

Evaluation of skin beetles (Coleoptera: Dermestidae) as model organisms for nutritional
bioavailability studies of companion animal foods

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Abstract

It has been shown over recent years that pet owners are becoming more aware of the influence that their pet's nutrition can play on the pet's life expectancy, wellness, and energy. Consequently, pet owners have been pushing these values onto the pet food industry, influencing the formulations of diets to incorporate novel ingredients, with better balanced nutrition, and more fortified for specific medical conditions or ages. All pet food companies must follow the guidelines of the Association of American Feed Control Officials (AAFCO) as have been adopted by local authorities and in partnership are enforced by the United States Food and Drug Administration (FDA) to certify the safety of food products, supplements, and veterinary products. The FDA's Federal Food, Drug, and Cosmetic Act ensures proper manufacturing, safety, and labeling of companion animal feeds. With more companies targeting pet owners with scientific claims for health benefits, more research is being conducted for pet foods that must be overseen by the Institutional Animal Care and Use Committee (IACUC) when taking place in university laboratories. But with the many restrictions implemented by IACUC, it has been difficult to fulfill these research demands. However, IACUC only places emphasis on research conducted using vertebrates or cephalopods, creating an opportunity for insects to become potential model organisms for pet food research.

As a result, in the first study, three different species of beetles (Coleoptera: Dermestidae) were investigated in this research: *Trogoderma variabile* (Ballion), *Trogoderma inclusum* (LeConte), and *Dermestes maculatus* (DeGeer). The larvae of all three species were fed a balanced pet food diet to determine their protein efficiency ratios (PERs) and either placed in an incubator or a countertop for six days. The results demonstrated that *D. maculatus* larvae were the most efficient at converting ingested protein into body weight gain when placed in the

incubator, with an average PER value of 1.439. Unfortunately, the molting of some larvae interfered with the body weights recorded before and after feeding. A second experiment was conducted using only *D. maculatus* larvae with a shorter time window of two days to prevent the time necessary to molt. Although there were fewer moltings, they still occurred, so another time window of 24 hours was tested and was found to be successful in preventing the larvae from molting and yielding an improved average PER value of 2.476.

In the second study, experiments were implemented to determine if *D. maculatus* larvae could detect the quality of different protein sources in prepared companion animal diets through the standard PER assessment. Seven different diets were tested, including a commercially available balanced pet food diet, one lacking protein (negative control), spray dried egg (a positive control known to have balanced amino acids for mammal companion animals), soy protein concentrate, corn gluten meal, fish meal, and pea protein concentrate. The highest average PER recorded came from the spray dried egg diet at 6.455, followed by fishmeal at 6.238. The two diets were not significantly different in ranking, possibly attributed to fish being a known food of choice for *D. maculatus*. The next highest PER values were soy protein concentrate > pea protein concentrate > balanced pet food diet, corn gluten meal > no protein. These results are supported by the general amino acid makeup of the ingredients, all known to have a different limiting amino acid and protein quality.

The results of both studies have shown similar outcomes to previous PER studies of companion animal feeds, suggesting that *D. maculatus* has potential as a model organism for pet food nutritional bioavailability studies in the future. Future experiments needed to support these findings would be to test prepared diets at various protein percentages, 0%, 5%, 10%, 15%, and 20%, to demonstrate the larvae's efficiency at utilizing the protein sources for growth, resulting

in higher PERs from higher protein percentage diets. Further studies should be conducted in determining the beetles' essential amino acid profiles to verify that they are similar in order of limiting factor to those of the companion animals that they would model for.

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**Chapter 1 - An Introduction to the Regulations, Development, and
Biology of Pet Foods**

Companion Animal Nutrition

One of the best ways for an owner to improve an animal's well-being is through proper balanced nutrition to prevent diseases, cancers, and health symptoms. Though primarily credited with regulating companion animal feeds, the Association of American Feed Control Officials (AAFCO), a private non-profit organization, standardizes commercial livestock feeds in order to guarantee "complete and balanced" diets (Beckman 2023). Commercial pet foods tend to cost more than commercial livestock feeds per pound. Unlike the livestock industry, which primarily uses raw or minimally processed ingredients such as hay, wheat, barley, oats, and corn, companion animal feeds are primarily formulated around the use of byproducts from consumer demands leading to excessive amounts of unnecessary nutrients (Swanson et al. 2013). Companion animal feeds require a nutritional adequacy statement, guaranteeing that it is "complete and balanced," either by meeting regulations of AAFCO nutrient profiles or by undergoing a feeding trial using AAFCO procedures (*Complete* 2020). Livestock feeds do not require adequacy statements like pet foods but are recommended to include a purpose statement for the feed that identifies the specific species and proper classes for the animal feed (AAFCO 2018). With the use of plain and unaltered ingredients, livestock feeds are commonly mixed together or formulated on site before use whereas companion animal feeds are more likely to be commercially processed for consumers. Below is an overview of current methods to study and evaluate nutrition of pet foods, followed by information on the potential for using insects to evaluate pet food nutrients.

Regulations and expectations for pet foods in the USA

The United States Food and Drug Administration (FDA) is a federal entity responsible for protecting the health of the public by certifying the safety and efficacy of food products,

drugs, supplements, cosmetics, medical devices, and veterinary products. Since its establishment in 1938, the FDA has regulated the safety, proper manufacturing, and adequate labeling of animal feeds, including pet foods, via the Federal Food, Drug, and Cosmetic Act (*FDA's 2022*). The FDA collaborates through partnership with AAFCO at state, national, and international levels to ensure proper law regulation enforcement regarding production, labeling, distribution, use, and sale of animal feed and feed ingredients (*Memorandum 2019*).

AAFCO was established in 1906 and its membership is comprised of state, dominion, or other governmental agencies of North America that enforce animal feed regulations, address issues in administering feed laws, create definitions and policies, and develop just and equitable standards (*Role 2023*). AAFCO is commonly compared to other private, non-profits, such as the European Pet Food Industry Federation (FEDIAF) and the National Research Council (NRC) with their strict guideline requirements and references based upon the European Union regulations (Dodd et al. 2021, Kazimierska et al. 2021, Zafalon et al. 2020). All required micronutrients, crude protein, and crude fat for companion animals have a minimum quota for caloric intake, while crude fiber and moisture have a maximum to prevent diluted calories and rancidity.

Food quality and quantity

Ingredients are often categorized into predominant constituent areas. For instance, potatoes and cereals are often considered carbohydrates due to their predominance of starch, and ingredients like butter and soybean oils are considered fats due to their predominance of lipids. Likewise, ingredients such as meat and bone meal, soybean meal, and corn gluten meal are considered “proteins” because they contain a predominance of crude protein. The level of crude protein or amino acids in an ingredient does not completely tell the entire story about the

protein's quality. The protein's quality is dependent upon its ability to be readily digestible and have bioavailability within the animal's digestive system to meet the animal's essential amino acid requirements. Three of the nine essential amino acids for a majority of mammals (dogs having ten with the addition of arginine and cats having eleven with the further addition of taurine) are recognized as branched chain amino acids (BCAAs). BCAAs are almost exclusively provided through animal-based protein sources due to a mammal's inability to synthesize them (Li and Wu 2023). Until 1973, the standard reference for amino acid scoring was egg proteins that are high in sulfur amino acids compared to others and the World Health Organizations (WHO) protein assumptions determine that this reference was inadequate (Mariotti 2017, WHO 1985). In 1989, the reference was further defined by the WHO and FAO that an adequate protein quality could be determined by expressing the content of the first limiting essential amino acid in a known reference protein (Schaafsma 2005). Protein quality is most often compared to the determined values of casein, although others such as albumin from eggs, milk, or certain meat sources also are acceptable as standards known to possess high quality (Mansilla et al. 2020, Hoffman and Falvo 2004, Brody 1999). Chapman et al. (1959) were first to suggest casein as a reference protein due to its common use as a standard for research. Casein was recognized by the Animal Nutrition Research Council, making it easily accessible and consistent for purposes of testing. Overall, there are still many different relationships between all the assorted amino acids functions that may influence the efficacy of a protein source under varying conditions, such as the efficiency of metabolites (Mariotti 2017). Animals are reared in different environmental conditions, develop at different rates, and the variation of nutrient utilizations may affect their overall amino acid requirements. The species used as a model may differ to aid research in maximizing animal health and quality products. Despite these differences, the order of their

essentiality may be somewhat consistent given the similarity amongst species for the basic functions of protein assimilation (Hou and Wu 2018).

Similarly, the quantity of protein intake plays just as much of a role in pet nutrition as the quality of the protein, both the overall amount of protein and the relative ratios of different amino acids within the food sources. The inclusion of plant-based proteins has long been debated among veterinarians, animal scientists, and animal nutritionists as beneficial for pets (Clark et al. 2023). Plant-based protein sources are more abundant and cheaper to implement than animal-based proteins, favoring a higher plant-based protein to animal-based protein ratio. Utilizing differing protein sources also aids in balancing different amino acid bioavailabilities from the sources varying amino acid compositions. Inclusion of plant-based proteins in the pet food industry has been facilitated by supplementation with vitamin-mineral mixes, isolated amino acids, or protein supplements to meet essential amino acid requirements (Pedrinelli et al. 2021, Amato et al. 1974, Schaafsma 2005). More recent studies have demonstrated the effects of protein sources on the pets' gut microbiome and thus their gut health and ability to digest their foods, likely to cause implementation of newer standards or higher expectations from consumers in the future (Sieja et al. 2023, Clark et al. 2023). However, there are disadvantages for plant-based protein digestibility including the presence of trypsin inhibitors, tannins, and lectins (Reilly et al. 2020). Quality still likely plays a higher role in consumer decisions as pet food companies often humanize pets and guarantee the freshest ingredients.

Humanization of pets

Humanization, sometimes known as anthropomorphosis, is attributing one's own human values, beliefs, and feelings onto non-human animals, often pets. The popularity of owning a pet has been amplified in recent years by younger consumers in response to increased costs of living,

leading to deferred child-rearing and deep attachment to their animals. This was most evident during the SARS-CoV-2 pandemic of 2019 which fueled the growth of the pet food industries (White 2022, Kumcu and Woolverton 2015). The pet food industry was estimated to be worth over \$42 billion in the United States in 2022, having a large growth from 2014 when the entirety of consumer pet spending was \$58 billion and include clothing, toys, food, treats, and supplements (Kumcu and Woolverton 2015, Schleicher 2019). The first noticeable shift to increased pet food sales began in the mid-1970's when certain pet food companies introduced "super premium" brands claiming better nutrition and health benefits at higher prices and partnering with educated veterinarians, breeders, pet stores, and kennels to sell to consumers (White 2022). However, some companies have tried to capitalize on humanization and have used reviews from human health studies to claim health outcomes for dogs or other companion animals leading to misinformation and potential toxicity. For example, reports that too much green tea extract might be toxic led to distrust by pet owners which in turn has led companies to demand more transparency and food safety (Clemens 2014, Kumcu and Woolverton 2015). Some of the highest priorities for current pet owners is to ensure premium high protein content and promotion of their pet's health, energy, life expectancy, resistance to diseases such as diabetes, and lifestyle needs (Clemens 2014, Kumcu and Woolverton 2015). All of these humanization influences have raised the stakes for commercial pet food companies to satisfy their clientele and address the needs of their pets as a primary consumer.

Protein Sources

Animal based vs plant based

It has long been debated which mixed protein sources are ideal for commercial animal feed products, as it is known that animal and plant sources vary in their amino acid

bioavailabilities. Proteins must balance amino acid requirements for animals to meet the nutritional needs of the primary limiting essential amino acid without exceeding the needs of the other amino acids. A primary limiting essential amino acid is the first amino acid that is lacking in quantity from a protein source or protein mixture to meet an organism's nutritional amino acid requirements for yield or growth (Schaafsma 2005). A formulation of a mixture of amino acids that are equally limiting for an organism with a minimal excess of nitrogen would be a model protein source for that organism to provide optimal nutrition (Boisen et al. 1999). Within the agricultural production industry, all animals that are mass-produced are mostly mammals, and thus mammals have been the most studied animals for their essential amino acid requirements. Studies have shown that mammals' most common limiting amino acid is lysine, especially in non-ruminants, followed by methionine, then sometimes threonine or tryptophan (Boisen et al. 1999; Rosenberg 1957). However, due to the formulations of mixed feeds, lysine or methionine could be the limiting amino acid if one is overprioritized over the other and given an excess supplementation.

Another consideration in contemplating the feed formulation and nutrition of pet foods is the possibility of nutrient bioavailability loss due to processing. It is well known that heat treatments can denature proteins and lower their bioavailability through Maillard products or oxidation, and both lysine and methionine are the most likely to be lost to such processes (Mariotti 2017, Boisen et al. 1999, Elango 2020). With mixed protein source contents, however, there is no primary limiting amino acid because they balance each other since the goal has been met to have complimentary essential limiting amino acids (Rao et al. 1968). These are some of the many reasons that the most commonly limiting amino acids are commercially available for supplementation within a feed to meet the nutritional requirements. To be an optimal diet,

supplementation must not provide more of the limiting amino acid than to balance with the second limiting amino acid where they become co-limiting (Rosenberg 1957).

Eggs: the “ideal animal source” for mammals

When conducting experiments to compare protein sources, a standardized reference is needed to juxtapose the other proteins being tested. A reference protein is the theoretically perfect protein that gets used at 100% efficiency taking advantage of all its amino acids without a limiting amino acid regardless of quantity (Bender 2006). The two best protein sources for animal nutrition are egg and milk proteins (albumin, whites, whey, and casein protein) known to support young animals with nutritionally adequate protein, carbohydrates, lipids, vitamins, and minerals with 90%+ efficiency (Bender 2006, Fevold 1951, Smith 1980, Hoffman and Falvo 2004). Although milk is a common protein source for mammalian livestock and pets, the most common animals targeted for nutritional studies, all animals begin their earliest development in an egg, making it the universal juvenile animal protein source. For this reason, chicken eggs have been considered the reference protein sources for these studies.

Plants proteins often lacking in sulfur-based proteins or lysine

Plant proteins used for commercial feeds fall into three broad categories: fresh vegetable, cereal, and legume proteins. The fresh vegetable and cereal proteins most commonly lack methionine, lysine, and threonine while legumes most commonly lack sulfur-based amino acids (methionine and cysteine) (Fevold 1951, Schaafsma 2005, Mariotti 2017, Zafalon et al. 2020). The lack of these amino acids can be further exaggerated from the heat used to sterilize the food particles before mixed into commercial food products (Donadelli et al. 2018). Variations in the amino acid composition of fresh vegetable, cereal, and legume proteins may complement one another when mixed to lower deficiencies and create a more complete protein source (Mariotti

2017). A study carried out by Reilly et al. in 2020 showed that adult dogs were reliably limited most by methionine when fed various pulses, a subfamily of legumes, by comparing the digestible indispensable amino acid scores and further supporting the previous findings. If plant protein sources lacking methionine are not fed in larger quantities, animal-based proteins will be needed in the diets.

Carbon and water footprints value plant-based proteins

While the pet food industry has become highly influenced by humanization of companion animals, awareness of the public's perception of a company's standing on various business, political, and environmental issues has been more scrutinized. More consumers aim to minimize their pet's food influence on the environment while still maximizing benefits to their companion animals. Both carbon and water footprints from the burning of fossil fuels and industrial overuse of fresh water, respectively play large roles in the environmental impacts of protein sources. With both cats and dogs, the most common companion animals, preferences for animal protein sources may influence their carbon and water footprints. Cattle and other ruminants are the most environmentally intensive culprits of general livestock due to the carbon dioxide from their digestions and manure degradation and also the methane and other greenhouse gases produced by ruminants (Nijam et al. 2012, Flachowsky et al. 2018). The ranking of carbon footprint intensity within livestock generally is listed as cattle at the highest, then swine, poultry, cow's milk, chicken eggs, seafood farming, and the lowest are vegetable sources (e.g. meat substitutes) (Nijdam et al. 2012). However, with modern machinery and farming practices, yields from livestock have greatly increased with a reduction in carbon footprint, although some protein sources are still as much as 100 times more eco-friendly than others both due to the animals within the system and various production practices (Nijdam et al. 2012). As anticipated,

livestock productions have higher carbon footprints not only as previously discussed, but also because of their feed and transport. The feeds of livestock can vary largely on the carbon footprint scale from 4 to well over 600 $\frac{\text{kg CO}_2\text{-equivalents of all greenhouse gases}}{\text{kg protein produced}}$ (Nijdam et al. 2012).

One of the overlooked aspects of a crop's carbon footprint is soil sequestration, which can be amplified in organic crops, yet they still have room for improvement. However, there is no guarantee that a smaller footprint can reduce environmental impact because of other factors including transportation, fertilization, and the types of fuel used (Knudsen 2013). With such large variations in the carbon footprint, prices of protein sources vary greatly, thus in return influencing the pricing and formulations of commercial pet food feeds.

