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**An Experimental Approach to Assessing Climate Sensitivity in
Juvenile Yellowbelly Flounder (*Rhombosolea leporina*):
Methodological Insights and Applications**

A thesis
submitted in partial fulfilment
of the requirements for the degree
of
Master of Science (Research) - Ecology & Biodiversity
at
The University of Waikato
by
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THE UNIVERSITY OF
WAIKATO
Te Whare Wānanga o Waikato

2026

Abstract

As anthropogenic CO₂ emissions continue to rise, marine ecosystems face the dual threats of ocean acidification and ocean warming. A number of studies have examined the effects of ocean acidification and warming on coastal marine fin fish species, but little attention has been paid to estuarine species, the assumption being that they are likely to be tolerant to fluctuations in seawater parameters given freshwater and large tidal dynamics. This thesis investigates the impacts of these stressors on the early life history of the estuarine yellowbelly flounder (*Rhombosolea leporina*), an ecologically and culturally significant taonga species in New Zealand.

Research was conducted in two phases; a larval pilot study focusing on the transition to exogenous feeding, and a behavioural study on juveniles assessing olfactory mediated responses to food and predator cues.

The larval pilot study, carried out in aquaria, revealed an extreme developmental bottleneck at approximately 14 days post-hatch (dph). Mass mortality occurred across all treatments, coinciding with the onset of metamorphosis and the “critical period” of first feeding. The extent of the mortality event masked any possible treatment effects imposed in terms of pH and temperature combinations. These results highlight the high physiological sensitivity of *R. leporina* during early ontogeny, independent of climate stressors.

In the second phase, juvenile flounder were exposed to a fully-crossed factorial design of pH (~7.3 & ~8.0) and temperature (20°C & 25°C). Behavioural trials using a two channel choice chamber measured the proportion of time spent in stimulus streams (*Perna canaliculus* food cues & *Notolabrus celidotus* predator cues). A three-way ANOVA revealed a significant main effect of stimulus type ($p = 0.017$) and temperature ($p=0.040$), but no significant effect of pH and no interactive effects. Interestingly, 66.7% of individuals remained in the neutral mixing zone, particularly during predator trials and under elevated temperatures.

These findings suggest that while *R. leporina* larvae remain highly vulnerable to intrinsic developmental bottlenecks, juveniles exhibit a degree of behavioural resilience to reduced pH, potentially due to their evolved tolerance of fluctuating estuarine environments. The preference for remaining stationary (crypsis) in the neutral zone is

interpreted as a natural, risk-averse strategy for this benthic species. This research underscores the importance of considering species-specific ecology and life stage transitions when predicting the impacts of climate change on New Zealand fisheries.

Overall, this research apparently represents the first study to experimentally examine the effects of ocean acidification and warming on estuarine flatfish species across critical early life stages. Although no clear pH-driven effects were detected in the juvenile behavioural trials, the study remains valuable in testing hypotheses around the resilience of marine species that naturally experience highly variable environmental conditions. In particular, species with significant estuarine habitat phases may possess inherent tolerance to fluctuations in seawater chemistry, yet may still face vulnerability during key developmental transitions. Investigations such as this are therefore important for improving our understanding of how climate change stressors interact with species-specific ecology and life history bottlenecks, and for informing future research on the adaptive capacity of estuarine-associated marine fishes.

Acknowledgements

First and foremost, I would like to express my deepest gratitude to my supervisor, Professor Chris Battershill. This thesis would quite simply not exist without his unwavering belief in my ability to balance the demands of being a full-time teacher and a part-time student. After nearly a year of setbacks, it was Chris's constant reassurance that kept me grounded. Thank you, Chris, for challenging me to think outside the box and for reminding me that while climate change research is inherently difficult, it is profoundly worthwhile. Most importantly, thank you for the literal "above and beyond" moments specifically, for heading out at 1:00 AM on zero sleep to catch baby flounder for my study when I was at my lowest point. Your mentorship kept me going when I was ready to give up. I am also incredibly grateful to Simon Muncaster. Thank you for your patience with my endless fish-related questions and for allowing me to conduct a trial run on your flounder broodstock; that opportunity was an essential part of my learning process.

A massive thank you must go to the technical team, without whom I simply would not have had an experimental setup. To Bonnie Lewis, thank you for spending weeks helping me configure my tanks and the highly complex CO₂ system, even when I managed to go through three solenoids in a single week. Your dedication was truly evident when you came in on a Saturday evening to collect fresh seawater and reset my tanks after an initial overflow. To Holly Fergusson, thank you for your incredible craftsmanship in creating the choice chamber from scratch and for your patience in adapting the design whenever I felt it needed to change; I could never have achieved such a watertight setup on my own. I am so grateful to you both for checking on my systems when my teaching schedule prevented me from leaving work until late.

To my student assistant, Jazmin Mitchell, thank you for keeping me sane. From the heartbreak of the pilot study to the grueling days of carrying out trials for hours on end and the constant stress of fish trying to jump out of buckets your support was invaluable. You challenged my ideas and forced me to refine my procedures, making

the final study much stronger. You have a bright future in marine studies should you choose to pursue it.

A sincere thank you to the University of Waikato for the scholarship support. This financial assistance was vital, providing me with the necessary flexibility to take time off work and focus on my research during critical periods.

Finally, to my family, thank you for being my constant sounding board. You may not always have known the intricacies of the research, but you understood the weight of it. Thank you for keeping me accountable and reminding me of how far I had come whenever I felt like two and a half years and multiple species changes were too much to overcome. I didn't give up because you wouldn't let me.

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Chapter 1 - General Introduction

1.1 Climate Change

The climate is changing. Over the course of the 21st century, New Zealand's marine environment is expected to undergo significant changes due to rising ocean temperatures, shifts in wind patterns, and increased intensity and frequency of extreme weather events (Mullan et al., 2008). Climate change poses significant threats to marine ecosystems due to rising ocean temperatures, acidification, sea level rise, and deoxygenation, all of which disrupt species, habitats, and food webs (Doney et al., 2012; Bijma et al., 2013). Coral bleaching, biodiversity loss, and the collapse of vulnerable habitats like reefs and polar ecosystems highlight the urgency of the issue (Hughes et al., 2017). These changes threaten global food security, economies reliant on fisheries and tourism, and the critical ecosystem services oceans provide, such as carbon storage and climate regulation (Pinkerton, 2017). Yet, the ocean remains a critical ally, with its ability to buffer against global warming and its impacts. Urgent action to mitigate emissions, restore habitats, and protect marine life is imperative to safeguard these blue frontiers and ensure the resilience of these vital ecosystems in the face of ongoing environmental change (Jiang et al., 2019). Various processes are linked to warming and acidification, and can interact to intensify each other's effects on ocean biology. For instance, global warming will enhance surface ocean stratification, consequently altering the light conditions at the surface and the nutrient influx from deeper layers, thereby influencing primary production (Rost & Wolf-Gladrow, 2008; Bopp et al., 2013).

Since the onset of the industrial revolution, global atmospheric CO₂ concentrations are increasing at an unprecedented pace due to ongoing and accelerated human activities, including fossil fuel combustion, cement production, and changes in land use (Bijma et al., 2013; Lönnstedt et al. 2013). The atmospheric CO₂ concentration has increased from pre-industrial levels of approximately 280 parts per million (ppm) to 419.3 ppm in 2023 (Forster et al., 2024), representing an increase of over 49%. While the oceans play a critical role in mitigating climate change by sequestering around 25% of human-generated CO₂ (DeVries, 2022; Gruber et al., 2023), this absorption leads to a fundamental shift in seawater carbonate chemistry and a reduction in pH levels, a process known as ocean acidification (Caldeira & Wickett, 2003). Equilibrium with the ocean has already resulted

in a pH decline of approximately 0.1 units (Friedlingstein et al., 2022). This trend is expected to continue over the next two centuries at a rate unprecedented in the past 300 million years, potentially leading to a pH decrease of up to 0.77 by the year 2300 under high emission scenarios (Meehl et al., 2007; Hönlisch et al., 2012). Surface ocean CO₂ closely follows atmospheric levels (Gregor & Gruber 2021), influenced by the ocean's buffering capacity mathematically described as the Revelle factor (Egleston, 2010; Jiang et al., 2019). Although air-sea exchange responds nearly linearly to atmospheric CO₂, ocean acidification has reduced the ocean's buffering capacity by approximately 10% since pre-industrial times (Orr et al., 2005; Jiang et al., 2019). This reduction in capacity creates a positive feedback loop; as the Revelle factor increases, the ocean becomes less efficient at absorbing additional CO₂ from the atmosphere.

The total number of marine heatwave (MHW) days per year has risen globally over the twentieth and early twenty-first centuries. On average, the number of MHW days per year during the period 1987–2016 was over 50% higher compared to the earlier period 1925–1954 (Smale et al., 2019). These prolonged periods of abnormally warm water disrupt ecosystem structure and can lead to mass mortality events, particularly for species already living near their physiological thresholds (Oliver et al., 2018).

Aquatic poikilothermic animals are naturally buffered against rapid or seasonal fluctuations in temperature due to the high thermal inertia of water. This stability in their environment allows them to thrive within a relatively narrow temperature range, making them more stenothermal compared to terrestrial animals (Blaxter 1992). However, this specialization comes at a cost: their limited ability to tolerate 'unusual' temperature variations in terms of ranges beyond their 'normal experiences', renders them potentially more vulnerable to long-term changes and acute thermal spikes, such as those induced by climate change (Blaxter 1992). Recent evidence suggests that marine ectotherms may be more vulnerable to global warming than terrestrial species because they often occupy nearly all of their available thermal safety margin, leaving little room for adaptation as oceans warm (Sunday et al., 2012; Pinsky et al., 2019).

1.1 Multiple Stressors

Multiple stressors here refers to two or more environmental, biological, or anthropogenic factors that simultaneously or sequentially affect an organism, population, community, or

ecosystem (Breitburg & Riedel, 2005). These stressors interact in complex ways, often leading to compounded or unpredictable effects on marine and terrestrial environments (Breitburg et al., 1998). The combined impacts of multiple stressors on coastal marine organisms remain poorly understood, limiting our ability to accurately predict future ecological consequences (Pirotta et al., 2022). Regional variability further complicates this understanding, as ecosystems experience distinct combinations of stressors depending on local environmental conditions (Heuer & Grosell, 2014). Consequently, findings from single-stressor experiments may not fully capture the dynamics observed in natural systems, where multiple stressors act concurrently.

