

Sex-Specific Transcriptomic Profiling of the Perfectly Synchronous Flashing Fireflies *Pteroptyx tener* (Coleoptera, Lampyridae)

(Pemprofilan Transkriptomik Khusus Jantina bagi Kelipan Sinkroni Sempurna Kelip-Kelip *Pteroptyx tener* (Coleoptera, Lampyridae))

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ABSTRACT

The firefly species *Pteroptyx tener* is well known for its precisely synchronous male flashing behaviour at night, while females do not exhibit this trait. However, the molecular mechanisms underlying this synchronisation by males remain largely unknown. To understand the genetic basis of this firefly synchronous flashing, RNA sequencing (RNA-seq) was performed on samples from males and females of *P. tener* collected during the day and at night, generating approximately 43 to 50 million reads per sample with over 90% of bases achieving a Phred quality score above 30. Due to the lack of a reference genome for this species, *de novo* assembly of the sequencing data was conducted resulting in 160,894 total transcripts. Raw data (FASTQ) were deposited in the NCBI Sequence Read Archive (SRA) with the accession number PRJNA1242504. The discovery of a hidden-break in 28S rRNA highlighted the need for revised RNA integrity assessment protocols for arthropod species, as standard RIN-based metrics might yield misleading results. Differential expression analysis showed a list of 151 genes consistently upregulated in male night samples compared to all other groups under a false discovery rate *p*-value threshold of less than 0.05. Within this group, the top 20 upregulated genes exhibited maximum log₂ fold change values between 10 and 12. The study indicated that the overall transcriptomic profile in *P. tener* was influenced by sex rather than by sampling time. This finding suggests potential sex-specific regulation of genes involved in bioluminescence and signalling. This identification of differentially expressed genes and key molecular pathways is potentially crucial for the firefly species with synchronous flashing behaviour, offering new avenues for future functional and evolutionary studies on firefly communication. Additionally, it offers insights into sex-specific transcriptomic differences that may influence other biological processes in *P. tener*.

Keywords: Fireflies; *Pteroptyx tener*; RNA-seq; sex; synchronous flashing

ABSTRAK

Kelip-kelip jantan spesies *Pteroptyx tener* terkenal dengan ciri kelipan sinkroni pada waktu malam, manakala kelip-kelip betina tidak menunjukkan ciri tersebut. Namun begitu, mekanisme molekul yang mendasari kelipan sinkroni oleh jenis jantan masih belum diketahui dengan jelas. Bagi memahami asas genetik kelipan sinkroni kelip-kelip, penjujukan RNA (RNA-seq) telah dijalankan ke atas sampel jantan dan betina *P. tener* yang diambil pada waktu siang dan malam, menghasilkan kira-kira 43 hingga 50 juta bacaan setiap sampel dengan lebih 90% bes mencapai skor kualiti Phred melebihi 30. Disebabkan ketiadaan genom rujukan bagi spesies ini, himpunan semula data penjujukan secara *de novo* telah dilakukan yang menghasilkan 160,894 transkrip. Data mentah (FASTQ) telah disimpan dalam

NCBI *Sequence Read Archive* (SRA) dengan nombor akses PRJNA1242504. Penemuan pecahan tersembunyi pada 28S rRNA menekankan keperluan untuk menyemak semula protokol penilaian kualiti RNA bagi spesies artropod, kerana metrik berasaskan RIN semata-mata mungkin memberikan keputusan yang mengelirukan. Analisis pembezaan pengekspresan menyenaraikan 151 gen dengan pengekspresan yang tinggi dalam sampel jantina pada waktu malam berbanding semua sampel lain dengan nilai p kadar penemuan palsu kurang daripada 0.05. Dalam kumpulan sampel ini, 20 gen teratas yang diekspreskan dengan tinggi menunjukkan nilai perubahan $\log_2$ kali ganda maksimum antara 10 hingga 12. Kajian ini menunjukkan bahawa profil transkriptom keseluruhan *P. tener* lebih dipengaruhi oleh faktor jantina berbanding masa pensampelan. Keputusan ini mencadangkan kemungkinan wujudnya pengawalaturan khusus jantina bagi gen yang terlibat dalam biopendarkilau dan pengisyaratan. Pengenalpastian gen yang diekspreskan secara berbeza dan tapak jalan molekul utama ini penting untuk spesies kelip-kelip yang mempunyai sifat sinkroni kelipan bagi membuka peluang baharu untuk kajian fungsian dan evolusi komunikasi kelip-kelip pada masa hadapan. Tambahan pula, keputusan ini turut memberikan pandangan baharu terhadap perbezaan transkriptom khusus jantina yang mungkin mempengaruhi proses biologi lain dalam *P. tener*.

