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Sexual selection in true fruit flies (Diptera: Tephritidae): transcriptome and experimental evidences for phytochemicals increasing male competitive ability

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Abstract

In male tephritid fruit flies of the genus *Bactrocera*, feeding on secondary plant compounds (*sensu lato* male lures = methyl eugenol, raspberry ketone and zingerone) increases male mating success. Ingested male lures alter the male pheromonal blend, normally making it more attractive to females and this is considered the primary mechanism for the enhanced mating success. However, the male lures raspberry ketone and zingerone are known, across a diverse range of other organisms, to be involved in increasing energy metabolism. If this also occurs in *Bactrocera*, then this may represent an additional benefit to males as courtship is metabolically expensive and lure feeding may increase a fly's short-term energy. We tested this hypothesis by performing comparative RNA-seq analysis between zingerone-fed and unfed males of *Bactrocera tryoni*. We also carried out behavioural assays with zingerone and cuelure-fed males to test if they became more active. RNA-seq analysis revealed, in zingerone-fed flies, up regulation of 3183 genes with homologues transcripts to those known to regulate inter-male aggression, pheromone synthesis, mating and accessory gland proteins, along with significant enrichment of several energy metabolic pathways and GO terms. Behavioural assays show significant increases in locomotor activity, weight reduction and successful

mating after mounting; all direct/indirect measures of increased activity. These results suggest that feeding on lures leads to complex physiological changes, which result in more competitive males. These results do not negate the pheromone effect, but do strongly suggest that the phytochemical-induced sexual selection is governed by both female preference and male competitive mechanisms.

Key Words: *Bactrocera tryoni***,** mating, genome, sequences, courtship, cuelure, metabolism, zingerone, EST, gene expression, mate choice, Red Bull, competition

Introduction

Insect-plant interactions, in addition to their importance for the ecology of most terrestrial systems (Schoonhoven *et al*. 2005), are central to the management of pests of agriculture and forestry (Price *et al*. 1980; Horn 1988). Many plant-insect interactions are driven, or at least modified, by plant secondary chemistry, of which plant chemicals detrimental to insects (e.g. tannins, toxins) are perhaps the best known (Rosenthal & Berenbaum 1992). However, such interactions can be more complex and beneficial to both plant and insect. For example, in the case of pollinator-plant associations involving South American orchids and euglossine bees, the orchids attract bee pollinators by offering phytochemicals which are used by the bees in their own sexual communication (Zimmermann *et al*. 2006). In plants of the genus *Senecio* the production of pyrolizidine alkaloids deter many generalist herbivores, but for specialist herbivores such as arctiid moths the chemicals are sequestered by the feeding larvae and subsequently used for their own anti-predators defence (Rothschild *et al*. 1979).

In true fruit flies (Diptera: Tephritidae), males of many *Bactrocera* Macquart species and *Ceratitis capitata* (Wiedemann) demonstrate strong, positive olfactory and gustatory responses to a

number of chemically related phytochemicals such as methyl eugenol, raspberry ketone (=cuelure) and zingerone (Drew & Hooper 1981; Tan & Nishida 2007), and these compounds are commonly involved in sexual selection by increasing male mating success (Raghu 2004; Shelly *et al*. 2004; Shelly 2010; Kumaran *et al*. 2013, but see Raghu & Clarke 2003). The prevailing hypothesis to explain this selection links the male mating success to a more attractive pheromone blend released by males after feeding on plant compounds (Hee & Tan 1998; Khoo & Tan 2000; Shelly 2010; Tan & Nishida 2012).

Our previous studies with the Queensland fruit fly, *Bactrocera tryoni* (Froggatt), showed enhanced mating success in males fed on cuelure and zingerone (Kumaran *et al*. 2013), and also changes in pheromonal compounds after lure feeding and subsequent enhanced female attraction to modified pheromone moieties of males fed on cuelure (Kumaran *et al*. 2014a). While these results support the pheromone hypothesis, females did not show enhanced attraction to the pheromone of males fed with zingerone, despite zingerone-fed males having a mating advantage over control males. Further, when lure-fed males (both lure types) were calling, mating occurred more quickly than for control males, i.e. the mating latency period was shorter (Kumaran *et al*. 2013). However, no such change in the latency period was seen when females were exposed to the male pheromone for either cuelure or zingerone; i.e. more females responded to the cuelure enhanced male pheromone than to the control pheromone, but they did not do so more quickly (Kumaran *et al*. 2014a). This result, combined with the mating success of zingerone-fed males in the absence of an enhanced pheromone blend, suggest that the male mating success observed in *B. tryoni* after lure feeding is partly governed by a male factor unrelated to female preference, as has been previously hypothesised.

An alternate (in the case of zingerone), or additive (in the case of cuelure) hypothesis to explain male mating advantage following lure feeding in males lies in possible metabolic effects of the lures. Raspberry ketone (the hydroxy equivalent of cuelure) and zingerone are known to be associated with enhanced energy metabolism in shrimp, fish and mammals (Venkatramalingam *et al*. 2007; Park 2010; Chang *et al*. 2012). If this also occurs in fruit flies, then the lures may act as energy boosters, making males more active after lure feeding. More active males may perform better in male-male competition, courtship displays, or in prevailing-over females resisting copulation, providing a possible explanation for the 'hidden' male mating effect. In *B. cacuminata* (Hering), no changes in primary nutritional reserves such as fat or carbohydrates were found following ingestion of methyl eugenol (Raghu *et al*. 2002); however, we speculate male lures provide a short term energy boost, akin to caffeine, which does not substantially change primary reserves (Graham *et al*. 2000). This hypothesis is not competing to the male pheromone enhancement hypothesis, but provides additional or alternate explanations on how males benefit by feeding on plant compounds.

In addition to pheromonal and hypothesised metabolic effects, phytochemical lures rendered indirect effects in female *B. tryoni* mated with lure fed males and offspring sired; those being increased fecundity, decreased remating receptivity and reduced longevity (Kumaran *et al*. 2013; Kumaran & Clarke 2013). These changes in female reproductive phenotype suggest that lure feeding may change the male ejaculate; as such post-copulatory female changes have been attributed to male accessory gland proteins (Acps) in *Drosophila* and *Bactrocera* (Chapman 2001; Radhakrishnan & Taylor 2007).

