Sex-specific molecular specialization and activity rhythm-dependent gene expression in honey bee antennae

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RESEARCH ARTICLE

Sex-specific molecular specialization and activity rhythm-dependent gene expression in honey bee antennae

Rikesh Jain[1,2,*,‡] and Axel Brockmann[1]

ABSTRACT

We performed an RNA-seq-based comparison of gene expression levels in the antennae of honey bee drones and time-trained foragers (workers) collected at different times of the day and different activity states. Interestingly, olfaction-related genes [i.e. odorant receptor (*Or*) genes, odorant binding protein (*Obp*) genes, carboxyl esterase (*CEst*) genes, etc.] showed stable gene expression differences between drone and worker antennae. Drone antennae showed higher expression of 24 *Or* genes, of which 21 belong to the clade X which comprises the receptor for the major queen pheromone compound 9-ODA. This high number of drone-biased *Or* genes suggests that more than previously thought play a role in sex-pheromone communication. In addition, we found higher expression levels for many non-olfaction-related genes including *nitric oxide synthase* (*NOS*), and the potassium channel *Shaw*. In contrast, workers showed higher expression of 67 *Or* genes, which belong to different *Or* clades that are involved in pheromone communication as well as the perception of cuticular hydrocarbons and floral scents. Further, drone antennae showed higher expression of genes involved in energy metabolism, whereas worker antennae showed higher expression of genes involved in neuronal communication, consistent with earlier reports on peripheral olfactory plasticity. Finally, drones that perform mating flight in the afternoon (innate) and foragers that are trained to forage in the afternoon (adapted) showed similar daily changes in the expression of two major clock genes, *period* and *cryptochrome2*. Most of the other genes showing changes with time or onset of daily flight activity were specific to drones and foragers.

KEY WORDS: *Apis mellifera*, Antennal transcriptome, Sexual dimorphism, Mating behaviour, Foraging, Circadian clock

INTRODUCTION

Honey bees (*Apis mellifera*) and other eusocial insects, which show an elaborate division of labour involving morphologically and physiologically specialized phenotypes, provide a unique system to study the molecular underpinnings of behaviour (Brockmann et al., 2007; Liang et al., 2012; Whitfield et al., 2006). Honey bee drones (males) and workers (females), for example, strongly differ in their behaviour, morphology and physiology. Drones have to find and mate with a virgin queen at the same time as outcompeting other drones (Brockmann et al., 2006; Gary, 1962; Koeniger et al., 2005; Ruttner, 1985), whereas workers do all the tasks needed to maintain the colony and organize the underlying division of labour; and as foragers, they have to learn and memorize odour mixtures that indicate different rewarding flowers (Frisch, 1967). The different behavioural functions have led to different evolutionary adaptations in the visual and olfactory systems (Esslen and Kaissling, 1976; Menzel et al., 1991).

Honey bee antennae are multimodal sense organs housing olfactory, gustatory and mechanosensory sensilla, as well as CO_2-, humidity- and temperature-sensitive sensilla. However, Esslen and Kaissling (1976) estimated that in workers, 96.9% and in drones, 99.6% of the antennal sensory neurons are olfactory. Regarding olfaction, drones are considered olfactory specialists and workers generalists (Masson and Mustaparta, 1990). Drone antennae have about 7 times more olfactory poreplate sensilla, each containing about 15–30 olfactory sensory neurons (Esslen and Kaissling, 1976). Furthermore, in drones, most of these sensory neurons are specialized to detect minute amounts of the queen's sex pheromone (Brockmann et al., 1998; Vareschi, 1971; Wanner et al., 2007). Electrophysiological recordings indicated that the sex pheromone-responsive sensory neurons have lower action potential thresholds and higher firing frequencies compared with sensory neurons responsive to floral odours (Vareschi, 1971). Compared with drones, workers have a more generalist olfactory system, probably with a broader odorant response profile. For example, worker antennae house hair-like olfactory sensilla (s. basiconica) that are almost absent on drone antennae, and worker antennal lobes are composed of about 170 isomorphic olfactory glomeruli, whereas drones have only 100 normal-sized and 4 macro glomeruli (Arnold et al., 1985; Brockmann and Brückner, 2001; Esslen and Kaissling, 1976; Flanagan and Mercer, 1989; Galizia et al., 1999; Kamikouchi et al., 2004; Kropf et al., 2014; Sandoz, 2006; Wanner et al., 2007). Given the different behavioural functions and the strong sexual dimorphism, drone and worker antennal sensory systems may exhibit different sensory reception strategies and molecular adaptations (Masson and Mustaparta, 1990; Sandoz et al., 2007; Zayed et al., 2012). Previously, Wanner et al. (2007) reported differences in the expression of odorant receptor (*Or*) genes between drones and workers and showed that one of the drone-expressed *Or* genes (*Or11*) binds 9-ODA, the major sex pheromone compound. However, the differences in the overall molecular equipment for odorant reception might be larger and more complex between drone and worker antennae, particularly if one considers the possibility of temporal and behavioural state-dependent changes in gene expression.

In this RNA-seq study, we compared the antennal transcriptomes of sexually mature drones and time-trained foragers collected at different times of the day and under different activity states (Jain and Brockmann, 2018; Naeger and Robinson, 2016). The goals of our project were 3-fold. First, we wanted to provide a more comprehensive description of gene expression differences between

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Received 26 October 2019; Accepted 5 May 2020

Journal of Experimental Biology (2020) 223, jeb217406. doi:10.1242/jeb.217406

drone and worker antennae. Most studies on the insect antennal transcriptome still focus on identifying and reporting genes involved in odorant binding and detection (Liu et al., 2016; McKenzie et al., 2016; Missbach et al., 2020). In contrast, we also aimed at identifying so far unrecognized molecules, not directly involved in odorant detection, but likely to play an important role in peripheral olfactory processing. Second, global gene expression differences between drone and worker antennae might provide information about differences in peripheral sensory processing strategies between a specialist and a generalist peripheral olfactory system (Amin and Lin, 2019; De Bruyne and Baker, 2008; Renou, 2014). Finally, we wanted to explore whether daily changes in gene expression and correlations between gene expression levels and behavioural activity might be a fruitful approach to identify additional genes involved in odorant reception.

