MOHAMAD IKMAL HAKIM BIN ALLAHUDIN MASTER OF SCIENCE 2025

BIOEFFICACY OF ENTOMOPATHOGENIC FUNGUS (*Metarhizium anisopliae)* STRAIN MET-GRA4 AGAINST GOLDEN APPLE SNAILS (*Pomacea canaliculata*)

MOHAMAD IKMAL HAKIM BIN ALLAHUDIN

MASTER OF SCIENCE
UNIVERSITI MALAYSIA TERENGGANU

2025

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MOHAMAD IKMAL HAKIM BIN ALLAHUDIN

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in the Faculty of Science and Marine Environment
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Dedicating this thesis to:

My all,

My backbone and my reasons to go on when times get tough:

Late Abah, dearest Mak, my sisters Kak Nora, Kak Fizah, Noin, Anie, Liana,

My closest friends, Nafi, Putri, and Aliff

This would not have been possible without your support

You have my utmost gratitude

May Allah S.W.T bless your lives always and forever.

Abstract of thesis presented to the Senate of Universiti Malaysia Terengganu in fulfilment of the requirements for the degree of Master of Science

BIOEFFICACY OF ENTOMOPATHOGENIC FUNGUS (*Metarhizium anisopliae*) STRAIN MET-GRA4 AGAINST GOLDEN APPLE SNAIL (*Pomacea canaliculata*)

MOHAMAD IKMAL HAKIM BIN ALLAHUDIN

JULY 2025

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Faculty	**:**	**Faculty of Science and Marine Environment**

Golden Apple Snails (GAS), *Pomacea canaliculata* is an invasive species in Malaysia causing millions of ringgit of damages to the paddy sector. Numerous strategies were implemented to develop greener molluscicides such as plant-derived extracts and biological control methods, however most are still in development. The utilization of biological control gained significant interest recently including entomopathogenic fungus. To develop a safer alternative to chemical molluscicides, nano-emulsion formulated conidia of *Metarhizium anisopliae* (strain Met-Gra4) were evaluated against GAS adults (n = 60) under laboratory conditions. The *M. anisopliae* (strain Met-Gra4) were cultured on rice media while pesticide free GAS adults were collected from UniSZA lake side were exposed to varying concentrations of conidia (10^5, 10^6, 10^7 and 10^8 conidia mL^{-1}) prepared in conidial suspension and emulsion-formulated forms for 24 hours and post-treatment observation for 14 days. Population of GAS exposed to emulsion-formulated conidia had significantly lower survival compared to conidial suspension ($p < 0.05$). The emulsion-formulated conidia exhibited LC_{50} 6.7 x 10^5 conidia mL^{-1} after 14 days, a lower concentration than conidial suspension with LC_{50} of 6.7 x 10^6 conidia mL^{-1} indicating higher efficacy. LT_{50} of emulsion-formulated treatments demonstrated significantly faster 50% mortality than conidial suspension except the highest concentration (6.7 x 10^8 conidia

mL^{-1}) ($p < 0.01$). The increased efficacy in emulsion-formulated treatments is due to the delivery system provided by nano-sized particles facilitating the uptake of fungus into the snails more effectively. Histopathological analysis revealed that digestive tubules of hepatopancreas exhibited severe alterations particularly hyperplasia of K corpuscles in both *M. anisopliae* treatments ($p < 0.05$). Histopathological analysis of gonads unveiled that exposure to *M. anisopliae,* especially emulsion-formulated caused significant castration evident by gamete disintegration and necrosis of reproductive tubules in testis and ovary ($p < 0.05$ respectively). Histopathological condition index scores obtained from the same tissue samples further supported the histopathological findings confirming emulsion-formulated conidia treatment caused the most significant histopathological alterations ($p < 0.05$) among all treatments. Overall, the results highlight emulsion-formulated *M. anisopliae* as a potential bio-molluscicide for effective control of GAS, offering an environmentally sustainable solution to severe rice pests.

Abstrak tesis yang dikemukakan kepada Senat Universiti Malaysia Terengganu sebagai memenuhi keperluan untuk Ijazah Sarjana Sains

KEBERKESANAN KULAT ENTOMOPATOGEN (*Metarhizium anisopliae*) STRAIN MET-GRA4 TERHADAP SIPUT GONDANG EMAS (*Pomacea canaliculata*)

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Siput gondang emas (GAS), *Pomacea canaliculata* merupakan spesies invasif di Malaysia menyebabkan kerosakkan mencecah jutaan ringgit kepada sektor padi. Pelbagai strategi dilaksanakan untuk mewujudkan racun siput yang mesra alam seperti ekstrak tumbuh-tumbuhan dan kawalan biologi tetapi kebanyakannya masih dalam perkembangan. Penggunaan kawalan biologi mendapat perhatian yang signifikan kebelakangan ini termasuk kulat entomopatogen. Bagi mewujudkan alternatif yang lebih selamat kepada racun siput kimia, formulasi emulsi-nano kulat *Metarhizium anisopliae* (strain Met-Gra4) telah diuji terhadap GAS dewasa dalam kondisi makmal (n = 60). Kulat *M. anisopliae* (strain Met-Gra4) telah dikultur menggunakan media beras manakala GAS dewasa yang bebas racun perosak telah dikutip dari persisiran tasik UniSZA telah didedahkan kepada pelbagai kepekatan konidia (10^5, 10^6, 10^7 & 10^8 konidia mL^{-1}) dalam bentuk suspensi konidia dan formulasi emulsi dalam keadaan makmal selama 24 jam and pemerhatian pasca-rawatan selama 14 hari. Kadar kelangsungan hidup bagi populasi GAS didedahkan kepada formulasi emulsi lebih rendah berbanding suspensi konidia ($p < 0.05$). Formulasi emulsi menunjukkan LC_{50} 6.7 x 10^5 konidia mL^{-1} selepas 14 hari, kepekatan lebih rendah berbanding suspensi konidia LC_{50} 6.7 x 10^6 konidia mL^{-1}, menunjukkan keberkesanan yang lebih tinggi. LT_{50} formulasi emulsi lebih pantas dalam mencapai 50% kematian berbanding

suspensi konidia kecuali pada kepekatan tertinggi (6.7×10^8 konidia mL^{-1}) ($p < 0.01$). Peningkatan keberkesanan pada rawatan formulasi emulsi disebabkan sistem penghantaran yang dibekalkan molekul bersaiz nano yang membantu pengambilan kulat yang lebih efektif ke dalam siput. Analisis histopatologi mendedahkan bahawa tubul pencernaan hepatopankreas menunjukkan perubahan teruk terutama peningkatan K korpusel dalam kedua-dua rawatan *M. anisopliae* ($p < 0.05$). Analisis histopatologi gonad mendedahkan bahawa pendedahan kepada *M. anisopliae*, terutamanya formulasi emulsi, mengakibatkan kesan pengembirian yang signifikan, jelas daripada penguraian gamet, dan nekrosis tubul pembiakan di testis dan ovari ($p < 0.05$, masing-masing). Markah indeks keadaan histologi yang diperoleh daripada sampel tisu yang sama selanjutnya menyokong dapatan histopatologi, mengesahkan rawatan formulasi emulsi menyebabkan perubahan histopatologi yang paling ketara ($p < 0.05$) berbanding semua rawatan lain. Secara keseluruhannya, hasil kajian menunjukkan bahawa formulasi emulsi *M. anisopliae* adalah racun siput yang berpotensi untuk mengawal GAS secara efektif dan memberi penyelesaian yang mesra alam kepada haiwan perosak padi yang teruk.

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APPROVALS

I certify that an Examination Committee has met on 24th July 2025 to conduct the final examination of Mohamad Ikmal Hakim bin Allahudin, on his Master of Science thesis entitled **"Bioefficacy of Entomopathogenic Fungus (*Metarhizium anisopliae*) Strain Met-Gra4 Against Golden Apple Snail (*Pomacea canaliculata*.)"** in accordance with the regulations approved by the Senate of Universiti Malaysia Terengganu. The Committee recommends that the candidate be awarded the relevant degree. The members of the Examination Committee are as follows:

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UMT or other institutions.

_____

MOHAMAD IKMAL HAKIM BIN ALLAHUDIN

Date:

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LIST OF ABBREVIATIONS

µL	Microliter
AChE	acetylcholinesterase
AKUATROP	Institute of Tropical Aquaculture & Fisheries
ANOVA	Analysis of Variances
c	C corpuscles
cfu	Colony-forming unit
CI	Confidence internal
CRAFS	Centre of Research and Field Service
cs	Conidial Suspension
cf	Degree of freedom
dH2O	Distilled water
do	Disintegrated oocytes
DOA	Department of Agriculture
ds	Disintegrate spermatocyte
dt	Digestive tubules
dtt	Disintegrated testis tubules
EF	Emulsion-formulated
EPF	Entomopathogenic fungus
es	Enlarged spermatocyte
GASs	Golden Apple Snail
ha	Hectare
hc	Haemocytic infiltration
HI	Histopathological condition index

HSD	Honestly Significant Difference
IPM	Integrated pest management
k	K corpuscles
l	lumen
LC50	Lethal concentration 50
LT50	Lethal time 50
°	degree
<	less than
>	more than
%	percentage
±	plus, minus
χ^2	Chi-square

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CHAPTER 1

INTRODUCTION

1.1 Background of the Study

Golden Apple Snails (GAS), *Pomacea canaliculata*, belong to the family Ampullariidae, a diverse group of freshwater snails. This group of snails possesses unique features such as an aerial-aquatic respiratory system with the development of pulmonary sac (lung) while retaining ancestral ctenidium (gills) (Rodriguez et al., 2019). GAS has the largest shells among other freshwater snails and some genera in the family having cleidoic eggs with calcareous eggshells (a feature typically unique to land dwelling organisms such as birds and reptiles) (Hayes et al., 2009; 2015).

GAS is the only aquatic snails listed as one of the world's 100 worst invasive alien species by the International Union for Conservation of Nature (Lowe et al., 2000; Tricarico et al., 2016). GAS is significant enough to also be listed among Malaysia's top ten worst invasive species and being the only species of molluscs included in the Malaysia National Action Plan on Invasive Alien Species (NAP IAS) 2021-2025 (DOA, 2021). Its initial introduction into many non-indigenous regions was intended as a source of supplemental income (Yang et al., 2018). However, due to inadequate research on market demands and the ecological impacts of these snails, soon after its introduction, it quickly became a pest for various aquatic crops (Hayes et al., 2008; Zheng et al., 2012; Matsukura et al.,2013). Most evidently affected by GAS include rice seedlings (Dong et al., 2011) and has severely threatened rice fields worldwide especially in Asia as the world's largest rice producer (Rajamoorthy et al., 2015).

As one of the rice-producing countries in the world, Malaysia has also been affected by the presence of GAS. GAS was first detected in Malaysia in tin mines' waterways in early 1991 (Yahaya et al., 2017) and have become prevalent rice pests, infesting nearly all rice growing areas in Malaysia ever since. According to the Department of Agriculture (DOA), the damages from GAS persisted until recent years with a total of 2,386.84 ha of paddy granary areas across 10 states in the peninsula were affected during main planting season of 2022 and 2023 (Damage Survey DOA, 2022). Due to their high survivability, there have been multiple strategies to overcome this problem, including chemical, cultural and biological control methods such as utilizing the ducks, fish and even using microbial agents like fungi.

Although multiple efforts have been made to control the populations of these GAS, there has been no success in completely eradicating these snails. Current control methods still heavily rely on chemical molluscicides; which is still insufficient to reduce the severity of the infestation as the snail populations continue to persist. Continuous application of chemical molluscicides can lead to the snails in the populations to develop resistance towards the chemicals and pose significant risks to the environment, humans, and non-target species. Other control efforts such as the use of multiple biochemical compounds derived from different plants, have also been explored. However, these methods remain in the developmental stages. Recent strategies have explored to utilize microbes as an alternative to mitigate the spreading and persistence of GAS. One of the microbial agents include the usage of the entomopathogenic fungus *Metarhizium anisopliae* as a potential molluscicide to control GAS. The application of *Metarhizium* spp. for biological pest control especially insect pests have been widely studied and well-established as a sustainable way to combat pest issues (Ullah et al., 2023).

As a molluscicide, *M. anisopliae* is still in the early stages of evaluation. Nevertheless, the fungus has demonstrated promising results thus far, exhibited by its capabilities in showing ovicidal properties against planorbid aquatic snails *Biomphalaria glabrata* (Duarte et al., 2015), terrestrial snails *Monacha obstructa* (Abo-Elwfa et al., 2024) and has been used widely as an insecticide. Despite the potential of *M. anisopliae* as an alternative bio-molluscicide and the capabilities to

control a wide range of insect pests, there is still limited study conducted on the potential applications against molluscan pest like GAS. Development and optimization of *M. anisopliae* through formulation such as incorporated in an emulsion formulation would enhance the virulence of the fungus, improve stability, and ensure effectiveness in the field (Cheong et al., 2022; 2023). There are several differences between emulsion-formulated conidia and conidial suspension that contributes to possible differences in efficacy. Emulsion formulation uses oil and surfactant that emulsifies into an emulsion, this emulsion contains nano-sized particles compared to conidial suspension only using water and Tween 80 in the solvent system (Preeti et al., 2023). The conidia are incorporated into an emulsion formulation containing nano-sized particles that will encapsulate the conidia of *M. anisopliae* and this encapsulation improves the pathogenicity by increasing rate of uptake, virulence of the conidia, the viability as well as the tolerance towards ecophysiological factors. Conidial suspension lacks the ability to keep the conidia separated and hence the conidia tend to clump as it is free-floating. With the establishment of the emulsion-formulated conidia, the question arises on which form of *M. anisopliae* treatment; whether in the form of conidial suspensions or suspended in the emulsion, is more efficacious in controlling GAS population under laboratory conditions. The comparison will be able to provide insights into which form of treatment will yield results on par with the chemical molluscicide with metaldehyde 5%, as an alternative to the current GAS control practice. The effects caused by the conidia on the tissues of GAS must also be studied to understand the mechanism of how the conidia operates to control the population of GAS and this can be done through histopathological analysis.

Histopathological analysis is an essential method to unveil the cytotoxicity effects on the targeted organisms, assessing the response towards stimuli since histopathological changes are oftentimes associated with complex responses, both biochemically and physiologically, of a targeted organism's response against any kind of stressors, pathogens, and diseases (Nur et al., 2021). Despite the inability of histopathological analysis to provide quantitative information, histopathological analysis is essential in unveiling biomarkers especially in samples exposed to contamination (Viana et al., 2013). However, there are methods involving the enumeration of histopathological conditions and alterations observed in tissues that

can alleviate this limitation (Arrighetti et al., 2022). Through this analysis, it can highlight the results of the intake and absorption of pesticides into the systemic circulation of the organisms and the effects it has on a cellular level, elucidating the histopathological changes in the tissue, integrity, and negative responses towards the applied treatment (Nur et al., 2021). With the histopathological analysis conducted, the potential of *M. anisopliae* as a molluscicide can be validated. The findings could possibly highlight its promising potential as part of the integrated pest management (IPM) practice of GAS which could ultimately benefit and contribute to the sustainability of the rice industry.

1.2 Problem Statement and Justification

Following diligent review conducted on the topic, the main gap that was identified in regards to the capabilities and efficacy of the entomopathogenic fungus *M. anisopliae* as bio-molluscicides for GAS (*Pomacea canaliculata*) which has not been fully evaluated. The difference in effectiveness between emulsion-formulated conidia and conidial suspension has not been evaluated. This can be achieved by comparing the lethal concentration 50 (LC_{50}) and lethal time 50 (LT_{50}) between the two forms of treatments to unveil the more efficacious form of treatment.

Another gap that was identified was on the effects of pathogenic infections on the anatomical and structural of GAS. Hence, this study aims to fulfil this gap by evaluating the pathogenicity effects of *M. anisopliae* particularly on the anatomical and structural of GAS by conducting histopathological analysis and enumerating the conditions observed through Histopathological Condition Index (HI). It is hypothesized that the entomopathogenic fungus *M. anisopliae* is highly toxic and has the potential as an effective bio-pesticide against GAS. It is also hypothesized that the cells of the snails will be vacuolized, experiencing hyperplasia and possibly necrosis under a microscopic examination as a response to the fungal infection by *M. anisopliae*. The histopathological analysis conducted on the infected tissues is crucial to confirm that *M. anisopliae* is a reliable control agent against GAS.

Current control practices for GAS in Malaysia rely heavily on chemical molluscicides which pose risks not only to humans but to crops and non-target organisms. In contrast, the greener potential alternative to the molluscicide such as *M. anisopliae* has received little attention. Information on the responsive pathways' interaction between *M. anisopliae* and the GAS remains unknown due to a lack of studies conducted on this topic up to that depth. Understanding the specific responsive pathways is vital to develop and achieve optimum molluscicidal capabilities of the fungus *M. anisopliae*. It is also important to understand the mode of action to be able to optimize the efficacy in order to be able to maximise the molluscicidal properties of the biocontrol agent and minimizing the risks of resistance development. Histopathological observation of infected GAS' organs will provide valuable insights into the infection mechanism and physiological impact as well as the changes to the overall constituents of the tissues and organs of GAS.

1.3 Research Questions

1. Between conidial suspension and emulsion-formulated conidia of *M. anisopliae* strain Met-Gra4, which is the more efficacious form of treatment against GAS under laboratory bioassays?
2. What is the lethal concentration 50 (LC_{50}) and lethal time 50 (LT_{50}) for both conidial suspension and emulsion-formulated conidia of *M. anisopliae* against GAS?
3. What are the internal organs of GAS affected by *M. anisopliae* infections and what are the histopathological alterations observed in the organs?

1.4 Objectives

This study has the following objectives: -

1. To compare the effectiveness of *M. anisopliae* conidial suspension and emulsion-formulated conidia against Golden Apple Snails (GAS) under laboratory conditions.
2. To determine the median lethal concentration (LC_{50}) and median lethal time (LT_{50}) of both conidial suspension and emulsion-formulated conidia of *M. anisopliae*.
3. To determine the effect of entomopathogenic fungus *M. anisopliae* on the organs and tissues of infected GAS tissues through histopathological analysis

1.5 Hypothesis

1. All concentrations of emulsion-formulated of *M. anisopliae* treatments are expected to cause 50% mortality within 14 days and achieve it faster compared to the conidial suspension.
2. Treatments of higher concentrations will generally result in faster mortality, leading to a lower LT_{50} value for both treatments meanwhile LC_{50} for emulsion-formulated treatment is expected to be lower.
3. Multiple alterations can be observed especially in the specific targeted organs and these changes in the tissues and organs affect the physiological processes of GAS that eventually contribute to mortality.

1.6 Limitation of Study

This study was conducted on a population of GAS that consisted mostly of females which was apparent after dissection and histopathological analysis. Sorting according to the sexes was not performed as determining the sex of the snails may cause unnecessary stress on the snails due to the operculum which requires to be forcefully pulled open to observe the genital complex. Morphological observation on sex determination was also not reliable as the descriptions for the morphology of the two sexes often overlap and are vague in nature, hence it was not possible to be accurately done.

Determining the species were also not conducted between *P. canaliculata* and *P. maculata* since the samples were retrieved from one isolated site which is separated from other bodies of water, hence minimizing the likelihood of interspecies mixing and morphological difference oftentimes overlap between the two species hence using morphological attributes to differentiate the two species is not viable. Both species have also been recorded to hybridize successfully hence the distinction of the two species was deemed unnecessary and have no influence over the results (Matsukura et al., 2013). Since *P. canaliculata* is the most prevalent species in Terengganu, and in Malaysia, the species tested is assumed to be *P. canaliculata* (Rao et al., 2018).

The experiments were conducted exclusively under laboratory conditions. Hence, for the same results to be expected in a different setting such as semi-field or field, a more thorough and extensive study on the different settings must be conducted first. For this treatment to be validated for field applications, further studies must investigate the efficacy of the conidia under natural conditions and potential impacts on other non-target organisms as the conidia could interact differently in different environmental conditions exposed to harsher and more unstable ecophysiological factors as compared to in the laboratory before large-scale implementations.

CHAPTER 2

LITERATURE REVIEW

2.1 Golden Apple Snails (GAS), *Pomacea* spp.

2.1.1 Life Cycle, Biology and Adaptations

Golden apple snails, GAS (Ampullariidae; *Pomacea* spp.) have a lifespan of about four years and reaches sexual maturity between three months old to two years old depending on the ambient temperature (Pratama et al., 2023). Their life cycle starts from conspicuously coloured bright pink eggs which are also calcareous that are laid in clutches containing up to 1000 eggs but generally having around 200-300 eggs per clutch (Pratama et al., 2023). Adults have thin shells approximately 35-60 mm in height and coiled to the right (dextral) (Suartini et al., 2021). GAS are characterized by conical shells that are rather round, cylindrical and in many varying colours such as yellow brown (possibly the origin of the name golden apple snail), greenish-brown to dark brown (oftentimes with bands). However, the shell of GAS by itself is not a reliable method to discern *Pomacea* species. About 15 species out of the 50 known species have channelled shells, which was thought to be a trait solely belonging to *Pomacea canaliculata,* the channelled apple snail (Pratama et al., 2023). The pigments present in the periostracum layer of the shell determine the colours of the shells (Andriani et al., 2023). GAS develop alternative phenotypes to help them adapt to different environmental conditions. The differences in shells can be influenced by ontogenetic or ecophenotypic factors of the GAS where significant differences in environmental conditions cause major differences in the phenotypes of the snails'

shells (Suartini et al., 2021). Unlike many snails that are hermaphroditic by nature (individuals having both male and female reproductive systems), GAS are dioecious, having only one set of reproductive organs per individual. Males have penile complex and females have vaginal opening right next the ctenidium (gills) on top of the left side of its head respectively (Tamburi et al., 2023). An operculum attached to the foot can close the aperture of the shell and the aperture is able to be closed or opened by the operculum through the foot contraction (Xu et al., 2018). On the other hand, the entire visceral mass of the body is pulled in by the contraction of the columellar muscle and vice versa for the relaxation of the muscle. GAS are well-known for their generalist nature and ability to feed on vascular plants, detritus, periphytons and even animal remains (Suartini et al., 2021). This factor alone helped increase the survivability of this invasive rice pest. GAS have many adaptations that have enabled this pest to thrive in drastically different environments across the globe.

