Understanding the role of Proteins and Carbohydrates in driving the feeding behavior of *Drosophila melanogaster*

A Thesis

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by

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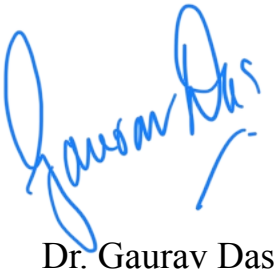
Certificate

This is to certify that this dissertation entitled ***"Understanding the role of Protein and Carbohydrate ratios in driving feeding behavior in Drosophila melanogaster"*** towards the partial fulfillment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by Shrutika Lokkapure at the National Centre for Cell Science (NCCS), Pune University under the supervision of Dr. Gaurav Das, Scientist E, National Centre for Cell Science (NCCS), during the academic year 2022-23.



Dr. Gaurav Das

Committee:

Dr. Gaurav Das

Dr. Raghav Rajan

To Aai-Dadaji & Mummy-Pappa
for creating my genome; their unconditional love and unwavering support in never limiting me from dreaming the impossible!

Tribute to the late Dr. Mangala Jayant Narlikar, the great woman mathematician and researcher for her blessings and being my humbling-inspiration to not give up on my pursuit and quest of science!

Declaration

I hereby declare that the matter embodied in the report entitled ***"Understanding the role of Protein and Carbohydrate ratios in driving feeding behavior in Drosophila melanogaster"*** are the results of the work carried out by me at the National Centre for Cell Science (NCCS), Pune University under the supervision of Dr. Gaurav Das and the same has not been submitted elsewhere for any other degree.

Shrutika Lokkapure

27 October 2023

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Abstract

<u>Short Abstract</u> (250 words):

In delving into *Drosophila* nutrition, through my master's thesis, I have explored the roles of proteins and carbohydrates in driving the *Drosophila* feeding behavior. Previous studies and extant literature have looked at this aspect but in the light of factors like age, sex, and lifespan. Moreover, they primarily use liquid diets, not reflecting the fruit flies' natural solid food intake. My master's thesis aims to fill this gap, establishing a foundation for measuring protein and carbohydrate intake in a solid food assay setup. Using the DIETS assay, I played around and made various ratios of P:C diets to track fly consumption in order to address this. A major challenge in this was to get the evaporation values for all of these diets to the permissible comparable levels and thus made for a major part of my thesis. This problem was overcome by doing repeated rounds of evaporation standardization with various parameters like humidity, distance from the food cup etc, for all these different diets and has been highlighted and summarized in the troubleshooting section of this master's thesis.

While existing literature leans toward the "Protein leverage hypothesis," my results consistently highlight carbohydrates, not proteins, being maintained across diverse diets. This contradicts the norm and led to our speculative "Carbohydrate leverage hypothesis." Results from two-cup-two-choice and CAFE assays and several other results from our lab align with this perspective, suggesting carbohydrates drive *Drosophila* feeding behavior. The thesis concludes by exploring the discussions, implications, reasons, and arguments for this novel hypothesis.

<u>Long Abstract</u> (515 words):

Protein, carbohydrates and fats are the 3 major sources of macronutrients for the normal functioning, growth, development and survival of any organism, but despite this fact, in recent times, the very few studies in *Drosophila melanogaster* that have looked at the exact role of protein (P) and carbohydrate (C) in feeding behavior have largely been scattered with most of them trying to look at protein and carbohydrate intake only in the context of some other parameters like age, sex, mating status, lifespan, egg production rate etc.
Most of these studies have only been carried out with liquid diets- but out in the wild and beyond the setting of the labs, fruit flies rarely get their foods in liquid forms and thus, it becomes even more important to look at the fruit-flies' feeding behavior in a much more naturalistic setting- a setup where we can mimic the solid food consumption in *Drosophila* and then track its feeding behavior in terms of its protein and carbohydrate consumption. None of the studies till date have explored these basic fundamental questions mentioned above, especially with the aspect of looking at the intake of protein and carbohydrates in flies in a solid food consumption setup.

Thus, my master's thesis project has been an attempt to bridge this gap in the basic understanding of the role of protein and carbohydrates by first laying out the foundations of how we can measure the protein and carbohydrate intake in a simple solid food assay setup. I have used a simple yet powerful solid food estimation and tracking assay called the DIETS developed in our lab to track how much protein and carbohydrates are being consumed by the flies. I have played around and made various P:C diets in order to do this. A major challenge in this was to get the evaporation values for all of these diets to the permissible comparable levels and thus made for a major part of my thesis. This problem was overcome by doing repeated rounds of evaporation standardization with various parameters like humidity, distance from the food cup etc, for all these different diets and has been highlighted and summarized in the troubleshooting section of this master's thesis. Results from the extant literature show that proteins (but not carbohydrates) are maintained in a narrow range and put forth a "Protein leverage hypothesis" to conclude that proteins drive the feeding behavior in most organisms including fruit flies. However, my results and results from our lab repeatedly show carbohydrates (and not proteins) being maintained in every kind of diet we make in our lab-different P:C ratio diets, protein rich or protein diluted diets, high sugar or high calorie diets. Even my results from two-cup-two-choice as well as CAFE assay verify this. All our results hint towards carbohydrates rather than proteins being the major factor driving and leveraging the *Drosophila* feeding behavior. We thus hypothesize and speculatively put forth a 'Carbohydrate leverage hypothesis' in light of our results. The reasons, speculations, arguments for this newly proposed hypothesis through my work have also been discussed at the end of this thesis.

Questions/Points addressed through Chapters of this thesis:

Questions addressed in Chapter-1: Background & Introduction

-Why Fly? Why use Drosophila in biological studies?
-What all feeding behaviour is Drosophila currently being used for?
-Why care for Protein and Carbohydrate ratios in diets?
-What has been known in the field?
-What is the previous/current literature on proteins and carbohydrates hinting at?
-What are the current Hypotheses in the field? Protein Leverage Hypothesis
-Summary of the works in proteins and carbohydrates-works of Raubenheimer, Simpson, Rushby and KP Lee
-what are the current unanswered questions in the field?
-Guiding questions of my Master's thesis to bridge this gap and setup a basic foundation for measuring, tracking and analyzing the proteins and carbohydrates in Drosophila feeding behavior

Questions addressed in Chapter-2: Methodologies, Assays and Experiments

-How is feeding measured in our lab? -Novel DIETS Assay
-What was my plan for checking the feeding in my fruit flies?
-What food/diet did I give my flies to look at their protein and carbohydrate intake?
-How did I prepare these diets? -the P:C ratio calculations and achieving P:C ratio to check feeding with our solid DIETS setup
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-Proof-of-concept/ working evidence for all performed Evaporation Standardization
-Plotting of Data and Analysis -Excel, Numbers and GraphPad Prism

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-Flies prefer high carb/ P:C ratio than high protein/P:C ratio

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-Different plausible speculations about my results
-What have been the limitations of my study?
-What are the parameters I have not looked at through my experiments?
-Other possible potential hypotheses from my results
-Other possible experiments to verify and confirm my current 'carbohydrate leverage hypothesis'
-What all further questions can be asked? What else can be checked?
-Why is it still early to make strong claims about 'Carbohydrate leverage hypothesis' and why the other possible hypotheses cannot be discarded
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-The speculated plausible role of IN1 interneurons
-Further speculations and possibilities from the latest studies-Taste of sugar v/s taste of proteins- the unexplored pre-ingestive and/or post-ingestive effects of these macronutrients
-Further biological implications to humans

Appendix and supplementary materials included in Chapter-5

1. **KEY RESOURCES TABLE: list of reagents with their source and catalog numbers, Excel sheets with all raw feeding data, calculations for different ratios of P:C diets, all the protein, carbohydrate, calories and 2-cup, 2-choice calculations and conversions for all plots**
2. **Contacts for reagents and resource sharing**
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4. **Quantification, Data Analyses and Software availability**
5. **Different trial and errors and calculations done before achieving the required 1:1, 1:2, 1:4, 1:8 and 1:16 diets!**
6. **Different trial and errors and evaporation standardization done before achieving the required 1:1, 1:2, 1:4, 1:8 and 1:16 diets!**
7. **Exact Evaporation Standardizations and Troubleshootings with 1/2xC+1xP, 1/2xP+1xC,1xC+1xP (basal control diet), 2xC+1xP, 2xP+1xC**

Acknowledgments

The journey of my master's thesis has been one of the most beautiful, satisfying, peaceful, rewarding, thrilling years of my IISER life despite also having some puzzling, confusing, scary and challenging moments! But I am super grateful for every bit of everything that happened to me and happened with me-I couldn't have asked for a more adventurous rollercoaster (academic+personal) master's thesis journey than this! All my time and experience at all four institutes building upon this term- IISER Pune, Rockefeller University, New York, National Centre for Cell Science (NCCS)/Pune University has been truly life changing and has shaped me in a way I never envisaged! I am very grateful to every bit of opportunity I got, all the people I crossed paths and connected with and most importantly all the fruitful and meaningful conversations and discussion I had! These academic years have greatly been a journey of me trying to grow independent in all aspects, me utterly enjoying my own work and company, me embracing my solitude and traveling places and in turn me discovering myself like never before!! Even though this master's thesis has been a largely independent journey of self-discovery, this piece of work and transforming years of my life would be incomplete without acknowledging the contributions from all the wonderful people who made this experience so much fun, rewarding, bearable and highly enriching! Here's to all of them!

First of all, I want to thank Dr. Leslie Vosshall and her Laboratory of Neurogenetics and Behaviour at Rockefeller for giving me the opportunity to work in neurogenetics of ATP receptor mosquito stylet neurons as a visiting master's student and a Foreign Research Intern in New York before I could start my current master's thesis. I am grateful for this place where I was first exposed to the neurogenetics and behavior of the very cool mosquitoes and their blood-feeding behavior, learning FISH technique and confocal microscopy and lastly being able to give a talk at the Kronauer-Vosshall symposium on my FISH results! My months at Rockefeller and New York paved the way and shaped me for everything that was coming ahead!

I want to begin with thanking my home institution, IISER Pune and all its amazing bio-faculties who have taught and helped me build my strong foundations and appetite for hardcore pure biology through the most beautiful core science subjects in such a bewitching and enchanting way over the years! But most importantly I am grateful for their open-mindedness, always-curiosity-driven temperament, highly contagious-love and enthusiasm for all sciences, constructive-feedbacks and rightly-questioning critique, clarity in communicating/discussing/teaching science, excitement in attending each and every science (and beyond!) talk/event, appetite for the right aptitude and attitude in science, humbling and welcoming kindness in academia that has remarkably impacted and shaped the way I think today! Their legacies through their teachings and values that they have conferred upon us has sculpted my outlook, positivity and enthusiasm towards science, research and life in general and has helped me build myself in order to be able to strive in and take on the challenges of the world today! The innumerable class discussions, the questions and exchange of science during and after the talks, the always-welcoming-despite-of-being-super-busy office chats, all the direct and indirect support, encouragement and motivating words, honest opinions and feedbacks, and the highly infectious science-is-hard-but-we-still-love-it temperament in all my Profs- Raghav, Anand Krishnan, Richa, Girish, Aurnab, Sutirth, Nagaraj, Nishad, Sudha, Subhedar sir, Satyajit, Vineeta, Nixon, Neeraja, Mridula, Santhanam, Arnab and Poornima ma'am have largely shaped and built both my sciency and non-sciency parts (or rather strengthened my non-sciency side to be able to take on the challenges and dark sides of science and vice-versa) today! These people have been pillars of strength in my academic journey at IISER and have been constant anchors of support when I was lost the most- they have enabled me to pursue my interest, love and passion for science (in general) and neuroscience (in particular) when I thought I was not good enough for it, my personality was not made for research or when I was on the brink of giving up on science and lab work in my domains of interests- these people helped me stand up and shake-off the dust every single time to be able to complete my final year of master's thesis with so much joy in exactly the field I wanted! Thanks to these people I now know and am completely sure that all those fears were baseless. Special thanks to Raghav for always being my super honest-supportive TAC and inspiring me to do my science right along with keeping myself chill and calm-minded just like him, in every circumstance!

Taking just a few names is doing great injustice to the several other fun and memorable conversations I had with so many other IISER people in the nooks and corners of our main-building, LHC and several other corridors throughout the campus! I always loved all my time at IISER Pune, but what it means to have IISER Pune as my 'home institution' is something I realized and truly understand only now after doing projects for my master's thesis outside IISER!

It has been real fun to be able to go outside IISER Pune for my work every single day and I have enjoyed every moment of being at the National Centre for Cell Science and in Pune University. Growing up in PCMC, I always fancied and was in love with the vibe of main Pune city but little did I know then that one day I would have an opportunity to work in Pune University itself! And how! I am super grateful to my fun-loving and always-creative on science boss-PI-mentor-guide Dr. Gaurav Das and his Brain and feeding behavior lab for taking me under his wings to be able to pursue my master's thesis in the field and the model organism I feel passionately for! He has always given me the right kind of liberty, freedom and the necessary push where I needed to grow the most! I am grateful to him to give me the right kind of mentor and support in the time I needed the most! Just like the way he puts it: he has tamed the energy in me- and I think he has been very rightly wise in helping me channelize my energy in the right way- by always encouraging me to ask the right questions to decode, by pushing me to go and read more and more, to allowing me to be process and not completely-results oriented (cause results will definitely follow when the process is right), by not allowing me to over-work and do 3-experiments in a day but rather produce efficient meaningful data that gives me more clarity or helping me build the habit of keeping my presentations, thought-process, ideas and words short, succinct and to the point! His most-unique temperament to go-figure-it-out or go-try-it-out has allowed me to widen my horizons of doing science in the most fun and innovative way! The liberty and space that he has given me to try out new things in my project and beyond has helped me become independent and open-minded like never before!
My acknowledgements will be nothing without thanking my mentor, Manik in the lab! His spontaneous nature, always ready-to-help-&-teach attitude, undwindling excitement about planning and looking at experiments, practice of plotting the data right away, eagerness to look at what the data is saying in order to plan the next day, smart-efficient work meticulousness, tendency to perfect one-experiment at a time yet juggling through different lab chores throughout the day- are all practices I have gotten into myself through him! His support and time through always being present in the lab- is exactly what I needed for building my core foundations and habits in my lab-academic life! His infinite time and patience, words of advice and encouragement, our philosophical discussions over academia, pep-talks when things didn't work have been a complete wholesome package in the form of an awesome mentor!
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I am also super grateful to Bhavna for being such an inspiring senior-colleague in always being ready to share the right strategies and wise ideas for getting things done in the lab, for allowing me to imbibe the right kind of lab-wisdom! Suhas, by being the tech-savvy, know-it-all person in the lab has been such a savior for finding things in the lab and always being available to teach all the cool lab equipment from soldering machine to pH meter in meticulous fun-way! I am glad to have overlapped with Prerna who has always told me to confidently be myself and inspired me in all my major academic milestones throughout the thesis! I thank Anindyo for always identifying when I needed some general-philosophical words of lab-encouragement by coming forth and reminding me to not take things like incubators/ideas/lab-meetings or interactions with sir personally when they went down south! Thanks to Radhika, Meghna and Tanzeel for being the fun peers with all the random fun talks, all the latest news and current affairs but for all the crazy jokes, comments and unsolicited cat pranks planted against me- they shall never be thanked! Thanks to Rashmi for small lab arrangements, sorting overlaps and words of concern and help every now and then! Asmita, in being my very first colleague-cum-friend during the initial days and first half of my project cannot be thanked enough for all the lab-life and life conversations we together shared!

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All these interactions with people in my lab and our intellectually stimulating and enriching fun lab meetings, all science talks at both NCCS and IISER that I have attended have not just allowed me to be a better version of myself in my academic and professional life but have also given me the environment to keep-alive and hone my curiosity, enthusiasm and love for science and lab work while being myself!

Utmost thanks to my seniors in Biology and IISER alumni who have been consistently with me throughout the thick and thins of the entire term of my master’s thesis even before it all started- Vaibhav, Chitnis, Shriya Hirve, Pratyush and Rishika. My solid support system from NYC- my roommates: Tarana & Fuhui, my go-to-advisors from Columbia: postdocs Dr. Surojit Sural & Dr. Mohita Tagore for believing in my academic abilities and reminding me of those when I needed them the most- for the exceptional number of phone calls, video calls and meetings we had discussing science, Biology, careers, mentoring styles, academics and research! If it was not for them, I would have never been able to grow the right mindset to look at academia for the way it is- for all its rewarding satisfying sides along with its truthful dark ugly sides. Special thanks to the most caring and open-minded- Arjun Ramakrishnan and Sharika, the neurobiologist couple from IIT Kanpur for their roles in kindling my love for Neuroscience and their undettering encouragement to keep up my positivity, optimism and energy in all our brain & O.U.R. P.S.Y.C.H.E. interactions till this day ever since covid! Thanks to all of these people in academia, I could build and never give up on the right mindset, temperament, aptitude and attitude in science and research to be able to complete this master’s thesis!

All these people in my lab along with the people from IISER have not just taught me to swim in the most challenging waters, but they have helped me build the confidence to be able to swim on the deepest sides of the swimming pool all by myself, and independently on my own! Through them, I have not just learnt to save myself from drowning but rather enjoy my swim- to be able to now know how to gather myself up again with the right strategies and control to continue swimming with my head held high!

My life and amazing support system outside the lab and workplace have been the reason to look forward and be excited about going to the lab every single day! I cannot thank my closest and dearest set of people in the hostel and home enough! It has been great to look forward to and to be able to return back to my closest friend-cum-roommate Chahana in the later half of my project- to be able to unwind the day with conversations about our days at work, our bosses, lab-mates, work and thesis- apart from the constant amazing unmatchable friendship that we have shared all through the 5 years at IISER! Her warm love and care, and talking to her about anything bothering me has always given me clarity and unknotted my issues of biggest concern! The sisters-like bond we share over friends and family is unequivocal-till death, do us part! Rajat, with his presence and absolutely sweet, kind and caring nature has been a blessing-in-disguise on the campus: through our serendipitous overlaps and interactions since the beginning of my project in January to the end of it- he has been the strong constant in-person support I always needed to be a better version of myself during this term! I will be forever grateful to him for always giving me a chance to correct my shortcomings, all of his direct and indirect life-work-life advice, his fresh out-of-the-box perspectives on life and all our philosophical exchanges! Emulating and getting inspired by his consistent discipline, professionalism, work-life balance, healthy lifestyle and a quest for knowledge and wisdom in everything has made me the best independent version of myself! Aviral, in being his unique quirky self and my listening-box, has been a phenomenal friend by being my safe ranting space enabling me to process anything and everything that has ever bothered me in work, academia, lab, project and personal life! Special shoutout to Akanksha for being such a savior in helping me resolve citation and technical glitches of this thesis!

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~Shrutika
(23/10/23)

Contributions

Contributor's Name	Contributor's Role in the thesis
Shrutika Lokkapure, Manikrao Thakare, Dr. Gaurav Das	Brainstorming & Conceptualisation of Ideas
Shrutika Lokkapure	Methodologies & Assays
Shrutika Lokkapure	Calculations and Tabulations
Shrutika Lokkapure	Diets Preparation
Shrutika Lokkapure	Experimentation
Shrutika Lokkapure	Troubleshooting and Standardisation
Shrutika Lokkapure	Data curation (collection and tabulation)
Shrutika Lokkapure & Manikrao Thakare	Software
Shrutika Lokkapure, Manikrao Thakare, Dr. Gaurav Das	Data Analysis
Shrutika Lokkapure, Manikrao Thakare, Dr. Gaurav Das	Data & Experiment Validation
Shrutika Lokkapure, Manikrao Thakare, Dr. Gaurav Das	All Investigations
Dr. Gaurav Das, Manikrao Thakare & Raj sir	Resources
Shrutika Lokkapure	Thesis writing & editing
Shrutika Lokkapure & Manikrao Thakare	Thesis reviewing
Shrutika Lokkapure, Manikrao Thakare, Dr. Gaurav Das	Visualization
Shrutika Lokkapure, Manikrao Thakare, Dr. Gaurav Das	Supervision
Shrutika Lokkapure & Dr. Gaurav Das	Project Administration
Dr. Gaurav Das	Funding Acquisition

Chapter-1
Background & Introduction

Questions that are addressed in this chapter:

-Why Fly? Why use Drosophila in biological studies?
-What all feeding behaviour is Drosophila currently being used for?
-Why care for Protein and Carbohydrate ratios in diets?
-What has been known in the field?
-What is the previous/current literature on proteins and carbohydrates hinting at?
-What are the current Hypotheses in the field? Protein Leverage Hypothesis
-Summary of the works in proteins and carbohydrates-works of Raubenheimer, Simpson, Rushby and KP Lee
-what are the current unanswered questions in the field?
-Guiding questions of my Master's thesis to bridge this gap and setup a basic foundation for measuring, tracking and analyzing the proteins and carbohydrates in Drosophila feeding behavior

1.1 *Drosophila* as a model organism in biological sciences
1.2 *Drosophila* as a model to study feeding behavior
1.3 Exact role of protein and carbohydrates in driving the feeding behavior in *Drosophila* has been largely unclear in the field
1.4 Review of the previous protein and carbohydrate studies in *Drosophila* in the literature
1.5 Guiding questions and Potential hypothesis for my project

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1.1 *Drosophila* as a model organism in biological sciences

For more than 100 years now, *Drosophila melanogaster*, the common fruit fly, has been employed as a powerful model organism to investigate different biological questions in a laboratory setting (Yamaguchi and Yoshida 2018)
Fruit flies continue to be a popular model organism as they are **small**, **easy to handle**, can be **maintained in large numbers**, are **highly cost-effective and affordable**, have a **short life span** (of just two months) and generation cycle (of just 10 days) -as shown in the animation below. Apart from this, they also have very **few legal restrictions** for research enabling us to start experiments right away (rather than having to wait for formal approvals which is the case for other larger model organisms like mice or rats). Moreover, due to the **ease of genetic manipulations** (because of the presence of just 4 chromosomes in the entire organisms) and already **generated and readily available lines** for carrying out crosses of interest, work in *Drosophila* has helped to unravel fundamental mechanisms in many areas of biology. This in turn has set up a solid foundation in core biological sciences as well as helped us progress our conceptual understanding in several key areas of biology like classical and modern genetics, developmental biology, neurobiology, neurogenetics, cancer and stem cell biology and behavioral sciences to name a few (Yamaguchi and Yoshida 2018).

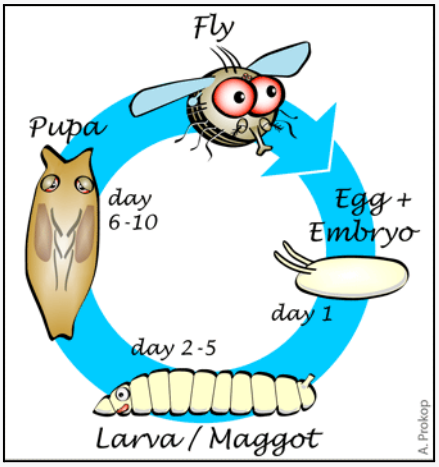

Image 1.1.1: a simple animation depicting the 10-day life cycle of a fly, from WhyFly

All this is possible because it has been found that the underlying genes and fundamental biological processes responsible for health and disease in humans are often very similar to those that can be studied in the *Drosophila melanogaster*. As the cartoon below summarizes, it has been estimated that about a whopping **"75% of known human disease genes have a corresponding recognizable match in the genome of fruit flies"** (Reiter et al. 2001). Not just that, all of the major organ systems of humans and vertebrates namely the digestive system, the nervous system, other physiological systems like circulatory, excretory and even connective tissues like the skeleton system and the muscular system along with the basic body organization and framework have been found to be homologously conserved from fruit flies all the way to the humans!

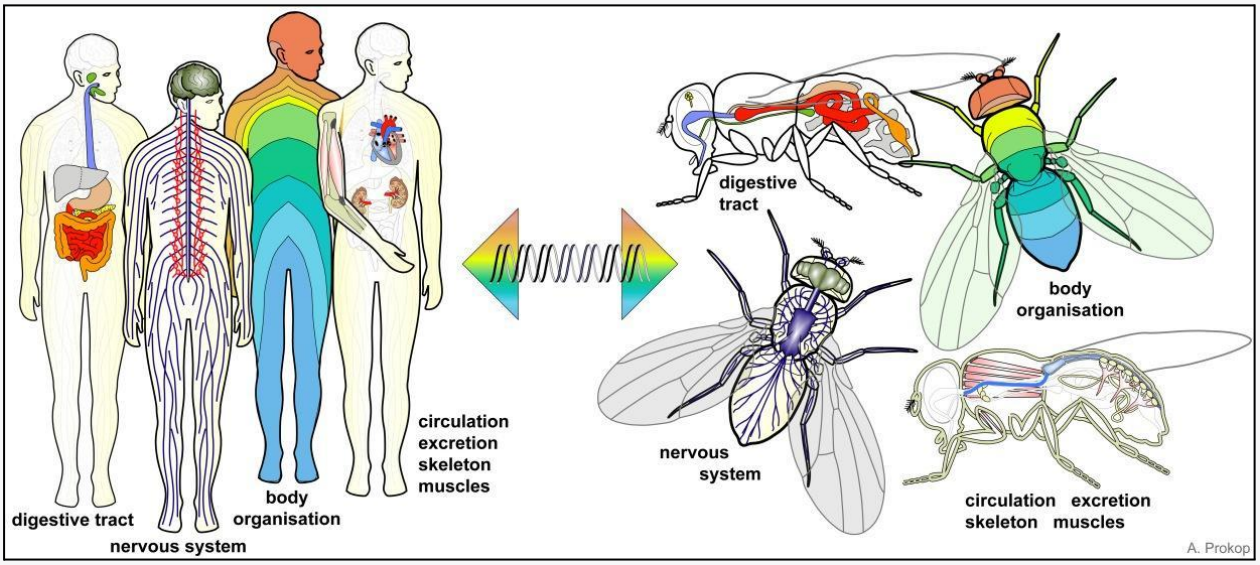

Image 1.1.2: A cartoon depicting the homologous similarity between humans and fruit flies, from WhyFly

Therefore, flies are fondly called “test tubes” to pioneer research as they help us study and understand the fundamental biological processes of our interest, followed by work aiming to determine whether the new understanding and discoveries achieved in *Drosophila* are further applicable in other mammals like mice or even humans! Taking all this even further, in recent years, with the studies of the connectome and neural circuitry in *Drosophila* and the latest AI-generated power tools like the Flywire (that has reconstructed a full hemi-brain connectome of the fruit fly), a greater number of investigations can be carried out now at even the level of synapse and circuitry than ever before and thus, help us make significant advances in our understanding of how the brain works by ultimately linking neuronal wiring with brain functions at a deeper nuanced level.

1.2 *Drosophila* as a model to study feeding behavior

Being a fantastic model organism to maintain, manipulate and study like we saw in the above section, *Drosophila melanogaster,* in recent times, has also largely been studied and extensively researched to investigate and better understand feeding behaviors in animals. Till date, the feeding behaviors in *Drosophila* have broadly studied neuronal regulation of feeding (Iijima et al. 2009; Xu, Zheng, and Sehgal 2008), hormonal regulation of feeding (Pool and Scott 2014; Mattila and Hietakangas 2017), sensory aspects of feeding (Scott 2018; Edgecomb, Harth, and Schneiderman 1994; Pool and Scott 2014; May et al. 2019), dietary restriction (Krittika and Yadav 2020; Oka et al. 2021; H.-Y. Lee et al. 2023), lifespan and longevity based studies (Krittika and Yadav 2020; Srivastava et al. 2022; Holvoet et al. 2023), learning and memory aspects of feeding (Mohandasan et al. 2022; Wu et al. 2018; Honda 2022) and feeding on high fat and high sugar diets (May et al. 2019; Skorupa et al. 2008). Like humans, a high-sugar diet is also known to produce obesity and insulin-resistance in wild type *Drosophila*. Thus, studying the effects of high sugar diets in the fruit flies can help us gain insights into how dietary sugar affects and influences various pathophysiological conditions in humans.(May et al. 2019; V. Dam et al. 2021; Baenas and Wagner 2022; E. van Dam et al. 2020)

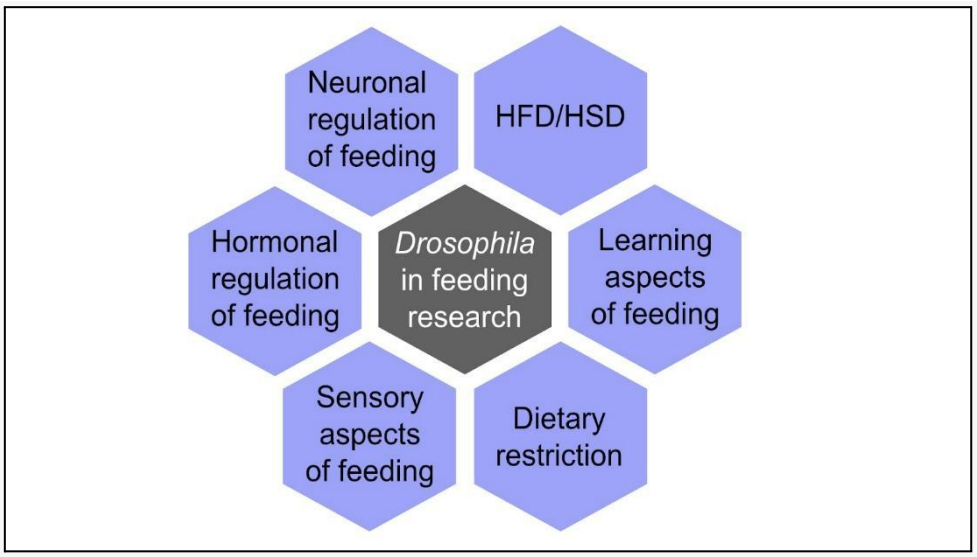

Image 1.2.1: A simple schematic describing the current feeding behavior research in *Drosophila*

1.3 Exact role of Protein and Carbohydrates in driving the feeding behavior in *Drosophila* has been largely unclear in the field

When I did a literature review/survey of all these feeding-associated studies in these various categories, I found that even though all these studies look at the various aspects of feeding behavior, a very few of these studies focus on what goes inside the system of the organism in the first place and what drives that feeding. We know that protein, carbohydrates and fats are the 3 major sources of macronutrients for the normal functioning, growth, development and survival of any organism. But why are proteins and carbohydrates important for an organism? Proteins and carbohydrates play crucial roles in animal feeding behavior. Proteins are essential for growth, tissue repair, and maintaining body structure, influencing animals to seek protein-rich sources. Carbohydrates provide a quick energy source, impacting animals' preference for foods with varying carbohydrate content. Together, they contribute to nutritional balance, influencing the feeding choices and behaviors of animals.
But despite these facts, in recent times, there have been only a few recent studies that have looked at the exact role of protein and carbohydrate in feeding behavior. Moreover, these studies have largely been scattered with most of them trying to look at protein and carbohydrate intake only in the context of some other parameters like age, sex, mating status, lifespan, egg production rate etc (as I briefly summarize below). With very limited studies, there has been a lack of general consensus about what we already know in the field and how we look at proteins and carbohydrates in driving the feeding behavior. There are many unanswered fundamental questions like is it the proteins or is it the carbohydrates that play a bigger role in driving the feeding behavior, or is it the combined ratio of both of these macronutrients- these aspects have not been explored completely and thus have largely remained unclear and not fully understood. Shedding some light on the technical aspects of these studies, most of these studies have only been carried out with liquid diets- but out in the wild and beyond the setting of the labs, fruit flies rarely get their foods in liquid forms and thus, it becomes even more important to look at the fruit-flies' feeding behavior in a much more naturalistic setting- a setup where we can mimic the solid food consumption in *Drosophila* and then track its feeding behavior in terms of its protein and carbohydrate consumption. None of the studies till date have explored these basic fundamental questions mentioned above, especially with the aspect of looking at the intake of protein and carbohydrates in flies in a solid food consumption setup.

Thus, my master's thesis has been largely about an attempt to bridge this gap in the basic understanding of the role of protein and carbohydrates by first laying out the basic foundations of how we can measure the protein and carbohydrate intake in a simple solid food assay setup (developed in our lab and described below in detail) where one can manipulate and track how much protein and carbohydrates are being consumed by the flies in order to better understand their feeding behavior.

1.4 Review of the previous protein and carbohydrate studies in *Drosophila* in the literature

The earliest and the most fundamental works that have looked at the entire Protein and Carbohydrate landscape in animals has been the works of two evolutionary biologists from University of Sydney: Professor Raubenheimer and Simpson. Their earliest work with locusts in the wild suggests that proteins and carbohydrates do indeed play a major role in how much of what an organism feeds, but mostly it's protein that drives this feeding behavior. In their experiment, they made 25 different ratios of P:C diets and provided each of the locusts with only one type of this diet for 24 hours and then measured feeding. Their results showed that the locusts that consumed too much of carbohydrates ended up eating too little protein and the locusts that ate too much of protein ate too little carbs- but these were not the healthiest locusts, the locusts that showed the best survival and growth (their parameters of maximum fitness) were supported by a diet that had about a balanced ratio of P:C i.e., not too much protein and not too much carbohydrates. With this and several other experiments in other animals like rodents and mammals (including humans), they formulated and put forth a 'Protein Leverage Hypothesis'. According to this hypothesis, protein is what leverages the other overall consumption of food- in fact protein is what drives even how much of the other macronutrients are consumed.

