rate (LMM, $F(8, 344.68) = 2.575$, $p = .010$) following a negative relationship (Figure 4.2.2.3a). During the period when temperatures were below 20 °C, growth was negatively affected by low pH (LMM, $F(8, 774.95) = 4.642$, $p < .001$) following a positive relationship (Figure 4.2.2.3b). During the last observation period, there was no significant effect of pH on the growth rate (LMM, $F(8, 344) = 2.172$, $p = .058$) (Figure 4.2.2.3c).


a
0,045
0,04
0,035
0,03
0,025
0,02
0,015
0,01
0,005
0
SGR1 (mm day-1)
7,15
7,25
7,35
7,45
7,55
7,65
7,75
7,85
7,95
pHT


b
0,001
0
-0,001
-0,002
-0,003
-0,004
-0,005
-0,006
-0,007
SGR2 (mm day-1)
7,15
7,25
7,35
7,45
7,55
7,65
7,75
7,85
7,95
pHT

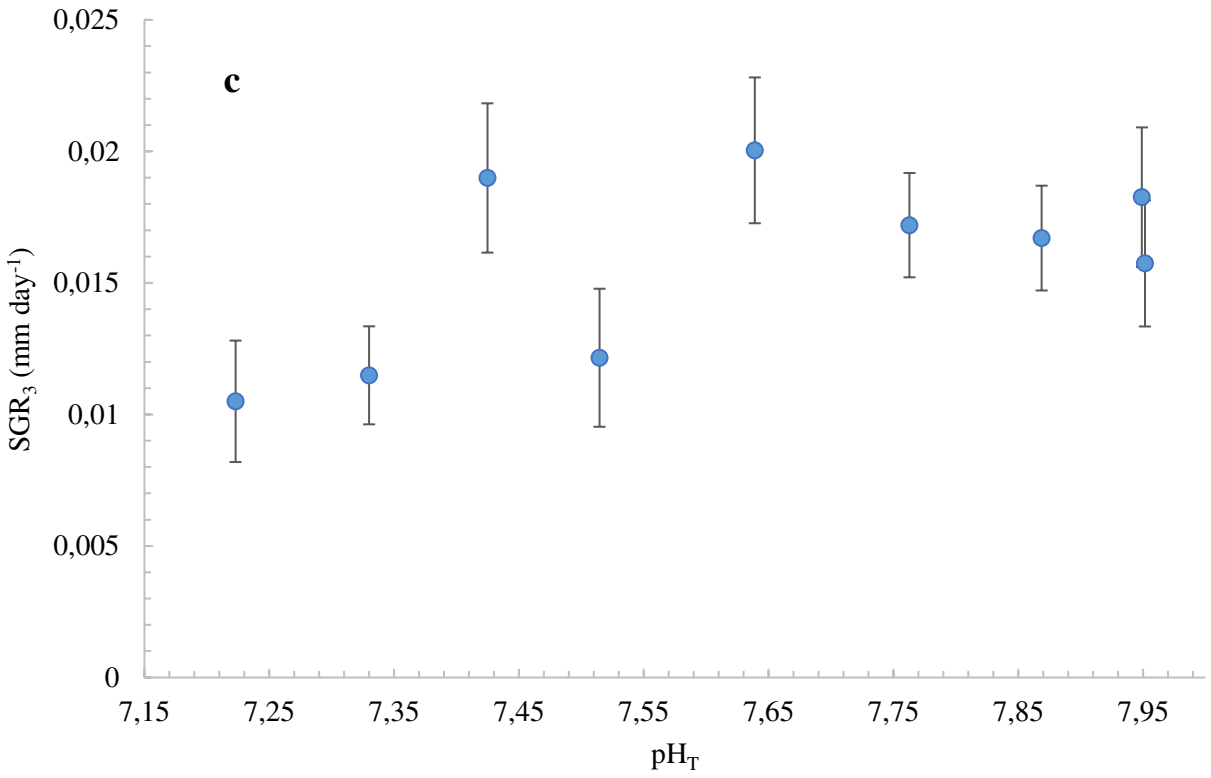


Figure 4.2.2.3 Relationship between estimated marginal means (EMMs ± SE) of shell growth rate (SGR, mm day^{-1}) of banded-dye murex, *Hexaplex trunculus*, and pH_T, for each observation period: a) between day 0–59 of the experiment – SGR_1, b) between day 59–236 of the experiment – SGR_2, and c) between day 236–310 of the experiment – SGR_3.

The effect of sex on the shell growth rate was compared for the 3 tested time periods (SGR_1, SGR_2, SGR_3). For the SGR_1 and SGR_2, there was no effect of sex on the shell growth rate (LMM, $F(1, 344.68) = 1.087$, $p = .298$ and $F(1, 774.95) = 0.033$, $p = .857$, respectively). However, in the last observation period (SGR_3), females had a significantly higher shell growth rate than males (LMM, $F(1, 344) = 15.253$. $p < .001$; $MD = .007$, $SE = .002$, $p < .001$). The effect of sex did not differ across pH (LMM, $F(8, 344) = .522$. $p = .840$). For this period, estimated marginal means (EMMs) of the shell growth rate for males and females in each pH_T are presented in Table 4.2.2.1. A summary of the statistical analysis is presented in Table 4.2.2.2.

Table 4.2.2.1 Estimated marginal means of the shell growth rate (EMM ± SE) for males and females in each pH_T for the third observation period (SGR_3).

				95 % Confidence Interval	
pH_T	Mean	SE	df	Upper	Lower
Male					
7.22	.013	.003	326	.007	.020

7.33	.011	.004	326	.004	.018
7.42	.012	.004	326	.005	.019
7.51	.016	.003	326	.009	.022
7.64	.016	.004	326	.008	.023
7.76	.011	.004	326	.004	.019
7.87	.019	.004	326	.012	.026
7.95	.008	.004	326	.001	.015
7.95	.006	.004	326	-.002	.014
Female					
7.22	.018	.003	326	.012	.025
7.33	.025	.003	326	.018	.031
7.42	.020	.003	326	.014	.026
7.51	.019	.004	326	.012	.027
7.64	.023	.003	326	.017	.029
7.76	.023	.007	326	.010	.036
7.87	.019	.003	326	.013	.026
7.95	.015	.003	326	.008	.022
7.95	.013	.003	326	.007	.019

Table 4.2.2.2 Linear mixed model on the shell length growth rate with pH and sex as fixed effects, and individual ID as a random effect variable. Significant effects are in bold.

Source	Numerator df	Denominator df	*F*	*p*
310 days				
Intercept	1	540	171.503	**.001**
pH	8	540	.763	.636
sex	1	540	.016	.899
sex * pH	8	540	.812	.592
SGR_1				

Intercept	1	344.68	342.302	**.001**
pH	8	344.68	2.575	**.010**
sex	1	344.68	1.087	.298
sex * pH	8	344.68	2.015	.044
SGR_2				
Intercept	1	774.95	124.925	**.001**
pH	8	774.95	4.642	**.001**
sex	1	774.95	0.033	.857
sex * pH	8	774.95	.985	.446
SGR_3				
Intercept	1	344	318.22	**.001**
pH	8	344	2.172	.058
sex	1	344	15.253	**.001**
sex * pH	8	344	.522	.840

4.2.3 Net calcification rate

Net calcification rates were calculated as the daily increase in shell weight between successive observation points (CR g day^{-1}). Due to technical difficulties, the calcification rate was measured from day 59 of the experiment. The net calcification rate in all pH treatments followed a similar trend over time, with lower calcification rates between days 172–236 (Figure 4.2.3.1) when the temperature was below 15 °C (Figure 4.2.3.2).

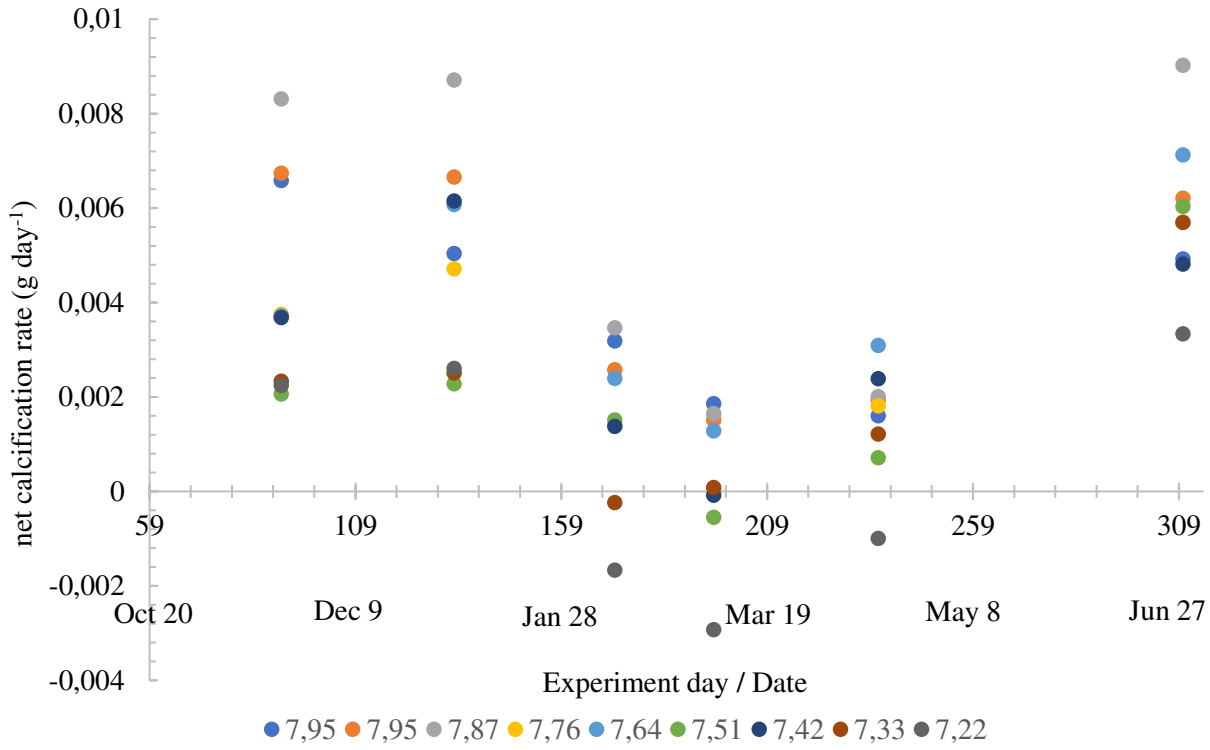


Figure 4.2.3.1 Net calcification rate (CR, g day^{-1}) of banded-dye murex, *Hexaplex trunculus*, over the course of the experiment (Experimental day / Date). Colored dots indicate respective pH_T.

Analysis of the net calcification rate was divided into three periods to account for the temperature effect: (i) 59–133 days of exposure with the average temperature of 18.56 °C (CR_1, T = 18.56 ± 3.68 °C), (ii) between 133 and 236 days with a temperature below 15 °C (CR_2, T = 13.09 ± 1.64 °C), and (iii) the last 74 days with a temperature above 20 °C (CR_3, T = 22.1 ± 3.9 °C).

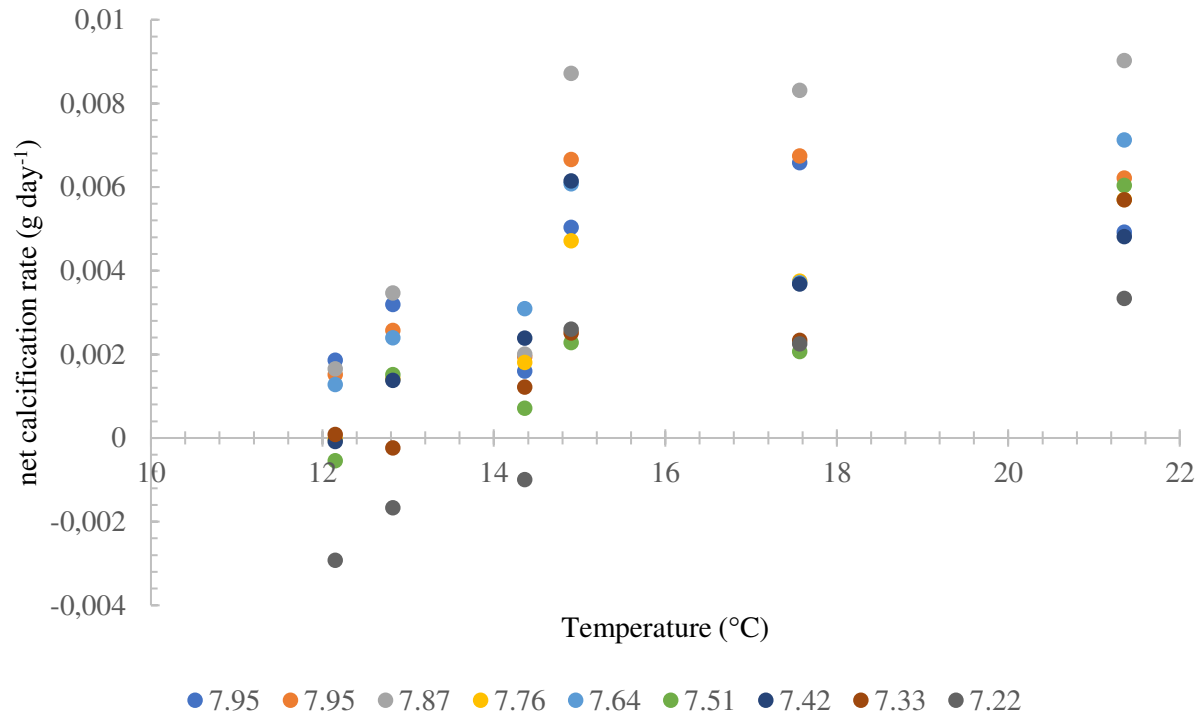
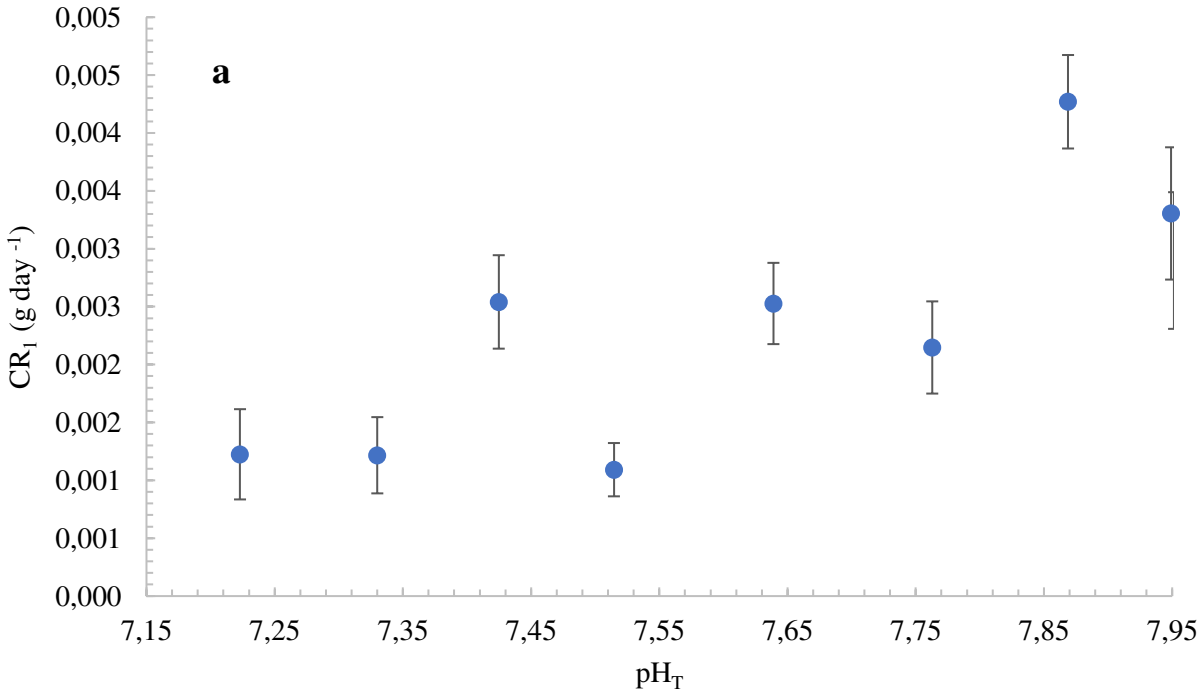
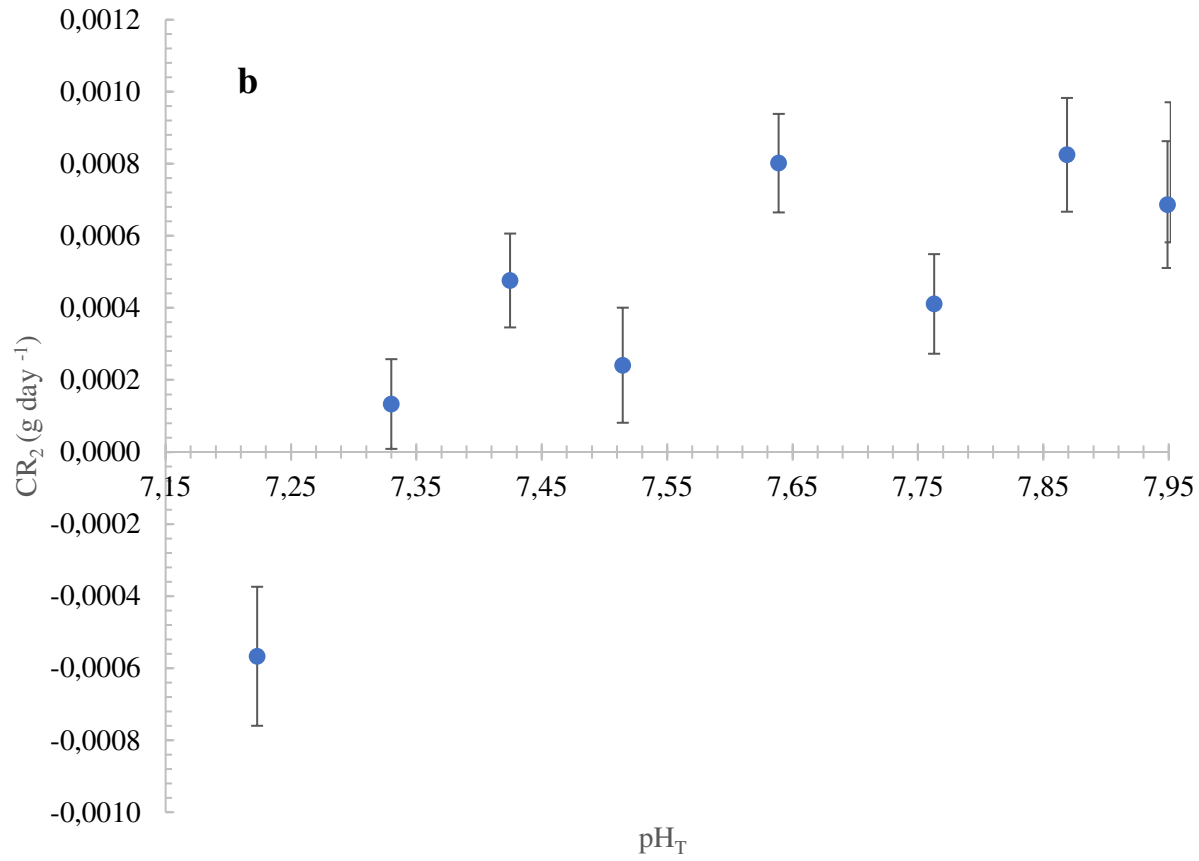


Figure 4.2.3.2 Relationship between net calcification rate (CR, g day^{-1}) of banded-dye murex, *Hexaplex trunculus*, in pH treatments (colored dots) and seawater temperature (T, °C).

The net calcification rate was negatively affected by pH for all three observation periods (LMM, $F(8, 346) = 6.46$, $p < 0.001$; $F(8, 340.73) = 8.48$, $p < 0.001$; $F(8, 346) = 2.28$, $p = .021$, respectively) (Figure 4.2.3.3).

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0,0006
0,0004
0,0002
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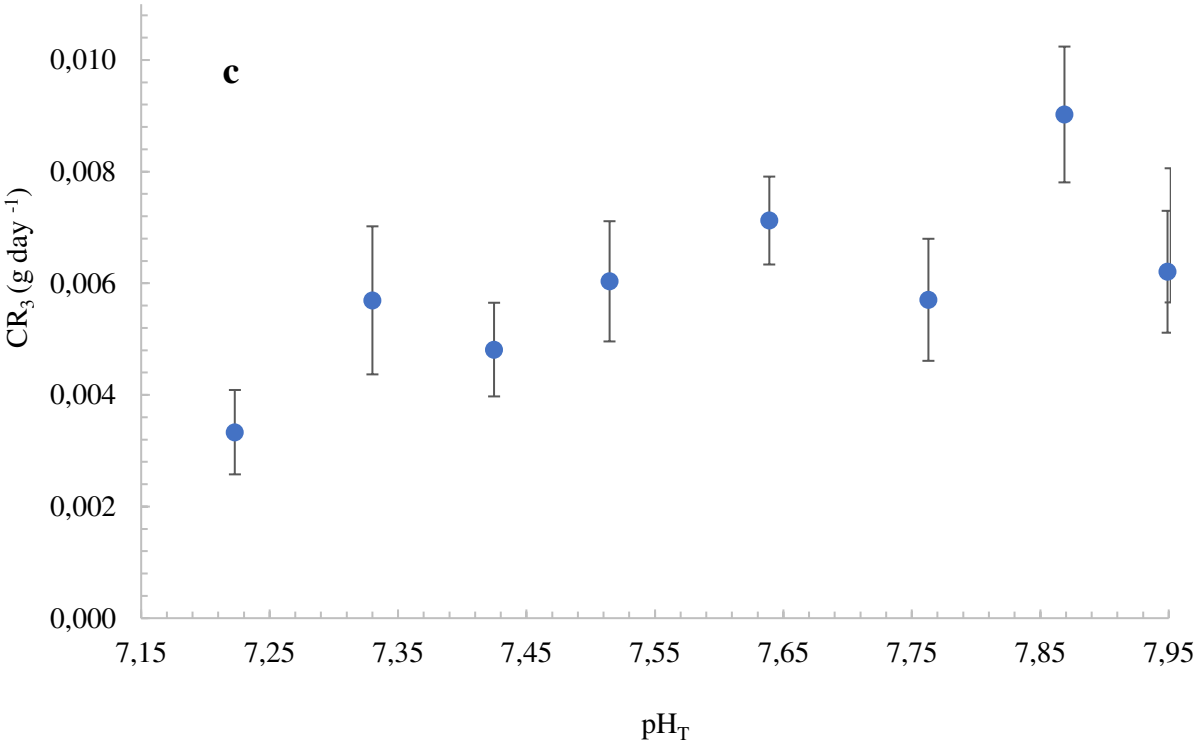
-0,0004
-0,0006
-0,0008
-0,0010
CR2 (g day -1)
7,15
7,25
7,35
7,45
7,55
7,65
7,75
7,85
7,95
pHT


c
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0,008
0,006
0,004
0,002
0,000
CR3 (g day -1)
7,15
7,25
7,35
7,45
7,55
7,65
7,75
7,85
7,95
pHT

Figure 4.2.3.3 Relationship between estimated marginal means (EMMs ± SE) of net calcification rate (CR, g day^{-1}) of banded-dye murex, *Hexaplex trunculus*, and pH_T, for each observation period: a) between day 59–133 of the experiment – CR_1, b) between day 133–236 of the experiment – CR_2, and c) between day 236–310 of the experiment – CR_3.

Sex had a significant effect on the net calcification rate only in the winter period, CR_1 (LMM, $F(1, 340.73) = 5.48$, $p = .02$), irrespective of pH (LMM, $F(1, 340.73) = 1.31$, $p = .24$),), with females having significantly higher net calcification rates than males ($MD = .027$, $SE = .009$, $p = .003$). Estimated marginal means for calcification rate in males and females and for each pH_T are presented in Table 4.2.3.1. A summary of the statistical analysis is presented in Table 4.2.3.2.

Table 4.2.3.1. Mean values of the net calcification rate (Mean ± SE) for males and females in each respective pH_T for the second observation period (CR_2).

				95 % Confidence Interval	
pH_T	Mean	SE	df	Upper	Lower
Male					
7.22	-.066	.018	-.102	-.030	-.066
7.33	.021	.020	-.018	.060	.021
7.42	.027	.021	-.014	.067	.027
7.51	.026	.017	-.007	.059	.026
7.64	.052	.021	.012	.093	.052
7.76	.048	.018	.012	.084	.048
7.87	.088	.020	.048	.127	.088
7.95	.059	.019	.021	.097	.059
7.95	.015	.022	-.028	.058	.015
Female					
7.22	-.050	.018	-.087	-.014	-.050
7.33	.008	.018	-.027	.043	.008
7.42	.065	.018	.031	.100	.065
7.51	.023	.021	-.018	.065	.023
7.64	.104	.017	.070	.137	.104

7.76	.036	.019	-.001	.073	.036
7.87	.083	.018	.047	.119	.083
7.95	.091	.019	.054	.128	.091
7.95	.117	.016	.084	.149	.117

Table 4.2.3.2 Linear mixed model on the net calcification rate with pH and sex as fixed effects, and individual ID as a random effect variable. Significant effects are in bold.

Source	Numerator df	Denominator df	F	p
Overall				
Intercept	1	318.18	282.36	**.001**
pH	8	318.18	4.94	**.001**
sex	1	318.18	1.31	.253
sex * pH	8	318.18	1.88	.062
CR_1				
Intercept	1	346.00	282.884	**.001**
pH	8	346.00	6.466	**.001**
sex	1	346.00	1.925	.166
sex * pH	8	346.00	1.479	.163
CR_2				
Intercept	1	340.73	52.493	**.001**
pH	8	340.73	8.484	**.001**
sex	1	340.73	5.488	**.020**
sex * pH	8	340.73	1.309	.238
CR_3				
Intercept	1	346.00	295.117	**.001**
pH	8	346.00	2.285	**.021**
sex	1	346.00	.002	.967
sex * pH	8	346.00	1.696	.098

4.2.4 Total weight growth rate

Total weight growth rates were calculated as the daily increase in the total body weight (the weight of the shell and soft tissue) between successive observation points (TWGR, g day^{-1}).

The total weight in all pH treatments followed a similar trend over the course of the experiment, with a higher weight rate at the beginning (Figure 4.2.4.1).

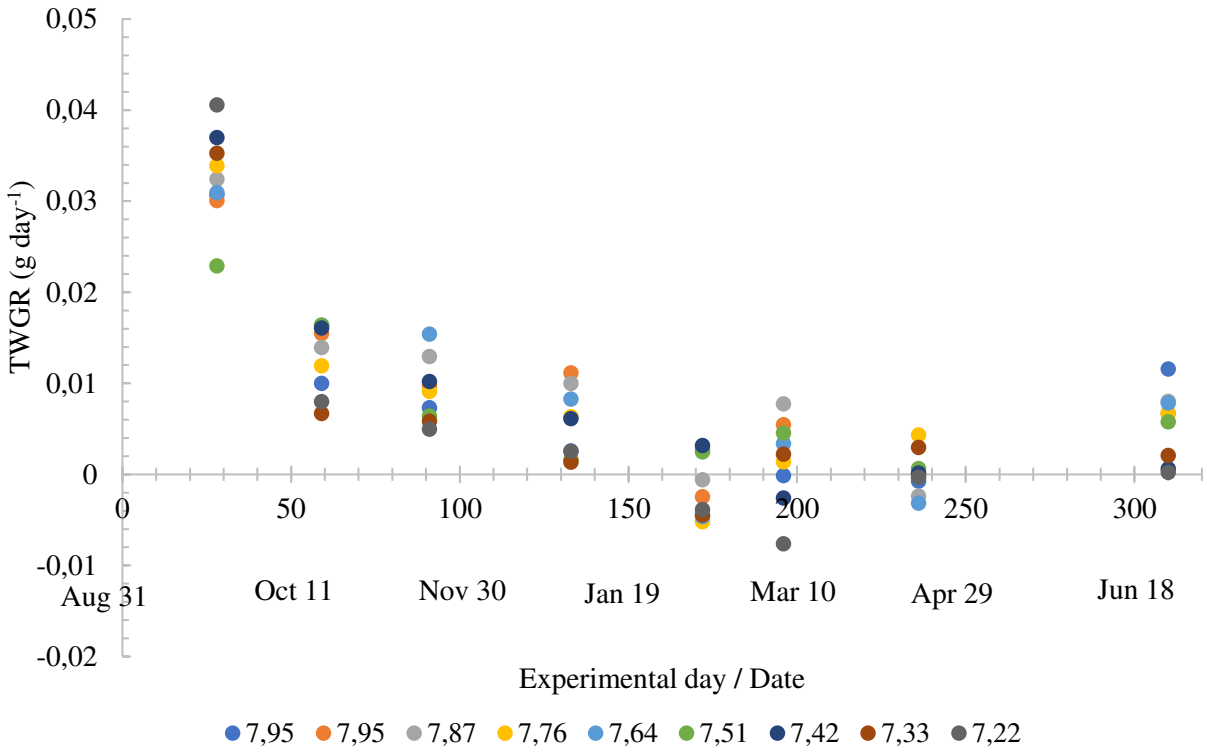


Figure 4.2.4.1 Total weight growth rate (TWGR, g day^{-1}) of *Hexaplex trunculus* over the course of the experiment (Experimental day / Date). Colored dots indicate respective pH_T.

The total weight growth rate was the highest at temperatures above 24 °C, and was only negative at temperatures below 15 °C, although not for all pH treatments (Figure 4.2.4.2). The analysis of total weight growth rate was divided into periods as follows: (i) the first 59 days of acute exposure to an average temperature above 20 °C ($TWGR_1$, T = 22.19 ± 2.37), (ii) day 59 to 133 to a temperature below 20 °C ($TWGR_2$, T = 18.56 ± 3.68°C), (iii) day 133 to 236 to a temperature below 15 °C ($TWGR_3$, T = 13.09 ± 1.64 °C) and (iv) the last 74 days to a temperature above 20 °C ($TWGR_4$, T = 22.1 ± 3.9°C).