One of those essential adaptations begins with its eggs. The aerial egg masses laid by these amphibious snails have multiple adaptations to ensure the survivability of the eggs since it is exposed to harsh environments and higher chance of predation. Eggs laid by GAS are calcareous in nature unlike the gelatinous eggs laid by most aquatic snails; this is to prevent the desiccation of the aerial eggs exposed to harsh environments (Giglio et al., 2018). The bright pink eggs of these snails are due to two types of perivitelline that act as the defense mechanism against predation. One of the two perivitelline is PcPV2, a neurotoxin storage lectin found to have a strong lethal effect on mice specifically affecting the neurons within the spinal cord (Dreon et al., 2014; Giglio et al., 2018). Another perivitelline that has been discovered in the perivitelline fluid (PVF) of the egg masses of GAS is PcOvo which is a storage carotenoprotein which is the compound responsible for the highly conspicuous colouration of the egg masses which is theorized to also serve as an aposematic signalling to predators. The defence mechanism of this PcOvo perivitellin involves the inhibition of predators' ability to digest proteins contained in the eggs due to its antinutritive and antidigestion properties (Dreon et al., 2014; Giglio et al., 2018). A study by Sun et al., (2012) conducted a proteomic analysis and discovered over 50 other proteins involved in the immunity of molluscs with some identified having potential against other types of pests (insects and fungi). These proteins coupled with

the visual signalling of the bright pink colouration of the egg masses must have been a successful strategy against predators as even in the native region, there is only one recorded predator of the GAS egg, which is the fire ant *Solenopsis geminata.*

GAS are also able to invade many regions across the globe with various ecophysiological factors such as fluctuating temperatures, changes of seasons and different community makeup of different regions. GAS are able to tolerate the fluctuating temperatures as well as changes in water level as severe as drought by undergoing a process called aestivation. Aestivation occurs when the surrounding conditions are unfavourable and GAS go into a period of dormancy or torpor that significantly reduces all physiological processes (Giraud-Billoud et al., 2022). This process allows these aquatic snails to withstand extended periods of dry spells and drought as 50% of mortality is only recorded after 10 months of induced aestivation with only a 30% of body mass reduction (Mueck et al., 2018). Upon termination of the induced aestivation, GAS were able to resume feeding and activity within 24 hours of reinstating favourable conditions with no additional mortality recorded. Mueck et al., (2019) found that the smaller GAS lost relatively more body mass compared to medium and large snails, and survival of aestivation seems to depend on preservation of body mass where the surviving GAS lost less body mass than those that did not survive the aestivation.

GAS are also unique as one of the bimodally breathing aquatic snails in the family Ampullariidae that oddly retained its gill while having a lung for air breathing. GAS, belonging to the genus *Pomacea,* differs from other genera in the family as members of *Pomacea* genus rely more on air-breathing as opposed to fully-aquatic snail species in the same family that rely more on their gills (Prieto et al., 2021). GAS changes the mode of respiration (either using gills or lung) based on the availability of dissolved oxygen in the water it inhabits (Chen et al., 2024). When the level of dissolved oxygen is low, GAS use their lung to respire and when the level of dissolved oxygen in the water is high, it uses gills to respire. This adaptation allows GAS to populate in poorly oxygenated water as they rely heavily on air-breathing hence allowing them to thrive in environments that would have otherwise been inaccessible to aquatic snails. Such versatile respiratory strategies enabled GAS to invade and

populate any water bodies including heavily contaminated water such as drainage channels further spreading its population.

2.1.2 History of Spreading and The Damages Incurred Worldwide and in Malaysia.

GAS are native to the tropical and subtropical regions of South America. Introduction of these snails was done deliberately and repeatedly to the East and South-East Asian rice-growing countries, which proved to be disastrous and negatively affected the agricultural sector of the introduced regions (Djeddour et al., 2021). The introduction of GAS from Argentina to Taiwan was initially intended to be a source of protein alternatives to various aquatic animals like fish and shrimps, as well as for the aquarium pet trade (Pertiwi & Saputri, 2020). However, the effort of exporting GAS as a food source quickly diminished due to their soft texture was unappealing to the taste of the locals and their roles as a vector host for rat lungworm parasite, *Angiostrongylus cantonesis* that is hazardous to human health (Amzah & Yahya, 2014). From Taiwan, the snails were distributed to other developing countries including Malaysia for economic purposes to generate side-incomes and to provide additional protein sources (Ahmed, 2015). The decrease in demand for GAS led to a sharp decrease in intentional productions as well as many projects being deserted which contributed to the excessive spreading and escaping of GAS into the waterways.

GAS caused damages to crops by consuming large amounts of economically important crops in the different regions, such as taro, water cress, tomato and most importantly paddy. These attacks lead to reduction of yield as the seedlings are completely consumed, causing an increase of production cost which include replanting and pest management cost. GAS prefers young rice plants and will attack the base so that the plants topple and fall over. Transplanted rice seedlings are susceptible up to 15 days after planting meanwhile direct-seeded seedlings are more susceptible until 30 days after planting (Kalau et a., 2018). Ambient conditions affect the populations of GAS in the fields especially the relative humidity and rainfall significantly contributing to the populations hence influencing the damage caused too (Arfan et al., 2016). According to Arfan, due to the pesticide applied during the beginning of

planting season (sowing stage), the population of GAS declined, however a general increase of population for all stages of maturity of GAS were seen during later periods of the planting season as the pesticide application stop and food are more readily available. As GAS are generalists, they can also rely on weeds alongside paddy seedlings to maintain their population in the field and have been recorded to have significant roles in weed control in the field (Cho et al., 2023). Some of the more common pesticides used in agriculture includes pyrethroids, dimethoate, malathion, 2,4-D-dimethylammonium and glyphosate-based pesticide which targets weeds and insect pests (Kamaruzaman et al., 2020). However, for snail pest like GAS, an increasingly common usage of the illegal fentin-acetate based molluscicide were being used to combat GAS infestation.

It has been recorded that even a single snail per meter squared (m^2) can cause damage up to 90% in which it is estimated that the losses due to GAS range from $806 - $2138 million USD (United States Dollar) annually just across South-East Asia alone. In Thailand, GAS have been responsible for serious damages in the last ten years to paddy causing damages up to $8.4 billion USD causing not only heavy economic losses to the country but also requiring a high cost in an effort to combat it (Nghiem et al., 2013; Win et al. 2018). Nghiem also recorded that Vietnam had an estimated $10.1 billion USD in damages to the paddy sector meanwhile the annual cost of this snail to Philiipines was estimated to be up to $2,064 million USD. In Myanmar, GAS started attacking water cress, tomato plants, and infesting rice paddy fields up to a point where some farmers lost 100% of their crops and rendered afraid to replant due to the infestation. In Malaysia, GAS was first detected in the waterways in an abandoned tin mine in Puchong, Selangor back in September 1991 and had quickly spread to be one of the most prevalent rice pests in this region (Amzah & Yahya, 2014). In Malaysia Yahaya et al., (2017) reported that the losses caused by GAS reached up to RM83 million (Ringgit Malaysia) in 2010. Based on a Damage Survey conducted by DOA (2021-2023), a total of 2,386.84 hectares of paddy plantation were damaged in 10 states of Malaysia in 2023. These figures highlight GAS as a prevalent pest in rice-growing countries, prompting control methods to prevent the situation from getting out of hand.

2.2 GAS Control Methods

Following the significant damages inflicted to the agricultural sector by GAS, various control methods have been explored which include biological, chemical, cultural control methods as well as the use of microbes as biocontrol agents against these invasive aquatic snails. Due to the many adaptations GAS have, controlling the populations have shown to be very challenging.

2.2.1 Cultural Control Methods

Cultural control method is one of the initial strategies employed to control the population of GAS. This control method utilizes manual labour in the field by physically removing the GAS and their eggs from the environment and involves agricultural practices to reduce the snail population and mitigate crop damage. One of the most common methods is handpicking snails by farmers. This method is often used alongside plant attractants as baits, for example papaya leaves, sweet potato and even newspapers, with the best attractant found to be ripe jackfruit skin (Amzah & Yahya, 2014). These baits attract or lure GAS, making them easier to locate and remove manually by hand, especially at the early stages of GAS that are much smaller and more challenging to locate through sight only (Rusli et al., 2023).

Another cultural control method applied in the field is the usage of older seedlings in transplanting and water level management. GAS are molluscs hence lacking teeth and instead has a rasping tongue called radula which is the autapomorphy of this taxon (Krings et al., 2022). As a result, GAS can only rasp on the softer parts of the paddy seedlings, hence transplanting older seedlings (usage of 21-day-old seedlings as opposed to 13-day-old seedlings) with harder culms has been proven to work to reduce damages caused by GAS. A study conducted on water level management, seedlings age and pest density revealed that damage done to paddy seedlings was most affected by the water level of standing water, followed by age of seedlings and the density of GAS in the plot (Teo, 2003). Since GAS are fully aquatic snails and use water as a medium to attack these paddy seedlings, they become less mobile in shallow waters as compared to a flooded paddy field consequently limiting

their mobility and range of paddy area they can attack. GAS also show a stronger preference towards direct-seeded and dapog-transplanted younger seedlings (a technique where seedlings grown on a mat compared to traditional flooded nursery) as opposed to seedlings that are older (21-day-old) and wet-bed-transplanted seedlings as evident in the damage recorded in the prior compared to the latter (Donayre et al., 2022).

In combination with other cultural control methods, establishing trenches around the edges of the plots in the field can also help reduce the attack of GAS on the seedlings (Rusli et al., 2023). Trenches also limit the mobility of the snails at the same time causing them to concentrate in one area making collection and disposal easier. Having the trenches establish a barrier between the crop and the surrounding area, which limit GAS' access to the crop hence reducing the damage inflicted. When this method is carried out together with other techniques such as using filter mesh on the water inlets and outlets can reduce ability of the snails to disperse and in the long run reduce the severity of infestations over time. However, despite the promising nature of cultural control methods, it is simply not feasible for small-scale farmers to implement such labour and cost-intensive control methods. For example, the mesh of the inlets oftentimes is blocked by vegetation and requires constant monitoring to ensure no blockage occurs. The typically large area of paddy plantation added to the number of trenches, inlets, and baits place require a lot more labour to upkeep making this method not feasible for the majority of small-scale farmers.

2.2.2 Biological Control Methods

Biological control agents on the other hand involve natural enemies of GAS including pathogens, parasites, predators, and competitors to predate on, outcompete and suppress populations of GAS, either the adults or eggs (Fahad et al., 2015). These natural enemies typically live in the same habitat as GAS such as birds, ducks, waders, kites, and egrets (Ranamukhaarachchi & Wickramasinghe, 2006). The list of potential predators of GAS is extensive as compiled by Wahizatul et al., (2022), ranging from

reptiles such as turtle and caimans, to insects and even mammals like rats. However, only a small number of the animals are scientifically important to slug control studies.

In the field, some of the promising applications of biological control agents would be fish-farming system and rice-duck farming showing promising success in controlling GAS. Fish-farming system and rice-duck farming theoretically uses the same principle of rearing the fish or ducks in situ on the paddy field. These organisms are intended to predate on GAS and control the population of GAS in the field. Some examples of fish used in this system are listed in Table 2.1 where most are aimed to control juvenile to sub-adult GAS due to the size of adults is typically too large to be consumed by the fish (Kelvin, 2014).

Fish-farming trials in the field have been conducted by constructing a plot enclosed by a net where transplanted rice seedlings are placed. Fish were then released into the plot at 30 fish per plot one month after transplanting following and one week after the snails were introduced to the plots (Teo, 2006). The effectiveness of the predatory capabilities of fish species was evaluated by examining the gut of three fish sampled from the population one week after the introduction of snails into the plots. Fish-farming have demonstrated that fish farming can effectively control the population of GAS in the field especially the common carp (*Cyprinus carpio*) and African catfish (*Clarias gariepinus*) with the latter being more suitable as a biocontrol agent in the field due to its capabilities to recover its population and persists in the field conditions (Teo, 2006). However, certain fish used as biocontrol agents such as the common carp are also grazers for macrophytes, which would also contribute to the damage onto surrounding macrophytes (Kelvin, 2014).

Table 2.1 Species of fish that have been studied as biocontrol agents for golden apple snails (GAS).

Biological control agent using fishes		
Type of fish	Species	References
Carps	*Cyprinus carpio*	Ben-Ami & Heller, 2001; Yusa et al., 2006; Kelvin et al., 2014
	Carassius gibelio	
	Mylopharyngodon piceus	
Chubs	*Zacco temmincki*	
	Zacco platypus	Yusa et al., 2006
Dace	*Tribolodon hakonensis*	Yusa et al., 2006
Green pufferfish	*Tetraodon nigroviridis*	Guo et al., 2016
Gudgeons	*Pseudogobio esocinus*	Yusa et al., 2006
	Pungtungia herzi	
	Pseudoborasbora parva	
Pearl cichlid	*Geophagus brasiliensis*	da Silva & Figueiredo, 2014
Sleeper	*Eleotris oxycephala*	Yusa et al., 2006
Tilapia	*Oreochromis niloticus*	Teo, 2006; Yusa et al., 2006; Halwart et al., 2014
	Clarias garienpinus	

Duck farming as a biocontrol agent for GAS in the open paddy fields have also yielded promising results (Qin et al., 2012; Liang et al., 2014). Several different duck varieties have been tested to evaluate the efficacy of different varieties of ducks in controlling populations of GAS including William Siam, Taiwan, Mallard, Peking, Cherry Valley, Khaki Campbell, and Muscovy ducks (Teo, 2001). The method employed was by letting ducks roam the plot of paddy field during the day at about five to ten ducks per 100 m^2, which proved to be efficacious as there were hardly any snails or egg clusters seen at the end of the trials (Liang et al., 2014). Even with availability of duck feed, the ducks exhibited strong predatory capabilities with the William Siam variant (a cross between Mallard x Khaki Campbell) showed the highest consumption of snails consuming around 124 snails per duck. The William Siam variant, despite being one of the smallest varieties (average weight around 1.70 kg), managed to consume significantly more than the number of snails the largest variety of duck consumed which was the Cherry Valley (average weight 2.80 kg) consuming only about 17 snails per duck (Teo, 2001). Differences in body size may be the reason why William Siam performed the best as the conditions in the paddy fields, which were muddy and wet, often caused mobility issues with ducks of larger sizes.

Despite the promising outcomes of duck-farming and fish-farming, it might still be unable to be applied commercially due to several limiting factors which boil down to limited accessibility to resources. Resources needed to successfully apply these biological control methods include labour cost, initial investment to obtain the biocontrol agents with the biggest issue being limited scalability. The initial investment of a large-scale paddy field may deter farmers from implementing this method of control agents as opposed to cheaper chemical alternatives. The constant monitoring of the biocontrol agents whether the number is sufficient to control the GAS population, the feed for the biocontrol agents, the timely-release of the biocontrol agent so that it does not damage the crops especially with duck-farming are all very labour-intensive steps that are not optimal for farmers to apply in real life conditions (Ng et al., 2017). Besides, the effectiveness of biocontrol agents may highly depend on external factors on the field such as water depth, temperature, and snail density as well as the availability of other food, foraging behaviours, and habitat preferences (Wang et al., 2015; Joshi et al., 2018). For example, suitable water level is needed to accommodate the population of fish to be implemented as control agents however paddy fields are not kept waterlogged all year round and this poses an issue. The issue lies with the costs of investment to employ fish as a biocontrol agent in the field, which needed to be repopulated in the fields with each paddy planting seasons, which will quickly cause the cost to accumulate. Although according to Capinha et al., (2013) biological control may be more economically vital compared to chemical control methods in some cases, the entirety cost of starting, maintaining and aftercare of the biocontrol method should be carefully assessed. Aside from the cost, the introduction of non-native species biocontrol agents or not typically found in the field may drastically alter the community structure and food web of the target habitat (Estebenet et al., 2012).

When it comes to other biocontrol agents compiled by Wahizatul et al., (2022) tabulated in Appendix 1 (see page 119), reptiles that have been studied as a biological control agent for GAS showed astounding capabilities to control populations of GAS as well (Yusa et al., 2006; Yoshie & Yusa, 2008; Dong et al., 2012; Guo et al., 2017a). However, the species of reptiles that have been studied in the literature are all non-native species to Malaysia hence, the introduction of non-native species may cause

more harm than good to the local ecological communities. There have yet to be a study of native reptiles as promising biocontrol agents for GAS in Malaysia so far which is a gap that can be filled with future studies. Multiple records have also reported cannibalistic tendencies of GAS especially the adults consuming younger juveniles (Yusa et al., 2006; Cowie et al., 2012; Hayes et al., 2015). However, considering adults of GAS as predators and biocontrol agents for juvenile GAS would be counterproductive as the number of juveniles consumed by the adults GAS are nowhere near the number of eggs the adults would be able to produce. The rapid reproductive rate and high fecundity of the adult GAS would offset any considerable dent in the population that is caused by cannibalism.

2.2.2.1 Utilization of Microbes in GAS Control Methods

Studies on the utilization of microorganisms as a control for GAS have been relatively low compared to other means of control such as chemical and biological control. However, in general there are several studies conducted on the utilization of microbes including *Metarhizium* species as a biocontrol agent for mollusc pests which includes *Pomacea* spp. Tabulated in Table 2.2 is the list of microbes evaluated for their capabilities to control molluscs including *Pomacea* species. An exhaustive compilation of literature is provided in Appendix 2 (see page 120).

Table 2.2 Microbe-based control studied for molluscs.

Microbe used	Target mollusc	Mechanism of control	References
Fungus			
Metarhizium anisopliae	*Monacha cantiana*	Moderate molluscicidal effect	Hendawy et al. 2015
	Monacha obstructa	Infection towards hosts, eventually forming fungal hyphae on the soft tissues of cadaver	Abo-Elwfa et al., 2024 `
	Pomacea spp.	Infection towards the host (excessive mucus production and decreased activity levels)	Allahudin et al., 2024
Metarhizium brunnerum	*Cornu aspersum*	Enzymatic digestion	Khoja et al., 2019
Nematode			
Angiostrongylus cantonensis	*Pomacea canaliculata*	Parasitic towards host snails	Yang et al., 2013
Phasmarhabditis hermaphrodita	*Pomacea canaliculata*	Change of protein expression (PC-bpi)	Montanari et al., 2020; Boraldi et al., 2021
Trematode			
Dietziella sp.	*Pomacea canaliculata*	Host to the parasitic trematodes, undergoing pathogenic castration	Martinez et al., 2024

2.2.3 Chemical Control Methods

The usage of chemicals is one of the most widely used and widely studied for managing the GAS populations and this could be attributed to several factors. The main factor that causes chemical control to be most favoured is largely due to the immediate effects observed especially when dealing with severe infestation (Lucero & Wilson, 2023). However, this high efficacy comes with potential risks that affect health and the environment as well as possibly be lethal to non-target organisms (Nicolopolou-Stamati et al., 2016).

Commercial molluscicides fall under the category of chemical control methods as the molluscicides utilize the active chemical ingredient in the formulation to act upon GAS to control its population. Currently, there are more than 200 registered molluscicides in the market used worldwide mainly consisting of active ingredients such as niclosamide, triphenyltin acetate, ethanolamine salt with metaldehyde being the active ingredient in 80% of the molluscicide pellets in the market (Wang et al., 2021). Widespread use of many formulations of molluscicide containing metaldehyde can be attributed to its potency and fast-acting molluscicidal capacity.

Metaldehyde have been reported to be able to induce the increase of acetylcholinesterase (AChE) activity in snails directly disrupting the metabolic detoxification capabilities of GAS, consequently inhibiting the energy supply leading to its death. It is also able to inhibit the function of the spiral ganglion muscle in GAS (Zhu et al., 2004; Zou et al., 2014; Zhang et al., 2016). Metaldehyde also works as a molluscicide by rapidly hydrolysing to acetaldehyde once ingested causing the mollusc to excessively produce mucus leading to dehydration and eventually die (Triebskorn et al., 1998). Metaldehyde is highly mobile in the soil due to its physico-chemical properties and once applied, can easily run-off under wet conditions into water bodies and subsequently into the soil (Castle et al., 2017). This raise concerns as metaldehyde is toxic to most organisms that ingest it (Castle et al., 2017). Although oral median lethal concentration (LC_{50}) is dose dependent, there are records of poisoning in livestock (O'Neil & Davies, 2001; Plumlee et al., 2006; Smith & Adams,

2009), house pets (Dolder, 2003; Cope et al., 2006; Buhl et al., 2013; Fenton & Thompson, 2015), earthworms and even human with recorded deaths from metaldehyde poisoning (Shih et al., 2004).

Bioactive derived from plant materials makes up the most of current literature on the control methods of GAS, Appendix 3 (see page 122) tabulates studies conducted on plant-derived bioactive compounds in the past decade (Wahizatul et al., 2022). Most chemical control proved to be beneficial and have promising capabilities with some chemicals have successfully been produced *en masse* as an agricultural product such as Nano-Emulsion-formulated conidia of Saponin produced by a study in Universiti Putra Malaysia (UPM) that incorporates the saponin of *Furcraea* plants to control GAS population in the field. The bioactive components studied from one species of plant to another vary significantly where some studies were conducted on extracts obtained from stem, flowers, leaves, essential oils etc. As most of the treatments used in literatures are liquid-based, it proved to be easier to administer the treatments to GAS either by spraying or simply by dissolving it in the aquatic environment of the GAS, whether in the lab, semi-field, or field conditions. This is why chemical control is much more popular compared to biological control or cultural control methods as chemicals can be implemented anytime without post-application monitoring such as with biological control agent (to ensure the livelihood of the biocontrol agents) and cultural control method (manually picking the snail attractants after a few hours maintaining the inlets). Chemicals are readily sold and used in liquid form to be sprayed or dried up powders and pellets containing niclosamide or metaldehyde as the active ingredients. .

The main concern when it comes to commercial applications of chemical molluscicide would be the long-term effects to the environments, the non-target species and human health. Hence, despite the promising results of the plant-derived bioactive compounds as alternative to the commercial molluscicide, large scale field application these compounds pose several challenges. Concern that arises when trying to apply these bioactive compounds in commercial fields is the approval of regulatory bodies and the interaction of the compounds with the environment as well as non-target organism. Evaluating the ecological impacts of the intended molluscicides may

help to minimize unintended consequences indicating importance of comprehensive ecological assessment risks when dealing with bioactive compounds (He et al., 2018). The long-term application of molluscicides may also prompt GAS to develop resistance towards that molluscicides which is also a vital factor to be taken into consideration when trying to apply molluscicides commercially over a long period of time (Cui et al., 2015). With thorough understanding of the mechanisms, behaviours, and interactions of the bioactive compounds with the environment that can be unveiled with more studies, many of these promising compounds may finally be reliable tools to control the population of GAS in the fields.

2.2.4 Histopathology and Defence Mechanism of GAS Exposed to Toxicants and Infections

Histopathological analysis is a valuable method to unveil alterations to organs and tissues caused by treatments exposed to the target organisms (Arrighetti et al., 2022). Histopathological changes can be observed in multiple organs in GAS that can serve as biomarkers of the living conditions it is living in that ultimately lead to mortality (Peña et al., 2017). Key organs that typically show histopathological changes when exposed to infections or toxicants are the kidneys (anterior and posterior), foot, gill, reproductive organs and most importantly digestive gland (hepatopancreas) (Peña et al., 2017; Martinez et al., 2024). The digestive gland of molluscs is an essential biomarker to indicate numerous environmental stressors (Bighiu et al., 2017; Fernández et al., 2020). This could be attributed to the fact that the digestive gland of a mollusc plays a role in digestion, absorption, storage of nutrients and excretion of metabolites. This organ also plays an integral role in detoxifying, metabolizing and is the accumulation point of pollutants as well (Marigómez et al., 2013; Arrighetti et al., 2018).

