Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biology

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Does the rate of plasticity in active regulation of hemolymph osmolality and salinity tolerance depend on temperature?

Master's thesis in Biology
Supervisor: Sigurd Einum
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Abstract

The increased attention to both the speed and scale of global warming has underscored the importance of comprehending how organisms react to environmental variations. The link between temperature and biological rates is established by multiple studies, nonetheless, the evidence of this effect on plasticity rates is limited. This study aims to investigate if temperature influences the plasticity rate in the active regulation of hemolymph osmolality and freshwater tolerance in the littoral amphipod *Echinogammarus marinus*. The study will not only test the effects of the temperature on plasticity rate but also quantify this rate for a trait that exhibits mixed plasticity (i.e. traits that are subject to both passive influences from the environment and active regulation) using a novel analytical method. Specimens were acclimated at 5°C and 15°C in 15‰ salinity for five days before being transferred to 2‰ salinity at those same temperatures. Individuals were sampled at different intervals following this transfer, and the chloride concentrations in their hemolymph were measured. This enabled me to assess how fast the organisms adjusted their osmoregulation at the two temperatures. The model provides a good fit to the trends in the data for the plasticity rate. However, the estimates of the parameters used to find the plasticity rate revealed substantial uncertainty, and the results indicate no significant difference in the plasticity rates between the temperature treatments. Yet, the results revealed that animals acclimated to 15°C exhibited lower hemolymph chloride concentrations compared to animals acclimated to 5°C. Following this, I examine how acclimation temperature and acclimation duration in low salinity water influence the tolerance to freshwater. Specimens were acclimated for different durations (96 hours, 48 hours, 24 hours, 12 hours, and 0 hours) to 2‰ salinity at two temperatures (5°C and 15°C). After the acclimation, the animals were transferred to freshwater, and survival was monitored. Survival analysis for animals exposed to freshwater revealed generally higher survival rates (measured as LT50) in animals acclimated at 15°C, although no consistent trends were observed concerning the acclimation time. However, variability in responses, potentially influenced by factors such as molt stage, gender and specimen size, prevents definitive conclusions. Some specimens survived over 100 hours in freshwater and for 5 days in low-salinity water (2‰), which in general underscore the amphipods' great resilience, capability to survive across a wide spectrum of environments, and their significant role in ecosystems.

Sammendrag

Både omfanget og hastigheten av global oppvarming har fått økt oppmerksomhet de siste årene, og det har blitt enda viktigere å undersøke og forstå hvordan ulike organismer responderer på variasjoner i miljøet. Flere studier har påvist en kobling mellom temperatur og biologiske hastigheter, men det finnes få bevis for denne effekten på plastisitetsrater. Denne studien har som mål å undersøke om temperatur påvirker plastisitetsraten i aktiv regulering av hemolymfe osmolalitet og ferskvannstoleranse hos amfipoden *Echinogammarus marinus*. I tillegg til å teste effekten av temperatur på akklimering, blir også hastigheten til et trekk som både demonstrerer aktiv og passiv plastisitet kvantifisert. Dette ble gjort ved å ta sammenhengende blodprøver over 5 timer av dyr som ble overført til 2‰ etter å ha vært akklimert i 15‰ i fem dager ved to ulike temperaturer (5°C og 15°C). Resultatene fra forsøket viste at modellene for å beregne plastisitetshastighet gir en god tilpasning til de målte dataene, men det var imidlertid ikke mulig å avdekke om det var en signifikant forskjell mellom de to akklimerings-temperaturene grunnet betydelig usikkerhet i estimatene. Likevel hadde dyr akklimert til 15°C lavere kloridkonsentrasjon i hemolymfen sammenliknet med dyr som var akklimert til 5°C. I den andre delen av studiet undersøkte jeg hvordan ulik akklimeringstemperatur og varighet påvirker toleranse og dermed overlevelse når dyrene eksponeres til ferskvann. Dette ble gjort ved å akklimere dyr i ulike varigheter i 2‰ vann ved to ulike temperaturer (5°C og 15°C), for deretter å eksponere individene for ferskvann. Overlevelsesanalysene viste at det ikke var mulig å se en effekt av akklimeringstid for noen av behandlingene. Det var imidlertid mulig å påvise en positiv effekt av temperatur på LT50 verdiene, der dyr akklimert i høyere temperaturer demonstrere høyere overlevelse sammenliknet med dyr akklimert i lavere temperatur. Det skal understrekes at var variasjon i responsene og i dataene generelt i begge deler av studiet, som gjør det utfordrende å trekke endelige konklusjoner. Generelt overlevde noen individer i over 100 timer i ferskvann og 5 dager i 2‰ som viser til hvor tolerant denne arten er til svingninger i miljøvariabler og deres sentrale rolle sett i økosystem-sammenheng.

Preface and acknowledgments

This master´s thesis is submitted as the end of the five-year teaching program at Norwegian University of Science and Technology (NTNU) in Trondheim. The scope of this thesis is 30 study points and corresponds to one semester. In my third year I chose marine biology as a primary course, alongside chemistry and pedagogy. My interest in this topic began when I had my current supervisor on previous biology courses, whose teaching and research left a positive impression on me. Consequently, when exploring the available master´s thesis topics, the one focusing on gammarids stood out as particularly interesting. As a soon-to-be biology teacher, I see the need to learn more about how species respond to rapid climate changes, and I found this thesis to be a perfect opportunity to work with this matter. Although I initially had little knowledge of gammarids, the steep learning curve has both been challenging and rewarding, and a good insight into how a scientist thinks and works.

First and foremost, I would like to express my gratitude to my supervisor, Sigurd Einum, for all the assistance provided throughout this thesis. His availability and support have been unique and have greatly enhanced my learning process during this semester. He has not only assisted with correcting the thesis, but also contributed to the collection of animals, rearing of animals, development of methods, and pilot experiments. I would also like to thank my co-supervisor, Tim Burton, for revision of the text and content. Additionally, I am grateful to my fellow students, Nora Thelma Jevne and Martine Camilla Graham whose companionship during night-work shifts, gammarid collecting and rearing made this thesis more manageable and entertaining.

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Introduction

Species can adapt to changed environmental conditions caused by tides, droughts, flooding, and land runoff in several different ways. While the quickest and easiest strategy for organisms to avoid the fluctuations is relocation, others must employ adaptive strategies that require modification of their physiological processes to overcome these challenges (Einarson, 1993). The slowest mechanism is evolution caused by selection for particular phenotypes, which will result in modification of the genetic variation in each population (Burton et al., 2022). Another mechanism involves the expression of phenotypic plasticity, wherein a single genotype can generate multiple phenotypes based on environmental conditions (DeWitt et al., 1998). Plasticity stands out as an essential and immediate adaptive mechanism, especially significant due to its rapid response, a feature particularly relevant in the context of sudden shifts in the environment. However, depending on the trait, an animal exposed to a new environment may simultaneously exhibit both active and passive plasticity whereby the only active component represents an adaptive process (Havird et al., 2020). An example of passive plasticity is the increase in respiration rates of ectotherms in response to rising temperatures. As time progresses, organisms might employ active plasticity responses to counteract this effect, as passive plasticity brings the trait away from its optimum (Kielland et al., 2017). The simultaneous expression of both active and passive plasticity, hereafter referred to as mixed plasticity, underscores the complexity of biological adaptability in nature.

