Quantifying Insect Cell Nutrition for Use in Cellular Agriculture

A thesis submitted by

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Abstract

Cellular agriculture is defined as creating and producing agricultural products such as meat by using cell culture instead of traditional agriculture involving animals. Meat produced via cellular agriculture is called cultured meat which is created by first isolating cells from a donor animal. These initial cells are then multiplied and grown in specific conditions to create meat relevant tissues. However, there are many challenges to creating a cost effective, scalable, nutritious final product using mammalian cells such as bovine. There is a different cell type which can circumvent these issues, insect cells. Insect cells are grown at lower temperatures and without CO_2 regulation similar to ambient conditions, have a slower buildup of toxic byproducts, do not require additional growth factors and in fact produce their own. These factors allow for cost reduction and easy scalability in comparison to mammalian cells. One aspect still to be quantified with primary insect cells for use in cellular agriculture products is nutritional value. Insects themselves are eaten around the world and are known to be more nutritious than steaks and other common meat cuts. It is assumed that insect cells follow this trend and are more nutritious than mammalian cells. Previous research into representative cell lines of insect and mammalian cells has shown insect cells are denser in protein, zinc, and iron. Here, the nutrition of a primary insect cell species, *Manduca sexta*, was compared to primary bovine satellite cells. *M. sexta* cells were found to be denser in protein, similar in zinc and iron to bovine cells, and comparable in protein to whole insects. While many more nutritional aspects are still to be quantified, insect cells show promise in the field of cellular agriculture to create meat to supplement the growing market demand.

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Chapter 1. Introduction

The demand for meat and animal related products is on the rise. By 2050, it is expected that meat consumption will rise by 73% (FAO, 2011). However, traditional agriculture methods will not be able to keep up. This increasing demand for resources and lack of necessary space means there is a need to find an alternative or supplementary source of food, specifically for meat which consumes a vast amount of limited resources and land in addition to producing waste and greenhouse gasses. One way to supplement this market is through cellular agriculture, an alternative to conventional meat production. Here, I review the current knowledge on whole insect nutrition and farming and insect cell culture compared to mammalian livestock nutrition and mammalian cell culture. In addition, I quantify relevant nutritional aspects of primary insect cells isolated from the *Lepidoptera, Manduca sexta,* and compare them to common meat cuts from cattle *Bos taurus*. Compared to mammalian cell culture, insect cells are more robust. They require fewer considerations, such as growth factors and frequent media changes, thrive in a wider range of conditions, pH, temperatures, and osmolarity, and are more easily adapted to alternative culture methods, serum-free and suspension. To support this growing demand for food, insect cells grown in-vitro will need to be characterized and the nutrition quantified and compared to current mammalian cells used for cultured meat.

1.1 Food sustainability and alternatives to traditional meat production

Currently, almost half of habitable land is used for agriculture, 77% of which is dedicated to livestock for meat and dairy (Ritchie, 2017) (Alexander et al., 2016). This meat and dairy livestock produce 61%, the majority, of the livestock sectors greenhouse gas (GHG) emissions (Gerber et al., 2013). This leads to a livestock contribution of 18% of the total GHG emissions produced per year (Gerber et al., 2013). This vast consumption of land and production of GHGs only contributes to 18% of the world's calorie supply and 37% of

the world's protein supply (Ritchie, 2017). With animal agriculture being a leading contributor to GHG emissions and global warming, many alternatives have been presented; plant-based meat being a popular option today as it emits 30%-90% less GHGs than traditional meat farming methods (GFI, 2019). Insect farming has also been proposed and is gaining attention worldwide, not just in Asia and Africa where insects are currently consumed (van Huis et al., 2013) (Bukkens, 1997) (Ramos-Eldorduy et al., 1997). These farmed insects offer several environmental and nutritional advantages over traditional farming methods, they emit fewer GHGs, use up less space, and are often rich in protein, zinc, and iron (van Huis et al., 2013). Insects could be consumed whole or turned into products with less bug characteristics. Cricket powder is gaining popularity as an alternative to wheat flour either as a gluten-free bread or a bread that offers more protein value (da Rosa Machado & Thys, 2019) (Osimani et al., 2018) (Goldin, 2015). Combining plant-based meat and insects leads to foods such as the Insecta burger which is made from ground buffalo worms mixed with a vegetable burger, the upcoming Ento burger which utilizes a plant-based burger where the protein comes from cricket powder mixed in, or the 'neatball' which utilizes root vegetables mixed with ground mealworms to mimic a meatball (*Dutch Retailer Jumbo Launches Edible Insects*, 2014) (ento, 2022) (SPACE10, 2018).

Yet, these methods have disadvantages. Plant-based meats are primarily eaten by vegetarians and vegans and consumers associate these plant-based products with 'disgust' (Michel et al., 2021). Consumers who eat meat want a meat alternative with similar attributes and plant-based meat currently falls short. Insect eating (entomophagy), an alternative solution to the rise in meat and food demands, is viewed with skepticism in Western cultures and does not offer a similar enough meat feel either due to texture and taste differences (Wendin & Nyberg, 2020). While bug burgers, such as Insecta in the Netherlands, are bought by consumers, a poll found most people only bought them once but did not return for more

due to not liking the taste, high cost, or low availability (House, 2016). Other options for supplementing the growing meat consumption demand that reduce the environmental impact and increase the similarity to current meat options are being pursued, such as cellular agriculture.

1.2. Cellular agriculture and mammalian cells

Cellular agriculture utilizes cells isolated from animals grown in a lab compared to the animal itself with conventional meat production. Cellular agriculture as a field is not only producing meat without the need for whole animals but is also exploring other agricultural products such as dairy and leather. Removing the animal from the equation creates a more humane and environmentally friendly process. Cellular agriculture, specifically meat production, has the potential to address environmental issues by producing 96% less GHG emissions, utilizing 99% less land, 45% less energy, and 96% less water than conventionally produced meat (Tuomisto & Teixeria de Mattos, 2011).

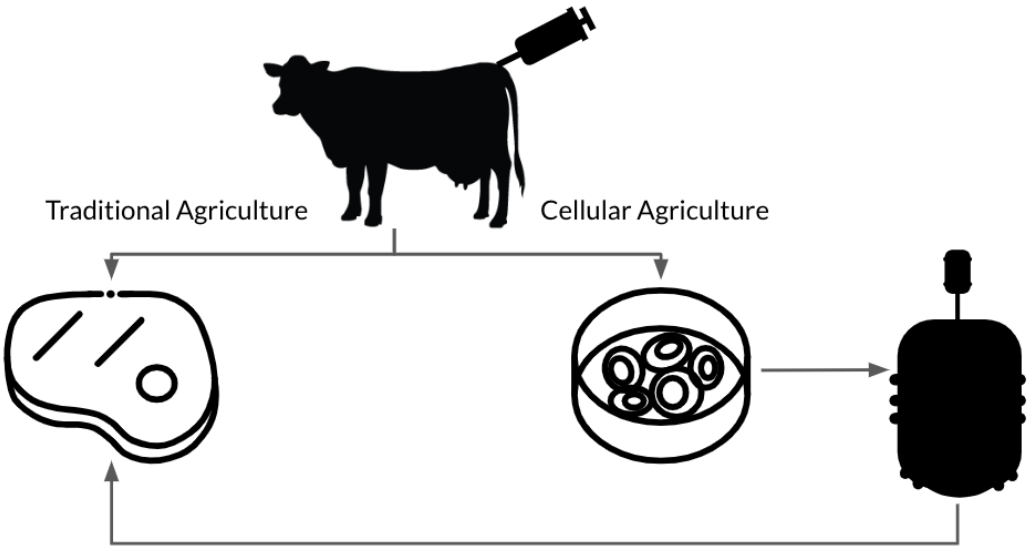

Figure 1. Cellular agriculture compared to traditional agriculture. In cellular agriculture, cells are obtained from the donor animal, expanded in the lab, and turned into meat relevant tissues.

The technology behind cellular agriculture meat products, dubbed cultured, cultivated, cell-based, or clean meat, is relatively new, the first cultured meat burger only being produced less than a decade ago in 2013 for about a quarter million dollars (Post, 2014)

(Alok Jha, 2013). Cellular agriculture and cultured meat have developed dramatically over the past few decades, yet producing cultured meat is still costly. This high cost of cultured meat is due in part to the strict temperature, pH, and CO_2 requirements; however, the main cost is the large media usage and need for expensive growth factors that mammalian cells, such as bovine, require (Konakovsky et al., 2016) (Specht, 2019) (Swartz, 2021) (Cohen et al., 2022). Cultured meat does not yet compare to current meat prices but potentially could in the next decade if more money is put into researching media alternatives and other cost-reducing methods (Swartz, 2021) (Cohen et al., 2022). To truly become an ethical solution to the growing demand for food, cultured meat should be accessible to people from all economic backgrounds (Stephens et al., 2018).

Over one hundred startups with a total investment of $1.93 billion have already been founded focusing on cultured meat (Cohen et al., 2022). Several of the culture meat startup companies are focusing on bovine, porcine, and avian meat, yet are encountering problems in cost, media usage, and scale up – often having to supplement cultured meat products with plant-based protein (Cohen et al., 2022). Mammalian cells, such as bovine, have strict requirements for temperature, CO_2, and pH which contribute to their high-priced production (Konakovsky et al., 2016). Yet, there is a cell type that has fewer requirements leading to reduced production costs, insect cells. Instead of focusing on reducing cost for mammalian cells, which work for traditional agriculture, why not shift to cell types which may be more suited for cellular agriculture? Cultured insect foods may offer a means to sustainably produce cost-effective products.

1.3. Insect cell culture

Insect cell culture is not a new idea, the first insect cell lines were created in 1962 (Grace, 1962). In the decades since then, hundreds of new insect cell lines have been created for a large variety of uses (Smagghe et al., 2009). The use of insect cells for cultured meat

products is not quite as old but still offers tantalizing solutions to current cultured meat issues, mainly cost and scalability: 1) They require fewer media changes as they have a slower build up toxic byproducts such as lactic acid and ammonium like mammalian cells do; 2) they are grown at a lower temperature; 3) they better tolerate changes in pH and osmolarity; 4) they are more easily adapted to alternative culture methods such as serum free media and suspension culture; 5) they are not contact inhibited; and 6) they require mechanical as opposed to enzymatic methods for detachment from the substrate when grown in adherent conditions (Table 1).

	Insect Cells	Mammalian Cells	References
Toxic Byproduct Build Up	Slowly (media change ~weekly)	Rapidly (media change ~two/three days)	Ferrance et al., 1993; Neerman & Wagner, 1996; Rubio et al., 2019b
Temperature	20-32℃	30-39℃	Sisken et al., 1965; Reuveny et al., 1993; Shao-Hua et al., 1998; Invitrogen, 2002; Gotoh et al., 2004; van Oerrs & Lynn, 2010; Rubio et al., 2019b; ThermoFisher, n.d.b
pH	6.0-7.0	6.9-7.9	Mackenzie et al., 1961; Grace, 1962; Ceccarini & Eagle, 1971; van Oerrs & Lynn, 2010; Wagner et al., 2014; ThermoFisher, 2016; Rubio et al., 2019b
Osmolarity	340-390 mosm/kg	290-330 mosm/kg	Waymouth 1970; Ozturk & Palsson, 1990; Zhang et al., 1994; van Oerrs & Lynn, 2010; Rubio et al., 2019b
CO_2 Regulation	No	Yes	Invitrogen, 2002; ThermoFisher, 2016;
Sensitivity to Changes in Temperature, pH, Osmolarity	Low	High	Agathos, 1991

Culture Methods	Suspension and attachment versatility Spontaneous immortalization Ease of serum free adaptability	Difficult to switch between suspension and attachment Immortalized cells are mostly transformed Serum free adaptability is time consuming	Invitrogen, 2002; Brunner et al., 2010; van Oerrs & Lynn, 2010; ThermoFisher, 2016; Luhur et al., 2018
Contact Inhibited?	No	Yes	Agathos, 1991
Detachment Method	Mechanical	Enzymatic	Agathos, 1991; Lynn, 2002; Rubio et al., 2019b

Table 1. Differences in insect and mammalian cell culture.

