Colouring the grey areas of insect mass-production to solidify their use as feed, food and biological control agents

by

Michael Josias Woods

Dissertation presented for the degree of

Doctor of Philosophy (Agricultural Science)

at

Stellenbosch University

Animal Sciences, Faculty of AgriSciences

Supervisor: Dr Elsje Pieterse

Co-supervisor: Prof Louwrens Christiaan Hoffman

Co-supervisor: Prof Desmond Edward Conlong

Co-supervisor: Dr Neil Jurgens Goosen

December 2019

Stellenbosch University https://scholar.sun.ac.za

DECLARATION

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated) that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Date: December 2019

Copyright © 2019 Stellenbosch University
All rights reserved

Stellenbosch University https://scholar.sun.ac.za

SUMMARY

The mass-production of various insect species for biocontrol methods and as feed and food is becoming common practice and several different rearing facilities have been established across the world. Although insect mass-production facilities have been set in place, the mass-production of insects is still in an infantile stage and research is necessary to optimise these systems. As with conventional intensive livestock production the success of these systems, as well as the costs implicated, can largely be contributed to the nutrition of the animals. Trials were conducted to try and establish the nutrient requirements of selected insect species based on the body composition technique, a method commonly used for conventional livestock. Positive results were obtained for all involved insect species and led to an increase in production output, as well as to a decrease in the cost of production. For mass-produced insects to reach their full potential they need to be tailored for their end use. A study was conducted where larvae were reared on a substrate rich in polyunsaturated fatty acids in attempt to manipulate the fatty acids that they deposited into their biomass, and subsequently to evaluate the effect this had on the production and meat quality parameters of the animals the larvae had been fed to. Promising results were observed as an increase in the omega-3 fatty acids were observed in the larval biomass and the same occurred in the meat of the quails the enriched larvae were fed to. Although, the ratio of polyunsaturated- to saturated fatty acids could not be changed. Insect processing is another field that needs attention and the fractionation of insect biomass into protein, fat and chitin can result in significant increase in the margins made both environmentally and economically. An enzymatic fractionation method was developed that resulted in the isolation of protein, fat, and chitin and greater total recoveries were obtained compared to previous studies. The subsequent quantification of the chitin content of insects has also been a problem in the past and based on chitin isolation from crustaceans, a gravimetrical protocol was developed to address this issue. The resulting protocol led to the most repeatable and accurate estimation of the chitin content of insect larvae to date. Overall, the results obtained from the series of studies were a step in the right direction to solidify the mass-production of insects for biocontrol methods as well as for feed and food purposes.

Stellenbosch University https://scholar.sun.ac.za

OPSOMMING

Die grootskaalse produksie van verskillende insekspesies vir die doel van biologiese beheer en as voer en voedsel raak algemene praktyk. ‘n Verskeidenheid produksie eenhede is al reeds gevestig reg oor die wêreld. Alhoewel grootskaalse eenhede alreeds in plek gestel is, is die grootskaalse produksie van insekte nog in ‘n aanvangs fase. Navorsing word benodig sodat hierdie eenhede hul produksie potensiaal kan bereik. Soos in die geval met intensiewe produksie van vee speel voeding ‘n groot rol in die sukses sowel as die koste van die produksie van insekte. Die grootskaalse produksie van verskillende insekspesies vir die doel van biologiese beheer en as voer en voedsel raak algemene praktyk. Proewe was uitgevoer om die nutriënt behoeftes van geselekteerde insek spesies te bepaal met behulp van die heel karkas mael metode, ‘n tegniek wat algemeen in die intensiewe produksie van vee gebruik word. Goeie resultate was aan die orde van die dag vir alle betrokke insek spesies en het ‘n toename in produksie, sowel as ‘n afname in produksie koste, tot gevolg gehad. Indien grootskaalse produksie van insekte sy volle potensiaal wil bereik moet insekte geproduseer word om by die spesifikasies van hul eind gebruik te pas. ‘n Studie was gedoen waar insekte op ‘n substraat ryk aan poli-onversadige vetsure geproduseer was om die insek biomassa dieselfde eienskappe te gee. Dit blyk dat die insekte meer omega-3 vetsure kon neerlê vanaf die verrykde substraat en so ook die diere wat hierdie insekte gevreet het in hulle vleis, alhoewel die verhouding van versadigde- tot onversadigde- vetsure nie gemanupileer kon word nie. Die prosessering van insekte is ‘n veld wat aandag kort en die fraksionering van insekte in hul individuele komponente naamlik proteïen, vet en chitien kan marges verhoog, beide in terme van ekonomie en die omgewing. ‘n Ensimatiese fraksionerings metode was ontwikkel wat die gedeeltelike skeiding van die insek komponente tot gevolge gehad het en groter fraksies was herwin in vergelyking met vorige studies. Die kwantifisering van chitien, afkomstig van insekte, is ‘n problem waarmee die industrie al ‘n hele ruk ‘n stryd mee voer. ‘n Protokol was ontwikkel, afkomstig van die skulpvis industrie, waarmee chitien geïsoleer kan word en die chitien inhoud van insekete dan gravimetries bepaal word. Die ontwikkelde protokol het die akkuraatste en herhaalbaarste resulte vir die kwantifisering van insek chitien, tot op hede, tot gevolg gehad. In geheel was die resultate van die reeks proewe ‘n stap in die regte rigting vir die grootskaalse produksie van insekte.

Stellenbosch University https://scholar.sun.ac.za

ACKNOWLEDGEMENTS

Firstly, I am grateful to my Heavenly Father, to whom I owe my very existence and all I have achieved in life. Without His grace, none of these things would be possible.

I wish to express my sincere gratitude and appreciation to the following institutions for funding my research and supporting my studies:

- The South African Sugarcane Research Institute. Any opinion, finding and conclusion or recommendation expressed in this material is that of the author(s) and the South African Sugarcane Research Institute does not accept any liability in this regard.
- The South African Research Chairs Initiative of the Department of Science and Technology and the National Research Foundation of South Africa (UID: 84633). Any opinion, finding and conclusion or recommendation expressed in this material is that of the author(s) and the National Research Foundation does not accept any liability in this regard.
- The Monogastric Research Fund of the Department of Animal Sciences of Stellenbosch University. Any opinion, finding and conclusion or recommendation expressed in this material is that of the author(s) and the Monogastric Research Fund does not accept any liability in this regard.
- The Department of Process Engineering of Stellenbosch University. Any opinion, finding and conclusion or recommendation expressed in this material is that of the author(s) and the Monogastric Research Fund does not accept any liability in this regard.

I would like to thank the following people, in no particular order, for their support and assistance during the completion of my PhD:

- For her guidance and support of my project, as well as for allowing me the freedom to work independently and instilling self belief, my supervisor Dr Elsje Pieterse.
- For his insight during the writing of my thesis, my co-supervisor Prof Louw Hoffman.
- For their willingness to assist and add expertise in this multi-disciplinary reaseach field, my co-supervisors Prof Des Conlong and Dr Neill Goosen.
- For performing the statistical analysis of my data, Prof Daan Nel from the Centre for Statistical Consultation at Stellenbosch University.
- For their assistance in the laboratory and constant good cheer, Ms Beverly Ellis, Mr Michael Mlambo and Ms Janine Booyse.

Stellenbosch University https://scholar.sun.ac.za

- For their assistance with all administrative issues, Mrs Adele Smith-Carstens and Mrs Talitha Mostert.
- For the use of their facilities and provision of larvae, Agriprotein (Pty, Ltd) (Cape Town, South Africa).
- For the provision of fish offal, I&J fisheries (Pty, Ltd) (Cape Town, South Africa).
- For the use of their facilities, Rino Cailotto ("La Colombara" Società Agricola, Castelnovo di Isola Vicentina, VI, Italy)
- For the use of their facilities, Quaja Veneta® Società Cooperativa Agricola (Malo, VI, Italy).
- For the provision of feed ingredients, FANIN Srl (San Tomio di Malo, VI, Italy).
- For the provision of larvae, Proticycle (Pty, Ltd) (Worcester, South Africa).

I would also like to mention a few special people:

- Willene, thank you for being my rock through this emotional rollercoaster. Grace and I look forward to sharing our lives with you for a long time yet.
- Oom Danie, thank you for all the 7h00 coffees and banter that we could engage in.
- Last but not least to my parents, Ivan and Zelda, for encouraging and supporting me to pursue my dreams no matter the cost. For the morals that you have instilled in me. For any better I could not have asked.

Stellenbosch University https://scholar.sun.ac.za

NOTES AND PUBLICATIONS

This thesis is presented in the format prescribed by the Department of Animal Sciences, Stellenbosch University. As each chapter has either been published or prepared for publication as a journal article, and was thus written as an individual entity, some repetition between chapters was unavoidable. Language, style and referencing are in accordance with the specifications of the *Journal of Insects as Feed and Food.*

Results from this dissertation have been published in or submitted for publication in the following journals:

- Woods, M.J., Conlong, D.E., Ngomane, N., Gillespie, D.Y., Hoffman, L.C. and Pieterse, E., 2019. The development of an improved artificial diet for the mass-rearing of *Eldana saccharina* Walker (Lepidoptera: Pyralidae). Journal of the Science of Food and Agriculture (in review). (Chapter 3)
- Woods, M.J., Conlong, D.E., Hoffman, L.C. and Pieterse, E., 2019. Marching on: A novel approach to formulating breeder diets for the mass-rearing of black soldier fly, *Hermetia illucens* (L.) (Diptera: Stratiomyidae). Journal of Insects as Food and Feed (in review) (Chapter 4)
- Woods, M.J., Cullere, M., Van Emmenes, L., Vincenzi, S., Pieterse, E., Hoffman, L.C. and Zotte, A.D., 2019. *Hermetia illucens* larvae reared on different substrates in broiler quail diets: Effect on apparent digestibility, feed-choice and growth performance. Journal of Insects as Food and Feed 5(2): 89-98. (Chapter 5)
- Cullere, M., Woods, M.J., Van Emmenes, L., Pieterse, E., Hoffman L.C. and Dalle Zotte, A., 2019. *Hermetia illucens* larvae reared on different substrates in broiler quail diets: effect on physicochemical and sensory quality of the quail meat. Animals 9(8): 525. (Chapter 6)
- Woods, M.J., Goosen, N.J. Hoffman, L.C. and Pieterse, E., 2019. A simple and rapid protocol for measuring chitin content of insect larvae. Journal of Insects as Food and Feed (in press). (Chapter 7)

Stellenbosch University https://scholar.sun.ac.za

CONTENTS

DECLARATION II
SUMMARY III
OPSOMMING IV
ACKNOWLEDGEMENTS V
NOTES AND PUBLICATIONS VII
CONTENTS VIII
CHAPTER 1: 1
General introduction 1
References 2
CHAPTER 2: 4
Insect mass-production and current shortfalls: a review 4
Introduction 4
Current shortfalls 6
Determination of insect nutrient requirements for mass-production 6
Insect processing 11
Tailoring insects for specific needs 15
Conclusion and future recommendations 18
References 19
CHAPTER 3: 28
The development of an improved artificial diet for the mass-rearing of *Eldana saccharina* Walker (Lepidoptera: Pyralidae) 28
Abstract 29
Introduction 30
Materials and methods 32
Treatment diets 32
Determination of ideal amino acid profile (IAAP) 38
Determination of proximate composition 38
Larval growth trials 39
Dietary costs analysis 40
Statistical analysis 40
Results and discussion 41
Physical and chemical properties 41
Larval development 43
Diet costs 47
Recommendations for future studies 48
Conclusion 48

Stellenbosch University https://scholar.sun.ac.za

References 50
CHAPTER 4: 54
Marching on: A novel approach to formulating breeder diets for the mass-rearing of black soldier fly, *Hermetia illucens* (L.) (Diptera: Stratiomyidae) 54
Abstract 55
Introduction 56
Materials and methods 57
Treatment diets 57
Determination of ideal amino acid profile (IAAP) 59
Larvae growth trials 60
Dietary costs analysis 61
Statistical analysis 61
Results and discussion 61
Physical properties 61
Larval development 63
Diet costs 66
Conclusion 67
References 68
CHAPTER 5: 71
Hermetia illucens larvae reared on different substrates in broiler quail diets: effect on apparent digestibility, feed-choice and growth performance 71
Abstract 72
Implications 72
Introduction 73
Material and methods 74
Insect farming 74
Performance trial 74
Digestibility trial 75
Feed choice test 75
Chemical analysis of the diets and the excreta 75
Slaughtering, carcass dissection and meat yields 76
Statistical analysis of results 76
Results and Discussion 80
Conclusion 84
References 86
CHAPTER 6: 90
Hermetia illucens larvae reared on different substrates in broiler quail diets: effect on physicochemical and sensory quality of the quail meat 90
Simple Summary 91
Abstract 91

Stellenbosch University https://scholar.sun.ac.za

Introduction 92
Materials and Methods 93
Physical meat quality 94
Chemical meat quality 94
Fatty acid profile of Hermetia illucens larvae, diets and meat samples 95
Sensory analysis 96
Retail-display trial 97
Statistical analysis 98
Results 98
Discussion 106
Conclusions 110
References 112
CHAPTER 7: 118
A simple and rapid protocol for measuring chitin content of insect larvae 118
Abstract 119
Introduction 119
Materials and methods 121
Larvae material and preparation 121
Moisture and ash content (minerals) 121
Lipid Content 121
Nitrogen content 121
Determination of chitin content 122
Statistical analysis 124
Results and discussion 124
Chemical composition of the larvae 124
Extraction of chitin 124
Conclusion 126
References 127
CHAPTER 8: 130
Enzymatic fractionation of protein, fat and chitin from *Hermetia illucens* (L.) (Diptera: Stratiomyidae) 130
Abstract 131
Introduction 132
Materials and methods 134
Experimental design 134
Larvae material 135
Proximate composition 136
Determination of amino acids 136
Determination of fatty acids profile by GC-FID 136
Determination of chitin 137

Stellenbosch University https://scholar.sun.ac.za

Fractionation method 138
Determination of yields of compounds 139
Determination of the integrity of protein fraction by degree of hydrolysis 140
Statistical analysis 141
Results and discussion 142
Degree of fractionation 142
Degree of hydrolysis 146
Proximate composition 147
Amino acid composition 149
Fatty acid composition 150
Conclusion 151
References 153
CHAPTER 9: 159
General conclusion 159

Stellenbosch University https://scholar.sun.ac.za

CHAPTER 1

General introduction

Over recent times academia and government have been making the case for innovation as a way of boosting productivity as well as sustainability in the agricultural sector. One such innovative concept is the mass-production of insects for feed, food and biocontrol purposes. The mass-production of insects for these uses is an area of transformative technology that could make a step-change breakthrough in efficient use of agricultural resources.

When mass-produced on biowaste, insects are known to have a lower impact in terms of global warming potential than conventional livestock production (Smetana *et al.,* 2016). Insects have feed conversion ratios that are twice as efficient as chickens, four times more efficient than pigs and twelve times more than cattle (Smil, 2002). Compared to conventional livestock, taxonomically insects are more distant from humans and therefore risks associated with the spread of zoonotic disease are very low (Van Huis, 2013). Insects use little to no water and water can actually be gained, through the production of water vapour, when insects are reared on biowaste (Pieterse, 2018). Considering the fact that a multi-tier system can be used to mass-produce insects, the production of insects can be significantly higher per hectare than that of current crop and livestock production (Alexander *et al.,* 2017).

It is has been notoriously articulated that if insects are going to be a serious contender as an alternative, more sustainable protein source as well as a solution to the over-exploitation of chemical pesticides, extremely large numbers needs to be produced (Ortiz *et al.,* 2016). In fact, great strides have been made over recent times and some of the first commercial mass-producing insect factories have been established. As example, BioBee Sde Eliyahu Ltd. is leading the way in mass-producing beneficial insects to form part of integrated pest management programs, with branches in the United Kingdom, Israel, Russia, Colombia, Chile and South Africa (https://www.biobee.com/). On the food and feed side, Protix B.V. has recently opened the biggest insect mass-production facility based on size to date, but the total production volumes are confidential at this stage (All About Feed, 2019). As of 2017, there were 37 companies mass-producing insects globally for feed and food and of these, 25 were willing to disclose their production capacity. Combined, these 25 companies currently have the capacity to produce ~ 232 metric tonnes of insect meal per day (All About Feed, 2017).

Indeed, great inroads have been made since the first hypotheses were made that mass-produced insects can be suitable for feed, food and biocontrol, but there is still much to learn about insect mass-rearing practises, irrespective of their end use. Fields such as insect nutrition have been neglected as very little research has focused on the nutrient requirements

Stellenbosch University https://scholar.sun.ac.za

for specific mass-produced species. Conventional livestock nutrition is miles ahead of their "sixed-legged' counterparts and guidelines for nutrient specifications for intensive mass-produced insects needs to be developed (Woods, 2016). Post-harvest processing of these insects is another discipline demanding attention as insect compounds i.e. protein, fat and chitin could be fractionated and sold in separate high-end markets, further increasing their value (Melgar-Lalanne *et al.,* 2019); currently very little research has been done in this field. Also, to further solidify insects for their use in agricultural industries, they can be tailored, both genetically and phenotypically by rearing practises, to meet the needs of their end application. However, collaboration between government, industry and academia will be crucial in determining the eventual success of insect-mass production.

Therefore, the aim of this collection of studies was threefold. Firstly, to develop a method to identify the nutrient requirements of specific mass-produced insect species in an attempt to initiate the first step to develop nutrient matrixes for these insects. Secondly, to develop a sustainable fractionation method to separate protein, fat and chitin as well as to develop a rapid and repeatable protocol to analyse for chitin. Lastly, as a proof of concept model, to investigate the manipulation of the fatty acid profiles of insects and to study the subsequent effect of the tailored insects on the production parameters and meat quality attributes of the animals they were fed to.

References

Alexander, P., Brown, C., Arneth, A., Dias, C., Finnigan, J., Moran, D. and Rounsevell, M.D., 2017. Could consumption of insects, cultured meat or imitation meat reduce global agricultural land use? Global Food Security 15: 22-32.

All About Feed, 2017. A-Z of insect protein/oil companies. (https://www.allaboutfeed.net/New-Proteins/Articles/2017/12/A-Z-of-insect-proteinoil-companies-162419E/) Accessed 25 Jul 2019.

All About Feed, 2019. New Protixplant: A transition in insect farming. (https://www.allaboutfeed.net/New-Proteins/Articles/2019/6/New-Protix-plant-A-transition-in-insect-farming-437555E/?dossier=23697&widgetid=0). Accessed 25 Jul 2019.

Melgar-Lalanne, G., Hernández-Álvarez, A.J. and Salinas-Castro, A., 2019. Edible Insects Processing: Traditional and Innovative Technologies. Comprehensive Reviews in Food Science and Food Safety 18(4): 1166-1191.

Ortiz, J.C., Ruiz, A.T., Morales-Ramos, J.A., Thomas, M., Rojas, M.G., Tomberlin, J.K., Yi, L., Han, R., Giroud, L. and Jullien, R.L., 2016. Insect mass production technologies. In: Dossey, A.T., Morales-Ramos, J.A and Rojas, M.G. (eds.) Insects as Sustainable Food Ingredients. Academic Press, pp. 153-201.

Stellenbosch University https://scholar.sun.ac.za

Pieterse, E., 2018. Insect protein: from waste to feed to food. AFMA Matrix 27(4): 21-25.

Smetana, S.M., Mathys, A. and Heinz, V., 2016. Life Cycle Assessment Perspectives of Insect Proteins for Feed and Food. In: Putting LCA into Practice. 10th International Conference on Life Cycle Assessment of Food 2016: Book of Abstracts (pp. 980-987). LCA Food 2016.

Smil, V., 2002. Eating meat: evolution, patterns, and consequences. Population and development review 28(4): 599-639.

Van Huis, A., 2013. Potential of insects as food and feed in assuring food security. Annual review of entomology 58: 563-583.

Woods, M.J., 2016. The determination of nutrient requirements and development of artificial diets for the mass rearing of insects of economic importance. MSc thesis, Stellenbosch University, Stellenbosch.

Stellenbosch University https://scholar.sun.ac.za

CHAPTER 2

Insect mass-production and current shortfalls: a review

Introduction

In the past insects were mostly only reared on laboratory scale to study their biology (Singh and Ashby, 1985) and for the most part, research focused on killing pest insects due to the fact that they caused significant damages to crops (Parker, 2005), with the rare exclusion of silkworms and bees due to the commodities they produce. In recent times, the benefits of insects have drawn attention and currently emphasis has shifted to mass-producing insects for various reasons. These reason include feed and food production (Rumpold and Schlüter, 2013), as well as for biocontrol agents (Sørensen *et al.,* 2012).

The first real application of insect mass-production was in the 1950's when the screwworm (*Cochliomyia hominivorax*) was mass-produced on an artificial diet for a sterile insect technique program (SIT) against the infestation of mentioned fly species in livestock production (Krafsur *et al.,* 1987). Ever since, various other insects have been mass-produced as biocontrol agents to form part of integrated pest management programs (IPM). The mass produced insects can either be released as a predator of the identified pest insect or invasive plant (Barbosa, 1998), or can be sterilised and allowed to mate with their wild counterparts with no resulting offspring (Dyck *et al.,* 2006). These methods have been adopted to conform to sustainable agricultural practises. Despite the fact that pesticides have undoubtedly played a pivotal role in increasing agricultural production worldwide, the approach is under pressure due to high levels of pesticide resistance in many pest species, pesticide residue, environmental toxicity, fishery losses, ground and surface water contamination, depletion of rhizosphere microflora, food safety hazards and human health concerns (Shera and Arora, 2015). Due to the mentioned sustainability concerns insects as biocontrol agents have enjoyed great interest. Sterile insect technique programs have successfully been implemented against various pest species such as screwworm (*Cochliomyia hominivorax*), Mediterranean fruit fly (*Ceratitis capitate*), tsetse flies (*Glossina*), codling moth (*Cydia pomonella*), false codling moth (*Thaumatotibia leucotreta*), pink bollworm (*Pectinophora gossypiella*) and the onion fly (*Delia antiqua*) (Krafsur, 1998). The effectiveness of SIT on numerous other pest species are being researched. Host specific mass-produced insects have also been successfully used as weed biocontrol agents where chemical and mechanical control were ineffective (Harris, 1993).

More recently, mass-produced insects have gained traction as future alternative for the production of animal protein due to reasons such as their nutritional value, feed conversion efficiency, low water and space requirements, their ability to convert organic side streams into

Stellenbosch University https://scholar.sun.ac.za

high-value products, and no real significant carbon footprint (Oritz *et al.,* 2016). The phylum of insects includes the largest species variety in the world and suitable species, providing high protein and sulphur containing amino acid concentrations, have been identified which can be successfully exploited in human and animal diets (Józefiak and Engberg, 2015). In this context, insects such as the yellow meal worm (*Tenebrio molitor*), the super worm (*Zophabas morio*) the house fly (*Musca domestica*), the black soldier fly (*Hermetia illucens*), and the house cricket (*Acheta domesticus*) have enjoyed most interest (Hopley, 2016).

The constant improvement of the genetic potential of livestock species has resulted in increased nutrient density of the diets, which limits the inclusion of low quality raw materials. Accompanied by the increased demand for sustainable intensification, the feed industry is in need of novel sources of highly digestible protein with desirable amino acid profile to supplement other limited unsustainable protein sources of animal origin such as fishmeal (Józefiak and Engberg, 2015). Due to the previously mentioned attributes of insects, they are a possible solution for the highlighted problem. In their natural environment, fish and chickens at all life stages readily eat insects, which indicates that they are evolutionary adapted for the inclusion of insects into their feed (Makkar, 2018). Ascending from this statement, it has been shown that insects are suitable feed ingredients in the diets of commercially farmed fish (Henry *et al.,* 2015), poultry (Pieterse and Pretorius, 2014) swine (Driemeyer, 2016) and rabbits (Martins *et al.,* 2018). Insects can also form part of dog and cat food (Bosch *et al.,* 2014).

As the world population grows, food security becomes an even greater challenge. Hunger and malnutrition is a never-ending problem in third world countries, where the majority of the population growth is predicted to occur (Wu *et al.,* 2019). As nutritional deficiency is the root cause of numerous other diseases, it is crucial that adequate nourishment be provided (Patel *et al.,* 2019). In this regard, mass-produced insects are seen by some to be the, or at least part of the solution (Van Huis, 2015). Insects are already being consumed as food in about 120 countries around the world, and it is estimated that more than 2000 insect species are edible (Jongema, 2017). Insects as food has also attracted much Western interest in recent years due to their nutritional and environmental advantages (Tan *et al.,* 2015). If the willingness of Western consumers to include insects in their everyday diets can be heightened, the consumption of mass-produced insects can strengthen the fragile food supply.

Insect mass-production systems resemble the same principles as conventional intensive livestock production systems in terms of nutrition, breeding and management practises. Considering insects as novel six-legged livestock, the main aim of this review is to discuss some of the current shortfalls regarding the mass-production of insects so that they can fulfil their potential as feed, food and biocontrol agents.

Stellenbosch University https://scholar.sun.ac.za

Current shortfalls

Determination of insect nutrient requirements for mass-production

In the past when insect nutrition was referred to, it was commonly held that entomologists did not respond enthusiastically to this topic (Slansky, 1982). However, the understanding and implementation of insect nutrition is showing an ever growing interest. This could be contributed to the fact that this field does not just entail basic nutritional requirements, but the amount and rates of feed eaten, digested, assimilated and converted to tissue, are all important for successful mass-production. Insect nutrition is a more sophisticated field than what it was thought to be. It is seen to be more than just common dietetics and involves many metabolic processes. Research on this topic determines essential nutrients and highlights metabolic pathways, genetic mechanisms and comparative biochemistry (Lipke and Fraenkel, 1956).

The use of artificial diets for rearing insects have been developed since the 1950's in order to meet the demand for large number of insects required in the fields of physiology, ecology and genetics, and for insect control techniques such as male sterilization, pathogen production, hormone and pheromone manipulations, and biological and integrated control programmes (Singh, 1976). More recently the demands have included feed and food purposes (Van Huis *al.,* 2013). Over the past 40 years much effort has been expended in attempting to combine the 40-50 nutrients common to most foodstuffs into an acceptable feed for insects. This has resulted in papers describing diets for more than 750 species of insect (Singh, 1976). Only recently has the focus turned to identifying and formulating feeds according to nutrient requirements of the different insect species. The success of entomology over the last century has a large part to do with the ability to rear insects on artificial diets and probably much of the future of entomology depends on this factor (Cohen, 2015).

As with the nutrition of any other animals, insect nutrition can be broken down into nutrient classes and diet components. These components include amino acids, carbohydrates, lipids, vitamins and minerals (Chapman, 2012). Most insects have qualitatively similar nutritional requirements because their chemical composition and metabolic capabilities are broadly uniform. However, certain insect species have specific associations with certain microorganisms and some insects have adapted to particular diets (Cohen, 2015). These factors contribute to variation in their nutritional requirements.

Amino acids are essential for the production of proteins which in turn is used for structural purposes and receptor molecules (Isralewitz *et al.,* 2001). Nitrogen concentration significantly influences the performance of insects. It has a major impact on weight gain and egg production rate (Joern and Behmer, 1997). Dietary protein is the main source of amino acids for most

Stellenbosch University https://scholar.sun.ac.za

insect species. Protein quality is defined as how well the essential amino acid profile of a protein source matches the requirements of the animal; the digestibility of the protein source and bioavailability of the amino acids also play a role. The nutritional value of proteins depends on the capability to meet the amino acid requirements of the animal in question without over- or undersupplying certain amino acids while considering digestibility, absorbency and mobilisability. It was determined that, when using artificial diets to rear insects, the nutritive effect of proteins on growth of insects after hatching is greatly dependent on the type of amino acids in the diet. When larvae are reared on diets containing proteins with a poor amino acid profile, haemolymph protein is decreased and uric acid secretion is accelerated immensely (Horie and Watanabe, 1983). Supplementation of essential amino acids are crucial in determining the success of artificial diets. Insects are not able to synthesize nine or ten amino acids which are called essential amino acids (Chapman, 2012). If essential amino acids are omitted from the diet the insect will not be able to grow whatever the total diet supplementary amino acids. Although certain non-essential amino acids can be synthesized from other amino acids through the process of transamination, insects are still dependent on precursor molecules derived from the diet. Sustained growth of insects is impaired by gross dietary imbalances and therefore it is of utmost importance that insects consume a balanced mix of dietary amino acids.

Carbohydrates, including simple sugars, starch and other polysaccharides, are important components of the insect diet. Carbohydrates make up roughly between 4 % and 27 % of the natural diet of insects (Joern and Behmer, 1997). They are the usual respiratory fuel, can be converted to lipids and through transamination can provide the carbon skeleton for the synthesis of various amino acids (Chapman, 2012). Insects generally do not have the capability to digest and utilise certain carbohydrates such as cellulose. Although certain insect species are not able to digest cellulose, it can be used as a filler in their diet and help to promote intestinal stability (Cohen, 2015). Most mass reared insects fail to perform on diets that contain less than 50 % carbohydrates and it is also important to keep in mind that the type of carbohydrate must be fitted to the specific insect species. Insects such as the screwworm fly, on the other hand, can grow without any carbohydrates and only from live animal tissue (Chapman, 2012). There may also be differences in the ability of larvae and adults to utilize carbohydrates (Huffaker & Gutierrez, 1999).

The value of lipids, including sterols, oils, fats and phospholipids have been underestimated in insect nutrition. Failing to provide the right amount and type of lipids has been one of the major failings in insect dietetics. In most circumstances lipids have been undersupplied (Cohen, 2015). The absorption and digestion of lipids by insects are similar to those of vertebrates but do differ in some important aspects. Fat metabolism in insects centres on the

Stellenbosch University https://scholar.sun.ac.za

lipid transport system; diacyglycerol is the major lipid transported in insect metabolism by means of a high-density lipoprotein called lipophorin. Lipophorin is a reusable shuttle that picks up lipid from the gut and delivers it to tissue for storage and utilization without using the endocytic processes common to vertebrate cells (Nicol *et al.,* 2002). All insects require dietary sterols, but because it is difficult to dissolve it is often omitted from their diets (Fraenkel & Blewett, 1946). Sterols are often given in the wrong form. Leaf eating insects are not able to digest sterols from animal origin such as cholesterol, and require plant sterols such as β-sitosterol or campesterol (Cohen, 2015). Lipids function as building-blocks of cell membranes, hormones (fatty acids are converted to juvenile hormone and sterols to moulting hormone), source of energy, nutrient transporters and as structural material for building other materials. Insects cannot produce sterols like vertebrates can and therefore sterols are essential nutrients to include into the diets of insects (Clayton, 1964).

Vitamins are organic compounds required in trace amounts for sustained growth. The general understanding of these organic structures in insects is frustratingly limited and our understanding of vitamin function in insects comes largely from knowledge as applicable to vertebrates. Vitamins can be classed in two distinct categories, water soluble or fat soluble. Water-soluble vitamins are readily excreted from the insect's metabolic pool and therefore they have a relatively short half-life compared to lipid-soluble vitamins which tends to accumulate in lipid stores.

Water-soluble vitamins include B vitamins, vitamin C (ascorbic acid), and some miscellaneous compounds. Vitamin C serves as an antioxidant and is an essential phagostimulant for many phytophagous insects (Vanderzant *et al.,* 1962). Ascorbic acid is most commonly present in its L-ascorbic form, a component that is found in high concentrations in several kinds of fresh fruits and green tissues of plants. For example, in broccoli and fresh green peppers one can expect to find amounts as high as 90 mg / 100 g (USDA, 2019). The concentrations of ascorbic acid in plant components that are not green or in fruit is very low or absent. Therefore, when formulating artificial diets using grains and seeds one should include ascorbic acid if the target insect has a requirement for ascorbic acid. Ascorbic acid functions both in the diet and as a factor in the metabolic pathway of the insect that has ingested it (Briggs, 1962). It is also thought that ascorbic acid promotes collagen synthesis in the extracellular matrix of insects (Hunter *et al.,* 1979). The B vitamins function as cofactors in various metabolic pathways. These metabolic pathways include decarboxylation (Vitamin B_1, thiamine), flavoproteins (Vitamin B_2, riboflavin), cytochromes in ATP production (Vitamin B_3, niacin), acyl group transfer reactions (vitamin B_5, pantothenate), amino acid metabolism (vitamin B_6, biotin) and one carbon transfer reactions (vitamin B_9, folic acid). The B_{12} vitamins (choline, carnitine and

Stellenbosch University https://scholar.sun.ac.za

cyanocobalamin) and lipoic acid are water-soluble vitamins that are required by insects in very small amounts (Chapman, 2012).

Insects only have requirements for two of the four lipid-soluble vitamins. They have requirements for the vitamin A complex (β-carotene and related carotenoids) and vitamin E (tocopherols), but not for vitamin D (calciferols) and vitamin K (phylloquinone). Vitamin A complex are essential for the formation of eye- and other pigments and for normal growth (House and Barlow, 1958). Carotenoids are extremely effective antioxidants and prevent damage to cell membranes and vacuoles (Goodwin, 1986). Vitamin E is important for reproduction of insects, including spermatogenesis and egg maturation (Meikle and McFarlane, 1965).

Mineral nutrition is the most poorly understood aspect of insect nutrition. This is due to the difficulty in performing definitive nutritional studies due to the uncertainty that ingredients in a diet are entirely free of the mineral in question (Cohen, 2015). Insects require sodium, potassium, phosphate and chloride to be added to their diets for cellular ionic balance. Insects do not have the same requirements for calcium and iron as vertebrates which utilise these minerals in bone and haemoglobin formation (Chapman, 2012).

The supply of the above mentioned nutrients must coincide in the correct balance. This balance is of utmost importance. Therefore, proteins, carbohydrates, lipids, vitamins and minerals must all be present in optimal quantities. The quantitative balance of nutrients is the dominant factor in the success of the diet (Singh, 1976). Although some growth can occur on foods that contain widely differing levels of nutrients, optimal performance will only be achieved if the nutrients are in the correct balance. Insects have the capability to select feedstuffs as is required to satisfy their nutrient demand and balance (Raubenheimer, 1992). An unsatisfactory nutrient balance may lead to nutritional diseases affecting growth, development, reproduction and other life processes. Compensation is therefore an important means whereby insects maintain nutritional homeostasis in the face of dietary imbalances or food shortages.

Various methods have been used to try and assemble all these nutrients to formulate insect diets. When reviewing previous literature, the exact logic behind these methods is contradictory and rationally unclear. This is unfortunate as it forces all other researchers to start from scratch when developing methods to formulate artificial diets, repeating the same mistakes predecessors have made. Previously artificial diets have been developed using the following strategies: diets developed for insects with similar feeding habits; use of food analysis as a basis for diet development; use of whole carcass analysis in diet development

Stellenbosch University https://scholar.sun.ac.za

(body composition); radioisotopes and diet deletion techniques; use of digestive enzymes as aids in diet development; nutrient self-selection and the eclectic approach.

Each species has its own feeding habits. When formulating diets, insects with the same feeding habits are likely to perform on similar diets. For example, leaf-feeding insects such as the cabbage looper (*Trichoplusia ni*) is more likely to be suitable to a generalist diet formulated for leaf feeding insects such as that for the armyworm (*Spodoptera exempta*) whereas a diet for carnivores or a phloem sap eater or even a specialist on other plant tissues such as an insect that consumes seeds or fruits, would be less suitable (Cohen, 2015). Diets that have previously been found to be suitable can then be used as a starting point for insects with the same feeding habits.

A nutrient analysis of the insect's natural feed could also be used to formulate diets using raw materials other than what would be eaten in their natural environment. The principle being that the artificial diet should mimic as closely as possible the general composition of the natural feed. This was found to play a significant role in determining the eventual success of the diet. In this method, the study of the exact feeding choices of the target species is important. This method could be misleading if the wrong assumptions are made on the natural feeding habits of the target species as well as faulty nutrient compositional analysis. The USDA nutrient data base (USDA, 2019) could be useful when the feeding habits of insects are known to be restricted to foods used by humans and whose nutritional composition are well documented. This approach has been successfully implemented for the development diets to mass-produce the false codling moth (*Thaumatotibia leucotreta*) (Woods *et al.,* 2019a).

Another method uses the whole-carcass analysis (body composition) to formulate artificial diets. It was thought that the composition of an insect's body reflects its nutritional needs (Rock & King, 1967). This method incorporates using the profile of various nutrients in a target insect's body as template for artificial diets. The body composition technique is a protocol used to estimate changes in the body composition of animals during an experiment. Although this method has been previously considered by Cohen (2015), entomologists lack basic understanding of mentioned technique. The body composition technique has been widely used by animal scientists to determine protein and energy requirements of various domesticated animals (Blaxter, 1967) and has recently enjoyed attention with diets for neonatal black soldier fly (*Hermetia illucens*) formulated according to this method (Woods *et al.,* 2019b).

Digestive enzymes are used as aids in diet development. When a digestive enzyme is present it indicates that the insect can utilize the substrate that the enzyme hydrolyses. When an insect is able to hydrolyse a certain substrate it in turn means that the insects may be prepared to

Stellenbosch University https://scholar.sun.ac.za

use the food material from which the substrate originates in its diet. The application of this basic technique has proven to be useful in the improvement of artificial diets for several species of insects (Cohen, 2015). This method aids in selecting raw materials but diets should still be formulated according to the nutrient needs of the target insect.

Nutrient self-selection has also been thought to be an adequate way to develop artificial diets. Insects are hatched with an innate “knowledge” about what is healthy or nutritional sound feed. If given a proper set of choices, the insect will select food or a combination of foods that completely fills its nutritional needs (Cohen *et al.,* 1987). Previous studies on the corn earworm / cotton bollworm / tomato fruitworm (*Helicoverpa zea*) showed that this species was capable of selecting an optimal mixture of protein and starch when offered diets with these components that were spatially separated (Cohen *et al.,* 1987). This method can only be used for insect species that do not live in their feed substrate.

The most robust approach in the development of artificial diets was found to be the “eclectic” approach. This method is described as using multiple strategies, combining all the previously discussed methods to develop an artificial diet. By combing all these strategies and principles one is most likely to formulate a diet that is most suited to the nutritional requirement of the target species. It will lead to biological parameters that indicate greater fitness. Higher fecundity, fertility and body weight, among many other biological parameters can be expected. Currently we are only at the beginning of understanding the special requirements of different insect species under intensive production conditions and much work has to be done to optimize the diets to support their optimal performance and nutrient composition.

Insect processing

As with conventional livestock, insects should not be perceived to be eaten “head, feet and tail” and the extraneous and unpalatable parts such as the gut, wings, legs and head (depending on the species) should be removed. The use of mass-produced insects as feed and food hinges on consumer acceptance and the post-harvest processing of insects can help tip the scales in favour of “six-legged livestock” as alternative protein sources in human and animal diets (Sogari *et al.,* 2019). Balzan *et al.* (2016) determined that the willingness of people to consume insects depends on the presented form of the products (Bessa *et al.,* 2017). Also, post-harvest processing of insects is an important technology readiness level for improving food and feed value and widening the utilisation options (Mtungi *et al.,* 2019, Bessa *et al.,* 2019). Understanding how different processing procedures affects physical material losses, nutrient content and bioavailability, as well as product safety and acceptability can offer crucial guidance to technological improvements. Currently post-harvest processing of mass-produced insects ranges from traditional processing of insects in Africa (Mtungi *et al.,* 2019)

Stellenbosch University https://scholar.sun.ac.za

to the first attempt to fractionate insects into their individual nutritional compounds (Caligiani *et al.,* 2018).

The application of insect-derived ingredients in feed and food formulation is facilitated by insect processing and protein extraction. Basic post-harvest processing of mass-produced insects entails heat treatment. Fresh insects contain high levels of microorganisms and spore forming bacteria that are typical with fresh food harvested from soil (Klunder *et al.,* 2012). Blanching of insects destined for feed and food for between 5 and 10 minutes, not only eliminated their microbial load, but deactivated enzymatic processes causing black discolouration (enzymatic browning) of the insects. However, Klunder *et al.,* (2012) observed an increase in the overall counts of viable bacteria after milling of the blanched *T. molitor* larvae, possibly indicating a release of microbiota from the gut. It was also determined that roasting of the insects for 10 minutes did not completely eliminate microbial load of the whole yellow mealworm (*T. molitor*) larvae. A study by Leni *et al.* (2019) investigated blanching compared to freezing of black soldier fly (*H. illucens*) prepupae as killing method and confirmed that blanching inhibits enzymatic degradation and subsequent enzymatic browning, whereas this was not the case for freezing. This study also indicated that prepupae that were not blanched were subject to changes in nutritional quality, with a loss of cysteine and lysine, likely involved in the process of melanisation. Another important finding was that blanching also increases the extractability of proteins in aqueous solution, avoiding essential amino acid loss, and improving enzymatic digestibility.

To successfully fractionate larvae into their functional compounds at an industrial scale current processing methods from traditional protein sources needs to be adapted to meet the specific requirements of edible insects as a raw material. The first attempt at extracting proteins from insects was carried out by Del Valle *et al.* (1982) on Mexican fruit fly (*Anastrepha ludens*). This study managed to produce a protein concentrate and isolate containing 65 % and 87 % protein respectively, with corresponding recoveries of 94% and 85% by precipitating the proteins at different pH levels. The isolated protein was found to possess negligible foaming and emulsifying properties. This study was followed up by Yi *et al.,* (2013) who aimed to extract the protein from five insect species: yellow mealworm (*Tenebrio molitor*) larvae, superworm (*Zophobas morio*) larvae, darkling beetle (*Alphitobius diaperinus*) adults, house cricket (*Acheta domesticus*) adults and Dubia roach (*Blaptica dubia*) adults via aqueous extraction methods. This resulted in protein purities based on dry matter from 50 - 75 % but it was concluded that research is needed to develop further extraction and purification methods. Bußler *et al.* (2016) aimed to identify the necessary process stages and extraction parameters required to maximize the yield of soluble intact proteins from yellow mealworm (*Tenebrio molitor*) and black soldier fly (*Hermetia illucens*) larvae building on the aqueous extraction

Stellenbosch University https://scholar.sun.ac.za

methods used by Yi *et al.* (2013). It was concluded that processing of both insect species by means of aqueous extraction affected composition, appearance, microbial load as well as techno-functional and protein properties of the recovered insect compounds. In the most recent study Caligiani *et al.* (2018) investigated multiple fractionation methods for *H. illucens* larvae, including two chemical methods as well as an enzymatic fractionation method. Caligiani *et al.* (2018) found that all the fractionation methods that were investigated had their advantages as well as certain drawbacks and therefore the methods could be improved or further methods could be explored.

Aqueous chemical extraction of protein derived from insects as well as the fractionation of their compounds, using solvents, acids and alkalis, has enjoyed the most interest over recent times and is the most reported in literature. These methods include salting out, isoelectric precipitation and solvent fractionation. Yi *et al.* (2017) has enjoyed the most success to date isolating 100 % of the protein soluble proteins from yellow mealworm (*Tenebrio molitor*) using 0.1 M NaCl at pH 10, but observed significant enzymatic browning at such an alkaline pH. The same study evaluated acid precipitation at pH 4 and could only recover 22 % of the total protein and it concluded that proteins from *T. molitor* larvae behave more or less the same as proteins from meat and fish with respect to aqueous extraction. None of these studies have looked at recovering the insoluble protein fractions so that the total protein of mass-produced insects can be isolated

Caligiani *et al.* (2018) was the first who aimed to recover, at the highest purity level, all compounds of *H. illucens* i.e. protein, fat and chitin. Firstly, the fat was isolated by means of solvent extraction with petroleum ether. Two parts petroleum ether was used for every one part insect material, hence the described method is not suited for commercial use as large quantities of solvent would be necessary and only 87 % of the fat was recovered by this means. This study investigated two chemical methods to isolate the protein with the first, similar to Yi *et al.* (2017) using a strong alkali, could recover 84 % of the protein and with a subsequent acid treatment could solubilise and recover an additional 12 % of the protein fraction. However, the extraction conditions were too harsh and protein modifications were observed that resulted in poor functionality and reduced nutritive value and also problems were encountered with the neutralising of the substrate and subsequent drying procedures due to the vast amount of solvents used. Trying to overcome these limitations, Caligiani *et al.* (2018) explored Osborne fractionation of the protein (Osborne, 1907), as a variant of the previous method. This method hinges on the different solubility's of proteins in different solvents and can be conducted under milder conditions that do not negatively modify the protein structure to the extent that the previously mentioned method did. However, this method consists of four sequential extraction steps and therefore is only envisaged for laboratory scale

Stellenbosch University https://scholar.sun.ac.za

use. Also, the fat was removed with petroleum ether and as previously mentioned this is not viable for commercial use.

Identifying a suitable method to defat insects is crucial to solidify their use in food and feed application. Insects contain high amounts of fat, which makes it difficult to include them as raw materials in feed formulations as well as to be managed by feed mills due to the high energy content, proneness to oxidation and a decrease in pellet stability (Henry *et al.,* 2015). Defatting of insects will also allow the raw materials to be more uniform in nature, as the fat and protein content of insects can range greatly within species, whilst increasing the crude protein content. Several methods have been studied to defat mass-produced insects, with some enjoying more success than other. Mechanical methods by means of a press (usually a screw press), as used in the soybean industry, is not as effective in isolating fat from insect larvae due to their chemical composition. Soybeans and other plant proteins contain significantly more fibrous matter allowing greater lipid extraction using mechanical methods compared to insects (Bredeson, 1983). Therefore, mechanical methods have only been successful to partially defat insects to date and a combination of mechanical and solvent extraction has been used to remove all the fat (Surendra *et al.,* 2016). Supercritical CO_2 extraction has also been explored at pilot-scale as a means to defat insect larvae. Purschke *et al.* 2016 achieved a 95 % defatted meal when applying this technology to *T. molitor* larvae and determined that defatting performance and fat composition were not substantially different to using hexane. These methods can be successfully implemented to produce fat and protein fractions from insects.

Enzymatic hydrolysis with subsequent centrifugation has also been explored to fractionate insect larvae, although only one study has been published to date. In feed / food science and technology enzymes are exploited to perform desired functions in processing and to facilitate the conversion of raw materials into higher quality, more desirable food and feedstuff (Kristinsson & Rasco, 2000). Enzymatic hydrolysis also allows for modification of the physiochemical, functional and sensory properties of proteins, without negatively effecting its nutritive value. Compared to chemical fractionation procedures, enzymatic fractionation can be conducted at milder conditions and do not produce hydrolytic degradation products via racemisation reactions (Gonzàlez-Tello *et al.,* 1994). A promising prospect for insects as food is that enzymatic processing can reduce the risk of these novel foods to evoke cross-reactivity and allergenicity in crustacean- and house dust mite- allergic patients (Linnemann *et al.,* 2019). Caligiani *et al.* (2018) used an enzymatically assisted fractionation protocol to isolate protein, fat and chitin from *H. illucens* larvae. Once the hydrolysis had been terminated and the substrate centrifuged, only 67 and 10 % of the protein and fat was recovered respectively, although this method was seen as more rapid and sustainable, demanding further research.

Stellenbosch University https://scholar.sun.ac.za

Centrifugation on its own has also been explored as fractionation method for *T. molitor*, but by this means only 31 to 58 % of the protein was recovered (Purschke *et al.,* 2018).

Like most foods and feeds, processing and extraction of compounds from mass-produced insects before consumption will also influence their nutritional composition (EFSA, 2015). Therefore insect processing should not just be focused on protein content and yield, but also pay close attention to the effect that various processing methods have on the quality of the isolated fractions. This is crucial for the successful implementation of insect compounds for commercial feed and food applications.

Tailoring insects for specific needs

Mass-produced insects is envisaged to have specialised characteristics ranging from superior fitness for bio-control programs to their application as “sustainable super-foods” in the human nutrition market. Therefore mass-produced insects as well as their farming practises needs to be tailored for their end application to further solidify their use in speciality industries.

It is known that insects can readily adapt to a range of environmental conditions and these include conditions used in artificial mass-rearing facilities. When mass-reared insects are applied as biocontrol agents and released to provide pest control in agricultural settings, adaptation to their artificial rearing environment can decrease their effectiveness (Bertin *et al.,* 2017). Artificial mass-rearing environments typically lack variation and complexity due to their strictly controlled nature and therefore selective pressures associated with variable conditions may be less. This reduces selection for tolerance associated with thermal extremes, desiccation and starvation (Hoffmann, 2001), as well as the ability of the insects to utilise a variety of food sources or oviposition substrates (Bravo & Zucolots, 1998). The success of the biocontrol programs using mass-produced insects hinges on the released individuals being able to survive, mate, predate and reproduce effectively in the wild. Although some researchers postulate that mass-produced strains behave similarly to their wild counterparts and can be representation of populations and species even after many years (Kölliker-Ott *et al.,* 2003; Jong *et al.,* 2017), measures need to be put in place for mass-producing insects of not only similar, but superior fitness to their wild counterparts.