When investigating plasticity there are two different parameters to consider: the capacity for plasticity and the rate of plasticity. The primary focus of research has revolved around explaining the differences in the degree of plasticity (i.e. capacity) observed among organisms. However, even if an organism can adapt to its environment, the effectiveness of this adaptation depends on the speed at which it occurs, or the rate of plasticity. For an organism to capitalize on the benefits of plasticity, its adaptive responses must occur instantaneously or closely with the pace of environmental change. Failure to do so could result in the organism struggling to effectively adapt to a new environment, creating a potential "lag" in its adaptive responses. Quantitative analyses reveal variations in plasticity rates among species, where terrestrial organisms tend to have higher rates compared to aquatic organisms (Einum & Burton, 2022). Thus, the experimental work on plasticity would likely benefit from incorporating measurements of rates in addition to the more traditional measurements of capacity.

The intertidal zone is influenced by fluctuations that alter physiochemical factors such as air, pressure, dissolved oxygen, mineral composition, and salinity. These variations are particularly interesting concerning organisms' plasticity, to better understand their adaptive capacity in dynamic environments. The total concentration of inorganic salts in the open ocean exhibits a relatively stable range of 33-37‰ (De Mora, 2007). In contrast, coastal environments have considerable variability in salinity influenced by regional, local, and seasonal factors. Additionally, fresh groundwater from inland mixes with the seawater and results in a broad salinity range (Liu et al., 2018). Moon phase and tidal variation further impact these conditions, causing shifts in the upper part of the ocean. These factors collectively expose marine life in tidal habitats to both sudden and repetitive shifts in their environmental conditions. Many of these organisms are osmoregulators, with mechanisms allowing them to mitigate diffusion gradients and reduce the ion-water permeability across their body surfaces (Kirschner, 1979).

This adaptive mechanism plays a pivotal role in facilitating biological functions, including growth, respiration, and locomotion (Torres et al., 2011; Whiteley & Taylor, 2015). For crustaceans the gills act as the main location where ion uptake processes occur, involving the precipitation of key proteins such as Na^+-K^+-ATPase and ion co-transporters such as Na^+-K^+-Cl^- symporter (Cieluch et al., 2007; Lucu & Towle, 2003; Torres et al., 2011). Alternatively, passive osmoregulation occurs as organisms naturally align the osmolality of their internal fluids with that of their surroundings, thus maintaining osmotic balance without the application of energy (Campbell & Jones, 1989). While passive osmoregulation is driven by concentration gradients, active osmoregulation requires the expenditure of energy to actively transport ions and molecules against the concentration gradients (Lignot & Charmantier, 2015).

Temperature is an important environmental variable that also affects the abundance and distribution of any given species (Sinclair et al., 2016). Temperature can impact various physiological mechanisms, including metabolism, cellular regulation, and reproduction. Ectothermic organisms, which fluctuate their internal temperatures based on external environmental conditions, are particularly susceptible to alterations in temperature (Gillooly et al., 2001). This susceptibility is evidenced by the impact of ambient temperature on metabolic rates, often assessed through measures such as oxygen consumption, primarily through passive plasticity. Traditional experimental biology typically concentrates on isolated tests, examining the organism's response to a single environmental variable at a time (e.g., temperature, salinity, UV). Additionally, reaction norms serve as a common method to quantify plasticity, revealing the relationship between a given trait, and an environmental factor like temperature. However, such singular tests may not capture the complex interactions organisms experience in natural conditions, considering simultaneous fluctuations at once. For instance, temperature and salinity are two environmental variables known to have substantial impacts on the physiology and behavior of aquatic organisms. Studies have confirmed that temperature can influence the sensitivity of ectotherms to salinity changes, consequently affecting their osmoregulatory abilities (Huey & Kingsolver, 1989).

In the littoral and sublittoral zones, short-term and long-term variations in both temperature and salinities occur due to tidal influences (Einarson, 1993). Furthermore, species residing in coastal regions are subject to the influence of freshwater inflow from rainfall (Simpson, 1997). Within this context, amphipods, specifically gammarids, hold ecological significance due to their diverse distribution and roles within marine food webs (Costa et al., 1998). Notably, more than 100 species of gammarids are found in freshwater, brackish, and marine environments (Cieluch et al., 2007), and factors like a broad dietary spectrum, adaptability in foraging, migration, and drift behavior, and high reproductive capacity have allowed their great ecological success (Gerhardt et al., 2011). *Echinogammarus marinus* is one of the most abundant species in intertidal communities on hard substrata stretching from Norway to Portugal (Maranhão & Marques, 2003). The amphipods in this area have been found in salinity and temperature ranges between 4–31‰ and 7–29 °C, and are therefore considered suitable for this present study (Marques & Nogueira, 1991).

The correlation between temperature and biological rates, as highlighted in Einum and Burton's (2022) meta-analyses, supports the idea of a link between temperature and rates of plasticity. Based on published data describing how temperature tolerance changes over time under acclimation to sub-lethal high temperatures they calculated the

rate of plasticity in temperature tolerance. One of their findings was that this rate of plasticity was higher in experiments that used higher acclimation temperature. However, direct experimental evidence for this effect is lacking. The main aim of the current study is to address this issue, and the study compromises two components. In the first part, I investigate the impact of temperature on the acclimation process to a change in salinity. Specifically, I investigate how two different acclimation temperatures (5°C and 15°C) affect the regulation of chloride in the hemolymph in *E. marinus* shifted from 15‰ salinity to 2‰ salinity. The hemolymph chloride concentration represents a trait exhibiting a mixed plasticity response whereby the phenotype shows both a passive response to environmental changes (through diffusion) and an active regulatory response. Previously, no method has been available to calculate the rate of plasticity for such traits, and my study represents a first attempt to apply an analytical method to such data. In the second part, I test how temperature and acclimation duration to a low salinity influence the survival of animals exposed to freshwater. This is done by acclimating the organisms for different durations (0, 12, 24, 48, and 96 hours) and temperatures (5°C and 15°C) in low salinity water (2‰), followed by freshwater exposure.

Methods and materials

Collection and rearing

E. marinus were collected in November 2023 and February 2024 from the upper tidal zone of Korsvika, Trondheimsfjorden, Norway (63.449705 °N, 10.431850 °E). The specimens were collected under stones during low tide. The animals were transported to the laboratory in containers filled with seawater from the collecting area and transferred to a cultivation tub (50L) filled with experimental seawater (30L tap water, 15‰ salinity, Aquavital seasalt, Aquarium Münster). The animals were acclimated in a temperature-controlled room (10°C) with an aquarium air pump (AM Top CR-10) to ensure suitable water quality. The amphipods were fed three times per week with fish food pellets (Tetra cichlid color) and kept in 24-hour dim light. The water in the cultivation tub was changed once per week and small stones were placed in the bottom of the tub to provide shelter.

