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ODUNAYO ABIGEAL TOVIHO

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Head of Doctoral School:
Prof. Dr. István Komlósi
professor

Supervisor:
Dr. Péter Bársony
assistant professor

Optimalization of the European perch (*Perca fluviatilis*) Digestion and the Utilization of the Yellow mealworm in the Perch Nutrition

Odunayo Abigeal Toviho
PhD candidate

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Written by **Odunayo Abigeal Toviho** certified Animal Husbandry Engineer
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Dissertation supervisor: Dr. Péter Bársony

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Abbreviation list

FAO	Food and Agricultural Organization
UN	United Nations
SO	Soybean oil
TM	*Tenebrio molitor* oil
ZM	*Zophobas morio* oil
CP	Crude protein
EAA	Essential amino acid
DBSFLM	Defatted Black soldier fly larvae meal
FM	Fish meal
WG	Weight gain
SR	Survival rate
K	Condition factor
PWG	Percentage of weight gain
DGR	Daily growth rate
SGR	Specific growth rate
BSF	Black soldier fly
FI	Feed intake
HSI	Hepatic somatic index
VSI	Visceral somatic index
YM	Yellow mealworm
DM	Dry matter content
CF	Crude fat
ADF	Acid detergent fibre
ADL	Acid detergent lignin
SA	Surface area
BM	Body mass
PAPs	Process animal proteins
RAS	Recirculating aquaculture systems
NFE	Nitrogen free extract
PER	Protein efficiency ratio
FCR	Feed conversion ratio
DNA	Deoxyribonucleic acid

PCV2	Porcine circovirus 2
PCVD	Porcine circovirus-associated diseases
PCVs	Porcine circoviruses
Rep	Replication-associated
Cap	Capsid
HCl	Hydrochloric acid
AAs	Amino acids
GHG	Greenhouse gases
PUFA	Polyunsaturated fatty acids
DBSFLM	Defatted Black soldier fly larvae meal
MM	Mealworm meal
Dph	Day post hatching
TCA	Trichloroacetic acid
NPV	Net protein value
FT	Crude fiber
PER	Protein efficiency ratio
TD	True digestibility
RGL	Relative gut length
IBW	Initial body weight

1. INTRODUCTION

The world population is projected to reach 9 billion in 2037 and 10 billion in 2057 from the current 7.9 billion (WORLDOMETER, 2022), and this poses a new challenge to researchers (BORIDEAN ET AL., 2020b). To provide enough food for the increasing population, it was projected that the current food production will need to almost double; while resources needed for increased production such as land and water resources are becoming scarce (VAN HUIS ET AL., 2013). Hence, there is a need to find alternative and sustainable ways of growing food (DA ROSA MACHADO & THYS, 2019). World population increase and increase in consumer demand for protein will sustainably make the production of protein a challenge in the future (ZHAO ET AL., 2016).

An increase in demand for fishmeal and soy as a result of fast-growing aquaculture production and consequently increase in price has led to new research in the use of insect protein in aquaculture, poultry, and pig feed (VAN HUIS ET AL., 2013). To face the challenge of protein production there is need for an alternative protein source that requires fewer resources to produce effectively and efficiently, whether it is for direct consumption or livestock feed production. The aquaculture industry is especially in dire need of an alternative protein source to produce complete feed for commercial aquaculture species because of the fast growth in the industry. Other than the economics and sustainability issues, the aquaculture industry is under pressure from consumer that are now more conscious not just of the quality of fish they eat but also of the ethics and environmental impact the production of the fish has. The aquaculture industry depends on capture fisheries for fishmeal, and this raises the question; How ethical, economical, or sustainable is the use of fish to produce fish? Although the aquaculture industry uses soybean meal as an alternative protein source there is still a need for animal protein sources, especially in carnivore species that are adapted to consumption of animal sources in their natural habitat.

Edible insects are consumed in parts of the world like Africa, Asia, and South America, but their consumption is still not widely accepted in the Western world. Because of the low level of acceptance, care must be taken in using it as the protein source in fish production because the fish will eventually be consumed by humans.

The use of edible insects has however been encouraged by FAO, and this has helped improve the acceptability of edible insects.

Edible insect importance and suitability as an alternative protein source can be linked to their high nutritional value, less production requirement, low environmental impact, and high feed conversion efficiency compared to other livestock.

Although there are over 2000 edible insects documented there are only 7 species (yellow mealworm, lesser mealworm, black soldier fly, housefly, house cricket, banded cricket, and field cricket) approved for use in pet, fish, poultry, and swine feed in the EU (EC2021/1372).

The yellow mealworm (YM) is one of the most bred and traded of these seven insects in the EU, it is an omnivore species and can be raised on any substrate, they are now being reared on “former foods”. They can convert waste to useful protein mass, but this practice is subject to regulations for the country or region. They have been used as fishmeal replacements in several fish species, but this is mainly for research purposes and has yielded good outcomes. YM has low saturated fatty acids and hence higher unsaturated fatty acid, studies have however shown that the growth rate and nutrient composition of insects can be influenced by their diet providing an opportunity to alter the nutrient composition of insects through their feed. Common duckweed *Spyrodela polyrhiza* is a fast-growing floating water plant with a high protein content of about 30% and it has been considered an innovative feedstuff in animal nutrition, especially for fish and poultry. There is still an ongoing search for high-value feed for the mass production of the yellow mealworm. The modification of the nutrient composition of the yellow mealworm based on the diet composition provides an opportunity to explore the effect of duckweed as a feeding substrate in yellow mealworm production.

The order Perciformes consists of about 240 species, they are found in fresh and brackish water mostly in the northern hemisphere. They are easily distinguished from the trout and salmon family by the absence of an adipose fin. The European perch is a Percidae and the closest relative are the pikeperch and the yellow perch. The European perch is a carnivore fish, they feed on zooplankton and insect larvae at the larval stage and as they grow, they begin to feed on insect crustaceans and eventually other fishes.

This fish has the potential to become an important species in European aquaculture especially because of the quality of their flesh. The flesh of this fish is white, has a mild taste, and has no bones, overall, the quality of the flesh is top-shelf quality, and it commands a premium price. The European perch has a low feeding rate, and the table size is about 150 g, when reared in a RAS it can reach this size in about 8 months. Currently, there is no specific feed developed or tailored to the nutritional a need of this species, hence there is need for one.

It has been established from literature that there is a need for an alternative protein source to supplement the current traditional protein source in feed formulation.

Edible insect is an innovative protein source in the feed industry, and we identify the opportunity to use yellow mealworm in the formulation of European perch feed since it is a new species with a lot of potential in European aquaculture. The feeding habit of this species makes it a good candidate for the use of yellow mealworms.

On the other hand, providing a nutritionally balanced feed to fish in the culture system is not enough for a good fish production cycle. The process of digestion also plays a key role in determining the success of feed, an understanding of the digestion process of the European perch species is therefore important. The knowledge of the feedstuff, European perch digestion, and the effect of rearing conditions on digestion is important for the effective optimization of the nutrition of this species.

1.1 Justification

The European perch is still currently undergoing domestication, and its contribution to European aquaculture is still very low. Most of the perch production comes from capture fisheries because it is limited by its slow feeding rate. There is a need for the production of a feed suited to the digestion process of the European perch and the protein source for this feed must be of very high quality and an animal protein source. In the wild, the European perch already consume up to 50% insects as part of their diet, this makes it a suitable species for insect meal-based feed. Producing insect meal-based European perch feed reduces the environmental impact of feed production and makes it more sustainable.

The process of this research is approached from two major perspectives, we divided the topic into major parts the yellow mealworm and European perch. First, we explored YM as shown in *Figure 1*.

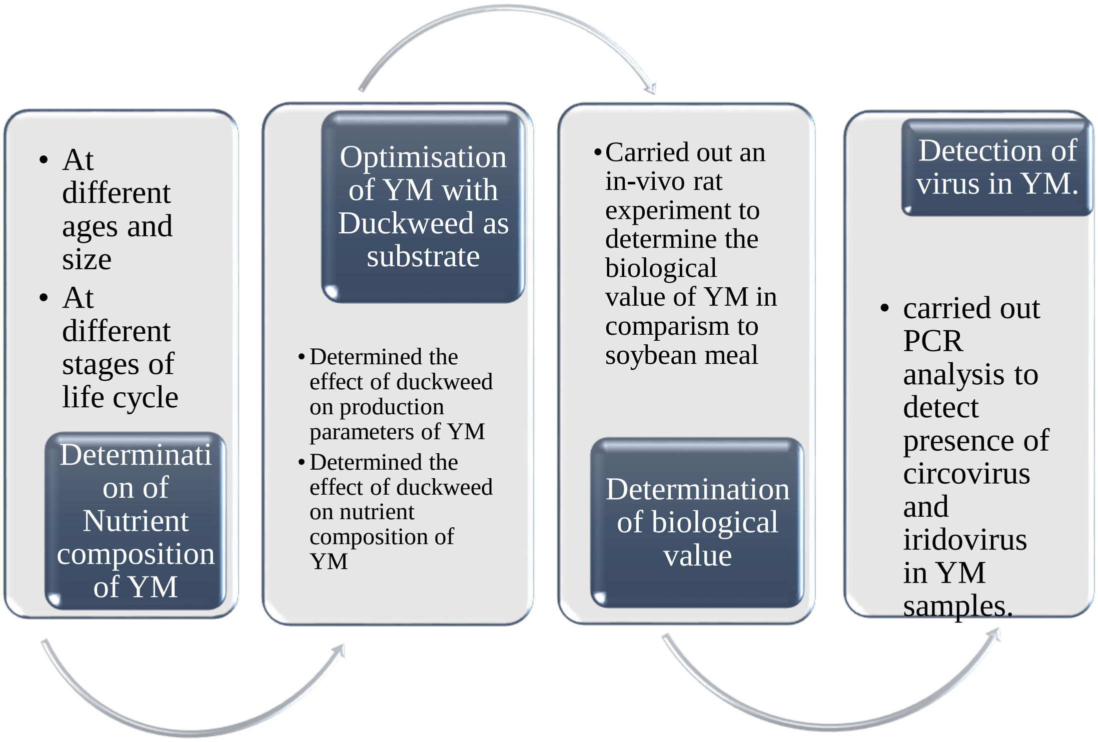
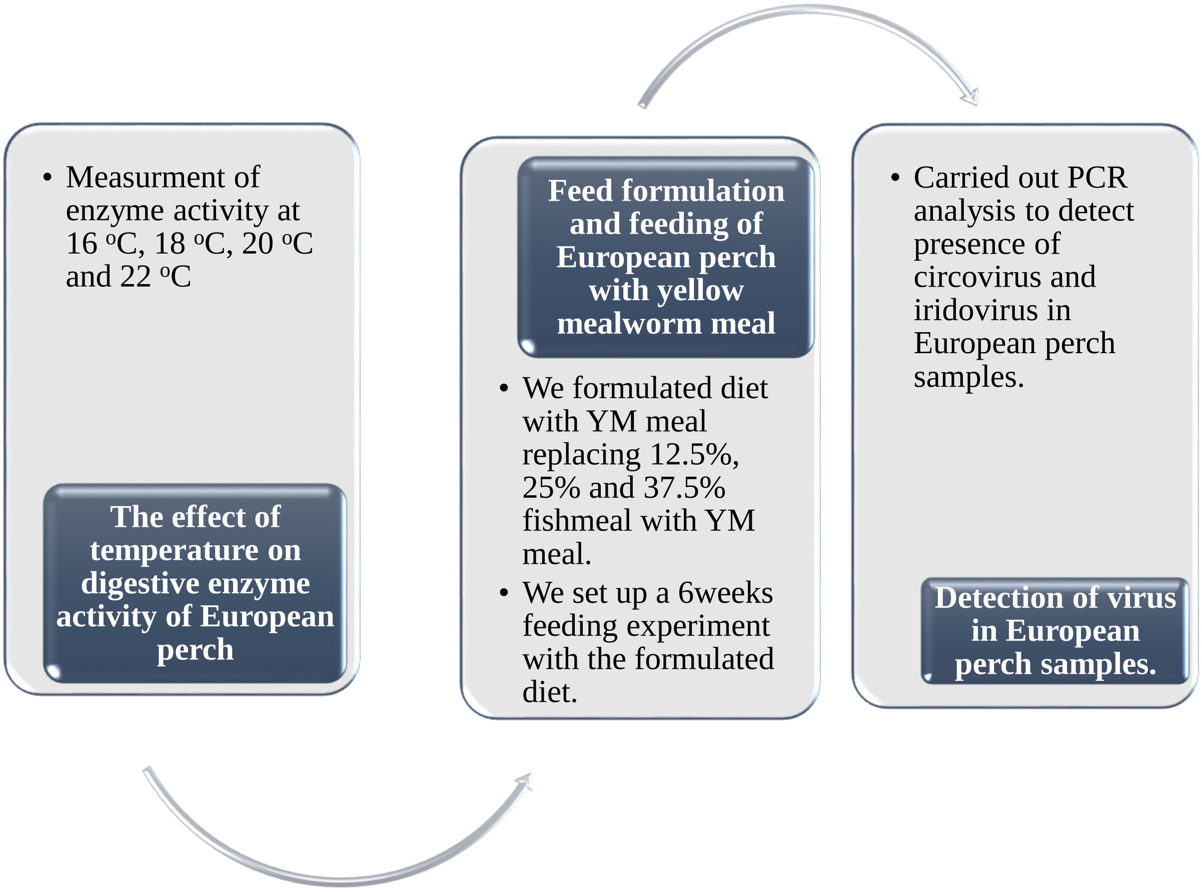


Figure 1: Workflow for mealworm experiment.

For the European perch, we explored different aspects of the digestion process, the European perch from the colder countries in the north are usually bigger because of this we analysed the enzyme activity of the European perch at different rearing temperatures lower than the reported 23 °C optimal temperature. The workflow for the experiments of the European perch is shown in *Figure 2*.



- Measurment of enzyme activity at 16 °C, 18 °C, 20 °C and 22 °C

- We formulated diet with YM meal replacing 12.5%, 25% and 37.5% fishmeal with YM meal.
- We set up a 6weeks feeding experiment with the formulated diet.

- Carried out PCR analysis to detect presence of circovirus and iridovirus in European perch samples.

Figure 2: European perch Experiments workflow

1.2 Objectives

- To determine the biological value of yellow mealworm using an Animal model
- To determine the nutrient composition of yellow mealworms at different ages sizes and stages of life cycle.
- To optimize the production parameters and nutrient composition of yellow mealworm using Duckweed (*Spirodela polyrhiza***)**.
- To briefly touch upon the viral safety concern of insect use.
- To determine the effect of different rearing temperatures on the digestive enzyme activity of European perch.
- To determine the effect of replacing fishmeal with yellow mealworm meal on the production parameters of European perch.

2. LITERATURE REVIEW

2.1. Insects

Insects have the highest diversity in the animal kingdom and there are about 1 million species that have been identified out of which around 2000 of them are currently considered edible (MITSUHASHI, 2017; STORK, 2018; TOVIHO & BÁRSONY, 2022). Edible insects were recommended by the United Nations Food and Agriculture Organization, as a fascinating, alternative nutritional resource for both animals and humans (VAN HUIS ET AL., 2013). The commonly consumed insects worldwide belong to the order; *Coleoptera* (31%), *Lepidoptera* (18%) and *Hymenoptera* (14%), *Orthoptera* (13%), *Hemiptera* (10%), *Isoptera* (3%), *Odonata* (3%), flies *Diptera* (2%) and others (5%). Culture and religion have a great influence on the practice of entomophagy, although insect is consumed in several parts of the world like Asia, Africa, South America, and Central America where they are farmed or harvested from the wild and are part of the traditional diet. Unfortunately, there is still a disgust factor surrounding it in the Western world (VARGAS-ABÚNDEZA ET AL., 2019; MANCINI ET AL., 2019; VAN HUIS ET AL., 2013). The majority of insects are consumed in Asia and Central America, where around 2 billion people in 113 nations consume insects as part of their cultural customs and diet (LESNIK, 2018, LICEAGA, 2021). Insects are often caught in the wild and sold in public marketplaces in these countries.

2.2. Edible insects

Edible insects are being consumed in some parts of the world as part of their diet, but their consumption is still influenced by disgust factors in other parts of the world especially Western countries (WENDIN & NYBERG, 2021). Edible insect has been suggested as a protein source because of their feed conversion efficiency and nutritional value (ZHAO ET AL., 2016).

If insects become a generally acceptable food and feedstuff in industrial countries there will be huge economic change (RAVZANAADII ET AL., 2012). Edible insects have been part of the local diets of about 2 billion people in the world (VAN HUIS ET AL., 2013).

There are about 2000 edible insects documented (JONGEMA, 2017), the United Nations (UN) Food and Agricultural Organization (FAO) began to promote insects in 2013 as a prospect for global food security and continue to do so.

This has sustained the interest of stakeholders in research, legislation, and process improvement geared toward sustainable insect production and acceptance (ARDOIN & PRINYAWIWATKUL, 2021). Edible insect has been suggested as a protein source because of their feed conversion efficiency and nutritional value (ZHAO ET AL., 2016). If insects become a generally acceptable food and feedstuff in industrial countries there will be huge economic change (RAVZANAADII ET AL., 2012). Edible insects have been part of the local diets of about 2 billion people in the world (VAN HUIS ET AL., 2013). It is difficult to give an actual number of edible insects worldwide but the inventory with the highest number of insects reported 2111 species of edible insect (JONGEMA, 2017).

Wasps, caterpillars, crickets, and locusts are now being marketed as expensive delicacies in high-end restaurants in Thailand, while ant larvae (escamoles) are also being served in high-end restaurants in Mexico, and consumers can experience a degustation plate of different insects in trendy food markets. The health and environmental arguments made in support of insects as an alternative protein source are compelling, but positive dining experiences are more likely to persuade consumers. (BERGER ET AL., 2018; LICEAGA, 2021). Furthermore, food regulations in Europe and the United States stipulate that insects intended for human consumption must be produced in certified insect farms rather than being caught in the wild.

Insects are considered food if that is the intended use, according to the US Food, Drug, and Cosmetic Act (Sec. 201(f)), hence insects raised for animal/pet feed or wild-crafted cannot be used for human use.Furthermore, given strong evidence that shellfish-allergic people may also be allergic to insects, insect-containing meals require allergen-warning labels. (LEWIS, 2015, LICEAGA, 2021).

The scholarly interest is also well-established. A search of the keyword "edible insects" on Web of Science (accessed 11 June 2021) yielded 48 hits between 1945 and 2010, and 805 finds between 2011 and 2020, with more than half in 2019 and 2020 alone. The amount of pet food firms creating insect-based products demonstrates that the industry is diversifying.

The European Union (EU) permitted the first product (dry powder of yellow mealworm, *Tenebrio molitor*) to be commercialized for food in early 2021, with more edible insect species goods expected in the future. This expansion and diversification may cause additional businesses to become involved in the industry (VAN HUIS 2021).

2.2.1. Nutrient composition of edible insect

Edible insects are exceptionally nutrient-dense, with high levels of protein and micronutrients including minerals and vitamins. The crude protein (CP) content of insects is species-dependent and varies from 40% to 60% (MAKKAR ET AL., 2014). It should be emphasized that the CP quality also depends on the production technology and feed composition for larval rearing. The protein content of insects is not just higher than plant sources, insects also have a balanced amino acid profile (BLÁSQUEZ ET AL., 2012) Fat is the second most represented constituent of the insect nutritional composition, after proteins. According to the literature, the fat content of insects varies by order and species (SÁNCHEZ-MUROS ET AL., 2014; RUMPOLD AND SCHLÜTER, 2013; DREASSI ET AL., 2017). Insects are rich in fat, vitamins, and minerals, and the quality of fatty acids is good especially long-chain omega-3 fatty acids like alpha-linolenic acid, and eicosapentaenoic acid. There is however need for caution as the fatty acid profile differs based on insect species (AKINNAWO & KETIKU, 2000; YANG ET AL., 2006; RAKSAKANTONG ET AL., 2010).

Edible insect contains insoluble chitin, which is present in their exoskeleton. FINKE (2007) analyzed the chitin content of some commercially raised insects species; *Acheta domesticus* (nymphs and adults), *Galleria mellonella* (larvae), and *Tenebrio molitor* (beetles, larvae), and found that their chitin content ranged from 2.7–49.8 mg/kg (wet weight) and 11.6–137.2 mg/kg (dry matter basis).

The proteins in chitin fibres have strong linkage and changes in chitin according to life stage causes estimations of the amounts of chitin and non-digestible protein in insect cuticles to be variable: hard cuticles have low chitin contents of 15–30%, whereas soft cuticles contain approximately 50% of chitin (NOGALES-MERIDA ET AL., 2019). Chitin is a controversial component of insect protein some studies have shown that it plays a role in the immune modulation of animals that consume feed with insects as a protein source protecting them against some parasitic infections.

However other studies have reported negative effects such as poor nutrient absorption hence growth reduction (MOLDAL ET AL., 2014), this is related to the effect of the number of amino acids (AAs) bound to chitin or scleroprotein on the digestibility of proteins/ (AAs) (ZAKI ET AL., 2015; NOGALES-MERIDA ET AL., 2019).

In sea bass fed with different levels of chitin (0.5, 1, 2, 3, and 4 g/kg) improved growth and feed efficiency performance was observed at 0.5 g/kg, 1 g/kg, and 2 g/kg compared to the control diet with no chitin inclusion after a 75-day experiment (NOGALES-MERIDA ET AL., 2019).

Despite the report of gastrointestinal bacteria and chitinase activity in some fish species, chitin digestibility is still very low or absent. Species reported with high chitinase activity include Cod (*Gadus morhua*), Yellowtail kingfish (*Seriola lalandi*), Japanese eel (*Anguilla japonica*), Cobia (*Rachycentron canadum*), and Red seabream (*Pagrus major*). However, to improve the utilization of chitin as a nutrient, chitin-degrading enzymes or bacteria could be integrated into diets (KROECKEL ET AL., 2012).

2.2.2. Edible insect farming

The process of mass production of insects is capital-intensive making the price of insect and insect products similar to meat. For example, the cost of feed used in commercial production of house cricket is between 0.17 to 2.04 €/kg while the market price per Kg of cricket is between 24–48 €/kg. This makes it look like commercial insect producers are making a lot of gains but in fact, the other cost of production like manual labor facilities and other resources reduces the profit margin.

The development of rearing, harvest, post-harvest processing technologies, and safety and quality monitoring for automated insect production will make large-scale commercial production of insects more attractive and competitive compared to meat. This will reduce its production costs, ensure food, and feed safety (RUMPOLD AND SCHLUTER, 2013). An up-scaled production in terms of automation and control in the production system is required to reduce manual labour and this is a challenge that insect farmers are faced with. Upscaling will result in improved competitiveness in the price of insects, this can be made possible as insect producers are attracting investors and have raised €350 million in 2018, and €600 in 2019, and it is expected to reach €2.5 billion by 2025 (IPIFF, 2018).

2.2.3. Safety concerns

There are food and feed safety concerns in the use of insects which includes; microbial risks, parasitological risks, and chemical hazards. When insects are produced as farm animals meant for feed, these feeds and safety concerns pose a risk to the health and welfare of humans and animals that consume them (BELLUCO ET AL., 2015, BELLUCO ET AL., 2015, VAN ET AL., 2018).

Information regarding the microbiology of insects and their potential microbial risk are mainly described in studies that consider insects as pests rather than food animals. These insects were investigated for their potentials to act as vectors of foodborne pathogens under farming conditions.

Hence, the value of this data is limited in the context of insects as farm animals fit for human consumption or feed production; however, they do provide some qualitative information. Escherichia coli O157:H7 proliferates in houseflies for at least 3 days after ingestion according to the studies of KOBAYASHI ET AL. (1999), suggesting a potential dissemination mechanism (BELLUCO ET AL., 2015).

Microbial risks that could be associated with fresh insects are greatly reduced or eliminated during heat treatment (RUMPOLD AND SCHLUTER, 2013). There has been no observation of specific prionic diseases in insects because of the lack of a PrP-encoding gene.

However, insects could act as mechanical vectors of prions gotten from at-risk substrates of ruminant origin with potential concerns for humans or susceptible animals according to the destination of insect-based products (EFSA, 2015; VAN ET AL., 2018). Arboviruses are a particular group of viruses that require investigation in insects as they cause human diseases and can replicate in insects. Other human viruses, which are taxonomically related to insects, are unable to replicate in insects (VAN ET AL., 2018). Some viruses associated with insects are Parvoviridae in European house cricket, Dicistroviridae, Iridoviridae, and Nudiviridae in field cricket, Hytrosaviridae, and Reoviridae in Housefly and Iridoviridae in Yellow mealworm but they are mainly an issue in rearing system causing loss to the farmer (MACIEL-VERGARA & ROS, 2017).

Chemical contaminants that are present in food and feed originally derived from insects occur as a result of the presence of such contaminants in the environment and/or the substrate as such the chemicals of concern vary. Chemical contaminants include but are not limited to, heavy metals, veterinary drug residues, organohalogen compounds, and pesticide residues.

There are no indications that insects being considered for use as food or feed in Europe produce reactive, irritating, or toxic substances in the life stages being considered for consumption. In general, insects harbor a wide variety of microorganisms, and some pathogenic bacteria may be present. (VAN ET AL., 2018).

2.2.3.1. Viral safety concerns in Insects

The role of natural selection of a virus is unpredictable causing widespread speculations about the direction of the evolution of a virus especially during an outbreak. The widespread claim that a virus will mutate to become more virulent while it can become less virulent is illustrative of this phenomenon. The evolution of the virus is more complex and has garnered extensive research. A mutation is a prerequisite for the spillover of a virus from an animal reservoir to a human or alternate arthropod vector for transmission (GRUBAUGH ET AL., 2020). Iridoviruses and circoviruses are viruses that have been reported in several hosts and they are continually being detected in new hosts.

