INVESTIGATING THE EFFECTS OF THE MALE LURES CUE-LURE AND ZINGERONE ON GENE EXPRESSION IN *BACTROCERA TRYONI* AND *BACTROCERA JARVISI* (DIPTERA: TEPHRITIDAE)

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Bactrocera jarvisi, *Bactrocera tryoni*, cue-lure, Dacini, differential gene expression, fruit flies, gene ontology, generational, male lure, phytochemicals, plant secondary metabolites, RNAseq, Tephritidae, transcriptome, zingerone

Abstract

True fruit flies are a globally significant group of insect pest species capable of severely damaging commercial crops, and thus they are actively managed. Plant secondary metabolites, including methyl eugenol, cue-lure, and zingerone, elicit a positive response in male fruit flies. These male lures are employed in trapping fruit flies, either for monitoring purposes or as part of a male annihilation technique aimed at suppressing the population of fruit flies. Research has demonstrated that male lures can have various biological effects in fruit flies, and that these effects can vary between and within species and lure types. Not only does investigating these effects improve the understanding of the complex relationship between male lures and fruit flies, but this knowledge has potential applications in pest management strategies for advantageously modifying fruit fly biology. The use of transcriptomics in this field is relatively limited, therefore, this study aimed to investigate the effects of lure feeding on gene expression across different lures, species, and generations. Two separate studies were conducted, the first investigated differential gene expression in *Bactrocera tryoni* and *Bactrocera jarvisi* fed cue-lure and zingerone, and the second investigated differential gene expression in *B. tryoni* fed cue-lure and their offspring. To our knowledge, this is the first report of viral upregulation in a fruit fly in response to a lure; across both studies iflavirus transcripts were found upregulated in cue-lure fed *B. tryoni*. In the first study, 262 genes were differentially expressed in cue-lure fed *B. tryoni*, 238 in zingerone fed *B. tryoni*, 159 in cue-lure fed *B. jarvisi*, and 176 in zingerone fed *B. jarvisi*. Transposable element-related genes were differentially expressed in all treatments and differentially expressed sensory-related genes (e.g. general odorant binding protein 56a-like and general odorant binding protein 99a-like) varied across lure type and species. In the second study, 282 genes were differentially expressed in cue-lure fed flies in *B. tryoni* parents and 102 in their offspring. Across cue-lure fed parents and offspring, 39 genes were consistently differentially expressed, indicating that these genes could be involved in the generational effects of cue-lure in *B. tryoni*. These two studies have been able to provide new insights into the effects of male lures at the gene expression level and identify candidate genes for further functional analyses. These results will help improve the understanding of male lures in fruit flies and may have implications in pest management.

Table of Contents

Keywords ii
Abstract iii
Table of Contents iv
List of Figures vii
List of Tables viii
List of Supplementary Figures ix
List of Supplementary Tables x
List of Abbreviations xi
Acknowledgements xii
1.0 Chapter 1: Introduction 1
1.1 Introduction 1
1.2 Tephritid fruit flies 1
1.3 Male lures 2
1.4 Biological effects of male lures 3
1.4.1 Mating advantage 4
1.4.2 Accelerated sexual maturation 7
1.4.3 Male aggregation 8
1.4.4 Predator deterrence 8
1.4.5 Reduced repeat lure feeding and other effects 9
1.4.6 Effects in females and offspring 9
1.5 Project aims 10
2.0 Chapter 2: Methods 12
2.1 Study 1: investigating the effects of cue-lure and zingerone on gene expression in *B. tryoni* and *B. jarvisi* 12
2.1.1 Insects 12
2.1.2 Chemicals 12
2.1.3 Male lure feeding 13
2.1.4 Sampling 13
2.1.5 Sample preparation and sequencing 14
2.1.6 Transcriptome assembly and functional annotation 14
2.1.7 Differential gene expression analysis 15
2.1.8 Subcluster and gene ontology analysis 15
2.1.9 Annotated differentially expressed genes of interest 15
2.2 Study 2: Investigating the effects of cue-lure feeding on gene expression across two generations in *B. tryoni* 16
2.2.1 Insects 16
2.2.2 Chemicals 16
2.2.3 Male lure feeding 16

2.2.4 Fly rearing 17
2.2.5 Sampling 18
2.2.6 Sample preparation and sequencing 18
2.2.7 Read mapping and functional annotation 18
2.2.8 Differential gene expression analysis 19
2.2.9 Gene ontology analysis 19
2.2.10 Differentially expressed genes of interest 19
3.0 Chapter 3: Results 20
3.1 Study 1: Investigating the effects of cue-lure and zingerone on gene expression in *B. tryoni* and *B. jarvisi* 20
3.1.1 Transcriptome assembly and functional annotation 20
3.1.2 Differential gene expression analysis 20
3.1.3 Subcluster and gene ontology analysis 22
3.1.4 Annotated differentially expressed genes of interest 25
3.2 Study 2: Investigating the effects of cue-lure feeding on gene expression across two generations in *B. tryoni* 33
3.2.1 Read mapping and functional annotation 33
3.2.2 Differential gene expression analysis 34
3.2.3 Gene ontology analysis 35
3.2.4 Differentially expressed genes of interest 38
4.0 Chapter 4: Discussion 43
4.1 Direct effects of cue-lure and zingerone 43
4.1.1 Iflavirus and immunity 44
4.1.2 Transposable element-related genes 45
4.1.3 Sensory-related genes 46
4.1.4 Comparison to Kumaran et al. (2014b) zingerone fed *B. tryoni* 47
4.1.5 Time point comparison in cue-lure fed *B. tryoni* 49
4.2 Generational effects of cue-lure 49
4.2.1 Generational effects of cue-lure feeding 49
4.2.2 Generational effects of laboratory rearing 50
4.3 Implications in pest management 51
4.4 Assumptions, limitations and considerations 51
4.5 Future directions 52
4.6 Concluding remarks 53
5.0 Reference list 54
6.0 Appendices 78
Appendix A: Preliminary lure response bioassays 78
Methods 78
Results and discussion 79
Appendix B: Supplementary figures 82

Appendix C: Supplementary tables..... 85

List of Figures

Figure 1. Heatmaps of differentially expressed genes in the *B. tryoni* transcriptome……..……21

Figure 2. Heatmap of differentially expressed genes and subcluster analysis in *B. tryoni* excluding genes with NCBI:txid2795009 = *B. tryoni* iflavirus 1……...…..23

Figure 3. Heatmap of differentially expressed genes and subcluster analysis in *B. jarvisi*……..….. 25

Figure 4. Heatmaps of sensory-related differentially expressed genes…….……..28

Figure 5. Heatmaps of immune-related differentially expressed genes……..…….30

Figure 6. Venn diagram of annotated differentially expressed genes from male lure treatments compared to the control……..……31

Figure 7. Heatmap of differentially expressed genes in the *de novo* assembled *B. tryoni* generation one transcriptome……34

Figure 8. Scatterplot of enriched gene ontology biological process terms between generation one and generation two……...36

Figure 9. Heatmap of the 39 differentially expressed genes in *B. tryoni* between two treatments: i) cue-lure fed flies and their offspring, and ii) control flies and their offspring……..…….39

Figure 10. Boxplots of individual expression of candidate genes across generation one cue-lure fed flies and their offspring, generation one control flies and their offspring……..……40

List of Tables

Table 1. Transcriptome assembly statistics for *B. tryoni* and *B. jarvisi*……...20

Table 2. Gene ontology enrichment analysis on differentially expressed genes in *B. tryoni* zingerone fed flies vs control flies…….…… 24

Table 3. Sensory-related genes differentially expressed in *B. tryoni* and *B. jarvisi* transcriptomes…....27

Table 4. Immune system-related genes differentially expressed in *B. tryoni* and *B. jarvisi* transcriptomes……...29

Table 5. Annotated differentially expressed genes from male lure treatments compared to the control in *B. tryoni* and *B. jarvisi*…….……..32

Table 6. Number of genes differentially expressed between all pairwise comparisons in *B. tryoni*…....35

Table 7. Gene ontology enrichment analysis on genes differentially expressed between generation one cue-lure fed flies and generation two cue-lure fed flies in *B. tryoni*……..……...…..37

Table 8. Gene ontology enrichment analysis on genes differentially expressed between generation one control flies and generation two control flies in *B. tryoni*…….....37

Table 9. Sensory and immune system-related genes differentially expressed in *B. tryoni* generation one cue-lure fed flies vs generation one control flies and generation two cue-lure flies vs generation two control flies……...……...…...42

List of Supplementary Figures

Figure A 1. Jitter boxplot of the response of male *B. tryoni* to different cue-lure concentrations……80

Figure A 2. Linear regression of abiotic effects on the number male *B. tryoni* responding to cue-lure……..……..81

Figure A 3. Sample correlation matrix of differentially expressed genes for *B. tryoni* with treatments cue-lure fed, zingerone fed, and control……..…….....82

Figure A 4. Sample correlation matrix of differentially expressed genes for *B. jarvisi* with treatments cue-lure fed, zingerone fed, and control……..…...83

Figure A 5. Sample correlation matrix of differentially expressed genes for *B. tryoni* across two generations with cue-lure fed flies and control flies……..….84

List of Supplementary Tables

Table A 1. Read counts for *B. tryoni* and *B. jarvisi* before and after trimming with Trimmomatic (Study 1)……...……....85

Table A 2. Gene ontology analysis on differentially expressed genes in *B. tryoni* cue-lure fed flies vs control fed flies…….86

Table A 3. Gene ontology analysis on differentially expressed genes in *B. tryoni* zingerone fed flies vs control fed flies…….87

Table A 4. Gene ontology analysis on differentially expressed genes in *B. tryoni* cue-lure fed flies vs zingerone fed flies…….88

Table A 5. Gene ontology analysis on differentially expressed gene subclusters in *B. tryoni* with treatments cue-lure fed, zingerone fed, and control……...89

Table A 6. Gene ontology analysis on differentially expressed genes in *B. jarvisi* cue-lure fed flies vs control fed flies…….…….91

Table A 7. Gene ontology analysis on differentially expressed genes in *B. jarvisi* zingerone fed flies vs control fed flies…….….92

Table A 8. Gene ontology analysis on differentially expressed genes in *B. jarvisi* cue-lure fed flies vs zingerone fed flies…….93

Table A 9. Gene ontology analysis on differentially expressed gene subclusters in *B. jarvisi* with treatments cue-lure fed, zingerone fed, and control…….……..95

Table A 10. Iflavirus genes in *B. tryoni* and *B. jarvisi* transcriptomes (study one)……...….……..97

Table A 11. Read counts for *B. tryoni* before and after trimming with Trimmomatic (Study 2)…..…100

Table A 12. *De novo* transcriptome assembly statistics for study two *B. tryoni* compared with the statistics for study one *B. tryoni* and *B. jarvisi de novo* transcriptome assemblies…….…..101

Table A 13. Iflavirus genes in the *B. tryoni* transcriptome (study two)…….…..……102

Table A 14. Gene ontology enrichment analysis on differentially expressed genes in *B. tryoni* between generation one and generation two……...…..…….104

Table A 15. Gene ontology enrichment analysis on differentially expressed genes in *B. tryoni* generation one cue-lure fed flies and generation two control flies……..106

Table A 16. Gene ontology enrichment analysis on differentially expressed genes in *B. tryoni* between generation one control flies and generation two cue-lure fed flies……..…….108

Table A 17. Transposition-related genes differentially expressed in *B. tryoni* generation one cue-lure fed flies vs generation one control flies and generation two cue-lure flies vs generation two control flies…….…….109

List of Abbreviations

BP	Biological Process
BTIV1/2/3	Bactrocera tryoni iflavirus 1/2/3
CC	Cellular Component
CDS	Coding Sequence
CL	Cue-lure
DEG(s)	Differentially Expressed Genes
DGE	Differential Gene Expression
DPT	Days Post Treatment
FDR	False Discovery Rate
FN	Fibronectin
G1	Generation 1
G2	Generation 2
GNE	Guanine Nucleotide-Exchange
GO	Gene Ontology
GR	Gustatory Receptor
IG	Immunoglobulin
jar	*Bactrocera jarvisi*
MAT	Male Annihilation Technique
MF	Molecular Function
mRNA	messenger RNA
NCBI	National Center for Biotechnology Information
OBP	Odorant Binding Protein
OR	Odorant Receptor
Prot	Protein
QUT	Queensland University of Technology
RNAseq	RNA sequencing
SIT	Sterile Insect Technique
try	*Bactrocera tryoni*
ZN	Zingerone

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Chapter 1: Introduction

1.1 INTRODUCTION

The relationship between insects and plants is complicated; in particular, insect interactions with plant secondary metabolites. True fruit flies are a globally significant group of insect pest species capable of severely damaging commercial crops, and thus they are actively managed. Plant secondary metabolites, including methyl eugenol, cue-lure, and zingerone, elicit a positive response in male fruit flies. These male lures are employed in trapping fruit flies, either for monitoring purposes or as part of a male annihilation technique aimed at suppressing the population of fruit flies (Tan et al., 2014). Research has demonstrated that male lures can have various biological effects in fruit flies, and that these effects can vary between and within species and lure type. Not only does investigating these effects improve the understanding of the complex relationship between male lures and fruit flies, but this knowledge has potential applications in pest management strategies for advantageously modifying fruit fly biology.

1.2 TEPHRITID FRUIT FLIES

Tephritid fruit flies (Diptera: Tephritidae) are a highly speciose insect family with over 4000 species across more than 450 genera (Norrbom, 2004). Although among tephritids a range of substrates are utilised for development, species that use fruit tissue are a severe threat to agriculture and are the primary focus of this review. These frugivorous species oviposit into ripening fruit tissue and larvae feed on the tissue before leaving the fruit to pupate and emerge as adult flies (Fletcher, 1987). Larval development directly damages the fruit tissue leading to decay and premature fruit drop and therefore these species are of major economic concern (Clarke et al., 2011). Australia's horticultural production was valued at over $15 billion from 2020 - 2021, with an estimated $6 billion at risk of fruit fly damage (National Fruit Fly Council, n.d.). Economic losses are not only caused by agricultural yield loss, but also through associated pest management costs, quarantine treatments, trade restrictions, and diminished market access (Clarke et al., 2011; Papadopoulos, 2014; Trombik et al., 2023). The tribe Dacini (Tephritidae: Dacinae) contains some of the most notorious pest species including *Bactrocera dorsalis* (Hendel), widely considered the most destructive pest, and *Bactrocera tryoni* (Froggatt), Australia's worst horticultural pest (Vargas et al., 2015). Dacini contains four genera (*Bactrocera*, *Zeugodacus*, *Dacus*, and *Monacrostichus*) and 932 species, of which, 10 % are recognised as pest species (Doorenweerd et al., 2018). Pest species are generally polyphagous such as *Bactrocera jarvisi* (Tryon), or highly invasive such as *Bactrocera oleae* (Gmelin), or a combination of both traits such as *B. dorsalis* and *B. tryoni* (Vargas et al., 2015).

In area-wide pest management of tephritid species, there are two commonly used techniques which are the male annihilation technique (MAT) and the sterile insect technique (SIT) (Vargas et al., 2015). MAT utilises male lure combined with insecticide within a trap which attracts and kills male fruit flies to reduce population size (Steiner, 1952; Vargas et al., 2014). While SIT involves the release of sterile male flies to mate with wild female flies resulting in inviable offspring, thus, reducing the population (Knipling, 1955; Klassen et al., 2021). Generally, MAT and SIT are applied consecutively to ensure sterile flies are not killed by MAT and to improve the likelihood of sterile flies mating with wild females. The simultaneous application of these techniques would greatly improve their effectiveness if sterile flies were not attracted to MAT traps (Barclay et al., 2014). Research has indicated that feeding sterile flies male lure prior to release can decrease the likelihood of flies being trapped with MAT and increase their mating competitiveness (Shelly et al., 2010; Khan et al., 2017; Shelly, 2020). Therefore, understanding the effects of male lures in fruit flies is important for improving the efficacy of pest management techniques.

1.3 MALE LURES

The term 'male lure' is used throughout Tephritidae literature to describe a group of chemicals that induce a positive chemotactic response in male fruit flies. Some male lures occur naturally in plants as secondary metabolites (e.g. cue-lure, raspberry ketone, methyl eugenol, zingerone) and others are exclusively man-made compounds (generally analogues of plant-derived male lures or chemical blends) (Tan et al., 2014). The majority of lure responsive Dacini species are attracted to either phenylbutanoids (e.g. cue-lure, raspberry ketone, zingerone) or phenylpropanoids (e.g. methyl eugenol) (Tan et al., 2014; Royer, 2015; Segura et al., 2018). The responsiveness and sensitivity of fruit flies to different male lures varies between species (Howlett, 1915; Wee et al., 2002; Fay, 2010; Fay, 2012; Royer, 2015; Royer et al., 2020; Wee et al., 2020). Species may also be attracted to multiple male lures, however, Dacini species responsive to phenylbutanoids will generally not respond to phenylpropanoids and vice versa (Drew, 1974; Fay, 2010; Royer, 2015; Royer et al., 2019b). Cue-lure and methyl eugenol have been and continue to be the most prominent male lures due to the high numbers of responsive species; of 932 Dacini species, 407 are responsive to cue-lure and 123 are responsive to methyl eugenol (Doorenweerd et al., 2018). There is a smaller group (but expanding with the discovery of new attractants) of species responsive to other male lures (Fay, 2010; Royer, 2015; Royer et al., 2019a; Royer et al., 2019b), the remaining Dacini species are either untested, have insufficient data, or no response to known lures (Doorenweerd et al., 2018).

The first male lure to be discovered was methyl eugenol (3,4-dimethoxy-allylbenzene) by Howlett (1912), who, during his search for a female fruit fly attractant, found male fruit flies covering his neighbours mosquito repellent: a handkerchief sprinkled with citronella oil. Howlett (1915) later

identified that the methyl eugenol component of the citronella oil was responsible for the attraction of *Bactrocera zonata* (Saunders) and *B. dorsalis*. These findings mark the beginning of male lure research, and would be instrumental in future pest management (Steiner, 1952). Methyl eugenol is one of the most widely distributed male lures in nature, occurring across various plant organs in over 450 plant species across 80 families (Tan & Nishida, 2012).

Over 40 years after the discovery of methyl eugenol, Barthel et al. (1957) identified anisyl acetone (4-(4-Methoxyphenyl)-2-butanone) as an attractant for *Zeugodacus cucurbitae* (Coquillett) through chemical screening. Soon after, Beroza et al. (1960) tested the attraction of related chemicals and identified that cue-lure (4-(4-Acetoxyphenyl)-2-butanone) was a more effective male lure for *Z. cucurbitae*. The hydrolysed form of cue-lure, raspberry ketone (4-(4-Hydroxyphenyl)-2-butanone) was also recognised as a strong attractant by Beroza et al. (1960) and independently identified as an attract in Australia in 1959 (Drew, 1974). Cue-lure and raspberry ketone are generally considered to elicit the same effects in fruit flies due to their structural similarity, however, cue-lure is generally considered a more attractive lure (Royer et al., 2020). Cue-lure was long considered an exclusively man-made compound until the identification of trace amounts occuring in the orchid *Bulbophyllum hortorum* (Nishida & Tan, 2016; Katte et al., 2020). Since this discovery, cue-lure has also been identified in the flowers of *Passiflora maliformis*, but in low levels (Park et al., 2020). On the other hand, raspberry ketone has been identified across several orchid species generally in larger quantities (Nishida et al., 1993; Tan & Nishida, 2005; Katte et al., 2020; Park et al., 2020; Tan et al., 2021).

More recently zingerone (4-(4-hydroxy-3-methoxyphenyl)-2-butanone) was discovered by Tan and Nishida (2000) in the orchid *Bulbophyllum patens* as an attractant for several fruit fly species including *B. dorsalis* and *Z. cucurbitae*. This finding was significant in that it was the first report of methyl eugenol responsive species (*B. dorsalis*) and cue-lure responsive species (*Z. cucurbitae*) being attracted to the same chemical; this attraction was attributed to the structural similarity of zingerone to both compounds. Continual screening efforts have facilitated the identification of several new attractants, some of which are more attractive lures in some species (Royer et al., 2019a; Royer et al., 2019b); however, methyl eugenol, cue-lure, raspberry ketone, and zingerone continue to be some of the most prominent lures in Dacini literature.

1.4 BIOLOGICAL EFFECTS OF MALE LURES

The feeding on (direct contact), or exposure to (no direct contact) male lures can have various biological effects in fruit flies. These effects are mostly investigated in the context of mating behaviour due to the sex-specific attraction and seminal works reporting unique pheromone components (Fitt, 1981; Nishida et al., 1988) and increased mating success (Shelly & Dewire, 1994) in lure fed males. To date, twelve Dacini species have been investigated for the effects of male lures (beyond confirming response to), all

of which demonstrate an effect on mating behaviour or related processes for at least one male lure. Although the proportion of species investigated is limited (12 of > 500 lure responsive Dacini species; Doorenweerd et al., 2018), pest species *B. dorsalis* and *B. tryoni* have been intensely researched which has greatly improved the understanding of the relationship between fruit flies and male lures. The reported effects of lures in male fruit flies include: i) mating advantage, ii) accelerated sexual maturation, iii) male aggregation, iv) predator deterrence, v) reduced repeat feeding on lure, and vi) increased lure response rate in offspring. In female fruit flies that have mated with lure fed males, reported effects include increased fecundity and reduced remating. These effects can vary between and within species and different lures, therefore it is important to review the literature thoroughly and critically. There have been several excellent literature reviews on this topic, including Shelly (2010) with a review of methyl eugenol and raspberry ketone/cue-lure in the genus *Bactrocera* (Diptera: Tephritidae: Dacinae: Dacini), and Tan et al. (2014) and Segura et al. (2018) with broader reviews of male lures in the family Tephritidae (Diptera). The following text will aim to provide an updated review of male lures in the tribe Dacini (Diptera: Tephritidae: Dacinae), therefore the genera *Ceratitis* and *Anastrepha* will not be discussed here. Additionally, the species *B. oleae* will not be discussed here due to its unique mating system compared to other Dacini species, see Segura et al. (2018) for a review of this species.

1.4.1 Mating advantage

The most common and investigated effect of male lures is a mating advantage in sexually mature male fruit flies; ten species (excluding *B. oleae*) have been tested and all exhibit increased mating success in lure fed males compared to lure denied males at least under certain conditions with one, or multiple male lures. Increased mating success has been heavily documented in *B. dorsalis* with feeding on methyl eugenol (Shelly & Dewire, 1994; Shelly, 1995; Shelly et al., 1996; Tan & Nishida, 1996, 1998; Shelly et al., 2000; Shelly & Nishida, 2004; Shelly et al., 2007; Shelly et al., 2008; McInnis et al., 2011; Ji et al., 2013; Obra & Resilva, 2013; Haq et al., 2018; Shelly, 2020), exposure to methyl eugenol (Haq et al., 2018; Shelly, 2020), and feeding on plants with natural sources of methyl eugenol (Shelly, 2000c; Shelly, 2001; Shelly & Edu, 2007; Shelly et al., 2007). *Bactrocera carambolae* Drew & Hancock also exhibits a mating advantage with feeding on and exposure to methyl eugenol (Shelly & Villalobos, 1995; Wee et al., 2007; Haq et al., 2014, 2015). Other species that have exhibited increased mating success after lure feeding are as follows: *Bactrocera umbrosa* (Fabricius) with methyl eugenol (Wee et al., 2018a), *Bactrocera cacuminata* (Hering) with methyl eugenol (Raghu & Clarke, 2003a), *Bactrocera zonata* with methyl eugenol (Rasool et al., 2023), *Bactrocera correcta* (Bezzi) with methyl eugenol and β-caryophyllene (Wee et al., 2018b), *B. tryoni* with cue-lure and zingerone (Kumaran et al., 2013; Kumaran et al., 2014b), *B. jarvisi* with raspberry ketone and zingerone (Wee & Clarke, 2020), *Z. cucurbitae* with cue-lure, raspberry ketone and zingerone (Shelly & Villalobos, 1995; Shelly, 2000a;

Shelly & Nishimoto, 2016; Inskeep et al., 2019; Shelly, 2019; Panduranga et al., 2023), and *Zeugodacus tau* (Walker) with zingerone (Shamshir & Wee, 2019).

Although all ten species exhibit a mating advantage after lure feeding, the temporal profile of this effect has been shown to vary between species. For example, methyl eugenol feeding in *B. dorsalis* conferred a mating advantage at 0, 2, 5, 10, 21, and 35 days post treatment (DPT) (Shelly & Dewire, 1994), whereas in *B. cacuminata* this effect was observed only at 16 and 32 DPT (not at 0, 1, 2, 4, or 8 DPT; Raghu & Clarke, 2003a). Moreover, the temporal profile of a mating advantage has been shown to vary between lure type. For example, cue-lure feeding in *B. tryoni* conferred a mating advantage at 0, 1, 2, and 3 DPT (not at 7, 14, 21, or 28 DPT), but with zingerone feeding this effect occurred only at 0 and 1 DPT (not at 2, 3, 7, 14, 21, or 28 DPT; Kumaran et al., 2013). This variation may explain why some studies report no mating advantage after lure feeding but did not test multiple DPT (*B. dorsalis* with exposure to natural methyl eugenol sources [Shelly, 2000c; Shelly, 2001]). In addition to differences between species and lure types, the dose of the lure can affect the temporal profile of a mating advantage. Wee and Clarke (2020) found increased mating success in zingerone fed *B. jarvisi* at a dose of 20 µg at 1 DPT (but not 3 DPT), 50 µg at 1, 3, and 7 DPT (but not 14 DPT), and 100 µg at 3 DPT (but not 1 or 7 DPT). The authors state that the low dose likely led to the rapid exhaustion of a mating advantage, but the mechanisms underlying the effects at medium and high doses are unknown. Moreover, Shelly and Dewire (1994) demonstrated that *B. dorsalis* fed on methyl eugenol for 30 seconds had a mating advantage from 0 DPT, but when fed for 2 hours (and presumably consumed a higher lure dose) a mating advantage did not begin until 2 DPT. The effect of lure dose on mating advantage can also be seen across studies using similar feeding methods: *B. dorsalis* exposed to methyl eugenol for 2 hours (none; Shelly & Dewire, 1994) vs 5 hours (significant; Haq et al., 2018), *B. carambolae* exposed to methyl eugenol for 2 hours (none; Shelly & Villalobos, 1995) vs 3 hours (significant; Haq et al., 2015). Although the underlying mechanisms are not well understood, these studies indicate a complex relationship between mating advantage, DPT, and lure dose at least in some species.

There are two prevailing hypotheses to explain the male lure mating advantage: i) pheromone enhancement, where the male pheromone is more attractive to females due to the incorporation of lures or lure derivatives into the pheromone blend, and ii) metabolic enhancement, where male courtship activities are increased due to increased metabolism and activity after lure feeding. Pheromone enhancement is supported by alterations to volatile emissions or rectal gland contents (where pheromone components are sequestered; Hee & Tan, 2005) and increased attraction of females to lure fed males irrespective of changes in activity. Whereas metabolic enhancement is supported by increased courtship signalling activities (i.e. wing fanning) or other measures of activity. These proposed mechanisms are not mutually exclusive, the occurrence of one or both appears to be species and lure specific. In *B. dorsalis* both mechanisms have been supported to explain the methyl eugenol mating

advantage. Several studies have demonstrated that methyl eugenol fed *B. dorsalis* accumulate additional compounds (2-allyl-4,5-dimethoxyphenol and (*E*)-coniferyl alcohol) in their rectal glands (Nishida et al., 1988; Tan & Nishida, 1996, 1998; Shelly & Nishida, 2004; Hee & Tan, 2006; Tan et al., 2010), and that females are more attracted to lure fed males regardless of wing fanning activity (Shelly & Dewire, 1994; Shelly, 2001). Moreover, multiple studies have reported increased wing fanning in *B. dorsalis* after feeding on methyl eugenol (Shelly & Dewire, 1994; Shelly et al., 1996; Shelly et al., 2000), but not always (Shelly, 2000c).

