Estimation and evaluation of protein metabolism in a freshwater snail *Planorbella duryi*

by

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## Abstract

Animal bioenergetics can be defined as the conversion chemical energy from food into forms that can be used *in vivo* for maintenance, development and growth as well as reproduction. Thus, metabolic strategies that maximize the efficiency of energy assimilation may be beneficial to animals, especially under changing environmental conditions like elevated temperatures. Protein metabolism is a major contributor to resting energetic costs in ectotherms and protein accretion is a central component of growth. Proteins are responsible as cellular catalysts, essential structural elements and are core to defense against heat damages. Hence, a thorough understanding of protein turnover can provide insights to how energy metabolism interacts with growth dynamics under temperature change. In this thesis, two developed methods are used to assess protein metabolism in adult as well as embryonic stages of the freshwater snail *Planorbella duryi*. Using a novel $D_5$-phenylalanine tracer approach to protein metabolism to flood endogenous phenylalanine pools we measure protein metabolism in the adult snails and test the effect of temperature acclimation on both the kinetics of protein synthesis and degradation. A novel open-chambered respirometer was utilized for the estimation of protein metabolism cost during embryonic development. While biochemical processes are often associated with a 2-fold to 3-fold change per 10°C change in temperature, protein metabolism following acclimation suggests marked compensation for temperature effects in the adults. Embryonic snails showed a higher commitment of energy metabolism to protein synthesis, as may be expected given the rapid cellular turnover and growth during this life-stage.

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## Chapter I. Introduction

Animals are heterotrophs, meaning that they must consume some external source of nutrients to meet their energetic needs. External energy supplies as food sources must be catabolized then are either integrated into molecules during anabolism or used for cellular work with ATP as an intermediate for most reactions in animals (summarized in Figure 1.1A). As described, the amount of assimilated energy is dependent on food uptake as primary energy sources; catabolism and mitochondria-related processes are responsible for energy conversion and most of the ATP production with heat loss can reduce the efficiency of conversion. After food digestion, decomposed macromolecules are oxidized as a source of electrons that generate covalent bonds and drive the mitochondrial electron transport system to fuel oxidative phosphorylation. Coupled reactions transport electrons through electron carriers and the complexes of the electron transport system and protons are translocated across the inner membrane of mitochondria to produce an electrochemical gradient or the protonmotive force. Oxidative phosphorylation relies on this protonmotive force to produce ATP. The reversible reaction of ATP hydrolysis to ADP releases energy in the body system for work including kinetic (movement) and synthetic (forming of new chemical bonds) processes. This aerobic cycle occupies approximate 90% of whole body $O_2$ flux. Noted that this conversion of energy forms is not 100% efficacy as the mean to generate ATP: uncoupled reactions or proton leakage usually act as a strategy to release energy often as a form of heat. (Divakaruni & Brand, 2011; Rolfe & Brown, 1997).

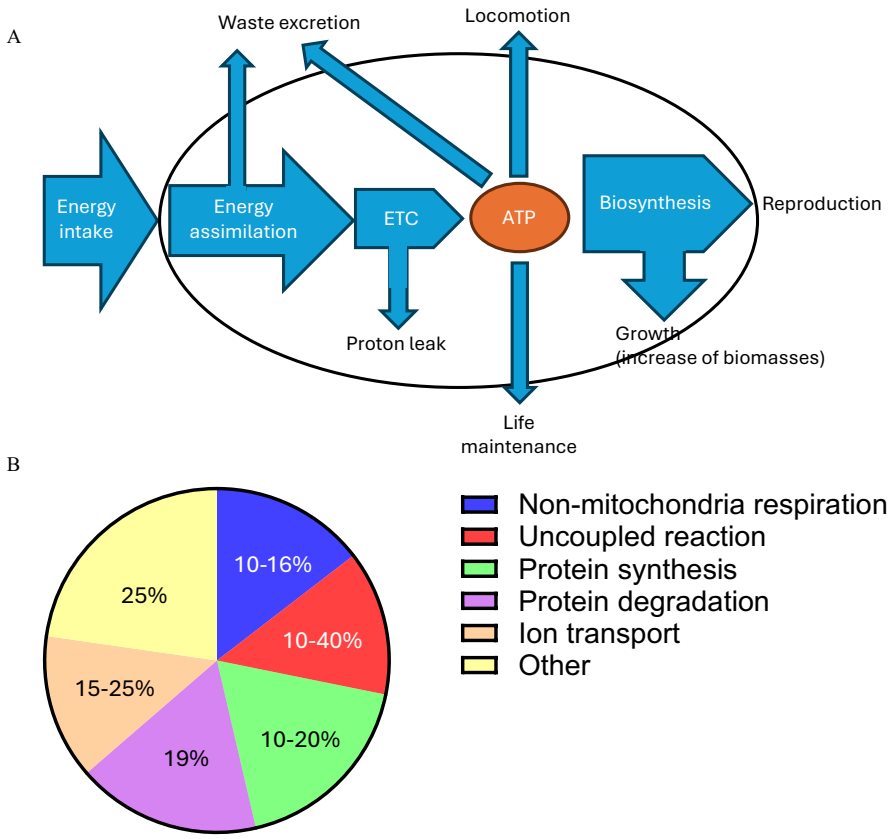



**Figure 1.1. Illustration of an animal' bioenergetics based on (A) flows of energy and matter or (B) relative contributions to whole animal oxygen flux.** In (A), energy intake reflects food consumption followed by losses due to food processing and losses from incomplete digestion (egestion) and excretion. Along the energy metabolism, heat release (not show) as a form of energy and defined as heat increment of feeding. Assimilated energy/matter is then either catabolized, primarily in the mitochondria electron transport complex (ETC), to generate ATP for cellular work, repair and homeostasis and kinetic work such as locomotory activity. Energy intake remaining after basal and active metabolic costs are met can be allocated to biomass as somatic growth and reproductive output. (B) Relative contributions of cellular processes to whole body oxygen demand in a typical aerobic ectotherm. Data in (B) summarized based on reviews by Carter & Houlihan, 2001 and Sokolova, 2021. Cost of protein degradation was converted based on protein loss of starved Atlantic cod in relation to the standard metabolic rate.

Metabolic demands, including the standard metabolic rate, are influenced by environmental temperature and therefore climate change may have profounds effect on ectotherms, including altered energy intake and *in vivo* catabolism (Sokolova, 2021). Disruption of food webs can potentially destabilize the established energy flow between trophic levels (Iles, 2014). While elevated temperature may be beneficial to some species, for instance, many microorganisms and algae can exploit resources better in warmer water temperatures, leading to algae blooms as reviewed by Griffith & Gobler, 2020. But on the opposing side, many species will have difficulties gaining sufficient energy if their prey species fail to thrive when the water temperature rises (Doney et al., 2014). Additionally, ectothermic animals' metabolic demands often increase with elevated external temperatures.

While dietary protein is a major source of energy in most animals, proteins are also required for cellular maintenance and thus are ubiquitous to life. Protein metabolism occupies 10-40% of basal metabolic cost in ectotherms with assortment between protein synthesis and degradation as illustrated in figure 1.1B (Data was summarized and calculated based on reviews from Carter & Houlihan, 2001 and I. Sokolova, 2021). Protein metabolism includes elevated expression of heat shock proteins by means to repair cellular damage, which is induced by high temperatures (Rosic et al., 2011).

Protein synthesis is the process to produce protein complexes. It starts at translation of mRNA sequences for making a polypeptide chain with the assembly of amino acids taken from the intracellular free amino acid pool during elongation of the polypeptide chain using tRNA and rRNA. In summary, energy demanded per unit peptide bond is 4 ATP equivalents and 40 mmol ATP equivalents are required per gram synthesized protein (Carter & Houlihan, 2001). Most proteins are used within the cells that produce them, but exceptions available such as vitellogenin or vitellogenin-like proteins enrolled in vitellogenesis, where protein that produced in the fat body of insects, or digestive glands in molluscs, transfers from the source tissue via the circulation  and is then deposited within the reproductive tissues and ultimately transported into oocytes following sexual maturation (Fahmy, 1949). Similarly, the mammalian liver produces substantial amounts of protein for secretion into the plasma, including albumin and globulins both of which turnover at a rate of rough 10-18% per day (Mcfarlane, 1963). On the other hand, protein degradation involves recycling of proteins back to individual amino acids as a way for cellular maintenance and saving energy (Fraser & Rogers, 2007). Proteins are prone to damages from chemical modifications and misfolding, both of which can cause loss of a protein molecule's function, in which requires regulation via various strategies as reviewed by Amm et al., 2014.

The metabolic costs of protein synthesis which can be quantified using both theoretical estimations depended the known biochemical reactions of transcription and translation processes, as described above, or pharmacological inhibitors like cycloheximide to measure the contribution of protein synthesis to cellular oxygen flux (Houlihan et al., 1993). Conversely, protein degradation occurs in variable sites that are dependent to the location when misfolded proteins are recognized by the cell. As reviewed by Amm et al., 2014, the major quality control processes in cytosol and nucleus to deal with malfunctional protein is the ubiquitin-proteasome system that is focused with endogenous protein and vacuolar (lysosomal) proteolysis to recycle wastes from both intra and intercellular spaces. Additional strategies include endoplasmic reticulum associated degradation and some site-specific control center, like nucleus and mitochondria. Hence, pharmacological methods cannot thoroughly estimate protein degradation.

In many fish, accounting for protein degradation has been determined based on the mass balance difference between total protein pool growth and the instantaneous rate of protein synthesis. The conclusion indicated 60% recycled protein products are retained and recycled *in vivo* during starvation (Houlihan et al., 1989). Hence, this estimation is logistically feasible to

quantify protein degradation. But the time frame that set the measurement between rate of synthesis to that of degradation is different, which could introduce increased confounding errors such as weight loss that occurs naturally, and this can only be visible at least by days or weeks; some slow-growing creatures might require months to be detected. In contrast, flooding dose approaches to calculate instantaneous rate of protein synthesis takes place on the scale of 4-12 hours. This way of measurement allows one bolus injection of labelled isotopes to flood the intracellular free amino acid pools, which are incorporated into the protein pool as part of protein synthesis. The rate of protein synthesis is obtained via the rate of uptake of this marked amino acid. Despite the ease of measurement, this method is effective for instantaneous estimation, but no periodical changes are considered during the design of the test. Overall, the evaluation of both rate estimations must be carefully considered due to the time gap.

**Table 1.1. Fractional rates of protein synthesis and degradation in ectotherms.**

| Classification | Rate of protein synthesis (%/day) | Rate of protein degradation (%/day) |
|---|---|---|
| Fish | 3.8±1.92 | 2.1±1.71 |
| Mollusc | 0.6±1.56 | 0.5±0.29 |
| Crustacean | 13.3 | 12.4 |

Note. Data summarized from literature reviewed by Fraser & Rogers, 2007 (see Fraser and Rogers, 2007 for complete list of citations) where one data of Crustacean is included as lobster *Homarus gammarus*. All species included are under standard living conditions. The table encompassed whole body measurements of 8 species of fish, 3 of Mollusc and 2 of Crustacean.

**Table 1.2. Comparison of tissue-specific rates of fractional protein synthesis in fish and invertebrates. Summary of protein metabolic rate for ectothermic tissues.**

| Fish tissues | #species of measurements | Rate of protein synthesis (%/day) | Invertebrate tissues | #species of measurements | Rate of protein synthesis (%/day) |
|---|---|---|---|---|---|
| White muscle | 18 | 0.6±0.27 | Muscle | 10 | 0.5±0.41 |
| Liver | 6 | 11.5±6.3 | Hepatopancreas (liver) | 8 | 1.9±1.22 |
| Heart | 2 | 1.3±0.1 | Heart | 4 | 2.25±0.44 |
| Gill | | 14.5±7.95 | Gill | 4 | 2.4±1.1 |
| Brain | 3 | 0.5±0.26 | | | |
| Intestine | 1 | 12.1±4.5 | | | |

Note. Data summarized from literature reviewed by Fraser & Rogers, 2007 (see Fraser and Rogers, 2007 for complete list of citations). All species included are under standard living conditions.

Despite the uncertainty of estimation on protein degradation, current understanding on rate of protein synthesis is available as a reference for metabolic cost of protein turnover summarized in table 1.1. Overall, protein synthesis rates vary markedly across tissues, for example, muscle fractional synthesis rates are typically much lower than visceral organs or respiratory epithelia in fishes (Table 1.2). Invertebrates are slower in synthetic rate versus that of fish for most tissues, and the energy assortment between tissue types is more even. But this could due to lack of data in invertebrate compared to fish and those measured invertebrates residing at temperatures being typically less than 10°C as summarized by Sokolova & Lannig (2008). Then, invertebrates homeostasis with environmental constraints under variable stressors need more energy under increased temperature to meet their costs in comparison with vertebrates (Kingsolver & Woods, 2016; Riemer et al., 2018; Sokolova & Lannig, 2008). Hence, this balance between these energy blocks potentially faces more pressure under climate change since the overall energy supply is limited or reduced.

