What stress sequence kills: different sequences of cold and freshening stress with varying latency periods and mortality of *Tigriopus californicus* copepodids.

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Table 2. The results of a general linear mixed model using template model builder on binomial data with tide pool number (10 total) nested in experiment number (2 total) as the random effects. A significance value of 0.05 was used and estimates represent the log odds value. The effects are the different stress sequences (cold-cold [CC], cold-fresh [CF], fresh-cold [FC], and fresh-fresh [FF]) and the interaction between the stress sequences and latency time.

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Abstract

Anthropogenic activities are increasing the number of extreme events in nature prompting researchers to study how multiple stressors impact animals. Currently, few studies have researched sequential stressors. However, in nature, stressors generally act one after another making sequential stressor studies increasingly more important to properly inform management practices. Responses of animals can change when stressors are applied in different sequences and with different amounts of time in benign conditions (latency time) between stressors. I test how mortality changes with different sequences of 12-hour cold (-15 °C change) and freshening (34% salinity of seawater) stress with three latency periods of 0, 12, and 24 hours. I use a model organism without laboratory rearing for physiology, *Tigriopus californicus*, which is an abundant harpacticoid copepod found in high intertidal splash pools. I find that hypo-salinity has a more severe effect than cooling disturbance and that stress sequence changes the mortality of *T. californicus*. In particular, there was high fatality in stress sequences that included freshening while individuals survived better in sequences that only had cold stress. The low recovery in hyposaline water shows that *T. californicus* had a poor capacity to osmo-regulate in 12 ppt hypoosmotic surroundings while the low mortality observed following cold stress indicates individuals were adapted for temperatures below freezing. Furthermore, there was a positive relationship between survival and latency time when hypoosmotic conditions were applied before low temperature stress showing that exposure to hypoosmotic extremes increases the ability of *T. californicus* to withstand cold. My results support the hypothesis that the sequence of extreme events and timing between stressors may change whether an organism lives or dies. This research adds to studies of multiple stressors and can be important for reference to future studies investigating sequence effects.

Keywords: multiple stressors, harpacticoid copepod, hypoosmolality

1. Introduction

Animal populations are exposed to an increasing number of abiotic and biotic stressors because of anthropogenic effects (Intergovernmental Panel on Climate Change, 2022). Stressors are a variable that causes negative impacts on organisms when they surpass the normal range that the animal is adapted to (Barrett *et al.*, 1976). Currently, stressors are the biggest threat to ecosystems including driving biodiversity change (Intergovernmental Panel on Climate Change, 2022). Thus, discovering organismal stress reactions is important for predicting how communities may change and allows us to take the appropriate management steps to prevent further change (Côté *et al.*, 2016). Model organisms are beneficial to use for understanding organismal stress responses as they could inform us about the impact of stressors on other species (Matthews *et al.*, 2020). Finally, to make the most informed conclusions regarding possible responses to stressors in the wild, studies must use stressors organisms would experience naturally (Gunderson *et al.*, 2016; Pirotta *et al.*, 2022).

Understanding how animal responses change when experiencing multiple stressors is important for developing management strategies to increase ecosystem health (Pirotta *et al.*, 2022). Stressors rarely act in isolation; therefore, multiple stressor studies are becoming increasingly more common across many disciplines (Dong *et al.*, 2014; Orr *et al.*, 2020). When stressors act alone organisms may be able to survive the extreme conditions; yet, when multiple stressors act either at the same time or sequentially, organisms may have a much harder time adapting (Gunderson *et al.*, 2016). Similarly, organismal responses to individual stressors cannot predict the response when both stressors are applied together making multiple stressor studies crucial (Kelly *et al.*, 2016).

Currently, most multiple stressor studies impose combinations of stressors simultaneously; however, stressors can also act independently at different times, falling sequentially (Gunderson *et al.*, 2016). For instance, in the summer, tide pools warm during the daytime, while pH reduces at night because of the creation of carbon dioxide due to respiration. Thus, dependent on tidal cycle timing, organisms can be exposed to both potential stressors sequentially (Morris and Taylor, 1983). The importance of researching sequential stressors is that responses to simultaneous stressors, which are currently the main area of focus in stressor research, cannot predict the response to stressors falling one after another and thus are uninformative (Figure 1; Bible *et al.*, 2017; Orr *et al.*, 2020). Two responses that are possible when stressors are applied in sequence but not simultaneously are cross-tolerance and cross-susceptibility. Cross-tolerance occurs when the first stressor increases the ability of an organism to deal with a second stressor thus increasing survival while cross-susceptibility has the opposite effect (Gunderson *et al.*, 2016). Antagonistic effects are commonly seen during cross-tolerance because the same internal stress response pathway is activated (Côté *et al.*, 2016). If the same proteins are created as a response to both stressors, then less energy will be expended to cope with the stressors because proteins would be present in the organisms' system and not have to be remade when exposed to the second stressor. By contrast, organisms experiencing cross-susceptibility are more likely to have synergistic reactions which happen when stressors impact separate internal mechanisms that interact with each other (Gunderson *et al.*, 2016). Synergistic responses to multiple stressors are greater than the separate responses to each stressor added together (Côté *et al.*, 2016). Discovering which stressors cause cross-tolerance and cross-susceptibility ultimately allows us to understand how sequential stressors impact animals (Fukami, 2001; Jackson *et al.*, 2021; Frishkoff *et al.*, 2018).

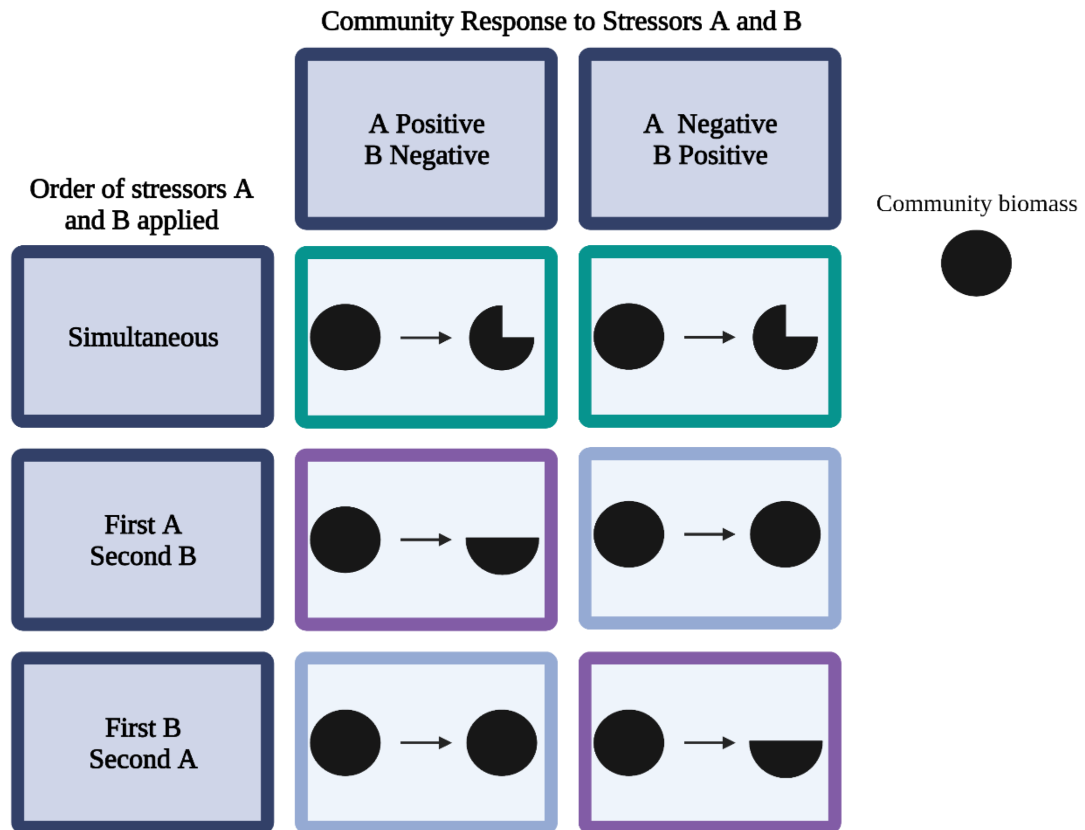

Figure 1. A conceptual diagram, adapted from MacLennan and Vinebrooke (2021), showing how community biomass changes with different orders of two stressors (A and B) when responses to one stressor is positive and the other is negative. The colours outlining boxes indicate that the same biomass change as a result of the stressors is seen. Created with BioRender.com.

The duration and timing of stressors can have an impact on an organism's stress response, and understanding these impacts can provide valuable insights into how organisms adapt to unsuitable conditions. When stressors are applied in sequence the amount of time in normal conditions between stressors is known as the latency period (Orr *et al.*, 2020). Depending on latency duration, stressors in sequence may be coupled or decoupled and cause a variety of

different animal responses. Coupled stressors are applied back-to-back or with time to recover partially from the first stressor but not fully and interact to determine an animal's stress response suggesting the reactions to the second stressor are reliant on the first stressor applied (Thompson *et al.*, 2018). Studies report that the most negative reactions are seen in back-to-back stressors (no or little latency) because the organism is under stress for a longer total duration time without any recovery period (Paine *et al.*, 1998; Gunderson *et al.*, 2016; Bible *et al.*, 2017). Stressors applied with time to recover but not enough for full recovery are likely to cause antagonistic reactions which is less than the additive effects of the two stressors. An organism adapts easier to a second stressor because of their previous experience with the first (Cheng *et al.*, 2015; Brooks and Crowe, 2019). On the other hand, decoupled stressors, which are far apart in time, do not interact and organismal response to a second stressor is not determined by an earlier stressor (Gunderson *et al.*, 2016). Usually, an additive effect is seen when stressors are decoupled because there is full recovery between the stressors thus causing an animal to respond to each stressor separately (Gunderson *et al.*, 2016; Bible *et al.*, 2017). Nonetheless, latency duration along with the type of unsuitable conditions applied, impacts an organism's survival and stressors just above or below normal ranges can have huge effects (Brooks and Crowe, 2019; Mayling *et al.*, 2018; Bible *et al.*, 2017). For example, in Bible *et al.*'s (2017) study, the mortality of Oysters (*Ostrea lurida*) depended on the sequence of low salinity stress, high-temperature exposure, and the latency duration. Latency time also determined whether hypoxic and warming or freshening stressors had an additive or synergistic impact on this species (Cheng *et al.*, 2015). Generally, researchers apply only one latency duration time (rather than testing for differences in duration) when looking at stressors in sequence, especially in studies that focus on the marine environment (Orr *et al.*, 2020; Gunderson *et al.*, 2016). However, highly informed conclusions can be made when testing stress

sequences with varying latencies because these studies provide substantial information about organismal stress reactions in a variety of different situations (Cheng *et al.*, 2015; Brooks and Crowe, 2019).

In the ocean, low salinity occurs via melting glaciers and extreme precipitation events which are caused by anthropogenic actions leading researchers to focus on the reactions of organisms to hypo-saline conditions (Cyr and Galbraith, 2021; Dotto *et al.*, 2018; Intergovernmental Panel on Climate Change, 2022; Lui *et al.*, 2022). Although hypoosmotic stress is heavily studied in the literature, it is usually combined with heat intensities (Gunderson *et al.*, 2016). Yet not all areas are likely to experience severe precipitation and heat at the same time of year and when identifying stressors for experimental treatments, the variables selected should be ecologically relevant to the study organism (McPhillips *et al.*, 2018; Pirotta *et al.*, 2022). For example, low salinity and cold conditions are common for North Pacific intertidal organisms in the winter and spring due to low temperatures, increased precipitation, and melting terrestrial ice (Johannessen *et al.*, 2019). At present, low temperature extremes are overlooked in research due to the abundance of heat stress studies. It is important to study winter stressors for accurate predictions of ecosystem change because organisms are still exposed to cold temperatures.