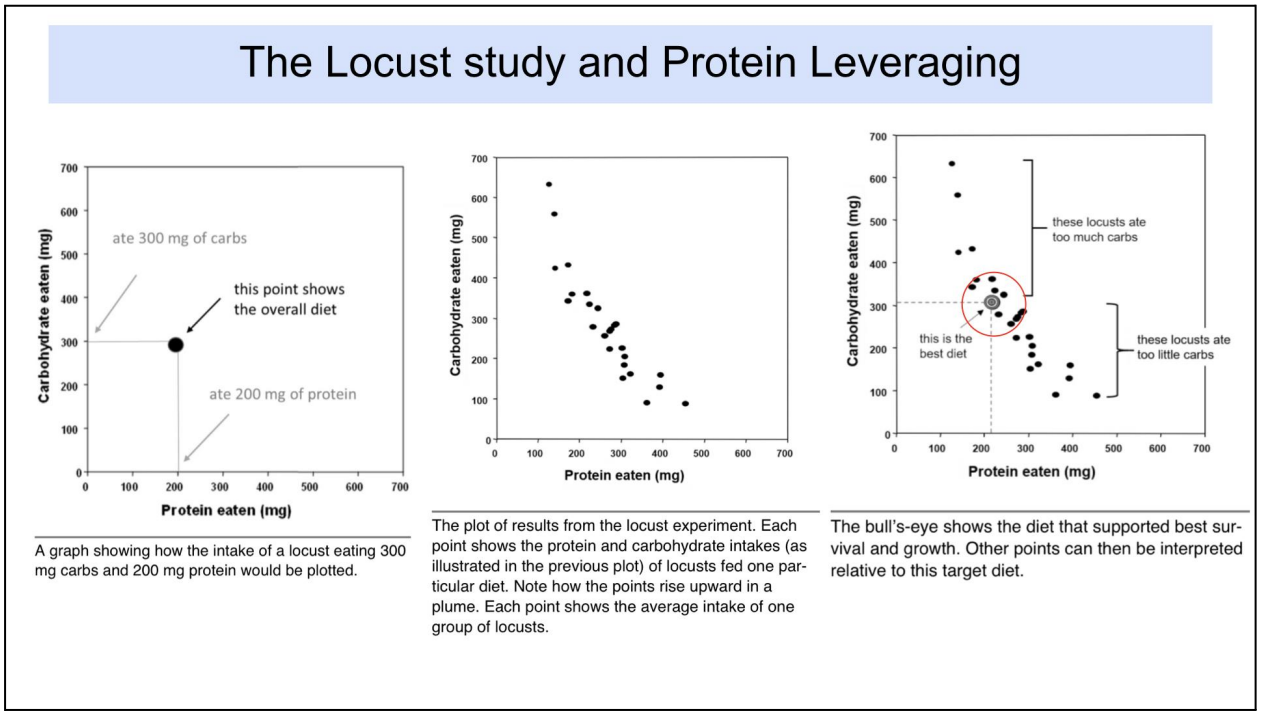

Image 1.4.1: Graphs from the earliest works of Raubenheimer and Simpson showing average carbohydrate and protein intake of locusts in the wild.

Too little protein in the diet leads to overconsumption of the other major macronutrient i.e., carbohydrates and in turn over consumption of the entire diet (this leads to the obesity-like phenotype and has also been speculated to be the root cause of the current obesity crisis).(Wells 2021; Raubenheimer, Simpson, and Couteur 2020).Whereas too much protein in the diet leads to the underconsumption of the available carbohydrates and also the overall food.

Similar studies have also been carried out in *Drosophila* that show an obesity-like phenotype when given a high sugar diet (which has a higher proportion of carbs than proteins) (S. J. Simpson and Raubenheimer 2005; Raubenheimer and Simpson 2019; A. K. Gosby et al. 2014; Stephen J. Simpson and Raubenheimer 2014; Raubenheimer and Simpson 2023; Alison K. Gosby et al. 2013). Other such studies have also looked at the effect of insulin resistance, cardiomyopathy and diabetes in the fruit flies when given high sugar/carb/calorie diets (as the phenotype of obesity, insulin resistance and diabetes has been well characterized in the flies)! (May et al. 2019; V. Dam et al. 2021; Baenas and Wagner 2022; E. van Dam et al. 2020)

A very recent study that has closely looked at the role of protein and carbohydrates in the context of *Drosophila* than ever before has been in the year 2023 by *Rushby et al*, where they have used various P:C diets in *Drosophila* with a focus of looking at specific factors like sex, age and mating status associated with protein consumption (Rushby et al. 2023). So, these studies do use various ratios of P:C but they use a previously developed, well known capillary-based liquid-food feeding assay called CAFE (Deshpande et al. 2014; Ja et al. 2007) (and thus, do not focus on the more-naturalistic solid food that the fruit flies actually consume out in the wild, in nature).

Moreover, in this paper, *Rushby et al* explore and focus on the impact of aging on protein utilization in fruit flies. Their study reveals a sex-specific decline in the ability of aged *Drosophila* to efficiently leverage protein for various physiological processes. Their findings suggest that the aging process in *Drosophila* is associated with sex-specific alterations in protein metabolism, providing valuable insights into the intricate dynamics of aging and nutritional regulation in this model organism.

To be precise, *Rushby et al* claim that Protein leveraging is exhibited in flies and that it is the proteins that drive feeding of the other macronutrients. As shown in their graphs below, they find that as the ratio of P:C in their liquid CAFE diet increases, the total food consumed also increases. Further, when they dissect how much of proteins and carbs are consumed in case of both males and females, they see that proteins are maintained in a narrow range, however carbs intake varies over a wide range, a range greater than that seen in case of protein- thus all of this leads them to conclude that protein is what drives the feeding behavior in flies and that it is protein that leverages the consumption of other macronutrients in the diet!

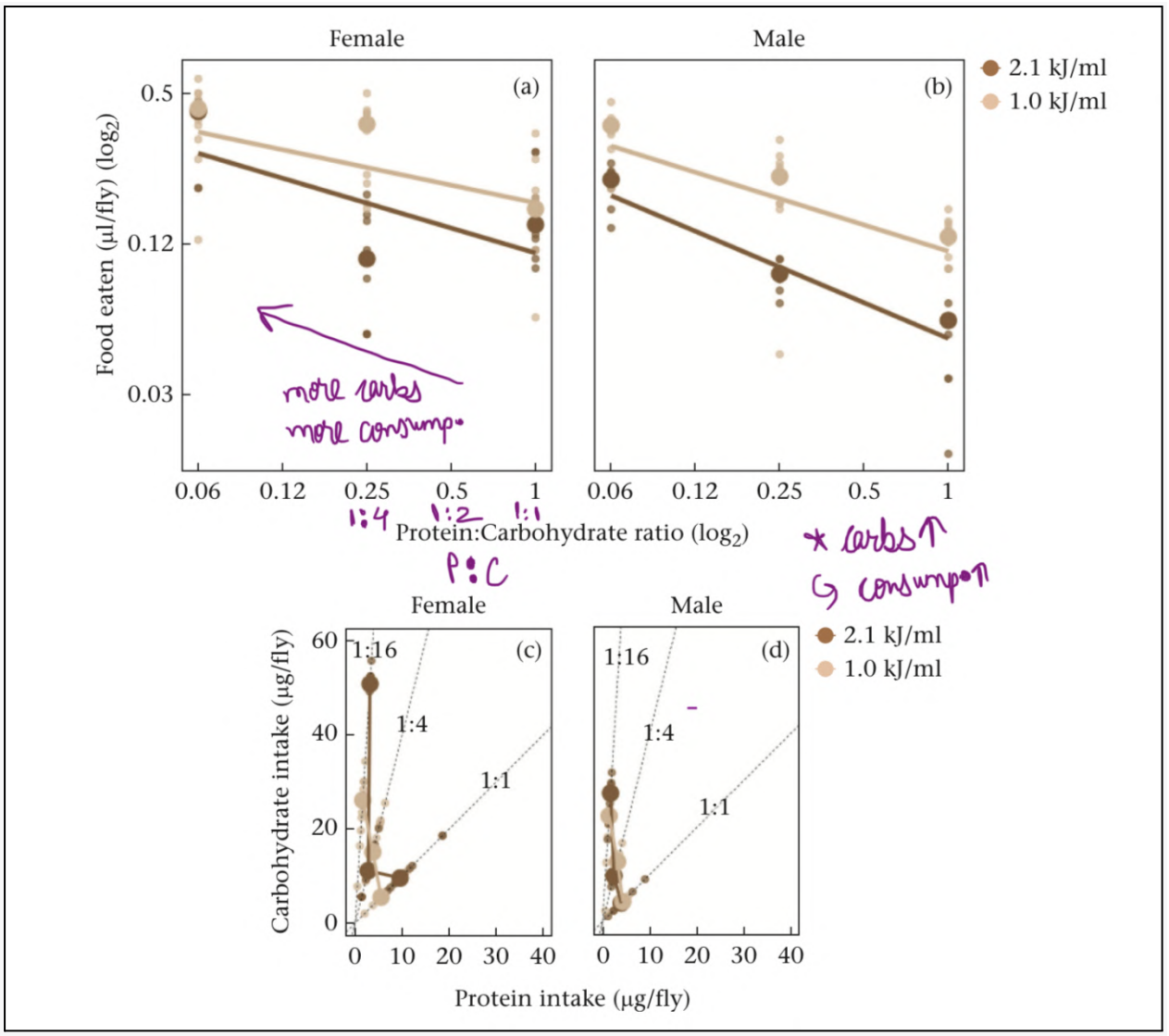

Image 1.4.2: Graphs from *Rushby et al* that demonstrates 1) in the upper panel- how the overall feeding increases as the ratio of P:C increases from 1:1 to 1:4 to 1:16 and 2) in the lower panel- how in spite of this overall increasing trend in feeding- the protein intake (ug/fly) on x-axis is maintained in a narrower range than the carbohydrate intake (ug/fly) on the y-axis! (Kindly note that the graphs have been annotated in purple to better highlight the trend)

However, it's also important to note how they prepare these liquid diets (as shown in their table summary below)- they highly decrease the amount of protein but increase the amount of carbohydrates - almost about exponentially from 64 g to 25 g to 7 g for proteins whereas 17 g, 83 g, to 115 g for carbohydrates in order to make each of the 1:1, 1:4 and 1:16 diets respectively (pay special attention to this table as this has also been one of the inspirations for me to prepare the diets for my own experiments and thus, this will be again referred to in the next chapter of this thesis-Methods)

Table 1
The high- and low-calorie 1:1, 1:4 and 1:16 P:C diets used in this study

		High calorie (2071 kJ/litre)	Low calorie (1038 kJ/litre)
1:1 P:C			
	Yeast	142.22	71.11
	Sucrose	17.07	8.54
	Protein	64.00	32.00
	Carbohydrates	64.00	32.00
1:4 P:C			
	Yeast	56.89	28.45
	Sucrose	83.63	41.82
	Protein	25.61	12.80
	Carbohydrates	102.40	51.20
1:16 P:C			
	Yeast	16.73	8.37
	Sucrose	115.00	57.5
	Protein	7.53	3.76
	Carbohydrates	120.52	60.26

The diets were produced by altering the amount (g) of sucrose and yeast in 1 litre of water.

Image 1.4.3: Table from *Rushby et al* summarizing the amount of Yeast and Sucrose, and thus the corresponding amount of protein and carbohydrate added for making different ratios of P:C in case of both high and low calorie liquid diets given to their flies

They also report that, as shown in the graph below, both mated and virgin females consume more food as the ratio of P:C increases (i.e., as seen in the table above, the amount of proteins in the diet is exponentially decreased but the amount of carbohydrates is substantially increased). Further, it's also seen that proteins drive feeding not just in male flies, but also in case of both mated and virgin females- in fact much more in case of mated females than the virgin females. Moreover, just like in the graphs above, proteins are maintained in a narrower range than compared to carbohydrates in both of these mating types- and thus, again, protein leverage feeding as proteins seem to be playing a major role in driving the feeding behavior of flies. Thus, this study shows that flies do exhibit protein leveraging- however this is largely in the context of age, sex and mating status of the flies. However, the limitation/drawback of this study is that it uses only liquid food and manages to fit and conclude this trend with an overall of only 3 types of diets- 1:1, 1:4 and 1:16- what happens to the trend with the intermediate ratios of 1:2 and 1:8 is an extrapolation here.

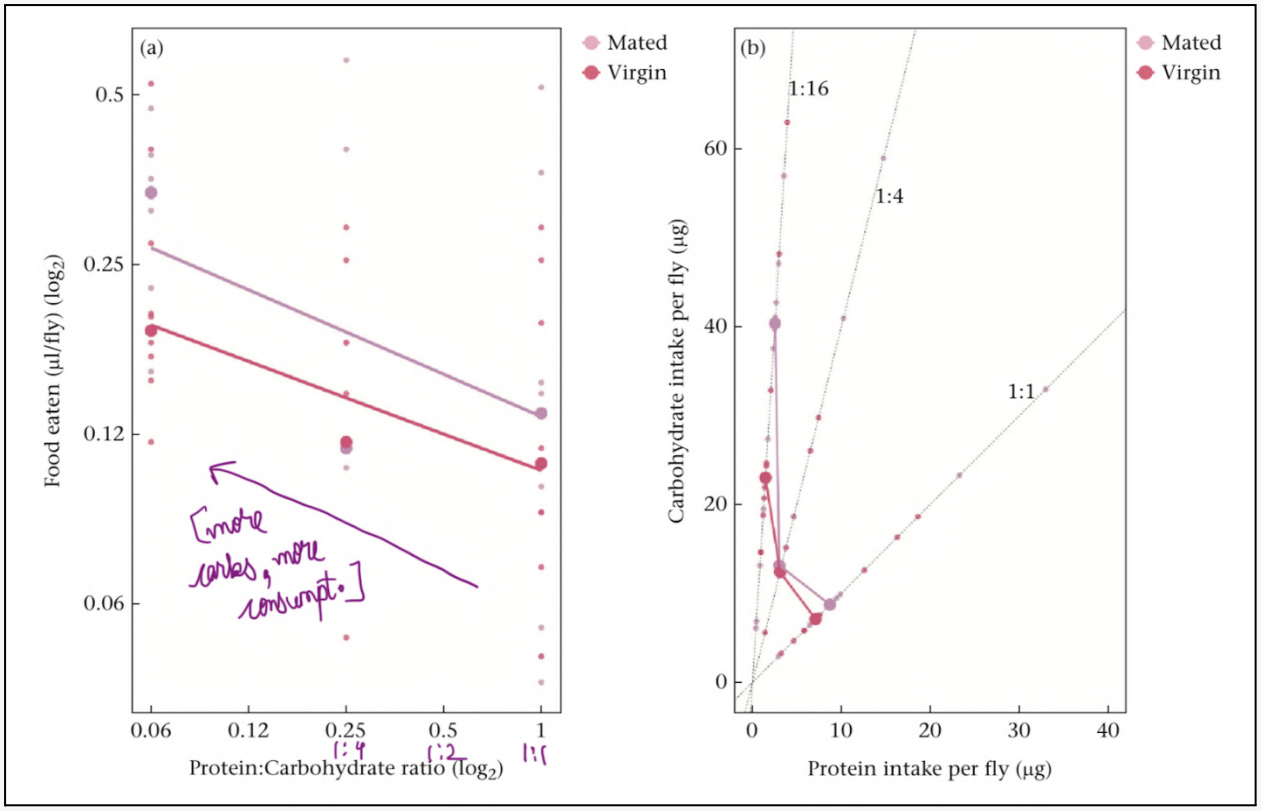

Image 1.4.4: Graphs from *Rushby et al* that demonstrates 1) in the left panel- how the overall feeding increases as the ratio of P:C increases from 1:1 to 1:4 to 1:16 for both mated (in light pink) and virgin females (in dark pink) and 2) in the right panel- how in spite of this overall increasing trend in feeding- the protein intake (ug/fly) on x-axis is maintained in a narrower range than the carbohydrate intake (ug/fly) on the y-axis for again both mated (in light pink) and virgin females (in dark pink) ! (Kindly note that the graphs have been annotated in purple to better highlight the trend)

Another key study that has looked at the role of protein and carbohydrates in driving the Drosophila's feeding behavior has been by K.P Lee, a graduate student in the lab of Raubenheimer and Simpson. In their paper, for the first time in literature, they study how much a fruit fly eats throughout its lifetime (K. P. Lee et al. 2008; K. P. Lee 2015). They wanted to see how much protein: carbohydrate intake best supports key aspects in drosophila's lifetime like the egg production rate, lifetime egg production and lifespan. For this they prepared 28 different ratios of P:C diets demonstrating total feeding and Carbohydrate v/s Protein intake (in ul/fly) for both male and female flies in liquid form (in capillaries) and gave an ad libitum choice between a combination of any of these diets. With these experiments, they lastly conclude that lifespan, lifetime egg production and egg production rate are maximized at different P:C ratios of 1:16, 1:4 and 1:2 respectively. Thus, again here the role of P:C ratios has been studied but again with liquid diet and also in the context of a few key parameters that define *Drosophila's* growth, survival and fecundity (K. P. Lee et al. 2008; K. P. Lee 2015)

1.5 Guiding questions and potential hypothesis for my project

Thus, with all of this knowledge from the extant literature, the focus of my Master's thesis has been on checking the role of fundamental macronutrients: protein and carbohydrate in driving the *Drosophila* feeding behavior.
The above summarized works of *Raubenheimer, Simpson, Rushby and Lee et al* point towards a protein leverage hypothesis and hint that protein rather than carbohydrates influence how much of other nutrients are consumed. We also began our work with a potential hypothesis that protein plays a major key role in driving the feeding behavior in *Drosophila*. With this, we expected that in any kind of protein-carbohydrate containing diet that I would give to my flies, if proteins are what drive feeding- i.e. the amount of protein consumed would be expected to lie in a narrow range or remain constant as the flies would try to maintain it (like we saw in the graphs from *Rushby et al* above), and the other macronutrient, namely carbohydrates- may or may not be maintained. With this, as my thesis title reads, I started my quest of trying to decipher the role of protein and carbohydrates in driving the feeding behavior in *Drosophila* with a special focus on solid diet that better mimics the naturalistic settings than providing liquid food in capillaries. I began this investigation by first learning the solid food consumption and tracking setup developed in our lab called the DIETS (described further below) to track *Drosophila's* feeding behavior and then preparing different kinds of protein and carbohydrate-containing diets to be given to the flies using this setup in order to further test my hypothesis and decode this protein and carbohydrates-based feeding behavior of *Drosophila*!

Chapter-2
Methodologies, Assays & Experiments

Questions that are addressed in this chapter:

-How is feeding measured in our lab? -Novel DIETS Assay
-What was my plan for checking the feeding in my fruit flies?
-What food/diet did I give my flies to look at their protein and carbohydrate intake?
-How did I prepare these diets? -the P:C ratio calculations and achieving P:C ratio to check feeding with our solid DIETS setup
-Achieving P:C comparable to P:C ratio in the literature (*Rushby*)
-Troubleshooting and Evaporation Standardization for different diets- Challenges, Tricks and Solutions
-Proof-of-concept/ working evidence for all performed Evaporation Standardization
-Plotting of Data and Analysis -Excel, Numbers and GraphPad Prism

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2.1 DIETS Assay

For measuring and quantifying feeding, I have used a novel assay called DIETS that has been developed in our lab. DIETS-**D**irect **I**ntake **E**stimation and long-term **T**racking of **S**olids is a simple yet powerful assay that has been originally devised by our lab to quantify and track food consumption in Drosophila. All of the data from the plots in the Results section has been measured and recorded using the DIETS setup. The schema for this assay has been explained in the diagram below. (Thakare et al 2023)
Newly eclosed flies that are around 2-3 days old are collected and sorted into males and females using CO_2 anesthesia sorting method. As per the requirement of the experiment, we use 12-22 male/female flies per vial. Post sorting, to recover from CO_2 anesthesia, the flies are habituated for 2 days in fresh agar vials that have a 1% agar (1gm agar dissolved in 100ml water) bed (that acts as source of water) along with a food cup (made out of cutting the caps of 1 ml Eppendorf tubes). These food cups are stuck to the walls of the vials with a double-sided tape and have the food of our choice that we want our flies to feed on as per the purpose of the experiment. Following these 2 days of habituation, at the start of the experiment (i.e., Day 0), food cups are freshly prepared with diets as per the particular experiment (the procedure that I use for preparing diets is described below) and weighed (using an analytical weighing balance) for recording their initial weight before they are stuck to the walls of new agar vials and flies are transferred into them.

We call these vials as DIETS vials. Flies are allowed to feed for exactly 24 hours on the given diet in these vials before the final weight reading is measured and recorded. Vials and food cups are freshly prepared every day and flies transferred into them at every 24 hours. The total consumption of food consumed by per fly per day is given as:

Consumption mg/fly/day =

$$\frac{\text{[(Initial weight - Final weight) - Average Evaporation value]}}{\text{Total number of flies}}$$

An important part of the experiment is the evaporation control. We account for the loss in weight due to evaporation (apart from the loss in weight due to the flies' feeding) by keeping evaporation control vials. Evaporation control vials are the vials that contain only the food cups without any flies in them, thus accounting for any kind of change in weight due to evaporation in the environment of the agar medium (that may occur for reasons that are independent of the flies' presence). This is subtracted from the change in weight to give the overall consumption reading. Graphs are plotted for the overall **Consumption (mg/fly/day)** v/s Days by first tabulating the initial and final weights, average of evaporation in excel sheet/Numbers application and then are plotted using the GraphPad Prism plotting software.

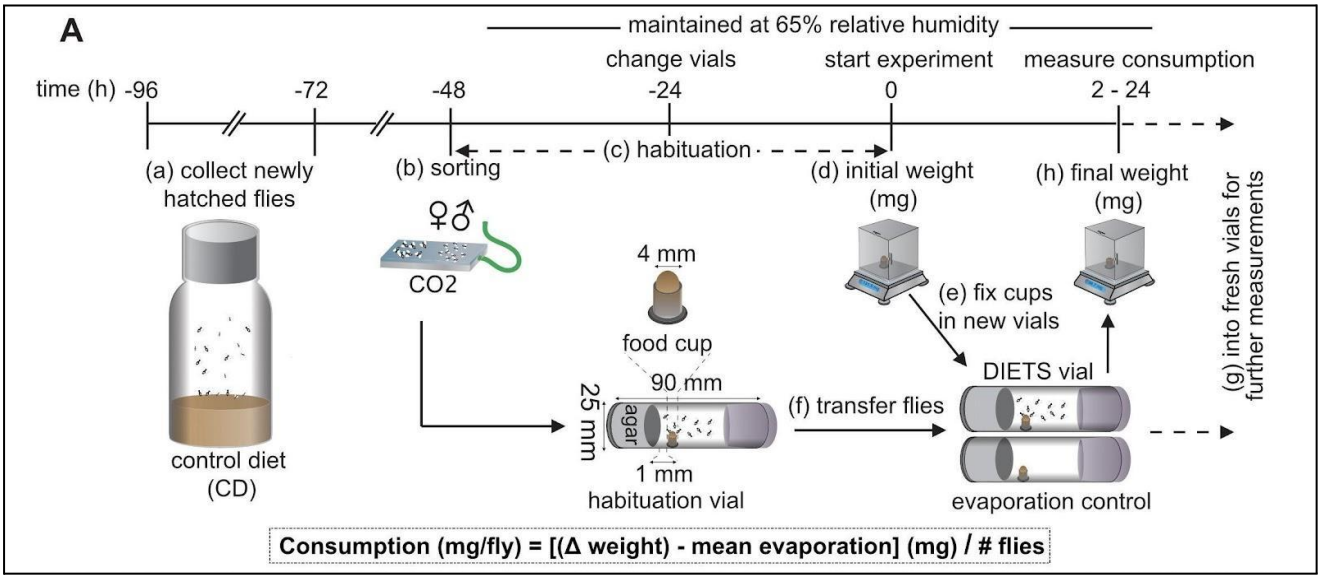


Image 2.1.1: Schema for the simple DIETS assay developed in our lab to measure and quantify solid food consumption in *Drosophila (Thakare et al 2023)*

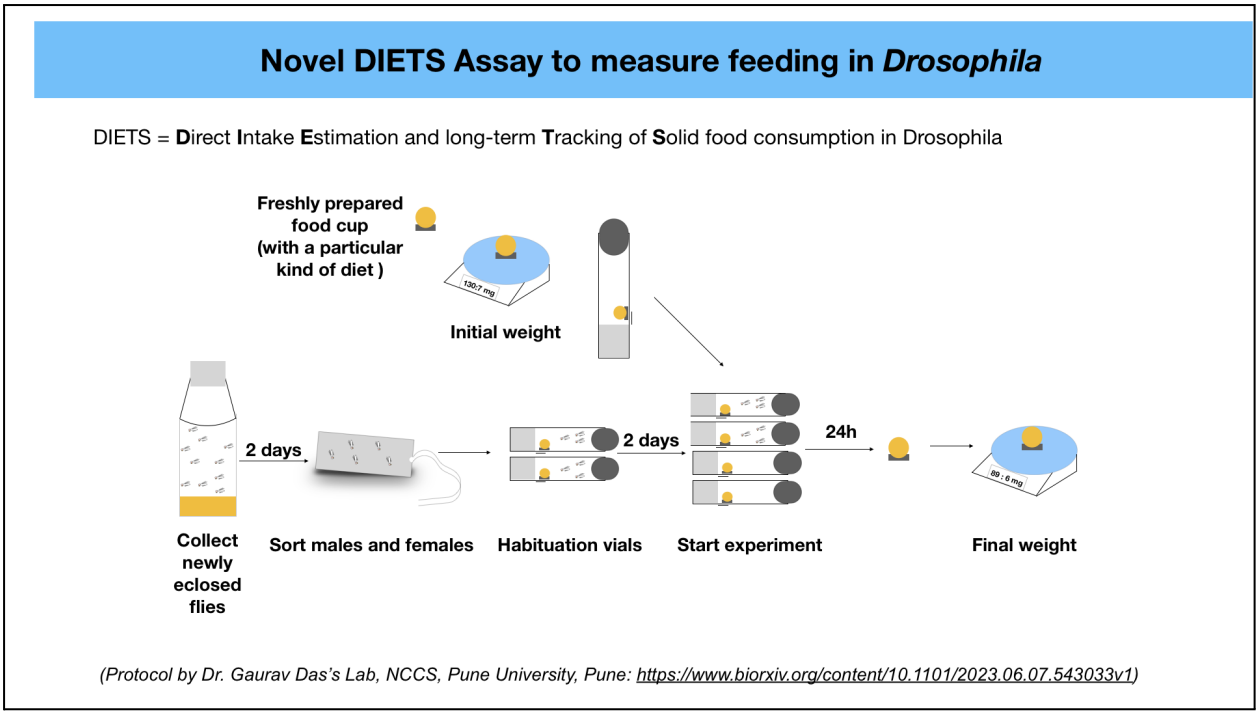


Image 2.1.2: A cartoon schematic demonstrating the DIETS assay in a simplified way

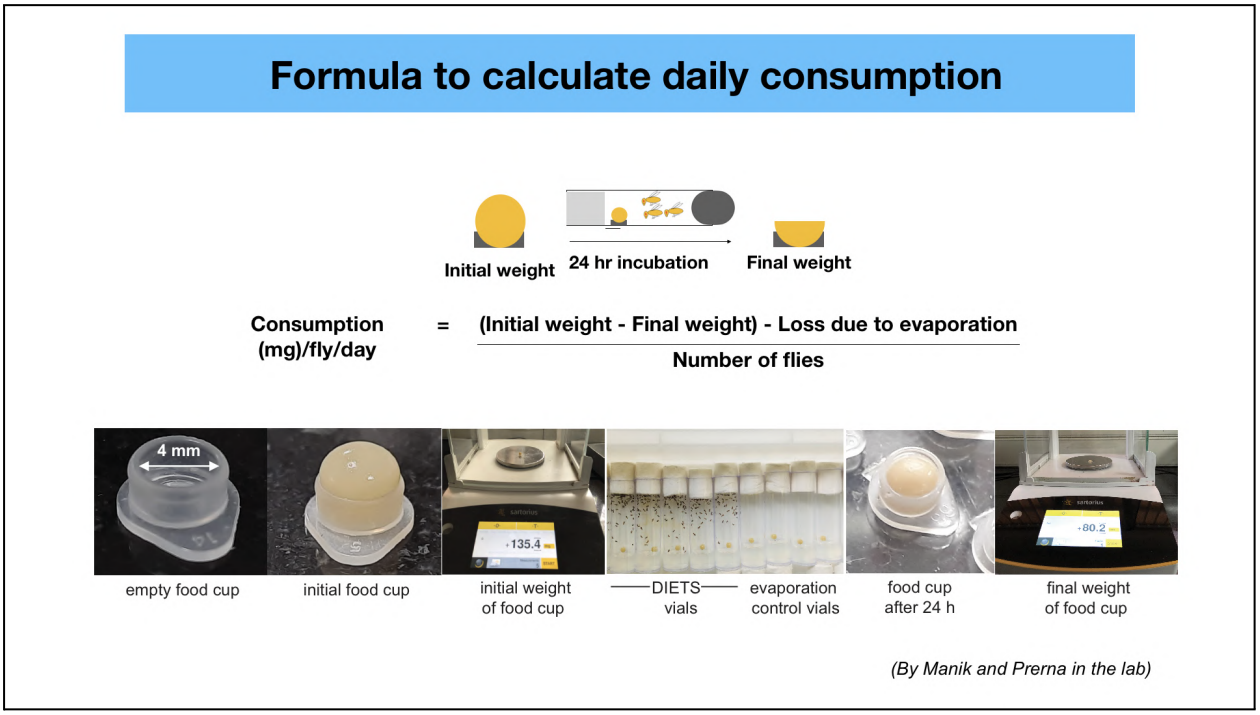


Image 2.1.3: above-Formula for total solid food Consumption (mg/fly/day) in the DIETS assay, below (inset)- a real-life picture demonstration of the DIETS assay

2.2 Preparing of various P:C ratio diets

In order to look at how proteins and carbohydrates play a role in driving the feeding behavior in fruit flies, the task at my hand was to prepare diets to provide to the flies such that the diet is:

1) **Nutritionally balanced**- i.e., the diet has all the major macronutrients (namely carbohydrates, proteins and fats) and micronutrients (minor amounts of vitamins and minerals) such that the flies are healthy and not deprived/starved of any of these major components in our diets
2) **Alterable in its protein and carbohydrate contents-** i.e. I wanted to select for a diet in which the protein and carbohydrate contents are well defined and standardized to support the growth of flies such that the components can be played around with/altered/manipulated easily in terms of its protein and carbohydrate contents such that I could achieve different P:C ratios in order to check feeding behavior
3) **Preservable**- i.e., the diet given to the flies has the right antifungals and antibacterials (which do not interfere with the taste/appetite of the diet) such that there is no bacterial or fungal growth in the food of our interest and thus, doesn't lead to any infections in our flies
4) **Solid**- i.e., I wanted to make a diet that is not liquid/semi-solid but one that can be given in our DIETS assay setup as mentioned above. I thus, wanted to have a solid form of the diet and in our lab I achieve this by using 1% agar (Agar-Agar Type I- see Methods section in Appendix) in all my diets (refer to the tables of diet contents that I mention below for a detailed description of how I prepared these diets
5) **Feasible to make, easy to handle and store**- i.e. I wanted to select for a diet with components that are easily accessible/orderable at an efficient cost, that is feasible to use in the cups of our DIETS setup, is possible to make in large quantities at one-go such that it is easy to store over days and can be reheated (in order to pour in the food cups to be given to the fruit flies for measuring their 24-hour feeding- refer to the DIETS assay section above)

With all of these necessities in mind, we surveyed the literature and found a diet that accounts for all the above-mentioned factors. This is called a semi-defined diet as mentioned in the table below (Musselman et al. 2011).
The components of this diet as exactly mentioned in/taken from (Musselman et al. 2011). Exactly 1L of this food has agar (solidifying agent, a substance that gives jelly-like consistency to the food), Brewer's yeast (a source of ~60% proteins and ~40% carbohydrates), Yeast extract and peptone (largely protein sources), sugar in the form of sucrose (purely carbohydrate), Magnesium sulfate and Calcium chloride (sources of micronutrients) and finally, propionic acid and mold inhibitor (antibacterial and antifungal agents) in the following standardized quantities (refer Appendix- star reagents for product numbers of each of these exact constituents used). Kindly note that the exact amount of proteins, carbohydrates and fats are specified in gms/L in each of these components and

also the total kcalories received from the consumption of these exact specified amounts from each of these components in the diets has been mentioned in kcalories/L

		carbs (g)	protein (g)	fat (g)	total kcal
control					
agar	10g	8.9		0.1	
brewers yeast	80g ←	32.0	36.0	0.8	
yeast extract	20g ←	3.3	10.8	0.0	
peptone	20g ←	0.1	14.6	0.0	
sucrose	51g	51.0	0.0	0.0	
$MgSO_{4\,x}\ 6H_2O$					
$CaCl_2$ x $2H_2O$					
propionic acid		6.0			
mold inhibitor		11.0			
% carbs	63.9%				
%fat	1.1%				
%protein	34.0%				
total grams		112.3	61.4	0.9	
total kcal		449.2	245.7	7.7	702.6

Table 2.2.1: Reference diet (*Musselman et al. 2011*) used for all dietary manipulations done in this thesis

Taking this standardized recipe for making diets from the literature (Musselman et al. 2011), I converted the above-mentioned table into the following form to calculate and note the P:C ratio. Observe that the P:C ratio of this diet comes out to be 1:1.8 ~ 1:2 and has 702.6 kcalories/L (kJ/L = kcalories/L) out of which 450 kcalories are coming from carbs and 245 kcalories are coming from proteins. The same table from (Musselman et al. 2011) has been depicted as:

	For 1L of diet:	Weight (gm/L)	Carbs (gm/L)	Proteins (gm/L)	Fats (gm/L)
Control Diet from Musselman et al	Agar	10	8.9		0.1
	Brewer's Yeast	80	32	36	0.8
	Yeast Extract	20	3.3	10.8	
	Peptone	20	0.1	14.6	
	Sucrose	51	51		
			112.3	61.4	0.9
	P:C	**0.546**			
	1:1.8				
	Total Calories (g/L or kJ/L)	**702.6**	449.2	245.6	7.65

Table 2.2.2: Calculating P:C ratio for Control diet from *Musselman et al. 2011*

But for the purpose of my experiments, my aim was to measure and quantify feeding in flies when given different P:C ratios, and thus, I wanted to achieve various ratios of P:C for this purpose. I took the above-mentioned Control diet as used in Musselman and played around with the calculations of various protein and carbohydrate ratios by altering the major sources of proteins and carbohydrates like Brewer's Yeast, Yeast extract and Peptone (refer to the tables and paragraphs above for exact constituent amounts of these). I wanted to check how the ratios of P:C get altered when these components are either increased or decreased by ½ folds, ¼ folds, 2 folds, 4 folds and so on. What happens when I decrease/increase these components even further, say to 1/16 times or 1/32 times? I was curious to look at how these components together play a role to give different P:C ratios. This is not straightforward because some of the constituents that go into our diets are not a pure source of protein/carbohydrates but have an equal or varied proportion of each of these- for e.g., Brewer's Yeast is 60% protein and 40% carbs but peptone and yeast extract are largely (~80-90% proteins). The source of sugar that is used here is sucrose which is always a pure source of carbohydrate(Jéquier 1994).
So, the question was how does the P:C ratio change when I alter these components by various factors? What happens to the P:C ratio when protein is decreased/increased from its basal amount as described above? What happens when I alter the pure source of carbohydrate that is sucrose? What ratios are achieved and what constituents do majorly govern and affect the P:C ratios?
How many times do I alter these well-defined constituents in order to get specific P:C ratios? I came across the answer to all of these questions when I played around with different quantities and calculations of the various components in the fixed diet referred from the literature (Musselman et al. 2011)(the record of which has been documented and described in the Appendix- Extra-supplementary Calculations of P:C section below). Please refer to the appendix section for viewing all the calculations I did and the different ratios I achieved to finally be able to decide on the following 5 ratios for the purpose of my experiments as mentioned below for checking drosophila feeding:

2.3 Choosing particular P:C ratios to use for my experiments

Based on previous literature (K. P. Lee et al. 2008; K. P. Lee 2015; Rushby et al. 2023), I decided to make the ratios that will range from lower P:C to higher P:C. In the literature, the selective few studies like Rushby et al use 1:1, 1:4 and 1:16 P:C ratio diets to measure carbohydrates and protein but it is not exactly the same kind of diet I use for exactly 2 reasons:

1) The diet they use is in liquid form and the feeding is measured using a CAFE setup (Deshpande et al. 2014; Ja et al. 2007).
2) The carbohydrates and protein intake that they measure is always in the context of a third parameter like lifespan, lifetime egg production and egg production rate. They always measure feeding so as to check the effects of protein and carbohydrates on one of these three parameters as mentioned above. And so, they are not just measuring and quantifying protein and carbohydrates intake alone for a 24 hours feeding.