To improve predictive accuracy, a comprehensive assessment must consider the interactive effects of multiple stressors, since acute CO₂ exposure alone fails to represent the complexity of real-world marine environments. The increase in atmospheric CO₂ not only drives ocean acidification but also contributes to global ocean warming (Kroecker et al., 2013; Stocker, 2014). These dual stressors, rising temperature and declining pH pose a significant challenge for marine ecosystems, including fish species during their early life stages (Munday et al., 2009).

Combined environmental stressors such as elevated temperature and CO₂ can interact to influence fish physiology in ways that differ from the effects of individual stressors. For example, juvenile blue rockfish (*Sebastes mystinus*) exposed to elevated CO₂ and hypoxia show unique gene expression responses under combined stressors that are not predictable from single-stressor experiments, indicating non-additive effects on energy metabolism and cellular processes (Cline et al., 2020). In other species, elevated CO₂ and warming together lead to increased metabolic costs and reduced energy available for growth and condition, with combined stressors producing greater physiological disruption than when experienced alone. For example, in European sea bass exposure to both elevated pCO₂ and higher temperature altered growth patterns and physiological performance compared with single-stressor treatments (Cominassi et al., 2020). Such interactions can be additive, where the total response approximates the sum of individual effects, or synergistic, where the joint impact exceeds expectations from single stressors (Breitburg & Riedel, 2005). These compounded physiological costs can reduce overall fitness and resilience, leaving fish more vulnerable to ecological pressures. Indeed, the co-occurrence of warming and acidification has been shown to impair fish behaviour more severely than either stressor alone (Munday et al., 2009).

1.3 The case for estuaries

While extensive research has explored the effects of ocean warming and acidification on tropical, coastal, and coral reef fishes, relatively little is known about species that inhabit estuarine environments. Estuaries are highly dynamic systems, with natural fluctuations in temperature, pH, and oxygen, meaning that estuarine fishes may respond differently to climate stressors than their marine counterparts. The combined influence of elevated temperature and acidification is particularly relevant to estuarine species such as flatfish and other demersal species, whose early life stages are already highly sensitive to environmental fluctuations (Pörtner et al., 2004; Chambers et al., 2014; Przeslawski et al., 2015). Ocean warming accelerates metabolism and developmental rates, often leading to smaller larval sizes and reduced fitness (Pörtner & Peck, 2010; Baumann et al., 2012), while ocean acidification reduces survival, causes tissue damage, and leads to skeletal deformities (Frommel et al., 2012; Chambers et al., 2014). When combined, these stressors can act synergistically to magnify physiological disruption, compromising both structural integrity and behavioural function. Elevated temperatures can exacerbate acidification-induced deformities and developmental delays, while acidification can intensify temperature-related stress by altering metabolic balance and oxygen transport efficiency (Pörtner & Peck, 2010).

In some cases, interactive effects may vary depending on life stage and species. For example, olive flounder larvae exposed to elevated CO₂ displayed enhanced growth in size and weight, but these apparent benefits were offset by negative physiological trade-offs such as reduced bone density (Kim et al., 2013, 2015). When warming occurs concurrently, such trade-offs may become more pronounced, as energy demands increase while resource allocation to skeletal development and tissue maintenance declines. Moreover, combined exposure to warming and acidification has been shown to disrupt neurological function and behaviour in larval fish more severely than either stressor alone. Impaired olfactory detection and predator avoidance behaviours under elevated CO₂ and temperature conditions significantly compromise survival and recruitment success (Munday et al., 2009; Dixon et al., 2010; Nilsson et al., 2012; Hamilton et al., 2014; Porteus et al., 2018).

Temperature is a particularly critical factor influencing the early development of estuarine fishes, including flatfish species, with effects on vertebral formation, hatching time, body size, growth, and muscle differentiation (Blaxter, 1992; Wargelius et al., 2005). In

estuarine flatfish, which often experience highly variable environmental conditions during early life stages, these developmental processes can be especially sensitive to the combined effects of warming and acidification, with interactions frequently producing non-linear and unpredictable outcomes (Kroeker et al., 2013). While substantial research has examined the responses of marine species to ocean acidification and warming (Kroeker et al., 2010; Wittmann & Pörtner, 2013), comparatively few studies focus specifically on estuarine fishes or flatfish, despite the ecological and commercial importance of these species. The reality that estuarine organisms regularly encounter multiple co-occurring stressors underscores the need to consider combined effects, as interactions between elevated CO₂ and temperature can generate synergistic or antagonistic impacts on growth, development, and survival (Kroeker et al., 2013; Danylchuk et al., 2023).

Overall, the limited research on estuarine fishes, and flatfish in particular, highlights a significant knowledge gap in understanding how these species respond to the combined effects of warming and acidification. For this reason, the focus of the present study is on flatfish as a representative estuarine species, providing insight into how key species of ecological and commercial concern may cope with future environmental stressors.

1.4 Flatfish ecology

Flatfish, belonging to the order Pleuronectiformes, represent a diverse and commercially significant group of marine teleosts recognised globally for their economic value and high-quality flesh (Nenadic, 1998; Robledo et al., 2017; Cheung & Oyinlola, 2018; Thomson, 2024). Their defining characteristic is a profound morphological asymmetry in adulthood, resulting from a specialised benthic existence (Schreiber, 2013; Robledo et al., 2017). This transition occurs during metamorphosis when one eye migrates to the opposite side of the head, allowing the flatfish to settle onto the seabed (Schreiber, 2013). These species are fundamentally bottom-dwelling (benthic), predominantly inhabiting soft-sediment environments such as sand flats, muddy coastal waters, estuaries, and harbours, utilizing camouflage for defence and exploiting the abundant benthic food resources (Nenadic, 1998; Gibson et al., 2014; Koverman, 2018). Following their developmental transition of metamorphosis, the ecological success of flatfish is largely dictated by their recruitment into shallow, estuarine nursery grounds. These habitats are selected through a combination of chemical and tactical cues, such as substrate grain size,

which signal areas of high prey density and thermal conditions conducive to rapid growth (Gibson et al., 2014). Within these environments, flatfish function as specialised “sit and wait” predators, utilising chromatophores to camouflage. This cryptic behaviour, paired with the ability to remain buried while maintaining visual of the water column, facilitates the efficient capture of benthic invertebrates (Nenadic, 1998; Gibson et al., 2014).

The flatfish life cycle begins with early life stages (ELS) eggs and larvae which are considered the most vulnerable period in the fish life cycle (Pörtner et al., 2004; Chambers et al., 2014). Reproduction often involves batch spawning during protracted seasons, typically initiated in cooler or offshore waters to minimise exposure to extreme environmental variability (Colman, 1973; Koverman, 2018). For instance, the incubation of greenback flounder (*Rhombosolea tapirina*) eggs is optimised around 12°C to 15°C, reflecting specific thermal requirements for early development (Hart & Purser, 1995, 1996). Following hatching, the larvae are initially symmetrical and planktonic, relying on endogenous yolk reserves before transitioning to external feeding (Dou et al., 2005; Schreiber, 2013). The metamorphosis from a symmetrical larva to an asymmetrical juvenile involves extensive cranial remodelling which is a critical developmental bottleneck highly susceptible to environmental disruption (Schreiber, 2013; Chambers et al., 2014).

Estuarine flatfish routinely experience highly variable salinity and pH due to freshwater inputs, tidal cycles, and seasonal rainfall, with salinity often ranging from near freshwater (~5 ppt) to fully marine conditions (~35 ppt) and pH fluctuating by 0.3–0.5 units over short timescales (Hart & Purser, 1996; Gibson et al., 2014; Koverman, 2018). This environmental variability suggests that estuarine species such as flatfish may possess broad physiological tolerance to pH and osmotic changes, which could overshadow or mask the effects of experimental ocean acidification studies if these natural tolerances are not considered (Pörtner et al., 2004; Chambers et al., 2014). Consequently, assuming outcomes based solely on marine or reef species risks misrepresenting the responses of estuarine taxa. In New Zealand, systematic investigations into the combined effects of pH and salinity variability on estuarine flatfish have not been carried out, highlighting the need to establish controlled experimental systems that can accurately simulate local estuarine conditions and provide insights directly applicable to native species and habitats (Nenadic, 1998; Dou et al., 2005).

Taken together, the relative paucity of information on estuarine fishes underscores the value of focusing on flatfish in this study, using them as a representative species to explore how ecologically and commercially important estuarine fishes may respond to future climate-driven stressors.

1.5 Study Species: Yellowbelly Flounder *Rhombosolea leporina*

The yellowbelly flounder, *Rhombosolea leporina*, is a significant New Zealand native flatfish belonging to the family Pleuronectidae, commonly known as the right-eye flounders (Last et al., 1983; Nenadic, 1998). It is one of four key *Rhombosolea* species found in New Zealand waters (Colman, 1973; Nenadic, 1998; Thomson, 2024). The common Māori name for the species is pātiki-tōtara, and flounder (pātiki) as a whole are recognised as taonga species holding significant cultural value to Māori. Historically, flatfishes provided a reliable food source (NIWA, 2017; Koverman, 2018).

Rhombosolea leporina is a vital component of New Zealand's marine economy, supporting robust commercial, recreational, and customary fisheries (MPI, 2016; Wynne-Jones et al., 2019). As a benthic, or bottom-dwelling species, it is predominantly found in shallow coastal environments, including muddy estuaries, sheltered harbours, and tidal inlets (McLay, 1976; Nenadic, 1998). Because they reside in these near-shore habitats which are often the first to experience the impacts of urban runoff, sedimentation, and pollution, yellowbelly flounders are recognised as sensitive bioindicators of environmental health (Nenadic, 1998).

The life cycle of the yellowbelly flounder is characterised by a seasonal spawning migration, typically occurring in the spring months between September and November (Colman, 1973; Hart & Purser, 1995). Like all flatfish, *R. leporina* begins life as a symmetrical larva before undergoing metamorphosis. This process involves a 90-degree rotation of the cranium, moving the left eye to the right side of the head, and extensive internal remodelling to suit a life spent on the seafloor (Schreiber, 2013). This metamorphosis represents a significant physiological "bottleneck." Research on related species, such as the greenback flounder (*R. tapirina*), highlights that these larvae have extremely narrow thermal and light requirements for survival (Hart & Purser, 1995). Furthermore, studies on the summer flounder (*Paralichthys dentatus*) and olive flounder (*P. olivaceus*) indicate that anthropogenic stressors, specifically ocean acidification and

rising sea temperatures, can induce cranio-facial deformities and severely reduce larval survival rates (Chambers et al., 2014; Kim et al., 2015). Given its reliance on shallow, human-impacted estuaries for nursery grounds, *R. leporina* is vulnerable to these shifting environmental parameters.