Intertidal animals are exposed to abiotic extremes making them great warning systems and model organisms for stressor studies (Helmuth *et al.*, 2006). Because organisms found between tide lines experience both marine and terrestrial abiotic effects, they are more likely to adapt to multiple stressors in combinations other creatures are not exposed to (Raffaelli and Hawkins, 1996). For instance, subtidal animals are unlikely to undergo adaptation to intense salinity and temperature changes that occur in tide pools on short (daily) and long (seasonally) time scales (McAllen and Block, 1997). An example of an intertidal invertebrate that is very tolerant to

extreme environmental conditions is the harpacticoid copepod *Tigriopus californicus* which has been seen to survive a wide range of salinity levels from near fresh (2 ppt) to hypersaline (100 ppt) and a temperature range of 6 to 33 °C in Barkley Sound (Raisuddin *et al.*, 2007; Burton and Feldman, 1983; Powlik, 1996). The *Tigriopus* genus, found in the intertidal, has become a model organism due to its coloured body (making it easy to see), sexual dimorphism, short development, and high abundance and is commonly used in stressor studies (Fraser, 1936; Raisuddin *et al.*, 2007; Tward *et al.*, 2019).

Tigriopus californicus has been commonly used to study responses to salinity and temperature extremes. For example, salinity tolerance latitudinal gradients have been discovered in *T. californicus* because northern populations adapt better to hypo-salinity (DeBiasse *et al.*, 2018), but other studies found no geographical trend and high variation between populations (Lee *et al.*, 2021). Furthermore, low salinity stress may be a main factor limiting *T. californicus* geographical movement because it has been seen to cause mortality instead of different stress reactions (Lee *et al.*, 2021). Currently, it is known that proteins including Proline and Alanine are made when exposed to hypo-osmolarity stress which helps with osmoregulation by stabilizing internal ion levels (Goolish and Burton, 1989; Lee *et al.*, 2021; Kelly *et al.*, 2016). Conversely to freshening stress, there is clear evidence of latitudinal trends in *T. californicus* temperature tolerance with northern populations being able to withstand cold better (Wallace *et al.*, 2014). To adapt to cold *T. californicus* may upregulate the transcription of AOX (alternate oxidase) genes, which make proteins that give animals the capability to adapt to extreme abiotic conditions by increasing oxygen consumption and in the case of *T. californicus*, to increase internal temperature levels when below optimal (Tward *et al.*, 2019). Overall, understanding how internal mechanisms

are used to adapt to stressors allows for increased insight as to why we see specific reactions to multiple stressors.

I performed mortality experiments on *T. californicus* to test how their stress responses change with different stress sequences and latency durations. Specifically, I used different sequences of fresh (35 ppt to 12 ppt) and low temperature (14°C to 1°C) stressors with three different latency duration times (the duration of time in normal conditions between the two stressors) of 0, 12, and 24 hours. Copepods were collected during winter in the North Pacific. This was an important factor in deciding the ranges and timing of cold and freshening stressors representing what these animals would experience in the natural habitat. I expected to see a change in mortality between the different sequences of stressors and latencies. Mainly, I proposed the response to the mixed stressors (cold-fresh and fresh-cold) to be more negative than the response to the single stressors (cold-cold and fresh-fresh) because the specimens would have to adapt to two different extremes. The 0-hour latency time was predicted to increase mortality because of no recovery between stressors, while 12- and 24-hour latency periods would give individuals time to adapt to a future unsuitable condition thus increasing survival. My results support the role of sequence and latency in changing mortality rates as a key consideration for predicting population persistence which gives information about possible future management.

2. Methods

2.1 Study organism

Tigriopus californicus is a harpacticoid copepod found year-round in high intertidal splash pools of the Pacific Northwest from Alaska to Mexico. Population abundances of *T. californicus* reach up to 20 individuals per millilitre (Powlik, 1996). Like all copepods, *Tigriopus* species have 12 life stages: 6 naupliar, 5 copepodite, and one adult stage with developmental time varying by species and temperature (*T. californicus* takes 30 days to fully develop in winter temperatures; Spaeth *et al.*, 1997). Males and females are sexually dimorphic with males being smaller and having modified antennules used for mate-guarding behaviour. Conversely, females have a wide abdomen used for protecting brooded eggs. Naupliar stage individuals are small, transparent, and circular while copepodids look like small adults without fully developed antennules or telsons (Spaeth *et al.*, 1997). Interestingly, *Tigriopus* species have differences in stress tolerances between sexes and life stages as well (DeBiasse *et al.*, 2018; Dahms *et al.*, 2017; Tangwancharoen and Burton, 2014). For example, females are generally more tolerant to unsuitable conditions than males, in particular when freshening occurs (Kadiene *et al.*, 2019; Foley *et al.*, 2019; Lee *et al.*, 2021). These differences make it important to focus on only one life stage/sex or block life stages and sexes separately. Currently, most studies use adults for research and overlook earlier life stages. However, studying juveniles can give important information about population structures after extreme events (Paiva et al., 2020). Here I used the copepodite life stage because they were the most abundant in my samples. Furthermore, field collected individuals were used directly in the experiments without laboratory rearing because they would display a more accurate response to the stressors (Sinclair *et al.*, 2015).

2.2 Field collection

Tigriopus californicus specimens were collected from 10 tide pools in the middle rock structure of the Arbutus Cove beach in Victoria, B.C. Canada. Arbutus Cove was selected because it has a high abundance of *T. californicus* due to having decreased extinction events because it is a sheltered beach (Powlik, 1998). In October 2022, the beach was surveyed to choose and picture tide pools for identification purposes. Sixteen tide pools were distinguished with individual codes to prevent repetition on following collection days and selected based on the visual presence of actively swimming *T. californicus* (only ten were collected from; Figure 1). During pilot experiments, the water of three tide pools containing *T. californicus* was measured for nitrite, nitrate, and ammonia levels, using API saltwater master test kit (included nitrate, nitrite, pH, and ammonia test solutions), to find what ranges of these chemicals *T. californicus* are naturally exposed to.

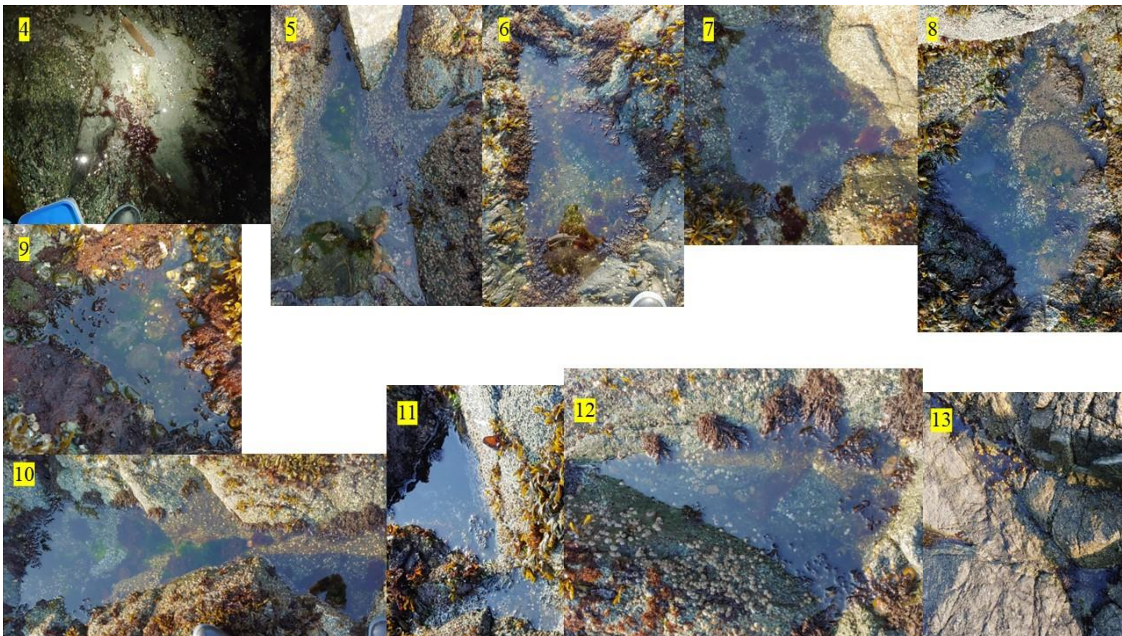

Figure 2. A picture of the 10 tide pools that *Tigriopus californicus* samples were collected from at Arbutus Cove Beach in Victoria, BC, labelled with the tide pool number.

Collections took place on the evenings of January 9th and 20th, 2023 when the tide fell below 1.6 meters. Before collection started the date, time, air temperature (°C), cloud coverage (percent), 24-hour precipitation amount (mm), and tide (m) were received from The Government of Canada (2023) and noted. Tide pool water data was collected in the field, specifically, with a turkey baser, 50 mL of water was collected from about 5 cm below the water surface and about 5 cm above the benthic surface from each tide pool and mixed. Temperature, salinity, and pH data from the tide pool water was collected, using an AZ water quality meter model 8371 and API saltwater high range pH test solution, and recorded. Specimens were collected from each tide pool using a 50 mL turkey baser, 53 µm mesh filter, and 250 mL wash bottle. As many individuals as possible were collected from each tide pool in a reasonable time frame and the samples were kept separated. After field collection, samples were held separately in containers in the laboratory at a controlled temperature of 14 ± 1.5 °C with a 12-hour light-dark cycle using a PHCbi MIR-154 cooled incubator before sorting took place.

2.3 Laboratory protocol

Artificial seawater made with dechlorinated freshwater from a filtered tap, Instant Ocean Reef Crystals Reef Salt, and API pH up and pH down was used along with a 12-hour light-dark cycle (controlled using a PHCbi MIR-154 cooled incubator) throughout the experiment. The pH up and pH down solutions were used to modify pH levels of the artificial seawater and freshwater (used for the low salinity treatment) to a pH of 8. All water used was kept at 14 °C in a cooler to prevent temperature shock to individuals when water was changed. Water salinity and temperature were measured using an AZ water quality meter model 8371. All water used tested nitrate, nitrite, pH, and ammonia levels using an API saltwater master test kit (included nitrate, nitrite, pH, and

ammonia test solutions) to confirm levels were in a safe range for survival, therefore not creating stressful conditions, (nitrate: = 0, nitrite: = 0, pH: 7.9-8.1, ammonia: <0.2) which was determined from field water testing. *Tigriopus californicus* individuals were held at normal conditions, 14 ± 1.5 °C, 12-hour light-dark cycle, 35 ppt, and 8 pH, before and after stress was applied as well as during the latency duration. The normal temperatures and light conditions (controlled with a PHCbi MIR-154 cooled incubator) were picked because they are reliable and non-stressful conditions for *T. californicus* in the laboratory, while a salinity of 35 ppt and pH of 8.0 are typical levels for marine water (Liguori, 2022).

