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(Anti)Nutritional, Techno-functional and Antimicrobial Properties of the Bush Cricket, *Ruspolia differens*

A comparative nutritional analysis with locusts and crickets

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Supervisor:
Prof. Dr. Jozef Vanden Broeck

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Dissertation presented in partial fulfillment
of the requirements for the degree of
Doctor of Science (Ph.D.) Biology

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&

baby

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ABSTRACT

The continuously growing human population and increasing food insecurity in sub-Saharan Africa stimulate the demand for affordable alternative and sustainable protein sources. Insects are among the most successful organisms on the planet. They have long been a traditional and cultural part of many African diets and are currently promoted as a mainstream source of energy and nutrients to tackle different forms of malnutrition.

Of the several insect species consumed in sub-Saharan Africa, *Ruspolia differens* (Serville, 1983) (Orthoptera, Tettigoniidae) stands out. In East Africa, large volumes of this swarming, cone-headed bush cricket are harvested from the wild. However, the availability of wild-harvested insects is seasonal, limiting their continuous inclusion in diets. In this context, this thesis focuses on the (anti)nutrient content, sustainable rearing, and functional properties of *R. differens* to enhance food security. Therefore, I investigated the nutritional, anti-nutritional, and techno-functional properties of *R. differens* after processing. These properties were further compared with crickets and locusts, edible species belonging to the same insect order (Orthoptera). Moreover, I developed a novel artificial diet and conducted experiments to optimize the rearing of *R. differens* in captivity. As a potential non-food application, I also explored the antimicrobial potential of its extracts.

Nutritional profiling after oven- and freeze-drying indicated that *R. differens* (on a dry matter basis) has a high lipid and protein content. Also, the presence of micronutrients, such as iron, zinc, manganese, and copper contributes to its nutritional quality. In terms of anti-nutrients, oven-drying, when compared to freeze-drying, resulted in significantly higher phytate contents in whole-insect and defatted flours obtained from *R. differens*, while the drying methods did not significantly affect the content of tannins.

To optimize rearing conditions, several biological traits and fitness parameters were assessed for *R. differens* exposed to two distinct light regimes (*i.e.*, <1h *versus* 12h photoperiods). The shortest developmental time (58 ± 3 days) and the highest survival rate (84 %) from hatchlings to adults were recorded when the insects were reared almost entirely in the dark (photoperiod

<1h). Also, their average final weight was significantly higher (0.43 ± 0.03 g) when compared to insects exposed to a 12h photoperiod. The occurrence of cannibalism was identified as an important challenge for mass-rearing of *R. differens*. This species also feeds on other insect preys, an observation that may contribute to the development of rearing strategies in the future.

Additionally, techno-functional properties of flours (water binding, foaming capacity and stability, and fat absorption capacity) and oils (acid value, iodine, saponification, and peroxide values) derived from processed (dried and defatted) insects were evaluated. Non-defatted or defatted insect flours could be included as ingredients in various food products to improve their functionality.

Abstract (Nederlands)

De continu groeiende menselijke bevolking en de toenemende voedselonzekerheid in sub-Sahara Afrika stimuleren de vraag naar betaalbare, alternatieve en duurzame eiwitbronnen. Insecten behoren tot de meest succesvolle organismen op aarde. Ze zijn lange tijd een traditionele voedselbron geweest in vele Afrikaanse culturen en worden momenteel gepromoot om verschillende vormen van ondervoeding aan te pakken.

Onder de insectensoorten die ten zuiden van de Sahara worden geconsumeerd neemt *Ruspolia differens* (Serville, 1983) (Orthoptera, Tettigoniidae) een vooraanstaande plaats in. In Oost-Afrika worden grote aantallen van deze zwermvormende sabelsprinkhaan uit het wild geoogst. De beschikbaarheid van deze uit het wild geoogste insecten is echter seizoensgebonden, waardoor hun continu gebruik in de voeding wordt beperkt. In deze context richt dit proefschrift zich op het bestuderen van de gehaltes aan (anti)nutriënten, de duurzame kweek en de functionele eigenschappen van *R. differens* om de voedselzekerheid te vergroten. Bijgevolg heb ik de nutritionele, anti-nutritionele en techno-functionele eigenschappen van *R. differens* na verwerking onderzocht. Deze eigenschappen werden verder ook vergeleken met krekels en sprinkhanen, eetbare soorten die behoren tot dezelfde insectenorde (Orthoptera). Bovendien heb ik een nieuw kunstmatig dieet ontwikkeld en experimenten uitgevoerd om de kweek van *R. differens* in gevangenschap te optimaliseren. Als mogelijke non-food toepassing heb ik tevens de antimicrobiële activiteit van extracten onderzocht.

Nutritionele analyse gaf aan dat *R. differens* (op basis van droge stof) na oven- en vriesdrogen hoge lipide- en eiwitgehaltes bezit. Ook de aanwezigheid van micronutriënten, zoals ijzer, zink, mangaan en koper, draagt bij aan de voedingskwaliteit. Bij analyse van anti-nutriënten aanwezig in ontvette en niet-ontvette poeders afgeleid van *R. differens* na behandeling in een droogoven werden, in vergelijking met vriesdrogen, significant hogere fytaatgehaltes vastgesteld, terwijl deze droogmethoden het gehalte aan tannines niet significant beïnvloedden.

Om de kweekomstandigheden te optimaliseren werden verschillende biologische eigenschappen en fitnessparameters beoordeeld voor *R. differens* blootgesteld aan twee verschillende belichtingsregimes (<1h *versus* 12h). De kortste ontwikkelingstijd van jonge nymf tot adult (58 ± 3 dagen) en het hoogste overlevingspercentage (84 %) werden geregistreerd

wanneer de insecten bijna volledig in het donker werden gehouden (fotoperiode <1h). Ook was hun gemiddelde eindgewicht significant hoger (0,43 ± 0,03 g) in vergelijking met de insecten die werden blootgesteld aan een fotoperiode van 12h. Kannibalisme werd bovendien geïdentificeerd als een belangrijke uitdaging voor de massakweek van *R. differens*. Deze soort voedt zich ook met andere prooien, een observatie die kan bijdragen aan het ontwikkelen van toekomstige kweekstrategieën.

Van poeders (waterbinding, schuimcapaciteit en stabiliteit, vetabsorberend vermogen) en oliën (zuur-, jodium-, verzepings-, en peroxidewaarden) afgeleid van verwerkte (gedroogde en ontvette) insecten werden tenslotte ook enkele techno-functionele eigenschappen onderzocht. Niet-ontvette of ontvette insectenpoeders zouden kunnen verwerkt worden als ingrediënten in diverse voedingsproducten om hun functionaliteit te verbeteren.

SUMMARY

The worldwide rise in demand for affordable alternative and sustainable protein sources has led to the FAO's continuous support for edible insects. In Sub-Saharan Africa (SSA), entomophagy centered on harvesting from the wild is already practiced. Still, it is associated with many challenges such as seasonal availability, sustainability issues, pathogen risks, and high perishability of the harvested insects.

Ruspolia differens (R. differens) (Serville) (Orthoptera: Tettigoniidae) is one of the most consumed insects out of the 500+ food insects described in Africa, especially in the Eastern African region, where it is commonly known as *Nsenene*. This katydid (also designated as "bush cricket" and previously known as "long-horned grasshopper") species is among the African edible insects that have been recommended as a food system that can help in alleviating food and nutrition insecurity in SSA. It is highly nutritious and is a crucial source of livelihood for many households. The species is mainly harvested seasonally from the wild, where its discontinuous availability hampers its supply.

The consumption of this locally occurring bush cricket, *R. differens*, is a significant part of the food culture in several regions of Kenya and other countries of East Africa and constitutes 5-10 % of the protein intake of the rural and urban population. For many households, commerce in edible insects is a significant source of income and considerably contributes to improvements in livelihood. Currently, *R. differens* are harvested in the wild in a non-sustainable and destructive manner. There is little if any knowledge in East Africa on optimizing harvesting practices to sustain the insect populations. Although rearing technologies for other Orthopterans (*e.g.*, crickets) are relatively advanced in Southeast Asia, equivalent knowledge for this African katydid species is lacking despite its popularity among locals. Alongside *Ruspolia differens* three other insects of the order Orthoptera (*Gryllus bimaculatus, Locusta migratoria,* and *Schistocerca gregaria*,) which are readily available and consumed in several African countries, are equally being introduced to fortify or complement staple foods. One of the challenges limiting the continued inclusion of edible insects in diets in Kenya and East Africa is that the availability of

these wild-harvested insects is seasonal. Additionally, the numerous traditional and rudimentary processing methods utilized in Africa to accomplish food security, lessen poisoning, and improve the nutritional quality of insects are currently still being practiced for insect consumption. There is a need to shift from these practices if the quality of the food must improve.

This thesis aims to:

(i) determine the effect of the drying method on the postharvest nutritional composition of *R. differens*; (ii) compare the nutritional quality of *R. differens* to that of other edible orthopteran insects; (iii) investigate the antinutrient attributes of *R differens* and other edible orthopteran insects; (iv) evaluate the effect of photoperiod as a rearing parameter on the biological fitness of lab-reared *R. differens* fed on an artificial diet aiming at mass production; (v) evaluate the phenomenon of cannibalism in lab-reared adult *R. differens*; (vi) assess the impact of processing (drying and defatting) on the physicochemical (techno-functional) properties of these insects' flours and oils; and finally, as a non-food application, (vii) explore the antibacterial (therapeutic) properties of *R. differens* using organic solvents as extractant.

Nutritional profiling studies indicated that *R. differens* (on a dry matter basis) has a high lipid content of 36 %, as well as significant protein levels ranging between 33 % and 46 % dry matter. Oleic acid (44 %) and palmitic acid (28 %) were the two dominant fatty acids. In comparison, the presence of arachidonic acid (0.6 %) and docosahexaenoic acid (0.21 %) suggests that *R. differens* is also a source of polyunsaturated fatty acids. The observed amino acid profile showed that all essential amino acids were present. The trace elements iron (217–220 mg/100 g), zinc (14.2–14.6 mg/100 g), manganese (7.4–8.3 mg/100 g), and copper (1.66 mg/100 g) were present, which suggests that inclusion of these katydids in human diets may aid in combatting micronutrient deficiencies. This implies that *R. differens* can contribute up to 44.6 - 88, 0.25 - 32.4, and 1 - 2.2-folds of recommended dietary allowance (RDAs) of protein, iron, and zinc, respectively, for all age groups. Oven-drying *R. differens* delivered the same nutritional quality as freeze-drying. Hence, both drying approaches could be satisfactorily used in insect-derived food products without noticeable nutritional changes.

In this thesis, the nutritional profiles of three widely consumed orthopterans, *Gryllus*

bimaculatus, Locusta migratoria, and *Schistocerca gregaria*, were evaluated after blanching and oven-drying. All three insect species had high protein (65.3, 54.2, and 61.4 % on a dry matter (DM) basis for *G. bimaculatus, L. migratoria,* and *S. gregaria*, respectively) and fat contents. Oleic (22.9–40.8 %) and palmitic acid (26.1–43.0 %) were the two most abundant fatty acids. All essential amino acids (in mg/100 g protein) were present, with glutamic acid (120–131), alanine (90.2–123), and leucine (82.3–84.6) being the most abundant. The levels (in mg/100 g dry matter) of the mineral elements potassium (796–1309) and phosphorus (697–968) were elevated for an animal protein source. Also, high concentrations (in mg/100 g dry matter) of the trace minerals iron (4.60–7.31), zinc (12.7–24.9), manganese (0.40–7.15), and copper (1.20–4.86) were detected in the samples. The vitamin B_{12} contents were higher than in other animal protein products (0.22–1.35 g/100 g dry matter). In terms of antinutrients (on a dry matter basis), oven-drying resulted in significantly higher phytate contents in whole insect flour and defatted insect flour obtained from *R. differens* (0.21 mg/100 g, 0.22 mg/100 g, respectively). On the other hand, drying methods did not significantly affect the *R. differens* content of tannins (mg/ 100 g), *i.e.*, 10.0 ± 0.41 and 7.8 ± 0.29 for the oven- and freeze-drying, respectively. However, the highest concentration was observed in oven-dried whole insect flour from *G. bimaculatus* (14.9 mg/100 g). Drying methods did not significantly affect the oxalate content (mg/100 g) of *R. differens* in either the full-fat flours (0.39 ± 0.01 and 0.39 ± 0.02) and defatted flours (0.26 ± 0.01 and 0.33 ± 0.01) for oven-dried and freeze-dried samples, respectively. All the analyzed insects were found to contain low levels of antinutrients. These values are much lower than corresponding ones from some common foods of plant origin. Thus, using these insects as food or food ingredients can be encouraged without fear of toxicity emanating from antinutrients associated with them.

Attempts to rear *Ruspolia differens* in the lab were carried out in this thesis. Here, biological fitness parameters were assessed using a novel artificial diet, as well as multiple performance traits, for *R. differens* reared at two light regimes, near-complete dark (< 1 h/day light) and 50 % light (~12 h/day light), from newly hatched nymphs to death of the adults. The proximate composition (on a dry matter basis) of this novel artificial diet was: 53.5 % carbohydrates, 21.0 % crude protein, 7.0 % ash, 5.0 % fat, and 4.5 % crude fiber. All essential amino acids and

mineral elements were also present. The highest nymphal survival rate (84 %) to the adult stage was recorded with the dark-reared insects. The mean final weight was significantly higher in the dark-reared insects (0.43 ± 0.03 g) with shorter developmental times (58 ± 2.9 days). For the light-reared insects, it took on average 8-9 molts to reach adulthood, instead of 6-7 molts for those reared in the dark. Adult longevity of the dark-reared insects was also significantly longer (37 ± 3.7 days) compared to the light-reared ones (10 ± 3 days). These findings strongly support the nocturnal behavior of these bush crickets and suggest that placing them in a predominantly dark environment for mass rearing would seem a profitable venture, as fewer energy demands in terms of lighting, as well as shorter developmental times, would be achieved. These conditions, in addition to the artificial diet, when fully optimized, will facilitate automation and reduce labor for feeding the insects in mass-rearing programs.

In addition to assessing photoperiod as a rearing parameter, measures aimed at reducing cannibalism by providing alternative prey to reared *R. differens* were evaluated using the new diet as a control. Two sets of experiments were set up: In the no-choice set-up, live or dead second instar larvae, each of *Hermetia illucens*, *Chilo partellus*, *Bactrocera invadens,* and *Schistocerca gregaria,* were administered separately to both male and female cages for six successive days. In the choice set-up, a mixture of twenty living prey was fed for twelve consecutive days. The control diets that included both the artificial diet only and the artificial diet plus corn leaves were provided for six days consecutively. The percentage of cannibalism was significantly reduced in both male and female *R. differens* cages where prey had been administered compared to the control diets. There were indications of hunting behavior, as the katydids tended to feed more on living than on dead insect prey. Therefore, the addition of live insect prey may reduce the prevalence of cannibalism, thus preventing colony collapse due to cannibalism and providing a safe means to eliminate insect pests, which could serve as prey.

This thesis also analyzed the influence of oven- and freeze-drying methods on the techno-functionality (water binding, foaming capacity and stability, and fat absorption capacity). Also, the antinutrient (phytates, oxalates, tannins) composition of processed insect flours from three edible orthopteran species, *Ruspolia differens*, *Gryllus bimaculatus,* and *Schistocerca gregaria* was evaluated. The acid value, iodine, saponification, and peroxide values of the extracted oils

were assessed. Our findings demonstrate that both non-defatted and defatted insect flours could serve as a suitable alternative nutrient-rich source of ingredients for inclusion in diets and other food matrices to improve their functionality.

To assess the local, traditional claims that *R. differens* may also be utilized to treat specific ailments, the antimicrobial properties of whole *R. differens* extracts were evaluated. Freeze-dried, finely ground whole flour of *R. differens* was extracted at ambient temperature with different solvents successively (hexane, ethyl acetate, methanol, and water) and tested for antimicrobial activity using the microdilution broth method against *Escherichia coli, Staphylococcus aureus,* and *Candida albicans.*

The hexane extract effectively inhibited the growth of *S. aureus* at a concentration of 0.01 % (v/v). The peaks with confirmed anti-*Staphylococci* properties suggested that the antibacterial activity might be due to a synergistic action of several fatty acids, including linoleic and linolenic acid, amongst others. The results provide evidence for the occurrence in *R. differens* extracts of a broad-spectrum antibacterial activity, which perhaps may explain why these katydids are utilized in African traditional medicine.

The various results provided in this thesis either indicate leads and lay foundations or offer ample knowledge about: (i) the influence of drying methods on the nutritional composition and the anti-nutritional composition of *R. differens*; (ii) a suitable artificial diet for rearing *R. differens*; (iii) the influence of light on its growth and development and the extent of cannibalism in *R. differens*; (iv) attempts to curb this phenomenon in mass rearing endeavors; (v) impact of drying and defatting on the techno-functional properties of some orthopterans and (vi) the antimicrobial properties present in hexane extracts of *R. differens.*

This knowledge can be used to optimize the processing of edible insect flours, validate protein quantity, and improve their quality and functionality as food ingredients. Additionally, this thesis offers emerging insight into the immense diversity of unique chemicals present in edible insects, beckoning more studies that could potentially arouse more interest in insect-based drug discovery and development. Finally, this thesis also lays the foundation for further investigations into designing and implementing strategies to optimize the diet and rearing conditions of *R. differens* and other edible insect species for mass production and commercialization.

LIST OF ABBREVIATIONS

AA	Amino Acid
AAR	Amino acid residue
ANOVA	Analysis of Variance
a_w	Water Activity
BSF	Black Soldier Fly
CAR/PDMS	Carboxen Polydimethylsiloxane
CFU	Colony Forming Unit
DG	Dichloran Glycerol Agar
DMDS	Dimethyl Disulphide
DMTS	Dimethyl Trisulphide
DNA	Deoxyribose Nucleic Acid
EAA	Essential Amino Acids
EU	European Union
EFA	Essential Fatty Acids
EFSA	European Food Safety Authority
FAO	Food and Agricultural Organization of the United Nations
FASFC	Federal Agency for the Safety of the Food Chain (Belgium)
FAMEs	Fatty Acid Methyl Esters
FCR	Feed Conversion Ratio
FFA	Free Fatty Acids
GC	Gas Chromatography
ICIPE	International Center for Insect Physiology and Ecology
ICP-OES	Inductively Coupled Plasma-Optical Emission Spectrometry
IPIFF	International Platform of Insects for Food and Feed
JKUAT	Jomo Kenyatta University of Agriculture and Technology
JOOUST	Jaramogi Oginga Odinga University of Science and Technology
KBS	Kenya Bureau of Standards
LOD	Limit of detection
LOQ	Limit of Quantification
MEGL	Methionine Gamma Lyase
MPa	Mega Pascals
MRS	Man Ragosa Sharpe agar
MS	Mass Spectrometry
MUFA	Mono-unsaturated fatty acid
NCBI	National Centre for Biotechnology Information
NFE	Nitrogen Free Extract
NGS	Next Generation Sequencing
NIST	National Institute of Standards and Technology
NT	Total Nitrogen
OECD	Organisation for Economic Co-operation and Development
OTU	Operational Taxonomic Unit

PCA	Plate Count Agar
PCR	Polymerase Chain Reaction
PUFA	Poly-unsaturated fatty acid
rRNA	Ribosomal Ribonucleic Acid
RSA	Radical Scavenging Activity
SD	Standard Deviation
SPSS	Statistical Package for Social Scientists
UN	United Nations
UPLC	Ultra-Performance Liquid Chromatography
VLIR-UOS	Vlaamse Interuniversitaire Raad Universitaire Ontwikkelingssamenwerking
VRBGA	Violet Red Glucose Bile Agar
WHO	World Health Organization

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Chapter ONE

General Introduction

Sections (written By Forkwa Fombong) of this chapter have been submitted for publication as:

*Tanga C. M, Kababu M., Nakimbugwe D., Egonyu J. P., Ndimubandi N., Kinyuru J., Holger K., Rutaro K., **Fombong T.F**., Matojo N., Ng'an'ga, J.K., Kipkoech C., Mbeche R., Mutibha L., Kidoido M., Ssepuuya G., Subramanian S. and Ekesi S. (2021) Ruspolia differens Serville as a delicacy in Sub-Saharan Africa: Nutritional status, safety, production technologies, marketing, and policy implications -A review. Food Control.*

1.1 Insects

The phylum of Arthropoda comprises more than 85 % of all animal species on Earth. Estimates put the total number of arthropod species up to 30 million, which is way above the total number of all other animals combined (Chapman et al., 2013). For this reason, arthropods are often referred to as the most successful animals on planet Earth. Insects form the most significant class (Insecta) of this phylum, with more than one million species on record. The class is represented in almost every terrestrial and freshwater habitat, and its members are extraordinarily diverse in their structures and food sources (Gullan & Cranston 2005).

Insects have principally three life stages: egg, juvenile, and adult. The change in the form that most insects undergo as they develop from juvenile to adult is called metamorphosis. Depending on the life cycle strategy during post-embryonic development, a distinction is made between two of the most dominant categories. On the one hand are holometabolous insects, which display a complete metamorphosis. Their life cycle occurs with egg, larva, pupa, and adult stages. Examples of holometabolous insect orders are the Lepidoptera (moths and butterflies), Coleoptera (beetles), and Diptera (flies). Hemimetabolous insects, on the other hand, undergo an incomplete or gradual metamorphosis, typically with successive immature nymphal stages that progressively resemble the adult. Examples of hemimetabolous insect orders are **Orthoptera** (locusts, grasshoppers, and crickets), Blattodea (cockroaches), and Isoptera (termites) (Gullan & Cranston, 2005; Chapman et al., 2013).

1.2 The global status of entomophagy

Worldwide approximately 842 million people (12 % of the global population) could not meet their dietary energy requirements from 2010 to 2013 (Van Huis et al., 2013). The vast majority of these (827 million) live in developing regions, where the prevalence of undernourishment was at 14.3 % in 2011–13. Africa remains the continent with the highest occurrence of undernourishment, with Sub-Saharan Africa's (SSA) figures at 24.8 % (Van Huis et al., 2013). SSA is exceptionally susceptible to frequent food crises and famines easily triggered by droughts,

floods, pest outbreaks, economic recessions, and rampant conflicts. SSA is the single region where hunger is expected to increase over the next two decades unless drastic measures reverse food insecurity. To effectively respond not just to the rapid population growth but also to other pressing challenges, including climate change and rising volatility of food prices, SSA needs to accelerate its agricultural productivity and efficacy without delay (Fombong et al., 2017).

In this context, the Food and Agricultural Organization of the United Nations (FAO) recommends using sustainable diets with low environmental impact to contribute to food and nutrition security for present and future generations. In the last decade, a high-profile review outlined the importance of insects in assuring food and feed security. Globally, it is estimated that about 1611 species of insects are used as food by at least two billion people (Van Itterbeeck et al., 2022), especially in South America, Africa, and Asia. Of the currently 1611 food insects (consumed worldwide, almost a quarter (500+ species) are from Africa (Morales- Ramos, & Rojas, 2016; Kelemu et al., 2015). Besides being a delicacy for some local communities, the other benefits of consuming insects above other foods cannot be overemphasized. Insects are highly nutritious in quantity and quality and are more environmentally sustainable in production (Van Huis et al., 2013; Zielińska et al., 2015) are harvested from the wild except for a few species of mealworms, crickets, and grasshoppers that are being reared on either a commercial or an experimental scale (Melgar-Lalanne, Hernández-Álvarez, & Salinas-Castro, 2019; Van Huis & Oonincx, 2017). Several insect species are comparable to conventional livestock meat in nutritional content but with the added benefit of converting a far higher feed ratio into high-quality protein for human consumption. This translates into a much better ecological footprint of mass-produced insects than beef and other livestock, with significantly lower greenhouse gas emissions and water requirements. This assertion is arguable as it depends on the insect species in question. Harvesting and cultivation of edible insects would therefore contribute to habitat conservation by using less space, as well as improving food security and livelihoods of the rural poor.

Thus, the demand for alternative sources of animal proteins to curb the global rise in food insecurity has sparked an interest in the utilization of insects as food and feed (van Huis 2015).

This heightened interest in demand for affordable unconventional protein sources had led to the FAO's initial support for edible insects. Recently, the Word bank has come in and is advocation insect farming and hydroponics. (Verner et al., 2021). Therefore, entomophagy promotion as a suitable substitute for protein and other nutrients is growing worldwide (Berger, Bärtsch, Schmidt, Christandl, & Wyss, 2018; Verner et al., 2021).

The *documented* use of insects as food dates over two thousand years ago *(*Dobermann et al., 2017). Nevertheless, for many years, insect consumption has been shunned and regarded as a traditional or cultural practice reserved for a few (Schabel 2010). However, the last couple of years has seen an increase in the demand and utilization of insects as food and feed and also as a source of income and livelihood for many families (Insights 2015; Van Huis 2020).

The EU and North American edible insects market is overshadowed by reared insects, predominantly black soldier flies, cricket, and mealworm species, due to their high nutritional value, easy farming, and processing, as well as increasing demand for incorporation into various food recipes and products (Meticulous Market Research Pvt. Ltd. 2019). However, there is an increasing middle class of insect consumers in Africa, and thus an urge for convenient, nutritious, and high-quality foods will grow the market even higher (Staaz, 2015).

The European Union (EU) is the leading region toward a legal regime that ensures the quality of edible insects produced within and imported into the region. These efforts so far include the EU regulation on novel foods as amended in 2015, *i.e*., (EU) No 2015/2283, a finalized draft to amend regulation 853/2004 to include a section on specific hygiene rules for insects intended for human consumption. Also, a guide on good hygiene practices for EU producers of insects for food and feed (International Platform of Insects for Food and Feed (IPIFF, 2019)). Recently, dried mealworm *Tenebrio molitor* and frozen locust *Locusta migratoria* have been officially approved as food in the EU (Turck et al., 2021). This EU guideline specifies the foodstuff and the applied processing, i.e., dried and frozen in these cases.

In African countries where, according to the FAOLEX database, food policies/ laws/ regulations are available, these are silent about the use of insects as food (FAOLEX, 2019). This implies that the current food policies/ laws/ regulations in Africa can be amended to promote edible insects. Notably, the edible insects' market growth is much higher in countries with a proper legal food

regime that, to a given extent, defines the minimum quality standards of a food (insect) product acceptable on the market. In Africa, these guidelines and regulations are often absent, and where they are available, there is often no competent and functional infrastructure to successfully implement them (Nakimbugwe & Boor, 2010; World Health Organisation [WHO], 2012). Nevertheless, Codex Alimentarius details general food hygiene practices, *i.e.*, CAC/RCP 1-1969 (CAC/RCP, 2003), that can be used to ensure proper hygiene practices in the value chain of wild-harvested and farmed edible insects (Sareen, 2014). Efforts are underway in Uganda and Kenya to develop standards for insects as food and food ingredients under the INSBIZ project, and possibly similar initiatives also exist in many other African countries.

1.3 Malnutrition & Edible insects in Kenya and East Africa

1.3.1 Malnutrition status in East Africa

Child Malnutrition (in all its forms) continues to be a severe health concern in sub–Saharan African (SSA) countries. Amongst them, protein-energy malnutrition and micronutrients deficiencies (especially iron, zinc, and vitamin A) are the most common forms reported (Bain et al., 2013; Akombi et al., 2017; Gassara et al., 2021).

Kenya ranks high amongst the twenty countries that account for up to 80 % of the world's malnourished children, where stunting, wasting, and underweight in children below five years have been estimated at 26 %, 4 %, and 11 %, respectively (Grace et al., 2012; Kimani-Murage et al., 2015; De Vita et al., 2019). One out of three child deaths in Africa are starkly related to protein-energy malnutrition and micronutrient deficiencies (Akombi et al., 2017; Gassara et al., 2021). Child malnutrition alone is responsible for up to 5 % of under-five child mortality, such a real danger to Africa's growth and development. Therefore, urgent, sustainable, and affordable solutions to this colossal menace are needed.

It is currently established by the volume of literature that these malnutrition challenges could be mitigated, at least in part, by exploring the vast diversity of insect species of African origin (Niassy et al., 2018; Weru et al., 2022).

1.3.2 Edible Insect Survey in Kenya and East Africa

Although about 500 insect species are consumed in Africa (van Huis, 2013; Kelemu et al., 2015), in East Africa, the most consumed insects include termites, grasshoppers, bush crickets, and 'true' crickets (Ayieko et al., 2011; 2012; Mbabazi et al., 2012). Termites form an essential part of the food culture within the Lake Victoria region (Defoliart, 1999; van Huis, 2017). Within the winged termites (*Macrotermes spp*), several species exist, including *Macrotermes nigeriensis*, *Macrotermes notalensis*, *Macrotermes subhylinus,* and *Macrotermes bellicosus*. However, the sizeable alate termite (*M. subhylinus*) is consumed chiefly within the Lake Victoria region and tends to emerge during the onset of long rains between March to May and October (Ayieko et al., 2011; Kinyuru et al., 2013). In addition, bush crickets (*i.e.*, katydids) are considered a delicacy by many tribes in East Africa. In particular, *Ruspolia differens* is non-destructive, as it causes no damage to crops and vegetation, which makes it unique from migrating locusts (Kelemu et al., 2015). In addition, *R. differens* exhibits swarming behavior during specific year periods (Bailey & McCrae, 1978). During the swarming seasons, most of the swarms are concentrated on streetlights because these katydids are typically attracted to light. During this period, the local communities arm themselves with baskets, polythene bags, and drums filled with these insects (Kinyuru et al., 2010; Ssepuuya et al., 2016). These edible katydids are usually wild harvested bi-annually during March-May and October-December and consumed as a traditional snack by many tribes in the region (Kinyuru et al., 2010; Mmari et al., 2017; Ssepuuya et al., 2016).

1.4 The case for orthopterans

The order Orthoptera represents a group of insects that include locusts, crickets, katydids, and grasshoppers. Orthopteran species include critical pest species, such as the desert locust*, i.e., Schistocerca gregaria (*Orthoptera*:* Acrididae*),* and the migratory locust*, i.e., Locusta migratoria (*Orthoptera*:* Acrididae). Several orthopteran species are traditionally eaten in Sub-Saharan Africa, including bush crickets*, i.e., Ruspolia differens* (Serville) (Orthoptera: Tettigoniidae), and crickets, *i.e.*, the house cricket *Acheta domesticus* (Orthoptera: Gryllidae) and the field cricket *Gryllus bimaculatus* (Orthoptera: Gryllidae), but not at all in Europe though their use as food or feed has excellent potential (Van Huis 2013).

For several reasons, locusts, grasshoppers, and (bush) crickets are favored for this purpose: they have a low feed conversion ratio, a short generation time, tolerance of high densities, a broad disease tolerance, and are not undergoing diapauses (Hanboonsong et al., 2013; Halloran et al., 2017; Lundy & Parrella 2015). Crickets are among the most widely farmed species for human consumption as food and high-quality protein ingredients for inclusion in livestock feeds in several world regions (Hanboonsong et al., 2013; Ayieko et al., 2016). Thus, crickets are increasingly considered a sustainable and attractive alternative to animal and plant protein sources. Nevertheless, their applicability in large-scale settings needs extensive pilot studies.

Despite the worldwide progress to achieve food security in recent years, Sub-Saharan Africa, including Kenya, is still marred with undernutrition and food shortage caused by several factors, including adverse climatic conditions and the rising cost of animal protein (Dorward 2012; FAO 2013b). As population growth is expected to increase very fast in this region (UN 2015), how to achieve food security remains a crucial question in need of answers. Establishing and improving the insect production sectors could be a sustainable option (Van Huis, 2003; Kelemu et al., 2015; Ayieko et al., 2016). Thus, the establishment of small-to-large-scale insect production sectors and developing the value chain can contribute to food security in East Africa and beyond, given that consumers show an encouraging enthusiasm to purchase insect-based foods. Orthopterans contain significant amounts of protein and other nutrients, which can increase the intake of animal protein and reduce micronutrient deficiency (Ramos-Elorduy, 1997; Christensen et al., 2006; Finke, 2007; Ayieko et al., 2016). Additionally, the production of some insect species generally contributes less to climate change and needs fewer inputs than livestock production (FAO 2013a). Among the edible orthopterans, *Ruspolia differens* is currently getting the most attention in East Africa (Fombong et al., 2021; Nyangena et al., 2020; Cheseto et al., 2020; Ssepuuya et al., 2020).

1.5 The biology and phenology of *R. differens* as a food source in Africa

1.5.1 Introduction

The bush cricket *Ruspolia differens (R. differens)* is arguably the most consumed insect in the Eastern African region, commonly known as *Nsenene* (Bailey & Macrae, 1978; Matojo and Yarro, 2010; Matojo & Njau, 2010). *R. differens* is highly nutritious and is an essential source of livelihood for many households. It is therefore considered a delicacy that was traditionally reserved for men and in-laws and mainly consumed due to respect for traditional culture, high nutrients, and its tastiness served as a 'multipurpose sauce '(Agea et al., 2008; Kinyuru et al., 2010; Mmari et al., 2017). The bush cricket is mainly harvested seasonally from the wild, where its supply is curtailed by its availability (Okia et al., 2017; Agea et al., 2008; Mmari et al., 2017). *R. differens* is highly nutritious and suggested to possess specific medicinal properties (Mmari et al., 2017). *R. differens* ranks high amongst the most traded edible insect species in East Africa, particularly in Uganda, Rwanda, Tanzania, and Burundi, with a very intricate value chain (Okia et al., 2017; Odongo et al., 2018; Agea et al., 2008). It typically begins with wild harvesting and then processing, and in rare instances rearing (Niassey & Van Huis, 2018; Kinyuru & Ndung'u, 2019).

1.5.2 Nomenclature and species description

Since first described in 1838, the species under study has been referred to by more than ten different names or synonyms (Matojo 2017). They include *Conocephaloides differens* (Serville, 1838); *Conocephaloides longipennis* (Redtenbacher, 1891); *Conocephalus albidonervis* (Redtenbacher, 1891); *Conocephalus differens* (Serville, 1838); *Conocephalus exiguus* (Stål, 1876); *Conocephalus lemur* (Redtenbacher, 1891); *Conocephalus longipennis* (Redtenbacher, 1891); *Conocephalus melanostictus* (Karny, 1907); *Conocephalus vicinus* (Walker, 1869); *Homorocoryphus albidonervis*; *Homorocoryphus differens* (Serville,

1838); *Homorocoryphus lemur*; *Homorocoryphus longipennis*; *Homorocoryphus mediotessellatus* (Karny, 1917); *Homorocoryphus melanostictus*; *Xiphidium vicinum* (Morse, 1901).

Such has been the confusion that a sister species, *Ruspolia nitidula*, has been erroneously referred to as *R. differens* (Agea et al., 2008; Ssepuuya et al., 2016; Fombong et al., 2017).

However, the name ***Ruspolia differens*** is accepted by the International Code of Zoological Nomenclature alongside the other recognized synonyms (Matojo 2017). The accepted taxonomic position and nomenclature are as follows:

Kingdom	Animalia
Phylum	Arthropoda
Sub-Phylum	Hexapoda
Class	Insecta
Order	**Orthoptera (grasshoppers, locusts, crickets)**
Sub-order	Ensifera (crickets, true crickets, bush crickets)
Family	**Tettigoniidae (bush crickets or katydids)**
subfamily	Copiphorinae (coneheads)
Genus	*Ruspolia*
Species	***Ruspolia differens***

R. differens is, therefore, a long-horned, cone-headed bush cricket predominantly found in sub-Saharan East, central and Southern Africa (Matojo & Hosea, 2013; Matojo, 2017). The *pseudo* grasshopper is primarily nocturnal and exhibits color polymorphism and sexual dimorphism (Matojo, 2017). It has up to eight morphs; namely, green, brown, purple, suffused brown, suffused green, purple-striped, brown, and purple striped, green, with the green and brown morphs being more dominant than the purple (Matojo & Hosea, 2013). The brown morph is male-biased, while all the green morphs are female-biased. The male adults are smaller than the females and have longer antennae and a pair of tongue-like metathoracic flaps that are not found in females. The female possesses a long slender ovipositor, whereas the male possesses

dorsoventrally bi-lobed cerci (Matojo & Yarro, 2010; Matojo, 2017; Matojo & Yarro, 2013; Matojo & Njau, 2010).

Figure 1. 1: *De-winged and de-legged R. differens color morph found in Kagera, Tanzania: a. Purple. b. green, purple stripes c. brown. d. brown, purple stripes, e. green. Source: Mmari et al., 2017*

1.5.3 Feeding and diet

R. differens is an opportunistic feeder reported on the most abundant available grasses in the target countries. It is predominantly found on grasses and hedges where they feed on inflorescences of grasses such as *Panicum maximum*, *Brachiaria ruziziensis*, *Hyparrhenia rufa*, *Chloris gayana*, *Pennisetum purpureum*, *Cynodon dactylon,* and *Sporobolus pyramidalis* (Opoke

et al., 2019).

Different diets have been tested to produce *R. differens* with the various blends ranging from single to mixed diets based on the host plants of the grasshopper to a wide array of artificial diets, including food crops or their by-products and animal feed (Valtonen et al., 2018). The experiments assessed the acceptance and preference of different diets, effects of diet on growth, development, reproductive performance, and effects of diet on the nutrient composition of the grasshopper. As stated by Valtonen et al., (2018), *R. differens* is a facultative oligophagous herbivore that can feed on the leaves and inflorescences of many host plants in the absence of their preferred host. According to Malinga et al., (2020), a varied diet mixture of the various host plants increases nymphal survival and shortens the developmental time of *R. differens.*

1.5.4 Swarming behavior

R. differens has a distinct swarming phase during wet seasons and a non-swarming phase during dry seasons (Matojo, 2017). The non-swarming phase has more males than females, with the reverse being confirmed in the swarming phase (Matojo & Yarro, 2010). Upon swarming, the adult bush crickets hide in local bushes, which later become their breeding grounds (Matoj & Njau, 2010). Swarms emanate from the non-swarming populations recruited from locally available suitable reproductive sites (Matojo & Njau, 2010; Opoke et al., 2019), unlike the widespread traditional beliefs that swarms emerged from heaven, Lake Victoria, dense clouds, or pine trees (Mmari et al., 2017). The swarms appear biannually during the long (March-May) and short (November-December) rainy seasons (Mmari et al., 2017; Opoke et al., 2019). Seasonal variations have been reported in the ratio of green and brown morphs and the population densities of the non-swarming *R. differens* (Matojo & Njau, 2010; Matojo & Yarro, 2013). The densities are reportedly low in the dry season but high soon after the rainy season. The high density after the rainy season is attributed to increased availability of quality food, related to increased egg production and faster development rate of immature stages (Matojo &

Yarro, 2010; Matojo & Njau, 2010).

1.5.5 Life cycle

The life cycle of *R. differens* is approximately 147 days, with egg development taking about 19 days. A recent study reported that the bush crickets have six nymphal stages for males and seven for females, with an average nymphal stage duration of, respectively, 46 and 56 days at 30 °C (Opoke et al., 2019). The adult females have a pre-oviposition and oviposition period of approximately 16 and 32 days, respectively. Adults have been reported to live for 50-90 days in the laboratory (Opoke et al., 2019). Peak fecundity and maturity occur in the middle of the swarming season. In contrast, minimum fecundity occurs at the end of the non-swarming season with a mean clutch size of 71-72 eggs and 7-18 eggs, respectively (Matojo & Njau, 2010).

1.5.6 Rearing *Ruspolia differens*

At the moment, *R. differens* is principally obtained from the wild during the two swarming seasons (Mmari et al., 2017; Agea et al., 2008). However, efforts have been made to rear this bush cricket under laboratory conditions at varying success levels. The following rearing parameters have been reported: temperature range of 22-32 °C, 40-78% relative humidity, and a photoperiod of 12 hours light: 12 hours dark (Lehtovaara et al., 2017; Malinga et al., 2020, 2018a; Rutaro et al., 2018; Ssepuuya et al., 2018; Leonard et al., 2021). The optimal temperature range for rearing *R. differens* with the shortest development time and the best survival rates have been proven to be 28-30 °C. (Lehtovaara et al., 2018; Ssepuuya et al., 2018; Leonard et al., 2021). However, only limited data exist on the effects of light and humidity on the rearing of these bush crickets. According to Ssepuuya et al., (2018), egg development time decreased with increased temperature, whereas the nymphal hatching weight decreased. The highest hatchability of eggs from the leaf sheath was obtained at 30 °C (Ssepuuya et al., 2018).

The species has been reared individually or in small plastic containers and wooden cages with great success (Rutaro et al., 2018; Ssepuuya et al., 2018). However, there was a lacuna of data on rearing containers/ cages and housing for *R. differens*. Effects of rearing environment and

density on *R. differens* have also been tested. According to Lehtovaara et al., (2019), different rearing environments were found to have minimal effects on the survival of the nymphs. The appropriate rearing density was established to be ≤ 36 nymphs per liter, but nymphal mortalities were found to increase densities above this level (Lehtovaara et al., 2019). Malinga et al., (2019) developed and tested different egg-laying substrates for *R. differens* and demonstrated that females laid significantly more eggs on the folded plastic cloth (artificial substrate) compared to the natural substrate (elephant grass). In addition, Egonyu et al. (2020) found that the katydid species prefers *Panicum* and maize over cotton and *Pennisetum* for oviposition.

Rearing of *R. differens* on grains and other diets (wheat bran, germinated finger millets, rice seed head, finger millet seed head, chicken feed egg booster, and sorghum seed head) showed high levels of acceptability (Malinga, Valtonen, Lehtovaara, Rutaro, Opoke, Nyeko, Roininen, et al., 2018). The effects of blending natural plant materials and artificial diets have been tested on the development, reproductive performance, and nutrient composition of *R. differens*. According to Malinga et al., (2018), diets with more diversified feed items led to a significant reduction in the nymphal developmental time, increased adult weight, fecundity, and survival. However, fatty acid content and composition of *R. differens* were observed to vary considerably when *R. differens* was subjected to different artificial diets but the overall composition of the insects was similar to that of their diets (Lehtovaara et al., 2017; Opoke et al., 2018). A similar trend in nutrient composition of *R. differens* has been observed when provided mixtures of their natural plant diets (Malinga et al., 2020; Rutaro et al., 2018; Malinga et al., 2018). However, bush crickets fed on more diversified diets based on their natural host plants showed similar fatty acid composition to their wild population counterparts (Rutaro et al., 2018). Additionally, protein-rich diets were found to significantly shorten the developmental time, increase survival, and increase weight gain.

Evidence indicates that *R. differens* can be reared successfully on various by-product diets (barley feed, barley mash, and potato protein diets) (Sorjonen et al., 2020). Based on the above study, increasing the dry matter protein levels in the diet up to 17 % enhanced growth, developmental time, and survival of *R. differens*. However, increasing the protein levels above

17 % did not show any further enhancement in the biological parameters of *R. differens*, except for a decrease in the feed conversion rate (Sorjonen et al., 2020).

1.6 Nutritional composition of *R. differens*

The nutritional composition of *R. differens* collected from different regions has been analyzed by different authors, with considerable variations observed with samples associated with the various geographic locations of collection, subtype/ color morphs, and processing methods, as illustrated in Table 1. **1**. The moisture content was between (47-66 %), the protein content of *R. differens* ranged between 34.2 and 72.7 %, carbohydrates (2.5-6.03 %), fat (33-54.3 %), dietary fiber (3.93-14 %), ash (1.79-5 %), (Table 1.1). Ssepuuya et al., (2017) found that the nutrient composition of *R. differens* varied with swarming season, geographical location, and subtype. The *R. differens* collected in March-May had significantly higher protein and dry matter content but much lower moisture content when compared to those harvested in the November-December season. Furthermore, the mineral content (especially phosphorus) varied with geographical location. The brown bush crickets had a significantly lower dry matter and higher moisture content when compared to the green morphs (Ssepuuya et al., 2017). The most abundant minerals (as dry matter) included phosphorous (426-673 mg/100 g), potassium (446-673 mg/100 g), and calcium (34.9-128 mg/100 g), though variations were observed across the seasons and geographical zones. The Vitamin B_{12} content ranged between 0.73-1.35 µg/100 g (Ssepuuya et al., 2019). Higher protein and ash content, as well as lower fat contents, were recorded in April-May compared to samples collected in November-December (Ssepuuya et al., 2019).

The effects of processing methods on nutrient composition of *R. differens* have also been reported and are discussed in greater detail in *section 1.8*. However, the comparable nutrient composition has been reported by other authors despite the processing method involved (Kinyuru et al., 2009; Ssepuuya et al., 2017). The macro minerals (as dry matter) included potassium (259-834 mg/100 g), phosphorous (120-680 mg/100 g), and calcium (24-1124 mg/100 g). The highest trace minerals included iron (13-258 mg/100 g) and zinc (12-17 mg/100

g), as illustrated in Table 1. **2**.