In molluscs, hepatopancreas as the digestive gland is regarded as the most important organ when it comes to metabolism of endogenous and xenobiotic compounds (Dummee et al., 2015). Due to this sensitive nature of this organ, it can easily be damaged by water-borne pollutants and frequently used as a bioindicator for toxicants (Zhang & Zou, 2020). Several studies have highlighted histopathological alterations that occur to the hepatopancreas when exposed to multiple different treatments as a reaction towards toxicants and stressors. The hepatopancreas is made up of numerous digestive tubules amongst other structures. Within the digestive tubules are the basophilic cell (K corpuscles), digestive cells (C corpuscles), vacuoles, epithelial cells, columnar cells, and pyramidal cells (Dummee et al., 2015; Peña et al., 2017). C and K corpuscles are thought to play different roles within the hepatopancreas with C corpuscles being involved in intracellular food digestion meanwhile K corpuscles are involved in extracellular digestion and metabolic regulation (Peña et al., 2017). Commonly observed histopathological alterations within the

hepatopancreas includes increase in basophilic cell size, hypervacuolisation, tubular secretion, disintegration of the digestive tubules, atrophy, hyperplasia of certain cells and even necrosis. It has been recorded that an increase in the number (hyperplasia) of K corpuscles has been linked to accumulation of toxicants to detoxify and minimize the effects on the body of the snails (Peña et al., 2017). Upon exposure to infections and xenobiotic, an increase of haemocytes in the haemolymph can be expected and this can be because the haemocytes are part of the defense mechanism. Haemocytes can recognize and phagocytize foreign bodies detected and entrap them through nodulation and if the foreign objects are too large to engulf, the haemocytes encapsulate them (Rodriguez et al., 2018).

Some biological control agents have exhibited a castration effect on the reproductive gonads of GAS where the gonads undergo histopathological changes to the gonads that reduce the fertility of the individuals to a varying degree from acute to severe (Martinez et al, 2024). The testis in male gonad, occupies the first two spiral at the end of the digestive glands, meanwhile the ovaries in the females are situated within the inner apex of the spiral rim. Martinez et al., (2024) reported that when exposed to digenean trematodes can lead to partial or total castration depending on severity of the infection. This is where total lysis of the testis was observed alongside haemocytic infiltration meanwhile for the females, the trematodes caused alterations with the connective tissues of the ovaries leading to atrophy of the tubules however no haemocytic infiltration was recorded. Deformation of tubules was also recorded during the study when severe infection occurred. According to previous studies (Martinez et al., 2024), the gonads are the primary organ infected by digenean trematodes followed by the digestive glands, hence it could be assumed that these two organs would be first to be affected by pathogenic presence. Histopathological alterations caused by the exposure to toxicants can be classified into three reaction patterns which are inflammatory response, regressive changes (the lowering of cellular function than normal) and progressive changes (increased level of activity of cell and/or tissues than normal) (Arrighetti et al., 2022).

The condition alterations observed during histopathological analysis can be assigned quantitatively through Histopathological Condition Index (HI) which

enumerates the significance of the alterations observed (weight) as well as the frequency of occurrence (score) (Costa et al., 2013). Under progressive change also includes the hyperplasia (an increase in the number of cells in organs or tissues) of K corpuscles in the hepatopancreas meanwhile regressive change would include flattened epithelium, major disruption in the muscular bundle in the foot, loss of cilia, folding and dilations of the filaments in the gill as well as a general loss of structural integrity of all the affected organs (Peña et al., 2017). Changes and alterations such as disorganisation of connective tissue, lipofuscin accumulation, atrophy of digestive epithelium, fibrosis and haemocytic infiltration are commonly associated with the impairment of the functions of the digestive gland commonly associated with exposure of GAS to infections and xenobiotics (Osterauer et al., 2010; Arigghetti et al., 2022).

2.3 *Metarhizium anisopliae*

2.3.1 Taxonomy, Ecology and Biology of *M. anisopliae*

Metarhizium anisopliae belongs to the genus *Metarhizium* which is one of the most important groups of entomopathogenic fungus (EPF) found worldwide (Li et al., 2010; Kumar et al., 2018; Mascarin et al., 2019). The genus *Metarhizium* (Order Hypocreales: Family Clavicipitaceae) comprises of many high interest, fundamental members in both pathogen interaction and development of microbial insecticides (Aw & Hue, 2017; Pedrini, 2018). Out of all the members of the genus, *Metarhizium robertsii* and *M. anisopliae* are the two species that can reproduce by cloning and are able to infect a wide range of hosts (Liao et al., 2014).

Metarhizium anisopliae functions as both an entomopathogenic as well as endophyte, commonly found in the soils (Iwanicki et al., 2019). *Metarhizium* colonizes roots of plants increasing the plants' tolerance against pests and diseases, while simultaneously infecting insects (Sasan & Bidochka, 2012; 2013). Amongst the members of the genus, *M. anisopliae* is one of the members that can undergo asexual reproduction alongside a few other species such as *M. acrididae, M. brunneum M.*

flavoviride, M. frigidum, M. globosum, M. guizhouense, M. lepidiote, M. majus, M. pingshaense, M. rileyi, and *M. robertsii* (Kepler et al., 2014). Asexual reproduction of *M. anisopliae* produces conidia which are a type of spore that can be produced through aerial reproduction and cultivation meanwhile blastospores are sexual spores that are produced by liquid fermentation (Gotti et al., 2023). Initial observation led to the believe that this species is only limited to insect hosts. However, due to more research conducted revealed that this fungus can infect a relatively broader range of hosts compared to other members of the genus and is considered as generalist (Liao et al, 2014; Mesquita et al., 2023).

2.3.2 Mode and Mechanism of Infection

As a generalist, *Metarhizium anisopliae* can indeed infect a wider range of hosts however the mechanism of infection against insects have been recorded to be similar which begins with the spore adhesion to the exoskeleton of the hosts, germination of the spore, cuticle penetration, mycelial spreading through the haemolymph, outgrowth on the cadavers and the production of green conidia completes the cycle on a typical host (Aw & Hue, 2017).

The initial adhesion of *M. anisopliae* conidia is like other pathogens in the mechanism of biophysical interactions which either involve electrostatic (charge between the conidia and the host cuticles drawing them together) or hydrophobic interaction (Leger & Wang, 2020). The attachment on the host is then further stabilized by the enzymatic actions, mucus and specialized surface-associated protein that is responsible for adherence called adhesins (Greenfield et al., 2014; Leger & Wang, 2020). On atypical hosts such as snails, the interaction between the hydrophobic conidia with the shells may be different than with the cuticle of an insect host. The shell of GAS and snails in general are comprised of calcium carbonate ($CaCO_3$) and calcium oxide (CaO), with the latter exhibiting a broader and stronger antimicrobial activity (Brakemi et al., 2024). Although calcium is unable to be readily degraded through enzymatic actions produced by *M. anisopliae*, metabolic activity of the fungus may alter the pH of the environment creating a more acidic environment that would

lead to the dissolution of the integrity of the calcium carbonate-based shell of GAS (Soído et al., 2009; Nualnisachol et al., 2023). The *M. anisopliae* conidia may also enter by other means such as through the respiratory system or the exposed soft body of the GAS since *M. anisopliae* conidia are also able to secrete lipases, proteases and chitinase alongside many carbohydrate-degrading enzymes and will be able to enter and adhere with the presence of the special adhesive protein adhesin (Leger & Wang, 2020). Once inside the body, *M. anisopliae* can avoid detection because it has a gene that is involved in host immune evasion (Leger & Wang, 2020). Moreover, *M. anisopliae* has a special yeast-like phase called blastospores that can avoid from being phagocytosed by the defense mechanism of hosts (Belanger et al., 2002). *Metarhizium anisopliae* is also able to form hyphae once inside the body which are harder to phagocytose compared to the conidial form (Leger & Wang, 2020). By evading the host immune system, *M. anisopliae* can colonize and consume the haemolymph of the host severely affecting the host and eventually leading to its death. The evasion from being detected by the host immune system may be the biggest contributing factor to the pathogenicity success of *M. anisopliae* as a generalist.

2.3.3 Use of Pathogenic Agents in Controlling Golden Apple Snails

Species within the genus *Metarhizium* are well-known for their virulence and entomopathogenicity, making this genus favourable for development into pesticides and used worldwide as a greener alternative to control pests particularly *M. anisopliae*. The usage of a more environmentally friendly pesticide such as conidia of *M. anisopliae* species have been acknowledged for the wide variety of sources, great potential for improvements, low risk of target organisms developing resistance, and low possibilities of affecting non-target organisms (Walia et al., 2017). *Metarhizium* species have been used to control multiple species of mostly insect hosts (Table 2.3). The hosts range from agricultural pests such as termites and locusts to more urban pest such as mosquitoes (Peng et al., 2022).

Table 2.3 *Metarhizium* species used as biocontrol agents. Bolded species are snail species that were involved as the target organisms of *Metarhizium.*

Species of *Metarhizium*	Host	Reference
Metarhizium acridum	*Cornops frenatum frenatum*	Schmidt et al., 2018
	Tropicadis collaris	Schmidt et al., 2018
	Parascopas obesus	Schmidt et al., 2018
	Rhammatocerus schistocercoides	Magalhaes et al., 2000
Metarhizium anisopliae	*Aedes aegypti*	Gomes et al., 2023
	Ceratitis capitata	Gava et al., 2021
	Deois flavopicta	Mascarin et al., 2019
	Duponchelia fovealis	Poitevin et al., 2018
	Glycaspis brimblecombei	Domingues et al., 2022
	Gonipterus platensis	Jordan et al., 2021
	Mahanarva fimbriolata	Mascarin et al., 2019
	Monacha cantiana	**Hendawy et al., 2015**
	Monacha obstructa	**Abo-Elwfa et al., 2024**
	Nasutitermes sp.	Diniz et al., 2021
	Pomacea canaliculata	**Allahudin et al., 2024**
	Thaumastocoris peregrinus	Soliman et al., 2019
	Triatoma infestans	Rangel et al., 2020
	Zulia entreriana	Mascarin et al., 2019
Metarhizium braziliense	*Dalbulus maidis*	Souza et al., 2021
Metarhizium brunnerum	*Aedes aegypti*	Prado et al., 2020
	Leucoptera coffeella	Franzin et al., 2022
	Cornu aspersum	**Khoja et al., 2019**
Metarhizium humberi	*Hedypathes betulinus*	Lopes et al., 2014; Luz et al., 2019
	Hemiptera: Cynidae	Rezende et al., 2015
	Scaptocoris castanea	Lopes et al., 2014; Luz et al., 2019
Metarhizium lepidiotae	*Aegopsis balboceridus*	Lopes et al., 2014
Metarhizium majus	*Oryctes rhinoceros*	Paudel et al., 2021
Metarhizium marquandii	*Nasutitermes* sp.	Diniz et al., 2021
Metarhizium pinghaense	*Cosmopolites sordidus*	Lopes et al., 2014
Metarhizium rileyi	*Anticarsia gemmatalis*	Lopes et al., 2020
	Chrysodeixis includens	Lopes et al., 2020
	Helicoverpa armigera	Loureiro et al., 2020
	Spodoptera frugiperda	Barros et al., 2021
Metarhizium robertsii	*Dalbulus maidis*	Iwanicki et al., 2020
	Leucoptera coffeella	Franzin et al., 2022

The use of entomopathogenic fungi as a biocontrol agent is being explored more frequently recently as EPFs are not only environmentally friendly but are also typically host-specific, oftentimes not affecting non-target species in the ecosystem (Abdul Qayyum et al., 2021). The virulence of *M. anisopliae* as one of the existing entomopathogenic fungi can be affected by factors that can be divided into biotic and abiotic factors (Sharma et al., 2020). Biotic factors consist of factors relating to the conidia of the EPF themselves such as host compatibility with the conidia of the EPFs, meanwhile abiotic factors are related to external ecophysiological factors such as ambient temperature (Bayissa et al., 2017; Cheong et al., 2022; 2023), sunlight (Fernandes et al., 2007), humidity and precipitation levels and soil properties (Inglis et al., 2001; Quesada Moraga et al., 2007). In a controlled laboratory, the external factors can be monitored and ensured to be relatively stable hence the optimization and testing of EPFs' virulence and pathogenicity conducted in laboratory can truly assess the unhindered capabilities of EPFs against targeted host before the uncontrollable ecophysiological factors in the field is incorporated into the assays. This can reveal the relationship between host and pathogen much more clearly as well as testing the biological capabilities of these EPFs including *M. anisopliae.*

On the other hand, the efficacy of *M. anisopliae* can be significantly affected by environmental conditions such as temperature, humidity, and the exposure to ultraviolet (UV) radiation (Gašić & Tanović, 2013; Peng et al., 2022). This prompts the development of emulsion-formulated conidia to encapsulate the conidia of *M. anisopliae* to overcome these limitations such as the one developed by Cheong et al., (2022: 2023). This formulation incorporates a system involving surfactant and oil to encapsulate the conidia in nano-sized particles. Nano-encapsulation techniques provide sustainable approach for pest management in agriculture through the formulation of this nano pesticide (Kim et al., 2018). Nano-emulsions can help to improve poorly water-soluble active ingredients or water-resistant components such as *Metarhizium anisopliae* by encapsulating the component in the emulsion particles preventing desiccation and imbibitional damage (Cheong et al., 2019). This allows for increased spreadability and mechanical stability aside from having good bio-availability as well due to high surface area that supports better diffusion (Karimi-Maleh et al., 2024). This can be a huge contributing factor for the development of

molluscicide since in the market molluscicides are either in pellets or wettable emulsion forms only, hence nano-sized emulsions can expand on the gap of existing molluscicides (Kim et al., 2018; Cheong et al., 2019; Karimi-Maleh et al., 2024). This formulation has been proven by Cheong et al., (2022) to improve not only the fungal bioefficacy of *M. anisopliae* but also improve the pathogenicity, improved the stability of the conidia, the viability and shelf life of the conidia, as well as improve the resistance of the conidia towards harsh heat stress and UV radiation. Although Cheong et al., (2022) was limited to in laboratory bioassays, the results are promising enough and warrant further evaluation in the semi-field and field conditions to verify the capabilities for field conditions effectiveness. Emulsion-formulated treatment allows for the components to be shelf-stable and will not separate for a much longer period compared to in conidial suspension form. This allows for ease of storage for the usage of everyday farmers and scalability. Cheong et al., (2019) also mentioned that emulsion-formulated conidia are able to stay virulent and viable for much longer than conidial suspension and the increased dispersion may be useful when applications in field are conducted in the future since the water in the field has many different components compared to distilled water in laboratory conditions that may affect dissolvability and affect the spreadability of the treatment.

The instance of epizootic by EPF such as *M. anisopliae* relies on the host-pathogen compatibility as well as the spore density, its persistence, genetics, and latency (Abdul Qayyum et al., 2021). Spore density directly influences the chances of an infection to occur as the concentration of pathogens increase, so will the chances of a successful pathogen intrusion into the host by the infective propagules (Butt et al., 2000). Hence, this is why knowing the minimum concentration of treatments (in this case pathogenic conidia) is essential in trying to induce infection. As mentioned, the abiotic factors can also affect the persistence of the fungal conidia, the virulence as well as the biological capabilities. The emulsion-formulation encapsulates the conidia of *M. anisopliae* allowing the fungal conidia to withstand higher ambient temperature, consequently increasing its persistence and viability as well as its virulence and biological performance (Cheong et al., 2022; 2023). It has been proven to allow the conidia to be suspended in the emulsion matrix encapsulated by shelf-stable

formulation that allowed the conidia to overcome the abiotic factors from affecting its efficacy.

Due to the initial assumption that *Metarhizium* species is only able to control insect pests, the majority of studies using *Metarhizium* species as biocontrol agents have focused on insect hosts as this is the typical host for the fungus. Upon further investigations involving mostly terrestrial snails as mentioned in Table 2.3, it can be deduced that *Metarhizium* in fact has molluscicidal potential that must be evaluated (Hendawy et al., 2015; Khoja et al., 2019; Abo-Elwfa et al., 2024). This highlights the clear gap in knowledge when it comes to true molluscicidal capabilities of *Metarhizium* species. A study conducted by Abo-Elwfa et al., (2024) showcased *M. anisopliae*'s capabilities as a molluscicide. The study reported at least 50% mortality recorded after exposure to *M. anisopliae* and white fungal hyphae were observed growing out of the cadaver further solidifying *Metarhizium* species molluscicidal capabilities and potential. By testing on more snail samples, more evidence on the molluscicidal potential can be evaluated and further tested in fields ultimately establishing this fungus to be a highly viable alternative as an integrated pest management (IPM) method to commercial molluscicide.

CHAPTER 3

METHODOLOGY

3.1 Collection and Maintenance of Golden Apple Snails Adults

The Golden Apple Snail (GAS) adults were collected from the lake and surrounding vicinity within Universiti Sultan Zainal Abidin (UniSZA) Besut Campus, located at 5.75489°N, 102.62608°E (Figure 3.1). The selected snails collected for the experiments had shell lengths of at least 20 ± 5 mm in accordance to criteria established by Pratama et al., (2023). This particular study site was chosen because it is free from exposure to commercial molluscicides or any other treatments unlike the surrounding paddy fields nearby. Sites exposed to such treatments may affect the viability, growth and vigour of the snails collected from this area and may risk collecting snails with increased resistance to molluscicides or shortened lifespan due to surrounding factors. The physical conditions of the shells and size of GAS also play a role in the selection of UniSZA as the sampling site since in other sites, GAS observed were significantly smaller with more significant cracking and bleached shells. The populations of GAS in this area were also present all-year round compared to populations in paddy fields that undergo aestivation during drier, off-season months.

Figure 3.1 Lake at Universiti Sultan Zainal Abidin (UniSZA) Besut campus for the sampling site of GAS.

The GAS collected were maintained under laboratory conditions (25 ± 5°C, 80-90% relative humidity, 12 h photoperiod) in plastic aquariums (60 x 45 x 45 cm) (Md Rejab et al., 2022). The entirety of the experimentation from acclimatization of GAS to the preparations of GAS organs for histopathological analysis was conducted in Fungus Laboratory Centre of Research & Field Service (CRAFS) of Universiti Malaysia Terengganu. GAS adults were fed ad libitum with lettuce, cucumbers, and mustard greens. The snails were allowed to acclimatize for at least one week to allow any displacement distress to subside before the bioassay was conducted and ensured that only healthy individuals, by observing the activeness of the snails, were selected (Bae et al., 2021). Viability and vigour of the snails were observed through behaviour indicator such as their appetite, response towards mechanical stimuli and overall activeness during acclimatization. Only snails that possessed intact shells that were not degraded with high appetites and were visibly active were included in the bioassays to minimize the number of external factors influencing the results obtained.

3.2 Cultivation and Mass Production of Entomopathogenic Fungus *Metarhizium anisopliae* Strain Met-Gra4

3.2.1 Subculturing and Maintenance of *M. anisopliae* (Strain Met-Gra4)

The source of *Metarhizium anisopliae* strain Met-Gra4 used for cultivation was supplied by Wahizatul A. A. This local strain of *M. anisopliae* was originally isolated by Grace et al., (2017) named Met-Gra4. Maintenance of the fungus *M. anisopliae* was conducted by periodically isolating cultures and subculturing onto Potato-Dextrose Agar (PDA) plates to ensure optimum pathogenicity and viability of the fungus used which can degrade naturally over time due to abiotic factors such as high temperature or low relative humidity (Cheong et al., 2022; 2023).

The subculturing was conducted by streaking the fungus onto a PDA plate using an inoculating loop and incubated for seven days in room conditions (25 ± 5°C, 80 - 90% relative humidity) (Najihah et al., 2021). All subculturing procedures were carried out in the Biological Safety Chamber (BSC) to provide a sterile environment (Figure 3.2). The inoculating loops used were sanitized after every plate streaked by using Bunsen burner to burn it bright red as part of standard aseptic techniques. PDA was selected due to its suitability for microbiological growth and was supplemented with chloramphenicol to prevent bacterial contaminations at the rate of 20 mL L^{-1} of PDA (Westphal et al., 2021). The PDA plates were inverted before incubation to prevent condensation that formed on the lid of the plates to drip down onto the medium (Sanders, 2012; Mollea et al., 2022). After seven days of incubation, the PDA plates containing *M. anisopliae* were observed and evaluated before proceeding with the mass production process. Only uncontaminated pure cultures of *M. anisopliae* were used in the mass production of conidia.

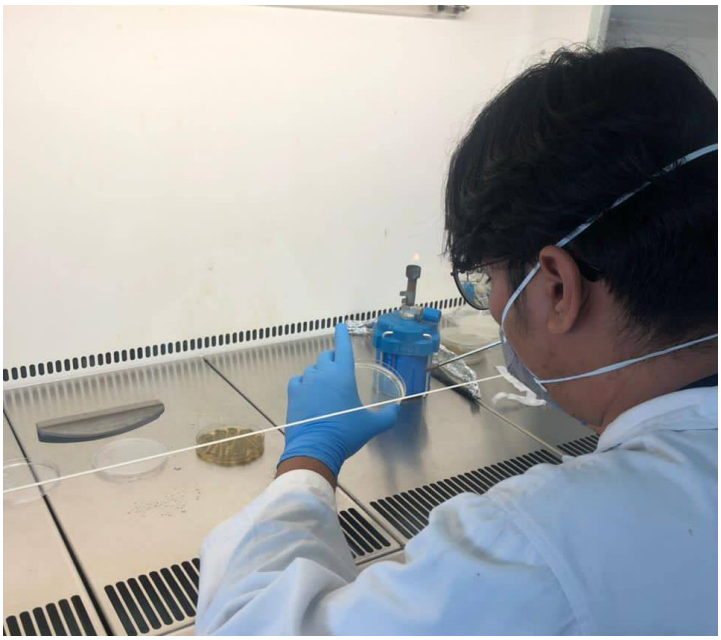

Figure 3.2 Subculturing process in the Biological Safety Chamber (BSC) under sterile conditions.

3.2.2 Solid-State Fermentation to Mass Produce *M. anisopliae* Conidia

Mass production of *M anisopliae* was carried out using solid-state fermentation technique with rice as the substrate. The methods for this mass-production were adapted from Najihah et al., 2021. For each mass production batch, three replicates of spawn bags with each containing 150 g of rice were produced. The rice was first weighed, washed, and dried before transferred into the autoclavable spawn bags. To each bag, 150 mL of autoclaved distilled water (dH_2O) was added along with 1.5 g of yeast as the nitrogen source meanwhile, the rice provided carbon and carbohydrates for the optimal growth of *M. anisopliae* (Jaronski & Jackson, 2012). The media were left to incubate for 18 hours in room conditions (25 ± 5°C, 80 - 90% relative humidity) (Najihah et al., 2021). After 18 hours, the media were cooked in a microwave for about 15 minutes before they were autoclaved for 25 minutes at 121 ˚C, 15 psi. The spawn bags containing cooked and autoclaved media were allowed to cool down before proceeding with the inoculation.