In order, the steps of my methods were: field collection, sort, 24-hour acclimation, stress one, latency time, stress two, 48-hour recovery, and determination of life status (Figure 2). First, individuals were sorted from the field sample, looked at under a microscope to confirm they were in the copepodite life stage, and randomly placed into a well in a Greiner Bio-One 24-well plate containing 3 millilitres of normal-condition water. To account for tank effects, one individual was placed in each well. Plates were labelled with experiment number, treatment group, and tide pool number before placement into normal conditions. A total of 280 copepodids underwent experimentation with the breakdown of sample size in each experiment, tide pool, and treatment group in Table 1.

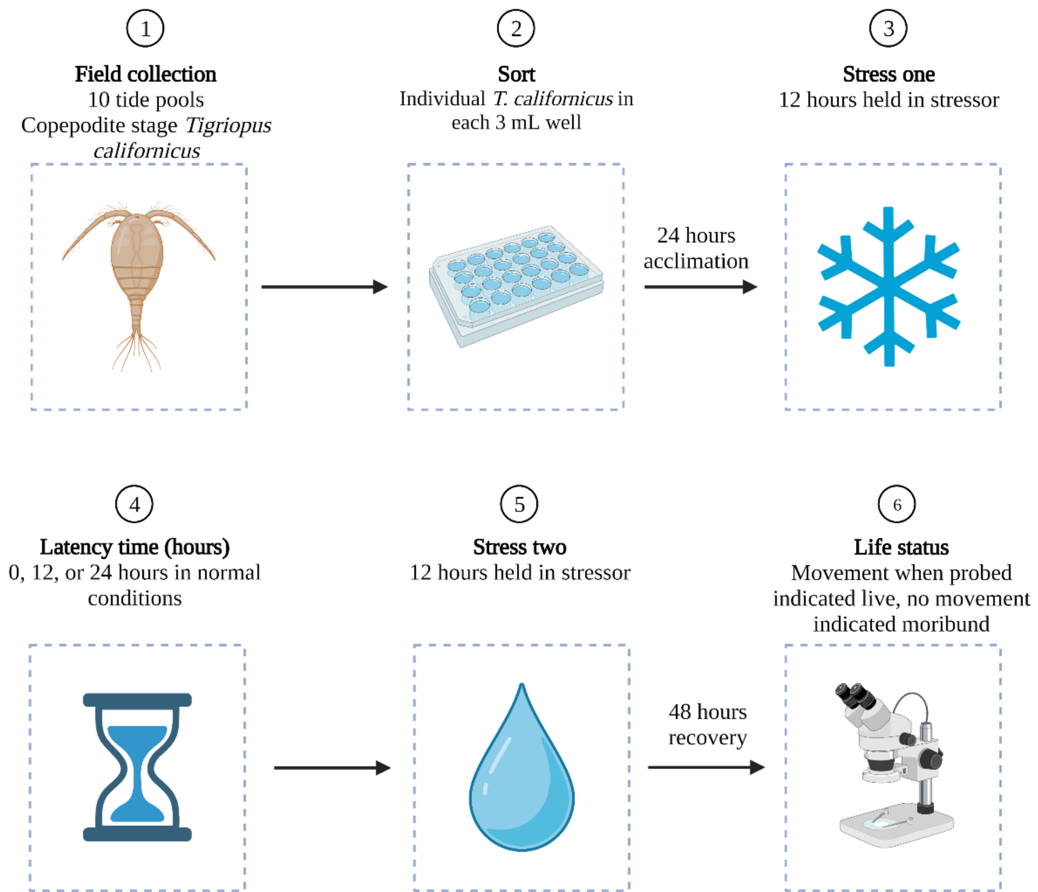

Figure 3. A visual diagram showing the steps of the methods. Individual *Tigriopus califonicus* copepodids were held in normal conditions (14 °C and 35 ppt) during the acclimation and recovery periods. Stress one and two were either cold stress (-1 °C) or freshening stress (12 ppt) dependent on the treatment group. Created with BioRender.com.

Table 1. The breakdown of the sample sizes of *Tigriopus californicus* copepodids for each of the 12 treatment groups (including stress sequence [CC: cold-cold, CF: cold-fresh, FC: fresh-cold, FF: fresh-fresh] and latency time in hours [0, 12, and 24]) and 13 tide pools (N = 280). Sample sizes are further grouped by experiment number and stress sequence.

Experiment Number		**1**				**2**						**Total**	
Tide pool Number		**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**		
	0	3	2	2	3	4	3	2	2	3	2	26	
CC	**12**	1	1	1	2	3	2	2	2	3	2	19	68
	24	1	2	2	2	3	4	2	3	3	1	23	
	0	2	1	0	1	5	4	2	3	3	4	25	
CF	**12**	1	2	3	3	5	4	2	2	3	2	27	74
	24	1	2	2	3	4	2	0	3	3	2	22	
	0	2	1	1	2	4	3	2	2	4	2	23	
FC	**12**	1	3	0	2	4	3	2	1	3	0	19	67
	24	2	3	2	2	3	3	0	3	4	3	25	
	0	2	2	2	3	4	3	2	2	3	1	24	
FF	**12**	1	2	2	3	4	2	2	2	2	2	22	71
	24	2	2	2	2	4	3	2	3	3	2	25	
Total		19	23	19	28	47	36	20	28	37	23	280	
		89				171							

Following sorting, all individuals were held for at least 24 hours at normal conditions for adjustment. Then individual life status of alive or moribund was determined and individuals that died during adjustment, due to handling effects, were removed. Live individuals were defined as copepods who were actively moving around or started moving when probed while moribund individuals did not move. Samples were placed into the first stressor for 12 hours, then held at normal conditions for the determined latency duration, and finally placed in the second stressor for 12 hours, conditions were changed by manipulating the well plates to prevent over handling (Figure 3). After stressors were completed, *T. californicus* specimens spent 48 hours in recovery, necessary to allow individuals to recoup from a dormant state, before the final life status of alive or moribund was determined. A 48-hour recovery time was determined from the results of Wallace *et al.* (2014) who found that the highest recovery of *T. californicus* succeeding freezing was after 48 hours in normal conditions and confirmed by me with pilot experiments. An entire experiment was completed with 6 days of sorting and 12 days of collection.

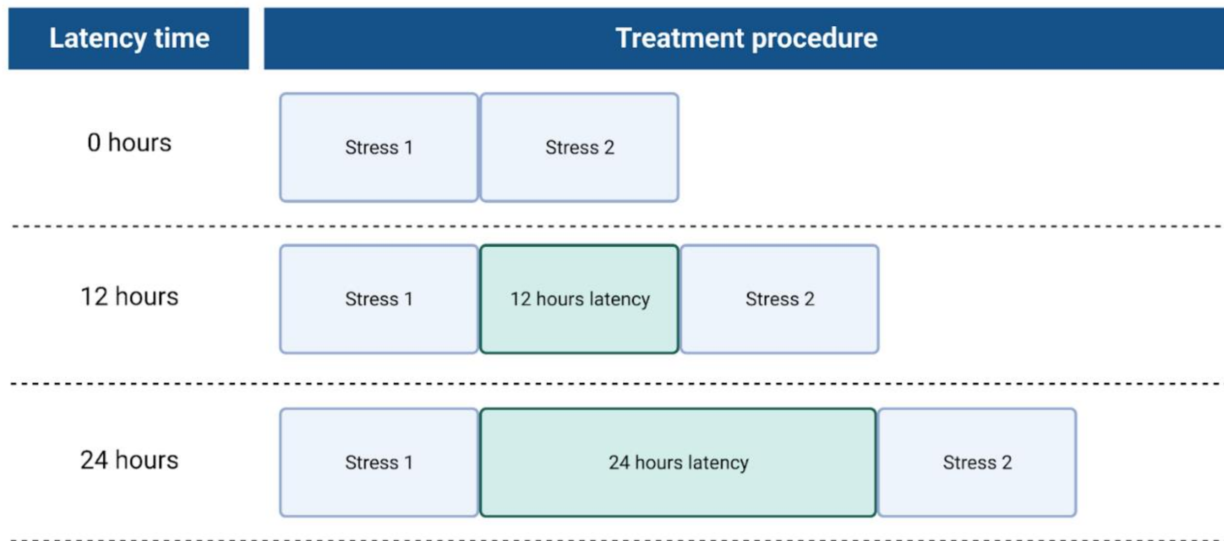

Figure 4. A visual representation of the treatment procedure with the three different latency times of 0, 12, and 24 hours. The stressors were applied for 12 hours. Created with BioRender.com.

2.4 Stressor treatments

Four stress sequence treatments including cold and freshening stress were used: cold-cold, cold-fresh, fresh-cold, and fresh-fresh, and combined with three different latency times: 0, 12, and 24 hours, to create a total of twelve treatment groups (Figure 4). Cold temperatures and hypo-salinity were chosen as stressors because individuals were collected in the winter. Resistance to stressors in *T. californicus* has been seen to change throughout the seasons thus the ecological relevance of the stressors chosen (Sinclair *et al.*, 2015). Latency times of 0, 12, and 24 hours and 12-hour stress periods were selected to mimic what specimens would experience during a tidal cycle.

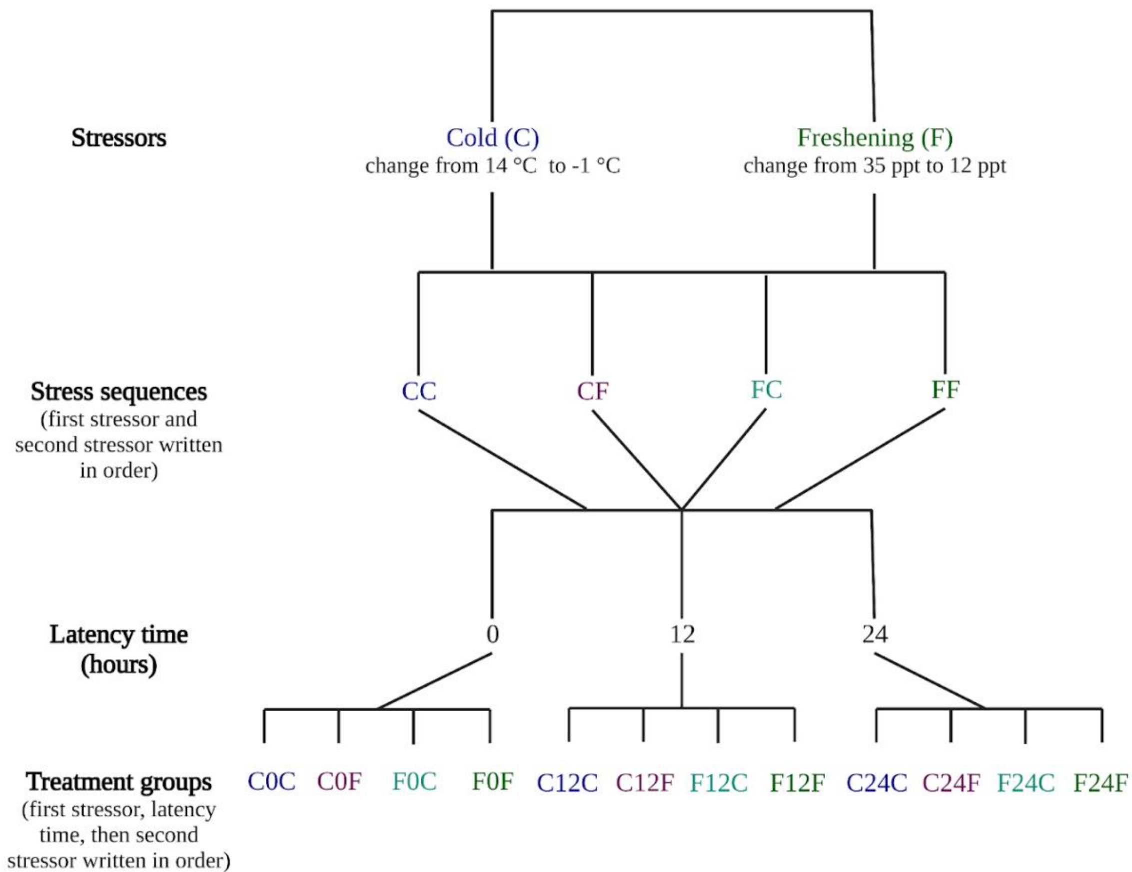

Figure 5. A visual breakdown representation for the 12 treatment groups used in the experiments. Created with BioRender.com.