Kata kunci: Jantina; kelipan sinkroni; kelip-kelip; penjujukan RNA; *Pteroptyx tener*

INTRODUCTION

Fireflies are a well-known bioluminescent insect from the family Lampyridae and order Coleoptera that evolved more than 100 million years ago (Fallon et al. 2018; Kazantsev 2015). There are an estimated 2000 different species of fireflies in 80 genera worldwide (Buck & Buck 1966; Lloyd 2008). Fireflies emit a spectrum of green to orange light from their light organ (Biggley, Lloyd & Seliger 1967; Seliger et al. 1964). While most fireflies are rover types that flash their light individually, there exists a subgroup capable of both individual and synchronised group flashing. Fireflies that flash synchronously while flying include certain species from the North American genus *Photinus* and *Photuris* (Copeland & Moiseff 2004; Faust 2010; Lloyd 2008; Moiseff & Copeland 2000), the genus *Luciola* sp. from Eastern Europe (Baldaccini, Fiaschi & Papi 1969) and Japan (Ohba 1984; Ohba, Numata & Kawano 2020). The Southeast Asian genus *Pteroptyx* (18 species), including *P. tener*, *P. malacca*, *P. cribellate*, and *P. validus* (Buck 1988; Dawood & Saikim 2016; Jusoh et al. 2018), on the other hand, are known to be stationary, congregating synchronous flashing fireflies (Copeland & Moiseff 2004). *P. tener* is widely distributed in Malaysia and can be frequently found perching on mangroves along tidal rivers across the states of Selangor, Johor, Negeri Sembilan, Perak, Terengganu, Sabah, and Sarawak (Jusoh, Hashim & Adam 2013).

The function of firefly flashing evolved from warding off predators to communication for mating (Oba et al. 2020). It was postulated that the synchronised flashing behaviour in male fireflies at night primarily aims to minimize visual clutter for females, facilitating their ability to distinguish among various species and sexes during the mating period (Buck 1988; Lewis & Cratsley 2008; Moiseff & Copeland 2010). Indeed, variations in flash characteristics have been well documented and comprehensively reviewed among different firefly species, highlighting the diversity of their signalling behaviours (Goh, Lee & Wang 2022; Lewis & Cratsley 2008; Ohba

2004). A distinct bioluminescent flashing pattern was previously observed in the *P. tener* population from Kuala Selangor, which has become an important ecotourism attraction (Khoo, Nada & Kirton 2016). The male *P. tener* fireflies exhibit two different types of flashing: non-display flashing and display flashing. During non-display flashing, which occurs while the males are stationary or walking, the emitted light is relatively dim. In contrast, display flashing produces brighter flashes that may last up to 300 milliseconds and often contain multiple peaks, resulting in an almost continuous pulsating light pattern. In both flashing modes, males maintain synchronisation with the surrounding swarm. Females, however, do not flash in synchrony with the males. Their flashes are typically single-peaked, lasting up to nearly one second, and are emitted at a slower and more irregular rate compared to the males' display frequency (Case 1980).

Despite extensive documentation of fireflies' synchronous flashing patterns, the underlying molecular mechanism that enables precise synchronisation specifically among the males remains unknown. Previously, it was proposed that the on or off mechanism of the firefly flashing depends on the gating of oxygen, which is required for the light reaction catalysed by the luciferase enzyme (Timmins et al. 2001; Tsai et al. 2014). This proposed oxygen-gating mechanism depends on the diffusion of oxygen regulated by tracheolar fluid level (Timmins et al. 2001) and the action of nitric oxide (NO) produced by nitric oxide synthase (NOS) (Trimmer et al. 2001; Tsai et al. 2014). In the study done on the firefly species *Photuris* sp., the removal of NO causes impairment in the firefly flashing (Trimmer et al. 2001), supporting the notion that NO inhibits the utilisation of oxygen by mitochondria surrounding the photocytes, thus freeing the oxygen to be used in the light reaction (Aprille et al. 2004; Trimmer et al. 2001). In addition to NOS, calcium signalling has been linked to the firefly flash control mechanism (Carlson 2004). Recently, calcium signalling components Calreticulin and Voltage-dependent Anion Channel

(VDAC) were found to be highly expressed in flashing firefly species (*Abscondita terminalis*) compared to glowing firefly species (*Lamprigera yunnana*), highlighting its importance in the flashing control (Zhang et al. 2020). It was proposed that calcium ion release triggered by the octopamine receptor activation induces NOS activity, thus allowing oxygen supplied by the trachea to reach the photocyte peroxisome for the light reaction (Aprille et al. 2004; Nathanson 1979; Zhang et al. 2020). However, whether these identified molecular components govern the synchronous flashing mechanism in *P. tener* is unknown, partly due to the lack of transcriptomic and genomic data of this species.

To further investigate the molecular components of this synchronised flashing, RNA sequencing (RNA-seq) analysis on male and female *P. tener* specimens collected during the day and at night was conducted. Since the genome of this species has not yet been fully sequenced, *de novo* RNA-seq approach was chosen to characterise its gene expression profile without the need for a reference genome. These transcriptomic profiles were then compared to narrow down potential molecular candidates responsible for the males' precise synchronous flashing phenomenon. This study presents the first transcriptomic dataset for the poorly studied firefly species *P. tener*. The generated transcriptomic profile offers valuable insights into differentially expressed genes that may regulate sex-specific bioluminescence behaviours and the possible mechanisms underlying synchronised flashing in *P. tener* males at night. Additionally, this dataset serves as a foundational resource for investigating other biological processes that differ between sexes in *P. tener* and can be leveraged for comparative studies in closely related firefly species.