Table 1. 1 *Proximate composition¤ of R. differens (expressed as % dry matter, except for moisture)*

Author, year	Morph	Energy (kcal/100 g)	Source					
		Moisture	Ash	Crude protein	Crude fat	Crude fiber	Carbohydrate	Energy (KJ)	Source
Kinyuru et al., (2010)	Green	66.4	2.8	43.1	48.2	4.0			Wild, Kenya
	Brown	71.2	2.6	44.3	46.2	4.0	-		
Ssepuuya et al., (2017)	Green	47.6-53.4	2.6-3.2	37.9-40.3	41.8-42.4	11.33-14.3	2.5-3.0		Wild, Uganda
	Brown	50.9-55.4	2.7-3.9	37.0-40.4	41.4-43.0	11.3-13.9	2.9-3.1		
Siulapwa et al., (2012)	Green	4.5	2.2	44.6	49.0	-	8.4	618	Wild, Zambia
Rumpold et al., (2013)	Mixed	-	4.6	72.7	46.2	6.3	-	-	Wild

¤No chitin contents were reported in these studies

Table 1. 2 *Mineral content of R. differens (in mg/100g) expressed as dry matter*

Author, year	Morph	Calcium	Magnesium	Potassium	Sodium	Phosphorus	Iron	Zinc	Manganese	Copper
Kinyuru et al., (2010)	Green	27.4	33.9	371	359	141	16.6	17.3	5.26	0.63
	Brown	24.5	33.06	259.7	229.7	121	13.01	12.4	2.46	0.47
Ssepuuya et al., (2019)	Mixed	34.9-128	40.8-69.0	446-673	18.6-41.9	429-627	32.4-69.0	8.11-15	1.41-7.23	1.60-2.23
Siulapwa et al., (2012)	Mixed	9.0	5.2	9.1	14.6		2.0	-	0.0	0.1
Rumpold et al., (2013)	Mixed	24.5	-	-	-	121.0	13.0	12.4	33.1	-

*=mg/kg

Table 1. 3 *Vitamin composition of R. differens (in mg/100g) expressed as dry matter*

		Type of vitamin*								
Author, year	morph	Retinol[¤]	α-tocopherol[¤]	Niacin	Riboflavin	Ascorbic acid	Folic acid	Pyridoxine	Vit B_{12}[¤]	Source
Kinyuru et al., (2010)	Green	2.12	201	2.12	1.2	0.07	0.99	0.44	-	Wild, Kenya
	Brown	2.75	152	2.36	1.36	0.14	0.92	0.16	-	
Ssepuuya et al., (2019)	Mixed	-	-	-	-	-	-	-	0.73-1.35	Wild, Uganda

[¤](µg/g)

The vitamin contents of *R. differens* are illustrated in Table 1.3, with clear indications that these insects are an excellent source of several vitamins (Kinyuru et al., 2009; Rumpold et al., 2013; Ssepuuya *et al.,* 2019). Kinyuru et al., (2010) reported α-tocopherol in *R. differens* in the range of 161 µg/g to 170 µg/g on a dry weight basis. Heat processing and solar drying have been shown to significantly reduce the level of retinol in brown bush crickets (Kinyuru et al., 2010). The total antioxidant activity of the grasshopper has been reported and shown to be comparable to that of many fruits and vegetables consumed worldwide (Ssepuuya et al., 2020). The total flavonoids and phenolic contents of the grasshopper were higher than that of several fruits and vegetables consumed in Burkina Faso and comparable to those reported in China (Ssepuuya et al., 2020).

The amino acids found in *R. differens* included aspartic acid, alanine, glutamic acid, leucine, lysine, valine, and tryptophan in different levels ranging from 5.18 - 123 mg/g protein, as illustrated in .

Table 1. 4 *Amino acid composition (mg/ g protein) of R. differens*

Author, year	Siulapwa et al., (2012)	Ssepuuya et al., (2019)
Morph	Green	-
Alanine (Ala	49.6	93.7-110
Arginine (Arg)	92.8	52.1-65.9
Aspartic acid (Asp)	91.3	ND
Cysteine (Cys)	1.3	6.3-7.9
Glutamic acid (Glu)	157.1	ND
Glycine (Gly)	48.5	52.3-58.2
Histidine (His)	82.2	20.9-26.5
Isoleucine (Ile)	48.6	46.5-49.0
Leucine (Leu)	49.8	80.9-88.5
Lysine (Lys)	107.0	54.0-69.8
Methionine (Met)	8.0	16.0-19.9
Phenylalanine (Phe)	48.6	36.1-38
Proline (Pro)	35.4	64.6-82.7
Serine (Ser)	48.3	42.1-47.7
Threonine (Thr)	53.3	39.7-43.1
Tryptophan (Trp)	0.6	6.8-9.7
Tyrosine (Tyr)	47.1	48.3-67.6
Valine (Val)	30.6	58.1-60.7

ND= None detected; (-) not mentioned

Table 1. 5 *Fatty acid composition of R. differens from literature (expressed as % of total fatty acids)*

Author, year	Kinyuru et al., (2010)		(Malinga et al., 2020)	(Ssepuuya et al., 2019)	(Rutaro et al., 2018)	(Rutaro et al., 2018)
Source	Wild, Kenya		Lab reared, Uganda	Wild, Uganda	Lab reared, Uganda	Lab reared, Uganda
Morph	Green	Brown				
Type of fatty acid						
Decanoic(C10:0)	0.36	0.22			0.09	
Lauric (C12:0)	-	-		0.04-0.37	0.04-0.11	
Myristic (C14:0)	0.97	0.77	0.25-0.49	0.66-0.82	0.58-0.91	2.8-11.9
Myristoleic (C14:1)					0.07-0.11	2.1-7.7
Palmitic (C16:0)	31.52	32.12	15.22-20.13	26.6-27.3	19.61-22.26	20.9-32.7
Palmitoleic (C16:1)	1.98	1.42	0.29-1.09	1.2-1.9	0.66-1.34	15.8-21.3
Stearic (C18:0)	5.49	5.99	6.24-8.98	6.67-7.5	7.69-9.20	5.7-7.9
Oleic (C18:1 n-9)	24.58	24.87	24.57-36.75	38.4-42.7	19.51-28.60	15.1-25.7
Linoleic acid (C18:2(n-6))	31.21	29.54	28.08-37.63	19.0-23.0	21.03-28.45	9.0-19.6
α-Linolenic C18:3(n-3)	3.21	4.23	3.79-16.78	0.96-1.5	12.36-16.34	0.9-2.6
Arachidic acid (C20:0)			0.49-2.13		0.94-1.41	0.5-2.3
Arachidonic (C20:4(n-6))				0.25-0.43		
Pentadecanoic (C15:0)			0.06-0.08	0.01-0.15	0.23-0.38	
Heptadecanoic (C17:0)				0.18-0.4		
Heptadecenoic (C17:1 n-7)			0.01-0.08		0.27-0.5	
9-oxoheptadecanoic acid (C17:2)			0.26-0.50			

11-Octadecenoic acid (C18:1n-7)			0.01-0.26			
Nonadecanoic acid (C19:0)			0.00-0.14			
Henicosanoic acid (C21:0)			0.00-0.02			
Eicosapentaenoic (C20:5(n-3))				0.09-0.4		
Docosanoic acid (C22:0)			0.11-0.57		0.36-0.51	
Tricosanoic acid (C23:0)					0.01-0.03	
Tetracosanoic acid (C24:0)					0.04-0.09	
Hexacosanoic acid (C26:0)					0.14-0.32	
Total SFA	38.34	39.10	24.57-29.79	35.1-35.8	32.1-43.0	31.0-48.4
Total USFA	60.98	60.06		64.2-64.9		
MUFA	26.56	26.29	25.68-38.25	40.4-44.6	45.65-55.19	38.6-46.4
PUFA	34.42	33.77	31.91-47.40	20.3-24.6	4.74-21.49	11.5-20.6

SFA=Saturated Fatty Acid; USFA=Unsaturated Fatty Acid; MUFA=Monounsaturated Fatty Acid; PUFA= Polyunsaturated Fatty Acid

The primary fatty acids found in *R. differens* include oleic, palmitic, palmitoleic, linoleic, α-linolenic, myristic, and stearic acids, as shown in (Kinyuru et al., 2010; Lehtovaara et al., 2017; Rutaro et al., 2018; Ssepuuya et al., 2019).

Studies have shown that the fatty acid composition of *R. differens* reared in the laboratory can be manipulated based on the diet provided. For example, according to Rutaro et al., (2018a), increasing the diversification of artificial diets by varying the inclusion of various food items from one up to six components in the composite diet significantly alters the fatty acid composition of *R. differens*. The proportion of linoleic and α-linolenic acids increased with diet diversification (Rutaro et al., 2018a). The most considerable variability in fatty acid composition was found in insects fed on the most diverse diet components (Rutaro et al., 2018a). Similarly, Lehtovaara et al., (2017) showed that the fatty acid composition of *R. differens* reared on protein and carbohydrate-rich diets compares well with that of the wild-caught populations. However, insects fed on diets rich in linoleic and α-linolenic acids showed considerable variation in their fatty acid composition (Lehtovaara et al., 2017). The fatty acid levels recorded (linoleic, α-linolenic acids, docosahexaenoic acids, and eicosapentaenoic acid) in *R. differens* were proportional to these of the diet (Lehtovaara et al., 2017). Nevertheless, Malinga et al., (2020) found that the fatty acid composition of *R. differens* was not altered when the insects were subjected to mixed dietary blends with host plant materials.

1.7 Harvesting, processing, preservation, and storage of *R. differens*

R. differens is mainly obtained from wild harvesting during the two annual swarming seasons, as depicted in this introductory chapter. Traditionally, harvesting of *R. differens* for household consumption and marketing was conducted in the fields away from the homes (Mmari et al., 2017; Agea et al., 2008). However, the situation has changed with most harvesting activities undertaken around the homes using bright light sources. Home collection is usually carried out in the morning hours before sunrise by women and children. However, the commercialization of this bush cricket has led to the development of locally assembled traps to optimize its harvesting (Mmari et al., 2017). The traps comprise folded iron sheets, large buckets, drums, and three very bright mercury lights (Okia et al., 2017). The iron sheets are folded into a cone

shape and arranged to fit directly in the collecting buckets or drums as the grasshoppers hit the iron sheets and slide into the containers (Agea et al., 2008; Mmari et al., 2017, Okia et al., 2017). Recent work by Sengendo et al., (2021a,b) resulted in an improved *R. differens* trap, which is safer, energy-efficient, and more profitable than the current commercial setups. The improved trap comprises collection drums fitted with a funnel to improve retention of the catch, meshes to filter by-catches, and light-emitting diode bulbs to replace potentially hazardous mercury lamps.

Freshly collected *R. differens* are known to have a short shelf life of 12-48 hours under temperature conditions of 24-28 °C. After harvesting, the bush crickets are manually removed from the drums and stored in aerated sisal, polythene, nylon, or cloth mesh bags. Occasionally, they are temporarily spread on mats before further processing (Mmari et al., 2017; Ssepuuya et al., 2019).

According to Agea et al., (2008), *R. differens* can be eaten raw, boiled, sun-dried, fried, and flavored with onions or soup. Processing of the bush cricket entails cleaning to remove unwanted body parts like appendages, wings, and ovipositor in females; sorting out nontarget insects and waste materials (such as grasses, wood ashes, cassava, and maize flour used during harvesting). This processing is done manually by women and children (Mmari et al., 2017; Ssepuuya et al., 2019). After cleaning, the bush crickets are processed by sautéing, boiling, smoking, toasting, deep frying, sun drying, and freezing (Mmari et al., 2017; Ssepuuya et al., 2016; Ssepuuya et al., 2017). Boiling is done by placing the bush crickets (*Nsenene*) in boiling salted water with or without spices (Mmari et al., 2017). Toasting is achieved by putting boiled or raw *Nsenene* in a hot pan and stirring till they turn brownish and produce a meat-like smell. Sun drying is commonly considered a preservation method and usually takes two days, preceded by storage in plastic buckets and sacks ready for consumption (Mmari et al., 2017). Deep-fried bush crickets are cooled, packed in cardboard boxes, and stored in clean and dried places free of rodents (Mmari et al., 2017; Ssepuuya et al., 2019). According to Mmari et al., (2017), traditionally preserved *nsenene* has an extended shelf life of approximately one year. Ssepuuya et al., (2017) further demonstrated that the sensory acceptability of dried and boiled *R. differens* reduced significantly within the first two weeks of storage. Acceptability scores for

dried and non-frozen bush crickets were also significantly reduced compared to chilled and frozen bush crickets (Ssepuuya et al., 2017). The authors further established that chilling and freezing were the most suitable option for ensuring the storage stability of the bush cricket, and vacuum packaging were unnecessary for maintaining the storability of dried ready-to-eat grasshoppers (Ssepuuya et al., 2016, 2017).

Processing has been shown to influence the nutrient composition of *R. differens,* with boiled bush cricket showing significantly higher percentages of chitin and protein but lower percentages of fat and ash than the raw ones (Ssepuuya et al., 2020). Roasting was shown to significantly increase the relative level of crude proteins and lowered fats (Ssepuuya et al., 2020). However, heat processing did not significantly affect carbohydrate content (Ssepuuya et al., 2020). Cognizant of the processing, storing, and preservation challenges facing this budding industry in East Africa, the stage was therefore set to undertake this Ph.D. research.

1.8 Knowledge gaps regarding antinutrient composition and techno-functional properties of *R. differens* and other orthopterans

1.8.1 Antinutrient composition of insects

The nutritional value of insects consumed as food is established by their nutritional and antinutritional composition. Antinutrients are more common in plant-based than in animal-based foods. The major antinutrients found in plant-based foods include phytates, tannins, lectins, oxalates, cyanogenic glycosides, protease inhibitors, lectins, and antivitamin factors (Popova & Mihaylova, 2019; Franceschi & Nakata, 2005; Chakravorty et al., 2016). Other natural toxicants that can loosely be classified as antinutritional factors include solanine, cyanides, glucosinolates, lathyrogens, mimosine, and nitrosamines (Popova & Mihaylova 2019; Petroski & Minich 2020).

Although sufficient data are available on the nutritional quality of insects, information regarding the antinutritional composition of insects is very limited (Adeoju & Omotayo, 2014; Musundire

et al., 2014). These antinutrients have been reported to elicit detrimental physiological effects. Oxalates prevent calcium absorption in the body by binding with it (Jiru et al., 1995). Tannins can inactivate enzymes that regulate protein absorption. Phytates, in some instances, cause diminished mineral absorption, while lectins are responsible for various reactions in the body (Gupta et al., 2015).

R. differens and other orthopterans naturally have a predominantly plant-based diet. The tendency to have copious amounts of antinutrients present in their gut could pose a health hazard. Though some studies have reported extremely low levels of antinutrients in some edible insects (Ekop et al., 2010; Omotoso, 2006; Adeduntan, 2005), there is a need to examine the levels of such undesirable components in the trendy insects consumed as food in East Africa.

1.8.2 Techno-functional properties of edible insects

Although insects are a well-known valuable alternative protein source to fish and beef in many countries, consumer acceptability remains a significant challenge, especially in the western world. However, consumer acceptance can be improved through processing into flours that can be used as high-quality food ingredients. There is a lack of information on the impact of processing methods on the techno-functional properties of most edible insects.

While consumer acceptability remains a predominant challenge to integrating Western food culture, edible insects as meals and defatted insect flour could decrease neophobia. Thus, edible insects can be consumed whole or disguised in other products, a trend that may highly influence their consumption (Bußler et al., 2016; Baiano, 2020; Hartmann & Siegrist, 2017; Sogari, Menozzi, and Mora, 2016). Recent studies have revealed that people may consume insects more when they are in a concealed form, such as protein extracts and concentrates incorporated in other food products (Baiano, 2020; Rumpold, 2013). Thus, the protein can be extracted for food applications to improve the nutritional, bioactive/ antioxidative, and techno-functional properties of targeted food products.

In the food industry, proteins are being used as integral sources not only because of their nutritive value but also because of their functional properties (Cabra et al., 2008). Some of the required functional properties of food proteins for application in food formulations include their

solubility, gelation ability, emulsifying activity, foaming capacity, and biological activity (Panyam and Kilara, 1996; Nongonierma and FitzGerald, 2017). Defatting raw food materials and by-products using organic solvents is a frequent method for producing protein-enriched ingredients (L'hocine et al., 2006; Capellini et al., 2020). Hexane remains the most popular solvent to remove lipid from the solid insect matrix owing to its high efficiency and availability (Russin et al., 2011). For instance, Kim et al., (2020) evaluated the impact of different organic solvents on the functional properties of defatted proteins extracted from *Protaetia brevitarsis* larvae. The authors found that hexane-defatted protein fractions had a better amino acid composition, protein solubility, and functional properties than the same fractions defatted with methanol and ethanol.

In the literature, however, only a few studies have investigated the functional properties (e.g., solubility, foaming, gelling, and emulsions) (Ndiritu et al., 2017; Zielińska, Karaś, & Baraniak 2018; Mishyna et al., 2019; Khatun et al., 2021) of insect protein powders as a function of processing methods, mainly blanching and drying, in order to optimize the ingredient quality (Gravel & Doyen, 2020; Gravel et al., 2021). Currently, data on the techno-functional properties of *R. differens* based flours are lacking. Also, the influence of processing such as drying, defatting, and storage on these functionalities for several insect species consumed in Africa is absent. There is, therefore, the need to investigate and fill such research gaps if insects are to be regarded as a natural alternative to well-known protein sources.

1.9 Objectives and outline of this thesis

One of the main challenges limiting the continued inclusion of edible insects in diets in Kenya and East Africa is that the availability of wild-harvested insects is seasonal and that the harvested edible insects have a limited shelf-life. This limitation is precisely the case for *Ruspolia differens*. While the post-harvest shelf-life of freshly harvested *R. differens* has been thoroughly investigated, studies on farmed *R. differens* are still lacking. The overall aim of this research project was to improve and add value to harvested *R. differens* vis-a-vis other popularly consumed orthopteran insects. In addition, this research intended to support and improve all-year-round availability and mass production of this bush cricket to meet the local market,

especially during the non-swarming seasons, by developing a sustainable methodology for rearing nutritionally rich *R. differens*. This Ph.D. dissertation aimed to establish processing effects on the nutritional, anti-nutritional, and techno-functional properties of a food ingredient. Finally, it set out to evaluate the antimicrobial attributes claimed to be responsible for the therapeutic uses of this edible bush cricket. To achieve these, the following specific objectives were set out, with a focus on the nutritional composition, sustainable rearing, and functional properties of *R. differens* to enhance food security:

1. To determine the effect of the drying method on the postharvest (anti)-nutritional composition of *R. differens.*
2. To compare the nutritional and antinutritional quality of *R. differens* to that of other edible orthopteran insects.
3. To evaluate the effect of photoperiod as a rearing parameter on the biological fitness of lab-reared *R. differens* fed on an artificial diet aiming at mass production.
4. To evaluate the extent of cannibalism in lab-reared adult *R. differens* and propose mitigating strategies.
5. To evaluate the influence of processing on the techno-functional attributes of *R differens* and other edible orthopteran insects.
6. To elucidate the antibacterial (potentially therapeutic) properties of *R. differens.*

Guided by the objectives listed above, the following dissertation outline was designed, as illustrated in Figure 1.2 below.

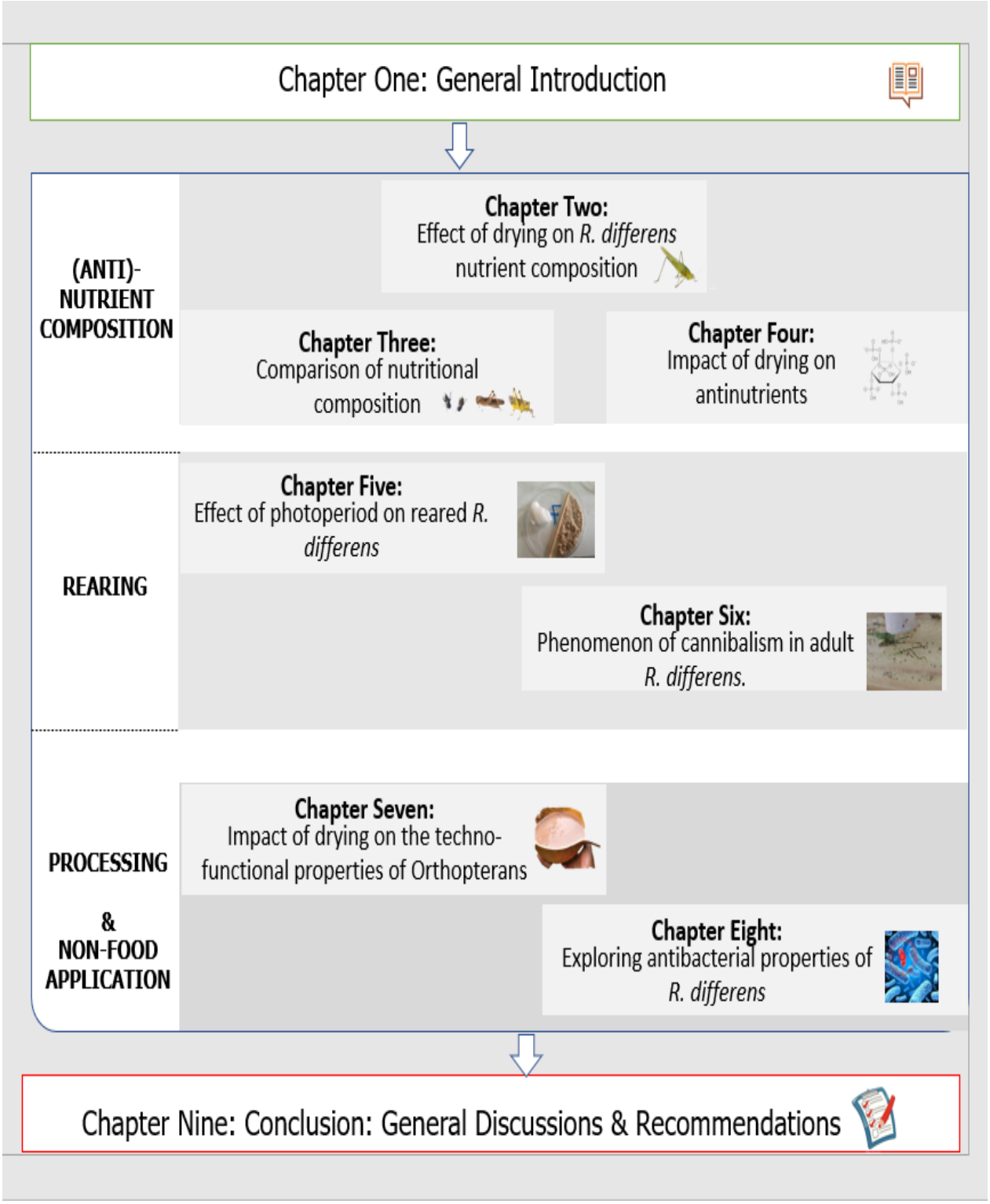


***Figure 1. 2**: Outline of the Ph.D. thesis chapter organization*

Chapter One constitutes this general introduction**. Chapter Two** examines the effect of two widespread drying methods, oven-drying, and freeze-drying, on the nutritional composition of wild-harvested *R. differens.*

In **Chapter Three,** a nutritional comparison is made between *R. differens* (Chapter two) and a few other edible insects of the same order (Orthoptera), *i.e.*, *Locusta migratoria, Schistocerca gregaria,* and *Gryllus bimaculatus*.

Here the more affordable oven-drying method (from chapter two) is applied post-blanching to characterize the nutritional composition of three orthopterans that are easier to rear and commonly consumed in Africa (*S. gregaria, L. migratoria,* and *G. bimaculatus*) alongside *R. differens.* Like *R. differens*, these insects are rich in proteins, essential amino acids, omega three fatty acids, trace minerals, and vitamin B_{12}.

Having established the nutritional composition of *R. differens* (chapters two and three), **Chapter Four** investigates some anti-nutritional properties (phytates, oxalates, and tannins) of *R. differens* alongside commonly consumed locust (*S. gregaria*) and cricket (*G. bimaculatus*) species. Having confirmed their nutritional status, and in order to reduce the impact of wild-harvesting, rearing was then attempted.

Therefore, **Chapter Five** dwells on rearing aspects of *R. differens*. In this chapter, the influence of two very distinct photoperiodic conditions using a novel artificial diet is explored.

The rearing experiments in chapter five revealed the occurrence of cannibalism among reared *R. differens.* **Chapter Six** looked more deeply into the aspect of cannibalism among the adults of these tettigoniids. Two strategies were employed to evaluate and potentially mitigate this phenomenon using insect prey and artificial diets.

Upon rearing, the influence of processing was investigated, and this was the subject matter of **Chapter Seven,** where the techno-functional properties of processed (dried and defatted) flours of *R. differens* and a few other edible insects (locusts and crickets) were determined, and compared.

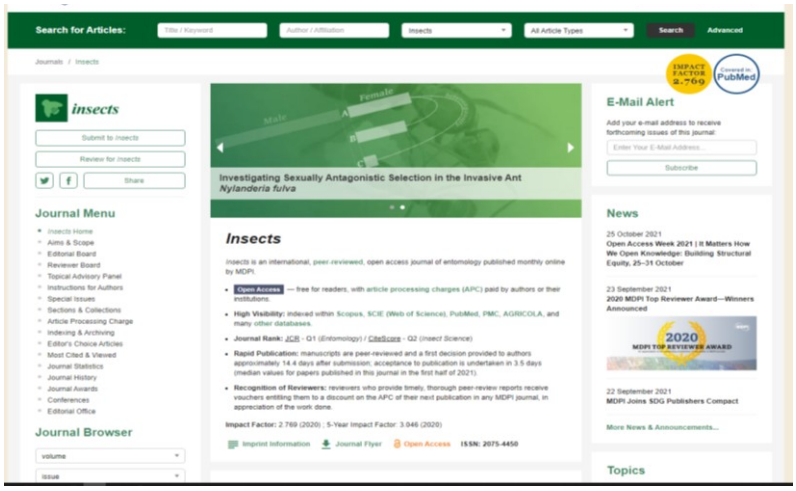
With the anti-nutritional, techno-functional, and processing of *R. differens* established, **Chapter Eight** rounds off with a non-food (medicinal) application that explored the potential anti-

microbial properties of *R. differens* extracts to evaluate the healing claims purported by local indigenous knowledge.

Finally, a general discussion, recommendations, and a glance into future directions are covered in **Chapter Nine.**

CHAPTER TWO

Determination of the Nutrient Composition of blanched *Ruspolia differens* after Freeze-Drying and Oven-Drying



The contents of this chapter were redrafted[1] after Fombong, F. T., Van Der Borght, M., & Vanden Broeck, J. (2017). Influence of freeze-drying and oven-drying post blanching on the nutrient composition of the edible insect *Ruspolia differens*. Insects, 8(3), 102. https://doi.org/10.3390/insects8030102

[1]The complete content of this paper (Fombong et al., 2017) was included in Chapter 2, with slight alterations to maintain the logical flow of this dissertation. As the first author, Fombong Forkwa contributed to the majority of the parts described in this work, from conceptualization, sample acquisition, experimental work, analysis, and interpretation of the data to the writing of the paper.

2.1 Introduction

As mentioned in chapter one, *R. differens* is a much sought-after delicacy (Van Huis, 2003; Agea et al., 2008; Kinyuru et al., 2010; Kelemu et al., 2015; Fombong et al., 2017). The swarming behavior of *R. differens* observed in several localities has revealed the broad diet of these insects comprising grains and grasses of the ubiquitous *Poaceae* family and their tendency to live together in high densities. Their nutrient content is therefore considered to be both high and variable (Van Huis 2013), which indicates tremendous potential for this edible insect to combat human nutritional deficiencies. However, preservation steps are crucial to their processing to extend their shelf-life beyond 24 hours (Ssepuuya et al., 2016).

Many insects are only seasonally available, and they are typically well-preserved for later consumption by drying in the sun, over ashes, or in the oven (Van Huis 2003). In Kenya, tettigoniids are traditionally consumed as a snack, either fresh, toasted, or sun-dried, depending on the season (Kinyuru et al., 2010a). While storing insects under deeply frozen conditions ensures food quality, drying allows storage at ambient temperatures.

Drying is a traditional method of food preservation used worldwide. Besides sun-drying, commercial-drying methods, such as drum, evaporation, spray, and freeze-drying, are used to preserve a vast array of vegetable- and animal-based foodstuffs. A typical drying process begins with a heating step (e.g., cooking or blanching) to inactivate most of the microorganisms and enzymes present, then the application of dry heat to remove any water contained in the foodstuff (Grabowski & Klein 2016). Two commonly-employed drying methods in the food industry are oven-drying and freeze-drying. For tilapia fish, for instance, the preferred method is freeze-drying, as it is known to cause the least damage to proteins (Chukwu 2009). Considering the high capital and running cost associated with the freeze-drying process, alternate drying methods (i.e., sun- or oven-drying) are more frequently used in developing nations for preserving insects. Research on other animals (e.g., tilapia fish) has shown that different drying methods had different effects on the nutritional composition, attributed to the chemical and physical changes caused by exposure to heating or freezing (Chukwu 2009). It seems logical that such effects would influence the nutrient composition of insects, as well. Nonetheless, investigations into the effect of different drying processes on the nutritional properties of

insects are limited.

In Kenya, the emergence and growth of the middle class, combined with increasing Westernization, is driving the demand for more sophisticated processed food products with greater variety and improved nutritional quality. Kenyan tastes and preferences are also increasingly influenced by foreign travel and broader access to global brands via the growth of modern grocery retailing (Euromonitor 2017). To convince consumers to accept insect products from different processing (drying) methods, investigations of their effects on the nutritional constituents are required.

The influence of processing methods, i.e., toasting and solar drying, on the *in vitro* protein digestibility and vitamin content of green and brown bush crickets consumed in Kenya has been studied by Kinyuru et al., (2010a). However, the authors did not investigate the influence of the cooking (toasting) and drying method (sun-drying) on the proximate, fatty acid, or mineral contents of *R. differens*. Furthermore, only approximate values were given for temperatures used for sun-drying. Consequently, a study that evaluates drying effects based on accurate measurements and includes a broader range of nutritional parameters is necessary. Oven- and freeze-drying remain the most popular methods of preserving *R. differens*. Surprisingly, research that investigates the effect of these two drying methods on the nutritional composition of this African tettigoniid has not been carried out.

Therefore, this study aimed to compare the nutritional properties of *Ruspolia differens* following two popular drying methods (i.e., oven-drying and freeze-drying).

2.2 Materials and methods

2.2.1 Sample acquisition and preparation

During the swarming season of October–November 2016, approximately 20 kilograms of bush crickets were harvested from the wild from Masaka and Kampala in Uganda; and Busia and Kisumu in Kenya. Bush crickets were pooled together in specially-designed cages and transported alive to the laboratory at the Food Technology department in Jaramogi Oginga Odinga University of Science and Technology (JOOUST) Bondo, Kenya.

Blanching

Upon arrival, they were sorted based on color (morph) before being blanched in boiling water for 5 minutes, drained and allowed to cool, placed into zip-lock freezer bags, and immediately frozen at −20 °C. Blanched samples were used for this study, and the justification here is that these fresh bush crickets deteriorate quickly with noticeable chemical changes (Ssepuuya et al., 2016; Ng'ang'a et al., 2018). Therefore, to begin on the same starting point, all samples for freeze or oven-drying were equally blanched. Another reason for using blanched samples instead of raw samples was that when used as a food, it is seldom eaten raw but mostly boiled or fried. Thus, blanching was a simple unit process that mimicked the boiling, roasting, or frying treatment usually given to wild-harvested *R. differens* after washing.

The frozen samples were then transported in an ice-packed thermos box to the Food Science department of Jomo Kenyatta University of Agriculture and Technology (JKUAT), Nairobi, for drying. They were then freeze-dried (FD) or oven-dried (OD), as described below.

Drying Methods

The freeze-drying process was performed using the freeze-dryer (CHRIST-ALPHA- LDPLUS-101541, Martin Christ, Osterode am Harz, Germany). Blanched green, brown, and purple bush crickets were frozen at −30 °C for 20 min and then freeze-dried in a vacuum in two steps: primary drying (−50 °C at 0.040 bars) for 48 h and final drying (−55 °C at 0.021 bars) for another 48 h.

Blanched green and brown bush crickets were oven-dried using a laboratory oven (Memmert UF 110, Memmert, Schwabach, Germany) at 60 °C for 24 h. Purple morphs were not oven-dried because they were not collected in sufficient amounts.

Dried samples were ground into flour using a two-speed Waring laboratory blender (Camlab, Over, UK). Samples were transported to Belgium and stored for a month in the dark in sealed plastic bottles prior to analysis. Compositional analyses of samples were performed in triplicate unless otherwise stated.

2.2.2 Chemical Analyses

Proximate composition

Moisture content was analyzed using a forced-air oven (UF 110, Memmert, Schwabach, Germany) at 105 °C for 24 h (Vandeweyer et al., 2017).

Crude protein content was measured from the total organic nitrogen content, which was determined by the Kjeldahl method using a steam distillation apparatus (Vapodest 20, Gerhardt, Königswinter, Germany). The dried samples were placed into destruction tubes containing K_2SO_4 (GPR RECTAPUR®, VWR Chemicals) and $CuSO_4$ (≥ 97 %, VWR Chemicals). After adding concentrated H_2SO_4, the sample was digested for three hours at 380 °C (Kjeldatherm, Gerhardt). After adding 33 % NaOH, the formed ammonia was distilled (Vapodest, Gerhardt) into a 4 % boric acid solution (GPR RECTAPUR, VWR Chemicals). Using a standardized 0.100 M HCl solution (TitriPUR®, Merck) and bromocresol green as an indicator, the nitrogen content of the samples was determined by titration. The method was verified using acetanilide (Sigma-Aldrich, St. Louis, MO, USA) as the reference standard; the method blank was also included. Protein content was calculated with 6.25 as the conversion factor.

$$Protein\ content = nitrogen\ content \times 6.25 \qquad \text{Eq. 2.1}$$

(where 6.25 = protein conversion factor).

Crude fat content was obtained by the Soxhlet method of Nielsen (Nielsen 2010) using petroleum ether as the solvent. The solvent was then removed using a rotary evaporator (Büchi, R-200) at 50 °C.

Ash content of the insect samples was determined gravimetrically (Marshall 2010) using a muffle furnace (B 180, Nabertherm, Lilienthal, Germany) overnight at 550 °C. After the determination of the ash content, the ashes were collected in a low-density polyethylene container and stored for mineral analysis.

Chitin content was measured gravimetrically after deproteinization using 1.0 M NaOH and

subsequent demineralization with 1.0 M HCl using the procedures outlined by Liu et al., 2012.

Nitrogen-free extract (w_{NFE}) was calculated as follows (FAO 2003):

$$w_{NFE} = 100 - (w_{proteins} + w_{lipids} + w_{ash} + w_{fibre} + w_{moisture}) \quad \text{Eq. 2.2}$$

where w = mass fraction (g/100 g dry matter)

Energy content (*EC* in Kcal/100 g dry matter) was estimated using meat/fish-specific factors according to the guidelines of the FAO food nutrition paper report (FAO 2003) using the following formula:

$$EC = w_{proteins} \times 4.27 \text{ kcal/g} + w_{lipids} \times 9.02 \text{ kcal/g} + w_{NFE} \times 3.87 \text{ kcal/g} \quad \text{Eq. 2.3}$$

Amino acid analysis

It has been suggested that diet and, by extension, drying methods have little or no effect on the amino acid composition of insects (Sealey et al., 2011). It was, therefore, decided to perform such analysis only on the FD samples. Furthermore, the close similarity in the values of the protein content of different colored morphs further confirmed the redundancy in repeating the analysis on OD samples.

Before determining the amino acid profile of blanched and FD insect specimens, 25 mg of the samples were subjected to acid hydrolysis using 10.0 mL of 6.0 M HCl in 20 mL test tubes. The method described by Hewitson (Hewitson et al., 2007) was employed to determine amino acids.

Amino acid profiles were determined after acid and alkaline (for tryptophan) hydrolysis of the dried and defatted samples, followed by separation and quantification using amino acid standards on a Waters Acquity UPLC H-class system, as described. Approximately 25.00 mg of the samples were subjected to acid hydrolysis using 6.0 M HCl TITRINORM at 110 °C for 22 h in an inert atmosphere to prevent oxidation. It should be noted that asparagine and glutamine were converted into aspartic acid and glutamic acid, respectively, during the acid hydrolysis step. Tryptophan determination was achieved separately after hydrolysis with 4.0 M LiOH, EXTRAPUR

for 22 h at 110 °C. The UPLC separation of these amino acids was performed on an Acquity UPLC (Waters, Milford, MA, USA), consisting of a PDA detector, column heater, sample manager, binary solvent delivery system, and an AccQ·TagTM Ultra column (2.1 mm i.d. x 100 mm; Waters). Sample derivatization was achieved using the Waters AccQ·Tag Ultra Chemistry Package. Gradient elution was applied according to Waters AccQ·Tag Ultra method (AccQ·Tag Ultra Eluent A Concentrate (10-times diluted) (Waters); AccQ·Tag Ultra Eluent B (Waters), as well as a flow rate of 0.7 $mL \cdot min^{-1}$ and column temperature of 60 °C. To quantify the amino acids in the sample, the system was calibrated using the analytical standards of amino acids (AAS18, Sigma- Aldrich, St. Louis, MO, USA). The data were processed using the Empower 2 software (Waters, Milford, MA, USA). Analyses of all samples were performed in triplicate.

Fatty acid analysis

Fatty acid methyl esters (FAMEs) were prepared from the lipid samples by esterification in a methanolic KOH solution (0.500 M) with the addition of a 20 % BF_3-methanol solution (Sigma-Aldrich, St. Louis, MO, USA) according to Joseph & Ackman (1992). In brief, 0.025 g of extracts of the insect oils were weighed. Using 0.500 M sodium methoxide (VWR International BVBA, Leuven, Belgium) and 10 % methanolic boron trifluoride (BF_3) solution (Sigma- Aldrich, St. Louis, MO, USA), the acylglycerols and free fatty acids in the sample were (trans)esterified into fatty acid methyl esters (FAMEs); 1.0 µL of the esterified samples or fatty acid methyl ester standards (Sigma-Aldrich, St. Louis, MO, USA) were injected into the gas chromatograph (Agilent 7820A-5977E GC-MSD, Agilent Technologies, Santa Clara, CA, USA). Using methyl tricosanoate (Sigma Aldrich, St. Louis, MO, USA) as the internal standard, the MassHunter software (Agilent Technologies, Santa Clara, CA, USA) was used to compute the concentrations of fatty acids as % of total fatty acids. Analyses of all samples were performed in triplicate.

Table 2. 1 *GC-MS parameter settings*

Parameter	Settings
Volume sample injected	1.0 µL
Ratio split injector	10:1
Carrier gas	Helium
Temperature	40 °C
Pressure	12 psi
Capillary column	Sigma-Aldrich SLB-IL60
Stationary phase (SP)	1,12-di(tripropylphosphonium)dodecane-*bis*-(trifluoromethylsulfonyl)imide
Length	30 m
Diameter	0.25 mm
Thickness SP	0.2 µm
Oven	
	Start: 4 minutes at 40 °C | during analyses: increase of 5°C/minute | end: 280 °C
Ion source MS	Electron impact
Scan type MS	Single ion monitoring
Software	MassHunter

Methyl tricosanoate was used as the internal standard to determine the fatty acid composition.

2.2.2.4 Mineral and trace elemental analysis

To determine the mineral composition, the ashes obtained during the determination of ash content were dissolved in 65 % HNO_3 (VWR Chemicals, Fontenay-sous-Bois, France) and then diluted ten-fold to appropriate concentrations based on the mineral element and the resulting calibration curve. The calibration curves were obtained using standard solutions from certified stock solutions containing 1000 ppm of the elements investigated (Chem Lab, Zedelgem, Belgium). The contents of each investigated element (sodium, calcium, phosphorus, potassium, magnesium, iron, zinc, manganese, copper, and selenium) were determined by inductively coupled plasma optical emission spectrometry (ICP-OES) measurements (Optima 4300™ DV ICP-OES, Perkin Elmer, Wellesley, MA, USA). The parameter settings for the ICP-OES are outlined in Table 2.1 below. Data were processed using the WinLab 32 software. For each element, two readings (replicates) were measured by the spectrometer, and the mean value was obtained. For each element, the limit of quantification (*LOQ*) was calculated as follows:

$$LOQ = 10 \times \frac{SE}{b}$$ Eq. 2.4

Where SE = the standard error of the regression for each element. b = the value of the slope of the calibration curve for each element.

Table 2. 2 *ICP-OES working conditions' settings*

Sampler		Spectrometer	
Parameter	Setting	Parameter	Setting
Plasma conditions	Same for each element	Pulsed gas flow	Normal
Type Aerosol	Wet	Spectral profiling	No
Start nebulizer	Directly	Resolution	Fixed (normal)
Sample flow (mL/min)	1.5	Reading time (s)	Automatic
Plasma sight (all)	Radial	Break time (s)	30
Plasma sight (element)	Axial	Replicas (#)	2
Source delay (s)	30	Software	WinLab 32
Flush time (s)	10		

Elemental limits of quantification (LOQ) in ppm: Na: 4.97; Ca: 2.16; K: 2.63; Mg: 4.83; Zn: 0.14; Fe: 0.22; P: 5.94; Cu: 0.02; Mn: 0.05. Se: 0.001 # = Number

2.2.3 Statistical analysis

To elucidate the effect of the drying method (post blanching) on the nutritional composition of the brown and green bush cricket morphs, a Mann–Whitney U-test ($\alpha = 0.05$) was performed on the mean values of oven-dried (OD) and freeze-dried (FD) bush cricket samples. The statistical package used was GraphPad Prism Version 5.00 for Windows (GraphPad Software, La Jolla, CA, USA). The purple sample was not included in the statistical analyses as the sample was too small to obtain significant amounts for both drying methods; as such, only FD purple samples were analyzed.

2.3 Results

2.3.1 Proximate composition

The FD materials had a moisture content of 4.55 %, 4.91 %, and 4.24 % for the green, brown, and purple-colored morphs, respectively. The final water content for the green and brown OD samples was 4.33 % and 4.50 %, respectively.

The changes in moisture content, crude fat, crude protein, chitin fibers, and ash are depicted in Table 2.3. In general, neither drying method seems to have had any significant effect on the proximate composition of the bush crickets.

Table 2. 3 *Proximate compositions of oven-dried (OD) and freeze-dried (FD) green, brown, and purple R. differens (in % on a dry matter basis). Each value expresses the mean ± SD of triplicate determinations.*

Samples	Green OD	Brown OD	Mean OD	Green FD	Brown FD	Purple FD ¤	Mean FD ö	Orthoptera §
Moisture content	4.33	4.50	4.42	4.55	4.91	4.24	4.42	Not available
Crude fat	34.95 ± 1.01	36.11 ± 0.34	**35.53 ± 0.82[a]**	33.28 ± 0.38	36.84 ± 0.62	38.8 ± 0.40	**35.56 ± 1.82[a]**	**13.41**
Crude protein	45.53 ± 0.84	52.90 ± 0.86	**47.7 ± 3.09[a]**	44.99 ± 0.74	47.83 ± 1.62	50.50 ± 0.35	**46.41 ± 2.01[a]**	**61.32**
Chitin	14.86 ± 0.58	14.22 ± 0.73	**13.4 ± 1.13[a]**	9.79 ± 1.06	10.81 ± 1.17	11.2 ± 1.11	**11.33 ± 0.74[a]**	**9.55**
Ash	3.93 ± 0.03	5.38 ± 0.00	**4.66 ± 1.03[a]**	4.92 ± 0.07	4.66 ± 0.13	4.32 ± 0.32	**4.79 ± 0.18[a]**	**3.85**
NFE	1.39	0.01	**0.7 ± 0.98[a]**	3.97	0.01	0.01	**1.99 ± 2.80[a]**	**12.98**
Energy (Kcal/g)	510	539	**524 ± 20.6[a]**	501	537	566	**519 ± 25[a]**	**426.25**

[a] Mean values (in bold) with the same superscript letter across rows are NOT significantly different at alpha = 0.05

¤ *Due to its small sample size, the purple-colored R. differens were only freeze-dried.*

§ *Orthoptera average values as compiled by Rumpold & Schluter 2013.*

OD=oven-dried, FD=freeze-dried; NFE, nitrogen-free extract.

It was found that apart from the purple morph, in which the protein, fat, and ash content differed, there was slight variation ($p > 0.99$) in the green and brown *R. differens* across both drying methods.

This study obtained average water contents of 53.72 g/100 g fresh weight before drying.

Overall, *R. differens* showed higher crude fat contents (35.5 %) than most orthopterans (13.41 %). However, this was compensated by lower average protein content (47.75 % for OD and 46.41 % for FD).

The average protein content of *R. differens* was found to be 46.41–47.7 %, Dry matter

The fiber value (predominantly chitin) is close to the mean values (13.4 % and 11.33 %) for either drying method obtained in the current study. As before, no significant differences were observed between morphs, but a slight influence of drying mode was noticed.

Average ash content after the oven and freeze-drying was 4.66 % and 4.79 %, respectively.

Nitrogen-free extract (NFE) levels, essentially representing carbohydrates (but not chitin), are usually low in insects. In the current study, NFE levels were low (0.7–1.99 %) but were highest in the green morphs (1.39–3.97 %).

Due to their high fat content, the average energy content (539 and 519 Kcal/g) for this study estimated by calculation (Finke 2002) was beyond the mean values reported for insects

When data are expressed on a dry matter basis, the sum of proximates should exceed the expected level of > 95 g/100 g edible portion (Charrondière et al., 2013). This interval was true for the sum of proximates calculated in the current study.

2.3.2 Fatty acid composition

The fatty acid composition of the samples examined is shown in Table 2.4 below. Similar values were obtained for all fatty acids irrespective of the drying method. Oleic (44 %), palmitic (28 %), and linoleic (14 %) acids were the major fatty acid components of *R. differens* contributing up to 86 % of the total fatty acids present. For FD and OD samples, respectively, the primary saturated fatty acids (SFA) were palmitic ((C16:0), 28.2 %, and 27.8 %) and stearic acid ((C18:0), 7.88 %, and 8.45 %). In contrast, the most dominant unsaturated fatty acids (UFA) were oleic ((C18:1), 44.3 % and 44.0 %) and linoleic acid ((C18:2), 14.0 % and 14.1 %).

