Addition of Apple Pomace to Feeding Substrate on Growth, Development, and Survival Rates of Yellow Mealworms (Tenebrio molitor) and Characterization of Their Proteins Extracted by a pH-Shift Process

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Addition of Apple Pomace to Feeding Substrate on Growth, Development, and Survival Rates of Yellow Mealworms (*Tenebrio molitor*) and Characterization of Their Proteins Extracted by a pH-Shift Process

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Thesis submitted to the Davis College of Agriculture, Natural Resources and Design at West Virginia University in partial fulfillment of the requirements for the degree of Master of Science in Nutritional and Food Science

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2023

Keywords: *Tenebrio molitor*, yellow mealworm, edible insect(s), feeding study, agricultural by-product, growth rate, development, proximate composition, protein quality, amino acid(s), solubility, isoelectric solubility/precipitation, ISP, SDS-PAGE, differential scanning calorimetry, DSC, mineral(s), color, freeze-dried, protein isolate

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ABSTRACT

Addition of Apple Pomace to Feeding Substrate on Growth, Development, and Survival Rates of Yellow Mealworms (Tenebrio molitor) and Characterization of Their Proteins Extracted by a pH-Shift Process

Michelle DuVall

The weight changes, survival/mortality rates, and proximate compositions of Tenebrio molitor (yellow mealworm) larvae whose diets were supplemented with apple pomace were analyzed after a 4-week feeding study to examine the nutritional quality of the insects for use as a future potential food source. Going forward, the terms "mealworm(s)", "yellow mealworm(s)", "T. molitor" will be used interchangeably and will always refer to the same species. The mealworm larvae were randomly divided into two groups: in addition to standard worm chow, one group was given 15 g/week of non-nutritive water-storing polymer crystals and the other was given apple pomace ad libitum which was made in-house. Weight changes were not significantly different between the two groups after four weeks (p > 0.05). Survival rates were similar; however, there was a correlation between increased rates of pupation and decreased survivability rates among the survivors (p < 0.05) in both groups. There were no significant differences in protein, fat, and ash between the two groups (p > 0.05; dry weight basis). The crude protein in water bead (WB) worms was 38.38 ± 0.05 g/100 g compared to apple pomace worms (AP) which contained 38.33 ± 0.07 g/100 g. The total fat in the WB worms was 51.94 ± 0.15 g/100 g, compared to 52.27 ± 0.09 g/100 g in the AP worms. The ash content was also similar between WB worms (0.20 ± 0.001) and AP worms (0.16 ± 0.002). On the other hand, the moisture content of the WB mealworms was greater than (66.89 ± 0.01 g/100 g) the AP mealworms (63.89 ± 0.01; p< 0.05). Results of this study show that mealworms are a significant source of protein, and when mealworm feed is supplemented with apple pomace, protein concentration is not affected.

The solubility, proximate composition, amino acid profile, SDS-PAGE summary, thermal abilities, mineral composition, and color of Tenebrio molitor (yellow mealworm) larvae were studied to determine the nutritional quality and functional properties of the insects. The mealworm larvae were tested in various forms (fresh, frozen whole mealworms; freeze-dried whole mealworms; fresh, frozen mealworm protein isolate; and freeze-dried mealworm protein isolate) to determine if there were any differences in the qualities of the mealworms when proteins were isolated via isoelectric solubilization/precipitation (ISP) or freeze-dried before various analyses. The greatest protein solubility was seen at a pH of 12. On a dry weight basis, the protein concentration of the fresh, frozen whole mealworms (75.30±0.02 g/100g) was greater than the freeze-dried mealworms (66.33±0.02 g/100g); (p<0.05) but not the mealworm protein isolate 70.27±0.07 g/100g for fresh, frozen protein isolate (PI) and 65.61±0.02 g/100g for freeze-dried MW (p>0.05). T. molitor contains all essential amino acids; however, tryptophan is limiting. Whole freeze-dried mealworm samples had significantly greater
concentrations of amino acids when compared to mealworm protein isolate samples. SDS-PAGE revealed that actomyosin was seen across the Mini-PROTEAN TGX precast Gel; it was effectively isolated and did not degrade when ISP was completed. Differential scanning calorimetry showed that mealworm protein isolates had more drastic transitions than freeze-dried whole mealworms. Mealworms are not a good source of calcium, but they do provide potassium, phosphorus, and especially magnesium. The fresh, frozen whole mealworms contained a significantly greater amount of these minerals than freeze-dried mealworm protein isolate samples (p<0.05). Lastly, protein separated from fresh, frozen mealworm was lighter, greener, and more yellow than freeze-dried mealworm protein isolate. Results of this study show that mealworms are a significant source of protein, and the protein quality is enhanced when the mealworms are fresh, frozen rather than freeze-dried. In addition, isolating the protein did not appear to improve protein quality when in comparison to whole mealworm samples. Insects such as *Tenebrio molitor* can provide a reliable protein option while possibly mitigating environmental impacts; therefore, it is imperative to further investigate the potential of insect proteins.
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Chapter 1

Review of Literature

1. **Weight Change, Development Time, and Survival Rate for Mealworms**

   Primary data to examine within these studies are the mealworms’ weight, development times, and survival rates of the insects. Though all studies utilized different feeding strategies, there were some variances in how the diet interventions were implemented.

   Several studies investigated many different types of insect feeds/substrates. Rumbos et al (2020) experimented with forty-four different products as a part of a mealworm feeding study. These included cereal flours/meals, non-flour/cereal commodities, legumes, and various feeds of both vegetative and animal origin. Potato slices were also given to the mealworms as a moisture source. Linear regression analysis did not show any significant correlations between the protein found in the food substrates and the total larval weight. Adding a water source significantly impacted the growth, development, and survival rates of mealworms. When considering the addition of a moisture source to mealworms’ diets, several studies utilized vegetable or fruit pomace to test for effects on mealworm growth, development, or survival rates. The sources included: carrots, olives, lettuce, and potatoes. Lower rates of mortality, speedier pupation times, and higher mealworm weights were observed when carrot pomace & potatoes were added to wheat bran (Rovai et al, 2021). Pomace from fruit sources also showed similar positive results. Ruschioni et al (2020) performed a study utilizing varying percentages of olive pomace. The pomace was mixed with wheat flour/middling and carrots for moisture. The addition of the olive and carrot pomace showed significantly increased larval/pupal weights, higher survival rates, and development times were reduced to optimal levels as determined by each study. Studies on fruit by-products were limited. There were no studies found on apples or apple pomace despite apples being among the most popular fruits in the world (Mala, 2020).

   According to a 2017 FAO statistic, 366 million tons of apples are produced each year, and along with that, there are 3 to 4.2 million tons of apple pomace by-products manufactured worldwide (Campos et al., 2020).

   Not all studies utilized vegetable or fruit pomace by-products. Several studies investigated grain by-products. Therefore, the majority of the studies examined for this review focused on wheat middling; however, other grains and industry by-products were assessed as well. Zhang et al (2019) tested mushroom spent corn stover, highly denatured soybean meal, spirit distiller’s grains (SDG), and utilized wheat bran as their control. The wheat bran had the highest (p<0.05) development rate, survivability, and mealworm weight gain; however, despite these results, the other three by-product substrates showed potential to be utilized as commercial mealworm diets.

   Kim et al (2017) refuted the previous study. Their study utilized brewer’s spent grain (BSG), distiller’s dried grain (DDG), and a mixed grain feed. Results showed that larval/pupal weights, larval development, and survival rate were highest when larvae were fed...
mixed diets (50% wheat bran mixed with either 50% BSG or DDG). Overall, the reviewed studies indicated that a mixture of grain and produce by-products combined with wheat bran were most effective for supporting mealworm growth, development, and survival.

2. **Crude Protein Analysis**

It is also important to determine the protein content of the mealworms and whether these amounts are comparable to other commonly eaten protein sources such as: lean meats, poultry, fish and seafood, eggs, dairy products, nuts, seeds, or legumes/beans. The amount of protein typically found in edible insects varies from 35-60% (dry weight) and 10-25% (fresh weight) (Melo et al, 2011; Schulter et al, 2017). These percentages are greater than many plant proteins such as soy, lentils, and cereal (Bukkens, 1997). Some studies found that insects contain more protein than animal products or chicken eggs (Mlcek et al, 2014). This review considered five different studies. All of the studies examined utilized wheat bran as their control diet. Three of the five studies examined vegetable or fruit pomace. Li et al (2012) utilized the inedible parts of cabbage leaves. The study found that the crude protein percentage was significantly higher in plant waste-fed mealworms (75.14% ± 0.74) than control diet (68.14% ± 0.66). Feeding mealworms carrot pomace decreased protein content to 16.5% whereas, feeding carrot pomace paired with wheat bran increased protein percentage to 19.57%. Based on the results two feed substrates when combined yielded a higher crude protein percentage (Rovai et al, 2021). Ruschioni et al (2020) mixed olive pomace with a control diet of wheat flour and organic middling. Similar to the other two studies, the protein content was statistically greater when mealworms were fed a mixture of olive pomace and wheat bran (47.58% ± 1.59) versus 37.78% ± 0.74 for the control of wheat flour and organic middling. Grain by-product feeding studies showed similar findings to the pomace feeding studies. Mixed grains were often utilized, for example, when mixed soy and maize crop stover residue were fed to mealworms; the mealworms fed the mixed grain diet had the highest protein concentration out of the tested substrates (19.93 grams per 100-gram sample) (Stull et al, 2019).

This finding was confirmed in another grain byproduct feeding study utilizing mushroom spent corn stover, highly denatured soybean meal, spirit distiller’s grains, and wheat bran (control). Mealworms fed mushroom spent corn stover had the highest (76.25% ± 0.77) crude protein among the substrates (Zhang et al, 2019). It should be noted that the researchers in this study found that the mushroom spent corn stover was also the cheapest by-product tested, providing yet additional advantage for by-product feeds for economical insect rearing. Collectively, the five studies evaluated showed that either by-product feeds or a combination of by-product feeds with wheat bran was effective for significantly increasing protein content in mealworms.

3. **Amino Acid Profile**

Mealworms (*Tenebrio Molitor*) contain eighteen different amino acids, eight of which
are considered essential to humans. The amino acids present in the highest concentrations were glutamic acid, aspartic acid, and alanine. The lowest were tryptophan, cysteine, and methionine (Yu et al, 2021). Methionine is an essential but limiting amino acid in mealworms (Panini et al, 2017). When feeding pomace by-products, Ruschioni et al (2020) discovered that a 3:1 mixture of wheat flour to olive pomace yielded the highest (p<0.05) percentages of amino acid content in mealworms. In contrast to Yu et al and Panini et al, the Ruschioni et al study found that one of the most abundant essential amino acids was methionine at 7.62-8.36% of the total amino acid composition. One possible explanation for this may be that amino acid content can differ depending upon which developmental phase mealworms are at the time tested, with the larval phase being the highest (Yu et al, 2021).

When comparing grain by-product studies, Zhang et al (2019) observed that the amino acid totals in mealworms were greater than or equal to the Food and Agriculture Organization (FAO) and World Health Organization (WHO) requirements for all diet treatments; however, in contrast with other studies, Zhang et al (2019) showed that histidine was the limiting amino acid in mealworms.

Additional studies concluded that when mealworms are fed mixed diets, their amino acid yield tends to be higher than when only fed wheat bran as a control diet. Stull et al (2019) confirmed this to be the case in mealworm-fed diets consisting of wheat bran, oats, brewer’s yeast, and corn maize stover. Further, Kim et al (2017) reported that the mealworms fed distiller’s dried grains (DDG) had 2.32 and 1.88 times more essential and non-essential amino acid concentrations, respectively, than the wheat bran control-fed group.

Overall, the studies showed that a mixture of by-product-supplemented feeds combined with wheat bran supplied the highest amounts of amino acids. Regarding which amino acids were greatest or least, there was variation within the studies, both in essential and non-essential amino acids; however, all studies agreed that the amino acids found within the insects can meet the human requirements for essential amino acids.