Compared to adults, embryos and larvae can show increased fragility to environmental challenge in many species because early developmental stages may lack well developed defensive strategies for example in sea urchins (Giudice et al., 1999). During early stages when, despite high metabolic rates and demands, periods of rapid development may be favoured over cellular maintenance and defence. Energy has been provided for embryos from the maternal source in the case of egg-laying ectothermic species, with nutrients located mainly in the egg yolk which then fuel metabolism and growth demands as the embryo develops and increases organismal complexity. Under this situation, proteins appear to have increased importance since it is required for embryogenesis development as well as account partially as body growth. Protein metabolism can occupy over 50% of the standard metabolic rate (van den Biggelaar, 1971; Pace & Manahan, 2006; Lee et al., 2016), which is almost double that found in adults. While as adults, many organisms can rely heavily on dietary carbohydrate as a major energy source, during embryogenesis metabolism is focused mainly on fatty acid and amino acids, which further emphasized the importance of protein synthesis for eggs (Ghosh et al., 2023). The study of protein metabolism in accordance with total energy cost *in vivo* is limited, primarily due to lack of effective strategies to manipulate embryos without introducing further stresses to the system because of the small size and fragility. While some fish larvae can use strategies similar to those measurements for adults (Houlihan et al., 1992); but as described previously, the flooding dose method has a major deficiency where it fails to evaluate protein synthesis and degradation rates on equal timescales, so potential confounding errors could occur. =Sessile embryos often rely on the estimation of the energetic content of macronutrients over the total dry weight of studied tissues, then convert this ratio using unit ATP costs. This way to address the cost of protein metabolism can lack precision especially when considering the effect of heat on the protein metabolism during embryogenesis which can be time-specific and dependent of the thermal responsiveness of the processes involved, both of which may vary between acute changes and acclimation. Thus, thus this method that relies on the estimation of the separate macronutrients (protein, lipid and carbohydrate) over the total dry weight of studied tissues can

possibly miss that targeted window due to lack of ways to properly detect the ongoing process. Recently, a novel open-chambered respirometer was developed offering the opportunity to test the cost of protein metabolism directly via estimation of $O_2$ consumption rate that refers to the mitochondria $O_2$ flux. A notable issue with relating $O_2$ flux to metabolic demand is that the ATP production of glycolysis is not included since this part of energy conversion does not directly contribute to $O_2$ consumption. This measurement uses 96-well culture plate allows for many 32 replicate measurements in a single experiment. Elongated timeline opens wider test window that is expected to reveal the activity of protein metabolism in details.

Aquatic ectotherms like the freshwater snail *Planorbella duryi* are a useful study organism for protein metabolism because their liquid environment can also be used as a delivery system for isotopes that can be used to track precursors and polypeptides. Additionally, for *P. duryi* specifically, which is hermaphroditic, a simple body structure allows for deconstruction of major physiological systems into three energy contributing blocks: the mantle and foot, which are mostly locomotory muscle, the gonad representing reproductive investment and the visceral organs responsible for most dietary processing and nutrient allocation to the other organs as well as excretion and egestion. Eggs of aquatic snails are enclosed in a clear protective aqueous sac, and the sac can hold on one surface to keep the embryos sessile. Studies on other snails show that snail embryogenesis is susceptible to temperature and exhibit thermal intolerance in which the level of heat damage is dependent to their developmental stage (Ghosh et al., 2023). This thesis used the freshwater snail *P. duryi* at both adult and embryonic stages to reveal details on metabolic costs of protein metabolism for different major life stages.

# Chapter II. Protein metabolism of adult snails

## 2.1 Abstract

Protein metabolism is a major contributor to resting energetic costs in ectotherms and protein accretion is a central component of growth. Hence, a thorough understanding of protein turnover can provide insights into how energy metabolism interacts with growth dynamics. Using $D_5$-phenylalanine to flood internal phenylalanine pools we measured protein metabolism in the freshwater snail *Planorbella duryi*. Adding the labelled tracer to the water bathing the snails allows for a sufficiently longer period of exposure to the isotope, compared to injections, to determine stabilization in the protein-bound pool, which should provide estimates of both synthesis and degradation kinetics. The enrichment of phenylalanine in the free amino acid pool needed 12hrs to stabilize using our approach the free pool enrichment can be maintained long enough for equilibration with the protein pool that took approximately 2-3 weeks proportionate to bathed temperature. Past studies compare the short-term (hours) rate of synthesis to whole body protein balance to estimate the rate of degradation; by using this longer exposure to the labelled amino acid we tested if these short-term synthetic rates are appropriate to compare with the pool of protein that turns over long-term by using the novel way of calculation that focused on the enrichment of the protein pool along with the assumption of approaching equilibrium between the free and protein bound pools over time. The results indicate short-term estimates of synthesis appear to reflect a pool of protein with faster turnover than the majority of the tissue protein pool followed for degradation estimates. The effect of temperature acclimation on protein turnover was evaluated using this new technique, however, increases and decreases of 5°C did not lead to clear changes in synthesis or degradation rates suggesting compensation for the effect of temperature on the biochemical process of protein maintenance. Overall, this chapter provides new insight on integration of protein metabolism. More experiments that use current methodology with variable of animals should assist on evaluation the established study.

## 2.2 Contributions

Credits are given to Dr. Jillian Detwiller for her generous supply of *P. duryi* snails. The author thanks Dr. Simon Lamarre from Universite de Moncton for assistance on the manipulation of GC-MS as well as some of the data analysis and suggestions for the experiments. Much appreciation goes to Dr. Nahid Tamanna for the instruction on HPLC and offering valuable technical support. The experiment was established based on the primitive idea from Dr. Jason Treberg with major methods achieved by the author Chenyi Wang under NSERC funding.

## 2.3. Introduction

An animals energy budget can be summarized as a series of inputs and outputs with a major distinction between endotherms and ectotherms being that ectotherms either are entirely passive thermoregulators where their internal temperature reflects the environment, or they behaviourally thermoregulate which contributes to the activity costs but there is no directed heat production to regulation internal temperatures like the condition in endotherms. Basal energetic costs include those which are required for cellular maintenance, of which, the cost of protein metabolism being a major contributors to the overall organismal energy budget occupying approximate 40% of the resting metabolic costs as shown in Figure 1.1B (Houlihan et al., 1995; Sokolova, 2021). Protein metabolism combines synthesis and degradation, and in ectotherms may be influenced by environmental features like ambient temperature because biochemical rates are typically temperature sensitive. The energetic protein metabolism requires considerable amount of energy supply (Hou et al., 2015), especially with the elevated expression of stress defensive strategies like chaperons including heat shock protein family is responsible for repair of cell damages, including misfolding of protein sequences that is proportional to heat stimuli (Csermely, 1999).

Many methods have been developed to test the rate of protein synthesis, where isotope-labeling is one of the most common ways (reviewed by Carter & Houlihan, 2001, and by Treberg et al., 2016). This method relies on the uptake of a large dose of labelled isotope into the animal, usually via injection and accounts for the fractional protein synthetic rate via tracing this labelled isotope into the protein 'bound' pool of amino acids within a tissue, or whole organism. Protein synthesis generally follows the sequence of gene transcription producing mRNA which is translated to polypeptides with successive addition of activated amino acids carried on tRNA, followed by folding of polypeptides with attachment of protein subunits to generate protein complexes. Key assumptions of measuring protein synthesis rates using isotopically labelled amino acids are as follows: 1) the isotopic tracer must quickly diffuse into the intracellular free amino acid pools and the enrichment should be stable over time; 2) while the free pool is highly enriched, the enrichment of the protein pool should increase linearly as labeled amino acids move from the free pool to the protein bound pool (developed by Garlick et al., 1980; Lamarre et al., 2015; Mcnurlan et al., 1979). Also, the results should not be biased via excess nutrients available for the body system including the increased amount of the amino acid used to administer the isotope. In other words, the addition of the isotopic carrier must not accelerate the protein synthesis rate of the animals. While a one time injection seems advantageous because the estimation of fractional rate of protein synthesis is a relatively fast reaction, but as described, current flooding dosage methods that estimate instantaneous protein synthetic rate could potentially be biased by the time-lag in the activation of heat defence strategies, which accounts by the proceeding cellular damages (Dietz & Somero, 1992).

Quantification of protein degradation seems to be difficult due to the complexity of protein degradation mechanisms. While protein degradation is necessary to recycle amino acids from

damaged or excess proteins. There are two major protein degradation mechanisms involved in animal tissues, the ubiquitin-proteasome system as well as the vacuolar (lysosomal) proteolytic pathways. Unfortunately, there are no pharmacological agents that are effective for complete blockage of both degradation pathways therefore measurement of protein degradation cannot be achieved using a simple single analysis (Amm et al., 2014). To overcome this analytic challenge, past research on ectotherms either estimate protein loss during starvation as means to calculate protein degradation, or estimate protein synthesis rates on short-term scales (generally less than a day) and net protein growth on the scale of weeks or more; the difference between synthesis and protein pool growth being assumed to be the protein degradation rate (Carter and Houlihan, 2001). This latter approach has been used frequently in aquatic ectotherms, but the assumption that synthesis rates measured over hours are truly reflective of protein dynamics on these much longer time scales for growth has not been tested directly.

Freshwater aquatic ectotherms may be particularly susceptible to environmental stressors since freshwater ecosystems are often limited in their capacity to escape their local ecosystem restricting the opportunity for large scale migration away from unfavourable conditions. Freshwater snails have simple body structures that can easily be separated as the muscle tissues that are responsible for locomotion, visceral tissues for growth, nutritional processing and excretion and finally gonad for reproduction. These listed physiological processes are energetically expensive and closely dependent on proteins as both cellular catalysts (enzymes) and structural elements.

Here, we design experiments to test if it is appropriate to use the short-term estimates of protein synthesis typical of studies on ectotherms with long-term estimates of protein degradation which are measured on much longer time scales. We chose to use the freshwater snail *Planorbella duryi* while adding the isotope to the water around them as a delivery system which has also been used successfully for larval fishes (Fauconneau, 1984; Houlihan et al., 1992). By bathing animals in labelled amino acid (phenylalanine is the typical choice), as opposed to injections, allows for long-term stabilization of an enriched free pool of amino acids by continual uptake on the surface epithelia as well as by the water uptake seen when snails are grazing (Frankel et al., 2020). Thus, water bathing strategy that adds isotopes into the water allows snails to absorb isotopes over longer periods time than a single injection would

Using this extended exposure to the isotopically labelled amino acids, it is possible to address if the assumed short-term rate estimates of protein synthesis by the initial rate of labelled incorporation compared to the rate determined over longer term isotopic incorporation into the protein pools. Following this methodological advancement, we aim to test the effect of acclimation temperature on both synthesis and degradation rates in *P. duryi*.

## 2.4. Hypotheses

Rates of protein metabolism, including both synthesis and degradation, are proportional to acclimation temperature because *P. duryi* snail is an ectotherm invertebrate which cannot control its body temperature.

## 2.5. Objectives

Testing the validity of short-term synthesis kinetics relative to degradation kinetics relies on the assumed constant rate of incorporation of the labelled amino acid into the bound protein pool, the latter of which also can turnover by degradation, returning labelled and unlabelled amino acids to the free pool as shown in figure 2.1:

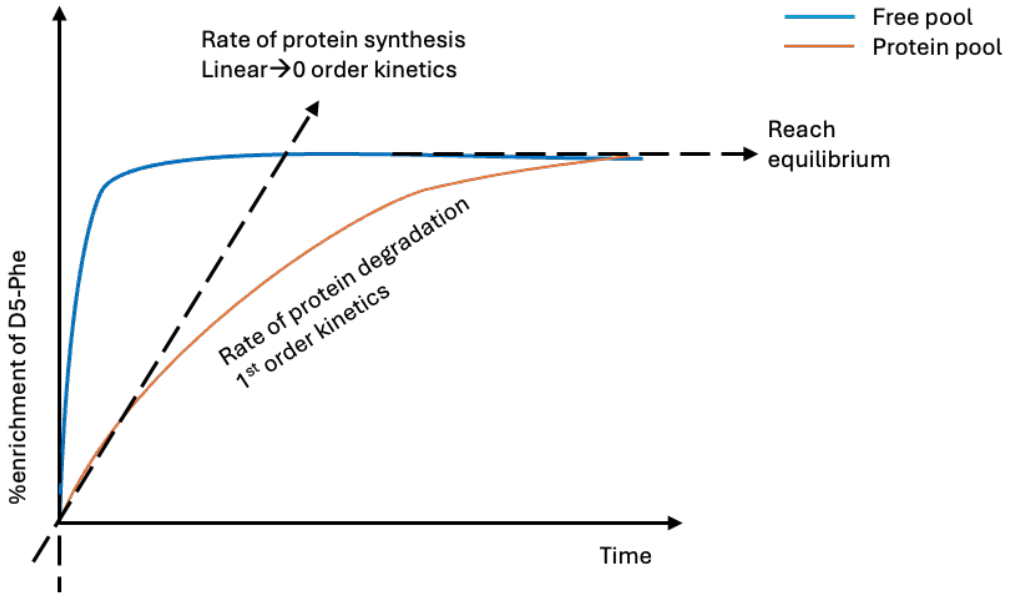



**Figure 2.1. Illustration of expected patterns of incorporation of D5-Pheneylalanine ((% enrichment of D5-Phe) into the intracellular free amino acid pool (blue) and the protein bound pool (orange) assuming a stable supply of label.** The free pool is expected to become rapidly enriched with the relative enrichment (% enrichment of D5-Phe) then remaining stable, while incorporation into the protein bound pool will initially show a near linear rate of apparent incorporation (pseudo-zero order kinetics) but as proteins are removed from the pool, the incorporation will show first order kinetics over longer periods of time for incorporation. Assuming the system remains in a stable for sufficient time, both the free and protein bound enrichments will approach the same value as they near equilibrium.

1). The verified isotope experiment needs to satisfy the assumptions listed in which the free amino acid pool can absorb the isotope following an approximately linear incorporation phase initially, and the protein synthetic rate constant can be calculated assuming linearity (zero order kinetics) relying on the incorporation of the isotope thru the free pool to the protein pool after this quick assimilation. Ultimately, the retained isotope in both the free and protein bound pools should reach equilibrium, meaning their relative enrichment becomes equal.

2) Prior to reaching equilibrium between free and bound pools, the non-linear region illustrates that as protein turnover has occurred, the curvature of the incorporation provides an estimate of the rate constant of protein degradation (which follows first order kinetics).

3) Using the validated means of estimating both protein synthesis and degradation kinetics tests if acclimation temperature alters protein metabolism in *P. duryi* muscle (foot), visceral organs or gonad.

## 2.6. Materials and methods

### *2.6.1. Animals*

*Planorbella duryi* snails were initially obtained from the colony kept in the University of Manitoba animal holding facility that was first established in 2015 (Friesen et al., 2024). We raised the snail colonies at 15, 20 or 25°C respectively in closed water systems. We provided Romaine lettuce (*Lactuca sativa*) twice to three times per week as the primary food source of snails. The water was changed every week to remove wastes and feces from the tanks. Light was kept for diurnal cycle (10h light with 14h dark) for the experiment. Snail size was measured as diameter (longest axis) using digital calipers (Spurtar) and snails that were 9.73±0.77mm in length were used for this experiment.

