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Identification of Insect Species in Feed by High Resolution Melting (HRM) Analysis

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Klementina Yakova BSc

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1. Introduction

Food authenticity and adulteration are critical issues in the food industry. Food authenticity refers to the assurance that food products meet the claims made on their labels, including their origin, composition, and production methods. Food adulteration involves the intentional or unintentional addition of inferior or misleading substances to food products, often to enhance profitability or alter characteristics (Brooks et al., 2021; Eurofins Scientific).

The globalization of food supply chains, combined with complex production processes, has made it increasingly difficult to accurately trace the origins and composition of food products. This has led to a rise in fraudulent practices, particularly for high-value products such as olive oil, honey, and wine. These are often targets of adulteration due to their economic significance (Brooks et al., 2021). To combat this challenge, the European Union has established the Alert and Cooperation Network (ACN), a system designed to facilitate rapid communication and collaboration between member states and relevant authorities to ensure food safety and prevent fraud. According to their annual report for 2023, a 26% increase in notifications was registered, totalling 758 suspected fraud cases. The most significant concern was the illegal trade of cats and dogs, accounting for 414 notifications. Other suspected cases involved meat substitution, honey adulteration, and falsely labelled olive oil (European Commission, 2024).

The legal basis for combating food fraud is outlined in Article 8 of Regulation (EC) No. 178/2002. For some foods that are rather susceptible to fraud, such as olive oil, wine, fruit juices, and honey, there are specific protocols for verifying their authenticity (European Commission). However, guidelines have not yet been established for all categories, making the assessment of food products and ensuring compliance with legal standards a challenge.

Recently, DNA molecules have become the focus for species identification due to their superior stability and abundance in most biological tissues. Most DNA-based methods for species identification in food involve the highly specific amplification of one or more DNA fragments using polymerase chain reaction (PCR). This technique is highly effective due to its speed, simplicity, sensitivity, and specificity (Mafra et al., 2008). For instance, researchers claim that compared to immunoprotein-based immunoassays, DNA-based methods are superior in distinguishing between closely related species. Additionally, these approaches can be used for meat species identification, detecting genetically modified organisms (GMOs), and detecting mutations (Furutani et al., 2017).

High-resolution melting (HRM) analysis is a logical progression from the real-time monitoring of PCR reactions, based on the melting profiles of PCR fragments tracked by measuring the decline in fluorescence after the dissociation of double-stranded DNA into single strands (Ririe et al., 1997). Over the past decades, HRM has been effectively implemented in mutation detection, methylation analysis and presequence scanning (R. Li et al., 2020).

The main subject of this master's thesis was to develop a method for distinguishing between seven legally allowed insect species in feed by implementing PCR and HRM analysis.

1.1 The dual challenge of agriculture

Modern agriculture faces significant challenges. The latest State of Food Security and Nutrition in the World (SOFI) report states that more than 700 million people faced hunger in 2023, equivalent to one in eleven people globally and one in five in Africa (FAO et al., 2024). According to the International Fund of Agricultural Development president, Alvaro Lario, the most effective way to alleviate global hunger is through investments in agriculture in rural areas (WHO, 2024). Research indicates that agricultural output may need to nearly double to meet the rising demand driven by population growth, and shifts in dietary preferences to a greater meat consumption, unless major changes in consumer habits occur (Foley et al., 2013).

On the other hand, the environmental footprint of this sector enhances biodiversity loss and resource depletion and contributes to climate change. Agriculture has already converted 70% of grasslands, 50% of savannas, 45% of temperate forests, and 27% of tropical forests, contributing to habitat destruction and species loss (Ramankutty et al., 2008). While the striking 75% of global agricultural land (3.73 billion hectares) is dedicated to raising livestock, almost 10% of that land is only used for growing animal feed (Foley et al., 2013). Additionally, there has been a significant shift toward diets that include more animal products, and this trend is expected to persist in the coming decades. Forecasts indicate an increase in the demand for meat and milk by 58% and 70%, respectively, by 2050 compared to values in 2010 (McLeod, 2011).

The primary concern regarding this is that allocating highly fertile cropland for animal feed, even when done efficiently, ultimately reduces the world's potential food supply. Furthermore, these crops require significant inputs of water, fertilizers, and energy, all of which have environmental costs. For instance, the use of fertilizers on feed crops contributes to nitrogen and phosphorus pollution, which can lead to water contamination and the creation of dead zones in coastal areas (MacDonald et al., 2011).

One possible approach to solving this issue is adjusting agricultural practices and dietary choices. In other words, redirecting crop production from livestock feed and other non-food uses could increase the global food supply without the need for further expansion of agricultural land (Foley et al., 2013).

1.2 Insects in feed - a possible solution?

Rearing insects may be a viable method to improve food and feed security (Huis, 2013). Although their consumption is rather controversial in the West, insects remain a part of the regular diet for over two billion people, especially in non-Western nations in Africa, Asia and Latin America (Kouřimská & Adámková, 2016).

The use of insect proteins from seven species was authorized in the European Union in 2017 – the black soldier fly (*Hermetia illucens*), housefly (*Musca domestica*), yellow mealworm (*Tenebrio molitor*), lesser mealworm (*Alphitobius diaperinus*), house cricket (*Acheta domesticus*), tropical house cricket (*Gryllodes sigillatus*), and Jamaican field cricket (*Gryllus assimilis*) – in feed for aquaculture, poultry, and swine (Regulation (EC) No. 2017/893). As of November 2021, the EU legislator added silkworm (*Bombyx mori*)

processed animal proteins (PAPs) to the list, expanding the number from seven to eight (Regulation (EC) No. 2021/1925).

Insects are highly nutritious, with their nutrient content varying significantly due to their wide diversity. Most edible species supply adequate energy and protein while rich in mono- and polyunsaturated fatty acids. Furthermore, some contain essential minerals such as copper, iron, magnesium, manganese, phosphorus, selenium, and zinc (Rumpold & Schlüter, 2013) and recently identified bioactive compounds could enhance gut health in animals (Choi et al., 2013a, 2013b). Besides, some studies suggest that insects can consume waste biomass and convert it into valuable food and feed resources when raised on organic by-products (Lakemond et al., 2019).

A study on the public acceptance of this proposition provided favourable results. The opinions of three population groups were investigated - farmers, stakeholders, and citizens, and two-thirds of all participants were open to the idea of insects being used in animal feed. While farmers were more critical than stakeholders and citizens, they still held a generally positive view. The resulting foods were considered more sustainable, nutritious, and healthy, though there were concerns about potential microbiological issues, off-flavours, and allergens (Verbeke et al., 2015).

Recent research has increasingly focused on five main groups: black soldier fly, housefly, mealworms, crickets, and silkworm.

1.2.1 Black soldier fly larvae (*Hermetia illucens*)

The black soldier fly is highly resilient, able to thrive under challenging environmental conditions like drought, food scarcity, or low oxygen levels (Diener et al., 2011). A key advantage of *Hermetia illucens* compared to other insects is that the adult flies do not feed, eliminating the need for special care and reducing the risk of disease transmission. Because of this, rearing this species has been proposed since the 1990s as an efficient method for processing organic waste by converting it into a protein- and fat-rich biomass that can be used for various applications, such as animal feed for all types of livestock (Diener et al., 2011; Huis et al., 2013).

Black soldier fly larvae are a valuable feed resource, packed with protein and fat. They consist of approximately 40–44% crude protein, while their fat content is highly variable, depending on their diet, and can range between 15% and 49% (Makkar et al., 2014).

Several studies have investigated the partial or full replacement of traditional feed with black soldier fly larvae meal in feed for different species. Its inclusion in the diets of growing pigs has been found beneficial, particularly due to its favourable amino acid, lipid, and calcium content, and proved to be as palatable as those based on soymeal. However, a supplementation of methionine, cysteine, and threonine is required to create balanced diets (Newton et al., 1977).

Black soldier fly larvae meal has been shown to support growth in chicks. Chicks fed this larvae meal (as a soymeal substitute) gained weight at 96% (insignificant) of the rate of those on the soymeal control diet but consumed only 93% (significant) of the feed, indicating improved feed conversion efficiency (Hale, 1973).

Multiple experiments have indicated that black soldier fly larvae can partially or fully replace fishmeal in fish diets. However, further studies are required to make an adequate conclusion, as reduced performance has been observed in some instances (Makkar et al., 2014).

1.2.2 Housefly (*Musca domestica*)

The housefly (*Musca domestica*) is the most prevalent fly species (*Diptera*) globally. It is considered a major vector for diseases, as both the larvae (maggots) and adult flies feed on manure and decaying organic matter. However, the ability of housefly maggots to thrive on a wide variety of substrates makes them valuable for converting waste into a protein- and fat-rich biomass.

Macronutrient composition is highly variable, with crude protein content ranging from 40% to 60% and lipid content being even more variable, falling between 9% and 26% (Makkar et al., 2014). The phosphate levels in housefly maggots are comparable to those in black soldier fly larvae, but the calcium levels are approximately 15 times lower.

Diets implementing the housefly have been tested on multiple types of chickens, such as rural chickens, broilers, and laying hens. Live maggots of the species could be a valuable addition to the diets of rural chickens - in Ghana, supplementing scavenging backyard chickens with 30–50 g of live maggots per day led to improved growth rates, and enhanced clutch size, egg weight, number of eggs hatched, and chick weight (Dankwa et al., 2002). Most trials on broilers suggest that it is advantageous to partially or even fully replace fishmeal, although the optimal inclusion rate is typically below 10% of the diet. Higher inclusion rates have led to decreased feed intake and performance, possibly due to the darker colour of the meal, which may be less attractive to chickens (Bamgbose, 1999). Housefly maggot meal replaced 50% of the fishmeal protein (5% of the diet) in 50-week laying hens without negatively impacting egg production or shell strength. Nevertheless, a complete 100% replacement was harmful to hen-egg production (Agunbiade et al., 2007).

1.2.3 Mealworms (*Tenebrio molitor, Alphitobius diaperinus*)

Mealworms are the larvae of two species of darkling beetles from the *Tenebrionidae* family: the yellow mealworm beetle (*Tenebrio molitor*) and the smaller, less common lesser mealworm beetle (*Alphitobius diaperinus*). *Tenebrio molitor* is considered a pest of grains, flour, and food storage, although they are omnivorous and can consume various plant materials along with animal products like meat and feathers (Ramos-Elorduy et al., 2002). *Alphitobius diaperinus* is more widely recognized as a pest than a beneficial insect, since it is one of the most prevalent insect pests in commercial poultry farms (Müller Richli et al., 2023).

Regarding macronutrient composition, mealworms have high levels of crude protein (47–60%) and fat (31–43%) (Makkar et al., 2014). They are relatively low in ash content (<5% dry matter) and therefore low in mineral content. Like other insects, mealworms have a low calcium content and calcium-to-phosphorus ratio. Because of this, feeding exclusively on mealworms can lead to calcium deficiency and further negative implications on bone health (Klasing et al., 2000).

One study on pigs fed *Alphitobius diaperinus* meal found that a substitution of up to 9% in their diet did not negatively impact daily gain or feed consumption, indicating it can replace soybean meal without compromising growth. Furthermore, meat quality traits, including lean meat content, were unaffected, though the polyunsaturated fatty acid content in back fat increased with higher incorporation levels. This observation suggests a change in the fatty acid profile without altering overall meat quality (Müller Richli et al., 2023).

Mealworms present a promising alternative source for poultry and broiler diets, particularly as a replacement for soymeal or fishmeal. Their protein quality is comparable to that of soymeal, yet the methionine and calcium content is limiting for poultry. Furthermore, dried mealworms comprising up to 10% of a broiler starter diet made with sorghum and soybean meal can be included without impairing feed consumption, weight gain, or feed efficiency (Klasing et al., 2000; Ramos-Elorduy et al., 2002).

The partial replacement of fishmeal with mealworms in the diets of various fish species, including rainbow trout (*Oncorhynchus mykiss*), African catfish (*Clarias gariepinus*), and European sea bass (*Dicentrarchus labrax*), has been examined. Nevertheless, the findings vary according to the specific species, although the majority indicate that a partial replacement of fishmeal is advantageous (Makkar et al., 2014).

1.2.4 Crickets (*Acheta domesticus, Gryllodes sigillatus, Gryllus assimilis*)

Crickets (*Gryllidae* family*)* are insects belonging to the order *Orthoptera*. They are generally edible, with over 80 species being a part of the human diet across Africa, South America, and Asia (Huis et al., 2013).

Acheta domesticus (house crickets), when measured in dry weight, are notably high in protein, with its content ranging from 48.06 to 76.19 g per 100 g of dry weight. Additionally, they are considered a good source of minerals and can also meet a substantial portion of the Daily Recommended Intakes (DRIs) for macrominerals, particularly for children aged 4–8 and healthy adults over 19. With the highest reported calcium content in crickets reaching 210 mg per 100 g of dry weight, 100 g of cricket flour can provide up to 21% of the DRIs for calcium in both age groups (Pilco-Romero et al., 2023).

Because of this optimal nutritional profile, the house cricket has also been researched as an alternative protein source for feed for broiler chickens. One study investigated the impact of a diet composed of maize and 25% house crickets, which were fed to broilers for two weeks. It was revealed that the cricket-based diets resulted in higher feed-to-gain ratios compared to the control diet, which was maize-soybean based, despite both diets having the same protein content. However, methionine and arginine were identified as limiting amino acids in diets incorporating the insect meal (Nakagaki et al., 1987).

The cricket species *Gryllodes sigillatus* possesses significant nutrient content, particularly lipids and fatty acids. Among edible crickets, *G. sigillatus* has been found to contain more lipids compared to other species, consisting of essential fatty acids, including polyunsaturated fatty acids (PUFAs). Additionally, *G. sigillatus* is especially high in linoleic acid, with levels reaching up to 30.76 g per 100 g of fat, and it

contains more PUFAs than widely consumed animal tissues such as beef and poultry. Furthermore, compared again to poultry, the calcium content of *G. sigillatus* dry matter is around 18 times higher- 130 mg/100 g dry matter versus 7 mg/100 g dry matter (Zafar et al., 2024).

On the other hand, another cricket species, *Gryllus assimilis,* has a rather low lipid content compared to other crickets- between 20 and 30 g per 100 g dry matter. The fatty acid profile consists of 33% monounsaturated and 15% polyunsaturated fatty acids and above 40% of saturated fatty acids, predominantly palmitic acid. The Jamaican cricket contains an exceptional amount of protein- between 60 and 70 g per 100 g dry matter, making it a valuable protein source relevant for future exploration. Its calcium levels, however, are significantly lower than those of the house cricket, standing at 45 mg compared to 130 mg per 100 g of dry matter (Soares Araújo et al., 2019).

1.2.5 Silkworm (*Bombyx mori*)

Silkworms are the larvae of moth species bred for silk production, with around 90% of global silk originating from the domesticated mulberry silkmoth (*Bombyx mori*), a species in the *Bombycidae* family. Large amounts of spent pupae, a by-product of silk production, are generated— approximately 8 kg of wet pupae for every 1 kg of raw silk. These spent pupae are often discarded, used as fertilizer. Moreover, research has shown that they can benefit the production of valuable oil for industrial uses, such as in paints, pharmaceuticals, soaps, candles and plastics (Patil et al., 2013).

Silkworm pupae meal is a protein-rich feed ingredient, with a crude protein content ranging from 52% to 72%. Similar to other insects, silkworm meal is low in calcium and has a low calcium to phosphate ratio. Its lysine (6–7% per 100 g crude protein) and methionine plus cysteine content (around 4%) are notably high. Additionally, silkworm oil is rich in PUFAs, particularly linoleic acid, which makes up between 11% and 45% of the total fatty acid content (Makkar et al., 2014).

Silkworm meal has been shown to effectively replace up to 33% of groundnut cake in calf fattening diets and serve as a substitute for other protein sources in sheep and lamb diets without compromising performance. Moreover, its use can result in cost-effective feed formulations, with improved protein digestibility compared to traditional protein sources (Narang & Lal, 1985).

In Brazil, non-defatted silkworm meal was able to completely replace soymeal in diets for growing and finishing pigs without diminishing growth performance or carcass quality (Sheikh et al., 2018). The results from studies done on fish species deliver similar results: a full replacement of fishmeal protein with silkworm meal in carp diets resulted in comparable growth and feed conversion. Moreover, silkworm pupae meal can also serve as a substitute for up to 75% of the protein in Asian stinging catfish diets without negatively impacting growth. On the other hand, the research on its implementation in feed for broiler chickens is less promising. Studies have demonstrated that substituting 50% of the primary protein source, typically fishmeal, with silkworm meal is generally safe, although mineral supplementation may be necessary, especially for calcium. Complete replacement is possible but often results in reduced performance (Makkar et al., 2014).

1.3 Identification of insects in food and feed

The identification of insect species in food and feed products is a developing area of research, with only a limited number of studies published thus far. The minimal amount of studies is possibly due to the fact that earlier insects were regarded mostly as pests, and large efforts were targeted at developing efficient methods to detect and therefore control their presence in the food chain (Choudhury et al., 2021; Solà et al., 2018).

DNA-based techniques such as real-time PCR have proven effective for qualitatively detecting the presence of some insect species, such as the black soldier fly (*Hermetia illucens*) (Zagon et al., 2018) and the yellow mealworm (*Tenebrio molitor*) (Debode et al., 2017). In another study investigating the yellow mealworm, housefly and house cricket, the genetic identification of insects relied on a fragment of the cytochrome c oxidase subunit I (COI) gene and colony PCR was employed (Moulistanos et al., 2023). Fragments of the same gene were also used by Korean researchers, who developed PCR assays combined with a microfluidic chip for the simultaneous detection of six edible insect species (Kim et al., 2019). Out of all the species analysed in this study, only two were relevant for the European market- the yellow mealworm (*Tenebrio molitor*) and the silkworm (*Bombyx mori*). A DNA metabarcoding method for insect differentiation was also recently developed, using a singleplex PCR assay to amplify a short DNA target region of the mitochondrial 16S rDNA gene. While the insect species currently relevant to food production were successfully detected, the researchers were unable to achieve clear species-level differentiation for all samples analysed *in silico* (Hillinger et al., 2023).

Other analytical approaches, such as microbiological methods, have been explored, where microscopic images revealed the distinct features of insects, including their cuticles, muscles, bristles, and tracheales (Weiner & Kwiatek, 2022). Proteomics have also gained attention as a possible technique for differentiating insect species in feed, with two studies providing molecular markers (peptides) that enabled the identification of the silkworm, the black soldier fly and the lesser mealworm. Additionally, these molecular markers were also successfully detected in complex matrices, such as in fish, pig, and poultry feeds (Leni et al., 2020; Stobernack et al., 2023).

2. Aims of the master´s thesis

The demand for food increases year by year, in line with the growth of the human population. A higher demand thus leads to the production of more livestock. Their feed includes a variety of protein sources, such as legumes, grains, animal by-products, etc. Over the past ten years, insects have been explored as an alternative ingredient in the feed industry, with the main argument being that they can replace other expensive high-quality feed materials like casein, soybean meal, or fishmeal.

Eight insect species are legally approved for use in animal feed in the EU: the black soldier fly (*Hermetia illucens*), the housefly (*Musca domestica*), the yellow mealworm (*Tenebrio molitor*), the lesser mealworm (*Alphitobius diaperinus*), the house cricket (*Acheta domesticus*), the tropical house cricket (*Gryllodes sigillatus*), the Jamaican field cricket (*Gryllus assimilis*), and the silkworm (*Bombyx mori*)—in feed for aquaculture, poultry, and pigs. However, some of them are genetically closely related, and their DNA sequences are very similar.

The aim of this master thesis was, therefore, to optimize a method that allows us to differentiate between legally allowed insect species in feed and to be able to identify an unknown insect species in a feed sample. The strategy consists of DNA extraction using an optimal DNA extraction kit, polymerase chain reaction (PCR), and high-resolution melting analysis (HRM). A recent study explored the application of the DNA metabarcoding approach for the distinction between insect species in food (Hillinger et al., 2023). One previous master student´s work verified that the primers from this study could also be used for the same purpose while using PCR-HRM analysis. However, additional primer pairs had to be designed to alter the curve profile of some closely related insect species (Wildbacher, 2023).

The same primers that were used for barcode sequencing are also present in the method developed in this master´s thesis. For closely related insect species, new primer pairs should be designed to modify the curve profiles and differentiate them from similar species. To achieve this, primers are to be created and tested both *in silico* and in practice. After having optimized the method for individual insect samples, the method must be tested on processed products—animal feed. Since three of the insect species allowed in human food are also allowed in feed, the method ought also to be tested on DNA extracts provided by the Austrian Agency for Health and Food Security (AGES) from both legal and illegal insect species in the EU for use in food or feed and from processed foods for human consumption containing the legally permitted species.

3. Theoretical background

3.1 Fluorimetry

Fluorimetry is an analytical technique for detecting and measuring fluorescence in compounds, where ultraviolet light excites the compounds, causing them to emit visible light. The emitted energy or light has a longer wavelength than the absorbed one. This analytical technique is preferred due to its exceptional sensitivity, high specificity, simplicity, and cost-effectiveness compared to other methods. Generally, it offers greater sensitivity than other detection methods, such as absorbance measurements, and is utilized in various applications, including environmental monitoring, medical diagnostics, forensics, genetic analysis, and biotechnology (Naresh, 2014).

The fluorescence DNA detection method employs a fluorimeter and a DNA-binding fluorescent dye that specifically attaches to double-stranded DNA (dsDNA), emitting fluorescence when excited. The positive correlation of the fluorescence intensity and the amount of intercalating dye enables the DNA concentration determination. Quantification is accomplished by relating the measured fluorescence to a standard curve, which is generated using reference solutions with pre-established DNA concentrations (X. Li et al., 2014).

3.2 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is an *in vitro* technique capable of producing tens of billions of copies (amplicons) of a specific DNA sequence from a DNA extract with the help of a DNA polymerase (U. S. National Library of Medicine). It was invented in 1985 by Kary Mullis, and this revolutionary discovery led to him winning the Nobel Prize in Chemistry in 1993 (The Nobel Prize).

PCR was the first target amplification technique to be developed and remains the most widely employed method to date. It has been extensively applied in various clinical domains, including the diagnosis of infectious diseases, identification of genetic disorders, and cancer prognostics. Furthermore, the emergence of PCR has driven advancements in research fields utilizing recombinant DNA technology, with substantial progress in gene expression analysis, DNA profiling, and the development of therapeutic strategies for genetic disorders. Beyond clinical and research applications, PCR has also been implemented in disciplines such as archaeology and forensic science, owing to its ability to amplify DNA from compromised or scarce samples (Shahzad et al., 2020).

The PCR takes place in a reaction mixture containing the template DNA, heat stable DNA polymerase, primers, which are complementary to known sequences of the target DNA, and an excess of the four deoxyribonucleoside triphosphates (dNTPs), which act as the building blocks for the DNA polymerase to create the resultant PCR product. All components are mixed within a buffer solution. The lanes holding this reaction mixture are exposed to repeated cycles of temperature changes in the heating block of a special DNA thermal cycler. A typical PCR consists of 20-40 amplification cycles (Tayyeb & Basit, 2024).

3.2.1 Principle of the PCR

The process is divided into the three following stages: denaturation, annealing and elongation.

The first step, known as the denaturation phase, occurs at 95°C. At this temperature, since hydrogen bonds cannot remain stable above 80°C, the double-stranded DNA separates into single strands.

The second stage called annealing or hybridisation is generally carried out between 40 and 70°C and is determined by the primers used. This procedure allows the specific primers to attach to the 3' end (3'-hydroxyl group) of the opposite strands of the single-stranded target DNA. Since primers are short single-stranded sequences, selected to be complementary to the regions flanking the target region, they exhibit greater annealing efficiency than the longer DNA strands. A higher annealing temperature increases the selectivity of this phase.

In sequence, the temperature is raised to 72°C, which is necessary for the elongation step. At this temperature, the DNA polymerase attaches to the primed single-stranded DNA sequences and proceeds to add nucleotides from 5' to 3' end (5'-phosphate group to 3'-hydroxyl group), advancing the replication. In subsequent cycles, the fragments produced in earlier cycles serve as templates, leading to the exponential amplification of new DNA products until the enzyme and reaction components are exhausted and a plateau is reached. The formula for calculating the number of DNA copies produced after a specific number of cycles is 2^n, where *n* represents the number of cycles. In reality, this theoretical amount of copies is not achieved due to several limiting factors, such as the limited amount of dNTPs, a decrease in the activity of the DNA polymerase, contaminants, etc. (Kadri, 2019; Shahzad et al., 2020; Tayyeb & Basit, 2024).

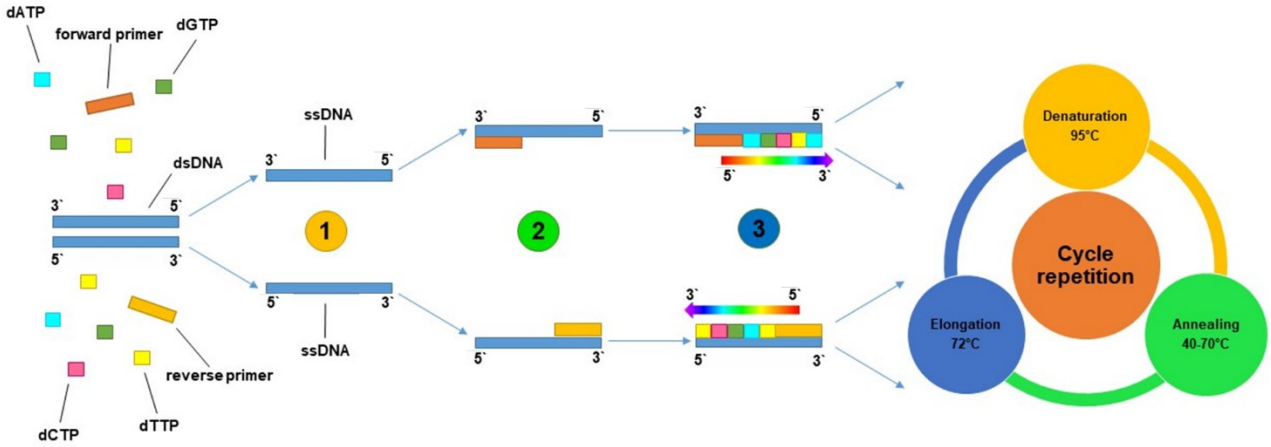


Figure 1: Schematic flow of PCR; dATP: adenosine triphosphate, dGTP: guanosine triphosphate, dCTP: cytidine triphosphate, dTTP: thymidine triphosphate.

3.2.2 Reaction components

Each PCR reaction mixture consists of template DNA, primers, DNA polymerase, dNTPs, and a buffer solution.

The quality of the template DNA is crucial for the successful outcome of the reaction- it should be intact and free of contaminants such as proteins, phenol, or other chemicals.

The DNA polymerase always exhibits a 5'→3' polymerase activity. One of the most commonly used enzymes in PCR is the Taq polymerase, which is derived from the extremophilic bacterium *Thermus*

aquaticus. Since the bacterium inhabits hot springs, this polymerase is able to withstand temperatures around 100°C, which are typically high enough to denature most proteins. *Thermus aquaticus* thrives at a comfortable temperature of 72°C, which is also the optimal temperature for the activity of its polymerase and therefore the chosen condition for the elongation stage of the PCR.

The primers are single-stranded DNA sequences that should complement both ends of the target sequence intended for amplification. One primer is designed to recognize a complementary sequence located upstream of the 5′–3′ strand of the target DNA (*forward* primer), while the other primer targets a complementary sequence located upstream on the 3′–5′ strand of the same DNA fragment (*reverse* primer).

The four dNTPs are the main elements from which the DNA polymerase synthesizes a new DNA strand. They include adenosine triphosphate (dATP), thymidine triphosphate (dTTP), cytidine triphosphate (dCTP) and guanosine triphosphate (dGTP).

The buffer solution provides a suitable chemical environment for the optimal activity and stability of the DNA polymerase. Additionally, bivalent cations such as magnesium Mg^{2+} in the form of $MgCl_2$ or $MgSO_4$ could be supplemented as a cofactor of the DNA polymerase. Their presence enhances enzyme activity and assists nucleotide binding, which improves DNA amplification. Furthermore, their availability in the reaction mixture supports the proper pairing of complementary bases, strengthening the precision. Typical magnesium ion concentrations used in PCR generally range from 1.0 mM to 2.5 mM. The amount is critical for optimal performance, since both too low or too high values can have negative implications. While excessively low concentrations can result in a reduced PCR yield and decreased DNA polymerase activity, exceedingly large concentrations can lead to an increase in non-specific amplifications and alter the melting temperatures significantly, therefore impairing the annealing phase of the PCR (Bell, 1989; Kadri, 2019; Müller & Prange, 2016).

3.2.3 Factors in primer design

Primer design is a crucial step in the PCR process, impacting the efficiency and specificity of DNA amplification. Several key factors must be considered for the optimal amplification of the target region.

Usually, the sequences are between 18 and 30 bases in length, due to the fact that short primers can lead to inaccurate and non-specific DNA amplification products, while long primers tend to hybridize at a slower rate. Primers are typically implemented at concentrations ranging from 0.1 to 1 μM. Excessively high primer concentrations, however, can lead to non-specific binding, resulting in generation of unintended products.

Another necessary parameter is the ratio of guanine-cytosine (GC) to adenine-thymine (AT), since it significantly influences the melting temperature (Tm) and consequently, the annealing temperature of the primers. It is recommended that primers maintain a GC content between 40% and 60%. A higher proportion of GC pairs leads to an increased melting temperature, while a lower proportion results in a decrease of the melting temperature. Furthermore, it's advisable to have a GC clamp at the 3' end, meaning the last two bases of the primer should consist of cytosine (C) and guanine (G) whenever

possible. This is important because GC pairs form three hydrogen bonds, while adenine-thymine (AT) pairs form only two. The presence of these three bonds at the primer's end provides a more stable starting point for the Taq polymerase, enhancing the efficiency of the DNA amplification process. Additionally, primer sequences should not contain runs of three or more identical bases due to an increased risk of “mispriming” (non-specific binding).

The melting temperature is the temperature at which 50% of the primers are bound to the template DNA, while the other 50% remain in single-stranded form. This parameter is a crucial factor in PCR because it dictates the temperature used during the annealing phase. In most cases, the optimal annealing temperature is about 5 °C lower than the Tm of the primers, however, it is best calculated using software tools such as the webserver OligoCalc. Preferably, Tm of the primers should meet two main criteria: ranging between 55 and 65 °C and Tm of forward and reverse primers differentiating by no more than five degrees.

Additionally, longer stretches of identical nucleotides are to be avoided, since these can result in self-binding. Secondary structures, such as hairpins, are to be excluded because of the possibility of disrupting the amplification process. The probability of these scenarios can be tested again employing webservers such as OligoAnalyzer for dimers and self-dimers and RNAfold WebServer for secondary structures (Shahzad et al., 2020; Tayyeb & Basit, 2024).

3.2.4 Real-time PCR (qPCR)

Real-time PCR, also known as quantitative PCR (qPCR), is a technique that enables the monitoring of the amplification of DNA in real time as the PCR process progresses. This method operates on the principle of amplifying DNA using primers, similar to standard PCR. The main difference lies in the inclusion of a fluorescent reporter molecule in each reaction well, which generates fluorescence proportional to the amount of DNA. During the analysis, the fluorescence emitted is measured in each amplification cycle to quantify the amount of PCR product generated, which allows for continuous monitoring of the DNA amplification process in real time.

The most straightforward and cost-effective approach to real-time PCR involves the use of DNA-binding dyes, such as *SYBR* Green. This dye emits fluorescence upon intercalating with DNA, leading to increased fluorescence with each amplification cycle as more dye binds to the growing DNA strands, until a saturation point is reached. However, highly specific primers are required when employing this method, since *SYBR* Green can insert itself into any DNA product. Another intercalating dye gaining popularity is EvaGreen, a third-generation double-stranded DNA (dsDNA) binding dye. Notably, it has proven to be less inhibitory in PCR reactions and can be employed under saturating conditions to produce stronger fluorescent signals. Additionally, EvaGreen is particularly well-suited for high-resolution melting (HRM) analysis, making it a versatile choice for various applications.

An alternative approach involves the use of fluorescently labeled DNA probes- short DNA sequences that are complementary to the target sequence located between the two primer sites. The key component in this method is the fluorescent molecule attached to the probe, which remains quenched until the exonuclease activity of Taq polymerase releases the fluorescent substance. As the PCR

product accumulates, the fluorescence signal increases correspondingly. The main advantage of this strategy is the enhanced precision it provides, since here three DNA sequences must bind to the target sequence in distinct locations instead of two (Avery et al., 2013; Navarro et al., 2015).

A widely employed technique for evaluation of the results of a qPCR is through melting curve analysis. The melt curve is generated by gradually increasing the temperature up to 95°C to denature the DNA in the presence of an intercalating dsDNA binding dye. As the DNA „melts", only dye bound to dsDNA emits fluorescence, which is continuously recorded by an optical system. Initially, the fluorescence is strong when the dye is still bound, but once the DNA strands separate, the dye is released, leading to a 1,000-fold decrease in fluorescence. This sharp decline occurs particularly near the melting temperature of the PCR product. The relationship between the fluorescence dye intensity and the temperature is the key to the identification of the target species (Miao et al., 2020).

3.2.5 High Resolution Melting (HRM) analysis

High resolution melting (HRM) analysis is an advanced, post-qPCR method designed to detect genetic variations in nucleic acid sequences. It was first introduced in 2002 by a collaboration between the University of Utah in the USA and the company Idaho Technology. It is a rapid and uncomplicated technique based on PCR melting curve principles. Factors such as GC content, sequence length, nucleotide composition, and heterozygosity can all contribute to the unique melt curve characteristics. Because of this, the resulting melting profile provides valuable insights into the composition of the amplicons. These unique profiles are important for various applications, including mutation screening, genotyping, methylation analysis, and other research purposes.

Similar to melt curve analysis, PCR is carried out in the presence of a fluorescent dye bound to dsDNA, which exhibits low levels of fluorescence when unbound, but high levels when in bound state. Commercially available fluorescent dyes such as EvaGreen or LCGreen Plus have proven to be highly efficient, possessing low PCR inhibition, sufficient fluorescence intensity and ability to generate melt curve profiles. The length of the amplicon is also a crucial factor for the efficiency of this analysis- ideally, it should not exceed 300 base pairs (bp). With longer amplicons, a small sequence difference becomes less evident. Additionally, longer amplicons may consist of several melting domains, resulting in a more complex melting profile.

After the final PCR cycle, the melting of the amplicons is performed by gradually increasing the temperature (e.g., 0.1°C every two seconds) up to 95°C. When this procedure is completed, melting curves are generated when plotting the diminishing fluorescence intensity against the temperature increase. During the analysis of the results, normalization of the absolute fluorescence levels before and after melting to 100% and 0% respectively can correct variations in the fluorescence and improve the repeatability of the method. Additionally, the melt curve can be converted to a melt peak by relating the negative derivative of the fluorescence with respect to temperature (-dF/dT) to the temperature increase (T). Generally, a longer amplicon would melt at a higher temperature than a shorter one due to the higher number of hydrogen bonds. Another crucial factor for the Tm is the amount of G or C bases in

the amplicon, since each G-C base pair possesses three hydrogen bonds, compared to two hydrogen bonds for A-T.

HRM analysis possesses several key advantages over other traditional gene scanning methods. Firstly, it is performed in the same well as the previous amplification step, which minimizes the risk of contamination and eliminates the need for any additional steps such as amplicon purification. Therefore, the procedure is better suited for high-sample throughput than other analytical methods, such as gel electrophoresis. Furthermore, it is a non-destructive approach, meaning that the amplicons can still be used for further investigation through sequencing or gel electrophoresis (Druml & Cichna-Markl, 2014; Nolan & Bustin, 2013; Radvanszky et al., 2015; Reed, 2007; Simko, 2016).

3.3 Agarose gel electrophoresis

Agarose gel electrophoresis is a common method employed in the analysis and separation of proteins, DNA or RNA. Its main principle is the utilization of an electric field to separate nucleic acid molecules according to their size. Negatively charged molecules migrate towards the positive pole (anode), and their movement speed is dependent on their size, since smaller weight molecules migrate faster than larger ones. The technique is routinely used in molecular diagnostics and genetic fingerprinting following PCR amplification, or as a purification method before other molecular procedures such as cloning.

The agarose concentration in the gel can be optimized according to the expected length of the DNA fragment to be analysed, ranging from 0.7 % for large DNA fragments (5-10 kb) to 3% for smaller DNA fragments (0.2-1 kb). However, higher agarose concentrations have the disadvantage of longer run times.

The procedure is performed in the presence of a buffer solution, such as Tris/ Acetate/ EDTA (TAE) or Tris/ Borate/ EDTA (TBE). Their presence is crucial for maintaining an ideal pH and supplying ions for efficient conductivity, and the solutions should not produce excessive heat, which could melt the gel.

The visualization of the results is possible when implementing a dye for nucleic acid visualization, which would make the “band” visible under ultraviolet (UV) light emitted from a UV Transilluminator. One of the most common dyes is ethidium bromide, with its first implementations dating as far back as the 1970s. However, since this compound is highly genotoxic, other alternatives have been explored, such as *SYBR* Green. Both *SYBR* Green and *SYBR* Green Safe, a less hazardous variant of *SYBR* Green, have exhibited similar or better sensitivity compared to ethidium bromide. Another possible replacement is GelRed, which has also proven superior to ethidium bromide in terms of safety without compromising staining sensitivity (Huang et al., 2010; Magdeldin, 2012).

4. Experimental part

4.1 Hygiene

Contamination is one of the most impactful systematic errors that can occur when working with DNA samples. Therefore, sufficient measures were taken to ensure the absence of such occurrences, and procedures were carried out in three different laboratories, wearing a different lab coat for each laboratory. In the first laboratory, DNA extraction was performed, and DNA samples were stored. In the second laboratory, the PCR-HRM master mix was prepared. In the third laboratory, the DNA extracts to be analysed were added to the prepared master mix. Additionally, DNA extracts and their respective dilutions, PCR-HRM master mix components, and PCR amplicons were separately stored in different freezers.

Before starting any procedure, the working surface was cleaned thoroughly with DNA Exitus spray. Additionally, in the second and third laboratories, the working stations were equipped with UV disinfection systems, which decontaminated the laminar flows for half an hour after every use.

4.2 Samples

4.2.1 Insect species samples

The insect species samples consisted of seven legally allowed in the EU species for use in feed (status as of 2024) and three illegal insect species. The DNA samples were provided by AGES in a homogenized form and are all listed in Table 1 with their origin and additional characteristics.

Table 1: List of insect samples obtained from AGES.

Insect	Latin name	Sample origin	Additional remarks	
Lesser mealworm	*Alphitobius diaperinus*	Snack-insects.com	juvenile stage, dried	allowed in feed
Mealworm	*Tenebrio molitor*	Six feet to eat	juvenile stage, dried	allowed in feed
Housefly	*Musca domestica*	Six feet to eat	pupae/larvae, original sample: insects on substrate (hay)	allowed in feed
Black soldier fly	*Hermetia illucens*	commercial import from Canada	juvenile stage, powder	allowed in feed
Silkworm	*Bombyx mori*	Thailand Unique	pupae, powder	allowed in feed
House cricket	*Acheta domesticus*	Six feet to eat	adult stage, dried	allowed in feed
Tropical house cricket	*Gryllodes sigillatus*	Six feet to eat	adult stage, dried	allowed in feed
Crazy red cricket	*Gryllus locorojo*	Fauna topics	adult stage, powder	not allowed in feed
Cockroach	*Blattodea*	Thailand Unique	flying termites, adult stage	not allowed in feed
Fruit fly	*Drosophila*	Reptilienkosmos.de	adult stage, unable to fly, small Drosophila, original sample: insects on substrate (sawdust), frozen at -80°C, lyophilized before shipping	not allowed in feed

4.2.2 Feed samples

All fifteen feed samples (Table 2) were commercially acquired, twelve of them containing one or more of the legally allowed insect species, and three samples without any declared insect content, serving as negative controls.

Table 2: List of commercially obtained feed samples.

Product label and remarks	Brand	Insect content
Nestlingfutter, dried insects	Dehner Natura	silkworm pupae, unknown amount
Meisenknödel, birdseed cakes	PREMIERE	mealworms, 4%
Insect hearts, snacks	Trixie	mealworms, 65%
Insect sticks, snacks	Trixie	mealworms, 65%
Erdnussbutter mit Mehlwürmer, peanut butter with mealworms	JR Farm	mealworms 2.5%, black soldier fly larvae 2.5%

Sensitive Mini Adult Insekt, granules	Select Gold	insect protein from black soldier fly larvae, 20%
Bugfutter Insekten, Karotte & Hefe, granules with insects, carrots and yeast	BugBell	insect flour from black soldier fly, 33%
Sensitive Adult, granules	MERA	insect protein from black soldier fly, 14%
Insekt mit saftigem Hühnchen, canned feed with chicken and insects	MjAMjAM	unknown insect, 32%
Eiweiß Cocktail, protein cocktail with dried insects	JR Farm	mealworms, dried, 33%
Insektenfutter, flakes	Sera Insect Nature	mealworms, 40%
Sensible Snack Insekt, sticks	RINTI	mealworms, 60%
Chicko Huhn, freeze-ried snacks from chicken meat	RINTI	not stated
Kulinarischer Hirsch & Wildschwein an Preiselbeeren, canned feed with wild boar	MjAMjAM	not stated
Hühnerherzen, chicken hearts snacks	Dokas	not stated

4.2.3 Meat samples

Three samples from different mammal species were obtained: pork, lamb and beef (Table 3). Pork and beef were bought from a local supermarket, while the lamb sample was acquired from a butcher store in an open market.