MATERIALS AND METHODS

SAMPLING

The adult fireflies of *P. tener* (Coleoptera, Lampyridae) were collected using a sweeping net method at Kampung Kuantan, Kuala Selangor (3°21'38.78"N 101°18'4.67"E). These fireflies were then separated according to sex, rapidly frozen in liquid nitrogen, and stored at -80 °C. The sampling was done twice per day at 1600 h (Day sample) and 2200 h (Night sample) for 3 days. In total, there were four different samples, assigned as Male Day, Female Day, Male Night and Female Night.

RNA EXTRACTION AND *de novo* ILLUMINA SEQUENCING

RNA extraction was carried out using the Qiagen RNeasy Plant Mini Kit (Qiagen) according to the protocol provided by the manufacturer. Whole bodies of approximately 10 adult male and female insects sampled from the day and night samplings (50 mg) were used per extraction. Extracted RNA was eluted with 30 µL

sterilised RNase-free distilled water. The quality of extracted RNA was analysed using gel electrophoresis and the Agilent 2100 Bioanalyzer with RNA 6000 Nano Labchip (Malaysian Genome and Vaccine Institute, MGVI). In brief, sample preparation for the Agilent 2100 Bioanalyzer involved heating the samples at 70 °C for 2 min to achieve denaturation in order to reduce the formation of secondary structure, followed by brief centrifugation and immediate cooling on ice.

Prior to the sequencing, cDNA library was prepared using Truseq RNA Sample Preparation kit (Illumina, San Diego, CA, USA) as described in the manufacturer's protocol. Total mRNA samples were purified using Poly-T magnetic beads (poly-A selection) followed by fragmentation of the purified mRNA. Then, pieces of the mRNA were converted into cDNA by Reverse Transcriptase enzyme and amplified through Polymerase Chain Reaction (PCR) to obtain the cDNA library. Clusters of cDNA library were later generated using cBOT clonal amplification system (Illumina, San Diego, CA, USA). *De novo* transcriptomic sequencing was carried out using the Illumina HiSeq 2000 platform (Illumina, San Diego, CA, USA; paired-end; 125 bp) (Macrogen, Korea).

De novo TRANSCRIPTOME ASSEMBLY AND TRANSCRIPT ANNOTATION

Raw paired-end Illumina reads were subjected to quality trimming using Trimmomatic v0.32 (Bolger, Lohse & Usadel 2014) to remove adapter sequences and low-quality bases before transcriptome assembly. Adapter clipping was performed using the TruSeq3-PE.fa adapter file as recommended for HiSeq (USADLLAB.org, Germany). The *de novo* assembly was carried out using the Trinity program (v2.0.6) (<http://trinityrnaseq.github.io>) (Haas et al. 2013) with a default k-mer size of 25. To evaluate the quality of the *de novo* transcriptome assembly, the original paired-end RNA-Seq reads for all samples were mapped back to the assembled transcripts using Bowtie v1.2.3 integrated within the Trinity pipeline. The assembled transcriptome was indexed using Bowtie, and alignments were performed with the parameters --all --best --strata -m 300 to exclude excessively repetitive mappings. The transcripts were then aligned against the NCBI non-redundant Swiss-Prot database (<http://www.uniprot.org/uniprot/>) performed by BLASTX and BLASTP via Trinotate to identify the transcripts based on the best match.

ESTIMATING TRANSCRIPT ABUNDANCE RSEM AND DIFFERENTIAL EXPRESSION ANALYSIS

For estimation of transcript abundance, paired-end RNA-Seq reads were aligned to the total *de novo* assembled Trinity transcriptome using Bowtie (Langmead et al. 2009) with parameters allowing for up to

300 multiple alignments per read pair and a maximum fragment length of 800 bp. The output was then converted into binary format, and unmapped reads were filtered using SAMtools (Li et al. 2009). Gene and transcript abundance estimation was performed via RNA-seq by Expectation-Maximization (RSEM) software (Li & Dewey 2011). Differential expression analysis at the gene level was performed using Empirical analysis of Digital Gene Expression in R (EdgeR) (Chen et al. 2025). An exact test for pairwise comparison was then used to identify differentially expressed genes between each sample. Genes were ranked according to their $\log_2$ fold change ($\log_2FC$) and false discovery rate (FDR)-adjusted p -values with a threshold value of ≤ 0.05 . Overlapping differentially expressed genes were visualized using the ggvenn package in R (<https://github.com/yanlinlin82/ggvenn>). Genes that were upregulated in male night samples relative to all other samples were extracted for subsequent enrichment analysis.

GO TERM ENRICHMENT

Gene set enrichment analysis (GSEA) was performed to identify enriched Gene Ontology (GO) terms among genes that were significantly differentially expressed in

male night samples compared with other sample groups. The analysis was conducted using the topGO package (Alexa & Rahnenführer 2016) with default parameters. Differentially expressed genes (DEGs) from the male night samples were used as the target set, while all annotated genes served as the background reference. Enrichment significance was evaluated using Fisher's exact test, and GO terms yielding $-\log_{10}(p\text{-value}) > 1.30$ were considered significantly enriched. The 'classic' algorithm implemented in topGO was applied for the analysis.

RESULTS AND DISCUSSION

P. tener FROM KUALA SELANGOR, MALAYSIA SPECIATION BASED ON MORPHOLOGY

The representative adult fireflies of male and female of *P. tener* (Coleoptera, Lampyridae) that were collected for this study are shown in Figure 1. The light organ of the *P. tener* male is located at the ventral of the sixth and seventh abdominal segments, while the female only has one segment of light organ located at the sixth abdominal segment (Figure 1). *P. tener* species were identified according to the speciation studies by Ballantyne (1987) and Jusoh et al. (2018).