### *2.6.2. Design of isotope experiment*

We used a water bathing strategy to introduce isotopes to the body system. The labelled isotope used in this experiment is $D_5$-Phenylalanine ($D_5$-Phe). We added 75μM $D_5$-Phe with 75μM Phenylalanine (Phe) with naturally occurring deuterium abundance (mass+ 0) to make the final concentration 150μM. During the experiment, three snails were placed in closed 250mL Maison jars contains isotope $D_5$-Phe with oxygen supply and Romaine lettuce as food sources. Their condition was checked daily and laid eggs were removed from the jars to keep the condition uniform.

*1). Test of degradation of dissolved $D_5$-Phe*

To confirm the stability of isotope when dissolved into water, we tested the rate of disappearance of the Phe in the water under 20°C. For one group, we kept four snails two days prior to the experiment start to mimic the actual environment for the snail experiment. The other group used clean jars. For every tested condition, four snails were available in two separated jars. The effect of the presence of snails and lettuce were tested under both water conditions to address the possible degradation of isotopes. The frequency of water exchange was assessed here to address the effect brought by the size of the flooding pool to the body system.

*2) Growth rate*

The growth rate of snails in water containing 150μM Phe with 50% $D_5$-Phe was collected constitutively in 7days in comparison with normal growth rate where snails were raised in

freshwater in order to confirm that the additional of Phe has no effect on growth rate prior to the start of isotope experiment. We measured the shell diameter for each of the four snails in each container. Water was exchanged at every second day. Snails were raised in water at either 15, 20 and 25°C respectively in preparation for later experiments.

*3) Water bathing strategy*

Snails were bathed with 150μM Phe with 50% $D_5$-Phe over time. The experiment started with one-week-trial, then replicated and extended to two weeks to meet possible equilibrium. Three snails were collected after 4, 8, 12hrs after exposing to the isotope at the 1$^{st}$ day, then everyday onward for the first trial. As of to the replicate, the collection time points were 12h, 24h and 48h; then day 5, 7, 9, 11, 13 after the start of the experiment. Remaining snails were removed after day 13 to freshwater in a clean jar, then collect as post exposure 24h afterward as day 14.

*2.6.3. Thermal test on protein synthesis*

We extended the application of the isotope experiment to investigate the effect of temperature on the protein metabolism. The experiment followed the steps of the previous experiments, but instead water was changed daily via switching of containers to keep the cleanliness of the experimental condition. Samples were collected 12hrs after exposing, then day 2, 3, 8, 11, 18, 21. The entire experiment lasted for three weeks under 15, 20 and 25°C respectively. Temperature was controlled via monitoring room temperature.

In parallel with the thermal test, we accounted the length, width and weight with shell (excess water on the shell were removed by Kimwipes) at day 0, 9, 15 and 21 after exposing to $D_5$-Phe to test the effect of temperature on growth, as well as to confirm excess isotope has no effects on growth. Nine snails were collected for every test group at each time points.

*2.6.4. Tissues processing and analysis of phenylalanine isotopic enrichment*

Tissues were collected by crushing the shells using a glass beaker to expose the soft tissues. The whole body was separated into i) the mantle and foot, which is mostly muscle tissue, ii) the visceral organ mass and iii) gonad tissues (i.e., the reproductive tissue). Tissues were frozen and stored at -80°C before use. Samples were weighed (Denver instrument with decimal point to 0.1mg) and then homogenized in 6 volumes of 0.6M Perchloric acid (PCA) using TissueLyser II (from QIAGEN®). Samples were centrifuged at 10000g for 5min under 5°C to separate the free amino acid pool (the supernatant) and the protein bound pool (the pellet). The free pool samples were then kept frozen before analysis. Residuals from the precipitated protein pellets were transferred carefully to a new 5mL capped glass tubes and added 2mL of 6M HCl, being held on heated blocks for 18hrs at 110℃ for hydrolysis.

Solid phase extraction (SPE) was used to collect amino acids from both the free and bound pools. Briefly, a C18 cartridge column (Bond Elut C18, 100mg 1mL, 40μm), was prepared with

1mL 100% Methanol followed by1mL 1M HCl to active the column followed by addition of 500 µL of a sample. Non-binding compounds were flushed from the column with 1mL of additional 1M HCl then the amino acids were eluted using 250µL of 30% methanol. Collected amino acid samples were then dried by placing the samples on the heating block at 110℃.

To determine the enrichment of the phenylalanine pools, gas chromatography mass spectrometry (GC-MS) was used to quantify the ratio of the naturally abundant (mass+ 0) phenylalanine and $D_5$-phenylalanine (mass+ 5) in each sample. To derivatize, samples were first dissolved in 75µL of MilliQ water (confirmed >18mOhms of resistance per cm). In a 2ml GC-MS vial, 20µL of 500 mM Phosphate buffer (pH=8.0) was added, then with 50µL of the redissolved samples followed by 130µL 100mM Pentafluorobenzyl Bromide (PFBBr) in acetone. Samples were incubated at 60℃ for 45min to allow derivatization after which 330µL of hexane was added to the vial and the organic phase (top layer) was collected in a glass insert prior to the GC-MS analysis. The GC-MS analyses were performed following the method established by Lamarre et al., 2015 where the mass-to-charge ratio is 300 for Phe and 305 for $D_5$-Phe.

High performance liquid chromatography (ultimate 3000 HPLC from Thermo Scientific) was used to assess the phenylalanine concentration in the bathing water. Note that the HPLC cannot separate phenylalanine with labelled $D_5$-phenylalanine. 200µL of water samples were mixed with 160µL Borate buffer (pH=9.5) and 200µL dinitrofluorobenzene (dissolved in Acetonitrile); then the solution was incubated at 60°C for 1h and filtered (20µM Nylon syringe filter from Wheaton®) into HPLC vials. The analysis using HPLC at 363nm was established using 50mM filtered N-methyl-morpholine as Pump A and 100% Acetonitrile as Pump B with detailed elution profile listed in table 2.1. The column used in this experiment was from ThermoFisher® with product number as 059132 (100 x 4.6 mm, with 3µm particle diameter). The retention time for Phe is at approximate 34min.

**Table 2.1. Elution program for HPLC (flow rate=1ml/min).**

| Time (min) | %B | %A |
|---|---|---|
| 0 | 0 | 100 |
| 14.2 | 15 | 85 |
| 32 | 30 | 70 |
| 40 | 80 | 20 |
| 43 | 0 | 100 |
| 45(wash) | 0 | 100 |

*2.6.5. Data analysis*

The calculations used in this experiment are as listed (Lamarre et al., 2015) where t indicates time in hours:

The enrichment of $D_5$-Phe into the free amino acid pool ($S_a$) or the body pool ($S_b$):

$$1.\ [D_5\text{-Phe}] \div ([\text{Phe}] + [D_5\text{-Phe}])$$

As indicated in figure 2.1, it is assumed that the rate of protein synthesis should follow 0 order kinetics as:

$$2.\ S_b = k_s * t$$

That over the rate of degradation increases the complexity of the system that alter the dynamic to behave as 1st order kinetics with equation:

$$3.\ S_b = \frac{k_S}{k_d} \times (1 - e^{-k_d t}).$$

Following the nature of protein turnover, the enrichment of both pools can reach equilibrium over time as indicated by the capacity of the protein metabolism, so that at infinite time, $Plateau\ S_b = Plateau\ S_a = \frac{k_S}{k_d}$. Equation then modifies to be for calculation of rate of protein degradation:

$$4.\ S_b = Pleateau\ S_a \times (1 - e^{-k_d t})$$

where this $S_a$ was obtained via integrated mean value of the free pool enrichment of $D_5$-Phe.

Based on this equation, the rate of protein synthesis can be traced reversely as:

$$5.\ k_S = S_b \times k_d$$

Other than this, the fractional rate of protein synthesis can be obtained via regular flooding dose method within 12h after the start of the experiment as follows:

$$6.\ k_{Synthesis} = \frac{S_b}{S_a} \times t \times 100$$

$$7.\ k_{degradation} = \frac{k_S}{k_d} \times (1 - e^{-k_d t})$$

.

*2.6.6. Statistical analysis*

Figures and data analysis was manipulated using Prism 10.0 with simple calculations was done with Microsoft excel. One way ANOVA (α=0.05) with repeated measurements on every

time points was used to compare the enrichment of $D_5$-Phe into the free amino acid pool under different frequency of water change. As of to the growth experiments, we used least-square linear regression to obtain the growth rate as the slope; then plotted in bar graphs with SEM and apply One way ANOVA ($\alpha$=0.05) without repeated measurements to address the results. Isotope experimental data was calculated using formulas described above, then displayed in a dotted graph. Fractional rate of protein synthesis between tissues was compared using One way ANOVA ($\alpha$=0.05) with repeated measurements on every time points. Results of thermal test was calculated based on the formulas with the parameter depends on integrated mean value of free pool enrichment as described above and degradation rates were compared based on overlap of the 95% confidence intervals for the estimated $k_d$ from the regression analysis.

## 2.7. Results

### *2.7.1. Isotope experiments*

*1). Testing for degradation of dissolved phenylalanine*

As shown in figure 2.2, the degradation of dissolved Phe started after 24hrs post the start of this experiment and was depleted after 48hrs by itself. The addition of other elements, especially with the continued use of tank water overtime, accelerated this process. Lettuce supply has no apparent effects on the disappearance of Phe. Paired t test ($\alpha$=0.05, df=2) was used to identify the level of isotopic enrichment in the free pool when the frequency of isotope supply changed in figure 2.3 for every tissue types. None of them differed in the free amino acid pool enrichment ($P$= 0.1992 for muscle, $P$ =0.6526 for visceral and $P$ =0.2460 for gonad), indicating that one or two-day-water exchange resulted in no differences on body pool supply.

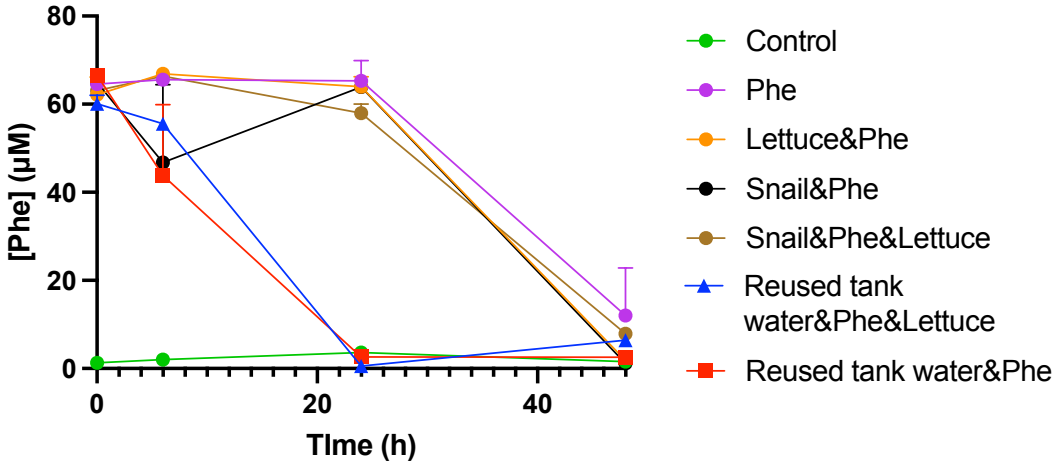



**Figure 2.2. The concentration of phenylalanine in the water over time under different experimental conditions.** Each conditions contained n=4 snails and 2 jars per treatment. 150µM of phenylamine containing 50% of $D_5$- phenylamine was added in the water except the control group and the data was computed via high performance liquid chromatography as descirbed in the methods seciton 2.6.4. Water temperature was 20°C for this experiment. Error bars indicate SEM.

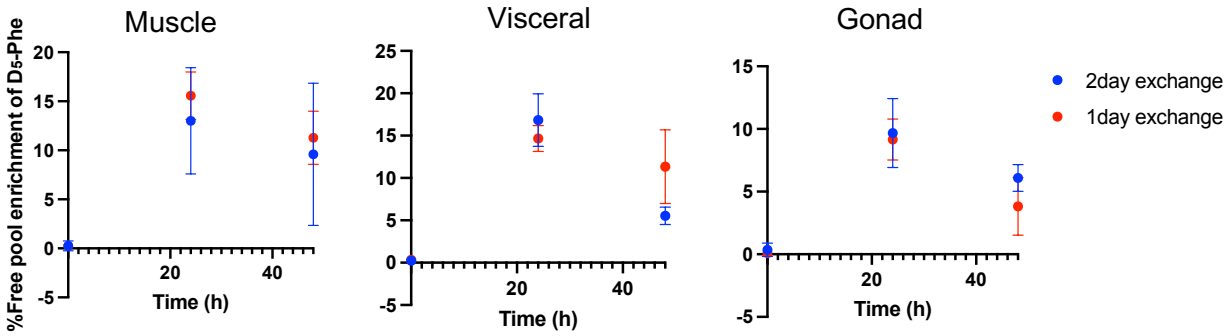



**Figure 2.3. The effect of replenishing phenylalanine in the bathing water every day or every other day on the free pool %enrichment of $D_5$-phenylalanine.** N=4 snails per each sampling point with 2 in each 250mL jar. 75µM of phenylamine and $D_5$- phenylamine (150 µM total phenylamine) was added in the water where snails were sat and the test group had one additional water change at 24hrs after the start of the experiment. The data was computed via gas chromatography mass spectrometry as described in the methods section 2.6.4. Water temperature was 20°C for this experiment. Error bars are SEM and were analyzed using paired t test ($\alpha$=0.05, df=2).

*2) Test of short-term growth rate*

The linear regression plot illustrated that possible growth is captured since the rate constant is positive (Figure 2.4.B). One way ANOVA test (α=0.05, df=2) applied on those growth rates fails to display statistical differences between the control versus Phe group within each temperature group ($P$ = 0.1634 for 15°C, $P$ =0.9012 for 20°C and $P$ = 0.2758 for 25°C). Hence, the addition of isotopes does not accelerate the growth of snails.

