UNIVERSITY OF GOTHENBURG

DEPARTMENT OF BIOLOGICAL AND ENVIRONMENTAL SCIENCES

MTDNA COPY NUMBER VARIATION IN MALE *LITTORINA SAXATILIS*

Association between mtDNA copy number and ecotypes

Elsa Snäckerström

Degree project for Bachelor of Science with a major in Biology
BIO603, Degree courses in Biology 30 hp
First cycle
Semester/year: Spring 2026
Supervisor: Erica Leder – Department of Marine Sciences
Examiner: Sam Dupont – Department of Biology and Environmental Sciences

Photo by Ameli Westling (March 4, 2026)

Index

Abstract

Mitochondria are not only the powerhouse of the cell, but the organelle also store its own genetic material, mitochondrial DNA (mtDNA). The amount of mtDNA is referred to as mtDNA copy number and has been found to reflect both energy demand and physiological adaptations of tissues. However, there are a limited number of studies that have examined if this pattern can be seen in marine invertebrates. To expand this knowledge, I examined how mtDNA copy number varies between tissues among males of a small marine invertebrate named *Littorina saxatilis*. Since the species *L. saxatilis* consists of different ecotypes that exhibit adaptation to different microhabitats, it was hypothesized that variation in mtDNA copy number would reflect these adaptive differences. DNA was extracted from different tissues (mantle, foot, head, seminal vesicles and testes), and mtDNA copy number was quantified in each tissue through qPCR. Comparisons were performed between ecotypes within a single population and between ecotypes across two different populations. The results were analyzed using a linear mixed effects model (LMM), which showed a significant effect of Tissue ($p < 0.001$) and Population ($p < 0.001$), but the effect of Ecotype was not significant. Significance was also shown in the interactions Ecotype x Tissue and Ecotype x Tissue x Population. Post-hoc comparisons using EMM showed the crab ecotype from Ängklåvbukten had significantly higher mtDNA copy number in the mantle and seminal vesicles. From Inre Arsklovet, the wave ecotype exhibited significantly higher mtDNA copy number in the head. Results showed variations in mtDNA copy number depend on the type of tissue, and that in turn is influenced by both ecotype and population.

Key words: mtDNA copy number, *Littorina saxatilis*, ecotypes, tissue-specific variation, qPCR.

Sammanfattning

Mitokondrier är inte bara cellens kraftverk, organellen innehåller också genetiskt material, mitokondriellt DNA (mtDNA). Mängden mtDNA kallas för mtDNA-kopienummer och har påvisats kunna återspegla både energibehov och fysiologiska anpassningar hos vävnader. Det är däremot ett begränsat antal studier som har undersökt om detta mönster kan ses hos marina evertebrater. För att utöka denna kunskap, undersökte jag hur mtDNA-kopienummer varierar mellan vävnader hos hanar av en liten marin evertebrat vid namn *Littorina saxatilis*. Eftersom arten *L. saxatilis* består av olika ekotyper anpassade till olika mikrohabitat, formulerades det en hypotes att variationen i mtDNA-kopienummer kan återspegla dessa adaptiva skillnader. DNA extraherades från olika vävnader (mantel, fot, huvud, sädesblåsor och testiklar), och mtDNA-kopienummer kvantifierades i varje vävnad genom qPCR. Jämförelser utfördes mellan ekotyper inom en enda population och mellan ekotyper från två olika populationer. Resultaten analyserades med hjälp av en Linear Mixed Effect Model (LMM), som visade en signifikant effekt av Tissue ($p <0.001$) och Population ($p < 0.001$), men effekten av Ecotype var inte signifikant. Signifikans visades också i interaktionerna Ecotype x Tissue och Ecotype x Tissue x Population. Post-hoc comparisons med EMM visade att krabb ekotypen från Ängklåvbukten hade signifikant högre mtDNA-kopienummer i manteln och sädesblåsorna. Från Inre Arsklovet påvisade våg ekotypen signifikant högre mtDNA-kopienummer i huvudet. Resultaten visade att variationer i mtDNA-kopienummer beror på vävnadstyp, och det i sin tur påverkas av både ekotyp och population.

Nyckelord: mtDNA kopienummer, *Littorina saxatilis,* ekotyper, vävnadsspecifik variation, qPCR.

1. Introduction

1.1 Background – mitochondria and energetics

Mitochondria are organelles present in nearly all eukaryotes and provide cells with one of the most fundamental sources of energy, ATP (Kühlbrandt, 2015). Mitochondria play a crucial role for cellular metabolism and are important when it comes to managing the overall health of cells (Spinelli et al., 2018). The number of mitochondria per cell can vary between different tissues, between different developmental stages and in response to stress. Additionally, mitochondria house their own genetic material, called mitochondrial DNA (mtDNA), which is contained in a circular chromosome. The number of copies of this chromosome can also vary in amount, and this amount is referred to as copy number (Cole, 2016). Levels of mtDNA copy number are closely correlated with the energy demands of cells (Dickinson et al., 2013), as well as being essential to maintain the respiratory chain, a key component in the OXPHOS system (Larsson et al., 1998). Mitochondrial DNA copy number can vary greatly between different types of cells, as well as between different types of tissues (Filograna et al., 2021). In addition, levels of mtDNA copy number can change in response to different stressors (Shimpi et al., 2024), which suggests that mtDNA copy number is a suitable biomarker to use for understanding how organisms respond when stressed. Studying variation in mtDNA copy number is therefore essential to understand how energy demands and stress response are regulated between different cells, tissues and organisms.

A study on mice by Veltri et al. (1990) showed that differences in mtDNA copy number can be organ specific. The brain was shown to have the lowest mtDNA copy number and the liver had the highest. A similar pattern was found in teleost's, demonstrating that mtDNA copy number can be specific to tissue. For example, the species *Pseudocaranx dentex* had higher mtDNA copy number in their heart tissue, which increases their oxygen transport and enables the species to be long-distance swimmers (Li et al., 2023). In *Drosophila melanogaster* there is sexual dimorphism in lifespan, where females have a longer life expectancy than the males. Kristensen et al. (2019) found that male *D. melanogaster* have a reduced stress tolerance as they age, which was shown by a reduction in their mtDNA copy number. In females, there is no reduction in either stress tolerance or mtDNA copy number, suggesting that variation in mtDNA copy number can be age specific, but also sex specific. Age-related changes of mtDNA have also been found in the species *Nothobranchius furzeri*, where it was shown that mtDNA copy number decreases with age in tissues such as the brain, the liver and muscle tissues (Hartmann et al., 2011).

The most used method today to estimate mtDNA copy numbers is quantitative real-time Polymerase Chain Reaction (qPCR). The results from a run of qPCR will show a ratio between mtDNA levels and levels of a selected nuclear gene (Kiliç et al., 2019).

1.2 Study system

There are a limited number of studies that have been done on mtDNA copy number variation in marine invertebrates. To contribute with new knowledge to this research field, I investigated variation of mtDNA copy number in a small marine invertebrate named *Littorina saxatilis*. The project was conducted at Tjärnö Marine Laboratory, located on the northern west coast of Sweden. The laboratory is in the Koster archipelago, and *L. saxatilis* can be found along intertidal shores among the many islands surrounding the laboratory.

Littorina saxatilis consists of different ecotypes, and the most commonly occurring ecotypes around Tjärnö, are the crab- and wave ecotype. These two ecotypes are adapted to different microhabitats. The crab ecotype inhabits rocky shores, where it lives more sheltered from waves and wind. However, they are subjected to predation from crabs. In contrast, the wave ecotype is

found on cliff sides, living more exposed while experiencing high wave action but no crab predation (Johannesson, 2015). Additionally, the two ecotypes are phenotypically different. The crab ecotype is large with thick shells, have relatively small apertures and high spires. According to Johannesson (1986), the shell-morphology of the crab ecotype is suggested to be an adaptation to better sustain crab predation, since crabs tend to crush the shell of the periwinkle or insert their chela into the aperture when predating on them. Wave ecotypes are smaller in size, have thinner shells and lower spires. Their aperture is larger and more rounded, leaving room for a bigger foot that helps them stay attached to cliff sides (Johannesson, 1986, Johannesson, 2015). These two ecotypes reside in different microhabitats, are exposed to different mechanical stressors and have different phenotypes. Both ecotypes exhibit adaptive differences (Johannesson, 2015), and these differences may cause variation in energy demand between different tissues which could potentially be detected through varying mtDNA copy number.

1.3 Aim

The aim of this project was to analyze variation in mtDNA copy number among tissues in males from two different ecotypes of the species *Littorina saxatilis*. It was also examined if mtDNA copy number would reflect adaptive differences between the two ecotypes. This project also aimed to provide a broader understanding of how mtDNA copy number can vary within a marine invertebrate species. Tissues (head, mantle, foot, seminal vesicles and testes) were dissected, DNA was extracted from each tissue and mtDNA copy number was quantified using qPCR.

1.4 Hypotheses

H_1: We expect that mtDNA copy number will vary among tissues in *Littorina saxatilis* with higher mtDNA copy number in higher energy demanding tissues. Both ecotypes reside in different microhabitats, showing adaptive differences through both morphological and behavioral properties. We therefore expect there to be a significant Tissue x Ecotype interaction with respect to mtDNA copy number.

- It is believed that the foot tissue will exhibit higher mtDNA copy number, reflecting the energy demand that is required for adhesion and locomotion. The wave ecotype lives in more exposed environments and is therefore more reliant on resisting dislodgement by waves (Le Pennec et al., 2017). It is expected the wave ecotype might exhibit a slightly higher mtDNA copy number in the foot, reflecting adaptation to more exposed environments.
- Another example is that the mantle will show a higher mtDNA copy number, since the tissue is responsible for shell maintenance (Fretter & Graham, 1962), a process that might reflect a higher energy demand, and in turn, a higher mtDNA copy number. The crab ecotype has a thicker and bigger shell, which is suggested to be an adaptation to crab predation (Johannesson, 1986). This could potentially mean they are more reliant on shell production, which could be reflected through a higher mtDNA copy number.
- In addition, it is expected that both reproductive tissues, the testes and seminal vesicles, will show higher mtDNA copy number across both ecotypes, reflecting the energy demand that is needed for processes involved in reproductive activity.