Treatment and sampling

Acclimation for both experiments was done in climate cabinets (Memmert IPP260plus incubator) under 24-hour dim light (intensity set to 1%).

Hemolymph chloride content

A total of 200 specimens were selected with 100 subjected to acclimation at 5°C and the remaining 100 at 15°C, all at a 15‰ salinity for 5 days (8L beakers filled with 4L water). During the acclimation phase, the animals were provided with food for four hours each day, and the water was changed after each feeding. There was a small stone placed in every glass to provide shelter and air was supplied by aquarium pumps to ensure appropriate oxygenation. After the acclimation period, the animals (n=190) were transferred to 2‰ salinity at their respective acclimation temperature, and individuals were obtained for hemolymph samples at three-minute intervals during the first hour, and then at six-minute intervals for the next 4 hours. Additionally, a final sample of individuals (n=10) was taken for hemolymph samples 24 hours post-transfer.

To measure the chloride concentration in the hemolymph, each specimen was rinsed with de-ionized water and dried on filter paper. Subsequently, the specimens were punctured on the dorsal side of the 2nd thorax segment using a syringe needle. The hemolymph was transferred into a glass capillary through capillary action (Micropipette, intraMARK, BLAUBRAND®, 125mm 1-5µl). Approximately 2 microliters of the hemolymph were collected from each individual. This was expelled into an Eppendorf tube pre-filled with 25 microliters of de-ionized water. The Eppendorf tube was then vortexed and frozen (-20°C) for subsequent analysis. Following the collection of hemolymph samples, the animals were weighed and underwent a drying process at 50°C for 48 hours to get the subsequent determination of dry weight. The concentration of chloride in the hemolymph was analyzed using a Sherwood Chloride Analyzer 926S.

Freshwater tolerance

The specimens were acclimated to 2‰ saltwater at varying time intervals, with high (15°C) and low (5°C) temperatures in 1L beakers containing 0.8 L water. Each beaker contained 30 animals, and there were 10 different treatments with one beaker per treatment. Five of the beakers were placed in a climate cabinet set at 5°C and were acclimated to 2‰ salinity for durations of 96 hours, 48 hours, 24 hours, 12 hours, and 0 hours. Before transfer into 2‰ salinity, the animals had been residing in water with a

salinity of 15‰ within the same beaker. The other five beakers were subjected to a temperature of 15°C, undergoing the same five salinity-treatment durations. Thus, animals were kept in the beakers for 96 hours in total for all treatments. During the acclimation phase, the animals were provided with food for four hours each day, and the water was changed after each feeding. The beakers had aeration (aquarium pump) during the acclimation to obtain appropriate oxygen levels.

Following the acclimation periods, individuals from each treatment were transferred to freshwater in identical beakers kept at 10°C. Survival was recorded every hour for the first nine hours. Subsequently, the interval between each observation was progressively extended until the final assessment, which took place 116 hours post-transfer. The deceased specimens, determined by the absence of responsiveness to disturbance, were removed for subsequent taxonomic verification.

Statistical analysis

All statistical analyses were performed using R (Version 2023.12.1+402)(Team, 2021). To analyze the measurements of the hemolymph chloride, I first applied a linear regression model with log hemolymph chloride content as the dependent variable to investigate the effects of temperature treatment (factor), interaction between temperature and time, individual dry weight, and log time acclimated in the 2‰ saltwater medium. By running the model with the *dredge* function from the *MuMin* package in R (Barton, 2009), the most suitable model was chosen. The verification of model assumptions was conducted through a visual analysis of the residual plots, checking for constancy of variance and normal distribution of residuals in the models.

A recently developed method for mixed plasticity traits (S. Einum, unpublished) was used to estimate the rate of plasticity in chloride regulation. The mathematical representation captures both the immediate passive response and the gradually increasing active response to a sudden change in the environment. The first equation,

$$\frac{dz}{dt} = k \cdot (E_2 - z(t)) \; - \; k \cdot E_1 \cdot (1 + \; c \cdot \; (1 - e^{(-\lambda \cdot t)}) \quad \text{(eq.1)}$$

describe how the rate of change in the trait (z) over time (t) is influenced by the new environment (*E2*) and the organism's active response to the shift from the initial environment (*E1*). Both E and Z are expressed in the same unit (concentration). Here, c is a unitless constant that modulates how much the active regulation can change, and λ represents how fast this regulation can adjust. The passive effect of E on z is directly proportional to their difference, expressed as $k \cdot (E_2 - z(t))$ where k is a constant. k is specific for both the environmental factor and the traits of the organism involved and has units of inverse time. Then we set z(0)=0 and the other values of z(t) and E relative to this initial value. This allows for setting z(t)=0 when solving the equation. The trait value as a function of time is given as:

$$z(t) = e^{-kt}(E_1 - E_2 + E_1 c - \left(\frac{E_1 ck}{k-\lambda}\right)) - e^{-kt}(e^{kt}(E_1 - E_2 + E_1 c) - \left(\frac{E_1 cke^{kt-\lambda t}}{k-\lambda}\right) \quad \text{(eq.2)}$$

By fitting this model, I estimated the parameters k, c and λ based on the measured data of the trait values using the function *nls_multstart* from the *nls.multstart* v.1.2.0 package (Padfield & Matheson, 2020). To evaluate whether there were significant differences in the estimated parameters (k, c and λ) between the two acclimation treatments, a bootstrap procedure was conducted on the dataset. A total of 1000 bootstrap samples were obtained (random sampling with replacement), and for each of these I estimated the value of the parameters (k, c or λ) for the two temperature treatments (using nls_multistart as described above). Finally, for each bootstrap sample, I calculated the difference between the two temperatures for each of the parameters.

The salinity tolerance data was analyzed using a logistic regression model (generalized linear model with a binomial distribution). The primary objective of this analysis was to establish the LT50 values, which is the time by which 50% of mortality occurs during exposure to freshwater, for the different acclimation groups. For every acclimation time and temperature combination, I used logistic regression models to fit binominal outcomes of the survival data. LT50 was calculated from the logistic regression coefficients (β0 and β1) using the following formula:

$$LT50 = -\frac{\beta 0}{\beta 1} \text{ (eq.3)}$$

Where β0 refers to the intercept and β1 refers to the slope in the regression model. From the model output the standard error (SE) of the LT50 is given as:

$$SE_{LT50} = \sqrt{\frac{var(\beta 1)}{\beta 1^2}} \text{ (eq.4)}$$

Confidence intervals were calculated as ±1.96 * SE around the LT50 estimate. To test the effect of temperature on plasticity rate to low salinity, a linear model was fitted with LT50 as the dependent variable, and acclimation time and acclimation temperature and their interaction as explanatory variables. By running the model with the *dredge* function from the *MuMin* package in R (Barton, 2009), the most suitable model was chosen.

