Two neuropeptides that promote blood-feeding in *Anopheles stephensi* mosquitoes

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Animals routinely need to make decisions about what to eat and when. These decisions are influenced not only by the availability and quality of food but also by the internal state of the animal, which needs to compute and give weights to these different variables before making a choice. Feeding preferences of female mosquitoes exemplify this behavioural plasticity. Both male and female mosquitoes usually feed on carbohydrate-rich sources of nectar or sap, but the female also feeds on blood, which is essential for egg development. This blood-appetite is modulated across the female's reproductive cycle, yet little is known about the factors that bring it about. We show that *Anopheles stephensi* females, a major vector for urban Malaria the Indian sub-continent and West-Africa, suppress blood-feeding between a blood-meal and until oviposition. Transcriptomics on blood-deprived and sated *An. stephensi* mid-brains allowed us to shortlist several candidate genes that might promote blood-feeding behaviour. Through dsRNA-mediated knockdown of nine of them, we identified two neuropeptides – short Neuropeptide F (sNPF) and RYamide - that promote blood-feeding. We find that these neuromodulators are expressed in non-overlapping subsets of neurons in the brain suggesting that blood-feeding behaviour is likely modulated by multiple mechanisms acting across different brain neurons.

Anopheles stephensi **| Blood-feeding | Host-seeking | Transcriptomics | sNPF | RYamide**

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An animal's dietary choice is an outcome of its internal physiological state and external stimuli. Its changing nutritional demands, reproductive state, and developmental history are integrated with external cues before a food-choice is made. For example, when deprived of a particular nutrient, like proteins, animals show a specific increase in protein-appetite (1–3). Mating influences food choices of female flies by increasing their preference for proteins, sugars, and salt for egg development and oviposition (4–8).

Changes in both sensory perception and central brain circuits have been shown to underlie such behavioural changes. For example, sensory neurons of starved animals become more sensitive to attractive cues and less sensitive to aversive ones, both in vertebrates and invertebrates (9–16). These changes are often brought about by neuromodulators and neuropeptides (9, 10, 17–22). In the central brain, internal nutrient or hormone sensors can monitor the animal's nutritional state. In mice, neurons in the hypothalamus express receptors for hunger and satiety signals like ghrelin and leptin (16, 23, 24). In insects, several neurons in the mushroom body, central complex, and subesophageal region have been shown to integrate hunger and satiety signals to regulate the choice between sugars or proteins, make the choice between food-source exploration or exploitation, or to initiate or terminate feeding (25–33).

Many female mosquitoes have a dual appetite for carbohydrate-rich nectar or sap – to meet energetic requirements – and protein-rich blood – for egg development. Consequently, the female mosquito oscillates between a state of heightened blood-appetite and its suppressed state through multiple reproductive cycles (figure 1A). What molecular signals controls these switches in dietary preference is of great interest not only because of its importance in public health, but also because understanding how the internal state influences dietary choice of an organism has implications in other organisms, and is of general importance. In *Aedes albopictus* anticipatory expression of the yolk protein precursor gene vitellogenin 2 in the fat body promotes host-seeking behaviour (34). In *Aedes aegypti*, neuropeptide-Y receptor (NPYLR1) suppresses blood feeding (35–37).

Here we describe the blood- and sugar-feeding patterns of *An. stephensi*, which is endemic to the Indian sub-continent but has now expanded its range into West Africa, putting millions more people at risk of Malaria. Neurotranscriptomics across the different conditions of blood-deprivation and -satiety allowed us to shortlist several candidate molecules that might promote blood-feeding. dsRNA-mediated knockdown of nine of these implicate two neuropeptides – short neuropeptide F (sNPF) and RYamide - in promoting blood-feeding. They are expressed in about different neurons in the brain suggesting that blood-feeding is regulated by multiple mechanisms acting across different cell-types in the brain.