Growth was evaluated in parallel as weight with shell (g/day) with the thermal test for 3 weeks and analyzed using one way ANOVA (α=0.05, df=26). The weight accretion rate is illustrated in figure 2.5A. No apparent growth is observed when comparing the initial values to the final dataset for 15°C groups, hence, no visible growth is captured for colder temperature test group ($P$ = 0.9857 for 15°C control, $P$ = 0.4037 for 15°C Phe). At 20°C, the control group grew, but not the Phe group ($P$ =0.0012 for 20°C control; $P$ =0.4475 for 20°C Phe), indicating that isotope had no effect on growth. At the highest temperature, both the control and Phe-exposed snails grew ($P$ = 0.0380 for 25°C control, $P$ = 0.0009 for 25°C Phe), but the same one way ANOVA test that compared the control group versus Phe group failed to reject the assumption where $D_5$-Phe can assist growth (α=0.05, df=2, $P$ =0.2002). Additionally, none of the simple linear regression lines in figure 2.5B about the tissue mass overtime is deviated from 0 ($P$>0.05) except the muscle tissues from 15 and 25°C, but their values are close to the margin. Hence, it is assumed that the protein pool size change is negligible during this test.

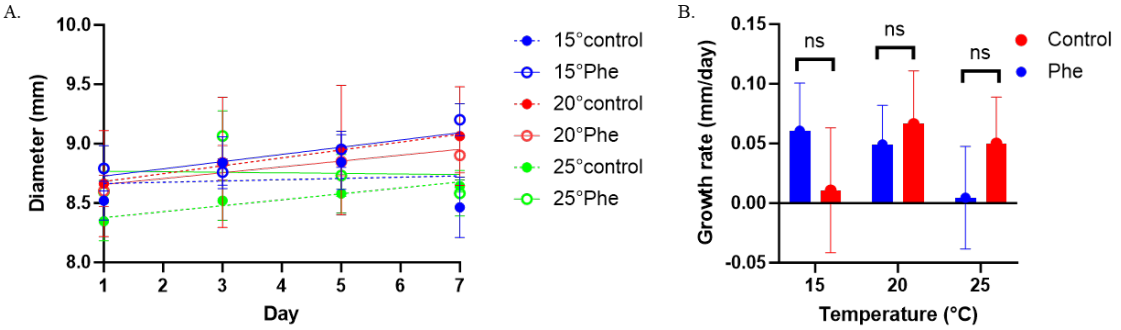



**Figure 2.4. (A.) Measurement of snail shell diameter over time and (B.) estimates of growth rates in snails at different temperatures, with (open) and without (closed symbols) supplemental phenylalanine in the water.** N=4 snails per each sampling point with 2 in each 250mL jar, and the concentration of phenylalanine is 150μM. Water was changed every two days to ensure the maintenance of high concentration of phenylalanine. Error bars are SEM and lines in A. are least-squares linear regression; the slope of each group is shown in B. as growth rate with standard error of the estimated slope. Data of A. was then analyzed using One way ANOVA (α=0.05) without repeated measurements and exhibited in B.

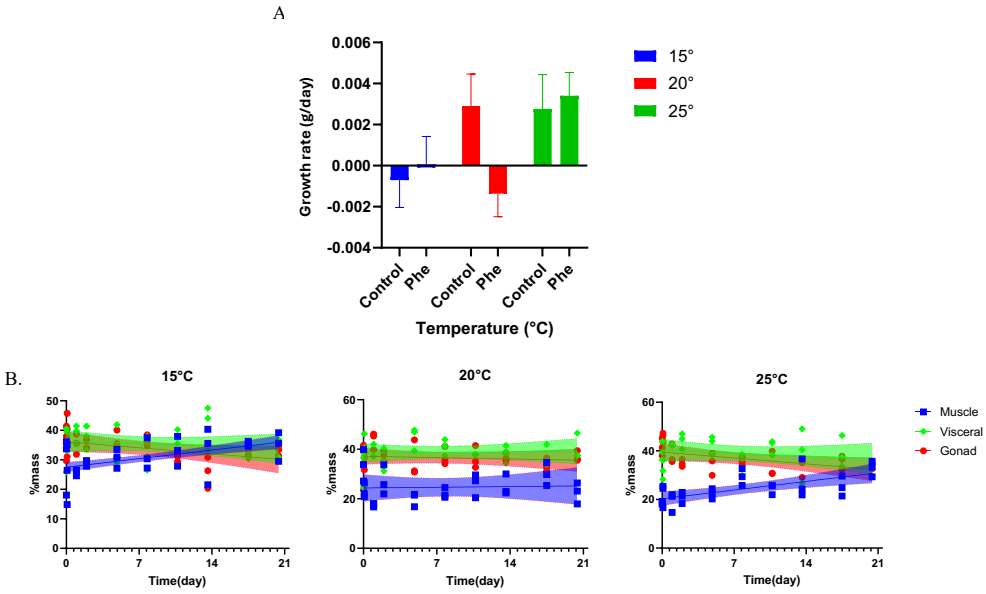



**Figure 2.5. Testing growth of snails over 3 weeks based on (A.) body mass with shell and (B.) specific tissue wet masses.** Error bars are standard deviation in (A.) and individual values are plotted along with least-squares linear regression over time in B., although in no cases were the slopes significantly different from zero (p>0.05). 4 snails were measured at every sampling point, and the concentration of phenylalanine is 150μM with 50% D5-Phe. Water was changed every day to ensure the maintenance of high concentration of phenylalanine. Snails were blotted dry with kim-wipe before weighing on an analytical balance (Denver Instruments, accuracy to 0.0001g). Raw data in A. were analyzed using one way ANOVA (α=0.05) without repeated measurements when considering each time points are a separate set of replicates.

*3) Determining D5-Phe dynamics and estimates for $k_s$ and $k_d$ at 20°C*

Figure 2.6A shows the tracking of the isotope $D_5$-Phe in vivo after the exposure via water bathing strategy. The free amino acid pool enriched shortly after the start of this experiment and this high level of isotopes maintained for the first 12 hrs (in figure 2.6B). With the assimilation of this large dosing supply of $D_5$-Phe, the protein pool gradually filled with this isotope via the process of protein synthesis and the turnover time allowing the protein pool to be near equilibrium with the free pool enrichment after approximately two weeks.

The $D_5$-Phe in both pools failed to reach 50% enrichment for the two-week-experiment that likely indicative of dietary phenylalanine contribution. By checking the rate constants in table 2.2 with figure 2.6, the calculated protein synthetic rate with its correspondence protein degradation rate gradually skews to the right with switched parameters from free pool to protein pool dependent. The fractional rate of synthesis that was determined using 12hr-data where the protein-bound pool enrichment increased linearly only calculated the instantaneous assimilation of isotope. Noticeably, there was high variance for both the free pool and the protein pool, which could be due to among individual variation, but may contribute to the dynamic equilibrium between pools, so that the enrichment is labile.

**Table 2.2. Sum of rate constant based on varied calculations.**

| Reference | Rate constant | Muscle | Visceral | Gonad |
|---|---|---|---|---|
| Plateau of %free pool enrichment (*Sa*) | *Sa* (%) | 8.36±3.19 | 7.42±3.40 | 6.81±3.10 |
| | *ks* | 0.59 | 0.70 | 1.30 |
| | *kd* | 0.07076 (0.063-0.079) | 0.09384 (0.079-0.111) | 0.1914 (0.15-0.245) |
| *ks* calculated as fractional rate from first 12hr-data | *ks* | 6.12±1.98 | 4.25±1.22 | 12.47±5.96 |
| | *kd* | 2.455 (1.937-3.320) | 1.418 (1.119-1.907) | 3.192 (2.490-4.395) |

Note. The unit for rate constant is %/day. Brackets enclosed the 95% confidence interval.

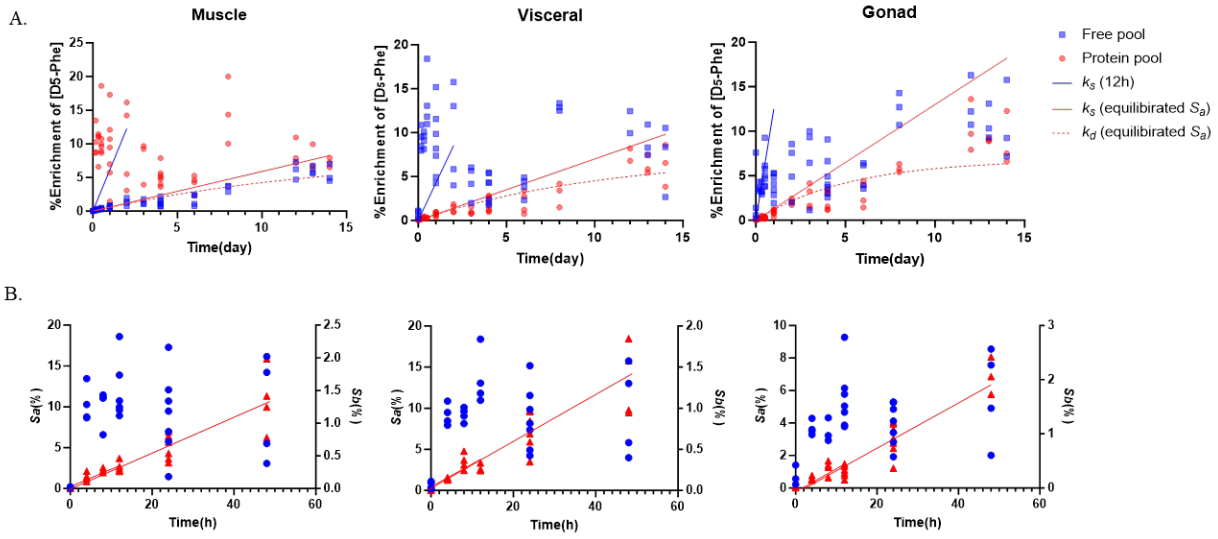



**Figure 2.6. The calculated enrichment of the isotope D5-Phenylalanine overtime for both free amino acid pool ($S_a$) and the body protein pool ($S_b$) for different tissue types in two weeks (A) with detailed 48hrs data (B).** N=3 snails per each sampling point with duplicates plotted in one figure (i.e. one-week-trial with 2-week-trial). 150μM of phenylalanine with 50% $D_5$-phenylalanine was added in the water where snails were sat and water changed every second day to refresh the isotope pool available for the snails. The enrichment of $D_5$-phenylalanine was computed via GC-MS with equation $D_5$-phenylalanine/($D_5$-phenylalanine+phenylalanine). Value of $S_a$ at equilibrium was collected via integrated mean value of the free pool data. $k_s$ (12h) was calculated based on the result of $S_a$ and $S_b$ within first 12hrs. Lines in set B indicates to least-squares regression of protein pool enrichment in 12hrs and extended to 48hrs.

*2.7.2 Thermal test on protein synthesis*

Based on figure 2.6 and 2.7, convergence between the free and protein pools, indicating approaching equilibrium between these pools, appears to occur for most tissues except under 15°C. The non-overlapping 95% confidence interval for $K_d$ in both muscle and visceral mass emphasize that *P. duryi* snails are more sensitive to colder environment in contrast with increased temperature; reproductive tissues remained to occupy fixed energy under variant temperatures (Table 2.3).

**Table 2.3. Sum of rate constant based on varied calculations for thermal test.**

| | | *Sa* (%) | *ks* | *kd* |
|---|---|---|---|---|
| Muscle | 15°C | 25±18.65 | 0.459 | 0.01836 (0.016-0.021) |
| | 20°C | 20.78±7.78 | 0.61343 | 0.02952 (0.027-0.032) |
| | 25°C | 24.53±12.08 | 0.71996 | 0.02935 (0.026-0.033) |
| Visceral | 15°C | 15.1±5.48 | 0.59237 | 0.03923 (0.034-0.045) |
| | 20°C | 14.12±2.62 | 0.78338 | 0.05548 (0.047-0.065) |
| | 25°C | 13.29±3.01 | 1.0212 | 0.07684 (0.065-0.09) |
| Gonad | 15°C | 11.49±7.79 | 0.921 | 0.08021 (0.064-0.101) |
| | 20°C | 12.33±5.08 | 1.26013 | 0.1022 (0.082-0.128) |
| | 25°C | 11.85±4.57 | 1.43741 | 0.1213 (0.095-0.157) |

Note. The unit for rate constant is %/day. Brackets enclosed the 95% confidence interval.