Results

Chloride concentration

The concentration of chloride in the hemolymph of *E. marinus* showed a declining trend over time following transfer from 15‰ to 2‰ salinity for both temperature treatments (figure 1). The 2‰ medium had a chloride concentration of 30mmol/L and the 15‰ had a concentration of 273mmol/L. The amphipod chloride concentrations in the hemolymph started at 378 ± 27.53 mmol/L for animals acclimated to 15°C, and 397 ±10.87 mmol/L for animals acclimated to 5°C (mean values± SE). Thus, the animals were slightly hyperosmotic to the 15‰ saltwater. After exposure for 24 hours in 2‰ salinity, the amphipods were highly hyperosmotic, with animals acclimated to 15°C having an average chloride osmolality of 194.9 ± 40.72 mmol/L and animals acclimated to 5°C having an average chloride osmolality of 251 ± 21.09 mmol/L.

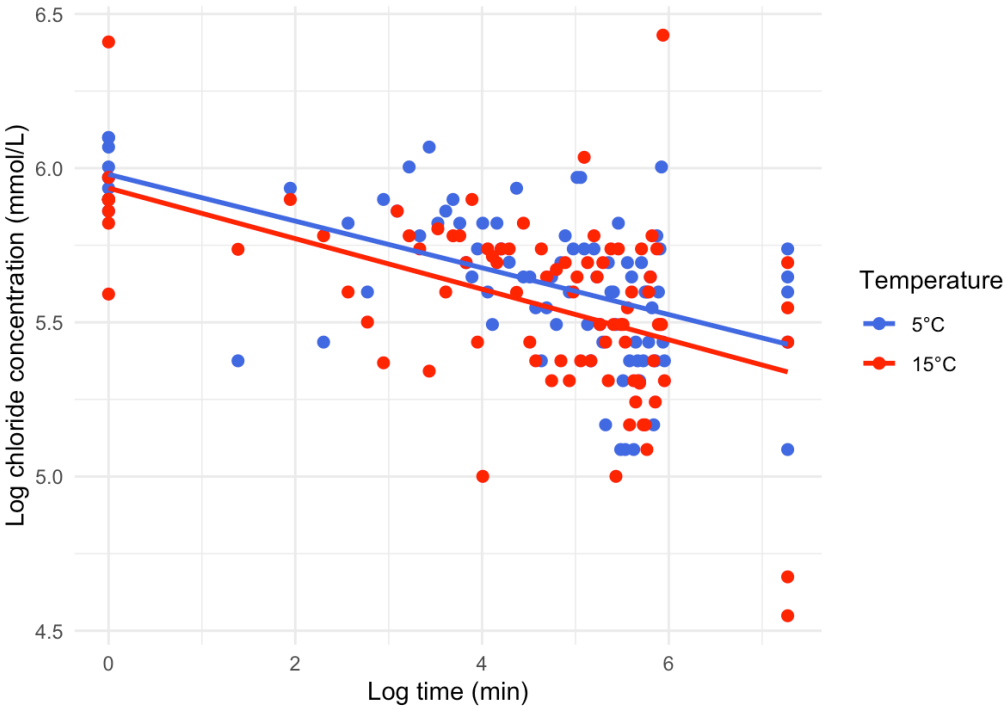

Figure 1: Chloride concentration in hemolymph of E. marinus acclimated to 5°C (blue dots) and 15°C (red dots) at different time points following transfer from 15 to 2‰ salinity medium. Each point represents one individual measurement of log-transformed chloride concentration at the corresponding log-transformed time. The lines represent predicted linear relationships from models fitted to the data points for each temperature group.

For the chloride data, the model comparison showed that the simplest model with a *Δ*AICc value less than 2 contained the main effects of time and temperature. Time remains as a significant predictor where increasing time leads to a decrease in hemolymph chloride concentration (table 2). There is also a tendency for an effect of temperature on chloride concentration, suggesting that animals acclimated to higher temperatures have lower chloride concentrations.

Table 1: AICc comparisons derived from using the dredge function on the linear regression model to investigate how hemolymph chloride concentration is influenced by various factors in *E. marinus* acclimated to 5°C and 15°C. The table present a summary of the top candidate models which include the effects of time, temperature, interaction between time and temperature, dry weight, and the wet/dry ratio as predictors of the log-transformed chloride concentration. ΔAICc represent the difference between the AICc of the best model and the presented model. W_i represents the relative likelihood of each model given the data.

Reduced dataset containing chloride osmolality data	K	AICc	ΔAICc	w_i
Temperature + Dry weight + Time + Wet/dry ratio + Temperature × time	6	-36.9	0.00	0.21
Temperature + Dry weight + Time	5	-36.5	0.40	0.17
Temperature + Time	4	-36.5	0.44	0.17
Temperature + Time + Wet/dry ratio	5	-36.8	1.06	0.13
Temperature + Dry weight + Time + Wet/dry ratio + Temperature × time	7	-36.8	2.07	0.08

Table 2: Regression coefficients from the model selected (table 1) following the dredge procedure with main effects along with their estimates, standard errors, t-values and p-values. The chloride concentration is regressed against the independent variables time (h) and temperature. The intercept is the chloride concentration when time is set to 0h and temperature at the reference level (5°C). Both chloride concentration and time are log-transformed.

Coefficients	Estimate	s.e.	t-values	p-values
Intercept	5.99	0.04	137.27	< 0.001
Log time	-0.078	0.008	-9.12	<0.001
Acclimation temperature	-0.099	0.033	-2.73	0.07

These results show a response that is both an effect of the active and passive response to changes in salinity. The isolated effect of active plasticity is therefore obtained by using equation 2. The parameters of k, c and λ were estimated with high relative errors, particularly for the λ estimate (5°C had a RE= 498.33% and 15°C had a RE= 756.19%) which suggests a large degree of uncertainty in the estimates (table 3). However, the model gives a good fit for the data for both treatments (figure 2). The results from the bootstrapping method demonstrate that the distribution for all estimated differences in the parameters are close to zero. Moreover, the confidence interval overlaps the zero marker, which implies no significant difference in parameter estimates between the two acclimation temperatures (figure 3).

Table 3: The estimated parameters *k*, *c* and *λ* along with their standard errors, t-values and p-values by using nonlinear leas squares (nls) regression analysis on the two temperature treatments on *E. marinus*. Animals were acclimated to 5°C and 15°C for five days in 15‰ salinity, and then transferred to 2‰ salinity to measure hemolymph chloride concentration. These values were used in equation 2 to obtain the plasticity parameters.