MATERIALS AND METHODS

Animals

In all experiments we used *Apis mellifera* Linnaeus 1758 colonies of naturally mated queens, which consisted of about 8000 workers (i.e. 8 frames with approximately 1000 workers) and hundreds of drones. Colonies were acquired from a local beekeeper and maintained on the campus of the National Centre for Biological Sciences – Tata Institute of Fundamental Research (NCBS-TIFR), Bangalore, India. All experiments including sample collections were performed under natural light–dark conditions during the months of October and November in Bangalore. During this time of the year, sunrise was at around 06:10 h and sunset was at around 18:00 h, which is close to 12 h of light and 12 h of darkness (LD 12:12) cycle (timeanddate.com).

Daily drone flight activity

Daily drone flight activity was determined for three colonies on three different days, i.e. three measurements for each colony, during the months of October and November. On the experimental days, the number of drones leaving the hive entrance was counted every half an hour for 10 min from morning (07:00 h) to evening (19:00 h) (LD 12:12; sunrise time 06:10 h, sunset time 18:00 h). It should be noted that drones and foragers of *A. mellifera* do not fly during the dark. On these days, we also recorded temperature and humidity changes every minute using a data logger (EQ-172, Equinox, Valli Aqua And Process Instruments, Chennai, India).

Collection of drones for antennal RNA-seq and qPCR

During daily mating flight activity under natural conditions, drones were caught at the hive entrance and colour marked on the thorax. The next day, colour-marked drones were collected at two different time points – 09:00 h (inactive) and 14:00 h (active/mating flight time) (see Naeger and Robinson, 2016) – from three different colonies (five drones per time point per colony). At 09:00 h, drones were collected from inside the colonies and at 14:00 h they were collected from the entrance before they started their mating flights. In addition, we collected colour-marked drones from one of the three colonies at six different time points: 06:00 h, 10:00 h, 14:00 h, 18:00 h, 22:00 h and 02:00 h (10 bees per time point) to determine daily expression changes of four major clock genes: *period* (*per*), *cryptochrome2* (*cry2*), *cycle* (*cyc*) and *clock* (*clk*). Night collections were done under dim red light. All collected drones were immediately flash frozen in liquid nitrogen.

Collection of time-trained foragers for antennal RNA-seq

An *A. mellifera* colony was transferred in an enclosed outdoor flight cage to entrain the foraging activity of the workers to a distinct time of the day. First, the colony was allowed to adjust to the new environment for 10 days. During this period, the sugar and pollen feeders were presented for the whole day. The sugar feeder was a yellow plastic plate surrounded by four filter papers containing a 5 µl drop of 100 times diluted linalool racemic mixture (Sigma-Aldrich, St Louis, MO, USA). Then, for time training, the sucrose reward (1 mol l^{-1} sucrose solution) was presented either from 08:00 h to 10:00 h (morning training) or from 13:00 h to 15:00 h (afternoon training) for 10 consecutive days (LD 12:12). Time for the afternoon training was chosen according to the drone flight time based on our behavioural data. Two different colonies were used for morning and afternoon training. Every day after the training time the feeder was cleaned with ethanol and linalool-scented filter papers were replaced with fresh unscented filter papers. This cleaned empty feeder was available for the rest of the day. On the 8th, 9th and 10th day of training, foragers visiting the feeder were marked on their thorax with different colours, one type of colour each day, to identify the frequently visiting foragers. On the 11th day, the feeder was not presented and the foragers that had all three colour marks were collected at 09:00 h and 14:00 h from both the colonies (10 bees per time point per colony). Collected foragers were immediately flash frozen in liquid nitrogen.

RNA isolation from the antennae

Collected honey bees were transferred from liquid nitrogen onto dry ice and the entire antennae (i.e. scape, pedicel and flagellum) were cut off. We pooled 10 antennae from five bees for RNA-seq samples and four antennae from two bees for qPCR samples. Total RNA was isolated using the Trizol® method (Invitrogen, Carlsbad, CA, USA). The tissue (i.e. antennae) was homogenized in Trizol, a solution of phenol and guanidinium isothiocyanate, at room temperature and then chloroform was added to separate the aqueous and organic phase. The aqueous phase, which contains RNA, was put into a new tube and total RNA was precipitated using isopropanol and sodium acetate. Samples were treated with DNaseI (Invitrogen) for 10 min to remove any possible DNA contamination. Final RNA concentration was measured using a Nanodrop and RNA quality was assessed by running samples on an agarose gel.

qPCR

To quantify the expression levels of clock genes using qPCR, around 800 ng of total RNA from each sample was converted into cDNA using Reverse Transcriptase Superscript III enzyme and oligo d(T)16 primers (Invitrogen). Then KAPA SYBR FAST qPCR Master Mix (Kapa Biosystems, Wilmington, MA, USA) in 7900HT Fast Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA) was used to perform qPCR. An internal control gene for normalization was selected after comparing the expression profiles of six different housekeeping genes: *tubulin* (XM_396338), *RpS18* (XM_625101), *actin* (NM_001185145), *ef-1a-f1* (NM_001011628), *gapdh* (XM_393605) and *ribosomal protein49* (*rp49*) (NM_001011587). *rp49* showed the highest stability (NormFinder; Andersen et al., 2004) in our experimental conditions (Fig. S3) and was used for normalization of clock gene expression data. All the primer sequences used in this study are available from figshare (https://doi.org/10.6084/m9.figshare.12089370.v1, Table S1). Triplicate reactions (10 µl reaction mix) for all the biological replicates of all six time point samples (n=5 per time point) were run in parallel on the same 384-well plate. This restricted us to analyse just one of the clock genes and *rp49* (internal control gene) per plate. We also ran a standard curve for both primers on the same plate using a separate stock cDNA. Final gene expression calculation was based

on the linear values interpolated from the standard curves. Efficiency of all the primers was between 95% and 100%. Reactions with a qPCR dissociation curve that did not support specificity of the PCR reaction were discarded from the analysis.

RNA-seq

Antennal transcriptomes of drones (n=3 per time point), morning-trained foragers (n=2 per time point) and afternoon-trained foragers (n=2 per time point) were sequenced for two different time points (09:00 h and 14:00 h). Total RNA from all the samples was shipped on dry ice to AgriGenome Labs (Kochi, India). RNA quality was further checked on Agilent Tapestation and Qubit. Libraries were prepared using TruSeq Ribo-Zero Gold+TruSeq Stranded mRNA Library Prep kit (Illumina, San Diego, CA, USA). Sequencing was performed on an Illumina HiSeq2500 platform and around 120 million (~7–10 million per sample) of 75 bp paired-end reads were generated.