The process of adding the inoculum to the media was conducted in the BSC where the workspace, and all apparatus to be used, were sanitized beforehand with 70% ethanol to ensure a sterile environment and to reduce risks of contamination. The fungal inoculum introduced to the media were cultures of *M. anisopliae* grown on PDA plates. The PDA containing cultures of *M. anisopliae* were cut into squares of 1 x 1 cm using an inoculating loop and inoculated onto the cooked, autoclaved, and cooled media (Figure 3.3). The inoculum was evenly distributed and the medium was slightly squeezed so that the inoculum was well-incorporated. For each bag of 150 g of rice medium, 7.5 squares (1 x 1 cm^2) of inoculums were used amounting to about 7.5 cm^2 of PDA (Najihah et al., 2021). Aseptic techniques were implemented between each bag of medium. The inoculating loop was sterilized by heating the loops with a Bunsen burner until the inoculating loop turned bright red. The inoculating loop was then ensured to have cooled down completely by testing the loop on an area other than the area of interest on the PDA before the cutting was conducted (Sanders, 2012). Once inoculated, the spawn bags were incubated for 14 days under ambient conditions (25 ± 5°C, 80 - 90% relative humidity). The spawn bags were monitored periodically to observe for any contamination or overgrowth of other microbes.

Figure 3.3 Inoculation of sterilised solid media (rice) in a spawn bag.

After 14 days of incubation, the spawn bags were carefully opened and the media were transferred to another container to dry the sporulated media. This procedure was also carried out inside the BSC to ensure the sterility of the working environment. The media were crumbled using a sanitized spatula to facilitate the drying and harvesting process. The containers were covered with three-layer thick tissue papers (prepared by folding the tissue three times before covering the container) and secured with rubber bands to dry for seven days as shown in Figure 3.4 (Najihah et al., 2021).

Figure 3.4 Drying process of sporulated solid media after incubation process, covered with triple-folded tissues in a sterile container (Method adapted from Najihah et al., 2021).

After seven days of drying, the fungal conidia were then harvested from the media. The conidia were mechanically harvested from the media by using a two-nested sieve shaker, consisting of an aperture 2.0 mm test sieve nested on another aperture 500 µm test sieve nested on a collection pan at the very bottom. The dried media were loaded on the test sieve mesh slowly to prevent excessive conidia from escaping into the air. Figure 3.5 shows the solid media after the drying process ready to be harvested. The test sieve shaker machine was run for two rounds for each replicate with one run

taking one hour. After the second round, the conidia were collected from the collection pan.

Figure 3.5 Sporulated solid media prior to mechanical harvesting process conducted using two-nested sieve shaker.

3.3 Preparation of Treatments

3.3.1 Viability test

The viability of the conidia was assessed to ensure the quality and effectiveness of the conidia harvested. The viability test was carried out by preparing a conidial suspension through a series of dilutions known as the serial dilution method adapted from Najihah et al., (2021). A stock solution was prepared by adding 0.15 g of conidia to 1 mL of dH_2O containing Tween 80. Tween 80 has the function to overcome the hydrophobic nature of fungus like *M. anisopliae* and allow the formation of a homogenous aqueous solution (Bukhari et a., 2011). This stock solution was then serially diluted ten-fold by taking 0.1 mL or 100 µL of the stock and diluting it in 0.9 mL or 900 µL of dH_2O containing Tween 80. This process was repeated upwards to

three times using an Ahn pipet4u pro manual single channel pipette with variable volumes to pipette the liquids into the respective microcentrifuge tubes. About 70 µL was taken from the third dilution which was then dispensed onto an improved Neubauer haemocytometer to calculate the concentration of conidia (Figure 3.6) following the method of Maketon et al., (2009). Approximately 50 µL of the third serial dilution was pipetted onto a PDA plate and allowed to incubate for 18 hours. This was repeated three times producing a total of three PDA plates containing 50 µL of the third serial dilution. After 18 hours of incubation, the plates were divided into three sections by drawing the sections with a marker to facilitate the counting process by forming a boundary for each section. The viability of conidia was calculated by counting the ratio of viable conidia which was characterized by having germinated germ tube with the length being twice the diameter of the conidia over the total number of conidia counted on the PDA plates (Herlinda, 2010).

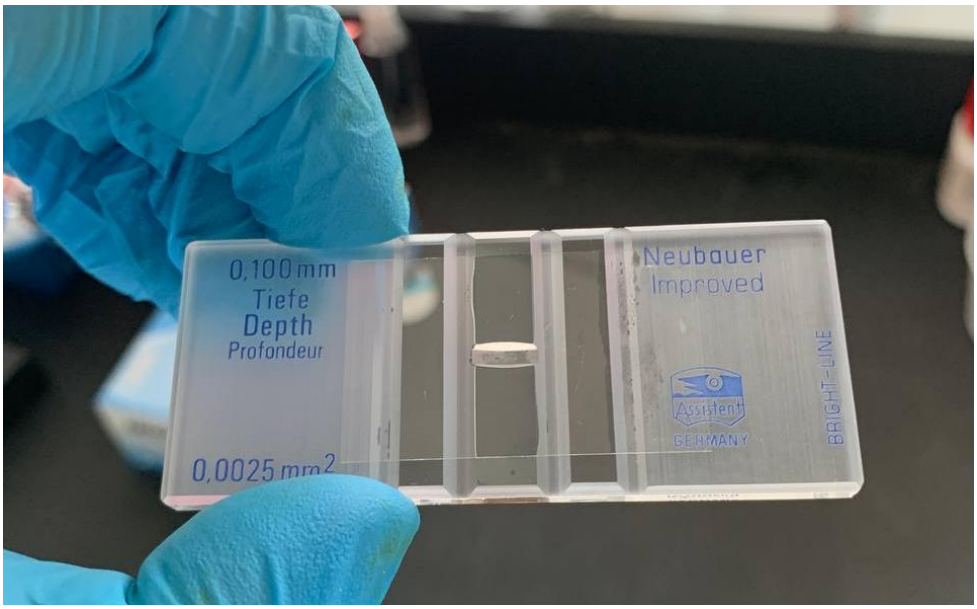

Figure 3.6 Neubauer haemocytometer with 3X serial dilution pipetted onto it for concentration calculation.

3.3.2 Preparations of Conidial Suspension and Emulsion-Formulated Conidia.

Conidial suspensions for bioassay were prepared by conducting serial dilution in a similar ratio to the viability test (ten-fold dilution). However, for conidial suspension used in bioassay, the initial stock suspension was prepared by suspending 1.5 g of conidia in 10 mL distilled water (dH_2O) with 0.05% Tween 80. The solutions were vortexed to achieve a homogenous suspension which produces the stock solution (Najihah et al., 2021). From the stock solution, 1 mL was pipetted and diluted into 9 mL dH_2O with 0.05% Tween 80 and serially diluted until three times (Figure 3.7). The third serial dilution (labelled 3X) had a concentration of 6.7 x 10^6 conidia mL^{-1} which was obtained by calculating using haemocytometer. After all the required stocks had been obtained, conidial suspension for the bioassay was produced by calculating the volume of stocks needed to produce the desired amount of concentration for the treatments. This was done by utilizing the formula $M_1V_1 = M_2V_2$ in which;

M_1 = the concentration of the stock
V_1 = the volume of stock to be used
M_2 = the desired concentration to be produced
V_2 = the volume of the treatment to be produced.

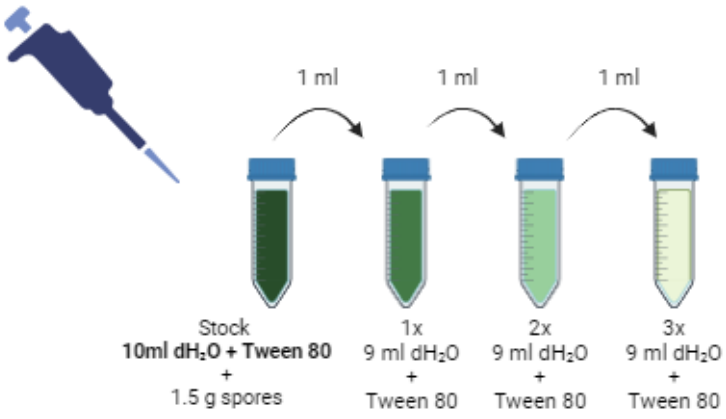

Figure 3.7 Illustration of the serial dilution procedure required for conidial suspension treatment for pathogenicity test.

The treatment volume was set at 350 mL for the container of size 17 x 11.4 x 6.2 cm, corresponding to a water depth of 5 cm which was found to be suitable for the snails (Md Rejab et al., 2022). In this experiment, conidial suspensions with the concentrations 6.7 x 10^5, 10^6, 10^7 and 10^8 conidia mL^{-1} were tested for their bioefficacy against GAS adults.

The emulsion-formulated conidia used in this experiment was supplied by Wahizatul A. A. The preparation of emulsion-formulated conidia followed the same procedure as the conidial suspension. A stock solution was prepared by dissolving 1.5 g of conidia into 10 mL of the emulsion-formulated conidia. Then, 1 mL of this emulsion-formulated conidia containing conidia was diluted in 9 mL of dH_2O with 0.05% Tween 80. A similar serial dilution as conidial suspension was conducted with the stock produced by incorporating conidia into the emulsion-formulated conidia (Cheong et al., 2022; 2023) (Figure 3.8). Treatments containing emulsion-formulated conidia were also prepared by utilizing the $M_1V_1 = M_2V_2$ formula to calculate the volume of stock required (V_1), with a predetermined concentration (M_1), to make the desired concentration of treatments (M_2), with the volume of 350 mL (V_2). The end products of this serial dilution had solutions with concentrations 6.7 x 10^5, 10^6, 10^7 and 10^8 conidia mL^{-1} which were also tested against GAS adults to unveil its efficacy. To ensure that the colony-forming unit (cfu) within both conidial suspension and emulsion formulation is as optimum as possible, viability test were conducted for each batch of conidia produced, carriers were used to prevent sedimentation and agglutination of conidia (such as emulsion particles and Tween 80), the media used for subculturing and mass production were kept constant, alongside the external conditions were also ensured to be consistent (relative humidity , temperature, photo period) (Sriram & Savitha, 2011). Proper serial dilution techniques were also implemented such as ensuring that the pipette tips flushing and tip-change were conducted properly (Thomas et al., 2015). The formulations prepared were also used within two to three days after preparations to avoid any drop in cfu or viability, and stored in the chiller to slow the metabolism and growth of the fungus as well as ensure stability of the formulations (Senaratne & Jayaweera, 2024).

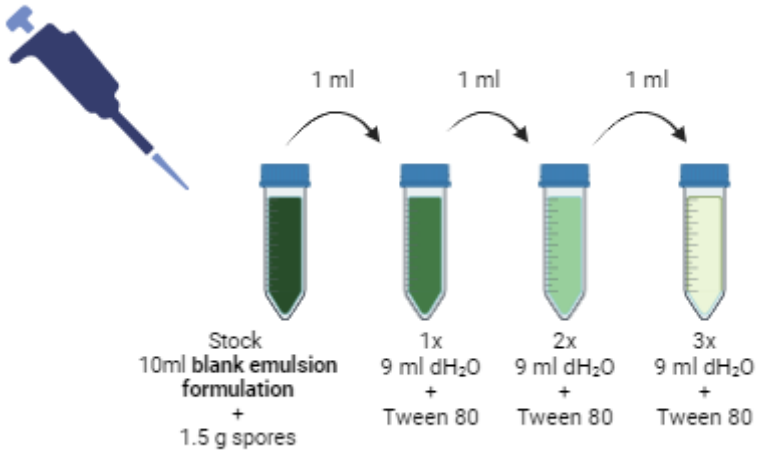

Figure 3.8 Illustration of the serial dilution procedure required for emulsion-formulated conidia for pathogenicity test.

3.4 Pathogenicity Tests

The procedures for pathogenicity test were adapted from the guidelines for testing molluscicides provided by the World Health Organisation (WHO, 2019). Pathogenicity test is based on a factorial bioassay with a Randomized Complete Design (RCD) which tested all possible combinations of the two factors (formulations types and concentrations) plus controls for three replicates for all treatments. The experiment comprised two formulation types, conidial suspension (CS) and emulsion-formulated (EF), tested at four concentrations (10^5, 10^6, 10^7, and 10^8 conidia mL^{-1}), together with a positive control (metaldehyde 5%) and a negative control (distilled water with Tween 80). Each treatment had three replicates, with one container of five snails per replicate, resulting a total of 30 containers (150 snails). Treatments were randomly assigned to eliminate positional or environmental bias.

Pathogenicity test was done by exposing GAS adults to both conidial suspension and emulsion-formulated conidia solution at four concentrations 6.7 x 10^5, 10^6, 10^7 and 10^8 conidia mL^{-1} for 24 hours. Five GAS adults of similar sizes were chosen and acclimatized for seven days under laboratory conditions to ensure that the snails selected were healthy and active before the treatment (Wang et al., 2023). The

GAS were exposed to 350 mL of suspension solution containing treatments (conidial suspension and emulsion-formulated conidia solution) prepared beforehand with concentrations ranging from 6.7 x 10^5, 10^6, 10^7 and 10^8 conidia mL^{-1} for 24 hours and food was still provided to the snails during experimentation, fed with lettuce, cucumbers and mustard green ad libitum (Figure 3.9). After 24 hours, the snails were removed from the solution, placed in new, different containers of the same size containing only distilled water and continued to be observed for the next 14 days. During this observation period, the snails were provided with fresh food daily, mortality and changes in behaviour were recorded. Each treatment concentration for both conidial suspension and emulsion-formulated conidia was replicated thrice, involving a total of 60 snails per treatment, and totalling up to 120 snails across both treatments. The sexes of the GAS chosen for bioassays were not discriminated as sex does not influence the parameters and histopathological analysis observed (Arigghetti et al., 2018).

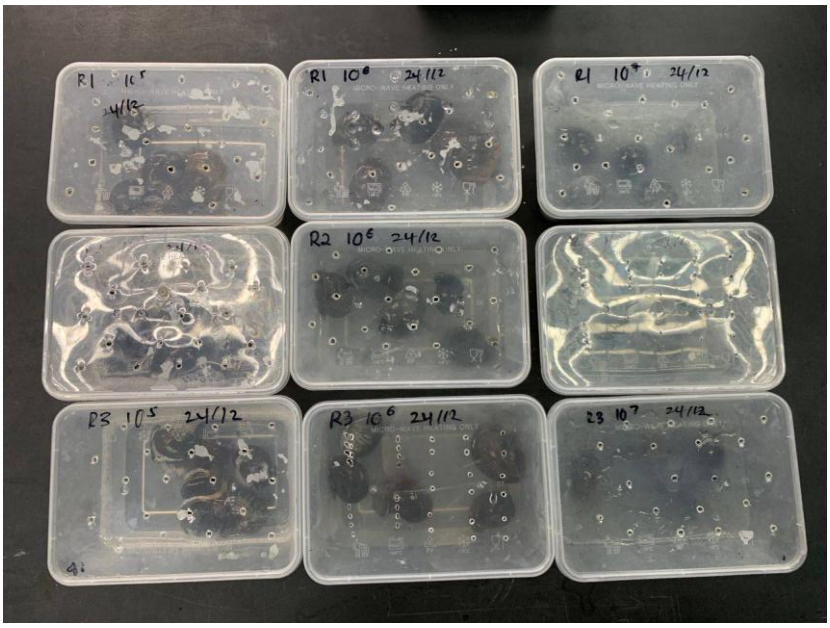

Figure 3.9 Plastic containers used to house GAS exposed to treatments.

For positive control (chemical molluscicide, metaldehyde 5%), the GAS was treated with a solution containing commercial molluscicide formulated in the form of granules with the active ingredient metaldehyde 5% (Figure 3.10). Based on the packaging instruction, 0.6 g of molluscicide was dissolved in 350 mL of dH_2O (Sisa

et al., 2016 with slight modifications), resulting in a concentration of 1.7 mg mL^{-1}. GAS exposed to this molluscicide also underwent this exposure for 24 hours before being removed from the treatment. For negative control, GAS were treated with dH_2O containing 0.05% Tween 80 for 24 hours. Any mortality or changes in behaviour were recorded. Negative and positive control (chemical molluscicide, metaldehyde 5%) treatments were replicated three times totalling up to 30 individuals of GAS for both treatments.

Figure 3.10 Chemical molluscicide, metaldehyde 5% used as positive control.

The snails were observed for 14 days where the snails were fed ad libitum with various vegetables such as lettuce, cucumbers, and mustard greens. Dead samples (were identified by snails hanging out of their shells, failure to exhibit response to mechanical stimulations as well as emitting foul odours), and these snails were dissected and fixed for further histopathological analysis (Meléndez & Capriles, 2002; Md Rejab et al., 2022).

3.4.1 Dissection and Sample Fixation

Samples of GAS adults that have died were carefully removed from their shells by cracking the shells along the sutures and cutting away at the columellar muscles to allow the entire cadaver to slide out easily (Figure 3.11). Dissections were done with utmost care to avoid damaging or accidentally puncturing the soft tissues and organs of the cadavers. The cadavers were then further dissected to extract the digestive gland (hepatopancreas) and the gonads. The digestive glands have been proposed as valuable biomarkers for the effects of environmental stressors owing to their essential functions in digestion, absorption, storage of nutrients, metabolism, and detoxification of pollutants (Arrighetti et al., 2022). The gonads were included in order to investigate if *M. anisopliae* has any castration effects on the population as it is proposed that the gonads are one of the primary sites of attacks by other pathogens such as trematodes (Martinez et al., 2024). The extracted organs were then fixed in Bouin's solution for 24 hours (Tamim et al., 2019). After fixation, the organs were transferred to 70% ethanol to dehydrate the tissues and organs in the specimens (Troiano et al., 2010) (Figure 3.12). Ethanol was changed daily until the yellow tint from the residue of Bouin's solution was completely absent and the ethanol remained clear.

Figure 3.11 Cadaver of a male golden apple snail (GAS) after removal from the shell.

Figure 3.12 Dissected organs of golden apple snails (GAS) soaked in ethanol for preparations of histopathological analysis.

3.4.2 Histopathological Glass Slide Preparation and Staining Protocol

Histopathological analysis from the tissue processing to the observation of samples under the microscope was conducted in Histology Laboratory of Institute of Tropical Aquaculture & Fisheries (AKUATROP). After the ethanol remained clear for 24 hours, the organs were processed in the tissue processor machine for 18 hours. Afterwards, the organs were embedded in paraffin wax (Roundfin Histoplast paraffin) using an embedding machine ensuring the positioning of the organ to be exactly as desired in order for the ribbons produced of the organs later to be able to showcase the entirety of the cut. The paraffin wax blocks produced were then serially cut into ribbons with 5 µm thickness using the microtome machine (Leica® HistoCore) and subsequently laid in a water bath set at 38˚C to allow any creases to smoothen before the ribbons were fished onto a glass slide. The glass slide with the cut samples were allowed to airdry for 24 hours before proceeding with the staining process.

After drying, the prepared glass slides were subjected to staining process conducted entirely in the laminar flow cabinet. The staining process began with the slides getting soaked into xylene for five minutes which has the purpose to clear the samples. After five minutes, the glass slides were removed from the xylene, put into another container, and dipped into xylene again for another five minutes. The slides were then further dehydrated by dipping into a series of graded alcohol, twice in 100% alcohol for five minutes each time. This was followed by being dipped into 95% alcohol for two minutes and 70% alcohol for two minutes. The slides were then run under tap water for two to three minutes. Afterwards, the slides were then soaked in haematoxylin (Leica®) for at least 15 minutes before being rinsed again under running tap water. By this point, the samples on the slides were checked under the microscope (Leica® DM750, ICC50 HD) to ensure that the purple stain of the haematoxylin has adequately stained the samples as desired.

Following that, the slides were dipped into acetone three times before rinsed under running water for another two minutes. The slides were subsequently soaked in 2% potassium acetate for three minutes before being rinsed under running tap water again for three minutes before soaked into eosin for 10 minutes. The slides were then observed under a light microscope (Leica® DM750, ICC50 HD) to check whether the sample had been adequately stained yellow by the eosin (Leica®) before soaking in 95% alcohol for 5 minutes, the soaking in alcohol was repeated twice. The samples were once again soaked in xylene for five minutes, and this was also repeated twice. Finally, the slides were mounted using DPX mountant (Merck) after being air-dried for about five minutes to ensure the xylene had evaporated and the samples were analysed under a light microscope (Leica® DM750, ICC50 HD).

The morphological changes seen in the tissues of the hepatopancreas and reproductive gonads were categorised according to the Histopathological Condition Index (HI index). This index account for the type of changes seen, the frequency of occurrence in the samples observed and the severity of each histopathological changes observed that might be attributed to the infection by *M. anisopliae*. Samples from the treatments (both conidial suspension and emulsion-formulated treatments), negative control and positive control (chemical molluscicide, metaldehyde 5%) treatments

were compared to elucidate the changes brought by infections to samples that died from commercial molluscicide and samples from the normal conditions of the organs and cells of healthy snails.

For each treatment, hepatopancreas from five individuals were observed under a microscope to determine the structure and integrity of the digestive tubules. The diameter of ten digestive tubules per sample, chosen at random, and the number of K corpuscles were recorded. In total, 50 digestive tubules were observed, measured, and randomly counted for each treatment. The hepatopancreas samples were then were subjected to the Histopathological Condition Index (HI) alongside the ovary and testis to enumerate the histopathological alterations observed in order to statistically differentiate the samples in different treatments.

Histopathological alterations observed under the microscope were described and classified according to the type of response exhibited, such as inflammatory responses, regressive changes (where cellular function was lowered compared to normal), and progressive changes (increased functional activity of the tissues and cells). With the enumeration of histopathological changes through the Histopathological Condition Index (HI), the conditions can be easily compared between treatments, providing a quantitative calculation method (Arrighetti et al., 2022). This index considers the biological significance of alterations observed (weight) as well as the frequency of appearance of the alterations (score) (Table 3.1). The weights of the alterations were classified according to the level of severity and reversibility of the damages or changes, with; 1 = indicating alterations with minimal importance and reversibility; 2 = indicating alterations of moderate importance, where alterations may reverse or subside post-exposure; 3 = indicating alterations of significant importance. The score of the index ranged from 0 to 3, with;

0 = no histopathology observed,
1 = mild histopathology (present in less than 25% of the observed samples),
2 = moderate histopathology (present in 25–75% of the observed samples),
3 = severe histopathology (present in more than 75% of the observed samples).

The index was based on the property of the affected samples and calculated according to the formula $HI_h = \Sigma^j(a_{jh} \times w_j)$, where HI_h is the histopathological index for individual h, wj is the weight of the j^{th} histopathological alteration, and a_{jh} is the score attributed to the j^{th} alteration observed in the h^{th} individual.