Table 2. 4 *Fatty acid composition (% of total fatty acids) of FD and OD R. differens. Values are expressed as the mean of triplicates ± SD*

Fatty Acid	Type	Green FD	Brown FD	Mean FD *	Brown OD	Green OD	Mean OD ¤
Decanoic Acid (C10:0)	SFA	0.07 ± 0.04	0.07 ± 0.03	**0.07 ± 0.01** [a]	0.06 ± 0.01	0.07 ± 0.04	**0.07 ± 0.01** [a]
Lauric acid (C12:0)	SFA	0.19 ± 0.01	0.19 ± 0.01	**0.19 ± 0.01** [a]	0.17 ± 0.01	0.16 ± 0.02	**0.17 ± 0.01** [a]
Myristic Acid (C14:0)	SFA	1.14 ± 0.10	1.13 ± 0.07	**1.14 ± 0.01** [a]	1.11 ± 0.06	1.08 ± 0.09	**1.10 ± 0.02** [a]
Kyriologic acid (C14:1)	MUFA	0.07 ± 0.04	0.08 ± 0.03	**0.08 ± 0.01** [a]	0.07 ± 0.01	0.07 ± 0.04	**0.07 ± 0.01** [a]
Pentadecanoic acid (C15:0)	SFA	0.11 ± 0.04	0.12 ± 0.03	**0.12 ± 0.01** [a]	0.11 ± 0.01	0.12 ± 0.04	**0.12 ± 0.01** [a]
Palmitic acid (C16:0)	SFA	28.2 ± 0.79	28.1 ± 0.65	**28.2 ± 0.11** [a]	27.3 ± 0.70	28.2 ± 1.09	**27.8 ± 0.62** [a]
Palmitoleic acid (C16:1)	MUFA	1.71 ± 0.16	1.68 ± 0.09	**1.70 ± 0.02** [a]	1.64 ± 0.11	1.62 ± 0.11	**1.63 ± 0.01** [a]
Heptadecanoic acid (C17:0)	SFA	0.15 ± 0.04	0.15 ± 0.03	**0.15 ± 0.01** [a]	0.14 ± 0.01	0.15 ± 0.05	**0.15 ± 0.01** [a]
Stearic acid (C18:0)	SFA	7.92 ± 0.67	7.84 ± 0.43	**7.88 ± 0.06** [a]	8.60 ± 0.63	8.30 ± 0.46	**8.45 ± 0.21** [a]
Oleic Acid (C18:1)	MUFA	44.4 ± 1.86	44.3 ± 0.72	**44.3 ± 0.11** [a]	43.7 ± 1.03	44.3 ± 1.48	**44.0 ± 0.42** [a]
Linoleic acid (C18:2) [n6]	PUFA	14.0 ± 1.50	14.0 ± 1.41	**14.0 ± 0.04** [a]	14.4 ± 1.63	13.9 ± 0.67	**14.1 ± 0.32** [a]
Linolenic Acid (C18:3) [n3]	PUFA	1.39 ± 0.14	1.44 ± 1.08	**1.42 ± 0.04** [a]	1.47 ± 1.16	1.43±0.13	**1.45 ± 0.03** [a]
Arachidonic acid (C20:4) [n6]	PUFA	0.39 ± 0.01	0.72 ± 0.56	**0.56 ± 0.23** [a]	0.93 ± 0.43	0.44 ± 0.02	**0.69 ± 0.35** [a]
EPA (C20:5) [n3]	PUFA	0.14 ± 0.09	0.23 ± 0.14	**0.19 ± 0.06** [a]	0.30 ± 0.17	0.15 ± 0.10	**0.23 ± 0.11** [a]
TOTAL SFA		37.82	37.58	37.7	37.52	38.08	37.8
TOTAL MUFA		46.19	46.02	46.11	45.43	46.00	45.72
TOTAL PUFA		15.99	16.4	16.20	17.05	15.92	16.49
TOTAL UFA		62.18	62.42	62.3	62.48	61.92	62.2
PUFA/SFA ratio		0.42	0.44	0.43	0.45	0.42	0.44
Total n6		14.46	14.73	14.60	15.28	14.34	14.81
Total n3		1.53	1.67	1.60	1.77	1.58	1.68
n6/n3 ratio		9.45	8.82	9.12	8.63	9.08	8.84
EFA		15.39	15.44	15.42	15.87	15.33	15.6

[a] Mean values (in bold) with the same superscript letter across rows are NOT significantly different at alpha = 0.05

OD = oven-dried; FD = freeze-dried; EPA = methyl-5, 8, 11, 14, 17-eicosapentaenoic acid; n6 = omega-6 fatty acid; n3 = omega-3 fatty acid; PUFA = polyunsaturated fatty acid; SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; UFA = unsaturated fatty acid; EFA = essential fatty acid = [C18:2 + C18:3]

¤: mean ± SD of combined color morphs

The present study revealed that the samples studied were rich in polyunsaturated fatty acids (PUFA), especially linolenic and linoleic acids. These essential fatty acids (EFA), including linoleic and alpha-linolenic acids, were present in appreciable quantities (15.42 % and 15.60 %) for FD and OD samples, respectively. The mean PUFA/SFA ratio was 0.44, *R. differens* and the n6:n3 ratio was 9:1 on average.

2.3.3 Mineral composition

Table 2.5 depicts the mineral composition of the *R. differens.* Again, there were no significant differences ($p > 0.88$) between drying methods. A trend evident in Table 2.5 was the higher mineral content in FD relative to OD samples, except for Zn (14.6 and 14.2 mg/100 g) and Cu (1.66 mg/100 g for both.

Average calcium levels were high, 895.7 and 1035 mg/100 g dry matter (DM) for OD and FD, respectively. Potassium was the next most abundant mineral (779 and 816 mg/100 g DM for OD and FD, respectively. The magnesium content was elevated at 145 and 161 mg/100 g DM for OD and FD, respectively. The samples evaluated in this study were also high in phosphorus 652.31–685.9 mg/100 g DM for OD and FD, respectively.

The trace minerals were also present in reasonable amounts (Table 2.5), particularly iron and zinc. Iron levels of between 216 and 220 mg/100 g were measured in the current study.

Also, zinc concentrations of 14.6 and 14.2 mg/100 g) were observed for the two drying methods.

Another trace metal, manganese (Mn), was present at levels ranging between 7.4 and 8.3 mg/100 g. *R. differens* was found to be a significant source of selenium in the brown morphs within 40–50 µg/100 g, which exceeds the adult RDI of 26–36 µg.

Table 2. 5 *Mineral composition (mg/100 g dry matter) of OD and FD R. differens. Results are expressed as the mean ± SD; (n = 2).*

Mineral	OB	OG	Mean OD	FG	FB	Mean FD
Sodium	50.79 ± 0.02	57.2 ± 0.01	**54.01 ± 4.55**	78 ± 0.01	60.2 ± 0.01	**69.1 ± 12.64**
Potassium	834.4 ± 0.04	724.0 ± 0.06	**779.19 ± 78.0**	806 ± 0.08	826.5 ± 0.06	**816.4 ± 14.27**
Calcium	1124 ± 0.05	967.6 ± 0.06	**895.67 ± 323**	1023 ± 0.08	1047 ± 0.10	**1034.7 ± 17.18**
Magnesium	168.5 ± 0.01	123.0 ± 0.01	**145.75 ± 32.1**	160 ± 0.01	161.8 ± 0.01	**161.0 ± 1.06**
Zinc	14.24 ± 0.01	15.0 ± 0.01	**14.63 ± 0.56**	13 ± 0.01	15.2 ± 0.01	**14.2 ± 1.46**
Iron	258.7 ± 0.01	174.4 ± 0.01	**216.56 ± 59.6**	217 ± 0.05	222.8 ± 0.01	**220.1 ± 3.83**
Phosphorus	693.9 ± 0.03	610.7 ± 0.06	**652.31 ± 58.8**	680 ± 0.05	692.1 ± 0.06	**685.9 ± 8.73**
Copper	1.67 ± 0.01	1.6 ± 0.01	**1.66 ± 0.01**	1 ± 0.01	1.8 ± 0.01	**1.66 ± 0.23**
Manganese	8.77 ± 0.01	6.0 ± 0.01	**7.40 ± 1.94**	8 ± 0.01	8.4 ± 0.01	**8.29 ± 0.21**
Selenium	0.05 ± 0.01	<LOQ	<LOQ	<LOQ	0.04 ± 0.02	<LOQ

[a] *. Mean values (in bold) with the same superscript letter across rows are NOT significantly different at alpha = 0.05*

< LOQ = below the limit of quantification

OG=oven-dried, green, OB=oven-dried, brown; FG=freeze-dried, green; FB=freeze-dried, brown

2.3.4 Amino acid composition

Table 2.6 comprises the amino acid content of the FD bush crickets, with the amino acids commonly found in proteins being identified in these samples. During acid hydrolysis, asparagine (Asn) and glutamine (Gln) were converted into aspartic acid (Asp) and glutamic acid (Glu), respectively.

As expected, given the similar overall protein content, amino acid compositions were similar. Except for methionine and cysteine, the essential amino acids were present at concentrations that met and surpassed the levels recommended for humans by the WHO/FAO. Glutamic acid (+ glutamine), alanine, and aspartic acid (+ asparagine) were the most abundant amino acids.

Table 2. 6 *Average amino acid content of the freeze-dried only green, brown and purple morph (mg/g protein). Results are expressed as the mean ± SD; (n = 3)*

Amino acid*	Green	Brown	Purple	WHO/FAO+
His	24.66 ± 0.12	25.80 ± 0.36	27.00 ± 0.43	15.0
Ser	48.05 ± 0.90	48.92 ± 0.63	50.59 ± 0.20	--
Arg	57.31 ± 3.49	55.04 ± 3.04	61.77 ± 3.99	--
Gly	60.72 ± 0.90	64.86 ± 0.79	59.38 ± 0.03	--
Asp	96.79 ± 3.20	92.95 ± 3.14	95.00 ± 3.67	--
Glu	123.3 ± 4.71	124.7 ± 4.22	122.6 ± 4.10	--
Thr	41.75 ± 0.58	42.98 ± 0.38	42.78 ± 0.45	23
Ala	117.0 ± 4.96	117.3 ± 4.46	104.73 ± 4.56	--
Pro	62.61 ± 1.65	64.63 ± 1.74	61.34 ± 1.64	--
Cys	5.18 ± 2.13	2.86 ± 2.53	6.88 ± 2.69	6.0
Lys	54.77 ± 3.89	53.80 ± 3.26	53.16 ± 3.29	45
Tyr	52.53 ± 4.46	49.57 ± 4.16	55.19 ± 4.15	--
Met	6.99 ± 8.76	1.39 ± 8.26	13.83 ± 8.82	16
Val	65.75 ± 0.89	67.06 ± 0.40	63.48 ± 0.07	39
Ile	47.61 ± 1.04	49.17 ± 1.20	46.91 ± 1.81	30
Leu	92.48 ± 1.84	95.22 ± 1.09	90.30 ± 1.52	59
Phe	33.79 ± 0.62	35.48 ± 0.83	36.88 ± 0.52	--
Trp	8.66 ± 0.28	8.26 ± 0.14	9.38 ± 0.32	6.0
E	376	379	384	
N	624	621	617	
E/N	0.60	0.61	0.62	
E + N	1000	1000	1001	
E/(E + N)	0.38	0.38	0.38	

** Amino acid analyses was not subject to statistics due to unavailability of oven-dried samples*
E = essential amino acid; N = nonessential amino acids; + amino acid requirements in humans (FAO, 2003)];
– data not available

2.4 Discussion

R. differens is a swarming bush cricket attracting much interest due to its nutritious reputation. This chapter explains the impact of both oven and freeze-drying after blanching on the proximate fatty acid, mineral, and amino acid compositions of this insect. These samples were wild-harvested. As such, what they had been feeding on was unknown at the time of analysis.

It was found that apart from the purple morph, in which the protein, fat, and ash content differed, there was little variation ($p > 0.99$) in the green and brown bush crickets across both drying methods. These observations agree with Kinyuru et al., (2010b), who reported similar values and found no difference in protein content and only slight variations in other parameters. The overall variation could also have arisen from the insects' diets since they were captured at different locations and pooled together. Since they were all caught in the adult stage, the stage of development could not have accounted for such variation, but the sex and reproductive state of insects have been shown to affect nutrient composition (Finke 2002).

The results for energy content, ash, and chitin presented here are consistent with the findings of Rumpold & Schluter (Rumpold & Schluter 2013), who compiled the proximate composition of 51 orthopterans (see Table 2.3) from the literature.

The moisture content for the insect morphs has been reported in other studies to vary between 66 % and 71 % (Kinyuru et al., 2010b), values slightly higher than those obtained in this study (53.72 g/100 g fresh weight). This variation could be explained by using different drying times and temperatures to yield different moisture values for the same species. Also, adult insects tend to have less moisture than their nymph counterparts. This trend was evident, as all samples evaluated were adults and had moisture contents lower than expected for grasshoppers (Aman et al., 2016). The low moisture content of the fresh bush crickets, when compared with more conventional meat, such as chicken and beef, would imply a higher content of dry matter. By extension, this means that *R. differens* constitutes a denser source of nutrients since a relatively more significant portion of its weight is represented by nutrients than other animal food sources. Based on laboratory experience, the drying conditions were set to obtain moisture below 5 % for the insect samples. These low values were carefully

chosen to prevent mold growth during long-term storage in case of power failures. Also, low moisture contents facilitated fat extraction using the Soxhlet procedure.

Fat is known to exude moisture evaporation during oven drying, which increases the effect of lipid and fatty acid losses. This phenomenon has been observed with oven-dried fish compared to other preservation methods, as outlined by (Chukwu 2009). However, the drying method had no significant effect on the crude fat content in our case. The absence of fat reduction during oven drying in our study is explained by the lower temperature used (60 ^{0}C, compared to 110 ^{0}C) in preparing dried tilapia fish (Chukwu 2009).

Overall, *R. differens* showed higher crude fat contents (35.5 %) than most orthopterans (13.41 %). However, this was compensated by lower average protein content (47.75 % for OD and 46.41 % for FD) in comparison to several other insects of this order (61.32 %) (Rumpold & Schluter 2013). In a review, data compiled by Aman et al., (2017) reveal that *R. differens* has the highest fat and lowest protein content that has been reported in the order of Orthoptera. The high lipid content of the mean value of *R. differens* morphs (35.50 %) accounts for the insects' palatability when fried or roasted, as mentioned by other authors (Womeni et al., 2012). This value is lower than the 46.2–48.2 % obtained by other researchers for the same species (Kinyuru et al., 2010b).

The lipid contents were higher than chicken, fish, and unprocessed milk but similar to raw chicken eggs (FAO 2012). When compared to beef or fish, these insects had high lipid contents and are therefore also good energy sources. Indeed, lipids are necessary for food because they increase palatability and retain the flavor of food, as well as enhance vitamin A, D, E, and K levels (FAO 2008).

The higher fat content of the FD purple morph compared to the other color morphs probably suggests why the purple bush crickets are anecdotally considered to be more delicious. However, larger sample sizes and more studies would be required to ascertain this fact.

The protein content of most insect species is very high, with many ranging from 60 to 85 % (Belluco et al., 2013; Finke, 1989). Orthopterans (crickets, grasshoppers, and locusts) averaged 60 % protein content, the highest among all edible insect Orders, compared to the isopteran (termites) with just 35 % (Rumpold & Schluter 2013). The average protein content of *R.*

differens was found to be 46.4–47.7 %, in agreement with similar studies on this species (43.1–44.3 %) (Kinyuru et al., 2010b). *R. differens* tends to contain midrange levels of protein relative to other edible insects. According to WHO/FAO 2007, the requirement for food to be labeled 'high in protein' is a 10 g/100 g edible portion. This limit, like for most insects, is far exceeded by *R. differens* proteins, and this edible insect can thus be considered a good protein source. The observed high protein and fat contents, which comprise more than 75 % of the dry mass, justify the cultural perception of the superior nutritional value attributed to *R. differens* (Kinyuru et al., 2010; Mmari et al., 2017; Fombong et al., 2017).

Insects are known to contain significant amounts of fiber (Finke 2008), and he suggested that the fiber in insects is predominantly composed of chitin. Chitin is present only in the insects' exoskeleton and is expected to be present in relatively small amounts. Rumpold & Schlüter (2013) reported 9.55 % as the average fiber content for grasshoppers. This fiber value (predominantly chitin) is close to the mean values (13.4 % and 11.33 %) for either drying method obtained in the current study. As before, no significant differences were observed between morphs, but a slight influence of drying mode was noticed. However, much lower values were reported by other colleagues (Kinyuru et al., 2010b) for the same species. *R. differens*' high chitin content might, therefore, present potential value to both the food and pharmaceutical industries (Liu et al., 2012).

The average ash contents were almost two-fold more than what has been previously reported for the same species (2.7 %) (Kinyuru et al., 2010b). The use of different analytical methods could explain this difference. Higher amounts of minerals were also observed, as shown in Table 2.5 but were nonetheless consistent with the average for 51 orthopterans compiled from the literature (Rumpold & Schluter 2013). Higher ash values of 8.55 % and 9.36 % in grasshoppers have also been mentioned in other studies (Aman et al., 2017; Rumpold & Schluter, 2013).

The average NFE values obtained for each drying method (i.e., 0.7 % and 2.0 % for OD and FD, respectively) were significantly lower than previously reported for most Orthoptera, being approximately 13 % (Rumpold & Schluter 2013). Ramos et al., (2012) obtained similarly low values for NFE in the house cricket, *Acheta domestica.* In contrast, extremely high values of up

to 63.20 % have been reported for the grasshopper *Zonocerus variegatus* (Banjo et al., 2006). Due to their high fat content, the average energy content (539 and 519 Kcal/g) for this study estimated by calculation (Finke 2002) was greater than the mean reported for insects. The above statement is true for other lipid-rich insects, particularly caterpillars, palm weevil larvae, and termites (Bukkens 1997). The values mentioned above obtained for proximate composition in our study were more consistent with those obtained for a related Ugandan species, *Ruspolia nitidula* (Ssepuuya et al., 2016).

The fatty acid composition of the samples examined is shown in Table 2.4. These fatty acids are mainly stored in the insect's fat body and comprise more than 90% of the total lipid content of the fat body (Finke 2013). Similar values were obtained for all fatty acids irrespective of the drying method. Oleic (44 %), palmitic (28 %), and linoleic (14 %) acids were the major fatty acid components of *R. differens* contributing up to 86% of the total fatty acids present; a trend that Kinyuru also observed (Kinyuru et al., 2010b) for the same species. For FD and OD samples, respectively, the primary saturated fatty acids (SFA) were palmitic (C16:0, 28.2 % and 27.8 %) and stearic acid (C18:0, 7.88 % and 8.45 %). In contrast, the most dominant unsaturated fatty acids (UFA) were oleic (C18:1, 44.3% and 44.0 %) and linoleic acid (C18:2, 14.0 % and 14.1 %). These values were again consistent with other studies (Bukkens, 1997; Finke, 2013; Yang et al., 2006). The present study revealed that the samples investigated were rich in polyunsaturated fatty acids (PUFA), especially linolenic and linoleic acids. Previous studies (Kinyuru et al., 2010b) did not detect lauric, arachidonic, or EPA fatty acids in their samples. They occur in low proportions and are not present in easily quantifiable amounts. PUFA/SFA ratios lower than 0.33 are not desirable as they likely lead to atherogenesis. In contrast, PUFA/SFA ratios greater than 0.8 are associated with desirable levels of cholesterol and reduced risk of coronary heart diseases (Mann, 1993). With a mean PUFA/SFA ratio of 0.44, *R. differens* was significantly higher in SFA than PUFA, though still above the cut-off point for triggering undesirable effects. Another health index associated with fatty acids is the omega-6/omega-3 fatty acid ratio (n6:n3), with a ratio of 3:1 considered optimal (Simopoulos, 2008). The current study gave an n6:n3 ratio of 9:1 on average, which is regarded as high. Lower ratios of omega-6/omega-3 fatty acids are desirable for reducing the risk for an array of chronic diseases of high occurrence

in both developed and developing countries (Mann 1993). Previous research revealed significant variation in n6:n3 ratios due to omega-3 fatty acid differences (Aman et al., 2017), thus confounding comparisons with existing literature. Essential fatty acids (EFA), including linoleic and alpha-linolenic acids, were present in appreciable quantities (15.42 % and 15.60 %) for FD and OD samples, respectively.

Finke (2002; 2013) concluded that, for a given insect species and developmental stage, the fatty acid composition is affected by environmental factors such as temperature, light, and humidity. These influences may also explain the variations observed in this study.

The mineral composition was quite variable, with high standard deviations were obtained for the average drying values of some minerals. This variability has been reported by other researchers, as well (Nowak et al., 2016, Oonincx & Dierenfeld, 2012) and attributed to a small sample size or contamination. The insects were high in most macro minerals, as well as trace minerals, which is true for most edible insects (Finke 2002; Banjo 2006; Christensen et al., 2006). Except for sodium (Na), the mineral values obtained in this study were higher than values previously reported (Kinyuru et al., 2010b) for this bush cricket species.

Average calcium (Ca) levels were high, being 895.7 and 1035 mg/100 g dry matter (DM) for OD and FD, respectively, i.e., values well below the recommended daily intake (RDI) for adult humans (1300 mg) (FAO 2007; FAO 2012). According to WHO/FAO 2007, foods containing Ca levels above 240 mg/100 g edible portion are considered 'high in calcium.' These results suggest that this edible insect could serve as an alternative source of calcium, especially for lactose intolerant or soy-allergic victims. This feat is entirely in contrast to statements made by other researchers that insects are low in Ca (Finke, 2002; Finke, 2013; Oonincx & Dierenfeld, 2012; Barker et al., 1998). The highest value previously reported for Ca in an edible insect was 2010 mg/100 g in the housefly *Musca domestica* (Linnaeus). However, some orthopteran species have been found to contain high Ca values (1290 mg/100 g in *Acheta domesticus* (Linnaeus), as well (Rumpold & Schluter, 2013). This could be attributed to the high Ca content of their gut (Ramos et al., 2012).

Potassium levels were found to be very high (779 and 816 mg/100 g DM for OD and FD, respectively) compared to those reported for other edible insects (Rumpold & Schluter, 2013),

though they did not meet the adult RDI of 4700 mg (FAO, 2012). These values were less than half those obtained for *Zonocerus variegatus* (2030 mg) (Ademolu et al., 2010), which are by far the highest recorded K values amongst the grasshopper family.

Magnesium was also elevated (145 and 161 mg/100 g DM) compared with previously reported data for *R. differens* (33.1–33.9 mg/100 g) (Kinyuru et al., 2010b). Comparable values for other orthopterans, such as cricket *Acheta domesticus*, have been reported in the literature (Ademolu et al., 2010). Adult RDI for Mg lies within 220–260 mg; thus, consuming about 200 g (100 fresh bush crickets) in a day will surpass the Mg RDI.

Like most edible insects, the samples evaluated in this study were also found to be high in phosphorus 652.31 – 685.9 mg/100 g DM. It has been suggested in the literature that a Ca : P ratio of 1:1 to 1:2 is acceptable in food and feed for most vertebrates (Anderson 2000). This ratio incorporates the one obtained in this study (1:1.5). Thus, the inclusion of *R. differens* in feed for other animals could balance the Ca : P ratio required for most diets.

The trace minerals were also present in reasonable amounts (Table 2.5), particularly Fe and Zn, which concurs with other reports (Anand et al., 2008; Christensen et al., 2006; DeFoliart, 1992). Consumption of mineral-rich insects could help mitigate Fe and Zn deficiencies, which are prevalent in developing countries (Christensen et al., 2006). That Fe was the most prominent trace mineral was also true for Kinyuru (Kinyuru et al., 2010b), though much higher Fe levels of between 216 and 220 mg/100 g were measured in the current study. The RDI of Fe for female adults stands between 20 and 59 mg, depending on bioavailability (FAO 2001). When compared to the Fe levels present in various red meats (1.1–3.3 mg/100 g) (Williams 2007), *R. differens* is a superabundant source of Fe. However, the bioavailability of Fe is not known.

Very similar zinc (Zn) concentrations (being 14.6 and 14.2 mg/100 g) were observed for the two drying methods, and these were comparable to levels reported previously (Kinyuru et al., 2010a; Oonincx & Dierenfeld, 2012). The RDI of Zn for adults is between 4.9 and 7.0 mg (moderate bioavailability) (FAO 2001); as such, either FD or OD preparation of *R. differens* would provide a relatively rich source of Zn.

Regarding the amino acid composition, except for methionine and cysteine, the essential amino acids were present at concentrations that met and surpassed the levels recommended

for humans by the WHO/FAO. Glutamic acid (+ glutamine), alanine, and aspartic acid (+ asparagine) were the most abundant amino acids.

DeFoliart (1992) showed that insect proteins are low in methionine and cysteine. This feature was confirmed in the current study. However, the opposing belief that insect proteins are high in threonine and lysine was not observed. Nonetheless, not all insects are the same in their nutritional contributions.

Protein quality and nutritional value are determined by the amino acid composition and digestibility of the protein fraction of foods (Van Huis, 2013; Charrondière et al., 2013). A crucial parameter for determining protein quality is the ratio of essential (E) and nonessential (N) amino acids. According to the FAO/WHO criteria, E/(E + N) should be about 40 % with E/N = 0.6 (FAO 2012). FAO/WHO/European food safety authority (EFSA) dietary criteria state that adults should consume 0.66 g/kg of body weight of protein daily (EFSA 2012). In the current study, an E/N ratio of 0.61 and E/(E + N) value of 38 % indicate that the amino acid composition of *R. differens* satisfies these criteria.

The *R. differens* amino acid profile was high in leucine, lysine, and threonine. In the predominantly cereal-based diet typical in developing nations, lysine and threonine are particularly limiting. Therefore, the inclusion of this insect species into the staple diets of these nations would be expected to improve the nutritional status of their population significantly.

In practice, freeze-drying would not be considered an economically feasible technique to implement in Sub-Saharan Africa, given its high investment costs compared to purchasing an oven dryer. So the fact that after blanching, both methods yield similar results would allow local farmers and food/feed manufacturers to use the more affordable oven-drying. An initial partial solar drying combined with oven drying could lower oven drying energy costs.

For the typical household struggling with undernourishment, of course, sun drying will always remain the most viable drying mode.

Future studies that would carry out unbiased hedonic tests to confirm the tastier nature of the purple morph are needed. Additionally, given the high amounts of essential amino acids, the umami taste may well contribute to the taste of *nsenene*, but more work is required to elucidate this as well.

2.5 Conclusions

Oven drying blanched *R differens* deliver the same nutritional (proximate, mineral, and fatty acid) quality and composition as freeze-drying; either drying method could be used to preserve nutrient properties. Differences in nutritional composition were attributed to the different morphs rather than the drying method employed. Importantly, all of the essential amino acids required by humans were present.

Insect samples from both drying approaches provided good sources of macronutrients, as well as minerals. They are, therefore, deemed suitable as alternative or complementary food sources to alleviate undernutrition, especially among vulnerable groups (i.e., women and children) in developing countries. The rare purple morph yielded similar nutritional values as the green and brown morphs, but slightly elevated fat content may explain anecdotal perceptions that this phenotype is 'tastier.'

CHAPTER THREE

Affordable Processing of Edible Orthopterans Provides a Highly Nutritive Source of Food Ingredients

This chapter was redrafted[2] after Fombong FT, Kinyuru J, Ng'ang'a J, Ayieko M, Tanga CM, Vanden Broeck J, Van Der Borght M.

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[2]The complete content of this paper (Fombong et al., 2021) was included in Chapter 3, with slight alterations to maintain a logical flow of this dissertation. As the first author, Fombong F.T. contributed to the majority of the parts described in this work, from conceptualization, sample acquisition, experimental work, analysis, and interpretation of the data to the writing of the paper.

3.1 Introduction

In the previous chapter, *R. differens* as a very nutritious tettigoniid was established. More so, it was determined that oven-dried *R. differens* yielded similar nutritional quality as the less affordable freeze-drying method. Also, given the known challenges of seasonality as explained in chapter one and the difficulty with rearing *R. differens* (captured later in chapter eight). Other easy-to-rear orthopterans are proposed as alternatives to eating *R. differens*. This chapter sets out to use only oven drying and compare the nutritional quality of three other orthopterans (*Schistocerca gregaria, Locusta migratoria, a*nd *Gryllus bimaculatus*) to that of oven-dried *R. differens* established in chapter two.

The desert locust, *Schistocerca gregaria* Forsskål, and the migratory locust, *Locusta migratoria* Linnaeus, are sporadic pests of historical importance in many countries across Africa, the Middle East, the Indo-Pakistan Peninsulas, and Europe, where they cause substantial crop losses during plagues (Cullen et al., 2017; Pener & Yerushalmi, 1998). They are commonly eaten as an essential food source by many marginalized communities (Meinzingen, 1993; Meyer-Rochow, 1975; Mohamed, 2016). For example, in Sudan, locusts are either eaten raw or prepared by boiling, frying, or sun-drying (Mohamed, 2016). The most recent locust outbreak started in the horn of Africa in December 2019 ("The Year of the Locust - The Mail & Guardian," 2020.), with countries in this region (Ethiopia, Kenya, Somalia, Uganda, and South Sudan) still battling their worst locust outbreak in decades (The Year of the Locust—The Mail & Guardian, 2020). Swarming locusts cause massive damage due to voracious feeding on crops, pastures, and any green vegetation, thereby significantly affecting the livelihoods of people. Studies have demonstrated that, in many countries, farmers forgo pesticidal control of locusts in favor of harvesting and selling them, generating much-needed income for their households compared to the sale of food crops (Jones, 2016).

Numerous orthopteran species with pest statuses are consumed in South America and Africa. For example, in Mexico, massive hand-picking of the chapuline grasshoppers (*Sphenarium purpurascens)* that infested alfalfa fields played a significant role in decreasing environmental damage while generating an additional source of nutrition and income through sales for human consumption (Cerritos & Cano-Santana, 2008). Several species of locusts are widely

eaten in Sub-Saharan Africa, including *S. gregaria*; *L. migratoria*; the red locust, *Nomadacris septemfasciata*; and the brown locust, *Locustana pardalina* (Van Huis, 2013). Large-scale wild harvesting of locusts for sale as a protein-rich ingredient for human food or animal feed may significantly contribute to their control and help to reduce the application of chemical pesticides and their associated environmental pollution (Khusro et al., 2012).

Currently, crickets are one of the most widely farmed insect groups for human consumption as food and high-quality protein ingredients for inclusion in livestock feeds in many regions of the world (Ayieko et al., 2016; Hanboonsong et al., 2013; Lundy & Parrella, 2015). Crickets are a widespread group of insects for this purpose for several reasons: an excellent feed conversion ratio, a short generation time, tolerance of high densities, being generalist feeders, broad disease tolerance, and not undergoing diapauses (Ayieko et al., 2016; Belluco et al., 2013; Wang et al., 2005). Thus, crickets are increasingly considered a sustainable alternative to animal and plant protein sources, with crude protein values above 58 % dry weight (Wang et al., 2005).

In Kenya, where there are currently large swarms of *S. gregaria*, beef is the most consumed protein source and retails between US$ 3 and 5 per kg. This study hypothesized that the consumption of locusts could provide adequate or superior nutritional needs compared to conventional livestock and crop sources. Despite the potential economic importance of these insects, very little attention has been given to the nutrient content of locust species in East Africa, where outbreaks are likely to become highly frequent (FAO, 2020.). Also, evaluating the nutritional content of insects using low-cost and affordable processing techniques need particular attention. Blanching followed by oven-drying before milling into flours is an affordable alternative delivering the same nutritional end-product in comparison to more costly processes such as freeze-drying (Fombong et al., 2017). When we compare the above straightforward processing to that of cereal grains (that involve soaking, fermenting, washing, peeling, drying, de-husking before milling into flour), this further justifies their introduction or promotion as healthy complements to local staple foods.

Several parameters that confer the nutritional attributes of proteins and lipids have been described (Livesey, 1987). However, the paucity of empirical data for less-known protein

quality attributes (such as protein efficiency ratio, nutritional index, and biological value) has inspired the use of other methods using several data-derived equations that can adequately predict these attributes (Alsmeyer & Cunningham 1974; Ijarotimi & Nathaniel, 2015; Oser, 1959). Recently, predicted nutritional quality parameters derived from amino acid and fatty acid values were used to predict the quality of proteins and lipids, respectively, in foods (Ijarotimi & Nathaniel, 2015). Previous studies have reported the content of various nutrients for *S. gregaria*, *L. migratoria*,and *G. bimaculatus* (Ghosh et al., 2017; Ramos-Elorduy et al., 1997; Wang et al., 2005). However, a complete nutritional breakdown has not been reported for any of these insects.

In addition, no studies comparing the nutrient potential of these two pest locusts to that of their more commonly reared orthopteran counterpart, the cricket, *Gryllus bimaculatus,* exist. Finally, no reports used predicted nutritional quality parameters to interpret the nutritional value of edible insects, including locusts and crickets. Therefore, a comparative analysis of the complete nutritional profiles (proximate composition, amino acids, fatty acids, minerals, and vitamins) and nutritional quality parameters of all three species, *G. bimaculatus*, *L. migratoria*, and *S. gregaria*, after blanching and oven-drying was performed.

3.2 Materials and methods

3.2.1 Rearing and maintenance of insect colonies

All three test species, *G. bimaculatus*, *L. migratoria*, and *S. gregaria*, were reared at KU Leuven's Zoological Institute (Department of Biology, Leuven, Belgium). The long-term colony of *S. gregaria* originated from the *Aquazoo* in Düsseldorf, Germany, which had a wild population in Nigeria. This colony has been maintained at the Laboratory of Molecular Developmental Physiology and Signal Transduction (KU Leuven, Belgium) for 35 years, whereas *L. migratoria's* original stock was purchased from the company *Sprinkhanenwinkel* in Someren (The Netherlands). Both locust species were reared under crowded conditions (> 200 locusts/cage) in perplex glass cages of 50 cm x 50 cm x 100 cm.Incandescent light bulbs (40 W) suspended in the cages provided extra warmth and light.Plastic cylindrical containers with open tops (diameter

of approximately 7.5 cm) containing sterile peat and sand were placed at the bottom of the cages for egg-laying. Vertical wire meshes were provided for extra perching space. Locusts were fed daily ad libitum with fresh cabbage leaves (*S. gregaria*) or ryegrass (*L. migratoria*), supplemented with dry oat flakes for both species. Two-spotted field crickets (*G. bimaculatus*) were purchased from a local pet shop and reared in small plastic boxes (50 cm x 50 cm x 50 cm) containing 50–60 crickets. Crickets were fed daily with dry fish flakes (sera® GVG-Mix Nature) from a pet shop and apples and carrots from local food vendors. Eggs were laid in moist double-layered cotton wool. The rearing room of the three insect species was maintained at a constant temperature of 30 ± 1 °C, relative humidity of 40–60 %, and a photoperiodof 14 h light/10 h dark.

3.2.2 Sample preparation

Approximately 1 kg of each locust species was sampled one week into adulthood, and 1 kg of crickets was sampled at adult stages. All insect samples were sacrificed by blanching in distilled water at 100 °C for 4 min before being cooled in ice water and drained. The insects were immediately dried using a laboratory oven Memmert UF 110, Memmert, Schwabach, Germany) at 60 °C for 24 h. The oven-dried samples were ground into fine flour using a laboratory blender (Camlab, Over, UK). Samples were stored at 20 °C for further analysis.

3.2.3 Nutritional composition parameters of the three insects

Proximate analyses

Total proximate compositions were obtained for moisture, crude protein, crude fat, ash, and chitin (fiber) using the methods outlined in chapter two, *section 2.2.2 'Chemical Analyses.'*

Amino acid analysis

Amino acid profiles were determined as in *section 2.2.2 of chapter two.*

Fatty acid analysis

Fatty acid profiles were determined as in *section 2.2.2* in the previous chapter.

3.2.3.4 Mineral and trace elemental analysis

The ash from ash analysis was used to estimate the mineral composition in all insect samples

based on the method described previously in *chapter two, section 2.2.2*

Vitamin B_{12} was quantified using a VitaFast® Vitamin B_{12} microtiter plate test kit (R-Biopharm, Darmstadt, Germany) according to the manufacturer's instructions and slight modifications as reported by Ssepuuya et al., 2019. Briefly, vitamin B_{12} was extracted from 1.000 g of a sample using sodium cyanide, and *taka diastase* enzyme (Sigma Aldrich, St. Louis, MO, USA) was added. To the selected wells of a microtiter plate, which were coated with *Lactobacillus delbrueckii subsp. lactis (leichmannii)*, the vitamin B_{12} assay medium, was added, followed by either the standard solution, sample, or blank. Incubation of the plates took place in the dark at 37 °C for 48 h. The intensity of metabolism or growth of *Lactobacillus delbrueckii* in reaction to the extracted vitamin B_{12} was measured as turbidity at 630 nm using a microtiter plate reader (VERSAmax EXTR, Molecular Devices, San Jose, CA, USA). The vitamin B_{12} content was then calculated by comparison to a standard curve using the SoftMax® Pro 5 software and expressed in µg/100 g dry mass.

3.2.4 (Predicted) nutritional quality parameters

1. Protein quality

The values obtained from the amino acid profiles were used to determine some key protein quality indicators, such as the essential amino acid and nutritional indices. Additionally, protein efficiency ratios and biological values (PER and BV) were predicted using values obtained from amino acid compositions.

Predicted protein efficiency ratio

The protein efficiency ratio measures the nutritive value of a protein source. It is based on the weight gain of a test subject divided by its intake of a particular food protein during the test period. Thus, the predicted Protein Efficiency Ratio (Predicted *PER*-value) estimates the valid PER was calculated using the following formula (Alsmeyer et al., 1974):

$$PER_{predicted} = -0.468 + 0.454 \times (\xi_{Leu}) - 0.105 \times (\xi_{Tyr}) - 0.1539 \quad \text{Eq. 3.1}$$

Where ξ = mass ratios of leucine (*Leu*) and tyrosine (*Tyr*) on insect protein (g/16 g N).

Essential amino acid index

The 'essential amino acid index' (EAAI) is the geometrical mean of the ratio of all essential amino acids in the evaluated protein source relative to their content in a highly nutritive reference protein source such as whole eggs. Thus ,in order to estimate the Essential Amino Acid index (*EAAI*), the formula below, developed by Oser et al., 1959, was used.

$$EAAI = \sqrt[9]{\frac{\xi_{Lys\ (i)}}{\xi_{Lys\ (r)}} \times \frac{\xi_{Trp\ (i)}}{\xi_{Trp\ (r)}} \times \frac{\xi_{Ile\ (i)}}{\xi_{Ile\ (r)}} \times \frac{\xi_{Val\ (i)}}{\xi_{Val\ (r)}} \times \frac{\xi_{Arg\ (i)}}{\xi_{Arg\ (r)}} \times \frac{\xi_{Thr\ (i)}}{\xi_{Thr\ (r)}} \times \frac{\xi_{Leu\ (i)}}{\xi_{Leu\ (r)}} \times \frac{\xi_{Phe+Tyr\ (i)}}{\xi_{Phe+Tyr\ (r)}} \times \frac{\xi_{Met+Cys\ (i)}}{\xi_{Met+Cys\ (r)}}} \quad \text{Eq3.2}$$

where $\xi_{AA\ (i)}$ *= mass ratio of insect amino acid on insect protein (mg/100 g protein) and*

$\xi_{AA\ (r)}$ *= mass ratio of reference amino acid on reference protein (mg/100 g protein).*

Egg protein was used as a reference.

Predicted biological value

Biological value (BV) is a measure of the proportion of protein from a food source that becomes absorbed and incorporated into the proteins of the organism's (human) body. It, therefore, gauges how efficiently a dietary protein source is transformed into body tissue.

The Predicted Biological Value, *BV* (predicted), can be estimated using essential amino acid indices. The predicted biological value is obtained by the linear relationship to the *EAAI* (Oser 1959).

$$BV_{predicted} = (1.09 \times EAAI - 11.7) \quad \text{Eq. 3.3}$$

Nutritional index

Another protein quality parameter is the Nutritional Index (*NI*, in %) which associates *EAAI* to the percent crude protein. The *NI* was calculated for all samples, according to Oser et al., (1959).

$$NI = \frac{EAAI \times w_{protein}}{100} \quad \text{Eq. 3.4}$$

2. Lipid quality

To evaluate the lipid quality of the oils from these insects, values obtained from the fatty acid profiles were used to compute the following health quality indices:

Atherogenic index

The Atherogenic Index (*AI*) is an index that can be used to estimate the risks for cardiac disorders. Its calculation is based on the content of the fatty acids of lauric acid (C12:0), myristic acid (C14:0), and palmitic acid (C16:0), and the groups monounsaturated fatty acid (*MUFA*) and polyunsaturated fatty acid (*PUFA*)

$$AI = \frac{w_{C12:0} + 4 \times w_{C14:0} + w_{C16:0}}{w_{MUFA} + w_{PUFA}} \quad \text{Eq. 3.5}$$

Where w = mass fraction of fatty acid(s) on total fatty acid content (mg/100 g fatty acids).

Polyunsaturated/Saturated fatty acid ratio

This is the ratio of the total polyunsaturated fatty acids (*PUFA*) content to that of the total saturated fatty acids (*SFA*) content or *P*/*S*:

$$P/S = \frac{w_{PUFA}}{w_{SFA}} \quad \text{Eq. 3.6}$$

Thrombogenic index

The thrombogenic index is a ratio that estimates the thrombogenicity (clot-forming ability) of a food (oil), as proposed by Ulbricht and Southgate (1991).

$$TI = \frac{w_{C14:0} + w_{C16:0} + w_{C18:0}}{0.5 \times w_{MUFA} + 0.5 \times w_{PUFA-n6} + 3 \times w_{PUFA-n3} + \frac{w_{PUFA-n3}}{w_{PUFA-n6}}} \quad \text{Eq. 3.7}$$

where w = mass fraction of fatty acid(s) on total fatty acid content (g/100 g fatty acids), TI = thrombogenic index, MUFA = monounsaturated fatty acids, SFA = saturated fatty acids, PUFA = poly-unsaturated fatty acids, UFA = unsaturated fatty acids, n6 = omega-6, and n3 = omega-3 fatty acids.

3.2.5 Statistical analyses

Data of proximate, amino acid, fatty acid, mineral composition, and vitamin B_{12} contents from the insect samples were subjected to Analysis of Variance (ANOVA) after testing for normality using a *Shapiro–Wilk* test. GraphPad Prism (GraphPad Software, La Jolla, CA, USA) version 8.4.3 for Windows was used. The difference between the means was separated using the Tukey post hoc test at 5 % (p = 0.05). The results are reported as the mean plus standard deviations of three technical replicates obtained from pooled samples. The standard deviation formulae for sums (*NFE*) and products (energy) were estimated using the equations derived by B.R. Scott (Scott, 2005).

3.3 Results

3.3.1 Proximate composition

The results for moisture content, crude fat, crude protein, chitin (fiber), and ash are shown in Table 3.1. Proteins and fat constituted the most abundant macronutrients, accounting for 80–86 % of total nutritional components. Protein content was significantly higher (p < 0.0001) in *G. bimaculatus* than in either locust species, for which *S. gregaria* contained significantly more protein than *L. migratoria* (p < 0.001). Fat content was significantly higher in *L. migratoria* (p < 0.001) than in either *G. bimaculatus* or *S. gregaria*, which had comparable fat contents. The fiber content of *L. migratoria* was significantly higher than that of *G. bimaculatus* and *S. gregaria*, which were similar. The ash contents of the three orthopteran species showed no significant differences. The available carbohydrate content expressed as a nitrogen-free extract (*NFE*) was significantly higher (p < 0.0001) in *S. gregaria* compared to that of *G. bimaculatus* and *L. migratoria*, and *NFE* did not significantly differ between these latter two species.

Table 3. 1 *Proximate composition of oven-dried G. bimaculatus, L. migratoria, and S. gregaria (based on a dry matter basis except for the moisture content of fresh samples): each value represents the mean ± standard deviation of triplicate determinations (except for moisture contents, n = 5)*

Parameter	*G. bimaculatus*	*L. migratoria*	*S. gregaria*
Moisture (fresh)	73.66 ± 2.27	77.77 ± 1.13	82.39 ± 2.20
Moisture (processed)	0.85 ± 0.16 [a]	1.46 ± 0.16 [b]	1.97 ± 0.18 [c]
Protein	65.34 ± 0.48 [a]	54.16 ± 0.93 [b]	61.41± 0.32 [c]
Fat (total lipids)	20.74 ± 0.16 [a]	30.52 ± 0.75 [b]	19.10 ± 0.10 [a]
Fiber (chitin)	5.80 ± 1.45 [a]	9.19 ± 0.32 [b]	6.61 ± 1.28 [a]
Ash	4.11 ± 0.01 [a]	3.08 ± 0.06 [a]	2.70 ± 0.13 [a]
NFE	4.80 ± 1.53 [a]	1.50 ± 1.25 [a]	6.50 ± 1.34 [b]
Energy (Kcal/g)	469.91 ± 44.09	512.34 ± 43.36	474.76 ± 47.40

DM = Dry matter; NFE = nitrogen-free extract. [a], [b], [c] Mean values in the same row with a different letter superscript are significantly different (p < 0.05)

3.3.2 Amino acid content

The amino acid profiles (in mg/100 g protein) of *G. bimaculatus*, *L. migratoria*, and *S. gregaria* are shown in Table 3.2. Glutamic acid was the most abundant amino acid recorded for *G. bimaculatus* (131 mg/100 g), *L. migratoria* (123 mg/100 g), and *S. gregaria* (120 mg/100 g). Methionine is the least abundant amino acid in *G. bimaculatus* (0.70 mg/100 g) and *S. gregaria* (3.90 mg/100 g). The highest total amino acid content was found in *S. gregaria*, followed by *G. bimaculatus* and *L. migratoria*. Individual amino acid concentrations varied widely across the three orthopteran species, except histidine, threonine, cysteine, isoleucine, leucine, and tryptophan. The differences in the concentrations of serine ($p < 0.0001$), arginine, ($p < 0.0001$), glutamic acid ($p < 0.0001$), alanine ($p < 0.0001$), and methionine ($p = 0.0012$) between the cricket (*G. bimaculatus*) and the two locusts species were particularly high.