Table 3: List of commercially obtained meat samples.

Meat type	Brand	Preparation
Pork	Ich bin Österreich	cut into rib steaks
Beef	Ich bin Österreich	minced
Lamb	not branded	minced

4.2.4 DNA extracts provided by AGES

Another master`s student, Michaela Wildbacher, previously worked on the development of a method for differentiating insect species in food for human consumption. The DNA extracts from the samples she analysed contained a broader variety of insect species, both legal and illegal for use in food and feed, and multiple food products containing one of the legally allowed insect species. Table 4 presents all of

her samples and the concentration of the DNA extract from that sample. All extracts were provided to her by AGES, and the concentration measurements were also completed by the agency beforehand (Wildbacher, 2023).

Table 4: List of insect and food DNA extracts, contributed by AGES (Wildbacher, 2023, modified).

Sample Nr.	Sample	Weight [g]	Concentration [ng/µL]
P-002	*Gryllus bimaculatus*, mediterranean field cricket	1.0	481.5
P-003	*Musca domestica*, housefly	1.0	148.0
P-004	*Gryllodes sigillatus*, tropical house cricket	0.8	356.8
P-005	*Gryllus locorojo*, crazy red cricket	1.0	3.6
P-006	*Bruchus maculatus*, cowpea seed beetle	<0.1	34.8
P-008	*Rhynchophorus ferrugineus*, red palm weevil	1.0	439.5
P-011	*Schistocerca gregaria*, desert locust	0.7	186.1
P-012	*Lucilia sericata*, common green bottle fly	0.1	264.5
P-015	*Drosophilia hydei*, fruit fly	<0.1	17.2
P-028	*Tenebrio molitor*, mealworm	<0.1	116.2
P-030	*Hermetia illucens*, black soldier fly larvae	0.6	0.9
P-031	*Galleria melonella*, greater wax moth	1.0	485.2
P-039	*Acheta domesticus*, house cricket	<0.1	3.1
P-042	*Zophobas atratus*, blind click-beetle	1.0	45.2
P-044	*Locusta migratoria*, migratory locust	0.2	121.1
P-049	*Alphitorius diaperinus*, lesser mealworm	0.5	82.9
P-050	Locusts, blanched and freeze-dried (*Locusta migratoria*)	0.6	250.8
P-054	*Cicadidae*, true cicada	0.9	1170.2
P-059	*Bombyx mori*, silkworm	0.4	132.8
P-067	*Tenebrio molitor*, mealworm	1.0	580.1
P-087	*Acheta domesticus*, house cricket	1.0	3.8
P-089	Fusilli with cricket flour (*A. domesticus*)	1.0	349.1
P-090	Cricket, fried *(A. domesticus)*	1.0	3.8
P-094	Ready-mix beetroot risotto with insect protein (*A. diaperinus*)	1.0	137.4
P-095	Ready-mix brownie cake with insect protein (*A. diaperinus*)	1.0	89.2
P-096	Ready-mix oat patty mix with insect protein *(A. diaperinus)*	1.0	258.0
P-098	Spiced house cricket, tomato	1.0	1504.3
P-099	Spiced house cricket, smoked	1.0	1003.4
P-102	ZIRP bar sour cherry (*A. diaperinus*)	1.0	121.3

P-103	ZIRP bar apple strudel (*A. diaperinus*)	1.0	169.2
P-104	ZIRP bar apricot (*A. diaperinus*)	1.0	88.2
P-116	Protein shake with buffalo insect (strawberry)	1.0	657.4
P-122	Peanut butter with buffalo insect	1.0	209.3
P-125	"Dschungelade", dark chocolate with roasted mealworms (*T. molitor*)	1.0	42.9
P-126	"Dschungelade", milk chocolate with roasted mealworms (*T. molitor*)	1.0	28.4
P-132	Cricket cracker, tomato & oregano (*A. domesticus*)	1.0	104.7
P-133	Cricket cracker, rosmarine & thyme (*A. domesticus*)	1.0	178.2
P-134	Cricket cracker, tumeric & smoked paprika (*A. domesticus*)	1.0	268.6

4.3 DNA extraction

Before beginning with any DNA extraction procedure, all working surfaces were cleaned with DNA Exitus spray. To prevent cross contamination, spatulas and spoons for weighing sample materials from different species were cleaned with DNA Exitus spray between the procedures, gloves were changed, and the mortar and pestle were cleaned with DNA Exitus in between if more than one sample was homogenized.

4.3.1 DNA extraction with NucleoSpin® Plant II Kit

DNA from all ten insect samples and the three meat samples, listed in Tables 1 & 3, was extracted using the NucleoSpin® Plant II Kit in a duplicate modifying the incubation time each time. Nine out of ten insect samples underwent this process a third time with again a different incubation time, with the exception of the housefly (*Musca domestica)*, since the sample material was not sufficient for a third extraction.

The working solutions included two lysis buffers, PL1 and PL2, a precipitation buffer PL3, a binding buffer PC, two wash buffers PW1 and PW2 and an elution buffer PE. The wash buffer W2 was a concentrate and had to be diluted with ethanol in a ratio 1:4. RNAse A was provided lyophilized and had to be dissolved with RNAse free water - for each mg RNAse A, 10 µL of H_2O were required.

The insect sample material was already partially homogenized by AGES and most samples required no further preparation. The only exception was *Musca domestica*, since parts of the exoskeleton were clearly distinguishable. Because of this, it was further homogenized using a mortar and pestle. The meat samples were cut into small pieces using a scalpel. For the DNA extraction, 150 mg of each sample were weighed into a 1.5 mL Eppendorf tube and 900 µL of PL2 buffer were pipetted. In the case of *Musca domestica*, the sample material was absorbing a large amount of the buffer solution, therefore, it was established that a further addition of 300 µL PL2 buffer is sufficient for a successful extraction (1200 µL PL2 total). After pulse-vortexing the samples for 15 seconds, 30 µL of RNAse solution was added, followed by another 15 seconds of pulse-vortexing. Subsequently, all Eppendorf tubes were

centrifuged in a VWR Galaxy Mini® centrifuge for a few seconds in order to collect all remaining sample from the lid of the tube. Afterwards, the samples were placed in a preheated thermal block at 65°C and 1400 rpm to be incubated, where the first batch was incubated for 30 minutes, the second batch for 90 minutes, and the third batch (without *Musca domestica*) overnight.

In sequence, 225 µL PL3 buffer were pipetted in the microcentrifuge tubes, followed by 15 seconds of pulse-vortexing and incubation on ice for five minutes. The samples were then centrifuged at 11 000 rcf for one minute to ensure sufficient precipitation. For each sample, a NucleoSpin® Filter (violet ring) was placed in a 2 mL collection tube where all of the lysate was pipetted and the tubes were then centrifuged at 11 000 rcf for two minutes. Afterwards, the filtered lysate from the collection tube was transferred to a 5 mL Eppendorf tube and 1350 µL PC buffer were added. The solutions were then pulse-vortexed for 15 seconds. NucleoSpin® Plant II Columns (green ring) were placed in a 2 mL collection tube for each sample for the DNA binding step, and all of the lysate from the previous stage was filtered through this column with the help of a centrifugation step, 11 000 rcf for two minutes. The filtration did not occur for the whole sample solution at once, since the column capacity was 700 µL. Because of this, 675 µL of the solution was pipetted in the column twice, and at last the remaining solution, centrifuging in between and discarding the collected filtrate after each step.

To wash the silica membrane, 400 µL PW1 buffer was added to the column and centrifuged at 11 000 rcf for one minute, disposing of the flow-through afterwards. The process was then repeated two more times, with 700 µL PW2 buffer and another 200 µL PW2 buffer, with the last centrifugation step being extended to two minutes instead of one to dry the silica membrane completely.

For the elution stage, the dried spin column was transferred to a new 1.5 mL microcentrifuge tube and then 50 µL PE buffer (preheated, 65°C) was pipetted directly onto the membrane. All tubes were then incubated for five minutes at 65°C on the thermal block. Finally, all tubes were centrifuged at 11 000 rcf for one minute to elute.

After performing DNA concentration measurements, the DNA extracts were stored at -20°C and the spin columns were disposed of.

4.3.2 DNA extraction with DNeasy® mericon® Food Kit

DNA from all insect species samples, meat samples and feed samples was extracted using the DNeasy® mericon® Food Kit once. In terms of sample preparation, *Musca domestica* was grinded using mortar and pestle, since the sample material was not homogenous enough. Meat samples were cut into small pieces with a scalpel. From the feed samples, Nestlingfutter, Insektenfutter and the two canned feed samples from MjAMjAM were used without any additional preparation. The Eiweiß Cocktail was grinded in mortar and pestle, since it consisted of whole dried mealworms and roughly cut other insects. The rest of the feed samples were cut into small pieces using a scalpel.

The working solutions consisted of Food Lysis Buffer, Proteinase K, QIAquick® Spin Columns, Buffer PB, one wash buffer AW2 as a concentrate (to be diluted with 96-100 % ethanol, as stated on the bottle

of the buffer) and an elution buffer EB. The Small Fragment Protocol (200 mg) for processed foods was performed for all of the named samples.

Firstly, 200 mg of the samples were weighed in 2 mL Eppendorf tubes. Food Lysis Buffer was then added to all samples, the volume depending on the texture of the sample. For most samples, 1 mL was sufficient, with the exception of the Eiweiß Cocktail, Hühnerherzen and Sensitive Mini Adult Insekt, which required the addition of another 1 mL (2 mL total). After 2.5 μL Proteinase K were added, all samples were incubated on a thermal block at 60°C and 1000 rpm for 30 minutes. Next, they were cooled on ice until room temperature was reached and then centrifuged at 2500 rcf for five minutes. In the meantime, new 2 mL microcentrifuge tubes were prepared, with 500 μL of chloroform in each one (this step was performed in the fume hood). After the five minutes' centrifugation was over, 700 μL of the clear supernatant of each sample was transferred into the 2 mL microcentrifuge tube with chloroform. If 700 μL were not available as much of the clear solution as possible was pipetted into the chloroform tube, without taking up any of the precipitate. All Eppendorf tubes were then pulse-vortexed for 15 seconds and then centrifuged at 14 000 rcf for 15 minutes. During that time, new 2 mL microcentrifuge tubes were set up, with 1 mL of PB buffer inside. After completing the centrifugation step, 250 μL of the upper aqueous phase in each centrifuged sample were pipetted into the newly arranged 2 mL microcentrifuge tubes. The new mixtures were then pulse-vortexed for another 15 seconds. Following that, QIAquick® Spin Columns with 2 mL collection tubes were prepared for each sample, and 600 μL of each mixture from the previous step were transferred to the spin columns. Then all columns were centrifuged at 17 900 rcf for one minute and the flow through was discarded. This step was to be repeated then with all of the remaining sample in each tube.

For the washing stage, 500 μL AW2 buffer was added to all of the spin columns, and then they were centrifuged at 17 900 rcf for one minute, disposing of the flow through afterwards. The centrifugation step was repeated again to dry the silica membrane.

For the elution, the spin columns were transferred to 2 mL Eppendorf tubes and 100 μL of EB buffer was pipetted directly on the membrane. All columns were then incubated at room temperature for one minute and then centrifuged at 17 900 rcf for one minute for sufficient elution.

After measuring the DNA concentration, the DNA extracts were stored at -20°C in the freezer, and the spin columns were discarded.

4.4 Concentration measurement of the DNA extracts

Fluorometric measurements of DNA concentrations for all sample DNA extracts were performed with the Qubit™ 4 Fluorimeter (Thermo Fisher Scientific) using the Qubit™ 1x ds DNA BR (broad range) Assay Kit. Firstly, the Qubit Working Solution was prepared in the amounts calculated by the fluorimeter, comprising of Qubit™ dye and Qubit™ buffer solution, in a ratio of 1:200, respectively. Two standard solutions with known concentration were provided in the kit for external calibration before the extract measurements.

For the analysis, Qubit™ Assay tubes were set up, with 190 µL working solution for the calibration standards and 198 µL working solution for the extracts. Since the final volume of all tubes had to be 200 µL, 10 µL of the Qubit™ standards were added to the 190 µL tubes and 2 µL of each DNA extract were added to the 198 µL tubes. After vortexing the prepared solutions for 2-3 seconds, they were incubated at room temperature for two minutes and then measured with the Qubit™ 4 Fluorimeter on the dsDNA BR (broad range) setting. All measured DNA concentrations are listed in Tables 18-21 in the Appendix.

Finally, the DNA extracts were stored at -20°C in the freezer, and all prepared solutions for the concentration determination were discarded.

4.5 PCR and HRM analysis

4.5.1 Primer design

The primer pair employed for the amplification of all insect species investigated was provided initially by AGES (Ins_fw and Ins_rev). The optimal annealing temperature of 58°C was determined in AGES.

Primer design was conducted with the software PyroMark Assay Design 2.0, and all primers sets were based on the DNA sequences obtained from the results of next-generation sequencing (NGS) performed by AGES. All DNA sequences used for the primer design are listed in Table 22 in the Appendix. Suitable regions were chosen after aligning the DNA sequences of closely related species using The Molecular Evolutionary Genetics Analysis (MEGA) software, where sections of the DNA sequences where the species differentiated from each other were identified. Next, the webserver OligoCalc was used to calculate the theoretical melt temperature (“salt adjusted” as a setting for the temperature in the webserver) of the primers. They were tested for secondary structure formation in the webserver RNA fold, both at 37.5°C and at a temperature 5°C lower than the theoretical melt temperature. Lastly, the primers were tested for self-dimers and dimers with other primers incorporated into the method with the help of the website Oligo Analyzer.

Eight primer pairs and two additional reverse primers were designed in total in the course of this master´s thesis. All were synthesized by Sigma-Aldrich®. In the first batch, four primer pairs were ordered, designed to bind to the DNA of house cricket (Kurzflügelgrille_1_fw and Kurzflügelgrille_1_rev), black soldier fly (Soldatenfliege_1_fw and Soldatenfliege_1_rev), silkworm (Seidenraupe_1_fw and Seidenraupe_1_rev) and fruit fly (Fruchtfliege_1_fw and Fruchtfliege_1_rev). Next, in the second batch another four primer pairs were acquired, two of which were meant to bind to DNA from housefly (Krullfliege_1_fw and Krullfliege_1_rev; Krullfliege_2_fw and Krullfliege_2_rev), one to DNA from mealworm (Mehlwurm_1_fw and Mehlwurm_1_rev) and one to DNA from crazy red cricket (Steppengrille_1_fw and Steppengrille_1_rev). In the last batch, two reverse primers were ordered, both meant to alter the melt profile of migratory locust (Wander_1_rev and Wander_2_rev).

All primers were delivered in a lyophilized form. They were dissolved in RNAse free water according to the manufacturer`s instructions, followed by vortexing and tipping over the primers every five minutes for a period of twenty minutes. Finally, 10 µL aliquots of each primer were prepared and stored in the freezer at -20°C.

4.5.2 PCR-HRM procedure

PCR amplification was performed employing two different thermal cyclers. In the beginning, Rotor-Gene Q (Qiagen) was the main choice for most amplification experiments, followed by an evaluation with the Rotor Gene Q Software 2.3.1 (Qiagen), where the melt profiles of the amplicons were examined using HRM analysis. Additionally, melt curves were normalized by setting two normalisation ranges, leading range and tailing range. The other thermal cycler, Quantstudio 5, possesses the advantage of being able to test multiple annealing temperatures in a single run. Therefore, it was firstly only utilized when primer sets had to be tested, which was performed by examining their functionality at three different temperatures- their theoretical melt temperature (Tm), Tm - 1°C and Tm - 3°C. Its respective software, QuantStudio™ Design & Analysis Software 1.4.3 (Thermo Fisher Scientific), provided information only on the melt peak, without visualizing the melt curves from the HRM analysis, which was the primary downside to this software.

However, during the course of the master´s thesis, Rotor-Gene Q experienced several operational issues, which resulted in a search for an alternative solution. For that reason, PCR amplifications after the beginning of the technical malfunctions were only operated on Quantstudio 5, and an additional software was used for HRM analysis - High Resolution Melt Software v3.2 (Applied Biosystems™, Thermo Fisher Scientific). Normalisation ranges were again applied for the evaluation of the melt profiles.

For the mastermix preparation, mostly Type-it HRM PCR Kit (Qiagen) was used, with the exception of two test runs for another kit - MeltDoctor™ HRM Master Mix (Thermo Fisher Scientific). Each mastermix solution comprised of 2x HRM PCR Master Mix/ MeltDoctor™ HRM Master Mix, primer solutions in the required concentration, RNAse free water and $MgCl_2$ solution in a concentration of 2 mM, unless stated otherwise. For most PCR runs, 18 µL of reaction mixture were set up in the respective tubes for the thermal cycler, with the exception of the last experiment, which required the use of 17 µL. For Rotor-Gene Q, Strip Tubes (Qiagen) were used, and for Quantstudio 5- MicroAmp™ Optical tubes. Additionally, if any DNA extract dilutions were necessary for the experiment, the required amount of RNAse free water was arranged on this working station. Prior to pipetting the solution, all working surfaces were cleaned with DNA Exitus spray, and UV light disinfection was performed after each mastermix preparation.

In a different laboratory, the addition of all DNA extracts was carried out, pipetting 2 µL of the DNA solution to the mastermix. If a No Template Control (NC) was prepared, 2 µL of RNAse free water were added instead. Both DNA extracts and NCs were analysed in duplicate. The final volume of the reaction mixture always had to be 20 µL. If any DNA extract dilutions had to be set up, the calculated volume of DNA extracts was pipetted to the respectively arranged microcentrifuge tube with RNAse free water from the previous step. The same hygiene protocol was applied for this working station as for the one where the mastermix was prepared.

PCR runs were generally executed according to the settings shown in Table 5, unless otherwise noted. The program was adopted from the master´s thesis of Michaela Wildbacher (Wildbacher, 2023).

Table 5: Temperature program for PCR-HRM analysis.

Step		Time	Temperature	Repeats
Hold 1	initial denaturation step	5 min	95°C	
Cycling 1	denaturation annealing elongation	15 s 30 s 30 s	94°C 57.4°C 72°C	Number of cycles: 50
Hold 2	final elongation	10 min	72°C	
Hold 3	denaturation	1 min	95°C	
Hold 4	strand pairing	1 min	40°C	
HRM	HRM	-	65 - 95°C	0.1°C/step; 90 s

4.6 Agarose gel electrophoresis

In order to visualize results from specific PCR runs and compare the length of different PCR products, agarose gel electrophoresis was performed. For the procedure, PowerPac HV (Bio-Rad), as a power supply, in combination with Mini-Sub Cell GT Cell (Bio-Rad), as an electrophoresis cell, was used.

Before the sample preparation, the loading dye (ROTI®Load DNA orange 1, 6x) was diluted with nuclease free water in a ratio 1:3, resulting in a 2x Loading Dye (LD). As a marker, the Low Molecular Weight DNA Ladder from New England Biolabs was diluted with nuclease free water in a ratio 1:4, and the 2x LD was added. All PCR amplicons were diluted in a ratio 1:1, pipetting 3.5 µL from the PCR product and 3.5 µL from the 2x LD.

For the buffer solution, 10x TBE buffer was diluted down to 0.5x TBE by measuring 50 mL of the 10x TBE and filling them up to 1000 mL with nuclease free water. 10x TBE buffer solution was already provided. According to Michaela Wildbacher, it was prepared by mixing 108 g Tris(hydroxymethyl)aminomethane and 55 g of boric acid, dissolving them in 750 mL distilled water, adding 7.5 g EDTA and filling up the solution with distilled water until reaching 1000 mL. The final buffer needed to have a pH value of approximately 8.3 (Wildbacher, 2023).

An agarose concentration of 3% was chosen for the gel and therefore, 3 g of agarose were weighed in an Erlenmeyer flask and 100 mL of the 0.5x TBE buffer and a magnetic stirrer were added. The mixture was heated on a heating plate at 100 - 150°C with magnetic stirring function activated for 20 - 30 minutes. After a clear solution was obtained, the solution was transferred while still hot in an arranged gel chamber with an inserted comb, either a 26 - well or a 30 - well comb, depending on the number of amplicons to be analysed. Next, the gel was left to solidify for 30 - 40 minutes, after which the comb was carefully removed. The chamber with the gel was then transferred to the electrophoresis cell and the cell was filled with 0.5x TBE buffer until the gel was sufficiently covered. Subsequently, 6 µL of either the prepared PCR product solutions or the diluted DNA Ladder were added to the lanes, with the first and last well always occupied by the ladder. After closing the electrophoresis cell, it was connected to the power supply and an electric current of 80 Volt was applied.

In 90 – 120 minutes, after the colour indicator reached around 1 cm distance from the end of the gel, the gel was laid in a plastic container, covered with 75 mL staining dye (GelRed Nucleic Acid Gel Stain) and incubated for 20 minutes in the dark. This dye had to be newly prepared after every three uses, mixing 45 μL 1000X GelRed NucleicStain with 150 mL distilled water. To decolorize, the gel was then incubated for another 20 minutes in the dark, this time covered with distilled water. Lastly, the results were visualized with the help of an UV Transilluminator (Herolab) at a wavelength of 312 nm and the gel was photographed under UV light for further evaluation. The obtained photographs were then edited in Microsoft Word to label the different PCR products.

5. Results and discussion

This section presents the results of all primer sets tested for the differentiation of the insect species allowed in the EU. Moreover, the impact of the DNA extraction protocol, the DNA concentration and the used mastermix on the melt profiles are addressed. The applicability of the PCR-HRM method was also tested on not legally permitted insect species and feed and food samples. All primer sets that were developed in the course of this thesis are listed in Table 6, with the corresponding primers, their optimized concentrations and the limitations of the method. Primer sequences, their target species and theoretical annealing temperatures are presented in Tables 7, 8, 10 & 16.

Table 6: Primer sets tested, primers included, their optimized concentrations and aims of the method.

Primer set	Primers included	Optimized concentrations	Aims
Primer set I	Ins_fw	0.4 µM	To amplify DNA from all insect species.
	Ins_rev	0.4 µM	
Primer set II	Ins_fw	0.4 µM	To resolve the first cluster and make melt curves for black soldier fly and silkworm distinguishable from each other; to impact the melt profile of silkworm.
	Ins_rev	0.4 µM	
	Seidenraupe_1_fw	0.2 µM	
	Seidenraupe_1_rev	0.2 µM	
Primer set III	Ins_fw	0.4 µM	To resolve two clusters and make melt curves for black soldier fly and silkworm; for housefly, crazy red cricket and mealworm distinguishable from each other; to impact the melt profiles of housefly, mealworm and silkworm.
	Ins_rev	0.4 µM	
	Seidenraupe_1_fw	0.2 µM	
	Seidenraupe_1_rev	0.2 µM	
	Mehlwurm_1_fw	0.4 µM	
	Mehlwurm_1_rev	0.4 µM	
	Krullfliege_1_fw	0.4 µM	
Primer set IV	Ins_fw	0.4 µM	To resolve two clusters and make melt curves for black soldier fly and silkworm; for housefly, crazy red cricket and mealworm differentiable; to impact melt profiles of silkworm, mealworm and crazy red cricket.
	Ins_rev	0.4 µM	
	Seidenraupe_1_fw	0.2 µM	
	Seidenraupe_1_rev	0.2 µM	
	Mehlwurm_1_fw	0.4 µM	
	Mehlwurm_1_rev	0.4 µM	
	Steppengrille_1_rev	0.4 µM	
Primer set V	Ins_fw	0.4 µM	To resolve two clusters and make melt curves for black soldier fly and silkworm; for housefly, crazy red cricket and mealworm differentiable; to impact melt profiles of silkworm, mealworm and housefly.
	Ins_rev	0.4 µM	
	Seidenraupe_1_fw	0.2 µM	
	Seidenraupe_1_rev	0.2 µM	
	Mehlwurm_1_fw	0.4 µM	
	Krullfliege_1_fw	0.4 µM	
Primer set VI	Ins_fw	0.4 µM	To resolve all three clusters and make the melt curves for black soldier fly and silkworm; mealworm, housefly and crazy red cricket; house cricket and tropical house cricket distinguishable from each other; to impact the melt profiles of silkworm, mealworm, crazy red cricket, house cricket and tropical house cricket.
	Ins_rev	0.4 µM	
	Seidenraupe_1_fw	0.2 µM	
	Seidenraupe_1_rev	0.2 µM	
	Mehlwurm_1_fw	0.4 µM	
	Steppengrille_1_rev	0.4 µM	
Primer set VII	Ins_fw	0.4 µM	To resolve all three clusters and make the melt curves for black soldier fly and silkworm; mealworm, housefly and crazy red cricket; house cricket and tropical house cricket distinguishable from each other; to impact the melt profiles of silkworm, mealworm, crazy red cricket, house cricket and tropical house cricket.
	Ins_rev	0.4 µM	
	Seidenraupe_1_fw	0.2 µM	
	Seidenraupe_1_rev	0.2 µM	
	Mehlwurm_1_rev	0.4 µM	
	Steppengrille_1_rev	0.4 µM	

Primer set VIII	Ins_fw	0.4 µM	To resolve all three clusters and make the melt curves for black soldier fly and silkworm; mealworm, housefly and crazy red cricket; house cricket and tropical house cricket distinguishable from each other; to impact the melt profiles of silkworm, mealworm, crazy red cricket, house cricket and tropical house cricket; to influence the melt profile of migratory locust and improve its distinguishability from the one of mealworm.
	Ins_rev	0.4 µM	
	Seidenraupe_1_fw	0.2 µM	
	Seidenraupe_1_rev	0.2 µM	
	Mehlwurm_1_rev	0.4 µM	
	Steppengrille_1_rev	0.4 µM	
	Wander_AS_fw_1	0.4 µM	
Primer set IX	Ins_fw	0.4 µM	To resolve all three clusters and make the melt curves for black soldier fly and silkworm; mealworm, housefly and crazy red cricket; house cricket and tropical house cricket distinguishable from each other; to impact the melt profiles of silkworm, mealworm, crazy red cricket, house cricket and tropical house cricket; to influence the melt profile of migratory locust and improve its distinguishability from the one of mealworm.
	Ins_rev	0.4 µM	
	Seidenraupe_1_fw	0.2 µM	
	Seidenraupe_1_rev	0.2 µM	
	Mehlwurm_1_rev	0.4 µM	
	Steppengrille_1_rev	0.4 µM	
	Wander_2_rev	0.6 µM	
Primer set X	Ins_fw	0.4 µM	To resolve all three clusters and make the melt curves for black soldier fly and silkworm; mealworm, housefly and crazy red cricket; house cricket and tropical house cricket distinguishable from each other; to impact the melt profiles of silkworm, mealworm, crazy red cricket, house cricket and tropical house cricket; to influence the melt profile of migratory locust and improve its distinguishability from the one of mealworm.
	Ins_rev	0.4 µM	
	Seidenraupe_1_fw	0.2 µM	
	Seidenraupe_1_rev	0.2 µM	
	Mehlwurm_1_rev	0.4 µM	
	Steppengrille_1_rev	0.4 µM	
	Wander_1_rev	0.6 µM	
Primer set XI	Ins_fw	0.4 µM	To resolve all three clusters and make the melt curves for black soldier fly and silkworm; mealworm, housefly and crazy red cricket; house cricket and tropical house cricket distinguishable from each other; to impact the melt profiles of silkworm, mealworm, crazy red cricket, house cricket and tropical house cricket; to improve repeatability of melt curves for food and feed samples through reduction of the number of primers.
	Ins_rev	0.4 µM	
	Seidenraupe_1_fw	0.2 µM	
	Seidenraupe_1_rev	0.2 µM	
	Steppengrille_1_rev	0.4 µM	

5.1 PCR-HRM with Primer set I

In this method, only one primer pair was employed, namely the primer pair provided by AGES, and the respective sequences are presented in Table 7. This primer pair was tested first in a study, where the researchers aimed to develop a DNA barcoding method to differentiate between insect species in food (Hillinger et al., 2023). Both primers were added in a concentration of 0.4 µM. DNA extracts from all insect species listed in section 4.2.1, Table 1 and all meat species listed in section 4.2.3, Table 3 were analysed. For this experiment, all DNA extracts with a concentration above 2.5 ng/µL were diluted down to this value. Extracts with an initial concentration < 2.5 ng/µL were tested without further dilution: silkworm, house cricket, crazy red cricket, cockroach for extracts obtained with NucleoSpin® Plant II Kit, and only house cricket for DNeasy® mericon® Food Kit (all DNA concentrations are presented in Tables 18 & 19 in the Appendix). DNA extracts with a cell lysis time of 30 minutes (Table 18, Appendix) obtained with the Nucleospin® Plant II Kit were used for this experiment. For the PCR run, the program from section 4.5.2, Table 5, was performed on Rotor-Gene Q. The aim was to investigate whether this one primer pair would be sufficient to differentiate the melt curves for the insect species, and secondly, to assess the impact of the extraction method on the melt curve profiles.

Table 7: Primer set I with Ins_fw and Ins_rev, sequence and optimal annealing temperature (Wildbacher, 2023). Fw: forward; rev: reverse.

Original primer name	Sequence 5' → 3'	Name used in this work	Optimal annealing temperature
16S_10_fwd_SHR	TWACGCTGTTATCCCTAAGG	Ins_fw	58.0 °C
16S_rev_SH1	GACGAGAAGACCCTATAGA	Ins_rev	

Figure 2 visualizes the amplification results of the meat and insect sample extracts under the named conditions. For the species black soldier fly and silkworm, no fluorescence signal was observed, indicating that no DNA amplification had taken place. Next, the amplification curves for crazy red cricket, housefly and mealworm exhibited an atypical form, instead of the smooth, exponential rise typically seen. The rest of the amplification curves, namely those for the house cricket, tropical house cricket, cockroach, lesser mealworm, and fruit fly, although for fruit fly the fluorescence signal was weaker, possessed a typical shape. Additionally, a fluorescence signal was also detected for all three meat species, although the primers were initially designed for insects.

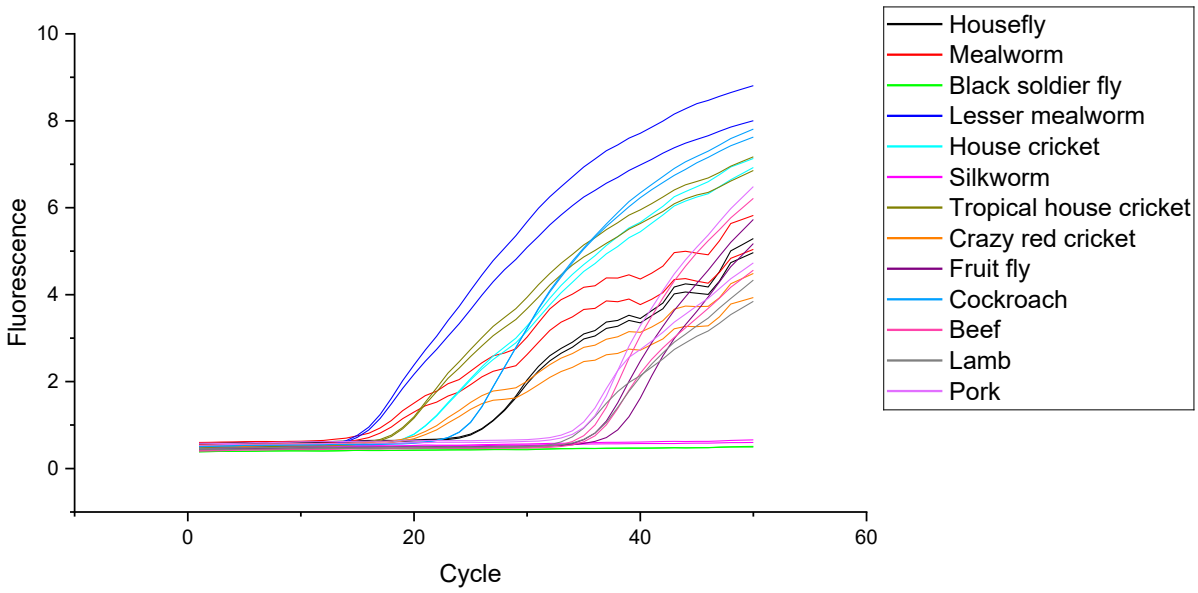


Figure 2: Amplification curves for all insect and meat samples when using primer pair Ins_fw and Ins_rev, DNA extracts obtained with NucleoSpin® Plant II Kit.

Figure 3 visualizes the amplification curves acquired after performing the PCR with the insect and meat DNA extracts, where the DNA isolated with the DNeasy® mericon® Food Kit was analysed. Similar to the results shown in Figure 2, the amplification curves for black soldier fly and silkworm suggested that the amplification process was unsuccessful. The fruit fly, housefly, mealworm and crazy red cricket amplification curves demonstrated again atypical curve form. However, the rest of the insect species displayed typical amplification results in terms of curve shape, and the DNA from meat species was also amplified.

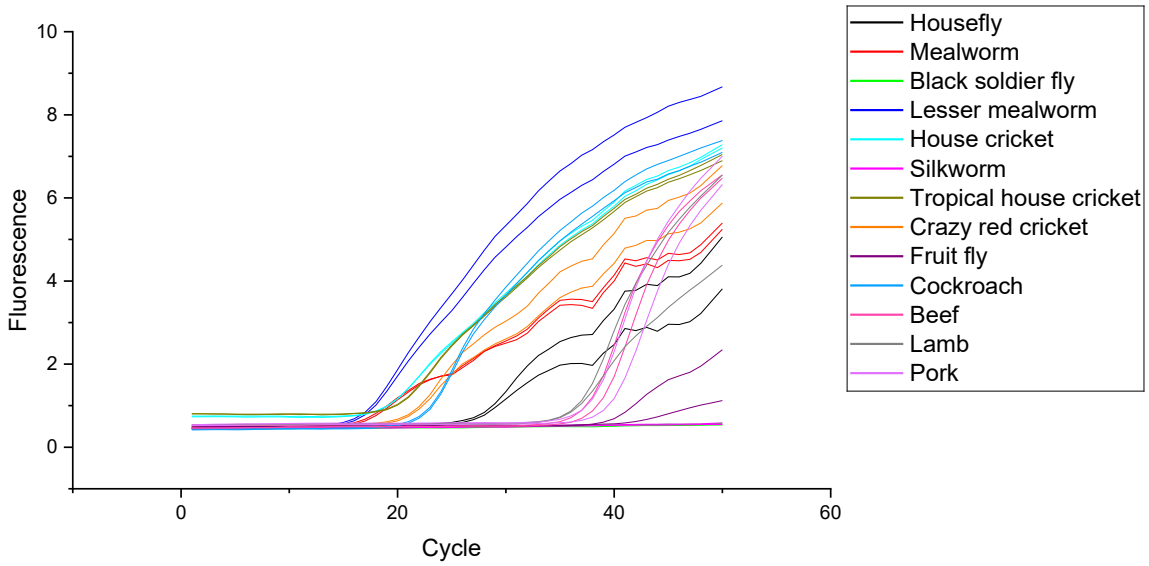


Figure 3: Amplification for all insect and meat samples when using primer pair Ins_fw and Ins_rev, DNA extracts obtained with DNeasy® mericon® Food Kit.

In Figure 4, the HRM results for DNA extracts obtained with the NucleoSpin® Plant II Kit are presented. Firstly, all insect species´ amplicons exhibited good repeatability Three clusters of melt curves were evident. The first cluster (blue circle), with melt temperatures ranging from 71-72 °C, consisted of the melt curve of silkworm and black soldier fly, which were not expected to exhibit a melt curve in the first place, based on the results from the amplification. The second cluster (red circle), with melt temperatures between 72.5 and 73 °C, included the melt curves of housefly, mealworm and crazy red cricket. Lastly, the third cluster (green circle), where melt temperatures ranged between 74 and 74.5°C, contained the melt curves of fruit fly and tropical house cricket. All of these three clusters contained insect species legally allowed in insect feed, indicating that the differentiation of their melt curves was a significant challenge to be faced in future experiments.

The rest of the insect species` amplicons, namely house cricket, cockroach, and lesser mealworm, melted at temperatures that made their melt curves more distinguishable. Looking at the meat species results, only the melt curves of beef were adequately repeatable while the melt curves of lamb and pork varied significantly. However, since the primers used were not designed with other species than insects in mind, the quality of these results was not unexpected.

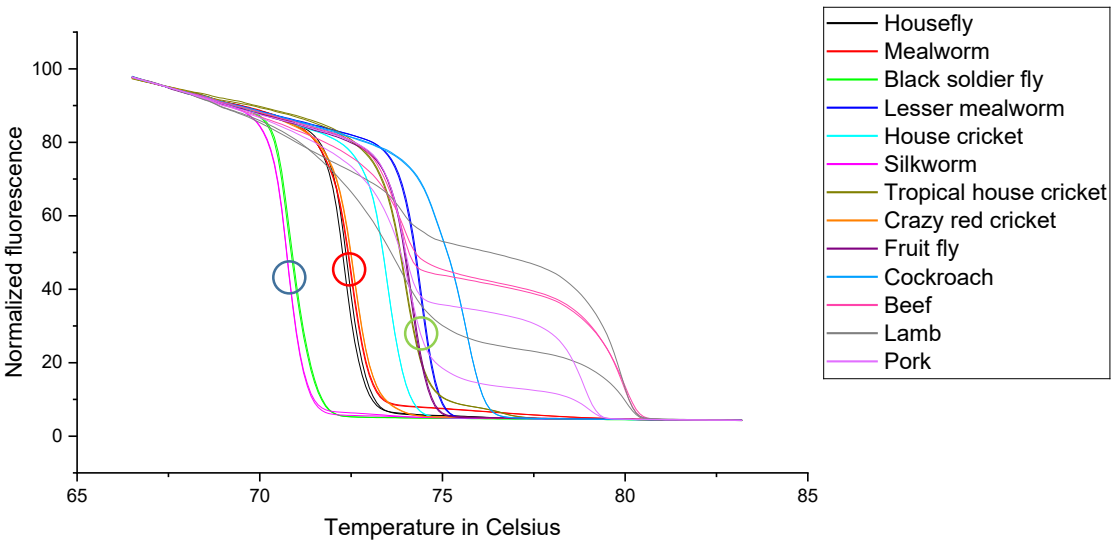


Figure 4: HRM analysis for all insect and meat samples when using primer pair Ins_fw and Ins_rev, DNA extracts obtained with NucleoSpin® Plant II Kit. Blue circle: first cluster, red circle: second cluster, green circle: third cluster.

Figure 5 displays the results of the experiment carried out with the DNA extracts acquired with the DNeasy® mericon® Food Kit. Overall, all melt curves were similarly repeatable compared to the results from the previous HRM analysis, with two exceptions. Firstly, the melt curves of fruit fly appeared at a different melt temperature in comparison to the prior experiment. Here, the amplicons melted between 71°C and 72°C, and previously they melted at 74-74.5°C. However, in the second experiment, the fluorescence signal of the fruit fly PCR product was reduced, indicating a decrease in amplification efficiency. Secondly, the other exception were the melt curves of pork, which were overlapping in the second experiment and were clearly distinguishable from those of lamb. In terms of clusters built, the first (silkworm and black soldier fly, blue circle) and second cluster (housefly, mealworm, crazy red cricket, red circle) appeared again at the described temperatures in the previous analysis. Additionally, with these DNA extracts, house cricket and tropical house cricket melt curves were less differentiable in contrast to the previous HRM, this time with melt temperatures between 73.6 and 73.9°C. With DNA isolated with NucleoSpin® Plant II Kit, the difference between their melt curves was 0.5°C, namely 73.6°C-74.1°C, which led to the formation of a third cluster (green circle). When analysing DNA obtained with DNeasy® mericon® Food Kit, tropical house cricket amplicons melted at a higher temperature. All clusters comprised insect species legally permitted in insect feed, highlighting the substantial challenge of differentiating their melt curves, which were to be addressed in future experiments.

To conclude, the melting behaviour of amplicons of DNA isolated with both methods did not differ greatly with some exceptions. However, these differences could be due to a number of additional factors. For instance, differences in the melting temperatures could be attributed to matrix effects, if the dilution was insufficient or not performed at all. Moreover, the amplification curves for six out of the ten insect species extracts analysed were unusual, which asked for further optimization of this step.