1.6 Effects of abiotic stressors on early life stages in fish

Egg and larval stages represent critical bottlenecks in the life cycle of many fish species, as survival during these early developmental periods strongly determines recruitment success and overall population dynamics (Blaxter, 1992). During these stages, individuals possess limited physiological capacity to regulate their internal environment, making them particularly vulnerable to environmental stressors associated with climate change (Falk-Petersen, 2005; Pörtner & Peck, 2010). As a result, flatfish populations are highly susceptible to environmental fluctuations during their early life stages (Pörtner et al., 2004; Chambers et al., 2014; Przeslawski et al., 2015).

Ocean warming (OW) increases metabolic rates and accelerates development in larval fish, often resulting in smaller body size and reduced fitness (Pörtner & Peck, 2010; Baumann et al., 2012). Temperature plays a fundamental role in determining embryonic duration, hatchling size, developmental rate, pelagic larval duration, and overall survival (Pankhurst & Munday, 2011). Rising ocean temperatures can also cause phenological shifts, which in turn lead to mismatches between larval hatching and the peak availability of food resources. When larvae hatch outside of optimal feeding windows, survival rates may decline, and broader food web dynamics can be disrupted (Blaxter, 1992; Poloczanska et al., 2013). In species such as the southern flounder, exposure to extreme temperatures has also been shown to trigger temperature-dependent sex determination (TSD), resulting in phenotypic sex reversal toward males. This imbalance has serious implications for both wild populations and aquaculture operations, particularly where female-biased populations are desired due to faster growth rates (Luckenbach et al., 2003).

Ocean acidification (OA), driven by increasing atmospheric CO₂ levels and the corresponding decline in seawater pH, poses another significant threat to early fish development. Embryonic and larval stages are especially sensitive to changes in pH, with exposure leading to developmental delays, morphological deformities, and physiological

impairments that may persist into adulthood (Heuer & Grosell, 2014). In summer flounder, for example, exposure to elevated CO₂ concentrations (1808 µatm) reduced embryo survival by approximately 52% (Chambers et al., 2014). Acidified conditions have also been linked to tissue damage, including necrosis in the liver, disruption of skeletal muscle fibers, and craniofacial deformities in developing larvae (Frommel et al., 2012). Although some species, such as the olive flounder, exhibit increased larval growth in size and weight under elevated CO₂ conditions, these apparent benefits are often offset by negative physiological trade-offs such as reduced bone density (Kim et al., 2013, 2015).

Beyond these structural effects, ocean acidification has been shown to interfere with the neurological function and behaviour of larval fish. Elevated CO₂ impairs sensory processing, reducing the ability of larvae to detect and respond to predator olfactory cues (Munday et al., 2009; Dixon et al., 2010; Nilsson et al., 2012; Hamilton et al., 2014; Porteus et al., 2018). Such disruptions in predator-prey recognition and avoidance behaviours can significantly compromise survival during the most vulnerable stages of development.

Overall, the early life stages of fish are highly sensitive to individual environmental stressors such as temperature increases and ocean acidification. These factors independently alter essential physiological and developmental processes, with consequences for growth, survival, and recruitment success. Understanding the distinct effects of each stressor provides an essential foundation for predicting how fish populations will respond to the combined pressures of climate change.

1.7 Predator avoidance

The ability to detect and avoid predators is crucial for survival, particularly during vulnerable early life stages of marine organisms (Lima & Dill, 1990, as cited in Dixon et al., 2010). However, exposure to elevated seawater carbon dioxide (CO₂), as predicted under future ocean acidification (OA) conditions, can significantly impair important innate antipredator behaviours in many fish species (Munday et al., 2009; Clements & Hunt, 2015). This disruption is thought to result from changes to the normal function of γ-aminobutyric acid type A (GABA A) receptors in the brain (Nilsson et al., 2012; Hamilton et al., 2014; Heuer & Grosell, 2014). These changes occur because fish must

regulate their internal acid–base balance in high CO₂ conditions, which alters the balance of key ions such as bicarbonate and chloride (Heuer et al., 2016; Schunter et al., 2018). A fundamental consequence is the impairment of sensory systems, preventing larval fish from distinguishing ecologically important olfactory cues, such as chemical alarm cues from injured conspecifics or predator odour (Dixon et al., 2010; Porteus et al., 2018). This sensory deficit often leads to maladaptive responses, characterised by a reversal from strong avoidance to dangerous attraction toward predator odour in affected species (Munday et al., 2009; Dixon et al., 2010; Welch et al., 2014). Beyond chemosensory interference, elevated CO₂ also compromises visual risk assessment and impairs kinematic escape responses, manifesting as increased latency before initiating escape and subsequent reduction in escape speeds (Ferrari et al., 2011; Allan et al., 2013; Watson et al., 2018).

These widespread behavioural deficits directly translate into severe ecological costs, resulting in substantially increased predation mortality in natural environments (Munday et al., 2009; Ferrari et al., 2011). However, vulnerability to OA is highly dependent on species, life stage, and context (Ferrari et al., 2011; Cattano et al., 2017). For instance, while increased anxiety is reported in Californian rockfish (Hamilton et al., 2014) and juvenile yellowtail kingfish (Jarrold et al., 2020), other species, like the temperate blacksmith damselfish, show robust behavioural tolerance (Kwan et al., 2017). Furthermore, OA affects species across trophic levels; predatory fish such as the brown dottyback exhibit a reduced attraction to injured prey odour (Cripps et al., 2011), sharks demonstrate impaired hunting capabilities (Pistevos et al., 2017), and cephalopods display altered predatory strategies, including increased latency to attack (Spady et al., 2018), alongside shifts in defensive behaviours toward energetically costly jet escapes over cryptic postures (Spady et al., 2014). Despite this variation, the fundamental compromise of sensory and cognitive functions across diverse marine animals in response to elevated CO₂ levels challenges that their ability to successfully perceive and respond to risk in future acidified oceans (Briffa et al., 2012; Nagelkerken & Munday, 2016). Despite these extensive findings across multiple taxa, no studies to date have examined the effects of ocean acidification on the antipredator behaviour of flounder or other estuarine flatfish, representing a critical gap in understanding how these ecologically and commercially important species may respond to future environmental change.

1.8 Purpose of research

The increasing concern over climate change and its impact on marine ecosystems has highlighted the need to understand how key environmental stressors, such as elevated temperatures and reduced pH levels, affect marine species, particularly during their early life stages. This research focuses on yellowbelly flounder (*Rhombosolea leporina*), an economically significant species in New Zealand's fisheries, to investigate how these stressors influence critical phases of early life history.

The early life stages of marine species are often the most vulnerable to environmental changes, making it crucial to study the impacts of climate change on these stages (Laubenstein et al., 2018). While there is a growing body of research on the effects of individual stressors, the synergistic effects of elevated temperatures and reduced pH on benthic, sedentary, mainly estuarine species like *R. leporina* remain less understood. This research aims to fill this knowledge gap, or at the very least, advance the field in examining estuarine fish responses, by providing empirical data on how these stressors interact to influence the sensory mediated behaviours of juvenile yellowbelly flounder.

Flounder are a vital component of New Zealand's commercial and recreational fisheries. By examining how climate-induced stressors affect this species during its most vulnerable stages, this research aims to provide insights that can inform sustainable fisheries management practices and help safeguard the future of these fisheries in the face of ongoing environmental change. In addition, a research methodology for application across a range of facilities, including those without specialised aquaria systems, is developed.

1.9 Ethics and permits

Flounder were caught under the Ministry for Primary Industries special permit 950 for University of Waikato. Animals were handled according to the requirements of Animal ethics NZ standards under protocol number 1262.

Chapter 2 - General Methods

2.0 Introduction

Two phases of research were embarked upon. The first, a pilot study, examined the possible effects of combined temperature and pH regimes on larval flounder (described in Chapter 3). The second involved early life history juvenile flounder in behavioral experiments detailed below. All experiments were conducted at the University of Waikato Coastal Marine Field Station at Sulphur Point in Tauranga. It is important to note that this facility did not have an adequate temperature-controlled environment within the general research and aquarium area, hence a research aquarium system for this study needed to be designed such that it was completely autonomous.

2.1 Study Species & location of collection

Juvenile yellowbelly flounder (*Rhombosolea leporina*) were collected at high tide at Matua Estuary on 13th January 2026 from 2am (Figure 1). 40 Juveniles were gently scooped into a butterfly net once located with an underwater light, then placed into buckets of seawater to be transported back to the Field station. Seawater temperature was approximately 21-23°C *in situ* and this was stable throughout the collection process and transport as it equated to the ambient air temperature at the time. The wild-caught juvenile flounder were collected under a Ministry for Primary Industries (MPI) permit. Once the flounder were back at the field station they were immediately placed randomly into 16 treatment tanks stocked at 2-3 per tank to acclimatise for 24 hours (noting that any circatidal rhythmicity in behaviour would be dampened during this time). Only juvenile flounder in the size range of 1-10cm in total length were collected.



Figure 2.1. Map of the Matua Estuary (Tauranga Harbour, New Zealand) indicating the primary sampling locations where yellowbelly flounder (*Rhombosolea leporina*) were collected for this study. Marker indicates site of sampling.



Figure 2.2. A juvenile yellowbelly flounder (*Rhombosolea leporina*) during the acclimation phase within the experimental chamber. The image illustrates the typical benthic posture.

2.2 Experimental Design

A fully crossed factorial design was used to investigate the effects of seawater pH and temperature on juvenile flounder responses to olfactory cues. Two pH treatments were applied: an ambient control (~ 8.0) and a reduced pH treatment (~ 7.3) based on future OA trend predictions (Law et al., 2018). These were crossed with two temperature treatments: ambient temperature ($\sim 20^\circ\text{C}$) and elevated temperature (~ 25

°C). Treatment levels were selected to reflect projected end-of-century ocean conditions under Representative Concentration Pathway (RCP) 8.5 scenarios (Collins et al., 2013).