The water footprint also influences availability of differing feed sources, especially as different parts of the world are experiencing large yield losses in response to climate change. The water footprint can be divided into three different sections: green water that naturally seeps into the soil, blue water that flows through rivers and aquifers, and grey water that is used to assimilate the load of pollutants (Mekonnen and Hoekstra 2010, Flachowski et al. 2018). One of the biggest losses related to the water footprint is from unused food waste (Flachowski et al. 2018). In comparison to feed production, animal management occupies a trivial amount (2%) in the livestock water footprint (Flachowski et al. 2018). Livestock water footprints mirror carbon footprints, with the highest in cattle and lowest in poultry (Flachowski et al. 2018). Up to 25% of the global blue water footprint would be reduced by lowering the water use on five crops (wheat, rice, cotton, fodder, and sugarcane), including excessively evaporated water from over watering (Mekonnen and Hoekstra 2020). Three other heavy crops include maize, soybean, and rapeseed, further drain aquifers around the world at an unstable rate that cannot be replenished, depleting renewable water sources (Mekonnen and Hoekstra 2020). The United States is a prime example

of overusing the aquifers and river basins available, accounting for 12% of the global unsustainable blue water footprint and increasing the nations' water scarcity (Mekonnen and Hoekstra 2020). To overcome these barriers, it is essential to improve watering practices, increase crop yields, and improve drought sensitivities. The water footprint is often overshadowed by the carbon footprint of differing food sources. Nonetheless, it is imperative to realize that they complement each other, especially when crops absorb carbon and animals require less water.

Nutritional Protein in Pet Foods

The protein efficiency ratio

In a study reported by Osborne et al. in 1919, a new protocol, known as the protein efficiency ratio (PER), was created to determine the best protein source for animal growth efficiency. The underlying principle for the PER method was that immature animals will consume food in proportion to their growth, and thus intake is diminished if their growth is limited. If multiple diets have an equal protein percentage, not all animals will grow at the same rate, likely attributed to genetics, other underlying health factors, or nutritional composition of the diet. When studying in a fixed timeframe, in the case of Osborne et al. (1919), albino rats grew slower when they were restricted to a suboptimal quantity of protein relative to their body weight and reported that the rats grew slower than normal. To test these findings the amount of protein ingested was determined based on the nitrogen content of the diets. Protein was the sole limiting nutrient for growth. The limiting protein intake slowed growth sufficiently to allow comparisons and separate the overall protein quality due to the growth. Thus, PER demonstrates limitations when an animal ingests copious amounts of food but has minimal gain. This causes the PER value to approach infinity from a larger numerator; however, this error should be

minimal when comparing proteins of significant nutritional values. The PER equation is as

$$\text{follows: PER} = \frac{\text{body weight gain (g)}}{\text{protein consumption (g)}}$$

The PER method was the first widely used bioassay to determine proper protein digestion and quality based on growth under a predetermined time window (Chapman et al. 1959, Schaafsma 2005, Brody 1999). The PER results are all relative rather than absolute values and gives equal importance to the “control” and test groups (Steinke 1977). There are numerous variables that can influence a PER value, including moisture, differences in particle sizes, overall fat content, or various ingredients that give the diet a high mineral content (Donadelli et al. 2018). Cramer et al. (2007) reported that ash content had a negative correlation with PER and, potentially, the total essential amino acid digestibility; however, the latter may be due to differing ash content varying based on the diets being either raw or rendered by-products. The ash effects affected the amino acid digestibility presumably because of the processing procedures degrading the amino acids.

There are many disadvantages in using PER for analysis of food nutrition. Steinke (1977) divided the shortcomings of PER into six general categories of life quality of an animal. These categories include: the animal’s stress levels, genetics, and overall health; consideration of the acclimation periods such that the longer they become acclimated, the higher the PER values; a diet’s potential instability (e.g., higher fat content can lead to rancidity or lowering palatability over time); missing nutritional balance (e.g., the vitamins and minerals need to be higher than the ash content which is not always the case in study diets); the diet’s water content, which can vary by diet and influence rancidity, palatability, or hardness/pain on teeth; and the diet needing to be as homogenous as possible to deter animals from eating around unpalatable ingredients. Other hurdles that must be considered include: the experimental subjects need to grow quickly to avoid

an extensively long test period; PER cannot determine protein requirements for maintenance; the experiments are often designed with regards to human nutrition but completed using animal models with differing caloric requirements; and the models have differing amino acid requirements to the subject (Mansilla et al. 2020, Schaafsma 2005, Brody 1999). Protein sources in the current era are often mixed, although PER is unable to determine the advantages (more balanced amino acid profiles) over a singular protein source (Woodham and Clarke 1975). On the other hand, the PER method is extremely simple, needs minimal equipment, is commonly used, allows for easy merit comparisons of diets, and is inexpensive (Mansilla et al. 2020). Based on these advantages, PER is a popular method, at least as a prerequisite to using any other method, for further investigation of nutritional value of an animal feed.

Protein Digestible Corrected Amino Acid Score

The Protein Digestible Corrected Amino Acid Score (PDCAAS) is another method developed to compare protein sources relative to the hosts amino acid needs. According to Mansilla et al. (2020), PDCAAS works by measuring indispensable amino acids by HPLC or other analytical techniques and then calculates the lowest indispensable amino acid score based on their established requirements and values for “true total tract protein digestibility” of the model animal used to compute a limiting amino acid value. The calculation can be determined as follows:

$$\text{PDCAAS (\%)} = \frac{\text{mg of first limiting amino acid in 1 g of test protein}}{\text{mg of the same amino acid in 1 g of reference protein}} * \text{total digestibility (\%)}$$
. The primary

model animals tested are rats under the procedure referenced, and this method has been approved by the Food and Agriculture Organization (FAO) and World Health Organization (WHO), of the United Nations (Mansilla et al. 2020, Schaafsma 2005). The PDCAAS method has demonstrated its importance, as it has been recognized in the United States as the method most commonly used

to determine protein qualities of foods (ingredients) to be fed to children younger than 4 years old (Mansilla et al. 2020). According to the PDCAAS formula, the first limiting amino acid is a critical factor for a protein to meet the nutritional needs, and the proteins only meet the nutritional needs based on the bioavailability of the amino acids (Shaafsma 2005). Therefore, PDCAAS may be used in a similar manner to PER for pet food formulations when testing individual protein source ingredients. However, it requires a digestibility score from an animal model for each protein evaluated and so it does not reduce or eliminate animal model use.

There are numerous limits of using PDCAAS, including that an amino acid's bioavailability can be overestimated due to the values being truncated to 1.0, meaning if the amino acids are supplied in excess, then it is assumed the unused portion will be catabolized (Mansilla et al. 2020, Schaafsma 2005). The PDCAAS has a time limiting factor of requiring a digestibility coefficient, which likely will not exist for all various ingredients possible within a diet and would need to be formulated (Mansilla et al. 2020). Using fecal excretion quantities instead of ileal digesta flow fails to consider for the nitrogen contribution from large intestinal microbial protein production. The PDCAAS can still be compared to a reference protein, and it relies upon the entire proteins' digestibility instead of the individual amino acids, and it loses reliability in determining any benefits of combining different protein sources (Schaafsma 2005, Mansilla et al. 2020). Advantages of PDCAAS are that it does not always rely on a physical experiment when it could be computed from values in literature is the chosen method for human protein requirements, with additive values, and simplicity (Mansilla et al. 2020).

Fecal vs ileal digestibility

Fecal digestibility studies depend on using the intake and digest flow at various points throughout the alimentary tract (ileal or fecal excretions) of the model organism by calculating

the amount of nutrients (protein) that is excreted and by difference computed as retained or digestible. The metabolism of microbes within the colon can cause variations in digestibility readings from interfering with the host's metabolism, often causing studies to overestimate the bioavailability of amino acids (Darragh and Hodgkinson 2000). All forms of fecal digestive bioassays have simple methodology, estimating fecal output, which can help differentiate the various sources of protein amino acids in terms of degradability (Darragh and Hodgkinson 2000, Mansilla et al. 2020).

Ileal digestibility is similar except that the comparison is between recovered digesta collected from the end of the ileum, the terminal section of the small intestine. It requires a cannula or in some cases post exsanguination harvest of intestinal tracts, and the food's initial nutrient contents. As with fecal digestibility studies, ileal digestibility studies also can use different model organisms including rats, swine, ruminants, and even horses. Although the methods differ somewhat; from euthanasia and dissection to collection via indwelling cannula (Schaafsma 2005, Darragh and Hodgkinson 2000). Regardless of method each has nondietary amino acids that interfere with the analysis, originating from the mucus, cells, enzymes, and bile within the ileal digesta and from microbe interactions within the feces (Darragh and Hodgkinson 2000, Mansilla et al. 2020). Ileal digestibility studies are more invasive and can have values determined in multitudes of ways compared to the fecal digestibility studies (Darragh and Hodgkinson 2000). Unlike fecal digestibility studies, ileal digestibility studies prevent the digesta from going through the colon and contacting a rich environment of fermentative microbiota that could break down dietary fiber, add antinutritional factors, and uptake some of the essential amino acids (Schaafsma 2005). These findings have led the FAO to recognize ileal digestibility as better to determine the true bioavailability of a diet compared to fecal

digestibility, which often overestimates the microbe's contribution (Mansilla et al. 2020). As a model species, swine are the models of choice for human nutrition due to their similar monogastric anatomy and physiology, they are a meal-eating species, and they do not partake in coprophagia as other models might (Schaafsma 2005). However, cannulized animal models may have an altered physiology, potentially leading to an overestimation of amino acid bioavailability (Darragh and Hodgkinson 2000, Mansilla 2020).

***In vitro* measurement of digestion**

There are several varieties of *in vitro* models separated into two primary categories: static and dynamic. Static models assume consistency throughout the entire experiment, containing few digestive stages such as oral, gastric, and intestinal, including which enzymes are used and the set pH of each step. Dynamic models are more complex and are regulated based on real time digestive enzymes, pH, and movement. The differing models such as batch cultures, chemostat simulators, and computer-controlled systems all vary in their complexities, use of enzymatic breakdown, and other microbial fermentative processes (de Godoy et al. 2016). Some models use microbial fermentation, and those studies are able to make predictions for both ruminants and non-ruminants (de Godoy et al. 2016). Over the years, dynamic models have become more popular due to more accurate outcomes and increased flexibility to accommodate future research questions. An example is the TNO Gastro-Intestinal Model (TIM), developed by the company TNO (Utrechtseweg 48, P.O. Box 360 3700 AJ Zeist, The Netherlands), modeling the multi-compartmental design of the digestive tract to simulate conditions in the lumen of the gastrointestinal tract, including the stomach, duodenum, jejunum, and ileum. The pH, bile, and digestive fluids are all computer-controlled to produce the most realistic conditions based on the foods and animals to be compared (Schaafsma 2005, Mansilla et al. 2020). In more recent years

the TIM models have been adapted to reflect a dog's digestive system and has been titled Functional gastroIntestinal Dog Model (FIDO), showing promise when compared to cannulated canines (de Godoy et al. 2016). As the models continue to progress, more reliable outcomes are anticipated to be compared to other studies and methods.

As potential drawbacks in *in vitro* methods, artificial membranes are unable to account for active transport and are unable to mimic the body's natural feedback which influences digestion. Therefore, these methods may have lower indices of nutritive values and may not accurately assess absorption for human nutrition (Mansilla et al. 2020, Buchanan 1969). Using the FIDO method could simulate the canine's digestive system sufficiently to provide meaningful evaluation of foods. However, there will always be minute changes and feedback disregarded in the gastrointestinal system of the animal that would be overlooked within the *in vitro* methods. Regardless, the benefits of *in vitro* studies outweigh the drawbacks. *In vitro* models are relatively inexpensive, bypass ethical constraints of using living models, have more consistent outcomes from bypassing biological differences between individuals, and accurately mimic the various digestive systems including those of cats and dogs (Mansilla et al. 2020, de Godoy et al. 2016).

Model Organisms for *In Vitro* Nutrient Analyses:

Rats

Rats, *Rattus norvegicus domestica*, were the first model organism used in PER studies and the most commonly used to determine protein qualities. The most common use for rat PER studies is in human nutrition, although rats have been shown to have differing needs compared to humans. Such differences involve basic morphology such as rats growing fur, and thus needing different amino acid profiles (50% more methionine and cysteine) to meet these requirements

(Schaafsma 2005). Other characteristics to consider are the sex, where females appear to require lower protein to achieve the same PER score, and the growth stage where adults only require maintenance while juveniles require approximately 15% more to reach maximal efficiency (Morrison and Campbell 1960, Matsuno et al. 1976). In an experiment conducted by Buchanan in 1969, heating and moistening the diets (dilution) lowered the PER values that were reestablished during lipid extraction, demonstrating that the diet compositions given to rats influences PER values. However, a strict methodology for using rats as a PER model is lacking for comparisons among studies.

The lack of experimental protocol with regard to strain of rat, sex, age, weaning, the amount of food fed, or the length of the experimental period is problematic (e.g., Matsuno et al. 1975, Buchanan 1969, Rao et al. 1968, Woodham and Clarke 1977, Chapman et al. 1959). There are many different strains of the domestic rat that have been used over the years to produce PER studies. These include the albino, hooded Norwegian strain, Rowlett hooded Lister strain, and Wistar strains, to name a few. Most studies used primarily male rats, with the exception of female rats in Buchanan (1969) and other studies using both sexes. Ages ranged from weanlings to 19-21 days of age, or based on body weight rather than physical age. While the majority of experiments fed *ad libitum*, some only meal fed at certain hours of the day or based on a percentage of body weight. Acclimation periods and testing periods varied as well. Most experiments lacked an acclimation period, although the Association of Official Agricultural Chemists procedure demonstrated that the longer a weanling rat is acclimated on a 10% control protein diet, the higher their weight gain and PER scores (Steinke 1977). The testing periods ranged from reaching maturity to a few days or a few weeks amongst the experiments.

Much like people, rats also have preferences when it comes to food that could interfere with their PER scores. Some rats may partake in coprophagia (fecal consumption), ingesting extra “food source”, or are rather picky when given a choice between two diets based on their sweetness or carbohydrate content rather than the protein content (Steinke 1977). Determining PER values using rats has been shown to be the most basic of methods (Chapman et al. 1959). However, differentiating the limiting amino acids in diets composed of multiple protein sources can be a source of error (Rao et al. 1968, Woodham and Clarke 1977).

Pigs

Swine, *Sus domesticus*, may be a high quality model for companion animals as they are similar in anatomy and physiology to other monogastric mammals and are raised commercially, making them easy to acquire. Ileally-cannulated pigs are recommended and more recently used as models for companion animals, including cats and dogs, after showing similar PER values in their digestion of the essential amino acids and determining protein quality to those of cecectomized roosters (de Godoy et al. 2016, Johnson et al. 1998). However, because of their affable omnivorous behavior, one could assume they would make for a better model of canines than felines who are obligate carnivores and have preferences based on colors, shapes, textures, and flavors (de Godoy et al. 2016). One partial advantage to using pigs is their long growth period until maturity. The pig can be used from the age of a weanling for the PER method with values until they achieve weights that are unmanageable (~195 pounds). They are often supplemented with soybean for growth unless bought before being integrated into the nurseries or growing farms (Geurin 1950). As seen in other animals the males require more protein, specifically lysine, than females, likely due to the increased muscle mass in males (Morrison and

Campbell 1960). There are no set methods as to the age, sex, breed, amount of feed fed, or length of the experiment when considering the use of swine as a model.

Dogs

Naturally, as a target species, at one point the animal of interest, the dog, *Canis familiaris*, was used. Using dogs and cats as their own models is innately the best scientific model to get accurate readings of their digestion of various protein sources (de Godoy et al. 2016). The beagle is considered the ideal dog model from their strong sense of smell and docile, human-loving nature, other breeds are often used for experiments. For example, in an experiment carried out in Norway, 12 huskies were used before euthanasia to test four diets provided daily with unlimited water intake for 10 days (Tjernsbekk et al. 2016). Cannulated dog studies have often compared their findings to those of cecectomized roosters and found they have similar digestibility of amino acids in various food sources, especially for lysine, methionine, threonine, and cysteine, including processed foods after heating or extrusion (e.g., de Godoy et al. 2016, Johnson et al. 1998, Cramer et al. 2007, de-Oliveira et al. 2011). Though most canine models use the beagle, published work varies in ages of the dogs, the use of different sexes, and inconsistent feeding regimens when experiments are compared to one another (de-Oliveira et al. 2011, Dust et al. 2005, and Tjernsbekk et al. 2016).

The largest hurdle when using a canine as a test model in the USA and other countries is to gain approval from the Institutional Animal Care and Use Committee (IACUC) within the organization doing the research. There are much stricter guidelines on cats and dogs than common laboratory and model animals such as rats, swine, or chickens. Approval of animal research is exacerbated by society's growing value for companion animals regarding their welfare, but also issues regarding facility maintenance and an insufficient number of available

canine models for studies (De Godoy et al. 2016, Cramer et al. 2007). Sometimes the number of replicates will be low from a lack of model canine supply that the age gaps can be over 10 years apart, thus bringing into question the validity of the studies. Dogs have rapidly become a rare model in companion animal nutrition studies, forcing laboratories to seek other options. After producing preliminary data in a comparable model animal, however, the findings can be verified with canines using the two most common methods. The first method is conducting a standard nutritional evaluation while the other is a two-bowl, free-choice test (Dust et al. 2005). The first method aims to validate the nutritional findings with those of previous model animals. The second method determines which differences in preparation of the food such as ingredients, heat treatment, and enrobing increase the palatability of the foods.