4. **Protein Solubility and Protein Recovery Yield in T. molitor**

Various studies have assessed protein solubility in *T. molitor*, and despite some differences between the studies, all reviewed did report solubility increased in alkaline conditions and decreased in acidic conditions. Bußler et al utilized non-defatted insect flour which was frozen before use (2016). Proteins in this study showed the highest solubility at pH 10, and the isoelectric point (pI) was found to be around pH 4. Other studies concurred with these findings at pH values of 12.4, 11, 9, and 11 (Ravichandran et al, 2019; Yi et al, 2017; Zhao et al, 2016; & Zielińska, 2018). Lowest solubility and thus, highest protein precipitation amounts were also consistent among studies at acidic pH values of 4, 2-4, 4-5, 5, and 3-5 (Bußler et al, 2016; Yi et al, 2017; Zhao et al, 2016; Zielińska, 2018; & Azagoh, 2016).

Despite the similarities in solubility findings between studies, there were often differences in the form of *T. molitor* utilized in tests and also the methods for which the insects were prepared for testing. Two studies utilized mealworm larvae flour (Bußler et al, 2016; & Ravichandran et al, 2019); however, Bußler et al worked with frozen insect flour, and Ravichandran et al boiled the insects and microwave-dried them before grinding them into
flour. The use of heat in preparing the mealworms for analysis does present concerns over whether the heat may denature the insects’ proteins. All other analyzed studies utilized mealworm larvae which were prepared by freeze-drying or liquid nitrogen (Purschke et al, 2018; Yi et al, 2017; Yi et al, 2013; Zhao et al, 2016; Zielińska, 2018; & Azagoh, 2016).

It is important to note specific unique aspects of some of these studies as well. Bußler et al showed that in general, defatting mealworms led to decreased protein solubility (2016). They also found that increasing ionic strength or extraction temperatures of protein solvents can increase solubility.

Purschke et al examined how pH, centrifugation speed, and time in the centrifuge affected protein recovery (2018). ANOVA revealed that the pH is certainly the most influential in determining protein recovery. Zhao et al discussed that protein solubility decreased with the addition of a NaCl solution (2016); however, this is in contrast to Bußler et al (2016).

The aim of this project was (i) to analyze the growth, weight, survival, and protein composition of mealworms (*T. molitor*) reared using an agricultural by-product to support mealworm production and (ii) to characterize the potential nutritional quality and solubility of the mealworm protein, specifically through the use of a pH-shift protein recovery process based on isoelectric point. Proximate composition, SDS-PAGE patterns, amino acid profiles, differential scanning calorimetry (DSC), mineral analysis, and color (L,a,b) analysis were also measured to determine the effects of mealworm protein isolate.
References


Chapter II

Addition of Apple Pomace to Feeding Substrate on Growth, Development, and Survival Rates of Yellow Mealworms (Tenebrio molitor)

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Abstract

The weight changes, survival/mortality rates, and proximate compositions of *Tenebrio molitor* (yellow mealworm) larvae whose diets were supplemented with apple pomace were analyzed after a 4-week feeding study to examine the nutritional quality of the insects for use as a potential food source. The mealworm larvae were randomly divided into two groups: in addition to standard worm chow, one group was given 15 g/week of non-nutritive water-storing polymer crystals and the other was given apple pomace ad libitum made in-house. Weight changes were not significantly different between the two groups after four weeks (p > 0.05). Survival rates were similar; however, there was a correlation between increased rates of pupation and decreased survivability rates among the survivors (p < 0.05) in both groups. There were no significant differences in protein, fat, and ash between the two groups (p > 0.05; dry weight basis). The crude protein in water bead (WB) worms was 38.38 ± 0.05 g/100 g compared to apple pomace worms (AP) which contained 38.33 ± 0.07 g/100 g. The total fat in the WB worms was 51.94 ± 0.15 g/100 g, compared to 52.27 ± 0.09 g/100 g in the AP worms. The ash content was also similar between WB worms (0.20 ± 0.001) and AP worms (0.16 ± 0.002). On the other hand, the moisture content of the WB mealworms was greater than (66.89 ± 0.01 g/100 g) of the AP mealworms (63.89 ± 0.01; p< 0.05). Results of this study show that mealworms are a significant source of protein, and when mealworm feed is supplemented with apple pomace, protein concentration is not affected.

KEYWORDS: *Tenebrio molitor*, yellow mealworm, edible insect(s), feeding study, agricultural by-product, growth rate, development, proximate composition, protein quality
1. Introduction

The Population Division of the UN of Economic and Social Affairs anticipates that the world’s population will increase in the next 30 years to approximately 9.7 billion people by 2050 (UN DESA, 2019). Given this increase, the global food supply must be able to support the growing population. Identifying protein sources that are affordable and sustainable is of utmost importance and finding solutions promptly is vital. Protein sources must be balanced but diverse, safe, and plentiful for reducing hunger and protein energy malnutrition (Webb et al., 2018). Insects are a progressively appealing food possibility. The ability to use these underutilized resources can have a positive economic impact on the agricultural industry. In many countries, consuming insects, or entomophagy is commonplace; however, in the United States, there remains stigma or “yuck factor” behind this idea. Insects are plentiful, have a smaller carbon footprint than many other commercial food sources, and offer an alternative to traditional sources of protein. Mealworms provide protein, including essential amino and fatty acids, and many other nutrients. In contrast to other protein sources (e.g. beef), mealworms require less water and also utilize less land to produce a similar amount of food (Wimer & Frank, 2018). Not only can this assist with protein-energy malnutrition, but it may also result in cost savings for insect farmers and food manufacturers by diverting discarded resources from food wastes back into the food supply. Insects have been shown to have high bioconversion proportions when fed agricultural or food by-products (Fowles & Nansen, 2019). However, insect-feeding studies using fruit by-products are limited, and a review of the literature revealed a gap in research that looked at apples or apple pomace as a source of feed, despite that apples are among the most popular fruits in the world (Mala, 2020). According to a 2017 FAO statistic, 366 million tons of apples are produced each year, and along with that, there are 3 to 4.2 million tons of apple pomace by-products manufactured worldwide (Campos et al., 2020). An opportunity exists to divert the otherwise discarded pomace back into the human food supply by using it as an insect feed; however, in the U.S., raising insects for food is still a new concept, and many challenges are expected to arise throughout the evolution of this idea (van Huis, 2016). Therefore, the objective of this study was to analyze the growth, weight,
survival, and protein composition of mealworms (*Tenebrio molitor*) whose diets were supplemented with apple pomace.

2. Methods

Insect studies were carried out between March and April 2022 with a one-week acclimation period and a four-week growth study (5 weeks total). This took place in the Food Science Core Laboratory in the Agriculture Sciences building at West Virginia University, Morgantown, West Virginia.

2.1 Materials

Medium mealworms were purchased (New York Worms, Long Island, NY) and shipped overnight to our facility. Two separate shipments were received to replicate the experiment at the shipment level. Each shipment (separated by several weeks) constituted a random replication like a blocking factor in statistical analysis. The first shipment contained 1,000 mealworms, and the second contained 3,000. Upon receipt of the mealworms, they were placed in one large holding container with the original shipping medium (crumpled-up newspaper) and dry mealworm feed (given ad libitum.) The container was covered with plastic cling wrap with air holes, and the mealworms were left to acclimate for 7 d in the dark at room temperature.

Apple pomace was made in-house to control the age and freshness of the pomace. Organic gala apples that were commercially grown in Washington were purchased from a local grocery store (Wal-Mart, Morgantown, West Virginia). Apples were washed, cored, and sliced which allowed for the removal of the apple seeds which contain amygdalin, a substance that can release cyanide when digested (Bolarinwa et al., 2015). Apples were loaded into a manual fruit crusher (Fruit Apple Crusher, EJWOX, Wilmington, NC) and twice crushed by spinning the side wheel that operated the steel blades. Apple pomace was collected in cheesecloth and hand-squeezed to remove excess moisture and juice. Approximately 25 grams of the pomace was placed into small snack bags (Ziplock, SC Johnson, Racine, WI) and stored at 0°C. All the apple pomace for the study was prepared as one batch and removed from freezer storage as needed.

2.2 Growth Parameters
After acclimation, worms were separated from the packing material and randomly assigned into 12 food-safe BPA-free polyethylene freezer containers (9 cm x 9 cm x 5 cm, Arrow Home Products, USA). Four containers were placed uncovered into each of the three incubators (Thermo Scientific 18L Low Temp Incubator & Benchmark Scientific H2200-H My Temp Mini Incubator). To avoid environmental discrepancies between incubators, the insect-rearing containers were rotated between incubators, and between shelves inside the incubators twice per week.

2.3 Growth Conditions

The air temperature inside the incubators was kept at ~27.8°C (range: 26.6-29.5°C), and the humidity was maintained at approximately 47% (range: 29.3-71.0% RH). Both air temperature and humidity levels were monitored with an electronic temperature/humidity hygrometer (model # 8541833457, Veanic mini) inside each incubator. The photoperiod in the room was mostly dark; with less than 1 hr/day of light for feeding and cleaning.

2.4 Feeding and Hydration Strategies

All mealworms were fed ad libitum a commercial mealworm bedding/chow which contained the following ingredients: wheat bran, corn flour, soybean flour, Brewer’s Yeast, bone meal, and multivitamins (Wormy Worms Premium Mealworm Superworm Bedding Chow, USA). The dry food/bedding was changed out weekly to remove the mealworm frass (mealworm excrement). The mealworm larvae were randomly divided into two groups: the control group (WB) was given 15 g/week of non-nutritive water-storing polymer crystals and the supplementation group (AP) was given apple pomace ad libitum. The apple pomace was changed out twice per week to reduce spoilage.

2.5 Growth Performance

Every 7 days, ten mealworms from each box were picked at random and weighed. Mealworms were also observed for mortalities in the supplement group, and for the amount of apple pomace consumed. After the predetermined growth period (1 week of acclimation and 4 weeks of treatment), food and water or apple pomace were removed to allow for gut cleaning 24 h before being freeze-dried (FreeZone 8L Tray Freeze Dryer, Labconco, Kansas City, MO) and stored at -40°C until analyses were conducted.
2.6 Proximate Composition

Proximate composition (moisture, crude protein, fat, and ash) was determined on the commercial chow, apple pomace, and mealworms after the growth study according to the methods described by the Association of Official Analytical Chemists (AOAC, 2000). Proximate analyses were conducted on a dry matter basis for protein, lipids, and ash and on a wet basis for moisture. Moisture was determined by spreading approximately 1 g samples in aluminum pans (Fisher Scientific) and placed into a drying oven at 110°C for 24 h (AOAC, 2000). Total fat content was determined using the Soxhlet indirect method (AOAC, 2000) where lipid was extracted using petroleum ether at a drip rate of 10 mL per minute for 24 h and dried in a drying oven at 110°C for an additional 24 h. Crude protein was measured using the Kjeldahl titration method (AOAC, 2000) and ash was determined by placing samples into ashing crucibles and incinerated in a muffle oven furnace for 24 h at 550°C (AOAC, 2000). 6.25% was the conversion factor utilized to convert nitrogen content into protein content.

2.7 Statistical design

Data were analyzed using JMP and SAS software (JMP®, Version Pro 16.0.0, SAS Institute Inc., Cary, NC, Copywrite ©2021; SAS®, Version 9.4, SAS Institute Inc., Cary, NC, Copyright ©2002-2012). Three methods of survival rate analysis were employed; repeated measures ANOVA that considered each box with multiple larvae as an experimental unit and allowed adjustment to additional covariates possibly affecting the survival; and two categorized survival data methods, such as the Life Table method (actuarial) and Kaplan-Meier (Stokes et al., 2012). A mixed model analysis of variance (ANOVA) was utilized, where the repeated factor was the week used with autoregressive covariance structure and the fixed effect was the treatment group (WB, AP). Covariates in the model were pupation rate, humidity, and temperature. In addition, the interaction of the rate of pupation and week, as well as treatment and week were examined.