Table 3.1 Weight of the alterations observed for all types of responses.

Histopathological alterations	Weight (W)
Inflammatory responses	
Haemocytic infiltration in the intertubular space	1
Progressive changes	
Enlargement of cells	2
Hypervacuolisation of the cells	2
Hyperplasia of cells	2
Regressive changes	
Disorganisation of connective tissues	1
Degeneration of cells	2
Tubule regression	2
Cell lysis	3
Necrosis	3

The severity score for the hepatopancreas (Table 3.2) and the gonads (ovary and testis) (Table 3.3) were adapted from Joshy et al., (2022). The severity score is calculated by calculating the maximum possible score obtainable for each organ, and classifying the score into quartiles based on the maximum score obtained. Since ovary and testis had the same categories of observation and alterations, the maximum possible score as well as the classifications has the same scores as below:

Table 3.2 Severity score of Histopathological Condition Index (HI) for hepatopancreas of Golden Apple Snail (GAS).

Hepatopancreas	
Score	Severity
1-8	Mild
9-16	Mild-Moderate
17-24	Moderate-Severe
25-33	Severe

Table 3.3 Severity score of Histiopathological Condition Index (HI) for gonads (ovary & testis) of Golden Apple Snails (GAS).

Ovary & Testis	
Score	Severity
1-9	Mild
10-18	Mild-Moderate
19-27	Moderate-Severe
28-36	Severe

3.5 Data Analysis

The cumulative mortality of samples exposed to conidial suspension, emulsion-formulated conidia and positive control (chemical molluscicide, metaldehyde 5%) were analysed in Statistical Package for Social Sciences (SPSS) 21.0 software using Kaplan-Meier test and followed up with pairwise comparison to see significant differences in survival of the different populations.

The median lethal time (LT_{50}) and median lethal concentration (LC_{50}), which indicated the time taken for 50% of the population to be killed and the concentration of treatment that can kill 50% of the population, respectively, were calculated using Probit regression in SPSS 21.0 software as well. Statistical Package for Social Sciences (SPSS) 21 software was also used to run the normality test and the homogeneity of variances test (Levene's test).

Two Way Analysis of Variances (ANOVA) was conducted to understand the relationship between the two factors: concentrations and formulations, when significant relationship was discovered, One Way Analysis of Variance (ANOVA) was used to further compare significant differences between the efficacies of different treatments used Singh et al., 2019a; Liu et al., 2020; Martinez et al., 2021) When one way ANOVA showed significant differences, Post Hoc Tukey Honestly Significant Difference (HSD) test was conducted to indicate which group has a significant different from the others with significance level of $\alpha = 0.05$. One-Way ANOVA was conducted in a way that all treatments were considered as separate groups since formulation x concentration showed significant interactions.

One-way ANOVA was conducted to determine significant differences between the treatment groups regarding the diameter of the digestive tubules followed by Tukey HSD post hoc test for multiple comparison. On the other hand, due to the failure to comply with the assumptions to perform parametric tests, determining differences in the number of K corpuscles in the digestive tubules between treatments led to applying the non-parametric Kruskal–Wallis's test followed by stepwise step-down post hoc (similar to Dunn's test) tests.

Data analysis for the Histopathological Condition Index (HI) of all organs was also conducted using SPSS 21 software with the non-parametric test because the dataset was not continuous and comparisons between more than two group required the Kruskal-Wallis' test to be used. Significant differences between the groups were subsequently determined using by stepwise step-down post hoc as well.

CHAPTER 4

RESULTS

4.1 Efficacy between Conidial Suspension and Emulsion-Formulated Conidia Treatments in Inducing Mortality of GAS

The Kaplan-Meier test used to analyse the survival of the populations of GAS revealed that the emulsion-formulated treatment caused mortality in more than 50% of the entire sample GAS population within the first four days post-treatment (marked by the intercept of lines at y=0.5), as opposed to the conidial suspension, only able to achieve 50% mortality approximately seven days after treatment (Figure 4.1). Two concentrations of emulsion-formulated conidia, 6.7×10^7 and 6.7×10^8 conidia mL^{-1}, achieved 100% mortality within the first 24 hours. In comparison, only the highest concentration of 6.7×10^8 conidia mL^{-1} of the conidial suspension showed similar potency.

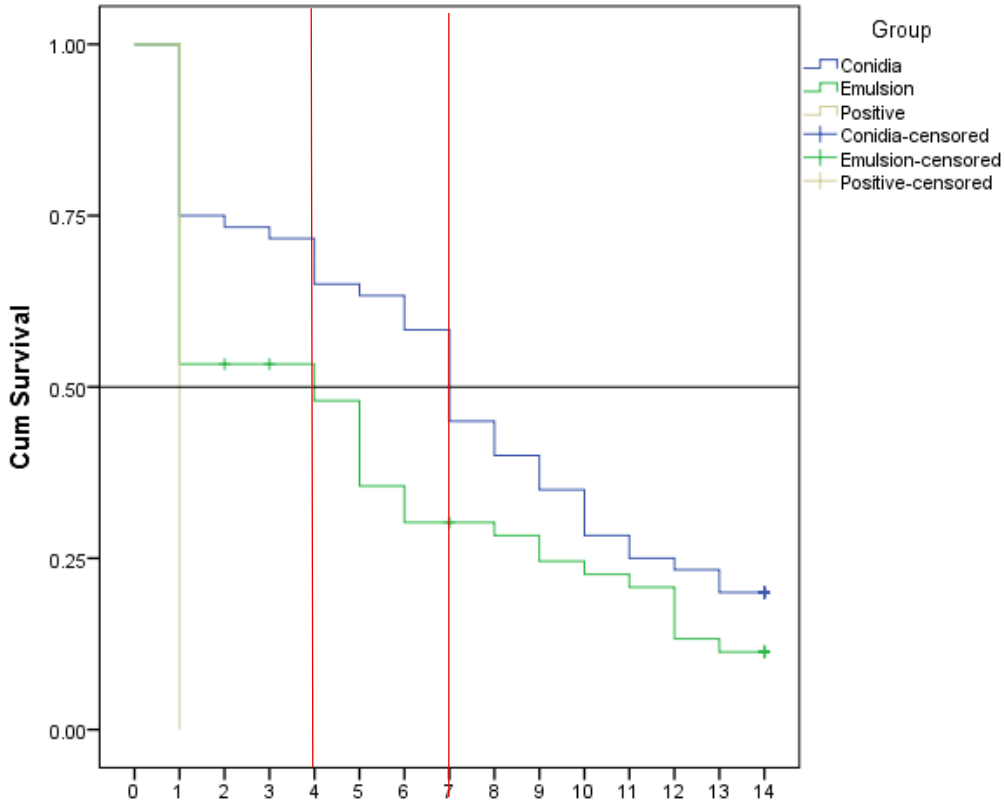

Figure 4.1 Kaplan-Meier test showing cumulative survival of samples treated with emulsion-formulated conidia, conidial suspension and positive control (metaldehyde 5%, 1.7 mg mL^{-1})

The test revealed that the median survival time for the entire populations including the positive control (metaldehyde 5%), conidial suspension and emulsion formulation, the median survival time was five days (95% confidence internal (CI): 3.5-6.48). When stratified according to treatment groups, median survival time for samples treated with emulsion-formulated conidia was four days (95% confidence interval (CI)) meanwhile the median survival time for samples treated with conidial suspension was seven days (95% CI). Samples treated with emulsion-formulated had consistently lower survivability compared to samples treated with conidial suspension from day 1 up until day 14. When log-rank test was conducted, it showed there is a significant difference within the samples (log-rank test, $\chi^2 = 25.70$; df = 2; $p<0.01$). Pairwise comparison showed that there is a significant difference between the positive control (metaldehyde 5%) to emulsion-formulated samples (Pairwise comparison; $\chi^2 = 13.77$; df = 1; $p<0.01$) and to conidial suspension (Pairwise comparison; $\chi^2 = 27.75$; df = 1; $p<0.01$). Log-rank test results of emulsion-formulated samples were also

significantly different to conidial suspension (Pairwise comparison; $\chi^2 = 4.55$; df = 1; $p<0.05$). Indicating that the positive control (metaldehyde 5%) had the highest molluscicidal capabilities hence the lowest survivability in the treated samples, followed by emulsion-formulated conidia and conidial suspension.

Both treatments were recorded to have less than 20% survivors, (more than 80% mortality) GAS populations within the time frame of 14 days of the test. However, the emulsion-formulated treatment consistently resulted in a higher percentage of daily mortality than the conidial suspension throughout the observation period. Positive control (metaldehyde 5%) achieved a staggering 100% mortality across all replicates within 24 hours of treatment.

4.2 Comparison of LT_{50} And LC_{50} Between Conidial Suspension and Emulsion-Formulated Conidia of *M. anisopliae* Against GAS

Table 4.1 shows the Two-Way ANOVA results to test the relationship between two factors: formulation and concentration. The test performed revealed that there is significant effect of formulation type ($F = 104.88$; df = 1; $p <0.01$). Concentration also had a significant effect on the results unveiled by the Two-Way ANOVA (($F = 142.42$; df = 3; $p <0.01$) and there is a significant interaction between treatment and concentration to the results obtained ($F = 14.04$; df = 3; $p <0.01$).

Table 4.1 Two-Way ANOVA test result between the formulation, concentration and the interaction between formulation and concentration.

Source of variation	df	F	p-value
Formulation	1	104.88	$p<0.01$
Concentration	3	142.42	$p<0.01$
Formulation * Concentration	3	14.04	$p<0.01$

Next, One-Way ANOVA was conducted to obtain the LT_{50} and LC_{50} of adult GAS treated with conidial suspension and emulsion-formulated conidia of *M.*

anisopliae and post-hoc Tukey Honestly Significant Difference (HSD) was also conducted to know which formulation x concentration were significantly different to one another (Table 4.2). No mortality was recorded in the negative control group which contained 0.05% Tween 80 in distilled water (dH_2O). The emulsion-formulated Met-Gra4 against adult GAS caused more significant toxicity than conidial suspension treatment, as no significant difference was observed in efficacy between the positive control (metaldehyde, 5%), 6.7×10^8 conidia mL^{-1}, and 6.7×10^7 conidia mL^{-1} treatments, with LT_{50} values of 0.5199, 0.449, and 0.6123 days, respectively (Tukey HSD; F: 205.91; df 8; $p<0.01$). In contrast, within the conidial suspensions, only the concentration of 6.7×10^8 conidia mL^{-1} exhibited potency similar to that of the positive control (metaldehyde, 5%).

Table 4.2 Median lethal time (LT_{50}) and median lethal concentration (LC_{50} of the emulsion-formulated conidia, conidial suspension, and positive control (metaldehyde, 5%) treatments. Different subscript letters indicate significant differences (Tukey HSD; F: 205.91; df 8; $p<0.01$).

Pathogenicity test					
Concentration (conidia mL^{-1})	LT_{50} (days)	Lower Boundary	Upper Boundary	Regression equation	Goodness of fit (R^2)
Emulsion-formulated conidia					
6.7×10^5	9.39 ^{d}	5.29	12.69	$y = 0.2756x - 0.1963$	0.9417
6.7×10^6	5.96^{b}	5.11	7.28	$y = 0.619x - 1.1905$	0.8711
6.7×10^7	0.61 ^{a}	0.50	0.73	$y = 0.209x + 0.5714$	0.9201
6.7×10^8	0.45 ^{a}	0.42	0.53	$y = 0.2421x - 0.1429$	0.8904
Conidial suspension					
6.7×10^5	15.33 ^{e}	13.27	17.44	$y = 0.2037x - 0.6227$	0.8953
6.7×10^6	8.57 ^{d}	7.50	10.32	$y = 0.4022x - 0.9451$	0.9453
6.7×10^7	7.72 ^{c}	6.88	8.45	$y = 0.4357x - 0.819$	0.9492
6.7×10^8	1.15 ^{a}	0.53	1.60	$y = 0.1163x - 0.7211$	0.8433
Positive control Esaro molluscicide (5% metaldehyde)	0.52^{a}	0.26	0.78	$y = 0.2487x - 0.6071$	0.9854
Treatment	LC_{50} (conidia mL^{-1})			Regression equation	Goodness of fit (R^2)
Emulsion-formulated conidia	6.7×10^5	3.47	5.79	$y = 3.4x - 11.1$	0.9323
Conidial suspension	6.7×10^6	5.82	6.70	$y = 4.3x - 19.7$	0.9968

These results indicate that the emulsion-formulated Met-Gra4 exhibited strong molluscicidal capability, comparable to a chemical molluscicide containing 5% metaldehyde (1.7 mg mL^{-1}), even at a low concentration of 6.7×10^7 conidia mL^{-1}. In contrast, the conidial suspension required a much higher concentration of 6.7×10^8 conidia mL^{-1} to achieve molluscicidal efficacy similar to the commercial molluscicide. When the two forms of treatments were compared statistically (Tukey HSD; F: 205.91; df 8; $p<0.01$), the LT_{50} of 6.7 x 10^5 conidia mL^{-1} of emulsion-formulated was significantly different to the LT_{50} of conidial suspension at the same concentration, indicating the higher molluscicidal efficacy of emulsion-formulated treatment. This observation was consistent when comparing across all the concentrations between the two forms of treatments, except for concentration 6.7 x 10^8 conidia mL^{-1} for the

conidial suspension and 6.7 x 10^7 & 6.7 x 10^8 conidia mL^{-1}. The largest difference can be seen when comparing both forms of treatments at the concentration 6.7 x 10^7 conidia mL^{-1}, where the emulsion-formulated treatment reached LT_{50} approximately seven days faster even at the same concentration. Even at the lower concentration of 6.7 x 10^6 conidia mL^{-1}, the emulsion-formulated treatment achieved LT_{50} faster at 5.9260 days approximately 1.7556 days faster than 6.7 x 10^7 conidia mL^{-1} in conidial suspension form.

The median LC_{50} for the emulsion-formulated treatment was calculated to be 6.7×10^5 conidia mL^{-1}, significantly lower than that of the conidial suspension, which required a concentration of at least 6.7×10^6 conidia mL^{-1} to achieve 50% mortality. Only one concentration of treatment which was 6.7 x 10^5 conidia mL^{-1} in the forms of conidial suspension failed to reach at least 50% mortality throughout the observation period of 14 days which highlighted the difference in potency. In general, all tested concentrations of the emulsion-formulated treatment successfully reached LT_{50} within the 14-day observation period and consistently exhibited higher potency and molluscicidal properties compared to treatment in the form of conidial suspension at the same concentrations.

4.3 Histopathological Alterations of GAS Exposed to *Metarhizium anisopliae* strain Met-Gra4

4.3.1 Histopathological Observation of Hepatopancreas.

As shown in Figure 4.2, histopathological analysis revealed several alterations that fell within the HI categories for the samples treated with *M. anisopliae* conidia (A: emulsion-formulated; B: conidial suspensions) and the chemical molluscicide (C: metaldehyde 5%), compared to samples exposed to the negative control (D). Specifically, GAS samples exposed to the negative control treatments exhibited digestive tubules that maintained the structural integrity of the epithelial cells, with numerous digestive cells (C corpuscles; lightly stained, smaller circular cells) and few basophilic cells (K corpuscles; characterized by dark and oval structures within the

digestive tubules). Six histopathological traits for the target organ, hepatopancreas, were selected from the list and observed using the weighted index (HI) approach, as shown in Table 3.1 in Section 3.4.2. Among these, the most evident alteration in the samples treated with the conidial suspension and emulsion-formulated conidia was the significant hyperplasia of K corpuscles. This change was evidenced when the K corpuscles count from 50 different digestive tubules for each treatment was counted and analysed, it showed that samples treated with emulsion-formulated conidia and conidial suspension were significantly higher than negative control, however samples treated with chemical molluscicide (metaldehyde 5%0 had a significantly reduced number of K corpuscles (Stepwise-stepdown; F: 153.109; df: 3; $p<0.01$). Increased number of K corpuscles in the digestive tubules were observable in Figures 4.2A and 4.2B. This alteration was observed in more than 75% of samples; hence, a maximum score of 3 under the HI classification was assigned to this progressive change. Hyperplasia of the K corpuscles within the digestive tubules was also observed alongside haemocytic infiltration and hypervacuolisation, where the cells enlarged and eventually caused the digestive tubules to disintegrate and lose their structural integrity. However, hyperplasia of K corpuscles was not observed in samples treated with chemical molluscicide (metaldehyde 5%); instead, the results revealed emptying of the digestive cells, as shown by the significant decrease in the number of K corpuscles. Another significant observation recorded was the disorganisation of the connective tissue within the digestive tubules that led to significant distension of the lumen. When a total of 50 digestive tubules from each treatment were compared, samples treated with emulsion-formulated conidia, conidial suspension and chemical molluscicide (metaldehyde 5%) all underwent significant increase in diameter of lumen compared to negative control (One-Way ANOVA; F: 134.94; df: 3: $p<0.01$).

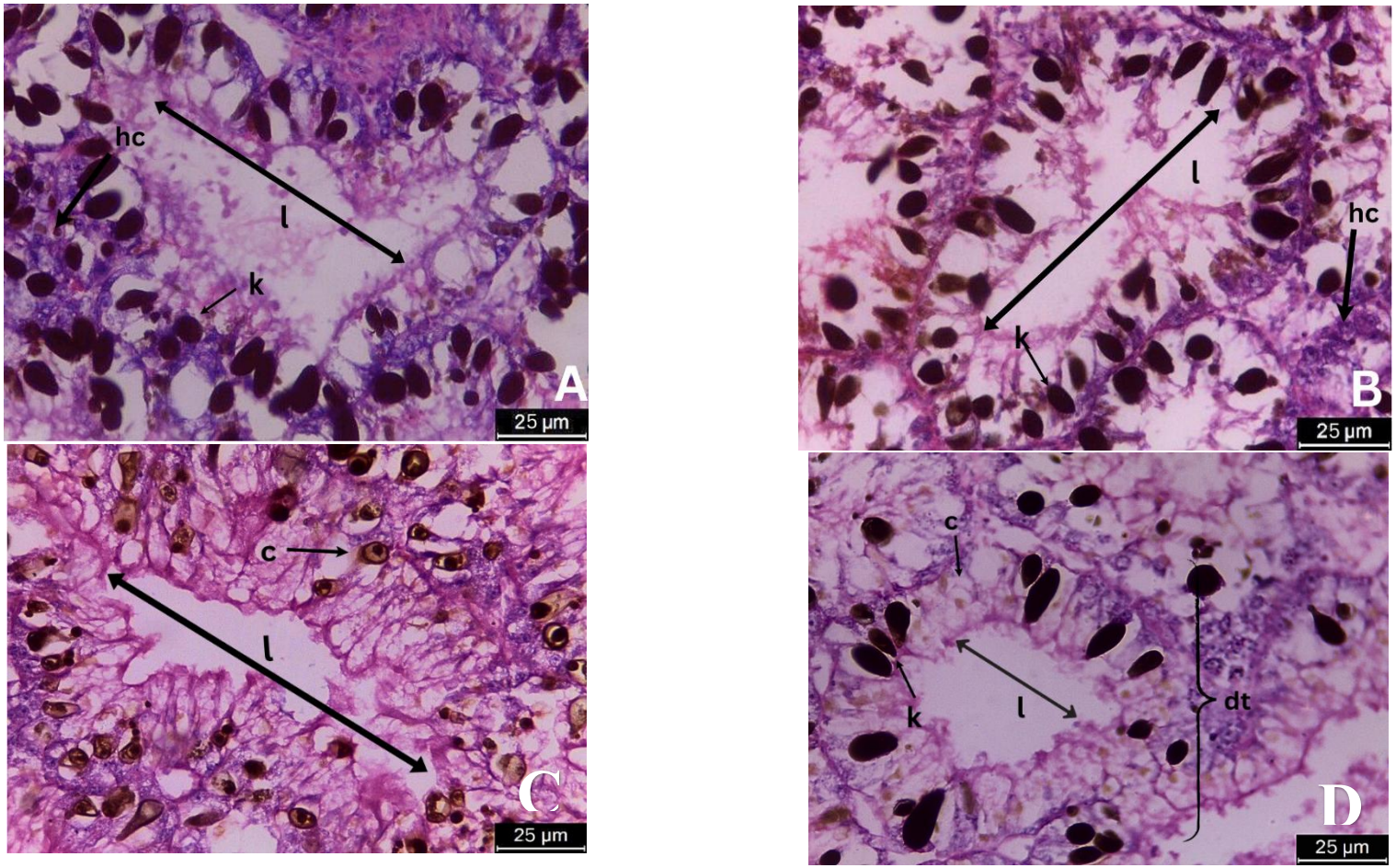

Figure 4.2 Digestive tubules of GAS treated with emulsion-formulated conidia (A), conidial suspension (B), and chemical molluscicide (metaldehyde 5%) (C) showing various histopathological alterations meanwhile negative control (D) showing normal conditions of digestive tubules. Legend: c, C corpuscles; dt: digestive tubules; hc: haemocytic infiltration; k: K corpuscles; l, lumen. Images were captured at 400× magnification.

4.3.2 Histopathological Observation of Reproductive Gonads (Ovary and Testis).

The reproductive glands of the samples were observed and compared across the different treatments to investigate the histopathological alterations that occurred in Figure 4.3 (A: emulsion-formulated; B: conidial suspension; C: chemical molluscicide (metaldehyde 5%)), showed varying degrees of alterations compared to ovary samples treated with negative control (D). In some oocytes in samples other than negative control, the vacuolisation of oocytes occurs where the oocyte seems to have increased in size compared to the oocytes in negative control; however, this occurrence is not widespread to all the ovarian tubules in the sample. The most evident alteration in female samples would be the degeneration of oocytes where rupturing of oocytes occurs and this is especially true for samples treated with conidia of *M. anisopliae*, both emulsion-formulated conidia and conidial suspension (A & B, respectively). Oocytes were severely fragmented and nuclei loss is widespread and the oocyte can be seen to have detached from the tubules. The disintegration is also recorded in samples treated with positive control (metaldehyde, 5%) however to a much lesser degree of severity of alterations to the oocytes and ovarian tubules (C).