Mass-rearing conditions can be manipulated to tailor insects with superior performance phenotypes for field release. Kristensen *et al.* (2008) studied the effect of developmental and temperature acclimation of mass-produced fruit fly (*Drosophila melanogaster*) and found that the flies could be manipulated with rearing temperatures to have greater performance in either extreme hot or cold field conditions. In accordance with this study, Chidawanyika and Terblanche (2011) concluded that temperature acclimation could be successfully exploited to improve field performance of codling moth (*Cydia pomonella*) used in SIT programmes and

Stellenbosch University https://scholar.sun.ac.za

thus can possibly enhance other biocontrol programmes seeking increased efficiency. The diets used for mass-production can also be manipulated to increase quality of the insects produced. Koštál *et al.* (2011) determined that an increased level of proline in the larval diet of drosophilid fly (*Chymomyza costata*) allowed the insect to survive extremely low temperatures. The inclusion of diapause in mass-production has also been implemented to increase the quality of the insects produced (Bloem *et al.,* 1997).

Field performance of mass-produced insects remains one of the greatest challenges for their use as biological control agents and therefore innovative rearing methods needs to be adopted to give them an edge over their field targets to ensure successful pest control. This can be done in an inexpensive manner based on a cross-disciplinary approach and through the application of relatively basic techniques.

Mass-produced insects used as feed and food can also be manipulated to have a superior nutrient composition. It is well studied that Western consumers are reluctant to indulge on insects as food due to the "disgust-factor" (Tan *et al.,* 2015). On the other hand, Western consumers are becoming more inclined to eat a healthy diet (Barrie, 2014). This phenomenon could be exploited, and insects could be marketed as a nutritionally superior food to try and overcome the negative perception of entomophagy in these countries (Schiermer *et al.,* 2018). Superfoods do not have a legal or scientific definition but can be loosely defined as foods that are thought to be particularly nutritious and energy dense (Sikka, 2016). The scope for insects as "sustainable superfood" can become a greater reality by further enhancing their nutrient profile through altering mass-production practises.

Mass-produced insects are often low in n-3 polyunsaturated fatty acids (PUFA) and have a suboptimal n-6 / n-3 PUFA ratio (Schiavone *et al.,* 2017). For optimal human health the ratio of n-6 / n-3 fatty acids should be around 5 (Gertser, 1998), whereas the diet of average western consumer currently has a ratio of ~ 16 (Simopoulos, 2009). Therefore, if insects are to be seen as a nutritionally superior food it would be advantageous if they contain relatively large proportions of n-3 PUFA, which would result in more favourable n-6 / n-3 ratios. Similar to monogastric animals, the fatty acid composition of mass-produced insects is dependent on the substrate that they are reared on and therefore their fatty acid composition could be manipulated (Van Broekhoven *et al.,* 2015). St-Hilaire *et al.* (2007) determined that rearing *H. illucens* on fish offal increased their n-3 fatty acid content. Liland *et al.* (2017) had similar findings when rearing the same species on a seaweed enriched media. In a more recent study Oonincx *et al.* (2019) looked at the incorporation of flaxseed oil into the diets of house crickets (*Acheta domesticus*), lesser mealworms (*Alphitobius diaperinus*) and black soldier flies (*Hermetia illucens*), and the subsequent accumulation of n-3 fatty acids into their biomass.

Stellenbosch University https://scholar.sun.ac.za

They found that all three insects species responded similarly to the inclusion of flaxseed oil in their diets and a 4 % addition increased the n-3 fatty acid content 10 - 20 fold and thereby strongly decreased n-6 / n-3 ratios from 18 - 36 to 0.8 - 2.4. This is very promising results for insects as food but no research has been done to investigate the flow of n-3 fatty acids from the substrate it is grown to the meat of the animals they are fed to.

The fat of insects, especially *H. illucens,* also contains large amounts of lauric acid (Gasco *et al.,* 2018), similar to that of coconut oil. Lauric acid is known to have profound antiviral and antibacterial activity (Lieberman *et al.,* 2006). It was determined that lauric acid derived from insects has a substantial effect against D-streptococci infections in pigs (Spranghers *et al.,* 2018) and Belghit *et al.* (2018) found that lauric acid reduced the occurrence of liver fat in freshwater Atlantic salmon (*Salmo salar*). The lauric acid content of insects can be increased by rearing the insects on substrates that consists of high amounts of starch (Spranghers *et al.,* 2017) which makes it a promising prospect for insect mass-production. Even more so if this phenomenon can be exploited and the fat can be used in the cosmetic industry and fulfil a similar role as coconut oil.

Insects also contain antimicrobial peptides and until now, 150 insect proteins with antimicrobial properties have been identified (Otvos, 2000). Insect-derived antimicrobial peptides have been seen to be a solution to the restricted use of antibiotics in the feed industry due to over exploitation and ever-growing resistance of bacteria to antibiotics. These antimicrobial peptides have been used successfully in the place of antibiotics as growth promotors in broiler production (Choi *et al.,* 2013). Animals living in pathogen infested environments are known to contain more antimicrobial peptides (Lee *et al.,* 2012). Therefore, mass-producing insects in pathogen-infested environments to contain higher concentrations of antimicrobial peptides is a possibility if it can subsequently be isolated.

An interesting finding by Oonincx *et al.* (2018) was that insects are able to synthesise vitamin D when exposed to ultraviolet b (UVb) light. Low irradiance of UVb tended to increase vitamin D_3 levels in *A. domesticus*, vitamin D_2 levels in *H. illucens* and vitamin D_2 and D_3 in *T. molitor*. Vitamin D_3 is essential to intensive broiler and layer production as they are not exposed to the sun and birds deprived from vitamin D_3 can experience severe skeletal problems (Edwards, 2000). If *H. illucens*, naturally high in calcium, can be tailored to contain significant levels of vitamin D_3 on large scale, it can be an invaluable raw material in monogastric nutrition. Liland *et al.* (2017) also observed that when *H. illucens* was reared on a substrate containing brown algae (*Ascophyllum nodosum*) they incorporated vitamin E into their biomass.

The ability of certain insect species to treat biowaste and generate protein rich feedstock should not go without mention and although insects cannot be specifically tailored for this

Stellenbosch University https://scholar.sun.ac.za

purpose, the ability and constraints of each species should be focused towards the waste that needs to be disposed of. For instance, *M. domestica* is ideally suited for breaking down abattoir waste, food waste and manure (Pretorius, 2011), whereas *H. illucens* are less suited for breaking down abattoir waste, but excellent for cycling nutrients from manure and kitchen waste (Spranghers *et al.,* 2017). The copper bottom blowfly (*Chrysomya chloropyga*), being a carnivore, is suited for abattoir waste while largely unable to break down kitchen waste and manure (Van der Merwe, 2018), and *T. molitor* can be applied for dry milling and semi-moist bakery waste (Ramos-Elorduy *et al.,* 2002). The efficiency with which these wastes are broken down depends largely on the environment in which this occurs. The closer the environment is to the ideal environmental conditions of the species being produced, the higher the efficiency of conversion (Pieterse, 2018). Further, the density of the insects will play a major role. Higher densities of insects in the waste will result in better bioconversion of waste but leaner larvae and *vice versa* (Paz *et al.,* 2015). It is also important to keep in mind that the use of biowaste as rearing substrate for the mass-production of insects will not necessarily lead to maximum production compared to insects grown on artificial diets, but environmental benefits and circular economics will be at the forefront.

All the discussed results confirm the plasticity of the nutritional make-up of insects, allowing them to accumulate both lipid- and water- soluble compounds. A more in-depth understanding of how different insect species incorporate different compounds from their rearing substrates into their biomass can help tailor insects into a nutrient profile suited for specific feed and food purposes.

Conclusion and future recommendations

For insects to reach their potential as feed, food and biocontrol agents we need the ability to produce them in sufficient quantities to supply the potential demands, of which there is many. Great inroads have been made over recent times with some of the first insect mass-production facilities, both for feed / food production as well as biocontrol agents, being established. Despite the sustainability attributes of mass-producing insects it is important that the use of insects as feed, food and biocontrol agents can economically compete with current practises and therefore needs to be timely and cost effective. At the moment this is not the case and there is still some work to be done. Identifying nutrient requirements of species that are mass-produced will not only lead to higher survivability and eventual yield, but also decrease time to harvest or adulthood. Feeding mass-produced insects, whether on waste streams or artificial diets, according to their specific nutrient needs will result in significant financial savings, higher bioconversions and greater production output. Research should focus on developing nutrient specifications for individual mass-produced insect species, as is common practise in conventional livestock nutrition. Feed formulation programs could subsequently be

Stellenbosch University https://scholar.sun.ac.za

used to formulate diets or blend waste streams to the exact needs of the insect. Also, more emphasis can be placed on the plasticity of insects to tailor them for specific needs, thereby distinguishing them as a cut above, demanding even more attention. Post-harvest fractionation of edible insects is still in an infant stage and processing methods should be adapted from conventional raw material processing. Successful fractionation of insects will allow the individual compounds to fetch higher prices in diversified markets, justifying the current high cost of insect mass-production. All-in-all insect mass-production is at an exciting stage with bright future.

References

Balzan, S., Fasolato, L., Maniero, S. and Novelli, E., 2016. Edible insects and young adults in a north-east Italian city an exploratory study. British Food Journal 118(2): 318-326.

Barbosa, P.A., 1998. Conservation biological control. Elsevier Science Publishers, Amsterdam, the Netherlands, 395 pp.

Barrie, J., 2014. Our protein obsession is getting out of control. Munchies. Available at: https://www.vice.com/en_us/article/nz9xmx/our-protein-obsession-is-getting-out-of-control. Accessed 24 July 2019.

Belghit, I., Waagbø, R., Lock, E.J. and Liland, N.S., 2019. Insect-based diets high in lauric acid reduce liver lipids in freshwater Atlantic salmon. Aquaculture nutrition 25(2): 343-357.

Bertin, A., Pavinato, V.A.C. and Parra, J.R.P., 2017. Fitness-related changes in laboratory populations of the egg parasitoid *Trichogramma galloi* and the implications of rearing on factitious hosts. BioControl 62(4): 435-444.

Bessa, L.W., Pieterse, E., Sigge, G. and Hoffman, L.C., 2019. An exploratory study into the use of black soldier fly (*Hermetia illucens*) larvae in the production of a vienna-type sausage. Meat and Muscle Biology doi: 10.22175/mmb2018.11.0038

Bessa, L.W., Pieterse, E., Sigge, G. and Hoffman, L.C., 2017. Insects as human food; from farm to fork. Journal of the Science of Food and Agriculture https://doi:10.1002/jsfa.8860

Blaxter, K.L., 1967. Techniques in energy metabolism studies and their limitations. Proceedings of the Nutrition Society 26(1): 86-96.

Bloem, S., Bloem, K.A. and Fielding, L.S., 1997. Mass-rearing and storing codling moth larvae in diapause: a novel approach to increase production for sterile insect release. Journal of the Entomological Society of British Columbia 94: 75-82.

Bosch, G., Zhang, S., Oonincx, D.G. and Hendriks, W.H., 2014. Protein quality of insects as potential ingredients for dog and cat foods. Journal of nutritional science, 3: e29

Stellenbosch University https://scholar.sun.ac.za

Bravo, I.S.J. and Zucoloto, F.S., 1998. Performance and feeding behavior of *Ceratitis capitata*: comparison of a wild population and a laboratory population. Entomologia experimentalis et applicata 87(1): 67-72.

Bredeson, D.K., 1983. Mechanical oil extraction. Journal of the American Oil Chemists' Society 60(2): 211-213.

Briggs, M.H., 1962. Some aspects of the metabolism of ascorbic acid in insects. Comparative biochemistry and physiology 5(4): 241-252.

Bußler, S., Rumpold, B.A., Jander, E., Rawel, H.M. and Schlüter, O.K., 2016. Recovery and techno-functionality of flours and proteins from two edible insect species: Meal worm (*Tenebrio molitor*) and black soldier fly (*Hermetia illucens*) larvae. Heliyon 2(12): p.e00218.

Caligiani, A., Marseglia, A., Leni, G., Baldassarre, S., Maistrello, L., Dossena, A. and Sforza, S., 2018. Composition of black soldier fly prepupae and systematic approaches for extraction and fractionation of proteins, lipids and chitin. Food research international 105: 812-820.

Chapman, R., 2012. The Insects: Structure and function. (5th ed). Cambridge University Press, New York, 771 pp.

Chidawanyika, F. and Terblanche, J.S., 2011. Costs and benefits of thermal acclimation for codling moth, *Cydia pomonella* (Lepidoptera: Tortricidae): implications for pest control and the sterile insect release programme. Evolutionary Applications 4(4): 534-544.

Choi, S.C., Ingale, S.L., Kim, J.S., Park, Y.K., Kwon, I.K. and Chae, B.J., 2013. An antimicrobial peptide-A3: effects on growth performance, nutrient retention, intestinal and faecal microflora and intestinal morphology of broilers. British poultry science 54(6): 738-746.

Clayton, R.B., 1964. The utilization of sterols by insects. Journal of lipid research 5(1): 3-19.

Cohen, A.C., 2015. Insect diets: Science and technology. (2nd ed) CRC press, Boca Raton, Florida, 473 pp.

Cohen, R.W., Heydon, S.L., Waldbauer, G.P. and Friedman, S., 1987. Nutrient self-selection by the omnivorous cockroach *Supella longipalpa*. Journal of Insect Physiology 33(2): 77-82.

Del Valle, F.R., Mena, M.H. and Bourges, H., 1982. An investigation into insect protein 1. Journal of Food processing and Preservation 6(2): 99-110.

Driemeyer, H., 2016. Evaluation of black soldier fly (Hermetia illucens) larvae as an alternative protein source in pig creep diets in relation to production, blood and manure microbiology parameters. MSc Thesis, Stellenbosch University, Stellenbosch.

Dyck, V.A., Hendrichs, J. and Robinson, A.S., 2006. Sterile insect technique: principles and practice in area-wide integrated pest management. Springer, Dordrecht, 760 pp.

Stellenbosch University https://scholar.sun.ac.za

Edwards Jr, H.M., 2000. Nutrition and skeletal problems in poultry. Poultry Science 79(7): 1018-1023.

EFSA Scientific Committee, 2015. Risk profile related to production and consumption of insects as food and feed. EFSA Journal 13(10): 4257.

Fraenkel, G. and Blewett, M., 1946. Linoleic acid, vitamin E and other fat-soluble substances in the nutrition of certain insects, Ephestia kuehniella, E. elutella, E. cautella and Plodia interpunctella (Lep.). Journal of Experimental Biology 22(3-4): 172-190.

Gasco, L., Finke, M. and van Huis, A., 2018. Can diets containing insects promote animal health?. Journal of Insects as Food and Feed 4(1): 1-4.

Gerster, H., 1998. Can adults adequately convert a-linolenic acid (18: 3n-3) to eicosapentaenoic acid (20: 5n-3) and docosahexaenoic acid (22: 6n-3)?. International Journal for Vitamin and Nutrition Research 68(3): 159-173.

Gonzàlez-Tello, P., Camacho, F., Jurado, E., Paez, M.P. and Guadix, E.M., 1994. Enzymatic hydrolysis of whey proteins: I. Kinetic models. Biotechnology and Bioengineering 44(4): 523-528.

Goodwin, T.W., 1986. Metabolism, nutrition, and function of carotenoids. Annual review of nutrition 6(1): 273-297.

Harris, P., 1993. Effects, constraints and the future of weed biocontrol. Agriculture, Ecosystems & Environment 46(1-4): 289-303.

Henry, M., Gasco, L., Piccolo, G. and Fountoulaki, E., 2015. Review on the use of insects in the diet of farmed fish: past and future. Animal Feed Science and Technology 203: 1-22.

Hoffmann, A.A., Hallas, R., Sinclair, C. and Partridge, L., 2001. Rapid loss of stress resistance in *Drosophila melanogaster* under adaptation to laboratory culture. Evolution 55(2): 436-438.

Hopley, D., 2016. The evaluation of the potential of *Tenebrio molitor*, *Zophobas morio, Naophoeta cinerea, Blaptica dubia, Gromphardhina portentosa, Periplaneta americana, Blatta lateralis, Oxyhalao duesta* and *Hermetia illucens* for use in poultry feeds. MSc thesis, Stellenbosch University, Stellenbosch.

Horie, Y. and Watanabe, K., 1983. Effect of various kinds of dietary protein and supplementation with limiting amino acids on growth, haemolymph components and uric acid excretion in the silkworm, *Bombyx mori*. Journal of Insect Physiology 29(2): 187-199.

House, H.L. and Barlow, J.S., 1958. Vitamin requirements of the house fly*, Musca domestica* L. (Diptera: Muscidae). Annals of the Entomological Society of America 51(3): 299-302.

Stellenbosch University https://scholar.sun.ac.za

Huffaker, C.B. and Gutierrez, A. P., 1999. The food of insects in: Ecological Entomoligy. (2nd ed). John Wiley & Sons, New York, pp. 83-123.

Hunter, B., Magarelli, J.P., Lightner, D.V. and Colvin, L.B., 1979. Ascorbic acid-dependent collagen formation in penaeid shrimp. Comparative biochemistry and physiology. B, Comparative biochemistry 64(4): 381-385.

Isralewitz, B., Gao, M. and Schulten, K., 2001. Steered molecular dynamics and mechanical functions of proteins. Current opinion in structural biology 11(2): 224-230.

Joern, A. and Behmer, S.T., 1997. Importance of dietary nitrogen and carbohydrates to survival, growth, and reproduction in adults of the grasshopper *Ageneotettix deorum* (Orthoptera: Acrididae). Oecologia. 112: 201–208.

Jong, Z.W., Kassim, N.F.A., Naziri, M.A. and Webb, C.E., 2017. The effect of inbreeding and larval feeding regime on immature development of *Aedes albopictus*. Journal of vector ecology 42(1): 105-112.

Jongema, Y., 2017. List of edible insects around the world. Available at: https://www.wur.nl/en/Research-Results/Chair-groups/Plant-Sciences/Laboratory-of-Entomology/Edible-insects/Worldwide-species-list.htm (Accessed July 2019).

Józefiak, D. and Engberg, R.M., 2015, August. Insects as poultry feed. In 20th European Symposium on poultry nutrition, pp. 24-27.

Klunder, H.C., Wolkers-Rooijackers, J., Korpela, J.M. and Nout, M.J.R., 2012. Microbiological aspects of processing and storage of edible insects. Food control 26(2): 628-631.

Kölliker-Ott, U.M., Bigler, F. and Hoffmann, A.A., 2003. Does mass rearing of field collected *Trichogramma brassicae* wasps influence acceptance of European corn borer eggs?. Entomologia experimentalis et applicata 109(3): 197-203.

Koštál, V., Zahradníčková, H. and Šimek, P., 2011. Hyperprolinemic larvae of the drosophilid fly, *Chymomyza costata,* survive cryopreservation in liquid nitrogen. Proceedings of the National Academy of Sciences 108(32): 13041-13046.

Krafsur, E.S., 1998. Sterile insect technique for suppressing and eradicating insect population: 55 years and counting. Journal of Agricultural Entomology 15(4): 303-317.

Krafsur, E.S., Whitten, C.J. and Novy, J.E., 1987. Screwworm eradication in north and central America. Parasitology Today 3(5): 131-137.

Kristensen, T.N., Hoffmann, A.A., Overgaard, J., Sørensen, J.G., Hallas, R. and Loeschcke, V., 2008. Costs and benefits of cold acclimation in field-released Drosophila. Proceedings of the National Academy of Sciences 105(1): 216-221.

Kristinsson, H.G. and Rasco, B.A., 2000. Fish protein hydrolysates: production, biochemical, and functional properties. Critical reviews in food science and nutrition 40(1): 43-81.

Stellenbosch University https://scholar.sun.ac.za

Lee, S., Siddiqui, R. and Khan, N.A., 2012. Animals living in polluted environments are potential source of antimicrobials against infectious agents. Pathogens and global health 106(4): 218-223.

Leni, G., Caligiani, A. and Sforza, S., 2019. Killing method affects the browning and the quality of the protein fraction of black soldier fly (*Hermetia illucens*) prepupae: A metabolomics and proteomic insight. Food research international 115: 116-125.

Lieberman, S., Enig, M.G. and Preuss, H.G., 2006. A review of monolaurin and lauric acid: natural virucidal and bactericidal agents. Alternative & Complementary Therapies 12(6): 310-314.

Liland, N.S., Biancarosa, I., Araujo, P., Biemans, D., Bruckner, C.G., Waagbø, R., Torstensen, B.E. and Lock, E.J., 2017. Modulation of nutrient composition of black soldier fly (*Hermetia illucens*) larvae by feeding seaweed-enriched media. PLoS One 12(8): p.e0183188.

Linnemann, D.E.L., Meinlschmidt, P., Purschke, B., Hofstetter, G., Monroy, F.A.R., Einhorn, L., Mothes-Luksch, N., Jaeger, H., Jensen-Jarolim, E. and Pali-Schoell, I., 2019. Novel foods: Enzymatic and thermal food processing make edible insects non-allergenic. Journal of Allergy and Clinical Immunology 143(2): p.AB35.

Lipke, H. and Fraenkel, G., 1956. Insect nutrition. Annual Review of Entomology 1(1): 17-44.

Makkar, H.P.S., 2018. Feed demand landscape and implications of food-not feed strategy for food security and climate change. Animal 12(8): 1744-1754.

Martins, C., Cullere, M., Dalle Zotte, A., Cardoso, C., Alves, S.P., Bessa, R.J.B., Freire, J.P.B. and Falcao-e-Cunha, L., 2018. Incorporation of two levels of black soldier fly (*Hermetia illucens* L.) larvae fat or extruded linseed in diets of growing rabbits: effects on growth performance and diet digestibility. Czech Journal of Animal Science 63(9): 356-362.

Meikle, J.E.S. and McFarlane, J.E., 1965. The role of lipid in the nutrition of the house cricket, *Acheta domesticus* L. (Orthoptera: Gryllidae). Canadian Journal of Zoology 43(1): 87-98

Mutungi, C., Irungu, F.G., Nduko, J., Mutua, F., Affognon, H., Nakimbugwe, D., Ekesi, S. and Fiaboe, K.K.M., 2019. Postharvest processes of edible insects in Africa: A review of processing methods, and the implications for nutrition, safety and new products development. Critical reviews in food science and nutrition 59(2): 276-298.

Nichol, H., Law, J.H. and Winzerling, J.J., 2002. Iron metabolism in insects. Annual review of entomology 47(1): 535-559.

Oonincx, D.G., Laurent, S., Veenenbos, M.E. and van Loon, J.J., 2019. Dietary enrichment of edible insects with omega 3 fatty acids. Insect science (in press).

Stellenbosch University https://scholar.sun.ac.za

Oonincx, D.G.A.B., van Keulen, P., Finke, M.D., Baines, F.M., Vermeulen, M. and Bosch, G., 2018. Evidence of vitamin D synthesis in insects exposed to UVb light. Scientific reports 8(1): 10807.

Ortiz, J.C., Ruiz, A.T., Morales-Ramos, J.A., Thomas, M., Rojas, M.G., Tomberlin, J.K., Yi, L., Han, R., Giroud, L. and Jullien, R.L., 2016. Insect mass production technologies. In: Dossey, A.T., Morales-Ramos, J.A and Rojas, M.G. (eds.) Insects as Sustainable Food Ingredients. Academic Press, pp. 153-201.

Osborne, T.B., 1907. The proteins of the wheat kernel. Carnegie institution, Washington, DC.

Otvos, Jr, L., 2000. Antibacterial peptides isolated from insects. Journal of peptide science: an official publication of the European Peptide Society 6(10): 497-511.

Parker, A.G., 2005 Mass-Rearing for Sterile Insect Release. In: Dyck V.A., Hendrichs J., Robinson A. (eds.) Sterile Insect Technique. Springer, Dordrecht, pp. 787.

Patel, S., Suleria, H.A.R. and Rauf, A., 2019. Edible insects as innovative foods: Nutritional and functional assessments. Trends in Food Science and Technology 86: 352-359.

Paz, A.S.P., Carrejo, N.S. and Rodríguez, C.H.G., 2015. Effects of larval density and feeding rates on the bioconversion of vegetable waste using black soldier fly larvae *Hermetia illucens* (L.), (Diptera: Stratiomyidae). Waste and Biomass Valorization 6(6): 1059-1065.

Pieterse, E. and Pretorius, Q., 2014. Nutritional evaluation of dried larvae and pupae meal of the housefly (*Musca domestica*) using chemical-and broiler-based biological assays. Animal Production Science 54(3): 347-355.

Pieterse, E., 2018. Insect protein: from waste to feed to food. AFMA Matrix 27(4): 21-25.

Pretorius, Q., 2011. The evaluation of larvae of *Musca domestica* (common house fly) as protein source for broiler production. MSc thesis, Stellenbosch University, Stellenbosch.

Purschke, B., Sanchez, Y.D.M. and Jäger, H., 2018. Centrifugal fractionation of mealworm larvae (*Tenebrio molitor,* L.) for protein recovery and concentration. LWT 89: 224-228.

Purschke, B., Stegmann, T., Schreiner, M. and Jäger, H., 2017. Pilot-scale supercritical CO_2 extraction of edible insect oil from *Tenebrio molitor* L. larvae–Influence of extraction conditions on kinetics, defatting performance and compositional properties. European Journal of Lipid Science and Technology 119(2): 1600134.

Ramos-Elorduy, J., González, E.A., Hernández, A.R. and Pino, J.M., 2002. Use of Tenebrio molitor (Coleoptera: Tenebrionidae) to recycle organic wastes and as feed for broiler chickens. Journal of economic entomology 95(1): 214-220.

Stellenbosch University https://scholar.sun.ac.za

Raubenheimer, D., 1992. Tannic acid, protein and digestible carbohydrate: Dietary imbalance and nutritional compensation in locusts. Ecology 73: 1012–1027.

Rock, G.C. and King, K.W., 1967. Qualitative amino acid requirements of the red-banded leaf roller, *Argyrotaenia velutinana* (Lepidoptera: Tortricidae). Journal of Insect Physiology 13(1): 59-68.

Rumpold, B.A. and Schlüter, O.K., 2013. Potential and challenges of insects as an innovative source for food and feed production. Innovative Food Science & Emerging Technologies 17: 1-11.

Schiavone, A., Cullere, M., De Marco, M., Meneguz, M., Biasato, I., Bergagna, S., Dezzutto, D., Gai, F., Dabbou, S., Gasco, L. and Dalle Zotte, A., 2017. Partial or total replacement of soybean oil by black soldier fly larvae (*Hermetia illucens* L.) fat in broiler diets: effect on growth performances, feed-choice, blood traits, carcass characteristics and meat quality. Italian Journal of Animal Science 16(1): 93-100.

Schiemer, C., Halloran, A., Jespersen, K. and Kaukua, P., 2018. Marketing Insects: Superfood or Solution-Food?. In: Halloran, A., Flore, R., Vantomme, P. and Roos, N. (eds.) Edible Insects in Sustainable Food Systems. Springer, Dordrecht, pp. 213-236.

Shera, P.S. and Arora, R., 2015. Biointensive integrated pest management for sustainable agriculture. Biological and molecular approaches in pest management. Scientific Publications, Jodhpur, pp. 373-429.

Sikka, T., 2016. Contemporary superfood cults. In: Gargill, K. (eds.) Food Cults: How Fads, Dogma, and Doctrine Influence Diet. Rowman & Littlefield, Lamham, Maryland, pp. 87-107.

Simopoulos, A.P., 2009. Omega–6/Omega–3 Essential Fatty Acids: Biological Effects. World Review of Nutrition and Dietetics 99: 1-16.

Singh, P., 1976. Artificial diets for insects, mites, and spiders. (2nd ed). P.D. Company, Ed., New York, pp. 594.

Singh, P., and M. D. Ashby. 1985. Insect rearing management. In: Singh, P. and Moore, R.F. (eds.) Handbook of insect rearing, Volume I. Elsevier Science Publishers BV, Amsterdam, The Netherlands, pp. 185–215.

Slansky, F., 1982. Insect nutrition: an adaptationist's perspective. The Florida Entomologist 65(1): 45-71.

Sogari, G., Amato, M., Biasato, I., Chiesa, S. and Gasco, L., 2019. The Potential role of insects as feed: A Multi-Perspective Review. Animals 9(4): 119.

Sørensen, J.G., Addison, M.F. and Terblanche, J.S., 2012. Mass-rearing of insects for pest management: challenges, synergies and advances from evolutionary physiology. Crop Protection 38: 87-94.

Stellenbosch University https://scholar.sun.ac.za

Spranghers, T., Michiels, J., Vrancx, J., Ovyn, A., Eeckhout, M., De Clercq, P. and De Smet, S., 2018. Gut antimicrobial effects and nutritional value of black soldier fly (*Hermetia illucens* L.) prepupae for weaned piglets. Animal Feed Science and Technology 235: 33-42.

Spranghers, T., Ottoboni, M., Klootwijk, C., Ovyn, A., Deboosere, S., De Meulenaer, B., Michiels, J., Eeckhout, M., De Clercq, P. and De Smet, S., 2017. Nutritional composition of black soldier fly (Hermetia illucens) prepupae reared on different organic waste substrates. Journal of the Science of Food and Agriculture 97(8): 2594-2600.

St-Hilaire, S., Cranfill, K., McGuire, M.A., Mosley, E.E., Tomberlin, J.K., Newton, L., Sealey, W., Sheppard, C. and Irving, S., 2007. Fish offal recycling by the black soldier fly produces a foodstuff high in omega-3 fatty acids. Journal of the World Aquaculture Society 38(2): 309-313.

Surendra, K.C., Olivier, R., Tomberlin, J.K., Jha, R. and Khanal, S.K., 2016. Bioconversion of organic wastes into biodiesel and animal feed via insect farming. Renewable energy 98: 197-202.

Tan, H.S.G., Fischer, A.R., Tinchan, P., Stieger, M., Steenbekkers, L.P.A. and van Trijp, H.C., 2015. Insects as food: exploring cultural exposure and individual experience as determinants of acceptance. Food Quality and Preference 42: 78-89.

United States Department of Agriculture (USDA), 2019. Food composition database. USDA, Beltsville, Maryland.

Van Broekhoven, S., Oonincx, D.G., Van Huis, A. and Van Loon, J.J., 2015. Growth performance and feed conversion efficiency of three edible mealworm species (Coleoptera: Tenebrionidae) on diets composed of organic by-products. Journal of insect physiology 73: 1-10.

Van der Merwe, A.J.W, 2018. *Chrysomya chloropyga* larvae meal as a protein source for broiler nutrition. MSc thesis, Stellenbosch University, Stellenbosch.

Van Huis, A., 2015. Edible insects contributing to food security? Agriculture & Food Security 4(1): 20.

Van Huis, A., Van Itterbeeck, J., Klunder, H., Mertens, E., Halloran, A., Muir, G. and Vantomme, P., 2013. Edible insects: future prospects for food and feed security (No. 171). Food and Agriculture Organization of the United Nations.

Vanderzant, E.S., Pool, M.C. & Richardson, C.D., 1962. The role of ascorbic acid in the nutrition of three cotton insects. Journal of Insect Physiology 8: 287-297.

Stellenbosch University https://scholar.sun.ac.za

Woods, M.J., Boersma, N., Hoffman, L.C. and Pieterse, E., 2019a. Development of reduced cost diets for the mass rearing of false codling moth (*Thaumatotibia leucotreta*). Journal of the Science of Food and Agriculture 99(8): 3976-3982.

Woods, M.J., Hoffman, L.C. and Pieterse, E., 2019b. Artificial diets for neonatal black soldier fly (*Hermetia illucens*) larvae. Journal of Insects as Food and Feed, 5(2): 99-105.

Wu, Y., Chen, R., Feng, Z. and Yang, Y., 2019. What if there were no food aid and food import: food insufficiency in Africa from the perspective of self-sufficiency. IOP Conference Series: Earth and Environmental Science 234(1): 012043.

Yi, L., Lakemond, C.M., Sagis, L.M., Eisner-Schadler, V., van Huis, A. and van Boekel, M.A., 2013. Extraction and characterisation of protein fractions from five insect species. Food chemistry 141(4): 3341-3348.

Stellenbosch University https://scholar.sun.ac.za

CHAPTER 3

The development of an improved artificial diet for the mass-rearing of *Eldana saccharina* Walker (Lepidoptera: Pyralidae)

In review as: Woods, M.J., Conlong, D.E., Ngomane, N., Gillespie, D.Y., Hoffman, L.C. and Pieterse, E., 2019. The development of an improved artificial diet for the mass-rearing of *Eldana saccharina* Walker (Lepidoptera: Pyralidae). Journal of the Science of Food and Agriculture.

Stellenbosch University https://scholar.sun.ac.za

Abstract

Background

This study determined the nutrient requirements of *Eldana saccharina* Walker (Lepidoptera: Pyralidae), a serious pest of sugarcane in South Africa, so as to develop a more efficient artificial diet for mass-rearing purposes for sterile moth production. Diets tested consisted of a minimum specification (MS) diet representing a diet formulated according to the minimum specification of a summary of published diets which yielded satisfactory results, an ideal amino acid profile (IAAP) diet, where amino acid composition was based on the profile of amino acids in the second (IAAP2) and fifth / sixth (IAAP5/6) instar larvae, and lastly two diets based on the nutrient composition of the natural diet, papyrus (PAP) and sugarcane (SC), of the insects. Six treatments with 50 replications were randomly allotted to 300 25 mL plastic screw top vials. Fifteen milliliters diet was dispensed into each vial and inoculated with two freshly hatched larvae. Larvae, pupae and moths were harvested at 28 days after inoculation. Overall survivability, pupal weight, sex ratio and rate of development was determined and compared to the diet currently in use at the South African Sugarcane Research Institute (CON). Physical characteristics of the diets such as pH and water holding capacity of the diets were also determined.

Results

The natural diets (PAP & SC) were not viable as they did not yield any results. Survivability was significantly higher (78 %) for the MS diet whilst IAAP2 and IAAP5/6 yielded the second highest survivability (74 %) compared to CON (68 %). There were no differences in male pupal weights between all treatment diets, as was the case for female pupae. Within dietary treatments, female pupae were heavier than male pupae for all treatment diets. CON (1.0 : 1.6) produced significantly less male than female pupae with MS (1.0 : 1.2), IAAP2 (1.0 : 1.0) and IAAP5/6 (1.0 : 1.1) all producing equal amounts of male and female pupae. The MS diet (16 %) yielded fourfold the number of moths after 28 days compared to CON (4 %) and IAAP2 (4 %) diets. IAAP5/6 yielded no moths after 28 days. The life stages thus developed fastest in the MS diet. The pH of all treatment diets remained stable for the entire duration of the trial. No biological contamination was observed through all diets. Differences in water holding capacity were observed between most diets with PAP and SC losing the most moisture whilst the MS and IAAP2 diets retained the most moisture.

Conclusion

The MS diet most closely represented the nutrient requirements of *E. saccharina*, leading to its faster development on this formulation which could be readily applied for large scale

Stellenbosch University https://scholar.sun.ac.za

production of this lepidopteran pest as an aid in the mass rearing of sterile males as part of the integrated pest management plan.

Keywords: nutrient requirements; survivability; pupal weight; rate of development; SIT

Introduction

Eldana saccharina Walker (Lepidoptera: Pyralidae), more commonly known as the African sugarcane stalkborer, occurs naturally in wetland sedges and indigenous grasses and has been identified as an indigenous pest species of graminaceous crops on the African continent for the past 100 years.[1,2,3] *Eldana saccharina* was first described in Sierra Leone[4] and since then has been recorded in east Africa and southwards down the east coast to the southern KwaZulu-Natal and Transkei coasts in South Africa.[1,2,5-8] The first report of *E. saccharina* as a crop pest in South Africa was in 1939, when a severe infestation of *E. saccharina* was found during harvesting of sugarcane on the Umfolozi flats in KwaZulu-Natal.[9]

Eldana saccharina is a major pest of sugarcane,[10] maize,[11,12] and to a lesser extent sorghum.[1,13] However, *E. saccharina* has been the most destructive in sugarcane production and in 1970 reached key pest status in South Africa.[14] It is estimated that *E. saccharina* causes direct losses of ZAR1 300 (USD 90) hectare^{-1} annum without taking into account indirect losses due to a reduced cropping duration.[9] Direct losses mainly result from a decrease in sugarcane quality due to a reduction of soluble solids, sucrose content and sucrose percentage in total solids present in the plant juice.[15] An increase in the fibre content is caused by a combination of *E. saccharina* and secondary fungal infestations which also has a negative effect on sugarcane quality. A cause for greater concern is the reduced cropping duration of 12 months caused by *E. saccharina* infestations, compared to 18 months that was the recommended practice for the eastern coastal areas of South Africa prior to the mid 1970's. This has further financial implications. A cropping period of 18 months in the absence of *E. saccharina* was estimated to increase revenue gain by ZAR 7 900 (USD 545) hectare^{-1} annum.[9] *Eldana saccharina* is thus a major concern for South African sugarcane farmers, and means of effectively managing the pest is the subject of numerous intensive research programs at the South African Sugarcane Research Institute (SASRI). Its eradication is not a possibility, due to it being indigenous to Africa with several wild host plants. An integrated pest management (IPM) strategy has thus been identified as the way forward to suppress the population of *E. saccharina* to below economic threshold limits.[9] The sterile insect technique (SIT), a branch of IPM, has recently drawn attention as a promising control method to assist existing programs and has therefore been included as part of SASRI's *area-wide integrated pest management program* (https://sasri.org.za/research-focus/). Sterile insect technique

Stellenbosch University https://scholar.sun.ac.za

(SIT) involves the mass-rearing, sterilisation and release of large numbers of insects in and around the infested crop. Mating of released sterile males with native females leads to a decrease in their reproductive potential and ultimately, if males are released in sufficient numbers over a sufficient period of time, control the pest species.[16] Sterile insect technique has successfully been implemented for various species in South Africa, such as *Ceratitis capitata* Wiedemann (Diptera: Tephritidae) (Mediterranean fruit fly), *Ceratitis rosa* Karsch (Diptera: Tephritidae) (Natal fruit fly), *Cydia pomonella* Linnaeus (Lepidoptera: Tortricidae)(codling moth) and *Thaumatotibia leucotreta* Meyrick (Lepidoptera: Tortricidae) (false codling moth).[15] Due to the fact that SIT programs have been successfully implemented for Lepidoptera species, and the biological similarities between *T. leucotreta* and *E. saccharina,*[15] it is envisaged that SIT could significantly help decrease wild *E. saccharina* numbers.

Sterile insect technique involves the mass-rearing of large quantities of insects on artificial diets under controlled conditions. Artificial diets thus play a crucial role in the eventual success of SIT programmes.[17] Feeding these insects according to their nutritional needs is of utmost importance for economical and efficient production since over- and undersupply of nutrients have the same detrimental effect in insect production as it does in domesticated animals. The undersupply or absence of nutrients could lead to a total break in production while marginal undersupply could lead to immune suppression, decreased efficiency of production or a reduction in fertility and fecundity.[18] On the other hand, an oversupply of nutrients could lead to the build-up of primary or secondary metabolites which could be toxic, antagonistic or result in imbalances that could lead to metabolic stress.[19 - 21] Therefore the identification of specific nutrient requirements for the mass-rearing of *E. saccharina* needs to be determined.

Diet development is a continuous process, as demonstrated in the *E. saccharina* IPM program. Atkinson,[22] Graham and Conlong,[23] Graham,[24] Gillespie[25] and Walton and Conlong[26] developed diets that continuously improved quality and production of the insects needed for the various IPM programs. A new and improved artificial diet based on one developed for *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae),[27] with lucerne meal supplied in rabbit pellets as the main ingredient, recently replaced the previous conventional sugarcane based diet used to routinely rear *E. saccharina,* as insects were produced faster, were of higher quality, and less expensive than those reared on the previously used diets.[28] All of these literature diets were based on ingredient composition and were not formulated based on the nutrient requirements of *E. saccharina* and therefore there is still a large margin for improvement.

Stellenbosch University https://scholar.sun.ac.za

The carcass composition technique is a technique used to determine the nutrient requirements of domesticated animals and has successfully been applied to determine baseline nutrient specifications for the mass-rearing of insects such as *Hermetia illucens* (Linneus) (Diptera: Stratiomyidae) (black soldier fly)[29] and *T. leucotreta*.[17] The development of a more effective artificial diet that meets the specific nutrient needs of *E. saccharina* larvae is crucial to solidify the prospect of SIT as a biological control method for the mentioned species. An artificial diet meeting the nutritional needs of the larvae in question will decrease mortality, increase fertility and fecundity, and effectively optimise production. The aim of this investigation was therefore to develop an artificial diet, using the body composition principle,[30] for *E. saccharina* that will result in maximum survival of larvae, optimal sex ratio and moths with high fertility and fecundity.

Materials and methods

Treatment diets

Table 1. A description of *Eldana saccharina* trial diets used

Treatment	Discription
CON	Diet currently in use at the SASRI insectary (control)
MS	Diet formulated according to the minimum nutrient specifications from published diets previously used to mass-rear *E. saccharina*
IAAP2	Diet formulated using carcass milling methods to resemble the amino acid compostion of 2nd instar larvae
IAAP5/6	Diet formulated using carcass milling methods to resemble the amino acid compostion of 5th/6th instar larvae
PAP	Diet formulated to resemble the nutrient composition of papyrus
SC	Diet formulated to resemble the nutrient composition of sugarcane

Table 1 defines the different diets tested to improve diet efficiency and cost. The diet currently in use to mass-rear *E. saccharina* at SASRI[28] was reverted to nutrient composition using Winfeed (EFGSoftware, 2017) and the calculated nutrient composition used as the control nutrient specifications (CON).

Stellenbosch University https://scholar.sun.ac.za

Table 2. Ingredient and calculated nutrient composition of previous diets used for rearing *Eldana saccharina*

	Gillespie, 1993	Walton & Conlong 2016	Ngomane *et al,* 2017
Ingredients (g kg^{-1})			
Crushed sugarcane	521.3	524.8	--
Ground chickpea	260.7	252.4	109.1
Yeast extract	7.8	7.9	7.3
Casein	44.7	45.0	--
Sodium propionate	23.8	24.0	18.6
Ascorbic acid	8.7	8.8	11.6
Calcium lactate	3.0	3.0	2.3
Ferric citrate	0.2	--	--
Tri-sodium citrate	6.0	6.0	4.7
Sodium chloride	1.5	1.5	1.2
Citric acid	6.0	6.0	4.7
Nipagin	5.2	--	11.6
Dithane M45	0.4	0.5	0.4
Formalin (40%)	9.1	--	--
Ethanol (70%)	91.2	--	--
Agar	10.4	13.1	8.1
Methyl-p-hydroxybenzoate	--	5.2	--
Denol (70%)	--	91.8	71.3
Lucerne pellets	--	--	436.5
Wheat bran	--	--	72.8
Full cream milk powder	--	--	48.0
Whole egg powder	--	--	58.2
Sucrose	--	--	116.4
Terralon LA	--	--	2.6
Acetic acid	--	--	14.6
Calculated nutritional value (g kg^{-1})			
Dry matter	956.4	944.6	943.2
Moisture	43.6	55.4	56.8
Crude protein	110.6	161.1	147.1
Crude fiber	130.8	55.3	146.7
Crude fat	20.3	65.8	44.7
Calcium	4.8	14.4	101.0
Available phosphorous	0.1	0.97	18.4
Sodium	1.1	3.4	2.1
Potassium	28.7	67.2	127.2
†DE (Pig) (MJ kg^{-1})	8.5	9.9	9.3
Lysine	25.8	11.0	29.9
Methionine	2.1	2.9	7.4
Sulphur amino acids	2.8	5.5	14.2
Tryptophan	1.0	4.8	6.8
Isoleucine	4.9	8.0	23.7
Leucine	9.2	14..0	42.4
Threonine	1.0	2.1	22.6

†DE: Digestible energy (MJ kg^{-1}) as determined for pigs
--: No available value

Stellenbosch University https://scholar.sun.ac.za

Ingredient compositions of three diets[25,26,28] which yielded satisfactory results for the rearing of *E. saccharina* were reverted to nutrient composition (16 nutrients) using Winfeed[31] (Table 2). No energy value could be found for insects and in order to maintain a standard and comparable method the digestible energy value of the raw materials for pigs were used (DE(pig)).[31] Since all of these diets yielded satisfactory results in rearing *E. saccharina*, the minimum value for each nutrient was used as nutrient specifications for a treatment diet (Minimum specification (MS) diet; Table 1).

Stellenbosch University https://scholar.sun.ac.za

Table 3. Amino acid composition and ratio of amino acids (in relation to lysine) of different instar *Eldana saccharina* larvae, and their natural host plants (papyrus and sugarcane), determined from the carcass milling technique

Amino acid	**2nd instar**		**5th / 6th instars**		**Papyrus**		**Sugarcane**	
	AA† content ($g \cdot 100\ g^{-1}$) sample	IAAP‡ Ratio (AA†:Lys§)	AA† content ($g \cdot 100\ g^{-1}$) sample	IAAP‡ Ratio (AA†:Lys§)	AA† content ($g \cdot 100\ g^{-1}$) sample	IAAP‡ Ratio (AA†:Lys§)	AA† content ($g \cdot 100\ g^{-1}$) sample	IAAP‡ Ratio (AA†:Lys§)
Histidine	2.06	0.26	2.27	0.61	0.10	0.02	0.10	0.04
Serine	1.151	0.14	1.68	0.45	0.12	0.02	0.09	0.04
Arginine	1.79	0.22	1.76	0.47	0.10	0.02	0.08	0.03
Glycine	1.80	0.22	2.12	0.57	0.16	0.03	0.15	0.06
Aspartine	2.55	0.32	2.92	0.78	0.49	0.09	1.23	0.49
Glutamine	3.04	0.38	3.27	0.87	0.27	0.05	0.24	0.10
Threonine	1.06	0.13	1.10	0.29	0.09	0.02	0.08	0.03
Alanine	1.95	0.24	2.36	0.63	0.16	0.03	0.11	0.04
Proline	2.42	0.30	2.77	0.74	0.21	0.04	0.11	0.04
Lysine	8.03	1.00	3.75	1.00	5.59	1.00	2.51	1.00
Tyrosine	2.05	0.26	2.94	0.78	0.12	0.02	0.09	0.04
Methionine	0.63	0.08	0.75	0.20	0.00	0.00	0.02	0.01
Valine	1.48	0.18	1.76	0.47	0.13	0.02	0.08	0.03
Iso-leucine	1.22	0.15	1.39	0.37	0.11	0.02	0.08	0.03
Leucine	2.09	0.26	2.47	0.66	0.22	0.04	0.15	0.06
Phenylalanine	1.21	0.15	1.65	0.44	0.10	0.02	0.07	0.03

†AA: Amino acid
‡IAAP: Ideal amino acid profile
§Lys: Lysine

Stellenbosch University https://scholar.sun.ac.za

The carcass milling technique was used to determine the amino acid profiles of the 2nd and 5th/6th instar larvae (Table 3). This represents the ideal amino acid profile (IAAP) of the insect stage being tested, and the supply of amino acids in this ratio in a diet will result in an amino acid profile most closely representing the requirements of the animal.[30] The IAAP of the different instars were used to formulate treatment diets IAAP2 and IAAP5/6 (Table 1).

Table 4. Proximate composition of the larval instars and natural diets of *Eldana saccharina* i.e. papyrus and sugarcane

Proximate composition (g kg^{-1})	**2nd instar**	**5th / 6th instars**	**Papyrus**	**Sugarcane**
Moisture	51.2	55.6	49.8	50.1
Ash	15.1	14.4	36.3	17.1
Crude fat	501.2	489.4	24.6	21.5
Crude fiber	--	--	305.6	202.3
Crude protein	294.0	380.8	37.3	41.1

--: No available value

The final two diets were formulated to resemble the nutrient composition of the natural diet of the larvae i.e. papyrus (PAP) (*Cyperus papyrus* Linneus) and sugarcane (SC) (Table 1) that was determined by the amino acid and proximate analyses (Tables 3 & 4).