The categorical methods (Life Table and Kaplan-Meier) enabled the generation of weekly probabilities of survival. Both categorical methods take each individual larvae into account and in both methods, the larvae that pupated were considered as a withdrawal count for mathematical purposes. The pupated insects were removed from the boxes at every
interval, thus the starting totals of living larvae in the boxes were changing at each interval both due to pupation and mortality. Both categorical strategies assume that the rate of pupation is independent of treatment and that the pupation rate is uniform throughout the interval. The categorical survival method was followed by the Log-Rank test of Equality of Strata; strata being the treatment groups. A significant $p$-value in Log-Rank indicates evidence of one group having a more favorable survival outcome (Stokes et al., 2012). The relationship between pupation rate and mortality was explored employing the nonparametric Spearman’s $\rho$ correlation analysis.

For growth (weight) and the proximate analysis variables (moisture, crude protein, fat, and ash), Shapiro-Wilk $W$ and Levene’s tests were used to check residuals for satisfying the assumption of normal distribution and homogeneity of variance, respectively. Protein required natural log transformation. Mixed model ANOVA was utilized to determine the differences between the treatment groups in growth (weight in grams). The repeated factor was the week used with autoregressive covariance structure, the fixed effect was the treatment group (WB, AP) and the interaction of treatment and week on growth was also examined. In addition to repeated measures, the effect of treatment on final larval weight at week 4 was examined, using the study replication as a random effect in the ANOVA.

For the proximate data analysis (percent ash, fat, moisture, and protein), samples were pooled across weeks and boxes into 3-9 replicates per treatment. The treatment effect was examined using a T-test.

In all statistical analyses, the significance criterion alpha was 0.05.

3. Results and Discussion

When mealworms are utilized for food, both the cost of production and the ability to maintain a supply chain that provides an inexpensive and abundant source of protein needs to be considered. If production facilities can reduce the growth period and increase weight and survival rates, then money and time would be saved and potentially improved protein quality. Therefore, the weight, survival/mortality rates, and proximate composition of mealworms whose diets were supplemented with apple pomace were compared to those reared on a standard ration.
3.1 Weight Change & Survival/Mortality Rate

Growth was measured by monitoring the average weight (g) for each group of mealworm larvae (water bead; WB and apple pomace; AP), and there were no significant differences in growth during the 4-week period (p > 0.05) (Table 1 and Figure 1.) The weight of the WB mealworm larvae increased by 84.99% ± 0.21 compared to the AP group which saw a weight increase of 81.86% ± 0.24.

When utilizing repeated measures ANOVA with adjustments for pupation rate, humidity, and temperature, the survival rate of the mealworms did not significantly differ (p > 0.05) between the AP mealworms and the WB mealworms. However, it is interesting to note that in both groups there was a correlation between the frequency of pupation and survival among the larvae that remained (p < 0.05; Table 2; as the pupation rates increased, the frequency of mortality increased. This was also seen when looking at the sequential test for the rate of pupation concerning mealworms’ treatment groups (p < 0.05). So, even though the treatment group on its own did not seem to affect mortality (p > 0.05) when considering the rate of pupation, apple pomace mealworms had a better survival rate than water bead mealworms.

The Life Table Statistical Method was utilized and found that the survival rate of mealworm larvae given water beads was 66.71% over four weeks and the apple pomace-fed mealworm larvae was 73.28% over the same four-week period (Table 2). The life test procedure tested for homogeneity of larvae survival curves for weeks over two treatment conditions. The Log-Rank Test of Equality over Strata was also utilized: \( \chi^2 = 12.7954, \ DF = 1, Pr > \chi^2 < 0.001, \) indicating a difference between AP mealworm and WB mealworm survivability. This demonstrates that apple pomace may have a defensive influence on the AP mealworm group, especially towards the end of the growing period when the pupation rate increases (see Table 2 and Figures 2-3). A positive correlation was observed between pupation rate and mortality every week (\( r = 0.81, p = 0.80, r = 0.65 \) week 3, \( r = 0.72 \) week 4, all \( p < 0.001 \)). Supplementing with apple pomace could have provided the mealworms with effective antioxidants which encourage the immune system in invertebrates (Vigneron et al., 2019).
It is important to note that even though the mealworms’ survival rate did not differ between the apple pomace worms and the water bead worms in the ANOVA statistical analysis when the effect was adjusted to temperature, humidity, and the pupation rate, there is still an interesting connection between a greater pupation rate and a greater death rate among the larvae that remained in the boxes. Possible explanations for this could be as simple as a larger population density may have led to increased competition, crowding, lower feeding opportunities, or even a change in immunity to illnesses or infections (Zaelor & Kitthawee, 2018). And again, after further analysis, we did see that increased pupation correlated with increased mortality, and the treatment groups were statistically different in that apple pomace mealworms had a greater survival rate than water bead mealworms as pupation increased. Though all studies mentioned utilized different feeding strategies, there were some variances in how the diet interventions were implemented. Rumbos et al. (2020) experimented with forty-four different products as a part of their feeding study. These included cereal flours/meals, non-flour/cereal commodities, legumes, and various feeds of both vegetative and animal origin. Potato slices were also given to the mealworms as a moisture source. Linear regression analysis did not show any significant correlations between the protein concentration of the food substrates and the total larval weight (Rumbos et al., 2020). Adding a water source significantly impacted mealworm growth, development, and survival rates. When considering the addition of a moisture source to mealworms’ diets, several studies utilized vegetable or fruit pomace to test for effects on mealworm growth, development, or survival rates. The sources included: carrots, olives, lettuce, and potatoes. Lower rates of mortality, speedier pupation times, and greater mealworm weights were observed when carrot pomace and potatoes were added to wheat bran (Rovai et al., 2021). Pomace from fruit sources also showed similar positive results. Ruschioni et al. (2020) performed a study utilizing varying percentages of olive pomace. The pomace was mixed with wheat flour/middling and carrots for moisture. The addition of the olive and carrot pomace showed significantly increased larval/pupal weights, greater survival rates and development times were reduced to optimal levels as determined by each study. Overall, a mixture of grain and produce by-products combined with wheat bran were most effective for supporting mealworm growth, development, and survival. In the instances
where there were no differences or the differences were not statistically significant, it does not appear that the addition of produce by-products inhibits mealworm growth, slows down development, or increases mortality rate unless the mealworms are only given by-products without the addition of any grain products.

3.2 Proximate Composition

The proximate composition of worm chow and apple pomace was confirmed and is reported in Table 3. The protein concentration of the chow was under 9% and the apple pomace had about 1.5% protein. The proximate composition of each group of mealworm larvae (water bead; WB and apple pomace; AP) is reported in Table 4. There were no significant differences in fat, protein or ash ($p > 0.05$); however, moisture content was greater in the mealworms given water beads than in mealworms fed apple pomace. A possible reason for this may be that the water beads were simply a more direct route for the mealworms to obtain moisture, whereas the apple pomace also contained other nutrients and fiber. By-product feeds or a combination of by-product feeds with wheat bran was effective for significantly increasing protein content in mealworms. It is important to consider the protein content of the mealworms and how this compares to other commonly eaten protein sources such as lean meats, poultry, fish and seafood, eggs, dairy products, nuts, seeds, or legumes/beans. The amount of protein typically found in edible insects varies from 35-60% (dry weight) and 10-25% (fresh weight) (Melo et al., 2011; Schulter et al., 2017). These concentrations are greater than many plant proteins such as soy, lentils, and cereal (Bukkens, 1997). Some studies found that insects contain more protein than animal products or chicken eggs (Mlcek et al., 2014). In this study, the average crude protein concentration of T. molitor larvae was $38.36 \pm 0.06 \text{ g/100 g}$, and there was no significant difference between larvae fed apple pomace (AP) and larvae only given water beads (WB) ($p > 0.05$; Table 4. In fact, aside from moisture, there were no significant differences in proximate composition between the two groups ($p > 0.05$) with the average fat and ash content (dry matter basis) of the mealworms $52.11 \pm 0.12 \text{ g/100g}$, and $0.18 \pm 0.00 \text{ g/100g}$, respectively. There were significant differences in moisture content, with the WB and AP larvae having $66.89 \pm 0.01 \text{ g/100 g}$ and $63.89 \pm 0.01 \text{ g/100 g}$ moisture, respectively ($p < 0.05$).
In this current study, supplementation of the control diet with apple pomace did not significantly impact the protein concentration of mealworms, which is not consistent with other studies where mealworm feed was supplemented with agricultural waste and by-products. For example, Li et al. (2013) fed mealworms the inedible parts of cabbage leaves in addition to wheat bran and found that the crude protein percentage was improved by 8% (dry matter basis). On the other hand, in a different study, when mealworms were fed only carrot pomace, protein content decreased to 16.5% whereas, when carrot pomace was paired with wheat bran, the protein percentage increased to 19.57% (wet weight) (Rovai et al., 2021). When Ruschioni et al. (2020) mixed olive pomace was mixed with a control diet of wheat flour and organic middling, the protein content was also improved by almost 10% from the control (Ruschioni et al., 2020). This shows that the addition of produce by-products can enhance the nutrients and proximate composition of the mealworms and only tend to cause a decrease in crude protein when all grain products are removed completely from mealworms’ diets.

The protein found in the grain portion of the mealworm diet is an important factor when it comes to the final protein concentration of the harvested mealworms. For example, feeding studies showed that in addition to wheat bran, supplementation with mixed grains increased the concentration of protein in harvested mealworms. Mixed grains were often utilized, for example, when mixed soy and maize crop stover residue were fed to mealworms; the mealworms fed the mixed grain diet had the highest protein concentration out of the tested substrates (19.93 g/100 g sample) (Stull et al., 2019). This finding was confirmed in another feeding study which compared mushroom spent corn stover, highly denatured soybean meal, spirit distiller’s grains, and wheat bran as the control. Mealworms fed mushroom spent corn stover had the greatest concentration of crude protein (76.25% ± 0.77) among the substrates tested (Zhang et al., 2019). It should be noted that the researchers also reported that the mushroom spent corn stover was also the least expensive by-product tested, providing yet additional advantage for by-product feeds for economical insect rearing.

The differences in protein concentration between the feeding strategies highlighted above are likely due to the protein concentration of the diet. The protein present in the grain-based feed serves to nourish them for survival, whereas the addition of produce by-products
may serve as an enhancement to their growth, either by increasing final weights, enabling greater survival rates, or increasing the final protein concentration of the mealworms. Interestingly enough, the proximate composition tends to be the area in which the most variation is seen. In this study, there were no significant differences in the concentration of protein, fat, and ash between the apple pomace and water bead-fed groups. Many of the other studies reported differences, particularly in protein, once again reiterating that the addition of produce by-products can enhance the nutrients and proximate composition of the mealworms and only tend to cause a decrease in proximate composition when all grain products are removed completely from mealworms’ diets.

4. Conclusions

The combination of wheat bran with food by-products, pomace or grain-based, has been shown to improve the protein content of mealworms; however, there were no significant differences in protein concentration seen in this present study. In addition, there were no significant differences in mealworm weights or survival rates; however, it was demonstrated that AP mealworms had a greater survival rate as pupation rates increased than WB mealworms did. The protective effect of the apple pomace during pupation should be noted as a positive result of the supplementation, especially since the rate of pupation is difficult to control. In additional studies, it would be advantageous to experiment with different amounts of supplemented produce by-products to see if the proportion of standard mealworm food to supplemented food shows any differences in weight, pupation times, survival rates, or proximate composition of the mealworms. Performing a cost comparison between feed supplementation products could also be beneficial. There are additional considerations to study as well. Both mealworm allergenic components (e.g. chitin) and microbial safety concerns must be addressed to ensure that mealworms can make a suitable protein alternative for human consumption. It may be recommended that further research be completed to address some of these topics before providing a general endorsement to add insect protein to human diets.

Overall, Tenebrio molitor has the potential for providing a high-quality protein source. Agricultural by-products can contribute to the mealworm-rearing industry and assist in
providing an alternative protein option with fewer environmental impacts. It is imperative to further investigate the potential of insect proteins.