Blood-, but not sugar-feeding, is modulated across the reproductive cycle. To assess the female *An. stephensi* appetite for blood through her reproductive cycle we designed a behavioural assay (figure 1B, S1A; see methods for details), and tested it on *Ae. aegypti*, a well-studied model. We were able to reproduce the previously reported results that mating enhances blood-feeding in *Aedes* and that once fed to repletion, females actively suppress blood-feeding until oviposition (38) (Figure 1C). Using this assay on *An. stephensi*, we found that these females were uninterested in blood when they emerge but that their appetite increased with age (magenta, D0-D5 in figure 1D). Like *Ae. aegypti*, *An. stephensi* suppress blood-feeding for at least four days after a blood-meal and until oviposition, after which, the suppression is lifted to pre-blood-meal levels (magenta, $D1^{B}$-$D2^{O}$ in figure

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1D). Unlike blood-feeding, sugar-feeding is not modulated similarly barring a brief suppression post-blood-meal, which was not dependent on oviposition (figure 1E; methods and S1F for details). Surprisingly, on the day of emergence, female mosquitoes were uninterested in both sugar- and blood-feeding (compare D0 in fig 1D, E).

To ascertain whether sugar and blood represent parallel appetites independent of each other we presented sugar-sated co-housed females a choice between the two. Under these conditions, most females chose blood and only some took both suggesting that the two are independent appetites (figure 1F). We next deprived co-housed females of sugars for a day before offering them the choice and found that most females now took both meals, while only some took just blood (figure 1F). This confirms that blood and sugar are independent appetites that exist in parallel. The males, as expected, fed well on sugar in both sated and starved conditions (grey, figure 1F).

As mating is known to promote blood-feeding in *Ae. aegypti*, we tested if this was true for *An. stephensi*. A post-hoc dissection of each female's spermatheca (where sperm is stored after mating) revealed that many of the blood-fed females in the above assay were unmated (figure 1G). To confirm this, we assayed blood-feeding behaviour in virgin females and found that virgin *An. stephensi*, unlike *Ae. aegypti*, have a robust appetite for blood and that mating enhances it only marginally (cyan, figure 1D). Interestingly, however, these virgins, unlike mated *An. stephensi (or Ae. Aegypti)* females, failed to suppress their blood-appetite after the first blood-meal. Instead, they continued to blood-feed for up to four days after their first blood-meal (cyan, $D1^B$ - $D4^B$ in figure 1D). A flipped order of the two behaviours - virgins were first given a blood-meal then allowed to mate – also resulted in suppression of the appetite (figure 1H). This suggests that the post-blood-meal suppression of blood-appetite in *An. stephensi*, is dependent upon mating status.

Taken together, our data suggest that female *An. stephensi* modulate their appetite for blood to ensure that this essential, but high-risk feeding activity is engaged in only when necessary. Feeding on sugars, which is not associated with such risks, does not appear to be regulated similarly, but might suffer some collateral modulation. We also find the two appetites are independent and that while mating promotes blood-feeding in *Ae. aegypti*, it suppresses it in *An. stephensi*.

Host-seeking is modulated like blood-feeding is in *An. stephensi*. The modulation of blood-feeding through the reproductive cycle could be due to 1) modulation in sensitivity to host kairomones, 2) modulation by central brain circuits, or 3) modulation in both peripheral and central circuit elements. Extensive data in other mosquito species suggest that sensitivity to host kairomones change as females age (39–41) and following a blood-meal (42–44). We tested this in *An. stephensi*.

Because of the multi-sensory nature of host-seeking, we used a two-choice behavioural assay where multiple host-cues were provided in one arm while the other had none. Mosquitoes had to make a choice between the two arms (figure 2A). When no human-cues are provided in either arm (blank) even blood-hungry females distribute equally between the two arms demonstrating that the assay shows no bias (black, figure 2B). Using this assay, we found that newly emerged females showed no preference for either the control or the host arm (figure 2B). Five days later, they were strongly attracted to the host-cues and this attraction was suppressed after their blood-meal and until oviposition (magenta, figure 2B). This general trend in attraction to host-cues tracks the trend in blood-feeding. Interestingly, the response of virgin females to host-cues in the Y-maze was highly variable under all conditions tested (cyan, figure 2B). Yet, like in the blood-feeding assay, blood-fed virgins were comparatively more attracted to human host-cues than were the mated blood-fed females (figure 2B). Importantly, in all conditions tested, participation in the assay was satisfactory (figure 2C).