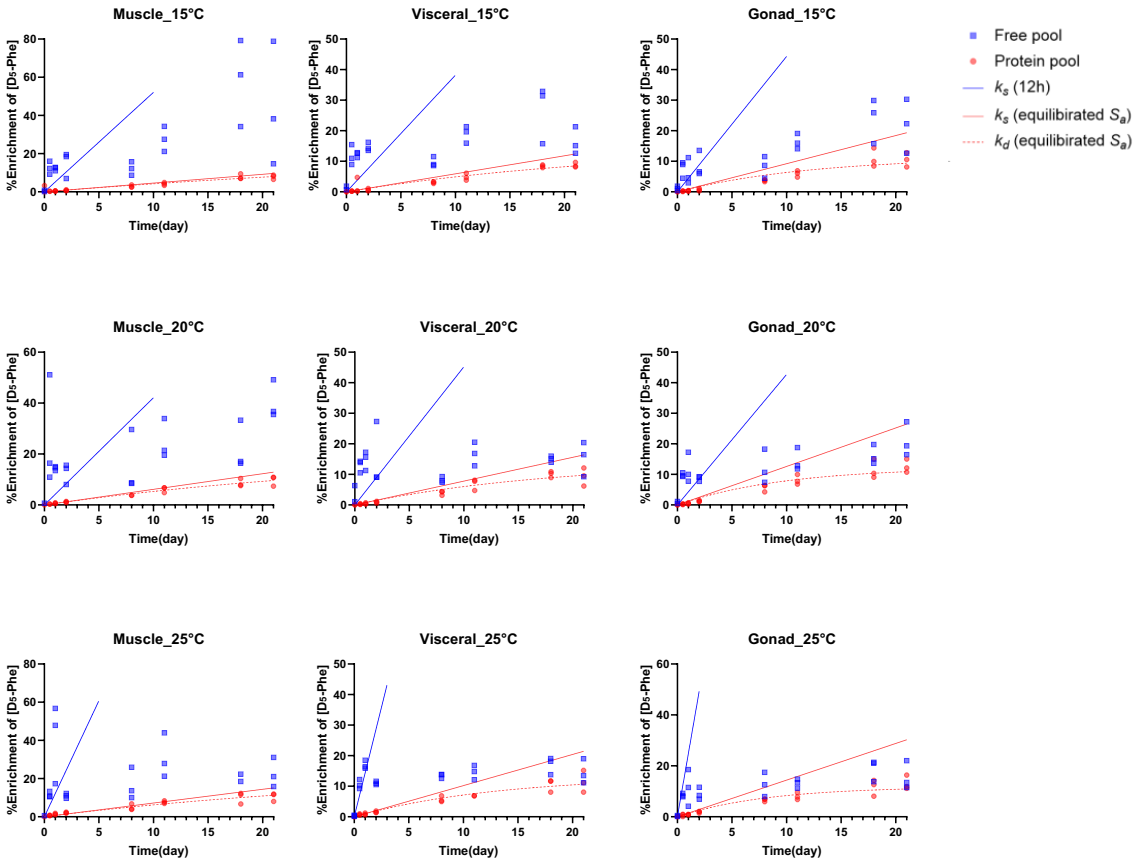



**Figure 2.7. The calculated enrichment of the isotope $D_5$-Phenylalanine ($D_5$-Phe) overtime for both free amino acid pool ($S_a$) and the body protein pool ($S_b$) for different tissue types in three weeks.** N=3 snails per each sampling point. 150μM of phenylalanine with 50% $D_5$- phenylalanine was added in the water where snails were housed and water changed every day to refresh the isotope pool available for the snails. The enrichment of $D_5$-phenylalanine was computed via GC-MS with equation $D_5$-phenylalanine/($D_5$-phenylalanine+phenylalanine). Value of $S_a$ at equilibrium was collected via integrated mean value of the free pool data. $k_s$ (12h) was calculated based on the result of $S_a$ and $S_b$ within first 12hrs.

## 2.8 Discussion

### *2.8.1. Design of isotope experiment*

The results shows that the experimental design had no apparent factors that potentially can bias the experiment under all temperature settings since the degradation of dissolved isotope can be solved via periodic (1 or 2 day) replacement of the D5-Phe in the bathing water. The frequency of this repeated exposure has no effect on the body pool isotopic enrichment in the free pool (Fig. 2.3), so the administered dosage might have reached the maximum assimilation of the body system. Because whole body growth was not measurable even over the 3-week-acclimation experiment (Fig 2.5), the protein pool size can be assumed to be similar, or the change is indiscernible, over the course of the protein metabolism experiments. Thus, bathing in 150μM Phe with 50% $D_5$-Phe does not measurably alter growth dynamics within the precision of our measurements.

### *2.8.2. Isotope methodology: determination of protein turnover rate constants $k_s$ and $k_d$*

Following the incorporation of $D_5$-Phe in the body system, the enrichment of isotope fails to reach 50% in the free pool. Thus, the lower enrichment of phenylalanine intracellularly compared to the $D_5$-Phe in the water indicates either isotope dilution, as the animals are still feeding, or low uptake, the latter of which isbe consistent with the low rate of protein synthesis as well as degradation in *P. duryi*.

When the $k_s$ is calculated following the typical short-term (hours) analysis of incorporation, in this case assuming the first 12hrs of this experiment fits closest to timing of a flooding dose method, the protein synthesis rates appear much faster than the long-term estimates of $k_s$ (Table 2.2). Hence, the previous methods commonly employed for testing the rate of protein metabolism were likely overly simplistic, thereby neglecting the synthesis of amino acids into the protein pool chronically and merely including the absorption of tracers into the body in the calculation. Having shown that the short-term (12 hrs) $k_s$ is much too high to match the long-term estimates needed for determining $k_d$, it is clear the widely used assumption that a short-term approach to measuring $k_s$ combined with long-term protein pool changes to estimate $k_d$ may be inappropriate (Fig 2.6A). Though it is true that these faster short-term estimates compare well to other aquatic ectotherms like Atlantic salmon (whole body $k_s$=3.3%/day) and rainbow trout (whole body $k_s$=4.4%/day) estimated via the use of $^3$H-Phe (Houlihan et. al., 2001). It is hard to conclude that this assumption is widely useful for other species without further tests using other organisms.

Although the initial experiment showed that the free and protein bound pools of phenylalanine approached a common enrichment of D5-Phe after two weeks (Fig 2.6A), which would indicate equilibrium between these pools of phenylalanine, the results of the three-week acclimation study were less clear, especially in the 15°C animals (Fig 2.7). While the long-term estimates of $k_s$ and $k_d$ in the colder group are still be comparable in general to the other

acclimation groups, the slower rates of protein metabolism in the 15°C animals failed to approach equilibrium between the free and bound pools of phenylalanine but the snails acclimated to 20°C and 25°C were approaching the equilibrium end-point between free and bound pools in most tissues after three weeks (Fig 2.7).

The protein metabolism rates of among the tissues differed, and this is presumably associated with the functions they perform (table 2.2). The rate in muscle tissue is the lowest among them, followed by visceral mass in the middle, and gonad has the highest proportion, signifying that protein metabolism is of greater significance in the gonad for snails than additional somatic growth. This finding is also consistent with previous studies in which allowance of early reproduction could reduce the rate of growth (Norton & Newman, 2016).

*2.8.3. Thermal test on protein synthesis*

Few apparent changes are observed for the fractional rates of protein synthesis, with gonad increasing between 20 and 25°C. With estimates of protein degradation, the non-overlapping 95% confidence intervals suggest that degradation rates are temperature sensitive in muscle as well as visceral mass (Table 2.3), indicating that some temperature sensitivity of protein metabolism was seen even with acclimation. However, overall, most rates showed limited absolute differences well below the anticipated $Q_{10}$ of 2-3 (DeWhatley & Alexander, 2018). The natural habitat for *P. duryi* is the Southern United States and does not include Canada, although they can be found in Canada as an exotic species; the furthest invasion of this species has been documented in Central Siberia, indicating their capacity to adapt to new environments (Johnson et al., 2013; Nekhaev et al., 2024). Three-week-trial has the same pattern between the protein degradation rate among tissues further supported the assumption that acclimation led to some compensatory responses limiting the impact of stress from changing thermal environments.

## 2.9. Conclusion

The introduction of isotopes via water bathing is an effective means to introduce isotopically labelled phenylalanine, and the enrichment indeed can be kept chronically stable *in vivo* long enough to assess protein degradation and synthesis rates in *P. dury*i. The current results indicate that assuming short-term $k_s$ values combined with protein growth dynamics to determine degradation rates should be done with caution. The isotope experiment and the thermal test experiment has no statistical differences on the enrichment of $D_5$-Phe in the free pool, thus the thermal test has minor approvements on the method steps, meaning that current methodology is reproducible although variation between individuals was high.

Overall, this set of experiment opens the view to the application of isotopes toward chronical protein metabolism investigation despite that many steps need further improvement to address individual variation in responses as well as to clarify the timeline.

## 2.10. Appendix

*2.10.1 Growth estimation in companied with thermal test*

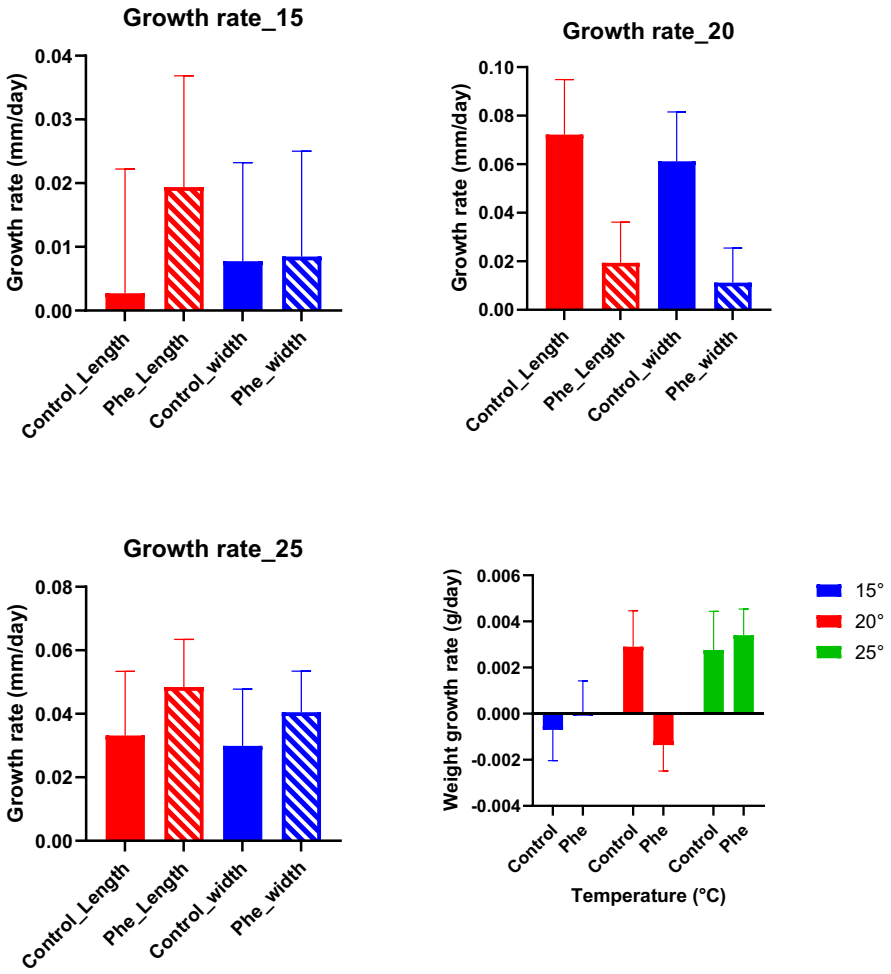



**Figure 2.9. The growth rate measurement of snails based on different parameters in 21days.** Each jar contains 4 snails, and the concentration of Phenylalanine is 150μM with 50% D5-Phe. Water was changed everyday to ensure the maintenance of high concentration of Phenylalanine. Snails were damped dry before estimation using an analytical balancer (Denver Instruments, accuracy =0.1mg). Error bars in figures indicate to standard error. Data was analyzed using one-way ANOVA (α=0.05) without repeated measurements when considering each time points are a separate set of replicates.

# Chapter III. Contribution of protein synthesis to metabolic activity in embryonic *Planorbella duryi*

## 3.1 Abstract

Climate change can introduce heat damage to snail body systems with emphasis on the impact to embryogenesis because eggs lack effective strategies for temperature defenses. Protein metabolism, as responsible for both development and growth, in together with possible repair of cell damage when subjected to change can be considerably energy costly under current situation. With the assistance of novel open-chambered respirometer Resipher, this chapter intended to integrate the cost of protein metabolism in relating to oxygen consumption with cycloheximide as pharmacological inhibitor protein synthesis. Other inhibitors were used in the experiment as means to validate the application of the Resipher. Under regular circumstances at 20℃, Resipher successfully achieved the experimental goals for most inhibitors. Eggs at middle life stage marked from late trochophore to early veliger stage consumes 0.2952pmol/s $O_2$. Protein metabolism occupies 47% of mitochondria-sensitive aerobic respiration, and the capacity of oxidative phosphorylation estimated via uncoupler CCCP is 105% higher than the regular state. Oligomycin fails to reactive steadily as to inhibit the ATP synthase located on the electron transport chain. Overall, Resipher is appropriate to address the oxygen consumption rate during embryogenesis.

### 3.2 Contribution

The author thanks to Lucid lab. Inc. for the assistance on the manipulation of Resipher and offering technical supports. The experiment was established based on the primary idea from Dr. Jason Treberg with major methods achieved by the author Chenyi Wang.

### 3.3. Introduction

Embryonic development for all animals requires the balancing of many metablic needs including rapid cellulr division and growth. Because embryos are under strain to develop quickly, alterations in their energy metabolism may be critical constraints and therefore the amount of metabolism directed towards protein metabolism is of particular interest at early stages of development since production of new protein is known to be energetically costly. Protein acts as both a fuel and the source of amino acids for cellular growth throughout embryogenesis (van den Biggelaar, 1971). Although widely recognized as important, there are very few measurements of how much protein synthesis contributes to overall energy budgets in developing ectotherm and with aquatic invertebrates being particularly understudied given their ecological importance. In sea urchins, the metabolic cost of protein synthesis during embryogenesis is up to 54 ± 8% (Pace & Manahan, 2006); 54-59% of total energy assimilation was consumed as protein synthesis in Pacific oyster larvae (Lee et al., 2016). Thus, protein synthesis itself can occupy approximate 50% of total oxygen consumption, and therefore protein metabolism is a major component of overall energy requirements however further species should be evaluated including those from freshwater environments.

Freshwater snails present an excellent group to study embryonic metabolism. These snails lay their eggs in a jelly-encolsed layer by means of protection within water. Embryo cells start to proliferate after the deposition of eggs, and this marks the morula stage. Blastomeres form afterward and initiate proliferation and differentiation prior to trochophore stage. Followed by mass cellular differentiation and specialization as well as secretion of shell, embryos growth centralizes on cleavage to generate organs during late trochophore to early veliger stage. During these stages, perivitelline fluid serves as energy supply that stores most nutrients (Bayne, 1966). Snail embryos enter the veliger stage at about 60hrs to 72hrs. At this stage, embryos gradually display the typical larval structure that bares shell and mantle feet: eyes are visible and heartbeat appears at day 5 (Boon-Niermeijer & van de Scheur, 1984; van den Biggelaar, 1971). Many snail species start to swim in the eggs, but the size of the embryo remains small. After 7 days post-deposition, embryos enter the hippo stage prior to hatching when metamorphosis ends indicated by the ind urated shell and expanded body size which fills the egg sac.