Iridoviruses

"Irido" is from the Greek Word Iris, which is the name of a Greek Goddess, whose essence was rainbow. Infected insect with high viral load has a rainbow-like iridescence due to the presence of matured virons collected in a huge paracrystalline arrangement in infected cells' cytoplasm. There are two subfamilies of Iridoviridae namely *Alphairidovirinae* (genera; *Ranavirus, Megalocytivirus,* and *Lymphocystivirus* primarily infecting cold-blooded vertebrates and *Betairidovirinae,* (genera; *Iridovirus, Chloriridovirus,* and *Decapodiridovirus* largely infecting invertebrates like insects and crustaceans).

In the mid-1950s, the first invertebrate iridovirus was reported in insects after which they were then identified in many other invertebrates, with only a few reported in mollusks, annelids, and nematode (PAPP AND MARSCHANG, 2019).

While most invertebrate iridoviruses (IIVs) have been found in poikilothermic vertebrates such as reptiles and amphibians, there have been more detected in invertebrates. Because invertebrate and vertebrate iridoviruses differ in host range and molecular characteristics, different molecular studies and bioassays have been used for the characterization and comparison of IIVs, as well as to assess their ability to infect vertebrates (PAPP AND MARSCHANG, 2019).

Chloriridovirus IIV-6's host range in insects is over 100 species that can be categorized under six orders; *Coleoptera, Diptera, Hemiptera, Hymenoptera, Lepidoptera,* and *Orthoptera,* according to WILLIAMS, 2008. A suggestion from reports is that *Chloriridovirus* IIV-31 is naturally able to infect several *Crustacea* and *Drosophilidae* (PAPP AND MARSCHANG, 2019).

Particles similar to IIVs have also been detected in marine invertebrates but the genomic information collected was not enough for classification. In an Infected host, the luminous blue color is the easily recognised sign of infection, this is due to the crystalline arrays of particles in majority of the cells. Patent IIV infections can be lethal, but covert infections are more common and may influence negatively the reproduction and longevity of infected insects and arthropods (INCE ET AL., 2018).

Circoviruses

Circoviruses are non-enveloped viruses with a covalently closed single-stranded DNA genome in an icosahedral capsid, with a genome size of about 2000 nt (TUBOLY, 2016; MANKERTZ, 2008). Circoviruses are the smallest viruses that can self-replicate in cells of eukaryotes (MANKERTZ, 2008). After the discovery of the first porcine circovirus (PCV), several other similar viruses have been discovered leading to the establishment of the family *Circoviridae* (TUBOLY, 2016). By 2006, ten circoviruses have been characterised; two of them were porcine circovirus that infects pigs, while others have been isolated from avian species. Interestingly, all circoviruses trigger diseases except PCV and can reduce the efficiency of the immune system and impact negatively on development. Interest in molecular-virological research of Circoviruses is due to their limited coding capacity, and high dependence on the host (MANKERTZ, 2008).

Since the identification of the first circovirus of porcine origin, the circoviruses described were avian species; not counting the pathogenic porcine variant (PCV2), (ALLAN, 1998) and canine circovirus.

The discovery of the first fish circovirus was in 2010 (LŐRINCZ ET AL., 2011), and this increased the number of hosts. The pathogenicity of fish circoviruses has not been established, but the pathogenicity of avian and porcine circoviruses can be extrapolated (TUBOLY, 2016), although the report from LŐRINCZ ET AL. (2011) was from fish stock with high mortality, the relationship between the virus and clinical signs could not be established. The mode of replication is the rolling circle replication model (MANKERTZ ET AL., 1998; CHEUNG, 2004), aided by circular, double-stranded (ds) replicative DNA intermediates.

Genomes of Circoviruses are ambisense and they contain two major genes *rep* and *cap* except for gyroviruses' negative-sense genomic arrangement, which encodes the replication-associated (Rep) and the capsid (Cap) proteins.

Reps function is the replication of the virus while Cap is responsible for block building of the capsid of the virions, in addition, they are the main targets of antigens for immune response.

CHEUNG (2003) identified a complex replication process for PCVs where 9–12 rep transcripts are generated, and a similar strategy is likely followed by other circoviruses.

Although there has been a report of PCV2 infection of other species, circoviruses are generally considered to be host-specific indicating they have a narrow host range. The virus’s transmission is through the fecal-oral route, but vertical transmission has also been known to occur.

The genome design is simple and because of this, the virus relies on the infected cell's replication machinery and must also be able to withstand the host's humoral and cellular immune responses, which primarily target the Cap protein. Circoviruses multiply in dividing cells, therefore young animals are more susceptible to infection. Because the pathogenesis of circoviral illnesses and the general outcome of infection are quite similar in birds and pigs, the consequence of infection in fish may be comparable as well. PCVs, for example, are involved in a wide range of disorders, most commonly known as porcine circovirus-associated diseases (PCVD), which appear in a variety of clinical settings (SEGALÉS, 2012), depending on the animal's age, viral load, and factors that affect the infected pig's immune system. It is common knowledge that cofactors are required for the formation of a PCVD.

When the diseases occur, they occur concurrently with secondary infections that are opportune by the reduced functionality of the immune system (ALLAN, 1998; ALLAN AND ELLIS, 2000; TODD, 2004; TUBOLY, 2016).

2.2.4. European Union regulation on the use insect as feedstuff

The first legislative breakthrough in the European insect industry was the approval of seven insect proteins for use in pet and aqua feed under Regulation (EU) 2017/893. This regulation is considered a "pioneering piece of legislation" because it established EU standards that are explicitly applicable to insect production for the first time.

In the EU Regulation on Transmissible Spongiform Encephalopathies, also known as the "TSE Legislation" - Annex IV section F of Regulation 999/2001, EU Regulation 2017/893 added a section for insects and insect products. This allows insect producers to obtain the same authorization as producers and processors of proteins derived from other non-ruminant animals, such as pigs and poultry, for feeding pets and aquaculture animals.

The criteria for using the seven approved insects is that they must be processed in facilities that have been approved for this purpose.

This is to ensure that producers adequately manage the potential microbial risks associated with these products. Clearance is given if the processing procedures outlined in the EU's 'animal by-products regulation,' i.e., Regulation 142/2011, are implemented.

The seven approved insect species are the black soldier fly, house fly, yellow mealworm (YM), lesser mealworm, house cricket, banded cricket, and field cricket.

Recently, COMMISSION REGULATION (EU) 2021/1372 of 17 August 2021 amended Annex IV to Regulation (EC) No 999/2001 of the European Parliament and of the Council. This amendment allows for the use of insect and insect-derived protein in the feeding of poultry and pigs, lifting the previous prohibition on feeding non-ruminant farmed animals, other than fur animals, with protein derived from animals.

2.2.5. Edible insect and the feed industry

Rising pricing and concerns about the sustainability of soybean and fishmeal, two of the most important protein sources for animal feed, are among the issues driving interest in alternate protein sources for animals. Insects can make a good source of high-protein animal feed (VAN HUIS ET AL., 2013). Insect meal, fishmeal, and soybean meal all have similar amino acid compositions, according to studies (BARROSO ET AL., 2014; VAN HUIS AND OONINCX, 2017). Furthermore, the environmental impact of producing and trading raw materials for conventional animal feed is far greater than that of insects, which can be a local, sustainable, and on-demand product (VAN HUIS AND OONINCX, 2017). Insect-based feed, on the other hand, can be more expensive than traditional feed from an economic standpoint (ARRU ET AL., 2019). Various studies offer various viewpoints on this (JOLY AND NIKIEMA, 2019). *Dipteran* (black soldier fly and house fly larvae) insect meal has a value at least twice that of soybean, but less than fishmeal, according to PROteINSECT (2016). PINOTTI ET AL. (2019), suggested that the pricing for insect-based feedstuff could be even higher, with the smaller mealworm (*Alphitobius diaperinus*) and black soldier fly (*Hermetia illucens*) costing six and nine times more per unit of protein, respectively than soybean-based feed.

The cost price of insect meal is predicted to fall as production volumes increase and the feed manufacturing chain becomes more optimized (ALL ABOUT FEED, 2016).

In 2018, the global market of insects as feed sources was worth USD 688 million, and it is predicted to grow to USD 1.4 billion by 2024. In 2018, the aquaculture industry accounted for more than half of the global insect feed market (GLOBE NEWSWIRE, 2020).

2.2.5.1. The role of insectmeal in the feed industry

Grain consumption in animal diets has become necessary due to the expansion of livestock production in recent years. The indirect production of human food (meat, eggs, and dairy products) using 35% of the world's crop production for animal feed makes this process less efficient. Systems for raising cattle demand a lot of land, energy, and water, and they also damage the soil and groundwater and produce a lot of greenhouse gases (GHG). According to calculations, the water needed by Brazilian broiler farms between 2000 and 2010 accounted for 99% of all water used for the indirect production of grain. The direct water use for chicken production was found to be less than 0.32% of the total water demand. (ALLEGRETTI ET AL., 2018).

Insects are a potential protein source in poultry (MAURER ET AL., 2016; BOVERA ET AL., 2016; ALLEGRETTI ET AL., 2018; KIEROŃCZYK ET AL., 2018); swine (JIN ET AL., 2016), fish (BRUNI ET AL., 2018; DIETZ & LIEBERT, 2018; FENG ET AL., 2019; HENRY ET AL., 2018; STENBERG ET AL., 2019; VARGAS-ABÚNDEZA ET AL., 2019; WANG ET AL., 2019), shrimp (PANINI ET AL., 2017) and companion animal nutrition (BOSCH ET AL., 2014). The crude protein (CP) content of insects is species-dependent and varies from 40% to 60% (MAKKAR ET AL., 2014). The CP quality can also be influenced by the production technology and feed composition for larval rearing. In the European Union, the usage of insect meal in livestock nutrition was banned by (Regulation (EC) No. 1069/2009) because these compounds are considered to be processed animal protein (PAP, Regulation (EC) No. 999/2001). The EU Standing Committee on Plants, Animal, Food, and Feed (SCoPaFF) amended the section banning the use of PAP in livestock nutrition in regulation EC 2017/893 allowing the use of insects as a protein source in the case of fish, mink, and pet-food nutrition.

Birds were fed soybean-maize diets developed by replacing 50g/kg of the basal diet with various fats i.e., soybean oil (SO) and *Tenebrio molitor* oil (Exp. 1), or Soybean oil, *Tenebrio molitor* oil, and *Zophobas morio* oil (Exp. 2). Overall, these results highlight the possibility of completely replacing soybean oil with *Tenebrio molitor* oil and *Zophobas morio* oil in broiler diets, without adverse influences on growth performance and nutrient digestibility.

Moreover, the results of the study suggest that *Tenebrio molitor* oil positively affects meat quality, which is a key factor for the modern consumer (KIEROŃCZYK ET AL., 2018).

In particular, *Hermetia illucens* or the Black Soldier Fly larvae (BSFL) belonging to the *Diptera* order is being contemplated as a promising substitute to replace the conventional protein sources to an extent.

Industrial rearing, processing, and valorization of BSFL present its challenges, yet their nature to aggregate the micro, and macro-nutrients (BARROSO ET AL., 2017; BARROSO ET AL., 2019) present in the feeding substrate thereby giving proteins, lipids, chitin derivatives, and bioactive peptides, and organic manure makes them an appealing candidate for a variety of uses (RAVI ET AL., 2019).

2.2.5.2. Edible insect in aquaculture industry

Fish meal (FM) has traditionally been the principal source of protein for the formulation of commercial aquatic feeds. (HENRY ET AL., 2015). The supply of FM is declining while prices are rising. These elements have sparked the quest for a sustainable substitute (WANG ET AL., 2019). In 2014, the production of FM and fish oil accounted for around 10% of all fish produced worldwide (including wild and farmed). Little marine fish that are captured in the wild and are typically judged unfit for direct human eating because they are high in bones and oil are used to make FM. FM is an extensively used, high-quality feed ingredient for pigs, poultry, and aquaculture. Yet it's becoming increasingly scarce and more expensive. Between 1988 and 2010, the poultry sector reduced its usage of fishmeal from 60% to 12%, while the aquaculture sector expanded its use of fishmeal from 10% to 56%. Lower fishmeal inclusion percentages in aquafeed have been driven by rising fishmeal prices, although this has not had a significant impact on fishmeal utilisation because of the aquaculture industry's explosive growth (VAN HIUS AND OONINCX 2017).

In the aquaculture industry, using insect meal rather than fishmeal is becoming more and more popular. Being the main source of nutritional protein, fishmeal is neither environmentally friendly nor sustainable. It is also getting more expensive.

Emerging problems including the increased demand for fish protein on a global scale, its scarcity, its high cost, and its negative effects on the ecology of fishing grounds have drawn and sustained emphasis on the need for substitute dietary protein sources (FAO, 2016, 2014).

Animal and non-animal protein from legumes and/or oil seeds or cereal gluten are now used as substitutes (FAO, 2014, TRAN ET AL., 2015). Regrettably, plant protein derivatives rarely have a balanced essential amino acid (EAA) profile, frequently have antinutritive elements, have a low level of palatability, and have significant levels of fibre and non-starch polysaccharides. Although chemical and mechanical processing can help to some extent to mitigate some of these limitations (SANCHEZ-MUROS ET AL., 2014; OLIVA-TELES ET AL., 2015; TRAN ET AL., 2015).

PAPs are regarded as a valuable substitute because it has a better EAA profile and is easier to digest than plant proteins; however, within the European Community, restrictions on the use of some processed animal proteins still exist as a preventive measure against the spread of spongiform encephalopathies (Regulation 68/2013/EC, 2013) (BRUNI ET AL., 2018).

Besides the earlier mentioned shortcoming of Plant feedstuffs, there is also the absence of certain FM components (i.e. taurine and hydroxyproline) leading to the potential problems of poor growth performance, intestinal inflammation, and decreased palatability. The value of insect protein as partial or complete replacements for FM has been studied before the new millennium (WANG ET AL., 2019). The European Commission first approved the use of processed insect protein in aquafeeds in 2017 (WANG ET AL., 2019; BRUNI ET AL., 2018). The EU Commission regulation (2017/893-24/05/2017) authorized the use of seven insects (2 flies, 2 mealworms, and 3 cricket species) in aquafeeds, this will further motivate the intensification of their production (HENRY ET AL., 2018).

Insects should be investigated as a potential ingredient for aquafeeds due to their negligible environmental impact when compared to the majority of traditional feed commodities. They are raised using environmentally sustainable, cost-effective farming techniques since they may be produced from waste or byproducts. They have a high lipid content in addition to being rich in proteins with an essential amino acid makeup comparable to FM. (VARGAS-ABÚNDEZ ET AL., 2019).

To increasing profitability and sustainability in finfish production, fishmeal (FM) and fish oil replacement in aquafeeds has already drawn a lot of attention. However, studies have concentrated on goods like oilseeds (particularly soybeans), meat byproducts (such as blood meal and bone meal), and microbial proteins. The complete replacement of fish oil and FM in feeds for finfish aquaculture have several downsides. Vegetable proteins, particularly for carnivorous fish, have an improper amino-acid balance, low protein digestion, and anti-nutritional factors. Necessitating research into the inclusion of other highly nutritious supplements such as microalgae and/or meat by-products. It's crucial to remember that these ingredients don't always adhere to the expected ecological, dietary, and financial requirements. Therefore, alternatives that ensure fish health and welfare standards for both finfish and ornamental aquaculture could be beneficial by offering appropriate feeding stimulants, proper levels of essential amino acids and polyunsaturated fatty acids (PUFAs), high nutrient and energy bioavailability, as well as reduced anti-nutritional factors. In the aquaculture industry of several countries, using insect meal instead of fishmeal is becoming more prevalent. (FAO, 2014).

Processed animal protein is considered a valuable alternative as it has a better EAA profile and is more digestible than plant proteins; however, there are restrictions on the use of certain processed animal proteins within the Europe Community, this serves to protect against transmissible spongiform encephalopathies (Regulation 68/2013/EC, 2013) (BRUNI ET AL., 2018). The increasing attention attracted recently by insects as a sustainable nutrient source for feed is not only in Europe but also around the world. Insects are a good source of EAA, lipids, vitamins, and minerals (HENRY ET AL., 2015; VAN HUIS ET AL., 2013). They grow and reproduce quickly and easily on low-quality organic waste and manure (VAN HUIS ET AL., 2013); they have a small ecological footprint and high feed conversion efficiency (MAKKAR ET AL., 2014), and can reasonably foster a circular bioeconomy (BRUNI ET AL., 2018).

According to PANINI ET AL. (2017), weight gain, specific growth rate, feed intake, feed conversion, survival, and protein retention of pacific white shrimp were not affected by replacing FM with mealworm meal (MM) at 0%, 25%, 50%, and 100%. There were no significant differences in protein content between the treatments. However, the moisture content showed a linear decrease with an increase in the fishmeal replacement level while the lipid content increased with MM inclusion level.

However, certain insect species might also serve as alternative protein sources without these drawbacks in plant protein sources, in particular, the black soldier fly *Hermetia illucens* (L.) (*Diptera*: *Stratiomyidae*). Studies conducted with Atlantic salmon in a 500L fiberglass tank showed that fishmeal could be completely replaced (i.e. 100% insect meal) in their diet without adverse effects on the net growth of the fish, histology, odor, flavor/taste, and texture. (LOCK ET AL., 2016). The study of GEBREMICHAEL ET AL. (2021) shows that replacing 50% and 100% fishmeal with BSFL meal had no adverse effect on weight gain (WG), specific growth rate (SGR), survival rate (SR), feed conversion ratio (FCR), protein efficiency ratio (PER), condition factor (K), relative gut length (RGL), and hepatosomatic index (HSI) during an 8weeks long experiment. The feed was formulated to have between 38-39% CP. Similarly, a meal made from the black soldier fly is a suitable protein source for a number of other farmed fish species, such as African catfish *Clarias gariepinus* (ADENIYI AND FOLORUNSHO, 2015), Channel catfish *Ictalurus punctatus*, and Blue tilapia *Oreochromis aureus* (BONDARI AND SHEPPARD, 1987). Another insect species, the Yellow mealworm (*Tenebrio molitor* L.; Coleoptera: *Tenebrionidae*), has also been evaluated. Yellow mealworm meal could partially (35%) replace fishmeal in the diet of European sea bass (*Dicentrarchus labrax*) juveniles without affecting mortality or growth (GASCO ET AL., 2016). However, replacing 70% of the fishmeal did depress growth.

A similar trial was conducted with Rainbow trout (*Oncorhynchus mykiss*) where 25% and 50% fishmeal were replaced by yellow mealworm in the experimental diet. The study found that weight gain was not affected at higher inclusion levels of yellow mealworm meal, while the protein content increased and lipid contents of fillets decreased, compared to the control (BELFORTI ET AL., 2015; VAN HUIS AND OONINCX, 2017).

The presence of chitin, which is likely to cause a general decrease in the digestibility of the diet, is one of the main issues associated with the use of insect meal in aquafeeds. Yet, due to conflicting findings, the effects of chitin in fish feeds are still not completely understood.

Although there have been reports of unfavourable effects, such as possible intestinal inflammation and a decreased ability to digest and assimilate nutrients, moderate inclusion of chitin has been shown to increase fish immune response and positively modulate the microbiota in other studies (VARGAS-ABÚNDEZA ET AL., 2019).

However, more current research indicates no evidence of severe intestinal inflammation by the histological analyses in any of the samples examined, apart from a reduction in intestinal fold length that was only shown in fish (clownfish juvenile: *Amphiprion ocellaris*) fed diets that included 50% and 75% of *Hermetia illucens* meal. (VARGAS-ABÚNDEZA ET AL., 2019). Shortening of intestinal folds has previously been associated with impaired nutrient absorption translating to growth reduction (MOLDAL ET AL., 2014); however, in this study, this was not observed and the GR and Heath Shock Protein 70kDa (HSP70) molecular markers involved in stress response showed no difference among groups fed insect meal and control group. These markers can be used to identify stress; inflammation, physiological reactions, and the lack of a significant difference across groups indicate fish welfare in general. The absence of intestinal inflammation may be due to the insect's fatty acid content (VARGAS-ABÚNDEZA ET AL., 2019).

Defatted Black soldier fly larvae meal (DBSFLM) has been shown as a promising FM substitute in diets for Turbot, Rainbow trout, Jian carp, Pacific white shrimp, and Atlantic salmon. WANG ET AL., 2019 examined DBSFLM as an alternative protein source in juvenile Japanese seabass (*Lateolabrax japonicus*) diets.

Five diets were formulated by replacing 0% (FM), 16%, 32%, 48%, and 64% fishmeal in a 56days feeding trial. Results showed that growth performance, somatic indexes, hepatic and intestinal histomorphology, and the intestinal antioxidant and immunity indexes of fish were not affected by dietary treatments. At 48% and 64% insect meal inclusion, there was higher feed intake (9.5% and 19% respectively), but lower whole-body ash content (6.3% and 7.6% respectively) and ash retention (14.7% and 30.3% respectively) than those fed 100% fishmeal.

At 48% and 64% insect meal inclusion lower serum concentrations of total cholesterol (10.8% and 12.5% respectively), triacylglycerol (10.9% and 10.9% respectively), high-density lipoprotein cholesterol (15.4% and 19.2% respectively), and malondialdehyde (31.1% and 34.5% respectively) were observed compared to those fed 100% fishmeal. The inclusion of insect meal did not alter activities of hepatic trypsin, lipase, and amylase, but increased the activity of intestinal lipase for fish fed 48% and 64% insect meal by 31.3% and 29.5% respectively than those fed 100% fishmeal (WANG ET AL., 2019).

To determine the effects of *Tenebrio molitor* protein on Macrobrachium rosenbergii growth performance, immunological parameters, and resistance against Lactococcus garvieae and Aeromonas hydrophila, four test diets formulated with 4%, 8%, 12%, and 16% *Tenebrio molitor* protein were compared with a control (0% *Tenebrio molitor* protein). Following a 10-week experiment, researchers discovered that the 12% *Tenebrio molitor* protein significantly outperformed the control, 4%, and 8% *Tenebrio molitor* protein in terms of weight gain (WG), percentage of weight gain (PWG), daily growth rate (DGR), specific growth rate (SGR), and protein efficiency ratio (PER), but was not statistically different from the 16% *Tenebrio molitor* powder. The survival rate (SR), feed conversion ratio (FCR), and condition factor (K) did not substantially differ across groups. As *Tenebrio molitor* protein quantities grew, lipid content in the prawn carcass and muscle reduced while protein content increased. (FENG, ET AL., 2019).

Insect meal (IM) produced from Black soldier fly larvae was used to replace IM in the diet of Seawater Atlantic salmon. Three different diets were formulated and used in the feeding trial that lasted for 8 weeks; a control diet (IM0, protein from fishmeal and plant-based ingredients (25:75) and lipid from fish oil and vegetable oil (33:66); and two insect-meal containing diets, IM66 and IM100, where 66% and 100% of the fishmeal protein was replaced with IM, respectively.

There were no significant effects of IM inclusion on final weight or any of the growth or feed intake parameters (SGR, FCR, feed intake (FI), hepatic somatic index (HSI) and visceral somatic index (VSI)) (STENBERG, ET AL., 2019).

Partly defatted *Hermetia* meal could replace soy protein up to 50% in Nile Tilapia, as shown in the result of a 56 day experiment where soybean protein was replaced by 25%, 50%, and 100% defatted *Hermetia* meal. Above 50% inclusion level showed a tendency to impair growth (DIETZ & LIEBERT, 2018).

European sea bass, *Dicentrarchus labrax*, fed with MM for 6 weeks showed significant anti-inflammatory responses (ceruloplasmin, myeloperoxidase, and nitric oxide).

Incorporation of insect larvae into the fish diet is a relatively new research area and to our knowledge, so far there has only been a few studies on the effect of insect meal on the immune system and antioxidant enzymes of the fish.

When Black carp (*Mylopharyngodon piceus*) fed with low doses (2.5%) of maggot (*Musca domestica*) for 60 days, showed increased serum lysozyme, serum complement, and liver superoxide dismutase and catalase activities and reduced liver Malondialdehyde suggesting an increased antibacterial activity and an antioxidant activity of the insect meal at a low dietary dose (MING ET AL., 2013).

The insect meal also protected these fish against a bacterial challenge with *Aeromonas hydrophila* (MING ET AL., 2013).

A similar study in Red seabream *(Pagrus major)* showed that the introduction of low doses of 0.75 and 7.5% of Housefly (*Musca domestica*) pupae in the diet of red sea bream for 10 days showed a significant increase of the phagocytic activity in peritoneal macrophages (IDO ET AL., 2015).

Interestingly, 5% of dietary housefly pupae for 2 months did protect (100% survival) the fish against the bacterial pathogen *Edwardsiella tarda* while all control fish died 12 days after the bacterial challenge (IDO ET AL., 2015). The protective effect of dietary insects was suggested to be either directly through the secretion of antimicrobial peptides by the insect or indirectly through the stimulation of the fish immune system by chitin (ESTEBAN ET AL., 2001; ESTEBAN ET AL., 2000; LEE ET AL., 2008) or by other insect components (IDO ET AL., 2015).

The first hypothesis involving the secretion of antimicrobial peptides by the insects seemed however unlikely as dead insects do not secrete any antimicrobial peptides. The indirect effect through the immune stimulation of the fish was, therefore, more likely (HENRY ET AL, 2018).

2.2.6. Yellow mealworm (*Tenebrio molitor*)

The yellow mealworm larvae (YM) (*Tenebrio molitor* L., *Coleoptera, Tenebrionidae*) have the potential for industrial food and feed production. Yellow mealworm is one of the most reared and traded insect species in Europe, they can convert substrates originating from the agricultural, baking, and brewing industries. Their ability to convert substrates to protein mass increases economic profit and reduces environmental pollution by reducing the quantity of waste generated (BORIDEAN ET AL., 2020b). Mandibulate insects have been observed to consume and ingest plastic bags and this has prompted research into plastic biodegradation by insects.