H_0: There is no significant variation between tissues of *Littorina saxatilis*, and variation in mtDNA copy number does not reflect adaptive differences between the two ecotypes.

2. Material and method

2.1 Sampling

The project was conducted at Tjärnö Marine Laboratory, and sampling was performed at two sites surrounding Tjärnö, Ängklåvbukten and Inre Arsklovet (**Figure 1**). At each site, 12 individuals per ecotype were sampled, resulting in 24 individuals per site and a total sample size of 48 individuals. After sampling was done, the sex of each snail was determined, whereas the males were identified by the presence of a penis. After determining the sex of all males, they were placed in aquaria.

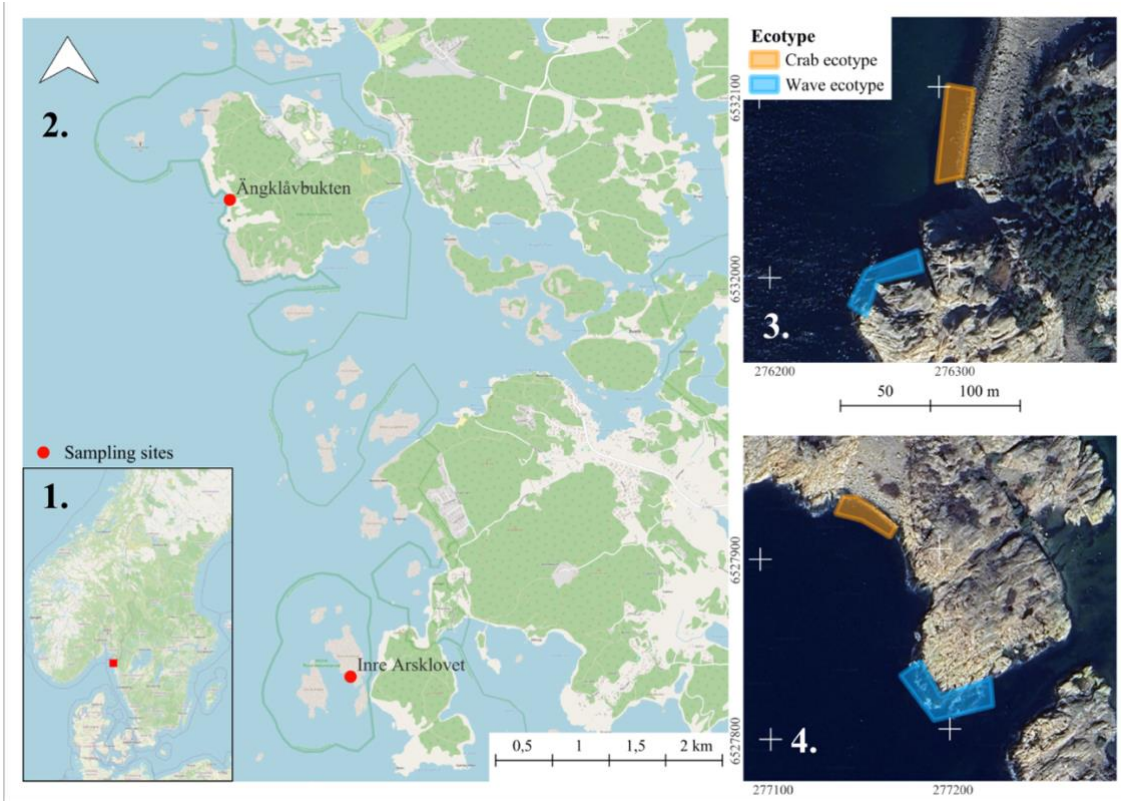


***Figure 1**. Overview map of sampling sites on the Swedish west coast, and detailed view of exact sampling locations. First panel (1) shows the location of the sampling area within Sweden (red polygon). Second panel (2) shows location of the two sampling sites, Änklåvbukten and Inre Arsklovet (red points). Panel three and four shows detailed maps of Ånkllåvbukten (3) and Inre Arsklovet (4). Transects used for sampling the crab ecotype (orange) and wave ecotype (blue) are shown in legend (3). Map data: © Google; © OpenStreetMap contributors*

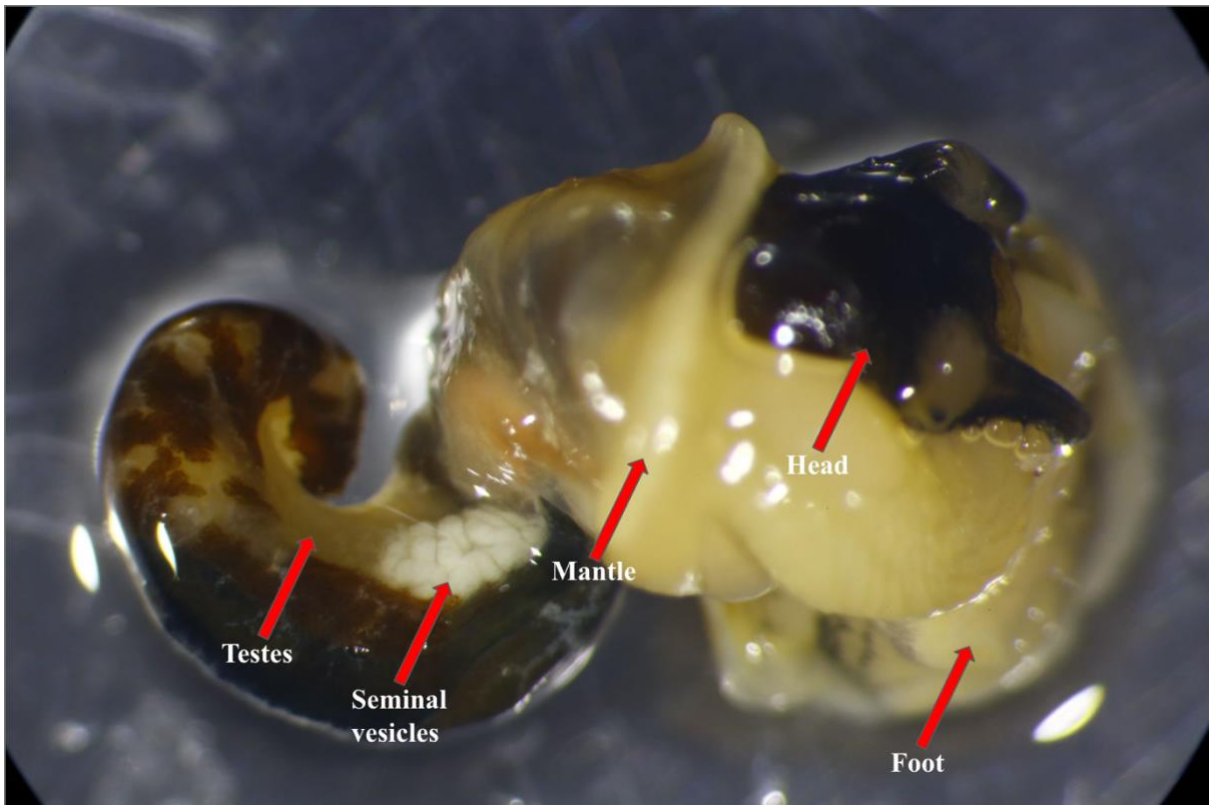


Figure 2. *Photo of naked male Littorina saxatilis, taken after cracking the shell and before dissection. Red arrows point out each tissue that was dissected.*

2.2 Dissecting

Before dissection began, photographs were taken of all individuals together with a millimeter paper and an ID-tag. The millimeter paper was used as a reference to enable measurement of the size, while the ID-tag made it possible to keep track of every individual throughout the whole project. The first step of dissection was to break the shell of the individual. Depending on the size and thickness of the shell, different tools were used. For the crab ecotype, which has a bigger and thicker shell, a nutcracker or pestle was used. The wave ecotype has a smaller and thinner shell, so the preferred tool for breaking their shell were pliers.

Position of each tissue is illustrated in figure 2. The seminal vesicles were removed by using a pair of tweezers, and the testes were dissected in a similar way. For the mantle, a small scissor was used to make a small cut at the juncture of the mantle edge. While cutting along the edge, a tweezer was used to hold on to the mantle until the acquired piece was obtained. The head was dissected with the help of a tweezer and a scalpel. With the scalpel, a cut was made below the head and then between the head and the foot. When the head was removed, only the operculum needed to be cut off to dissect the foot. To facilitate digestion of the tissue during DNA extraction, the foot was cut into smaller pieces. Each sample was put in Eppendorf tubes and stored in a -80 degrees Celsius freezer.

2.3 DNA extraction

DNA extraction for the head, mantle, testes and seminal vesicles, was done by using the DNeasy Blood & Tissue Kit and the QIAmp DNA Micro kit from Qiagen. The extractions were performed

according to the respective protocols (Qiagen, n.d.). After DNA extraction, the samples were stored in a refrigerator at +4 degrees Celsius until performing qPCR.

Many invertebrates have been found to secrete mucopolysaccharides, a carbohydrate that inhibits endonucleases, enzymes needed to digest tissues before extraction (Whitmore, 1983). To prevent this inhibition, the foot tissue was treated with collagenase IV, an enzyme that breaks down collagen found in connective tissue (Thermo Fisher Scientific, n.d.). Prior research has also demonstrated that the foot tissue has inconsistent results if not treated this way. The foot tissue was digested for 2 hours in a solution of physiological saline buffer (PBS) and 2% collagenase IV.