Evaluating bioenergetics in embryonic ectotherms such as developing snails is challenging because of their small size and fragile physiology. However, because the animals are obligate aerobes and must still respire, oxygen consumption rates (OCR) can be used as an indirect measure of metabolic activity. (Thornton, 1917). By using specific inhibitors of metabolic processes, the relative change in OCR can inform on cellular energetics, as has been shown for intact hepatopancreas cells isolated from the land snail *Helix aspersa* (Bishop and Brand, 2000) This chapter investigates the aerobic metabolic activities by measuring the OCR using a multi-well open respirometer to test for the relative contribution of protein synthesis to the overall energy metabolism of embryonic *P. duryi*. To do so, first optimization and validation of this open respirometer with the embryos was conducted to determine an appropriate stage of development

to investigate. Following these optimization assays, pharmacological manipulations were optimized to provide a quantitative estimate of how much of the OCR was committed to protein synthesis.

### 3.4. Hypothesis

The number of mitochondrial complexes in relating to aerobic activities should be proportional to $O_2$ demand in which inhibiting properties on protein metabolism can reduce $O_2$ consumption rate (OCR). Under different temperature, these actions should exacerbate its effect on aerobic activities since snails belong to ectotherms.

### 3.5. Objectives

1). Establish proper method for using the Resipher respirometer to quantify mitochondrial-related energy activities with emphasis on protein metabolism.

2). Utilize this developed methodology to investigate the effect of temperature change on the protein metabolism of snails at embryonic stages.

### 3.6. Methods

#### *3.6.1. Animals*

Snails were raised at a temperature of 20°C in aerated, recirculating tanks with 70% water exchange every week to remove feces and other wastes. Lettuce was provided twice per week as food and cuttlefish keels were added as supplementary calcium sources. Polyethylene bags weighted with ceramic pestles to keep then sunk and in place over the bottom of the tank were placed in each tank for egg collections. Eggs were then placed into petri dishes containing tank water from their respective tank. As the initial experiment was to categorize OCR over embryonic development, three stages intending to capture major events were used. Eggs at morula and trochophore stage were marked as the early experimental group. Middle test group contained snail eggs at late trochophore to early veliger stage. Late life stage referred to eggs that nearly hatched (late veliger to hippo stage) (refer to the appendix I for detailed growth of snails). Revamping and determination of life stages of egg clusters were manipulated under 10x with a stereo-microscope prior to the start of experiments.

#### *3.6.2. Use of Resipher as respirometry equipment*

##### *3.6.2.1. Instrument setup*

Resipher was used in this experiment as an open-chamber respirometer using 96-well plates as the base and top sensing lid where a fluorescent-sensor that measures oxygen concentration is attached to a post that hangs within the test well (refer to figure 3.1A for detailed setting). The sensor lid moves vertically within the column of liquid medium covering the biological material at the bottom of the well. Thus, as the cells at the bottom of the well consume

oxygen this instrument can convert the pressure gradient ($pO_2$) generated along the height of the culture medium to an $O_2$ consumption rate (OCR) that can refer to the mitochondria $O_2$ flux *in vivo*.

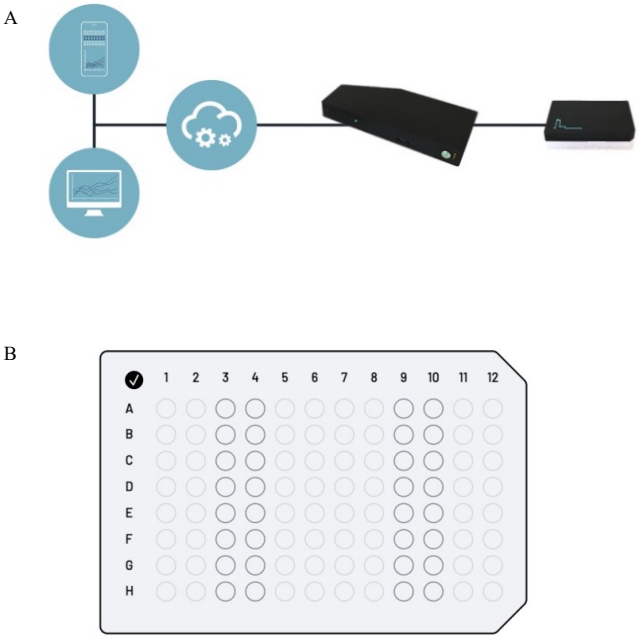



**Figure 3.1. Brief illustration to the Resipher instrument as an open-chamber respirometer.** Figures were collected from Lucid lab manual on instruction (from https://www.lucidsci.com/docs/LucidScientific_Resipher_Guide.pdf). A indicates to the general protocol on the use of Resipher where data collected from the hub can store in cloud storage and view online. B is the mapping view of one experiment in which 32 wells can be used over 96-culture well plate.

*3.6.2.2.Culture plate setup*

Falcon 96-well-culture plate (flat round bottom with diameter = 0.33cm, code 353072) was selected for this experiment and 32 wells were available for work despite that water filled to every well to increase humidity as well as to eliminate evaporation during the test as shown in figure 3.1B. Culture media used in this experiment was 200µL filtered tank water from the snails original tank environment (filter size = 0.22µm) to ensure the bathing environment for eggs was the same as that over embryonic development but has removed bacteria and other particulate. Egg clusters were peeled from the plastic by gently folded the bag and transferred to settle against the bottom of the well. Gravity and surface tension between the egg sac and the culture plate held the egg sac at the bottom of the well, which is sufficient to resist disturbance from the measurement lid. Egg numbers were confirmed once the egg sac had settled on the bottom of the well and each well contained a unique egg cluster revamped from one egg sac. The culture plate and reading device lid were housed in an incubator to minimize temperature deviations over the course of the experiment.

### *3.6.3. Establishment of the proportionality of $O_2$ flux is a function of embryo number and developmental stage*

This series of experiments used embryos at all three life stages to measure a routine OCR overtime. Each culture plate well is considered an experimental unit and experiments were manipulated using multiple culture plates to remove confounding errors. 66, 43, 14 culture plate wells as experimental units were used respectively from early to late test group with each well containing one of 4, 6 or 8 eggs enclosed in a sac; every experimental trial lasted for 20hrs to generate sufficient data.

After showing proportional OCR with added number of eggs, toxicity experiments to optimize inhibitor concentrations used 6 eggs per plate well at the middle life stage to ensure a detectable $O_2$ flux during the test.

### *3.6.4. Application of pharmacological agents*

#### *3.6.4.1. Rationale behind OCR measurements to determine protein synthesis costs*

Figure 3.2 illustrates the rationale behind the series of pharmacological additions, which in all cases started with measuring a 2-hr-baseline OCR which becomes the routine rate for the reference relative to the response of the respective pharmacological agent. Inhibitors were dissolved in Dimethyl sulfoxide (DMSO) to avoid effects of ethanol or other metabolizable solvents. Uncoupler Carbonyl cyanide m-chlorophenyl hydrazone (CCCP) was introduced to test mitochondrial maximum OCR; cycloheximide used in this interferes with mRNA translational elongation, which subsequently inhibits protein synthesis. The incubation of these additional inhibitors lasted for 5hrs to ensure fully reaction, indicating as the plateau of OCR. Myxothiazol and rotenone were then added in the end to fully block the function of electron transport chain (Bishop & Brand, 2000). Therefore, the remaining detectable $O_2$ flux is considered non-mitochondria and is subtracted from all other rates. Additional inhibitors like oligomycin were

enrolled to try to estimate the contribution due to proton leak by means to validate and evaluate the use of the instrument.

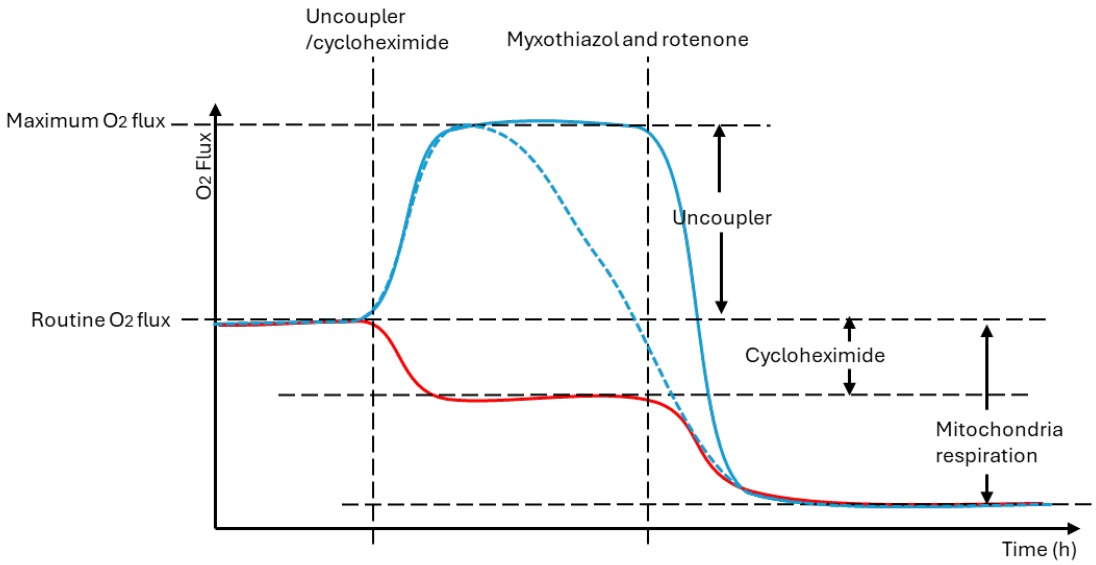



**Figure 3.2. The schematic view of the toxicity test.** The experiment started with a 2-hr-establishment of baseline, acting as the positive control for each individual test groups. Additional inhibitors were added afterward to disintegrate possible hallmarks enrolled in mitochondria respiration. The effect of cycloheximide should indicate the proportion of routine Oxygen flux due to protein synthesis. Elevated O2 flux after the application of uncoupler is expected as indicated by the solid blue line, however, in many systems optimized uncoupling concentrations tend to have a time-dependent decline following the maximal stimulation after the addition of the uncoupler. (illustrated as the dotted blue line). Additional of myxothiazol and rotenone can block the electron transport chain, and the rest of the detectable activities from the non-mitochondrial oxygen consumpiton.

**Table 3.1. Population size and replicates for toxicity test.**

| | Dosage | # of culture plate wells |
|---|---|---|
| DMSO | 1-8μL | 4 |
| Myxothiazol and rotenone | 0.5:2μM – 2:8μM | 3-8 |
| CCCP | 0.25-2μM | 5-17 |
| Oligomycin | 0.158μM-404.5μM | 8 |
| Cycloheximide | 0.1-10mM | 11-14 |

The appropriate dosage of the application of these inhibitors are essential to achieve the goal in which the desired cascades are completely restricted, but no apparent side effects are triggered that exacerbate the result or lead to early defeat. To achieve this, a discrete concentration of inhibitors was tested in sequence and results were evaluated as well as the effect

of sole DMSO to the body system. The population sizes varied between inhibitor types with more than three culture plates as replicates as detailed in table 3.1.

*3.6.5. Statistical analysis*

Data on OCR were collected using the Resipher built-in software, which is programmed to analyze data assuming $O_2$ solubility at 37°C. The collected data required a correction using a scale factor to correspond with the actual experimental temperatures (please refer to the appendix for programmable scale factor generated by Resipher lab). Statistical analyses were done on temperature corrected rates of OCR using GraphPad 10.0.

The linearity of data from the test of OCR in relation to number of eggs in the well was checked using linear regression for all stages of development. Then the OCR per egg number was calculated accordingly, and the results between life stages were compared using one way ANOVA ($\alpha$=0.05). To assist structuring of the toxicity data set as well as to remove noises brought by sample variances, the 95% confidence interval was calculated using this routine OCR with linear regression; individuals that were away from this data range were excluded (2.84pmol/s> OCR>0.2373pmol/s) and outliers were removed (ROUT method with Q = 1%).

To account for variations between clusters of eggs, the effect of inhibitors on mitochondria respiration data were normalized according to the initial routine value of OCR. This change in percentage based on the rule in which the routine measurement was set as 100% and the negative control was 0%. For most type of inhibitors, this formula assumes 100% to be the referenced OCR estimated at the initial 2hrs and 0% was fixed to the OCR captured at Time = 15h when the rest measures the OCR from non-mitochondrial works. In the myxothiazol and rotenone test, where no additional inhibitors were added, 100% inhibition was set similarly to the other toxins, but then 0% indicated that OCR was equal to 0.

3.7. Results

*3.7.1. Validation of the routine O2 flux*

Refer to figure 3.3A, Although there was substantial variation, the OCR was found to increased proportionally with the number of eggs in the well. Moreover, the embryonic developmental stage had a marked effect on the OCR, with early embryoes having a much lower $O_2$ demand than the middle and late stages and the late stage having approximately 30% greater OCR than the middle stage (Fig. 3.3B). Because the early stage had OCR too low to allow for much accuracy to measure inhibitor effects and late stage eggs have increased variances that may contribute to the extent of mobility of snails. As the most consistent stage, with sufficently high OCR, the middle stage of development was selected for the remaining experiments.

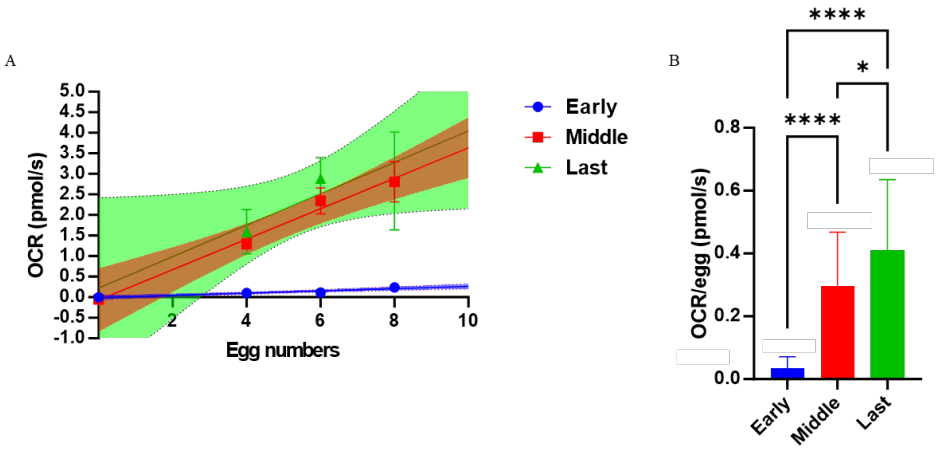



**Figure 3.3. The Oxygen consumption rate (OCR) of snail embryos at different embryonic stage (A) with unit rate calculation in B.** An open respirometer made by Lucid lab was used to collect the OCR data. Embryo sacs were cut open to fix the egg numbers. Early stage referred to morula to trochophore; middle was late trochophore to veliger; last stage was late veliger to hippo. Error bars shown on the graph is SEM. Experiments were replicated on multiple culture plates with N>8 culture wells for every experiment. Lines indicate a linear regression analysis of each life stages and extend to x=0. Asterisk (*) indicates to the result of one-way ANOVA using GraphPad (α=0.05) where **** means P≤0.0001.