Flounder were collected from an estuarine environment where ambient pH at the time of collection was 7.5. To ensure a meaningful experimental contrast and simulate ocean acidification conditions, pH in treatment tanks was reduced below collection levels, as maintaining pH similar to that of the collection site would not have provided a strong experimental treatment. Reduced pH treatment tanks were gradually adjusted to a mean pH of 7.3 via controlled CO₂ dosing (see below). In control pH tanks, pH was not experimentally altered and was allowed to naturally buffer back to ambient seawater conditions, which averaged 8.3 over the experimental period.

All tanks were maintained under a controlled photoperiod of 14 h light : 10 h dark to replicate natural summer conditions.

2.3 Seawater Carbonate Chemistry

Seawater was collected from a header tank then filtered into two separate 100L sumps, each assigned to one of the two pH treatments (ambient control and reduced pH). From each sump, seawater was pumped up, through a heat exchanger and distributed to eight 12 L juvenile conditioning tanks. Each tank contained a plastic mesh basket to confine fish while allowing unrestricted water flow.

Temperature treatments were applied within each pH treatment. Elevated temperature tanks were maintained at 25°C using Aqua One 100 W glass heaters, while ambient temperature tanks were regulated at 20°C via a chiller. Fine-scale temperature control was maintained within $\pm 1.6^\circ\text{C}$.

Seawater pH was regulated using an APEX Fusion Neptune Systems controller, which dosed CO₂ into the sump water via a solenoid-controlled injection system. CO₂ was introduced as a slow gas stream into a powerhead positioned at the base of the reduced pH sump, ensuring effective dissolution through vigorous mixing. CO₂ dosing was automatically triggered when seawater pH exceeded the target set point which was 7.35 and turned off when the pH reached 7.25.

Carbonate chemistry was monitored following best practices for seawater CO₂ experiments (Dickson et al., 2007), consistent with methodologies used in comparable studies (e.g. Laubenstein et al., 2018; Watson et al., 2018; Munday et al., 2019; Jarrold et al., 2020). Seawater pH (total scale), temperature, dissolved oxygen (%) and salinity (ppt) were measured daily in each conditioning tank using a YSI multiparameter meter calibrated with fresh standard buffers. Behaviour and mortality were monitored throughout the experiment.



Figure 2.3. Configuration of the experimental rearing system. Individual mesh-lined baskets are suspended within the primary tanks using securing fasteners (pegs), with submersible heaters positioned at the base to ensure uniform thermal regulation across treatments.

2.4 Choice chamber construction

The choice chamber used in this experiment was constructed from grey and clear acrylic (PMMA) and measured 70 cm in length, 15 cm in width, and 7 cm in height, with a total working volume of approximately 7 L. The chamber was designed as a two-channel flume to allow fish to choose between two laminar water streams.

A sponge was placed over the outflow weir to prevent individuals from being swept into the outflow section. Two 20 L buckets were connected to the inflow end of the flume, one containing conditioned water and the other containing control seawater. Water was delivered to the chambers using two Aqua One Maxi Power Head 102 submersible pumps, each supplying one channel of the flume.

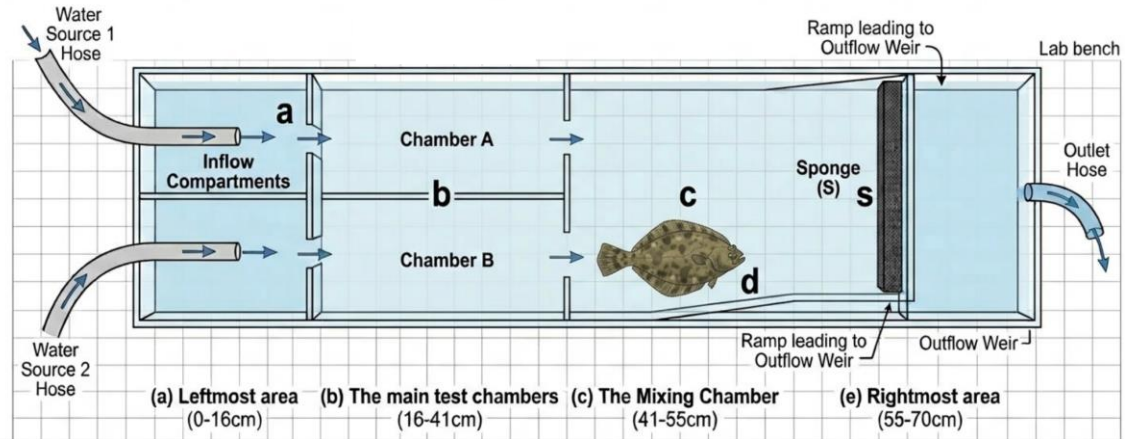


Figure 2.4. Choice chamber schematic (made using Gemini 1.5 Pro), overall size 70 x 15, water depth ~5cm. (a) Inflow compartments (16cm long), two water sources can be switched manually by moving the two hoses. (b) Chambers separated by barrier (25cm long), (c) mixing chamber where fish are placed at the start of each trial. (d) Ramp to outflow weir and sponge (s) to contain test fish; (e) outflow weir and outlet hose.

Prior to behavioural trials, a dye test was conducted to verify hydrodynamic separation within the choice chamber. The test confirmed that the flume supported two discrete, laminar water streams that remained largely separated along the length of the chamber, with minimal turbulence or eddy formation. Mixing of the two water masses occurred primarily within the central mixing area, which was therefore designated as a neutral zone for behavioural observations. As illustrated in Figure 2.5, the two water flows remained predominantly distinct even within this region, ensuring that fish were exposed to clearly-defined olfactory cues when occupying either side of the chamber.

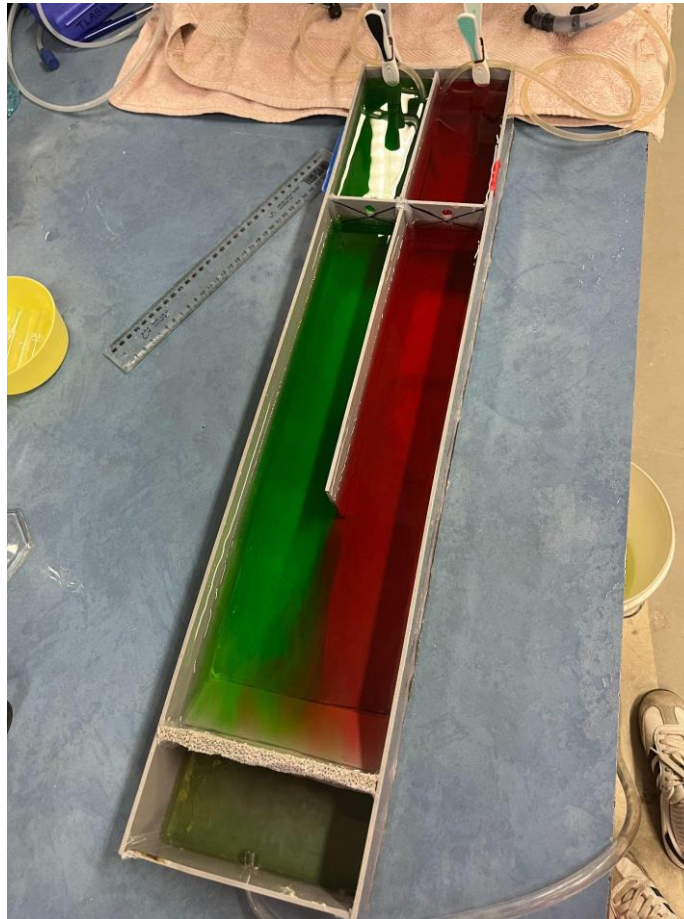


Figure 2.5. Visualization of laminar flow pathways via dye injection; independent water streams maintain discrete paths without cross-contamination until reaching the primary mixing chamber.

2.5 Experimental procedure: Behavioural trials

Behavioural trials were conducted during daylight hours under natural ambient light to assess juvenile flounder behavioural responses to chemical food cues and predator olfactory cues under different pH and temperature treatments. The primary behavioural response measured in all trials was space use within the flume, quantified as the proportion of time spent in each section of the choice chamber in response to chemical cues. Time spent in the stimulus water stream relative to control water was interpreted as attraction (food trials) or avoidance (predator trials).

Upon collection, juvenile flounder were measured (total length) and assigned to one of three size classes: small (< 5 cm), medium (6–7 cm), and large (> 8 cm). Juveniles were gently scooped using a small net and transferred into treatment tanks. For behavioural trials, fish were scooped into a net and gently poured into the flume to

minimise handling stress, and then poured back into their original conditioning tanks following each trial. Two to three fish were stocked into each 2 L mesh basket, which was suspended within a 12 L conditioning tank. Fish were allowed to acclimate to experimental conditions for 24 h prior to behavioural testing. The experimental design consisted of four treatment combinations, each replicated across four independent tanks (16 tanks total).

2.5.1 Food cue trials

Food cue trials were conducted to assess attraction to chemical cues associated with prey. Food-conditioned water was prepared by soaking two whole mussels in 50 L of control seawater for 1 h. This water was used as the food stimulus, with control seawater used as the comparison stream.

For each trial, one fish from a single treatment replicate was placed into the central mixing area of the flume with both water sources running. Fish were allowed a two-minute acclimation period before observations commenced. A white lid was placed over the mixing area to minimise disturbance. Fish position was recorded for five minutes, with location recorded at 30 s intervals (11 time points per trial). The flume was divided into three sections (A–C), corresponding to food cue, control water, and the central mixing zone respectively.

Attraction to food cues was quantified as the proportion of time spent in the food cue section relative to the control and mixing sections. Increased occupancy of the food cue section was interpreted as stronger attraction and cue detection ability.

Food cue trials were conducted once per week for three consecutive weeks (Days 1, 7, and 13 following introduction to experimental treatments). The order of trials was randomised using a random number generator.

2.5.2 Predator cue trials

Predator cue trials were conducted to assess avoidance behaviour in response to chemical cues from a natural predator. In this case the wrasse (*Notolabrus celidotus* or spotty) was selected. It is acknowledged that this is arguably not the best predator to be selected (their most common predators in this habitat are stingrays *Myliobatis tenuicaudatus*, kahawai *Arripis trutta*, snapper *Pagrus auratus*, shags *Phalacrocorax*

carbo novaehollandiae, and kingfishers *Todiramphus sanctus*), however, limitations in the size of the experimental regime and logistics associated with collecting ‘predator cued stimuli’ precluded use of these species. Predator-conditioned water was prepared by turning off water flow to spotty holding tanks for 1 h, allowing predator chemical cues to accumulate. Following this period, 50 L of tank water was collected and used as the predator stimulus. Control seawater was used as the comparison stream.