Stellenbosch University https://scholar.sun.ac.za

Table 5. Ingredient and calculated nutrient composition of the experimental diets used for rearing *Eldana saccharina*

	CON	MS	IAAP2	IAAP5/6	PAP	SC
Ingredients (g kg^{-1})						
Full fat milk powder	45.3	15.0	15.0	15.0	48.9	15.0
Yeast extract	6.8	--	--	--	--	296.7
Ascorbic acid	12.9	12.9	12.9	12.9	12.9	12.9
Nipagin	12.9	12.9	12.9	12.9	12.9	12.9
Lucerne meal	432.6	500.0	500.0	50.0.0	100.0	100.0
Chickpea	113.2	106.0	109.2	170.5	162.6	169.5
Acetic acid	16.1	16.1	16.1	16.1	16.1	16.1
Trisodium citrate	5.2	5.2	5.2	5.2	5.2	5.2
Citric acid	5.2	5.2	5.2	5.2	5.2	5.2
Whole egg powder	56.6	56.6	56.6	56.6	56.6	56.6
Sodium propionate	20.6	20.6	20.6	20.6	20.6	20.6
Oxytetracycline	4.0	4.0	4.0	4.0	4.0	4.0
Carrageenan gel	30.0	30.0	30.0	30.0	30.0	30.0
Wheat bran	113.2	111.6	90.5	12.2	81.5	--
L-Lysine HCL	--	--	--	--	50.0	--
†Vit+min premix	1.5	1.5	1.5	1.5	1.5	1.5
Sucrose	122.5	102.5	120.3	137.3	266.0	140.5
Sodium Chloride	1.4	--	--	--	126.0	9.44
Calculated nutritional value (g kg^{-1})						
Dry matter	943.2	947.4	947.7	943.4	954.5	946.4
Moisture	56.8	52.6	52.3	56.6	41.5	53.6
Crude protein	157.1	132.4	132.3	131.9	128.5	213.2
Crude fiber	166.7	158.4	158.2	156.0	52.5	49.9
Crude fat	44.7	39.7	40.7	40.6	41.4	45.9
Calcium	101.0	108.2	108.3	114.1	29.6	37.9
Phosphorous	18.4	0.8	0.8	0.2	0.9	1.6
Sodium	2.1	1.4	1.4	1.5	50.0	42.6
Potassium	127.2	136.1	136.4	147.8	50.0	50.0
‡DE (Pig) (MJ kg^{-1})	9.3	8.8	8.8	8.8	9.4	8.9
Lysine	20.7	30.7	30.9	34.0	55.9	25.1
Methionine	7.4	7.6	7.7	8.1	3.8	4.9
*TSSA	14.2	14.7	14.8	15.6	7.3	10.3
Tryptophan	6.8	7.1	7.1	7.4	3.0	4.4
Isoleucine	23.7	24.7	24.8	26.5	11.3	17.4
Leucine	42.4	44.3	44.4	47.6	20.5	28.6
Threonine	22.6	23.7	23.8	25.3	10.4	16.2

CON: Control diet
MS: Diet formulated according to the minimum nutrient specifications previously used to mass-rear *E. saccharina*
IAAP2: Diet formulated to resemble the amino acid composition of 2nd instar larvae
IAAP5/6: Diet formulated to resemble the amino acid composition of 5th/6th instar larvae
PAP: Diet formulated to resemble the nutrient composition of papyrus
SC: Diet formulated to resemble the nutrient composition of sugarcane
†Vit+min premix: Broiler starter vitamin and mineral premix
‡DE: Digestible energy (MJ kg^{-1}) as determined for pigs
--: No available value
*TSSA: Sulphur amino acids

Stellenbosch University https://scholar.sun.ac.za

Diets were formulated according to the novel nutrient specifications using similar ingredients to those currently in the diet used for rearing *E. saccharina* at SASRI. However, the use of agar as gelling agent, was substituted with carrageenan gel as a cheaper alternative (Table 5).

Determination of ideal amino acid profile (IAAP)

Larvae were grown in 4 L plastic trays on the diet currently in use at SASRI in a temperature controlled environment at 27 ± 1 °C and relative humidity of 65 ± 5 %. Fifty gram grab samples of 2nd and 5th/6th instar larvae were taken from ten of these respective trays boxes and thoroughly mixed, but keeping the 2nd instars separated from the 5th/6th instars. Larvae were killed with steam, dried in an oven at 60 °C for 24 h (Labcon FSOH 16), homogenised using a mill (KN 195 Knifetec™), subsamples taken and the amino acid profile determined.[32] During this process, a sample weighing 0.1 g was placed in a specialized hydrolysis tube. Six mL hydrochloric acid (HCl) solution and 150 mL L^{-1} phenol solution was then added to the sample. The tubes were then vacuated and nitrogen (N) added under pressure. The tubes were subsequently sealed off with a blue flame and the samples left to hydrolyse at 110˚C for 24 hours. After hydrolysis, the samples were transferred to 1.5 mL Eppendorf tubes and refrigerated until sent to the Central Analytical Facility of Stellenbosch University (https://www.sun.ac.za/english/faculty/science/CAF/units/mass-spectrometry), where amino acid composition was determined by means of the Waters AccQ Tag Ultra Derivatization method (http://www.waters.com/waters/en_US/Amino-Acid-Analysis/nav.htm?cid=514511&locale=en_US).

Papyrus and sugarcane were sampled from crops at SASRI where after they were dried in an oven at 60 °C for 24 h (Labcon FSOH 16), homogenised using a mill (KN 195 Knifetec™), subsamples taken and the amino acid profile determined as described above for the larvae.

Determination of proximate composition

The proximate chemical composition was determined for the natural diet of *E. saccharina* (sugarcane and papyrus) as well as for the 2nd and 5th/6th instar larvae, with moisture, protein and ash being analysed according to the methodology of the Association of Official Analytical Chemists.[33]

For the determination of the moisture content, 2.5 g of the homogenised sample was dried at 100 - 105 °C for 24 hours and re-weighed.[33] This dried sample was then incinerated at 500 °C for at least 6 hours, cooled and weighed to provide an estimate of the ash content (AOAC method 942.05).[33]

Stellenbosch University https://scholar.sun.ac.za

The total lipid content was determined using a rapid solvent extraction method,[34] with a 1: 2 chloroform : methanol solution being used for the plant samples due to their relatively low fat content (< 50 g kg^{-1}, USDA, 2019). An initial test determination of the lipid content of *E. saccharina* found it to be above 50 g kg^{-1}, and a 2:1 chloroform: methanol solution was consequently used for these samples.

The dried, defatted samples were ground and used for the determination of the total protein content using the LECO combustion or Dumas method.[33] A 0.5 g aliquot of each sample was weighed into a LECO foil cup, which was incinerated and analysed for nitrogen content, using EDTA for calibration (LECO Corporation, St Joseph, MI, USA). The nitrogen content was converted to a protein content by multiplying by 6.25.

Larval growth trials

E. saccharina eggs were obtained from SASRI (Mount Edgecombe, South Africa), placed in tissue paper on top of ice packs and shipped overnight via airfreight, to the laboratories of the Department of Animal Sciences, Stellenbosch University (Stellenbosch, South Africa). Eggs were kept in an incubator at 27 ± 1 °C until they emerged. In contrast to the trail conducted by Ngomane *et al.*[28] the treatment diets were inoculated with freshly hatched larvae instead of eggs, due to the lack of ability to accurately count *E. saccharina* eggs, as they are laid in clumps.

Six different diets were formulated, mixed and moisture standardized at 750 g kg^{-1} by the addition of boiling water. Acetic acid was added to the water after boiling at a 16.1 g kg^{-1} (dry matter) inclusion level. The diets were placed in a 2 L open glass bowl and cooked for one minute using a 1000W microwave oven (Defy DM0357 34L). Diets were then mixed by hand to ensure that it cooked evenly and placed in the microwave for a further minute. Six treatments with 50 replications were randomly allotted to 350 25 mL plastic screw top vials. The top of the screw top lid was cut out and replaced by a fine filter nylon mesh. Based on previous trials conducted at SASRI, 15 mL of hot diet was dispensed into each vial by means of a 50 mL syringe before the diet could cool down and set. After the diet had set, the surface was scarified and 2.5 g of sago was placed on top of the diet. The temperature of the diet was brought to 27 ± 1 °C by leaving the diet in a controlled-environment (Laboratory, Stellenbosch University; 27 ± 1 °C, 65 ± 5 %) over-night, before placing the neonate larvae, kept at the same conditions, on the diet. This was done to ensure that the metabolic rate of the larvae did not slow down[35] and that the diet was not too hot for the larvae. Freshly hatched larvae were randomly allotted to one of the six treatment diets, with two larvae assigned to a vial. Larvae were individually transferred to the vials by means of a fine paintbrush dipped in water. Larvae were placed onto the sago and not directly onto the diet as sago absorbs excess moisture and

Stellenbosch University https://scholar.sun.ac.za

acts as dispersal medium to prevent them from drowning. Vials were placed randomly in a controlled-environment room with a constant temperature of 27 ± 1 °C, relative humidity of 65 ± 5 % and zero light: 24 hour dark photophase.

Harvesting took place 28 days after the larvae were placed on the diet. Number of larvae, larval weight, number of pupae, pupal weight, pupal sex and number of moths were determined on day 28. Larvae survivability was determined by adding the total number of viable larvae, pupae and moths obtained after the 28 day trial period (empty pupal cases were ignored in the instances where moths were observed). Pupae and larvae were separated from the remaining diet by hand and counted. Pupae were carefully removed from their cocoons and sexed under a digital microscope based on the different structures on the ventral surface of their last abdominal segment[2] whereafter they were weighed. The pH of the different treatments diets were measured at larval placement as well as on days 7, 14, 21 and 28 after larval placement. At a more acidic pH microbial growth and contamination is known to be suppressed.[36] Moisture content was also determined at day 28 using using a moisture analyser (Radwag PMX 50/1/NH) to evaluate the ability of the treatment diets to retain water over the trail period.

Dietary costs analysis

A cost analysis was undertaken to determine whether the newly formulated treatment diets were more cost effective than the current diet used at SASRI. The cost analysis was based on diet ingredient prices, amounts of ingredients used in the diets, and the results obtained from the experiment.

Statistical analysis

Analysis of variance was performed on larval, pupal and moth yield data, as well as on pH and moisture content, using the ANOVA procedures of SAS (2009) with treatment (diet) as the main effect. All the parameters were tested for normality and homoscedasticity before analysis. Significance was declared at $P \leq 0.05$. Significant means were separated with Bonferroni *post hoc* test (SAS, 2009). The results were used to compare the yield of larvae between different treatments.

Stellenbosch University https://scholar.sun.ac.za

Results and discussion

Physical and chemical properties

Table 6. Moisture content and pH of treatment diets used to rear *Eldana saccharina*

	Moisture content (g kg^{-1})		pH				
	Day 1	Day 28	Day 1	Day 7	Day 14	Day 21	Day 28
CON	775.0^{a}	624.5^{b}	4.86^{a}	4.95^{a}	4.81^{a}	4.90^{a}	4.90^{a}
MS	775.0^{a}	700.7^{a}	4.65^{a}	4.63^{a}	4.59^{a}	4.64^{a}	4.68^{a}
IAAP2	775.0^{a}	695.4^{a}	4.51^{b}	4.51^{b}	4.41^{b}	4.53^{b}	4.56^{b}
IAAP5/6	775.0^{a}	545.8^{c}	4.83^{a}	4.80^{a}	4.71^{a}	4.73^{a}	4.71^{a}
PAP	775.0^{a}	401.2^{d}	4.87^{a}	4.84^{a}	4.77^{a}	4.70^{a}	4.62^{a}
SC	775.0^{a}	446.7^{d}	4.87^{a}	4.60^{a}	4.65^{a}	4.60^{a}	4.62^{a}

^{a,b,c,d} Means within columns with different superscripts differ significantly ($P < 0.05$)
CON: Control diet
MS: Diet formulated according to the minimum nutrient specifications previously used to mass-rear *E. saccharina*
IAAP2: Diet formulated to resemble the amino acid composition of 2nd instar larvae
IAAP5/6: Diet formulated to resemble the amino acid composition of 5th/6th instar larvae
PAP: Diet formulated to resemble the nutrient composition of papyrus
SC: Diet formulated to resemble the nutrient composition of sugarcane

The physical properties of the diets (pH and moisture content) are presented in Table 6. No significant differences were observed for the initial pH's of the diets except for the diet formulated to resemble the amino acid profile of the 2nd instar larvae (IAAP2) which had the lowest pH of 4.51 ($P < 0.05$). The pH of all treatment diets remained stable for the entire duration of the trial. This suggests that the ingredients added as buffers (trisodium citrate and citric acid) effectively buffered any pH changes due to the formation of secondary metabolites by the larvae. The pH of artificial diets is important for numerous reasons due to the fact that it influences flavour, palatability, texture and most chemical reactions involving digestive enzymes. In addition pH also regulates microbial action on the prepared diet and a more acidic pH is known to suppress microbial growth.[36] The ideal pH would be similar to that of the host plant and therefore treatment diets were formulated to resemble the initial pH of sugarcane juice, which is acidic in nature (4.5 – 5.5).[37] The pH of all of the treatment diets were in this range and remained so for the duration of the trial.

No biological contamination was observed in any of the treatment diets. Therefore, the preservatives, anti-fungal, bacterial and viral agents such as nipagin (methyl paraben), ascorbic acid, acetic acid, oxytetracycline and sodium propionate (added in equal amounts to all diets; Table 5) maintained a sterile environment for the duration of the 28 day growth period. These ingredients play an important role in the success of the diets but are more expensive compared to the other ingredients. The inclusion levels of these ingredients could be lowered until the minimum level is reached and some even omitted as certain ingredients have the same function; this could result in significant savings. However, this needs to be researched further. The substitution of agar (ZAR 408 kg^{-1} ; 28 USD kg^{-1}), used conventionally as a gelling

Stellenbosch University https://scholar.sun.ac.za

agent for *E. saccharina* diets,[28] with carrageenan gel (ZAR 110 kg^{-1} ; USD 8 kg^{-1}), resulted in significant financial savings and did not seem to have a negative effect on the results.

Differences ($P < 0.05$) in water holding capacity were observed between most treatment diets (Table 6). At day 28, PAP and SC lost the most moisture whilst the MS and IAAP2 diets retained the most moisture. The control diet performed intermediate alongside IAAP5/6. Lepidopteran larval legs are much reduced, so they cannot easily move around in the diet, the moisture content of the diets should thus remain high enough for the duration of the trial for the larvae to move freely in the diet. On appearance the PAP and SC diets had a watery-like texture at day 28, even though it had lost the most moisture, which suggest that there was more free water present in these diets than the other treatment diets. This supports the evaporation of water due to the lack of PAP and SC to bind water. Most plant-feeding insects require a high content of moisture in their diets, but in their natural foods the water is retained within cell walls and bound to cellular constituents. However in artificial diets a nutritionally inert substance is required to bind water.[38]

Stellenbosch University https://scholar.sun.ac.za

Larval development

Table 7. *Eldana saccharina* production parameters from the different experimental diets measured 28 days after neonate larval placement

Treatment	Larval yield (%)	Avg. larval weight (g) ± S.E	Pupal yield (%)	Male pupae (%)	Avg. male pupal weight (g) ± S.E	Female pupae (%)	Avg. female pupal weight (g) ± S.Er	Ratio M:F	†Moth yield (%)	Diet survivability (%)
CON	0^{b}	--	64^{c}	39	$0.1329^{b} \pm 0.0073$	61	$0.2063^{a} \pm 0.0078$	$1:1.6^{a}$	4^{b}	68^{c}
MS	0^{b}	--	62^{c}	45	$0.1331^{b} \pm 0.0081$	55	$0.1944^{a} \pm 0.0067$	$1:1.2^{b}$	16^{a}	78^{a}
IAAP2	0^{b}	--	70^{b}	49	$0.1224^{b} \pm 0.0044$	51	$0.1991^{a} \pm 0.0085$	$1:1.0^{b}$	4^{b}	74^{b}
IAAP5/6	0^{b}	--	74^{a}	47	$0.1192^{b} \pm 0.0073$	53	$0.1932^{a} \pm 0.0088$	$1:1.1^{b}$	0^{c}	74^{b}
PAP	4^{a}	0.0172±0.0011	0^{d}	0	--	0	--	--	0^{c}	4^{d}
SC	0^{b}	--	0^{d}	0	--	0	--	--	0^{c}	0^{d}

[a,b,c] Means within columns with different superscripts differ significantly ($P < 0.05$)
CON: Control diet
MS: Diet formulated according to the minimum nutrient specifications previously used to mass-rear *E. saccharina*
IAAP2: Diet formulated to resemble the amino acid composition of 2nd instar larvae
IAAP5/6: Diet formulated to resemble the amino acid composition of 5th/6th instar larvae
PAP: Diet formulated to resemble the nutrient composition of papyrus
SC: Diet formulated to resemble the nutrient composition of sugarcane
--: No available value
† Moths found in vials at harvest (28 days) were counted and used to asses rate of development

Stellenbosch University https://scholar.sun.ac.za

The effects that the different diet formulations had on larval development through to the pupal and adult stages (survivability) are presented in Table 7. Differences ($P < 0.05$) in survivability at 28 days of age were detected between most treatment diets. Survivability was significantly higher (78 %) for the MS diet whilst the diets formulated to resemble the amino acid profile of the 2nd and 5th/6th instar larvae (IAAP2 & IAAP5/6) yielding the second highest survivability (74 %) compared to the control (CON) and natural (PAP & SC) diets. No differences were observed between IAAP2 and IAAP5/6. The control diet performed intermediate with a survivability of 68 % whereas PAP and SC yielded no results indicating that none of the larvae being reared solely on their natural diet survived. The treatment diets with the highest (MS & IAAP2) and lowest (PAP & SC) survivability also had the strongest and weakest ability to hold water, respectively. Therefore, it is suggested that the ability of the diet to hold water, or the lack thereof, is directly correlated to the success of the diet. Diets PAP and SC lost ~30 g kg^{-1} moisture over the trial period compared to the diets that yielded the highest survivability (MS & IAAP2) that only lost ~7.0 g kg^{-1} (See Table 6). Although all treatment diets were set in carrageenan gel and cooked using a microwave, which in turn allows for little to no free water, the water holding capacities of the individual raw materials need to be taken into account when formulating diets for insects.[17] When the moisture content of a diet falls too low, the insect cannot gain access to the nutrients,[38] therefore the poor performance of the natural diets (PAP & SC) cannot necessarily be attributed to the under or oversupply of nutrients *per se,* as in all probability these food sources under natural conditions would contain sufficient water.

Also, fibre enhances water holding capacity and aerates the diets allowing the insect to freely move and feed in the substrate.[38] The fibre content of PAP (52.5 g kg^{-1}) and SC (49.9 g kg^{-1}) were a mere third compared to that of CON (166.7 g kg^{-1}), MS (158.4 g kg^{-1}), IAAP2 (158.2 g kg^{-1}) and IAAP5/6 (156.0 g kg^{-1}) diets (Table 5). The relatively low fibre content and its interaction with water holding capacity could explain the lack of results shown by the natural diets.

It was observed that the decreased nutrient specification diet (MS) increased the survivability of the larvae to pupae by 10 % compared to that of the presently used control (CON) diet (Table 7). The lower protein content of the MS diet (132.4 g kg^{-1}) compared to CON (157.1 g kg^{-1}) (Table 5) could have contributed to the increase in survivability of the MS diet. Insects, being uricotelic, excrete excess nitrogen in the form of uric acid. If high concentrations of protein is fed to insects, it results in increased uric acid secretion,[39] which has high energy cost implications to the insect, negatively impacting on the insect’s performance.

Stellenbosch University https://scholar.sun.ac.za

Table 8. Comparison of amino acid profiles of the IAAP of the *Eldana saccharina* 2nd and 5th/6th instar larvae and the amino ratio of the formulated diets

Amino acids	IAAP 2nd instar larvae	IAAP 5/6th instar larvae	Percentage supply expressed as IAAP		
			CON	IAAP2	IAAP5/6
Lysine	1.00	1.00	1.00	1.00	1.00
Methionine	0.08	0.20	0.36	0.25	0.24
TSAA	0.10	0.10	0.69	0.48	0.46
Isoleucine	0.15	0.37	1.14	0.81	0.78
Leucine	0.26	0.66	2.05	1.44	1.40
Threonine	0.13	0.29	1.09	0.77	0.74

CON: Control diet
TSAA: Total Sulphur containing amino acids (methionine and cysteine)
IAAP2: Diet formulated to resemble the amino acid composition of 2nd instar larvae
IAAP5/6: Diet formulated to resemble the amino acid composition of 5th/6th instar larvae

When comparing the amino acid ratios of IAAP2 and IAAP5/6 larvae to that supplied in the CON and IAAP2 and IAAP5/6 diets, expressed as a ratio to lysine (Table 8), it clearly seems that the ratios of the amino acids are such that most of the amino acids are oversupplied in the diets. Issues that are observed are the extreme oversupply of methionine in CON and also threonine in CON, IAAP2 and IAAP5/6. Oversupply of these amino acids could lead to toxicity which cannot be alleviated except by removal through the formation of uric acid,[18] this combined with the high crude protein level (Table 5) of these diets could explain why the MS diet yielded the best results. This does not however imply that the ratio of amino acids are incorrect but that protein is oversupplied. It would thus be worthwhile to evaluate lower levels of these specific amino acids while maintaining the IAAP amino acid profile which should closely represent the ideal amino acid profile of the animal.[40]

The lower fat content of MS (39.7 g kg^{-1}) compared to that of CON (44.7 g kg^{-1}) (Table 5) could also have resulted in more favourable physical characteristics for the MS diet. Although insects cannot synthesize sterols *de novo*[41-44] and therefore these must be included as a dietary component, the higher fat content of CON could have caused it to have a more putty-like texture, decreasing the mobility of the insect in and around the diet.[29] Future studies should evaluate the rheological properties of these diets to gain a better understanding of the effect that this could have on mobility and survivability of the larvae.

The effects that the diets had on rate of development are presented in Table 7. The MS diet (16 %) yielded fourfold the number of moths compared to CON (4 %) and IAAP2 (4 %) treatment diets with IAAP5 yielding no moths after 28 days (Table 7) indicating that the larvae fed on the MS diet had a faster rate of development. These results also indicated a faster rate

Stellenbosch University https://scholar.sun.ac.za

of development than what is currently observed in intensive mass-rearing of *E. saccharina* at SASRI. Another factor to take into consideration is that the age of the adult moths were not known at harvest, as the moths could have emerged before the 28 days were completed. The time to adulthood could thus have been even shorter. This implies that the nutrient requirements were more accurately met with a less nutrient dense diet. Relatively good uniformity as pertaining to the level of development was seen throughout the CON, MS, IAAP2 and IAAP5/6 treatment diets as all the insects had all reached either pupal or moth stages and no larvae were observed after 28 days (Table 7).

The effects that diet had on male and female pupal weights are presented in Table 7. There were no differences in male pupal weights between all treatment diets, as was the case for female pupae. Within dietary treatments, female pupae were heavier than male pupae for all treatment diets ($P < 0.05$). This supports previous results obtained.[28] Female pupal weight is directly proportional to their fecundity.[45] Therefore heavier female pupal weight will result in a bigger adult, providing more eggs laid.

The effects that diet had on male : female sex ratio is presented in Table 7. These results indicates that CON (1.0 : 1.6) produced significantly less male than female pupae with MS (1.0 : 1.2), IAAP2 (1.0 : 1.0) and IAAP5/6 (1.0 : 1.1) all producing equal amounts of male and female pupae. These findings are in line with that of Sampson & Kumar[46] who obtained an 1.0 : 1.0 sex ratio for laboratory reared *E. saccharina.* With natural infestations a ratio of 42 : 51 is expected[46] but a sex ratio of more males than females is favourable when it comes to SIT programs due to the fact that only males undergo radiation to render them infertile and subsequently released to mate with wild females making the eggs infertile, and thus decreasing wild *E. saccharina* numbers. MS, IAAP2 and IAAP5/6 outperformed CON in this regard.

Stellenbosch University https://scholar.sun.ac.za

Diet costs

Table 9. Total dry dietary costs (ZAR) of the different *Eldana saccharina* experimental diets

Dietary treatment	Cost (kg DM‡)	
	ZAR	USD†
Modified *Ostrinia nubilalis* diet (Ngomane)[28]	41.37^{a}	2.85^{a}
CON	36.04^{b}	2.51^{b}
MS	34.49^{c}	2.38^{c}
IAAP2	34.79^{c}	2.40^{c}
IAAP5/6	36.38^{b}	2.51^{b}

^{a,b,c} Means within columns with different superscripts differ significantly ($P < 0.05$)
CON: Control diet
MS: Diet formulated according to the minimum nutrient specifications previously used to mass-rear *E. saccharina*
IAAP2: Diet formulated to resemble the amino acid composition of 2nd instar larvae
IAAP5/6: Diet formulated to resemble the amino acid composition of 5th/6th instar larvae
‡DM Dry matter
†USD Exchange rate of 27 /03 / 2019 1USD = 14.52 ZAR

Diet costs (exchange rate from ZAR to USD as per 27 / 03 / 2019) as applicable to South African conditions are presented in Table 9. MS (ZAR 34.49 kg^{-1} ; USD 2.38 kg^{-1} and IAAP2 (ZAR 34.79 kg^{-1} ; USD 2.40 kg^{-1}) were cheaper than CON (ZAR 36.38 / kg; USD 2.51 kg^{-1}) with no difference in cost between the two. CON and IAAP5/6 were intermediately priced compared to MS and IAAP2 treatment diets. When comparing treatment diets to that previously used by Ngomane *et al.*[28] (ZAR 41.37 kg^{-1} ; USD 2.85 kg^{-1}) to rear *E. saccharina*, all were significantly cheaper. The MS diet, which yielded highest survivability, was ZAR 6.88 (USD 0.47) kg^{-1} cheaper that the diet used by Ngomane.[28] The yield of insects from the diet should also be taken into account though, and not just the dry dietary costs, as often a diet with a higher dry dietary cost can produce many more insects than a cheaper diet, thus making the cost per insect obtained from the diet significantly less.[28]

Although the rearing of *E. saccharina* presently only occurs on laboratory scale, the sterilisation of males has been shown to be successful[15] and intensive mass-rearing should commence for SIT to be used as part of the IPM approach against *E. saccharina.* Mass-rearing facilities such as X Sterile Insect Technique (Pty) Ltd., which currently implements a successful SIT program in citrus orchards against *T. leucotreta,* use 70 ton of feed to produce approximately 170 x 10^{6} moths per month.[17] Hence, a saving of ZAR 6.88 (USD 0.47) kg^{-1} could lead to an overall saving of ZAR 481 600.00 (USD 33 214.00) per month, once production scales for commercial application are developed at the same scale for *E. saccharina,* as that for *T. leucotreta*.

Stellenbosch University https://scholar.sun.ac.za

Considering the fact that mass-rearing of insects is essentially similar to intensive animal production systems, the scenario is not much different with current SIT facilities and systems being faced with similar challenges seen such as feed costs typically seen in intensive animal production.[47]

Recommendations for future studies

Further studies on low protein diets are warranted, both to see if production would be improved as well as the cost. Futher diets could also include micronutrients, such as sterols, that are known to play a vital role in insect development. Unlike other animals, insects do not have the ability to synthesize sterols *de novo*, including cholesterol. Sterols are important to insects in three ways: they are precursors of many hormones, they are important components of cellular membranes and they play a role in regulating genes involved in the developmental processes. Also nutrients such proline and trehalose, could be added to the diet of *E. saccharina* to possibly give them an advantage over their wild counterparts when used in SIT programs. It is postulated that the combination of this amino acid and sugar could improve cold hardiness of mass-reared *E. saccharina.* The addition of dyes such as Calco Red and Sudan Red, that help distinguish between mass-reared sterile males and their wild counterparts caught in traps, could be investigated. Lastly, more attention should be given to the physical properties of diets. If poor growth results from unsatisfactory forms of the diet, the failure should not only be attributed to poor nutrition.

Conclusion

The nutrient requirements of *E. saccharina* larvae were more accurately met with a less nutrient dense diet compared to that currently used. In these trials, this led to a 10 % increase in survivability, as well as a faster rate of development for *E. saccharina* larvae compared to that experienced currently in the SASRI insect unit. The reduced nutrient specifications determined for *E. saccharina* larvae could be used as matrix for further studies.

Certain key observations were made during the trail that may affect the survivability of the larvae and should be considered in future studies. The ability of the diet to hold water was a major factor determining feed utilization by the larvae. Larvae live within their feed and therefore their direct environment is determined by the feed provided. The fibre content of the natural diets was postulated to be too low and an optimal inclusion level needs to be determined. It was once again confirmed that the texture, water holding capacity and density of the feed plays a crucial role in determining the success of the diets and that the nutrient composition of the diet is not the only factor contributing to its success. Rheological properties of insect diets have been found to be one of the major differences between conventional livestock and insect nutrition, and poses the greatest challenge to overcome. Physical

Stellenbosch University https://scholar.sun.ac.za

properties of insect diets need to be understood to bridge the gap between conventional livestock and “six-legged livestock” nutrition.

Stellenbosch University https://scholar.sun.ac.za

References

1. Girling DJ, *Eldana saccharina* Wlk. (Lepidoptera: Pyralidae), a pest of sugarcane in East Africa. *Proc In Soc Sugarcane Technologists* **14**:429 – 434 (1972).
2. Atkinson PR, On the biology, distribution and natural host-plants of *Eldana saccharina* Walker (Lepidoptera: Pyralidae). *Proc S Af. Sug Technol.Ass* **43**:171 – 194 (1980).
3. Atkinson PR, Distribution and natural hosts of *Eldana saccharina* Walker in Natal, its oviposition sites and feeding patterns. *Proc S Afr Sug Technol Ass* **53**:111 – 115 (1987).
4. Walker P, List of specimens of Lepidopterous insects in the collection of the British Museum. British Museum (Natural History). Part 32. Suppl. Part 2: 632 – 633 (1865).
5. Girling DJ, The distribution and biology of *Eldana saccharina* Walker (Lepidoptera: Pyralidae) and its relationship to other stem borers in Uganda. *Bull Entomological Res* **68**:740 – 488 (1978).
6. Atkinson PR, Carnegie AJM and Smaill, RJ, A history of the outbreaks of *Eldana saccharina* Walker in Natal. *Proc S Afr Sug Techno. Ass* **55**: 111–115 (1981).
7. Conlong DE, Search for *Eldana saccharina* and its parasitoids in sugarcane and natural hosts at the East Pondoland Sugar Project, Transkei (23-26/6/86). Internal Report 8.1.45 (267), (unpublished) (1986).
8. Leslie GW, The arthropod predators of *Eldana saccharina* Walker (Lepidoptera: Pyralidae), their identification and relative importance. M.Sc. Thesis. University of Natal, Pietermaritzburg (1986).
9. Rutherford RS, IPM for Eldana Control: An Integrated Pest Management (IPM) approach for the control of the stalk borer *Eldana saccharina* Walker (Lepidoptera: Pyralidae). Unpublished Report of the South African Sugarcane Research Institute. 170 Flanders Drive, Mount Edgecombe, 4300. pp. 6-86 ISBN: 1-874903-41-7 (2015).
10. Carnegie AJM, Report on a visit to Ghana, West Africa, 14-24 June, 1976. Unpublished report. South African Sugarcane Association, Mt Edgecombe, Natal (1975).
11. Kaufman T, Behavioural biology, feeding habits and ecology of three species of stem borers: *Eldana saccharina* (Lepidoptera: Pyralidae), *Sesamia calamistis* and *Busseola fusca* (Noctuidae) in Ibadan Nigeria, West Africa. *Georgia Entomol Soc* **18**: 259 – 272 (1983).
12. Bosque-Perez NA and Mareck JH, Effect of the stem borer *Eldana saccharina* Walker (Lepidoptera: Pyralidae) on the yield of maize. *Bull Entomological Res* **81**: 243 – 247 (1991).

Stellenbosch University https://scholar.sun.ac.za

13. Waiyaki JN, The biology and control of the principle Lepidopterous borers associated with sugar cane at the Tanganyika Planting Company, Arusha Chini. Miscellaneous report No. 653 (Unpublished) (1968).
14. Conlong DE, A study of pest-parasitoid relationships in natural habitats: An aid towards the biological control of *Eldana saccharina* Walker (Lepidoptera: Pyralidae) in sugarcane. *Proc S Afr Sug Technol Ass* **64**:111 – 115 (1990).
15. Potgieter L, Van Vuuren, JH and Conlong DE, A reaction-diffusion model for the control of *Eldana saccharina* Walker in sugarcane using the sterile insect technique. *Ecol Model* **250**:319 – 328 (2013).
16. Dyck VA, Hendrichs J and Robinson AS, Sterile Insect Technique – Principles and Practice in Area-Wide Integrated Pest Management. Springer, Dordrecht (2005).
17. Woods MJ, Boersma N, Hoffman LCH and Pieterse E, Development of reduced cost diets for the mass rearing of false codling moth (*Thaumatotibia leucotreta*). J. S. Food Agric. **99**: 3976-3982 doi: 10.1002/jsfa.9623 (2019).
18. Waldbauer GP, The Consumption and Utilization of Food by Insects. *Adv. Insect Physiol.* **5**:229 – 288 (1968).
19. D'mello JPF and Lewis D, Amino acid interactions in chick nutrition: 3. Interdependence in amino acids requirements. *Br Poult Sci* **11**:367 – 85 (1970).
20. D'mello JPF and Lewis D, Amino acid interactions in chick nutrition: 2. Interrelationships between leucine, isoleucine and valine. *Br Poult Sci* **11**:313 – 23 (1970).
21. D'mello JPF and Lewis D, Amino acid interactions in chick nutrition: 1. The interrelationship between lysine and arginine. *Br Poult Sci* **11**:299–311 (1970).
22. Atkinson PR, Mass rearing and artificial infestation methods for *Eldana saccharina* Walker. *Proc S Afr Sug Technol Ass* **52**: 143–145 (1978).
23. Graham DY and Conlong DE, Improved laboratory rearing of *Eldana saccharina* (Lepidoptera: Pyralidae) and its indigenous parasitoid *Goniozus natalensis* (Hymenoptera: Bethylidae). *Proc S Afr Sug Technol Ass* **62**:116 – 119 (1988).
24. Graham DY, Development of mass rearing methods for *Eldana saccharina* (Lepidoptera: Pyralidae) I: Control of fungal contamination on the artificial diet. *Proc S Afr Sug Technol Ass* **64**:116 – 119 (1990).
25. Gillespie DY, Development of mass-rearing methods for the sugarcane borer *Eldana saccharina* Walker (Lepidoptera: Pyralidae) II: Diet gelling agents. *Proc S Afr Sug Technol Ass* **67**:127 – 130 (1993).
26. Walton AJ and Conlong DE, General biology of *Eldana saccharina* Walker (Lepidoptera: Pyralidae): A target for the sterile insect technique. *Fla Entomol* **99**:30 – 35 (2016)

Stellenbosch University https://scholar.sun.ac.za

27. Nagy B, Rearing of the European corn borer (*Ostrinia nubilalis* Hbn.) on a simplified diet. *Acta Phytopathol Acad Sci Hung* **5**:73 – 79 (1970).
28. Ngomane NC, Gillespie DY and Conlong DE, The effect of an improved artificial diet formulation on *Eldana saccharina Walker*, growth and development. *Proc S Afr Sug Technol Ass* **90**: 66 – 77 (2017).
29. Woods MJ, Hoffman LCH and Pieterse E, Artificial diets for neonatal Black Soldier Fly (*Hermetia illucens*) larvae. *Journal of Insects as Feed and Food* **5**:99 – 105 (2019).
30. Gous RM, Measurement of response in nutritional experiments. Nutrient Requirements of Poultry and Nutritional Research, pp. 41–57. (Fisher, C. and Boorman K.N. Eds.), Butterworths, London (1986).
31. EFGSoftware. Pietermaritzburg: EFG Software, Available from: http://efgsoftware.net (2017)
32. Cunico R, High sensitivity amino acid analysis using a novel automated precolumn derivativation system. *Bio-Chromatography* **1**: 6 – 12 (1986).
33. A.O.A.C. International. Official methods of analysis (17th ed.), Arlington, Virginia, USA: Association of Official Analytical Chemists Inc. (2002)
34. Lee CM, Trevino B. and Chaiyawat M, A simple and rapid solvent extraction method for determining total lipids in fish tissue. *J AOAC Int* **79**:487 – 492 (1996).
35. Čičková H, Newton GL, Lacy RC and Kozánek M, The use of fly larvae for organic waste treatment. *Waste Management* **35**:68 – 80 (2015).
36. Cohen AC, Insect diets: Science and technology. (2nd ed) CRC press (2015).
37. Chauhan OP, Singh D, Tyagi SM and Balyan DK, Studies on preservation of sugarcane juice. Int. J. Food Prop. **5**:217 – 229 (2002)
38. Vanderzant ES, Physical aspects of artificial diets. *Ent Exp & apll* **12**:642 – 650 (1969)
39. Horie Y and Watanabe K, Effects of various kinds of dieatry protein and supplementation with limiting amino acids on growth, haemolymph components and uric acid excretion in the silkworm, *Bombyx mori*. *J Insect Phsyiol* **29**:187 – 199 (1983).
40. Pieterse E, Siebrits F, Gloy E, Mphuloane AK, The effect of protein inclusion level in diets formulated to contain an ideal amino acid composition for growing pigs. *S Afr J Anim Sci* **30**:57 – 61 (2000).
41. Clarck AJ and Bloch K, Function of sterols in *Dermestes vulpinus*. *J Biol Chem* ***234**:2583 – 2588* (1959).
42. Clayton RB, The Utilization of Sterols By Insects. *J Lipid Res* **15**:3 –1 9 (1964).
43. Dwivedy AK, Dietary cholesterol requirements of housefly, *Musca domestica*, larvae. *J Insect Physiol* **21**:1685 – 1690 (1975).
44. Behmer S and Nes W, Insect Sterol Nutrition and Physiology: A Global Overview. *Adv*

Stellenbosch University https://scholar.sun.ac.za

Insect Physiol **31**:1 – 72 (2003).

45. Honěk A, Intraspecific variation in body size and fecundity in insects: a general relationship. *OIKOS* **66**:483 – 492 (1993).
46. Sampson MA, Kumar R, Life history, development and behaviour of *Eldana saccharina* Walker on sugar-cane in southern Ghana. *Int J Trop Insect Sci* **6**:135 – 143 (1985).
47. Teguia A and Beynen AC, Alternative feedstuffs for broilers in Cameroon. *Livest Res Rural Dev* **17**:1 – 6 (2005).

Stellenbosch University https://scholar.sun.ac.za

CHAPTER 4

Marching on: A novel approach to formulating breeder diets for the mass-rearing of black soldier fly, *Hermetia illucens* (L.) (Diptera: Stratiomyidae)

In review as: Woods, M.J., Conlong, D.E., Hoffman, L.C. and Pieterse, E., 2019. Marching on: A novel approach to formulating breeder diets for the mass-rearing of black soldier fly, *Hermetia illucens* (L.) (Diptera: Stratiomyidae). Journal of Insects as Food and Feed.

Stellenbosch University https://scholar.sun.ac.za

Abstract

This study aimed at developing a more efficient larval breeder diet for the mass-rearing of *Hermetia illucens*. The body composition technique was used to develop five diets (B1 – B5), where amino acid composition of the diets were based on the profile of amino acids in *H. illucens* larvae (IAAP), formulated with alternative raw materials. Eight-hundred grams of diet was added to 1 L containers and inoculated with 254 four-day old larvae. Larvae, prepupae and pupae were harvested at 16 days after inoculation. Survivability, larval-, prepupal- and pupal weights, rate of development and bioconversions were determined and compared to larvae reared on a commercial layer mash (CON). Differences in water holding capacity were observed with CON losing ~ 7 % more moisture compared to the IAAP diets. B1 and B5 had the highest bioconversion with CON yielding the poorest results. The higher crude fiber content of diets B1 and B5 (~ 7 %) compared to CON (2.36 %), improved the water holding capacity and in turn also improved bioconversion. Survivability was significantly higher for all IAAP diets. Diets B1 – B5 had ~ 10 % higher survivability compared to CON (85.81 %) with no differences being observed between these diets. The use of bloodmeal or poultry by-product as raw materials, at both 2 % and 5 % inclusion levels, had no effect on the overall survivability. At day 16 only 3.79 % of the larvae fed CON had pupated compared to other treatment diets where ~ 50 % of the larvae had pupated, indicating a faster rate of development. CON yielded heavier larvae compared to all treatment diets, where B1 – B5 yielded heavier pupae. B4 was ~ 50 % cheaper than CON. This study developed an artificial diet that can be used at commercial scale to increase total production efficiency and output of *H. illucens* breeders.

Keywords: nutrient requirements; survivability; pupation; bioconversion

Stellenbosch University https://scholar.sun.ac.za

Introduction

The search for sustainable feed and food ingredients has enjoyed great interest in recent times, fuelled by the environmental impact and sustainable production of soybean meal and fish meal (Smetana *et al.,* 2019). *Hermetia illucens* (L.) (Diptera: Stratiomyidae) has the ability to transform food industry side streams into intermediate products suitable for feed and food purposes (Bruno *et al.,* 2019), a process also known as up-streaming of waste products. *Hermetia illucens* larvae stop feeding before prepupation and are therefore very efficient at storing nutrients, which is then convenient to harvest (Bertinetti *et al.,* 2019). Due to their omnivorous nature *H. illucens* larvae can be reared rapidly on a wide range of substrates and are thus the preferred choice of insect for companies in the insect-mass rearing sector to work with (Meneguz *et al.,* 2018).

Breeder *H. illucens* larvae are typically reared on soaked poultry layer mash for their entire life cycle to help with uniformity and ensure high quality adults that lay large egg batches (Diener *et al.,* 2009). Mortality of larvae was found to vary between 20% and 60% when larvae where grown on soaked poultry layer mash (Woods *et al.,* 2019a). This was deemed suboptimal and could be attributed to both physical and nutritional characteristics of the diet. Poor feeding and absence of growth and eventually death is associated with insufficient or unavailable nutrients (Cohen, 2015). The nutrition of insects is thus of utmost importance for economical and efficient production, since over- and undersupply of nutrients have the same detrimental effect in production as it does in domesticated animals. The undersupply of nutrients could lead to a total break in production, while marginal undersupply could lead to immune suppression, decreased efficiency of production or a reduction in fertility and fecundity (Waldbauer, 1968). On the other hand, an oversupply of nutrients could lead to the build-up of primary or secondary metabolites which could be toxic, antagonistic or result in imbalances that could lead to metabolic stress (D'mello and Lewis, 1970a, 1970b, 1970c). Although these interactions have been widely published and are deemed to be common knowledge in animal science; it has not been investigated or published in insect nutrition.

A method that has recently drawn attention in developing artificial diets for the mass-production of insects is the body composition (whole-carcass or carcass milling) technique. (Cohen, 2015). It is thought that the composition of an insect's body reflects its nutritional needs (Rock and King, 1967). This method incorporates using the profile of various nutrients in a target insect's body as template for artificial diets. The body composition technique is a protocol used to estimate changes in the body composition of animals during an experiment. Although this method has been previously considered by Cohen (2015), most researchers lack the basic understanding of this technique and few diets have been published using this method. The body composition technique has been widely used by animal scientists to

Stellenbosch University https://scholar.sun.ac.za

determine protein and energy requirements of various domesticated animals (Blaxter, 1967). More recently this technique has been used to formulate more efficient artificial diets for *H. illucens* neonates (Woods *et al.,* 2019a), *Thaumatotibia leucotreta* (Woods *et al.,* 2019b) and *Eldana saccharina* (Woods *et al.,* 2019c).

The development of a sustainable, effective diet that meets the specific nutrient needs of the breeder *H. illucens* larvae is crucial to solidify the prospect of *H. illucens* larvae replacing conventional protein sources such as fish and soya bean meal as a sustainable option in animal diets. A breeder diet meeting the nutritional needs of the larvae in question will decrease larval mortality and cost, increase uniformity in development and effectively optimise production. Therefore, the aim of this investigation was to develop a cheaper breeder diet, using more sustainable raw materials, which will increase survivability and quality of larvae destined to become breeder *H. illucens*.

Materials and methods

Treatment diets

Table 1. A description of *Hermetia illucens* trial diets used

Treatment	Discription
CON	Diet formulated according to nutrient specifications of the post peak Hyline brown hen with feed intake of 120 g per day (control)
B1	Diet formulated to resemble ideal amino acid profile of BSF larvae
B2	Diet formulated to resemble ideal amino acid profile of BSF larvae with the inclusion of 5% poultry by-product
B3	Diet formulated to resemble ideal amino acid profile of BSF larvae with the inclusion of 2% poultry by-product
B4	Diet formulated to resemble ideal amino acid profile of BSF larvae with the inclusion of 5% blood meal
B5	Diet formulated to resemble ideal amino acid profile of BSF larvae with the inclusion of 2% blood meal

Table 1 defines the different diets tested to improve diet efficiency and cost. Since layer feed is suitable for raising *H. illucens* (Sheppard *et al*., 2002), the nutrient composition of this feed was used as the control. As such, a diet was formulated according to the nutrient specifications of a Hyline brown layer consuming 120g of feed per day (CON) using Winfeed (EFGSoftware, 2017). The diet contained only plant protein sources.

Stellenbosch University https://scholar.sun.ac.za

Table 2 Amino acid composition and ratio of amino acids (in relation to lysine) of *Hermetia illucens* larvae, determined from the body composition technique

Amino acid	***Hermetia illucens* larvae**	
	AA† content (g/100g) sample	IAAP‡ Ratio (AA†:Lys§)
Histidine	1.39	0.64
Serine	1.81	0.83
Arginine	2.07	0.95
Glycine	1.81	0.83
Aspartine	2.89	1.33
Glutamine	4.62	2.12
Threonine	1.42	0.65
Alanine	2.03	0.93
Proline	1.83	0.84
Lysine	2.18	1.00
Tyrosine	1.24	0.57
Methionine	0.60	0.28
Valine	1.72	0.79
Isoleucine	1.34	0.61
Leucine	2.16	0.99
Phenylalanine	1.14	0.52
Tryptophan	0.55	0.25
Cysteine	2.13	0.98

†AA: Amino acid
‡IAAP: Ideal amino acid profile
§Lys: Lysine

Based on the study by Woods et al., (2019a), the body composition technique was used to identify the amino acid profiles of *H. illucens* larvae (Table 2). This represents the ideal amino acid profile (IAAP) of the insect being tested, and the supply of amino acids in this ratio in a diet will result in an amino acid profile most closely representing the requirements of the animal (Gous, 1986).

Stellenbosch University https://scholar.sun.ac.za

Table 3 Ingredient and calculated nutrient composition of the experimental breeder diets used for rearing *Hermetia illucens*

	CON	B1	B2	B3	B4	B5
Ingredients (%)						
Maize	76.82	27.69	24.48	26.54	30.70	30.49
Wheat bran	--	59.95	62.78	61.10	60.63	60.19
Soybean 46	18.75	8.41	4.19	6.60	--	3.52
Blood meal	--	--	--	--	5.00	2.00
Poultry by-product meal	--	--	5.00	2.00	--	--
Dl methionine	0.65	0.31	0.27	0.30	0.31	0.32
†Vit+min premix	0.15	0.15	0.15	0.15	0.15	0.15
Limestone	1.61	1.90	1.28	1.67	2.14	2.07
Sodium chloride	0.25	0.24	0.20	0.22	0.22	0.23
Monocalcium phosphate	2.12	1.17	1.50	1.29	0.69	0.84
Sodium bicarbonate	0.24	0.18	0.15	1.17	0.17	0.18
Calculated nutritional value (%)						
Dry matter	87.60	88.13	88.16	88.14	87.99	88.01
Moisture	12.40	11.87	11.84	11.86	12.01	11.99
Crude protein	15.22	13.82	13.91	13.207	13.56	13.56
Crude fiber	2.36	7.02	7.15	7.07	6.74	6.87
Crude fat	3.20	3.58	4.20	3.83	3.64	3.64
Calcium	1.00	1.00	1.00	1.00	1.00	1.00
Phosphorous	0.87	1.14	1.33	1.21	1.00	1.50
Sodium	0.18	0.18	0.18	0.18	0.18	0.18
Potassium	0.64	1.01	0.99	1.00	0.87	0.93
‡DE (Pig) (MJ kg-1)	14.03	11.30	11.30	11.30	11.30	11.30
Lysine	0.73	0.69	0.70	0.69	0.86	0.72
Methionine	0.38	0.35	0.32	0.34	0.34	0.35
*TSAA	1.38	1.45	1.77	1.57	1.52	1.42
Tryptophan	0.26	0.20	0.20	0.20	0.21	0.19
Isoleucine	0.75	0.58	0.62	0.60	0.45	0.50
Leucine	1.53	1.09	1.13	1.10	1.38	1.17
Threonine	0.79	0.54	0.58	0.55	0.59	0.54

CON: Control diet
B1: IAAP
B2 IAAP with the inclusion of 5% poultry by-product
B3: IAAP with the inclusion of 2% poultry by-product
B4: IAAP with the inclusion of 5% blood meal
B5: IAAP with the inclusion of 2% blood meal
†Vit+min premix Broiler starter vitamin and mineral premix
‡DE: Digestible energy (MJ kg^{-1}) as determined for pigs
--: No available value
*TSAA Total Sulphur containing amino acids

The IAAP was used to formulate treatment diets B1, B2, B3, B4, and B5, with B1 using conventional raw materials, B2 / B3 including 5 % / 2 % poultry by-product and B4 / B5 including 5 % / 2 % blood-meal respectively (Table 3).