These insect cells would not be used to create novelty foods or to recreate whole insects, but to create burgers and other meat products consumers eat often. Insect cells would allow for the creation of a final product, such as a burger, with a lower cost than mammalian cells currently offer by reducing energy, media, and personnel expenses.

1.4. Nutrition of insects

Insects have played, and currently play, an important role as a nutrition source in several countries (Bukkens, 1997) (Ramos-Eldorduy et al., 1997). Almost 2000 species are eaten in 113 different countries by 2 billion people (MacEvilly, 2000) (Yen, 2009) (van Huis et al., 2013) (Madau et al., 2020). The most common insects consumed are *Coleoptera* (beetles), *Lepidoptera* (caterpillars and butterflies), *Hymenoptera* (ants and bees), *Orthoptera* (crickets and locusts), and *Heteroptera* (cicadas), with most being consumed in the larval stage (Figure 2) (van Huis et al., 2013) (Hadi & Brightwell, 2021). With such diversity in insect's species and types, there is diversity within their nutrition. Insects themselves vary greatly in nutrition between species and even life stages (Tang et al., 2019) (Oonincx & Finke, 2020) (Xiaoming et al., 2010) (Kouřimská & Adámková, 2016) (Mwangi et al., 2018) (Hlongwan et al., 2020).

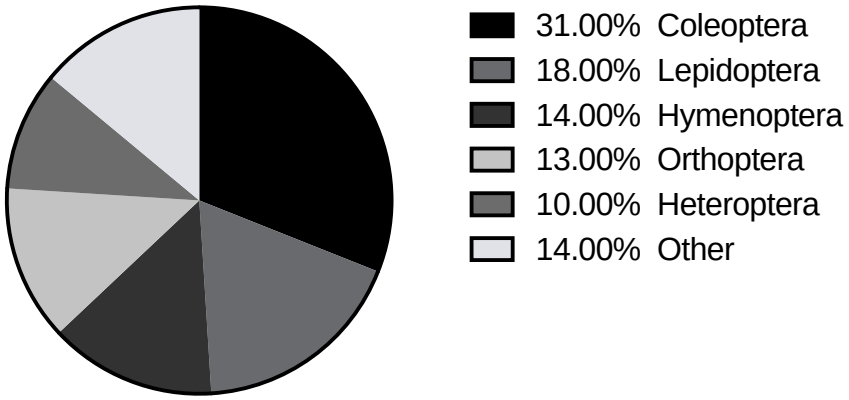

Figure 2. Commonly eaten insect orders. (van Huis et al.,2013; Hadi & Brightwell, 2021).

The protein percent of dry weight (DW), iron milligrams per 100 grams DW, and zinc milligrams per 100 grams DW of several orders of insects is described below (Table 2) (Table 3). The protein percent DW of each class, while it varies, have similar averages around 50%. Comparing this to the protein content of fresh beef cuts (21%-41%), insect have a higher average protein content (Greenwood et al., 1951).

Order	Examples	Stage	Protein content (% DW)	Average protein (% DW)
Coleoptera	Beetles	Adult, larvae	23-66	50
Lepidoptera	Butterflies	Pupae, larvae	14-68	45
Hymenoptera	Bees, wasps, ants	Adult, pupae, larvae, egg	13-77	47
Orthoptera	Grasshoppers, locust, crickets	Adult, nymph	23-65	44
Heteroptera	Cicadas, aphids, bed bugs	Adult, larvae, egg	42-74	53
Odonata	Dragonflies	Adult, naiad	46-65	59
Artiodactyla	Cows	Fresh cut	21-41	30

Table 2. Protein percent dry weight of several insect orders compared to a fresh beef cut (Greenwood et al., 1951; Xiaoming et al., 2010; Kouřimská & Adámková, 2016).

Scientific name	Common name	Iron (mg/100g DW)	Average iron (mg/100g DW)	Zinc (mg/100g DW)	Average zinc (mg/100g DW)
Orthoptera	Grasshoppers, locust, crickets	0.4-910.0	64.8	2.3-1300.0	143.3
Coleoptera	Beetles	2.3-30.9	12.0	2.3-26.8	13.9
Hymenoptera	Bees, wasps, ants	10.4-25.2	17.8	5.7-11.1	7.5
Lepidoptera	Butterflies	1.8-64.0	15.4	8.6-14.0	10.6

Table 3. Iron and zinc content per dry weight of several insect orders (Mwangi et al., 2018; Hlongwan et al., 2020)

The zinc and iron content in milligrams per 100 grams of commonly eaten insect orders (brown) is greater than common beef and pork cuts (red) (Figure 3). These values are for fresh meat cuts and non-dried insects.

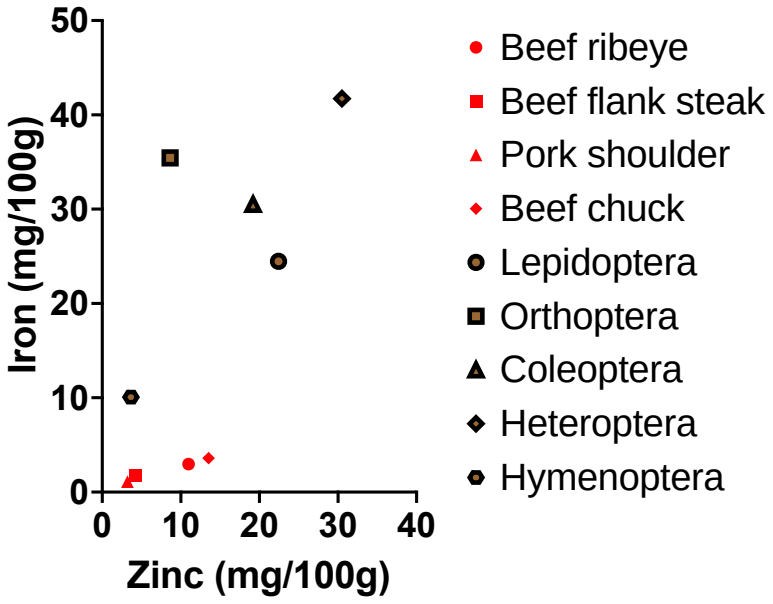

Figure 3. Zinc and iron content of meat cuts and insect orders. Mammalian meat cuts in red, insects in brown (Lategan, 2019; Haytowitz et al., 2019).

Outside of protein, zinc, and iron, whole insects have amino acid profiles comparable to conventional meat; yet the protein quality, the digestibility of proteins (i.e. how well the

protein can be used by the body) and the quantity of amino acids in ratios necessary for humans, is higher in insects; in addition, insects appear to have less fat and more minerals than conventional meat (Figure 4) (Tang et al., 2019) (Verkerk et al., 2007) (Rubio et al., 2019b). While insects may have a higher or relatively comparable nutritional value to conventional meat, are there isolated and cultured cells the same? Insect cells grown in the lab may not have the exoskeleton or the same diet which contributes to and can alter the final nutritional value (Zhao et al., 2019) (Oonincx & van der Poel, 2011) (Oonincx et al., 2015).

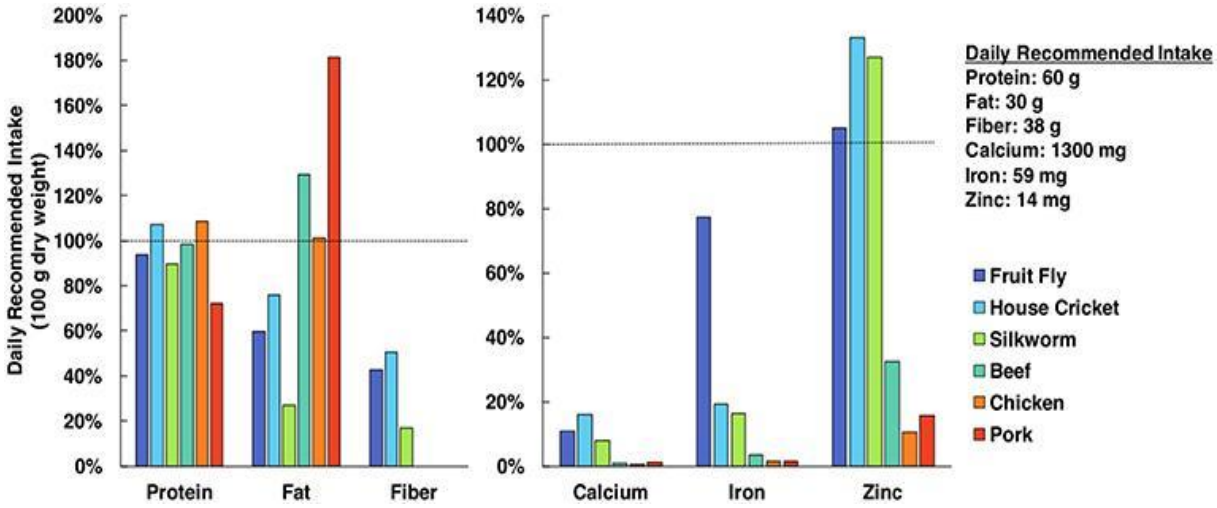

Figure 4. Nutrient profiles of several insect, mammalian, and avian species. (Rubio et al., 2019b) (License: CC BY 4.0).

While insects themselves are known to be nutritious, it is assumed that this translates over to insect cells. Preliminary research has been done with representative cell lines of mammalian and insect cells looking at protein, zinc, and iron quantity which showed drosophila cells had higher concentrations in all three nutritional aspects tested than murine cells (Rubio et al., 2019a). Insects themselves vary greatly in nutrition between species and even life stages meaning more research is needed into other insect cells to make this preliminary evidence stronger (Tang et al., 2019) (Oonincx & Finke, 2020). In addition, primary cells as opposed to cell lines need to be investigated.

1.5. Insect farming and western markets

Until recently at the turn of the twentieth century, the most common way to harvest insects was by collecting them from the wild (Mikkola, 2020). Yet, this is becoming harder to do as land is being converted and urbanized and the wild insects are being overexploited (Mbata et al., 2002). The farming of insects helps to create a dedicated food source while leaving the wild population alone to lessen the environmental impact. There are several ways for insect farming to occur, at the household level, local level, or commercial level. Yet not all species are able to be farmed at a commercial scale, the nutritious mopane worm of the emperor moth are easily impacted by disease leading to a need for several smaller scale farms (van Huis et al., 2013).

Insect farming offers a scalable option to help supplement the growing need for food, however, the issue comes in cost-effectiveness. Currently, there is little data or analysis about the cost-effectiveness of insect farming especially on a large scale. The main cost is the feed required by the insects (Tanga et al., 2021). This can be seen in the price discrepancy between farmed ($10.9-$18.2/kg) and wild caught ($2.8-4.5/kg) crickets (Tanga et al., 2021). Out of the numerous cricket species available, only two cricket species are economically farmed, due mainly to their short life cycle (van Huis et al., 2013). There is the potential to reduce this cost by automating farming processes and investing in the development of lower cost feed.