The freshening treatment applied was created by hand-dropping freshwater into wells to decrease normal seawater salinity (35 ppt) by 66% (12 ppt). The treatment salinity level was determined by running pilot experiments and chosen because it put high stress on individuals without causing full fatality. To decrease the salinity, first, 2 mL of artificial seawater (at 35 ppt) was removed from each well. Then, slowly, over an hour, 2 mL of dechlorinated freshwater was hand-dropped into each well creating a water salinity of around 12 ppt. The conditions were kept at 12 ppt for 11 hours before being changed to 35 ppt immediately. The instantaneous salinity manipulation back to normal conditions reflected an incoming tide. Salinity was returned to 35 ppt by removing 2.5 mL of 12 ppt water from each well and adding in 2.5 mL of 40 ppt artificial seawater.

The cold treatment temperature was chosen based on winter temperatures in Victoria which regularly drop below 0 °C and freeze tide pools (McAllen and Block, 1997). However, freezing samples was not feasible because changing stress conditions from cold to fresh with a 0-hour latency time would not have been possible. Therefore, the lowest temperature before seawater freezes, -1 ± 1.5 °C, was chosen . Specimens were placed in a PHCbi MIR-154 cooled incubator where the air temperature was reduced over an hour from 14 ± 1.5 °C to -1 ± 1.5 °C at a rate of -0.25°C/minute (selected as it is a typical cooling rate [Sinclair *et al.*, 2015]) creating an overall -15°C temperature change. The incubator temperature was then held at -1 °C for 10 hours and 50 minutes before being raised back up to 14 °C in 10 minutes (the quickest speed that the incubator could increase the temperature).

2.5 Statistics

I used a general linear mixed model with template model builder to determine significance of the data. The response variable was binomial to indicate life status while the explanatory variables were the factor stress sequence and the integer latency time. I used the tide pool number nested in the experiment number as my random effects, both values were numeric. All statistical tests were completed in R software (R Core Team, 2021). The glmmTMB function in the glmmTMB package was used to make the model (Brooks *et al.*, 2017). After the model was made, the simulate residuals function in the DHARMA package confirmed the model was a good fit for the data (Hartig, 2022). Finally, the confidence intervals were calculated using the confint function in R base (R Core Team, 2021). Graphs were made in GraphPad Prism 9 or in R using the ggplot2 package and conceptual diagrams were made in BioRender (GraphPad Software; Wickham, 2016; BioRender.com).

3. Results

3.1 Stress sequences

In the treatment with two consecutive cold exposures, there was high survival compared to the stress sequences with either a single or double freshening stress event (Figure 5). The logarithmic odds ratio (± s.e.) of survival was found to be 0.807 ± 0.318 ($n = 68$, $z = 2.54$ $p = 0.0111$), -1.32 ± 0.369 ($n = 74$, $z = -3.58$, $p = 0.000346$), -1.04 ± 0.375 ($n = 67$, $z = -2.78$, $p = 0.00543$), and -1.54 ± 0.373 ($n = 71$, $z = -4.08$, $p = 0.000045$) for cold-cold, cold-fresh, fresh-cold, and fresh-fresh respectively (Figure 6; Table 2).

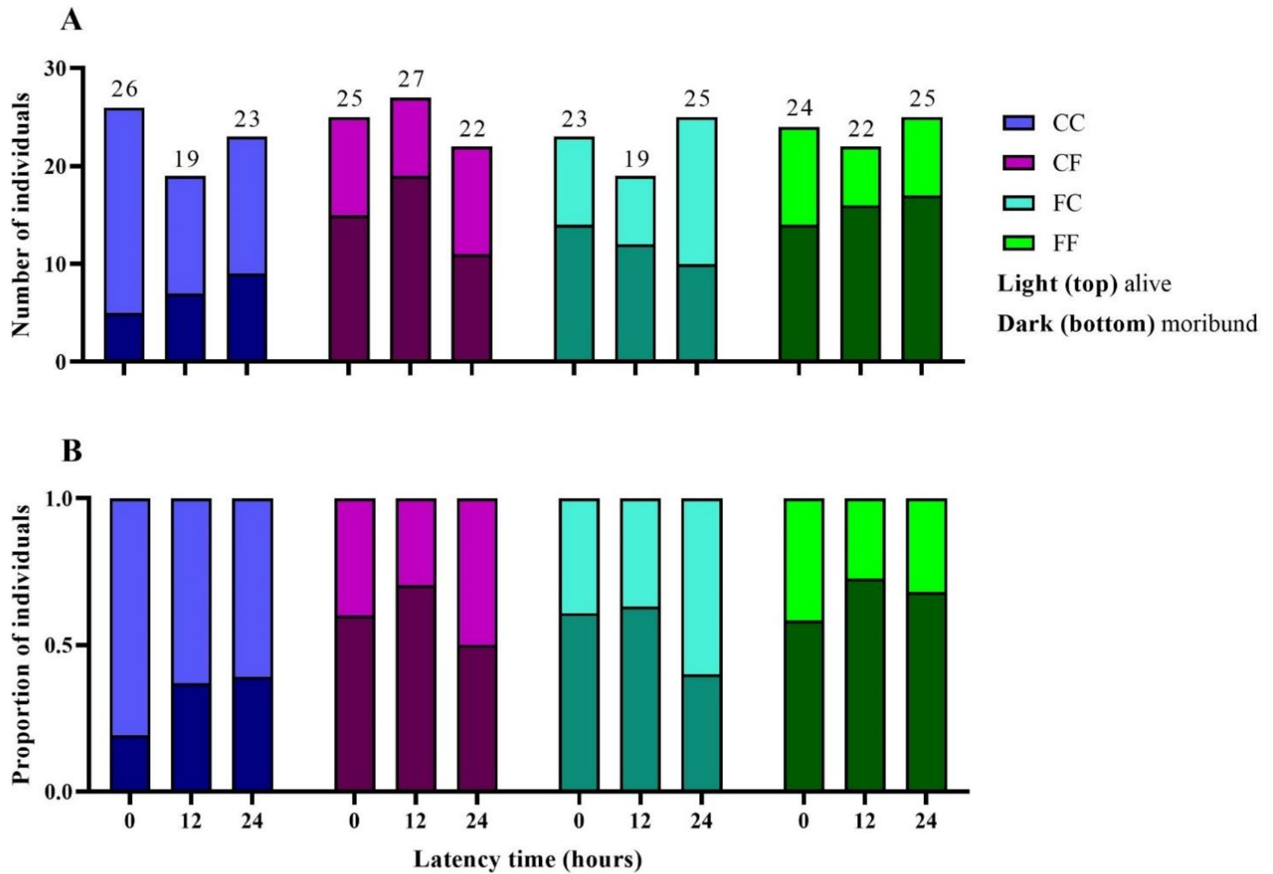

Figure 6. The number (A) and proportion (B) of *Tigriopus californicus* alive (light) and moribund (dark) for each latency time (hours) in each stress sequence. Stress sequences are differed by colour (cold-cold [CC]: blue, cold-fresh [CF]: purple, fresh-cold [FC]: turquoise, fresh-fresh [FF]: green). The numbers above the columns in panel A represents the treatment group sample size (N = 280).

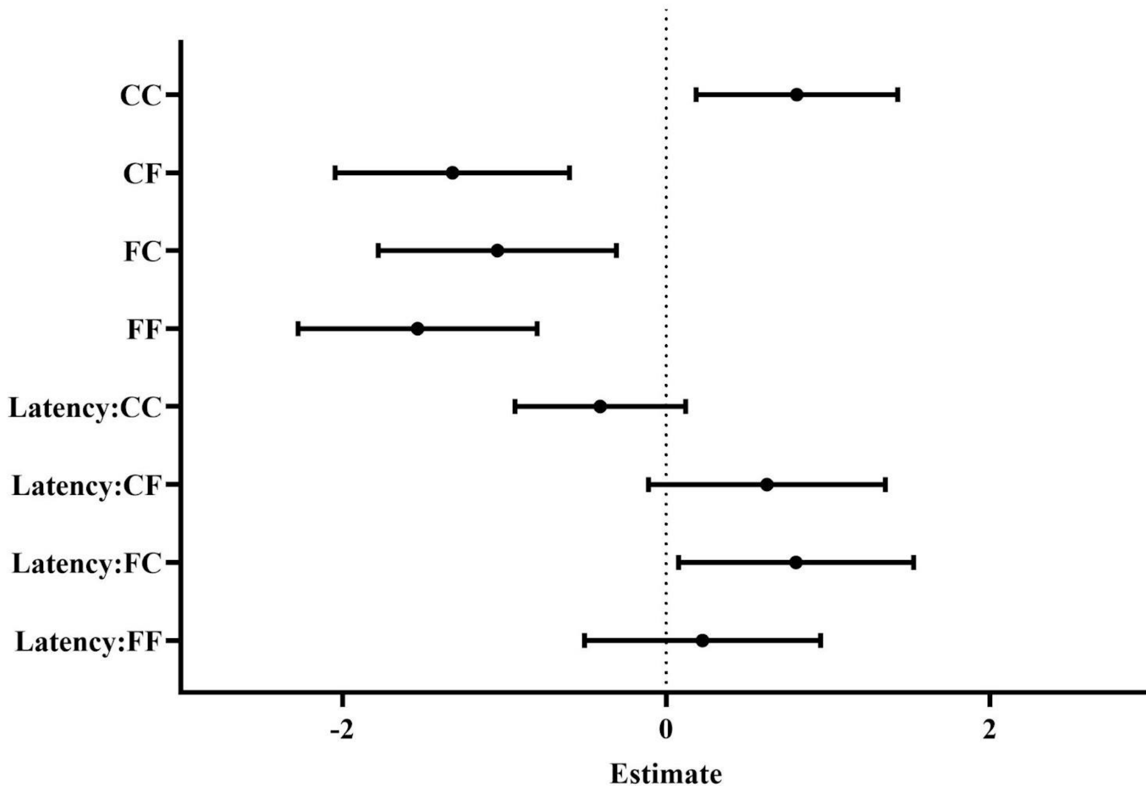

Figure 7. The results from a general linear mixed model using template model builder on binomial data with tide pool number (10 total) nested in experiment number (2 total) as the random effects. A significance value of 0.05 was used and estimates represent the log odds value. Error bars are the 2.5% to 97.5% confidence intervals. There was a significant difference in survival of *Tigriopus californicus* copepodids between the cold-cold and the other three stress sequences (cold-cold [CC]: n = 68, z = 2.539, p = 0.011101; cold-fresh [CF]: n = 74, z = -3.578, p = 0.000346; fresh-cold [FC]: n = 67, z = 2.780, p = 0.005433; fresh-fresh [FF]: n = 71, z = -4.079, p = 0.000045). Fresh-cold also had a significant interaction between latency and stress sequence while the three other sequences did not (CC: n = 68, z = -1.514, p = 0.13007; CF: n = 74, z = 1.664, p = 0.09602; FC: n = 67, z = 2.166, p = 0.03033; FF: n = 71, z = 0.603, p = 0.54677).