Determination of ideal amino acid profile (IAAP)

Larvae were grown on commercial layer mash in 37 L trays (648 x 348 x 209 mm) in a temperature controlled environment at 27 ± 1 °C and relative humidity of 65 ± 5 %. Fifty gram

Stellenbosch University https://scholar.sun.ac.za

grab samples of larvae were taken from ten of these respective boxes and thoroughly mixed. Larvae were removed from their feed and purged for six hours where after they were killed with steam, dried in an oven at 60 °C for 24 h (Labcon FSOH 16), homogenised using a mill (KN 195 Knifetec^{TM}), with the amino acid profile determined on subsamples (Cunico, 1986). For the amino acid analyses, a sample weighing 0.1 g was placed in a specialized hydrolysis tube. Six mL hydrochloric acid (HCl) solution and 15 % phenol solution was then added to the sample. The tubes were then vacuated and nitrogen (N) added under pressure. The tubes were subsequently sealed off with a blue flame and the samples left to hydrolyse at 110 ˚C for 24 hours. After hydrolysis, the samples were transferred to 1.5 mL Eppendorf tubes and refrigerated and sent to the Central Analytical Facility (https://www.sun.ac.za/english/faculty/science/CAF/units/mass-spectrometry) of Stellenbosch University, where the amino acid composition was determined by means of the Waters AccQ Tag Ultra Derivatization method (http://www.waters.com/waters/en_US/Amino-Acid-Analysis/nav.htm?cid=514511&locale=en_US).

Larvae growth trials

Trials were conducted at the AgriProtein (Pty, Ltd) insect production facility (Cape Town, South Africa). Six different diets were formulated with Winfeed (EFGSoftware, 2017), the dietary components mixed and moisture standardized at 72 ± 1 % by the addition of boiling water. Six treatments with ten replications were randomly allotted to 60 1 L plastic containers (156 x 166 x 77 mm). Eight-hundred grams (wet weight) of diet was added to each container. The temperature of the diet was brought to 27 ± 1 °C by leaving the diet in a controlled-environment (Laboratory, AgriProtein (Pty, Ltd); controlled at 27 ± 1 °C, relative humidity of 65 ± 5 %) overnight, before placing the larvae into the containers where after the larvae were reared under the same conditions on the diet. These experimental growth conditions were selected so as to ensure that the metabolic rate of the larvae would not slow down (Čičková *et al.,* 2015) or that the diet would be too hot or cold. Four-day old larvae, reared on the neonate diet developed by Woods *et al.* (2019a), were counted by hand and randomly allotted to one of six treatment diets (ten replicates per diet), with 254 larvae assigned to a container, the latter being placed randomly in the controlled-environment room.

Harvesting took place 16 days after the larvae were placed on the diet. Number of larvae, larval weight, number of prepupae, prepupal weight, number of pupae and pupal weight were determined. Overall survivability was determined by adding the total number of viable larvae, prepupae and pupae after the 16 day trial period. Larvae, prepupae and pupae were separated from the remaining diet by hand, weighed and counted. The remaining diet was weighed, dried at 100 °C for 24 hours in an oven, and used to determine the bioconversion rate of the diet on

Stellenbosch University https://scholar.sun.ac.za

a dry matter basis. Moisture content of the diets was also determined at harvest using a moisture analyser (Radwag PMX 50/1/NH) to evaluate the ability of the treatment diets to retain water over the trail period (water holding capacity).

Dietary costs analysis

A cost analysis was undertaken to determine whether the newly formulated treatment diets, according to the novel nutrient specifications, were more cost effective than the current commercial layer mash used to mass-rear breeder *H. illucens* larvae. The cost analysis was based on diet ingredient prices, amounts of ingredients used in the diets, larval yield and the results obtained from each diet.

Statistical analysis

Analysis of variance was performed on larval, prepupal and pupal yield and mass data, as well as on moisture holding capacity and bioconversion of diets, using the ANOVA procedures of STATISTICA (2018) with treatment (diet) as the main effect. All the parameters were tested for normality and homoscedasticity before analysis. Significance was declared at $P \leq 0.05$. Significant means were separated with Bonferroni *post hoc* test (SAS, 2009). The results were used to compare the larval, prepupal and pupal yields and weights, as well as pH and bioconversion between different treatments.

Results and discussion

Physical properties

Table 4. Moisture content and bioconversion rate of treatment diets used for rearing *H. illucens* breeder larvae

	Moisture content (%)		**Bioconversion (%) ± †SD**
	Day 1	Day 28	
CON	73	61^{b}	$62.47^{c} \pm 4.59$
B1	73	68^{a}	$71.62^{a} \pm 4.71$
B2	73	67^{a}	$66.87^{ab} \pm 5.15$
B3	73	68^{a}	$66.81^{ab} \pm 4.22$
B4	73	70^{a}	$67.84^{ab} \pm 3.95$
B5	73	71^{a}	$69.59^{a} \pm 4.22$

† Standard deviation
a,b Means within columns with different superscripts differ significantly (*P < 0.05*)
CON: Control diet
B1: IAAP
B2: IAAP with the inclusion of 5% poultry by-product
B3: IAAP with the inclusion of 2% poultry by-product
B4: IAAP with the inclusion of 5% blood meal
B5: IAAP with the inclusion of 2% blood meal

The physical properties of the diets (moisture and bioconversion) are presented in Table 4. Differences ($P < 0.05$) in water holding capacity was observed between CON and the rest of the treatment diets at the end of the trial period. CON lost the most moisture over the trial

Stellenbosch University https://scholar.sun.ac.za

period compared to all other treatment diets. No differences in water holding capacity were observed between B1, B2, B3, B4 and B5. CON lost ~ 10 % moisture compared to the other treatment diets that lost ~ 3 %. In nature, larvae have access to feed of high water content that in turn serves as their water source. Not only is a high moisture content important as source of water for the larvae, it also influences the texture of the diet. Texture is important as the larvae of *H. illucens* feed within the substrate and are legless, moving mainly by circular motion. Therefore, if the moisture content of the diet falls too low, larvae will not be able to access the nutrients provided (Woods *et al.,* 2019a). On the other hand, the presence of free water could be detrimental to the development of *H. illucens* larvae. Free water is detrimental to insect larvae that cannot move easily or for newly hatched larvae as they may drown (Vanderzant, 1969). Therefore it is important that the water incorporated in the diet is bound by the raw materials.

Differences ($P < 0.05$) in bioconversion of the diets were observed between most treatment diets (Table 4). Diets B1 and B5 had the highest bioconversion with CON yielding the poorest results. No differences were observed between diets B2, B3 and B4 with all these diets performing intermediately. Dadd (1960), McGinnis and Kasting (1960, 1966) and Mukaiyama and Ito (1962) all reported that there is direct correlation between the amount of cellulose in the diet and the bioconversion of the diet; the more cellulose in the diet the more is consumed. Diets B1 to B5 all had a higher crude fiber content (~ 7 %) compared to that of CON (2.36 %). This was due to the strategic inclusion of wheat bran at a ~ 60 % level (Table 3), as suggested by Woods *et al.* (2019a). The higher crude fiber content of diets B1 (7.02 %), B2 (7.15 %), B3 (7.07 %), B4 (6.74 %) and B5 (6.87 %) compared to CON (2.36 %) (Table 3), improved the water holding capacity of the diets and in turn also improved the bioconversion of the diets in question (Table 4). The higher bioconversions of these diets led to less wastage which will allow for cheaper and more efficient production. The incorporation of fiber into the diets of *H. illucens* breeders allowed for physical modification of the diets. As fiber is not readily digested by insects, its main purpose was to add texture to the diets so that the larvae would feed. Additionally, the fiber provided roughage which aided the passage of feed material through the gut of the larvae (Neville and Luckey, 1962).

Stellenbosch University https://scholar.sun.ac.za

Larval development

Table 5. *Hermetia illucens* production parameters from the different experimental diets measured 16 days after larval placement

Treatment	Mean Number of Larvae	Mean larval weight (g)	Mean Number of Prepupae	Mean Prepupal weight (g)	Number of Pupae	Mean Pupal weight (g)	Mean Diet Survivability (%) ± †SD	Mean Prepupation (%) ± SD	Mean Pupation (%) ± SD
CON	3.14^{a}	0.3419^{a}	216.29^{a}	0.2827	6.00^{c}	0.2132^{b}	85.81^{b} ± 8.45	84.94^{a} ± 10.33	3.79^{c} ± 8.62
B1	2.50^{b}	0.1885^{b}	128.50^{b}	0.2578	122.50^{ab}	0.2560^{a}	95.87^{a} ± 4.88	52.95^{b} ± 8.02	46.00^{ab} ± 8.82
B2	2.30^{b}	0.2576^{ab}	125.00^{b}	0.2516	126.75^{a}	0.2361^{a}	96.54^{a} ± 3.68	50.86^{b} ± 9.56	48.18^{ab} ± 9.90
B3	1.67^{b}	0.1994^{b}	120.78^{b}	0.2625	128.22^{a}	0.2408^{a}	98.69^{a} ± 3.84	48.08b^{b} ± 13.76	51.25^{a} ± 13.57
B4	1.80^{b}	0.1647^{b}	119.10^{b}	0.2604	126.30^{a}	0.2458^{a}	97.32^{a} ± 6.27	48.12b^{b} ± 9.97	51.16^{a} ± 10.41
B5	1.60^{b}	0.1703^{b}	138.20^{b}	0.2692	101.00^{ab}	0.2491^{a}	94.80^{a} ± 5.62	57.36b^{b} ± 14.07	41.96^{b} ± 14.24

† Standard deviation
a,b,c Means within columns with different superscripts differ significantly (*$P < 0.05$*)
CON: Control diet
B1: IAAP
B2: IAAP with the inclusion of 5% poultry by-product
B3: IAAP with the inclusion of 2% poultry by-product
B4: IAAP with the inclusion of 5% blood meal
B5: IAAP with the inclusion of 2% blood meal

Stellenbosch University https://scholar.sun.ac.za

The effects that the different diet formulations had on larval development through to the pupal and adult stages (survivability) are presented in Table 5. Differences ($P < 0.05$) in survivability at 20 days of age were detected between most treatment diets. Survivability was significantly higher for all newly formulated breeder diets compared to that of CON. No differences between diets B1, B2, B3, B4 and B5 were observed; these diets all had ~ 10 % higher survivability compared to CON (85.81 %). The use of blood-meal or poultry by-product as more sustainable raw materials, at both 2 % and 5 % inclusion levels, had no effect on the overall survivability indicating that these raw materials can be included at 5 % in the diets of breed *H. illucens* larvae with no adverse effects on survivability. Derived from abattoir waste, poultry by-product and blood-meal have crude protein contents of 60 % (Dong *et al.*, 1993) and 81 % (El-Sayed, 1998), respectively. Currently due to legislative restrictions they have limited application in conventional animal nutrition. Therefore these raw materials could be more effectively utilized in the formulation of *H. illucens* diets.

The inclusion of ~ 60 % wheat bran in the newly formulated diet diluted the nutrient specifications; especially in the case of energy and protein content (Table 3). In previous studies by Woods et al. (2019a, 2019b, 2019c), it was observed that insects grow more efficiently on a lower nutrient specification diet. This could be due to the fact that the oversupply of nutrients lead to metabolites which could be toxic or creates imbalances that cause metabolic stress (D'mello and Lewis, 1970a, 1970b, 1970c). Cammack and Tomberlin (2017) found that feeding *H. illucens* larvae unbalanced diets with a high protein / carbohydrate content had a negative effect on larval performance. This study was backed up by Bruno *et al.* (2019) that concluded that a diet high in protein content causes gut dysbiosis which may have contributed to reduced larval performance. Therefore, it is suggested that when formulating artificial diets for *H. illucens* the protein content of the diet should not exceed 13 %. Also, when *H. illucens* larvae are reared on biowaste, with a protein content exceeding 13 %, it should be blended with substrates with a lower protein content for dilution purposes to ensure optimum production.

Stellenbosch University https://scholar.sun.ac.za

Table 6. Comparison of amino acid profile of the IAAP of *Hermetia illucens* larvae and the amino acid ratio of the formulated diets

Amino acids	IAAP larvae	Percentage supply expressed as IAAP					
		CON	B1	B2	B3	B4	B5
Lysine	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Methionine	0.28	0.52	0.51	0.46	0.49	0.40	0.49
†TSAA	1.25	1.89	2.10	2.53	2.28	1.77	1.97
Tryptophan	0.25	0.36	0.29	0.29	0.29	0.24	0.26
Isoleucine	0.61	1.03	0.84	0.89	0.87	0.52	0.69
Leucine	0.99	2.10	1.58	1.61	1.59	1.60	1.63
Threonine	0.65	1.08	0.78	0.83	0.80	0.69	0.75

CON: Control diet
†TSAA: Total Sulphur containing amino acids (methionine and cysteine)
B1: IAAP
B2: IAAP with the inclusion of 5% poultry by-product
B3: IAAP with the inclusion of 2% poultry by-product
B4: IAAP with the inclusion of 5% blood meal
B5: IAAP with the inclusion of 2% blood meal

Moreover, not only is the protein content of the diet important for optimal production, the amino acid profile should resemble the needs of the insect. A comparison of the IAAP of *H. illucens* larvae to that supplied in the CON and B1, B2, B3, B4, B5 diets, expressed as a ratio to lysine (Table 6), indicated that most of the amino acids are oversupplied in the CON diet whilst the newly formulated diets more closely resemble that of the IAAP. Kasting and McGinnis (1960) determined that nutritional essential amino acids for Calliphoridae fly species (blow flies) are lysine, phenylalanine, valine, isoleucine, leucine and threonine, as they could not synthesize these amino acids from precursor molecules, although the order of limitation and ratio was not determined. The oversupply of most amino acids by the CON diet could lead to toxicity that cannot be alleviated except by removal by the larvae through the formation of uric acid (Waldbauer, 1968). This combined with the high crude protein level (Table 3) of the CON diet could explain why the IAAP diets yielded optimum results.

The effects that the diets had on the rate of development of the larvae are presented in Table 5. At 20 days of age, only 3.79 % of the larvae fed CON had pupated compared to that of B3 where 51.25 % of the larvae had pupated. There were no significant differences in the number of larvae that had pupated between B1, B2, B3 and B4, with B5 yielding an intermediate number of pupae and CON performing the worst. These results indicate that diets B1, B2, B3, B4 and B5 caused a much faster rate of development of the larvae, as most of the larvae in the CON treatment had only reached the prepupal stage, whereas with diets B1, B2, B3, B4 and B5 almost half of the larvae had reached their pupal stage. This implies that the nutrient requirements were more accurately met with a less nutrient dense diet.

Stellenbosch University https://scholar.sun.ac.za

The effects that diet had on larval, prepupal and pupal weights are presented in Table 5. CON yielded heavier larvae compared to all treatment diets, but this was not the case for the pupal weight. In contrast diets B1, B2, B3, B4 and B5 all yielded heavier pupae compared to CON ($P < 0.05$), with no significant difference among them. Female pupal weight is directly proportional to fecundity and therefore heavier pupae will result in a larger female and higher fecundity (Honěk, 1993), although this aspect warrants further research. No differences in prepupal weights were observed between all treatment diets (Table 5).

Diet costs

Table 7. Total dry dietary costs of the different *Hermetia illucens* experimental diets.

Dietary treatment	**Cost (kg / DM‡)**	
	ZAR	**USD†**
CON	7.48	0.52
B1	4.37	0.30
B2	3.70	0.25
B3	4.08	0.28
B4	3.06	0.20
B5	3.51	0.24

a,b,c Means within columns with different superscripts differ significantly (*P < 0.05*)
CON: Control diet
B1: IAAP
B2: IAAP with the inclusion of 5% poultry by-product
B3: IAAP with the inclusion of 2% poultry by-product
B4: IAAP with the inclusion of 5% blood meal
B5 IAAP with the inclusion of 2% blood meal
‡DM Dry matter
†USD Exchange rate of 28 / 05 / 2019 1USD = 14.50 ZAR

Diet costs (exchange rate from ZAR to USD as per 28 / 05 / 2019) as applicable to South African conditions are presented in Table 7. B1 (ZAR 4.37 / kg; USD 0.30 / kg), B2 (ZAR 3.70 / kg; USD 0.25 / kg), B3 (ZAR 4.08 / kg; USD 0.28 / kg), B4 (ZAR 3.06 / kg; USD 0.20 / kg) and B5 (ZAR / kg; USD/kg) were all cheaper than CON (ZAR 7.48 / kg; USD 0.52 / kg) with diet B4 being the cheapest. Diet B4, which yielded higher survivability and had a faster rate of development, was more than half the price compared to CON. The yield of insects from the diet should also be taken into account though, and not just the dry dietary costs, as often a diet with a higher dry dietary cost can produce many more insects than a cheaper diet, thus making the cost per insect obtained from the diet significantly less. When taking survivability into account the feed cost per larvae from day four for diet B4 was R 0.0037 (0.00026 USD) compared to R 0.0077 (0.00053 USD) for CON.

Due to the wide variety of substrates that *H. illucens* can consume it is currently the preferred insect species to mass-rear by commercial producers for feed and food (Dossey *et al.,* 2016). Although for the most part larvae are reared on biowaste, a breeding colony has to be maintained and reared on artificial diets to maximise production. High feed cost is a challenge

Stellenbosch University https://scholar.sun.ac.za

for conventional intensive animal production systems and considering the fact that mass-rearing of insects is essentially similar to these practices it is a challenge that needs to be overcome (Teguia and Beynen, 2005).

Conclusion

The nutrient requirements of *H. illucens* breeder larvae were more accurately met with a less nutrient dense diet, formulated according to the IAAP of the larvae, rather than the nutrient specifications of a Hyline brown layer consuming 120g of feed per day conventionally used as *H. illucens* breeder feed. The high inclusion level of wheat bran as source fiber in the novel diets, favourably influenced the physical properties of the diets allowing for more moisture to be retained and higher bioconversion ratios. Also, diets formulated according to the IAAP of *H. illucens* larvae were cheaper, had higher survivability and faster rate of development compared to layer mash. The study thus developed an artificial diet for the mass-rearing of *H. illucens* breeders that can be used at a commercial scale to increase total production efficiency and output. Further research could evaluate the fertility and fecundity of the *H. illucens* breeders reared on these specific diets.

Stellenbosch University https://scholar.sun.ac.za

References

Association of Official Analytical Chemists (AOAC), 2002. Official methods of analysis (17th ed.), AOAC, Arlington, Virginia, USA. Inc.

Atkinson, P.R., 1978. Mass rearing and artificial infestation methods for *Eldana saccharina* Walker. Proceedings of the South African Sugar Technologists' Association-June: 143.

Bertinetti, C., Samayoa, A.C., and Hwang, S.Y., 2019. Effects of Feeding Adults of *Hermetia illucens* (Diptera: Stratiomyidae) on Longevity, Oviposition, and Egg Hatchability: Insights into optimizing egg production. Journal of Insect Science 19(1): 19.

Blaxter, K.L., 1967. Techniques in energy metabolism studies and their limitations. Proceedings of the Nutrition Society 26(1): 86-96.

Bruno, D., Bonelli, M., De Filippis, F., Di Lelio, I., Tettamanti, G., Casartelli, M., Ercolini, D. and Caccia, S., 2019. The intestinal microbiota of *Hermetia illucens* larvae is affected by diet and shows a diverse composition in the different midgut regions. Applied and Environmental Microbiology 85(2): e01864-18.

Cammack, J. and Tomberlin, J., 2017. The impact of diet protein and carbohydrate on select life-history traits of the black soldier fly *Hermetia illucens* (L.) (Diptera: Stratiomyidae). Insects 8(2): 56.

Čičková, H., Newton, G.L., Lacy, R.C. and Kozánek, M., 2015. The use of fly larvae for organic waste treatment. Waste management 35: 68-80.

Cohen, A.C., 2015. Insect diets: science and technology. CRC press.

Cunico, R., 1986. High sensitivity amino acid analysis using a novel automated precolumn derivativation system. Bio-Chromatography 1: 6-12.

Dadd, R.H., 1960. Observations on the palatability and utilisation of food by locusts, with particular reference to the interpretation of performances in growth trials using synthetic diets. Entomologia experimentalis et applicata *3*(4): 283-304.

Diener, S., Zurbrügg, C. and Tockner, K., 2009. Conversion of organic material by black soldier fly larvae: establishing optimal feeding rates. Waste Management and Research 27(6): 603–610. doi: 10.1177/0734242X09103838.

D'mello, J.P.F. and Lewis, D., 1970a. Amino acid interactions in chick nutrition: I. The interrelationship between lysine and arginine. British Poultry Science 11(3): 299-311.

D'mello, J.P.F. and Lewis, D., 1970b. Amino acid interactions in chick nutrition: 2. Interrelationships between leucine, isoleucine and valine. British Poultry Science 11(3): 313-323.

D'mello, J.P.F. and Lewis, D., 1970c. Amino acid interactions in chick nutrition: 3. Interdependence in amino acid requirements. British Poultry Science 11(3): 367-385.

Stellenbosch University https://scholar.sun.ac.za

Dong, F.M., Hardy, R.W., Haard, N.F., Barrows, F.T., Rasco, B.A., Fairgrieve, W.T. and Forster, I.P., 1993. Chemical composition and protein digestibility of poultry by-product meals for salmonid diets. Aquaculture 116(2-3): 149-158.

Dossey, A.T., Tatum, J.T. and McGill, W.L., 2016. Modern insect-based food industry: current status, insect processing technology, and recommendations moving forward. In *Insects as Sustainable Food Ingredients* 113-152. Academic Press.

EFGSoftware, 2017. Pietermaritzburg: EFG Software, Available from: http://efgsoftware.net

El-Sayed, A.F., 1998. Total replacement of fish meal with animal protein sources in Nile tilapia, *Oreochromis niloticus* (L.), feeds. Aquaculture research 29(4): 275-280.

Gous, R.M., 1986. Measurement of response in nutritional experiments. Nutrient Requirements of Poultry and Nutritional Research, pp. 41–57. (Fisher, C. and Boorman K.N. Eds.), Butterworths, London.

Honěk, A., 1993. Intraspecific variation in body size and fecundity in insects: a general relationship. Oikos: 483-492.

Kasting, R. and McGinnis, A.J., 1960. Use of glutamic acid-U-C14 to determine nutritionally essential amino acids for larvae of the blow fly, *Phormia regina*. Canadian Journal of Biochemistry and Physiology 38(11): 1229-1234.

McGinnis, A. J. and Kasting, R., 1960. Nutrition of the pale western cutworm, *Agrotis orthogonia* Morr. (Lepidoptera: Noctuidae): III. Lypohilized sprouts and leaves of wheat as a basal diet for larvae, and effects of supplementation with L-Leucine. Canadian Journal of Zoology 38(3): 585-592.

McGinnis, A.J. and Kasting, R., 1966. Comparison of tissues from solid-and hollow-stemmed spring wheats during growth-IV. Apparent dry matter utilization and nitrogen balance in the two-striped grasshopper, *Melanoplus bivittatus* (Say). Journal of Insect Physiology 12(6): 671-678.

McGinnis, A.J. and Kasting, R., 1967. Dietary cellulose: effect on food consumption and growth of a grasshopper. Canadian Journal of Zoology 45(3): 165-167.

Meneguz, M., Schiavone, A., Gai, F., Dama, A., Lussiana, C., Renna, M. and Gasco, L., 2018. Effect of rearing substrate on growth performance, waste reduction efficiency and chemical composition of black soldier fly (*Hermetia illucens*) larvae. Journal of the Science of Food and Agriculture 98(15): 5776-5784.

Mukaiyama, F. and Ito, T., 1962. Digestion experiments in the silkworm, *Bombyx mori*, by means of artificial diets. The Journal of Sericultural Science of Japan 31(6): 398-406.

Neville, P.F. and Luckey, T.D., 1962. Carbohydrate and roughage requirement of the cricket, *Acheta domesticus*. The Journal of Nutrition 78(2): 139-146.

Stellenbosch University https://scholar.sun.ac.za

Rock, G.C. and King, K.W., 1967. Quantitative amino acid requirements of the red-banded leaf roller, *Argyrotaenia velutinana* (Lepidoptera: Tortricidae). Journal of Insect Physiology 13(2): 175-186.

Sheppard, D.C., Tomberlin, J.K., Joyce, J.A., Kiser, B.C. and Sumner, S.M., 2002. Rearing methods for the black soldier fly (Diptera: Stratiomyidae). Journal of Medical Entomology 39(4): 695-698.

Smetana, S., Schmitt, E. and Mathys, A., 2019. Sustainable use of *Hermetia illucens* insect biomass for feed and food: Attributional and consequential life cycle assessment. Resources, Conservation and Recycling 144: 285-296.

STATISTICA 13 (TIBCO Software Inc. 2018). Statistica (data analysis software system), version 13. http://tibco.com.)

Teguia, A. and Beynen, A.C., 2005. Alternative feedstuffs for broilers in Cameroon. *Livestock* Research for Rural Development 17: 3.

Vanderzant, E.S., 1969. Physical aspects of artificial diets. Entomologia Experimentalis et Applicata 12(5): 642-650.

Waldbauer, G.P., 1968. The consumption and utilization of food by insects. In Advances in insect physiology 5: 229-288. Academic Press.

Woods, M.J., Hoffman, L.C. and Pieterse, E., 2019a. Artificial diets for neonatal black soldier fly (Hermetia illucens) larvae. Journal of Insects as Food and Feed 5(2): 99-105. Doi: 10.3920/JIFF2018.0028

Woods, M.J., Boersma, N., Hoffman, L.C. and Pieterse, E., 2019b. Development of reduced cost diets for the mass rearing of false codling moth (*Thaumatotibia leucotreta*). Journal of the Science of Food and Agriculture, 99, 3976-3982. doi: 10.1002/jsfa.9623

Woods, M.J., Conlong, D.E., Hoffman, L.C. and Pieterse, E., 2019c. The development of an improved artificial diet for the mass-rearing of *Eldana saccharina* Walker (Lepidoptera: Pyralidae). Journal of the Science of Food and Agriculture (In Review).

Stellenbosch University https://scholar.sun.ac.za

CHAPTER 5

Hermetia illucens larvae reared on different substrates in broiler quail diets: effect on apparent digestibility, feed-choice and growth performance

Published as: Woods, M.J., Cullere, M., Van Emmenes, L., Vincenzi, S., Pieterse, E., Hoffman, L.C. and Zotte, A.D., 2019. *Hermetia illucens* larvae reared on different substrates in broiler quail diets: Effect on apparent digestibility, feed-choice and growth performance. Journal of Insects as Food and Feed 5(2): 89-98.

Stellenbosch University https://scholar.sun.ac.za

Abstract

This research is aimed at improving the fatty acid profile of *Hermetia illucens* larvae and evaluating the effect of its inclusion on the apparent nutrient digestibility, feed choice, growth performance and slaughter traits of growing broiler quails (*Coturnix coturnix japonica*). *Hermetia illucens* larvae (IM) were reared on two different substrates: layer mash (IM1) and 50 : 50 layer mash : fish offal (IM2). For the digestibility and feed choice trials, a total of sixty 16-day-old quails were assigned to three dietary groups: commercial diet (Control = C), a diet including 10 % IM1 (IM1D), and a diet including 10 % IM2 (IM2D). For the growth performance trial, a total of three hundred 10-day-old birds were allocated to the three dietary groups and fed the experimental diets until slaughter. Results of the digestibility trial showed a higher apparent metabolizable energy (AME) for larvae fed quail (14.0 and 13.9 MJ/kg DM *vs* 12.9 MJ/kg DM, ($P < 0.001$). The IM2D quails also showed higher apparent digestibility for dry matter and organic matter. Feed choice results indicated that quails preferred the C diet compared to diets including *Hermetia illucens* dried larvae. Productive performance, mortality and carcass traits were in line with commercial standards except for the IM2 quails which exhibited lower slaughter weight compared to C and IM1 fed quails. Based on the results of the present study, a 10 % dietary inclusion of *Hermetia illucens* larvae reared on a substrate rich in n-3 FA did not negatively affect the apparent digestibility of nutrients, mortality, nor carcass yield. However, feed choice, growth rate and final carcass weight were negatively influenced by the IM2 diet. This result requires further investigations which should include the addition of an anti-oxidant.

Keywords: black soldier fly; substrate modulation; insect protein; larvae meal

Implications

Hermetia illucens larvae are a promising candidate in the perspective to reduce the feed-food competition and to improve the sustainability of the livestock sector. Moreover, when such larvae are farmed on a n-3 enriched diet, their nutritional quality can be further enhanced which is important to provide healthy meat for consumers. Results of this research show that the inclusion of n-3 enriched larvae in the diet for broiler quails is technically feasible and provides positive productive results, but less satisfactory compared to those given by quails fed with larvae farmed on a conventional substrate. Therefore, further research efforts are required.

Stellenbosch University https://scholar.sun.ac.za

Introduction

Soybean is one of the main feed ingredients for livestock and, for the poultry sector, it is used both as a protein and a fat source. However, soy requires substantial land and water to grow and therefore has a strong environmental impact (Charlton *et al.,* 2015). Furthermore, the soy currently used in European poultry farming is mainly imported from the United States, Argentina and Brazil (Boerema *et al.,* 2016) and, soy also being a food source for humans, the feed-food competition is predicted to increase soon; hence prices for this commodity will increase. This is caused by the growing world population and the consequent perspective of a growing global agricultural output to satisfy the increasing demand for food and livestock products (van Huis, 2013). For all the above-mentioned reasons, the poultry sector is currently looking towards alternative feed ingredients.

In the context of an impellent search for new, sustainable and valuable feed ingredients for livestock and poultry, insects represent a great opportunity. Among insect species, the black soldier fly (*Hermetia illucens*) is considered one of the most promising to be used for industrial production. *Hermetia illucens* larvae meal showed to be an excellent source of apparent metabolizable energy and digestible amino acids for broilers (De Marco *et al.,* 2015; Schiavone *et al.,* 2017a). In fact, when different inclusion levels of defatted black soldier fly larvae meals replaced fish meal in the diet for laying quails (Widjastuti *et al.,* 2014) and substituted soybean oil/meal in the diet for broiler quails (Cullere *et al.,* 2016 and 2017) or Barbary partridge (Loponte *et al.,* 2017), no negative effects on the studied variables were observed.

Recent trials were also conducted to evaluate the potential of the fat extracted from *H. illucens* larvae to replace vegetable oils. Specifically, encouraging results were observed in broiler chickens fed with *H. illucens* prepupae fat in substitution to the soybean oil (Schiavone *et al.,* 2017b and 2017c).

However, a drawback related to the use of *H. illucens* larvae or the derived products (meal and oil) in poultry diets was highlighted in all these studies when the fatty acid profile of the poultry meat was analysed. In fact, the fatty acid profile of *H. illucens* larvae is peculiar, being notably rich in saturated fatty acids and with a high n-6/n-3 ratio. Consequently, poultry species being monogastric animals, the fatty acid profile of meat derived when fed with diets containing *H. illucens* is sub-optimal in providing healthy meat for the modern consumer (Cullere *et al.,* 2017; Schiavone *et al.,* 2017c).

Overall, the lipid content of insects is largely dependent on their diet and stage of development. Moreover, the chemical composition of *H. illucens* larvae can theoretically be manipulated through diet as they are considered monogastric animals. Consequently, an appropriate

Stellenbosch University https://scholar.sun.ac.za

growth substrate as well as the time of harvest could hypothetically improve their nutrients composition, which was partly demonstrated by some recent studies (Tschirner and Simon, 2015; Spranghers *et al.,* 2017). In addition, in the only two trials aiming to improve the fatty acids profile of *H. illucens* larvae by modulating the rearing substrate, it was shown that the larvae could successfully incorporate EPA and DHA n-3 fatty acids (St-Hilaire *et al.,* 2007; Barroso *et al.,* 2017). However, no data deriving from *in vivo* studies on poultry species fed with larvae enriched in n-3 fatty acids have been conducted until now.

Starting from these premises, the present research studied the effect of a dietary inclusion of *H. illucens* dried larvae reared either on a conventional substrate or on a substrate enriched in n-3 polyunsaturated fatty acids on broiler quails' apparent nutrient digestibility, feed choice, growth performance, slaughter traits, carcass and meat yields.

Material and methods

Insect farming

Black soldier fly (*H. illucens*) larvae were harvested at Agriprotein (Pty) Ltd (Cape Town, South Africa). The larvae were reared on two different substrates: a commercial chicken layer mash (IM1), and a substrate made of 50 % chicken layer mash : 50 % fish offal (IM2). Fish offal was provided by I&J commercial fishing company (Cape Town, South Africa) and analysed for heavy metals content on an ICP-MS as described by Bosch *et al.* (2016). After 18 days feeding, larvae were harvested, separated from the remaining residue by using a saturated salt solution in which the boxes were emptied, and collected (further details can be found in the Supplementary materials file). Subsequently, larvae were thoroughly washed with water to remove any residual salt and then dried in a commercial oven set at 65 °C for 16 hours. Dried larvae were then finely ground, vacuum-sealed and couriered to the Department of Animal Medicine, Production and Health (MAPS) of the University of Padova (Italy). The chemical composition, gross energy (GE) content, amino acids profile and main fatty acid classes of the larvae reared on IM1 and IM2 substrates are shown in Table 1.

Performance trial

The production trial was conducted at a private quail farm ("La Colombara" Società Agricola, Castelnovo di Isola Vicentina, Italy): for the experiment, a total of three hundred 10-day-old broiler quails (*Coturnix coturnix japonica*) of both sexes were weighed, marked and housed in batteries in an environmentally controlled room. The chicks (20 per cage) were allocated to 15 cages and received three dietary treatments (five replicates per treatment) until being slaughtered, at 29 days of age: a Control diet (C) which was formulated according to the common grower diet used on the farm, IM1 and IM2 diets in which conventional protein/fat sources were partly substituted with 10 % IM reared on the two different substrates. All diets

Stellenbosch University https://scholar.sun.ac.za

were formulated to meet the minimum requirements for Japanese quails (National Research Council, 1994). Ingredients, chemical composition and energy content of the diets are shown in Tables 2 and 3, respectively. Mashed feeds and water were provided *ad libitum*. Mortality was monitored daily. Individual live weight (LW) was measured at the end of the experimental period whereas feed intake (FI) was measured within replicate throughout the trial. Body weight gain (BWG) and feed conversion ratio (FCR) were then calculated.

Digestibility trial

A total of sixty 16-day-old broiler quails were randomly selected from a large commercial flock and destined to an *in vivo* digestibility trial. These quails were different to those used for the performance trial. Digestibility cages were provided by the MAPS Department of Padova University (Italy). Quails were individually weighed and divided into three experimental feeding groups (C, IM1D and IM2D) with five replication each (four quails/replication). Each replication had similar mean live weight and standard deviation (592 ± 16.2 g) and was balanced for gender. Quails were then subjected to a 10-day adaptation period to the experimental diets during which individual feed intake was determined. At the end of adaptation period and after 12 h fasting, quails were weighed again and then fed their corresponding experimental diet for 3 days followed by another 12 h of fasting, so that the feed intake and excreta could be accurately determined. The excreta samples were collected daily from each cage, carefully cleaned of feathers and feed, weighed, then promptly chilled. The total excreta, separated by cage, were freeze-dried, ground and stored at +4 °C until further analysis.

Feed choice test

After the digestibility trial, a total of fifteen 31-day-old male quails of similar LW were selected from the same 60 birds involved in the digestibility trial, individually caged, and simultaneously provided with three feeders containing C, IM1D and IM2D diets. After 10 days of adaptation to the new feeding condition, a 10-day feed choice trial was carried out. Feed and water were provided *ad libitum*. Feeders were placed in complete randomized order and their position within the cage was changed every 3 days. At the end of the experiment, the feed consumed from each feeder was measured on the cage basis. Free choice was expressed as grams of dry matter (DM) per 100 g of LW. Birds used for the digestibility trial and free choice test were returned to the farmer after completion of the experiments.

Chemical analysis of the diets and the excreta

Analyses of IM1 and IM2 dried larvae, experimental diets and freeze-dried excreta were carried out in duplicate using AOAC (2012) methods to determine DM (method 934.01), crude protein (CP, method 2001.11), crude fibre (CF, method 978.10), ash (method 967.05) and starch (amyloglucosidase-alpha-amylase, method 996.11) contents. Crude fat was

Stellenbosch University https://scholar.sun.ac.za

determined after acid hydrolysis (EC, 1998). The methods to analyse the mineral content of the IM1, IM2 larvae and of the experimental diets were: method 968.08 (Ca and Mg), method 995.11 (P), method 956.01 (Na), method 975.03 (K), and method 943.01 (Cl) of the AOAC (2012). Gross energy of IM1, IM2 larvae, experimental diets and freeze-dried excreta was analysed with an adiabatic bomb calorimeter (ISO, 1998). The amino acid content of IM1 and IM2 larvae was analysed with a method developed by Grace Davison Discovery Sciences using High Performance Liquid Chromatography (HPLC). Lipid extraction and the subsequent fatty acid profile analysis of IM1 and IM2 larvae were performed following the method described by Cullere *et al.* (2016) for the defatted *H. illucens* larvae meal.

Crude protein content of excreta was corrected for uric acid content which was analysed according the procedure described by Fievez *et al.* (2001) with the modification reported in Cullere *et al.* (2016). Urinary nitrogen was estimated at 1.2 times uric acid content (Terpstra and de Hart, 1973). Experimental diets and freeze-dried excreta were analysed in triplicate for chitin content following the method by Zhang and Zhu (2006) with the modifications described in the Supplementary materials file.

Slaughtering, carcass dissection and meat yields

At 29 days of age, after feed removal, quails of the performance trial were individually weighed (slaughter weight, SW) and transported to a commercial slaughterhouse (Quaja Veneta® Società Cooperativa Agricola, Malo, VI, Italy) located 8 km from the farm. After 6 hours fasting (from feed withdrawal until slaughtering), all birds were electrically stunned and processed under commercial conditions. Carcasses were bled, plucked, eviscerated and the head, neck, shanks and abdominal fat were removed. After one hour in the refrigeration tunnel (+2 °C), all carcasses were transported in chilled conditions to the MAPS Department of the University of Padova and stored at +2 °C.

The following day, carcasses were individually weighed (CW) and dressing percentage was calculated as a percentage of the SW. Breast muscle was excised and the yield as a percentage of CW was then determined.

Statistical analysis of results

Growth performance, carcass and breast meat yields, nutrients' apparent digestibility and nutritive values of the diets were subjected to a one-way analysis of variance (ANOVA) with experimental diet (Control, IM1D and IM2D) as fixed effect, following the general linear model (GLM) procedure of the SAS 9.1.3 statistical analysis software for Windows (SAS Institute, 2008). The experimental unit was the cage. A chi-squared test with the Marascuilo (1966) procedure was performed on mortality to detect differences among treatments. For nutrients' apparent digestibility and the nutritive value of the diets, the model considered the

Stellenbosch University https://scholar.sun.ac.za

experimental diet as main factor. An one-way ANOVA of the GLM procedures of SAS was then performed that studied the effect of the experimental diet on the individual feed consumption. Differences were considered significant when $P \leq 0.05$.

Stellenbosch University https://scholar.sun.ac.za

Table 1. Chemical composition, gross energy content, amino acid concentration (g/kg as fed) and main fatty acid classes (% of total fatty acids) of the two *Hermetia illucens* dried larvae (IM1, IM2) reared on different substrates

	IM1[1]	IM2[2]
Dry matter (DM)	934.9	940.1
Crude protein	336.6	343.1
Crude fat	342.0	366.9
Crude fibre	55.4	51.3
Chitin	24.8	24.2
Ash	135.2	99.2
Ca	43.4	29.1
P	5.91	5.75
Gross Energy (MJ/kg)	22.63	23.36
Indispensable amino acids:		
Arginine	19.1	37.9
Histidine	13.4	21.2
Isoleucine	13.1	15.3
Leucine	20.4	21.8
Lysine	18.8	22.4
Methionine	5.0	6.3
Phenylalanine	13.9	21.4
Threonine	13.3	16.5
Valine	17.3	21.2
Dispensable amino acids:		
Alanine	20.6	15.3
Aspartic acid	30.3	6.8
Glycine	45.3	26.7
Glutamic acid	13.4	21.2
Proline	17.6	32.0
Serine	13.3	18.1
Tyrosine	16.0	17.6
Fatty acid classes:		
Saturated fatty acids (SFA)	68.9	72.0
Monounsaturated fatty acids (MUFA)	17.7	18.7
Polyunsaturated fatty acids (PUFA)	12.6	6.99
n-6	12.1	5.42
n-3	0.53	1.57
n-6/n-3	22.9	3.45

[1] IM1 = Dried and ground *Hermetia illucens* larvae reared on layer mash.

[2] IM2 = Dried and ground *Hermetia illucens* larvae reared on 50:50 layer mash and fish offal.

Heavy metals content of fish offal (mg/kg): Al = 39.34; Cr = 1.02; Mn = 169.6; Fe = 378.85; Cu = 10.31; Zn = 106.77; As = 4.89: Cd = 0.17; Hg = 0.14; Pb = 0.22.

Stellenbosch University https://scholar.sun.ac.za

Table 2. Ingredients of the experimental diets[1] (g/kg as fed)

Ingredients	Experimental diets[1]		
	C	IM1D	IM2D
Ground corn	435.6	450.5	434.8
Soybean meal	460.4	377.0	373.5
Dried *Hermetia illucens* larvae (IM)	0.0	100	100
Whole wheat	23.5	42.5	61.7
Calcium carbonate	21.5	20.0	20.0
NaCl	2.70	2.70	2.70
L-Lysine	0.50	0.50	0.50
DL-Methionine	1.80	1.80	1.80
Vitamin-Mineral premix[2]	5.00	5.00	5.00
Soybean oil	49.0	0.0	0.0

[1] Control = control diet; IM1D = diet supplemented with 10 % of dried *Hermetia illucens* larvae reared on layer mash; IM2D = diet supplemented with 10 % of dried *Hermetia illucens* larvae reared on 50:50 layer mash and fish offal.

[2] Vitamin and mineral premix provided the following per kg of diet: vitamin A, 20,000 IU; vitamin D3, 6000 IU; vitamin E (α-tocopherol acetate), 90 mg; vitamin K3, 7 mg; vitamin B1, 3.5 mg; vitamin B2, 16 mg; niacinamide, 100 mg; vitamin B6, 8 mg; Vitamin B12, 0.04 mg; biotin, 0.4 mg; folic acid, 2.5 mg; Ca-panthotenate, 27.78 mg; Fe, 80 mg; Mn, 200 mg; Cu, 50 mg; Zn, 200 mg; Ca-iodate, 2 mg/kg; Se, 0.4 mg; E 1604 Endo 14, 2200 U; Endo 1, 3000 FTU; Sepiolite, 175 mg.

Table 3. Chemical composition, mineral and gross energy contents of the experimental diets (g/kg as fed)

	Experimental diets[1]		
	Control	IM1D	IM2D
Dry matter (DM)	901.1	899.6	901.0
Crude protein	243.3	238.9	239.8
Crude fat	59.8	56.9	55.9
Chitin	0.0	3.93	3.32
Ash	63.2	71.8	65.3
Starch	282.1	314.4	312.6
Ca	12.1	17.4	14.1
P	3.80	3.78	3.55
Na	1.46	1.81	2.01
Cl	2.24	4.06	4.00
K	12.2	10.2	9.40
Mg	2.45	2.27	2.13
Gross Energy (MJ/kg)	17.43	17.85	17.81

[1] Control = control diet; IM1D = diet supplemented with 10 % of dried *Hermetia illucens* larvae reared on layer mash; IM2D = diet supplemented with 10 % of dried *Hermetia illucens* larvae reared on 50:50 layer mash and fish offal.

Stellenbosch University https://scholar.sun.ac.za

Results and Discussion

The results of the chemical composition of the two dried *H. illucens* larvae reared on different substrates partly confirmed the previous findings (Tschirner and Simon, 2015; Spranghers *et al.,* 2016); crude fat and ash contents of the larvae were modified by the growing substrate, whereas their crude protein content remained constant. The crude protein contents of the IM1 and IM2 larvae were in line with values found by Tschirner and Simon (2015), but slightly lower compared to the range provided by Makkar *et al.* (2014) and Spranghers *et al.* (2016).

The amino acid profile of the two dried *H. illucens* larvae reared on different growing substrates differed markedly, and also differed from that reported by Spranghers *et al.* (2016) considering chicken feed, digestate, vegetable waste and restaurant waste as growing substrates. In fact, the addition of fish offal improved the amino acid content of black soldier fly larvae (Treatment IM2), as it led to higher amounts of all the indispensable amino acids, when compared to the IM1 larvae, reared on a substrate made of 100 % layer mash. As for the amino acid profile of the dried IM1 larvae, it is similar to values reported by De Marco *et al.* (2015) for a full-fat *H. illucens* larvae meal, for both indispensable and dispensable amino acids.

The average chitin content of both IM1 and IM2 larvae was only 24.5 g/kg as fed, lower than values (56-67 g/kg as fed) commonly found in literature (Spranghers *et al.,* 2016), which is important for better absorption of nutrients from the diet. This result can probably be ascribed to the fact that larvae were collected before the prepupae instar, thus having less chitin in the exoskeleton.

The addition of fish offal to the normal growing substrate influenced the main fatty acid classes of the larvae. The saturated fatty acids (SFA) proportion increased (72.0 *vs* 68.9 % SFA for IM2 and IM1 larvae, respectively), whereas total polyunsaturated fatty acids (PUFA) decreased (6.99 *vs* 12.6 % PUFA for IM2 and IM1 larvae, respectively). However, such reduction involved only the n-6 PUFA whereas the n-3 PUFA fraction showed a three-fold increase (1.57 *vs* 0.53 % n-3 PUFA for IM2 and IM1 larvae, respectively), resulting in a nutritionally valuable improvement of the n-6/n-3 ratio (3.45 *vs* 22.9 for IM2 and IM1 larvae, respectively). Thus, the main objective of the substrate modulation has been successful. Such findings were coherent with those presented by St-Hilaire *et al.* (2007) who found that adding different proportions of fish offal from rainbow trout to cow manure, substantially enriched the larval content of n-3 fatty acids. Despite this, the enrichment of n-3 PUFA was much lower in the present study compared to the previous cited (0.23 *vs* 2.99 % n-3 PUFA for larvae fed on 100 % cow manure substrate *vs* larvae fed on 50 % cow manure + 50 % fish offal, respectively). Such difference could be explained by the different fish offal used in the two

Stellenbosch University https://scholar.sun.ac.za

studies, with that of the present research being an undefined mixture coming from different marine fish species.

A further key aspect that should be considered, when using fish offal as growing substrate for insects, is its heavy metals content. In fact, Directive 2002/32/EG (EC, 2002) allows a maximum amount in feedstuffs of 2 mg/kg feed for Cd and 10 mg/kg feed for Pb. The bioaccumulation of some heavy metals by black soldier fly larvae was previously studied by Diener *et al.* (2015) and it was observed that Cd readily accumulates into the insect body through the Ca^{2+} channel, as Cd^{2+} ions have very similar ionic radii. Conversely, Zn seemed to be actively regulated and Pb tended to be transported and immobilised in the exoskeleton. In the present experiment, the concentration of heavy metals in fish offal was 0.17, 106.8 and 0.22 mg/kg for Cd, Zn and Pb, respectively. These concentrations are far below the lower levels tested in the above cited experiment on bioaccumulation, and therefore they can be considered acceptable and safe.