However, the main issue with insect farming is the ‘ick’ factor. Insects face a strong cultural barrier to come to Western markets, and despite the large benefit insects hold over livestock in nutrition, it is not enough (Deroy et al., 2015). Insects within the United States are currently sold as novelty snack foods as most consumers are not willing to buy foods with visible insects – even in scenarios where there is a drastic price difference and foods with visible insects are cheaper (Collins et al., 2019). Collins et al. (2019) found a strong

correlation between the visibility of insects and the desirability of a food suggesting foods that use insects but don't 'look like bugs' are more viable in the Western market.

1.6. Aims of this study

The aims of this study were to (1) quantify and compare insect cell nutrition to mammalian cell nutrition of protein, zinc, and iron of representative primary cells from the tobacco hornworm, *Manduca sexta,* and a common meat cattle, *Bos taurus*; (2) quantify the effect on protein of differentiation with a naturally occurring ecdysteroid hormone of the isolated primary *M. sexta* cells to help determine the cell type; and (3) isolate new insect cells from *Gryllodes sigillatus* to later quantify nutrition.

Chapter 2. Materials and Methods

2.1. Primary cell isolation

An isolation protocol similar to Harvard postdoctoral researchers Sepp & Bai (n.d.) was followed. Insect cells were isolated from the eggs of the tobacco hornworm, *Manduca sexta*. *M. sexta* eggs were collected from a foam sponge soaked in a tobacco infusion hung in an adult *M. sexta* cage. The tobacco infusion in the sponges attracts the moths as in the wild they lay their eggs under tobacco leaves (Zhou et al., 2017). The sponges were left in the cage for 1.5-2 hours after which the sponges were removed from the cage and the eggs gently scraped off by hand. The eggs are small, around 0.7 mm to 0.8 mm in diameter, and a light green in color (Casanova & Gaud, 1975). The eggs were placed in a small sealable cup and incubated at 27°C overnight for ~19 hours (which is when myogenesis occurs in *M. sexta*).

Culture media was prepared with M3 Shields and Sang Insect Medium (M3; Sigma-Aldrich #S8398) dissolved in distilled water with 2.5 g/L bacto peptone (BP; VWR #90000-264), 1 g/L yeast extract (YE; Sigma-Aldrich #Y1000), and 10% heat inactivated fetal bovine serum (HI-FBS; ThermoFisher #10438-018). Growth media was prepared by adding 100 μg/ml primocin (InvivoGen #ant-pm-1) to the culture media and plating media was prepared by adding 100 μg/ml primocin and 3 mM ethylene glycol-bis(2-aminoethyl)-N,N,N′,N′-tetraacetic acid (EGTA; Sigma-Aldrich #03777). Primocin, a common antimicrobial agent for primary cells, reduces the risk of contamination (Primocin®, 2016). EGTA, a calcium chelator, separates myoblasts based on adhesion (Bernstein et al., 1978). Media was 0.2 μm sterile filtered before use.

After incubation, the eggs were sterilized to remove bacteria on the outside of the egg without disturbing the cells within. The eggs were washed in distilled water, sterilized in 50% bleach for 5 minutes, washed twice in distilled water, and finally washed once in plating media. The eggs were then transferred to a Dounce homogenizer in 5 ml of plating media.

The cells were released from the eggs by gently moving the plunger up and down 5 times. This homogenate was filtered through a 100 μm filter to remove egg casings. The filtered mixture was centrifuged at 40 g for ten minutes at room temperature (RT). The supernatant was then removed and saved. The pellet was resuspended in 5 ml of plating media and counted using an R3 cell counting protocol on the NC-200 automated cell counter (Chemometec). The supernatant of the first centrifugation was centrifuged at 380 g for five minutes at RT. The pellet was resuspended in 5 ml of plating media and the cells were counted using an R3 cell counting protocol. The cells from the first and second round of centrifugation were seeded at 100k/cm^2 and incubated for two hours at 27°C. Next, the plates were shaken for ten minutes at 100 rpm and non-adherent cells were transferred using a pipette to a new plate. The plating media was replaced with growth media for the adherent cells. Four plates, first pellet adherent, second pellet adherent, first pellet non-adherent, second pellet non-adherent cells were sealed with parafilm, and incubated at 27°C. The first pellet adherent cells yielded the proliferative cells used in these experiments. This protocol can be seen in Figure 5.

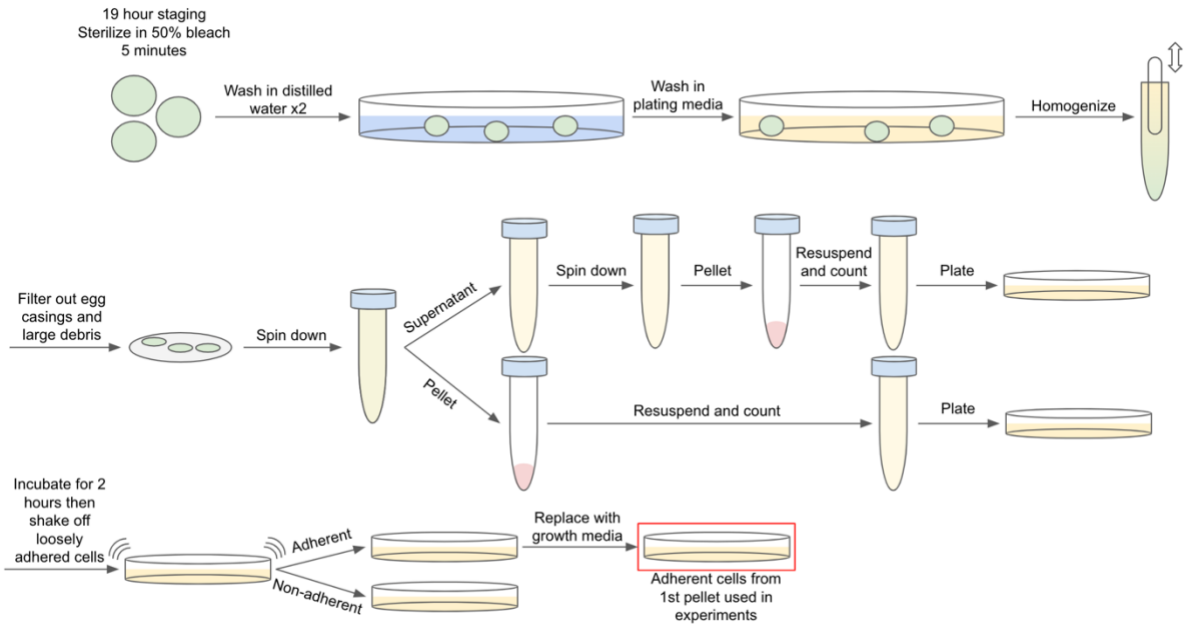

Figure 5. Isolation procedure for *M. sexta* primary cells from eggs.

2.2. Insect cell culture

The isolated M. sexta insect (MSPC) cells were grown at 27℃ with seven days between passaging. They were grown in culture media without primocin and EGTA. Myogenic differentiation was attempted to be induced by adding either 100 μg/ml or 20 μg/ml 20hydroxyecdysone (20HE; Sigma-Aldrich #H5142) as was done with 20 μg/ml in Baryshyan et al. (2012). 100 μg/ml 20HE was used as a higher value as previous research has shown that the addition of 100 μg/ml led to 100% of the myoblasts, isolated from abdominal ganglia in one day old pupae and diapausing pupae, being in S phase after 30 hours (Champlin et al., 1999). Values of 2000 μg/ml or more of 20HE led to very low levels of myoblasts in S phase and thus why a higher level of 20HE was not included in this experiment (Champlin et al., 1999).

The MSPC cells were passaged using a standard protocol. Briefly, the cells were scraped off using a cell scraper and moved to a falcon tube. These cells use mechanical detachment as opposed to enzymatic detachment. A small amount of fresh culture media was used to wash any remaining cells off the flask and put in the same falcon tube. These cells were mixed thoroughly in the media they were previously grown in, conditioned media containing growth factors produced by the cells, and counted. Cells were spun down at 1200 rpm for five minutes at RT and resuspended if necessary to achieve 1:1 conditioned media to fresh media. Cells were seeded at 100k/cm^2 for all experiments. High passage MSPCs at P34, low passage MSPCs at P6, all other MSPCs cells P27.

2.3. Mammalian cell culture

First, bovine satellite cell (BSC) media containing DMEM Glutamax (ThermoFisher #10566024, Waltham, MA, USA) with 20% FBS (ThermoFisher #26140079), 1% Antibiotic/Antimycotic 100X (Anti-Anti; ThermoFisher #15240062), and 1 ng/ml human FGF basic (ThermoFisher #68-8785-63)) was prepared. Media was 0.2 μm sterile filtered

before use. A 0.2 μg/cm^2 iMatrix recombinant laminin-511 coating (Nippi #892 021) was added in with the BSC media when seeding. The BSCs were grown at 37℃ with two to three days between passaging. Myogenic differentiation of BSCs was induced by growing the cells to confluence over three days and then through serum starvation for six days. Serum starvation does not require other forms of serum such as horse serum reducing the need for extra costly ingredients and, through lab recommendations, is known to work similar to differentiation media with horse serum.

The cells were passaged using a standard protocol. Briefly, the media was removed, and the cells washed in phosphate buffered saline (PBS). 0.25% Trypsin (ThermoFisher #25200056) was added and incubated at 37℃ for four minutes to allow for detachment. The trypsin was neutralized with FBS by adding BSC media and spun down at 1200 rpm at RT for five minutes. The cell pellet was resuspended in BSC media and counted using the NucleoCounter NC-200. Cells were seeded at 3k/cm^2. All BSCs P3-P4.

2.4. Cell lysis

A cell lysis protocol from lab recommendations was used. First, the cells were grown to 80% confluency. They were then scraped and washed off as with passaging for MSPCs or trypsinized off as with passaging for BSCs. The cells were then spun down at 1200 rpm for ten minutes at RT, resuspended in cold PBS and spun down again at 1200 rpm for ten minutes at RT. This was repeated twice. Cold PBS was used as this can reduce the activity of proteases. The cells were then lysed by being resuspended in chilled Pierce RIPA Lysis and Extraction Buffer (Thermo Fisher #89900) with 1 mM phenylmethylsulfonyl fluoride (PMSF; ThermoFisher #36978) protease inhibitor added. A protease inhibitor was added to further reduce protein degradation. These samples were stored at -80℃ until analysis. They were thawed to RT and spun down at 13000 g for five minutes at RT to separate the cell

debris from the proteins and micronutrients of interest. The supernatant was then collected and used for analysis.

2.5. Nutrient quantification assays.

Nutrient quantifications were determined by using; Pierce BCA protein kit (BCA assay; ThermoFisher #23227), zinc kit (abcam ab102507), and iron kit (abcam ab83366). Each assay was carried out in 96-well plates according to the manufacturer's protocol.