Table 2. The results of a general linear mixed model using template model builder on binomial data with tide pool number (10 total) nested in experiment number (2 total) as the random effects. A significance value of 0.05 was used and estimates represent the log odds value. The effects are the different stress sequences (cold-cold [CC], cold-fresh [CF], fresh-cold [FC], and fresh-fresh [FF]) and the interaction between the stress sequences and latency time. One asterisks indicates a p-value below 0.05, two asterisks indicates a p-value below 0.01, and three asterisks indicates a p-value below 0.001.

Effect	Estimate	Standard error	2.50% Confidence interval	97.5% Confidence interval	Z Value	P Value
CC	0.8065	0.3176	0.184052	1.48986	2.539	0.011101*
CF	-1.3214	0.3693	-2.045320	-0.597566	-3.578	0.000346***
FC	-1.0434	0.3753	-1.779030	-0.307839	-2.780	0.005433**
FF	-1.5358	0.3765	-2.273871	-0.797917	-4.079	0.000045***
Latency: CC	-0.4076	0.2693	-0.935373	0.120129	-1.514	0.130069
Latency: CF	0.6215	0.3734	-0.110332	1.353371	1.664	0.096016
Latency: FC	0.8017	0.3702	0.076199	1.527221	2.166	0.030325*
Latency: FF	0.2240	0.3717	-0.504587	0.952610	0.603	0.546774

3.2 latency effects

There was a linear relationship between survival and latency time in all stress sequences (Figure 7). Specifically, a positive interaction between stress sequence and latency was found for the fresh-cold sequence. The interaction log odds value (± s.e.) for cold-cold was -0.408 ± 0.269 ($n = 68$, $z = -1.51$, $p = 0.130$), for cold-fresh was 0.621 ± 0.373 ($n = 74$, $z = 1.66$, $p = 0.960$), for fresh-cold was 0.802 ± 0.370 ($n = 67$, $z = 2.17$, $p = 0.0303$), and for fresh-fresh was 0.224 ± 0.372 ($n = 71$, $z = 0.603$, $p = 0.547$; Figure 6; Table 2).

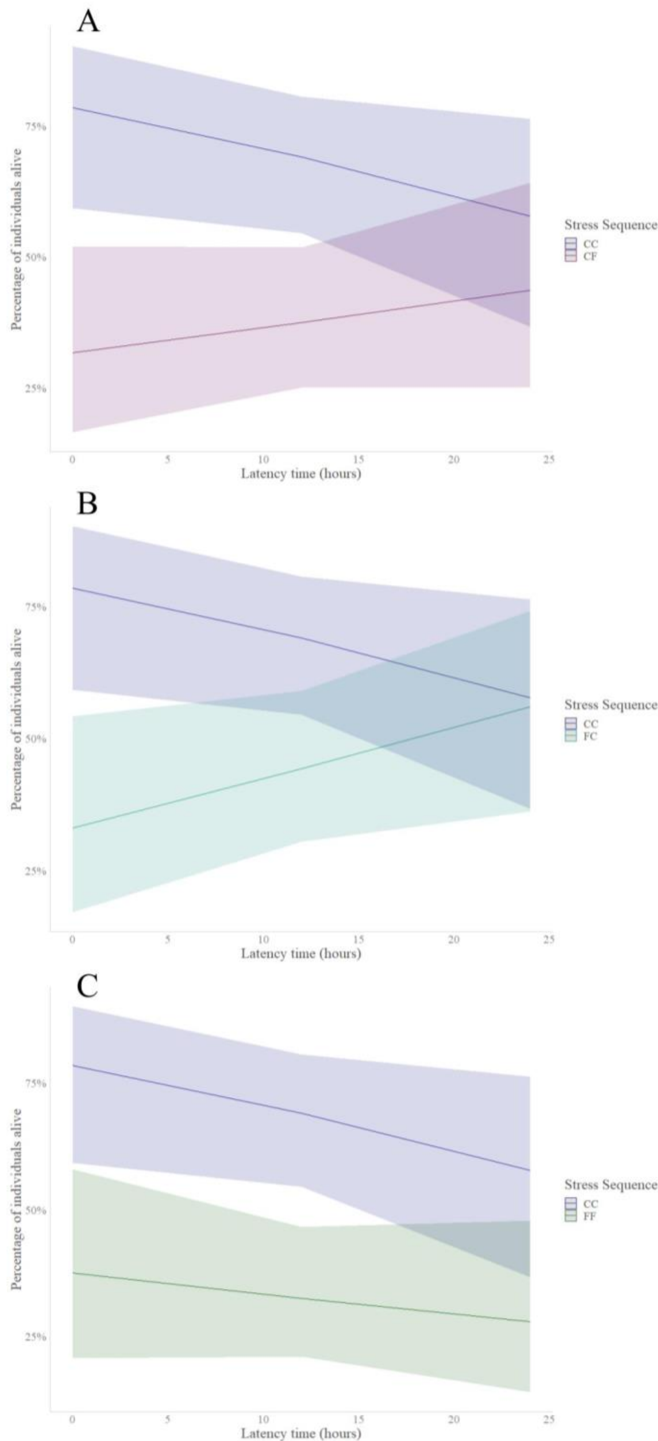

Figure 8. A visual representation of the regression lines calculated in a general linear mixed model using template model builder on binomial data with tide pool number nested in experiment number as the random effects. The percentage of individuals alive was plotted against the latency time (hours) to create regression lines, based on the best fit model, for visualization of the interaction between stress sequence and latency time. The figure compares the regressions of the stress sequences cold-cold (CC) to cold-fresh (CF; A), fresh-cold (FC; B), and fresh-fresh (FF; C) differed by colour (CC: blue, CF: purple, FC: turquoise, FF: green). Error bars are the 2.5% to 97.5% confidence intervals. Fresh-cold also had a significant interaction between latency and stress sequence while the three other sequences did not (CC: $n = 68$, $z = -1.514$, $p = 0.13007$; CF: $n = 74$, $z = 1.664$, $p = 0.09602$; FC: $n = 67$ $z = 2.166$, $p = 0.03033$; FF: $n = 71$, $z = 0.603$, $p = 0.54677$). A significance value of 0.05 was used.

3.3 Blocks

Overall, individual mortality partially depended on which tide pool they originated from (Figure 8). The tide pool number nested in experiment number explained 14 ± 2% (s.e.) while the experiment number explained 1 ± 1% (s.e.) variability of the results. The breakdown of the number of individuals found alive and moribund in each tide pool is indicated in Table 3.

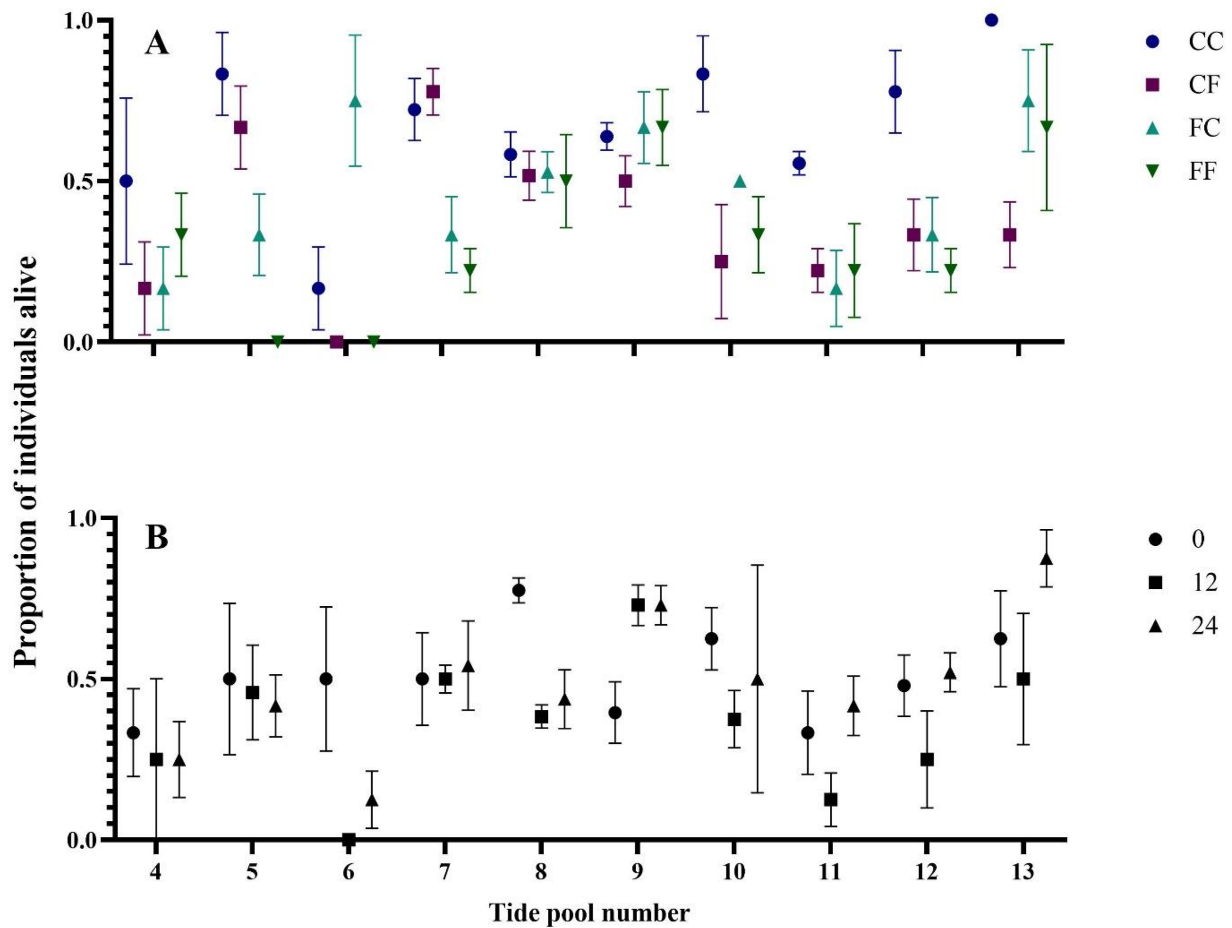

Figure 9. The proportion of *Tigriopus californicus* copepodids alive in the different stress sequences, differentiated by shape and colour (cold-cold [CC]: blue circle, cold-fresh [CF]: purple square, fresh-cold [FC]: turquoise triangle, and fresh-fresh [FF]: green inverted triangle; A), and latency periods in hours, differentiated by shape (0: circle, 12: square, 24: triangle; B), for each tide pool (4: n = 19, 5: n = 23, 6: n = 19, 7: n = 28, 8: n = 47, 9: n = 36, 10: n = 20, 11: n = 28, 12: n = 37, 13: n = 23). The error bars represent the standard error of the proportion. There was a 14% variation in the results due to the random effect of tide pool.

Table 3. The number of *Tigriopus californicus* copepodids alive (top) and moribund (bottom) for each tide pool in each treatment group which included stress sequence (cold-cold [CC], cold-fresh [CF], fresh-cold [FC], and fresh-fresh [FF]) and latency time in hours (0, 12, and 24).