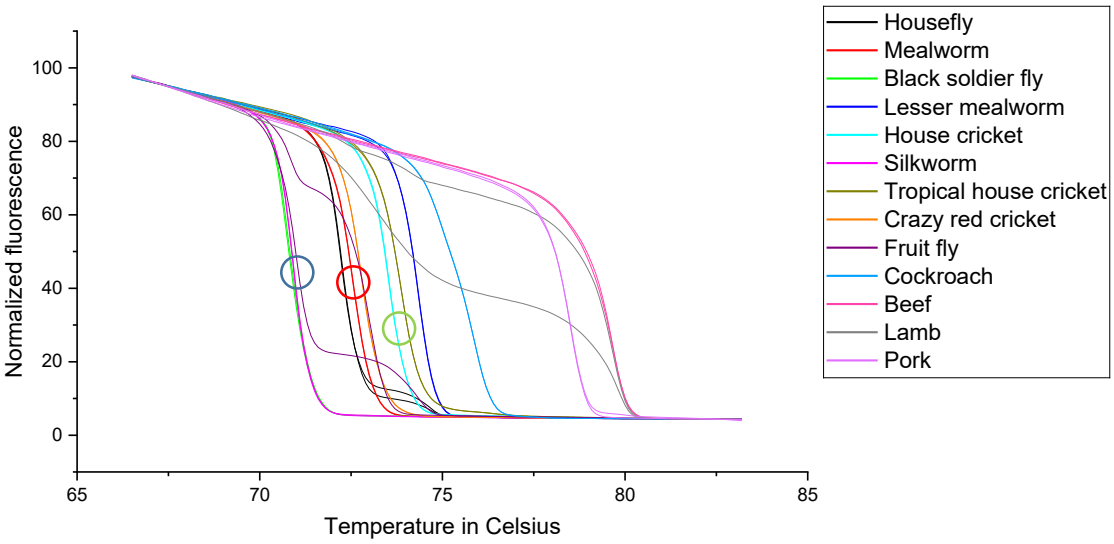


Figure 5: HRM analysis for all insect and meat samples when using primer pair Ins_fw and Ins_rev, DNA extracts obtained with DNeasy® mericon® Food Kit. Blue circle: first cluster, red circle: second cluster, green circle: third cluster.

5.1.1 PCR amplification optimization

A potential solution to the issue of atypical DNA amplification curves of housefly, mealworm, black soldier crazy red cricket and fruit fly and the absence of detectable DNA amplification curves of silkworm and black soldier fly was the addition of $MgCl_2$ to the mastermix solution. In the master´s thesis of Michaela Wildbacher, the supplementation of the reaction solution with 2 mM $MgCl_2$ improved the amplification (Wildbacher, 2023). As stated in section 3.2.2, in the presence of Mg^{2+} ions the DNA polymerase exhibits improved efficiency. For this experiment, $MgCl_2$ was added in two different concentrations, 1 mM and 2 mM. DNA extracts of six insect species, which demonstrated atypical melt curves (fruit fly, housefly, mealworm and crazy red cricket) or where a lack of fluorescence signal was observed (black soldier fly and silkworm), were analysed on Rotor-Gene Q. The same DNA extracts dilutions were used as in the previous experiment: DNA extracts with a concentration above 2.5 ng/µL were diluted to a concentration of 2.5 ng/µL, and extracts with a concentration < 2.5 ng/µL were tested without further dilution. Both the DNA extracts obtained with NucleoSpin® Plant II Kit and with DNeasy® mericon® Food Kit were tested to assess the influence of the extraction method on the amplification efficiency. Primers Ins_fw and Ins_rev in a concentration of 0.4 µM were used.

In Figure 6, the amplification curves for all DNA extracts from NucleoSpin® Plant II Kit are presented. With the addition of $MgCl_2$ to the reaction mix, the amplification curves for mealworm, crazy red cricket and housefly demonstrated a typical shape. Additionally, both black soldier and silkworm demonstrated amplification curves instead of the flat lines observed in Figure 3, which previously indicated inadequate amplification. These findings indicate improved amplification efficiency for these species, since the PCR products generated a sufficient fluorescence signal. The amplification curves for fruit fly seemed to be unaffected by the substitution of $MgCl_2$ and remained similar.

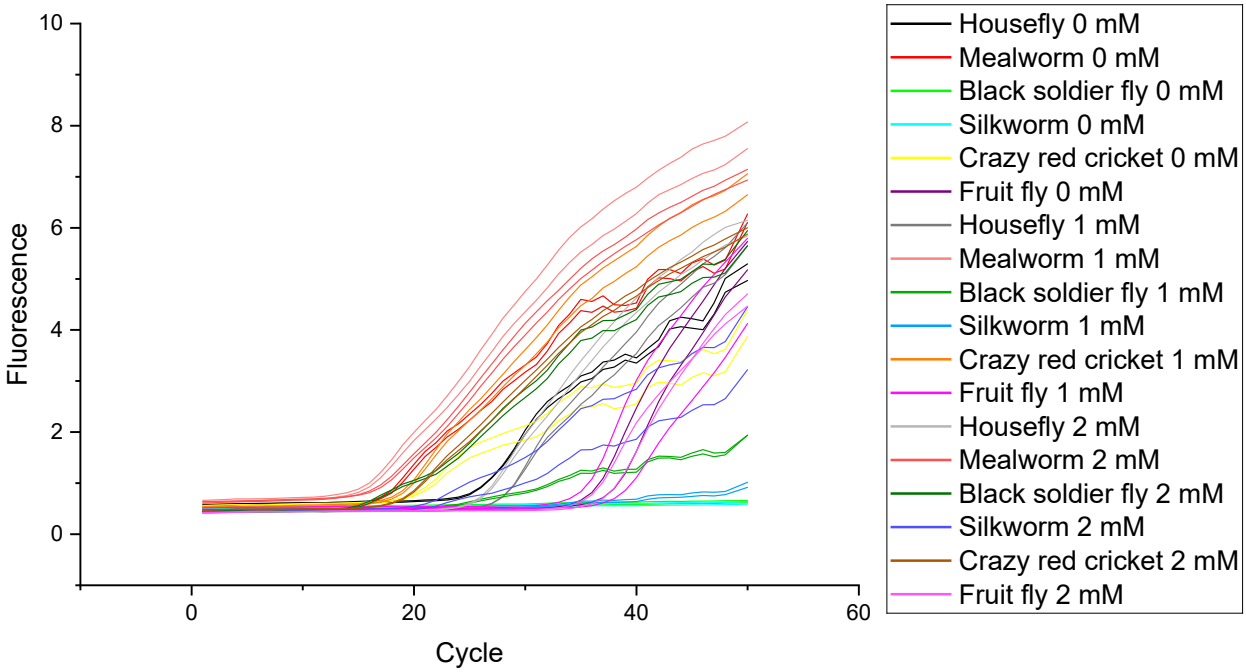


Figure 6: Amplification curves (NucleoSpin® Plant II Kit) for six insect species without $MgCl_2$ and with the addition of 1mM and 2 mM $MgCl_2$ to the mastermix.

Figure 7 displays the amplification results for the same six insect species with the addition of 1 mM and 2 mM $MgCl_2$, investigating the DNA extracts obtained with DNeasy® mericon® Food Kit. Overall, the same changes in the shape of the amplification curves were observed, with previously irregular curves (for mealworm, crazy red cricket and housefly) obtaining the typical shape and black soldier fly and silkworm demonstrating an amplification curve, although its form was not smooth. Additionally, the fluorescence signal was most intense when 2 mM $MgCl_2$ were added. Fruit fly DNA amplification was again not influenced by the presence of $MgCl_2$.

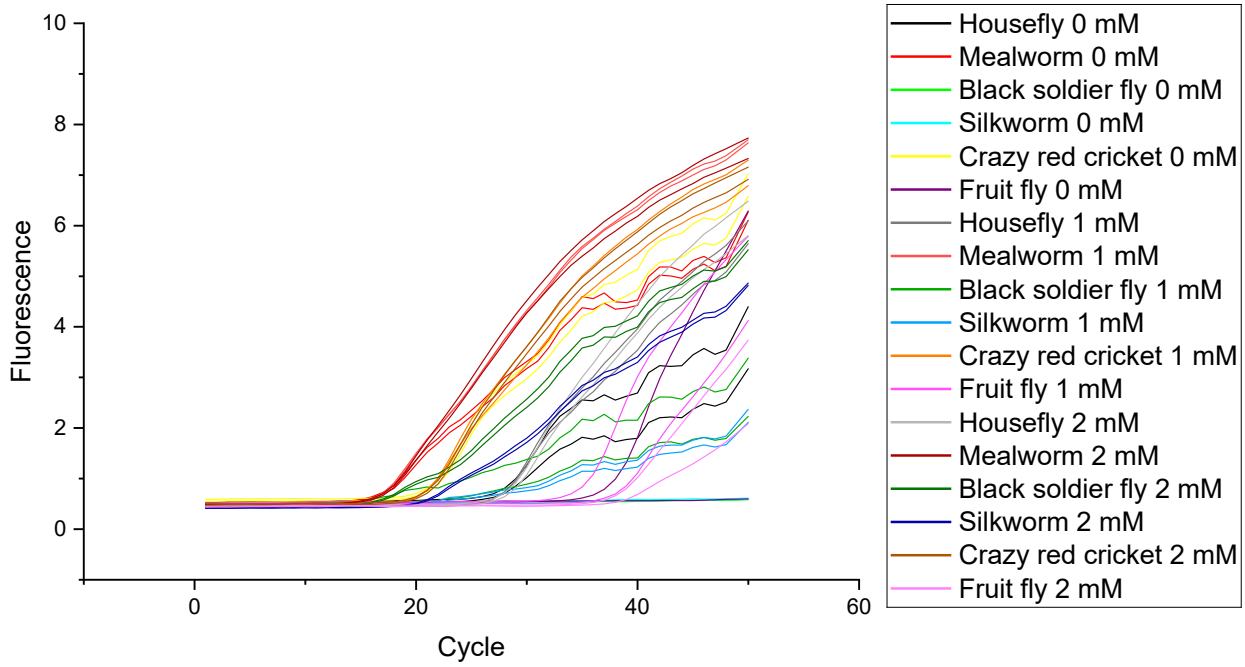


Figure 7: Amplification curves (DNeasy® mericon® Food Kit) for six insect species without $MgCl_2$ and with the addition of 1mM and 2 mM $MgCl_2$ to the mastermix.

Due to the positive impact of the addition of $MgCl_2$ in the reaction mix on the amplification of DNA from five out of six problematic insect species, it was further proceeded with a concentration of 2 mM $MgCl_2$. There were no indications that the DNA extraction method had any impact on the amplification process.

5.1.2 Assessment of matrix interferences

To investigate whether the DNA extraction protocol influenced the melting profile, a subsequent experiment was conducted on Rotor-Gene Q, analysing DNA extracts of all insects obtained with both methods. As a result of this test, the melt curves for four insect species (house cricket, silkworm, crazy red cricket and cockroach) did not overlap completely after performing HRM analysis, as shown in Figure 8. Among all insect DNA extracts of these species, only three were diluted to 2.5 ng/µL – the silkworm, cockroach and crazy red cricket extracts, acquired with the DNeasy® mericon® Food Kit. All of the extracts obtained with the NucleoSpin® Plant II Kit and the house cricket DNA extract from the DNeasy® mericon® Food Kit were analysed undiluted, since their concentration was < 2.5 ng/µL. Moreover, they were the only undiluted extracts analysed in the whole experiment. Therefore, matrix influences were a possible reason for these observations.

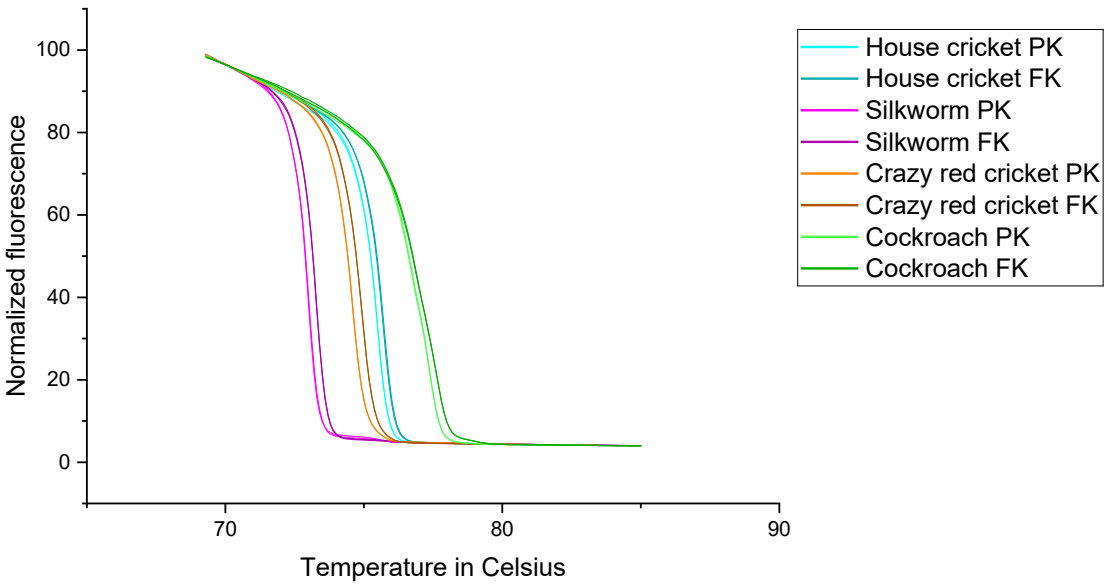


Figure 8: HRM curves for house cricket, silkworm, crazy red cricket and cockroach. DNA was extracted both with NucleoSpin® Plant II Kit (PK) and DNeasy® mericon® Food Kit (FK). Primers Ins_fw and Ins_rev in a concentration of 0.4 µM were used.

In a consecutively executed experiment, dilutions with nuclease free water were prepared from house cricket, silkworm, crazy red cricket and cockroach DNA extracts in ratios 1:10, 1:20 and 1:50 and then subjected to PCR-HRM analysis. The aim was to assess potential matrix effects. Figure 9 visualizes the HRM melt profiles for all dilutions. Since all melt curves of a single species overlapped consistently, regardless of the dilution factor, it became evident that the variations in the melt profiles observed in the previous PCR run were attributable to matrix interferences. However, the silkworm melt curves presented an exception, as the repeatability of the PCR products' melt curves for the DNeasy® mericon® Food Kit was suboptimal for the 1:10 and 1:50 dilutions.

The results of this test clearly indicated that the matrix affected the melt profiles. As a precaution for subsequent experiments, a minimum dilution factor of 1:10 was adopted for DNA extracts with an initial concentration below 2.5 ng/µL.

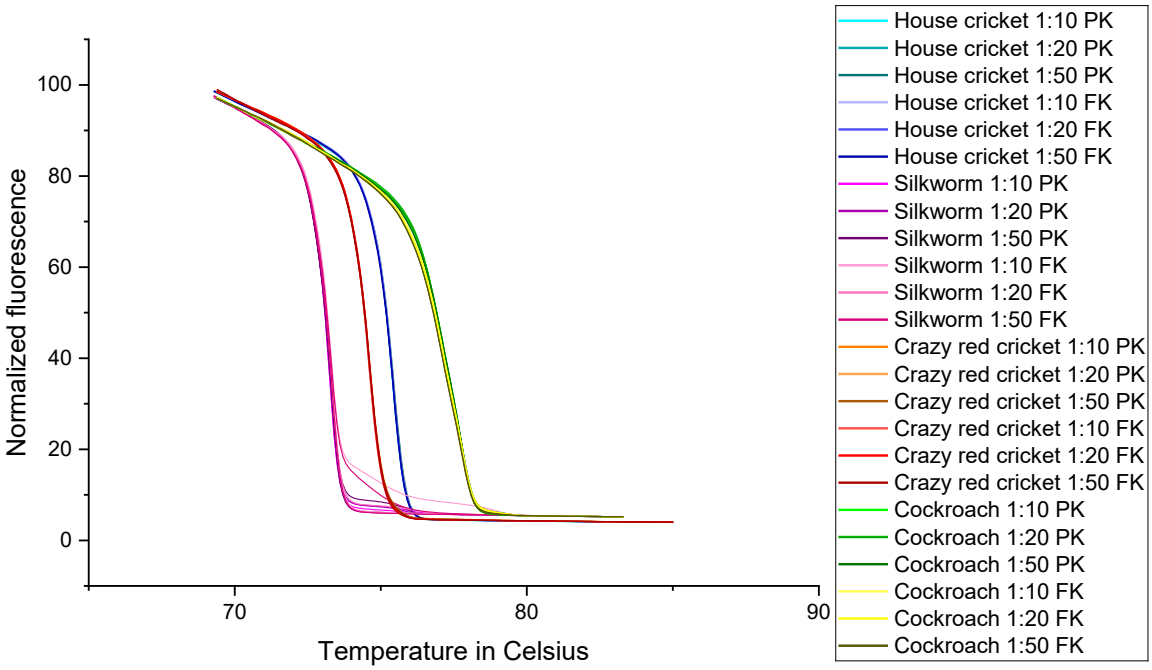


Figure 9*:* HRM curves for dilutions of DNA extracts from house cricket, silkworm, crazy red cricket and cockroach (1:10, 1:20, 1:50), extracted both with NucleoSpin® Plant II Kit (PK) and DNeasy® mericon® Food Kit (FK). Primers Ins_fw and Ins_rev in a concentration of 0.4 µM were used.

5.2 Development of Primer set II for the differentiation of silkworm and black soldier fly

Based on the observations from previous PCR runs, it became evident that additional primers were necessary to modify the melt profiles of one or more insect species within the identified clusters. The primary focus of further work was on the first cluster described in section 5.1, involving silkworm and black soldier fly and the third cluster which comprised of melt curves for tropical house cricket and house cricket, as depicted in Figure 4. Moreover, the inefficient amplification of fruit fly DNA, as seen in Figure 2, prompted optimization efforts for this task.

Primer pairs for the species tropical house cricket, black soldier fly, silkworm, and fruit fly were designed employing the method explained in section 4.5.1 and all primers are listed in Table 8. Optimal regions of the NGS DNA sequences for the procedure were chosen when closely related species were aligned with the help of the MEGA software. The silkworm NGS sequence was aligned with the one of black soldier fly, and the tropical house cricket NGS sequence with the one from house cricket. For fruit fly, the NGS sequence was not compared to any other DNA sequences, since the main objective was to improve its amplification. Table 9 presents the target regions in each NGS sequence for the respective insect species, and shows that these target regions were supposed to be different for each insect species.

All primers were designed to supplement the Ins_fw and Ins_rev from the previous PCR runs, therefore, the annealing temperature from the implemented PCR program, 57.4°C, was also the goal temperature for the newly designed primers, with an acceptable variation of ± 2°C.

Table 8: Primer pairs for tropical house cricket, black soldier fly, silkworm, and fruit fly. Fw: forward; rev: reverse.

Primer name	Sequence 5' → 3'	Theoretical melt temperature (salt adjusted)	Target insect species
Kurzflügelgrille_1 _fw	CAATCATCATAGTCCAATTTAAAG	56.6°C	tropical house cricket
Kurzflügelgrille_1 _rev	CCCTATAGATCTTTATACATTGG	57.6°C	tropical house cricket
Soldatenfliege_1_fw	GCGCCCTTGAATGATCTTC	57.5°C	black soldier fly
Soldatenfliege_1_ rev	GATAGGCCTCTGTTTTGGC	57.5°C	black soldier fly
Seidenraupe_1_fw	GCAGAAATACCAGATATAAATATAG	57.6ºC	silkworm
Seidenraupe_1_rev	CTCCTACTCCTGTTTCTGC	57.5°C	silkworm
Fruchtfliege_1_fw	CGCTGTTATCCCTAAAGTAAC	57.5°C	fruit fly
Fruchtfliege_1_rev	GACGAGAAGACCCTATAAATC	57.5°C	fruit fly

Table 9: Target regions for primer pairs for black soldier fly, silkworm, tropical house cricket and fruit fly. Target region sequence is presented in the 5' → 3' direction.

Species	English name	Primer pair	Primer pair bound to target region (red: fw, green: rev)
Hermietia illucens	black soldier fly	Soldatenfliege_1_fw/rev	GCGCCCTTGAATGATCTTCCTAACTTAAAATTAGGGTTGTAGT TAATTATAACATTTGATTTGCATTCAAAAAGTACTATGCTTTA GTCTTCCTTGAATATGAAGCGATTTATTGCAATTAGTTTCGAC CTAATCTTAGATAACTTTTATCCTTATTCTTTAATTGAAGCCA AAACAGAGGCCTATC
Bombyx mori	silkworm	Seidenraupe_1_fw/rev	GCAGAAATACCAGATATAAATATAGTTAAAATAGATAAAAATA ATAAAACCTTAAAAAAAAATATATCAATCAATAAATAATTAAA ACGAATTAATAAATATACACCGGCCGTAACCAAAGTAGAAGAA TGAACTAAAGCAGAAACAGGAGTAGGAG
Gryllodes sigillatus	tropical house cricket	Kurzflügelgrille_1 _fw/rev	CAATCATCATAGTCCAATTTAAAGAAGTTTTTTTATTCTCTCG TCACCCCAACCAAATACAATAAATCAATATTCAAGTCATAATA CTATCCTTAATATATTAATCCAATGTATAAAGATCTATAGGG
Drosophila	fruit fly	Fruchtfliege_1_fw/rev	CGCTGTTATCCCTAAAGTAACTTAAATTTTTAATCATTATTAA TGGATCAATTATTCATAAATTAATGAATTATTACAATTAAAAG TTTTTTAAATTTTAATATCACCCCAATAAAATATTTTATTTTA TTATAATAAAATTAATCTTTACAATTAAAATAAATAAAATATA AAGATTTATAGGGTCTTCTCGTC

All four primer sets were subsequently tested on Rotor-Gene Q with DNA extracts from all insect species, obtained using the NucleoSpin® Plant II Kit following a 30-minute cell lysis incubation. DNA extracts from house cricket, silkworm, crazy red cricket, and cockroach were analysed at a 1:50 dilution of their initial concentrations, while the remaining DNA extracts were tested at a concentration of 2.5 ng/µL.

The concentrations for the new primer sets were determined in a PCR run on Quantstudio 5, where each primer pair was tested solely on the species for which it was designed for and in two concentrations - 0.2 and 0.4 µM, in the presence of 0.4 µM Ins_fw and Ins_rev. For the house cricket and black soldier fly, there was not a big difference between the amplification with the different primer concentrations. On the other hand, for both fruit fly and silkworm the alternating concentrations had a large impact on the

amplification. In the case of fruit fly, at 0.4 μM the Ct (Cycle threshold) value was higher (29.9 for 0.2 μM and 35.6 for 0.4 μM), suggesting that the DNA amplification was better at a lower primer concentration. For silkworm primers, the change of the melt profile (Figure 16) was also observed at the lower concentration. Furthermore, the melt peaks of possible primer dimers were less intense compared to the ones at the higher concentration, as shown in Figure 17. As a result of this analysis, primer pairs for tropical house cricket and black soldier fly were implemented in a concentration of 0.4 μM, and those for fruit fly and silkworm in 0.2 μM.

Figure 10 visualizes the results from the HRM analysis of the test of the primer pair for tropical house cricket, namely Kurzflügelgrille_1 _fw and Kurzflügelgrille_1 _rev in a concentration of 0.4 μM. While the melt curve for the target species was not clearly distinguishable from that for the house cricket, several other melt profiles were impacted by this change in the method. The repeatability of the mealworm melt curves was negatively influenced, and the melt curves of housefly, silkworm and fruit fly were indicating that the DNA amplification was inefficient, especially when comparing their melt curves with the one of the negative control.

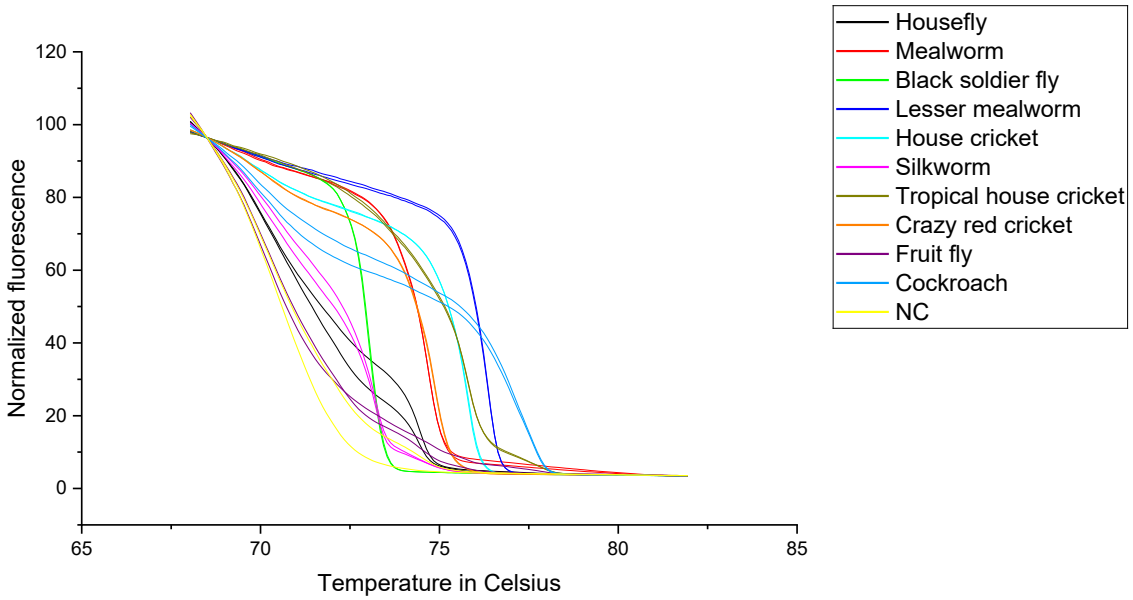


Figure 10: HRM analysis of DNA from housefly, mealworm, black soldier fly, lesser mealworm, house cricket, silkworm, tropical house cricket, crazy red cricket, fruit fly, cockroach, and no template control (NC) with primer pair for tropical house cricket and primer pair from AGES.

Figure 11 demonstrates that the DNA amplification of the three species in question was indeed inadequate, particularly in comparison to the no template control. These results were not satisfactory, since the DNA amplification of the legally permitted insect species in feed is crucial for the development of this method, and while fruit fly is not a part of the legislation, housefly and silkworm are included.

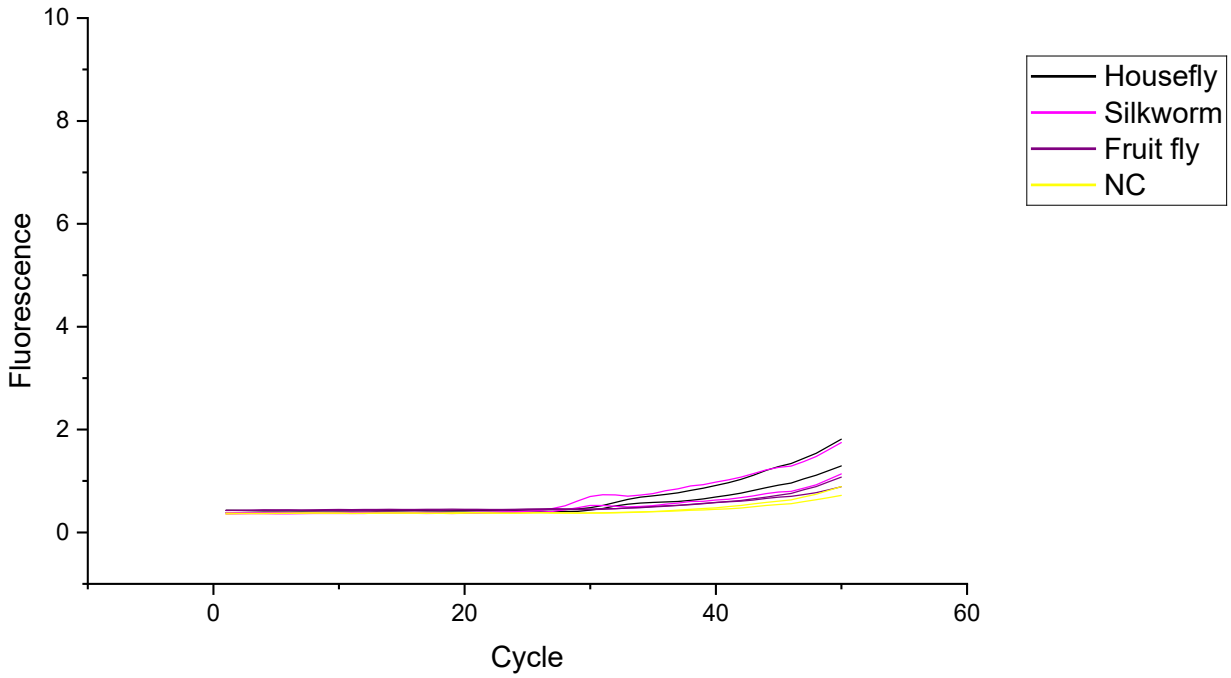


Figure 11: Amplification curves of housefly, silkworm and fruit fly DNA and no template control (NC), when testing primer pair for tropical house cricket and primer pair from AGES.

In Figure 12, the results of the HRM analysis when assessing the primer pair for black soldier fly (Soldatenfliege_1_fw and Soldatenfliege_1_rev, concentration 0.4 μM) are illustrated. The melt profile for black soldier fly was undoubtedly altered- in this PCR run, the black soldier fly amplicons melted at a temperature around 77°C compared to around 73°C in other experiments (Figures 14 & 16). However, the melt curves for several other insect species were negatively impacted by this addition to the reaction mix, with the repeatability of the melt curves of cockroach and crazy red cricket decreasing. Another negative consequence was the decrease in fluorescence signal for housefly, silkworm and fruit fly compared to the results, as shown in Figure 13 and when compared to the results in Figure 7. Corresponding to the results from the test of the tropical house cricket primer pair, the primer pair designed for black soldier fly produced results that excluded its further implementation in the advancement of the method.

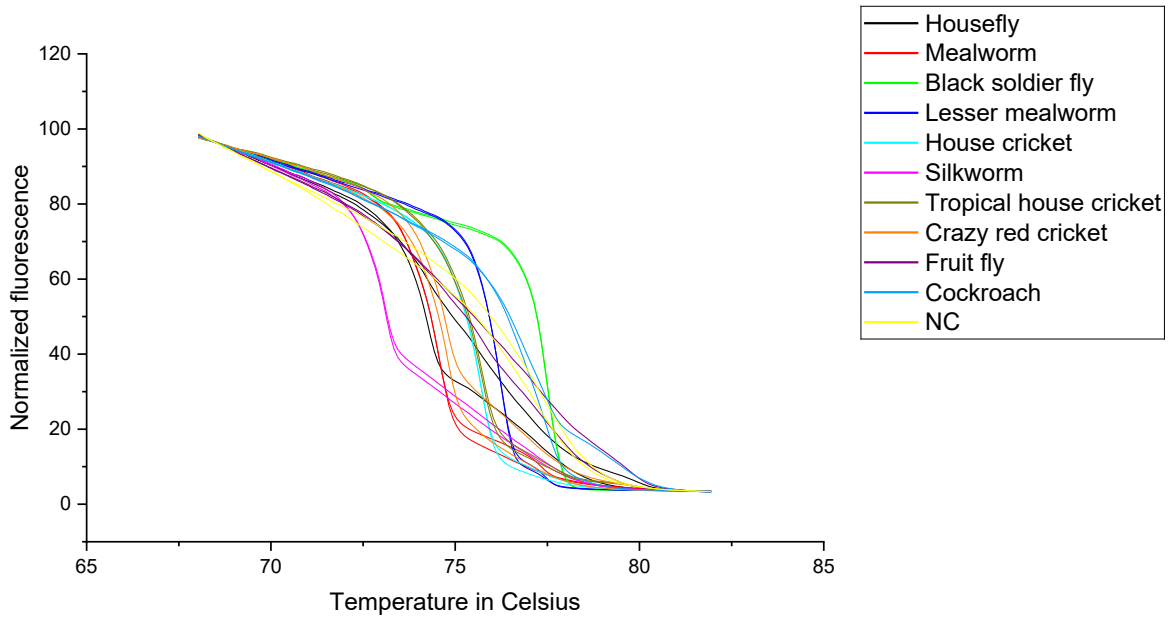


Figure 12: HRM analysis of DNA from housefly, mealworm, black soldier fly, lesser mealworm, house cricket, silkworm, tropical house cricket, crazy red cricket, fruit fly, cockroach, and no template control (NC) with primer pair for black soldier fly and primer pair from AGES.

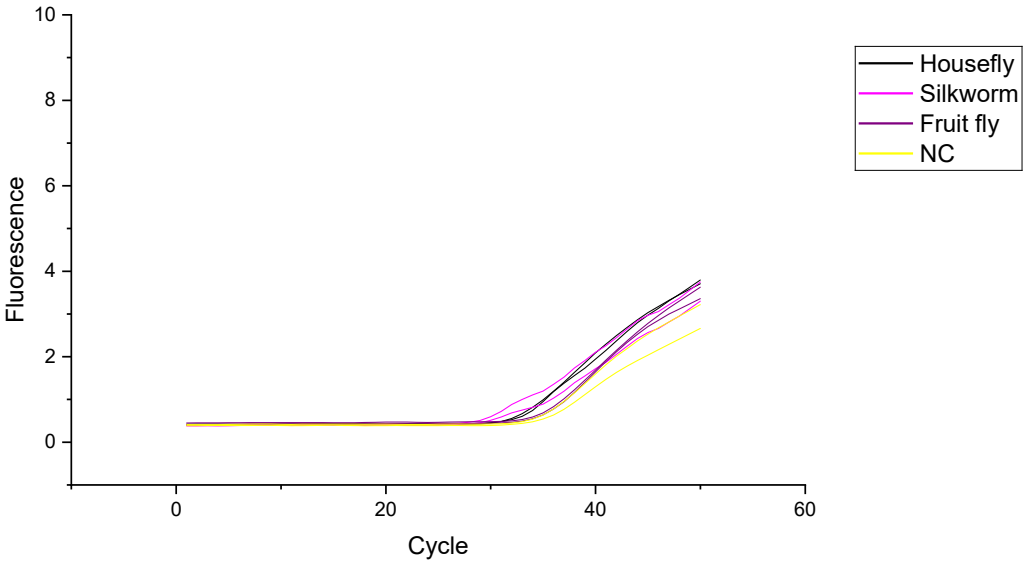


Figure 13: Amplification curves of housefly, silkworm and fruit fly DNA and no template control (NC), when testing primer pair for black soldier fly and primer pair from AGES.

Figure 14 visualizes the results of the HRM analysis when testing the primer pair Fruchtfliege_1_fw and Fruchtfliege_1_rev in a concentration of 0.2 μM, designed for improving the amplification of fruit fly DNA. While the repeatability of the melt curves of all insect species DNA was good, and the negative control induced no fluorescence signal, the fruit fly melt profile remained inconclusive with insufficient repeatability. In Figure 15, it was evident that DNA amplification of this species occurred, especially after comparing its amplification curves to the no template control. However, the melt behaviour continued to

be unsatisfactory for proper analysis. Therefore, this primer pair was not further implemented in future experiments. The inefficient analysis of fruit fly DNA was generally not a priority in following experiments, since this insect species is not legally permitted for use in feed in the EU.

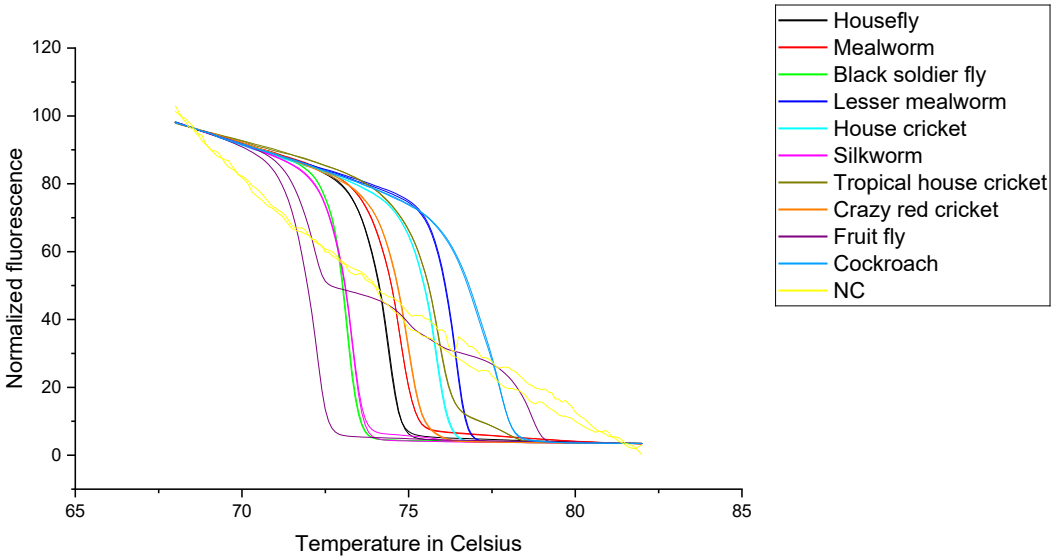


Figure 14: HRM analysis of DNA from housefly, mealworm, black soldier fly, lesser mealworm, house cricket, silkworm, tropical house cricket, crazy red cricket, fruit fly, cockroach, and no template control (NC) with primer pair for fruit fly and primer pair from AGES.

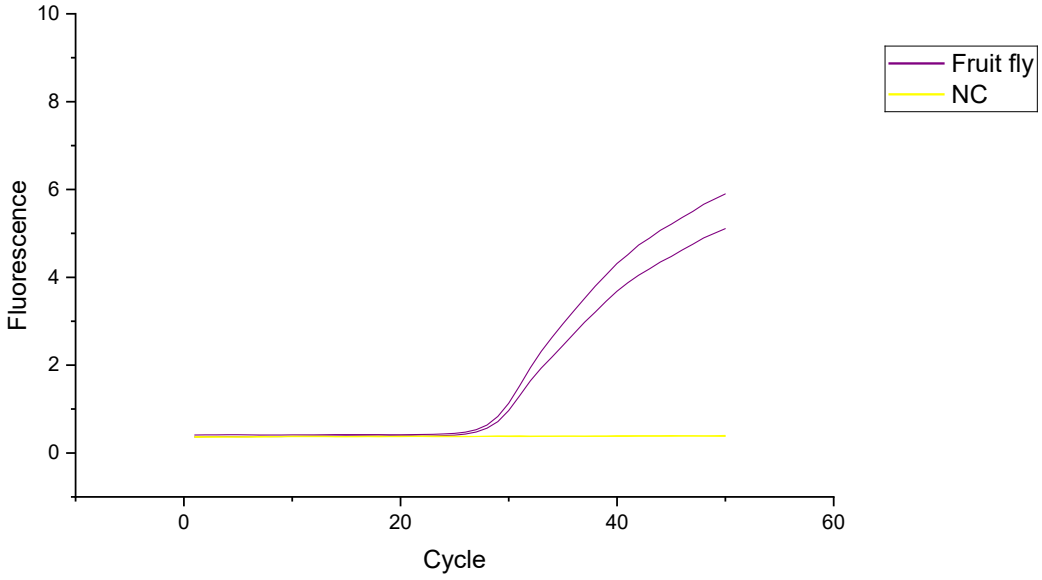


Figure 15: Amplification curves of fruit fly DNA and no template control (NC), when testing primer pair for fruit fly and primer pair from AGES.

In Figure 16, the results of the HRM analysis when including the primer pair Seidenraupe_1_fw and Seidenraupe_1_rev in a concentration of 0.2 µM are presented. All melt profiles, with the exception of fruit fly, demonstrate good repeatability. Furthermore, out of all melt profiles, an alteration was only observed with the one for silkworm when compared to previous results (Figure 9). This shift was indeed the desired outcome, since it improved its differentiation from the melt curve of black soldier fly. Figure

18 demonstrates that a second PCR product for silkworm was generated, which induced a second less intense melt peak in the HRM analysis.

Additionally, the no template control induced a fluorescence signal, resulting in a broad flat peak at a lower temperature, as shown in Figure 17. In comparison, when implementing only Ins_fw and Ins_rev (Primer set I, Table 6), this was not detected. However, the presence of primer dimers did not impair the repeatability of the melt profiles for any insect species or the amplification performance.

As a result of this experiment, the first cluster mentioned in 5.1, consisting of the melt curves of black soldier fly and silkworm, was resolved and the melt curves of these species were now distinguishable from each other. For this reason, this primer pair, Seidenraupe_1_fw and Seidenraupe_1_rev, was included in subsequent tests.

Moreover, the primers from this pair were also tested separately (either 0.4 µM Seidenraupe_1_fw or Seidenraupe_1_rev + 0.4 µM Ins_fw/rev) to examine whether the implementation of just one primer would induce the same effect. However, this test was unsuccessful and the second PCR product shown in Figure 17 was not formed.