Table 3. 2 *Amino acid profile (in mg/100 g protein) recommended daily intake and predicted protein quality indicators of G. bimaculatus, L. migratoria, and S. gregaria: each value represents the mean ± standard deviation of triplicate determinations*

Amino Acid	*G. bimaculatus*	*L. migratoria*	*S. gregaria*	Recommended Daily Intakes (WHO/FAO) ¤
Histidine	25.79 ± 0.51 [a]	27.23 ± 1.40 [a]	26.32 ± 0.93 [a]	15.0
Serine	55.84 ± 0.34 [a]	40.71 ± 0.76 [c]	44.73 ± 3.29 [b]	----
Arginine	74.21 ± 1.32 [a]	59.12 ± 2.86 [b]	58.32 ± 1.90 [b]	----
Glycine	55.14 ± 1.10 [a]	68.92 ± 2.47 [b]	65.11 ± 1.33 [c]	----
Aspartic acid	104.45 ± 1.52 [a]	81.15 ± 1.70 [b]	80.53 ± 1.56 [b]	----
Glutamic acid	131.04 ± 2.86 [a]	122.45 ± 1.19 [b]	120.29 ± 2.05 [b]	----
Threonine	41.31 ± 0.34 [a]	39.62 ± 0.68 [a]	39.47 ± 0.77 [a]	23.0
Alanine	90.23 ± 3.84 [b]	122.96 ± 1.17 [a, b]	120.10 ± 2.05 [a]	----
Proline	64.73 ± 1.68 [a]	76.35 ± 0.96 [a]	74.28 ± 0.13 [a, b]	----
Cysteine	4.40 ± 0.11 [a]	5.67 ± 1.71 [a]	7.36 ± 0.23 [a, b]	6.6
Lysine	57.20 ± 0.15 [a]	47.84 ± 3.84 [b]	45.69 ± 2.18 [b]	45.0
Tyrosine	51.36 ± 1.36 [c]	56.60 ± 4.88 [b]	66.66 ± 3.07 [a]	----
Methionine	0.70 ± 0.49 [b]	3.91 ± 2.36 [b]	9.49 ± 4.90 [a]	16.0
Valine	64.59 ± 1.44 [a, b]	72.11 ± 0.79 [a]	68.68 ± 0.35 [b]	39.0
Isoleucine	46.08 ± 0.13 [a]	46.18 ± 0.29 [a]	46.25 ± 0.82 [a]	30.0
Leucine	83.47 ± 0.06 [a]	84.56 ± 0.26 [a]	82.30 ± 1.7 [a]	59.0
Phenylalanine	38.90 ± 0.41 [a]	34.9 ± 1.5 [a]	36.3 ± 1.73 [a]	----
Tryptophan	10.52 ± 0.76 [a]	9.71 ± 1.42 [a]	8.20 ± 0.41 [a]	6.0
∑ Amino acids (%) [ö]	55.93 ± 2.65 [a]	47.74 ± 5.27 [a]	58.46 ± 3.46 [a]	
Essential (E)	368.57 ± 1.06 [a]	366.06 ± 2.84 [a]	362.65 ± 3.16 [a]	
Non-Essential (N)	631.40 ± 1.08 [a]	633.94 ± 2.88 [a]	637.36 ± 3.19 [a]	
E/N	0.58 ± 0.00 [a]	0.58 ± 0.01 [a]	0.57 ± 0.01 [a]	
E + N	1000 ± 0.00 [a]	1000 ± 0.00 [a]	1000 ± 0.00 [a]	
E/ (E + N)	0.37 ± 0.00 [a]	0.37 ± 0.00 [a]	0.36 ± 0.00 [a]	
PER $_{predicted}$	2.32 ± 0.12 [a]	2.39 ± 0.29 [a]	2.42 ± 0.23 [a]	
EAAI	69.23 ± 0.96 [a]	73.00 ± 1.25 [b]	76.10 ± 1.82 [c]	
BV $_{predicted}$	63.76 ± 1.05 [a]	67.87 ± 1.36 [b]	71.25 ± 1.98 [c]	
Nutritional Index $_{predicted}$	45.24 ± 0.94 [b]	39.53 ± 0.23 [a]	46.73 ± 1.11 [c]	

[a, b, c] Mean values in the same row with a different superscript are significantly different ($p < 0.05$).

[ö] Expressed as percent 100 g per insect sample.; — data are not available.

¤ Food and Agriculture Organization (FAO)/WHO Amino acid requirements in humans (mg/100 g protein).

The ratio of essential to non-essential amino acids was reasonably constant (0.36–0.37)and did not vary among the three species. The Essential Amino Acid Index (*EAAI*) and Nutritional Index (NI) were significantly different at α = 0.05 for all three species. The highest *EAAI* and *NI* values were recorded for *S. gregaria* (76.0 and 46.7 %), while *G. bimaculatus* hadthe lowest values. By contrast, *L. migratoria* had the lowest *NI* (39.5 %). The biological value (*BV*) of the three species ranged between 63.7–71.3 % and varied significantly.

3.3.3 Fatty acid content

The fatty acid compositions of *G. bimaculatus*, *L. migratoria*, and *S. gregaria* are presented in Table 3.3. The predominant fatty acids in all three species were palmitic and oleic, while eicosapentaenoic acid (EPA) was the least abundant. Linoleic acid was the most abundant polyunsaturated fatty acid in all three species. Of the monounsaturated fatty acids, oleic acid had the highest concentration in *S. gregaria* and *G. bimaculatus,* while myristoleic acid had the lowest concentrations in both species. Myristic and lauric acid were low in all three species.

Table 3. 3 *Fatty acid profile (in g/100 g fatty acid) of G. bimaculatus, L. migratoria, and S. gregaria: each experimental value represents the mean ± standard deviation of triplicate determinations*

Fatty Acid	Class	*G. bimaculatus*	*L. migratoria*	*S. gregaria*
Decanoic acid (C10:0)	*SFA*	0.10 ± 0.02 [a]	1.08 ± 1.25 [a]	0.15 ± 0.11 [a]
Lauric acid (C12:0)	*SFA*	1.60 ± 0.09 [a]	1.30 ± 1.17 [a]	0.41 ± 0.06 [a]
Myristic acid (C14:0)	*SFA*	0.74 ± 0.45 [a]	3.63 ± 0.56 [a]	2.66 ± 0.25 [a]
Myristoleic acid (C14:1)	*MUFA*	0.10 ± 0.02 [a]	1.25 ± 1.54 [a]	0.15 ± 0.13 [a]
Pentadecanoic acid (C15:0)	*SFA*	0.19 ± 0.09 [a]	0.40 ± 0.35 [a]	0.22 ± 0.18 [a]
Palmitic acid (C16:0)	*SFA*	27.73 ± 2.42 [a]	43.01 ± 1.45 [b]	26.14 ± 1.21 [a]
Palmitoleic acid (C16:1)	*MUFA*	1.05 ± 0.30 [a]	1.60 ± 0.96 [a]	1.34 ± 0.10 [a]
Heptadecanoic acid (C17:0)	*SFA*	0.29 ± 0.02 [a]	1.14 ± 0.83 [a]	0.34 ± 0.13 [a]
Stearic acid (C18:0)	*SFA*	7.36 ± 0.82 [a]	6.31 ± 6.01 [a]	7.02 ± 0.67 [a]
Oleic acid (C18:1)	*MUFA*	31.76 ± 0.82 [a]	22.85 ± 6.07 [b]	40.87 ± 1.76 [c]
Linoleic acid (C18:2) (*n*6)	*PUFA*	27.33 ± 0.41 [a]	9.32 ± 0.90 [b]	6.85 ± 0.18 [b]
α-Linolenic acid (C18:3) (*n*3)	*PUFA*	1.02 ± 0.75 [a]	4.85 ± 0.56 [a]	13.11 ± 0.55[a]
Arachidonic acid (C20:4)) n6)	*PUFA*	0.59 ± 0.06 [a]	1.40 ± 0.96 [a]	0.43 ± 0.16 [a]
Eicosapentaenoic acid (C20:5) (*n*3)	*PUFA*	0.13 ± 0.12 [a]	1.89 ± 1.35 [a]	0.29 ± 0.30 [a]
Total Lipids		20.74 ± 0.16 [b]	30.52 ± 0.75 [c]	19.10 ± 0.10 [a]
Total *SFA*		38.02 ± 1.15 [a]	56.85 ± 4.64 [b]	36.95 ± 1.43 [a]
Total *MUFA*		32.91 ± 0.53 [a]	25.70 ± 4.72 [a]	42.37 ± 1.77 [b]
Total *PUFA*		29.54 ± 0.40 [b]	17.45 ± 3.54 [a]	20.68 ± 1.77 [a]
Total UFA		62.46 ± 0.34 [b]	43.15 ± 4.64 [a]	63.05 ± 1.89 [b]
Total essential (C18:2 + C18:3)		28.82 ± 0.45 [b]	14.17 ±1.23 [a]	19.96 ± 0.58 [a]
Total *n*6		27.92 ± 0.39 [b]	10.71 ± 1.79 [a]	7.28 ± 0.24 [a]
Total *n*3		1.15 ± 0.86 [a]	6.74 ± 1.83 [b]	13.40 ± 0.63 [c]
*n*6/*n*3 ratio		17.19 ± 0.58 [c]	1.63 ± 0.26 [b]	0.54 ± 0.01 [a]
PUFA/*SFA* (*P*/*S*) ratio		0.78 ± 0.02 [b]	0.31± 0.07 [a]	0.56 ± 0.03 [b]
Atherogenic index (*AI*)		0.48 ± 0.02 [a]	1.06 ± 0.12 [a]	0.44 ± 0.03 [a]
Thrombogenic index (*TI*)		1.11 ± 0.07 [a]	3.19 ± 0.62 [a]	1.56 ± 0.10 [a]

[a, b, c] *Mean values in the same row with a different letter superscript are significantly different ($p < 0.05$). MUFA = Monounsaturated fattyacids, SFA = Saturated fatty acids, PUFA = Poly-unsaturated fatty acids, UFA = Unsaturated fatty acids, n6 = omega-6, and n3 = omega-3fatty acids.*

3.3.4 Mineral composition and vitamin B12 content

The mineral compositions of the three orthopteran species are shown in Table 3.4. There were significant differences ($p < 0.001$) observed for all the mineral elements tested in all three species. The highest potassium concentration was recorded in *S. gregaria*, *G. bimaculatus*, and finally, *L. migratoria*. Phosphorus, the next abundant mineral, was highest in *S. gregaria* and least in *L. migratoria.* Sodium was highest in *G. bimaculatus*, *S. gregaria*, and least in *L. migratoria*.

Table 3. 4 *Recommended daily intakes, mineral content (mg/100 g dry matter), and vitamin B12 content (µg/100 g dry matter) of G. bimaculatus, L. migratoria, and S. gregaria: each value represents the mean ± standard deviation of triplicate determinations*

Mineral or Vitamin B_{12}	*G. bimaculatus*	*L. migratoria*	*S. gregaria*	Recommended Daily Intakes (WHO/FAO) ¤
Potassium	1025.14 ± 0.03 [a]	796.01± 0.01 [b]	1309.46 ± 0.66 [c]	4700
Phosphorus	882.38 ± 0.09 [a]	697.17± 0.02 [b]	968.65 ± 0.51 [c]	700
Sodium	383.25 ± 0.01 [a]	221.89 ± 0.01 [b]	285.14± 0.15 [c]	1500
Calcium	191.16 ± 0.01 [a]	129.79 ± 0.01 [b]	80.48 ± 0.04 [c]	1300
Magnesium	110.57 ± 0.01 [a]	86.01 ± 0.01 [b]	128.29 ± 0.07 [c]	260
Zinc	13.76 ± 0.01 [a]	12.70 ± 0.01 [b]	24.88 ± 0.01 [c]	3–14
Manganese	7.15 ± 0.01 [a]	0.40 ± 0.01 [b]	1.26 ± 0.01 [c]	1.8–2.6
Iron	4.60 ± 0.01 [a]	6.59 ± 0.01 [b]	7.31 ± 0.01 [c]	7.5–59
Copper	1.84 ± 0.01 [a]	1.20 ± 0.01 [b]	4.86 ± 0.01 [c]	0.9–1.3
Ca/P	0.22 ± 0.09	0.19 ± 0.02	0.08 ± 0.52	
‡ Vitamin B_{12} ¥	1.35 ± 0.14 [a]	1.10 ± 0.06 [b]	0.22 ± 0.01 [c]	0.40–1.8

[a, b, c] Mean values in the same row with a different superscript are significantly different ($p < 0.05$).
¥ = value expressed in µg/100 gdry matter.
¤ FAO/WHO (values in mg/day except for selenium).
‡ FAO 2001 (human vitamin and mineral requirements (for infants and children)).

The three least abundant elements were copper (significantly higher in *S. gregaria*) and manganese (recorded significantly higher amounts in *G. bimaculatus*). The ratio of calcium to phosphorus (Ca/P) (0.08–0.22) did not differ significantly among the three species. Amongst the micro minerals, zinc was the most abundant (12.7–24.9 mg/100 g), and the least in all three species was selenium (0.033–0.125 mg/100 g).

The vitamin B_{12} (cobalamin) content for the three species is shown in Table 3.4. Vitamin B_{12} levels in the three orthopterans varied significantly, with the highest values recorded in *G. bimaculatus*.

3.4 Discussion

Despite the economic importance of locusts, very little research has directly compared their nutritional profile to that of the more commonly reared and eaten crickets like *G. bimaculatus*, which are frequently used as a food ingredient in East Africa. It has been demonstrated that powdered preparations of both locust species are of comparable nutritional quality to *G. bimaculatus*. Also, given the widespread consumption across Sub-Saharan Africa and the display of swarming behavior to another orthopteran, *R. differens*, nutritional comparisons are frequently made throughout the discussion between the latter and the insects of the present study. The contrast is further established because all four insects (*L. migratoria*, *S. gregaria, G. bimaculatus*, and *R. differens*)were subjected to the same processing, analyses, and measuring equipment. The simplicity of the processing outlined in this paper, from washing to blanching, then oven-drying and blending to flour, exemplifies their ease for inclusions and more frequent use as a food ingredient or dietary supplement. Therefore, this chapter also aims to strengthen advocacy for the use of such minimally processed insect flours as processed food ingredients that could affordably be incorporated into other food products (Cheseto et al., 2020; Messina et al., 2019; Choi et al., 2020). The current literature suggests evidence to support that oven-dried insect are comparable to freeze-dried ones. At the same time, oven-drying, in comparison with freeze-drying, reduces energy input costs and improves specific physicochemical attributes such as reducing lipid oxidation and improving protein solubility (Fombong et al., 2017; Kröncke et al., 2019; Lautenschläger et al., 2017). Additionally, the nutritional value of the cricket and locust proteins after oven-drying at higher

temperatures is high, as was also the case for mealworms and lesser mealworms as reported by other studies (Jensen et al., 2019).

The protein content of edible insect species is high, ranging from 35 % (in termites) up to 77 % (in grasshoppers) (Finke et al., 1989; Belluco et al., 2013), a range that is consistent with that of the current findings. However, the crude protein content in *L. migratoria*, *S. gregaria*, and *G. bimaculatus* is significantly higher than that of *R. differens* (47.7 %) measured in the same study (Fombong et al., 2017). Our findings agree with that of previous studies that reported that the crude protein levels of all orthopterans ranged between 43.9–77.1 % (Ramos-Elorduy & José Manuel, 2012; FAO/GoK & FAO/Government of Kenya, 2018; Rumpold & Schluter 2013). The protein values reported in *L. migratoria*, *S. gregaria*, and *G. bimaculatus* are superior or comparable to most conventional protein sources. For instance, beef consumed in Kenya with dry matter protein within the range of 40.5 – 71.2 g/100g (FAO; Government of Kenya). These comparable values are an indication that these insect flours can serve as an alternative protein source to conventional beef proteins.

The lipid content of the locusts and cricket analyzed in the present study were quite variable and significantly lower than the values reported for *R. differens* (35.60 %) (Fombong et al., 2017), though slightly higher than the averages reported for other orthopterans (13.41 %) (Rumpold & Schluter, 2013). The high lipid content of *R. differens* can be attributed to their highly polyphagous nature and their ability to store higher quantities of lipids during the early stages of life (Finke 2002). However, *L. migratoria* in this study showed higher quantities of lipids than that of soybean (a commercial lipid source), with approximately 20 % lipids on a dry weight basis (Messina et al., 1999). Thus, the locusts *L. migratoria* and *S. gregaria* could serve as alternative sources of oils after defatting and can be used as substitutes for the expensive fish oil in animal feeds or human food (Cheseto et al., 2020). The high fat content reported for orthopterans is a direct indication of energy. The energy values recorded in the current study are within the range reported in the literature for other species (400–500 Kcal/g) (Rumpold & Schluter 2013). This implies that the consumption or inclusion of these insect flours in food products would contribute to meeting energy requirements in human and animal nutrition (Joint, F.A.O, 1985).

The crude fiber contents of *G. bimaculatus*, *L. migratoria*, and *S. gregaria* are lower than *R. differens* (Table 3.1) (Fombong et al., 2017). These crude fiber results are similar to that reported in the literature for other orthopterans (1–22 %) (Blásquez et al., 2012; Rumpold & Schlüter, 2013). Studies have shown that adequate plant fiber intake confers some health benefits, such as lowering serum cholesterol levels, the risk of coronary heart diseases, hypertension, constipation, and diabetes (Ishida et al., 2000). The high fiber content of *L. migratoria* is comparable to that of *R. differens* and might confer more excellent health benefits for humans and animals. The crude fiber in insects is composed of chitin (Finke 2008). This serves as a raw material frequently used in the industrial production of chitosan, oligosaccharides, and glucosamine (Finke 2008). Whether the health benefits of plant fibers count for chitin is still to be studied

The ash contents of *G. bimaculatus* and *S. gregaria* were comparable to *R. differens* (4.66 %) (Fombong et al., 2017). Extremely low ash contents have been reported in other orthopterans such as *Sphenarium mexicanum* with 0.34 % (Rumpold & Schlüter 2013). It is important to note that the ash content reported for the three species in this study is consistent with previous findings for Orthoptera (0.34–9.36 %) (Rumpold & Schlüter 2013).

In the present study, the three orthopteran species had all the essential amino acids (except methionine) in adequate amounts required for human and animal nutrition (Joint, F.A.O, 1985). Nutritionally, protein-based food materials are acceptable when biological values are above 70 % and the essential amino acid index is higher than 0.70 (Nakai & Modler 1996). *PER* is the ratio of the weight gain of a test group or sample to the total proteins consumed (Nakai & Modler 1996). $PER_{predicted}$ is a calculated parameter that accurately estimates the real or actual *PER* values (Alsmeyer et al., 1974). A *PER* below 1.5 connotes a low-quality protein that between 1.5–2.0 has an intermediate quality and that above 2.0 is considered adequate to high quality (Nakai & Modler 1996). As such, the following familiar protein sources are widely considered high quality: casein (2.5), beef muscle, fish and poultry (2.7), and eggs (3.1) (Canadian Food Inspection Agency, 2020). The $PER_{predicted}$ values calculated from the amino acid compositions of the insects in our study (2.3–2.4) were above the 2.0 threshold and compared favorably with the above traditional proteinsources and, therefore, can be classified

as high-quality proteins. The mean *EAAI* of the blanched and oven-dried *L. migratoria*, *S. gregaria*, and *G. bimaculatus* is comparable to that of the chicken egg, estimated to be 1.0 (Oser 1959). Apart from *G. bimaculatus*, the *EAAI* of *L. migratoria* and *S. gregaria* were higher than 70 %, similar to *R. differens* (72 %) (Fombong et al., 2017), thus confirming the high protein nature of the insects. The calculated *EAAI*s of *L. migratoria* and *S. gregaria* are higher than those reported for soybean (65–72 %) (Oser 1959). Thus, the EAAI values recorded in the present study meet the standard requirements for adequate human nutrition (Livesey 1987). The lysine and threonine levels in *L. migratoria* and *S. gregaria* were reasonable compared to predominantly cereal-based diets (3.7–4.2 mg/100 g and 3.2–3.4 mg/100 g protein, respectively) of most African rural communities (Charrondiere et al., 2013). These levels could indicate that the inclusion of locust meals in food products in plague-affected regions would positively impact the household nutritional status of the various communities.

One limitation of this study is the lack of protein digestibility data, which could predict the overall efficiency of protein utilization using the Protein Digestibility-Corrected Amino Acid Score (PDCAAS). This evaluation method comprises the Biological Value (BV) and the digestible value of the proteins concerned. Studies on other insects have shown that industrial drying methods like oven-drying have minimal influence on PDCAAS (Jensen et al., 2019). The FAO/WHO (Leser, 2013) recommended PDCAAS as the standard protein quality evaluation method, but in 2013, they endorsed a new evaluation method, the Digestible Indispensable Amino Acid Score (DIAAS). Therefore, additional studies relating predicted biological values and protein efficiency ratios to either the PDCAAS or DIAAS would be of more relevance to human nutrition.

Lauric acid (C12:0) and myristic acid (C14:0) were present in the three orthopteran species in minute amounts. The presence of both fatty acids has been implicated in raising harmful cholesterol levels in the blood serum (Grundy, 1994). Therefore, being present in small amounts, including these insects in foods, would not create any adverse cholesterol implications. In contrast to the abovementioned saturated fatty acids, the inclusion of poly- or mono-unsaturated fatty acids such as oleic acid (C18:1), linoleic acid (C18:2), and α-linolenic acid (C18:3) in diets is recommended for preventing cardiovascular diseases (Alfaia et al., 2009). The presence of linoleic and α-linolenic acid as essential polyunsaturated fatty acids in all

three orthopteran species was remarkable. These fatty acids are crucial in developing children under five and women of reproductive age (Grundy, 1994). For optimal growth and development of children's brains, polyunsaturated acids such as eicosatetraenoic acid (20:4) and docosahexaenoic acid (22:6), which are synthesized from their respective essential fatty acid precursors, linoleic acid (18:2) and α-linolenic acid (18:3), are required (Grundy, 1994). Deficiency in some essential fatty acids in children can lead to malnourishment and several clinical problems. These include increased risk of infection, impaired wound healing, fatty liver, and psychomotor changes coupled with growth retardation (Ulbricht and Southgate, 1991; Grundy, 1994; Dobermann et al., 2019).

The nutritional qualities of *G. bimaculatus*, *L. migratoria*, and *S. gregaria* were demonstrated by the presence of saturated (*SFA*), monounsaturated (MFA), and polyunsaturated fatty acids (*PUFA*) *n*6 *PUFA*, *n*3 *PUFA;* (*n*6/*n*3); and saturation (*P*/*S*), atherogenic (*AI*), and thrombogenic (*TI*) indexes (Ulbricht and Southgate 1991). *G. bimaculatus* exhibited a very high *n*6/*n*3 ratio compared to *L. migratoria* and *S. gregaria* lipids that had negligible levels of *n*6/*n*3. A good *n*6/*n*3 ratio lies between 1:1–and 1:6; however, there is contention regarding these ratios among researchers (Aman et al., 2017). This would suggest that consuming the fats from these two locusts would positively affect human cardiovascular health. Furthermore, studies have suggested that a high *n*6/*n*3 ratio (>20:1) in the diet might be linked to the development of a variety of physiological disorders such as cancer and coronary heart disease (Aman et al., 2017). The polyunsaturated to saturated fatty acid (*P*/*S*) ratio is one of the most significant markers of lipid composition in a healthy diet. It is recommended to consume a diet with a P/S ratio close to 1. A high P/S ratio (3) in the diet may promote tumor formation, while a low P/S ratio (0.33) in the diet could be atherogenic (Turley & Thompson, 2015). Interestingly, *G. bimaculatus* lipids had a P/S closest to 1 (0.76), which means they contain more of the "desirable" polyunsaturated than the "less desirable" saturated fatty acids. At the same time, the locusts performed poorly in this parameter, though less than 0.33, which is the cut-off mark to be considered atherogenic. Ulbricht and Southgate (1991) proposed the term "atherogenic index" (*AI*) for lipids as a nutritional index for the risk of cardiovascular diseases. The *AI* index was obtained using the Table 3.3 values for lauric (C12:0), myristic (C14:0), and

palmitic (C16:0) acids and unsaturated fatty acids. An increase in the *AI* index increases the risk of the incidence of cardiovascular diseases in humans. The *AI*s of the diets of the Eskimo (0.39), British (0.93), and Danish (1.29) (Ulbricht & Southgate, 1991) suggest that consuming 100 g dry weight of any of the examined insects (except for *L. migratoria*) would fall within the recommended value for these countries. Additionally, our study revealed atherogenic index values for *G. bimaculatus* and *S. gregaria*, which were significantly lower and compared favorably with those for other animals such as sheep (0.7–0.9), beef (0.7), pork (0.6), and poultry (0.5) (Camacho et al., 2017; Richard, J & Charbonnier, 1994; Stajić et al., 2011). Consequently, the consumption of food products composed of *G. bimaculatus* and *S. gregaria* with lower atherogenic index values may lead to a decrease in the total cholesterol and the LDL cholesterol in human blood plasma. Additionally, the lower atherogenic index observed in our study highlights that *G. bimaculatus* and *S. gregaria* had low concentrations of the saturated fatty acids lauric (C12:0), myristic (C14:0), and palmitic (C16:0) acids in comparison to other lipid sources (Alfaia et al., 2009). Thehigh risk of cardiovascular diseases in humans warrants a more thorough look into the fatty acids of some insects as a possible replacement for other lipid sources (Ulbricht & Southgate, 1991).

Minerals play vital roles in numerous biological processes, and in many developing nations, micronutrient deficiencies are still widespread. Such micronutrient deficits, especially for trace minerals, can have severe health outcomes affecting growth and development. In this study, the high potassium values recorded for the three orthopteran species agree with other related studies (724–834 mg/100 g DM) (Rumpold & Schlüter, 2013; Fombong et al., 2017; Kinyuru et al., 2010). It can be ascribed to the food plants known to contain high levels of potassium (Aremu et al., 2014).The phosphorus contents of *G. bimaculatus*, *L. migratoria*, and *S. gregaria* were high, thus influencing the calcium-phosphorus ratio, which was less than one. The phosphorus in most insects is readily available, as shown for *Musca autumnalis* puparia with 92 % availability (Aremu et al., 2014). *S. gregaria* had the highest amounts of Mg, Zn, Fe, K, and Cu than *G. bimaculatus* and *L. migratoria*. The Ca/P ratio of *G. bimaculatus*, *L. migratoria*, and *S. gregaria* was within the recommended range between 0.1–2.0 for this ratio. The Ca/P ratio is a tool to indicate the facilitation of bone and teeth formation in children, and this ratio also

signals the incidence of osteoporosis in adults (Williams 2007).

Such a ratio implies that consumption of any of the orthopteran species might help to improve bone and teeth formation in infants and reduce osteoporosis in adults (Williams 2007).

The vitamin B_{12} content observed in *G. bimaculatus*, *L. migratoria*, and *S. gregaria* is comparable to that reported for *R. differens (*0.88–1.35 µg/100 g) (Fombong et al., 2017) but much less than that reported for *Acheta domesticus* adults (5.4 µg/100 g) and nymphs (8.7 µg/100 g) by another study (Van Huis et al., 2013). When compared to other animal-based food sources, the vitamin B_{12} content recorded in the three orthopteran species was, however, lower than that documented for pork and poultry meat (9.3 µg/100 g) (Green & Miller 2007). Nonetheless, the consumption of *G. bimaculatus, L. migratoria*, and *S. gregaria* would be capable of providing an adequate amount of vitamin B_{12}, known to play a valuable role in DNA synthesis, regenerating methionine for protein synthesis and methylation, and in preventing homocysteine accumulation (Meyers et al., 2006; Allen 2009). The recommended dietary allowance (in mg/day) of vitamin B_{12} is 0.4 mg for the first six months of life, 0.5 mg for 6–12 months., 0.9 mg for 1–3 years, 1.2 mg for 4–8 years, 1.8 mg for 9–13 years, and 2.4 mg for 14 years through old age. In pregnancy, 2.6 mg is recommended, and in lactation, 2.8 mg is recommended (Meyers et al., 2006). Thus, vitamin B_{12} fortification of flours with these insects is highly recommended in developing countries, especially those that decisively evade all animal source foods (Allen 2009).

Based on the nutritional values computed in this chapter, it is estimated that the consumption of 100 g of dry locusts or crickets contributes around 0.39 – 0.82 g/kg of the daily protein requirement of 0.8 - 1.0 g/kg body weight (Ssepuuya et al., 2016). These insects can therefore contribute to improving the low daily per capita protein consumption of 55 - 65 g/person/day in most of sub-Saharan Africa compared to approximately 60 -100 g/person/day in the developed countries (OECD/FAO, 2016). With an average adult locust fresh weight of 2 g and mean water content north of 60%, a 100g dry weight locust serving would mean roughly consuming about 120 fresh insects.

3.5 Conclusions

It can be concluded that the three orthopteran species, *G. bimaculatus*, *L. migratoria*, and *S. gregaria,* can be considered as potential high-quality, affordable food sources, given that the protein values are comparable or superior to those of most plants and animal sources commonly consumed as foods. For the first time, the high quality of insect proteins was confirmed with predicted protein quality parameters such as *PER* and *BV*. Additionally, their high mineral content, good amino acid quality, suitable fatty acid quality, and the presence of vitamin B_{12} make them a worthy replacement or substitute for meat. Our findings revealed that the inclusion of these insect flours in food products might help to mitigate most of the energy and protein insufficiency issues observed in most Sub-Saharan African countries. The highlight of this study is to promote the use of dried and processed insects instead of highly perishable fresh insects.

Furthermore, the blanched and oven-dried insect flour can be incorporated into other foodstuffs, enhancing nutritional value in this way. Thus, by minimally processing these insects into their flours post-harvesting through simple processes including blanching, oven-drying, and blending, they provide a nutrient-rich and affordable food ingredient. Of these three insects examined, the study recommends that *S. gregaria* be the most consumed since it fared better in nutritional attributes and is the most devastating locust pest that could save a lot on insecticides. These insects can be easily mass-harvested from the wild, implying that incorporating them into local diets would help to combat food and nutritional insecurity, would assist with job creation to generate much-needed household income and would improve livelihoods. Therefore, in the event of natural swarms (provided they are insecticide-free), this study recommends the safe harvesting and processing of locusts for food and feed as an integrated approach for the management of these ravaging pests in affected areas.

CHAPTER FOUR

Evaluation of Antinutrients Compositions in *Ruspolia differens*, Crickets (*Gryllus bimaculatus*), and Locusts (*Schistocerca gregaria*)

4.1 Introduction

The previous chapters demonstrated the nutritional properties of *Ruspolia differens* and how well they perform with respect to other insects of the same Orthoptera order. This chapter follows up on this by evaluating non-nutritional constituents, which can constitute a health menace when consumed in copious amounts. These are a class of secondary plant metabolites frequently referred to as antinutrients. Antinutrients or antinutritional factors are natural or synthetic compounds that interfere with the digestion and absorption of nutrients. Their general mode of action is by binding to essential nutrients and minerals or else by interfering with the efficiency of digestive enzymes or transport systems in the gut, thus rendering their absorption by the human gut complex (Gemede & Ratta, 2014).

Over the last decade, tremendous strides have been made to advance the use of insects as food and feed. Recently, the European Food Safety Authority (EFSA, 2019) paved the way for insects as food in Europe and globally. However, the report also highlighted the paucity of safety data for effective risk assessment for novel foods that include parts or whole insects. Among others, data relating to allergies, mycotoxins, heavy metals contamination, and antinutritional factors are conspicuously lacking. The FAO (2003) has recommended that data for these components should be included for the relevant foods. The same report further emphasizes that data for these natural components should be incorporated into the reference food database.

The nutritional value of foods (including edible insects) largely depends on their nutritional and antinutritional composition. While data on nutritional composition are on the rise, the same cannot be said about research on antinutrients in insects. The major antinutrients found in plant-based foods include phytates, tannins, lectins, oxalates, cyanogenic glycosides, protease inhibitors, lectins, and antivitamin factors (Gemede & Ratta, 2014). Other natural toxicants that can loosely be classified as antinutritional factors include solanine, cyanides, glucosinolates, lathyrogens, mimosine, and nitrosamines (Gemede & Ratta, 2014; Popova & Mihaylova, 2019). The concentration of antinutrients in food classes is quite variable (Table 4.1), and phytates (phytic acid), tannins, and oxalates are among the most popular in plant-based foods. Some constituents of these antinutrients and toxicants have undesirable physiological effects. For instance, oxalates prevent calcium absorption in the body by binding with it (Jiru et al., 1995).

Tannins, which are predominant in tea, wine, some fruit, and chocolate, when consumed in excess, can bind with dietary and endogenous proteins and digestive enzymes, thereby interfering with normal digestion (Chang et al.,1994). Phytates that are present in grains, nuts, seeds, white peppers, and eggplants may lead to lower mineral absorption (Gupta et al., 1987, Gupta et al., 2015).

Table 4. 1*. Antinutrients sources in different foods (expressed as dry matter)*

Source	Type of Antinutrient	Amount
Legumes (soy, lentils, chickpeas, peanuts, beans)	*Phytic acid*	386-714 mg/100g
	Saponins	106-170 mg/100g
	Cyanide	2-200 mg/100g
	Tannins	0.018-0.18 mg/100g
	Trypsin inhibitor	6.7 mg/100g
	Oxalates	8 mg/kg
Grains (wheat, barley, rye, oat, millet, corn, spelt, kamut, sorghum)	*Phytic acid*	50-74 mg/g
	Oxalates	35-270 mg/100g
Pseudo-grains: quinoa, amaranth, wheat, buckwheat, teff	*Phytic acid*	
	Lectins	0.5-7.3 g/100g
	Saponins	0.04-2.14 ppm
	Goitrogens	
Nuts: almonds, hazelnut, cashew, pignola, pistachio, brazil nuts, walnuts, macadamia	*Phytic acid*	150-9400 mg/100g
	Lectins	37-144 µg/g
	Oxalates	40-490 mg/100g
Seeds: sesame, flaxseed, poppy seed, sunflower, pumpkin	*Phytic acid*	1-10.7 g/100g
	Alpha-amylase inhibitor	0.251 mg/mL
	Cyanide	140-370 ppm
Tubers: carrot, sweet potato, Jerusalem artichoke, manioc (or tapioca), yam	*Oxalates*	0.4-2.3 mg/100g
	Tannins	4.18-6.72 mg/100g
	Phytates	0.06-0.08 mg/100g
Nightshades: potato, tomato, eggplant, pepper	*Phytic acid*	0.82-4.48 mg/100g
	Tannins	0.19 mg/100g
	Saponins	0.16-0.25 mg/100g
	Cyanide	

Source: Adapted from Popova & Mihaylova, 2019

To date, edible insects have been promoted as a healthy animal protein source, but little is known about their antinutrient content. However, edible insects have been shown to contain some antinutrients and allergens, which could be a potential risk to consumers. Pupae of the African silkworm (*Anaphe venata*), for example, contain a heat-resistant thiaminase and, therefore, can cause thiamine deficiency (Rumpold & Schluter, 2013). When four types of edible insects (Cricket - *Gymnogryllus lucens*; Yam beetle - *Heteroligus meles*; Palm weevil - *Rhynchophorus phoenicis*; and grasshopper - *Zonocerus variegatus*) were analyzed for the

antinutrients, i.e., hydrocyanide, oxalate, phytate, and tannin, these were found to be generally far below the toxic levels for human consumption (Ekop, 2010). A different study on anti-nutritional components of the Westwood larvae, *Cirina forda,* yielded low levels of oxalate and phytic acid within nutritionally accepted values, and tannin was not present (Omotoso, 2006). Adeduntan (2005) reported the tannin percentage in an *unnamed* grasshopper harvested in Ondo state (Nigeria) to be 1.05 %, lower than the 2.5 % observed in *Oedaleus abruptus*. Other studies have also reported the presence of antinutritional factors such as tannins, oxalates, phytate, and hydrogen cyanide (Jonathan et al., 2012; Shantibala et al., 2014), thiaminases (Nishimune et al., 2000), and protein inhibitors (Eguchi, 1993) in edible insects.

In this study, we examined antinutrient levels in three of the most widely consumed edible insect species across Sub-Saharan Africa, *i.e.*, the desert locust *Schistocerca gregaria*, the field cricket *Gryllus bimaculatus,* and the bush cricket *R. differens* (Order: Orthoptera) (Fombong et al., 2017; 2021, Kelemu et al., 2015, Kinyuru et al., 2020). We found that the main reasons behind the wide acceptance of these insects, apart from their taste and flavor, were that they were locally abundant throughout the year (crickets) or that they could be gathered in large amounts during their swarming periods (bush crickets and locusts). However, whereas there exist data on their nutritional profile (Kinyuru et al., 2010; 2020; Cheseto et al., 2015; 2020, Fombong et al., 2017; 2021), studies on their antinutrient composition are very limited in the literature. Additionally, although it has been postulated that toxic components, such as antinutrients in insects, may get detoxified during food processing steps, such as frying, boiling, and roasting, there is little evidence in scientific literature to support this. Phytates, oxalates, and tannins are considered the most abundant in plant-based foods, which orthopterans feed on (Popova & Mihaylova 2019). Therefore, this study aimed to determine the antinutrient (phytate, oxalate, and tannin) contents of three popular edible insects (*Ruspolia, Schistocerca,* and *Gryllus*) and to evaluate the impact of the drying technique on these contents.

4.2 Materials and methods

4.2.1 Insect samples

The desert locust (*S. gregaria*) and bush cricket (*R. differens*) were mass-reared at the Animal Rearing and Containment Unit (ARCU) of the International Centre of Insect Physiology and Ecology (icipe) in Nairobi (Kenya).

The colonies of *S. gregaria* were maintained at a temperature of 30 ± 2 °C, 65 ± 5% relative humidity (RH), and a photoperiod of 12 hours: 12 hours light: darkness. The colonies were fed on a diet consisting of wheat seedlings, corn leaves, wheat bran, harvested on maturity

R. differens colonies were fed on *Panicum sp*. shoots and maize seedlings supplemented with broiler chicken feed. The rearing unit was kept at 28.8 ± 2 °C, 50 ± 5% relative humidity, and a photoperiod of 12 h light and 12 h dark.

Both adult insect samples were harvested and transported immediately to JKUAT (Nairobi, Kenya) and processed further as described in *Section 2.2*

Reared field crickets (*G. bimaculatus*) were acquired from the insect rearing facility of the Jomo Kenyatta University of Agriculture and Technology (JKUAT) in Nairobi (Kenya). Colonies were kept at ambient temperature in a non-controlled environment, with light bulbs used for heating at night. They were fed with chicken mash for the first four weeks upon hatching. After that, the chicken mash was supplemented with leafy vegetables such as pumpkins and cassava leaves (Kinyuru and Kipkoech, 2018). Upon attaining maturity, 5 kg of adult crickets were harvested and processed as outlined in *Section 2.2.*

Given that these insects are processed for consumption in East Africa without prior starvation, the samples of this study were equally not starved.

4.2.2 Drying and defatting of insects

All insect samples were transported to the Food Biochemistry Laboratory of JKUAT, Kenya, for analysis. Insect batches were separated equally into two portions of approximately 2.50 kg each and subjected to either oven-drying or freeze-drying.

Drying process

The freeze-drying process was performed using a freeze-dryer as described in chapter two, *section 2.2*. Freeze-dried samples were coded as FD. Similarly, the other portions were oven-dried using a laboratory oven (Memmert UF 110, Memmert, Schwabach, Germany) at 60 °C for 24 h and coded as OD. All dried samples were ground into flour using a two-speed Waring laboratory blender (Camlab, Over, UK).

The resulting flour of each insect (FD or OD) was then sub-divided into two equal portions. One portion was labeled as the "Whole insect flour" (W), and the other portion was defatted and referred to as "Defatted insect flour" (D).

Defatting process

The defatting was done based on the procedure of Nderitu and colleagues (Nderitu et al., 2017). Each FD and OD flour was mixed with hexane (Laboratory Reagent, ≥95%, Sigma-Aldrich, Saint Louis, MO, USA) in a ratio of 1:5. The mixture was stirred using a mechanical lab shaker (Spectrum, *Camida* Ireland) at 200 rpm for 16 h and filtered using a Whatman # 1 filter paper. The residue was washed with the same hexane to remove any traces of oil. The mixture was filtered again, and the now defatted powder was dried under the fume hood at room temperature (25 °C). The dried powder was sieved through a 500 µm screen, and the sieved residue (flour) was stored at 4 °C until analysis.

Moisture determination

To determine the moisture (dry matter content) of the dried insect flours, samples were subjected to oven drying at 105 °C for 48 h using a UF30 Memmert (GmbH, Schwabach Germany) convection oven.

4.2.3 Antinutrient analyses

Determination of phytic acid

Analysis of phytic acid in the dried insect samples was done according to Tanjendjaja et al., (1980), with modifications as detailed by Camire & Clydesdale (1982). One gram (1.00 g) of the sample was weighed into a 125 mL Erlenmeyer flask, extracted with 25.0 mL of a 3.0 %(v/v) sulphuric acid solution on a shaker bath (Labortechnik KS 250b, Germany) at 100 rpm at a temperature of 30 °C for 30 min. The slurry was filtered through a filter paper (Whatman #41) and rinsed using a fine jet stream with a small extract solvent volume. The filtrate was transferred to a 50.0 mL screw-cap centrifuge tube and placed in a boiling water bath for 2 - 5 min (to aid in the precipitation of ferric phytate) before the addition of 3.0 mL of a ferrous chloride solution containing 6.00 mg ferric iron per mL in 3.0 % sulphuric acid. The tubes were heated in a boiling water bath for 45 minutes to complete the precipitation of the ferric phytate complex. This was centrifuged with a high-speed centrifuge (800d centrifuge, China) at 12,000 g for 10 min, and the supernatant was discarded, while the residue was washed once with 30.0 mL of distilled water centrifuged again, and the supernatant was removed.

To the remaining content in the tube, 3.0 mL of 1.5 M NaOH and 1.0 mL of distilled water were added. The residue ‘cake’ was broken up and then sonicated to disperse the precipitate, then topped to 30 mL thoroughly. The samples were cooled, centrifuged, and the supernatant was quantitatively transferred to a 50.0 mL volumetric flask. The precipitate was rinsed once with approximately 10 mL of distilled water and added to the mark.

A stock solution containing 10.00 mg/mL of sodium phytate in distilled water was prepared. Serial dilutions were made to obtain concentrations from 1.00 mg/100 mL to 100 mg/100 mL based on previous lab analyses. The sample and standard dilutions were injected into the HPLC using a 20-µl sample loop. The analysis was performed using an HPLC (Model LC-10AS, Shimadzu Corp., Kyoto,

Japan) equipped with a UV detector at 205-340 nm filter, 250 mm x 4.6 mm ID column containing spherisorb ODS C18 10 µm packing, and the oven temperature was 35 °C. The mobile phase was an aqueous 0.005 M sodium acetate solution; the flow rate was 0.5 mL/min, and the injection volume was 20 µl. The *Shimadzu* software was used to calculate the peak areas.

Determination of tannins

Tannin content was determined by the Folins-Denis colorimetric method described by Kirk & Sawyer (1998). Exactly 5.00 g were dispersed in distilled water and shaken. The mixture was allowed to stand for 30 min at 28 ºC, then filtered through filter paper (Whatman #42). Then, 2.0 mL of the extract and the standard tannin solution (tannic acid) 0, 0.1, 0.2, 0.3, 0.4 and 0.5 mg/mL were dispensed into a 50 mL flask. Similarly, 2.0 mL of distilled water was put in a separate volumetric flask as a blank to calibrate the instrument to zero. Then, 2.0 mL of Folins Denis reagent was added to each flask, followed by 2.5 mL of saturated Na_2CO_3 solution.

The content of each flask was adjusted to 50.0 mL by adding distilled water and then allowed to incubate at 28 °C for 90 min. Their respective absorbance was measured in a UV-vis spectrophotometer (UV mini 1240 model, Shimadzu Corp., Kyoto, Japan) at 760 nm. The results were expressed as the level of tannic acid in mg/mL of extract.

Determination of oxalates

The analysis was made according to Libert & Franceschi (1981) with modifications (Yu et al., 2002). Aliquots of 0.5 of the sample were homogenized in 4.0 mL of 0.5 M HCl. The homogenate was heated at 80 °C for 10 min with intermittent shaking. Distilled water was added to the homogenate to a volume of 25.0 mL. About 3.0 mL of the solution was withdrawn and centrifuged (800d centrifuge, China) at 12000 g for 10 min. 1.0 mL of supernatant was passed through a Whatman filter paper (0.45 µm) before HPLC analysis. Standards were prepared at varying concentrations for quantification.

The sample and standard dilutions were injected into the HPLC using a 20-µl sample loop. The analysis was performed using an HPLC (Model LC-10AS, Shimadzu Corp., Kyoto, Japan) equipped with a UV detector at 220 nm filter, 250 mm x 4.6 mm ID column containing spherisorb ODS C18 10 µm packing, and the oven temperature was 35 °C. The mobile phase was a solution containing 0.5 % KH_2PO4 and 0.5 mM TBA (tetrabutylammonium hydrogen sulfate) buffered at pH 2.0 with orthophosphoric acid. The flow rate was 1 mL/min, and the injection volume was 20 µl. Again, the Shimadzu software was used to calculate the peak areas.

4.3 Statistical analyses

The dry matter values of the antinutrients were reported as means and standard deviations of three replicates. To determine the effect of the drying methods and defatting on the antinutrient content, after testing for normality using a Levine's test, data were analyzed using a two-way analysis of variance (ANOVA) using drying method and type of insect flour (whole or defatted) as the two independent factors. A posthoc test using the Tukey method at $p \leq 0.05$ separated differences among the mean values. Data analysis was performed using GraphPad Prism Version 9.02 for Windows (GraphPad Software, La Jolla, CA, USA).

4.4 Results

4.4.1 Phytate content of whole insect flour and defatted insect flour

Table 4.2 shows the phytate content of whole insect flours and defatted insect flours after drying. Oven-drying resulted in significantly higher (p = 0.004*)* phytate contents in whole insect flours and defatted insect flours obtained from *R. differens* (range: 0.21 – 0.22 mg/100 g) and *G. bimaculatus* (range: 0.18 – 0.19 mg/100 g). By contrast, insect flour type (whole versus defatted) had no significant differences *(*p = 0.4779) in the phytate content of whole insect flours and defatted insect flours when either of the drying methods was applied.