Acclimation treatment			*5°C*				*15°C*	
Parameters	*Estimate*	*s.e.*	*t-value*	*p-value*	*Estimate*	*s.e.*	*t-value*	*p-value*
k	0.01	0.0096	1.45	0.150	0.02	0.05	0.50	0.61
λ	0.12	0.59	0.20	0.841	0.10	0.79	0.13	0.89
c	0.79	0.09	8.18	<0.001	1.10	0.10	10.49	<0.001

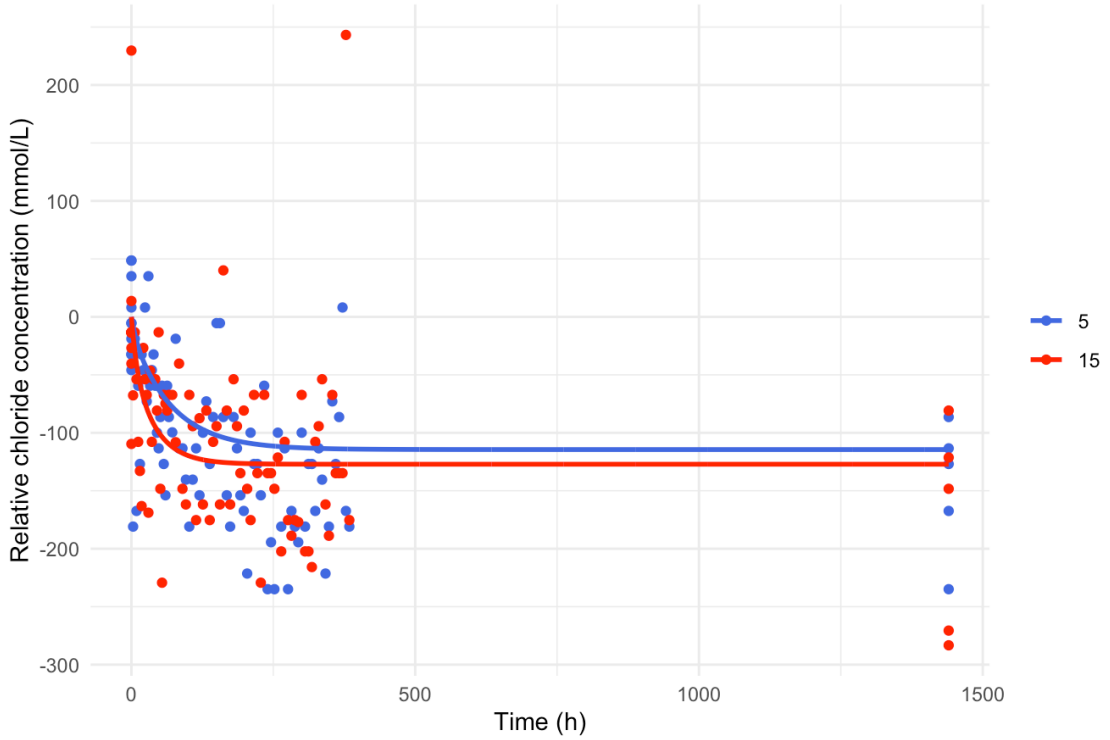

Figure 2: Change in hemolymph chloride concentration over time after transfer from 15‰ to 2‰ salinity in *E. marinus*. The dots represent the actual measured values of the chloride concentration in animals acclimated to 5°C (blue) and 15°C (red). The lines show the rate of plasticity for the two acclimation treatments and are based on the estimates of the parameters *k*, *λ*, and *c* from equation 2. The amphipods acclimated to 5°C show a slower plasticity rate over time compared to amphipods acclimated to 15°C. The initial environmental value was 273 mmol/L, and the new environmental value was 30mmol/L with the start value for time being relative.

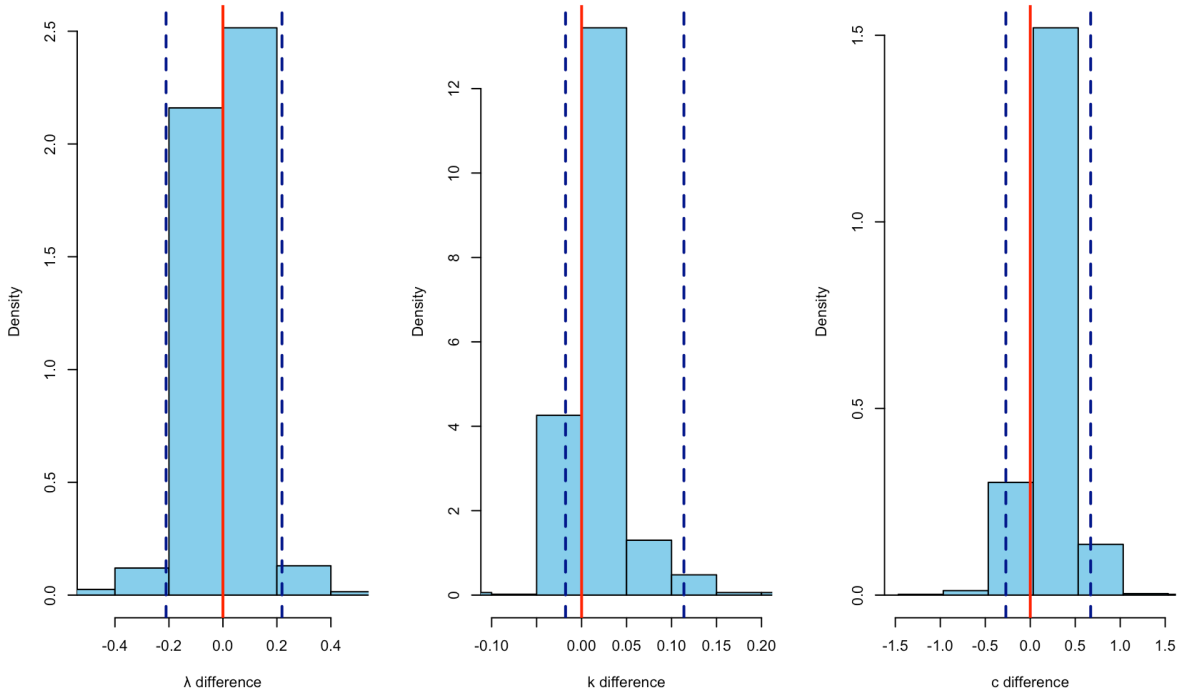

Figure 3: Distributions of differences in estimated plasticity rate parameters λ, k and c for the 5°C and 15°C acclimation temperature groups. The magnitude of the difference is presented along the x-axis while the y-axis indicates the density (frequency) for occurrences for each range of difference values. The vertical dashed blue lines indicate the 95% confidence intervals for each parameter, while the solid red line represents the zero-difference marker highlighting the point of no effect.

Freshwater tolerance

The survival curve for the ten trials exposed to freshwater showed a sigmoid shape (figure 4). The average lethal time for 50% mortality for the animals acclimated to 15°C was 50 hours, while the average LT50 for 5°C was 25 hours. The mean across all trials was calculated to be 38 hours, however, there was a substantial range with the maximum LT50 estimate reaching 75 hours (96h, 15°C), while the minimum LT50 estimate was 11 hours (96h, 5°C).