The foot tissue was then extracted through salt extraction, which was performed according to a protocol presented by Aljanabi and Martinez (1997). A few modifications were done. The buffer recipe was adjusted to create a concentrated solution (2X) so it was at normal strength when added to the collagenase-digested tissue. The amount of buffer added to each sample was 200 µL, and the NaCl added after incubation was 5M. The first step of centrifugation was at 12 000 x g, and the second step at 14 000 x g. After the pellet was washed with ethanol, it was centrifuged for 5 min at 8 000 x g. The ethanol was then poured out, and the pellet was dried from the ethanol with the help of a Kimwipe. Lastly, the samples were eluted with nuclease-free water. After salt extraction was performed, the extracted foot tissue was analyzed using a NanoDrop spectrophotometer. The NanoDrop was used to measure the purity of the samples, including contamination from RNA and proteins, and to determine the final concentration of all extracted DNA samples, a Qubit fluorometer was used. The Qubit can measure double-stranded DNA concentration and is more sensitive and accurate, especially at low concentrations.

2.4 qPCR

Quantitative PCR (qPCR) was performed using a MicroAmp Fast optical 96-well reaction plate. Each sample was analyzed in triplicates for two primer sets, resulting in a total of six reactions per sample. A total of 16 samples could be analyzed per plate, which equals a total of 96 reactions per plate. To check consistency between samples and qPCR runs, 21 replicates were performed.

Two primer sets were used to quantify the relative abundance of mtDNA compared to nDNA. The ND1 primer set amplified a 219-base segment of subunit 1 of the mitochondrial NADH dehydrogenase gene. The TPX1 primer set amplified a 322-base segment of the nuclear reference gene T-box transcription factor 1.

A primer mix was prepared for each set, containing equal amounts of the forward and the reverse primer diluted to a working concentration of 2.5 µmol/L. This resulted in a final primer concentration of 0.3 µmol/L per reaction well. Each reaction was performed in a volume of 12 µL containing:

Each reaction well contained:

- 6 µL PowerUp SYBR Green Master Mix (2X)
- 1.4 µL Primer mix
- 4.6 µL DNA template

To ensure the amplified products of both primer sets fell in the required range for detection, it was determined that each reaction needed to contain 3.5 ng of DNA. Therefore, a DNA-mix was prepared for each sample by diluting the DNA with nuclease-free water to a concentration of 0.76

ng/µL, enabling 4.6 µL of the DNA-mix to be added to each reaction. Since the required volume of DNA for each reaction was too small to pipette accurately, DNA-mixtures of multiple reactions (7-12 reactions depending on the DNA-concentration of the sample) were prepared and diluted with nuclease-free water to achieve the target concentration.

For each run of qPCR, a master mix was prepared for each primer set. The master mix contained the PowerUp SYBR Green PCR Master Mix and the primer mix. The volume of each master mix needed to be enough for 48 reactions, but extra was made to prevent insufficient volumes during pipetting. The recipe of each master mix was as follows (recipe for approximately 50 reactions):

- SYBR Green: 6 x 50 = 300 µL
- Primer mix (ND1 or TPX1): 1.4 x 50 = 72 µL

DNA dilutions were prepared and distributed in strip-tubes. Then, all components were systematically dispensed in the 96-well plate. First, 4.6 µL of each DNA mix was added to reduce the risk of contamination, and afterwards, 7.4 µL of the master mix was added to each reaction well. To make the pipetting more efficient, a repeater pipette with a 0.2 mL tip was used to dispense the master mix.

After the plate was loaded, it was sealed with a MicroAmp optical adhesive film, centrifuged, before being placed in the StepOnePlus Real-Time PCR instrument. A plate layout was designed beforehand and entered in the software before the qPCR run could start. Each qPCR run lasted approximately 1.5 h and consisted of 40 cycles with the following protocol:

Holding stage:

1. 98 °C for 2 min

Cycling stage:

1. 98 °C for 15 s
2. 58 °C for 15 s
3. 72 °C for 1 min

Each qPCR run generates an amplification curve for each sample, consisting of an initiation, exponential and plateau phase. During early stages of amplification, the levels of fluorescence from the SYBR green is low, which sets a baseline. As amplification progresses, it reaches the exponential phase, where DNA starts to amplify significantly, and fluorescence signals become at their highest. A threshold is set within this phase, and the cycle at which amplification crosses the threshold is recorded as the cycle threshold (Ct) value for each sample (Adams, 2020). A threshold was set for each primer in each qPCR run, but to enable comparison of data between samples, the thresholds were adjusted manually, resulting in both primers having the same threshold. Errors of samples could occur and were identified based on deviating amplification curves and by high standard deviations among replicates. These samples were rerun. From the qPCR, each sample gave two mean Ct values, where both means were averaged across the three replicates for each sample. One mean value for the ND1 (mtDNA) targets, and one mean value for the TPX1 (nDNA) targets. With the Ct-mean values, ΔCt could be calculated according to the following formula (Rooney, 2015):

$$\Delta Ct = Ct_{nDNA} - Ct_{mtDNA} = Ct_{TPX} - Ct_{ND1}$$

Relative mtDNA copy number was calculated using the following formula (Rooney, 2015):

$$2 \times 2^{\Delta Ct}$$

This calculation was used due to the amplifications being exponential and each cycle doubles the amount of DNA (Leuthner et al., 2021).

2.5 Statistical method

2.5.1 Model

Statistical analysis of the data was done using RStudios version 2024.12.1+563. The packages *readxl, tidyverse*, *dplyr, car, lmerTest, emmeans, effectsize, performance* and *broom* were used for modelling of the data. The values on mtDNA copy number were $\log_{10}$-transformed, improving normality and reducing homoscedasticity. mtDNA copy number where the numeric response variable, while ecotype, tissue and population were the categorical predictor variables. Visualization of the data was done using *geom_boxplot* together with *geom_jitter* from the library *ggplot2*.

To analyze differences in log-transformed mtDNA copy number between the two ecotypes, a linear mixed-effects model (LMM) was constructed. The model included Tissue, Ecotype and Population as fixed effects, together with their interaction terms. Since multiple tissues were sampled from the same individual, the measurements were not independent. ID was therefore included in the models as a random effect. A GLMM was constructed for the data, as well as an additional LMM without the three-way interaction term (Ecotype x Tissue x Population). Both models were compared against the LMM with the three-way interaction term through Akaike's Information Criterion (AIC), whereas the LMM with the three-way interaction had the lowest AIC and was therefore used for further analysis.

Normality of residuals were assessed through a Q-Q plot, and additionally through a Shapiro-Wilk test (Ghasemi & Zahediasl, 2012). Homogeneity of variance was analyzed visually through a scatterplot of the residuals versus fitted values from the LMM. The explanatory power of the LMM was estimated with the help of marginal and conditional R^2 values, and further model diagnostics were analyzed using *check_model*. Statistical significance of each factor, as well as the interactions between them, was analyzed using type III analysis of variance (ANOVA). Type III ANOVA tests statistical significance for every term, while accounting for all others (Langsrud, 2003). For comparative purposes, type II ANOVA was performed on the model as well, but gave similar results.

2.5.2 Post-hoc comparison

Post-hoc comparisons using estimated marginal means (EMMs) were calculated for ecotype differences within each Tissue x Population combination, enabling comparison between all tissues within each ecotype. A pairwise comparison was made between the two ecotypes using Tukey's method for honest significant difference (HSD), whereas the method controls for Type I Errors

when generating p-values (Kim, 2015). Confidence intervals for the EMMs were also checked and effect sizes using Cohens d were calculated for the differences between ecotypes within each Tissue x Population combination. Lastly, group means were analyzed using the function *aggregate*.

3. Results

3.1 Model comparison

Model comparison was performed using Akaike's Information Criterion (AIC). The AIC showed that a linear mixed effects model (LMM) was more suitable to use for the data, than a generalized linear mixed model (GLMM) (AIC = 252.8 vs. AIC = 1839.0). Further evaluation of the model using AIC showed that keeping the three-way interaction (Ecotype x Tissue x Population) in the LMM improved the model fit (AIC = 196.5 vs. AIC = 201.0).

3.2 Model diagnostics

In the final LMM, the fixed effects explained 57.7 % of the variance (marginal $R^2 = 0.577$), and when including the random effect of ID, 71.8% of the variance could be explained (conditional $R^2 = 0.718$). The intraclass correlation coefficient revealed that 33.2% of the variance could be explained by differences among individuals (ICC = 0.332) in the final LMM. Variation of residuals was low (RMSE = 0.270, $\sigma = 0.305$), and the Shapiro-Wilk test showed the residuals to be normally distributed (W = 0.994, p = 0.494). The Q-Q plot showed no clear deviations from normality, and the residuals showed no clear violation against homoscedasticity. Variance Inflation Factors (VIFs) revealed high collinearity between some of the interaction terms in the LMM, which was expected due to including the three-way interaction. Additional diagnostics using *check_*model revealed no further violations against the model assumptions.

3.3 Type III ANOVA

Type III ANOVA showed a highly significant effect of Tissue on log-transformed mtDNA copy number ($F = 89.40$, $p < 0.001$). The effect of Population showed significance ($F = 12.80$, $p < 0.001$), whereas Ecotype was not significant ($F = 3.38$, $p = 0.072$). The interaction Ecotype x Tissue was significant ($F = 12.42$, $p < 0.001$), as well as the three-way interaction Ecotype x Tissue x Population ($F = 2.94$, $p = 0.022$; **Figure 3**). The interaction Ecotype x Population was not significant ($F = 1.97$, $p = 0.167$) and the same applied for the interaction Tissue x Population ($F = 1.43$, $p = 0.225$).

3.4 Post-hoc analysis

Post hoc comparisons using estimated marginal means (EMMs) showed that in Ängklåvbukten, the crab ecotype had a significantly higher mtDNA copy number in the mantle (estimate = 0.53 ± 0.15 SE, t = 3.50, $p < 0.001$), and in the seminal vesicles (estimate = 0.69 ± 0.15 SE, t = 4.57, $p < 0.001$), compared to the wave ecotype. No significant differences were observed in the foot (estimate = -0.03 ± 0.15 SE, t = -0.20, p = 0.844), the head (estimate = -0.20 ± 0.15 SE, t = -1.35, p = 0.179) or the testes (estimate = 0.18 ± 0.15 SE, t = 1.22, p = 0.224) between the two ecotypes (**Figure 4**).