Similarly, both pheromone and metabolic enhancement has been supported to explain the cue-lure mating advantage in *B. tryoni*, however, the zingerone mating advantage has only been supported via metabolic enhancement. Cue-lure and raspberry ketone fed *B. tryoni* both accumulate the additional compounds raspberry ketone and 4-(4-hydroxyphenyl)-2-butanol in their rectal glands (Tan & Nishida, 1995; Kumaran et al., 2014a). Kumaran et al. (2014a) demonstrated that cue-lure fed *B. tryoni* release raspberry ketone with their endogenous pheromones during the courtship period, and importantly, that females were more attracted to the extracted rectal glands from cue-lure fed flies. Further work by Kumaran et al. (2014b) demonstrated that cue-lure fed males had increased locomotor activity and increased weight loss, which is indicative of increased metabolism and activity; therefore, both mechanisms can be supported with this lure in this species. On the other hand, zingerone fed *B. tryoni* were found to accumulate zingerone and raspberry ketone in their rectal glands and release these compounds during the courtship period, but females were not more attracted to their extracted rectal glands (Kumaran et al., 2014a). However, zingerone fed *B. tryoni* did demonstrate the same indicators of increased metabolism and activity as cue-lure fed *B. tryoni*, and a transcriptome analysis revealed zingerone fed *B. tryoni* had upregulation of several metabolic genes and enrichment of metabolic processes (Kumaran et al., 2014b); therefore, metabolic enhancement but not pheromone enhancement can be supported with this lure in this species.

Like cue-lure fed *B. tryoni*, cue-lure fed *Z. cucurbitae* accumulate raspberry ketone in their rectal glands (Nishida et al., 1993). However, only metabolic enhancement has been supported in cue-lure fed *Z. cucurbitae*. Shelly and Villalobos (1995) demonstrated that increased female sightings to cue-lure fed *Z. cucurbitae* males could not be explained by a more attractive pheromone, but that wing-fanning was significantly increased. Again, like zingerone fed *B. tryoni*, zingerone fed *Z. tau* accumulate zingerone in their rectal glands (Nakahira et al., 2018). However, both mechanisms have been supported in zingerone fed *Z. tau*. Shamshir and Wee (2019) demonstrated that females were significantly attracted to the extracted rectal glands of zingerone fed *Z. tau* males and that wing fanning was also significantly increased in these males. While other species have recorded changes in the chemical compositions of their rectal glands after lure feeding (e.g. *B. carambolae* [Wee et al., 2007], *B. umbrosa* [Wee et al., 2018b], *B. frauenfeldi* (Schiner) [Wee et al., 2020], *B. correcta* [Tokushima et al., 2010]), female attraction to only the altered pheromone blend has not been tested in these species and therefore the

underlying mechanisms cannot be fully interpreted. From the species tested, there is no clear pattern between lure types or species and whether pheromone and/or metabolic enhancement can explain the male lure mating advantage.

An interesting case of a species with no clear mechanism/s to explain male lure attraction and mating advantage is *B. cacuminata*. Raghu and Clarke (2003a) demonstrated that methyl eugenol fed males exhibited a mating advantage in small cages at 16 and 32 DPT, and not at 0, 1, 2, 4, or 8 DPT; but no mating advantage was found in large field cages. This delay in mating advantage is much greater than other species, where the second longest delay that has been recorded is 3 DPT in *B. carambolae* (Wee et al., 2007; Haq et al., 2015). Although not directly tested, Raghu and Clarke (2003a) suggest that pheromone enhancement is unlikely to explain the delayed mating advantage due to the rapid transformation of phytochemicals as seen in other fruit fly species (Wee & Tan, 2007) and other insects (Tillman et al., 1999). Likewise, it would be surprising if metabolic enhancement was underlying the mating advantage as increased activity is observed shortly after lure consumption in other fruit fly species (Shelly & Dewire, 1994; Shelly & Villalobos, 1995; Kumaran et al., 2014b; Shamshir & Wee, 2019). Raghu et al. (2002) also investigated the physiological consequences of methyl eugenol in *B. cacuminata*; while some significant differences were observed in protein and carbohydrate reserves at various DPT (but not in weight or lipid reserves), the authors suggest that a relationship to the mating advantage is unclear. Further research by Raghu and Clarke (2003b) supported that methyl eugenol serves as a mating rendezvous site in *B. cacuminata* as more mating pairs were observed at methyl eugenol compared to host plant, sugar, and protein sources in a field cage. However, Drew et al. (2008) identified *B. cacuminata* mating activities occurring on the host plant in the wild and did not detect methyl eugenol present in the host plant, therefore, the authors argued against methyl eugenol serving as a mating rendezvous site for *B. cacuminata*. For now, the mechanisms underlying *B. cacuminata* methyl eugenol attraction and the delayed mating advantage (at least in small cages) are unknown.

1.4.2 Accelerated sexual maturation

The male lure mating advantage is investigated mostly in sexually mature flies as this is generally when flies exhibit increased attraction to the lure (Wong et al., 1989; Wong et al., 1991; Wee & Tan, 2000; Wee et al., 2018a; Wee et al., 2018b; Wee et al., 2018c; Rasool et al., 2023); however, some studies have demonstrated that feeding on lure at young ages can increase mating success, and thereby accelerate sexual maturation. Akter et al. (2017b) found that *B. tryoni* fed on a raspberry ketone supplemented diet within the first three days of emergence exhibited a significantly higher number of matings at 4 – 10 days of age compared to lure denied flies. Moreover, Khan et al. (2019) demonstrated that incorporation of raspberry ketone into the adult diet of sterile *B. tryoni* was associated with the onset of sexual maturity approximately one day earlier than lure denied flies. On the other hand, no

effect on the rate of sexual maturation was reported for raspberry ketone feeding in *Z. cucurbitae* (Fezza & Shelly, 2018), or methyl eugenol feeding in *B. dorsalis* (Shelly et al., 2008). The mechanisms underlying the effect of male lures on fly development are not well understood; they could be similar to those proposed for the mating advantage in sexually mature flies, or there may be entirely different mechanisms involved.

1.4.3 Male aggregation

In addition to increased female attraction to lure fed males, male flies can be more attracted to lure fed males and thus lures may promote male aggregation. The increased attraction of males to lure fed males has been identified in *B. dorsalis* with methyl eugenol (Tan & Nishida, 1996; Hee & Tan, 1998), *Z. cucurbitae* with cue-lure and zingerone (Khoo & Tan, 2000), *B. carambolae* with methyl eugenol (Wee et al., 2007), and *B. umbrosa* with methyl eugenol (Wee et al., 2018a). Male-male attraction also occurred earlier with lure fed males compared to lure denied males in *B. dorsalis* (Hee & Tan, 1998), *Z. cucurbitae* (Wee et al., 2007), and *B. carambolae* (Wee et al., 2007). Moreover, Wee et al. (2007) observed males surrounding methyl eugenol fed *B. carambolae* during courtship and feeding on their anal secretions (containing the lure derivative and endogenous pheromones); as did Wee et al. (2018a) with methyl eugenol fed *B. umbrosa*. Tan and Nishida (1996) suggested that the attraction of males to lure fed males may divert the attention of lure denied males from potential female mates during courtship. Wee et al. (2007) also speculated that lure fed males may benefit, in part, from increased mating success due to lure denied males being distracted during courtship by the anal secretions of lure fed males. Alternatively, Wee et al. (2018a) suggested that lure denied males may benefit from feeding on the anal secretions of lure fed males as lure derivatives can be incorporated directly into their pheromone without the cost of transforming the lure. Further studies are required to substantiate these hypotheses.

1.4.4 Predator deterrence

There has been some evidence to suggest that methyl eugenol is associated with predator deterrence in *B. dorsalis*. Tan and Nishida (1998) and Wee and Tan (2001) demonstrated through no choice tests that the consumption of male *B. dorsalis* by geckos (*Hemidactylus frenatus* and *Gekko monarchus*, respectively) was significantly lower in methyl eugenol fed flies compared to lure denied flies. When *H. frenatus* were offered a choice of lure fed males and lure denied females, the number of flies consumed did not increase from the no choice male test, suggesting a potential benefit to female flies in the proximity of lure fed males (Tan & Nishida, 1998). Moreover, some *G. monarchus* that consumed lure fed males exhibited tumour like growth in their liver (Wee & Tan, 2001). There has also been some indirect evidence to suggest that male lures can reduce predation. Nishida et al. (1988) demonstrated

that sparrows consumed much fewer rice grains when they were applied with methyl eugenol. Tan (2000) demonstrated that house flies applied topically with raspberry ketone deterred consumption by *H. frenatus*. While these studies indicate a predator deterrence effect of male lures, there has been no further investigations into this topic in recent years; as highlighted by Kumaran (2014) there needs to be investigation into the natural predators of fruit flies like spiders or pray mantis to confirm that predator deterrence occurs in the natural system.

1.4.5 Reduced repeat lure feeding and other effects

Male lure feeding has also been associated with a subsequent decrease in lure response, and has been reported in *B. dorsalis* (Shelly & Dewire, 1994; Shelly, 1995; Shelly et al., 1996; Manoukis et al., 2018; Shelly, 2020), *Z. cucurbitae* (Chambers et al., 1972; Shelly & Villalobos, 1995), and *B. tryoni* (Akter et al., 2017a; Khan et al., 2017; Khan et al., 2021). This reduced repeat lure feeding has been investigated mostly in pest species and in the context of pest management as this effect is especially important for the simultaneous application of MAT and SIT (Barclay et al., 2014). The earlier onset of courtship activities has been observed in several species reporting an increased mating advantage (Wee et al., 2007; Kumaran et al., 2013; Wee et al., 2018a; Shamshir & Wee, 2019), this effect likely contributes the mating advantage however it does not clearly align with the currently proposed mechanisms. Increased mortality has also been reported in lure fed males (Kumaran et al., 2013), but not in all conditions (Raghu et al., 2002; Shelly & Nishimoto, 2016; Akter et al., 2017b; Inskeep et al., 2019).

1.4.6 Effects in females and offspring

As demonstrated, male lures can have various biological effects in male flies, but they can also have effects in female flies and their offspring; knowledge of these effects (or the lack thereof) is particularly important to help inform potential evolutionary drivers of the male lure response. Three species have been tested for male lure effects in female flies and offspring, however, these effects have only been identified in *B. tryoni*. Kumaran et al. (2013) found that female *B. tryoni* mated with lure fed males (cue-lure and zingerone) exhibited increased fecundity, reduced remating propensity, and reduced longevity. Transcriptome analysis of mated female *B. tryoni* revealed 80 genes differentially expressed only in females mated with zingerone fed males and not in females mated with control flies, further supporting that female *B. tryoni* undergo unique changes after mating with lure fed males (Kumaran et al., 2017). In addition to female effects, Kumaran and Clarke (2014) found that the offspring of cue-lure fed *B. tryoni* were more responsive to cue-lure and zingerone than the offspring of control flies; interestingly, the number of lure responsive offspring from zingerone fed *B. tryoni* was not significantly different from the control, the number was however intermediate between cue-lure and control

offspring. On the other hand, Shelly (2000b) did not find significant differences in fecundity, fertility (successful egg hatches), or longevity in *B. dorsalis* females mated with methyl eugenol fed males. Shelly (2021) also found no significant differences in the proportion of lure responsive flies in the offspring of methyl eugenol fed *B. dorsalis*. Like *B. dorsalis*, *Z. cucurbitae* females mated with cue-lure fed males demonstrated no significant differences in fecundity or longevity, but fertility was significantly reduced (Shelly & Nishimoto, 2016). Whereas *Z. cucurbitae* females mated with zingerone fed males demonstrated no significant differences in fecundity or fertility, but longevity was reduced (Inskeep et al., 2019). These studies in *B. tryoni*, *B. dorsalis*, and *Z. cucurbitae* have demonstrated that: i) female and/or offspring benefits (increased fecundity and lure response) have only been observed in *B. tryoni*, ii) adverse female effects (reduced longevity or fertility) were observed in *B. tryoni* and *Z. cucurbitae*, and iii) these effects are not necessarily consistent across lure types.

1.5 PROJECT AIMS

The biological effects of male lures in fruit flies are complex, varying between and within species and lure types. Consequently, the mechanisms underlying these complexities are not well understood. This project sought to further explore these effects through a transcriptomics approach to broaden the understanding of the genetic mechanisms involved in the fruit fly male lure response. Transcriptomics is an underused approach in male lure research that can provide information regarding the cellular processes affected by male lures in fruit flies. Moreover, this approach can be used to identify candidate genes for further exploration and potential applications in pest management strategies. This project was partitioned into two studies:

The objective of study one was to investigate differences in gene expression between two species of fruit flies: *B. tryoni* and *B. jarvisi*; and two male lures: cue-lure and zingerone. These species were chosen as they exhibit inverse attraction to the lures, in that *B. tryoni* are strongly attracted to cue-lure but weakly attracted to zingerone, and the reverse is true for *B. jarvisi*. This study aimed to identify differentially expressed genes between the treatments. If differentially expressed genes were identified, the study aimed to compare and contrast gene expression between the two species, between the two lure types, and between the two levels of attraction (strongly and weakly responsive). Additionally, the study aimed to identify specific genes of interest, including those related to sensory functions.

The objective of study two was to extend on previous research by Kumaran and Clarke (2014) and investigate gene expression in cue-lure fed *B. tryoni* and their offspring. This study aimed to identify differentially expressed genes between cue-lure and control flies in the parental and offspring generation. If differentially expressed genes were identified, the study aimed to identify consistent patterns of differential gene expression in *B. tryoni* fed cue-lure and their offspring, and compare and

contrast differential gene expression between the generations. Additionally, the study aimed to identify specific genes of interest, including those related to sensory functions.

Chapter 2: Methods

2.1 STUDY 1: INVESTIGATING THE EFFECTS OF CUE-LURE AND ZINGERONE ON GENE EXPRESSION IN *B. TRYONI* AND *B. JARVISI*

2.1.1 Insects

Bactrocera tryoni pupae (10th generation) and *B. jarvisi* pupae (18th generation) were obtained from a laboratory-reared culture maintained by the Department of Agriculture and Fisheries from Brisbane and Cairns, Queensland, respectively. The pupae were moved into a mesh sleeved cage (BugDorm-4F3030, 32.5 x 32.5 x 32.5 cm, MegaView Science Co., Ltd., Taiwan) to emerge and were provided with water, sugar cubes (CSR® White Sugar Cubes, Australia), and hydrolysed yeast (MP Biomedicals, CAT 103304, USA) as required. The cage was kept in a dedicated laboratory maintained at 25.5 °C and 65 % RH with natural light and fluorescent lighting between 0700 and 1800 hours. Emerged F10 *B. tryoni* were used directly for sampling, whereas F18 *B. jarvisi* were reared for 3 generations before sampling. Rearing followed the protocol of Heather and Corcoran (1985) with some modifications. The carrot media used for rearing was made with carrot granules (H. J. Langdon, USA), water, torula yeast (H. J. Langdon, USA), hydrochloric acid, and nipagin (Sigma-Aldrich, CAS 99-76-3, India). When the flies were sexually mature (≥ 14 days old), small pin holed containers filled with carrot media were placed in the cage for females to oviposit. The egging containers were removed after ≤ 3 days and the contents were transferred into a cup with additional carrot media. The cup was kept in a larger container with vermiculite and covered with a mesh lid. Once the larvae had pupated in the vermiculite (~ 1 week), the vermiculite was sieved, and pupae were transferred into a mesh cage with water, sugar cubes, and hydrolysed yeast. For male lure feeding, flies were sex separated into new mesh cages after ≤ 4 days of emergence to eliminate the bias of previous matings on lure response.

2.1.2 Chemicals

Analytical standard cue-lure (4-(4-Acetoxyphenyl)-2-butanone) ≥ 95 % purity (Sigma-Aldrich, CAS 3572-06-3, Germany) and analytical standard zingerone (4-(4-hydroxy-3-methoxyphenyl)-2-butanone) ≥ 98 % purity (Sigma-Aldrich, CAS 122-48-5, China) were diluted stepwise with absolute ethanol (≥ 99.5% purity, Sigma-Aldrich, CAS 64-17-5) to achieve concentrations of 500 ng/µL and 400 ng/µL, respectively. The concentration of cue-lure was selected based on preliminary trials (Appendix A) to achieve approximately 50 % *B. tryoni* response and the concentration of zingerone was selected based on Wee et al. (2018c) to achieve approximately 50 % of *B. jarvisi* response. Additionally, considerations were made so the lure concentration would elicit feeding in the weakly responsive species, and that the

concentration would represent, to a limited extent, the amount naturally available to the fruit flies (Park et al., 2020).

2.1.3 Male lure feeding

The approach used for male lure feeding was based on Wee et al. (2002), Wee et al. (2018c), and Wee and Clarke (2020) with similar fruit fly experiments. Lure feeding was conducted in a shaded outdoor area during late December and early January (summer) in Brisbane, Queensland, and performed under clear weather. Sexually mature virgin male *B. tryoni* (17 – 20 days old) and *B. jarvisi* (16 – 20 days old) were used for lure feeding. The species were fed lure on different days between 0830 – 1130 hours. For both species flies were either fed cue-lure or zingerone, cue-lure feeding was conducted first on the day followed by zingerone feeding to avoid cross-contamination. Fly colonies were moved from the laboratory to outside (separate area from experimental site) one hour prior for acclimatisation to the environment. Flies were transferred from the main male colony into mesh cages (32 x 32 x 32 cm) at the experimental site in 10 mL clear tubes and allowed five minutes to exit the tube before being prompted. Lure feeding was conducted with 10 flies in a cage, two cages were set up to allow 20 flies to be fed at once. In each cage, an inverted container was placed in the centre of the cage floor, 10 µL of cue-lure or zingerone was pipetted onto a glass slide and given 30 seconds for solvent evaporation before being placed atop the container. Flies were allowed one instance of lure feeding, that is, they were allowed to feed for any duration and were carefully captured with a tube when they walked or flew off the glass slide (no repeat feeding). This ensured the flies used for RNA extraction had been observed to feed on the lure, rather than assuming. Captured lure fed flies were transferred into a separate mesh cage. If flies had stopped approaching the lure after 10 minutes, the remaining nonresponsive flies were removed, and the feeding process was repeated with new flies. Flies that were still feeding were allowed to continue (no flies remained after 20 minutes). This process was repeated until there were ≥ 20 lure fed flies for each lure type. New cages were used between cue-lure and zingerone feeding, a new glass slide was used for every cage and the inverted container was cleaned with 70 % ethanol between different lures. Control flies were collected from the same fly colonies that treated flies were sampled from to ensure they were subject to the same handling and environmental exposure.

2.1.4 Sampling

Flies were sampled seven hours after feeding for each male lure, control flies (no lure exposure) from the main male colony were sampled at the same times. Sampled flies were sedated at – 20 °C for 10 minutes, afterwards, the flies were pierced with entomological tweezers and added to sample tubes with

RNA*later*® (CAS 7783-20-2) stabilisation solution and stored at – 80 °C until required for RNA extraction.

2.1.5 Sample preparation and sequencing

Total RNA was extracted from fly heads to allow gene expression patterns of the brain, antennae, maxillary palps, mouthparts, and other sensory organs to be investigated. For each species, four fly heads were pooled together for each sample, and each treatment (cue-lure fed, zingerone fed, and control) had three replicate pools. Tissue was homogenised using a TissueLyser II (Qiagen, CAT 85300) with 1mL of TRIzol™ reagent (Invitrogen, CAT 15596026). Total RNA was extracted using TRIzol™ Reagent following the manufacturers protocol. RNA quantity and quality was assessed using agarose gel electrophoresis, NanoDrop™ (Thermo Scientific, CAT ND-2000), Qubit™ RNA High sensitivity assay (Invitrogen, CAT Q32851), and the Fragment Analzyer System (5200, Agilent). Due to low 260/230 NanoDrop™ results, three samples were cleaned using RNA Clean & Concentrator kit (Zymo Research, CAT R1013) and a Qubit™ assay was performed again. Library preparation was performed on extracted total RNA using the Stranded mRNA Prep, Ligation kit (Illumina, CAT 20040534) which uses poly-A tail enrichment to capture mRNAs. Library preparation was conducted as per the manufacturers protocol (1000000124518 v02), with an additional 0.8 X AMPure XP bead clean up at the final Clean Up Library stage. Libraries were sequenced with 100 bp paired end sequencing on a NovaSeq 6000 (Illumina).

2.1.6 Transcriptome assembly and functional annotation

De novo transcriptome assembly was performed for *B. tryoni* and *B. jarvisi* as there was no publicly available *B. jarvisi* genome suitable for gene mapping. Initial quality control results were provided by BaseSpace (Illumina) when retrieving the data. The quality of raw reads was evaluated using FastQC v0.11.7 (Andrews, 2010), low quality reads ($Q < 30$) and adapters were removed using Trimmomatic v0.39 (Bolger et al., 2014) and the quality of the remaining reads ($Q > 30$) was confirmed with FastQC. Filtered reads were assembled into contiguous sequences (contigs) separately for *B. jarvisi* and *B. tryoni* using the Trinity short read *de novo* assembler v2.13.2 (Haas et al., 2013). To remove redundant contigs from the transcriptome assemblies, cd-hit-est from CD-HIT v4.8.1 (Fu et al., 2012) was used to cluster contigs ≥ 95 % similarity. Assembly statistics were calculated using the Trinity TrinityStats.pl and contig_ExN50_statistic.pl Perl scripts. BUSCO v4.0.5 (Manni et al., 2021) was used to assess the completeness of the transcriptomes to Diptera and Metazoa lineages (diptera_odb10 and metazoan_odb10). Transcripts were queried against the UniRef90 database (Suzek et al., 2015; downloaded 01/06/2022) using MMseqs2 Release 13-45111 (Steinegger & Söding, 2017). Annotated transcripts were filtered for the top 10 hits with an E-value threshold of 1e-5 and associated Gene

Ontology (GO) terms were retrieved for the best hit (based on UniProt idmapping.selected.tab) using a custom script (https://github.com/zkstewart/Genome_analysis_scripts/blob/master/annotation_table/sub_annot_pipeline.sh, accessed February 2023). Ancestral GO terms for the best hits were retrieved with the Python library GOATOOLS (Klopfenstein et al., 2018) using the custom script.

2.1.7 Differential gene expression analysis

Differential gene expression (DGE) analysis was conducted following the Trinity pipeline using Trinity scripts (https://github.com/trinityrnaseq/trinityrnaseq/wiki, Haas et al., 2013). RSEM v1.3.3 (Li & Dewey, 2011) was used for transcript quantification which aligned reads to assembled contigs using Bowtie 2 (Langmead & Salzberg, 2012) and calculated count matrices and TMM normalised expression libraries. Pairwise differential gene expression was calculated from the RSEM count matrices using the DESeq2 v1.26.0 R package (Love et al., 2014). Differentially expressed genes (DEGs) where the p-value < 0.05 were extracted and clustered using TMM normalised expression. Trinity Perl-to-R scripts (https://github.com/trinityrnaseq/trinityrnaseq/wiki/QC-Samples-and-Biological-Replicates, accessed February 2023) were used to generate initial DGE heatmaps, principal component analysis (PCA) plots and sample correlation matrices. DGE was initially visualised using centred log2 fold change to observe the absolute expression of genes (parameters: --heatmap --log2 --min_colSums 0 --min_rowSums 0 --gene_dist euclidean --sample_dist euclidean --center_rows – heatmap_scale_limits "-2,2"). Abnormally high expression was observed in the initial DGE heatmap without heat map scale limits for *B. tryoni* and this group of transcripts all had a functional annotation with NCBI:txid2795009 = *B. tryoni* iflavirus 1. Due to the contamination of iflavirus, all transcripts with 2795009 taxon ID were removed from the transcriptome, and the DGE analysis was repeated.

2.1.8 Subcluster and gene ontology analysis

Subclusters were generated from the extracted DEGs by cutting the hierarchically clustered tree at 40 % of its height. GO enrichment analysis was performed using GOseq v1.38.0 R package (Young et al., 2010) on the differentially expressed genes and subclusters with clear patterns across replicates, an FDR value < 0.05 was used to determine significance. REVIGO (Supek et al., 2011) was used with SimRel semantic similarity (0.5) to cluster and reduce redundant GO terms.

2.1.9 Annotated differentially expressed genes of interest

To improve the annotation of specific genes of interest, differentially expressed transcripts (including the genes annotated with NCBI:txid2795009 = *B. tryoni* iflavirus 1) were queried against the NCBI nr

database (Sayers et al., 2023; downloaded 18/02/2023) using blastx from BLAST+ v2.13.0 (Camacho et al., 2009). The isoform with the highest abundance was selected to represent the gene. Best hits from both databases were used to inform gene annotations; where the UniRef90 annotation was non-specific or absent, best hits to the nr database were used. The DEGs were searched for genes of interest, in particular potential sensory-related and immune-related genes. The expression of genes of interest was visualised using pheatmap v1.0.12 R package (Kolde, 2022) to create heatmaps of log2 transformed values with Z scaling applied to each gene. The average expression visualised in the gene heatmaps was calculated with the average normalised TMM value across replicates before log2 transformation.

2.2 STUDY 2: INVESTIGATING THE EFFECTS OF CUE-LURE FEEDING ON GENE EXPRESSION ACROSS TWO GENERATIONS IN *B. TRYONI*

2.2.1 Insects

Bactrocera tryoni pupae (3rd generation) were obtained from a laboratory-reared culture maintained by the Department of Agriculture and Fisheries from Brisbane, Queensland. The pupae were moved into a mesh sleeved cage (BugDorm-4F3030, 32.5 x 32.5 x 32.5 cm, MegaView Science Co., Ltd., Taiwan) to emerge and were provided with water, sugar cubes (CSR® White Sugar Cubes, Australia), and hydrolysed yeast (MP Biomedicals, CAT 103304, USA) as required. The cage was kept in a dedicated laboratory maintained at 25.5 °C and 65 % RH with natural light and fluorescent lighting between 0600 and 1700 hours. Flies were sex separated into new mesh cages after ≤ 5 days of emergence to eliminate the bias of previous matings on lure response.

2.2.2 Chemicals

Analytical standard cue-lure (4-(4-Acetoxyphenyl)-2-butanone) ≥ 95 % purity (Sigma-Aldrich, CAS 3572-06-3, Germany) was diluted stepwise with absolute ethanol (≥ 99.5% purity, Sigma-Aldrich, CAS 64-17-5) to achieve a concentration of 500 ng/µL. The concentration of cue-lure was selected based on preliminary trials (Appendix A) to achieve approximately 50 % *B. tryoni* response and to represent, to a limited extent, the amount naturally available to the fruit flies (Park et al., 2020).

2.2.3 Male lure feeding

The approach used for male lure feeding is similar to the protocol outlined in Section 2.1.2, based on Wee et al. (2002), Wee et al. (2018c), and Wee and Clarke (2020) with similar fruit fly experiments. Lure feeding was conducted in a shaded outdoor area during mid-November (spring). Sexually mature

virgin male *B. tryoni* (F3, 17 – 19 days old) were fed between 0800 – 1700 hours. The male *B. tryoni* colony was moved from the laboratory 30 mins prior for acclimatisation to the environment (separate area from experimental site). Flies were transferred from the main male colony into mesh cages (32 x 32 x 32 cm) in 10 mL clear tubes and allowed five minutes to exit the tube before being prompted. Lure feeding was conducted with 10 flies in a cage, two cages were set up to allow 20 flies to be fed at once. In each cage, an inverted container was placed on the centre of the cage floor, 10 µL of 500 ng/uL cue-lure was pipetted onto a glass slide and given 30 seconds for solvent evaporation before being placed atop the container. Flies were allowed one instance of feeding, that is, they were allowed to feed for any duration and were carefully captured with a tube when they walked or flew off the glass slide (no repeat feeding). This ensured the flies used for RNA extraction had been observed to feed on the lure, rather than assuming. Captured lure fed flies were transferred into a separate mesh cage. If flies had stopped approaching the lure after 10 minutes, the remaining nonresponsive flies were removed, and the feeding process was repeated with new flies. Flies that were still feeding were allowed to continue (no flies remained after 20 minutes). This process was repeated until there were > 70 lure fed flies. Lure feeding experiments were completed at 1700 hours, and the lure fed flies and main male colony were moved back into the laboratory. During lure feeding, a new glass slide was used for every cage and the inverted container was cleaned with 70 % ethanol.

2.2.4 Fly rearing

The photoperiod and light source for fly rearing was the same as outlined in section 2.2.1. Male flies were transferred into female cages 24 hours after lure exposure (1700 hours) due to the peak of increased male mating success in cue-lure fed flies one day after feeding (Kumaran et al., 2013). Forty lure fed males and 40 control males were released into separate cages containing 110 sexually mature virgin female flies (17 – 19 days old). The next day (0930 hours) small containers filled with carrot media with pinholes were placed in the cage for females to oviposit. The egging containers were removed the next day at 1700 hours; additional egging containers were added at 1730 hours and removed the following day at 1700 hours to ensure there would be enough eggs. Once egging containers were removed from the lure fed and control fly cages, the contents were transferred into separate cups with additional carrot media. The cups were kept in separate larger containers with vermiculite and covered with a mesh lid. The vermiculite was sieved once larvae had pupated (~ 1 – 2 weeks) and pupae were transferred into separate mesh cages with water, sugar cubes and hydrolysed yeast provided. Emerged male flies were separated into new mesh cages after 4 days of emergence to keep conditions consistent between the generations. When the first generation (F1) flies were 15 – 20 days old, they were moved outside between 0800 – 1700 hours to replicate the conditions of the parental generation.