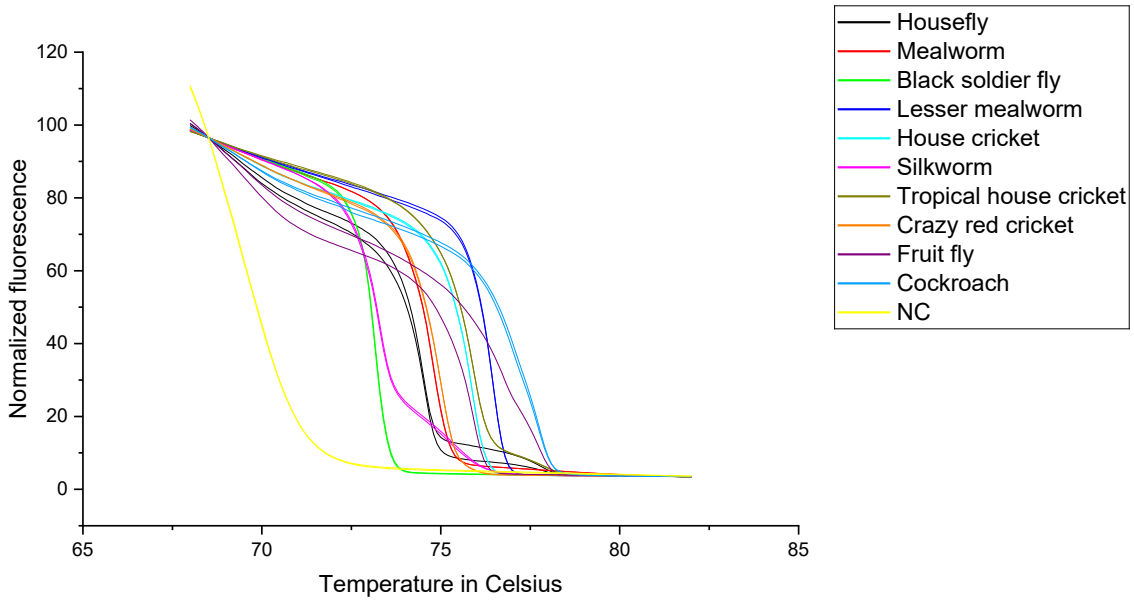


Figure 16: HRM analysis of DNA from housefly, mealworm, black soldier fly, lesser mealworm, house cricket, silkworm, tropical house cricket, crazy red cricket, fruit fly, cockroach, and no template control (NC) with primer pair for silkworm and primer pair from AGES (Primer set II).

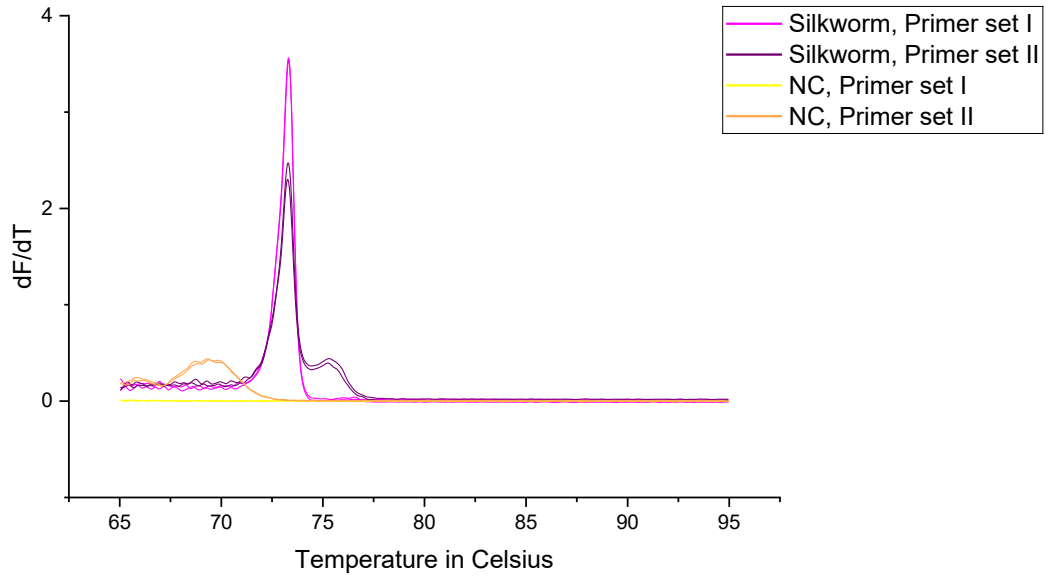


Figure 17: Melt plot of silkworm DNA and no template control (NC) when using only Ins_fw and Ins_rev (Primer set I) and with the addition of the primer pair for silkworm (+ Seidenraupe_1_fw and Seidenaupe_1_rev, Primer set II). On the y-axis, dF/dT refers to the derivative of fluorescence (F) with respect to temperature (T).

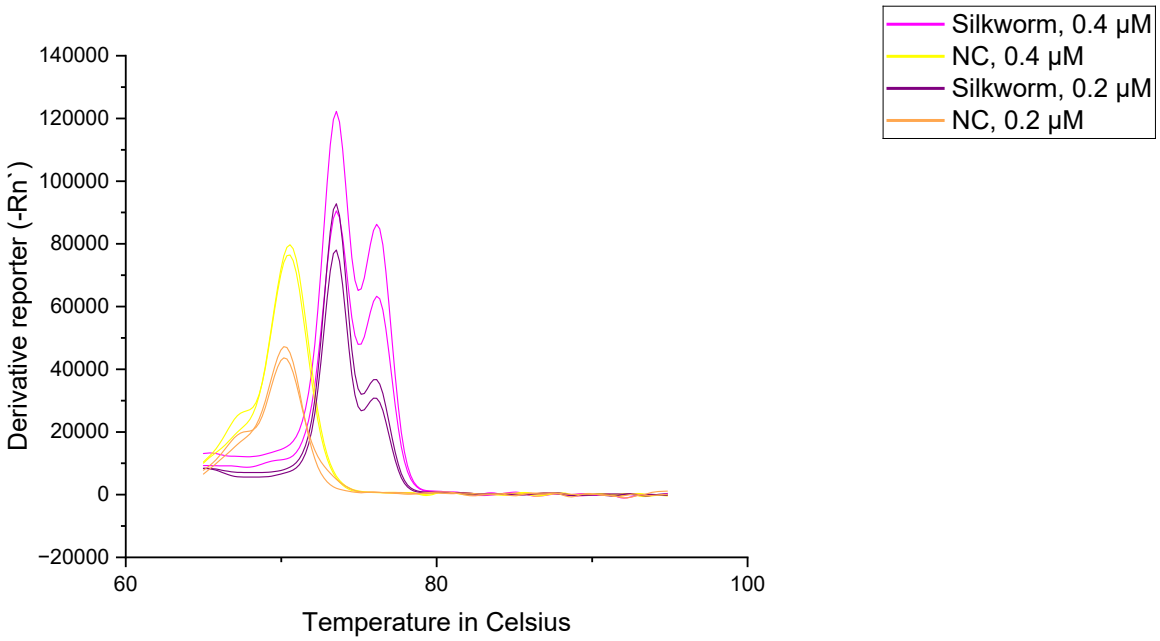


Figure 18: Melt plot for silkworm when implementing primer pair Seidenraupe_1_fw/rev in concentrations 0.4 µM and 0.2 µM, tested on silkworm DNA and no template control (NC) in the presence of 0.4 µM Ins_fw/rev.

5.2.1 PCR-HRM with feed samples with Primer set II

Next, DNA extracts from commercially sourced feed samples (DNeasy® mericon® Food Kit, listed in Table 2) were analysed using a duplex method that incorporated two primer pairs: Ins_fw/rev at a concentration of 0.4 µM and Seidenraupe_1_fw/rev at 0.2 µM (Primer set II, Table 6). The extracts were diluted to 2.5 ng/µL, and DNA extract concentrations are presented in Table 21 in the Appendix.

For comparative purposes, DNA from insect species present in the feed samples was also included in this PCR run, using DNA extracts from the NucleoSpin® Plant II Kit, similarly diluted. These were new extracts obtained from an additional DNA extraction between experiments to assess the effect of varying cell lysis durations—90 minutes and overnight. It became evident that longer incubation times significantly improved DNA yield, with overnight incubation resulting in the highest concentrations, consistently exceeding 2.5 ng/µL (listed in Table 18, Appendix). Consequently, the DNA concentrations became more uniform, and the resulting dilutions were used in subsequent experiments. Housefly DNA extracts with 30 minutes' cell lysis time were further used because of insufficient sample material to perform the cell lysis overnight.

In Figure 19, the HRM curves of all feed samples and the respective insect species contained are presented. Three feed samples are not included in this graph, namely the three samples where insect content was not stated. Both DNA extracts from the freeze-dried snacks from chicken meat and the chicken hearts snacks exhibited Ct mean values close to the one of the negative control template- 40 and 37 for the chicken feed DNA and 40 for the NC. DNA of canned feed containing wild boar was successfully amplified with a Ct mean value of 29, but its melt curve did not appear close to any of the other ones in this analysis.

Three main clusters, corresponding to the three insect species contained in these feed samples, were built. The first cluster (blue circle) consisted of the melt curves of black soldier fly and all feed samples containing it as an ingredient: Sensitive Mini Adult (granules), Bugfutter (granules), Sensitive Adult (granules) and the canned feed with chicken and insect, where the exact insect species was not stated. All melt curves demonstrate good repeatability and overlap, enabling the identification of the black soldier fly DNA in all sample extracts. These results suggested that the not declared insect kind in the canned feed was black soldier fly, since its melt curve aligned with the rest from this group.

The second cluster (red circle) is composed of the silkworm DNA and Nestlingfutter DNA, where the inclusion of silkworm pupae was stated. The melt curves overlap with great repeatability, and are also clearly distinguishable from those of the extracts containing black soldier fly.

In the third cluster (green circle), melt curves of mealworm DNA and its feed samples DNA were present: Insect hearts, Insect sticks, Protein Cocktail, Insektenfutter (flakes) and Sensible Snack (sticks). They overlapped repeatedly, allowing the identification of the species in all DNA extracts.

Two amplicons melted in a different way compared to the three clusters formed: those resulting from birdseed cakes and peanut butter. Both are also presented in Figure 20, where the melt peaks for these two problematic DNA extracts were compared to the ones of mealworm and black soldier fly. Firstly, the melt curve for birdseed cakes was supposed to align with the mealworm group, which suggested that an alternative PCR product was generated. This notion is further confirmed by the HRM analysis in Figure 20, where a second melt peak is evident, possibly due to primer binding to another DNA sequence in the original sample. For peanut butter, although its melt curve was anticipated to be unusual due to its composition (2.5% black soldier fly and 2.5% mealworm), it more closely aligned with the

mealworm group. A second melt peak, typically expected when multiple species are present, was absent, prompting further investigation through a spike experiment later in the thesis.

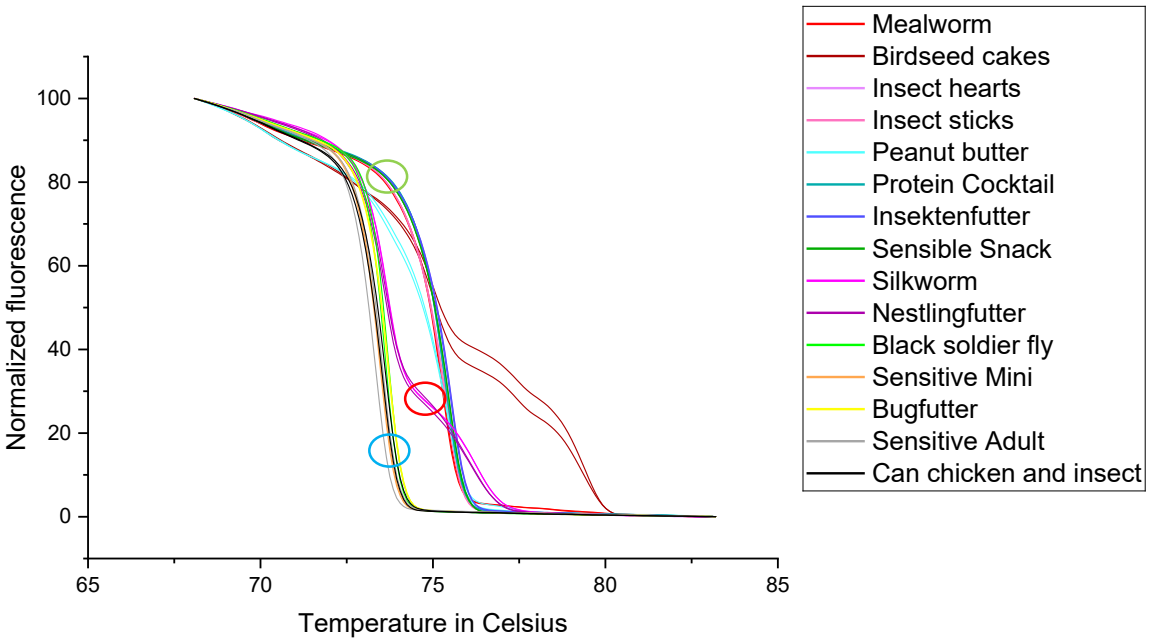


Figure 19: HRM analysis for all feed with insect, along with the three respective species present in the original samples- mealworm (green circle cluster), black soldier fly (blue circle cluster) and silkworm (red circle cluster) with Primer set II.

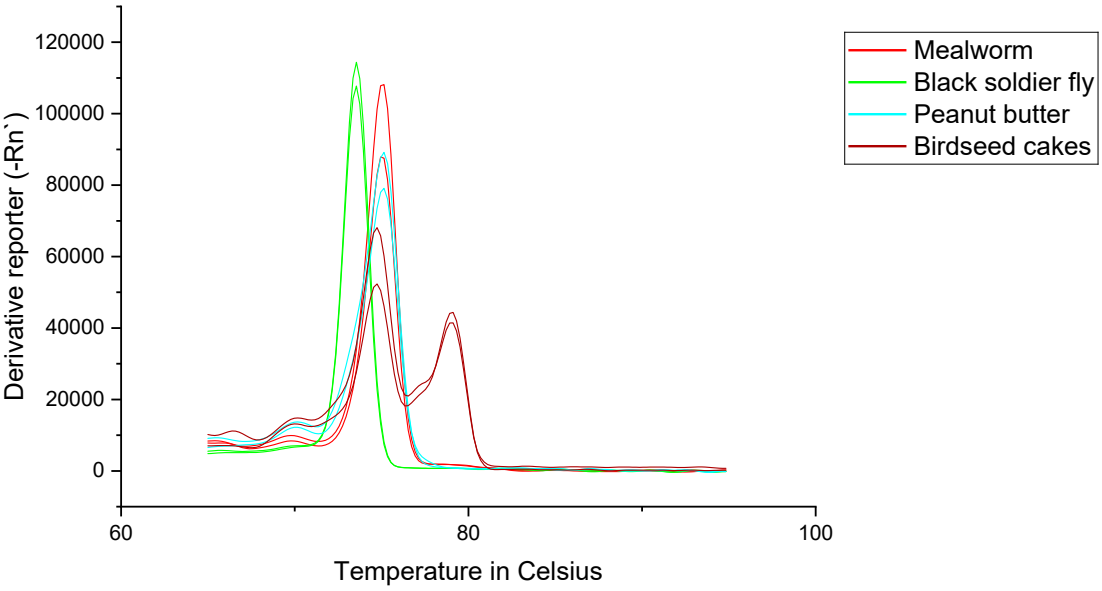


Figure 20: Melt plot for peanut butter and birdseed cakes, compared to mealworm and black soldier fly with Primer set II.

5.2.2 PCR-HRM with MeltDoctor™ HRM Master Mix

In a subsequent experiment, the performance of the method with four primers was tested when employing a different mastermix, namely the MeltDoctor™ HRM Master Mix, which was designed to be compatible with the thermal cycler used in all following experiments- Quantstudio 5. For this PCR run, a mastermix was prepared implementing this reaction mix and the primers Ins_fw/rev in 0.4 µM and

Seidenraupe_1_fw/rev in 0.2 μM. Ten insect species´ DNA extracts were analysed: the housefly, cockroach, fruit fly, crazy red cricket, tropical house cricket, silkworm, house cricket, lesser mealworm, black soldier fly and mealworm in a concentration of 2.5 ng/μL (NucleoSpin® Plant II Kit, cell lysis incubation overnight, for housefly 30 minutes). This PCR run revealed that this mastermix was not suitable for the analysis of these DNA extracts, since the amplification of the insect DNA was inadequate in all cases compared to previous experiments. The DNA extracts from cockroach, tropical house cricket and lesser mealworm exhibited a mean Ct (Cycle threshold) value between 30 and 40, those from housefly, crazy red cricket, silkworm and mealworm above 40, and fruit fly and black soldier fly were classified by the software as undetected. In contrast, in previous experiments with Type-it HRM PCR Kit, the highest mean Ct value (with the exception of fruit fly DNA, since it does not amplify well) was observed for the housefly and was measured to be 24. The rest of the insect species DNA all exhibited mean Ct values below 20. A possible reason for this observation could be the supplementation of $MgCl_2$ in the reaction mix, since too high concentrations could lead to non-specific binding and interfere with DNA polymerase activity, as mentioned in 3.2.2. A further experiment without its addition to this reaction mix was conducted later in the course of the master´s thesis, and some of the PCR amplicons obtained in this PCR run were subjected to gel electrophoresis (see section 5.3.1) to gather further information on the products generated.

5.3 Development of Primer sets (III-VI) for the differentiation of housefly, silkworm and crazy red cricket

In section 5.1, three clusters of melt curves were described, which could not be easily distinguished: the first comprising black soldier fly and silkworm, the second including housefly, crazy red cricket, and mealworm, and the third consisting of tropical house cricket and house cricket. Following the incorporation of the Seidenraupe_1_fw/rev primer pair, differentiation between black soldier fly and silkworm was achieved. However, the remaining clusters required the development of additional primers. Using the method outlined in section 4.5.1, primers were designed by aligning NGS sequences in MEGA software and targeting regions where the DNA sequences of the species would have multiple different bases. Housefly, mealworm, and crazy red cricket sequences were aligned to determine optimal regions for primer design. The optimal target regions are presented in Table 11 with the corresponding primer pair.

Additionally, the theoretical melt temperature of the primers had to be as close as possible to 57.4°C, with an acceptable variation of ± 2°C. Two primer sets were developed for housefly, one for mealworm and one for crazy red cricket, all listed in Table 10.

Table 10: Primer pairs for housefly, mealworm and crazy red cricket. Fw: forward; rev: reverse.

Primer name	Sequence 5' → 3'	Theoretical melt temperature (salt adjusted)	Target insect species
Krullfliege_1_fw	CGTTAATAACGGATCAATTATTCAT	57.6ºC	housefly
Krullfliege_1_rev	GGATTTTTTTTGTTGTGATTGTTG	56.6ºC	housefly
Krullfliege_2_fw	CGGATCAATTATTCATAAATTAATG	55.9ºC	housefly
Krullfliege_2_rev	GTGATTGTTGATAATATTTTATTGG	55.9ºC	housefly
Mehlwurm_1_fw	GGATCATAAACTCAAAAATAAATGT	55.9ºC	mealworm
Mehlwurm_1_rev	GTTTTGGTATTTTGCTGGGG	56.4ºC	mealworm
Steppengrille_1_fw	CCATAATAATAGGATCAATTAACC	56.6ºC	crazy red cricket
Steppengrille_1_rev	GGTGACAAGGGAATAATTTAAC	56.4ºC	crazy red cricket

Table 11: Target regions for primer pairs for housefly, mealworm and crazy red cricket. Target region sequence is presented in the 5' → 3' direction.

Species	English name	Primer pair	Primer pair bound to target region (red: fw, green: rev)
Musca domestica	housefly	Krullfliege_1_fw/rev	CGTTAATAACGGATCAATTATTCATAAATTAATGATTTAATTA ATTAAAAGTTTTTTAAATTTTAATATCACCCCAATAAAATATT ATCAACAATCACAACAAAAAAAATCC
		Krullfliege_2_fw/rev	CGGATCAATTATTCATAAATTAATGATTTAATTAATTAAAAGT TTTTTAAATTTTAATATCACCCCAATAAAATATTATCAACAAT CAC
Tenebrio molitor	mealworm	Mehlwurm_1_fw/rev	GGATCATAAACTCAAAAATAAATGTAAAAATAAAAGAAAAGTT AAATAAATTTTCTAACCGCCCCAGCAAAATACCAAAAC
Gryllus locorojo	crazy red cricket	Steppengrille_1_fw/rev	CCATAATAATAGGATCAATTAACCAATCATCATAGTAAAATAT AAAGAAGTTAAATTATTCCCTTGTCACC

Each primer pair (fw + rev) was subsequently tested on Quantstudio 5 separately with a concentration of 0.4 μM, in the presence of 0.4 μM Ins_fw/rev and 0.2 μM Seidenraupe_1_fw/rev. DNA extracts from the species from this cluster were analysed: housefly, mealworm and crazy red cricket, extracts obtained with NucleoSpin® Plant II Kit, cell lysis incubation overnight (except housefly, cell lysis 30 minutes), diluted to a concentration of 2.5 ng/μL.

After performing HRM analysis, it was observed that the no template controls of each primer set differed significantly in melt behaviour, as shown in Figure 21. The presence of additional melt peaks complicated the assessment of which products were synthesized when introducing new primers into the reaction mixture. To correctly interpret these results, an agarose gel electrophoresis was required to compare the length of the PCR products from the insect DNA and possible primer dimers.

Additionally, the HRM analysis showed that the melt peak form for mealworm DNA was successfully altered, as shown in Figure 22. A wider, two-shoulder peak was now observed, compared to the previous single peak. all

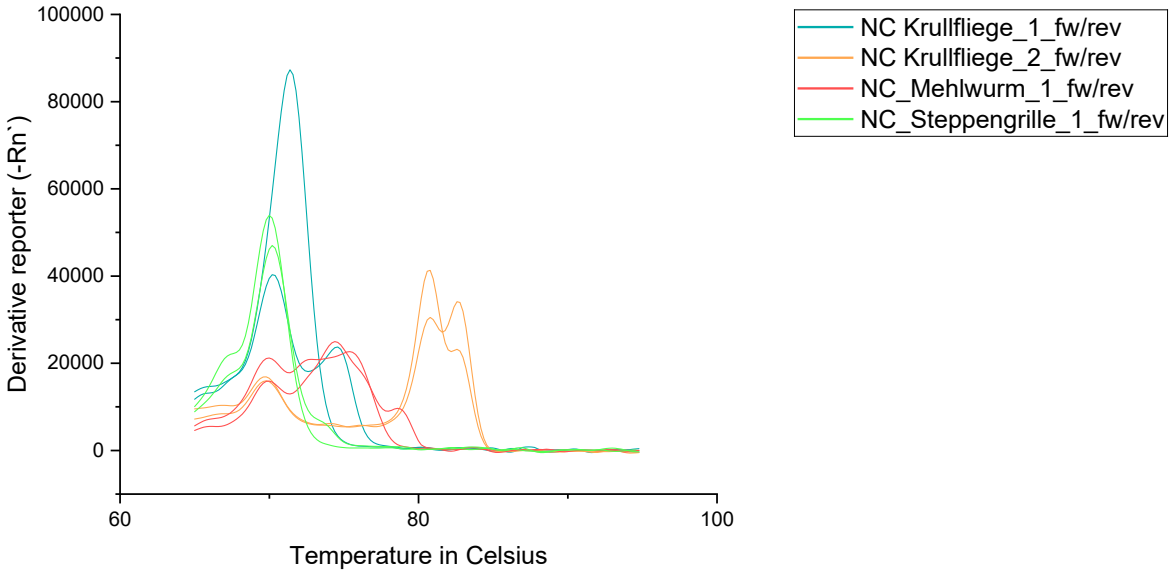


Figure 21: Melt plot for no template controls (NCs) for mastermixes with primer pairs Ins_fw/rev + Seidenraupe_1_fw/rev (Primer set II, Table 6) and either Krullfliege_1_fw/rev, Krullfliege_2_fw/rev (both for housefly), Mehlwurm_1_fw/rev (for mealworm) or Steppengrille_1_fw/rev (for crazy red cricket).

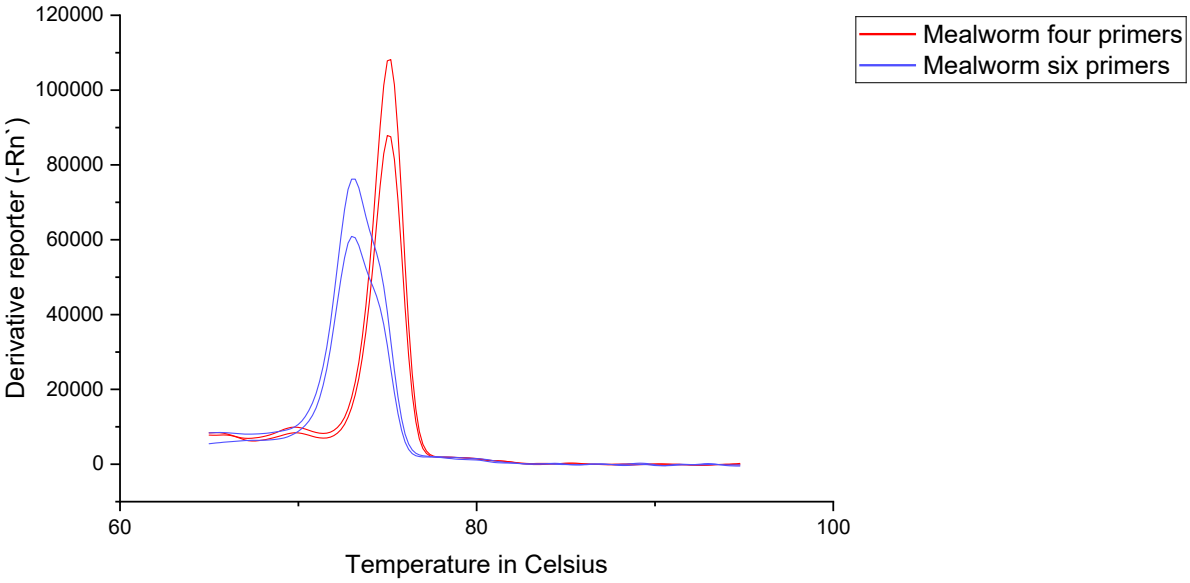


Figure 22: Mealworm DNA melt peak when in the presence of four primers (Ins_fw/rev + Seidenraupe_1_fw/rev) versus six primers (Ins_fw/rev + Seidenraupe_1_fw/rev + Mehlwurm_1_fw/rev).

5.3.1 Agarose gel electrophoresis with PCR products

In sequence to the primer test of the four primer pairs (Ins_fw/rev + Seidenraupe_1_fw/rev + either Krullfliege_1_fw/rev, Krullfliege_2_fw/rev, Mehlwurm_1_fw/rev or Steppengrille_1_fw/rev), an agarose gel electrophoresis was performed to assess the length of the PCR products acquired in this run. Of highest importance was the appearance of the no template controls, which exhibited concerning melting behaviour, as shown in Figure 21. One amplicon from each duplicate along with the corresponding negative controls for the PCR run were analysed, and in one case for the primer pair Krullfliege_1_fw/rev both negative controls were analysed due to their inadequate repeatability. To compare, amplicons of the named species with the corresponding no template controls were added from a previous run where only two primer pairs were used: Ins_fw/rev and Seidenraupe_1_fw/rev (Primer set II). Additionally, amplicons of five species from the experiment with MeltDoctor™ HRM Master Mix in 5.2.2 were included: housefly, mealworm, crazy red cricket, lesser mealworm and silkworm, where silkworm and lesser mealworm amplicons were added due to their usually efficient amplification. For adequate comparison, the amplicons of silkworm and lesser mealworm from the PCR run with two primer pairs (Primer set II) and Type-it HRM PCR Kit were added. The scheme for all lanes and their corresponding amplicon and the primer sets used are presented in Table 12, and a photograph of the gel (under UV light), labelled with well numbers and DNA ladder product lengths is shown in Figure 23.

Table 12: Lane scheme for analysed amplicons. All cells containing amplicons from the same PCR run are shown in the same colour. NC: no template control, Seid: Seidenraupe, Mehl: Mehlwurm, Kr: Krullfliege. M1/4: DNA Ladder, diluted 1:4.

Lane	Well	Sample	PCR condition	PCR date	Volume [µL]	
1	-	M 1/4	-	-		
2	A1	Housefly	0.4 µM Ins_fw/rev + 0.2 µM Seid_1_fw/rev	10.06.2024		
3	A7	Crazy red cricket	0.4 µM Ins_fw/rev + 0.2 µM Seid_1_fw/rev	10.06.2024		
4	B7	Mealworm	0.4 µM Ins_fw/rev + 0.2 µM Seid_1_fw/rev	10.06.2024		
5	E3	NC	0.4 µM Ins_fw/rev + 0.2 µM Seid_1_fw/rev	10.06.2024		
6	A3	Housefly	0.4 µM Ins_fw/rev + 0.2 µM Seid_1_fw/rev + 0.4 µM Step_1_fw/rev	18.06.2024		
7	C3	Crazy red cricket	0.4 µM Ins_fw/rev + 0.2 µM Seid_1_fw/rev + 0.4 µM Step_1_fw/rev	18.06.2024		
8	B3	Mealworm	0.4 µM Ins_fw/rev + 0.2 µM Seid_1_fw/rev + 0.4 µM Step_1_fw/rev	18.06.2024		
9	D3	NC	0.4 µM Ins_fw/rev + 0.2 µM Seid_1_fw/rev + 0.4 µM Step_1_fw/rev	18.06.2024		
10	E3	Housefly	0.4 µM Ins_fw/rev + 0.2 µM Seid_1_fw/rev + 0.4 µM Mehl_1_fw/rev	18.06.2024		
11	G3	Crazy red cricket	0.4 µM Ins_fw/rev + 0.2 µM Seid_1_fw/rev + 0.4 µM Mehl_1_fw/rev	18.06.2024		
12	F3	Mealworm	0.4 µM Ins_fw/rev + 0.2 µM Seid_1_fw/rev + 0.4 µM Mehl_1_fw/rev	18.06.2024		
13	H3	NC	0.4 µM Ins_fw/rev + 0.2 µM Seid_1_fw/rev + 0.4 µM Mehl_1_fw/rev	18.06.2024		
14	A1	Housefly	0.4 µM Ins_fw/rev + 0.2 µM Seid_1_fw/rev + 0.4 µM Kr_1_fw/rev	18.06.2024		
15	C1	Crazy red cricket	0.4 µM Ins_fw/rev + 0.2 µM Seid_1_fw/rev + 0.4 µM Kr_1_fw/rev	18.06.2024	6.0	LD orange
16	B1	Mealworm	0.4 µM Ins_fw/rev + 0.2 µM Seid_1_fw/rev + 0.4 µM Kr_1_fw/rev	18.06.2024		
17	D1	NC	0.4 µM Ins_fw/rev + 0.2 µM Seid_1_fw/rev + 0.4 µM Kr_1_fw/rev	18.06.2024		
18	D2	NC_2	0.4 µM Ins_fw/rev + 0.2 µM Seid_1_fw/rev + 0.4 µM Kr_1_fw/rev	18.06.2024		
19	E1	Housefly	0.4 µM Ins_fw/rev + 0.2 µM Seid_1_fw/rev + 0.4 µM Kr_2_fw/rev	18.06.2024		
20	G1	Crazy red cricket	0.4 µM Ins_fw/rev + 0.2 µM Seid_1_fw/rev + 0.4 µM Kr_2_fw/rev	18.06.2024		
21	F1	Mealworm	0.4 µM Ins_fw/rev + 0.2 µM Seid_1_fw/rev + 0.4 µM Kr_2_fw/rev	18.06.2024		
22	H1	NC	0.4 µM Ins_fw/rev + 0.2 µM Seid_1_fw/rev + 0.4 µM Kr_2_fw/rev	18.06.2024		
23	A1	Housefly	0.4 µM Ins_fw/rev + 0.2 µM Seid_1_fw/rev : MeltDoctor	17.06.2024		
24	A7	Crazy red cricket	0.4 µM Ins_fw/rev + 0.2 µM Seid_1_fw/rev : MeltDoctor	17.06.2024		
25	B7	Mealworm	0.4 µM Ins_fw/rev + 0.2 µM Seid_1_fw/rev : MeltDoctor	17.06.2024		
26	B3	Lesser mealworm	0.4 µM Ins_fw/rev + 0.2 µM Seid_1_fw/rev : MeltDoctor	17.06.2024		
27	B3	Lesser mealworm	0.4 µM Ins_fw/rev + 0.2 µM Seid_1_fw/rev	10.06.2024		
28	A11	Silkworm	0.4 µM Ins_fw/rev + 0.2 µM Seid_1_fw/rev : MeltDoctor	17.06.2024		
29	A11	Silkworm	0.4 µM Ins_fw/rev + 0.2 µM Seid_1_fw/rev	10.06.2024		
30	-	M1/4				

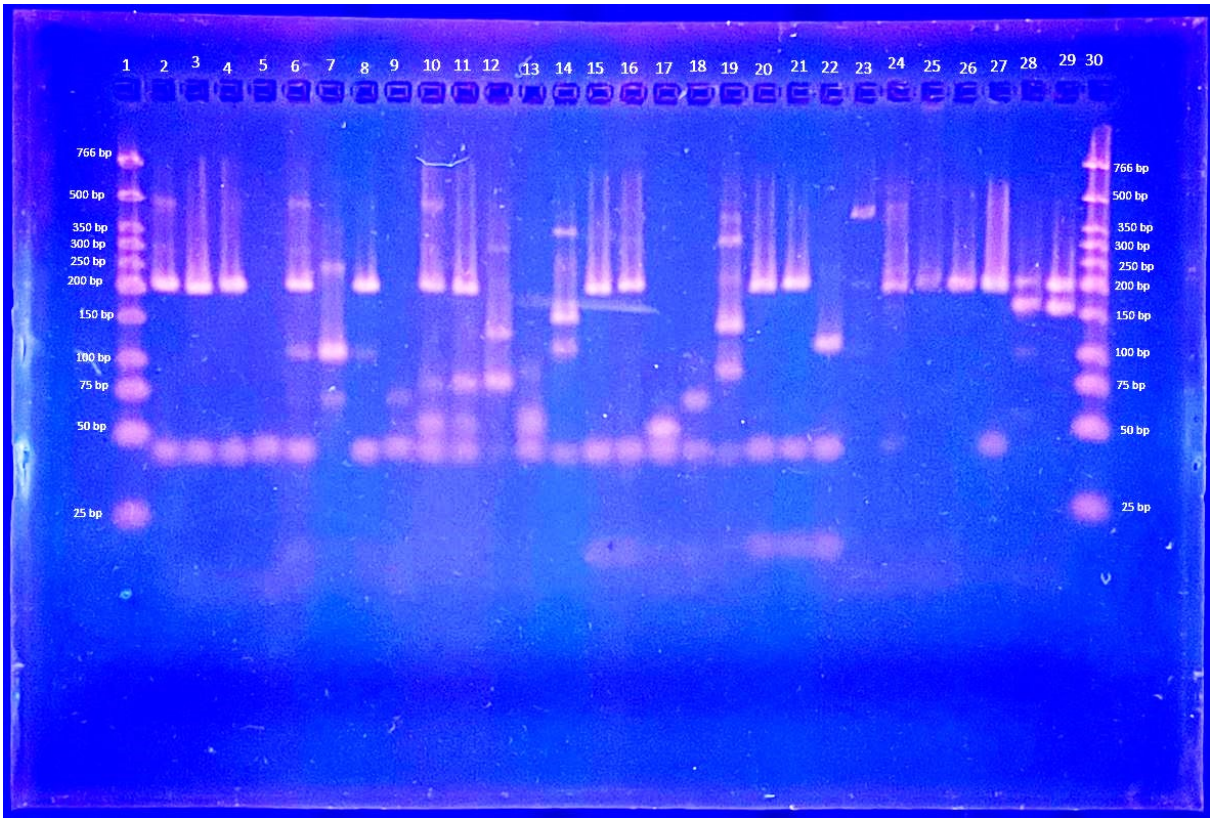


Figure 23: Gel, loaded according to Table 12, with added amplicon lengths of the DNA Ladder, bp = base pairs.

Lanes 2, 3, 4, and 5, containing amplicons of housefly, crazy red cricket, mealworm, and a no template control respectively, indicated that the three insect species cannot be clearly distinguished from each other. This could be due to the similar PCR products generated, all around 200 base pairs (bp) long. Additionally, the no template control in lane 5 contained a short PCR product of around 50 bp, which was also present in lanes 2-4 where amplicons of insect DNA were analysed. These short DNA products could be the primer dimers that occur when Primer set II is implemented (discussed in section 5.2).

The second four lanes (6-9) included the amplicons of the same three insect species, but with the supplementation of a third primer pair: Steppengrille_1_fw/rev, which was designed for the DNA sequence of crazy red cricket. This primer pair managed to alter the PCR product without impacting the other two species or the no template control significantly, only inducing the amplification of a second PCR product for crazy red cricket around 100 bp in length.

Lanes 10-13 were occupied with the three insect species amplicons and a negative control with the addition of the primer pair for mealworm: Mehlwurm_1_fw/rev. Here, the no template control revealed the amplification of more short PCR products compared to the previous primer set, 50-75 bp long, possibly primer dimers. On the other hand, out of the three insect species, only DNA from mealworm was influenced by this supplementation and completely different PCR products were created, 100-150 bp long.

Lanes 14-18 comprised of the amplicons of the three insect species and two no template controls when the primer pair for housefly Krullfliege_1_fw/rev was included. The primer set successfully altered the

produced PCR products only for housefly, with the current PCR products being approximately 75 and 150 bp long. The no template controls consisted of two different amplification products, as expected from the melt curve analysis, shown in Figure 21, one around 50 bp and the other 75 bp long.

Lanes 19-22 incorporated the amplicons of the three insect species and a no template control when adding the second primer set for housefly: Krullfliege_2_fw/rev. While the primer pair efficiently amplified different parts of the DNA sequence, resulting in products 75 bp and between 100-150 bp long, the no template control contained a PCR product of 100-150 bp. A negative control of such length would be too similar to the products of the target DNA, and this primer set was excluded from any future experiments.

Lanes 23-25 included the amplicons of the same three insect species, analysed with the primer pairs Ins_fw/rev in 0.4 μM and Seidenraupe_1_fw/rev in 0.2 μM in the presence of the MeltDoctor™ HRM Master Mix. While housefly DNA was indeed not efficiently amplified, mealworm and crazy red cricket PCR products exhibited the same appearance as the ones obtained with the Type-it HRM PCR Kit, although with less intense fluorescence (lanes 2-4). The same phenomenon was observed for silkworm and lesser mealworm amplicons (lanes 26-29).

5.3.2 Test of Primer sets III & IV

In the following experiment, it was tested whether only the forward or the reverse primer of the primer pairs Krullfliege_1_fw/rev and Steppengrille_1_fw/rev would induce an alteration in the melt profile. The main objective was to reduce the number of primers in the multiplex assay without impairing the differentiation. Both mealworm primers were kept for this experiment because of the two-shoulder peak they induced in mealworm DNA, shown in Figure 22.

Each primer was supplemented separately and in a pair, always in a concentration of 0.4 μM, to a mastermix containing three primer pairs: Ins_fw/rev in a concentration of 0.4 μM, Seidenraupe_1_fw/rev in 0.2 μM and Mehlwurm_1_fw/rev in 0.4 μM. DNA extracts from housefly, mealworm and crazy red cricket were analysed, obtained with the NucleoSpin® Plant II Kit, cell lysis incubation overnight, only for housefly 30 minutes. All DNA extracts were diluted to 2.5 ng/μL. The PCR-HRM was performed on Quantstudio 5.

Two primer combinations were selected due to the high repeatability of the HRM curves and the enabled differentiation of the three insect species. Their discrimination power for the melt curves for mealworm, housefly and crazy red cricket was tested.

Primer set III: Ins_fw/rev + Seidenraupe_1_fw/rev + Mehlwurm_1_fw/rev + **Krullfliege_1_fw** (Figure 24)

Primer set IV: Ins_fw/rev + Seidenraupe_1_fw/rev + Mehlwurm_1_fw/rev + **Steppengrille_1_rev** (Figure 25).

The results obtained using both primer sets were consistent and of high quality. For Krullfliege_1_fw/rev, the repeatability demonstrated was less consistent when employing either both primers from the set or

only the Krullfliege_1_rev. For Steppengrille_1_fw/rev the result was very similar, and the melt curves did not overlap properly when implementing either both primers or only the forward primer.

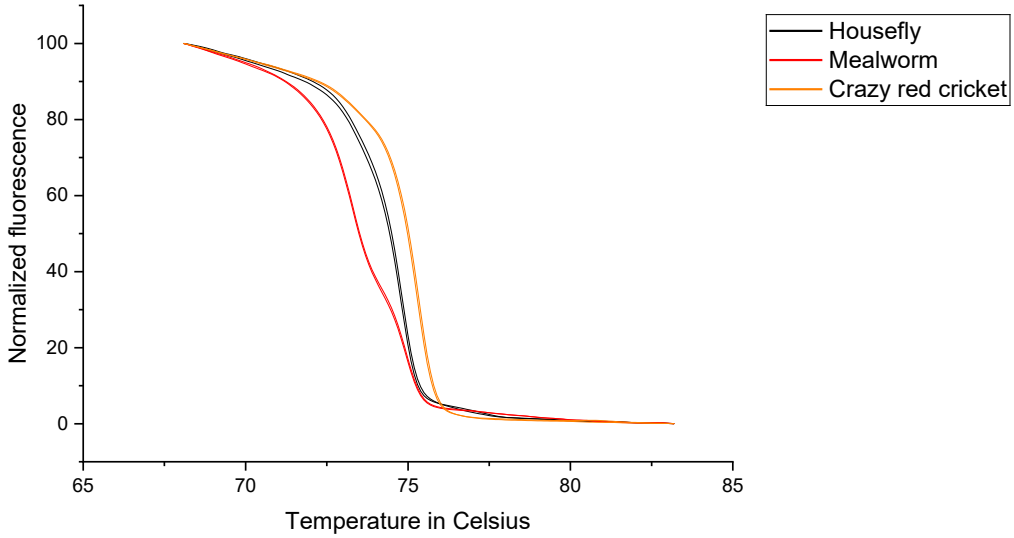


Figure 24: HRM analysis for housefly, mealworm and crazy red cricket DNA when implementing the primers Ins_fw/rev + Seidenraupe_1_fw/rev + Mehlwurm_1_fw/rev + Krullfliege_1_fw (Primer set III).