In Inre Arsklovet, the EMMs showed that the wave ecotype had a higher mtDNA copy number in the head (estimate = −0.49 ± 0.15 SE, t = -3.20, p = 0.002), compared to the crab ecotype. No significant differences were observed in the mantle (estimate = 0.27 ± 0.15 SE, t = 1.74, p = 0.083),

the seminal vesicles (estimate = 0.16 ± 0.15 SE, t = 1.02, p = 0.307), the testes (estimate = -0.05 ± 0.15 SE, t = -0.33, p = 0.740) or the foot (estimate = 0.27 ± 0.15, t = 1.79, p = 0.075) between the two ecotypes (**Figure 5**).

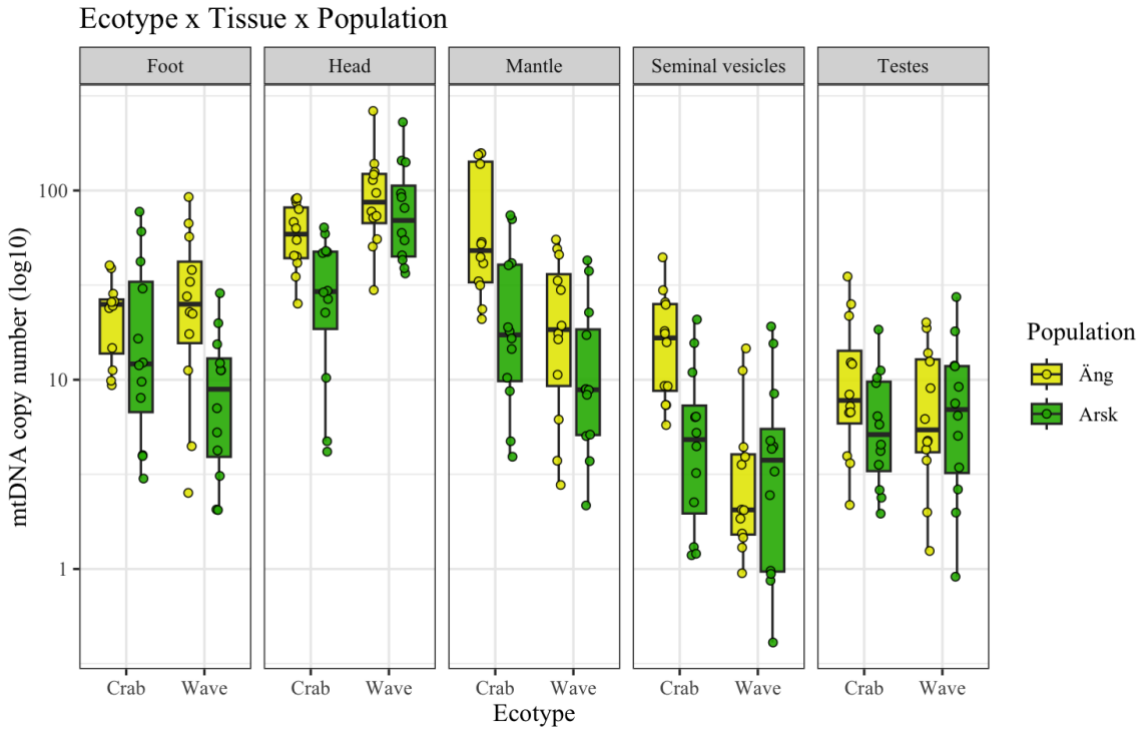


Figure 3. *Log-transformed mtDNA copy number in Littorina saxatilis across tissues, ecotypes and populations. Boxplots show mtDNA copy number (log10) in foot, head, mantle, seminal vesicles and testes for the crab- and wave ecotype from Ängklåvbukten (Äng = Yellow boxplots) and Inre Arsklovet (Arsk = Green boxplots). Yellow jitter show distribution for individuals from Ängklåvbukten and green jitter show distribution for individuals from Inre Arsklovet. Significant differences were detected in the mantle and seminal vesicles between ecotypes from Ängklåvbukten, and in the head between ecotypes from Inre Arsklovet (Post hoc comparison using EMM, $p < 0.05$).*

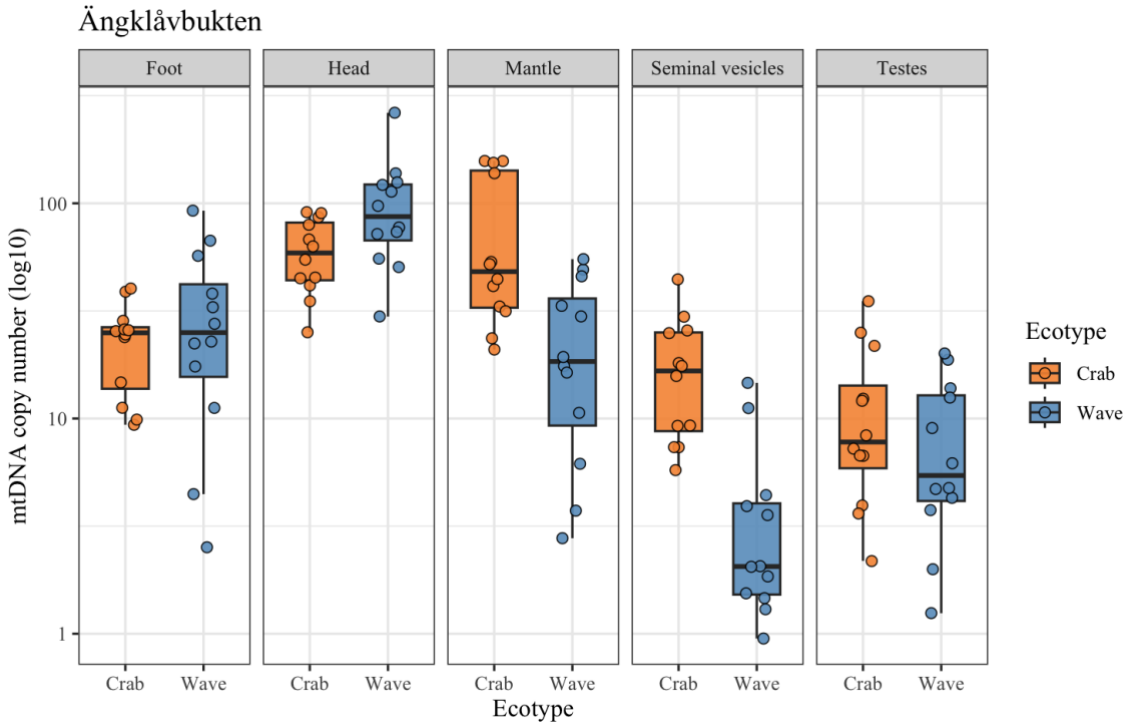


Figure 4. *Log-transformed mtDNA copy number in Littorina saxatilis across tissues between ecotypes in the population from Ängklåvbukten. Boxplots show mtDNA copy number (log10) in the foot, head, mantle, seminal vesicles and testes for the crab ecotype (orange) and the wave ecotype (blue). The distribution of data points for each ecotype within each tissue is shown by the jitter. The crab ecotype showed higher mean mtDNA copy number in the mantle, seminal vesicles and testes. The wave ecotype showed higher mean mtDNA copy number in the head and foot.*

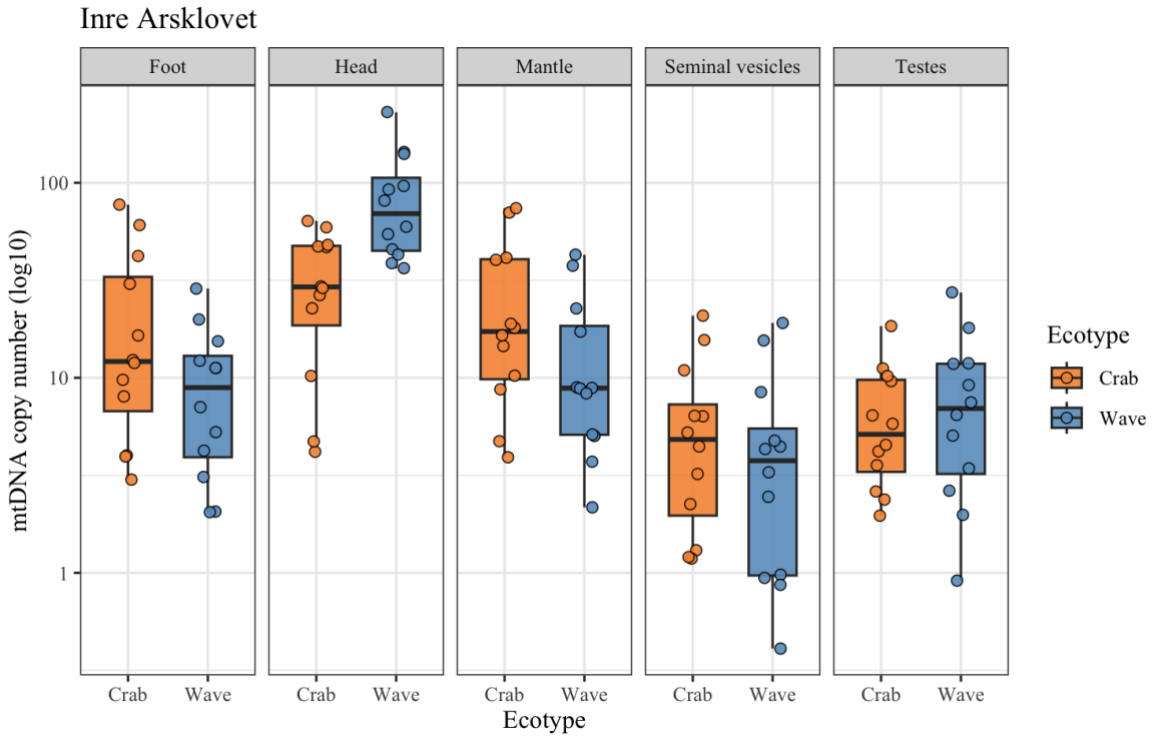


Figure 5. *Log-transformed mtDNA copy number in Littorina saxatilis across tissues between ecotypes in the population from Inre Arsklovet. Boxplots show mtDNA copy number (log10) in the foot, head, mantle, seminal vesicles and testes for the crab ecotype (orange) and the wave ecotype (blue). The distribution of data points for each ecotype within each tissue is shown by the jitter. The crab ecotype showed higher mean mtDNA copy number in the foot, mantle and seminal vesicles. The wave ecotype showed higher mean mtDNA copy number in the head and testes.*

3.5 Effect size

In Ängklåvbukten, the effect size was larger for the crab ecotype in the mantle (d = 1.73, SE = 0.502, 95% CI [0.74, 2.72]), and in the seminal vesicles (d = 2.25, SE = 0.505, 95% CI [1.26, 3.25]). Smaller effect sizes applied for the head (d = -0.67, SE = 0.496, 95% CI [-1.65, 0.31]), the testes (d = 0.60, SE = 0.495, 95% CI [-0.37, 1.58]), and the foot (d = -0.10, SE = 0.493, 95% CI [-1.07, 0.88]).