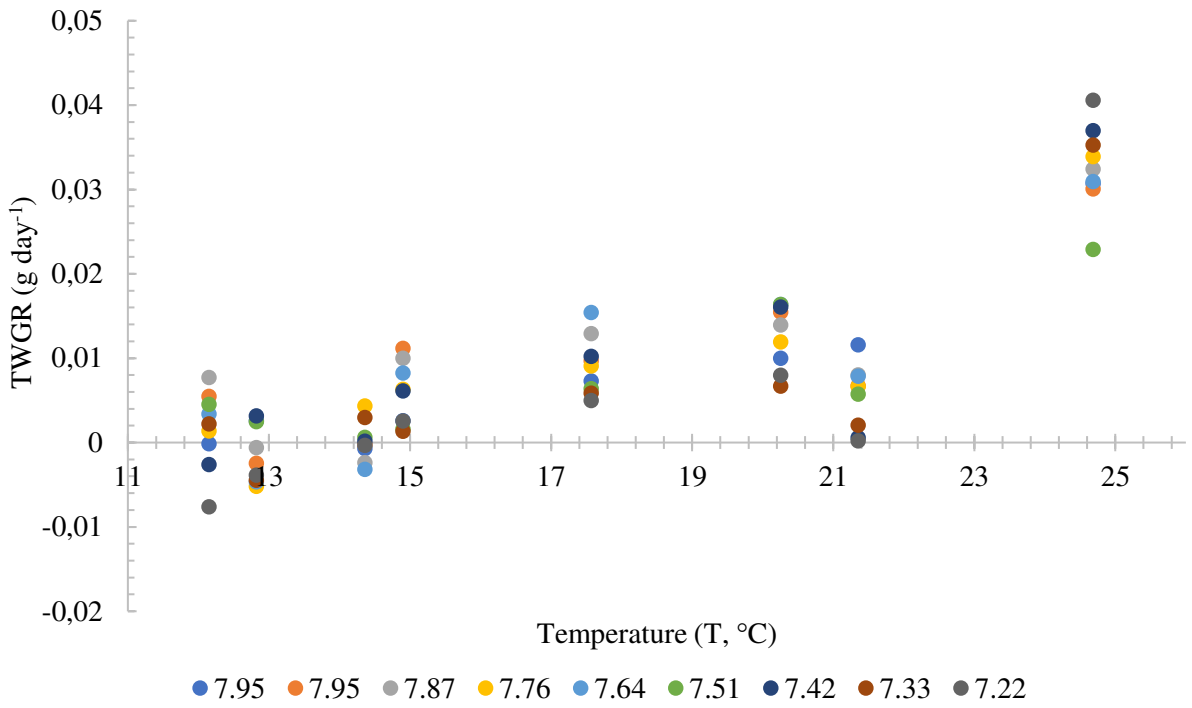
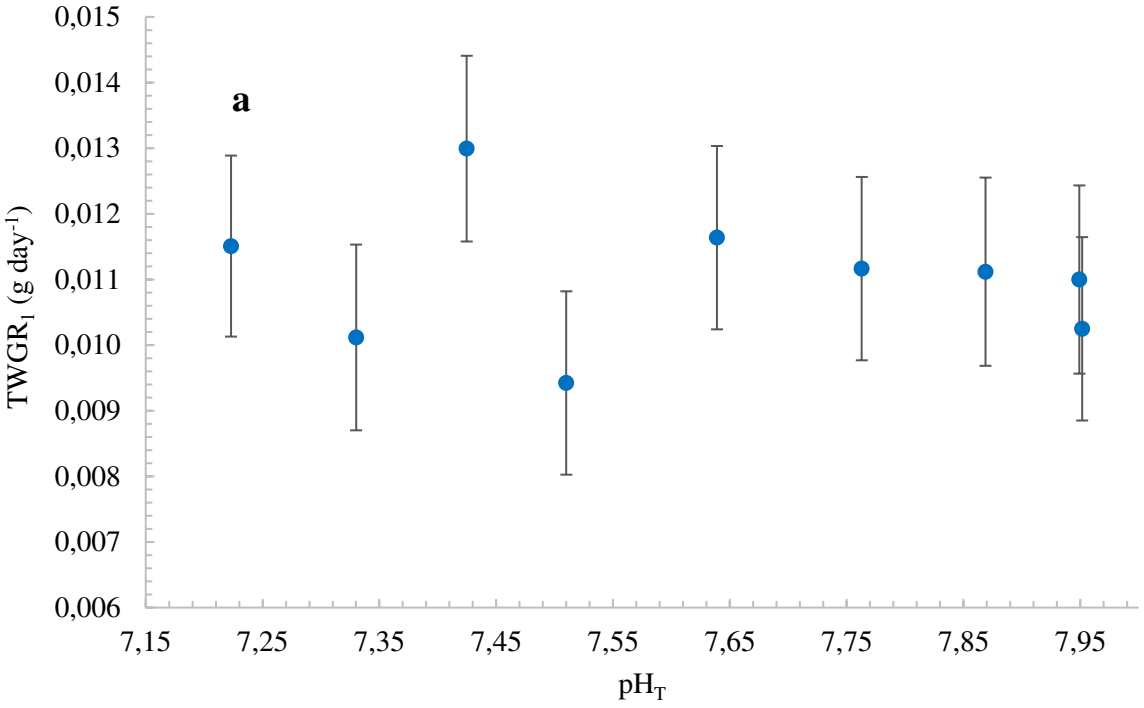
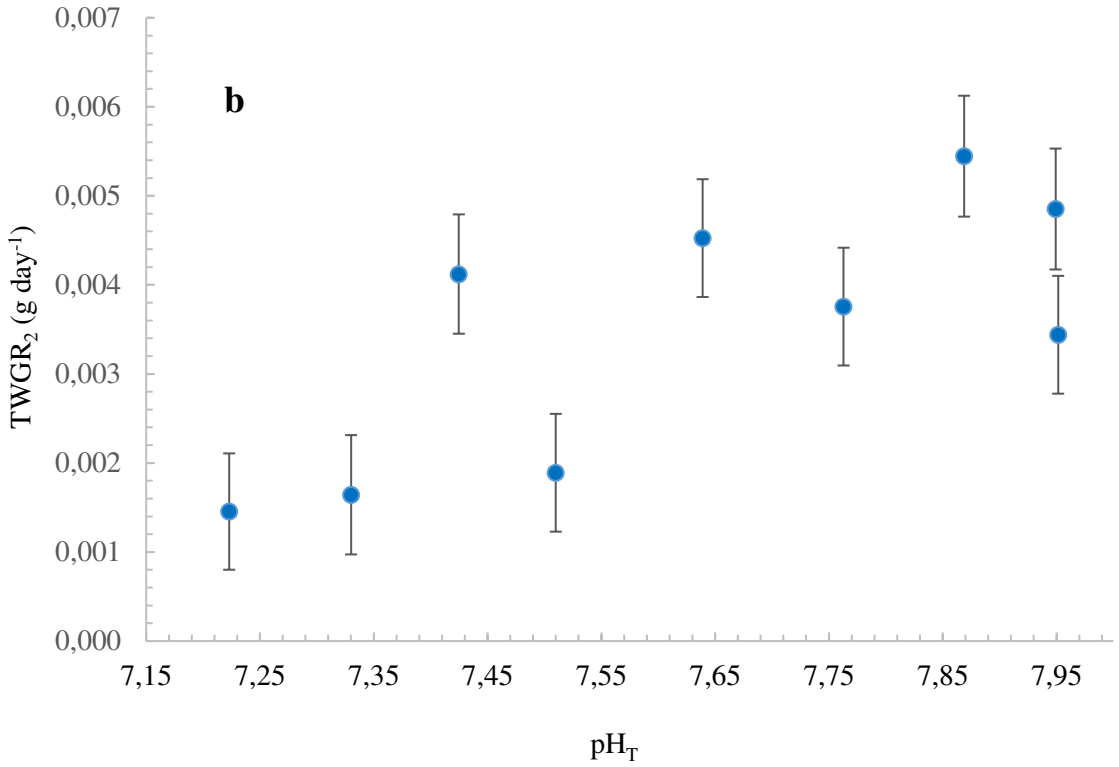


Figure 4.2.4.2 Relationship between total weight growth rate (TWGR, g day^{-1}) of banded-dye murex, *Hexaplex trunculus,* in pH treatments (colored dots) and seawater temperature (T, °C).

Over the period of acute exposure ($TWGR_1$), pH had no significant effect on the total weight growth rate (LMM, $F(8, 346) = .597$, $p = .780$), but for the remaining three periods ($TWGR_2$, $TWGR_3$ & $TWGR_4$) of the experiment the total weight was negatively affected by low pH following a positive relationship (LMM, $F(8, 346) = 3.295$, $p < .001$), $F(8, 456.45) = 4.47$, $p < .001$), $F(8, 346) = 6.312$, $p < .001$; respectively) (Figure 4.2.4.3).


a
0,015
0,014
0,013
0,012
0,011
0,010
0,009
0,008
0,007
0,006
TWGR1 (g day-1)
7,15
7,25
7,35
7,45
7,55
7,65
7,75
7,85
7,95
pHT


b
0,007
0,006
0,005
0,004
0,003
0,002
0,001
0,000
TWGR2 (g day-1)
7,15
7,25
7,35
7,45
7,55
7,65
7,75
7,85
7,95
pHT

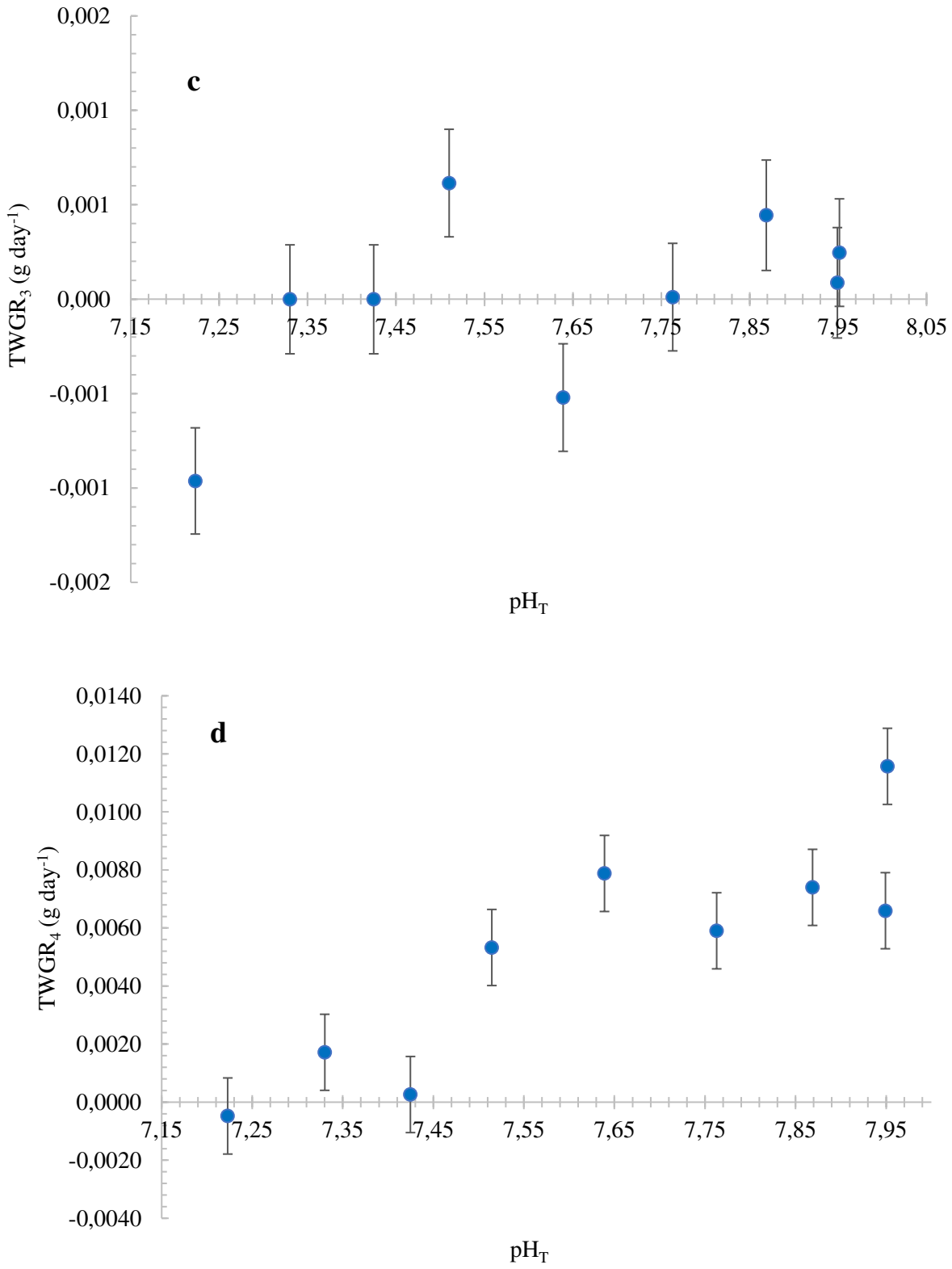


Figure 4.2.4.3 Relationship between estimated marginal means (EMMs ± SE) of the total weight growth rate (TWGR, g day^{-1}) of banded-dye murex, *Hexaplex trunculus*, and pH_T, for each observation period: **a)** day 0–59 of the experiment – $TWGR_1$, **b)** day 59–133 of the experiment – $TWGR_2$, **c)** day 133–236 of the experiment – $TWGR_3$ and **d)** day 236–310 of the experiment – $TWGR_4$

Sex had a significant effect on the total weight growth rate only in the winter period (LMM, $F(1, 456.45 = 11.917$, $p = .001$), irrespective of pH (LMM, $F(8, 456.45) = .707$, $p = .685$), with females having significantly higher growth rates in total weight than males ($MD = .056$, $SE = .02$, $p = .005$). The estimated marginal means of the total weight growth rate for males and females in each pH_T are presented in Table 4.2.4.1. A summary of the statistical analysis for all observation periods is presented in Table 4.2.4.2.

Table 4.2.4.1 Mean values of the total weight growth rate (Mean ± SE) for males and females of banded-dye murex *H. trunculus*, in each respective pH_T for the third observation period ($TWGR_3$).

				95 % Confidence Interval	
pH_T	Mean	SE	df	Upper	Lower
Male					
7.22	-.130	.041	-.210	-.050	-.130
7.33	-.018	.044	-.105	.069	-.018
7.42	-.053	.046	-.143	.036	-.053
7.51	.062	.037	-.011	.135	.062
7.64	-.099	.046	-.189	-.010	-.099
7.76	.010	.041	-.070	.090	.010
7.87	-.002	.044	-.088	.085	-.002
7.95	-.031	.043	-.116	.053	-.031
7.95	-.065	.049	-.161	.031	-.065
Female					
7.22	-.068	.041	-.148	.012	-.068
7.33	.014	.040	-.064	.092	.014
7.42	.039	.039	-.038	.115	.039
7.51	.086	.047	-.006	.179	.086
7.64	-.044	.038	-.118	.031	-.044
7.76	-.008	.042	-.090	.074	-.008
7.87	.086	.041	.006	.166	.086
7.95	.047	.042	-.035	.129	.047

7.95	.076	.037	.004	.148	.076

Table 4.2.4.2 A linear mixed model for the total weight growth rate of banded-dye murex, *Hexaplex trunculus*, with pH and sex as the fixed effects, and individual ID as a random effect variable. Significant effects are in bold.

Source	Numerator df	Denominator df	F	p
Overall				
Intercept	1	334.53	401.29	**.001**
pH	8	334.53	1.99	**.047**
sex	1	334.53	2.296	.131
sex * pH	8	334.53	1.599	.124
$TWGR_1$				
Intercept	1	346.000	579.014	**.001**
pH	8	346.000	.597	.780
Sex	1	346.000	1.463	.227
sex * pH	8	346.000	.894	.591
$TWGR_2$				
Intercept	1	346	161.63	**.001**
pH	8	346	3.295	**.001**
Sex	1	346	.455	.501
sex * pH	8	346	.962	.466
$TWGR_3$				
Intercept	1	456.45	.112	.738
pH	8	456.45	4.47	**.001**
Sex	1	456.45	11.917	**.001**
sex * pH	8	456.45	.707	.685
$TWGR_4$				
Intercept	1	346	109.175	**.001**
pH	8	346	6.312	**.001**
sex	1	346	.035	.852
sex * pH	8	346	.557	.813

4.2.5 Soft tissue weight growth rate

Soft tissue weight growth rates were calculated as a difference in the total weight growth rate and shell weight growth rate (TWGR – CR = STWGR g day^{-1}) between successive observation periods. The soft tissue weight growth rate also followed a similar trend for all pH treatments over the course of the experiment (Figure 4.2.5.1)

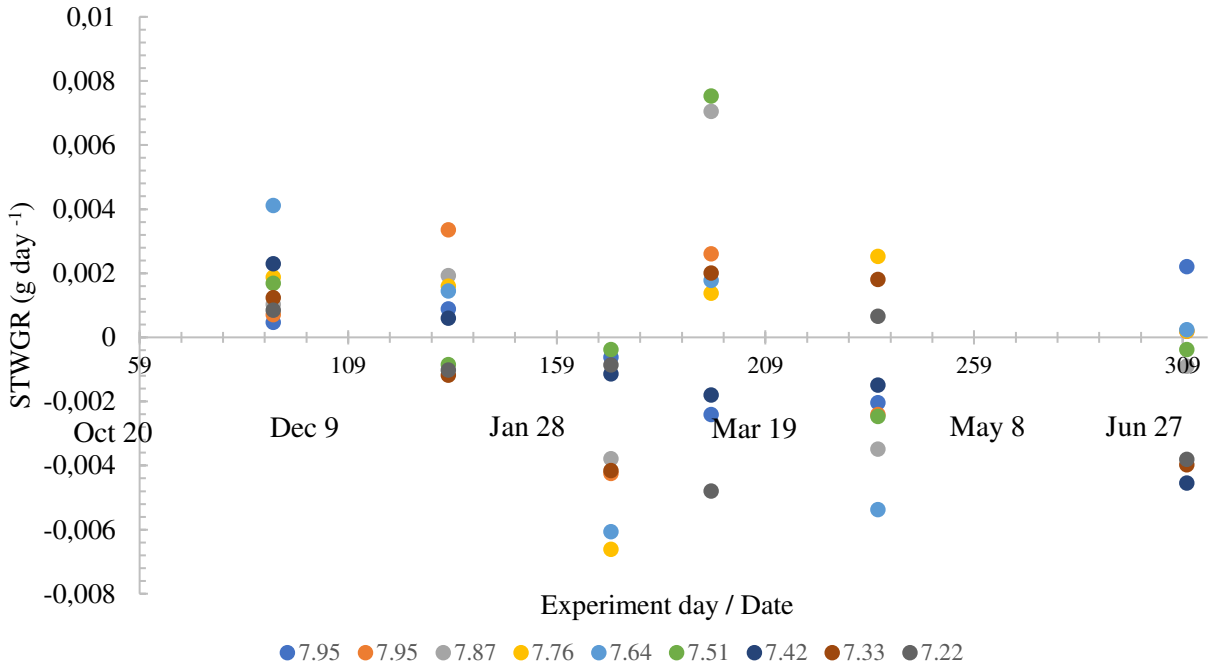


Figure 4.2.5.1 Soft tissue weight growth rate (STWGR, g day^{-1}) of banded-dye murex, *Hexaplex trunculus*, over the course of the experiment (Experiment day / Date). Colored dots indicate respective pH_T.

Soft tissue weight growth rate and temperature followed a non-linear relationship (Figure 4.2.5.2). For a better interpretation of gastropods' growth, the analysis of the soft tissue weight growth rate was divided into the same three periods as the net calcification rate: (i) day 59–133 of the exposure to an average temperature of 18.56 °C ($STWGR_1$, T = 18.56 ± 3.68 °C), (ii) day 133–236 to a temperature below 15 °C ($STWGR_2$, T = 13.09 ± 1.64°C), and (iii) the last 74 days to a temperature above 20 °C ($STWGR_3$, T = 22.1 ± 3.9°C).

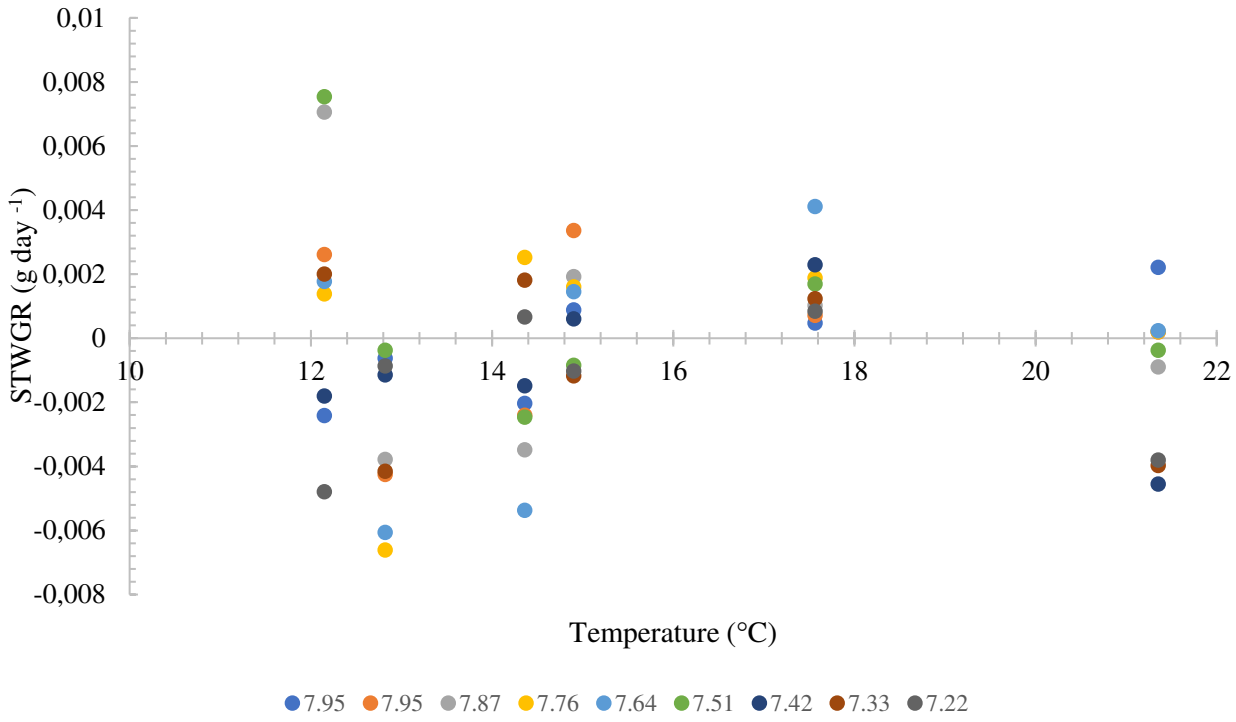
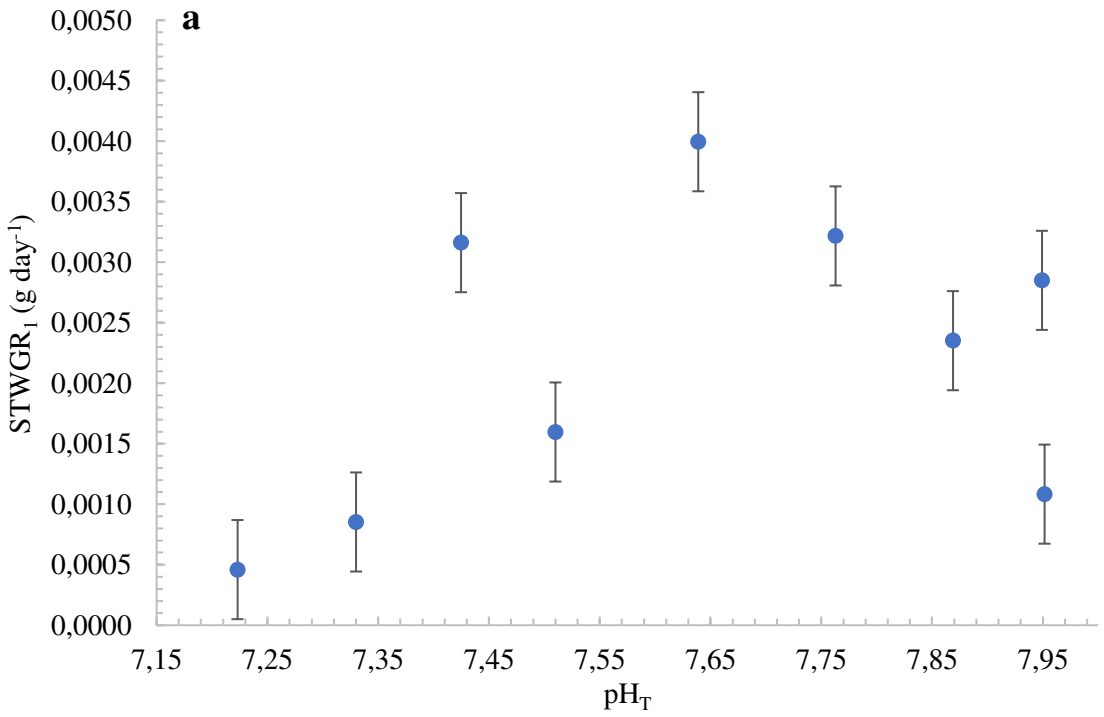
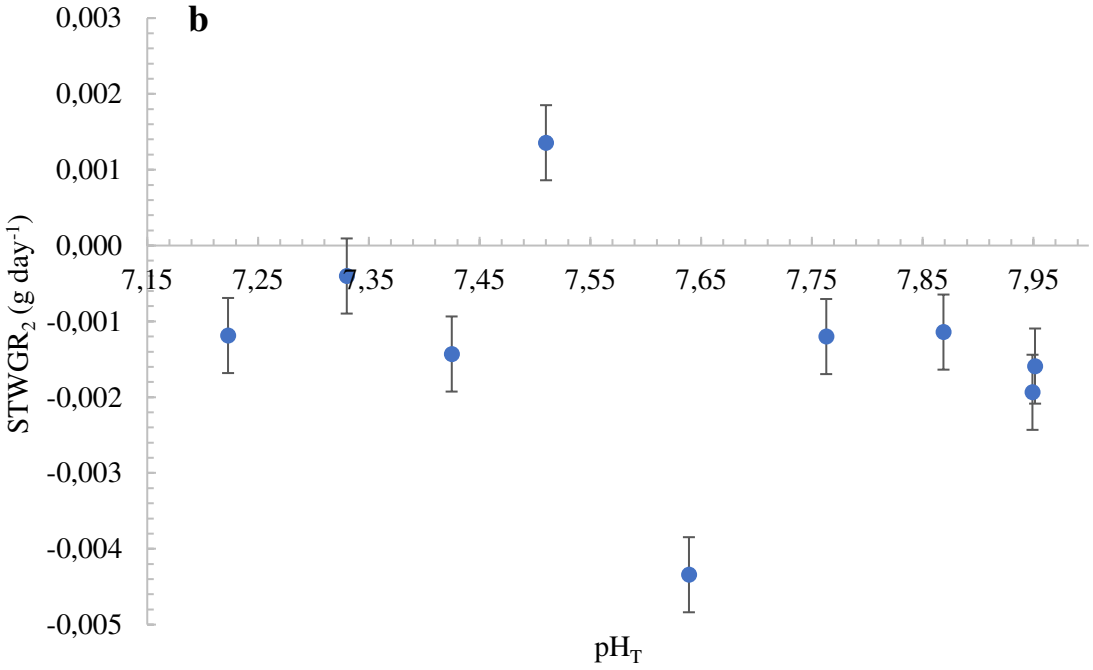


Figure 4.2.5.2 Relationship between soft tissue weight growth rate (STWGR, g day^{-1}) of banded-dye murex, *Hexaplex trunculus*, in pH treatments (colored dots) and seawater temperature (T, °C).

The soft tissue weight growth rate was not affected by pH for the first two observational periods ($STWGR_1$ & $STWGR_2$, $F(8, 346) = 1.117$, $p = .351$, $F(8, 487.55) = 1.263$, $p = .428$; respectively), but in the last observational period, $STWGR_3$, gastropods from lower pH had a significantly lower weight growth rate of their soft tissue ($F(8, 346) = 4.742$, $p = .001$) (Figure 4.2.5.3).


a
0,0050
0,0045
0,0040
0,0035
0,0030
0,0025
0,0020
0,0015
0,0010
0,0005
0,0000
STWGR1 (g day-1)
7,15
7,25
7,35
7,45
7,55
7,65
7,75
7,85
7,95
pHT


b
0,003
0,002
0,001
0,000
-0,001
-0,002
-0,003
-0,004
-0,005
STWGR2 (g day-1)
7,15
7,25
7,35
7,45
7,55
7,65
7,75
7,85
7,95
pHT

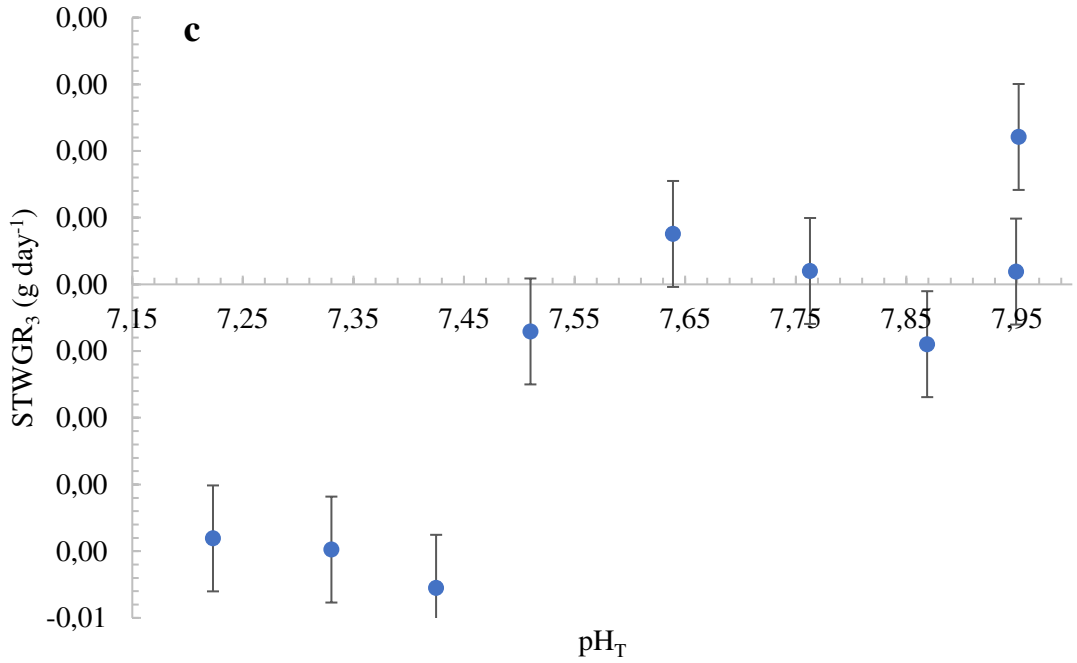


Figure 4.2.5.3 Relationship between estimated marginal means (EMMs ± SE) of the soft tissue weight growth rate (STWGR, g day^{-1}) of banded-dye murex, *Hexaplex trunculus*, and pH_T, for each observation period: **a)** day 59–133 of the experiment – $STWGR_1$, **b)** day 133–236 of the experiment – $STWGR_2$, and **c)** day 236–310 of the experiment – $STWGR_3$.

A significant effect of sex was observed in the winter period (LMM, $F(1, 487.55) = 5.562$, $p = .019$), irrespective of pH (LMM, $F(8, 487.55) = .336$, $p = .952$), with females having significantly higher growth rates than males ($MD = .039$, $SE = .02$, $p = .049$). The estimated marginal means of the soft tissue weight rate for males and females in each pH_T are presented in Table 4.2.5.1. A summary of the statistical analysis for all observation periods is presented in Table 4.2.5.2.

Table 4.2.5.1 Mean values of the soft tissue weight growth rate (Mean ± SE) for males and females of banded-dye murex, *Hexaplex trunculus*, in each respective pH_T for the second observation period ($STWGR_2$).

				95 % Confidence Interval	
pH_T	Mean	SE	df	Upper	Lower
Male					
7.22	-.0064	.0040	-.143	.015	-.064
7.33	-.0039	.0044	-.124	.047	-.039

7.42	-.0080	.00045	-.168	.008	-.080
7.51	.0036	.0037	-.035	.108	.036
7.64	-.0152	.0045	-.240	-.064	-.152
7.76	-.0038	.0040	-.117	.040	-.038
7.87	-.0089	.0044	-.174	-.004	-.089
7.95	-.0090	.0042	-.173	-.007	-.090
7.95	-.0080	.0048	-.174	.014	-.080
Female					
7.22	-.0018	.0040	-.096	.061	-.018
7.33	.0006	.0039	-.071	.083	.006
7.42	-.0027	.0038	-.102	.048	-.027
7.51	.0063	.0046	-.028	.154	.063
7.64	-.0147	.0037	-.221	-.074	-.147
7.76	-.0044	.0041	-.125	.037	-.044
7.87	.0003	.0040	-.076	.082	.003
7.95	-.0044	.0041	-.125	.037	-.044
7.95	-.0041	.0036	-.111	.030	-.041

Table 4.2.5.2 Linear mixed model on the soft tissue weight growth rate of banded-dye murex, *Hexaplex trunculus*, with pH and sex as the fixed effects, and individual ID as a random effect variable. Significant effects are in bold.

Source	Numerator df	Denominator df	F	p
Overall				
Intercept	1	768.37	2.55	.111
pH	8	768.37	2.53	**.010**
sex	1	768.37	1.53	.216
sex * pH	8	768.37	1.10	.360
$STWGR_1$				
Intercept	1	346.00	33.809	.001

pH	8	346.00	1.117	.351
sex	1	346.00	.004	.947
sex * pH	8	346.00	.969	.460
$STWGR_2$				
Intercept	1	487.55	26.357	**.001**
pH	8	487.55	1.263	.428
sex	1	487.55	5.562	**.019**
sex * pH	8	487.55	.336	.952
$STWGR_3$				
Intercept	1	346.00	7.004	**.009**
pH	8	346.00	4.742	**.001**
sex	1	346.00	.037	.847
sex * pH	8	346.00	1.257	.265

4.2.6 Respiration rate of females

The respiration rate of *H. trunculus* females was calculated as mg L^{-1} O_2 min^{-1} g TW^{-1}. The respiration rate in all pH treatments followed a similar trend over the course of five measurements (days 88, 149, 181, 209, 240) with lower respiration rates present at lower seawater temperatures (Figure 4.2.6.1)

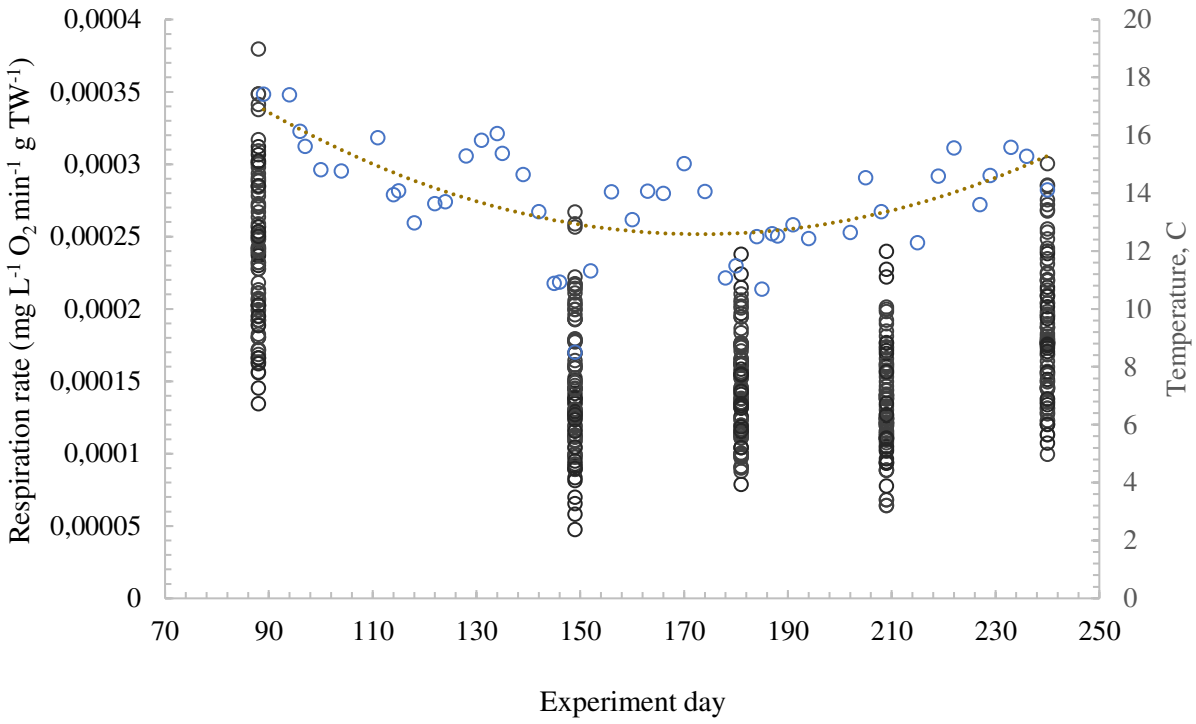
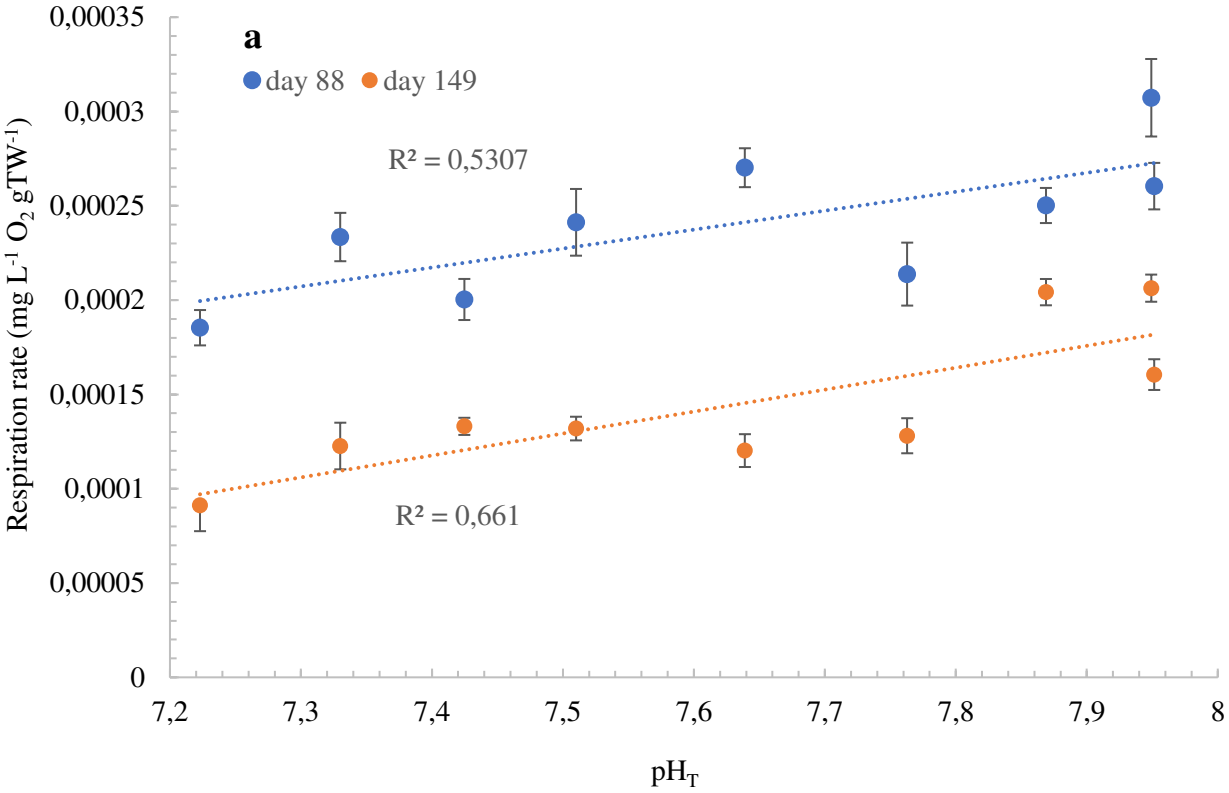


Figure 4.2.6.1 *Hexaplex trunculus* female respiration rate (mg L^{-1} O_2 min^{-1} g TW^{-1}) over the course of the experiment (days). Blue dots represent measured temperature in the respective period.