It has also raised the possibility that gut microbes of insects could serve as a potential source for screening plastic-degrading bacteria. Certain insects and their gut microbes have been tested and confirmed to have the ability to degrade polystyrene and polyethylene, the yellow mealworm is one of these insects (LOU ET AL., 2021).

Insects can tolerate temperature fluctuations, while the optimal rearing temperature for YM is 28 °C but they easily survive 15 °C for 48 h. However, they cannot tolerate high humidity, they die very quickly when exposed to humidity conditions higher than 70%. Yellow mealworm larvae are the most widely reared for human food in Europe. Mealworm is one of the largest beetles (cca 15 mm long) that infest stored food products.

It damages the total mass and nutritive value of plant products by feeding on them and contaminating them with exudates, excrements, body parts, and whole dead insects. Mealworms are commonly stored product pests, particularly in mills, facilities for storing plant products, grocery shops, and warehouses. They are easy to rear, highly nutritious, and commonly used as feed for pets and some other exotic animals to which they are usually served live, canned dried, or lyophilized (JAJIC ET AL., 2019).

Yellow mealworm undergoes four life stages: egg, larva, pupa, and adult (*Figure 3*). Larvae measure about 2.5 cm, whereas adults have a 1.25–1.8 cm length. The optimum breeding conditions include temperatures between 25–35 °C and soft ground where they can lay their eggs. The beetles are found in decaying and rotting bark of deciduous trees. The larvae and adult beetles feed on dead organisms and cereal products in pantries, poultry farms, and dovecotes (BOZEK ET AL 2017).

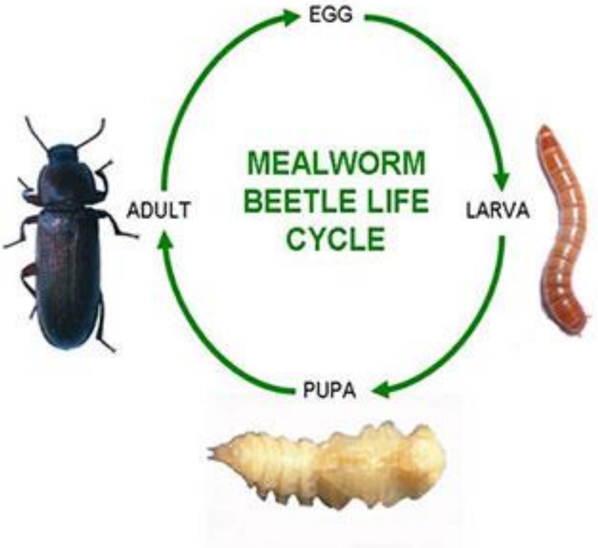


Figure 3: Yellow mealworm life cycle (https://www.breedinginsects.com/yellow-mealworm-life-cycle/)

Yellow mealworm is one of the most promising insect species suitable for mass production because it is easy to breed and feed. Mealworm larvae fed on plant by-product diets grow well and have a short life cycle: the egg stage lasts 3 to 9 days, the larval stage lasts 26 to 76 days, and the pupal stage lasts 5 to 17 days (REMA ET AL., 2019). It is commonly produced on mixed grain diets. Its mass production for human and livestock feed use has been well documented (CHOI ET AL., 2018).

The adult dark beetles lay eggs, small larvae hatch from eggs and the larval stage commences, the larva becomes a pupa before it becomes an adult beetle. The whole process takes place in the same ecosystem. Environmental conditions such as diet, relative humidity, temperature, and population density are the main factors contributing to the dark beetle mealworm's developmental stages.

T. molitor is a species in the family *Tenebrionidae*, usually referred to as darkling beetles. The adult darkling beetle female lays an average of 400-500 eggs in a substrate (SPENCER AND SPENCER, 2006). The young adult will begin oviposition after emerging from the pupa stage in just 3 days (MANOJLOVIC, 1987). Females produce a 4-methyl-1-nonanol pheromone to attract males during the mating phase (TANAKA ET AL., 1986).

Females are also attracted to odours produced by outbred males, according to PÖLKKI ET AL. (2012), indicating that inbreeding decreases the desirability of sexual signaling. The adult stage is recorded to last from 16 to 173 days developing as white beetles and gradually darkening.

When beetles are exposed at 30 °C compared to 20 °C or 25 °C respectively, the length of adult life is shorter (FIORE, 1960; SELALEDI ET AL., 2020). Young beetle parents' offspring have been reported to have a longer adult life than adult beetle parents' offspring (TRACEY, 1958; SELALEDI ET AL., 2020).

Before eventually turning into a pupa, worms go through around 15-23 instars or molts. They are susceptible to dehydration after each molt, so it is necessary to maintain adequate moisture and wet food. Often, they are more vulnerable to higher temperatures. From the eggs, the tiny larvae emerge, and the larval stage begins. According to PARK ET AL. (2014), the larval stage of the mealworm goes through instar stages where a new skin replaces the old skin; the process will take 17 instars before a pupa comes. With each successive instar, the body length gradually increases during this process. However, the length of the body after the 17th instar begins to decrease (PARK ET AL., 2014). The larval length can be between 12 and 32 mm (HILL, 2002). The larvae of the first instar of the yellow mealworm are white; after the second instar, it gradually becomes brown.

The larvae begin a short period of inactivity after the larval stage, acquiring a "C" shape before transforming into a pupa, in a process called metamorphosis (SELALEDI ET AL., 2020). A larva provides chickens and other captive animals with a decent source of protein (BIASATO ET AL.2016; SELALEDI ET AL., 2020). Compared to larvae from old parents, larvae from young parents develop faster (FIORE, 1960; SELALEDI ET AL., 2020).

The pupa has no mouth or anus, so it does not eat; its helpless body is constantly turned around. The length of the pupal stage ranges from 6 to 20 days before a beetle becomes (KIM ET AL., 2015; COTTON, 1927; SELALEDI ET AL., 2020).

The period of the pupal stage is therefore not determined by the parental age of the mealworm beetle (SELALEDI ET AL., 2020).

Previous studies have assessed different diets for mass-producing *Tenebrio molitor*. For example, dry potato flour, dry egg white, soy protein, peanut oil, canola oil, and salmon oil were examined as nutritional supplements to the *T. molitor* diet. Results showed that a diet with increased protein and lipid content, compared to a carbohydrate-rich diet, significantly improved various biological parameters.

Building on these findings, a diet consisting of 80% wheat bran and 20% of a supplement composed of dry potato, dry egg white, and soy protein in a 17:2:1 ratio was developed to investigate the effect of larval density on food utilization efficiency. Another similar diet, composed of 80% wheat bran and 20% supplement consisting of dry potato, dry egg white, soy protein, and peanut oil in an 80:10:5:5 proportion, was used to analyze *T. molitor* instars morphometrically. However, existing information is not comprehensive, and data on the impact of individual feeding substrates as diet components for *T. molitor* are limited. Obtaining data on the growth and performance of *T. molitor* on single-component diets could enhance our understanding of its nutrition and contribute to the development of nutritionally balanced diets for mass-production. This is particularly important due to the wide range of food preferences of *T. molitor*, making it challenging to find suitable dietary components compared to other stored-product insects with narrower diets. While *T. molitor* has been associated with 51 different types of stored commodities, there is currently no comparative study evaluating a broad range of feeding substrates for potential inclusion in a mass production diet. Therefore, the objective of this study was to establish baseline information on the suitability of a diverse spectrum of feeding substrates of both plant and animal origin as oviposition and larval development substrates for *T. molitor*. Furthermore, the effect of the selected feeding substrates on the nutrient composition of *T. molitor* larvae was also investigated (RUMBOS ET AL., 2020).

2.3. European Perch (*Perca fluviatilis*)

2.3.1. Taxonomy, habitat, feeding habit.

Percids belong to the family *Percidae* (RAFINESQUE, 1815), Order *Perciformes*, and Class *Actinopterygii. Percidae* containing 11 genera, 266–275 identified species. Percids are freshwater fishes with only a few species in brackish waters. Morphological characteristics of percids include ctenoid scales, two dorsal fins, thoracically located pelvic fins, Spines on the opercula, villiform teeth, and bicuspid teeth on the maxilla (*Figure 4*).

The origin of the *Percidae* family was believed to be in Europe, but North America has more species (KESTEMONT ET AL., 2015).

There are three species of Perca; *Perca fluviatilis* Linnaeus (European perch) *Figure 4*, *Perca flavescens* Mitchill (yellow perch), and *Perca schrenki* Kessler (Balkhush perch) and they are biologically similar (TONER & ROUGEOT, 2008).

Individual percid development is important because it may influence reproductive capacity, mortality, maturation, future growth, and foraging. Potential harvest yields can also be determined by fish growth and foraging behavior. Percid growth varies significantly depending on location and sex; also, Perca species have a slower development rate and lower peak size than Sander species. Percids show sexual dimorphism in terms of growth, in favour of females before and after maturation, possibly because of females' superior growth efficiency (KESTEMONT ET AL., 2015). Food competition results in slower growth rate, weight, and size diversity in European perch compared to other Percid species. Seasonally, the pace of growth changes, with a high rate towards the end of spring, a low pace in summer, or even a full halt at the end of autumn and through winter. The total length is 20 to 35cm, with a total length of 51cm, and the average weight is 0.3 to 2kg, with a maximum weight of 5kg. Perch females grow 20% quicker than males, demonstrating a sexual growth dimorphism (PIMAKHIN ET AL., 2015).

Perch is a carnivorous species that prefers to prey on fish. Daybreak and twilight are the best times to feed. Percid species have similar ontogenetic eating habits, ranging from zooplankton through benthic prey and finally piscivory, as they get larger. Perca employs a broad-based foraging strategy.

Temperature, dissolved oxygen, light, prey availability, and harvest rates are all elements that influence diversity in growth and foraging behavior within species (KESTEMONT ET AL., 2015).

Cannibalism is prominent in this specie with onset in fingerlings (1.5 cm) (TONER & ROUGEOT, 2008).

In temperate waters, the European perch is an important predator for both commercial and recreational fisheries (POPOVA & SYTINA, 1977; YAZICIOĞLU ET AL., 2016). Consumers in Central Europe prefer European perch to other freshwater fish because of its white flesh, delicate texture, and mild flavor (CECCUZI ET AL., 2010). The protein content of perch fillets ranges from 18.5 g (wild) to 20.1 g (reared) per 100 g of edible portion. (JANKOWSKA ET AL., 2007; SKURIKHIN & TUTELIAN, 2007; PIMAKHIN ET AL., 2015).

Figure 4 : European Perch (*Perca fluviatilis*)

Photo: Abigeal Toviho (2020)

2.3.2. Importance of European perch

European perch (*Perca fluviatilis* L.) is a predatory species that feeds on invertebrates and fish. They were originally only found in temperate waters of the northern hemisphere but have now been introduced to Australia, New Zealand and South Africa. It attains maturity in 2-3 year with body length of 15-25 cm (ORBAN ET AL., 2007). Intensive culture of the carnivorous freshwater European perch (*Perca fluviatilis* L.) is becoming more popular in recirculating aquaculture systems (RAS) and is a growing segment of commercial fish farming in Europe. Despite this, productivity is minimal because it is a relatively new aquaculture species.

It is typically fed feed designed for salmonids or other marine fish species. Carnivorous animals' diets are high in protein, which is obtained from marine fishmeal (FM), which is considered ideal due to its balanced nutritional makeup. Plant protein sources, particularly soybean meal, are currently being employed in aquaculture to reduce reliance on FM and feed costs, as the cost and unsustainability of FM have increased.

Due to an imbalance in essential amino acid (EAA) content, limited feed acceptance, and the presence of anti-nutritional elements, high levels of plant protein in feeds might impair growth performance or cause fish health issues.

Process animal proteins (PAPs) such as chicken by-product meal, meat meal, and meat and bone meal are acceptable proteins for aquaculture diets, but their use is restricted by legislation. (STEJSKAL ET AL., 2020a).

Perch has been recognized as a prospective option for intensive aquaculture because of its high nutritional value, particularly beneficial fatty acids, and growing market demand. (STEJSKAL ET AL., 2020b, TRAN ET AL., 2021).

Freshwater fish accounted for 43.64 percent of global aquaculture production in 2020, with carp accounting for 25.65 percent of total production by volume (FAO, 2022). The European perch was the most harvested *Percidae* species in 2020 accounting for 60.41% of harvested *Percidae*. European perch is mostly produced in Europe, accounting for 47.99% of *Percidae* from aquaculture production after the pike perch with a total of 52.01%. The Russian Federation (17 991 t) and Finland (5 369 t) were the countries with the most European perch catches (FAO, 2022). Switzerland and France are the primary importers of European perch, with Switzerland serving as the primary consumer market. The European perch market is undersupplied (WATSON, 2008).

Perch fillets have a high protein level of 18.5 g per 100 g of the edible portion (20.1 g in cultured perch) (JANKOWSKA ET AL., 2007; SKURIKHIN AND TUTELIAN, 2007).

The main limiting feature of the commercial culture of European perch is its modest growth rate. The rate of growth and sexual development of perch varies by geographical region and is influenced by climatic conditions and food availability. Perch larvae are now obtained mostly by controlled reproduction of wild broodstock, with no opportunity for genetic selection that could increase survival and growth under culture conditions. Variations in the biological properties of perch from different geographical regions can be caused by climate and differences in available feed (PIMAKHIN ET AL., 2015).

2.3.3. European perch ongrowing

The European perch is cultured in 3 different production systems, the traditional system is the extensive polyculture system.

They are also now cultured in semi-intensive cage culture and RAS. Suitable conditions for intensive aquaculture have been investigated, this has helped to develop the intensive European perch culture in RAS for predictable production, especially in countries like France, Ireland, and Switzerland. European perch production under extensive pond monoculture in Western European takes about 3-4 years with a stocking density of 120000 fish/hectare, harvesting of ponds takes place twice per year in autumn and spring. European perch are stock with or without prey fish (such as roach, topmouth gudgeon, or small cyprinids). Stocking of European perch with prey fish has been shown to have no beneficial effect but the presence of macrophytes which have a positive effect on the main food (macroinvertebrates) of reared perch is beneficial to production. The survival rate of perch under extensive pond culture is between 12%-36% and an SGR of 1.3%/day. Under extensive polyculture systems, they are mostly rear in culture systems with cyprinids species such as common carp, and Chinese carps (bighead carp or grass carp). Only 0.25-1% of European perch is stocked while the remaining 99% are cyprinids because they help regulate carp production in ponds ((KRATOCHVÍL 2012; BLÁHA ET AL. 2013; POLICAR ET AL., 2015).

The ongrowing of European perch in the semi-intensive cage or pond culture is affected by suboptimal ambient temperature leading to low growth and survival rate, and bacterial infection. Because of these drawbacks, this production system is usually not used for European perch production.

The use of semi-intensive pond and RAS culture have been employed in the culture of the European perch, the larval and juvenile were reared up to 1.2-3 g in pond system and the ongrowing phase is completed in the RAS. RAS provides optimal conditions for growth, a shorter production cycle, and a better survival rate of the European perch.

The optimum temperature for European perch culture is between 22-24°C, when the temperature is maintained at the optimal it reaches a market size of 130-150 g in 14 months including the larval rearing phase. When 0.5 g of European perch is stocked and maintained at 23°C market size of 100 g can be reached in 9 months.

Optimal light conditions for rearing of the European perch are 12L:12D OR 18L:8D light regime, 200-1100 1x light intensity in white, grey, or black tanks. Salinity, ammonia, and nitrite concentrations should be maintained at >4%, >0.3 mg/l, and >0.5 mg/l respectively.

Stocking density of 10-20 kg/m^3 for 10 g fish and 60-70 kg/m^3 for 150 g fish (KESTEMONT & MÉLARD 2000; STEJSKAL ET AL., 2010; ÖBERG 2012; POLICAR ET AL., 2015).

2.4. Digestive enzymes and enzyme activity in carnivore fishes

The European perch is sensitive to environmental conditions especially temperature such that even a 1˚C change in temperature can affect their feeding or even make them cease feeding. Fishes are ectotherm in nature and their environmental temperature determines their body temperature, the temperature of fish determines the rate of all biochemical processes hence physiological processes. Temperature is so important that it is termed the abiotic ecological master factor. At temperatures outside the optimal, nutrient digestibility is reduced and this can be partly attributed to the effect of temperature on the digestive enzyme activity (VOLKOFF & RØNNESTAD, 2020)

They are also sensitive to stress from handling, and stocking density, which can also affect their feed intake and growth (ZARE ETAL., 2021). The type and quantity of digestive enzymes determine the utilization of nutrients. There are several enzymes required to break down different nutrient classes into absorbable units in the gastrointestinal tract of fish. Various factors, including feeding behavior, the biochemical composition of food, and the onset of sexual maturity, among others, influence the changes in digestive enzyme activity (KUZMINA, 1996). The enzyme activity differs between fish species, and this is thought to be based on their feeding habits, some studies have however debunked this belief, they found that the relationship between feeding habits and proteolytic and ∝ amylase activity cannot be vividly described. The presence of ∝ amylase activity has been detected in carnivore fishes, though they do not consume starch in their natural diet. The domestication process of the European perch affects the digestive enzymes present in the digestive system, the digestive enzymes present in wild European perch were significantly modified during the process of domestication (PALIŃSKA-ŻARSKA ET AL., 2020).

Various factors, including feeding behavior, the biochemical composition of food, and the onset of sexual maturity, among others, influence the changes in digestive enzyme activity (KUZ'MINA, 1996). Total enzyme activity increase with fish age and this is linked to the increase in intestinal size and mucosal weight.

The differences in brush border enzymes according to age in fish vary from one species to another. Alkaline phosphate activity increased slightly with age in Pike perch and European perch. Sucrase activity did not change during ontogenesis in pike perch, but it increased 2 folds in European perch peaking during sexual maturity (KUZ'MINA, 1996).

The knowledge of the digestive enzyme activity of fish species can help in the effective selection of diet ingredients for the formulation of complete feed, which is suitable for the fish.

Studies on digestive enzymes have helped define the limits of the dietary inclusion of protein and carbohydrates (LANGELAND ET AL., 2013).

2.4.1 Development percid digestion

After the absorption of the yolk sac when feeding begins, the digestive tract of percids is basic with a non-functional stomach, because of this the absorption capacity is low. The digestive tract is underdeveloped and cannot function like those of juveniles and adults of the same species, so there is a need to understand the digestive function at different life stages to determine the nutritional needs and recommend an appropriate feed and feeding technique. The development of the European perch's digestive tract is similar to that of pikeperch with only some differences in the time it takes for the digestive tract to reach full functionality.

The digestive tract at hatching is a tube-like structure with the mouth and anus closed and the esophagus unlinked to the intestine. The different parts of the digestive tract cannot be distinguished, the pancreas is undetectable, and the liver is above the anterior intestine extending outwards. On the first day of hatching, the gut lengthens, and some folds become visible. On the second day, the pancreas becomes visible beneath the stomach (KESTEMONT ET AL., 1996).

On the 5th to 7th day post-hatching (dph), the mouth and the esophagus open, and the connection between the anterior intestine is formed, the rudimentary stomach is formed, and it is distinguishable because of the absence of the mucus cells responsible for the production of acid mucus (OSTASZEWSKA, 2005).

The stomach area and the pyloric sphincter are not yet formed. In European perch, the stomach differentiation starts earlier, on 2nd dph the inner surface of the stomach is smooth with a border of the mucus layer that increases in number and size with age. By the 6th dph the liver is vascularized and already shows storage functions (KESTEMONT ET AL., 1996). According to DIAZ AND CONNES (1991), glycogen is produced at the same time as hepatocyte development.

The first proenzyme granules in the pancreas cells appear and increase to a considerable number in 2 days. After 2days of the appearance of the proenzyme granule, the islet of Langerhans appears.

The activity of the pancreas increases at the start of the endo-exogenous feeding (RIBEIRO ET AL., 1999; ZAMBONINO & CAHU, 2001). At 1st dph, the valve between the median and posterior intestine is formed in the European perch (KESTEMONT ET AL., 1996).

The intestinal valve plays an important role in the early larval stage, preventing the escape of enzymes from the intestine. WATANABE (1984) suggests that before the growth of the stomach, acidophilic granules contained in this portion of the epithelium indicate successful intracellular digestion required for protein assimilation.

Between 15-20 dph stomach growth occurs with the appearance of the gastric gland and pyloric sphincter. The stomach morphology is complete at about 20dph. The intestine also elongates and forms the first loop. The stomach is Y-shaped with the blind sac, the mucosa consists of cylindrical single-layered epithelium, the muscle layer of the cardia and blind sac, and the lamina propria. The gastric gland is located in the lamina propria and surrounded by loose connective tissue of the mucosa of the blind sac and cardia. At 20-30 dph the mouth and the pharynx are lined with squamous epithelium with multiple taste cells.

The teeth appear on the pharynx; the esophagus is lined with multilayered cubic cells near the stomach. There are signs of pepsinogen secretions. The intestine begins with the pyloric sphincter, including the pyloric caeca, the anterior portion of the stomach points downwards.

The development of the pyloric caeca in the *Perca* species is the last of the digestive tract’s morphological growth. The cross-section of the Pyloric caeca lumen is star-like; reports show that there 3 pyloric caeca in the *Perca* species (BISBAL & BENGTSON, 1995). Carbohydrate compounds are the key component of bowel mucus in vertebrates, according to the same researchers.

GRAU ET AL. (1992), reported that intestinal neutral mucous compounds are involved in enzymatic food digestion, food mass formation, and absorption. Intestinal mucus plays an important defensive function in fish and mammals (DOMENEGHINI ET AL., 1998).

Mucous cells are very abundant in the whole intestine in pikeperch juveniles after metamorphosis, but their numbers are different in different sections. In the posterior gut, they are the most numerous. The high density of mucous cells in the colon is vital for easy defecation (DOMENEGHINI ET AL., 1998). The mucus produced by the mucous cells of fish plays the same role in protecting the mucosa of the digestive tract as in mammals (SCOCCO ET AL., 1998).

2.4.2. European perch nutrition

Intensive culture European perch are reared mostly on salmonid feeds, which contain high-quality protein formulated from fishmeal derived from marine species (STEJSKAL ET AL., 2020b).

Compared with other carnivore species the feeding rate of the European perch is low, the feeding rate decreases as they grow. For the European perch to be successful and live up to its potential in the European aquaculture industry, there needs to be a dedicated feed formula and it will be an advantage if this species does not depend on fishmeal and fish oil as a source of protein and essential fatty acids. Hence, the use of insect meals could benefit the nutrition of European perch. Several literatures have studied the effect of replacing fishmeal with insect meal, majority of this study focuses on the use of BSF and YM as these are the most bred and traded insect meals. There are many studies regarding the use of insect meal in commercial species but there is very little information regarding the use of insect meal in *Perciformes*.

However, there is some data on the use of insect meal in *Perciformes*, YM meal can replace the fishmeal in the diet of European sea bass up to 25% without any negative effect on growth and mortality. This result was obtained in a 70-day growth trial when fishmeal was replaced with 25% and 50% full-fat yellow mealworm meal (GASCO ET AL., 2016).

Similarly, the effect of replacing fishmeal with BSF meal in the diet of juvenile pike perch was observed in a 12-weeks experiment where 3 experimental diets were formulated replacing 25%, 50%, and 100% fishmeal with partially defatted black soldier fly meal. The result showed that fishmeal can be replaced by partially defatted black soldier fly meal up to 50% without any detrimental effect on growth performance (STEJSKAL ET AL., 2023). There are studies reporting the effect of the use of YM meal in European perch nutrition (TRAN ET AL., 2021; TRAN ET AL., 2022), while the study of TRAN ET AL., (2021) addressed the effect from a stable isotope point of view, the TRAN ET AL., (2022) study considered the effect on production parameter, nutrient digestibility, serum biochemistry, fillet composition, intestinal microbiota, and environmental impacts. 3 experimental diets were formulated replacing 25%, 50%, and 100% fishmeal with defatted yellow mealworm meal.

The study in 2022 found that replacing 25% fishmeal with defatted YM meal in European perch feed did not have any effect on the growth performance of 50% and 75% had lower growth performance compared to the control diet with 100% fishmeal (TRAN ET AL., 2022).

2.5. Summary of literature review

The static marine catch has necessitated the practice of aquaculture in a controlled environment to meet the demand gap for fish worldwide. The demand for fish keeps increasing as the population is becoming more aware of the health benefit of fish consumption.

On the other side consumers awareness of the health benefit of fish, they are also now aware of the fish producers' responsibility to produce responsibly and sustainably. Currently, aquaculture depends on wild catch for the best quality protein source (fishmeal), although soybean has also now been used as an alternative protein source the attendant effect is that the feed industry now has to compete with the food industry for these protein sources. Consumers and other stakeholders are voicing their concern about the dependence on wild catch for protein sources. This has put the feed industry under constant pressure in search of a sustainable protein source. Protein is the most expensive feed component and feed alone takes up to 50% of the production cost and sometimes more depending on the species and stage of life cycle. Soybean has proved to be a good alternative protein source, but it is also not sustainable and has an imbalanced amino acid profile and this has led to a continuous search for other alternatives with a more balanced amino acid set.

The practice of entomophagy is an age-long practice in humans and animals, this practice has been neglected and is not widely accepted as part of human diet in the Western world. However, this practice is still well practiced, and insects are part of the staple diets in Africa, Asia, and South America. Insects are now being considered and studied as an alternative protein source in the food and feed industry to meet the demand of the growing world population. There are several reasons why insects are being considered as an alternative protein source, from the lower environmental footprint and better feed conversion efficiency compared to conventional livestock. Edible insects have also been shown to have high nutrient composition, but this varies based on the species, stage of lifecycle, and diet.

The nutrient composition of insects cannot be generalized but the main components are protein and fat. The fact that the nutrient composition of insects depends on their diet allows insect producers to use feeding substrates to optimize the nutrient composition of insects and make them into functional foods.