Data analysis

qPCR

We used the cosinor package (https://github.com/sachsmc/cosinor) in R (http://www.R-project.org/) to fit a 24 h cosine model {y=intercept+amplitude×cos[2×π(x−acrophase)/24]} (Nagari et al., 2017) in the circadian gene expression data. We also performed a non-parametric Jonckheere–Terpstra–Kendall (JTK) cycle analysis (Hughes et al., 2010; Patton et al., 2014) to detect the phase and amplitude of clock gene expression profiles.

RNA-seq

Approximately 7–10 million pairs of 75 bp reads per sample were mapped to the *A. mellifera* genome (NCBI Assembly Amel_HAv3.1; Wallberg et al., 2019) using STAR RNA-seq aligner (Dobin et al., 2013). The alignment rate was more than 75% (75.15–86.82%) for all the samples. The number of reads aligning to each gene was counted using featureCounts, a program specifically designed for read summarization on genomic features (Liao et al., 2014). The read counts for each gene were normalized with the respected library size and differences in gene expression between the samples were calculated using DESeq2 (Love et al., 2014). Genes showing expression differences with adjusted P-values (P_{adj}) less than 0.05 (Wald test) were called differentially expressed genes (DEGs). The Pathview package (Luo and Brouwer, 2013) in R was used to map the above differential gene expression data to relevant pathway graphs from the Kyoto Encyclopedia of Genes and Genomes (KEGG) and to visualize. In addition, the GAGE package (Luo et al., 2009) in R was used for gene-set analysis (GSA) using normalized count data from featureCounts and the KEGG pathway database (Kanehisa and Goto, 2000). Gene ontology (GO) enrichment analysis was done using g:Profiler (Raudvere et al., 2019) with an alpha of 0.05 as the cut off for significance.

RESULTS

Drone and worker antennae showed characteristic differences in their transcriptomic profiles (Fig. 1), and the 14 RNA-seq samples (6 drones and 8 workers) separated into two distinct sex-specific clusters (Fig. 1B). We could detect and compare the expression levels of 10,635 genes between drone and forager antennae using our RNA-seq data. Overall, we identified 4004 DEGs (P_{adj}<0.05, Wald test), of which 1814 were more highly expressed in drones and 2190 were more highly expressed in foragers (Fig. 1A; see also figshare: https://doi.org/10.6084/m9.figshare.12089370.v1, Table S2).

Expression differences in olfaction-related genes

We were able to detect expression of most of the annotated olfaction-related genes, i.e. we detected 146 out of 167 *Or* genes, 15 out of 21 odorant-binding protein (*Obp*) genes, all 6 chemosensory protein (*CSP*) genes, 13 out of 17 carboxylesterase (*CEst*) genes, 8 out of 10 gustatory receptor (*Gr*) genes, 34 out of 45 cytochrome P450 (*CYP*) genes, 7 out of 8 glutathione *S*-transferase (*Gst*) genes and 42 out of 51 other cytochrome-related genes. Of the 146 detected *Or* genes, 91 showed significant expression differences (P_{adj}<0.05, Wald test) between drone and forager antenna, of which 24 (13 with $\log_2$ fold-change>1) were more highly expressed in drones and 67 (54 with $\log_2$ fold-change>1) were more highly expressed in foragers (Fig. 1C; see also https://doi.org/10.6084/m9.figshare.12089370.v1, Table S3). In addition, we found significant expression differences (P_{adj}<0.05, Wald test) for 13 *Obp* genes (4 drone biased and 9 forager biased), 4 *CSP* genes (all higher in foragers), 12 *CEst* genes (5 drone biased and 7 forager biased), 5 *Gr* genes (2 drone biased and 3 forager biased), 22 *CYP* genes (8 drone biased and 14 forager biased), 15 *Gst* genes (all higher in drones) and 26 other cytochrome-related genes (22 drone biased and 4 forager biased) between drone and forager antennae (Fig. 1C,D; see also https://doi.org/10.6084/m9.figshare.12089370.v1, Table S3).

Regarding the differentially expressed *Or* genes, 21 out of the 24 drone biased *Or* genes belong to the prominent clade X (hymenopteran subfamily L) which comprises the 9-ODA-sensitive *Or11* (https://doi.org/10.6084/m9.figshare.12089370.v1, Table S4; see Karpe et al., 2016; Robertson and Wanner, 2006). The 67 forager-biased *Or* genes belong to 13 different *Or* clades, but most of them are members of 4 clades: clade XXI (n=20, hymenopteran subfamily J, a bee expanded clade), clade X (n=16, the '9-ODA clade'), clade XI (n=11, 9 exon – putative CHC receptors) and clade XVIII (n=5, hymenopteran subfamily H, putative floral scent receptors) (see https://doi.org/10.6084/m9.figshare.12089370.v1, Table S4).

Finally, we found almost all olfaction-related genes that were previously reported to be differentially expressed between drones and workers were among the DEGs with the highest expression differences: *Or10*, *Or11* (the 9-ODA odorant receptor), *Or18*, *Or170* and *CEst1* (higher in drones; Wanner et al., 2007); *Or63*, *Or81*, *Or109*, *Or150*, *Or151*, *Or152*, *Obp2*, *Obp4*, *Obp11*, *Obp16*, *Obp19*, *Obp21*, *CSP6* and *Cyp6BE1* (higher in foragers; Forêt and Maleszka, 2006; Wanner et al., 2007) (see Fig. 1D; see also https://doi.org/10.6084/m9.figshare.12089370.v1, Table S3).

Expression differences in 'non-olfaction-related' genes

Besides the genes that are directly involved in odorant detection, we found a number of differentially expressed genes ($\log_2$ fold-change>1, P_{adj}<0.05, Wald test) that are probably involved in olfactory sensory neuron processing or other sensory functions of the antennae (Fig. 1A; see also https://doi.org/10.6084/m9.figshare.12089370.v1, Table S2). Drone antennae showed higher expression of the two major sex-determining genes, *complementary sex determiner* (*csd*) and *feminizer* (*fem*), as well as *nitric oxide synthase* (*NOS*), and the potassium channel *Shaw* (Shaker cognate w; see Hodge, 2009).