For the first two measurements (days 88 & 149), the respiration rate was negatively affected by low pH (SLR, R^2= .530, $F(1, 8) = 7.915$, $p = 0.026$; $R^2 = .661$, $F(1, 8)= 13.649$, p= .007, respectively) following a positive relationship (Figure 4.2.6.2a). For the next three measurements (days 181, 209 & 240), there was no significant relationship between the respiration rate and pH (SLR, R^2= .031, $F(1, 8) = .228$, $p = .647$; R^2= .369, $F(1, 8) = 4.095$, $p = .082$; R^2= .208, $F(1, 8) = 1.840$, p= .217, respectively) (Figure 4.2.6.2b).


a
day 88
day 149
R² = 0,5307
R² = 0,661
Respiration rate (mg L-1 O2 gTW-1)
0,00035
0,0003
0,00025
0,0002
0,00015
0,0001
0,00005
0
7,2
7,3
7,4
7,5
7,6
7,7
7,8
7,9
8
pHT

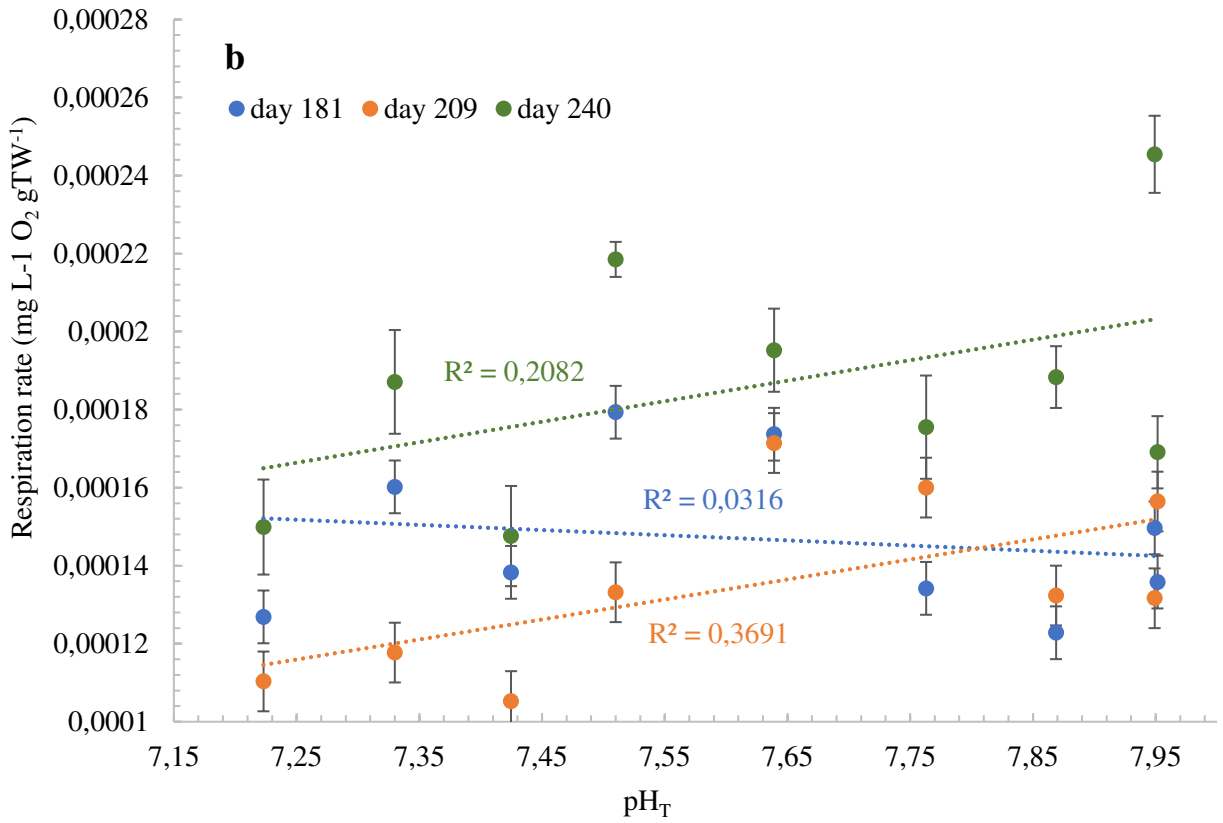


Figure 4.2.6.2 Respiration rate of *Hexaplex trunculus* females (mg L^{-1} O_2 min^{-1} g TW^{-1}) for **a)** the first two measurements (days 81 and 149) indicating the effect of pH on the respiration rate, and **b)** the last three measurements (days 181, 209 and 240) with no effect on pH. Error bars indicate a standard error.

4.3 Effect of long-term exposure to different pHs on the reproduction and intracapsular development of *H. trunculus*

4.3.1 Spawning

Temperatures reached above 20 °C on 22 May which was a trigger for banded-dye murex to start spawning. The start of spawning of the marked females happened on 31 May 2021 (pH_T 7.949) and was denoted as day 1. Other females continued to spawn three days after, with two to six females starting spawning per day. The peak of the spawning was on the 13th day after the first spawning event occurred when nine females started to spawn. The last spawning was recorded on the 16th day (Figure 4.3.1.1).

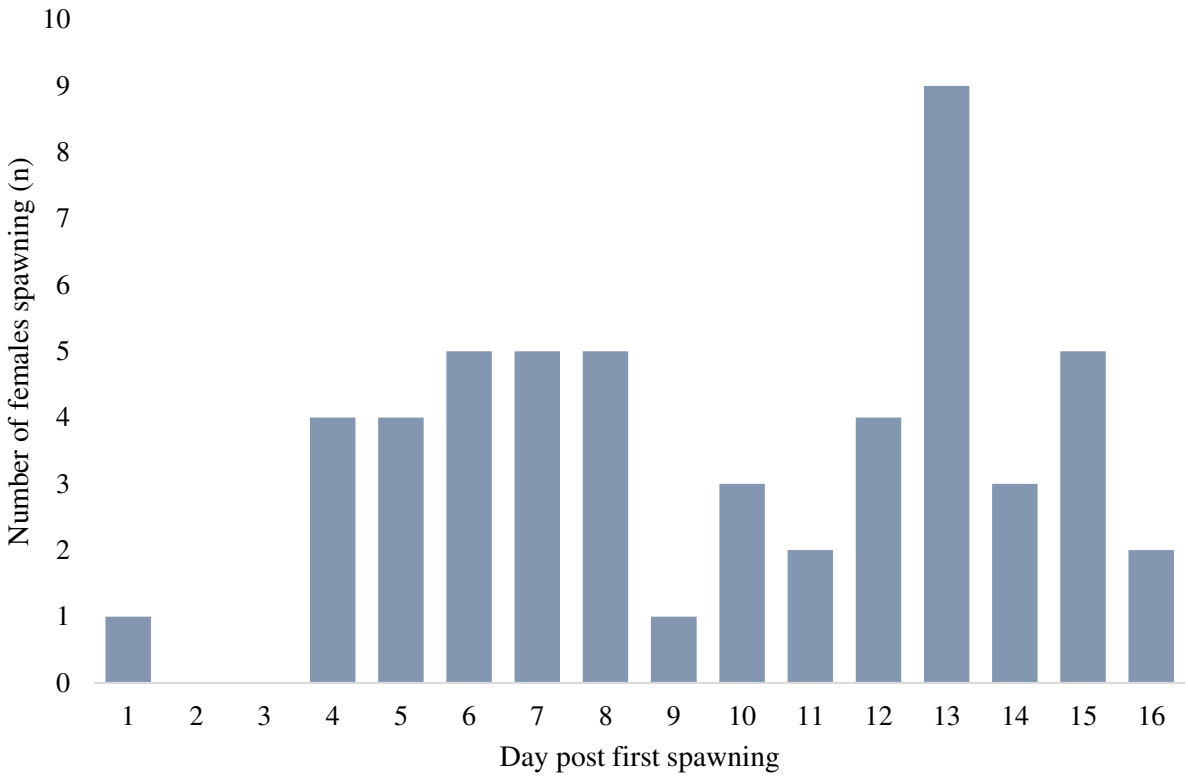


Figure 4.3.1.1 Number of tagged females of banded-dye murex, *Hexaplex trunculus*, spawning on each day during the overall spawning duration. The day when the first spawning event was recorded was denoted as day 1.

There was no relationship between the day when the spawning started and pH_T (Figure 4.3.1.2).

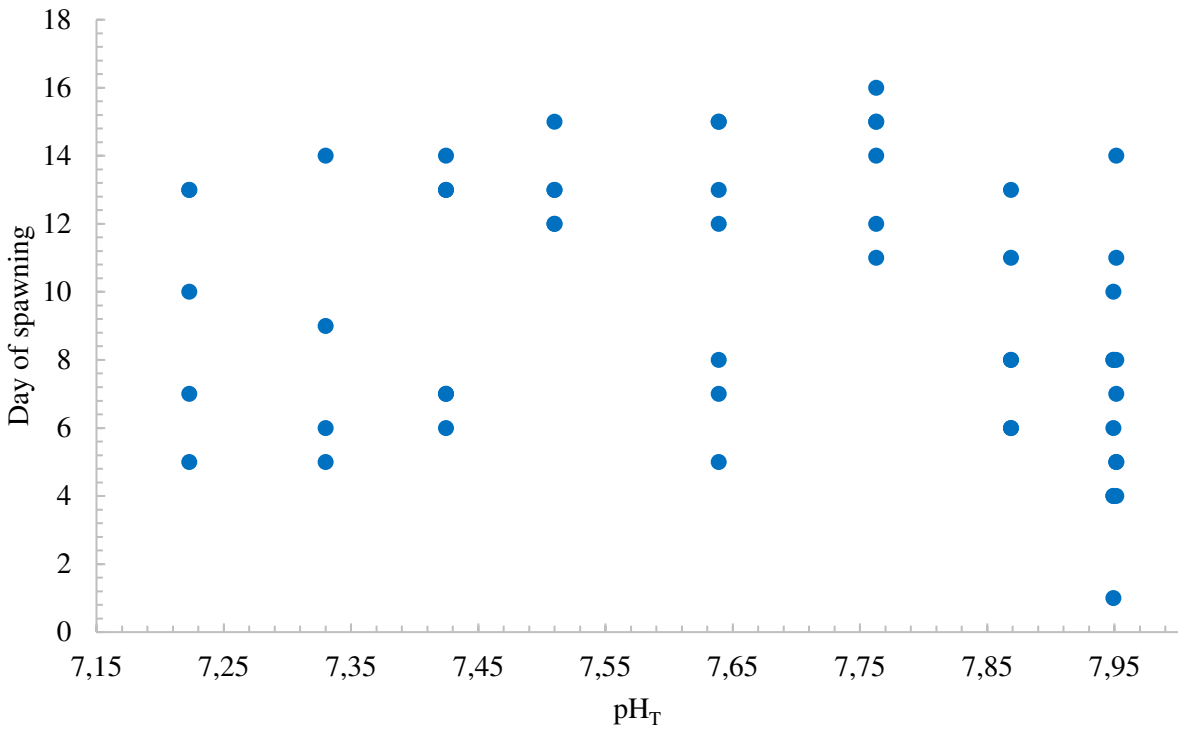


Figure 4.3.1.2 Relationship between pH_T and the day when the spawning started for each banded-dye murex, *Hexaplex trunculus*, female per pH_T.

The relationship between pH_T and the likelihood of females spawning was not statistically significant (BLR, ($\chi^2(1) = 2.168$. $p = .141$). The average spawning duration for all pH_T treatments was 2.92 ± 0.87 days, with a maximum of five days (pH_T 7.22) and a minimum of one day (pH_T 7.51). There was no relationship between the number of spawned capsules and pH_T (SLR, $R^2 = .001$, $F(1, 52) = .003$, $p = .953$) (Table 4.3.1.1).

Table 4.3.1.1 Number of females of banded-dye murex, *Hexaplex trunculus*, spawning (N), mean spawning duration in days and the number of spawned capsules (N) per pH_T (mean ± standard deviation).

pH_T	N females spawning	Mean duration (days)	N capsules
7.95 ± 0.07	7	3.20 ± 1.30	196 ± 60.78
7.95 ± 0.08	7	2.25 ± 0.50	244 ± 97.87
7.87 ± 0.08	6	3.33 ± 0.81	222 ± 100.87
7.76 ± 0.07	6	1.80 ± 0.83	272 ± 110.85

7.64 ± 0.07	7	3.00 ± 0.81	218 ± 89.68
7.51 ± 0.07	5	3.33 ± 0.51	197 ± 54.30
7.42 ± 0.07	6	2.83 ± 0.98	234 ± 57.45
7.33 ± 0.06	4	3.29 ± 0.48	266 ± 132.84
7.22 ± 0.07	5	2.86 ± 0.69	210 ± 55.67
Average	5.9 ± 1.05	2.92 ± 0.87	228 ± 83.38

Ten capsules from each spawn (530 in total) were measured for length, width and thickness, showing no significant relationship with pH (SLR, R^2= .014, $F(1, 529) = 5.729$. p = .017; R^2 = .022, $F(1, 529) = 9.306$, p = .002; R^2 = 0.008, $F(1, 529) = 4.122$, p = .043, respectively). The mean measured capsule length was 4.76 ± 0.54 mm, mean measured capsule width was 4.13 ± .53 mm and the mean thickness was 1.53 ± 0.26 mm.

Five capsules from each spawn were carefully opened to count the number of eggs (the total of 265 capsules). There was no significant relationship between the mean number of eggs per spawn and pH (SLR, R^2 = 0.007, $F(1, 52) = 0.364$, p = 0.549). The mean length, width and thickness of capsules, as well as the number of eggs per capsule in each pH are presented in Table 4.3.1.2.

Table 4.3.1.2 *Hexaplex trunculus* capsule length, width and thickness, and the number of eggs per capsule (N eggs) per pH_T (mean ± standard deviation).

pH_T	Length (mm)	Width (mm)	Thickness (mm)	N eggs
7.95 ± 0.07	4.57 ± 0.49	3.88 ± 0.36	1.52 ± 0.26	255 ± 71
7.95 ± 0.08	4.78 ± 0.43	4.02 ± 0.51	1.52 ± 0.22	251 ± 85
7.87 ± 0.08	4.93 ± 0.48	4.21 ± 0.49	1.45 ± 0.20	287 ± 33
7.76 ± 0.07	4.35 ± 0.54	4.13 ± 0.45	1.51 ± 0.29	291 ±80
7.64 ± 0.07	4.66 ± 0.52	3.96 ± 0.40	1.49 ± 0.23	250 ± 86
7.51 ± 0.07	4.89 ± 0.50	3.94 ± 0.46	1.45 ± 0.29	269 ± 22
7.42 ± 0.07	4.70 ± 0.44	4.27 ± 0.65	1.58 ± 0.27	273.6 ± 84

7.33 ± 0.06	4.97 ± 0.54	4.37 ± 0.49	1.57 ± 0.28	273.45 ± 75
7.22 ± 0.07	4.61 ± 0.62	3.93 ± 0.59	1.56 ± 0.26	279 ± 115
Average	4.76 ± 0.54	4.13 ± 0.53	1.53 ± 0.26	271 ± 62

4.3.2 Intracapsular development

Intracapsular embryonic development started with fertilized eggs, followed by the development of trochophore, early veliger, veliger and pediveliger larvae. Four days after spawning, the capsules were sampled to measure the diameter of the fertilized eggs. For each spawn, a minimum of two capsules were carefully opened and eggs were photographed under the microscope. A diameter of a minimum of 100 eggs per spawn was measured with Fiji software (Figure 4.3.2.1). Embryos that already started cell divisions were not measured. There was no significant relationship between the average egg diameter per spawn and pH_T (SLR, R^2 = .029, $F(1, 52) = 1.525$, p = .222; Figure 4.3.2.2).

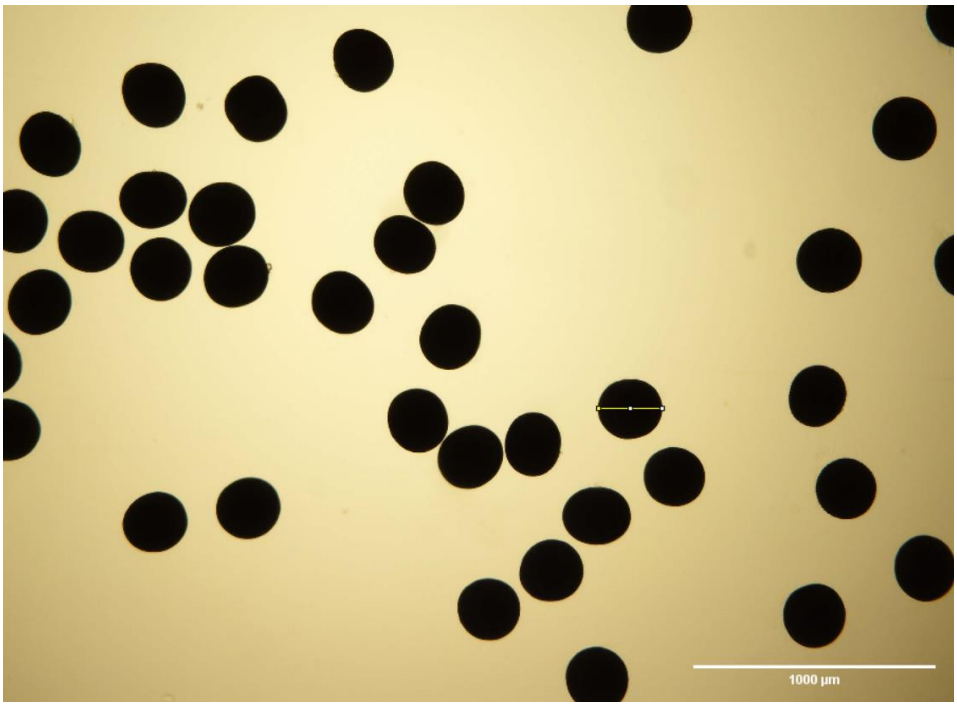


Figure 4.3.2.1 Measurement of *Hexaplex trunculus* egg diameter with Fiji software (scale bar = 1000 µm).

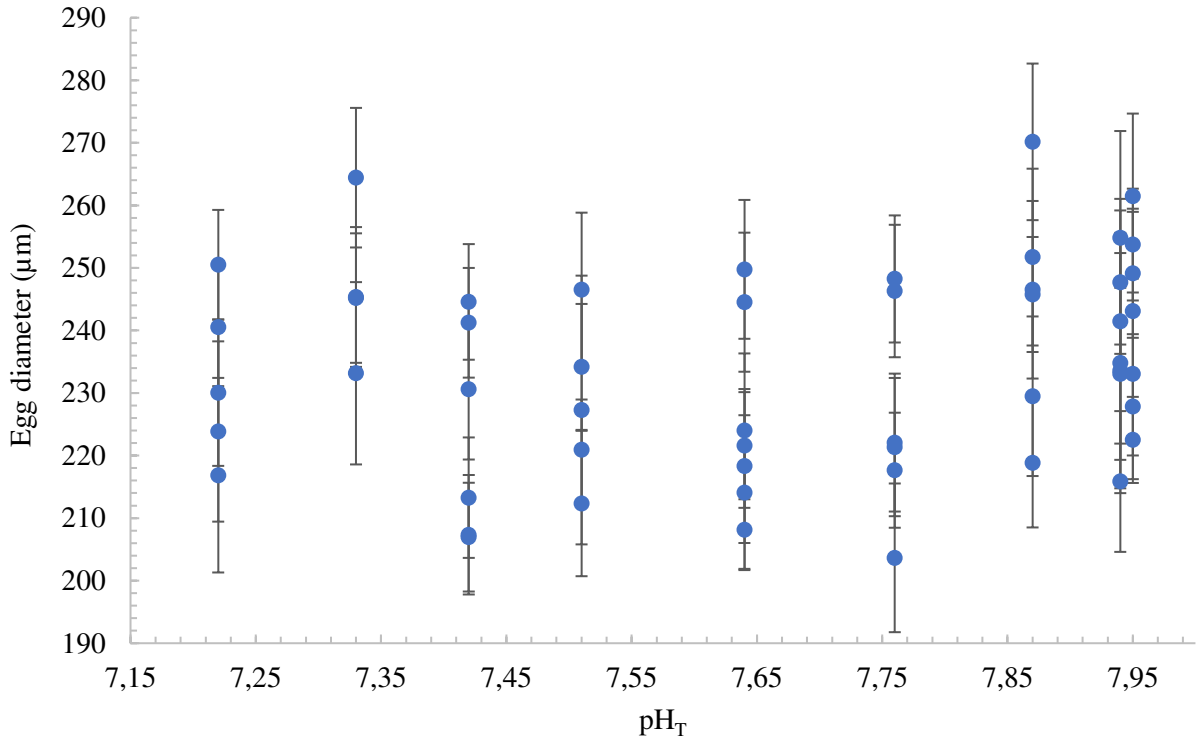


Figure 4.3.2.2 Relationship between egg diameter of banded-dye murex, *Hexaplex trunculus*, at day 4 and pH_T. Each dot represents the mean egg diameter (µm) per spawn.

After the initial sampling of the newly deposited capsules from each spawn, further capsules were randomly sampled at minimum four times over the duration of their intracapsular development, except for the spawns where development was arrested. Spawns were not sampled on the same day post-spawning. In several spawns, embryos developed into the next stage between the two capsules sampling, therefore, not all developmental stages were measured for each spawn. however, this does not indicate that the spawn that did not go through that stage (Figure 4.3.2.3).

pH	ID	DPS	1-4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
7.95	7.95_F3		fert_egg		trochophore						veliger						veliger					pediveliger		
7.95	7.95_F7		fert_egg								veliger						veliger							
7.95	7.95_F9		fert_egg				veliger						veliger								veliger			
7.95	7.95_F1		fert_egg	trochophore											veliger						veliger			
7.95	7.95_F6		fert_egg												fert_egg						early_veliger			
7.95	7.95_F8		fert_egg						veliger							veliger								
7.95	7.95_F10		fert_egg			trochophore						veliger								veliger				
7.94	7.94_F1		fert_egg										veliger					veliger						
7.94	7.94_F2		fert_egg								veliger						veliger							
7.94	7.94_F4		fert_egg					early_veliger							veliger									pediveliger
7.94	7.94_F5		fert_egg										veliger								veliger			
7.94	7.94_F9		fert_egg			trochophore				veliger							veliger							pediveliger
7.94	7.94_F10		fert_egg														veliger				veliger			
7.94	7.94_F6		fert_egg			trochophore						veliger									veliger			
7.87	7.87_F1		fert_egg					early_veliger				veliger								veliger				
7.87	7.87_F2		fert_egg	trochophore						veliger								veliger						
7.87	7.87_F5		fert_egg				early_veliger					veliger								pediveliger				
7.87	7.87_F6		fert_egg							veliger								veliger						
7.87	7.87_F7		fert_egg						veliger								veliger					pediveliger		
7.87	7.87_F8		fert_egg				early_veliger					veliger								pediveliger				
7.76	7.76_F1		fert_egg				early_veliger							veliger							veliger			
7.76	7.76_F2		fert_egg	trochophore						veliger								veliger						
7.76	7.76_F5		fert_egg	trochophore						veliger								veliger				AD		
7.76	7.76_F6		fert_egg			early_veliger						veliger								pediveliger				
7.76	7.76_F9		fert_egg					early_veliger								veliger								
7.76	7.76_F10		fert_egg		trochophore											veliger								
7.64	7.64_F1		fert_egg	trochophore					veliger								veliger							
7.64	7.64_F3		fert_egg							veliger								veliger						
7.64	7.64_F5		fert_egg						veliger								veliger							pediveliger
7.64	7.64_F9		fert_egg						veliger								veliger							
7.64	7.64_F4		fert_egg				early_veliger							veliger										
7.64	7.64_F8		fert_egg				veliger											veliger						
7.64	7.64_F10		fert_egg									veliger							veliger					
7.51	7.51_F2		fert_egg			early_veliger								veliger								pediveliger		
7.51	7.51_F4		fert_egg		early_veliger							veliger									veliger			
7.51	7.51_F8		fert_egg			early_veliger								veliger								pediveliger		
7.51	7.51_F10		fert_egg					early_veliger									veliger							
7.51	7.51_F9		fert_egg							veliger														
7.42	7.42_F2		fert_egg						early_veliger								veliger							
7.42	7.42_F3		fert_egg						early_veliger									veliger				AD		
7.42	7.42_F4		fert_egg							veliger									veliger					
7.42	7.42_F5		fert_egg						early_veliger										veliger					
7.42	7.42_F6		fert_egg		trochophore							veliger									veliger			
7.42	7.42_F7		fert_egg							veliger								veliger						
7.33	7.33_F2		fert_egg									veliger									veliger			
7.33	7.33_F5		fert_egg								veliger									veliger				
7.33	7.33_F6		fert_egg					early_veliger									veliger							
7.33	7.33_F7		fert_egg							veliger									veliger					
7.22	7.22_F2		fert_egg						early_veliger									veliger						
7.22	7.22_F8		fert_egg							veliger														
7.22	7.22_F4		fert_egg				trochophore							veliger								veliger		
7.22	7.22_F7		fert_egg					early_veliger							veliger									
7.22	7.22_F9		fert_egg							veliger							veliger							

Figure 4.3.2.3 Overview of capsules'sampling during the intracapsular development and determination of developmental stage. ID – individual spawn from marked female (F1-10) in each pH (7.95 – 7.22), DPS (1-35) day post spawning when capsules were sampled, AD – arrested development. Spawn in pH 7.95, female 6 (in red) was excluded from analysis.

pH	ID	DPS	26	27	28	29	30	31	32	33	34	35
7.95	7.95_F3						hatchling					
7.95	7.95_F7			hatchling								
7.95	7.95_F9					hatchling						
7.95	7.95_F1							hatchling				
7.95	7.95_F6		AD									
7.95	7.95_F8		pediveliger									hatchling
7.95	7.95_F10					AD						
7.94	7.94_F1				pediveliger			hatchling				
7.94	7.94_F2		pediveliger						hatchling			
7.94	7.94_F4					hatchling						
7.94	7.94_F5				pediveliger							hatchling
7.94	7.94_F9								hatchling			
7.94	7.94_F10							pediveliger				hatchling
7.94	7.94_F6											pediveliger - AD
7.87	7.87_F1			pediveliger						AD		
7.87	7.87_F2	pediveliger					hatchling					
7.87	7.87_F5			hatchling								
7.87	7.87_F6	hatchling										
7.87	7.87_F7		hatchling									
7.87	7.87_F8		hatchling									
7.76	7.76_F1								hatchling			
7.76	7.76_F2					hatchling						
7.76	7.76_F5				AD							
7.76	7.76_F6											
7.76	7.76_F9											hatchling
7.76	7.76_F10	AD										
7.64	7.64_F1				pediveliger					AD		
7.64	7.64_F3	pediveliger				hatchling						
7.64	7.64_F5							hatchling				
7.64	7.64_F9								hatchling			
7.64	7.64_F4							pediveliger				AD
7.64	7.64_F8											pediveliger AD
7.64	7.64_F10						hatchling					
7.51	7.51_F2						pediveliger			AD		
7.51	7.51_F4		veliger				D					
7.51	7.51_F8				AD							
7.51	7.51_F10						pediveliger					AD
7.51	7.51_F9					pediveliger				hatchling		
7.42	7.42_F2	veliger					hatchling					
7.42	7.42_F3											
7.42	7.42_F4		AD									
7.42	7.42_F5				AD							
7.42	7.42_F6						AD					
7.42	7.42_F7		veliger						AD			
7.33	7.33_F2					veliger				AD		
7.33	7.33_F5			AD								
7.33	7.33_F6	veliger										hatchling
7.33	7.33_F7	AD										
7.22	7.22_F2				veliger				AD			
7.22	7.22_F8							veliger				AD
7.22	7.22_F4						veliger			AD		
7.22	7.22_F7	veliger					veliger					
7.22	7.22_F9			veliger						hatchling		AD

Figure 4.3.2.3 Continued.

In the samples between the fifth- and ninth-day post-spawning (DPS), trochophore larvae were recorded for every pH_T, except for 7.51 and 7.33. Trochophore larvae were reached on average on day 6.91 ± 1.16 with a mean trochophore length of 331.94 ± 29.02 µm. The pH had no effect on the time to reach the trochophore stage (SLR, R^2 = .038. $F(1, 11)$ = .395, p = .544). There was no difference in the average trochophore length among pH treatments (one-way ANOVA, $F(6, 35)$ = 1.632. p = .174; Figure 4.3.2.4).

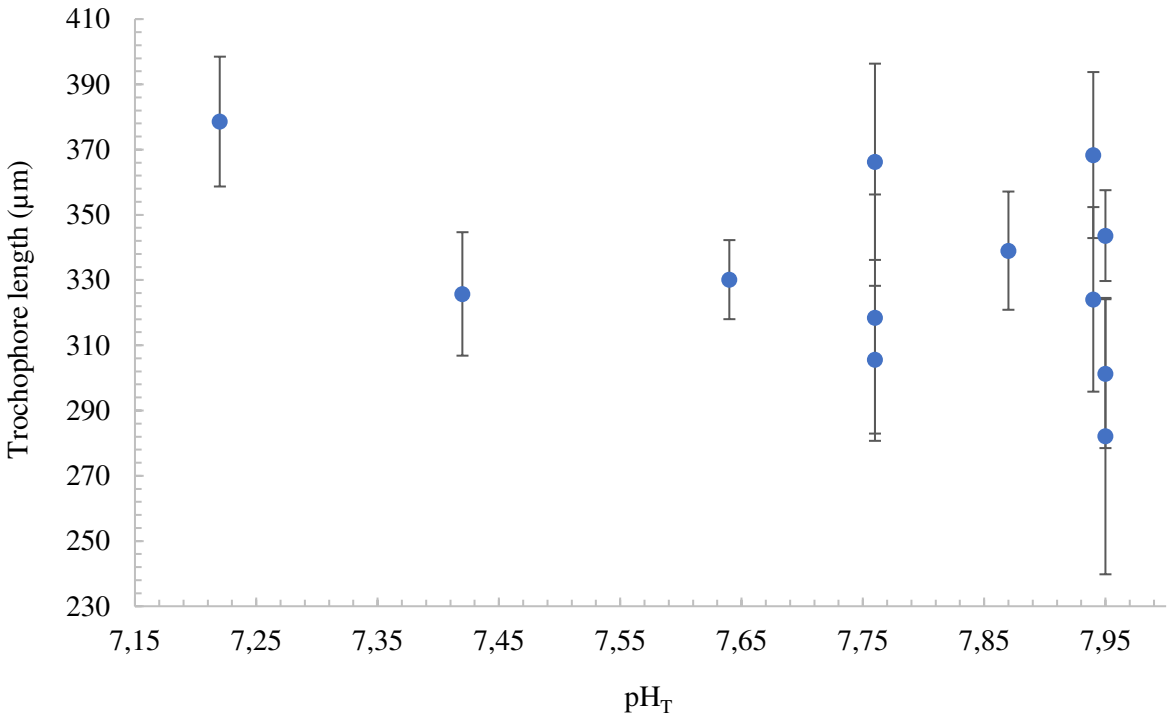


Figure 4.3.2.4 Relationship between pH_T and mean trochophore length (µm) per spawn of banded-dye murex *Hexaplex trunculus*.