In summary, we hypothesize there are potentially at least three complex mechanisms underpinning lure induced sexual selection in *Bactrocera*, including: (i) more attractive pheromones;

and/or (ii) physiologically more active males; and/or (iii) modified male Acps. While direct behavioural and pheromonal studies provide strong evidence for the first of these (Hee & Tan 1998; Khoo & Tan 2000; Shelly 2010; Tan & Nishida 2012), evidence for the last two is still indirect and based on inference. To explicitly test the second mechanism, and indirectly the third, in this paper we developed two RNA-seq libraries, one for males fed on zingerone and the other for males deprived of zingerone. We present differentially expressed candidate genes, and undertake gene set enrichment analysis of differentially expressed genes to determine if genes and gene ontology (GO) groups that are known to be, or possibly involved in inter-male aggression, pheromone emission, courtship, mating, Acps and general metabolism are enriched in zingerone-fed males. In addition, to test if males become more active following lure feeding, we studied four parameters related to physical fitness and energy use, *viz*., (i) increase in wing beat frequency and calling bout duration during courtship (Mankin *et al*. 2008); (ii) increase in locomotor activity; (iii) increase in metabolic activity; and (iv) successful copulations after male mounting, a measure of male quality in prevailing-over resisting females (Myers 1952; Barton Browne 1957). While transcriptome studies used only zingerone, behavioural assays were performed using cuelure and zingerone.

Methods

Insect source

Bactrocera tryoni were obtained as pupae from the rearing facility at the [Queensland Government] Department of Agriculture Fisheries and Forestry, Brisbane. Emerged flies were provided with water, sugar and protein hydrolysate *ad libitum*, and maintained at 27°C and 70% RH in a room illuminated with natural light in addition to fluorescent lighting between 07:00 and 16:00 hours every day. Flies were sexed within two days of emergence, and sexually mature virgin flies were used for all studies. To obtain lure-fed males, flies were provided with 1.5ml of cuelure (International pheromone systems Ltd., >95% purity) or zingerone (10ug/µl of 95% ethanol, Sigma-

Aldrich, CHEME, GmbH, Germany, >96% purity) on a cotton wick placed on inverted Petri dish for 2 h. The concentrations, dilutions and presentation methods are based on previous studies on other *Bactrocera* flies (Shelly & Villalobos 1995; Hee & Tan 1998).

Experiment 1: Differential expression of candidate genes

RNA isolation, cDNA library synthesis, EST assembly and annotation

A total of 20, 14 day-old virgin male flies from each group (zingerone-fed and zingerone-deprived) were collected and snap frozen in liquid nitrogen six hours after lure exposure during time of sexual activity. Total RNA was extracted from whole bodies using Trizol and purified with a Qiagen RNeasy kit following the manufacturer's instructions. Detailed methodology on isolation, library construction, assembly and annotation, along with sequence files, contigs, unigenes and sequence assembly are presented in the companion paper Arthofer *et al*. (2014).

Analysis of differentially expressed genes

Sequencing reads were mapped to unigenes from two samples and converted to Fragments Per kb per Million fragments (FPKM) to determine which genes were differentially expressed (DEGs) between lure-fed and unfed conditions. P value threshold for the analysis was determined using False Discovery Rate (FDR). Smaller FDR value and larger FPKM ratio shows a greater difference in expression levels. In this analysis, we chose FDR ≤ 0.001 and FPKM ratio larger than two for significant expression abundance between lure-fed and unfed flies. We undertook gene set enrichment analysis to determine if particular GO categories were over represented in the DEGs. For the identification of significantly enriched metabolic pathways or signal transduction pathways, we mapped all DEGs to terms to the KEGG database using enzyme codes. Pathways with Qvalue ≤ 0.05 were considered significantly enriched in DEGs.

Validation of gene expression profile by qRT-PCR

Four genes were randomly chosen for the qRT-PCR analysis to confirm the DEG data. Qiagen RT-PCR kit was used with Taqman fluorophores and quenchers following manufacturer's protocol with RNA extracted as described for the DGE library preparation and sequencing. The 16s rRNA was used as an internal control. The primers, fluorophores and quenchers used for qRT-PCR detection are listed in Table S1. Primers were designed with *primer 3* using the same settings as Prentis *et al*. (2010). The 20 µL PCR reaction contained 10 µL of RT-PCR master mix, 0.2 µL of QuantiFast RT mix, 1 µL each of two target genes, 1 µL of internal control and 6.8 µL of template RNA and RNase-free water combination for triplex assay. Two-step reaction was performed under the following hold and cycling conditions: Hold 1: 50 °C for 20 min; Hold 2: 95 °C for 5 min; Cycling 1: 95 °C for 15 sec; and Cycling 2: 60 °C for 30 sec with 40 cycles. The relative gene expression data were analyzed using the comparative $2^{-\Delta\Delta Ct}$ method. All quantitative PCR analyses were repeated in three biological samples.

Experiment 2: Behavioural assays

Wing beat frequency and calling bout duration

Acoustic signals were recorded by releasing a virgin male and female pair (14 – 17 days old) into a glass bottle (6 cm high and 3.5 cm diameter) plugged with sponge. The sponge was hollow in the centre to tightly hold a studio condenser microphone (Behringer B-5, Japan) to record wing beating. The apparatus was placed within a clear Perspex cage to minimize external noise. When male calling (*sensu* Tychsen 1977) was observed, the buzzing sound produced was received through the microphone and recorded using a pocket recorder (Pocketrak, Yamaha Corporation, Japan). A preamplifier (Xenyx502, Behringer, Germany) provided +48V phantom power for the microphone input connected via mono channels. The activity of flies was simultaneously filmed using a digital HD camcorder (Canon LEGRIA HV40, Japan). The recording of courtship wing beat was done until male calling activity ceased or copulation ensued.

After recording was completed, audio and video files were synchronized and the duration of male calling recorded. Sound files were further analysed using Raven 1.2 software (Cornell Lab of Ornithology, Ithaca, NY). Fundamental frequencies of calling bouts and of single calling bout durations (CBD) (*sensu* Webb *et al*. 1976; Mankin *et al*. 2008) were determined from the audio file (Fig. 1). Audio information from at least 20 males for each treatment group (cuelure-fed, zingerone-fed and lure unfed) was captured, with an average of six calling bouts produced per male (375 calling bouts in total from the three groups). Fundamental frequency and CBD were calculated for each calling bout.

Locomotor activity

To measure the locomotor activity, a 14 to 16 day-old unmated male was transferred into a 15 cm long test-tube with the open end plugged with cotton-wool. The test-tube was placed against a cm rule and direct observations were made over a 10 min period on: (i) total distance walked; (ii) the number of times the test-tube midpoint was crossed by the fly; and (iii) total resting time. Observations were made between 10:00 and 16:00 hours and rotated across the three treatment groups to minimize temporal effects. Twenty males were observed for each of the three treatments. This methodology was adapted from Dominiak *et al*. (2014), who considered locomotor activity a useful quality measure test for fruit flies mass reared for the sterile insect technique.