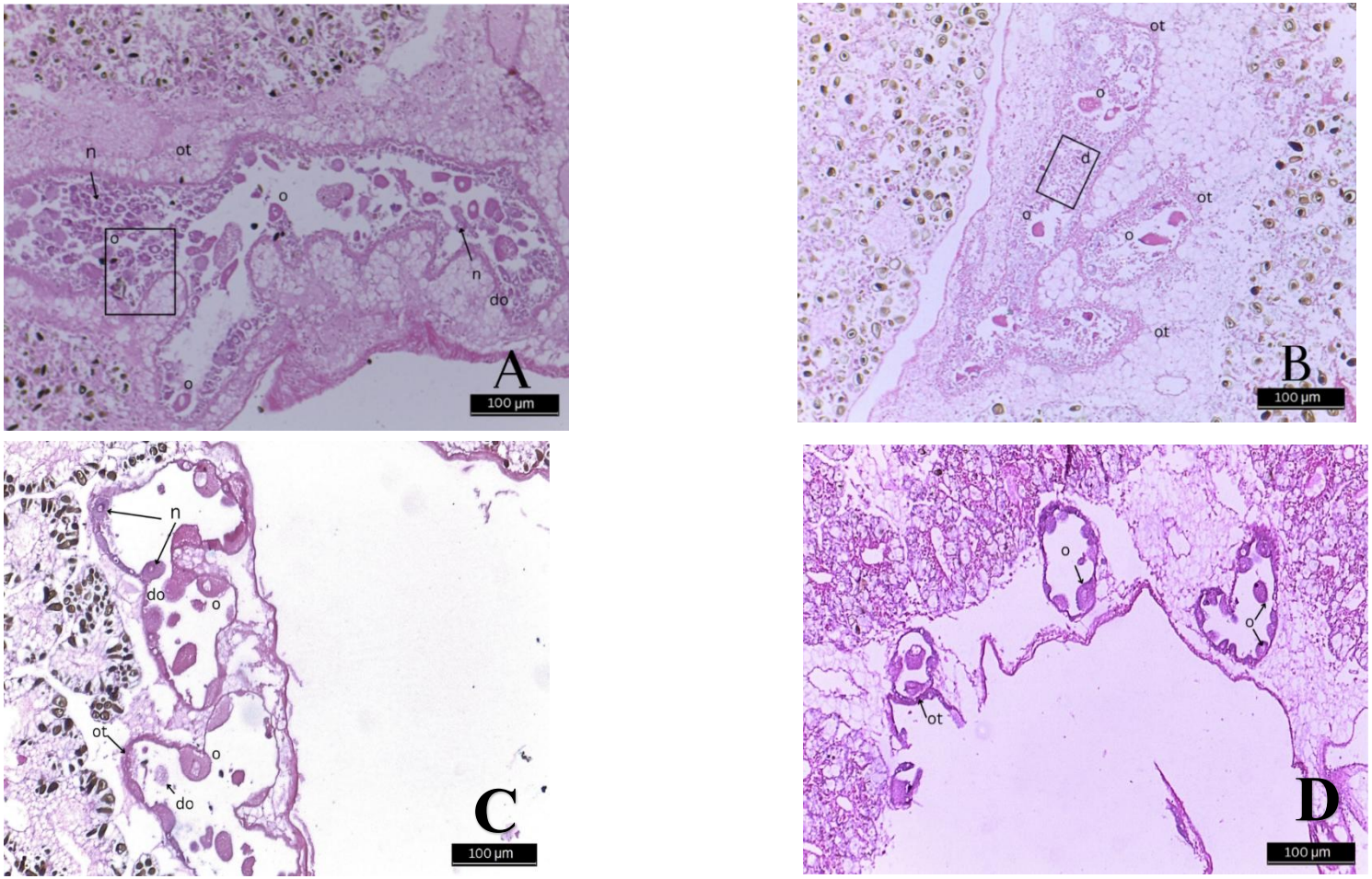

Figure 4.3 Ovary tubules of GAS treated with emulsion-formulated conidia (A), conidial suspension (B), and chemical molluscicide (metaldehyde 5%) (C) showing various histopathological alterations meanwhile negative control (D) showing normal conditions of ovary tubules. Legend: do, disintegrated oocytes; n, nucleus; o, oocytes; ot, ovarian tubules. Images were captured at 100× magnification.

When it comes to the ovarian tubules, the ovarian tubules in all the remaining treatments were still able to produce oocytes since the ovarian tubules observed were not devoid of oocytes. However, the oocytes were heavily fragmented and disintegrated, which causes heavy amounts of debris to be within the tubules which is indicative of cell lysis and necrosis to have occurred since the gametes have lysed. Tubules of samples treated with emulsion-formulated conidia, conidial suspension and chemical molluscicide have lysed and connected with the neighbouring tubules as seen in Figure 4.3(A, B & C, respectively). The oocytes that were not heavily fragmented were also recorded to have lost their nuclei which is also indicative of necrosis. These conditions were extensively observed within the tubules of samples treated with conidia of *M. anisopliae* in both forms (conidial suspension and emulsion-formulated conidia). The fragmentation of oocytes and loss of nuclei can also be recorded in samples exposed to positive control (metaldehyde, 5%) however it is not as widespread and extensive as the samples treated with conidia of *M. anisopliae*. The ovarian tubules also had disorganisation of the connective tissue and peeling of the connective tissues can be seen in samples treated with emulsion-formulated conidia (A), conidial suspension (B) and chemical molluscicide (C). This disorganisation was not observed in the samples in the negative control as tubules remained intact and without any lifting of the connective tissues (D).

In the male samples, the histopathological alterations observed include mild disorganisation of the testis tubules. The tubules of testis in samples exposed to treatments containing conidia of *M. anisopliae* (A: emulsion-formulated; B: conidial suspension; C: positive control (metaldehyde, 5%)) showed notable differences compared to negative control (D). In contrast, the samples exposed to negative control treatment did not exhibit any consistent notable alterations. The tubules of samples exposed to negative control remain intact with the spermatocytes existing in complete conditions with the head and tails present (D). Interestingly, testis samples exposed to treatments with emulsion-formulated conidia and conidial suspension of *M. anisopliae* (A & B, respectively) and positive control (metaldehyde, 5%) (C) showed a mild disintegration of the tubules, observable by the distended shape of the tubules as well as lifting of the outer cells of the tubules. However, the samples in negative samples did not exhibit the same as the other treatments. Another notable observation that was made was on the cells within the tubules themselves. The spermatocytes within the testis tubules were found to undergo enlargement of the head where the head of the spermatocyte seems to have been inflated compared to negative control. This inflated head of spermatocyte is observable in all the treatments aside from samples in the negative control but it is most widely observed in samples in positive control (metaldehyde, 5 %). Another alteration to the spermatocyte that can observed is the loss of tails of the spermatocyte to a varying degree. Certain spermatocytes have shortened tails meanwhile some completely lacked tails. This alteration is especially more severe and widespread in samples exposed to positive control (metaldehyde, 5%) and emulsion formulated conidia of *M. anisopliae*.

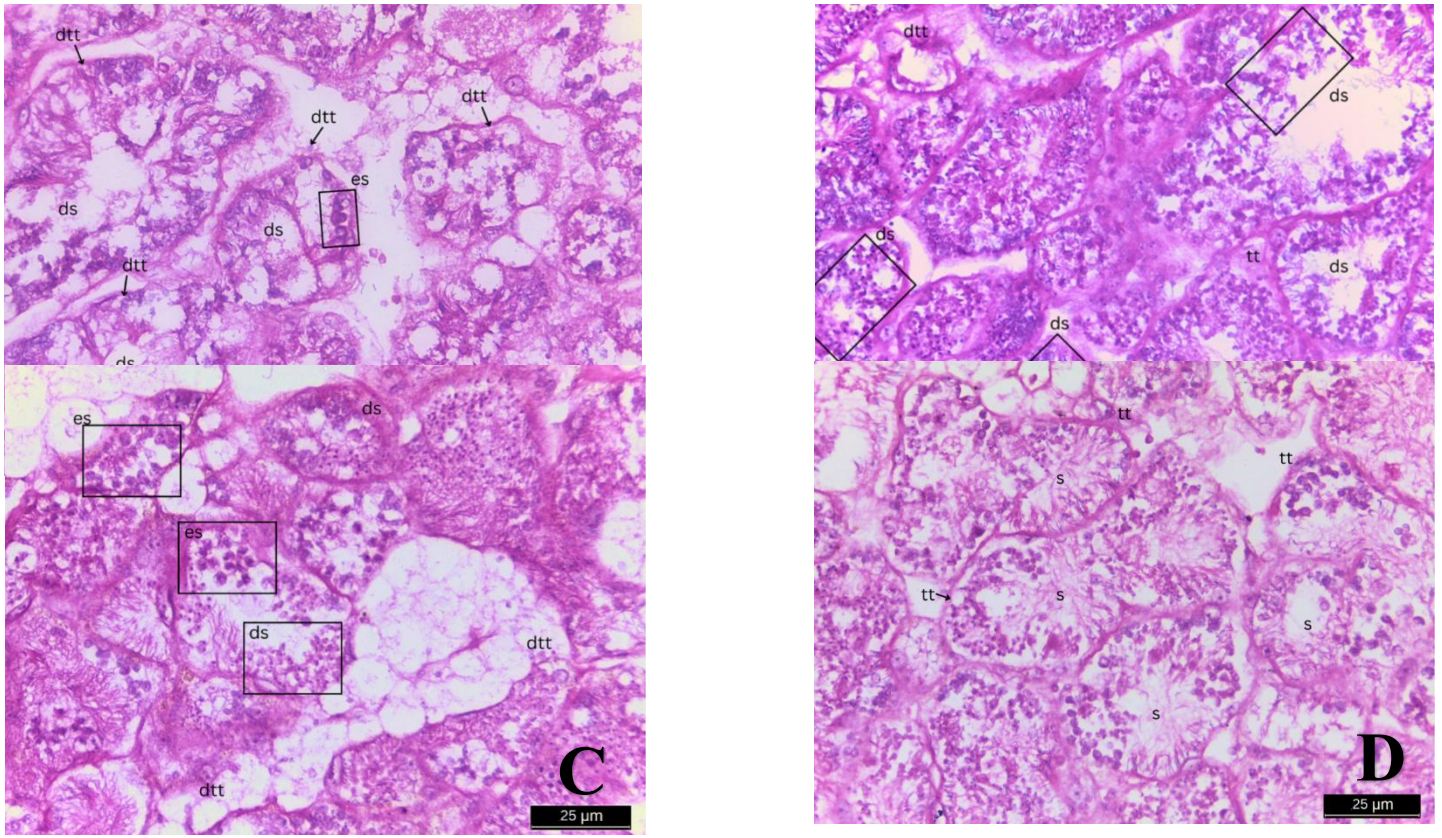

Figure 4.4 Testis tubules of GAS treated with emulsion-formulated conidia (A), conidial suspension (B), and chemical molluscicide (metaldehyde 5%) (C) showing various histopathological alterations meanwhile negative control (D) showing normal conditions of testis tubules. Legend: ds, disintegrated spermatocyte; es, enlarged spermatocyte; dtt, disintegrated testis tubules s, spermatocyte; tt, testis tubules. Images were captured at 400× magnification.

Atrophy of tubules was also prevalent in samples exposed to emulsion-formulated conidia treatment (Figure 4.4A). The atrophy of testis tubules is evident from the lack of spermatocytes within many of the testis tubules. This is true across all treatments containing conidia of *M. anisopliae* as well as positive control (metaldehyde, 5%) to a certain degree, however this alteration is most widely observed in samples treated with emulsion-formulated conidia whereas atrophy is not observed in negative control. Testis tubules were also seen to undergo lysis where the tubules appear fragmented and disintegrated; however, the fragmentation is not widespread and can be seen in approximately 25% of the populations for samples treated with conidial suspension (Figure 4.4B) and chemical molluscicide (metaldehyde 5%) (Figure 4.4C). Testis tubules of samples exhibit the most severe lysis with a large percentage of tubules being disintegrated and atrophied. Meanwhile tubules regression and lysis are not observed in the negative control (Figure 4.4D). Necrosis of the spermatocyte and gametes were obviously seen for samples in positive control (metaldehyde, 5%), conidial suspension as well as emulsion formulated conidia. Necrosis can be seen by the loss of nuclei of the spermatocyte causing them to appear empty as well as the disintegration of the gametes themselves which can be seen by the fragmentation that is observed within the tubules and is the most severe in emulsion formulated samples

4.4 Histopathological Condition Index (HI)

4.4.1 Histopathological Condition Index (HI) of Hepatopancreas

The list of alterations of hepatopancreas differs slightly from ovary and testis hence the relevant alterations were tabulated in Table 4.3. The table shows the histopathological condition index (HI) values of all treatments according to the reaction pattern, alterations, weight of each alteration, and the mean HI value of each treatment. The calculation revealed that for the progressive reaction pattern, negative control group did not exhibit any observable alterations and only showed an inflammatory response and regressive changes. In the negative control, only one type of regressive change was observed: disorganisation of connective tissues. Positive control (metaldehyde, 5%) recorded inflammatory and regressive alterations but did not exhibit any observable progressive changes.

Table 4.3 Histopathological Condition Index (HI) values of GAS hepatopancreas samples treated according to the type of reaction pattern observed. Different letters indicate significant difference across groups (Stepwise step-down; F: 17.99; df: 3; $p < 0.05$).

Reaction pattern	Alteration	Weight (W)	HI values			
			Negative control	Chemical molluscicide (metaldehyde 5%)	Conidial suspension	Emulsion-formulated conidia
Inflammatory response	Haemocytic infiltration	1	0.08 ± 0.45^{a}	2.4 ± 0.54^{b}	2.8 ± 0.45^{b}	2.8 ± 0.45^{b}
Progressive changes	Hypervacuolisation of cells	2	0	0.4 ± 0.90^{a}	5.2 ± 1.10^{b}	5.6 ± 0.89^{b}
	Hyperplasia of cells	2	0	0	4.8 ± 1.10^{b}	5.6 ± 0.89^{b}
Regressive changes	Disorganisation of connective tissues	1	0.2 ± 0.45^{a}	2.6 ± 0.55^{b}	2.5 ± 0.55^{b}	2.4 ± 0.55^{b}
	Tubule regression	2	0	5.6 ± 0.89^{b}	6.0^{b}	5.6 ± 0.89^{b}
	Necrosis	3	0	$7.8 \pm 1.64^{b, c}$	6.6 ± 1.34^{b}	9.0^{c}
Total			1 ± 0.71^{a}	18.8 ± 0.84^{b}	28.00 ± 1.22^{c}	31.4 ± 0.89^{d}

In contrast, treatments containing conidia of *M. anisopliae* strain Met-Gra4 induced all types of alterations. When comparisons were made according to each reaction pattern, these two treatments were not significantly different within almost all categories, except for necrosis, in which samples treated with the emulsion-formulated conidia showed more severe and widespread necrosis than those treated with the conidial suspension. The treatments containing *M. anisopliae* Met-Gra4 conidia showed similar histopathological alterations among all other alterations observed. The calculated total HI value revealed significant differences across all four treatments (Figure 4.5). The HI value of the negative control samples was the lowest at 1.0 ± 0.71, followed by the positive control (metaldehyde, 5%) (18.8 ± 0.84), conidial suspension (28.0 ± 1.22), and emulsion-formulated conidia (31.4 ± 0.89). The non-parametric test Kruskal–Wallis and post hoc comparison stepwise step-down tests revealed a significant difference in HI values between each of the treatments conducted (Stepwise step-down; F: 17.99; df: 3; $p < 0.05$). The treatment with the highest HI value was the emulsion-formulated conidia, indicating that the most widespread and severe histopathological alterations were observed in the samples in this particular treatment. The severity category is based on Table 3.2, where HI of emulsion-formulated conidia is in the severe category, conidial suspension also in severe category although significantly lower than emulsion-formulated conidia. Followed by positive control in moderate-severe category and negative control in mild HI category.

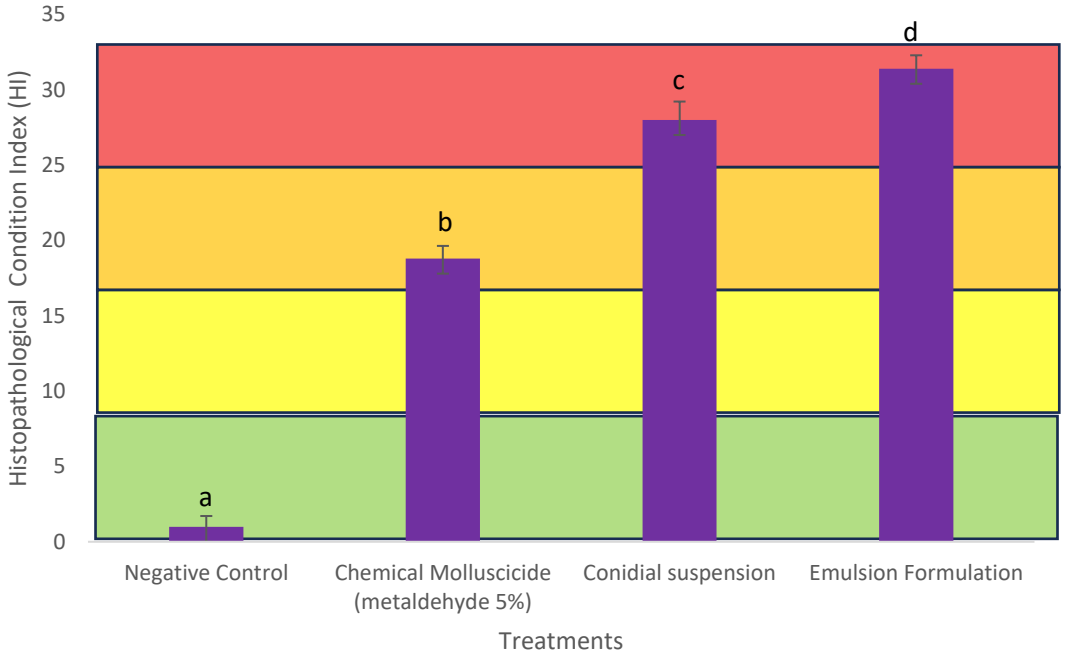

Figure 4.5 Comparison between the Histopathological Condition Index (HI) of hepatopancreas for all treatments conducted. Different letters indicate significant differences (Stepwise step-down; F: 17.99; df: 3; $p < 0.05$).

4.4.2 Histopathological Condition Index (HI) of Reproductive Gonads (Ovary and Testis)

Histopathological Condition Index (HI) conducted on the ovaries revealed that samples treated with conidia of *M. anisopliae* exhibited the most severe histopathological alterations. Both conidial suspension and emulsion-formulated treatments were able to cause alterations that are significantly more than the samples treated with negative control and positive control (metaldehyde, 5%) with 21.67 ± 0.88 and 22.67 ± 0.33, respectively as illustrated in Figure 4.6 (Stepwise stepdown, F: 6.305; df: 2; $p<0.05$). The severity category is based on Table 3.3, where HI of emulsion-formulated conidia and conidial suspension are not significantly different from each other (Stepwise stepdown, F: 6.06; df: 2; $p<0.05$) and in the same moderate-severe category of HI values. Followed by positive control in mild-moderate category.

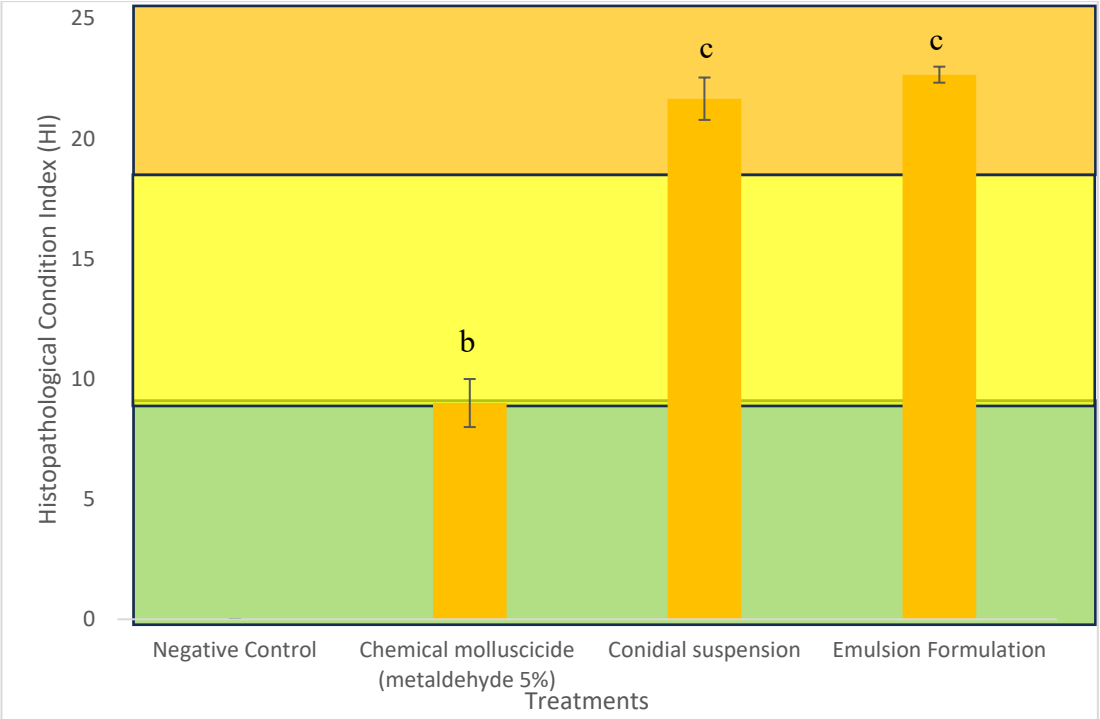

Figure 4.6 Comparison of Histopathological Condition Index (HI) of ovary from samples treated with negative control, positive control (metaldehyde, 5%), conidial suspension and emulsion formulated conidia. Different letters indicate significant differences (Stepwise stepdown, F: 6.06; df: 2; $p<0.05$).

Ovaries of GAS treated with conidia of *M. anisopliae* had the most severe histopathological alteration and were significantly different (Stepwise stepdown, F: 6.06; df: 2; $p<0.05$) compared to samples of ovaries treated with negative control and positive control (metaldehyde, 5%) (Table 4.4). Both the conidial suspension and emulsion formulated conidia managed to incite histopathological alterations in both progressive and regressive changes. Sample of ovaries exposed to negative control did not exhibit any histopathological changes, which is at least 25% to be assigned the frequency of 1. Hence, all ovaries exposed to negative control had negligible alterations that only occurred in very minor instances.

In contrast, the alterations observed in samples of positive control (metaldehyde, 5%), conidial suspension and emulsion-formulated conidia were comparable to each other and only negative control did not exhibit any considerable alterations. The most significant alteration was observed in degeneration of cells, cell

lysis and necrosis in ovary samples in conidial suspension and emulsion-formulated samples. Both conidial suspension and emulsion-formulated conidia were recorded to cause considerable amount of degeneration of cells, specifically oocytes within the ovaries that are significantly more than positive control (metaldehyde, 5%) and negative control. Following the degeneration of cells, a significantly higher degree of cell lysis was also observed in samples treated with conidial suspension and emulsion formulated conidia (Stepwise stepdown, F: 6.06; df: 2; $p<0.05$). With the increased cell lysis and degeneration of cells highlighting a significant degree of necrosis in samples from both treatments containing conidia of *M. anisopliae.*

Table 4.4 The Histopathological Condition Index (HI) of ovary of GAS samples treated according to the type of reaction pattern observed. Different letters indicate significant differences going across groups (Stepwise stepdown, F: 6.06; df: 2; $p<0.05$).