Together these data suggest that, like blood-feeding, host-seeking is also modulated through the female's reproductive cycle, and that mating influences this modulation by enhancing it initially and suppressing it post-blood-meal. The variable response that virgin females show to host-cues also suggests that mating, in general, enhances the female's attraction to a host.

Neurotranscriptomics reveals several candidate genes that promote blood-feeding. While sensitivity to host kairomones may modulate blood-feeding, it is also possible that central brain circuitry contributes to it. We reasoned that cues from mating, ovaries, or the fat body could act on brain circuitry to modulate blood-feeding. To determine the molecular underpinnings of this behavioural modulation, we performed RNA sequencing from the midbrains of mosquitoes at different stages of their reproductive cycle (consequently different states of blood-deprivation or satiety). This included females at emergence (D0), 5-days after emergence (D5; sugar-fed, blood-deprived), virgins and mated females one day after a blood-meal ($D1^B$), and females one day after oviposition ($D1^O$). Age matched males were included for the earliest two time points (figure 3A).

For each of our samples, 81-87percent of about 20-30 million reads aligned to the *An. stephensi* genome (45). The variance between our replicates was low as represented in the principal component analysis (PCA) (figure 3B). In the PCA, older female brains, regardless of mating-, blood-feeding-, or oviposition- status clustered together while the youngest female and the male brains stood apart on PC1 and PC2 suggesting that age and sex contribute most significantly to the variance in our data (figure 3B). The expression pattern of some hallmark genes showed the expected trends. Genes involved in eclosion behaviours – *eclosion hormone* (46) and *partner of bursicon*(47) – showed the highest expression in the D0 samples and were not expressed later. Male-specific genes, *HMG-B-13-like*, were expressed only in males (48), and genes involved in vitellogenic stages of egg-development – *vitellogenins*, *EcR*, and *E-20-monooxygenase* – were expressed only in post-blood-meal samples (figure 3C) (49).