Chickens

Chickens, *Gallus gallus domesticus*, are perhaps the most frequently used model for current pet food digestibility studies. The previous uses of cecectomized roosters and knowledge gained of their digestive tracts over the years made the use of the younger chicks a prime opportunity. As anticipated in a cecectomized model, roosters showed differences in metabolites compared to intact roosters, with 10% more protein digesta likely attributed to hind gut fermentation (Cramer 2007). While even cecectomized roosters have been used to compare results with a PER, they have passed their ideal growth period for the best PER scores, though they did point to using chicks as an alternative model for companion animal feeds in protein quality (de Godoy et al. 2016). Chicks are a common livestock animal, available even in more suburban areas, and are inexpensive to raise.

Some potential shortcomings to use of chickens as a model are that they require more essential amino acids at 65% compared to the anticipated 50% and similar to what was found in

rats (Matsuno et al. 1975). Since chicks are avian rather than a mammal, it is understandable that their essential amino acid requirements may differ compared to companion animals if for no other reason than to support feather production. In particular, chicks require a higher intake of the sulfur-containing amino acids (methionine and cysteine) than most mammals, resulting in different limiting amino acids (Brede et al. 2018; Donadelli et al. 2019). However, chicks grow at an exceptionally faster rate than the mammals, allowing for quicker detection of limiting amino acids via weight change over time, and the potential to test ingredients considered unsafe for certain mammal species (Donadelli et al. 2019, Cramer et al. 2007, de Godoy et al. 2016). Other benefits could include the small size of each subject allowing for higher data output with more replicates, less experimental feed, and saving on experimental supplies and space.

Mink

The American mink, *Neovison vison*, is a more recent contender as a potential model organism for PER studies with few laboratories using them to evaluate companion animal feed. Tjernsbekk et al. (2016) found that mink kits require a higher intake of amino acids than adult dogs. To further support their studies, they included comparisons of the protein quality measurements obtained from the mink kits to those of adult dogs while using extruded foods. Although there were variations between the diets tested on the minks, the results were still adequate for the requirements of a dog food, due to AAFCO's lack of requirements regarding crude protein and amino acid availability on pet food labels. All these findings pointed toward mink kits being a potential model for dogs, although they are not very common in laboratories around the world.

Protein Analysis Techniques for Pet Foods

Amino acid analysis

Partitioned column chromatography, first described by Martin and Synge (1941), allows for both separation and determination of the amino acid make up of a protein. The first amino acid analyzer was created in 1958 to automate the labor-intensive chromatographic procedures by using injectors for columns, timed buffer releases, and integrating the use of photometers to aid in analysis (Moore et al. 1958). In 1963, Moore and Stein (1963) advanced the automation further by developing accurate automatic recording apparatuses for the determination of the amino acids within amino acid analyzers after fragmenting proteins. When peptide bonds are hydrolyzed, however, some amino acids have been shown to be destroyed in the process and thus are underestimated (Maehre et al. 2018). These analytical processes remain in use and are a key part in nutritional studies of animals. Determining the amount of an available amino acid is necessary in creating a balanced diet in comparison to the limiting amino acids of the specific animal that the diet is formulated for.

Spectrophotometry

Spectrophotometrics are analytical techniques used to determine the functional groups within a protein based on the light absorbed between the wavelengths of 200-800 nm (UV and visible light). Unlike other methods, spectrophotometry does require the extraction of proteins from the sample prior to processing (Maehr et al. 2018). The first mass spectrometer was built in 1940 by Alfred Nier and was used to separate gases and isotopes, and he promoted its use in the biological fields with organic molecules. However, it was not until the 1980's that mass spectrometry was popularly used for proteins due to their large size and complex structures. Two new methods are electrospray ionization mass spectrometry (ESI-MS) originally created by

Malcolm Dole in 1968 but adapted by John Fenn, and matrix-assisted laser desorption-ionization mass spectrometry (MALDI-MS) created by Franz Hillenkamp, Michael Karas, and co-workers (Griffiths 2008; Dole et al. 1968; Yamashita and Fenn 1984; Karas et al. 1985). These two methods are still primarily used in labs for analyzing peptides and proteins.

Nitrogen Determination

One of the most common methods of determining the amount of protein in a food source is to measure the amount of nitrogen released when burned into a gas. Johan Kjeldahl reported in 1883 the use of a sulfuric acid solution rather than an alkaline solution to form ammonia by using 1g of raw sample through hydrolysis and heating until just under the boiling point to create more accurate, easier results than the common procedures of the time (Veibel 1949, Maehre et al. 2018). This method became known as the Kjeldahl method, which is still widely used today in high fat foods. However, the first recorded method of nitrogen determination was created by Jean-Baptiste Dumas in 1826 by combusting the materials at temperatures over 800°C, releasing carbon dioxide, water, and nitrogen, and this has become automatized and standardized with controls of compounds with known nitrogen content like glycerin for calibration in recent years. Neither of these methods are perfect, as they can record any contaminant form of nitrogen and not solely that of protein and rely upon correction factors (Ariton et al. 2022). The Dumas' modernized method has now become more common than the Kjeldahl method in determining protein percentage.

The protein analysis method used in this research was the LECO FP928 Nitrogen and Protein Analyzer (LECO Co., St. Joseph, MI, USA). The nitrogen in proteins makes up approximately 16% of its structure and is not found in carbohydrates or fats, with a crude protein coefficient of 6.25 for proteins ($100\%/16\% = 6.25$). To measure the nitrogen content of the food,

the protein coefficient was input into the LECO machine. The food was then automatically deposited into the machine that combusted the food to read the nitrogen content of the gases released. Seven different diets were used throughout the experiments and all were tested for their protein quantity. After control rounds using both EDTA of known nitrogen content and blanks for calibration, each diet had three samples of approximately $3.0 \text{ g} \pm 1.0 \text{ g}$ within the closest decimal to each other that were placed into the provided ceramic weigh boats designed for the machine. The ceramic boats were placed on the machine's loader and then the machine deposited the samples for testing automatically. The sample was burned off at a temperature of 1100°C and a thermal conductivity cell then detected the nitrogen content of the gases to determine what percentage of the sample was protein. The protein percentages were then averaged from the three samples and were used in the calculations for the PER of the respective diet.

Model Insects Proposed for Pet Food Nutrient Analyses

Dermestid beetles

To our knowledge, this is the first report of using insects as a model organism for evaluating the nutritional qualities of companion animal foods and ingredients. One significant factor to consider is the high protein percentage that is associated with cat and dog foods that would need to be readily ingested by the model organism. While the idea may seem unlikely, the range of insects in the world means that diversity could contain a good model organism to fit with this type of scientific inquiry. Specifically, the insect in question would ideally be accustomed to consuming high levels of protein, have a long enough growth cycle to fit within the PER growth duration, and be easily reared in a commercial/laboratory setting. Dermestid beetles (Coleoptera: Dermestidae), commonly called carpet beetles, larder beetles, or hide beetles, are well known for their infestations of grocery stores, pet food facilities, processing

facilities, museums, and natural fabrics (Roesli et al. 2003, Larson et al. 2003, Timm 1982). The family Dermestidae is composed of over 800 species, many of which are cosmopolitan, specializing in drier climates and surviving on animal materials or grains as larvae and nectar or pollen as adults (Kiselyova and McHugh 2006). Dermestidae were postulated to originate in the late Cretaceous period by Kiselyova and McHugh in 2006 after revisiting other scientists' data with the first undeniable evidence of a fossilized modern dermestid specimen dating back 20-30 million years ago. However, other specimens of ancestral dermestids have dated back to the Cretaceous period when they are hypothesized to have diversified.

Dermestid beetles are able to withstand low humidity, making them adept at infesting stored grains, dried meats, animal hides, and dried fish (Kiselyova and McHugh 2006). Most dermestid larvae are able to undergo diapause, or a prolonged time of development as a larva or pupa, allowing them to survive unfavorable circumstances involving the photoperiod, lack of food, overcoming certain insecticides, overcrowding, or surviving low temperatures or humidity, making them one of the most resilient pests in grain products (Abdelghany et al. 2015). These adaptations have also made dermestids great at surviving voyages across a wide array of trade and migration routes around the globe, leading to many becoming cosmopolitan.

With the spread of dermestid beetles, some have become very well-established pests, primarily in their larval stages. There are more than 40 different species that are known to infest durable stored food products (Abdelghany et al. 2015). Two genera are particularly well-known for their development in modern stored products, *Trogoderma* and *Anthrenus* (Kiselyova and McHugh 2006). With increased relevance over time as they have become more prevalent, more morphological taxonomic keys have been developed to aid in identifying the pests present in both their larval and adult stages (Kiselyova and McHugh 2006). However, some studies and

practice point to *Trogoderma* larvae being nearly indistinguishable from other dermestids, relying solely on molecular analysis of DNA for identification (Domingue 2023). Although much research has been done on specific species regarding stored product concerns, most species of Dermestidae remain understudied in their ecology, biology, and physiology (Kiselyova and McHugh 2006).

Warehouse Beetle

The warehouse beetle, *Trogoderma variabile* (Ballion), is a well-established cosmopolitan economic pest of concern. *Trogoderma variabile* is considered the second most serious Dermestidae pest after the khapra beetle, *Trogoderma granarium* (Everts). Originating from central Asia, *T. variabile* already had become widespread within the United States by 1954 (Partida and Strong 1975, Beal 1954, Gerken and Campbell 2018). The lifecycle of *T. variabile* averages 34 days from egg to adult, with adult females laying up to 90 eggs in their short weeklong lifespan (Merchant 2018). In their natural habitat, *Trogoderma spp.* are known to colonize near nests of wasps, bees, other insects, birds, mammals, spider webs, and under bark (Linsley 1944). When raised on stored grains, seeds, or pet foods, adults eat an insignificant amount of food whereas larvae do most of the damage (Abdelghany et al. 2015). Commonly confused with its close relative, *T. inclusum* (LeConte), *T. variabile* can be differentiated by lacking a postcoxal line on the first visible abdominal sternite that *T. inclusum* displays (Holloway and Turner 2019).

Larger Cabinet Beetle

The larger cabinet beetle, *Trogoderma inclusum* (LeConte), is another well-established cosmopolitan economic pest. The identification of *T. variabile* and *T. inclusum* are commonly confused, although they can be differentiated by *T. inclusum* having the inner margin of the eye

emarginate and a yellowish-cream color integument (Beal 1956). Ironically, *T. inclusum* was originally synonymous with *T. versicolor* (Beal 1956), but they were distinguished as two separate species by examining their eye margins, the base of male antennae, genitalia, and characteristics of the first abdominal sternite (Strong 1975). The species *T. inclusum* may have originated from within the United States or somewhere in Europe; however, both *Trogoderma* spp. feed on similar food sources and have similar natural habitats with broader host ranges (Beal 1956, Domingue 2023, Linsley 1944). The life cycles are similar, but *T. inclusum* larvae mature slightly faster (by approximately 5 days) in comparison to *T. variabile* giving an average of 29 days. It is likely that *T. inclusum* could be limited in its natural distribution within the United States based on the relative humidity; however, it too can be found in many processed foods, animal feeds, dry feeds, storage facilities, and agricultural products (Strong 1975).

Hide Beetle

The hide beetle, *Dermestes maculatus* (DeGeer), is a lesser studied cosmopolitan economic pest. The species *D. maculatus* is believed to have originated from continental North America and Hawaii (Shaver and Kaufman 2009). The average lifespan varies greatly based on environmental factors, lasting anywhere from 35 to 70 days to reach adulthood, 5 to 11 instars, and adults living up to a year in ideal conditions, allowing females to lay up to 800 eggs (Lyon 2001, Shaver and Kaufman 2009). They are known to have natural habitats around carrion or discarded animal products where aggregation pheromones are released to induce colonization (Shaver and Kaufman 2009, Archer and Elgar 1998). According to Archer and Elgar (1998), *D. maculatus* are cannibalistic, especially the larvae, eating exposed pupae for nutrients even when the environment has not reached full carrying capacity and there are other nutritional sources available. Like other Dermestidae, *D. maculatus* can undergo diapause. Studies indicate there is

often a tradeoff of potential survival with limited food sources since larvae that lose body mass may have decreased success in reproduction as an adult, due to being exposed to potential disease in an exposed pupal state, or a lack of time to find a food source once they reach adulthood (Shaver and Kaufman 2009, Archer and Elgar 1998).

Advantages and Impediments of Insects for Nutritional Analysis

Insects are ectothermic, relying on their environmental temperatures to regulate their body temperature, further influenced by the insect's size (Chown and Nicolson 2004, Wilson and Fox 2021). An animal's body heat can influence their metabolism rates, thus influencing their water conservation, starvation resistance, growth, and reproductive rates (Chown and Nicolson 2004, Lemoine and Shantz 2016, Contreras and Bradley 2010). Any of the affected outcomes of metabolism can also become a limiting factor to an animal's metabolism, depending on where the energy is directed influences the body temperature (Glazier 2009). Further reductions in metabolic rates also have been shown to reduce water loss, likely as a byproduct of retaining water metabolites, as well as respiratory rates (Chown and Nicolson 2004, Contreras and Bradley 2010).

Temperature not only influences insect metabolism but also respiration rate. In an experiment performed by Lemoine and Shantz (2016), a protein limitation caused by the denaturing of protein at high temperatures influenced the insects to seek more carbohydrate-rich foods to feed their bodies' intense need of energy to compensate for their respiration. Wilson and Fox (2021) corroborated that study when analyzing climate change effects on insects, showing that when insects are not in an optimal temperature, the bioavailability of nutrients is lost; yet when temperature is too high, insects attempt to ingest to support their respiratory rates. A common byproduct of respiration is water vapor. In my experiments, the laboratory prepared

diets are not enrobed in lipids, which is done by industry to prevent rancidity and water absorption while also increasing palatability. So, the more an insect respire, the more the diet will retain the water exhaled, increasing its weight.

Reared insects will require the use of an incubator to aid in their temperature, osmoregulatory processes, and, indirectly, in their containment, allowing more of the insects' energy to be used for feeding and growth than maintenance. However, this too would increase the amount of water vapor in contact with the diet, further adding to its water retention and influencing its weight.

One of the first instances of determining the essential amino acids in animal diets occurred in 1952 when scientists fed carbon-14 sucrose (Kasting and McGinnis 1958). These experiments have been repeated with various insects throughout the years, including varieties of Diptera, Coleoptera, and Lepidoptera, with all showing similar outcomes to those of mammals with the same ten limiting amino acids (arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine), though more recent studies do not list arginine as an essential amino acid for mammals even though it is necessary for cats and dogs (Kasting and McGinnis 1958, House 1961, Brodbeck and Strong 1987, Mitsuhashi 1978, Smith 1980, National Research Council 2006). In a study conducted by Mitsuhashi in 1978, the researchers found that to support the growth of Diptera and Lepidoptera cell lines, the addition of cysteine, glutamine, proline, serine, and tyrosine were added to the essential amino acids to allow for proper growth, and without cysteine the cells depreciated. Even with this knowledge, however, all species of animal have varying primary limiting amino acids in comparison to one another or even have exceptions to this rule (Hou and Wu 2018, House 1961). With the advancements of technology and methods since these previous studies have been conducted, the

determination of essential amino acids and their necessary quantity should be relatively easy to perform for any individual species of interest.

Few insects have been analyzed for their amino acid composition

Such possibilities in analyzing the protein requirements and retention of insects will be more routine soon as entomophagy is becoming more common practice and are also used as a feedstock for livestock. According to studies conducted by Brede et al. in 2018, supplementation of insect meal to the diet aided in reaching the necessary amino acid requirements of chickens that were lacking sulfur-containing amino acids. As more commonly accepted “edible insects” such as crickets and mealworms become popular, their nutritional values will require labeling which could influence future nutritional studies.

Unlike the digestive system of a monogastric mammal such as the dog or cat, the insect digestive system is more comparable to that of a chicken, which has already been used as a model for companion animal feeds. In both insects and chickens, the food travels down the esophagus into the crop, next into the proventriculus, then, unlike in the chicken where it goes to the ventriculus, the food continues into the midgut (equivalent to the chicken’s duodenum), followed by the ileum and rectum, respectively (Holtof et al. 2019). The general functions can be related to those of the companion animals after seeing the similarities with chickens. The foregut, composed of the esophagus, crop, and proventriculus, allows for ingestion, initial breakdown of food, and storage. Next, the midgut’s primary functions are digestion and nutrient absorption. Lastly, the hindgut, comprised of the ileum, colon, and rectum allow for water absorption (retention) and excretion of metabolic waste.

Unlike larger mammals, it would be impossible to cannulize or obtain the digesta from an insect before it reaches the hindgut. Few studies have focused solely on insect protein absorption,

although solute carriers 6, 7, and 15 intake different amino acids into different cells (Miller et al. 2008, Boudko 2012, Holtof et al. 2019). Another study conducted by Jing et al. in 2020 showed *Cryptorhynchus lapathi* (Linnaeus), a poplar-and-willow weevil (Curculionidae), had midgut symbionts for amino acid biosynthesis and protein digestion. True digestibility would disregard any digestion that occurs from the hindgut by microbes.