The early veliger stage was recorded in the samples between the ninth- and eleventh-day post-spawning (9.50 ± 1.20 DPS) in every pH_T, except for 7.95. The mean length of early veliger larvae was 504.33 ± 92.64 µm. pH had no effect on the day post-spawning when the early veliger stage was reached (SLR, R^2 = .099, $F(1, 17)$ = 1.764. p = .203). There was no difference in the average early veliger length among pH treatments (one-way ANOVA, $F(7, 17)$ = .625. p = .726; Figure 4.3.2.5).

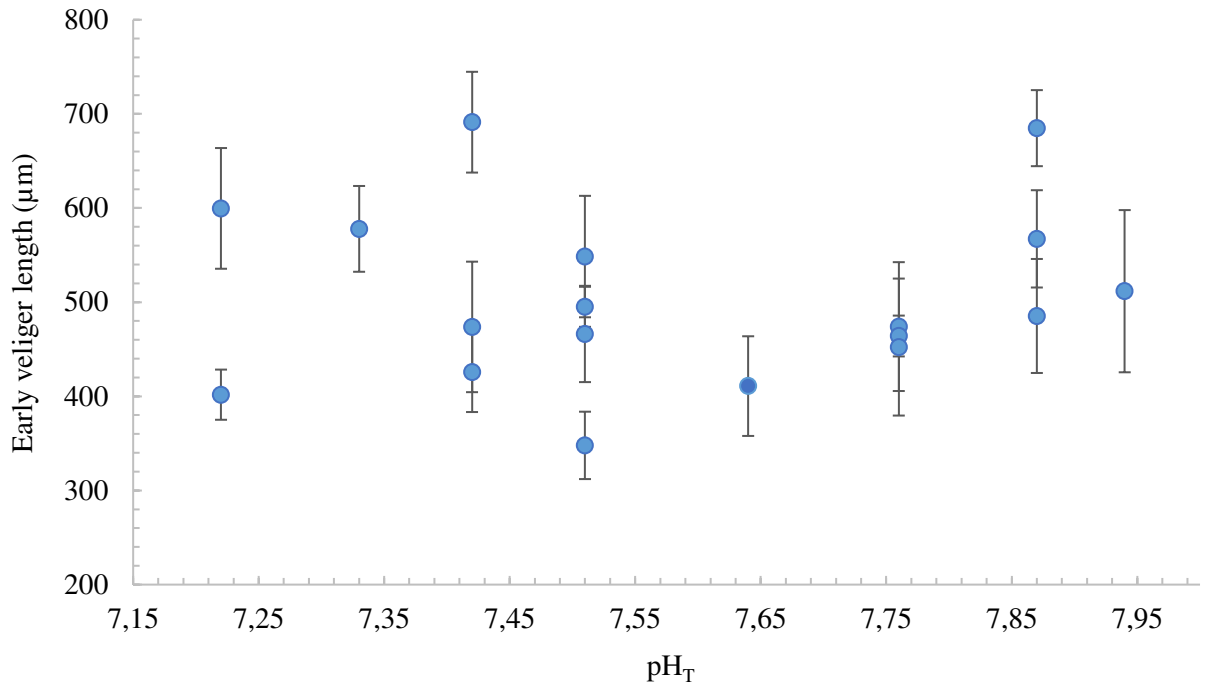


Figure 4.3.2.5 Relationship between pH_T and mean early veliger length (µm) per spawn of banded-dye murex *Hexaplex trunculus*.

Veliger larvae were first recorded in the samples between the ninth- and twentieth-day post-spawning (14.06 ± 2.61) with a mean length of 825.88 ± 106.92 µm. pH had no effect on the day post-spawning when the veliger stage was reached (SLR, R^2 = .0008. $F(1, 37)$ = .028. p = .867). There was no significant difference in the average veliger length among pH treatments (one-way ANOVA, $F(8, 37)$ = 1.330, p = .268; Figure 4.3.2.6).

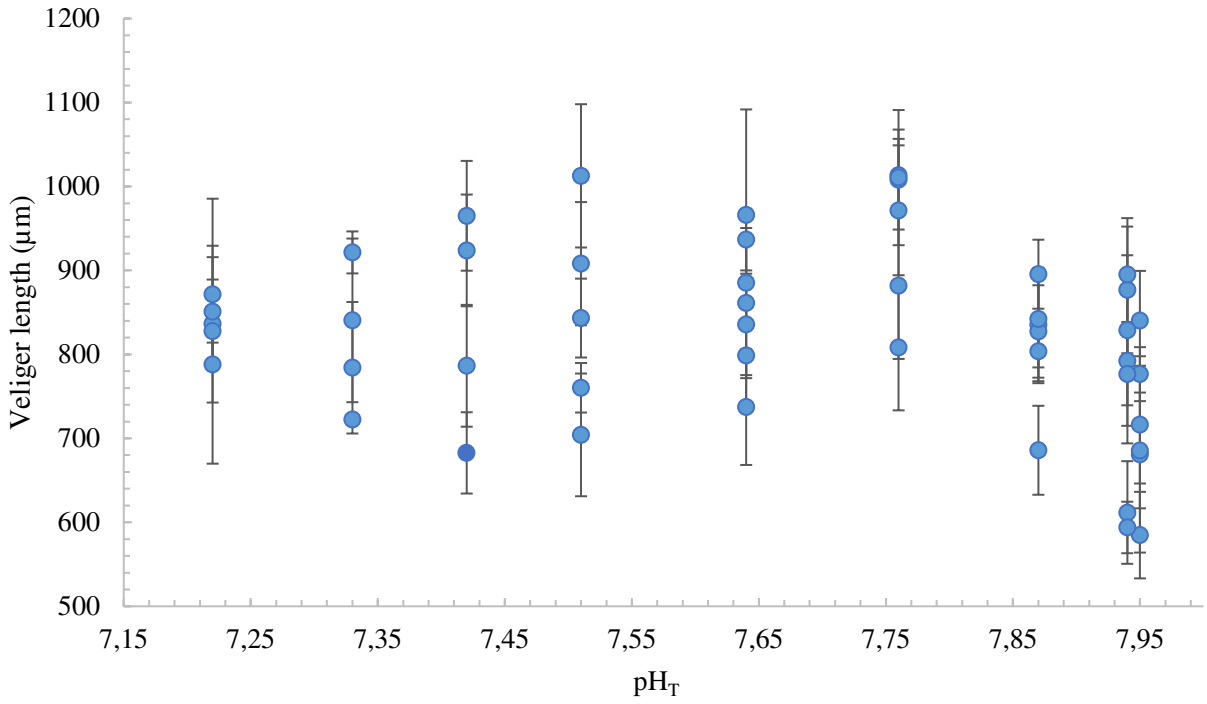


Figure 4.3.2.6 Relationship between pH_T and banded-dye murex, *H. trunculus*, mean early veliger length (µm) per spawn.

After reaching the veliger stage, a notable difference in development was observed in pH_T 7.51 – 7.22. The veliger stage lasted until the 22nd day post-spawning in pH_T 7.95–7.67, while in the lower pH_T, viable veliger larvae were sampled until the maximum of the 32nd day post-spawning with no further change in size (Figure 4.3.2.7).

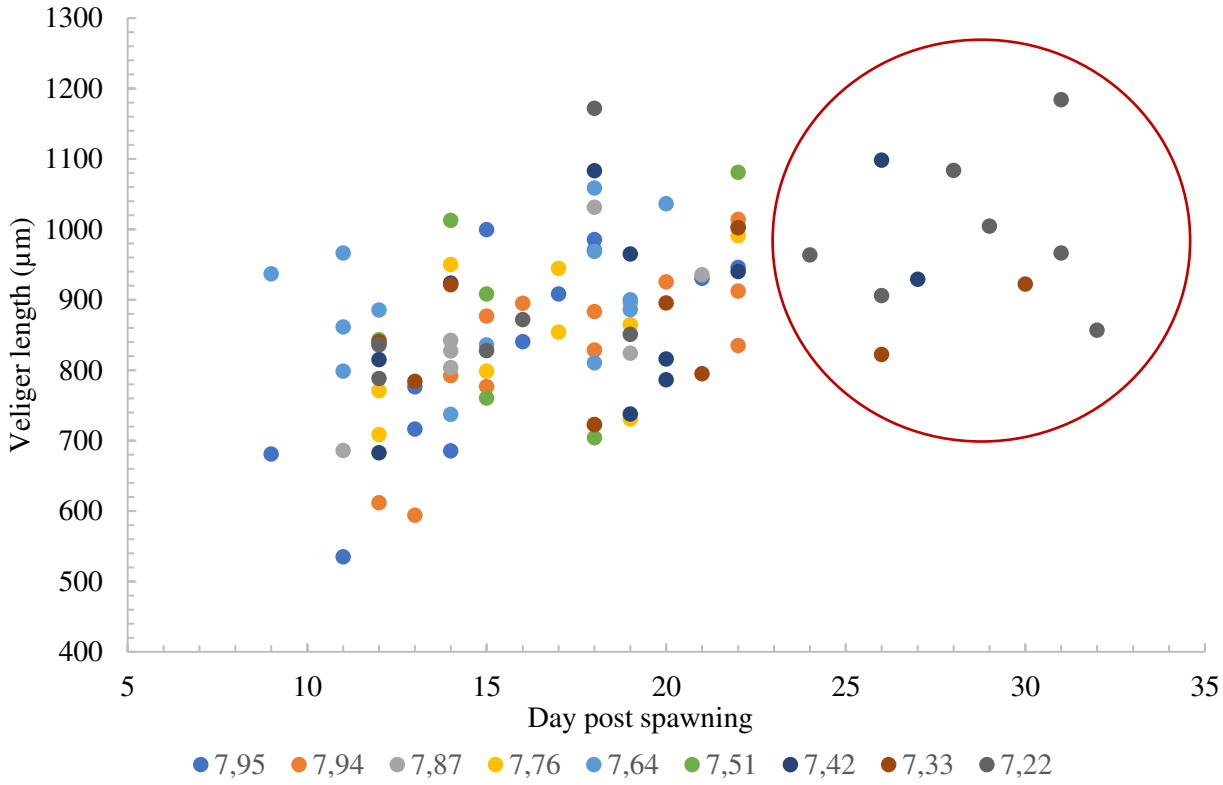


Figure 4.3.2.7 Relationship between the veliger length and the day post-spawning of banded-dye murex, *Hexaplex trunculus*, for each pH (colored dots) indicating longer veliger development time for pH_T 7.51–7.22 (DPS 22–32, red circle).

Pediveliger was not sampled in pH_T 7.42–7.22, although there was one spawn per each pH_T that reached hatching. Presented data for pediveliger larvae range from pH_T 7.95–7.51. The pediveliger stage was reached on average 27.23 ± 4.12 DPS. The mean pediveliger length was 1133.05 ± 83.27 µm. There was no difference in the average pediveliger length among pH treatments (one-way ANOVA, $F(5, 24) = .895$, $p = .504$; Figure 4.3.2.8). pH had no effect on the time when they reached the pediveliger stage (SLR, $R^2 = .029$, $F(1, 24) = .682$. $p = .417$).

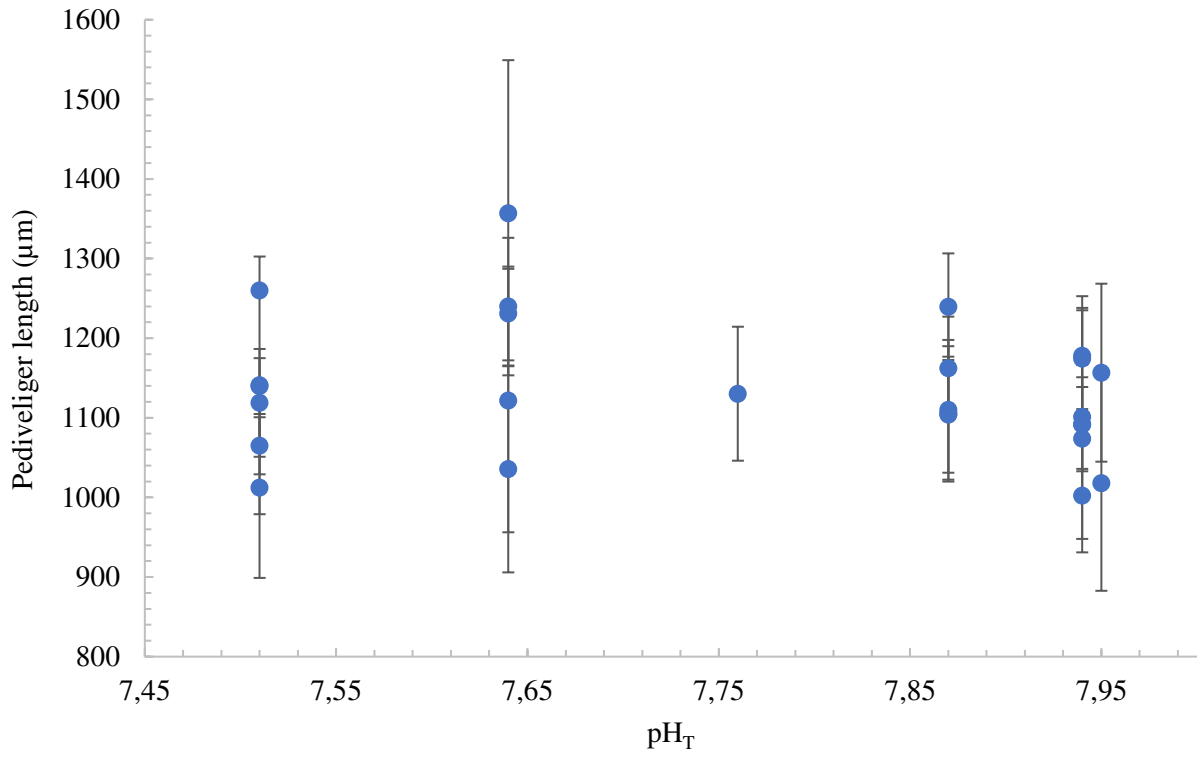


Figure 4.3.2.8 Relationship between pH_T and mean pediveliger length (µm) per spawn of banded-dye murex, *Hexaplex trunculus*.

Hatching started on average on day 31.46 ± 2.66 post-spawning, with an average hatchling length of 1412.08 ± 112.85 µm. pH had no effect on DPS when the hatching started (SLR, R^2 = .098, $F(1, 28) = 2.955$, $p = .097$) and there was no significant difference in the hatchling length among pH treatments (one-way ANOVA, $F(8, 27) = .482$. $p = .854$; Figure 4.3.2.9).

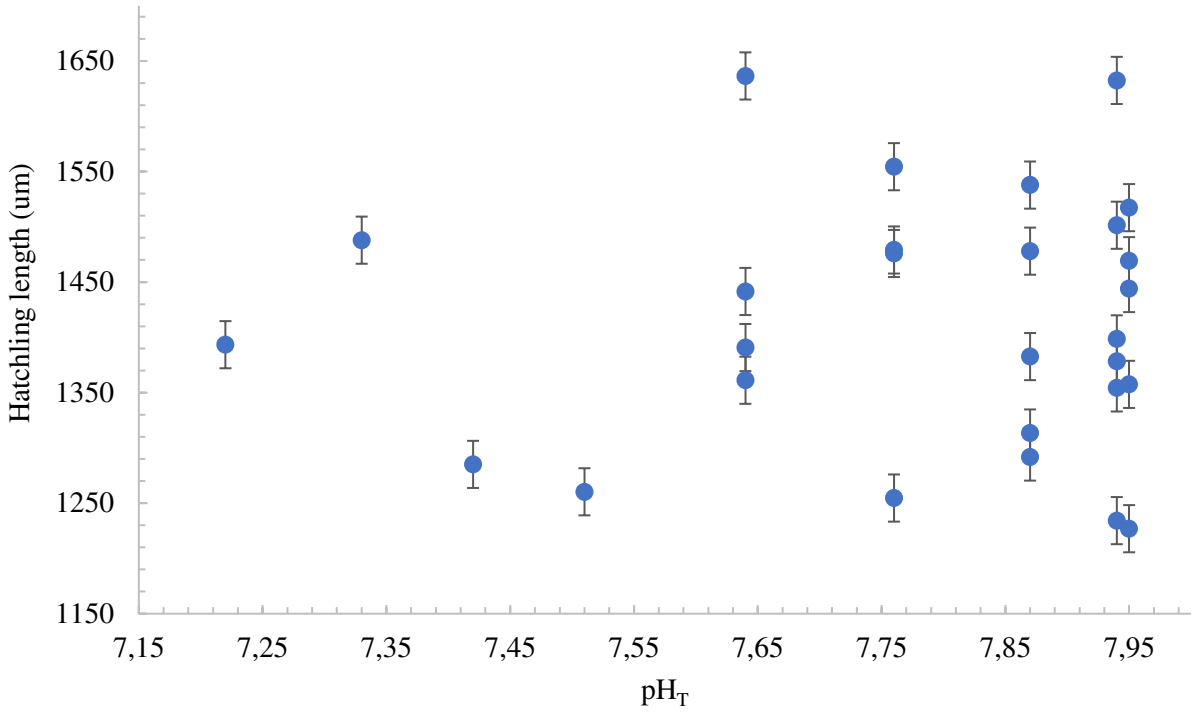


Figure 4.3.2.9 Relationship between pH_T and average hatchling length (µm) per spawn of banded-dye murex, *Hexaplex trunculus*.

The spawns of banded-dye murex in all pH treatments have reached trochophore, early veliger and veliger stage, except for one spawn from the control treatment (pH_T 7.95) that arrested at the beginning of embryonic development and was therefore excluded from further analysis. The proportion of spawns that have reached a certain developmental stage was calculated out of the initial number of spawns in each pH_T (Figure 4.3.2.10). There was a decline in the number of spawns that reached the pediveliger and hatchling stage from pH_T 7.51–7.22. The binary logistic regression model applied to determine if pH had a significant effect on the likelihood of reaching the pediveliger and hatchling stage of development was statistically significant ($\chi^2(1) = 11.852$. $p < .001$; $\chi^2(1) = 10.637$, $p = .001$. respectively). For every 0.1 decrease in pH, a 0.001 decrease in the log-odds of reaching the pediveliger stage is expected ($p = .001$, 95% CI [3.260E-5, 0 .059], whereas log-odds for reaching the hatchling stage decrease by 0.01 ($p = .001$, 95% CI [.001, .160].

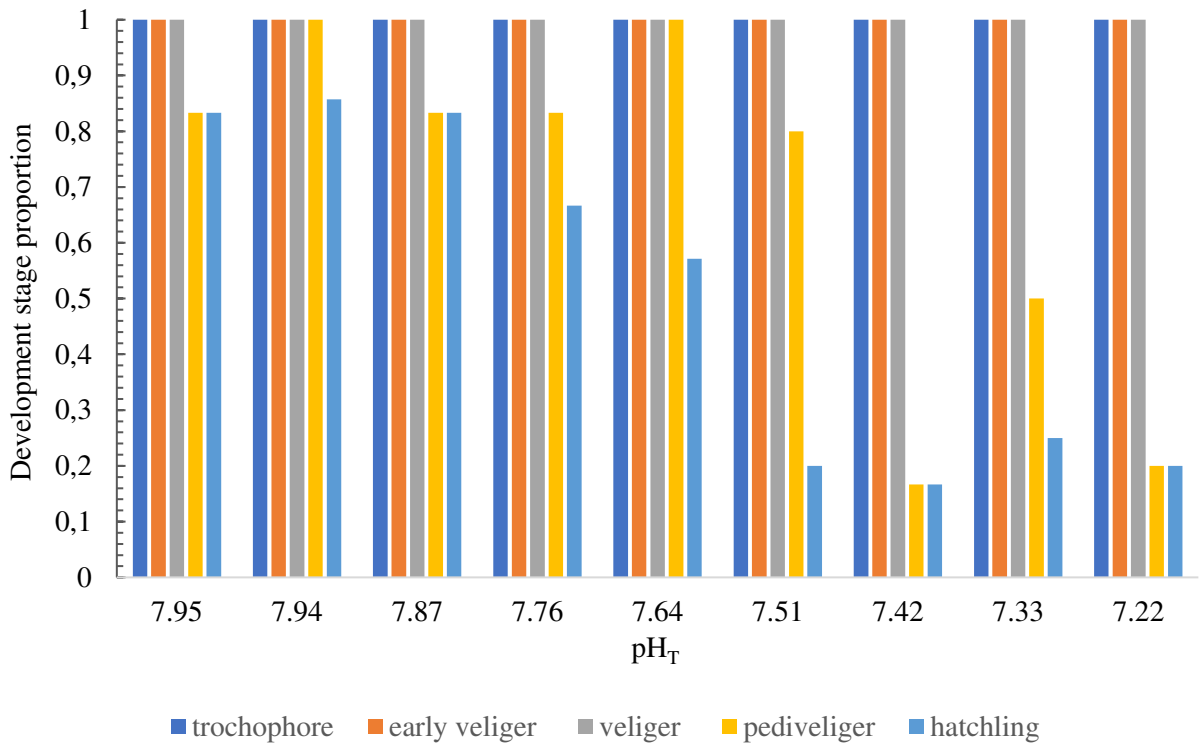


Figure 4.3.2.10 Proportion of spawns of banded-dye murex, *Hexaplex trunculus*, that reached the respective development stage (colored columns) per pH$_T$. The proportion of each developmental stage was calculated from the initial number of spawns in each pH$_T$.

The intracapsular growth rate for all pH followed a log-linear relationship with time (days post-spawning) (Figure 4.3.2.11).

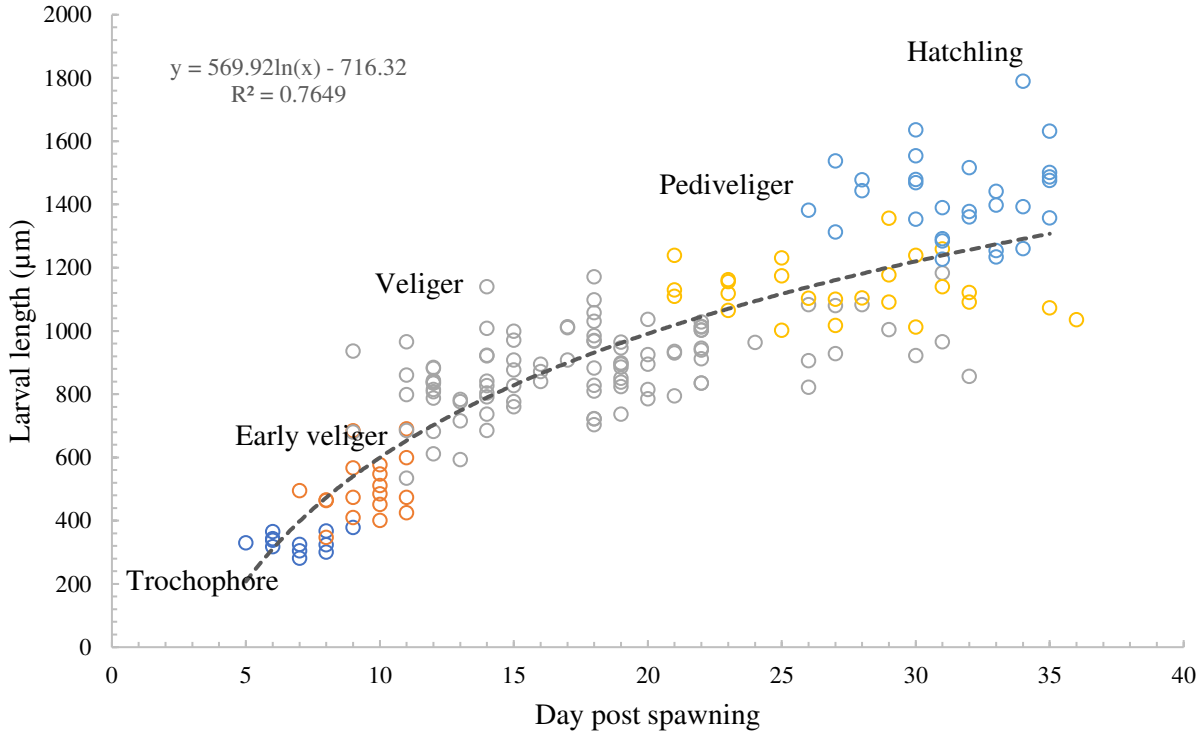


Figure 4.3.2.11 Relationship between the stage length (µm) and days post-spawning of banded-dye murex *Hexaplex trunculus*.

The intracapsular growth rate for each pH_T was calculated from the log-linear relationship between the developmental stage length and development time (µm log day^{-1}). The calculated average growth rate was then plotted against pH_T. There was a significant positive relationship between the growth rate and pH (SLR, $R^2 = .675$, $F(1, 8) = 14.603$, $p = 0.006$; Figure 4.3.2.12).

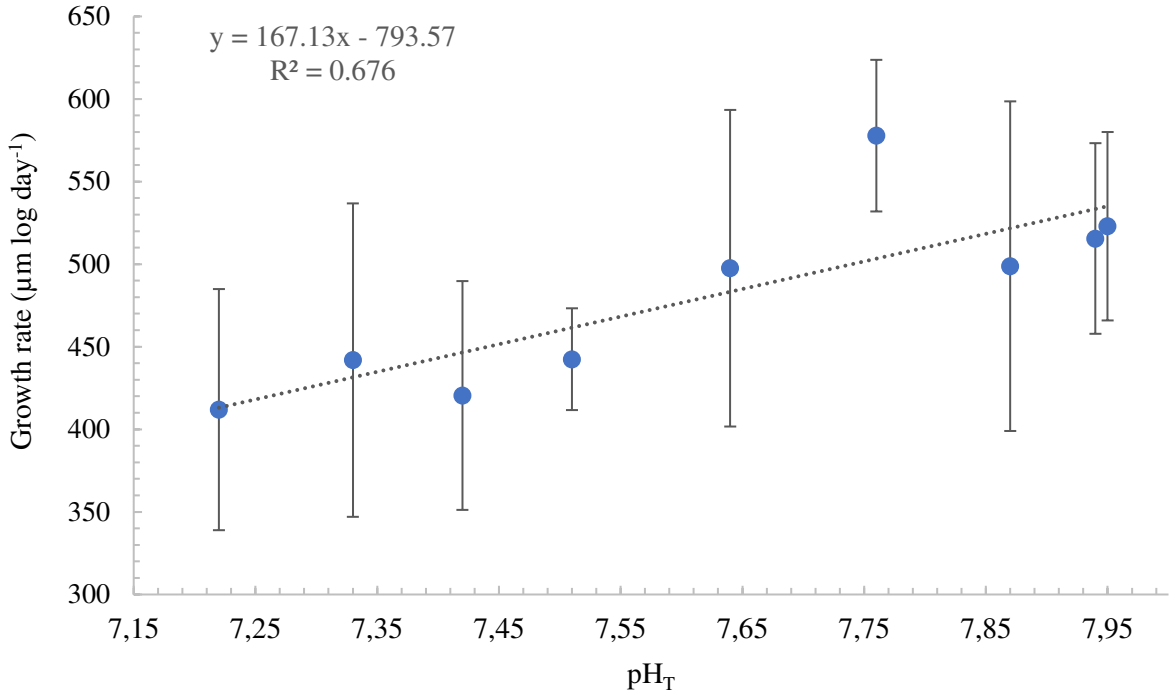


Figure 4.3.2.12 Relationship between average growth rate (µm log day^{-1}) of banded-dye murex *Hexaplex trunculus*, and pH_T.

4.3.3 Carryover effect

4.3.3.1 Cross-transplantation between pH_T 7.95 & 7.22

Spawns of banded-dye murex from pH_T 7.95 (females F1, F3 & F6) were cross-transplanted with pH_T 7.22. Intracapsular development was arrested at the trochophore stage in spawn from female F6, therefore it was excluded from further analysis. Overall, the spawns transplanted from pH_T 7.95 to 7.22 had a lower mean length than spawns that remained in the pH_T

7.95 (LMM, $F(1, 462) = 44.037$, $p = .001$; $MD = 25.90$, $SE = 6.385$, $p = .001$) (Figure 4.3.3.1.1, Table 4.3.3.1.1).

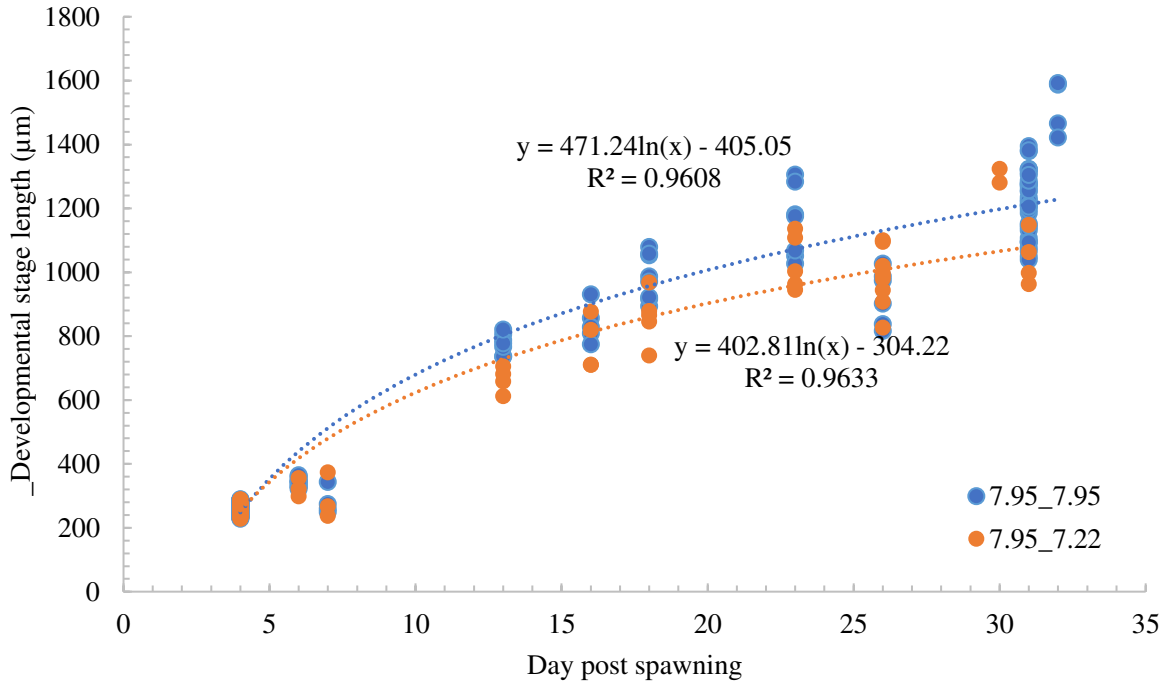


Figure 4.3.3.1.1 Growth rate (length, µm) of developmental stages of banded-dye murex, *Hexaplex trunculus*, spawned and developed in pH_T 7.95 (blue circle) and transplanted from pH_T 7.95 to 7.22 (orange circle).

Both spawns in pH_T 7.95 have reached the hatchling stage, while the transplants in pH_T 7.22 only reached pediveliger (Figure 4.3.3.1.2). A negative effect of a low pH on the growth rate (in length, µm) was observed in the spawn from female 3 (ANCOVA, $F(1, 291) = 9.101$, $p = .003$), with larvae transplanted in pH_T 7.22 having a lower mean length ($MD = 23.83$, $SE = 7.90$, $p = 0.003$). No significant difference in the mean length between the transplants was observed for the spawn from female 1 (ANCOVA, $F(1,169) = .634$, $p = .427$).