Table 4. 2 *Characterization of freeze-dried and oven-dried field crickets (G. bimaculatus) and bush crickets (R. differens) and desert locust (S. gregaria) by means of their phytate contents. Values are expressed as mean ± standard deviation (n = 3).*

	G.bimaculatus		*R. differens*		*S. gregaria*	
	Oven-dried	Freeze-dried	Oven-dried	Freeze-dried	Oven-dried	Freeze-dried
Whole $w_{Phytate}$ (mg/100)	0.19 ± 0.01^{aA}	0.13 ± 0.01^{aA}	0.21 ± 0.03^{aA}	0.13 ± 0.02^{bA}	0.15 ± 0.01^{aA}	0.13 ± 0.06^{aA}
Defatted $w_{Phytate}$ (mg/100)	0.18 ± 0.03^{aA}	0.13 ± 0.02^{aA}	0.22 ± 0.03^{aA}	0.10 ± 0.01^{bA}	0.13 ± 0.04^{aA}	0.12 ± 0.04^{aA}

where w = mass fraction (g/100 g dry matter)
Values in the same row with different lowercase superscripts and those in the same column with different uppercase superscripts represent significant ($p<0.05$) differences among the treatments of the dried insect flours species

4.4.2Tannin content of whole insect flour and defatted insect flour

The tannin content of whole insect flours and defatted insect flours of the three edible insects are shown in table 4.3. There was a significant effect of defatting (p = 0.0127) on the tannins' content. No significant differences (p = 0.9306) were observed in individual insect species when samples were either oven-dried or freeze-dried. However, the highest concentration was observed in oven-dried whole *G. bimaculatus* flour (on average, 14.9 mg/100g). The highest tannin level was measured in freeze-dried *R. differens* defatted insect flour (on average, 16.4 mg/100g).

Table 4. 3 *Characterization of freeze-dried and oven-dried field crickets (G. bimaculatus) and bush crickets (R. differens) and desert locust (S. gregaria) by means of their tannin contents. Values are expressed as mean ± standard deviation (n = 3)*

	G.bimaculatus		*R. differens*		*S. gregaria*	
	Oven-dried	Freeze-dried	Oven-dried	Freeze-dried	Oven-dried	Freeze-dried
Whole $W_{tannins}$ (mg/100)	14.9 ± 0.05 ^{aA}	11.5 ± 0.35 ^{aA}	10.0 ± 0.41 ^{aA}	7.8 ± 0.29 ^{aA}	9.0 ± 0.11 ^{aA}	9.3 ± 0.01 ^{aA}
Defatted $W_{tannins}$ (mg/100)	13.6 ± 0.47 ^{aA}	13.1 ± 0.07 ^{aA}	11.6 ± 0.31 ^{aA}	16.4 ± 0.25 ^{aB}	12.0 ± 0.16 ^{aB}	12.5 ± 0.22 ^{aB}

where w = mass fraction (g/100 g dry matter)

Values in the same row with different lowercase superscripts and those in the same column with different uppercase superscripts represent significant (p<0.05) differences among the treatments of the dried insect flours species.

4.4.3 Oxalate content of whole insect flour and defatted insect flour

Table 4.4 depicts the oxalate content of whole insect flours and defatted insect flours after drying. Drying methods did not affect the oxalate content of whole insect flours and defatted insect flours of individual insect species. The levels measured in all insect samples were less than 0.40 mg/100g regardless of the drying method.

Table 4. 4 *Characterization of freeze-dried and oven-dried crickets (G. bimaculatus) and bush crickets (R. differens) and desert locust (S. gregaria) by means of their oxalate contents. Values are expressed as mean ± standard deviation (n = 3).*

	G.bimaculatus		*R. differens*		*S. gregaria*	
	Oven-dried	Freeze-dried	Oven-dried	Freeze-dried	Oven-dried	Freeze-dried
Whole Oxalate (mg/kg)	0.35 ± 0.01 ^{aA}	0.17 ± 0.01 ^{aA}	0.39 ± 0.01 ^{aA}	0.39 ± 0.02 ^{aA}	0.33 ± 0.01 ^{aA}	0.29 ± 0.00 ^{aA}
Defatted Oxalate (mg/kg)	0.20 ± 0.00 ^{aA}	0.25 ± 0.02 ^{aA}	0.26 ± 0.01 ^{aA}	0.33 ± 0.00 ^{aA}	0.29 ± 0.00 ^{aA}	0.36 ± 0.02 ^{aA}

where w = mass fraction (g/100 g dry matter)

Values in the same row with different lowercase superscripts and those in the same column with different uppercase superscripts represent significant (p<0.05) differences among the treatments of the dried insect flours species.

4.5 Discussion

Our previous study on the chemical composition (overall protein, fat, and carbohydrate percentages and, more specifically, amino acids, fatty acids, and mineral levels) of these insects (*R. differens*, *G. bimaculatus* and *S. gregaria*) confirmed that these species represent healthy and nutritious food items (Fombong et al., 2021), and this was in agreement with what other researchers had reported previously for *R. differens* (Kinyuru et al., 2010; Ssepuuya et al., 2019), *S. gregaria* (Kinyuru et al., 2020), and *G. bimaculatus* (Udomsil et al., 2019). Besides nutritionally beneficial ingredients associated with these insects, the presence of antinutrients, such as phytates, tannins, and oxalates, ought to be better documented. Generally, antinutritional factors are usually present in plant materials, but many phytophagous insects have been identified to retain these materials in detectable amounts (Idowu et al., 2019).

Most processing methods, such as drying, soaking, frying, and boiling, have lowered tannins, phytates, and oxalates in plant materials (Camire & Clydesdale, 1982). However, this has been rarely investigated in edible insects. These reductions have mainly to do with increasing endogenous enzyme activities that break down these molecules. The phytate compositions of insects in this study were lower than those reported by other studies for crickets (2.8 mg/100 g), grasshoppers (2.8 mg/100 g), yam beetle (2.8 mg/100 g), and palm weevil larva (2.89 mg/100 g) (Ekop et al., 2010). Other authors reported comparable levels of phytate for crickets *Gryllus assimilis* (10.3 mg/100g), grasshoppers *Melanoplus foedus* (19.0 mg/100 g), and termites *Macrotermes nigeriensis* (9.0 mg/100 g) (Oibiokpa 2017). In the present study, the phytate levels in all insects analyzed were generally low compared to what was reported in the literature, which could be attributed to their diets. Phytates are very heat stable up to 100^{o}C and therefore not removed by conventional heat treatment like drying (Schlemmer et al., 2009). Other processes such as germination, soaking, and malting that release or activate *phytases* also reduce phytate contents. The presence of phytate in food reduces the bioavailability of mineral elements, such as iron, calcium, magnesium, manganese, and copper (Groff et al., 1995). The phytate contents of insects in this study are below permissible levels of *22 mg/100 g* (WHO, 2003). Therefore, they may not significantly interfere with the absorption of mineral elements in the body when consumed as food or food ingredients.

Although drying methods had little effect on the tannin content in the insects analyzed (all less than 16.0 mg/100 g), this was significantly lower compared to what was reported by others (Oibiokpa 2017) for *G. assimilis* (49 mg/100 g), *M. foedus* (52 mg/100 g), *M. nigeriensis* (47 mg/100g) and *Cirina forda* (48 mg/100 g). Chakravorty et al., (2016) reported even higher levels averaging 141.23 mg/100 g for *Oecophylla smaragdina* and 615.0 mg/100 g for *Odontotermes* sp. In contrast, low levels of tannins in the cricket *Gymnogryllus lucens* (3.3 mg/100 g), yam beetle *Heteroligus meles* (3.8 mg/100 g), palm weevil *Rhynchophorus phoenicis* (4.1 mg/100g) and grasshopper *Zonocerus variegatus* (4.30 mg/100 g) were observed (Ekop et al., 2010). When present in food materials, tannins can form insoluble complexes with protein, thereby interfering with their bioavailability, and a high tannin level in diets is ascribed to its astringent property (Ifie & Emeruwa, 2011). The quantity of tannins was below the permissible limits *(76 - 90 g/kg)* in all three insects analyzed (WHO, 2003). The presence of these antinutrients may not significantly affect the digestibility of proteins found in these insects when used as food.

In the same vein, the oxalate levels in the present study were generally low for all insects tested (less than 0.4 mg/100 g). In the literature, significantly higher levels have been reported. Ekop et al., (2010) observed oxalate contents to be between 13.20 mg/100 g for crickets to 28.40 mg/100 g for yam beetle. Similarly, when *G. assimilis*, *M. foedus,* and *M. nigeriensis* were analyzed, phytate levels ranged between 20.25 mg/100 g and 25.65 mg/100 g (Oibiokpa, 2017). Oxalates, just like phytate, limit the availability of some notable minerals, such as magnesium, iron, and even calcium (Groff et al., 1995). The oxalate values of this study were equally more negligible than the tolerable limits ranging between *20 and 50 mg/ kg* (Pearson, 1973). Thus, these insects reared using similar diets can be consumed without concerns frequently associated with oxalate toxicity.

Generally, oven-dried or freeze-dried insects had low levels of antinutrients in conformity with the reports of other researchers. In addition, the phytic acid and oxalate levels in the three edible insects analyzed in this study were much lower than corresponding ones from some common foods of plant origin in Table 4.1 (Schlemmer et al., 2009). Likewise, tannin levels were lower than in millet, rye, oat, sorghum, and broad bean (Khan et al., 1979).

The fact that all insects in this study were reared in the labs could suggest that specialized diets

low in antinutrients were used in the first place, thus explaining the exceedingly low antinutrient values. These minuscule values could be the reason why significant differences were not detected at ($p< 0.05$) upon defatting and using different drying modes, irrespective of insect species. Equipment sensitivity could also contribute to this insignificance, as more advanced chromatographic equipment has been shown to affect results (Martin & Guiochon, 2005). For instance, it has been reported that in physiological samples such as tissues and cells where the concentration of phytate and other inositol phosphate is deficient compared to food samples, highly sensitive methods and adequate procedures for the sample preparations from different matrices were required (Schlemmer et al., 2009).

Based on this study, it is insufficient to know if the antinutrients determined were present in their gut upon feeding or accumulated over time; It is therefore expected that this study prompts such questions leading to further research on insects antinutrients gut accumulation.

The effect of different diets or other factors that could affect antinutrient composition like seasonal/geographical variations were beyond the scope of this present study. Nevertheless, investigating these other effects could add value to this study as we cannot overlook the impact of such variations on the antinutrient composition of insects. If time allowed, the investigation of a suite of all possible antinutrients (lecithin, cyanogenic glycosides, protease inhibition factors, alkaloids) in these insects would have been worthwhile.

The correct categorizing of the three assessed compounds as either antinutrients or bioactive agents remains a vital issue of contention to date. Despite possessing no nutritional value, antinutrients may not always be harmful, depending on the dosage. Consequently, they could either be considered as antinutrients bearing negative consequences or as non-nutritive compounds that display positive health effects. Therefore, finding the right dosage balance between favorable and harmful effects of antinutrients remains a massive gap yet to be filled.

4.6 Conclusion

The analyzed insects contained low levels of the tested antinutrients, *i.e.,* tannin, phytate, and oxalate. The observed values were much lower than corresponding ones in some common foods of plant origin. Drying methods and the defatting process had little effect on the antinutrient levels. Introducing a drying step with the sole purpose of reducing antinutrients in insects would therefore seem unnecessary and too costly. More so, the rearing methods and insect diets described in this study did not adversely elevate the antinutrients levels we evaluated. Thus, under these specific diet and rearing conditions, the use of these three insects as food and food ingredients should be encouraged without contamination stemming from the above-examined antinutrients

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CHAPTER FIVE

Effect of Light and Dark Rearing Conditions on Fitness Parameters of Farmed *Ruspolia differens* Fed on a novel Artificial Diet

5.1 Introduction

So far in this thesis, *R. differens* used have been captured from the wild during the swarming months. However, in the course of this Ph.D., the lack of adequate *R. differens* samples was a perennial challenge, and there was the need to have a constant supply of these insects. The first option was to establish viable colonies of this insect. For any insect rearing system to be effective, a suitable diet, amongst other factors, is a prerequisite. Before this study, other research collaborators had recently established suitable oviposition substrates, rearing temperatures, and humidity for *R. differens*. Therefore, armed with this newfound knowledge, this chapter describes the first attempts to rear *R. differens* in captivity entirely on a novel artificial diet.

In Sub-Saharan Africa, entomophagy has expanded from the usual farm to fork via value chains that involve commercialization to improve household food security and income (Niassy et al., 2018; Mariod, 2020; Kinyuru and Ndung'u, 2019). Black soldier flies, (bush) crickets, mealworms, and grasshoppers top the list of the currently farmed insects, with profit-making as the primary target.

As explained already in chapter One, *R. differens* Serville is a crucial edible insect that is known to provide an extra family income, especially in Uganda, Cameroon, Tanzania, and Kenya (Agea et al., 2008; Ssepuuya et al.,2016; Kinyuru et al., 2010; Valtonen et al., 2013; Mmari et al., 2017; Kekeunou et al., 2020; van Itterbeeck et al., 2019; Matojo and Njau 2010).

It was established in the opening chapter and throughout this thesis that these bush crickets are delicious and nutritious (Fombong et al., 2017; Mmari et al., 2017; Kinyuru et al., 2010; Ssepuuya et al., 2016). While these studies have further established the insects' nutritive, ostentatious value in the regions and countries where they are consumed, there still exists the need to fill in the gaps of supply that occur during the non-swarming periods. Therefore, mass rearing has recently been considered a sustainable way to relieve the pressure on natural populations and habitats and generate a constant supply of edible insects for eager consumers.

Some researchers have attempted the mass rearing of *R. differens* in laboratory settings (Lehtovaara et al., 2018; 2019); however, expected outputs achieved have been far from successful. Some authors (Malinga et al., 2018a,b; Rutaro et al., 2018; Valtonen et al., 2018)

have independently shown that *R. differens* can feed on different grasses and substrates. They have even proposed the most likely plants for sustainable mass rearing, albeit developmental times recorded have been prolonged on such diets. The most common plants that have been reported as diets for rearing *Nsenene* include; maize leaves, millet, guinea grass, sorghum heads, ryegrass, elephant (*Napier*) grass (Malinga et al., 2018a). In another study the researchers assessed the acceptance and preference of different plant materials, their effects on growth, development, reproductive performance, and the effects of diet on the nutrient composition of the grasshopper (Anu Valtonen et al., 2018). The problems associated with such plant diets include unavailability during spells of drought, low protein content, and an amino acid score to meet the insects' nutritional requirements.

Furthermore, the labor-intensive nature of daily removal and replacement of such a perishable diet, as well as the susceptibility to microbial spoilage/contamination (*my observation*), were apparent. Thus, there is a clear need to find a suitable diet for rearing *R. differens*. Artificial diets, on the other hand, can be available globally and remain unaffected by prevailing local climatic severities. Their nutritional content can easily be modified to meet specific needs. Such ease in manipulating diet composition can enable the inclusion of other 'non-nutrient' ingredients, such as antimicrobial and anticaking agents (Cohen, 2005). The addition of such ingredients would generally result in longer shelf lives than 'natural' diets.

Artificial diets have long been used in the past to rear insects in the laboratory with great success (Cohen, 2001; Cohen, 2005). Such achievements have gone a long way to cancel out the cost of developing artificial diets. From a practical point of view, using an artificial diet would be more profitable for mass-rearing programs, which undoubtedly is the current upward trend for rearing edible insects across the globe.

In addition, amongst the many factors that determine the fitness parameters of insects, the photoperiod or the duration of light exposure can have a significant influence on their growth, development, and survival rate. *R. differens* is a nocturnal insect species. Its swarming and feeding activity mainly occurs at night (Agea et al., 2008; Kinyuru et al., 2010). Also, during the daytime, bush crickets are usually found under canopies in forests or deep down in tall grass bushes; rarely do they spend much of their time exposed to sunlight (Hartley, 1967; Robinson

and Hartley, 1978; Bailey and McCrae, 1978). Therefore, it seems logical that any attempts to rear these insects in a laboratory environment should at least try to simulate some considerable time in dark or dim light conditions.

Against this knowledge gap, our study aimed to develop an affordable mass-rearing artificial diet for *R. differens* and determine the influence of photoperiod. Thus, the effects of two light regimes, a "dark" condition, *i.e.,* with a very short photoperiod (≤ 1 h of light per 24 h), and a "light" condition, *i.e.,* with a photoperiod of 12 h (12 h of light per 24 h), on survival rate (mortality), developmental time, weight gain, feed conversion ratio, number and duration of molts to reach the adult stage, and longevity of adult *R. differens* fed on this artificial diet were examined. Also, the complete nutritional profile of an artificial diet developed for rearing *R. differens* is reported.

5.2 Materials and methods

5.2.1 Collection of wild *R. differens* adults and rearing of parent stock

The parent population of *R. differens* was collected from the wild near the Makerere University Agricultural Research Institute in Kabanyolo (MUARIK) (Uganda) (0°27'03 N and 32°36'42 E). We selected equal numbers of adult males and females and placed them into ten plastic containers (24 cm length x 18 cm width x 12.5 cm height) (Thermopak Limited, Nairobi, Kenya). Each container housed ten males and ten females to increase the chances of mating and oviposition (Brits and Thornton, 1981). Wet folded tissue paper was used as a source of water. Four small, round plastic jars with moistened cotton wool were placed at the corners of each plastic container as a substrate for oviposition. Once laid, the eggs were collected onto small, round plastic jars (5.3 cm width x 7.1 cm height) containing sieved moistened sand and cotton wool (50:50), which were sprayed daily with water, and incubated for 2-3 weeks at 31 °C and 70% RH until hatching.

5.2.2 Artificial diet preparation and composition

The new artificial diet was developed based on the reference in-house diet used for cricket supplementation at the ICIPE insect rearing facility. The ingredients which were readily available locally are listed in Table 5.1. They consisted of cornmeal, soy meal, wheat bran, bone meal, vitamin premix, lysine, methionine, and limestone (which were obtained from a local veterinary shop). Sugar, fishmeal (*omena*), and salt were purchased from a local supermarket chain, Naivas, Kenya. All above ingredients were weighed as shown in Table 5.1 using a balance (AB204-N, Mettler Toledo, Boston, USA), homogenized, and blended to a fine particulate size of approximately 10 mm as measured using a plastic sieve mesh. To this dry mixture, citric acid (antimicrobial agent) and nipagin (antifungal agent) supplied by Sigma Aldrich (Merck, Cape Town, South Africa) were added. Given it was the first attempt to develop an artificial diet for rearing *Ruspolia differens*, the ingredients were chosen to ensure a nutrient-dense diet that would limit the chances of failure. The goal was that, if successful, then the pricier components in this novel diet would be replaced by more affordable kitchen and agro-waste materials.

Table 5. 1 *Artificial diet composition and quantity on a dry matter basis*

Ingredient	Amount (g) per 100g
Wheat Bran	16.0
Cornmeal	4.0
Sugar	20.0
Soy meal	40.0
Fish meal	8.0
Nipagin (antifungal agent)	2.0
Citric acid (antimicrobial agent)	2.0
Bone meal (crushed cow bones)	2.0
Limestone	2.0
Salt (NaCl)	2.0
Vitamin premix*§	1.0
Lysine§	0.5
Methionine§	0.5

* *Ingredients as provided by manufacturer (per 50 kg) vitamin A: 10,000 I.U.; iron: 15 mg; zinc: 40 mg; manganese:*

20 mg; magnesium: 50,000 mg; vitamin D: 2,000 I.U.; vitamin E: 2,000 I.U.; iodine: 20 mg; selenium: 40 mg; cobalt: 400 mg; copper: 10 mg; calcium: 566 mg; antioxidant: 6,000 mg.

[§]The vitamin and amino acid premixes, (IS 723 FBF-V10), purchased from the local veterinary vendor were supplied by DSM Nutritional Products South Africa (Pty) Ltd. (Johannesburg, South Africa).

5.2.3 Experimental set-up and Insect Feeding

The experiment was conducted at International Centre for Insect Physiology and Ecology (*icipe*), Duduville campus in Nairobi (Kenya). The nymphs originated from a parent stock collected at the Makerere University Agricultural Research Institute (Kabanyolo, Uganda) in 2015 and 2016, as described in *section 5.2.1* above. The experiments were conducted between April 2018 to September 2018. During the experiments, the newly hatched nymphs were collected from the colony and weighed using tiny capsules. Afterward, they were reared individually in Petri dishes, on which one side was replaced with wire mesh (for ventilation). After the third molt, they were then transferred to larger containers which were all adequately ventilated at the top. This progression evolved to even bigger containers when mating pairs were put together. (Figure 5.1)

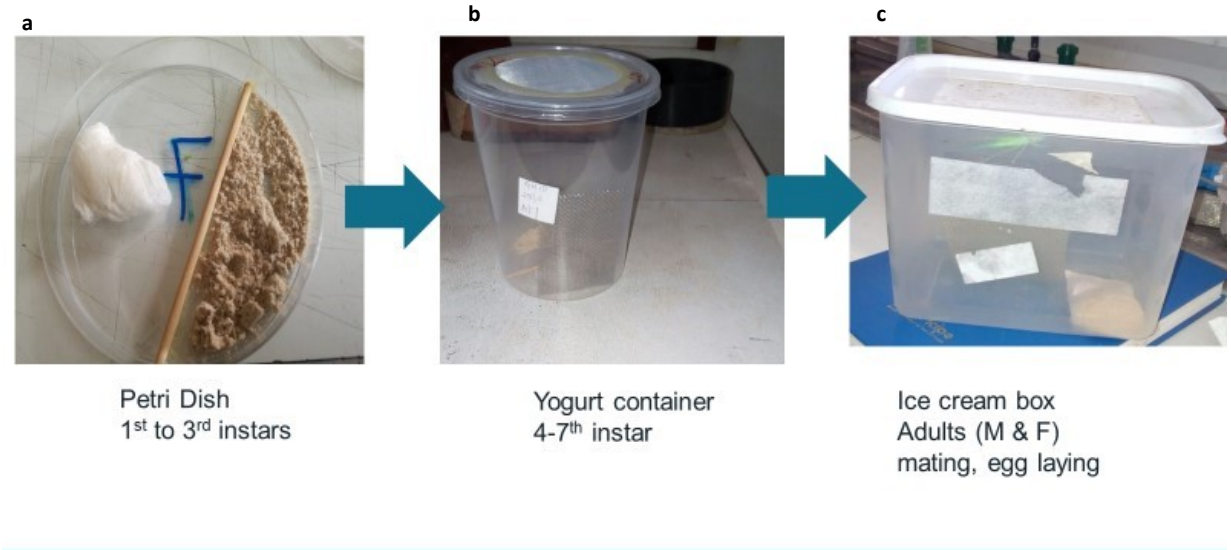

Figure 5. 1 *Progression of rearing containers (a) Petri dish with diet and cotton wool for first to third instar nymphs (b) Yoghurt container: for four[th] to the seventh instar with wire mesh included for molting and perching (c) Ice-cream box for mating adult pairs equipped with double-layer moist cotton wool as an oviposition substrate.*

A total of 120 insects were initially used with 60 nymphs in each setup and placed in semi climate-controlled rooms of similar dimensions L: 3 m x W: 2 m x H: 2.2 m. These insects were randomly placed on three blocks of 20 insects. Their positions were rotated weekly to allow equal exposure to sunlight in the light setup. Automated thermometers at 28 °C were installed in both rooms to sustain this fixed temperature; Relative humidity was maintained between 60- and 70 % and was measured by a *TFA* digital hygrometer placed on the benchtop.

In each case, the insects were placed on lab benchtops' supported on antivibration stands and equipped with anti-ant shoe glue (from a local shoe cobbler) on each table foot to ward-off ant predation.

Two light regimes were used for this study. A control, the typical 12L:12D regime (hereafter referred to as the lightroom or setup), was used; meanwhile, for the treatment condition, a predominantly dark regime (or dark room with 1L: 23D). Therefore, the light regime consisted of 12 h and 12 h dark, while the dark was 1 h light and 23 h dark.

The room of the light setup had large glass windows to provide ample sunlight during the day, supplemented by two 180 cm long fluorescent TL lamps (36W, 3000 lumens)120 cm. The darkroom, on the other hand, was similarly designed, but the windows were blocked with light impenetrable plywood sheets. The rooms' pre-installed fluorescent TL lamps were only used during daily monitoring and feeding, which lasted about an hour. Entry into this 'dark room' was strictly restricted to this one-hour window to avoid light from the corridors.

One gram of the artificial diet was fed weekly to each insect nymph and was replaced then. The uneaten rest was measured for its pH (see below). The leftover food was also analyzed for its moisture content and weighed to estimate how much was eaten. The diet was monitored daily to check for molds and replaced accordingly. To provide water, moistened cotton wool was added to each container (see figure 5.1).

Additionally, daily monitoring of the individually-reared nymphs was ensured to capture molting, mortality, and fecundity data and recorded accordingly.

5.2.4 Determination of body growth parameters

Survival was calculated as the percent of insects alive at the time of measurement to the total number of insects at the onset of the experiment. Developmental time was calculated as the time (in days) from egg hatching to adulthood (with the appearance of functional wings). This value corresponds to the nymphal (larval) developmental time since bush crickets are hemimetabolous insects with no pupal stage. The growth index was estimated according to the following formula:

$$\text{Growth index.} = \frac{percent\ survival}{developmental\ time\ (days)} \quad \text{Eq. 5.1}$$

During the feeding period, feed intake was calculated as the difference between the amount of feed offered and substrate residue. *R. differen*'s feces dropped in the substrate were carefully removed before weighing the substrate residue. The total amount of feed consumed during rearing was recorded. These data were then used to determine the feed conversion ratio (FCR), calculated as the proportion of dry feed consumed to the bodyweight gained (Lundy and Parrella, 2015).

$$\text{F. C. R.} = \frac{Feed\ consumed\ (1g)}{Animal\ weight\ gain\ within\ a\ week\ (days)} \quad \text{Eq. 5.2}$$

Adult longevity was calculated in males or females as the number of days elapsed after the final (adult) molt until death occurred. For analyzing fecundity, adult mating pairs were transferred to separate containers and provided with extra perching material. Double-layered wet cotton wool was provided as an oviposition substrate. The substrate was observed daily for eggs till the death of the female, which occurred quite frequently by cannibalism by the male (or vice versa). The number of eggs deposited per female was counted.

5.2.5 Intrinsic parameters and nutritional composition analyses

The water activity of the artificial diet was determined using a water activity meter (LabMaster aw, Novasina, Lachen Switzerland) at a constant temperature of (25 °C) until the water activity value was stable for 5 min.

The pH was measured using a bench-top digital pH meter (Mettler Toledo FE20-Kit FiveEasy™, coupled with a LE409 pH electrode, Columbia, MD, USA). Briefly, 1.0 g of artificial diet was dissolved in 5.0 mL distilled water, mixed thoroughly using a sterile glass rod, and measured in triplicate.

The proximate, fatty acid, mineral, and amino acid analyses of the artificial diet were performed as earlier described in *section 2. 2 Chemical Analyses* of chapter two

5.2.6 Data analysis

A Mann-Whitney non-parametric test was performed to compare the mean values of the biological fitness parameters (for at least five biological replicates for each parameter) observed for the insects reared under light and dark conditions. Survival analysis was done using the Kaplan Meier test. A correlation test between mortality and molting was carried out using the Pearson correlation test. All statistical analyses were performed using GraphPad Prism version 8.0 for Windows (GraphPad Software, San Diego, California, USA, www.graphpad.com).

5.3 Results

5.3.1 Nutritional composition of the artificial diet

Water activity and proximate composition

Table 5.2 shows the water activity and proximate contents of the artificial diet on a dry matter basis. Water activity was 0.49 at 25 °C. The moisture content of the undried artificial diet was 8.5 %. Proximate analysis revealed that the protein content had the highest concentration of 21.1 %, followed by the ash content (7.29 %). The other vital class of macromolecules, *i.e*., fat, was 5.15 %, while crude fiber was at least 4.53 %.

Table 5. 2 *Water activity and proximate composition (%) of the artificial diet on a dry matter basis (per 100 g) Data are the means ± standard deviations of three replicates. The values of moisture, protein, fats, ash and crude fiber represent mass fractions*

Parameter	Artificial Diet
Water activity	0.49 ± 0.01
Moisture content	8.48 ± 0.08
Protein	21.1 ± 0.64
Fat	5.15 ± 0.21
Carbohydrate¤	53.5 ± 0.21
Crude fiber	4.53 ± 0.02
Ash	7.29 ± 0.08
Energy (KJ)	374 ± 8.65

¤ Calculated as 100 minus the sum of mass fractions of moisture, protein, fats, ash, and fiber | See equation 2.2 in *section 2.2*

Fatty acid composition of the artificial diet

Table 5.3 displays the fatty acid profile of the artificial diet. The most abundant saturated fatty acids were stearic and palmitic acid in decreasing order. The two essential polyunsaturated fatty acids (PUFA) were present, with linoleic acid eight times more abundant than linolenic acid. The monounsaturated fatty acid (MUFA), oleic acid, was slightly lower than linoleic acid. The latter observation accounted for the higher PUFA levels (31.2 %) than the MUFA levels (27.31 %).

Table 5. 3 *Fatty acid composition (% total fatty acids) of the artificial diet. Data are the means ± standard deviations of three replicates*

Fatty acid	
decanoic acid	0.04 ± 0.02
Lauric acid	0.10 ± 0.01
myristoleic acid	0.91 ± 0.08
myristic acid	0.23 ± 0.21
pentadecanoic acid	0.16 ± 0.04
palmitic acid	11.5 ± 0.16
palmitoleic acid	1.71 ± 0.09
heptadecanoic acid	0.03 ± 0.01
stearic acid	32.0 ± 3.43
oleic acid	24.7 ± 1.24
linoleic acid	26.0 ± 0.89
linolenic acid	3.64 ± 0.27
arachidonic	0.12 ± 0.06
eicosapentaenoic acid	1.37 ± 0.52

Amino acid composition of the artificial diet

The amino acid profile of the diet reveals the presence of all ten essential amino acids required for insect growth and development (Table 5.4), which make up 46 % of the total amino acids present. Glutamic acid, leucine, glycine, and asparagine, respectively, in reducing order, make up the top four most abundant. At the same time, tryptophan and cysteine were all less than 1 % and comprised the least abundant amino acids in the artificial diet.

Table 5. 4 *Amino acid composition (mg/ g protein) of artificial diet on a dry matter basis. Data are the means ± standard deviations of three replicates*

Amino acid	
Histidine	3.05 ± 0.27
Arginine	5.67 ± 0.53
Serine	6.15 ± 0.18
Glycine	9.32 ± 0.34
Aspartic acid	8.41 ± 0.75
Glutamic acid	11.1 ± 0.79
Threonine	4.94 ± 0.05
Alanine	7.55 ± 0.4
Proline	7.55 ± 0.22
Lysine	4.71 ± 0.93
Cystine+cysteine	0.73 ± 0.05
Tyrosine	3.5 ± 0.48
Methionine	2.33 ± 0.12
Valine	5.81 ± 0.2
Isoleucine	4.91 ± 0.23
Leucine	9.44 ± 0.18
Phenylalanine	4.03 ± 0.81
Trptophan	0.81 ± 0.14

Mineral levels and vitamin B_{12}

Table 5.5 shows the mineral content of the diet. The mineral elements including Na, K, P, Ca, and Mg and essential trace minerals Mn, Cu, Fe, and Zn all comprised the mineral profile of the artificial diet. Ca and Fe were the most abundant major and trace minerals, respectively, while Cu (trace) and Na (major) recorded the lowest mineral concentrations. The vitamin B_{12} concentration was 1.62 µg per 100 g of the dry mass of the artificial diet.

Table 5. 5*: Mineral composition (in mg/100 g) and Vitamin B12 (µg/100 g) of artificial diet on a dry matter basis*

Mineral	Artificial diet
Sodium	0.73 ± 0.05
Potassium	5.67 ± 0.44
Calcium	13.32 ± 0.82
Magnesium	2.87 ± 0.17
Phosphorus	6.85 ± 0.38
Trace	
Zinc	0.14 ± 0.01
Iron	0.25 ± 0.01
Copper	0.04 ± 0.00
Manganese	0.08 ± 0.01
Vitamins	
Vitamin B_{12}	1.62 ± 0.56

5.3.2 Influence of light on biological fitness parameters

Table 5.6 summarizes the effects of light and dark conditions on some critical biological fitness parameters for *R. differens* when reared on an artificial diet.

Table 5. 6 *Life Table of fitness performance and intrinsic diet properties of R. differens reared under light and dark conditions and artificial diet intrinsic parameters. Values represent the mean ± standard error of the mean*

Description	Stage| sex | state	LIGHT	DARK
Fitness Parameters			
% Percent survival (n = 60) ¤		76[a]	84[a]
Developmental time of nymphs (days) (n = 10)	nymph	71 ± 1.38 [a]	58 ± 2.98 [b]
Growth index[µ] (n = 10)		1.07[a]	1.45 [b]
Average number of molts to reach adult (days)	nymph	8.9 ± 0.29 [b]	7.1 ± 0.29 [a]
Average duration of instars (n=10)	1st Instar	9.8 ± 1.2 [b]	7.2 ± 0.92 [a]
	Last instar	18.0 ± 1.0 [b]	10 ± 2.4 [a]
Sex ratio (M/F)* (n = 10)	Adult	1/1 [a]	1/1.2 [a]
Color ratio G:B: P (n = 10)		6: 1: 0	9: 1: 0.25
Initial weight (mg) (n = 10)	Nymph	4.1 ± 2.26 [a]	4.8 ± 1.30 [a]
Final (adult) weight (g) (n = 10)	Adult	0.35 ± 0.05[a]	0.43 ± 0.03 [a]
Adult longevity (days)	Male (n = 5,7)	12.5 ± 5.58 [a]	39.3 ± 5.77 [b]
	Female (n = 6,9)	22.0 ± 10.48 [a]	35.8 ± 5.05 [a]
Fecundity (No of eggs laid) (n=5)		0.60 ± 0.40 [a]	23.6 ± 7.38 [b]
Intrinsic diet properties			
Mean pH of diet after one week of feeding (n=3)*	Initial: 5.22 ± 0.1 [a]	5.48 ± 0.54 [a]	5.72 ± 0.37 [b]
Mean moisture of diet after one week of feeding (n=3)*	Initial: 7.31 ± 0.09 [a]	10.5 ± 0.99 [a]	14.3 ± 1.37 [b]

[a b] *Mean ± standard error in a row followed by the different letters were significantly different between insects reared under light versus dark conditions*
** Values represent mean ± standard deviation*
¤ 5 escaped midway through the dark experiment
Value in parenthesis () indicates the number of counts

Effect of light regime on the survival rate and mortality

There was no significant (p > 0.5) influence of photoperiod on the survival rate (Table 5.6). The overall survival rate was 76 % in light and 84 % in dark conditions. The mortality recorded coincided with the molting of the nymphs between instars, and the survival rate decreased as nymphs turned into adults.

The final adult male weight was almost twice as high for animals reared in the dark than for those reared in the light. Also, the females had higher weights upon adult wing emergence. A comparison of adult male and female longevity showed significantly more extended longevity in the dark-reared insects as opposed to those reared under light conditions.

Effect of light regime on development of *R. differens* nymphs

Developmental time was markedly higher in light-reared insects than in their dark-reared counterparts. This remark corresponded to significantly higher growth indices for insects reared in the dark (1.45) when compared to the light-reared ones (1.07). At $\alpha = 0.05$, there was a significantly greater number of molts (p < 0.002) in the light-reared insects, which took an average of 8.9 molts to reach adulthood compared to 7.1 molts needed for the insects reared in the dark (Table 5.6). Correspondingly, the instar duration (in days) was longer for the light-exposed insects, especially in the last instar stages, with the latter being most significant, as compared to the dark-reared ones (Table 5.6). Short instar durations were reported for the L2 stage (4 days for the dark condition), while the most prolonged instar duration was 18 days for the L7 stage of light-reared insects. Considering both setups, during the entire nymphal life stage, the mean time between two successive molts was 9.8 days.

Effect of light regime on body weight and feed conversion ratio

Table 5.7 shows the final body weight and feed conversion ratio of *R. differens* reared on an artificial diet. The feed conversion ratio was significantly higher in the dark-reared insects (4.09) than in the light-reared ones (3.13). Also, the mean weight gain and amount of food eaten in the dark were higher than in light conditions. The final adult male weight was almost twice as high for insects reared in the dark than for light.

Table 5. 7 *Effect of light regime on body weight and feed conversion ratio of R. differens reared on the artificial diet. Values are reported as means ± standard error (n = 5)*

	Mean initial weight (mg)	Mean final weight(mg)	Weight gained (mg)	Amount eaten (mg)	Feed conversion ratio
Light	4.25 ± 1.25	49.0 ± 2.0	44.75 ± 0.25	140 ± 1.0	3.13
Dark	4.36 ± 0.90	63.0 ± 1.02	58.64 ± 1.28	240 ± 2.5	4.09

Effect of light regime on fecundity and sex and color ratios

The number of eggs laid by the surviving females was significantly higher in the dark than in light conditions. The insects reared in the dark had a longer fecundity (more than 40 days). On average, 23 eggs were laid per female in the dark condition, whereas only 15 eggs were deposited in the light one. Very similar sex ratios were recorded between the two light regimes. The appearance of color morphs seemed very similar for both light regimes. In both set-ups, the green morph was the dominant one, comprising over 95 %. Purple morphs were not observed.

Effect of light regime on moisture content and pH change in artificial diet

To effectively measure feed conversion ratio, diets were weighed before and after feeding, as well as changes in moisture content and pH. A significant increase (7 %) in moisture content of the diet left over from the insects in the darkroom, as opposed to a 3 % increase in the light-exposed room, was observed (Table 5.6). The moisture contents from both setups were not significantly different between diets after two weeks post-feeding. Additionally, the pH was also slightly raised in the diet of insects reared in the dark (Table 5.6).

5.4 Discussion

R. differens is one of the most popular edible insects in Sub-Saharan Africa. However, its large swarms only have a seasonal occurrence. Therefore, there have been several attempts to mass-rear these delicacies to make them available all year round. However, despite the numerous studies that have reported different diets for mass-rearing of *R. differens* (Malinga et al., 2018a; b), there still exists a clear need for developing suitable artificial diets made up of affordable and locally available materials. Since *R. differens* is a nocturnal insect (Kinyuru et al., 2010), we hypothesized that its development would be faster in predominantly dark conditions than when reared in more lighted conditions.

The artificial diet developed involved mixing different food materials in different proportions (Table 5.1). In the diet, soy meal comprised the highest proportion (40 g/100 g: Table 5.1), resulting in high protein content in the artificial diet (Table 5.2). In addition, soy meal, wheat bran, and fish meal are known to be rich in essential amino acids, fatty acids, and minerals, and this justifies their inclusion in the diet, as shown in Table 5.2 and Table 5.3, respectively. This report is the first study that has evaluated the complete nutritional profile of an artificial diet developed for *R. differens,* and its performance was assessed during rearing. In general, diet mixing, *i.e.*, eating different food types, is a common feeding habit among polyphagous insect herbivores. It is reported that it allows individuals to balance the intake of various nutrients, which is considered beneficial for performance-related factors, such as survival oviposition and growth (Singer & Bernays, 2003). In comparison to previous works (Malinga et al., 2018a; b), an artificial diet was developed in this study that provided an improved means of keeping laboratory populations of *R. differens*.

The nymphal survival rate of *R. differens* did not significantly differ between the two light regimes. However, it was slightly higher (over 80 %) in dark-reared insects, and the mortality recorded coincided with the molting of the nymphs between instars. In the literature, when *R. differens* nymphs were fed with star grass (*Cynodon dactylon*) and guinea grass (*Panicum maximum*) (12 h light and 12 h) to adulthood, the highest survival rate was observed was 53.3 % (Ssepuuya et al., 2018). In a different study, when *R. differens* nymphs were supplied with a highly diversified diet, the nymphal survival to adulthood was significantly higher (38.1 %) than

when nymphs were fed with a single diet (rice seed heads) in the same 12 h light and 12 h dark regime (Malinga et al., 2018b). Therefore, it could be argued that the highly nutritious artificial diet developed in this study significantly improved the survival rate of nymphs compared to studies previously reported in the literature.

The total nymphal development time (in days) differed significantly between the two light regimes. In general, dark-reared insects took a shorter time (58 days) to reach adulthood than light-reared ones. This high rate of development in dark-reared insects is also supported by a lower number of molts and a shorter duration of instars than in light-reared insects. Similarly, Ssepuuya et al., (2018) also reported a short development time (approximately 60 days) of *R. differens* nymphs to adults when fed with wild millet. On the other hand, Malinga et al., (2018b) found that a diversified diet significantly reduced the development time of *R. differens* nymphs to adults (approximately 75 days) compared to a single diet with a development time of more than 130 days in a typical 12 h light and 12 h light regime. The improved performance with mixed diets could be due to an improved balance of high-quality nutrients (Unsicker et al., 2008). Studies have shown that grasshoppers (and bush crickets) perform better on high-quality diets, such as the artificial diet in this study, determining their growth and developmental rate (Berner et al., 2005; Miura and Ohsaki, 2004).

Recently a study that formulated diets for *R. differens* based on host plants specific to the insect was carried out (Leonard et al., 2022). In their study, these authors investigated the effects of diets mixed with the host plants (*Digitaria gayana, Cynodon dactylon, Megathyrsus maximus,* and *Ageratum conyzoides*) on developmental time, survival, longevity, and reproduction of *R. differens* at the lab-scale. Results of their study with host plants revealed the best values for developmental time (88.8 days), survival (47.5 %), and longevity (43 days for females and 34 days for males). The aforementioned biological fitness parameters were outperformed by the artificial diet in this study, as shown in Table 5.5.

In the present study, the rate of nymphal development and the adult bodyweight of *R. differens* were significantly increased in the dark condition. Usually, when the feeding conditions are favorable and less stressful (dark conditions for nocturnal insects), the nymphs do feed voraciously if the diet is palatable, and this probably contributed to the increased body weight

of dark-reared insects. Similar weight gains were reported when larvae were reared on more diversified diets than single resource diets in a light regime (Malinga et al., 2018). A higher feed conversion ratio was observed in the dark-reared insects. Also, the slight increase in moisture content and pH change could have influenced diet palatability, stability, preservatives activity of preservatives, and the solubility of nutrients, which may have favored a higher feed conversion ratio in this set-up. In general, the diet composition is the main factor determining the feed conversion efficiency depending on the insect species (Oonincx et al., 2015). Animals with a low feed conversion ratio are considered efficient feed users. In the literature, much lower feed conversion ratio values of 1.47 and 1.80 were reported when house crickets (*Acheta domesticus*) were fed poultry feed and food waste, respectively (Lundy & Parrella, 2015). By contrast, Oonincx et al., (2015) reported much higher values ranging from 2.3 to 10.0 when *A. domesticus* were fed four types of feed (beet molasses; potato steam peelings; spent grains and beer yeast; bread remains and cookie remains) formulated from food by-products. The higher feed conversion ratio values obtained in the current study may also be explained by overfeeding, which has been known to increase the ratio. Feed conversion ratio values for livestock reared in the US for broiler chicken, egg chicken, turkey, swine, beef cattle, and dairy cow were 1.98, 2.68, 3.58, 18.9, and 0.85, respectively (Mekonnen et al., 2019).

Furthermore, our experimental results indicated that the dark rearing condition greatly improved female fecundity. Thus, based on these results, it is evident that the dark-rearing condition, combined with the high nutritional value supplied with the artificial diet, was superior to the 12 h photoperiodic regime: this accelerated nymphal development, increased adult body weight, and higher fecundity. Similarly, in the 12 h light and 12 h dark regime, female fecundity was significantly improved when adult *R. differens* were fed with mixed diets than a single diet (Malinga et al., 2018b). In addition, the current study has also demonstrated that the sex ratio and the occurrence of color morphs were not affected under the photoperiodic conditions tested.

From the applied point of view, our results demonstrate how a cost-effective rearing program for *R. differens* could be designed with reduced labor. If proper care is taken to eliminate feces from the leftover food, much lower and improved FCR values would be obtained.

The novel artificial diet was affluent in proteins. Its amino acid and fatty acid profiles revealed the presence of essential amino acids and fatty acids, which are crucial for successful insect growth and development (Cohen 2003). Casein, yeast, wheat germ, and soybean continue to be the most popular sources of proteins for insect diets, with the latter comprising up to 40 % of this diet. It has been proposed that artificial diets used for rearing insects, high-grade animal proteins are suitable (Scheneider, 2009). Therefore this plant protein-based diet was augmented with amino acids and fishmeal (Table 5.1). The artificial diet had ten essential amino acids (arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine) necessary for the insects' development. While the addition of these amino acids and vitamin premixes would increase the cost of a mass-rearing diet, the initial idea was, to begin with, a nutrient-dense and balanced artificial diet using ingredients accessible to local farmers. Once this diet was established, the following steps were then to start knocking off one by one each 'expensive' ingredient and replacing them with waste-based alternatives. For instance, brewer's waste (spent grains) replaced soy and fishmeal in the waste-optimized diet. This effort has proved promising, though the results are not part of this thesis.

To provide essential fatty acids in artificial diets, vegetable oils (sunflower, olive, cottonwood, corn, safflower, and linseed oils) are most utilized (Scheneider, 2009). The C-18 polyunsaturated fatty acids - linoleic and alpha-linolenic acids are essential for insects of the order Orthoptera and were present in this diet. The fishmeal component would have been the most likely source of these fatty acids.

Amongst the macro elements, calcium and phosphorus were the most supplemented, as some insects have high calcium requirements. Also, the trace minerals (zinc, iron, copper, manganese) which serve as co-factors for various enzymatic reactions were assumed to be adequately present. Studies that specify the minimum levels of trace minerals required in insect diets are lacking.

One crucial aspect that was intended to be included in this study but was not achievable was to evaluate the diet over several generations. Due to cannibalism, the mated pairs ate up each other before a colony could be established. Based on this unexpected event that truncated this study, a can of worms was opened, which led to the discussion for the next chapter.