At some stage of the research into the use of insect chitin which is a component of insects that is not present in other traditional protein sources was stigmatized as an anti-nutritional component, it was even suggested that it could impair digestion or cause an overestimation of the protein content of insects.

It was later confirmed by several studies that chitin is a component of insect that makes it stand out as an alternative protein because they have an immunomodulatory effect. This functionality is especially beneficial in shrimp culture because they have no innate immunity.

Despite the disgust factor surrounding entomophagy in the Western world stakeholders are taking necessary steps and several approvals for the use of edible insects both as food and feed are coming up. The FDA has approved the use of edible insects provided it was raised for consumption.

In Europe the first approval of edible insects was in 2017 when 7 insects were approved for pet and aquaculture fed, since then EU has approved these insects in poultry and swine feeding and the yellow mealworm has also been approved to be marketed for food purposes. Although research on edible insects has been going on for decades the production in terms of volume and the price of edible insects is still not competitive in comparison to the traditional protein sources. The yellow mealworm is the most traded of the approved insect in the EU, we intend to explore the nutrient composition of yellow mealworm based on the most common feed source used in the production at the different life stages and determine the effect of using duckweed as a feeding substrate. We will also determine the biological value based on rat assay.

The European perch is a very small species in terms of body size and has a very low feeding rate compared to other commercial species, their market size is between 100 and 150g. This is a new commercial aquaculture species in Europe and is still undergoing domestication. The European perch is preferred by consumers in Western and Northern Europe for the quality of their flesh. It has white flesh, lean meat, and a mild taste, and currently goes for a premium price of 20-30€ per Kg.

This carnivore species requires a high percentage and high-quality animal protein. Currently, there is very little information about the feeding and digestion of the European perch, and information concerning its digestion is based mostly on the closely related percidae, pikeperch. The gut content of wild-caught European perch consists of about 54% insect, making it a candidate for the use of insects as an alternative protein source.

However, to effectively try to incorporate insects as a protein source in the feeding of the European perch it is important to examine the digestion of this species since there is very little information known. In this research, we will explore the digestive enzyme activity based on rearing temperature.

Feed producers and entrepreneurs are investing in the commercial production of insects but still there is a long way to go in terms of the volume of production and market price of insect meals to compete effectively with traditional protein sources.

Despite decades of research, currently, insect meal can only supplement other protein sources or at best be used as a functional feed component because the market price is not competitive.

Currently, most of Europe’s insect meal production is used in aquaculture, and the utilization will continue to increase.

3. MATERIALS AND METHODS

3.1. Yellow mealworm experiments

3.1.1 Determination of biological value of the yellow mealworm meal

To determine the biological value of the yellow mealworm (YM) meal, we carried out an in-vivo rat experiment with Wistar rats, to compare the protein efficiency ratio (PER), net protein ratio (NPR), and true digestibility (TD) of YM meal with soybean meal. The goal of this experiment is to determine the suitability of YM meal as a protein source using soybean meal as a reference point. The experiment was carried out at the Food Science research institute ÉKI by carrying out the feeding trial for 10 days.

3.1.1.1 Experimental design

Wistar male rats were housed in cages (*Figure 5*). The rats were weighed at the beginning of the experiments and divided into 5 rats/groups. Each group represents 1 treatment, and each treatment has 2 replicates.

Figure 5: Experimental set up of cages for housing experimental wistar rat.

Photo: András Nagy (2021)

3.1.1.2. Experimental diet

After lyophilisation and grinding the yellow mealworm (YM) (*Figure 6*) semi-synthetic diet was prepared. The rats were fed with an iso-energetic and iso-protein semi-synthetic diet containing 10% of protein (*Table 1*). Two different amounts of mealworms were added to the test diets. In one group, 50% of the protein content, in the other group 25% of the protein content comes from YM, while the rest of the protein comes from the extracted soy.

In the soy control group, the protein consists of only extracted soybeans. The determination of metabolic and endogenous protein was determined by feeding a separate group a diet containing 4 percent egg protein (FMK).

Figure 6: Lyophilisation of yellow mealworm to produce meal for diet formulation.

Photo: András Nagy (2021)

Table 1.

The composition of experimental diet (%)

INGREDIENTS	**FMK**	**SOY**	**Mealworm meal 50%**	**Mealworm meal 25%**
Protein source	4.88	-	10.48	5.24
Extracted soybean	-	25.16	12.65	18.90
Sunflower oil	7.00	6.34	5.90	6.12
Vitamin and mineral premix	5.00	5.00	5.00	5.00
NaCl	5.00	0.50	0.50	0.50
Sucrose	8.00	8.00	8.00	8.00
Celullose	5.00	4.02	2.33	3.17
Corn starch	69.62	50.99	55.22	53.10

3.1.1.3. Experimental procedure

The individual weight of experimental rats was measured at the beginning of the experiment, we ensure that among the average weight of each group, there was no significant difference at the start of the experiment (78.8g). Feed and water were given ad-libitum and feed consumption was determined by weighing the remaining feed for each group at the end of each day. Individual weight was measured daily throughout the experiment to determine the daily weight gain. We calculated the protein content of the feed to be;

FMK = 4.41%

Test diets= 10.15%

We calculated the total protein consumption per animal from the protein content of the feed and total feed consumption. We collected the faeces of test animals daily, weighed and determined the protein content and nitrogen content.

3.1.1.4. Calculations

Protein intake

$$\boldsymbol{Protein\ intake((g/animal)/day)} \\ = \boldsymbol{feed\ consumption} \times \left(\boldsymbol{protein\ content\ of\ feed}/\boldsymbol{100}\right)$$

Protein Efficiency Ratio

$$\boldsymbol{PER(g/g) = \frac{M_{t10} - M_{t0}}{I}}$$

M_{t10}= Weight on day 10

M_{t0}= Weight on day 0

I= Protein intake

Net Protein Ratio

$$\boldsymbol{NPR\left(g/_{g}N\right) = \left(\frac{((M_{t10} - M_{t0}) + (M_{c10} - M_{c0}))}{I}\right)}$$

M (and M_c)=average body weight of test (and control) group (g)

I (and I_c)= average intake nitrogen of test (and control) group (g)

True Digestibility:

$$\boldsymbol{TD}(\%) = \frac{\boldsymbol{I} - (\boldsymbol{F} - \boldsymbol{F_K}) \times \mathbf{100}}{\boldsymbol{I}}$$

Where;

I= Nitrogen intake of test animal

F= Feacal nitrogen of test group

F_K= Feacal nitrogen of control group

Statistical Analysis

Results were analysed with IBM SPSS 22.0. The homogeneity of the data was checked by Levene test. One-way analysis of variance (ANOVA) Tukey test ($P<0.05$) was used to evaluate the results.

3.1.2. Determination of the nutrient composition of Yellow mealworm at different ages and stages of life cycle

3.1.2.1. The rearing of the yellow mealworm

The yellow mealworms (YM) were fed a mixture of semolina, flour, and oat flakes. Potatoes, carrots, and apples were used as water sources. All stock received uniform feed and there was no variation in the type and quantity of feed received. The feed is replaced every 2 weeks and the vegetables are replaced every 2 days to prevent mold.

The mealworms and darkling beetles were kept in a transparent container 12 and 24 inches respectively. The temperature was maintained at 20-26 ˚C. Eggs are collected from the darkling beetle box every 2 weeks and reared for 105 days (*Table 2*). 3 different batches were maintained for this experiment.

Table 2.

The duration of mealworm development during the experiment

	Egg	**Larvae**	**Pupae**	**Bug**
Days	1-21	22-105	106-114	115-198
Survival Rate %	-	98.9	98.7	-

Samples were collected at weeks 8, 10, and 12. The YM were separated by size into large-sized and small-sized groups by passing them through a sieve (mesh 10; diameter: 2 mm) at week 8. The ones that passed through were considered small, while the ones that remained in the sieve were the large ones. The large- and small-sized groups were maintained in separate containers. All samples were collected in three replicates; the sample collection was limited to three replicates because the temperature in the insect room rose during the summer months.
Throughout the sample collection 200 pcs of each group were counted and weighed to determine the average body weight. Samples of pre-moult YM, newly moulted YM, cuticles, and pupae were collected at week 12. We determined the body mass/surface area ratio by measuring the length and circumference of 50 mealworms from each group for calculation of the surface area. Samples were stored at - 20 ˚C until analysis. *Table 3* shows the sample collection schedule.

*Table 3***.**

Sample collection schedule of the different sizes and life stages of yellow mealworm

	Larvae (small)	**Larvae (large)**	**Moulted**	**Pre-moult**	**Pupae**	**Cuticle**
Week 8	✓	✓	x	X	X	x
Week 10	✓	✓	x	X	X	x
Week 12	✓	✓	✓	✓	✓	✓

All samples were collected in triplicates.

✓Collected.

x Not collected.

3.1.2.2 Proximate analysis

Samples were analyzed to monitor changes in nutrient composition with age and size. The dry matter was determined in gravimetric measurement after drying (AOAC, 2000). The weight was determined as an average of two measurements. Crude protein content was determined according to the Kjeldahl method (AOAC, 2000). Total crude fat was determined gravimetrically after acid hydrolysis and solvent extraction (AOAC, 2000). A laboratory-scale Soxtec unit (Soxtec 2050, Foss Electric, Denmark) was used to extract the fat component from the sampled powders. Samples (5 ± 0.03 g each) corresponding to each sample were put into tarred cellulose thimbles which were then loaded in the Soxtec (the unit temperature at 135 ºC). Extractions were carried out in duplicates.

After completion of the extraction process, sample cups were dried in a pre-heated oven at 100 °C for 10 min and transferred to a desiccator and cooled to ambient temperature.

Crude fibre was determined by Fibertec method (FOSS, Hilleroed, Denmark) according to ISO 6865:2000

Chitin was determined according to HAHN ET AL. (2018) as subtraction of the acid detergent lignin content (ADL) from the acid detergent fibre (ADF) that both measured by gravimetric method according to ISO 13906:2008 standard method.

The samples were defatted by hexane solvent extraction and ground into particle size <0.5mm as sample preparation.

1g of defatted sample was suspended in 100 ml of 0.5 mol/L H_2SO_4 and 20 g/L cetyl trimethylammonium bromide (CTAB) and boiled under reflux for 1h. The suspension was transferred to a fritted disc crucible and filtered under vacuum. The retentate was then suspended in 50 ml of 80 °C demineralized water for 5mins. The suspension was filtered under vacuum and the washing step was repeated two times. An additional washing step with 50 ml acetone was also done twice. The sample was dried and weighed. The ADF content was expressed as the percentage of mass fraction of the dry defatted biomass relative to the applied dry mass before the process of lipid extraction.

After the determination of ADF, the residuum was treated further with 12 mol/L H_2SO_4 for 3 hours, then it was filtered under vacuum and washed with water. After drying and weighing the residuum, it was cremated at 525 °C ±15 °C in an incineration furnace and weighed again for ADL calculation (HAHN ET AL., 2018). Chitin content was obtained by subtraction of the ADL content from the calculated ADF value and expressed as chitin of the dry weight of insect larvae.

Crude ash was determined gravimetrically (AOAC, 2000).

NFE was calculated by subtracting the sum of DM, CP, CF, fibre, and ash content from 100.

3.1.2.3. Statistical analysis

Results were analysed with IBM SPSS 22.0. The homogeneity of the data was checked by Levene test. One-way analysis of variance (ANOVA) Tukey test ($P<0.05$) was used to evaluate the results of growth. Mean values of nutrient composition were compared using univariate analysis of variance Tukey test ($P < 0.05$).

3.1.3. Optimization of Yellow mealworm nutrient composition using feeding substrate.

The purpose of this study was to determine the effect of feeding yellow mealworm (YM) with a substrate containing a mixture of semolina and duckweed at different percentages on the production parameters and nutrient composition of YM. Yellow mealworms used for this experiment were obtained from a commercial farm and transported to the Aquaculture Laboratory of the University of Debrecen. The experiment was set up in a biological chamber at the laboratory at 25 °C and 67% humidity (*Figure 7*).

Figure 7: Biological chamber

Photo: Abigeal Toviho (2022)

3.1.3.1 Substrate composition

Duckweed was harvested form the pond at the Aquaculture laboratory of the University of Debrecen, they were dried in a dehydrator and milled into fine powder (*Figure 8*).

Figure 8: A. Duckweed (*Spirodela polyrhiza*) on pond surface, B. Milling of duckweed, C. Powdered duckweed

Photo: Abigeal Toviho (2022)

Semolina was purchased from the store and the nutrient composition of both duckweed and semolina was determine (*Table 4*).

Table 4.

Nutrient composition of semolina and Duckweed (% DM)

	Dry matter	**Crude protein**	**Crude fat**	**Crude fiber**	**Ash**	**Carbohydrate**
Semolina	88.3	15.4	2.60	2.32	0.93	79.3
Duckweed	10.22	28.56	6.03	13.09	19.15	40.03

A feeding substrate was prepared with different composition of duckweed and semolina as shown in *Table 5*.

Table 5.

Substrate composition

Semolina	**Duckweed**	**Total weight of substrate (g)**	**Label**
100%(20g)	0%	20g	S
75%(15g)	25%(5g)	20g	S75D25
50%(10g)	50%(10g)	20g	S50D50
25%(5g)	75%(15g)	20g	S25D75
0%	100%(20g)	20g	D

3.1.3.2. Experimental set up

There were 5 treatments in the experiment (S, S75D25, S50D50, S25D75, and D), with 4 replicates each. The experiment was set up in a transparent 0.15-liter box. 40 mealworms were stocked per box, biomass=2.6 g (Average body weight= 0.065) (*Figure 9*).

Figure 9: Experimental setup

Photo: Abigeal Toviho (2022)

The experiment lasted 6weeks and we measured the growth rate, FCR and checked the survival rate every week for the duration of the experiment. The larvae were then stored at -20 °C until further analysis. The production parameters were calculated as follows;

$$\boldsymbol{Growth\,rate = \frac{weight\ of\ YM\ at\ end\ of\ experiment}{weight\ of\ YM\ at\ start\ of\ experiment} \times 100}$$

$$\boldsymbol{FCR = \frac{Feed\ consumed}{weight\ gain}}$$

$$\boldsymbol{survival\ rate = \frac{number\ of\ yellow\ mealworm\ at\ end\ of\ experiment}{number\ of\ yellow\ mealworm\ at\ start\ of\ experiment} \times 100}$$

3.1.3.3. Proximate analysis

At the end of the experiment the yellow mealworm (YM) was analysed for different nutrient compositions (DM, CP, CF, FB, ash and NFE), Dry material was determined in gravimetric measurement after drying (AOAC, 2000). The weight was determined as an average of two measurements. Crude protein content was determined according to the Kjeldahl method (AOAC, 2000).

Total crude fat was determined gravimetrically after acid hydrolysis and solvent extraction (AOAC, 2000). Crude fibre was determined by Fibertec method (FOSS, Hilleroed, Denmark) according to ISO 6865:2000.

Chitin was determined according to HAHN ET AL., 2018 as subtraction of the acid detergent lignin content (ADL) from the acid detergent fibre (ADF) that both measured by gravimetric method according to ISO 13906:2008 standard method. Crude ash was determined gravimetrically (AOAC, 2000). NFE was calculated by subtracting the sum of DM, CP, CF, fibre, and ash content from 100 (see details in 3.1.2.2.).

3.1.3.4. Statistical analysis

Results from the calculation of the production parameter were plotted on a graph using Microsoft Excel 2016. Results were analysed with IBM SPSS 22.0 of nutrient composition. The homogeneity of the data was checked by Levene test. One-way analysis of variance (ANOVA) Tukey test ($P<0.05$) was used to evaluate the results.

3.1.4. Virus determination in Yellow mealworm and European perch

3.1.4.1. Sample collection

The purpose of this experiment is to highlight one aspect of the safety concerns in the use of insects as feed, this aspect of this research is limited by the scope and available resources. Mealworm samples were purchased from a commercial farm and transported live for analysis of circovirus and iridovirus. The fish were fed commercial feed and 10 fish were collected from the rearing tank and euthanized. The fishes were dissected, and samples collected from the kidney, liver, and spleen, samples from individual fish were pooled in a single Eppendorf tube (*Figure 10*). The samples were stored at -20 °C before analysis.

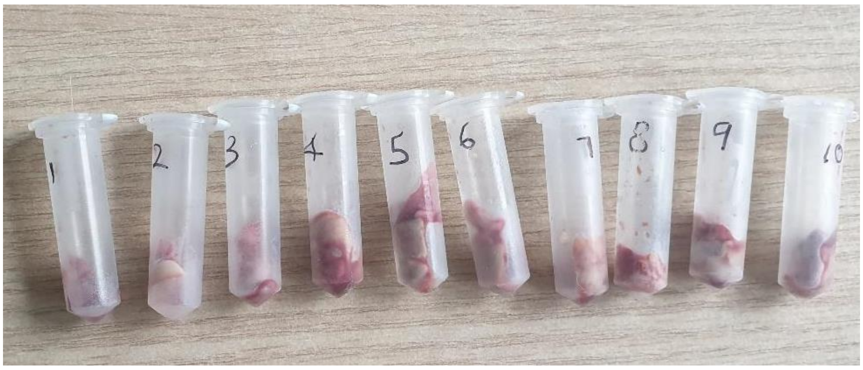


Figure 10: Kidney, liver and spleen for individual fish pooled in each Eppendorf tube.

Photo: Abigeal Toviho (2021)

3.1.4.2. PCR detection of virus (*Circovirus* and *Iridovirus*)

Sample preparation and nucleic acid digestion for PCR (Polymerase chain reaction) assays were performed with the NucleoSpin DNA RapidLyse kit (Macherey-Nagel GmbH & Co.) according to the manufacturer's instructions. PCR proposed for the general detection of circoviruses (Circoviridae family) was used (HALAMI ET AL., 2008). The primers were used to amplify the Rep gene of circoviruses. They allow for the multiplication of a 300 bp, highly preserved section. To detect iridoviruses of invertebrate host species (genus Iridovirus) that may also occur in vertebrates (PAPP AND MARSCHANG, 2019), we designed our primers (forward: 5'- GGN TTY ATH GAY ATH GCN AC-3 '; reverse: 5'- TCN CKD ATN ARR TTR TGC AT). DreamTaq DNA polymerase enzyme (Thermofisher) was used for PCR. The final reaction volume of 50 µl consisted of the following components: 21 µl of distilled water, 25 µl of 2X DreamTaq MasterMix, 1–1 µl of primer (50 pmol / µl), and 2 µl of sample DNA solution. In the PCR program, the initial denaturation was 3 minutes at 94 °C, followed by 45 cycles with the following parameters: 94 °C 30 sec; 46 °C 30 sec; 72 °C 60 sec. The final synthesis was 5 minutes at 72 °C. PCR products were analyzed by 1% agarose gel electrophoresis.

3.2 European perch experiments

3.2.1 The effect of temperature on digestive enzyme activity of fish

The purpose of this experiment is to gain insight into the changes in enzyme activity of European perch based on the rearing temperature.

The choice of temperature used in this experiment was based on the information from literature that European perch captured from the colder countries attain a larger size compared to warmer countries. This, therefore, informed our choice of temperatures lower than the optimal temperature (23 ˚C) reported.

3.2.1.1 Fish rearing

European perch were maintained in a 150-liters aquarium at the Fish Biology Laboratory on the University of Debrecen Agrar Campus. They were maintained at four different temperatures 16 °C ± 0.2, 18 °C ± 0.2, 20 °C ± 0.2, and 22 °C ± 0.2 which represented the treatment. The different temperatures were maintained using individual heaters in each aquarium (Tetra GmbH Herrenteich, 78 D-49324 Melle, Germany- HT 100). Each temperature had 4 replicates each with 25 fishes in each aquarium making a total of 100 fishes per treatment.
Fish used were of uniform stock hence uniform age. Fish weight range from 45- 60 g. The experimental design was completely randomized (*Table 6*).

Table 6.

The Experimental design

16 °C	**18 °C**	**20 °C**	**22 °C**
22 °C	**16 °C**	**18 °C**	**20 °C**
20 °C	**22 °C**	**16 °C**	**18 °C**
18 °C	**20 °C**	**22 °C**	**16 °C**

3.2.1.2 Feeding

Experimental fish were kept in the aquarium without feeding for 48 hours for acclimatization and emptying of the digestive tract before the commencement of feeding. The fish were fed with commercial feed containing 48% protein and 13% fat content. The fish were fed a total of 1.5% of their body mass for the experiment. The fish were fed twice, to ensure that the whole digestive tract was filled with feed and ensure that there will be detectable enzyme activity. At first feeding, the fish were fed 0.75% of their body mass, after 6 hours the fish were fed another 0.75% of their body mass.

3.2.1.3 Sampling and sample collection

The fish were collected from the rearing tank and euthanized. Each fish was weighed before dissecting and the weight of the whole digestive tract was also measured.
Samples in each replicate were pooled. Samples were stored at -80 °C for transportation and -20 °C until processing into analytical samples.

3.2.1.4 Sample preparation (gastric and intestinal enzyme extracts)

Analysis was carried out at the Food Science research institute ÉKI. Digestive enzyme extracts were prepared from the gastrointestinal tract of *Perca fluviatilis* with an approximate weight of 60 g from each replicate. Before the preparation of enzyme extract, they were thawed on ice batteries, at approximately 4 °C (VILLANUEVA-GUTIÉRREZ ET AL., 2020). We separated the stomach and intestine apart from each other at the line of the pylorus (KOLKOVSKI ET AL., 2000). The stomach was cut longitudinally, as described by ALMEIDA ET AL. (2018), then its content was washed out with 1 ml (in the case of each fish specimen) of distilled water adjusted with 0.1 M HCl (pH 2.5) *(Figure 11)* (KOLKOVSKI ET AL., 2000, DE MELO OLIVEIRA ET AL., 2014) to prepare the crude enzyme extract.

Figure 11: Filled and empty stomach

Photo: Krisztina Takacs (2021)

The contents of the intestine were pushed out manually, and then the intestine was cut longitudinally and washed with 1 ml (in the case of each fish specimen) of the 50 mM Tris-HCl buffer containing 0.5 M NaCl and 20 mM CaCl (pH 7.8), recommended by FUCHISE ET AL. (2011) (*Figure 12*).

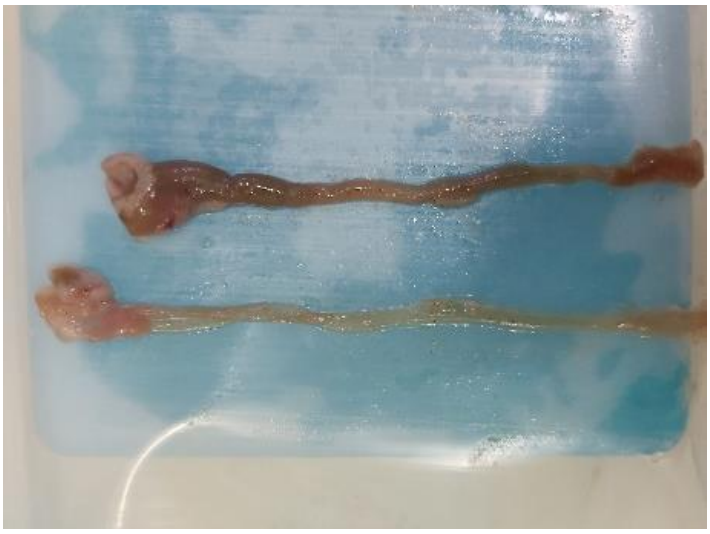


Figure 12: Filled and empty intestine.

Photo: Krisztina Takacs (2021)

3.2.1.5 Extraction of enzyme

The obtained mixture of the stomach/or intestine content (includes digested food and digestive fluids) (*Figure 13*) and the applied buffer represents the crude digestive enzyme extracts maintaining the fish’s physiological conditions.

The gained 25-25 gastric or intestinal enzyme extracts for each ambient temperature (16 °C, 18 °C, 20 °C, 22 °C) were pooled to have representative samples that can be used for further enzyme extracts preparation.

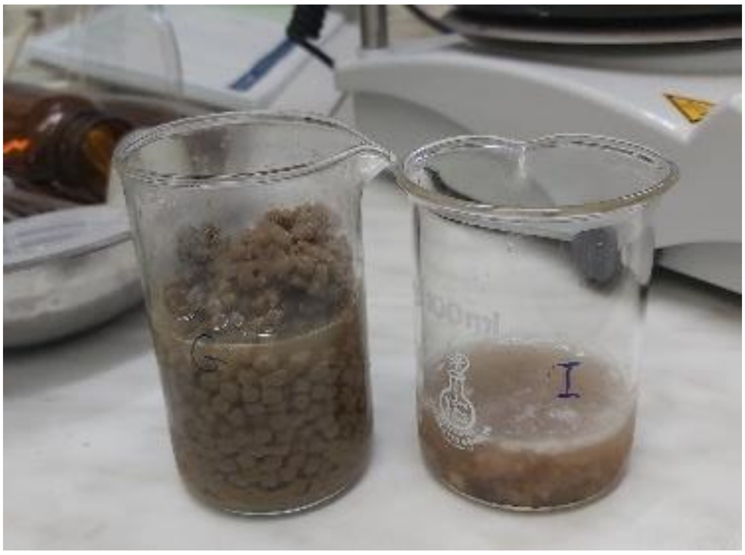


Figure 13: Gastric digesta and intestinal digesta (crude enzyme extract).

Photo: Krisztina Takacs (2021)

The preparation of the crude enzyme extracts was performed on ice batteries as a precaution to keep the samples chilled, to avoid enzyme denaturation and autolysis (KOLKOVSKI ET AL., 2000; DE MELO OLIVEIRA ET AL., 2014). The crude enzyme extracts were homogenized (30 sec) with Ultra-Turrax T25 (Janke & Kunkel Labortechnik) in beakers (VILLANUEVA-GUTIÉRREZ ET AL., 2020; DE MELO OLIVEIRA ET AL., 2014).