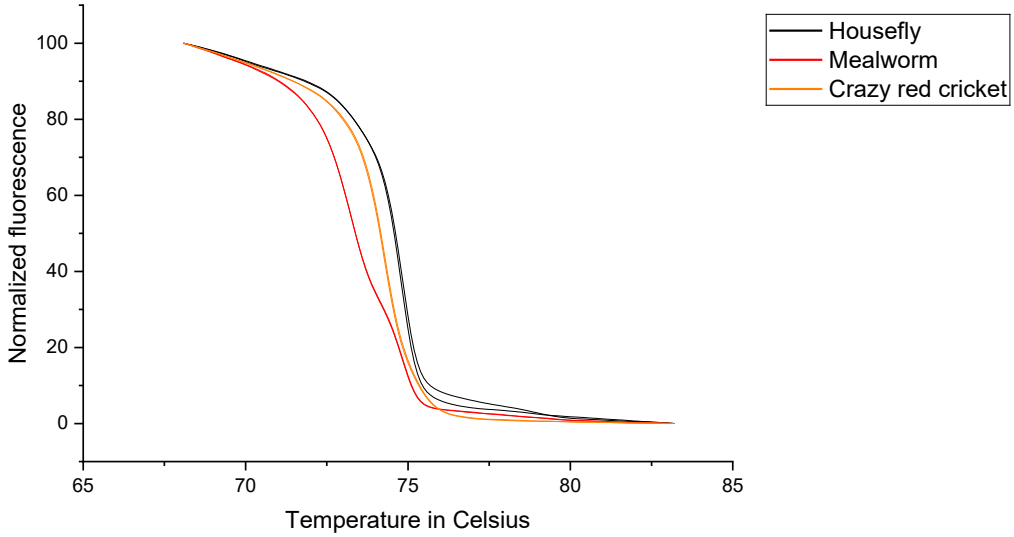


Figure 25: HRM analysis for housefly, mealworm and crazy red cricket DNA when implementing the primers Ins_fw/rev + Seidenraupe_1_fw/rev + Mehlwurm_1_fw/rev + Steppengrille_1_rev (Primer set IV).

5.3.3 Test for single primer efficacy of pair Mehlwurm_1_fw/rev

The purpose of this experiment was to determine whether only one of the two primers in the pair for mealworm would influence the melt profile and make the curve distinguishable from those of housefly and crazy red cricket. Primers from the pair were separately tested on the three insect species DNA in the presence of Primer set II: Ins_fw/rev in concentration of 0.4 μM and Seidenraupe_1_fw/rev in 0.2 μM. The newly added primers were both supplemented in a concentration of 0.4 μM. DNA extracts

obtained with NucleoSpin® Plant II Kit were analysed, cell lysis incubation overnight, only for housefly 30 minutes. All DNA extracts were diluted to 2.5 ng/µL. The experiment was carried out on Quantstudio 5.

When implementing only the forward primer, the melt curve of mealworm DNA had lost its signature shape from the experiment when both primers from the pair were present, as shown in Figure 26. Correspondingly, the melt peak of mealworm did not demonstrate the two-shoulder appearance anymore, but had returned to a single peak. However, the mealworm melt curve was still distinguishable from the other two, which led to the conclusion that a change of the melt temperature was induced without altering the curve shape. Melt curves for housefly and crazy red cricket could not be clearly differentiated as expected, since none of the primers designed for them were included in this PCR run.

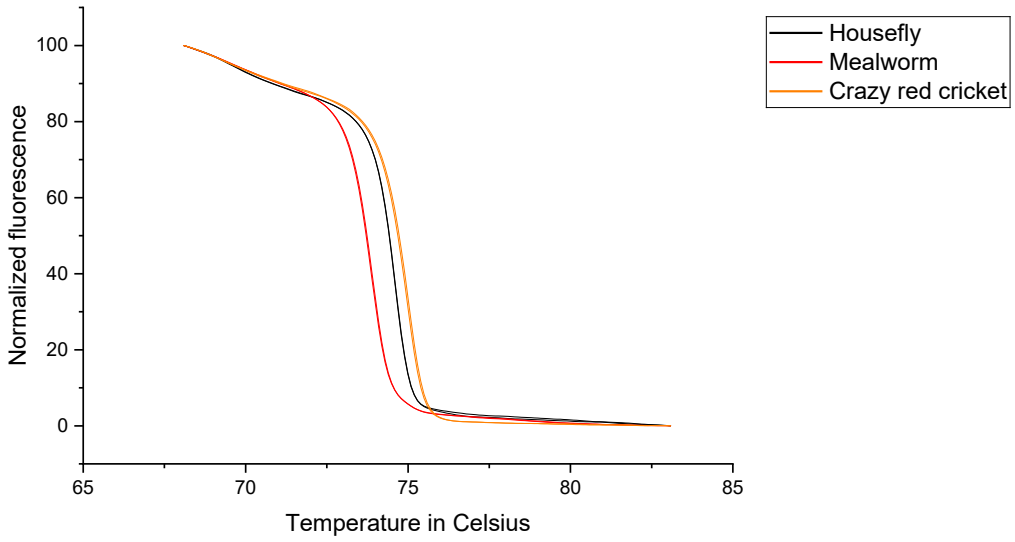


Figure 26: HRM analysis for housefly, mealworm and crazy red cricket DNA when implementing the primers Ins_fw/rev + Seidenraupe_1_fw/rev + Mehlwurm_1_fw.

When supplementing only the reverse primer, the HRM curves of the three species DNA were overlapping, which indicated that this primer was not suitable for further experiments.

5.3.4 Test of Primer sets V & VI

The purpose of this test was to determine whether the results obtained with the primer Mehlwurm_1_fw for mealworm from section 5.3.3 would be retained if one of the primers: Krullfliege_1_fw or Steppengrille_1_rev, was added. The desired outcome was the efficient differentiation of crazy red cricket, mealworm and housefly with as few primers as possible to minimize interactions between them.

Two methods were tested and DNA extracts obtained with NucleoSpin® Plant II Kit were analysed, cell lysis incubation overnight, only for housefly 30 minutes. All DNA extracts were diluted to 2.5 ng/µL, and the test was performed on Quantstudio 5.

Primer set V: 0.4 µM Ins_fw/rev + 0.2 µM Seidenraupe_1_fw/rev + 0.4 µM Mehlwurm_1_fw + 0.4 µM Krullfliege_1_fw;

Primer set VI: 0.4 µM Ins_fw/rev + 0.2 µM Seidenraupe_1_fw/rev + 0.4 µM Mehlwurm_1_fw + 0.4 µM Steppengrille_1_rev.

In Figure 27, the results from the HRM analysis of the test with Primer set V are visualized. Fruit fly melt curves were not included in this graph, since its amplification was again inefficient. All melt curves demonstrated good repeatability and seven out of the nine insect species melt curves were distinguishable from each other, which suggested that the newly added primers did not bind to any of the other species´ DNA sequences and did not influence their melt profiles. However, one cluster, namely the third one firstly mentioned in 5.1, remained unresolved- melt curves of house cricket and tropical house cricket overlapped. Both insect species are allowed for use in feed, which makes them of high importance for the development of this method.

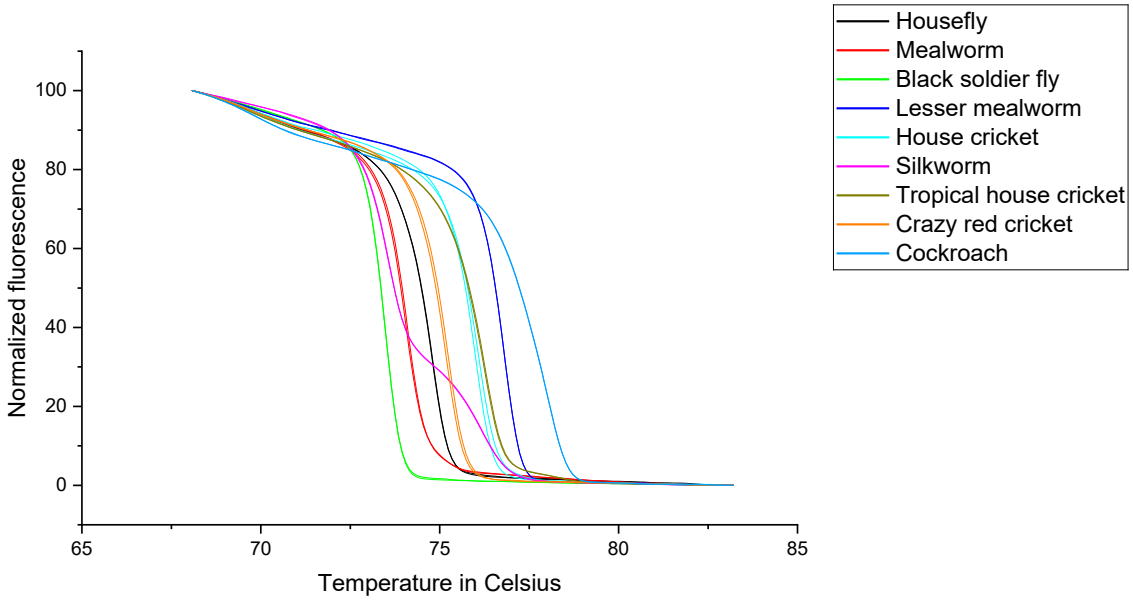


Figure 27: HRM analysis of nine insect species DNA, employing the method with 0.4 µM Ins_fw/rev + 0.2 µM Seidenraupe_1_fw/rev + 0.4 µM Mehlwurm_1_fw + 0.4 µM Krullfliege_1_fw (Primer set V).

Figure 28 presents the results of the HRM analysis of the test with Primer set VI, again with the exception of fruit fly due to its inadequate amplification. In comparison to Primer set V, housefly, silkworm and tropical house cricket melt curves showed slightly worse repeatability. Two main observations were made regarding the differentiation of the species. Firstly, both house cricket and tropical house cricket melt profiles were impacted by the addition of the Steppengrille_1_rev primer, improving their distinguishability. Secondly, the mealworm melt curve now overlapped with that of crazy red cricket, due to Steppengrille_1_rev primer binding to the crazy red cricket DNA sequence. This phenomenon could be explained by the fact that all three cricket species belong to the cricket family *Gryllidae*, and are more closely related than to other of the sample insect species. The sequences of the three cricket species are presented in Table 13.

Table 13: NGS sequences from AGES for tropical house cricket, house cricket and crazy red cricket. The base sequences highlighted in green represent regions in the DNA barcode with identical bases.

Species	English name	DNA barcode
Gryllodes sigillatus	tropical house cricket	TATCTTTATCTTAATTCCATAATACAGGATCTATTATCCAATCATC ATAGTCCAATTTAAAGAAGTTTTTTTATTCTCTCGTCACCCCAACC AAATACAATAAATTAATATTCAAGTCATAATACTATCCTTAATATA TTAATCCAATGTATAAAGA
Acheta domesticus	house cricket	TATCTTTATCTTCATTCCATAATACAGGATCAATTAACCAATCATC ATAGTTAAACATAAAGAAGTTAAATTATTCTCTTGTCACCCCAACC AAATACAATATATTAACATCTATAAAAAACCACCAAACATAAATTT CAATATAATAATATTAAGA
Gryllus locorojo	crazy red cricket	TATCTTTATCTTCATTCCATAATAATAGGATCAATTAACCAATCAT CATAGTAAAATATAAAGAAGTTAAATTATTCCCTTGTCACCCCAAC AAAACATTAAATAATAAAACATATTAAATACTCCTAAACATACATT TTAATAAATTAATATTAAGA

Since both improvements and new obstacles appeared in the test of the second method, further alterations in the conditions had to be implemented to optimize the differentiation of the insect species. Because of the improvement in differentiation of house cricket and tropical house cricket melt curves, the Steppengrille_1_rev was chosen for future experiments, and the Krullfliege_1_fw primer was no longer investigated.

Mehlwurm_1_fw was tested in a concentration of 0.8 µM in a subsequent PCR run on Quantstudio 5 in the presence of 0.4 µM Ins_fw/rev, 0.2 µM Seidenraupe_1_fw/rev and 0.4 µM Steppengrille_1_rev. The three insect species as in the previous primer tests were analysed: housefly, mealworm and crazy red cricket, DNA extracts obtained with NucleoSpin® Plant II Kit, cell lysis incubation overnight, only for housefly 30 minutes. All DNA extracts were diluted to 2.5 ng/µL However, this experiment was unsuccessful, and no differentiation between mealworm and crazy red cricket melt curves was achieved.

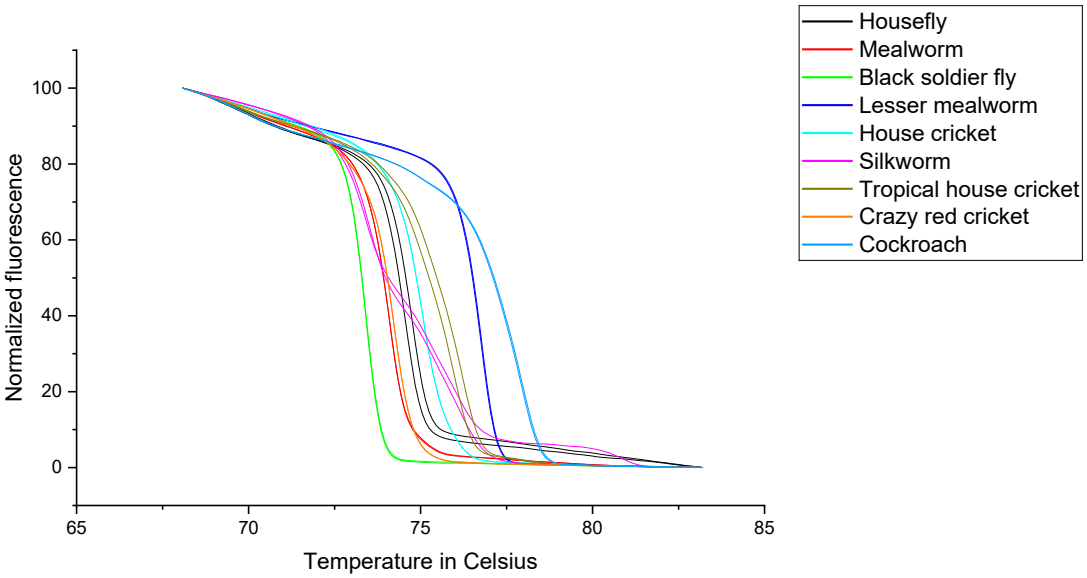


Figure 28: HRM analysis of nine insect species DNA, employing the method with 0.4 µM Ins_fw/rev + 0.2 µM Seidenraupe_1_fw/rev + 0.4 µM Mehlwurm_1_fw + 0.4 µM Steppengrille_1_rev (Primer set VI).

5.3.5 PCR-HRM with feed and meat samples and Primer set IV

In this experiment, a Primer set IV (Table 6) was tested on all insect species, feed samples and meat samples DNA extracts. Mealworm primers (Mehlwurm_1_fw/rev) were both added to the mastermix because of the findings described in 5.3.4, where it was established that in the presence of the primer Steppengrille_1_rev, the inclusion of a single mealworm primer does not result in differentiable melt curves for mealworm and crazy red cricket.

Insect and meat DNA used was isolated with NucleoSpin® Plant II Kit, cell lysis incubation overnight, for housefly 30 minutes. Feed DNA extracts were obtained with DNeasy® mericon® Food Kit. All DNA extracts were diluted to a concentration of 2.5 ng/µL, and the PCR was run on Quantstudio 5.

Primer set IV: 0.4 µM Ins_fw/rev + 0.2 µM Seidenraupe_1_fw/rev + 0.4 µM Mehlwurm_1_fw/rev + 0.4 µM Steppengrille_1_rev.

Figure 29 presents the results of the HRM analysis of all insect species with the exception of fruit fly, since its DNA was not adequately amplified. As expected, all species could now be distinguished from each other, because the mealworm melt curve regained its exclusive shape due to the implementation of the whole primer pair Mehlwurm_1_fw/rev. This alteration enabled its differentiation from the melt curve of crazy red cricket.

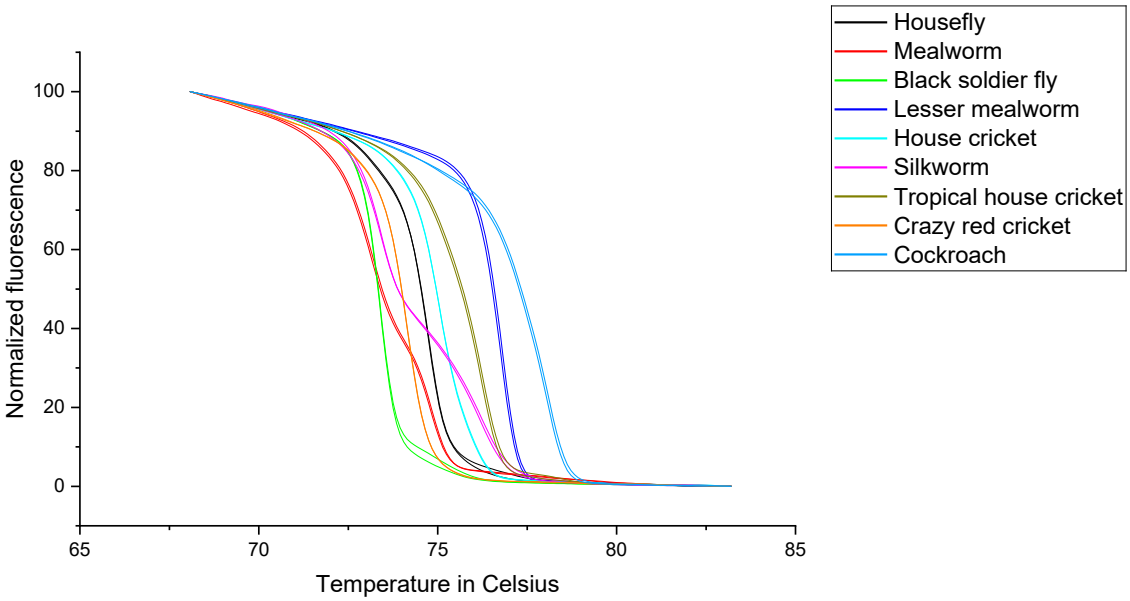


Figure 29: HRM analysis of nine insect species DNA, employing the method with 0.4 µM Ins_fw/rev + 0.2 µM Seidenraupe_1_fw/rev + 0.4 µM Mehlwurm_1_fw/rev + 0.4 µM Steppengrille_1_rev.

Figure 30 visualizes the results of the HRM analysis of all feed samples and the respective insect species contained, as well as the three meat samples and the canned feed with wild boar, which was the only one of the feed samples without insect content which had sufficient amplification. Both DNA from the freeze-dried snacks from chicken meat and the chicken hearts snacks were not successfully amplified, similar to the results from the test with four primers presented in section 5.2.1. Firstly, the alteration in the melt curve shape of mealworm DNA and its respective feed samples occurred again, which was the goal of the supplementation of both mealworm primers. However, overall repeatability for most melt curves was diminished in the HRM, compared to the method with Primer set II. If a supporting melt peak evaluation is conducted, the identification of insect DNA would be simplified since the melt peaks for all insect feed samples align with the melt peak for the corresponding insect (see Figure 31, 32, 33). The only exception was DNA from birdseed cakes with mealworms, which exhibited again a second peak, similar to the results shown in 5.2.1.

In Figure 31, it was evident that feed samples DNA differ in the melt peak shape compared to the one of mealworm DNA. With mealworm DNA, the left shoulder of the peak was higher, while with all feed samples the opposite was observed. The reason for this phenomenon was further investigated in future experiments, since multiple scenarios were possible. For instance, the DNA extraction method of feed samples and the insect sample were different, and although dilutions were performed, this factor could still impact the results.

As shown in Figure 30 and Figure 34, with this multiplex method the meat species DNA was again amplified and produced similar melt curves as in 5.2.1. Additionally, the melt curve produced by the feed sample with wild boar resembled the one of the pork DNA, and their melt peaks were overlapping.

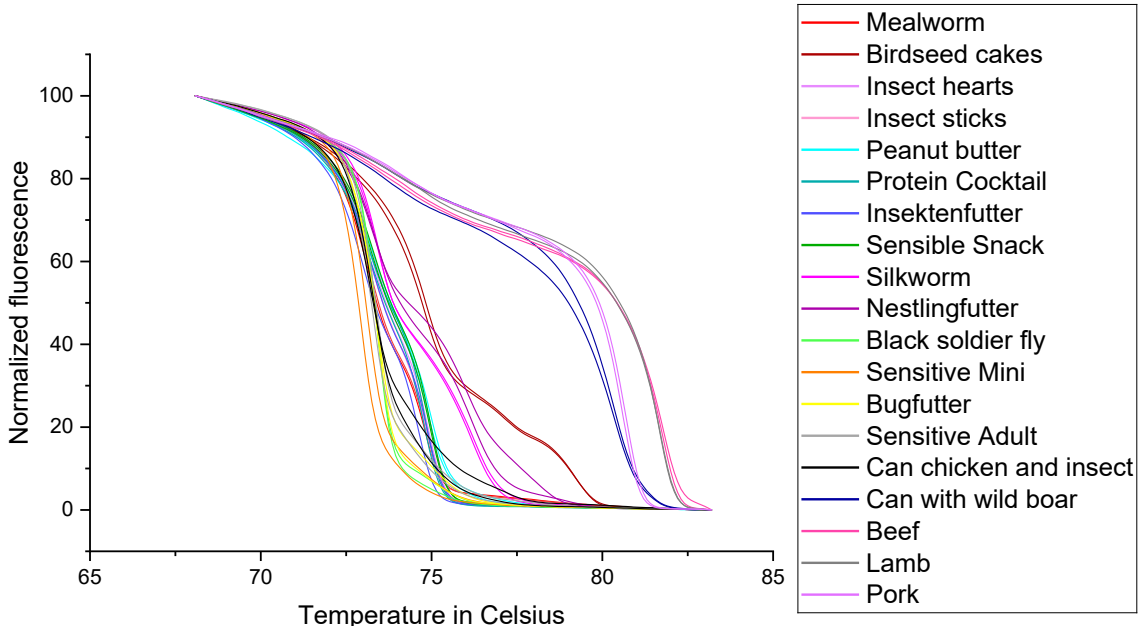


Figure 30: HRM analysis for all feed samples with insects, along with the three respective species present in the original samples- mealworm, black soldier fly and silkworm; three meat species extracts and the feed sample from canned feed with wild boar.

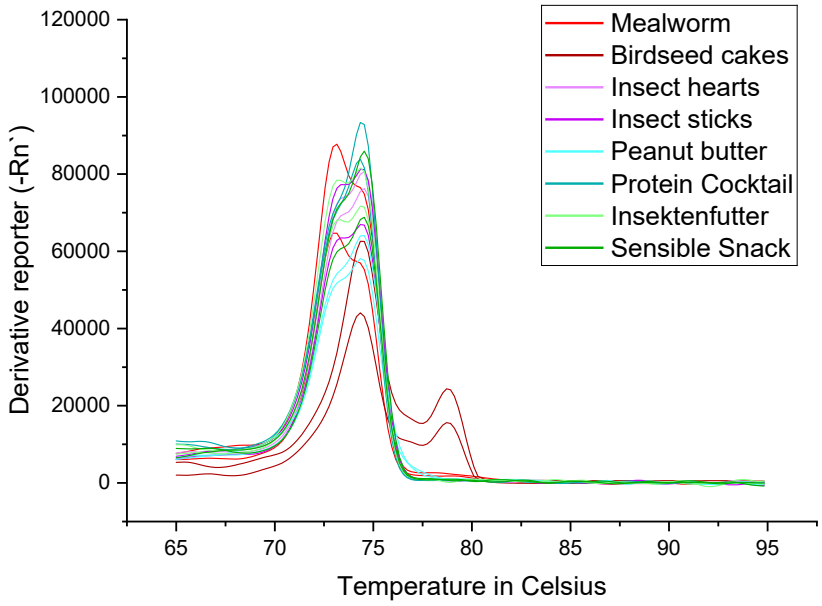
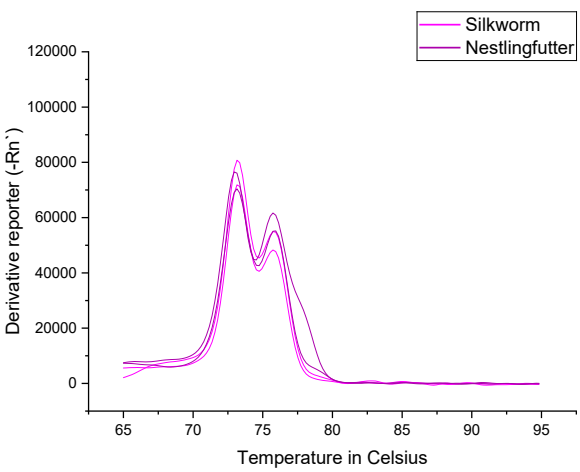


Figure 31: Melt plot for mealworm and all feed samples containing this species.



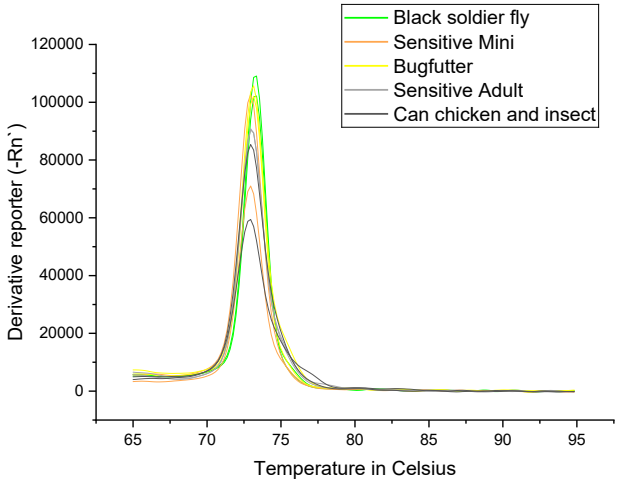


Figure 32 (left): Melt plot for silkworm and feed sample containing silkworm.
Figure 33 (right): Melt plot for black soldier fly and all feed samples containing this insect species.

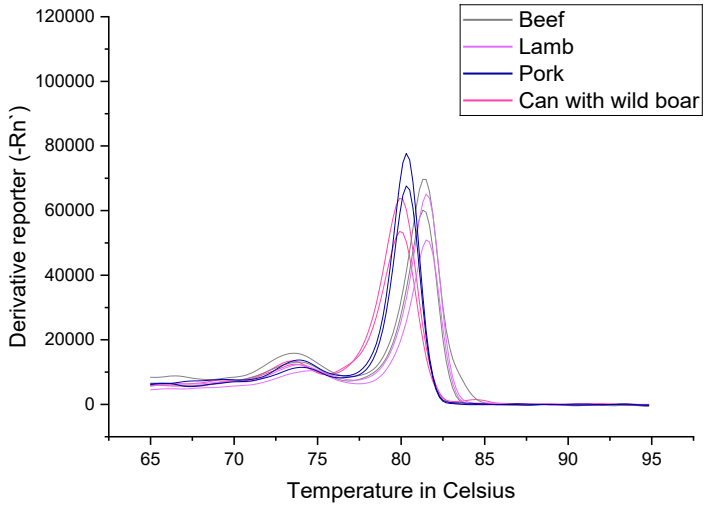


Figure 34: Melt plot for beef, lamb and pork and feed sample containing wild boar.

5.3.6 Optimized PCR-HRM with MeltDoctor™ HRM Master Mix

In this experiment, the mastermix MeltDoctor™ designed to be compatible with Quantstudio 5, was tested in a combination with the developed method with Primer set IV, without the addition of $MgCl_2$. DNA extracts from all ten insect species, both permitted and not legally permitted for use in feed in the EU, were analysed. All DNA extracts were obtained with NucleoSpin® Plant II Kit, cell lysis incubation overnight, for housefly 30 minutes. The extracts were diluted to a concentration of 2.5 ng/µL. However, most insect species´ PCR products exhibited a mean Ct value of above 30. Only one extract made an exception - lesser mealworm DNA, which led to a mean Ct value of 25. In a previous PCR run, where the Type-it HRM PCR Kit was used, the same DNA extract had a mean Ct value of 13. Therefore, it was concluded that the elimination of $MgCl_2$ did not improve the compatibility of this mastermix with the method and this issue was not further investigated.

5.3.7 Assessment of the repeatability of melt profiles for mealworm when using Primer set IV

In the following experiment, all available DNA extracts containing mealworm DNA were tested with Primer set IV: 0.4 μM Ins_fw/rev + 0.2 μM Seidenraupe_1_fw/rev + 0.4 μM Mehlwurm_1_fw/rev + 0.4 μM Steppengrille_1_rev. DNA extracts of insect material, extracted both with NucleoSpin® Plant II Kit (with all three incubation periods) and with DNeasy® mericon® Food Kit, feed extracts with mealworm and both insect DNA and food extracts were analysed. The main objective of this run was to investigate whether the two-shoulder peak form observed for mealworm was consistent, and if the extraction method had an impact on the size of the peak shoulders, as suspected in 5.3.5. The experiment was performed on Quantstudio 5.

Figure 35 and 36 demonstrate the HRM analysis of the three DNA extracts obtained with the NucleoSpin® Plant II Kit. The results were inconclusive, since the two-shoulder melt peak appeared only with the DNA extract obtained with a 30 minutes incubation period, and the other two displayed a single peak. These findings indicated that the primer pair was not consistent in the generation of the desired PCR product, since in the two previous PCR runs, when employing the DNA extract with incubation time overnight, the two-shoulder peak was present. Consequently, the HRM curves did not overlap as well, although DNA from the same sample was amplified.

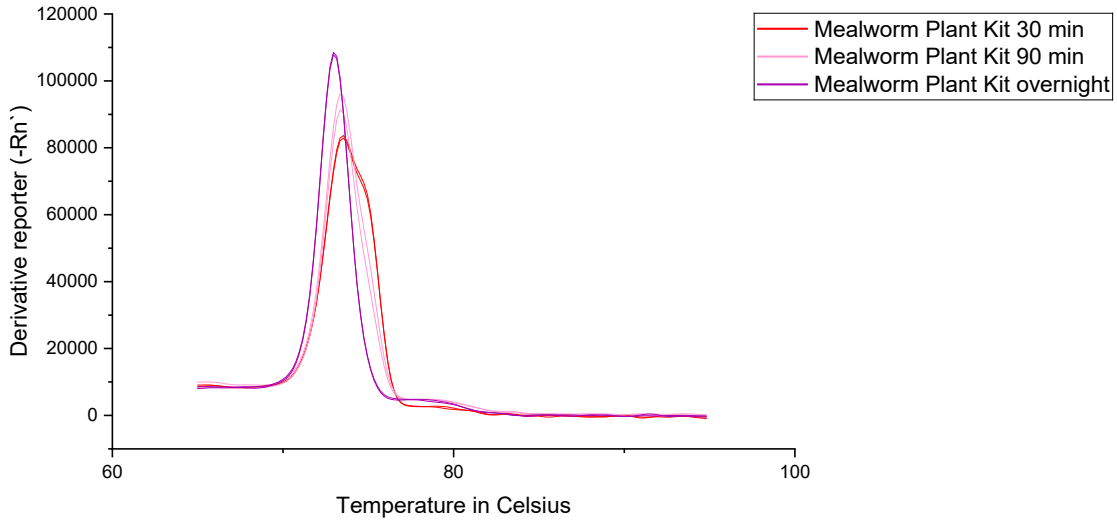


Figure 35: Melt plot of three mealworm DNA extracts, acquired with NucleoSpin® Plant II Kit, with three different incubation periods for cell lysis.

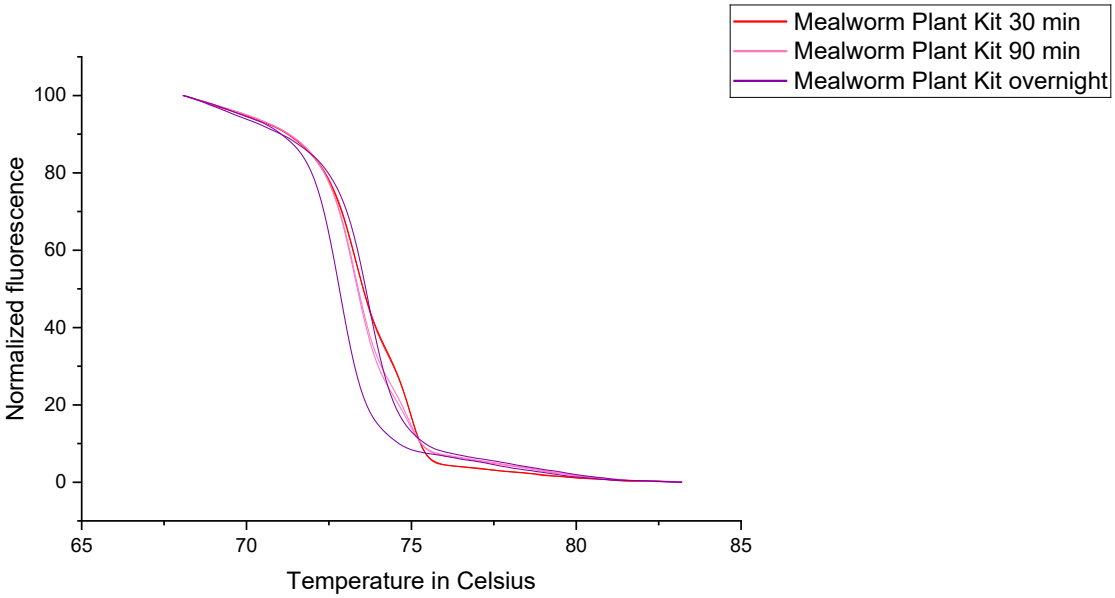


Figure 36: HRM analysis of three mealworm DNA extracts, acquired with NucleoSpin® Plant II Kit, with three different incubation periods for cell lysis.

In Figure 37 and 38, the HRM analysis of all DNA extracts with mealworm DNA obtained with the DNeasy® mericon® Food Kit are presented. The feed sample Birdseed cakes was not included in this analysis, since it resulted in the formation of different PCR products than the rest of the samples and two melt peaks were again present, similar to the results in Figure 31. Here, comparable to the results shown in this figure from the previous run, most peaks possessed a higher right shoulder, which suggested that this peak shape could be characteristic for this DNA extraction method. On the other hand, two feed DNA extracts made an exception: DNA from insect sticks and insect hearts produced peaks with a higher left shoulder, which emerged only when analysing extracts from the NucleoSpin® Plant II Kit. As a consequence, the HRM curves for insect sticks and insect hearts were not aligned with the rest of the curves. Therefore, results attained with the second extraction method were not uniform as well.

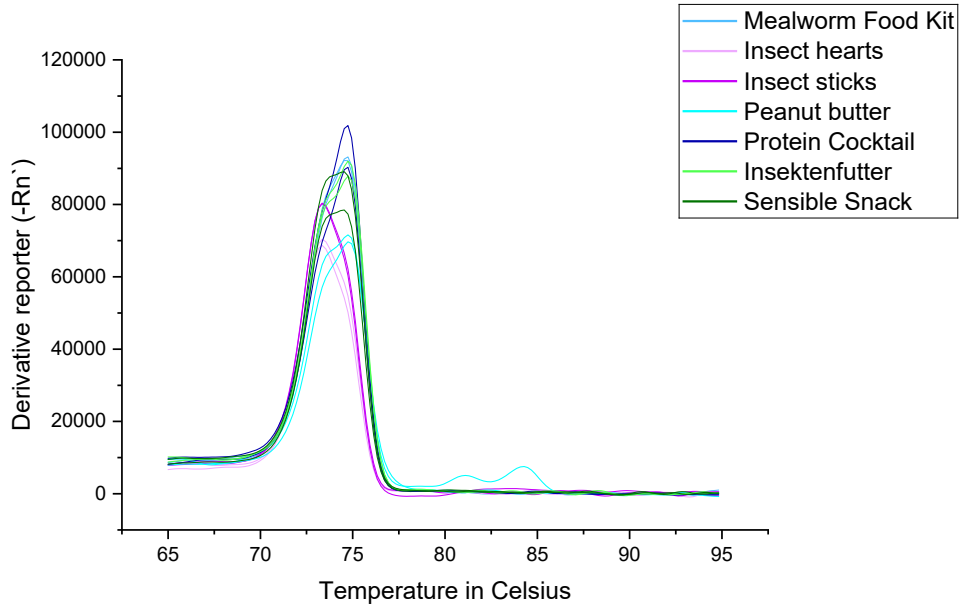


Figure 37: Melt plot of all insect and feed extracts containing mealworm DNA, isolated with DNeasy® mericon® Food Kit.

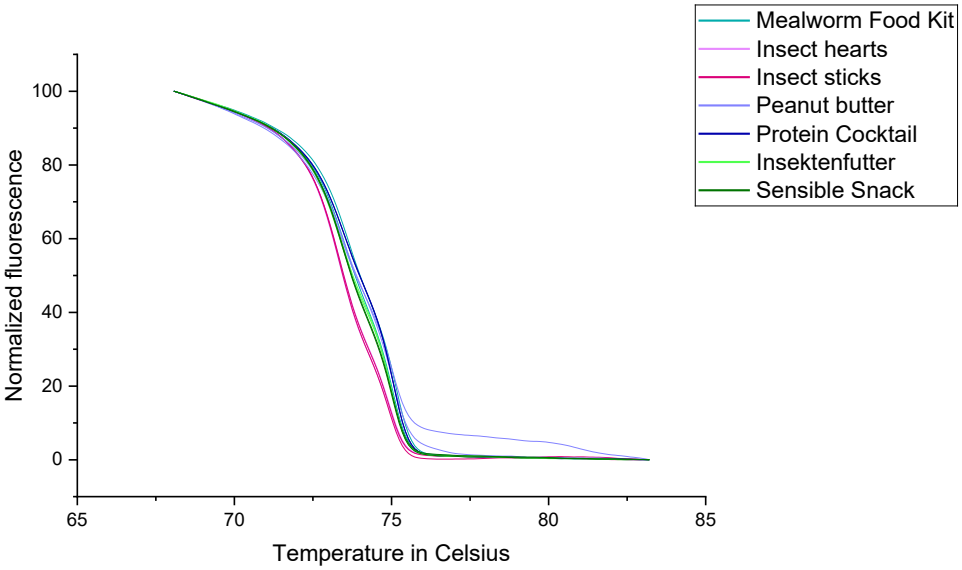


Figure 38: HRM analysis of all insect and feed extracts containing mealworm DNA, isolated with DNeasy® mericon® Food Kit.

In Figure 39 and 40, HRM analysis results with all food DNA extracts containing mealworm are visualized. Here, all DNA extracts resulted in identical melt peak shapes and therefore overlapping HRM curves. The melt peak form, with a higher right shoulder, was resembling that of most DNA extracts from the DNeasy® mericon® Food Kit. These DNA extracts were isolated in AGES with a CTAB method, different from the other two implemented in this thesis.

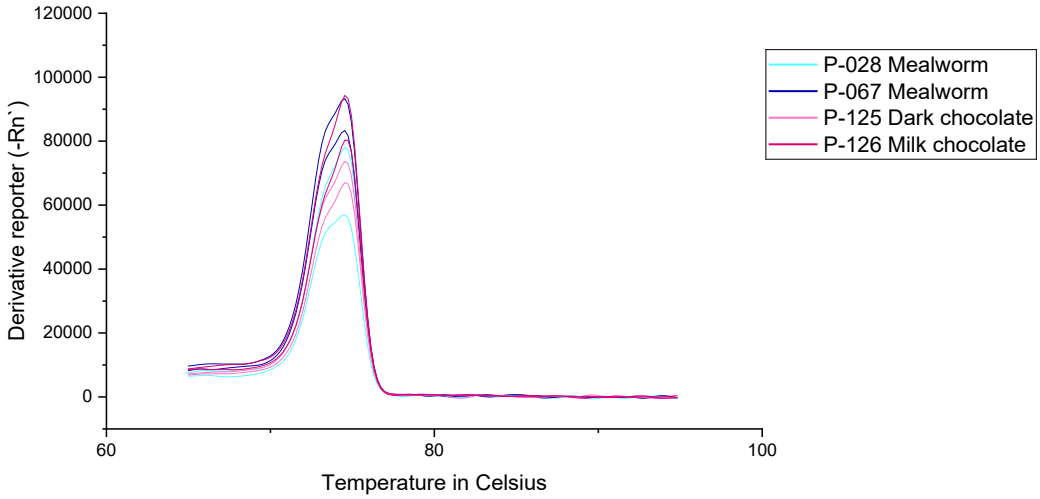


Figure 39: Melt plot of all food DNA extracts containing mealworm.