Weight loss and water content

To evaluate changes in energy level/metabolism after lure feeding, we monitored fly weight change over a 14 day period as reduction is weight is positively correlated with increased energy metabolism and aggression (Schuster & Levitsky 1982). Two-hundred and forty virgin male flies for each treatment were kept in small cages (60 flies per cage), where they had *ad libitum* access to

sugar, water and protein. On a given day, between 13:00 and 15:00 hours, 30 males (14 - 27 day-old) from each group were cooled in a refrigerator and weighed (fresh weight) using a Sartorius analytical balance with 0.0001 g precision. The flies were then rapidly killed in a freezer and dried in an oven at 42 °C for two days, after which dry weights were obtained. Percent water content was calculated as ([Wet weight – Dry weight]/Wet weight) * 100. Weight was measured one day prior to exposure to lures, on the day of exposure, and 1, 3, 7, 10 and 14 days after exposure. While a starvation diet regime may have detected weight changes due to increased metabolic rate more easily than a non-starvation regime, we fed the test flies as a more accurate representation of what might happen in the field and any observed result can be considered a conservative test.

Proportion of successful mating attempts versus failed attempts

Bactrocera tryoni exhibit lek mating in which advertising males mount on encountered females (Tychsen 1977); however, females very commonly reject mounting males by actively moving their legs and wings to dislodge them (Myers 1952; Barton Browne 1957). We hypothesized that if lure feeding increased male activity and metabolic rate, then lure-fed males may be better able to resist female rejection following mounting, and so achieve greater rates of successful copulation. To test this, 15 virgin males and 15 virgin females (14 - 17 day-old) were released into a small Perspex cage 2 h prior to dusk and we recorded the total number of mounting attempts and the number of subsequent successful copulations. Eight replicate cages for each lure treatment were run.

Statistical analyses

The means of all behavioural parameters recorded were compared using one-way ANOVA with a Tukey's *post-hoc* analysis at a probability level of $\alpha = 0.05$. Data were tested for assumptions

on homoscedasticity and any violations were corrected using appropriate transformations. For descriptive analysis mean ± 1SE are presented.

Results

Experiment 1: Differential expression of candidate genes

Sequencing and sequence assembly

RNA sequencing of zingerone-fed and zingerone-deprived male flies resulted in 53,482,202 high quality reads that resulted in 4,813,398,180 bp of sequence data. Clean nucleotides obtained from zingerone-fed and zingerone-deprived libraries are 2,475,846,000 and 2,337,552,180 respectively. Trinity assembled the clean reads into contigs and further refinement of this assembly was done using paired end information to obtain unigenes. The contig size distribution of fed and unfed libraries is presented in Fig. S1. Refer the companion paper, Arthofer *et al*. (2014), for information on complete assembly statistics.

Annotation of predicted proteins

Unigene sequences from the overall assembly were used as BLASTx and BLASTn queries against the GenBank NR and NT databases, Swissprot, KEGG, COG and GO with a cut-off E-value of 10^{-5}. A total of 25,389 sequences, representing approximately 68.5 % of contigs, matched known genes with distinct biological functions. The majority of sequences with blast hits (66.2%) had strong homology with *Drosophila* proteins (Fig. 2a). The other sequences made up 33.8% and had hits with other insect species including the dipterans *Anopheles gambiae* Giles*, Musca domestica* L.*, Glossina morsitans morsitans* Westwood and *Bactrocera dorsalis* (Hendel). Identity distribution analysis showed most of the hits (31.6%) had 60 - 80% identity with other insects (Fig. 2b).

Gene ontology (GO), clusters of orthologous group (COG) classification and metabolic pathway analysis

Gene ontology terms were assigned for sequences that received successful blast hits that contained functional annotations. In total, 148,570 GO terms were assigned to unigenes and were categorised into 52 functional groups with 25 biological process, 15 molecular function and 12 cellular component categories (Fig. S2). The most commonly assigned biological processes GO terms were cellular, metabolic and developmental processes, response to stimulus, signalling and biological regulation. The GO terms most frequently assigned from cellular components GO category were cell, cell part and organelle. In the case of molecular function GO category, binding, catalytic activity and transporter activity are the most commonly assigned GO terms.

Assignment of clusters of orthologous groups (COG) were used to predict and classify possible functions of unigenes. Based on sequence homology, 18,001 unigenes were annotated and divided into 25 COG categories with the most common category being general function followed by transcription, and carbohydrate transport and metabolism (Fig. S3).

The unigene metabolic pathway analysis was done using KEGG annotation system. In total 16,268 unigenes were mapped to 247 pathways. Metabolic pathways contained 2,160 (13.28%) unigenes, which was followed by pathways in cancer (659 – 4.05%), regulation of actin cytoskeleton (606 – 3.73%), focal adhesion (561 – 3.45%) and purine metabolism (503 – 3.09%).

Enriched pathways and GO terms

To investigate over-representation of gene categories, libraries from fed and unfed males were tested for enrichment of GO terms and pathways. There were several GO terms and KEGG pathways identified from transcripts of zingerone-fed and zingerone-deprived libraries (Dryad Digital Repository, doi:10.5061/dryad.v6qj0). Within the GO terms, there were several transcripts related to energy and metabolism enriched in zingerone-fed libraries (Fig. 3). The most significantly enriched GO terms include metabolic processes, oxidation reduction process, mitochondrion, oxido-reductase activity and catalytic activity that are presented in Table 1. Likewise, within the differentially expressed transcripts in KEGG pathways, several enriched pathways had transcripts related to energy metabolism in zingerone-fed libraries. The highly significantly enriched pathways include metabolic pathways, proteasome and cytochrome P450. The most enriched pathways and number of associated transcripts are presented in Table 2.

Differentially expressed genes

Relative transcript abundance per gene was determined to understand expression patterns of functionally annotated genes between zingerone-fed and zingerone-deprived groups. Among the annotated genes, 3,198 transcripts were found to be differentially expressed (DEG) after FDR correction. DEGs were found in all GO classes belonging to cellular component, molecular functions and biological functions. Out of 3,198 transcripts, 3,183 transcripts (99.5%) were up regulated in male flies fed with zingerone.

Because of behavioural and physiological changes reported in previous studies, we were specifically interested in examining differential expression of transcripts related to male ability (male aggression), pheromone release, courtship, mating and Acps. In addition to enrichment in metabolic

pathways and GO terms, several transcripts that underlie traits involved in inter-male aggression (a proxy for male ability) were found up regulated in the zingerone-fed library (Table 3). Transcripts that had significant homology with *Drosophila* male aggression genes include *obp99c, GI11981, GA28881, GI15924, GI13022, GI12367* and *GK17103*. A number of transcripts were identified from genes implicated in pheromone release, courtship and mating in other Diptera that were up regulated. Genes known to be involved in pheromone release had significant sequence homology with *obp*, *obp3* and *obp99c* transcripts (Table 4). Eleven up regulated transcripts had significant hits with courtship regulating genes such as *takeout, membrane transporter* and *white eye protein* gene of *Bactrocera* spp and *lingerer* of *Drosophila* (Table 5). Two over-expressed sequences had best hit homology with *timeless* and *GK23075* that encode circadian rhythm and male mating behaviour in *Neobelleria* and *Drosophila* respectively.