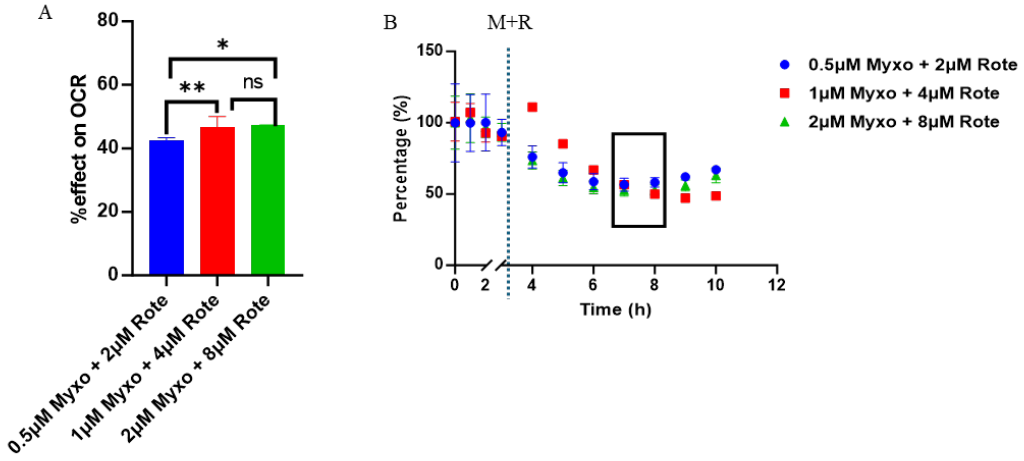



**Figure 3.4. The Oxygen consumption rate (OCR) of snail embryos with the addition of myxothiazol and rotenone at different concentrations.** Date presented in normalized form (B). An open respirometer made by Lucid lab was used to collect the OCR data. Embryo sacs were cut open to fix the egg numbers to be 6. On triplicate plates, 0.5uM myxothiazol and 2uM rotenone had 3 culture wells, 1uM myxothiazol had 6 culture wells and 2uM myxo had 8 culture wells. Error bars shown on the graph is SEM. A is calculated using average data of B at T= 7-8h with formula (100- captured effective region in %) as enclosed in rectangular. Asterisk(*) indicates the result of a one-way ANOVA using GraphPad (α=0.05) where ** means P≤0.01.

*3.7.2. Determination of inhibitor dosages*

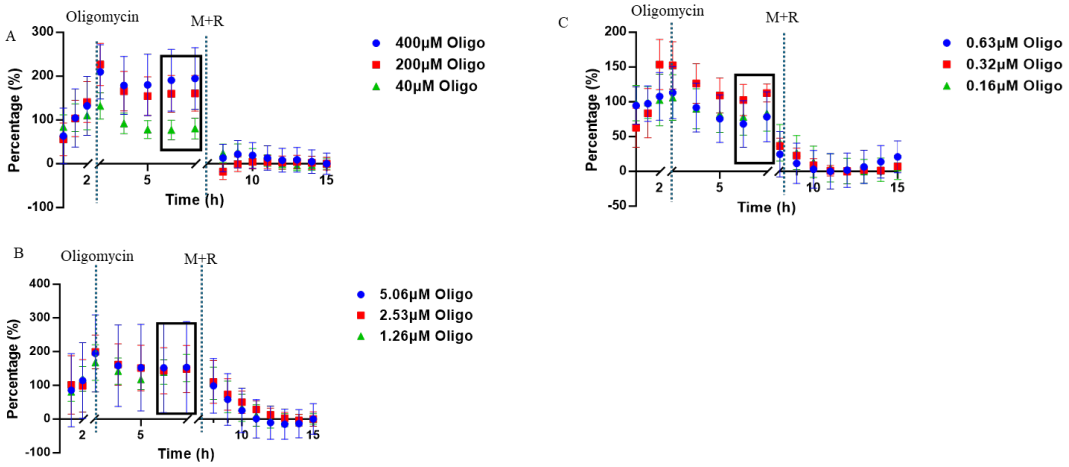
The effect of inhibitors to the body system varied by categories and their correspondence reaction time, which requires to be determined respectively. The predominant response, seen as a maximum response, was most clear after data normalization to each well's routine OCR for every chemical. Myxothiazol and rotenone reached the plateau at Time = 7-8h post exposure; uncoupler CCCP was functional at Time = 2h when the OCR reaches the peak; the responsive zone of oligomycin or cycloheximide was captured at Time = 6-7h relying on the reduction of OCR. Data at these time points were compared among all groups within each inhibitor types to determine the appropriate dosage for later thermal test and the %effect of these inhibitors on mitochondria activities were calculated to determine their energetic cost to the current system.

Although the majority of respiratory OCR was inhibited by the lowest dose tested, the minimum dosage to fully block mitochondrial electron transport was 1μM myxothiazol and 4μM rotenone (Fig 3.4A). Hence, 1μM myxothiazol and 4μM rotenone is sufficient to completely restrict the normal function of mitochondria-sensitive respiration. The rest of the detectable OCR includes the non-mitochondrial process and can be quantified as 53% by subtracting the mitochondrial part from the routine OCR.

Oligomycin was added intending to estimate proton leak by interfere with $F_0$ of ATP synthase as previously emphasized by the use with the hepatopancreas cells of snails (Bishop & Brand, 2000). However, the effect of oligomycin on the OCR is low as shown in figure 3.5. The rountine measurement that used average of all data within this 2-h-interval seems gives the appearance of an increasing trend before the addition of oligomycin; however, the was no significant difference over the time-points used to determine the routine OCR and thus the trend seen was ignored. The responsive curve of oligomycin immediately raised after the dosage, and reduced overtime. The end measurements remain to be higher than routine OCR (marks as 100% in normalized graphs) for most concentrations despite that of 0.632 and 0.158μM and the result has no consistency over at least two discrete concentrations other than to stimulate the system, in which fails to follow the expected inhibiting properties of oligomycin..

Other than the issues of oligomycin, the rest of inhibitors function following expectation. CCCP is a chemical uncoupler that leads to elevated $O_2$ consumption, but collapses the system in the end presumably via dissipation of the protonmotive force, ultimately impairing substrate uptake. Hence, the dosage of CCCP has to be carefully managed as any concentration beyond the appropriate range can lead to malfunction of mitochondria rather than acelerating the OCR. As seen in figure 3.6, this experiment result narrows the functional zone of CCCP to between 1.25μM to 0.25μM, and 1μM CCCP consumes most abundant of $O_2$ where the OCR is raised to 105% compared to the basal respiration rate. Thus, 1μM of CCCP is considered as maximized uncoupling effect in this experiment.

Cycloheximide is commonly used to arrest mRNA translation and in turn protein synthesis, in which reduces the ATP cost as well as OCR. Thus, the change of OCR can reflect the amount of oxygen flux commited to protein synthesis. As referred to figure 3.7, 0.1mM of cycloheximide failed to prevent protein synthesis as the OCR was inhibted but remained stable; 10mM cycloheximide has the largest reduction on $O_2$ consumption to 73% but failed to stablize indicating an inappropriate amount of this inhibitor. . Also, the pattern of this response curve is similar to that of 2μM CCCP in figure 13A in which the $O_2$ flux continues to drop and approach closely to 0% till the end of the reaction region, raising doubts that this high dosage of cycloheximide might interfere with other system components such that cycloheximide can reduce lipid absorption via lymphatic pathways in parallel with its major prohibiting function to protein synthesis (Al Nebaihi et al., 2023). Thus, higher concentration of cycloheximide potentially can accelerate system defeat, leading to an inaccurate estimation of the cost of protein synthesis. The responsive curve of 1mM cycloheximide has a relatively flat region that occupies 44% of mitochondria-sensitive respiration starting from 5h to 7h prior to the addition of




myxothiazol and rotenone

**Figure 3.5. The Oxygen consumption rate (OCR) of snail embryos with the addition of oligomycin at different concentration.** Data presented in normalized form (A, B and C). Normalized 0% for graph A referred as the averaged data at Time=15h, that of B and C was the smallest value after additional Myxothiazol and rotenone. An open respirometer made by Lucid lab was used to collect the OCR data. Embryo sacs were cut open to fix the egg numbers at 6. On triplicate plates, every dosage of oligomycin had 7 culture wells except 5.06μM had 5 wells. Error bars shown on the graph is SEM.

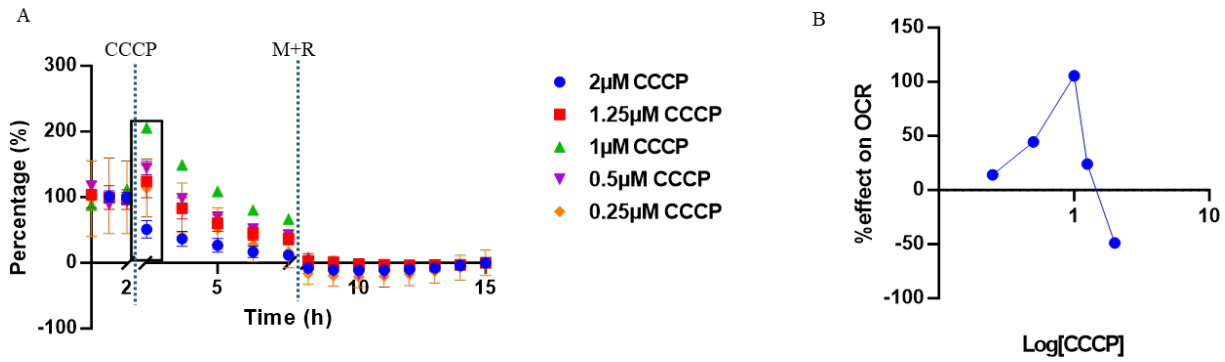



**Figure 3.6. The Oxygen consumption rate (OCR) of snail embryos with the addition of CCCP at given concentrations**. Data presented in normalized form (A). An open respirometer made by the Lucid lab was used to collect the OCR data. Embryo sacs were cut open to fix the egg numbers at 6. On triplicate plates, every dosage of CCCP had 5- 17 culture wells. Error bars shown on the graph is SEM. B is calculated using average data of A at T= 7-8h with formula [100- captured effective region in %] as enclosed in rectangular**.**

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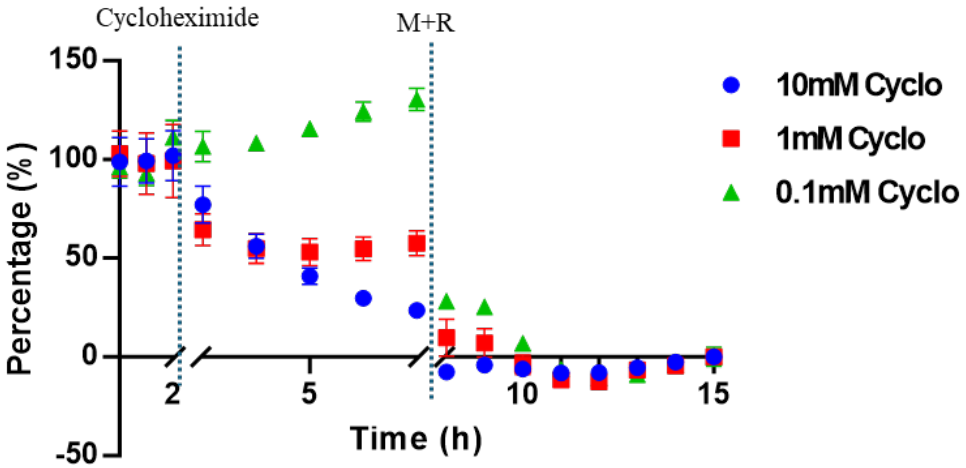


**Figure 3.7. The Oxygen consumption rate (OCR) of snail embryos with the addition of cycloheximide at different concentrations.** Data presented in normalized form. An open respirometer made by the Lucid lab was used to collect the OCR data. Embryo sacs were cut open to fix the egg numbers at 6. On triplicate plates, every dosage of cycloheximide had at least 11 culture wells. Error bars shown on the graph is SEM**.**

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## 3.8. Discussion

### *3.8.1. Validation of routine OCR*

Differentiation of  cells and changes in morphology occupy a large portion of energy demands via mitochondria during growth with clear difference based on developmental stage (figure 3.3). When comparing the OCR of early to middle stage and the last stage where major cell fates have been determined, rates in the early stage are much lower. This result is consistent with previous literature that stated most energy storage in the perivetillite fluid is transformed for use at approximate veliger stage when organs initially form (Heras et al., 1998). Other studies that used *Drosophila*, frogs (*Xenopus laevis*), fish and beetles also stated the peak use of energy either measured as OCR or heat dissipation targeted at the early development before gastrulation (Dunkel et al., 1979; Nagano & Ode, 2014; Rodenfels et al., 2020; Song et al., 2019); then the increase of metabolic activities may mark the end of the first differentiation that iniates the establishment of the major cell lineages. Hence, by comparing with the above literatures, the use of Resipher is validated to be appropriate to estimate OCR along embryogenesis for snail eggs since the activities that reflected as OCR is following the growth of embryos.