5.5 Conclusion

In this chapter, we have attempted for the first time to assess the effect of two very different photoperiodic conditions on the growth and development of *R. differens* using a novel diet. This novel artificial diet for *R. differens* was designed to be rich in proteins, essential amino acids, polyunsaturated fatty acids, linoleic and linolenic acids, and trace minerals required for insect diets. The study revealed that *R. differens* could grow entirely on an artificial diet. Most strikingly, the outcome revealed that on the same diet, temperature, and relative humidity, *R. differens* grows faster in the dark when compared to the 50: 50 light-dark regime typically applied when reared in captivity. This feature aligns with the predominantly nocturnal nature reported for this tettigoniid. It was equally demonstrated that using this artificial diet, the developmental time from hatchling to adult ranged from 60 to 75 days covering on average 6-8 molt cycles. Labor-intensive feed preparations, microbial contaminations, especially molds, and pH changes constitute a solid deterrent for feeding these insects. However, with an initial moisture content of 7 % and water activity a_w of 0.45, the novel artificial diet, which is analyzed now, could last for several months of the duration of the study without noticeable deterioration.

CHAPTER SIX

Cannibalism among Adult *Ruspolia differens* when Fed on Different Insect Prey and Artificial Diet

6.1 Introduction

In the previous chapter, the rearing experiments using two light regimes ended prematurely due to mated adult *R. differens* pairs eating each other up, bringing to an abrupt end the feeding trials on the new artificial diet. This was more evident in the light-reared insects, where fecundity was extremely low as the males ate up their paired female counterparts. As discussed earlier, recent attempts aimed to develop and improve methods for the mass-rearing of *R. differens* have been reported (Lehtovaara et al., 2018; Lehtovaara et al., 2019) Opoke et al., 2019; Malinga et al., 2018a,b; Rutaro et al., 2018; Valtonen et al., 2018). In all the above instances, these were laboratory-based experiments. Nonetheless, the ability to mass-rear these bush crickets and, at the same time, be economically feasible is a precondition for a sustainable *R. differens* farming activity. However, it has been shown that rearing these insects at high densities often results in high mortality rates, principally due to cannibalism among conspecifics (personal observation; Lehtovaara et al., 2019). Cannibalism in animals is a widespread feeding strategy, particularly among bush crickets, locusts, and Mormon crickets, and can be a significant cause of mortality (Elgar & Crespi, 1992). This mostly happens from behind or the side, resulting in injuries causing denervation and immobilization, significantly increasing cannibalism (Bazazi et al., 2008). It is often attributed to the unnatural conditions of laboratory environments, especially during grasshopper rearing.

On the other hand, cannibalism may enhance the fitness of arthropods in natural habitats by providing access to essential nutrients, minimizing competition, and regulating population density. Nonetheless, it may also be detrimental under some circumstances, such as when the cannibal is injured by its intended prey or acquires pathogens from the victim (Richardson et al., 2009). For instance, cannibalism within swarming Mormon crickets (*Anabrus simplex*) in the United States is rife and is usually driven by the need to find nutrients, such as protein and salt. If individuals fail to continue moving, they are likely to be attacked and risk becoming another cricket's source of these essential resources (Bazazi et al., 2008).

So far, to the best of our knowledge, studies to curb cannibalism in *R. differens* have not been

documented. Besides this, studies that attempt to use other insect prey in colonies to curb cannibalism are missing. Against this backdrop, the objectives of the present chapter were to (i) determine if the introduction of different insect prey influenced cannibalism in *R. differens,* (ii) capture any portrayal of hunting behavior (to find out if they would prefer live or dead prey), (iii) determine the extent of cannibalism exhibited by males and females (to find out if cannibalistic behavior might be sex-linked), (iv) compare the proportion of cannibalism (irrespective of sex or live/dead status) when fed a mixture of insect prey in different experimental setups.

6.2 Materials and methods

6.2.1 Study location

This study was performed at International Centre for Insect Physiology and Ecology (*icipe*), Duduville campus, in Nairobi (Kenya).

6.2.2 Insect samples

All insects were obtained from colonies reared at the Animal Rearing and Containment Unit (ARCU) at icipe. A brief description of their rearing is explained below.

6.2.3 *R. differens* stock colony

The 200 young adult *R. differens* used in this experiment were obtained from the *icipe* insectary. These progenies were the F3 generation of adults captured in the wild from Kampala (Uganda) in 2016. Prior to the experiments, the laboratory colony was maintained for at least 48 months. The insects were reared in polystyrene and plexiglass cages (50 cm x 50 cm x 50 cm) containing holes in the walls covered with fine mesh for aeration. Humidified absorbent paper or cotton wool was placed inside the cages as a water source. The animals were fed on corn leaves and supplemented with an artificial diet composed of soy and cornmeal. The colony was maintained at 28 ± 1 °C, 60 ± 5 % relative humidity (RH), and a photoperiod of 12 h (12 h light:12 h darkness).

6.2.4 Insect preys

Black soldier fly(BSF)-*Hermetia illucens* larvae

The larvae were maintained at the insectary in icipe, according to Chia et al., (2018). Wild harvested egg clusters were transferred to metal trays (76 cm x 27.5 cm x 10 cm) containing brewers' spent grains hydrated to approximately 70 ± 2 % moisture content. The culture was monitored daily for larval development. The second instar larval stages after self-dispersal from the substrate were then transferred to a transparent rectangular plastic container (21 cm x 14 cm x 15 cm) containing moist wood shavings (sawdust) as pupation substrate. The container had an opening (14.5 cm x 8.3 cm) on the lid of each container and was covered with fine netting organza material to prevent larval escape. The second instar larvae were sieved out from this substrate and fed to *R. differens,* while the other half (200 g) was harvested and kept aside for nutritional (protein content) analyses. The rearing room conditions were maintained at 28 ± 1 °C, 70 ± 2 % RH, and a photoperiod of 12 h (12 h light:12 h darkness).

Stemborer (*Chilo partellus)* larvae

The larvae of the exotic stemborer *Chilo partellus* colonies were rejuvenated twice a year with field-collected larvae. A cotton pad moistened with water was placed inside the container to maintain relative humidity > 80 %. The insects were kept in a rearing room at 25 ± 0.05 °C, 58.5 ± 0.4 % RH., and a photoperiod of 12 h (12 h light:12 h darkness).

Fruit fly (*Bactrocera invadens)* larvae

Bactrocera invadens larvae were fed a diet consisting of three parts sugar and one-part enzymatic yeast hydrolysate and water on pumice granules. All experiments were conducted in a room maintained at 28 ± 1 °C and 50 ± 8 % RH and a photoperiod of 12 h (12 h light:12 h darkness).

Desert locust (*Schistocerca gregaria*) nymphs

About 200 - 250 gregarious phase desert locusts (*Schistocerca gregaria*) were reared in aluminum cages (50 cm x 50 cm x 50 cm). They were fed on a diet consisting of wheat seedlings and wheat bran in a well-aerated, dedicated room (4.5 m x 4.5 m) which was maintained at a temperature of 30 ± 4 °C, 40–50 % RH, and a photoperiod of 12 h(12 h light:12 h darkness).

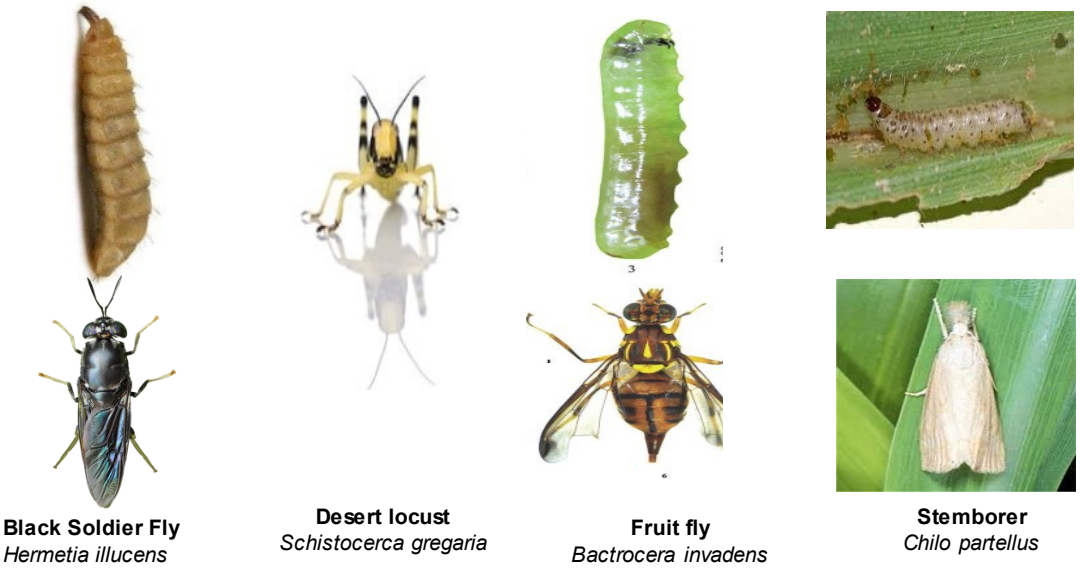

Figure 6. 1 *Insect preys fed to R. differens. The top pictures are the larval stages that were provided as food, *except for the desert locust where larval and adult instars looked alike*

6.2.5 Experimental set-up

A completely randomized design was applied during the experiment. Ten (10) young adult females or males of *R. differens* were kept separately in well-aerated cages (50 cm x 50 cm x 50 cm). Two experimental set-ups were designed, a no-choice and a choice, respectively.

In the no-choice set-up, ten *R. differens* males and females were subjected to the same insect prey. That is 20-second instar larvae each of black soldier fly (*Hermetia illucens*), Stemborer (*Chilo partellus*), fruit fly (*Bactrocera invadens*), and desert locust *Schistocerca gregaria*) in succession were administered. This feeding was carried out separately in male and female cages for six successive days. For the first three days, they were fed living prey and, following three days, dead prey. A 24-hour washout period between each prey diet change was introduced. In other words, each cage of 10 males and another cage of 10 female *R. differens* were fed 20 live black soldier fly larvae for three days, and then 20 dead black soldier flies for another three days. After a washout period (period whereby insects were given a feeding break), the same procedure was repeated for the other three insect preys. An overview of the feeding scheme is shown in Supplementary Table S6.1**.**

In the choice set-up, a mixture of 20 living preys, five each of (black soldier fly, Stemborer, fruit fly, and desert locust) were fed all at once for 12 consecutive days to each of ten males and females adult *R. differens* in separate cages. For the controls, four cages were used, with each having ten male and ten female *R. differens*. The first control setup constituted two cages, each with ten males and females, and fed in one cage with an artificial diet only. The second control had a male and female cage again with the same artificial diet but now supplemented with corn leaves provided each to ten males and females separately for six days consecutively.

In all setups, whenever *R. differens* was cannibalized or found dead in cages, new adults were replaced after each diet change to maintain the number of predators to ten in each cage.

In each case, the proportion of cannibalism was recorded by counting the initial and final number of *R. differens* daily. Also, the proportion of prey eaten was recorded by counting the number of left-over prey (dead or alive).

Cannibalism was evidenced by death with clear signs of body parts having been eaten or the

complete disappearance of the victim (Figure 6.2). Such cannibalized insects were immediately picked up from the cages and stored at -20 °C for nutritional analyses.

6.2.6 Feeding scheme

The experiment was carried out according to the feeding scheme outlined in supplementary Table S6.1.

The ten males and females in each cage were fed with twenty prey (dead and alive): This was the case for both the choice and the no-choice experiments.

6.2.7 Chemical analyses

Moisture and Crude fat determination of insect prey

Moisture content for the insect prey was determined gravimetrically by oven-drying (See chapter two, *section 2.2*).

Crude fat was gravimetrically determined using the Soxhlet extraction procedure as earlier described in *section2.2*

Crude protein determination of insect prey

Crude protein content was determined by the Kjeldahl method (Fombong et al., 2017) and is described fully in section *2.1 of chapter two*.

Determination of mineral content of cannibalized *R. differens*

To determine the mineral content of the cannibalized victims, the samples were incinerated, and the crude ash was dissolved in nitric acid. After that, its mineral components were determined using the inductively coupled plasma-optical emission spectrometry (ICP-OES) technique as outlined by Fombong et al., (2017). The details are described fully in *section 2.2* of chapter two. For each sample, extraction, detection, identification, and quantification were performed in triplicate.

Determination of fatty acid profile of cannibalized *R. differens*

Fatty acid methyl esters were prepared from *R. differens* oil extracts and separated and identified by gas chromatography (GC) coupled with mass spectrophotometry (Fombong et al., 2017). Details are described elsewhere in chapter two, *section 2.3.* For each sample, detection, identification, and quantification were performed in triplicates.

6.3 Statistical analysis

To evaluate the proportion of cannibalism in each case using the count data, a binomial logistic regression model was fitted to the data with insect type, the number of prey and prey eaten as factors using the logit function. The dispersion parameter for the binomial family was taken to be 1. Additionally, to determine the preference for either dead or alive prey, a quasi-binomial model was applied. Data analyses were carried out at $\alpha = 0.5$ using R statistical software version 3.5.3 (R Core Team, 2019).

6.4 Results

6.4.1 Crude fat and crude protein of insect prey

The crude fat and crude protein of insect prey are shown in Table 6.1. The crude fat in Stemborer was significantly higher compared to that of other insect prey. No significant difference was observed between the fat content of *BSF larvae* and *S. gregaria.* On average, artificial diet (AD) fed insects had the lowest fat content compared to insect prey. The crude protein content differed significantly among the insect preys. *BSF larvae* and *S. gregaria* had the highest protein content, though no significant difference was observed between the two insect preys. Like crude fat, the artificial diet had the lowest crude protein compared to insect prey (Table 6.1).

Table 6. 1 *Crude fat and crude protein of insect prey used in the experiment (± SD, n = 3)*

	Black soldier fly	Stemborer	Fruitfly	Desert locust	Artificial diet
Moisture content	7.32 ± 1.21	5.73 ± 0.64	12.50 ± 0.69	6.33 ± 0.58	8.48 ± 0.08
Crude Fat	29.10 ± 2.10[c]	45.34 ± 1.58[d]	35.67 ± 1.71[e]	19.0 ± 2.10[b]	5.15 ± 0.21[a]
Crude protein	52.50 ± 1.14[c]	25.57 ± 1.66[a]	26.64 ± 2.11[a]	44.5 ± 0.98[b,c]	21.1 ± 0.64[a]
Crude Fibre	5.06 ± 1.44[a]	8.11 ± 0.90[b]	7.95 ± 0.01[b]	6.25 ± 0.25[a, b]	4.53 ± 0.02[a]
Ash	8.42 ± 0.63[b]	7.28 ± 0.72[b]	7.02 ± 0.55[b]	5.47 ± 0.21[a]	7.29 ± 0.08[b]
carbohydrate ¤	< 0.1	7.97 ± 2.46	10.20 ± 2.27	18.49 ± 1.84	53.5 ± 0.21

[a,b,c] Means on the same row having different superscripts are significantly different ($p < 0.05$)
¤ Calculated as 100 minus the sum of mass fractions of moisture, protein, fats, ash, and fiber | See equation 2.2 in *section 2.2*

6.4.2 Mineral content of cannibalized *R. differens*

Table 6.2 shows the mean content of minerals (in mg/100 g dry matter) examined in cannibalized *R. differens*. The mineral levels were variable, as expected. The mean potassium (975 mg/100 g and phosphorous (782 mg/100 g) levels were the highest. The trace minerals zinc (16.08 mg/100 g) and copper (7.69 mg/100 g) were exceptionally high. Selenium was not detected. When values were compared to *R. differens* harvested from the wild (Fombong et al., 2017), sodium, zinc, and copper were higher. In contrast, the other mineral elements were much lower, particularly calcium and magnesium concentrations.

Table 6. 2*. Mineral composition (mg/100 g dry matter) of cannibalized R. differens. Results are ± standard deviation of duplicate readings*

Mineral		Wild harvested (from chapter 2)
Sodium	179 ± 0.01	57.2 ± 0.01
Potassium	975 ± 0.06	724.0 ± 0.06
Calcium	127 ± 0.00	967.6 ± 0.06
Magnesium	85.64 ± 0.00	123.0 ± 0.01
Zinc	16.08 ± 0.00	15.0 ± 0.01
Iron	15.54 ± 0.00	174.4 ± 0.01
Phosphorus	782 ± 0.01	610.7 ± 0.06
Copper	7.69 ± 0.00	1.6 ± 0.01
Manganese	2.61 ± 0.00	6.0 ± 0.01
Selenuim	<LOQ	<LOQ

6.4.3 Fatty acid profile of cannibalized *R. differens*

The fatty acid profile of cannibalized *R. differens* is shown in Table 6.3. In general, the saturated fatty acids that included stearic acid and palmitic acid were dominant. On the other hand, unsaturated fatty acids, including oleic acid, linoleic acid, and linolenic acid, were significantly more abundant. Other fatty acids were present at considerably lower levels.

Table 6. 3*. Fatty acid profile (% of total fatty acids) of cannibalized R. differens. Results are ± standard deviation of triplicate measurements*

Fatty acid		Wild harvested (from chapter 2)
Decanoic acid (C10:0)	0.21 ± 0.01	0.07 ± 0.01
Lauric acid (C12:0)	0.80 ± 0.05	0.17 ± 0.01
myristoleic acid (C14:0)	0.54 ± 0.06	1.10 ± 0.02
myristic acid (C14:1)	1.61 ± 0.07	0.07 ± 0.01
pentadecanoic acid (C15:0)	0.04 ± 0.02	0.12 ± 0.01
palmitic acid (C16:0)	16.7 ± 0.48	27.8 ± 0.62
palmitoleic acid (C16:1)	2.59± 0.04	1.63 ± 0.01
heptadecanoic acid (C17:0)	0.02 ± 0.01	0.15 ± 0.01
stearic acid (C18:0)	28.7 ± 1.18	8.45 ± 0.21
oleic acid (C18:1)	23.7 ± 1.16	44.0 ± 0.42
linoleic acid (C18:2)	14.1 ± 0.43	14.1 ± 0.32
linolenic acid (C18:3)	5.12 ± 0.07	1.45 ± 0.03
Arachidonic (C20:4)	0.77 ± 0.02	0.07 ± 0.01
Eicosapentaenoic acid (C20:5)	5.04 ± 0.19	0.17 ± 0.01

6.4.4 Levels of predation

R. differens in the cages were provided with live or dead insect preys. The number of prey eaten by either male or female *R. differens* was recorded. Table 6.4 shows the mean number of prey eaten in both the no-choice and choice experiments.

There was a strongly significant difference in insect prey type ($p < 0.00001$) for the response to eating. From the analysis of the means, there was minimal evidence of a difference ($p = 0.00544$) between the prey eaten alive or dead. From the analysis of deviance (dead = 0.7574680 versus live = 0.8606544), there is a slight difference in whether the predator (*R. differens*) prefers alive or dead prey. Thus the predators tended to feed more on living than dead insect prey. (Table 6.4). Among the no-choice prey, BSF larvae were eaten the most,

followed by stemborer larvae. The desert locust nymphs were the least eaten. There was a significant increase in the number of prey eaten in the choice setup compared to the no choice. Adding an artificial diet (control) to the mixed prey did not significantly alter the mixed prey eaten.

Table 6. 4 *Levels of predation of the average (alive and dead) number of prey (alive and dead) eaten by male and female R. differens. Values are mean count ± standard deviation. Means with different superscript letters along the column are significant at p = 0.05*

INSECT TYPE	Mean number of prey eaten (n=6)
No choice	
Black soldier fly	18 ± 1.63 [c]
Stem borer	15.15 ± 1.47 [c]
Fruit Fly	12.2 ± 1.11 [b]
Desert locust	9.5 ± 1.77 [a]
Choice	
Mixed Prey*	19.1 ± 0.39 [c]
Mixed prey* + artificial diet	18.5 ± 0.86 [c]
Controls	
Artificial diet only	NA
Artificial diet + corn leaves	NA

** Mix of all four-insect prey (black soldier fly, stem borer, Fruitfly, and desert locust)*
NA= Not Applicable (No insect prey, only powdered diet (+ corn leaves)

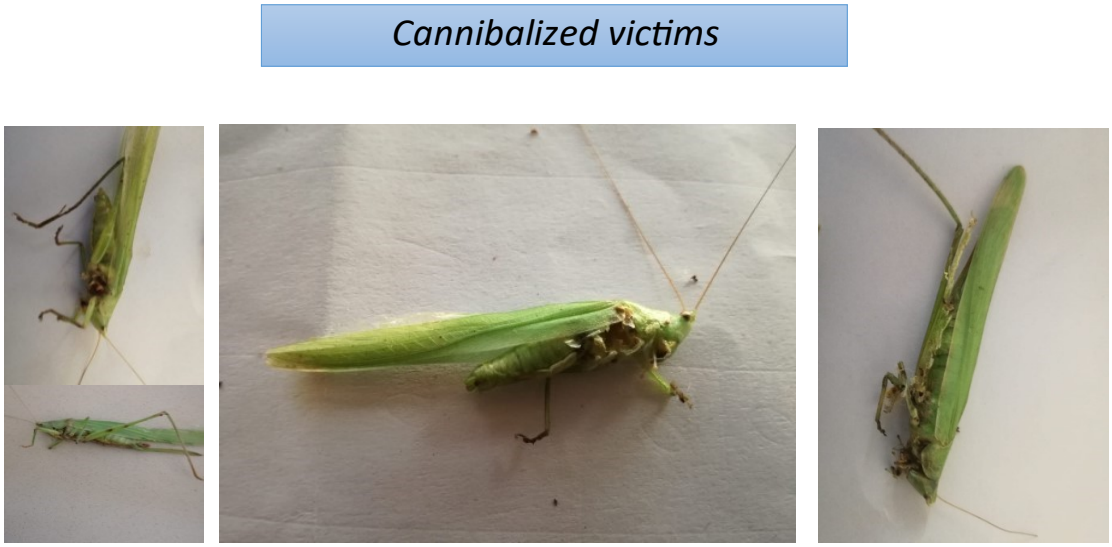

Figure 6. 2*. Cannibalized 'deceased' victims showing portions of bitten-off parts*

Figure 6. 3 *R. differens adult feeding on insect prey (stemborer larva).In this particular instance, the above bush cricket could eat up to 20 living larvae after a 24 h fasting period within four hours.*

6.4.5 Cannibalism in no choice set-up

Table 6.5 showed the percentage of cannibalism when either male or female *R. differens* were fed with living or dead insect prey. In general, cannibalism was significantly reduced (p = 0.0002494) when either male or female *R. differens* were fed with living insect prey than dead prey. Conversely, there was no significant difference (p = 0.4330812) between sex with response to cannibalism. When both male and female *R. differens* were fed with living BSF larvae, no cannibalism was observed. However, when other insect preys (dead or alive were provided to female *R. differens*, significant levels of cannibalism were observed, the highest on dead Stemborer and live fruit fly (Table 6.5). On the other hand, when male *R. differens* were supplied with dead BSF larvae and living Stemborer and fruit fly, no cannibalism was observed. However, significant levels of cannibalism were observed when dead prey (Stemborer, fruit fly, and desert locust) were supplied as feed (Table 6.5).

Significant levels of cannibalism were observed in both male and female *R. differens* when artificial diet only and artificial diet + corn leaves were supplied as feed compared to insect prey. For female *R. differens*, cannibalism was the highest in artificial diet and differed significantly from artificial diet + corn leaves (Table 6.5). Cannibalism in male *R. differens* did not vary when either artificial diet only or artificial diet + corn leaves were supplied as feed (Table 6.5).

Table 6. 5 *Percentage of cannibalism in male and female cages concerning live and dead prey in the no-choice and choice set-ups when compared to the controls (artificial diets)*

The values are mean ± standard error of given replicates.

INSECT TYPE	Status (live/dead)	% cannibalism Male cages(n=3)	% cannibalism female cages(n=3)
No choice			
Black soldier fly	live	0.00 ± 0.00 [a]	0.00 ± 0.00 [a]
	dead	0.00 ± 0.00 [a]	6.67 ± 3.33 [a]
Stem borer	live	6.67 ± 3.33 [a]	6.67 ± 3.33 [a]
	dead	13.33 ± 3.33 [b]	13.33 ± 3.33 [b]
Fruit Fly	live	13.33 ± 3.33 [b]	6.67 ± 3.33 [a]
	dead	6.67 ± 3.33 [a]	6.67 ± 3.33 [a]
Desert locust	live	5.00 ± 4.08 [a]	0.00 ± 0.00 [a]
	dead	6.67 ± 3.33 [a]	6.67 ± 3.33 [a]
Choice			
Mixed Prey*	live	10.00 ± 1.48 [b]	10.71 ± 4.31 [b]
Mixed prey* + artificial diet	live	17.00 ± 2.13 [c]	11.00 ± 1.00 [b]
Controls			
Artificial diet only	NA	16.67 ± 5.16 [c]	23.33 ± 5.58 [d]
Artificial diet + corn leaves	NA	11.67 ± 3.07 [b]	16.67 ± 13.33 [c]

** Mix of all four-insect prey (black soldier fly, stem borer, Fruitfly, and desert locust)*
NA= Not Applicable (No insect prey, only powdered diet (+ corn leaves)

6.4.6 Cannibalism in the choice set-up

The levels of cannibalism in the choice set-up are shown in Table 6.5. The percentages of cannibalism did not differ significantly between *R. differens* males and females fed with a mixture of prey. However, with the addition of corn leaves to the artificial diet, cannibalism decreased significantly. Like in the no-choice experiment, providing an artificial diet only resulted in a significant rise in cannibalism, and female *R. differens* displayed a significantly higher level than males. For the case of male *R. differens*, mixed prey and artificial diet + corn resulted in similar levels of cannibalism (Table 6.5).

6.5 Discussion

R. differens is a swarming bush cricket distributed all over SSA (Kelemu et al., 2015; Jongema, 2015), harvested chiefly from the wild (Mmari et al., 2017; Kinyuru et al., 2010; Agea et al., 2008). Farming of *R. differens* in a controlled environment has been proposed to reduce the pressure on wild populations. However, rearing them at high densities results in high mortality rates principally due to cannibalism among conspecifics (Lehtovaara et al., 2019; Egonyu et al., 2021). This study highlights cannibalism in *R. differens* adults reared in captivity and, for the first time, compares the proportion of cannibalism when fed live or dead insect preys with respect to an artificial diet supplemented with or without corn leaves. Although the mechanisms driving collective movements are poorly understood, these phenomena are typically associated with actual or anticipated depletion of food resources after increasing population density (Simpson et al., 2006).

In this study, insect preys fed to adult *R. differens* had a higher protein content than the artificial diet (Table 6.2). Thus, it could be argued that the higher preference for insect prey was due to their higher protein content, as demonstrated previously in Mormon crickets *Anabrus simplex* (Simpson et al., 2006). From our study, BSF larvae had the highest protein content (Table 6.2). They were most preferred by both male and female *R. differens,* which resulted in significantly lower levels of cannibalism in the no-choice set-up. The protein content in Stemborer and fruit fly of insect prey were quite similar, male *R. differens* highly preferred them*,* and cannibalism was not observed. However, in these two insect preys, significant levels of cannibalism were observed in female *R. differens*.

S. gregaria prey had a higher protein content than the fruit fly and Stemborer. However, the latter insect prey, being less mobile than the desert locust, were consumed more, as their chances of being eaten increased with less mobility. In the literature, lack of protein as the leading cause of cannibalism has been reported. For instance, Mormon crickets (*A. simplex*) were deprived of two essential nutritional resources, *i.e.*, protein and salt, and placed in clean buckets with a live but freshly incapacitated cricket of equivalent size and left for 50 min, a high level of cannibalism was observed. This behavior was associated with a lack of dietary protein (Simpson et al., 2006). In this study, cannibalism was still encountered despite feeding the

insects with prey that had very high protein contents (Table 6.1). Other trials (not described here) in which insect prey was dried, blended, and added together with other diet components and fed *R. differens* still exhibited some degree of cannibalism. A likely explanation could be that besides protein demands, these bush crickets are still 'on the march' for specific amino acids or other micronutrients. When a comparative examination of the mineral composition of victims of cannibalism with that of *R. differens* captured from the wild is made (Table 6.2), we realize a striking difference. Sodium and calcium were lower and higher in the wild than in those reared in this study. Perhaps, the fact that these lab-reared *R. differens* had sodium contents more than triple that of wild-harvested ones could also have enhanced the eating of conspecifics and the predation on prey as a strategy to increase sodium uptake (as seen in the elevated values).

The essential fatty acid linoleic was pretty constant in both reared and wild-harvested *R. differens*, but not the case for linolenic acid, which was higher in lab-reared counterparts. These two fatty acids are needed as precursors for eicosapentaenoic acid (EPA), which was higher. Could cannibalism also be due to the quest for essential fatty acids? These current results cannot ascertain. Other notable differences were higher stearic acid and lowered palmitic and oleic acids, respectively, in the cannibalized ones compared to wild-harvested bush crickets. Alternatively, cannibalism could simply be an innate behavioral pattern used as a survival means given their seasonal occurrence to brave the dry season spells where plants and flower inflorescences are scarce.

Figure 6.2 showed that when cannibalism took place, there was a preference for biting off from the thorax region. This feature has been reported by other authors (Egonyu et al., 2021). A possible explanation could be that damage to thoracic nerves may help quickly immobilize the victims. At the same time, the skeletal muscles, which are abundant in the thorax, also contain high protein levels.

Among the several factors reported by Hartley (1967) to trigger cannibalism in tettigoniids, the availability of weak, newly molted nymphs was critical. Thus, to eliminate this weakness due to molting, this study focused only on adults. Hartley (1967) had described that by providing *R. differens* (previously known as *Homorocoryphus nitidulus vicinus*) with sufficient food and

water, adults could show less cannibalism. Our results suggest the contrary; even with adequate insect prey, there was still evidence of cannibalism in all cases.

In addition, this study has demonstrated that adult *R. differens* slightly preferred live prey over dead prey. This phenomenon could be associated with hunting behaviors of *R. differens*. Other investigators (Egonyu et al., 2021) reported that cannibalism occurred predominantly at night in the dark when *R. differens* went hunting for prey. Video observations showed that potential victims (BSF larvae, desert locust, Stemborer, and fruit fly) repelled possible attacks by predators (*R. differens*) by moving away, especially desert locusts, but the predators' movements were aggressive. Although predation of dead BSF larvae by male *R. differens* was higher and resulted in low cannibalism, in all other dead insect preys, high levels of cannibalism were still observed, and this could be connected to the release of necrotic secretions from these dead prey. More so, high levels of cannibalism were observed when artificial diets only and artificial diets with corn leaves were introduced to both male and female *R. differens* in the no-choice set-up. This could be associated with the relatively low protein content (21 %) in these diets. Since *R. differens* are higher in protein content and fat (Fombong et al., 2017) than this artificial diet, it could explain the preferential taste of their conspecifics (Lehtovaara et al., 2019).

In comparison to control diets, mixed prey resulted in a low level of cannibalism in males when compared to female *R. differens*. This reduced cannibalism could be associated with their voracious habit of feeding high protein diets (Lehtovaara et al., 2019) and likely explain the lesser extent of cannibalism when a variety of prey was provided. The preference for mixed diets having better performance traits in *R. differens* has already been demonstrated (Malinga et al., 2020; 2018).

Up until now, *R. differens* was considered by many researchers as a selective grass feeder. However, this research, coupled with the challenges encountered in lab-rearing this bush cricket entirely on grass, has proven otherwise. This highlights why when found hanging on tall grasses; these insects can be observed nibbling on the inflorescence containing the anthers (pollen) and seeds rather than the leaves, indicating their inclination toward more proteinaceous diets. In

one notable instance (fig 6.2), one *R. differens* alone could eat up to 20 living stemborer larvae within 4 h post a 24 h fasting period. The findings of this study have likewise demonstrated that feeding living insect preys to *R. differens* reared on a lab-scale reduces the incidence of cannibalism. This knowledge can be exploited in commercial endeavors where farmers could target the improvement or manufacture of special artificial diets using parts or whole dry, powdered portions of other insects as feed for *R. differens*. Another suggestion that could have vital practical implications, especially in mass-rearing programs, is to separate the adults as much as possible from younger nymphs in breeding cages. It will therefore be essential to supply such isolated adults not only with high-protein and mineral-rich feeds but also with diverse nutritional components if cannibalism is to be eliminated.

One inherent limitation of this study was the shortage of a sizeable *R. differens* parent stock to increase the sample size due to cannibalism and thus difficulty rearing them *en masse* during their non-swarming season. Also, the fact that new adults were replaced after each death in cages to maintain the number of predators may have introduced more fit adults into the ongoing setup.

Additional studies which would unravel the role played by specific essential amino acids, fatty acids, and other micronutrients in diets for *R. differens* species in cannibalism would be precious. Likewise, It will be interesting to understand what will happen if *R. differens* is provided a synthetic diet containing more proteins and fats. Future comparison of the cannibalized and non-cannibalized predators in terms of their nutritional profile could be useful if time and other resources would allow.

Discussions on the implications of processing these insects to use as either complementary foods or to fortify less-nourishing foods as a means to redress food security from an East African point of view are in the next chapter.

6.6 Conclusions

Cannibalism was significantly reduced in cages where prey had been administered compared to the control diets. Live insect preys with high protein contents were more preferred by both male and female *R. differens.* There were indications of a hunting behavior as the predators tended to feed more on living than on dead insect prey. The addition of live insect prey could reduce the prevalence of cannibalism, thus preventing colony collapses due to cannibalism. This practice could also provide a safe means to eliminate insect pests serving as prey. Therefore, it is recommended to include (portions of) other insects when formulating artificial diets as this will have a similar effect of increasing the protein and fat content of the artificial diet. This action would, in turn, lessen death by cannibalism in adult *R. differens* farming set-ups.

CHAPTER SEVEN

Influence of Drying on the Techno-Functional Properties of Three Edible Insects: *Ruspolia differens, Gryllus bimaculatus,* and *Schistocerca gregaria*

7.1 Introduction

Throughout the literature reviewed in chapter one, it was evident that insects are excellent sources of protein, fats, oils, and other nutrients for improved nutrition and health of humans and animals. In chapters two and three of this thesis, his trend was strongly substantiated. In chapter four, it was shown that three popular insects were low in antinutrients and that drying does not affect these compositions. As a result, this current chapter focuses on the processing of those insects. Therefore, the same insect samples were subjected to the exact defatting and drying conditions, albeit this time evaluated for their techno-functional properties.

Malnutrition still remains one of the significant public health challenges throughout Sub-Saharan Africa. In Kenya, for instance, up to 26 % of children under 5 years are stunted, 4 % are wasted, and 11 % are underweight (KNBS 2014) due to food and nutrition insecurity. Despite their glaring nutritional benefits, many communities have not embraced entomophagy as they view insect consumption as primitive or even show disgust. In Kenya, for example, only the communities within the Western and Nyanza regions are known to consume insects, especially termites and crickets. Thus, there is a need to create more awareness of edible insects' health and economic benefits, as well as promote them to mitigate malnutrition (Kinyuru et al., 2015). One of such ways is to use insects as complementary foods. Typical porridges are usually fortified with soybean or milk, which are increasingly expensive protein sources (Kipkoech et al., 2019). In their study, Kipkoech et al., (2019) showed that a 5 % inclusion of crickets into porridge compared well with milk-based porridge. Additionally, they proved that cricket-based porridge improved the nutritional status of children and recognized the need to develop cricket-based products for child feeding. To properly formulate such insect-based products, studies of their physicochemical or techno-functional properties are crucial.

In the western world, where insect-eating is rare, the aversion towards entomophagy is even worse. Studies have reported that edible insects can be consumed whole or concealed in other products (Bußler et al., 2016), a culture that may highly influence their consumption (Baiano,

2020; Hartmann & Siegrist, 2017; Sogari et al., 2016). Recent studies have revealed that people may consume insects more in a concealed form, such as protein extracts and concentrates incorporated in other food products (Baiano, 2020; Bußler, 2016). Thus, protein can be extracted for food applications to improve the nutritional, bioactive/ antioxidative, and techno-functional properties of targeted food products.

In the food industry, proteins are being used as integral sources not only because of their nutritive value but also because of their functional properties (Méx et al., 2008). Some of the required functional properties of food proteins for application in food formulations include their solubility, gelation ability, emulsifying activity, foaming capacity, and biological activity (Panyam & Kilara, 1996; FitzGerald et al., 2006; Nongonierma & FitzGerald, 2017). Defatting raw food materials and by-products using organic solvents is a frequent method for producing protein-enriched ingredients (L'hocine et al., 2006; Capellini et al., 2020). Extraction with hexane remains a popular method to remove lipids from the solid insect matrix for its high efficiency and availability (Russin et al., 2011).

For instance, Kim et al., (2020) evaluated the impact of different organic solvents on the functional properties of defatted proteins extracted from *Protaetia brevitarsis* larvae. The authors found that hexane-defatted protein fractions had increased amino acid content, protein solubility, and functional properties than the same fractions defatted with methanol and ethanol (Kim et al., 2020).

In the literature, however, only a few studies have reported on the functional properties, *e.g.*, solubility, foaming, gelling, and emulsions (Ndiritu et al., 2017; Zielińska et al., 2018; Mishyna et al., 2019; Stone et al., 2019) of insect protein flours as a function of processing methods, mainly blanching and drying, in order to optimize the ingredient quality (Borremans et al., 2020; Gravel & Doyen, 2020). Thus, the techno-functionality of defatted insect flours could differ based on postharvest treatments of edible insects, which may impact the adoption of these concentrates in food formulations or applications. Overcoming this challenge is therefore paramount for the upscaling and utilization of insect protein isolates in the food industry. To improve functionality or bioactivity, new drying methods, such as microwave oven drying and freeze-drying, should be tested.

Based on local availability, three edible insects, *i.e*., the desert locust (*Schistocerca gregaria*), the field cricket (*Gryllus bimaculatus*), and the African bush-cricket (*R. differens*) (Order: Orthoptera) that are widely spread across several countries within Sub-Saharan Africa (Fombong et al., 2017; Fombong et al., 2021; Kelemu et al., 2015; Kinyuru et al., 2020) were considered for this study. A recent, massive invasion of the desert locust, *S. gregaria,* continues to be experienced in the horn of Africa, with many parts of the affected countries undergoing substantial economic losses and food insecurity. While some communities are consuming locusts as food, others are skeptical. *R. differens* continue to be associated with regular seasonal swarms during rainy seasons, thus allowing mass harvesting at night under bright lights for food. *G. bimaculatus*, on the other hand, is currently being actively farmed across Kenya and Uganda. Transforming these insects into an unrecognizable powder form and using them as food ingredients that can be incorporated into diets could increase insect consumption. Their recent success in incorporating them into complementary foods for children is an added motivation. Whereas several studies have emphasized their nutritional contents (Kinyuru et al., 2010; Ssepuuya et al., 2019; Fombong et al., 2017; Kinyuru et al., 2020, Cheseto et al., 2015, 2020, Fombong et al., 2021), studies on techno-functional properties of these insects' defatted flours are limited. Additionally, scarcely reported in the literature is the influence of the drying method on the associated techno-functional properties. Consequently, this study aims to compare and determine the impact of drying methods post-hexane-defatting on a few techno-functional properties (water holding capacity, foaming capacity and stability, and fat absorption capacity) of the three edible insect species mentioned above. This chapter also sheds some light on the impact of oven-drying and freeze-drying on selected properties (free fatty acid, iodine, saponification, and peroxide values) of the extracted oils from these whole insect flours. These properties were selected based on the availability of resources for their determination.

7.2 Materials and methods

7.2.1 Insect samples

The insect samples were reared as previously described in chapter four, *section 4.2.1*

7.2.2 Drying of insect samples

All insect samples were dried, either oven-drying or freeze-drying processes as described in chapter four, section 4.2.2

Freeze-dried samples were coded as **FD**. and coded as **OD**.

The dried samples were rendered into flour using a food blender (Argos, UK). The resulting flour of each insect (FD or OD) was then sub-divided into two equal portions. One portion was denoted as the Whole Insect flour (**W**), and the other portion was defatted (see below) and referred to as Defatted insect flours (**D**).

Therefore, for each insect sample, there were six sub-samples, i.e., a freeze-dried (FD) and an oven-dried (OD) portion, and for each drying mode, samples were either whole (W) or defatted (D).

7.2.3 Defatting of insect flour

The oils from the insect samples were extracted, as explained earlier in *section 54.2.3* in chapter four.

7.2.4 Determination of techno-functional properties of insect flours

<u>Water holding capacity and fat absorption capacity</u>

Water holding capacity (WHC) and fat absorption capacity (FAC) of the insect flours were determined in triplicate according to the method of Bußler et al., (2016) with slight modifications. Briefly, 0.50 g of each insect flour was weighed into a pre-weighed centrifuge beaker to which either 3.0 mL of distilled water (for water holding capacity) or 3.0 mL of commercial rapeseed oil (for fat absorption capacity) had been added. The mixtures were

stirred (for 60 s or two times 60 s for water holding capacity and fat absorption capacity, respectively). The stirring was done using a propeller stirrer and an overhead agitator (Yellowline® OST Basic, IKA®, Wilmington, NC, USA). It was then centrifuged (Eppendorf 5810R, Eppendorf AG, Hamburg, Germany) at 3,900 g for 20 min. The samples were re-weighed after discarding the supernatant. The differences in weight (i.e., the precipitate) were calculated, and the results were presented as grams of water or oil absorbed per gram of insect flour sample.

$$WHC\ or\ FAC\ \left(\frac{g_{\ water\ or\ oil*}}{g_{\ insect\ flour}}\right) = \frac{\left(m_{initial\ sample}\ (g)\ - m_{final\ sample}\ (g)\right)}{m_{,DM,initial\ sample}\ (g)} \qquad \text{Eq. 7.1}$$

** oil in the case of FAC*

Where *m* is the initial and final weight of the dry insect flour sample (in g) $m_{,\ DM}$ is the initial weight of the sample based on dry mass (in g).

Foaming capacity and stability

The foaming capacity and foaming stability determinations in 0.25 % protein suspensions were prepared at pH 7.0. Briefly, each sample was suspended in 100 mL of distilled water, and the pH was adjusted to 7.0 with analytical grade 1.0 M NaOH or 1.0 M HCl (Sigma Aldrich, Saint Louis, MO, USA).

The solutions were stirred (INFORS HT labotron, MS-L GmbH, Wiesloch, Germany) at 100 rpm for 30 min at room temperature and centrifuged (Eppendorf 5810R, Eppendorf AG, Hamburg, Germany) for 20 minutes (4 °C, 10,000 g). The supernatants were collected and stored at 4 °C until subsequent analyses. The foaming properties were determined fivefold by the method described by Zielinska et al., (2018), with modifications. Twenty milliliters (20.0 mL) of supernatant were transferred into a 250 mL beaker, and each sample was individually beaten in a high shear homogenizer mixer (16,000 rpm, 2.0 min, Ultra turrax, IKA, Staufen, Germany). The whipped insect sample was immediately transferred into a graduated cylinder, and the total volume was read at times zero and 30 min after whipping. Foaming capacity (FC) was calculated using the formulas described by Zielinska et al., (2018).

$$FC\ \left(\frac{mL}{mL\ fat}\right) = \frac{\left(V_{initial\ sample}\ (mL)\ -\ V_{final\ sample}\ (mL)\right)}{V_{initial\ sample}\ (mL)} \times 100 \qquad \text{Eq. 7.2}$$

where FC = Foaming Capacity, V = volume of insect sample

7.2.5 Determination of oil functional properties

Oil samples resulting from the *n*-hexane defatting process were subjected to chemical analyses adapted from Nielsen's food analyses handbook (Nielsen 2015).

Peroxide value

Peroxide values (PV) are a measure of the level of hydroperoxides, which gives an indication of lipid autoxidation and rancidity in an oil/fat sample.

In 0.50 g of the extracted fat, 6.25 mL of a 3:2 ratio of acetic acid, ReagentPlus®, and -chloroform ReagentPlus®, Sigma Aldrich, Saint Louis, M.O, USA) and 0.25 mL of saturated potassium iodide (ACS Reagent, Sigma Aldrich, Saint Louis, M.O, USA) were added and stored in the dark for 10 min. Then, 7.50 mL of distilled water was added. The resulting mixture was immediately titrated against a standard 0.01 N sodium thiosulfate ($Na_2S_2O_3$) (Titripur, Sigma Aldrich, Saint Louis, M.O, USA) solution using 1 % potato starch as an indicator.

The peroxide value was then calculated as follows:

$$PV\ \left(\frac{meq\ O_2}{kg\ fat}\right) = \frac{\left(V_{Na_2S_2O_3,sample}\ (mL) - V_{Na_2S_2O_3,blank}\ (mL)\right) \times N_{Na_2S_2O_3}\left(\frac{meq}{mL}\right)}{m_{sample}\ (g)} \times 1000 \qquad \text{Eq. 7.3}$$

Where, $V_{Na_2S_2O_3,sample}$ is the volume of sodium thiosulphate added, $V_{Na_2S_2O_3,blank}$ is the volume of sodium thiosulphate for the blank, $N_{Na_2S_2O_3}$ is the normality of the standard thiosulphate solution, and m_{sample} is the mass of the fat sample

Saponification value

The saponification value corresponds to the mass in mg of potassium hydroxide needed to neutralize the free fatty acids and saponify the esters contained in a gram of fat. In 0.50 g of the extracted fat, 6.30 mL of 0.50 N alcoholic potassium hydroxide (Titrisol®, Sigma Aldrich, Saint Louis, M.O., USA) was added and refluxed for 30 min at 60 °C. Then, 0.50 mL of phenolphthalein indicator (ACS, Reag. Sigma Aldrich, Saint Louis, M.O, USA)
was added and titrated with 0.50 N HCl (Titripur, Sigma Aldrich, Saint Louis, M.O, USA) until the pink color disappeared. The saponification value was calculated as:

$$SV\left(\frac{mg\ KOH}{g\ fat}\right) = \frac{\left(V_{KOH,sample}\ (mL)\ -\ V_{KOH,blank}\ (mL)\right) \times N_{KOH}(\frac{meq}{mL})}{m_{sample}\ (g)} \times 56.1 \qquad \text{Eq. 7.4}$$

Where, SV= saponification value (mg KOH per g sample), B = volume of Blank titrant, (mL), S = volume of Sample titrant (mL), N = normality of HCl (in mEq/L), W = sample mass (g) and 56.1 = molecular weight of KOH (kg/mol).