Transcript analogues of Acps such as *Acp 26Ab, Acp29AB, Acp62F, AttB*, and *CecA1*were also identified and were up regulated. Specific transcript information for these best hit Acp unigenes is presented in Table 6. In addition to Acps that regulate female post mating physiology, we also found transcripts encoding *spinster* (CL965. Contig3) and *GF12703* (unigene14143) of *Drosophila* spp that may regulate female mating receptivity and oviposition. Of the DEGs, several odorant binding proteins, reproduction and adult longevity related transcripts had up regulation that are presented in Tables S2, S3 and S4 respectively. The up regulated transcripts include odorant binding proteins such as *Obp, Obp 3, Obp22* and *Obb56a*, reproduction transcripts such as *GA28408, GK11687 and GH25061*, and longevity transcripts *viz*., *hsp70, hsp11, hsp12, hsp27OS, takeout* and *SOD*.

Verification of gene expression profile

The expression level of four genes *viz.*, *doublesex, period, Obp99c* and *unigene266* were analysed by qRT-PCR with 16srRNA as internal control to further analyse the DGE library. Expression of transcripts showed similar magnitude and direction of expression as the DEG data. All the genes were enriched in flies fed with zingerone and validated the DEG analysis (Dryad Digital Repository, doi:10.5061/dryad.v6qj0). Positive $\Delta\Delta C_T$ values obtained for *doublesex, period, Obp99c* and *unigene266* that are 1.33 ± 0.15, 1.13 ± 0.09, 0.34 ± 0.21 and 0.98 ± 0.37 respectively.

Experiment 2: Behavioural assays

Wing beat frequency and calling bout duration

Fundamental wing beat frequency during male courtship calling did not differ among treatments ($F_{2, 374}$ = 2.50; P = 0.083), at 318.63 ± 0.67, 320.83 ± 0.73 and 320.32 ± 0.72 Hz in cuelure-fed, zingerone-fed and unfed males, respectively. Calling bout duration also did not differ among treatments ($F_{2, 374}$ = 2.04; P = 0.816), at 147.93 ± 40.52, 150.56 ± 52.73 and 147.04 ± 43.13 ms for cuelure-fed, zingerone-fed and unfed males, respectively.

Locomotor activity

There was a significant difference in distance walked between lure-fed and unfed males ($F_{2, 59}$ = 5.62; P = 0.006), with cuelure and zingerone-fed males walking longer distances in a 10 min period compared to unfed males. There was no significant difference between cuelure and zingerone-fed males (Fig. 4a). Resting time also differed significantly among treatments ($F_{2, 59}$ = 5.00; P = 0.010), with unfed males resting for significantly longer than either of the lure-fed treatments, which were not different from each other (Fig. 4a). The number of midpoint crosses did not differ

among treatments ($F_{2, 59}$ = 0.587; P = 0.560), at 35.8 ± 2.7, 38.6 ± 3.4 and 33.4 ± 4.1 in cuelure-fed, zingerone-fed and unfed males, respectively.

Weight loss and water content

Lure-fed males showed significant weight loss compared to unfed males on days 1, 3, 7 and 14 after exposure, but the difference was not significant on days 5 and 10 (Day 0: $F_{2, 89}$ = 3.27; P = 0.048; Day 1: $F_{2, 89}$ = 10.40; P < 0.001; Day 3: $F_{2, 89}$ = 6.64; P = 0.003; Day 5: $F_{2, 89}$ = 0.83; P = 0.443; Day 7: $F_{2, 89}$ = 6.44; P = 0.004; Day 10: $F_{2, 89}$ = 1.93 P = 0.157; Day 14: $F_{2, 89}$ = 4.57; P = 0.016): there were no differences between cuelure and zingerone. Both lure-fed treatments showed increased weight on the day of lure feeding and then the weight rapidly dropped on days 1 and 3 after lure feeding, after which weight changes across all three lure treatments followed similar patterns (Fig. 4b).

Changes in percent water content of the flies did not differ among treatments throughout the observation period (Day 0: $F_{2, 89}$ = 1.23; P = 0.304; Day 1: $F_{2, 89}$ = 0.02; P = 0.998; Day 3: $F_{2, 89}$ = 0.26; P = 0.770; Day 5: $F_{2, 89}$ = 0.47; P = 0.628; Day 7: $F_{2, 89}$ = 0.23; P = 0.796; Day 10: $F_{2, 89}$ = 2.40; P = 0.104; Day 14: $F_{2, 89}$ = 2.13; P = 0.131).

Proportion of successful mating attempts versus failed attempts

The proportion of successful to failed mating attempts differed significantly among male types ($F_{2, 23}$ = 12.47; P < 0.0001). Lure-fed males were significantly more successful in progressing from female mounting to copulation than were unfed males. There was no significant difference between the two lures (Fig. 4c).

Discussion

Summary of results

We have generated a functionally diverse collection of EST contigs for *B. tryoni* and assigned putative functions based on sequence homology with other genomes. The data are larger than that reported for other tephritid fruit flies such as *B. dorsalis* and *C. capitata* (Gomulski *et al*. 2008; Shen *et al*. 2011). The results revealed enrichment of transcripts related to energy metabolism, metabolic pathways and enrichment of energy related GO terms in zingerone-fed libraries suggesting perhaps lures are involved in energy metabolisms.

The differentially expressed genes (DEG) data revealed that 3,198 transcripts had differential abundance between zingerone-fed and zingerone-deprived males. Of the DEGs, 99% of transcripts were up regulated with 1%, mostly heat stock proteins, down regulated in zingerone-fed males. The up regulated transcripts include homologous genes that govern male aggression (male ability), pheromone release, courtship and mating. Within the DEGs, several metabolic gene classes were enriched, which further supports that lures might elicit metabolic effects. There were several other differentially expressed transcripts that could not be assigned definitive functions, possibly because *B. tryoni* is a non-model species.

In behavioural assays, flies fed with lures showed increased locomotor activity; for the first three days after lure feeding lure-fed flies had greater weight loss (an indirect measure of increased metabolic rate) than control flies; and for lure-fed flies the proportion of successful copulations following mounting was almost doubled, suggesting that males become hyperactive after lure feeding. No differences in male calling bouts were detected between lure-fed and unfed flies.

Zingerone used in all studies *sensu stricto* is zingerone diluted in ethanol. While previous studies on other *Bactrocera* species evidenced no significant effect on behaviour (Hee & Tan 1998), ethanol induces shorter development time and influences lipid biosynthesis in *Drosophila melanogaster* through alcohol dehydrogenase (Deltombe-Lietaert *et al*. 1979; Geer *et al*. 1985). We acknowledge that flies may have ingested traces of ethanol along with zingerone, and we cannot contravene the possibility that the effects produced in zingerone-fed flies in part are due to ethanol. However, increase in metabolic rate and up regulation of associated genes is well documented for zingerone and related compounds across a range of taxa (Venkatramalingam *et al*. 2007; Park 2010; Pulbutr *et al*. 2011; Chang *et al*. 2012; Yue *et al*. 2013), and hence, we regard it more likely that the effects demonstrated here are the result of zingerone rather than ethanol.