Thus, for the purpose of my experiments, where my aim was to measure and quantify feeding for checking how much proteins and carbohydrates are consumed within 24 hours of feeding and more importantly whether or not these constituents affect how much of a particular kind of total diet is consumed by an organism for 24 hours using the novel DIETS assay developed in our lab (details of which have been described above).

So, with these goals in mind and the known background from the literature,
I decided to make 1:1, 1:2, 1:4, 1:8 and 1:16 P:C ratios. I made the additional 1:2 and 1:8 P:C ratios too as I wanted to take a closer look at the feeding trend even in these intermediate diets. These intermediate ratios would also enable me to look at the feeding in a further nuanced manner.

Also, if one wonders, like even I did before making these diets, why cannot we just give pure sources of proteins and carbohydrates alone to our flies and check feeding? Wouldn't it be easier then to see how much protein and carbohydrates they consume? Wouldn't it directly tell us how much protein and carbohydrates they need every day? And like all other answers in biology, this too, is not that straightforward. Flies need both proteins and carbohydrates to survive. In fact, we know this empirically in the lab through our experiments, that flies do not survive in a pure protein diet (without any source of carbohydrates) at all beyond 1-2 days. Thus, for this very reason we know that flies need both proteins and carbohydrates for their healthy survival and growth. (Wells 2021; Raubenheimer, Simpson, and Couteur 2020; S. J. Simpson and Raubenheimer 2005; Raubenheimer and Simpson 2019; K. P. Lee et al. 2008)

Now, to achieve these exact ratios, I first thought to alter the quantities of various components in the diet of our interest in the literature (cite Musselman) mentioned above. I realized that the diet in (Musselman et al. 2011) has a P:C ratio of roughly 1:1.8 i.e., ~1:2. Thus, I thought I should have a basal control diet that first has equal proportions of P:C and so, I thought of starting out with making a 1:1 P:C diet. With simple math and logic, I achieved the basal 1:1 diet by doubling all the constituents of protein in the known (Musselman et al. 2011) diet as it had exactly half the proportion of proteins than carbohydrates. Thus, keeping the pure carbohydrate source (sucrose) constant as it is- 51 gm/L, I doubled all the sources of protein in the known diet (Musselman et al. 2011) namely Brewer's Yeast (from 80 to 160 gm/L), Yeast Extract and Peptone (both from 20 to 40 gm/L). And then, with all this, the exactly 1:1 diet that I get (with all its exact amounts of constituents given in the table below) can now be called as the new 'Basal Control Diet-P:C 1:1' as follows:

	For 1L of diet:	Weight (gm/L)	Carbs (gm/L)	Proteins (gm/L)	Fats (gm/L)
Basal Control Diet- P:C (1:1)	Agar	10	8.9		0.1
	Brewer's Yeast	160	64	72	1.6
	Yeast Extract	40	6.6	21.6	
	Peptone	40	0.2	29.2	
	Sucrose	51	51		
			130.7	122.8	1.7
	P:C	0.9395562357			
		1.064332248			
	1:1				
	Total Calories (g/L or kJ/L)	1028.45	522.8	491.2	14.45

Table 2.3.1: Achieving P:C ratio of 1:1- Basal diet (1L)

Now, to achieve the other 1:2, 1:4, 1:8 and 1:16 diets, I took this new Basal Control 1:1 diet and reduced all the protein constituents by 3 folds, 7 folds, 15 folds and 32 folds respectively (keeping the pure carbohydrate source, sucrose, to the constant 51 gm/L amount). How did I know I had to reduce protein constituents by exactly these amounts? I tried dividing and reducing proteins by all different whole numbers sequentially starting from 2, 3,....all the way till 32 and checked what happens to the ratio of P:C This has been shown in the forms of tables in the Appendix section of this thesis below. Kindly refer to the Appendix for all the other calculations made and further for further clarity and understanding on this.

	For 1L of diet:	Weight (gm/L)	Carbs (gm/L)	Proteins (gm/L)	Fats (gm/L)
P/3, C constant	Agar		8.9		0.1
	Brewer's Yeast	53.3	21.33	24	0.533
	Yeast Extract	13.3	2.2	7.2	
	Peptone	13.3	0.06	9.733	
	Sucrose	51	51		
			83.49	40.933	0.633
	P:C	0.4902742843			
		2.03967459			
	1: 2				
	Total Calories (g/L or kJ/L)	503.0725	333.96	163.732	5.3805

Table 2.3.2: Achieving P:C ratio of 1:2 by doing P/3 and keeping C constant from the 1:1 basal diet (1L)

	For 1L of diet:	Weight (gm/L)	Carbs (gm/L)	Proteins (gm/L)	Fats (gm/L)
P/7, C constant	Agar	10	8.9		0.1
	Brewer's Yeast	22.857	9.142857	10.2857	0.22857
	Yeast Extract	5.7142857	0.942857	3.0857	
	Peptone	5.7142857	0.028571	4.1714	
	Sucrose	51	51		
			70.014285	17.5428	0.32857
	P:C	0.2505602964			
		3.991055305			
	1: 4				
	Total Calories (g/L or kJ/L)	353.021185	280.05714	70.1712	2.792845

Table 2.3.3: Achieving P:C ratio of 1:4 by doing P/7 and keeping C constant from the 1:1 basal diet (1L)

	For 1L of diet:	Weight (gm/L)	Carbs (gm/L)	Proteins (gm/L)	Fats (gm/L)
P/15, C constant	Agar	10	8.9		0.1
	Brewer's Yeast	10.66666	4.2	4.8	0.106
	Yeast Extract	2.666	0.44	1.44	
	Peptone	2.666	0.0133	1.9466	
	Sucrose	51	51		
			64.5533	8.1866	0.206
	P:C	0.1268192331			
		7.885239293			
	1: 8				
	Total Calories (g/L or kJ/L)	292.7106	258.2132	32.7464	1.751

Table 2.3.4: Achieving P:C ratio of 1:8 by doing P/15 and keeping C constant from the 1:1 basal diet (1L)

	For 1L of diet:	Weight (gm/L)	Carbs (gm/L)	Proteins (gm/L)	Fats (gm/L)
P/32, C constant	Agar	10	8.9		0.1
	Brewer's Yeast	5	2	2.25	0.05
	Yeast Extract	1.25	0.20625	0.675	
	Peptone	1.25	0.00625	0.9125	
	Sucrose	51	51		
			62.1125	3.8375	0.15
	P:C	0.06178305494			
		16.18566775			
	1:16				
	Total Calories (g/L or kJ/L)	265.075	248.45	15.35	1.275

Table 2.3.5: Achieving P:C ratio of 1:16 by doing P/32 and keeping C constant from the 1:1 basal diet (1L)

The above quantities were for 1L food, but now, to achieve the ratios 1:1, 1:2, 1:4, 1:8 and 1:16 diets for 50 ml of food, I did the corresponding calculations as follows:

	For 50 ml of diet:	Weight (gm/50 ml)	Carbs (gm/50ml)	Proteins (gm/ 50ml)	Fats (gm/50ml)
Basal Control Diet- P:C (1:1)	Agar	0.5	0.445		0.005
	Brewer's Yeast	8	3.2	3.6	0.08
	Yeast Extract	2	0.33	1.08	
	Peptone	2	0.01	1.46	
	Sucrose	2.55	2.55		
			6.535	6.14	0.085
	P:C	0.9395562357			
		1.064332248			
	1:1				
	Total Calories (g/L or kJ/L)	51.4225	26.14	24.56	0.7225

Table 2.3.6: Achieving P:C ratio of 1:1- Basal diet (50 ml)

	For 50 ml of diet:	Weight (gm/50 ml)	Carbs (gm/50 ml)	Proteins (gm/50ml)	Fats (gm/50 ml)
P/3, C constant	Agar	0.5	0.445		0.005
	Brewer's Yeast	2.665	1.0665	1.2	0.02665
	Yeast Extract	0.665	0.11	0.36	
	Peptone	0.665	0.003	0.48665	
	Sucrose	2.55	2.55		
			4.1745	2.04665	0.03165
	P:C	0.4902742843			
		2.03967459			
	1: 2				
	Total Calories (g/L or kJ/L)	25.153625	16.698	8.1866	0.269025

Table 2.3.7: Achieving P:C ratio of 1:2 by doing P/3 and keeping C constant from the 1:1 basal diet (50 ml)

	For 50 ml of diet:	Weight (gm/50 ml)	Carbs (gm/50 ml)	Proteins (gm/50ml)	Fats (gm/50 ml)
P/7, C constant	Agar	0.5	0.445		0.005
	Brewer's Yeast	1.14285	0.45714285	0.514285	0.0114285
	Yeast Extract	0.285714285	0.04714285	0.154285	
	Peptone	0.285714285	0.00142855	0.20857	
	Sucrose	2.55	2.55		
			3.50071425	0.87714	0.0164285
	P:C	0.2505602964			
		3.991055305			
	1: 4				
	Total Calories (g/L or kJ/L)	17.65105925	14.002857	3.50856	0.13964225

Table 2.3.8: Achieving P:C ratio of 1:4 by doing P/7 and keeping C constant from the 1:1 basal diet (50 ml)

	For 50 ml of diet:	Weight (gm/50 ml)	Carbs (gm/50 ml)	Proteins (gm/50ml)	Fats (gm/50 ml)
P/15, C constant	Agar	0.5	0.445		0.005
	Brewer's Yeast	0.533333	0.21	0.24	0.0053
	Yeast Extract	0.1333	0.022	0.072	
	Peptone	0.1333	0.000665	0.09733	
	Sucrose	2.55	2.55		
			3.227665	0.40933	0.0103
	P:C	0.1268192331			
		7.885239293			
	1: 8				
	Total Calories (g/L or kJ/L)	14.63553	12.91066	1.63732	0.08755

Table 2.3.9: Achieving P:C ratio of 1:8 by doing P/15 and keeping C constant from the 1:1 basal diet (50 ml)

	For 50 ml of diet:	Weight (gm/50 ml)	Carbs (gm/50 ml)	Proteins (gm/50ml)	Fats (gm/50 ml)
P/32, C constant	Agar	0.5	0.445		0.005
	Brewer's Yeast	0.25	0.1	0.1125	0.0025
	Yeast Extract	0.0625	0.0103125	0.03375	
	Peptone	0.0625	0.0003125	0.045625	
	Sucrose	2.55	2.55		
			3.105625	0.191875	0.0075
	P:C	0.06178305494			
		16.18566775			
	1:16				
	Total Calories (g/L or kJ/L)	13.25375	12.4225	0.7675	0.06375

Table 2.3.10: Achieving P:C ratio of 1:16 by doing P/32 and keeping C constant from the 1:1 basal diet (50 ml)

"I take all this and cook"
But in Lab language I will rather say:

Once these desired ratios are achieved, I follow the following procedure and protocol used in our lab for preparing and cooking these diets:

1) Take 5 different 50 ml falcons and label each of them as **1:1 P:C, 1:2 P:C, 1:4 P:C, 1:8 P:C and 1:16 P:C** (apart from my name initials and date on each label)
2) Weigh all of these specified quantities of agar, Brewer's yeast, Yeast extract, peptone and sucrose (which are in powder form) as mentioned above using an analytical balance

3) Add all these components one by one as per their specific quantities in each of the differently labeled 50ml falcons and mix
4) Once the powdered forms of all of these components are added in the right quantities to the 50 ml falcon, add RO water to make up for the remaining volume till the 50ml mark in the falcon!
5) Now take a glass beaker and keep each of your falcons into it (without the caps on so that the heat and pressure is not built while heating/cooking the food)
6) Keep the falcons along with these beakers in the microwave at 900 watt power setting for about 20-30 secs
7) Continuously monitor the simmering of food and keep your eye until the food boils (the food will simmer and start to boil out of the falcon)
8) Stop the microwave as soon as you observe the boiling of the diets
9) Take the beakers along with the falcon containing diets out and let them cool for 10-15 mins until room temperature
10) Once the food is back to normal room temperature, add the following micronutrients, antifungal and antibacterial agents in the specific quantities as mentioned below:

Name of the Reagent	Usage/Role in the diet	Amount to be used (/30 ml) of diet	Amount to be used (/10 ml) of diet	Amount to be used (/50 ml) of diet
Propionic Acid	Antimicrobial/Antibacterial	180 ul	60 ul	300 ul
Methyl Paraben	Antibacterial/Antifungal	300 ul	100 ul	500 ul
$CaCl_2$	Micronutrients-Calcium & Chlorine	102 ul	34 ul	170 ul
$MgSO_4$	Micronutrients-Magnesium & Sulphur	60 ul	20 ul	100 ul

Table 2.3.11: Antibacterial and antifungal agents that have to be added to the diets to increase their shelf-life

- The anti-fungal and antibacterial agents like propionic acid and methyl paraben mentioned above are important to be added to the food because they increase the shelf-life of the diets which enables us to store the food prepared for *Drosophila* for as long as 1-2 months when refrigerated at 4°C
- Further, reagents like **$CaCl_2$** and **$MgSO_4$** are added to supplement micronutrients like calcium and magnesium to the diets

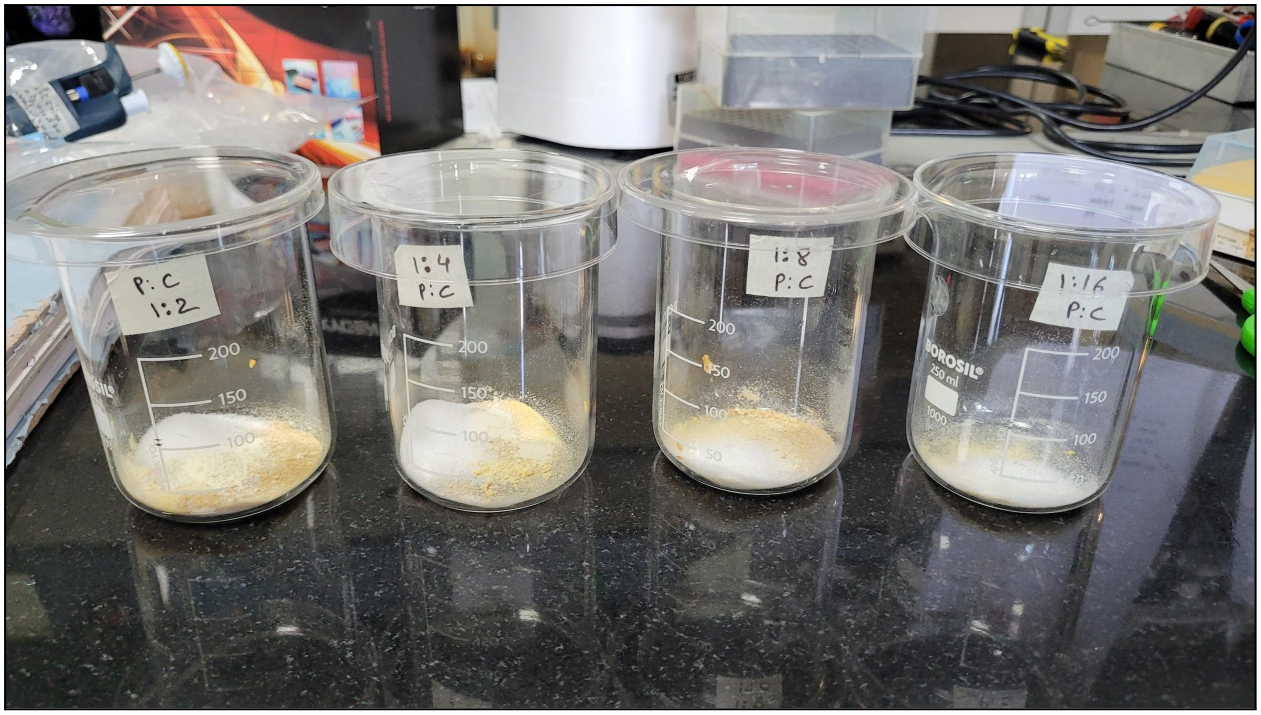


Image 2.3.1: Picture showing the making of different ratios of P:C diets- beakers with all the above tabulated contents for the various 1:2, 1:4, 1:8 and 1:16 P:C ratio diets to be made up for 100 ml volume of water. Note how the 1:2 P:C diet has the maximum amounts of all the contents as compared to 1:4, 1:8 or 1:16 P:C diets in a descending order.

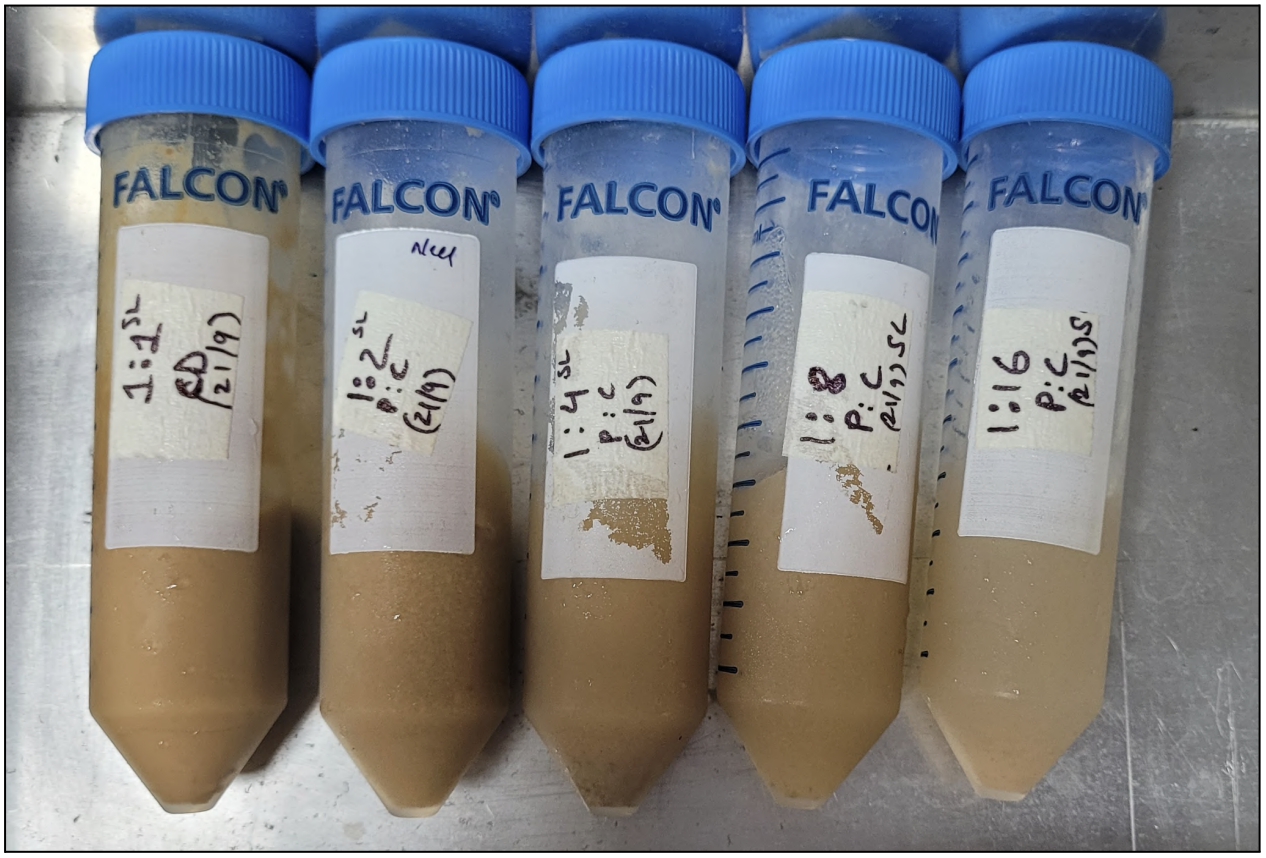


Image 2.3.2: Picture showing 50 ml falcons with the various 1:1, 1:2, 1:4, 1:8 and 1:16 P:C ratio diets to be used in the DIETS assay

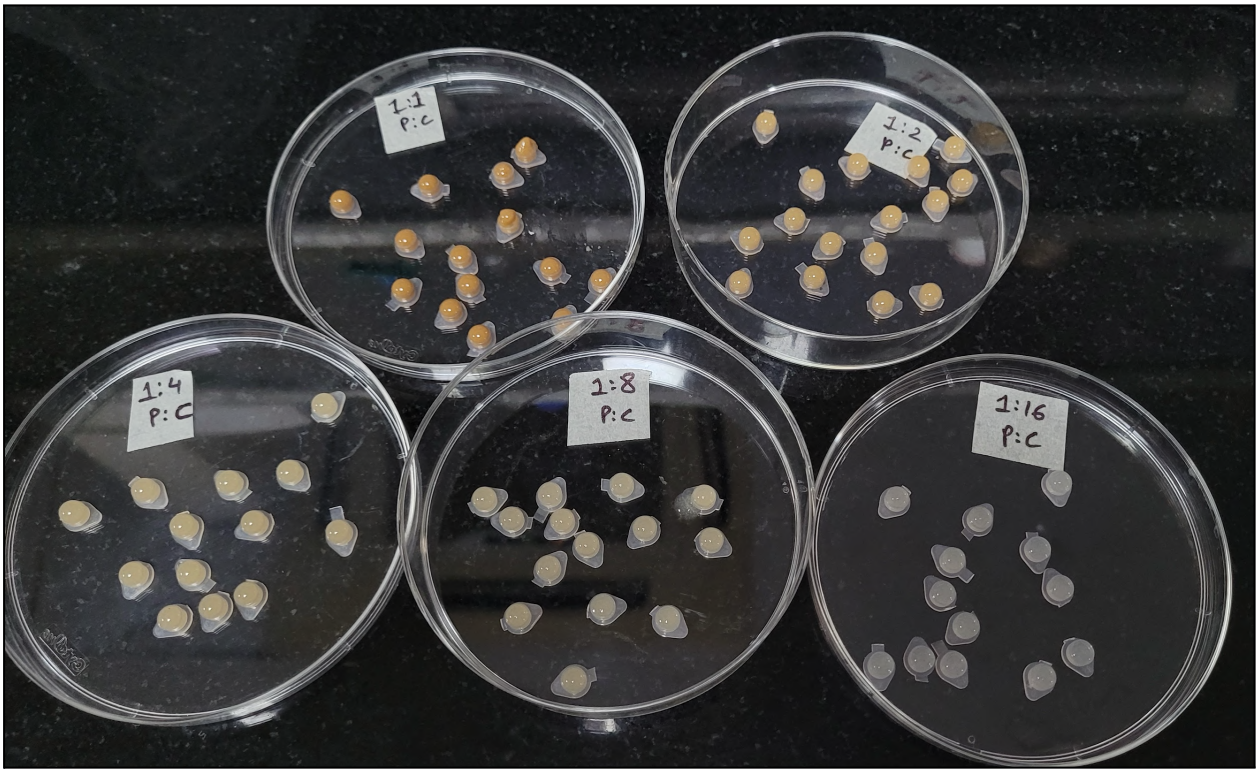


Image 2.3.3: Picture showing food cups (made out of 1 ml eppendorf tube caps) with the various 1:1, 1:2, 1:4, 1:8 and 1:16 P:C ratio diets to be weighed and given to the flies in the DIETS assay

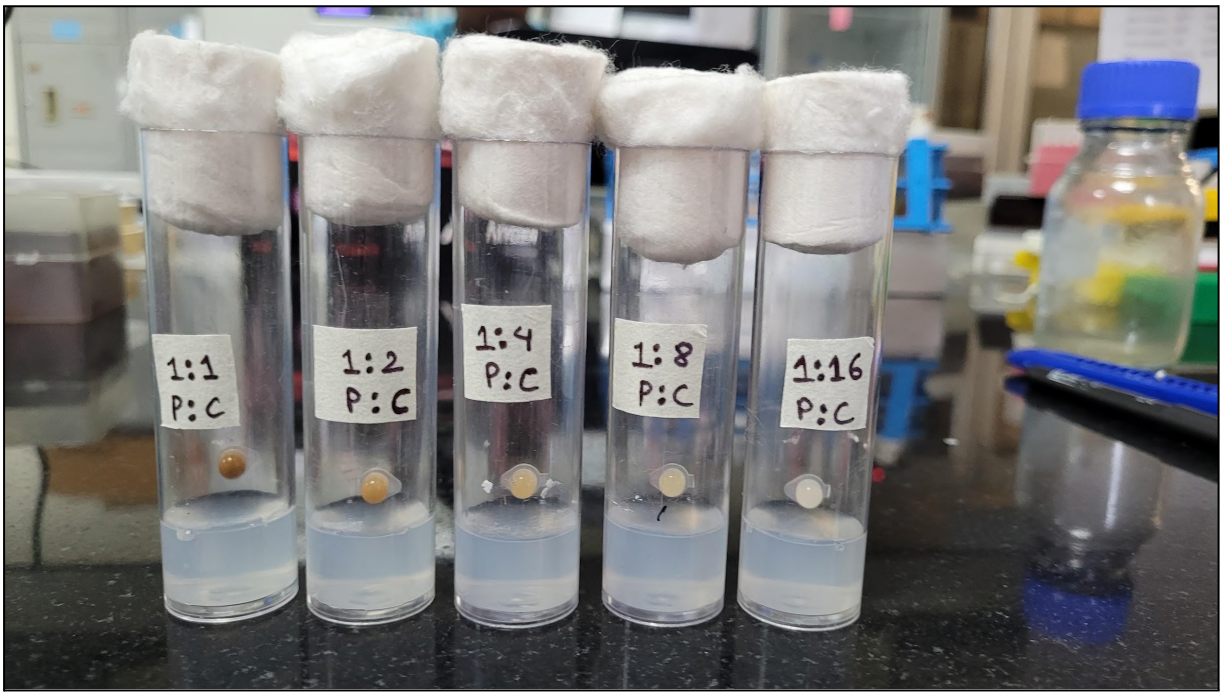


Image 2.3.4: Picture depicting how the weighed food cups with various labeled 1:1, 1:2, 1:4, 1:8 and 1:16 P:C ratio diets are stuck to the walls of the vials in a DIETS setup to be given to the flies

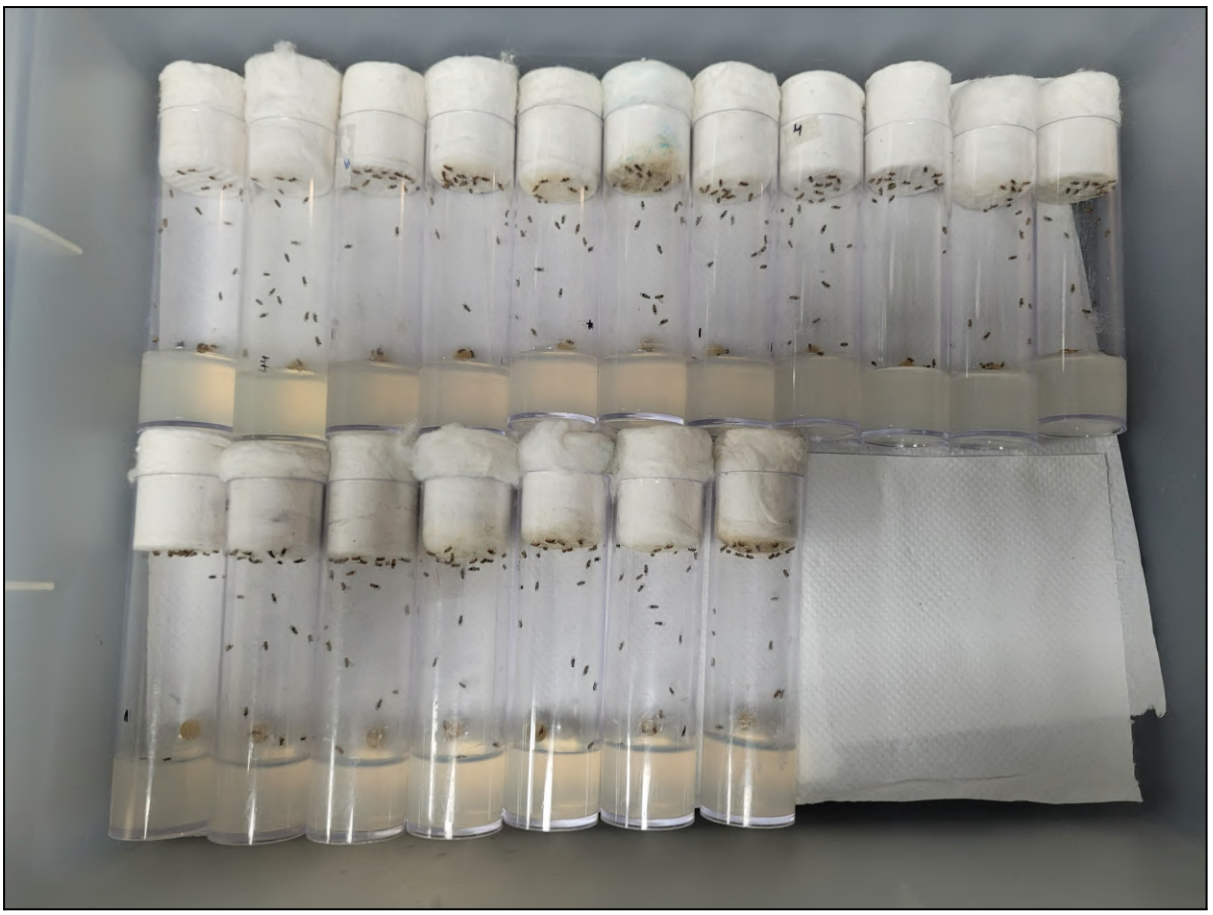


Image 2.3.5: Picture showing the DIETS vials with 20-22 male flies given the various ratios of P:C food cups

2.4 Evaporation Standardization and Troubleshooting (Major Challenge and Obstacle in the project)

The problem/challenge:

A major part (2-3 months) of my Master's thesis term post mid-year was about troubleshooting and standardizing the evaporation values of my diets. This was especially tricky because empirically (through my experience as well as the experience of other members in the lab working on other different kinds of diets) and with the basic understanding of chemistry, we would expect that each kind of diet would have a different evaporation value based on its consistency, texture and number of molecules present in the diet. Thus, by simple logic and basic principles of chemistry:

- a diet that is thick/dense has a higher concentration i.e., higher number of constituent molecules per unit volume and so, will allow for lesser escape of water molecules (nothing but evaporation) per unit volume of diet. Thus, a thick/dense diet will always have a lower rate of evaporation. In fact, by experience of working with other diets in our lab: we know that diets that have a greater number of molecules and are thicker like the high sugar diet (Control diet+0.5 M sugar) tends to be hygroscopic- i.e., it absorbs higher amounts of water

- as opposed to a diet that is thin/less dense/highly diluted in terms of its constituents will have a lower concentration i.e., a smaller number of molecules per unit volume (as compared to a thicker diet) and so, will allow for much easier escape of water molecules (nothing but evaporation) per unit volume of diet. Thus, a thin/less dense/highly diluted diet will always have a higher rate of evaporation than a thick/dense diet.