Forager antennae showed higher expression of several genes involved in biogenic amine signalling (*Dop1*, *Dop3*, *DopR2*, *5-HT2alpha*, *5-HT2beta*, *TyrR*), glutamate signalling (ionotropic glutamate receptor, glutamate receptor 1, vesicular glutamate transporter 1, metabotropic glutamate receptor 1 and glutamate decarboxylase 1 genes), enzymes of the tyrosine/dopamine metabolic pathway (tyrosine hydroxylase and tyrosine

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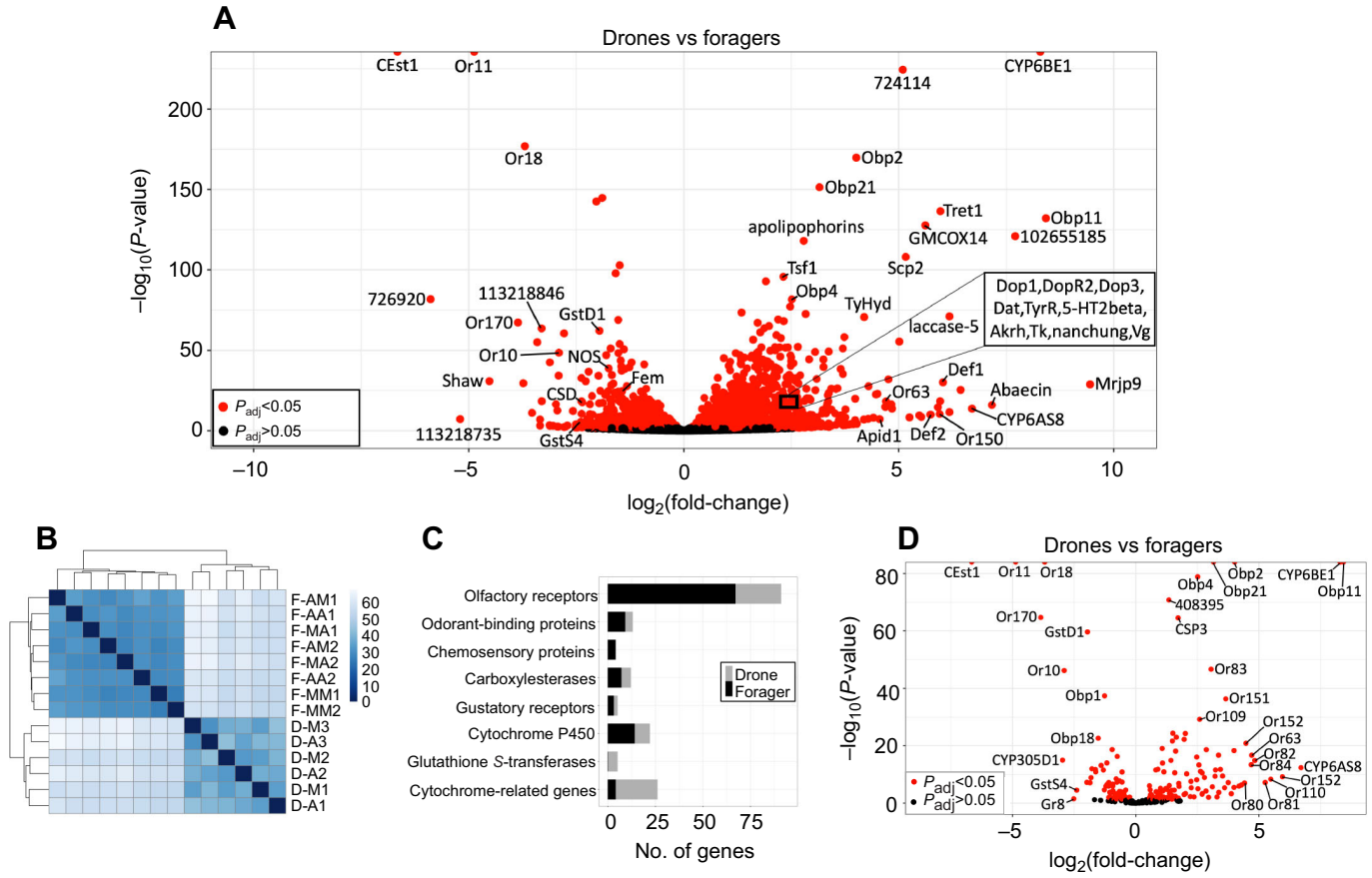

Fig. 1. Sexual differences in the antennal transcriptome. Drones (males) and time-trained foragers (females) were collected from *Apis mellifera* colonies at different times of the day and their antennal transcriptome was sequenced. (A) Overall differences between the antennal transcriptome of drones (n=6) and foragers (n=8). Genes with negative $\log_2$ fold-change values (left) were more highly expressed in drones, and genes with positive $\log_2$ fold-change values (right) were more highly expressed in foragers. Red circles indicate genes with significantly different expression (adjusted P-value, P_{adj}<0.05, Wald test, DESeq2); black circles indicate genes with non-significantly different expression (P_{adj}>0.05). (B) Sample distance heatmap based on variance stabilized RNA-seq read-count (DESeq2). Samples that are closer to each other in the dendrogram have more similar transcriptome profiles. Row names are the sample names in which the first letter, D or F, stands for drone or forager. In the drone samples, the next letter, M (morning) or A (afternoon), indicates the time when the sample was collected; in forager samples, the next letter is the training time and the last letter is the collection time (e.g. in F-AM1, A is the training time and M is the time when the sample was collected). The number indicates the replicate number. Drone and forager samples separated into two clear clusters suggesting huge transcriptome-wide differences. (C) Number of olfaction-related genes differentially expressed between drones and foragers. (D) Differentially expressed olfaction-related genes across drone versus forager antennae.

aminotransferase genes), as well as several genes involved in neuropeptide signalling [adipokinetic hormone receptor (*Akhr*), tachykinin (*Tk*), prohormone-2 and neprilysin-4 genes].

Further, the TRPV channel nanchung showed a higher gene expression in forager antennae. *nanchung* was reported to be expressed in the Johnston's organ and involved in hearing and gravity perception (Ai et al., 2007; Sun et al., 2009). Mondet et al. (2015) previously showed a higher expression of *nanchung* in forager antennae compared with nurse antennae.