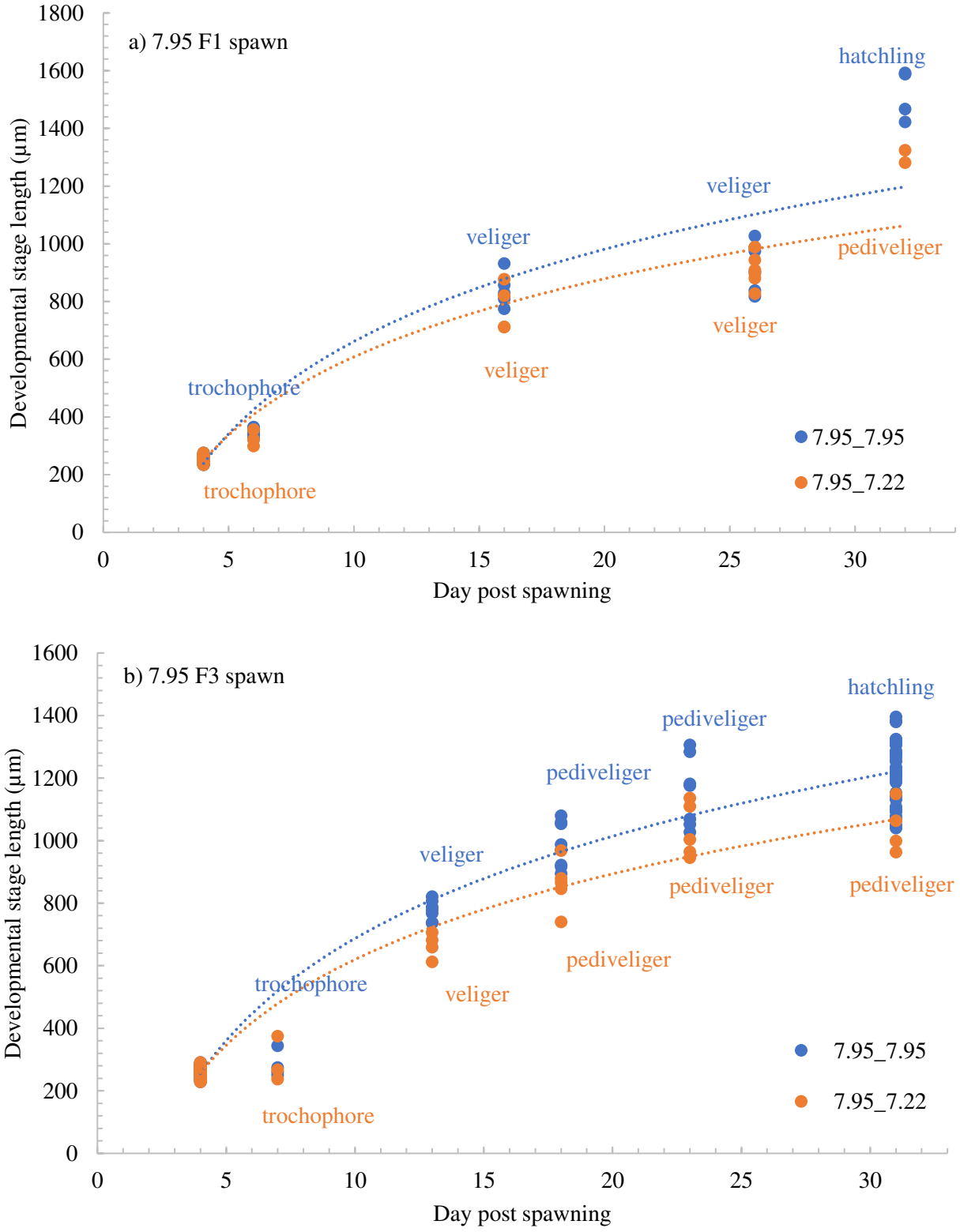


Figure 4.3.3.1.2 Relationship between the developmental stage of length (µm), the developmental stage (trochophore, veliger, pediveliger, hatchling) and the day post-spawning for half of the spawn from banded-dye murex, *Hexaplex trunculus*, a) female 1 & b) female 3, in pH_T 7.95 (blue) and the transplanted in pH_T 7.22 (yellow).

Spawns from pH_T 7.22 (females F4, F7 & F9) were cross-transplanted with pH_T 7.95. No significant difference was observed in the growth rate between the spawns transplanted to pH_T 7.95 and spawns that remained in 7.22 (LMM, $F(1, 426) = 2.639$, $p = .105$) (Figure 4.3.3.1.3).

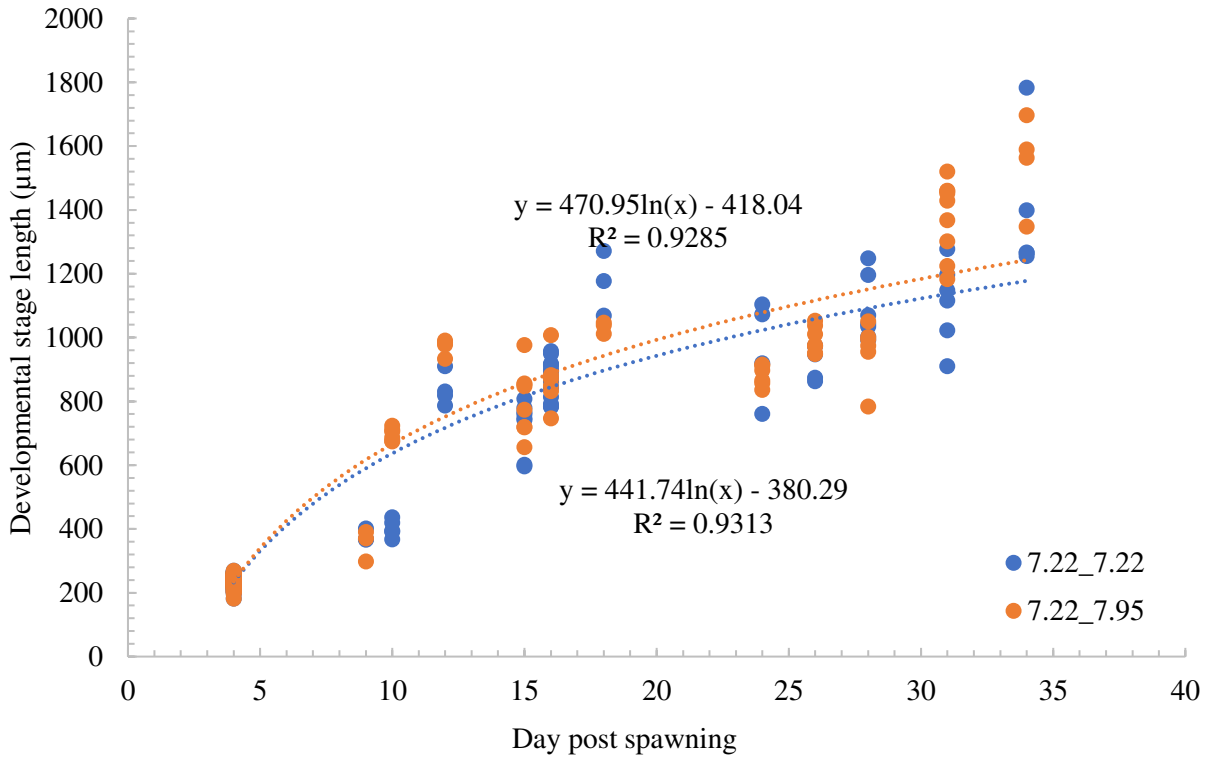
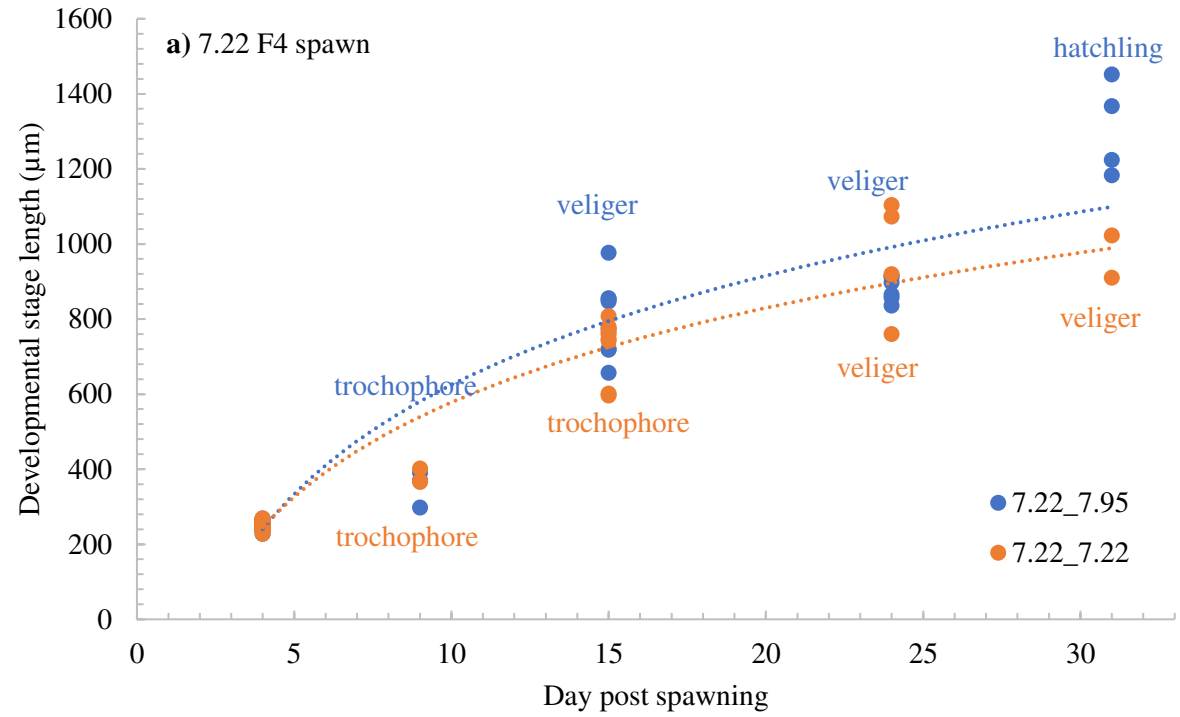
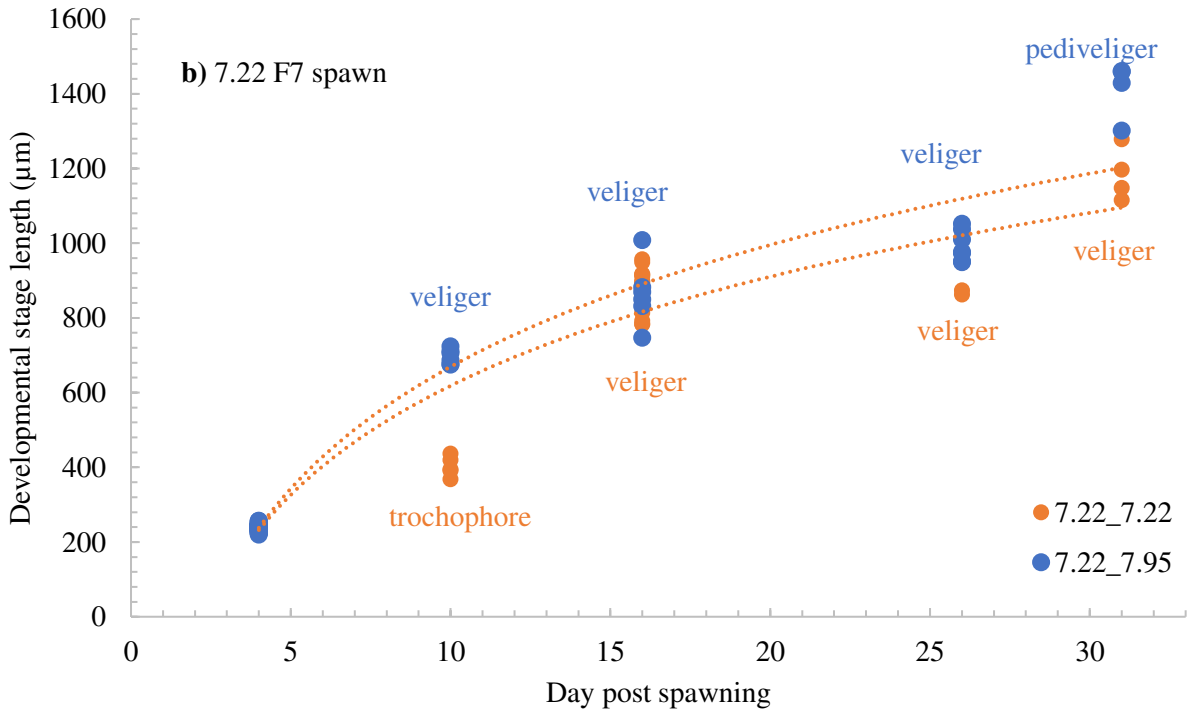


Figure 4.3.3.1.3 Growth rate (length, µm) of the developmental stages of banded-dye murex, *Hexaplex trunculus*, spawned and developed in pH_T 7.22 (blue circle) and transplanted from pH_T 7.22 to 7.95 (orange circle).

Despite no observed difference in larval length, the spawns from females F4 and F7 in pH_T 7.22 reached the veliger stage and did not develop further, while their transplants in pH_T 7.95 reached the hatchling and pediveliger stage, respectively (Figure 4.3.3.1.4 a, b). Only the spawn from female F9 reached the hatchling stage in both pH_T 7.22 and 7.95 (Figure 4.3.3.1.4 c). An analysis of individual spawns and their respective transplants revealed a significant difference in the larval length between transplants only for F7 spawn (ANCOVA, $F(2, 169) = 6.534$, $p = .001$), with larvae transplanted in pH_T 7.95 having a higher mean length than in pH_T 7.22 ($MD = 28.186$, $SE = 11.027$, $p = .011$). A summary of the statistical analysis for spawns transplanted between 7.95 ⇔ 7.22 is presented in Table 4.3.3.1.1.


a) 7.22 F4 spawn
Developmental stage length (µm)
Day post spawning
hatchling
veliger
veliger
veliger
veliger
trochophore
trochophore
trochophore
7.22_7.95
7.22_7.22


b) 7.22 F7 spawn
Developmental stage length (µm)
Day post spawning
pediveliger
veliger
veliger
veliger
veliger
veliger
veliger
trochophore
7.22_7.22
7.22_7.95

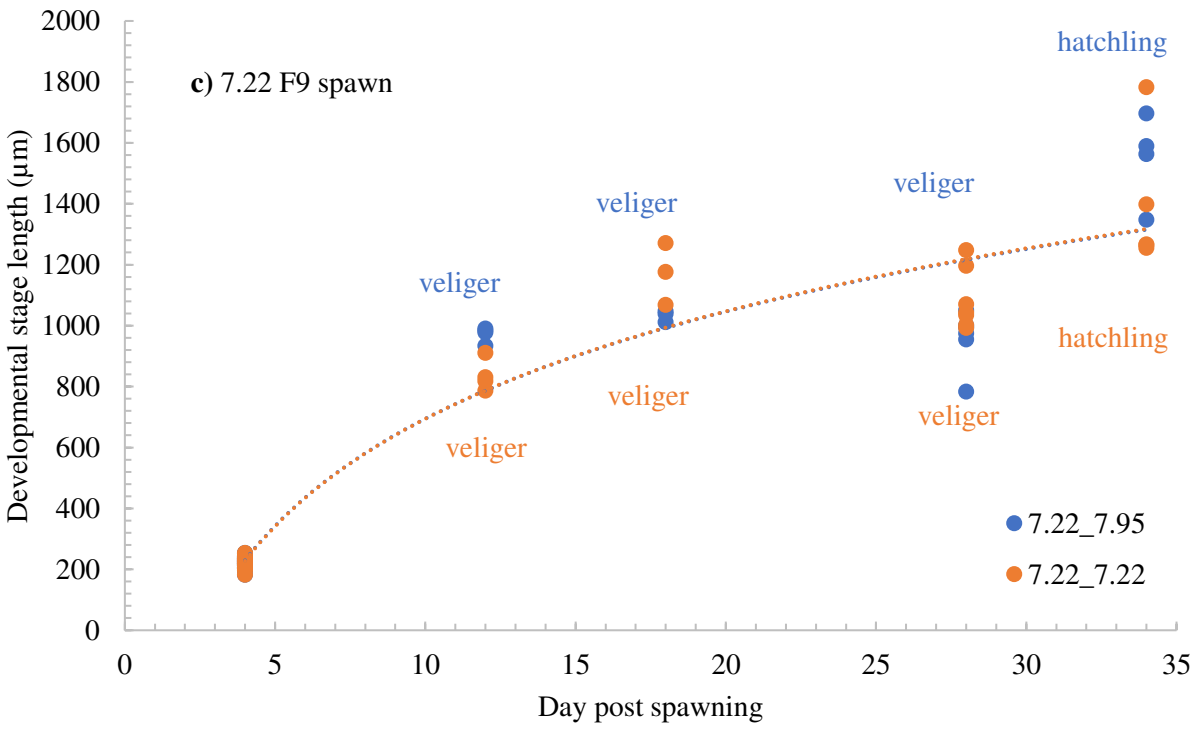


Figure 4.3.3.1.4 Relationship between the developmental stage of length (µm), the developmental stage (trochophore, veliger, pediveliger, hatchling) and the day post-spawning for half of spawn of banded-dye murex, *Hexaplex trunculus*, from **a)** female 4, **b)** female 7 & **c)** female 9, that was transplanted to pH_T 7.95 (blue) and that remained in pH_T 7.22 (orange).

Table 4.3.3.1.1 Summary of statistical analysis for the developmental stage length of banded-dye murex *H. trunculus*, (length, µm) with the day post-spawning (DPS) between transplants in pH_T 7.95 ⇔ 7.22. (SS – Sum of squares, df – degrees of freedom, MS – mean square, F – test statistics, p – probability value). Significant effects are in bold.

	SS (Type III)	df	MS	F	p
7.95 → 7.22; F1					
ANCOVA	1311501.1	2	6557506.5	1996.25	**.001**
DPS	13053109.3	1	13053109.3	1983.11	**.001**
pH_T	4175.03	1	4175.033	.634	.427
Corrected total	14214229.04	169			
7.95 → 7.22; F3					
ANCOVA	38079776.8	2	1903988.39	4540.761	**.001**
DPS	35120210.46	1	35120210.46	8375.705	**.001**

	SS (Type III)	df	MS	F	p
pH_T	38160.29	1	38160.29	9.101	**.003**
Corrected total	39291584.17	291			
7.22 → 7.95; F4					
ANCOVA	10193751.5	2	5096875.750	789.030	**.001**
DPS	10094466.14	1	10094466.14	1562.691	**.001**
pH_T	11767.178	1	11767.178	1.822	.180
Corrected total	11014129.53	129			
7.22 → 7.95; F7					
ANCOVA	17669187.3	2	8834593.639	1714.651	**.001**
DPS	17669072	1	17669072.17	3429.279	**.001**
pH_T	33665.973	1	33665.973	6.534	**.011**
Corrected total	18529640.71	169			
7.22 → 7.95; F9					
ANCOVA	21387716.3	2	10693858.13	914.094	**.001**
DPS	21369456.97	1	21369456.97	1826.627	**.001**
pH_T	25.712	1	25.712	0.002	.963
Corrected total	22826676.41	125			

Due to the observed variability in the larval growth rate originating from the same pH_T treatment, an effect size relative to the control pH_T was calculated to further assess the magnitude of the difference in intracapsular growth among transplants:

$$\text{Relative effect size} = -(\text{growth rate}_{(\text{control pHT})} - \text{growth rate}_{(\text{pHT treatment})}) / \text{growth rate}_{(\text{control pHT})}.$$

The control pH_T were the treatments where the spawning occurred, while the pH_T treatments were considered to be where the other half of the spawn was transferred. The effect size relative to the control was calculated for each replicate and plotted against the control growth rate (Figure 4.3.3.1.5). A positive effect size indicates that the embryo growth rate was higher in the transferred pH_T than the control, and a negative effect size indicates the opposite – the growth rate was lower

in the transferred pH_T than the control. Larvae with a higher growth rate in pH_T 7.95 were more affected when transferred to low pH, and larvae with a lower growth rate in pH_T 7.22 had higher growth rates when transferred to 7.95.

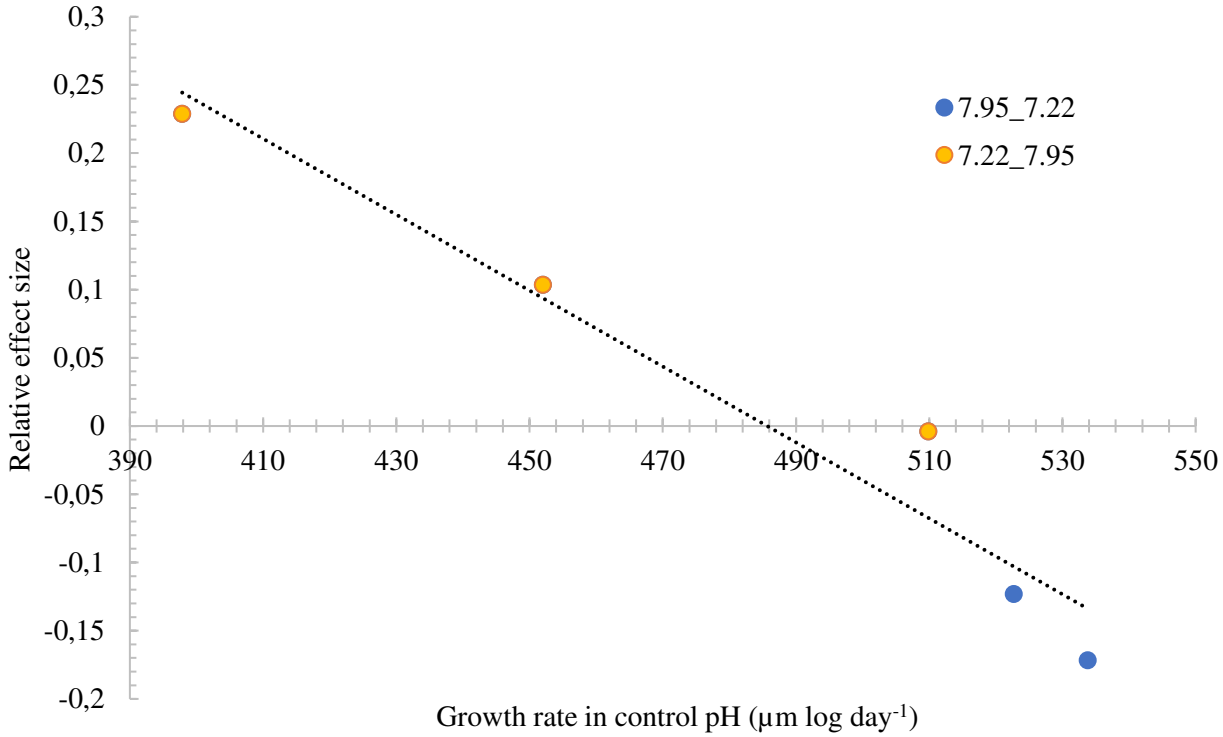


Figure 4.3.3.1.5 Relationship between initial growth rate of banded-dye murex, *Hexaplex trunculus* in control pH_T (µm log day^{-1}) and relative effect size for transplants between 7.95 ⇔7.22 (blue dots – initial pH_T 7.95, transplanted to 7.22; orange dots – initial pH_T 7.22, transplanted to 7.95)

4.3.3.2. Cross-transplantation between pH_T 7.95 & 7.64

Spawns from females 8 and 10 in pH_T 7.95 were transplanted to pH_T 7.64. Additionally, spawn from pH_T 7.94 (manipulated treatment, female 6) was chosen due to a lack of suitable spawns in 7.95 (unmanipulated treatment). Intracapsular development from female 10 arrested at the veliger stage, and therefore was excluded from further analysis. Overall, no significant difference in growth rate was observed between the spawns that remained in pH_T 7.95 and that were transplanted to pH_T 7.64 (LMM, $F(1, 252) = .033$, $p = .857$; Figure 4.3.3.2.1).

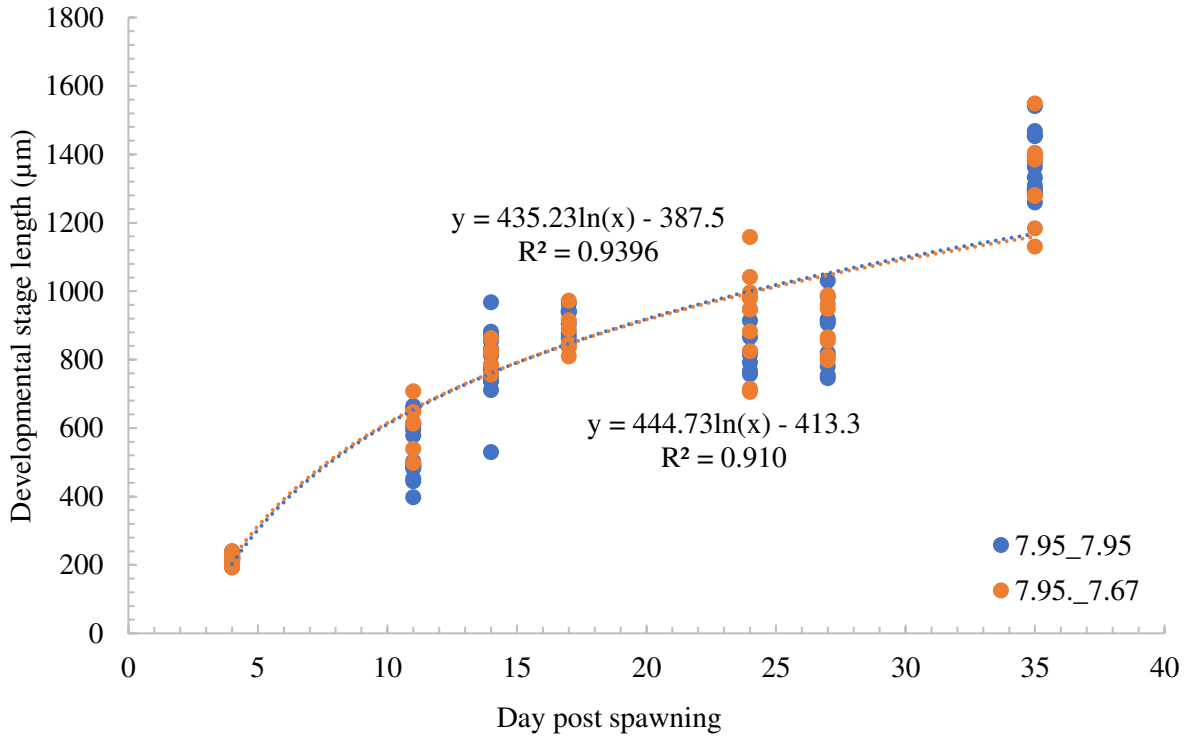
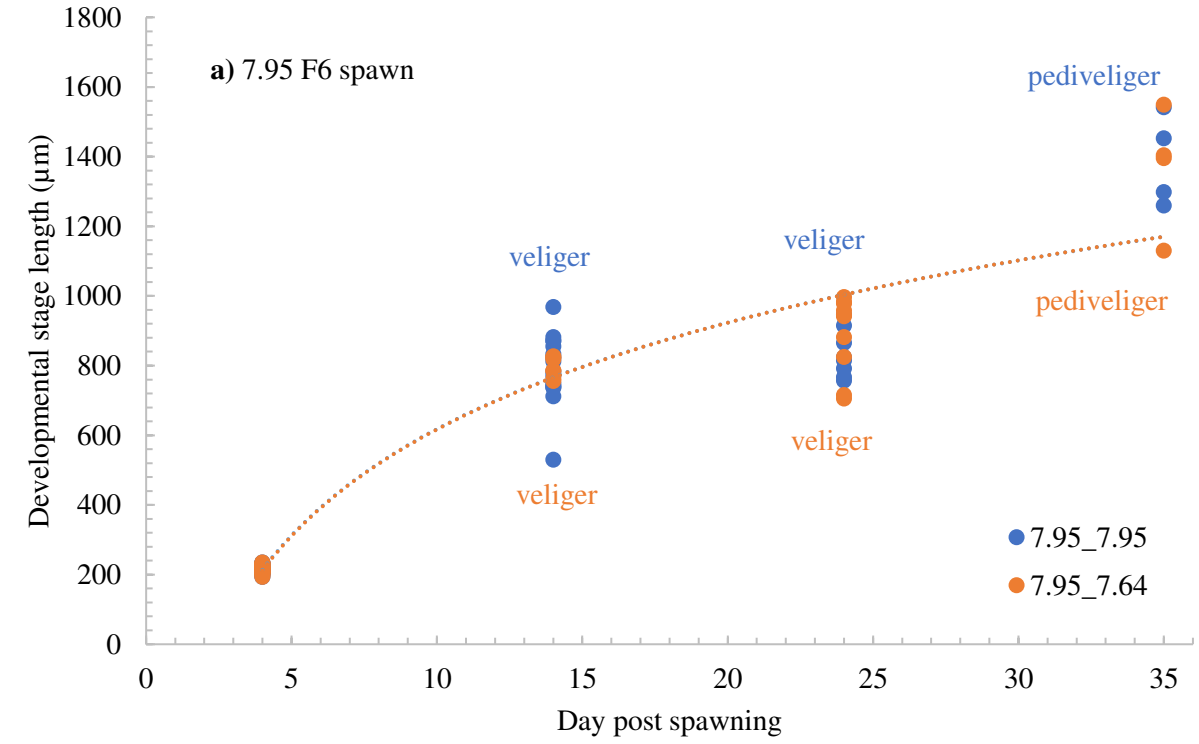


Figure 4.3.3.2.1 Growth rate (length, µm) of developmental stages of banded-dye murex, *Hexaplex trunculus*, spawned and developed in pH_T 7.95 (blue circle) and transplanted from pH_T 7.95 to 7.64 (orange circle).

Spawn from female F6 reached the pediveliger stage in both pH_T 7.95 and 7.64 (Figure 4.3.3.2.2 a), while the spawn from female 8 reached the hatchling stage in both pH_T (Figure 4.3.3.2.2 b) with no observable difference in the growth rate (ANCOVA, $F(1,102) = .003$, $p = .959$; $F(1,141) = .24$, $p = .877$; respectively).



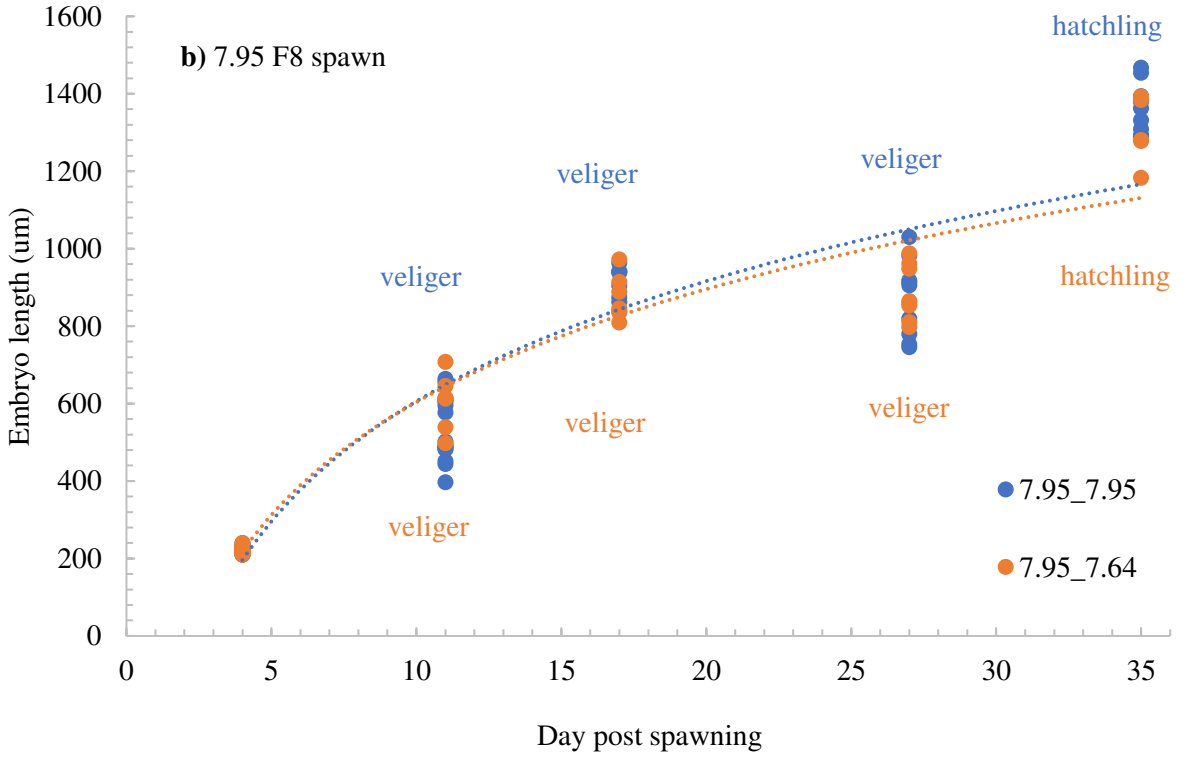


Figure 4.3.3.2.2 Relationship between developmental stage length (µm), developmental stage (trochophore, veliger, pediveliger, hatchling) of banded-dye murex, *Hexaplex trunculus*, and the day post-spawning for half of the spawn from **a)** female 6 & **b)** female 8, that was transplanted to pH_T 7.64 (orange) and that remained in pH_T 7.95 (blue).

The spawns from females F4, F8 and F10 in pH_T 7.64 were selected for cross-transplantation to pH_T 7.95. Larvae from female F8 with arrested development at the veliger stage and were excluded from the further analysis. In general, there was a significant difference in the larval length during development between spawns that remained in pH_T 7.64 and that were transplanted to 7.95 (LMM, $F(1,289) = 9.381$, $p = .002$, Figure 4.3.3.2.3).

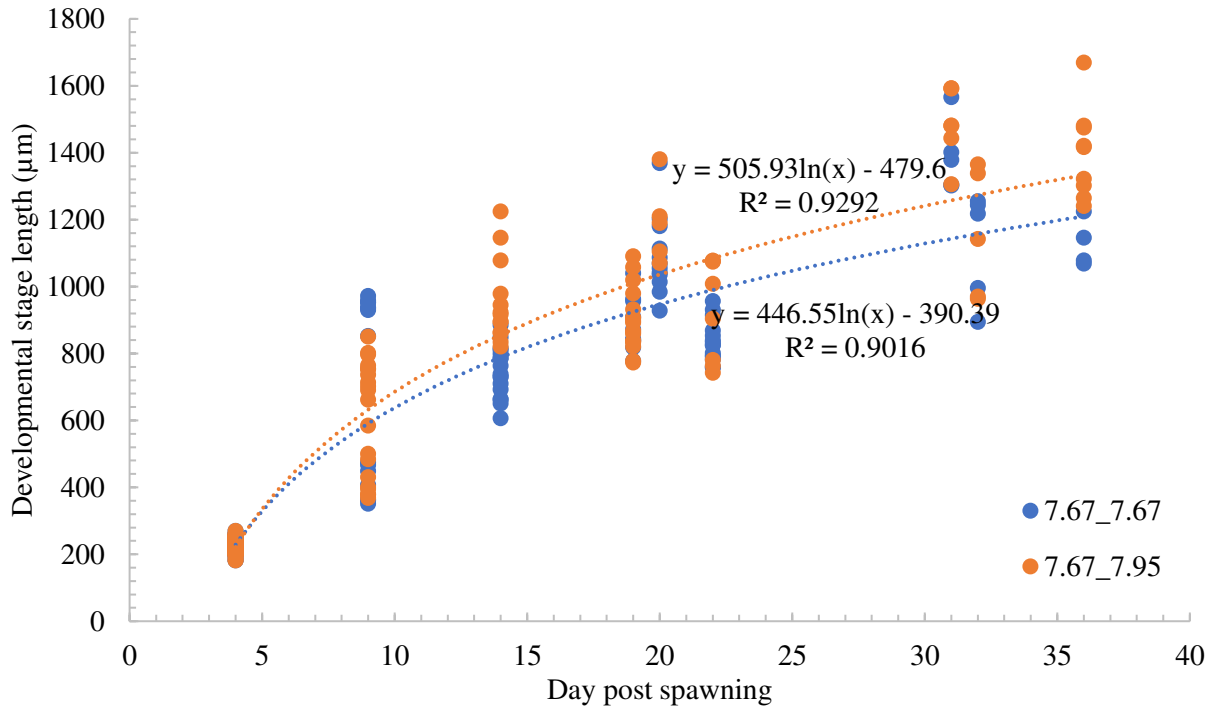


Figure 4.3.3.2.3 Growth rate (length, µm) of developmental stages of banded-dye murex, *Hexaplex trunculus*, spawned and developed in pH_T 7.64 (blue circle) and transplanted from pH_T 7.64 to 7.95 (orange circle).