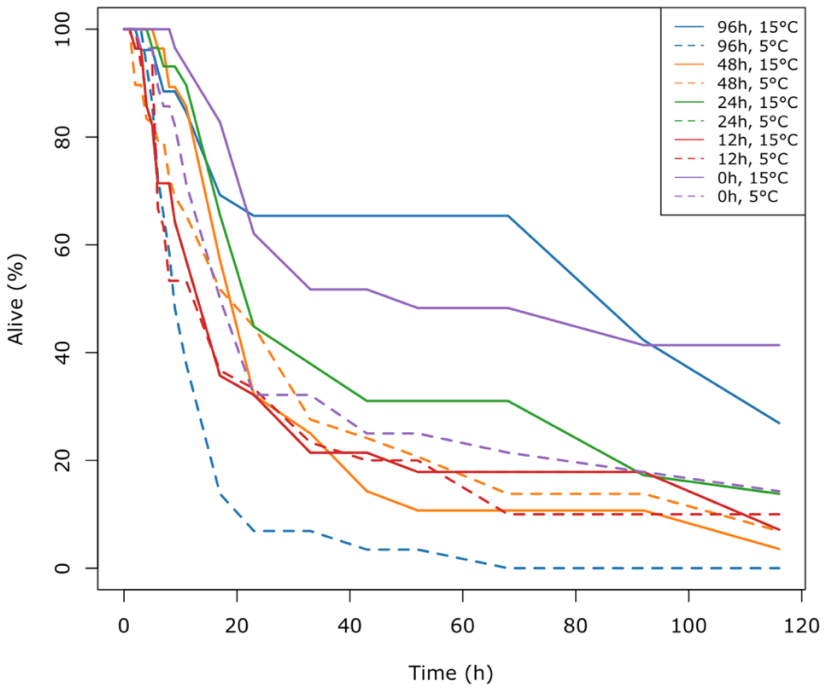

Figure 4: Survival curves for E. marinus when exposed to freshwater. The curve displays the percentage of organisms surviving over time across various acclimation periods (0, 12, 24, 48, 96 hours) in 2‰ saltwater reared in two different temperatures (5°C and 15°C).

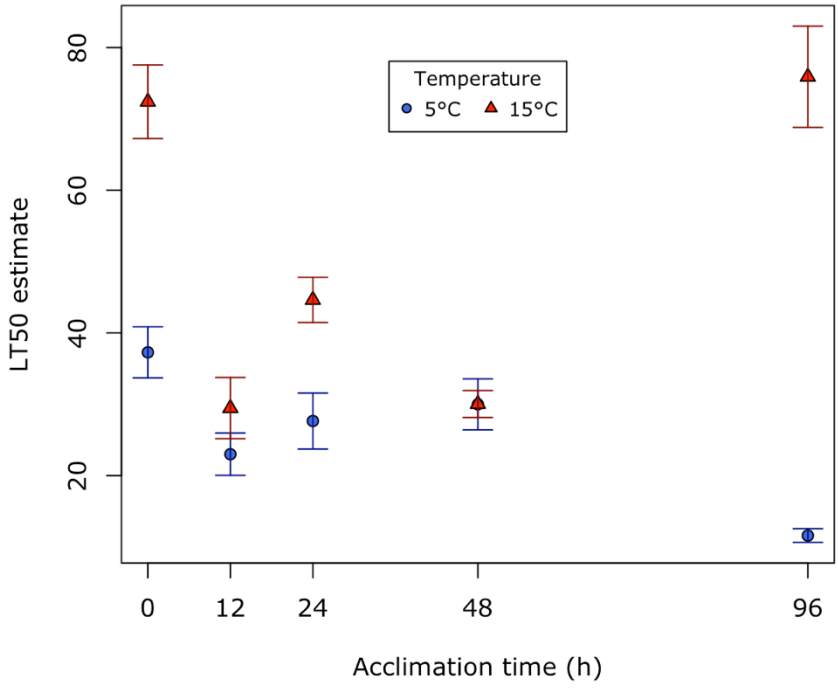

Figure 5: Lethal time estimates (LT50) for E. marinus acclimated in 2‰ saltwater for different acclimation times (0, 12, 24, 48, 96 hours) followed by freshwater exposure. The amphipods were acclimated at two different temperatures: 5°C (blue circles) and 15°C (red triangles). Error bars represent a 95% confidence interval and are derived from the standard errors of the LT50 estimates from the logistic regression model.

The animals show varying survival based on the different acclimation periods, although the longer acclimation period (96h) appears to provide higher probability of survival compared to shorter acclimation periods (48, 24 and 12h) for animals acclimated to 15°C. Contrastingly, the 5°C acclimated amphipods exhibit a declining probability of survival, with the lowest LT50 estimates at 96 hours acclimation. Moreover, there was a substantial difference between the two temperature treatments at 96-hour acclimation, where it has for the 15°C group led to increased survival, whereas the 5°C group demonstrated lowest survival. Despite this, the statistical analysis does not reveal an interaction between acclimation time and acclimation temperature (table 4).

Upon evaluating the AICc values from the model comparison process, the simplest model with the lowest delta ΔAICc was chosen (ΔAICc= 0.47 between the full model and the best fitting model). The linear model included the acclimatation temperature as a predictor against LT50 values as response value (table 5). Results indicate that temperature significantly affects the LT50 estimate, with higher temperatures leading to higher LT50 values.

Table 4: AICc comparisons from the top candidate models from model that investigate the effects of acclimation time and acclimation temperature on the LT50 estimates. ΔAICc represent the difference between the AICc of the best model and the presented model. W_i represents the relative likelihood of each model given the data.

Reduced dataset containing LT50 data	k	AICc	ΔAICc	w_i
Acclimation time + Acclimation temperature + Acclimation time × Acclimation temperature	5	255.8	0.00	0.49
Acclimation temperature	3	256.3	0.47	0.39
Acclimation time + Acclimation temperature	4	258.9	3.13	0.10

Table 5: Results from linear model including main effect of acclimation temperature on the LT50 values with their estimates, standard errors, t-values, and p-values. The intercept shows the LT50 estimate when acclimation is set to 0h and temperature at the reference level (5°C). Acclimation is the time when the animals have been acclimated to 2‰ salinity and temperature gives the estimates for the 15°C acclimation.

Coefficients	Estimate	s.e.	t-values	p-values
Intercept	25.90	4.12	6.27	<0.001
Temperature	24.58	5.83	4.21	<0.001

Discussion

The aim of this study was to examine the influence of temperature on the rate of plasticity in the regulation of hemolymph osmolality and salinity tolerance in *E. marinus.* This was accomplished through two approaches. In the first part, I took measurements of hemolymph chloride concentration following transfer from 15‰ to 2‰ salinity in individual amphipods acclimated to different temperatures. From these measurements, a novel mathematical method was utilized to quantify the plasticity rate in the two temperature treatments. In the second part, I assessed the survival during exposure to freshwater in individuals that had been previously acclimated to 2‰ for various durations at two different temperatures (5°C and 15°C).