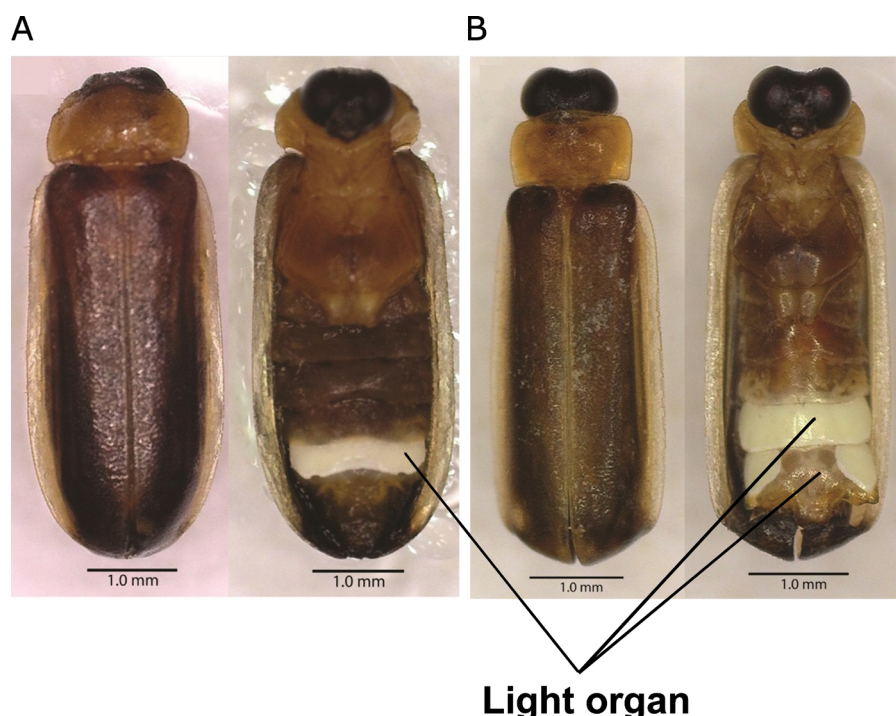


FIGURE 1. The dorsal and ventral views of both (A) female and (B) male *P. tener* fireflies. Male *P. tener* possesses yellowish-white light organs that are large and located on the sixth and seventh sternites (a sclerite of the sternum of an insect, especially a ventral sclerite of an abdominal segment). The light organ of the sixth sternite covers the entire sternite, whereas the light organ on the seventh sternite is bipartite, consisting of a pair of light organs that are located on the left and right sides of the sternite (Ballantyne & Menayah 2002)

BREAKDOWN OF *P. tener* 28S rRNA UPON HEATING

In this study, RNA quality was assessed by gel electrophoresis and BioAnalyzer (Agilent 2100 BioAnalyzer) analysis of intact 18S rRNA, 28S rRNA, and total RNA extracted. BioAnalyzer analysis employs a specific software algorithm to produce an RNA integrity number (RIN), which indicates the quality of the RNA. RIN can be ranked from the lowest to the highest quality with a score of 1.0 up to 10.0, respectively. Low RIN or unavailable scores are typically indicative of degradation that causes the failure to calculate the rRNA ratio (Mueller, Lightfoot & Schroeder 2004).

Based on Table 1, all *P. tener* heated RNA samples generated either low RIN scores or were unable to be computed. The RIN score results alone indicated that the RNA extracted was not of good quality, likely caused by the degradation of either the 28S rRNA or the 18S rRNA. However, the electropherogram (The Supplementary Material 1 has been provided) showed the absence of

unspecific peaks usually observed in degraded RNA samples, but instead exhibited only a small peak of 5S rRNA and a higher peak with the size of 18S rRNA for every sample. It is interesting that when the same samples were not heated prior to the BioAnalyzer analysis, the RIN scores obtained were high (Table 1), indicating high-quality RNA samples. The electropherograms produced for the non-heated samples also illustrated an intact rRNA profile with a higher peak of 28S rRNA (approximate size of 4000 bp) and a lower peak of 18S rRNA (approximate size of 2000 bp) (Supplementary Material 1-data not shown) for each sample. In support of the results from the BioAnalyzer analysis, gel electrophoresis of the heated Male Day RNA sample generated a single band with the size of 18S rRNA but the non-heated sample generated double bands with the size of 28S rRNA and 18S rRNA (Figure 2). Neither sample produced smearing bands as commonly occurred with degraded RNA.