2.2.5 Sampling

The parental generation of male flies were sampled 24 hours after lure exposure (1700 hours) at 18 – 20 days old. Twelve flies were sampled for both lure fed and control treatments. The F1 generation were sampled when 16 – 21 days old at 1700 hours. Twelve male flies were sampled for the sons of lure fed and control treatments. For both generations, sampled flies were sedated at – 20 °C for 10 minutes, afterwards, the flies were pierced with entomological tweezers and added to sample tubes with RNA*later*® (CAS 7783-20-2) stabilisation solution and stored at – 80 °C until required for RNA extraction.

2.2.6 Sample preparation and sequencing

Total RNA was extracted from fly heads to allow gene expression patterns of the brain, antennae, maxillary palps, mouthparts, and other sensory organs to be investigated. Four fly heads were pooled together for each sample and each treatment (lure fed, control, lure fed sons, and control sons) had three replicate pools. Tissue was homogenised using a TissueLyser II (Qiagen; CAT 85300) with 1mL of TRIzol™ reagent (Invitrogen; CAT 15596026). Total RNA was extracted using TRIzol™ Reagent following the manufacturers protocol. RNA quantity and quality was assessed using agarose gel electrophoresis, NanoDrop™ (2000; Thermo Scientific; CAT ND-2000), Qubit™ RNA High sensitivity assay (Invitrogen; CAT Q32851), and the Fragment Analzyer System (Agilent 5200). Library preparation was performed on extracted total RNA using the Stranded mRNA Prep, Ligation kit (Illumina; CAT 20040534) which uses poly-A tail enrichment to capture mRNAs. Library preparation was conducted as per the manufacturers protocol (1000000124518 v02), with an additional 0.8 X AMPure XP bead clean up at the final Clean Up Library stage.

2.2.7 Read mapping and functional annotation

Genome-guided transcriptome assembly was performed using the publicly available *B. tryoni* genome and gene models (CSIRO_BtryS06_freeze2; accession GCA_016617805.2) from NCBI GenBank. Low quality reads (Q < 30) and adapters were removed from the raw reads using Trimmomatic v0.36 (Bolger et al., 2014). Filtered reads were aligned to CDS *B. tryoni* gene models (including all alternative isoforms) using a custom script (DEW; https://github.com/alpapan/DEW; commit 3fb77e5). DEW was configured to use the kanga aligner from BioKanga (https://github.com/csiro-crop-informatics/biokanga; commit 57f94e3). The script produced read counts associated with genes using the samtools idxstats function (Li et al., 2009) and isoform read counts with a shared locus were summed to provide a single locus-level count. Gene models were queried against the UniRef90 database (Suzek et al., 2015; version 2021_04) using MMseqs2 Release 13-45111 (Steinegger & Söding, 2017). Annotated transcripts were filtered for the top 10 hits with an E-value threshold of 1e-5 and associated

Gene Ontology (GO) terms were retrieved for the best hit (based on UniProt idmapping.selected.tab) using a custom script (https://github.com/zkstewart/Genome_analysis_scripts/blob/master/annotation_table/sub_annot_pipeline.sh, accessed February 2023). Ancestral GO terms for the best hits were also retrieved with the Python library GOATOOLS (Klopfenstein et al., 2018) using the custom script. Due to the identification of iflavirus in the *B. tryoni* transcriptome in Study 1, a *de novo* transcriptome assembly was conducted using the methods outlined in Section 2.1.6, and DGE was analysed for generation one and heatmaps were created using the methods outlined in Section 2.1.7. Additionally, the genes were queried against the NCBI nr database using the methods outlined in Section 2.1.9.

2.2.8 Differential gene expression analysis

Genes with low abundance (normalised read count < 10 across 2 samples) were removed prior to analysis. Differential gene expression was analysed using the DESeq2 v1.26.0 R package (Love et al., 2014), significance was defined as an adjusted p-value < 0.05.

2.2.9 Gene ontology analysis

GO analysis was performed using GOseq v1.38.0 R package (Young et al., 2010) on differentially expressed genes, an FDR value < 0.05 was used to determine significance. REVIGO (Supek et al., 2011) was used with SimRel semantic similarity (0.5) to cluster and reduce redundant GO terms. REVIGO was also used to create a semantic similarity plot by exporting the scatterplot R script and the figure was further adjusted in R using ggplot2 (Wickham et al., 2016).

2.2.10 Differentially expressed genes of interest

The expression of genes of interest was visualised using pheatmap v1.0.12 R package (Kolde & Kolde, 2018) to create heatmaps of log2 transformed values with Z scaling applied to each gene. The average expression visualised in the gene heatmaps was calculated with the average median of ratios value across replicates before log2 transformation. Boxplots of individual gene expression were created using ggplot2 (Wickham et al., 2016). The DEGs were searched for potential sensory-related genes (GO:0005549 odorant binding, and GO:0007600 sensory perception), immune-related genes (GO:0002376 immune system process), and transposition-related genes.

Chapter 3: Results

3.1 STUDY 1: INVESTIGATING THE EFFECTS OF CUE-LURE AND ZINGERONE ON GENE EXPRESSION IN *B. TRYONI* AND *B. JARVISI*

3.1.1 Transcriptome assembly and functional annotation

The number of reads sequenced across samples for *B. tryoni* ranged from 30,230,808 to 58,947,409 ($\bar{x}$ = 45,611,139, σ = 9,090,103) and for *B. jarvisi* from 40,954,984 to 53,394,291 ($\bar{x}$ = 45,633,821, σ = 3,773,307). Initial BaseSpace (Illumina) quality control results demonstrated that 91.3% of all reads were ≥ Q30. After read trimming there was an average read loss of 3.77×10^{-2} % for *B. tryoni* and 2.69×10^{-2} % for *B. jarvisi* (see Supplementary Table A 1 for the full list of read counts). The assembled transcriptomes were comparable in gene count, N50, E90N50 and E90 transcript counts (Table 1). BUSCO completeness scores for Metazoa and Diptera lineages were 98.7% and 92.5% for *B. tryoni* and 98.7% and 92.3% for *B. jarvisi*, respectively. The percent of transcripts that retrieved a functional annotation was 47.58% for *B. tryoni* and 44.7% for *B. jarvisi*.

Table 1. Transcriptome assembly statistics for *Bactrocera tryoni* and *B. jarvisi*.

	B. tryoni	***B. jarvisi***
Transcript count	129687	140039
Gene count	90008	93614
N50[1]	1555	1415
E90N50[2]	2565	2508
E90 transcripts	5669	5959

[1] Based on longest isoform per gene. [2] N50 calculation based on the highly expressed transcripts.

3.1.2 Differential gene expression analysis

The heatmap generated from all DEGs in the assembled *B. tryoni* transcriptome revealed a group of 30 genes with consistent extreme expression (Figure 1). The group of genes were annotated as *B. tryoni* iflavirus 1 (NCBI:txid2795009) genome polyprotein. These genes were mostly upregulated in cue-lure fed flies and one replicate of control flies compared to zingerone fed flies and the other control flies. In total 52 transcripts with hits to NCBI:txid2795009 were removed from the *B. tryoni* transcriptome to repeat DGE analysis without contamination. After the removal of *B. tryoni* iflavirus 1 transcripts, a total of 500 genes were found differentially expressed across all treatments. Pairwise DGE analysis revealed

262 genes differentially expressed between cue-lure fed flies and control flies (145 upregulated and 117 downregulated in cue-lure fed flies), 238 between zingerone fed and control flies (134 upregulated and 105 downregulated in zingerone fed flies), and 101 between the lure treatments. The sample correlation plot (Supplementary Figure A 3) showed that replicates mostly clustered together without overlap with other treatments, indicating consistent replicates.

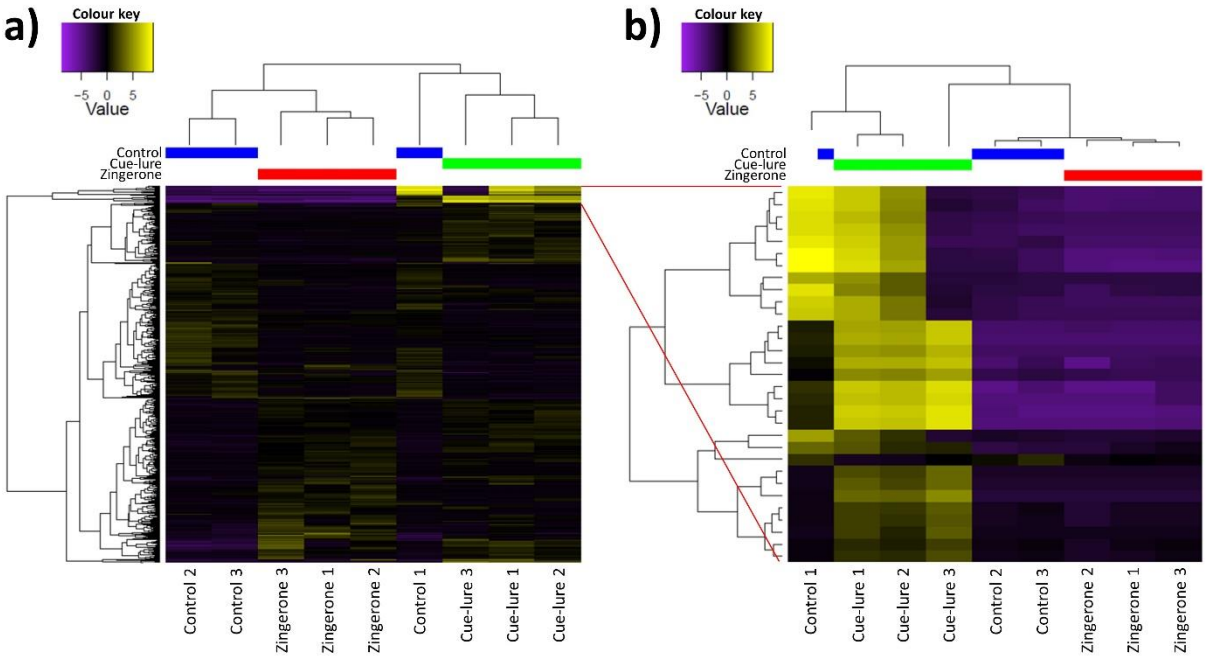

Figure 1. Heatmaps of differentially expressed genes in the *Bactrocera tryoni* transcriptome without scale limits (log2 centred TMM values; treatments = zingerone feeding, cue-lure feeding, control; replicates = 1, 2, 3) **a)** Heatmap of all differentially expressed genes (500 genes). **b)** Heatmap of extracted differentially expressed genes with NCBI:txid2795009 = *B. tryoni* iflavirus 1 (30 genes).

In the *B. jarvisi* assembled transcriptome, a total of 466 genes were found differentially expressed across all treatments. Pairwise DGE analysis calculated 159 genes differentially expressed between cue-lure fed flies and control flies (93 upregulated and 66 downregulated in cue-lure fed flies), 176 between zingerone fed flies and control flies (100 upregulated and 76 downregulated in zingerone fed flies), and 196 between the lure treatments. The sample correlation plot (Supplementary Figure A 4) demonstrated that replicates mostly clustered together without overlap with other treatments, indicating consistent replicates. Note that 12 transcripts annotated as *B. tryoni* iflavirus 1 (NCBI:txid2795009) genome polyprotein were also found in the *B. jarvisi* transcriptome, however they were not differentially expressed therefore removal was not necessary.

3.1.3 Subcluster and gene ontology analysis

Ten subclusters (> 5 transcripts) were generated from the *B. tryoni* heatmap of DEGs with five subclusters demonstrating generally consistent mean expression across replicates (subclusters 5, 6, 7, 9, 10; Figure 2). However, individual gene expression profiles highlight variation in some genes between replicates. GO analysis for the DEGs between zingerone fed flies and control flies revealed some enriched GO terms (with FDR < 0.05) associated with response to stimulus, immune system process, and interspecies interaction between organisms (Table 2). For DEGs between cue-lure fed and control flies, between the lure treatments and in the subclusters, no GO terms were significantly enriched; the full list of enriched GO terms including results with FDR > 0.05 is available in Supplementary Tables A 2, 3, 4 and 5.

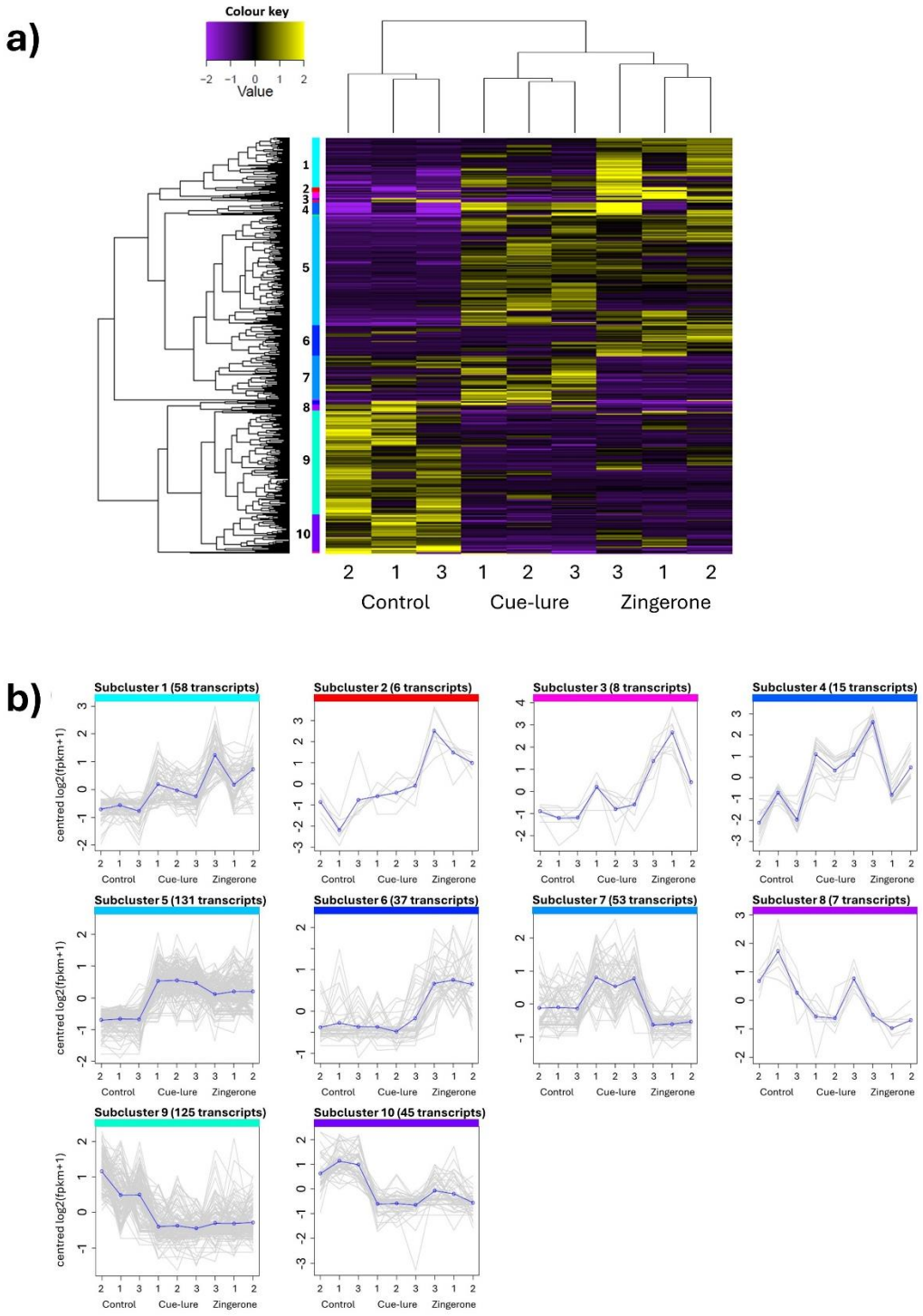

Figure 2. Heatmap of differentially expressed genes and subcluster analysis in *Bactrocera tryoni* (treatments = zingerone feeding, cue-lure feeding, control; replicates = 1, 2, 3) excluding genes with NCBI:txid2795009 = *B. tryoni* iflavirus 1. **a)** Heatmap of differentially expressed genes (500 genes) with -2, 2 scale limits (log2 centred TMM values). **b)** Subclusters (transcript count > 5) extracted from differentially expressed genes. The trendlines of mean expression profiles are blue and individual gene expression is grey.

Table 2. Gene ontology enrichment analysis on differentially expressed genes in *Bactrocera tryoni* zingerone fed flies vs control flies (reduced with REVIGO; FDR < 0.05).

Enriched GO terms			
Ontology	ID	Term	p-value
BP	GO:0006952	defence response	8.61×10^{-6}
BP	GO:0006959	humoral immune response	3.71×10^{-7}
BP	GO:0009605	response to external stimulus	6.04×10^{-5}
BP	GO:0009607	response to biotic stimulus	8.03×10^{-6}
BP	GO:0009617	response to bacterium	2.89×10^{-7}
BP	GO:0044419	biological process involved in interspecies interaction between organisms	7.73×10^{-6}

BP = biological process

Four subclusters (> 5 transcripts) were generated from the *B. jarvisi* heatmap of DEGs with generally consistent mean expression across replicates (Figure 3); notably, individual gene expression profiles highlight variation in some genes between replicates. GO analysis on the DEGs and subclusters revealed no GO terms significantly enriched with FDR < 0.05; the full list of enriched GO terms including results with FDR > 0.05 is available in Supplementary Tables A 6, 7, 8 and 9.

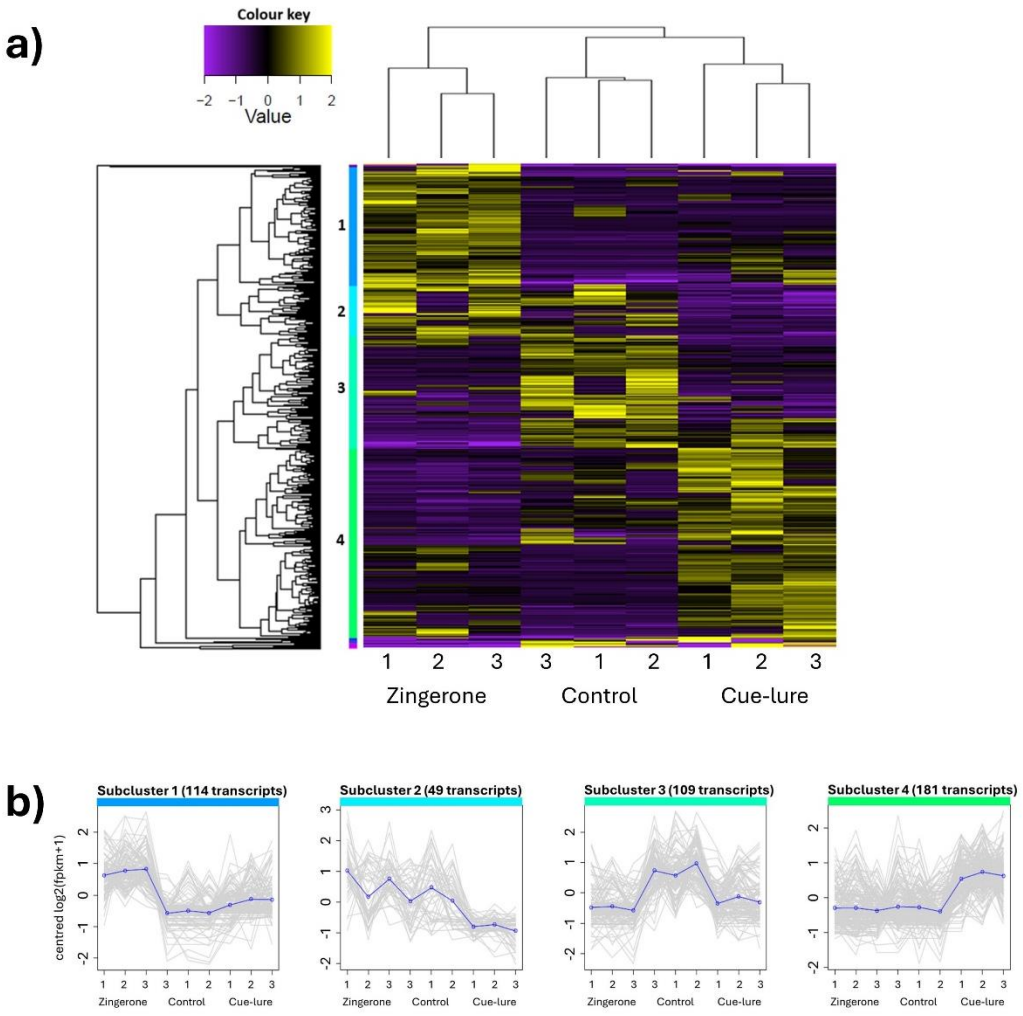

Figure 3. Heatmap of differentially expressed genes and subcluster analysis in *Bactrocera jarvisi* (treatments = zingerone feeding, cue-lure feeding, control; replicates = 1, 2, 3). **a)** Heatmap of differentially expressed genes (466 genes) with -2, 2 scale limits (log2 centred TMM values). **b)** Subclusters (transcript count > 5) extracted from differentially expressed genes. The trendlines of mean expression profiles are blue and individual gene expression is grey.

3.1.4 Annotated differentially expressed genes of interest

To expand the annotation coverage for genes of interest, differentially expressed transcripts were searched against the NCBI non-redundant protein (nr) database in addition to the whole transcriptome annotation to the UniRef90 database. Best hits from both databases were used to inform gene annotations; where the UniRef90 annotation was non-specific or absent, best hits to the nr database were used. Annotation coverage for DEGs increased from 42.8 % (UniRef90) to 47.8 % (UniRef90 and nr) for *B. tryoni* and from 35.19 % (UniRef90) to 41.2 % (UniRef90 and nr) for *B. jarvisi*. For the NCBI hits for genes annotated with NCBI:txid2795009 (*B. tryoni* iflavirus 1 genome polyprotein) in both species, see Supplementary Table A 10. The DEGs and corresponding proteins identified by Kumaran

et al. (2014b) in intermale aggression, pheromone, courtship, odorant binding protein, reproduction, and longevity gene groups were searched for in the annotated DEGs of the present study. One protein was identified that was consistently differentially expressed across the studies: odorant binding protein (OBP) 56a. Kumaran et al. (2014b) identified OBP 56a (*Obp56a* gene) upregulated in zingerone fed flies compared to control flies in *B. tryoni*. Interestingly, in the present study, general OBP 56a-like was found downregulated in zingerone fed flies compared to control flies in *B. tryoni*. No other genes (or proteins) from the gene groups of interest were identified in the *B. tryoni* or *B. jarvisi* annotated DEGs.

To investigate genes possibly involved in male lure perception, annotated DEGs were searched for sensory-related protein hits. In *B. tryoni* five potential sensory-related genes were identified: two odorant binding proteins (OBPs), one gustatory receptor (GR), and two proteins possibly involved in visual perception (Phosrestin-2 and opsin Rh4) (Table 3, Figure 4a). General OBP 99a-like was upregulated in zingerone and downregulated in cue-lure fed flies, but not significantly differentially expressed in either male lure treatment compared to the control. Whereas general OBP 56a-like was downregulated in zingerone fed flies compared to the control. Gustatory receptor for sugar taste 64b was downregulated in both male lure treatments compared to the control. Phosrestin-2 was upregulated in both male lure treatments compared to the control and opsin Rh4 was upregulated only in cue-lure fed flies compared to the control.

In *B. jarvisi* five potential sensory-related genes were also identified: one OBP, two odorant receptors (ORs), and two proteins possibly involved in visual perception (retinin-like and opsin Rh1) (Table 3, Figure 4b). General OBP 99a-like was upregulated in cue-lure fed flies compared to the control. Both genes with hits to odorant receptor 7a-like were upregulated in cue-lure and downregulated in zingerone fed flies, but not significantly differentially expressed in either male lure treatment compared to the control. Retinin-like was downregulated in cue-lure fed flies compared to the control and opsin Rh1 was downregulated in zingerone fed flies compared to cue-lure fed and control flies.

Table 3. Sensory-related genes differentially expressed in *Bactrocera tryoni* and *B. jarvisi* transcriptomes (treatments = zingerone feeding, cue-lure feeding, control). The best hit annotation to the most abundant isoform was selected to represent the gene. Annotation was performed against Uniref90 and NCBI nr databases.

Bactrocera tryoni						
Protein name	Accession	E-value	Identity %	Length bp	Taxon	Transcript ID
Gustatory receptor for sugar taste 64b	XP_049313798.1*	5.77E-24	94.231	52	*Bactrocera dorsalis* 27457	TRINITY_DN52032_c0_g1_i1
General OBP 56a-like	A0A0K8VDV4	2.063E-104	97.6	495	Dacini 43871	TRINITY_DN481_c0_g1_i10
General OBP 99a-like	XP_050326260.1*	4.05E-69	87.5	128	*Bactrocera neohumeralis* 98809	TRINITY_DN19949_c0_g1_i2
Phosrestin-2	A0A034W3M3	7.070E-13	90.4	1089	Acalyptratae 43741	TRINITY_DN48560_c0_g1_i6
Opsin Rh4	A0A034VX19	1.666E-17	96.3	546	*Rhagoletis* 28609	TRINITY_DN23366_c4_g7_i1
Bactrocera jarvisi						
Protein name	Accession	E-value	Identity %	Length bp	Taxon	Transcript ID
Retinin-like	XP_039971357.1*	4.23E-54	96.226	159	*Bactrocera tryoni* 59916	TRINITY_DN19793_c0_g2_i3
General OBP 99a-like	XP_039947469.1*	1.11E-56	75.806	124	*Bactrocera tryoni* 59916	TRINITY_DN40641_c0_g1_i3
Opsin Rh1	P06002	9.750E-83	84.9	1119	Cellular organisms 131567	TRINITY_DN10620_c28_g1_i1
OR 7a-like	XP_050327913.1*	4.46E-114	97.076	171	*Bactrocera neohumeralis* 98809	TRINITY_DN11855_c0_g1_i7
OR 7a-like	XP_050331452.1*	0	86.75	400	*Bactrocera neohumeralis* 98809	TRINITY_DN9433_c0_g1_i2

OBP = odorant binding protein. OR = odorant receptor. * Best hit from the NCBI nr database. Taxon contains the classification of the best match and the corresponding NCBI taxon code.

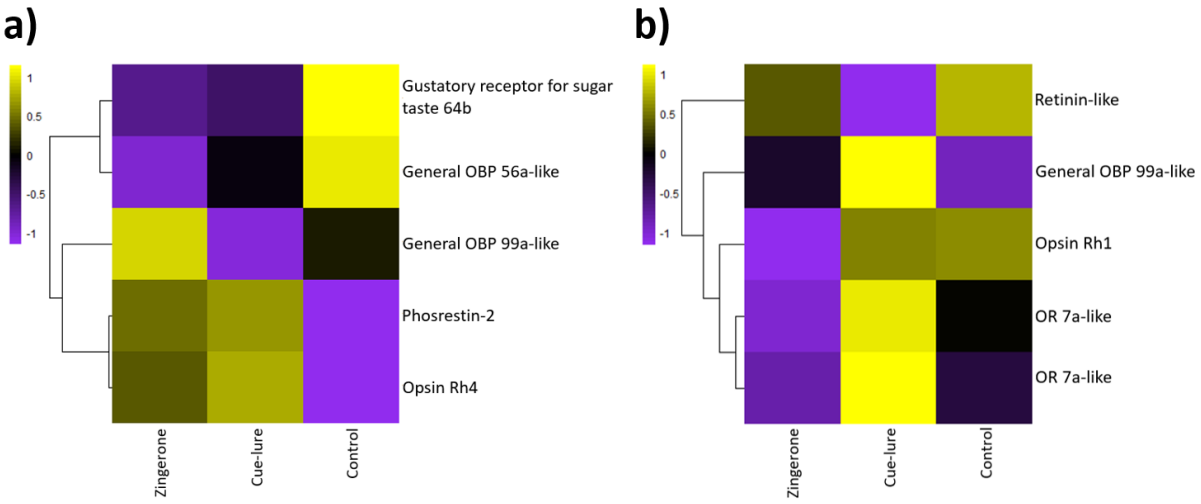

Figure 4. Heatmaps of sensory-related differentially expressed genes (log2, z-scaled TMM values averaged across replicates). **a)** Differentially expressed genes in the *Bactrocera tryoni* transcriptome. **b)** Differentially expressed genes in the *B. jarvisi* transcriptome. OBP = odorant binding protein. OR = odorant receptor.