Productive performances of growing broiler quails were overall consistent with commercial standards but slightly different compared to those presented by Cullere *et al.* (2016). At the end of the trial, quails fed the C and IM1D diets showed higher slaughter weight and body weight gain (BWG) ($P < 0.0001$) compared to quails fed the IM2D diet. However, no differences ($P > 0.05$) for feed intake (FI), feed conversion ratio (FCR), and for overall mortality, were observed among the three experimental groups (Table 4). The lower performance of quails fed the IM2 diet compared to the other dietary treatments was not expected. In fact, the chemical composition of the two diets with dried *H. illucens* larvae were very close to each other in terms of crude protein, crude fat, starch and overall gross energy contents. Results from the feed choice trial highlighted that the C diet was the most preferred by quails, but no difference was observed between IM1D and IM2D ($P < 0.001$). Therefore, it was hypothesized that the marked fish aroma of the IM2 made the IM2D less palatable compared to the other two diets, and results of the performance trial seemed to corroborate this hypothesis, as IM2D quails exhibited a tendency ($P = 0.0529$) towards a reduced feed intake. It was also observed that IM1D and IM2D had a darker colour compared to C which could have negatively affected feed choice. Since the IM2 larvae showed a three-fold increase in n-3 PUFA it could have been more prone to oxidative damages and therefore off-odours. The avian taste system is generally well developed but it can have a great variability among different species because of various feeding patterns and strategies. As reviewed by Roura *et al.* (2013), such differences reflect on a different number of taste buds and the size of the bitter taste receptors gene repertoire. Variability could also be observed within the same bird species, as different chicken breeds can display different sensitivities to the bitter taste in function of the total number of taste buds. Chickens have been reported to perceive also

Stellenbosch University https://scholar.sun.ac.za

sweet, salty, sour, fat taste and calcium, with avoidance, preference and lowest-highest limits depending on several factors i.e. the age of the bird, physiological status, etc. The experimental diets of the present trial also differed in starch content and some minerals such as Na, K and Cl thus probably affecting the palatability of feed. However, such hypothesis found no confirmation in previous literature, as data on taste patterns of Japanese quail are scarce. Only a preference for sucrose was reported in a previous study considering this avian species (Harriman and Milner, 1969), thus suggesting that sugars may play a key role in the feeding strategy of quails.

Table 4. Effect of the dietary inclusion of dried *Hermetia illucens* larvae reared on two different substrates on the live performance and mortality of broiler quails

	Experimental groups			*P*-value	RSD[1]
	Control	IM1D	IM2D		
No. (initial)	100	100	100		
LW (g)					
Initial weight (10 days)	77.8	77.7	77.7	0.9785	4.07
Slaughter weight (29 days)	240.6[a]	247.6[a]	228.7[b]	<0.0001	22.83
BWG (g/day)	9.0[a]	9.4[a]	8.4[b]	<0.0001	1.21
FI, (g/day)[2]	29.1	29.9	27.5	0.0529	1.39
FCR[2]	3.4	3.4	3.5	0.8434	0.28
Mortality (%)	1	1	3	0.6388	1.63

[1] RSD= Residual Standard Deviation.

LW = live weight.

[2] Five replicates per treatment.

[a,b] Means in a row with different superscripts differ significantly.

At the end of the digestibility trial, quails showed the same final LW, DM and water intake, however experimental groups differed in terms of excreta production (Table 5). In fact, IM1D and IM2D quails produced less excreta than Control birds ($P < 0.05$). Moreover, IM2D quails showed a higher apparent digestibility of DM ($P < 0.05$) and organic matter ($P < 0.05$) compared to C, with IM1D being intermediate. Starch was better digested in IM1D compared to C ($P < 0.01$) but did not differ from IM2D. On the other hand, IM2D quails showed a lower EE apparent digestibility than C ($P < 0.05$) but did not differ from IM1D. Quails belonging to the IM1D and IM2D groups displayed the highest gross energy apparent digestibility ($P < 0.05$) with the same trend being observed for the metabolizable energy ($P < 0.001$). Analysing these results, a further hypothesis to explain the lower productive performance of IM2D quails compared to the other groups is suggested. The IM2D quails exhibited a tendency ($P = 0.0556$) towards a lower apparent digestibility of chitin compared to IM1D which probably had an effect in determining a lower apparent digestibility of the ether extract. Chitin is known to have a negative effect on the digestibility of protein and lipid fractions, which is particularly evident with increasing absolute chitin content or when animals have no chitinolytic activity

Stellenbosch University https://scholar.sun.ac.za

(Kroeckel *et al.,* 2012). In fact, even though it was not significant (P = 0.3068), the apparent digestibility of crude protein was ~10.5 % lower in IM2D quails compared to IM1D ones. Another factor that could have contributed to the low digestibility of ether extract in the IM2D could be related to the different fatty acids composition of the larvae which had higher saturated fatty acids (SFA) and lower polyusaturated fatty acids (PUFA) compared to those in the IM1D. For instance, it is known that the assimilation of dietary fats by chickens and turkeys is higher for unsaturated (UFA) than for SFA. This phenomenon is due to UFA spontaneously forming mixed micelles with monoglycerides and conjugated bile salts which are then easily transported to the mucosal surface and finally absorbed by the small intestine (Tancharoenrat *et al.,* 2014). On the other hand, SFA are non-polar, therefore requiring appropriate amount of bile salts for an efficient emulsification. Because of this, their incorporation into micelles is slower compared to UFA (Zhang *et al.,* 2011).

Table 5. Effect of the dietary inclusion of dried *Hermetia illucens* larvae reared on two different substrates on the quail nutrients' apparent digestibility and nutritive value of diets and feed choice

	Experimental groups			*P*-value	RSD[1]
	Control	IM1D	IM2D		
No.	20	20	20		
Initial live weight (LW) (g)[2]	600	596	579	0.0904	14.30
Final LW (g)	703	698	682	0.3066	21.33
Average LW (g)	651	647	631	0.1866	17.58
Dry Matter (DM) intake (g)	314.5	309.6	314.6	0.8651	16.57
DM intake (g/100 g LW)	48.3	47.9	49.9	0.3018	2.10
Total water intake (g)	957	865	817	0.1071	97.10
Excreta (g DM)	141.2^{a}	127.7^{b}	127.0^{b}	0.0184	7.52
Apparent digestibility (%):					
Dry matter	55.0^{b}	58.7^{ab}	59.6^{a}	0.0374	2.63
Organic matter	58.1^{b}	62.5^{ab}	62.9^{a}	0.0193	2.50
Crude protein	45.9	46.8	41.9	0.3068	5.02
Ether extract	93.0^{a}	91.9^{ab}	87.2^{b}	0.0274	3.11
Starch	90.3^{b}	95.5^{a}	93.2^{ab}	0.0018	1.76
Gross energy	66.6^{b}	70.6^{a}	70.4^{a}	0.0104	1.94
Chitin	-	55.3	45.3	0.0556	7.11
Nutritive value:					
Metabolizable energy (MJ/kg DM)	12.9^{b}	14.0^{a}	13.9^{a}	0.0008	0.38
Metabolizable protein (g/kg feed)	123.6	124.2	111.6	0.2812	13.43
Feed choice trial:					
Feed intake (g DM/100 g LW)	48.9^{a}	23.2^{b}	27.9^{b}	<0.0001	8.87

[1] RSD = Residual Standard Deviation;

[2] Four quails/replicated cage

[a,b] Means in a row with different superscripts differ significantly.

Stellenbosch University https://scholar.sun.ac.za

Independently to the above cited results, the dietary inclusion of black soldier fly larvae meal in both IM1D and IM2D groups did not negatively affect the overall apparent digestibility of nutrients. In fact, even if DM and organic matter (OM) apparent digestibility showed lower values than those previously reported by Sahin *et al.,* (2006), the digestibility of CP, EE and starch for all treatments was consistent with values found on broiler quails by Cullere *et al.* (2016).

Interestingly, quails from the IM1D group performed better than C and IM2D groups, exhibiting the highest carcass weight (CW) ($P < 0.0001$) and breast meat weight ($P < 0.01$), with more favourable carcass and breast yields ($P < 0.01$) (Table 6). Even though IM2D birds showed the lightest carcasses, their carcass and breast yields were comparable to those of the C group. Interestingly, considering results about performance, digestibility and carcass and meat traits, quails fed the IM1D diet performed the best. Moreover, despite the lower growth rate and ether extract digestibility, IM2D showed the same carcass yield, breast meat quantity and yield as the Control group.

Table 6. Effect of the dietary inclusion of dried *Hermetia illucens* larvae reared on two different substrates on the carcass and breast meat traits of broiler quails

	Experimental groups			*P*-value	RSD[1]
	Control	IM1D	IM2D		
No.	99	99	97		
Carcass weight CW (g)	150.9^{b}	159.2^{a}	145.1^{c}	<0.0001	15.41
Carcasss yield (% CW)	62.7^{b}	64.2^{a}	63.1^{b}	0.0039	3.01
Breast meat (g)	45.9^{b}	49.8^{a}	43.9^{b}	<0.0001	6.50
Breast meat yield (% CW)	30.3^{b}	31.3^{a}	30.2^{b}	0.0044	2.53

[1] RSD = Residual Standard Deviation.

^{a,b,c} Means in a row with different superscripts differ significantly.

Conclusion

The overall outcomes from the present research which was conducted under intensive conditions and using dried black soldier fly larvae, when compared to recently published results testing a defatted *H. illucens* larvae meal on broiler chickens (Schiavone *et al.,* 2017a), Barbary partridge (Loponte *et al.,* 2017), broiler quails (Cullere *et al.,* 2016), and laying quails (Widjastuti *et al.,* 2014), as well as a further research testing black soldier fly prepupae fat in the diet for broiler chickens (Schiavone *et al.,* 2017b and 2017c), showed that productive performance, apparent digestibility of nutrients and carcass traits were always satisfactory. Such findings confirm once more the potential of the tested insect species as an alternative ingredient for poultry feeding, which could help in alleviating the competition between poultry species and humans for soya in the food-feed debate. Furthermore, results of the present research indicated that an appropriate substrate modulation is a key tool to improve the

Stellenbosch University https://scholar.sun.ac.za

chemical composition of the black soldier fly, thus helping to meet the nutrients requirements of different poultry species. Further research is necessary to investigate the exact reason why quails fed black soldier fly larvae reared on a substrate enriched with fish offal exhibited the lowest growth rate. This research should include the evaluation of the level of lipid oxidation in the feed, and if required, the addition of an anti-oxidant. Moreover, the complete chemical composition and sensory traits of the derived meat needs further research.

Stellenbosch University https://scholar.sun.ac.za

References

Association of Official Analytical Communities (AOAC), 2012. Official methods of analysis, 19th edition. Association of Official Analytical Chemists, Arlington, VA, USA.

Barroso, F.G., Sánchez-Muros, M.J., Segura, M., Morote, E., Torres, A., Ramos, R. and Guil, J.L., 2017. Insects as food: Enrichment of larvae of *Hermetia illucens* with omega 3 fatty acids by means of dietary modifications. Journal of Food Composition and Analysis 62: 8-13.

Boerema, A., Peeters, A., Swolfs, S., Vandevenne, F., Jacobs, S., Staes, J. and Meire, P., 2016. Soybean Trade: Balancing Environmental and Socio-Economic Impacts of an Intercontinental Market. PloS one 11, e0155222. doi:10.1371/journal.pone.0155222

Bosch, A.C., O'Neill, B., Sigge, G.O., Kerwatch, S.E. and Hoffman, L.C., 2016. Heavy metal accumulation and toxicity in smoothhound (*Mustelus mustelus*) shark from Langebaan Lagoon, South African Food Chemistry 190: 871-878.

Charlton, A.J., Dickinson, M., Wakefield, M.E., Fitches, E., Kenis, M., Han, R., Zhu, F., Kone, N., Grant, M., Devic, E., Bruggeman, G., Prior, R. and Smith, R., 2015. Exploring the chemical safety of fly larvae as a source of protein for animal feed. Journal of Insects as Food and Feed 1: 7-16.

Cullere, M., Tasoniero, G., Giaccone, V., Acuti, G., Marangon, A. and Dalle Zotte, A., 2017. Black soldier fly as dietary protein source for broiler quails: meat proximate composition, fatty acid and amino acid profile, oxidative status and sensory traits. Animal doi:10.1017/S1751731117001860

Cullere, M., Tasoniero, G., Giaccone, V., Miotti-Scapin, R., Claeys, E., De Smet, S. and Dalle Zotte, A., 2016. Black soldier fly as dietary protein source for broiler quails: apparent digestibility, excreta microbial load, feed choice, performance, carcass and meat traits. Animal 10: 1923-1930.

De Marco, M., Martínez, S., Hernandez, F., Madrid, J., Gai, F., Rotolo, L. and Kovitvadhi, A., 2015. Nutritional value of two insect larval meals (*Tenebrio molitor* and *Hermetia illucens*) for broiler chickens: Apparent nutrient digestibility, apparent ileal amino acid digestibility and apparent metabolizable energy. Animal Feed Science and Technology 209: 211-218.

Diener, S., Zurbrüg, C. and Tockner, K., 2015. Bioaccumulation of heavy metals in the black soldier fly, *Hermetia illucens* and effects on its life cycle. Journal of Insects as Food and Feed 1: 261-270.

EC European Commission, 2002. Directive 2002/32/EC of the European Parliament and the Council on undesirable substances in animal feed. Official Journal of the European Union L140: 1-29.

Stellenbosch University https://scholar.sun.ac.za

EC European Commission, 1998. Commission Directive 98/64/EC of 3 September 1998 establishing Community methods of analysis for the determination of amino acids, crude oils and fats, and olaquindox in feeding stuffs and amending Directive 71/393/EEC. Official Journal of the European Union 19.9.1998, L257/14.

Fievez, V., De Fauw, K., Notteboom, K. and Demeyer, D., 2001. Effect of level and origin of rumen degradable nitrogen on rumen microbial growth and nitrogen utilization efficiency of animals fed maize silage at maintenance. Reproduction Nutrition Development 41: 349-364.

Harriman, A.E. and Milner, J.S., 1969. Preference for sucrose solutions by Japanese quail (*Coturnix coturnix japonica*) in two-bottle drinking test. The American Midland Naturalist 81:98 575-578.

International Organisation for Standardisation (ISO), 1998. Animal feeding stuffs, animal products and faeces or urine. Determination of gross calorific value – Bomb calorimetric method. Reference number 9831.

Kroeckel, S., Harjes, A.G., Roth, I., Katz, H., Wuertz, S., Susenbeth, A. and Schulz, C., 2012. When a turbot catches a fly: Evaluation of a pre-pupae meal of the Black Soldier Fly (*Hermetia illucens*) as fish meal substitute—Growth performance and chitin degradation in juvenile turbot (*Psetta maxima*). Aquaculture 364: 345-352.

Loponte, R., Nizza, S., Bovera, F., De Riu, N., Fliegerova, K., Lombardi, P., Vassalotti, G., Mastelloni, V., Nizza, A. and Moniello, G., 2017. Growth performance, blood profiles and carcass traits of Barbary partridge (*Alectoris barbara*) fed two different insect larvae meals (*Tenebrio molitor* and *Hermetia illucens*). Research in Veterinary Science 115: 183-188.

Makkar, H.P.S., Tran, G., Heuzé, V. and Ankers, P., 2014. State-of-the-art on use of insects as animal feed. Animal Feed Science and Technology 197: 1-33.

Marascuilo, L.A., 1966. Large-sample multiple comparisons. Psychological Bulletin 65: 280–290.

National Research Council (NRC), subcommittee on Poultry Nutrition, 1994. Nutrient Requirements of Ring-Necked Pheasants, Japanese Quail, and Bobwhite Quail. In Nutrient requirements of poultry (9th revised edition, National Academy of Sciences), pp 44-45. National Academy of Sciences, Washington, DC

Roura, E., Baldwin, M.W. and Klasing, K.C., 2013. The avian taste system: Potential implications in poultry nutrition. Animal Feed Science and Technology 180: 1-9.

Sahin, K., Onderci, M., Sahin, N., Gursu, M.F., Khachik, F. and Kucuk, O., 2006. Effects of lycopene supplementation on antioxidant status, oxidative stress, performance and carcass characteristics in heat-stressed Japanese quail. Journal of Thermal Biology 31: 307–312.

Stellenbosch University https://scholar.sun.ac.za

Schiavone, A., De Marco, M., Martínez, S., Dabbou Renna, M., Madrid, J., Hernandez, F., Rotolo, L., Costa, P., Gai, F. and Gasco, L., 2017a. Nutritional value of a partially defatted and a highly defatted black soldier fly larva (*Hermetia illucens* L.) meal for broiler chickens: apparent nutrient digestibility, apparent metabolizable energy and apparent ileal amino acid digestibility. Journal of Animal Science and Biotechnology 8: 51.

Schiavone, A., Dabbou, S., De Marco, M., Cullere, M, Biasato, I., Biasibetti, I., Capucchio M.T., Bergagna, S., Dezzutto, D., Meneguz, M., Gai, F., Dalle Zotte, A. and Gasco, L., 2017b. Black soldier fly (*Hermetia illucens* L.) larva fat inclusion in finisher broiler chicken diet as an alternative fat source. Animal doi:10.1017/S1751731117003743

Schiavone, A., Cullere, M., De Marco, M., Meneguz, M., Biasato, I., Bergagna, S., Dezzutto, D., Gai, F., Dabbou, S., Gasco, L. and Dalle Zotte, A., 2017c. Partial or total replacement of soybean oil by black soldier fly larvae (*Hermetia illucens* L.) fat in broiler diets: Effect on growth performances, feed-choice, blood traits, carcass characteristics and meat quality. Italian Journal of Animal Science 16: 93-100.

Spranghers, T., Ottoboni, M., Klootwijk, C., Ovyn, A., Deboosere, S., De Meulenaer, B., Michiels, J., Eeckhout, M., De Clercq, P. and De Smet, S., 2017. Nutritional composition of black soldier fly (*Hermetia illucens*) prepupae reared on different organic waste substrates. Journal of the Science of Food and Agriculture 97: 2594-2600.

Statistical Analysis Software for Windows (SAS), 2008. Statistics version 9.1.3 ed. SAS Institute, Cary, NC, USA.

St-Hilaire, S., Cranfill, K., McGuire, M.A., Mosley, E.E., Tomberlin, J.K., Newton, L. and Irving, S., 2007. Fish Offal Recycling by the Black Soldier Fly Produces a Foodstuff High in Omega-3 Fatty Acids. Journal of the World Aquaculture Society 38: 309-313.

Tancharoenrat, P., Ravindran, V., Zaefarian, F. and Ravindran, G., 2014. Digestion of fat and fatty acids along the gastrointestinal tract of broiler chickens. Poultry Science 93: 371-379.

Terpstra, K. and de Hart, N., 1973. The estimation of urinary nitrogen and faecal nitrogen in poultry excreta. Journal of Animal Physiology and Animal Nutrition 32: 306-320.

Tschirner, M. and Simon, A., 2015. Influence of different growing substrates and processing on the nutrient composition of black soldier fly larvae destined for animal feed. Journal of Insects as Food and Feed 1: 249-259.

Van Huis, A., 2013. Potential of insects as food and feed in assuring food security. Annual Review of Entomology 58: 563-583.

Stellenbosch University https://scholar.sun.ac.za

Widjastuti, T., Wiradimadja, R. and Rusmana, D., 2014. The effect of substitution of fish meal by Black Soldier Fly (*Hermetia illucens*) maggot meal in the diet on production performance of quail (*Coturnix coturnix japonica*). Animal Science Journal 57: 125-129.

Zhang, B., Haitao, L., Zhao, D., Guo, Y. and Barri, A., 2011. Effect of fat type and lysophosphatidylcholine addition to broiler diets on performance, apparent digestibility of fatty acids, and apparent metabolizable energy content. Animal Feed Science and Technology 163: 177-184.

Zhang, J. and Zhu, K.Y., 2006. Characterization of a chitin synthase cDNA and its increased mRNA level associated with decreased chitin synthesis in *Anopheles quadrimaculatus* exposed to diflubenzuron. Insect Biochemistry and Molecular Biology 36: 712-725.

Stellenbosch University https://scholar.sun.ac.za

CHAPTER 6

Hermetia illucens larvae reared on different substrates in broiler quail diets: effect on physicochemical and sensory quality of the quail meat

Published as: Cullere, M., Woods, M.J., Van Emmenes, L., Pieterse, E., Hoffman L.C. and Dalle Zotte, A., 2019. *Hermetia illucens* larvae reared on different substrates in broiler quail diets: effect on physicochemical and sensory quality of the quail meat. Animals 9(8): 525.

Stellenbosch University https://scholar.sun.ac.za

Simple Summary

Hermetia illucens (HI) is one of the most promising insect species that can be exploited as alternative and sustainable feed ingredient in poultry farming. HI larvae can grow on a wide range of decomposing organic substrates, thus transforming organic waste into high-value feed products. When HI is included in diets of different poultry species, they provide positive results both in terms of productive performance and health status of the animals, and overall product quality. However, a common drawback highlighted by most studies was a sub-optimal quality of the meat lipids of HI-fed poultry (high amount of saturated fatty acids (SFA) to the detriment of the unsaturated fraction (UFA)). The present experiment tested the effect of a dietary inclusion of HI larvae farmed on two different substrates in the diets of meat quails. The two substrates were: 100% layer mash (conventional rearing substrate) and 50:50 layer mash:fish offal, aiming to increase the UFA content of the larvae. Overall results showed that it is possible to improve the fatty acid profile of the larvae by modulating the rearing substrate that in turn, improves the lipid quality of the meat obtained from quails fed on diets containing such larvae.

Abstract

This research aimed at improving the fatty acid (FA) profile of *Hermetia illucens* larvae (HI) and evaluating the effect of its inclusion into growing broiler quails' diet on the quail's meat physicochemical quality, including detailed amino acid (AA) and FA profiles, sensory traits and retail display. HI were reared on two different substrates: layer mash (HI1) and 50:50 layer mash:fish offal (HI2). A total of three hundred 10-day-old quails were allocated to the 3 dietary groups (5 replicates/each) and fed the experimental diets until slaughter. A soybean meal-based diet was formulated (Control), and two other diets were formulated including either 10% HI1 or HI2. Diets were formulated to be isonitrogenous and isoenergetic. Breast meat quality was affected by the dietary treatments which displayed different proximate composition, AA and FA profiles. Meat physical quality, sensory profile and retail display remained unaffected for the most part. Overall, results showed that it is possible to improve the FA profile of the HI-fed quails' meat and thus lipid quality through substrate modulation of the HI's diet.

Keywords: Black soldier fly; substrate; quail; insect meal; alternative protein source; animal feeding; meat quality; proximate composition; fatty acids; amino acids.

Stellenbosch University https://scholar.sun.ac.za

Introduction

Quails are birds of economic importance due to a series of positive features such as high meat quality and egg production, as well as fast return on investments, which is possible due to their early sexual maturity, rapid growth, short generation interval, high laying rate, limited feed and space required/bird [1]. As for other poultry species, commercial feed formulations mainly rely on soybean as protein and fat sources which requires vast amount of water and land to grow thus having a significant environmental impact [2]. Due to the growing demographic trends and the consequent augmenting pressure on the natural resources such as water and land (deforestation), as well as an ever-increasing feed-food competition, the search for novel feed ingredients for poultry diets with the potential to improve the sustainability of the sector, is of utmost importance. In this perspective, insects have been recognized as one of the possible candidates to solve this issue. This was internationally emphasized for the first time with the first FAO International Conference on insects for food and feed in 2014 and, in the following years, research studies on this topic as well as innovations and applications of techniques by the industry have grown tremendously. Also the European legislative frameworks regarding the use of insects in animal nutrition has been updated which led to the publication of the Regulation No 2017/893, allowing the use of seven insect species in aquaculture. Despite this key success, more research efforts are required to allow their utilization in poultry feeding, where insect consumption already falls within their natural dietary habits, particularly in free-range production systems [3].

One of the most promising insect species in this sense is the black soldier fly (*Hermetia illucens*), a Diptera of the Stratyomidae family. Together with their un-doubtfully interesting nutritional profile [4] and suitability for mass production, *H. illucens* larvae can exploit a wide range of decomposing organic material for their growth, thus representing an opportunity to recycle organic matter into valuable nutrients [5]. Independently to the inclusion level, when tested in the diets of different animal species including fish [6], poultry [7-10], pig [11] and rabbit [12,13] species, overall results such as health status of the animals, productive performances as well as overall quality of the derived animal products (i.e meat and eggs) proved to be satisfactory. However, a constant nutritional drawback emerged related to the fatty acid (FA) profile of the animal product derived from animals fed with larvae of this Dipteran. In fact, *H. illucens* larvae are naturally rich in saturated fatty acids (SFA; mainly lauric, myristic and palmitic) and poor in unsaturated FA [14]. For this reason, they are sub-optimal in providing healthy food for modern consumers. As previous research demonstrated that the rearing substrate can have a great impact on the chemical composition of the larvae [15], the hypothesis is that the FA profile of *H. illucens* larvae could be improved by rearing

Stellenbosch University https://scholar.sun.ac.za

them in a substrate enriched with a source of *n*-3 polyunsaturated FA (PUFA), recycling a relevant nutrient source which is considered a by-product: fish offal. The ultimate goal is the successful application of such larvae into poultry nutrition aiming to obtain a meat with a healthy FA profile. The only research on this topic published to date found promising results [16]. However, the study concerned a possible application of *n*-3 enriched larvae in fish diets, whereas no scientific works are available on poultry species. Therefore, the present research project studied the inclusion of 10% HI larvae reared on a conventional substrate or on a substrate enriched with *n*-3 PUFA in the feed for broiler quails. In the first report of this study [17] the total tract nutrients' digestibility and feed-choice as well as the quails' productive performance and carcass traits were studied: despite feed-choice and carcass weight were not in favor of the group including the *n*-3 enriched larvae, nutrients' digestibility, mortality and carcass yield were satisfactory. In this, the second part of the study, detailed meat quality aspects including physical characteristics, proximate composition, cholesterol, heme-iron and amino acid contents, FA profile, sensory traits and storage stability of the quails fed these diets were evaluated.

Materials and Methods

The present trial was conducted after the approval by the veterinary authority and according to Article 2, DL 4 March 2014, No. 26 of the Official Journal of the Italian Republic (http://www.gazzettaufficiale.it/eli/id/2014/03/14/14G00036/sg), implementing the EU Directive 2010/63/EU on the protection of animals used for scientific purposes.

Experimental design

For the present experiment, three dietary treatments were tested on broiler quails from 10 to 29 days of age: a control diet (Control) was formulated according to the conventional grower diet used on the farm, and two diets containing 10% of dried and ground black soldier fly (*Hermetia illucens*) larvae as a partial substitution of conventional protein/fat sources. The larvae included in the two diets differed as they were farmed on two different growth media: the first was a conventional substrate made of 100% chicken layer mash (HI1), whereas the second was made of 50% chicken layer mash and 50% fish offal (HI2), aiming at increasing larvae n-3 PUFA content. Detailed information about insect farming, diets formulation and chemical composition, quails farming conditions, live performances and slaughtering procedure are provided in Woods *et al.* [17]. Each experimental group consisted of 100 quails (20 birds/cage, 5 cages/dietary treatment). All slaughtered quails (n=99, n=97 and n=97 from the C, HI1 and HI2 groups, respectively) were considered for meat quality evaluations. From each slaughtered bird, breasts were excised and analyzed at the Laboratory of the Department

Stellenbosch University https://scholar.sun.ac.za

of Animal Medicine, Production and Health - MAPS - of the University of Padova (Italy). Each breast was labelled, vacuum-packaged in polyethylene bags (water vapor transmission rate: 3.5 ± 1 g/m^2 day at 23 °C and 85 ± 2% relative humidity) using a CSV-41n ORVED machine (99% vacuum level) and stored at −40 °C until analytical determinations. For every experimental dietary group, 24 breasts were dedicated to the analysis of the proximate composition, cholesterol content, amino acid and fatty acid (FA) profiles, 10 breasts for heme-iron content, 16 breasts for the sensory profile, and 12 breasts for a retail-display trial. The remaining breasts were dedicated to physical evaluations.

Physical meat quality

After 2 weeks of frozen storage, quail breasts were weighed, allowed to thaw for 12 h at 4 °C and weighed again to calculate the thawing loss (%). Color measurements were performed on the cranial and caudal part of the *Pectoralis major* muscle using a RM200QC colorimeter (X-Rite Co, Neu-Isenburg, Germany. Measuring Area: 8 mm; Measuring Geometrics: 45/0 Image Capture; Illuminant/Observer: D65/10) and considered lightness (L*), redness (a*) and yellowness (b*) indexes [18]. In the same parts of the breast considered for color determination, pH was measured with a portable pH meter (FG2-Five GoTM; Mettler Toledo, Greifensee, Switzerland) calibrated at pH 4.0 and 7.0. Color and pH values represented the average of two repeated measurements. Breasts were then vacuum-sealed using the above-mentioned equipment and cooked in a water bath set at 80 °C, until the core temperature reached 77 °C. Then, samples were cooled in an iced bath, gently dried by dabbing with a paper towel and weighed to compute the cooking and the total losses (%). Shear force was assessed with a TA-HDi Texture Analyzer (Stable Macro System, London, UK) on four cooked meat cores (diameter 1.25 cm) per sample, sheared perpendicularly to the muscle fibre direction with a Warner-Bratzler cell (100 kg load cell, 2 mm/s crosshead speed) fitted on the texturometer. Warner-Bratzler shear force (WBSF) was calculated by averaging four measurements/sample.

Chemical meat quality

The He-iron content of fresh breast meat samples was determined following the method described by Hornsey [19], and was expressed as mg He-Fe/kg of fresh tissue. Breasts dedicated to the other chemical determinations were randomly paired (to guarantee enough material to perform the scheduled analyses) and ground with a Retsch Grindomix GM 200 (7000 g for 10 s); in this way, 12 samples/experimental group were obtained. Once ground, meat samples were frozen at −40 °C, freeze-dried and ground again (7000 g for 5 s) to obtain a fine powder which was used to determine proximate composition, amino acid and FA profiles

Stellenbosch University https://scholar.sun.ac.za

and cholesterol content. The proximate composition of breast meat samples was analyzed in accordance with the AOAC [20] methods. The cholesterol content was determined through absolute quantitative analysis using HPLC, following the method described by Casiraghi et al. [21]. The amino acid content of the experimental diets as well as that of meat samples was analyzed according to the methods described by Cunico [22]. During this process, a sample weighing 0.1 g was placed in a specialized hydrolysis tube. Six mL hydrochloric acid (HCl) solution and 15 % phenol solution was then added to the sample. The tubes were then vacuated and nitrogen (N) added under pressure. The tubes were subsequently sealed off with a blue flame and the samples left to hydrolyze at 110 ˚C for 24 hours. After hydrolysis, the samples were transferred to Eppendorf tubes and refrigerated until sent to the Central Analytical Facility of Stellenbosch University. Amino acid composition was determined by means of the Waters AccQ Tag Ultra Derivatization method. The quantitative results are expressed as g/100 g meat.

Fatty acid profile of Hermetia illucens larvae, diets and meat samples

The lipid extraction was performed by Accelerated Solvent Extraction (M-ASE) using different solvents according to the specific matrix: petroleum ether for HI larvae and diets, and chloroform:methanol 1:2 for breast meat samples. Total lipid content was determined gravimetrically after the removal of the solvent by evaporation under nitrogen stream at 50 °C. Samples were subsequently trans methylated using a methanolic solution of H_2SO_4 (4%) in order to determine fatty acid methyl esters (FAME). A biphasic separation was obtained by adding 0.5 ml of distilled water and 1.5 ml of N-heptane to each sample. FAME were quantified by gas chromatography (Shimadzu GC17A), equipped with an Omegawax 250 column (30 m x 0.25 µm x 0.25 µm) and FID detector. Helium was used as the carrier gas at a constant flow of 0.8 mL/min. The injector and detector temperatures were 260 °C. Peaks were identified based on commercially available FAME mixtures (37-Component FAME Mix, Supelco Inc., Bellefonte, PA). The results are expressed as % of total detected FAME. The FA composition of the *H. illucens* larvae and diets are presented in Table 1.

Stellenbosch University https://scholar.sun.ac.za

Table 1. Fatty acid profile (% of total FAME) of the dried *Hermetia illucens* larvae (HI) reared on layer mash (HI1) or on 50:50 layer mash and fish offal (HI2), and of the experimental diets: Control, and Control diet including either 10% HI1 or HI2.

Fatty acids	**Dried larvae**		**Experimental diets**		
	HI1	**HI2**	**Control**	**HI1**	**HI2**
C10:0	0.95	1.02	0.00	0.62	0.68
C12:0	44.2	48.3	0.06	29.4	33.1
C14:0	7.98	6.88	0.09	5.15	4.66
C15:0	0.09	0.03	0.00	0.08	0.13
C16:0	13.6	13.4	11.4	12.8	12.9
C17:0	0.09	0.11	0.10	0.14	0.11
C18:0	1.74	2.06	2.64	1.80	1.97
C20:0	0.23	0.04	0.07	0.07	0.06
C22:0	0.02	0.02	0.14	0.07	0.06
C23:0	0.00	0.02	0.05	0.04	0.05
C24:0	0.00	0.12	0.06	0.00	0.17
Total SFA	68.9	72.0	14.6	50.1	53.8
C14:1	0.16	0.20	0.00	0.11	0.13
C15:1	0.05	0.02	0.00	0.00	0.00
C16:1	2.39	3.82	0.10	1.60	2.73
C17:1	0.13	0.16	0.11	0.09	0.17
C18:1 *n*-9	13.7	13.3	24.3	17.3	16.9
C18:1 *n*-11	1.11	1.05	1.39	0.71	0.87
C20:1 *n*-9	0.11	0.09	0.39	0.24	0.21
C22:1 *n*-9	0.08	0.03	0.34	0.11	0.10
C24:1 *n*-9	0.00	0.00	0.47	0.19	0.12
Total MUFA	17.7	18.7	27.1	20.4	21.2
C18:2 *n*-6	11.0	4.28	51.3	25.1	18.8
C18:3 *n*-6	0.02	0.05	0.07	0.04	0.04
C18:3 n-3	0.44	0.19	4.37	1.16	1.35
C20:2 *n*-6	0.08	0.63	0.05	0.06	0.04
C20:3 *n*-6	0.02	0.04	0.05	0.05	0.04
C20:3 *n*-3	0.02	0.07	0.07	0.06	0.06
C20:4 *n*-6	0.05	0.12	0.06	0.06	0.09
C20:5 *n*-3	0.07	1.00	0.08	0.05	0.58
C22:2 *n*-6	0.96	0.29	0.40	0.64	0.22
C22:6 *n*-3	0.00	0.32	0.00	0.00	0.10
Total PUFA	12.6	6.99	56.4	27.3	21.3
UFA/SFA	0.44	0.36	5.68	0.95	0.79
n-6	12.1	5.42	51.9	26.0	19.2
n-3	0.53	1.57	4.51	1.26	2.09
n-6/*n*-3	22.9	3.45	11.5	20.6	9.20
Identified FA (%)	99.7	97.9	98.2	97.8	96.4

FAME= fatty acid methyl esters; MUFA= monounsaturated fatty acids; PUFA = polyunsaturated fatty acids; SFA = saturated fatty acids; UFA = unsaturated fatty acids; FA= fatty acid.

Sensory analysis

Meat was analyzed at the Sensory Analysis Division of the “Istituto per la Qualità e le Tecnologie Agroalimentari, Laboratorio Analisi Sensoriale (Veneto Agricoltura)”, Thiene, Vicenza, Italy. Quail breasts were subjected to a descriptive sensory analysis, to detect

Stellenbosch University https://scholar.sun.ac.za

possible differences among the dietary treatments (C vs. HI1 vs. HI2). The sensory analysis was performed by an eight-member trained panel, which were qualified as experts according to ISO 8586 and had experience with descriptive tests (ISO 13299) on various food matrixes. All judges who perform tests with accredited methods undergo training every 3 years. Panelists underwent two pre-test training sessions of 1 h each to familiarize themselves with the matrix and select appropriate descriptors, possible off-odors and off-flavors, also drawn from the literature. The meat used for the training sessions (quail breasts) was purchased at a local supermarket and it was processed, stored, handled and cooked in the same manner as the samples which were used for the subsequent sensory analysis. The panel received a list of descriptors to score on numerical and continuous scales from 0 (the lowest score for each attribute) to 10 (the highest score for each attribute). Olfactory, gustative and textural aspects were evaluated. The descriptors were: odor intensity, off-odor intensity, flavor intensity, off-flavor intensity, juiciness, toughness, chewiness, fibrousness. The chosen off-odors and off-flavors were: game meat, metallic, liver, oil/fat, peanut/hazelnut, rancid. For the experiment, a total of 16 quail breasts/treatment were used and 2 days of analysis were scheduled (8 quail breasts/treatment per session). After 1 month of frozen storage at −40 °C, quail breasts were allowed to thaw for 16 h at +4 °C. Each sample was then placed on a cooking plate (model GR6010 XL Health Comfort, 2400 Watt; Rowenta, Erbach, Germany) set at thermostat position '2' and cooked for 5 min/side, until the core temperature reached 74 °C. Subsequently, samples were individually wrapped in aluminum foil, placed in aluminum trays and served to the panel in random sequence. Samples were identified by a random three-digit code. The evaluation sheet, distribution of samples to the judges and data acquisition were performed using FIZZ software (Biosystèmes France, St-Ouen l'Aumône, France) installed in eight terminals in the tasting booths of the laboratory. In addition, for each sample, panelists were asked to indicate if and which of the listed off-odors and off-flavors they could recognize. Still water at room temperature and unsalted crackers were available to panelists for palette cleansing throughout each sensory session.

Retail-display trial

Quail breasts were individually wrapped with food-grade polyvinyl chloride (PVC) film (thickness: 8.5 μ +/- 8%; breaking load: > 17 N/mm; extension: > 135%; temperature range: -30 °C/ +40 °C) to prevent direct air contact and stored in a refrigerator for an eight-day retail display trial. The refrigerator was set at 4 °C ± 1 and continuous cold fluorescent light illumination (L58 W⁄ 20 Osram, Germany, at 870 lux - MT 940, Major) was provided for the whole period to simulate commercial retail conditions. Analyses were performed at days 0 (24h post mortem) and 8 of refrigerated storage. For storage drip loss calculation, the quail

Stellenbosch University https://scholar.sun.ac.za

breasts were removed from the refrigerator, unwrapped and weighed. The pH and L*a*b* color values were measured (in duplicate) on the same breast portions using the same instruments described previously. After physical evaluation, the breasts were ground with a Retsch Grindomix GM 200 (7000 g for 10 s) and used to determine the extent of muscle lipid oxidation. The latter was evaluated with a spectrophotometer (Hitachi U-2000; Hitachi, Mannheim, Germany) set at 532 nm, that measured the absorbance of thiobarbituric acid-reactive substances (TBARs) and a 1,1,3,3-tetraethoxypropane calibration curve [23]. Oxidation products were quantified as malondialdehyde (MDA) equivalents (mg MDA/kg muscle).

Statistical analysis

Meat physical traits, proximate composition, He-iron, cholesterol, and amino acid contents and FA profile data were subjected to a one-way ANOVA with experimental diet (C, HI1 and HI2) as fixed effect, following the GLM procedure of the SAS 9.1.3 Statistical Analysis Software for Windows [24]. A mixed model (PROC MIXED) was used to detect any dietary influence on sensory analysis scores, considering experimental diet and the eight panelists as fixed and random effects, respectively. Least square means were obtained using the Bonferroni test and the significance was calculated at a 5% confidence level. A χ^2 test using the Marascuilo [25] procedure was performed on off-odors and off-flavors characterization to detect the differences among treatments.

Results

Overall, quail breast derived from birds fed with diets containing 0% or 10% dried *H. illucens* larvae reared on two different growing media (HI1 and HI2) showed similar physical meat quality traits to that of the quails fed the control diet (Table 2). Specifically, breasts displayed similar pH ($p > 0.05$), CIE L*a*b* color values ($p > 0.05$) and thawing loss ($p > 0.05$). The only exception was the cooking loss percentage, which was highest in quail breasts of the HI2 group ($p < 0.05$); however, this did not affect the total loss percentage ($p > 0.05$) nor the meat toughness ($p > 0.05$) which was similar for the three experimental groups.

Stellenbosch University https://scholar.sun.ac.za

Table 2. Effect of the dietary inclusion of *Hermetia illucens* dried larvae farmed on different growth substrates (HI1, HI2) on the physical traits of broiler quails' breast meat

	Experimental groups[1]			***p*-value**	**RSD**[2]
	C	**HI1**	**HI2**		
No.	37	39	39		
pH	5.62	5.59	5.63	0.2346	0.14
L*	49.6	49.9	50.1	0.6205	3.02
a*	2.44	2.28	2.11	0.2374	1.21
b*	9.32	9.48	9.42	0.8382	1.66
Thawing loss, %	11.3	10.9	10.9	0.9173	3.25
Cooking loss, %	20.3^{b}	20.0^{b}	22.0^{a}	0.0380	3.36
Total loss, %	31.6	30.9	32.9	0.2393	4.65
WBSF, kg/cm^2	11.3	10.9	10.4	0.0680	1.72

[1] HI1= Control diet incorporated with 10% dried *Hermetia illucens* larvae reared on a substrate made of 100% layer mash; HI2: Control diet incorporated with 10% dried *Hermetia illucens* larvae reared on a substrate made of 50% layer mash and 50% fish-offal; [2]Residual standard deviation; L*: lightness; a*: redness; b*: yellowness; WBSF: Warner-Bratzler Shear Force; [a,b] Values within a row with different superscripts differ significantly at $p < 0.05$.

The chemical composition of quail breasts was significantly affected by the tested experimental diets (Table 3). Breasts of the HI1 group displayed the highest lipid content (5.27 vs. 4.89 and 4.93 % for HI1, C and HI2 breast meat, respectively; $p < 0.01$) to the detriment of the protein fraction ($p < 0.05$) which was lower than that of C but not different from HI2. Independently to the growing substrate used to farm *H. illucens* larvae, their inclusion in the quails' diets increased the cholesterol content of the breast meat compared to that of the C group ($p < 0.01$). Water, ash and heme-iron contents were not affected by the dietary treatment.

Stellenbosch University https://scholar.sun.ac.za

Table 3. Effect of the dietary inclusion of *Hermetia illucens* dried larvae farmed on different growth substrates (HI1, HI2) on the proximate composition (%), cholesterol and heme-iron contents (mg/100 g meat) of broiler quails' breast meat

	Experimental groups[1]			***p*-value**	**RSD**[2]
	C	**HI1**	**HI2**		
No.	12	12	12		
Water	74.5	74.5	74.6	0.4220	0.25
Protein	23.9^{a}	23.5^{b}	23.7^{ab}	0.0143	0.26
Lipids	4.89^{B}	5.27^{A}	4.93^{B}	0.0003	0.23
Ash	2.31	2.29	2.29	0.9777	0.26
Cholesterol	68.7^{Bb}	74.1^{A}	73.6^{ABa}	0.0031	3.96
Heme-iron[3]	0.64	0.60	0.66	0.3044	0.87

[1] HI1= diet supplemented with 10% of dried *Hermetia illucens* larvae reared on layer mash; HI2= diet supplemented with 10% of dried *Hermetia illucens* larvae reared on 50:50 layer mash and fish offal; [2] RSD= Residual Standard Deviation; [3] Heme-iron analysis was conducted on No.=10 fresh breast meat samples/treatment. ^{a,b} Means in a row with different superscripts differ significantly ($p < 0.05$); ^{A,B} Means in a row with different superscripts differ significantly ($p < 0.01$).

The protein percentage as well as the majority of the essential and non-essential amino acids (g/100 g meat) of quails' breast meat were affected significantly by the dietary treatments (Table 4). The 10% dietary inclusion of HI1 lowered the protein percentage (on a dry mass, defatted basis) of quails' breast meat compared to C, whereas HI2 meat showed an intermediate result (81.4, 83.4 and 89.5 % for HI1, HI2 and C breast meat, respectively; $p < 0.05$). The above-mentioned result was mainly attributable to the low amounts of the essential amino acids histidine, leucine, methionine and phenylalanine. The rearing substrate of the larvae played a role in determining the overall quality of the protein provided by the quails' breast meat: in general, HI2 meat showed a slightly better amino acid profile than HI1, especially considering arginine and threonine ($p < 0.05$) among the essential amino acids, and glycine, glutamic acid and serine ($p < 0.05$) among the non-essential amino acids.

Stellenbosch University https://scholar.sun.ac.za

Table 4. Effect of the dietary inclusion of *Hermetia illucens* dried larvae farmed on different growth substrates (HI1, HI2) on the amino acids content (g/100 g meat) of broiler quails' breast meat

	Experimental diets			Experimental groups[1]			*p*-value	RSD[2]
	C	HI1	HI2	C	HI1	HI2		
No.				12	12	12		
% protein	92.6	80.6	87.0	89.5^{a}	81.8^{b}	83.4^{ab}	0.0226	6.62
Essential amino acids:								
Arginine	1.52	1.21	1.49	1.46^{a}	1.31^{b}	1.33^{ab}	0.0238	0.14
Histidine	0.52	0.45	0.52	0.85^{A}	0.74^{B}	0.74^{B}	0.0003	0.07
Isoleucine	0.87	0.89	0.90	0.96	0.90	0.95	0.0710	0.07
Leucine	1.85	1.75	1.77	2.01^{Aa}	1.76^{B}	1.83^{ABb}	0.0017	0.16
Lysine	0.61	0.66	0.82	0.88	0.87	1.00	0.1862	0.18
Methionine	0.25	0.23	0.19	0.54^{A}	0.47^{B}	0.45^{B}	0.0008	0.05
Phenylalanine	1.58	1.32	1.28	1.81^{A}	1.22^{B}	1.25^{B}	0.0006	0.38
Threonine	0.80	0.68	0.78	0.95^{a}	0.86^{b}	0.87^{ab}	0.0429	0.09
Valine	0.87	0.83	0.89	0.98	0.91	0.97	0.0609	0.07
Non-essential amino acids:								
Alanine	1.12	1.07	1.12	1.32	1.24	1.28	0.2052	0.12
Aspartic acid	2.49	1.85	2.14	2.10	1.88	1.94	0.0582	0.22
Glycine	1.25	1.10	1.15	1.23^{a}	1.12^{b}	1.12^{ab}	0.0175	0.11
Glutamic acid	4.10	3.08	3.60	3.16^{a}	2.86^{b}	2.91^{ab}	0.0357	0.30
Proline	1.54	1.44	1.37	0.93	0.90	0.94	0.5473	0.08
Hydroxiproline	0.42	0.33	0.34	0.54^{a}	0.48^{ab}	0.46^{b}	0.0287	0.07
Serine	1.25	1.00	1.16	0.95^{a}	0.86^{b}	0.86^{ab}	0.0250	0.09
Tyrosine	1.01	0.97	1.04	0.96	0.89	0.89	0.2037	0.11

[1] HI1= diet supplemented with 10% of dried *Hermetia illucens* larvae reared on layer mash; [2] HI2= diet supplemented with 10% of dried *Hermetia illucens* larvae reared on 50:50 layer mash and fish offal; [3] RSD= Residual Standard Deviation; [a,b] Means in a row with different superscripts differ significantly ($p < 0.05$); [A,B] Means in a row with different superscripts differ significantly ($p < 0.01$).

The FA profile of quails' breasts (% of total FAME) showed remarkable differences depending on the presence and the type of HI included in the experimental diets (Table 5). The inclusion of HI larvae increased the total saturated fatty acids (SFA) proportion compared to the C diet, with HI2 breasts showing a greater SFA proportion, also compared to HI1 breasts (32.0 vs. 40.3 vs. 42.8 % for C, HI1 and HI2 breast meat, respectively; $p < 0.001$). The higher SFA content of quail breasts derived from HI-fed quails compared to C was attributable to the increasing percentages of C12:0, C14:0, and C16:0 ($p < 0.001$) FA (Table 1). Also, the monounsaturated FA (MUFA) proportion increased in the breasts of HI-fed quails with differences attributable to the different growing substrates used to farm *H. illucens* larvae (18.8 vs. 22.7 and 22.2 % for C, HI1 and HI2 breasts, respectively; $p < 0.001$). The MUFA affected

Stellenbosch University https://scholar.sun.ac.za

by the dietary treatments to the greatest extent were C14:1, C15:1 and C16:1 ($p < 0.01$) FA, although these specific FAs were present at low concentrations. As a result of the dietary treatment (Table 1), the overall polyunsaturated FA (PUFA) percentage differed in C, HI1 and HI2 groups (Table 5). Specifically, C showed the highest level compared to HI1 and HI2 breasts, which did not differ from each other (44.1 vs. 32.0 and 30.4 % for C, HI1 and HI2 breast meat, respectively; $p < 0.001$). Despite this, the two HI breasts showed different PUFA compositions: HI1 breasts highlighted a consistent decrease in both the *n*-6 as well as the *n*-3 PUFA fractions compared to the C group ($p < 0.001$), whereas HI2 breasts exhibited the most intense lowering of the *n*-6 PUFAs but a consistent increase of the *n*-3 PUFA fraction, which was similar to that of the C group. The loss of *n*-6 and the concomitant increase of the *n*-3 PUFAs consistently reduced the *n*-6/*n*-3 ratio which was the lowest in HI2 breasts and the highest in the HI1 breasts (11.2 vs. 14.0 vs. 6.56 *n*-6/*n*-3 for C, HI1 and HI2 breasts, respectively, $p < 0.001$).