Trial procedures were identical to those used in food cue trials. For each trial, one fish from a single treatment replicate was introduced to the mixing chamber, allowed a two-minute acclimation period, and observed for five minutes with position recorded at 30 s intervals. Avoidance of predator cues was quantified as reduced occupancy of the predator cue section relative to the control and mixing sections.

Predator cue trials were conducted once per week for three consecutive weeks (Days 2, 8, and 14 following introduction to experimental treatments). To minimise repeated handling stress, fish used in food cue trials were not preferentially selected for predator cue trials on the following day where possible. Trial order was randomised using a random number generator.

2.5.3 Trial controls and bias minimisation

All water sources (control and treatment water) were maintained at the same temperature (23°C) to prevent density-driven flow differences or mixing artefacts within the flume. Prior to experimental trials, a pilot test was conducted to assess whether fish showed a preference for water of differing pH. A random subset of six fish was exposed to two water streams of differing pH (same pH of treatment tanks) in the flume, with all individuals spending the majority of time in the mixing chamber, indicating no inherent pH preference.

To control for potential side bias, the positions of stimulus and control water sources were switched after trial 8. In total, sixteen trials were conducted per stimulus type, with one fish tested from each tank replicate. Predator-conditioned water was supplied to section A in eight trials and to section B in eight trials. Trials were conducted without replacement within a single day, and fish were returned to their original conditioning tanks following each trial.

2.6 Data analysis

The data were collated and analysed in R studio. The proportion of time spent in each chamber was calculated for each individual fish by dividing the number of time intervals spent in the stimulus chamber by the total number of intervals recorded. Fish that remained exclusively in the neutral mixing chamber for the full trial were identified, and the proportion of non-participating fish was calculated both overall and across tank conditions and stimulus types.

A Spearman's rank correlation was used to test for a relationship between fish size (Small, Medium, Large) and the proportion of time spent in the stimulus chamber. This non-parametric method was chosen due to the ordinal nature of fish size and potential non-normality of the data.

The effects of tank conditions (pH: Control vs Low; Temperature: Control vs High) and stimulus type (Predator vs Food) on the proportion of time spent in stimulus chambers were analysed using a three-way ANOVA, testing for main effects and interactions. Separate two-way ANOVAs were conducted for Predator and Food stimuli to examine tank condition effects within each stimulus type. Post hoc analyses were not required, as no significant interactions were detected.

Participation in the choice chamber (i.e., fish leaving the neutral mixing chamber) was analysed using percentage calculations, both overall and by tank condition and stimulus type. Fish that remained in the mixing chamber for the full trial were classified as non-participating, while those that entered either stimulus chamber were classified as participating. Percentages of non-participating fish were reported in tables and visualised in a grouped bar graph (Figure 5). This approach follows methods used by Dixon et al. (2010) and Radford et al. (2012) for assessing activity and participation in choice chambers.

All statistical analyses were conducted at an alpha level of 0.05, and figures were generated to visualise patterns in fish behaviour, including variation across fish sizes, stimulus types, and tank conditions.

Chapter 3 - Pilot study

3.1 Introduction

Early life stages of marine fishes are widely recognised as among the most sensitive phases to environmental stressors, yet the majority of experimental work examining the impacts of ocean acidification (OA) has focused on adult or juvenile life stages of coastal and oceanic species. Comparatively little research has examined the physiological responses of estuarine fishes, despite these environments experiencing some of the most dynamic and extreme fluctuations in carbonate chemistry. Estuaries regularly experience large diel and seasonal shifts in pH driven by freshwater inputs, biological productivity, and restricted water exchange, suggesting that species inhabiting these systems may either possess unique tolerance mechanisms or face significant physiological challenges under future OA scenarios. Despite this ecological importance, experimental OA research has largely centred on reef or open-coast species such as damselfish, clownfish, and pelagic larvae, with only limited investigation into estuarine taxa.

A brief review of the literature highlights this disparity. Numerous experimental studies have investigated behavioural or physiological responses to elevated CO₂ in marine species including coral reef fishes and commercially important pelagic species (e.g., Munday et al., 2009; Dixon et al., 2010; Welch et al., 2014). In contrast, relatively few studies have explored OA effects in estuarine species, and even fewer have examined responses during early ontogenetic stages when organisms are often most vulnerable to environmental stressors. This gap is particularly notable because larval stages represent a critical window in fish life history where survival and recruitment can be strongly influenced by environmental conditions.

Although flatfish development and metamorphosis have been studied in several aquaculture species, there remains very limited research examining how OA conditions may influence these early ontogenetic processes, particularly in estuarine species such as flounder. Understanding larval responses in these taxa is important because many flatfish species rely on estuarine habitats during early life stages, where they may experience naturally variable but increasingly acidified conditions.

The aim of this component of the study was to experimentally investigate the response of larval estuarine flounder to altered carbonate chemistry during a critical early life-history stage. Although complete larval mortality prevented assessment of treatment effects, the study represents an ambitious and worthwhile attempt to address a clear gap in the literature. Importantly, the successful design and implementation of the experimental system demonstrates its suitability for future larval rearing trials, providing a strong methodological foundation for extended investigations by others.

3.2 Methods

This pilot study was designed to develop and validate an experimental system capable of maintaining stable temperature and pH conditions representative of projected end-of-century climate scenarios, prior to its application in larval fish trials. The original experimental design was centred on the use of kingfish larvae; however, their availability was ultimately constrained by factors beyond the authors control. As a result, larval flounder were used opportunistically to test the functionality of the system and the feasibility of conducting larval exposure experiments, and in the realisation that a study on an estuarine species would be of value in progressing this field of research.

Substantial effort was directed toward the establishment and stabilisation of the experimental system. Over a period of several weeks prior to larval introduction, temperature and pH conditions were continuously monitored and iteratively adjusted to achieve consistent and reliable treatment levels. Real-time monitoring was conducted using the APEX control system and associated mobile application, allowing continuous tracking of system performance. These measurements were supplemented by daily manual validation using a handheld YSI probe to ensure accuracy and consistency of pH and temperature across tanks.

To further assess system stability, independent temperature logging was conducted using HOBO data loggers deployed in selected tanks. These loggers were used to quantify diel and overnight temperature fluctuations within the experimental space, which was located in a semi-controlled “hangar” environment at the University of Waikato Coastal Marine Field Station. Data from these loggers informed adjustments to system configuration and insulation to minimise environmental variability and improve thermal stability.

Larval flounder (~14 days post-hatch; approximately 1–2 mm in length) sourced from another experimental program examining bred-in-captivity larval survivorship and development using broodstock fish caught using drag nets near the Manukau Harbour heads (closing the flounder life cycle in aquaria systems) were carefully transferred into experimental tanks using methods designed to minimise handling stress. Due to their small size and fragility, larvae were gently collected from the water column using a 200 μm mesh. Individuals retained on the mesh were then carefully rinsed into a beaker using a squeeze bottle containing distilled water, allowing transfer without direct physical handling. Larvae were subsequently introduced into treatment tanks at a stocking density of 50–60 individuals per tank.



Figure 3.1. Image of larvae flounder showing size (~1.7mm) and development when placed into pilot study experimental setup.

The final experimental setup consisted of multiple replicate tanks maintained under controlled pH and temperature treatments. Once stable conditions were achieved, larval flounder were introduced to the system, providing a practical test of system performance under live experimental conditions. Across all treatments, larvae were provided daily rotifer feed with algal enrichment aimed at improving prey visibility.

3.3 Results and discussion

Despite consistent husbandry practices and provision of live food, larval mortality was rapid and severe (as indeed it was with the same source stock of larval fishes in separately located large-scale holding tanks associated with another study). After 24 hours, fewer than half of the stocked larvae remained alive in all tanks, with no discernible relationship between experimental treatment and survival. By 48 hours post-stocking, complete mortality occurred in all tanks, preventing further data collection.

The observed mortality pattern corresponded with a pronounced die-off in the original broodstock at approximately 14-dph, suggesting a developmental bottleneck in aquaria bred larvae, rather than a treatment effect. The metamorphosis from a symmetrical larva to an asymmetrical juvenile involves extensive cranial remodelling, which represents a critical developmental bottleneck highly susceptible to environmental disruption (Schreiber, 2013; Chambers et al., 2014). Research on larval development also shows that the thyroid hormone surge required to trigger cranial remodelling begins significantly earlier than actual eye migration and creates a high metabolic demand. Some studies have noted this could begin as early as 7-14 dph specifically in flatfish species like *Solea senegalensis* where the onset of metamorphic behavioural and physiological shifts is recorded as early as 9-dph (Yúfera & Darias, 2007; Blanco-Vives et al., 2012; Boglione et al., 2013). This period is often marked by a “point of no return” where physiological stress or inadequate energy reserves leads to an irreversible decline (Yúfera & Darias, 2007).

Because the larvae were very small and near-transparent, visual counts were challenging, introducing uncertainty into exact survival estimates; nonetheless, the overarching pattern of mass loss was clear and consistent across all replicates. Although the experiment did not yield interpretable treatment effects, the observed outcomes align with well-documented biological phenomena affecting larval fish survival during early ontogeny. Larval fish in the first weeks after hatching typically experience extremely high mortality rates due to intrinsic limitations in physiology and feeding performance, often referred to as the “critical period” of larval life history. Mortality rates during this transition often exceed 90% in both wild and hatchery settings due to the precise timing required for successful prey capture before yolk reserves are depleted (Hjort, 1914; Houde, 2008). This period is characterised by the transition from endogenous yolk

feeding to exogenous feeding and is universally recognised as a major bottleneck for survival in both wild and cultured contexts (Garrido et al., 2016). By situating the observed mass mortality within the established 'critical period' framework, these findings demonstrate that the experimental failure was likely driven by universal ontogenetic bottlenecks rather than specific treatment variables, highlighting the extreme physiological sensitivity of flounder during the transition to exogenous feeding.

One mechanistic explanation for high mortality in first-feeding larvae relates to hydrodynamic constraints on feeding performance (Holzman et al., 2015). Newly feeding larvae operate at low Reynolds numbers, where viscous forces dominate fluid interactions, limiting effective prey capture even under high prey densities (China & Holzman, 2014). This phenomenon, termed hydrodynamic starvation, can substantially reduce feeding success and lead to starvation despite the presence of abundant food in the environment. Experimental evidence shows that hydrodynamic constraints may limit the larval capacity to capture prey because their feeding apparatus and swimming ability are not yet sufficiently developed to overcome the physical limitations imposed by fluid viscosity at very small body sizes (China et al., 2017).