Thus, with this simple logic and the above given explanation, I could expect the rate/amount of evaporation trend in my diets as follows:

Trend in consistency/thickness/density in my diets of interest (from the most dense to the most diluted) = 1:1 P:C < 1:2 P:C <1:4 P:C < 1:8 P:C <<<1:16 P:C

Thus, the trend in amount/rate of evaporation in my diets of interest = 1:1 P:C < 1:2 P:C <1:4 P:C < 1:8 P:C <<<1:16 P:C

We have already seen in the Consumption (mg/fly/day) formula (mentioned below) that the real catch and game-changer in the formula is the value of evaporation! This is because with different evaporation rates in my diets, the consumption value that I would get would also be highly variable and may even hinder my actual consumption values.

$$\textbf{Consumption (mg)/fly/day} = \frac{\textbf{[(Initial weight - Final weight) - Average Evaporation value]}}{\textbf{Total number of flies}}$$

Note that we can neither have evaporation too high nor too low than the amount of consumption because:

- If the evaporation is higher/greater than the actual consumption value it will lead to a resultant negative value of total consumption and total food consumed cannot be negative or less than 0!
- If the evaporation is highly lower/lesser than the actual consumption value, this too is impossible and would be too realistic!

Thus, only a small value of evaporation that is not greater than the total food consumed is possible! But this statement is still vague: how much evaporation exactly is too much or too little? What if the flies eat and consume different/same amounts of food in each of these diets, then how will we address the problem of evaporation in that case? How much evaporation is okay and how much is not okay?

With all this, it was first important to make sure that the evaporation of each of these diets is brought down in a similar comparable range such that we would be able to make accurate feeding estimations and comparisons for all of these diets.

This challenge was very well tackled by deciding the evaporation value to be allowed only to be in fixed threshold value for all the diets. The fixed threshold value was decided to be in an acceptable range of <20% of the total food consumed for every individual diet! This rule of thumb has also been used and adapted from the previous DIETS papers from our lab. (Thakare et al 2023)

The solution:

$$\text{Consumption (mg/fly/day)} = \frac{[(\text{Initial weight} - \text{Final weight}) - \text{Average Evaporation value}]}{\text{Total number of flies}}$$

Threshold of evaporation allowed for each of the Diets = <= 20% of Total Consumption in each of the particular diets

Now, after deciding this allowed threshold of evaporation theoretically, the real challenge was to actually get the evaporation values of each of these diets within the permissible limits. How would I achieve that? What are the possible options I could deploy such that I am actually able to execute my experiments in a feasible manner without torturing/affecting the normal behavior of the flies? After many sessions of brainstorming, discussions with my mentor and PI, referring to previous literature on DIETS in the lab (Thakare et al 2023) different endless sessions of trials and errors, and experimenting with various parameters, I was able to come up with 3 potential feasible solutions to increase/decrease evaporation in a DIETS session:

1) Alteration at the level of quantity/concentration of water source (agar bed) inside the Vials:
    - Since % of agar used determines how much water is available to the flies, a higher % of agar used could provide more water within the vials and thus, decrease the rate/amount of evaporation from the food cup placed within this vial
    - However, the usage of the % of agar makes substantial difference only for other kinds of diets used in our lab like the Holidic diet (Piper et al. 2014) (an extensively cumbersome/difficult to use, type of well defined diet that is used to employ in sterile conditions as unlike my diets of interest, it doesn't have any processed sources of proteins like the Brewer's yeast /yeast extract/peptone but only direct sources of amino-acids), but for my kinds of diets, through the empirical experience of my mentor and others in the lab, it has been well established that % of agar only minimally changes the evaporation rates in complex diets such as the ones adapted from (Musselman et al. 2011) and like the ones I have prepared for my experiment
    - Thus, altering % of agar didn't seem to be very feasible and doable option in my case

2) Alteration at the level of distance at which the food cup is placed with respect to the water source i.e., agar bed:
    - Through previous work by my mentor in the lab, and through the diets paper from our lab, (Thakare et al 2023), it has been well established that distance of the food cup from the agar bed increases/decreases evaporation from the food without affecting/influencing the feeding behavior of the fruit flies (yay, this is great!)
    - By simple common logic, increasing the distance of the food cup from the agar bed increases the rate/value of evaporation whereas decreasing the distance from the agar bed (i.e., placing the food cup extremely close ~at the most the feasible and technically possible distance of 1 mm decreases/minimizes evaporation to the lowest possible value
    - Thus, I could use this logic and place my food cups containing the diets with the highest rates of evaporation/i.e., the most diluted diets at the closest distance from the agar bed.
    - I used this option to deploy my troubleshooting further. This has been shown in the execution and the proof-of-concept sections below:

3) Alteration/modification to the humidity levels of the entire environment in which the vials and the DIETS setup is kept/stored at the start/end of the experiment
    - A huge challenge was the humidity controller in the fly incubators of our lab: the humidity in all the three per-existing incubators in my lab were not regulating and maintaining humidity in a constant range
    - The humidity was highly variable and giving rising to highly fluctuating evaporation as well as consumption values repeatedly in all of these incubators

- The humidity was highly variable and ranged from 60% all the way to 90%- which is very high (refer appendix for data I plotted to verify this fluctuating humidity giving rise to the high variability in my feeding data!)

With all this brainstorming, and trials and errors (based on my previous evaporation standardization experience working with a different ratio of 1/2xC, 1/2xP, 1xC, 1xP, 2xC and 2xP diets- as mentioned and summarized in the Appendix section at the end of this thesis), I used the following strategies to keep the evaporation values to <20% of the average food consumption in the Panasonic incubator (Low Humidity mode-40% humidity) in each of the cases:

1) Placing the **1:1 P:C food cup (most concentrated food) at a measured distance of exactly 3 mm** from the agar bed to avoid gain of water in the food cups (trials with all 1 mm, 2 mm and 3 mm distances with the highest concentration of diet shown in the Appendix section)
2) Placing the **1:2, 1:4, 1:8 P:C ratio food cups at the least distance possible from the agar beds i.e., at a measured distance of exactly 1 mm** along with an **elevated surface made out of pipette tip box stages inside a tray containing 100 ml** RO water (as shown in the images below) to reduce the evaporation under these DIET vials
3) Placing the **1:16 P:C ratio food cup (most dilute and hence having the highest amount of evaporation) at the least distance possible from the agar beds i.e., at a measured distance of exactly 1 mm** along with an elevated surface made out of pipette tip box stages inside a tray containing **100 ml RO water along with a plastic covering the entire tray** to reduce the evaporation further under these 1:16 P:C DIET vials
4) An open tray with 200 ml RO water to maintain constant humidity in the environment of the entire incubator

Note that 100 or 200 ml RO water was added by empirical experience: always 100 ml beneath an elevated surface and 200 ml when in an open surface in the tray!

The actual execution and successful proof-of-concept of all of this is as depicted with the help of a series of pictures and a table summarizing this evaporation standardization and troubleshooting below:

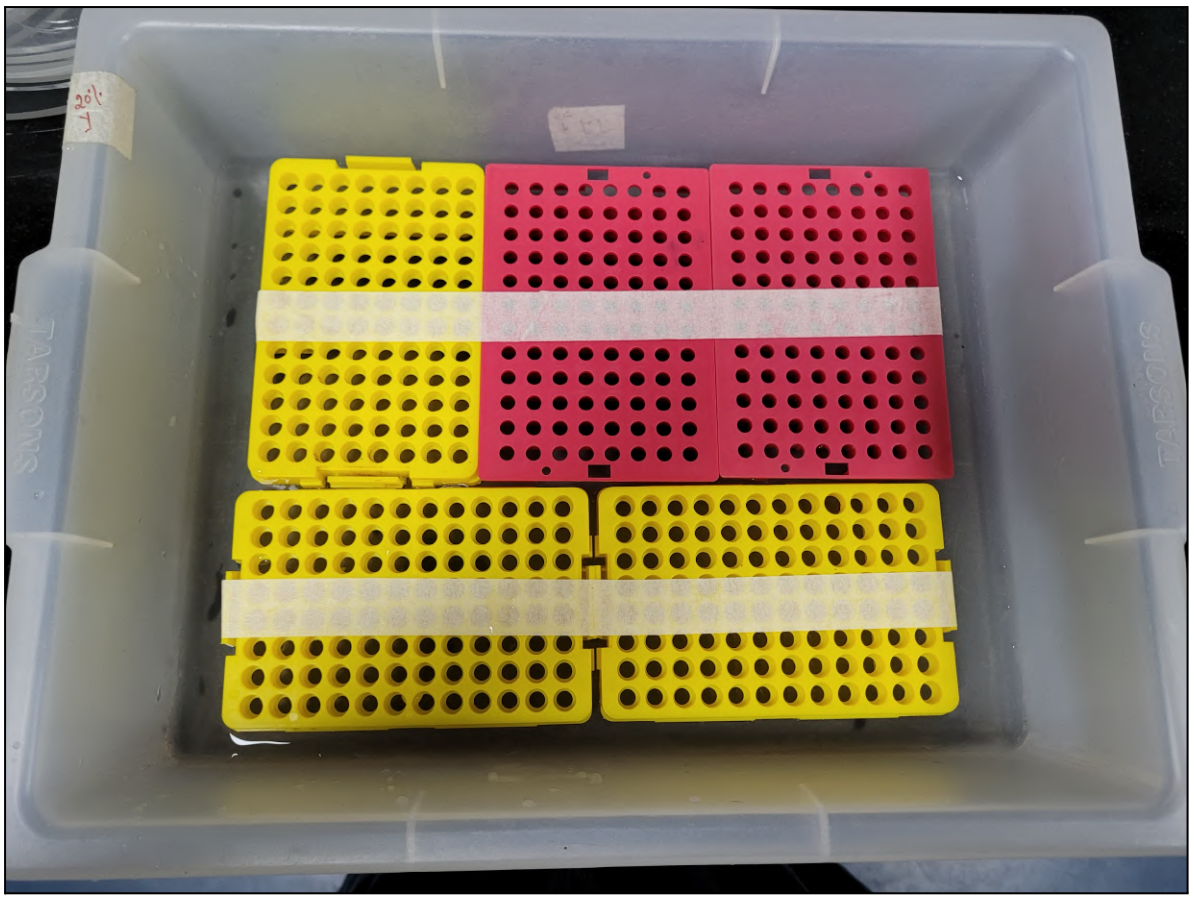


Image 2.4.1: Picture depicting the elevated surface made out of pipette tip box stages with 100 ml RO water beneath to reduce the evaporation such that the DIET vials do not get wet but have the humidity maintained through the big holes present in these stages

Image 2.4.2: Picture depicting the 10 serially labeled DIET vials containing 1:1 P:C food cups at a measured distance of exactly 3 mm from the agar beds. Note that these vials are not kept with any water/elevated stage beneath it as the purpose is to increase (and not decrease) evaporation in this case of highly concentrated P:C diets!

Image 2.4.3: Picture depicting the 10 serially labeled DIET vials for each of the containing 1:2 and 1:4 P:C food cups respectively at a measured distance of exactly 1 mm from the agar beds with an elevated surface and 100 ml RO water beneath.

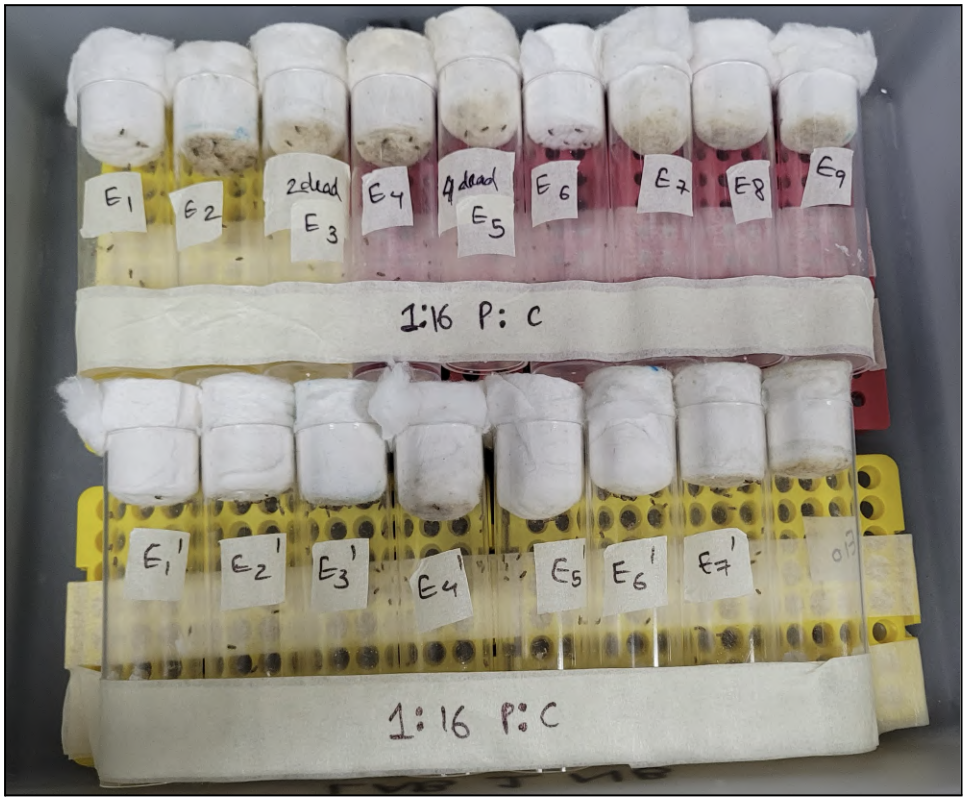


Image 2.4.4: Picture depicting the 20 serially labeled DIET vials for 1:16 P:C food cups at a measured distance of exactly 1 mm from the agar beds with an elevated surface and 100 ml RO water beneath. Plastic covering this tray will be added to reduce the evaporation down to the permissible threshold further. Extra vials have been kept for further evaporation standardization trials with this high evaporation-prone highly diluted food ratio of 1:16

Image 2.4.5: Picture depicting the Panasonic incubator shelf with all of the differently labeled and placed DIET vials for each of the 1:1, 1:2, 1:4, 1:8 and 1:16 P:C ratios with an elevated surface and 100 ml water beneath for all conditions except the 1:1 P:C. Note the 200 ml water in an open tray below to maintain a constant humidity in the entire incubator. 1:16 condition is not visible as it is covered in a plastic bag beneath.

*Also, the number of dead flies if any at all (permissible number of deaths/escapees being 1-2 only) are also noted and recorded in all of these vials.

2.5 Proof of concept/ working evidence for Evaporation Standardization:

A table summarizing the successful working proof-of-concept for all the above discussed strategies for the evaporation standardization and troubleshooting has been given below. Several trial and errors for all the different conditions and conclusions helped keep the evaporation values to the permissible threshold of <20% average consumption in all of the 1:1, 1:2, 1:4, 1:8 & 1:16 P:C cases as tabulated below:

P:C ratio type	Average Consumption (per mg/fly)	Average evaporation	Is the avg. Evaporation <20% avg. Consumption?	Conclusion!
1:1 P:C (3 mm distance)	12.6916666666667	0.675000000000001	**DEFINITELY!**	3mm distance of food cups works perfect for this protein rich diet!
1:2 P:C (1 mm distance)	16.5833333343333	0.549999999999997	**DEFINITELY!**	Elevated surface with 100ml water directly beneath in the same tray helps bring down evaporation!
1:4 P:C (1 mm distance)	16.625	2.675	**DEFINITELY!**	Elevated surface with 100ml water directly beneath in the same tray helps bring down evaporation!
1:8 P:C. (1 mm distance)	20.5583333333333	2.425	**DEFINITELY!**	Elevated surface with 100ml water directly beneath in the same tray helps bring down evaporation!
1:16 P:C. (1 mm distance)	14.675	4.02500000000001	**NO**	EVAPORATION STILL VERY HIGH! Troubleshoot by 200ml water.

Table 2.5.1: Proof of concept/working evidence for achieving evaporation standardization <=20% of average feeding for all of the 1:1, 1:2, 1:4 and 1:8, except for 1:16 P:C ratio diets

The evaporation value for the 1:16 P:C condition was brought down to the permissible threshold further as mentioned in the section and depicted in the images above by using a transparent plastic bag covering the tray containing the 1:16 condition DIET vials apart from

the regular 1 mm distance of food cup from the agar bed along with 100 ml water under the elevated surface of these DIET vials.

Chapter-3
Observations, Results and Conclusions

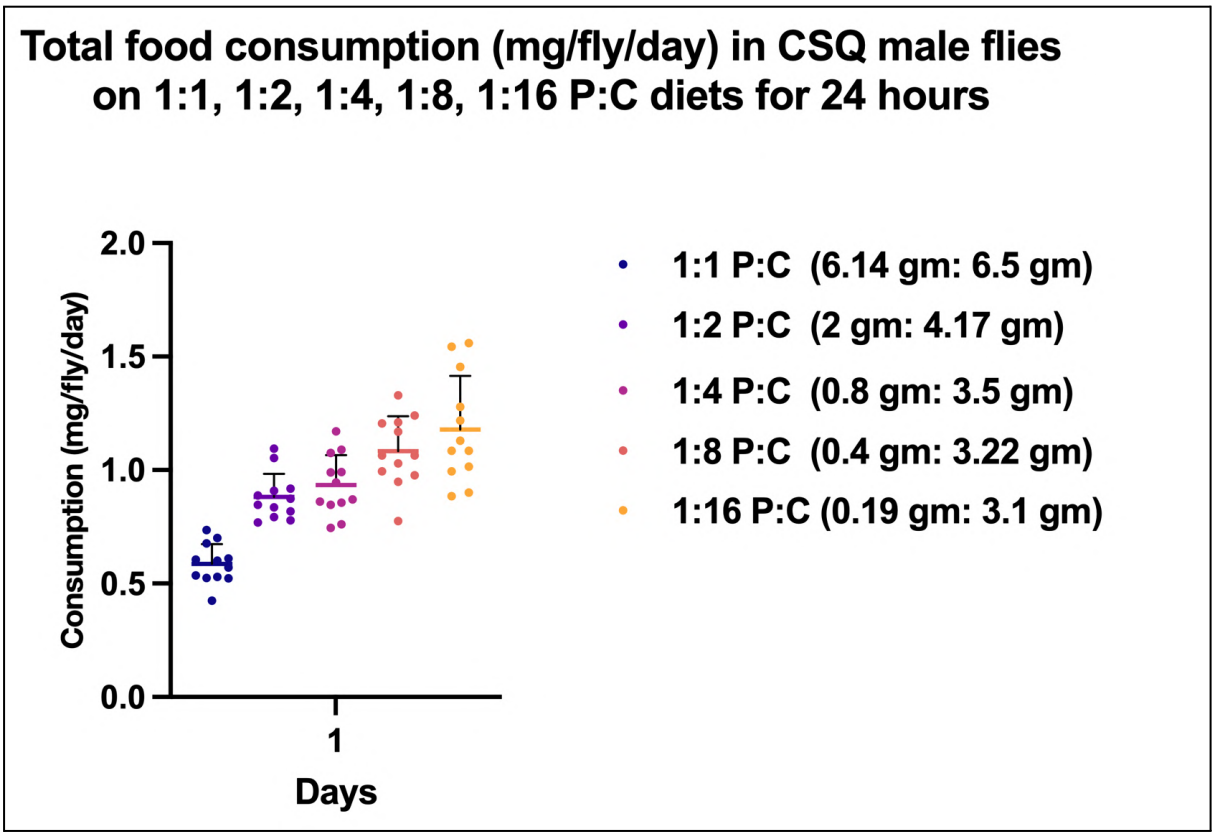
Observations, Results and key take-aways from this chapter:

-Total feeding or consumption plot-Trend in total feeding
-Comparison of trend in total feeding with *Rushby*
-Total carbohydrates consumption plot- Carbohydrates maintained
-Protein consumption plot- Proteins show a decreasing trend
-Total carbohydrates and protein consumption plot comparisons with *Rushby*
-Carbohydrates (but not proteins) maintained in our results
-Total calories consumed plot
-Total calories from Carbs/Proteins plot
-Preliminary data from 2-cup 2-choice Assay plots
-Total Carbohydrates consumed per vial in the 2-cup 2-choice Assay
-Total Proteins consumed per vial in the 2-cup 2-choice Assay
-clear preference for High P:C (but low carbs/mg diet over high carb/mg diet)
-Flies prefer high carb/ P:C ratio than high protein/P:C ratio

3.1 The total consumption of food (mg/fly/day) increases as the ratio of P:C increases
3.2 Feeding trend is comparable to the trend seen in *Rushby et al*
3.3 The total carbohydrates consumed in the different P:C diets seem to be more or less constant i.e., carbohydrate intake is maintained across the different P:C diets!
3.4 Proteins show a decreasing trend with each of the increasing P:C diets and unlike carbohydrates, proteins do not seem to be maintained across the various P:C diets
3.5 Comparison of Carbohydrate and Protein intake graphs with *Rushby et al.*
3.6 Total calories consumed do not seem to be playing a role in driving the feeding behavior in *Drosophila melanogaster*
3.7 Like carbohydrates, even the calories coming from carbohydrates seem to be more or less constant i.e. calories coming from carbohydrates are maintained!
3.8 Calories from Proteins show a decreasing trend with each of the increasing P:C diets and unlike carbohydrates, proteins do not seem to be maintained across the various P:C diets
3.9 Next-In Line Experiment: Preliminary Data and Results from the 2-cup, 2-choice Assay
3.10 Summary, conclusions & quick take-aways from the chapter!

Now, with the various P:C diets made above and the feeding experiments performed along with proper evaporation standardization (getting evaporation value <=20% of total feeding) for all the conditions, the feeding data in the form of Consumption (mg/fly/day) is plotted as below. (The exact feeding and evaporation values can be found in the form of a link to a excel sheet with all the calculations in the Appendix section at the end of this thesis)
The results obtained from the 24 hours feeding performed using the DIETS setup in CSQ male flies is as follows:

3.1 The total consumption of food (mg/fly/day) increases as the ratio of P:C increases



Graph 3.1.1: As the P:C ratio increases from 1:1 to 1:16 P:C, the overall feeding given by Consumption (mg/fly/day) also increases. **[22 male flies/vial, n of 12, Biological replicates]**

Type of Diet (P:C)	Amount of Sucrose (in gm/L)	Amount of Carbs (in gm/L)	Amount of Proteins (in gm/L)	Total calories (in gm/L)
	Amount of Sucrose (in gm/ 50 ml)	Amount of Carbs (in gm/ 50 ml)	Amount of Proteins (in gm/ 50 ml)	Total calories (in gm/ 50 ml)
1:1	51	130	122	1028
	2.55	6.5	6.14	51.4
1:2	51	83.49	40.933	503
	2.55	4.17	2	25.15
1:4	51	70	17	353
	2.55	3.5	0.8	17.65
1:8	51	64	8	292
	2.55	3.22	0.4	14.63
1:16	51	62	3	265
	2.55	3.1	0.19	13.25

Table 3.1.1: Summary of the exact amounts of sucrose, carbs and proteins for different types of diets calculated from the tables above in both gm/L and gm/50 ml.

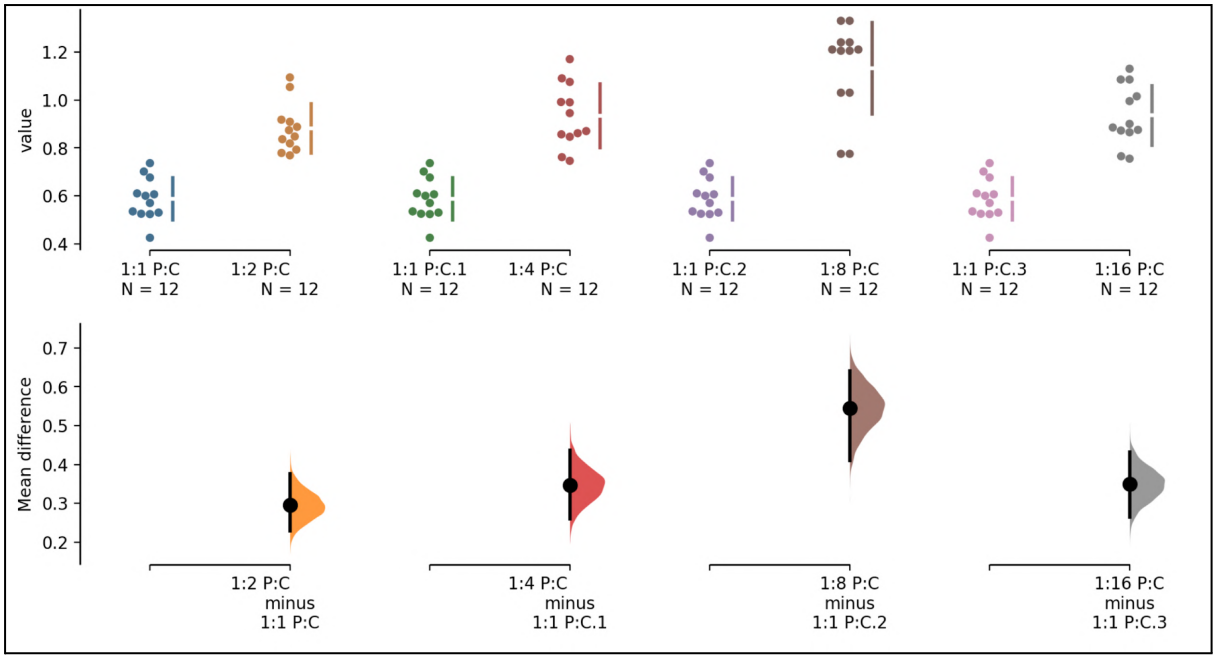
As I give different ratios of P:C diets to the flies per vial in the DIETS setup (as mentioned in the Methods section above)- 1:1, 1:2, 1:4, 1:8 and 1:16 to be particular, I observe a trend of linear increase in feeding as the ratio of P:C increases.

This is interesting as this tells us the following:

1) Flies on high P:C ratio diets feed more as compared to the flies on low P:C diets.
2) The comparative ratio of Protein: Carbohydrate is high in the high P:C ratio diets like 1:4, 1:8 & 1:16 as compared to the low P:C ratio diets like 1:1 & 1:2 which logically implies that as the ratio of P:C increases from 1:1 to 1:16, the relative proportion of carbohydrates in the diet as compared to the proportion of protein increases. But it is important to note the fact that even though the P:C ratio increases, the amount of Carbohydrate/mg is high in the low P:C ratio diet as compared to the high P:C ratio diet (for eg, 1:1 P:C diet has 122 ug/mg of carbohydrates as compared to just 63 ug/mg in the 1:16 P:C diets-refer to the tables that summarize the various constituents of P:C in the Methods section above). Thus, a low P:C ratio diet has low proportion of carb:protein as compared to the high P:C diets (that has highly diluted amounts of protein) but in terms of carbs/mg, the same diet (i.e. low P:C diet) has high carb/mg as compared to the high P:C diets.
3) Similarly, a low P:C diet even though has low proportion of protein for every part of carbohydrate as compared to the other diets (1:1 P:C has one part of protein for every 1 parts of carbohydrates, but high P:C say 1:8 or 1:16, has just 1 part protein as compared to 8 or 16 parts of carbohydrates). But again, the amount of protein/mg is high in a low P:C diet as compared to a high P:C diet (for e.g. 122 ug/mg for 1:1 P:C as opposed to just 15 ug/mg for 1:16 P:C).
4) We observe that flies consume less total food mg/fly/day on a low P:C diet- for eg 0.5-0.6 mg/fly/day on 1:1 P:C as opposed to about 1.4-1.5 mg/fly/day on 1:16 P:C diet.

But then out of this total food consumed, how much of it is carbohydrates and how much of it is protein? What is the amount of carbohydrates/proteins intake for each of these diets? For this, I plotted the following individual carbohydrate and protein intake graphs from this overall total feeding.