15.) Growing at a slower pace, world population is expected to reach 9.7 billion in 2050 and could peak at nearly 11 billion around 2100 | UN DESA Department of Economic and
20.) Zaelor J, Kithawee S. Growth response to population density in larval stage of darkling beetles (Coleoptera; Tenebrionidae) Tenebrio molitor and Zophobas atratus. Agriculture and Natural Resources. 2018 Dec 1;52(6):603–6.
Tables & Figures

Table 1: Growth rate of *Tenebrio molitor* larvae over a 4-week period where a standard wheat-based worm chow was supplemented with apple pomace in place of standard water beads. Values are the LS Means (mg ± SE) of 10 randomly selected mealworms. There were no significant differences in weight gain over time between apple pomace and water bead (control) treatments (ANOVA, p > 0.05).

<table>
<thead>
<tr>
<th></th>
<th>Water Bead (WB)</th>
<th></th>
<th>Apple Pomace (AP)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Larval wt. (mg)</td>
<td>Δ wt. (mg)</td>
<td>Larval wt. (mg)</td>
<td>Δ wt. (mg)</td>
</tr>
<tr>
<td>Initial</td>
<td>5.66 ± 0.32</td>
<td></td>
<td>5.50 ± 0.32</td>
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<tr>
<td>1</td>
<td>8.37 ± 0.32</td>
<td>2.71</td>
<td>8.06 ± 0.32</td>
<td>2.65</td>
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<tr>
<td>2</td>
<td>8.97 ± 0.32</td>
<td>0.60</td>
<td>8.89 ± 0.32</td>
<td>0.83</td>
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<tr>
<td>3</td>
<td>9.55 ± 0.32</td>
<td>0.56</td>
<td>9.17 ± 0.32</td>
<td>0.27</td>
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<tr>
<td>4</td>
<td>10.5 ± 0.32</td>
<td>0.90</td>
<td>10.0 ± 0.32</td>
<td>0.82</td>
</tr>
<tr>
<td>% Weight gain</td>
<td>84.99 ± 0.21</td>
<td></td>
<td>81.86 ± 0.24</td>
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</tr>
</tbody>
</table>

*a, b, c, d* Designates significant increases in growth by week by Tukey’s HSD (p<0.05).
Table 2. Survival/Mortality data on *Tenebrio molitor* larvae after 4-week supplementation study.

<table>
<thead>
<tr>
<th>Interval (weeks)</th>
<th>Mortality %</th>
<th>Survival %</th>
<th>Mortality %</th>
<th>Survival %</th>
<th>Pupation Count</th>
<th>Mortality Count</th>
<th>Contour</th>
<th>Contour</th>
<th>Contour</th>
<th>Contour</th>
<th>Contour</th>
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<tbody>
<tr>
<td></td>
<td>Life Table Method</td>
<td><em>Kaplan-Meier</em></td>
<td><em>Kaplan-Meier</em></td>
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</table>

**AP = Apple Pomace; WB = Water Beads; TRT GRP = Treatment Group

* Chi-square = 12.79, p=0.0003

** Log-Rank Test of Equality over TRT GRP

<table>
<thead>
<tr>
<th>TRT GRP</th>
<th>AP</th>
<th>WB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interval (weeks)</td>
<td>Pupation/Mortality/Survival Counts</td>
<td>Pupation/Mortality/Survival Counts</td>
</tr>
<tr>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Interval (weeks)</th>
<th>Survival Count</th>
<th>Mortality Count</th>
<th>Contour</th>
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<th>Interval (weeks)</th>
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Significant *p*-value indicates evidence of one group having a more favorable survival outcome.

Both categorical survival methods were followed by the Log-Rank test of Equality over Strata, strata being the Treatment Groups. **An Tests were supplemented by the baseline for the measurements.** Week 0 represents the end of the acclimation period and the baseline for the measurements.

<table>
<thead>
<tr>
<th>Interval (weeks)</th>
<th>Survival Count</th>
<th>Mortality Count</th>
<th>Contour</th>
</tr>
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<tbody>
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</table>
Table 3. Proximate Composition of Worm Chow & Apple Pomace (dry matter basis).

<table>
<thead>
<tr>
<th>Material</th>
<th>Moisture (g/100g)</th>
<th>Protein (g/100g)</th>
<th>Fat (g/100g)</th>
<th>Ash (g/100g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wormy Worm Chow</td>
<td>10.95 ± 0.25</td>
<td>8.46 ± 0.12</td>
<td>6.13 ± 0.09</td>
<td>1.71 ± 0.21</td>
</tr>
<tr>
<td>Apple Pomace</td>
<td>85.86 ± 0.89</td>
<td>1.20 ± 0.10</td>
<td>3.19 ± 2.22</td>
<td>0.04 ± 0.04</td>
</tr>
</tbody>
</table>

Data are given as means ± SD.
**Table 4.** Proximate composition (dry weight basis for protein, fat, ash; wet basis for moisture) of *Tenebrio molitor* larvae after 4-week supplementation study

<table>
<thead>
<tr>
<th>Species</th>
<th>Moisture (g/100g)</th>
<th>Protein (g/100g)</th>
<th>Fat (g/100g)</th>
<th>Ash (g/100g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mealworm (AP)</td>
<td>63.89 ± 0.01(^a)</td>
<td>38.33 ± 0.07</td>
<td>52.27 ± 0.09</td>
<td>0.16 ± 0.002</td>
</tr>
<tr>
<td>Mealworm (WB)</td>
<td>66.89 ± 0.01(^b)</td>
<td>38.38 ± 0.05</td>
<td>51.94 ± 0.15</td>
<td>0.20 ± 0.001</td>
</tr>
</tbody>
</table>

Data are given as means ± SD. Mean values within columns with different letters indicate significant differences (p< 0.05).
**Figure 1**: *Tenebrio molitor* larval weight gains over a 4-week period where a standard wheat-based worm chow was supplemented with apple pomace in place of standard water beads. Values are LS Means (mg +/- SE). There were no significant differences in weight gain over time between apple pomace and water bead (control) treatments (ANOVA, p > 0.05).
Figure 2: Probability of survival of *Tenebrio molitor* (live larvae, pupae censored) over a 4-week period where a standard wheat-based worm chow was supplemented with apple pomace in place of standard water beads (Life Test Estimation of Survival Rates). There was a significant difference in probability of survival between apple pomace and water bead (control) treatments (Log-Rank Test, $\chi^2 = 12.7954$, DF = 1, Pr > $\chi^2 = 0.0003$).
**Figure 3:** Number of live, dead, and pupated mealworm larvae fed a standard wheat-based worm chow supplemented with apple pomace (left) in place of standard water beads (right).
Chapter III

Characterization of Mealworm (*Tenebrio molitor*) and Mealworm Proteins Extracted by a pH-Shift Process

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Abstract

The solubility, proximate composition, amino acid profile, SDS-PAGE summary, thermal abilities, mineral composition, and color of Tenebrio molitor (yellow mealworm) larvae were studied to determine the nutritional quality and functional properties of the insects for possible utilization as a future alternative food resource. The greatest protein solubility was seen at a pH of 12 so protein separation was attempted using a pH-shift process (ISP) based on solubilizing proteins at pH 12 and precipitating them at their isoelectric point (pH 4.0). Protein concentration of the fresh, frozen whole mealworms was 75.30±0.02 g/100g, which was greater than the freeze-dried mealworms (66.33±0.02 g/100g; p<0.05). Protein concentration was not increased using the pH-shift process (p<0.05). T. molitor contains all essential amino acids; however tryptophan is limiting. Whole freeze-dried mealworm samples had significantly greater concentrations of amino acids when compared to mealworm protein recovered using ISP. SDS-PAGE revealed that actomyosin was effectively isolated via ISP and ISP protein samples showed a more drastic thermal transition than freeze-dried whole mealworms. Mealworms are not a good source of calcium, but they do provide potassium, phosphorus, and especially magnesium. The fresh, frozen whole mealworms contained a significantly greater amount of these minerals than ISP protein samples. Fresh, frozen ISP-recovered protein was lighter, greener, and more yellow than freeze-dried ISP protein. Results show that mealworms are a significant source of protein, and the protein quality is enhanced when the mealworms are fresh, frozen rather than freeze-dried. In addition, isolating the protein using the pH-shift process did not appear to improve protein quality when in comparison to whole mealworm samples.

KEYWORDS: Tenebrio Molitor, yellow mealworm, edible insect(s), proximate composition, protein quality, amino acid(s), solubility, isoelectric solubility/precipitation, ISP, SDS-PAGE, differential scanning calorimetry, DSC, mineral(s), color, freeze-dried, protein isolate
1. Introduction

The Food and Agriculture Organization (FAO) of the United Nations reported that nearly one in three people in the world (2.37 billion) did not have enough food in 2020; an increase of approximately 320 million people since 2019 (FAO, 2021). In addition, as the population increases protein malnutrition will also continue to be a global problem as approximately one billion people have inadequate protein intake (FAO, 2021). Simply increasing the production of our current sources of protein is not sustainable. For example, protein from animal sources has high nutritional quality; however, studies have implicated meat and poultry production with adverse environmental impacts such as water pollution, water scarcity, environmental contamination, and increased greenhouse gas emissions, as well as adverse health implications from red meat like heart disease and carcinogens (González et al., 2020). Therefore, we need to identify alternative and often underutilized sources of protein that are both high quality and sustainable.

In the Western world, human consumption of insects is not typical of any standard or customary dietary eating pattern; however, in many other places in the world, they are routinely eaten and serve as a source of protein. Even the FAO has considered the potential of insects as a human food source and to assist with food security (FAO, 2010). Dried yellow mealworm (Tenebrio molitor) larvae were the first insect species to receive EU approval for human consumption (Turck et al., 2021) and it is important to determine whether the amounts of protein in mealworms are comparable to other commonly eaten protein sources such as lean meats, poultry, fish and seafood, eggs, dairy products, nuts, seeds, or legumes/beans. The amount of protein typically found in edible insects varies from 35-60% (dry weight) and 10-25% (fresh weight) (Melo et al., 2011; Schulter et al., 2017). These concentrations are greater than many plant proteins such as soy, lentils, and cereal (Bukkens, 1997). Some studies found that insects contain more protein than animal products or chicken eggs (Mlcek et al., 2014). Typically, edible insects can have higher protein content than many plant sources, and in some cases, they can provide at least comparable amounts or even more protein than some meat and egg sources (Bukkens, 1997).
When insect proteins are studied, the starting material that researchers start with is most often lyophilized or freeze-dried (Azagoh et al., 2016; Brogan et al., 2021; Kim et al., 2020; Ruschioni et al., 2020; Zhang et al., 2019; Zhao et al., 2016). There is still much that is unknown about the effects of freeze-drying on proteins. Freeze-drying proteins can cause them to undergo molecular changes which could lead to inactivation, and the freeze-drying process is not completely harmless in many instances (Roy & Gupta, 2004). The Roy & Gupta study revealed conformational alterations and clumping during freeze-drying, and they also determined that when moisture is present in a sample, these freeze-dried proteins can experience substitutions in disulfide bonds. This can lead to the proteins becoming inactive. The process should be fully comprehended and utilized with care, especially when considering protein sources for human consumption.

The main objectives of the research were to characterize *Tenebrio molitor* larvae protein in several different states to determine the potential nutritional quality and solubility of the protein. Specifically, the protein of fresh, frozen whole mealworms; freeze-dried whole mealworms; fresh, frozen mealworm protein separated using a pH-shift process (ISP) based on solubilizing proteins and precipitating them at their isoelectric point pH 4.0); and freeze-dried mealworm protein separated using ISP. Protein content (e.g. proximate composition), protein pH-solubility, and SDS-PAGE patterns were analyzed to determine differences between samples. Full amino acid profiles and differential scanning calorimetry (DSC) were also examined between freeze-dried whole worms and protein separated from freeze-dried mealworm. Mineral analysis and color were also tested.