Transplants in both pH_T 7.64 and 7.95 from females F4 and F10 reached the pediveliger and hatchling stage, respectively (Figure 4.3.3.2.4 a, b). A significant difference in the larval length during the intracapsular development was observed only for the spawn from female 10, with larvae in pH_T 7.95 having a higher growth than in 7.64 (ANCOVA, F (1, 167) = 18.251, $p = .001$; MD = 54.196, SE = 12.686, p = .001). A summary of the statistical analysis is presented in Table 4.3.3.2.1.

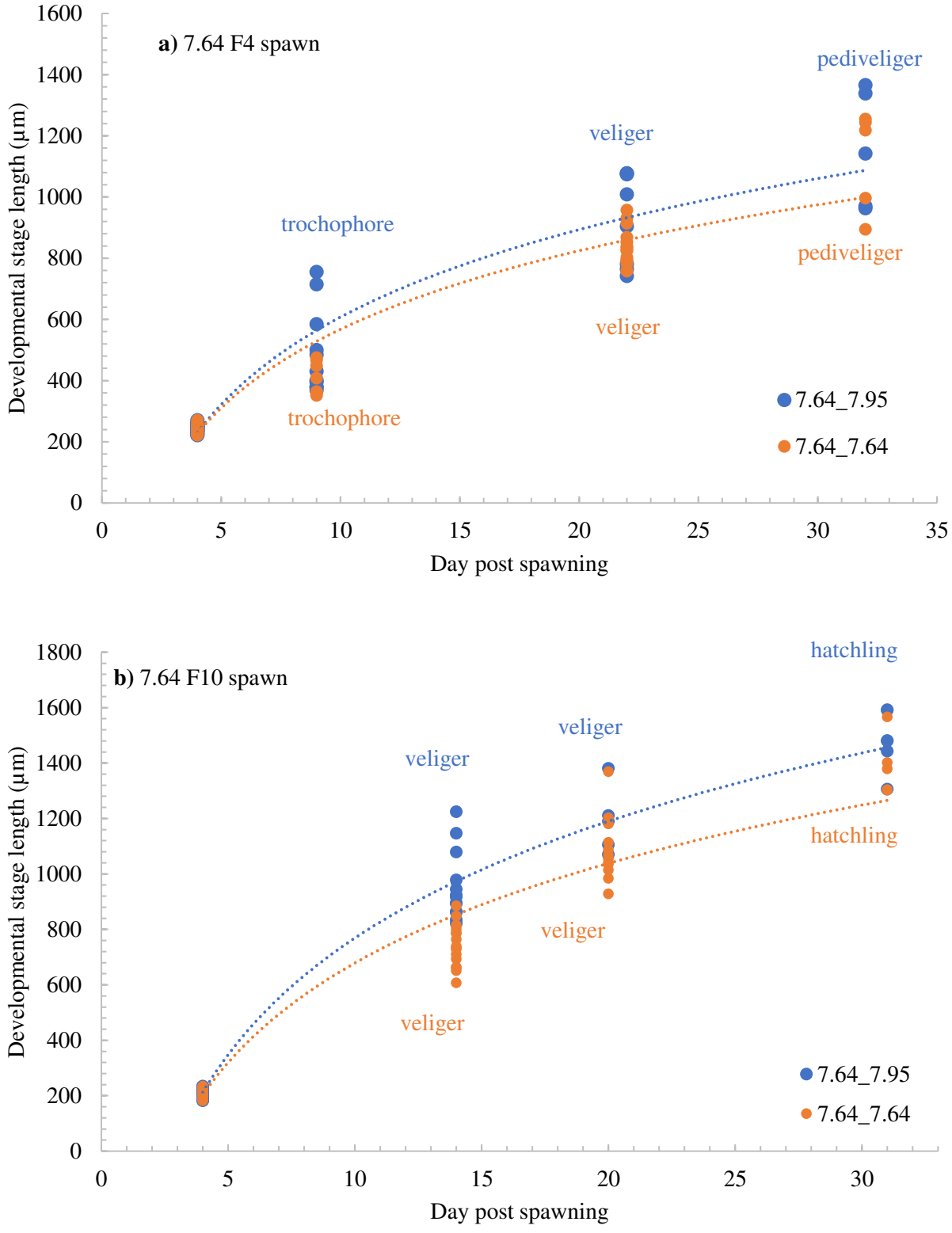


Figure 4.3.3.2.4 Relations among the developmental stage length (µm)of banded-dye murex, *Hexaplex trunculus*, developmental stage (trochophore, veliger, pediveliger, hatchling) of banded-dye murex, *Hexaplex trunculus*, and day post-spawning for half of the spawn from **a)** female 4 & **b)** female 10, that was transplanted to pH_T 7.95 (blue) and that remained in pH_T 7.64 (orange).

Table 4.3.3.2.1 Summary of statistical analysis for the developmental stages growth of banded-dye murex, *H. trunculus* (length, µm) with day post-spawning (DPS) between transplants in pH_T 7.95 ⇔ 7.64. (SS – Sum of squares, df – degrees of freedom, MS – mean square, F – test statistics, p – probability value). Significant effects are in bold.

	Sum of Squares (Type III)	df	Mean Square	F	p
7.95 →7.64; F6					
ANCOVA	13993495.9	2	6996747.92	605.51	**0.001**
DPS	13981735.79	1	13981735.7	1210.01	**0.001**
pH_T	29.999	1	29.999	0.003	0.959
Corrected total	15148998.58	102			
7.95 →7.64; F8					
ANCOVA	19549049.1	2	9774524.54	843.634	**0.001**
DPS	19363482.71	1	19363482.7	1671.25	**0.001**
pH_T	277.398	1	277.398	0.24	0.877
Corrected total	21159533.44	141			
7.64 → 7.95, F4					
ANCOVA	11436897.2	2	5718448.58	677.938	**0.001**
DPS	11428402.624	1	11428402.6	1354.86	**0.001**
pH_T	22316.475	1	22316.475	2.646	0.106
Corrected total	12432234.39	120			
7.64 → 7.95, F10					
ANCOVA	28512490.6	2	14256245.3	2128.00	**0.001**
DPS	28510947.53	1	28510947.5	4255.77	**0.001**
pH_T	122272.31	1	122272.531	18.251	**0.001**
Corrected total	286617883.28	167			

The relative effect size for transplants in pH_T 7.95 ⇔ 7.64 was calculated as previously described for each replicate. Effect size was very small (between -0.05 & 0.09) and there was no observable relationship between the initial growth rate and the growth rate of the transplanted spawns (Figure 4.3.3.2.5).

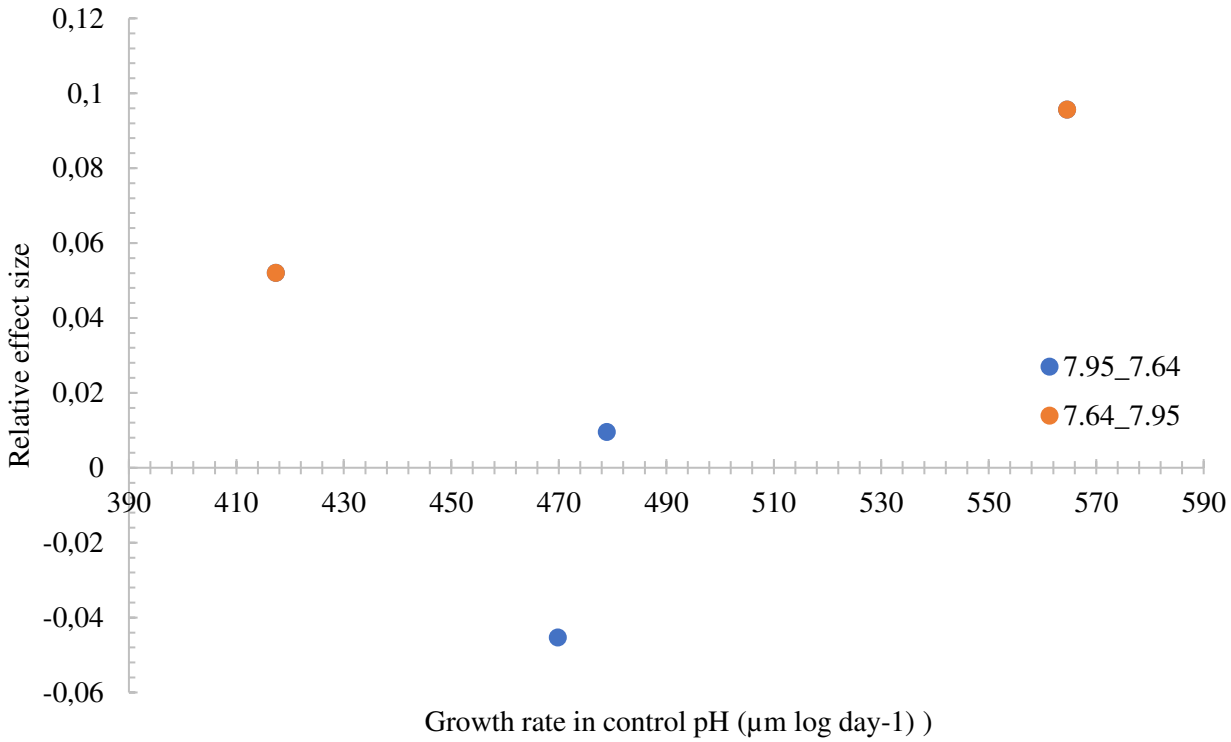


Figure 4.3.3.2.5 Relationship between the initial growth rate in control pH_T (µm log day-1) and relative effect size for transplants of banded-dye murex, *Hexaplex trunculus*, between 7.95 ⇔ 7.64 (blue dots – initial pH_T 7.95, transplanted to 7.64; orange dots – initial pHT 7.64, transplanted to 7.22).

5. DISCUSSION

5.1 Effect of long-term exposure to different pHs on the performance of *H. trunculus*

All physiological processes in heterotrophic organisms, such as reproduction, growth, activity, maintaining homeostasis etc., require energy (Clements & Darrow, 2018). When coping with environmental stressors such as ocean acidification, organisms shift energy between these physiological and behavioral functions, depending on the species (Pan et al., 2015). Although many coastal species are already adapted to variable environmental factors, the ongoing increase in variability of global drivers could increase energetic costs associated with acclimatization or adaptation, and therefore decrease species' fitness (Foo & Byrne, 2016). To evaluate a species response to environmental stressors, a broader perspective that accounts for variability in various physiological responses should be taken.

Feeding performance is certainly one of the most important aspects to consider, as it directly influences the energy reserves needed for life-maintaining functions and indirectly impacts ecosystem function through possible shifts in community structure. A recent review revealed that calcifying marine invertebrates can respond differently in their feeding behavior under low pH (Clements and Darrow, 2018). In this study, the amount of food consumed by banded-dye murex varied throughout the experiment, but mainly as a function of temperature. Lower consumption rates were observed during the cold winter months when metabolic activity is known to be naturally suppressed (Seibel, 2007). A significant increase in mussel consumption was observed during weeks 36-38 of the experiment, corresponding to both the increase in seawater temperature above 17 °C and a period prior to spawning (2-3 weeks). Females of *H. trunculus* are known to stop feeding during the spawning season (Vasconcelos et al., 2004), so presumably higher pre-spawning energy requirements resulted in higher consumption rates in all pH treatments. Although ocean acidification has been shown to negatively affect the feeding behavior of some species (e.g. Vargas et al., 2013; Houlbrèque et al.,2015; Vargas et al., 2015), or the feeding rates to increase as compensation for extra energy costs (Towle et al., 2015), the consumption rates of banded-dye murex were not affected by pH. Similarly, no change in the feeding rate was observed in another gastropod species *Austrocochlea constricta* (Lamarck, 1822) (Leung et al., 2017). Although short- and medium-term exposure to ocean acidification, in general, affects the feeding of many calcifying invertebrates, over long-term exposure (> 100 days) they show the ability to acclimate with no significant effect on the feeding rate itself (Clements & Darrows, 2018). Maintaining the same feeding rate does not necessarily imply acclimation in other

life traits, as higher energy needs for e.g. maintaining the acid-base regulation, could lead to less energy being available for reproduction or calcification.

To assess the feeding habits of a species, not only feeding rates but also other behaviors related to feeding should be studied, such as the ability to detect the food or the movement speed. After being exposed to a range of pH for 60 weeks, banded-dye murexes from three representative pH treatments (pH_T 7.95, 7.64 and 7.22) reached their food with equal success, although individuals from a pH of 7.22 took significantly less time to reach their food. This may indicate that individuals from the low pH treatments were more affected by starvation and had higher hunger levels than those from the high pH treatments, although there was no difference in the amount of food consumed during the experiment. Chatzinikolaou et al. (2019) observed the negative effects of low pH (pH_{NBS} 7.6) on successful foraging by the *H. trunculus* population inhabiting the Gournes coast in the northern Crete in a long-term study that lasted 2.5 years. Temperature was controlled and kept constant at 20 °C, while in this study temperature followed natural fluctuations, so the data are not directly comparable, considering that in this study it was demonstrated that *H. trunculus* foraging is directly affected by temperature throughout the year. For another muricid also, *R. clavigera*, no difference in consumption rate was observed, but its time for prey search increased at low pH (Xu et al., 2017; Li et al., 2020), with no effect on the movement speed (Li et al., 2020). In fact, banded-dye murex in this study did not need to search for food because it was directly in front of them. A longer exposure time probably resulted in higher energy expenditure and consequently higher hunger levels for gastropods in low pH, so their activity increased when food was detected.

Seasonal variations in the shell growth rate, soft body weight, and calcification are well-known in molluscan species, with the temperature being an important factor contributing to annual variation (Ekaratne & Crisp, 1984; Takada, 1995). Within the temperature tolerance range of a species, metabolic and activity rates generally increase with increasing temperature (Ekaratne & Crisp, 1984). In this experiment, the growth rate of the shell length, total and soft body weight, and net calcification was highest at the beginning and end of the experiment when the temperature was above 20 °C, regardless of pH, and decreased significantly in winter (temperature below 15 °C), indicating a lower metabolic rate, which is also supported by a lower food consumption rate over this period. In addition, for banded-dye murex, winter is a time of gonadal development (Vasconcelos et al., 2008), which is known to impair or even temporarily arrest growth in marine invertebrates (Ekaratne & Crisp, 1984; Takada 1995).

Long-term exposure to low pH had no significant effect on the average shell growth rate of banded-dye murex, although an effect was observed at the time of acute exposure and energy-limiting periods. During the first period of the experiment (temperature above 20 °C), the shell growth rates were positively affected by low pH. An increase in metabolism under low pH is well documented, such as in sea urchin *Strongylocentrotus purpuratus* (Stimpson, 1857) where metabolic rates were increased under elevated pCO_2 (Stumpp et al., 2011) or, as demonstrated in sea urchin *S. droebachiensis,* where respiration rates increased with decreasing pH (Dorey et al., 2013), and may translate into increasing growth rates under non-limiting energetic conditions. In this experiment, the organisms were fed *ad libitum* and were then likely able to compensate for the additional energetic costs associated with exposure to low pH (e.g., regulation of acid-base balance) and increase their shell growth rate along with an increase in metabolism. In winter, when the temperature decreased below 20 °C, a negative growth rate was observed for all treatments, with a significant negative effect of low pH. The combination of lower metabolic rates in general during winter, increased energetic costs for gametogenesis in banded-dye murex (Vascocenlos et al., 2008), and the observed lower food intake may explain both the negative growth in banded-dye murex rates and the inability to compensate for the additional energetic costs associated with the exposure to low pH. During the third observation period (temperature above 20 °C), positive shell growth rates were observed with no effect of pH. Despite similar temperature conditions, the effect of pH on the growth rate was strikingly different between the first and last experimental periods. This may be a result of acclimation over a longer period of time (Form & Riebesell, 2012; Lee et al., 2020; Maboloc & Chan, 2021). The time required to acclimate to a new environment can be species-specific. For example, the adult Antarctic sea urchin *Sterechinus neumayeri* (Meissner, 1900) required six to eight months to acclimate to the combined stressors of altered pH and temperature (Suckling et al., 2015), but in the green sea urchin *S. droebachiensis*, 16 months passed before full acclimation was observed (Dupont et al., 2013). Similarly, in a 10-week study with the snail *Austrocochlea constricta* (Lamarck, 1822), there was no effect of pH on somatic growth, but it did have a positive effect on shell length, although with a decreased inner shell density (Leung et al., 2017).

Furthermore, the growth in shell length is directly related to the calcification process. Net calcification is generally reduced by ocean acidification, which is due to a reduction in gross calcification or an increase in gross dissolution that may occur on the outer or inner surface of the shell, or a combination of both (Hurd et al., 2020). Low pH had a negative effect on the net calcification rate of banded-dye murex for the experimental period. During the period of colder

winter temperatures, a negative net calcification rate was observed at pH 7.22, presumably due to a combination of higher dissolution rates and a lower ability to form calcified structures. Among the other traits examined, the net calcification rate was the only one negatively affected by pH throughout the whole period of exposure, suggesting that the gastropods did not have enough energy to sustain this energy-consuming process. Interestingly, the observed negative effect on net calcification did not translate to the shell length growth rate after prolonged exposure when no difference was observed. Similarly, no difference in the average shell length was observed between *H. trunculus* individuals collected at the CO_2 vent site, although the outer shell showed visible signs of pitting and erosion and the inner shell had decreased toughness (Duquette et al., 2017). This may be explained by phenotypic plasticity in the shell morphology in response to low pH. Shell alterations in response to marine pollution have been previously reported for *H. trunculus*, when exposure to pollutants resulted in remodeling of the shell layer, but did not affect shell thickness (Lahbib et al., 2022). In addition, it has previously been observed that low pH can negatively affect only one component of growth. For example, in the dominant reef-building coral *Porites* sp., skeletal density was significantly reduced, but linear growth was not affected (Mollica et al., 2018). Another gastropod species, *Nucella ostrina* (A. Gould, 1852), was able to maintain both calcification and shell growth at low pH, with the shell weakened only due to dissolution (Barclay et al., 2020), once again demonstrating the different responses of the same process. Newly produced shells under low pH could be thinner and more fragile, without affecting the shell length (Byrne and Fitzer, 2019), which may also be the case for *H. trunculus* in this study. While the building and maintenance of calcified structures depend on the balance between calcification and dissolution (Byrne & Fitzer, 2019), it is also important to note that it is not possible to distinguish gross calcification and dissolution with the buoyant weight technique used in this study. Nonetheless, the observed data suggest that the shell of banded-dye murex is strongly affected by a decrease in pH. Since one of the most important functions of mollusk shells is protection from predators, compromising shell integrity and structure may have consequences for future ecosystem functioning (Kroeker et al., 2014; Duquette et al., 2017). *H. trunculus* is one of the most important predators in benthic communities, and as such, greater vulnerability to predation can have cascading effects on the diversity of habitat for this species.

The total weight of gastropods is composed of both soft body and shell weight. In this study, only data on the total weight are available for the acute exposure period (59 days), showing no effect of pH, which suggests that the banded-dye murex somatic can cope with acute exposure to low pH in terms of somatic growth and calcification. As shown in previous studies, for example

on cold-water coral *Lophelia pertusa* (Form & Riebsell, 2012), commercial bivalve species *Chamelea gallina* (Sordo et al., 2021) or gastropods *Nassarius conoidalis* (Zhang et al., 2015) and *O. erinaceus* (Mardones et al., 2022), acute exposure cannot provide a definite answer on how the species will respond to future ocean acidification in the long term. For the remainder of the observation period in this experiment (59-310 days), pH had a negative effect on the growth rate of the total weight of *H. trunculus*. To understand what impacts the change in the total weight, both changes in somatic growth and calcification should be analyzed. While low pH had a negative effect on the net calcification rate throughout the experiment, changes in the soft tissue weight rate were not affected until the last observation period when a pronounced negative effect was observed. In terms of the significant negative effect of pH on the total weight, this means that the effect for days 59–236 was primarily due to changes in the shell weight rather than soft tissue weight. Interestingly, in the last observation period, which corresponds to the period prior and after spawning, gastropods from lower pH treatments (7.43–7.22) had a significantly lower soft tissue growth rate indicating that, after a prolonged period of exposure, they are unable to maintain their somatic growth despite having the same feeding rates as in the higher pH treatments. There was also no effect of pH on somatic growth in a 10-week study with the snail *A. constricta* (Leung et al., 2017), nor in the mussel *M. galloprovincialis* after 84 days of exposure (Range et al., 2012). The results of this study highlight the importance of the duration of exposure to detect effects over time. Individuals exposed to lower pH presumably used all of their energy reserves for reproduction, and after spawning was completed, they were unable to invest energy in somatic growth.

The unbalanced ratio of male to female individuals of *H. trunculus* randomly sampled for this study was 1:1.08, which is consistent with previously reported data on population structure, with females predominating over males (Gharsallah et al., 2010; Elhasni et al., 2010). Over the winter period, a significant increase in somatic growth and net calcification rate was observed for females in all treatments, while in the last observation period females had higher growth rate of shell length, irrespective of pH. Female gastropods of many species generally have higher growth rates than males (Cob et al., 2009; Stella & Raghunathan, 2009; de los Rios et al., 2020), including *H. trunculus* (Elhasni et al., 2010), but intra-annual growth differences between sexes have not received as much attention. The maturation of banded-dye murex gonads occurs in the winter period, and females' gonads are in general more voluminous and have more weight than males' (Vasconcelos et al., 2008). Moreover, the gametogenic cycle of banded-dye murex is slightly asynchronous – males release gametes two to three months prior to spawning. Their reproductive

strategy allows females to store spermatozoids in a special structure, *receptaculum seminis*, and fertilize oocytes when the sea temperature is favorable (Vasconcelos et al, 2008; Lahbib et al., 2010). It is very likely that these two events occurred during the second observation period of this study, which is explains females' higher soft tissue growth over the winter. After the completion of spawning, there was again no effect of sex on the somatic weight, confirming the previous conclusion. Furthermore, as females stop feeding during the spawning period, there is a possibility they consume more food than males during the winter period in order to store energy reserves for the upcoming reproductive activity. The increased consumption may have also resulted in more available energy for the calcification process, resulting in a higher net calcification rate of females over the winter period, which converted into a higher shell length growth rate afterward. In general, gastropods can accumulate lipid and glycogen reserves when food is abundant, as in this study where they had access to an unlimited amount of food, and use them for energy-consuming processes or during times of food scarcity (Dimitriadis & Hondros, 1992). Although both males and females are capable of storing and using these energy reserves, differences in lipid content between the sexes were demonstrated during the year. For example, the males of the muricid *Nucella lapillus* (Linnaeus, 1758) had stable lipid reserves with no significant variation across seasons, while females had periods of extensive accumulation and depletion of lipid droplets (Gonçalves & Lobo-da-Cunha, 2013). Females may store more energy reserves at a certain time of the year, for example during periods when no energy-consuming processes occur, and use them when energetic requirements are higher (e.g. spawning, ovarian maturation), which may also explain the variation in the response of males and females in this study.

Although there is a lack of studies in the field of ocean acidification that account for sex in the experimental design (Ellis et al., 2017), there are indications that sex can modulate the response of a species, particularly when it comes to reproduction, gonadal tissue, and gamete quality (Ellis et al., 2014; Schram et al., 2014; Uthicke et al., 2014). Interestingly, in this study, sex does not appear to have a significant effect on the shell length growth rate, net calcification rate, or somatic growth of banded-dye murex associated with pH treatments. This can possibly be explained by the species' traits studied. For example, in the Antarctic limpet *Nacella concinna* (Strebel, 1908), females exhibited a relatively greater increase in the lipid content in the ovaries compared to the testes after six weeks of exposure to high temperature and low pH, but no differences were observed in the wet body weight or shell weight (Schram et al., 2014). Similarly, the sea urchin *P. lividus* was studied for a variety of physiological, biochemical, and immunological responses, and was also found to have a significant sex-specific response that was highly dependent on the process

tested (Marčeta et al., 2020). In addition, sex differences in oxidative status were examined in a top shell *Trochus histrio* Reeve, 1842, using lipid peroxidation, heat shock protein response and antioxidant enzyme activity under combined exposure to elevated temperature and low pH. Males and females of that species use different physiological strategies to cope with oxidative stress, with females apparently being more successful (Grilo et al., 2018). It appears that the initial difference in sex-specific response to low pH occurs mostly at the cellular and molecular level, which may translate, for example, into reduced growth or impaired reproduction after a prolonged period of exposure (Grilo et al., 2018). Therefore, how males and females of the banded-dye murex will cope with future ocean acidification remains an important question to be considered by including an analysis of the stress response at the biochemical levels.

The respiration rate, a common indicator of the metabolic rate in marine organisms (Killen et al., 2021), can be influenced by increased CO_2 concentration that results in lower pH of seawater. This change may demand additional energy for maintaining acid-base balance, diverting resources otherwise provided for processes such as calcification or reproduction (Marčeta et al., 2020, Melzner et al., 2009; Gazeau et al., 2013). Some species can increase their metabolic rates to compensate for the energy expended on restoring the acid-base balance. For example, in the experiment which lasted 135 days, a gastropod from the Muricidae *O. erinaceus* family increased oxygen consumption at low pH during the first 40 days, but after 95 days, the respiration rate was no longer affected by low pH (Mardones et al., 2022). On the contrary, in the Australian gastropod *Diloma concameratum* (W. Wood, 1828), an elevated respiration rate persisted after eight weeks of exposure to low pH, suggesting a potentially longer acclimation period (Leung et al., 2020). Species-specific differences in the time required to restore the acid-base balance have also been observed in other taxonomic groups such as echinoderms. Studied sea urchin species mostly increased their respiration rates during acute exposure to low pH, with acclimation occurring after a variable period, ranging from 40 to 140 days (Stumpp et al., 2011; Moulin et al., 2014; Taylor et al., 2014; Uthicke et al., 2014, Marčeta et al., 2022). In some studies, a negative effect of pH on the respiration rate was observed during short-term exposure (Navarro et al., 2013; Pimentel et al. 2014; Leung et al., 2017), suggesting a reduced metabolic rate – a known adaptive mechanism to conserve energy during an acute exposure to stress (Pörtner et al., 2004; Calosi et al., 2013; Gazeau et al., 2013). The initial decrease in the respiration rate of *H. trunculus* in this study during the first 149 days of the experiment suggests reduced metabolic rates to restore the acid-base balance. This adaptive mechanism can have a negative effect on the overall fitness of the species in the long term due to the reallocation of energy (Calosi et al., 2013), but *H. trunculus* showed no difference

in the respiration rate between pHs from day 181 until the last measurement on day 240, indicating acclimation in terms of internal acid-base homeostasis. The literature suggests that species from variable environments may be more tolerant to acute exposure to low pH (Kurihara et al., 2020) although they may already exhibit energetic trade-off in the physiological processes, making them more at risk over prolonged exposure (Lagos et al., 2016; Osores et al., 2017).

The population of banded-dye murex used in this study originates from the small enclosed Bistrina Bay, where temperature and salinity are known to fluctuate widely (Pećarević et al., 2020). Therefore, a degree of adaptation to low pH is expected, as observed on the regulation of the metabolic rate after longer exposure to low pH. However, trade-offs have been observed in terms of the previously discussed inhibited somatic growth and reduced net calcification rate.

5.2 Effect of long-term exposure to different pHs on the reproduction and intracapsular development of *H. trunculus*

The reproductive cycle of *H. trunculus* is well documented to be strongly dependent on temperature, as evidenced by the spawning periods of different populations such as in Tunisia (early April, 22 °C), Portugal (late April, 20 °C) or Turkey (May, 19 °C) (Vasconcelos et al., 2004, Lahbib et al., 2009a, Gùler & Lök, 2014). In this study, banded-dye murex began spawning ten days after the temperatures reached 20 °C, coinciding with the observations in the natural population in Bistrina Bay (personal observation). The spawning lasted about three days, a duration reported in previous studies (Vasconcelos et al., 2004; Lahbib et al., 2010). Although the spawned capsules were slightly smaller than in other populations studied, the size of the capsules has previously been reported to vary, presumably due to different environmental factors (Lahbib et al., 2010). *H. trunculus* population from northern Tunisia had a smaller average capsule size (Lahbib et al., 2009a, b; Lahbib et al., 2010) than populations from the Aegean Sea (Gùler & Lök, 2014) and Portugal (Vasconcelos et al., 2004). The average number and size of eggs in this study is consistent with previous findings (Lahbib et al., 2009a, b; Lahbib et al., 2010), and so are the observed sizes of each developmental stage from fertilized egg to hatchling (Lahbib et al., 2010; Gùler & Lök, 2014). No observable effect of pH on these reproductive traits was noted. A successful development of gonads and spawning under ocean acidification does not necessarily indicate that there is no effect of pH on the reproductive traits. Assessing the reproductive performance through a variety of mechanisms is critical to understanding the potential impact of future ocean acidification on a species' fitness. There are a variety of coping mechanisms that a species may employ to deal with low pH, impacting various stages of the reproductive process. Some species may even fail to fully invest in reproduction (Kimura et al., 2011), while others may

produce larger eggs but in lower quantities (Glass et al., 2023). There are examples of successful gametogenesis, but insufficient energy reserves for successful fertilization or hatching (Kimura et al., 2011) which may also be the case in this study where pH had no effect on the fertilization or spawning of *H. trunculus*, or on the larval size until a later stage of intracapsular development. In pH_T 7.95 to 7.51, veliger larvae continued to develop into pediveliger larvae. However, at lower pH (pH_T 7.42 – 7.22) the number of spawns that reached pediveliger decreased significantly. Delay in development was observed with live veligers being sampled until the 32nd day post-spawning with no further change in size, and fewer than 25% of spawns managed to develop to the hatchling stage. Similarly, longer developmental time and reduced viability of encapsulated embryos exposed to low pH were observed for intertidal snail *Littorina obtusata* (Ellis et al., 2009), while the barnacle *A. improvisus* successfully developed gonads but failed to produce viable embryos and larvae after eight months of exposure to low pH (Panch et al., 2018).

Early life stages are generally considered more sensitive to ocean acidification than later stages, likely due to higher energy requirements to maintain homeostasis (Stumpp et al., 2012; Bergman et al., 2018; Kriefall et al., 2018; Lee et al., 2020). However, parental exposure to stressful conditions may influence offspring resilience or sensitivity, but the impact is still largely unknown due to differences in responses among taxa (Chirgwin et al., 2018; Lee et al., 2020). For example, while elevated pCO_2 had a negative effect on the oyster larvae *S. glomerata*, preconditioning the parent generation to elevated pCO_2 resulted in a positive carryover effect on larvae in the form of larger and faster-developing larvae, even though the larvae of both parent generations had similar survival rates (Parker et al., 2010). The phenotypic characteristics of another oyster larva*, C. hongkongensis*, also improved following parental exposure to low pH. (Lim et al., 2021). In contrast, parental exposure to low pH had no effect on the sea anemone *N. vectensis* offspring's performance, but it did affect the parental gamete production and physiology (Glass et al., 2023). In this study, the intracapsular development of *H. trunculus* larvae preconditioned to ambient pH_T was negatively affected by low pH_T 7.22, as evidenced by a reduced ability to reach the hatching stage, unlike half of the spawn that remained in the ambient. Exposure of the parents to pH_T 7.22 had even more pronounced negative effects on the larvae, which mostly developed to the veliger stage. Negative carryover effects of the parental exposure to low pH have also been reported in previous studies, such as reduced larval survival in the sea urchin *P. lividus* (Marčeta et al., 2022), reduced larval size and impaired development in the sea star *Asterias rubens* Linnaeus, 1758 (Hu et al., 2018), and negative effects on overall fitness in the North Atlantic bivalves *Mercenaria mercenaria* (Linnaeus, 1758) and *A. irradians* (Griffith &

Gobler, 2017). These differences in carryover effects depend primarily on the mechanisms employed by the parent generation to cope with ocean acidification. Negative carryover effects observed in this study could be due to a reduced energy transfer between the parent generation and their offspring, as observed in common sea star *A. rubens* where parental pre-acclimation to low pH negatively affected the larval size and development (Hu et al., 2018). When organisms are exposed to low pH, their energy requirements to maintain homeostasis increase, which could result in less energy being provided for gametogenesis (Pörtner & Farrell, 2008). While this did not affect the reproductive performance or egg size of *H. trunculus* in this study, it could have affected the egg's nutritional quality (Allen et al., 2008). For example, in two abalone populations, a relationship between larval performance and maternal provisioning was demonstrated, with the maternal provisioning of lipids to offspring varying across populations and a positive correlation between lipid concentrations and survival at low pH (Swezey et al., 2020). *H. trunculus* larvae from only one spawn exposed to parental pH_T 7.22 managed to hatch in the same pH_T and did not show significant differences in length compared to the larvae hatched in the control group, indicating variability in response within the population. In addition, the larvae from the spawn preconditioned to pH_T 7.22 and transferred to ambient pH demonstrated that a negative effect could be reversed by reaching either the pediveliger or the hatching stage. This suggests that removal from stressful conditions leaves larvae with more available energy for the development of larval structures, likely because less energy is expended on maintaining the acid-base balance (Byrne, 2011). In contrast to the ability to reach a particular developmental stage, the effect on average length between the spawns was much more variable, showing either positive or negative effects or no effect at all for each pH transplant. The differential responses of spawns indicate a possible interplay between parental exposure and offspring sensitivity, as well as a stronger influence of parental exposure on certain individuals. Intraspecific variation in response to ocean acidification has already been reported for several species (Kurman et al., 2017; Sekizawa et al., 2017; Kurihara et al., 2018), highlighting the importance of considering variability when assessing a species' ability to persist. For example, both intra- and inter-specific variation in calcification and photosynthetic efficiency was observed in two branching corals, *Montipora digitata* (Dana, 1846) and *Porites cylindrica* Dana, 1846 (Sekizawa et al., 2017). Within-population variability in this study was supported by the relative effect size, which showed that the better the larval performance was in pH_T 7.95, the more it worsened when transferred to pH_T 7.22, while larvae with poorer performance in pH_T 7.22 thrived better when transplanted to pH_T 7.95. Smith et al. (2019) observed similar changes, but in the fertilization success rate of two sea urchins, *Lytechinus pictus* (Verrill, 1867) and *Heliocidaris erythrogramma* (Valenciennes, 1846), under ambient and

future pH conditions. In their study, the individuals with high fertilization success under the current pH conditions had a significantly lower success at low pH, while the individuals with low fertilization success actually improved their performance. If there is sufficient genetic variability within the population of a given species, adaptive responses to ocean acidification could be aided by profiling genotypes that are more resilient than others (Kurman et al., 2017).