In addition to sensory-related proteins, annotated DEGs were searched for immune-related proteins due to the enrichment of immune-related GO terms in zingerone fed *B. tryoni* (Table 2). In *B. tryoni* seven potential immune-related genes were identified: attacin-A, three cecropin-1-like proteins, protein spaetzle 3, lysozyme B-like, and an immunoglobulin-like protein (Table 4, Figure 5a). Notably, the first three proteins listed exhibit a general pattern of strong upregulation in zingerone and weaker upregulation in cue-lure as shown in Figure 5a. Attacin-A and two cecropin-1 genes were upregulated in zingerone fed flies compared to cue-lure fed and control flies. Protein spaetzle 3 and one cecropin-1 gene were upregulated in zingerone fed flies compared to the control. Lysozyme B-like was downregulated in zingerone fed flies compared to the control and the immunoglobulin-like protein was downregulated in cue-lure fed flies compared to the control. In *B. jarvisi*, seven potential immune-related genes were also identified: cecropin-1-like, diptericin, sarcotoxin-2A, attacin-A, an immunoglobulin-like protein, lysozyme-P-like, and apidaecin (Table 4, Figure 5b). Diptericin, sacrotoxin-2A, attacin A and the immunoglobulin-like protein were all downregulated in zingerone fed flies compared to the control. Cecropin-1-like was downregulated in zingerone fed flies compared to cue-lure fed and control flies. Lysozyme-P-like was upregulated in zingerone and downregulated in cue-lure, but not significantly differentially expressed in either male lure treatment compared to the control. Apidaecin was downregulated in cue-lure fed flies compared to the control.

Table 4. Immune system-related genes differentially expressed in *Bactrocera tryoni* and *B. jarvisi* transcriptomes (treatments = zingerone feeding, cue-lure feeding, control). The best hit annotation to the most abundant isoform was selected to represent the gene. Annotation was performed against Uniref90 and NCBI nr databases.

Bactrocera tryoni						
Gene name	Accession	E-value	Identity %	Length bp	Taxon	Transcript ID
Attacin-A	A0A0K8UBV1	2.113E-143	91.4	717	Dacini 43871	TRINITY_DN1502_c0_g1_i11
Cecropin-1-like	XP_036322705.1*	7.96E-19	92.857	42	*Rhagoletis pomonella* 28610	TRINITY_DN951_c0_g1_i3
Protein spaetzle 3	XP_050319955.1*	1.42E-30	99.225	129	*Bactrocera neohumeralis* 98809	TRINITY_DN10265_c0_g1_i3
Cecropin-1-like	A0A6I9VKP7	6.785E-30	88.6	288	*Bactrocera dorsalis* 27457	TRINITY_DN1954_c0_g1_i10
Cecropin-1-like	A0A6I9VKP7	1.488E-29	88.1	288	*Bactrocera dorsalis* 27457	TRINITY_DN9965_c0_g1_i1
Lysozyme B-like	A0A6I9VB01	1.376E-89	99.0	423	Endopterygota 33392	TRINITY_DN14649_c0_g1_i3
IG-like and FN type-III domain-containing protein C25G4.10 (Fragment)	A0A0K8W4U3	5.7E-35	97.3	3189	*Bactrocera* 47832	TRINITY_DN13707_c0_g1_i1
Bactrocera jarvisi						
Gene name	Accession	E-value	Identity %	Length bp	Taxon	Transcript ID
Lysozyme P-like	XP_049307629.1*	4.11E-90	97.222	144	*Bactrocera dorsalis* 27457	TRINITY_DN9006_c0_g1_i1
Diptericin	A0A0K8V7U2	1.480E-59	95.6	309	*Bactrocera* 47832	TRINITY_DN6291_c0_g1_i1
Sarcotoxin-2A	A0A0A1WP34	2.586E-176	88.3	906	Dacini 43871	TRINITY_DN11029_c0_g2_i5
Cecropin-1-like	A0A6I9VKP7	1.853E-19	92.3	288	*Bactrocera dorsalis* 27457	TRINITY_DN10247_c1_g1_i1
Attacin-A	A0A0K8UBV1	2.777E-145	92.3	717	Dacini 43871	TRINITY_DN3448_c0_g1_i2
IG-like and FN type-III domain-containing protein C25G4.10 (Fragment)	A0A0K8VDA5	4.951E-57	99.7	4503	Dacinae 164860	TRINITY_DN34778_c0_g1_i1
Apidaecin	A0A034W104	2.144E-134	90.6	681	*Bactrocera* 47832	TRINITY_DN20096_c0_g1_i1

IG = immunoglobulin. FN = fibronectin. * NCBI nr database annotation. Taxon contains the classification of the best match and the corresponding NCBI taxon code. ^ This gene had two best hits with identical E-values.

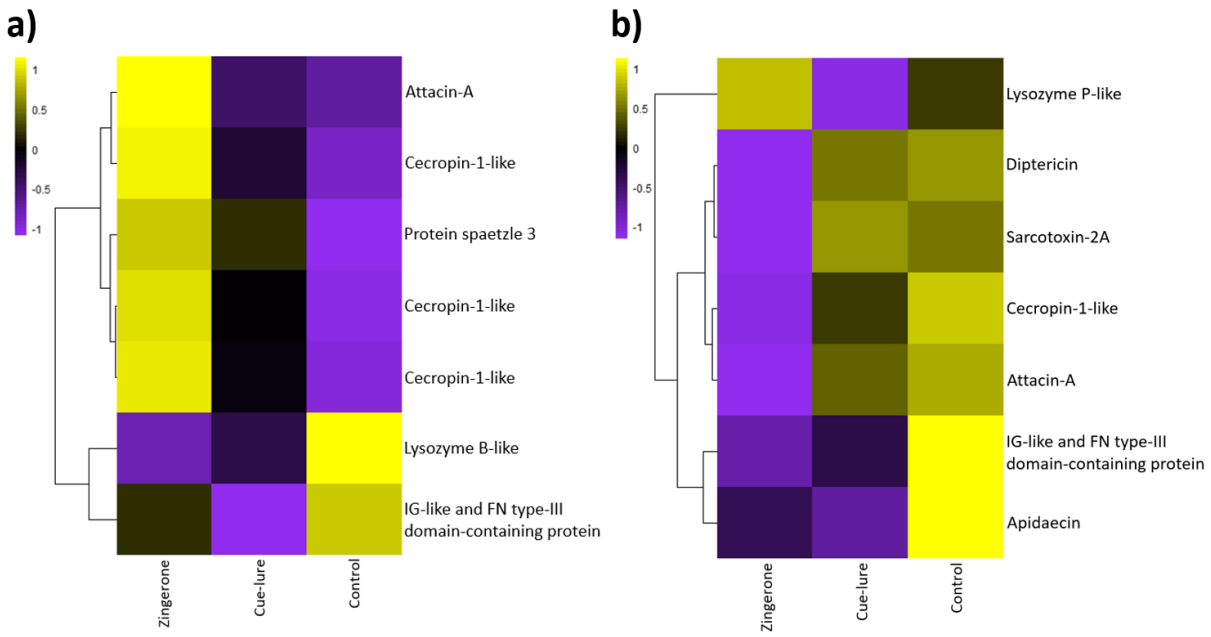

Figure 5. Heatmaps of immune-related differentially expressed genes (log2, z-scaled TMM values averaged across replicates). **a)** Differentially expressed genes in the *Bactrocera tryoni* transcriptome. **b)** Differentially expressed genes in the *B. jarvisi* transcriptome. IG = immunoglobulin. FN = fibronectin.

To determine intra- and inter-specific similarities in gene expression across treatments consistently, DEGs between treatments and species were investigated. For these comparisons, only genes that were differentially expressed between either male lure treatment and the control flies were considered. Additionally, only the UniRef90 protein name was considered, meaning that DEGs with the same protein name were considered one gene; this was necessary as several genes were annotated with the same protein name but different accessions. Across cue-lure and zingerone fed flies in both *B. tryoni* and *B. jarvisi*, four genes were consistently differentially expressed that potentially function in transposition: putative DD34D transposase, putative DD41D transposase, reverse transcriptase, and reverse transcriptase domain-containing protein (Figure 6, Table 5). The pairwise comparison with the most annotated DEGs in common was *B. tryoni* cue-lure and *B. tryoni* zingerone fed flies (24 unique protein hits), followed by *B. tryoni* zingerone and *B. jarvisi* zingerone fed flies (15 unique protein hits), and *B. tryoni* cue-lure fed and *B. jarvisi* zingerone fed flies (14 unique protein hits). The remaining pairwise comparisons had ≤ 9 unique protein hits in common. Proteins that were differentially expressed across three of the pairwise comparisons were craniofacial development protein 2 (fragment), RNA-directed DNA polymerase, RNA-directed DNA polymerase from mobile element jockey (fragment), tax protein (fragment), and transposase.

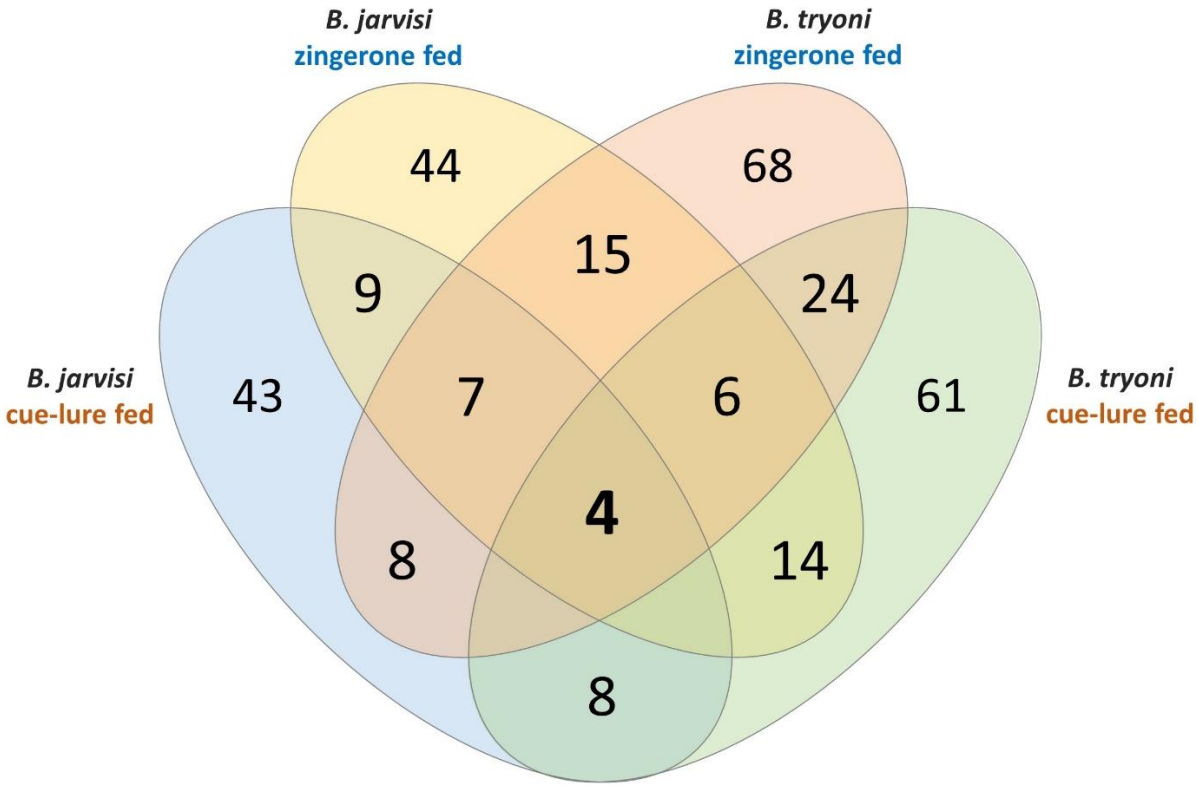

Figure 6. Venn diagram of annotated differentially expressed genes (adjusted p-value < 0.05) from male lure treatments (zingerone and cue-lure feeding) compared to the control (no lure) in *Bactrocera tryoni* and *B. jarvisi*. Genes annotated with the Uniref90 database; only unique protein name annotations contributed towards the total count.

Table 5. Annotated differentially expressed genes (adjusted p-value < 0.05) from male lure treatments (zingerone and cue-lure feeding) compared to the control (no lure) in *Bactrocera tryoni* and *B. jarvisi*. Coloured cells indicate that the gene was differentially expressed in both treatments compared to their respective control. Genes annotated with the UniRef90 database; only unique protein name annotations contributed. Uncharacterised protein, uncharacterised protein (fragment), polyprotein (fragment) and hypothetical protein were excluded.

Protein name	*jar CL vs try CL*	*jar CL vs try ZN*	*jar CL vs jar ZN*	*jar ZN vs try ZN*	*try ZN vs try CL*	*try CL vs jar ZN*
Putative DD34D transposase						
Putative DD41D transposase						
Reverse transcriptase						
Reverse transcriptase domain-containing protein						
Craniofacial development protein 2 *						
RNA-directed DNA polymerase						
RNA-directed DNA polymerase from mobile element jockey *						
Tax protein *						
Transposase						
Reverse transcriptase domain-containing protein *						
Dimer_Tnp_hAT domain-containing protein						
Histone-lysine N-methyltransferase SETMAR *						
HTH_48 domain-containing protein						
Uncharacterized protein LOC105230095						
Attacin-A						
Cecropin-1-like						
Insulin receptor *						
Putative nuclease HARBI1						
Transposable element Tc3 transposase						
Adenylate cyclase type 8 *						
Angiopoietin-related protein 7						
Fat-body protein 1						
Gustatory receptor *						
MADF domain-containing protein *						
Metallothionein-1						
Phosrestin-2						
Secreted protein *						
SWIB domain-containing protein						
Transcription factor Adf-1						
Transposon TX1 uncharacterized 149 kDa protein *						
Uncharacterized transposon-derived protein F52C9.6 *						
Variant surface glycoprotein 1125 *						
Voltage-dependent L-type calcium channel subunit beta-1 ^						
(Mediterranean fruit fly) hypothetical protein						
CG12239 *						
Farnesol dehydrogenase						
IG-like and FN type-III domain-containing protein C25G4.10 *						
Retrovirus-related Pol polyprotein LINE-1 *						
Transposable element Tcb1 transposase						

jar = *B. jarvisi*. try = *B. tryoni*. CL = cue-lure. ZN = zingerone. * (fragment). ^ isoform X1. IG = immunoglobulin. FN = fibronectin.

3.2 STUDY 2: INVESTIGATING THE EFFECTS OF CUE-LURE FEEDING ON GENE EXPRESSION ACROSS TWO GENERATIONS IN *B. TRYONI*

3.2.1 Read mapping and functional annotation

The number of reads sequenced across samples for *B. tryoni* ranged from 24,611,627 to 43,441,024 ($\bar{x}$ = 38,598,563, σ = 5,166,755). Initial BaseSpace (Illumina) quality control results demonstrated that 91.3% of all reads were ≥ Q30. After read trimming there was an average loss of 9.49×10^{-3} % of reads. The read alignment success rate to *B. tryoni* gene models ranged from 14.66 % to 72.12 % ($\bar{x}$ = 55.29 %, σ = 21.89 %) (see Supplementary Table A 11 for the full list of read counts and read alignment success rates). In addition to the genome-guided transcriptome assembly, a *de novo* assembly was performed to investigate if the *B. tryoni* iflavirus 1 observed in study one was also present in the current *B. tryoni* transcriptome. The transcriptome assembly statistics can be found in Supplementary Table A 12, the transcript count and E90N50 were comparable to the *B. tryoni* assembly statistics from study one. DGE was analysed for generation one only. The heatmap of differentially expressed genes from generation one (cue-lure fed and control flies) revealed a group of seven genes with consistent extreme expression like the pattern observed in study one, Figure 1 (Figure 7). These genes were upregulated in two replicates of cue-lure fed flies compared to control flies and the other cue-lure replicate. The group of genes were annotated as *B. tryoni* iflavirus 1 (NCBI:txid2795009) genome polyprotein, which indicates that the same virus was present in the *B. tryoni* transcriptomes from both studies (both were sourced from the Department of Agriculture and Fisheries but from different breeding lines). Additionally, there is a similar expression pattern between the studies of upregulation in cue-lure fed flies compared to the control (study one: upregulated in 3/3 cue-lure replicates + 1 control replicate; study two: upregulated in 2/3 cue-lure replicates). For the NCBI hits for these genes, see Supplementary Table A 13.

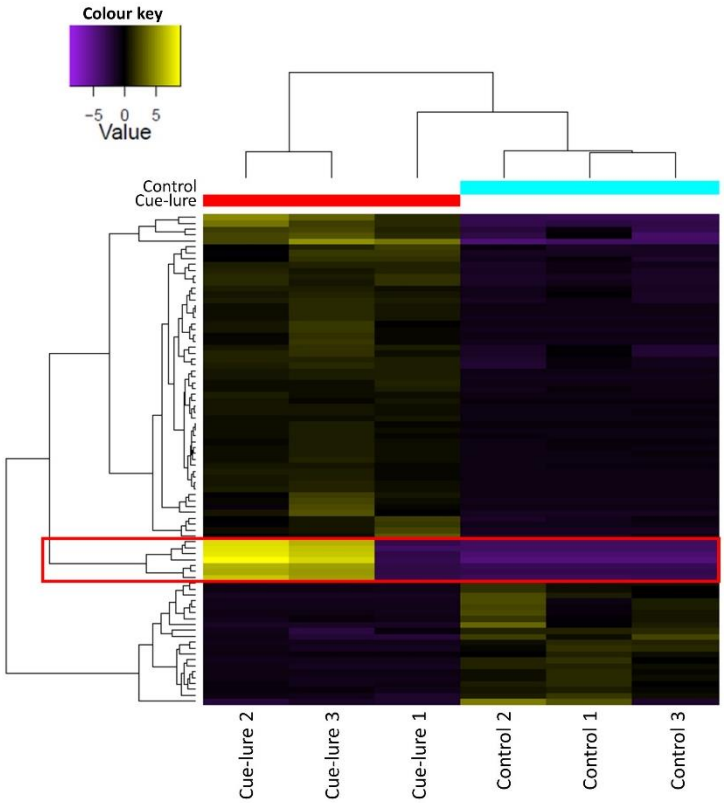

Figure 7. Heatmap of differentially expressed genes in the *de novo* assembled *Bactrocera tryoni* generation one transcriptome without scale limits (treatments = cue-lure feeding and control; replicates = 1, 2, 3; log2 centred TMM values). The red box surrounds genes annotated with NCBI:txid2795009 = *B. tryoni* iflavirus 1.

3.2.2 Differential gene expression analysis

DGE analysis was conducted on all pairwise comparisons between the four groups as well as between generations (treatments combined) and treatments (generations combined) to investigate these variables separately. Note, unless the generation is explicitly stated, the term 'treatment' is used to describe two groups: i) cue-lure fed flies and their offspring, hereafter referred to just as 'cue-lure', and ii) control flies and their offspring, hereafter just 'control'. Likewise, unless the treatment is explicitly stated, the term 'generation' is used to describe two groups: i) generation one cue-lure flies and control flies, hereafter just 'generation one' and ii) generation two cue-lure and control flies, hereafter just 'generation two'. A total of 3647 genes were found differentially expressed across all treatments and generations. Pairwise DGE analysis revealed 282 genes differentially expressed between generation one (G1) cue-lure fed and control flies (157 upregulated and 125 downregulated in cue-lure fed flies), 102 between generation two (G2) cue-lure and control flies (76 upregulated and 26 downregulated in cue-lure flies), 3100 between G1 and G2, and 39 between cue-lure and control flies (see Table 6 for the number of genes differentially expressed between all pairwise comparisons). The sample correlation

plot demonstrated clustering between G1 and G2, however, not all treatment replicates clustered together (Supplementary Figure A 5).

Table 6. Number of genes differentially expressed between all pairwise comparisons in *B. tryoni*. Four groups = generation one cue-lure fed flies, generation one control flies, generation two cue-lure offspring, generation two control offspring.

Pairwise comparison	Total DE genes	Upregulated genes	Downregulated genes
G1 cue-lure vs G1 control	282	157	125
G2 cue-lure vs G2 control	102	76	26
G1 cue-lure vs G2 cue-lure	1044	685	359
G1 control vs G2 control	843	557	286
G1 cue-lure vs G2 control	1800	1132	668
G1 control vs G2 cue-lure	576	431	145
G1 vs G2	3100	1792	1208
Cue-lure vs control	39	28	11

G1 = generation 1. G2 = generation 2. Upregulated and downregulated genes in the first group defined in the pairwise comparison compared to the second group defined.

3.2.3 Gene ontology analysis

GO analysis on the DEGs revealed significantly enriched and/or depleted GO terms between all G1 and G2 comparisons, but not between exclusively cue-lure and control comparisons within the same generation. The GO analysis between G1 and G2 retrieved the largest number of enriched GO terms (104; before REVIGO reduction) across the three major GO groups: 60 biological process (BP), 23 cellular component (CC), and 21 molecular function (MF) GO terms. BP GO terms were plotted in semantic space where more related GO terms are positioned more closely together (Figure 8). Four clusters of terms can be seen in Figure 8, generally related to regulation, metabolic processes, transport and behaviour. The enriched BP GO terms were associated with parental terms including metabolic process, cellular process, biological regulation, localization, homeostatic process, behaviour and developmental process. The top five enriched GO terms (REVIGO reduced) by p-value were structural constituent of ribosome (GO:0003735; MF), ribosome (GO:0005840; CC), translation (GO:0006412; BP), cellular amide metabolic process (GO:0043603; BP), and organonitrogen compound biosynthetic process (GO:1901566; BP). The top five depleted GO terms (REVIGO reduced) by p-value were olfactory receptor activity (GO:0006259; MF), DNA metabolic process (GO:0006259; BP), structural constituent of cuticle (GO:0042302; MF), ATP-dependent activity acting on DNA (GO:0008094; MF), and DNA repair (GO:0006281; BP). See Supplementary Table A 14 for the full list of enriched and

depleted terms between the generations. The GO analysis between G1 cue-lure and G2 cue-lure flies retrieved 45 GO terms (before REVIGO reduction) and was dominated by BP terms: 28 BP, 11 CC, and 6 MF GO terms (Table 7). The enriched BP GO terms were associated with the parental terms metabolic process, cellular process, biological regulation, and homeostatic process. The top five enriched GO terms (REVIGO reduced) by p-value were structural constituent of ribosome (GO:0003735; MF), translation (GO:0006412; BP), cellular amide metabolic process (GO:0043603; BP), organonitrogen compound biosynthetic process (GO:1901566; BP), and ribosome (GO:0005840; CC). Whereas the GO analysis between G1 control and G2 control flies retrieved only 7 GO terms (before REVIGO reduction) and was dominated by MF terms: 0 BP, 1 CC, 7 MF (Table 8). The enriched MF GO terms were associated with the parental terms catalytic activity and molecular function regulator. The enriched GO terms (REVIGO reduced) were endopeptidase inhibitor activity (GO:0004866; MF), oxidoreductase activity (GO:0016491; MF), and extracellular space (GO:0005615; CC). The enriched and depleted GO terms between G1 cue-lure and G2 control, and G1 control and G2 cue-lure are available in Supplementary Tables A 15 and 16, respectively.

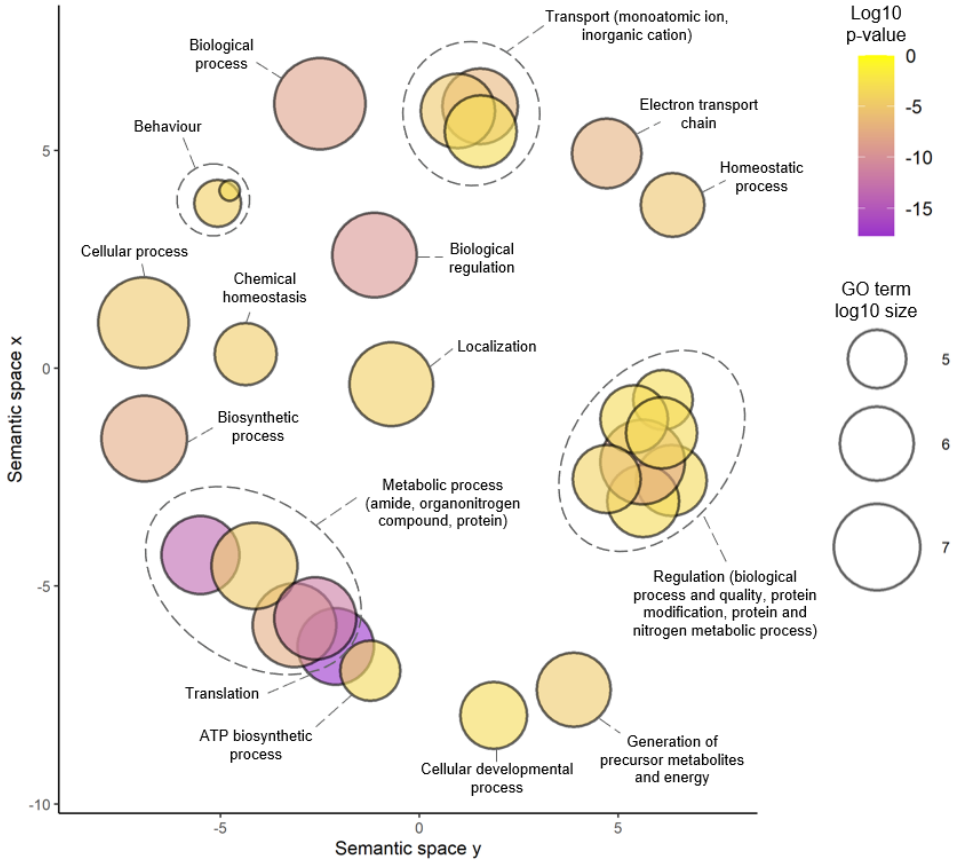

Figure 8. Scatterplot of enriched gene ontology biological process terms (FDR < 0.05) between generation one and generation two. REVIGO was used to reduce GO terms, generate semantic similarity and produce the figure. Groups of GO terms were placed within a dashed lined circle if they shared

similar GO terms, these were labelled with general function and more specific information in brackets. GO term log10 size represents the percentage of genes annotated with the term in the whole UniProt database. See Supplementary Table A 14 for the full list of enriched GO terms.

Table 7. Gene ontology enrichment analysis on genes differentially expressed between generation one cue-lure fed flies and generation two cue-lure fed flies in *Bactrocera tryoni* (reduced with REVIGO; FDR < 0.05).

Enriched GO terms			
Ontology	ID	Term	p-value
MF	GO:0003735	structural constituent of ribosome	1.57×10^{-18}
BP	GO:0006412	translation	2.77×10^{-18}
BP	GO:0043603	cellular amide metabolic process	2.46×10^{-14}
BP	GO:1901566	organonitrogen compound biosynthetic process	7.24×10^{-11}
CC	GO:0005840	ribosome	2.57×10^{-10}
BP	GO:0019538	protein metabolic process	5.92×10^{-8}
MF	GO:0005198	structural molecule activity	1.05×10^{-7}
BP	GO:0065007	biological regulation	7.45×10^{-6}
CC	GO:1990904	ribonucleoprotein complex	8.79×10^{-6}
BP	GO:0009058	biosynthetic process	1.06×10^{-5}
MF	GO:0106310	protein serine kinase activity	1.47×10^{-5}
MF	GO:0004725	protein tyrosine phosphatase activity	3.35×10^{-5}
BP	GO:1901564	organonitrogen compound metabolic process	4.04×10^{-5}
CC	GO:0098796	membrane protein complex	5.16×10^{-5}
BP	GO:0050794	regulation of cellular process	5.22×10^{-5}
CC	GO:0043228	non-membrane-bounded organelle	6.72×10^{-5}
CC	GO:0098803	respiratory chain complex	6.90×10^{-5}
CC	GO:0070069	cytochrome complex	9.80×10^{-5}
BP	GO:0022904	respiratory electron transport chain	2.81×10^{-4}
CC	GO:0098798	mitochondrial protein complex	3.04×10^{-4}
BP	GO:0048878	chemical homeostasis	3.5×10^{-4}

BP = biological process. CC = cellular component. MF = molecular function.

Table 8. Gene ontology enrichment analysis on genes differentially expressed between generation one control flies and generation two control flies in *Bactrocera tryoni* (reduced with REVIGO; FDR < 0.05).