Tide pool Number		4	5	6	7	8	9	10	11	12	13
CC	0	3	2	1	2	3	2	2	1	3	2
		0	0	1	1	1	1	0	1	0	0
	12	1	1	0	1	1	1	1	1	3	2
		0	0	1	1	2	1	1	1	0	0
	24	0	1	0	2	2	3	2	2	1	1
		1	1	2	0	1	1	0	1	2	0
CF	0	1	1	0	1	3	1	1	1	1	0
		1	0	0	0	2	3	1	2	2	4
	12	0	1	0	2	1	3	0	0	0	1
		1	1	3	1	4	1	2	2	3	1
	24	0	1	0	2	3	1	0	1	2	1
		1	1	2	1	1	1	0	2	1	1
FC	0	0	0	1	0	3	1	1	1	1	1
		2	1	0	2	1	2	1	1	3	1
	12	0	1	0	1	2	2	1	0	0	0
		1	2	0	1	2	1	1	1	3	0
	24	1	2	1	1	1	3	0	0	3	3
		1	1	1	1	2	0	0	3	1	0
FF	0	1	0	0	1	4	1	1	1	1	1
		1	2	2	2	0	2	1	2	2	0
	12	0	0	0	1	2	2	1	0	0	0
		1	2	2	2	2	0	1	2	2	2
	24	1	0	0	0	0	2	0	2	1	2
		1	2	2	2	4	1	2	1	2	0

4. Discussion

4.1 Survival of single stressors

Currently, whether anthropogenic actions are increasing the occurrence of low temperature extremes is still being debated but cold stressors should not be overlooked as a disturbance organisms may experience (Stott *et al.*, 2016; Cohen *et al.*, 2014). For example, tropical organisms that are moving north into temperate zones to avoid warming will experience chilled temperatures outside of their normal range thus creating stress (Vergés *et al.*, 2014). Also, there is evidence of adaption to warming, caused by climate change, making it harder for animals to withstand cooler temperatures (McAlpine-Bellis *et al.*, 2021). Finally, in a marine environment, there are many moving parts and there is evidence of increasing cold and freshening stress due to melting glaciers (Fox *et al.*, 2022; Dotto *et al.*, 2018; Cyr and Galbraith, 2021). These examples show cold stressors should not be overlooked during research as we do not know how habitats are changing and marine animals will be exposed to low temperatures outside of their adapted range (Cyr and Galbraith, 2021).

Across the marine ecosystem, many animals osmo-conform to adapt to cold stress (Nordlie, 2009). For example, the more saline a solution, the lower the freezing temperature is. So, in chilled water, marine bony fishes will concentrate salts in their body, lowering solution freezing temperatures to avoid internal freezing (Nordlie, 2009). Similarly, when saltwater freezes only water particles freeze while salt particles do not. Instead, salt particles group together between frozen water and create hypersaline solution. Some small organisms, including *Tigriopus brevicornius*, avoid internal freezing by seeking out hypersaline water and osmo-conforming to increase internal salinity levels (Figure 9; McAllen and Block, 1997; Wallace *et al.*, 2014). Here I found that specimens were able to withstand cold intensities well, which is consistent with a

previous study showing that there is 70-80% recovery of northern *T. californicus* populations after exposure to freezing (Wallace *et al.*, 2014). It is predicted that these copepods resist freezing in a similar way to *Tigriopus brevicornius* via osmo-conforming (Wallace *et al.*, 2014; McAllen and Block, 1997). However, researchers have discovered that *T. californicus* osmo-regulates internal salinity when exposed to hypersaline water instead of osmo-conforming, but it is feasible that individuals are more likely to survive high internal salinity levels over internal freezing, and so they adapt to cold temperatures via the former (Nordlie, 2009). Furthermore, a knock-down (dormant) response, which *T. californicus* uses as a stress response to avoid mortality, has been observed when *T. californicus* goes through both hyper-salinity and cooling intensities (DeBiasse *et al.*, 2018; Lee *et al.*, 2021). The knock-down-like behaviour which increases the survival of *T. californicus* in low temperatures was previously attributed to a cold-coma state that is a common freezing response in insects, yet the reaction could be due to hyper-salinity (Wallace *et al.*, 2014; Sinclair *et al.*, 2015). Future research should investigate how *T. californicus* adapts to freezing temperatures for a better understanding of the internal mechanisms in use.

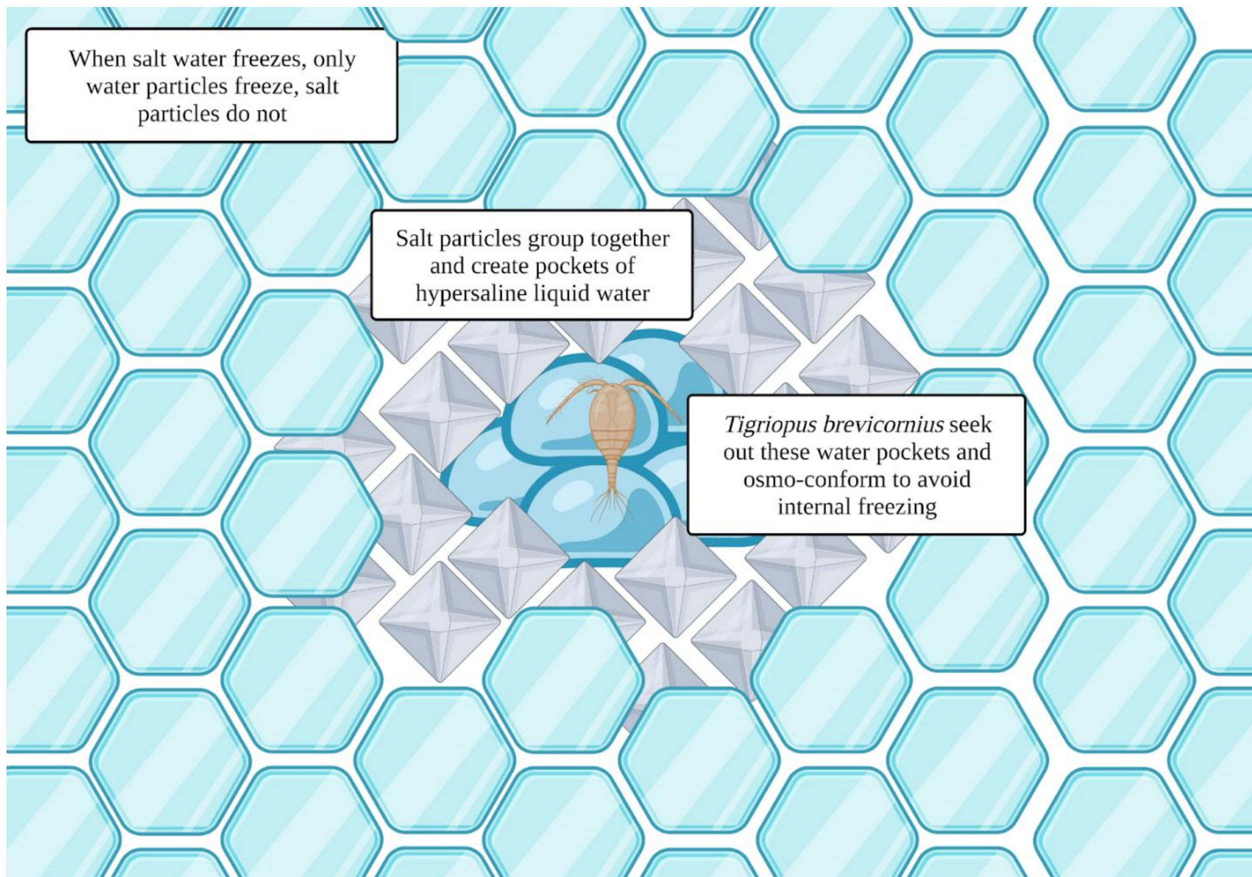

Figure 10. A conceptual diagram showing how *Tigriopus brevicornius* avoids internal freezing to adapt to cold stress as explained by McAllen and Block (1997). It is predicted that *T. californicus* uses the same method to adapt to cold stress (Wallace *et al.*, 2014). Frozen water particles are indicated by the blue hexagon and salt particles are indicated by the silver diamonds. Created with BioRender.com.

Low tolerance to freshening is common in marine animals (McKnight *et al.*, 2021). For example, during experimentation, both Sea Hares and Olympia Oysters had extremely low resilience to hypo-salinity which caused high mortality, compared to the other stressor applied (cold in Sea Hares [McAlpine-Bellis *et al.*, 2021] and heat or hypoxia in Oysters [Cheng *et al.*, 2015]). Some studies have found that there are increased metabolic rates in limpets (Morritt *et al.*, 2007) and flat worms (Rivera-Ingraham *et al.*, 2016) and decreased growth in sea cucumbers (Yu

et al., 2013) when exposed to hyposaline surroundings. Similarly, I saw a weak capacity of *T. californicus* to survive hypoosmotic stress with close to double the mortality amount compared to low temperature extremes. When compared to other *T. californicus* populations in a nearby area (Barkley Sound) that endured low salinity down to 2 ppt (Powlik, 1996), the population I studied did not have a high ability to adapt to hypo-salinity because of high fatality in 12 ppt and full mortality in 5 ppt water (personal observation). The high tides that Arbutus Cove beach experiences may be the reason for my samples' low salinity tolerance. For instance, multiple studies have concluded that resistance of *T. californicus* to salinity changes is based on the conditions of the beach where the population is found (tide lines, wave and sun exposure, runoff, etc.) and other factors like salinity level (Lee *et al.*, 2021; Foley *et al.*, 2019; Liguori, 2022). The high tides at Arbutus Cove may have caused the sampled *T. californicus* population to be covered by ocean water (35 ppt) for about half of a day. Therefore, they would not be exposed to rainfall, causing freshening, for long periods of time nor build up resilience to fresh conditions. This might be why I found such a high mortality of my specimens under freshening stress conditions.

Few studies examine the changes in stressor tolerances of invertebrates throughout development; however, this type of research is important because juveniles' stress responses determine how a population and ecosystem may be structured in the future (Orr *et al.*, 2020; Paiva *et al.*, 2020). Nonetheless, some studies have found that younger marine animals generally have a worse capability to withstand stressors (Lee *et al.*, 2020; Paiva *et al.*, 2020). The low tolerance to freshening stress seen in my results might be because the copepodite stage was used. For example, smaller individuals have decreased resistance to salinity extremes in copepods (Lee *et al.*, 2007) and limpets (Nagaraj, 1988). Furthermore, Kim *et al.* (2022) found that copepodids and naupliar *T. kingsejongensis* individuals had lower resilience to hypo-salinity (Morritt *et al.*, 2007).

Copepodids have a larger surface area to volume ratio than adults due to their smaller size which is a disadvantage when exposed to osmolarity stress because the individual has a harder time maintaining a stable internal environment (Spaeth *et al.*, 1997; Kim *et al.*, 2022). Correspondingly, Dahms *et al.* (2017) suggested that decreased flexibility to withstand metal pollutants in earlier life stages of *T. californicus* was due to their thin exoskeleton. Salts in a solution would also easily move over the thin exoskeleton due to the large surface area of *T. californicus* copepodids and change internal salinity concentration. In freshening stress this means that salts would leave the body and decrease internal salinity levels below optimum. Individuals would then undergo stress, increasing energy output to regulate internal salinity levels and lead to mortality as reflected in my results.