Development of allergies

Dermestid beetles are well-known as stored product pests and pests of museums and biological specimens. These beetles also cause allergies including itching, hives, weakness, fever, headaches, eye or respiratory irritation or nausea in response to touching particles of their cast skins or bodily hairs (Abdelghany et al. 2015; Timm 1982). It is common for museum curators, forensic entomologists, and stored product warehouse workers to develop dermestid allergies, as they work in areas common place for dermestid infestation. One such case occurred in 2009 when a healthy 42-year-old male who worked in a pet food factory had been exposed to *T. variabile* exoskeletons when dust became airborne during cleaning (Bernstein et al. 2009). The more frequently a person is exposed to the exoskeletons or urticating hairs of larvae, the more likely the person is to develop an allergy.

With the risk of developing allergies, working with dermestid beetles can be bothersome. For best practices, those who are exposing themselves to dermestid beetles should wear gloves and a mask to prevent direct interactions or inhaling any exuviae or hair particles from the colonies. Handwashing after removing the gloves is essential to protect all workers. Since dermestids are a common house-dwelling species, such as the varied carpet beetle *Anthrenus verbasci* (Linnaeus), exposure may also occur in commonplace locations.

Insects can save space, time, and money

Dermestid beetles grow no larger than 15 mm, making them the smallest proposed model organism of interest for PER studies. Their small size means that they would require minimal space, much less diet and resources, and would grow at a considerably faster rate than all previous animal models.

Possible drawback of inbreeding

Though initially foreseen as a benefit, one potential drawback of colonizing dermestid beetles is inbreeding. As the generations continue overtime, the beetles will become more similar to one another genetically and lack the variability that occurs naturally. A potential solution to such cases is the periodical introduction of externally obtained beetles to integrate new genetic material into the colony, or simply to maintain multiple colonies from differing points of acquirement to test comparatively to one another.

IACUC does not regulate use of insects

The IACUC was founded under the growing concern for the rights of the animals used in scientific experiments. Harvey-Clark (2011) goes deep into detail regarding IACUCs general stances and what constitutes a concern for the committee members. The committees are found at any institution that takes part in scientific research and are primarily concerned with the animal's welfare regardless of their phylogenetic position. However, regulation standards are lacking for animals that are not a vertebrate, cephalopod, or decapod crustacean since it leads to a moral dilemma of whether they require the same treatment. In the scientific community, pain is not only defined by the presence of nociceptors, but also by the body's response to stimuli anatomically, physiologically, and behaviorally to indicate distress. Although invertebrates have nociceptors, the debate arises as to whether they accurately indicate proper stimulus response of trying to withdraw from a painful stimulus. From these assumptions being made that

invertebrates do not show proper behavioral responses to indicate “pain”, at least to an average person, it is assumed they do not require IACUCs approval and therefore require minimal effort to gain approval. However, more research over recent years and the growth of animal welfare is beginning to further investigate pain and suffering within invertebrates. With a long-standing routine of minimal effort, due to lack of criticism, many entomologists often overlook the ethical stance of using the 3 R’s of reducing, replacing, and refining in research (Fischer and Larson 2019). If IACUC begins to enforce more stringent standards among the use of invertebrates, it will likely still be more relaxed than those for commonly used mammal models and take time to be integrated.

Though IACUC has remained active since 1985, it does not mean there are no pitfalls or shortcomings of its structure. IACUC has taken over both the animal care professionals’ jobs and an advisory position in decision-making. Steneck 1997 goes into detail about the committees’ responsibilities and relations to the research they oversee. The largest concerns regarding IACUC involve members’ lack of expertise, diverting resources, conflicts of interest, and restricting academic freedom. The IACUC is composed of a minimum of five committee members per institution. The five members include a veterinarian (DVM), an experienced animal researcher, a nonscientific member, someone who is unaffiliated with the institution, and a faculty member with research experience taking lead as the chair. At first glance the committee may be balanced, but the members may be lacking proper training in the welfare and care of animals within a research setting. Due to the increased demand for animal welfare, there have been more stringent research guidelines, paperwork, laboratory visits, and ethics reviews, all without truly adding funding to research budgets placing stress on IACUC members. According to Steneck (1997), all these responsibilities regarding proper animal care stretch members thin.

In addition, there are no checks and balances with IACUC ensure reasonable regulations as they are the only committee given oversight on the matter. Lastly, the nonscientific member and unaffiliated member may compromise the judgement of the entire IACUC committee regarding scientific research due to their lack of knowledge in certain matters and protocols. IACUC has moral values to protect the researchers from civil issues of overlooking animal welfare, however, there may be much responsibility put on one committee. Therefore, Steneck (1997) believes that animal care professionals should resume their previous positions while IACUC becomes the advisory board alone.

Benefits of insects in food quality research

Insects make up a majority of animals on earth. Since insects have relatively fast generation times, allowing for faster genetic alterations to occur, sometimes in more specific life stages than others, then presumably, this would allow for more genetic mutations to occur over a shorter time period (Thomas et al. 2010, Danks 2006). Thomas et al. further tested this in an experiment and found similarities across various classes of invertebrates, verifying the assumption. With their smaller sizes, dermestids are able to multiply and create a larger population. Other factors can affect their growth rates including the size of their enclosure, the temperature, relative humidity, photoperiod, having ample diet available, and preventing overcrowding.

With considerably dense colonies and maintenance of multiple colonies, there could be an exceptionally larger number of replicates in comparison to the vertebrate models previously used. This creates a more robust statistical model that can show the natural variations between individuals that is often lost in studies using vertebrates with fewer than six replicates versus

insects with more than 30 replicates. When too few replicates are incorporated, it could lead to false conclusions, be influenced by an outlier, or falsely be assumed to be statistically normal.

While having more replicates aids in the statistical analyses of the data, it also allows for more individual observations to be made. Variations in behavior, size, sex, or any other undesignated variable to the experiment could potentially be observed to show correlations to the results.

Study Objectives and Justification

The growing demands of consumers for nutritionally sound, healthy, and appetizing companion animal foods are overwhelming. Scientifically designed diets are growing in popularity as owners desire the healthiest pet foods that have undergone testing to make sure the formulations meet requirements, and that the industry has ways to further improve them. Many aspects revolve around companies finding alternative and novel ingredients to provide the proteins necessary for our pets as the companies struggle to compete with the excessive meat consumption of humans that is limiting their ingredients of choice. With the novel ingredients, such as alligator, kangaroo, duck, cassava, and sweet potato, being added to the resource pool for pet food formulators, a faster, more predictable, and more vigorous evaluation method would be necessary for approving or removing the ingredients from their potential list of diet components. The pet food companies and university laboratories are limited in which model organisms they can use for nutritional studies, primarily by IACUC, limited space, limited time, funding restraints, and the number of model organisms available per study. Dermestid beetles are known to be common pests within the pet food industry and to ingest high protein diets like those of cats and dogs and have potential to serve as model organisms.

The broad objective of the research described in Chapter 2 and 3 is to determine if a dermestid beetle may be used as a model organism in companion animal bioavailability studies rather than vertebrates to forgo the stringent regulations of IACUC and to expand our capacity and capability to evaluate protein ingredients in a timely and routine manner. Two experiments will be conducted. First, three species of dermestid beetles, *T. variabile*, *T. inclusum*, and *D. maculatus* will be evaluated on their ability to efficiently transform the energy they ingest from a nutritionally balanced pet food into growing at a measurable rate in the larval stage to work as a model for PER studies. The best determined model of the three dermestids tested will then be the species used in the following experiments. The second experiment will utilize the model species as determined from the first experiment and place them on various diets, varying only by the protein sources and amounts, to find the most efficient protein source for growth. The most efficient proteins are anticipated to be of egg or milk origin based on previous PER studies.

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Chapter 2 - Potential for Using *Trogoderma variabile*, *Trogoderma inclusum*, and *Dermestes maculatus* (Coleoptera: Dermestidae) As Model Organisms to Determine Nutrient Bioavailability for Companion Animals

Introduction

Proteins play a tremendous role in companion animal nutrition and well-being. The amino acid composition can influence both the food's digestibility and the proteins' contribution to numerous body functions of the pet. For these reasons many pet foods contain various protein sources to form a more complete profile, typically favoring animal sources (Wernimont et al. 2020, Dust et al. 2005). In recent years the pet food industries have been making large changes in their formulations due to consumer perceptions and worry about the purposes and health claims for various ingredients (Clemens 2014, Schleicher et al. 2019).

Some consumers may overlook the nutritional adequacy statement from the manufacturer that is printed on pet food packaging as required by law under the Federal Food, Drug, and Cosmetic Act (FDA 2022). The Food and Drug Administration (FDA) acknowledges that the Association of American Feed Control Officials (AAFCO) has developed the "Model Regulations for Pet Food and Specialty Pet Food" that follow regulations and requirements already set forth in the Federal Food, Drug, and Cosmetic Act (FDA 2020). According to the United States Department of Health and Human Services (USDHHS), the bioavailability of nutrients in a food source is based upon the amount of the food that can be absorbed into the body and reach tissues after being digested, whereas the digestibility is the amount that is available to be broken down for absorption in the intestines (USDHHS n.d.). For this reason, some pet food companies will scientifically test their products rather than simply formulate above the minimum requirements necessary to reach the AAFCO nutritional guidelines. Scientific diets base their statements on bioavailability feeding studies by stating "Animal feeding tests using AAFCO procedures substantiate that... provides complete and balanced nutrition..." rather than only using formulation calculations stating "... is formulated to meet the

nutritional levels established by AAFCO...” (IAMS 2022, Clinical Nutrition Team 2020, Dzanis 2008). According to Kumcu and Woolverton (2015), the most prevalent components considered by consumers as to meeting the criteria of being a premium pet food are the quality of food inputs, the cost of the product, and its protein content. Using a protein efficiency ratio (PER) experiment to determine the bioavailability of protein within prospective pet food formulations can allow labeling that can provide assurance to the pet owners that they have a higher value of product.

The first use of PER occurred in 1919 with albino rats by Osborne, Mendel, and Ferry to investigate the ability to compare various growth rates by limiting the growth of a negative control litter with protein-free milk, and soon the method became the standard of assessing protein quality. It allows for the various protein sources to be sorted based on their bioavailability of essential amino acids. Although PER was initially developed to study protein digestibility for human foods, it has since been adapted for use in agricultural productions and later still to include pet foods. Prange et al. carried out similar experiments as early as 1928 for growing chickens, which are still used today. Other used models include pigs, dogs (usually beagles), and minks (de Godoy et al. 2016, de-Oliviera et al. 2012, Tjernsbekk et al. 2016, Mansilla et al. 2020). Although all these models have various benefits and similar or dissimilar dietary requirements by the target pets of interest, they all must conform to the laboratory regulations of the Institutional Animal Care and Use Committee (IACUC, Silverman 2015). Insects have not been used to determine protein quality in foods. Insects as a model organism for protein quality determination are not regulated by the limitations set by IACUC, are easier to rear, can be raised in larger populations to provide more replicates, and have a faster growth rate compared to vertebrates used for PER experiments.

Here I report a study on the potential to use the feeding larval stages of three common stored product insects as models for estimating PER of commercial pet foods, and as substitutes for vertebrate model organisms. Insect species included three different species of beetles (Coleoptera) in the family Dermestidae: *Trogoderma variabile* (Ballion), *Trogoderma inclusum* (LeConte), and *Dermestes maculatus* (DeGeer). Dermestid beetles are a family of well-studied stored product pests known to infest pet foods, stored grains, dried meats, and sometimes carrion (Hodgson 2008, Roesli et al. 2003). These three dermestid species may have potential as invertebrate alternatives to vertebrates for determining PER in pet foods because of their known dietary needs and behavioral habits of scavenging for carrion and they infest pet food and eat foods high in protein in their larval stages for growth.

Both *T. variabile* and *T. inclusum* are well-known economic pests and are found in various facilities including retail grocery and department stores, pet food stores, and food processing facilities (Roesli et al. 2003, Larson et al. 2008, Hodgson 2008). These insects typically feed on various food sources including animal-based fabrics like wool and silk, pet foods, stored grains, and museum exhibits with animal or grain derivatives. Both *Trogoderma* species are morphologically similar but can be distinguished by discrete morphological differences that require expertise to recognize, like the notching of the interior eye margin of *T. inclusum*, or genetic testing (Banks 1994). However, development and behaviors can sometimes be used for identification. *Trogoderma variabile* larvae develop slightly slower than *T. inclusum* larvae, taking approximately 34 days to reach full maturity, after which females can lay up to 90 eggs in their short week-long adult life span (Merchant 2018). When their normal mating behaviors are disrupted, *T. inclusum* will typically lay eggs longer than *T. variabile* leading to more eggs being laid and more progeny overall (Gerken and Campbell 2018).

Dermestes maculatus are an economic pest that is less studied than the other two species being investigated. They are considered pests of products including fish, pet foods, the silk industry, fur, and museum exhibits (Timm 1982). Their life cycle differs in length with a variable number of instars (from 5 to 13) for development that are highly reliant on environmental factors including humidity, moisture content of their food source, and the type of nutrition provided (Scoggin and Tauber 1951). This variation can cause larvae to take anywhere from 40 to 70 days to reach adulthood. The adults can survive for up to a year and a female can lay up to 800 eggs (Lyon 2001).

Based on these lifecycles, it is anticipated that *D. maculatus* would be the most efficient of the three dermestid species as a model organism for companion animal bioavailability studies. In this study, dietary bioassays were carried out for larvae of *T. variabile*, *T. inclusum*, and *D. maculatus* to compare their PER values and feed conversion rates that are analogous to other PER model organisms in the scientific analysis of pet food studies. There were multiple refinements made throughout the trials to account for unforeseen issues in the experimental set up.

Materials and Methods

Insect colonies:

Colonies of *T. variabile* and *T. inclusum* were acquired from the Center for Grain and Animal Health Research (CGAHR) in Manhattan, KS in 2017. Both *Trogoderma* species were reared separately on Purina Complete Puppy Chow diet (Nestlé Purina PetCare Co., St. Louis, MO, USA) with a thin layer of rolled oats (The Kroger Co., Cincinnati, OH, USA) inside 1 L mason jars (Ball Co., Broomfield, CO, USA), with the lids replaced with metal mesh to prevent

escape and allow exchange of fresh air and humidity. Crumpled paper towels also were placed in the colony jars to provide extra surface space and shade for the insects. The colony of *Dermestes maculatus* also was obtained from CGAHR and originated from wild-captured beetles infesting powdered blood at a pet food processing plant in 2009. *D. maculatus* colonies were reared with a centimeter thick layer of Purina One Lamb and Rice diet (Nestlé Purina PetCare Co.) topped with a thin layer of rolled oats inside 6 qt (30 cm x 15 cm x 9 cm) plastic bins (Sterilite Co., Townsend, MA, USA) with 3 cm diameter holes drilled into the lid and covered with metal mesh to allow air flow but prevent insect escape. Each bin also had a 1 sq in faux fur fabric placed upside down near the end of the container as described by Fontenot et al. (2014) to promote egg laying, as well as two folded paper towels for shade and increased surface area. Each colony was rotated on a weekly basis. The rotations involved mating 60 adults in the next week's colony container for one day and then removing the adults after 24 hours so that all larvae would be within one day of the same age.

All colonies were maintained in a Percival I-66VL incubator (Percival Scientific Inc., Perry, IA, USA) set to 27°C with a 16:8 light:dark photoperiod and maintained at a relative humidity of ~60%. Small second to third instar (two-week old) larvae were used from each species in bioassays.

Protein content of diets:

Two diets, one as a positive control and the other a negative control, were used in the following experiment to test the three beetle species for their PER. The positive control was Purina One Lamb & Rice diet (PLR, Nestlé Purina PetCare Co., St. Louis, MO, USA), available commercially, which was complete and balanced for adult dogs with normal activity levels. The negative control diet (NC) was composed of cornstarch and dextrose (added in a 2:1 percentage

ratio), mineral premix (5.365%), soybean oil (5.365%), choline chloride (0.22%), and vitamin premix (0.203%), completely devoid of any added protein source. The NC diet was made from raw solid ingredients that were dehydrated and broken down into a fine powder.

Nitrogen typically constitutes approximately 16% of a protein's structure and has a crude protein coefficient of 6.25 ($100\%/16\% = 6.25$). To measure the nitrogen content of the food, the crude protein coefficient was input into a nutrient composition instrument machine, LECO FP928 Nitrogen and Protein Analyzer (LECO Co., St. Joseph, MI, USA) that combusts the food to read the nitrogen content of the gases. Three samples of 3.0 g of both test and NC diets were placed into the ceramic weigh boats designed for the machine, which were placed on the machine's loader that deposited the sample for testing. The sample was then burned off at a temperature of 1100°C, and a thermal conductivity cell was used to detect the nitrogen content of the burned off gas to determine what percentage of the sample was protein. The protein percentages were then averaged from three samples and used in the calculations for the PER of the respective diet.