Finally, *vitellogenin* (*Vg*), several genes of the major royal jelly and yellow proteins (*Mrjp1*, *Mrjp3*, *Mrjp4*, *Mrjp6*, *Mrjp8*, *Mrjp9*, *Y-h*, *Y-y*, *Y-e3*, *Y-f*), all likely to be involved in sex- and caste-specific behaviours (Buttstedt et al., 2013; Drapeau et al., 2006), as well as the immune response genes *Def1*, *Def2*, *abaecin*, *Apid1* and *Tsf1* were more highly expressed in worker antennae.

KEGG pathway and GO analyses of DEGs

GSA using the KEGG pathway database revealed significant (q<0.05, GAGE) upregulation of 67 biological pathways in drone antennae and 3 biological pathways in forager antennae (see https://doi.org/10.6084/m9.figshare.12089370.v1, Table S5). Two of the most significant pathways (lowest q-values) in drone antennae were oxidative phosphorylation (ame00190) and protein processing in the endoplasmic reticulum (ame04141) (Fig. S1). In contrast, in worker antennae, the two most significant pathways were neuroactive ligand-receptor interaction (ame04080) and tyrosine metabolism (ame00350) (Fig. S2).

GO enrichment analysis using DEGs (P_{adj}<0.05, g:Profiler) with more than 2-fold expression differences (539 DEGs in drones and 973 in foragers) showed significant enrichment of 57 and 95 GO terms in drones and foragers, respectively (P<0.05; see https://doi.org/10.6084/m9.figshare.12089370.v1, Table S6). Some of the top GO terms in drones were catalytic activity (GO:0003824), oxidoreductase activity (GO:0016491), metabolic process (GO:0008152), protein folding (GO:0006457), mitochondria (GO:0005739) and mitochondrial protein complex (GO:0098798). In foragers, some of the significantly enriched GO categories were signalling receptor activity (GO:0038023), molecular transducer activity (GO:0060089), regulation of cellular process (GO:0050794), cell communication (GO:0007154),

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integral component of membrane (GO:0016021) and extracellular region (GO:0005576).

Similarity in clock gene expression patterns between drones and foragers

Drones of all three *A. mellifera* colonies performed mating flights between 13:00 h and 15:00 h on all three observation days (Fig. 2A). The drone flight activity did not differ among colonies and experimental days. During these 2 h, the temperature was about 25°C and the relative humidity was around 60–70% (Fig. 2B).

The antennae of drones performing mating flights showed significant 24 h daily rhythms (JTK cycle analysis, P_{adj}<0.05) in the mRNA levels of major clock genes (n=5 per time point; Fig. 3 and Table 1). *cry2* and *per* mRNA levels peaked during the early morning, while the *cyc* mRNA level was highest during the afternoon. *clk* mRNA levels did not change. *cry2* levels oscillated with higher amplitude than those of *per* and *cyc*. Interestingly, this expression pattern of clock genes is similar to that of afternoon-trained foragers (Jain and Brockmann, 2018; Spangler, 1972).

Activity state and time of day affect antennal gene expression in drones and foragers

Here, we analysed the changes in antennal transcriptome with activity state and time of day. In drone antennae, we found 78 genes that were differentially expressed (P_{adj}<0.05, Wald test) (Fig. 4A; see also https://doi.org/10.6084/m9.figshare.12089370.v1, Table S7) between morning and afternoon. As drones fly out only in the afternoon, these 78 DEGs represent a combined list of genes affected by time of day and activity state. In foragers, morning and afternoon feeder time training allowed separation of time of day and activity state effects on gene expression differences. First, by comparing gene expression differences between the active forager group (i.e. morning-trained foragers collected in the morning and afternoon-trained foragers collected in the afternoon) and in-active forager group (i.e. morning-trained foragers collected in the afternoon and afternoon-trained foragers collected in the morning), we identified 17 DEGs (P_{adj}<0.05, Wald test) that are likely to be regulated by activity state (Fig. 4A; see also https://doi.org/10.6084/m9.figshare.12089370.v1, Table S7). Second, by comparing gene expression differences between morning and afternoon irrespective of activity state or training time, we identified 50 DEGs (P_{adj}<0.05, Wald test) that were associated with the time of day (Fig. 4A; see also https://doi.org/10.6084/m9.figshare.12089370.v1, Table S7). Finally, we also compared the combined transcriptomes of the morning-trained bees (collected in the morning and afternoon) against the combined transcriptomes of the afternoon-trained bees (collected in the morning and afternoon), but did not find any DEGs, suggesting there were no colony differences or differences due to morning and afternoon training itself.

Comparing the identified DEGs of these workers with those of the drones, we found that 2 out of the 17 activity state-regulated worker DEGs (*per* and *LOC107966102*) also showed a difference in expression between the drone samples. Likewise, 10 out of the 50 time of day-regulated worker DEGs (including *cry2*) showed a

Table 1. Non-parametric JTK cycle analysis for time-series qPCR data

Gene name	Phase	Amplitude	P_{adj}
cryptochrome2	04:00 h	6.62	7.96E−10
period	04:00 h	3.19	2.02E−08
cycle	10:00 h	0.53	3.54E−04
clock	06:00 h	0.01	1

JTK, Jonckheere–Terpstra–Kendall algorithm (Hughes et al., 2010); Phase, approximate time of day at which the gene expression cycle reaches its maximum; Amplitude, amplitude of the mRNA expression cycle; P_{adj}, adjusted *P*-values.