The respective gastric and intestine suspensions were mixed (BioSan Multi Bio RS-24) for 1 hour in a refrigerator at 4 °C for homogenizing the samples and to enhance proper extraction of the enzymes. The homogenized samples were centrifuged at 4100 rpm for 10 minutes at room temperature.

Furthermore, the obtained supernatants were transferred into 2-2 ml Eppendorf tubes and then were centrifuged again for 15 min at 4 °C at 12000 rpm. This way, the cell debris, lipids, feed, and other materials could be discarded from the supernatant. The remaining feed residue was stored lyophilized (digested feed) and will be used to determine in vivo biological accessibility of protein, lipid, and carbohydrate. The supernatants, which contained the enzymes, were stored at -20 °C until enzyme activity measurements.

3.2.1.6 Enzyme activity measurement

Protein measurement was done according to BRADFORD (1976) while pepsin, trypsin, total alkaline protease, lipase, and amylase activities were measured according to the protocol described by MINEKUS ET AL., (2014) with details in the electronic supplementary material (ESI). Pepsin and amylase activities were determined using the spectrophotometric stop reaction method, trypsin and total alkaline protease activities were determined using the continuous spectrophotometric rate determination method while lipase activity was determined according to the pH-stat reaction method.

Protein content measurement

We measured the protein content according to BRADFORD (1976).

Reagents and Equipment

- Bovine Serum Abumin (BSA) (Sigma-Aldrich)
- Bradford reagent (Sigma B6916)
- Spectrophotometer Genesys 6. Thermo Electron Corporation Madison, USA

Procedure

The Bradford Reagent was gently mixed in the bottle and brought to room temperature. After this the protein standards were prepared in appropriate concentrations using the same buffer as the unknown samples. We created a standard by serially diluting 2 mg/ml BSA protein standard. The protein standards range from 0.1–1.4 mg/ml. We created a standard assay table. After adding 3 ml of Bradford Reagent to each tube, they were vortexed gently for thorough mixing. The total liquid volume in each tube is 3.1 ml. Samples were then incubated at room temperature for 5–45 minutes and transferred into cuvettes. The absorbance was measured at 595 nm. We recorded the absorbance of the samples before the 60 minutes time limit and within 10 minutes of each other. The protein concentration was determined by comparing the unknown samples to the standard curve prepared using the protein standards.

Measurement of Pepsin Activity

Materials

- Substrate: 2% w/v haemoglobin (bovine blood haemoglobin, ref H2500 Sigma- Aldrich). To prepare the solution we disperse 0.5 g haemoglobin in 20 mL purified water, we then diluted this solution in 5 mL of 300 mM HCl to get a solution at 2% w/v haemoglobin at pH 2
- Enzyme: we prepared the enzyme by dissolving a stock solution of Pepsin (porcine pepsin, ref P6887 Sigma-Aldrich) in 150 mM NaCl adjusted to pH 6.5 using 100 mM
- NaOH. The stock solution was stored on ice or refrigerated at 4°C as prescribed. Just before the assay, a range of 5 to 10 concentrations of pepsin in 10 mM HCl was prepared, we diluted the pepsin at 5, 10, 15, 20, 25, and 30 μg/mL.
- Bench top shaking incubator
- Spectrophotometer Genesys 6. Thermo Electron Corporation Madison, USA
- Centrifuge Jouan BR4i (Saint-Herblain, France)

Procedure

We prepared Eppendorff tubes for each enzyme concentration by diluting the pepsin solution (1 mg/kg) in 10 mM HCl (*Table 7*):

Table 7.

Enzyme concentrations

Pepsin concentration (μg/mL)	**Volume of 1 mg/mL (μL)**	**HCl (10 mM) μL**
5	10	1990
10	20	1980
15	30	1970
20	40	1960
25	50	1950
30	60	1960

We utilized 2 mL Eppendorf tubes for each enzyme concentration to be tested, as well as for the respective blanks. To each tube, including the blanks, 500 µL of the haemoglobin solution was pipetted.

The tubes were then incubated at 37 °C in a shaking thermo bloc for approximately 3-4 minutes. Next, 100 µL of each enzyme concentration (except for the blanks) was added to the respective tube, and the tubes were incubated for exactly 10 minutes. To stop the reaction, 1 mL of 5% w/v trichloroacetic acid (TCA) was added to every tube, including the blank. After that, 100 µL of each enzyme concentration was added to the respective blank tube. All tubes were then centrifuged at 6000 g for 30 minutes to precipitate haemoglobin. The supernatant was transferred to new Eppendorf tubes for each enzyme concentration and blank, and they were incubated at 20 °C to reach temperature equilibration. The absorbance was measured at 280 nm using a quartz cuvette. For blank tests, the same procedure was followed, but the pepsin was added after the addition of TCA to stop the reaction. The absorbance of the blank was noted as A280 Blank. A linear curve was aimed to be obtained during the assay.

Calculation

$$\boldsymbol{Units/mg} = \frac{[\boldsymbol{A280\,Test} - \boldsymbol{A280\,Blank}] \times \mathbf{1000}}{(\Delta \boldsymbol{t} \times \boldsymbol{X})}$$

Where:

Δt: duration of the reaction, *i.e.* 10 minutes

X: concentration of pepsin powder in the final reaction mixture (quartz cuvette) [mg/mL]

Trypsin enzyme measurement

Materials

- Substrate: TAME (p-Toluene-Sulfonyl-L-arginine methyl ester) (ref T4626 Sigma-Aldrich) at 10 mM is prepared and dissolved in purified water.
- Enzyme: Prepare 1 mM HCl to dissolve the enzyme. Dilute Trypsin (porcine trypsin, ref T0303 Sigma-Aldrich) at least at 2 concentrations ranging between 10-20 µg/mL in 1 mM HCl.
- Prepare a 46 mM TRIS/HCl buffer, containing 11.5 mM CaCl2 adjust its pH at 8.1 at 25 °C.
- Spectrophotometer Genesys 6. Thermo Electron Corporation, Madison, USA

Procedure

The spectrophotometer was adjusted to 247 nm and 25 °C. A mixture consisting of 1.3 mL of Tris-HCl buffer (pH 8.1) and 150 µL of the substrate (10 mM TAME) was pipetted into a quartz cuvette and gently mixed by inversion. The cuvette was then placed in the spectrophotometer and incubated at 25 ºC for 3-4 minutes to allow for temperature equilibration. Subsequently, 50 µL of the trypsin solution(s) was added to the mixture and mixed by inversion. Absorbance was measured at 247 nm at 10-second intervals for a total of 10 minutes. The resulting data of absorption (y-axis) plotted against time (x-axis) in minutes was used to determine the slope (ΔA247) from the initial linear portion of the curve. For blank assays, the protocol is similar, but no enzyme was added, and the absorbance is similarly recorded, however it stabilises quicker (within 5 min).

Calculation

$$\boldsymbol{Units/mg} = \frac{[(\Delta \boldsymbol{A247\ Test} - \Delta \boldsymbol{A247\ Blank}) \times \mathbf{1000} \times \mathbf{3}]}{(\mathbf{540} \times \boldsymbol{X})}$$

Where:

A_{247}= slope of the initial linear portion of the curve, [unit absorbance/minute] for the Test (with enzyme) and Blank

540= molar extinction coefficient of TAME at 247 nm.

3= Volume (in millilitres) of reaction mix (Tris-HCl + TAME + Enzyme)

X= quantity of trypsin in the final reaction mixture (quartz cuvette) [mg]

Check that the same activity is obtained for the tested concentrations.

Lipase activity measurement

We conducted the pancreatic lipase activity assays with the pH-stat technique using tributyrin as substrate. The free fatty acids released by the lipase are titrated at a constant pH by sodium hydroxide (NaOH, 0.1N) during the course of the hydrolysis.

Materials

Assay solution: Tris 36 mg/mL with electrolytes (NaCl, CaCl2) and biliary salts (sodium taurodeoxycholate). Containing; 200 mL solution containing 7.2 mg of Tris- (hydroxymethyl) aminomethane, 1800 mg of NaCl, 40 mg of CaCl2 and 420 mg of sodium taurodeoxycholate.
Titration solution: 0.1 N sodium hydroxide
- Prepare a (NaOH) titration solution by dissolving 2 g of NaOH in 500 mL of purified water.

Enzyme solutions: lipase and colipase
- Dissolve 5 mg of enzyme powder in 5 mL of purified water. Store on ice. At least 2 amounts of enzyme solution (1 mg/mL) must be tested.

Mechanical stirrer MR HEI-STANDARD, EKT Hei-Con (sensor) SCHWABACH, Germany
Thermo-regulated pH stat device

Procedure

Blank: We poured 15 mL of the aqueous phase and add 0,5 mL of tributyrin into a titration vessel.
We ensured that the volume of the assay was high enough for adequate pH- measurement (microelectrode should be correctly immersed). We then switch on magnetic stirring to disperse the tributyrin and let equilibrate at 37 °C (Figure 14). We monitored the delivery rate of titrant solution (NaOH) at a pH of 8 at 37 °C. We added 14.5 mL of assay solution and 0.5 mL of tributyrin to a titration vessel and switched on the mechanical stirrer and let it equilibrate at 37 °C. We measured the pH and monitor the volume of NaOH needed to keep the pH at 8 at 37 °C, for 10 minutes, this will be the blank. We added 10-100 μL of the lipase solution and start monitoring the amount of NaOH required to compensate the liberations of fatty acids and keep the pH at 8 at 37 °C, for 10 minutes.

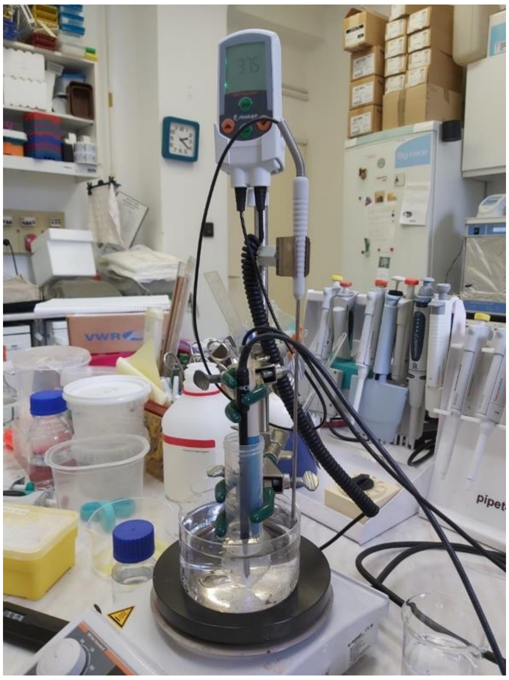


Figure 14: Titration vessel on a mechanical stirrer

Photo: Krisztina Takacs (2021)

Calculations

$$\frac{\boldsymbol{Units}}{\boldsymbol{mg\ Powder}} = \frac{\boldsymbol{R(NaOH) + 1000}}{\boldsymbol{V \times [E]}}$$

Where:

R(NaOH): Rate of NaOH delivery in µmol NaOH per minute, *i.e.*, µmol free fatty acid released per minute

v: volume [µL] of enzyme solution added in the pHstat vessel

[E]: concentration of the enzyme solution [mg powder/mL]

Check that the same activity (U/mg) is obtained for both tested enzyme amounts

α-amylase Enzyme determination

Materials

- Substrate: the substrate is soluble potato starch. We prepared 100 mL of a 20 mM sodium phosphate buffer containing 6.7 mM NaCl and adjusted it to pH 6.9 at 20 °C with 1 M NaOH. We then prepared a 1.0% w/v soluble starch solution in the sodium phosphate buffer pH 6.9 this was done by dissolving 0.25 g soluble potato starch (ref S2630 Sigma-Aldrich) 20 mL initial buffer volume.

To avoid evaporation of water we covered the beaker to bring to a boil while stirring and maintaining the temperature just below boiling for 15 minutes. We cooled to room temperature and added 5 mL of purified water to complete the starch solution to 25 mL volume.

- Colour reagent solution: We prepared a 5.3 M sodium potassium tartrate solution in 2 M NaOH. To do this we dissolved 0.8 g NaOH in 10 mL purified water, heated the solution to a temperature between 50 to 70 °C. 12.0 g of sodium potassium tartrate tetrahydrate (MW: 282.22 g/mol) was then dissolved in 8.0 mL of warm 2 M NaOH solution. We maintained the temperature so as not to bring it to a boil while constantly stirring to dissolve.
- We prepared a 96 mM 3,5-dinitrosalicylic acid (MW: 228.12 g/mol) solution by dissolving 438 mg of acid in 20 mL of purified water. Heated the solution up to a temperature between 50 to 70 °C. We maintained the temperature so as not to bring it to a boil while constantly stirring to dissolve.
- We heated 12 mL of purified water to 60 °C and added slowly 8 mL of the 5.3 M Sodium Potassium Tartrate Solution. We then added 20 mL of the 96 mM 3,5-dinitrosalicylic acid solution and stired until complete dissolution.
- 10 mL of 0.2% w/v maltose standards (Figure 15) (for instance ref M5885 Sigma- Aldrich).
- Enzyme: We prepared a solution containing approximately 1 unit/mL of α- amylase in purified water just before use.
- Bench top shaking incubator
- Spectrophotometer Genesys 6. Thermo Electron Corporation Madison, USA

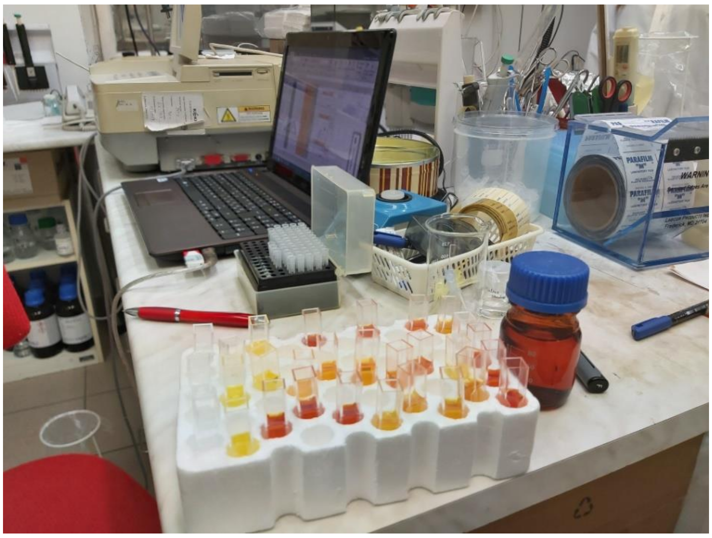


Figure 15: Maltose standards in test tubes

Photo: Krisztina Takacs (2021)

Procedure

We Prepared a standard curve by diluting the maltose solution (0.2% w/v) in purified buffer (*Table 8*)

Table 8.

Maltose standard concentrations

Standard	Volume maltose stock(mL)	Volume of buffer pH 6.9(mL)	Maltose Standard solution	Maltose standard concentration
0	0	1	0	0
1	0.025	0.975	0.05	0.005
2	0.1	0.9	0.2	0.02
3	0.2	0.8	0.4	0.04
4	0.3	0.7	0.6	0.06
5	0.4	0.6	0.8	0.08
6	0.5	0.5	1	0.1
7	1	0	2	0.2

We set the spectrophotometer at 540 nm and 20 °C. The bench top shaking incubator was fitted with sample holder at 20 °C. We heated a water bath at 100 °C, fit it with a sample holder to stop the reaction. We prepared a container filled with ice to cool the samples. 1 mL of substrate was pippeted into 15 mL cap covered containers, mixed by swirling and incubated at 20 °C for 3-4 minutes to achieve temperature equilibration.

Test: We added 500 µl to 1 mL of enzyme solution and mixed by swirling. We incubated at 20 °C for exactly 3 minutes, 1 mL of colour reagent solution was added to stop the solution. Reaction vessel was capped and place in the boiling water bath. We added 500 µL of enzyme to complete the volume of enzyme added to 1 mL. We boiled for exactly 15 minutes and cool on ice for a few minutes.

We added 9 mL of purified water and mixed by inversion. 3 mL of the reaction mixture was pipetted into a cuvette of spectrophotometer, and we recorded the absorbance at 540 nm, noted A540 Test.

Blank: For blank assays, the protocol is similar, but no enzyme is added before the 3 minutes incubation.

It is only after the addition of the colour reagent and after putting the reaction vessel in the boiling bath, that 1 mL of enzyme solution is added. After blank procedure, the absorbance, A540 Blank is similarly recorded.

Calculations

ΔA540Standard = A540 Std. - A540 Std. Blank

Plot the ΔA540nm of the Standards vs. quantity of maltose [mg]. Establish the corresponding linear regression:

$$\Delta \boldsymbol{A540\ Standard} = \boldsymbol{a} \times [\boldsymbol{maltose}] + \boldsymbol{b}$$

Enzyme activity determination:

ΔA_{540} Sample = A_{540} Test - A_{540} Test Blank

$$\frac{\boldsymbol{Units}}{\boldsymbol{mg\ Powder}} = \frac{[(\boldsymbol{A_{540}\ Test} - \boldsymbol{A_{540}\ Test\ Blank}) - \boldsymbol{b}]}{(\boldsymbol{a} \times \boldsymbol{X})}$$

Where:

a: slope of the linear regression established for ΔA540nm of the Standards vs. quantity of Maltose (mg)

b: intercept of the linear regression established for ΔA540nm of the Standards vs. quantity of Maltose (mg)

X: quantity of amylase powder (mg) added before stopping the reaction

Total alkaline protease activity measurement.

We also determined the total alkaline protease activity (PEÑA ET AL., 2015; GARCÍA-ORTEGA ET AL., 2000). We estimated the Alkaline protease activity using casein as substrate. The composition of the enzymatic reaction mixtures is; 250 µL of 0.1 mol L-1 Tris-HCl buffer, 0.01 M (pH 9) CaCl2, 100 µL of enzymatic extract and 250 µL of 1% casein in Tris-HCl buffer. Incubation was done at 37 °C and the reaction was stopped by adding 600 µL of 8% (w/v) TCA.

3.2.1.7 Statistical analysis

Shapiro-Wilk Normality Test and Bartlett Test of Homogeneity of Variances in R (Team R Core 2021) was used to verify the normality and homogeneity of variances of our results.

ANOVA tests were applied (TEAM R CORE 2021) according to ALKARKHI AND ALQARAGHULI (2018) to find out whether the environmental temperature has a significant effect on pepsin, trypsin, total alkaline proteolytic, lipase and α-amylase activity, respectively.

Tukey Honest Significant Difference method in R (TEAM R CORE 2021) was used to determine whether there is a significant difference between the effect of 16 °C, 18 °C, 20 °C and 22 °C regarding pepsin, trypsin, total alkaline proteolytic, lipase and α-amylase activity.

3.2.2 European perch feeding experiment with Yellow mealworm meal diets

This experiment was carried out to determine the effect of replacing fishmeal with YM meal at different percentages on the production parameters of European perch. European perch with average body weight 40 g was used for the experiment, the fishes were reared from larvae stage at the Aquaculture Laboratory of the University of Debrecen. The experiment was carried out in a RAS system, 40 pieces of fish were stocked in each 350liter circular tank.

The water parameters were temperature: 20±0.8°C DO: 7.5±1.4 mg/l, PH 6.7±0.4, ammonia 0.02±0.003 mg/l. The experiment was carried out for a period of 6weeks, sampling was done every week for the duration of the experiment. The fish were feed 1.5% body weight in 2times daily feeding. They were fed in the morning and in the evening.

3.2.2.1 Experimental diet formulation

Four experimental diets were prepared for this experiment (*Table 9*). The control diet did not contain YM meal while the other 3 experimental diets contained 12.5%, 25% and 37.5% YM meal respectively.

Table 9.

Diet Formula (Kg)

INGREDIENTS	Control	Yellow Mealworm 12.5%	Yellow Mealworm 25%	Yellow Mealworm 37,5%
Blood meal	2	2	2	3
JPC 56 soy cocncentrate	3	3	5	5
Fishmeal	72	63	54	45
Mealworm meal	0	9	18	27
Vitamin premix	2	2	2	2
Dextrose	1	1	1	1
Vitamin C	0.001	0.001	0.001	0.001
Fish oil	6.4	4.3	2.1	
Novilpel	1	1	1	1
Lysine	0.8	1.4	1.4	1.6
Methionine	0.5	0.6	0.7	0.9
Treonin	0.5	1	1.17	1.17
Tryptophan	0.07	0.08	0.08	0.08
Wheat meal	10.73	11.62	11.55	12.25
Total	100.001	100.001	100.001	100.001

Preparation of Experimental diets

Live YM was purchased from a commercial farm, it was dehydrated at 43 °C and milled. The different feed ingredients were homogenised using a mixer and the mixture was made into 3 mm pellets using a pelletizer after which it was dried in the dryer (*Figure 16*).

Figure 16: Feed preparation

Photo: Abigeal Toviho (2023)

A: Feed mixture in pelleting machine,

B: Pelettes from pelleting machine

C: Feed pellets in dryer.

Statistical Analysis

Results were analysed with IBM SPSS 22.0. The homogeneity of the data was checked by Levene test. One-way analysis of variance (ANOVA) Tukey test ($P<0.05$) was used to evaluate the results.

4. RESULTS

4.1. Yellow mealworm experiments

4.1.1. Determination of the biological value of the yellow mealworm meal

4.1.1.1. The growth of the experimental rats

The growth of test animals is expressed in our result as the average daily weight and average weight gain of test animals in the different groups.

Average daily weight

Figure 15. shows the average daily weight of Wistar rats from the 4 different test groups. Each group were fed different diets, the rats fed diets containing yellow mealworm meal and soy had similar daily weight gain during the period of the experiment.

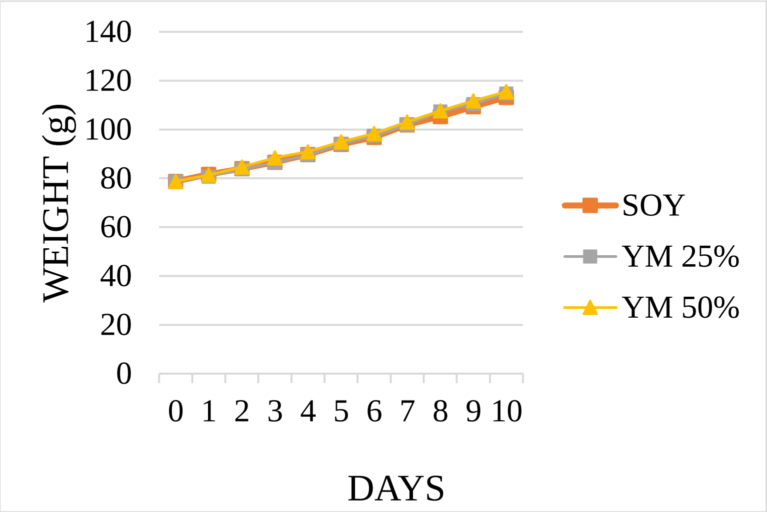


Figure 17: Average daily weight of rats during the In-vivo animal experiment

Soy: 100% soy diet

YM 25%: 25% yellow mealworm meal diet

YM 50%: 50% yellow mealworm meal diet

Average weight gain

Figure 18. shows the average weight gain of rats in the different experimental groups. The weight gain observed in the different groups follows a trend, there was no statistically significant difference ($p<0.05$) between the control diet (soy), YM 25%, and YM 50% groups.

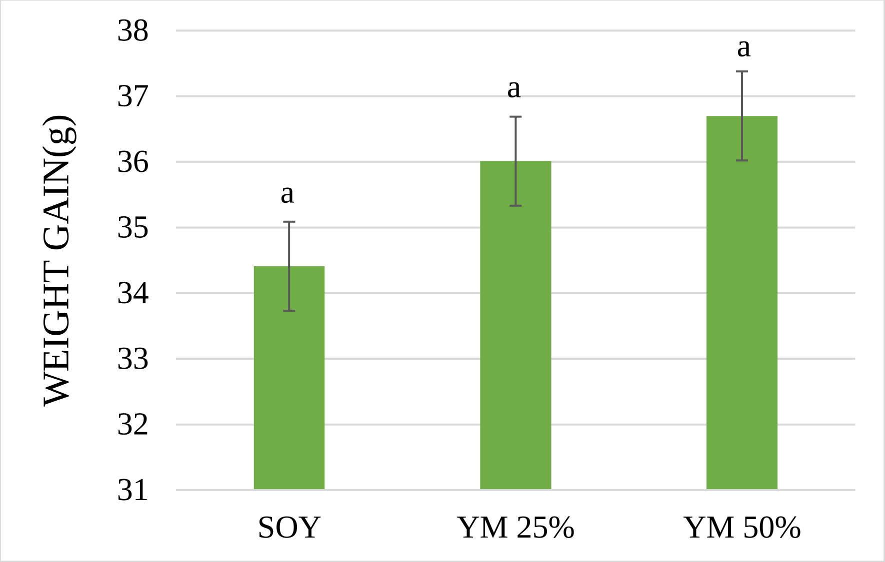


Figure 18: Average weight gain of wistar rat feed experimental diets

Soy: 100% soy diet

YM 25%: 25% yellow mealworm meal diet

YM 50%: 50% yellow mealworm meal diet

4.1.1.2. Biological value determination of the Yellow mealworm

The result of the biological value determination was shown through the PER, NPR, and TD values. It was important for us to determine the biological value to know the nutritive value, quality, and bioavailability of the protein in yellow mealworm meal as there have been suggestions that although insects have high protein content the nutritive value could be low due to the presence of chitin. We determined the NPR as a backup for the PER as it was recommended (MITCHELL ET AL., 1989).

Protein Efficiency Ratio (PER)

Figure 19. shows the PER value for the test diet, the tendency observed was that the PER increase with the increase in inclusion level of yellow mealworm meal. However, there was no statistical difference ($p<0.05$) between the PER of all 3 test diets.