Evolutionary significance of lure response

Lures induce enhanced mating success in male flies in most *Bactrocera* species studied (Shelly 2010), while in *B. tryoni* they also reduce longevity and remating receptivity in females mated with lure-fed males (Kumaran *et al*. 2013). While a pheromonal basis underpinning enhanced male mating success through female preference is well supported, alternative hypotheses associated with lures increasing male activity and modifying male Acps have not been directly tested. For *B. tryoni* males fed with zingerone, the transcriptome data showed significant up regulation of transcript analogues possibly regulating inter-male aggression, courtship, pheromone release, mating and Acps. This supports our hypothesis that complex physiological mechanisms may under-pin phenotypic changes recorded in male and female *B. tryoni* following direct or indirect lure exposure and in the following sections we elaborate further upon these issues.

The 'Red Bull'® effect

Genes from several metabolic and catabolic processes were found to be significantly enriched after males had fed on zingerone. These included oxidation-reduction, catabolic process and significant enrichment in molecular functions such as oxidoreductase activity, nutrient reservoir activity and ATPase activity (Table 1). Both cuelure (raspberry ketone) and zingerone cause complex metabolic and neural changes, such as lipolysis and fat oxidation, in a wide variety of organisms (Morimoto *et al*. 2005; Pulbutr *et al*. 2011; Yue *et al*. 2013) and, in general, they have effects very similar to caffeine or green tea that are known to increase metabolic rate and decrease appetite (Westerterp-Plantenga *et al*. 2006). Indeed, so well-known is this effect that "raspberry ketone diet" as an internet search term will turn up pages of information on a 'fad' human diet regime that is used for weight reduction or obesity control.

The weight change data also strongly supports cuelure and zingerone having metabolic effects in addition to enriched metabolism GOs and pathways. The observed weight loss for the first three days after feeding, and disappearance of effects thereafter (Fig. 4b), directly correlates with the three-day mating success observed in *B. tryoni* and some other *Bactrocera* species (Shelly & Villalobos 1995; Shelly 2000). While a proposed link between increased metabolic rate and short term weight loss is highly consistent with other systems (Morimoto *et al*. 2005; Pulbutr *et al*. 2011; Yue *et al*. 2013), we do recognise that weight loss is an indirect mechanism of increased metabolic rate. However, when combined with other phenotypic measures such as increased locomotor activity and increased progression from mounting to copulation, we believe that data supports lures having what we refer to as a 'Red Bull'® effect on fruit flies, our analogy to the well-known caffeine energy drink which provides short term energy 'hits' and lifts general activity.

Transcriptional changes in courtship and mating genes

Transcripts encoding pheromone release, courtship interactions and mating showed differential transcript abundance. *Takeout* and *white eye protein* that regulates courtship in *B. dorsalis* and *Drosophila* showed up regulation (Kimura *et al*. 2008). Mutations in *takeout* lead to reduced or no courtship interactions (Dauwalder *et al*. 2002), which suggest its role in courtship displays. Odorant binding protein transcripts (*Obp, Obp3* and *Obp99c*) that are possibly involved in pheromone release, as has been found in both *B. dorsalis* and *Delia antiqua* (Meigen) (Mitaka *et al*. 2011; Zheng *et al*. 2013), were also up regulated. *Obp* proteins play a role in olfactory perception, releasing and modulating chemical messages and present the stimulus molecule in a particular way to the receptor proteins to facilitate signal transduction.

Timeless, a gene governing mating behaviour, was up regulated in zingerone-fed males. *Timeless* regulates mating activity rhythm and acts as part of an autoregulatory feedback loop in conjunction with the *period* (per) gene product (Nishinokubi *et al*. 2006). Similarly *lingerer*, which encodes a cytoplasmic protein that regulates the initiation and termination of copulation in *Drosophila grimshawi* (Kuniyoshi *et al*. 2002), had differentially enriched transcript abundance. In *D. melanogaster*, *lingerer* was found to be expressed in the central nervous system of third instar larva, where it functions to regulate normal copulation after adult eclosion (Kuniyoshi *et al*. 2003). Up regulation of *timeless* and *lingerer* in zingerone-fed males is consistent with a hypothesis that lure feeding, through general transcript up regulation, contribute to enhanced mating success and quicker copulation initiation in lure-fed males.

Indirect genetic effects in females

Female post-mating physiology has been shown to be modified after mating with lure-fed males (Kumaran *et al*. 2013); these changes include reduced receptivity for remating, enhanced fecundity and reduced longevity. These changes have been documented in various insect species and are largely driven by male Acps transferred during copulation (Avila *et al*. 2011; Chapman 2001). Hence, Acps that lure-fed males transfer to females perhaps triggered female genes associated with remating, fecundity and longevity. This scenario implicates that males gain mating benefits through indirect genetic effects in females.

Transcriptome analysis revealed differential expression of Acps that had significant homology with Acps in other organisms including *Acp62F, Acp26Ab* and *Acp29AB* of *Drosophila*. *Acp62F* is a trypsin protease inhibitor that localizes to the sperm storage organs of mated females (Mueller *et al*. 2008); it also enters the female's circulation and is toxic to flies upon repeated ectopic expression (Lung *et al*. 2002), suggesting a possible role in life span. This gene could therefore be related to the increased mortality observed in *B. tryoni* females mated with lure-fed males (Kumaran *et al*. 2013). *Acp26Ab* also codes for accessory gland protein, however its function is unknown (Chapman 2001). *Acp29AB* encodes a 234-amino-acid protein, which is associated with resisting sperm displacement during sperm competition (Wong *et al*. 2008). Besides Acps, we found enrichment of *spinster* and *GF12703* homologues that might possibly regulate female receptivity and oviposition (Ferveur 2010).

Conclusion

Phytochemical lures have been associated with sexual selection and increased male mating success in *Bactrocera* fruit flies and appear to do so through a suite of complex mechanisms. The

best known of these is that increased male mating success is associated with an enhanced pheromone composition. This paper does not reject that hypothesis, but provides evidence that there are potential alternate mechanisms when a pheromone effect is not recorded or an additive effect when a pheromone enhancement is involved. Lure feeding in *B. tryoni* resulted in a suite of gene expression changes in a number of different pathways. Male lures also enhanced physical activity, probably through increased metabolic rate, and we believe this may well explain the increased mating success of zingerone-fed *B. tryoni*, for which no enhanced pheromone effect has been found. While this work is specific to *B. tryoni*, the physiological effects of cuelure and zingerone occur across a diverse range of animal taxa and, as such, we see no reason why the general transcriptome enrichment patterns we recorded should not also apply to other *Bactrocera* species. The next step of this work will be to develop tissue specific libraries to aid identification of the specific genes underlying the physiological and behavioural differences in lure-fed and unfed male flies.