Enriched GO terms			
Ontology	ID	Term	p-value
MF	GO:0004866	endopeptidase inhibitor activity	1.33×10^{-6}
MF	GO:0016491	oxidoreductase activity	3.84×10^{-6}
CC	GO:0005615	extracellular space	1.07×10^{-6}

Depleted GO terms			
Ontology	ID	Term	p-value
BP	GO:0090304	nucleic acid metabolic process	3.05×10^{-6}

BP = biological process. CC = cellular component. MF = molecular function.

3.2.4 Differentially expressed genes of interest

The DEGs and corresponding proteins identified by Kumaran et al. (2014b) in intermale aggression, pheromone, courtship, odorant binding protein, reproduction, and longevity gene groups were searched for in the DEGs of the present study; specifically, between treatments, G1 cue-lure fed vs G1 control flies, and G2 cue-lure vs G2 control flies. Two proteins were identified that were consistently differentially expressed across the studies, OBP 56d and protein takeout. Kumaran et al. (2014) identified OBP 56d (*GK15692* and *GJ21407* genes) and protein takeout (*takeout* gene) upregulated in zingerone fed flies compared to control flies in *B. tryoni*. In the present study, general OBP 56d and general OBP 56d-like were both upregulated in generation two cue-lure compared to the control. Protein takeout was also upregulated in generation two cue-lure compared to the control. No genes (or proteins) from the gene groups of interest were identified in the DEGs between treatments or G1 cue-lure fed vs G1 control flies.

To find genes potentially involved in multigeneration effects of cue-lure, the expression of the DEGs between the treatments was further investigated. The heatmap of average gene expression demonstrates that most genes are upregulated in cue-lure (39 total; 28 upregulated, 11 downregulated) (Figure 9). Most of the upregulated genes are strongly upregulated in G1 cue-lure, but less in G2 cue-lure. However, generally the expression of G2 cue-lure is still much higher than the G2 control. There are several genes in the heatmap that demonstrate consistent expression level across the generations, and with boxplot gene expression visualisation, demonstrated minimal or no overlap in the whiskers; these genes were selected as candidate genes for the multigenerational effects of cue-lure. In total, 9 genes were selected from the 39 differentially expressed between the treatments: LOC120772642 ([Mediterranean fruit fly] hypothetical protein), LOC120782759 (uncharacterised protein), LOC120767653 (maltase 2), LOC120768368 (protein bicaudal C), LOC120774457 (tolloid-like protein 2), ATP8 (ATP synthase protein 8), LOC120776780 (acid sphingomyelinase-like phosphodiesterase 3b), LOC120771949 (galpha49B), and LOC120777068 (uncharacterised protein LOC109579818). Boxplots of individual gene expression for these candidate genes can be seen in Figure 10.

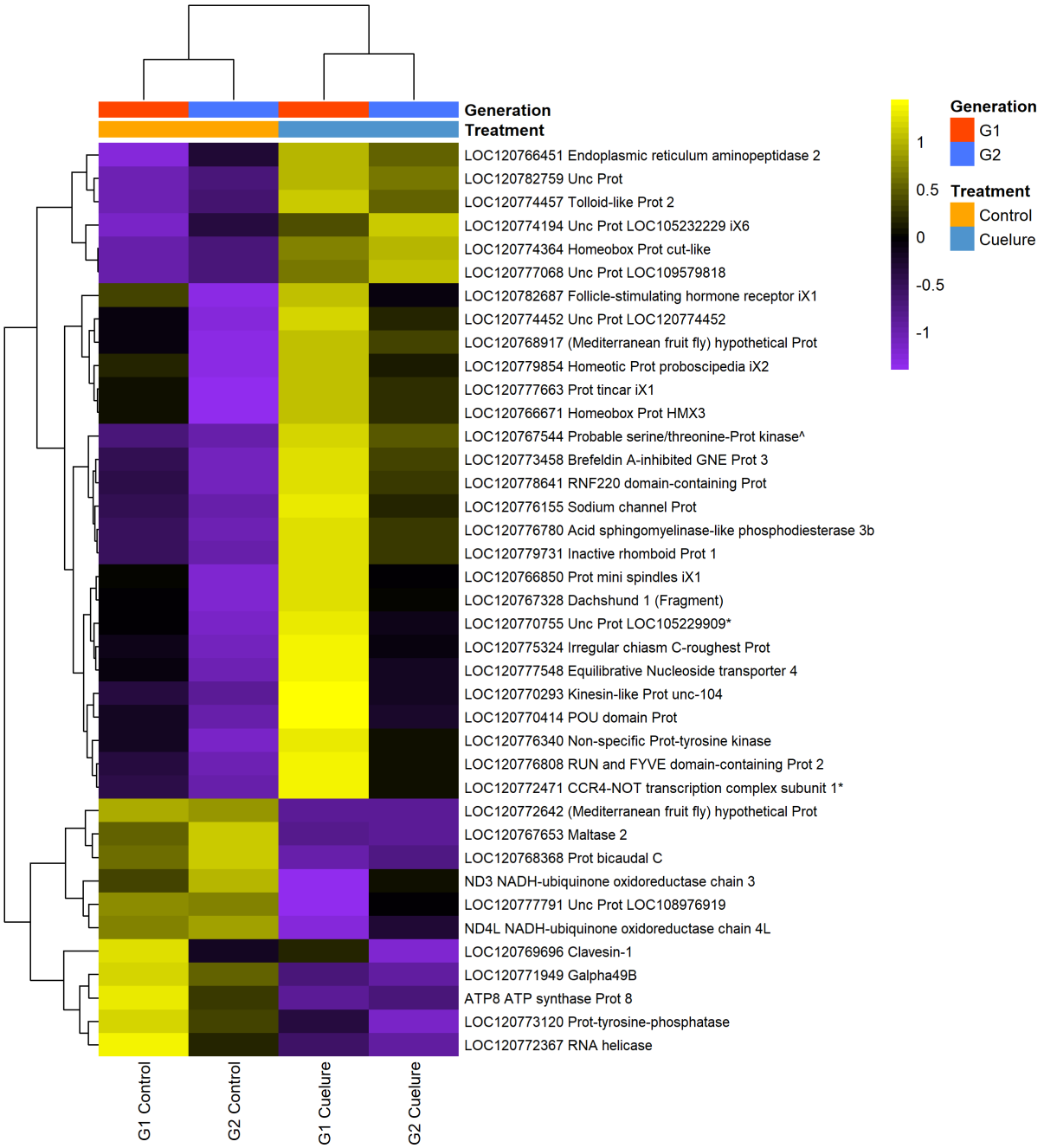

Figure 9. Heatmap of the 39 differentially expressed genes in *Bactrocera tryoni* between two treatments: i) cue-lure fed flies and their offspring, and ii) control flies and their offspring (log2, z-scaled, median of ratios values averaged across replicates). G1 = generation 1. G2 = generation 2. Prot = protein. Unc = uncharacterised. GNE = guanine nucleotide-exchange. * low quality protein. ^ DDB_G0282963.

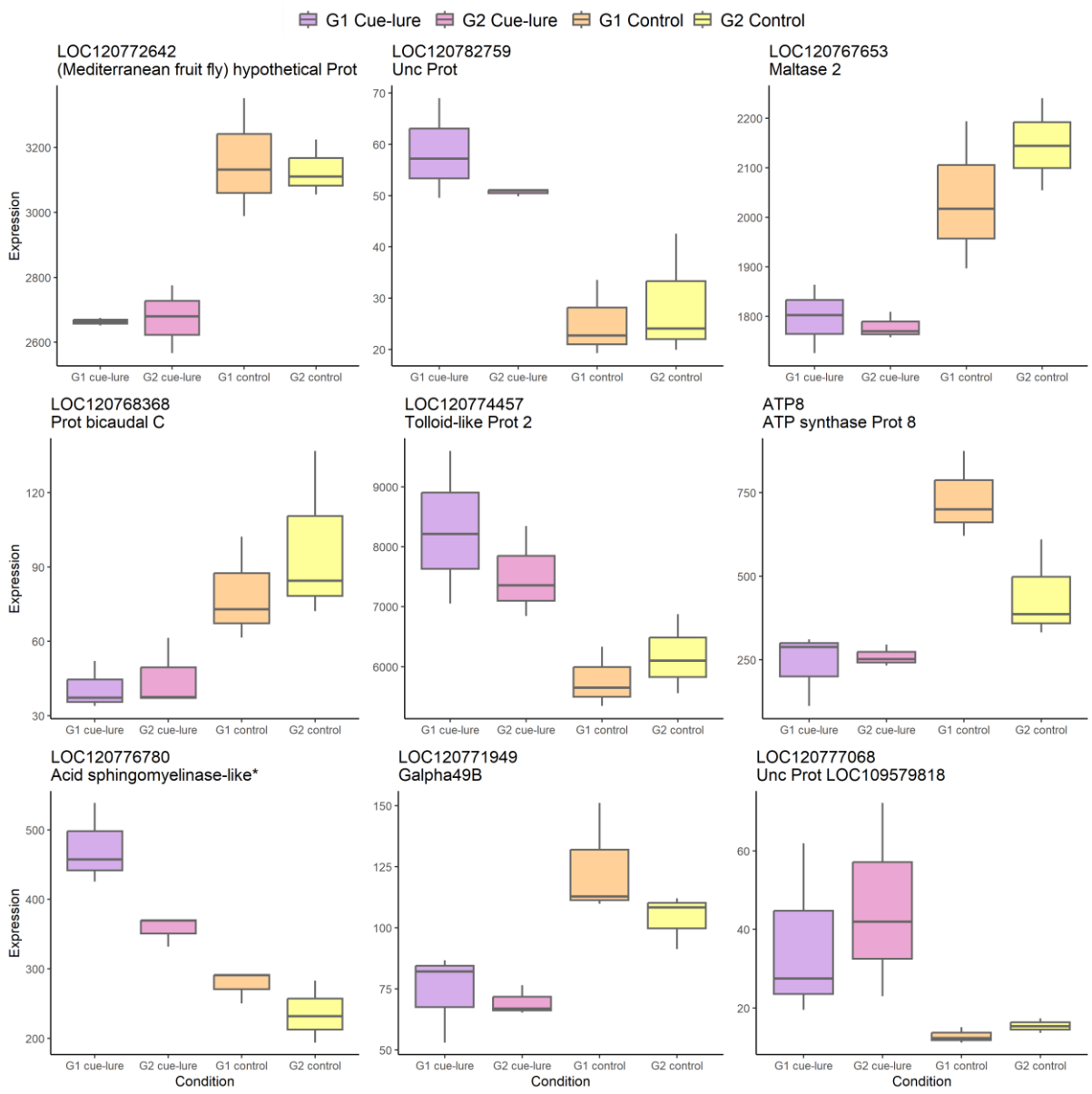

Figure 10. Boxplots of individual expression (median of ratio values) of candidate genes across generation one cue-lure fed flies and their offspring (generation two), generation one control flies and their offspring (generation two). Each plot is labelled with the gene and protein name. G1 = generation 1. G2 = generation 2. Prot = protein. Unc = uncharacterised. * phosphodiesterase 3b.

To further investigate the DEGs between G1 cue-lure vs G1 control and G2 cue-lure vs G2 control, annotated genes were searched for GO terms or protein names of interest. In G1 cue-lure vs control, one potential sensory-related gene was upregulated in cue-lure: neuropeptide SIFamide receptor (Table 9). Four potential immune-related genes were downregulated in cue-lure: peptidoglycan-recognition protein SB1, peptidoglycan-recognition protein, Ras-like GTP-binding protein Rho1, and uncharacterized protein LOC105231288 (Table 9). One potential transposition-related gene was upregulated in cue-lure: transposable element Tc3 transposase (Supplementary Table A 17). In G2 cue-lure vs control, four potential sensory-related genes were upregulated in cue-lure: two general OBP 56d, OBP 5-1, and OBP19b (Table 9). One potential immune-related gene was upregulated in cue-lure: lucifensin-like (Table 9). Seven potential transposition-related genes were identified both up and downregulated: six tigger transposable element-derived proteins and piggyBac transposable element-derived protein 4 (Supplementary Table A 17). Additionally, DEGs from study one and study two were compared to investigate the differences between *B. tryoni* fed cue-lure sampled 7 hours after feeding (study one), and *B. tryoni* fed cue-lure 24 hours after feeding (study two). Unique UniRef90 protein name annotations were compared in the DEGs between study two G1 cue-lure vs G1 control and study one *B. tryoni* cue-lure vs control. Only two proteins were consistently differentially expressed between the studies, transcription factor Adf-1 and transposable element Tc3 transposase (excluding uncharacterised and hypothetical protein annotations).

Table 9. Sensory and immune system-related genes differentially expressed in *Bactrocera tryoni* generation one cue-lure fed flies vs generation one control flies and generation two cue-lure flies vs generation two control flies. The best hit annotation was selected to represent the gene. Annotation was performed against the Uniref90 database.

G1 cue-lure vs G1 control						
Gene name	Accession	E-value	Identity %	Length bp	Taxon	Gene ID
Neuropeptide SIFamide receptor[1]	A0A6I9V224	0.000E+00	88.7	2202	*Bactrocera* 27456	LOC120777660
Peptidoglycan-recognition protein SB1[2]	A0A6I9W3J7	9.460E-151	98.5	687	*Bactrocera* 47832	LOC120766467
Peptidoglycan-recognition protein[2]	A0A4D6C7Q3	2.370E-125	98.8	576	*Bactrocera* 47832	LOC120774927
Ras-like GTP-binding protein Rho1[2]	P48148	4.189E-121	96.2	576	Neoptera 33340	LOC120781251
Uncharacterized protein LOC105231288[2]	A0A6I9W260	6.318E-186	80.8	1059	*Bactrocera dorsalis* 27457	LOC120776486
G2 cue-lure vs G2 control						
Gene name	Accession	E-value	Identity %	Length bp	Taxon	Gene ID
General OBP 56d[1]	A0A0K8UPA1	6.419E-66	77.6	414	*Bactrocera latifrons* 174628	LOC120771526
General OBP 56d[1]	A0A6I9V3V6	2.185E-74	87.4	414	*Bactrocera* 47832	LOC120771524
OBP 5-1[1]	A0A866W2K7	1.263E-69	92.4	369	*Bactrocera* 47832	LOC120771671
OBP 19b[1]	A0A0G2UET6	2.221E-83	86.8	462	*Bactrocera* 47832	LOC120773564
Lucifensin-like[2]	A0A6I9VF33	5.607E-45	97.5	282	*Bactrocera tryoni* 59916	LOC120770463

OBP = odorant binding protein. [1] sensory-related proteins. [2] immune-related proteins.

Chapter 4: Discussion

To broaden the understanding of genetic mechanisms involved in the fruit fly male lure response, two studies were conducted to investigate the effects of lure feeding on gene expression across different lures, species, and generations. Firstly, the effects of cue-lure and zingerone were investigated in *B. tryoni* and *B. jarvisi*, which exhibit inverse attraction to the lure types. Secondly, the heritability of cue-lure feeding effects was investigated in *B. tryoni* across a generation. The results demonstrated that the effects of lure feeding on gene expression vary intra- and inter-specifically, with few genes consistently expressed between lure types or species. Further, the results indicate potential novel immune system effects of lure feeding in *B. tryoni*. Several candidate sensory genes associated with lure response were identified, as well as several candidate genes associated with the generational effects of lure feeding. These two studies have been able to provide new insights into the effects of male lures at the genetic level and identify genes for further functional analyses.

4.1 DIRECT EFFECTS OF CUE-LURE AND ZINGERONE

The direct effects of cue-lure (or raspberry ketone) and zingerone in some male fruit flies include a mating advantage through increased courtship behaviours and activity (Shelly & Villalobos, 1995; Kumaran et al., 2013; Kumaran et al., 2014b; Shamshir & Wee, 2019), and/or pheromone enhancement (Khoo & Tan, 2000; Kumaran et al., 2014a; Shamshir & Wee, 2019); additionally, both lures can increase mortality over time (Kumaran et al., 2013), but not always (Shelly & Nishimoto, 2016; Akter et al., 2017b). Cue-lure/raspberry ketone has also been shown to accelerate sexual maturation (Akter et al., 2017b; Khan et al., 2019) and decrease repeat lure feeding (Chambers et al., 1972; Shelly & Villalobos, 1995; Shelly, 2000a; Akter et al., 2017a; Khan et al., 2017; Khan et al., 2021). The results of the current study suggest that feeding on these lures may also affect the immune system of *B. tryoni*. The upregulation of a common virus was detected in cue-lure fed *B. tryoni*, which could reflect an adverse effect of cue-lure on the fly immune system. On the other hand, zingerone may also affect the immune system as some immune-related genes were upregulated in *B. tryoni* but others were downregulated in *B. jarvisi*. Transposable element-related genes were differentially expressed in all treatments and sensory-related genes differentially expressed varied across lure type and species. Notably, our results were very different to Kumaran et al. (2014b) who conducted a similar study. The direct effects of lure feeding observed on gene expression will be discussed below and compared with the results of Kumaran et al.'s (2014b) study.

4.1.1 Iflavirus and immunity

De novo transcriptome assembly revealed iflavirus present in both *B. tryoni* and *B. jarvisi* transcriptomes; the virus was found differentially expressed in *B. tryoni* but not in *B. jarvisi*. Iflavirus belongs to the family *Iflaviridae* of positive sense single stranded arthropod RNA viruses in the order *Picornavirales* (Valles et al., 2017). The most abundant iflavirus across all transcriptomes was Bactrocera tryoni iflavirus 3 (BtIV3). In addition to BtIV3, Bactrocera tryoni iflavirus 2 (BtIV2), Bactrocera tryoni iflavirus 1 (BtIV1), and Bactrocera bryoniae iflavirus 1 were also found differentially expressed in *B. tryoni*. Sharpe et al. (2021) recently identified these fruit-fly-specific iflaviruses. The viruses were found to be prevalent in both *Bactrocera* laboratory and field populations, with 90 – 100 % of sampled individuals having at least one virus present. Although the BtIV3 variant was not tested, BtIV1 was present in all sampled individuals from one of the *B. tryoni* laboratory populations tested, indicating that the iflavirus detected in the current study is not unexpected. Sharpe et al. (2021) suggest that some of the identified viruses are likely active infections in part due to the relatively high expression of viral transcripts. In the current study iflavirus had very high transcript abundance in *B. tryoni* across all treatments, therefore it is likely that *B. tryoni* had an active iflavirus infection; however, the same molecular diagnostic tests as Sharpe et al. (2021) were not conducted. While it is probable that *B. tryoni* had an active infection, it is unlikely that *B. jarvisi* did as there was very low viral transcript abundance. The iflavirus in *B. jarvisi* may have been present on the flies without infection. Morrow et al. (2023) demonstrated that BtIV2 can be horizontally transmitted (through co-habitation); if other variants share the same transmission mode, it is likely *B. jarvisi* was exposed to the virus through non-sterile cleaning procedures, sharing equipment between species, and the proximity of fly colonies.

An interesting finding in the current study was the general upregulation of iflavirus transcripts in cue-lure fed *B. tryoni* compared to control flies in both studies. These results indicate that cue-lure may affect the immune system of *B. tryoni* is some way, directly or indirectly, allowing iflavirus to proliferate. In *B. tryoni* iflavirus infection is considered covert and persistent due to the lack of obvious symptoms and the transmission across generations (Morrow et al., 2023). These types of infections can still affect host fitness and viral replication can be influenced by environmental and stress factors (de Miranda & Genersch, 2010; Williams et al., 2017). As cue-lure can increase activity in *B. tryoni* (Kumaran et al., 2014b), resources may be diverted from the immune system for energy metabolism and thus allow iflavirus to proliferate. Nutrient based trade-offs have been observed in crickets, where *Teleogryllus commodus* reared on a high protein diet exhibited increased reproductive effort (requires increased metabolic energy) but consequently had a reduced life span (Hunt et al., 2004). Moreover, in *Gryllodes sigillatus* high carbohydrate intake compared to protein increased reproductive effort, whereas high protein intake compared to carbohydrate increased aspects of immune function (Rapkin et al., 2018). It is possible that a similar type of dietary trade-off mechanism occurs in activity (energy metabolism) and immunity when *B. tryoni* feeds on cue-lure. Additionally, although no enriched or

depleted immune-related GO terms were observed in cue-lure fed *B. tryoni*, four immune-related genes were downregulated in flies sampled 24 hours after feeding (study two). This downregulation could suggest an immune system effect of cue-lure feeding. Further, as the abdomen and thorax are important sites for immune system organs in *Drosophila melanogaster* (Buchon et al., 2014), other immune gene expression may not have been captured in the current study as only head tissue was investigated.

Although iflavirus was upregulated in cue-lure fed *B. tryoni*, it was not upregulated in zingerone fed *B. tryoni*; however, zingerone fed *B. tryoni* exhibited enriched immune-related GO terms and the upregulation of five immune-related genes. As cue-lure and zingerone both increase activity in *B. tryoni* (Kumaran et al., 2014b), if immunity is a trade-off with activity, iflavirus upregulation in zingerone fed *B. tryoni* would also be expected. The lack of viral upregulation in zingerone fed flies could indicate that i) increased activity is not a trade-off for immune system function, ii) the lure concentrations tested do not elicit the same increased activity as Kumaran et al. (2014b), or iii) zingerone may have properties beneficial to the immune system to counteract negative effects. In mammals, zingerone has demonstrated a wide range of pharmacological effects including anti-inflammatory, antioxidative, and antimicrobial properties (Ahmad et al., 2015). Though there is limited research in invertebrates, Chang et al. (2012) demonstrated that shrimp fed a zingerone diet had increased immunity and disease resistance against a bacterial pathogen. Feeding on zingerone may also enhance components of the immune system in *B. tryoni*, supported by the enrichment of immune-related GO terms and the upregulation of some immunity-related genes. If immunity is a trade-off with activity, it is possible that zingerone could enhance immune system function and counter the negative effects of increased activity, hence the lack of iflavirus upregulation. However, like cue-lure, there is no behavioural data on zingerone at the concentration tested and therefore the potential effects of activity increases are purely speculative. The differential expression of immune-related genes in cue-lure and zingerone fed *B. tryoni* and in zingerone fed *B. jarvisi* does however suggest some immune system effects of these male lures in these species.

4.1.2 Transposable element-related genes

In study one, most differentially expressed genes were not consistent between species or lure type, however, a group of transposable element-related genes were found commonly differentially expressed in all lure fed flies compared to the control. Transposable elements are DNA sequences capable of replicating or moving from one location in the genome to another via an RNA intermediate (class I) or directly as DNA (class II). Class I transposable elements encode a reverse transcriptase to facilitate transposition, whereas class II transposable elements encode a transposase (Wicker et al., 2007). In the current study, the proteins putative DD34D transposase, putative DD41D transposase, reverse transcriptase and reverse transcriptase domain-containing protein were differentially expressed in all

lure treatments compared to the control. The expression of transposases and reverse transcriptases suggests that transposable elements are affected by cue-lure and zingerone feeding in *B. tryoni* and *B. jarvisi*. Although, there were no class I transposable elements found commonly differentially expressed which would be expected as mRNA is utilised in transposition (McCullers & Steiniger, 2017). Transposable elements are recognised to have roles in adaptive evolution (Schrader & Schmitz, 2019) and gene regulation (Chuong et al., 2017). Moreover, stress conditions have been associated with the activation and repression of transposable elements, where they can consequently affect nearby gene expression and have beneficial or adverse host effects (Horváth et al., 2017). The common expression of transposable element-related genes indicates that transposable elements are affected in some way, either in response to or as a consequence of lure feeding in *B. tryoni* and *B. jarvisi*. Whether this expression is related to adaptive, regulatory, stress, or other mechanisms is worth further exploration.

4.1.3 Sensory-related genes

Most of the differentially expressed sensory-related genes identified were different between species and lure type. The only protein found differentially expressed in both species was general OBP 99a-like, however, the pattern of expression across treatments was not consistent. In *B. tryoni* the expression of general OBP 99a-like was highest in zingerone fed flies and lowest in cue-lure fed flies, whereas in *B. jarvisi* general OBP 99a-like was expressed highest in cue-lure fed flies and lowest in the control. OBP99a has previously been associated with host perception (Zhang et al., 2018; Yao et al., 2021), attractant perception (Ruiz-May et al., 2020), and mating behaviour (Zhang et al., 2018) in Tephritid fruit flies. Zhang et al. (2018) demonstrated that *OBP99a* silencing in *B. dorsalis* decreased mating propensity in males, but increased mating in females. Due to this sexually dimorphic behaviour, the authors suggested that *OBP99a* could be involved in male lure attraction. In the current study, the highest OBP 99a-like expression was observed in the weakly responsive lure for both species; this could indicate that OBP 99a-like is involved in the differential male lure attraction of cue-lure and zingerone in *B. tryoni* and *B. jarvisi*.

The other OBP found differentially expressed was general OBP 56a-like in *B. tryoni*, which was downregulated in zingerone fed flies compared to the control. Like OBP99a, OBP56a has been associated with mating in some Dipteran species (McGraw et al., 2004; Zhou et al., 2009; Nakamura et al., 2016; Campanini et al., 2017; Kumaran et al., 2017). OBP56a has also been associated with male lures in *B. tryoni* (Kumaran et al., 2014b; Kumaran et al., 2017; Khan et al., 2021). In contrast to the results of the current study, Kumaran et al. (2014b) found that *OBP56a* was upregulated in zingerone fed *B. tryoni*. Both studies shared similar sampling methods, however, we tested expression only in the fly head with a lower dose of zingerone. This could indicate that general OBP 56a-like expression is dependent on lure concentration or tissue type. Lure concentration has been shown to influence repeat

lure feeding in some species, whereby less flies exhibit repeat feeding when fed a high concentration of methyl eugenol compared to a low concentration in *B. dorsalis* (Tan, 2020). Moreover, Khan et al. (2021) suggest the involvement of OBP 56a in repeat lure feeding for cue-lure as they demonstrated that OBP 56a was upregulated in the head tissue of *B. tryoni* cue-lure non-responders reared on raspberry ketone compared cue-lure responders reared on standard diet; although in the current study no significant expression of general OBP56a-like was observed in cue-lure fed *B. tryoni*, it was lower than the control. General OBP 56a-like could be involved in the regulation of zingerone attraction in *B. tryoni* post feeding, hence the different expression pattern in the current study compared to Kumaran et al. (2014b); perhaps in *B. tryoni* the downregulation of OBP56a correlates with repeat zingerone feeding and upregulation correlates with no repeat zingerone feeding.

There was no OBP differentially expressed exclusively in *B. jarvisi*, but an OR was identified; OR7a-like was found upregulated in cue-lure compared to zingerone fed *B. jarvisi*. OR7a has been associated with oviposition and aggregation behaviours in *D. melanogaster* (Lin et al., 2015). Additionally, a *Bactrocera minax* OR7a homolog was shown to respond weakly to methyl eugenol (Liu et al., 2020), which corresponds to the weak attraction recorded for the species (Drew et al., 2007). The results of the current study also suggest that OR7a-like is associated with male lures, specifically in *B. jarvisi* but not *B. tryoni*. Moreover, the differential expression observed between cue-lure and zingerone fed flies but not the control indicates that OR7a-like may be involved in differential male lure attraction in *B. jarvisi*.

Several sensory-related genes were also identified that have not previously been associated with male lures. In *B. tryoni* GR for sugar taste 64b (GR64b) was downregulated in both male lure treatments compared to the control. In *Drosophila*, GR64b has been shown to be involved in sugar perception (Slone et al., 2007; Fujii et al., 2015) and more recently, in proteostasis under proteotoxic stress (Baumgartner et al., 2022). Our results also demonstrated that the vision-related genes phosrestin-2, retinin-like, opsin RH4 and opsin RH1 were differentially expressed in both species. While male lures are usually investigated in relation to chemoreception, these differentially expressed genes could reflect an effect on visual processes.

4.1.4 Comparison to Kumaran et al. (2014b) zingerone fed *B. tryoni*

A component of this study aimed to build upon work performed by Kumaran et al. (2014b), who investigated the effects of zingerone feeding on whole body gene expression in *B. tryoni*. The authors found that zingerone upregulated mating related genes and enriched energy and metabolic GO terms and pathways. In addition to behavioural data and previous work (Kumaran et al., 2013; Kumaran et al., 2014a), the results indicate that zingerone feeding in *B. tryoni* increases energy metabolism, which increases activity and thus enhances mating ability. In the current study the effects of a lower zingerone dose were tested in only the head tissue of *B. tryoni* with otherwise similar sampling methods to

Kumaran et al. (2014b). In our study, 0.005 mg of zingerone was administered to 10 flies for one feeding instance (no more than 20 minutes), whereas Kumaran et al. (2014b) administered 15 mg of zingerone to flies for two hours. Though the number of flies was not specified, if 200 flies were present in the cage, there would still be a 150 fold increase in zingerone dose administered compared to our study. Interestingly, our results were very different to Kumaran et al. (2014b). In the current study, 238 genes were found differentially expressed between zingerone fed and control *B. tryoni*, whereas Kumaran et al. (2014b) found over 3000 genes differentially expressed. We identified few enriched GO terms, none of which were involved in energy or metabolic processes. There was one gene from the genes of interest (e.g., OBP, courtship, male aggression) that was differentially expressed in both studies, but the expression pattern was not consistent (discussed in Section 4.1.3). Although DGE analysis was performed differently between the studies, it is likely that these differences are related to experimental parameters, such as the lure dose and tissue type.