As a result of the changes in the FA profile of quails' breasts belonging to the different dietary treatments, the health indexes of the quail's breasts were also influenced (Table 5): the atherogenicity index (AI) and the thrombogenicity index (TI) increased ($p < 0.001$), thus worsened, with the dietary inclusion of HI (both types). Furthermore, according to the different growing substrates used to farm larvae, different magnitudes in these changes were observed: HI1 breasts showed a lower AI, but a higher TI compared to HI2 breasts. As a result of a growing SFA proportion in HI breasts compared to C, the hypocholsterolemic/hypercholesterolemic ratio (HH ratio) decreased, thus worsened, and the peroxidability index (PI) decreased too, thus making the meat of HI1 and HI2 quails less susceptible to oxidative phenomena than those of the C group (61.7 and 66.5 vs. 81.9 for HI1, HI2 and C breast meat, respectively; $p < 0.001$).

Table 5. Effect of the dietary inclusion of *Hermetia illucens* dried larvae farmed on different growth substrates (HI1, HI2) on the fatty acid profile (% of total FAME) of broiler quails' breast meat

	Experimental groups[1]			*p*-value	RSD[2]
	C	**HI1**	**HI2**		
No.	12	12	12		
C10:0	0.01^{C}	0.11^{B}	0.14^{A}	<0.0001	0.03
C12:0	0.22^{C}	4.58^{B}	6.49^{A}	<0.0001	1.07
C14:0	0.31^{B}	2.48^{A}	2.79^{A}	<0.0001	0.37
C16:0	16.6^{B}	19.2^{A}	19.5^{A}	<0.0001	0.93
C17:0	0.06	0.04	0.04	0.5978	0.06
C18:0	13.1^{a}	12.3^{ab}	12.0^{b}	0.0109	0.88

Stellenbosch University https://scholar.sun.ac.za

C20:0	0.11	0.08	0.10	0.5825	0.06
C22:0	0.13	0.11	0.13	0.1049	0.03
C23:0	0.57^{B}	0.74^{A}	0.37^{C}	<0.0001	0.13
C24:0	0.87^{B}	0.60^{C}	1.12^{A}	<0.0001	0.83
Total SFA	32.0^{C}	40.3^{B}	42.8^{A}	<0.0001	1.86
C14:1	0.00^{B}	0.31^{A}	0.30^{A}	<0.0001	0.05
C15:1	0.06^{B}	0.08^{AB}	0.12^{A}	0.0012	0.03
C16:1	1.02^{B}	3.16^{A}	3.09^{A}	<0.0001	0.45
C17:1	0.20	0.17	0.19	0.2548	0.04
C18:1 *n*-9	15.4^{b}	17.0^{a}	16.3^{ab}	0.0136	1.30
C18:1 *n*-11	1.71^{A}	1.50^{B}	1.80^{A}	<0.0001	0.29
C20:1 *n*-9	0.34	0.30	0.31	0.8064	0.13
C22:1 *n*-9	0.12	0.12	0.12	0.9615	0.05
Total MUFA	18.8^{B}	22.7^{A}	22.2^{A}	<0.0001	1.64
C18:2 *n*-6	32.0^{A}	22.1^{B}	20.4^{C}	<0.0001	1.21
C18:3 *n*-6	0.12	0.10	0.13	0.4937	0.05
C18:3 *n*-3	1.46^{A}	0.48^{B}	0.54^{B}	<0.0001	0.13
C20:2 *n*-6	0.17	0.20	0.23	0.3251	0.09
C20:3 *n*-6	0.42	0.46	0.40	0.1811	0.08
C20:3 *n*-3	0.36^{B}	0.61^{A}	0.56^{A}	<0.0001	0.12
C20:4 *n*-6	7.50^{A}	6.74^{A}	5.03^{B}	<0.0001	0.99
C20:5 *n*-3	0.26^{B}	0.21^{B}	0.99^{A}	<0.0001	0.13
C22:2 *n*-6	0.21	0.21	0.16	0.5621	0.13
C22:6 *n*-3	1.59^{Ab}	0.89^{B}	1.97^{Aa}	<0.0001	0.34
Total PUFA	44.1^{A}	32.0^{B}	30.4^{B}	<0.0001	2.24
n-6	40.5^{A}	29.8^{B}	26.4^{C}	<0.0001	1.88
n-3	3.66^{A}	2.19^{B}	4.06^{A}	<0.0001	0.50
n-6/*n*-3	11.2^{B}	14.0^{A}	6.56^{C}	<0.0001	1.63
PI	81.9^{A}	61.7^{B}	66.5^{B}	<0.0001	7.51
AI	0.29^{C}	0.62^{B}	0.71^{A}	<0.0001	0.07
TI	0.74^{B}	1.04^{Aa}	0.94^{Ab}	<0.0001	0.09
HH ratio	3.46^{A}	2.20^{B}	2.03^{B}	<0.0001	0.23
Identified FA, %	95.0	95.0	95.4		

[1] HI1= diet supplemented with 10% of dried *Hermetia illucens* larvae reared on layer mash; [2] HI2= diet supplemented with 10% of dried *Hermetia illucens* larvae reared on 50:50 layer mash and fish offal; [3] RSD= Residual Standard Deviation; PI= Peroxidability index; AI= Atherogenicity index; TI= Thrombogenicity index; HH ratio= hypocholsterolemic/hypercholesterolemic ratio; ^{a,b} Means in a row with different superscripts differ significantly ($p < 0.05$); ^{A,B} Means in a row with different superscripts differ significantly ($p < 0.01$).

Overall, the dietary inclusion of HI larvae reared on different growing substrates into quails' diets did not negatively impact the sensory profile of the breast meat which showed similar traits among the three groups (Table 6). The only exception regarded some specific textural attributes: juiciness and fibrousness. Specifically, the HI2 meat had the lowest juiciness compared to C and HI1 (4.53 vs. 5.01 and 4.90 for HI2, C and HI1 breast meat, respectively; $p < 0.001$). As a consequence, HI2 meat exhibited also the highest fibrousness, followed by

Stellenbosch University https://scholar.sun.ac.za

HI1 and C meat (5.28 vs. 5.05 vs. 4.79 for HI2, HI1 and C breast meat, respectively; $p < 0.001$). As indicated in Table 7, the off-odors and off-flavors were not affected by the experimental diets.

Table 6. Effect of the dietary inclusion of *Hermetia illucens* dried larvae farmed on different growth substrates (HI1, HI2) on the sensory scores of broiler quails' breast meat

	Experimental groups[1]			***p*-value**	**RSD**[2]
	C	**HI1**	**HI2**		
No.	16	16	16		
Odour:					
Odor intensity	5.10	5.31	5.48	0.1446	0.52
Off-odor intensity	0.90	1.15	1.03	0.1748	0.37
Flavour:					
Flavor intensity	5.13	5.52	5.29	0.1232	0.52
Off-flavor intensity	1.15	1.14	1.11	0.9725	0.46
Texture:					
Juiciness	5.01^{A}	4.90^{A}	4.53^{B}	0.0008	0.33
Toughness	4.76	4.53	4.55	0.2638	0.44
Chewiness	6.24	6.22	6.41	0.1471	0.28
Fibrousness	4.79^{Bb}	5.08^{ABa}	5.28^{A}	0.0006	0.31

Sensory attributes were scored on numerical and continuous scales from 0 (the lowest score for each attribute) to 10 (the highest score for each attribute); N=6 breasts of the C group were used for the training sessions; [1] HI1= diet supplemented with 10% of dried *Hermetia illucens* larvae reared on layer mash; [2] HI2= diet supplemented with 10% of dried *Hermetia illucens* larvae reared on 50:50 layer mash and fish offal; [3] RSD= Residual Standard Deviation; ^{a,b} Means in a row with different superscripts differ significantly ($p < 0.05$); ^{A,B} Means in a row with different superscripts differ significantly ($p < 0.01$).

Stellenbosch University https://scholar.sun.ac.za

Table 7. Effect of the dietary inclusion of *Hermetia illucens* dried larvae farmed on different growth substrates (HI1, HI2) on the off-odors and off-flavors (% on total tested samples) of broiler quails' breast meat

	Experimental groups[1]			*p*-value	RSD[2]
	C	HI1	HI2		
No.	16	16	16		
Off-odors:					
Game meat	18.8	6.25	6.25	0.5926	1.79
Metallic	6.25	0.00	0.00	1.0000	2.04
Liver	31.3	50.0	31.3	0.5930	1.60
Oil/fat	6.25	25.0	31.3	0.2788	3.28
Peanut/Hazelnut	12.5	0.00	6.25	0.7650	2.13
Rancid	0.00	0.00	12.5	0.3110	4.17
Off-flavors:					
Game meat	6.25	18.8	18.8	0.6810	1.34
Metallic	18.8	0.00	0.00	0.1000	6.40
Liver	37.5	75.0	56.3	0.1152	4.57
Oil/fat	6.25	25.0	6.25	0.3342	3.43
Peanut/Hazelnut	12.5	12.5	6.25	1.0000	0.45
Rancid	0.00	0.00	0.00	-	-

N=6 breasts of the C group were used for the training sessions. [1] HI1= diet supplemented with 10% of dried *Hermetia illucens* larvae reared on layer mash; [2] HI2= diet supplemented with 10% of dried *Hermetia illucens* larvae reared on 50:50 layer mash and fish offal.

The results of the retail display trial (Table 8) showed that the dietary treatment did not play a role in affecting the broiler quails' breast meat traits: drip loss percentage, overall colorimetric characteristics and oxidative status showed similar results throughout the retail display trial. The only exception was the lightness (L*) value measured at day 0 of retail display, when HI2 meat had a higher value compared to C, with HI1 being intermediate (51.6 vs. 50.6 vs. 49.0 for HI2, HI1 and C breast meat, respectively; $p < 0.05$).

Stellenbosch University https://scholar.sun.ac.za

Table 8. Effect of the dietary inclusion of *Hermetia illucens* dried larvae farmed on different growth substrates (HI1, HI2) on a 7-day retail display trial of broiler quails' breast meat

	Experimental groups[1]			*p*-value	RSD[2]
	C	HI1	HI2		
No.	12	12	12		
Drip loss, %	6.05	4.83	6.67	0.0525	1.63
Day 0:					
pH	5.60	5.54	5.54	0.4075	0.10
L*	49.0^{b}	50.6^{ab}	51.6^{a}	0.0357	2.15
a*	2.07	2.83	3.02	0.2025	1.17
b*	10.3	10.6	11.5	0.2161	1.54
TBARs, mg MDA/kg meat	1.52	1.51	1.53	0.2179	0.02
Day 8:					
pH8	5.90	5.90	6.01	0.1424	0.14
L*	47.7	46.4	46.7	0.6531	3.27
a*	2.68	4.06	3.46	0.1207	1.44
b*	10.4	10.7	11.7	0.4268	2.26
TBARs, mg MDA/kg meat	1.84	1.75	1.87	0.6852	0.30

[1] HI1= diet supplemented with 10% of dried *Hermetia illucens* larvae reared on layer mash; [2] HI2= diet supplemented with 10% of dried *Hermetia illucens* larvae reared on 50:50 layer mash and fish offal; [3] RSD= Residual Standard Deviation; [a,b] Means in a row with different superscripts differ significantly ($p < 0.05$).

Discussion

Physical breast meat traits observed in the present research (Table 2) were overall satisfactory and in line with values reported for quails [26]. The highest cooking loss shown by HI2 meat was similar to that observed by Cullere *et al.* [7]. In the latter, breast meat derived from quails fed with the highest dietary inclusion level (15%) of a partly defatted *H. illucens* larvae meal, showed the highest cooking loss. In this case, a lower meat pH was also observed and it was hypothesized as causative agent of the higher cooking loss: as the pH value approaches the isoelectric point of proteins, their water holding capacity reduces thus generating a higher moisture loss. However, HI2 meat in the present trial had the same pH of the other experimental groups, thus a causative agent for the observed different cooking loss percentage is to be searched elsewhere.

Even if the experimental diets HI1 and Control of the present experiment had a slightly different absolute protein contents (23.9 and 24.3 g/100 g feed for HI1 and Control diets, respectively) [17], the lower protein content observed in the breast meat of the HI1 quails compared to those of the Control group could not be directly attributable to this factor. It is now well established that not all nitrogen present in insects originates from protein: insects' exoskeleton contain chitin, a polysaccharide containing N atoms which typically falls within the amount of nitrogen quantified by the common Kjeldahl method. On the other hand, during the larvae's sclerotization phase of development, different cuticolar proteins harden the cuticle

Stellenbosch University https://scholar.sun.ac.za

(exoskeleton) by linking the chitin fibres. This particular structural arrangement of the insect cuticle makes such proteins indigestible to animals [27]. Up to a couple of years ago, these factors were ignored in the formulations of animals' diet including insects, thus technically resulting in diets which were only apparently iso-nitrogenous because the protein content of insects was generally overestimated [28]. To solve this drawback, further research on this topic resulted in the recent recommendation to use of a specific nitrogen conversion factor for insects of 5.62 [29]. This above-mentioned issue could be the main reason for the observed difference in the protein content of HI1 and Control quail breasts. Interestingly, for HI2 meat the same finding was not applicable. It is known that different rearing substrates can determine diverse development rates in *H. illucens* larvae [30], HI2 larvae could have been slightly less developed than HI1 larvae and this could have led to a different sclerotization stages of the cuticle in the two HI larvae. As a consequence, the amount of indigestible protein could have been different in the two HI larvae, thus leading to the observed results in breast meat protein content.

Results about the amino acid content of breast meat (Table 4) further emphasized the above-mentioned speculations: HI1 meat had an absolute lower amino acid content compared to Control meat (19.3 compared to 21.6 g/100 g meat), which determined the lower protein percentage in the HI1 group compared to the Control meat. However, in the previous study by Cullere et al. [8] the same finding was not observed, as quails receiving increasing dietary inclusion levels (up to 15%) of a partly defatted *H. illucens* larvae meal had similar protein and thus amino acids contents. This apparent discrepancy could be explained by the fact that, in Cullere et al. [8], the tested insect source was a defatted meal derived from same aged larvae that had been fed the same diets. Previous literature showed that the defatting process concentrates protein and amino acids therefore determining a higher amount of amino acids compared to full-fat larvae [31]. When defatted *H. illucens* larvae were incorporated into different avian species such as quail [8], chicken [32] and Barbary partridge [9], protein quantity of the derived meat was similar in the experimental groups.

The inclusion of a dietary ingredients with cholesterol up to a certain threshold in poultry diets, should only moderately affect that of meat because liver lipid metabolism can decrease cholesterol *de novo* synthesis as well as increase transformation and transportation rate as a response to the dietary level; this mechanism is essential to guarantee optimal animal health, welfare and growth [33]. Despite this, independently to the growing substrate, the inclusion of 10% HI larvae into quail diets increased the cholesterol content of the breasts compared to the Control group. Literature studies considering this aspect are still limited and available results are controversial: in the work by Cullere et al. [34] testing *H. illucens* fat as soybean oil replacer in finisher broiler chickens, a 100% substitution produced breast meat with the same

Stellenbosch University https://scholar.sun.ac.za

cholesterol content as the Control group. A similar finding was observed by Cullere *et al.* [8] on broiler quails fed either with 10 or 15% *H. illucens* meal. In the latter, however, numerical, but not significant increase in meat cholesterol contents going from the lowest to the highest inclusion level (71.6, 73.3, 74.9 mg/100 g meat in 0%, 10% and 15 % inclusion levels, respectively) was noted. In past experiments studying quail metabolism, it emerged that these birds respond particularly quickly and intensely to dietary cholesterol content and that they can develop atherosclerosis relatively fast, with relevant differences depending on sex and genetics [35,36]. Based on such speculations, it would be interesting to investigate the effects of genetics, sex and type of insect product fed to quails on their cholesterol metabolism.

The fact that HI1 meat showed the highest meat lipids content was unexpected, especially considering that the dietary fat content was similar in the three experimental groups, and almost identical in the HI1 and HI2 diets [17]. A possible reason to explain this could come from the results of the digestibility trial [17] where the HI1 quails showed the highest apparent digestibility of starch and metabolizable energy which could explain the higher meat lipids content observed in the present research. However, this finding is not confirmed by previous studies on quail [8], chicken [32,37], or Barbary partridge [9] fed with different inclusion levels of *H. illucens* larvae protein meal or fat.

Results of the present research demonstrate once more that the FA composition of the *H. illucens* larvae can only be moderately affected by the farming substrate. In this case, exploiting fish offal waste to modulate the larvae growing media, improved the healthiness of their FA profile. In fact, the *n*-3 proportion increased (+34% compared to HI1 larvae), almost exclusively due to the eicosapentaenoic (EPA) and docosaesaenoic (DHA) acids, which are FA typically found in marine fish sources and which have important implications in human health mainly due to their positive effects on cardiometabolic risk factors [38]. Despite this, the overall FA profile was similar in HI1 and HI2 larvae and coherent to the typical FA profile which is reported in literature for this insect species: rich in lauric (C12:0), myristic (C14:0) and palmitic (C16:0) FAs for the SFA fraction, and with oleic (C18:1 *n*-9) and linoleic (C18:2 *n*-6) FAs being the main MUFA and PUFA, respectively [9,34]. The successful increase of the *n*-3 proportions in the *n*-3 HI fed larvae is in agreement with previous studies testing the inclusion of fish-offal [16], fishmeal [39], or brown algae (*Ascophyllum nodosum*), in the growing substrate [40]. The high SFA content of *H. illucens* larvae is linked to their peculiar adult stage: in fact, after pupation, the buccal apparatus regresses thus adults cannot feed anymore. For this reason, to survive they rely on energy reserves accumulated during their larval stage. Furthermore, it seems that dietary PUFA are primarily used by the larvae as energy source (oxidation) and that extra energy is stored in their body mainly as SFA and MUFA [39], thus explaining why their FA profile is poorly represented by PUFA. As a direct effect of larvae FA

Stellenbosch University https://scholar.sun.ac.za

profile, when the 10% IM was included into the quails' diets their FA profile was characterized by a 28% SFA increase and an overall reduction in MUFA (-5.5%) and PUFA (-57%) proportions. This had a direct effect on the FA composition of the meat obtained from quails fed with the experimental diets. In fact, SFA increased by 30% and PUFA decreased by 29%. Even if dietary SFA intake in humans should be moderate as it is associated to increased cardiovascular disease risk [41], it must be stressed that SFAs play important roles in normal cellular and tissue metabolism and function as, among others, they are essential constituents of cell membrane phospholipids, structurally part of important cell signaling molecules, involved in gene expression, and in the modulation of cholesterol, fatty acid and triacylglycerol synthesis and lipoprotein assembly, secretion, and clearance [42]. Specifically referring to lauric acid, which accounts for the greatest part (about 65%) of the larvae's SFA percentage and for about the 13% of the meat SFA proportion, in the human or animal body it is converted to monolaurin which in turn aids in preventing viral, bacterial and protozoal infections [43]. Furthermore, 6-12 medium-chain FA in general are efficiently absorbed, digested and beta-oxidized in the gut, thus being very efficient energy substrates for both livestock and humans [44], and they were reported to improve the gut's health thanks to their intrinsic antibacterial and antiviral properties [45]. The addition of HI to the quails' diets reduced the overall dietary MUFA content, but the observed trend in the meat was not coherent in this sense: meat of HI1 and HI2 groups had about +15% MUFA compared to the Control group. This outcome agreed with previous observations on quails fed with increasing levels of a partly defatted *H. illucens* larvae meal [8] but not with those reported on broiler chickens whose dietary content of soybean oil was replaced by 50% and 100% with *H. illucens* fat [34]. This could highlight, on the one hand, a particular intrinsic capability of quails to desaturate and elongate SFA into MUFA. On the other hand, it further corroborates the hypothesis that diets characterized by low-fats and high carbohydrates have a role in the up-regulation of the activity of the lipogenic enzyme $\Delta 9$ desaturase [46]. In fact, the Control diet of the present study slightly differed in terms of fat and starch contents compared to those including the 10% HI larvae [17]. Regarding meat PUFA, it was observed that modulating HI larvae substrate with fish offal was a successful way to improve the *n*-3 PUFA proportion of meat of HI-fed quails and thus to lower the breasts' *n*-6/*n*-3 ratio which is considered a sort of target index for a meat to be considered as healthy [47]. Specifically, as a result of the HI substrate modulation, in the present experiment a reduction of ~53% and ~41% of the *n*-6/*n*-3 ratio in HI2 quails compared to the HI1 and Control quails, respectively was noted.

Research dealing with the effect of meat obtained from insect-fed poultry on its sensory characteristics is still limited. Despite the fact that *H. illucens* larvae meal has been characterized by a peculiar flavor [48], existing knowledge seems to indicate that the impact

Stellenbosch University https://scholar.sun.ac.za

of this emerging feed ingredient on the sensory traits of animal products is limited [10]. The sensory profile of quail meat and that of eggs obtained from quails fed with *H. illucens* larvae meal (up to the 15% inclusion level) did not differ from the Control group without insect meal [8,14]. Also, the use of *H. illucens* fat as alternative fat source for broiler chickens did not affect the sensory traits of the derived meat compared to chicken fed with a conventional soybean-oil diet [34]. In this context, the present research is the first highlighting significant changes in the textural traits of quail meat; as meat of the HI2 treatment displayed the highest cooking loss), it was not surprising that meat juiciness and fibrousness were the lowest and the highest, respectively. This is because, independently to the considered animal species, cooking loss has a great influence on the juiciness of meat [49]. Diversely, it was unexpected the higher fibrousness indicated for the HI1 meat compared to that of the Control. Furthermore, HI1 meat was also the richest in lipids which should have had a positive effect on meat sensory characteristics, including textural and sensory perception [50].

Results on the quail breasts subjected to the retail display trial are in agreement with Schiavone et al. [51] who evaluated the impact of feeding *H. illucens* fat to poultry on their meat quality evaluated during refrigerated storage. Meat physical traits are responsible for its visual appearance which has a key impact on consumers' choice at purchase [52]. In this view, the fact that meat of the three dietary treatments showed similar drip loss and comparable colorimetric characteristics is of utmost importance for marketing purposes. The highest L* value at day 0 of refrigerated storage observed in the HI2 meat was not expected since the same result was not noted when studying the physical breast meat characteristics. In past studies, it was found that *H. illucens* larvae contain carotenoids (about 2.00-2.15 mg/kg), which can positively affect yolk yellowness of eggs produced by quails and hens fed with this larvae meal [14,53]. No mention about a possible effect on meat color/lightness is however found in literature considering poultry species fed with different inclusion levels and products derived from the *H. illucens* larvae. Interestingly, despite the intense modifications in the FA profile of the quail meat, its oxidative status remained unaffected throughout the retail-display trial, ensuring meat, in this sense, that is completely satisfactory from the qualitative point of view.

Conclusions

The present experiment showed that it is possible to exploit a valuable nutrient source which is generally considered a waste to partly improve the fatty acid profile of *H. illucens* larvae: in fact, the use of fish offal to farm *H. illucens* larvae provided a substantial enrichment of the HI with *n*-3 fatty acids. This, in turn, lead to a lower *n*-6/*n*-3 ratio of broiler quails' meat which was the primary objective of the present work. The main remaining drawback, however, is still the SFA proportion that remains the preponderant larvae lipid fraction and which directly reflects on the FA profile of quails' meat. Therefore, results suggest that it would be interesting to test

Stellenbosch University https://scholar.sun.ac.za

the application of a defatted HI meal combined with the direct inclusion of another PUFA-rich feed source to produce meat with a lipids profile that fully meets the health requirements of modern consumers. Based on present findings, further research to investigate the role of this insect source in poultry metabolism of fats and cholesterol is also warranted.

Stellenbosch University https://scholar.sun.ac.za

References

1. Silva, W.J.D.; Gouveia, A.B.V.S.; Sousa, F.E.D.; Santos, F.R.D.; Minafra-Rezende, C.S.; Silva, J.M.S.; Minafra, C.S. Turmeric and sorghum for egg-laying quails. *Ital. J. Anim. Sci.* **2018**, *17*, 368-376, DOI: 10.1080/1828051X.2017.1360160.
2. Charlton, A.J.; Dickinson, M.; Wakefield, M.E.; Fitches, E.; Kenis, M.; Han, R.; Zhu, F.; Kone, N.; Grant, M.; Devic, E.; Bruggeman, G.; Prior, R.; Smith, R. Exploiting the chemical safety of fly larvae as a source of protein for animal feed. *J. Insects Food Feed* **2015**, *1*, 7-16, DOI: 10.3920/JIFF2014.0020.
3. Biasato, I.; De Marco, M.; Rotolo, L.; Renna, M.; Lussiana, C.; Dabbou, S.; Capucchio, M.T.; Biasibetti, E.; Costa, P.; Gai, F.; Pozzo, L.; Dezzutto, D.; Bergagna, S.; Martínez, S.; Tarantola, M.; Gasco, L.; Schiavone, A. Effects of dietary *Tenebrio molitor* meal inclusion in free-range chickens. *J. Anim. Physiol. An. N.* **2016**, *100*, 1104–1112, DOI: 10.1111/jpn.12487.
4. Makkar, H.P.S.; Tran, G.; Heuzé, V.; Ankers, P. State-of-the-art on use of insects as animal feed. *Anim. Feed Sci. Technol.* **2014**, *197*, 1-33, DOI: 10.1016/j.anifeedsci.2014.07.008.
5. ur Rehman, K.; Rehman, A.; Cai, M.; Zheng, L.; Xiao, X.; Somroo, A.A.; Wang, H.; Li, W.; Yu, Z.; Zhang, J. Conversion of mixtures of dairy manure and soybean curd residue by black soldier fly larvae (*Hermetia illucens* L.). *J. Clean. Prod.* **2017**, *154*, 366-373, DOI: 10.1016/j.jclepro.2017.04.019.
6. Renna, M.; Schiavone, A.; Gai, F.; Dabbou, S.; Lussiana, C.; Malfatto, V.; Prearo, M.; Capucchio, M.T.; Biasato, I.; Biasibetti, E.; De Marco, M.; Brugiapaglia, A.; Zoccarato, I.; Gasco, L. Evaluation of the suitability of a partially defatted black soldier fly (*Hermetia illucens* L.) larvae meal as ingredient for rainbow trout (*Oncorhynchus mykiss* Walbaum) diets. *J. Anim. Sci. Biotechno.* **2017**, *8*, 57, DOI: 10.1186/s40104-017-0191-3.
7. Cullere, M.; Tasoniero, G.; Giaccone, V.; Miotti-Scapin, R.; Claeys, E.; De Smet, S.; Dalle Zotte, A. Black soldier fly as dietary protein source for broiler quails: apparent digestibility, excreta microbial load, feed choice, performance, carcass and meat traits. *Animal* **2016**, *10*, 1923-1930, DOI: 10.1017/S1751731116001270.
8. Cullere, M.; Tasoniero, G.; Giaccone, V.; Acuti, G.; Marangon, A.; Dalle Zotte, A. Black soldier fly as dietary protein source for broiler quails: Meat proximate composition, fatty acid and amino acid profile, oxidative status and sensory traits. *Animal* **2018**, *12*, 640-647, DOI: 10.1017/S1751731117001860.

Stellenbosch University https://scholar.sun.ac.za

9. Secci, G.; Moniello, G.; Gasco, L.; Bovera, F.; Parisi, G. Barbary partridge meat quality as affected by *Hermetia illucens* and *Tenebrio molitor* larva meals in feeds. *Food. Res. Intl*. **2018**, *112*, 291-298, DOI: 10.1016/j.foodres.2018.06.045.
10. Pieterse, E, Erasmus, S.W., Uushona, T., Hoffman, L.C. Black soldier fly (*Hermetia illucens*) pre-pupae meal as a dietary protein source for broiler production ensures a tasty chicken with standard meat quality for every pot. *J. Sci. Food Agr.* **2019**, *99*, 893-903, DOI: 10.1002/jsfa.926.
11. Yu, M., Li, Z., Chen, W., Rong, T., Wang, G., Li, J., Ma, X. Use of *Hermetia illucens* larvae as a dietary protein source: Effects on growth performance, carcass traits, and meat quality in finishing pigs. *Meat Sci.* 2019, DOI: 10.1016/j.meatsci.2019.05.008.
12. Martins, C.; Cullere, M.; Dalle Zotte, A.; Cardoso, C.; Alves, S.P.; de Bessa, R.J.B.; Freire, J.P.B.; Falcão-e-Cunha, L. Incorporation of two levels of black soldier fly (*Hermetia illucens* L.) larvae fat or extruded linseed in diets for growing rabbits: effects on growth performance and diet digestibility. *Czech J. Anim. Sci.* **2018**, *63*, 356-362. DOI: 10.17221/22/2018-CJAS.
13. Dalle Zotte, A.; Cullere, M.; Martins, C.; Alves, S.P.; Freire, J.P.B.; Falcão-e-Cunha, L.; Bessa, R.J.B. Incorporation of two levels of black soldier fly (*Hermetia illucens* L.) larvae fat or extruded linseed in diets for growing rabbits and their effects on meat quality traits including detailed fatty acid composition. *Meat Sci.* **2018**, *146*, 50-58. DOI: 10.1016/j.meatsci.2018.08.002.
14. Dalle Zotte, A.; Singh, Yazavinder.; Michiels, J.; Cullere, M. Black soldier fly (*Hermetia illucens*) as dietary source for laying quails: live performance, and egg physic-chemical quality, sensory profile and storage stability. *Animals* **2019**, *9*, 115, DOI: doi:10.3390/ani9030115.
15. Spranghers, T.; Ottoboni, M.; Klootwijk, C.; Ovyn, A.; Deboosere, S.; De Meulenaer, B.; Michiels, J.; Eeckhout, M.; De Clerq, P.; De Smet, S. Nutritional composition of black soldier fly (*Hermetia illucens*) prepupae reared on different organic waste substrates. *J. Sci. Food Agr.* **2017**, *97*, 2594-2600. DOI: 10.1002/jsfa.8081.
16. St-Hilaire, S.; Cranfill, K.; McGuire, M.A.; Mosley, E.E.; Tomberlin, J.K.; Newton, L.; Sealey, W.; Sheppard, C.; Irving, S. Fish offal recycling by the black soldier fly produces a foodstuff high in omega-3 fatty acids. *J. World Aquacult. Soc.* **2007**, *38*, 309-313, DOI: 10.1111/j.1749-7345.2007.00101.x.
17. Woods, M.J.; Cullere, M.; Van Emmenes, L.; Vincenzi, S.; Pieterse, E.; Hoffman, L.C.; Dalle Zotte, A. *Hermetia illucens* larvae reared on different substrates in broiler quail diets: effect on apparent digestibility, feed-choice and growth performance. *J. Insects Food Feed* **2019**, 1-10, DOI: 10.3920/JIFF2018.0027.

Stellenbosch University https://scholar.sun.ac.za

18. Commission Internationale de l'Éclairage. Official recommendations on uniform colour space, colour difference equations and metric colour terms. CIE Publication no. 15 (E-1.3.1), suppl. 2. Bureau Central de la CIE, Paris, France, **1976**.
19. Hornsey H.C. The colour of cooked cured pork. I. Estimation of the nitric oxide-haem pigments. *Journal of the Science of Food and Agriculture* **1956**, *7*, 534–540, DOI: 10.1002/jsfa.2740070804.
20. AOAC. Official methods of analysis, 19th edition. Association of Official Analytical Chemists, Arlington, VA, USA, **2012**.
21. Casiraghi, E.; Lucisano, M.; Pompei, C.; Dellea, C. Cholesterol determination in butter by high performance chromatography. *Milchwissenschaft* **1994**, *49*, 194–196, WOS: A1994NM43000004.
22. Cunico R., Mayer, A.G., Wehr, C.T., Sheehan, T.L. High sensitivity amino acid analysis using novel automated precolumn derivativation system. *Biochromotography* **1986**, *1*, 6-14.
23. Botsoglou, N.A.; Fletouris, D.J.; Papageorgiou, G.E.; Vassilopoulos, V.N.; Mantis, A.J.; Trakatellis, A.G. Rapid, sensitive, and specific thiobarbituric acid method for measuring lipid peroxidation in animal tissue, food, and feedstuff samples. *J. Agr. Food Chem.* **1994**, *42*, 1931–1937. DOI: 10.1021/jf00045a019.
24. Statistical Analysis Software for Windows. Statistics version 9.1.3. SAS Institute, Cary, NC, USA, **2008**.
25. Marascuilo LA. Large-sample multiple comparisons. *Psychol. Bull*. **1966**, *65*, 280–290. DOI: 10.1037/h0023189.
26. Tavaniello, S.; Maiorano, G.; Siwek, M.; Knaga, S.; Witkowski, A.; Di Memmo, D.; Bednarczyk, M. Growth performance, meat quality traits, and genetic mapping of quantitative trait loci in 3 generations of Japanese quail populations (*Coturnix japonica*). *Poultry Sci.* **2014**, *93*, 2129-2140, DOI: 10.3382/ps.2014-03920.
27. Andersen, S.O.; Hojrup, P.; Roepstorff, P. Insect cuticular proteins. *Insect Biochem. Mol. Biol.* **1995**, *25,* 153-176, DOI: 10.1016/0965-1748(94)00052-J.
28. Jonas-Levi, A.; Martinez, J.J.I. The high level of protein content reported in insects for food and feed is overestimated. *J. Food Compos. Anal.* **2017**, *62*, 184-188, DOI: 10.1016/j.jfca.2017.06.004.
29. Nery, J.; Gasco, L.; Dabbou, S.; Schiavone, A. Protein composition and digestibility of black soldier fly larvae in broiler chickens revisited according to the recent nitrogen-protein conversion ratio. *J. Insects Food Feed* **2018**, *4*, 171-177, DOI: 10.3920/JIFF2018.0006.

Stellenbosch University https://scholar.sun.ac.za

30. Tschirner, M.; Simon, A. Influence of different growing substrates and processing on the nutrient composition of black soldier fly larvae destined for animal feed. *J. Insects Food Feed* **2015**, *1*, 249-259, DOI: 10.3920/JIFF2014.0008.
31. Schiavone, A.; De Marco, M.; Martínez, S.; Dabbou, S.; Renna, M.; Madrid, J.; Hernandez, F.; Rotolo, L.; Costa, P.; Gai, F.; Gasco, L. Nutritional value of a partially defatted and a highly defatted black soldier fly larvae (*Hermetia illucens* L.) meal for broiler chickens: apparent nutrient digestibility, apparent metabolizable energy and apparent ileal amino acid digestibility. *J. Anim. Sci. Biotechno.* **2017**, *8*, 51, DOI: 10.1186/s40104-017-0181-5.
32. Schiavone, A.; Dabbou, S.; Petracci, M.; Zampiga, M.; Sirri, F.; Biasato, I.; Gai, F.; Gasco, L. Black soldier fly defatted meal as a dietary protein source for broiler chickens: Effects on carcass traits, breast meat quality and safety. *Animal* **2019**, 1-9, DOI: 10.1017/S1751731119000685.
33. Liu, J.; Duan, Y.; Hu, Y.; Sun, L.; Wang, S.; Fu, W.; Ni, Y.; Zhao, R. Exogenous administration of chronic corticosterone affects hepatic cholesterol metabolism in broiler chickens showing long or short tonic immobility. *Comp. Biochem. Phys. A* **2016**, *191*, 53-58, DOI: 10.1016/j.cbpa.2015.09.020.
34. Cullere, M.; Schiavone, A.; Gasco, L; Dalle Zotte, A. Meat quality and sensory traits of finisher broiler chickens fed with black soldier fly (*Hermetia illucens* L.) larva fat as alternative fat source. *Animals* **2019**, *9*, 140, DOI: 10.3390/ani9040140.
35. Morrissey, R.B.; Donaldson, W.E. Rapid accumulation of cholesterol in serum, liver and aorta of Japanese quail. *Poult. Sci.* **1977**, *56*, 2003-2008, DOI: 10.3382/ps.0562003.
36. Bavelaar, F.J.; Beynen, A.C. The relation between diet, plasma cholesterol and atherosclerosis in pigeons, quails and chickens. *Int. J. Poultry Sci.* **2004**, *11*, 671-683, DOI: 10.3923/ijps.2004.671.684.
37. Schiavone, A.; Cullere, M.; De Marco, M.; Meneguz, M.; Biasato, I.; Bergagna, S.; Dezzutto, D.; Gai, F.; Dabbou, S.; Gasco, L.; Dalle Zotte, A. Partial or total replacement of soybean oil by black soldier fly larvae (*Hermetia illucens* L.) fat in broiler diets: effect on growth performances, feed-choice, blood traits, carcass characteristics and meat quality. *Ital. J. Anim. Sci.* **2017**, *16*, 93-100, DOI: 10.1080/1828051X.2016.1249968.
38. Innes JK, Calder PC. The differential effects of eicosapentaenoic acid and docosahexaenoic acid on cardiometabolic risk factors: a systematic review. *Int. J. Mol. Sci.* **2018**, DOI: 10.3390/ijms19020532.
39. Barroso, F.G.; Sánchez-Muros, M.J.; Segura, M.; Morote, E.; Torres, A.; Ramos, R.; Guil, J.L. Insects as food: Enrichment of larvae of *Hermetia illucens* with omega 3 fatty

Stellenbosch University https://scholar.sun.ac.za

acids by means of dietary modifications. *J. Food Compos. Anal.* **2017**, *6*2, 8-13, DOI: 10.1016/j.jfca.2017.04.008.

40. Liland, N.S.; Biancarosa, I.; Araujo, P.; Biemans, D., Bruckner, C.G.; Waagbø, R.; Torstensen, B.E.; Lock, E.J. Modulation of nutrient composition of black soldier fly (*Hermetia illucens*) larvae by feeding seaweed-enriched media. *PloS one* **2017***, 12*, e0183188, DOI: 10.1371/journal.pone.0183188.
41. Kris-Etherton, P.M.; Petersen, K.; Van Horn, L. Convincing evidence supports reducing saturated fat to decrease cardiovascular disease risk. *BMJ Nutr. Prev. Health* 20 **2018**, *0*, 1-4, DOI: 10.1136/bmjnph-2018-000009.
42. Calder, P.C. Functional roles of fatty acids and their effects on human health. *JPEN-Parenter. Enter.* **2015**, *39*, 18S-32S, DOI: 10.1177/0148607115595980.
43. Enig, M.G. Coconut: in support of good health in the 21st century. In 36th meeting of APCC, September **1999**, http://citeseerx.ist.psu.edu/viewdoc/download?doi=10.1.1.602.2612&rep=rep1&type=pdf, Accessed July 2019.
44. Dayrit, F.M. The properties of lauric acid and their significance in coconut oil. *J. Am. Oil Chem. Soc.* **2015**, *92*, 1-15, DOI: 10.1007/s11746-014-2562-7.
45. Liu, Y. Fatty acids, inflammation and intestinal health in pigs. *J. Anim. Sci. Biotechno.* **2015**, *6*, 41, DOI: 10.1186/s40104-015-0040-1.
46. Poureslami, R.; Turchini, G.M.; Raes, K.; Huyghebaert, G.; De Smet, S. Effect of diet, sex and age on fatty acid metabolism in broiler chickens: SFA and MUFA. *Brit. J. Nutr.* **2010**, *104*, 204-213, DOI: 10.1017/S0007114510000541.
47. Wood, J.D.; Richardson, R.I.; Nute, G.R.; Fisher, A.V.; Campo, M.M.; Kasapidou, E.; Sheard, P.R.; Enser, M. Effects of fatty acids on meat quality: a review. *Meat Sci.* **2004**, *66*, 21-32, DOI: 10.1016/S0309-1740(03)00022-6.
48. Wang, Y.S.; Shelomi, M. Review of black soldier fly (Hermetia illucens) as animal feed and human food. *Foods* **2017**, *6*, 91. DOI: 10.3390/foods6100091.
49. Aaslyng, M.D., Bejerholm, C., Ertbjerg, P., Bertram, H.C., Andersen, H.J. Cooking loss and juiciness of pork in relation to raw meat quality and cooking procedure. *Food Qual. Prefer.* **2003**, *14*, 277-288, DOI: 10.1016/S0950-3293(02)00086-1.
50. Fernandez, X.; Monin, G.; Talmant, A.; Mourot, J.; Lebret, B. Influence of intramuscular fat content on the quality of pig meat—2. Consumer acceptability of *m. longissimus lumborum. Meat Sci.* **1999**, *53*, 67-72, DOI: 10.1016/S0309-1740(99)00038-8.
51. Schiavone, A., Cullere, M., De Marco, M., Meneguz, M., Biasato, I., Bergagna, S., Dezzutto, D., Gai, F., Dabbou, S., Gasco, L., Dalle Zotte, A. Partial or total replacement of soybean oil by black soldier fly larvae (*Hermetia illucens L.*) fat in broiler diets: effect

Stellenbosch University https://scholar.sun.ac.za

on growth performances, feed-choice, blood traits, carcass characteristics and meat quality. Ital. J. Anim. Sci. **2017**, *16*, 93-100, DOI: 10.1080/1828051X.2016.1249968.

52. Ressurreccion, A.V.A. Sensory aspects of consumer choices for meat and meat products. *Meat Sci.* **2004**, 66, 11-20, DOI: 10.1016/S0309-1740(03)00021-4.
53. Secci, G.; Bovera, F.; Nizza, S.; Baronti, N., Gasco, L.; Conte, G.; Serra, A.; Bonelli, A.; Parisi, G. Quality of eggs from Lohmann Brown Classic laying hens fed black soldier fly meal as substitute for soya bean. Animal 2018, 12, 2191-2197, DOI: 10.1017/S1751731117003603.

Stellenbosch University https://scholar.sun.ac.za

CHAPTER 7

A simple and rapid protocol for measuring chitin content of insect larvae

In review as: Woods, M.J., Goosen, N.J. Hoffman, L.C. and Pieterse, E., 2019. A simple and rapid protocol for measuring chitin content of insect larvae. Journal of Insects as Food and Feed.

Stellenbosch University https://scholar.sun.ac.za

Abstract

The study reports on a simple gravimetrical analysis to determine the chitin content of insect larvae. *Hermetia illucens* larvae was used as sample material. The method of analysis comprised of a defatting treatment by means of rapid solvent extraction (2 : 1 chloroform : methanol), followed by a treatment with 1 M HCL (demineralisation) and 1 M NaOH (deproteinisation). The nitrogen content of the obtained chitin was determined and compared to that of the nitrogen content of pure chitin (6.89). The chitin content of *H. illucens* larvae was determined to be 5.68 % ± 0.15 with a nitrogen content of 6.43 ± 0.038 (mean ± standard error). The average nitrogen content of the isolated chitin was lower than the theoretical value calculated for pure chitin. This indicated that there was still a small amount of inorganic compounds present in the chitin of the insect larvae after applying the developed analytical procedures. This was confirmed by the ash value of the isolated chitin (1.50 % ± 0.06) (mean ± standard error). The developed analysis is a simple and accurate method, which gives repeatable results, for the determination of the chitin content of insect larvae. Further studies regarding the demineralisation treatment could improve the accuracy of the method due to the removal of all inorganic components.

Keywords: gravimetrical analysis, defatting, demineralisation, deproteinisation

Introduction

Insects are gaining popularity as source of feed and food due to a growing population, increasingly demanding consumers and a limited amount of agricultural land (Van Huis *et al.,* 2013). Insects have been identified as an alternative source of protein to supplement the predicted increase in demand for protein of 75% by 2050 from the current status (Herrero *et al.,* 2015). Insects can be farmed in high densities with small space requirements and have high bioconversion ratios (Oonincx & de Boer, 2012). Moreover, there are numerous insect species that can be reared on biowaste, which assists in recycling refuse and keeps the environmental footprint low (Smetana *et al.,* 2016).

In most cases the larval stage of insects was found to be the most nutritious (Kouřimská & Adámková, 2016). Insect larvae consist mainly of protein, fat, ash and chitin present in the cuticle (Finke & Oonincx, 2014). Chitin is the second most abundant polymer in the world after cellulose and is a natural polysaccharide of major importance (Rinaudo, 2006). Chitin has many commercial applications in the biotechnological, biomedical and cosmetic industries, to name but a few (Rinaudo, 2006). Chitin has been found in various other living organisms such as shrimps, crabs, and tortoises, as well as in the cell walls of fungi, internal structures of invertebrates and exoskeleton of arthropods (Rinaudo, 2006; Vincent & Wegst, 2004; Kadokawa, 2011). Up until now, despite its widespread occurrence, the main commercial

Stellenbosch University https://scholar.sun.ac.za

sources of chitin have been crab and shrimp shells. Limited data in literature exist stating the chitin content of whole insect larvae but from an enzymatic assay, Cauchie (2002) reported that insect larvae contain between 2.9 – 10.1 % chitin on a dry matter basis.

In recent times, interest surrounding the exact chitin content of larvae has grown for numerous reasons. Firstly, in feed and food application it was determined that the protein content of the larvae is over estimated due to the fact that chitin contains nitrogen and not all nitrogen found in insects should be allocated to protein (Janssen *et al.,* 2017). Secondly, the quantification of chitin found in insects is important to be able to conduct accurate animal digestibility studies. Even though the enzyme chitinase is found in human and animal gastric juices (Paoletti *et al.,* 2007), chitin is considered as an indigestible fibre. It is postulated that the enzyme might be inactive although it is thought that there an active chitinase response in the body prevails among tropical countries where the consumption of insects has a long-term tradition (Muzzarelli *et al.,* 2001). Hence, the removal of chitin will improve the digestibility of insect protein (Finke, 2007). Lastly, the body composition technique has been used to identify the nutrient requirements of insects and formulate artificial diets for the mass-rearing of insects for various applications (Woods *et al*., 2019a,b) as it is thought that the composition of an insect's body reflects its nutritional needs (Rock & King, 1967). This method incorporates using the profile of various nutrients in a target insect's body as template for artificial diets and thus further highlights the demand to accurately determine the chitin content of insects.

It has been found that the chitin content of shellfish can be determined by acid detergent fiber (ADF) and crude fiber (CF) analyses. This is not the case for chitin in insects as there is a structural difference between the chitin in the insect cuticle and that found in shellfish. The chitin in shellfish is usually composed of chitin in a matrix with protein and minerals (Johnson & Peniston, 1982; No *et al.,* 1989), whereas the cuticle of insects is composed of chitin in a matrix with cuticular protein, lipids and other compounds (Kramer *et al.,* 1995; Nation, 2002). Therefore, the aim of this study was to develop a simple and repeatable analysis to accurately determine the chitin content of insect larvae.

Stellenbosch University https://scholar.sun.ac.za

Materials and methods

Larvae material and preparation

Hermetia illucens larvae were obtained from ProtiCycle (Pty) Ltd, a mass-rearing factory (Worcester, South Africa), 16 days after hatching. Larvae were grown on a formulated diet (Woods *et al.,* 2019b) for the first four days, where after they were transferred to bio-waste for the remaining 14 days. Larvae were purged for six hours and killed by blanching for three minutes at 95 ˚C and allowed to air dry. The larvae were then dried at 60 °C for 24 hours and homogenised using a mill (KN 195 Knifetec^{TM}). The samples were vacuumed sealed and placed at -40 ˚C until analysed.

Moisture and ash content (minerals)

For the determination of the moisture content, 2 g of the homogenised sample was dried at 100 - 105 °C for 24 hours and re-weighed (AOAC method 934.01). This dried sample was incinerated at 500 °C for at least 6 hours, cooled and weighed to provide an estimate of the ash content (AOAC method 942.05).

Lipid Content

The total lipid content was determined using a rapid solvent extraction method, as described by Lee *et a*l., (1996), with a 2 : 1 chloroform : methanol solution being used for the larvae samples due to their relatively high fat content (Spranghers *et al.,* 2017).

Nitrogen content

The larvae material, as well as the isolated chitin (see method below), were ground and used for the determination of the total nitrogen content using the LECO combustion/Dumas method (AOAC method 992.15). A 0.1 g aliquot of each sample was weighed into a LECO foil cup, which was incinerated and analysed for nitrogen content, using EDTA for calibration (LECO Corporation, St Joseph, MI, USA).