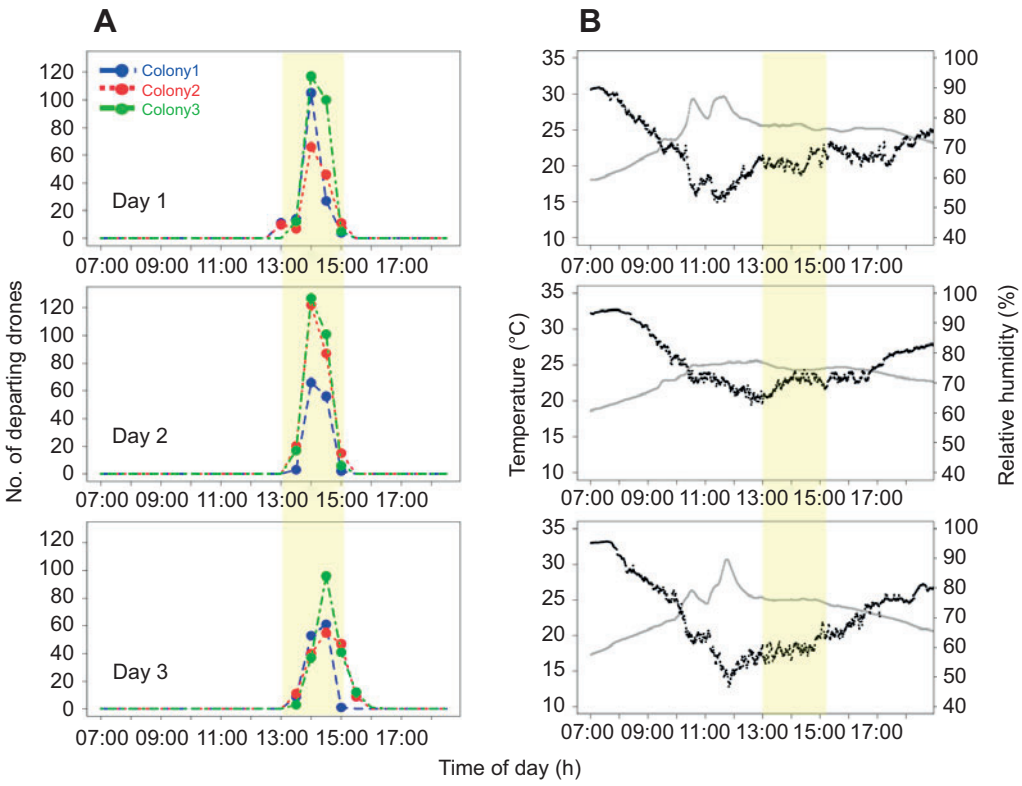

Fig. 2. Drone flight timing of *A. mellifera* in Bangalore. (A) The number of departing drones was counted for the first 10 min every half an hour from morning to evening (07:00 h to 19:00 h) over 3 days from three different colonies (see key). Sunrise was at around 06:10 h and sunset was at around 18:00 h. Counting was performed only during the day as *A. mellifera* drones do not fly at night. (B) Temperature (grey) and humidity (black) were recorded every minute on all 3 days. Yellow bars indicate the time of the drone flight.

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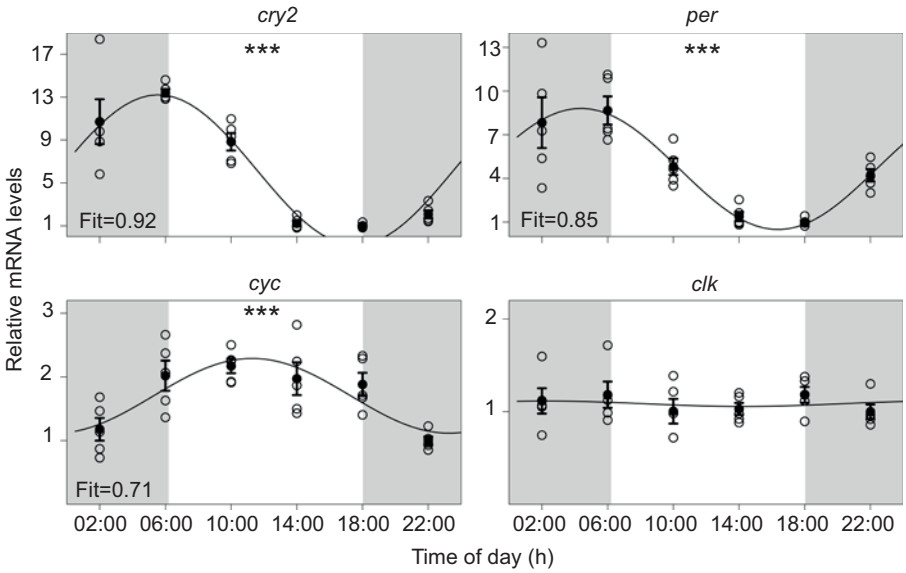

Fig. 3. Clock gene mRNA rhythm in drone antennae. During daily mating flight activity under natural conditions, mature flying drones were colour marked and collected the next day at 6 different time points from the colony. Open circles indicate individual qPCR measurements from 4 pooled antennae per sample (n=5 per time point), normalized against the internal reference gene, *rp49*. Filled circles represent the mean±s.e.m. Curved lines through the data points correspond to the best fitted 24 h cosine models. Fit values for the cosine models are indicated at the bottom left of the plots. Statistical significance of daily mRNA rhythms is indicated by asterisks (***P_{adj}<0.005, JTK cycle analysis) at the top of each plot. Grey shading indicates the night time (sunrise at 06:10 h and sunset at 18:00 h).

difference in expression between the drone samples (Fig. 4A,B). Furthermore, 8 out of these 12 common DEGs showed expression changes in the same direction in drones and foragers (Fig. 4B). *per* was more highly expressed during the inactive state and *cry2* was more highly expressed during the morning in both drones and foragers. Similarly, *LOC107966102*, *CUBN*, *DNAH3*, *LOC100576934*, *low-density lipoprotein receptor-related protein 2* and *RNA-binding protein 1* showed changes in the same direction in both drones and foragers (Fig. 4B). In contrast, the expression changes in the remaining 4 common DEGs (*hsp60A*, *hsp90*, *ebony*, *AAEL008004*) were in the opposite direction in drones and foragers (Fig. 4B).

Despite these similarities, most of the DEGs showing differences with respect to activity state or time of day were either drone or worker specific. Sixty-six DEGs showed changes in expression only in the drone antennae (see https://doi.org/10.6084/m9.figshare.12089370.v1, Table S7). Among these genes were *jun-related antigen* (*Jra*), *Hr38*, *endoplasmin*, *SIFamide receptor* (*SIFR*), *foraging* (*for*), *dopa decarboxylase* (*Ddc*), *kruppel homolog-1*, *semaphorin-2A*, *prohormone-2* and few heat shock protein (*hsp*) genes. In foragers, we found 15 and 40 unique DEGs associated with time of day and activity state, respectively (DEGs associated with activity state include: *bruchpilot*, *semaphorin-1A*, *translation initiation factor 2* (*IF-2*) and *histone H1*; DEGs associated with time of day include: *takeout*, *ataxin-2 homolog*, *Cdk4*, netrin receptor gene *UNC5B* and non-coding nuclear RNA gene *Ks-1*; see also https://doi.org/10.6084/m9.figshare.12089370.v1, Table S7).