Starvation, therefore, remains a leading cause of larval mortality when larvae begin exogenous feeding, and high mortality early in rearing trials even with sufficient prey offered has been observed in many marine species. For example, models and empirical studies on marine larvae indicate that starvation accounts for the largest portion of mortality prior to early development stages in species such as tuna and sardine larvae, with significant larval death occurring within 48 hours of first feeding (Garrido et al., 2016).

The consistent occurrence of mass mortality at around 14 dph in both this experimental cohort and the original rearing batch further suggests that intrinsic developmental thresholds may be at play; many marine fish species experience significant mortality during the transition from yolk dependency to sustained exogenous feeding regardless of rearing conditions (Zambonino-Infante et al., 2008; Xu et al., 2023).

Although the trial did not yield interpretable treatment effects, this study functions effectively as a pilot experiment that reveals critical challenges in the larval rearing of flounder at the targeted developmental stage. The development of an experimental

system that was autonomous and robust in a crude experimental precinct (the University of Waikato Marine Field Stations' 'hangar') was successful and lends itself to future work on early life history stages of aquatic fish and invertebrates.

Chapter 4 - Behavioural Experiment Results

4.1 Effect of Fish Size on Stimulus Chamber Use

Spearman's rank correlation revealed a very weak, non-significant positive relationship between fish size and the proportion of time spent in the stimulus chamber (all stimuli combined) ($\rho = 0.087$, $p = 0.40$). This indicates that fish size did not meaningfully influence stimulus preference, with Small, Medium, and Large fish showing broadly similar patterns of behaviour (Figure 4.1).

However, when examining the data more closely, Medium and Large fish were more often observed within the stimulus chambers, whereas Small fish tended to spend more time in the neutral mixing chamber. While these trends were not statistically significant, they may suggest biologically meaningful patterns. For example, smaller fish could have been more affected by handling and capturing stress, leading them to remain in the mixing chamber, whereas larger fish may have been more exploratory and better able to navigate the experimental setup.

These observations highlight a potential size-related difference in activity and exploratory behaviour, which, although not statistically confirmed in this experiment, could be a useful focus for future studies examining behavioural responses of fish under experimental conditions.

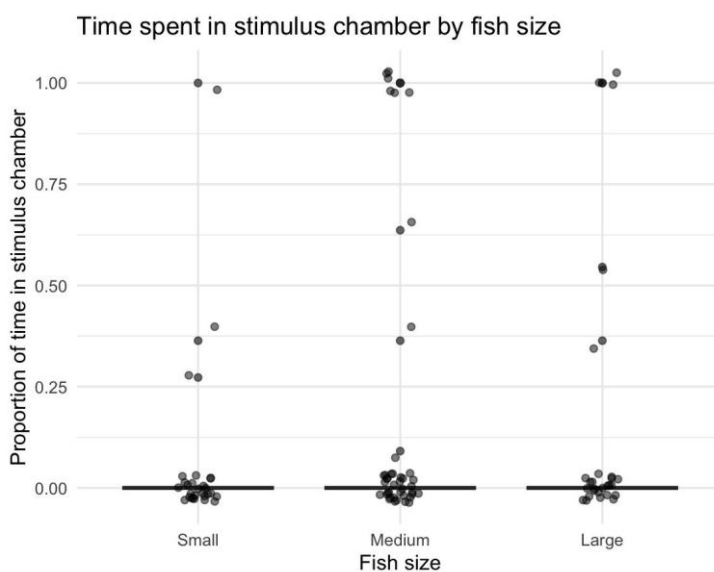


Figure 4.1. Proportion of time spent in the stimulus chamber by fish size (Small, Medium, Large). Boxplots show median and interquartile range, with individual points overlaid. No meaningful differences were observed between fish sizes.

4.2 Effect of Tank Conditions on Stimulus Chamber Use

To visualise the effects of tank conditions, separate boxplots were generated for predator and food stimuli.

For predator stimulus fish exhibited broadly similar chamber use across all four tank conditions (Control pH $\times$ Control Temp, Control pH $\times$ High Temp, Low pH $\times$ Control Temp, Low pH $\times$ High Temp), with no substantial differences in median or variability (Figure 4.2). A notable exception was observed in the Low pH $\times$ Control Temp treatment. In this group, three individual fish spent over 80% of the trial time in the Predator stimulus chamber (Figure 4.2). While the overall median for this condition was not statistically different from other treatments, these high-response individuals suggest that some fish may be particularly sensitive to predator cues under low pH conditions.

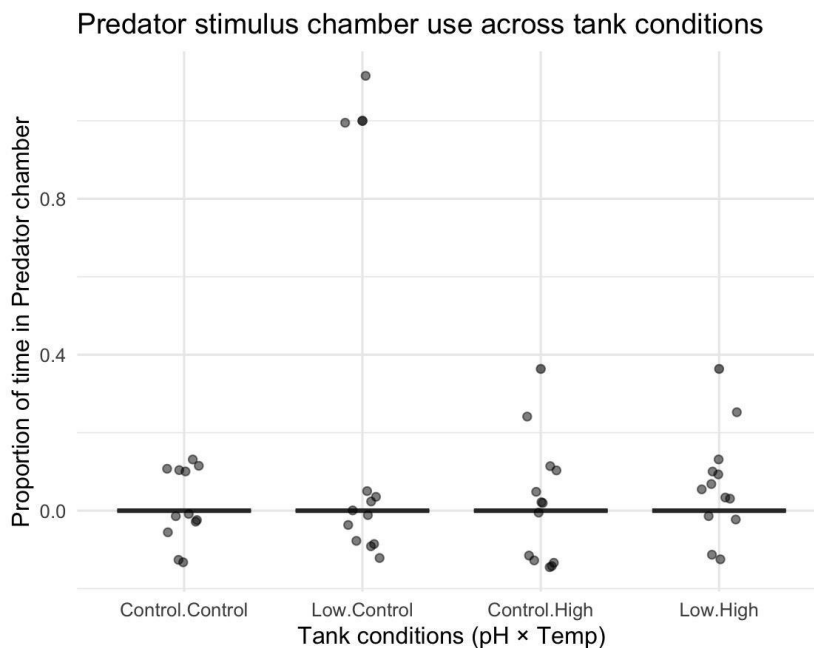


Figure 4.2. Proportion of time spent in Predator stimulus chambers across four tank conditions. Boxplots show median and interquartile range, with individual points overlaid. Behaviour was broadly consistent across conditions.

For food stimulus, fish behaviour was largely consistent across tank conditions, although high-temperature tanks showed a weak, non-significant trend toward higher

time spent in Food chambers (Figure 4.3). The pH and temperature control fish appear to be randomly dispersed throughout the chamber based on their times within it, which would be expected. The fact that the low pH and control temperature fish were distinctly separated to either no time or a significant amount of time in the food chamber indicates an effect in response to pH albeit insignificant given the wide spread of data. The trend does however indicate cause for further examination in future work. All other treatments also demonstrated a partitioning of responses.

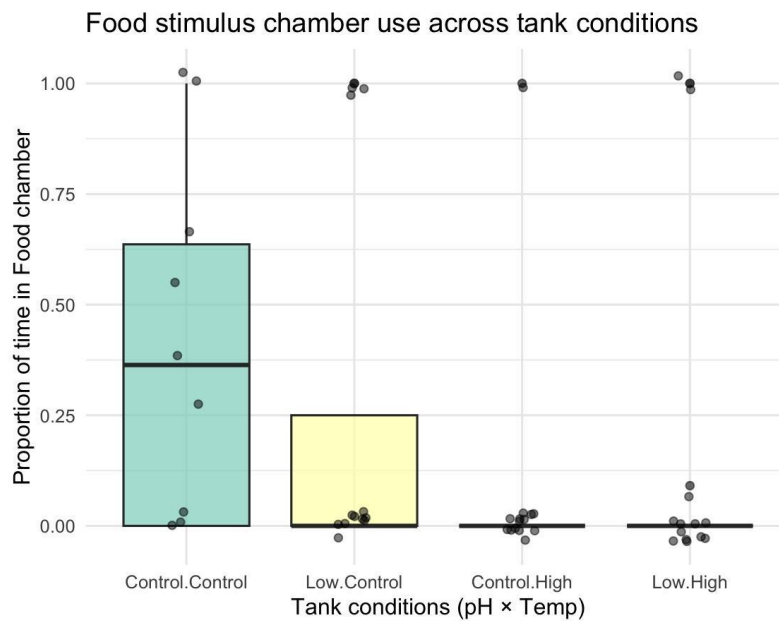


Figure 4.3. Proportion of time spent in Food stimulus chambers across four tank conditions. Boxplots show median and interquartile range, with individual points overlaid. Behaviour was variable across treatments, with Low pH × Control Temp fish showing a distinct separation between individuals spending very little or substantial time in the Food chamber, indicating potential pH effects.

4.3 Statistical Analysis

A three-way ANOVA testing the effects of stimulus type, pH, and temperature confirmed these observations. Results are summarised in Table 4.1. There was a significant main effect of stimulus type (Predator vs Food; $F_{1,88} = 5.87$, $p = 0.017$), indicating that, across all tank conditions, fish spent a greater proportion of time in Predator stimulus chambers compared with Food chambers. This suggests that predator cues elicited a stronger behavioural response than food cues, independent of pH or temperature. Temperature also had a significant effect (Control vs High; $F_{1,88} = 4.36$, $p = 0.040$), with fish in high-temperature tanks spending slightly less time in the stimulus

chambers overall, indicating that overall activity was mildly reduced under higher temperature conditions. There were no significant effects of pH ($F_{1,88} = 0.39$, $p = 0.532$) and no significant two-way or three-way interactions (all $p > 0.20$), indicating that the effect of stimulus type was consistent across tank conditions given the degree of variation observed. The three-way interaction of Stimulus type $\times$ pH $\times$ Temperature was near-significant ($F_{1,88} = 2.99$, $p = 0.087$), suggesting a potential trend whereby the combined effects of stimulus type, pH, and temperature may influence fish behaviour. Although not statistically significant, this result indicates that predator versus food responses could be subtly modulated by environmental conditions, highlighting a possible direction for further investigation.