Reaction pattern	Alteration	Weight (W)	HI values			
			Negative control	Chemical molluscicide (metaldehyde 5%)	Conidial suspension	Emulsion-formulated conidia
Progressive changes	Enlargement of cellular components	1	0	1^{b}	1^{b}	1^{b}
Regressive changes	Disorganisation of connective tissues	1	0	1^{b}	1.33 ± 0.47^{b}	1.67 ± 0.47^{b}
	Degeneration of cells	2	0	2^{b}	4.67 ± 0.94^{c}	4.67 ± 0.94^{c}
	Tubule regression	2	0	0	2^{b}	2.67 ± 0.94^{b}
	Cell lysis	3	0	3^{b}	6^{c}	6^{c}
	Necrosis	3	0	2 ± 1.41^{b}	6^{c}	6^{c}
Total			0	9 ± 1^{b}	21.67 ± 0.88^{c}	22.67 ± 0.33^{c}

The testis on the other hand is slightly different when it comes to reaction patterns and histopathological alterations that can be observed across different treatments. Figure 4.7 illustrates the Histopathological Condition Index (HI) of testis samples of all the treatments, showing significant differences in the HI between testis samples observed. The HI revealed that there are no significant differences between testis samples from GAS treated with positive control (metaldehyde, 5%) (HI value 19.00 ± 0.47) and emulsion-formulated conidia (HI value 17.00 ± 0.82. The histopathological alterations of samples treated with conidial suspension (HI value 13.67 ± 09.72) on the other hand is significantly more severe when comparing to negative control but not as severe as the previous two treatments (Stepwise stepdown, F: 6.31; df: 2; $p<0.05$). The severity category is based on Table 3.3, where HI of emulsion-formulated conidia and positive control (chemical molluscicide, metaldehyde 5%) are not significantly different from each other (Stepwise stepdown, F: 6.31; df: 2; $p<0.05$) and both bordering the moderate-severe and the mild-moderate category of HI values.

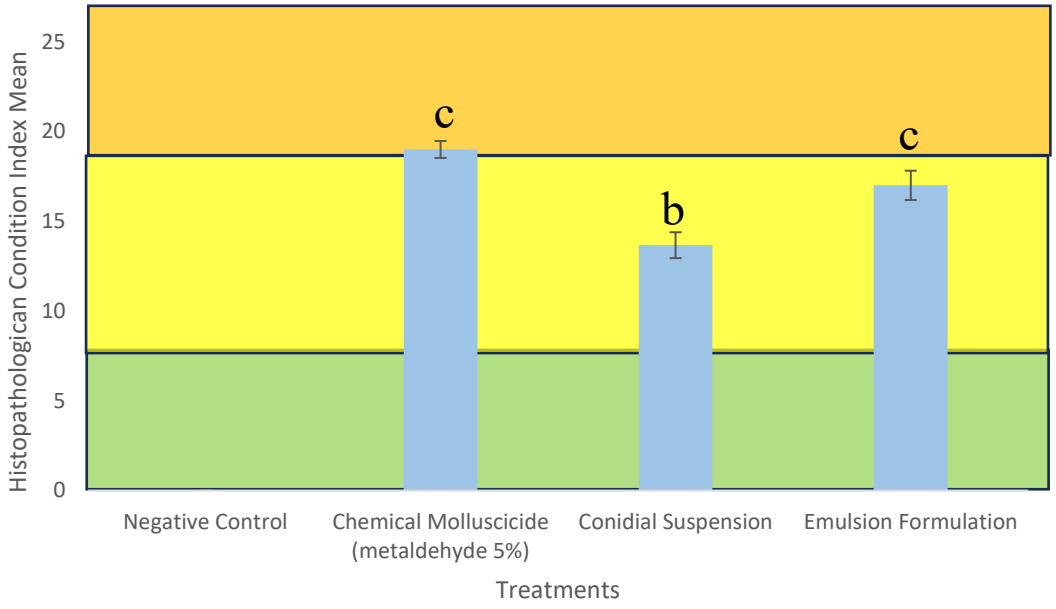

Figure 4.7 Histopathological Condition Index (HI) of testis samples from all the treatments showing significant differences between conidial suspension, negative control and positive control (metaldehyde, 5%) and emulsion-formulated conidia. Different letters indicate significant differences (Stepwise stepdown, F: 6.31; df: 2; $p<0.05$).

Table 4.5 tabulates the different histopathological alterations to the testis observed for all treatments. It was observed that negative control did not exhibit any considerable alterations to the testis of GAS. Meanwhile for the samples treated with positive control (metaldehyde, 5%), conidial suspension, and emulsion-formulated conidia all showed significant alterations (Stepwise stepdown, F: 6.31; df: 2; $p<0.05$). Enlargement of cells was observed in all three treatments with the increased in the size of the spermatocyte heads observable in all treatments however it was not widespread and only occurred in about 25% of the samples. Some more notable alterations that occurred with higher frequency within the samples include degeneration of cells, observable by the disintegrated tails of the spermatocytes, observed in all treatments, at about the same frequency in all three treatments. Tubule regression observed by the reduced functionality of the tubules in producing spermatocytes, was also recorded in all the remaining three samples in a similar frequency and was calculated to not be significantly different from one treatment to another. Samples of testis treated with positive control (metaldehyde, 5%) exhibited a more widespread and severe cell lysis and necrosis compared to conidial suspension and emulsion-formulated conidia. In general, the HI of testis samples from individuals treated with positive control (metaldehyde, 5%) and emulsion-formulated conidia achieved similar comparable alteration index.

Table 4.5 The Histopathological Condition Index (HI) of testis of GAS samples treated according to the type of reaction pattern observed. Different letters indicate significant differences going across groups (Stepwise stepdown, F: 6.31; df: 2; $p<0.05$).

Reaction pattern	Alteration	Weight (W)	HI values			
			Negative control	Chemical molluscicide (metaldehyde 5%)	Conidial suspension	Emulsion-formulated conidia
Progressive changes	Enlargement of cellular components	1	0	1.33 ± 0.47^{b}	1^{b}	1^{b}
Regressive changes	Disorganisation of connective tissues	1	0	1^{b}	1^{b}	1^{b}
	Degeneration of cells	2	0	2^{b}	2.67 ± 0.92^{b}	4^{b}
	Tubule regression	2	0	2.67 ± 0.92^{b}	2^{b}	4^{b}
	Cell lysis	3	0	6^{b}	$4\pm1.41_{b}$	4 ± 1.41^{b}
	Necrosis	3	0	6^{c}	3^{b}	3^{b}
Total			0	19 ± 0.47^{c}	13.67 ± 0.72^{b}	17 ± 0.82^{c}

CHAPTER 5

DISCUSSION

5.1 Pathogenicity and Efficacy of Conidial Suspension and Emulsion-Formulated Conidia of *Metarhizium anisopliae.*

Results obtained in this study revealed that the daily cumulative survival of population treated with emulsion-formulated treatment was consistently significantly lower compared to cumulative survival of population treated with conidial suspension (Pairwise comparison; $\chi^2 = 4.55$; df = 1; $p<0.05$). This could be attributed to the conidia in suspension form have a weaker adhesion capability to aquatic organisms as opposed to terrestrial hosts which could lead to the conidia in suspension having more time to establish fatal infections (Greenfield et al., 2014). In contrast, the emulsion-formulated *M. anisopliae* conidia were able to overcome this problem as the emulsion contained nano-sized particles that encapsulate the conidia, enhance the dissolvability of the treatment in water (Cheong et al., 2022; 2023), which directly showed the increased efficacy against GAS in this study. This difference in efficacy can also be attributed to the delivery system of each treatment as the efficacy of a treatment depends not only rely on its intrinsic properties of the treatment but also depends heavily on the formulation of the treatment among other parameters (Buzzetti, 2017). Findings of this study demonstrate that emulsion-formulation is faster and more efficacious in controlling GAS under laboratory conditions. Although when treatments containing conidia of *M. anisopliae* were compared to the positive control (metaldehyde, 5%), which utilized a commercial molluscicide containing 5% metaldehyde at a concentration of 1.7 mg mL^{-1}. The positive control (metaldehyde, 0.05%) was significantly more potent (Log-rank test; χ^2: 25.70; df: 2; $p<0.01$), achieving 100%

mortality on the first day of the bioassay. Ultimately, the populations treated with emulsion-formulated conidia had higher mortalities (lower survival rates) than populations treated with conidial suspension.

The differences in the efficacy of the delivery system provided by each form of treatments were further reflected in the LT_{50} and LC_{50} of the treatments. Emulsion-formulated treatments showed greater molluscicidal capabilities as two concentrations of 6.7×10^7 and 6.7×10^8 conidia mL^{-1}, showed molluscicidal capabilities comparable to commercial molluscicide containing 5% metaldehyde at the concentration 1.7 mg mL^{-1} achieving LT_{50} in 0.6123 and 0.4549 day respectively. Meanwhile for conidial suspension treatment of Met-Gra4, only the highest concentration (6.7×10^8 conidia mL^{-1}) achieved LT_{50} comparable to commercial molluscicide (LT_{50} 0.51 day; $p<0.05$, Tukey HSD). This finding is supported by Buzzetti, (2017) that the efficacy of a pesticide should also take into consideration the delivery agent of pesticide in order to fully gauge the efficacy.

The increased efficacy of emulsion-formulated can be attributed to the physico-chemical properties of the nano particles in the emulsion-formulated conidia which improve uptake of the conidia into the snails' body (Yassin et al., 2021) as it provides a better conidia delivery system into the organism's body. With the increase in uptake of the conidia into the organism, the treatment is able to yield more potent results at a lower concentration. A clear trend that was observed in this study was the LT_{50} is conversely proportional to the concentration of treatment. As the concentration increased, LT_{50}, the time to reach 50% mortality of the population is decreased. This observation is true across both forms of treatments and hence shows that the capabilities of treatments of higher concentration can be explained to the number of conidia contained in the treatment. Treatments with higher number of pathogens has higher probabilities of successful attachment because the probability of pathogens attaching to host is directly influenced by the concentration of pathogens (Leger & Wang, 2020). This goes to show that the concentration of conidia is pivotal in achieving successful attachments to hosts as the higher the number of conidia contained within the treatment, the faster the LT_{50} is achieved.

The median lethal concentration (LC_{50}) for conidial suspension was calculated to be 6.7 x 10^6 conidia mL^{-1} indicating that the lowest concentration tested (6.7 x 10^5 conidia mL^{-1}) did not achieve 50% mortality (LT_{50}) within 14 days of observation. The LC_{50} for emulsion formulated treatments was calculated to be 6.7 x 10^5 conidia mL^{-1}. This is a lower concentration than the LC_{50} for conidial suspension with conidial suspension requiring at least 6.7 x 10^6 conidia mL^{-1} to kill 50% of the population. The difference in LC_{50} value indicates that even when using the same organism as the biocontrol agent, the difference in the formulation and the delivery system of the treatments (nano-sized particles versus water), made an impact on the results in obtaining 50% mortality even when other factors (temperature, exposure time, humidity, photoperiod) were kept the same. Thus, this supports that the efficacy of a pesticide does not only rely on the intrinsic attributes of the treatment, however the formulation also plays a role (Buzzetti, 2017). The time taken to reach LT_{50} by treatments of lower concentrations is longer could be due to the defence mechanism of GAS trying to fight off infections by excreting an increased amount of mucus once exposed to the pathogens indicating that the GAS are trying to dilute and expel the toxicants from their bodies. Excessive excretion of mucus has been recorded to be the defence mechanism of snails against pathogens due to its antimicrobial properties (Nantarat et al., 2019). Similar occurrence of GAS producing excessive mucus paired with visible signs of inactivity post-treatment was observed during this study, which could indicate the defence mechanism of GAS trying to utilize the anti-microbial properties of the mucus to fight the infection alongside physically removing the conidia from the body with the mucus. Even though production of mucus is highly expensive when it comes to energy expenditure (Zolovs et al., 2024), GAS were seen to do excrete excessive amounts during the exposure towards the conidia of *M. anisopliae* as well as after removal from the treatments, especially the first three to four days after removal from treatments. This could explain why concentration 6.7 x 10^5 conidia mL^{-1} failed to reach 50% mortality of the population as GAS are able to remove the attachments of the conidia on the body though this excessive mucus production Leger & Wang (2020) emphasized that attachment to the host is the most important step of infection and is highly dependent on the concentration of pathogens. The nano-sized particles in emulsion-formulated treatment were able to provide a more effective delivery system and increase the uptake of the fungus into the snails' body hence overcoming this problem successfully as revealed in this study. The decreased

activity and anti-feedant effect of treatment containing *M. anisopliae* were observed to be continuing even after the removal of GAS from the treatment and into new clean containers with distilled water and even in the presence of food. The anti-feedant effect was observed from the amount of food left in the container. After 24 hours, samples treated with negative control was able to finish the food meanwhile surviving samples from the treatments were not showing much interest in the food provided for the first three to four days after the treatment and only started to show considerable increase in both appetite and activity after the four-day mark. This excessive drop in energy may be attributed to the internal usage of energy to fend off infections as well as excessive energy used to excrete mucus in trying to combat pathogens causing a drop in activity levels and appetite (McDermott et al., 2021; Zolov et al., 2024).

When compared to a previous study by Allahudin et al., (2024), which tested Met-Gra4 conidial suspension on GAS, the study demonstrated 46.62% faster than the current study in reaching LT_{50} where LT_{50} was achieved within 5.30 days for the previous study than the 7.717 days observed in this current study. Even though the concentration used in this current study is stronger with 6.7×10^7 conidia mL^{-1} instead of the lower concentration used in the previous study (3.0×10^7 conidia mL^{-1}), the conidial suspension treatment in the previous study was still effective in terms of time to mortality. This could be because the snails obtained and used in the previous study possibly had reduced viability and vigour as it was collected from a paddy field that was treated with commercial molluscicide, as compared to this current study that collected the individuals from a non-treated body of freshwater in UniSZA, Besut Campus. Typically, the viability, resistance, and vigour of snails such as GAS depend on several factors such as the size of the individuals, the condition of the shells and the shell strength. Since the samples used in the previous study were obtained from treated areas such as paddy fields and nearby waterways, the occurrence of degraded shells was more prominent due to environmental conditions. This degradation could be attributed to the pH of the water as acidification of aquatic environments can degrade snail shells that are calcium carbonate-based, reducing survivability since GAS shells starts to erode at pH level of ≤ 5.0 (Singh et al., 2019b; Jesús-Navarrete et al., 2023). According to Jesús-Navarrete et al., (2023), the integrity and structure of shells are highly dependent on the levels of calcium available in the water for the snails to grow

the shells properly and maintain its structure. Hence, the weakened shells increase the GAS' susceptibility to infection and external environments (Erknano, 2021). Conversely, the samples of GAS obtained for this current study came from an untreated sampling site that is excluded from any paddy fields or treated waterways and independent of the waterway system of any paddy fields hence the shell integrity, shell strength and with the availability of food all year round, could attribute to a more persistent population of GAS.

Another contributing factor could be the size of GAS individuals tested. GAS in this current study measured 25 ± 5 mm in shell height whereas those in the previous study used GAS individuals of size 15 ± 5 mm. These size differences when comparing populations of GAS likely attributed to nutrient availability in the lake that is available all year round versus in the paddy is only available during paddy season and the snails having to undergo aestivation during drier months. The size difference between the samples of GAS used may have affected the rate at which LT_{50} was reached as size affects the ability of snails to withstand infection with larger snails being able to withstand a longer period of infection before dying compared to smaller hosts (Graham, 2003; Kalinda et al., 2017). This indicated that the pathogen load required to cause mortality in larger individuals are much higher than the pathogen load needed to kill smaller individuals. Larger snails are generally more resistant to infection.

In general, all concentrations tested for emulsion-formulated treatments were able to reach the median lethal time (LT_{50}) within the stipulated time of 14 days. This shows that even the lowest concentration of emulsion-formulated conidia (6.7×10^5 conidia mL^{-1}) was able to cause 50% mortality of the population within the stipulated time of 14 days as compared to conidial suspension that was unable to do so. Hence, this indicates that the lowest concentration of emulsion-formulation is also capable to be used to control populations of GAS proving the higher efficacy of treatments that is due to the improved efficacy of the delivery system for the treatments. However, although the results of treatments containing conidia of *M. anisopliae* yielded promising results in the laboratory conditions, applications in the filed require must more meticulous preparation and considerations. Even though *M. anisopliae* is generally regarded as non-toxic and poses little harm towards non-target organisms

(Abonyo et al., 2016; Vivekanandhan et al., 2020; Sousa et al., 2025) however there are records of non-target organisms that are sensitive towards *M. anisopliae* such as temporary changes in the stomach and intestines of birds, rats and frogs (Donovan-Peluso et a., 1980), tissue damage in rats (Shadduck et al., 1982) inducing mortalities in fishes (*Melatonia dubuolayi, Ulmerophlebia* spp., and *Ceriodaphnia dubia*) (Milner et al., 2002),inducing mortalities in *Apis mellifera* especially in higher concentrations (Butt et al., 1994), causing death of embryos and larvae of shrimps (*Palaemonetes pugio*) (Genthner et al., 1997) having effects toward arthropods *Daphnia magna* (Tanner et al., 2015). Hence, even though the consensus generally reveals that *M. anisopliae* is safe for non-target organisms and the records were in studies more than 10 years ago, caution must be exercised before field trials to avoid unwanted harmful effects to the non-target organisms and the environment.

5.2 Histopathological Alterations Induced by the Infection of *Metarhizium anisopliae*

The hepatopancreas is one of the most important organs in GAS for detoxification (Yang et al., 2023). As such, any exposure to toxic agents in this organ may be evident through histopathological analysis of the hepatopancreas. The histopathological analysis conducted in this study revealed various alterations that differed across treatments, with the most prominent alteration being an increase in the appearance of the dark granules, which were the basophilic cells (K corpuscles) (Figures 4.2A & B).

The K corpuscles, which are arranged concentrically around the perimeter of the digestive tubules and known as basophilic cells, can accumulate toxicants and have detoxification capability (Peña et al., 2017). K corpuscle hyperplasia has been theorized to be associated with enzyme secretion, detoxifying activity, and phagocytosis of dying cells in gastropods (Arrighetti et al., 2022). Hence, the hyperplasia of K corpuscles could be a defence mechanism in an attempt to phagocytose dying cells and pathogens in the hepatopancreas, which could eventually lead to mortality. The excessive energy required to phagocytose both dying cells and pathogens may explain why GAS exhibited immobility and non-responsiveness

towards stimuli during the 14-day observation period. This eventually led to death due to anti-feedant behaviour. This alteration was expected to occur as the hyperplasia of K corpuscles was linked to a pathogenic presence, such as the entomopathogenic fungus *M. anisopliae*. This alteration was also consistent with the theory that K corpuscles are lysosomal residual bodies, which typically increase in the occurrence of toxicant accumulation (Peña et al., 2017). K corpuscles are usually excreted in faeces and within mucus strings of GAS (Giraud-Billoud et al., 2018). Notably, the number of K corpuscles observed under the microscope during and after exposure to *M. anisopliae* (strain Met-Gra4) was more abundant than usual. However, further investigation must be carried out to confirm this observation.

There is evidence that these corpuscles are morphs of a prokaryotic organism akin and highly similar to cyanobacteria. Pure cultures of *P. canaliculata* have been reported to cause significant histopathological alterations within the hepatopancreas of other snails. This damage is attributed to the generation of reactive oxygen species, which cause oxidative stress and antioxidative damage (Sui et al., 2024). Oxidative stress and antioxidative damage have been theorized to be caused by the release of microcystins, a type of hepatotoxin typically released by cyanobacteria (Sui et al., 2024). Therefore, there may be linkages between hyperplasia of K corpuscles and the rate of mortality observed. The excessive K corpuscles produced and excreted into the environment may have contributed to the mortality of GAS due to oxidative stress caused by the microcystins (Sui et al., 2024). This may be the reason why mortality was observed rather rapidly in GAS, as *M. anisopliae* was able to cause GAS to produce and excrete more K corpuscles into the environment, which not only causes exhaustion but also increases the probability of oxidative stress due to microcystin presence.

Another notable alteration that occurred histopathologically in GAS exposed to metaldehyde, conidial suspension, and emulsion-formulated conidia was the widening and dilation of the lumen within the digestive tubules (Figure 4.2A, B & 4.3). Widening of the lumen in the digestive tubules of GAS is a characteristic sign of atrophy (Arrighetti et al., 2022). This indicates that the positive control (metaldehyde, 5%), conidial suspension, and emulsion-formulated conidia treatments caused atrophy

within the digestive tubules. This is evident by the significantly widened lumens of the digestive tubules and disintegration of the digestive tubule structure. Widening of the lumen in the digestive tubule is also a histopathological effect observed in response to environmental stressors, such as the presence of pathogens. This could indicate that the treatments created an environment that caused stress to the GAS and internal disintegration of the digestive tubules, causing mortality similar to exposure to heavy metals such as copper, cypermethrin, and even metaldehyde (Dummee et al., 2015; Peña et al., 2017; Arrighetti et al., 2022). A study conducted by Liu et al. (2024) revealed that metaldehyde disrupts the activity of acetylcholine (Ach) causing behavioural changes in snails, as observed in the current study where GAS were less active post-exposure to *M. anisopliae*. Severe rupture of the digestive tract leads to the loss of functionality of the digestive tubule, leading to necrosis of digestive tubules and eventually mortality.

On the other hand, the diverse and extensive histopathological alterations that were observed in both the female and male reproduction organs indicate that exposure to conidia of *M. anisopliae* induces changes that would reduce or in severe cases, induce castration of GAS. The most notable alteration observed on female reproductive organs was the disintegration of gametes. Samples of GAS exposed to conidial suspension, emulsion-formulated conidia, and positive control (metaldehyde, 5%) treatments all had disintegration of gametes with the most severe alterations observed in emulsion-formulated conidia and positive control (metaldehyde, 5%). This finding aligns with the finding of Wongsiri et al., (2023) where the oocytes that are typically round, large, with nucleus that stains and situated near the edge of the ovary were now seen undergoing severe disintegration with loss of nuclei, increased in size and have been severely fragmented within the ovarian tubules.

The disintegration of oocytes was seen in an increased frequency in all treatments when compared to the negative control but it was the most severe in samples treated with conidial suspension and emulsion-formulated conidia. Increasing rate of disintegration in oocytes and nucleus revealed in this study is in line with literature where samples of *Lymnaea glabra* exhibit multiple disintegration of oocytes and

nucleus paired with lifting of the connective tissues when the *L. glabra* snails are exposed to molluscicides (Rondelaud & Gilles, 1996; Wongsiri et al., 2023).