GSA revealed significant enrichment (q<0.05, GAGE) of 3 pathways (Fig. 4C; see also https://doi.org/10.6084/m9.figshare.12089370.v1, Table S8): oxidative phosphorylation (ame00190), protein processing in endoplasmic reticulum (ame04141) and ribosome (ame03010). The oxidative phosphorylation pathway (ame00190) showed significant enrichment only in foragers and was strongly associated with their activity state (Fig. 4C). ame04141 and ame03010 were found to be significantly enriched in both foragers and drones (Fig. 4C). In foragers, both gene-sets were up-regulated during active states (during their feeder training time) while in drones, ame04141 was up-regulated during the active state (in the afternoon during their mating flight time) and ame03010 was up-regulated during the inactive state (in the morning).

DISCUSSION

So far, most studies on sexual dimorphism in antennal gene expression have focused solely on olfaction-related genes (Liu et al., 2016; McKenzie et al., 2016; Missbach et al., 2020) and have only used antennae collected at a single time of the day, neglecting that antennal gene expression might be temporally dynamic. Here, we performed an extensive comparison of gene expression levels between the antennae of honey bee drones and foragers collected at different activity states, i.e. resting versus mating flight activity and resting versus foraging flight activity. Altogether, we detected 4004 DEGs (P_{adj}<0.05), with 1814 more highly expressed in drones and 2190 more highly expressed in foragers (Fig. 1A; see also https://doi.org/10.6084/m9.figshare.12089370.v1, Table S2).

Interestingly, none of the canonical olfaction-related genes showed significant expression changes with activity state or time of day. Thus, drone and worker antennae show stable sex-specific molecular specialization which nicely correlates with the already described morphological and physiological differences (Brockmann and Brückner, 2001; Esslen and Kaissling, 1976; Vareschi, 1971). We identified 20 new drone-biased *Or* genes, all but one of which belong to the clade X that comprises the 9-ODA-sensitive *Or11* (Karpe et al., 2016; Robertson and Wanner, 2006; Wanner et al., 2007). This finding suggests that more than just 4 *Or* genes (as previously suggested) might be involved in honey bee sex-pheromone communication. For workers, we found 67 more highly expressed *Or* genes. Intriguingly, most of these genes belong to the bee expanded clade (clade XXI) as well as *Or* clades that are associated with the perception of mandibular gland pheromone (clade X) cuticular hydrocarbons (clade XI), and floral scents (clade XVIII; see Karpe et al., 2016; Robertson and Wanner, 2006). Regarding the other olfaction-related genes (*Obp*, *CSP*, *CEst*, *Gr*, *CYP*, *Gst* and cytochrome-related genes), most were more highly expressed in foragers with the exception of the *Gst* and cytochrome-related genes, which were generally more highly expressed in drones. *Gst* and cytochrome-related genes are assumed to be involved in signal (pheromone) inactivation, which is very important for fast pheromone-guided flight navigation (Leal, 2013; Rogers et al., 1999).

Among the non-olfaction-related genes showing a higher expression in drone antennae were *csd*, *fem*, *NOS* and, most interestingly, the potassium channel gene *Shaw* (Shaker cognate w).

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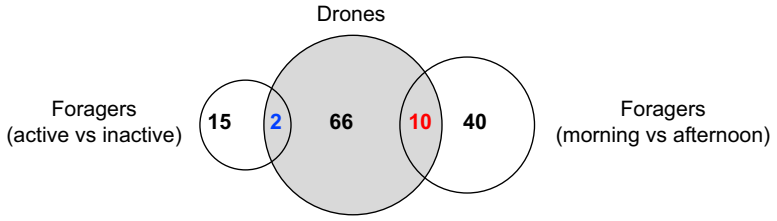
A



B

Entrez ID	Gene name	Foragers		Drones
		Activity (A)	Time (T)	A/T
406112	*period* (*per*)	Inactive	–	Inactive
107966102	*LOC107966102* (ncRNA)	Active	–	Active
410197	*cryptochrome2* (*cry2*)	–	Morning	Morning
411053	*cubilin* (*CUBN*)	–	Morning	Morning
412230	*dynein heavy chain 3* (*DNAH3*)	–	Morning	Morning
100576934	*LOC100576934*	–	Morning	Morning
552358	*low-density lipoprotein receptor-related protein 2*	–	Afternoon	Afternoon
413835	*RNA-binding protein 1*	–	Afternoon	Afternoon
409384	*heat shock protein 60A* (*hsp60A*)	–	Morning	Afternoon
408928	*hsp90*	–	Morning	Afternoon
409109	*mycosubtilin synthase subunit C* (*ebony*)	–	Afternoon	Morning
550828	*elongation of very long chain fatty acids protein* (*AAEL008004*)	–	Afternoon	Morning

C

KEGG ID	Pathway name	Foragers		Drones
		Activity (A)	Time (T)	A/T
ame00190	Oxidative phosphorylation	Active	–	–
ame04141	Protein processing in endoplasmic reticulum	Active	–	Active
ame03010	Ribosome	Active	–	Inactive

Fig. 4. Gene expression changes in drone and worker antennae with flight activity and time of day. (A) The number of differentially expressed genes (DEGs) in drones and foragers that showed changes with activity state and time of day. In drones, there were a total of 78 (66+2+10) DEGs that showed changes in expression between morning (inactive state) and afternoon (active state). In foragers, there were 17 (15+2) DEGs that showed changes with activity state and 50 (40+10) DEGs that showed changes with time of day, of which 2 and 10 DEGs, respectively, were common with drones. (B) All 12 common DEGs from the Venn diagram in A (colour coded) are listed along with the activity state or time of day when their expression was higher. (C) Significantly enriched gene sets (q<0.05, GAGE) are listed with the activity state when their average expression was higher.

It is not entirely clear why the two major sex-determining genes *csd* and *fem* were more highly expressed in the drone antennae, but Naeger and Robinson (2016) also found a higher expression of these two genes in the mushroom bodies of drones compared with those of workers. There is some evidence that *NOS* modulates responses of olfactory sensory neurons in insects (Wasserman and Itagaki, 2003). In contrast, as far as we know, there is no report regarding a possible function of *Shaw* in insect olfaction. However, there is evidence that the sensillum lymph contains high K^+ concentrations and the membrane potential of olfactory sensory neurons is regulated by the movement of K^+ ions (Küppers and Thurm, 1979; Mohapatra and Menuz, 2019; Stengl et al., 1999). Also, *Shaw* was demonstrated to play a role in modulating the activity of clock neurons (Hodge and Stanewsky, 2008).