Iodine value

The iodine value (or iodine number) is a measure of the degree of unsaturation, which is the number of carbon-carbon double bonds in relation to the amount of fat or oil. Iodine value is defined as the grams of iodine absorbed per 100 g of sample. The higher the amount of unsaturation, the more iodine is absorbed and the higher the iodine value.

Iodine value was determined by adding 2.50 mL of the chloroform (ReagentPlus®, Sigma Aldrich, Saint Louis, M.O., USA) into the 0.50 g of the oil/fat extracted. This was followed by adding 6.25 mL of Wij's iodine solution (ReagentPlus®, Sigma Aldrich, Saint Louis, M.O., USA) and stored in the dark for one hour. Then, 25.0 mL of distilled water was added.

The resulting mixture was titrated against 0.10 N sodium thiosulphate standard solution (Titrisol®, Sigma Aldrich, Saint Louis, M.O., USA), and starch was added when a faint yellow color appeared. Titration continued until a blue color appeared with vigorous shaking during each titration.

Calculation of the iodine value of each sample was as follows:

$$IV\ \left(\frac{mg\ I_2}{g100\ fat}\right) = \frac{\left(V_{IODINE,sample}\ (mL)\ -\ V_{IODINE,blank}\ (mL)\right)\ \times\ N_{IODINE}(\frac{meq}{mL})}{m_{sample}\ (g)} \times 126.9\ \times 100 \qquad \text{Eq. 7.5}$$

Where, IV= Iodine value (g iodine (I_2) per 100g sample), B = volume of blank titrant, (mL), S = volume of sample titrant (mL), N = normality of $Na_2S_2O_3$ (in mEq/L), W = sample mass (g) and 126.9 = molecular weight of I_2 (g/mol).

Free fatty acid value

The free fatty acid (FFA) value (or acid number or acidity) is the mass of potassium hydroxide (KOH) in milligrams that is required to neutralize one gram of oil. This number is a measure of the number of carboxylic acid groups in fatty acids.

The free fatty acid was determined by adding a 2:1 ratio of 25 mL of diethyl ether and ethanol (ReagentPlus®, Sigma Aldrich, Saint Louis, M.O., USA) to 0.50 g of the oil extracted sample and 0.5 mL of phenolphthalein indicator (ACS, Reag. Sigma Aldrich, Saint Louis, M.O, USA). The resulting mixture was shaken well and titrated against 0.1 N alcoholic 95% potassium hydroxide (Titrisol®, Sigma Aldrich, Saint Louis, M.O, USA) with brisk shaking until endpoint indicated by a pale pink color. Since the FFA is measured by titration against a standard KOH solution, the conversion of the titration to a weight of fatty acid depends on the molecular weight of the fatty acid concerned. Most often, oleic acid (molecular weight 282) is taken as representing the average molecular weight of the acids being estimated.

Therefore, the % free fatty acid (expressed as oleic acid) was then calculated as:

$$FFA\ (\%) = \frac{\left(V_{KOH,sample}\ (mL)\ \right)\ \times\ N_{Na_2S_2O_3}\left(\frac{meq}{mL}\right) \times\ 282}{m_{sample}\ (g)} \times 100 \qquad \text{Eq. 7.6}$$

Where, % FFA = percent free fatty acid (g/100 g insect fat), expressed as *oleic acid*, V = volume of KOH titrant, N = normality of KOH (in mEq/L), W = sample mass (g) and 282 = molecular weight of Oleic acid (g/mol). KOH = Potassium hydroxide

7.3 Statistical analyses

The data were reported as means and standard deviations of triplicate measurements. To determine the effect of the drying methods and on the techno-functional properties of insect flours and oil functional properties, data were analyzed using a two-way analysis of variance (ANOVA) after passing Levine's test for normality. The drying method and type of insect flour (whole or defatted) are the two variable factors. Additionally, to elucidate the effect of defatting on techno-functional properties of the oven- and freeze-dried insect flours, data were subjected to a two-way analysis of variance (ANOVA) with main factors dried insect type and defatting mode (D or W). A mixed model with interaction was used in either case, and a post hoc test using Tukey's method at $p \leq 0.05$ revealed differences among the mean values. Data analysis was performed using GraphPad Prism Version 9.02 for Windows (GraphPad Software, La Jolla, CA, USA).

7.4 Results

7.4.1 Effect of drying and defatting on techno-functional properties on insect flours

Effect on the water holding capacity

Table 7.1 shows the water holding capacity of whole insect flours and defatted insect flours after drying procedures, *i.e*., oven drying (OD) and freeze-drying (FD). There was a significant difference between whole versus defatted insect flour types ($p < 0.0001$). The drying mode was equally significant ($p = 0.0257$); however, the interaction effect was also significant ($p = 0.0257$); the above significant effects are challenging to interpret. Across each drying method for all insects, water holding capacity was higher in the defatted flours. In the case of whole insect flours, the water holding capacity of *R. differens* and *G. bimaculatus* were not significantly different when either of the drying methods was applied. In contrast, when the FD method was applied to whole insect flour of *S. gregaria* (range: 3.74 – 3.95 g/g dry matter, a significantly higher level ($p = 0.0061$) of water holding capacity was observed compared to the OD method.

The highest water holding capacity was recorded on *S. gregaria* (range: 3.74 – 4.16 g/g dm). In contrast, dried *R. differens* recorded the lowest water holding capacities (range: 1.77 – 1.85 g/g dm).

Table 7. 1 *Characterization of freeze-dried and oven-dried field crickets (G. bimaculatus), bush crickets (R. differens), and desert locust (S. gregaria) by means of their Water holding capacity (g water/ g DM. Values are expressed as mean ± standard deviation (n = 3). Values in the same row with different lowercase superscripts and those in the same column with different uppercase superscripts represent significant (p < 0.05) differences among the treatments of the dried insect flour species.* DM = dry matter

	G.bimaculatus		*R. differens*		*S. gregaria*	
	Oven-dried	Freeze-dried	Oven-dried	Freeze-dried	Oven-dried	Freeze-dried
Whole	2.12 ± 0.18^{aA}	2.19 ± 0.08 ^{aA}	1.77 ± 0.18^{aA}	1.85 ± 0.08 ^{aA}	3.03 ± 0.08^{bA}	3.87 ± 0.11 ^{bA}
Defatted	2.67 ± 0.17 ^{aB}	2.32 ± 0.14 ^{aA}	1.82 ± 0.27 ^{aA}	2.07 ± 0.33 ^{aB}	3.74 ± 0.46 ^{aB}	4.16 ± 0.61 ^{bB}

Effect on the fat absorption capacity

Table 7.2 presents the fat absorption capacity of whole insect flours and defatted insect flours after drying. The effect of defatting was considered to be highly significant between whole and defatted insect flour types (p = 0.0008). The impact of the drying mode was also significant (p = 0.0313). A very significant (p = 0.0012) interaction effect of both drying and defatting effects, thus rendering their interpretation challenging. Generally, defatting increased the fat absorption capacities except for freeze-dried *R. differens*. The fat absorption capacity of whole insect flour derived from *R. differens* was significantly higher (p = 0.0054) when freeze-drying (FD) was applied than oven drying (OD) and ranged between 1.42 and 2.67 g oil/g dm. On the other hand, the fat absorption capacity of whole insect flours derived from *G. bimaculatus* and *S. gregaria* did not differ significantly when either freeze-drying or oven-drying was applied. The highest fat absorption capacity was recorded in defatted freeze-dried *S. gregaria* (3.56 g oil/g DM). *R. differens* whole powders that were oven-dried had the least fat absorption capacity (1.42 g oil/g DM).

Table 7. 2 *Characterization of freeze-dried and oven-dried field crickets (G. bimaculatus), bush crickets (R. differens), and desert locust (S. gregaria) by means of their fat absorption capacity (g oil/g DM) Values are expressed as mean ± standard deviation (n = 3). Values in the same row with different lowercase superscripts and those in the same column with different uppercase superscripts represent significant (p < 0.05) differences among the treatments of the dried insect flour species*

	G.bimaculatus		*R. differens*		*S. gregaria*	
	Oven-dried	Freeze-dried	Oven-dried	Freeze-dried	Oven-dried	Freeze-dried
Whole	2.64 ± 0.42^{bA}	2.36 ± 0.18^{bA}	1.42 ± 0.05^{aA}	2.67 ± 0.06^{bA}	1.98 ± 0.14^{bA}	2.84 ± 1.77^{bA}
Defatted	2.47 ± 0.23^{aB}	2.52 ± 0.78^{aB}	3.43 ± 0.49^{aB}	2.59 ± 0.08^{aB}	2.75 ± 0.21^{aB}	3.56 ± 0.41^{aB}

DM = dry matter

Effect on foaming capacity

The foaming capacity of whole insect flours and defatted insect flours of the three edible insects are shown in Table 7.3. Similar to the two previous properties, there was an enormously significant difference between whole versus defatted insect flour types ($p < 0.0001$). The drying method was also significant ($p < 0.0001$). The interaction effect was also significant ($p < 0.0001$); thus, the significance of the main treatment effects - drying and defatting, cannot be adequately interpreted. In the case of whole insect flours, the foaming capacity of the whole insect flour from *R. differens* and *G. bimaculatus* differed significantly when either FD or OD procedure was applied.

Similarly, the foaming capacity of defatted insect flours derived from *R. differens* and *G. bimaculatus* was significantly higher in oven-dried than in freeze-dried conditions. At the same time, no significant differences were observed between oven- and freeze-dried whole insect flours or defatted insect flours derived from *S. gregaria*. Defatting decreased the foaming effect of all insects for both drying methods. The highest foaming capacity was found in the whole oven-dried *G.bimaculatus* (17.10 %), with the least value occurring in defatted *R. differens* samples (1.42 %) that were freeze-dried.

Table 7. 3 *Characterization of freeze-dried and oven-dried field crickets (G. bimaculatus), bush crickets (R. differens), and desert locust (S. gregaria) by means of their foaming capacity (%). Values are expressed as mean ± standard deviation (n = 3). Values in the same row with different lowercase superscripts and those in the same column with different uppercase superscripts represent significant (p < 0.05) differences among the treatments of the dried insect flour species*

	G.bimaculatus		*R. differens*		*S. gregaria*	
	Oven-dried	Freeze-dried	Oven-dried	Freeze-dried	Oven-dried	Freeze-dried
Whole	17.10 ± 1.54^{bC}	4.30 ± 1.03 ^{aA}	17.00 ± 2.88^{bC}	3.10 ± 1.07 ^{aA}	6.00 ± 2.85^{aA}	4.02 ± 1.09 ^{aA}
Defatted	12.10 ± 3.52 ^{bB}	4.20 ± 1.05 ^{aA}	16.60 ± 2.17 ^{bC}	2.40 ± 2.12 ^{aB}	4.00± 0.01 ^{aA}	3.70 ± 1.90 ^{aA}

7.4.2 Effect of drying on techno-functional properties of insect oils

The results of the influence of oils obtained from oven-dried and freeze-dried insects on specific physicochemical parameters are summarized in Table 7.4 below.

Table 7. 4 *Effect of drying method on saponification (mg KOH/g), peroxide (mEq O2/Kg), iodine (g I2/100 g), and free fatty acid (% oleic acid) values of field (G. bimaculatus), bush crickets (R. differens) and desert locust (S. gregaria). Values are expressed as mean ± standard error (n = 3). For each parameter, values in the same row with different lowercase superscripts represent significant (p < 0.05) differences among the treatments for insect species oil.*

	G.bimaculatus		*R. differens*		*S. gregaria*	
	Oven-dried	Freeze-dried	Oven-dried	Freeze-dried	Oven-dried	Freeze-dried
Saponification Value (mg KOH/ g)	35.0 ± 3.19^{a}	99.7 ± 1.35^{b}	193 ± 1.31 ^{a}	112 ± 4.83 ^{b}	17.6 ± 0.01 ^{a}	19.1 ± 1.38 ^{a}
Peroxide Value (mEq O_2/Kg)	25.9 ± 3.24^{a}	23.3 ± 1.86^{a}	24.8 ± 2.69^{b}	13.5 ± 0.14^{a}	23.7 ± 0.65^{a}	29.7 ± 2.55^{a}
Iodine Value (g I_2/100 g)	46.9 ± 0.34^{a}	49.4 ± 1.46^{a}	41.6 ± 1.30^{a}	43.9 ± 1.41^{a}	59.8 ± 0.47^{c}	55.6 ± 1.68^{b}
Free fatty acid Value (% oleic acid)	6.89 ± 0.09^{a}	56.6 ± 2.36^{b}	13.8 ± 0.24^{a}	52.3 ± 3.76^{b}	58.6 ± 0.36^{b}	94.5 ± 4.77^{c}

Effect of drying method on the saponification value

Table 7.4 shows the saponification values of extracted oils from three edible insects that were either oven- or freeze-dried. Globally, the effect of drying was considered significant (p = 0.0201). The saponification value of freeze-dried *R. differens* oil differs significantly, with the oven-dried samples showing the higher values, ranging between 191.0 and 194.0 mg KOH/g. On the other hand, the saponification value of oil derived from oven-dried *G. bimaculatus* differs significantly, with the higher values observed in the freeze-dried samples. Neither freeze-drying nor oven-drying impact the saponification value of oil extracted from *S. gregaria*. Apart from *R. differens* oil, freeze-drying resulted in high saponification values.

Effect of drying method on the peroxide value

The peroxide values of oils extracted from the three edible insects after the oven and freeze-drying are shown in Table 7.4. These values were significantly different (p = 0.0267) for either drying technique. In general, oven-dried *R. differens* oil recorded a significantly higher peroxide value than freeze-dried extracted oil. By contrast, the peroxide values of oils extracted from *G. bimaculatus* and *S. gregaria* were not significantly different when either freeze-dried or oven-dried procedure was applied. However, on average, oven-dried samples recorded higher values except for the case of *S. gregaria*. Peroxide values ranged from 13.5 mEq O_2/kg in *R. differens* to 29.7 mEq O2/kg for freeze-dried samples.

Effect of drying method on the iodine value

Table 7.4 shows the iodine values of extracted oils from three edible insects that were either freeze-dried or oven-dried. Overall, drying had a very profound significance (p < 0.0001) on the insects' oils' iodine values. The iodine value difference of oils extracted from *R. differens* and *G. bimaculatus,* either oven-dried or freeze-dried, was insignificant. However, a significant difference was observed in oil obtained from *S. gregaria,* with the freeze-dried samples displaying the higher values, ranging from 55.6 to 59.8 g I_2/100 g. In general, all the samples

analyzed recorded an iodine value above 40 g I_2/ 100g.

Effect of drying method on the free fatty acid value

The free acid values of oils extracted from the three edible insects after applying both drying modes are depicted in Table 7.4. The free acid value differed significantly ($p < 0.0001$) in all insect species that were either oven-dried or freeze-dried. In all insect species, the oil derived from freeze-dried samples produced the highest free acid value. Oils derived from oven-dried *G. bimaculatus* and *R. differens* recorded the lowest free acid value of less than 10 % and 15 %, respectively.

7.5 Discussion

The inclusion of edible insects in human diets is increasingly promoted as a sustainable source of proteins with high nutritional value (Van Huis et al., 2013). However, consuming insects is still unfamiliar in many countries due to food neophobia (Sogari et al., 2017; Orsi et al., 2019). Recent consumer studies suggest that introducing insect-derived products in unrecognized forms into food may enhance consumer awareness, reduce neophobia and make the idea more palatable (Gould & Wolf, 2018). The extraction and application of defatted insect flours are among the most promising ways of overcoming consumer neophobia regarding insect consumption (Yoon et al., 2019). In this study, the desert locust (*S. gregaria*), field cricket (*G. bimaculatus*), and bush cricket (*R. differens*) were selected because they are widely consumed in several countries in Sub-Saharan Africa (Kelemu et al., 2015). This was an effort to assess how drying methods may affect techno-functional properties of the protein profiles of hexane-defatted or non-hexane-defatted whole insect flours and defatted insect flours, as well as to evaluate their potential applicability in food formulations. In chapter two, we showed that neither oven-drying nor freeze-drying had a significant impact on the nutritional composition of *R. differens* (Fombong et al., 2017). Thus, there was a need to evaluate the same drying methods for their possible influence on techno-functional properties.

The water holding capacity of a protein concentrate is an important characteristic when considering its use as a food ingredient (Hall et al., 2017). The water holding capacity of whole

insect flours was slightly lower compared to their defatted insect flours, and this could be attributed to the high relative protein levels in the concentrates. Oven-dried (OD) whole insect flour derived from *S. gregaria* showed a markedly lower water holding capacity than freeze-dried (FD) ones, and this could be a result of the continuous denaturation of protein molecules at increased drying temperatures (Yeomans et al., 2008). The water holding capacity of defatted insect flours from all insect species was similar regardless of the drying method. In the literature, the water holding capacity values of the defatted insect flours derived from *S. gregaria* and *G. bimaculatus* were close to 2.38 mL/g reported for a *Gryllidae* species (Bußler et al., 2016). Those of *R. differens* exhibited water holding capacity values close to 1.87 mL/g reported for yellow mealworm protein extract (Zhao et al., 2016). Water holding capacity plays a vital role in the texture quality of various foods, especially meat products. Also, it is essential in maintaining the consistency and bulking of products, as well as in baking applications (Tan et al., 2015).

The fat absorption capacity of freeze-dried whole insect flours derived from *R. differens* was significantly higher than oven-dried ones. However, the fat absorption capacity of the remaining whole insect flours was similar, regardless of the drying method adopted, but the latter values were above 300 %. Irrespective of the drying method adopted, the fat absorption capacity of defatted insect flours of all three edible insects was similar but well above 350 %. The fat absorption capacity of the defatted insect flour was higher than 178.7 % reported in *Cirinia forda* (Osasona et al., 2010) and 233 % reported in yellow mealworm protein extract (Bußler et al., 2016). Likewise, the fat absorption capacity exhibited by defatted insect flours derived from the three edible insects in this study was significantly higher compared to commonly used defatted insect flours, such as whole egg powder (260 %), egg yolk powder (60 %), and egg white powder (50 %) (Ndife et al., 2010). The high fat absorption capacity exhibited by the defatted insect flours in this study shows the potential of defatted insect flours in enhancing the flavor characteristics of processed foods and improving the mouthfeel of foods (Osasona et al., 2010).

The foaming capacity of a protein is measured as the amount of interfacial area that can be created by whipping the protein (Osasona et al., 2010). Typically, as the structure unfolds,

hydrophobic sites are exposed, making it possible for the protein to adsorb more quickly to air-water interfaces and lower interfacial tension, thus trapping more air and increasing the foaming capacity. The foaming capacity of OD whole insect flours (*R. differens* and *G. bimaculatus*) was significantly higher (more than 15 %) than FD samples. In comparison, *S. gregaria* recorded less than 10 % when two drying methods were adopted. Similar observations were revealed in defatted insect flours when both drying methods were applied. The foaming capacity and foam stability of the defatted insect flours were considerably lower than those reported for *Tenebrio molitor*, *Allomyrina dichotoma*, and *Protaetia brevitarsis seulensis* (more than 100 % and 20 %, respectively) when extracted using 0.02 % ascorbic acid and 0.58 M saline solution. In addition, other commonly used products, such as soy protein isolates recorded higher foaming capacity and stability values (61 and 21.2%, respectively) compared to the defatted insect flour in this study (Chove et al., 2007). Foam stability is highly influenced by many factors such as protein structure, protein concentration, pH, viscosity, and ionic strength, and the insect protein structure could explain the relatively low foam stability (Yi et al., 2013). This low foaming capacity could also be due to the blanching process prior to drying, as extensive heat denaturation of proteins decreases their ability to form foams. The low foaming capacity of these insect flours would make them unsuitable for bakery products that need aeration, such as cakes and muffins.

The saponification value or number relates to all fatty acids present in the insect oil, both free and esterified. This value depends on the molecular weight and the percentage concentration of fatty acid components present in fatty acid methyl esters of oil, and it is used to determine the average relative molecular mass of oils and fats. A high saponification value of oven-dried oil extracted from *R. differens* was observed, while an increased value was observed in freeze-dried oil from G*. bimaculatus*. Generally, low saponification values were only observed in *S. gregaria* regardless of the drying method. An elevated saponification value is an indication that oils contain triglycerides and can provide an estimation of the average molecular weight of the lipids. The higher the molecular weight, the smaller its saponification value is (Onyeike & Acheru, 2002). A higher saponification value was observed for oven-dried *R. differens* freeze-dried ones. This elevated value is probably because of a higher degree of saturation and a

shorter chain length (Onyeike & Acheru, 2002). The ranges of saponification values (in mg KOH /g) of some common oils are as follows: olive (187–196), soybean (189–195), palm (200–205), coconut (242–263), sunflower (186–194). Elsewhere it was reported that the saponification value of groundnut oil at 28 °C was 89.52, and this rose to 296.57 when heated at 200 °C (Onyeike & Acheru, 2002). Differences in the composition of the saturated and unsaturated fatty acids among the insect oils could explain the varied response in saponification value upon drying for the different insect species.

The peroxide value is among the most common chemical method of measuring the oxidative deterioration of oils and rancidity. A high peroxide value indicates the increased formation of hydroperoxides or their reduced decomposition. Regardless of the drying method applied, slightly elevated peroxide values of extracted oils were recorded (all above 15 meq O_2/kg). According to the *Codex Alimentarius* (2006), oil with a peroxide value above 10 meq O_2/kg has an intermediate oxidation state. Based on this cut-off value, the oils in these studies would taste rancid. This is, however, expected as the oils were extracted from processed and not fresh insects. The peroxide values were similar to the range for mealworms, 55.57 meq O_2/kg – 125.13 meq O_2/kg (Lenaerts et al., 2018). In contrast, when compared to other cricket species- *Acheta domesticus* and *Gryllus assimilis,* the values obtained in this study were much higher (0.96 and 0.67 meq O_2/kg), respectively. (Khatun et al., 2021). Diet is known to affect fatty acid composition (Rutaro et al., 2018); thus, the diet of these insects could have contributed to the elevated values. Also, high temperature, visible light, and oxygen will promote primary oxidation, which increases the peroxide value. Thus, the heating methods and air exposure applied in this study may have resulted in some significant oxidation levels. Oils with higher peroxide values may predispose consumers to adverse health effects like stimulating cardiovascular and inflammatory diseases. Additionally, oils with higher peroxide values will have a shorter shelf life and become rapidly unsuitable for consumption. (Lobo et al.,2010). Thus, to correctly use these insect oils, further fractionation, a suitable choice of diet, and optimized processing and storage conditions should be carefully investigated to ensure their marketability for culinary or industrial oil use. Typical ranges of peroxide values in meq O_2/kg are soybean (0.05 -19.69), rapeseed (1.00 -19.67), and sunflower (1.40-31.96) (Wealleans et al.,

2021).

The Iodine value is related to the degree of unsaturation and therefore provides an estimation of the oxidative stability of the oil in question: the greater the iodine value, the more unsaturation and the higher the susceptibility to oxidation. The iodine value of oil extracts was not significantly affected by the drying method except for *S. gregaria.* This suggests that the drying conditions did not alter the degree of unsaturation of the insect oils of *R. differens* and *G. bimaculatus*. However, the high iodine value recorded in all oil extracts indicates a relatively high level of unsaturation and probably high oxidation susceptibility, but with high nutritional value. Iodine values for *R. differens* (41.6 – 43.9 g I_2/ 100g) in this study were much lower than figures obtained for *R. differens* (86.97 g I_2/ 100g) in another study (Kinyuru et al., 2020). This could be attributed to the difference in diet that in turn affects the fatty acid composition, especially the amount of unsaturated fatty acids. Typical iodine values for some popular oils (in g I_2/ 100g) include peanut (82–107), corn (103–128), cottonseed (99–113), coconut (7.7–10.5), palm (44–54) or butter (25–42) oils (Caballero et al., 2003). In the literature, heating of various plant-derived oils (palm oil, coconut oil, and groundnut oils) up to 200 °C was reported to have no significant effect on the iodine values compared to the unheated oils (Gharby et al., 2016). Currently, the iodine value is calculated from fatty acid composition using specific factors for each unsaturated fatty acid (Kyriakidis & Katsiloulis, 2000). In such cases, the percentage of each unsaturated fatty acid would be multiplied by a predetermined constant, and all results are added up (Barrera et al., 2019).

The free fatty acid value, or acid value for short, determines the amount of free fatty acids in fat. Drying methods significantly influenced the free acid values of all oil extracts, with freeze-drying resulting in higher free acid values in all insects. As oils rancidify, triglycerides are converted into fatty acids and glycerol, causing an increase in acid number. Thus, freeze-drying which lasted 72 h, resulted in a high degree of degradation of the oil quality resulting from hydrolysis of triacylglycerols of the oils compared to oven-drying (Gharby et al., 2016). Oils used for biodiesel are usually classified based on their free fatty acid (FFA). Refined oils like soybean, sunflower, or canola have FFA < 1.5 %; the low free fatty acid yellow oils are FFA < 4 %, whereas high free fatty acid oils and animal fats, have FFA ≥ 20 %.

Peroxide values only indicate the level of primary oxidation products (hydroperoxides), which are further converted into secondary products. As such, they may not reflect the actual extent of lipid oxidation and only measure the oxidation products produced by the initial stages of oxidation. However, a more useful measure is the *p*-anisidine value (*p*AV) which measures the aldehyde and ketonic breakdown products of peroxides. This, then, is a more accurate measure of the chemicals responsible for the rancidity of oils. Due to a lack of resources, this measurement was not carried out and should be included in future studies. Furthermore, the determination of fatty acid composition using gas chromatography-mass spectrometry rapidly replaces these oil physicochemical parameters. Although the fatty acid profiles of these insect fats are already discussed (chapters 2 and 3), physicochemical attributes still add value to the oil characteristics. However, in a hypothetical scenario where insect oils might be considered for biodiesel use, the measurements of the peroxide, iodine, and free fatty acid values could serve as a quick pointer for which insect oil could perform best as a biofuel. Other beneficial physicochemical properties of these processed insect flours, such as taste, smell, and color, which were not assessed due to limited time, would be a valuable addition to this study.

7.6 Conclusion

The results showed that the techno-functional properties of edible insects differed depending on insect species and drying methods. Defatted insect flour and whole insect flour derived from *S. gregaria* exhibited superior water holding capacity. The fat absorption capacity of defatted insect flours of all three edible insects was better than the whole, regardless of the drying method. The foaming capacity and stability of oven-dried whole insect flours (*R. differens* and *G. bimaculatus*) were higher than in freeze-dried samples. At the same time, *S. gregaria* recorded the least foaming capacity when two drying methods were adopted. The oil properties were significantly influenced by drying methods, especially the free acid values in all insects. Thus drying and defatting insect flours significantly alter the techno-functional properties in diverse ways. Based on the above, the more affordable oven-drying is recommended coupled with defatting yielding more concentrated protein flour for use in complementary foods while at the same time generating oil as an additional product.

CHAPTER EIGHT

Solvent Extracts of *Ruspolia differens* Show Antimicrobial Activities Against Clinical Pathogens: An exploratory study.

8.1 Introduction

In the opening chapters, *Ruspolia differens* were collected from the wild and subjected to different drying methods. During these field expeditions, there was regular contact and communication with locals. In several of such discussions, they kept on mentioning the fact that they used these insects not only as food but also as medicine. A few villagers mentioned using the oil as an eye droplet. Others said they used the crushed skin to heal wounds, yet others indicated that they boiled the insects and extracted the water, which, together with the ashes, were used to treat fever and headaches. How accurate were their claims? This chapter sought to (dis)prove that assertion in a first of its kind exploratory study.

Several natural products (particularly plant origin) are well-established and proven drugs and medicinally relevant substances. Of all the drugs available on the market, more than 70% are either based on or derived from natural compounds (Harvey, 2000). Due to their huge success, natural products are likely to remain an essential source of commercial drugs. However, the search for new antimicrobials in modern drug discovery is urgently necessary, given the continuous emergence of new pathogens and various antimicrobial-resistant pathogens (Ventola, 2015, WHO, 2022; Ancilloti et al., 2022). The WHO has declared that antimicrobial resistance is one of the top-ten global public health threats affecting humans. More so, the global burden of bacterial infections remains excessive and is exacerbated by increasing resistance to multiple antibiotics. Hence, this has necessitated seeking alternatives that can effectively remedy problematic infections. Notorious for its ability to resist antibiotics is the gram-positive bacterium *Staphylococcus aureus,* which ranks high among challenging diseases. *S. aureus* infections are an excellent example that would benefit from discovering and developing new antimicrobials (DeLeo & Chambers, 2009, WHO, 2022).

Insects remain the vastest group of living organisms, and it is estimated that more than one million species of insects have sufficiently been described, comprising about 70% of all known metazoans (Gullan & Cranston 2005). Insects constitute a very substantial food source in many countries, as more than 70 families and 200 genera of insects are eaten up by people from

different places of the world. In developing countries, the traditional use of insects as food continues to be widespread as it provides substantial nutritional, economic, and ecological benefits for rural communities (Meyer-Rochow 2017). On the contrary, little attention has been given to medically important terrestrial arthropods as a source of bioactive compounds. However, they have long been reported to play critical roles in treating diseases (Meyer-Rochow, 2017).

Alongside their nutritional status, in which edible insects research has positioned them as a sustainable food source, they also display the potential to provide bioactive compounds with health benefits for humans (Harvey 2000). Various recent studies have indicated such bioactivity in different insect species. The effects of chitin, lauric acid, and anti-microbial peptides (AMPs) provided by insects have been reported (Ma et al., 2019; Rončević et al., 2019; Dhayakaran et al., 2015; Brady et al., 2019; Djeussi et al., 2013; Shahidi et al., 1999). Insects produce the most extensive repertoire of AMPs, which can be explored as alternatives to conventional antimicrobial medicines. Globally, the use of medicinal insects has been captured and comprehensively reviewed. In Asia (India and China) and Latin America (Brazil), the utilization of insects as medicine is well documented (Dossey, 2010; V. Benno Meyer-Rochow, 2017; Ma et al., 2019; Feng et al., 2009; Wilsanand et al., 2007; Costa-Neto 2005). Amongst the African edible insects that have been reported to be used in traditional medicine, the most common are termites (Wilsanand et al., 2007; Jideani & Netshiheni, 2017; Coutinho et al., 2009), bagworms (Van Huis, 2019; Nonaka 1996; Fasoranti 1997), crickets (Fasoranti 1997) and grasshoppers (Costa-Neto 2005; van Itterbeeck et al., 2019).

Over the past decade, much research on *Ruspolia differens* has focused on the nutritional (Kinyuru et al., 2010; Siulapwa et al., 2012; Fombong et al., 2017, safety (Ssepuuya et al., 2019; Ng'ang'a et al., 2019) and rearing (Malinga et al., 2018a; Rutaro et al., 2018a; Rutaro et al., 2018b; Lehtovaara et al., 2019; Malinga et al., 2018b) aspects of *R. differens*. Current indigenous knowledge rumors about the utilization of this insect as traditional medicine for specific ailments (Mmari et al., 2017; Van Itterbeeck et al., 2019). However, there has been apparent neglect of the scientific validation of indigenous traditional knowledge regarding insects in Sub-Saharan Africa. To the best of our knowledge, no study has scientifically evaluated the

antimicrobial properties of edible bush crickets *R. differens* of African origin. Therefore, the present chapter aims to explore the antimicrobial properties of wild-harvested *R. differens* extracted successively with different solvents (hexane, ethyl acetate, methanol, and water).

8.2 Materials and Methods

8.2.1 Materials

Insect samples

The insect samples were obtained as previously described in *section 2.2* of chapter two.

8.2.2 Methods

The present study aimed at unraveling natural antimicrobial compounds from insects through a bioassay-guided method. Bioassay-guided fractionation is a procedure whereby extract is chromatographically fractionated and re-fractionated until a pure biologically active compound is isolated. Each fraction produced during the fractionation process is evaluated in a bioassay system, and only active fractions are fractionated. Bioassay-guided isolation techniques incorporate the separation of compounds in a crude extract using an HPLC column. It begins with testing the extract to confirm the presence of bioactivity. If present, there is a crude separation of the compounds into crude fractions. Further fractionation is carried out on the fractions that are found to be active against the target microbe at a concentration of 1 mg/mL (Jamil et al., 2012).

8.2.2.1 Sample Preparation

Drying methods

The bush crickets were freeze-dried using the method described in chapter two, *section 2.1.* Drying was essential to prevent the growth of fungi, molds, bacteria, or other microorganisms (Panda et al., 2016). The dried insects were ground into flour using a laboratory blender (Camlab, Over, UK) and transported to Belgium, and stored in the freezer at -20 °C.

Extract preparation

Extraction was carried out in two steps using the method described by Panda et al. (2017).

This first preliminary screening step was done to find out if there was any antibacterial activity in any of the four solvent extracts before proceeding to a large scale (see later).

One gram of dried insect powder was transferred into each of four 15 mL sterile polypropylene tubes with screwcaps, and 10 mL of sterile water, hexane, ethyl acetate, and methanol were added, respectively. Extraction was performed at ambient temperature with the aid of repeated vortexing and sonication (4 × 15 min over a 24 h period) in a sonicator water bath (Branson, USA). After 1 day, the tubes were centrifuged for 10 min at 4500g (Hettich Rotanta 46R, C4810, Germany), and the supernatant was transferred in 1 mL aliquots to 1.5 mL Eppendorf tubes. The hexane and ethyl acetate extracts were dried at room temperature under a forced convection fume-hood chamber. In contrast, the evaporation of the water and ethanol extracts took place in a Savant SpeedVac Concentrator (SVC 200H, Stratech Scientific, London, UK).

The dried residue of 1 mL extract was re-dissolved in 200 µL water (for the aqueous extract) or 200 µL DMSO for the hexane, ethyl acetate, and methanol extracts. The samples were stored at 4 °C until further antimicrobial testing (described below)

After the screening, the hexane extract showed activity and was selected for a large-scale extraction using 5.0 grams of insect powder and following the same steps outlined above.

Liquid-Liquid separation (Hexane-Acetonitrile)

Upon drying the hexane extract, It was observed that the oil-containing hexane extract was a very oily and complex mixture and couldn't be loaded into the *Sunfire* HPLC column for the bioassay-guided purification . So the oil was dissolved in acetonitrile using a 50 mL tube and 1 mL dried concentrated oil, added acetonitrile and mixed thoroughly, and let it stand for 24 h. Two separate layers were formed, one layer the *R. differens* hexane extract and the other layer the acetonitrile. Both layers were tested for bioactivity, and all active metabolites were found to be in the acetonitrile layer. So this active acetonitrile fraction was dried, and then a tiny portion (50 µL) was collected . One portion, 10 µL, was added to 190 µL DMSO and retested, confirming

activity in the acetonitrile fraction as described above. The remainder of the 40 µL acetonitrile fraction in which antimicrobial activity was present was then further subjected to HPLC bioassay-guided purification (described below). Acetonitrile was selected as the solvent for the liquid-liquid separation based on the laboratory restrictions in drying other aggressive solvents in the SpeedVac concentrator.

8.2.2.2 Antimicrobial Test

Microbial strains

Microbes were selected based on availability in the lab and also to each represent a gram-negative bacteria, gram-positive bacteria, and a fungi species. Bacterial strains *E. coli (DH10B), S. aureus* (ATCC 65385) (Rosenbach), and fungal strain *Candida albicans* (SC5314) (American Type Culture Collection, Manassas, Virginia, USA) (Fonzi and Irwin, 1993) were used for the antibacterial and antifungal test, respectively. Existing lab cultures for each of the examined microbes stored in Petri dishes at -20°C were used as starting material. For bacteria, colonies were inoculated on Mueller-Hinton (MH) agar plates (Panda et al., 2017). In contrast, for *C. albicans*, colonies were inoculated on YPD agar, and after overnight growth, both plate types were sealed with parafilm and stored in a cold room at 4 °C (Panda et al., 2017).

Preparation of pre-culture

For the fungi, a single colony of *C. albicans* was inoculated from an agar plate to 5 mL of YPD broth (1 % yeast extract, 2 % peptone, and 2 % dextrose), while for *E. coli* and *S. aureus*, single colonies of each bacterium were inoculated in MH broth in separate reaction tubes. The tubes were then incubated overnight while shaking at 200 rpm at 37 °C.

Antibacterial activity test (Microdilution Broth) protocol

Antimicrobial activity was assessed as described previously using a 96–well microdilution (Panda et al., 2017). Extracts were stored at 4°C and brought to ambient temperature right before the start of the experiment. After thorough vortexing of the tubes with stored extracts were prepared in DMSO using sterile 96 flat-bottom microdilution plates. A standardized inoculum was obtained by growing the test organisms overnight in MH broth and diluting the suspension with MH broth to turbidity of optical density (OD) = 0.003 at 620 nm on a Perkin Elmer UV/VIS spectrometer Lambda 20 (typically approximately 100-fold). Each well of a microdilution plate was inoculated with 190 µL of the diluted suspension, and 10 µL of the test solution was added. Control wells were prepared with 190 µL MH broth and 10 µL extract to correct any absorption due to extracting components. In every experiment, three wells of each plate were filled with 190 µL of the diluted suspension and 10 µL of DMSO or MilliQ as solvent control, while the antibiotic ciprofloxacin (stock 100 µg/mL) in DMSO was used as a positive control. After mixing, the plates were sealed off with parafilm.

The microdilution plates were placed in a shaker incubator at 37°C for 18 h and then read on a Mithras LB 940 Multimode Microplate Reader (Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany) at 620 nm with lamp energy of 13,000 using the *MikroWin 2000* software package. The extent of growth in the wells containing the test compound or extract was compared to the extent of growth in the solvent-control wells (containing the appropriate solvent). For a test to be considered valid, acceptable growth (≥ 0.5 OD) must occur in the solvent control well and none (OD < 0.05) in the well with a medium containing solvent. All OD values of wells with *R. differens* extract were corrected for the absorption contributed by the extract. The tests were done in duplicate. The relative inhibition (%) of the test sample was calculated as follows

$$Inhibition\ (\%) = 100 - \left(\frac{A-B}{C} \times 100\right)$$ (Panda et al., 2017) Eq. 8.1

Where A is the OD value of a well with the microbial culture and test sample, B is the OD value of the corresponding negative control well with a mixture of pure broth and test sample, and C is the OD value of the average of two or more solvent control wells (OD = Optical density).

Antifungal test

Antifungal activity was tested against *C. albicans,* similarly to bacteria (Panda et al., 2017). Instead of MH broth, YPD broth was used with a smaller insect extract (to keep the final DMSO concentration below 2 %). For antifungal activity, each well of a microdilution plate was inoculated with 196 µl of the diluted yeast suspension, and 4.0 µl of the test solution was added. Control wells were prepared with 196 µL YPD broth and 4.0 µl extract to correct for any absorption due to extracting components, or 4.0 µl of DMSO or MilliQ (solvent control), or the antifungal miconazole (200 µg/mL) in DMSO (positive control). Tests were carried out in duplicate.

8.2.2.3 Chromatographic Analyses

HPLC-DAD analysis

The active acetonitrile fractions that tested for activity against *S. aureus* were dissolved in 95 % acetonitrile (5 % water) and injected into the HPLC. The HPLC analysis was performed on a Shimadzu, City, Contry, LC-20AT system (model DGU 20A3) equipped with an LC-20AT quaternary pump, a DGU-20A3/DGU-20A5 online degasser, an SPD-20A photodiode array detector, and a CBM-20A/20A interface as described earlier (Kouakou et al., 2019) using conditions in Table 8.1. The chromatography data were acquired and processed using the *Lab Solution* software.

During the 30 min run time of the HPLC, 1mL sub-fractions were manually collected each minute, dried in the SpeedVac concentrator as before, and to each of these 30 subfractions, DMSO was added and tested for bioactivity as described previously.

Table 8. 1 *Analytical conditions of HPLC for analysis*

Parameters	Conditions		
Column	Sunfireprep C18 column (10 mm × 250 mm, 5 µm) (Waters, Ireland).		
Flow rate, temperature	4 mL/min, 20 °C		
Injection volume	2 mL (filtered using a CHROMAFILTER Xtra H-PTFI filter (pore size 0.45 µm, filter 13 mm, MACHEREY-NAGEL, Germany)		
UV detection	190 to 280 nm		
Run time	30 minutes		
Gradient	Time (min)	% A (H_2O with 0.1 % trifluoroacetic acid, Acros Organics)	% B (acetonitrile with 0.1 % trifluoroacetic acid)
	0	5	95
	5	5	95
	22	2	98
	25	0	100
	30	0	100

The HPLC was then re-run under the same conditions, and the chromatogram peaks were then linked to the corresponding active fractions that were previously manually collected.

After linking the active fractions to their corresponding peaks by aligning the activity profile with the corresponding chromatogram (Figure 8.1), those peaks with activity found to be higher than 30 % were then esterified for gas chromatography-mass spectrometry (GC-MS). These were 12 peaks (that represented sub-fractions at least 30 % or more active against *S. aureus*. Hence only the activity of fatty acids was tested in these sub-fractions. These dried sub-fractions corresponding to these peaks were resuspended in hexane and sent for fatty acid determination using gas chromatography-mass spectrometry (GC-MS).

<u>GC-MS Determination of the fatty acid composition of 12 active sub-fractions</u>

Fatty acid methyl esters (FAMEs) of the twelve collected sub-fractions (with activity above 30 %) were prepared from the lipid samples by esterification in a methanolic KOH solution (0.500 M) with the addition of a 20 % BF_3-methanol solution (Sigma-Aldrich, St. Louis, MO, USA) following the procedure *section 2.2* (Fombong et al., 2017). The fatty acid composition was obtained with an Agilent 7820A-5977E GC-MSD (Agilent Technologies, Santa Clara, CA, USA) using the settings laid out in *chapter two* (Table 2.2)

8.3 Results

8.3.1 Percent yield of flour extracts and bioassay tests

The percent yield of whole *R. differens* flour extracts after sequential extraction with four different solvents (according to polarity) is shown in Table 8.2. The hexane extract, which contained much oil, gave the most abundant yield, with a reported crude fat content of between 33-44 % fat per 100 g of dry weight (Kinyuru et al., 2010; Siulapwa et al., 2012; Fombong et al., 2017). The water extract preceded this, the most polar solvent containing the protein fractions.

Table 8. 2 *Percent yield of R. differens flour extracts g/100 g dry matter*

Type of extract	Percent yield
Hexane	23.60
Methanol	6.25
Ethyl acetate	11.90
Water	19.10

The *in-vitro* screening of crude extracts of the different solvents from *R. differens* showed differential antimicrobial activities against the three clinical strains (*E. coli*, *S. aureus*, and fungal strain *C. albicans*) tested as depicted in Table 8.3. The hexane extract was the most potent, showing almost 100 % planktonic growth inhibition against *S. aureus.* This result contrasts its effect on *E. coli* bacteria, which seemingly favored a 25 % growth of these strains. Complete growth inhibition was also observed against all test strains with the water extract, which upon further dilution, seemed to be contaminated with spoilage microorganisms. Additionally, when tested after filter sterilization with a membrane filter (0.22 µm), the aqueous extracts lost their activity. As a result, the water extracts were not included in further analyses. The positive controls, ciprofloxacin, and miconazole, were effective against bacteria and yeast, respectively (Table 8.3).

Table 8. 3 *Activity (% growth inhibition) of extracts in various solvents of R. differens extracts. Negative values indicate growth of microorganisms (bacteria or fungi) was favored, while positive values indicate growth inhibition of the pathogen in question*

Solvent Extracts†	*E. coli*	*S. aureus*	*C. albicans*
Hexane	-25.6 ± 0.4	99.7 ± 0.6	38.7 ± 1.0
Methanol	-49.5 ± 0.6	-61.6 ± 2.6	5.4 ± 4.0
Ethyl acetate	-21.4 ± 1.9	-26.9 ± 0.8	4.9 ± 0.4
Water	99.9 ± 1.0	75.1 ± 0.4	99.7 ± 1.5
Control (ciprofloxacin)	91.2 ± 1.4	95.0 ± 1.1	---
Control (miconazole)	---	---	99.0 ± 1.0

†*Concentration of extract: 1.00 mg/mL, for extracts, the activity is 0.05 %*
Negative(-) values indicate microbial growth support.
--- Indicates not tested. Values represent the mean percentage ± standard deviation

8.3.2 HPLC chromatogram of hexane fractions showing bioactivity against *S. aureus*

Given that the hexane extract was the most abundant and showed almost 100 % inhibition against *S. aureus*, it was further partitioned using an acetonitrile – hexane mixture. Few well-resolved peaks were recorded at 190 nm, as shown in the chromatogram corresponding to antibacterial property (Figure 8.1).