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References

Arthofer W, Clarke AR, Kumaran N *et al*. (2014) Genomic Resources Notes accepted 1 December 2013–31 January 2014. *Molecular Ecology Resources*, **14**, 664-665.

Avila FW, Sirot LK, Laflamme BA *et al*. (2011) Insect seminal fluid proteins: identification and function. *Annual Review of Entomology*, **56**, 21-40.

Barton Browne L (1957) An investigation of the low frequency of mating of the Queensland fruit fly *Strumeta tryoni* (Frogg.). *Australian Journal of Zoology*, **5**, 159-163

Berenbaum MR, Favret C, Schuler MA (1996) On defining" key innovations" in an adaptive radiation: Cytochrome P450s and Papilionidae. *American Naturalist*, **148**, S139-S155.

Chang YP, Liu CH, Wu CC *et al*. (2012) Dietary administration of zingerone to enhance growth, non-specific immune response, and resistance to *Vibrio alginolyticus* in Pacific white shrimp *Litopenaeus vannamei* juveniles. *Fish Shellfish Immunology*, **32**, 284-290.

Chapman T (2001) Seminal fluid mediated fitness traits in Drosophila. *Heredity*, **87**, 511-521.

Dauwalder B, Tsujimoto S, Moss J *et al*. (2002) The Drosophila takeout gene is regulated by the somatic sex-determination pathway and affects male courtship behavior. *Genes & Development*, **16**, 2879-2892.

Deltombe-Lietaert MC, Delcour J, Lenelle-Monfort N *et al*. (1979) Ethanol metabolism in *Drosophila melanogaster*. *Experientia*, **35**, 579-581.

Dominiak BC, Fanson BG, Collins SR *et al*. (2014) Automated locomotor activity monitoring as a quality control assay for mass reared tephritid flies. *Pest Management Science*, **70**, 304-309.

Drew RAI, Hooper GHS (1981) The responses of fruit fly species (Diptera: Tephritidae) in Australia to various attractants. *Australian Journal of Entomology*, **20**, 201-205.

Ferveur JF (2010) *Drosophila* female courtship and mating behaviors: sensory signals, genes, neural structures and evolution. *Current Opinion in Neurobiology,* **20**, 764-769.

Geer BW, McKechnie SW, Bentley MM *et al*. (1988) Induction of alcohol dehydrogenase by ethanol in *Drosophila melanogaster*. *The Journal of Nutrition*, **118**, 398-407.

Gomulski L, Dimopoulos G, Xi Z *et al*. (2008) Gene discovery in an invasive tephritid model pest species, the Mediterranean fruit fly, *Ceratitis capitata*. *BMC Genomics*, **9**, 243.

Graham TE, Helge JW, Maclean DA *et al*. (2000) Caffeine ingestion does not alter carbohydrate or fat metabolism in human skeletal muscle during exercise. *The Journal of Physiology*, **529**, 837-847.

Hee AKW, Tan KH (1998) Attraction of female and male *Bactrocera papayae* to conspecific males fed with methyl eugenol and attraction of females to male sex pheromone components. *Journal of Chemical Ecology*, **24**, 753-764.

Horn DJ (1988) Ecological approach to pest management. Guilford Press, New York. 285p.

Khoo CCH, Tan KH (2000) Attraction of both sexes of melon fly, *Bactrocera cucurbitae* to conspecific males–a comparison after pharmacophagy of cue-lure and a new attractant–zingerone. *Entomologia Experimentalis et Applicata*, **97**, 317-320.

Kimura KI, Hachiya T, Koganezawa M *et al*. (2008) Fruitless and doublesex coordinate to generate male-specific neurons that can initiate courtship. *Neuron*, **59**, 759-769.

Kumaran N, Balagawi S, Schutze MK *et al*. (2013) Evolution of lure response in tephritid fruit flies: phytochemicals as drivers of sexual selection. *Animal Behaviour*, **85**, 781-789.

Kumaran N, Clarke AR (2014) Indirect effects of phytochemicals on offspring performance of Queensland fruit fly, *Bactrocera tryoni* (Diptera: Tephritidae). *Journal of Applied Entomology*, **138**, 361-367.

Kumaran N, Hayes RA, Clarke AR (2014a) Cuelure but not zingerone make the sex pheromone of male *Bactrocera tryoni* (Tephritidae: Diptera) more attractive to females. *Journal of Insect Physiology*, **68**, 36-43

Kumaran N, Prentis P, Clarke AR (2014b) "Transcriptome sequencing of the Queensland fruit fly, *Bactrocera tryoni* (Diptera: Tephritidae)" in Genomic Resources Notes accepted 1 December 2013 to 31 January 2014. *Molecular Ecology Resources*, **14**, 664–665. http://dx.doi.org/10.1111/1755-0998.12255.

Kuniyoshi H, Baba K, Ueda R *et al*. (2002) *lingerer*, a *Drosophila* gene involved in initiation and termination of copulation, encodes a set of novel cytoplasmic proteins. *Genetics*, **162**, 1775-1789.

Kuniyoshi H, Usui-Aoki K, Juni N *et al*. (2003) Expression analysis of the *lingerer* gene in the larval central nervous system of *Drosophila melanogaster*. *Journal of Neurogenetics*, **17**, 117-137.

Lung O, Tram U, Finnerty CM *et al*. (2002) The *Drosophila melanogaster* seminal fluid protein Acp62F is a protease inhibitor that is toxic upon ectopic expression. *Genetics*, **160**, 211-224.

Mankin R, Lemon M, Harmer A *et al*. (2008) Time-pattern and frequency analyses of sounds produced by irradiated and untreated male *Bactrocera tryoni* (Diptera: Tephritidae) during mating behavior. *Annals of the Entomological Society of America*, **101**, 664-674.

Mitaka H, Matsuo T, Miura N *et al*. (2011) Identification of odorant-binding protein genes from antennal expressed sequence tags of the onion fly, *Delia antiqua*. *Molecular Biology Reports*, **38**, 1787-1792.

Morimoto C, Satoh Y, Hara M *et al*. (2005) Anti-obese action of raspberry ketone. *Life Sciences*, **77**, 194-204.

Mueller JL, Linklater JR, Ram KR *et al*. (2008) Targeted gene deletion and phenotypic analysis of the *Drosophila melanogaster* seminal fluid protease inhibitor *Acp62F*. *Genetics,* **178**, 1605-1614.

Myers K (1952) Oviposition and mating behaviour of the Queensland fruit fly, *Dacus tryoni* and the solanum fruit fly *Dacus cacuminatus*. *Australian Journal of Biological Sciences*, **5**, 264-281.

Nishinokubi I, Shimoda M, Ishida N (2006) Mating rhythms of *Drosophila*: rescue of tim01 mutants by *D. ananassae* timeless. *Journal of Circadian Rhythms*, **4**, 4.

Park KS (2010) Raspberry ketone increases both lipolysis and fatty acid oxidation in 3T3-L1 adipocytes. *Planta Medica*, **76**, 1654.