Table 4.1: F-values and degrees of freedom from three-way ANOVA testing the effects of stimulus type, pH, and temperature on proportion of time spent in stimulus chambers. Significant effects are marked with *.

Effect	F	df	p-value
Stimulus type (Predator vs Food)	5.874	1, 88	0.017*
pH (Control vs Low)	0.394	1, 88	0.532
Temperature (Control vs High)	4.359	1, 88	0.040*
Stimulus type $\times$ pH	0.666	1, 88	0.417
Stimulus type $\times$ Temp	1.647	1, 88	0.203
pH $\times$ Temp	0.134	1, 88	0.715
Stimulus type $\times$ pH $\times$ Temp	2.994	1, 88	0.087

To visualise the main effect of stimulus type, a bar plot of mean proportion time per stimulus was generated (Figure 4.4). There is a significant difference in the main factors (Predator vs Food $p=0.017$), that clearly shows the experiment can robustly detect behavioural responses in the test animals. Furthermore it is clear that despite pH or Temperature treatments examined in this work, predators significantly deterred fish

behaviours thus validating that experimental regime (predator attraction or avoidance) in the trials conducted.

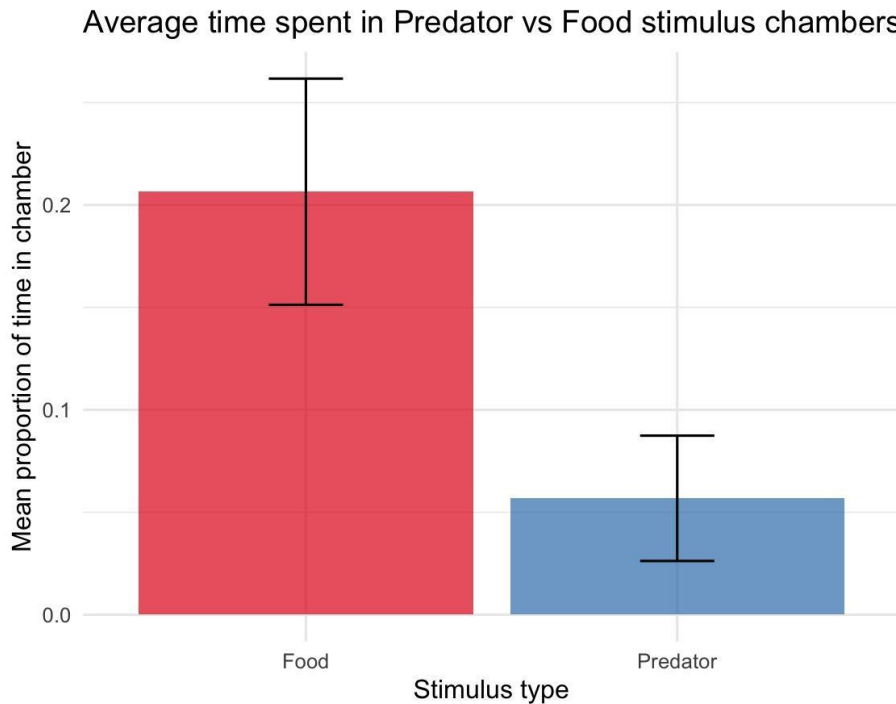


Figure 4.4. Mean proportion of time spent in Predator vs Food stimulus chambers across all tank conditions. Bars represent the mean $\pm$ standard error. Fish spent slightly different proportions of time in the two stimuli, consistent with the significant main effect of stimulus type observed in the ANOVA.

4.4 Time Spent in the Mixing Chamber

Many fish did not enter either stimulus chamber and remained in the neutral mixing chamber for the duration of the trial. Overall, 66.7% of fish stayed in the mixing chamber across all trials.

The proportion of fish staying in the mixing chamber varied depending on tank condition and stimulus type (Table 4.2, Figure 4.5). Fish were generally more likely to remain in the mixing chamber when exposed to predator cues compared to food suggesting possibly that the avoidance of a predator was a stronger stimulus than attraction to food. Tank conditions also influenced the likelihood of remaining in the mixing chamber, with Control pH, High Temp having the highest proportion of fish staying in the mixing chamber. There was also a suggestion (not statistically significant) that lower numbers of fish stayed in the mixing zone in Predator treatments for all non-temperature/pH control treatments. That is, there may be some influence of pH and or

temperature increase that dampened the juvenile flounder's natural avoidance response to predators. The opposite was the case for the Food treatments.

Table 4.2: Percentage of juvenile flounder remaining within the mixing chamber across varying environmental treatments (pH and temperature) during food-seeking and predator-avoidance trials.

Tank condition	Food (%)	Predator (%)
Control pH, Control temp	33.3	83.3
Control pH, High temp	84.6	75
Low pH, Control temp	50	66.7
Low pH, High temp	64.3	66.7

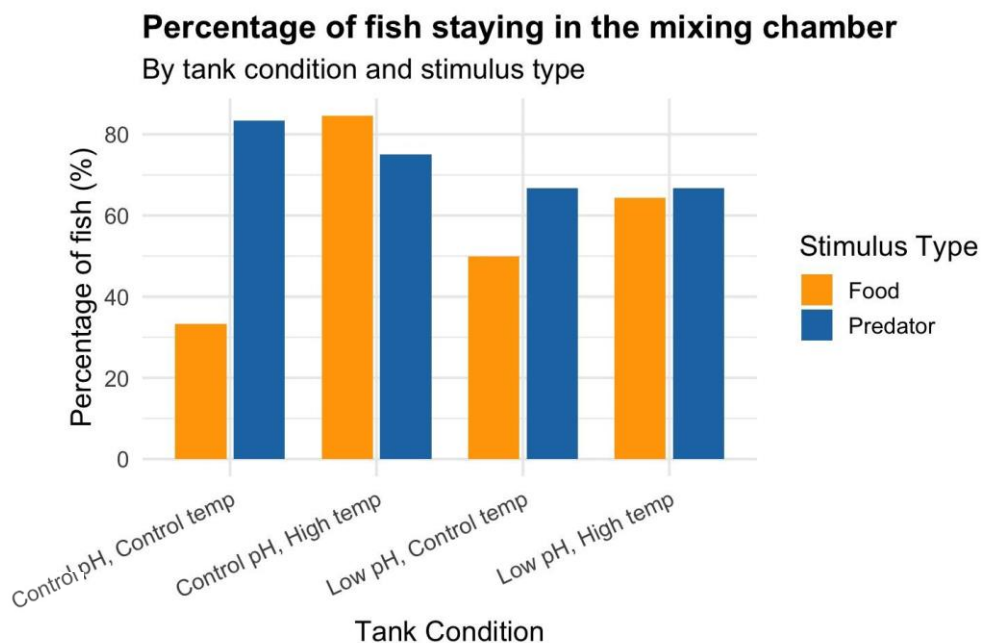


Figure 4.5. Percentage of fish that remained in the mixing chamber for the full test under different tank conditions and stimulus types. Bars are grouped by tank condition and coloured by stimulus type (orange = Food, blue = Predator). Fish were generally more likely to stay in the mixing chamber when exposed to predator cues, with the highest proportion observed in Control pH, High Temp conditions.

4.5 Overall Patterns

Across all analyses, fish size had minimal influence on chamber use, with no significant relationship detected between size and time spent in stimulus chambers (Figure 4.1). However, Medium and Large fish were more frequently observed within stimulus chambers, while Small fish more often remained in the neutral mixing chamber. Although not statistically significant, this pattern suggests potential size-related differences in exploratory behaviour or sensitivity to experimental handling.

Tank conditions showed subtle but noteworthy effects on behaviour (Figure 4.2). Temperature had a significant influence, with fish in high-temperature treatments generally spending less time in stimulus chambers, indicating a reduction in overall activity. In contrast, pH alone had no significant effect; however, visual patterns in the data suggest that low pH may still influence behaviour in more complex ways. For example, under Low pH $\times$ Control Temp conditions, fish responses to both predator and food stimuli appeared more variable and, in some cases, more extreme, with individuals either strongly engaging with or avoiding stimulus chambers.

Fish responses differed between stimulus types, with a significant overall difference in the proportion of time spent in Predator versus Food chambers. Inspection of the data (Figure 4.4) shows that fish spent more time in Food stimulus chambers than Predator chambers across treatments. This suggests that food cues elicited a stronger behavioural response than predator cues, potentially reflecting attraction to food resources or avoidance of predator-associated areas. This finding is further supported by the high proportion of fish remaining in the mixing chamber during predator trials, suggesting that predator cues may induce cautious or avoidance behaviour rather than active exploration.

A substantial proportion of fish (66.7%) remained in the neutral mixing chamber for the entire trial, highlighting generally low levels of participation. This tendency was influenced by both tank condition and stimulus type, with the highest proportions

observed under Control pH $\times$ High Temp conditions and during predator exposure. There was also a suggestion that under altered environmental conditions (low pH and/or elevated temperature), fewer fish remained in the mixing chamber during predator trials, potentially indicating a disruption or dampening of natural avoidance responses.

Although many of these patterns were not statistically significant, the near-significant three-way interaction between stimulus type, pH, and temperature suggests that fish behaviour may be influenced by the combined effects of environmental conditions. Overall, the results indicate that while stimulus type and temperature had measurable effects on behaviour, pH and fish size showed more subtle influences, and individual variability played a substantial role in shaping observed responses. These trends highlight important areas for further investigation, particularly regarding how environmental stressors interact to influence behavioural responses during early life stages.

Chapter 5 - Discussion

5.1 General Discussion

Understanding how key physical drivers of climate change particularly ocean warming and acidification affect marine organisms has become a central focus of contemporary marine science; however, this body of work is heavily biased toward oceanic and reef-associated species, with comparatively little attention given to estuarine systems. This is a notable gap, as estuarine species are routinely exposed to highly dynamic and often extreme environmental conditions, yet their responses to future climate scenarios remain poorly resolved. Addressing this gap, this study focused on juvenile flounder, an ecologically relevant estuarine species, to examine how reduced pH and elevated temperature influence behavioural responses to ecologically important chemical cues.