4.2 Survival of mixed stressors

If a species has a very low tolerance for a specific stressor and it is combined in sequence with another stressor, the order of the stress sequence will not change the organism's reaction (MacLennan and Vinebrooke, 2021). For example, a study on Sea Hares found that hypo-salinity stress caused more mortality than cold conditions. If these invertebrates survived hypo-salinity stress and then were exposed to low temperature extremes, hypo-salinity stress did not increase or decrease their ability to survive cold (McAlpine-Bellis *et al.*, 2021). Equivalently, I found similar survival amounts between the mixed stressor sequences and the fresh-fresh sequence. (The cold-cold stress sequence had significantly higher survival.) This indicates that *T. californicus* survival in cold-fresh and fresh-cold depended mainly on their capacity to survive hypo-salinity, not cold stress, independent of stress sequence because it was the more severe stressor. My prediction that mixed stressors would have more mortality than single stressors was proven wrong because of the

dominance of freshening stress. My findings support those of Velasco *et al.* (2018) who saw that freshening has the strongest negative impact in multiple stressor studies. However, when latency time is applied between the stressors, the stress sequence does matter.

Responses to simultaneous stressors cannot predict responses to sequential stressors with a latency because the reactions are different (Mayling *et al.*, 2018). Concerning my study organism, *T. californicus* would likely have low survival when hypoosmotic and cold stressors are applied simultaneously. Hypo-osmolarity stress would decrease *T. californicus* survival in cooling when applied at the same time because less saline solution would be available for osmo-conforming to avoid internal freezing. Currently, no studies have investigated cold and low salinity stressors at the same time in *T. califonicus*. However, my results can be compared to the response of *T. brevicornius* to simultaneous freshening and low temperature stress which caused decreased recovery (McAllen and Block, 1997). Because the same methods are likely used to adapt to low temperature and salinity extremes in these two closely related species, it is also likely that *T. californicus* would show increased mortality. In my results we will see that freshening stress increases survival of cold extremes when latency time is considered and thus overall survival which is opposite to the response likely seen if the stressors were applied simultaneously.

4.3 The interaction between stress sequence and latency time effecting survival

Previous studies have found reactions to stressors in sequence was determined by latency time (Brooks and Crowe, 2019; Cheng *et al.*, 2015; Mayling *et al.*, 2018). This is consistent with my results where there was a significant positive impact of latency time on the survival of *T. californicus* specimens in the fresh-cold stress sequence. The 24-hour latency treatment had less mortality compared to the 0- and 12-hour latency treatments. Undergoing freshening may increase

the adaptability of *T. californicus* to cold extremes and their overall recovery showing cross-tolerance (Gunderson *et al.*, 2016). As described before, *T. californicus* may withstand low temperature extremes via increasing internal salinity levels and undergoing osmolarity stress; thus, using the same internal pathway as when exposed to low salinity water (Wallace *et al.*, 2014; DeBiasse *et al.*, 2018). For example, Kim *et al.* (2022) showed that a closely related species, *Tigriopus kingsejongensis* used the same proteins to adapt to hypo- and hyper-salinity. Furthermore, studies have found that when *T. californicus* was exposed to low salinity for multiple hours, they prepared for a rapid increase in salinity (which happens when the tide comes in) by prematurely making proteins commonly used for adaption to hyper-salinity (DeBiasse *et al.*, 2018; Lee *et al.*, 2021). Finally, it is also likely that *T. californicus* has adapted to cooling and low salinity together because these stressors are commonly applied at the same time of year. Being exposed to one stressor may have indicated to the specimens that they were soon going to experience the other stressor (Liguori, 2022). These examples show there is a high possibility that the proteins *T. californicus* makes when exposed to freshening stress are also used to withstand cold stress. Therefore, individuals would not have to expend extra energy to create new proteins and cross-tolerance is seen. However, in my study latency duration was only seen to increase survival in the fresh-cold stress sequence with 24 hours between stressors, contradicting my prediction that there would be an increase in survival with a 12-hour latency time as well. Possibly there may not have been enough time for recovery in the 0- and 12-hour latency durations because the first and second stressors were too close together. This could have caused individuals to be in an elevated stress state for an extended period, thereby disallowing cross-tolerance. Future studies using *T. californicus* should use increased latency times for understanding the full degree of the relationship between stress sequence and latency.

The trend between an increasing latency time and an organism's negative stress response should be parabolic. At first the stress response of the organism would be highly negative because of back-to-back stressors. Then, the negative effects on the organism would decrease as recovery time is allowed during latency. Finally, an organism's negative stress response would increase again because additive effects are present. Currently, researchers have found that increasing latency times changes the degree of a stress response in an organism but not the direction of the linear relationship because they are finding a specific slope along the parabola (Thompson *et al.*, 2018). Studies have mainly discovered a positive relationship between the latency duration and the negative effects of stressors because they focused on latency times that decoupled stressors (Brooks and Crowe, 2019; Cheng *et al.*, 2015; Orr *et al.*, 2020). My results show that increased latency time decreased the negative effects of the stressors due to my short latency times not decoupling the stressors. For instance, the positive trends of stress sequences that included hypo-salinity indicate that if the time between the two stressors was increased, we may have seen an antagonistic effect. Moreover, the linear relationship between latency time and survival that I found, allows for prediction about *T. californicus* survival with a chosen latency time between 0 and 24 hours (Orr *et al.*, 2020). This shows that using regression to understand varying organismal stress responses between latencies allows for easier predictions of animals' stress responses leading to more informed management practices (Orr *et al.*, 2020; Côté *et al.*, 2016). Future studies should use many latency times on both short and long scales to confirm the parabolic relationship between latency time and species' stress responses.

4.4 Survival variation due to tide pools and collection times

In a population, exposure differences to abiotic extremes can lead to different subpopulations adapting to specific stressors. This creates asymmetrical tolerances to stressors across a population (Varpe, 2017). In my results, there was high variation (14%) of survival depending on which tide pools the individuals came from. This shows that stress responses of *T. californicus* individuals within a single tide pool are more similar to each other than between tide pools. It is unlikely that the variation was due to genetic differences because there is lots of movement of *T. californicus* individuals between tide pools in a single rock outcrop making subpopulations genetically similar (Burton and Swisher, 1984; Liguori, 2022; Burton, 1987). The most likely reasons for the differences in recovery between tide pools are the characteristics of the tide pool causing similar previous experience. For example, Sokolova *et al.* (2000) found that the distance of the tide pool from the tide line was a factor in determining salinity tolerances in different *Littorina* species. Studies have also found that the characteristics of the beach determined the survivability of *T. californicus* in hypo-osmolarity stress (Lee *et al.*, 2021; Foley *et al.*, 2019; Liguori, 2022) and the same could be true for cold stress. A tide pool in the lower part of the Arbutus Cove rock cropping would be exposed for less time than the tide pools in the high intertidal, meaning these *T. californicus* subpopulations would spend more time in hyposaline or cooling circumstances and thus become better adapted to withstand them. Overall, the variability in my results shows that to reflect natural variation, wide-scale samples and the collecting of *T. californicus* from multiple different tide pools in the same area is important.

Stress resistances have been seen to change throughout different generations of *T. californicus* (Liguori, 2022), however, epigenetic material has also been seen to change stress response in this species (Kelly *et al.*, 2017). Samples in this experiment were collected two weeks

apart suggesting that different generations were experimented on because individuals only spend two weeks in the copepodite life stage (Spaeth *et al.*, 1997). Only a 1% variation in the data was explained by the collection date of *T. californicus*, suggesting that individuals were exposed to similar conditions in the field. Yet, the first experiment had a substantially lower sample size per tide pool than the second experiment; this was not expected because we tried to collect as many individuals as possible suggesting a mass mortality event happened before the first collection. An extreme cooling event, temperatures in Victoria dropping down to -15 °C, about two weeks prior to the first collection (at the end of December 2022), may have been the reason for the depleted population size in the first sample. Individuals surviving this cold would have been better adapted to low temperatures. This previous experience could have increased survival in cold stress during my experimentation. On a related note, the second sample would have been expected to have higher mortality than the first sample because they did encounter this cooling event, but this contrasts with the low variability found in my data. Epigenetic maternal effects may explain this disparity because they have been observed to increase the survival of *T. californicus* offspring that underwent the same stress as their mothers (Kelly *et al.*, 2017). Therefore, individuals collected for the second experiment had mothers that withstood the severe cooling event and passed on genetic material that increased the offspring's ability to deal with low temperatures creating no differences between the two samples.

Studies on field organisms are important for predicting the actual stress responses of animals in the wild which experimentation on laboratory-reared animals may miss out on (Dong *et al.*, 2014). Many studies, like mine, have found a high variation of *T. californicus* stress responses between populations and subpopulations and maternal effects impacting the recovery of individuals (Kelly *et al.*, 2016; Lee *et al.*, 2021; Kelly *et al.*, 2017). Stress tolerances of *T.*

californicus change based on which laboratory-reared generation is used, showing that laboratory-reared animals do not display proper natural variation (Liguori, 2022). Most researchers using species of the *Tigriopus* genus, rear individuals in the laboratory. If only a few laboratory-reared individuals of the *Tigriopus* genus are taken from a population, sub population, or female, the full picture of population variation may not be noticed because individuals will be more similar to each other (genetically and with regards to previous exposure). When conducting stressor studies, using specimens collected directly from the field should not be overlooked because these specimens will display the natural variation of the population giving a better overall understanding of the animals' stress reactions. With my results, I filled a gap in the understanding of variation in *T. californicus* stress response by experimenting on specimens taken directly from the field.

4.5 Conclusion

My research showed the interaction between the sequence of stressors and the latency duration changes animals' stress responses which is consistent with other studies (Brooks and Crowe, 2019; Cheng *et al.*, 2015). I used multiple stressors in sequence with varying latency times which helped to fill a research gap in stressor studies. Additionally, my results demonstrated the importance of studying stressors that organisms will experience in nature for proper determination of animal reactions (Gunderson *et al.*, 2016; Côté *et al.*, 2016). Collecting *T. californicus* from multiple tide pools presented natural variation within a single population of this invertebrates' survival when exposed to stressors and indicated the relevance of using non-laboratory-reared individuals. Freshening had a more detrimental effect on *T. californicus* than cold stress which agrees with studies showing hyposaline water is severely dangerous to marine animals (McKnight *et al.*, 2021). Furthermore, evidence that the survival of *T. californicus* copepodids depended on

the interaction between stress sequence and latency time was seen when low salinity stress came before cold stress. Particularly, hypoosmotic stress increased individuals' capacities to withstand cooling, possibly due to related proteins being used for adaptation to both stressors. The differences in survival seen between the varying stress sequences can ultimately lead to changes in population structure. In the case of copepods, mortality can affect overall community function because of this clade's important position as a trophic link in the marine food web (Schmidt *et al.*, 2003). Overall, my results highlight the importance of ecologically relevant stressor studies and proposes that future research should focus on testing stressors in sequences with varying latency durations.

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Appendix A: Controls

Methods

I used two control treatments during experiment two, a normal and water control, with the sample size breakdown per tide pool in Figure 10. Individuals were randomly placed into one of the two treatments after sorting, a 24-hour adjustment period, and removal of deceased individuals (due to handling complications) after acclimation. In the normal control, copepodids were placed in normal conditions (14 ± 1.5 °C, 12-hour light-dark cycle, 35 ppt, and 8 pH) for the entire experiment (84 hours) and not moved until the life status of alive or moribund was determined. During the water control, samples were kept in normal conditions, but the well water was changed four times, the same number as the maximum amount of water changes in the treatment groups (the fresh-fresh sequence with 12- and 24-hour latency). Water changes included removing 2 mL of water from each well and replacing it with 2 mL of normal conditioned artificial seawater at 14°C (to prevent temperature shock). The water was changed at the same time as the fresh-fresh and 12 latency time treatment, every 12 hours for a 36-hour duration. Finally, the life status of the control groups was determined at the same time as the final treatment groups (minimum 109 hours after sorting). There were no controls used in experiment one due to low sample sizes. No statistical tests were performed on the control data.