At Inre Arsklovet, the wave ecotype showed higher mtDNA copy number in the head, which was shown by a large negative effect size (d = -1.60, SE = 0.505, 95% CI [-2.60, -0.60]). The mantle (d = 0.87, SE = 0.501, 95% CI [-0.12, 1.86]), the seminal vesicles (d = 0.51, SE = 0.500, 95% CI [-0.48, 1.50]), the foot (d = 0.90, SE = 0.501, 95% CI [-0.09, 1.89]), and the testes (d = -0.17, SE = 0.500, 95% CI [-1.15, 0.82]) all had smaller effect sizes.

3.6 Mean mtDNA copy number

In Ängklåvbukten the crab ecotype showed higher mean mtDNA copy number in the mantle (**1.762** vs. 1.233), the seminal vesicles (**1.171** vs. 0.483) and the testes (**0.949** vs. 0.765) compared to the wave ecotype. The wave ecotype had a marginally higher mean mtDNA copy number in the head (**1.954** vs. 1.750) and the foot tissue (**1.349** vs. 1.319).

The crab ecotype from Inre Arsklovet exhibited a slightly higher mean mtDNA copy number in the mantle (**1.256** vs. 0.990), the foot (**1.144** vs. 0.871) and seminal vesicles (**0.638** vs. 0.482). Differences in mean mtDNA copy number in the testes was minimal between the two ecotypes (0.733 vs. 0.784). The wave ecotype showed a higher mean mtDNA copy number only in the head tissue (**1.873** vs. 1.385).

Across both ecotypes from Ängklåvbukten, variation in mean mtDNA copy number among tissues had the following order: head (1.846) > mantle (1.492) > foot (1.229) > testes (0.871) > seminal vesicles (0.808). A similar pattern was observed at Inre Arsklovet: head (1.629) > mantle (1.123) > foot (1.007) > seminal vesicles (0.758) > testes (0.560). When averaged across all tissues, mean mtDNA copy number was slightly higher in the crab ecotype compared to the wave ecotype from Ängklåvbukten (1.349 vs. 1.153). In Inre Arsklovet, the averaged mean was more similar between the two ecotypes (1.031 vs. 1.000).

4. Discussion

Mitochondrial DNA copy number varied within individuals and between individuals of *Littorina saxatilis*. Variation could also be analyzed between tissues, ecotypes and populations, with Tissue having the strongest significant effect on mtDNA copy number. Significance was also shown in the interaction Ecotype x Tissue, which illustrated that patterns of varying mtDNA copy number were not consistent between the two ecotypes. Across both ecotypes, the head tissue had the highest mean mtDNA copy number, followed by the mantle tissue and foot tissue. Both reproductive tissues exhibited lower mean mtDNA copy number across both ecotypes compared to the somatic tissues.

4.1 Tissue effects

According to an article by Vaishnavi et al. (2010), neural activity may promote mitochondrial growth. Many of the ganglia that constructs the central nervous system can be found in the head

tissue of *Littorina saxatilis* (Fretter & Graham, 1962). These structures might cause high levels of neural activity, which in turn could entail a higher energy demand, as well as a higher density of mitochondria. According to Filograna et al. (2021), higher mitochondrial density can indicate a higher mtDNA copy number. The ganglia in the head are connected to various sensory organs, including the eyes, the statocyst and the tentacles (Fretter & Graham, 1962). Sensory organs are considered to need a significant amount of energy to process and respond to external stimuli (Laughlin, 2001). In other marine invertebrate species, it has been shown that tissues requiring more energy contain a higher mtDNA copy number (Li et al., 2023). Neural activity together with the sensory organs could explain why the head tissue had the highest mtDNA copy number across all tissues. The wave ecotype from exhibited a slightly higher mtDNA copy number in the head. They live in more exposed microhabitats (Johannesson, 2015), dealing with a high frequency of mechanical stressors, such as waves and wind. The wave ecotype could be more reliant on using their sensory organs to respond to external stimuli, requiring higher amounts of energy (Laughlin, 2001), which possibly could explain the higher mtDNA copy number in the head.

In the mantle cavity of *Littorina saxatilis*, shell formation and shell repair occur, and the shell mainly consists of calcium carbonate (Fretter & Graham, 1962). Palmer (1992) estimated that the cost of calcification equals 410% of the energy that marine molluscs invest in reproduction. Growth and maintenance of the shell occurring in the mantle could imply significant energy requirements of that tissue. In marine invertebrates, higher energy demanding tissues have been linked to contain a higher mtDNA copy number (Li et al., 2023), possibly explaining why the mantle was the tissue with the second highest mtDNA copy number. The visualization of the mtDNA copy number in the mantle shows a large variance between data points, which may indicate individuals being in different stages of shell growth or individuals might have different mechanisms for repairing the shell. This interpretation is supported by the Intraclass Correlation Coefficient (ICC), saying that 33.2% of the variance in mtDNA copy number is explained by differences between individuals. The crab ecotype had a slightly higher mean mtDNA copy number in their mantle. The shell morphology of the crab ecotype is an adaptation to better withstand predation from crabs (Johannesson, 1986), which could indicate them being more dependent on maintaining their shell. Since calcification is such a high energy demanding process (Palmer, 1992), maintaining a thicker and bigger shell could require more energy, possibly explaining why we saw a higher mtDNA copy number in the mantle of the crab ecotype. This supports one of the hypotheses formulated for this project, that mtDNA copy number can reflect adaptative differences between the two ecotypes.

The foot of marine gastropods is mainly used for movement and attachment (Miller, 1974). The smooth muscle cells responsible for locomotion within the foot require significant amounts of ATP from the mitochondria (Pavlova, 2006). To sustain adhesion, gastropods secrete protein-rich mucus that enables attachment to different substrates (Newar et al., 2021). The foot being responsible for processes regarding both locomotion and adhesion could indicate a high energy demand, possibly reflecting why the tissue had the third highest mtDNA copy number. The shell morphology of the wave ecotype is constructed to make room for a bigger and more rounded foot (Johannesson, 1986, Johannesson, 2015), and their foot enables them to better resist dislodgement by waves (Le Pennec et al., 2017). The wave ecotype in Ängklåvbukten showed a minimally higher mean mtDNA copy number in their foot compared to the crab ecotype. However, from Inre Arsklovet, it was instead the crab ecotype that had a higher mean mtDNA copy number in the foot tissue. For this project, it was believed the wave ecotype would exhibit higher mtDNA copy number in their foot tissue, reflecting adaptation to its microhabitat. Since the difference is minimal, the hypothesis cannot be supported.

Both the testes and seminal vesicles showed lower mean mtDNA copy number compared to the somatic tissues. In another species of gastropods, *Onchidium struma*, the number of mitochondria increases during spermatogenesis, to provide with energy for sperm to mature (Chen et al., 2015).

Sampling could have been performed during a time when reproductive activity was lower, causing a reduced activity of spermatogenesis, and a reduced energy demand. However, the energy demand of reproductive tissue does not necessarily correlate with mtDNA copy number, since mtDNA copy number can be regulated independently from mitochondrial abundance. In sperm cells, it has been shown that mtDNA copy number becomes reduced during late stages of spermatogenesis (Bagdonaitė et al., 2025), which is hypothesized to be due to prevent inheritance of mtDNA from males to the offspring (DeLuca & O'Farrell, 2012). For this project, it was believed the reproductive tissues would show higher mtDNA copy number, since mitochondria are an essential component of sperm cells (Boguenet et al., 2021). Since both tissues exhibited lower mtDNA copy number, the hypothesis cannot be supported. Within the testes of *Littorina saxatilis*, lobular testes containing diploid spermatogonia and spermatocytes, as well as haploid spermatids and spermatozoa can be found (Demin, 2019). The testes of *L. saxatilis* contain a mixture of haploid and diploid cells (Demin, 2025) and the seminal vesicles are where male *Littorina saxatilis* store the sperm (Kumpitsch et al., 2025). To obtain mtDNA copy number, it is calculated through a mtDNA:nDNA ratio (Kiliç et al., 2019), indicating that different ploidy levels most likely affects the estimations of mtDNA copy number. Across all groups the seminal vesicles exhibited the lowest mtDNA copy number. However, the seminal vesicles for the crab ecotype at Ängklåvbukten showed higher mtDNA copy number. Certain individuals of the crab ecotype in Ängklåvbukten could have been more reproductively active at the time of sampling, which could be supported by the ICC, which indicates that 33.2% of varying mtDNA copy number is due to differences between individuals. Although, this interpretation is highly speculative since many factors could have influenced the reproductive state of every individual.