The differences between our study and Kumaran et al. (2014b) may be due to exclusively investigating expression in the head, or the lower lure dose, or both; it is unfortunately difficult to discern which variable contributed to the differences in gene expression. Wee and Clarke (2020) demonstrated that lure dose impacts the biological effects seen in *B. jarvisi*, in particular, zingerone dose demonstrated variable effects on the temporal profile of the mating advantage without any clear pattern. Moreover, Shelly and Dewire (1994) demonstrated that the permitted time of methyl eugenol feeding (consequently affecting the dose consumed) impacted mating success on the day of feeding in *B. dorsalis*, where 30 seconds of feeding resulted in increased mating success, but two hours did not. The concentration of male lures likely impacts the biological effects in other species, including *B. tryoni*. Here, it is possible that the concentration of male lure did not elicit the same metabolic and energy enhancement seen with a higher dose, hence the lack of enriched related GO terms. On the other hand, it is also possible that the majority of this expression occurs in other tissue types in *B. tryoni*. In *Drosophila*, the abdomen and thorax are the primary sites of the fat body, muscles, and digestive system which play important roles in energy metabolism (Chatterjee & Perrimon, 2021). It is likely that *B. tryoni* also rely on these organs for energy metabolism, therefore the metabolic enhancement observed by Kumaran et al. (2014b) may have occurred primarily in the abdomen and thorax and thus not observed in our study. Additionally, testing only head tissue likely contributed to the overall reduction in differentially expressed genes. Khan et al. (2021) conducted a differential gene expression analysis of *B. tryoni* reared on raspberry ketone, or not, in response to cue-lure (but no direct feeding). The authors found 269 genes (of 30,137 assembled transcripts) differentially expressed in the head tissue between responsive and non-responsive flies. While this study is different to the current study, the number of differentially expressed genes is more similar to our study (238 – 262 genes in *B. tryoni* for both lures of 129,687 assembled transcripts) than Kumaran et al. (2014b) (3198 transcripts of 37,098

assembled contigs) and could reflect that head tissue generally has a lower number of genes that are in some way affected by male lures.

4.1.5 Time point comparison in cue-lure fed *B. tryoni*

In study one flies were sampled seven hours after feeding, whereas in study two (generation one) flies were sampled 24 hours after feeding, this provides some insight into temporal changes in gene expression after cue-lure feeding in *B. tryoni*. The differentially expressed genes observed in study one were almost completely different to study two. In comparison to study one, study two had less sensory-related and transposable element-related genes differentially expressed, but more immune-related genes differentially expressed. Given that the effects of lure feeding such as mating advantage fluctuate with DPT, (Shelly & Villalobos, 1995; Kumaran et al., 2013; Shamshir & Wee, 2019; Wee & Clarke, 2020), and that gene expression may be changing based on circadian rhythm, it is unsurprising that such large changes in gene expression were observed between the studies.

4.2 GENERATIONAL EFFECTS OF CUE-LURE

Not all individuals from a species will respond to male lures, however the proportion of lure responsive flies can be increased (Kumaran & Clarke, 2014; Yazdani, 2022) or decreased (Ito & Iwahashi, 1974; Shelly, 1997; Guo et al., 2010; Yazdani, 2022; Mandanayake & Hee, 2023) through artificial selection. Kumaran and Clarke (2014) demonstrated in *B. tryoni* that the percentage of cue-lure responsive offspring increased when parental males fed on cue-lure compared to parental males that did not. Here, several candidate genes were identified that may be involved in the generational effects of cue-lure feeding in *B. tryoni*. Interestingly, an odorant binding protein and a courtship regulating protein were consistently differentially expressed between treatments in the offspring of the current study and zingerone fed *B. tryoni* from Kumaran et al. (2014b). Large changes in gene expression were also observed between the generations unrelated to the treatments. The generational effects of cue-lure feeding and laboratory rearing will be discussed below.

4.2.1 Generational effects of cue-lure feeding

Thirty-nine genes were found differentially expressed between cue-lure and control flies and nine candidate genes were selected that demonstrated consistent expression in both generations relative to the control. The expression of these genes suggests they were affected by cue-lure feeding in *B. tryoni* and may be involved in selection for cue-lure response. To our knowledge, these candidate genes (Figure 9) have not previously been implicated in male lure response in fruit flies, providing a new avenue to investigate the generational effects of lure feeding. Given that these genes have not been

associated with lures in other fruit fly species, their expression may be a novel lure response in *B. tryoni*. In *B. dorsalis* techniques such as RNA interference and CRISPR/Cas-9 have been applied to investigate methyl eugenol response (Zheng et al., 2012; Wu et al., 2016; Liu et al., 2017; Liu et al., 2018; Chen et al., 2021; Xu et al., 2022). These techniques could also be applied in *B. tryoni* to manipulate the expression of candidate genes and determine the effects on lure response across generations. Additionally, it is important to investigate the heritability of the gene expression patterns, such as through epigenetic sequencing; this would confirm the involvement of the candidate genes in male lure response and selection.

Most of the differentially expressed genes between treatments in the parental generation were different from those in the offspring. More potential sensory-related genes and genes of interest from Kumaran et al. (2014b) were found differentially expressed in the offspring; OBP5-1, OBP19b, general OBP56d, and protein takeout were upregulated in cue-lure offspring compared to control offspring. The upregulation of OBP5 has been identified in exposure to protein bait (Idrees et al., 2017), as well as methyl eugenol and a female biased sex pheromone (Hu et al., 2021). This could suggest a change in odour perception in the current study between cue-lure and control offspring. Moreover, OBP19b has been associated with amino acid perception (Rihani et al., 2019) and fruit odour perception (Arya et al., 2010). It is interesting that OBP5-1 and OBP19b were not differentially expressed in the parental generation or in study one, this reflects changes in the offspring potentially related to odour perception that were not observed in the parents who directly fed on cue-lure. Similarly, general OBP56d and protein takeout were found differentially expressed in the offspring, but not in the parental generation or study one. Kumaran et al. (2014b) demonstrated that general OBP56d (genes *GJ21407* and *GJ21407*) and *takeout* were upregulated in zingerone fed *B. tryoni*. OBP56d has been associated with mating in some fruit fly species (Campanini et al., 2017), and *takeout* has been implicated in mating behaviour in *D. melanogaster* (Dauwalder et al., 2002). Given that general OBP56d and protein takeout were not found differentially expressed in *B. tryoni* directly fed cue-lure, the involvement of these proteins in *B. tryoni* male lure response is unclear when considering the results of Kumaran et al. (2014b). Further investigation of these proteins in male lure response across generations is needed to better understand their role.

4.2.2 Generational effects of laboratory rearing

An inadvertent outcome from the design of this study was the comparison of gene expression unrelated to treatments between the parental generation and their offspring. Domestication and mass-rearing of fruit flies has been shown to affect their behaviour and physiology (Meats et al., 2004; Weldon, 2005; Weldon et al., 2010; Schutze et al., 2015). The results of the current study demonstrated that approximately 18 % (3100 genes) of all genes were differentially expressed between the generations

when treatments were combined, the enriched parental GO terms included metabolic process, biological regulation, and developmental process. This large proportion of differential gene expression is likely associated with the changes in behaviour and physiology from environmental and rearing selection pressures. Hull et al. (2023) investigated the effects of domestication on gene expression in *Hermetia illucens* (black soldier fly) and found 898 genes were differentially expressed between the larvae of F2 and F3 generations. Similarly, when treatments were investigated separately in the current study, 1044 and 843 genes were differentially expressed in the cue-lure and control line (F3 and F4), respectively. Notably, the number of differentially expressed genes was higher in the cue-lure line which could reflect the additional selection pressure of lure feeding in the parental generation.

4.3 IMPLICATIONS IN PEST MANAGEMENT

The pre-release exposure of sterile flies to male lures can improve male quality and cost effectiveness due to the effects observed of i) increased mating competitiveness, ii) accelerated sexual maturation, and iii) reduced repeat lure feeding. Consequently, these effects could improve the SIT by i) increasing sterile male mating success, ii) decreasing the pre-release holding time, and iii) allowing MAT and SIT to run simultaneously (Pereira et al., 2021). These biological effects have been observed in *B. tryoni* (Kumaran et al., 2013; Akter et al., 2017a; Akter et al., 2017b; Khan et al., 2017; Khan et al., 2019; Khan et al., 2021), which indicates that implementing cue-lure/raspberry ketone feeding could greatly improve the effectiveness of the technique. The results of the current study indicate that cue-lure feeding was associated with the upregulation of iflavirus in *B. tryoni*, but the effects of this viral upregulation on the fly were not investigated. It is important to determine any short and long term effects of iflavirus upregulation before implementing lure feeding in the SIT for *B. tryoni* to ensure there are no adverse effects on the quality of individuals. In *C. capitata*, a higher abundance of nora virus (order *Picornavirales*, the same as iflavirus) was associated with a shorter lifespan (Llopis-Giménez et al., 2017). Moreover, other insect iflaviruses such as the deformed wing virus in honeybees can cause severe deformities and reduce lifespan (Martin & Brettell, 2019). The current study also identified several genes of interest potentially related to sensory functions and the generational effects of lure feeding. These genes may provide useful targets for modifying the response of flies to male lures, particularly for *B. tryoni*, as sterile flies non-responsive to male lures would allow the simultaneous application of MAT and SIT.

4.4 ASSUMPTIONS, LIMITATIONS AND CONSIDERATIONS

There were some assumptions and limitations in the methodology and results of both studies that are important to consider. The male lure feeding method employed in the current study was different to previous studies investigating the effects of lure feeding in fruit flies. In most studies a group of flies is

provided with a male lure and allowed to feed for an allocated period of time; lure feeding for all individuals can be assumed (e.g. Shelly & Dewire, 1994; Kumaran et al., 2014b; Wee & Clarke, 2020) or directly observed (e.g. Hee & Tan, 1998; Shamshir & Wee, 2019). In both cases, the time spent feeding by each individual within the allocated period is unknown and consequently the amount of lure consumed is unknown. In the current study, lure feeding was directly observed, but flies were allowed to feed until they left the lure source rather than for a fixed period of time. This method was chosen to impose a constant of one feeding instance per individual, however, the time spent feeding and amount of lure consumed still varied between individuals (within the maximum 20 minute assay duration). Although four flies were pooled for each replicate to reduce individual variation, it is possible that the differences in time spent lure feeding affected gene expression. Additionally, ethanol was used as a solvent to dilute the male lures as per previous studies (Kumaran & Clarke, 2014; Kumaran et al., 2014b; Kumaran et al., 2017; Wee et al., 2018a; Shamshir & Wee, 2019; Wee & Clarke, 2020). Ethanol exposure has been shown to affect a wide range of gene expression in *Drosophila* including genes involved in olfaction and the immune system (Kong et al., 2010). However, in the current study, male lures were given time for solvent evaporation which was observable on the glass slides due to the low volume of male lure.

Our results demonstrate that male lures can affect gene expression at low concentrations, however, behavioural effects have not been tested at these concentrations. This is important to consider for study two as this investigation was an extension of research by Kumaran and Clarke (2014) who found the offspring of cue-lure fed *B. tryoni* had an increased response rate to cue-lure; however, the lure concentrations used in the current study were lower and cue-lure response rate was not tested. While the gene expression results are not directly interpreted to explain the behavioural effects identified by Kumaran and Clarke (2014), there is still an underlying assumption that the genes identified could be involved in the selection of the lure response trait. The lack of behavioural data is also important to consider for study one, as some results are discussed in relation to known behavioural effects such as increased mating success and activity, but these effects have not been tested at the lure concentrations used. Moreover, the differential gene expression results of study one and two are interpreted as the effects of male lure feeding; however, it is unknown how much of the total gene expression may be explained by the response of *B. tryoni* to the increase in iflavirus load, rather than to the lure.

4.5 FUTURE DIRECTIONS

The finding of iflavirus upregulation with cue-lure feeding has uncovered questions about the interactions between iflavirus, cue-lure, and *B. tryoni*. Investigating gene expression, behaviour and physiology in cue-lure fed *B. tryoni* from iflavirus infected and uninfected populations would allow the effects of cue-lure feeding to be distinguished from the effects of iflavirus upregulation. Further

investigating the effects of a high abundance of iflavirus in *B. tryoni* without cue-lure feeding would allow the effects of all three variables to be differentiated. This would better the understanding of the relationship between cue-lure, iflavirus, and *B. tryoni* and could have implications for the SIT. Additionally, investigating how male lures affect the expression of viruses in other species would help determine whether this is a unique effect in *B. tryoni* or a common effect of lure feeding.

We identified several candidate genes for lure response in *B. tryoni* and *B. jarvisi* and in the generational effects of cue-lure in *B. tryoni*. Functional analyses of these genes would validate their involvement in lure response; techniques such as RNA interference or CRISPR/Cas9 could be used to modify the expression of genes and determine the effect on fly response to male lures. Moreover, investigating gene expression across several generations, the effects of lure reinforcement, and how lure response changes in the offspring is important to better understand the mechanisms underlying this effect. This research would improve the understanding of the genetic mechanisms involved in the intra- and inter-species differences in response to male lures and could provide target genes for pest management.

Large differences were observed in cue-lure fed *B. tryoni* sampled at two different times after feeding, these results highlight the need for a time course RNA-seq analysis to investigate the effects of male lures on gene expression. This research would be particularly valuable in conjunction with a corresponding behavioural and physiological study to provide a more complete representation of the effects of male lures in fruit flies. Additionally, our results for zingerone fed *B. tryoni* were vastly different to Kumaran et al. (2014b), which emphasises the importance of lure concentration and tissue type. Further investigating the effects of different male lure concentrations across tissue types such as head, thorax and abdomen would greatly improve the understanding of male lures in fruit flies.

4.6 CONCLUDING REMARKS

This study aimed to further explore the relationship between male lures and fruit flies by investigating the effects of cue-lure and zingerone on gene expression in *B. tryoni* and *B. jarvisi*. The results demonstrated that cue-lure feeding was associated with viral upregulation in *B. tryoni*, which to our knowledge, is the first report of this effect in fruit flies. Moreover, the results revealed potential immune system effects of lure feeding in the species tested, which warrants further investigation. Candidate genes for further functional analysis in *B. tryoni* and *B. jarvisi* were also identified. These results will help improve the understanding of male lures in fruit flies and may have implications in pest management. The outcomes of this study highlight the potential of using such an approach in other species, particularly those with evidenced attraction but no known biological effects in line with other species. Male lures and fruit flies provide a unique system to investigate the relationship between phytochemicals and insects, and the complexities of the system are worth further exploration.

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Appendices

APPENDIX A: PRELIMINARY LURE RESPONSE BIOASSAYS

The sensitivity of *Bactrocera* spp. to male lures can be measured by the number of male flies responding to a range of lure concentrations within a given time. The response of *Bactrocera* spp. has previously been investigated with methyl eugenol (Metcalf et al., 1975; Metcalf et al., 1979; Wee et al., 2002; Hee et al., 2015), raspberry ketone, and zingerone (Wee et al., 2018c); cue-lure has not yet been investigated. Therefore, this study aimed to investigate the responsiveness of *B. tryoni* to cue-lure.

Methods

Insects

Bactrocera tryoni pupae (10th generation) were obtained from a laboratory-reared culture maintained by the Department of Agriculture and Fisheries, Brisbane, Queensland. The pupae were moved into a mesh sleeved cage (BugDorm-4F3030, 32.5 x 32.5 x 32.5 cm, MegaView Science Co., Ltd., Taiwan) to emerge and were provided with water, sugar cubes (CSR® White Sugar Cubes, Australia), and hydrolysed yeast (MP Biomedicals, CAT 103304, USA) as required. The cage was kept in a dedicated laboratory maintained at 25.5 °C and 65 % RH with natural light and fluorescent lighting between 0600 and 1700 hours. Flies were sex separated into new mesh cages after ≤ 4 days of emergence to eliminate the bias of previous matings on lure response.

Chemicals

Analytical standard cue-lure (4-(4-Acetoxyphenyl)-2-butanone) ≥ 95 % purity (Sigma-Aldrich; CAS 3572-06-3) was diluted stepwise using absolute ethanol (≥ 99.5% purity, Sigma-Aldrich; CAS 64-17-5) to achieve concentrations of 2000, 1000, 500, 100, 10, and 5 ng/µL. These concentrations were selected based on pilot testing between 100 – 10,000 ng/uL to provide a range of response.

Cue-lure sensitivity bioassay

Metcalf et al. (1979) defined the positive response of *B. dorsalis* to methyl eugenol as a behavioural sequence of searching, arrestment, and feeding. Following this, a positive response of *B. tryoni* to cue-lure was recorded when the fly approached the lure (flying or walking), made direct contact and extended its probiscis to feed on the lure. The methods for the cue-lure sensitivity bioassays were based on the protocol of Wee et al. (2018c) with some modifications. Bioassays were conducted in a shaded location outside during October and November (spring/summer) in Brisbane, Queensland, and performed in clear weather. Sexually mature virgin male *B. tryoni* (15 – 24 days old) were tested across

3 days between 0800 – 1100 hr. Male fly colonies were moved from the laboratory to outside (separate area from experimental site) one hour prior for acclimatisation to the environment. Flies were transferred from the main male colony into mesh cages (32 x 32 x 32 cm) at the experimental site in 10 mL clear tubes and allowed five minutes to exit the tube before being prompted. The bioassays were conducted with 20 flies in a cage, two trials were run concurrently > 1 meter apart. In each cage, an inverted container was placed in the centre of the cage floor, 10 µL of cue-lure (2000, 1000, 500, 100, 10, 5 ng/µL) or ethanol (control) was pipetted onto a glass slide and given 30 seconds for solvent evaporation before being placed atop the container. Flies were monitored during the test duration and flies with a positive response were carefully captured with a 10 mL tube avoiding contact with the glass slide. After 10 minutes, remaining non-responsive flies were removed from the cage and discarded. A fresh batch of unexposed flies and new glass slides were used for every assay, the inverted container was cleaned with 70 % ethanol for 5 assays then replaced. The mesh cages were cleaned between different lure concentrations and days, all surfaces of the cage inside and outside were cleaned with 70 % ethanol, then cleaned with pressurised hot water and left in the sun for 12 hrs. There were seven replicates for each cue-lure concentration and five replicates for the control, the order of trials was randomised to remove potential positional effects. At the beginning of each bioassay, the light, humidity, temperature and time were recorded using an environmental meter (Extech Instruments; model 45170) to determine if these variables affect fly response. Wind was also recorded however this was not included in the analyses as most bioassays had 0 km/hr of wind. To visualise the data, boxplot and linear regression graphs were created with ggplot2 R package v3.4.1.

Results and discussion

Male *B. tryoni* response to different cue-lure concentrations was unexpectedly low. The number of responsive flies increased from 5 ng/µL – 100 ng/µL, however plateaued in response from 100 ng/µL – 2000 ng/µL (See Figure A 1). At 100 ng/µL, an average of 36 % ($\overline{x}$ = 7.3, σ = 3.99) of flies were responsive, and at 2000 ng/µL an average of 44 % ($\overline{x}$ = 8.71, σ = 2.71) of flies were responsive; from the pilot studies it was expected that response rate at 2000 ng/µL would be ~ 80 %. As demonstrated in Figure A 1, the response of flies at each concentration is highly variable; at 100 ng/µL one of the trials resulted in 0 responsive flies. There was no clear effect of time, temperature, humidity, or light on the number of responsive flies (Figure A 2). Plots A, B, C, and D (Figure A 2) show that most confidence intervals overlap, and each concentration group generally covered the same range in the abiotic variables. Therefore, it can be inferred that the abiotic factors did not introduce bias in response for any concentration group. Plots E, F, G, and H (Figure A 2) demonstrate no clear relationship between any of the abiotic variables and the number of flies responding. While the line of best fit suggests a negative relationship of fly response with time and light, and a positive relationship with humidity, the data is

too highly dispersed (red points) and is generally not linear; therefore, a linear relationship cannot be accurately inferred. Investigating abiotic effects on fruit fly response was not the aim of this study, if this objective were to be tested, it would be useful to expand the time frame and weather conditions for conducting bioassays.

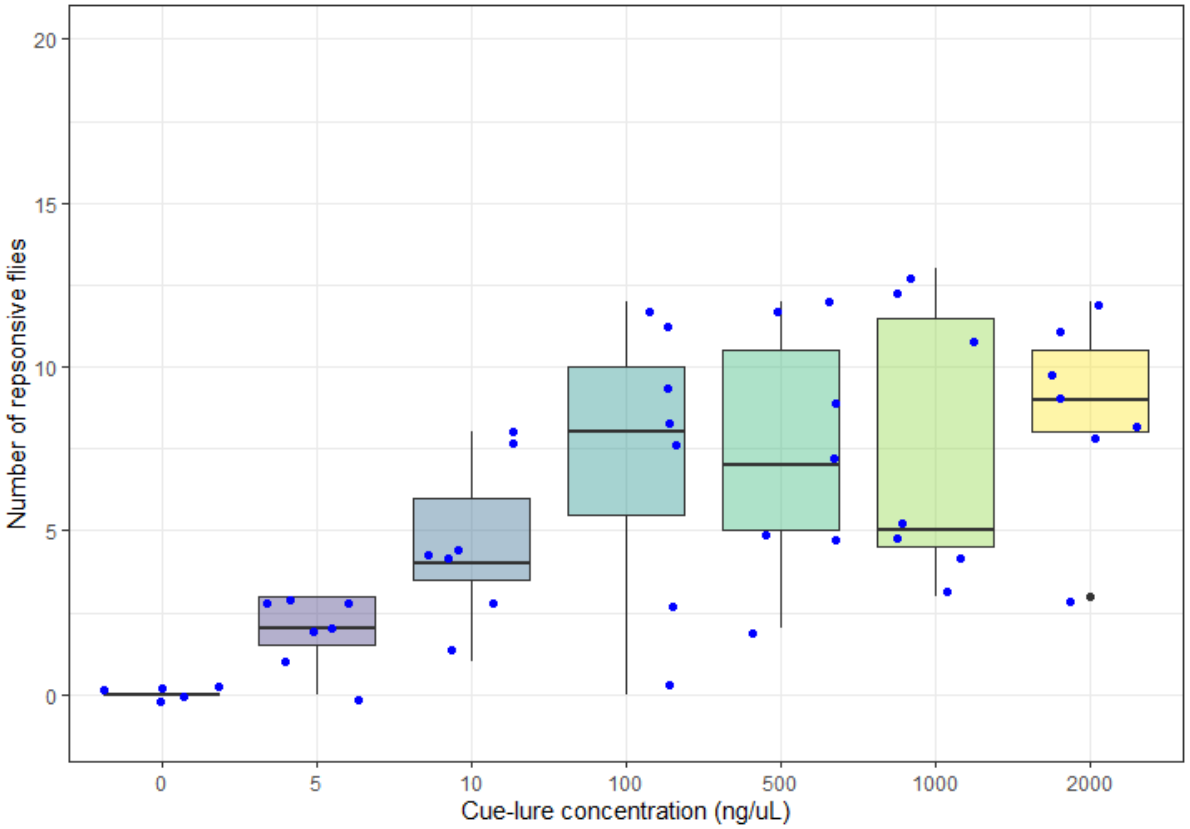

Figure A 1. Jitter boxplot of the response of male *B. tryoni* to different cue-lure concentrations.

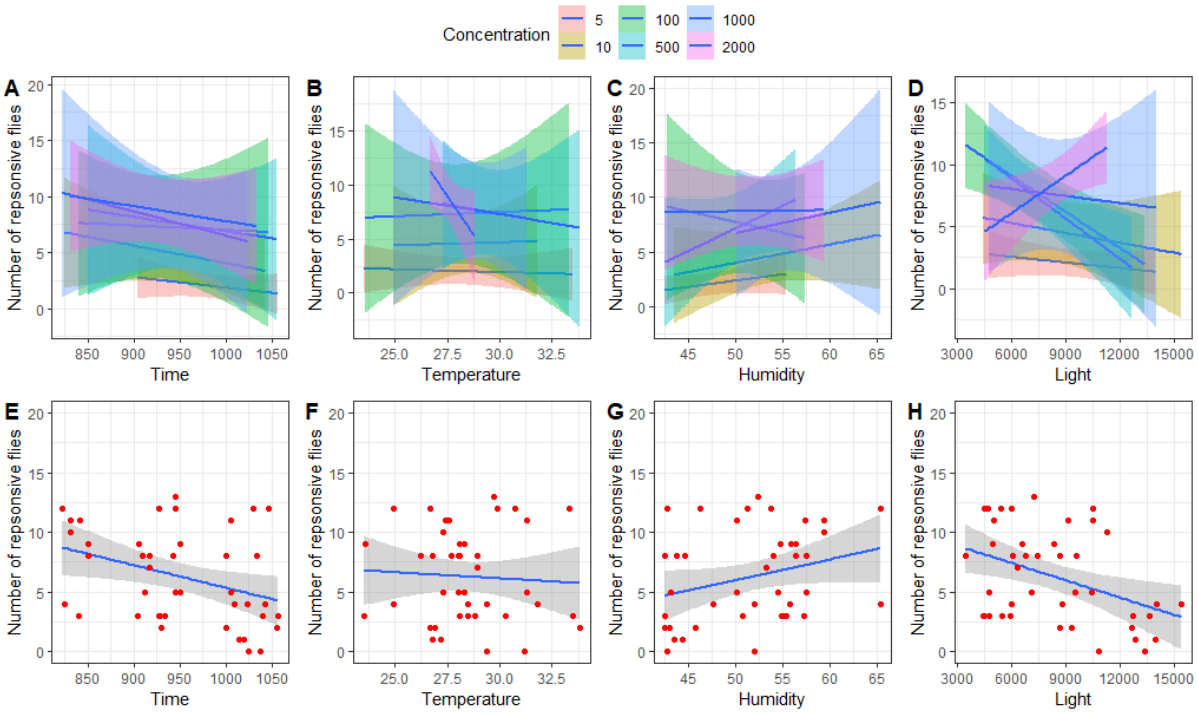

Figure A 2. Linear regression of abiotic effects on the number male *B. tryoni* responding to cue-lure. Plots A, B, C, and D demonstrate the effects of the abiotic variables on the repsonsive flies separated by the concentration. Plots E, F, G, and H demonstrate the effects of the abiotic variables on the responsive flies across all concentrations tested. The line of best fit was generated using geom_smooth(method = lm). The shaded area surrdouning the line of best fit represents 95 % CI.

Overall, the repsonse of *B. tryoni* to cue-lure was expected to be much higher. The presence of variable abiotic factors as a result of being outside may have reduced fly response. Laboratory reared flies are kept in stable temperature, humidity and light conditions, therefore the introduction to a new environment (even with time for acclimatisation) likely impacted the response rate. Pilot studies were conducted in a tempeature and humidity controlled environment, however this was unavailable for the bioassays. The difference in the expected (~ 80 % at 2000 ng/µL) compared to the actual response rate (44 % at 2000 ng/µL) indicates that abiotic variables may considerably affect the response of *B. tryoni* to cue-lure. This could be because the volatility of the chemical changes with abiotic changes, or that sensory functions in *B. tryoni* are impacted by abiotic changes, or a combination of both. Additionally, the age range of the flies may have been too large (15 – 24 days) for a concise result. While the results suggest that experimental refinement is necessary to measure *B. tryoni* response to cue-lure, they also suggest that investigating fruit fly response in laboratory conditions may not accurately reflect the response rate of species in the wild. Unfortunately, the sensitivity of *B. tryoni* to natural lure sources in the wild is largely unknown and probably very difficult to determine.