The BCA protein assay works by first reducing copper and forming a green colored complex with protein in an alkaline environment where peptides with three or more amino acids form the colored complex. Next, the color develops by bicinchoninic acid (BCA), which gives the kit its name, reacting with the reduced copper cation to form a soluble complex with a purple color. This purple complex is 100 times more sensitive than the copper and blue colored complex first formed. This kit looks the peptide backbone and is heavily influenced by four amino acid residues (tyrosine, tryptophan, cysteine, and cystine). The influence of the backbone helps to reduce variability which may be caused by the different composition of proteins (*Pierce™ BCA Protein Assay Kit*, n.d.).

For the BCA assay, standards were prepared with bovine serum albumin from the kit and distilled water ranging from 2000 μg/ml to 0 μg/ml. The working reagent (WR) was also prepared by mixing Reagent A and Reagent B in a 50:1 ratio. 25 μl of the samples and each standard were pipetted into a 96 well plate in triplicate. 200 μl of the WR were added to each well. This was then mixed for 30 seconds on a shaker, covered, and incubated for 30 minutes at 37℃. The plate was removed from the incubator and allowed to cool down to RT. The absorbance of the plate was read at 562 nm (*Pierce™ BCA Protein Assay Kit*, n.d.).

The zinc assay works by first deproteinizing the sample with trichloroacetic acid (TCA) which forms a white precipitate of proteins. The supernatant containing fewer proteins is then used to analyze the zinc. A reaction mix containing sodium dimethylglyoxime, 2-(5-Bromo-

2-pyridiylazo)-5-[N-n-propyl-N-(3-sulfopropyl)amino]phenol, and salicylaldoxime masks other metal reactions such as iron, copper, and nickel while binding with zinc to form a red colored complex with a known absorbance (Taylor, 1997) (5-Br-PAPS | Dojindo, n.d.).

For the zinc assay, standards were prepared utilizing the 50 mM Zinc Standard of zinc chloride in the kit and distilled water. The standards ranged from 5 nmol/well to 0 nmol/well. The reaction mix was prepared by mixing Zinc Reagent 1 to Zinc Reagent 2 in a 4:1 ratio. The samples required additional preparation. After spinning down, the supernatant was deproteinized by adding 7% TCA from the kit in a 1:1 ratio of sample to TCA. This was spun down at 13,000 g for 5 minutes at RT and 50 μl of this was added to wells in a 96 well plate in triplicate. 200 μl of the reaction mix was added to each sample and standard. This was incubated at RT for ten minutes and the absorbance read at 560 nm (*Zinc Assay Kit (ab102507)* | *Abcam*, n.d.).

The iron assay works in an acidic environment where a ferric carrier protein dissociates into ferric in the solution. This acid pH allows the iron and ferric ions to be released into solution. A chelate is included in the Assay Buffer to block interference from copper. Ferric iron is reduced to ferrous iron and ferrous iron reacts with the Iron Probe containing 3-(2-pyridyl)-5,6-di(2-furyl)-1,2,4-triazine-5′,5″-disulfonic acid disodium salt, a chromagen, to produce a colored complex with known absorbance (Hedayati et al., 2018).

For the iron assay, standards, ranging from 10 nmol/well to 0 nmol/well, were prepared with 1 mM Iron Standard of iron (III) chloride and the Assay Buffer in the kit. 50 μl of each sample was added to the wells of a 96 well plate. The volume was brought up to 100 μl by adding Assay Buffer. 5 μl of Iron Reducer was added to each well, samples and standards. The samples were cloudy so the recommended 5 μl of 1 M sodium dodecyl sulfate was added. The samples were then mixed and incubated for 30 minutes at 37°C. 100 μl of the Iron Probe was added to samples and standards, covered, and left to incubate again for 60 minutes

at 37°C. Absorbance was then read at 593 nm (*Iron Assay Kit (Colorimetric) (Ab83366/K390-100) | Abcam*, n.d.).

2.6. Cricket cell isolation

In addition to isolating *M. sexta* cells, *Gryllodes sigillatus* banded cricket cells were also attempted. A similar procedure to the *M. sexta* cells was followed. Four attempts were made with the procedure differing slightly each time. First, growth media was prepared with M3 2.5 g/L BP, 1 g/L YE, 10% HI-FBS, 100 μg/ml primocin, and 1% Anti-Anti. Plating media was prepared by adding 3 mM EGTA to the growth media. Media was 0.2 μm sterile filtered before use.

Cricket eggs were generously donated from Ovipost. The cricket eggs were shipped in peat moss at RT. Before isolation, the cricket eggs, which have a similar appearance to white rice, needed to be separated from the peat moss by tweezers. The first attempt used seven day old eggs. The peat moss was further rinsed off with PBS. First, two sterilization method was examined as cricket eggs may not possess the same protective coating as *M. sexta* eggs. 100% Lysol disinfectant for ten minutes was assessed as Lysol has previously been used to sterilize eggs (Brundage et al., 2016). 50% bleach in PBS was also assessed. The viability was tested, and the outcome was relatively similar thus 50% bleach was used as it is a similar method to the 50% bleach in distilled water used for the *M. sexta*. Once sterilized, the eggs were washed twice in PBS and once with plating media. The eggs were moved to the Dounce homogenizer, crushed, filtered to remove large debris, centrifuged, and split same as the *M. sexta* eggs. The cells were seeded at 100k/cm^2, incubated at 27°C for two hours, shaken at 100 rpm for ten minutes, and the non-adherent cells transferred to a new plate. A LIVE/DEAD assay (ThermoFisher #R37601) was performed on day fourteen.

The second attempt utilized the same procedure as the first with two differences, the eggs were younger, five days old as opposed to seven, and they were only centrifuged once at 380 g for ten minutes.

The third attempt utilized the same procedure as the second with one difference, the eggs were again younger, three days old as opposed to five or seven days old.

The fourth attempt differed greatly; it utilized the same sterilization procedure but instead of homogenizing the whole egg the eggs were cut open. After sterilization, the eggs seven day old eggs were dipped in 100X Anti-Anti, washed in distilled water twice and growth media once without EGTA. The EGTA helps select for muscle cells which were desired but not the only cell type within the eggs, thus was omitted to improve the chance of isolating any cells from the cricket eggs. After washing, two to three eggs were placed in a well with growth media of a 24 well plate and crushed against the side of the well with a sterile P200 tip to cut open the egg. The cells were not shaken and moved as no EGTA had been used thus muscle cells were not adhered differently the other cell types.

2.7. Statistical analysis

Statistical analysis was performed with GraphPad Prism 9 software. All data was analyzed via one-way ANOVA. Multiple comparisons of all analyses were performed with Tukey's HSD post-hoc test. P values ≤ 0.05 were treated as significant. **** $p \leq 00001$, *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$, not significant (ns) $p > 0.05$. Outlier detection was performed by the ROUT method with Q=1%. Errors are given as ± standard deviation.

Chapter 3. Results

3.1. Protein content

The Pierce BCA protein assay showed that per cell, there is a statistical difference between the differentiated BSC, and the other cell types tested; BSC, low passage MSPCs, and high passage MSPCs (Figure 6). As differentiated BSCs are the only cells with known muscle characteristics, the cells having more protein makes sense. However, insect cells are often smaller and weigh less than mammalian cells. The MSPCs, both high and low passage, are smaller than they BSCs. A common metric for food is grams per kilogram (g/kg). Correcting for cell weight shows protein between 150-650 grams of protein per kilogram of cell dry weight (DW) (Figure 7). The low passage MSPCs offer the most grams of protein per kilogram of cell DW.

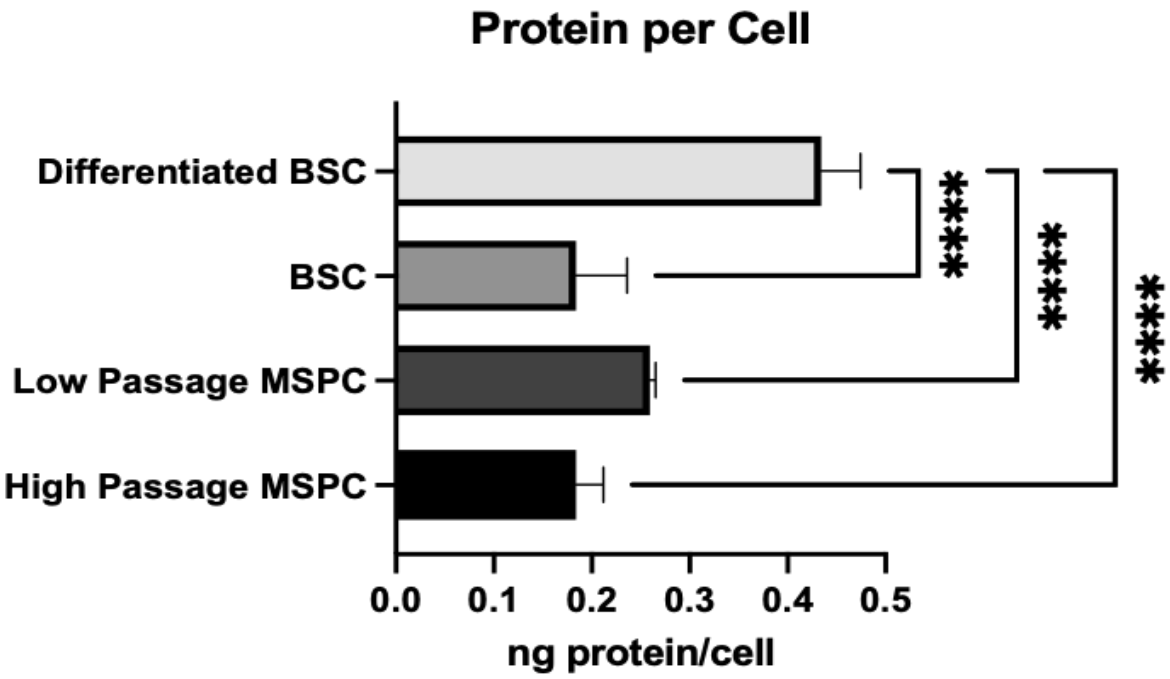

Figure 6. Protein per cell of MSPC and BSC.

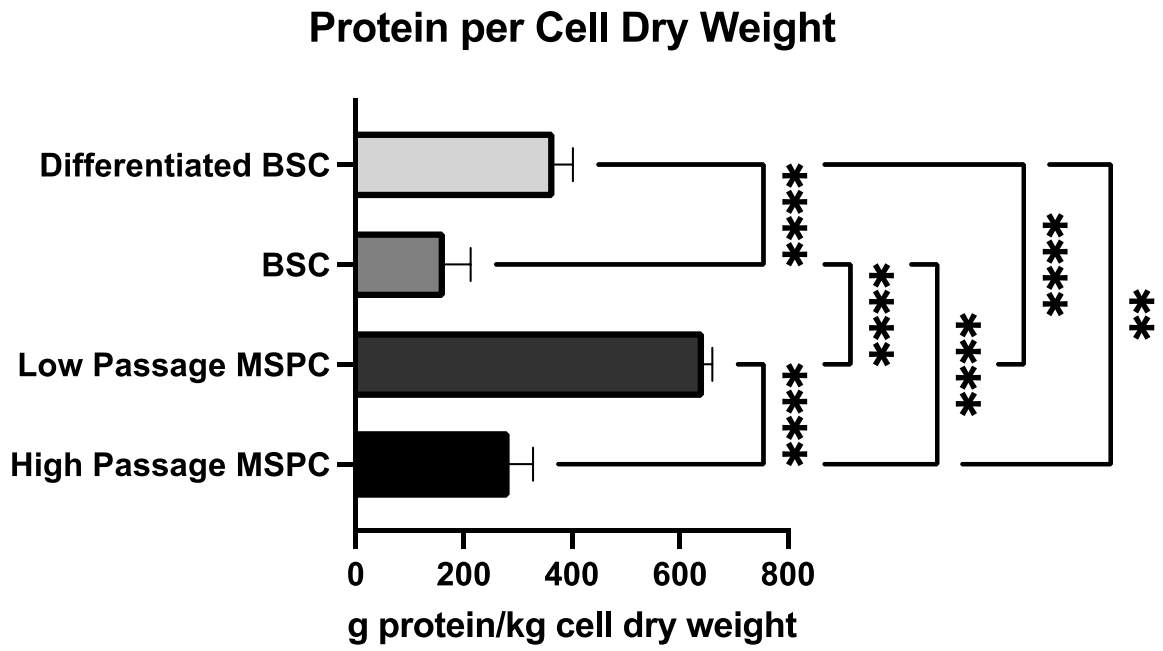

Figure 7. Protein weight per dry cell weight of MSPC and BSC.