4.2 Ecotype and Population effects

Littorina saxatilis has been found to experience strong local selection, enabling the species to diverge over small geographic distances. Differing levels of exposure to biotic and abiotic factors can create different selective pressures on individuals (Johannesson et al., 2010, Johannesson, 2015) During field work, it was observed that Ängklåvbukten may be more exposed than Inre Arsklovet. Since there was a significant effect of Population on mtDNA copy number, it may suggest that environmental conditions between Ängklåvbukten and Inre Arsklovet are different and that possibly, the environmental effects driving the ecotypes are not as strong at Inre Arsklovet. When looking at the visual representation of the data on mtDNA copy number, and by analyzing mean values, differences in mean mtDNA copy number were slightly smaller between the two ecotypes from Inre Arsklovet. It could reflect a weaker selection pressure between the two ecotypes at Inre Arsklovet, or gene flow might be stronger. Simultaneously, it may indicate that the two ecotypes at Ängklåvbukten have differentiated more from each other than the two ecotypes at Inre Arsklovet (Johannesson et al., 2010, Johannesson, 2015). There was a non-significant effect in the interaction between Ecotype x Population, which tells us that mtDNA copy number averaged across all tissues exhibited similar patterns between both ecotypes in both populations. In addition, the Tissue x Population interaction was not significant, which indicates that mtDNA copy number averaged across both ecotypes showed similar patterns in all tissues in both populations. Lastly, the effect of Ecotype was not significant, suggesting there to be no overall difference in mtDNA copy number between ecotypes when averaged across all tissues and both populations. However, despite the non-significant main effect of Ecotype, there was a significant effect in the three-way interaction Tissue x Ecotype x Population. This suggests that differences in mtDNA copy number between ecotypes depend on both which tissue is being analyzed and which population this tissue represents. As previously mentioned, *L. saxatilis* experience strong local selection, and degrees of divergence between ecotypes can vary between populations, which can depend on local environmental conditions (Johannesson et al., 2010).

Thus, differences in selective pressure between populations may affect how large a difference there is in mtDNA copy number across tissues between these two ecotypes. In addition, mtDNA copy number has been suggested to reflect energy demand of cells and physiological adaptation (Li et al., 2023) and can also change due to stress response (Shimpi et al., 2024). Therefore tissue-specific variations in energy demand and stress-exposure may contribute to differences between ecotypes and populations. In conclusion, variations in mtDNA copy number in *L. saxatilis* is likely influenced by ecotype, tissue and population, possibly reflecting interactions between local environmental conditions, energy demands and degrees of divergence within populations.

4.3 Limitations

There is little known about the physiology of *Littorina saxatilis*. Research regarding physiology has been done on another species in the *Littorina* genus, *Littorina littorea*. In the discussion, it was therefore assumed that *L. saxatilis* shared similar physiological properties as *L. littorea*.

The mtDNA copy number in a tissue might not only reflect energy demand and physiological adaptations. According to Ryu et al. (2024) varying mtDNA copy number can also reflect specialization of mitochondria. This could be an additional explanation of why mtDNA copy number varies between tissue-types.

Sampling was performed around the late stages of winter and early signs of spring. During this time of year, weather conditions can fluctuate and be somewhat harsh. Sokolova (2018) concluded that mitochondria of intertidal animals obtain a high tolerance against environmental stress factors. However, how high this "tolerance" goes is unclear. There is little knowledge regarding the mechanisms *Littorina saxatilis* have against colder temperatures, so it is unknown if colder conditions influence the energy demand of certain tissues, as well as the mtDNA copy number.

Sampling of all individuals was not performed at the same time. The crab ecotype from Ängklåvbukten was sampled in late February, while sampling of the remaining individuals was performed in early March. Weather conditions changed between these two occasions. As previously mentioned, it is unclear if the mtDNA copy number of *Littorina saxatilis* changes as the weather gets warmer.

The sample size for this project was 12 individuals per ecotype per population, resulting in a total sample size of 48 individuals. A larger sample size and sampling from additional populations might have increased the statistical power, as well as exposed clearer patterns regarding variation in mtDNA copy number between ecotypes. However, compared to a previous study, that used 8 individuals instead of 12, the statistical power did not improve much. This may be due to there being a lot of individual variation.

The method of sampling might have been stressful for individuals of *Littorina saxatilis*. Plucking and putting them in containers, transporting them, identifying the sex and putting them in aquaria might have induced a stress response in the individuals. Stress responses can affect the resulting mtDNA copy number (Cole, 2016). However, we tried to minimize this effect by treating the samples in the same manner and perform sampling quickly.

The species *Littorina saxatilis* is small and different tissues are positioned close to each other. There is a possibility that some tissues were dissected differently between individuals, resulting in a variation in tissue composition between samples. Mitochondrial DNA copy number can vary greatly between different types of cells, and between different types of tissues (Filograna et al., 2021), which could have affected the resulting mtDNA copy number.

Different methods were used for DNA extraction for the foot tissue compared to the other tissues, and this may have caused variation in DNA extraction yield between the samples, if they for some reason biased mtDNA over gDNA. This in turn could have affected the resulting mtDNA copy number, affecting the resulting data.

4.4 Future research

If this project were to be performed again, the first thing to reconsider is the sample size. A larger sample size might increase the statistical power, as well as decrease the risk of type II errors even more. Besides a larger sample size, a smaller time frame for sampling of individuals is a suggestion. If sampling occurs within a shorter time frame, the conditions become more equal for all individuals.

For this project, the time of dissection was relatively equal for every individual. Individuals spent a maximum of two to three days in the aquariums. If further research were to be done, a suggestion might be to try and dissect all individuals within a shorter time frame, or perhaps perform dissection of *Littorina saxatilis* as a group effort.

An additional change for further research within this area is to make sure all tissues are extracted through the same extraction method. Using the same method will cause a similar DNA extraction yield between all samples.

5. Conclusion

From the results, we found that Tissue had the strongest effect on mtDNA copy number. Across both ecotypes and populations, the head tissue had the highest mtDNA copy number, followed by the mantle tissue and the foot tissue. Meanwhile, the testes and the seminal vesicles both exhibited lower mtDNA copy number compared to the somatic tissues. The resulting mtDNA copy number of the somatic tissues supports the hypothesis that mtDNA copy number varies between tissues, with higher mtDNA copy number in higher energy demanding tissues such as the mantle and the foot. However, both the testes and the seminal vesicles showed lower mtDNA copy number, which contradicts the expectation that both reproductive tissues would reflect a higher energy demand. This could indicate that other biological factors need to be taken in consideration when estimating mtDNA copy number. Varying ploidy levels, mitochondrial specialization and different stages of reproductive activity are a few of possibly many factors that can influence variation in mtDNA copy number.

The results showed a significant effect of the Ecotype x Tissue interaction, supporting the hypothesis that mtDNA copy number may reflect adaptation, as illustrated by the crab ecotype showing a higher mtDNA copy number in the mantle. Furthermore, the significant effect of Population, and the significant three-way interaction suggests that mtDNA copy number is not only affected by the type of tissue. Variation in mtDNA copy number can also be explained by differences between ecotypes, as well as by differences between populations.

Variation in mtDNA copy number likely reflects differences in energy demands and physiological adaptations, suggesting it to be an informative biomarker to use for analyzing factors that contribute to variation among cells, tissues and organisms. Since previous studies regarding mtDNA copy number variation in marine invertebrates are limited, results obtained from this project hopefully provide new knowledge, while also encouraging to conduct further research.

6. Acknowledgements

First and foremost, I would like to give a huge thanks to my supervisor, Erica Leder, for providing me with this opportunity. Through this project, I have acquired many new skills, including dissecting, extracting DNA and performing qPCR. I never thought myself to be a person enjoying laboratory work, but Erica has successfully converted me. Thank you for your patience, guidance and support throughout my project.

A huge thanks to Gabriella Malmqvist who's also given me so much guidance and support. From climbing up cliffs to help me during field work, to teaching me about how to identify species and genders. Thank you for all you have taught me. To both Erica and Gabriella, thank you for all this new knowledge, and for being such inspiring and dedicated mentors.

Additionally, I would like to thank Kerstin Johannesson for joining us during fieldwork, as well as answering the many questions about *Littorina saxatilis*. Thank you, Tamara Tasi, for answering my many questions and thank you, Arne Nygren, for being such great company in the top-lab.

Thank you, Ameli Westling, for being my partner in crime during this project. Your constant presence made this project ten times more enjoyable than it already was. Thank you for all your support and all your help.

Lastly, I'd like to thank my family. Thank You for teaching me how to appreciate animals and nature and setting me on the right course towards becoming a biologist. If it weren't for your constant support and interest in what I do I would never be where I am today.

7. Reference list

Adams, G. (2020). A beginner's guide to RT-PCR, qPCR and RT-qPCR. *The Biochemist*, *42*(3), 48–53. https://doi.org/10.1042/BIO20200034

Aljanabi, S. M., & Martinez, I. (1997). Universal and rapid salt-extraction of high quality genomic DNA for PCR-based techniques. *Nucleic Acids Research*, *25*(22), 4692–4693. https://doi.org/10.1093/nar/25.22.4692

Bagdonaitė, L., Mauvisseau, Q., Johnsen, A., Lifjeld, J. T., & Leder, E. H. (2025). Sperm mtDNA copy number is not associated with midpiece size among songbirds. *Ecology and Evolution*, *15*(3), e71055. https://doi.org/10.1002/ece3.71055

Boguenet, M., Bouet, P.-E., Spiers, A., Reynier, P., & May-Panloup, P. (2021). Mitochondria: Their role in spermatozoa and in male infertility. *Human Reproduction Update, 27*(4), 697–719. https://doi.org/10.1093/humupd/dmab001

Chen, S. H., Xia, L. P., Dahms, H. U., Peng, X., & Ying, X. P. (2015). The ultrastructural characteristics of spermatogenesis in *Onchidium struma* (Pulmonata: Onchidiidae) and its functional adaptation. *Italian Journal of Zoology*, *82*(4), 489–498. https://doi.org/10.1080/11250003.2015.1062149

Cole, L. W. (2016). The Evolution of per-cell organelle number. *Frontiers in Cell and Developmental Biology, 4*, 85. https://doi.org/10.3389/fcell.2016.00085

DeLuca, S. Z., & O'Farrell, P. H. (2012). Barriers to male transmission of mitochondrial DNA in sperm development. *Developmental Cell, 22*(3), 660–668. https://doi.org/10.1016/j.devcel.2011.12.021

Demin, S. I., Mikhailova, N. A., Granovitch, A. I., & Bogolyubov, D. S. (2025). Non-canonical male meiosis in a marine gastropod, *Littorina saxatilis*. *Biology*, *14*(11), 1572. https://doi.org/10.3390/biology14111572

Demin, S. I., Stefanova, V. N., Granovitch, A. I., & Mikhailova, N. A. (2019). Spermatogenesis and lobular cyst type of testes organization in marine gastropod *Littorina saxatilis* (Olivi, 1792). *Cell and Tissue Research*, *376*, 457–470. https://doi.org/10.1007/s00441-019-03004-y

Dickinson, A., Yeung, K. Y., Donoghue, J., Baker, M. J., Kelly, R. D. W., McKenzie, M., Johns, T. G., & St. John, J. C. (2013). The regulation of mitochondrial DNA copy number in glioblastoma cells. *Cell Death & Differentiation*, *20*(12), 1644–1653. https://doi.org/10.1038/cdd.2013.115

Filograna, R., Mennuni, M., Alsina, D., & Larsson, N.-G. (2021). Mitochondrial DNA copy number in human disease: the more the better? *FEBS Letters*, *595*(8), 976–1002. https://doi.org/10.1002/1873-3468.14021

Fretter, V., & Graham, A. (1962). *British prosobranch molluscs: Their functional anatomy and ecology*. Ray Society.