TABLE 1. RIN scores obtained from BioAnalyzer analysis for heated and non-heated samples in BioAnalyzer sample preparation

RNA sample	Condition	RIN score
Male Day	Heated	3.10
Male Day	Non-heated	8.50
Female Day	Heated	NA
Female Day	Non-heated	9.0
Male Night	Heated	NA
Male Night	Non-heated	8.7
Female Night	Heated	NA
Female Night	Non-heated	8.4

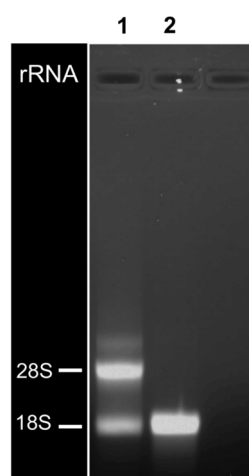


FIGURE 2. Gel image from the electrophoresis run of heated and non-heated samples of Male Day total RNA. Lane 1 contains the non-heated RNA sample, whereas Lane 2 contains the heated RNA sample

The lower RIN score was likely due to the missing 28S rRNA peak caused by the heat introduced during the BioAnalyzer sample preparation. This observation, known as the hidden-break of 28S rRNA was also previously reported in a study conducted on a bee species *Apis mellifera* (Winnebeck, Millar & Warman 2010). Despite the low RIN score in this study, the samples proceeded to be used for RNA-seq based on the observation of the non-heated samples and the electrophoresis results (Figure 2).

One of the pivotal aspects of successful RNA-seq methodology is determining the quality of the RNA samples. Through the data obtained, we have accidentally observed the presence of a hidden-break phenomenon in the 28S rRNA of *P. tener* when the RNA samples are subjected to heat. The hidden-break of 28S rRNA is a phenomenon observed in many protostomes, particularly arthropods (DeLeo et al. 2018). In species such as *P. tener*, the 28S rRNA (~4000 bp) may consist of two hydrogen-bonded subunits, 28Sa and 28Sb (~2000 bp each), which separate upon heating, producing a single rRNA peak and lower RIN values. Hidden-breaks also occur in some mammals (small rodents) and higher plants (McCarthy, Dugon & Power 2015). Hence, our results suggested that the RNA quality assessment, in particular for samples from arthropod species, must not rely solely on the RIN score, as it can be misleading. The mechanism behind the hidden-break is not yet clear until now, nonetheless, a computational approach has been developed to identify species containing hidden-

breaks without having to depend on RNA extraction and electrophoresis (Natsidis et al. 2019). This approach is, however, limited to species with available transcriptomic data.

ILLUMINA SEQUENCING AND *de novo* ASSEMBLY OF PAIRED-END READS

To obtain the universal transcriptomic profile of *P. tener*, four different RNA-seq data from different time points and sexes, namely PtMD (male fireflies sampled during the day), PtFD (female fireflies sampled during the day), PtMN (male fireflies sampled at night) and PtFN (female fireflies sampled at night), were obtained using the Illumina Hiseq 2000 RNA sequencing platform paired-end sequencing. Good quality sequencing data was obtained with more than 90% of all the bases sequenced, producing Phred quality of above 30 for all four datasets. Overall, the total number of sequence reads was approximately between 43 and 50 million reads per sample, which was sufficient to provide the overall gene profile of *P. tener*. A summary of the sequencing output is shown in Table 2. Raw RNA-seq data in FASTQ format have been deposited in the NCBI Sequence Read Archive (SRA) under accession number PRJNA1242504. The data for each sample are available under the following accession numbers: PtMD (SRX28154934; [https://www.ncbi.nlm.nih.gov/sra/SRX28154934\[accn\]](https://www.ncbi.nlm.nih.gov/sra/SRX28154934[accn])), PtFD (SRX28154936; [https://www.ncbi.nlm.nih.gov/sra/SRX28154936\[accn\]](https://www.ncbi.nlm.nih.gov/sra/SRX28154936[accn])), PtMN (SRX28154935; [https://www.ncbi.nlm.nih.gov/sra/SRX28154935\[accn\]](https://www.ncbi.nlm.nih.gov/sra/SRX28154935[accn])), PtFN (SRX28154937; [https://www.ncbi.nlm.nih.gov/sra/SRX28154937\[accn\]](https://www.ncbi.nlm.nih.gov/sra/SRX28154937[accn])).

TABLE 2. Summary of the sequencing output from Illumina Hiseq 2000 RNA sequencing.

Sample	Total bases	Read count
PtMD	6,421,018,968	50,960,468
PtFD	6,346,522,728	50,369,228
PtMN	5,481,532,224	43,504,224
PtFN	5,773,136,544	45,818,544

PtMD (*P. tener* Male Day); PtFD (*P. tener* Female Day); PtMN (*P. tener* Male Night); PtFN (*P. tener* Female Night)

TABLE 3. The output from the *de-novo* assembly of the paired-end reads from all four samples was obtained by using the Trinity software

Trinity output result	Total bases
Total Trinity genes	119,591 bp
Total Trinity transcripts	160,894 bp
GC percentage	38.52%
Contig N50	2,014 bp
Median length	470 bp
Average length	1,024 bp
Total assembled bases	164,824,045 bp

[www.ncbi.nlm.nih.gov/sra/SRX28154935\[accn\]](http://www.ncbi.nlm.nih.gov/sra/SRX28154935[accn])) and PtFN (SRX28154937; [https://www.ncbi.nlm.nih.gov/sra/SRX28154937\[accn\]](https://www.ncbi.nlm.nih.gov/sra/SRX28154937[accn])).