For confirming the statistics of these graphs, I have used Estimation statistics. Going beyond the conventional Hypothesis testing that accepts or rejects a particular null hypothesis and confirms “whether or not the difference significant”, Estimation stats tells us “by how much” is the difference significant in any particular data by calculating the difference of the means for the test and control groups. All these estimation statistics graphs are as plotted below. (Moving beyond P values: data analysis with estimation graphics)



Estimation statistics plotting the difference of the means for overall feeding (mg/fly/day) of the respective groups with 1:1 P:C (control group). This confirms that the difference is significant and increases as the ratio of P:C goes from 1:1 to 1:16. **[22 male flies/vial, n of 12, Biological replicates]**

3.2 Feeding trend is comparable to the trend seen in *Rushby et al*

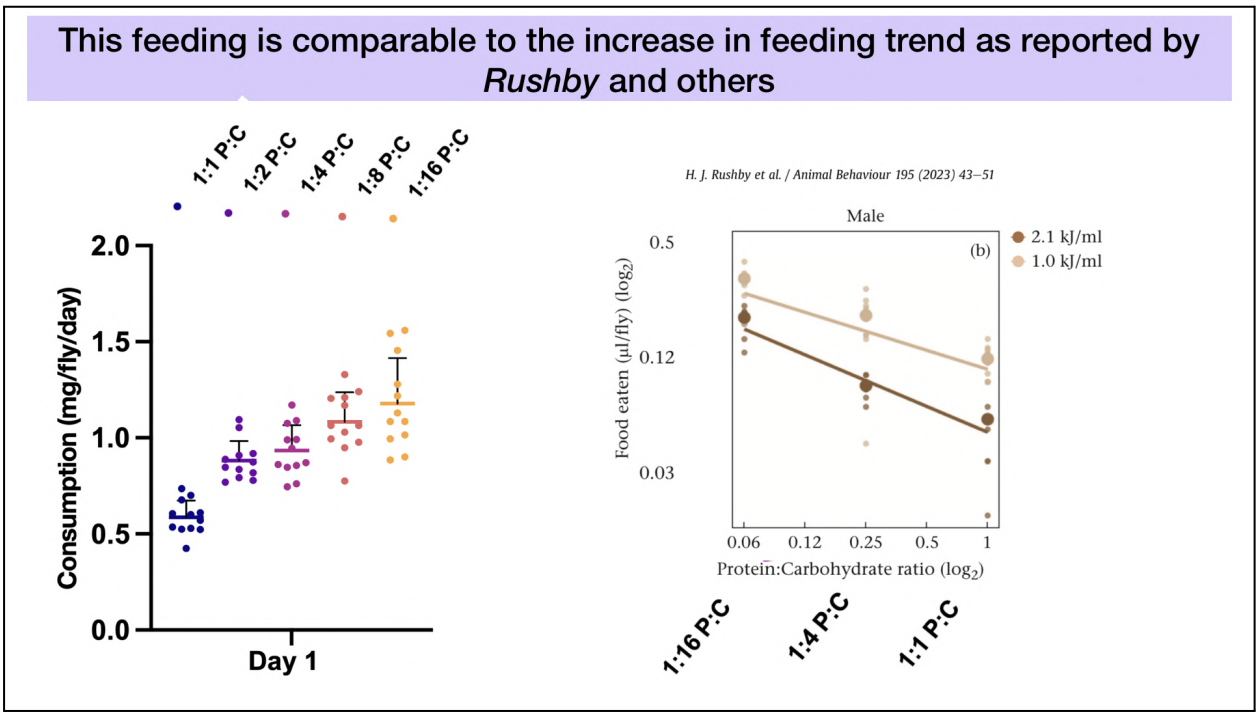


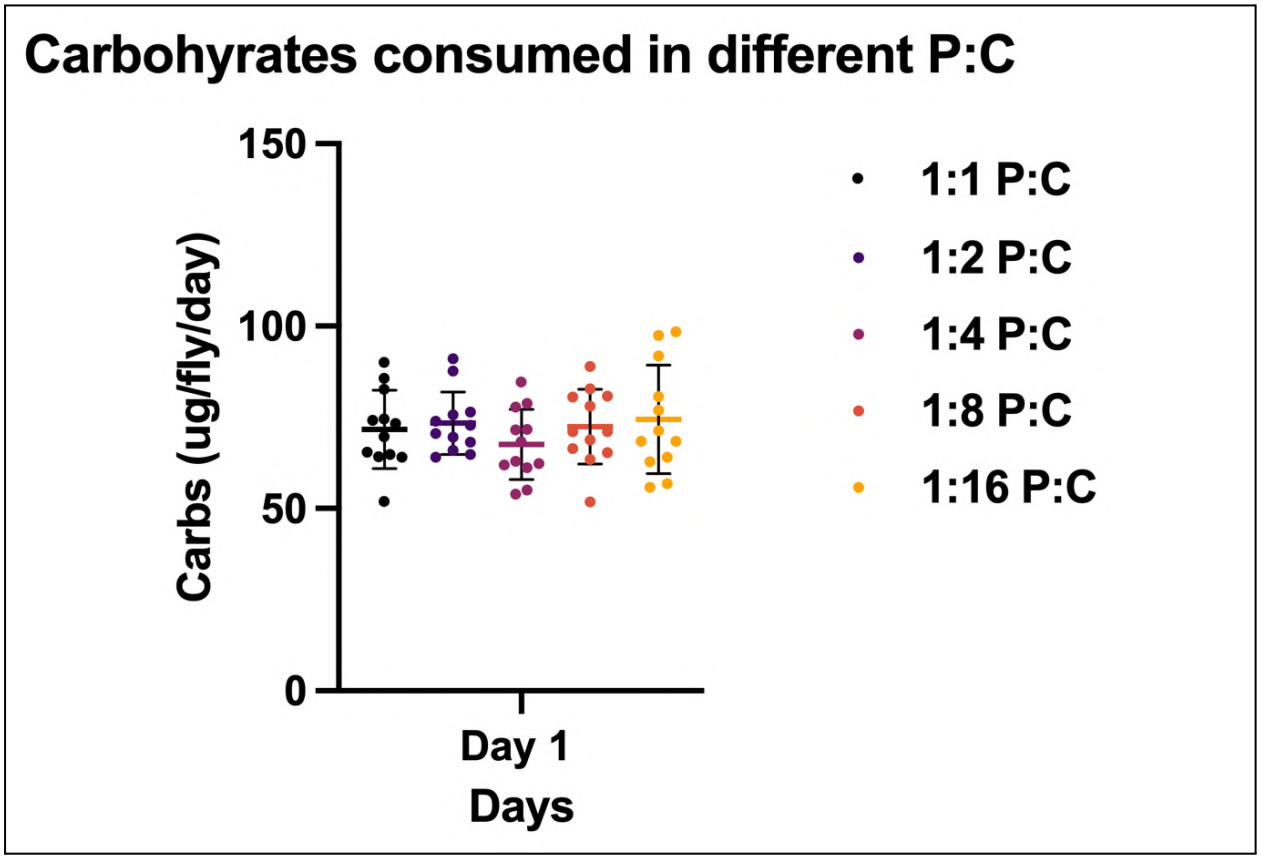
Image 3.2.1: The increasing feeding trend that I observe with different P:C ratios (solid food) that I give to my flies is comparable to the increasing feeding trend in case of the total liquid food eaten in the ratios of 1:1, 1:4 and 1:16 P:C in case of *Rushby et al.* Note that *Rushby et al* uses only 3 ratios of 1:1, 1:4 and 1:16 P:C and fits the line with an extrapolation for the intermediate ratios of 1:2 and 1:8, whereas, the ratio I have made include all five ratios including the intermediate 1:2 and 1:8 diets such that I get even a further nuanced trend. **[22 male flies/vial, n of 12, Biological replicates]**

As compared in the graphs above, *Rushby et al* find that as the ratio of P:C in their liquid CAFE (Deshpande et al. 2014; Ja et al. 2007) diet increases from 1:1, 1:4 & 1:16 P:C, the total food consumed also increases. Even though the diets used in *Rushby* are liquid diets given using a CAFE which is different from the solid food given in case of my DIETS assay- the trend is comparable as the ratio of P:C are the same.

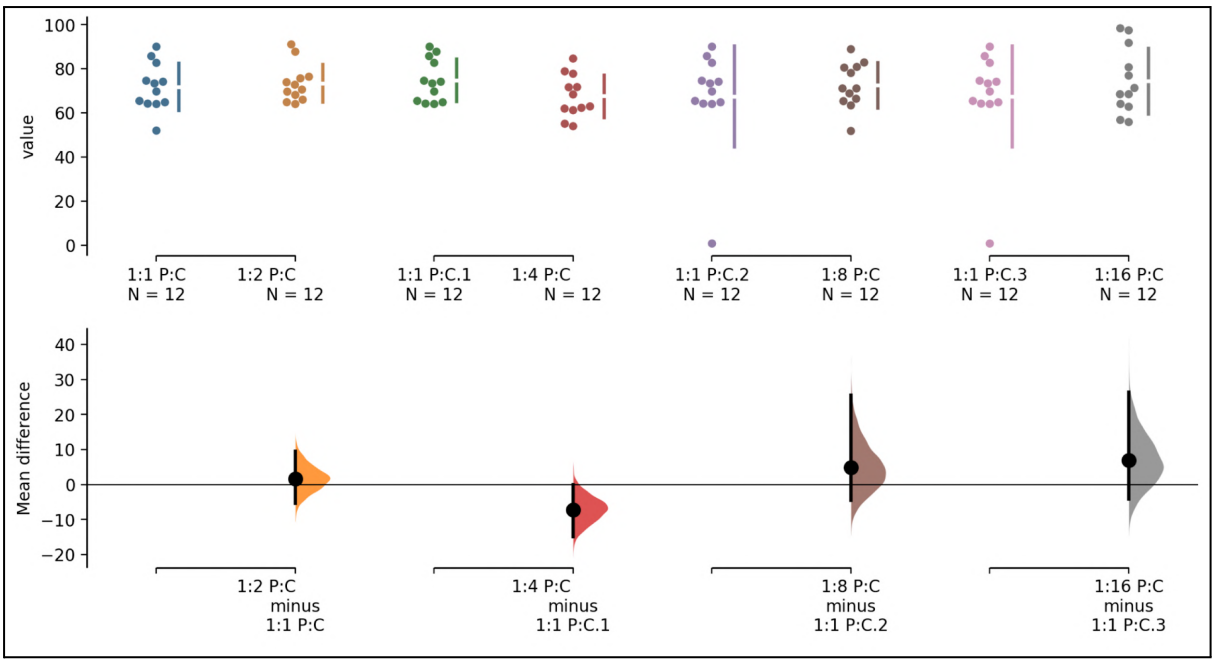
3.3 The total carbohydrates consumed in the different P:C diets seem to be more or less constant i.e., carbohydrate intake is maintained across the different P:C diets!

Next, in order to dissect out of the total feeding, how much of the total food comes from carbohydrates and how much of the total food comes from proteins, I converted the feeding plots into carbohydrate and protein intake based on how much carbohydrates and proteins these diets have (from all the tables listed in the Methods chapter above). I also did the same for calories in order to understand the consumption and trend in calories. Excel Sheet recording all the protein, carbohydrate, calories calculations and conversions for plotting can be found in the form of a link in the Appendix section at the end of this thesis.

After converting the total consumption and feeding data into total amount of carbohydrates consumed, the plot I got is as follows:



Graph 3.3.1: Plot for the total carbs consumed in ug/fly/day from the total overall food consumption plot above for all the 1:1, 1:2, 1:4, 1:8 and 1:16 P:C diets. Carbohydrates intake seem to be near-about the same, i.e., carbohydrates are maintained near about constant for all the different P:C ratios. **[22 male flies/vial, n of 12, Biological replicates]**



Estimation statistics plotting the difference of the means for total carbohydrates consumed (mg/fly/day) of the respective groups with 1:1 P:C (control group). This confirms that the difference is almost 0 for all the groups and thus, not significant as the ratio of P:C goes from 1:1 to 1:16. **[22 male flies/vial, n of 12, Biological replicates]**

Even though there is an increasing trend in the total amount of food consumed (mg/fly/day) from 0.5-0.6 mg/fly/day for 1:1 P:C all the way up to 1.4-1.5 mg/fly/day on 1:16 P:C diet, however the amount of carbohydrates consumed in each of these diets is near about the same! This trend is initially surprising- how are fruit flies that are eating less and the ones that are eating more, both maintaining an equal carbohydrate intake when fed on different ratios of P:C? But this trend makes sense when we look at the total carbohydrate content of each of these diets. A lower P:C diet has a higher content of carbs/mg than a higher P:C diet that has a comparatively lower content of carbs/mg. This is absolutely different from and should not be confused with the proportion of carbs in the diet- proportion of carbs tells us the relative proportion of carbs as compared with proteins- this corresponds to the exact P:C ratio- a lower P:C ratio has a lower proportion of carbs as compared to protein as opposed to a higher P:C ratio that has a higher proportion of carbs as compared to the proportion of proteins within the same diet!

Thus, when we multiply the total food consumed in mg/fly/day with the total carbs/mg in a particular diet, we get this trend.
Thus,

lower total food consumed (in mg/fly/day) x higher carbs/mg

=

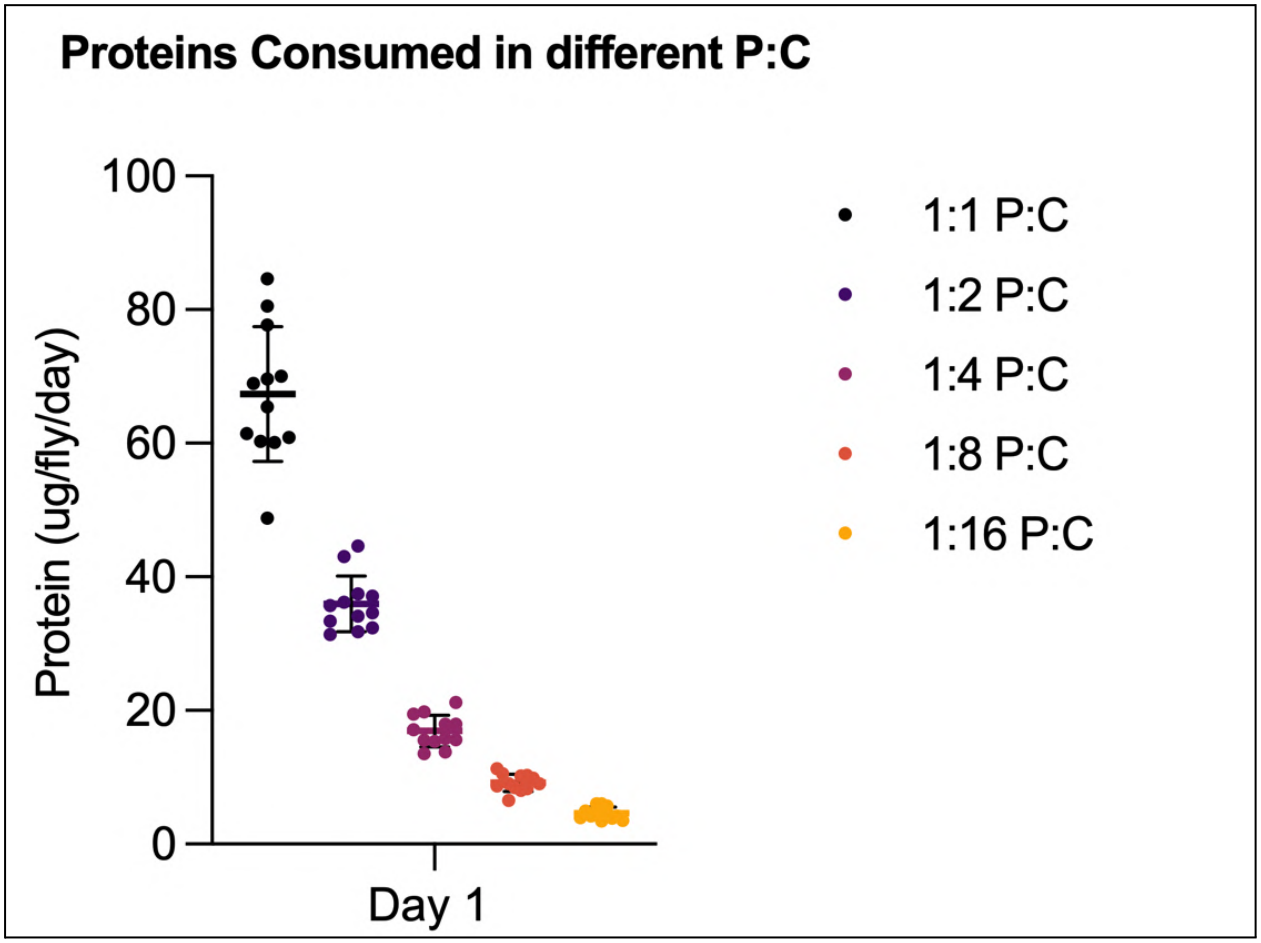
Higher total food consumed (in mg/fly/day) x lower carbs/mg

With this, it looks like fruit flies are consuming food so as to maintain their carbohydrate demands. Or the carbohydrates seem to be driving the feeding behavior of the fruit flies.

This is clearly elucidated in the **'total carbohydrates consumed'** table in the appendix section below. In order to understand what exact calculations I did behind plotting this graph, please refer to this table for the specific numbers of total food consumed as well as the total carbohydrates consumed from each of these different P:C diets.

3.4 Proteins show a decreasing trend with each of the increasing P:C diets and unlike carbohydrates, proteins do not seem to be maintained across the various P:C diets

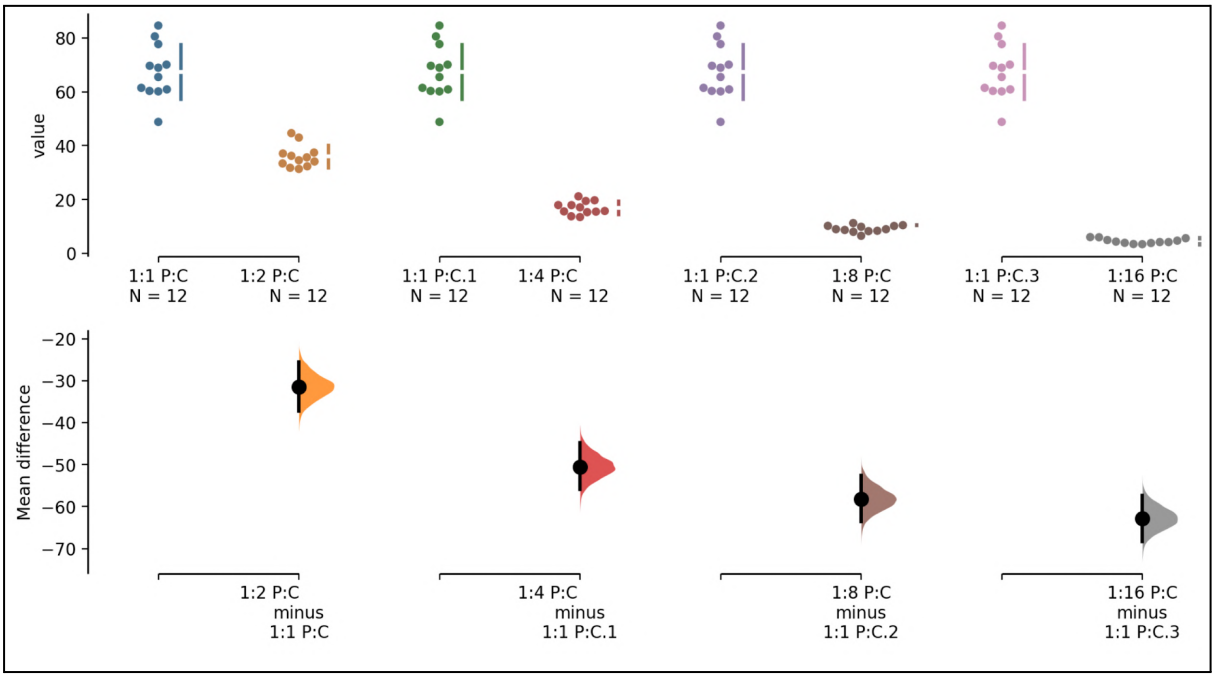
Just like I did for the carbohydrates, after converting the total consumption and feeding data into total amount of proteins consumed, the plot I got is as follows:



Graph 3.4.1: Plot for the total proteins consumed in ug/fly/day from the total overall food consumption plot above for all the 1:1, 1:2, 1:4, 1:8 and 1:16 P:C diets. Protein in take seem to be decreasing at an exponential rate as the ratio of P:C in the diet increases. **[22 male flies/vial, n of 12, Biological replicates]**

As the protein content decreases from 1:1 to 1:16 P:C diet, in both the terms of proportion (in comparison with carbs) as well as the protein/mg content, quite expectantly, even the total proteins consumed per fly per day decreases with a similar trend. This makes complete sense as flies have such less protein to get from the high P:C ratio diets even though they consume large quantities of these diets.

This is clearly elucidated in the **'total protein consumed'** table in the appendix section below. In order to understand what exact calculations I did behind plotting this graph, please refer to this table for the specific numbers of total food consumed as well as the total protein consumed from each of these different P:C diets.



Estimation statistics plotting the difference of the means for total proteins consumed (mg/fly/day) of the respective groups with 1:1 P:C (control group). This confirms that the difference follows a negative exponential curve as well indicating a decreasing trend and thus, is significant for all the groups as the ratio of P:C goes from 1:1 to 1:16. **[22 male flies/vial, n of 12, Biological replicates]**

3.5 Comparison of Carbohydrate and Protein intake graphs with *Rushby et al.*

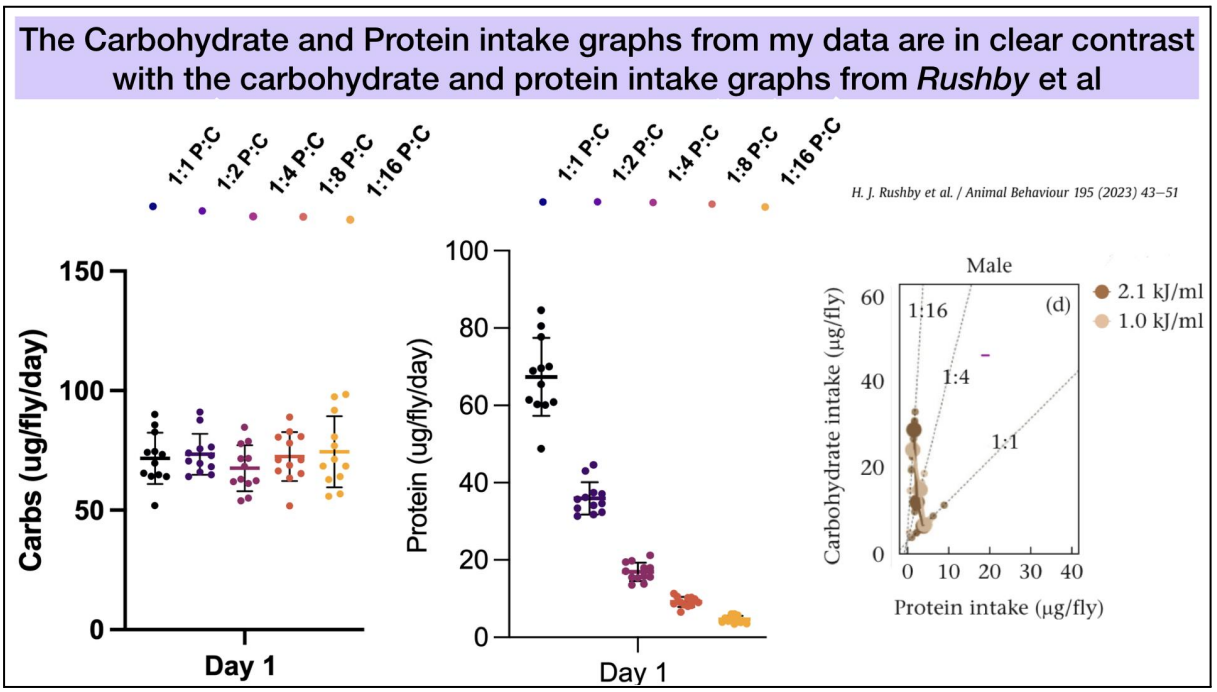
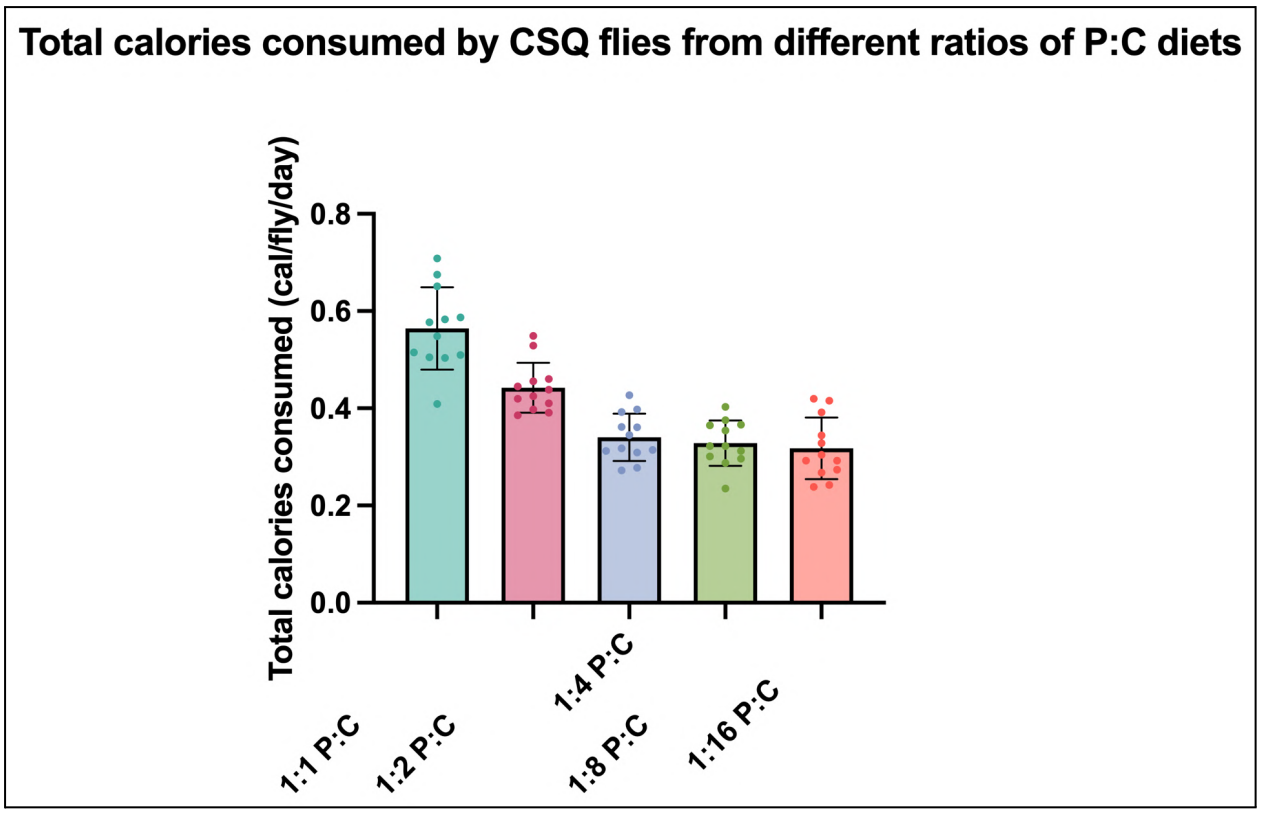


Image 3.5.1: Comparison between carbohydrate and protein graphs from my data (in left) and *Rushby's* Carbohydrate v/s Protein intake data (in right) shows complete contrast. Plots from my data show carbohydrates (but not proteins) maintained over a constant range of 60-65 ug/fly/day for all the different P:C ratio diets whereas *Rushby's* plot shows protein maintained over a narrower range but carbohydrates are not! (Note- all plots are for male flies). **[22 male flies/vial, n of 12, Biological replicates]**

Further, when I compare my carbohydrate and protein intake plots with *Rushby's* carbohydrate v/s protein intake plots, I observe a complete contrast in the trends of how carbohydrates and proteins are maintained and in what range. Plots from my data show carbohydrates are maintained over a constant range of 60-65 ug/fly/day for all the different P:C ratio diets but proteins show a stark exponential decline and thus, are not maintained across the different P:C ratios.
Whereas, *Rushby's* plot for carbohydrates v/s protein intake shows that proteins are maintained in a tighter narrower range, however carbs intake varies over a wider range, a range greater than that seen in case of proteins. Thus, this is in complete contrast to the trend I observe in my data where carbohydrates are maintained over a narrower range but proteins are not!

3.6 Total calories consumed do not seem to be playing a role in driving the feeding behavior in *Drosophila melanogaster*

Just like carbohydrates and proteins, now after converting the total consumption and feeding data into total amount of calories consumed, the plot I got is as follows:



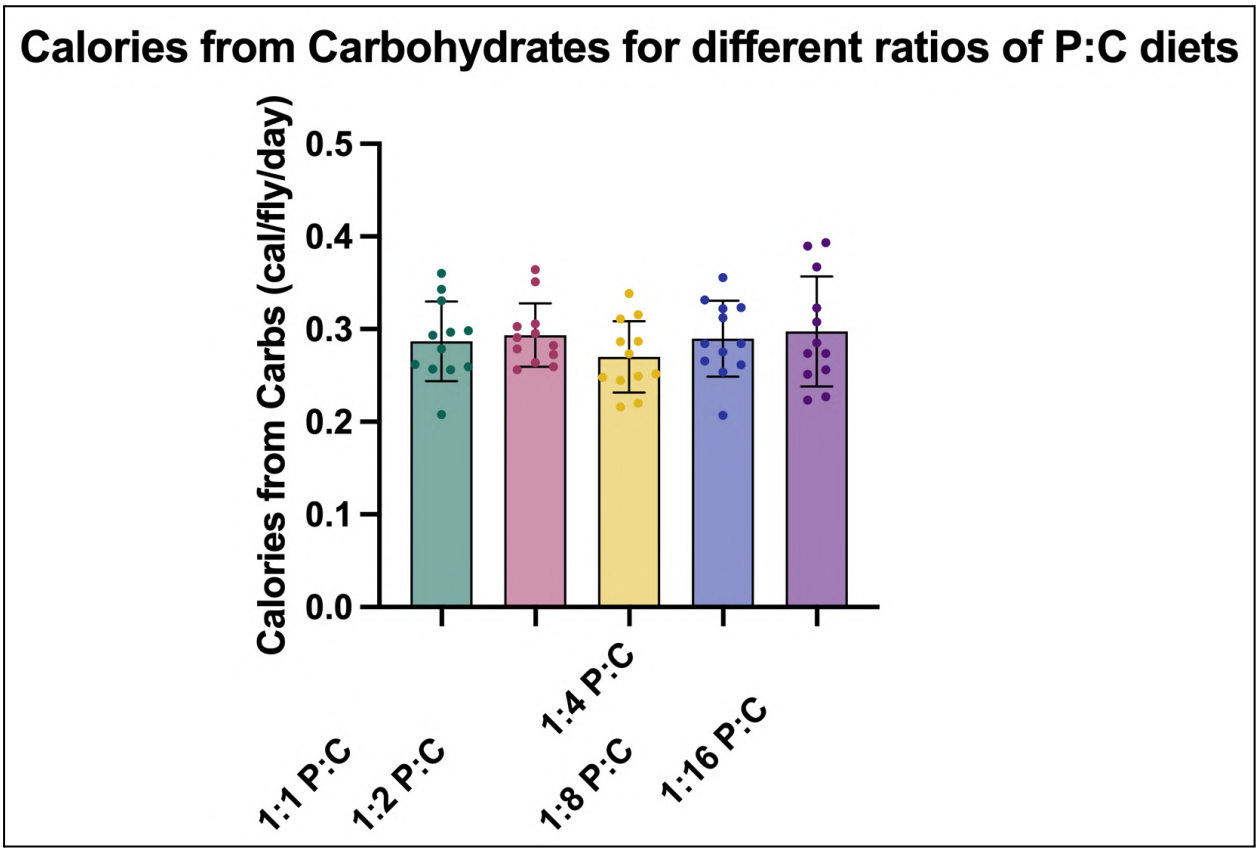
Graph 3.6.1: Feeding (Consumption-mg/fly/day) data converted into total calories (cal/fly/day) consumed. Trend in total calories (cal/fly/day) consumed by the flies shows a decreasing trend from 1:1 P:C to 1:16 P:C- as the calories in these foods decreases, the total calories consumed by the flies also decreases! **[22 male flies/vial, n of 12, Biological replicates]**

As the total calorie content decreases substantially from 1:1 to 1:16 P:C diet, 1028 kcalories/L to about 265 kcalories/L, the total amount of calories consumed also shows a decreasing trend with the same logic! As the total calories in the food is extremely high in a 1:1 P:C diet, even after consuming a small amount of this diet, the total calories consumed becomes very high. Similarly, as the total calories in the food is comparatively very low for 1:16 diet as compared to a high calorie diet say 1:1, but eating large quantities of low calorie diet i.e. 1:16 makes up for this low calories and thus shows a near-about average value of calories! The last three bars in the above depicted graph are almost the same because the calorie content of these ratios of diets is also almost the same (353, 292 and 265 kcalories/L for 1:4, 1:8 and 1:16 ratio diets)!

This is clearly elucidated in the **'total calories consumed'** table in the appendix section below. In order to understand what exact calculations I did behind plotting this graph, please refer to this table for the specific numbers of total food consumed as well as the total calories consumed from each of these different P:C diets.