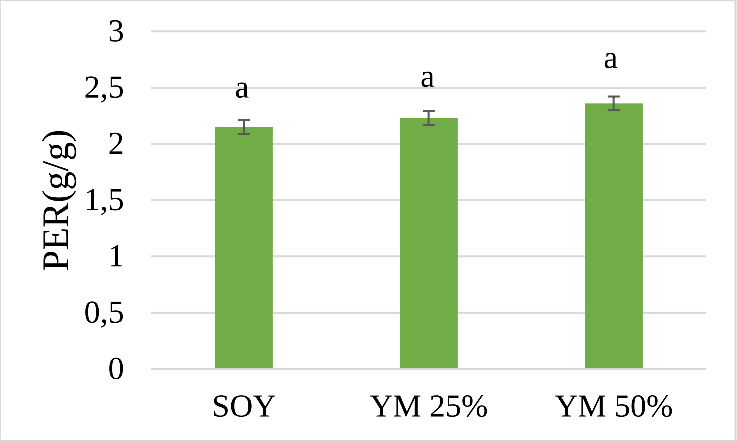


Figure 19: Protein efficiency ratio of wistar rat fed experimental diets.

Soy: 100% soy diet

YM 25%:25 % yellow mealworm meal diet

YM 50%: 50% yellow mealworm meal diet

Net protein ratio (NPR)

Figure 20. shows the NPR of the test groups, the tendency observed was that the NPR increase with the increase in inclusion level of yellow mealworm meal There was no statistical difference ($p<0.05$) between the test groups.

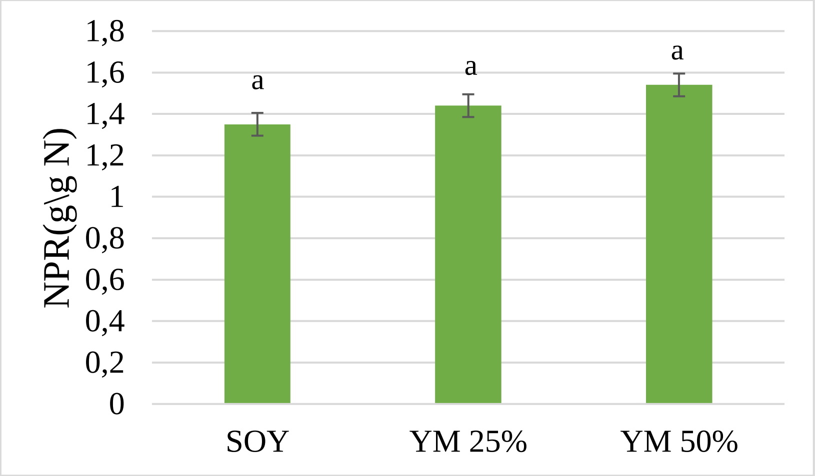


Figure 20: Net Protein Ratio of wistar rat fed experimental diets.

Soy: 100% soy diet

YM 25%: 25% yellow mealworm meal diet

YM 50%: 50% yellow mealworm meal diet

True digestibility

Figure 21. shows the true digestibility of Wistar rat groups fed different experimental diets containing soy, YM 25%, and YM 50% as protein sources. There was no trend observed between the TD of the different groups. There was no statistical difference ($p<0.05$) in the TD of all test diets.

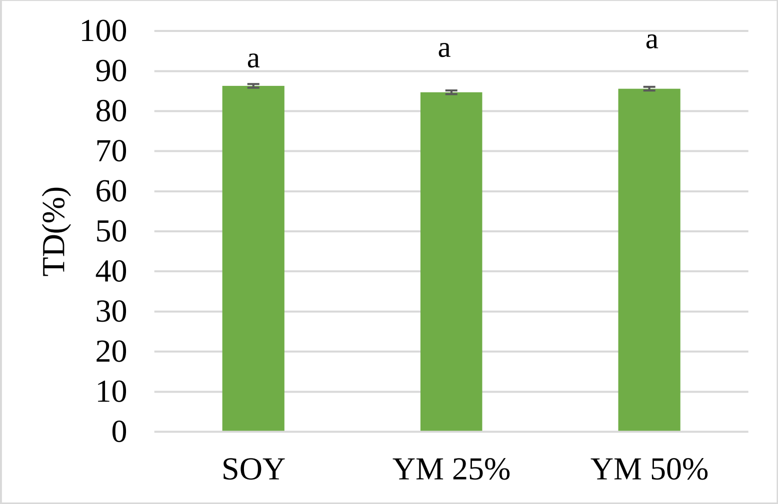


Figure 21: True digestibility (TD) of wistar rats feed experimental diets.

Soy: 100% soy diet

YM 25%: 25% yellow mealworm meal diet

YM 50%: 50% yellow mealworm meal diet

4.1.1.3 Discussion

The rats fed diets with YM meal diets had the best liveweight of all the treatments. The lowest daily weight was observed in FMK (control) as they were only provided protein for maintenance. The average weight gain of test diets was significantly higher than FMK (control), YM 50% had the highest weight gain. Although YM 50% had the highest average weight gain there was no significant difference ($p<0.05$) among the groups (soy, YM 25%, and YM 50%). This shows that the YM 25% and YM 50% had no adverse effect on the growth of the test animals, and it was comparable to soy as a protein source.

When we considered the biological value of the test diets using the PER and NPR the result followed the same trend YM 50% had the highest PER and NPR while soy had the lowest. All the diets were however statistically the same, showing that the protein quality of the diet with 50% replacement of soy with YM meal produced feed with the same protein quality.

The TD of all 3 diets did not differ statistically soy had the highest TD, the result did not follow a particular trend like PER and NPR. These results show that replacing 25% and 50% soy with yellow mealworm meal in the feed kept the quality of protein in the feed and growth response of the test animals the same as when 100% soy was used as the protein source. This information provides us with the basis for the formulation of our experimental diet with the aim of including YM meal as a protein source for the European perch. This guided our decision on the percentage inclusion of YM meals during the diet formulation.

4.1.2. Determination of the nutrient composition of Yellow mealworm at different ages and stages of life cycle

4.1.2.1. Growth of Yellow mealworm

Overall growth of Yellow mealworm

Table 10 displays the weight changes and percentage increases in YM's weight during the experiment. The only statistical difference was detected between weeks 10 and 12, despite the weight of 8-, 10-, and 12-week-old insects steadily increasing.

Table 10.

Yellow mealworm overall average weight (g)

	Week 8	**Week 10**	**Week 12**
Weight	0.074±0.006^a	0.084±0.005^a	0.104±0.007^b

Means in the same row with different superscripts differ significantly ($p < 0.05$)

Growth of different age and size groups of Yellow mealworm

Table 11 displays the weight gain of mealworms in two sizes (large and small). While there was a nearly 3-fold difference in the average body weight between the two groups, at the beginning of the measurements (week 8; large: 0.111 g; small: 0.036 g), it was only slightly more than 2-fold at the conclusion of the trial (large: 0.139 g; small: 0.067 g).

Table 11.

Average weight by size and age (g) of the yellow mealworm

	Week 8	Week 10	Week 12
Large	0.111±0.007^{a}	0.121±0.009^{ab}	0.139±0.009^{b}
Small	0.036±0.008^{c}	0.046±0.005^{c}	0.067±0.015^{d}

Means with no common letters in their superscript differ significantly ($p < 0.05$).

Growth rate

The growth rate for Large YM was 9%, while the growth rate for small YM was 11%. Mealworm weight grew by 23.8% between weeks 10 and 12 compared to only 13.5% between weeks 8 and 10 (*Figure 22*). Similar to how the growth rate from week 10 to week 12 is higher than from week 8 to week 10, the growth rate is higher for small YMs than for large sizes.

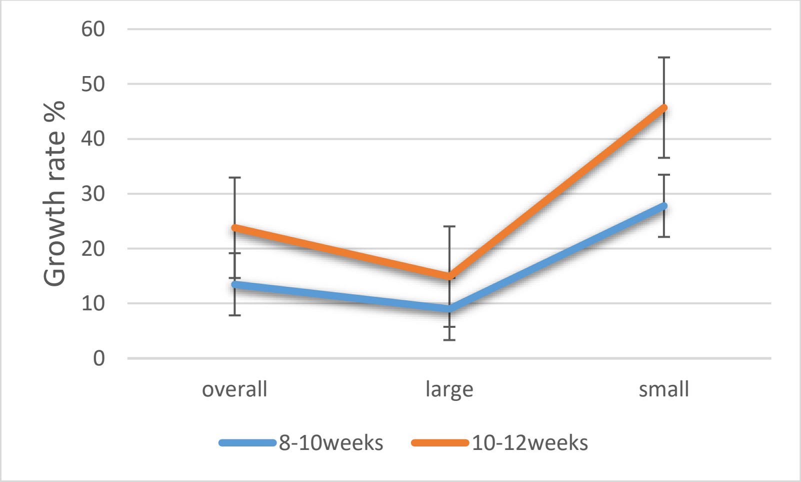


Figure 22: Growth rate of the Yellow mealworm.

Surface area/body mass ratio, length and width of Yellow mealworm at week 12.

Table 12. displays the length, width, and surface area/body mass ratio (SA:BM) of yellow mealworms at week 12. There was no statistically significant difference between the ratios of large- and small-sized worms. Although there was no significant difference between the width values, the average length of the large-sized mealworm stock was statistically higher than that of the small-sized stock.

Table 12.

Average surface area (SA)/ body mass (BM) ratio, length and width of yellow mealworm at week 12

	Large	**Small**
SA:BM	1:55±9^{a}	1:62±24^{a}
Length (cm)	2.7±0.20^{a}	1.9±0.10^{b}
Width (cm)	0.4±0.05^{a}	0.3±0.05^{a}

Means in the same row with different superscript differ significantly ($p< 0.05$). SA: surface area BM: body mass

4.1.2.2 Nutrient composition

Nutrient composition at different ages

Nutrient compositions of YM at different ages are shown in *Table 13*. There was no significant difference between all the nutrient component measured.

Table 13.

Average nutrient composition of yellow mealworm at different ages (%)

	Week 8	**Week 10**	**Week 12**
DM	37.42± 1.33^{a}	37.27±1.88^{a}	36.57±1.47^{a}
Crude Protein	43.35±0.67^{a}	44.72±2.16^{a}	44.93±1.75^{a}
Crude Fat	39.47±0.61^{a}	38.02±1.68^{a}	37.85±1.78^{a}
Crude fibre	11.30±0.04	11.38±0.05	11.52±0.06
Chitin	22.73±1.00^{a}	22.25±1.07^{a}	21.68±1.03^{a}
Crude Ash	3.64±0.05^{a}	3.66±0.06^{a}	3.68±0.05^{a}
NFE	2.29±0.03^{a}	2.26±0.13^{a}	2.10±0.10^{a}

Means in the same row with different superscripts differ significantly ($p< 0.05$).

Nutrient composition of Yellow mealworm at different ages and sizes (%)

With age, both the large- and small-sized mealworms' dry matter, fat, and chitin contents declined while their protein contents increased (*Table 14*). The sizes rather than the ages show significant variations. Independent of age, the dry matter contents of the large yellow mealworms were consistently and significantly larger than those of the small-sized groups; nevertheless, the chitin and NFE contents of the small-sized groups were of significantly higher value. In all ages, the large-sized mealworm consistently had a higher ash content than the small size. In all samples, there was no discernible trend in the NFE.

Table 14.

Average nutrient composition of Yellow mealworm at different ages and sizes (%)

	Week 8		Week 10		Week 12	
	Large	**Small**	**Large**	**Small**	**Large**	**Small**
DM	38.60 ± 0.30^{a}	36.23 ± 0.40^{b}	38.53 ± 1.60^{a}	36.00 ± 1.22^{b}	37.50 ± 1.13^{a}	35.63 ± 1.25^{b}
Crude Protein	43.70 ± 0.50^{a}	43.00 ± 0.70^{a}	45.23 ± 2.57^{a}	44.20 ± 2.06^{a}	45.13 ± 2.38^{a}	44.73 ± 1.34^{a}
Crude Fat	39.60 ± 0.53^{a}	39.33 ± 0.77^{a}	38.10 ± 2.00^{a}	37.93 ± 1.74^{a}	37.80 ± 2.23^{a}	37.90 ± 1.71^{a}
Fibre	11.53 ± 0.61^{a}	11.00 ± 0.10^{b}	11.83 ± 0.15^{a}	10.93 ± 0.25^{b}	12.13 ± 0.25^{a}	10.90 ± 0.26^{b}
Chitin	21.93 ± 0.56^{b}	23.53 ± 0.50^{a}	21.40 ± 0.61^{b}	23.10 ± 0.55^{a}	20.87 ± 0.58^{b}	22.50 ± 0.56^{a}
Crude Ash	3.65 ± 0.08^{a}	3.62 ± 0.05^{a}	3.64 ± 0.05^{a}	3.62 ± 0.05^{a}	3.64 ± 0.01^{a}	3.60 ± 0.06^{a}
NFE	1.52 ± 0.03^{b}	3.05 ± 0.10^{a}	1.2 ± 0.01^{b}	3.32 ± 0.09^{a}	1.3 ± 0.13^{b}	2.87 ± 0.02^{a}

Means in the same row with different superscripts differ significantly ($p< 0.05$).

Nutrient composition of Yellow mealworm at different development stages and nutrient composition of the cuticle

Table 15 shows the nutritional contents of yellow mealworms at various developmental stages. Comparing the pupa stage to the moult and premoult stages, the pupa stage revealed decreased amounts of dry matter, crude fat, and chitin. Significant differences could be seen in the pupae's crude fat, dry matter, and chitin compositions. There were no statistically significant differences between the nutrient composition of the premoult and moult. The cuticle's nutritional makeup was entirely distinct from those of the three developmental phases. In comparison to mealworms, the dry matter content was significantly higher (97.5%), and it also contained more crude protein, fibre, chitin, ash, and NFE but significantly less crude fat.

Table 15.

Average nutrient compositions of Yellow mealworm at different development stages and average nutrient composition of the cuticle (%)

	Pre-moult	Moult	Pupae	Cuticle
DM	40.15 ± 0.14^{b}	39.13 ± 0.98^{b}	37.12 ± 0.11^{c}	97.5 ± 1.32^{a}
Crude Protein	44.18 ± 0.03^{b}	43.97 ± 0.02^{b}	44.00 ± 0.08^{b}	53.40 ± 0.17^{a}
Crude Fat	39.38 ± 0.03^{a}	39.29 ± 0.01^{a}	38.25 ± 0.04^{b}	12.20 ± 0.13^{c}
Crude Fibre	10.53 ± 0.03^{b}	10.82 ± 0.02^{b}	11.9 ± 0.06^{b}	19.8 ± 0.21^{a}
Ash	3.66 ± 0.04^{b}	3.65 ± 0.02^{b}	3.62 ± 0.05^{b}	9.8 ± 0.19^{a}
NFE	2.25 ± 0.03^{b}	2.27 ± 0.02^{b}	2.23 ± 0.03^{b}	4.4 ± 0.20^{a}
Chitin	23.82 ± 0.01^{b}	23.65 ± 0.03^{b}	23.42 ± 0.04^{c}	35.60 ± 0.26^{a}

Means in the same row with different superscripts differ significantly ($p < 0.05$)

4.1.2.3 Discussion

Growth

Considering the overall growth, although there was an increase in growth among each age group, the growth was statistically different only between week 10 and week 12. This demonstrates that it is preferable to wait until week 12 of the development in order to collect a much bigger amount of biomass. Waiting until week 12 to collect the biomass will be advantageous in terms of size growth across age groups as well since only the 12-week-old larvae exhibit a statistically higher weight compared to the 8-week-old ones. The weight of the small-sized larvae nearly doubled from week eight to week twelve (86.1%) while the weight of the large-sized larvae only grew by 25.2%. This demonstrates that the weight disparities between small and large-sized individuals get smaller over time. Consequently, week 12 is also the optimal harvesting time for the small-sized larvae.

The average body weight of the large-sized YM in our experiment was higher than the 0.040–0.111g range described in another investigation. The fact that our study only reached a weight of 0.134 g by week 12 whereas their experiment terminated at week 9, according to their report, may be due to the different feed. In the study, industrial waste was YM's feeding substrate. The weight achieved at week 8 of that trial was similar (0.075g) to the weight obtained at week 8 of our investigation (BORDIEAN ET AL., 2020b).

4.1.2.4. Nutrient composition

Nutrient composition based on age and size

When examining the DM, CP, CF, fibre, chitin ash content and NFE contents, the nutrient composition of mealworms showed no significant difference among the age groups. The 36.5% reported by YI ET AL. (2013) is within the range of the DM content we observed, though the DM content recorded was slightly greater than the 32.2% reported by JONES ET AL. (1972).

The NFE we observed was lower than what was reported by the same study, while the ash content we measured was higher than what was reported by the studies of FINKE (2002). Contrary to what GHOSH ET AL. (2017) stated, the ash content measured in our study was lower while the NFE is higher.

Compared to the 46% and 49% reported by RAVZANAADII ET AL. (2012) and FINKE (2002), respectively, the CP value observed in this research was lower.

In comparison to our findings, the CP content of 52% was reported by JONES ET AL. (1972), YI ET AL. (2013), and ZHAO ET AL. (2016) was greater. In spite of the fact that all of the studies previously mentioned observed lower chitin concentrations (15% and 8.5% by FINKE (2002) and JONES ET AL. (1972), respectively), they also reported higher protein contents. This was caused by the crude protein's and chitin's overlapping amino acid composition. This may be an overestimate of the bioavailable amino acids (JONES ET AL., 1972; FINKE, 2002; RAVZANAADII ET AL., 2012; YI ET AL., 2013; HAHN ET AL., 2018; ZHAO ET AL., 2016; BORDIEAN ET AL., 2020b). In general, the protein content and ash content found in our investigations increased with age, whereas the amounts of fat decreased, which is consistent with the findings of MEYER-ROCHOW ET AL (2021). The measurement of chitin content showed a higher chitin content than the 15% reported in the study by HAHN ET AL (2018). While the methods used to assess chitin were the same, it is possible that the differences in chitin contents between our study and that of HAHN ET AL. (2018) are due to the various environmental factors and feeding practices. Notwithstanding the fact that the chitin content was higher than the 5.6% reported by FINKE (2007), the reason for the discrepancy may be related to the different chitin measurement techniques. The study adjusted the ADF for the amino acid content to determine the estimated chitin content instead (FINKE 2007).

It showed that amino acids represented 10% to 55% of the ADF (JONES ET AL., 1972; FINKE, 2002; FINKE, 2007; RAVZANAADII ET AL., 2012; YI ET AL., 2013; HAHN ET AL., 2018; ZHAO ET AL., 2016).

The CF measured in our experiment was within the range described by FINKE (2002). However, the fat content was higher than the values of 35.4% and 32.9% reported by JONES ET AL. (1972), and ZHAO ET AL. (2016), respectively, which could be due to the different feeding and environmental conditions.

The YM used in the study of Jones and Zhao were sourced from commercial insect farms, while the study of ZHAO ET AL. (2016). fed wheat, wheat bran and carrot there is no available data on the feed used in the study of JONES ET AL., (1972) (JONES ET AL., 1972; FINKE, 2002; FINKE, 2007; RAVZANAADII ET AL., 2012; YI ET AL., 2013; HAHN ET AL., 2018; ZHAO ET AL., 2016).

Contrary to DM, fibre, chitin, and NFE, which differed significantly, the CP and CF values of the various size groups did not show significant differences.

The large-sized group had higher levels of DM and fibre than the small-sized ones, while the smaller sizes had higher levels of chitin content and NFE.

This is explained by the fact that smaller individuals have a higher surface area to body mass ratio than larger individuals, who have a lower surface area to body mass ratio. This indicates that the exoskeleton makes up a larger percentage of the smaller size than the larger size. Increasing the average body weight during harvest offers the chance to boost DM and decrease chitin content. The ash content was lower than 4.9% and 4.0%, respectively but higher than the 2.4% (FINKE, 2007; ZHAO ET AL., 2016; GHOSH ET AL., 2017).

Nutrient composition based on the developmental stages and of the cuticle

The DM, CP, and chitin levels did not differ significantly between the moult and premoults nutrient compositions. Fat content, however, was an exemption as it was higher for the premoult. The argument presented by FINKE in 2002 that the sclerotization of protein is more significant than chitin in determining the physical properties of the insects' cuticle is further supported by the absence of significant difference between the moult and the premoult (FINKE, 2007). There are no benefits to employing ecdysterone to synchronise moulting, according to the lack of substantial changes between the dark brown premoult and the white newly moulted YM. (SUBRAMANIAN AND SHANKARGANESH, 2016).

However, the pupae exhibit significant differences in the DM, CF, and chitin levels, which were lower than those seen in the moult and premoult stages. In contrast, the CP values are largely constant throughout the three stages and do not exhibit any significant differences.

This might be accounted for by the fact that the YM does not feed during the pupal stage. Quantitative information on the chitin composition of whole insects is scarce. CAUCHIE (2002), however, showed that aquatic insect larvae had 2.9 to 10.1% chitin on a dry weight basis (KLASING, 1998; FINKE, 2002). It is consistent with the findings of DREASI ET AL. (2017), who evaluated the fat content of YM reared on 6 different diets, that pupae have less fat than larvae. Except for two instances, the pupae consistently had less fat than the larvae (DREASSI ET AL., 2017).

Given that most of the moisture content is contained in the larvae's bodies and is shielded by the fat found in the cuticle, the high DM content in the cuticle is self-evident.

However, since the cuticle only makes up a little portion (0.4%) of the overall weight of the larvae, this had no impact on the DM of the premoult and moult. When DM increased, the amount of crude protein, chitin, fibre, ash, and NFE increased while the amount of fat decreased. Our findings were in line with the literature's conclusions that chitin and protein make up the majority of the insect cuticle's principal component. (TAJIRI, 2017, MONDOTTE AND SALEH, 2018).

4.1.3 Optimization Yellow mealworm nutrient composition using feeding substrate.

This chapter the effect of inclusion of duckweed in the feeding substrate of European perch can be shown. First, we show the effect of the different composition on production parameters and then the modifications that can be seen in the nutrient composition. It is important that from the following result that the effect of duckweed on the production parameters need to be considered together in order to draw accurate conclusion.

4.1.3.1 Production parameters

Average body weight

Figure 23. shows the average body weight of yellow mealworms in the different treatments. S75D25 had the highest average body weight, followed by S50D50. There was no statistical difference between the average body weight of yellow mealworms fed diet S and S25D75.

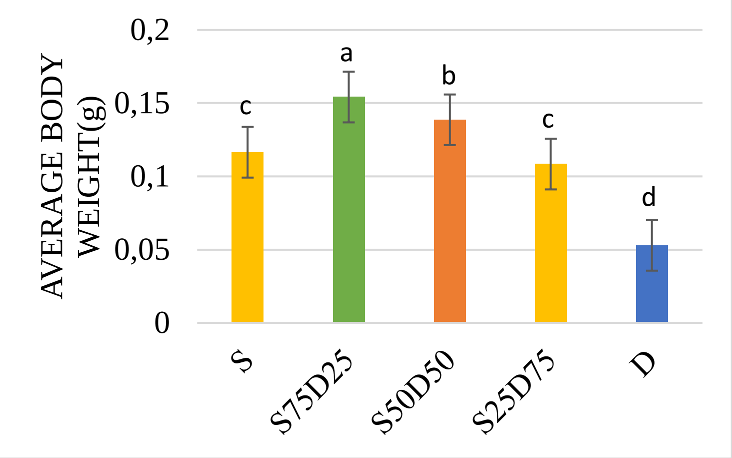


Figure 23: Average body weight of yellow mealworm fed different inclusion level of duckweed.

S: 100% semolina
S75D25: 75% semolina + 25% duckweed
S50D50: 50% semolina +50% duckweed
S25D75: 25% semolina + 75% duckweed
D: 100% duckweed

FCR value

Figure 24. shows the FCR of yellow mealworms reared on different substrates, there was no statistically significant difference in the FCR observed in yellow mealworms fed diets S, S75D25, and S50D50.

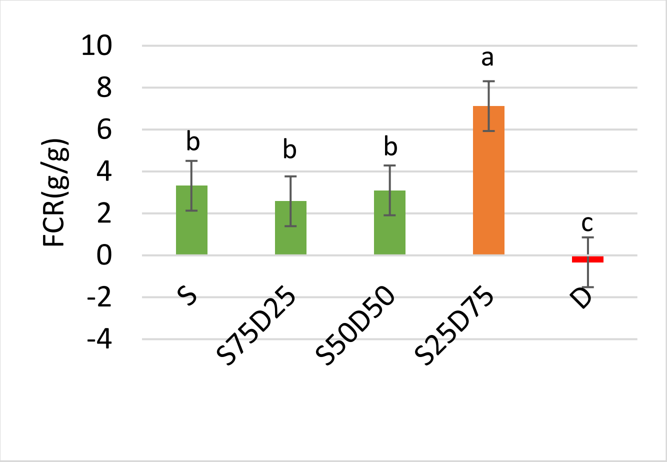


Figure 24: FCR values of yellow mealworm fed different duckweed inclusion level.

S: 100% semolina

S75D25: 75% semolina + 25% duckweed

S50D50: 50% semolina +50% duckweed

S25D75: 25% semolina + 75% duckweed

D: 100% duckweed

Survival rate

Figure 25 shows the survival rate of the different treatments, S and S75D25 had the highest survival rate of all the treatments.