### *3.8.1. Application of pharmacological agents*

Mitochondrial-sensitive respiration achieved by the blockage of complex I and III can reduce the OCR to half of the regular state, which is consistent with isolated hepatopancreas cells from a land snail where non-mitochondrial OCR was 35% of the routine rate using only myxothiazol (Bishop & Brand, 2000). One potential confounding errors that could obey current proportion is the available of glycolysis as alternative way of ATP production. The degree of partitioning of glycolysis is hard to quantify under current experimental setting though the differentiation of mesenchymal stromal cells during human early neurogenesis mainly relies on glycolysis as way to generate adequate ATP (Guntur et al., 2018). But since snails prefer lipid as primary energy source in contrast with many organisms utilize Carbohydrates (Heras et al., 1998), the effect of glycolysis is considered low.

Most types of inhibitors functional as expected following their commonly known properties other than oligomycin that ought to restrict the generation of ATP via ATP synthase. Normalization is benefitical when considering to maximize the change brought by inhibitors, but on the other hand, it could potentially exacerbate the variaton of data as emphasized as the rountine estimation of oligomycin since only two trials out of the oligomycin-engaged experiments have unexpected fluctuation during routine OCR interval. Due to the lack of addtioanl effective strategy to exclude those two datasets, they were presented as current format. A recent study on mammalian cells found that at high concentrations oligomycin also has an uncoupling ability beyond blockage of ATP synthase, which could partially explain the result of this experiment (Hearne et al., 2020).

The calculated cost of protein synthesis that is mitochondria-sensitive is 54% out of total mitochondrial OCR, which is consistent with other organisms. Few studies can be found on the quantification of cost of protein metabolism and the results indicate that the general cost of protein synthesis is about 50% (summarized from Houlihan et al., 1992; Lee et al., 2016; Pace & Manahan, 2006). Some exception available as the cost of RNA, DNA as well as protein polymerization occupies only 10% out of total ATP consumption in *Drosophila*, but this result can be explained by either too primitive embryonic structure are presented at the stage of measurement (2-6h post fertilization), or cells as the estimating unit complicate the calculation (Song et al., 2019). Other researchers have documented that 20% of maternal protein was utilized during embryogenesis of barnacles, which accounts for 75% of total energy expenditure (Lucas & Crispt, 1987). In embryogenesis, the dry mass composition of protein in the Chilean scallop is 50% of total weight (Farías et al., 1998). This is much higher compared to that of the embryos of the snail *P. duryi* which occupies about 10-15% at the stage of development used here (Heras et al., 1998).

Overall, this experiment has identified some major energy combustion events in relating to mitochondria. Mitochondrial electron transport can be elevated by as much as 47% with uncoupler, and 54% of the aerobic respiration is subjected to protein synthesis. The maximum capacity of electron transport chain as referred to the peak $O_2$ assimilation brought by uncoupler CCCP is 205% to its regular size.

### *3.8.2. Thermal test on embryo $O_2$ consumption*

Temperature has a strong impact on the embryogenesis of snails and result in energy allocation of these tested major energy sources. Heat damages can result in the maldevelopment of snail eggs, starting from the retardation of growth caused by failure during morphogenesis; induced gene mutations ultimately can lead to early death of eggs (B.S. Khangarot & Sangita Das., 2010). After exposure to heat damages, eggs at morula to trochophore stages of *Lymnaea stagnalis* have the highest survival rate that was then vastly reduced to below 10% at late trochophore to early veliger stage (Boon-Niermeijer & van de Scheur, 1984). This result is consistent with the experimental observation as seen in the appendix figure 3.8. where the growth of acclimated 15°C embryos are extremely restricted so that the hatching requires doubled the time in comparison to the 20°C embryos. Hence, additional stresses such as thermal damages with inhibitors could potentially lead to early failure of the body system prior to measurement.

### *3.8.3. The use of Resipher to test mitochondrial activities*

One major downside of using open-chamber respirometer is that the instrument requires longer than other closed-chamber machines to equilibrate with $O_2$ supply and self-titrate the system for the next measurement when additions like inhibitors are applied via pausing the running system. This calibration usually takes approximate 1h, which leads to difficulties on data integration. Increasing temperature from 20 to 25°C may also increase the speed of entry of

inhibitors into body system. Again, the actual response curve could possibly be missing due to the self-correction of instrument, then only displays the gradual disspation of proton motive force beyond to the peak for uncoupler.

### 3.9. Conclusion

There are very few estimates of protein metabolism costs in developing ectotherm embryoes and in this chapter an open respirometer was used to estimate this in *P. duryi*. Measurements of OCR were found to be proportional to the number of embryoes added to the repirometer and developmental stage was clearly influential on the the observed OCR per embryo. Using the trochophore to veliger stage of embryoes it was possible to measure the routine, uncoupler induced maximum and cyclohexiamide-sensitive OCR in 20°C acclimated embryoes, indicating 30-50% of routine OCR is directed toward protein synthesis. Because further work on embryonic bioenergetics are warranted, the multiwell open respirometer design may be well suited for future studies on embryonic metabolism in small aqautic ectotherm species with the exchange of variable sized culture plates. This instrument works best with biological material with little to no mobility or are sessile to ease anchoring to the bottom of the culture plate wells as the way to generate $PO_2$ differences. Other than those experiments that aims to create standard for further experiment in which no additional manipulations are included after the initiation of the test, the time needs to be carefully addressed.

## 3.10 Appendix

### *3.10.1 Snail embryonic stage recording*

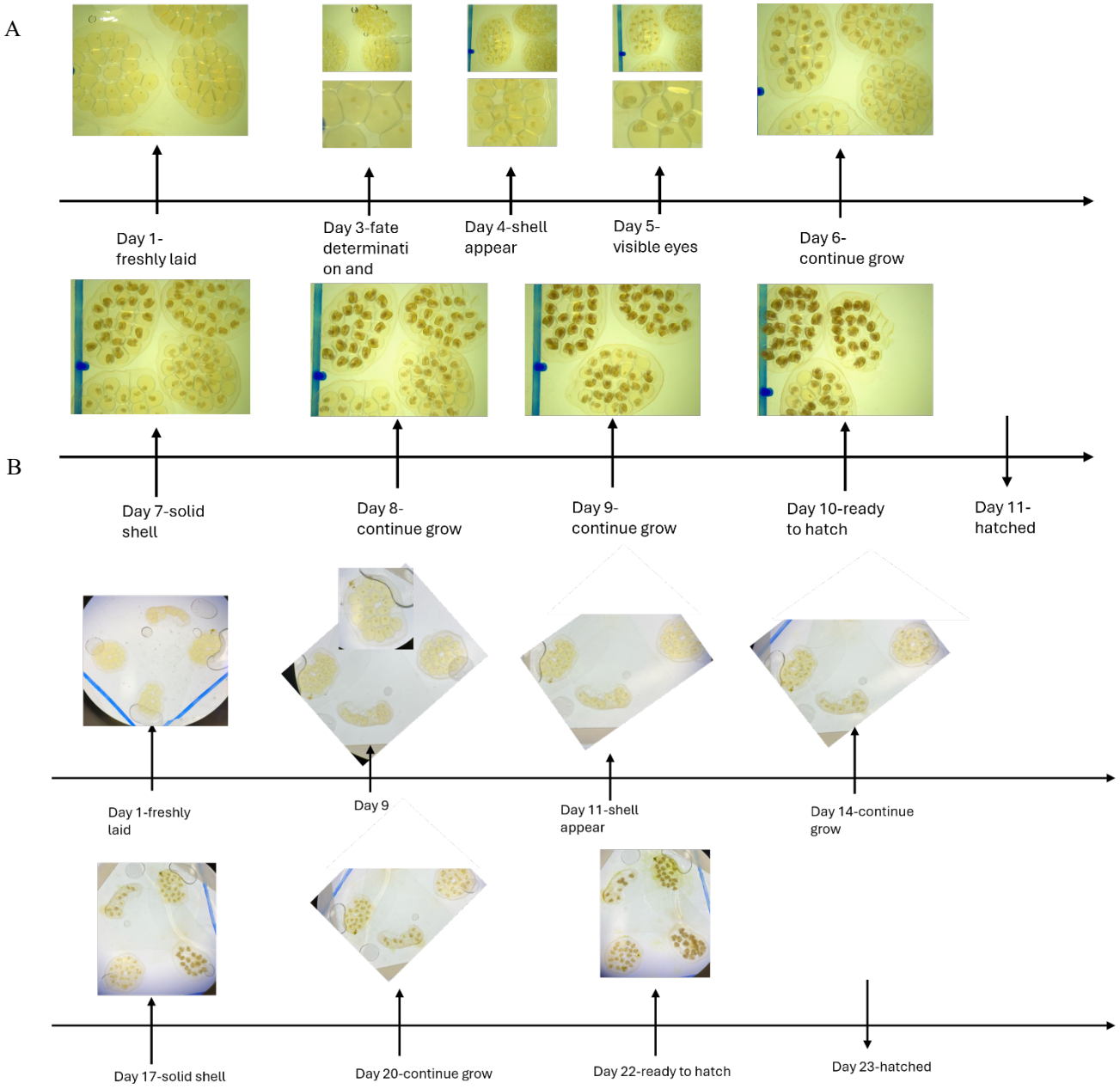


**Figure 3.8. Embryonic life stages for snail eggs.** Picture was obtained via microscope (10x) with attached Leica camera. Snails were housed at 20°C (A) or 15° (B) with filtered tank water. Pictures were turned to exhibit better resolution.

*3.10.2 Scale factor of resipher (established by Resipher Lab.)*

**Table 3.2. Scale factor for Resipher data conversion**

| temperature | scale_factor |
|---|---|
| 10 | 1.93 |
| 11 | 1.85 |
| 12 | 1.79 |
| 13 | 1.73 |
| 14 | 1.67 |
| 15 | 1.62 |
| 16 | 1.57 |
| 17 | 1.52 |
| 18 | 1.48 |
| 19 | 1.44 |
| 20 | 1.4 |
| 21 | 1.37 |
| 22 | 1.34 |
| 23 | 1.31 |
| 24 | 1.28 |
| 25 | 1.25 |
| 26 | 1.22 |
| 27 | 1.2 |
| 28 | 1.17 |
| 29 | 1.15 |
| 30 | 1.13 |
| 31 | 1.11 |
| 32 | 1.09 |
| 33 | 1.07 |
| 34 | 1.05 |
| 35 | 1.03 |
| 36 | 1.02 |
| 37 | 1 |
| 38 | 0.99 |
| 39 | 0.97 |

## Chapter IV. Conclusion

This thesis investigated protein metabolism in the freshwater snail *Planorbella duryi* with integration of the current isotope method for adults and use of OCR as reference to reflect the contribution of protein metabolism to aerobic energy expenditure. Overall, this snail species appears to be an excellent study organism because of its simplicity of body structure, and because it is an ectotherm it is subject to temperature change as well as being hermaphroditic such that no bias exists for sex differentiation though *Lymnaea stragnalis* snails have male organs develop first (Fodor et al., 2020). High variances are discovered during these experiments for both adult snails and embryos and part of this might contribute to phenotypic plasticity and the nature of the results such as acclimation to varying temperatures in adults.

Two major experimental mechanisms were developed. $D_5$-Phe was used as the tracer isotope to quantify protein turnover on a unified time sequence and the result suggest that the previous way to estimate these parameters via the flooding dose method could potentially exaggerate the actual rate of synthesis relevant to the protein degradation rate. Given the complexity of protein metabolism, especially when the estimated unit is not individual organelles, but rather a functional complex that is responsible for growth, reproduction and mobility; the interpretation to this result is feasible. Issues raised mainly contribute to the high variances brought by individual samples, which may contribute to phenotypic plasticity, but further study is warranted to address this. Protein transportation can cause some concerns as described in the introductory chapter; however this may not interfere with the embryogenesis as much as the adulthood but requires additional attention when interpreting the result. Multiple calculations are included when integrating the dataset to offer the assumed actual rate of protein metabolism in parallel with validation strategies. This study adds to the limited data on aquatic ectotherm protein metabolism, but additional study species such as fish or aquatic organisms where the addition of tracer to the environment would allow for comparison to the flooding dose method should be considered to further validate these findings as well as to expand the use of this methodology. Other than the shifted result comparing to literature consequences, the experimental setting can possibly be expanded to use in the field as the experimental apparatus is simple and free of radiation. The actual experimental settings and equipment might require additional fine-tuning based on specific experimental circumstances such as the size of the equipment where housing of the study organisms must be accommodated based on their body size.

As to the protein metabolism of embryogenesis, the result is consistent with other literature, hence the use of current methodology is appropriate to address certain energy metabolism questions, especially when the study organisms are small in size. Resipher as an open-chambered respirometer is promising on the estimation of standard cost of protein metabolism. Further introduction of thermal stresses is viable since drastic changes are observed in respective to 5°C temperature differences. As refer to the use of other inhibitors, like

oligomycin that requires specific reactive time window, must be used with caution due to the long time required for self-calibration. Thus, improvements relating to the instrument programing are appreciated. Variation between individuals is expected, and part of this could contribute to the natural mechanisms of the instrument in which allowed the access of surrounding environment. The result needs to be carefully evaluated before giving conclusions, again, more datasets of the application of this equipment on different species would be an asset. From another perspective, the utilization of current apparatus to detect the oxygen consumption rate of snail eggs is more consistent with the regular living environment of snails. Meanwhile, it will not cause excessive damages to the embryos in comparison with closed respirometer that often requires stir of testing chamber, thereby not influencing the experimental results with extra bias. In summary, this newly invented instrument is beneficial because of its portability, and because its offers cloud storage that can suit a wide set of experimental conditions located both in lab and in the field.

The effect of temperature seems to be smaller than would be expected assuming biochemical rates double with every 10°C change in temperature, suggesting acclimation leads to compensatory responses to changing temperature in *P. duryi*. This acclimation response would be consistent with snails having a robust defense system capable of adjusting to life over a relatively wide range of environmental conditions. Embryos appear more susceptible to temperature change; their growth might be restricted under prolonged exposure to coldness. Overall, this experiment discovers many details about the cost of protein metabolism, many of them acquires additional experiments to provide further validation.

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