APPENDIX B: SUPPLEMENTARY FIGURES

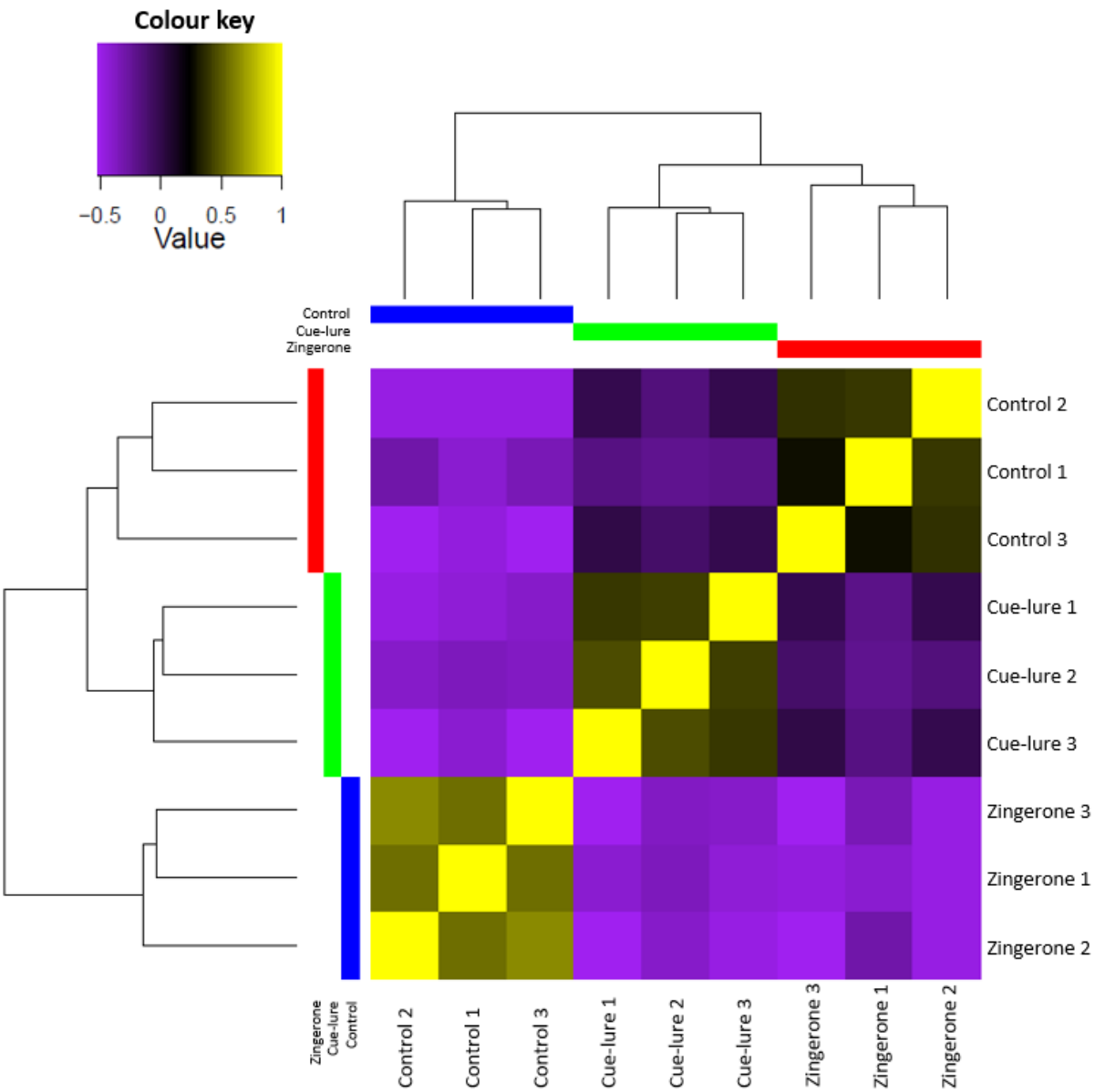

Figure A 3. Sample correlation matrix of differentially expressed genes for *B. tryoni* with treatments cue-lure fed, zingerone fed, and control (replicates = 1, 2, 3). TMM log2 centred values plotted.

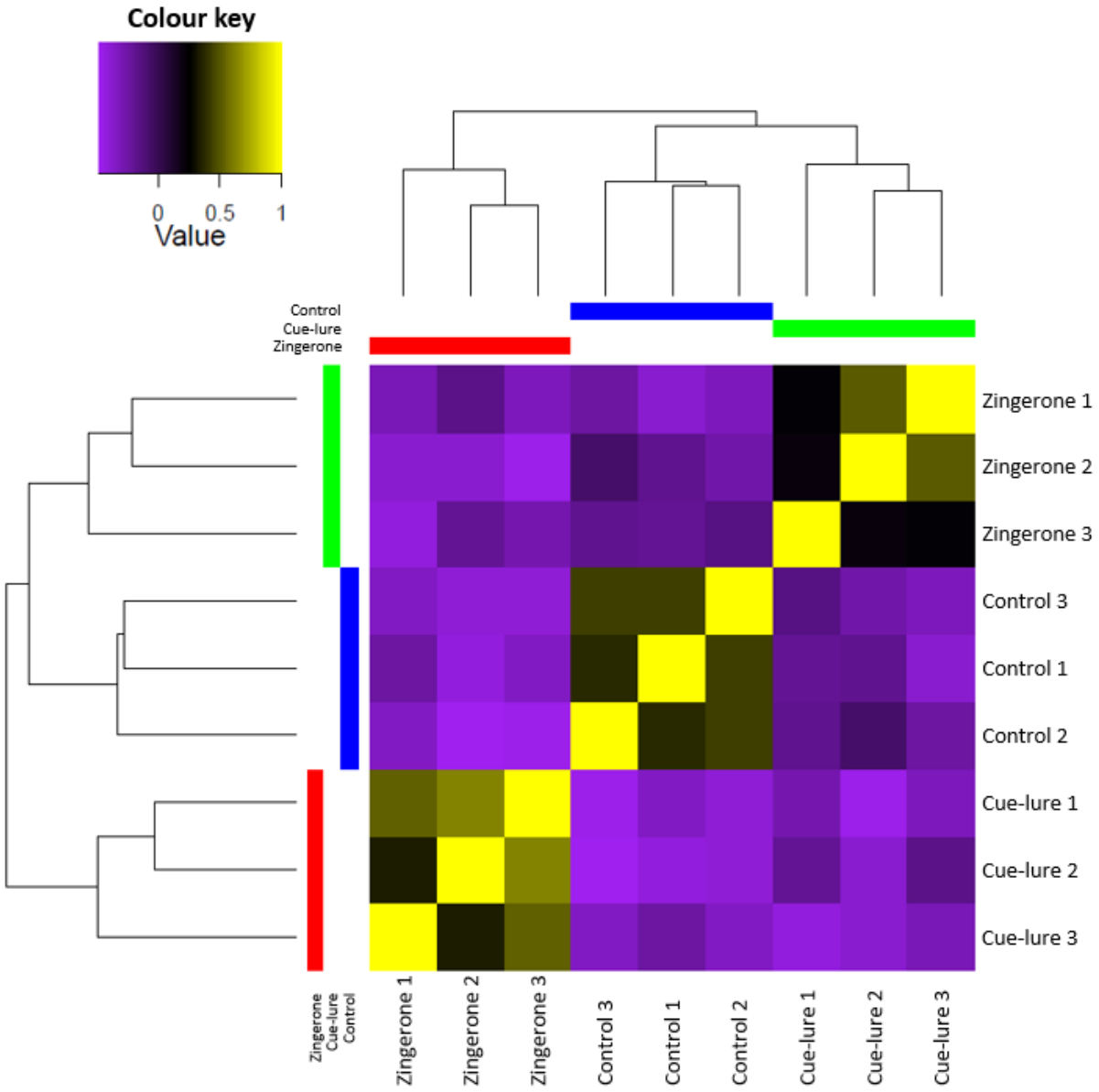

Figure A 4. Sample correlation matrix of differentially expressed genes for *B. jarvisi* with treatments cue-lure fed, zingerone fed, and control (replicates = 1, 2, 3). TMM log2 centred values plotted.

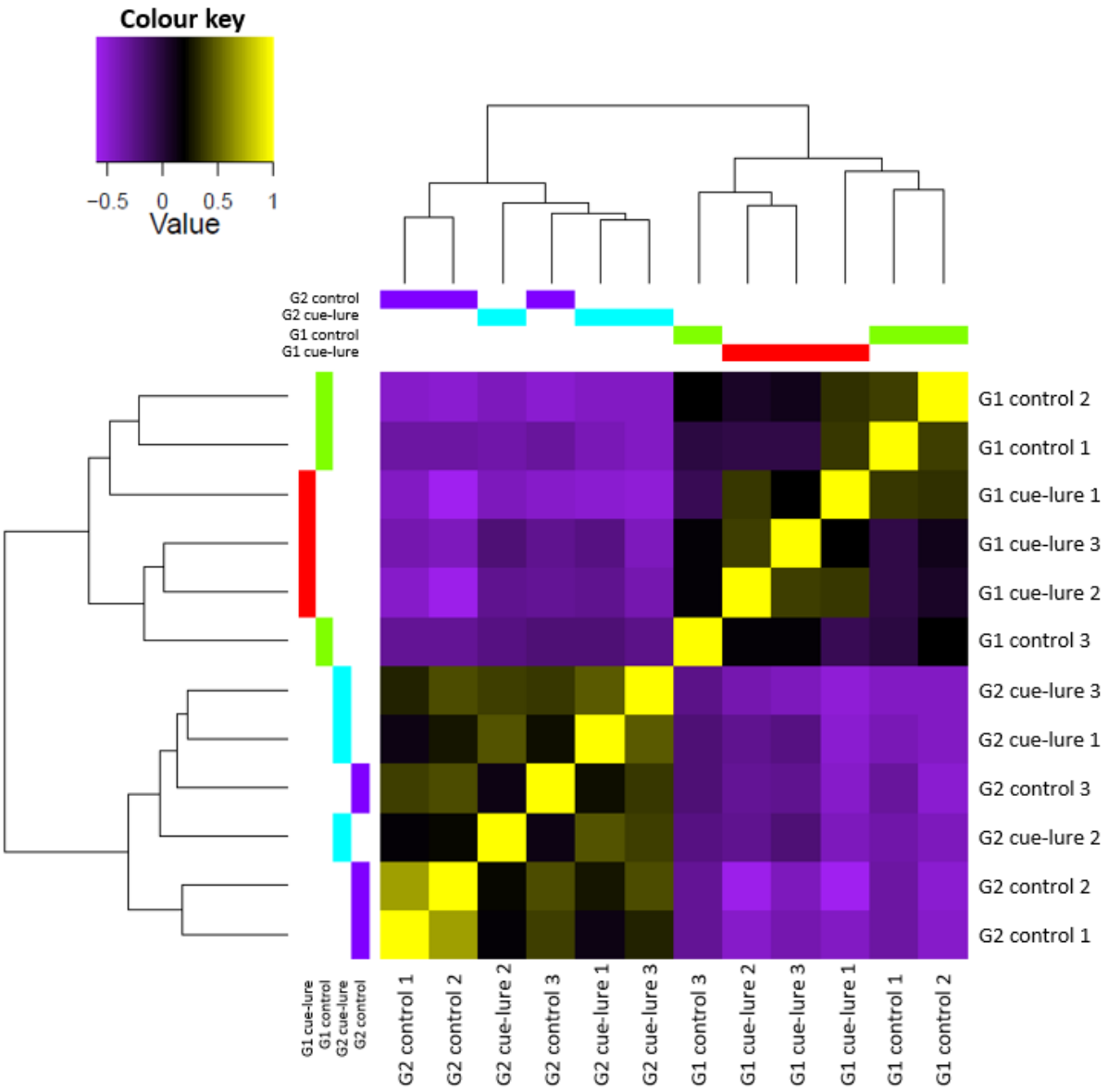

Figure A 5. Sample correlation matrix of differentially expressed genes for *B. tryoni* across two generations with cue-lure fed flies and control flies (replicates = 1, 2, 3). G1 = Generation 1. G2 = Generation 2. DESeq2 median of ratios log2 centred values plotted.

APPENDIX C: SUPPLEMENTARY TABLES

Table A 1. Read counts for *Bactrocera tryoni* and *B. jarvisi* before and after trimming with Trimmomatic (Study 1).

Bactrocera tryoni

Treatment and replicate	Raw read count	Read count after trimming	% of reads lost after trimming
Control 1	42,400,112	42,371,474	6.75×10^{-2}
Control 2	30,230,808	30,211,167	6.5×10^{-2}
Control 3	32,873,273	32,817,396	1.7×10^{-1}
Cue-lure 1	51,046,954	51,037,623	1.83×10^{-2}
Cue-lure 2	47,971,515	47,963,546	1.66×10^{-2}
Cue-lure 3	46,194,512	46,188,751	1.25×10^{-2}
Zingerone 1	58,947,409	58,942,135	8.95×10^{-3}
Zingerone 2	44,293,015	44,289,375	8.22×10^{-3}
Zingerone 3	56,542,649	56,524,185	3.26×10^{-2}
Average	45,611,139	45,593,961	3.77×10^{-2}
Standard deviation	9,090,103	9,099,469	4.68×10^{-2}

Bactrocera jarvisi

Treatment and replicate	Raw read count	Read count after trimming	% of reads lost after trimming
Control 1	41,338,826	41,317,865	5.07×10^{-2}
Control 2	42,556,478	42,534,997	5.04×10^{-2}
Control 3	44,628,472	44,598,309	6.76×10^{-2}
Cue-lure 1	53,394,291	53,386,294	1.5×10^{-2}
Cue-lure 2	49,382,500	49,379,113	6.9×10^{-3}
Cue-lure 3	40,954,984	40,951,513	8.5×10^{-3}
Zingerone 1	45,035,924	45,027,055	1.97×10^{-2}
Zingerone 2	47,145,351	47,140,397	1.05×10^{-2}
Zingerone 3	46,267,564	46,258,305	2×10^{-2}
Average	45,633,821	45,621,539	2.69×10^{-2}
Standard deviation	3,773,307	3,776,846	2.11×10^{-2}

Table A 2. Gene ontology (GO) analysis on differentially expressed genes in *Bactrocera tryoni* cue-lure fed flies vs control fed flies. GO terms reduced with REVIGO.

Enriched terms				
Ontology	ID	Term	p-value	FDR
BP	GO:0050794	regulation of cellular process	0.003251	1
BP	GO:0052652	cyclic purine nucleotide metabolic process	0.004283	1
MF	GO:0004016	adenylate cyclase activity	0.004489	1
BP	GO:0065007	biological regulation	0.006565	1
BP	GO:0006351	transcription, DNA-templated	0.00712	1
BP	GO:0009187	cyclic nucleotide metabolic process	0.008272	1
CC	GO:0043189	H4/H2A histone acetyltransferase complex	0.009658	1
MF	GO:0009975	cyclase activity	0.011056	1
MF	GO:0016849	phosphorus-oxygen lyase activity	0.011107	1
MF	GO:0019992	diacylglycerol binding	0.011471	1
BP	GO:0007268	chemical synaptic transmission	0.022792	1
BP	GO:0007267	cell-cell signaling	0.023515	1
BP	GO:0023052	signaling	0.024238	1
BP	GO:0007154	cell communication	0.027267	1
MF	GO:0008020	G protein-coupled photoreceptor activity	0.03218	1
MF	GO:0009881	photoreceptor activity	0.03218	1
BP	GO:0072521	purine-containing compound metabolic process	0.032675	1
BP	GO:0009582	detection of abiotic stimulus	0.034001	1
BP	GO:0051606	detection of stimulus	0.03543	1
BP	GO:0007601	visual perception	0.035781	1
MF	GO:0016829	lyase activity	0.039135	1

BP = biological process; CC = cellular component; MF = molecular function; FDR = False discovery rate

Table A 3. Gene ontology (GO) analysis on differentially expressed genes in *Bactrocera tryoni* zingerone fed flies vs control fed flies. GO terms reduced with REVIGO.

Enriched terms				
Ontology	ID	Term	p-value	FDR
BP	GO:0009617	response to bacterium	2.89E-07	0.000355
BP	GO:0006959	humoral immune response	3.71E-07	0.000355
BP	GO:0044419	interspecies interaction between organisms	7.73E-06	0.004124
BP	GO:0009607	response to biotic stimulus	8.03E-06	0.004124
BP	GO:0006952	defense response	8.61E-06	0.004124
BP	GO:0009605	response to external stimulus	6.04E-05	0.026711
BP	GO:0002376	immune system process	0.000273	0.098153
CC	GO:0005576	extracellular region	0.002071	0.661086
BP	GO:0009190	cyclic nucleotide biosynthetic process	0.006812	1
BP	GO:0009187	cyclic nucleotide metabolic process	0.006899	1
MF	GO:0003796	lysozyme activity	0.008615	1
CC	GO:0043189	H4/H2A histone acetyltransferase complex	0.00967	1
MF	GO:0016829	lyase activity	0.0351	1
MF	GO:0061783	peptidoglycan muralytic activity	0.04225	1
BP	GO:0006950	response to stress	0.045636	1

BP = biological process; CC = cellular component; MF = molecular function; FDR = False discovery rate

Table A 4. Gene ontology (GO) analysis on differentially expressed genes in *Bactrocera tryoni* cue-lure fed flies vs zingerone fed flies. GO terms reduced with REVIGO.

Enriched terms				
Ontology	ID	Term	p-value	FDR
BP	GO:0009617	response to bacterium	0.000275	0.789481
BP	GO:0044419	interspecies interaction between organisms	0.001599	1
BP	GO:0009607	response to biotic stimulus	0.001635	1
BP	GO:0006952	defense response	0.001673	1
BP	GO:0009605	response to external stimulus	0.004715	1
BP	GO:0006959	humoral immune response	0.006748	1
BP	GO:0006351	transcription, DNA-templated	0.012353	1
MF	GO:0016779	nucleotidyltransferase activity	0.015322	1
MF	GO:0003964	RNA-directed DNA polymerase activity	0.017964	1
MF	GO:0003676	nucleic acid binding	0.0239	1
CC	GO:0005576	extracellular region	0.02683	1
MF	GO:0016772	transferase activity, transferring phosphorus-containing groups	0.031826	1
MF	GO:0140640	catalytic activity, acting on a nucleic acid	0.044635	1
MF	GO:1901363	heterocyclic compound binding	0.047139	1
MF	GO:0097159	organic cyclic compound binding	0.047173	1
Depleted terms				
Ontology	ID	Term	p-value	FDR
BP	GO:0008152	metabolic process	0.005183	0.999998
BP	GO:0071704	organic substance metabolic process	0.008236	0.999998
BP	GO:0044238	primary metabolic process	0.009	0.999998
BP	GO:0009987	cellular process	0.010193	0.999998
BP	GO:0006807	nitrogen compound metabolic process	0.011043	0.999998
BP	GO:0044237	cellular metabolic process	0.0126	0.999998
BP	GO:0043170	macromolecule metabolic process	0.01462	0.999998
BP	GO:0008150	biological_process	0.023082	0.999998
BP	GO:0034641	cellular nitrogen compound metabolic process	0.029425	0.999998
BP	GO:1901360	organic cyclic compound metabolic process	0.03394	0.999998
BP	GO:0006725	cellular aromatic compound metabolic process	0.034298	0.999998
BP	GO:0046483	heterocycle metabolic process	0.034843	0.999998
BP	GO:0090304	nucleic acid metabolic process	0.04246	0.999998
BP	GO:0044260	cellular macromolecule metabolic process	0.049053	0.999998

BP = biological process; CC = cellular component; MF = molecular function; FDR = False discovery rate

Table A 5. Gene ontology analysis (GO) on differentially expressed gene subclusters in *Bactrocera tryoni* with treatments cue-lure fed, zingerone fed, and control. Subclusters with consistent patterns across replicates were analysed, see Figure 2 (Results) for plots of subclusters 5, 6, 7, 9 and 10. GO terms reduced with REVIGO.

Subcluster 3: Enriched terms				
Ontology	ID	Term	p-value	FDR
BP	GO:0009190	cyclic nucleotide biosynthetic process	0.001949	1
BP	GO:0009187	cyclic nucleotide metabolic process	0.001984	1
MF	GO:0003796	lysozyme activity	0.004693	1
MF	GO:0016829	lyase activity	0.012388	1
BP	GO:0035556	intracellular signal transduction	0.01852	1
BP	GO:0055086	nucleobase-containing small molecule metabolic process	0.022671	1
MF	GO:0061783	peptidoglycan muralytic activity	0.023855	1
MF	GO:0008527	taste receptor activity	0.028421	1
BP	GO:0034654	nucleobase-containing compound biosynthetic process	0.029747	1
BP	GO:0019438	aromatic compound biosynthetic process	0.030794	1
BP	GO:0018130	heterocycle biosynthetic process	0.031056	1
BP	GO:1901362	organic cyclic compound biosynthetic process	0.031362	1
MF	GO:0003824	catalytic activity	0.043676	1
BP	GO:0019637	organophosphate metabolic process	0.044554	1
MF	GO:0004016	adenylate cyclase activity	0.046045	1

Subcluster 5: Enriched terms				
Ontology	ID	Term	p-value	FDR
CC	GO:0043189	H4/H2A histone acetyltransferase complex	0.006384	1
BP	GO:0006355	regulation of transcription, DNA-templated	0.037681	1
BP	GO:0032940	secretion by cell	0.045856	1
BP	GO:0140352	export from cell	0.045856	1

Subcluster 6: Enriched terms				
Ontology	ID	Term	p-value	FDR
MF	GO:0003676	nucleic acid binding	0.027605	1

Subcluster 8: Enriched terms				
Ontology	ID	Term	p-value	FDR
MF	GO:0003964	RNA-directed DNA polymerase activity	0.000767	1
CC	GO:0005615	extracellular space	0.02399	1
MF	GO:0016779	nucleotidyltransferase activity	0.024073	1
MF	GO:0016740	transferase activity	0.025624	1
MF	GO:0008017	microtubule binding	0.032184	1
MF	GO:0140097	catalytic activity, acting on DNA	0.033195	1
MF	GO:0003777	microtubule motor activity	0.040099	1
MF	GO:0016772	transferase activity, transferring phosphorus-containing groups	0.041281	1

Subcluster 8: Enriched terms *continued*				
Ontology	ID	Term	p-value	FDR
CC	GO:0099513	polymeric cytoskeletal fiber	0.044229	1
MF	GO:0003774	motor activity	0.049238	1
BP	GO:0007018	microtubule-based movement	0.049242	1
MF	GO:0140640	catalytic activity, acting on a nucleic acid	0.049999	1

Subcluster 11: Enriched terms				
Ontology	ID	Term	p-value	FDR
MF	GO:0019992	diacylglycerol binding	0.002543	1
BP	GO:0007268	chemical synaptic transmission	0.004526	1
BP	GO:0007267	cell-cell signaling	0.004858	1
BP	GO:0023052	signaling	0.005191	1
BP	GO:0007154	cell communication	0.006255	1
CC	GO:0045202	synapse	0.012897	1
BP	GO:0052652	cyclic purine nucleotide metabolic process	0.016444	1
MF	GO:0004016	adenylate cyclase activity	0.016623	1
BP	GO:0009187	cyclic nucleotide metabolic process	0.023442	1
MF	GO:1901363	heterocyclic compound binding	0.026464	1
MF	GO:0097159	organic cyclic compound binding	0.026484	1
MF	GO:0009975	cyclase activity	0.029135	1
MF	GO:0016849	phosphorus-oxygen lyase activity	0.029431	1
CC	GO:0030054	cell junction	0.031306	1
MF	GO:0003674	molecular_function	0.031776	1
MF	GO:0005488	Binding	0.038141	1
MF	GO:0008289	lipid binding	0.045879	1

BP = biological process; CC = cellular component; MF = molecular function; FDR = False discovery rate

Table A 6. Gene ontology (GO) analysis on differentially expressed genes in *Bactrocera jarvisi* cue-lure fed flies vs control fed flies. GO terms reduced with REVIGO.

Enriched terms				
Ontology	ID	Term	p-value	FDR
MF	GO:0042302	structural constituent of cuticle	3.49E-05	0.198441
MF	GO:0005198	structural molecule activity	0.002481	1
CC	GO:0071797	LUBAC complex	0.016565	1
MF	GO:0004714	transmembrane receptor protein tyrosine kinase activity	0.017397	1
BP	GO:0043248	proteasome assembly	0.018767	1
CC	GO:0000502	proteasome complex	0.023839	1
MF	GO:0060090	molecular adaptor activity	0.031548	1
Depleted terms				
Ontology	ID	Term	p-value	FDR
MF	GO:0016787	hydrolase activity	0.002875	0.999999
BP	GO:0008150	biological_process	0.023596	0.999999
MF	GO:0016788	hydrolase activity, acting on ester bonds	0.043977	0.999999
BP	GO:0009058	biosynthetic process	0.04493	0.999999
BP	GO:0008152	metabolic process	0.044961	0.999999
CC	GO:0043226	organelle	0.045891	0.999999
BP	GO:1901576	organic substance biosynthetic process	0.046019	0.999999
BP	GO:0009987	cellular process	0.04821	0.999999

BP = biological process; CC = cellular component; MF = molecular function; FDR = False discovery rate

Table A 7. Gene ontology (GO) analysis on differentially expressed genes in *Bactrocera jarvisi* zingerone fed flies vs control fed flies. GO terms reduced with REVIGO.

Enriched				
Ontology	ID	Term	p-value	FDR
BP	GO:0009605	response to external stimulus	7.09E-05	0.150143
BP	GO:0009617	response to bacterium	7.91E-05	0.150143
BP	GO:0044419	interspecies interaction between organisms	0.000335	0.227128
BP	GO:0006952	defense response	0.000348	0.227128
BP	GO:0009607	response to biotic stimulus	0.000359	0.227128
CC	GO:0005576	extracellular region	0.001591	0.905668
CC	GO:0000938	GARP complex	0.004986	1
MF	GO:0004335	galactokinase activity	0.010732	1
BP	GO:0016482	cytosolic transport	0.013846	1
MF	GO:0008378	galactosyltransferase activity	0.017806	1
MF	GO:0016263	glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase activity galactosyltransferase activity	0.017806	1
BP	GO:0006493	protein O-linked glycosylation	0.018506	1
BP	GO:0006012	galactose metabolic process	0.01857	1
BP	GO:0007200	phospholipase C-activating G protein-coupled receptor signaling pathway	0.020756	1
BP	GO:0009453	energy taxis	0.020756	1
CC	GO:0016027	inaD signaling complex	0.020756	1
BP	GO:0042331	phototaxis	0.020756	1
BP	GO:0043052	thermotaxis	0.020756	1
BP	GO:0046668	regulation of retinal cell programmed cell death	0.020756	1
BP	GO:0048667	cell morphogenesis involved in neuron differentiation	0.020756	1
CC	GO:0097038	perinuclear endoplasmic reticulum	0.020756	1
BP	GO:0104004	cellular response to environmental stimulus	0.022151	1
MF	GO:0140103	catalytic activity, acting on a glycoprotein	0.022411	1
BP	GO:0051093	negative regulation of developmental process	0.024625	1
BP	GO:0006119	oxidative phosphorylation	0.025842	1
CC	GO:0110165	cellular anatomical entity	0.028193	1
CC	GO:0099023	tethering complex	0.028529	1
BP	GO:0050896	response to stimulus	0.02886	1
MF	GO:0008020	G protein-coupled photoreceptor activity	0.037351	1
MF	GO:0009881	photoreceptor activity	0.037351	1
BP	GO:0006959	humoral immune response	0.03886	1
MF	GO:0140658	ATP-dependent chromatin remodeler activity	0.040565	1
BP	GO:0051606	detection of stimulus	0.044755	1
BP	GO:0010941	regulation of cell death	0.046262	1
BP	GO:0005996	monosaccharide metabolic process	0.047618	1
CC	GO:0031984	organelle subcompartment	0.048127	1

BP = biological process; CC = cellular component; MF = molecular function; FDR = False discovery rate

Table A 8. Gene ontology (GO) analysis on differentially expressed genes in *Bactrocera jarvisi* cue-lure fed flies vs zingerone fed flies. GO terms reduced with REVIGO.