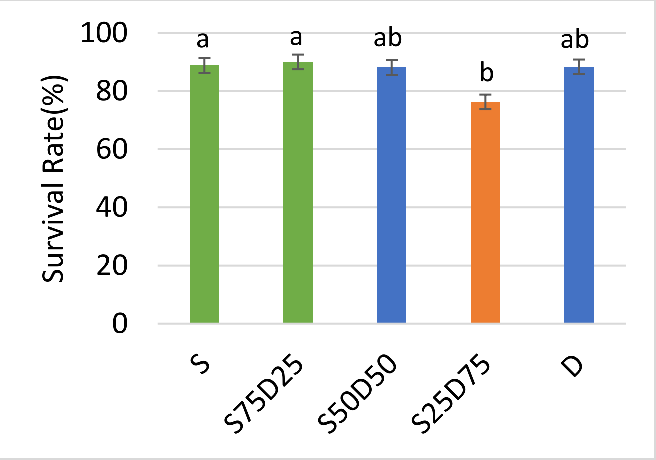


Figure 25: Survival rate yellow mealworm fed different duckweed inclusion level.

S: 100% semolina

S75D25: 75% semolina + 25% duckweed

S50D50: 50% semolina +50% duckweed

S25D75: 25% semolina + 75% duckweed

D: 100% duckweed

4.1.3.2 Nutrient composition

Table 16 shows the nutrient composition of the yellow mealworm, the CP, CF, FB, and ash were significantly affected by every 25% inclusion of duckweed. The chitin did not show a trend and the NFE was the same for all treatments.

Table 16.

Average nutrient composition of yellow mealworm fed different duckweed inclusion level (%)

	S	**S75D25**	**S50D50**	**S25D75**	**D**
Dry Matter	38.3 ± 0.26^{b}	39 ± 0.36^{a}	37.3 ± 0.10^{c}	37.3 ± 0.02^{c}	37.7 ± 0.17^{d}
Crude protein	45.7 ± 0.18^{e}	50.8 ± 0.22^{d}	55.8 ± 0.18^{c}	61.1 ± 0.18^{b}	69 ± 0.22^{a}
Crude fat	43.7 ± 0.22^{a}	37.9 ± 0.28^{b}	30.5 ± 0.27^{c}	24.2 ± 0.14^{d}	14.8 ± 0.20^{e}
Crude fiber	7.4 ± 0.22^{e}	7.9 ± 0.18^{d}	9.4 ± 0.18^{c}	10 ± 0.18^{b}	11.1 ± 0.18^{a}
Crude ash	2.8 ± 0.18^{e}	3.4 ± 0.14^{d}	4.2 ± 0.18^{c}	4.6 ± 0.12^{b}	5.0 ± 0.23^{a}
Chitin	21.5 ± 0.17^{a}	20.0 ± 0.12^{b}	21.5 ± 0.28^{a}	21.8 ± 0.20^{a}	20.8 ± 0.12^{b}

Means in the same row with different superscripts differ significantly ($p< 0.05$).

S: 100 % semolina

S75D25: 75 % semolina + 25 % duckweed

S50D50: 50 % semolina +50 % duckweed

S25D75: 25 % semolina + 75 % duckweed

D: 100 % duckweed

4.1.3.3 Discussion

Production parameters

The highest average body weight was achieved with the S75D25 substrate composition, also the average body weight of the S50D50 was higher than that of S but below 50% inclusion of semolina, the average body weight was lower than that obtained from semolina. This is understandable because the yellow mealworm is a pest of stored grains and flour (BORDIEAN ET AL., 2020a; HAGSTRUM ET AL., 2013), which are rich in carbohydrates. Semolina is rich in starch, which requires less energy to digest into absorbable nutrients.

FCR value

The duckweed has a negative FCR and this was because this substrate contained only duckweed and the mealworm was not able to effectively utilize substrate and convert it to absorbable nutrients and eventually body mass. This can be seen when the average body weight data is considered together with the FCR, the mealworms fed the duckweed substrate did not increase in body weight rather the body weight reduced from what was measured at the beginning of the experiment. The FCR 2.58 obtained in S75D25 is higher than the 1.57 to 2.08 obtained in the studies of BORDIEAN ET AL. (2020b), in mealworms fed chicken feed and wheat bran but lower than the 4.42 obtained in mealworms fed willow leaf sunflower. The FCR obtained in semolina and S50D50 is also lower than those obtained from mealworms fed willow leaf sunflower. The FCR of S75D25 (2.58) is similar to the FCR in chicken (expressed in Kg of feed/Kg of live weight) but the FCR of semolina and S50D50 is higher. All FCR in our experiment is lower than FCR in swine (4.5) and cattle (10) (BORDIEAN ET AL., 2020b).

Survival rate

The survival rate in the different treatments did not follow a particular trend but the highest survival rate was S75D25 while the lowest was obtained in the S25D75. This shows that of all the feeding substrates the S75D25 had the best production parameters.

Nutrient composition

The CP, FB, and ash content followed the same trend increasing with every 25%increase in duckweed inclusion while the CF decrease with every 25% increase in the duckweed composition in the substrate. The protein content of the D100% is the best, but this substrate had the worse production parameters. Considering both the production parameters and nutrient composition, the combination of 25% duckweed and 75% semolina is the most suitable substrate to significantly improve the production parameters and Nutrient composition. These results support what has been reported in other studies that the nutrient composition of yellow mealworm can be enhanced by high nutritional value feedstuff (DREASSI ET AL., 2017; BORDIEAN ET AL., 2020b; & LAWAL ET AL., 2021).

4.1.4. PCR detection of virus

Due to the limitations and scope of this study, we ran PCR detection for 2 viruses, the circovirus, and the iridovirus. These two viruses were selected because they keep emerging in new species over the decade.

4.1.4.1. virus detection in Yellow mealworm samples

Circovirus in Yellow mealworm sample

In *Figure 26.* the second well is the positive well. Comparing the second well to all the 10 samples no other sample shows the same band on the second well indicating none of the samples are positive for circovirus.

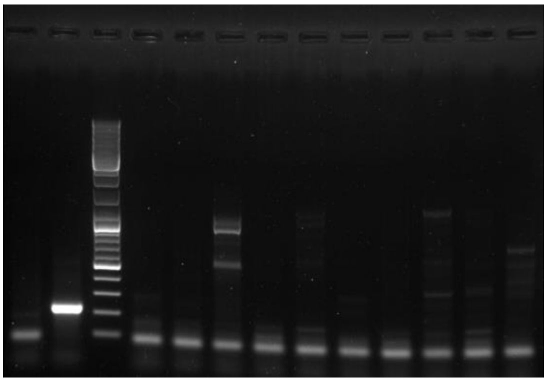


Figure 26: Circovirus PCR: 1st well negative, 2nd well positive, 3rd well marker, and 10 samples (Mealworms)

Iridovirus in Yellow Mealworm Sample

Figure 27. shows the first well shows the negative control, well 2 is the well maker followed by the 10 samples while the last well is the negative control. None of the samples is positive for iridovirus.

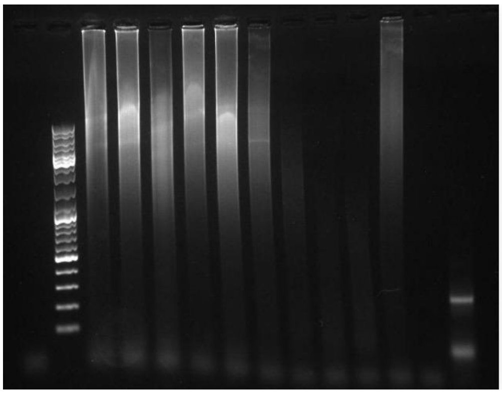


Figure 27: Iridovirus PCR: 1st well negative control, 2nd well marker, then 10 samples (mealworms) and the positive control.

4.1.4.2. Virus detection in European Perch sample

Circovirus in European Perch sample

Figure 28. shows the PCR result of the circovirus test in European perch kidney, liver, and spleen mixed samples. The first well show the negative control and all samples from well 3 to well 12 shows a similar band as the negative control.

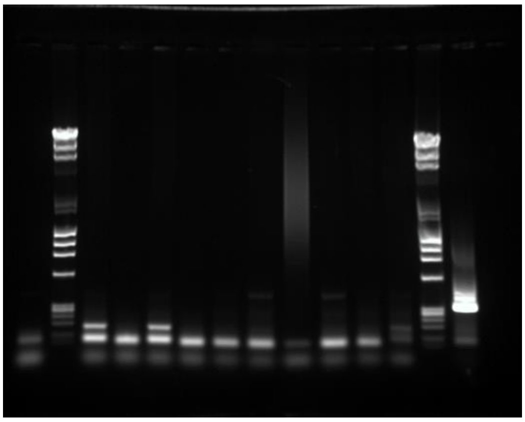


Figure 28: Circovirus PCR: 1st well negative control, 2nd well marker, then 10 samples (perch) and the positive control

Iridovirus in European perch Sample

Figure 29. shows the PCR result of the iridovirus test in European perch kidney, liver, and spleen mixed samples. The first well shows the negative control and all samples from well 3 to well 12 show similar band to the negative control.

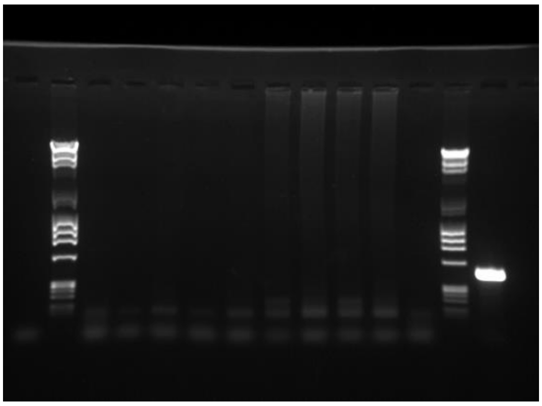


Figure 29: Iridovirus PCR: 1st well negative control, 2nd well marker, then 10 samples (Perch) and the positive control

4.1.4.3. Discussion

Circoviruses are emerging continuously, although there are only 2 that have been directly connected to a disease, the immune-suppressing nature of this virus cannot be trifled with (TARJÁN ET AL., 2014). This virus has now been detected in barbel catfish (LŐRINCZ ET AL., 2011), European eel (BORZÁK ET AL., 2017), common native toad and wild fish (common bream and monkey goby) from the Danube (TARJÁN ET AL., 2014). Although DENNIS ET AL. (2018), reported the novel circovirus sequences in invertebrate genome assemblies there was no circovirus detected in both yellow mealworm and European perch samples.

Iridovirus has been identified in insects and has also been shown to cause clinical interest in fish (WILLIAMS ET AL., 2005). This is the reason we decided to investigate the presence of this virus in both our insect and fish sample, in order to establish the safety of our samples concerning iridovirus.

Our results show that these viruses are not present in our mealworm samples and European perch samples. Hence, they may not be a safety concern in the use of insects as fishmeal replacement.

4.2. Nutrition experiments with European perch

The goal of these experiments was to ultimately exchange/ complement, the current plant and animal-based protein sources in European perch (*Perca fluviatilis*) feed with yellow mealworm meal. The European perch has a low feeding rate compared to other species, for example, the yellow perch juvenile will consume up to 4% of their body weight but the European perch juvenile will only consume up to 2.2% of their body weight and this percentage reduces as they grow (FIOGBE & KESTEMONT, 2003). Their feeding rate, therefore, requires that high-quality protein be provided in their diet for them to reach their growth potential. This will not only help the digestive processes of fishes, but it would also be a more economical and sustainable protein source for fish feed source. We intended to investigate the digestibility of commercially available feed with crude extracts of perch stomach and intestine content, taking into consideration two variables: the time that has passed since feeding the investigated specimens, and the effect of ambient temperature (16-22 °C) on digestive enzyme activity and digestibility. The protein digestibility protocol developed according to the results of the previous experiments will allow us to optimize the quantity and quality of alternative protein in perch feed– from the perspective of optimal protein availability.

According to the standardized human INFOGEST protocol (BRODKORB ET AL., 2019), the most important factors that have to be determined in case of fish are temperature, feed-digestive fluid ratio, and the time of passage through the gastrointestinal tract, and the activity of the digestive enzymes.

4.2.1. The effect of temperature on digestive enzyme activity of fish

Figure 30. shows the changes in enzyme activities at different rearing temperatures the enzyme activity increased at 18 °C, peaked at 20 °C and declined at 22 °C. One of the main factors that determine protein bioaccessibility is digestive enzyme activity. As the ambient temperature influences enzyme activity, we investigated the effect of water temperature (16 °C, 18 °C, 20 °C and 22 °C) on digestive enzyme activity of fish.

Our goal was to determine whether the ambient temperature alteration between 16-22 °C can have a significant effect on the digestive physiology of perch.

The impact of 16 °C, 18 °C, 20 °C and 22 °C on pepsin, trypsin, total alkaline proteolytic, lipase, and amylase activity is presented in *Figure 30*.

The pepsin activity was the lowest at 16 °C and at 22 °C (40480.14 and 40236), 32 U/mg protein, respectively). At 18 °C it increased to 69245.91 U/mg protein, while at 20 °C it reached an average of 79305.86 U/mg protein.

Trypsin activity increased progressively (but not significantly) between 16-20 °C (it was 7.77; 9.88; and 11.12 U/mg protein), then it was diminished at 22 °C to 5.42 U/mg protein.

Total alkaline proteolytic activity was 1.47 U/mg protein at 16 °C; 1.14 U/mg protein at 18 °C; 1.25 U/mg protein at 20 °C; and 0.84 U/mg protein at 22 °C.

We measured the highest lipase activity, 1.162 U/mg protein at 18 °C. The lipase activity of the intestinal extract of fish kept at 16 °C was 0.40 U/mg protein. The lipase activity at 20 °C and 22 °C was very similar, being 0.72 U/mg protein and 0.76 U/mg protein on average.

The amylase activity increased between 16-22 °C, from 0.29 U/mg protein at 16 °C to 1.17 U/mg protein at 22 °C. The amylase activity measured at 16 °C, was not significantly different from the one at 18°C, which was 0.40 U/mg protein. At 20 °C, the amylase activity was 0.98 U/mg protein and did not differ significantly from the average activity at 22 °C.

The pepsin, trypsin, total alkaline proteolytic, lipase, and amylase activity were significantly dependent on the ambient temperature of *P. fluviatilis* when we applied the ANOVA test for data concerning all the investigated temperatures (16 °C, 18 °C, 20 °C, 22 °C).

The pepsin activity at 18 °C and 20 °C is significantly higher than the one measured at 16 °C and 22 °C (Tukey HSD, $p<0.05$).

The trypsin activity of the European perch is significantly (Tukey HSD method, $p<0.05$) higher at 18 °C and 20 °C, than at 22 °C.

The trypsin activity at 16 °C was lower than in case of perch kept at 18 °C or 20 °C. In contrast, in case of total alkaline proteolytic activity (PEÑA ET AL. 2015; GARCÍA-ORTEGA ET AL. 2000), -casein hydrolysis, measured at pH 8.5- the activity was higher at 16 °C than at 18 °C or 20 °C. In case of perch kept at 16 °C or 22 °C, the trypsin activity did not differ significantly, while 16 °C induced significantly higher total proteolytic activity than 22 °C, according to the Tukey HSD test ($p<0.01$).

There was no difference in total alkaline proteolytic activity at 16 °C, 18 °C, and 20 °C with Tukey HSD Test. 18 °C, 20 °C, and 22 °C also did not differ significantly according to Tukey HSD method.

The lipase activity at 18 °C was significantly higher (Tukey HSD Test, $p<0.01$) than 16 °C, 20 °C, and 22 °C. Although the lipase activity at 20 °C and 22 °C was lower than 18 °C, it was still significantly higher (Tukey HSD method, $p<0.05$) than at 16 °C.

The perch kept at 20 °C and 22 °C had significantly higher α-amylase activity than those that were cultured at 16 °C and 18 °C (Tukey HSD Test $p<0.001$).

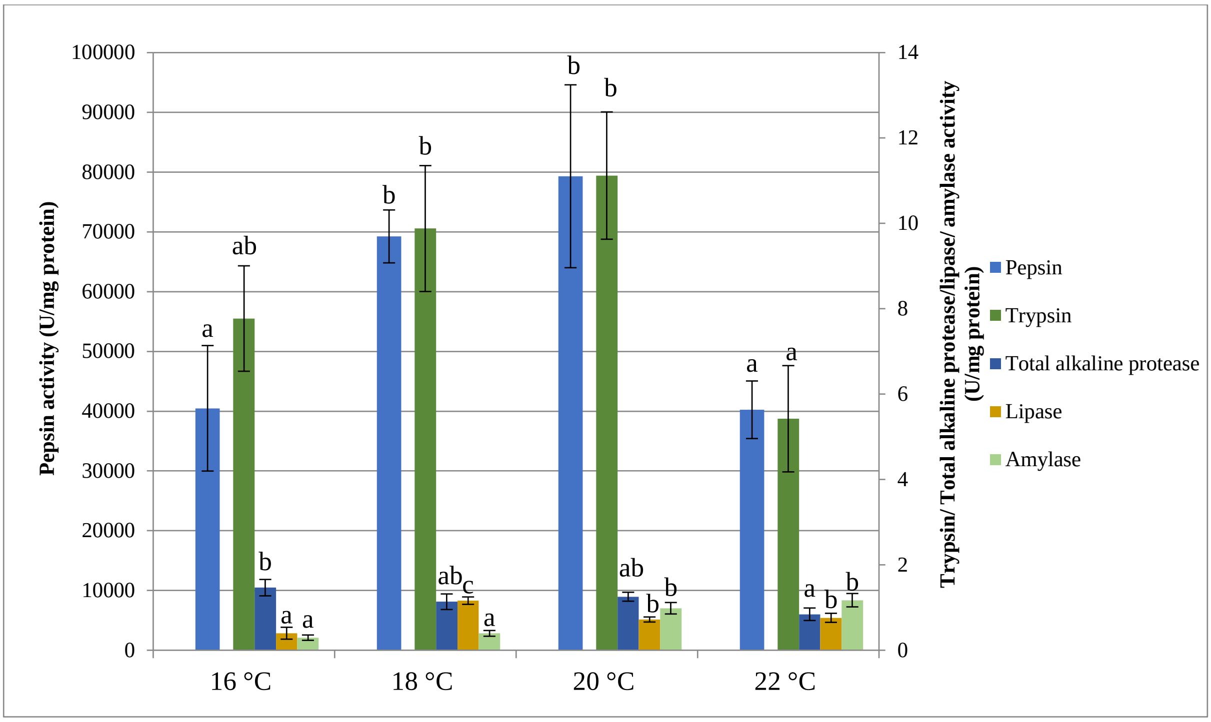


Figure 30: Digestive enzyme activity measurements of perch (*Perca fluviatilis*) kept at different water temperature.

Different letters designate significant difference (TukeyHSD method; $p<0.05$). The left axis of this figure represents pepsin activity, while the activities of the other enzymes are shown on the right axis.

4.2.1.1. Discussion

In the cavity of fish intestine proteins are digested by trypsin, chymotrypsin, elastase, collagenase, aminopeptidases, carboxypeptidases, the proteases of intestinal microbiota, and enzymes originating from live prey (KUZMINA, 2008; KUZMINA ET AL., 2015).

In our case, lysosomal cathepsins and cytosolic caspases from live prey do not contribute to the intestinal casein-lytic activity because the *Perca fluviatilis* specimens, which we were investigating, did not consume live prey. Studies of various fish species describe trypsin, chymotrypsin, carboxypeptidases, aminopeptidases, elastases, and collagenases as the most relevant casein-lytic proteases (PEÑA ET AL., 2015). The intestinal microbiota can also hydrolise casein (KUZMINA ET AL., 2015).

According to EINARSSON (1993) the most important endopeptidases in the fish digestive tract are trypsin and chymotrypsin; the quantity of other endopeptidases (e. g. elastase, collagenase) – if they are present- is low. Erepsin, which represents exopeptidases from the intestinal wall and lumen (SHREEVE, 2009), and is designated as the collective name of aminopeptidases, di- and tripeptidases (FÄNGE AND GROVE, 1979; HOLICKY, 1989) can hydrolyse casein (KENYON, 1925), so it may contribute to the total proteolytic activity measurement protocol based on WALTER (1984). Therefore, the total alkaline protease activity represents the sum of the activity of trypsin, chymotrypsin, carboxypeptidases, erepsin (aminopeptidases, di- and tripeptidases), proteases of the intestinal microbiota, and - in a less dominant manner, than the other does- enzymes elastase and collagenase.

Thus, our hypothesis that even a slight change in water temperature (between 16 °C and 22° C) can significantly influence digestive activity was proven to be right. Still, the maximal activity (in this temperature range) was not the same for all enzymes. When choosing the optimal temperature, we must take into consideration the activity of all measured digestive enzymes (pepsin, total alkaline proteolytic, trypsin, lipase, α-amylase). Probably, out of these enzymes, α-amylase is the least important, because perch usually consumes invertebrates and fishes, and amylase activity is minimal (GOLOVANOVA ET AL., 2013; GOLOVANOVA, 2010).

Summarizing our results, we can say, that the ambient temperature affected all investigated digestive enzyme activities significantly. Pepsin and trypsin activities were the highest at 18 °C and 20 °C. Total alkaline proteolytic activity was significantly higher at 16 °C than at 22 °C, but it did not differ significantly from the activity at 18 °C and 20 °C. According to these results, 18 °C and 20 °C are the most favourable for proteolysis.

Protein digestibility is also influenced by the degradation of the food matrix. Lipase activity was the highest at 18 °C, while 20 °C and 22 °C were more favourable to amylase activity.

According to literature, although the feeding habits of fish may influence enzyme activities, and in some carnivorous species low carbohydrase activities can be detected, it was proven that some carnivorous species (like perch) have α-amylase activities (LANGELAND ET AL., 2013). Still, the lipase activity of perch is much higher than the carbohydrase activity, because carnivorous fish consume a diet rich in fat (LANGELAND ET AL., 2013).

Thus, if the preponderant part of the feed is made of lipids, we recommend keeping the stock at 18 °C. From a technological point of view, 18 °C is more advantageous, because it is more stable, and has a better O_2 storing capacity because oxygen has greater solubility in colder water (HARVEY ET AL., 2011).

Water temperature can influence the synthesis, secretion, and catalytic properties of a digestive enzyme and fish can adapt to environmental temperature with various isoenzymes (HANI ET AL., 2018). An alteration of the environmental temperature can also affect food intake and/or gastrointestinal transit time, which in turn may also modify digestive enzyme activity (HANI ET AL., 2018; MIEGEL ET AL., 2010). Thus, the evaluation of the effect of water temperature on fish digestive enzyme activity cannot be described by one simple interaction. Ambient temperature influences digestibility by modifying the activity of the digestive enzymes (VOLKOFF AND RØNNESTAD, 2020). The determination of the proper ambient temperature for cost-effective European perch rearing can be supported by our results regarding the effect of water temperature (16 °C, 18 °C, 20 °C, 22 °C) on the digestive enzyme activity of *Perca fluviatilis*.

Protease activity determination is especially important, because trypsin and chymotrypsin activity was correlated with the growth of fish (LEMIEUX ET AL., 1999; RUNGRUANGSAK-TORRISSEN ET AL., 2006).

Numerous studies have established, that trypsin has an outstanding importance in the growth of fish through the digestion of feed proteins (RUNGRUANGSAK-TORRISSEN ET AL., 2006).

According to our results 18 °C and 20 °C seemed to be the most appropriate for maximal protease (pepsin, total alkaline proteolytic, trypsin) activity. XIONG ET AL. (2011) reported that Pepsin activity was highest at lower pH of 1.5 found in the stomach of sirsord catfish, which is more than five times the proteolytic activity at pH 7.5 found in the intestine (XIONG ET AL., 2011). The higher protease activity in comparison to lipase and amylase correlates with the result from our study.

Trypsin activity was highest at 20 °C according to our results but peak trypsin activity was reported at 17 °C and 18 °C in seabass and threespine-stickleback respectively (PEREIRA ET AL., 2018).

The temperature dependence of trypsin activity at 16 °C and 20 °C, is not reflected in the tendency of total alkaline proteolytic activity (at pH 8.5) because the latter is made of several components. In our case, the most important components of total proteolytic activity –besides trypsin- probably are chymotrypsin, aminopeptidases (erepsin), carboxypeptidases, and intestinal microbiota proteases. Applying enzyme-specific measurements could help us elucidate which are the main components of the total proteolytic activity at pH 8.5.

The highest lipase activity was detected at 18 °C, this is important since lipids are more relevant (LANGELAND ET AL., 2013) in the feed of carnivorous fishes because they are able to better digest lipids, than carbohydrates (TAŞBOZAN AND GÖKÇE, 2017).

PEREIRA ET AL., 2018 reported a peak lipase activity in seabass at 23 °C, although seabasses are also carnivores like perch there are species differences in enzyme activities but generally, they follow the same trend. XIONG ET AL., (2011) examined the digestive enzyme activity of carnivore sisorid catfish and found an average of 0.61 U/mg protein in the intestine, which is lower than the lipase activity in our experiment. The temperature at which the fishes were caught from the wild was between 12 °C -14 °C that shows the effect of temperature on lipase activity. 18 °C is more advantageous for increasing lipase activity and hence improved digestion according to our findings. We recommend the simulation of in vitro conditions at 18 °C.

We proved that rearing temperature (16 °C, 18 °C, 20 °C and 22 °C) influences digestive enzyme activity significantly. This fact should be also taken into consideration in perch industry management practices and during the evaluation of feed components with a static in vitro digestion model.

4.2.2. European perch feeding experiment with Yellow mealworm meal diets

The results shown in this chapter how the partial replacement of fishmeal with YM meal at different inclusion levels impacted the weight gain FCR and survival rate.

Table 17. show the weight gain, FCR, and survival rate of European perch fed with YM, there was no significant difference between all production parameters of all treatments compared to the control.

Table 17.

Average weight gain, FCR and survival rate of European perch

	CONTROL	YM 12.5%	YM 25%	YM 37.5%
IBW	39.75±0.76^{b}	40.98±0.76^{ab}	41.39±0.76^{a}	39.99±0.76^{ab}
Weight gain(g)	16.1±0.53	16.2±0.41	16.3±0.53	16.2±0.56
FCR(g/g)	1.41±0.05	1.46±0.03	1.47±0.04	1.42±0.04
Survival rate(%)	99.4±1.4	99.7±0.6	99.8±0.3	99.5±1.1

Means in the same row with different superscripts differ significantly ($p< 0.05$).