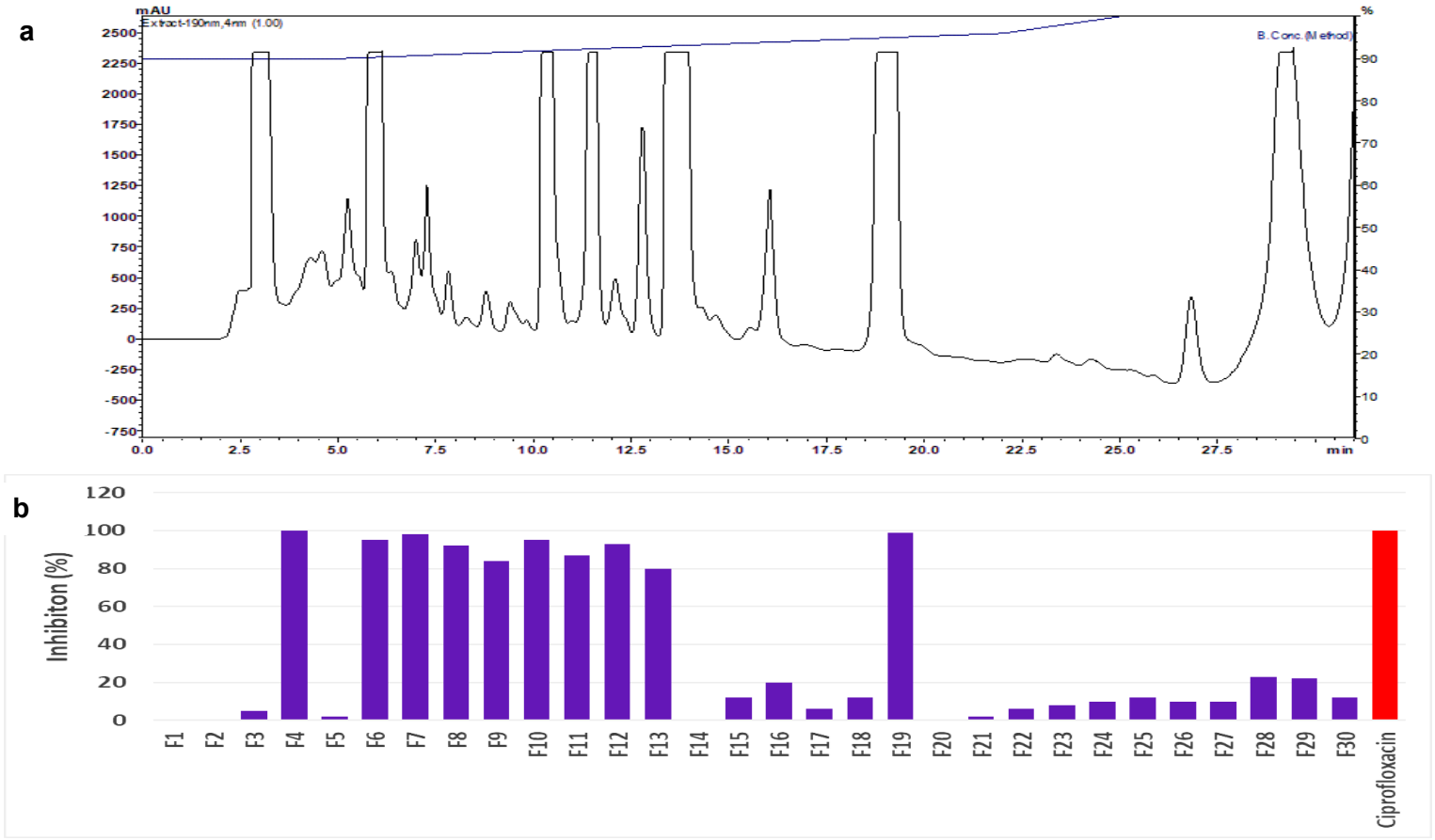

Figure 8. 1 *(a) HPLC chromatogram of 30 collected acetonitrile subfractions (F1 - F 30) of C-18 column; sub-fractions were collected every minute, and 12 resolved peaks were tested for bioactivity, (b) portions of the resolved peaks showing bioactivity against S. aureus. Of the 30 sub-fractions, 12 sub-fractions (renamed peak 1-12) that showed bacterial inhibition activity above 30 % were resuspended in hexane for fatty acid analyses*

The profile reveals the absence of short-middle chain fatty acids; from decanoic acid (C10:0) to myristic acid (C14:0) and palmitic acid (C16:0), these saturated fatty acids do not contribute to the bioactivity of hexane-extracted oils of *R. differens*. Conversely, a greater proportion of the long-chain fatty acids stearic acid (C18:0) to eicosapentaenoic acid, EPA (C20:5), is observed. Apart from peaks 6 and 11, all others contained varying amounts of all the fatty acids examined.

8.3.3 GC-MS fatty acid profiles of active peaks

The C-18 column nicely separated different fatty acids as sown in the peaks; however, due to leaching, small amounts of other fatty acids were found in the peaks. From the 30 collected fractions, twelve purified active peaks (assumed to be pure fractions and renamed peaks 1- 12) were resuspended in hexane, derivatized, and subjected to GC-MS. Table 8.4 reveals the GC-MS fatty acid profiles of these extracted peaks post fractionation, separation, and derivatization into their corresponding fatty acid methyl esters. Overall the essential fatty acid, *i.e.,* alpha-linolenic acid, ALA, (C18:3), was the most predominant fatty acid in most peaks. This was closely followed by heptadecanoic (C17:0) and oleic acids (C18:1). Other essential fatty acids, linoleic and arachidonic acids, were dominant among the fractions (Table 8.4).

A closer look into the specific profiles of each peak reveals a similar trend in the pattern, albeit in varying amounts of individual fatty acids. In terms of abundance, peaks 8 and 10 contained the highest concentrations (percent total fatty acids), while the least concentrations were observed in peaks 7 and 4 in that order. Except for peak 9, from peaks 8 to 12, the fatty acid eicosapentaneoic acid was not detected. No fatty acids were equally detected in peaks 6 and 11, as their concentrations were below the detection limit. All the short-chain fatty acids C10:0 to C14:0 were not detected in any purified subfraction peak. This can be seen when a comparison is made with the hexane fatty acid profile that was not subjected to testing, fractionation, and purification. As expected, the medium and long-chain fatty acids present in both profiles were higher in the unpurified extracts. The dominant fatty acids prior to separation and purification were linoleic (44 %) and palmitic acid (27.8 %). Compared to a few peaks with long-chain fatty acids (C20:4 and C20:5), the percentages were much lower in the untested hexane extracts.

Table 8. 4 *GC-MS Fatty acids (% fatty acid) profiles of hexane resuspended subfractions (peaks) from resolved fractions that showed bioactivity against* *S. aureus (n=3, ± standard error).*

Peaks 6 and 11 were left out as no fatty acids were detected. Values with zero (0.00) indicate concentrations below the detection limit

Fatty acid (% of fatty acids)	peak 1	peak 2	Peak 3	Peak 4	Peak 5	Peak 7	peak 8	Peak 9	Peak 10	peak 12	Profile before separation (Ch. 2)
decanoic acid(C10:0)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.07 ± 0.01
lauric acid(C12:0)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.17 ± 0.01
myristic acid(c14:0)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.10 ± 0.02
pentadecanoic acid (C15:0)	4.71 ± 0.89	5.97 ± 0.31	5.20 ± 0.39	1.11 ± 0.36	2.16 ± 1.52	1.42 ± 0.82	6.16 ± 0.07	5.54 ± 0.40	6.10 ± 0.20	6.12 ± 0.39	0.07 ± 0.01
palmitic acid(C16:0)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.12 ± 0.01
palmitoleic acid(C16:1)	1.79 ± 0.28	1.77 ± 0.03	1.53 ± 0.10	0.33 ± 0.13	0.60 ± 0.47	0.40 ± 0.23	1.73 ± 0.01	1.54 ± 0.10	1.73 ± 0.08	1.73 ± 0.05	27.8 ± 0.62
heptadecanoic acid(C17:0)	13.34 ± 2.45	16.89 ± 0.87	14.71 ± 1.12	3.09 ± 0.94	6.09 ± 4.31	4.02 ± 2.32	17.51 ± 0.19	16.90 ± 1.14	17.29 ± 0.62	17.51 ± 1.12	1.63 ± 0.01
stearic acid(C18:0)	9.80 ± 0.82	12.39 ± 1.41	12.64 ± 0.77	1.38 ± 0.03	4.65 ± 4.01	3.59 ± 2.07	12.76 ± 0.12	11.42 ± 0.87	12.63 ± 0.17	12.76 ± 0.71	0.15 ± 0.01
oleic acid(C18:1)	14.44 ± 6.65	17.59 ± 1.39	13.48 ± 0.77	6.42 ± 4.14	8.62 ± 2.50	4.39 ± 2.53	14.39 ± 0.63	12.19 ± 0.90	14.14 ± 1.32	14.39 ± 2.99	8.45 ± 0.21
linoleic acid(C18:2)	13.17 ± 0.80	14.77 ± 0.91	11.80 ± 0.80	1.39 ± 0.00	4.10 ± 3.85	3.21 ± 1.85	13.38 ± 0.17	12.79 ± 0.89	13.38 ± 0.40	13.38 ± 0.288	44.0 ± 0.42
linolenic acid(C18:3)	15.67 ± 2.78	17.53 ± 1.44	17.32 ± 1.31	3.54 ± 1.04	7.32 ± 5.01	4.90 ± 2.83	20.52 ± 0.20	19.71 ± 1.32	20.26 ± 0.68	20.52 ± 1.64	14.1 ± 0.32
arachidonic(C20:4)	10.42± 1.85	13.10 ± 0.67	11.40 ± 0.86	2.35 ± 0.70	4.69 ± 3.36	3.11 ± 1.79	13.54 ± 0.14	13.04 ± 0.88	13.38 ± 0.46	13.54 ± 0.84	1.45 ± 0.03
eicosapentaenoic acid (C20:5)	4.77 ± 4.77	0.00 ± 0.00	17.91 ± 0.19	4.30 ± 3.24	7.84 ± 5.29	4.49 ± 2.59	0.00 ± 0.00	6.45 ± 0.45	0.00 ± 0.00	0.00 ± 0.00	0.07 ± 0.01

8.4 Discussion

One of the main reasons consumers like these bush crickets is because they taste delicious, and it is well known that fats can trap flavor and aroma compounds, making them very palatable (Savell & Cross, 1988; Premjit et al., 2021) . Despite receiving tremendous attention in its role in complementing meals as a snack or part of a whole meal and the large volume of nutritional studies, the same cannot be said regarding research on nutraceutical and pharmacological potentials. This study is a pioneering one that aimed to explore on the one hand and attempt to evaluate the antimicrobial activity of extracts from the bush cricket *R. differens* found in East Africa. In the literature, results of proximate analyses have shown that the fat content of *R. differens* bush crickets ranks very high among edible insects (Rumpold & Schlüter, 2013; Kinyuru et al., 2010; Siulapwa et al., 2012; Lehtovaara et al., 2017; Fombong et al., 2017). Therefore, it could be expected that the hexane extract would yield copious amounts of oils. Hexane has long been used as a defatting solvent for different food processes, especially during upstream operations in extracting proteins. These oils are liquid at room temperature but solidify quickly at temperatures between 10 and 15 °C (Kinyuru et al., 2010).

Previous studies have demonstrated that the most predominant fatty acids present in *R. differens* are oleic (44 %), palmitic (28 %), and linoleic (14 %) acids making up more than 85 % of the total fatty acids (Kinyuru et al., 2010; Fombong et al., 2017; Rutaro et al., 2018). In comparing the fatty acid profiles in the literature with those of this current study, linoleic, oleic, and heptadecanoic were the most abundant. Probably, oleic and linoleic acid, which showed the same elevated trend (together with possibly other undetected compounds), may have contributed the most to the anti-staphylococcal properties observed. Our results are corroborated by work from other researchers (Saiki et al., 2021; Kim et al., 2015), who showed the presence of antimicrobial activities in methanol and ethanol extracts containing similar fatty acids from the Asian grasshopper *Oxya spp*. The latter insect is not a popular food insect in Africa, though.

The present study isolated eight fatty acids in the hexane-acetonitrile extract, which was principally responsible for the antibacterial effects observed. Thus, it is possible that (some of)

these fatty acids, *i.e.*, pentadecanoic, heptadecanoic, stearic, oleic, linoleic, a-linolenic, arachidonic, and eicosapentaenoic acid, may possess anti-staphylococcal properties. By contrast, palmitic acid, which was not detected, had no anti-staphylococcal effect when tested at a concentration of 0.01 %. Undocumented cultural use of *R. differens* in Siaya county, western Kenya, indicated that *R. differens* oil is utilized in droplets against eye infections. Lipids originating from animals have in the past been demonstrated to possess natural antimicrobial properties against a broad range of bacteria and fungi due to their free fatty acids. For example, caprylic acid (C8:0) present in breast milk and bovine milk can inhibit *Cronobacter sakazakii* (Juneja, Dwivedi, and Yan, 2012; Davidson, Critzer, and Taylor, 2013). In other studies, Singh et al., (2014) have used the oils from insects to treat skin diseases caused by *Mycobacterium tuberculosis.* Also, the oil obtained from the larvae of the May Beetle, *Melolontha vulgaris,* was applied topically to wounds and used as a cure for rheumatism. Elsewhere (Shrivastava et al., 2018), it has been shown that insect-derived unsaturated fatty acids can protect the skin and help decrease blood clots. This could likely explain why the oils of the insects mentioned above are applied topically.

The essential fatty acids Linoleic acid (LA) and α-linolenic acid (ALA) have long been known to have potent antimicrobial properties (Desbois and Smith, 2010). Linoleic acid, an omega-6 fatty acid, and α-linolenic acid, an omega-3 fatty acid, are considered essential since humans cannot synthesize them. The long-chain omega-3 fatty acids, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) can be produced from ALA. As omega-6 and omega-3 fatty acids are essential structural components of cell membranes, they act as precursors to bioactive lipid mediators and provide a source of energy. Long-chain omega-3 polyunsaturated fatty acids (PUFA), in particular, exert anti-inflammatory effects (Jeliazkov et al., 2016). It can, therefore, be suggested that the synergistic action of essential polyunsaturated fatty acids in the hexane extract of wild *R. differens* species possibly may have been responsible for the observed bioactivity against *S. aureus.* One of the biggest challenges to *R. differens* consumption is their short shelf life of fewer than 24 h post-harvesting. Unprocessed *R. differens* is subject to hydrolytic rancidity resulting in the formation of free fatty acids (FFAs) (Ssepuuya et al., 2016). Some FFAs possess the capacity to kill or inhibit the growth of bacteria to varying degrees

(Desbois & Smith, 2010). Though not fully understood, their most probable mode of action against Gram-negative bacteria is by targeting their actions on cell membranes. These FFA's bind to lipopolysaccharides and essential proteins of the outer membrane, thus disrupting the outer membrane synthesis of Gram-negative bacteria. It is believed this disrupts the electron transport chain and oxidative phosphorylation, interferes with cellular energy production, inhibits enzyme activity, disrupts the uptake of nutrients, or even directly lyse bacterial cells (Desbois & Smith 2010). For Gram-positive bacteria, such as *S. aureus*, cell wall biosynthesis is, however, inhibited due to the binding of AMPs to lipid II, a cell wall synthesis precursor molecule. These antimicrobials also indirectly target the cell wall by triggering autolysis of bacterial cells, that cleaves the peptidoglycan layer, resulting in the bacterial cell's destruction (Desbois & Smith 2010; Omardien et al., 2016). The antifungal activity of fatty acids occurs when they insert themselves into the lipid bi-layer of the fungal membranes and physically disturb their membrane, leading to increased fluidity of the membrane, conformational changes in membrane proteins and eventually cell disintegration. They may also inhibit β-oxidation, triacylglycerol synthesis and sphingolipid synthesis and they may inhibit topoisomerase activity (Pohl et al., 2011; Pinto et al., 2017).

In general, insects produce a large variety of antimicrobial substances in the form of lipids, diphenols, hydrocarbons as well as their chitin exoskeleton. These antimicrobial substances serve as their first line of defense against infections and as part of their innate immunity (Dossey, 2010). Plant-eating insects can sequester toxic plants as a defense mechanism to become unpalatable to their predators. While toxic to their predators, these compounds could exert antimicrobial properties when extracted in their crude forms. In the order of Orthoptera, to which *R. differens* belongs, some species are capable of sequestering: (i) aromatic compounds such as in the acridid *Romalea guttata* which sequestered 2,5-diphenol, (ii) pyrrolizidine alkaloids, for example, in the grasshopper *Zonocerus variegatus,* (iii) sulfur-containing quinones as in *Romalea guttata,* and (iv) cardiac glycosides as sequestered by the grasshopper *Poekilocerus bufonuis* from its host plants (Opitz, Muller, & Müller 2009). No reports on compounds sequestered by *R. differens* can be found in the literature, but this does not entirely exclude their existence.

Our study presents insights into the potential use of insects as a functional food and as a natural source of novel bioactive agents that could be explored further. One major limitation of our exploratory study is that there was no complete identification of the non-fatty acid components in the hexane extract, especially in subfraction peaks 6 and 11, which yielded no fatty acids but showed antibacterial properties. We only tested the major peaks, as minor peaks were not tested, and these could contain other no-fatty acid metabolites responsible for combined bioactivity.

Future work should consider a more detailed chemical investigation to isolate and characterize compounds that contributed to the observed bioactivities.

8.5 Conclusions

R. differens is an oil-rich katydid whose crude fat fraction, when esterified, contains fatty acids. These fatty acids occur in concentrations that can easily be extracted and isolated by the guided bioassay techniques already described. The hexane extracts (post acetonitrile fractionation) of *R. differens* showed activity against *Staphylococcus aureus,* possibly due to a synergistic action of a combination of these fatty acids. Given that this was an exploratory study, it is theorized that *R. differens* hexane extracts show a broad-spectrum antibacterial activity for the investigated bacteria. Perhaps this might be the reason why *R. differens* is not only used as food but also as a medicinal treatment in African traditional culture(s). This study can also serve as a snippet view into a vast yet unexploited potential for new drug discovery from extracts of different edible insects consumed in Africa.

CHAPTER NINE

General Discussion and Recommendations for Future Research

9.1 General Conclusions

This study set out to explore the influence of processing on nutritional, techno-functional, and antimicrobial properties regarding the rearing possibilities of the edible insect *R. differens*. The research investigated the extent to which freeze-drying and oven drying affected nutritional and antinutritional parameters, as well as functionalities of the insect's flour.

Ruspolia differens is by far the most consumed non-farmed insect across Sub-Saharan Africa (Kinyuru et al., 2010; Kelemu et al., 2015; Fombong et al., 2017; Ssepuuya et al., 2019; Fombong et al., 2021). In chapter one, a literature review affirmed its position as one of the most nutritious and highly fatty insect foods, packed with impressive quantities of essential amino acids, minerals, and vitamins (Ssepuuya et al., 2016; Fombong et al., 2017; Rutaro et al., 2018; Opoke et al., 2019). It is, therefore, no surprise that this insect continues to be consumed as a delicacy due to its oily and tasty matrix.

Its impressive swarming phenomenon allows for a large quantity to be harvested, leaving locals with tons of fresh raw products (Kinyuru et al., 2010; Ssepuuya et al., 2016; Mmari et al., 2017; Sengendo et al., 2021). Nevertheless, if immediate and sufficient post-harvest processing techniques are not applied, massive deterioration is suffered (Kinyuru et al., 2010; Ssepuuya et al., 2016; Ng'ang'a et al., 2018). Amongst the several processing techniques that can be applied, drying them appeared to be the most convenient and affordable, especially sun drying. Given its limitations (low, variable temperatures and its susceptibility to seasonality), however, sun drying needs to be substituted with other conventional drying methods. We have shown the standard drying methods (oven and freeze-drying) for *R. differens* processing in chapter two. These two drying techniques have remained the mainstay of this thesis when making comparisons of *R. differens* to crickets and locusts for their (anti)nutritional and techno-functional attributes.

As observed in chapter one, several researchers took an interest in the same insect and studied various aspects of its nutrition, rearing, safety, and much more. The potential for overlap with previous research interests shaped the outcome of this doctoral study to create a delicate balance between novelty and avoiding repetition. As a result, mention was constantly made to compare the findings of *R. differens* to that of three other orthopterans (*Schistocerca gregaria, Locusta migratoria,* and *Gryllus bimaculatus*). Besides their availability, in the rearing labs, these three insects were chosen because of their ease of rearing as opposed to *R. differens*. Furthermore, on the one hand, the pest status of the locusts was a reason to work with an insect that is a menace to food security in Africa. On the other hand, crickets are underway to improve food security as they are currently the most farmed insects for food and feed in the Sub-Saharan region.

Additionally, because of the seasonality and challenges to mass-rear *R. differens*, comparison with the above orthopterans seemed an attractive and reasonable strategy. However, in dealing with these insects and conducting research across two continents, carrying samples in unprocessed forms across international borders proved an uphill task. Therefore, there was the need to fall back to insects of the same species, albeit reared under slightly different conditions.

9.1.1 Objective 1: To determine the effect of the drying method on the postharvest nutritional composition of *R. differens*

R. differens were wild-harvested in Uganda and Kenya. Based on cuticular coloration, three morphs, *i.e.*, green, brown, and purple, were identified. The three morphs proved to be highly nutritious, with high values of protein and fat. Balanced levels of fatty acids, essential amino acids, and trace mineral elements such as iron zinc manganese may combat micronutrient deficiencies in human diets.

In chapter two, we demonstrated that oven drying blanched *R. differens* morphs delivers the same nutritional (proximate, mineral, and fatty acid) quality and composition as freeze-drying.

Both drying approaches provided *R. differens* with good sources of macronutrients and minerals and are therefore deemed suitable as alternative or complementary food sources to relieve undernutrition, especially among vulnerable groups (*e.g.*, elderly and children) in developing countries.

Computations for the cost estimations for both applied drying methods were beyond the scope of this study. Most labs and small-scale farmers in East Africa cannot secure the substantial capital investments to set up freeze dryers. Thus, the sheer massive amounts required to purchase a freeze-dryer in comparison to an oven-dryer is a considerable obstacle that makes the sustainable use of freeze-drying in a low-income country unlikely. This dissertation recommends using more affordable oven-drying, which would be faster and safer than sun drying, especially when using these insects for food formulations as end products. Oven-drying, though more affordable than freeze-drying, is able to deliver dried insects with comparable nutritional quality.

Future Research

Although sun drying is 'free' and the most used drying method for many *R. differens* harvesters, given its limitations of inconsistency and time-consuming nature, there was a need to investigate other conventional drying methods.

A more sustainable and cost-effective approach would be a kind of integrated drying system, where insect samples are first partially sun-dried and then drying is completed using an oven dryer. This approach would attract some level of critique as sun drying cannot be controlled.

Regarding the quantified nutrients, particularly vitamins and minerals, their bioavailability should be assessed. Further studies of the true ileal protein digestibility during transit through the dynamic *in vitro* gastrointestinal model should be conducted. Also, a correct evaluation of the nitrogen digestibility expressed as a percentage of the total nitrogen intake, including non-protein nitrogen, would provide insights into how bio-accessible these insect proteins are. In adherence to the recommendations by FAO, the protein quality should be determined through the Digestible Indispensable Amino Acid Score (DIAAS), using the child, adolescent, and adult group (3–10 years) as the reference value. The DIAAS value will allow meaningful comparisons of how well these insect proteins fare compared to other highly digestible ones like casein.

After several rearing generations of *Ruspolia differens* at different institutions across different countries (KU Leuven, Belgium; Makerere University, Uganda; JKUAT and JOOUST, Kenya), researchers have failed to come close to replicating neither the occurrence nor relative

abundance of the rare purple morph in their colonies.

As such, phylogenetic and molecular level studies that could explain at molecular and gene-level the occurrences of the several color morphs would be worthwhile. Considering that the purple morphs are rare and fetch a higher market value in Tanzania, ruling out environmental factors responsible for the cuticular coloration would already throw light into this grey area. On the flip side, studies that manipulate the environment with resultant color changes could also disprove emphasis on a genetic predisposition for *R. differens* cuticle color.

9.1.2 Objective 2: To compare the nutritional quality of *R. differens* to that of other edible orthopteran insects

In chapter three, it was revealed that in addition to *R. differens*, *Gryllus bimaculatus* (GB), *Locusta migratoria* (LM), and *Schistocerca gregaria* (SG) are three possible orthopteran alternatives to conventional edible insects consumed in East Africa. In general, the nutritional profile of the three orthopterans compared very well with that of *R. differens*.

One key advantage of these three over *R. differens* is that they are easy to rear. The research question sought to understand if these three orthopterans are as nutritious as the already consumed *R. differens* in East Africa. Results indicated that these insects (GB, LM, and SG), on dry matter, have 40 – 50 % higher protein content than *R. differens,* exceeding the protein content of most consumed foods. On the other hand, *R. differens* had significantly higher fat content than these alternative orthopterans. Oleic acid and palmitic acid were the two most abundant fatty acids.

In contrast, the presence of arachidonic acid and docosahexaenoic acid in SG and LM suggests that these insects are a source of polyunsaturated fatty acids. All essential amino acids were present, with glutamine, alanine, and leucine being the most abundant. Trace mineral elements and vitamins of public health concern in East Africa, such as iron, zinc, copper, and vitamin $B_{12,}$ were present. The studies here aligned well with previous studies for similar and related orthopteran species.

Locust swarms were predominant in the previous years (2019-2020), and one could argue that capturing these pests and processing them using simple, affordable means would provide a much-needed protein source both for humans and livestock.

In areas where spraying insecticides is not possible or affordable, the design and construction of rudimentary traps similar to those of *Nsenene* could be employed to harvest tons of these devastating armies. How practical this can be is arguable, but lessons can be drawn from Mexico and how they harvest *chapuline* grasshoppers that otherwise are crop pests.

Adding these orthopteran alternatives to human diets could mitigate macro-and micronutrient deficiencies. However, the presence of pesticide residues should be continually and carefully monitored since locust swarms can migrate over very long distances.

Future Research

The amount of macro and micronutrients in *R. differens* and the other three orthopterans are known from research. However, concentrations of specific elements such as fat-soluble vitamins, some water-soluble vitamins, antioxidant compounds (phenolic compounds, peptides, carotenoids, vitamins A and E), and cholesterol levels were not determined. In addition to determining these, the protein quality and digestibility of these orthopterans should be determined using the Digestible Indispensable Amino Acid Score (DIAAS).

This study did not consider the effect of diet or fasting prior to investigating and comparing their nutritional qualities. Cognizant that these insects in their natural habitats have different feeding habits, our attempts to rear these insects on a single artificial diet are still ongoing. Proposals that could enhance the development of one 'super' diet meal-*OrthoptoMeal*, that could be used to rear all three or even other acridid and gryllid insect species, are encouraged. The effect fasting of emptying the guts of these insects on their nutritional qualities would be an exciting outcome.

The possibility to design affordable and straightforward mass-harvesting equipment would seem a worthy investment to depopulate the swarms quickly. Therefore, there is a need for research into innovative yet indigenous tools that can sufficiently quench these swarming storms. However, care must be taken to avoid consuming insecticide-infected insects.

9.1.3 Objective 3: To evaluate the influence of drying on some antinutrients of R differens and other edible orthopteran insects

In the fourth chapter, the influence of drying methods (oven and freeze-drying) and the defatting process on antinutrient content (phytates, tannins, and oxalates) are associated with *Schistocerca gregaria*, *Gryllus bimaculatus,* and *Ruspolia differens* are presented. Oven drying resulted in higher phytate content than freeze-drying in whole insect flour and defatted insect flour obtained from *R. differens*. On the other hand, drying methods and defatting did not affect tannin content in individual insect species. Similarly, drying methods and defatting did not affect the oxalate content of whole insect flour, and defatted insect flour of individual insect species and all levels measured were less than 0.04 mg/kg. In general, insects contained low antinutrients, with values much lower than corresponding ones from some common foods of plant origin. Our results broadly concur with those of other researchers who consistently found low to undetectable levels of antinutrients in insects. Consequently, the use of these insects as food and food ingredients can be encouraged without fear of toxicity emanating from antinutrients associated with them. The study has highlighted the antinutrient content in dried and defatted insect flours only. This was due to constraints encountered with the storage of fresh samples. The antinutrient content on the raw, unprocessed forms was not performed. Future work that could include these other forms is required.

The fact that antinutrient contents in both the whole and defatted powders were very low and comparable could be attributed to their diets. These insects were reared on chicken diets that are low in antinutrients due to the heat treatments applied, and the addition of degrading enzymes (for instance, phytases that break down phytates) in some poultry feeds (Erdaw & Beyene, 2018).

Future Research

As already highlighted, studies that can finally put to rest the debate between either calling these compounds antinutrients or bioactive compounds (with positive human effects) are considered a priority. These studies will benefit not just insects used as food and feed but generally for foods as a whole.

Further studies on orthopteran content of other potential antinutrients such as heat-resistant thiaminase, protein inhibitors, and antivitamin factors (which could be a potential risk to insect consumers) are recommended. An assessment of antinutrient content on the raw, unprocessed forms is advised. Alongside the insect samples, the diets the insects fed should equally be analyzed in the same study.

Additional studies on the influence of diet (predominantly plant-based) on the insects' antinutrient contents should be evaluated. Such studies would enable a comparison to be made between reared and wild-captured insects in terms of their antinutrient contents. Other bioactive compounds such as anthocyanins and other phenolic compounds occasionally categorized as antinutrients should also be examined. A thorough study that will investigate processing steps that could eliminate elevated antinutrients without destroying valuable nutrients is advised. Currently, it is not known if the presence of antinutrients in these insects is just a result of feeding (presence in the gut) or of an accumulation over a longer term. Therefore, more work that compares insect batches that are fasted with those with full guts should be investigated.

9.1.4 Objective 4: To evaluate the effect of photoperiod as a rearing parameter on the biological fitness of lab-reared *R. differens*.

This objective was dealt with in chapter five, which sought to establish the optimal light rearing conditions of *R. differens*. Two light regimes, 'dark' (23 h dark and 1 h light) and 'light' (12 h light and 12 h dark), were applied to rear newly hatched nymphs until the death of the adult *R. differens* were assessed. Artificially formulated diet principally composed of wheat bran, fishmeal, cornmeal, sugar, bone meal, and soy flour was fed to *R. differens*. Our results revealed that the highest nymphal survival rate for adults was recorded in the dark-reared insects. The mean weight of adult *R. differens* was significantly higher in the dark-reared insects, which occurred in a shorter developmental time. The study also demonstrated that adult longevity in the dark-reared insects was longer (37 ± 3.7 days) than in light-reared ones.

Our findings are consistent with other research, which confirmed that *R differens* fared better on a mixed diversified diet and could be fed artificial diets. An immediate benefit and implication of these findings would be providing a window of opportunity into insect feed formulators. These 'insectipreneurs' could use these diets as a base to improve, optimize and commercialize a marketable version not only for *R. differens* but for related insect species.

The study also provided sufficient evidence supporting the nocturnal behavior of *R. differens* as reported in the literature. It suggests that feeding should be done mostly at night and that rearing them in dark rooms for mass production would be more cost-effective due to energy savings from less lighting.

The use of chicken feed as a base ingredient for insects' artificial diets can be contentious given the ever-soaring prices of its soy and maize components. Nevertheless, the argument presented here is that insects have a better feed efficiency ratio than poultry, yielding more proteins per kg of the edible portion (Van Huis, 2013). This higher protein yield outweighs the initial feed costs given that protein in feed accounts for up to 80 % of the total feed cost, and feed, in turn, accounts for 60-70 % of production costs (Okello et al., 2021). Hence, local farmers engaged in poultry farming that uses a similar base diet for their chicken feeds could purchase this alongside their livestock feed. Lastly, testing with diets that have already proven successful in other insects is a quick step in gradually replacing the more expensive components (soy,

fishmeal, corn, sugar) with cheaper ones such as kitchen, brewery, and agricultural waste. Additional experiments with these different waste streams using this novel artificial diet as a control diet have been carried out with different levels of success. These experiments are still ongoing, and, as such, the results are not included in this thesis.

The inability to continue to several generations due to unsuitable egg-laying substrates led to decreasing progeny, and most notably, cannibalism among mated pairs eventually caused a colony collapse.

Feed conversion rates (FCRs) in this chapter were remarkably high, a significant concern for having a novel artificial diet. These high FCRs for this insect were likely due to the depositing of frass, which at the nymphal stages were hard to distinguish from the grains of the artificial diet.

Future Research

Adjustments to the current experimental set-up in which a separate rearing box with food only to measure the changes in moisture content of the diet due to exposure to air would improve the interpretation of FCR results.

If successful rearing over several generations is achieved, it would be interesting to see the long-term impact of using less light and artificial diets to improve biological fitness parameters.

Research on diets that could appeal nutritionally to all three other orthopterans investigated throughout this dissertation would be ideal. Additionally, the impact of processing on the quality, nutritional changes, and its effect on the growth and development of insects would be valuable.

In formulating artificial diets for insects, proper care and attention should be provided to suitable egg-laying substrates. Some of their natural diets also serve as egg-laying sites.

Studies that will also analyze the antinutrients or deterrents present in foods meant for insects would add more knowledge to this field.

The use of waste, especially agro and household organic waste, to formulate diets would not only provide a more sustainable and circular economy but also cut costs on insect feeds, which are already proving to be a barrier to upscaling in this sector.

9.1.5 Objective 5: To evaluate the extent of cannibalism in lab-reared adult *R. differens* and propose mitigating strategies

Cannibalism in animals is a widespread feeding approach, particularly among bush crickets and locusts, leading to high mortality. For *R. differens*, attempts to mass-rear them have proven an uphill task due to their cannibalistic behaviors. To curb cannibalism, reared *R. differens* were provided alternative insect prey (*Hermetia illucens*, *Chilo partellus*, *Bactrocerca invadens,* and *Schistocerca gregaria*) in no-choice and choice set-ups. Cannibalism was reduced in both male and female *R. differens* cages where prey had been administered compared to the control (artificially formulated diet used in the previous objective). It was also observed that *R. differens* tended to feed more on living than on dead insect prey, a hunting behavior by these predators. The finding also suggests that harvesting *R. differens* for human consumption before or after adult emergence could circumvent cannibalism. These findings were in harmony with that of other researchers regarding the extent of cannibalism in crowded spaces despite sufficient food, as well as the choice of biting off the thorax region of their victims.

Our attempts to pinpoint a mineral element that could be key in cannibalism behavior did not yield convincing results. It, however, could be hypothesized that besides minerals and proteins, as is the case for the Mormon cricket (Simpson et al., 2006), specific essential amino acids could be what they are after. If this is the case, it would be interesting to look into known essential amino acids in the metazoans. Genomic studies have revealed a conserved functional role of enzymes involved in de novo biosynthesis pathways of essential amino acids. (Costa et al., 2015). Therefore, assays using different test diets with varying amounts of these essential amino acids reported for other animals could play a key role in understanding the presumed amino acid deficiency triggering cannibalism in *R. differens.*

Being the first study that tests mitigating cannibalism in lab colonies using other insect prey, precedence is therefore set to seek out and formulate diets based on the most preferred eaten prey. This study could also point towards finding out what nutritional constituents in conspecifics or other insects *R. differens* seek to obtain.

We infer that the appropriate live prey could be introduced into *R. differens* rearing cages to reduce the prevalence of cannibalism. However, given that *H. illucens* - black soldier fly (BSF)

larvae were the most preferred, it would be tricky to see farmers feeding *R. differens* these BSF larvae, given their already ostentatious value as an animal feed. Nevertheless, in the last years, locust swarms in the horn of Africa could serve as an excellent playground to test the efficacy of cannibalism reduction while limiting the spread of this locust plague. A dual purpose of preventing colony collapse due to cannibalism and providing a safe means to eliminate insect pests that could serve as prey is ensured. The practicalities of how this can be effectively carried out are beyond the scope of this dissertation.

Future Research

The possible roles played by mineral salts, fatty acids, and amino acids in cannibalism urgently require further research if *R. differens* is ever to be mass-produced for commercial purposes. The impact of cannibalism not only in adults but among all stages of this insect should be evaluated. As such, the influence of molting on cannibalism can be made more evident.

Besides nutrient content, cannibalism in insects could also be impacted by several other aspects. Other factors such as the influence of seasonality, color morph, and biological rearing parameters (temperature, humidity, photoperiod), need to be investigated. Filling such knowledge gaps will buttress the work initiated in this thesis and provide more concrete answers to any *R. differens* cannibalization. This would pave the way to designing cost-effective and scalable means to curb this devastating phenomenon in farmed colonies.

The effect of space and shelter on the extent of cannibalism would be a relevant addition to this field. This will help future farmers allocate sufficient space in their colonies to prevent collapse due to cannibalism. More studies investigating the impact of adding wild-harvested insects to the rearing stock would be beneficial. Behavioral assays that would correctly map the exact physical actions before, during, and after an attack of a victim or prey would inform researchers on the mechanics of cannibalism in this insect.

9.1.6 Objective 6: To evaluate the influence of drying on techno-functional attributes of R differens and other edible orthopteran insects

Although edible insects exhibit excellent nutritional qualities and protein digestibility, consumers are often bothered by their appearance. However, consumer acceptance can be improved through processing into flours for use as high-quality food ingredients. Thus, chapter six investigated the influence of oven and freeze-drying methods and defatting on the techno-functional properties (water-holding, foaming capacity and stability, and fat absorption capacity) of *Ruspolia differens*, *Gryllus bimaculatus,* and *Schistocerca gregaria*. The study revealed some significant differences in the influence of the drying method on the techno-functional properties of the whole insect flours investigated. However, there was variation for most of the techno-functional properties in the defatted insect flour and non-defatted portions among the different insect species. Irrespective of the drying method, there was a clear distinction in the quality of non-defatted insect flours and their defatted counterparts, which directly reflects the techno-functional properties of the target insects. Thus depending on the target end product, a choice could be made between non-defatted and defatted insect flour for inclusion in diets and other food matrices to improve their functionality.

The current study could not make any case in favor or against defatting to improve some functional protein properties used in food applications. However, given the extra cost of a defatting step, it would be crucial for the industry to carefully count the cost if the sole purpose is to improve protein functionality. In any case, the gains obtained from an additional product, the oils that can be commercialized, are worthwhile.

Future Research

An obvious addition to this study is the influence of several other techno-functional properties such as bulking, solubility, emulsion capacity, gelation, and other rheological properties. Supplementary studies using different solvents (for defatting) and different extraction methods and how they in turn influence techno-functional properties are needed.

Insect oils have been shown to confer some exciting antioxidant properties (Cheseto et al., 2020). Still, before these insects can be used for cooking or frying, proper research on the influence of decolorizing and deodorizing on the final quality of these oils should be investigated. Primary and secondary oxidation products of insect oils (fats) should be explored using more advanced techniques such as GC-MS to complement or replace outdated methods used in this thesis involving peroxide, iodine, and saponification values.

The influence of raw versus blanched insect samples, besides the drying technique, would provide more insight into the impact of both oven and freeze-drying as unit processes on the examined techno-functional properties. Studies that will distinguish the composition and impact of protein concentrate, protein isolates, and hydrolysates on techno-functional properties would complement the current study. If insects are to replace animal proteins as functional ingredients in human diets, it is crucial to evaluate insect protein digestibility, as already mentioned, to appropriately evaluate their protein quality and functional properties.

9.1.7 Objective 7: To elucidate the antibacterial properties of *R. differens*

Chapter eight's research question was born from the recurrent narrations from locals during our harvesting and field surveys, recounting how they use these insects for healing purposes. Because of the current indigenous knowledge rumors about using *R. differens* as traditional medicine for specific ailments, this chapter presents the antimicrobial properties of *R. differens* extracts against common clinical pathogens. At a concentration of 0.01 %, hexane extracts showed complete growth inhibition of *S. aureus*. This study purported that these extracts show a broad-spectrum antibacterial activity as they were active against gram-positive and gram-negative bacteria as well as fungi and perhaps underline why the bush crickets are utilized in African traditional medicine. These findings are broadly in line with several studies, especially in China and East Asia, acknowledging the use of insects and grasshoppers for healing purposes. Given the limitation of time and other resources, the study focused only on the fat contents of these bush crickets, as fats are the most dominant components after proteins. We isolated and tested active peaks using the GC-MS method already established for fatty acid methyl esters. It was, therefore, not possible to identify other fatty acids present in the hexane extract that could have contributed to the bioactivity beyond those which were esterified. Also, the methanolic boron trifluoride (BF_3) derivatization method used prior to the GC-MS trans-esterifies both free and bound fatty acids into their methyl esters. As such, making a distinction as to whether it was free or bound fatty acids yielding activity was inconclusive.

It would have been interesting to test within the same assay if commercial fatty acids identified here would exert similar bioactivity. Although reports of *in vitro* antimicrobial activity of fatty acids exist, a similar study of their synergistic actions *in vivo* (especially in insect cells and tissues) is rare.

The methods used by indigenes in communities where these insects are used as medicine are very rudimentary and crude. They consist of grinding into a paste, boiling and drinking the water extract, or frying and roasting to extract the oil (Meyer-Rochow, 2017; Mmari et al., 2017; Van Itterbeeck et al., 2019). In extreme cases, the insects are entirely incinerated, and the ash is used. Applications are usually topical or oral, thus limiting their 'healing' actions via the skin and

gastrointestinal portals. This study, therefore, advocates the need for additional research into the different methods in which the said active ingredients are extracted and their possible mode of action. This could invariably lead to novel nutraceuticals that potentially could pave the way for new drugs from different extracts of the diverse edible insects consumed in Africa.

Future Research

One avenue for further study would be to analyze the entire lipid profile and not just the fatty acids only of these insects and test each fraction for bioactivity. The aqueous fraction also tested active against the pathogenic fungus *Candida albicans* but was discontinued due to contamination. It is recommended that further attention is required to identify compounds displaying such bioactivity in the water extracts.

Additionally, the use of a combination of both sequential and non-sequential extraction procedures using different solvents should be pursued. Going a step further to calculate the minimum inhibitory concentration (MIC) value and IC_{50} value for the fractions responsible for bioactivity would be a step in the right direction. The influence of drying method, defatting level, chemical composition, developmental stage of the insects, and type of post-harvest processing on their resulting bioactivity, should be exciting themes for researchers to tackle.

The occurrence, diversity, and identification of antimicrobial peptides (AMP) were beyond the scope of this exploratory study. A few studies have revealed the medicinal potential of AMP from insects (Tonk & Vilcinskas, 2017; Wu et al., 2018; Sahoo et al., 2021; Manniello et al., 2021). Insects are the largest class of organisms in the animal kingdom, owing to their enormous biodiversity. Part of their humoral immune response to microbial infections is the release of these AMP into their hemolymph. Antimicrobial peptides have been reported in other katydids (Torres-Castillo et al., 2015). *R. differens* is already used as a wound healing ailment; it is therefore hypothesized that this wound-healing ability could be due to AMP present in its hemolymph. It is the expectation that such a fascinating area of research be picked up to complement this current exploratory study of the antimicrobial activity of its hexane and water extracts.

9.2 Implications of research findings in Kenya and East Africa

Valuable research and scientific evidence now exist in East Africa that supports the importance of edible insects as nutritious food and animal feed.

Agriculture is the cornerstone of the Kenyan rural economy, and small-scale farms of edible insect rearing are projected to be integral to a broad-based, poverty-reduction strategy. Overall, 75 % of Kenya's population derives part of their livelihoods from agriculture, including livestock and pastoral activities. At the same time, only one-fifth of the country's land is suitable for farming, and productivity remains low. Rearing insects for food, which requires far less space than traditional livestock, would reduce poverty. Implementing sustainable insect breeding projects can be leveraged to invest in gender equality and women's empowerment.

The indigenous knowledge formalized during this research would benefit the local community where the bearers of such knowledge live and work. The local partner universities would also use this as a foundation for more research, leading to more relevant and high-impact results in high-quality journals.

9.3 Final words

Substantial research has already been carried out on *R. differens.* New research grounds have continuously been broken in East Africa to unravel its biology and boost the optimization of mass rearing technologies. This dissertation has provided additional information to further this field of study. The nutritional composition of *R. differens* has been adequately documented in this dissertation to explain why this species is receiving growing attention as a promising insect to mitigate foreseen food and protein shortages. It can therefore be anticipated that the inclusion of *R. differens* products in human diets would be an excellent strategy to combat micronutrient malnutrition and significantly contribute towards the achievement of the 'zero hunger' sustainable development goal target.

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SUPPLEMENTARY DATA

S.6. SUPPLEMENTARY DATA FOR CHAPTER SIX

Supplementary Table S6.1: Feeding scheme for both male and female cages as well as mixed and control groups

Period (24h)	INSECT type	Prey status
Day 1	BSF ALIVE	ALIVE
Day 2	BSF ALIVE	ALIVE
Day 3	BSF ALIVE	ALIVE
Day 4	BSF DEAD	DEAD
Day 5	BSF DEAD	DEAD
Day 6	BSF DEAD	DEAD
	WASHOUT	
Day 7	SB-Alive	ALIVE
Day 8	SB-Alive	ALIVE
Day 9	SB-Alive	ALIVE
Day 10	SB-dead	DEAD
Day 11	SB-dead	DEAD
Day 12	SB-dead	DEAD
	Washout	
Day 13	FF Alive	ALIVE
Day 14	FF Alive	ALIVE
Day 15	FF Alive	ALIVE
Day 16	FF-dead	DEAD

Day 17	FF-dead	DEAD
Day 18	FF-dead	DEAD
	WASHOUT	
Day 19	SG-Alive	ALIVE
Day 20	SG-Alive	ALIVE
Day 21	SG-Alive	ALIVE
Day 22	SG-dead	DEAD
Day 23	SG-dead	DEAD
Day 24	SG-dead	DEAD
	WASHOUT/ CONTROL A	
Day 25	AD ONLY	n.a
Day 26	AD ONLY	n.a
Day 27	AD ONLY	n.a
Day 28	AD ONLY	n.a
Day 28	AD ONLY	n.a
Day 29	AD ONLY	n.a
	WASHOUT/ CONTROL B	
Day 30	AD+Grass/corn leaves	n.a
Day 31	AD+Grass/corn leaves	n.a
Day 32	AD+Grass/corn leaves	n.a
Day 33	AD+Grass/corn leaves	n.a
Day 34	AD+Grass/corn leaves	n.a
Day 35	AD+Grass/corn leaves	n.a
washout		
Day 36	BSF+SB+FF+SG	ALIVE
Day 37	BSF+SB+FF+SG	ALIVE
Day 38	BSF+SB+FF+SG	ALIVE
Day 39	BSF+SB+FF+SG	ALIVE
Day 40	BSF+SB+FF+SG	ALIVE

Day 41	BSF+SB+FF+SG	ALIVE
Day 42	BSF+SB+FF+SG	ALIVE
Day 43	BSF+SB+FF+SG	ALIVE
Day 44	BSF+SB+FF+SG	ALIVE
Day 45	BSF+SB+FF+SG	ALIVE
Day 46	BSF+SB+FF+SG	ALIVE
Day 47	BSF+SB+FF+SG	ALIVE
Day 48	BSF+SB+FF+SG	ALIVE
Day 49	BSF+SB+FF+SG	ALIVE