Effect of protein availability on dermestid larval growth:

Three separate biological replicates of the experiment were performed, each in a different location of the laboratory. The replicates included 10 larvae of each species for both positive control and negative control. Each individual 3-dram 15 mL (20 x 65 mm) glass shell vial (Thermo Fisher Scientific Inc., Waltham, MA) was weighed individually, without the snap cap, and then tared before adding $0.5\text{ g} \pm 0.005\text{ g}$ of the diet using an Accuris Instruments W3100A-210 scale (Accuris Instruments, Edison, NJ, USA). Each larva was weighed on a Cahn 28 automatic electro-balance (C.Y. Scientific LLC, Irvine, CA, USA) and placed individually into one of the vials containing 0.5 mg of either the positive or negative control diets. Once all the

vials were filled with diet and an individual larva, they were capped and placed either on the laboratory countertop (20°C, 40% R.H.), the top rack of the incubator (27°C, 60% R.H.), or the middle rack of the incubator (27°C, 65% R.H.), all recorded using HOBO's (Honest Observer By Onset) (Model: UX100-003, ONSET Computer Corp, Bourne, MA, USA) in each environment. The incubator had a fan at the top, giving more direct air flow to the vials located on the top rack and a lower humidity. After 6 days, larvae were weighed again individually within the vial after the snap cap was removed, and the remaining food and the glass shell vials were weighed together.

Data analysis:

Each larva was evaluated separately. To calculate the PER, the following formula was used:

$$PER = \frac{\text{larval weight gain (g)}}{\text{protein availability for absorption}} = \frac{\text{final larval weight} - \text{initial larval weight}}{[(\text{initial diet weight} + \text{vial weight}) - (\text{final diet weight} + \text{vial weight})] * (\text{Protein \% decimal})}$$

Data collected was analyzed on a single variable of insect species as a randomized complete block design using the GLM procedure of Statistical Analysis Software OnDemand for Academics Studio (SAS Institute Inc., 3.81 Enterprise Edition, 2022). When analyzing the interaction between both the placement and insect species the data was analyzed using JMP Pro Software (SAS Institute Inc., version 16.0.0, 2021) and SAS. The insect species were considered the experimental unit with three replicates each in three environmental placements as the blocks. The Bonferroni multiple comparison method for preplanned comparisons was used to control the fixed comparisons of differences between the experimental units of count data. Figures were graphed using GraphPad Prism Software (GraphPad, version 10.2.1, 2024).

Results

The larvae from each of the three dermestid species readily consumed the provided diets in each environmental setting, as evidenced from the diet consumed (Table 2.1). When all species were analyzed together and only the locations of the larvae were considered, the location played a significant role in their PER values ($P < 0.05$). However, although differences between the two *Trogoderma* species were not significant with respect to location, *D. maculatus* had significant differences in PER among locations, with lower PER in insects reared on the counter, and higher PER when they were reared on the top rack of the incubator. On an individual species basis, the location in the rearing chamber had no effect on *T. variabile* ($P = 0.1969$) and *T. inclusum* ($P = 0.8853$), whereas *D. maculatus* was affected by chamber location ($P = 0.0003$). When comparing the individual species to one another, the two *Trogoderma* species had significantly different ($P < 0.05$) PER values; however, there were no significant differences between PER values of *Trogoderma* species and *D. maculatus* (Figure 2.1, Table 2.1).

The positioning and insect species were compared together in an interaction analysis (Figure 2.1). When comparing under the method of a mean \pm 3 interquartile, there were 4 outliers for both *Trogoderma* species and none for *D. maculatus*. *Dermestes maculatus* also had the smallest range in PER value among all three species. However, the P -values of the full model, species, and placement interactions were all non-significant ($P > 0.05$). Outliers were caused by larval molting and the diets absorbing moisture, reflecting a lower ingestion rate or a higher weight gain, respectively, compared to others in that group. When a larva molted, it negated time from ingesting food and thus lowered their weight gain, lowering the PER value. The water absorbed by the diets sometimes caused a reading of the replicates having more food

present after the experiment than what was recorded before the experiment took place, increasing the PER value. Note the major outlier in *T. variabile* on the middle rack, caused due to low food intake, lowering the denominator value, while molting to the next instar, increasing the numerator value, thus skewing the data. Once the major outliers were removed from the assay, species and placement interactions were significantly different ($P < 0.001$, Figure 2.2). On an individual basis, the only species that was significantly different in PER value was *D. maculatus* ($P < 0.0001$). All placements had significant differences ($P < 0.05$), with the PER values of insects reared on the counter and top rack of the incubator having the most significant difference ($P < 0.001$).

Results from Figure 2.1 and Figure 2.2 indicated that the most ideal species of the three tested for use in further experiments was *D. maculatus*. Unlike both *Trogoderma* species, *D. maculatus* had reliably positive PER values eliminating the need to run an excessive number of replicates, since it is impossible to have a negative PER in ideal conditions with a subject eating a positive amount of food and gaining weight. Both *Trogoderma* species struggled in gaining weight compared to the rate of *D. maculatus* (Table 2.1). The PER results from *D. maculatus* had the qualities sought for a PER model organism, with efficient conversion rates of energy intake to growth rates. However, an issue observed among all three species was larval molting, which needed to be addressed as it interfered with the anticipated weight gain recorded. The weight of the exoskeleton is not considered within the PER formula, and the larvae do not eat and are inactive during the molting process.

To address the problems associated with larval molting, a follow-up experiment was performed only using *D. maculatus*. In this experiment, adults were placed in a colony plastic bin for three days to lay eggs. The two-week old larvae were placed on either a positive control diet

or an absence of diet for a shorter feeding period of 48 hours in the incubator to determine if the time frame would be adequate to detect a weight gain. In all cases, larvae reared for 48 hours had a decrease in overall molts with positive PER values. The data displayed variation in weight in the shorter timeframe depending on whether insects were fed or not. However, there were still too many molts in this tested 48 h timeframe to effectively serve as a model for PER determinations.

A further follow-up experiment was performed with *D. maculatus* larvae placed on either a positive or a negative control diet for 24 hours. The negative control diet was used for comparison this time, because any time frame shorter than 24 hours did not demonstrate enough weight difference to provide adequate PER readings (data not shown). At 24 hours of feeding, measurable PER values were obtained, and no molting was observed (Table 2.3).

Discussion

In this study, three dermestid beetle species, *T. variabile*, *T. inclusum*, and *D. maculatus*, were assessed for their ability to substitute as a PER model organism while fed different diets at different protein levels. To our knowledge, no previous studies have been reported using insects for PER bioassays. This dearth of reports and limited knowledge about their protein requirements or growth patterns led to multiple shortcomings in our procedures that required additional experimentation to refine the methods in this study. Such issues included (Table 2.1), factors such as the larvae that were in the incubator gained more weight in a six-day experiment than those on the counter. Ostensibly this was due to the insects being ectothermic, and temperature has an impact on growth and reproduction. More specifically, these results were likely due to the insects in the incubator being able to ingest food with higher humidity (60% R.H.) that provided

the hydration necessary to aid in digestion. Insects on the countertop may have lost water weight from the drier air (~40% R.H.) and used more energy for homeostasis, thus impacting their post-feeding weight. Another factor was assuring that there was lower variation in data by having insects at the same life stage, and thus colonies were rotated on a weekly basis. An additional problem encountered was due to insect larvae molting during the experimental period, which created a loss in weight that was probably not due to diet intake. To counter the problems with molting, consideration of the temperature (whether it was inside an incubator or not) and decreasing the feeding timeline of the bioassay were addressed as potential issues. All data collected through the three experiments suggested that *D. maculatus* was the best of the three Dermestidae species tested for purposes of this PER assay because of the consistency and higher weight gain, protein consumption, and PER values compared to those from the *Trogoderma* species. Thus, it can be concluded that out of the three species tested, *D. maculatus* has the highest likelihood to become a quality model organism in protein bioavailability studies to evaluate ingredients used in companion animal diets.

Proteins are essential to the nutrition and health of animals. Influencing structure and function down to the genetic level (nutrigenomics) has become critical in livestock applications, such as the influence on milk yield and fatty acid profile in muscle tissue which could eventually influence companion animal health research (Nowacka-Woszuik 2020). Although overlooked by many studies, proteins also provide nutrition to the gut's microbiome after being digested by the host, which in turn can influence other health effects including inflammation, kidney disease, diabetes, heart disease, allergens, and oral health. The full scope of a protein's influence on the animal's health will likely never be fully understood or implemented within the pet food industry's research. With the growing concerns of pet owners for their companions, many

companies aim to aid in health and nutrition with direct claims on the front of their packages to reassure and inveigle consumers, often claiming higher quality and quantity of protein sources.

Throughout the most recent years of the SARS-CoV-2 viral pandemic, and the years leading up to the pandemic, there were rising numbers of pet owners, people bonding with their pets, and a tendency to anthropomorphize their pets, leading to advances of the pet food industry (White 2022, Schleicher et al. 2019). Global pet food sales, predominantly for cats and dogs, have been vastly increasing over the past decade with a nearly 70% increase from \$78.1 billion to \$114.8 billion from 2011 to 2021, respectively (Watson et al. 2023). With an ever-growing market and an increasing clientele, pet food companies have been following trends in the human food industry to appeal to the owners, including vegetarian, vegan, and “premium diets” (which aim to target the owners’ lifestyles including non-GMO, sustainability, or natural ingredients); some of which claim to be healthier (Kumcu and Woolverton 2015, Watson et al. 2023). With owners growing a deeper connection to their pets they have been favoring brands that claim more health benefits and better nutrition compared to previous years, especially those claiming higher quality and quantity of protein. For these reasons, it is important to consider the costs, regulations, and management of the bioassays used for testing pet food ingredients, and use of insects in protein analyses could certainly satisfy these needs.

This study and subsequent research may influence the pet food industry to reassess methods to analyze formulations. Future experiments will need to refine the process further and test other variables, such as different insect species models, humidity, temperature, processing, amino acid analyses, and even the type of packaging since some incorporate insecticides to protect the product during storage. If insect testing is validated, a major benefit to companies is to reduce the use of vertebrates such as mice, chickens, cats, or dogs as the preliminary screening

tool to evaluate protein quality. This benefit alone could improve overall public perception of pet food processing and how the products are made (Kwak and Cha 2021).

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Figure 2.1 Comparison of all three species, *Trogoderma variabile*, *Trogoderma inclusum*, and *Dermestes maculatus*, on Protein Efficiency Ratios (PER) interaction in relation to locations (counter, top rack, or middle rack) over a six-day period using the standard mean, +/- 3 interquartile range. All treatments had ten individual larvae. The full model interaction has an $F_{4,82} = 1.37$ and $P\text{-value} = 0.2522$, analysis of the species alone has an $F_{2,84} = 0.98$ and $P\text{-value} = 0.3783$, and analysis of placement alone has an $F_{2,84} = 0.96$ and $P\text{-value} = 0.3884$.

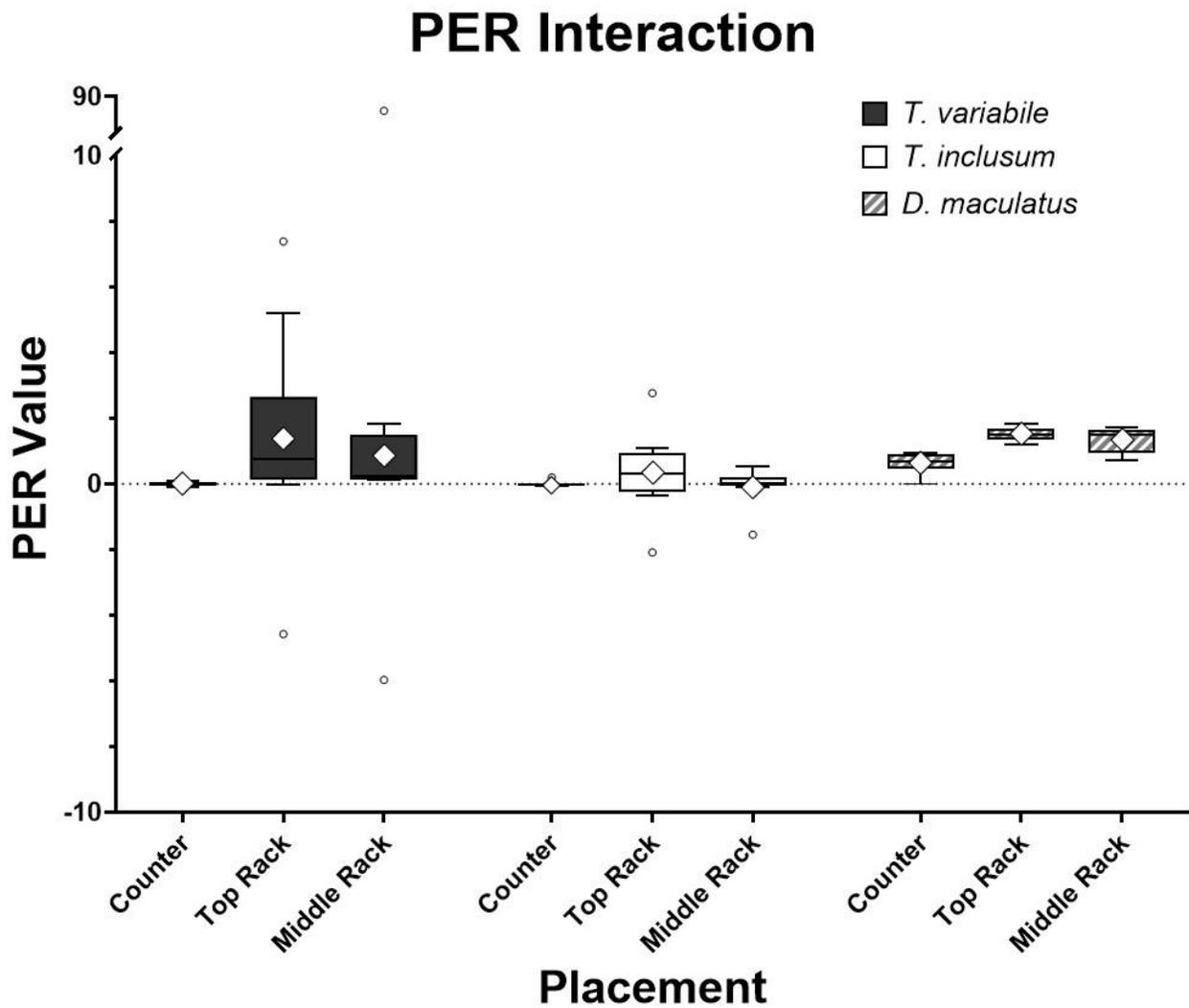


Figure 2.2 Comparison of all three species, *Trogoderma variabile*, *Trogoderma inclusum*, and *Dermestes maculatus*, on Protein Efficiency Ratios (PER) interaction in relation to locations (counter, top rack, or middle rack) over a six-day period using the standard mean, +/- 3 interquartile range after removing six major outliers. All treatments had ten individual larvae. The full model interaction has an $F_{4,76} = 21.64$ and $P\text{-value} < 0.0001$, analysis of the species alone has an $F_{2,76} = 21.55$ and $P\text{-value} < 0.0001$, and analysis of placement alone has an $F_{2,76} = 10.5$ and $P\text{-value} = 0.0001$. Note that none of the *Dermestes maculatus* data deviated from the results in Figure 1.

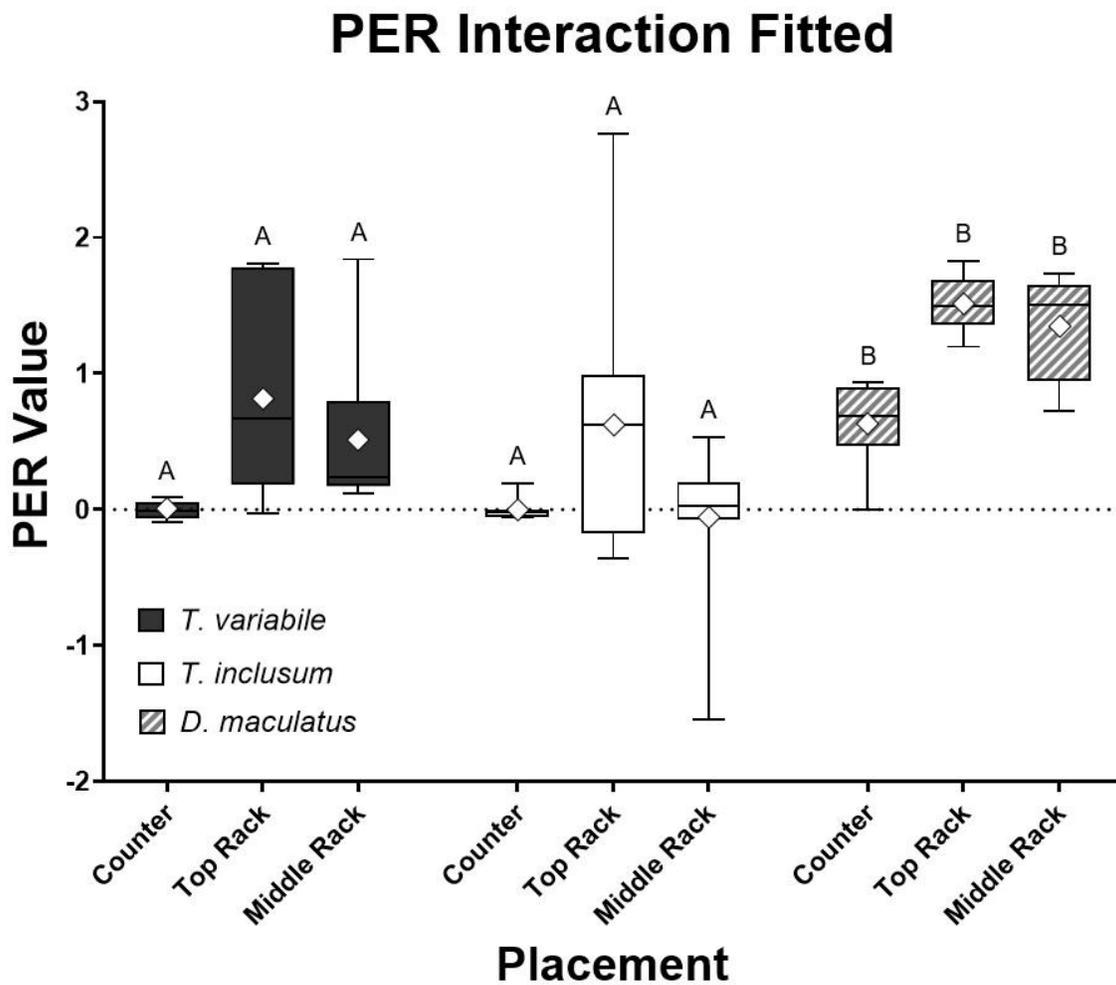


Table 2.1 The growth performance and protein efficiency ratio (PER) of three different dermestid species in three different environments (n=10 per row).