IBW: initial body weight

4.2.4.1Discussion

There was no significant difference between all production parameters when 12.5%, 25%, and 37.5% of fishmeal was replaced with YM meal except for the IBW but this did not reflect on the weight gain and FCR. This shows that replacing up to 37.5% of fishmeal had no effect on the growth of European perch, hence reducing the quantity of fishmeal required in producing feed. The more sustainable protein source YM meal can therefore be included in European perch feed, improving the sustainability of the protein source.

A similar study was carried out to check the growth performance of European perch fed the experimental diet with 25%, 50%, and 75% replacement of fishmeal with defatted YM meal. The results showed that there was no significant difference in the growth performance between the control diet and 25% defatted YM meal which is similar to the result observed in our study. At 50% and 75% inclusion of defatted YM meal had detrimental effects on the growth performance.

This study associated the declining growth performance with the increasing chitin content with the increasing inclusion rate of defatted YM meal, which leads to reduced nutrient digestibility. The survival rate observed in our study is similar to the >98% reported in this study as well (TRAN ET AL., 2022).

Another study reported the effect of partial replacement of fishmeal (20%, 40% & 60%) with partially defatted BSF in European perch feed.

The results from the studies show that growth performance was not affected at 20% and 40% inclusion which is consistent with the result from our study, but there was reduced growth performance at 60% inclusion. There is a unanimous conclusion to be drawn from the results of this study and that of STEJSKAL ET AL., (2020a) and TRAN ET AL., (2022) that insect meal inclusion rate above 50% has a detrimental effect on the growth performance of European perch. The FCR obtained in the current study at 25% (1.47) is higher than the 1.19 obtained in the study of TRAN ET AL., 2022 at a 25% inclusion rate, this could be because defatted YM meal was used as opposed to the full-fat YM meal used in this current study.

5. CONCLUSIONS

Yellow mealworm has the potential to become one of the key protein sources in feed production, hence there is a need for in-depth knowledge of the protein content and quality. Using rats as test animal show that a diet containing 100% soy as a protein source produce a statistically similar result in growth, PER, NPR, and TD as the same diet with 50% and 25% replacement of soy with yellow mealworm meal. This result shows that the protein quality was not adversely affected by the inclusion of yellow mealworm meal. This result cannot directly be used as an interpretation for the effect when a different animal is used as the test animal e.g. fish. However, in this study, it served as a guide for the formulation of test diets for the European perch. It can also be applied in similar nutrition studies as a guide for the formulation of new test diets.

Yellow mealworm has great potential as an alternative protein source in the aquaculture industry and the European perch nutrition too. This study shows that there is no significant difference in the composition of YM larvae of different age groups. Our results show that the size influences the DM, fibre, chitin contents, and NFE, therefore, by exploring feed substrates that can cause a rapid increase in the average body weight, insect farmers can achieve a shorter production time, a higher dry matter content, a lower chitin content, and a lower NFE, and ultimately a better digestibility. Considering the difference in growth rate observed between the large and small yellow mealworms it is advisable that yellow mealworm farmers practice partial harvesting so that the larger-sized mealworms are harvested while the smaller-sized mealworms have more time to grow. The higher DM and lower chitin content of large-sized larvae, compared with the lower DM and higher chitin content of small-sized ones, indicates an inverse relationship between the DM and chitin. The difference in the chitin content is in line with our hypothesis that the higher the surface area/body mass ratio, the higher the chitin content.

There are several studies aimed at finding a cost-effective diet for the commercial production of yellow mealworm, there needs to be more research into choosing an appropriate diet to improve the growth rate for a better body mass/body surface ratio. Duckweed is effective in improving the production parameters and nutrient composition at the right inclusion rate (25-50%).

Although with 100% duckweed feeding substrate the crude protein content is by far the highest, this result needs to be considered side by side with the result of the production parameters to arrive at a viable result.

The conclusions drawn from the results show that the maximum inclusion level of duckweed to still achieve a significantly higher crude protein content is 50%, although the highest growth rate is achieved at 25% inclusion of duckweed it is important for farmers to decide whether the highest growth rate is more important for their objective or a balance between a significantly higher growth rate and crude protein content. This result shows that the inclusion of duckweed is promising in the diet of yellow mealworm, it will therefore be worth it to consider using the duckweed with other widely used feeding substrates for yellow mealworms such as wheat bran. This could be necessary to determine if the combination of duckweed and wheat bran could yield better results. Another research that could prove to be beneficial to the use of duckweed is to consider the use of duckweed concentrate as part of the feeding substrate of the yellow mealworm, protein concentrate from greater duckweed is about 64.6% (FASAKIN, 1999)

The safety of using insect meal is yet to be explored but within the limitation of this research, we touch on one of the risks associated with its use. We found that although the circovirus and iridoviruses continue to be reported in new species they are not a safety risk in the yellow mealworm and European perch samples used in this experiment. This result barely scratches the surface concerning the microbial risk associated with the use of insect meal, hence more research needs to be done in this area. Other viruses need to be researched to ensure that they will not pose a safety risk in the use of yellow mealworms as food or feed. Another plausible safety concern is the less understood prions which are mainly transmitted through animals and can cause disease in animals and humans. The scope of this study does not cover the topic of prions and there is an opportunity for research to determine if they pose a safety risk to the use of yellow mealworm as a novel food and feed source. Results obtained from the enzyme activity measurement in the temperature experiment provide a new consideration for the optimal rearing temperature.

This data shows that temperatures between 18 ℃ and 20 ℃ is optimal for European perch digestion providing farmers a basis for reconsidering the 23 ℃ optimal temperature for the rearing of the European perch. Our results support the implementation of feeding and rearing procedures that employ the optimal protein digestibility parameters regarding temperature.

From our result, we propose that farmers take advantage of the opportunity farmers make a better choice regarding rearing temperature. This may result in a reduction of energy consumption by reducing the rearing temperature and could help maximise the effectiveness of the feed provided to the fish.

This could make a huge difference in the effectiveness of the feeding technology in this species because of the lower buffer capacity of feed compared to species with higher feeding rates. Another possibility to improve the digestibility of yellow mealworms in this species because of their low feed buffer capacity could be to incorporate the use of chitinase enzyme in other to utilize the chitin present in yellow mealworms and other insects.

The results of the nutrition experiment where we replaced 12.5%, 25%, and 37.5% of fishmeal with yellow mealworm meal shows that yellow mealworm meal can replace fishmeal up to 37.5% without any adverse effect on growth, FCR, and survival rate of the European perch.

6. NEW SCIENTIFIC RESULTS

1. The protein efficiency ratio, net protein ratio and true digestibility (biological value) of feed containing 100% soy as a protein source is statistically the same as the same feed when 25% and 50% of the soy is replaced by yellow mealworm meal in an in-vivo rat experiment. The protein efficiency ratio is 2.15 g/g, 2.23 g/g and 2.36 g/g for 100% soy, 25% yellow mealworm and 50% yellow mealworm diet respectively. The net protein ratio is 1.35 g/g, 1.44 g/g and 1.54 g/g for 100% soy, 25% yellow mealworm and 50% yellow mealworm diet respectively. The true digestibility is 86.3%, 84.7% and 85.6% for 100% soy, 25% yellow mealworm and 50% yellow mealworm diet respectively.
2. Yellow mealworm chitin content is affected by the size. The larger sized larvae had a chitin content of 21.93%, 21.40%, and 20.87% at week 8, week 10 and week 12 respectively and this significantly lower than the chitin content of the smaller sized larvae 23.53%, 23.10% and 22.50% at week 8, week 10 and week12 respectively.
3. Our result shows 25% duckweed and 75% semolina as a feeding substrate of yellow mealworm can increase the average body weight by 25% after 6weeks compared to a feeding substrate made up of 100% semolina. Similarly, 50% duckweed and 50% semolina substrate can improve the average body weight of yellow mealworm by 16.7% compared to 100% semolina.
4. 100% duckweed as a feeding substrate for yellow mealworm increased Crude Protein by 48.57% while it reduced Crude Fat by 66.67%, the yellow mealworm larvae however did not grow on this feeding substrate.
5. Yellow mealworm meal can replace fishmeal in the diet of European perch up to 37.5% without any adverse effect on growth, feed conversion ratio and survival rate.
6. Our data shows that highest pepsin, and lipase activity can be achieved at temperature between 18 °c and 20 °c indicating an optimal digestion at this temperature. When rearing temperature was increased from 16 °c to 18 °c pepsin, and lipase activity increased by 71.06%, and 190% respectively. When the temperature was increased from 18 °c to 20 °c the pepsin increased by 14.53%.

7. PRACTICAL RESULTS

1. The rat in-vivo experiment provides information on the quality of the yellow mealworm protein and how it compares to soybean meal as a protein source, this provides feed producers with information that can serve as a guide when using yellow mealworm meal in feed formulation.
2. Results from analysis of YM nutrient composition can provide insight to commercial insect farmers that there is an opportunity to explore diets that can shorten the life cycle of YM as a result of faster and better growth without loss of quality in terms of nutrient composition. It also provides information to farmers regarding moult and premoult larvae, it informs farmers that it is futile to synchronise moulting in YM larvae as this does not affect the chitin content of the larvae.
3. Results from the optimization of YM nutrient composition and production parameters can be used by insect farmers to achieve faster, better growth while also improving the nutrient composition of YM by using duckweed as part of the feeding substrate.
4. The results obtained from the enzyme activity at different rearing temperatures can help European perch farmers reconsider the optimal rearing temperature in order to maximize protein digestion and ultimately effective utilization of feed, thereby reducing the cost of production. These results can also help farmers reduce the energy cost by reducing rearing temperature from 23 ℃ to 20 ℃.

8. ÖSSZEFOGLALÁS

A világon a halak és a belőlük készült haltermékek iránti kereslet folyamatosan nő. Részben ennek köszönhetően is, az akvakultúra az egyik leggyorsabban növekvő állattenyésztési szektor, Újabb és újabb fejlesztések jelennek meg az ágazatban, de még mindig sokat tehetünk a hatékonyabb termelésért. Az akvakultúrás termelés költségeinek legnagyobb részét a takarmányozási költségek teszik ki. A takarmányozáson belül pedig a legdrágább takarmányösszetevők a fehérjehordozók. Célunk, hogy a rovarfehérjét alternatív fehérjeforrásként alkalmazzuk, különösen ragadozó halfajok esetében ahol a fehérjeszükséglet kifejezetten magas.

A Phd kutatásaim során a közönséges lisztbogár lárvára (*Tenebrio Molitor*) más néven lisztkukacra (Yellow mealworm) fókuszáltam, mint kiegészítő fehérjeforrásra a szóba jöhető rovarfajok köszül. Vizsgáltam a közönséges lisztkukac tápanyag-összetételét, optimalizáltam a termelését, megvizsgáltam a vírusterheltségét. A lisztkukac esetében a fehérjehatékonysági arányt (PER), nettó fehérje arányt (NPR) és valódi emészthetőséget (TD) patkány kísérletek segítségével végeztük el, így a lisztkukac fehérjéjének a minősége összehasonlíthatóvá vált más fehérjeforrásokkal.

A halas kísérletek célállata a sügér (*Perca fluviatilis*) volt. Elsősorban azért ezt a halfajt választottuk, mert egyrészt nagy értéket képvisel, másrészt pedig a rovar a természetben is a táplálékbázisát képezi. Alapvetően két kísérletet végeztünk. Egyrészt annak érdekében, hogy a sügér takarmányozása során emésztésbiológiai szempontból a legjobb környezetet tudjam biztosítani beállítottam egy kísérletet arra vonatkozóan, hogy megtudjam, milyen hőmérsékleten emészt a sügér a legjobban, ezzel is optimalizálva a takarmányozását. Az optimális hőmérséklet megtalálása után összehasonlítottam különböző arányú lisztkukac és halliszt tartalmú tápok hatását a sügér növekedésére és takarmányhasznosítására.

Valamennyi hal és lisztkukac nevelési kísérletet a Debreceni Egyetem Halbiológiai Laboratóriumában állítottuk be. Az emésztési és emészthetőségi kísérletet a KÉKI-vel (Központi Élelmiszer-tudományi Kutatóintézet) közösen, a vírusok kimutatását pedig az Állatorvostudományi Kutatóintézet munkatársaival végeztük.

A lisztkukac tápanyag-összetételét vizsgáltuk különböző életkorokban, méretekben és életciklusokban is. Az eredmény alapján megállapíthatjuk, hogy a lárvák életkor alapján nem különböznek, de a méret hatással van a tápanyag-összetételükre. A nagyobb méretű lárváknak kisebb volt a felület/tömeg aránya, ezáltal kisebb volt az exoskeleton aránya (alacsonyabb kitintartalom) és magasabb a szárazanyagtartalma.

Az in vivo patkány kísérletek eredménye azt mutatta, hogy a 25% és 50%-os arányban bekevert lisztkukac liszttel etetett kísérleti patkányok növekedési tendenciája magasabb volt, mint a szójaliszttel etetett patkányoké, bár statisztikailag nem volt eltérés. Ez azt jelenti, hogy a szója akár 50%-os lisztkukac liszttel való helyettesítése kedvezően hat a kísérleti állatok súlygyarapodására, és a fehérje minősége sem különbözött, ahogyan azt a PER, NPR és TD értékek mutatják.

Az egyik lisztkukacos kísérlet során békalencsét (*Spirodela polyrhiza*) is használtunk takarmányozási szubsztrátként, hogy meghatározzuk annak hatását a lisztkukac termelési paramétereire és tápanyag-összetételére. Az eredmények azt mutatják, hogy a békalencse 50% feletti részaránya a takarmányozási szubsztrátban már rosszabb termelési paramétereket eredményezett. A termelési paraméterek és a tápanyag-összetétel eredményét figyelembe véve ebben a kísérletben az 25%-os békalencse tartalom volt a legkedvezőbb.

A lisztkukac és a süger mintákat cirkovírusok és iridovírusok szempontjából elemeztük, és megállapítottuk, hogy ez a két vírus nem jelent biztonsági kockázatot. A vizsgálatok során minden minta negatív volt e két vírusra nézve.

Az enzimaktivitás különböző hőmérsékleteken mért eredménye azt mutatja, hogy a legmagasabb enzimaktivitási érték 18 °C és 20 °C között van, 20 °C-on tetőzik, majd ezt követően csökken. Ugyanebben a fejezetben található az európai süger 12,5%, 25% és 37,5%-ban hallisztet lisztkukac liszttel helyettesítő tápokkal történő kísérletnek az eredménye is. A vizsgálat azt mutatja, hogy a különböző helyettesítési szinteken nincs jelentős különbség a termelési paraméterekben, így akár a legmagasabb arányban (37,5%) is helyettesítheti a lisztkukac liszt a hallisztet anélkül, hogy káros hatással lenne a süger növekedési ütemre, az takarmányozási együtthatóra (RTE) re vagy a halak megmaradási százalékára.

9. SUMMARY

The demand for fish and fish products is increasing daily, although the aquaculture industry is the fastest-growing animal production industry there is still a lot to be done concerning reaching the efficiency of the livestock industry. The cost of feeding takes the highest part of aquaculture production cost; protein sources are the most expensive feed ingredient. Insects have been proposed as an alternative protein source and they are especially well suited for carnivore species because European perch already consume more than 50% insects in their natural diet. To develop an effective feed formulation an understanding of the digestive processes is important.

The research conducted during this dissertation explored the nutrient composition of yellow mealworm (YM), optimization of production, and nutrient composition of YM and also compared the growth, protein efficiency ratio (PER), net protein ratio (NPR), and true digestibility (TD) of diets containing 25% and 50% yellow mealworm meal to soybean meal using a rat assay. We continued to gain a proper understanding of the digestion processes of the European perch, by investigating the enzyme activity at different temperatures lower than the reported optimal. The various experiment provided a basis for the formulation of a diet where various percentages of fishmeal were replaced with yellow mealworm meal, this diet was used in a feeding experiment.

All fish and YM rearing experiments were carried out at the fish laboratory of the University of Debrecen. All digestion and digestibility experiments were carried out in conjunction with ÉKI (Élelmiszer-tudományi Kutatóintézet). The virus detection was carried out at Állatorvostudományi Kutatóintézet.

In Chapter 4.1. we explored the nutrient composition of YM at different ages, sizes, and stages of life cycle. The result shows that larvae do not differ based on age, but the size has an effect on the nutrient composition, the larger-sized worm had lower SA hence a lower proportion of exoskeleton and higher dry matter content.

We also used duckweed as a feeding substrate to determine its effect on production parameters and nutrient composition of YM, the result indicates that above 50% inclusion of duckweed in the feeding substrate result in worse production parameters. When we considered the result of the production parameters and the nutrient composition in this experiment the S75D25 feeding substrate is the best.

From the rat, in-vivo experiment the growth of test rats, feed 25% and 50% YM meal replacement was higher than those fed soybean meal although not statistically and the biological values were also not statistically different.

This means that up to 50% replacement of soy with YM meal is beneficial to the weight gain of the test animals and the quality of protein was no different either as seen in the PER, NPR, and TD.

We analysed YM and European perch samples for circoviruses and iridoviruses and found that these 2 viruses are not a safety concern for the use of YM meal in European perch feeding in our experiment. All samples tested were negative for these 2 viruses.

The result of enzyme activity at different temperatures shows that the highest enzyme activity is between 18 °C and 20 °C, the enzyme activity peaks at 20 °C after which there is a decline in the enzyme activity. In the same chapter, the result of feeding European perch with feed formulated replacing 12.5%, 25%, and 37.5% fishmeal with YM meal was shown. The result shows that at all 3-inclusion levels, there is no significant difference in production parameters, hence YM can replace fishmeal up to 35.7% without adverse effects on the growth rate, FCR, and survival rate.

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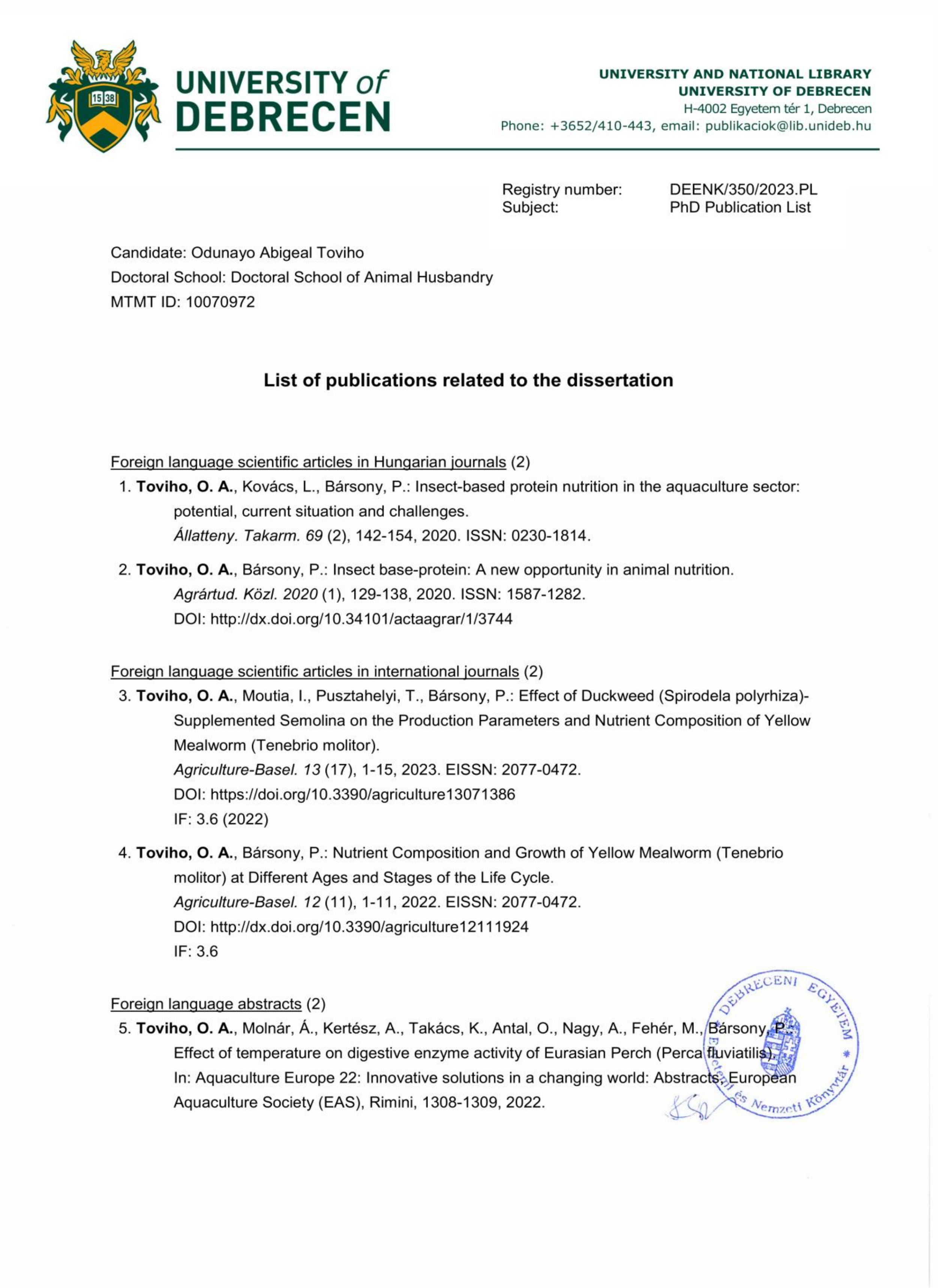
11. PUBLICATIONS IN THE FIELD OF RESEARCH



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Registry number: DEENK/350/2023.PL
Subject: PhD Publication List

Candidate: Odunayo Abigeal Toviho
Doctoral School: Doctoral School of Animal Husbandry
MTMT ID: 10070972

List of publications related to the dissertation

Foreign language scientific articles in Hungarian journals (2)

1. **Toviho, O. A.**, Kovács, L., Bársony, P.: Insect-based protein nutrition in the aquaculture sector: potential, current situation and challenges.
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2. **Toviho, O. A.**, Bársony, P.: Insect base-protein: A new opportunity in animal nutrition.
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   DOI: http://dx.doi.org/10.34101/actaagrar/1/3744

Foreign language scientific articles in international journals (2)

3. **Toviho, O. A.**, Moutia, I., Pusztahelyi, T., Bársony, P.: Effect of Duckweed (Spirodela polyrhiza)-Supplemented Semolina on the Production Parameters and Nutrient Composition of Yellow Mealworm (Tenebrio molitor).
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   DOI: https://doi.org/10.3390/agriculture13071386
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4. **Toviho, O. A.**, Bársony, P.: Nutrient Composition and Growth of Yellow Mealworm (Tenebrio molitor) at Different Ages and Stages of the Life Cycle.
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Foreign language abstracts (2)

5. **Toviho, O. A.**, Molnár, Á., Kertész, A., Takács, K., Antal, O., Nagy, A., Fehér, M., Bársony, P.: Effect of temperature on digestive enzyme activity of Eurasian Perch (Perca fluviatilis).
   In: Aquaculture Europe 22: Innovative solutions in a changing world: Abstracts, European Aquaculture Society (EAS), Rimini, 1308-1309, 2022.

6. **Toviho, O. A.**: Nutrient composition and growth of Yellow Mealworm (Tenebrio Molitor) at different ages.
   In: XXV. Tavaszi Szél Konferencia 2022 : Absztraktkötet. Szerk.: Molnár Dániel, Molnár Dóra, Doktoranduszok Országos Szövetsége, Budapest, 82, 2022. ISBN: 9786158205481

List of other publications

Foreign language scientific articles in Hungarian journals (3)

7. Molnár, Á., **Toviho, O. A.**, Fehér, M.: Investigation of the production parameters, nutrient and mineral composition of mealworm (Tenebrio molitor) larvae grown on different substrates.
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8. Homoki, D., **Toviho, O. A.**, Minya, D., Kovács, L., Lelesz, J. É., Bársony, P., Fehér, M., Kövics, G., Stündl, L.: Effect of dissolved oxygen on common carp (Cyprinus carpio) and basil (Ocimum basilicum) in the aquaponics system.
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9. Kovács, L., Minya, D., Homoki, D., **Toviho, O. A.**, Molnár, Á., Fehér, M., Stündl, L., Bársony, P.: Comparision of growth of mature all-female and mixed-sex Common carp (Cyprinus carpio L.) stocks in RAS.
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Foreign language scientific articles in international journals (1)

10. Kovács, L., Minya, D., Homoki, D., **Toviho, O. A.**, Fehér, M., Stündl, L., Bársony, P.: Effect of different water temperatures on sex ratio, gonad development and production parameters of common carp (Cyprinus carpio L.).
    Aquac. Res. 51 (2), 858-862, 2019. ISSN: 1355-557X.
    DOI: http://dx.doi.org/10.1111/are.14407
    IF: 1.748

Total IF of journals (all publications): 8,948
Total IF of journals (publications related to the dissertation): 7,2

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

19 July, 2023

12. STATEMENTS

STATEMENT

I wrote this thesis in the framework of the University of Debrecen Animal Science Doctoral School for the purpose of obtaining a doctoral degree (Ph.D.) at the University of Debrecen.

Deberecen, 2023.08.08

……………………………...

Signature of the candidate

STATEMENT

I hereby certify that the doctoral candidate Odunayo Abigeal Toviho has carried out his/her work under my/our supervision within the framework of the above-mentioned Doctoral School between 2019-2023. The candidate has made a decisive contribution to the results of the thesis through his/her independent creative work, and the thesis is the candidate's independent work. I/we recommend that the thesis be accepted.

Debrecen, 2023.08.08

……………………………...

Signature of the supervisor

13. ANNEXES

I thank God for His protection, guidance, and provision from the beginning of this programme.

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