The effect of parental exposure may depend on the specific pH conditions. In this study, the larvae preconditioned to ambient pH_T 7.95 showed no difference in their growth rate and ability to reach the developmental stage (pediveligers and hatchlings) when transferred to 7.64. The lowest reported pH_T in Bistrina Bay, the natural habitat of the studied population, was pH_T 7.73, so the banded-dye murex is likely able to cope with 0.1 units lower pH in the short term. The larvae of the parents preconditioned to pH_T 7.64 showed different responses, with the larvae of one spawn again demonstrating no difference whether they developed in 7.64 or were transferred to the ambient. In contrast, the larvae of the other spawn grew significantly faster at pH_T 7.95 (even faster than the larvae of the parents that were conditioned to pH 7.95), although the transplanted spawns hatched at both pHs. Despite the overall trend, this significant difference in the growth rate of one spawn, suggests that individual sensitivity may override the potential mitigating effects of parental exposure. Small effect sizes suggest that the effect of the parental exposure on offspring sensitivity is subtle in the pH_T 7.64 transplants. The subtlety of the effect could be due to the moderate pH change, and larger effect sizes in certain cases (such as the female 10 spawn) highlight the importance of individual variation. This suggests that a pH of 7.64 is not detrimental to the banded-dye murex, and that it already has coping mechanisms to deal with it, although variability within populations in terms of growth is present. *H. trunculus* populations from the Adriatic Sea have already been shown to be significantly genetically and epigenetically differentiated (Šrut et al., 2023), suggesting that the observed differences could appear due to different genotypes or, – more likely – explained by variation in phenotypic plasticity traits expressed through epigenetic mechanisms. However, the possible presence of tolerant genotypes suggests that *H. trunculus* has a potential for adaptation (Foo et al., 2012), although a larger sample size as well as the study of subsequent life stages is needed to draw more precise conclusions.

6. CONCLUSIONS

This thesis contributes to a better understanding of the response of marine gastropods, specifically *H. trunculus*, to future ocean acidification. Furthermore, it emphasizes the importance of simultaneously studying various biological processes of a species in a long-term experiment and underscores the complexity of an organism's adaptive strategies under low pH. It has demonstrated that *H. trunculus* employs a diversity of trade-off mechanisms to cope with stress under low pH. While some physiological and morphological features remained unaffected, others varied in relation to other factors such as temperature, metabolic activity and the longevity of exposure. Including natural pH variability and a broader range of pH conditions enhances our ability to assess a species' possible resilience for a given habitat. This study also contributes to the present knowledge gap of intraspecific variation in response, highlighting the importance of including individual variability and parental influence on specific individuals when evaluating species sensitivity.

The potential resilience of *H. trunculus* as a generalist predator to low pH raises concerns about its impact on community structure and ecosystem functioning. Since it is a key player in the predator-prey dynamic, any shifts in the behavior and physiology of banded-dye murex could have cascading effects on the diversity and abundance of its prey. In addition, it could possibly pose a risk for bivalve aquaculture in this area, specifically for the *O. edulis* and *M. galloprovincialis*, suggesting the implementation of precautionary measures, such as the adaptation of farming structures to mitigate the risk of *H. trunculus* predation. However, the impact of low pH on the physiology of other species in Mali Ston Bay still remains to be studied as well as the predator-prey interactions involving banded-dye murex, to understand the broader implications of the projected future low pH levels and designing effective mitigation strategies.

The conducted research resulted in the following conclusions:

- the feeding rates of *H. trunculus* are not affected by low pH
- the exposure to low pH does not affect *H. trunculus* ability to reach food, although individuals from low pH took less time to reach their food. Further research is needed to understand the specific mechanisms responsible for the faster movement in the low pH treatment
- An acute exposure to low pH has a positive effect on the shell growth rate, presumably as a result of increased metabolism

- over the energy-limiting periods (winter period), the shell growth rate of *H. trunculus* is negatively impacted by low pH
- *H. trunculus* can acclimate in terms of the shell growth rate after a prolonged period of exposure to low pH, although with a possible negative effect on the inner shell structure
- the net calcification rate of *H. trunculus* was negatively impacted by low pH for the whole duration of the experiment
- *H. trunculus* was able to maintain the total body weight rate only over the period of acute exposure to low pH; for the remaining period of the experiment, the total body weight decreased with low pH
- the soft tissue body weight rate of *H. trunculus* is not affected by low pH until the completion of spawning when a pronounced negative effect of low pH is observed, indicating the lack of energy for somatic growth after investing in reproductive processes
- the males and females of *H. trunculus* show no difference in their response to low pH in terms of their shell length growth rate, total weight growth rate, soft tissue weight growth rate and net calcification rate
- the females of *H. trunculus* exhibit reduced metabolic rates as a response to low pH for the first 145 days, but no difference in the oxygen consumption rate was noted between pHs for the remaining period, which indicates a restoration of the acid-base balance
- pH has no effect on the timing and duration of spawning, the number of the spawned females nor the size and quantity of the spawned capsules
- pH has no effect on the embryo length nor on the time needed to reach each developmental stage during *H. trunculus* intracapsular development
- intracapsular development was impaired after the veliger stage in pH_T 7.51–7.22 as demonstrated by a delayed development (spending more time in the veliger stage and arresting the overall development and survival afterward) and fewer larvae reached the pediveliger and hatchling stage
- an acute exposure of spawns to pH_T 7.22 has a negative effect on *H. trunculus* intracapsular development
- parental exposure of *H. trunculus* to pH_T 7.22 generally has a negative effect on intracapsular development, but individual variability in response was also observed
- negative effects can be reversed to some extent, demonstrated with a better growth of embryos from the parents preconditioned to pH_T 7.22 when transferred to the pH_T 7.95

- an acute exposure of spawns to pH_T 7.64 has no effect on *H. trunculus* intracapsular development
- generally, parental exposure of *H. trunculus* to pH_T 7.64 has no negative effect on the intracapsular development, but subtle differences between spawns highlight the importance of individual variation

7. LITERATURE

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8. PROŠIRENI SAŽETAK

UVOD

U posljednjih 250 godina koncentracija ugljikovog dioksida (CO_2) u atmosferi povećala se za 50% u odnosu na predindustrijsko razdoblje – od 280 ppm (eng. *parts per million*) do 417 ppm u 2022. godini što se prvenstveno smatra posljedicom izgaranja fosilnih goriva (Friedlingstein i sur., 2022).Osim globalnog zagrijavanja, povećana koncentracija CO_2 uzrokuje i velike promjene u karbonatnoj kemiji morske vode (Doney i sur., 2009) jer oceani i mora apsorbiraju oko 27% ugljikovog dioksida iz atmosfere (Hurd i sur., 2018). Povećanje parcijalnog tlaka ugljikovog dioksida (pCO_2) u moru uzrokuje niz kemijskih reakcija – istovremene promjene koncentracije vodikovih iona (H^+), otopljenog ugljikovog dioksida (CO_2), bikarbonatnih (HCO_3^-) i karbonatnih (CO_3^{2-}) iona (Hurd i sur., 2018). Posljedica ovih promjena je zakiseljavanje ili acidifikacija mora. Ovisno o kolebanju antropogenih emisija CO_2 u budućnosti, parcijalni tlak CO_2 morske vode otvorenog mora koji trenutno iznosi 40.53 Pa, do 2100. godine bi mogao dosegnuti vrijednosti od 60.80 do 101.33 Pa (Caldeira & Wickett, 2005), što znači da će se pH vrijednost spustiti od 0,08 do 0,37 jedinice na pH ljestvici u razdoblju od 2081. do 2100. godine u odnosu na razdoblje od 2006. do 2015. godine (Cooley i sur., 2022).

Kada se ugljikov dioksid iz atmosfere otopi u morskoj vodi, reagira s vodom i nastaje ugljična kiselina koja je nestabilna i brzo disocira na vodikov i bikarbonatni ion, te sljedećom reakcijom bikarbonatni ion disocira na karbonatni:

$$CO_{2(atm)} + H_2O \rightleftarrows H_2CO_3 \rightleftarrows H^+ + HCO_3^- \rightleftarrows 2H^+ + CO_3^{2-}.$$

Reakcije u morskoj vodi su povratne i blizu ravnotežnog stanja. Pri salinitetu 35 i pH vrijednosti 8,1 koja je uobičajena u površinskom sloju morske vode, 90% anorganskog ugljika čine bikarbonatni ioni, 9% karbonatni ioni, a svega 1% je otopljeni ugljikov dioksid. Relativni udjeli ovih vrsta anorganskog ugljika reguliraju pH morske vode tijekom kraćih i dužih vremenskih perioda. Dodatnim apsorbiranjem CO_2 iz atmosfere povećava se koncentracija otopljenog CO_2, bikarbonata i iona vodika, a ioni vodika snižavaju pH ($pH = -\log_{10}[H^+]$) (Orr i sur., 2005, Doney i sur., 2009). Tijekom dužih vremenskih razdoblja, sposobnost apsorbiranja atmosferskog CO_2 mora i oceana ovisi i o stupnju otapanja kalcijevog karbonata ($CaCO_3$) u stupcu vode ili sedimentu:

$$CaCO_3 + CO_2 + H_2O \rightleftarrows Ca^{2+} + 2HCO_3^-.$$

Kalcijev karbonat u moru potječe iz ljuštura i skeleta morskih organizama. U pelagijalu karbonati tonu kroz stupac vode i pri tom se ili otope ili nakupljaju u plitkim i dubokomorskim sedimentima. Kalcijev karbonat, koji se u moru nalazi uglavnom u dva polimorfna oblika – aragonita i kalcita, otapa se u morskoj vodi reakcijom s ionima vodika i tvori ione kalcija i bikarbonata. Formiranje kalcijevog karbonata i stopa otapanja mijenjaju se ovisno o zasićenju (Ω) koje je definirano kao ionski produkt koncentracije kalcijevih i karbonatnih iona:

$$\Omega = [Ca^{2+}]\ [CO_3^{2-}]/\ K'sp,$$

pri čemu je K'sp – produkt konstante topivosti koji ovisi o temperaturi, salinitetu, tlaku i određenoj mineralnoj fazi; aragonit je 50% topiviji od kalcita (Feely i sur., 2004).

Formiranje ljuštura i skeleta organizama nastupa u području u kojem je $\Omega > 1{,}0$, a njihovo otapanje počinje pri $\Omega < 1{,}0$, osim u slučajevima u kojima su ljušture i skeleti zaštićeni, primjerice organskim pokrovom. Zasićenje kalcijevim karbonatom najveće je u plitkim, toplim tropskim područjima, a najmanje na hladnijim, višim geografskim širinama i na većim dubinama. Dakle, topljivost kalcijeva karbonata raste sa smanjenjem temperature i povećanjem tlaka (Feely i sur., 2004).

Acidifikacija mora osobito dolazi do izražaja u obalnim područjima zbog nižeg puferskog kapaciteta mora na tom području i prirodnih procesa kojima voda bogata ugljikovim dioksidom dospijeva iz srednjeg na površinski sloj mora (Osborne i sur., 2020). Od izrazite je važnosti razumjeti kako će promjene u karbonatnoj kemiji mora utjecati na ključne vrste i na cijeli ekosustav. Širok raspon utjecaja uzrokovanih promijenjenom karbonatnom kemijom opažen je kod organizama na svim trofičkim razinama. Određivanje utjecaja acidifikacije mora na biocenoze i ekosustave dodatno je otežano zbog interakcija s drugim lokalnim i globalnim promjenama, od eutrofikacije do povećanja površinske temperature mora, a svaka od tih promjena ima širok raspon utjecaja na život u moru (Hurd i sur., 2018).

8.1.1 Svrha i ciljevi istraživanja

Radi mogućnosti uspoređivanja eksperimentalnih podataka o utjecaju acidifikacije mora na organizme, Riebesell i suradnici (2011) predložili su postavljanje eksperimenta tako da se obuhvate tri scenarija acidifikacije mora, odnosno vrijednosti $p(CO_2)$ – danas, 2050. i 2100. godine u skladu s procjenama Međuvladinog panela za klimatske promjene (IPCC – *International Panel for Climate Change)* o očekivanim promjenama koje će nastupiti do kraja stoljeća. Međutim, navedene projekcije odnose se na otvoreno more, a većina do sad proučavanih organizama

nastanjuje obalna staništa poput područja plime i oseke, pješčanih i kamenitih staništa, koraljnih grebena, estuarija ili fjordova u kojima uvjeti okoliša znatno kolebaju. Zbog promjena okoliša u kojemu žive, može se očekivati adaptacija lokalnih populacija, što može imati značajnu ulogu pri osjetljivosti vrste na promjene pH mora (Vargas i sur., 2017). Dosadašnja istraživanja bioloških i ekoloških značajki organizama pokazala su da organizmi reagiraju na različite načine na snižavanje pH, ovisno o tipu eksperimenta i vremenu aklimatizacije organizma na eksperimentalne uvjete. Da bi se odredio učinak acidifikacije na pojedine organizme, potrebno je znati varijaciju vrijednosti pH u staništu istraživane populacije te na temelju tih podataka postaviti eksperiment (Dupont i sur., 2013).

Rezultati dosadašnjih kratkotrajnih istraživanja koja su bila usmjerena na jedan životni stadij neke vrste ne uzimaju u obzir selekciju, aklimatizaciju i transgeneracijski učinak između uzastopnih životnih stadija (Dupont i sur., 2013). Za očekivati je da vrsta koja se može aklimatizirati i/ili adaptirati, može i opstati u uvjetima acidifikacije. Aklimatizacija podrazumijeva fenotipske fiziološke, morfološke ili ekološke odgovore koji pomažu održati kondiciju vrste u novom, promijenjenom okolišu, dok adaptacija podrazumijeva selekciju na genetskoj razini koja pomiče prosječni fenotip prema boljoj kondiciji vrste (Kroeker i sur., 2017). Potencijal za adaptaciju ovisi o veličini populacije i generacijskom vremenu, te se najveće stope adaptacije očekuju kod vrsta koje imaju velike populacije i kratko generacijsko vrijeme (Dupont i sur., 2013). Aklimatizacija može ublažiti odgovor populacije na trenutne učinke acidifikacije pa jedinke imaju više vremena za adaptaciju, što može biti osobito važno za organizme s dugim generacijskim vremenom. S druge strane, aklimatizacija može iziskivati energetski trošak koji će preusmjeriti energiju potrebnu za druge procese npr. razvoj gonada (Sunday i sur., 2014). Prema Hurdu i suradnicima (2018) te Sabai i suradnicima (2019) buduća istraživanja utjecaja acidifikacije na organizme u moru trebala bi biti dugotrajna, a eksperimenti bi trebali obuhvatiti više od jedne generacije. Osim toga, nužno je eksperimentiranje s većim brojem različitih pH vrijednosti te ispitivanje sposobnosti adaptacije organizama na acidifikaciju aklimatizacijom i fenotipskim promjenama te konačno genetskom adaptacijom (Kroeker i sur., 2017).

Iako nijedan pojedinačni eksperiment ne može predvidjeti što će se dogoditi s budućim generacijama neke vrste, akumulacija empirijskih dokaza od iznimne je važnosti za donošenje općenitijih procjena odgovora pojedinih vrsta (Baumann, 2019). Jedna od rupa u znanju je unutrašnja varijabilnost osjetljivosti vrste ili populacije neke vrste te važnost varijabilnosti za adaptivni potencijal pojedinih morskih organizama (Baumann, 2019). Iako su u nekim eksperimentima populacije iste vrste koje nastanjuju stabilno stanište pokazale veću toleranciju na

acidifikaciju od populacija koje nastanjuju varijabilno stanište (Osores i sur., 2017; Kurihara i sur., 2020), brojna istraživanja ukazuju da te populacije mogu biti pod većim rizikom jer dugoročno izlaganje acidifikaciji može prouzročiti zakidanje energije za druge životne procese kao što je smanjenje kalcifikacije (Lagos i sur., 2016; Osores i sur., 2017).

Sukladno takvim preporukama, glavni cilj ovog istraživanja je određivanje utjecaja raspona pH vrijednosti na biološko-ekološke značajke predatorske vrste puža *Hexaplex trunculus* (Linnaeus 1758), u kontekstu očekivane acidifikacije. Naglasak ovog rada je na populaciji kvrgavog volka koja nastanjuje ekonomski i ekološki važno područje Malostonskog zaljeva na jugoistoku Jadranskog mora. U istraživanje dugotrajnog utjecaja acidifikacije mora na kvrgavog volka uključena su i prirodna kolebanja pH vrijednosti u Malostonskom zaljevu i vrijednosti predviđene za blisku budućnost.

Ova disertacija predstavlja prvo istraživanje utjecaja acidifikacije mora na populaciju neke vrste iz istočne obale Jadranskog mora. Osim toga, ovo je prvo istraživanje intrakapsularnog razvoja vrste iz razreda Gastropoda, koje uključuje dugotrajnu izloženost roditeljske generacije rasponu uvjeta niskog pH tijekom gametogeneze i mriješćenja. Rezultati ovog rada doprinijet će razumijevanju odgovora organizama s karbonatnim vanjskim skeletom na potencijalnu acidifikaciju mora. Konkretno, analizom odgovora vrste *H. trunculus*, jedne od ključnih vrsta bentoskih zajednica u istraživanom području, moguće je bolje razumjeti i potencijalno ublažiti učinke acidifikacije u istraživanom području.

Ciljevi ovog doktorskog istraživanja su:

- utvrditi utječe li acidifikacija mora na stopu prehrane kvrgavog volka *H. trunculus*
- laboratorijskim eksperimentom procijeniti sposobnost pronalaska hrane nakon dugotrajne izloženosti kvrgavog volka *H. trunculus* uvjetima sniženog pH
- utvrditi utječe li snižena vrijednost pH na stopu rasta kućice kvrgavog volka *H. trunculus*
- odrediti i usporediti neto stopu kalcifikacije kućice kvrgavog volka *H. trunculus* pri uvjetima sniženog pH
- odrediti stopu rasta mekog tkiva i ukupne mase kvrgavog volka *H. trunculus* pri uvjetima sniženog pH
- utvrditi postoji li razlika između mužjaka i ženki kvrgavog volka *H. trunculus* u stopi rasta kućice, mase mekog tkiva, ukupne mase i netokalcifikacije pri uvjetima sniženog pH
- mjerenjem respiracije odnosno potrošnje kisika u jedinici vremena, odrediti metaboličku aktivnost ženki kvrgavog volka *H. trunculus* pri uvjetima sniženog pH

- utvrditi utječe li acidifikacija mora na broj ženki kvrgavog volka *H. trunculus* koje će se izmrijestiti, te na broj i veličinu izmriješćenih kapsula
- utvrditi postoje li razlike u intrakapsularnom razvoju pri uvjetima sniženog pH određivanjem embrionalnih i ličinačkih stadija kvrgavog volka *H. trunculus*
- utvrditi postoji li učinak izlaganja roditeljske generacije sniženom pH na intrakapsularni razvoj embrija i ličinki kvragov volka *H. trunculus*

MATERIJALI I METODE

Područje istraživanja Malostonski zaljev nalazi se na istočnoj obali Jadranskog mora, između poluotoka Pelješca i kopna. Područje je zaštićeno na nacionalnoj razini kao Posebni rezervat u moru (Narodne novine 124/13), te je također područje ekološke mreže Europske unije Natura 2000 (https://www.bioportal.hr/gis/). Malostonski zaljev je od antičkih vremena poznat po uzgoju školjkaša (Benović, 1997). Zaljev je dug 21 km, maksimalne širine 2,2 km, a dubina koleba između 7 i 28 metara. Dno je većinom pjeskovito – muljevito, s kamenitom obalom (Benović i sur., 2004). Temperatura mora varira između 12 i 29 °C, a salinitet između 25 i 38 zbog pritoka slatke vode iz rijeke Neretve te mnogobrojnih podzemnih izvora (Pećarević i sur., 2020). Jedini dostupni podaci o pH morske vode datiraju iz 1970. godine, te navode da je prosječni pH_{NBS} 8,12 (minimum 7,98, maksimum 8,27) (Stjepčević i sur., 1981).

Prikupljanje uzoraka i aklimatizacija za uvjete u laboratoriju Odrasle spolno zrele jedinke puževa prikupljene su u kolovozu 2020. godine, u uvali Bistrina koja je dio Malostonskog zaljeva (42°52'19.1 "N 17°42'02.3 "E) pomoću plastičnih zamki napunjenih mediteranskim dagnjama, *Mytilus galloprovincialis* Lamarck 1819. Zamke su postavljene na dubinu od pet metara i pričvršćene konopom za obalu. Nakon 24 sata prikupljeno je ukupno 360 puževa. Digitalnim pomičnim mjerilom izmjerena im je duljina kućice,. Puževi su preneseni u laboratorij i pažljivo očišćeni od obraštajnih organizama. Nasumično je odabrano 40 jedinki za svaku eksperimentalnu pH vrijednost. Za analizu mriješćenja i embrionalnog razvoja odabrano je po deset ženki za svaku eksperimentalnu pH vrijednost. Kod kvrgavog volka nema spolnog dimorfizma, te su puževi anestetizirani u otopini magnezijevog klorida heksahidrata ($MgCl_2$ x 6 H_2O) da bi se mogao odrediti spol (Gibbs, 1999). Laganim povlačenjem operkuluma određen je spol ženki po odsustvu penisa i prisutnosti vaginalnog otvora iza desnog očnog tentakula. Svi puževi su označeni s brojčanim oznakama za pčele koje su zalijepljene za kućicu poliakrilatnim ljepilom (Henry & Jarne, 2007). Prije početka eksperimenta, puževi su ostavljeni dva tjedna u protočnom sustavu s

filtriranom, ambijentalnom morskom vodom kako bi se prilagodili na laboratorijske uvjete. Hranjeni su *ad libitum* mediteranskom dagnjom.

Postavljanje eksperimenta Eksperiment je proveden u Laboratoriju za marikulturu Sveučilišta u Dubrovniku na Bistrini. U zatvorenom prostoru postavljen je protočni sustav od devet spremnika volumena 130 litara kroz koje protječe filtrirana, UV-sterilizirana i prozračena morska voda. Za eksperiment je odabrano devet različitih uvjeta pH, u rasponu od pH_T 7,95 do 7,22. Eksperimentalni uvjeti nisu replicirani. Nasumično je izabrano po 40 jedinki za svaki spremnik. Ciljanim pH vrijednostima morske vode upravljalo se upuhivanjem ugljikovog dioksida u svaki spremnik. Na svaki spremnik bio je spojen regulator pH i elektromagnetska sklopka spojena cijevima na bocu s ugljikovim dioksidom. Sonda za mjerenje pH spojena je na regulator kojim se kontinuirano mjerio pH, a elektromagnetska sklopka otvarala je dotok CO_2 kada se zadana pH vrijednost povisi i zatvara kada se snizi. Za vrijeme trajanja eksperimenta mjerene su vrijednosti saliniteta, temperature, koncentracije otopljenog kisika, pH na ukupnoj ljestvici i ukupnog alkaliniteta morske vode, te se pH vrijednost koju su bilježili regulatori provjeravala stolnim laboratorijskim pH-metrom i po potrebi se korigirala.

Određivanje parametara karbonatne kemije morske vode Standardnim operativnim postupcima za istraživanja acidifikacije mora određivali su se pH na ukupnoj ljestvici (pH_T) i ukupni alkalinitet (TA, µmol kg^{-1}) (Dickson i sur., 2007). Uz mjerenje navedenih parametara uz poznate vrijednosti saliniteta i temperature, te korištenje odgovarajućih konstanti disocijacije, uz pomoć računalnog programa CO2SYS izračunati su parametri koji opisuju karbonatnu kemiju morske vode-pCO_2, Ω_{Ca} i Ω_{Ar} (Lewis & Wallace, 1998).

Određivanje pH na ukupnoj ljestvici, pH_T Postoje četiri ljestvice koje se obično koriste pri mjerenju pH morske vode. pH_{NBS} (eng. *National Bureau of Standards*), poznata i kao IUPAC-ova ljestvica (eng. *International Union of Pure and Applied Chemistry*) iskazuje aktivnost iona vodika, dok pH_{sw} (morska voda), pH_F (slobodni ioni vodika) i pH_T (ukupni ioni vodika) ljestvice iskazuju koncentracije iona vodika, ali se razlikuju po iskazivanju različitih disociranih protona (Dickson, 1984). Za mjerenje pH morske vode najprikladnija je pH_T ljestvica jer se u morskoj vodi nalaze visoke koncentracije iona sulfata. pH_T uključuje koncentraciju slobodnih iona vodika i protona koji disocira od hidrogen sulfata (HSO_4^-), stoga se za mjerenje pH morske vode preporuča kalibriranje elektroda standardiziranim puferima napravljenim u umjetnom moru poput 2-amino-2-metil-1,3-propandiola (TRIS) (Dickson, 1993b; Millero i sur., 1993). Tijekom ovog istraživanja pH_T je određivan prema standardnom protokolu za potenciometrijsko određivanje pH morske vode

na ukupnoj skali (Dickson i sur., 2007). Za kalibraciju je korišten TRIS pufer napravljen u sintetičkom moru kako bi ionska snaga uzorka i pufera bile slične. TRIS pufer napravljen je u laboratoriju prema metodologiji za volumetrijsku pripremu TRIS pufera (Paulsen & Dickson, 2020). Za potvrdu točnosti izračuna pH_T vrijednosti nakon kalibracije sonde s puferom napravljenim u laboratoriju, napravljena je usporedba izračuna nakon kalibracije s TRIS puferom nabavljenim iz Scripps laboratorija (Serija T37). Izračunata pH_T vrijednost nakon kalibracije puferom napravljenim u laboratoriju iznosila je u prosjeku 0,0217 ± 0,0001 više od izračuna nakon kalibracije TRIS T37 puferom. Tijekom provedbe eksperimenta, pH_T je mjeren svakih dva dana. Nakon kalibracije sonde, uzimani su uzorci morske vode iz svakog spremnika te su mjereni elektrodni potencijal, temperatura i salinitet te je pomoću dobivenih podataka izračunat pH_T. Dva puta mjesečno mjeren je pH_T mora u uvali Bistrina, pri čemu je morska voda za izračun uzorkovana ispred usisa pumpe vode za eksperimentalni sustav.

Ukupni alkalinitet morske vode Ukupni alkalinitet morske vode određivan je potenciometrijskom dvostupanjskom titracijom uzorka 0,1 M klorovodičnom kiselinom napravljenom u 0,6 M otopini natrijevog klorida. Titrant i uzorak dovedeni su na istu temperaturu uranjanjem u vodenu kupelj. Izvagana masa uzorka morske vode titrirana je inicijalnim volumenom 0,1 M klorovodične kiseline dovoljnim da snizi pH uzorka malo iznad 3,5, nakon čega se uzorak ostavio na magnetnoj miješalici oko šest minuta da bi izreagirali ugljikov dioksid izašao iz uzorka. Titracija se nastavila sve do pH 3,0 uzorka, pri čemu se nakon svake dodane kapi titranta zabilježio elektrodni potencijal i temperatura. Postupak titracije pratio se pH sondom, a ukupni alkalinitet izračunat je nelinearnom metodom najmanjih kvadrata iz volumena titranta i izmjerenog elektrodnog potencijala pri svakoj točki titracije.

Učinak dugotrajne izloženosti rasponu pH na kvrgavog volka, *Hexaplex trunculus*

Prehrana Volci u svakom pH tretmanu hranjeni su 40 tjedana *ad libitum* mediteranskom dagnjom, *M. galloprovincialis* (duljina ljušture 67,1 0 ± 0,83 mm). Dagnje iz spremnika zamjenjivane su svježima jednom tjedno. Za svaki tretman zabilježen je broj praznih ljuštura, te podijeljen s brojem jedinki u spremniku. Stopa prehrane izračunata je kao prosječan broj dagnji konzumiranih tjedno po jedinci (dagnja tjedan^{-1}). Nakon 60 tjedana izloženosti puževa rasponu različitih pH vrijednosti, izabrane su tri vrijednosti pH za testiranje postotka jedinki koje će uspješno pronaći hranu i vrijeme koje im je potrebno za pronalazak hrane. Plastične posude (52x35x20 cm) napunjene su morskom vodom iz spremnika s eksperimentalnim jedinkama. Četiri dagnje su otvorene i postavljene na jednu strane posude. Četiri nasumično odabrane jedinke postavljene su u posudu na suprotnu

stranu. Vrijeme potrebno da jedinke pronađu hranu mjerilo se najdulje sat vremena, te je bilježen broj jedinki koji su uspješno pronašle hranu. Postupak je ponovljen tri puta s tri nove, nasumično izabrane jedinke za svaki pH tretman.

Stopa rasta duljine kućice Duljina kućice mjerena je kao maksimalna duljina duž centralne osi, od vrha kućice do kraja sifonalnog kanala (Vasconcelos i sur. , 2016). Svaki puž je mjeren devet puta tijekom trajanja eksperimenta digitalnim pomičnim mjerilom, preciznošću 0,01 mm. Stopa rasta duljine kućice za svakog puža (SGR, mm dan^{-1}) izračunata je iz promjena duljine kućice nakon uzastopnih mjerenja.

Neto stopa kalcifikacije Neto kalcifikacija definirana je kao bruto kalcifikacija kućice umanjena za otapanje kalcificiranih struktura (Smith & Key, 1975) i predstavlja neto promjenu kalcijevog karbonata u kućicama i skeletnim strukturama morskih organizama (Findlay i sur., 2009). Za određivanje neto stope kalcifikacije korištena je neinvazivna metoda mjerenja mase u uzgonu (eng. *buoyant weight*) (Fitzer i sur., 2019). Metoda se temelji na razlici specifične mase kućice i mase mekog tkiva (Palmer, 1982). Da bi se izračunala točna masa kućice, potrebno je napraviti regresijsku analizu suhe mase kućice i mase uronjene jedinke. Za tu svrhu prikupljeni su puževi reprezentativne veličine u uvali Bistrina, te je mjerena njihova masa na zraku i to masa kućice i masa mekog tkiva te masa jedinki uronjenih u morsku vodu. Nakon mjerenja, odvojeno je meko tkivo od kućice, a kućica je sušena do konstantne mase na 80 °C. Koeficijenti regresijske analize suhe mase kućice i mase uronjene jedinke korišteni su za korekciju pri određivanju stvarne mase kućice nakon vaganja uronjenih jedinki. Za vaganje je korištena laboratorijska analitička vaga s donjom kukom Mettler toledo JL602-G/L na koju je pričvršćen držač uronjen u posudu s morskom vodom. Nakon tariranja vage, jedinka je postavljena na držač i očitana masa (0,01 g). Mjereni su svi puževi iz pH tretmana. Postupak je ponovljen sedam puta tijekom trajanja eksperimenta. Neto stopa kalcifikacije za svaku jedinku (CR, g dan^{-1}) izračunata je iz promjene mase kućice nakon uzastopnih mjerenja.

Stopa rasta ukupne mase Ukupna masa svake jedinke mjerena je pomoću laboratorijske vage Mettler toledo JL602-G/L, ukupno devet puta tijekom trajanja eksperimenta. Prije mjerenja mase puževi su ostavljani na staničevini 20 minuta da bi se uklonio višak vode. Stopa rasta ukupne mase za svaku jedinku (TWGR, g dan^{-1}) izračunata je iz promjene ukupne mase nakon uzastopnih mjerenja.

Stopa rasta mase mekog tkiva Ukupna masa puževa sastoji se od mase kućice i mase mekog tkiva. S dobivenim vrijednostima ukupne mase puža i mase kućice, izračunata je masa mekog

tkiva. Stopa rasta mase mekog tkiva (STWGR, mm dan^{-1}) izračunata je iz razlike stope rasta ukupne mase i stope rasta mase kućice nakon uzastopnih mjerenja.