Ghasemi, A., & Zahediasl, S. (2012). Normality tests for statistical analysis: A guide for non-statisticians. *International Journal of Endocrinology and Metabolism, 10*(2), 486–489. https://doi.org/10.5812/ijem.3505

Hartmann, N., Reichwald, K., Wittig, I., Dröse, S., Schmeisser, S., Lück, C., Hahn, C., Graf, M., Gausmann, U., Terzibasi, E., Cellerino, A., Ristow, M., Brandt, U., Platzer, M., & Englert, C. (2011). Mitochondrial DNA copy number and function decrease with age in the short-lived fish *Nothobranchius furzeri*. *Aging Cell*, *10*(5), 824–831. https://doi.org/10.1111/j.1474-9726.2011.00723.x

Johannesson, B. (1986). Shell morphology of *Littorina saxatilis* Olivi: The relative importance of physical factors and predation. *Journal of Experimental Marine Biology and Ecology*, *102*(2–3), 183–195. https://doi.org/10.1016/0022-0981(86)90175-9

Johannesson, K. (2015). What can be learnt from a snail? *Evolutionary Applications, 9*(1), 153–165. https://doi.org/10.1111/eva.12277

Johannesson, K., Panova, M., Kemppainen, P., André, C., Rolán-Alvarez, E., & Butlin, R. K. (2010). Repeated evolution of reproductive isolation in a marine snail: unveiling mechanisms of speciation. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *365*(1547), 1735–1747. https://doi.org/10.1098/rstb.2009.0256

Kiliç, H. B., Bulduk, B. K., & Kocaefe, Y. Ç. (2019). A single-tube multiplex qPCR assay for mitochondrial DNA (mtDNA) copy number assessment. *Turkish Journal of Biochemistry*, *44*(6), 769–777. https://doi.org/10.1515/tjb-2018-0372

Kim, H. -Y. (2015). Statistical notes for clinical researchers: post-hoc multiple comparisons. *Restorative Dentistry & Endodontics*, *40*(2), 172–176. https://doi.org/10.5395/rde.2015.40.2.172

Kristensen, T. N., Loeschcke, V., Tan, Q., Pertoldi, C., & Mengel-From, J. (2019). Sex and age-specific reduction in stress resistance and mitochondrial DNA copy number in *Drosophila melanogaster*. *Scientific Reports*, *9*, 12305. https://doi.org/10.1038/s41598-019-48752-7

Kumpitsch, L., Johannesson, K., Havenhand, J. N., & Leder, E. H. (2025). Variation in eusperm length may reflect reproductive barriers and differences in sperm competition intensity among *Littorina* snails. *Ecology and Evolution*, *15*(8), e71865. https://doi.org/10.1002/ece3.71865

Kühlbrandt, W. (2015). Structure and function of mitochondrial membrane protein complexes. *BMC Biology, 13*, 89. https://doi.org/10.1186/s12915-015-0201-x

Langsrud, Ø. (2003). ANOVA for unbalanced data: Use Type II instead of Type III sums of squares. *Statistics and Computing*, *13*, 163–167. https://doi.org/10.1023/A:1023260610025

Larsson, N.-G., Wang, J., Wilhelmsson, H., Oldfors, A., Rustin, P., Lewandoski, M., Barsh, G. S., & Clayton, D. A. (1998). Mitochondrial transcription factor A is necessary for mtDNA maintenance and embryogenesis in mice. *Nature Genetics, 18*(3), 231–236. https://doi.org/10.1038/ng0398-231

Laughlin, S. B. (2001). Energy as a constraint on the coding and processing of sensory information. *Current Opinion in Neurobiology, 11*(4), 475–480. https://doi.org/10.1016/S0959-4388(00)00237-3

Le Pennec, G., Butlin, R. K., Jonsson, P. R., Larsson, A. I., Lindborg, J., Bergström, E., Westram, A. M., & Johannesson, K. (2017). Adaptation to dislodgement risk on wave-swept rocky shores in the snail *Littorina saxatilis*. *PLOS ONE*, *12*(10), e0186901. https://doi.org/10.1371/journal.pone.0186901

Leuthner, T. C., Hartman, J. H., Ryde, I. T., & Meyer, J. N. (2021). PCR-based determination of mitochondrial DNA copy number in multiple species. *Methods in Molecular Biology*, *2310*, 91–111. https://doi.org/10.1007/978-1-0716-1433-4_8

Li, B., Wang, H., Jiang, C., Zeng, X., Zhang, T., Liu, S., & Zhuang, Z. (2023). Tissue distribution of mtDNA copy number and expression pattern of an mtDNA-related gene in three teleost fish species. *Integrative Organismal Biology*, *5*(1), obad029. https://doi.org/10.1093/iob/obad029

Miller, S. L. (1974). Adaptive design of locomotion and foot form in prosobranch gastropods. *Journal of Experimental Marine Biology and Ecology*, *14*(2), 99–156. https://doi.org/10.1016/0022-0981(74)90021-5

Newar, J., Verma, S., & Ghatak, A. (2021). Effect of metals on underwater adhesion of gastropod adhesive mucus. *ACS Omega*, *6(*24), 15580–15589. https://doi.org/10.1021/acsomega.0c06132

Palmer, A. R. (1992). Calcification in marine molluscs: how costly is it? *Proceedings of the National Academy of Sciences, 89*(4), 1379–1382. https://doi.org/10.1073/pnas.89.4.1379

Pavlova, G. A. (2006). The velocity of gliding of a freshwater gastropod depends on its muscular activity. *Doklady Biological Sciences*, *410*, 358–360. https://doi.org/10.1134/S0012496606050024

QIAGEN. (n.d.). *DNeasy Blood & tissue handbook*. Retrieved March 30, 2026, from https://www.qiagen.com/en-US/resources/download/KitHandbook/hb-2987-004-hb-qiawave-dna-blood-tissue-0525-ww

QIAGEN. (n.d.). *QIAamp DNA Micro handbook*. Retrieved April 22, 2026, from https://www.qiagen.com/en-US/resources/download/KitHandbook/hb-0333-005-1090260-hb-qiaamp-dna-micro-1214-ww-lr

Rooney, J. P., Ryde, I. T., Sanders, L. H., Howlett, E. H., Colton, M. D., Germ, K. E., Mayer, G. D., Greenamyre, J. T., & Meyer, J. N. (2015). PCR based determination of mitochondrial DNA copy number in multiple species. *Methods in Molecular Biology*, *1241*, 23–38. https://doi.org/10.1007/978-1-4939-1875-1_3

Ryu, K. W., Fung, T. S., Baker, D. C., Saoi, M., Park, J., Febres-Aldana, C. A., Aly, R. G., Cui, R., Sharma, A., Fu, Y., Jones, O. L., Cai, X., Pasolli, H. A., Cross, J. R., Rudin, C. M., & Thompson, C. B. (2024). Cellular ATP demand creates metabolically distinct subpopulations of mitochondria. *Nature*, *635*, 746–754. https://doi.org/10.1038/s41586-024-08146-w

Shimpi, G. G., Vargas, S., Bentlage, B., & Wörheide, G. (2024). Mitochondrial DNA damage, repair and copy number dynamics of *Sclerophytum* sp. (Anthozoa: Octocorallia) in response to short-term abiotic oxidative stress. *Journal of Experimental Marine Biology and Ecology, 580*, 152051. https://doi.org/10.1016/j.jembe.2024.152051

Sokolova, I. (2018). Mitochondrial adaptations to variable environments and their role in animals' stress tolerance. *Integrative and Comparative Biology, 58*(3), 519–531. https://doi.org/10.1093/icb/icy017

Spinelli, J. B., & Haigis, M. C. (2018). The multifaceted contributions of mitochondria to cellular metabolism. *Nature Cell Biology*, *20*, 745–754. https://doi.org/10.1038/s41556-018-0124-1

Thermo Fisher Scientific. (n.d.). *Collagenase, type IV, powder (Cat. No. 17104019)*. Retrieved April 22, 2026, from https://www.thermofisher.com/order/catalog/product/17104019

Vaishnavi, S. N., Vlassenko, A. G., Rundle, M. M., Snyder, A. Z., Mintun, M. A., & Raichle, M. E. (2010). Regional aerobic glycolysis in the human brain. *Proceedings of the National Academy of Sciences*, *107*(41), 17757–17762. https://doi.org/10.1073/pnas.1010459107

Veltri, K. L., Espiritu, M., & Singh, G. (1990). Distinct genomic copy number in mitochondria of different mammalian organs. *Journal of Cellular Physiology*, *143*(1), 160–164. https://doi.org/10.1002/jcp.1041430122

Whitmore, D. H. (Ed.). (1983). *Electrophoretic and isoelectric focusing techniques in fisheries management*. CRC Press.