Insect Species	Location	Avg. (SE) Body	Avg. (SE) Diet	Avg. (SE)	Avg. (SE)	Avg. (SE) PER
		Weight Gain (mg)	Consumed (mg)	Gain:Feed Ratio	Crude Protein Intake (mg)	
<i>T. variabile</i>	Counter	-0.021 ± 0.04	10.28 ± 2.02	-0.002 ± 0.01	2.724 ± 0.54	-0.0063 ± 0.02
<i>T. variabile</i>	Incubator top rack	1.028 ± 0.29	3.71 ± 0.91	0.363 ± 0.27	0.983 ± 0.24	1.3684 ± 1.01
<i>T. variabile</i>	Incubator middle rack	0.847 ± 0.30	3.60 ± 1.08	2.871 ± 2.99	0.954 ± 0.29	10.8366 ± 11.28**
<i>T. inclusum</i>	Counter	-0.014 ± 0.04	8.35 ± 0.69	-0.002 ± 0.01	2.213 ± 0.18	-0.0047 ± 0.02
<i>T. inclusum</i>	Incubator top rack	0.158 ± 0.08	2.93 ± 1.80	0.092 ± 0.11	0.776 ± 0.48	0.3464 ± 0.40
<i>T. inclusum</i>	Incubator middle rack	0.284 ± 0.11	15.31 ± 6.14*	0.014 ± 0.05	4.058 ± 1.63	-0.0568 ± 0.18
<i>D. maculatus</i>	Counter	2.985 ± 0.51	16.99 ± 1.18	0.167 ± 0.03	4.502 ± 0.31	0.6313 ± 0.09
<i>D. maculatus</i>	Incubator top rack	8.791 ± 0.79	22.51 ± 2.53	0.401 ± 0.02	5.965 ± 0.67	1.5130 ± 0.07
<i>D. maculatus</i>	Incubator middle rack	7.969 ± 1.27	22.59 ± 2.42	0.350 ± 0.03	5.985 ± 0.64	1.3490 ± 0.13

*This calculation includes one major outlier with 53.1 mg of diet consumed.

** This calculation includes one major outlier with PER of 89.591.

Table 2.2 The growth performance and protein efficiency ratio (PER) of *Dermestes maculatus* in three different environments (n=10 per diet/location combination) for two days. Any of the larvae that had molted were discarded from the statistics.

Diet	Location	Number of Used Values	Avg. (SE) Body Weight Gain (mg)	Avg. (SE) Diet Consumed (mg)	Avg. (SE) Gain:Feed Ratio	Avg. (SE) Crude Protein Intake (mg)	Avg. (SE) PER
Purina One Lamb and Rice	Top left rack	7	3.085 ± 0.46	6.243 ± 0.66	0.466 ± 0.05	1.654 ± 0.18	1.7588 ± 0.20
Purina One Lamb and Rice	Top right rack	8	3.336 ± 0.81	5.55 ± 0.75	0.531 ± 0.13	1.471 ± 0.20	2.0042 ± 0.48
Purina One Lamb and Rice	Middle rack	3	2.709 ± 0.35	4.967 ± 0.87	0.557 ± 0.04	1.316 ± 0.23	2.1004 ± 0.13
None	Middle rack	8	-0.4671 ± 0.05	0 ± 0.00	-	-	-

Table 2.3 The growth performance and protein efficiency ratio (PER) of *Dermestes maculatus* in the middle rack of the incubator (n=20 per diet).

Diet	Avg. (SE) Body Weight Gain (mg)	Avg. (SE) Diet Consumed (mg)	Avg. (SE) Gain:Feed Ratio	Avg. (SE) Crude Protein Intake (mg)	Avg. (SE) PER
Purina One Lamb and Rice	1.572 ± 0.13	2.36 ± 0.19	0.656 ± 0.03	0.625 ± 0.05	2.4759 ± 0.13
Negative Control	0.142 ± 0.02	-1.105 ± 0.08	-0.163 ± 0.04	0 ± 0.00	-

**Chapter 3 - Determining Pet Food Protein Quality Formulated with
Different Protein Sources Using Hide Beetle, *Dermestes maculatus*
DeGeer (Coleoptera: Dermestidae), as a Model Organism**

Introduction

With pet owners in developed nations growing ever more aware of what ingredients go into their companion's food, pet food companies continue to investigate novel ingredients, aim to improve pet health, and provide a more balanced, nutritious meal (Polyn 2022). The protein efficiency ratio (PER) method is commonly used to determine protein quality of individual ingredients, including those that may not be safe for certain animals but are for others based upon which model organism is used (Donadelli et al. 2019, Cheng et al. 2022). PER studies have become a more common practice since 2010 to accommodate the demands of companion animal owners. Model vertebrate animals are commonly used for PER studies, but non-vertebrate models need to be investigated as alternatives.

Hoffman and Falvo (2004) determined that casein provided an excellent reference PER value of 2.7 when the experiments were carried out in rats, *Rattus norvegicus*, (Cheng et al. 2022). Even though the first model organism was the rat in 1919 (Osborne et al. 1919), the models have evolved over time. The most used model organism currently, at least in the United States, are domestic chickens, *Gallus gallus domesticus* (Reilly et al. 2020, Donadelli et al. 2019, Cheng et al. 2023, Molnar et al. 2016). Donadelli et al. (2019) found that low-temperature fluid bed air-dried chicken had the second highest PER value, and that vegetable proteins required complimentary amino acid supplementation to be nutritionally adequate in comparison to the spray-dried egg. In a separate study, Molnar et al. (2016) evaluated innovative preparations of chicken, including spray-dried, fluid-bed-dried, chicken meal, and chicken by-product meal and found that fluid-bed-dried chicken had the best PER compared to spray-dried egg, suggesting better ways to retain protein quality. The novel sources researched by Cheng et al. (2020) and Reilly et al. (2020) were black soldier fly larvae meal, *Hermetia illucens* (Linnaeus) (Diptera:

Stratiomyidae), and potato proteins, respectively. A 50% replacement of soybean meal with fortified black soldier fly larvae meal were found to be comparable in growth to soybean meal and have the highest PER value and better metrics than all other diets in the study (Cheng et al. 2020). Another study using *H. illucens* larvae indicated no limiting amino acids regarding feline nutritional needs, thus showing promise in its use as a novel protein (Do et al. 2020). Potato protein was found to be an easily digestible, energy-dense ingredient that had a higher PER value than pea protein and was determined to be safe for both dogs and cats as a novel ingredient, although to become balanced it would need to be supplemented with methionine and cysteine from cereal grains (Reilly et al. 2020). In a PER study using rats, rats chose the soy, chicken breast, and offal compared to whole chicken; however, the whole chicken had the highest PER of all the diets (Rajkumar et al. 2021). Standardizing methodologies and minimizing inter-animal comparisons between experiments can aid in refining protocols and allow for more accurate replication (Schapiro and Everitt 2006).

In a preferential study with dogs and different diets, the diets of choice tended to have lower PERs and could depend on various factors, such as the ingredients, texture, or humidity, which has also been observed in rats (Figuroa et al. 2016, Rajkumar et al. 2021). There often are trade-offs in pet food formulations between the nutrition and preference of the diets for target pets, leading companies to determine which is more important, or to make varieties of diets to cover all preferences and nutrient needs. Most pet food companies tend to focus primarily on dog and cat foods. A majority of nutritional studies are based upon canine nutrition, with few studies done for felines. Industry generally requires that the digestibility of amino acids should be greater than 90%, so that amino acid digestibility differences between cats and dogs may be negligible (Reilly et al. 2020). However, it is critical to remember that felines have an extra

essential amino acid, taurine, compared to canines, in order to meet the pet food formulation requirements (Li and Wu 2023).

Pet food companies either formulate their diets according to AAFCO guidelines or by validating their diets through nutritional studies for target species. Animal studies are regulated by the Institutional Animal Care and Use Committee (IACUC). IACUC commonly questions publicly funded laboratories in the USA to justify the use of animals instead of carrying out an *in vitro* study, using a computer simulation, or using a less sentient animal (Silverman 2015). Many people, as well as government regulators, do not recognize insects as research animals and therefore do not have the same regulatory concerns as for vertebrate animals. With the lack of moral and ethical concern for insects, IACUC approval is not necessary when using insects as a model organism in studies that may lead to the death of insects or negatively affect their survival (Fischer and Larson 2019). The largest concerns to be considered by IACUC after the Animal Welfare Act are proposed to be Brambell's five freedoms, defined as being free from starvation or thirst; minimizing any physical or psychological inconvenience; being free from pain, injury, or disease; reducing fear and stress; and decreasing any limitations in the animals' natural behaviors, which also are suggested to be considered when using insects in studies by De Goede et al. (2013).

Benefits for using insects as ideal model organisms for PER studies include their fast generation times and the ability to conduct many more replicates than could be financially and practically feasible with vertebrates. The generation times in ectotherms has been linked to body size and is affected by their surrounding temperature, resource availability, latitude of distribution, season, or day lengths (Thomas et al. 2020). The generation time of insects also is highly dependent upon protein and energy intake, population size affecting competition for food,

and insect metabolism. Adult body size and longevity have a negative relation to the generation time (Jensen et al. 2017). Many external factors, such as daylight, temperature, and humidity can be artificially controlled for insects adapted to laboratory rearing with a controlled environment incubator.

Results from work reported in Chapter 2 demonstrated that larvae of the beetle species *Dermestes maculatus* are suitable invertebrate alternatives to vertebrates for determining the protein quality of different protein sources for companion animal diets. The objective of this chapter was to determine the effect of different diet ingredients on the PER variables relative to results previously obtained in studies using rats, dogs, or chickens. To aid in the accessibility of PER studies in future pet food studies, the use of *D. maculatus* larvae in PER bioassays will be conducted to determine if they can serve as a model organism in comparison to the target species and (or) previously used vertebrate models.

Materials and Methods

Insect colony:

The colony of *D. maculatus* used for this work were reared from wild-captured beetles collected in 2009 that were found in powdered blood used for manufacturing at a pet food processing plant and then maintained by scientists at the Center for Grain and Animal Health Research (CGAHR) in Manhattan, KS. The sub-colony used for this series of studies was reared with a centimeter thick layer of Purina One Lamb and Rice diet (Nestlé Purina PetCare Co., St. Louis, MO, USA) topped with a thin layer of rolled oats (The Kroger Co., Cincinnati, OH, USA) inside 6-quart (30 cm x 15 cm x 9 cm) plastic bins (Sterilite Co., Townsend, MA, USA). The lids of each bin were ventilated with two 3 cm diameter holes that were covered with a fine metal

mesh to allow air flow but prevent beetle escape. Each bin also had a 1 square-inch faux fur fabric placed upside down near the end of the container as described by Fontenot et al. (2014) to promote egg laying as well as two folded paper towels for shade and surface area. The colony was maintained in a Powers Scientific small insect incubator (Model: DROS33SD3, Powers Scientific Inc., Doylestown, PA, USA) set to 25°C and 16:8 light:dark photoperiod at a relative humidity of ~65%. Colonies were rotated every week, with 90 adults placed in the container for 24 hours to lay eggs and then removed, to have larvae of the same relative age and instar for PER experiments. For the experiment, small second-instar to third-instar (two-week old) active larvae were used.

Protein content of diets:

Seven diets were used in this experiment to test the protein efficiency ratio (PER) of *D. maculatus*. The balanced diet (which met all nutrient requirements of an adult dog) was Purina One Lamb & Rice diet (PLR) (Nestlé Purina PetCare Co., St. Louis, MO, USA), available commercially, which had 22% protein. All remaining diets were made in the Kansas State University Pet Food lab, courtesy of Alaina Mooney. The negative control diet (NC) was composed of cornstarch and dextrose (added in a 2:1 ratio), mineral premix (5.365%), soybean oil (5.365%), choline chloride (0.22%), and vitamin premix (0.203%), completely devoid of any added protein source. All laboratory prepared diets were made from ingredients that were dehydrated and broken down into a fine powder.

The KSU diets were made in reference to the negative control. To account for the protein in the diets, the 2:1 cornstarch to dextrose mix was removed to account for a total of 10% protein. The reference protein diet used was spray-dried egg (SDE) as 21.21% of the total diet makeup. The remaining tested protein sources were formulated as follows: soy protein isolate

(SPI) at 14.04%, corn gluten meal (CGM) at 15.94%, menhaden fish meal (FM) at 16.75%, and pea protein concentrate (PPC) at 19.76%. Not all the diets were as homogenous as the commercially prepared diet. The three plant-based protein diets, SPI, CGM, and PPC, all had small masses within the powdered diet, likely from the durability of the plant material.

Nitrogen is an element found in proteins, making up approximately 16% of its structure and is not found in carbohydrates or fats. Thus, there is a crude protein coefficient of 6.25 for proteins ($100\%/16\% = 6.25$). To measure the nitrogen content of the food, the protein coefficient was input into a nutrient composition instrument, the LECO FP928 Nitrogen and Protein Analyzer (LECO Co., St. Joseph, MI, USA) that combusts the food to read the nitrogen content of the resulting gases. Three samples of 3.0 g of each of the seven diets were placed into the instrument's ceramic weigh boats and then placed on the machine's loader where the sample was deposited for testing. The sample was burned off at a temperature of 1100°C and a thermal conductivity cell detected the nitrogen content of the air to determine what percentage of the sample was protein. The protein percentages were then averaged from the three samples and used in the calculations for the PER of the respective diet.

Effect of protein availability on dermestid larval growth:

Three separate replicates of the experiment were performed in the middle rack of the incubator. The replicates included 10 larvae in individual glass vials for all seven diets, fasted for 24 hours while held in the incubator to expend any food within the digestive tract that may interfere with the PER calculations. Single 3-dram 15 mL (20 x 65 mm) glass shell vials (Thermo Fisher Scientific Inc., Waltham, MA, USA) were weighed on a Denver Instrument Company A-160 analytical balance (Denver Instrument Company, Arvada, CO, USA) and then tared before adding $0.5 \text{ g} \pm 0.005 \text{ g}$ of the diet. Vials with diet were then placed in an Equatherm

oven (Curtin Matheson Scientific Inc., Elk Grove Village, IL, USA) at 70°C. After 24 hours, the diets were re-weighed on a dry matter basis and placed in a refrigerator at 4°C to cool rapidly to room temperature. Beetle larvae were all weighed individually on a Cahn 28 automatic electro-balance (C.Y. Scientific LLC, Irvine, CA, USA) and placed into a vial containing one of the seven diets. Once all the vials received the diet and a larva, they were capped and placed on the middle rack of the Powers Scientific small insect incubator (Model: DROS33SD3, Powers Scientific Inc., Doylestown, PA, USA) set to 25°C and 16:8 light:dark photoperiod at a relative humidity of ~65% for 24 hours. Larvae were all individually weighed again and the remaining food and the glass shell vial were weighed and placed back in the oven for another 24 hours to dry the diet and remove any water vapor that was absorbed. Lastly, the diets were weighed again.

Data analysis:

Each individual larva was assessed separately. To calculate the PER, the following formula was used:

$$PER = \frac{\text{larval weight gain (g)}}{\text{protein availability for absorption}} = \frac{\text{final larval weight} - \text{initial larval weight}}{[(\text{initial diet weight} + \text{vial weight}) - (\text{final diet weight} + \text{vial weight})] * (\text{Protein \% decimal})}$$

Data collected was analyzed on a single variable of the protein of the diets (treatment) as a one-way ANOVA in a randomized complete block design using JMP Pro Software (SAS Institute Inc., version 16.0.0, 2021). The treatments were considered the experimental unit with three replicates each as the blocks. After the one-way ANOVA showed significance between treatments, the data was then put through a post hoc Tukey-Kramer honest significance test to compare the means of each treatment to identify any differences greater than an expected error. This method is conservative when the sample sizes become unequal. Outliers in this study were

defined as being any point of data that lies over 1.5 inter-quartile ranges below the first quartile or above the third quartile in a data set. Figures were graphed using GraphPad Prism Software (GraphPad, version 10.2.1, 2024).