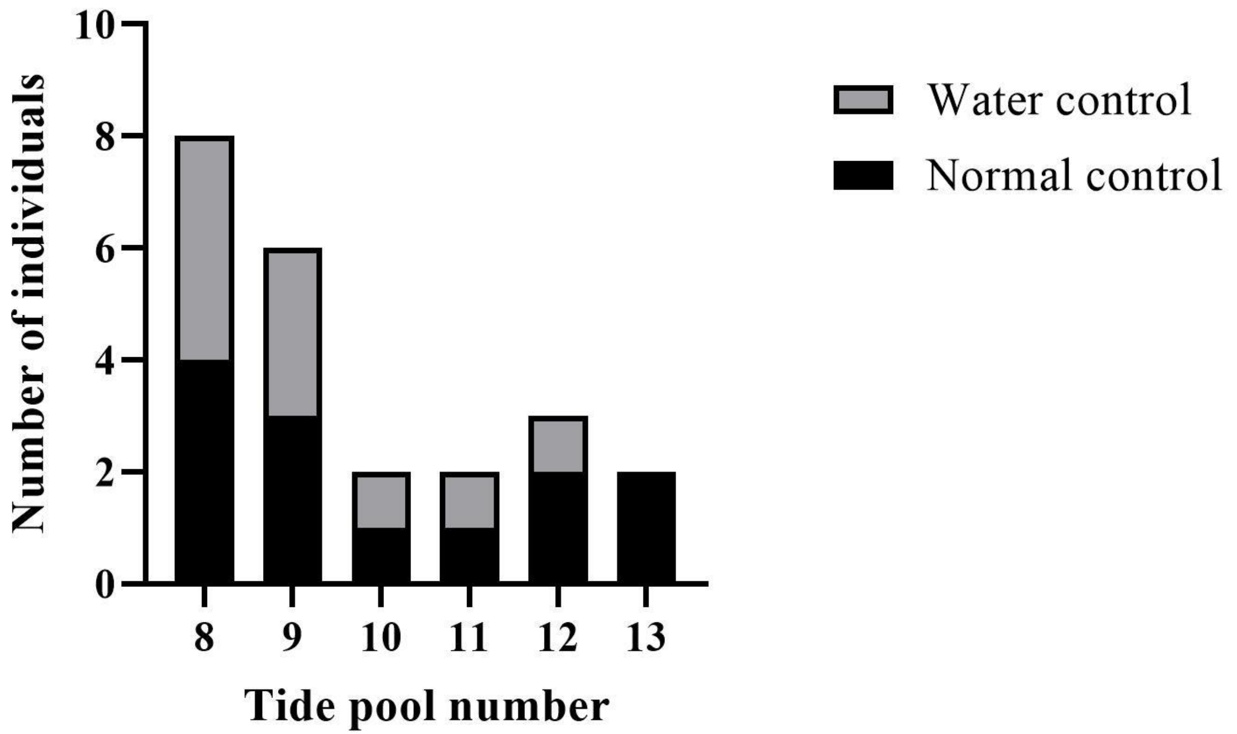

Figure 11. The number of copepodite life stage *Tigriopus californicus* individuals that the normal control (dark) and water control (light) were applied to from tide pools 8 to 13. Individuals collected from tide pools 4 to 7 did not undergo control treatments.

Results

There was high survival in the controls with only two individuals not recovering in the normal control (Figure 11). In particular, one copepodid from tide pools 8 and 12 was moribund when the normal control was applied.

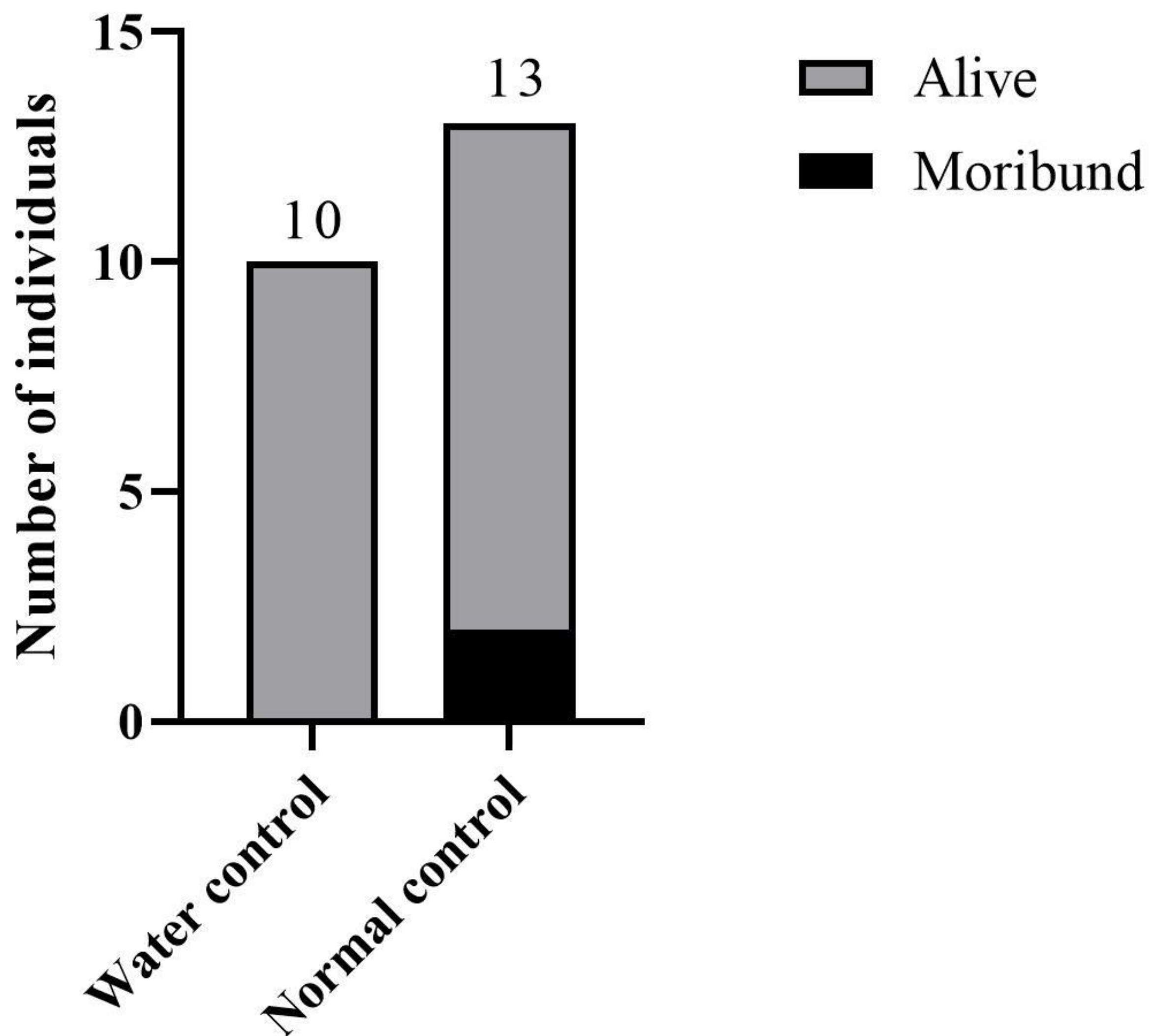

Figure 12. The number of *Tigriopus californicus* copepodids alive (light) and moribund (dark) in the water and normal control treatments. The sample size for each treatment is indicated on top of the bars.

Discussion

Control results were not used in experimental data analysis because of unlikely mortality created by laboratory conditions. The results show, there was fatality in the normal but not the water control. A source of mortality could have been starvation due to the lack of bacterial food in the well plates. For example, Spaeth *et al.* (1997) found that "clean" dishes increased the mortality of *T. californicus* individuals in control conditions. Yet, death due to lack of food would have been expected in the water control. Furthermore, *T. californicus* can survive 7-10 days without food which is longer than the length of time experiments took, showing that starvation was unlikely to cause mortality in the normal control (Spaeth *et al.*, 1997). The most likely reason for fatality in the normal control treatment was hypoxic surroundings created by the closed well plate. Hypoxia would have been created in the wells because oxygen could not flow through the sample water due to plates not being moved or opened. No moribundity was seen in the water control because proper oxygenation would have occurred during water changes and plate movement. The stressor treatments would have also experienced water changes (in the freshening treatments) and plate movement (in the cold treatments) and thus not be exposed to hypoxic conditions which could have increased mortality as seen in the normal control. Therefore, I conclude that the mortality seen in the normal control likely would not have occurred in the experimental treatments.

My control data agree with other studies that found *Tigriopus* species are good model organisms (Raisuddin *et al.*, 2007; Fraser, 1936). Because of the high survival of *T. californicus* in control conditions, I show that field-fresh, non-laboratory-reared individuals can be used in stressor experiments. Particularly, I showed that *T. californicus* can withstand artificial seawater which is important for use in salinity manipulation experiments to control for confounding variables. Furthermore, the high survival of this invertebrate makes them a reliable organism to

keep in the laboratory for experiments. Overall, these factors add to the previously identified variables that show *T. californicus* is a model organism.

Appendix B: Collection Observations

Personal observations

During the beach survey of Arbutus Cove in October 2022, I observed many *T. californicus* individuals actively swimming around the tide pools. However, when collecting in January, there was no swimming individuals visible, and instead, some were seen hanging onto algae. I found that the samples with more sediment had increased numbers of *T. californicus* and juveniles were more prevalent than adults overall. Furthermore, most adults in the samples, excluding gravid females, were paired in mate guarding position.

Discussion

Overall, I observed some interesting behaviours that should be noted for collection considerations in future studies. For example, the lack of *T. californicus* visibly swimming in the tide pools, making collection more difficult, could be due to the time of day that collections took place. The first collection started after the sun went down and the second during sundown therefore, during darkness, individuals may not actively swim around and rest on the benthic surface instead. Also, collections took place right after tide pools were exposed, suggesting that specimens may cling to sediment or algae during a receding tide to avoid being washed away. An issue with collecting specimens from algae or sediment is that the tide pools are disturbed by taking algae or mixing up the benthic surface (Powlik, 1998). Therefore, when planning collection, the time of day and tidal height should be considered for increased efficiency and decreased pool disturbance.

Similar to other studies, I found that most adults were in mate-guarding pairs which prevented me from using adults in my experiments (Spaeth *et al.*, 1997; Powlik, 1998). In pilot

studies, I found it very difficult to separate mate-guarding pairs without killing the individuals, so I opted to use copepodids. This an important consideration because to keep sexes separate from each other they must be laboratory-reared which does not reflect natural variation as previously discussed. An interesting study in the future could research the differences in stress survival of the sexes when separated or in mate-guarding pairs to highlight the ecological relevance of *T. californicus* adult stress studies. Another reason I used individuals in the copepodite life stage was that they were more abundant in my samples compared to the adults. The high abundance of copepodids opposes a trend found by Powlik (1998) who indicated that adults were most abundant in samples throughout all seasons in Barkley Sound. The contrasting results of Powlik (1998) and my observations shows that distinct areas may have different population structures of life stages. When a collection of *T. californicus* takes place the population structure of the beach should be assessed beforehand so that the wanted life stage will be available based on the study question of interest.

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