We next explored this data for insights into the molecular un-

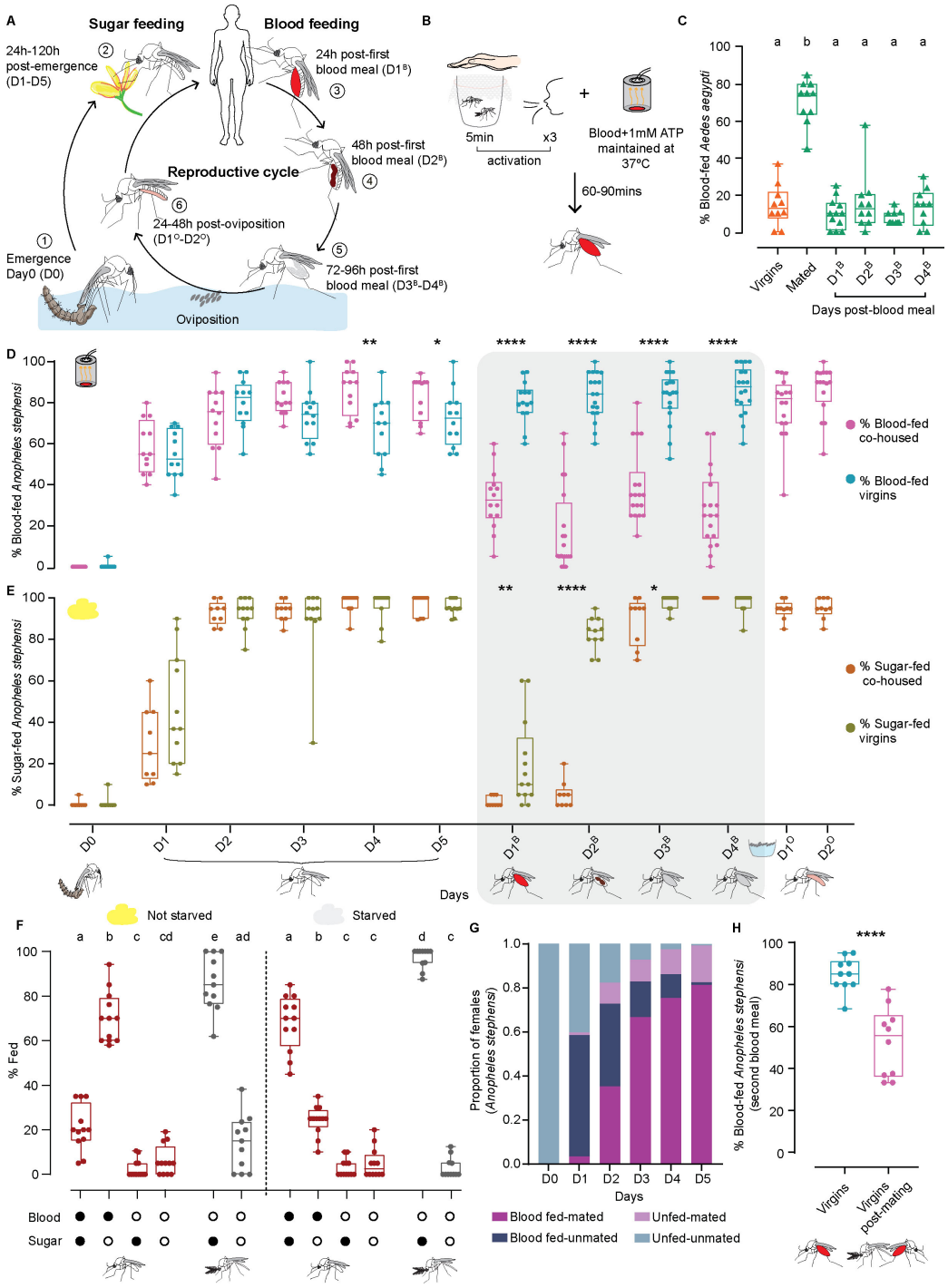

Fig. 1. Feeding behaviours of *An. stephensi*. A. Reproductive cycle of *An. stephensi*. Upon emergence [1, D0-D5] females have a dual appetite for protein-rich blood and carbohydrate-rich sources of sugar [2,3]. After a blood-meal [3,4,5, $D1^B$-$D4^B$], the eggs mature and are laid in water [6]. The next reproductive cycle can now begin. B. Blood-feeding assay. 0-7h old mosquitoes were collected in cups and aged appropriately on sugar. Prior to the test, mosquitoes were 'activated' for 5 minutes by presenting a human hand, followed by 3 exhalations. Blood-meals were presented through a Hemotek perfumed with human skin odours. Number of blood-fed mosquitoes were visually scored, irrespective of the quantity fed. C. Blood-feeding behaviour of *Ae. aegypti*. 8-days old virgin and mated females were assessed for their first blood-meal. Fully fed mated females were assessed for second blood-meal ($D1^B$-$D4^B$). Each dot represents a single trial with 18-21 females, n=10-12trails/group. Data labelled with different letters are significantly different. Kruskal-Wallis, with Dunn's multiple correction test, p<0.05. D. Blood-feeding behaviour of *An. stephensi*. Co-housed and virgin females were tested on the day of emergence (D0) and each day for the next 5 days (D1-D5). $D1^B$-$D4^B$ represents their blood-feeding 1-4 days after their first-blood meal and $D1^O$-$D2^O$ represents their blood-feeding 1-2 days after oviposition (see methods for assay details). 18-21females/trial, n=9-20trials/group. Two-tailed Mann-Whitney U test, p<0.05.*p<0.05, **p<0.01, ****p<0.0001. E. Sugar-feeding behaviour of *An. stephensi*. Females of similar conditions as in D were tested for sugar-feeding. 18-21females/trial, n=9-20trials/group. Two-tailed Mann-Whitney U test, p<0.05.*p<0.05, **p<0.01, ****p<0.0001. F. Choice assay for blood and sugar. Sugar-sated females were given a choice between blood and sugar and most took blood (left of dashed line). Sugar-starved females were given a choice between blood and sugar and most took both (right of dashed line). Males fed on sugar in both conditions. 17-24 mosquitoes/trial; 11-12 trials/condition. One-way Anova with Holm-Šídák multiple comparison test, p<0.05. G. Mating and blood-feeding. The mating status of co-housed females of D0-D5 assayed in figure 1D were determined post-hoc by dissecting their spermatheca. 232-239 females were analysed for each day. Many blood-fed females were unmated. H. Mating after blood-feeding. Blood-fed virgins were allowed to mate and then offered a second blood-meal. A suppression in blood-appetite was observed. 15-20females/trial, n=10 trials. Unpaired t-test, p<0.05.****p<0.0001.