3.2. Trace minerals - zinc and iron content

The zinc assay showed that when comparing zinc against dry cell weight, there is no statistically significant difference between any of the cell groups (Figure 8). The zinc per cell has been provided as a table for reference (Table 4).

The iron assay showed that when comparing total iron (II + III) against dry cell weight, there is no statistically significant difference between any of the cell groups (Figure 9). The iron per cell has been provided as a reference (Table 4). The standard deviations for this are very large and are mainly accounted for in one row of the microplate, for three of the cell types, that row did not produce an outlier but for the other the data point is classified as an outlier. The data without this row is also provided in picograms per cell and grams of iron per kilograms of cell dry weight (Table 4) (Figure 10). Without this row there is a statistically significant difference between the low and high passage MSPCs with low passage MSPCs having more iron per dry cell weight.

The main source of zinc and iron available to these cells comes from the FBS. FBS contains zinc in a concentration around 40 μM and iron in a concentration around 40 μM

(Cho et al., 2007). The BSC media contained 20% FBS while the MSPC media contained 10% HI-FBS. While the average composition of heat inactivated FBS has not been specifically explored, heat inactivation affects the complement proteins in the FBS and should not affect the zinc concentration. The basal media of the BSC media also had a 2.5 μM concentration of ferric nitrate (ThermoFisher, n.d.a). No data is available on the specific YE or BP used in the MSPC media, but the YE likely contains zinc, iron, and other trace elements in small concentrations. This means the BSC media had double the concentration of zinc and more than double the concentration of iron when compared to the MSPC media. However, the zinc and iron weight of the BSCs is larger yet not double the zinc and iron weight of the MSPCs.

	Zinc (pg/cell)	Iron (pg/cell) all data	Iron (pg/cell) with row removed
Differentiated BSC	8.30 ± 1.09	10.60 ± 5.71	8.32 ± 4.22
BSC	9.11 ± 3.20	14.74 ± 11.05	8.27 ± 3.66
Low passage MSPC	2.96 ± 1.04	6.14 ± 2.10	6.14 ± 2.10
High passage MSPC	3.64 ± 1.62	5.90 ± 3.27	4.82 ± 1.75

Table 4. Zinc and iron (II + III) per cell.

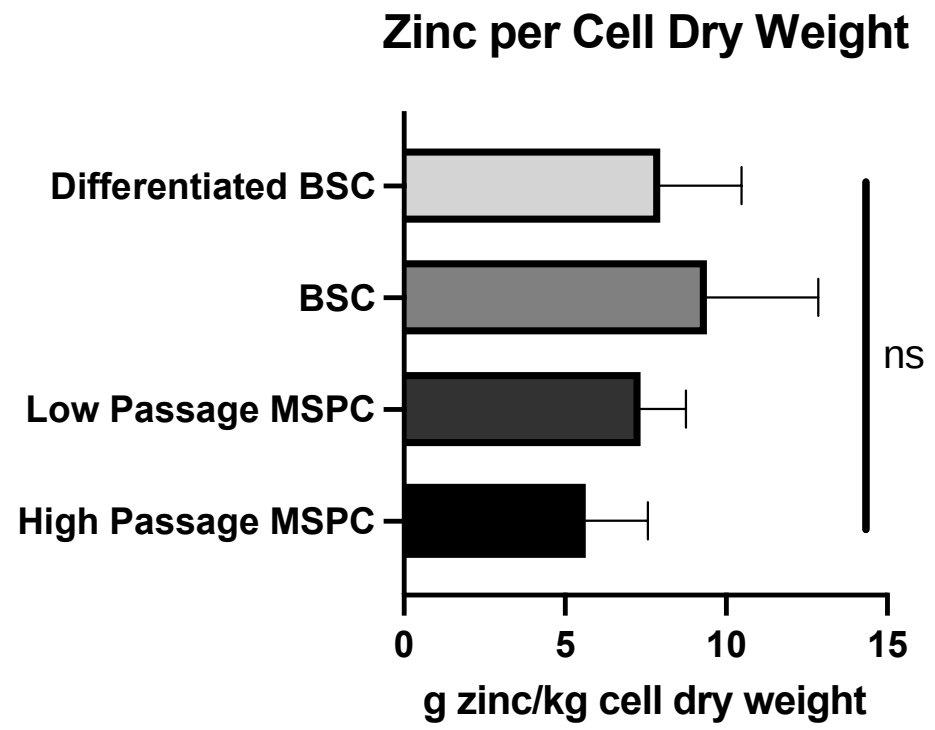

Figure 8. Zinc weight per dry cell weight of MSPC and BSC.

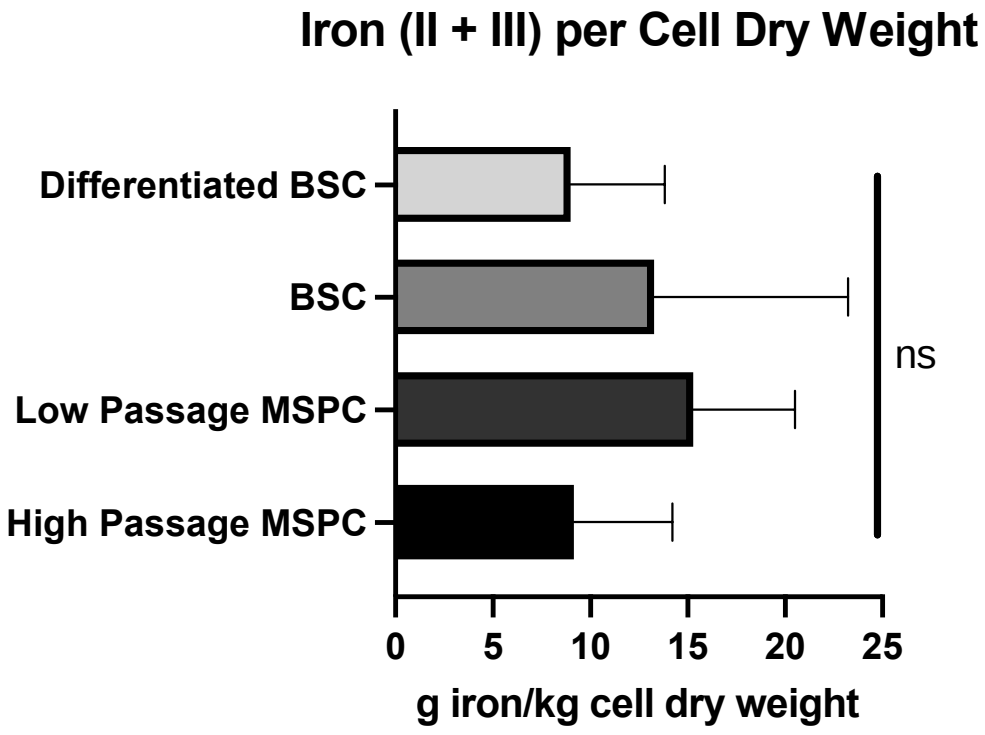

Figure 9. Iron (II + III) weight per dry cell weight of MSPC and BSC.

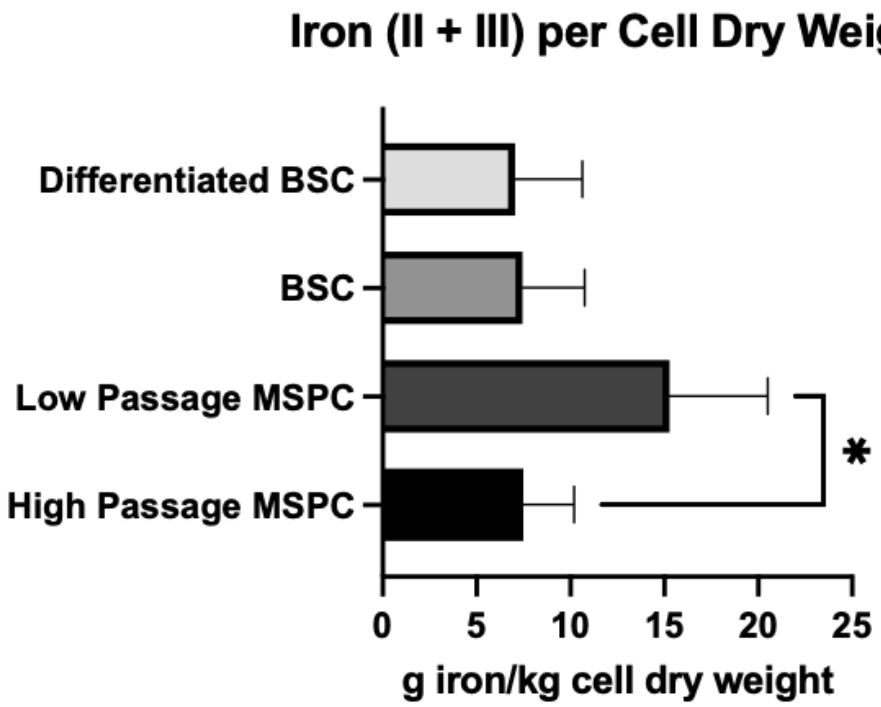

Figure 10. Iron (II + III) weight per dry cell weight of MSPC and BSC with row removed.

Comparing these cell values to whole insect shows drastically higher values. *Lepidoptera* have an average of 15.4 milligram of iron and 10.6 milligram of zinc per 100 grams which equates to 0.154 grams of iron and 0.106 grams of zinc per kilogram as seen in Table 3, a difference of about 50-fold to the values in Figures 8, 9, and 10.

3.3. 20HE effect on protein

20HE does not have an effect on protein of MSPCs that is statistically significant (Figure 11). The average does appear to slightly increase but not significantly and not linearly. The error bars also are quite large which could be due to cell clumping. The MSPC cells, which normally aggregate some, were in more aggregates in this experiment with and without the 20HE added which could affect the counted cell number.