2. Methods
2.1 Materials

*Tenebrio molitor* mealworm larvae were purchased (New York Worms, Long Island, NY) and shipped overnight to the Food Science laboratory at West Virginia University. Mealworm larvae were placed into a larger holding container with the shipping medium from New York Worms (crumpled newspaper), plastic cling wrap was used to cover the holding container, and air holes were poked into the cling wrap. Mealworm larvae acclimated 7 d in the dark at room temperature in this holding container before further experimentation was performed. After the
7-d acclimation treatment, mealworms were either frozen at -40°C or freeze-dried (FreeZone 8L Tray Freeze Dryer, Labconco, Kansas City, MO) and stored until needed for further analysis. With the freeze-dried samples, the vacuum was configured to a set point of 0.04 mbar and solidification/eutectic temperatures between -30 to -40°C. Feeding was not a part of the process for this study, and upon contact with New York Worm Company, they stated that the shipping medium was the only feed that the mealworms were given at the time they left the warehouse (NYWorms, 2022).

2.2 Solubility Study of Protein (Solubility Curve)

Approximately 2 g of fresh, frozen mealworm larvae, and 20mL distilled, deionized water was mixed using Teflon stir bars in 6 individual 250 mL beakers. The beakers were kept cool in an ice bath (4°C), and the homogenous mixtures were continuously mixed for fifteen minutes for stabilization before the initial pH was measured and values were adjusted. An Oakton pH 11 handheld meter with a pH probe was calibrated with calibration solutions before the tests. The pH of each beaker was adjusted utilizing hydrochloric acid (HCl) or sodium hydroxide (NaOH). Concentrated 12 M HCl or 1 M NaOH were used initially for larger pH changes, and 0.1 M concentrations were utilized for minor adjustments to pH 2, 4, 6, 8, 10, or 12 (+/- 0.05 for each). Again, the beakers were continuously stirred for 15 minutes for stabilization at the respective pH readings before recording the final pH.

The adjusted solutions were transferred to plastic tubes and centrifuged for 20 min at 5,000 x g (Eppendorf Centrifuge Model 5430R, Hamburg, Germany). After centrifugation, each tube presented with three separate layers. The lipids in the top layer were removed and discarded. The middle layer contained the protein-rich supernatant which was poured through a paper filter to separate it from the bottom layer which contained the unusable parts of the mealworms. The supernatant from each tube was freeze-dried separately, and the protein concentration in the powder residues from each pH was determined using the Kjeldahl method.

2.3 Protein Recovery via Isoelectric Solubilization and Precipitation (ISP)

Protein recovery using the ISP method was completed on previously fresh, frozen mealworms at -40°C and mealworms that were freeze-dried to compare protein properties between samples (Labconco FreeZone 8L Tray Freeze Dryer). Before the protein recovery
process, the fresh, frozen mealworms were crushed while frozen to form a thick paste in a sanitized (70% ethanol) Kinematica Polytron dispersing and mixing homogenizer. Protein was separated using the pH-shift method described in Figure 1 by Paker et al. (2013). Briefly, 10 g of freeze-dried mealworm or mealworm paste was mixed with 100 mL distilled, deionized water in a beaker to form a homogenous mixture. The beaker was placed in a 4°C ice bath. The pH was adjusted to the target pH of 12 using concentrated NaOH (1 M). The mixture was homogenized until the pH value reached stable numbers (approximately 15 min). After which the homogenized mealworm mixture was pipetted into plastic centrifuge tubes and spun at 5,000xg for 20 min at 4°C. Much like the solubility study, after centrifugation, each tube presented three separate layers. The top layer (lipids) was removed from the tube, freeze-dried, weighed on a dry-matter basis, and stored at -40°C for further studies. The supernatant (middle layer) was decanted into cheesecloth and then through a paper filter. The pH of the filtered supernatant was adjusted to the isoelectric point (pH 4.0) using concentrated HCl and homogenized until the pH value was steady for 15 min. Centrifugation was repeated at 5,000xg for another 20 min at 4°C resulting in two distinct layers, the supernatant at the top and the protein fraction on the bottom. The supernatant was discarded, and the pellet was either stored at -40°C or freeze-dried.

2.4 Proximate Composition

The proximate composition of fresh, frozen mealworms (FF), freeze-dried mealworms (FD), fresh, frozen mealworm protein recovered by ISP (FF ISP), and freeze-dried mealworm protein recovered by ISP (FD ISP) were determined according to the methods described by the Association of Official Analytical Chemists (AOAC, 2000). Proximate analysis was conducted in triplicate on a dry matter basis for protein, lipids, and ash and a wet basis for moisture. Samples (1 g) were placed in aluminum pans (Fisher Scientific), dispersed equally across the pan, and placed into a drying oven (110°C for 24 h) (AOAC, 2000). Total fat content was determined by the Soxhlet indirect method (AOAC, 2000). Samples (1 g) were extracted using petroleum ether at a drip rate of 10 mL per minute for 24 h. Before the samples were weighed for total fat content, the samples were also placed in the drying oven at 110°C for an additional 24 h. Crude protein was determined using the Kjeldahl titration method (AOAC, 2000). Ash was determined
by placing samples into ashing crucibles and incinerating them overnight (24 h) at 550° C (AOAC, 2000).

2.5 Amino Acid Profile Analysis

Complete amino acid profile evaluation was performed on fresh, frozen mealworms (FF), freeze-dried mealworms (FD), fresh, frozen mealworm protein isolate (FF MWPI), and freeze-dried mealworm protein isolate (FD MWPI) by the Agricultural Experiment Station Chemical Laboratories at the University of Missouri-Columbia according to the AOAC method (982.30). Briefly, samples were subjected to acid hydrolysis, acid oxidation, and alkaline hydrolysis before amino acids were quantified using a Beckman Amino Acid Analyzer (model 6300, Beckman Coulter, Inc., Fullerton, GA).

2.6 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Both the fresh, frozen whole mealworm and the freeze-dried mealworm protein isolate samples were weighed (~0.008 g each) and placed into 1.5 mL plastic tubes. The samples were dissolved into a freshly prepared 1,000 µL of 20 mM Tris/HCl, 2 mM EDTA (pH 8.0) buffer solution. Samples were homogenized via vortex, and 5 µL of each sample was removed and placed into new tubes with 4.75 µL of Laemmli sample buffer (Bio-Rad, Hercules, CA, USA). Tubes were again vortexed (approx. 15 sec), 0.25 µL of β-mercaptoethanol (Bio-Rad, Hercules, CA, USA) was added, and vortexed again. The tubes were then placed in plastic bags and submerged in a hot water bath (100°C) for five min to cause protein denaturation. A gel electrophoresis chamber was readied and loaded with both 1x Tris/glycine/SDS buffer and a Mini-PROTEAN TGX precast Gel® (Bio-Rad, Hercules, CA, USA). The gel had ten wells each in it. Once the hot water bath time was completed, sample tubes were vortexed for fifteen sec. To create a standard, 5 µL of bovine serum albumin was loaded into the gel in lanes one and ten so that each end of the gel could be used to compare the samples. Once the gel was loaded with all of the samples, electrophoresis was run at 200 volts for 50 min and again for another 50 min to ensure that the bands would be dark and easier to read. The gel was withdrawn from the electrophoresis chamber. The gel was placed in a sanitized dish with distilled, deionized (DDI) water for rinsing. The dish was placed on an orbital shaker for five min. This rinsing process was completed three times with fresh DDI water each time. After rinsing, the DDI water was
discarded, and the gel was placed in 50 mL of Bio-Safe™ colloidal Coomassie Blue G-250 (Bio-Rad, Hercules, CA, USA) stain. The gel was placed on the orbital shaker for ninety min, and then the gel was destained with DDI water. The destaining process was carried out for thirty min on the orbital shaker.

2.7 Differential Scanning Calorimetry (DSC)

Differential Scanning Calorimetry (DSC) was performed using a (TA Instruments DSC) Q20. This was completed for the freeze-dried whole mealworms, and freeze-dried mealworm protein isolate samples which were dried in a freeze-dryer to vaporize any water/solvent before analysis. Samples were run in triplicate, and 4-6 mg were added to TZero low-mass pans and lids (Part #901670.901 & Part #901671.901 respectively). Lids were crimped onto pans with a crimper, masses were taken for each pan, and the pans were placed one at a time into the inner lid with reference pans beside them. The temperature inside the DSC equipment was increased from 5° to 90°C at a rate of 10° C per min^-1. Data were obtained by utilizing DSC software. Results were exported and comparisons between freeze-dried whole mealworm samples and freeze-dried mealworm protein isolate samples were carried out.

2.8 Mineral Analysis (K, P, Mg; & Ca)

Mineral analysis of potassium, phosphorus, magnesium, and calcium was performed by the National Research Center for Coal and Energy (NRCCE) analytical laboratory on the Evansdale campus of West Virginia University in Morgantown, WV in duplicate. A sample of 1.5469 grams of fresh, frozen crushed mealworms was sent for mineral analysis along with 1.2142 grams of freeze-dried mealworm protein isolate. The NRCCE utilized the EPA method 200.7 (Determination of metals and trace elements in water and wastes by inductively coupled plasma-atomic emission spectrometry). Analytes were first solubilized with nitric acid, allowed to cool, made up to volume, and centrifuged before analysis. The solution is then run through an instrument designed to measure atomic-line emission spectra via optical spectrometry. Specific wavelengths are measured and processed for the determination of the various minerals (EPA, 1994).

2.9 Color Analysis (L,a,b)
A Konica Minolta Chroma Meter (CR-400) was utilized to establish the color of the mealworm samples (fresh, frozen whole protein isolate and freeze-dried protein isolate). The equipment was calibrated against the white calibration tile available in the equipment’s storage box as per the directions in the manual. L* values designated lightness, a* values indicated color on a red/green scale, and b* values denoted color on a yellow/blue scale. All experiments were performed in duplicate.

2.10 Statistical Design

One-way analysis of variance (ANOVA) and Tukey’s honestly significant differences (HSD) test were used for the overall analysis of variance and mean separation where \( \alpha = 0.05 \). One-way ANOVA analysis and a pooled t-test were performed for amino acid and mineral composition. SDS-PAGE, DSC, and color analysis were examined and qualitative results were reported; no statistical analyses were completed for these.

3.0 Results and Discussion

3.1 Proximate Composition of Whole Insect Samples

Table 1 shows the proximate composition of *T. molitor* larvae for fresh, frozen whole insect samples (FF) and whole insect samples after freeze-drying (FD). There were significant differences (\( p < 0.05 \)) in crude protein content (dry matter basis) between FF mealworms (75.30±3.31 g/100 g) and FD mealworms (66.33±3.88 g/100 g). Other studies reported 65.6%±1.5 dry matter crude protein percentage in whole freeze-dried mealworm larvae (Azagoh et al., 2016) and 68.14%±0.66 dry matter crude protein in freeze-dried mealworm larvae (Li et al., 2013). It is important to determine whether the amounts of protein in mealworms are comparable to other commonly eaten protein sources such as lean meats, poultry, fish and seafood, eggs, dairy products, nuts, seeds, or legumes/beans. The amount of protein typically found in edible insects varies from 35-60% (dry weight) and 10-25% (fresh weight) (Melo et al., 2011; Schulter et al., 2017). These percentages are greater than many plant proteins such as soy, lentils, and cereal (Bukkens, 1997). Some studies found that insects contain more protein than animal products or chicken eggs (Mlcek et al., 2014). Typically, edible insects can have higher protein content than many plant sources, and in some cases, they can provide at least comparable amounts or even more protein than some meat and egg sources (Bukkens, 1997).
FF mealworm larvae had a total fat content of 20.45±3.67 g/100 g, whereas the FD mealworm larvae had total fat of 30.82±5.97 g/100 g; however, these differences were not significant (p > 0.05). This is consistent with other studies that reported that mealworms have a fat concentration of approximately 24% (Zielińska et al., 2015 and Azagoh et al., 2016). Ash content was minimal for both groups as well; 1.08±0.57 g/100 g and 2.17±1.51 g/100 g for FF mealworm larvae and FD mealworm larvae, respectively. These results were slightly lower than the findings of Zielińska et al. (2015) at 3.62%±0.6 and Zhao et al. (2016) at 4.9%±0.07.

The FF mealworms had 70.21±0.46 g/100 g moisture. Azagoh et al. (2016) reported mealworm moisture content at 68.8% ± 0.1; which is consistent with this present study. Freeze-drying the mealworms in the present study did not appear to remove all of the moisture, the concentration was confirmed at 14.14±2.83 g/100 g. When the freeze-dried mealworms were oven dried after freeze-drying, an additional 10.91% moisture was detected.