After raw reads filtering and trimming were completed, clean reads were assembled using the Trinity software pipeline. To create an overall reference sequence, all four samples were assembled, and the nucleotide bp outcomes is as shown in Table 3. Assembly evaluation using Bowtie showed that more than 85% of the total reads for all of the samples were successfully aligned to the assembled transcriptome, indicating that the assembly effectively represents the original sequencing data. RNA-seq effectively captures molecular biological processes but poses challenges for species without a reference genome, such as *P. tener*, which can be overcome using Trinity *de novo* assembly to reconstruct sequences from short reads (Paszkiwicz & Studholme 2010).

TRANSCRIPT QUANTIFICATION AND CORRELATION MATRIX

Using the quantified transcript, $\log_2$ fold change between different samples was determined for the sample correlation matrix (Figure 3) and differential expression analysis. Transcript abundance across samples is shown

in the heatmap in Figure 4. The sample correlation matrix and the heatmap indicate a high degree of similarity within the same sample group, minor variations between fireflies of the same sex, and substantial differences between sexes and across different sampling times. The comparison of the transcript abundance suggested that differences between sexes have a stronger influence on gene expression patterns rather than the temporal variation observed between day and night sampling. These results offer a useful foundation for subsequent studies on the molecular mechanisms underlying flashing behaviour and mating activity. Additional research is required to determine the functional relevance of the highly upregulated genes in PtMN, that are potentially linked to bioluminescence. As synchronous flashing in *P. tener* occurs exclusively in males at night, the transcriptomic profile of PtMN was compared with PtMD, PtFD, and PtFN to gain an overview of the molecular mechanisms that may underlie and regulate this coordinated flashing behaviour.

GENE EXPRESSION PROFILING

Differentially expressed genes were identified based on genes with a False Discovery Rate (FDR)-adjusted p -value ≤ 0.05 . The representation of the number of

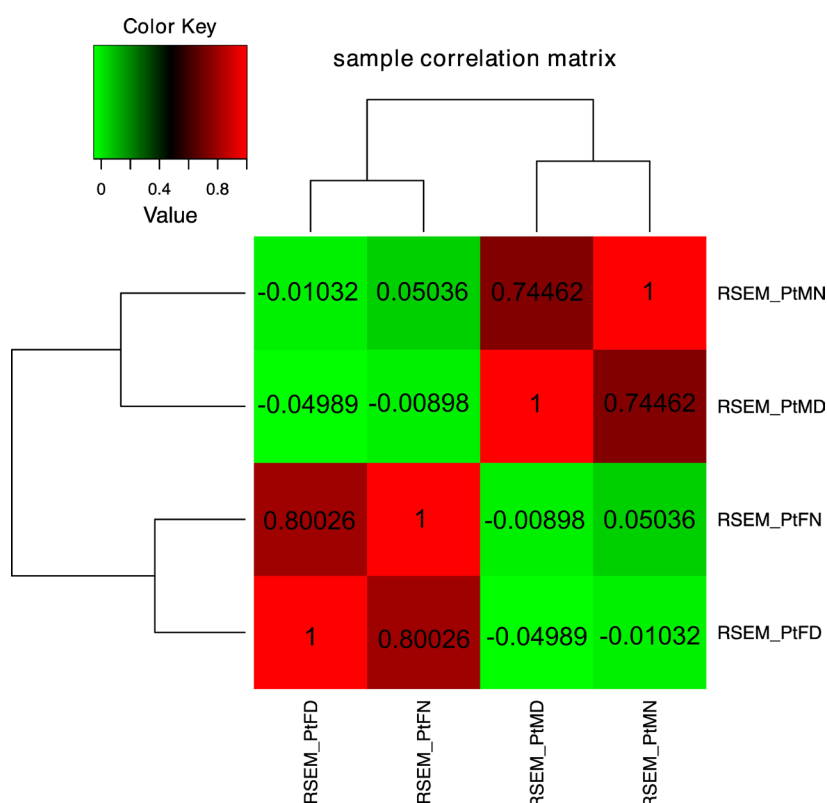


FIGURE 3. Heat map of the correlation between PtMD, PtFD, PtMN, and PtFN. The color shown by each sample corresponds to the correlation with values ranging from less than 0.4 (less correlated) to 1 (correlated). The distance between each sample corresponds to the log fold change between each sample

upregulated genes in PtMN compared to other samples is shown in Figure 5. An overlap of 151 genes was consistently upregulated in PtMN compared to PtMD, PtFD and PtFN samples (Figure 5). The list of the 151 overlap genes is provided in The Supplementary Material 2 has been provided.

A closer examination of the top 20 differentially upregulated genes with the highest $\log_2$ FC value in PtMN samples relative to other groups in this study showed molecular adaptations potentially associated with genes necessary to support high energy expenditure, locomotor function, and hormonal signalling. The high expression of specific transport proteins indicated that the capacity for rapid glucose and trehalose delivery is paramount. Facilitated trehalose transporter TRET1, which carries the primary haemolymph sugar in insects, is highly expressed in the male night sample with a maximum $\log_2$ FC value of 11.2. TRET1 functions to transport trehalose from storage organs, such as the fat body, to peripheral tissues with high energetic demand, including flight muscles, the central nervous system, and the photocytes (Kanamori et al. 2010; Mattila & Hietakangas 2017; Volkenhoff et al. 2015). In other insect systems, TRET1 expression is known to increase dramatically under energy-demanding conditions, such as mating or environmental stress (Hertenstein et al. 2021; Huo et al. 2020). Complementary to this, the upregulation of the Sugar Transporter Sweet 1 (maximum $\log_2$ FC = 11.1), a type of insect sugar transporter, underscores the need for high-flux carbohydrate transport for energy expenditure (Chaudhari, Pradhan & Joshi 2025).