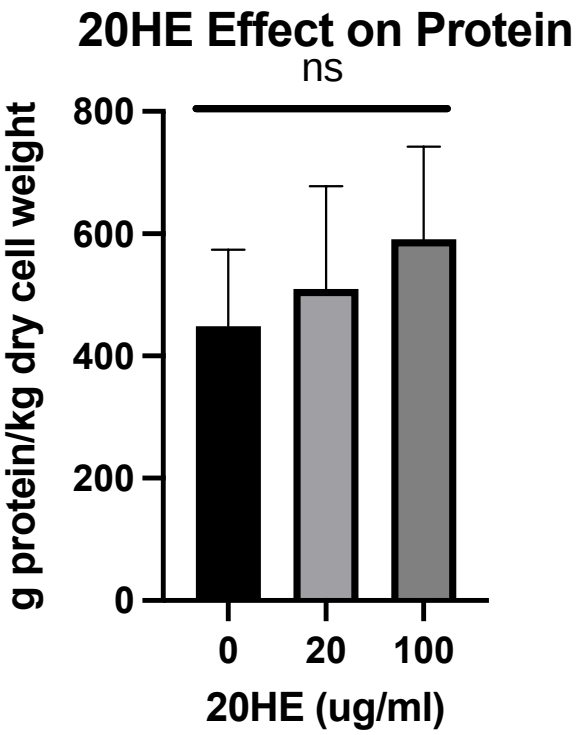

Figure 11. 20HE effect on protein weight per dry cell weight of MSPC.

3.4. Protein content over time

Protein amount in grams per kilogram of dry cell weight does not statistically significantly change as the cells multiply, become confluent, and stack (Figure 12). As MSPCs are not contact inhibited, they should continue to grow even when coming into contact with each other, unlike BSCs. However, the difference, shown as percent increase from day 0, between day 7 and day 14 is not statistically significant (Figure 13). In addition, the difference between days 2 and 4 is not statistically significant.

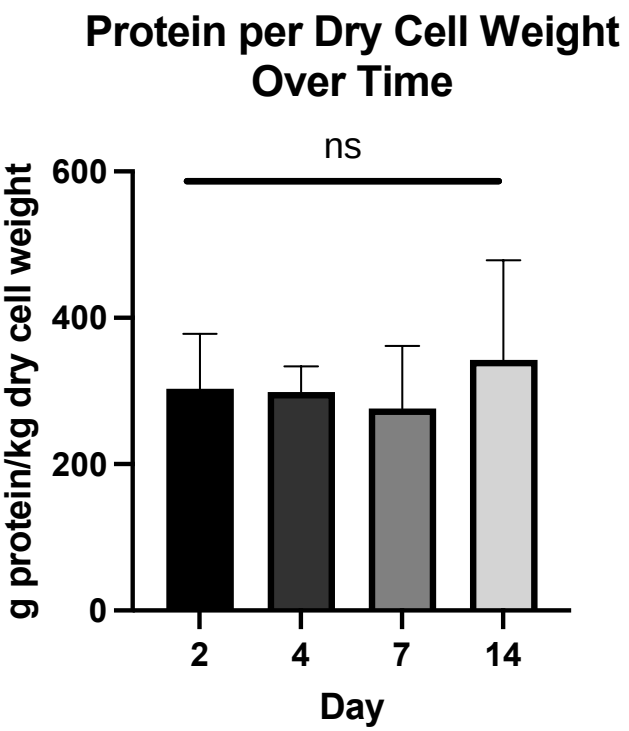

Figure 12. Protein per kilogram dry cell weight of MSPC over time.

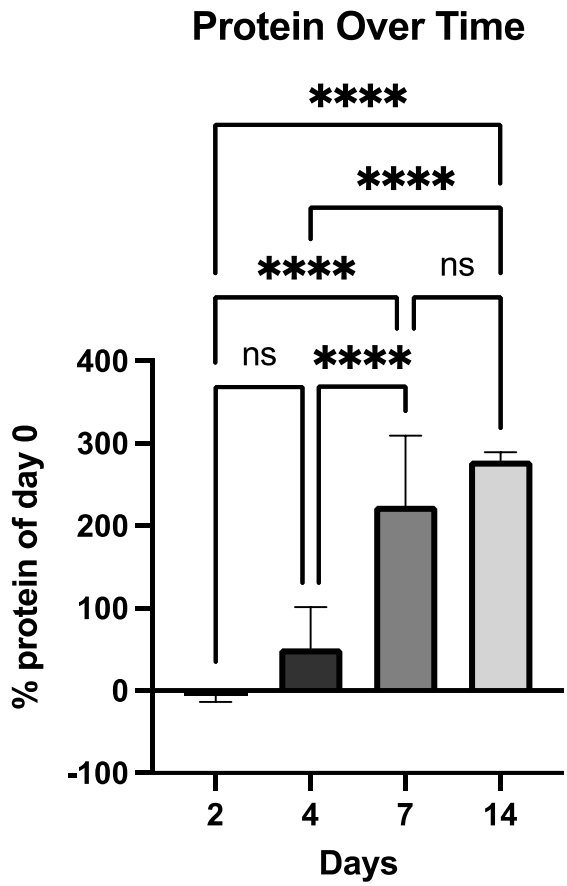

Figure 13. Protein of MSPC over time as a percentage of day 0.

3.5. Cricket cell isolation

Cricket cell isolations were attempted but more work is needed to continue. Four attempts were made with three following a similar protocol to the *M. sexta* isolation procedure and one attempting to cut open the eggs as opposed to homogenize them. The first isolation had some wells become contaminated, mainly the second centrifugation pellet. Of the non-contaminated wells, a LIVE/DEAD assay was performed on the first cell pellet (Figure 14). There were no cells, live or dead, detected after fourteen days. The Chemometec cell counter counted cells and they were plated at 100k/cm^2 initially so cells, dead or alive, were expected.

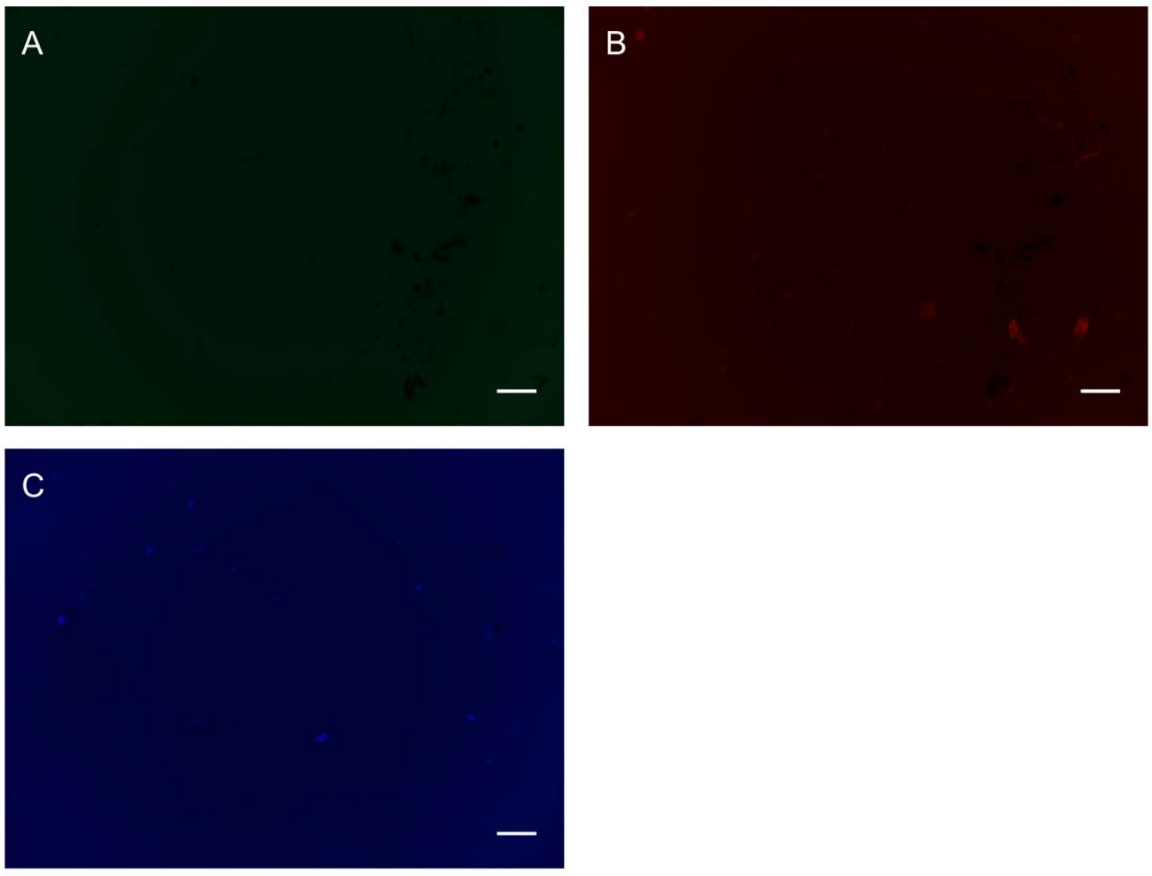

Figure 14. LIVE/DEAD of first cricket isolation attempt. A) live; B) dead; C) DAPI. Scale bar = 200 um.

As the eggs were seven days old, it is possible that they were too developed already and may have stated to develop their immune system which has made isolations in the past from adult insect or larvae hard as the cells clump. The cells may have clumped and been filtered

out with the egg casings. The next attempt utilized slightly younger five day old eggs. Again however, this was unsuccessful as was the third attempt with even younger three day old eggs. A different protocol was then used where instead of homogenizing the eggs, they were cut open in addition to being extra sterilized in 100X Anti-Anti. This fourth attempt yielded different results. Since the eggs were merely cut open, some portion of the cricket embryo remained inside the eggs and continued to develop. Some of the eggs appeared to be punctured with what appeared to be cells coming out and adhering to the tissue culture plastic of the well while others were more distinct cricket shapes and were fully or partially outside of the egg casing (Figure 15).

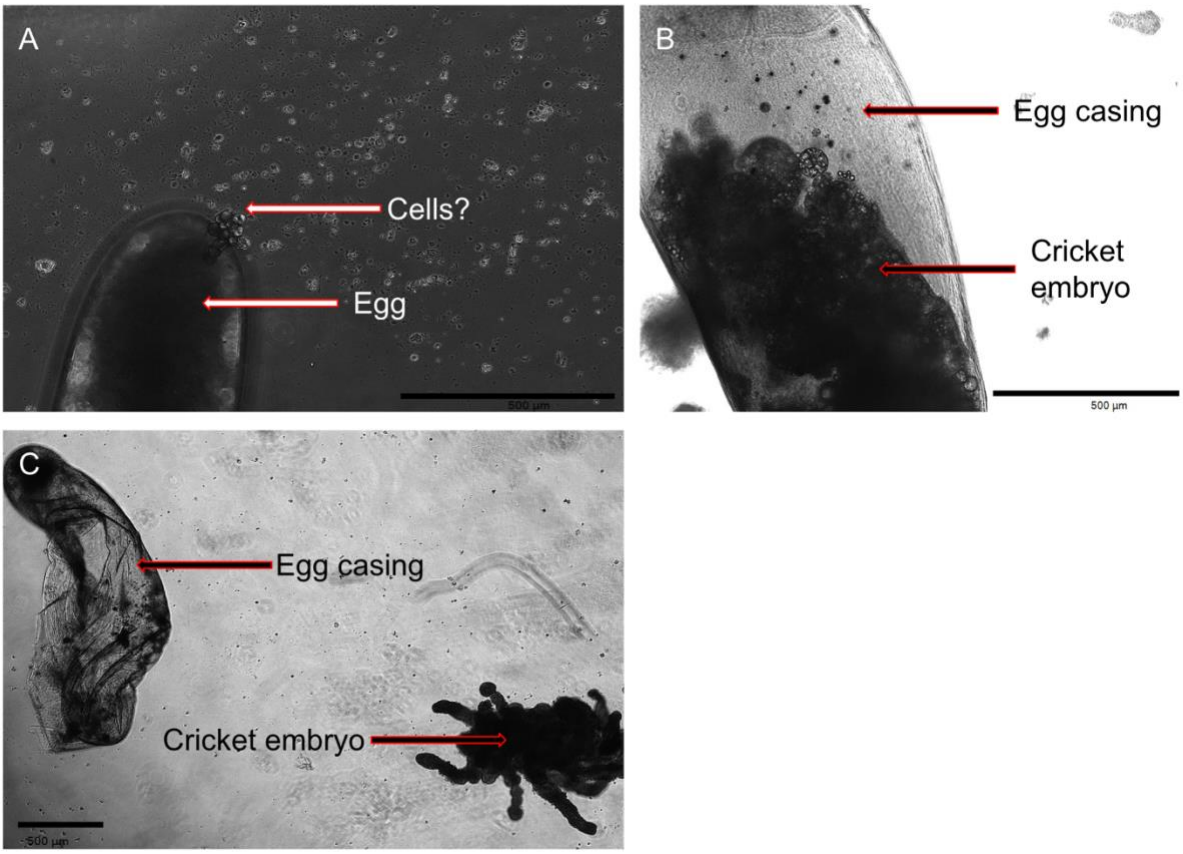

Figure 15. Fourth attempt at cricket cell isolation utilizing new crushing technique. Nine days in culture. A) egg with a hole in the side with potential cells coming out and attaching to the well; B) cricket embryo partially in egg casing; C) cricket embryo fully outside egg casing. Scale bar = 500 um.