Lastly, In the current study, freeze-drying the mealworms created more ash when compared to the fresh, frozen whole mealworms, but these differences were not statistically significant.

The nutrient composition of insects will vary based on their diets, species, and stages of development (van Huis et al., 2013). For example, the larval stage of the mealworm is often studied for nutrient analysis, and even during the larval stage, there are differences in composition. Insects’ development is often defined by their instars or the stage of the insects between successive molts. Yu et al found that fat content is greatest in the pupal stage, but crude protein is higher in the early larval stage (9th to 10th instar) (2021). That same study also found that a higher feed utilization rate is seen with younger mealworm larvae (Yu et al, 2021). Larger mealworm larvae have been shown to contain more carotenoids than younger, smaller mealworm larvae, which was attributed to gene encoding that occurs during the late larval stage (Finke, 2015). Another study showed how the developmental stage of the insect can point to different bacteria within the insect’s gut microbiome (Yun et al., 2014). Even though this paper was published before human gut microbiome studies were very common, insect gut microbiota may be important to consider when analyzing insects for human food sources. Another study monitored major nutrition components in various developmental stages of
Tenebrio molitor and concluded that pupal and adult stages of T. molitor are not suitable sources of protein because amino acid concentrations decrease as the worms mature (Yu et al., 2021).

3.2 Amino Acid Profile Analysis

Mealworms contain eighteen different amino acids, eight of which are considered essential to humans (Ghaly & Alkoaik, 2009). For a reference point, the Food and Agriculture Organization of the United Nations has established a standard for adult maintenance of essential amino acid intake patterns (Table 2) (FAO, 2013).

Amino acid profiles were analyzed on both freeze-dried whole mealworms, and also on freeze-dried mealworm protein isolate after ISP. Tests were performed in duplicate, and means were reported for each amino acid (Table 2). Of the essential amino acids, histidine, phenylalanine + tyrosine, and threonine in whole, freeze-dried mealworms all met the Food and Agriculture Organization (FAO) requirements for adults. All the other essential amino acids met at least 50% of the required FAO recommendation. The two amino acids lowest in the whole mealworm samples were tryptophan (3 g/kg versus FAO requirement of 6 g/kg) and methionine + cysteine (6.7 + 5.6 g/kg versus FAO requirement of 22 g/kg). This is consistent with other studies (Azagoh et al., 2016; Zhao et al., 2016; Finke, 2002 & 2015; and Ravzanaadid et al., 2012). In contrast, Ruschioni et al. (2020), Yi et al. (2013), and Zielinska et al. (2015) found that methionine was not a limiting amino acid in Tenebrio molitor. The Ruschioni et al. study found that one of the most abundant essential amino acids was methionine at 7.62-8.36% of total amino acid composition (2020). One possible explanation for this may be that amino acid content can differ depending on which developmental phase mealworms are in at the time tested, with the larval phase being the highest (Yu et al., 2021). Azagoh et al. reported that the proteins found in mealworm larvae contained enough essential amino acids to meet the FAO and World Health Organization (WHO) requirements; however, they also reported a deficiency in methionine (2016). Another possible reason for some of the discrepancies among studies could be attributed to whether the insects were defatted before amino acid analysis. Kim et al. found that when insects were defatted, their amino acid makeup increased significantly, and in some cases, it increased over three times the original composition (2020). The mealworms in
this current study were not defatted before amino acid analysis. In addition, some studies utilized frozen (or freeze-dried) worms for amino acid analysis; however, others such as Ravzanaadi et al. (2012) and Zielińska et al. (2015) heated their samples before amino acid analysis. This is of noteworthy attention because heat can denature proteins. Methionine is a non-polar hydrophobic amino acid, and the heat that was applied to the mealworms in Ravzanaadi et al. and Zielińska et al.’s studies could have disturbed hydrogen bonds and these non-polar hydrophobic interactions allow for the denaturation of the proteins in the mealworms (Ophardt, 2003).

Even though a limitation of tryptophan was noted in this study, other studies examined indicated that this was not the case. Zhao et al. reported that the amount of tryptophan in defatted yellow mealworm larvae was two times as much as the FAO-recommended amount (2016). Zhang et al. observed that the total amino acid in mealworms was greater than or equal to the FAO and WHO requirements; however, in contrast with other studies, that research showed histidine was the limiting amino acid in mealworms (2019). Non-essential amino acids were also examined in this study, and many of the non-essential amino acids were also found in the mealworms (see Table 2). Overall, the whole, freeze-dried mealworms analyzed in this study contained a sum of 256 g/kg of essential amino acids. The FAO adult essential amino acid requirements total 277 g/kg (2013). Therefore, the results of this study indicate that yellow mealworms can meet 92.4% of the FAO adult essential amino acid requirements. It is also interesting to note that when insect protein is compared to other readily available protein types, yellow mealworms have a greater concentration of histidine, phenylalanine + tyrosine, and threonine amounts than soybean protein (Yi et al., 2013).

The comparison in amino acid compositions between whole freeze-dried mealworms and freeze-dried mealworm protein isolate is provided in Table 2. The only amino acid that did not show a statistically significant difference between whole freeze-dried mealworms and freeze-dried mealworm protein isolate was tryptophan; however, it is also worth noting that tryptophan was the essential amino acid with the lowest percentage in both whole freeze-dried form and freeze-dried protein isolate form. Yi et al performed a study in which insects were not defatted, and they found tryptophan to be 12 g/kg; however, the method by which they
measured tryptophan content was different from all other amino acids (2013). Instead of analyzing amino acids using ion exchange chromatography following standard ISO 13903:2005 for tryptophan, the study utilized reversed-phase C_{18} HPLC following standard ISO 13904:2005 (Yi et al, 2013). The study’s methods did not elaborate on why they utilized a different procedure for tryptophan; however, it does allow us to question their measurement when it was obtained differently than the other amino acids. In this study, both whole, freeze-dried and freeze-dried mealworm protein isolate samples found tryptophan to be a limiting amino acid for *Tenebrio molitor*.

3.3 Solubility Study of Protein and Protein Recovery via ISP

There are numerous ways to measure protein quality in potential food sources to enhance comprehension of their potential use in products. One of the ways that we can find out more about a new food source is to determine the solubility of the proteins in that food, in this case, in mealworms. This can be done through the use of isoelectric solubilization and precipitation (ISP), also referred to as the “pH-shift” method. If we change the pH of a protein sample, the charges on the surface of that protein will change. When mealworm proteins take on a charge (whether that charge is positive or negative), these proteins begin to initiate ionized interactions with water. When an acid (such as HCl) is added, the protein becomes positively charged. In contrast, when a base (such as NaOH) is added, the protein becomes negatively charged. In acidic conditions, there is a net positive charge; water and protein interact via weak hydrogen bonds. In basic conditions, protein-water interactions are maximized, and the proteins accumulate more water around them as they are more polar, and the proteins become soluble in water. On the other hand, if the number of positive charges equals the number of negative charges on a protein, the protein molecule has a net zero charge and loses its solubility. This is the protein’s isoelectric point (pI). When a protein is at its pI, the hydrophobic protein-to-protein connections are supported, and so the proteins display minimal solubility and will normally precipitate (Gehring et al., 2011).

A pH-shift process that utilizes these principles is used to concentrate functional proteins. For example, when protein solutions are shifted to extreme acid or base conditions, the protein will solubilize. When the solutions are then centrifuged, following homogenization,
three distinct layers will emerge (lipids, supernatant with soluble compounds, and insoluble compounds, such as insects’ legs and their chitinous exoskeletons). The supernatant is collected after the first centrifugation is returned to its pl, allowing for once-soluble proteins to precipitate after the second centrifugation (Brogan et al., 2021). Factors such as water-holding capacity (WHC), ability to form gels, texture, emulsifying ability, foaming capacity, color, and nutritional quality can be examined for optimal levels once these functional proteins are isolated from lipids, water, and other insoluble materials during the process of ISP (Purschke et al., 2018).

Solubility results are shown in Table 3 and Figure 2. The results of the solubility study consistently favored alkaline solutions over acidic solutions. The lowest solubility was found at a pH of 4 and the greatest was found at pH 12 for T. molitor; however, there was also greater solubility at pH 2 as well, creating a positive, or right-skewed inverted bell-shaped curve.

Various studies have assessed protein solubility in T. molitor, and despite some differences between the studies, all reported that the solubility increased in alkaline conditions and decreased in acidic conditions. Bußler et al. (2016) tested previously frozen, non-defatted insect flour. Proteins in that study showed the greatest solubility at pH 10, and the pl was around pH 4. Other studies concurred with these findings at pH values of 12.4, 11, 9, and 11 (Ravichandran et al., 2019; Yi et al., 2017; Zhao et al., 2016; & Zielińska, 2018). The lowest solubility and thus, the greatest protein precipitation amounts were also consistent among studies at acidic pH values of 4, 2-4, 4-5, 5, and 3-5 (Bußler et al., 2016; Yi et al., 2016; Zhao et al., 2016; Zielińska, 2018; & Azagoh, 2016).

Once the protein solubility study was completed, protein isolation and concentration were attempted using the pH-shift method based on optimum pH values obtained from the solubility study. Both the previously fresh, frozen mealworms and the freeze-dried mealworms were tested, and proximate analysis, amino acid profile analysis, SDS-PAGE, DSC, and mineral analysis were conducted on the recovered fraction.

3.4 Proximate Analysis on Isolate (MWPI)

The proximate compositions (dry matter basis) of Tenebrio molitor after ISP (fresh, frozen MWPI and freeze-dried MPWI) are shown in Table 1. The protein concentrations
between samples were only statistically different when comparing fresh, frozen mealworms to freeze-dried mealworms (p<0.05). It is important to note that even though statistical significance was not found when comparing fresh, frozen mealworms to freeze-dried MWPI, the lower percentage of protein in the MWPI could be explained by the results of the process of isoelectric solubilization and precipitation. The maximum solubility for fresh, frozen whole mealworms was 45.65% while the minimum solubility was 10.54%. Therefore, ISP should have been able to recover nearly half of the total protein from fresh, frozen whole mealworms. The other half that does not dissolve is not recoverable. It is most likely not a functional protein; and thus, of low value. *Tenebrio molitor* contains soluble proteins, structural/muscle proteins, and metabolic proteins (such as enzymes) (Barre et al., 2019). Some protein fractions pH-dissolve; other protein fractions do not. The proteins that do dissolve at pre-determined pH levels are valuable because they often have functional properties such as emulsification or foaming abilities and can form gels which are often used in commercial food products (Zielińska et al., 2018). The fractions that do not pH-dissolve can still be used, but are often utilized in lower-value applications, like animal feed or pet food (Stamer, 2015). Nutritional quality is typically assessed by utilizing the Protein Digestion Corrected for Amino Acid Score (PDCAAS). It is possible that these protein fractions that do not pH-dissolve would score lower on the PDCAAS scale than the proteins which do pH-dissolve.

All other protein comparisons were not statistically different even though fresh, frozen (in both whole and MWPI) had a greater protein concentration than freeze-dried samples from either sample (p>0.05). This can be attributed to the fact that freeze-drying the samples removed the majority of the moisture. So, the protein concentration in a drier sample was greater than in a “wetter” sample; however, all measurements were based on dry matter percentages. Similar results can be seen when comparing the dry matter fat percentages and ash percentages. There were no statistically significant differences in total fat in fresh, frozen mealworms or MWPI (either fresh, frozen or freeze-dried). In addition to pH-solubility affecting protein concentration, the amount of fat that a sample has can also affect the protein. According to Kim et al. (2020), defatting insect samples before extraction processes can increase protein yield and even improve protein quality. Additional studies have also shown
that defatting insect samples could decrease crude fat and increase crude protein (Choi et al., 2017; Ribeiro et al., 2019). In this study, insect samples were not defatted; however, in both whole fresh, frozen mealworms and fresh, frozen MWPI the concentration of fat in the freeze-dried samples was greater than it had been in the fresh, frozen samples. The freeze-dried samples have a more concentrated percentage of lipids when the moisture is removed from the sample.