The model utilized to estimate the rate of plasticity for the two acclimation treatments provided a good fit to the observed trends in the data (figure 3). The animals acclimated to 5°C exhibited a higher chloride concentration compared to the animals acclimated to 15°C, suggesting a potential advantageous effect of acclimation in higher temperatures. However, the measurements of chloride concentrations along with the estimation of the parameters k, λ, and c showed high relative errors. Consequently, it was not possible to establish a significant effect of temperature on the estimated plasticity rates. It may seem that the method utilized for estimating the plasticity-rates requires data with a low degree of noise. The substantial residual variation in the data from this study can therefore explain the high uncertainty in the estimated parameters. The observed variability in the measured chloride concentrations in the hemolymph could arise from either true biological variation or measurement uncertainty. The experiments utilized a random sample of specimens, without any sorting based on molt stage, gender, or age. Previous research indicates that ion contents in newly molted specimens can vary within the stages of the molting cycle for different amphipod species (Lockwood & Inman, 1973). Consequently, this could result in unexplained variation, introducing uncertainty in the estimated parameters. Other studies also demonstrated that the molt cycle is temperature-dependent, with more frequent molting at higher temperatures (Lopes et al., 2020; Pöckl, 1992). The acclimation temperature may therefore introduce additional uncertainty in the data for specimens acclimated to 15°C, because there could be a higher ratio of the newly molted individuals present compared to the animals in the 5°C treatment. Additionally, variation in blood ions may correlate with differences in size within the same gender. Dorgelo (1977) reported that small male specimens of *E. marinus* exhibited elevated blood sodium content compared with larger ones. The specimens used in the chloride experiment differed in size, with wet weights varying from 19.6 mg to 131 mg. However, the results did not reveal any significant effect of size, but gender was not accounted for in this study, so the potential influence of this factor cannot be completely excluded. Finally, measurement error could not be accounted for due to the limited blood volume, which prevents the possibility of taking multiple replicates from the same individual. Ideally, there should been taken measurements of the hemolymph from single individuals both before and after transitioning to a new environment. However, the animals died after the hemolymph samples was taken, making it difficult to monitor each individual's chloride concentration before and after the transfer.

Both temperature treatments indicated that the amphipods had higher concentrations of chloride in their hemolymph than in the surrounding water (hyperosmotic), which corresponds with previous studies of gammarids exposed to variable salinities (Dorgelo,

1977). Additionally, samples collected after 24 hours exposure to 2‰ salinity confirmed that the amphipods remained strongly hyperosmotic. This pattern corresponds with observations made in other gammarids such as *Gammarus oceanicus*, which exhibit an increased osmotic gradient between the hemolymph and its increasingly diluted environment (Einarson, 1993). Further evidence of the broad range in salinity tolerance between gammarids is documented in a study by Dorgelo (1977). He investigated three gammarids; *E. marinus*, *Gammarus tigrinus* and *Gammarus fossarum*, and documented distinct differences in salinity tolerance optima among these species. *E. marinus* is a littoral species, *G. tirgrinus* is an oligohaline species and *G. fossarum* is a freshwater species, each adapted to different salinity conditions. The study highlighted that the *E. marinus* is particularly adaptable, capable of hypo- and hyper-regulating in response to salinities around the optimum at 31‰. Both the ability to survive and the hyperregulation of the hemolymph in *E. marinus* acclimated to dilute seawater serve some adaptive function, given they have been observed to tolerate salinities as low as 2.5% in natural environments (Segerstråle, 1959). Furthermore, this osmoregulatory ability may be advantageous during freshwater runoff or heavy rainfalls that lead to extended exposure (Gerhardt et al., 2011).

The gammarids were able to survive low salinities (2‰) for more than 120 hours, and in freshwater for 100 hours (36 of 275 animals lived when ending the experiment). The survival of animals exposed to freshwater decreased over time, which is expected as *E. marinus* is a littoral species. The positive effect of acclimation time in low salinity water before exposure to freshwater was not possible to establish. This could arise from the low number of replicates in the experiment which limits the reliability of the results. With only one replicate for each treatment, it could be that the observed effects are due to random effects or uncontrolled variables than the treatment itself. Therefore, it will not be possible to distinguish between variation caused by the treatment and variation caused by other sources. Additionally, the observed substantial variation between replicates that is not related to the treatments further complicates the interpretation of the results. However, there was a significant effect of temperature on the LT50 estimates, with a higher temperature leading to higher LT50 values. This points towards an advantageous effect of acclimating to warmer temperatures before exposure to freshwater. This aligns with a study done by Torres et al. (2021) where they found that increased temperatures enhanced the capacity to osmoregulate in both zoea and megalope. The larva they exposed to higher temperatures (21-24°C) exhibited better response to lower salinities than those acclimated at 15°C. Additionally, they demonstrated that the temperature has a positive effect on both biochemical reactions and metabolic rate. These findings can explain the positive relationship between acclimation at higher temperatures and the increased capacity for osmoregulation and thereby survival in freshwater during the experiment.

Einum and Burton (2022) investigated the temperature-dependent plasticity rate using data from various studies on the acclimation of the tolerance to extreme in ectotherms. They found that the plasticity rate was generally low among crustaceans, although the rate increased with higher acclimation temperatures. The effect of temperature on the plasticity-rate could not be established in the present study. This raises the question of whether the findings from Einum & Burton (2022) reflects differences in plasticity rates among different species rather than an effect of temperature per se. Specifically, animals that naturally live in warmer environments would typically be exposed to higher acclimation temperatures when running experiments of the type that their data were

based on. The apparent positive effect of acclimation temperature on plasticity rate would then not necessarily be a direct result of the temperature itself but could rather represent an evolutionary adaptation to their natural habitat. However, the large uncertainty in estimates of the plasticity rate in the current experiment precludes a strong conclusion regarding this role of temperature.

In conclusion, the study explored the effects of temperature on the plasticity of *E. marinus*, revealing that the species can adapt to varying salinity levels. The findings for the freshwater tolerance highlight a greater survival and plasticity in animals acclimated to 15°C. Additionally, the model used to calculate the plasticity rate in a trait that exhibit mixed plasticity resulted in a good fit. However, the considerable variability observed in the chloride concentration measurements and the LT50 values suggest that there may be influence from other factors. Moreover, there were limitations in establishing the effects of temperature and acclimation duration due to the statistical challenges posed by biological variability and potential measurement error. Future studies should aim to control factors such as size, gender, and molt stage, either through stratified sampling or a larger sample size. Additionally, incorporating systematic analysis over a broader acclimation period and temperature range could give higher reliability. However, the study extends our understanding of amphipod salinity tolerance by confirming hyperosmotic regulation across different salinity exposures, consistent with other gammarids. Most organisms in their natural habitat detect and respond to multiple environments at the same time, yet much remains uncertain about how reactions to one environment may affect responses to others. Therefore, it is crucial to further explore how organisms respond to multiple environmental conditions to understand the origins in variability in phenotypic plasticity. Although this extends beyond the scope of the present report, the methodology and insights can stimulate future research in this area.