Results

All individual *D. maculatus* larvae readily ingested their respective diets while being held in consistent conditions within the incubator and remained healthy during the experiment (Figure 3.1). The larval body weight gain was significantly more ($P < 0.05$) for those fed PLR, SDE, and FM (2.013, 1.895, and 1.173 mg, respectively). The PLR and SDE had a statistically larger amount of weight gain compared to FM ($P < 0.05$). All other experimental treatments of SPC, PPC, NC, and CGM resulted in lower weight gains (0.32, 0.317, 0.268, and 0.231 mg, respectively) all of which were insignificant ($P > 0.05$) compared to the PLR, SDE, and FM. All larvae gained weight, likely due to their digestive tracts having diet present. However, if the bioassay were to continue, the NC larvae would likely either lose weight or remain stagnant from the lack of protein necessary or by avoiding diet intake for proper growth. Larvae on SPC, PPC, and CGM experimental treatments would continue to grow, though at slower rates compared to PLR, SDE, and FM experimental treatments.

Interestingly, the overall protein consumption did not directly correlate to the findings of diet ingestion, though it did follow the general trends in the amounts ingested (Figure 3.2). The amount of protein consumed was highest in larvae fed the PLR experimental treatment (0.689 mg), as expected since the experimental treatment had more than twice the amount of protein compared to the laboratory made treatments. The amount of protein in larvae fed PLR was significantly more ($P < 0.05$) than those fed SDE (0.288 mg). The amount of protein ingested by

larvae fed SDE also was significantly different ($P < 0.05$) compared to those fed FM (0.180 mg). Larvae fed SPC, CGM, and PPC had a low protein intake and were not statistically different from one another ($P > 0.05$). However, larvae fed SPC, CGM, and PPC had significantly less protein ingestion ($P < 0.05$) than those fed FM. NC experimental treatment had significantly less ($P < 0.05$) protein consumption compared to all other diets since there was no protein intake.

Dermestes maculatus larvae reared on each diet had various PER value's (Figure 3.3). The PER values did not follow the trend seen before based on the diet intake or protein consumed (Mooney et al., n.d. personal communication). The two highest PER values were from larvae fed SDE and FM experimental treatments ($P < 0.05$). Next, larvae fed PPC had a PER value similar to those fed SPC but different from all other experimental treatments ($P < 0.05$). The PER value of larvae fed SPC was comparable to CGM ($P > 0.05$). Similarly, PER values of CGM, SPC, and PLR were similar ($P > 0.05$). However, PER values of larvae fed PLR were significantly different compared to all other experimental treatments ($P < 0.05$). Larvae fed the NC experimental treatment had an undefined PER due to no ingested protein and therefore was significantly different than the PER of all other diets ($P < 0.05$).

The PER in five of the diets had outliers which, when accounted for in the averages, had some slight impact on the data (for PLR, SPC, CGM, FM, and PPC). The diet with the most outliers was FM (four), followed by CGM (two) and PLR, SPC, and PPC each with one, making a total of nine datapoints that were removed (Figure 3.4). Overall, the study only showed 4% of the outcomes falling outside of a normal 95% confidence interval statistical range. With each experimental treatment having 30 replicates, there were still enough to compare the normal distribution of the PER values to other studies that often have fewer replicates. The outlier values were removed within the statistics that were then re-run.

With outliers, there was an increase in statistical variances (Figure 3.3, Figure 3.4). Outliers were caused by larvae preparing to molt and from certain diets clinging to the larval hairs. When a larva prepared to molt, it negated time from ingesting food and thus lowered their weight gain, lowering the PER value. When the diets clung to the larval hairs, it increased their post-feeding weight increasing the PER value. The two highest PER values still obtained from larvae fed SDE and FM experimental treatments and were significantly higher than the rest ($P < 0.05$). PER values from larvae fed SPC and PPC were significantly different from those from all other diets ($P < 0.05$). Larvae fed the two plant-based isolates had similar PER values ($P > 0.05$). PER values were relatively low from larvae fed PLR and CGM experimental treatments and were similar ($P > 0.05$). The range in PER values was lowest in larvae fed the PLR experimental treatment, anticipated from a commercially produced product. The NC diet had an undefined PER due to the formula used (weight gained divided by zero mg of protein ingested). Therefore, the NC diet was statistically significant in relation to the PER of all other diets ($P < 0.05$). Values are shown as the standard mean +/- 3 interquartile range after removing nine outliers (Figure 3.3). After removal of outliers, the replicates varied among treatments, with PLR (n = 29), NC (n = 30), SDE (n = 30), SPC (n = 29), CGM (n = 28), FM (n = 26), and PPC (n = 29), all using different larvae. The interaction between diet and PER was $F_{5,169} = 42.144$ and P -value < 0.0001 . Results from Figures 3.1 - 3.4 are summarized within Table 1.

Discussion

In this study, *D. maculatus* was assessed as a model to determine the quality of seven different protein sources. The highest PER values were in larvae fed the positive control protein, SDE, and FM, a known animal feed of choice for *D. maculatus* in Africa (Ugwu et al. 2005). Although previous studies have demonstrated FM as a high-quality protein, the PER values of

FM typically are not close to matching the PER value of SDE (Folador et al. 2006). *Dermestes maculatus* are pests of dried fish, and fish products are some of their most common food sources (Osuji 1975). Larvae reared on both the SPC and PPC experimental treatments had moderate PER values. Plant protein concentrates retain the dietary fiber from the plant source potentially improving stool quality and satiety but lacking in caloric energy (Reilly et al. 2020). Larvae fed PLR and CGM experimental treatments had low PER values in comparison to the other diets. The PLR experimental treatment, with a protein percentage more than double the other experimental treatments, resulted in a low PER value, likely due to the high protein allowing the larvae to feel satiated. The CGM-fed larvae had the lowest body weight gain, which could be caused in part due to the diet not being a homogenous powder from the thick pericarp of corn and thus allowing the larvae to potentially eat around the protein-rich areas of diet.

In previous PER studies using vertebrates such as rats, dogs, cats, and chicks, the positive control diets have PER values of spray dried egg of ~5 or casein of ~2.5, both occurring with animals consuming a 10% protein diet (Donadelli et al. 2019, Cheng et al. 2023, Osborne et al. 1919, Molnar et al. 2016, Hoffman and Falvo 2004). The present study had a much higher PER with the outlier, 9.07, from PPC-fed larvae. However, the highest average PER value of all seven experimental treatments without the outliers was that of SDE at 6.45, much closer to the previous scale of a relative value of 5. These results potentially may be explained by the variations in insect digestion, their size, their gut microbiota, their genetics, or from heterogenous lab-prepared diets. The two most consistent PER values were those of larvae fed PLR or FM experimental treatments, both of which are a homogenous and commercially prepared diet or a naturally known diet of the insect, respectively.

The next step to further investigate *D. maculatus* as a potential model organism would be to test prepared diets at various protein percentages, 0%, 5%, 10%, 15%, and 20%, to demonstrate the larval efficiency in utilizing increasing amounts of the same protein for growth, resulting in higher PERs from higher protein percentage diets. This will provide additional data for PER studies and allow researchers to rank the protein ingredients and evaluate the ability to supply essential amino acids to the model, as was done in this experiment. The protein sources that showed the best bioavailability to *D. maculatus* in this study were SDE > FM > PPC > SPC > PLR > CGM > NC.

A potential issue in using insects as a new model organism for companion animal feed studies could be the difference between the insect digestive system and the monogastric dog and cat. In mammals, proteins begin their transport in enterocytes of the small intestinal epithelium and decline after reaching maturity, when there is no longer a need to rely upon milk as a primary feed source (Jeffers and Roe 2008). The proteins may go through either a mediated or a non-mediated general transport between epithelial cells (Jeffers and Roe 2008). An overwhelming number, over 55 subtypes, of solute carrier (SLC) families have been studied in mammals (He et al. 2009). A well-studied transporter family in mammals are the SLC7's, which are known to allow passage of most essential amino acids, specifically cationic amino acids, and are aided by heterodimeric amino acid transporters (Holtorf et al. 2019). The mammal's gut microbiota begins to establish itself within the first few milk feedings and develops over time as in the intestines as a symbiotic environment. Insects undergo metamorphosis and may have different microbiota in their life stages and leading to variation in their microbiomes (Schmidt and Engel 2021). Certain bacteria can enhance protein absorption, specifically the branched-

chain amino acids within the hemolymph, which should be investigated to determine if it occurs in dermestids (Engel and Moran 2013).

In the insect digestive system, digestive enzymes are active in the midgut, where proteins are hydrolyzed to allow for absorption of peptides through the peritrophic membrane (Terra 1990, Holtof et al. 2019). Insects have different pH values within different regions of their digestive systems that affect the proteolytic activity within their gut (Hosseininaveh et al. 2007, Holtof et al. 2019). Both endopeptidase and exopeptidase enzymes are found in the gut lumen and in the enterocytes to break down proteins into smaller peptides and amino acids (Hosseininaveh et al. 2007, Holtof et al. 2019). Insect SLC6 transport neutral amino acids across the apical membrane of the epithelial cells of the midgut, SLC7 transportation of cationic amino acids into the fat body cells, and in parallel to mammals SLC15 transportation of oligopeptides (Holtof et al. 2019, Boudko 2012). With the large variations in diet across all the class Insecta, different insects ingest more certain amino acids than others based on their diet, thus having a varying expression and abundance of these transporters. Dermestid beetles lack cysteine proteinase activity, unlike some other beetles, and rely mostly on serine peptidases for digestion (Hosseininaveh et al. 2007). In a closely related species, *Dermestes frischii* (Kugelann), there was a highly conservative fragment of cysteine protease peptides that was anticipated to serve as a protease activity inhibitor, which could account for the low cysteine proteinase activity (Papisova et al. 2011). Yellow mealworms, *Tenebrio molitor*, have been tested and show that both the larvae and adults have similar enzymatic activities in similar gut regions suggesting parallel digestion patterns throughout the life stages (Terra 1990). Many coleopteran insects have an acidic gut, however, the dermestid beetle *T. granarium* has midgut luminal extracts within an alkaline range of pH 8-11 (Hosseininaveh et al. 2017). Digestive enzymes are complex

depending upon numerous factors including temperature, pH, abundance, and catalysts to be efficient and reach optimum capacities.

In reference to satisfying IACUC's standards, the often-cited work of Russell and Birch (1959) defined the three R's of humane experimental techniques. The three R's are known as replacement (substituting with either a lower ranking organism or *in vitro*), reduction (lowering the number of replicates or animals used), and refinement (decreasing the amount of inhumane procedures carried out on the subjects). To become successful in the future, replacements will need to continue to be developed and be adapted to new areas of research (Hubrecht and Carter 2019). With this newly proposed use of insects as models for companion animal feed studies, the replacement aspect of the three R's would be satisfied. However, there are varying conditions on what would be acceptable in differing regions of the world. According to Sneddon et al (2017), Brazil, China, and India consider "all" animals to be protected, though many people fail to realize that insects are classified as animals. Most other regions would consider the use of any invertebrate that is not a cephalopod or crustacean to be adequate in replacing vertebrates. In terms of refinement, the use of stored product insects, such as *D. maculatus*, would amply meet their handling needs since they have been reared for decades in differing laboratories and show abundant husbandry.

There are other potential insect models for future studies. Insects with chewing mouthparts that are relatively carnivorous and have been found to infest pet food or similar products could all be considered as potential models. A recent study investigated the feed conversion rates of *Tenebrio molitor*, the most commonly traded and cultivated insect in Europe, when fed various diets to improve the colony performance as feed for other animals (Bordiean et al. 2020). Other examples of probable models include the use of red-legged ham beetle larvae,

Necrobia rufipes (DeGeer); Indianmeal moth larvae, *Plodia interpunctella* (Hubner); red flour beetle larvae, *Tribolium castaneum* (Herbst); cigarette beetle larvae, *Lasioderma serricorne* (Fabricius); or black soldier fly larvae, *Hermetia illucens*; all stored product insects that have been heavily studied due to their infestations in warehouses, grain bins, and products within stores.

One of the future aims of my research to implement the use of insects as a model organism for companion animal feed would be to encourage entomophagy. Entomophagy has been in practice since ancient times and is still practiced regularly by over 2 billion people worldwide (Bordiean 2020). The main drivers for people's likelihood to accept entomophagy are their high nutritional value, improved gut health, their pet's routine ingestion, and low allergenicity, though links have been seen between those with allergies to shellfish and allergies to insects (Kepinska-Pacelik and Biel 2022, El-Wahab et al. 2021). According to Nyberg et al. (2021), entomophagy is a learned behavior of children from their upbringing as young kids show curiosity and an intention to try new things, including entomophagy. If entomophagy begins to integrate into the newer generations, it will become more accepted and less frowned upon for both human and companion animal consumption.

From the public's continued interest in their companion animals' pet food ingredients, we may assume that the public would become more open to eating insects after they become a more commonly seen ingredient, such as their integration into companion animal feeds. The public, in developed countries, have expressed that they would be more accepting of insect-based pet food if prescribed by a veterinarian, even though many studies have shown dogs and cats prefer the insect-based diets (Siddiqui et al. 2023, El-Wahab et al. 2021). However, according to Rajkumar et al. (2021), even with promising entomophagy studies, there are some countries such as India

where the public do not find the protein qualities of companion animal feeds to be essential, as the industry framework is lacking for growth and research. The values of companion animal feeds vary greatly based upon the cultural upbringings, ingredient availability, and scientific data available to the people. To better understand pet food innovation within developed countries, there have been recent studies to explore the incorporation of insect meals into their formulation. Land use around the world is reaching its maximum with livestock as the global population continues to increase, leading to strains in meat production and access for food companies. Both the scientific community and public have shown interest in using insects for their sustainability, novelty, and potential economic benefits (El-Wahab et al. 2021). With insects' low spatial, food, and water requirements, many studies are starting to take place in Europe to integrate insects into pet food, which could also benefit the developing countries (Kepinska-Pacelik and Biel 2022, Bordiean et al. 2020, Siddiqui et al. 2023). The largest hinderance regarding implementing entomophagy is neophobia, often caused by the insects look and as a learned behavior in childhood from adults and has prevented its commercialization in many countries (Kepinska-Pacelik and Biel 2022, Bordiean et al. 2020, Siddiqui et al. 2023, Nyberg 2021). It is anticipated that by the end of 2029, the market for insect animal feed could grow as high as \$2.386 million USD within Europe (Kepinska-Pacelik and Biel 2022). If companion animal owners begin to feed their animals insect-based diets, it may lead to the public overcoming neophobia and implementing more sustainable, novel, and nutritious practices.

This study may be the impetus to create new protocols to be implemented that limit impediments in analyzing companion animal diet formulations. The *D. maculatus* results were comparable to chicks in ranking the quality of protein sources using the same ingredients. In general, animal-based proteins had larger PER values over plant-based proteins. Digestion of

SDE and FM by *D. maculatus* larvae had the highest PER, whereas SPC and PPC experimental treatments had moderate values, and PLR and CGM had relatively low values. Future studies should investigate potential model insect's life histories, preferred foods or protein sources, and the insects' essential amino acid requirements and compare to those of the companion animal. With customers becoming more aware of their impact on the world's resources, properly implementing the use of insects as a model organism to evaluate pet food protein quality could greatly increase public perception of companion animal foods.

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Figure 3.1 Comparison of weight gained by individual *Dermestes maculatus* larvae on all seven diets: Purina One Lamb and Rice (balanced), negative control lacking any protein (NC), positive control using spray dried egg (SDE), soy protein concentrate (SPC), corn gluten meal (CGM), fish meal (FM), and pea protein concentrate (PPC). Values are shown as the standard mean \pm 3 interquartile range. All diets represent 30 biological replicates. The interaction between diet and larval weight gain (mg) had an $F_{6,209} = 109.9696$ and P -value < 0.0001 .