The total ash content was similar with only fresh, frozen MWPI and freeze-dried MWPI showing a slight difference (p<0.05). The moisture percentages between the four different sample types showed the most variation. In three out of four comparisons, this difference was statistically significant, and the only comparison where a difference was not seen in moisture content is between fresh, frozen MWPI and freeze-dried MWPI (p>0.05).

3.5 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is utilized to assist in determining the different molecular weights of the proteins. The SDS-PAGE gel image showed varying results for fresh, frozen mealworm larvae; freeze-dried mealworm larvae; fresh, frozen MWPI; and freeze-dried MWPI. In fresh, frozen mealworm samples, apparent protein clusters were found in the ranges of 25-50kDa; and also at approximately 220kDa (Figure 3). We suspect that the bands seen at approximately 27kDa are indicative of cuticle proteins. These proteins are what make up insects’ exoskeletons and often work together with chitin (Andersen et al., 1995). The protein faction conveyed at ~41kDa is suspected to be arginine kinase, which is an enzyme seen in insects and often in crustaceans. Liu et al., identified arginine kinase in a study completed on Bombyx mori (silkworm) larvae as a major allergen (2009). The other band that showed up right above arginine kinase at ~42kDa was identified as actin, and the band at ~220kDa was determined to be a myosin-heavy chain. These two protein fractions often work together in muscle proteins. When examining the myofibril protein in T. molitor, Yi et al. found that the myosin-heavy chain presented a molecular weight of 262.3kDa (2016). These actin-myosin fibrils work together as a part of the actomyosin complex, and it can be observed that the myosin band is visible on the SDS-PAGE gel all the way across, from the fresh, frozen mealworm sample to the freeze-dried MWPI. Based on this SDS-
PAGE gel, it can be concluded that the actomyosin at ~200kDa is not degraded and in fact, can be isolated with ISP. It is also important to note that the bands that are visible in the fresh, frozen mealworm samples around 40-50kDa appeared to be similar to the freeze-dried mealworm sample as well as the freeze-dried MWPI. The cuticle proteins (~27kDa) also appeared to be seen in the freeze-dried MWPI sample lane. The fresh, frozen MWPI lane appears quite dark, almost as if the protein fractions in this sample were very saturated. Because ISP did remove much of the lipids, insoluble fractions (such as insects’ exoskeletons, legs, etc.), and supernatant/water from the sample, it does seem logical to assume that the protein fractions left in that pellet were so concentrated that they could not show up individually on the gel. This does become more complex when comparing that sample to the freeze-dried MWPI though as those lanes do appear clearer and seemed to show some of the fractions that could be seen in the fresh, frozen mealworm and freeze-dried mealworm samples. The functionality of proteins could be affected by either freeze-drying or by isolating proteins via ISP. One study by Yi et al. discusses how losing protein fractions over 75kDa could cause negative functional effects for some proteins (2013). In our SDS-PAGE gel, it can be noted that there appear to be few bands seen between ~50kDa and 250kDa except for one band around 150kDa. Lee et al. propose that bands above ~95kDa in protein pellet samples might be a vitellogenin-like protein that enhances the synthesis of melanin, and that protein has a molecular weight of around 160kDa (2000).

3.6 Differential Scanning Calorimetry (DSC)

Differential Scanning Calorimetry (DSC) is utilized to establish thermal denaturation, gelation, and the texture of the proteins within food products, respectively (Benjakul et al., 2004; Yongswatdigul & Park, 2004; Chen & Jaczynski, 2007a, b). The dips and spikes visible on DSC graphs are indicative of protein transitions that result from breaking the bonds that stabilize native protein structure; thus, protein denatures or transitions to a different state and structure. Also, protein transitions occur when new bonds are generated; therefore, protein coagulates or gels. Protein coagulation and gelation are examples of when proteins transition from one state to another. These transitions collectively represent endo- or exothermic reactions that happen when bonds in proteins are broken (denaturation) or generated
(coagulation and gelation). DSC is a valuable analysis because it can determine how novel proteins such as the insect proteins studied here behave in response to heat.

When comparing a freeze-dried whole mealworm sample to a freeze-dried MWPI sample, it can be noted that there was a marked upward trend in the curve at a temperature of around 75°C in the freeze-dried MWPI (Figure 4). According to Lee et al., there is a point between 60 and 70°C where the accumulation of protein extracted from mealworms increases (Lee et al., 2019). These higher temperatures can affect the structure of protein chains, and the proteins become less stable as hydrogen bonds within the molecule are broken. This reaction can be seen in Figure 4 where an endothermic peak was shown after the temperature was increased from approx. 75°C to almost 90°C. The net heat energy (represented as enthalpy, ΔH) needed to break those hydrogen bonds is signified by the area underneath this peak (Tahergorabi et al., 2012). For protein samples with a distinctly recognized relationship between their organization and arrangement, utilizing DSC can help with emerging food product creation and trends.

3.7 Mineral Analysis

In addition to quality protein, insects can also provide a good source of certain minerals necessary for daily consumption in the human diet. After a thorough review of various studies on mineral composition in T. molitor, the four minerals that are generally listed with the greatest concentration consistently are potassium, phosphorus, magnesium, and calcium (Finke, 2002; Finke, 2015; Ravzanaadid et al., 2012; Siemianowska et al., 2013). For further examination within this study, only those four minerals were analyzed in the samples. The fresh, frozen whole mealworm larvae mineral content was compared to that of the freeze-dried mealworm protein isolate obtained after isoelectric precipitation was completed. Potassium, phosphorus, and magnesium concentrations in fresh, frozen whole mealworms were significantly more concentrated than in freeze-dried MWPI (p<0.05). While the statistical difference for calcium was not significant (p<0.0570), it was relatively close to falling within that same significance as the other minerals examined in the study. (See Table 4) The calcium content of Tenebrio molitor (158.92±6.82 mg/kg) in this study was similar to both studies completed by Finke (2002; 2015). Finke found a calcium concentration of 169 mg/kg and 156
mg/kg respectively in his 2002 and 2015 publications. It must be noted that insects are generally not a rich source of calcium as they do not have skeletal systems; however, the calcium concentration may be increased if mealworms are fed higher-calcium diets (Hunt et al., 2001). Based on the recommended dietary allowance (RDA) for calcium, most adults need between 1,000-1,300 mg/day (Institute of Medicine, 2011). Relying on insect protein alone would prove to be quite difficult for human calcium requirements, and this is even more deficient when utilizing the calcium from freeze-dried MWPI.

Potassium is found in *T. molitor* in much greater concentrations than calcium. This study found a potassium concentration of 3,255.05±24.67 mg/kg in fresh, frozen whole mealworms, and this concentration is similar to other studies which looked at potassium content. Finke’s two studies showed 3,410 and 3,350 mg/kg (2002; 2015). Ravzanaadii et al. measured 9,479.73 mg/kg in their study (2012). Even after protein isolation via ISP and freeze-drying the sample in this study, there was still 1,868.39±31.11 mg/kg of potassium present. While that is less concentrated than fresh, frozen whole mealworms, it is still over thirty times more concentrated in the MWPI than calcium.

Phosphorus content within the samples in this study was less concentrated than in other studies examined (855.73±23.83 for fresh, frozen whole mealworms and 153.41±4.20 for freeze-dried MWPI). Other studies showed phosphorus concentrations at 2,850; 2,640; & 7,060.7 (Finke, 2002; Finke, 2015; Ravzanaadii et al., 2012) respectively. It is not completely clear why this study showed lower concentrations; however, there is a complex metabolic association between phosphorus in mealworms’ feed and the amount of phosphorus in the mealworms’ bodies when tested. While calcium in mealworm feed tends to increase calcium content in yellow mealworms, this is not the case with phosphorus, and Oonincx et al. demonstrated this in their study (2015). While the mealworms in our study were not given a special diet, Finke utilized a specialized diet with enhanced nutrient contents (Finke, 2015). Ravzanaadii et al. fed the mealworms in their study wheat bran with the addition of cabbage, radish, or carrots twice a week, and this could also have affected the mineral content of the mealworms (2012).
Magnesium is the last mineral to discuss; however, it is possibly the mineral within *T. molitor* that has the best chance of meeting human daily requirements. Magnesium ingested by eating mealworms could meet daily mineral requirements better than other protein sources as is shown in a study by Siemianowska et al. which measured fresh mealworms at 87.5±5.34 mg/100g and ground mealworms at 144.6±4.65 mg/100g (2013). This was compared to conventional protein sources of chicken (26 mg/100g), eggs (12 mg/100g), beef (26 mg/100g), pork (24 mg/100g), and rainbow trout (25 mg/100g) (Siemianowska et al., 2013). In our study, magnesium content was the most concentrated mineral (both in fresh, frozen whole mealworms and also in freeze-dried MWPI). Currently, the recommended daily allowance (RDA) for magnesium in adults is 400-420 mg/d for men and 310-320 mg/d for women (Institute of Medicine, 2011). Based on these figures, if a human were to ingest ~200 g of mealworms in a day, this would exceed the RDA (Selaledi & Mabelebele, 2021).

With all four minerals examined for this study, it was more advantageous to consider the fresh, frozen whole mealworm samples as opposed to the freeze-dried MWPI samples as the mineral concentrations are greater in the fresh, frozen whole mealworm samples. Further research could also be done to determine the bioavailability of these minerals within the samples.

### 3.8 Color Analysis (L,a,b)

One of the additional properties examined in this study was the color analysis of *T. molitor*. This study analyzed L*, a*, and b* values for fresh, frozen mealworm protein isolate, and freeze-dried mealworm protein isolate (see Table 5). Prior studies have indicated that different pH conditions may establish which colors are visible during spectrophotometry as oxidation of those colorants is possible as is protein accumulation (Atkinson et al., 1973). The fresh, frozen MWPI had a greater L* value than the freeze-dried MWPI; therefore, it was lighter, and the freeze-dried MWPI was darker. The a* value was greater in the freeze-dried MWPI, and therefore that sample was redder than the fresh, frozen MWPI. The b* value was greater in the fresh, frozen MWPI indicating a more yellow sample when compared to the freeze-dried MWPI. A 2020 study by Kim et al. examined color values for mealworms in three different states (ground, defatted, and extracted). The L*, a*, b* results of the extracted
*Tenebrio molitor* samples were different from our findings. The ground mealworm and defatted mealworm samples had a greater L* value than their extracted samples; however, the extracted samples had an L* value of 30.25±0.14, and this is less than our values of 51.76 ± 1.15 (fresh, frozen MWPI) and 48.92 ± 0.03 (freeze-dried MWPI) (Kim et al., 2020). Similar results can be seen for the a* and b* values as well. The Kim et al. study was completed and L*, a*, b* values were analyzed with extracted protein that was at a pH of 6.81±0.01. The present study’s extracted protein samples were at a pH reading of approximately 4.0. Because this is more acidic, it is possible that conditions affected the color changes and essentially L*, a*, b* values. Color changes are significant when considering future processing or utilization of insects for food as these color values could be utilized as guides for the insects’ mechanical properties (Kim et al., 2020).