Određivanje spola Nakon završetka eksperimenta prikupljene su jedinke iz svih tankova i zamrznute do daljnje obrade u laboratoriju. Da bi se odredio spol jedinki, kućice su slomljene ručnom stegom kako bi se pristupilo mekom tkivu puževa. Mužjaci su određeni prisutnošću penisa iza desnog očnog tentakula, a ženke prema vaginalnom otvoru. Ukupno je identificirano 162 mužjaka i 183 ženki, što čini odnos spolova 1:1,08. Kako bi se testiralo utječe li spol na stopu rasta duljine kućice, neto stopu kalcifikacije, stopu rasta ukupne mase i mase mekog tkiva, te da bi se testiralo postoji li razlika u utjecaju spola na mjerene značajke u različitim pH uvjetima, zabilježen je spol svake obilježene jedinke.

Metabolička stopa ženki kvrgavog volka Za određivanje metaboličke stope ženki, mjerena je potrošnja kisika u jedinici vremena ukupno pet puta tijekom eksperimenta. Pojedinačne jedinke stavljane su u hermetički zatvorene posude napunjene morskom vodom zasićenom zrakom iz određenog pH tretmana. Pozadinska potrošnja kisika (kontrolne posude) mjerena je u tri prazne posude, te je srednja vrijednost korištena za korekciju izračuna potrošnje kisika puževa. Koncentracija otopljenog kisika mjerena je na početku i kraju inkubacije, maksimalno 2 sata, pomoću mikrosonde za kisik UMS Oxyscan 300 Lab. Vrijednosti potrošnje kisika standardizirane su prema ukupnoj masi puževa (TW, g). Ukupna masa puževa mjerena je nakon mjerenja potrošnje kisika.

Reprodukcija i intrakapsularni razvoj Očekivano vrijeme mriješćenja kvrgavog volka je krajem travnja (Vasconcelos i sur., 2004), odnosno mriješćenje započinje s povišenjem temperature mora. Ženke kvrgavog volka se okupljaju na jedno mjesto za vrijeme mriješćenja i pričvršćuju kapsule jednu na drugu pa su ženke iz tankova odvajane da bi se osigurali individualni mrijestovi za analizu. Ženke su razdvojene u plastične posude volumena pet litara početkom svibnja 2021. godine. Da bi se omogućio protok vode, na nasuprotnim stranama posude napravljen je otvor koji je prekriven mrežicom. Na vrh posude je postavljena mrežica da bi se spriječili prebjezi ženki. Ženke su hranjene mediteranskom dagnjom sve do početka mriješćenja. Za svaku ženku zabilježeni su početak i kraj mriješćenja. Nakon završetka mriješćenja ženke su vraćene u tankove s ostalim jedinkama, a mrijest je ostavljen u posudi. Zabilježen je broj izmriještenih ženki u svakom pH. Odmah nakon mriješćenja pažljivo je odstranjeno deset nasumično odabranih kapsula svakog mrijesta, te je digitalnim pomičnim mjerilom izmjerena duljina (cl, mm), širina (cw, mm) i debljina (ct, mm) kapsula (D'Asaro, 1970, 1986). Pet kapsula je pažljivo otvoreno

skalpelom, a jaja iz kapsule su ispražnjena na predmetno stakalce i izbrojana pod lupom (Olympus SZ40). Metoda praćenja intrakapsularnog razvoja prilagođena je prijašnjim istraživanjima embrionalnog razvoja kvrgavog volka (Vascocnelos i sur., 2004, Lahbib i sur., 2010., Gűller i Lok, 2014). Četiri dana nakon mriješćenja odvajane su najmanje po dvije kapsule sa svakog mrijesta. Oplođena jaja su stavljena na predmetno stakalce i fotografirana digitalnom kamerom na svjetlosnom mikroskopu (Olympus DP72, Olympus BX51). Promjer najmanje 50 jaja iz svake kapsule mjeren je pomoću programa Fiji. Za određivanje stadija intrakapsularnog razvoja uzorkovane su kapsule minimalno četiri puta od mriješćenja do izvaljivanja. Razvojni stadiji određeni su prema prepoznatljivim karakterističnim strukturama: stadij ličinke trohofore određen je prema malim cilijama na anteriornoj strani ličinke (Lahbib i sur., 2010), rani veliger stadij prema kratkom velumu s dva režnja, pojavljivanju očiju i viscelarne mase (Lahbib i sur., 2010, Gűller i Lok 2014). Daljnji razvojni stadij veliger ličinke određen je začetkom formiranja kućice i izražajnijim režnjevima veluma (Vasconcelos i sur., 2004). Razvoj stopala i velikog veluma s četiri režnja pokazatelj je stadija pediveliger ličinke. Pri kraju intrakapsularnog razvoja velum se počinje povlačiti, a kućica poprima žuto-smeđu pigmentaciju. Kada dođe vrijeme za izvaljivanje, ličinke probuše membranu koja se nalazi na otvoru kapsule i ispužu van (Vasconcelos i sur., 2004, Lahbib i sur., 2010).

Transgeneracijski učinak Za određivanje transgeneracijskog učinka izloženosti roditeljske generacije rasponu pH na osjetljivost embrionalnih stadija napravljeni su recipročni transplanti mrijesta između pH tretmana (pH_T 7,95, 7,64 i 7,22). Mrijestovi su odabrani prema veličini (broju kapsula) i dostupnosti u posudi. Recipročno su transplantirana po tri mrijesta iz pH_T 7,95 i 7,64, te po tri mrijesta iz pH_T 7,95 i 7,22. Svaki mrijest je pažljivo odstranjen iz posude i prerezan na pola, te je polovica mrijesta ostavljena u pH_T tretmanu iz kojeg je uzet, a polovica prebačena u previđen pH_T tretman. Minimalno četiri puta tijekom intrakapsularnog razvoja odstranjene su po dvije kapsule sa svake polovice mrijesta i konzervirane u 4% fomaldehidu te spremljene za daljnju analizu u laboratoriju, odnosno za određivanje razvojnih stadija i mjerenje duljine.

Statistička analiza Statistička analiza napravljena je u programu SPSS Statistics v.26. Srednje vrijednosti razlike temperature i saliniteta između pH tretmana testirane su jednostranom analizom varijance (one-way ANOVA). Veza između pH_T i stope hranjenja testirana je jednostavnom linearnom regresijom (SLR), dok je srednja razlika u vremenu potrebnom za pronaći hranu testirana jednostranom analizom varijance (one-way ANOVA). Utjecaj pH na stopu rasta duljine kućice, neto stopu kalcifikacije, stopu rasta ukupne mase i mase mekog tkiva testiran je linearnim miješanim modelom (LMM) s pH i spolom kao fiksnim varijablama, a individualnom oznakom

svakog puža (ID) kao nasumičnom varijablom da bi se uzelo u obzir ponavljajuća mjerenja na svakoj jedinci tijekom vremena. Očekivano je da su vremenski bliža mjerenja u većoj korelaciji nego ona vremenski udaljena, te je stoga primijenjena auto-regresivna struktura kovarijance (AR-1). Testirani su glavni učinci fiksnih faktora (pH i spol) na zavisnu varijablu, a razlika utjecaja spola na zavisnu varijablu u ovisnosti o pH analizirana je uključivanjem pojma interakcije između spola i pH u model (spol*pH). Veza između stope potrošnje kisika i pH_T testirana je jednostavnom linearnom regresijom. Model binarne logističke regresije je primijenjen za određivanje ima li pH značajan utjecaj na vjerojatnost mriješćenja i na vjerojatnost dostizanja određenog razvojnog stadija, pri čemu se koeficijent regresije (β) interpretira kao predviđena promjena u logaritmu omjera za svaku jedinicu povećanja pH. Veza između pH_T i duljine, širine i debljine kapsula, broja izmriještenih ženki i promjera jaja testirana je jednostavnom linearnom regresijom. Srednja vrijednost razlike duljine razvojnih stadija između pH je testirana s jednostranom analizom varijance, a veza između dana nakon mriješćenja u kojem je dosegnut određen razvojni stadij i pH_T testirana je jednostavnom linearnom regresijom. Srednja stopa rasta tijekom unutarkapsularnog razvoja izračunata je iz log-linearne veze između duljine razvojnih stadija i vremena potrebnog za razvoj (μm log dan^{-1}). Stope rasta su ucrtane na graf u odnosu na pH_T da bi se testiralo postoji li veza. Intrakapsularna stopa rasta za svaku polovicu transplantiranog mrijesta također je izračunata iz log-linearne veze između duljine razvojnog stadija i vremena potrebnog za razvoj. Duljina stadija između transplanata uspoređena je jednostranom analizom kovarijance (ANCOVA) log-lineariziranih podataka, s vremenom potrebnim za razvoj kao kovarijantom. Prije analize testirano je zadovoljavaju li podaci uvjete normalnosti ostataka s Q-Q grafom ili Shapiro-Wilk testom, te uvjet jednakosti varijanci Leveneovim testom. Podaci su zadovoljili uvjete. Granica značajnosti postavljena je na $p < 0,05$. Kada je opažen značajan učinak, primijenjen je post-hoc Tukey test između parova s Boferroni korekcijom za višestruke usporedbe. Procijenjene granične srednje vrijednosti dobivene iz modela korištene su da bi se dalje istražio smjer veze između pH_T i zavisne varijable.

REZULTATI

Parametri morske vode Vrijednosti temperature i saliniteta kolebale su tijekom eksperimenta, te je vrijednost temperature iznosila između 8,4 i 26,6 C, a salinitet između 22,6 i 35,3. Vrijednost pH nije imala utjecaja na temperaturu i salinitet, stoga su za daljnju analizu uzeti svi podatci. Koncentracija otopljenog kisika nije padala ispod vrijednosti 6,28 mg L^{-1} O_2. pH_T morske vode u tretmanu u kojem se nije upravljalo s pH kolebao je između 7,75 i 8,05 tijekom eksperimenta, odgovarajući mjerenim vrijednostima pH mora u uvali Bistrina. Morska voda je bila nezasićena

kalcitom samo u pH_T 7,22, dok je nezasićenost aragonitom nastupila već od pH_T 7,42. Vrijednosti ukupnog alkaliniteta kretale su se između 2976 ± 216 i 2851 ± 184 (mmol kg^{-1}). Vrijednost parcijalnog tlaka ugljikovog dioksida kretala se između 692 ± 18 u pH_T tretmanu u kojem se nije upravljalo s pH do 3221 ± 25 u pH_T 7,22.

Prehrana Količina konzumirane hrane varirala je tijekom eksperimenta ovisno o temperaturi morske vode. Manja je stopa konzumacije bila tijekom hladnijeg vremenskog razdoblja, od prosinca do ožujka. Prosječan broj konzumiranih dagnji po jedinki puža tijekom 40 tjedana iznosi 3,5 ± 0,17 dagnji. Zabilježena je povećana konzumacija dagnji između 7. i 21. svibnja. Nije uočena značajna povezanost između pH_T i stope prehrane. Nakon 60 tjedana izloženosti uvjetima niskog pH, zabilježen je značajan utjecaj pH na vrijeme koje potrebno da dođu do hrane – puževima u pH_T 7,22 bilo je potrebno manje vremena da dođu do hrane nego puževima u pH_T 7,95, iako se broj puževa koji su uspješno došli do hrane nije razlikovao, s postotkom uspješnosti 66,7 % u svim tretmanima.

Stopa rasta duljine kućice Stopa rasta duljine kućice imala je sličan trend u svim pH uvjetima tijekom trajanja eksperimenta, s tim da je rast bio viši na početku i na kraju eksperimenta, a u ovisnosti o temperaturi. Rast duljine kućice pozitivan je samo pri temperaturama iznad 20 °C. Analiza stope rasta duljine kućice podijeljena je na tri razdoblja kako bi se uzeo u obzir učinak temperature: a) prvih 59 dana s temperaturom iznad 20 °C i pozitivnom stopom rasta, b) između 59. i 236. dana s temperaturom ispod 20 °C i c) posljednjih 74 dana eksperimenta s temperaturom iznad 20 °C. Prvih 59 dana pH je imao značajan pozitivan utjecaj na stopu rasta duljine kućice. Tijekom razdoblja u kojem je temperatura bila ispod 20 °C, stopa rasta je bila pod negativnim utjecajem pH, te tijekom posljednjeg razdoblja nije zabilježen utjecaj pH na stopu rasta. Tijekom prvog i drugog razdoblja spol nije imao utjecaja na stopu rasta duljine kućice, dok su u zadnjem razdoblju ženke imale značajno veću stopu rasta od mužjaka. Utjecaj spola se nije razlikovao između jedinki izloženih različitim pH vrijednostima.

Neto stopa kalcifikacije Zbog tehničkih poteškoća, neto stopa kalcifikacije mjerena je od 59. dana eksperimenta. Zabilježen je istovjetan trend neto kalcifikacije tijekom trajanja eksperimenta u svim pH uvjetima, s nižim stopama neto kalcifikacije između 172 i 236 dana eksperimenta, kada su temperature bile ispod 15 °C. Analiza neto stope kalcifikacije podijeljena je na tri razdoblja da bi se uzeo u obzir učinak temperature: a) između 59. i 133. dana izloženosti, b) između 133 i 236 dana izloženosti i c) posljednjih 74 dana eksperimenta. Neto stopa kalcifikacije je bila pod negativnim utjecajem pH tijekom sva tri razdoblja. Spol je imao značajan utjecaj na neto stopu

kalcifikacije samo tijekom zimskog razdoblja, bez obzira na pH, te su ženke imale značajno veću neto stopu kalcifikacije nego mužjaci.

Stopa rasta ukupne mase Stopa rasta ukupne mase imala je sličan trend u svim pH uvjetima tijekom trajanja eksperimenta, s višim stopama rasta na početku eksperimenta. Najviše vrijednosti rasta mjerene su u razdoblju kada je temperatura bila 24 °C, a negativne su bile samo za temperature ispod 15 °C, iako ne u svim pH uvjetima. Analiza stope rasta ukupne mase podijeljena je na tri perioda: a) prvih 59 dana akutne izloženosti s prosječnom temperaturom iznad 20 °C, b) između 59 i 133 dana eksperimenta s temperaturama ispod 20 °C, c) između 133 i 236 dana eksperimenta s temperaturom ispod 15 °C, i d) posljednjih 74 dana s temperaturom iznad 20 °C. Tijekom razdoblja akutne izloženosti pH nije imao utjecaja na stopu rasta ukupne mase, dok je tijekom ostala tri razdoblja pH imao negativan utjecaj. Spol je imao utjecaj na stopu rasta ukupne mase samo tijekom zimskog razdoblja, neovisno o pH, pri čemu su ženke imale značajno više stope rasta ukupne mase od mužjaka.

Stopa rasta mase mekog tkiva Zabilježen je sličan trend rasta mase mekog tkiva za sve pH vrijednosti kojima su jedinke bile izložene tijekom trajanja eksperimenta. Stopa rasta mase mekog tkiva i temperature nisu imale linearan trend. Za bolju interpretaciju rasta puževa, analiza mase mekog tkiva podijeljena je u ista tri razdoblja kao i stopa neto kalcifikacije. Tijekom prva dva razdoblja nije zabilježen utjecaj pH na stopu rasta mase mekog tkiva, dok su u zadnjem razdoblju puževi izloženi nižem pH imali značajno manju stopu rasta mase mekog tkiva. Značajan utjecaj spola zabilježen je samo u zimskom razdoblju, neovisno o pH, pri čemu su ženke imali značajno veće stope rasta od mužjaka.

Metabolička stopa ženki Stopa potrošnje kisika imala je sličan trend u svakom pH tijekom pet mjerenja (dan eksperimenta: 88, 149, 181, 209, 240), s manjom stopom respiracije kada su temperature mora bile niže. Tijekom prva dva mjerenja (88. i 149. dan eksperimenta) pH je imao negativan utjecaj na stopu respiracije. Prilikom sljedećih tri mjerenja nije bilo značajne razlike u potrošnji kisika između pH uvjeta.

Reprodukcija i intrakapsularni razvoj Temperatura mora iznad 20 °C izmjerena je 22. svibnja, što je bio okidač za početak mriješćenja kvrgavog volka. Početak mriješćenja označenih ženki bio je 31. svibnja 2021. i obilježen je kao dan 1. Mriješćenje preostalih ženki nastavilo se nakon sljedeća tri dana (dan 3), vrhunac mriješćenja bio je 13-og dana nakon prvog zabilježenog, kada se 9 ženki započelo mrijestiti. Zadnja mriješćenja zabilježena su 16-og dana. Vrijednost pH nije imala utjecaja na dan kada su ženke započele mriješćenje, niti na vjerojatnost mriješćenja ženki.

Prosječno trajanje mriješćenja iznosilo je 2,92 ± 0,87 dana, s maksimalnim trajanjem od 5 dana u pH_T 7,22 i minimalnim od jednog dana u pH_T 7,51. pH nije imao utjecaja na broj izmriješćenih kapsula. Za deset kapsula po mrijestu, ukupno 530, izmjerene su duljina, širina i debljina, bez značajnog utjecaja pH na mjerene značajke kapsula. Srednja duljina kapsula iznosila je 4,76 ± 0,54, širina 4,13 ± 0,53 i debljina 1,53 ± 0,26 mm. Pet kapsula po svakom mrijestu, ukupno 265 kapsula, pažljivo je otvoreno i izbrojan je broj jaja. Nije zabilježena značajna veza između prosječnog broja jaja po kapsuli po mrijestu i pH. Četiri dana nakon mriješćenja uzorkovane su kapsule za mjerenje promjera oplođenih jaja. Uzorkovano je najmanje dvije kapsule po mrijestu te su jaja fotografirana ispod svjetlosnog mikroskopa. Programom Fiji izmjeren je promjer 100 jaja po mrijestu. Nije bilo značajne veze između prosječnog promjera jaja po mrijestu i pH_T. Nakon početnog uzorkovanja svakog mrijesta, sljedeće kapsule su nasumično uzorkovane minimalno četiri puta tijekom trajanja intrakapsularnog razvoja, osim kod mrijesta gdje je razvoj prekinut. Kod nekoliko mrijestova, embriji su se razvili u daljnji razvojni stadij između dva uzorkovanja, stoga nisu svi razvojni stadiji zabilježeni za svaki mrijest, s naglaskom da to ne znači da ti stadiji nisu bili razvijeni. U uzorcima između petog i devetog dana nakon mriješćenja (6,91 ± 1,16) zabilježena je ličinka trohofora u svakom pH_T, osim 7,51 i 7,33. Srednja duljina ličinke iznosila je 331,94 ±29,02 µm. pH nije imao utjecaja na vrijeme kada je dosegnut stadij trohofore, te nije bilo razlike u prosječnoj duljini između pH. Stadij rane veliger ličinke zabilježen je u svim uzorcima između devetog i jedanaestog dana nakon mriješćenja (9,50 ± 1,20), osim pH_T 7,95. Srednja duljina ranih veliger ličinki iznosila je 504,33 ± 92,64 µm. pH nije imao učinka na dan kada je dosegnut stadij te nije bilo razlike u prosječnoj duljini između pH. Stadij veliger ličinke dosegnut je između 9 i 20 dana nakon početka mriješćenja (14,056 ± 2,61) s prosječnom duljinom 825,88 ± 106,92 µm. pH nije imao utjecaja na dan kada je dosegnut stadij veliger ličinke i nije bilo razlike u prosječnoj duljini ličinki između pH. Nakon dosizanja stadija veliger ličinke, zabilježena je razlika u razvoju od pH_T 7,51 i niže. U pH_T 7,95 – 7,67 stadij veliger ličinke trajao je do 22 dana nakon mriješćenja, dok su u nižem pH vijabilne veliger ličinke zabilježene u uzorcima do 32 dana nakon mriješćenja, ali bez razlike u veličini. Stadij pediveliger ličinke dosegnut je 27,23 ± 4,12 dan nakon mriješćenja, s prosječnom srednjom duljinom od 1133,05 ± 83,27 µm, bez razlike između pH. Pediveliger ličinka nije zabilježena u uzorcima iz pH_T 7,42 – 7,22. Izvaljivanje je započelo u prosjeku 31,46 ± 2,66 dana nakon početka mriješćenja, s prosječnom duljinom izvaljenih ličinki od 1412,08 ± 112,85 µm, bez utjecaja pH. Mrijestovi u svakom pH tretmanu dosegli su stadij trohofore, rane veliger i veliger ličinke, osim jednog mrijesta iz pH_T 7,95 u kojem je razvoj prekinut na početku intrakapsularnog razvoja te je isključen iz daljnje analize. Postotak mrijesta koji je dosegnuo svaki razvojni stadij izračunat je od inicijalnog broja mrijestova u

svakom pH tretmanu. Smanjenje broja mrijestova koji su dosegli stadij pediveliger ličinke i stadij izvaljivanja zabilježeno je od pH_T 7,51 do 7,22. Intrakapsularna stopa rasta za svaki pH_T izračunata je iz log-linearne veze između duljine razvojnog stadija i vremena potrebnog za razvoj (μm log day^{-1}). Izračunata prosječna stopa rasta je dovedena u vezu s pH_T te je primijećena značajna pozitivna veza između stope rasta i pH_T.

Transgeneracijski utjecaj. Transplantacija mrijesta između pH_T 7,95 i 7,22. Mrijestovi iz pH_T 7,95 (ženke označene F1, F3 i F6) su unakrsno transplantirani s pH_T 7,22. Intrakapsularni razvoj stao je u stadiju trohofore u mrijestu ženke F6 te je isključen iz daljnje analize. U prosjeku su polovice mrijestova transplantirane u 7,22 imale manju prosječnu duljinu stadija nego polovice mrijestova ostavljene u pH_T 7,95. U oba mrijesta iz pH_T 7,95 nastupilo je izvaljivanje ličinki, dok su transplanti u pH_T 7,22 došli samo do stadija pediveliger ličinke. Negativan utjecaj niskog pH na stopu rasta zabilježen je kod ženke F3, ličinke u pH_T 7,22 imale su manju prosječnu duljinu. Nije zabilježena značajna razlika u prosječnoj duljini između transplanta od ženke F1. Mrijestovi iz pH_T 7,22 (ženke F4, F7 i F9) su unakrsno transplantirani s 7,95. Nije zabilježena značajna razlika u prosječnoj stopi rasta između transplantiranih polovica mrijestova. Iako nije zabilježena razlika u duljini ličinačkih stadija, mrijest ženki F4 i F7 u pH_T 7,22 dosegli su stadij veliger ličinke i nisu se dalje razvijali, dok su njihovi transplanti u pH_T 7,95 dosegli stadij pediveliger ličinke i izvaljivanja. Jedino je mrijest ženke F9 dosegao stadij izvaljivanja u oba pH tretmana. Analiza pojedinačnih mrijestova i njihovih transplanata pokazala je značajnu razliku u duljini ličinki između transplanata samo za mrijest ženke F7, gdje su ličinke u pH_T 7,95 imale veću prosječnu duljinu nego u pH_T 7,22. Zbog opažene varijabilnosti u stopi rasta ličinki iz istog pH, izračunata je veličina učinka u odnosu na kontrolnu vrijednost pH_T 7,95 da bi se odredila magnituda razlike intrakapsularnog razvoja između transplanata. Relativna veličina učinka izračunata je tako da se oduzme vrijednost stope rasta u niskom pH od negativne vrijednosti stope rasta u kontrolnom pH tretmanu te podijeli sa stopom rasta u kontrolnom pH tretmanu. Veličina učinka izračunata je za svaki mrijest i uspoređena sa stopom rasta u kontrolnom pH tretmanu. Pozitivna vrijednost stope učinka ukazuje da je stopa rasta ličinki viša u niskom pH nego u kontroli, a negativna veličina učinka ukazuje da je stopa rasta manja u niskom pH nego u kontrolnom. Ličinke s većom stopom rasta u kontrolnom pH prebačene u niski pH imale su veću vrijednost veličine učinka, a ličinke s manjom stopom rasta u 7,22 imale su veće stope rasta kada su prebačene u pH 7,95.

Transplantacija mrijesta između pH_T 7,95 i 7,64. Mrijestovi ženki F8 i F10 iz pH_T 7,95 transplantirani su u pH_T 7,64. Dodatno je transplantiran mrijest iz pH_T 7,94 ženke F6 zbog nedostatka odgovarajućih mrijestova iz pH_T 7,95. Intrakapsularni razvoj mrijesta ženke F10

zaustavio je razvoj u stadiju veliger ličinke, te je stoga isključen iz daljnje analize. Nije zabilježena značajna razlika u stopi rasta između mrijestova koji su ostali u pH_T 7,95 i polovice prebačene u 7,64. Mrijest ženke F6 dosegao je stadij pediveliger ličinke u oba pH, dok je mrijest ženke F8 dosegao stadij izvaljivanja u oba pH bez značajne razlike u stopi rasta. Mrijestovi ženki F4, F8 i F10 iz pH_T 7,64 izabrani su za unakrsnu transplantaciju u pH_T 7,95. Ličinke mrijesta ženke F8 zaustavile su razvoj u veliger stadiju, te su stoga isključene iz daljnje analize. Opažena je statistički značajna razlika u duljini ličinki tijekom razvoja između mrijesta ostavljenog u pH_T 7,64 i 7,95. Mrijestovi ženki F4 i F10 dosegli su stadij pediveliger ličinke i stadij izvaljivanja u oba pH spomenutim redom. Statistički značajna razlika u duljini ličinki zabilježena je samo kod mrijesta ženke F10, pri čemu su ličinke u pH_T 7,95 imale veću stopu rasta od ličinki u pH_T 7,64. Izračunata je veličina učinka, te nije zabilježena povezanost između početne stope rasta i stope rasta transplanata.

ZAKLJUČCI

Ovo istraživanje doprinosi boljem razumijevanju odgovora morskih puževa, konkretno, *H. trunculus*, na očekivanu acidifikaciju mora. Dobiveni rezultati naglašavaju važnost istovremenog proučavanja različitih bioloških procesa unutar vrsta tijekom dugotrajnih eksperimenta i naglašavaju složenost strategija prilagodbe organizama u uvjetima niskog pH. Pokazano je da *H. trunculus* upotrebljava različite mehanizme za nošenje sa stresom uzorkovanim niskim vrijednostima pH. Pojedine fiziološke i morfološke značajke nisu bile pod utjecajem pH, druge su druge kolebale u korelaciji s čimbenicima poput temperature, metaboličke aktivnosti i duljine izloženosti. Uključivanje vrijednosti prirodnih kolebanja pH u proučavanom staništu i uključivanje većeg raspona pH vrijednosti tijekom postavljanja eksperimenta povećava mogućnost procjene otpornosti vrste za određeno stanište. Ovo istraživanje doprinosi nedostatku znanja i poznavanja intraspecifične varijacije u odgovoru organizama te naglašava važnost uključivanja proučavanja utjecaja izloženosti roditeljske generacije stresu na pojedinačne jedinke potomaka kada se procjenjuje osjetljivost vrste. Potencijalna otpornost kvrgavog volka kao predatora na vrijednosti pH mora niže od današnjih ukazuju na moguće posljedice na strukturu zajednice i funkcioniranje ekosustava. Kao ključni sudionik u predator-plijen dinamici istraživanog područja, promjene u ponašanju i fiziologiji kvrgavog volka bi mogle imati kaskadni utjecaj na raznolikost i abundanciju niza vrsta koje su njegov plijen. Poremećeni odnosi unutar hranidbene mreže mogu predstavljati rizik za uzgoj školjkaša u ovom području, i za mediteransku dagnju *M. galloprovincialis* i za europsku plosnatu kamenicu *O. edulis*, pa je potrebno razmotriti potrebu za uspostavljanje preventivnih mjera za smanjivanje rizika. Međutim, utjecaj niskih pH vrijednost mora na

fiziologiju drugih vrsta koje nastanjuju Malostonski zaljev nije poznat, kao niti predator-plijen interakcije. Stoga je, da bi se razumjele šire implikacije projiciranih budućih vrijednosti pH te osmislile učinkovite strategije ublažavanja, potrebno proširiti postojeća istraživanja.

Zaključci ovog istraživanja su sljedeći:

- pH nema utjecaja na stopu prehrane kvrgavog volka *H. trunculus*
- izloženost niskom pH nije utjecala na sposobnost kvrgavog volka *H. trunculus* da pronađe hranu, iako su jedinke iz niskog pH trebale manje vremena da dođu do nje. Da bi se utvrdili specifični mehanizmi odgovorni za brže kretanje jedinki iz niskog pH potrebna su daljnja istraživanja
- akutna izloženost niskom pH ima pozitivan učinak na stopu rasta duljine kućice kvrgavog volka *H. trunculus*, vjerojatno zbog povećanja stope metabolizma
- tijekom razdoblja ograničene dostupne energije u zimskom razdoblju, stopa rasta duljine kućice kvrgavog volka *H. trunculus* je pod negativnim utjecajem pH
- nakon dugotrajne izloženosti niskom pH kvrgavi volak *H. trunculus* ima sposobnost aklimatizacije u kontekstu stope rasta duljine kućice
- neto stopa kalcifikacije kvrgavog volka *H. trunculus* je pod negativnim utjecajem smanjenog pH tijekom cijelog razdoblja eksperimenta
- kvrgavi volak *H. trunculus* pokazuje sposobnost održavanja stope rasta ukupne mase samo tijekom akutne izloženosti rasponu pH, ostatak eksperimentalnog razdoblja utjecaj je negativan, čime se rast ukupne mase smanjuje proporcionalno smanjenju pH
- pH nema utjecaj na stopu rasta mase mekog tkiva kvrgavog volka *H. trunculus* sve do razdoblja nakon mriješćenja kada je opažen značajan negativni utjecaj ukazujući na sinergijski učinak slabe kondicije nakon mriješćenja i niskog pH
- nema razlike utjecaja pH na stopu rasta duljine kućice, ukupne mase, mase mekog tkiva i neto stope kalcifikacije mužjake i ženke kvrgavog volka *H. trunculus*
- ženke kvrgavog volka *H. trunculus* pokazuju smanjenje metaboličke aktivnosti kao odgovor na niski pH tijekom prvih 145 dana izloženosti, a nakon toga dolazi do ponovnog uspostavljanja kiselo-bazne ravnoteže organizma
- pH nema utjecaja na vrijeme početka i sveukupno trajanje mriješćenja kvrgavog volka *H. trunculus*, na broj ženki koje se mrijeste ni na veličinu i broj izmriješćenih kapsula
- pH nema utjecaja na duljinu ličinki vrste *H. trunculus* niti na vrijeme potrebno da se dosegne određeni razvojni stadij tijekom intrakapsularnog razvoja

- intrakapsularni razvoj je narušen nakon stadija veliger ličinke za pH_T 7,51 – 7,22, što je demonstrirano odgođenim razvojem (ličinke provode više vremena u veliger stadiju te zaustavljaju razvoj i preživljavanje), te manje ličinki koje dosegnu stadij pediveliger ličinke i stadij izvaljivanja
- akutna izloženost mrijesta pH_T 7,22 ima negativan utjecaj na intrakapsularni razvoj kvrgavog volka *H. trunculus*
- roditeljska izloženost kvrgavog volka *H. trunculus* niskom pH općenito ima negativni utjecaj na intrakapsularni razvoj, ali je opažena varijacija u pojedinačnim odgovorima
- negativni učinci mogu do neke mjere biti povratni – opažen je veći rast ličinki iz mrijesta čiji su roditelji bili izloženi niskom pH, kada se prebace u pH_T 7,95
- akutna izloženost mrijesta pH_T 7,64 nema utjecaja na intrakapsularni razvoj kvrgavog volka *H. trunculus*
- roditeljska izloženost pH_T 7,64 generalno nema negativan utjecaj na intrakapsularni razvoj, ali suptilne razlike između mrijestova naglašavaju važnost individualne varijacije u odgovoru kvrgavog volka *H. trunculus*

9. BIOGRAPHY

Sanja Grđan was born on 17 June 1986, in Zagreb. In the academic year 2013/2014, she enrolled in the undergraduate study program Aquaculture at the University of Dubrovnik. She completed her graduate studies in Mariculture at the University of Dubrovnik, defending her thesis titled "Macrozoobentos of Mrtvo more (Lokrum Island)" in the academic year 2018.

On 15 November 2018, she started working as an assistant at the University of Dubrovnik, where she participated in teaching activities for the undergraduate study program Applied Marine Ecology and the graduate study program Mariculture. In the academic year 2019/2020, she enrolled in the postgraduate university study program Applied Marine Sciences at the University of Split and the University of Dubrovnik.

Sanja also actively contributed to various scientific and professional projects, including MARine Litter cross-border awarenESS and innovation actions (MARLESS), Development of a control and defense system for ports against the introduction of foreign species (ProtectAS), Knowledge Exchange in Sustainable Fisheries Management and Aquaculture in the Mediterranean Region (FISHAQU), Reconnect Science with the Blue Society (Blue-connect), Monitoring of eels in inland waters as part of the National Plan for collecting data in the fisheries of the Republic of Croatia, Preservation of the noble pinna (*Pinna nobilis*) in the southern part of the Adriatic Sea and Development of Aquaculture and Fisheries Education for Green Deal in Armenia and Ukraine (AFISHE).

She participated in the Science Festival in 2019 with a workshop titled “Colors as Indicators in Natural Sciences”. Additionally, she attended two summer schools on the topics of Modeling in Marine Ecology organized by ESMTB in Sicily and Sustainable Blue Economy in the Euro-Mediterranean Region organized by the University of EMUNI, Slovenia, and the OGS Institute, Italy. She was invited as a lecturer to the Basic Training Course on Ocean Acidification held at Kristineberg Marine Station, Sweden. Sanja also delivered an invited lecture at the workshop “Blue Bioeconomy Approach to Wasted Marine Biomass” organized as part of the MARIFERT project.

For the capacity-building program of the International Atomic Energy Agency (IAEA), she recorded publicly available video materials on the preparation of TRIS buffers used in laboratory research, as well as calibration and sampling in biological experiments. She also co-authored the accompanying manual titled "How to Measure pH_T in Biological Experiments".

She co-authored four scientific papers indexed in Current Content database. She has participated in several international and national scientific conferences with a total of 12 contributions.

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