Stellenbosch University https://scholar.sun.ac.za

Determination of chitin content

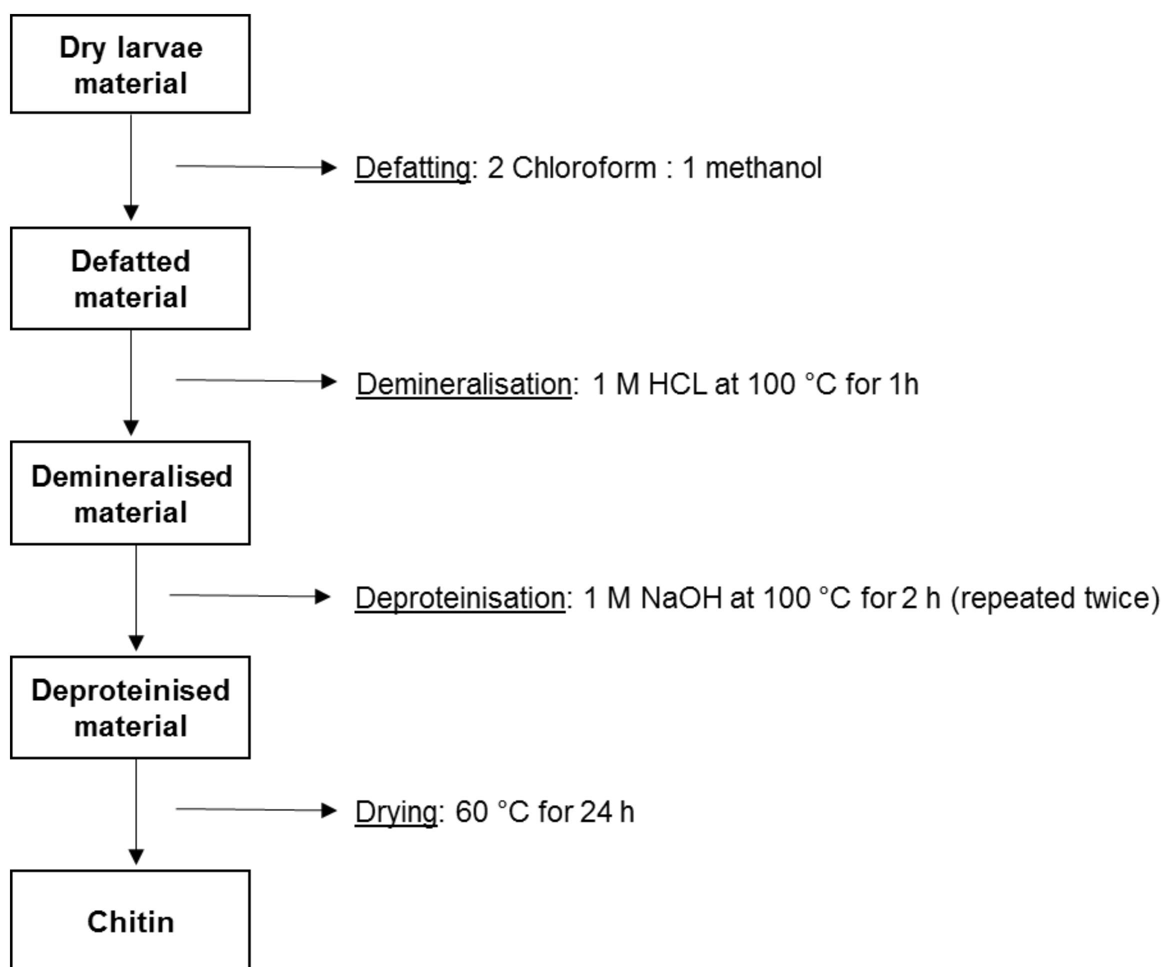

Figure 1: Flow diagram illustrating stepwise protocol for chitin determination of insect larvae

The chitin content of *H. illucens* larvae was determined gravimetrically after chemical digestion and solubilisation of other compounds present (fat, protein and minerals) (Figure 1). This method consisted of firstly removing the fat by means of solvent extraction where after the protein and mineral fractions were removed with acid and alkali respectively (see below). The remaining residue was determined to be chitin. The purity of the chitin was evaluated by analysing for nitrogen content (%) and comparing it to the theoretical value (6.89%) calculated for completely acetylated chitin. The analysis for the determination of the chitin content of the larvae material was repeated eight times. Repeatability of the chitin value, as well as the nitrogen value obtained, was evaluated between the eight runs.

Defatting

Firstly, the fat fraction of *H. illucens* larvae was removed by means of a rapid solvent extraction method. Five grams of sample (weighed accurately) was placed into a 600 mL glass beaker. Fifty millilitres of 2 : 1 chloroform : methanol solution was added to the glass beaker and the contents thoroughly mixed for one minute using a hand blender (Kenwood HB510). After

Stellenbosch University https://scholar.sun.ac.za

mixing, the solvent and solid fraction were separated by means of filtration (1 qualitative circles 150mm Ø filter paper). The solvent was allowed to completely filter through and the residue remaining on the filter paper was scraped of and dried at 60 °C for 24 hours.

Demineralisation

Two grams of the residue obtained after defatting, consisting mostly of protein and chitin, was subjected to a demineralisation treatment using hydrochloric acid (HCL). This was done using a VELP FIWE6 Raw Fiber Extractor (Cat# F30520200). Five grams of moisture and fat free sample was weighed accurately to four decimal places into a crucible with a glass filter. Demineralisation was performed using an acid treatment with 250 mL, 1 M HCL at 100 °C for one hour. After demineralisation was complete the residue was washed three times with 250 mL of boiling distilled water to achieve a neutral pH.

Deproteinisation

Deproteinisation was performed using an alkaline treatment with 250 mL, 1 M sodium hydroxide (NaOH) solution at 100 °C for two hours. After two hours the solvent was filtered out under vacuum and the treatment was repeated. The residue remaining in the crucible was dried at 60 °C for 24 hours where after the sample was weighed back. The difference in begin and end mass accounted for the chitin content as determined

Calculation

The difference in begin and end mass (dry matter basis) accounted for the chitin content of the defatted sample. The fat fraction removed before demineralisation and deproteinisation was taken into account when calculating the chitin content of the whole sample. The chitin content of the sample was calculated using Equation 1 and 2: where A is the weight of the residue remaining after demineralisation and deproteinisation, B is the weight of the sample before demineralisation and deproteinisation, C is the value obtained from Equation 1 and D is the fat content (dry matter basis) of the original sample

$$Chitin\,\%\,(defatted) = \frac{A}{B}\,x\,100$$

Equation 1

$$Chitin\,\% = \frac{C(100 - D)}{100}$$

Equation 2

Stellenbosch University https://scholar.sun.ac.za

Statistical analysis

Summary statistics was performed on chitin, nitrogen and ash yield data using the Descriptive Statistics procedures of STATISTICA (2018). Medians and means were used as the measure of central location for ordinal and continuous responses. Standard error and coefficient of variation were used as indicators of spread.

Results and discussion

Chemical composition of the larvae

Table 1 The proximate composition, 16 day old *H. illucens* larvae with regard to moisture, ash, fat and protein on dry matter basis

	Hermetia illucens **larvae**
Proximate composition (%)	
Moisture	67.65
Crude protein	37.06
Crude fat	31.08
Ash	6.95

The proximate chemical composition of 16 day old *H. illucens* larvae used to isolate chitin, with regard to moisture, protein, fat and ash was determined on dry matter basis (Table 1). Detailed composition of *H. illucens* larvae was important as a basis to design and evaluate the chitin isolation procedure. All values obtained were comparable to that found in literature (Finke, 2013; Spranghers *et al.,* 2017; Meneguz *et al.,* 2018).

Extraction of chitin

Table 2 Percentage chitin, nitrogen and ash obtained from defatted, deproteinised and dried chitin for each of the eight replications to confirm repeatability

Replication	**Chitin (%)**	**Nitrogen (%)**	**Ash (%)**
1	6.10	6.21	1.32
2	5.21	6.43	1.52
3	5.67	6.42	1.64
4	6.45	6.60	1.18
5	5.55	6.43	1.53
6	5.37	6.39	1.58
7	5.36	6.45	1.59
8	5.69	6.49	1.61
Mean ± Std. Error	5.68 ± 0.15	6.43 ± 0.038	1.50 ± 0.06
CV	7.34	1.69	10.78

The isolation and subsequent quantification of the chitin content of *H. illucens* larvae is unlike the traditional sources of these biopolymers (crustacean shells) due to the unique features of

Stellenbosch University https://scholar.sun.ac.za

the chitin found in insect larvae. Differences that exist between the chitin found in the cuticles of crustaceans and that of insects are that it first exist in a matrix with protein and minerals (Johnston & Peniston, 1982; No *et al.,* 1989) and the second in a matrix with proteins, fats and other compounds (Kramer *et al.,* 1995; Nation, 2002). Due to the high lipid content of larvae it requires a preliminary defatting treatment that is not applicable for crustaceans. Khayrova *et al.* (2019) found that in *H. illucens* larvae that had not been defatted, but blanched and dried, the chitin could not be extracted. This was attributed to the lipids preventing the interaction of phosphoric acid with the cell walls due to hydrophobic repulsion. Defatting of *H. illucens* larvae by means of solvent extraction (2 : 1 chloroform : methanol) effectively removed the lipids and the residue remaining was viable for further chitin isolation.

The chitin content of experimental *H. illucens* larvae was determined to be 5.68 % ± 0.15 (Table 2). A small variation in chitin content determined was observed between the eight values with a coefficient of variation of 7.34 (Table 2). With regards to *H. illucens*, there is limited research available to compare the obtained chitin value with. Previous studies either used crude fiber as estimate for chitin content (Finke, 2013) or the values obtained were not repeatable, as was the case with Caligiani *et al.* (2018) who achieved a chitin content of 9%. Studies conducted by Khayrova *et al.* (2019) and Waśko *et al.* (2016) leaned more on the characterisation of the physicochemical structure of chitin and isolation of chitosan and did not quantify the chitin content of *H. illucens* larvae. The average chitin content obtained in this study is comparable to that obtained by Kaya *et al.* (2016) for *Vespa crabro* larvae (6.2%).

A small variation in experimental nitrogen content (%) was observed between each of the eight values, with a coefficient of variation of 1.69 (Table 2). These values were all lower than the theoretical value calculated for pure chitin. Pure chitin, i.e. fully acetylated, has a nitrogen value of 6.89% and is considered to be an indicator of the purity of chitin (Liu *et al.,* 2012; Majtán *et al.,* 2007; Sajomsang & Gonil, 2010). Nitrogen levels lower that 6.89% indicate that there may still be some inorganic compounds present in the sample. This is confirmed by the ash value of the isolated chitin (1.50 ± 0.06%) (Table 2). Hence, the demineralisation treatment was not sufficient to completely remove all inorganic compounds, although it was reduced from 6.95 (Table 1) to 1.50 ± 0.06% (Table 2). However, it is also known that biopolymers contain bound moisture and if this property is taken into account the experimental results become more consistent with the theoretical value (Khayrova *et al.,* 2019). Since the nitrogen content is also indicative of the residual protein still present in the chitin, a low experimental value indicates the minimum amount of protein left and proves that the deproteinisation treatment was efficient. The average nitrogen value (6.43 ± 0.038% per DM) obtained by isolating chitin by means of the above described method is closer to the theoretical value than that obtained by various previous studies isolating chitin from insects. Majtán *et al.* (2006)

Stellenbosch University https://scholar.sun.ac.za

achieved a nitrogen value of 5.92 for chitin isolated from bumblebees (*Bombus terresttris*). Waśko *et al.* (2016) isolated chitin from *H. illucens* pupal exuviae and imagoes and achieved nitrogen values of 3.73 and 3.90, respectively. The nitrogen content of the chitin isolated in this study was comparable to that obtained by Kaya *et al.* (2016) who reported a nitrogen content of 6.50 % for chitin isolated from *Vespa crabo* larvae.

It should be noted that the above method developed to determine chitin content was only developed for larvae. Most pupae and adults are highly pigmented, as is the case for *H. illucens,* and contain high concentrations of melanin. Although the accuracy of the analysis was not tested for pupae or adults it is known that chitin is strongly bound to melanin, which decreases the solubility of chitin in acids and negatively affects the accuracy of the analysis. (Khayrova *et al.,* 2019).

Conclusion

This study demonstrated a simple and repeatable method for quantifying chitin obtained from insect larvae. Previous methods described by Waśko *et al.* (2016) and Caligiana *et al.* (2018) to quantify chitin from *H. illucens* larvae aim to remove non-chitin compounds from the chitin containing raw material, similar to the methods used in this study, but did not result in pure chitin or repeatable results. The developed analysis can contribute to standardised results for chitin determination of insect larvae and therefore chitin values can be comparable amongst future studies.

Stellenbosch University https://scholar.sun.ac.za

References

Association of Official analytical Chemists (AOAC), 2002. Official methods of analysis (17th Ed.), Arlington, Virginia, USA.

Caligiani, A., Marseglia, A., Leni, G., Baldassarre, S., Maistrello, L., Dossena, A., and Sforza, S., 2018. Composition of black soldier fly prepupae and systematic approaches for extraction and fractionation of proteins, lipids and chitin. Food research international 105: 812-820.

Cauchie, H.M., 2002. Chitin production by arthropods in the hydrosphere. Hydrobiologia 470:63–96.

Finke, M.D., 2007. Estimate of chitin in raw whole insects. Zoo Biology: Published in affiliation with the American Zoo and Aquarium Association 26(2): 105-115.

Finke, M.D., 2013. Complete nutrient content of four species of feeder insects. Zoo biology *32*(1): 27-36.

Finke, M.D. and Oonincx, D., 2014. Insects as food for insectivores. In Mass Production of Beneficial Organisms (pp. 583-616). Academic Press.

Herrero, M., Wirsenius, S., Henderson, B., Rigolot, C., Thornton, P., Havlík, P., de Boer, I. and Gerber, P.J., 2015. Livestock and the environment: what have we learned in the past decade? Annual Review of Environment and Resources 40: 177-202.

Janssen, R.H., Vincken, J.P., van den Broek, L.A., Fogliano, V. and Lakemond, C.M., 2017. Nitrogen-to-protein conversion factors for three edible insects: *Tenebrio molitor*, *Alphitobius diaperinus*, and *Hermetia illucens*. Journal of agricultural and food chemistry 65(11): 2275-2278.

Johnson, E.L. and Peniston, Q.P., 1982. Utilization of shell waste for chitin and chitosan production. In: Martin RE, Flick GH, Hebard CE, Ward DR, eds. Chemistry and biochemistry of marine food products. West Port, CT: AVI Publishing Co. p 514–22.

Kadokawa, J.I., 2011. Precision polysaccharide synthesis catalyzed by enzymes. Chemical Reviews 111(7): 4308-4345.

Kaya, M., Sofi, K., Sargin, I. and Mujtaba, M., 2016. Changes in physicochemical properties of chitin at developmental stages (larvae, pupa and adult) of *Vespa crabro* (wasp). Carbohydrate polymers 145: 64-70.

Khayrova, A., Lopatin, S. and Varlamov, V., 2019. Black Soldier Fly *Hermetia illucens* as a Novel Source of Chitin and Chitosan. International Journal of Sciences 8(4): 81–6. Available from: http://dx.doi.org/10.18483/ijSci.2015

Kouřimská, L. and Adámková, A., 2016. Nutritional and sensory quality of edible insects. NFS Journal 4: 22-26.

Stellenbosch University https://scholar.sun.ac.za

Kramer, K.J., Hopkins, T.L. and Schaefer, J., 1995. Applications of solids NMR to the analysis of insect sclerotized structures. Insect Biochemistry and Molecular Biology 25(10): 1067-1080.

Lee, C.M., Trevino, B. and Chaiyawat, M., 1996. A simple and rapid solvent extraction method for determining total lipids in fish tissue. Journal of AOAC International *79*(2): 487-492.

Liu, S., Sun, J., Yu, L., Zhang, C., Bi, J., Zhu, F., Qu, M., Jiang, C. and Yang, Q., 2012. Extraction and characterization of chitin from the beetle *Holotrichia parallela motschulsky*. Molecules 17(4): 4604-4611.

Majtán, J., Bíliková, K., Markovič, O., Gróf, J., Kogan, G. and Šimúth, J., 2007. Isolation and characterization of chitin from bumblebee (*Bombus terrestris*). International journal of biological macromolecules 40(3): 237-241.

Meneguz, M., Schiavone, A., Gai, F., Dama, A., Lussiana, C., Renna, M. and Gasco, L., 2018. Effect of rearing substrate on growth performance, waste reduction efficiency and chemical composition of black soldier fly (*Hermetia illucens*) larvae. Journal of the Science of Food and Agriculture 98(15): 5776-5784.

Muzzarelli, R.A.A., Terbojevich, M., Muzzarelli, C., Miliani, M. and Francescangeli, O., 2001. Partial depolymerization of chitosan with the aid of papain. Chitin Enzymology 405-414.

Nation Sr, J.L., 2015. *Insect physiology and biochemistry*. CRC press.

No, H.K., Meyers, S.P. and Lee, K.S., 1989. Isolation and characterization of chitin from crawfish shell waste. Journal of Agricultural and Food Chemistry 37(3): 575-579.

Oonincx, D.G. and De Boer, I.J., 2012. Environmental impact of the production of mealworms as a protein source for humans–a life cycle assessment. PloS one 7(12): e51145.

Paoletti, M.G., Norberto, L., Damini, R. and Musumeci, S., 2007. Human gastric juice contains chitinase that can degrade chitin. Annals of Nutrition and Metabolism 51(3): 244-251.

Rock, G.C. and King, K.W., 1967. Quantitative amino acid requirements of the red-banded leaf roller, *Argyrotaenia velutinana* (Lepidoptera: Tortricidae). Journal of Insect Physiology 13(2): 175-186.

Rinaudo, M., 2006. Chitin and chitosan: properties and applications. Progress in polymer science 31(7): 603-632.

Sajomsang, W. and Gonil, P., 2010. Preparation and characterization of α-chitin from cicada sloughs. Materials Science and Engineering: C 30(3): 357–363.

Smetana, S., Palanisamy, M., Mathys, A. and Heinz, V., 2016. Sustainability of insect use for feed and food: Life Cycle Assessment perspective. Journal of Cleaner Production 137: 741-751.

Stellenbosch University https://scholar.sun.ac.za

Spranghers, T., Ottoboni, M., Klootwijk, C., Ovyn A., Deboosere, S., De Meulenaar, B., Michiels, J., Eeckhout, M., De Clercq, P., and De Smet, S., 2017. Nutritional composition of black soldier fly (*Hermetia illucens*) prepupae reared on different organic waste substrates. Journal of the Science of Food and Agriculture 97(5): 2594-2600

STATISTICA 13 (TIBCO Software Inc. 2018). Statistica (data analysis software system), version 13. http://tibco.com.)

Van Huis, A., Van Itterbeeck, J., Klunder, H., Mertens, E., Halloran, A., Muir, G. and Vantomme, P., 2013. Edible insects: future prospects for food and feed security (No. 171). Food and Agriculture Organization of the United Nations.

Vincent, J.F. and Wegst, U.G., 2004. Design and mechanical properties of insect cuticle. Arthropod structure & development 33(3): 187-199.

Waśko, A., Bulak, P., Polak-Berecka, M., Nowak, K., Polakowski, C. and Bieganowski, A., 2016. The first report of the physicochemical structure of chitin isolated from *Hermetia illucens*. International journal of biological macromolecules 92: 316-320.

Woods, M.J., Boersma, N., Hoffman, L.C. and Pieterse, E., 2019a. Development of reduced cost diets for the mass rearing of false codling moth (*Thaumatotibia leucotreta*). Journal of the Science of Food and Agriculture doi: 10.1002/jsfa.9623

Woods, M.J., Hoffman, L.C. and Pieterse, E., 2019b. Artificial diets for neonatal black soldier fly (*Hermetia illucens*) larvae. Journal of Insects as Food and Feed 5(2): 99-105.

Stellenbosch University https://scholar.sun.ac.za

CHAPTER 8

Enzymatic fractionation of protein, fat and chitin from *Hermetia illucens* (L.) (Diptera: Stratiomyidae)

Stellenbosch University https://scholar.sun.ac.za

Abstract

Insects have the ability to convert biowaste into valuable functional compounds, such as proteins, fat and chitin. Currently, unlike the dairy-, fishmeal-, soy- and various other industries, there are few fractionation methods to isolate these compounds for diversification of use. This study aimed at fractioning *Hermetia illucens* larvae, into protein, fat and chitin using an enzymatic hydrolysis method. A central composite design was used to help identify the optimum hydrolysis conditions for fractionation. These conditions, using SEBDigest F59 P, were determined to be: E : S 0.6; pH 5.8; temperature 38 °C for 30 minutes. The expected yields for the fat (FF), protein (PF) and chitin (CF) containing fractions under these conditions were ~32 %, ~ 61 % and ~ 22 %, respectively. The degree of hydrolysis of PF at the optimum conditions was 22.21 %. Fat recovery was ~ 81 %; substantially more than previous studies. The fatty acid profile of FF stayed unchanged from the original larvae material (WL). The protein recovered in PF was ~ 57 % and although not optimal, it was on par with previous studies. The amino acid profile of PF differed from WL with a slight decrease in the ratio of essential to total amino acids. The proteins that were not recovered in PF were mostly insoluble and concentrated in CF. Future studies could investigate washing-and-sieving as means to recover the insoluble proteins from CF. Also, the spray drying of the protein hydrolysate produced and its functional properties could be investigated. Enzymatic hydrolysis is a particularly attractive technique to fractionate *H. illucens* larvae due to the milder process conditions required, the relative ease to control the reaction and minimal formation of by-products. This technology allows for possible diversification in use as well as the improvement of *H. illucens* functional compounds.

Keywords: Central composite design, Degree of fractionation, Degree of hydrolysis

Stellenbosch University https://scholar.sun.ac.za

Introduction

The growing global population has placed stress on the environment and sustainable intensification of production will need to be implemented at a higher rate than present to ensure food security in the coming years (Van Huis, 2013). An increase of 70% in food production is expected by 2050 (OECD/FAO, 2019) with an increased consumer demand for protein. Therefore, the search for alternative, more sustainable, protein sources has enjoyed interest lately. Insect production as source of protein has emerged as promising alternative and is reported to be 2 to 5 times more environmentally friendly than that of conventional protein sources (Smetana *et al.,* 2016, 2019).

Closely linked to the increased protein demand, is the accumulation and treatment of biowaste that has become a major health and environmental concern and involves costly processes to dispose of (Stehel *et al.,* 2019). *Hermetia illucens* Linnaeus 1758 (Diptera: Stratiomyidae), more commonly known as the black soldier fly, has been identified as a possible solution to treat biowaste due to the ability of their larvae to incorporate waste nutrients into their biomass and render functional compounds such as protein, fat and chitin (Miranda *et al.,* 2019). *Hermetia illucens* larvae have a high protein and fat content and have been successfully used as food (Bessa *et al.,* 2018, 2019) and feed for fish (Belghit *et al.,* 2019), pig (Biasato *et al.,* 2019) and poultry (Woods *et al.,* 2019a), as well as pet food (Lei *et al.,* 2019).

Whole *H. illucens* larvae do not have an optimal protein : fat : chitin ratio for formulation into animal feeds at high inclusion levels (Józefiak and Engber, 2015; Danieli *et al.,* 2019). The demand for defatted insect meal has increased in order to facilitate and improve the use of insect meal in feed formulations (Gasco *et al.,* 2019). The high fat content makes feed processing difficult and limits inclusion in feed for poultry (Kawasaki *et al.,* 2019). The chitin on the other hand, acts as a fibre and decreases nutrient digestibility, while diluting protein and energy, thus limiting the inclusion of whole insect meals into animal feeds (Gariglio *et al.,* 2019). In addition, storage of *H. illucens* larvae meal has proven to be a challenge due to the high fat content oxidising in a short period of time (Woods *et al.,* 2019a). However, separately these components have high functional value. The protein fraction is rich in essential amino acids with total tract digestibility in poultry in excess of 87% (Uushona, 2015). The fat fraction contains high levels of natural lauric acid and can be included into animal feed (Cullere *et al.,* 2019a). *Hermetia illucens* fat, due to its saturated nature, could also find application in the cosmetic (Sangduan, 2018) and biofuel (Surendra *et al.,* 2016) industries. Chitin has many commercial applications, including feed, food, cosmetics, textile and pharmaceutical products (Rinaudo, 2006).

Stellenbosch University https://scholar.sun.ac.za

Moreover, when using insects as foodstuff, it should be taken into account that western consumers are more likely to eat insects when they are not visible in food (Balzan *et al.,* 2016; Bessa *et al.,* 2018; Schösler *et al., 2012*). Insects have never played a substantial role in western food culture and therefore consumer acceptance is a major challenge. With this being the case, it would be beneficial to transform the whole *H. illucens* larval biomass into insect powders as has been done successfully with for example, crickets (Duda *et al.,* 2019). In order to meet the requirements of the food sector and western consumer preferences, fractionation processes have to be developed or adapted from conventional raw materials to produce high-quality, standardised, and tailored insect protein as an alternative to established protein sources. Microbiological safety issues could also be addressed through fractionation by deactivation of the bacteria during the drying and extraction steps (Lalander *et al.,* 2013). The fractionated compounds could also fetch higher prices when marketed individually compared to selling the whole insect biomass.

Currently there are few fractionation methods for the total separations of insect larvae into their main functional compounds described. Most of the studies focuses on the extraction of protein and not the complete recovery of all fractions (Del Valle *et al.,* 1982; Yi *et al.,* 2013; Bußler *et al.,* 2016). Caligiani *et al.,* (2018) assessed various fractionation methods for *H. illucens* larvae, mainly focusing on chemical separation on a laboratory scale. Their chemical fractionation method used recovered 96% protein, but the integrity of protein was compromised; the quality of the protein was negatively affected by the harsh fractionation conditions and further methods were investigated such as enzymatic hydrolysis. Even though this approach recovered the lowest amounts of protein, fat and chitin, Caligiani *et al.* (2018) found promising results and suggested further in-depth studies. The use of enzymatic hydrolysis as mean of fractionation method has many advantages compared to that of chemical methods. Chemical processes are harsh, usually carried out at high temperatures and may cause protein modification and possibly also chitin depolymerisation which affects the polymer properties. The use of enzymes is also a more sustainable, environmentally friendly and faster approach compared to that of chemical methods (Da Silva, 2018). The hydrolysate obtained from enzymatic hydrolysis can be tailored for different purposes such as high digestibility and bioavailability and hypoallergenic protein supplements for feed, food, thickening agents, texturisers and foaming agents (Tapal & Tiku 2019).

The aim of the present study was to fractionate *H. illucens* larvae into their functional compounds by means of enzymatic hydrolysis for use in various industries. The efficiency of fractionation and the resulting compound properties are highly dependent on the hydrolysis conditions, as these conditions strongly influence enzymatic working. Therefore, the objectives of this study were threefold. Firstly, to investigate the effect of different experimental

Stellenbosch University https://scholar.sun.ac.za

conditions (enzyme concentration, pH and temperature) on fractionation efficiency of *H. illucens* larvae and to determine optimal conditions *via* a central composite design. Secondly, to determine the degree of hydrolysis of the protein containing fraction; and lastly to investigate the chemical compositions and characterisation of the recovered *H. illucens* fractions by means of proximate-, amino- and fatty acid analyses.

Materials and methods

Experimental design

The main objective of the experiment was to obtain the optimum hydrolysis conditions that maximises the fractionation of *H. illucens* larvae into fat - (FF), protein - (PF) and chitin containing fractions (CF), using an endo-protease. Secondly, the experiment also evaluated the degree of hydrolyses (DH) of the protein containing fraction as there might be a correlation between the degree to which proteins are hydrolysed, and the liberation of fat and chitin (Ivanovs and Blumberga, 2017).

Table 1: Central composite design matrix for three factors and five level settings

Factors	Levels				
Coded levels	-1.68	-1	0	+1	+1. 68
Parameters			Actual values		
[1]E : S (X_i ; %)	0.00	0.10	0.30	0.50	0.60
pH (X_{ii})	5.80	6.50	7.50	8.50	9.20
Temperature (X_{iii}; °C)	40.0	45.0	55.0	65.0	70.0

[1] Enzyme : substrate ratio

Table 2: Central composite experimental design used to determine optimal fractionation and degree of hydrolyses conditions for *H. illucens* larvae

Run	[1]E : S (%)	pH	Temperature (°C)
1	0.10	6.50	45.0
2	0.10	6.50	65.0
3	0.10	8.50	45.0
4	0.10	8.50	65.0
5	0.50	6.50	45.0
6	0.50	6.50	65.0
7	0.50	8.50	45.0
8	0.50	8.50	65.0
9	0.00	7.50	55.0
10	0.60	7.50	55.0
11	0.30	5.80	55.0
12	0.30	9.20	55.0
13	0.30	7.50	40.0
14	0.30	7.50	70.0
15 [2](C)	0.30	7.50	55.0
16 [2](C)	0.30	7.50	55.0

[1] Enzyme : substrate ratio
[2] Centre point replicates

Stellenbosch University https://scholar.sun.ac.za

The data were generated using a rotatable (α = ± 1.68) Central Composite design (CCD), with three factors each at five levels. Table 1 show the levels for each independent variable. A CCD with a total of sixteen experimental runs (Table 2), of which two were centre point replicates, was performed. Once the optimum conditions were determined a validation run at the reported optimum conditions was done to validate the results.

The factors or independent variables were chosen as temperature (T), pH and enzyme to substrate ratio (E : S), with the degree of fractionation and degree of hydrolysis as the dependent or response variables. SEBDigest F59 P (Advanced Enzymes Technologies Ltd.), a food grade enzyme that demonstrates endo-protease activity, was used to hydrolyse the *H. illucens* larvae. SEBDigest F59 P rapidly hydrolyses the peptide bonds in protein molecules liberating polypeptides and peptides of various lengths, with an active range of pH 6 to 10, and T of 30 to 70 °C. The recommended dosage of SEBDigest F59 P is in the range of 0.05 to 0.5 % (w/w basis) of the substrate concentration.

This design was chosen as enzymatic hydrolysis takes place within the enzyme's working / active range. Central Composite designs are more efficient than normal Factorial designs, as they decrease the number of experimental runs required and the response variables can be mathematically modelled (Equation 1) as a function of the independent variables over the experimental range studied (Montgomery, 2017). In addition, CCD permits the estimation of main effects and interactions at numerous levels of the other independent variables, resulting in conclusions that are valid over a wide range of experimental conditions (Montgomery, 1997).

$$Y_i = \beta_0 + \sum_i \beta_i X_i + \sum_{ii} \beta_{ii} X_i^2 + \sum_{ij} \beta_{ij} X_{ij} \qquad \text{(Equation 1)}$$

Larvae material

Hermetia illucens larvae were obtained from ProtiCycle (Pty) Ltd, a mass-rearing factory (Worcester, South Africa), 16 days after hatching. Larvae were grown on a formulated diet (Woods *et al.,* 2019b) for the first four days, whereafter they were transferred to biowaste, consisting mostly of fruit pulp and yeast, for the remaining 12 days. Larvae were removed from their feed substrate for six hours to empty their digestive tract and subsequently killed by submerging the larvae in water at 100 ˚C for two minutes. The larvae were allowed to cool to ambient temperature, before being minced using a commercial meat mincer. The minced larvae were vacuum sealed and stored at -40 ˚C until being subjected to hydrolysis.

Stellenbosch University https://scholar.sun.ac.za

Proximate composition

The proximate chemical composition was determined for the whole larvae (WL), as well as for the different fractions obtained after hydrolyses at the determined optimum conditions. These fractions included a fat containing fraction (FF), a protein containing fraction (PF) and a chitin containing fraction (CF). Moisture, protein and ash were analysed according to the methodology of the Association of Official Analytical Chemists (AOAC, 2002).

For the determination of the moisture content, 2.5 g of the homogenised sample was dried at 100 - 105 °C for 24 hours and weighed (AOAC method 934.01). This dried sample was then incinerated at 500 °C for at least 6 hours, cooled and weighed to provide an estimate of the ash content (AOAC method 942.05).

The fat contents were determined using a rapid solvent extraction method, as described by Lee *et al.* (1996). A 1 : 2 chloroform : methanol solution was used for PF and CF due to their low fat content (< 5 %). *Hermetia illucens* larvae have a fat content ranging between 7 and 39 % DM (Barragan-Fonseca *et al.,* 2017), Hence a 2 : 1 chloroform : methanol solution was used to determine the fat content for WL and FF (Lee *et al*., 1996).

The protein content of WL as well as FF, and PF were determined using the LECO combustion / Dumas method (AOAC method 992.15). A 0.5 g aliquot of each sample was weighed into a LECO foil cup, which was incinerated and analysed for nitrogen content. EDTA was used for calibration for WL and PF whereas alfalfa was used for FF (LECO Corporation, St Joseph, MI, USA). The nitrogen content was converted to a protein content by multiplying by 6.25 for FF and PF, and 5.60 for WL and CF.

All proximate chemical components are reported as percentages of the dry weights (dry matter).

Determination of amino acids

Samples were prepared for analysis as described by Woods *et al.* (2019b) and sent to the Central Analytical Facility of Stellenbosch University. Amino acid composition of WL and PF were determined by means of the Waters AccQ Tag Ultra Derivatization method (Cunico, 1986). The quantitative results are expressed as a percentage of the total amino acids.

Determination of fatty acids profile by GC-FID

Whole larvae (1 g) and FF (after hydrolyses at optimum conditions) (0.5 g) samples were extracted in 50 ml 2 : 1 (v / v) chloroform : methanol solution (Folch *et al.,* 1957), which contained 0.01 % butylated hydroxytoluene as an antioxidant. The samples were homogenised for 30 seconds at 10 000 rpm using a polytron mixer (IKA® T18 digital ULTRA TURRAX®), after which they were passed through an extraction funnel fitted with a glass

Stellenbosch University https://scholar.sun.ac.za

microfibre filter paper (Whatman, GF/A, diameter 47 mm, Cat no. 1820-047). A heptadecanoic acid internal standard (catalogue number H3500, Sigma-Aldrich, Gauteng, South Africa) was used to allow the quantification of the individual FA, with 0.5 ml of the 10 mg/ml solution being added to each sample prior to homogenisation.

A 250 µl aliquot of the fat extract solution was evaporated in a water bath (45 °C, *ca.* 10 minutes) and subsequently transmethylated at 70 °C for 2 h using 2 ml of a 19 : 1 (v / v) methanol : sulphuric acid solution as the transmethylating agent. After allowing the resultant solution to cool to room temperature, the FA methyl esters (FAMEs) were extracted with water and hexane. This entailed adding 1 ml distilled water and 2 ml hexane to the transmethylated solution, mixing thoroughly, allowing to settle and transferring the top phase (FAME-containing hexane) to a clean glass tube. The hexane extract was dried in a water bath at 45 °C under a flow of nitrogen, where after the dried FAME sample was re-suspended in 100 µl hexane and transferred to a gas chromatography (GC) vial for analysis.

The FAMEs were analysed using a Thermo TRACE 1300 series gas-chromatograph equipped with a flame-ionisation detector (GC-FID) and coupled to a CTC Analytics PAL autosampler. Separation was performed using a polar ZB-WAX (30 m, 0.25 mm ID, 0.25 µm film thickness) Zebron 7HG-G007-11 capillary column. A sample volume of 1 µl was injected in a 5 : 1 split ratio and helium was used as the carrier gas at a flow rate of 1 mL / minute. The injector temperature was maintained at 250 °C. The oven temperature was programmed as follows: held at 50 °C for 2 minutes, then ramped up to 180 °C at a rate of 25 °C/minute for 5 minutes, followed by a ramping rate of 3 °C / minute for 2 minutes until 260 °C.

The FAMEs in each sample were identified by comparing the retention times to those of a standard FAME mixture (Supelco™ 37 Component FAME mix, Cat no. CRM47885, Supelco, USA), and quantified by comparing the integrated areas to that of the internal standard. The quantitative results are expressed as a percentage of the total FAME content, and as the *cis* and *trans* isomers of C18:1n-9 and C18:2n-6 co-eluted in the majority of the samples, they are reported as single combined values.

Determination of chitin

Chitin content was determined as per the method described by Woods *et al.,* 2019c. The chitin content of WL, as well as CF, were determined gravimetrically after chemical digestion and solubilisation of other compounds present (fat, protein and minerals). This method consisted of firstly removing the fat by means of solvent extraction, where after the protein and mineral fractions were removed with acid and alkali respectively. The remaining residue was determined to be chitin.

Stellenbosch University https://scholar.sun.ac.za

Fractionation method

To obtain the optimum fractionation conditions hydrolysis was carried out in a 600 ml reaction vessel with an overhead stirrer (600 rpm), automatic temperature control and automatic pH control as shown in Figure 1.

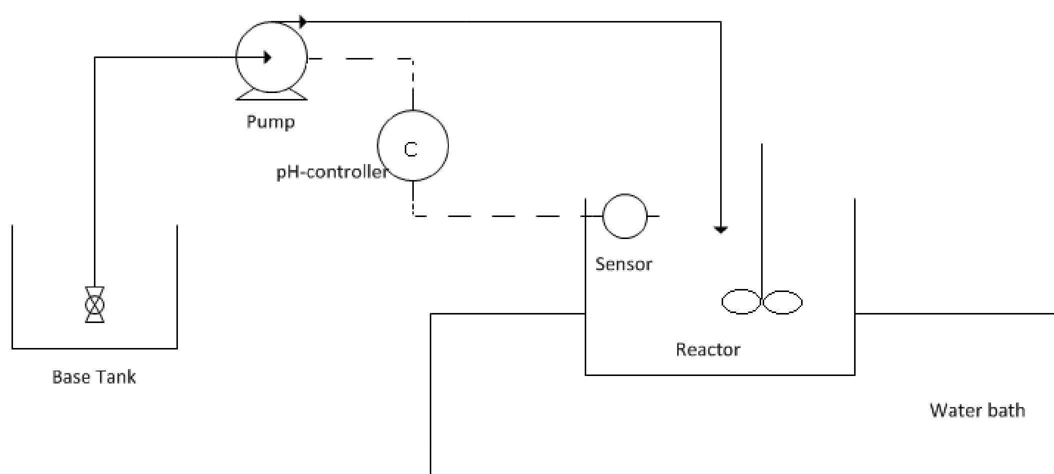

Figure 1: Experimental setup for enzymatic fractionation of *Hermetia illucens* larvae

Two-hundred grams of minced larvae was added to the reactor without the addition of extra water. Before hydrolysis, the solution was preheated to the reaction temperature as indicted in the experimental design (Table 2), where after the pH was adjusted to the required starting pH using either 1 M KOH or 1 M HCl. The hydrolysis was initiated with the addition of the enzyme, dissolved in 10 mL distilled water, at the rate determined in the experimental design. As the hydrolysis was conducted, the temperature was controlled using a water bath and pH was maintained by addition of HCl using an automatic pH controller as shown in Figure 1**Figure**. Two millilitre samples were taken at set time intervals (0, 10, 15, 20, 30, 60, 120 minutes) and transferred to two mL Eppendorf tubes. The enzymatic reaction within these samples was inactivated as soon as the samples were taken by heating the samples to 100 °C for two minutes, using a hotplate with boiling water, where after the samples were centrifuged for 4 minutes at 14.5 x 1000 rpm. Lastly, the samples were cooled to room temperature before storing at - 40 °C until analysed. The hydrolyses were carried out for 120 min at the E : S, temperature and pH range as set out in the experimental plan (Table 2). Figure 2 shows the stepwise proceedings for the fractionation of *H. illucens* larvae from raw material to the different fractions.

Stellenbosch University https://scholar.sun.ac.za

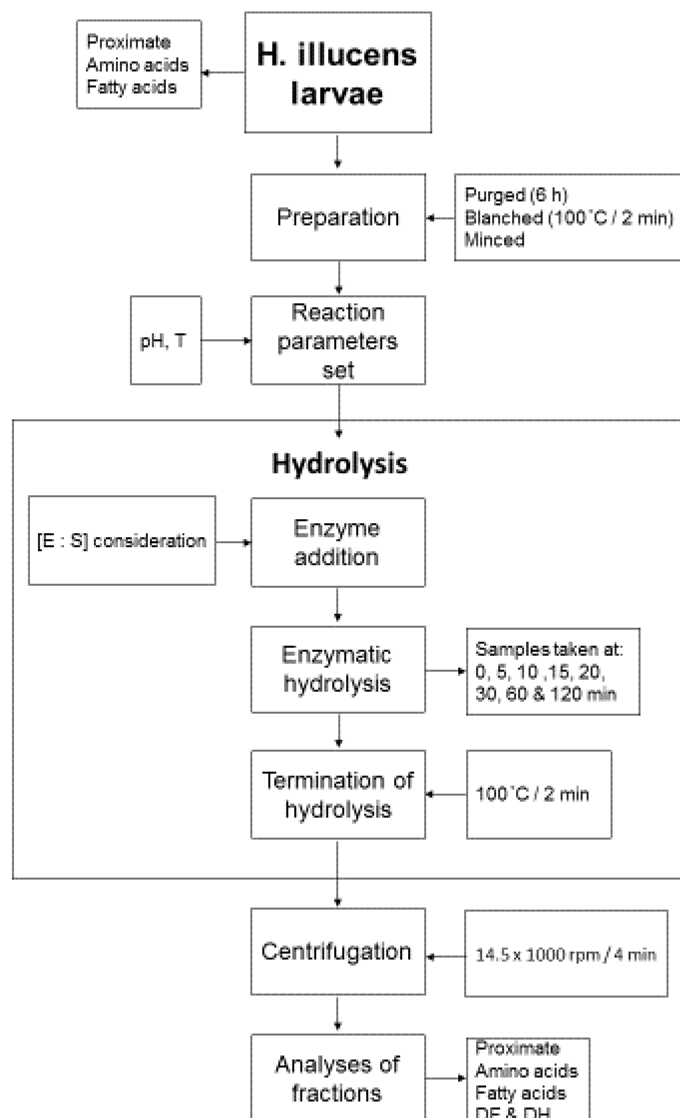

Figure 2: Flow sheet of enzymatic hydrolysis of *Hermetia illucens* larvae

Determination of yields of compounds

To determine the ratio / yields of fat : protein : chitin containing fractions, a digital camera was secured with a tripod on a stable surface at a constant distance. The centrifuged Eppendorf tube containing the fractionated *H. illucens* sample was placed before the camera and a photo was taken. As seen in the image (Figure 3) the fat would mostly rise to the top phase, the dissolved protein to the middle phase and the chitin to the insoluble bottom phase. The scale at the side of the image (Figure 3) was a calibration aid for the computer software program (ImageJ, National Institutes of Health, Wayne Rashand, 1997) that was used to calculate the surface area of each fraction. The ratio of FF : PF : CF fraction was subsequently determined.

Stellenbosch University https://scholar.sun.ac.za

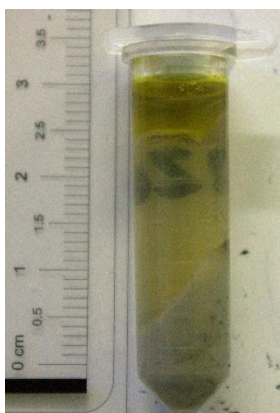

Figure 3: Image of the fractionated *H. illucens* sample used to determine FF : PF : CF with ImageJ

Determination of the integrity of protein fraction by degree of hydrolysis

The degree of hydrolysis of PF obtained after enzymatic fractionation was determined using the OPA (o-phthaldialdehyde) method of Nielsen *et al.* (2001) and Valencia *et al.* (2016). OPA reagent was prepared by completely dissolving 7.620 g di-Natetraborate decahydrate and 200 mg Na-dodecyl-sulphate (SDS) in 150 ml of deionized water, whereafter 160 mg o-phthaldialdehyde powder was dissolved in 4 ml ethanol and mixed with the above solution. Finally, 176 mg dithiothreitol (DTT) was added then rinsed with deionized water to make a 200 ml solution. A serine standard was prepared by dissolving 50 mg of serine powder in 500 ml of deionized water (0.9516 meqv / L).

The following procedure was followed to measure the absorbance of the samples, standard and blank. Blanks were prepared by adding 400 µl of distilled water to 3 ml OPA reagent. The samples and serine standard were prepared by the adding 400 µl of hydrolysates sample to 3 ml of OPA reagent into a cuvette. The absorbance values of the solutions were measured at 340 nm using an AE-S60-4U UV VIS spectrophotometer. The readings for the reaction of the amino acids with OPA reagent was taken after 2 min to equilibrate and after 10 min and average absorbance values were used to calculate the degree of hydrolysis. Finally, the degree of hydrolysis was calculated using Equation 2.

$$DH = \frac{h}{h_{tot}} \times 100\% \qquad \text{(Equation 2)}$$

Where h_{tot} represent the total number of peptide bonds per protein molecule and determined as 8.8 meqv /g protein according to Adler-Nissen (1986). h is defined as the number of hydrolysed bond at a certain time and calculated using Equations 3 and 4.

$$h = \frac{(Serine - NH_2) - \beta}{\alpha} \qquad \text{(Equation 3)}$$

Stellenbosch University https://scholar.sun.ac.za

$$Serine - NH_2 = \left(\frac{A_{sample} - A_{blank}}{A_{standard} - A_{blank}}\right)\frac{C_o V_s}{m} \quad \text{(Equation 4)}$$

Where Serine-NH_2 = meqv serine NH_2/g protein, m is the mass of the protein in gram in the sample taken, β = 1 and α = 0.4 are constants for whey protein (Adler-Nissen, 1986), V_s is the sample volume in L (litre), C_o is the standard concentration in meqv/L and A_{sample}, A_{blank} and $A_{standard}$ are the sample absorbance, blank absorbance and standard absorbance, respectively.

Statistical analysis

The experimental data obtained from measuring the degree of fractionation was used to identify the factor effects and interaction and find optimum reaction conditions. The degree of fractionation data was used to validate the mathematical model. STATISTICA (2018) was used to conduct statistical analyses. The optimum conditions were obtained from the desirability plots. The desirability for fat and protein was maximised and that for chitin minimized. The effect of the factors on the degree of fractionation was determined using analysis of variance (ANOVA).