Implications for UN sustainability goals and climate changes

Anthropogenic climate change has become an increasing concern over the last decade, and it is shown to have measurable effects on ecosystems, communities, and populations. Over the past 100 years the earth´s climate has warmed by approximately 0.6 °C (Walther et al., 2002). Marine systems may be affected by this pattern in at least three ways: 1) temperature increase, 2) sea level increase and 3) decreasing salinities (Houghton et al., 2001). Additionally, the Intergovernmental Panel on Climate Change (IPPC) predicts that there will be more extreme weather hazards such as extreme temperatures, heavy rainfall, drought and tropical cyclones (Clarke et al., 2022; Lee et al., 2023; Pörtner et al., 2019). The adaptability of natural systems and organisms in response to challenges posed by climate change is well documented. However, with the increasing speed and magnitude of these changes, we now risk that the changes may surpass organisms´ physiological limits and adaptive capability, a concern highlighted by Duarte et al. (2012).

If the effects of temperature and salinity are relevant to fitness, they also affect how the species will survive and distribute over large areas. The amphipods hold great ecological significance within marine and aquatic ecosystems due to their diverse distribution and ecological roles within food webs (Costa et al., 1998). Gammarus spp. consist of more than 100 species distributed in both freshwater, brackish and oceans (Cieluch et al., 2007) and knowing how alternations in the environment affect the animals may reveal how animals will deal with future changes. Additionally, gammarids are detrivores, shredders and grazers and play a crucial role in the carbon cycle and in food chains in general (Cottin et al., 2012). Anger (2003) has demonstrated that salinity stress can significantly impact crustaceans, leading to disruptions in developmental processes, reduced survival rates, alterations in feeding and growth rates, variations in metabolic allocation and modifications in behavioral patterns. A consequence of this could be that the gammarids may face challenges in coping with substantial changes in the environment, which could impact survival in the future. In an educational context, this research serves as a good example of understanding nature as a complex system, demonstrating why an isolated study of a single factor can miss crucial interactions. Moreover, it aligns with one of the three cross-curricular themes, "sustainable development", where it emphasizes the importance of each species contribution in a healthy ecosystem. The research resonates with the United Nations sustainable development goals 13 and 14, which mention the importance of conservation of the natural environment, and sustainable management of the oceans and marine resources (UN, 2016). Additionally, the research enlightens how climate change can impact nature and underscores the importance of our awareness and reaction.

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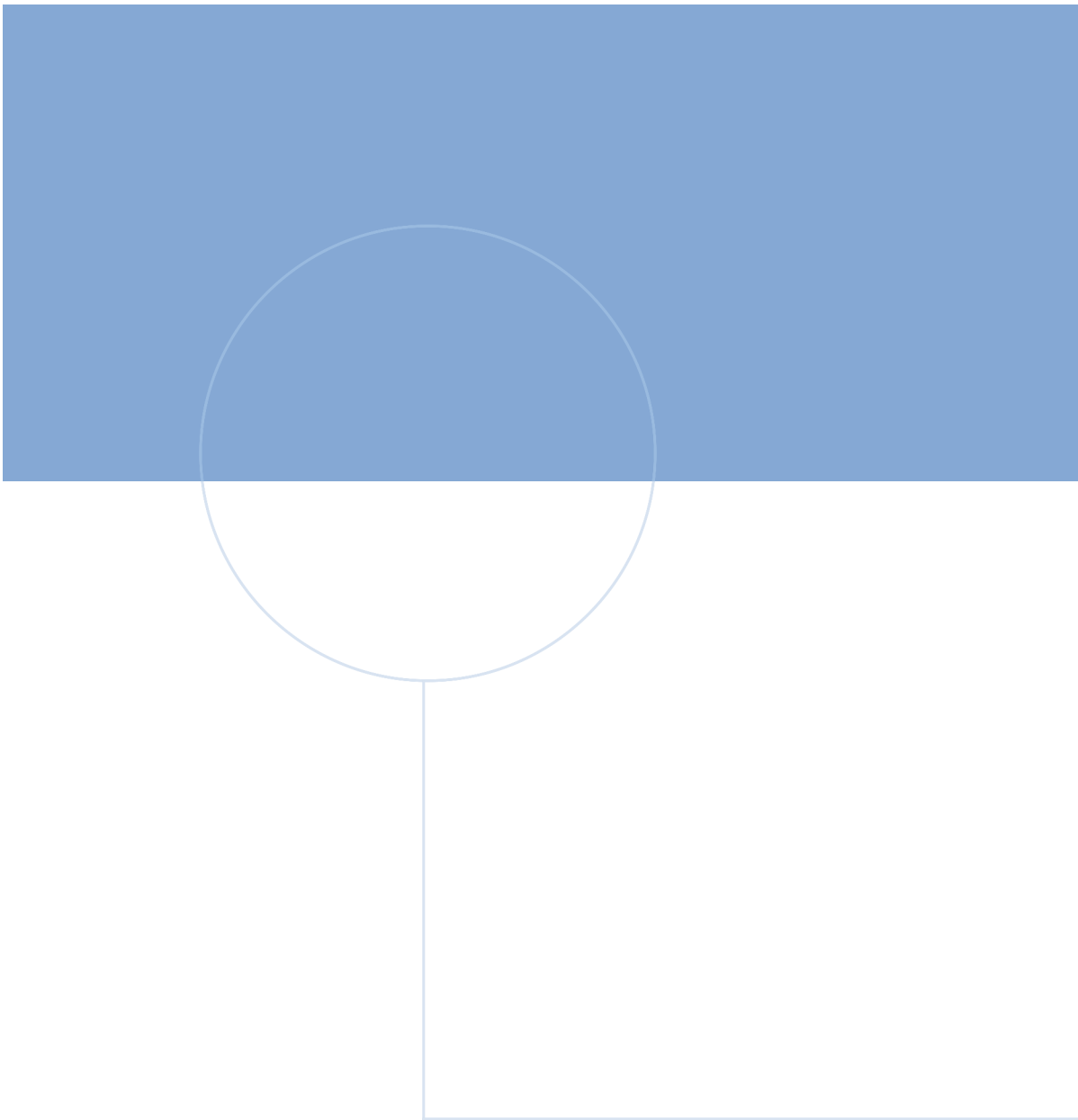
Attachments

Appendix 1: Codes

Summary of the functions from R that have been utilized in the statistical analysis (Team, 2013; Viechtbauer, 2010).

- "nls_multstart": to fit nonlinear least squares models with multiple starting parameters

- Variance Inflation Factor (VIF) from the car package: to assess multicollinearity amongst predictors within the linear models.
- "dredge": from the "MuMin"-package to carry out model selection.
- "glm": to make generalized linear models.



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