Similar observations can be seen on testis samples obtained from individuals treated with conidia of *M. anisopliae* resulting in a multitude of alterations such as disorganization of connective tissues of the testis tubules, enlargement of cellular components with spermatocytes undergoing disintegrated tails were also in line with a study conducted by Wongsiri et al., (2023). Wongsiri et al., (2023) found that when GAS was exposed to isolated saponins of *Camellia oleifera* brought histopathological alterations that were severe enough in the testis tubules to suggest the castration of the samples. The lifting of sperm membrane was an alteration that can be seen even in other species of snails when exposed to different treatments. A study on *Indoplanorbis exutus* snails' testis were conducted, and a similar reaction pattern involving the disintegration of spermatocytes and spermatids, as well as shrinking of the spermatocyte tails was observed (Wangsomnuk et al., 2000). This indicates that *M. anisopliae* were able to incite some degree of castration effect to a moderate level in the male individuals exposed to treatments containing conidia of *M. anisopliae* possibly resulting in a reduced fertility which could possibly extend to individuals that were exposed to treatments but were able to survive.

This parasitic castration effect may be able to reduce the overall reproductive success of the population if applied in the field as parasitic castration in invertebrate is an ideal way to control overpopulation issue that is one of the contributing factors to the invasiveness of GAS (Choubia, 2022). Parasitic castration is defined by the partial or definitive reduction in fecundity with two mechanisms: direct organ-damage by parasites to the reproductive glands, or indirectly by reducing energy reserve levels of hosts (Faro et al., 2013). As seen in the histopathological analysis, it is revealed that the infection of *M. anisopliae* caused severe damage to the reproductive organs hence it is obvious that the parasitic castration occurred. However, during pathogenicity testing, it is also observed that there are anti-feedant behaviours that were observed in samples possibly hinting that *M. anisopliae* may also have indirect effects as it had affected the overall energy levels of GAS in its presence.

It is theorised that both the gonads and the hepatopancreas provide a continuous supply of energy alongside playing crucial roles in facilitating the growth of snails, hence these organs exhibit alterations that corresponded with pathogenic infection as revealed in this study. These two organs were also reported to be affected by pathogenic infection by digenean larvae. Similar to the findings in this study, the gonads of samples infected with digenean larvae are also affected with the lysis of tubules, loss of tissue integrity and necrosis indicating that the castration effects of samples exposed to conidia of *M. anisopliae* exhibit castration effects just as described by Martinez et al., (2024).

The Histopathological Condition Index (HI) values also revealed that the overall alterations that occurred in the hepatopancreas of GAS significantly differed across treatments in this study. This shows that different treatments elicited various reactions within the most important organs involving detoxification, such as the hepatopancreas. Specifically, the conidial suspension and emulsion-formulated conidia elicited the most significant differences overall. However, when compared according to each type of change, such as inflammatory responses, progressive changes, and regressive changes, both showed comparable results in most categories of observable histopathological alterations observed, highlighting the potential of *M. anisopliae* strain Met-Gra4 as a potent molluscicide.

On the other hand, when it comes to the HI of gonads, only samples treated with emulsion-formulated conidia were able to cause the highest significant changes to the samples in both testis and ovary samples indicating the higher efficacy of conidia formulated in emulsion form over the standard conidial suspension. This also highlights the importance of the formulation and delivery mechanism when it comes to maximizing treatment efficiency, as the same conidia concentration yielded significantly different HI values owing to the differences in the delivery agents that could enhance their efficacy.

Despite these promising results documented in this study, it is important to recognise that this study only tested the bioefficacy of Met-Gra4 under controlled conditions of a laboratory with closely monitored parameters (ambient temperature,

humidity, and photoperiod). This study also only worked with GAS population from a specific site which means the results can vary across different populations of GAS from different environmental conditions as these GAS are highly adaptive to their surroundings oftentimes having enhanced resistance (Liu et al., 2020). In order to fully gauge the true molluscicidal potential of *M. anisopliae* strain Met-Gra4 on the field, a more extensive study needs to be conducted and further observations has to be done on the interactions of Met-Gra4 on the field with harsher ecophysiological conditions as well as the effects on other non-target organisms to fully understand the suitability of Met-Gra4 as a green alternative to commercial molluscicide.

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CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

This study investigated and elucidated the pathogenicity of the isolated native strain *M. anisopliae* (strain Met-Gra4) against GAS samples under laboratory conditions and the histopathological alterations resulting from conidial exposures. Despite the efficacy and viability of EPF being questionable due to its limited reports as biocontrol agents against snails which some may consider an unconventional application to explore, it prompted an investigation that expanded the capabilities of this locally isolated strain of fungus beyond initial limitations. Combining the improved viability, tolerance, and delivery system provided by the emulsion-formulation used to prepare the treatments, it elucidated a potential bio-molluscicide that may eventually be used to alleviate worries regarding the excessive usage of commercial molluscicides. In which the excessive usage of commercial molluscicides arouse concerns for its effects on sustainability and safety as well as help to provide an alternative to the issues regarding the invasion by the invasive GAS in the paddy fields.

In the first objective, this study is the first record comparing the pathogenicity of two form of treatments containing *M. anisopliae,* the conidial suspension and the emulsion-formulated conidia. Results clearly indicated that the emulsion-formulated treatments exhibited stronger and superior molluscicidal capabilities compared to the conidial suspension treatment. The emulsion-formulated treatment was also able to maintain the viability as it is more shelf-stable compared to the conidial suspension treatment hence proving once more that it is the more favourable treatment against the population of GAS under laboratory conditions. The prowess of emulsion-formulated

treatment was elucidated when statistically compared to the other treatments especially positive control (metaldehyde, 5%) where two of the highest concentrations (6.7×10^7 & 6.7×10^8 conidia mL^{-1}) managed to exhibit molluscicidal properties on par with commercial molluscicides. It is also evident that preparing conidia of *M. anisopliae* suspended in the emulsion-formulated conidia matrix has proven to be worth the extra steps to prepare the treatment as even the lowest concentration was able to reach 50% mortality within 14 days, being significantly faster than the treatment in conidial suspension form.

The second objective was to unveil the LT_{50} for conidial suspension treatments at concentrations of 6.7×10^5, 10^6, 10^7 and 10^8 conidia mL^{-1} which were recorded as 15.3299, 8.5656, 7.7176 and 1.1500 days respectively. On the other hand, for emulsion-formulated conidia treatments with concentrations of 6.7×10^5, 10^6, 10^7 and 10^8 conidia mL^{-1} were recorded to be 9.3874, 5.9620, 0.6123, and 0.4549 days respectively. These results indicated that emulsion-formulated conidia exhibit a stronger molluscicidal capabilities and are able to kill 50% of the GAS population within time frames comparable to those achieved by commercial molluscicide. The level of efficacy was observed at a lower concentration of 6.7×10^7 conidia mL^{-1} compared to conidial suspension requiring a much higher concentration of 6.7×10^8 conidia mL^{-1} to attain similar results.

The median lethal concentration (LC_{50}) for the emulsion formulated treatments was revealed to be lower than conidial suspension treatment which was 6.7×10^5 conidia mL^{-1} as opposed to LC_{50} of conidial suspension being 6.7×10^6 conidia mL^{-1}. This clearly reveals that all emulsion-formulated treatments were capable to kill off at least 50% of the population of GAS. Conclusively, the ability of emulsion-formulated conidia treatment of Met-Gra4 being already capable of killing at least 50% of the population at a lower concentration indicates that the emulsion-formulated treatment is the most efficacious form of treatment tested in this study on GAS under laboratory conditions.

The third objective revealed the effects of exposure and infection of *M. anisopliae* on two critical organs in GAS that act as biomarkers which were the

hepatopancreas and the reproductive organs, the key to its invasiveness. It was unveiled that histopathological alterations were endured by the GAS leading to impairment to the function of the hepatopancreas such as dilation of digestive tubules, atrophy, and necrosis comparable to commercial molluscicide in both treatments containing conidia of *M. anisopliae* leading to their deaths. Even though both form of treatments containing conidia of *M. anisopliae* were able to elicit histopathological alterations in the hepatopancreas of GAS tested, emulsion-formulated treatments, especially higher concentrations (6.7×10^7 and 6.7×10^8 conidia mL^{-1}) showed a more widespread and severe histopathological alterations that is statistically significant when compared to other treatments. Taking into consideration the roles hepatopancreas play as a biomarker organ as well as the digestive glands of GAS, these responses that were recorded were able to showcase the degree of damage and the severity of infections caused by conidia of *M. anisopliae* that would explain the molluscicidal effects observed and recorded in the first objectives.

Similar results were observed in the reproductive organs of GAS, where samples treated with emulsion-formulated conidia generally had consistently higher Histopathological Condition Index (HI) paired with a more severe and widespread alterations compared to other treatments such as negative control, positive control (metaldehyde, 5%), and conidial suspension. Exposure to emulsion-formulated conidia induced severe histopathological alterations within reproductive tubules in GAS that can be categorised as parasitic castration that may reduce fecundity of GAS and subsequently decrease their invasiveness in the long run.

In order to test the capabilities of conidia of *M. anisopliae* thoroughly, there are several aspects that future studies should take into consideration in order for the perfect concentration and formulation of treatment to be conclusively chosen. The promising results need to be replicated under open field applications. A more thorough study must be conducted to further investigate the replicability of the test results across different environmental factors. Future studies should especially emphasize the focus on the behaviour and interaction of emulsion-formulated conidia of *M. anisopliae* as well as conidial suspension of *M. anisopliae* with the environment and more importantly with non-target species. The stability of the emulsion-formulated conidia

may also have to be altered to accommodate the unpredictable and widely ever-changing ecophysiological factors of the field should there be a further study conducted on this. The optimised formulation may help to relief the issue of spore longevity by extending its viability in the field. Aside from improving the formulation to withstand the harsher environments, scanning for more virulent strains can also help to produce an alternative molluscicide with even stronger molluscicidal capabilities. With microbial control agents, even different strains of the same species may perform very differently with different formulations, reacting very differently when it comes to longevity, virulence and stability when prepared in different formulations. Since the potential of EPF as molluscicides is still heavily understudied, many aspects must be understood when it comes to the mechanism, the behaviours, and interactions on the field between the fungal conidia and the target as well as non-target species. This is essential to completely understand the true capacity and viability of EPF specifically *M. anisopliae* strain Met-Gra4 in controlling GAS populations in the field.

Histopathological alterations observed in this study were also found to be substantial evidence of severe pathogenic infection of *M. anisopliae.* Samples of hepatopancreas and gonads obtained were from dead samples of GAS exposed to treatments, hence in the future studies, the effect of time exposure against histopathological alterations may be conducted. This is to observe whether the period of exposure to conidia of *M. anisopliae* plays a role in inducing the histopathological alterations observed and whether parasitic castration occurs even in samples that survive the treatment. The establishment of this green alternative may alleviate the over-reliance on chemical molluscicides and may contribute to the rice industry in Malaysia. This fungal-based solution could play a pivotal role in Integrated Pest Management (IPM) strategies in handling pests for a more sustainable agricultural practice.

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APPENDICES

Appendix 1

Table 1 Animals that have been used as biocontrol agents.

Biological Control Agent		Stage of Predation	References
Reptile			
Turtle	*Chinemys reevesii*	Eggs, juvenile, adult	Yoshie & Yusa, 2008
	Mauremys japonica	Juvenile, adult	Yusa et al., 2006
	Pelodiscus sinensis	Eggs, juvenile	Dong et al., 2012
	Trachemys scripta elegans	Eggs, Juvenile	Guo et al., 2017a
Caiman lizard	*Dracaena* spp.	Eggs	Perera & Walls, 1996.
Annelida			
Leech	*Whitmania pigra*	Eggs	Guo et al., 2017b
Bird			
Asian Openbill	*Anastomus oscitans*	Eggs	Sawangproh & Poonswad, 2010
Chinese Pond Heron	*Ardeola bacchus*	Juvenile, Adult	Yusa et al., 2015.
Common Moorhen	*Gallinula chloropus*		Mochida, 2004
Duck	*Anas platyrhyncos*		Liang et al., 2014
	Anas zonorhyncha		Ramakrishnan et al., 2010
	Cairina moschata		Liang et al., 2014
Crustaceans			
Crab	*Eriocheir japonica*	Juvenile	Yusa et al., 2006
	Esanthephulsa nimoafi	Juvenile	Carlsson et al., 2004
	Geothelphusa dehaani	Adult snail	
Crayfish	*Procambarus clarkii*	Juvenile, adult	Yusa et a., 2006
Shrimps	*Macrobrachium formonsense*	Adult	Savaya-Alkalay et al., 2018
Insect			
Ants	*Solenopsis geminata*	Eggs	Yusa, 2001
	Pheidologeton spp.		
Diving Bees	*Cybister japonicus*	Small juvenile	Yusa et al., 2006
Aquatic Dragonflies	*Anax parthenope*	Small juvenile	Yusa et al., 2006
	Macromia amphigena		
	Pantala flavescens		
Stink bug	*Dindymus pulcher*	Eggs	Yusa et al., 2006
Water bug	*Sphaerodema molestum*	Juvenile	Yusa et a., 2006
Mammals			
Rats	*Rattus norvegicus*	Juvenile, sub adult	Yusa et al., 2000

Appendix 2

Table 3 Microbe-based control studied for molluscs. Studies conducted on GAS (*Pomacea* spp.) and *Metarhizium* species are bolded to highlight.

Microbe used	Target mollusc	Mechanism of control	References
Fungus			
Aspergillus terreus	*Biomphalaria alexandrina*	Release of mycotoxin	Saad et al., 2014
Beauvaria bassiana	*Amphimelania holandrii*	Cause vacuolation in digestive cell causing degeneration of secretory cells	Abdel Wareth et al., 2019
	Biomphalaria glabrata	Attack juvenile and eggs	Duarte et al., 2015
	Monacha cantiana	Fungus infection	Hendawy et al., 2015
Metarhizium anisopliae	***Pomacea* spp.**	**Infection towards the host (excessive mucus production and decreased activity levels)**	**Allahudin et al., 2024**
Metarhizium brunnerum	***Cornu aspersum***	**Enzymatic digestion**	**Khoja et al., 2019**
	Derocerus reticulatum	Toxicity of fungal volatile organic compounds	Khoja et al., 2019
Paecilomyces lilanicus	*Amphimelania holandrii*	Cause vacuolation in digestive cell causing degeneration of secretory cells	Abdel Wareth et al., 2019
Penicillium janthinellum	*Biomphalaria alexandrina*	Release of mycotoxin	Saad et al., 2014
Nematode			
Alloionema appendiculatum	*Arion vulgaris*	Toxicity in bacterial associates of the nematodes	Nermut' et al., 2019
Angiostrongylus cantonensis	***Pomacea canaliculata***	**Parasitic towards host snails**	**Yang et al., 2013**
Caenorhabditis briggsae	*Achantina fulica*	Parasite to the host snails	Guerrero et al., 2018
Heterorhabditis bacteriophora	*Biomphalaria glabrata*	Physiological alteration	Santos et al., 2022
	Parmacella ibera	Nematodes infection	Saeedizadeh & Niasti, 2020
Heterorhabditis baujardi	*Lymnaea columellar*	Changes the level of galactogen	Tunholi et al., 2017
	Pseudosuccinea columella	Physiological alteration (increased haemolymph activities of aminotransferase and lactate dehydrogenase)	Bitencourt Vidal et al., 2021
Heterorhabditis indica	*Bradybaena similaris*	Physiological alteration	Tunholi et al., 2014
Hugotdiplogaster neozelandia	*Athoracophorus bitentaculatus*	Parasite to the host slug	Sudhaus. 2018

Table 3 continues

Neoalloionema tricaudatum	*Cyclophoridae* spp.	Parasite towards juvenile snails	Ivanova et al., 2016
Hasmarhabditis californica	*Theba pisana*	Genetic variation in the bacterial associates of the nematode	Tandingan De Ley et al., 2020
Pellioditis hermaphrodita	*Neohelix albolabris*	Parasite to the host snails	Sudhaus, 2018
Phasmarhabditis hermaphrodita	*Achantina fulica*	Infect and kill the egg of snails	Azzam & El-Abd, 2021
	Deroceras invadens	Nematode infection	Cutler & Rae, 2020
	Deroceras reticulatum	Nematode infection	Tandingan De Ley et al., 2014
		Larval parasite	Mc Donell et al., 2020
		Inhibition of feeding	Cutler & Rae, 2020
	Eobania vermiculata	Infect and kill eggs of snails	Azzam & El-Abd, 2021
	Lehmannia valentiana	Nematode infection	Tandingan De Ley et al., 2014
	Limax flavus	Infect and kill eggs of snails	Azzam & El-Abd, 2021
	Parmacella ibera	Nematode infection	Saeedizadeh & Niasti, 2020
	Pomacea canaliculata	**Change of protein expression (PC-bpi)**	**Montanari et al., 2020; Boraldi et al., 2021**
	Theba pisana	Genetic variation in bacterial associates of the nematodes	Tandingan De Ley et al., 2020.
Phasmarhabditis papillosa		Nematode infection affecting herbivory	Laznik et al., 2020
	Theba pisana	Genetic variation in bacterial associates of the nematodes	Tandingan De Ley et al., 2020.
Rhabditella axei	*Archachatina achatina*	Nematode infection (gastrointestinal tract)	Olofintoye & Olorunniyi, 2016.
Steinernema feltiae	*Archachatina marginata*		
	Parmecella ibera	Nematode infection	Saeedizadeh & Niasti, 2020.
Trematode			
Schistosoma haematobium	*Biomphalaria alexandrina*	Affected physiological processes	Dokmak et al., 2024
	Bulinus truncates	Affected physiological processes	Dokmak et al., 2024
Schistosoma mansoni	*Biomphalaria alexandrina*	Affected physiological processes	Dokmak et al., 2024
	Bulinus truncates	Affected physiological processes	Dokmak et al., 2024

Appendix 3

Table 2 Plant-derived bioactive compounds studied as molluscicide for GAS.

Botanical name	Scientific name	Bioactive compound used	References
African Dream Herb	*Entada rheedii*	Flavonoid, glycosides, saponins, tannins, terpenoids	Nur Suraya et al., 2017
Cassava	*Manihot esculenta*	Aqueous extract	Osborne et al., 2022
Cinnamon	*Cinnamomum verum*	Essential oil extracts	Idris et al., 2020
Citrronella	*Cymbopogan nardus (L.) Rendle*	Essential oil extracts	Idris et al., 2020
Clove	*Syzygium aromaticum*	Essential oil extracts	Idris et al., 2020
Coffee	*Coffee liberica*	Ethanolic extracts	Khalid, 2020
Devil's horsewhip	*Achyranthes aspera*	Aqueous extract	Mandefro et al., 2017
Garlic	*Allium sativum*	Alkaloids, tannins, skin, stem extracts	Garin et al., 2019; Khalid, 2020
Ginger	*Zingiber officianale*	Crude extract, essential oil extract, rhizome	Prabhakaran et al., 2017; Idris et al., 2020; Ismail & Musa, 2021
Ground Cherry	*Physalis minima linn*	Ethanolic extraction	Malana & Salvador, 2020
Groundnut	*Archis hypogaea*	Skin extract	Khalid, 2020
Herb plants	*Glinus lotoides*	Aqueous extracts	Kiros et al., 2014
Kluwak	*Pangium edule*	Aqueous extracts	Manopo, 2017
Lantana	*Lantana camana*	Essential oils	Huy Hung et al., 2021
Madar	*Calotropis gigantean*	Aqueous extract	Osborne et al., 2022
Neem	*Azadiracha indica A. Juss*	Kernel oil	Prabharakan et al., 2017
Nerium	*Nerium indicum Mill.*	Leaves extract	Prabhakaran et al., 2017

Nipple fruit	*Solanum mammosum*	Extracts (alkaloids, phenols, tannins, saponin)	Quijano et al., 2014

Table 2 continues

Oleander	*Nerium oleander*	Aqueous extract	Osborne et al., 2022
Papaya	*Carica papaya*	Methanolic extracts	Rosli et al., 2021
Piper	*Piper nigrom L.*	Seeds	Prabharakan et al., 2017
Pongamia	*Pongamia pinnata L.*	Seed oil	Prabhakaran et al., 2017
Ragweed	*Ambrosia artemisiifolia*	Psilotachyin, psilotachyin B., axillaxin	Ding et al., 2018
Sweet potato	*Ipomoea batatas*	Hexane, methanolic and chloroform extracts	Noorshilawati et al., 2020
Spiny aralia	*Aralia armata*	Oleanolic acid saponin	Nguyen et al., 2021
Tobacco	*Nicotiana tabacum L.*	Leaves extract	Prabhakaran et al., 2017
Tuba	*Derris elliptica*	N-hexane & ethanol extracts (roots)	Manopo, 2017.
Variegated Sword Lily	*Furcraea selloana var. marginata*	Saponin extracts	Ramli et al., 2019
Wallich seed	*Derris elliptic*	Aqueous extracts (tannins, saponin)	Manoppo, 2017
Water spinach	*Ipomoea aquatic*	Stem aqueous extracts	Khalid, 2020
Western soapberry	*Sapindus saponaria*	Extracts (alkaloids, phenols, tannins, saponins)	Quijano et a., 2014
Wintersweet	*Chimonanthus praecox cv. Luteus*	Fatty acids	Zhang & Zou, 2020
	Chimonanthus nitens	Flower extracts	Li & Zou, 2019
Yellow Flamboyant	*Peltophorum pterocarpum*	Methanolic extracts	Rosli et al., 2021

BIODATA OF AUTHOR

Mohamad Ikmal Hakim is an individual whom has nurtured his passion for science especially Biology since he was a child. The passion initially started during his primary school years at Sekolah Kebangsaan Bandar Utama Damansara 2 where he first realised his affinity towards science subjects.

After Ujian Penilaian Sekolah Rendah, his passion for science grew. Sekolah Menengah Kebangsaan Bandar Utama Damansara 2 was where he continued to further explore his passion in science amongst his peers and decided that Biology was his favourite,

Following his now established passion in Biology, he pursued of what would be the beginning of his tertiary studies in Sekolah Menengah Kebangsaan Bandar Utama to challenge himself to take Sijil Tinggi Pelajaran Malaysia (STPM). STPM proved to be the hardest challenge in his life thus far with complicated syllabi crunched into three semesters.

In 2019, he received the unexpected opportunity to redeem himself and to reignite the dimmed passion at Universiti Malaysia Terengganu to enrol as a bachelor's student for Biodiversity Conservation and Management course. His passion burned brighter than ever before. Thriving in this favourite field of his, every class was exciting, every lab session was eventful. By the grace of God Almighty, he managed to graduate top of his class. Intrigued by challenges, he was presented with another unpassable opportunity; to continue is postgraduate here in Terengganu in 2022. Nervous and excited to explore new prospects within the research and laboratory field, with little experience in either of those. It was the passion, determination, endless support from the people around him that got him through to the finish line.

Publications

Molluscicidal Activity of Entomopathogenic Fungus, *Metarhizium anisopliae* (Hypocreales: Clavicipitaceae) against Golden Apple Snails, *Pomacea canaliculata* (Architaeniglossa: Ampullariidae).