Prentis PJ Woolfit M, Thomas-Hall SR *et al*. (2010) Massively parallel sequencing and analysis of expressed sequence tags in a successful invasive plant. *Annals of Botany*, **106**, 1009-1017.

Price PW, Bouton CE, Gross P *et al*. (1980) Interactions among three trophic levels: influence of plants on interactions between insect herbivores and natural enemies. *Annual Review of Ecology and Systematics*, **11**, 41-65.

Pulbutr P, Thunchomnang K, Lawa K (2011) Rats and high fat diet-fed rats. *International Journal of Pharmacology*, **7**, 629-634.

Radhakrishnan P, Taylor PW (2007) Seminal fluids mediate sexual inhibition and short copula duration in mated female Queensland fruit flies. *Journal of Insect Physiology*, **53**, 741-745.

Raghu S (2004) Functional significance of phytochemical lures to dacine fruit flies (Diptera: Tephritidae): an ecological and evolutionary synthesis. *Bulletin of Entomological Research*, **94**, 385-399.

Raghu S, Clarke AR (2003) Sexual selection in a tropical fruit fly: role of a plant derived chemical in mate choice. *Entomologia Experimentalis et Applicata*, **108**, 53-58.

Raghu S, Clarke AR, Yuval B (2002) Investigation of the physiological consequences of feeding on methyl eugenol by *Bactrocera cacuminata* (Diptera: Tephritidae). *Environmental Entomology*, **31**, 941-946.

Rosenthal GA, Berenbaum MR (1992) Herbivores: Their interactions with secondary plant metabolites: *Ecological and Evolutionary Processes*, vol 2. Access Online via Elsevier,

Rothschild M, Aplin R, Cockrum P *et al*. (1979) Pyrrolizidine alkaloids in arctiid moths (Lep.) with a discussion on host plant relationships and the role of these secondary plant substances in the Arctiidae. *Biological Journal of the Linnaean Society*, **12**, 305-326.

Schoonhoven LM, Loon JV, Dicke M (2005) Insect-plant biology. Vol Ed. 2. Oxford University Press, Oxford 440p.

Schuster JA, Levitsky DA (1982) Insensible weight loss as an indicator of metabolic rate. *Physiology & Behavior*, **28**, 381-384.

Shelly TE (2000) Effects of raspberry ketone on the mating success of male melon flies (Diptera: Tephritidae). *Proceedings of Hawaiian Entomological Society*, **34**, 163-167.

Shelly TE (2010) Effects of methyl eugenol and raspberry ketone/cue lure on the sexual behavior of *Bactrocera* species (Diptera: Tephritidae). *Applied Entomology and Zoology*, **45**, 349-361.

Shelly TE, McInnis DO, Pahio E *et al*. (2004). Aromatherapy in the Mediterranean fruit fly (Diptera: Tephritidae): sterile males exposed to ginger root oil in prerelease storage boxes display increased mating competitiveness in field-cage trials. *Journal of Economic Entomology*, **97**, 846-853.

Shelly TE, Villalobos EM (1995) Cue lure and the mating behavior of male melon flies (Diptera: Tephritidae). *Florida Entomologist*, **78**, 473-482.

Shen GM, Dou W, Niu JZ *et al*. (2011) Transcriptome analysis of the oriental fruit fly (*Bactrocera dorsalis*). *PloS One*, **6**, e29127.

Tan KH, Nishida R (2007) Zingerone in the floral synomone of *Bulbophyllum baileyi* (Orchidaceae) attracts *Bactrocera* fruit flies during pollination. *Biochemical Systematics and Ecology*, **35**, 334-341.

Tan KH, Nishida R (2012) Methyl eugenol: Its occurrence, distribution, and role in nature, especially in relation to insect behavior and pollination. *Journal of Insect Science*, **12**, 1-60.

Tychsen PH (1977) Mating behaviour of the Queensland fruit fly, *Dacus tryoni* (Diptera: Tephritidae), in field cages. *Australian Journal of Entomology*, **16**, 459-465.

Venkatramalingam K, Christopher JG, Citarasu T (2007) *Zingiber officinalis* an herbal appetizer in the tiger shrimp *Penaeus monodon* (Fabricius) larviculture. *Aquaculture Nutrition*, **13,** 439-443.

Webb J, Sharp J, Chambers D *et al*. (1976) Analysis and identification of sounds produced by the male Caribbean fruit fly, *Anastrepha suspensa*. *Annals of the Entomological Society of America*, **69**, 415-420.

Westerterp-Plantenga M, Diepvens K, Joosen AM *et al*. (2006) Metabolic effects of spices, teas, and caffeine. *Physiology & Behavior*, **89**, 85-91.

Wong A, Albright SN, Giebel JD *et al*. (2008) A role for Acp29AB, a predicted seminal fluid lectin, in female sperm storage in *Drosophila melanogaster*. *Genetics*, **180**, 921-931.

Yue HY, Jiang CY, Fujita T *et al*. (2013) Zingerone enhances glutamatergic spontaneous excitatory transmission by activating TRPA1 but not TRPV1 channels in the adult rat substantia gelatinosa. *Journal of Neurophysiology*, **110**, 658–671.

Zheng W, Peng W, Zhu C *et al*. (2013) Identification and Expression Profile Analysis of Odorant Binding Proteins in the Oriental Fruit Fly *Bactrocera dorsalis*. *International Journal of Molecular Sciences*, **14**, 14936-14949.

Zimmermann Y, Roubik DW, Eltz T (2006) Species-specific attraction to pheromonal analogues in orchid bees. *Behavioral Ecology and Sociobiology*, **60**, 833-843.

Data accessibility

Transcriptome sequence data: NCBI Sequence Read Archive: SRX388867 (http://www.ncbi.nlm.nih.gov/sra/?term=SRX388867)

Contigs, unigenes, sequence assembly, contig annotation and BLAST hits: 10.5061/dryad.b21r5 (Kumaran *et al*. 2014b)

Differentially expressed genes, RT-PCR results, enriched pathways, GO terms and data from behavioural assays: http://dx.doi.org/10.5061/dryad.v6qj0

Authors' contribution

NK, PJP and ARC conceived and designed the study. NK and PJP performed RNA-seq study and transcriptome analyses. NK and KPM performed behavioural assays with data analyses done by NK and MKS. NK wrote the paper with others contributing to revisions.

Figure captions

Figure 1. Oscillogram (a) and spectrogram (b) pattern for sound produced by male *Bactrocera tryoni* during calling. Fundamental frequency, calculated at the midpoint of each calling bouts is indicated

with an arrow and calling bout duration (CBD), measured from the start and end point of each calling bout is designated as CBD.

Figure 2. Species (a) and similarity (b) distribution of transcriptome sequences of *Bactrocera tryoni*. Percentage values are distribution of *B. tryoni* transcripts among known transcripts of reference species and proportionate similarities.