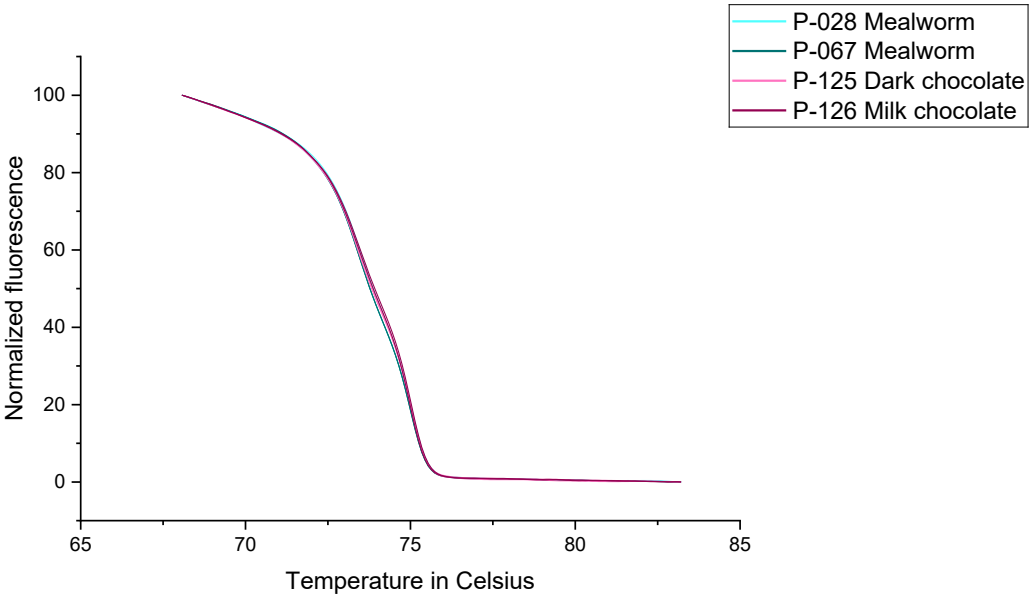


Figure 40: HRM analysis of all DNA extracts containing mealworm.

The results of this experiment suggested that the DNA extraction method was not the main reason for the alternating melt behaviour. Another factor could be the PCR products generated, if the primer amplified different parts of the DNA sequence in the separate PCR runs. To further investigate this possibility, a gel electrophoresis was performed with the amplicons from this run.

5.3.8 Agarose gel electrophoresis with mealworm amplicons obtained with Primer set IV

The purpose of this experiment was to visualize the amplicons generated in the previous PCR run with all available DNA extracts with mealworm content, since the melt peak shapes were inconsistent. A single amplicon from each duplicate for the samples and both amplicons from the duplicate of the no template control and peanut butter feed sample were analysed. Primer set IV was employed in that PCR run: 0.4 µM Ins_fw/rev + 0.2 µM Seidenraupe_1_fw/rev + 0.4 µM Mehlwurm_1_fw/rev + 0.4 µM

Steppengrille_1_rev. All DNA extracts were used in a concentration of 2.5 ng/µL. The scheme for all lanes is shown in Table 14, and a photograph of the gel under UV light is presented in Figure 41.

Table 14: Loading scheme for analysed amplicons. All amplicons originate from the same PCR run. NC: no template control. M1/4: DNA Ladder, diluted 1:4, Plant Kit: NucleoSpin® Plant II Kit, Food Kit: DNeasy® mericon® Food Kit.

Lane	Well	Sample	PCR condition	PCR date	Volume [µL]	
1	-	M 1/4	-	-		
2	A1	Mealworm Plant Kit 30 min	0.4 µM Ins_fw/rev + 0.2 µM Seid_1_fw/rev + 0.4 µM MehI_1_fw/rev + 0.4 µM Step_1_rev	31.07.2024		
3	B1	Mealworm Plant Kit 90 min	0.4 µM Ins_fw/rev + 0.2 µM Seid_1_fw/rev + 0.4 µM MehI_1_fw/rev + 0.4 µM Step_1_rev	31.07.2024		
4	D1	Mealworm Plant Kit overnight	0.4 µM Ins_fw/rev + 0.2 µM Seid_1_fw/rev + 0.4 µM MehI_1_fw/rev + 0.4 µM Step_1_rev	31.07.2024		
5	C1	Mealworm Food Kit	0.4 µM Ins_fw/rev + 0.2 µM Seid_1_fw/rev + 0.4 µM MehI_1_fw/rev + 0.4 µM Step_1_rev	31.07.2024		
6	E1	P-028 Mealworm	0.4 µM Ins_fw/rev + 0.2 µM Seid_1_fw/rev + 0.4 µM MehI_1_fw/rev + 0.4 µM Step_1_rev	31.07.2024		
7	F1	P-067 Mealworm	0.4 µM Ins_fw/rev + 0.2 µM Seid_1_fw/rev + 0.4 µM MehI_1_fw/rev + 0.4 µM Step_1_rev	31.07.2024		
8	G1	Insect sticks	0.4 µM Ins_fw/rev + 0.2 µM Seid_1_fw/rev + 0.4 µM MehI_1_fw/rev + 0.4 µM Step_1_rev	31.07.2024		
9	H1	Insect hearts	0.4 µM Ins_fw/rev + 0.2 µM Seid_1_fw/rev + 0.4 µM MehI_1_fw/rev + 0.4 µM Step_1_rev	31.07.2024		
10	A3	Peanut butter	0.4 µM Ins_fw/rev + 0.2 µM Seid_1_fw/rev + 0.4 µM MehI_1_fw/rev + 0.4 µM Step_1_rev	31.07.2024		
11	A4	Peanut butter	0.4 µM Ins_fw/rev + 0.2 µM Seid_1_fw/rev + 0.4 µM MehI_1_fw/rev + 0.4 µM Step_1_rev	31.07.2024		
12	B3	Insektenfutter, flakes	0.4 µM Ins_fw/rev + 0.2 µM Seid_1_fw/rev + 0.4 µM MehI_1_fw/rev + 0.4 µM Step_1_rev	31.07.2024		
13	C3	Sensible Snack	0.4 µM Ins_fw/rev + 0.2 µM Seid_1_fw/rev + 0.4 µM MehI_1_fw/rev + 0.4 µM Step_1_rev	31.07.2024		
14	D3	Protein cocktail	0.4 µM Ins_fw/rev + 0.2 µM Seid_1_fw/rev + 0.4 µM MehI_1_fw/rev + 0.4 µM Step_1_rev	31.07.2024		
15	E3	P-125 Dark chocolate	0.4 µM Ins_fw/rev + 0.2 µM Seid_1_fw/rev + 0.4 µM MehI_1_fw/rev + 0.4 µM Step_1_rev	31.07.2024	6.0	LD orange
16	F3	P-126 Milk chocolate	0.4 µM Ins_fw/rev + 0.2 µM Seid_1_fw/rev + 0.4 µM MehI_1_fw/rev + 0.4 µM Step_1_rev	31.07.2024		
17	G3	Birdseed cakes	0.4 µM Ins_fw/rev + 0.2 µM Seid_1_fw/rev + 0.4 µM MehI_1_fw/rev + 0.4 µM Step_1_rev	31.07.2024		
18	H3	NC	0.4 µM Ins_fw/rev + 0.2 µM Seid_1_fw/rev + 0.4 µM MehI_1_fw/rev + 0.4 µM Step_1_rev	31.07.2024		
19	H4	NC	0.4 µM Ins_fw/rev + 0.2 µM Seid_1_fw/rev + 0.4 µM MehI_1_fw/rev + 0.4 µM Step_1_rev	31.07.2024		
20	-	M1/4				

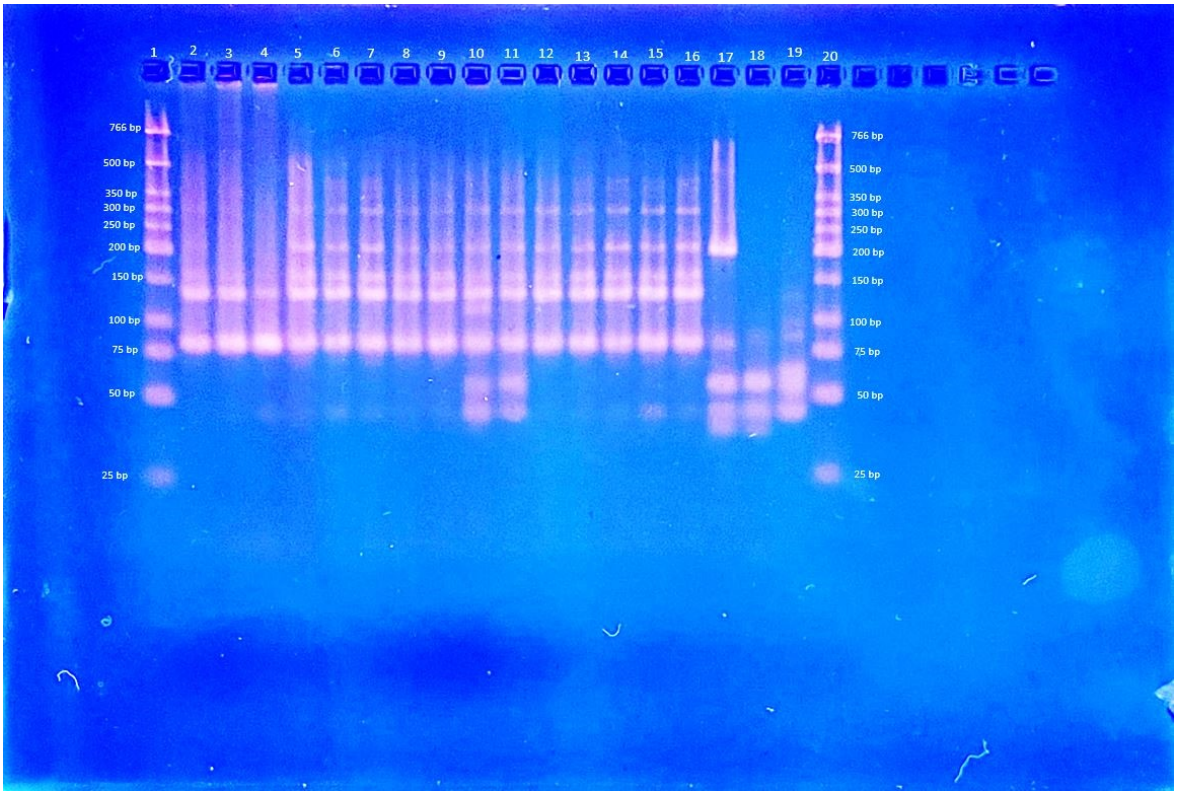


Figure 41: Gel, loaded according to Table 14, with added amplicon lengths of the DNA Ladder, bp = base pairs.

Lanes 1-3 included the three amplicons for DNA extracts obtained with the NucleoSpin® Plant II Kit. Although they exhibited different melt peak shapes (Figure 35), the length of the PCR products were identical. This finding suggested that both melt peak and HRM analysis would be unfit to identify mealworm DNA when employing this extraction method and the said PCR conditions.

Lanes 5-16 consisted of PCR products highly similar in appearance, with the only exception of the peanut butter amplicons, which were added to lanes 10-11. On the label of the original sample was

stated that black soldier fly was also present, and mealworm and black soldier fly were supplied in a ratio of 1:1 in the product. Therefore, the generation of a second PCR product from the black soldier fly DNA in the peanut butter sample is highly likely, which could be the reason behind the additional bands observed in lanes 10-11.

Apart from these amplicons, the rest were indistinguishable from each other, although DNA was isolated with different extraction methods: both with DNeasy® mericon® Food Kit and a CTAB method carried out by AGES. Moreover, lanes 8 and 9 consisted of the two feed amplicons which varied in peak shape in the previous experiment, namely Insect sticks and Insect hearts. However, their PCR products were identical to the rest of this group.

Lane 17 consisted of the amplicon of the feed sample Birdseed cakes, which always differed from the rest in terms of peak shape, with two melt peaks and no shoulder peak, as shown in Figure 31.

In conclusion, the findings from this analysis demonstrated that despite variations in melt peak shapes, DNA extracts prepared with the same extraction kit consistently produced identical PCR products. Unfortunately, under the current PCR conditions, these amplicons did not reliably yield consistent melt peak shapes and HRM curves. Therefore, it was necessary to modify the primers to enhance the method's accuracy and reduce interferences. Furthermore, amplicons generated from the DNeasy® mericon® Food Kit resembled results from the amplicons generated with the CTAB extracts from AGES, improving the uniformity of this protocol. Consequently, extracts obtained with the NucleoSpin® Plant II Kit were excluded from future experiments.

5.4 Test of Primer sets VI & VII

The next experiments aimed to reduce the number of primers required in the method and maintain the ability to differentiate the insect species. The main strategy was to eliminate either the Mehlwurm_1_fw primer or the Mehlwurm_1_rev primer in order to obtain repeatable results, which was not the case when both primers were present (with Primer set IV), as observed in section 5.3.7. Two sets of six primers were tested on Quantstudio 5:

Primer set VI: 0.4 μM Ins_fw/rev + 0.2 μM Seidenraupe_1_fw/rev + 0.4 μM Mehlwurm_1_fw + 0.4 μM Steppengrille_1_rev.

This set was already tested in section 5.3.4 and the melt curves for mealworm and crazy red cricket were overlapping, as shown in Figure 28. However, in that experiment, the DNA extracts obtained with NucleoSpin® Plant II Kit were analysed, and here, DNA extracts from DNeasy® mericon® Food Kit were the subject of analysis, which could impact the results.

Primer set VII: 0.4 μM Ins_fw/rev + 0.2 μM Seidenraupe_1_fw/rev + 0.4 μM Mehlwurm_1_rev + 0.4 μM Steppengrille_1_rev.

In sequence of the results of the previous gel electrophoresis, DNA extracts obtained with DNeasy® mericon® Food Kit were analysed from this moment on, and the dilution protocol was altered to further minimize matrix interferences. All DNA extracts with an initial concentration of above 25 ng/μL were

diluted to 2.5 ng/µL, and those below 25 ng/µL were diluted in a ratio 1:10. The purpose of this change was to unify the dilution factors as much as possible, and this protocol was consistently applied in all subsequent tests. In this PCR run, DNA extracts of mealworm, housefly and crazy red cricket were used, similar to the primer tests when developing Primer set IV.

Primer set VI produced unsatisfactory results, since no alteration of the melt profile of mealworm DNA was achieved and its HRM curve aligned with the one of crazy red cricket, resembling the results from Figure 28 when the same primer set was tested. Primer set VII delivered HRM curves with improved discrimination power, altering the HRM curve of mealworm and solving the issue at hand, as shown in Figure 42. Melt peaks were not impacted, and mealworm DNA maintained its single peak. Moreover, when analysing DNA isolated with DNeasy® mericon® Food Kit, the melt curve for housefly changed shape at higher temperature, and this was also observed in future experiments.

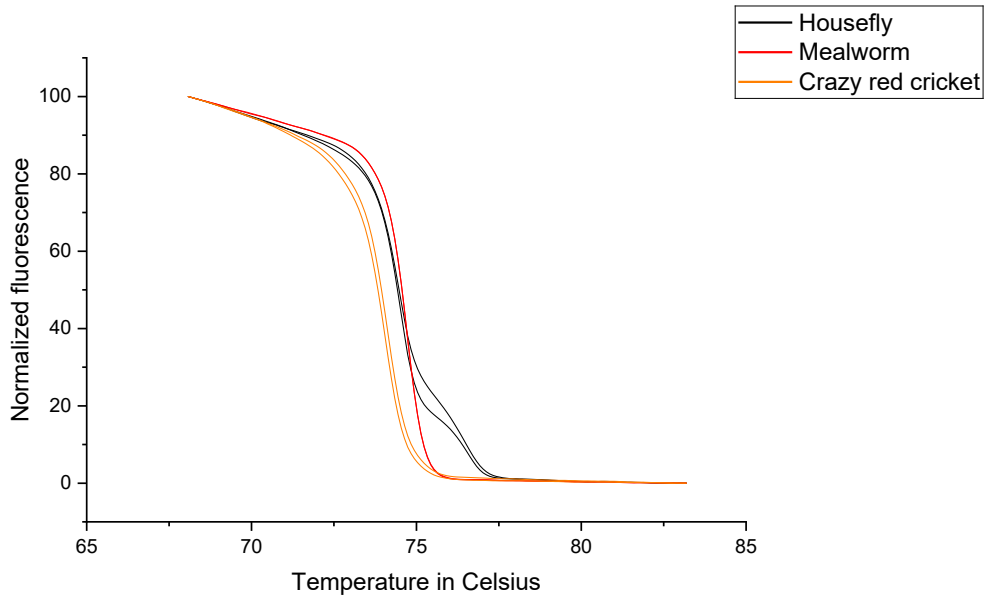


Figure 42: HRM analysis for housefly, mealworm and crazy red cricket when employing Primer set VII: 0.4 µM Ins_fw/rev + 0.2 µM Seidenraupe_1_fw/rev + 0.4 µM Mehlwurm_1_rev + 0.4 µM Steppengrille_1_rev.

5.4.1 PCR-HRM with insects, feed and meat DNA using Primer set VII

In the following experiment, DNA extracts of insects, feed and meat samples isolated with the DNeasy® mericon® Food Kit were analysed, with the above mentioned dilution protocol: all DNA extracts with an initial concentration of above 25 ng/µL were diluted to 2.5 ng/µL, and those below 25 ng/µL were diluted in a ratio 1:10. The PCR run was carried out on Quantstudio 5.

Primer set VII: 0.4 µM Ins_fw/rev + 0.2 µM Seidenraupe_1_fw/rev + 0.4 µM Mehlwurm_1_rev + 0.4 µM Steppengrille_1_rev.

Figure 43 presents the HRM analysis for nine out of the ten insect species. Fruit fly melt curves were as usual not included due to inadequate amplification. Most insect species´ melt curves, with the exception of silkworm and housefly, demonstrated ideal repeatability. Additionally, the melt curve shape of black soldier fly was altered similar to the one of housefly, with a distinct change in the lower region of the

curve at a temperature between 73.9-74°C. This phenomenon could either be attributed to the DNA extraction method or the new primer set. Despite that, all insect species´ melt curves were distinguishable from each other.

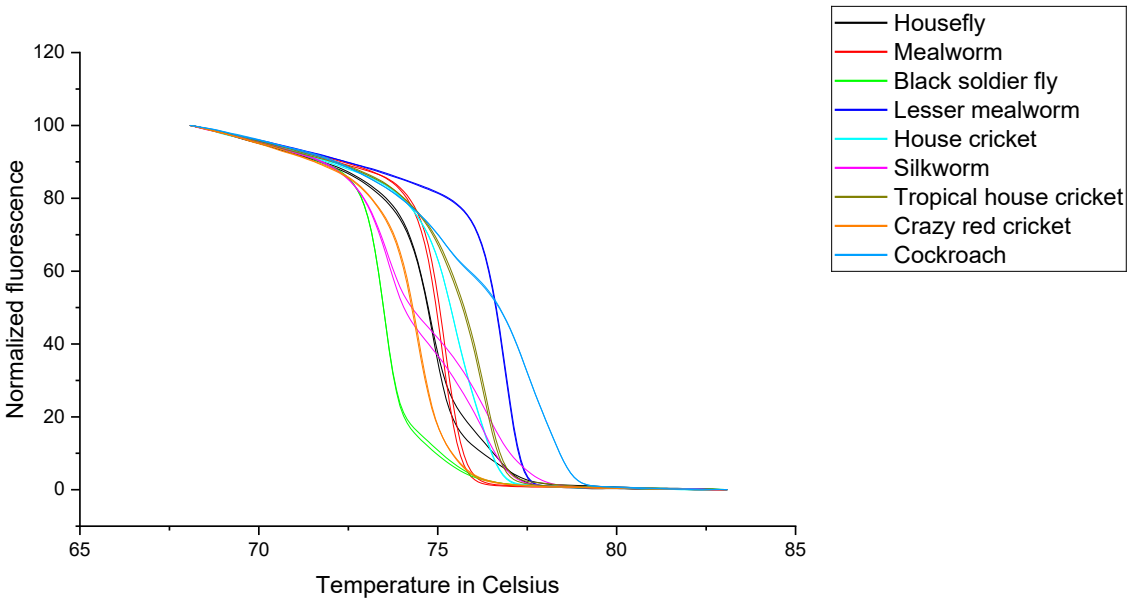


Figure 43: HRM analysis for nine insect species when amplified with Primer set VII: 0.4 µM Ins_fw/rev + 0.2 µM Seidenraupe_1_fw/rev + 0.4 µM Mehlwurm_1_rev + 0.4 µM Steppengrille_1_rev.

In Figure 44, the HRM analysis results for all feed samples and their corresponding insect, along with the three meat and one feed sample from canned feed with wild boar, are presented. In comparison to Figure 30, which visualized the same results with Primer set IV, the distinguishability between clusters of samples from the same species was greatly improved. The HRM curve of Birdseed cakes was not included in the HRM analysis, since its melt curve differed from all other form the mealworm group and the melt analysis showed again the presence of two peaks. The DNA from feed samples with chicken, namely the freeze-dried snacks from chicken meat and the chicken hearts snacks, was not adequately amplified.

The first cluster (blue circle) consisted of all extracts containing black soldier fly DNA. All melt curves possessed good repeatability, with the exception of the one for the can with chicken and insect. In the second cluster (red circle), both silkworm DNA and the feed sample Nestlingfutter demonstrated the typical curve shape, although they were not completely aligned. The third cluster (green circle) comprised of all melt curves from mealworm DNA, which displayed the best repeatability out of all clusters. Lastly, mammal DNA was again successfully amplified, and amplicons as before at higher temperatures than the rest. All three meat DNA products exhibited great repeatability, and beef and lamb melt profiles were not distinguishable as observed in previous experiments. Additionally, the melt curves for canned feed with wild boar did not show sufficient repeatability, possibly due to other components in the matrix apart from the wild boar feed sample.

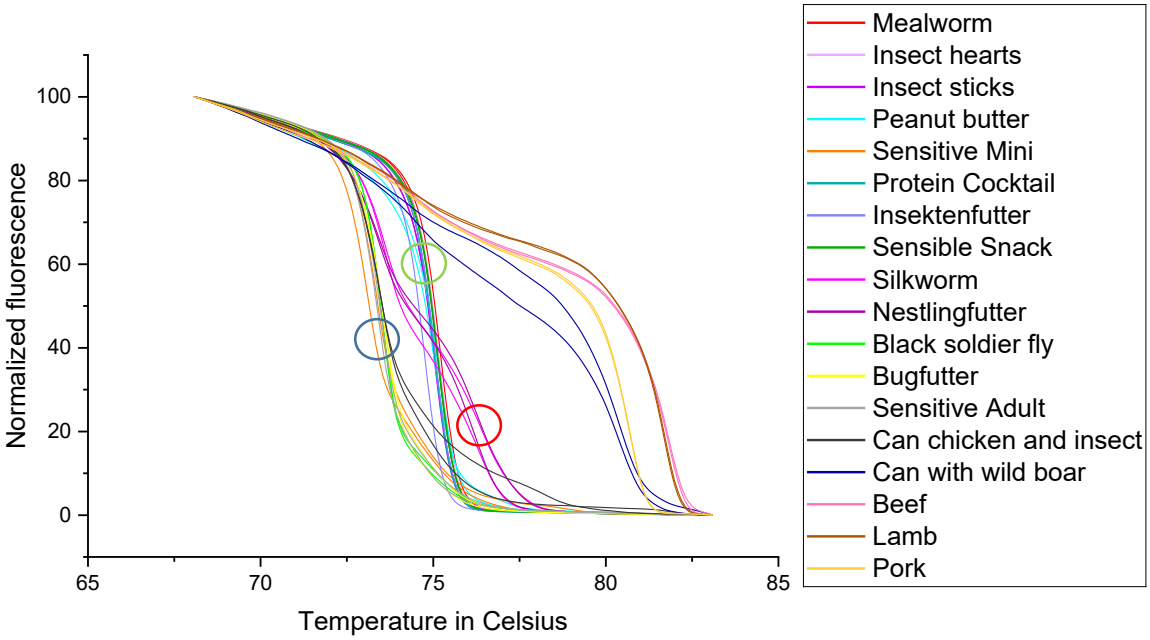


Figure 44: HRM analysis for all insect feed samples, along with the three respective species present in the original samples- mealworm (green circle cluster), black soldier fly (blue circle cluster) and silkworm (red circle cluster); three meat species and the feed sample from canned feed with wild boar.

5.4.2 PCR-HRM with food DNA extracts using Primer set VII

In the next test, all DNA extracts from Table 4 were analysed with Primer set VII: 0.4 µM Ins_fw/rev + 0.2 µM Seidenraupe_1_fw/rev + 0.4 µM Mehlwurm_1_rev + 0.4 µM Steppengrille_1_rev. Extracts were diluted according to the developed protocol: all DNA extracts with an initial concentration of above 25 ng/µL were diluted to 2.5 ng/µL, and those below 25 ng/µL were diluted in a ratio 1:10. The PCR run was carried out on Quantstudio 5.

In Figure 45, HRM analysis for all insect species permitted either for use in food or feed is presented. Firstly, housefly DNA was not efficiently amplified when working with the extracts from food, obtained with the CTAB method, with a mean Ct value of 31.3, while the no template control had a mean Ct value of 31.9. These observations indicated that either the extraction method failed to effectively isolate DNA from the sample or the sample material was unsuitable for this analysis. Additionally, similar obstacles were faced in the master thesis of Michaela Wildbacher, although different primer pairs were employed (Wildbacher, 2023). All other DNA extracts exhibited satisfactory amplification and good repeatability. Notably, one insect species permitted in food, the migratory locust, yielded a melt curve very close to the ones of mealworm DNA. This newly found issue inspired further efforts to optimize the primer set, in order to enable the differentiation of all legally allowed insect species both in food and feed. This improvement would prevent their use interchangeably and help maintain efficient quality control.

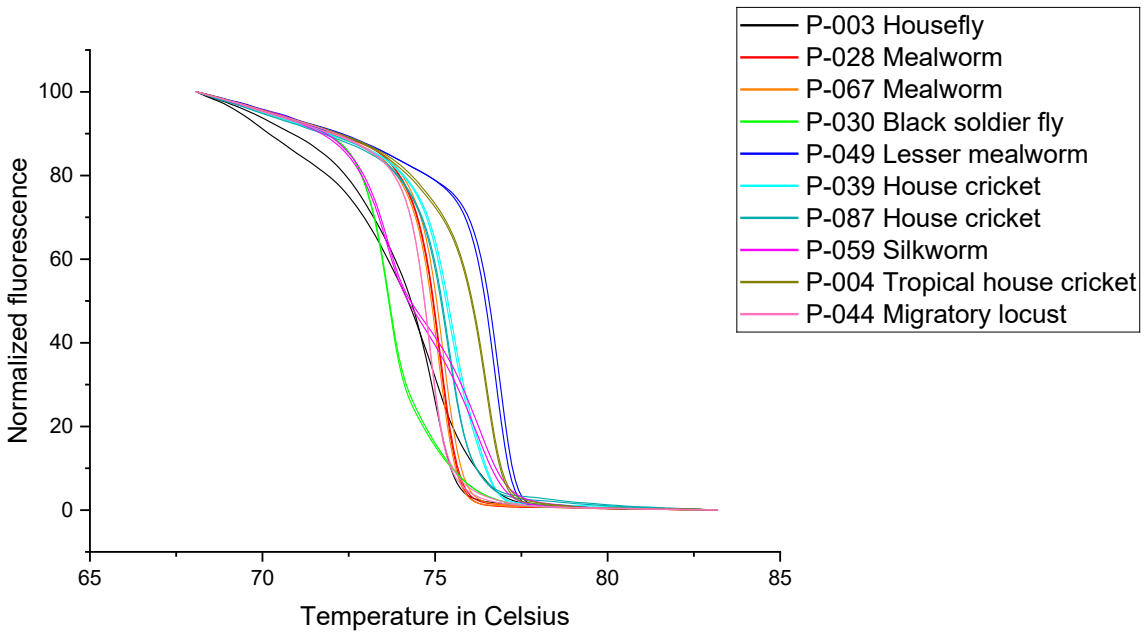


Figure 45: HRM analysis for all insect species (DNA extracts from AGES) permitted either in food or feed.

5.5 Optimization of the distinction between mealworm and migratory locust melt curves using Primer sets VIII-X

One possible strategy to combat this challenge was to incorporate a new primer in the set which would impact the melt curve of migratory locust. In the master´s thesis of Michaela Wildbacher, she had designed two forward primers for the said species, and the applicability of one of them in this method was tested. The second forward primer was not investigated, since according to the results she had obtained, it did not produce any alteration in the melt curve of migratory locust. Additionally, two new reverse primers were developed employing the method outlined in section 4.5.1, after having checked if they were compatible with the rest of the primers from the set and verifying that no homo- or heterodimers would occur. Moreover, their theoretical melt temperature had to be as close as possible to 57.4°C, which was the annealing temperature set in the PCR program, with an acceptable variation of ± 2°C. All primers created for migratory locust are presented in Table 16, and the regions in the DNA sequence where they bind are presented in Table 15.

Table 15: Primers for migratory locust and their respective DNA binding regions. DNA binding regions are presented in 5' → 3' direction.

Species	English name	Primer	Primer bound to DNA sequence (red: fw; green: rev)
Logusta migratoria	migratory locust	Wander_1_rev	TAACTTAATCTTATAATCACAAATTATGGATCAAATAAACATA AATTAATGATTTTATAATGAAGAGTTTAATTATTCTTCATGTC ACCCCAACAAAACAATAAAAATTAACATTAAAAAATAAACTAT ATAATAAAAATTAATTTATAAATGTAAAGC
		Wander_2_rev	TAACTTAATCTTATAATCACAAATTATGGATCAAATAAACATA AATTAATGATTTTATAATGAAGAGTTTAATTATTCTTCATGTC ACCCCAACAAAACAATAAAAATTAACATTAAAAAATAAACTAT ATAATAAAAATTAATTTATAAATGTAAAGC
		Wander_AS_fw_1 (Wildbacher, 2023)	TAACTTAATCTTATAATCACAAATTATGGATCAAATAAACATA AATTAATGATTTTATAATGAAGAGTTTAATTATTCTTCATGTC ACCCCAACAAAACAATAAAAATTAACATTAAAAAATAAACTAT ATAATAAAAATTAATTTATAAATGTAAAGC

Table 16: Primers designed for migratory locust (Wildbacher, 2023). Fw: forward; rev: reverse.

Primer name	Sequence 5' → 3'	Theoretical melt temperature (salt adjusted)
Wander_AS_fw_1 (Wildbacher, 2023)	CAAATTATGGATCAAATAAACATAAA	55.2ºC
Wander_1_rev	GTTAATTTTTATTGTTTTGTTGGGG	57.6ºC
Wander_2_rev	TGTTTTGTTGGGGTGACATG	56.4ºC

5.5.1 Development and assessment of Primer set VIII

Firstly, the following primer set was tested: 0.4 µM Ins_fw/rev + 0.2 µM Seidenraupe_1_fw/rev + 0.4 µM Mehlwurm_1_rev + 0.4 µM Steppengrille_1_rev + 0.8 µM Wander_AS_fw_1. The concentration for Wander_AS_fw_1 was chosen as a sequence of the tests ran by Michaela Wildbacher, where this primer concentration generated the largest alteration in the targeted melt curve. DNA extracts from all permitted insect species in both food and feed, extracted with DNeasy® mericon® Food Kit were analysed, along with the DNA extract of migratory locust from AGES. DNA extracts with an initial concentration of above 25 ng/µL were diluted to 2.5 ng/µL, and those below 25 ng/µL were diluted in a ratio 1:10. The PCR run was carried out on Quantstudio 5.

Figure 46 visualizes the results of the HRM analysis. The desired outcome was, however, not achieved: the melt profile of migratory locust was indeed altered, but the melt curve now aligned with the one of housefly. Since both insect species are allowed for use either in food or feed, these findings were critical for the development of the method. Furthermore, the differentiability of tropical house cricket and house cricket was diminished, and the repeatability of tropical house cricket had declined. Consequently, the primers Wander_AS_fw_1 and Steppengrille_1_rev were tested for heterodimers on Oligo Analyzer, and it was established that their interactions were the reason for this phenomenon.

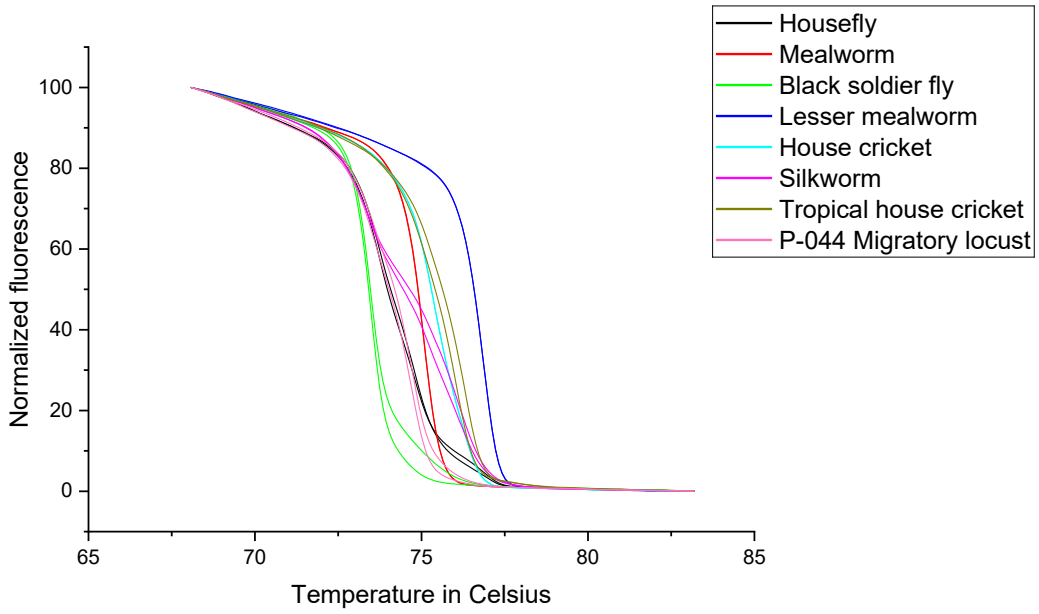


Figure 46: HRM analysis for all legally permitted in feed insect species extracted with DNeasy® mericon® Food Kit and insect sample P-044 from AGES, when using : 0.4 µM Ins_fw/rev + 0.2 µM Seidenraupe_1_fw/rev + 0.4 µM Mehlwurm_1_rev + 0.4 µM Steppengrille_1_rev + **0.8 µM Wander_AS_fw_1.**

Subsequently, Wander_AS_fw_1 was tested in two more concentrations to investigate whether that would produce a suitable HRM profile change. The same primer set was employed with the concentrations of 0.6 µM and 0.4 µM for Wander_AS_fw_1, and the same DNA extracts were used as in the previous run.

When in a concentration of 0.6 µM, the primer Wander_AS_fw_1 generated similar results as when added in 0.8 µM. On the other hand, a concentration of 0.4 µM appeared to improve the differentiability of mealworm and migratory locust melt curves without aligning with housefly. Besides, the repeatability and distinction of tropical house cricket and house cricket melt curves were maintained, as shown in Figure 47.

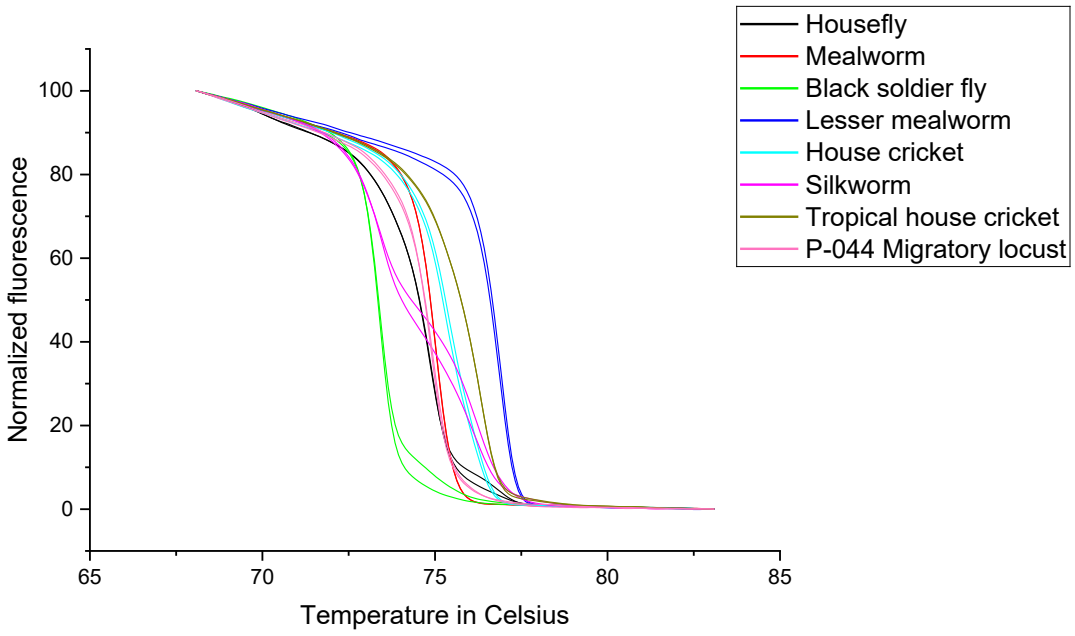


Figure 47: HRM analysis for all legally permitted in feed insect species extracted with DNeasy® mericon® Food Kit and insect sample P-044 from AGES, when using : 0.4 µM Ins_fw/rev + 0.2 µM Seidenraupe_1_fw/rev + 0.4 µM Mehlwurm_1_rev + 0.4 µM Steppengrille_1_rev + **0.4 µM Wander_AS_fw_1.**

In the next experiment, the newly developed primer set was tested on all insect DNA extracts, obtained with the DNeasy® mericon® Food Kit, both permitted and non-permitted species, plus the DNA extract for migratory locust. DNA extracts with a concentration above 25 ng/µL were diluted to 2.5 ng/µL, and those below 25 ng/µL were diluted in a ratio 1:10. The PCR was carried out on Quantstudio 5.

Primer set VIII: 0.4 µM Ins_fw/rev + 0.2 µM Seidenraupe_1_fw/rev + 0.4 µM Mehlwurm_1_rev + 0.4 µM Steppengrille_1_rev + 0.4 µM Wander_AS_fw_1.

Figure 48 presents an HRM analysis of all insect species analysed in this trial. In this run, the melt curves for housefly and migratory locust were aligned once again, similar to the results delivered by the primer set with 0.8 µM Wander_AS_fw_1. This occurrence was unexpected since the PCR conditions were not changed in comparison to the previous run. Therefore, migratory locust melt curves from both experiments were aligned with the software Origin to assess the repeatability of the method in between runs. Consequently, it became evident that the melt profile was not consistently altered, similar to the situation with the mealworm primer pair explained in section 5.3.7. This variation is demonstrated in an alignment in Figure 49. Therefore, Primer set VIII was excluded from future experiments.