P. tener nocturnal courtship necessitates acute visual processing and motor execution. The expression of Microtubule-associated protein Jupiter was upregulated in PtMN (maximum $\log_2$ FC = 11.5). In the model insect *Drosophila*, Jupiter is essential for maintaining the dynamics of cellular microtubules, and its homologs are known to be highly concentrated in the insect nervous system, including photoreceptor precursors (Karpova et al. 2006). This high expression likely supports the rapid maintenance and restructuring of the extensive neural network required for continuous visual processing and high-speed motor control during aerial manoeuvres and signalling.

The elevated expression of Cytochrome P450 9e2 in PtMN (maximum $\log_2$ FC = 12.0) highlighted the role of hormones during mating rituals. Cytochrome P450 enzymes are heme-containing oxidases involved in the metabolism of hormones, pheromones, and secondary metabolites (Feyereisen 2005). In insects, several P450s are associated with the biosynthesis and degradation of juvenile hormone (JH) (Feyereisen 2005), which is important for courtship behaviour in male *Drosophila* (Wijesekera, Saurabh & Dauwalder 2016). This may reflect enhanced hormonal activity associated with courtship or mating behaviours.

Carboxypeptidase B (CPB) (maximum $\log_2$ FC = 11.5) has been previously identified as a candidate seminal fluid protein (SFP) gene in *Ophraella communa*, where it exhibits strong male-biased expression, suggesting a potential reproductive role that may be conserved in male *P. tener* (Chen et al. 2023). Interestingly, CPB has been shown to efficiently hydrolyse the luciferin conjugate D-luciferyl-L-N α -arginine, rapidly liberating free D-luciferin, the substrate required for luciferase-catalysed light emission (Miska & Geiger 1987). This raises the intriguing possibility that CPB-mediated hydrolysis of potential conjugated D-luciferin could serve as a molecular control mechanism governing the timing and rapid onset of the firefly flash.

A previous hypothesis suggested that nitric oxide (NO), produced by nitric oxide synthase (NOS), regulates oxygen availability for luciferase activity, thereby controlling the flashing pattern (Trimmer et al. 2001; Tsai et al. 2014). However, studies on other synchronous flashing fireflies *Nipponoluciola cruciata* showed low NOS expression in the adult lantern, raising questions about this proposed mechanism (Ohtsuki et al. 2014). The present findings indicated that although NOS transcripts were detectable, they were not significantly upregulated in PtMN. Similarly, Calreticulin and VDAC transcripts were also detected but not significantly upregulated in PtMN samples compared to all other samples. These results might be due to the use of whole-body samples, which could have masked tissue-specific expression differences, particularly within the light organ, as observed in studies on *N. cruciata* larvae (Ohtsuki et al. 2014).

GENE ONTOLOGY (GO)

A total of 151 DEGs that were upregulated in PtMN were analysed for GO term enrichment to provide an overview of the functional categories overrepresented in this sample group. Table 5 lists the enriched GO terms across the three GO domains. Owing to the novelty of the species, fewer than 50% of the assembled transcripts could be annotated with GO terms. Consequently, the enrichment analysis was limited to annotated transcripts, which may have led to the exclusion of novel genes without known functional annotations.

The enrichment of gap junction (GO:0005921) in PtMN sample suggested a strong activation of electrical synaptic communication in the male central nervous system during courtship signalling. Gap junctions, composed of innexin proteins in insects, form intercellular channels that enable direct and rapid ionic and metabolic coupling between neurons. This allows for the millisecond-scale synchronisation of neural activity compared to chemical synapses (Aldworth & Stopfer 2022). Such electrical connectivity likely underlies the precise temporal coordination for producing synchronous light flashes during courtship displays.

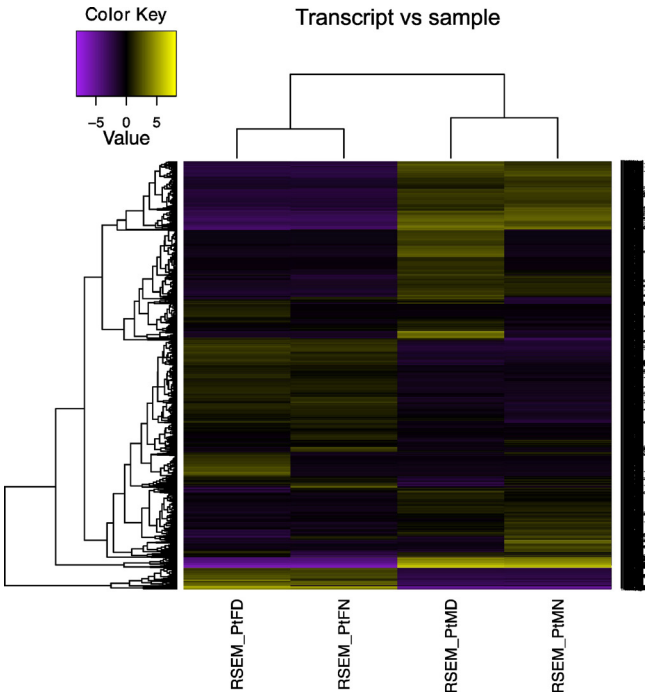


FIGURE 4. Heat map depicting the transcript abundance across PtFD, PtFN, PtMD, and PtMN. The color gradient represents log-transformed RSEM values, with lower abundance (below -5) shown in light purple and higher abundance (above 5) in light yellow. The distance between each sample corresponds to the log fold change between each sample