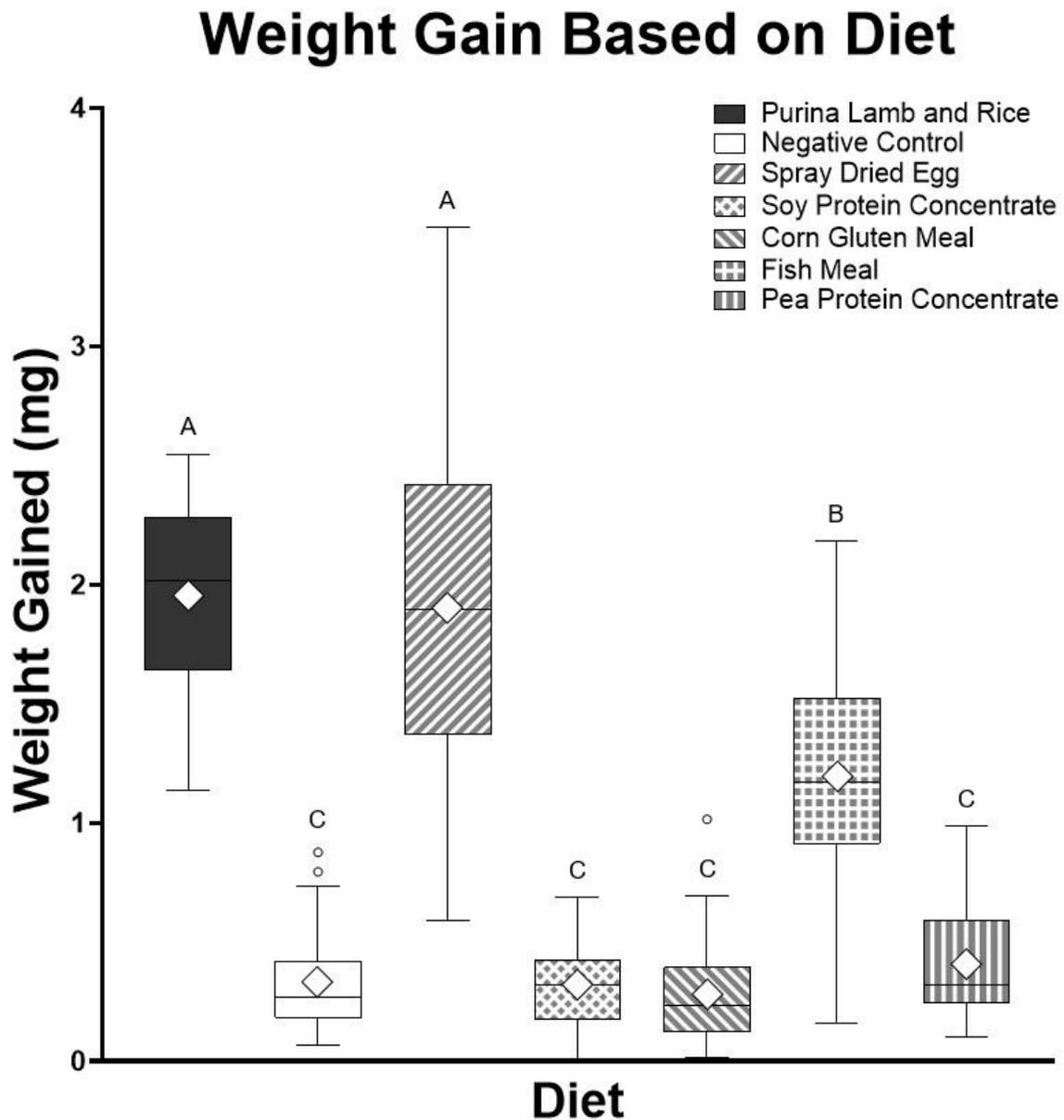


Figure 3.2 Comparison of protein consumed by individual *Dermestes maculatus* larvae on all seven diets: Purina One Lamb and Rice (balanced), negative control lacking any protein (NC), positive control using spray dried egg (SDE), soy protein concentrate (SPC), corn gluten meal (CGM), fish meal (FM), and pea protein concentrate (PPC). Values are shown as the standard mean \pm 3 interquartile range. All diets represent 30 biological replicates. The interaction between diet and protein consumed (mg) had an $F_{6,209} = 348.2604$ and P -value < 0.0001 .

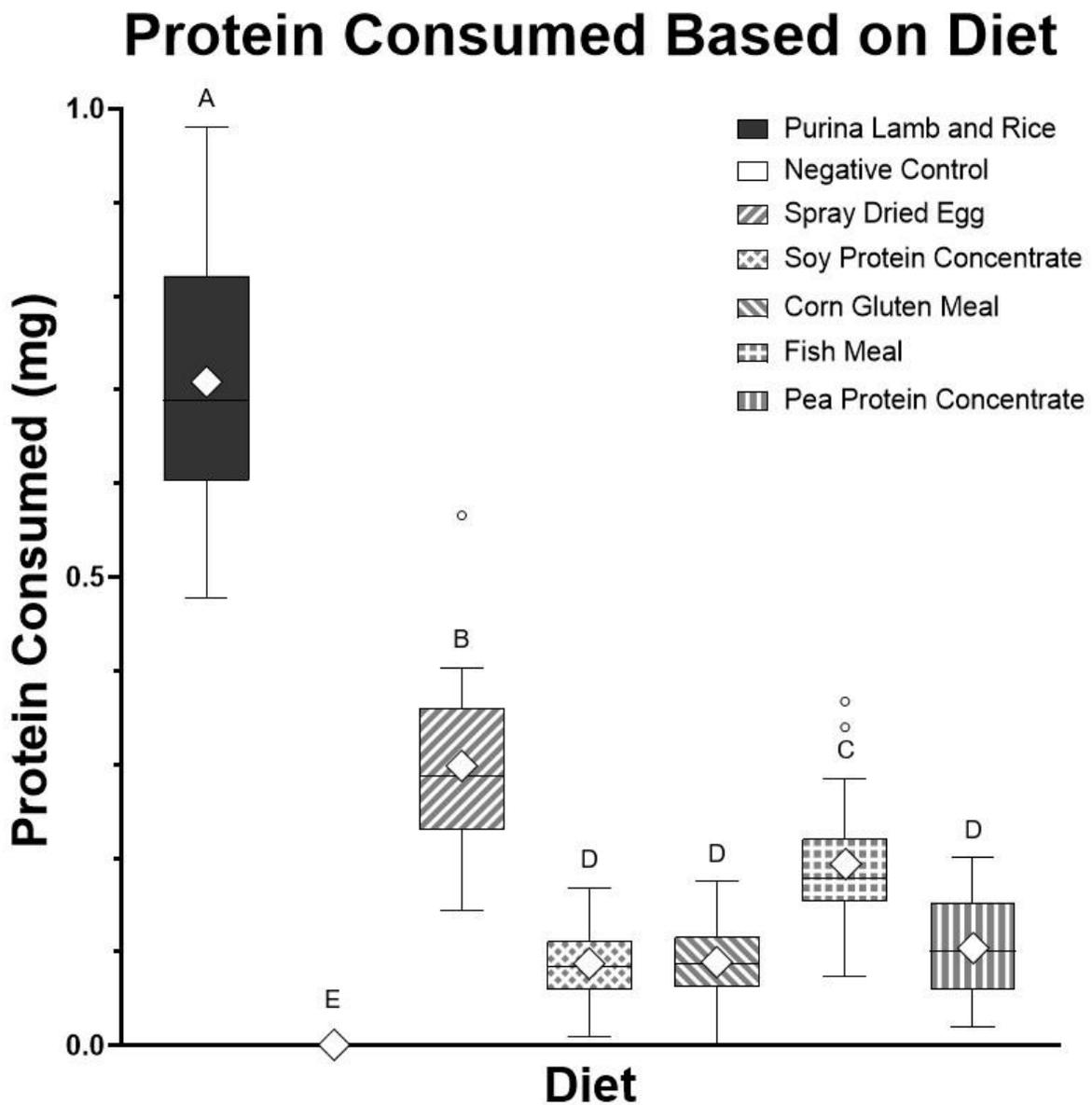


Figure 3.3 Comparison of protein efficiency ratios (PER) by individual *Dermestes maculatus* larvae on all seven diets: Purina One Lamb and Rice (balanced), negative control lacking any protein (NC), positive control using spray dried egg (SDE), soy protein concentrate (SPC), corn gluten meal (CGM), fish meal (FM), and pea protein concentrate (PPC). Values are shown as the standard mean \pm 3 interquartile range. All diets represent 30 biological replicates. The interaction between diet and PER had an $F_{5,178} = 20.1578$ and P -value < 0.0001 . Note the major outliers from the different diets.

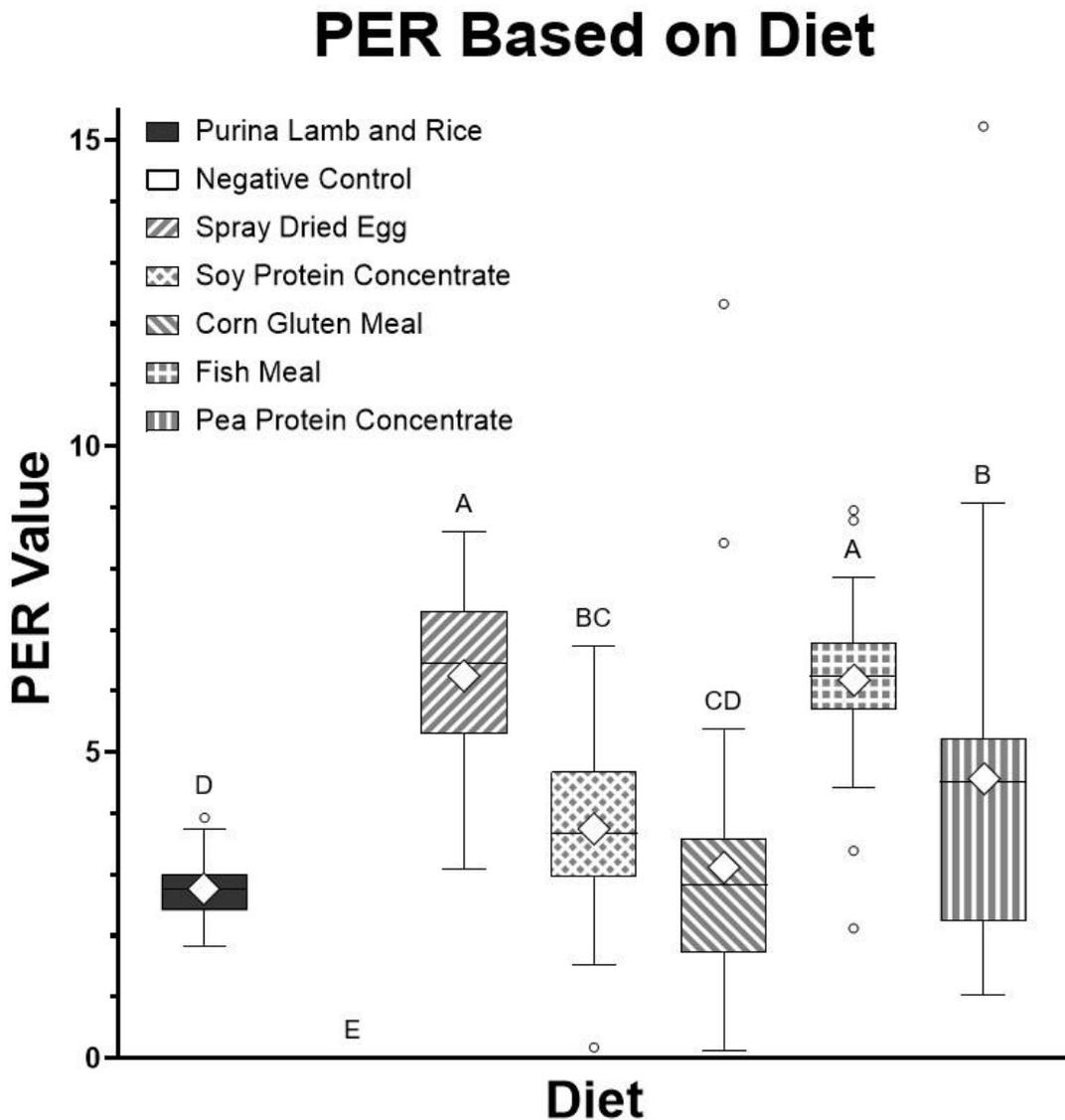


Figure 3.4 Comparison of protein efficiency ratios (PER) by individual *Dermestes maculatus* larvae on all seven diets: Purina One Lamb and Rice (balanced), negative control lacking any protein (NC), positive control using spray dried egg (SDE), soy protein concentrate (SPC), corn gluten meal (CGM), fish meal (FM), and pea protein concentrate (PPC). Values are shown as the standard mean +/- 3 interquartile range after removing nine outliers as seen in Figure 3.3. Biological replicates varied, with PLR (n=29), NC (n=30), SDE (n=30), SPC (n=29), CGM (n=28), FM (n=26), and PPC (n=29). The interaction between diet and PER had an $F_{5,169} = 42.144$ and $P\text{-value} < 0.0001$.

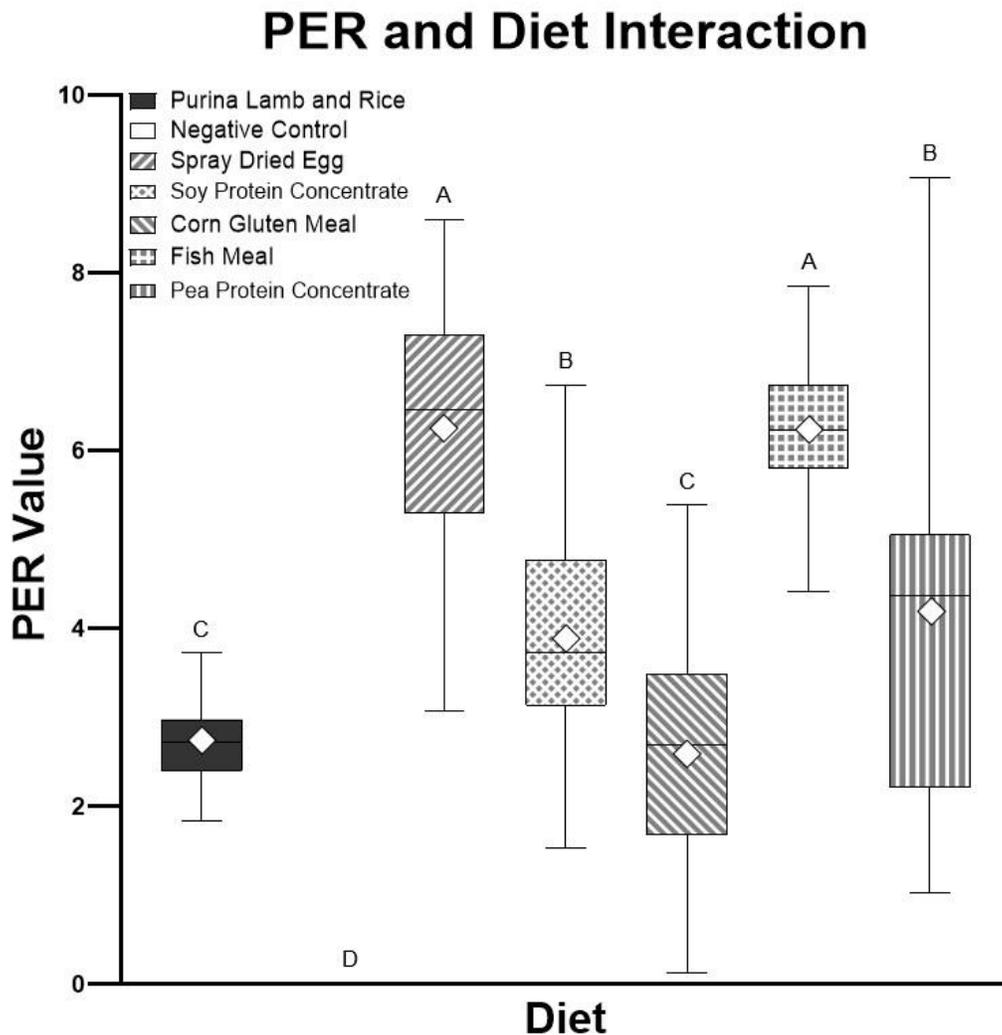


Table 3.1 The growth performance and protein efficiency ratio (PER) of *Dermestes maculatus* larvae on seven different diets (n=30).

	Avg. (SE)	Avg. (SE) Diet	Avg. (SE)	Avg. (SE)	Avg. (SE)	Avg. (SE) PER	PER
Diet	Body Weight	Consumed	Gain:Feed	Crude Protein	PER Value	Value Without	% of
	Gain (mg)	(mg)	Ratio	Intake (mg)	With Outliers	Outliers	SDE
Purina One Lamb and Rice	1.952 ± 0.07	2.673 ± 0.09	0.738 ± 0.02	0.708 ± 0.02	2.7870 ± 0.09	2.7475 ± 0.08	44.0
Negative Control	0.336 ± 0.04	1.010 ± 0.08	0.354 ± 0.04	0 ± 0.00	-	-	-
Spray Dried Egg	1.900 ± 0.14	3.110 ± 0.17	0.600 ± 0.02	0.298 ± 0.02	6.2513 ± 0.25	6.2513 ± 0.25	100.0
Soy Protein Concentrate	0.3223 ± 0.03	0.877 ± 0.06	0.371 ± 0.03	0.087 ± 0.01	3.7624 ± 0.26	3.8861 ± 0.23	62.2
Corn Gluten Meal	0.283 ± 0.04	0.863 ± 0.07	0.322 ± 0.05	0.089 ± 0.01	3.0194 ± 0.45	2.5866 ± 0.24	41.4
Fish Meal	1.201 ± 0.08	2.100 ± 0.13	0.568 ± 0.02	0.193 ± 0.01	6.1809 ± 0.26	6.2375 ± 0.16	99.8
Pea Protein Concentrate	0.411 ± 0.05	1.040 ± 0.10	0.458 ± 0.05	0.104 ± 0.01	4.5687 ± 0.52	4.2012 ± 0.37	67.2