Of the several eggs of this fourth attempt, two had visible muscle movement within them. This muscle movement was either a rhythmic ‘beating’ of the egg similar to a cardiac muscle or a slower more sporadic contraction like movement, potentially of a leg muscle

(Supplement 1) (Supplement 2). One egg with muscle movement was attempted to be dissociated with trypsin to hopefully have distinct muscle cells. This did not yield cricket muscle cells.

Chapter 4. Discussion

The purpose of this study was to compare insect and mammalian cell nutrition using primary cells and also to explore the protein content of *M. sexta* cells more specifically.

4.1. Protein, zinc, and iron comparison between MSPC and BSC

Previous research has shown that an insect cell line contained more protein, zinc, and iron than a mammalian cell line (Rubio et al., 2019a). Because of this preliminary data, the first investigation in this study explored primary cells of insect and mammals by quantifying the protein, zinc, and iron. Statistical analysis showed that low passage MSPCs contain more protein per kilogram of cell DW than BSCs, differentiated BSCs, and high-passage MSPCs further supporting the previous data that insect cells are denser in protein.

Kingdom/Species/Class/Cell Type	Protein Percent DW (%)
Lepidoptera Butterflies/Moths	14-68
Low Passage MSPC	64.5 ± 1.5
High Passage MSPC	28.5 ± 4.3
Artiodactyla Cow	21-41
Differentiated BSC	36.8 ± 3.4
BSC	16.6 ± 4.7
Eubacteria Bacteria	50-65
Fungi	30-45
-phyta Algae	40-60
-cota Yeast	45-55

Table 5. Protein percent dry weight comparison between cells and whole insect or meat cut. (Greenwood et al., 1951; Xiaoming et al., 2010; Kouřimská & Adámková, 2016; Bharti et al., 2014; Miller & Litsky ,1976).

Converting to percent DW and comparing cells to whole animals and meat cuts shows the low passage MSPCs have a higher average protein percent DW than whole insects of larvae or pupae from the same class (Table 5). These values are comparable to whole insects

within the same class or lean meat cuts. Low passage MSPCs contained an average of 64.5% protein DW while pupae and larvae within the same class, *Lepidoptera*, contain an average of 45% protein DW but range from 14% to 68%. The high-passage cells contained only 1/2.3 of the protein of their low-passage version at 28.5%. The differentiated BSCs contained 36.8% protein DW which is within the range of common meat cuts 21% to 41% with an average of 30%. The lowest protein percent DW was of the non-differentiated BSCs at 16.6%. This protein percent DW for the BSCs was below the lowest level of common meat cuts.

However, meat is not just made from one cell type, it is a combination of muscle and fat. The percent DW of protein from BSCs would be more comparable to a lean meat cut with a low fat percentage thus a protein percent DW of around 41% meaning differentiated BSCs have similar protein to lean fresh meat cuts. Insects too are a combination of muscle and fat cells and have an exoskeleton made up of chitin, an amino sugar polymer which is considered an animal fiber. Chitin in insects ranges from 11.6 to 137.2 milligrams per kilogram DW (Kouřimská & Adámková, 2016). This low level of chitin in whole insects does not significantly contribute to overall weight and thus can be considered to be made up primarily of muscle and fat similar to common meat cuts. *Lepidoptera* caterpillars have the highest fat content and edible insects as a whole containing 10% to 60% fat DW (Kouřimská & Adámková, 2016). As the protein content of the low-passage MSPC cells is on the higher end of the protein content range, the fat content is likely near the lower end making the MSPCs vastly different from their caterpillar counterparts.

In addition, other protein sources from bacteria, fungi, algae, and yeast contain medium to high amounts of protein 30% - 65% (Bharti et al., 2014) (Miller & Litsky ,1976). The low passage MSPCs are similar to a high protein bacterium while the differentiated BSCs are most similar to an average fungus. Fungi also contain chitin the same as the exoskeletons in whole insects (Daraghmeh et al., 2011). One option to alter the protein content is to combine

cells with a scaffold. Higher passage MSPCs could have increased protein above 28.5% by being combined with a fungal scaffold for example.

The zinc and iron of the MSPCs and BSCs was not significantly different unlike the protein. Contrary to previous results, there is no statistical difference between MSPCs and BSCs in zinc or iron per kilogram of cell DW. The values quantified here are also different from previous research into zinc and iron of mouse myoblast cell line C2C12 and *Drosophila melanogaster* adult muscle progenitor-like cell line which had an even higher value of picograms per cell (12-25 pg iron per cells compared to these values of 6-15 picograms iron per cell, and 46-156 picograms zinc per cell compares to these values of 3-9 picograms zinc per cell) (Rubio et al., 2019a). Some potential reasons for this discrepancy are that different species and classes of animals may have a different uptake of iron and zinc, the different media used, or simple error.

According to the FDA, the daily recommended intake of protein, iron, and zinc are 50 grams, 18 milligrams, and 11 milligrams respectively (CFSAN, 2020). Per 100 grams of DW, the low passage MSPC meet these requirements, differentiated BSC and high passage MSPC would need about 200 grams consumed to meet the daily requirements, and BSC at the lowest with about 300 grams consumed (Figure 16). All of the cells had a high content of iron and zinc ranging from about 500% to 850% of the daily recommended value. These recommended values and daily values have been updated as of 2020, with zinc decreasing to 11 milligrams from 15 milligrams; protein and iron remained some of the five nutrients daily values unchanged (CFSAN, 2020).

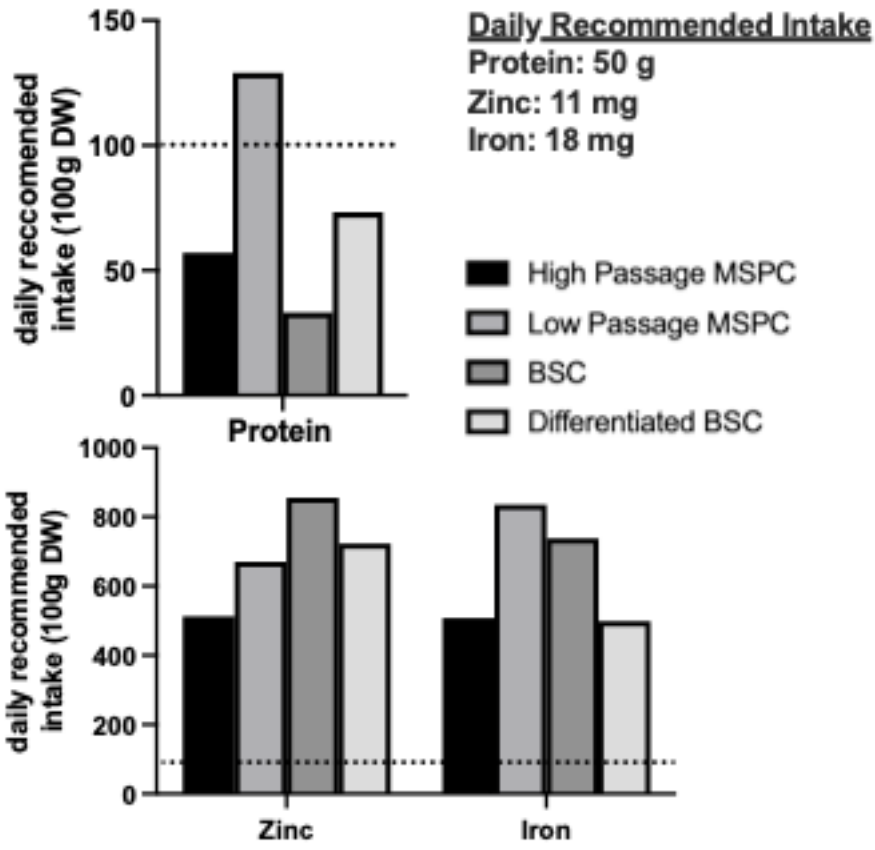

Figure 16. Nutrient profiles of MSPC and BSC according to the daily recommended intake of protein, zinc, and iron from the FDA (CFSAN, 2020).

These values of zinc and iron are with normal MSPC and BSC media which does not have zinc or iron additives. The main source of trace minerals comes from the FBS. Rubio et al., (2019a) found that using iron-fortified serum in the media increased the iron density in the *Drosophila* insect cell line. Thus, these MSPC cells could potentially be made denser in these trace minerals by the addition of supplements into the media. These findings provide initial insight into insect cells as a cellular agriculture product, but still require further and deeper analysis to understand the nutritional value of MSPCs and primary or cell line insect cells compared to mammalian.

4.2. 20HE differentiation of MSPC

The protein of MSPCs was shown to not be significantly affected by the addition of 20HE to induce myogenic differentiation at different treatment levels of 20 ng/ml and 100 ng/ml. As the MSPCs were not significantly affected by the 20HE, this points towards the

MSPCs cells as possibly not being muscle or muscle precursor cells. Currently, the MSPC cells are being labelled as embryonic cells since they were isolated from eggs. However, the proliferative cell type may not be a muscle-progenitor cell. Despite no differentiation being seen with the addition of 20HE, the MSPC cells still have a high percentage of protein. In addition, the error bars are quite large, even for the untreated MSPCs, suggesting potentially unhealthy cells. While the average increases slightly, nothing is significant because of the large error bars.

All of the values of the protein percent DW of the MSPCs treated and untreated with 20HE were within the range and near the upper end of the range seen in whole *Lepidoptera* (Table 6). The untreated 0 μg/ml 20HE, 44.9%, was closest to the average protein percent DW of *Lepidoptera* at 45%.

Class/Hormone Treatment	Protein Percent DW (%)
Lepidoptera Butterflies/Moths	14-68
0 μg/ml 20HE	44.9 ± 12.5
20 μg/ml 20HE	51.0 ± 16.8
100 μg/ml 20HE	59.1 ± 15.1

Table 6. 20HE effect on protein of MSPC cells compared to *Lepidoptera* (Xiaoming et al., 2010; Kouřimská & Adámková, 2016).