8. Appendix 1

8.1 Laboratory instructions

8.1.1 DNA extraction - QIAGEN

Procedure

1. Add 180 µL Buffer ATL
2. Add 20 µL Proteinase K. Mix thoroughly by vortexing and incubate at 56°C until the tissue is completely lysed. Vortex occasionally during incubation.
3. Vortex for 15 s. Add 200 µL Buffer AL to the sample and mix thoroughly by vortexing.
4. Then add 200 µL ethanol (96–100%), invert samples, and mix thoroughly by vortexing.
5. Pipette 600 µL of the mixture from step into the DNeasy Mini Spin Column placed in a 2 mL Waste Tube.
6. Centrifuge at ≥ 6000 x g for 1 min. Discard the flow-through and reuse the Waste Tube.
7. Place the DNeasy Mini Spin Column back in the Waste Tube, add 500 µL Buffer AW1, and centrifuge for 1 min at ≥ 6000 x g. Discard the flow-through and reuse the Waste Tube.
8. Place the DNeasy Mini Spin Column in the Waste Tube, add 500 µL Buffer AW2, and centrifuge for 3 min at 20,000 x g to dry the DNeasy membrane. Discard the flow-through and Waste Tube.
9. Place the DNeasy Mini Spin Column in a clean 1.5 mL or 2 mL Eppendorf tube and add the required amount of nuclease-free water.
10. Incubate sample for 10 minutes, and centrifuge for 1 min at ≥ 6000 x g in order to elute the sample.

8.1.2 Salt extraction

Day 1

1. Pipette 200 µL of salt extraction buffer and 40 µL 20% SDS into the 1.5 mL Eppendorf tube with the sample.
2. Add 8 µL of Proteinase K to the tube, mix by inverting several times.
3. Digest at 58 ◦C until tissue is dissolved (260 rpm).

Day 2

1. Add 300 µL 5M NaCl to the tube - mix well by inverting the tube.
2. Centrifuge at 12 000 x g for 30 minutes.
3. Carefully pipette out 600 µL of the liquid into a clean tube.
4. Add 600 µL of isopropanol, gently invert the tube several times.
5. Place the sample in a -20 ◦C freezer for 20 minutes.
6. Centrifuge at 14 000 x g in 4 ◦C for 20 minutes.
7. Pour out the isopropanol, not the pellet.

8. Add 500 µL 70% ethanol, roll tube to “wash” pellet.
9. Centrifuge at 8000 x g for 5 minutes.
10. Pour out the ethanol and make sure the pellet dries.
11. Add nuclease-free water, incubate samples at 54 °C for 1 hour.

8.1.3 Collagenase IV

Example recipe for 24 samples:

1. 12.25 mg Collagenase IV
2. 4 900 µL PBS

Procedure:

3. Foot tissues treated with 200 µL collagenase IV
4. Incubate samples for 2h at 260 rpm (no temperature)

8.2 Statistical analysis

8.2.1 Type III ANOVA

Effect	NumDF	DenDF	F value	Pr(>F)
Ecotype	1	46.931	3.380	0.072
Tissue	4	175.516	89.399	0.000
Population	1	46.931	12.799	0.001
Ecotype:Tissue	4	175.516	12.415	0.000
Ecotype:Population	1	46.931	1.967	0.167
Tissue:Population	4	175.516	1.432	0.225
Ecotype:Tissue:Population	4	175.516	2.938	0.022

8.2.2 *Post-hoc analysis*

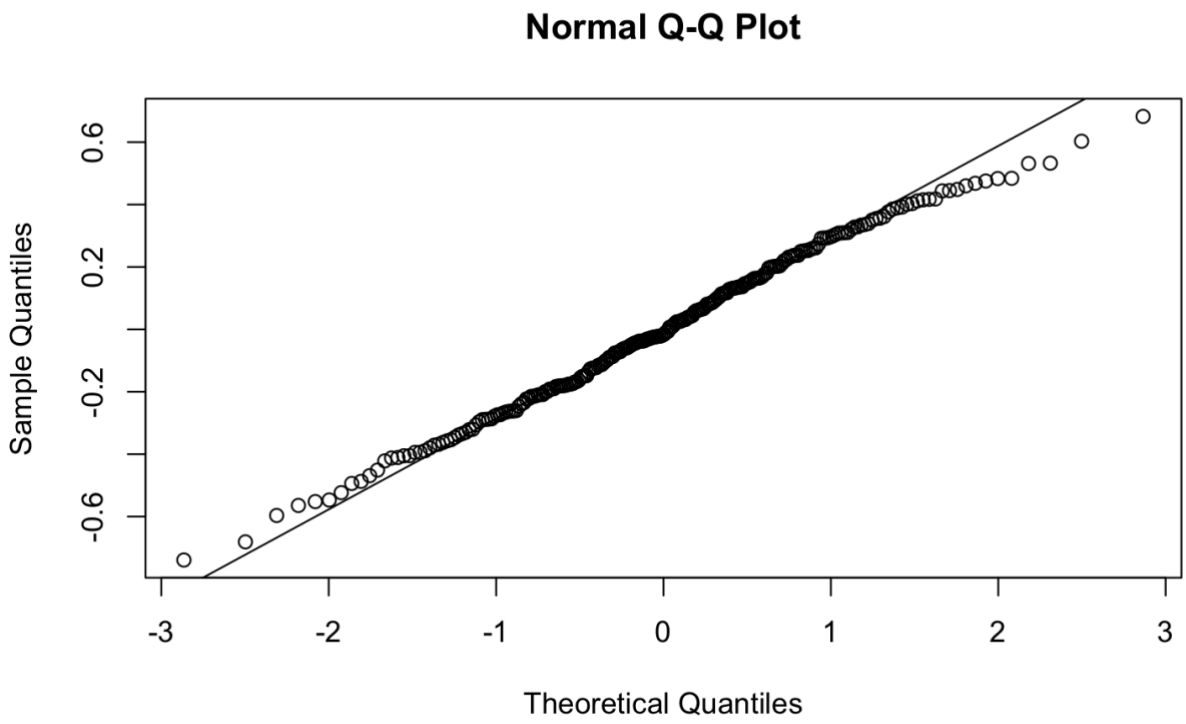
Population	Tissue	estimate	SE	t.ratio	p.value
Äng	Foot	−0.03	0.15	−0.20	0.84
Äng	Head	−0.20	0.15	−1.35	0.18
Äng	Mantle	0.53	0.15	3.50	0.00
Äng	Seminal vesicles	0.69	0.15	4.57	0.00
Äng	Testes	0.18	0.15	1.22	0.22
Arsk	Foot	0.27	0.15	1.79	0.07
Arsk	Head	−0.49	0.15	−3.20	0.00
Arsk	Mantle	0.27	0.15	1.74	0.08
Arsk	Seminal vesicles	0.16	0.15	1.02	0.31
Arsk	Testes	−0.05	0.15	−0.33	0.74

8.2.3 *Effect size*

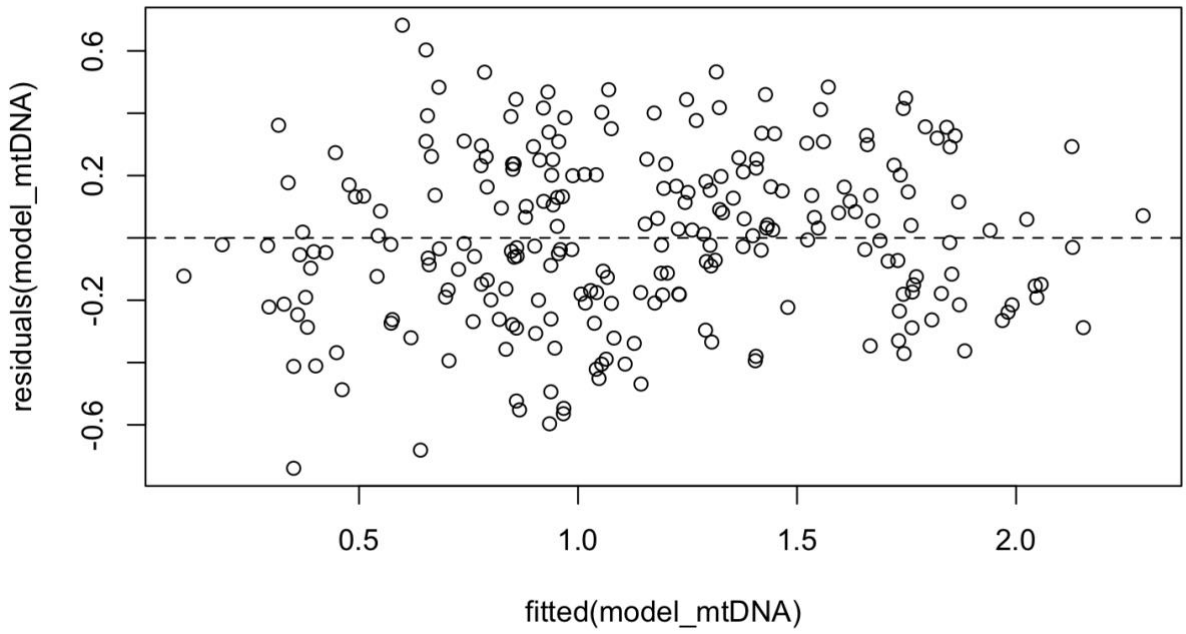
Tissue	Population	effect.size	SE	lower.CL	upper.CL
Foot	Äng	-0.10	0.49	-1.07	0.88
Head	Äng	-0.67	0.50	-1.65	0.31
Mantle	Äng	1.73	0.50	0.74	2.72
Seminal vesicles	Äng	2.25	0.51	1.26	3.25
Testes	Äng	0.60	0.50	-0.37	1.58
Foot	Arsk	0.90	0.50	-0.09	1.89
Head	Arsk	-1.60	0.51	-2.60	-0.60
Mantle	Arsk	0.87	0.50	-0.12	1.86
Seminal vesicles	Arsk	0.51	0.50	-0.48	1.50
Testes	Arsk	-0.17	0.50	-1.15	0.82

8.3 Model diagnostics

8.3.1 Q-Q plot



8.3.2 Residual vs. fitted values



8.3.3 Check_model