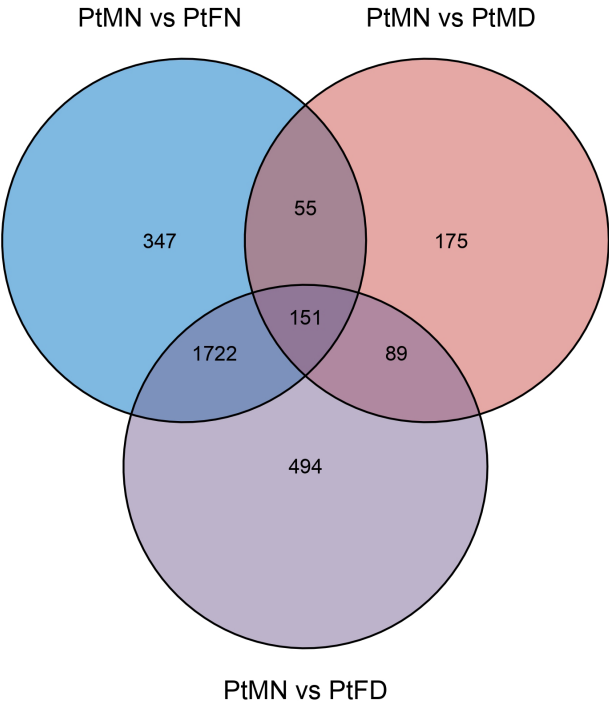


FIGURE 5. Venn diagram illustrating the overlap of differentially expressed genes (DEGs) that were upregulated in *PtMN* samples relative to *PtMD*, *PtFN*, and *PtFD* samples in pairwise comparisons. The central overlapping region represents 151 DEGs consistently upregulated in *PtMN* across all comparisons

TABLE 5. Significantly enriched Gene Ontology (GO) terms were identified among genes upregulated in PtMN samples compared with other sample groups. Only GO terms with $-\log_{10}(p\text{-value}) > 1.30$ are shown

Category	Go term	Description	$-\log_{10}(p\text{-value})$
Biological Processes	GO:0035999	tetrahydrofolate interconversion	1.80
	GO:0006563	L-serine metabolic process	1.59
	GO:0046653	tetrahydrofolate metabolic process	1.51
	GO:0006730	one-carbon metabolic process	1.43
	GO:0006544	glycine metabolic process	1.41
Cellular Component	GO:0005921	gap junction	1.89
Molecular Function	GO:0004372	glycine hydroxymethyltransferase activity	1.89
	GO:0004659	prenyltransferase activity	1.57
	GO:0046873	metal ion transmembrane transporter activity	1.52
	GO:0016742	hydroxymethyl-, formyl- and related transferase activity	1.48
	GO:0004197	cysteine-type endopeptidase activity	1.42
	GO:0005326	neurotransmitter transmembrane transporter activity	1.42
	GO:0005328	neurotransmitter:sodium symporter activity	1.42
	GO:0016741	transferase activity, transferring one-carbon groups	1.42
	GO:0022890	inorganic cation transmembrane transporter activity	1.39
	GO:0022857	transmembrane transporter activity	1.36
	GO:0015318	inorganic molecular entity transmembrane transporter activity	1.33
	GO:0015370	solute:sodium symporter activity	1.32

Genes involved in neurotransmitter transport, including neurotransmitter:sodium symporter activity (GO:0005328), were also enriched. Firefly flashes are regulated by the neurotransmitter octopamine (OA) (Copeland & Robertson 1982; Trimmer et al. 2001), which activates nitric oxide (NO) suggested to control oxygen availability for light production (Aprille et al. 2004; Greenfield 2001). The overrepresentation of neurotransmitter transporters, which could function to transport OA for perception or release from the receptor, suggests the functional system possibly involving OA that enables the quick switching on and off of light emission in *P. tener* (Rosikon, Bone & Lawal 2023).

influenced by sex rather than by the timing of sample collection. PtMN GO enrichment of gap junction and neurotransmitter transporter activities suggested a neural system optimized for rapid flash synchronisation. Future studies should focus on experimentally characterising the highly expressed candidate genes in PtMN as well as genes potentially involved in octopaminergic signalling, such as VDAC, Calreticulin and NOS, specifically from the lantern organ, as they may coordinate the initiation and termination of flashes. Combining transcriptomic data with functional analyses will deepen the understanding of the molecular mechanisms driving synchronous flashing behaviour in *P. tener* and other bioluminescent insects.

CONCLUSIONS

This study provided the first transcriptomic overview of the synchronous firefly *P. tener*, showing genes potentially responsible for its precise and energetically demanding bioluminescent courtship display. Overall, the transcriptomic profile of *P. tener* appeared to be

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