After looking at the calorie plot, I next wanted to dissect how much of these calories are coming from carbohydrates and proteins separately, and thus, after converting the feeding data and plots from carbohydrates and proteins into calories coming from carbohydrates and proteins (Excel Sheet links with the entire calculations and conversions of how I did this can be found in the Appendix section), the plots I got are as follows:

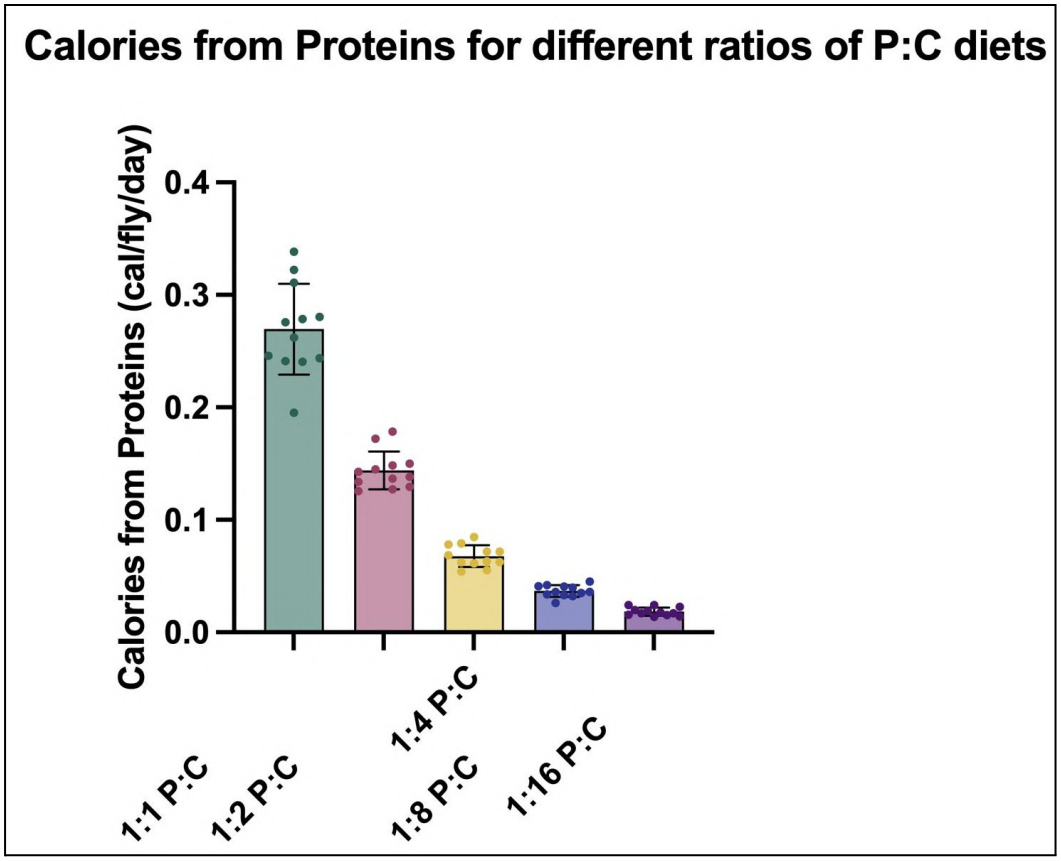
3.7 Like carbohydrates, even the calories coming from carbohydrates seem to be more or less constant i.e. calories coming from carbohydrates are maintained!



Graph 3.7.1: Carbohydrate feeding (carbs consumed in ug/fly/day) data converted into calories from carbohydrates (cal/fly/day) consumed. Trend in calories from carbs (cal/fly/day) consumed by the flies shows the same trend as the carbohydrate feeding data- as carbohydrates are maintained all across the different P:C ratio diets- even calories coming from carbohydrates are maintained and show a similar trend as expected. **[22 male flies/vial, n of 12, Biological replicates]**

This is in accordance with what one would expect, if the carbohydrates consumed in all these different diets is constant, then the calories coming from carbohydrates should also show a similar trend- and it does! From one graph above, we saw that the total calories consumed shows a decreasing trend, but here we find that the calories coming from carbohydrates are almost more or less the same and thus, maintained!

3.8 Calories from Proteins show a decreasing trend with each of the increasing P:C diets and unlike carbohydrates, proteins do not seem to be maintained across the various P:C diets



Graph 3.8.1: Protein feeding (proteins consumed in ug/fly/day) data converted into calories from protein (cal/fly/day) consumed. Trend in calories from proteins (cal/fly/day) consumed by the flies shows the same trend as the protein feeding data- as proteins show a stark exponential decreasing trend as the ratio of P:C ratio increases- even calories coming from proteins show a similar stark exponential decreasing trend as expected. **[22 male flies/vial, n of 12, Biological replicates]**

Like proteins, even the calories coming from proteins show a decrease in trend and unlike the carbohydrates and calories coming from the carbohydrates, calories coming from the proteins are not maintained but show a stark decreasing trend! This is exactly as one would expect from the consumption of total protein graphs!

This is clearly elucidated in the **'total calories coming from carbohydrates' and the 'total calories coming from proteins'** table in the appendix section below. In order to understand what exact calculations I did behind plotting this graph, please refer to this table for the specific numbers of total calories consumed as well as the total calories consumed from each of these two macronutrients for all the different P:C diets used for the experiment.

But do flies prefer carbohydrates over even a high protein diet? In order to answer this question, I thought of performing a comparative 2-cup 2-choice assay further, the results of which have been summarized below:

3.9 Next-In Line Experiment: Preliminary Data and Results from the 2-cup, 2-choice Assay

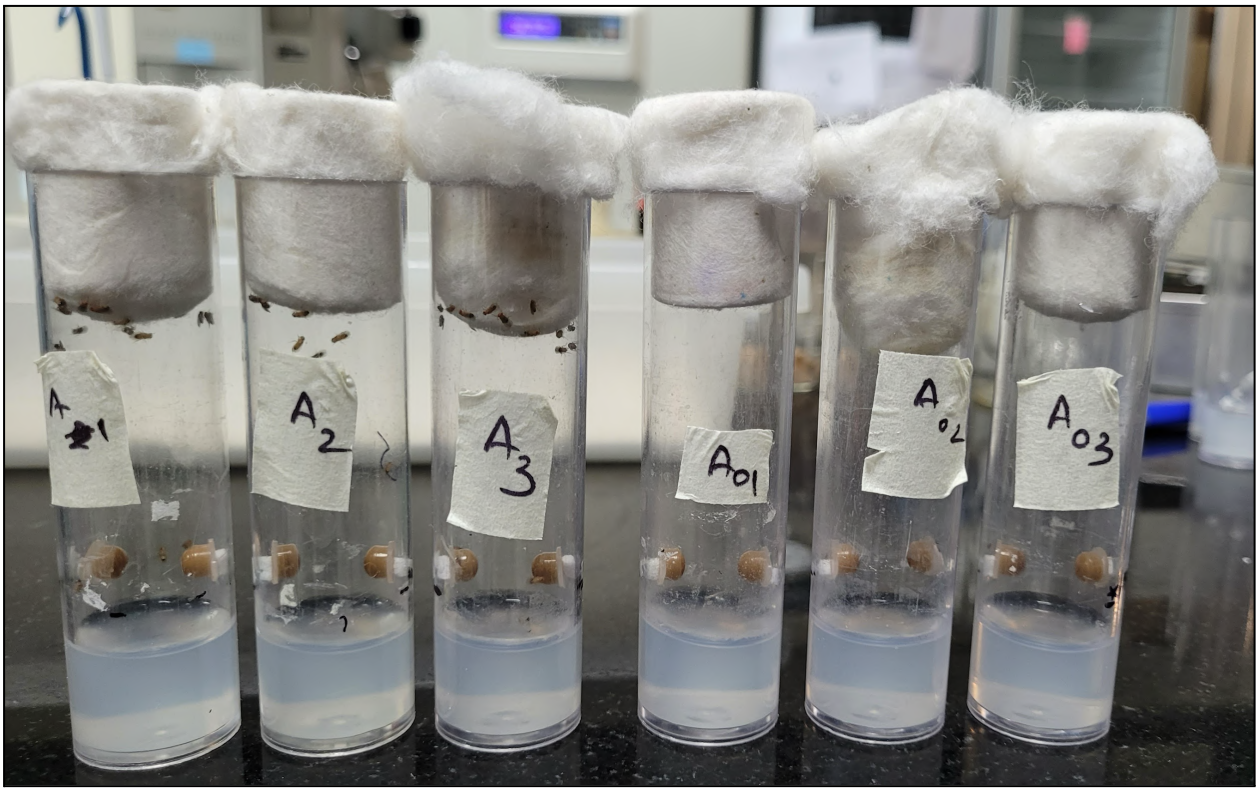


Image 3.9.1: A real-life depiction of a 2-cup-2-choice assay containing 2 cups for comparison and testing feeding preference instead of 1 cup in the typical DIETS setup. **[12 male flies/vial, n of 3, Biological replicates]**

2-cup 2-choice DIET assay is a simple modification of the regular DIETS assay- instead of 1 food cup we can place 2 food-cups diametrically opposite to each other in order to check food preference of the flies!

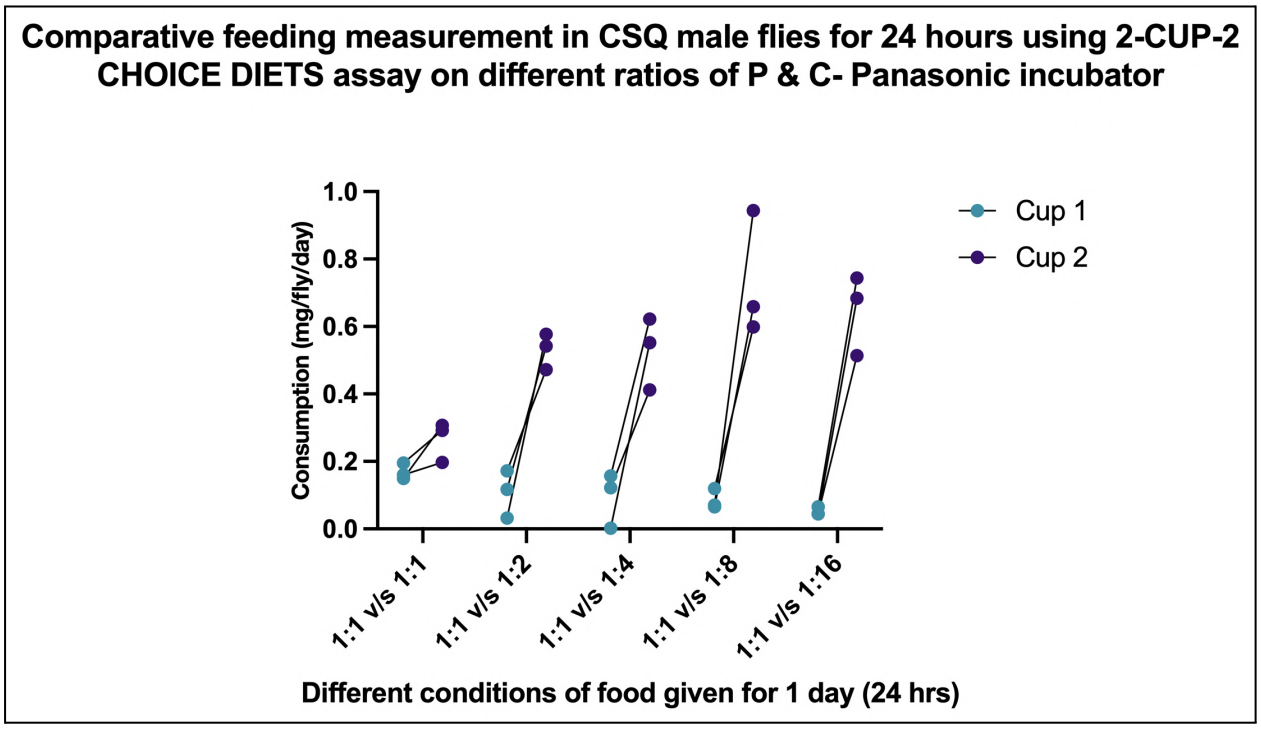
For my purpose, the control diet that I used for comparison was a 1:1 diet as it is highly rich in proteins and I wanted to check whether the flies prefer a high protein diet or high sugar diet or rather high carbohydrate diet.

Thus, the basal control diet was 1:1 v/s 1:1.
And all other experimental diets were 1:1 v/s any of the other 4 P:C ratio diets-
1:1 v/s 1:2
1:1 v/s 1:4
1:1 v/s 1:8
& 1:1 v/s 1:16

The challenge here is again evaporation standardization in fact even trickier as now I have to take care of diets with two different consistencies in the same vial- and so I need to be careful about the evaporation levels of both types of food cups and not just 1 cup that goes into the diet vials. That's why the following results are just the preliminary results from the pilot experiment conducted with n of just 3.

(Even the preliminary data and all the calculations and conversions done for the following 2-cup 2-choice plot can be found in the form of a link to the excel sheet in the Appendix section below).

The preliminary data for the total feeding in 2-cup, 2-choice assay is as follows:

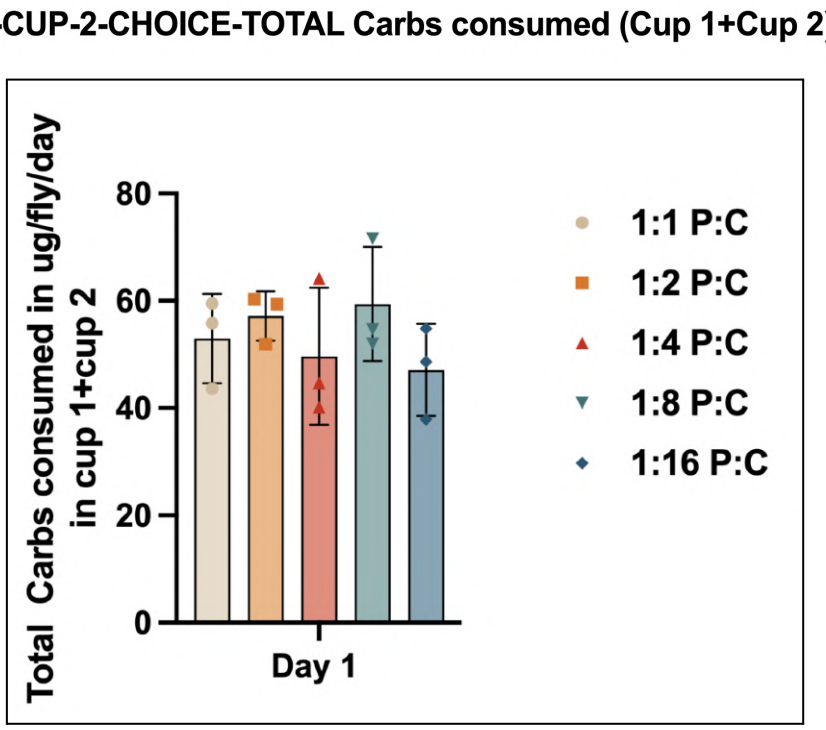


Graph 3.9.1: Preliminary data from the 2-cup-2-choice plot depicting clear preference for high P:C ratio (i.e. cup with higher amount of carbohydrates than proteins). **[12 male flies/vial, n of 3, Biological replicates]**

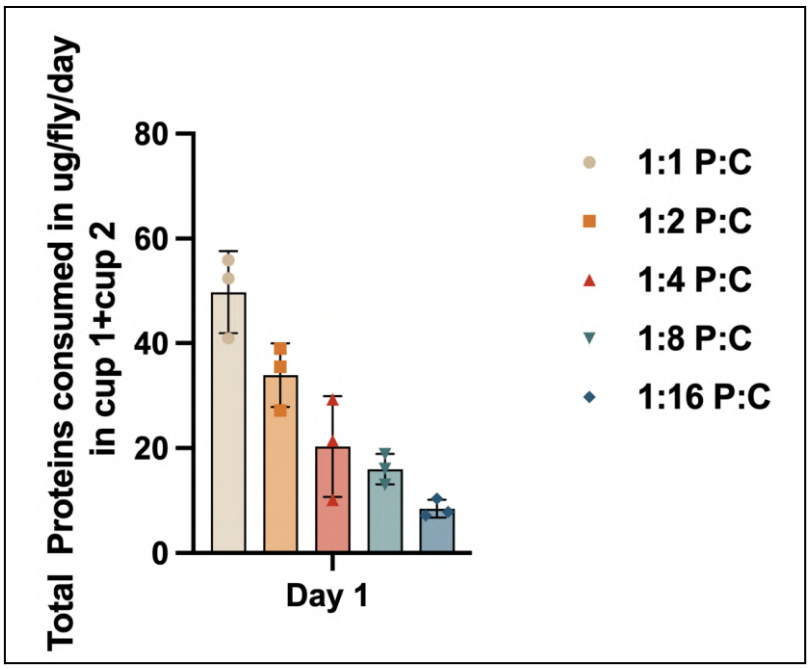
Even though these are just the preliminary results, in the above graph, I see a clear preference for high sugar or high P:C diet or high carbohydrate proportion diets over a highly protein rich diet. That means even when given an ad libitum access (unlimited choice and access) for the two types of diets, flies very clearly seem to not care in fact they chuck high protein diets for high carb diets even though high carb diets have such low amounts of proteins. So, clearly protein is not the one that drives feeding behavior in *Drosophila* but rather its carbohydrates that influences and even determines how much do flies feed! But this is also just the preliminary data and has high variability and so I would want to repeat this experiment.

I next wanted to reconfirm what the total consumption of proteins in each of these vials is in order to

1) check whether the same trend as we have seen in the graphs above is followed even when there is a choice of 2 cups with 2 different foods now
2) does the presence of another food source deter the flies from maintaining the overall carbohydrate levels for both the individual as well combined food source



Graph 3.9.2 Plot for total carbohydrates consumed (cup 1+cup 2) in the entire vial for different P:C ratio diets. Note that this plot again mimics the trend seen in the total carbohydrates consumed (ug/fly/day) for the various P:C ratio diets where the carbohydrates consumed is near-about maintained in the same range for all the diets. **[12 male flies/vial, n of 3, Biological replicates]**



Graph 3.9.3: Plot for total proteins consumed (cup 1+cup 2) in the entire vial for different P:C ratio diets Note that this plot again mimics the trend seen in the total proteins consumed (ug/fly/day) for the various P:C ratio diets where the proteins consumed show a stark decreasing trend as the ratio of P:C increases! **[12 male flies/vial, n of 3, Biological replicates]**

Through both of these graphs that plot the total combined levels of proteins and carbohydrates from the consumption values in each of the two cups, we see that the total carbohydrate consumed in a vial are still maintained to be near about the same in spite of giving a choice between high protein v/s high carb diets. However, just like the case earlier, the trend is not maintained for proteins but rather shows a decreasing curve indicating that it's again the carbohydrates that seem to be maintained and thus, in turn are responsible for driving the feeding behavior in *Drosophila melanogaster.* And this is in complete contrast with the Protein-leverage hypothesis that I started out with (in Chapter-1, Introduction). Thus, through my series of experiments (and also my mentor's in our lab), we have been repeatedly finding that carbohydrates and not proteins are being maintained in a narrow range as opposed to the graphs of *Rushby et al*. Thus, through my experiments and our work in our lab, we propose a **"Carbohydrates leverage Hypothesis"** rather than a protein leverage hypothesis because it looks like it's the carbohydrates but not proteins that are maintained and thus, drive the overall feeding behavior in the fruit flies!

3.10 Summary, conclusions & quick take-aways from the chapter!

1.1 Various ratio of P:C ratios diets (1:1, 1:2, 1:4, 1:8 and 1:16 to be particular) have been achieved to measure and quantify feeding using the DIETS assay setup

1.2 Evaporation standardization and successful troubleshooting by getting the evaporation values to a permissible comparable range in the Panasonic incubator was the key challenge to get the correct feeding data and measurements for all the diets with different consistencies and densities

1.3 Total feeding plots, total carbohydrate as well as protein feeding plots from my results reveal that Carbohydrates (and NOT proteins) are maintained and thus, seem to drive/leverage feeding in the fruit flies

1.4 Proteins or calories may play a role in the total feeding but neither proteins nor calories seem to be maintained across the various ratios of diets and thus, do not seem to drive the total feeding in flies

1.5 Flies also show a clear preference for carbohydrates over proteins and again total carbohydrates (but NOT proteins) are maintained as revealed through the 2-cup-2-choice DIETS assay.

1.6 These results from our lab repeatedly hint towards a potential ‘Carbohydrate leverage hypothesis’ and are in complete contrast with the proposed ‘Protein leverage hypothesis’ in the literature.

Chapter-4
Discussions & Future Directions

Discussions & Future Directions raised in this Chapter:

-Different plausible speculations about my results
-What have been the limitations of my study?
-What are the parameters I have not looked at through my experiments?
-Other possible potential hypotheses from my results
-Other possible experiments to verify and confirm my current 'carbohydrate leverage hypothesis'
-What all further questions can be asked? What else can be checked?
-Why is it still early to make strong claims about 'Carbohydrate leverage hypothesis' and why the other possible hypotheses cannot be discarded
-Major missing from the picture- underlying Neural circuitry and neuronal mechanisms
-The speculated plausible role of IN1 interneurons
-Further speculations and possibilities from the latest studies-Taste of sugar v/s taste of proteins- the unexplored pre-ingestive and/or post-ingestive effects of these macronutrients
-Further biological implications to humans

To summarize the thesis, I have observed and I speculate with all of my results (as we saw in the chapter-3 above), that it is mainly the carbohydrates that are maintained all across feeding in the various ratios of P:C (Protein:Carbohydrate) diets given to the flies. Even though the feeding in each of the various P:C diets varies and ranges largely from 0.5 mg/fly/day to a 3-fold value of 1.5 mg/fly/day in 1:1 P:C diet to 1:16 P:C diet respectively, however, the carbohydrate intake for each of these conditions remains more or less constant in other words- carbohydrates are maintained all across the various ratios.

Thus, through this thesis, we have seen that it is the carbohydrates that are maintained (as concluded through the various graph results in the earlier chapters) and so, carbohydrates largely seem to drive the feeding behavior in the *Drosophila melanogaster*. We call this the "Carbohydrate Leverage Hypothesis", drawing inspiration from the previously known and famous "protein leverage hypothesis"- where the proteins are maintained in a narrow range and it is the proteins that drive the overall feeding behavior apart from driving the feeding of other macronutrients (S. J. Simpson and Raubenheimer 2005; Raubenheimer and Simpson 2019; Rushby et al. 2023, K. P. Lee et al. 2008; K. P. Lee 2015)

The current extant studies that have used the various P:C ratios in the diets have largely looked at the P:C consumption in a backdrop of a third parameter like age, sex, mating status (Rushby et al. 2023) or survival, lifetime egg production and lifespan (K. P. Lee et al. 2008; K. P. Lee 2015). However, my work has largely been about laying out the basics of the feeding behavioral paradigm for the various P:C consumption in a DIETS setup. I have not measured feeding in the context of any of the other parameters like age, sex, lifespan or survival- and thus, it makes me wonder whether or not it is important to look at feeding in different diets in these contexts. What then will be the best ratio of diet that supports the maximum survival and growth? But this would then include first fixing upon a fixed intake target/ threshold in each of these different diets that supports the best survival and growth in the context of our diets. This would be tricky as well as interesting to look at as it would vary with both male and female flies and would need me to design a whole different set of experiments giving rise to a different project altogether!

However, with the current experiments that I have performed during the term of my master's thesis, I have barely scratched the surface of the field that tries to understand what exactly drives the feeding behavior in *Drosophila melanogaster.* Refuting and completely disapproving the well-known protein leverage hypothesis entirely would still be a long haul- I would want to check feeding with even more ratios of proteins and carbohydrates and dissect the role of carbohydrates and proteins further in those diets to make a stronger claim that carbohydrates drive feeding in the fruit flies. I can think of doing this by making other P:C diets like the reciprocal ratios: 2:1, 4:1, 8:1 and 16:1 P:C ratios and then repeating the same experiments that I mention in this thesis. However, making these diets would not be as straightforward as it sounds here. This is because sources of proteins in my diets have not been through a single source but have been rather complex and thus, completely altering proteins and achieving these ratios wouldn't be easy, but would definitely be worth attempting to play around with. Further, evaporation standardization of these complex diets will again have to be done separately before starting the main experiment since making diets highly rich in proteins makes them very thick and dense and thus, makes them hygroscopic (like we saw in the troubleshooting section in Chapter-2 above). Another challenge of using high protein diets is that flies do not survive on just protein diet beyond ~3-4 days whereas they can survive upto 3-4 weeks on a carbohydrate rich diet (as carbohydrate is a source of energy), thus the proteins cannot be decoupled from carbohydrates even though carbs can be decoupled from proteins!

Apart from this, there could have been various other ways of achieving different P:C ratio diets, and one could have done this in many different ways to measure and quantify the drosophila feeding behavior. For example, one could try keeping protein constant and largely increasing carbohydrates in the diets (this is what my mentor has already done in the lab and feeding on high sugar or high calorie diets makes for

the key central theme of his PhD thesis), or even increasing proteins so many folds rather than decreasing proteins with large amounts (I have done the latter during the course of my master's thesis) but this would pose an additional challenge and wouldn't be feasible as we know through literature and even empirical observation in our lab and like mentioned in the paragraph above: flies majorly need carbohydrates (source of energy) rather than proteins for their survival: and flies do not survive on high protein diets beyond a few 4-5 days as opposed to surviving for 15-21 days and beyond easily on even pure sucrose diets! Thus, checking the feeding behavior and dissecting the role of proteins is harder and logistically challenging in flies at least in the current settings of our lab! But it would definitely be very interesting and exciting to be able to dissect both of these together as well as in separate conditions and then look at their interplay in driving the feeding behavior!

Before making the strong claim that carbohydrates drive feeding entirely, I would also want to check the total feeding and the protein and carbohydrates consumed trend with the other diets that are also made and largely used in our lab (like the Holidic diet (Piper et al. 2014) and the simple Yeast-Sucrose diet). I would want to do these experiments with both DIETS setup and also validate the same using CAFE setup before making these strong claims. The effect of group v/s isolation housing effect in feeding behavior and playing a role in this P:C based feeding would also be another interesting aspect to further investigate as the extant literature already shows some evidence that flies feed more when they are in isolation than in a group setting (Li et al. 2021). I have already made some preliminary attempts to standardize group v/s isolation-based feeding by designing and troubleshooting evaporation in a mini-CAFE setup. However, these have only been more of tinkering and playing around with the CAFE setup and so the main experiments will have to be planned and standardized further before being able to claim anything about this!

Another point of view and speculation that emerged out of the discussion in our lab over these results was whether or not our flies get their normal threshold of protein requirements fulfilled (if there is such a threshold in the first place). We speculated that maybe our flies are already protein rich, and thus seem to be not regulating their protein intake (almost as if they do not care for the proteins at all). But this was not the case as even if we gave them extremely diluted or extremely protein rich diets as compared to the relative quantities of carbohydrates in the diet (like I did through the range of various P:C diets that I gave the flies), they showed the same trend-our flies continued to maintain carbohydrates more and better than the proteins. Moreover, when given a choice between high protein and high carbohydrate diet (through the 2-cup 2-choice experiments mentioned in chapter-3), the flies continued to choose carbohydrates over proteins- hinting that our flies are not protein deprived/starved in the first place! With these speculations being discarded and proteins not being maintained, we cannot deny our 'Carbohydrate leverage hypothesis'.

But before all this, another obvious question that arises when looking at these feeding trends is the palatability and the taste-based differences of both protein and carbohydrate rich diets. We already know through literature and also through common knowledge that proteins are bitter tasting and carbohydrates are largely sweet-tasting. So, what if the proteins are largely aversive to the flies in the first place and lead to this non-preference over highly palatable and appetitive carbohydrates!
This would entail separating the nutrition with the taste value of these macronutrients. I think this can be further tackled by designing experiments using non-sweet sources of sugar like sorbitol, xylitol, mannitol, erythritol, and lactitol (Zhang et al. 2020) or even artificial sugars like the locally available various well-known types of artificial sugars in the market [artificial sugar scientific name (their market brand name)]: like Aspartame (NutraSweet, Equal), Neotame (Newtame), Saccharin (Sweet'N Low), Sucralose (Splenda) while preparing various diets to be given to the flies. As for the proteins- for separating the nutrition value from the taste of proteins- I am currently not sure how this can be done completely and whether this is possible in the first place as proteins alone do not allow the flies to survive as mentioned above! But again, just pondering over these possibilities is exciting!

My current master's thesis has also largely been only about setting up the basic behavioral foundations and laying out the groundwork for trying to understand the simple feeding behavior in fruit flies in the light of proteins and carbohydrates; however, what has been largely untouched and completely missing from picture is understanding the underlying neural circuitry and the neuronal mechanisms that would govern such a fundamental separation of detection/response as well as subsequent intake of these two very distinct yet highly important macronutrients- carbohydrates and protein. With the current emerging connectome of the fly brain and the latest live brain calcium imaging studies that look at the neural circuitry in real time, the most recent upcoming studies have demonstrated the role of interneurons such as IN-1 interneurons in playing a key role in sucrose response and uptake. These IN-1 interneurons have been found to respond to sucrose in a state-dependent (hungry v/s fed state) as well as qualitative manner (concentration of sugars) to be able to control the rate, volume and timing of ingestion (Yapici et al. 2016).
There are 60 types of these interneurons and some of them have also shown to be responding to some basic amino acids, and thus, the interconnected inter-play of response of these interneurons to both proteins and carbohydrates has been speculated, however these are just speculations for now and exact literature has not been out yet (Yapici et al. 2016).

There have been some preliminary studies looking at the underlying neural circuitry that may play a pure role in just protein uptake and regulation, and the works of the Ribeiro group from Portugal hint towards this (Piper et al. 2014)

What would also be crucial to begin with would be to also look at whether the processing of both of these macronutrients is pre-ingestive or post-ingestive. There have been some studies in the past that have looked at both pre- and post-ingestive neural circuitry and sensors for both carbohydrates and proteins (Yang et al). But looking at this aspect of feeding as well as going till the neural circuitry level has been beyond the scope of my current project due to limited time and resources (knocking down or over-expressing specific genes or looking at the neural circuitry has not been possible due to the unavailability of specific fly lines and techniques like 2-photon calcium imaging currently). But again, all of these have just been initial studies and these are just preliminary ideas and hypotheses. Looking at all these aspects by designing and planning specific experiments to decode these questions further would be super exciting! This plausible series of the above-mentioned experiments would then further culminate into concrete studies and papers on these that would definitely revolutionize the field. This would further help us to better understand and apply this knowledge from the fruit flies to the dietary intake of humans-maybe we can one day have a freely customizable central one-diet-fits-all P:C diet for every human that would be ready to consume and altered in its P:C content just before its consumption as per the particular specific needs and requirements of the consumer humans. This might help us better treat, prevent or even completely eradicate a lot of diet-based diseases and eating disorders like diabetes, obesity, thyroid, diarrhea, hypertension etc. to name a few. Thus, some of these questions will be definitely interesting to dive deeper into and further leaves the feeding behavioral neuroscientists with even more exciting questions and wider possibilities than ever before!

Chapter-5
Appendix and Supplementary Material

The STAR METHODS of this Appendix Chapter include the following supplementary materials -

1. **KEY RESOURCES TABLE: list of reagents with their source and catalog numbers, Excel sheets with all raw feeding data, calculations for different ratios of P:C diets, all the protein, carbohydrate, calories and 2-cup, 2-choice calculations and conversions for all plots**
2. **Contacts for reagents and resource sharing**
3. **Experiment model subjects and rearing details**
4. **Quantification, Data Analyses and Software availability**
5. **Different trial and errors and calculations done before achieving the required 1:1, 1:2, 1:4, 1:8 and 1:16 diets!**
6. **Different trial and errors and evaporation standardization done before achieving the required 1:1, 1:2, 1:4, 1:8 and 1:16 diets!**
7. **Exact Evaporation Standardizations and Troubleshootings with 1/2xC+1xP, 1/2xP+1xC,1xC+1xP (basal control diet), 2xC+1xP, 2xP+1xC**

This section has all the supporting extra information, other additional experiments, results, troubleshootings and discussions associated with and alluded to in the main text that have been carried out throughout the term of my master's thesis work. I hope that this section aids to the better understanding of the content of my master's thesis for the reader!

STAR METHODS-

Star Methods of this Thesis includes the following:

1. KEY RESOURCES TABLE:
    - The following reagents along with their corresponding Product numbers and source were used for making of all the diets

Name of the Reagent used for making the different P:C diets	Source	Product Number/Identifier/ Catalogue Number
Sucrose	ANJ Biomedicals	#100314
Agar Agar Type I	HIMEDIA	#GRM666-500G
Yeast Extract	SIGMA-ALDRICH	#70161-500G
Peptone (Bacteriological)	HIMEDIA	#RM001-500G
Brewer's Yeast	MP Biomedicals	#11425722

Appendix Table 5.1.1: Table listing all the ingredients with their catalog numbers used for making all different types of P:C diets made in this thesis.

- Excel Sheet recording all the raw feeding data and calculations done can be found here:
    - https://docs.google.com/file/d/1sq8kvI4om0sHmG220pXoqZ3P5vVGVSPD/edit?usp=docslist_api&filetype=msexcel

- Excel Sheet recording all the calculations done for making all of different ratios of diets can be found here:
    - https://docs.google.com/spreadsheets/d/15qaL62rCEB3fZhrIJFWNyD8iP8-Ogr0L0rvzrNb3isA/edit

- Excel Sheet recording all the Protein, Carbohydrate, calories and 2-cup, 2-choice plots calculations and conversions for plotting can be found here:
    - https://docs.google.com/file/d/1S0DIAUJYQ_0pSH2AqZ4-02F1sbZnDQRN/edit?usp=docslist_api&filetype=msexcel

2. CONTACT FOR REAGENT AND RESOURCE SHARING
    - Shrutika Lokkapure (shrutika.lokapure@students.iiserpune.ac.in) can be contacted for any type of doubts/clarifications/resources/queries/information in any part of the thesis, Manikrao Thakare (mrdthakare@gmail.com) and Gaurav Das (gauravdas.dm@gmail.com / gauravdas@nccs.res.in) can be contacted for sharing of resources or any further queries/information regarding the thesis.