Figure 3. Significantly enriched GO terms and number of unigenes significantly over expressed under individual GO terms in *Bactrocera tryoni* males fed on zingerone (ccob-cellular component organization or biogenesis; nrbp- negative regulation of biological process; prbp- positive regulation of biological process; nabtfa- nucleic acid binding transcription factor activity; pbtfa- protein binding transcription factor activity).

Figure 4. Comparison of male physical parameters among cuelure-fed, zingerone-fed and unfed males of mature, virgin *Bactrocera tryoni*: (a) distance walked and total time rested in a 10 minute period; (b) weight change (mean + 1 SE) over a 14 days period; (c) proportion of successful copulations (mean + 1 SE) following female mounting in no-choice (i.e. non-competitive) arenas. Different letters on adjacent bars indicate significant difference ($P < 0.05$) between lure-fed and unfed males as tested by Tukey's HSD.

Table 1. GO terms highly significantly enriched in zingerone-fed *Bactrocera tryoni* males compared to males deprived of zingerone

GO class	Gene Ontology term	Corrected P-value
Biological process	Small Molecule Metabolic Process	4.28e-10
	Metabolic Process	1.12e-08
	Oxidation-Reduction Process	1.63e-07
	Cellular Amino Acid Metabolic Process	1.72e-07
	Carboxylic Acid Metabolic Process	4.60e-06
	Oxoacid Metabolic Process	4.60e-06
	Organic Acid Metabolic Process	5.13e-06
	Cellular Ketone Metabolic Process	1.39e-05
Cellular component	Mitochondrion	7.21e-14
	Mitochondrial Part	1.86e-12
	Proteasome Complex	3.64e-09
	Cytoplasm	1.36e-08
	Mitochondrial Matrix	5.40e-08
	Proteasome Core Complex	2.82e-07
	Cytoplasmic Part	2.76e-06
	Ribosomal Subunit	5.04e-06
	Ribosome	2.96e-05
Molecular function	Oxidoreductase Activity	6.65e-14
	Catalytic Activity	1.97e-08
	Lyase Activity	3.35e-07
	Threonine-Type Endopeptidase Activity	5.04e-05
	Threonine-Type Peptidase Activity	5.04e-05
	Carbon-Halide Lyase Activity	5.93e-05

Table 2. Pathways highly significantly enriched in zingerone-fed *Bactrocera tryoni* males compared to males deprived of zingerone

#	Pathway	No. of transcripts enriched	P value	Q value	Pathway ID
1	Metabolic pathways	344	1.267948e-18	3.131832e-16	ko01100
2	Proteasome	22	6.433674e-11	7.945587e-09	ko03050
3	Cytochrome P450	30	8.450217e-08	6.957345e-06	ko00982
4	Glutathione metabolism	31	2.863320e-07	1.768100e-05	ko00480
5	Retinol metabolism	27	6.353373e-07	3.138566e-05	ko00830
6	Metabolism of xenobiotics by cytochrome P450	27	9.989888e-07	4.112504e-05	ko00980
7	Ribosome	27	7.701827e-06	2.717645e-04	ko03010
8	Tyrosine metabolism	25	9.130035e-06	2.818898e-04	ko00350
9	Oxidative phosphorylation	45	1.169743e-05	3.210295e-04	ko00190
10	One carbon pool by folate	12	1.312132e-05	3.240966e-04	ko00670
11	beta-Alanine metabolism	19	4.596838e-05	9.874920e-04	ko00410
12	Pentose phosphate pathway	17	4.797532e-05	9.874920e-04	ko00030

Table 3. Inter-male aggression transcripts differentially enriched in *Bactrocera tryoni* males fed on zingerone compared to males deprived of zingerone

Gene	Contig/Unigene ID	Length (bp)	E-value	Reference species
obp99c	Unigene19499	669	1e-21	*Drosophila jakuba*
GI11981	CL2785	3813	0	*D. mojavensis*
GA28881	CL367	1246	5e-102	*D. pseudoobscura*
GI15924	Unigene24786	574	3e-94	*D. mojavensis*

GI13022	CL2995	1326	2e-54	*D. mojavensis*
GA28881	CL367	1533	7e-102	*D. pseudoobscura*
GI12367	Unigene2358	933	7e-93	*D. mojavensis*
GK17103	Unigene23129	202	4e-30	*D. willistoni*

Table 4. Pheromone transcripts differentially enriched in *Bactrocera tryoni* males fed on zingerone compared to males deprived of zingerone

Gene	Contig/Unigene ID	Length (bp)	E-value	Reference species
obp	Unigene19467	637	1e-81	*Bactrocera dorsalis*
obp 3	CL2556.Contig2	775	8e-62	*Delia antiqua*
obp99c	Unigene19499	669	0	*Drosophila melanogaster*
GJ13150	Unigene15918	555	3e-23	*D. virilis*
GG22675	Unigene16791	524	1e-60	*D. erecta*
GF17311	Unigene15498	891	5e-33	*D. ananassae*

Table 5. Courtship regulating transcripts differentially enriched in *Bactrocera tryoni* males fed on zingerone compared to males deprived of zingerone

Gene	Contig/Unigene ID	Length (bp)	E-value	Reference species
takeout	Unigene1359	944	6e-47	*B. dorsalis*
membrane transporter	Unigene18143	1817	0	*B. tryoni*
takeout	Unigene13516	787	4e-62	*B. dorsalis*
white eye protein	Unigene2375	859	3e-156	*B. cucurbitae*

calmodulin	Unigene19964	635	5e-67	*Drosophila pseudoobscura*
GG22675	Unigene16791	524	1e-60	*D. erecta*
GI21715	CL544.Contig4	3229	0	*D. mojavensis*
slowpoke	Unigene19771	411	1e-72	*D. pseudoobscura*
GJ20581	Unigene6329	815	7e-159	*D. virilis*
GI21724	Unigene17902	546	6e-49	*D. mojavensis*
lingerer	CL175. Contig2	2422	0	*D. grimshawi*

Table 6. Accessory gland protein transcripts differentially enriched in *Bactrocera tryoni* males fed on zingerone compared to males deprived of zingerone

Gene	Contig/Unigene ID	Length (bp)	E-value	Reference species
Acp29AB	Unigene266	939	4e-21	*Drosophila simulans; D. melanogaster*
Acp26Ab	Unigene2125	790	1e-35	*D. sechellia*
Acp62F	CL1136.Contig1	441	4e-06	*D. melanogaster*
CG4847	CL2150	1269	1e-117	*D. melanogaster; D. simulans*
CG8093	Unigene18589	638	6e-74	*D. melanogaster*
AttB	CL1222	730	5e-81	*D. melanogaster*
cecA1	Unigene28537	226	8e-12	*D. melanogaster; D. sechellia*
CSSFP003	Unigene16248	1424	2e-10	*Chilo suppressalis*
CSSFP002	Unigene22160	313	2e-36	*C. suppressalis*
HACP027	Unigene23842	227	9e-13	*Heliconius hortense*