Enriched terms				
Ontology	ID	Term	p-value	FDR
BP	GO:0009605	response to external stimulus	0.000121	0.379232
BP	GO:0044419	interspecies interaction between organisms	0.000497	0.379232
BP	GO:0006952	defense response	0.000516	0.379232
BP	GO:0002376	immune system process	0.000523	0.379232
BP	GO:0009607	response to biotic stimulus	0.000533	0.379232
MF	GO:0061783	peptidoglycan muralytic activity	0.001468	0.89326
BP	GO:0050896	response to stimulus	0.00211	1
MF	GO:0015085	calcium ion transmembrane transporter activity	0.002465	1
CC	GO:0034702	ion channel complex	0.007141	1
CC	GO:0098796	membrane protein complex	0.011326	1
MF	GO:0004984	olfactory receptor activity	0.012846	1
CC	GO:1990351	transporter complex	0.013792	1
MF	GO:0003796	lysozyme activity	0.018258	1
MF	GO:0004035	alkaline phosphatase activity	0.018638	1
CC	GO:0005576	extracellular region	0.019801	1
MF	GO:0008378	galactosyltransferase activity	0.020533	1
MF	GO:0016263	glycoprotein-N-acetylgalactosamine 3-beta-	0.020533	1
BP	GO:0006493	protein O-linked glycosylation	0.021246	1
BP	GO:0007200	phospholipase C-activating G protein-coupled receptor	0.023408	1
BP	GO:0009453	energy taxis	0.023408	1
CC	GO:0016027	inaD signaling complex	0.023408	1
BP	GO:0042331	phototaxis	0.023408	1
BP	GO:0043052	thermotaxis	0.023408	1
BP	GO:0046668	regulation of retinal cell programmed cell death	0.023408	1
BP	GO:0048667	cell morphogenesis involved in neuron differentiation	0.023408	1
CC	GO:0097038	perinuclear endoplasmic reticulum	0.023408	1
BP	GO:0104004	cellular response to environmental stimulus	0.025081	1
MF	GO:0140103	catalytic activity, acting on a glycoprotein	0.026213	1
MF	GO:0005549	odorant binding	0.027713	1
BP	GO:0051093	negative regulation of developmental process	0.02829	1
CC	GO:0005769	early endosome	0.031314	1
BP	GO:0000270	peptidoglycan metabolic process	0.037061	1
BP	GO:0006959	humoral immune response	0.042434	1
MF	GO:0060089	molecular transducer activity	0.046087	1
MF	GO:1901702	salt transmembrane transporter activity	0.047722	1
Depleted terms				
Ontology	ID	Term	p-value	FDR
NA	GO:0140640	catalytic activity, acting on a nucleic acid	0.01014	0.999994
MF	GO:0016772	transferase activity, transferring phosphorus-containing	0.0124	0.999994
MF	GO:0016740	transferase activity	0.013194	0.999994
MF	GO:0003964	RNA-directed DNA polymerase activity	0.015622	0.999994

Depleted terms *continued*				
Ontology	ID	Term	p-value	FDR
MF	GO:0140097	catalytic activity, acting on DNA	0.015642	0.999994
MF	GO:0043167	ion binding	0.024278	0.999994
MF	GO:0016779	nucleotidyltransferase activity	0.027161	0.999994
BP	GO:0044237	cellular metabolic process	0.043523	0.999994
MF	GO:0003824	catalytic activity	0.047512	0.999994

BP = biological process; CC = cellular component; MF = molecular function; FDR = False discovery rate

Table A 9. Gene ontology (GO) analysis on differentially expressed gene subclusters in *Bactrocera jarvisi* with treatments cue-lure fed, zingerone fed, and control. Subclusters with consistent patterns across replicates were analysed, see Figure 3 (Results) for plots of subclusters 5, 6, 7, 9 and 10. GO terms reduced with REVIGO.

Subcluster 1: Enriched terms				
Ontology	ID	Term	p-value	FDR
CC	GO:0000938	GARP complex	0.004796	1
MF	GO:0004984	olfactory receptor activity	0.010173	1
BP	GO:0016482	cytosolic transport	0.013036	1
MF	GO:0005549	odorant binding	0.0217	1
CC	GO:0099023	tethering complex	0.027314	1
Subcluster 1: Depleted terms				
Ontology	ID	Term	p-value	FDR
MF	GO:0003824	catalytic activity	0.004185	0.999991
MF	GO:0016740	transferase activity	0.009101	0.999991
NA	GO:0140640	NA	0.018553	0.999991
MF	GO:0043167	ion binding	0.019697	0.999991
MF	GO:0003964	RNA-directed DNA polymerase activity	0.020585	0.999991
MF	GO:0016772	transferase activity, transferring phosphorus-containing groups	0.022338	0.999991
MF	GO:0140097	catalytic activity, acting on DNA	0.026578	0.999991
CC	GO:0005575	cellular_component	0.034683	0.999991
MF	GO:0016787	hydrolase activity	0.041978	0.999991
MF	GO:0016779	nucleotidyltransferase activity	0.043296	0.999991
Subcluster 2: Enriched terms				
Ontology	ID	Term	p-value	FDR
BP	GO:0009605	response to external stimulus	0.008735	1
CC	GO:0071797	LUBAC complex	0.01125	1
BP	GO:0007200	phospholipase C-activating G protein-coupled receptor signaling pathway	0.013026	1
BP	GO:0009589	detection of UV	0.013026	1
CC	GO:0016027	inaD signaling complex	0.013026	1
BP	GO:0046668	regulation of retinal cell programmed cell death	0.013026	1
BP	GO:0048667	cell morphogenesis involved in neuron differentiation	0.013026	1
CC	GO:0097038	perinuclear endoplasmic reticulum	0.013026	1
MF	GO:0004714	transmembrane receptor protein tyrosine kinase activity	0.013347	1
BP	GO:0043248	proteasome assembly	0.013518	1
BP	GO:0042330	taxis	0.013553	1
BP	GO:0104004	cellular response to environmental stimulus	0.014079	1
BP	GO:0051093	negative regulation of developmental process	0.015866	1
CC	GO:0005769	early endosome	0.01754	1
CC	GO:0000502	proteasome complex	0.017965	1
BP	GO:0006959	humoral immune response	0.023796	1
MF	GO:0060090	molecular adaptor activity	0.023974	1
MF	GO:0008020	G protein-coupled photoreceptor activity	0.023983	1

Subcluster 2: Enriched terms *continued*				
Ontology	ID	Term	p-value	FDR
MF	GO:0140658	ATP-dependent chromatin remodeler activity	0.026756	1
BP	GO:0051606	detection of stimulus	0.028911	1
BP	GO:0010941	regulation of cell death	0.03089	1
CC	GO:0031984	organelle subcompartment	0.031052	1
BP	GO:0050793	regulation of developmental process	0.039288	1
BP	GO:0009628	response to abiotic stimulus	0.039386	1
CC	GO:0005576	extracellular region	0.040819	1
Subcluster 4: Enriched terms				
Ontology	ID	Term	p-value	FDR
MF	GO:0004335	galactokinase activity	0.007092	1
MF	GO:0008378	galactosyltransferase activity	0.012455	1
MF	GO:0016263	glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase activity	0.012455	1
BP	GO:0006493	protein O-linked glycosylation	0.012995	1
BP	GO:0006012	galactose metabolic process	0.013218	1
MF	GO:0140103	catalytic activity, acting on a glycoprotein	0.016459	1
BP	GO:0000270	peptidoglycan metabolic process	0.023015	1
MF	GO:0008745	N-acetylmuramoyl-L-alanine amidase activity	0.023015	1
MF	GO:0061783	peptidoglycan muralytic activity	0.03348	1
BP	GO:0005996	monosaccharide metabolic process	0.035907	1
Subcluster 4: Depleted terms				
Ontology	ID	Term	p-value	FDR
MF	GO:0140640	catalytic activity, acting on a nucleic acid	0.019038	0.999979
MF	GO:0140097	catalytic activity, acting on DNA	0.026227	0.999979
MF	GO:0016779	nucleotidyltransferase activity	0.038626	0.999979

BP = biological process; CC = cellular component; MF = molecular function; FDR = False discovery rate

Table A 10. Iflavirus genes in *Bactrocera tryoni* and *B. jarvisi* transcriptomes (study one). All genes were initially queried against the UniRef90 database, genes with NCBI:txid2795009 (Bactrocera tryoni iflavirus 1) were further queried against the NCBI nr database. Results from the NCBI database are presented below.

	B. tryoni				
Gene name	Accession	E-value	Identity %	Length bp	Gene ID
B. bryoniae iflavirus 1 RNA helicase, RdRp gene for 8043	HG993808.1	0	99	8043	TRINITY_DN65214_c11_g1_i1
B. bryoniae iflavirus 1 RNA helicase, RdRp gene for 8043	HG993808.1	1E-124	100	8043	TRINITY_DN65218_c14_g1_i1
B. bryoniae iflavirus 1 RNA helicase, RdRp gene for 8043	HG993808.1	1E-84	100	8043	TRINITY_DN18780_c0_g1_i1
B. bryoniae iflavirus 1 RNA helicase, RdRp gene for 8043	HG993808.1	0	100	8043	TRINITY_DN15440_c0_g1_i1
B. bryoniae iflavirus 1 RNA helicase, RdRp gene for 8043	HG993808.1	2E-142	100	8043	TRINITY_DN20854_c1_g1_i3
B. bryoniae iflavirus 1 RNA helicase, RdRp gene for 8043	HG993808.1	0	99	8043	TRINITY_DN15940_c0_g2_i3
B. bryoniae iflavirus 1 RNA helicase, RdRp gene for 8043	HG993808.1	0	100	8043	TRINITY_DN15940_c0_g2_i8
B. tryoni iflavirus 1 strain c polyprotein gene, complete cds	MW208810.1	0	97	10113	TRINITY_DN197_c0_g1_i16
B. tryoni iflavirus 1 strain c polyprotein gene, complete cds	MW208810.1	0	97	10113	TRINITY_DN197_c0_g1_i8
B. tryoni iflavirus 1 strain c polyprotein gene, complete cds	MW208810.1	3E-58	96	10113	TRINITY_DN11570_c0_g1_i1
B. tryoni iflavirus 2 Capsid proteins, RNA helicase, RdRp gene for 8133	HG994125.1	7E-102	100	8133	TRINITY_DN65218_c15_g1_i1
B. tryoni iflavirus 2 Capsid proteins, RNA helicase, RdRp gene for 8133	HG994125.1	0	100	8133	TRINITY_DN74460_c9_g1_i1
B. tryoni iflavirus 2 Capsid proteins, RNA helicase, RdRp gene for 8133	HG994125.1	9E-81	100	8133	TRINITY_DN15440_c5_g1_i1
B. tryoni iflavirus 2 Capsid proteins, RNA helicase, RdRp gene for 8133	HG994125.1	9E-141	100	8133	TRINITY_DN15440_c7_g1_i1
B. tryoni iflavirus 2 Capsid proteins, RNA helicase, RdRp gene for 8133	HG994125.1	0	100	8133	TRINITY_DN5765_c0_g1_i1
B. tryoni iflavirus 2 Capsid proteins, RNA helicase, RdRp gene for 8133	HG994125.1	0	100	8133	TRINITY_DN15940_c21_g1_i1
B. tryoni iflavirus 2 Capsid proteins, RNA helicase, RdRp gene for 8133	HG994125.1	0	98	8133	TRINITY_DN32968_c0_g1_i2
B. tryoni iflavirus 2 Capsid proteins, RNA helicase, RdRp gene for 8133	HG994125.1	0	100	8133	TRINITY_DN67977_c8_g1_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	1E-57	99	8113	TRINITY_DN65295_c2_g1_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	0	78	8113	TRINITY_DN197_c0_g1_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	0	98	8113	TRINITY_DN197_c0_g1_i29
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	0	94	8113	TRINITY_DN197_c0_g1_i6
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	0	100	8113	TRINITY_DN197_c2_g2_i1

B. tryoni *continued*					
Gene name	Accession	E-value	Identity %	Length bp	Gene ID
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	0	99	8113	TRINITY_DN197_c2_g4_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	0	100	8113	TRINITY_DN197_c2_g5_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	1E-158	99	8113	TRINITY_DN197_c2_g6_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	0	100	8113	TRINITY_DN197_c3_g5_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	3E-100	100	8113	TRINITY_DN197_c3_g6_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	0	100	8113	TRINITY_DN197_c3_g7_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	2E-103	100	8113	TRINITY_DN197_c6_g1_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	0	99	8113	TRINITY_DN197_c8_g2_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	0	100	8113	TRINITY_DN197_c8_g7_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	0	100	8113	TRINITY_DN197_c8_g8_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	0	100	8113	TRINITY_DN197_c8_g10_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	2E-163	100	8113	TRINITY_DN3591_c0_g1_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	0	99	8113	TRINITY_DN3765_c3_g1_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	7E-76	97	8113	TRINITY_DN20804_c0_g1_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	0	100	8113	TRINITY_DN20854_c0_g4_i10
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	1E-93	99	8113	TRINITY_DN37771_c0_g2_i2
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	2E-97	100	8113	TRINITY_DN6035_c0_g1_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	9E-121	100	8113	TRINITY_DN15940_c2_g1_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	1E-119	100	8113	TRINITY_DN15940_c22_g1_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	3E-128	100	8113	TRINITY_DN32968_c0_g1_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	0	100	8113	TRINITY_DN32968_c0_g1_i8
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	2E-56	100	8113	TRINITY_DN32968_c2_g1_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	0	100	8113	TRINITY_DN27850_c0_g1_i2
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	4E-152	100	8113	TRINITY_DN46395_c0_g2_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	4E-59	98	8133	TRINITY_DN67974_c8_g1_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	6E-63	100	8113	TRINITY_DN58926_c8_g1_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	7E-123	100	8113	TRINITY_DN58935_c4_g1_i1

***B. tryoni** continued*					
Gene name	Accession	E-value	Identity %	Length bp	Gene ID
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	7E-69	100	8113	TRINITY_DN58912_c9_g1_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	8E-75	100	8113	TRINITY_DN71238_c2_g1_i1
B. jarvisi					
Gene name	Accession	E-value	Identity %	Length bp	Gene ID
B. kraussi iflavirus 1 RdRp gene for 10130	HG993805.1	1E-80	97	10130	TRINITY_DN77034_c0_g1_i1
B. tryoni iflavirus 1 strain c polyprotein gene, complete cds	MW208810.1	3E-22	97	10113	TRINITY_DN72287_c0_g1_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	8E-141	100	8113	TRINITY_DN66996_c0_g1_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	8E-161	100	8113	TRINITY_DN73561_c0_g1_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	2E-128	100	8113	TRINITY_DN63187_c0_g1_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	0	100	8113	TRINITY_DN68999_c0_g1_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	5E-97	100	8113	TRINITY_DN74702_c0_g1_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	1E-158	100	8113	TRINITY_DN43777_c0_g1_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	2E-123	100	8113	TRINITY_DN43777_c0_g2_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	0	100	8113	TRINITY_DN43777_c0_g3_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	8E-101	100	8113	TRINITY_DN43777_c0_g4_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	2E-102	100	8113	TRINITY_DN43783_c0_g1_i1

B. = Bactrocera

Table A 11. Read counts for *Bactrocera tryoni* before and after trimming with Trimmomatic (Study 2).

Treatment and replicate	Raw read count	Read count after trimming	% of reads lost after trimming
Generation 1 cue-lure 1	24,611,627	24,609,873	7.13×10^{-3}
Generation 1 cue-lure 2	42,523,846	42,517,506	1.5×10^{-2}
Generation 1 cue-lure 3	39,858,601	39,852,566	1.51×10^{-2}
Generation 1 control 1	42,208,755	42,202,818	1.41×10^{-2}
Generation 1 control 2	42,519,133	42,512,532	1.55×10^{-2}
Generation 1 control 3	37,939,400	37,937,260	5.64×10^{-3}
Generation 2 cue-lure 1	34,176,738	34,174,809	5.64×10^{-3}
Generation 2 cue-lure 2	42,491,641	42,489,036	6.13×10^{-3}
Generation 2 cue-lure 3	41,281,882	41,277,594	1.04×10^{-2}
Generation 2 control 1	36,648,116	36,645,723	6.53×10^{-3}
Generation 2 control 2	43,441,024	43,438,337	6.19×10^{-3}
Generation 2 control 3	35,481,996	35,479,667	6.56×10^{-3}
Average	38,598,563	38,594,810	9.49×10^{-3}
Standard deviation	5,166,755	5,165,657	4.02×10^{-3}

Table A 12. *De novo* transcriptome assembly statistics for study two *Bactrocera tryoni* compared with the statistics for study one *B. tryoni* and *B. jarvisi de novo* transcriptome assemblies.

	***B. tryoni* study two**	***B. tryoni* study one**	***B. jarvisi* study one**
Transcript count	123145	129687	140039
Gene count	84545	90008	93614
N50[1]	1964	1555	1415
E90N50[2]	2246	2565	2508
E90 transcripts	2031	5669	5959

[1] Based on longest isoform per gene. [2] N50 calculation based on the highly expressed transcripts.

Table A 13. Iflavirus genes in the *Bactrocera tryoni* transcriptome (study two). All genes were initially queried against the UniRef90 database, genes with NCBI:txid2795009 (Bactrocera tryoni iflavirus 1) were further queried against the NCBI nr database. Results from the NCBI database are presented below.

Gene name	Accession	E-value	Identity %	Length bp	Gene ID
B. tryoni iflavirus 1 strain c polyprotein gene, complete cds	MW208810.1	5E-110	98	10113	TRINITY_DN7629_c0_g1_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	0	74	8113	TRINITY_DN101_c0_g1_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	0	98	8113	TRINITY_DN101_c0_g2_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	0	100	8113	TRINITY_DN101_c4_g1_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	0	100	8113	TRINITY_DN101_c4_g2_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	0	99	8113	TRINITY_DN101_c4_g3_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	0	100	8113	TRINITY_DN101_c4_g4_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	0	99	8113	TRINITY_DN101_c4_g5_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	0	100	8113	TRINITY_DN101_c4_g6_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	0	100	8113	TRINITY_DN101_c4_g7_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	0	100	8113	TRINITY_DN101_c4_g8_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	2E-63	100	8113	TRINITY_DN28493_c0_g1_i11
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	0	100	8113	TRINITY_DN28493_c0_g2_i2
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	0	99	8113	TRINITY_DN28493_c10_g1_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	0	100	8113	TRINITY_DN28493_c19_g1_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	3E-127	99	8113	TRINITY_DN28861_c1_g1_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	4E-79	100	8113	TRINITY_DN41364_c0_g3_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	2E-122	100	8113	TRINITY_DN41364_c7_g1_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	1E-111	100	8113	TRINITY_DN4479_c0_g1_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	7E-129	100	8113	TRINITY_DN45000_c0_g2_i3
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	1E-65	100	8113	TRINITY_DN57192_c9_g1_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	4E-120	100	8113	TRINITY_DN57199_c4_g1_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	1E-73	99	8113	TRINITY_DN57199_c5_g1_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	0	100	8113	TRINITY_DN57202_c2_g1_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	3E-88	100	8113	TRINITY_DN57202_c6_g1_i1

Continued

Gene name	Accession	E-value	Identity %	Length bp	Gene ID
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	4E-159	100	8113	TRINITY_DN57241_c1_g1_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	2E-117	100	8113	TRINITY_DN60006_c0_g1_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	4E-79	100	8113	TRINITY_DN60029_c1_g1_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	2E-142	100	8113	TRINITY_DN62860_c1_g1_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	7E-136	100	8113	TRINITY_DN65744_c0_g1_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	7E-142	100	8113	TRINITY_DN65800_c1_g1_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	4E-120	100	8113	TRINITY_DN65800_c3_g1_i1
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	0	100	8113	TRINITY_DN71488_c9_g1_i2
B. tryoni iflavirus 3 partial Capsid protein, RNA helicase, RdRp gene for 8113	HG994126.1	2E-62	100	8113	TRINITY_DN71562_c6_g1_i1

B. = Bactrocera

Table A 14. Gene ontology enrichment analysis on differentially expressed genes in *Bactrocera tryoni* between generation one and generation two (reduced with REVIGO; FDR < 0.05).

Enriched GO terms			
Ontology	ID	Term	p-value
MF	GO:0003735	structural constituent of ribosome	3.38E-21
CC	GO:0005840	ribosome	1.49E-18
BP	GO:0006412	translation	1.85E-18
BP	GO:0043603	cellular amide metabolic process	2.69E-14
BP	GO:1901566	organonitrogen compound biosynthetic process	1.86E-12
CC	GO:0005575	cellular_component	2.44E-12
CC	GO:0043228	non-membrane-bounded organelle	6.00E-12
CC	GO:0098796	membrane protein complex	2.00E-10
BP	GO:0065007	biological regulation	3.35E-10
BP	GO:0008150	biological_process	3.36E-09
BP	GO:0050789	regulation of biological process	1.62E-08
BP	GO:0009058	biosynthetic process	2.28E-08
CC	GO:0032991	protein-containing complex	6.61E-08
CC	GO:0110165	cellular anatomical entity	7.91E-08
BP	GO:0022900	electron transport chain	1.00E-07
MF	GO:0003674	molecular_function	1.08E-07
MF	GO:0003723	RNA binding	1.95E-07
BP	GO:0006811	ion transport	2.06E-07
CC	GO:0098798	mitochondrial protein complex	1.39E-06
BP	GO:0019538	protein metabolic process	3.14E-06
MF	GO:0015078	proton transmembrane transporter activity	3.86E-06
MF	GO:0009055	electron transfer activity	5.30E-06
BP	GO:0042592	homeostatic process	6.03E-06
BP	GO:0009987	cellular process	1.04E-05
BP	GO:0006812	cation transport	1.26E-05
MF	GO:0004672	protein kinase activity	1.30E-05
BP	GO:0006091	generation of precursor metabolites and energy	1.40E-05
BP	GO:1901564	organonitrogen compound metabolic process	1.93E-05
MF	GO:0008289	lipid binding	2.64E-05
BP	GO:0048878	chemical homeostasis	2.88E-05
MF	GO:0005198	structural molecule activity	3.13E-05
CC	GO:1990904	ribonucleoprotein complex	4.27E-05
BP	GO:0007610	behavior	5.30E-05
BP	GO:0051179	localization	5.40E-05
MF	GO:0015453	oxidoreduction-driven active transmembrane transporter activity	5.49E-05
CC	GO:0043226	organelle	6.50E-05
MF	GO:0005488	binding	8.66E-05
BP	GO:0065008	regulation of biological quality	9.35E-05
MF	GO:0019843	rRNA binding	0.000152
Enriched GO terms *continued*			
Ontology	ID	Term	p-value

MF	GO:0044877	protein-containing complex binding	0.000203
CC	GO:0098803	respiratory chain complex	0.000209
CC	GO:0031090	organelle membrane	0.000224
CC	GO:0005739	mitochondrion	0.000247
BP	GO:0006754	ATP biosynthetic process	0.000304
BP	GO:0048518	positive regulation of biological process	0.000328
CC	GO:0070469	respiratory chain	0.000371
BP	GO:0007626	locomotory behavior	0.00043
MF	GO:0016740	transferase activity	0.000453
BP	GO:0051173	positive regulation of nitrogen compound metabolic process	0.000545
BP	GO:0048869	cellular developmental process	0.000625
BP	GO:0048519	negative regulation of biological process	0.000633
BP	GO:0031399	regulation of protein modification process	0.000663
MF	GO:0005515	protein binding	0.000696
BP	GO:0098662	inorganic cation transmembrane transport	0.00074
MF	GO:0005543	phospholipid binding	0.00078
BP	GO:0051246	regulation of protein metabolic process	0.00157

Depleted GO terms

Ontology	ID	Term	p-value
MF	GO:0004984	olfactory receptor activity	4.94E-07
BP	GO:0006259	DNA metabolic process	5.64E-07
MF	GO:0042302	structural constituent of cuticle	7.73E-07
MF	GO:0008094	ATP-dependent activity, acting on DNA	7.84E-07
BP	GO:0006281	DNA repair	1.10E-05
BP	GO:0051276	chromosome organization	4.29E-05
MF	GO:0004252	serine-type endopeptidase activity	9.92E-05

BP = Biological process. CC = Cellular component. MF = Molecular function.

Table A 15. Gene ontology enrichment analysis on differentially expressed genes in *Bactrocera tryoni* generation one cue-lure fed flies and generation two control flies (reduced with REVIGO; FDR < 0.05).

Enriched GO terms			
Ontology	ID	Term	p-value
MF	GO:0003735	structural constituent of ribosome	6.43E-19
BP	GO:0006412	translation	2.57E-16
CC	GO:0005840	ribosome	3.23E-12
BP	GO:0043603	cellular amide metabolic process	5.87E-12
BP	GO:0022904	respiratory electron transport chain	1.05E-09
BP	GO:1901566	organonitrogen compound biosynthetic process	1.26E-09
BP	GO:0019538	protein metabolic process	2.95E-08
BP	GO:0065007	biological regulation	3.02E-08
CC	GO:0043228	non-membrane-bounded organelle	4.39E-08
MF	GO:0009055	electron transfer activity	6.96E-08
CC	GO:0005575	cellular_component	1.36E-07
CC	GO:0005743	mitochondrial inner membrane	1.49E-07
MF	GO:0015453	oxidoreduction-driven active transmembrane transporter activity	2.48E-07
BP	GO:0006091	generation of precursor metabolites and energy	5.73E-07
MF	GO:0005198	structural molecule activity	2.35E-06
MF	GO:0004672	protein kinase activity	2.39E-06
MF	GO:0015078	proton transmembrane transporter activity	5.22E-06
BP	GO:0050794	regulation of cellular process	5.97E-06
BP	GO:1901564	organonitrogen compound metabolic process	1.87E-05
BP	GO:0008150	biological_process	2.59E-05
CC	GO:0070469	respiratory chain	3.86E-05
MF	GO:0016655	oxidoreductase activity, acting on NAD(P)H, quinone or similar compound as acceptor	6.41E-05
MF	GO:0016651	oxidoreductase activity, acting on NAD(P)H	6.58E-05
CC	GO:0043226	organelle	9.86E-05
BP	GO:0042592	homeostatic process	0.000102
MF	GO:0016791	phosphatase activity	0.000121
BP	GO:0065008	regulation of biological quality	0.000126
CC	GO:0005739	mitochondrion	0.000155
CC	GO:0110165	cellular anatomical entity	0.00016
CC	GO:0098796	membrane protein complex	0.000196
CC	GO:1990904	ribonucleoprotein complex	0.000211
CC	GO:0045261	proton-transporting ATP synthase complex, catalytic core F(1)	0.000214
BP	GO:0006777	Mo-molybdopterin cofactor biosynthetic process	0.000259
BP	GO:0051189	prosthetic group metabolic process	0.000259
BP	GO:0007626	locomotory behavior	0.00029
BP	GO:0006812	cation transport	0.000403
MF	GO:0019843	rRNA binding	0.000415
Enriched GO terms *continued*			
Ontology	ID	Term	p-value

CC	GO:0032991	protein-containing complex	0.000422
CC	GO:0070069	cytochrome complex	0.000495
BP	GO:0006811	ion transport	0.000635

BP = Biological process. CC = Cellular component. MF = Molecular function.

Table A 16. Gene ontology enrichment analysis on differentially expressed genes in *Bactrocera tryoni* between generation one control flies and generation two cue-lure fed flies (reduced with REVIGO; FDR < 0.05).

Depleted GO terms			
Ontology	ID	Term	p-value
CC	GO:0005634	nucleus	3.32E-07
CC	GO:0043226	organelle	3.30E-06
BP	GO:0090304	nucleic acid metabolic process	8.28E-06
BP	GO:0043170	macromolecule metabolic process	4.78E-05
MF	GO:0003676	nucleic acid binding	5.03E-05

BP = Biological process. CC = Cellular component. MF = Molecular function.

Table A 17. Transposition-related genes differentially expressed in *Bactrocera tryoni* generation one cue-lure fed flies vs generation one control flies and generation two cue-lure flies vs generation two control flies. The best hit annotation was selected to represent the gene. Annotation was performed against the Uniref90 database.

G1 cue-lure vs G1 control						
Gene name	Accession	E-value	Identity %	Length bp	Taxon	Gene ID
Transposable element Tc3 transposase	A0A0A1X466	5.705E-283	86.7	1455	Dacini 43871	LOC120770412
G2 cue-lure vs G2 control						
Gene name	Accession	E-value	Identity %	Length bp	Taxon	Gene ID
Tigger transposable element-derived protein 1 (Fragment)	A0A0A1XAN0	9.611E-192	87.3	942	Dacini 43871	LOC120769715
Tigger transposable element-derived protein 1 (Fragment)	A0A0A1XAN0	5.429E-193	86.4	942	Dacini 43871	LOC120774861
Tigger transposable element-derived protein 1 (Fragment)	A0A0A1XAN0	6.312E-191	87.0	942	Dacini 43871	LOC120776068
Tigger transposable element-derived protein 1-like	A0A6J2YL06	3.401E-251	86.0	1686	Endopterygota 33392	LOC120781831
Tigger transposable element-derived protein 6	A0A2S2PW91	1.051E-132	56.1	1365	Aphididae 27482	LOC120779334
Tigger transposable element-derived protein 6	A0A2S2PW91	1.669E-130	58.5	1365	Aphididae 27482	LOC120779332
PiggyBac transposable element-derived protein 4	A0A4C1UXE0	1.159E-169	52.7	2091	*Eumeta japonica* 151549	LOC120770630