### 4.0 Conclusions

This study examined the essential properties of the proteins in *Tenebrio molitor* in several different formats. Looking further into the nutritional content and properties of these proteins (both in their whole format and in proteins that had been isolated via ISP) enabled us to determine the value of isolating the proteins from their whole forms. This analysis also examined proteins after they were freeze-dried to determine whether this freeze-drying treatment affected the proteins' nutritional quality and functional properties. The greatest protein solubility was found at pH 12 for *T. molitor*. By detecting the solubility of proteins, we can provide information on mealworm protein functionality (e.g.: texture, fat- and water-binding abilities, or gelation capabilities). The pH-shift protein recovery process was completed by first increasing the pH to 12, separating insoluble insect parts and lipids, and then decreasing the pH to an acidic value of ~ 4.0 to precipitate the mealworm protein for further analysis. Protein concentration was greatest in fresh, frozen whole mealworms and freeze-drying the mealworms did decrease the crude protein concentration significantly. While the protein isolate samples did have a lower crude protein percentage than the fresh, frozen samples, the difference was not significant. *Tenebrio molitor* contains all essential amino acids; however, tryptophan appears to be the limiting amino acid. Whole freeze-dried mealworms had a much greater concentration of amino acids (both essential and non-essential) when compared to
freeze-dried MWPI. While tryptophan is a limiting amino acid in *T. molitor*, the concentration of this amino acid in the protein isolate was the only one that was not statistically significant in its comparison to whole protein. SDS-PAGE gels revealed that actin, myosin, arginine kinase, and cuticle proteins could be seen in fresh, frozen whole mealworm samples and freeze-dried MWPI. In the freeze-dried whole mealworm samples and fresh, frozen MWPI samples, the bands were not as prominent; however, the actomyosin seen at ~200kDa was seen across the gel and was not degraded. It was isolated effectively with ISP. This would be useful when generating information about proteins’ molecular weights and possible yield percentage or quantity. DSC revealed that the freeze-dried MWPI had a definitive upward curve in its temperature and heat flow curve around 75°C. This signifies a greater transition to different protein states in the freeze-dried MPWI. Proteins can coagulate or gel as they transition. So, knowing when this point occurs is instrumental when working with these proteins, especially if considering future food product development. *Tenebrio molitor* is not a significant source of calcium; however, mealworms are a good source of potassium, phosphorus, and magnesium. The freeze-dried MWPI did however have a much lower concentration of these minerals than the fresh, frozen mealworms. Color analysis revealed that fresh, frozen MWPI was lighter, greener, and more yellow than freeze-dried MWPI which was blacker, redder, and bluer. pH can affect the *L*, *a*, *b* values within samples, and these color designations can give further insight into the additional mechanical properties of *T. molitor*.

If insects such as *Tenebrio molitor* can provide an alternative protein option for those who are protein malnourished while possibly mitigating environmental impacts, it is imperative to further investigate the potential of insect proteins. There are additional considerations to study as well. Both mealworm allergenic components (e.g. chitin) and microbial safety concerns must be addressed to ensure that mealworms can make a suitable protein alternative for human consumption. It may be recommended that further research be completed to address some of these topics before providing a general endorsement to add insect protein to human diets.
References


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45.) Tahergorabi R, Sivanandan L, Jaczynski J. Dynamic Rheology and endothermic transitions of proteins recovered from chicken-meat processing by-products using isoelectric


Table 1: Proximate composition of *Tenebrio molitor*; Values are reported as means ± SD (dry matter weights).

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Whole Mealworm (MW)</th>
<th>Protein Isolates (PI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sample</td>
<td>g/100g</td>
</tr>
<tr>
<td>Crude Protein</td>
<td>Fresh MW</td>
<td>75.30±0.02&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Freeze-dried MW</td>
<td>66.33±0.02&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total Fat</td>
<td>Fresh MW</td>
<td>20.45±0.03</td>
</tr>
<tr>
<td></td>
<td>Freeze-dried MW</td>
<td>30.82±0.03</td>
</tr>
<tr>
<td>Total Ash</td>
<td>Fresh MW</td>
<td>1.08±0.01</td>
</tr>
<tr>
<td></td>
<td>Freeze-dried MW</td>
<td>2.17±0.01</td>
</tr>
<tr>
<td>Moisture</td>
<td>Fresh MW</td>
<td>70.21±0.01&lt;sup&gt;Ab&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Freeze-dried MW</td>
<td>14.14±0.01&lt;sup&gt;Bb&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>A, B</sup> Different letters within a fraction in a column indicate significant differences (Tukey’s HSD, p<0.05).

<sup>a, b</sup> Different letters within a row indicate significant differences (Tukey's HSD, p<0.05).
<table>
<thead>
<tr>
<th>Essential Amino Acids (EAA):</th>
<th>Yi et al, 2013(^1)</th>
<th>DuVall, 2022 Whole FDMW</th>
<th>DuVall, 2022 FD MWPI</th>
<th>Adult FAO, 2013(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histidine</td>
<td>29.0</td>
<td>19.0±0.36</td>
<td>5.0±0.36</td>
<td>15</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>43.0</td>
<td>26.3±0.63</td>
<td>9.8±0.63</td>
<td>30</td>
</tr>
<tr>
<td>Leucine</td>
<td>73.0</td>
<td>40.7±0.64</td>
<td>16.5±0.64</td>
<td>59</td>
</tr>
<tr>
<td>Lysine</td>
<td>54.0</td>
<td>35.4±0.33</td>
<td>13.1±0.33</td>
<td>45</td>
</tr>
<tr>
<td>Met +</td>
<td>6.7±0.16</td>
<td></td>
<td>2.8±0.16</td>
<td></td>
</tr>
<tr>
<td>Cys (non-ess)</td>
<td>26.0</td>
<td>5.6±0.05</td>
<td>1.7±0.05</td>
<td>22</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>18.5±0.30</td>
<td></td>
<td>8.3±0.30</td>
<td></td>
</tr>
<tr>
<td>Tyr +</td>
<td>100.0</td>
<td>38.9±0.46</td>
<td>13.7±0.46</td>
<td>38</td>
</tr>
<tr>
<td>Thr (non-ess)</td>
<td>39.0</td>
<td>23.1±0.47</td>
<td>8.5±0.47</td>
<td>23</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>12.0</td>
<td>3.0±0.16</td>
<td>2.2±0.16</td>
<td>6</td>
</tr>
<tr>
<td>Valine</td>
<td>61.0</td>
<td>38.8±0.81</td>
<td>11.7±0.81</td>
<td>39</td>
</tr>
<tr>
<td><strong>Sum of EAA</strong></td>
<td>437.0</td>
<td>256±4.37</td>
<td>93.3±4.37</td>
<td>277</td>
</tr>
<tr>
<td>Non-Essential Amino Acids (NEAA):</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>70.0</td>
<td>45.9±0.59</td>
<td>13.7±0.59</td>
<td>---</td>
</tr>
<tr>
<td>Arginine</td>
<td>54.0</td>
<td>31.1±0.47</td>
<td>10.5±0.47</td>
<td>---</td>
</tr>
<tr>
<td>Asparagine</td>
<td>80.0</td>
<td>42.5±0.83</td>
<td>18.3±0.83</td>
<td>---</td>
</tr>
<tr>
<td>Glutamine</td>
<td>109.0</td>
<td>56.2±0.39</td>
<td>24.4±0.39</td>
<td>---</td>
</tr>
<tr>
<td>Glycine</td>
<td>50.0</td>
<td>30.7±0.35</td>
<td>10.0±0.35</td>
<td>---</td>
</tr>
<tr>
<td>Proline</td>
<td>66.0</td>
<td>40.3±0.57</td>
<td>9.1±0.57</td>
<td>---</td>
</tr>
<tr>
<td>Serine</td>
<td>44.0</td>
<td>22.8±0.54</td>
<td>8.3±0.54</td>
<td>---</td>
</tr>
<tr>
<td><strong>Sum of Total AA</strong></td>
<td>910</td>
<td>524.9±8.11</td>
<td>187.6±8.11</td>
<td>---</td>
</tr>
</tbody>
</table>

\(^1\)Yi et al., 2013

\(^2\)Food and Agriculture Organization of the United Nations, 2013

**Bold italic** type indicates that there were no significant differences (ANOVA, pooled t-test; p<0.05) between whole FDMW and FD MWPI (±SE, n=2) within the same row.
**Table 3:** Protein solubility of *Tenebrio molitor* (mealworm) as is in distilled, deionized water pH adjusted to pH2-12 with HCl and NaOH.

<table>
<thead>
<tr>
<th>pH</th>
<th>Mealworm Solubility (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>42.21</td>
</tr>
<tr>
<td>4</td>
<td>10.54</td>
</tr>
<tr>
<td>6</td>
<td>16.84</td>
</tr>
<tr>
<td>8</td>
<td>24.06</td>
</tr>
<tr>
<td>10</td>
<td>37.32</td>
</tr>
<tr>
<td>12</td>
<td>45.65</td>
</tr>
</tbody>
</table>

**Table 4:** Mineral Analysis of *Tenebrio molitor*; Values (mg/kg sample) are reported as means ± SD.

<table>
<thead>
<tr>
<th>Mineral</th>
<th>Mealworm</th>
<th>Mealworm Protein Isolate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium</td>
<td>158.92±6.82</td>
<td>58.64±34.73</td>
</tr>
<tr>
<td>Potassium</td>
<td>3,255.05± 24.67\textsuperscript{A}</td>
<td>1,868.39± 31.11\textsuperscript{B}</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>855.73±23.83\textsuperscript{A}</td>
<td>153.41±4.20\textsuperscript{B}</td>
</tr>
<tr>
<td>Magnesium</td>
<td>3,314.55± 5.00\textsuperscript{A}</td>
<td>2,849.05± 40.13\textsuperscript{B}</td>
</tr>
</tbody>
</table>

\textsuperscript{A,B} ... Different letters indicated significant differences (ANOVA, pooled t-test; \( p < 0.05 \)).

**Table 5:** Color Analysis of *Tenebrio molitor* (*fresh, frozen MWPI and freeze-dried MWPI*); Values are reported as qualitative measurements and observations without statistical analysis.

<table>
<thead>
<tr>
<th>Sample</th>
<th>L</th>
<th>a</th>
<th>b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mealworm</td>
<td>51.76 ± 1.15</td>
<td>2.55 ± 1.11</td>
<td>7.08 ± 0.67</td>
</tr>
<tr>
<td>Mealworm Protein Isolate</td>
<td>48.92 ± 0.03</td>
<td>2.87 ± 1.14</td>
<td>6.63 ± 1.02</td>
</tr>
</tbody>
</table>
Figure 1: Isoelectric Solubilization/Precipitation (ISP) Procedures Workflow

1. T. molitor sample
2. 1:10 (Mealworm: Distilled Water) dilution
3. Homogenization
4. Isoelectric Solubilization w/ NaOH (pH value Approx. 12; highest solubility)
5. Centrifugation at 5,000 rpm at 4°C for 20 min
   - Bottom layer: Insoluble insect parts
   - Middle layer: Soluble proteins
   - Top layer: Lipids
6. Collected & Stored
7. Filter through cheesecloth/filter paper
8. Isoelectric Precipitation w/ HCl (pH value Approx. 4; lowest solubility)
9. Centrifugation at 5,000 rpm at 4°C for 20 min
   - Pellet: Mealworm Protein
   - Supernatant: Water
10. Tested further...
Figure 2: Protein solubility* of *Tenebrio molitor* (mealworm) as a function of pH. Protein solubility is expressed as the % soluble protein of crude protein.

*Data are given as mean values (n=3).
Figure 3: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) Tris-HCl gel identification of major protein fractions using literature on *Tenebrio molitor* (mealworm) as is with protein concentrated at 8 mg/mL with a BSA 10-250 kDa reference protein.
**Figure 4:** Differential scanning calorimetry (DSC) thermograms of freeze-dried whole mealworms and mealworm protein isolate.
During phase one of this study, 1,350 mealworms were utilized. During the second phase, 3,424 were used to attempt to obtain a larger yield for testing after the growth study.

There were approximately 112-113 mealworms placed in each container (phase 1), and 285-286 were placed in each container for phase two.

Using small pieces of wet paper towels placed in the incubators twice per week. In instances where humidity levels were above average, paper towels were removed, and the moisture from the apple pomace within the incubators was high enough to keep the humidity at optimal levels.

There were times when researchers had to go into the lab to obtain materials, and lights were turned on momentarily for this purpose. There were no efforts to maintain 100% darkness in the room; however, a sign was posted asking other researchers to turn off the artificial lighting as soon as they were finished in the room.

All mealworms were fed ad libitum (boxes 1,2,5,6,9; & 10) were given commercial mealworm bedding/chow which contained the following ingredients: wheat bran, corn flour, soybean flour, Brewer’s Yeast, bone meal, and multivitamins (Wormy Worms Premium Mealworm Superworm Bedding Chow, USA).

Boxes 3,4,7,8,11; & 12 were given apple pomace ad libitum.