Although worker antennae have only one-seventh the number of sensory neurons of drones, their antennal tissue shows a higher expression of many genes involved in neural modulation (e.g. biogenic amine signalling, glutamate and neuropeptide signalling). In addition, our GO enrichment analysis suggested higher expression of genes involved in secondary messenger cascades, cell signalling and extracellular matrix in forager antennae. Both findings suggest a higher degree of signalling plasticity in the worker antennae. Plasticity in antennal processing might play a role in division of labour and in learning and memory. Previous studies in honey bees (McQuillan et al., 2012; Vergoz et al., 2009) demonstrated that the expression of dopamine and tyramine receptors is age and task dependent and can be modulated by social pheromones.

Further, we found a higher expression of the TRPV channel gene *nanchung* in worker antennae. *nanchung* was reported to be expressed in the Johnston's organ and involved in hearing and gravity perception (Ai et al., 2007; Sun et al., 2009). Mondet et al. (2015) previously showed a higher expression of *nanchung* in forager antennae compared with nurse antennae. The higher expression of *nanchung* might indicate a more sensitive or even more elaborate functional organization of the worker Johnston's organ, which is involved in several worker-specific tasks and communication (Ai et al., 2007, 2009; Frisch, 1967; Sun et al., 2009; Towne and Kirchner, 1989).

Finally, our GSA showed that genes involved in oxidative phosphorylation are predominantly more highly expressed in drone antennae. Correspondingly, we found that in workers, metabolic genes are regulated with respect to activity state. Drone antennae have a much higher number of sensory neurons than worker antennae and coding in sensory neurons is based on generating action potentials, which are energy expensive (Attwell and Laughlin, 2001; Niven and Laughlin, 2008). Furthermore, a higher expression of genes involved in protein folding and protein processing might be a consequence of a higher protein turnover rate associated with higher neural activity in drone antennae (Dörrbaum et al., 2018).

Similar to worker antennae, drone antennae exhibit robust daily oscillation of the two major clock genes *per* and *cry2*. More interestingly, the daily oscillations of *per* and *cry2* expression in the drone antenna were similar to those in the antennae of afternoon-trained foragers (Jain and Brockmann, 2018; Sasaki, 1990; Spangler, 1972). Given that drone mating flight times are species specific and innately activated but foraging time of workers can be shifted, it is highly likely that these daily oscillations are differently regulated. Furthermore, the comparison of expression levels between time of day and activity state suggest that the expression of the two clock genes is sensitive to different environmental factors. Expression of *per* seems to be sensitive to activity state (rest–activity cycle), whereas *cry2* is more stably associated with time of day (light–dark cycle). Unfortunately, not much is known about the plasticity of the circadian clock with respect to individual activity and changing light–dark cycles (Saunders, 2002).

In addition to the clock genes, we identified a total of 133 genes that show changes with time and activity state in the antennae of drones and foragers. Twelve of these genes (Fig. 4B) were common and 121 genes were different between drones and foragers, suggesting a strong sexual dimorphism in the set of genes that are regulated by the circadian clock and behavioural activity. Certainly, a more detailed (e.g. more collection time points) study that identifies which genes are innately regulated by the circadian clock and which genes can come under the control of the circadian clock by time training will be very instructive.

Finally, the findings of our RNA-seq study corroborate that the functional organization of insect antennae might be more complex than just detecting odorants and transmitting sensory signals to the brain (Andersson et al., 2010; Couto et al., 2005; Getz and Akers, 1994, 1995). Drone antennae are optimized to detect small amounts of the queen's pheromone and quickly respond to changes in pheromone concentration (Brockmann et al., 1998; De Bruyne and Baker, 2008), whereas forager antennae are predominantly involved in context-dependent detection and discrimination of complex odour mixtures, which might require flexible filtering of sensory signals sent to the brain (Conchou et al., 2019; Gadenne et al., 2016; Getahun et al., 2013). Previous extracellular recordings from the olfactory poreplate sensilla indicated that there might be physiological interactions between the olfactory sensory neurons within one sensillum (Getz and Akers, 1994, 1995). Furthermore, inhibitory interactions between olfactory sensory neurons had been suggested to sharpen and filter the sensory signal sent to the brain (Andersson et al., 2010; Couto et al., 2005). Our finding that worker antennae show higher expression of genes involved in neural modulation nicely corresponds with the many reports of context-dependent plasticity in peripheral sensory processing in worker honey bees (Bigot et al., 2012; Gadenne et al., 2016; Grosmaitre et al., 2001; McQuillan et al., 2012; Vergoz et al., 2009; Watanabe et al., 2014).

Acknowledgements
We are grateful to W. Roessler and J. Spaethe for constructive discussion regarding the project and experimental procedure during a research stay of R.J. at the University of Wuerzburg. We thank S. Unnikrishnan, W. Roessler, J. Spaethe, T. Tanimura and F. Marion-Poll for valuable and instructive comments on an earlier version of the manuscript.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: R.J., A.B.; Methodology: R.J.; Validation: R.J.; Formal analysis: R.J.; Investigation: R.J.; Resources: A.B.; Data curation: R.J.; Writing - original draft: R.J.; Writing - review & editing: R.J., A.B.; Visualization: R.J., A.B.; Supervision: A.B.; Project administration: A.B.; Funding acquisition: A.B.

Funding
We acknowledge support of the Department of Atomic Energy, Government of India [under project no. 12-R&D-TFR-5.04-0800]. R.J. was supported by an Indian Council of Medical Research (ICMR) fellowship and a DAAD (German Academic Exchange Service) 'A New Passage to India' travel fellowship; A.B. was supported by NCBS/TIFR (National Centre for Biological Sciences/Tata Institute of Fundamental Research) institutional funds [12P4167].

Data availability
Raw RNA-seq data are available at https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-8489. Additional data (Tables S1–S8) have been deposited in figshare: https://doi.org/10.6084/m9.figshare.12089370.v1

Supplementary information
Supplementary information available online at http://jeb.biologists.org/lookup/doi/10.1242/jeb.217406.supplemental

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