Stellenbosch University https://scholar.sun.ac.za

Results and discussion

Degree of fractionation

Optimum hydrolysis conditions for the fractionation of *H. illucens* larvae were obtained at: E : S 0.6; pH 5.8; temperature 38 °C after 30 minutes (Figure 4). Desirability's were set to maximise the fat (FF) and protein containing (PF) fractions and to minimise the chitin containing fraction (CF). The desirability's were set to resemble the proximate composition of *H. illucens* larvae which consists of significantly more fat and protein compared to that of chitin (Spranghers *et al.,* 2017). Therefore, when fractionated a greater amount of FF and PF is expected compared to CF. It was determined that applying the set conditions, for all treatment runs, for 30 minutes resulted in the highest fractionation of *H. illucens* larvae. It was observed that the degree of fraction increased from 0 to 30 minutes and decreased as the hydrolysis progressed after this period. The identified hydrolysis period of 30 minutes is significantly shorter compared to that used by Caligiani *et al.* (2018), where *H. illucens* prepupae where hydrolysed for 16 hours. This significantly shorter hydrolysis period compared to that of Caligiani *et al.* (2018) implies a cheaper fractionation method and a greater possibility to upscale. The expected yields for FF, PF and CF at these conditions are ~32 %, ~ 61 % and ~ 22 % respectively (Figure 4) and can be described by Equations 5 to 7 where X_i can be defined as E : S, X_{ii} as pH and X_{iii} as temperature:

$$FF\ (\%\ yield) = 6.28 + 4.02Xi^2 + 0.12Xi + 3.73Xii^2 + 0.80Xii + 3.72Xiii^2 - 0.61Xiii + 3.72XiXii - 0.29XiXiii - 1.62XiiXiii \quad \text{(Equation 5)}$$

$$PF\ (\%\ yield) = 38.82 - 0.10Xi^2 + 5.21Xi + 3.02Xii^2 - 4.26Xii - 2.31Xiii^2 + 3.58Xiii + 3.93XiXii - 1.15XiXiii + 1.77XiiXiii \quad \text{(Equation 6)}$$

$$CF\ (\%\ yield) = 54.89 - 3.92Xi^2 - 5.33Xi - 6.76Xii^2 + 3.46Xii - 1.41Xiii^2 - 2.96Xiii + 4.02XiXii + 1.44XiXiii - 0.15XiiXiii \quad \text{(Equation 7)}$$

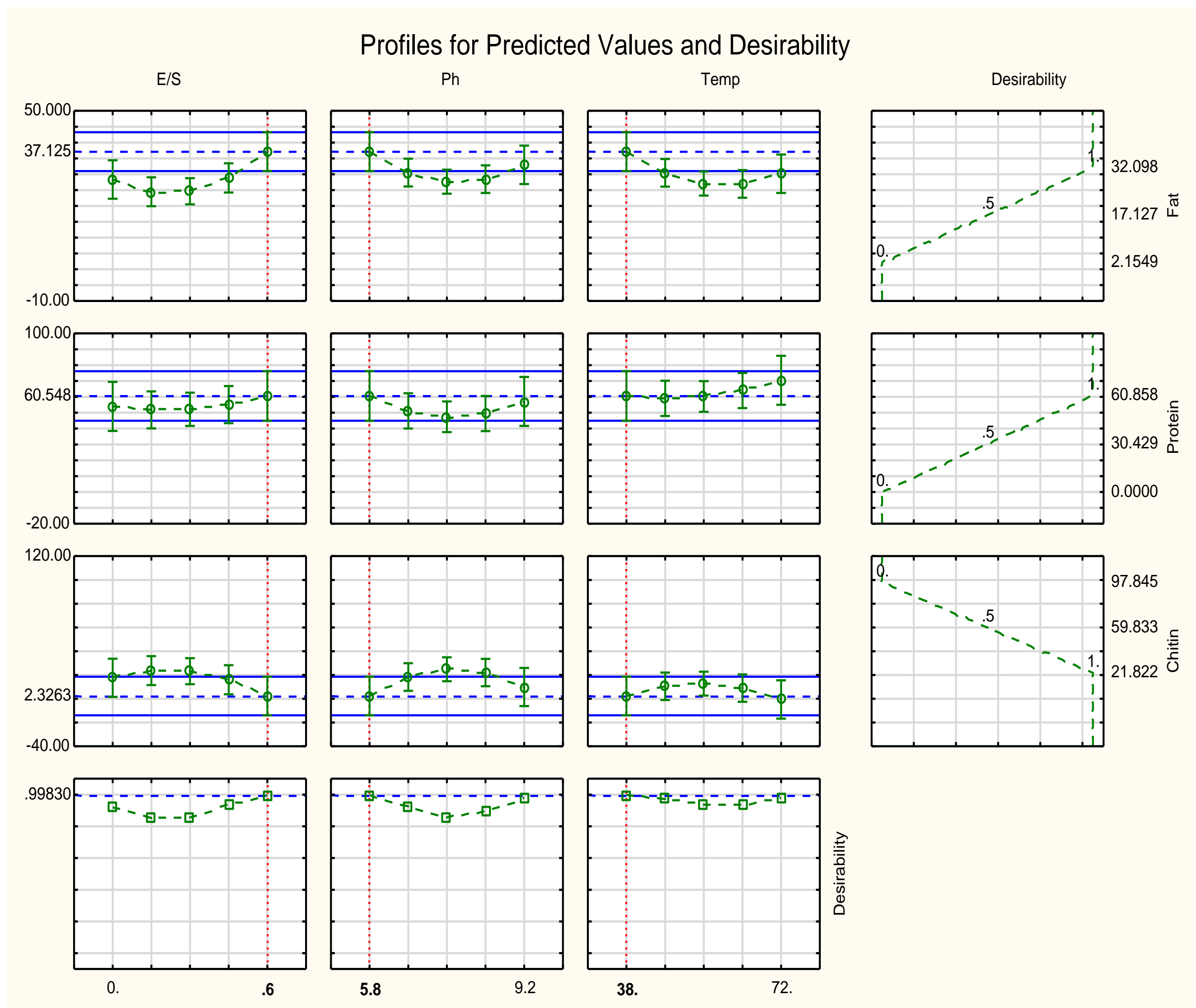

Figure 4: Profiles for predicted conditions and desirability at 30 minutes to maximise protein and fat containing fractions and minimise chitin containing fraction

As seen in Figure 5, the set desirability's were achieved at the outer limits of the experimental conditions. Therefore, the upper and lower limits for the experimental conditions (E : S , pH and temperature), using SEBDigest F59 P, could be reassessed in future studies.

An interesting observation was made during the hydrolysis process. It is expected that when a protein is hydrolysed under neutral or alkaline conditions the pH of the substrate will decrease due to the liberation of protons from free amino groups being formed (Camacho *et al*., 2001). Therefore, the pH tends to decrease as protein hydrolysis progresses and would need to be regulated through the addition of a base. However, this was not the case with the hydrolysing of *H. illucens* larvae; the pH of the substrate actually increased as the protein hydrolysis progressed, necessitating the addition of an acid to keep the pH constant. A possible explanation for this phenomenon is that the hypodermis of *H. illucens* larvae secrete and contain $CaCO_3$ (Johannsen, 1922; Barros-Cordeiro *et al.,* 2014), and during the hydrolysis process causes the pH to rise due to the formation of CO_2 and OH^-.

Stellenbosch University https://scholar.sun.ac.za

The use of an enzymatic hydrolysis is an alternative to chemical methods to fractionate *H. illucens* larvae. Enzymatic hydrolysis significantly decreases the use of harsh organic solvents and acid / alkali solutions. These chemical processes destroy the functional and nutritional value of the protein fractions (Kristinsson and Rasco, 2000) and causes protein modification and possibly chitin depolymerisation, affecting the functional properties of chitin. Caligiani *et al.,* (2018) investigated the use of acid / alkali solutions to fractionate *H. illucens* prepupae at the mildest conditions possible, yet still observed modifications in the structure of the protein such as racemisation, and formation of lysinoalanine and other cross-linked compounds. Racemisation of L-amino acids produce D-amino acids; the latter are not absorbed by humans, nor chickens or pigs. Also, the formation of cross-linked compounds such as lysinoalanine, ornithinoalanine, lanthionine and β-amino are toxic and highly undesirable in foods (Lahl, 1994). The use of solvents such as isopropanol, ethanol and ethyl dichloride to isolate protein results in protein with poor functionality, off-flavours, high costs of production and traces of solvents in the final product (Finch, 1977).

The fractionation method studied identified the optimum conditions for an enzymatic hydrolysis using the protease SEBDigest F59 P achieving fractionation of the functional compounds, i.e. protein, fat and chitin, of *H. illucens* larvae. Unlike the study by Bußler *et al.* (2016), the fractionation method was carried out on the minced larvae without any pre-treatment such as defatting of the larvae by means of hexane extraction. After hydrolysis, three fractions were obtained: a fat containing fraction (FF), a supernatant liquid underneath FF containing mostly hydrolysed proteins (PF) and a pellet containing mostly chitin and some insoluble proteins (CF).

Stellenbosch University https://scholar.sun.ac.za

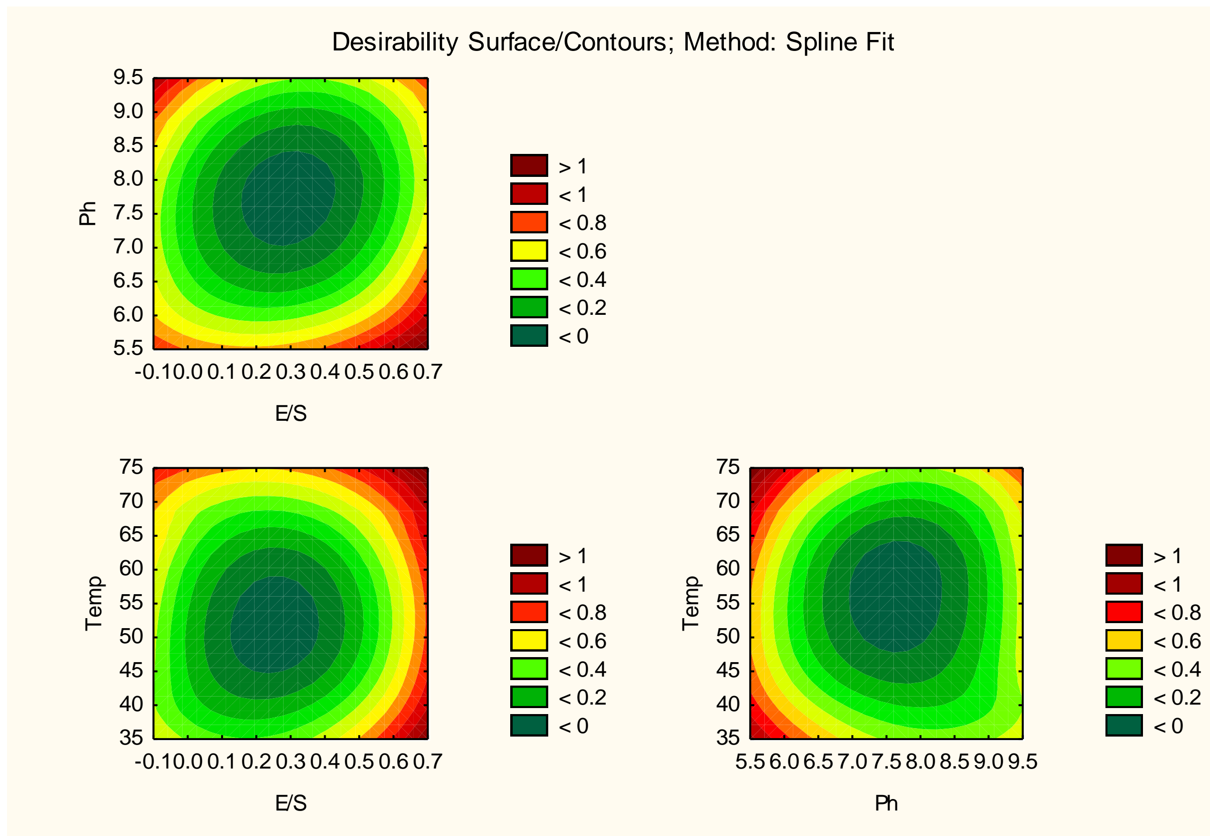

Figure 5: Desirability surface by means of fine split showing optimum conditions applied for 30 minutes to fractionate *Hermetia illucens* into functional compounds

Stellenbosch University https://scholar.sun.ac.za

Degree of hydrolysis

Table 3: Degree of hydrolysis (DH) of the protein containing fractions over a two hour period for each of the trial runs

Time (minutes)	Degree of hydrolysis (%)							
Run	0	5	10	15	20	30	60	120
1	3.21	7.40	7.06	6.79	7.35	7.57	7.52	9.58
2	5.38	6.03	5.38	6.58	7.83	7.89	11.8	12.3
3	6.67	9.07	8.25	10.3	13.0	16.6	18.4	24.1
4	8.27	8.69	10.3	10.4	11.4	11.9	12.1	--
5	8.45	8.61	7.49	7.64	9.57	10.2	9.83	12.1
6	4.59	4.59	7.57	7.66	8.17	8.26	8.66	11.2
7	7.32	7.49	8.61	8.87	9.83	10.2	11.0	12.1
8	10.3	10.8	11.7	11.8	12.1	16.5	16.5	18.6
9	10.1	9.89	9.87	10.5	9.74	10.2	10.0	9.68
10	6.61	7.79	13.0	13.3	18.0	18.4	19.6	19.7
11	5.17	6.82	6.87	6.87	7.28	7.71	7.88	8.27
12	4.51	6.89	8.46	9.79	9.48	9.65	9.83	10.2
13	8.52	11.6	11.3	11.2	10.9	11.0	14.4	22.7
14	7.83	7.43	21.1	26.8	32.8	39.4	39.6	39.9
15	8.38	8.46	17.0	27.5	32.4	--	--	--
16	7.97	27.9	28.9	28.9	32.1	--	--	--
†DOC	5.98	9.01	11.9	14.0	21.3	22.2	22.6	22.6

† Determined optimum conditions
-- No available value

The degree of hydrolysis (% DH), indicating the number of peptide bonds cleaved in relation to the total bonds, was calculated on the proteins present in the PF after enzymatic hydrolysis (Table 3). The % DH, after 30 minutes at the determined optimum conditions for fractionation of *H. illucens* larvae, was 22.21 %. The low % DH is desirable due to the fact that extensively hydrolysed proteins have the disadvantage of developing a bitter taste. Yu and Fazidah (1994) found a direct correlation between the degree of hydrolysis and the intensity of bitterness when hydrolysing Bighead carp (*Aristichthys nobilis*). This bitter taste is caused by the enzyme exposing buried hydrophobic peptides, which are able to readily interact with the taste buds resulting in the detection of bitterness (Kristinsson & Rasco, 2000). The protein hydrolysate produced from *H. illucens* larvae can provide highly digestible- and bioactive peptide molecules to grant nutritional and physiological functions when consumed by animals, as well as humans (Hou *et al.,* 2017). These bioactive peptides have antimicrobial, antioxidant, antihypertensive and immunomodulatory roles (Gasco *et al.,* 2018). They also help improve intestinal morphology, function and resistance to disease and in turn enhance the health and well-being of the end consumer (Shin and Park, 2019).

Stellenbosch University https://scholar.sun.ac.za

Insects, including *H. illucens,* are known to contain antimicrobial peptides (Józefiak and Engberg, 2017). These antimicrobial peptides can be used in functional food and feed applications as promising alternatives to antibiotics (Xiao *et al.,* 2015). The use of antibiotics has gained negative traction due to its misuse and the subsequent development of antibiotic resistance in various microbes, both in humans and animals. Therefore, the identification of alternatives to the use of antibiotics is of importance. A mechanism by which antimicrobial peptides exert their activity involves the destruction of the bacterial cell envelope (Józefiak and Engberg, 2017). This action requires a high antimicrobial peptide concentration. The hydrolysis and subsequent fractionation of the larvae may concentrate these antimicrobial peptides in the PF and could therefore increase their effectives.

The use of enzymatic hydrolysis *versus* chemical hydrolyses limits the catabolism of amino acids. The optimum hydrolyses conditions for this study were very mild and therefore preserved the functional properties of the proteins. The fact that no harsh acids were used for hydrolysis, means that the hydrolysate is better suited to further processing for feed and food purposes.

Proximate composition

Table 4: Proximate composition (% sample) on a dry matter basis of the whole *H. illucens* larvae, as well as the protein-, fat- and chitin containing fractions obtained after hydrolysis at optimum conditions

	WL	**FF**	**PF**	**CF**
Proximate composition (%)				
Crude fat	37.8	98.4	0.09	0.96
Crude protein	37.1	0.25	91.0	20.3
Ash	14.9	0.13	7.89	40.5
Chitin	5.68	0.00	0.00	36.9

WL Whole larvae
FF Fat containing fraction
PF Protein containing fraction
CF Chitin containing fraction

The proximate composition of the *H. illucens* larvae material used in this study, as well as of the different fractions obtained after hydrolysis, was important as a basis to design and evaluate the subsequent enzymatic fractionation procedure (Table 4).

The moisture content of the larvae was found to be 60.6 %, which is in accordance to the results of previous studies (Meneguz *et al.,* 2018). The relatively high moisture content of the larvae meant that no external moisture needed to be added to the experimental setup, to

Stellenbosch University https://scholar.sun.ac.za

achieve a medium where the enzyme could be homogenously distributed for optimal hydrolysis.

The high fat content of the larvae (37.8 %) has been a hurdle in previous attempts to fractionate *H. illucens* larvae (Bußler *et al.,* 2016) and multi-step approaches have been suggested, with the initial removal of the fat by means of solvents. Fat recovery, assuming the FF to be 32 % of the fractionated sample, was ~ 81 % on a dry matter basis. This is substantially more than the ~ 27 % obtained by Caligiani *et al.* (2018) also using enzymatic hydrolysis as fractionation method. The low fat recovery obtained by Caligiani *et al.* (2018), using enzymatic hydrolysis, could be explained by the lengthy hydrolysis period of 16 hours. The lengthy exposure of the fat to a high temperature could have emulsified the fat making it harder to separate from the protein and chitin. In the same study, stepwise chemical fractionation of the larvae recovered 86 % of the total fat.

The protein content, as determined by Dumas combustion, is generally calculated from the nitrogen percentage of the total sample using a standard nitrogen-to-protein conversion factor of 6.25. However, the total nitrogen content in insects cannot be attributed to protein alone as chitin, a polymer of N-acetylglucosamine and large constituent of insects, also contains nitrogen. Hence, in order to obtain a more accurate estimate of the protein content of the larvae a conversion factor of 5.60 was determined from the total amino acids. This took into account the non-protein- nitrogen (chitin) fraction so as to accurately determine the protein content of the larvae (Janssen *et al.,* 2017). Using this method, the protein content of the *H. illucens* larvae used for this trial was 27.1 %. This is lower than previously reported by Tschirner & Simon (2015) who used a conversion factor of 6.25, but in line with that reported by Caligiani *et al*. (2018) using the revised conversion factor of 5.60. The protein recovered, assuming the PF to be 60 % of the fractioned sample, was ~ 57 % on a dry matter basis. This is on *par* with that obtained by Caligiani *et al.* (2018) using enzymatic hydrolysis to isolate the protein. However, when Caligiani *et al.* (2018) exposed the defatted sample to 1 M NaOH for 1 hour they were able to recover 84 % protein. Even though it yielded high protein recovery, this method is not ideal as a solvent was used to isolate the protein. This alters the protein backbone and is a costly process to remove which limits upscaling. Also Caligiani *et al.* (2018) precipitated the protein with trichloroacetic acid to isolate it from the solvent, which decreased the final protein recovered to 73 %. The protein hydrolysate isolated by the current study had a moisture content of 71.7 % and contained the hydrolysed, as well as the soluble proteins. The proteins that were not recovered in PF were mostly insoluble. An additional washing step, using the aqueous PF, could be applied to CF where the insoluble proteins could be sieved out from the relatively larger chitin particles. Spray drying is envisaged as possible option to dry the protein hydrolysate obtained from *H. illucens* larvae. Spray drying is the most popular

Stellenbosch University https://scholar.sun.ac.za

drying technology used within the food, feed, chemical and pharmaceutical industries to prepare protein powder (Chen *et al.,* 2012). The fact that no external moisture was added to the fractionation method will reduce the cost of spray-drying significantly, as no unnecessary water is introduced into the hydrolysis procedure.

The chitin content of the larvae, determined gravimetrically, was 5.68 %. The chitin recovered, assuming the CF to be 22 % of the fractioned sample, was ~ 96 % on a dry matter basis. The chitin recovery using enzymatic hydrolysis was high, although the CF also contained the mineral fraction of the larvae as well as some insoluble proteins. The protein can be recovered as mentioned above and subsequently treating the CF with 1M HCl, as described in Woods *et al.* (2019c), the minerals could be removed and chitin isolated.

Amino acid composition

Table 5: Amino acid composition (% of total amino acids) of the whole *H. illucens* larvae (WL), as well as the protein containing fraction (PF) obtained after hydrolysis at optimum conditions

Amino acid	**Amino acid content (% of total amino acids)**	
	WL	**PF**
Essential		
Arginine	4.90	7.10
Cysteine	5.04	8.41
Histidine	3.48	4.67
Isoleucine	3.57	2.06
Leucine	6.13	2.99
Lysine	5.96	3.74
Methionine	1.99	0.93
Phenylalanine	6.10	2.80
Threonine	4.14	3.18
Tryptophan	1.30	2.24
Valine	5.61	2.80
Non-essential		
Alanine	11.2	17.2
Aspartine	8.78	6.17
Glutamine	12.6	17.3
Glycine	0.52	4.11
Proline	4.45	4.49
Serine	5.11	3.93
Tyrosine	9.04	5.73
Amino acid ratios		
Total EAA	48.2	40.9
Total NEAA	51.7	59.0
Total EEA : Total AA	0.48	0.41

WL Whole larvae
PF Protein containing fraction
EAA Essential amino acids
NEAA Non-essential amino acids
AA Amino acids

Stellenbosch University https://scholar.sun.ac.za

The amino acid compositions of WL and PF are presented in Table 5. The amino acid composition of the PF differed from that of the WL. This was due to the more soluble amino acids being concentrated in the aqueous PF and the less soluble amino acids in the CF. The amino acid composition of PF included all the essential amino acids (EAA), but in a lower total concentration to that present in WL. Isoleucine, leucine, lysine, methionine, phenylalanine and valine all decreased in PF, whereas arginine, cysteine, histidine, and tryptophan all increased. The least common EAA in PF was methionine (0.93 %), however, *H. illucens* larvae are known to be deficient in methionine (Newton *et al.,* 1977) The most abundant non-essential amino acid (NEAA) in both WL (12.6) and PF (17.3) was glutamine. This is in accordance with previous studies (Liland *et al.,* 2017).

Fatty acid composition

Table 6: Fatty acid (FA) composition (% total FAME) of the whole *H. illucens* larvae, as well as the fat containing fraction obtained after hydrolysis at optimum conditions

FAME (% of total fatty acids)		**Sample**	
Common name	**Abbreviation**	**WL**	**FF**
Capric	C10:0	0.97	0.99
Lauric	C12:0	56.7	57.4
Myristic	C14:0	10.7	10.9
Myristloleic	C14:1c9	0.21	0.19
Palmitic	C16:0	13.6	13.4
Palmitoleic	C16:1c9	3.36	3.32
Margaric	C17:0	0.06	0.06
Stearic acid	C18:0	1.88	1.85
Oleic	C18:1c9	7.50	7.17
Linoleic	C18:1c9 (n-6)	4.24	4.01
Arachidic	C20:0	0.05	0.03
A-Linolenic	C18:3c9,12,15 (n-3)	0.52	0.48
Conjugated linoleic acid	C18:2c9t11 (CLA)	0.08	0.08
Fatty acid ratios			
Total saturated FA	SFA	84.0	84.7
Total mono unsaturated FA	MUFA	11.0	10.6
Total polyunsaturated FA	PUFA	4.85	4.56
Total omega-6 FA	n-6	4.32	4.08
Total omega-3 FA	n-3	0.56	0.48
PUFA : SFA		0.06	0.05
n-6 : n-3		8.25	8.57
PUFA : MUFA		0.44	0.43

WL: Whole larvae
FF: Fat containing fraction
[a] Means in the same row with different superscript letter differ ($P \leq 0.05$)

No differences were observed between the fatty acid profile of WL and FF (Table 6). Lauric acid (56.7 %) was the highest constituent of the WL, followed by palmitic (13.6 %), myristic (10.7 %), oleic (7.50 %), linoleic (4.24 %), palmitoleic (3.36) and stearic acid (1.88 %) (Table 6). The fractionation of the larvae had little effect on the fatty acid profile of the FF as the profile

Stellenbosch University https://scholar.sun.ac.za

stayed mostly unchanged after enzymatic hydrolysis. A possible explanation for this could be the saturated nature of the fat making the fat less prone to oxidation at the optimum temperature for fractionation (38 °C). The fatty acid profiles were exceptionally rich in saturated fatty acids (84.0 %) and very poor in polyunsaturated fatty acids (4.85 %). The fatty acid profiles obtained for the WL were in line with that of previous studies (Ushakova *et al.,* 2016; Caligiani *et al.,* 2018; Danieli *et al.,* 2019). Cullere et al., (2019b) showed that the manipulation of the ratio of saturated to unsaturated fatty acid profile of *H. illucencs* larvae through their diet is difficult.

Lauric acid, the main constituent of the fatty acid profile of *H. illucens* larvae, has profound antiviral and antibacterial activity (Lieberman *et al.,* 2006). Lauric acid is particular active against gram positive bacteria present in the gut microbiota (Dierick *et al.,* 2002). It is postulated that the lauric acid content of the larvae can be manipulated by the rearing conditions or substrate that the larvae are grown on. In suboptimal rearing conditions and / or when larvae are grown on suboptimal substrates it is said that the lauric acid content of *H. illucens* larvae increase (Schiavone *et al.,* 2018). This phenomenon could be exploited to produce larvae high in lauric acid and, subsequently fat with the desired high lauric acid content can be isolated by means of the fractionation method developed.

Due to the saturated nature of the fatty acid profile of the larvae, it is not an ideal fat source to include into animal feeds for consumers concerned with meat that contains a high proportion of saturated fats. When fed to broiler chickens it increased the proportion of saturated fatty acids, to the detriment of the polyunsaturated fraction, in the breast meat (Cullere *at al.,* 2019b). However, an increase in the lauric acid content of the breast meat was observed which could be exploited for its health benefits. Therefore, the advantages and disadvantages need to be weighed up. Schiavone *et al.,* (2018) also reported that the inclusion of fat derived from *H. illucens* larvae into the diets of broiler chickens did not negatively affect the growth performance or carcass yields. Therefore, it cannot be discarded as a fat source to be used in animal nutrition. The best outcome would be to try and manipulate the larvae to contain more poly unsaturated fatty acids by means of feeding them a substrate high in omega-3, as proven possible by St-Hilaire (2007). The saturated nature of the fatty acid profile of the larvae could be exploited elsewhere, such as in the biofuel, cosmetic and nutraceutical industries (Wong *et al.,* 2019).

Conclusion

This study determined that the fractionation of *H. illucens* larvae by means of enzymatic hydrolysis was a feasible option. It was determined that almost all the fat could be recovered by means of enzymatic hydrolysis. Although it was not as efficient in isolating protein from

Stellenbosch University https://scholar.sun.ac.za

chitin, compared to chemical methods, the aqueous protein fraction had a high purity and future studies could investigate washing-and-sieving methods as mean to recover the insoluble proteins from the chitin fraction. Also, the spray drying of the protein hydrolysate produced and their functional properties could be investigated.

The fractionation method for *H. illucens* larvae acquired by this study diversifies the possible use of its functional compounds. The protein hydrolysate obtained can find application in the sport supplementation or similar industries where high quality proteins are required. This is due to the purity of the protein and important biological effects such as an increased rate of absorption, which is more effective in stimulating skeletal muscle protein synthesis, as well as antioxidant, -hypertensive and -inflammatory effects. *Hermetia illucens* fat, high in lauric acid, could be used to partially replace or supplement antibiotics in animal nutrition, as lauric acid has profound antiviral, -bacterial and –fungal effects. This property could also be exploited in the cosmetic industry as an alternative to coconut oil. The possibilities for the use of chitin are endless and one of these is the making of eco-friendly plastic to replace single-use plastics and their negative impact on the environment. The fractionation of *H. illucens* larvae, by enzymatic means, allows for higher margins to be made, both environmentally as well as financially, compared to the use of the “intact” biomass.

This study laid the foundation for the fractionation *H. illucens* larvae by means of enzymatic hydrolysis and future studies could refine the process. Enzymatic hydrolysis is a particularly attractive technique to fractionate *H. illucens* larvae due to the milder process conditions required, the relative ease to control the reaction and minimal formation of by-products. This technology allows for, possible diversification in use, as well as the improvement of *H. illucens* functional compounds. The elimination of the excessive use of harsh chemicals is also a promising prospect concerning sustainability and aligns with the ideology of *H. illucens* production as a means to recycle / upscale biowaste.

Stellenbosch University https://scholar.sun.ac.za

References

Association of Official analytical Chemists (AOAC), 2002. Official methods of analysis (17th Ed.), Arlington, Virginia, USA.

Balzan, S., Fasolato, L., Maniero, S., and Novelli, E., 2016. Edible insects and young adults in a north-east Italian city: An exploratory study. British Food Journal 118(2): 318-326.

Barragan-Fonseca, K.B., Dicke, M. and van Loon, J.J., 2017. Nutritional value of the black soldier fly (*Hermetia illucens* L.) and its suitability as animal feed–a review. Journal of Insects as Food and Feed 3(2): 105-120.

Barros-Cordeiro, K.B., Báo, S.N. and Pujol-Luz, J.R., 2014. Intra-puparial development of the black soldier-fly, *Hermetia illucens.* Journal of Insect Science 14(83): 1-10. https://doi.org/10.1093/jis/14.1.83

Belghit, I., Liland, N.S., Gjesdal, P., Biancarosa, I., Menchetti, E., Li, Y., Waagbø, R., Krogdahl, Å. and Lock, E.J., 2019. Black soldier fly larvae meal can replace fish meal in diets of sea-water phase Atlantic salmon (*Salmo salar*). Aquaculture 503: 609-619.

Bessa, L.W., Pieterse, E., Sigge, G. and Hoffman, L.C., 2018. Insects as Human Food; From Farm to Fork. Journal of the Science of Food and Agriculture https://doi:10.1002/jsfa.8860

Bessa, L.W., Pieterse, E., Sigge, S., Hoffman, L.C., 2019. An exploratory study into the use of black soldier fly (*Hermetia illucens*) larvae in the production of a vienna-type sausage. Meat and Muscle Biology (In Press). https://doi: 10.22175/mmb2018.11.0038

Biasato, I., Renna, M., Gai, F., Dabbou, S., Meneguz, M., Perona, G., Martinez, S., Lajusticia, A.C.B., Bergagna, S., Sardi, L. and Capucchio, M.T., 2019. Partially defatted black soldier fly larva meal inclusion in piglet diets: effects on the growth performance, nutrient digestibility, blood profile, gut morphology and histological features. Journal of animal science and biotechnology 10(1): 12. https://doi.org/10.1186/s40104-019-0325-x

Bußler, S., Rumpold, B.A., Jander, E., Rawel, H.M., Schlüter, O.K., 2016. Recovery and techno-functionality of flours and proteins from two edible insect species: Meal worm (*Tenebrio molitor*) and black soldier fly (*Hermetia illucens*) larvae. Heliyon 2: e00218.

Caligiani, A., Marseglia, A., Leni, G., Baldassarre, S., Maistrello, I., Dossena, A. and Sforza, S., 2018. Composition of black soldier fly prepupae and systematic approaches for extraction and fractionation of protein, lipids and chitin. Food Research International 105: 812-820.

Stellenbosch University https://scholar.sun.ac.za

Camacho, F., González-Tello, P., Páez-Dueñas, M.P., Guadix, E.M. and Guadix, A., 2001. Correlation of base consumption with the degree of hydrolysis in enzymic protein hydrolysis. The Journal of dairy research 68(2): 251-265.

Chen, C., Chi, Y.J. and Xu, W., 2012. Comparisons on the functional properties and antioxidant activity of spray-dried and freeze-dried egg white protein hydrolysate. Food and Bioprocess Technology 5(6): 2342-2352.

Cullere, M., Schiavone, A., Dabbou, S., Gasco, L. and Dalle Zotte, A., 2019a. Meat Quality and Sensory Traits of Finisher Broiler Chickens Fed with Black Soldier Fly (*Hermetia Illucens* L.) Larvae Fat as Alternative Fat Source. Animals 9(4):140. https://doi.org/10.3390/ani9040140

Cullere, M., Woods, M.J., Van Emmenes, L., Pieterse, E., Hoffman, L.C. and Dalle Zotte A., 2019b. *Hermetia illucens* larvae reared on different substrates in broiler quail diets: effect on meat quality and sensory traits. Animals (Under review).

Cunico, R., 1986. High sensitivity amino acid analysis using a novel automated precolumn derivativation system. Bio-Chromatography 1: 6-12.

Da Silva, R.R., 2018. Enzymatic synthesis of protein hydrolysates from animal proteins: exploring microbial peptidases. Frontiers in microbiology 9: 735. https://doi.org/10.3389/fmicb.2018.00735

Danieli, P.P., Lussiana, C., Gasco, L., Amici, A. and Ronchi, B., 2019. The Effects of Diet Formulation on the Yield, Proximate Composition, and Fatty Acid Profile of the Black Soldier Fly (*Hermetia illucens* L.) Prepupae Intended for Animal Feed. Animals 9(4): 178. https://doi.org/10.3390/ani9040178

Del Valle, F. R., Mena, M. H. and Bourges, H., 1982. An investigation into insect protein. Journal of Food Processing and Preservation 6(2): 99-102.

Dierick, N.A., Decuypere, J.A., Molly, K., Van Beek, E. and Vanderbeke, E., 2002. The combined use of triacylglycerols containing medium-chain fatty acids (MCFAs) and exogenous lipolytic enzymes as an alternative for nutritional antibiotics in piglet nutrition: I. In vitro screening of the release of MCFAs from selected fat sources by selected exogenous lipolytic enzymes under simulated pig gastric conditions and their effects on the gut flora of piglets. Livestock Production Science 75(2): 129-142.

Duda, A., Adamczak, J., Chełmińska, P., Juszkiewicz, J. and Kowalczewski, P., 2019. Quality and nutritional/textural properties of durum wheat pasta enriched with cricket powder. Foods 8(2): 46. https://doi.org/10.3390/foods8020046

Finch, R., 1977. Whatever happened to fish protein concentrate? Food Technology 31: 44

Gariglio, M., Dabbou, S., Biasato, I., Capucchio, M.T., Colombino, E., Hernández, F., Madrid, J., Martínez, S., Gai, F., Caimi, C. and Oddon, S.B., 2019. Nutritional effects of the dietary inclusion of partially defatted *Hermetia illucens* larva meal in Muscovy

Stellenbosch University https://scholar.sun.ac.za

duck. Journal of animal science and biotechnology 10(1): 37. https://doi.org/10.1186/s40104-019-0344-7

Gasco, L., Biasato, I., Dabbou, S., Schiavone, A. and Gai, F., 2019. Quality and Consumer Acceptance of Products from Insect-Fed Animals. In Edible Insects in the Food Sector (pp. 73-86). Springer, Cham.

Gasco, L., Finke, M. and Van Huis, A., 2018. Can diets containing insects promote animal health? Journal of Insects as Food and Feed 4(1): 1-4.

Hou, Y., Wu, Z., Dai, Z., Wang, G. and Wu, G., 2017. Protein hydrolysates in animal nutrition: industrial production, bioactive peptides, and functional significance. Journal of animal science and biotechnology 8(1): 24. https://doi.org/10.1186/s40104-017-0153-9

Ivanovs, K. and Blumberga, D., 2017. Extraction of fish oil using green extraction methods: A short review. Energy Procedia 128: 477-483.

Janssen, R.H., Vincken, J.P., van den Broek, L.A., Fogliano, V. and Lakemond, C.M., 2017. Nitrogen-to-protein conversion factors for three edible insects: *Tenebrio molitor*, *Alphitobius diaperinus*, and *Hermetia illucens*. Journal of agricultural and food chemistry 65(11): 2275-2278.

Johannsen, O.A., 1922. Stratiomyiid larvae and puparia of the North Eastern States. Journal of the New York Entomological Society 30(4): 141-153.

Józefiak, D. and Engberg, R.M., 2015, August. Insects as poultry feed. In 20th European Symposium on poultry nutrition 24-27.

Józefiak, D. and Engberg, R.M., 2017. Insect proteins as a potential source of antimicrobial peptides in livestock production. A review. Journal of Animal and Feed Sciences 26(2): 87-99.

Kawasaki, K., Hashimoto, Y., Hori, A., Kawasaki, T., Hirayasu, H., Iwase, S.I., Hashizume, A., Ido, A., Miura, C., Miura, T. and Nakamura, S., 2019. Evaluation of Black Soldier Fly (*Hermetia illucens*) Larvae and Pre-Pupae Raised on Household Organic Waste, as Potential Ingredients for Poultry Feed. Animals 9(3): 98. https://doi.org/10.3390/ani9030098

Kristinsson, H.G. and Rasco, B.A., 2000. Fish protein hydrolysates: production, biochemical, and functional properties. Critical reviews in food science and nutrition, 40(1): 43-81.

Lahl, W.J., 1994. Enzymatic production of protein hydrolysates for food use. Food Science 48: 68-71.

Lalander, C., Diener, S., Magri, M. E., Zurbrügg, C., Lindström, A. and Vinnerå, B., 2013. Faecal sludge management with the larvae of the black soldier fly (*Hermetia illucens*) – From a hygiene aspect. Science of The Total Environment 458: 312-318.

Lee, C.M., Trevino, B. and Chaiyawat, M., 1996. A simple and rapid solvent extraction method for determining total lipids in fish tissue. Journal of AOAC International *79*(2): 487-492.

Stellenbosch University https://scholar.sun.ac.za

Lei, X.J., Kim, T.H., Park, J.H. and Kim, I.H., 2019. Evaluation of supplementation of defatted black soldier fly (*Hermetia illucens*) larvae meal in beagle dogs. Annals of Animal Science 1(In Press). https://doi.org/10.2478/aoas-2019-0021

Lieberman, S., Enig, M.G. and Preuss, H.G., 2006. A review of monolaurin and lauric acid: natural virucidal and bactericidal agents. Alternative & Complementary Therapies 12(6): 310-314.

Liland, N.S., Biancarosa, I., Araujo, P., Biemans, D., Bruckner, C.G., Waagbø, R., Torstensen, B.E. and Lock, E.J., 2017. Modulation of nutrient composition of black soldier fly (*Hermetia illucens*) larvae by feeding seaweed-enriched media. PloS one 12(8): p.e0183188.

Meneguz, M., Schiavone, A., Gai, F., Dama, A., Lussiana, C., Renna, M. and Gasco, L., 2018. Effect of rearing substrate on growth performance, waste reduction efficiency and chemical composition of black soldier fly (*Hermetia illucens*) larvae. Journal of the Science of Food and Agriculture 98(15): 5776-5784.

Miranda, C.D., Cammack, J.A. and Tomberlin, J.K., 2019. Life-History Traits of the Black Soldier Fly, *Hermetia illucens* (L.)(Diptera: Stratiomyidae), Reared on Three Manure Types. Animals 9(5): 281. https://doi.org/10.3390/ani9050281

Montgomery, D.C., 2017. Design and analysis of experiments. John Wiley & Sons.

Newton, G.L., Booram, C.V., Barker, R.W. and Hale, O.M., 1977. Dried *Hermetia illucens* larvae meal as a supplement for swine. Journal of Animal Science 44(3): 395-400.

Nielsen, P. M., Petersen, D. and Dambmann, C., 2001. Improved method for determining for determining food protein degree of hydrolysis. Journal of food Science 66(5): 642-646.

OECD/FAO, 2019. OECD-FAO Agricultural Outlook 2019-2028, OECD Publishing, Paris. Food and Agriculture Organization of the United Nations, Rome. https://doi.org/10.1787/agr_outlook-2019-en

Rinaudo, M., 2006. Chitin and chitosan: Properties and applications. Progress in Polymer Science 31: 603-632.

Sangduan, C., 2018. Skincare product containing *Hermetia illucens* extract. U.S. Patent Application 15/981,689.

Schiavone, A., Dabbou, S., De Marco, M., Cullere, M., Biasato, I., Biasibetti, E., Capucchio, M.T., Bergagna, S., Dezzutto, D., Meneguz, M. and Gai, F., 2018. Black soldier fly larva fat inclusion in finisher broiler chicken diet as an alternative fat source. Animal 12(10): 2032-2039.

Schösler, H., Boer, J.D., and Boersema, J.J., 2012. Can we cut out the meat of the dish? Constructing consumer-oriented pathways towards meat substitution. Appetite 58 (1): 39-4.

Stellenbosch University https://scholar.sun.ac.za

Shin, H.S. and Park, S.I., 2019. Novel attacin from *Hermetia illucens*: cDNA cloning, characterization, and antibacterial properties. Preparative Biochemistry and Biotechnology 49(3): 279-285.

Smetana, S., Palanisamy, M., Mathys, A. and Heinz, V., 2016. Sustainability of insect use for feed and food: Life Cycle Assesment perspective. Journal of Cleaner production 137: 741-751.

Smetana, S., Schmitt, E. and Mathys, A., 2019. Sustainable use of *Hermetia illucens* insect biomass for feed and food: Attributional and consequential life cycle assessment. Resources, Conservation and Recycling 144: 285-296.

Spranghers, T., Ottoboni, M., Klootwijk, C., Ovyn, A., Deboosere, S., De Meulenaer, B., Michiels, J., Eeckhout, M., De Clercq, P. and De Smet, S., 2017. Nutritional composition of black soldier fly (*Hermetia illucens*) prepupae reared on different organic waste substrates. Journal of the Science of Food and Agriculture 97(8): 2594-2600.

STATISTICA 13 (TIBCO Software Inc. 2018). Statistica (data analysis software system), version 13. http://tibco.com.)

Stehel, V., Maroušková, A., Kolář, L., Strunecký, O. and Shreedhar, S., 2019. Advances in dry fermentation extends biowaste management possibilities. Energy Sources, Part A: Recovery, Utilization, and Environmental Effects (In Press). https://doi.org/10.1080/15567036.2019.1587066

St-Hilaire, S., Cranfill, K., McGuire, M.A., Mosley, E.E., Tomberlin, J.K., Newton, L., Sealey, W., Sheppard, C. and Irving, S., 2007. Fish offal recycling by the black soldier fly produces a foodstuff high in omega-3 fatty acids. Journal of the World Aquaculture Society 38(2): 309-313.

Surendra, K.C., Olivier, R., Tomberlin, J.K., Jha, R. and Khanal, S.K., 2016. Bioconversion of organic wastes into biodiesel and animal feed via insect farming. Renewable energy 98: 197-202.

Tapal, A. and Tiku, P.K., 2019. Nutritional and Nutraceutical Improvement by Enzymatic Modification of Food Proteins. In Enzymes in Food Biotechnology (471-481). Academic Press.

Tschirner, M. and Simon, A., 2015. Influence of different growing substrates and processing on the nutrient composition of black soldier fly larvae destined for animal feed. Journal of Insects as Food and Feed 1(4): 249-259.

Ushakova, N.A., Brodskii, E.S., Kovalenko, A.A., Bastrakov, A.I., Kozlova, A.A. and Pavlov, D.S., 2016. Characteristics of lipid fractions of larvae of the black soldier fly *Hermetia illucens*. Doklady Biochemistry and Biophysics 468(1): 209-212.

Stellenbosch University https://scholar.sun.ac.za

Uushona, T., 2015. Black Soldier Fly (*Hermetia illucens*) Pre-Pupae as a Protein Source for Broiler Production. MSc thesis, University of Stellenbosch, Stellenbosch.

Valencia, P. L., Flores, S. A., Pinto, M. J., Almonacid, S. F., 2016. Analysis of the operational strategies for enzymatic hydrolysis of food proteins in batch reactor. Journal of Food Engineering 176: 121-127

Van Huis, A., 2013. Potential of insects as food and feed in assuring food security. Annual Review of Entomology 58(1): 563-583.

Wong, C.Y., Rosli, S.S., Uemura, Y., Ho, Y.C., Leejeerajumnean, A., Kiatkittipong, W., Cheng, C.K., Lam, M.K. and Lim, J.W., 2019. Potential Protein and Biodiesel Sources from Black Soldier Fly Larvae: Insights of Larval Harvesting Instar and Fermented Feeding Medium. Energies 12(8): 1570. https://doi.org/10.3390/en12081570

Woods, M.J., Cullere, M., Van Emmenes, L., Vincenzi, S., Pieterse, E., Hoffman, L.C. and Zotte, A.D., 2019a. *Hermetia illucens* larvae reared on different substrates in broiler quail diets: effect on apparent digestibility, feed-choice and growth performance. Journal of Insects as Food and Feed 5(2): 1-10.

Woods, M.J., Hoffman, L.C. and Pieterse, E., 2019b. Artificial diets for neonatal black soldier fly (*Hermetia illucens*) larvae. Journal of Insects as Food and Feed 5(2): 99-105.

Woods, M.J, Goosen, N.J., Hoffman, L.C. and Pieterse, E., 2019c. A simple and rapid protocol for measuring chitin content of insect larvae. Journal of Insects as Food and Feed (In Review).

Xiao, H., Shao, F., Wu, M., Ren, W., Xiong, X., Tan, B. and Yin, Y., 2015. The application of antimicrobial peptides as growth and health promoters for swine. Journal of animal science and biotechnology 6(1): 19. https://doi.org/10.1186/s40104-015-0018-z

Yi, L., Lakemond, C.M.M., Sagis, L.M.C., Eisner-Schadler, V., Van Huis, A. and Van Boekel, M.A.J.S., 2013. Extraction and characterisation of proteins from five insect species. Food Chemistry 141: 3341-3348.

Yu, S.Y. and Fazidah, S., 1994. Enzymic hydrolysis of proteins from *Aristichthys nobilis* by protease P'Amano'3. Tropical science 34: 381-386.

Stellenbosch University https://scholar.sun.ac.za

CHAPTER 9

General conclusion

The primary objective of this collection of studies was to shed some light on current issues hindering insect mass-production from scaling up to even greater heights. These issues ranged from the substrates / diets that the insects are reared on, to post-harvest processing for feed and food, and possibly tailoring the insects for specific demands / markets. The results obtained were a step in the right direction to more efficiently produce insects at a commercial level.

The formulation of diets more suitable to the nutritional needs of *Eldana saccharina* led to a ~ 10 % increase in survivability of the larvae from neonate to pupal stage and a faster rate of development. Similar findings were observed for breeder *Hermetia illucens* diets. When the nutrient needs of breeder larvae were more accurately met, a ~ 10 % increase in survivability was observed. Higher bioconversion were also observed along with a ~ 46 % increase in developmental rate and heavier pupae.

A strong correlation between the physical properties of the diets, such as water holding capacity, and the eventual success of the diets was determined. It was also noted that the inclusion of fiber at significant levels into the diets of mass-produced insects positively correlated to the ability of these diets to rear large numbers of high quality insects. Although most insect are not able to digest fiber without any pre-treatment, fiber plays an important textural role as it allows for the aeration of the substrate and increases water holding capacity. For the most part, larvae that live within their feeding substrate are legless, moving mainly by circular motion, therefore the density and moisture content of the diets are important. It does not do any justice to provide the insects with a balanced diet, but due to physical constraints of the substrate, they cannot access these nutrients. Also, insects need aerobic environments to survive.

The use of the body composition (whole-carcass or carcass milling) technique, was successfully adopted from conventional livestock nutrition to determine ideal amino acid profiles for the mass-production of all insect species it was applied to (*Thaumatobia leucotreta*, *Hermetia illucens*, *Eldana saccharina*, *Conopomorpha cramerella*, *Chilo sacchariphagus*). It was also determined that for the most part insects are currently being over provided with nutrients. This reduces the performance of the insects because an oversupply of nutrients leads to the build-up of primary and / or secondary metabolites which could be either toxic, antagonistic or result in imbalances that leads to metabolic stress. In addition, diets with high nutrient specifications are expensive and with reduced nutrient specification diets, significant

Stellenbosch University https://scholar.sun.ac.za

savings were made. Therefore, not only can the production output of the mass-produced insects be increased, but the cost to do so can also be lowered. Building on this, response curves for the determination of energy : protein ratios could be studied in future.

In an attempt to further the profitability as well as the acceptability of insects as feed and food enzymatic assisted fractionation of *H. illucens* larvae was conducted. This fractionation protocol was done at a much larger scale than what was previous reported in literature, also enjoying more success. Fat recovery of ~ 81 % was substantially more than what was previously achieved. Protein solubility was on *par* with previous studies and determined at ~ 57 %, with the insoluble proteins found in the pellet fraction along with the chitin. Further studies are suggested to investigate a washing-and-sieving step to separate these two fractions, and this could result in the most promising fractionation method for commercial application. Currently there are thousands of commercial enzymes available on the market, most with different active parameters, and therefore there is still a lot of scope to improve the hydrolysis efficiency of *H. illucens* larvae and subsequently the degree of fractionation. The unique functional properties of the protein hydrolysate is also an exiting avenue to explore in future research.

The repeatability of chitin quantification of insects has been a stumbling block in the insect mass-production industry. A simple and rapid protocol was developed for measuring chitin content of insect larvae, based on the procedure to isolate chitin from crustaceans. Alterations had to be made due to structural differences between the chitin in insect cuticles and that found in crustaceans, as well as due to the high fat content of insect larvae. A stepwise method to isolate chitin from larvae was developed and the highest insect-chitin purity to date was achieved. Subsequently a gravimetrical comparison was made and chitin content of the larvae was determined with high and repeatable accuracy. The developed analysis can contribute to standardised results for chitin determination of insect larvae and therefore chitin values can be comparable across future studies. Also, it would be worth studying the application of the isolation protocol to the chitin containing fraction obtained after enzymatic fractionation, omitting the defatting step, and accessing this as a possible way of obtaining pure chitin on a commercial scale.

Aiming to tailor the fatty acid (FA) composition of *H. illucens* larvae for feed- and food- health benefits, it was determined the ratio of saturated- (SFA) to polyunsaturated- (PUFA) fatty acids cannot be altered substantially by the rearing of larvae on a substrate rich in PUFA. Although, the ratio of n-6 to n-3 FA was positively altered, and in this way seen as a healthier option to larvae reared on conventional substrates. It was determined that larvae, fed on a substrate rich in PUFA, did not negatively influence production parameters when fed to poultry. The

Stellenbosch University https://scholar.sun.ac.za

subsequent breast meat also had a more favourable n-6 to n-3 ratio and contained significant amounts of lauric acid, directly stemming from the inclusion of *H. illucens* larvae in the diets of the birds. Lauric acid is also known to have significant health benefits and thereby feeding monogastric animals larvae tailored to be rich in this n-3 FA, could result in firstly, better health status for the animals and secondly, healthier meat for humans. The larvae could also be directly used in food applications as a "sustainable superfood".

The mass-production of the insects has a huge role to play in ensuring food security and the biocontrol of pest species. With the global population estimated to increase, mostly in developing countries by one fourth of the total population within the next decade, insects as food, feed and biocontrol agents are a more than plausible solution to address food and feed security, as well as the over exploitation of chemical pesticides. The mass-production of insects can address numerous other problems such as waste management, the over exploitation of antibiotics in animal production systems, deforestation and reduce the use of extremely large quantities of water by agricultural industries. The inroads made by the collections of studies in this thesis can help solidify these prospects.