3. EXPERIMENTAL MODEL, SUBJECT AND REARING DETAILS
    - *Drosophila melanogaster* male flies [Wild type Canton-S (CS-Q) fly strain] were used for all the experiments.
    - All flies were reared on modified Bloomington's semi-defined food (**Musselman et al. 2011**) at 22-25 °C and low humidity mode in the Panasonic incubator (MIR-154-PE) with the specific evaporation conditions (as mentioned in the Chapter-2-Methods), with a 12 h:12 h light:dark cycle inside incubators.
    - I collected male flies under CO_2 anesthesia on days 1-3 after eclosion. After collection, flies were aged for an additional 1-2 days in agar vials with the corresponding type of food for habituation to nullify the effect of CO_2 anesthesia before starting the experiments (with the same type of corresponding particular food for each condition)

4. METHOD DETAILS (Already mentioned in Chapter-2 Methods of this thesis):
    - DIETS Assay (Thakare et al 2023)

- Troubleshooting for evaporation standardization

5. QUANTIFICATION, DATA ANALYSES AND SOFTWARE AVAILABILITY
   - Numbers Application in Mac along with Excel Application in Windows was used for tabulating all the tables presented in this thesis and recording the raw 24 hour feeding data in flies
   - GraphPad Prism version 8.3.1 was used for plotting of all the graphs in this thesis
   - All pictures and images taken in this thesis are original and have been taken by the author herself.

6. Different trial and errors and calculations done before achieving the required 1:1, 1:2, 1:4, 1:8 and 1:16 diets!

With the purpose of achieving 1:1, 1:2, 1:4, 1:8 and 1:16 P:C ratio diets, I wanted to check dividing the proteins with what factor would give me these ratios. With that in mind, I thought of dividing and reducing proteins by all different whole numbers sequentially starting from 2,3,...all the way till 32 and checked what happens to the ratio of P:C. The exact calculations, manipulations and the final constituents and numbers are shown in the forms of tables below. Very interestingly, I realized that dividing the proteins by a difference of factor of exactly 1 whole number increases the P:C ratio by exactly a factor of 2.5. For example, to make this trend clear: doing P/2 makes the P:C ratio 1:1.5, doing P/3 makes the P:C ratio 1:2, doing P/4 makes the ratio 1:2.5 and so on. Thus, this trend is continued all the way for all whole numbers that we divide our proteins with and is very very stark as shown below. So, this is how I then achieved all the main ratios of 1:1, 1:2, 1:4, 1:8 and 1:16 P:C that I have used for all my experiments in this thesis. Follow below, observe and enjoy!

	For 1L of diet:	Weight (gm/L)	Carbs (gm/L)	Proteins (gm/L)	Fats (gm/L)
Basal Control Diet- P:C (1:1)	Agar	10	8.9		0.1
	Brewer's Yeast	160	64	72	1.6
	Yeast Extract	40	6.6	21.6	
	Peptone	40	0.2	29.2	
	Sucrose	51	51		
			130.7	122.8	1.7
	P:C	0.9395562357			
		1.064332248			
	1:1				
	Total Calories (g/L or kJ/L)	1028.45	522.8	491.2	14.45

Appendix Table 5.1.2: Achieving P:C ratio of 1:1- Basal diet (1L)

	For 1L of diet:	Weight (gm/L)	Carbs (gm/L)	Proteins (gm/L)	Fats (gm/L)
P/2, C constant	Agar	10	8.9		0.1
	Brewer's Yeast	80	32	36	0.8
	Yeast Extract	20	3.3	10.8	
	Peptone	20	0.1	14.6	
	Sucrose	51	51		
			95.3	61.4	0.9
	P:C	0.6442812172			
		1.552117264			
		1:1.55			
	Total Calories (g/L or kJ/L)	634.45	381.2	245.6	7.65

Appendix Table 5.1.3: Achieving P:C ratio of 1:1.55 by doing P/2 and keeping C constant from the 1:1 basal diet (1L)

	For 1L of diet:	Weight (gm/L)	Carbs (gm/L)	Proteins (gm/L)	Fats (gm/L)
P/3, C constant	Agar	10	8.9		0.1
	Brewer's Yeast	53.3	21.33	24	0.533
	Yeast Extract	13.3	2.2	7.2	
	Peptone	13.3	0.06	9.733	
	Sucrose	51	51		
			83.49	40.933	0.633
	P:C	0.4902742843			
		2.03967459			
		1: 2			
	Total Calories (g/L or kJ/L)	503.0725	333.96	163.732	5.3805

Appendix Table 5.1.4: Achieving P:C ratio of 1:2 by doing P/3 and keeping C constant from the 1:1 basal diet (1L)

	For 1L of diet:	Weight (gm/L)	Carbs (gm/L)	Proteins (gm/L)	Fats (gm/L)
P/4, C constant	Agar	10	8.9		0.1
	Brewer's Yeast	40	16	18	0.4
	Yeast Extract	10	1.65	5.4	
	Peptone	10	0.05	7.3	
	Sucrose	51	51		
			77.6	30.7	0.5
	P:C	0.3956185567			
		2.527687296			
	1:2.5				
	Total Calories (g/L or kJ/L)	437.45	310.4	122.8	4.25

Appendix Table 5.1.5: Achieving P:C ratio of 1:2.5 by doing P/4 and keeping C constant from the 1:1 basal diet (1L)

	For 1L of diet:	Weight (gm/L)	Carbs (gm/L)	Proteins (gm/L)	Fats (gm/L)
P/5, C constant	Agar	10	8.9		0.1
	Brewer's Yeast	32	12.8	14.4	0.32
	Yeast Extract	8	1.32	4.32	
	Peptone	8	0.04	5.84	
	Sucrose	51	51		
			74.06	24.56	0.42
	P:C	0.3316230084			
		3.015472313			
	1: 3				
	Total Calories (g/L or kJ/L)	398.05	296.24	98.24	3.57

Appendix Table 5.1.6: Achieving P:C ratio of 1:3 by doing P/5 and keeping C constant from the 1:1 basal diet (1L)

	For 1L of diet:	Weight (gm/L)	Carbs (gm/L)	Proteins (gm/L)	Fats (gm/L)
P/6, C constant	Agar	10	8.9		0.1
	Brewer's Yeast	26.666	10.66	12	0.2666
	Yeast Extract	6.66	1.1	3.6	
	Peptone	6.66	0.03	4.866	
	Sucrose	51	51		
			71.69	20.466	0.3666
	P:C	0.2854791463			
		3.50288283			
	1: 3.5				
	Total Calories (g/L or kJ/L)	371.7401	286.76	81.864	3.1161

Appendix Table 5.1.7: Achieving P:C ratio of 1:3.5 by doing P/6 and keeping C constant from the 1:1 basal diet (1L)

	For 1L of diet:	Weight (gm/L)	Carbs (gm/L)	Proteins (gm/L)	Fats (gm/L)
P/7, C constant	Agar	10	8.9		0.1
	Brewer's Yeast	22.857	9.142857	10.2857	0.22857
	Yeast Extract	5.7142857	0.942857	3.0857	
	Peptone	5.7142857	0.028571	4.1714	
	Sucrose	51	51		
			70.014285	17.5428	0.32857
	P:C	0.2505602964			
		3.991055305			
	1: 4				
	Total Calories (g/L or kJ/L)	353.021185	280.05714	70.1712	2.792845

Appendix Table 5.1.8: Achieving P:C ratio of 1:4 by doing P/7 and keeping C constant from the 1:1 basal diet (1L)

	For 1L of diet:	Weight (gm/L)	Carbs (gm/L)	Proteins (gm/L)	Fats (gm/L)
P/8, C constant	Agar	10	8.9		0.1
	Brewer's Yeast	20	8	9	0.2
	Yeast Extract	5	0.825	2.7	
	Peptone	5	0.025	3.65	
	Sucrose	51	51		
			68.75	15.35	0.3
	P:C	0.2232727273			
		4.478827362			
	1: 4.5				
	Total Calories (g/L or kJ/L)	338.95	275	61.4	2.55

Appendix Table 5.1.9: Achieving P:C ratio of 1:4.5 by doing P/8 and keeping C constant from the 1:1 basal diet (1L)

	For 1L of diet:	Weight (gm/L)	Carbs (gm/L)	Proteins (gm/L)	Fats (gm/L)
P/15, C constant	Agar	10	8.9		0.1
	Brewer's Yeast	10.66666	4.2	4.8	0.106
	Yeast Extract	2.666	0.44	1.44	
	Peptone	2.666	0.0133	1.9466	
	Sucrose	51	51		
			64.5533	8.1866	0.206
	P:C	0.1268192331			
		7.885239293			
	1: 8				
	Total Calories (g/L or kJ/L)	292.7106	258.2132	32.7464	1.751

Appendix Table 5.1.10: Achieving P:C ratio of 1:8 by doing P/15 and keeping C constant from the 1:1 basal diet (1L)

	For 1L of diet:	Weight (gm/L)	Carbs (gm/L)	Proteins (gm/L)	Fats (gm/L)
P/16, C constant	Agar	10	8.9		0.1
	Brewer's Yeast	10	4	4.5	0.1
	Yeast Extract	2.5	0.4125	1.35	
	Peptone	2.5	0.0125	1.825	
	Sucrose	51	51		
			64.325	7.675	0.2
	P:C	0.1193159736			
		8.381107492			
	1: 8.4				
	Total Calories (g/L or kJ/L)	289.7	257.3	30.7	1.7

Appendix Table 5.1.11: Achieving P:C ratio of 1:8.4 by doing P/16 and keeping C constant from the 1:1 basal diet (1L)

	For 1L of diet:	Weight (gm/L)	Carbs (gm/L)	Proteins (gm/L)	Fats (gm/L)
P/32, C constant	Agar	10	8.9		0.1
	Brewer's Yeast	5	2	2.25	0.05
	Yeast Extract	1.25	0.20625	0.675	
	Peptone	1.25	0.00625	0.9125	
	Sucrose	51	51		
			62.1125	3.8375	0.15
	P:C	0.06178305494			
		16.18566775			
	1:16				
	Total Calories (g/L or kJ/L)	265.075	248.45	15.35	1.275

Appendix Table 5.1.12: Achieving P:C ratio of 1:16 by doing P/32 and keeping C constant from the 1:1 basal diet (1L)

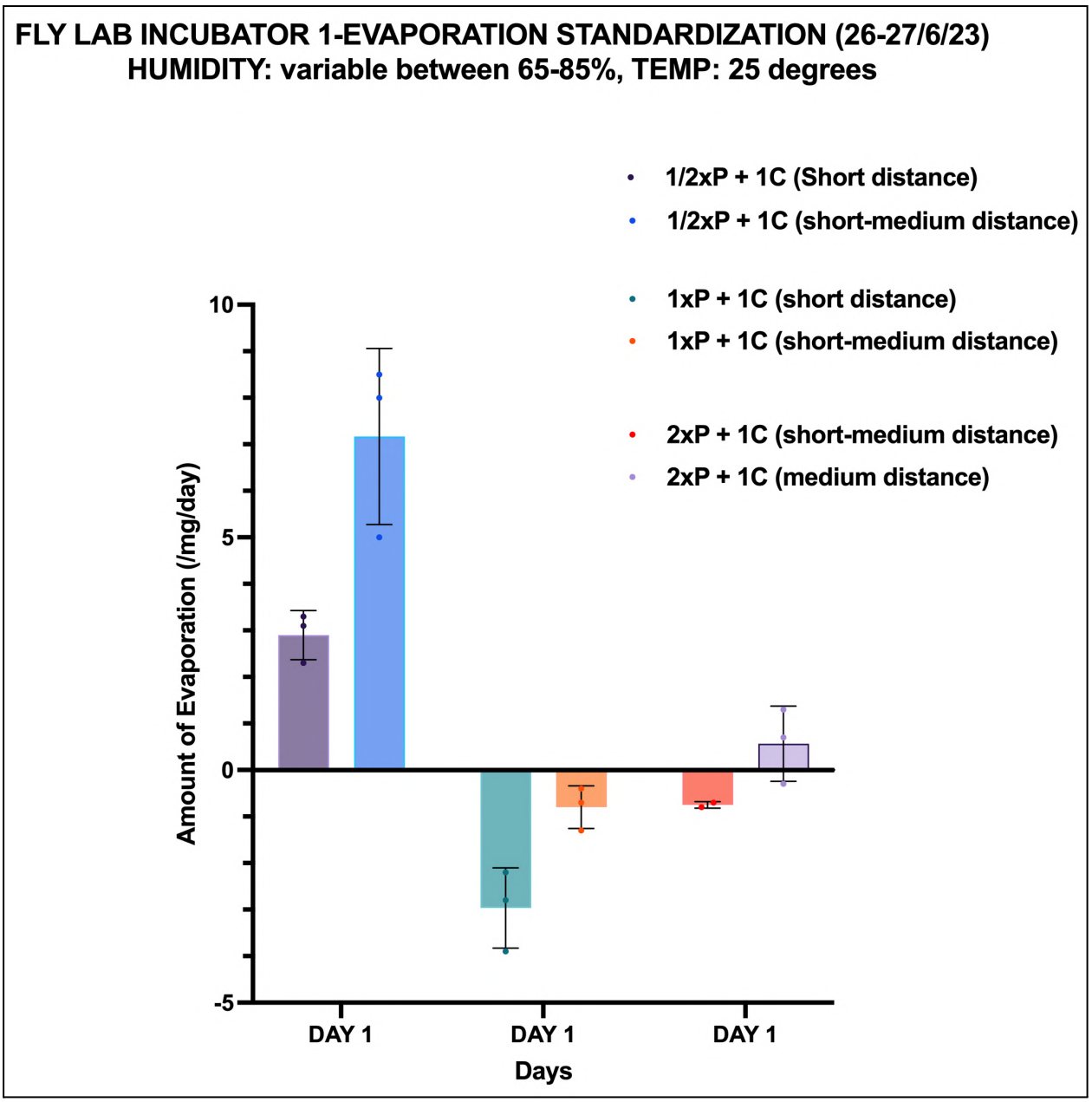
7. Different trial and errors and evaporation standardization done before achieving the required 1:1, 1:2, 1:4, 1:8 and 1:16 diets!

One of the main challenges of using various different P:C diets was to have evaporation values of each of these diets in a permissible comparable threshold of <20% of average consumption in that particular diet (Thakare et al. 2023)
But like I discussed in the 'Troubleshooting' section of the main text-keeping the evaporation to the permissible threshold was successfully possible because of several other evaporation standardization experiments I carried out with the other different concentrated and diluted diets of proteins and carbohydrates.

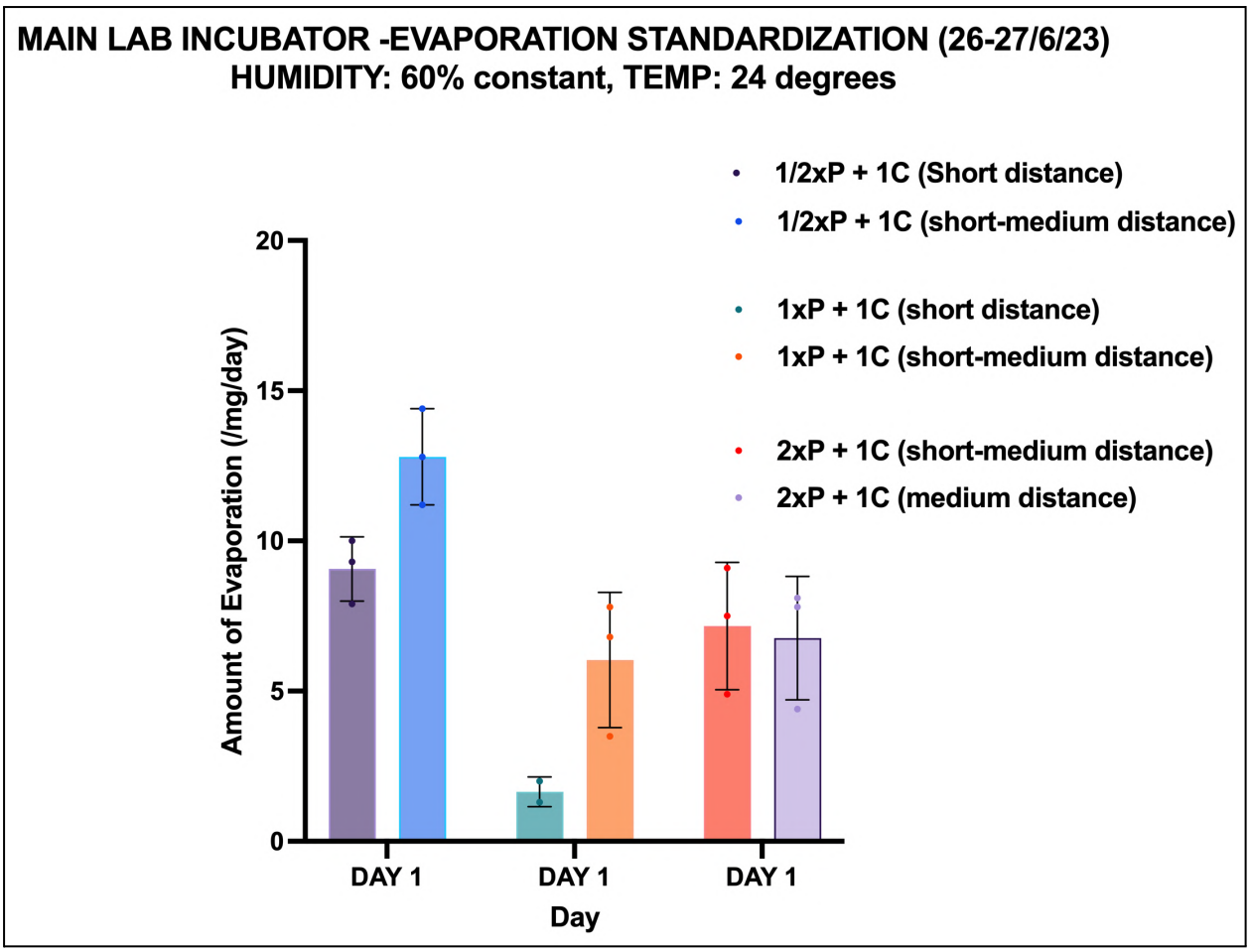
Like discussed in the main text, a major chunk of my master's thesis was thus, evaporation standardization and troubleshooting.

This included the following logical steps performed in chronology where every next evaporation standardization step is built upon the outcomes/results of the previous one. All of these experiments were carried out in diets that were rich/concentrated in carbohydrates and proteins separately (like 2xP1xC, 2xC1xP) or dilute in carbohydrates and proteins separately (like 1/2xP1xC, 1xP1/2xC) or the basal control diet being 1xC1xP. Thus, following are the steps I performed to be able to finally reach substantial solutions to crack troubleshooting and evaporation standardization in my main largely varied P:C ratio diets:

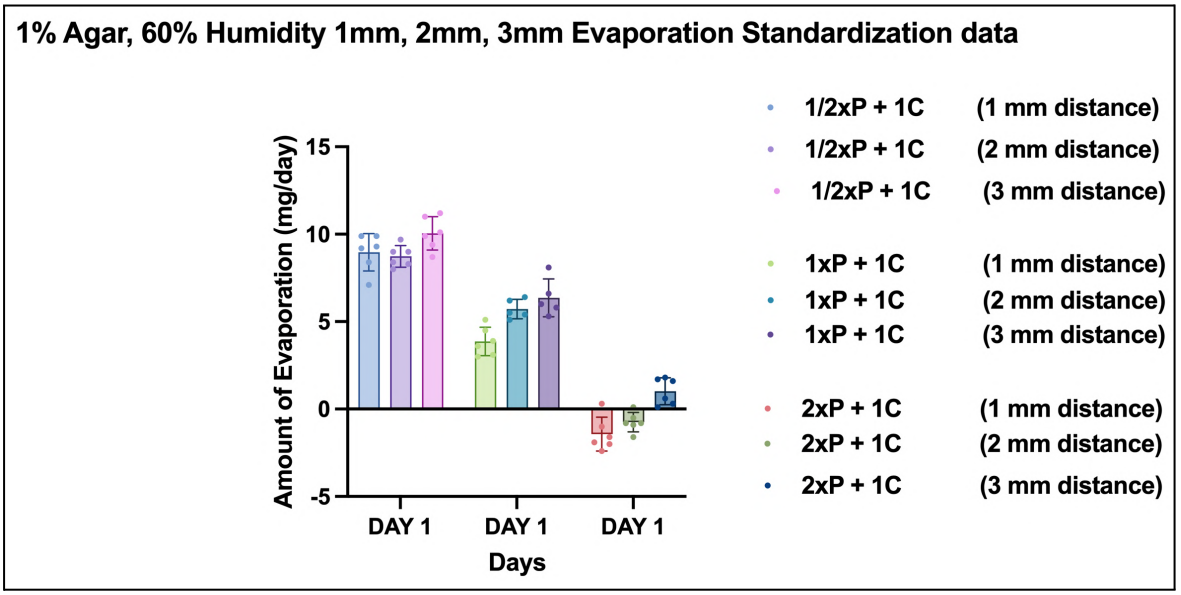
1) Standardizing for short, short medium distances for various food cups
2) Further nuancing the short, medium and long distance into the exactly measured 1 mm, 3 mm and 5 mm
3) 3 mm distance being apt for highly concentrated diets whereas when kept at any lower distance than that- the evaporation becomes negative
4) Whereas for diets that are highly dilute, the evaporation cannot be reduced further by changing the distance of the food cup as it already is at the most minimum distance of 1 mm from the agar bed, in such cases options like placing wet tissue paper beneath the vials brings down the evaporation but makes it highly variable and even negative sometimes.



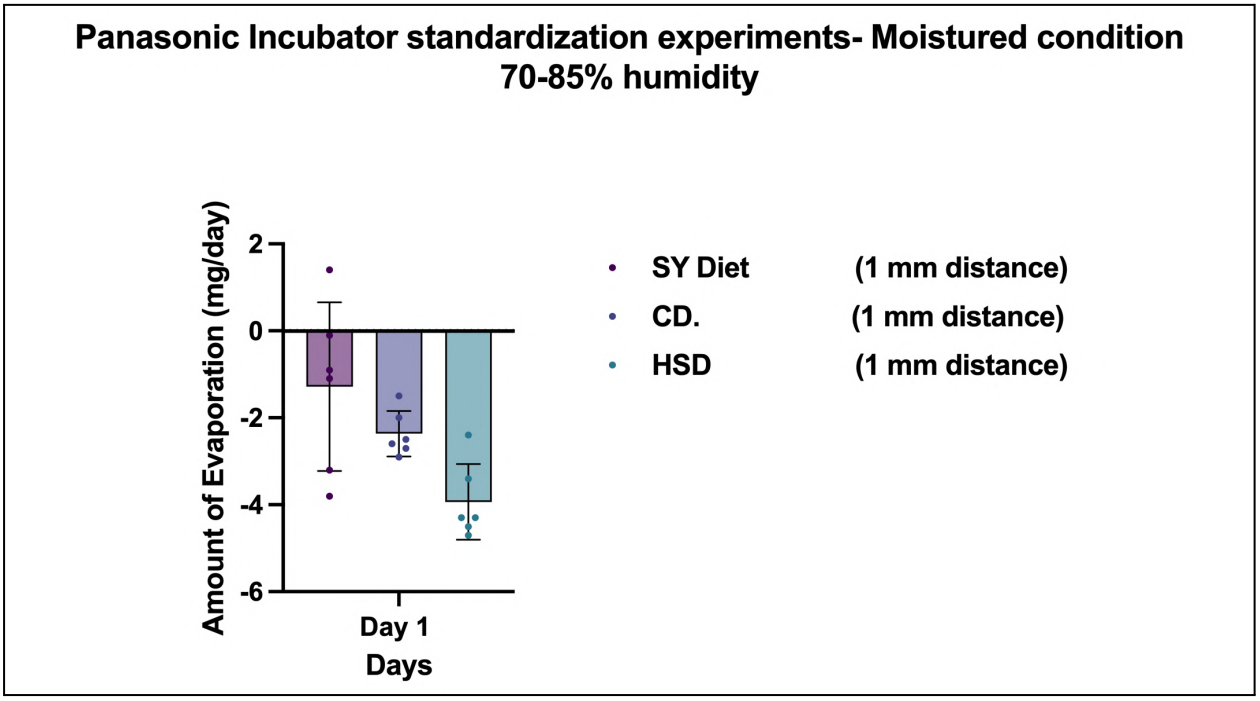
Appendix Graph 5.1.1- To minimize the evaporation to a small positive number, only 1/2xP + 1C at short-medium distance seems to have achieved it as the rest of the values are either negative or too high



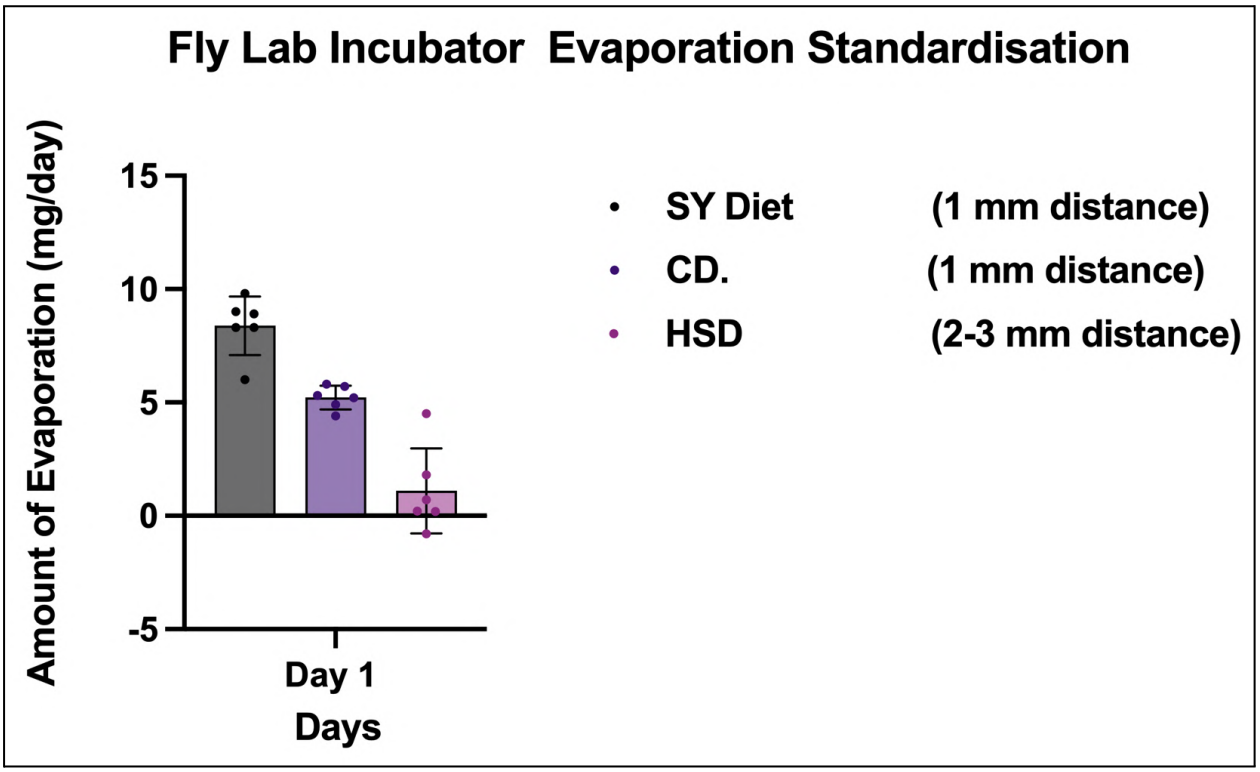
Appendix Graph 5.1.2- To minimize the evaporation to a small positive number, 1/2xP + 1C at short distance, 1xP +1xC basal at short distance and high density diet of 2xP + 1xC seems to be positive But the major challenge in this was that short, .medium and long are still vague terms and not completely defined!



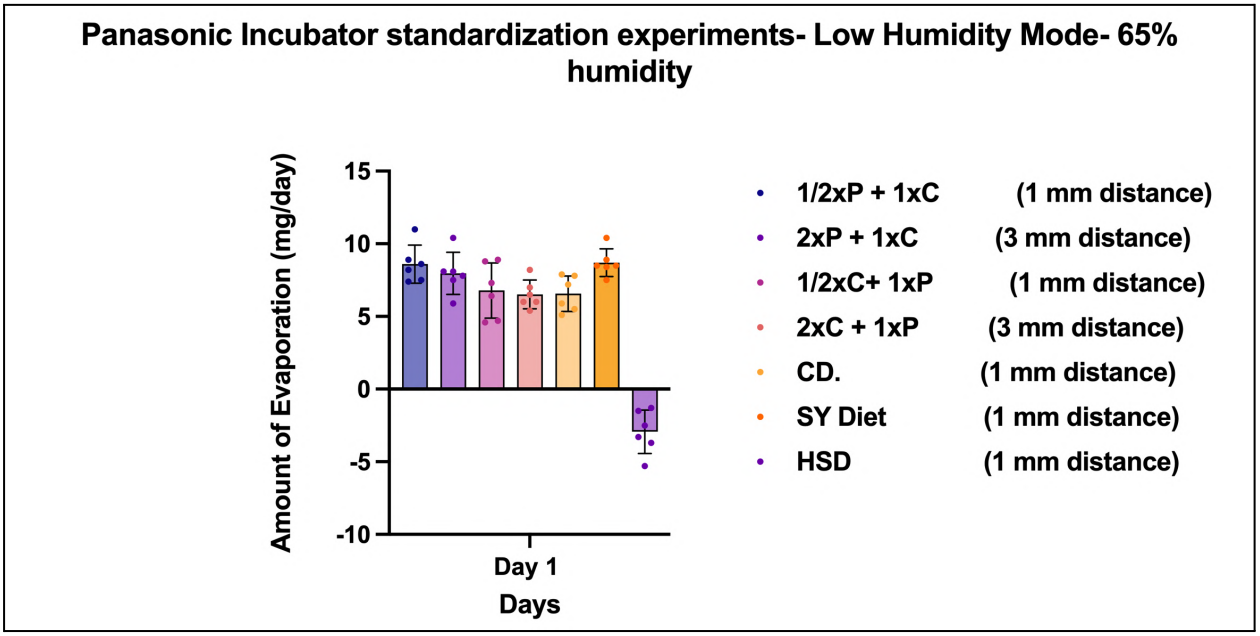
Appendix Graph 5.1.3- Now using the proper distances of 1, 2 and 3 mm, the evaporation values are positive but still very high



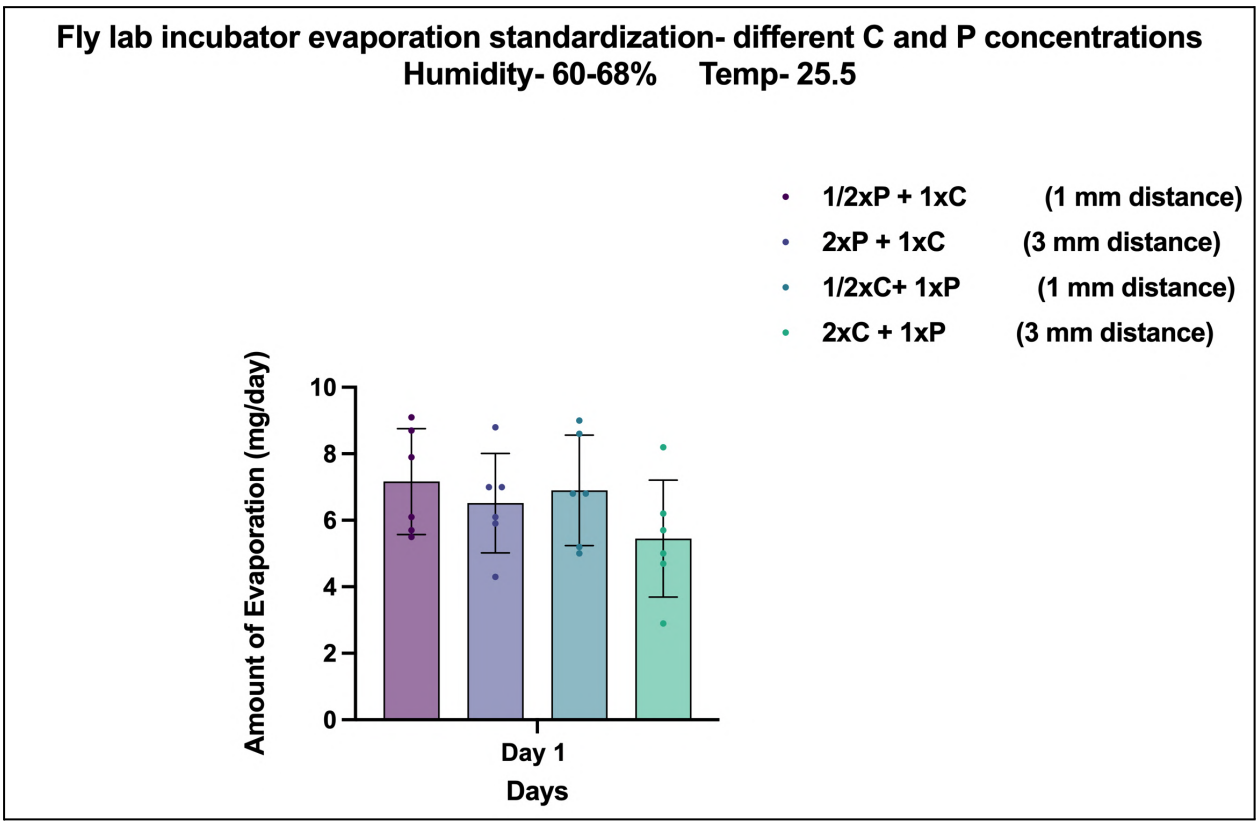
Appendix Graph 5.1.4- Now to bring down the evaporation from the graph above further, a wet-moistened tissue is kept underneath- but it leads all the values to become negative for simple Yeast-sugar diet (YS), High sugar diet (HSD) and the basal even control diet (CD) inside the Panasonic incubator.