4.3. Protein over time of MSPC

Over time, the protein per kilogram of dry cell weight, essentially the protein density, for MSPCs does not change as the cells multiply and become more confluent, stack on top of each other, and potentially differentiate. The protein as the cells multiply does increase but the cells seem to stop multiplying when they reach confluence despite MSPCs not being contact inhibited. This could be due to the higher passage number of the cells, P27. Potentially, these cells were losing the capability to grow as they aged. While insect cells are more likely to spontaneously immortalize than mammalian cells, this does not mean it always

occurs. The MSPC cells utilized have been grown to a very high passage meaning they are most likely immortalized, but this spontaneous event could have occurred at a later passage meaning the P27s here were not yet immortalized.

The low percent growth for day 2 may show that the MSPCs were slow to start growing and multiplying or that they were unhealthy (Figure 13). They were cells that had undergone two passages after being thawed and before being plated, thus should have had adequate time to recover from cryopreservation.

4.4. Cricket cell isolation

The rounds of cricket cell isolation were unsuccessful in obtaining a proliferative cell type. This isolation of cricket cells will be continued with different cricket species and at earlier timepoints.

4.5. Insect cultured meat

While insect cells as a whole offer a solution to cost and scalability problems often seen with mammalian cells, the nutrition of primary cells was unknown. A previous study by Rubio et al. (2019a) has shown that an insect cell line is higher in protein, zinc, and iron, than a mammalian cell line. But how primary cells of insects and mammals compared was not yet explored. Low passage MSPCs have more protein and comparable zinc and iron to differentiated and non-differentiated BSCs per DW meaning MSPC cultured meat products would be more protein rich than those of BSCs. In addition, low passage MSPCs offer the recommended daily protein intake in 100 grams of DW final product, the only cell tested to do so. MSPCs, being easier to culture and more cost effective than BSCs, offer an attractive option for cultured meat products from a nutritional point of view.

Chapter 5. Future work

Further examination into more components of nutrition, exploring different insect species and their isolated cells, and examining taste, flavor, and desirability of insect based cultured meat products.

5.1 Quantifying amino acid profiles of insect and mammalian cells

One interesting nutritional component to further explore is amino acid profiles. Insect cells often use yeast extract and/or bacto peptone as a media supplement which are rich in amino acids. If insect cells often have these amino acid rich mediums, are they then richer in amino acids than mammalian cells which often do not have additional amino acids added?

Aim: Quantify the amino acid profiles of several insect and mammalian species and compare amino acid profiles between species.

Objectives: 1) Grow multiple cell types of insect and mammalian species and prepare for amino acid analysis; 2) send off to have amino acid analysis done from an outside lab; and 3) compare values between species, specifically phenylalanine and tyrosine which are known to be in high levels in insects (Kouřimská & Adámková, 2016).

5.2 Increasing MSPC insect cell zinc and iron through media additives

Another aspect to look at regarding nutrition is can the nutrition be modified by adding components to the culture media. The addition of iron or zinc supplements offers the insect cells more for them to take up, potentially increasing the final amount of iron and zinc in these cells.

Aim: Quantify the difference in nutrition of MSPC insect cells with and without iron and zinc media supplements.

Objectives: 1) Grow MSPC insect cells and treat with different medias; normal growth media (final concentration 4 μM iron, 4 μM zinc), normal growth media with high (10 μM iron) and low (6 μM iron) levels of iron, normal growth media with 2.5x high (10 μM zinc) and 1.5x

low (6 μM zinc) levels of zinc, normal growth media with both high iron and zinc levels (10 μM iron, 10 μM zinc), and normal growth media with both low iron and zinc levels (6 μM iron, 6 μM zinc); 2) quantify iron and zinc in each scenario via colorimetric assays; and 3) compare iron and zinc values per cell DW between iron and zinc levels to see if additives can increase the values of the trace minerals.

5.3 *Polypedilum vanderplanki* nutritional analysis

Besides investigating more nutrition of one species, other species should also be explored as insect species vary greatly in nutrition. A species to look into is *Polypedilum vanderplanki*, the sleeping chironomid, an insect from Africa which can survive anhydrobiosis, being dried, and is revived after being rehydrated in addition to surviving long term exposure to outer space (Sakurai et al., 2008) (Gusev et al., 2010). This species is currently being explored for use in food for space travel making nutrition an important factor to quantify.

Aim: Quantify the nutrition of *Polypedilum vanderplanki* for use in food in space

Objectives: 1) Purchase or isolate *P. vanderplanki* cells and representative insect and mammalian cells such as BSC and *Drosophila*; 2) dry and rehydrate some *P. vanderplanki* cells 3) grow and lyse cells including fresh and dried/rehydrated *P. vanderplanki* cells; 4) quantify protein, fat, and carbohydrates via colorimetric assay; 5) compare values between all species and conditions.

5.4 Heavy metal and toxin buildup differences in-vitro and in-vivo

Insects are known to bioaccumulate contaminants found in their feed or environment (Schrögel & Wätjen, 2019). In the United States, three heavy metals are currently regulated in food, lead, mercury, and arsenic, all of which insects can bioaccumulate, the factor depending on species and life stages (EPA, 2022) (Meyer et al., 2021). In addition to heavy metals, insects can also bioaccumulate chemicals and toxins, especially in fatty tissues (Fries,

1995). Specifically, dioxin and polycyclic aromatic hydrocarbons (PAHs) if fed on supermarket food waste (Meyer et al., 2021).

Aim: Quantify contaminant bioaccumulation in-vitro to compare to in-vivo MSPC

Objectives: 1) Grow MSPC insect cells and treat with different medias; normal growth media, media with levels under the EPA requirements of the heavy metals, media with levels over the EPA requirements of heavy metals (EPA maximum contaminant level in drinking water: lead – 0.015 mg/L, mercury – 0.002 mg/L, arsenic – 0.01 mg/L, dioxin – 0.00000003 mg/L, and PAHs – between 0.0001 and 0.0004 mg/L) (US EPA, 2015) (ATSDR, 2021); 2) quantify bioaccumulation of contaminates in the different conditions; 3) compare in-vitro and in-vivo bioaccumulation

5.5 Characterization of MSPC

MSPC cells come from *M. sexta* eggs and thus are classified as embryonic cells. However, from the experiments conducted here, they do not appear to have the ability to differentiate into muscle. Further characterization of the MSPC cells to determine the exact cell type is necessary. MSPC cells, comping from the *Lepidoptera* order, are hard to characterize as most cell lines within this order are polyploidy and have small chromosomes (Tabachnick & Knudson, 1980) (Lynn, 1996). Thus, electrophoresis is preferred; four enzymes were found to be useful for characterizing insect cells (phosphoglucose mutase (PGM), phosphoglucose isomerase (PGI), malic enzyme (ME), and isocitrate dehydrogenase (IDH)) (Lynn, 1996).

In addition to characterizing the isoenzyme pattern which helps to distinguish between species, staining for relevant markers can help determine the cell type. However, few markers are known within insects and *M. sexta* specifically. Some stains work across a wide variety of species and phyla while others are specific to a certain order or genus.

Aim: Characterize the MSPC cells and determine cell type(s)

Objectives: 1) Grow MSPC cells; 2) perform electrophoresis on lysed cells; 3) stain for individual enzymes; 4) determine isoenzyme pattern; 5) grow MSPC cells; 6) fix and stain for relevant markers to determine cell type (examples: Myosin heavy chain, Act88F, Tropomyosin, alpha tubulin – muscle cells, horseradish peroxidase antisera – neuronal cells, Oil Red O – fat cells, and ZO-1 –endothelial cells)

5.6 Myogenesis time point in cricket eggs

To increase the chance of isolating muscle cells, staging the eggs when these muscle cells are forming in the embryo is crucial. When myogenesis occurs is known in some insects, but no data has been found focusing on crickets. A specific enzyme or hormone that regulates myogenesis in crickets could be quantified, but more work would need to be done to determine one. Another way to analyze myogenesis is to determine when gastrulation (3.5 hours) has begun as this is closely linked with myogenesis in *Drosophila* which occurs at 4 hours (Bernstein et al., 1978).

Aim: Determine when myogenesis occurs in crickets

Objectives: 1) Breed crickets and collect eggs; 2) analyze eggs under a microscope at different timepoints (2, 4, 8, 12, 18, hours, 1, 1.5, 2, 3, 4, 5, 6 days); 3) note timepoint when germ band extension takes place as this may occur before gastrulation; 4) note timepoint when abdominal segmentation takes place as this may occur after gastrulation; 5) if needed perform analysis again with more timepoints focusing around the previous germ band extension and abdominal segmentation timepoints; 6) isolate cells from cricket eggs between the two timepoints to determine which timepoint yields cells with muscle characteristics

5.7 Serum free and suspension culture of MSPC

Alternative culture methods such as serum free and suspension culture are easier to adapt to with insect cells. Serum free culture is crucial in lowering the price of cultured meat and suspension culture helps to produce more cells in a smaller volume.

Aim: Adapt MSPC cells to serum free and suspension culture

Objectives: 1) Adapt cells to serum free culture by slowly reducing the serum in the media in a 1:1 (conditioned:fresh serum free media) manner each passage with the viability high (>85%) and doubling times unchanging until the serum level is below 0.05% (with the current MSPC media containing 10% FBS this would take 8 passages); 2) adapt cells to suspension culture at 130 rpm by starting at 90-100 rpm in a shaker and slowly increase by 5 rpm each subculture as long as viability remains high (~90%), if viability decreases (<80%), decrease the shaking speed until the viability increases again (>80%).

5.8 Blind taste tests of cultured insect meat

Another important aspect of food beyond nutrition is the taste, flavor, or desirability of the food. If the nutrition of insect cells is similar to mammalian cells, how does the taste compare? Could the flavor of cultured insect meat be altered by adding certain compounds such as Impossible burger has done with heme (*Heme + The Science Behind Impossible™*, 2022)? Blind taste tests between cultured insect burgers and conventional beef burgers would offer more insight into consumer preferences regarding taste and flavor.

Aim: Gather information about consumer views on taste, texture, and smell of cultured insect burgers.

Objectives: 1) Purchase/prepare cultured insect burgers, cultured mammalian cell burgers, plant-based burgers, insect-based burgers made from plant-based meat and ground insects, and conventional burgers; 2) provide burgers to consumers in a blind taste test and acquire numerical values of similarity to conventional meat and "enjoyability" in taste, texture, and smell, 3) compare values between burgers normalized to the conventional burger.

5.9 Consumer acceptance of cultured insect meat

Beyond flavor is the willingness to consume the final product. How do consumers feel about eating cultured insect meat? Further exploration into consumer preferences regarding

insects or cultured meat, specifically cultured meat from insects, is needed if it will become a viable option on the market.

Aim: Estimate consumer acceptance of cultured insect burgers through opinions

Objectives: 1) Create a survey asking about factors that impact buying decisions, likelihood of purchasing cultured insect meat in any form, cultured mammalian meat, and conventional meat, knowledge about cultured meat and insect cultured meat, provide information about cultured insect meat and ask likelihood of purchasing again; 2) Provide survey to consumers across demographics within the United States; 3) quantify and compare likelihood of buying between cultured insect meat, cultured mammalian meat, and conventional meat; 4) determine primary factors affecting purchasing behavior to take into account for advertising cultured insect meat.

Chapter 6. References

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