This study explored how reduced pH and elevated temperature, under projected end-of-century climate scenarios, influenced juvenile flounder behavioural responses to food and predator olfactory chemical cues. Overall, behavioural responses were weak and highly variable, with fish size and pH having little apparent influence on swimming toward or away from chemical cues (food or predators). In contrast, stimulus type and temperature had small but statistically significant effects on cue-directed behaviour. Fish spent a greater proportion of time in predator cue chambers compared to food chambers, suggesting that predator cues elicited a stronger behavioural response overall. Interestingly, a substantial proportion of individuals remained within the neutral mixing chamber for the duration of trials, particularly during predator cue exposure and under elevated temperature conditions, raising questions about how sedentary species express behavioural responses in experimental assays. For a flatfish species that relies on crypsis and remaining stationary to avoid detection, a "no-choice" result in a flume may actually represent a natural defensive strategy rather than a lack of sensory perception (Killen et al., 2017). There were however interesting trends in the data that suggested some response to these stimuli, even given the basic experimental setup that was developed and acknowledging the low replicate number possible of experimental fish.

Contrary to expectations from previous studies suggesting that ocean acidification can impair olfactory discrimination (Lecchini et al., 2017), reduced pH alone did not seem to alter flounder swimming toward or away from either food or predator chemical cues.

Could this indicate that juvenile flounder possess some degree of behavioural resilience to moderate acidification? Given that flounder naturally experience substantial diel and tidal pH fluctuations in estuarine environments, where pH can vary by ~0.3–0.7 units over short time scales and temperatures can fluctuate several degrees daily depending on tidal state and solar input (Hofmann et al., 2011; Baumann et al., 2015), and this environmental variability may precondition them to cope with moderate reductions in pH that would otherwise disrupt the neurosensory systems of open-ocean fish. In addition, estuarine habitats are characterised by pronounced salinity fluctuations driven by freshwater inflow and tidal mixing, meaning juvenile flounder are routinely exposed to simultaneous changes in multiple physicochemical parameters (Elliott & Quintino, 2007). This environmental variability may precondition them to cope with moderate reductions in pH that would otherwise disrupt the neurosensory systems of open-ocean fish. (Feugere et al., 2021; Heuer & Grosell, 2014), it is not surprising that these experiments returned some inconclusive results. However, subtle patterns in the data suggest that pH effects may not be entirely absent. For example, under Low pH × Control temperature conditions, responses to both predator and food cues appeared more polarised, with individuals either showing strong responses or little to none. Similarly, several individuals in low pH treatments spent a high proportion of time in predator chambers. While not statistically significant, these patterns suggest that low pH may alter behavioural variability or individual responsiveness rather than producing a consistent directional effect. However, our short-term exposure was insufficient to elicit detectable behavioural changes (Heuer & Grosell, 2014), and longer or chronic exposure could produce different responses. The subtle trends identified above are intriguing and warrant further investigation.

Temperature, on the other hand, had a small but significant effect on cue-directed behaviour. Higher temperatures may have increased metabolic demand, resulting in reduced exploratory behaviour and less time spent in stimulus chambers overall. Higher temperatures may have increased metabolic demand or general activity, subtly influencing movement toward chemical cues (Pankhurst & Munday, 2011; Volkoff & Rønnestad, 2020). However, the effect was weak and did not interact with pH or stimulus type, suggesting that elevated temperature may alter general activity more than the ability to detect or respond to chemical cues. This suggests that elevated temperature may constrain movement rather than enhance it, potentially reflecting energetic trade-offs under thermal stress. This raises the question: to what extent do metabolic constraints influence cue responsiveness in sedentary species such as flounder (Pankhurst & Munday,

2011; Killen et al., 2017). The near-significant three-way interaction between stimulus type, pH, and temperature suggests that behavioural responses to predator versus food cues may be subtly modulated by environmental conditions. Although not statistically significant, this trend indicates that combined stressors could influence behaviour in more complex ways than single-factor effects alone.

Fish spent slightly more time in predator than food stimulus chambers overall, suggesting that predator cues elicited a stronger behavioural response. Yet, the high proportion of fish remaining in the neutral mixing chamber prompts us to consider whether remaining stationary is itself a meaningful behavioural response (Andrade et al., 2018). Staying in the neutral zone likely reflects a cautious, risk-averse strategy rather than a lack of cue detection (Mitchell et al., 2020). For a naturally sedentary species like flounder, this seems plausible; the neutral chamber may have acted as a perceived refuge, particularly during predator cue exposure or under elevated temperature, allowing individuals to balance exploration with risk avoidance (Wilson & Godin, 2010). This interpretation highlights the importance of considering species-specific ecology when designing behavioural experiments. As noted by Ferrari et al. (2010), directional swimming toward a cue is not the only way to express "detection" of that cue; for cryptic, benthic species, remaining stationary is often the most adaptive anti-predator response.

Fish size did not appear to meaningfully influence swimming toward or avoiding chemical cues, with small, medium, and large juveniles showing broadly similar patterns. However, there was a tendency for medium and large individuals to more frequently enter stimulus chambers, while smaller fish more often remained in the neutral zone. While not statistically significant, this may reflect differences in stress tolerance or exploratory behaviour, with smaller fish potentially more affected by handling or experimental conditions. This raises interesting questions about the ontogeny of cue use and risk sensitivity in juvenile flounder. While sensory acuity and swimming abilities generally increase along ontogeny (Morais et al., 2017), early juvenile stages often prioritise general directional orientation over fine-tuned, size-dependent risk assessment (Faillettaz et al., 2020). The narrow size range in this study may have limited our ability to detect size-dependent differences, but it is also possible that flounder rely more on individual behavioural tendencies—often described as "boldness" or "exploration" traits—which can be independent of body size or sex in early life stages (Andrade et al., 2018; Fraser et al., 2001).

The high use of the neutral mixing chamber (66.7% of fish across trials) underscores both the challenges of studying sedentary species and the importance of interpreting stationary behaviour as a valid response. In mobile animal populations, it is common to observe "leptokurtic" movement distributions, where most individuals remain at or near a starting point while only a few move long distances (Fraser et al., 2001). Could remaining in the neutral chamber represent a conservative behavioural strategy, reflecting an intrinsic trade-off between exploration and safety, particularly under perceived predation risk or thermal stress (Andrade et al., 2018; Mitchell et al., 2020)? Future experiments could test this hypothesis by modifying flume design, reducing the size of the neutral zone, or adding structural complexity to encourage movement, while still recognising that stationary behaviour is meaningful for sedentary species.

The weak effects of pH and modest effects of temperature observed here suggest that juvenile flounder may retain functional cue-mediated behaviour under near-future climate conditions over the short term. However, the tendency to reduce movement toward stimulus chambers and remain in the neutral zone under elevated temperature likely stems from physiological trade-offs; as temperatures rise, the standard metabolic rate (SMR) increases, which can lead to shifts in energy allocation away from active foraging or risk-assessment behaviours (Schwieterman et al., 2019; Volkoff & Rønnestad, 2020). Reduced directional movement might lower encounter rates with prey, potentially affecting growth and survival over longer timescales (McLeod et al., 2013). This raises broader questions about how climate stressors might interact with the natural sedentary behaviour of flounder to shape ecological outcomes in estuarine systems.

5.2 Limitations and future recommendations

While this research provides valuable insight into the resilience and behavioural strategies of yellowbelly flounder, several limitations must be considered. The total mortality observed in the pilot study highlights the extreme difficulty of rearing *R. leporina* through the "critical period" of metamorphosis (Chambers et al., 2014). This prevented the assessment of how climate stressors impact the transition from symmetrical larvae to asymmetrical juveniles.

This experiment used stable pH and temperature treatments. However, *R. leporina* inhabit estuaries which are highly variable environments where pH can swing by >0.5 units daily

(Feugere et al., 2021). The lack of behavioural response to reduced pH may be due to an evolved physiological tolerance to variability in water pH which was not fully represented in the lab setting of this experiment.

Traditional choice chamber experiments assume that behavioural choice is through active movement. For a cryptic, benthic species the high non-participation rate of 66.7% suggests that the experimental apparatus may conflict with the species natural defence instinct to remain stationary when stressed or sensing a predator (Wilson & Godin, 2010). Future research should move away from standard flow-through flumes and towards open-field mesocosms with natural substrates such as sand. This could then allow for the measurement of choice through crypsis efficiency or burial depth which is more relevant for sedentary fish (Seibel & Drazen, 2007; Killen et al., 2017).

This study focused on acute responses due to time constraints. Longer-term studies are needed to investigate whether chronic exposure to acidified water leads to gradual acclimation or if the metabolic cost of maintaining pH balance eventually impacts growth and survival (Kroeker et al., 2013).

Finally, the experimental apparatus that was constructed was ambitious and constituted a major undertaking in this research program. It was designed and constructed out of necessity as the operating environment (an open hangar) created significant challenges to running experiments manipulating fine scale differences in temperature and pH. It is a system that could now be utilised in other research in remote locations, mindful that improvements can always be made.

5.3 Conclusions

This research investigated the interactive effects of ocean acidification and climate warming on the early life stages of the yellowbelly flounder (*Rhombosolea leporina*), a taonga species. By simulating end of century scenarios (pH ~7.3 and +5°C), this study highlights that the early life stages of New Zealand flatfish are highly sensitive to shifting marine chemistry and thermal regimes.

Juvenile flounder demonstrated notable resilience to reduced pH, maintaining the ability to discriminate between food and predator olfactory cues even under projected end of

century scenarios. This suggests that as osmoregulators, bony fishes may possess a moderate degree of resilience to elevated CO₂ however, elevated temperature acted as a significant behaviour modifier, subtly altering activity levels and increasing the likelihood of fish remaining stationary. Overall, given the lack of significance in analyses, the results highlight that while acidification may not impair sensory perception, rising temperatures may force energetic trade-offs that favour risk aversion and inactivity over active foraging (Volkoff & Ronnestad, 2020). Having said that, some interesting trends in the data suggest some alterations may be occurring, especially in damping aversion to predators. While not significant, the fact that there was some variation in behaviours even to relatively low changes in temperature and pH (compared to what one would expect flounder to be exposed to in a highly tidal estuary), warrant further attention.

For a taonga species like the yellowbelly flounder, these findings offer a degree of optimism regarding chemical resilience but signal a clear warning regarding thermal thresholds. As coastal waters continue to warm, the metabolic costs of occupying shallow nurseries may increase, potentially altering the recruitment dynamics of this culturally and economically vital fishery (Munday et al., 2010; Volkoff & Ronnestad, 2020). To ensure the continued resilience of this species, fisheries management must move beyond single stressor models and account for the complex, stage specific interactions between a warming ocean and the unique benthic ecology of New Zealand's flatfish.

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