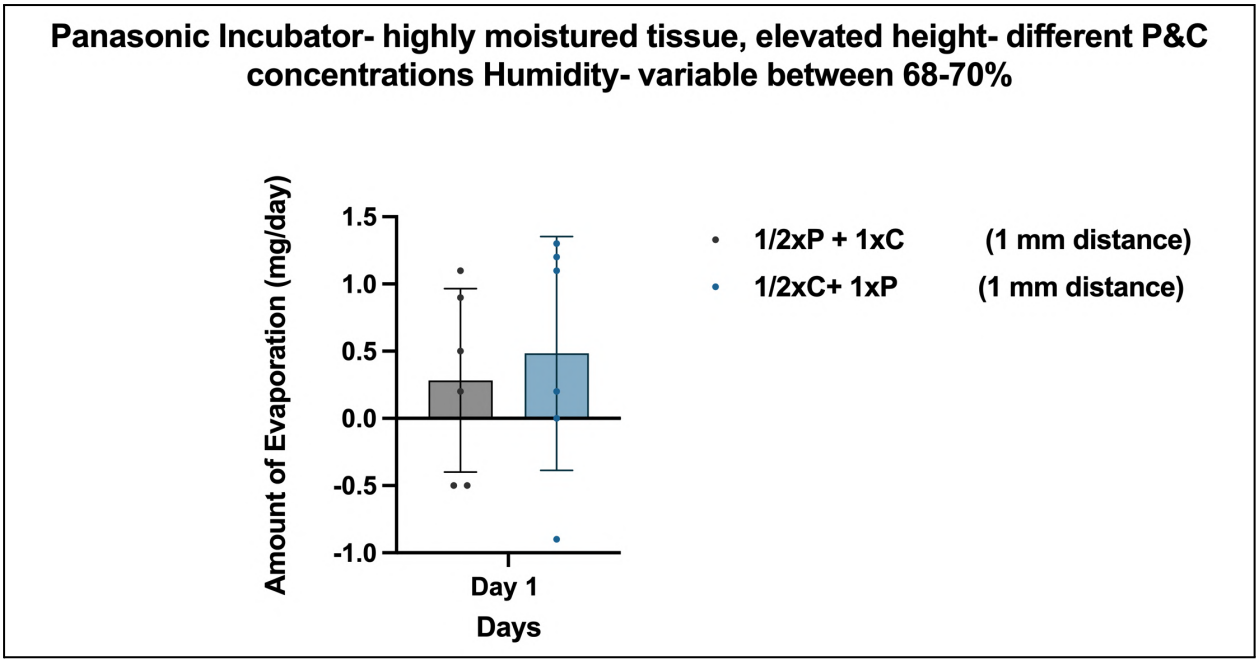
Appendix Graph 5.1.5- now the above mentioned negative evaporation was corrected by placing high sugar diet food at 2-3 mm distance from the agar bed but keeping the sucrose-yeast diet and basal control diet at the same minimal distance of 1 mm from the agar bed.



Appendix Graph 5.1.6- Reconfirming the fact with empirical evidence that high density diets like High sugar diet (HSD) or 2xC+1xP or 1xC+2xP, need to be placed at 3 mm for optimum evaporation



Appendix Graph 5.1.7- Reconfirming that food cups need to be placed at 3 mm for 2xP or 2xC conditions or at 1 mm for highly diluted foods like ½ x C and ½ xP. However the value of evaporation is still very high and way beyond the small permissible value.



Appendix Graph 5.1.8- Thus, to bring down the evaporation values for the highly diluted foods like ½x,P + 1xC placed at 1 mm like shown above even further, a moisturized tissue environment helps by decreasing the evaporation and maintains a good humidity!

8. Exact Evaporation Standardizations and Troubleshootings with 1/2xC+1xP, 1/2xP+1xC,1xC+1xP (basal control diet), 2xC+1xP, 2xP+1xC

Before making of different ratios of my main P:C ratios of 1:1, 1:2, 1:4, 1:8 and 1:16 mentioned throughout this thesis, I had first made the following basic **1/2xC+1xP, 1/2xP+1xC, 2xC+1xP, 2xP+1xC from the basal control diet of 1xC+1xP (Musselman et al 2011)** by only manipulating and altering just the Yeast extract and not brewer's yeast as we earlier thought that if this was both a source of carbohydrate and protein, then we should not alter it. But we were wrong, as this hardly helped us change the basal P:C ratio of 1:1.8 as can be seen below. The P:C ratios of all these diets have hardly changed from the 1:2 ratio and thus, I realized that calling these ratios by these names is actually a misnomer. Thus, these ratios did not help me decipher and decode anything further or decode any trend about proteins and carbohydrates as these were hardly altered- but these diets strongly helped me with one thing for sure: evaporation Standardization and troubleshooting with these diets in the Panasonic incubator. These diets and repeated rounds of trial and errors with these experiments gave me an idea of how to crack the evaporation standardization and troubleshooting with different consistencies (thick/thin) of diets as discussed in the main evaporation standardization and troubleshooting section of the chapter-2 Methods of this thesis!

Making of 1/2, 1 and 2x Protein and Carbohydrate diets…!

1/2xP + 1xC (Halved Yeast Extract and Peptone only)	*For 1L of diet:*	Weight (gm/L)
	Yeast (Brewer's Yeast + Yeast Extract)	80+10 = 90
	Peptone	10
	Sucrose	51
	Proteins (gm/L)	51.915
	Carbs (gm/L)	102.85
	P:C	**0.504**
	1:1.98	
	Calories (g/L or kJ/L)	**605.635**

1xP + 1xC	*For 1L of diet:*	Weight (gm/L)
	Yeast (Brewer's Yeast + Yeast Extract)	80+20 = 100
	Peptone	20
	Sucrose	51
	Proteins (gm/L)	61.4
	Carbs (gm/L)	112.3
	P:C	**0.546**
	1:1.8	
	Calories (g/L or kJ/L)	**702.6**

2xP + 1xC (Doubled Yeast Extract and Peptone only)	*For 1L of diet:*	Weight (gm/L)
	Yeast (Brewer's Yeast + Yeast Extract)	80+40 = 120
	Peptone	40
	Sucrose	51
	Proteins (gm/L)	80.37
	Carbs (gm/L)	131.2
	P:C	**0.61257**
	1:1.63	
	Calories (g/L or kJ/L)	**896.53**

1/2xC + 1xP (Halved only sucrose)	*For 1L of diet:*	Weight (gm/L)
	Yeast (Brewer's Yeast + Yeast Extract)	80+20 = 100
	Peptone	20
	Sucrose	24.75
	Proteins (gm/L)	61.4
	Carbs (gm/L)	86.05
	P:C	**0.7135**
	1:1.4	
	Calories (g/L or kJ/L)	**601.01**

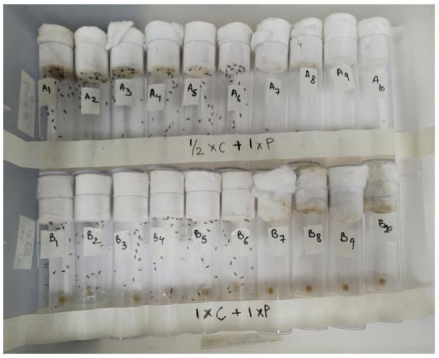
2xC + 1xP (Doubled only Sucrose)	*For 1L of diet:*	Weight (gm/L)
	Yeast (Brewer's Yeast + Yeast Extract)	80+20 = 100
	Peptone	20
	Sucrose	100
	Proteins (gm/L)	61.4
	Carbs (gm/L)	161.3
	P:C	**0.3806**
	1:2.62	
	Calories (g/L or kJ/L)	**892.23**

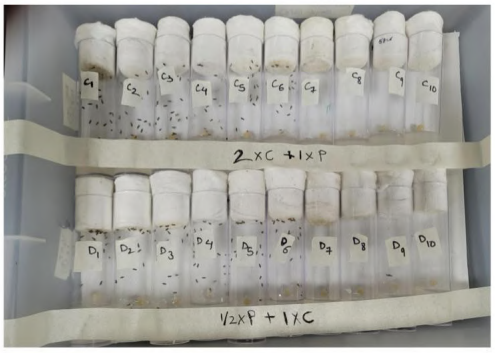
Appendix Image 5.1.13:**1/2xC+1xP, 1/2xP+1xC, 2xC+1xP, 2xP+1xC from the basal control diet of 1xC+1xP (Musselman et al 2011)** by only manipulating and altering just the Yeast extract and not brewer's yeast

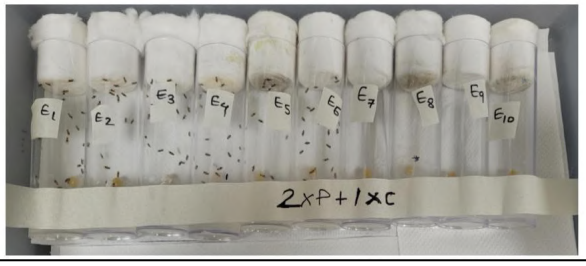
The following images are self-explainable with steps taken and evaporation solutions achieved as follows. The real-life summary with actual photos and tables that I made for this purpose and actual procedure followed-help put forth how the entire evaporation standardization and troubleshooting made for an important chunk of my work and was cracked successfully in my master's thesis project! With all this prior data and troubleshooting experience, it was thus possible to perform calculated troubleshooting with evaporation standardization for all the different diets of P:C as mentioned in the main text.

Continuation of DIETS with varying concentration of P & C along with Evaporation standardization for each condition (vials kept horizontally, without any water source in the incubator)

DAY 1





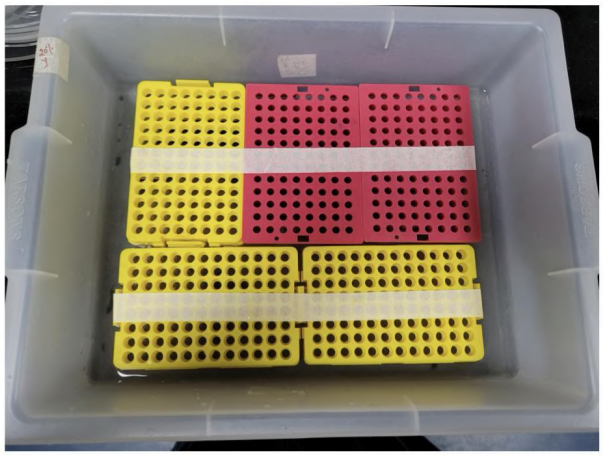


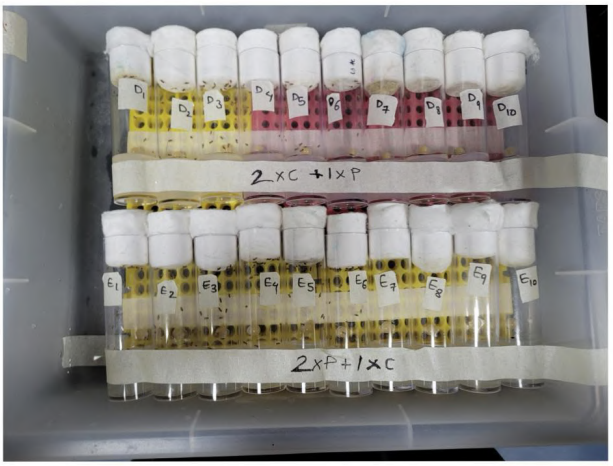
Day-1 DIETS Conclusion: simple horizontal vials setup in the Panasonic incubator without any water source!

DAY	Condition	Average Consumption (mg/fly)	Average Evaporation	Is avg. Evaporation <20% avg. Consumption?	Solution
DAY 1 (all normal open tray conditions)	**1/2xC + 1xP (1mm)**	27.8	5.225	YES	
	1xP + 1xC (1mm)	18.033	2.325	YES	
	2xC + 1xP (3mm)	17.583	**6.35**	**NO: bring evaporation down to 2**	**Constant water underneath with an elevated platform**
	1/2xP + 1xC (1mm)	25.1166	6.075	Almost	
	2xP + 1xC (3mm)	16.5833	**6.05**	**NO: bring evaporation down to 2**	**Constant water underneath with an elevated platform**

DIETS post corrective measures- elevated surface with 200ml water underneath for the denser diets (i.e. 2x conditions)

DAY 3





Day-3 DIETS Conclusions: usage of 200ml of water in an open tray in the entire Panasonic incubator helps maintain and bring down the evaporation of all other conditions too!

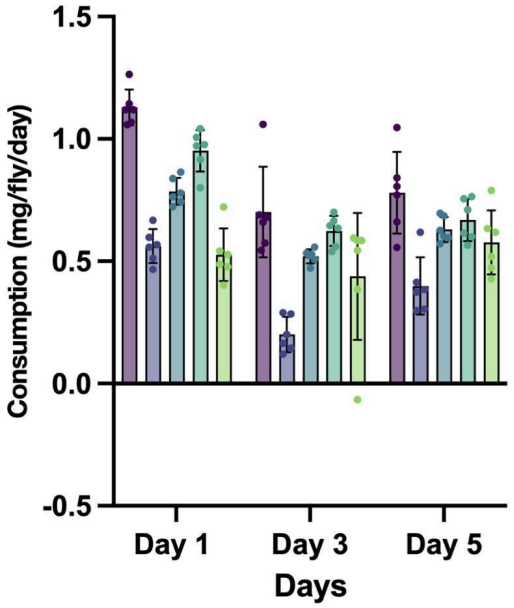
DAY	Condition	Average Consumption (mg/fly)	Average Evaporation	Is avg. Evaporation <20% avg. Consumption?	Conclusion	Solution
DAY 3 (with elevated surface and 200ml water beneath:only for 2x conditions)	**1/2xC + 1xP (1mm)**	16.33	2.6	YES		
	1xP + 1xC (1mm)	11.45	1.05	YES		
	2xC + 1xP (3mm)	4.8166	**-1.95**	**NO: -ve, get it upto 2**	Direct layer of 200ml water got down the evaporation directly from 6 to -ve. But having this tray of water also got down the other evaporation values from 6 to 2-3 which is great!	**Constant (200ml) water in an open tray for the entire environment in the Panasonic incubator with 2x conditions adjacent to this tray**
	1/2xP + 1xC (1mm)	16.15	3.6	Almost		
	2xP + 1xC (3mm)	10.516	**-1.6**	**NO: -ve, get it upto 2**	Direct layer of 200ml water got down the evaporation directly from 6 to -ve. But having this tray of water also got down the other evaporation values from 6 to 2-3 which is great!	**Constant (200ml) water in an open tray for the entire environment in the Panasonic incubator with 2x conditions adjacent to this tray.**

Thus, with all observed changes and conditions, tweaking the conditions finally… this is what my evaporation standardization Panasonic incubator setup looks like!

Finally, getting the evaporation<20% of average consumption and comparable for all the 5 conditions! Yay! Evaporation Standardization worked! Incubator Troubleshooting successful!

DAY	Condition	Average Consumption (mg/fly)	Average Evaporation	Is avg. Evaporation <20% avg. Consumption?	Conclusion
DAY 5 (with 200ml water in an open tray adjacent to the 2x conditions)	**1/2xC + 1xP (1mm)**	19.2833	3.675	YES	**Woohoo, all evaporation values are in a comparable range and <20% of the total consumption! Yay!**
	1xP + 1xC (1mm)	15.4666	2.875	YES	
	2xC + 1xP (3mm)	10.2	**2.225**	**YES**	
	1/2xP + 1xC (1mm)	16.866	3.5	Almost	
	2xP + 1xC (3mm)	14.5666	**3.025**	Almost	

Thus, with all the evaporation standardisation corrective measures, DIETS data reveals…



- 1/2xC + 1xP (1mm distance)
- 2xC + 1xP (3mm distance)
- 1xC + 1xP [CD] (1mm distance)
- 1/2xP + 1xC (1mm distance)
- 2xP + 1xC (3mm distance)

1. Compensatory feeding evident in 1/2xC as expected across all days
2. Feeding in 2xP is more than 2xC
3. Different P concentrations give near about same feeding: no change in P.

Chapter-6

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Notes from the Writer

-Reflections from my Master's Thesis journey

These last couple of years culminating towards my Master's thesis have been a complete roller-coaster ride and this has been hell of a journey- an extremely rewarding one! With getting the opportunity to do this project, the conception of the ideas, formulating of the right questions, facing different challenges in trying to get the experiments right, brainstorming for the possible solutions, executing all the troubleshooting and standardizations and then finally overcoming the obstacles with the most relevant sustainable ideas: I have seen myself grow substantially and immensely throughout! It has been beautiful to go through all the phases of working on a question from scratch! I couldn't have envisaged a better Master's thesis with all the personal, professional and academic growth it has conferred upon me! And thus, with that, I want to briefly record to the best of my abilities the reflections from this journey. Read on, if you had fun reading this so far!

The most fun has been in doing the whole bunch of typical lab routines- back-to-back weekly maintenance and flipping of flies, thinking and planning the most efficient experiments, rightly executing the daily-to-do lists building onto those experiments, taking timely readings, sticking to the plan, not sticking to the plans, things working and things NOT working! Getting dejected over the failures of the experiments but again gathering myself up together for putting more experimental plans, re-working those work plans, plotting and getting out that crisp set of data, NOT getting that expected data, plotting graphs, analyzing what the graph/plot and the trend is saying, analyzing what the data is NOT saying, looking at how the experiments failed, and then again going back to the process to try and fail better this time- the cycle has been vicious but has only gotten better with time and practice!

With this endless cycle, I think this entire journey of my master’s thesis process has given rise to, shaped and further polished my understanding of science and how I think it is rightly done in a biological laboratory setting. I now better understand the philosophy that better science grows from good science. That science and scientific temperament is cultivated from how rightly we think about our questions and then put together all our resources (both intellectual and physical) in what we call our experiments- in order to pursue and attempt to decode the underlying mysteries of the questions that intrigue us!

Through reading history of science and the scientific discoveries made in the past, I was just aware that the foundation of all of science is not understanding what’s happening around us, knowing what’s not known, a drive and curiosity to decipher these events and a quest to deeper our knowledge and understanding of nature and it’s known and unknown natural phenomenons. But now, through this Master’s thesis project and through this opportunity and exposure of doing real independent science for the very first time in my life, attempting to look at something novel in a way that has not been looked at exactly the same way before (at a post-undergrad level), I only now (at the end of substantially meaningful period of 10-12 months) fully understand that all of science is based on failure. Failing, understanding how you failed and above everything being comfortable with failure is inherent and inseparable from the process of doing science, especially the science that is done in the labs. Like it’s famously said in the scientific community, failing everyday but knowing how you can fail better today than yesterday and knowing how to not repeat how you failed, but rather fail newly and uniquely than the last time and learn from those unique failures every single time is the entire premise of building science! Failing and still being able to get up only to fail again, but fail better this time is at the very core of science.

Only now do I fully understand what Issac Newton meant when he said and I quote, “If I have seen any further than others, it is by standing upon the shoulders of giants.” I only knew and vaguely understood what it meant earlier, but only now after producing my own real data and contributing my small bit to what we already know in our lab, I completely understand his quote and what it is like to try to climb and search for the already known giants, their works and thus, their shoulders!

Recently, just a few days ago, with 10 months into my project and with a now-slightly better understanding of how one looks at data and produces meaningful results that helps progress our science, I penned down this small quote with my understanding and perception of it all.

I think it very well summarizes an undergrad's newly molded perception of science once they have gotten their hands dirty with the real hard-core, you-have-been-thrown-at-the-deeper-end-of-the-swimming-pool and now you-must -figure-out-swimming-by your-own kind of scary yet thrilling and liberating independent science that most of us are experiencing for the first time in this very-transitional and crucial phase of their academic/scientific/professional journeys. This cartoon sketch continues to adorn my lab-desk and never fails to remind me of what/why and how I am doing what I am doing!

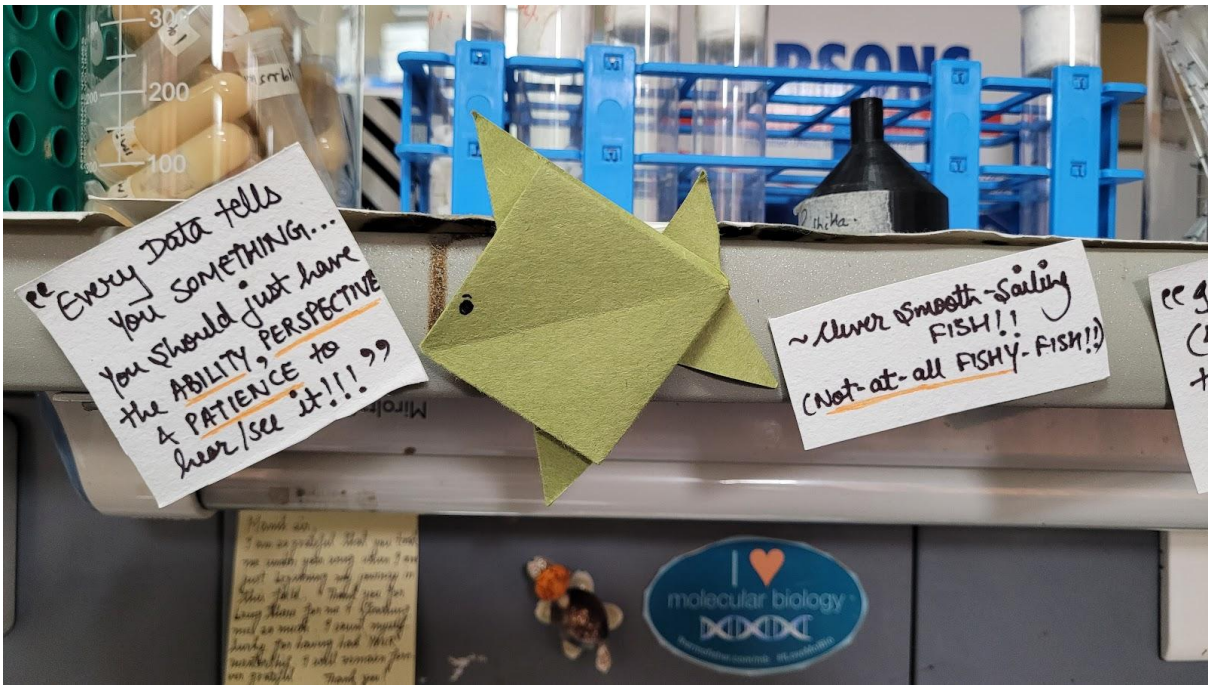

Image: My recent origami and cartoon meme depiction of what I think doing science in a lab is at the end of 10 months of my Master's thesis project!
(Kindly Note: these were not made with the purpose and intention of putting it in my Master's thesis, but I think they aptly summarize my thoughts in a fun way to be included in this 'reflections from the writer' section!)

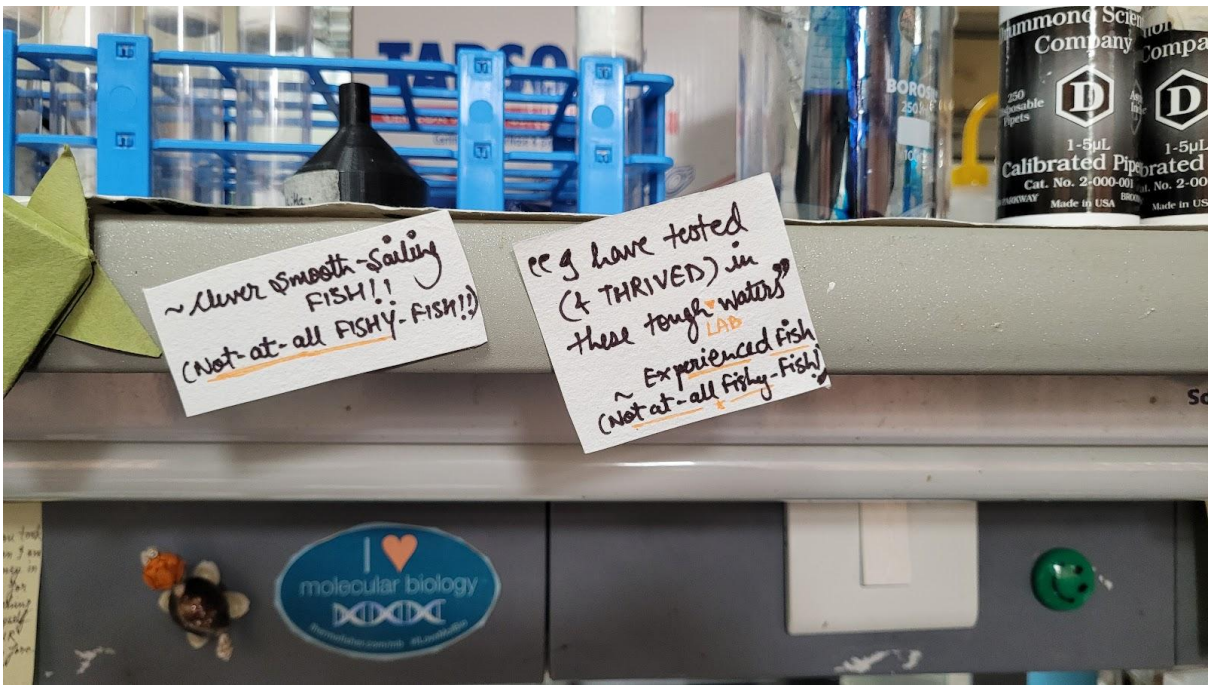

Image: A fun meme depiction of my reflections on what spending meaningful time in my lab, enjoying my work and science truly was like!

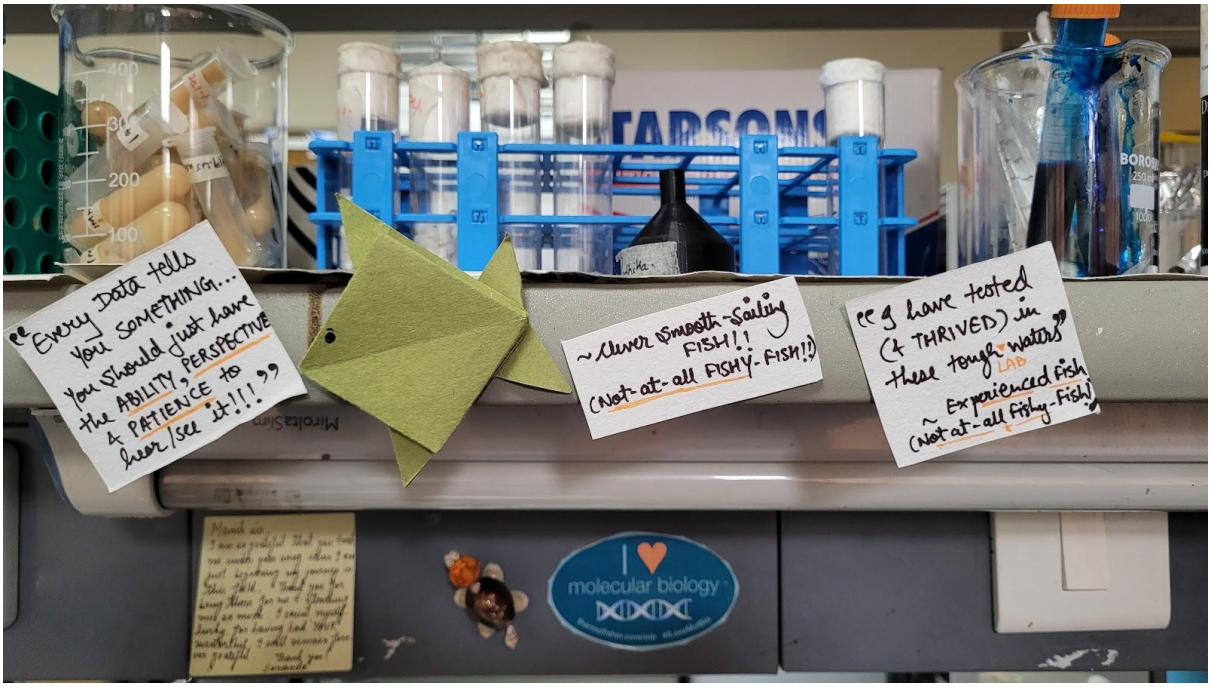

Image: At the end of 10 months of my Master's thesis project, this is what my desk now looks like! The quote is a constant reminder of how I want to do my science the right way.

And only now at the completion of writing down my thesis- I can say that the process has been extremely rewarding and fulfilling! To be able to gather all my meaningful work together and put forth my science in a clear, concise and easy-to-understand way has further helped me develop my science writing and presentation skills! The process of starting and coming back to every section till the science is rightly conveyed to the finest and most relevant detail- has been highly satisfying and rewarding at the end of it all! Designing my own unique thesis

framework based on what has been relevant to my work, thinking about the easiest way to convey my science and results in the most simplified ways, adding key sections, coming back and refining the content that I have added has been one of the most intensive science writing experiences till date- as it should have been! And thus, I am highly grateful to have gotten the opportunity to put together my very first piece of scientific work in the form of this thesis!

Without mentioning the reflections from my thesis work and the thesis writing journey I think, my Master's thesis or rather any piece of scientific work will be highly refined and just results oriented which would be great but it would also nullify and shadow over the details of doing science and as a result fail to reflect the nuances of the journey and the failures. This would in turn put across a false incomplete picture- that the science that has been done has been all hunky-dory and an easy straightforward linear journey and a cake-walk. For it is not. For this is hardly ever the case. Science in the lab is rarely just the “Introduction, Background, Methods, Results, Conclusions and Future Directions”. Even though these are extremely important to be able to reflect the scientific process, they fail to reflect what happens between and beyond all of this. This would be a traditional and typical way of recording which must be done, yes, definitely. We definitely have to present how science has been done objectively, but I believe, and this is of course my personal opinion, that going beyond the subjective-objective debate when scientific writings are also supplemented with how the process has been done in its honest form and as perceived by doer/writer of the process at the individual level- it can be highly empowering for both the writer as well as the reader/consumer of scientific writing.
This would make for a more effective, modern, futuristic and profoundly insightful way to record and capture (in whatever way possible, and in whatever capacity) the process of how the science has been done beyond the predefined categories. I have taken the liberty and freedom of writing and expression in order to do so in the way that is most meaningful to me as a writer and doer of my science.

Any writer can choose to do this in the way they think doing this will be their version of the holistic approach- a true reflection of their work and their journeys in the way they want to capture it. I truly believe that if we supplement all of our writings especially semester project reports, Master's and PhD thesis at the undergraduate and graduate levels (the major scientific writings of our concern in a Research institute) with special notes from the authors/doer/executor of science, it will enrich our scientific writing-reading-and recording experiences and in turn benefit all of us in the scientific community. This may even revolutionize how we write, read, record and do our science.

With all this, this has been nothing short of a phenomenal experience and a journey towards self-discovery!Writing this special and not-so-common 'Note from the author' has been my way of expressing, remembering and paying tribute to this journey. It has been extremely satisfying to pen down my thesis and all of this down and be able to include these special pages as a part of my thesis work, and thus I hope the reader has as much fun and enjoyment in reading this thesis as I had in putting everything together and articulating it all! I have kept my writing simple, easy-to-understand and to the point for even a layperson to read. As any piece of scientific writing should be, I have presented my thesis in such a way that all the work carried out during the term and mentioned in this thesis is completely reproducible by anyone in the world (provided they want to and have the specific resources)! With that, I sincerely hope that I have done a decent job of conveying a small-yet-significant part of my life-journey and thus, in the process have been able to convey and pass on the joy, excitement and love of doing core biological science research to the keen readers through all these pages and chapters of this thesis! Over to the reader!