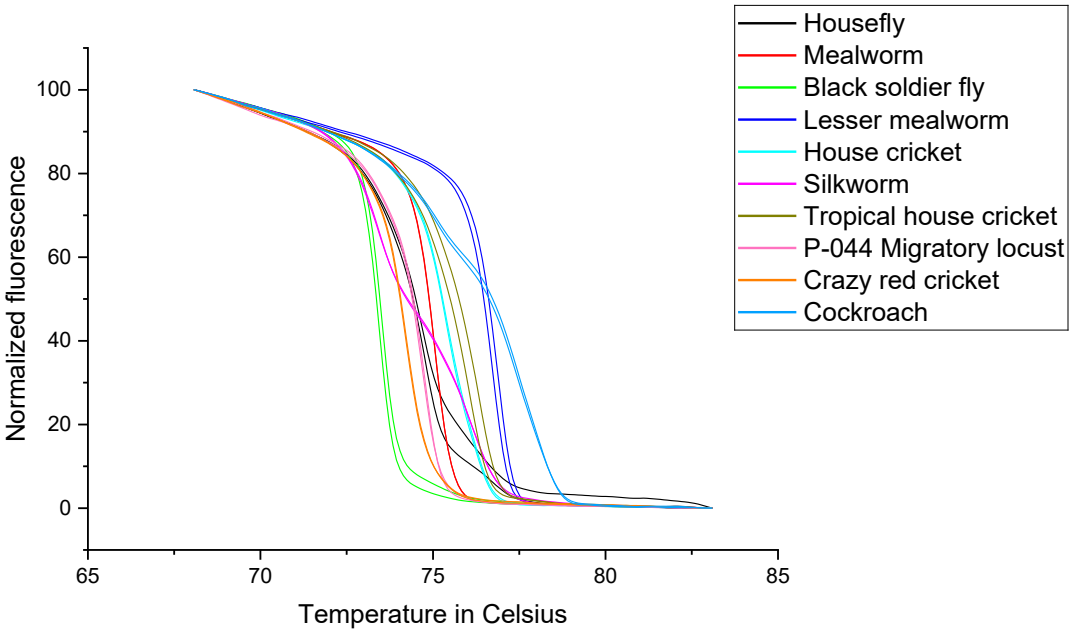


Figure 48: HRM analysis for all insect species extracted with DNeasy® mericon® Food Kit and insect sample P-044 from AGES, when using Primer set VIII: 0.4 µM Ins_fw/rev + 0.2 µM Seidenraupe_1_fw/rev + 0.4 µM Mehlwurm_1_rev + 0.4 µM Steppengrille_1_rev + **0.4 µM Wander_AS_fw_1.**

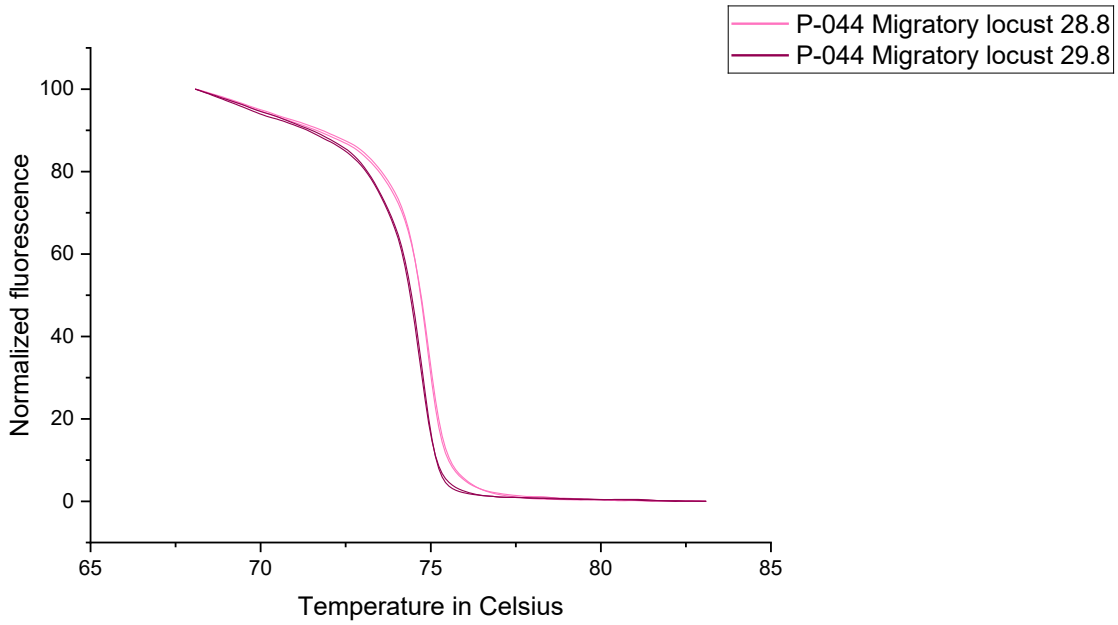


Figure 49: HRM curves alignment for migratory locust from experiments performed on two subsequent days. Primer set VIII: 0.4 µM Ins_fw/rev + 0.2 µM Seidenraupe_1_fw/rev + 0.4 µM Mehlwurm_1_rev + 0.4 µM Steppengrille_1_rev + **0.4 µM Wander_AS_fw_1.**

5.5.2 Development of Primer sets IX & X

The two newly designed reverse primers (Wander_1_rev and Wander_2_rev) were tested separately in concentrations of 0.4, 0.6 and 0.8 µM, in the presence of Primer set VII: 0.4 µM Ins_fw/rev + 0.2 µM Seidenraupe_1_fw/rev + 0.4 µM Mehlwurm_1_rev + 0.4 µM Steppengrille_1_rev. Only the migratory locust DNA extract was analysed, in a concentration of 2.5 ng/µL. The PCR was carried out on Quantstudio 5.

Figure 50 and Figure 51 visualize the HRM analysis of the tests for both primers. Both primer sets delivered similar results, shifting only the position of the melt curve to a lower melt temperature without changing its shape. Additionally, the repeatability of the melt curves was most optimal when they were applied in a concentration of 0.6 µM. Therefore, this concentration was also implemented in future experiments, and the following primer sets were established:

Primer set IX: 0.4 µM Ins_fw/rev + 0.2 µM Seidenraupe_1_fw/rev + 0.4 µM Mehlwurm_1_rev + 0.4 µM Steppengrille_1_rev + 0.6 µM Wander_2_rev (Figure 51);

Primer set X: 0.4 µM Ins_fw/rev + 0.2 µM Seidenraupe_1_fw/rev + 0.4 µM Mehlwurm_1_rev + 0.4 µM Steppengrille_1_rev + 0.6 µM Wander_1_rev (Figure 50).

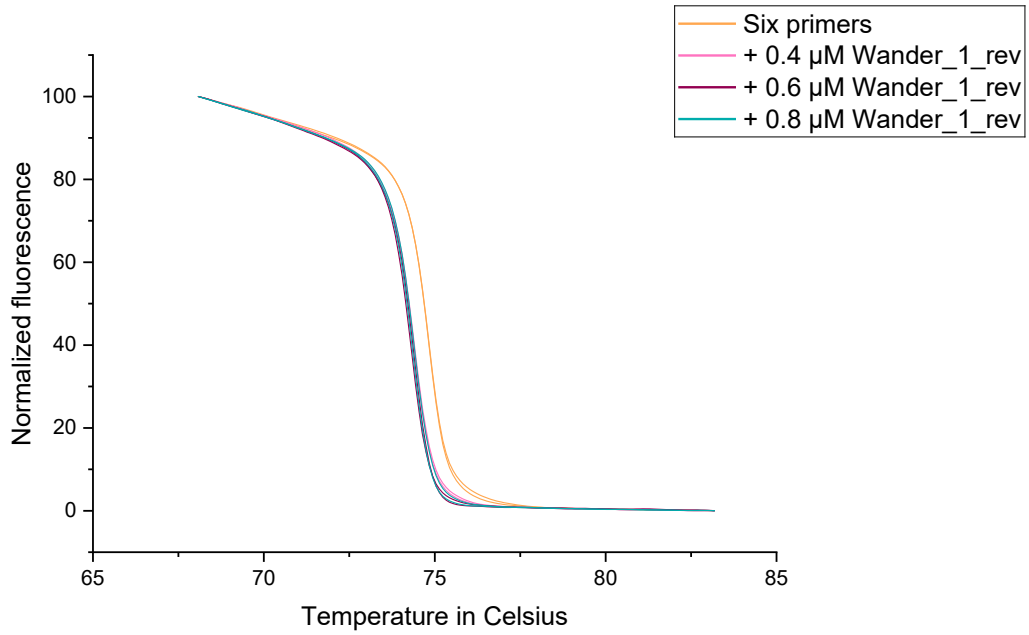


Figure 50: Alignment of melt curves for migratory locust when tested in the presence of Wander_1_rev in concentrations 0.4, 0.6 and 0.8 µM and a six primers set (Primer set VII): 0.4 µM Ins_fw/rev + 0.2 µM Seidenraupe_1_fw/rev + 0.4 µM Mehlwurm_1_rev + 0.4 µM Steppengrille_1_rev.

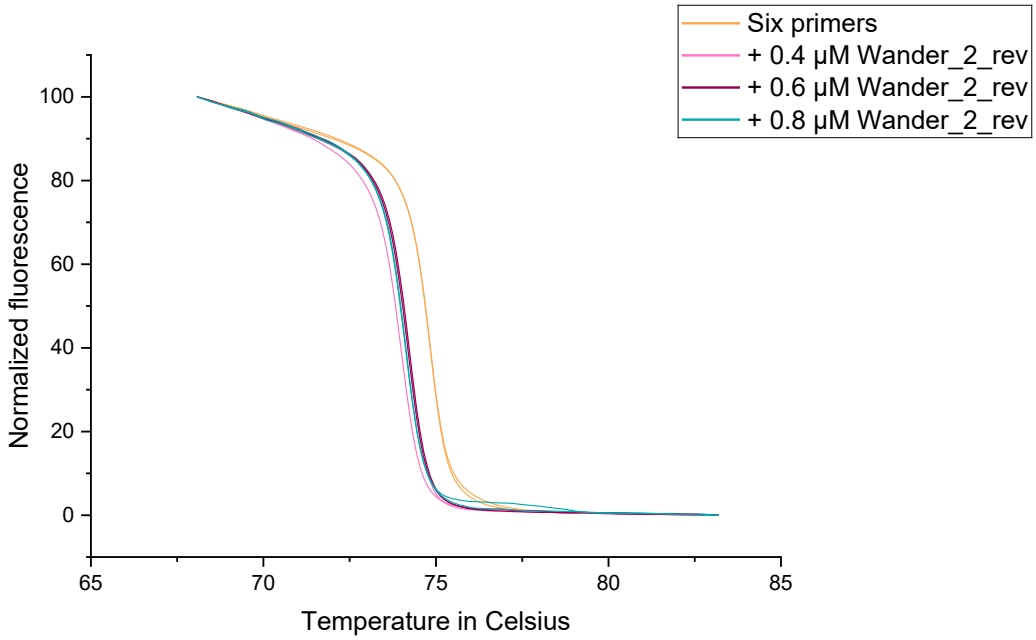


Figure 51: Alignment of melt curves for migratory locust when tested in the presence of Wander_2_rev in concentrations 0.4, 0.6 and 0.8 µM and a six primers set (Primer set VII): 0.4 µM Ins_fw/rev + 0.2 µM Seidenraupe_1_fw/rev + 0.4 µM Mehlwurm_1_rev + 0.4 µM Steppengrille_1_rev.

5.5.3 PCR-HRM with insects and food DNA with Primer set IX

In the following experiment, DNA from all permitted insects in feed, isolated with DNeasy® mericon® Food Kit, plus the DNA extracts containing migratory locust were analysed. The usual dilution protocol as in the previous runs was applied, and the analysis was performed on Quantstudio 5.

Primer set IX: 0.4 µM Ins_fw/rev + 0.2 µM Seidenraupe_1_fw/rev + 0.4 µM Mehlwurm_1_rev + 0.4 µM Steppengrille_1_rev + 0.6 µM Wander_2_rev.

Figure 52 presents the normalized melt curves for all permitted insects in feed and migratory locust and two important new observations were made. Firstly, the melt curve of housefly had lost its significant shape in its lower part, which led to it resembling those of migratory locust and mealworm. Next, the tropical house cricket melt curves were again exhibiting worse repeatability compared to the rest, which could occur because of interactions between the primers in the set. Other than that, the rest of the melt curves demonstrated great repeatability. However, this new implication concerning the housefly melt curve supported the decision not to include Wander_2_rev in subsequent experiments.

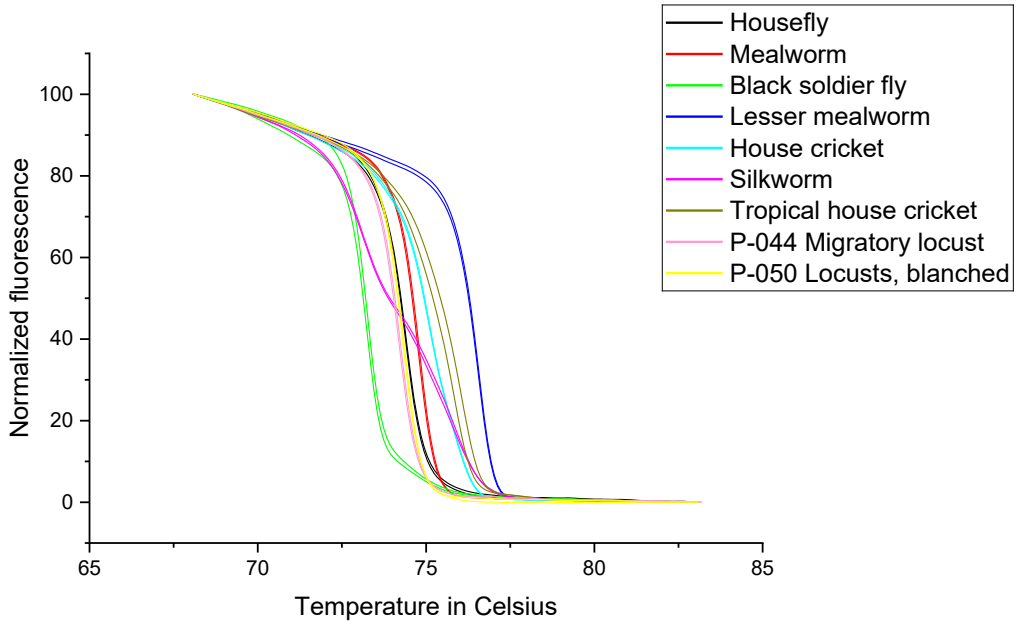


Figure 52: HRM analysis for all permitted insects in feed, isolated with DNeasy® mericon® Food Kit, plus the DNA extracts containing migratory locust from AGES.

5.5.4 PCR-HRM with insects and food DNA with Primer set X

Next, an identical experiment to the one in section 5.5.3 was carried out, but with the addition of 0.6 μM Wander_1_rev instead of 0.6 μM Wander_2_rev. DNA extracts of all permitted insects in feed, acquired with DNeasy® mericon® Food Kit, plus the DNA extracts containing migratory locust from Michaela Wildbacher were tested. The standard dilution protocol was applied and the experiment was performed on Quantstudio 5.

Primer set X: 0.4 μM Ins_fw/rev + 0.2 μM Seidenraupe_1_fw/rev + 0.4 μM Mehlwurm_1_rev + 0.4 μM Steppengrille_1_rev + 0.6 μM Wander_1_rev.

Figure 53 illustrates the results of the HRM analysis, and it mirrored the one presented in Figure 51. Although most insect species melt curves displayed good repeatability, the melt curve shape of housefly had again lost its distinctive feature and was now harder to differentiate from the ones of migratory locust and mealworm. Additionally, the melt curves of tropical house cricket demonstrated again diminished repeatability in comparison to the rest, reducing the gap between itself and the house cricket melt curve. These discoveries led to the decision to eliminate Wander_1_rev from the primer set for future experiments.

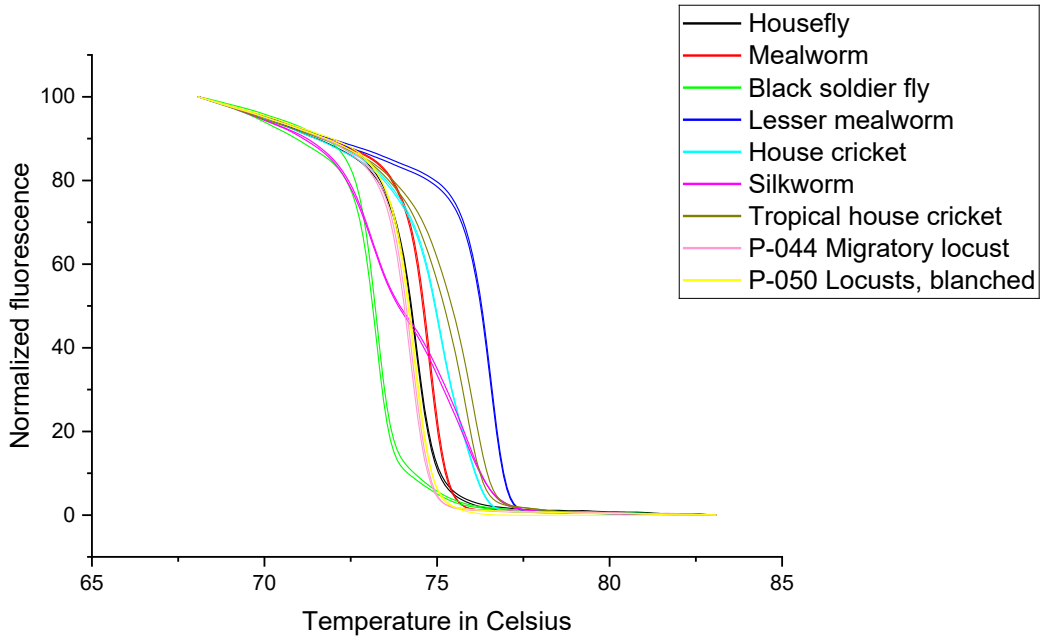


Figure 53: HRM analysis for all permitted insects in feed, isolated with DNeasy® mericon® Food Kit, plus the DNA extracts containing migratory locust from AGES.

5.6 Test and finalization of Primer set XI

After these unsuccessful attempts to improve the distinction between all insect species allowed both in food and feed, another strategy was employed, which required the removal of one of the primers from the set. This action would hopefully alter the melt curve profile of mealworm slightly, making it distinguishable from that of the migratory locust. The new primer set consisted of the following five primers in the said concentrations: 0.4 µM Ins_fw/rev + 0.2 µM Seidenraupe_1_fw/rev + 0.4 µM Steppengrille_1_rev. The reverse primer for mealworm, namely Mehlwurm_1_rev was excluded in comparison to the methods described in sections 5.5, 5.4 and 5.3. Another motivation for this decision was the possibility of an optimization in the repeatability and precision of the method, since it was expected that less primers would result in less interferences between them.

5.6.1 PCR-HRM with insect, feed and meat samples using Primer set XI

In the following experiment, all ten insect and three meat DNA extracts, and all feed DNA extracts obtained with DNeasy® mericon® Food Kit were analysed. Additionally, the DNA extracts of migratory locust from AGES were included. All DNA extracts were used in a dilution of 2.5 ng/µL if the initial DNA extract possessed a concentration above 25 ng/µL, and in a dilution of 1:10 if the initial DNA concentration was below 25 ng/µL.

Figure 54 represents the results of the HRM analysis for all insect species, except for fruit fly, since its DNA was not adequately amplified. The desired outcome was achieved: mealworm melt curve was still distinguishable from the migratory locust, and the housefly melt curve had retained its typical shape, which made it distinguishable from mealworm, migratory locust and crazy red cricket. All insect species´ melt curves demonstrated good repeatability, except for the one of tropical house cricket, which exhibited again varying melt curves, similar to all previous runs, shown in Figures 48, 52 and 53. This

caused its melt curve to closely resemble that of the house cricket, thereby reducing the ability to effectively discriminate between the two. However, in a subsequent run it was determined that this phenomenon was due to the extract employed and not the methodology, as previously thought (Figure 57, section 5.6.2). When the 1:10 dilution of the DNA extract was freshly prepared, this problem was not observed and the melt curves were ideally repeatable.

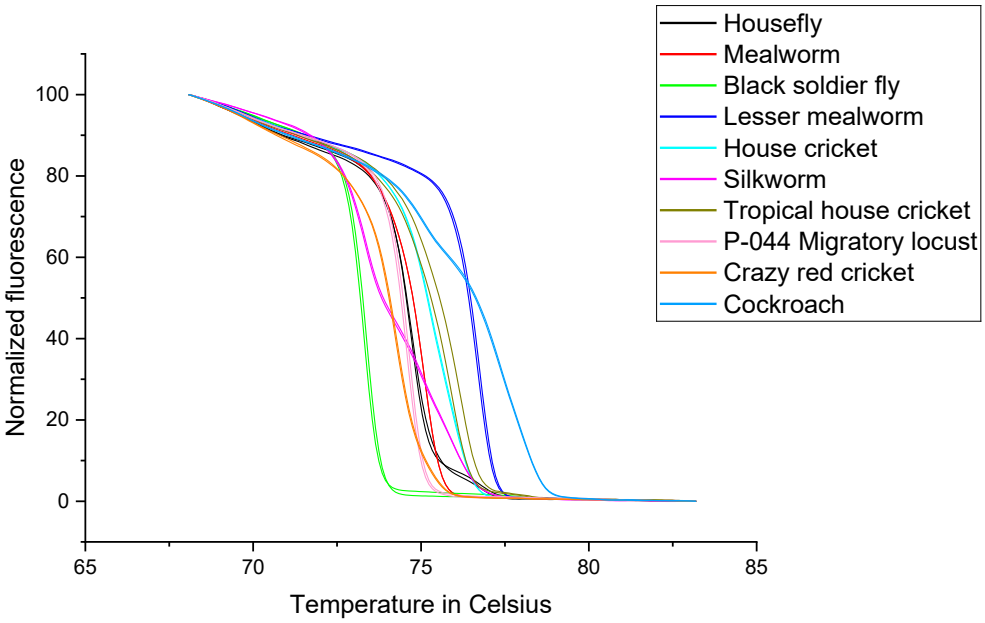


Figure 54: HRM analysis for all permitted insects in feed, isolated with DNeasy® mericon® Food Kit, plus the DNA extracts containing migratory locusts from AGES.

In Figure 55, the HRM analysis for all feed samples and the corresponding insect, along with the three meat samples and their respective one feed sample, is visualized. Repeatability of the melt curves was enhanced, especially when compared to the results from previous trials with the same DNA extracts, shown in Figures 30 and 44. The main difference was the improvement in the repeatability of all black soldier fly DNA melt curves (blue circle in Figure 55), which possessed more variance in other experiments (Figure 44, section 5.4). Mealworm DNA melt curves (green circle in Figure 55) aligned ideally, with the exception of the one for Birdseed cakes, which again exhibited its typical melt curve shape, similar to previous runs. Moreover, the melt curve of peanut butter was not perfectly aligned neither with that of the mealworm nor with black soldier fly, which could be due to the sample containing both insect species. Melt curves for silkworm (red circle, Figure 55) overlapped sufficiently. The three meat amplicons had again melted at higher temperatures than the rest of the samples, including their respective sample, the canned feed with wild boar. Two feed melt profiles were not presented, those of the freeze-dried chicken hearts and the snacks from chicken meat, since their DNA was not sufficiently amplified. Both feed samples were intended to function as negative controls, as they are labeled to contain no insect content. Therefore, these results were anticipated.

The positive results of this experiment influenced the decision to use this primer set for the next experiments. When analysing feed samples DNA, this method had delivered the most differentiable melt curves and most aligned clusters of melt curves for both the feed samples and the original insect sample.

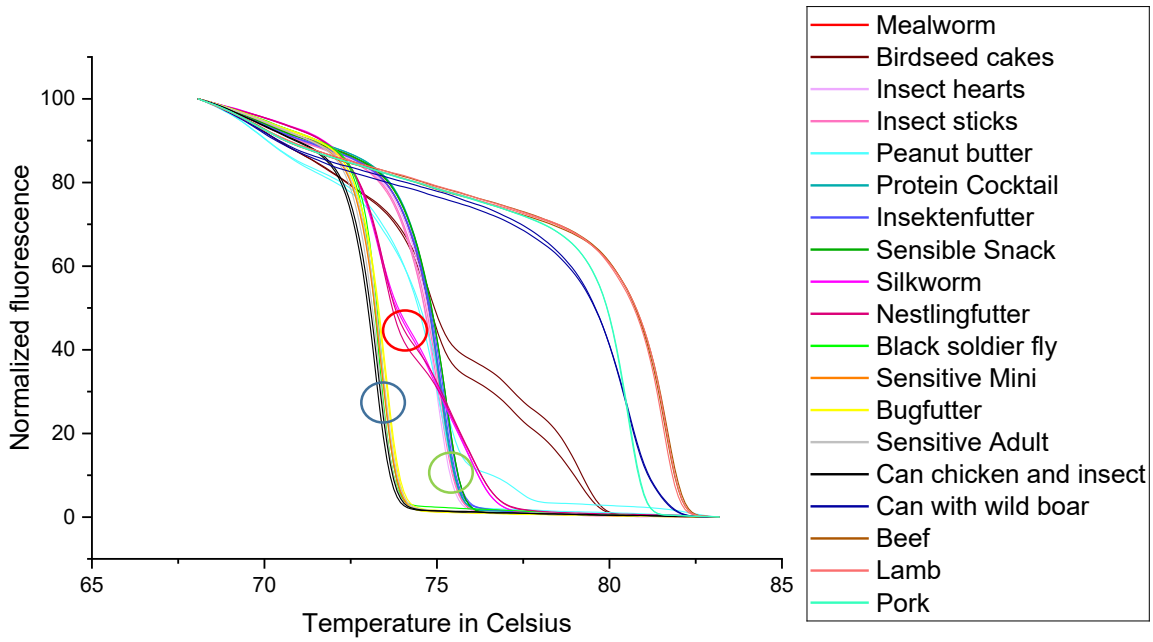


Figure 55: HRM analysis for all insect feed samples, along with the three respective species present in the original samples- mealworm (green circle cluster), black soldier fly (blue circle cluster) and silkworm (red circle cluster); three meat species extracts and the feed sample from canned feed with wild boar.

5.6.2 PCR-HRM with DNA extracts from food with Primer set XI

The next trial consisted of the HRM analysis of all DNA extracts from AGES, which contained both insect DNA and DNA from food samples. The same dilution protocol was applied as for previous PCR runs, namely dilutions of 2.5 ng/µL if the initial DNA extract possessed a concentration above 25 ng/µL, and a dilution of 1:10 if the initial DNA concentration was below 25 ng/µL. Additionally, due to the diminished repeatability of tropical house cricket melt curves in the last few experiments (Figures 46, 48, 52, 53, 54), more of the available DNA extracts of these species were included in this run: a 1:10 dilution of the extract with DNeasy® mericon® Food Kit, this same extract in a concentration of 2.5 ng/µL, the NucleoSpin® Plant II Kit extract with 30 minutes incubation time in a concentration of 2.5 ng/µL and the NucleoSpin® Plant II Kit extract incubated overnight again in a concentration of 2.5 ng/µL. All of these DNA extracts were analysed to determine whether this decrease in repeatability was due to the PCR-HRM method or the specific DNA extract used in the previous runs.

Figure 56 presents the results of the HRM analysis for all legally allowed in food or feed insect species. Apart from housefly DNA, the rest of the insect DNA exhibited good repeatability and sufficient amplification. Since the Ct mean value of housefly was 31.5 in this experiment, and the one of the no template control 40, it could be assumed that some DNA was amplified, but still not enough to produce two overlapping melt curves. To compare, the rest of the insect species DNA in this graph resulted in a Ct mean value below 25. Despite those observations, the rest of the melt profiles resembled those delivered from the analysis of the extracts from DNeasy® mericon® Food Kit.

In Figure 57, the HRM analysis of all DNA extracts of tropical house cricket and the two extracts of house cricket is visualized. The results suggest that the DNA extraction kit had an influence on the melt profiles for this insect species, which could impact the differentiation between the melt curves of tropical

house cricket and the house cricket. The amplicon of the DNA extract obtained with the CTAB method (P-004 Tropical house cricket) melted at a temperature around 75.7°C, while the rest of the amplicons melted at temperatures ranging between 75.1 and 75.3°C. However, all of the melt curves for tropical house cricket were distinguishable from both of the melt profiles for house cricket.

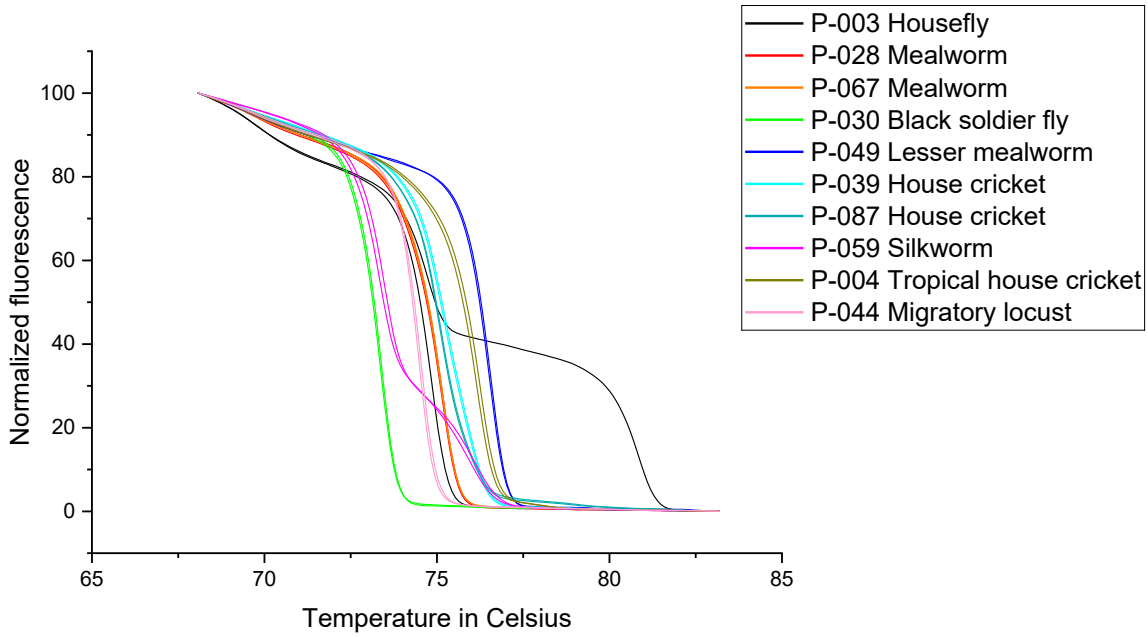


Figure 56: HRM analysis for all insect DNA extracts from AGES from species permitted either in food or feed.

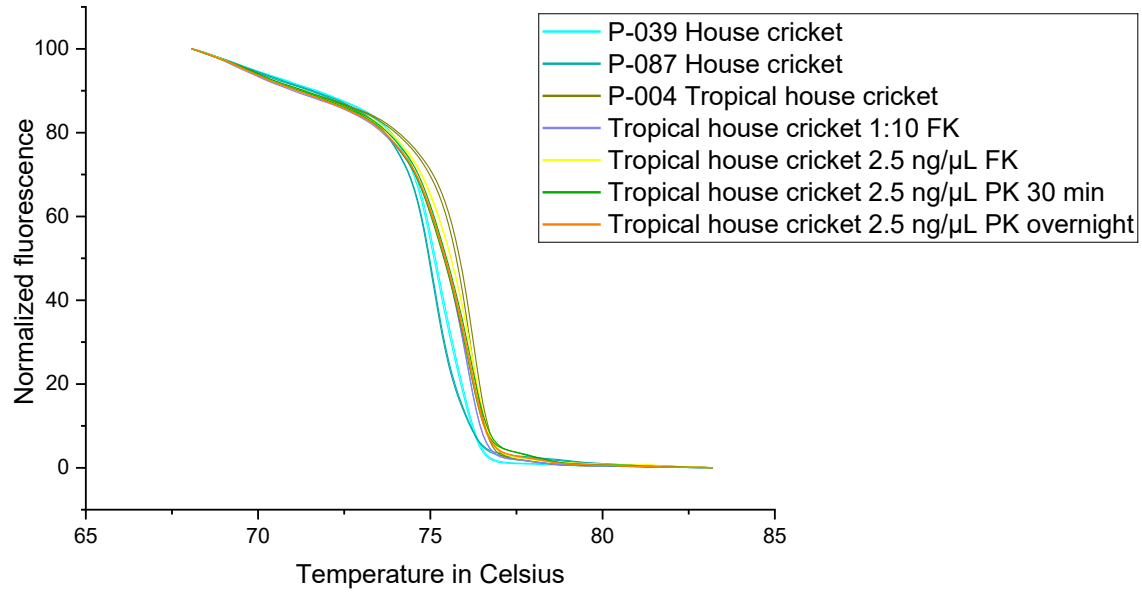


Figure 57: HRM analysis of DNA extracts from tropical house cricket and house cricket. FK: DNeasy® mericon® Food Kit; PK: NucleoSpin® Plant II Kit.

Figure 58 shows the HRM analysis for mealworm (green circle), lesser mealworm (red circle) and migratory locust (blue circle) and their respective food samples. Both mealworm and lesser mealworm melt curves for insect and food samples demonstrated great repeatability, with melt curves for the specific species overlapping completely. Migratory locust DNA melt curves were not overlapping as consistently as with the other two insect species, with the food sample varying slightly from the insect

DNA melt curve. On the other hand, in Figure 59, it was evident that food samples with house cricket DNA were less aligned than any of the other insect species. This could be due to the processing and different matrices of all food samples- for instance, it was observed that the amplicons of all three food samples labelled as Cricket Cracker with different flavours had all melted earlier than the rest of the house cricket amplicons. Moreover, the same issue was observed in the master´s thesis of Michaela Wildbacher, regardless of the fact that primers designed specifically for house cricket were implemented (Wildbacher, 2023).

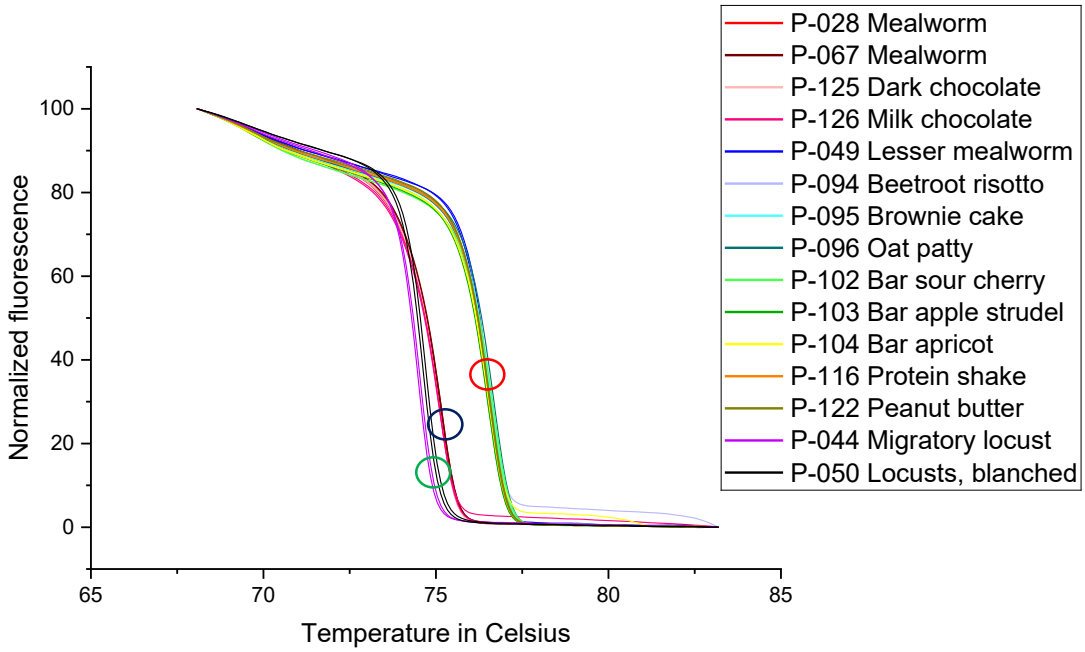


Figure 58: HRM analysis of DNA extracts from AGES containing mealworm (green circle), lesser mealworm (red circle) and migratory locust (blue circle) DNA.

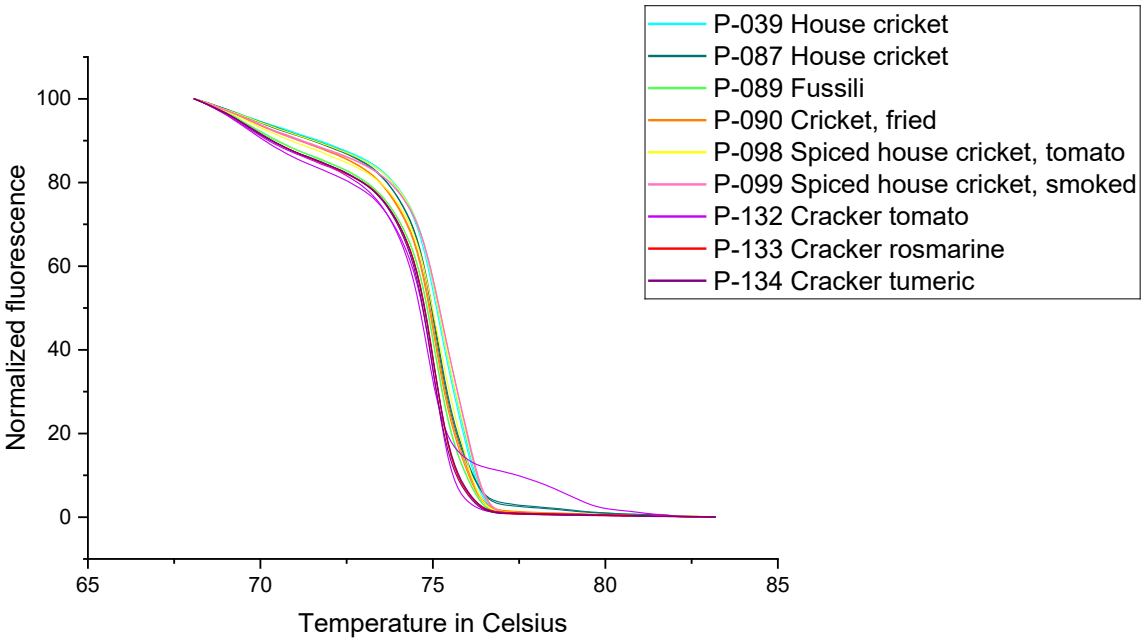


Figure 59: HRM analysis of DNA extracts from AGES containing house cricket DNA.

Figure 60 visualizes the results of the HRM analysis for all insects not permitted for use in food or feed. Two species were not included in the graph, since their amplification was inadequate with Ct mean values above 35- the fruit fly and the common green bottle fly. Two species exhibited some variety in the duplicates- greater wax moth and true cicada. Moreover, five insect species melt curves built a cluster and were indistinguishable from each other: crazy red cricket, Mediterranean field cricket, cowpea seed beetle, red pam weevil, blind click-beetle and the true cicada. However, their melt curves did not align with any of those of the legally permitted insect species in feed in the EU.

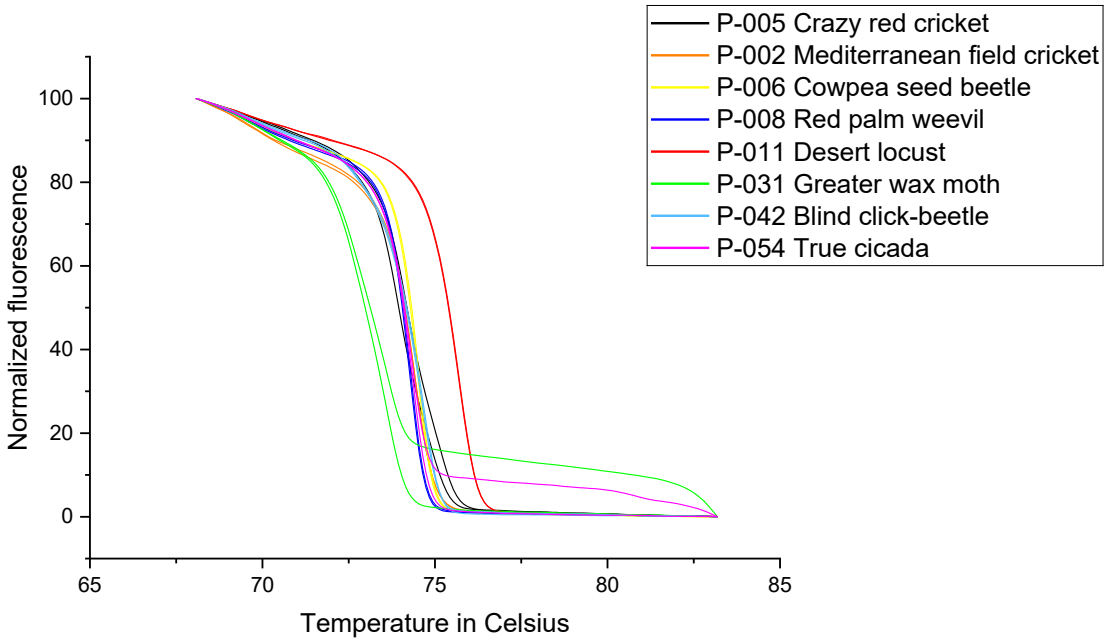


Figure 60: HRM analysis of DNA extracts from AGES containing not permitted insect species.

5.6.3 Spiking experiment with peanut butter feed sample (analysis with Primer set XI)

After having finalized the primer set, the next step was to investigate one of the feed samples- peanut butter, which contained both black soldier fly and mealworm. Although they were present in a ratio 1:1 according to the ingredients list on the product label, the melt curve and the corresponding melt peak were rather aligned with those of mealworm DNA than those of black soldier fly DNA (Figures 18 and 19). Secondly, in this scenario, a melt profile with two peaks would be expected, and here only one peak was observed with all methods tested. Because of this, an experiment was conducted where both the feed sample and mealworm DNA were analysed in the presence of different amounts of black soldier fly DNA. The DNA concentration of both the peanut butter feed sample and the mealworm DNA was 2.5 ng/μL, and the experiment was performed on Quantstudio 5. Five spike levels were chosen:

Level 1: + 0.25 pg black soldier fly DNA;

Level 2: + 2.5 pg black soldier fly DNA;

Level 3: + 25 pg black soldier fly DNA;

Level 4: + 250 pg black soldier fly DNA;

Level 5: + 2500 pg black soldier fly DNA.

Each amount of black soldier fly DNA was substituted to 5000 pg of either peanut butter or mealworm DNA. DNA of the two insect species in question (mealworm and black soldier fly) and the peanut butter DNA were also analysed non-spiked to enable a comparison. Figures 61-64 present the results of the HRM analysis of all components. As usual, the melt peak for non-spiked peanut butter aligned with the one for mealworm (Figure 61). However, after the addition of a small amount such as 0.25 pg black soldier fly DNA, a two-shoulder peak was observed, which was the expectation for the original feed sample. At the next spike level, + 2.5 pg black soldier fly DNA, the peak had shifted to the right and the two-shoulder form was no longer present. Additionally, it did not overlap completely with any of both insect melt peaks, and the melt peak was broader compared to the black soldier fly melt peak. When supplementing more DNA, the peak form of the spiked samples was aligned with the peak of black soldier fly in terms of both position and shape. On the other hand, mealworm DNA remained with an intact peak shape and position for four out of the five spike levels, and the peak had become wider at the highest level, + 2500 pg black soldier fly DNA (Figure 62). Therefore, it became evident that the peanut butter sample did indeed contain black soldier fly DNA, since it required a 10^5 of the amount needed to impact the peanut butter to even slightly alter the melt peak of mealworm DNA. The results of the HRM analysis, shown in Figures 63 and 64, further confirmed the observations and conclusion made from the melt peak visualization. Nevertheless, the precise proportion of black soldier fly and mealworm DNA in the feed sample remained uncertain. A potential explanation for this observation is the inconsistency in homogeneity of the material used for the DNA extraction, suggesting uneven DNA distribution in the peanut butter sample. Before coming to a definite conclusion, further experiments

should be conducted, for instance with multiple new DNA extractions to assess the homogeneity of the sample.

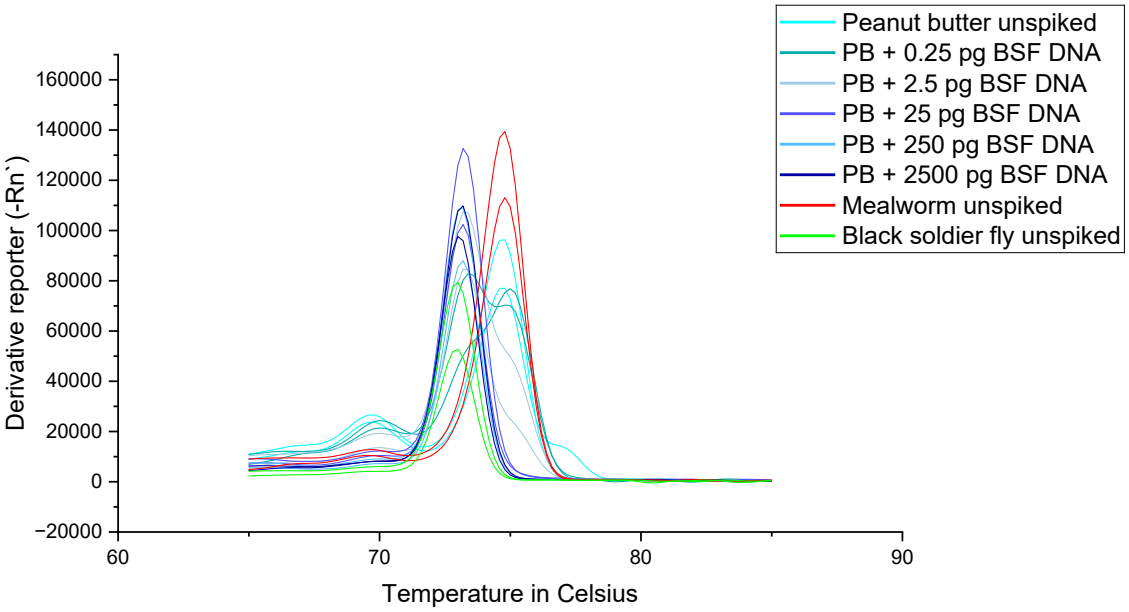


Figure 61: Melt plot for all five spike levels of peanut butter DNA, along with non-spiked mealworm and black soldier fly DNA; PB: peanut butter, BSF: black soldier fly.

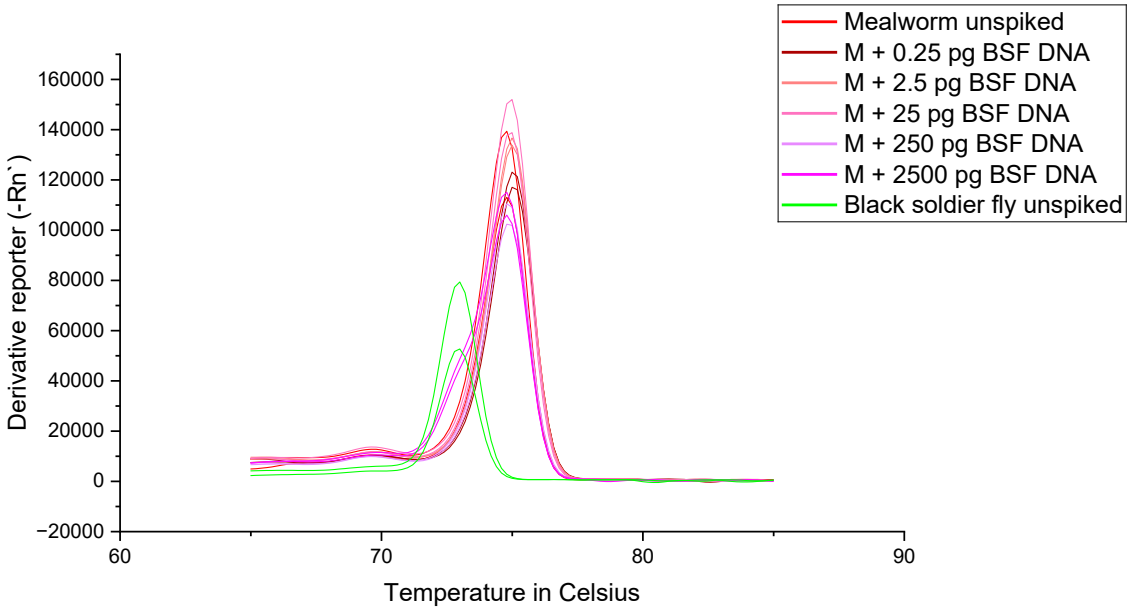


Figure 62: Melt plot for all five spike levels of mealworm DNA, along with non-spiked black soldier fly DNA; BSF: black soldier fly, M: mealworm.

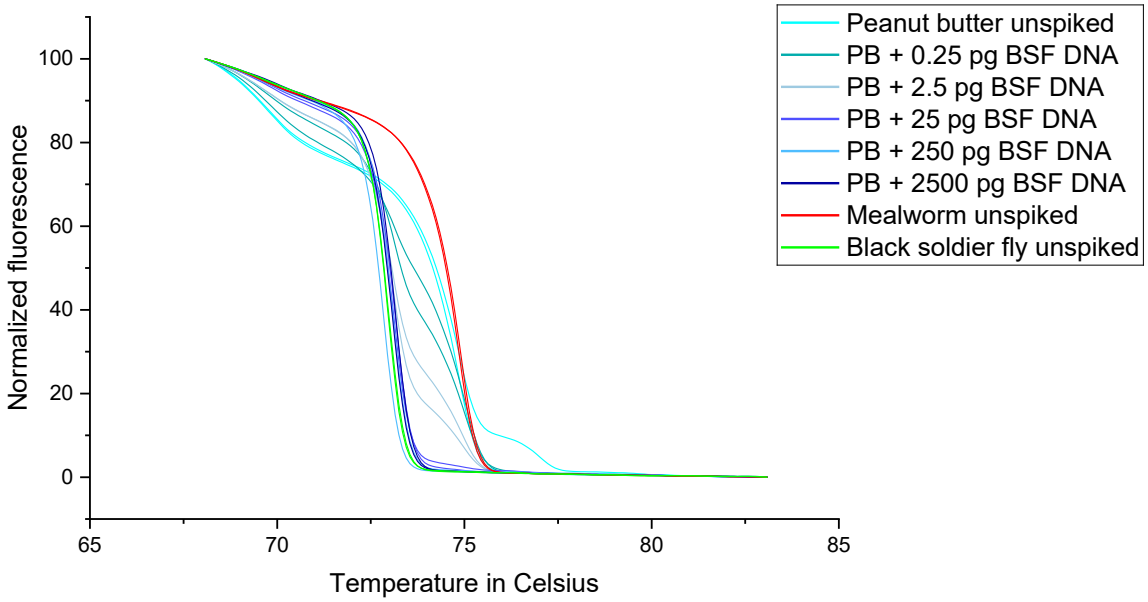


Figure 63: HRM analysis for all five spike levels of peanut butter DNA, along with non-spiked mealworm and black soldier fly DNA; PB: peanut butter, BSF: black soldier fly.

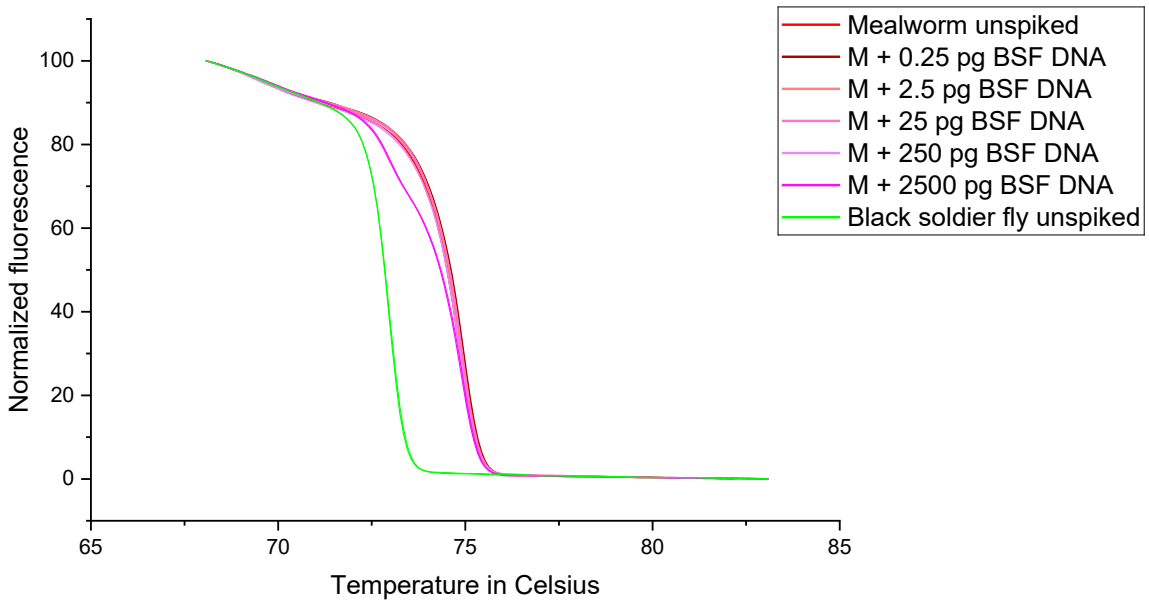


Figure 64: HRM analysis for all five spike levels of mealworm DNA, along with non-spiked black soldier fly DNA; BSF: black soldier fly, M: mealworm.

5.7 Summary of all primer sets and their limitations

In Table 17, all tested primer sets, already presented in Table 6, are listed along with their optimized concentrations and the limitations of the method.

Table 17: All primer sets tested in this master´s thesis.

Primer set	Primers included	Optimized concentrations	Limitations
Primer set I	Ins_fw	0.4 µM	Three clusters of melt curves for insect species were identified: black soldier fly + silkworm, mealworm + housefly + crazy red cricket, tropical house cricket + house cricket.
	Ins_rev	0.4 µM	
Primer set II	Ins_fw	0.4 µM	First cluster was resolved: melt curves for black soldier fly and silkworm were distinguishable from each other, silkworm melt profile was impacted. Two more clusters remained: crazy red cricket + mealworm + housefly and tropical house cricket + house cricket.
	Ins_rev	0.4 µM	
	Seidenraupe_1_fw	0.2 µM	
	Seidenraupe_1_rev	0.2 µM	
Primer set III	Ins_fw	0.4 µM	Two clusters were resolved: melt curves for black soldier fly and silkworm were differentiable, silkworm melt profile was impacted; melt curves for housefly, crazy red cricket and mealworm were distinguishable from each other, the housefly melt profile was impacted; the third cluster remained: tropical house cricket + house cricket.
	Ins_rev	0.4 µM	
	Seidenraupe_1_fw	0.2 µM	
	Seidenraupe_1_rev	0.2 µM	
	Mehlwurm_1_fw	0.4 µM	
	Mehlwurm_1_rev	0.4 µM	
	Krullfliege_1_fw	0.4 µM	
Primer set IV	Ins_fw	0.4 µM	Three clusters were resolved: melt curves for black soldier fly and silkworm were differentiable, silkworm melt profile was impacted; melt curves for housefly, crazy red cricket and mealworm were distinguishable from each other, the crazy red cricket melt profile was impacted; the third cluster was resolved: melt profiles for tropical house cricket and house cricket were differentiable, both were impacted. There was no consistency in the repeatability of melt profiles for mealworm.
	Ins_rev	0.4 µM	
	Seidenraupe_1_fw	0.2 µM	
	Seidenraupe_1_rev	0.2 µM	
	Mehlwurm_1_fw	0.4 µM	
	Mehlwurm_1_rev	0.4 µM	
	Steppengrille_1_rev	0.4 µM	
Primer set V	Ins_fw	0.4 µM	Two clusters were resolved: melt curves for black soldier fly and silkworm were differentiable, silkworm melt profile was impacted; melt curves for housefly, crazy red cricket and mealworm were distinguishable from each other, the housefly melt profile was impacted; the third cluster remained: tropical house cricket + house cricket.
	Ins_rev	0.4 µM	
	Seidenraupe_1_fw	0.2 µM	
	Seidenraupe_1_rev	0.2 µM	
	Mehlwurm_1_fw	0.4 µM	
	Krullfliege_1_fw	0.4 µM	
Primer set VI	Ins_fw	0.4 µM	First cluster was resolved: melt curves for black soldier fly and silkworm were distinguishable from each other, silkworm melt profile was impacted. Second cluster was not impacted, melt curves for mealworm and crazy red cricket overlapped. The third cluster was resolved: melt profiles for tropical house cricket and house cricket were differentiable.
	Ins_rev	0.4 µM	
	Seidenraupe_1_fw	0.2 µM	
	Seidenraupe_1_rev	0.2 µM	
	Mehlwurm_1_fw	0.4 µM	
	Steppengrille_1_rev	0.4 µM	
Primer set VII	Ins_fw	0.4 µM	Three clusters were resolved: melt curves for black soldier fly and silkworm were differentiable, silkworm melt profile was impacted; melt curves for housefly, crazy red cricket and mealworm were distinguishable from each other, the crazy red cricket melt profile was impacted; the third cluster was resolved: melt profiles for tropical house cricket and house cricket were differentiable, both were impacted. Melt curves for mealworm and migratory locust, an insect species permitted for use in food in the EU, overlapped.
	Ins_rev	0.4 µM	
	Seidenraupe_1_fw	0.2 µM	
	Seidenraupe_1_rev	0.2 µM	
	Mehlwurm_1_rev	0.4 µM	
	Steppengrille_1_rev	0.4 µM	

Primer set VIII	Ins_fw	0.4 µM	Three clusters were resolved: melt curves for black soldier fly and silkworm were differentiable, silkworm melt profile was impacted; melt curves for housefly, crazy red cricket and mealworm were distinguishable from each other, the crazy red cricket melt profile was impacted; the third cluster was resolved: melt profiles for tropical house cricket and house cricket were differentiable, both were impacted. Melt curves for migratory locust were not repeatable.
	Ins_rev	0.4 µM	
	Seidenraupe_1_fw	0.2 µM	
	Seidenraupe_1_rev	0.2 µM	
	Mehlwurm_1_rev	0.4 µM	
	Steppengrille_1_rev	0.4 µM	
	Wander_AS_fw_1	0.4 µM	
Primer set IX	Ins_fw	0.4 µM	Three clusters were resolved: melt curves for black soldier fly and silkworm were differentiable, silkworm melt profile was impacted; melt curves for housefly, crazy red cricket and mealworm were distinguishable from each other, the crazy red cricket melt profile was impacted; the third cluster was resolved: melt profiles for tropical house cricket and house cricket were differentiable, both were impacted. Melt curves for migratory locust were not repeatable.
	Ins_rev	0.4 µM	
	Seidenraupe_1_fw	0.2 µM	
	Seidenraupe_1_rev	0.2 µM	
	Mehlwurm_1_rev	0.4 µM	
	Steppengrille_1_rev	0.4 µM	
	Wander_2_rev	0.6 µM	
Primer set X	Ins_fw	0.4 µM	Three clusters were resolved: melt curves for black soldier fly and silkworm were differentiable, silkworm melt profile was impacted; melt curves for housefly, crazy red cricket and mealworm were distinguishable from each other, the crazy red cricket melt profile was impacted; the third cluster was resolved: melt profiles for tropical house cricket and house cricket were differentiable, both were impacted. Melt curves for migratory locust were not repeatable.
	Ins_rev	0.4 µM	
	Seidenraupe_1_fw	0.2 µM	
	Seidenraupe_1_rev	0.2 µM	
	Mehlwurm_1_rev	0.4 µM	
	Steppengrille_1_rev	0.4 µM	
	Wander_1_rev	0.6 µM	
Primer set XI	Ins_fw	0.4 µM	All clusters were resolved: melt curves for black soldier fly and silkworm were differentiable, silkworm melt profile was impacted; melt curves for housefly, crazy red cricket and mealworm were distinguishable from each other, the crazy red cricket melt profile was impacted; the third cluster was resolved: melt profiles for tropical house cricket and house cricket were differentiable, both were impacted. Melt curves for insects, feed and food samples exhibited good repeatability, with the exception of house cricket samples.
	Ins_rev	0.4 µM	
	Seidenraupe_1_fw	0.2 µM	
	Seidenraupe_1_rev	0.2 µM	
	Steppengrille_1_rev	0.4 µM	

6. Conclusion

The aim of this master´s thesis was to develop a method to differentiate between insect species permitted for use in feed in the EU with the help of PCR followed by an HRM analysis. Sample material from ten different insects was delivered by AGES. Seven of them are legally permitted for use in feed in the EU: lesser mealworm (*Alphitobius diaperinus)*, mealworm (*Tenebrio molitor*), housefly (*Musca domestica*), house cricket (*Acheta domesticus*), tropical house cricket (*Gryllodes sigillatus)*, silkworm (*Bombyx mori*) and black soldier fly (*Hermetia illucens*). The remaining three insect species: cockroach (*Blattodea*), crazy red cricket (*Gryllus locorojo*) and fruit fly (*Drosophila*) are not legally allowed for use in feed in the EU. Additionally, three commercially obtained meat samples from pork, beef and lamb meat were acquired. DNA from insect and meat samples was extracted employing two different extraction kits, NucleoSpin® Plant II Kit and DNeasy® mericon® Food Kit. The second one proved to be superior in terms of DNA yield and repeatability of the results. Because of this, this extraction protocol established itself as more universal and its extracts became the primary choice for most of the experiments. However, it was confirmed that if the DNA extracts were sufficiently diluted in a minimal ratio of 1:10 to eliminate any matrix effects, melt curves of DNA extracted with both protocols overlap.

For the amplification of insect species DNA, the primer pair Ins_fw and Ins_rev was tested and was found to be efficient. To further enhance the DNA amplification process and generate amplification curves for the species black soldier fly and silkworm, an addition of 2 mM $MgCl_2$ was performed. This substitution improved overall the amplification of all insect species DNA except for fruit fly, which remained inadequately amplified. This issue persisted for all subsequent experiments, regardless of the primer set employed. Furthermore, DNA from the three meat kinds was amplified as well, although the generated PCR products always melted at higher temperatures than the insect PCR products, which made them consistently distinguishable from the rest.

In the first experiments, three clusters of melt curves were identified: black soldier fly and silkworm; house fly, crazy red cricket and mealworm; house cricket and tropical house cricket. The main objective of future experiments was to be able to differentiate between the melt curves of the species in those clusters. Initially, three primer pairs were designed for the resolving of the first one: Kurzflüglegrille_1_fw/rev for tropical house cricket, Soldatenfliege_1_fw/rev for black soldier fly and Sedienraupe_1_fw/rev for silkworm. Additionally, the primer pair Fruchtfliege_1_fw/rev for fruit fly was designed to improve its amplification. All primer pairs were supposed to be implemented in a primer set together with Ins_fw/rev. Out of all primer pairs tested, only the pair Seidenraupe_1_fw/rev delivered satisfactory results, altering the melt profile of silkworm and creating a characteristic double peak. This way, silkworm DNA could be differentiated from black soldier fly DNA when performing the HRM analysis and the first cluster was resolved.

Next, four more primer pairs were designed for the second cluster: Krullfliege_1_fw/rev and Krullfliege_2_fw/rev for housefly, Mehlwurm_1_fw/rev for mealworm and Steppengrille_1_fw/rev for crazy red cricket. After testing different combinations of the Ins_fw/rev, Seidenraupe_1_fw/rev and the new primer pairs, it was concluded that only Steppengrille_1_rev would remain in the primer set. This

primer changed the melt profile of not only crazy red cricket, but also of tropical house cricket and house cricket, resulting in the settlement of the issue of not one, but two clusters. Together with Ins_fw/rev and Seidenraupe_1_fw/rev, a primer set of five primers was finalized.

The addition of other primers to this method was tested in multiple trials, but resulted in a decline in repeatability or distinction of the melt curves of different species. The primer pair Mehlwurm_1_fw/rev produced a characteristic two shoulder peak for mealworm DNA, which unfortunately did not occur in every PCR run. The substitution of the reverse primer alone was implemented as well, yet it did not alter the mealworm melt profile to this extent and possessed worse repeatability than the five primer method. Moreover, new primers were designed for the species migratory locust (*Locusta migratoria*), which is legally allowed in food and its melt curve appeared close to the one of mealworm. However, the two primers, Wander_1_rev and Wander_2_rev, diminished the ability to distinguish between housefly and mealworm melt curves.

Fifteen different feed samples were commercially obtained and analysed in the course of this thesis. Twelve of them contained insects and the rest were tested as negative controls. Seven included mealworm DNA, four possessed black soldier fly DNA, one contained silkworm DNA and on one product label the specific insect species was not stated. The vast majority of feed DNA melt curves aligned with those of the respective insect DNA it included, with the exception for two samples. One sample, Birdseed cakes, demonstrated two peaks when performing HRM analysis, suggesting another DNA segment was amplified. Another sample, peanut butter, which contained both black soldier fly and mealworm in a ratio 1:1, presented one peak, which was closer to that of mealworm than of black soldier fly DNA. To investigate this issue, a spike experiment was conducted, which indicated that both insect species were indeed present in the feed sample, although a single melt peak was observed instead of two. Further experiments are required to assess this problem, preferably with samples consisting of more than one legally allowed insect species. Out of the three non-insect feed samples, only the one DNA from canned feed with wild boar was amplified, and its PCR product melted at higher temperatures, similar to pork DNA.

The applicability of the method was tested on insect and food DNA extracts provided by AGES, and it was established that the five primers set could be implemented when analysing food samples. Additionally, a number of not legally allowed insect species were included in this trial, and none of them aligned with any of the melt curves of the seven investigated species permitted in feed. This finding suggested that this PCR-HRM possessed great potential as a screening method for the legally allowed in EU insect species, since it delivered distinguishable melt curves from a variety of species. The only exception were samples containing DNA from house cricket, which were less aligned and their melt curves could potentially overlap with those of other insects, such as tropical house cricket. Additional trials are needed to investigate this issue, and possibly primer set for house cricket should be designed to produce more precise results.

Based on the results presented in this thesis, PCR-HRM analysis has significant potential for use in feed authenticity testing. However, more work is required, particularly in testing on a larger number of

samples, especially complex matrices with multiple insect species included. Once optimized, PCR-HRM analysis could serve as a simple, fast, and cost-effective alternative to other DNA-based methods.

7. Appendix

7.1 Abstract

PCR-HRM analysis is a powerful tool for food adulteration screening due to its ability to rapidly detect DNA sequence variations without the need for subsequent processing. This makes it highly suitable for differentiating species in complex food matrices, securing food authenticity and quality. In this master´s thesis, the aim was to develop a PCR-HRM method to distinguish between seven insect species legally permitted in EU feed: black soldier fly, silkworm, house cricket, tropical house cricket, housefly, lesser mealworm and mealworm. DNA from ten insect species and three meat species was extracted using two different kits, with the DNeasy® mericon® Food Kit providing superior and more consistent results.

After performing multiple primer designs and optimizations, a five-primer set was developed that allowed for the successful differentiation of melt curves for the insect species. Initially, melt curves appeared in three clusters: black soldier fly and silkworm; mealworm, crazy red cricket and housefly; tropical house cricket and house cricket. The key challenge was to resolve these melt curve clusters, particularly between species with similar DNA sequences, such as black soldier fly and silkworm, and mealworm and housefly. The method was further applied by analyzing commercially available feed samples, where insect DNA was successfully detected in twelve samples where the presence of insect content was stated. However, the feed sample with mixed insect DNA from black soldier fly and mealworm displayed challenges in identifying the species present, suggesting that the method requires further refinement for mixed samples analysis.

The applicability of the PCR-HRM method was also demonstrated using DNA extracts obtained with a CTAB extraction method, and the results confirmed that this approach could provide reproducible and distinct melt curves for various insect species. Nevertheless, additional work is needed to improve its robustness in complex, highly processed products and to improve the repeatability for species such as house cricket, whose melt profiles exhibited more variability in food samples.

To conclude, PCR-HRM analysis shows great potential as a rapid, simple, and cost-effective tool for feed authenticity testing. With further optimization, it could effectively replace more complex DNA-based techniques, providing dependable detection and differentiation of legally permitted insect species in feed products.

7.2 Zusammenfassung

Die PCR-HRM-Analyse ist ein leistungsfähiges Verfahren für das Screening von Lebensmittelverfälschungen, da sie DNA-Sequenzvariationen schnell nachweisen kann, ohne dass eine nachfolgende Verarbeitung erforderlich ist. Sie eignet sich daher hervorragend für die Differenzierung von Spezies in komplexen Lebensmittelmatrizes und gewährleistet die Authentizität und Qualität von Lebensmitteln. Ziel dieser Masterarbeit war die Entwicklung einer PCR-HRM-Methode zur Unterscheidung von Insektenarten, die in der EU als Futtermittel zugelassen sind: Soldatenfliege, Seidenraupe, Heimchen, Kurzflügelgrille, Krullfliege, Buffalowurm und Mehlwurm. Die DNA von zehn Insektenarten und drei Fleischsorten wurde mit zwei verschiedenen Kits extrahiert, wobei das DNeasy® mericon® Food Kit mehr konsistente Ergebnisse lieferte.

Durch Designs von mehreren Primern und Optimierungen wurde ein Set von fünf Primern festgelegt, das eine erfolgreiche Differenzierung der der Schmelzkurven der Insektenarten ermöglichte. Anfangs erschienen die Schmelzkurven in drei Clustern: Soldatenfliege und Seidenraupe; Mehlwurm, Steppengrille und Krullfliege; Kurzflügelgrille und Heimchen. Die größte Herausforderung war die Auflösung von diesen Schmelzkurven-Clustern, insbesondere zwischen Arten mit ähnlichen DNA Sequenzen wie der Soldatenfliege und der Seidenraupe sowie dem Mehlwurm und der Krullfliege. Die Methode wurde weiter validiert, indem Futtermittelproben analysiert wurden, wobei in zwölf Proben erfolgreich Insekten DNA nachgewiesen werden konnte. Insbesondere die Futtermittelprobe mit gemischter Insekten DNA aus Soldatenfliege und Mehlwurm zeigte Schwierigkeiten bei der Identifizierung der enthaltenen Insektenart, was darauf hindeutet, dass die Methode für die Analyse gemischter Proben weiter verfeinert werden muss.

Die Anwendbarkeit der PCR-HRM-Methode wurde anhand von DNA-Extrakten demonstriert, die mit einer CTAB-Extraktionsmethode gewonnen wurden, und die Ergebnisse bestätigten, dass dieser Ansatz reproduzierbare und unterscheidbare Schmelzkurven für verschiedene Insektenarten liefern kann. Es sind jedoch weitere Experimente erforderlich, um die Robustheit der Methode in komplexen, stark verarbeiteten Produkten zu verbessern und die Wiederholbarkeit für Spezies wie Heimchen zu verbessern, dessen Schmelzprofile eine größere Variabilität in Lebensmittelproben aufwiesen.

Letztendlich zeigt die PCR-HRM-Analyse ein großes Potenzial als schnelles, einfaches und kosteneffizientes Instrument für die Authentizitätsprüfung von Futtermitteln. Bei weiterer Optimierung könnte sie komplexere DNA-basierte Verfahren effektiv ersetzen und einen zuverlässigen Nachweis und eine Differenzierung von zugelassenen Insektenarten in Futtermitteln ermöglichen.

7.3 DNA concentration measurements of DNA extracts

Table 18: List of all insect DNA extracts obtained with NucleoSpin® Plant II Kit, their incubation conditions, sample weight and DNA concentrations.

Species	Incubation time	Sample weight [g]	DNA concentration [ng/µL]
Lesser mealworm	30 min	0.1630	30.5
Mealworm	30 min	0.1555	6.13
Housefly	30 min	0.1537	27.7
Black soldier fly	30 min	0.1467	50.3
Silkworm	30 min	0.1510	1.55
House cricket	30 min	0.1606	1.75
Tropical house cricket	30 min	0.1522	41.8
Crazy red cricket	30 min	0.1544	1.99
Cockroach	30 min	0.1566	1.06
Fruit fly	30 min	0.1521	7.75
Lesser mealworm	90 min	0.1530	23.9
Mealworm	90 min	0.1531	6.86
Housefly	90 min	0.1555	70.0
Black soldier fly	90 min	0.1418	51.1
Silkworm	90 min	0.1575	too low
House cricket	90 min	0.1595	1.65
Tropical house cricket	90 min	0.1454	103
Crazy red cricket	90 min	0.1581	1.94
Cockroach	90 min	0.1636	too low
Fruit fly	90 min	0.1500	15.2
Lesser mealworm	overnight	0.1567	33.1
Mealworm	overnight	0.1533	13.2
Black soldier fly	overnight	0.1462	89
Silkworm	overnight	0.1632	18
House cricket	overnight	0.1500	4.16
Tropical house cricket	overnight	0.1570	22.9
Crazy red cricket	overnight	0.1577	5.96
Cockroach	overnight	0.1595	4.95
Fruit fly	overnight	0.1524	14.1

Table 19: List of all insect DNA extracts obtained with DNeasy® mericon® Food Kit, their incubation conditions, sample weight and DNA concentrations.

Species	Incubation time	Sample weight [g]	DNA concentration [ng/µL]
Lesser mealworm	30 min	0.2013	59.7
Mealworm	30 min	0.2090	41.3
Housefly	30 min	0.2007	46.7
Black soldier fly	30 min	0.2048	46.3
Silkworm	30 min	0.2100	17.4

House cricket	30 min	0.2152	1.61
Tropical house cricket	30 min	0.2114	14.6
Crazy red cricket	30 min	0.1931	3.42
Cockroach	30 min	0.2054	4.74
Fruit fly	30 min	0.2112	31.4

Table 20: List of all meat DNA extracts, the extraction kit, their incubation conditions, sample weight and DNA concentrations.

Species	DNA extraction kit	Incubation time	Sample weight [g]	DNA concentration [ng/µL]
Pork	NucleoSpin® Plant II Kit	30 min	0.1587	14.6
Beef	NucleoSpin® Plant II Kit	30 min	0.1676	187.0
Lamb	NucleoSpin® Plant II Kit	30 min	0.1574	23.9
Pork	DNeasy® mericon® Food Kit	30 min	0.1958	12.8
Beef	DNeasy® mericon® Food Kit	30 min	0.2030	63.0
Lamb	DNeasy® mericon® Food Kit	30 min	0.2094	28.4

Table 21: List of all feed DNA extracts obtained with DNeasy® mericon® Food Kit, their incubation conditions, sample weight and DNA concentrations.

Feed sample	Incubation time	Sample weight [g]	DNA concentration [ng/µL]
Nestlingfutter	30 min	0.2075	32.9
Meisenknödel	30 min	0.2148	35.7
Insect hearts	30 min	0.2098	25.5
Insect sticks	30 min	0.2027	11.7
Erdnussbutter	30 min	0.2014	38.9
Sensitive Mini	30 min	0.2140	4.19
Bugfutter Insekten	30 min	0.2036	4.02
Sensitive Adult	30 min	0.1940	19.7
Insekt mit Hühnchen	30 min	0.2106	21.1
Eiweiß Cocktail	30 min	0.2028	113
Insektenfutter	30 min	0.2040	38.1
Sensible Snack	30 min	0.2115	12.5
Chicko Huhn	30 min	0.1933	108
Kulinarischer Hirsch	30 min	0.2058	22.9
Hühnerherzen	30 min	0.1998	352

7.4 DNA sequences for primer design

Table 22: DNA sequences, obtained with NGS in AGES, employed for the design of all primer pairs in the thesis.

Species	English name	DNA-barcode
Gryllodes sigillatus	tropical house cricket	TATCTTTATCTTAATTCCATAATACAGGATCTATTATCCAATCAT CATAGTCCAATTTAAAGAAGTTTTTTTATTCTCTCGTCACCCCA ACCAAATACAATAAATTAATATTCAAGTCATAATACTATCCTTAA TATATTAATCCAATGTATAAAGA
Hermietia illucens	black soldier fly	TAACTTAAATTACTAATCATTACTAATGGATCAATTATTCATTAA TTTATGTTTAATTTATATAAGAGTTTATTAAATCTTATTATCACCC CAATAAAATAATCCTTTATATAAAATTTTAAATATTCTTTATAAAC TTAATATATCAATTATCAAGA
Drosophila	fruit fly	TAACTTAAATTTTTAATCATTATTAATGGATCAATTATTCATAAAT TAATGAATTATTACAATTAAAAGTTTTTTAAATTTTAATACCACC CCAATAAAATATTTTATTTTATTATAATAAAATTAATCTTTACAAT TAAAATAAATAAAATATAAAGA
Bombyx mori	silkworm	TAATTTTTTCTTTTAATCAATAAAATTGGATCATTTAATCACTTAT TAATGTAAACTATTAAAAAAAGTTAATTAAATTTTTTTATCACCC CAACAAAATAATTTAAAATTATAAAAATATAAAAATAAAAATATTT ACAAAATAAATTATAAAAC
Musca domestica	housefly	TAACTTAAATTTTTAATCGTTAATAACGGATCAATTATTCATAAA TTAATGATTTAATTAATTAAAAGTTTTTTAAATTTTAATATCACCC CAATAAAATATTATCAACAATCACAACAAAAAAAATCCACAAAAT TGTAATTATCTAAATATAAAGA
Tenebrio molitor	mealworm	TAATTTAATCTTAAAATCAATAAAAAGGATCATAAACTCAAAAAT AAATGTAAAAATAAAAGAAAAGTTAAATAAATTTTCTAACCGCC CCAGCAAAATACCAAAACAAAAAATCTAAATTAAAAACATAAAA TAAATAAATTATTATAATATTAAAC
Gryllus locorojo	crazy red cricket	TATCTTTATCTTCATTCCATAATAATAGGATCAATTAACCAATCA TCATAGTAAAATATAAAGAAGTTAAATTATTCCCTTGTCACCCC AACAAAACATTAAATAATAAAACATATTAAATACTCCTAAACATA CATTTTAATAAATTAATATTAAGA
Logusta migratoria	migratory locust	TAACTTAATCTTATAATCACAAATTATGGATCAAATAAACATAAA TTAATGATTTTATAATGAAGAGTTTAATTATTCTTCATGTCACCC CAACAAAACAATAAAAATTAACATTAAAAAATAAACTATATAATA AAAATTAATTTATAAATGTAAAGC

7.5 List of utensils

7.5.1 Chemicals and kits

Agarose High Resolution	*Carl Roth GmbH + Co. KG*
Boric acid	*Merck*
CoralLoad Concentrate 10x	*Qiagen*
Chloroform	*VWR*
DNA-ExitusPlus™	*IF PanReac AppliChem ITW Reagents*
DNeasy® mericon® Food Kit	*Qiagen*
Ethylendiaminetetraacetic acid (EDTA)	*Merck*
EvaGreen Dye 20x	*Qiagen*
GelRed Nucleic Acid Gel Stain	*Biotium*
Low Molecular Weight DNA Ladder	*New England BioLabs GmbH*
2x Type-It® HRM PCR Kit	*Qiagen*
Polyvinylpyrrolidon	*Sigma-Aldrich*
Primers	*Merck, Sigma-Aldrich*
2x PyroMark PCR Master Mix	*Qiagen*
NucleoSpin® Plant II Kit	*Macherey-Nagel*
RNase A (Ribonuclease A) solution	*Macherey-Nagel*
ROTI®Load DNA-orange 1 (with Glycerol)	*Carl Roth GmbH + Co. KG*
Proteinase K solution	*Macherey-Nagel*
RNase free water	*Qiagen*
Qubit™ 1x ds DNA broad range Assay Kit	*Invitrogen Thermo Fisher Scientific*
Tris(hydroxymethyl)aminomethane (Tris)	*Sigma-Aldrich*

7.5.2 Consumables

Biosphere® Filter Tips: 0.5-20 µL, 2-20 µL, 20-200 µL, 100-1000 µL, 200 µL	*Sarstedt*
MicroAmp™ Optical 8-Tube Strip	*Applied Biosystems by Thermo Fisher Scientific*
Qubit™ Assay vessel	*Invitrogen Thermo Fisher Scientific*
Strip tubes and lids, 100 µL	*Qiagen*
Falcon tubes, 50 mL	*Sarstedt*
Eppendorf tubes: 1.5 mL, 2.0 mL, 5.0 mL	

7.5.3 Equipment

Centrifuge	Mini Spin Plus	*Eppendorf*
Centrifuge	Galaxy mini	*VWR*
Centrifuge	Mini Star Silverline	*VWR*
Electrophoresis cell	Sub-Cell GT Gel Caster	*Bio-Rad Laboratories*
Fluorimeter	QubitTM 4.0 Fluorimeter	*Thermo Fisher Scientific*
Gel comb	Sub-Cell® GT MP comb	*Bio-Rad Laboratories*
	26-well and 30-well	
PCR Working Station	PCR Workstation	*VWR*
Pipettes	0.5-10 µL	*Eppendorf*
	2-20 µL	
	20- 200 µL	
	100- 1000 µL	
Power Supply	PowerPac HV Power Supply	*Bio-Rad Laboratories*
Scalpel		*Braun™*
Thermal shaker	Thermomixer comfort	*Eppendorf*
Thermal cycler	Rotor Gene Q Series	*Qiagen*
Thermal cycler	QuantStudio 5	*Thermo Fisher Scientific*
		Herolab
Transilluminator	UVT-20 M	*VELP Scientifica*
Vortex Mixer	Classic Advanced	*Janke & Kunkel GmbH & Co KG*
Vortex Mixer	VF2	*VWR*
Vortex Mixer	VV3	

7.5.4 Software

Molecular Evolutionary Genetics Analysis (MEGA) 11	
Microsoft Office 365 (Word, Excel, Powerpoint)	*Microsoft*
QuantStudio 5 Design & Analysis Software 1.4.3	*Thermo Fisher Scientific*
PyroMark Assay Design 2.0	

7.5.5 Webservers

OligoAnalyzer	https://eu.idtdna.com/calc/analyzer
OligoCalc	http://biotools.nubic.northwestern.edu/OligoCalc.html
RNAfold WebServer	http://rna.tbi.univie.ac.at/cgibin/RNAWebSuite/RNAfold.cgi

7.6 List of abbreviations

µL	microliter
µM	micromolar
A	adenine
ACN	Alert and Cooperation Network
AGES	Austrian Agency for Health and Food Safety
AT	adenine-thymine
bp	base pairs
C	cytosine
COI	cytochrome c oxidase subunit I
Ct	cycle treshold
DNA	deoxyribonucleic acid
dsDNA	double stranded DNA
dATP	adenosine triphosphate
dCTP	cytidine triphosphate
dGTP	guanosine triphosphate
dNTPs	deoxyribonucleoside triphosphates
DRI	daily recommended intake
dTTP	thymidine triphosphate
EC	European Commission
EFSA	European Food Safety Agency
EU	European Union
FAD	Food and Agriculture Organization

fw	forward
g	gram
G	guanine
GC	guanine-cytosine
HRM	High Resolution Melting
mg	milligrams
mM	millimolar
ng	nanograms
NGS	Next Generation Sequencing
nm	Nanometers
NC	No Template Control
PAPs	processed animal proteins
PCR	Polymerase Chain Reaction
pg	picograms
PUFAs	polyunsaturated fatty acids
qPCR	quantitative PCR (real-time PCR)
rcf	relative centrifugal force
rev	reverse
rpm	compute revolutions per minute
RNA	ribonucleic acid
SOFI	State of Food Security and Nutrition in the World
T	thymine
Tm	melt temperature
UV	ultraviolet
WHO	World Health Organization

7.7 List of tables

7.8 List of figures

8. References

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