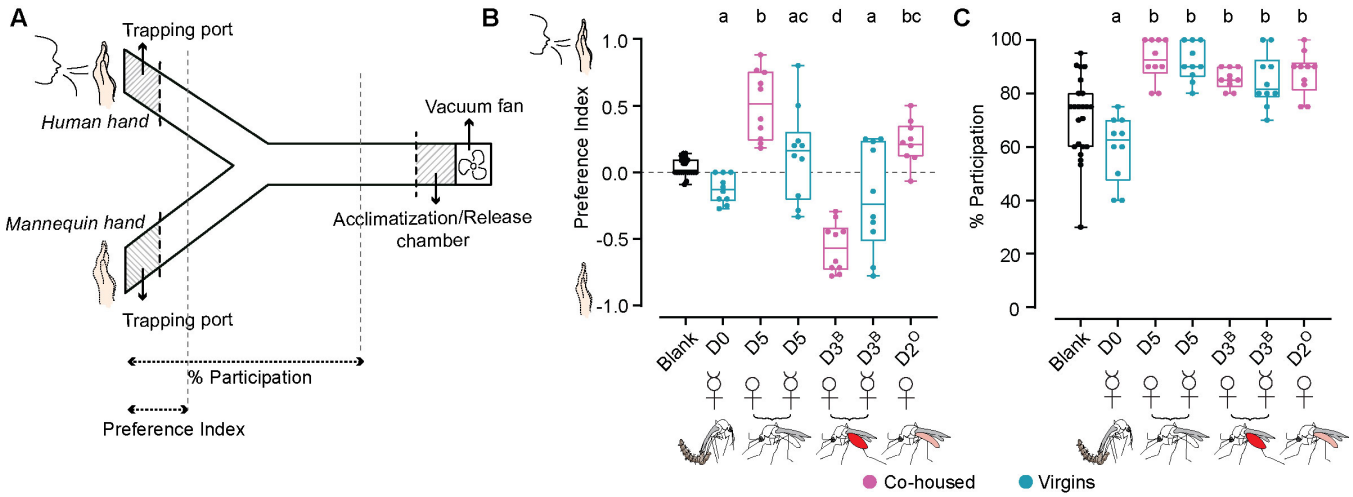

Fig. 2. Host-seeking is modulated like blood-feeding is in *An. stephensi*. A. Schematic of the Y-maze used to assess host-seeking behaviour of female An. stephensi. Females were allowed to acclimatise in the chamber for 5 minutes while being exposed to host kairomones that was presented in a test arm. A fan sucked the air from both test and control arms at 0.3-0.6m/s. Mosquitoes were released after acclimatisation and allowed to choose between the two arms. Preference index (B) and percent participation (C) of virgin and co-housed females in the Y-maze were calculated at the indicated time points. 17-21 females/trial, n=10 trials/group. Data labelled with different letters are significantly different. One-way Anova with Tukey's multiple comparison test (B) and Kruskal-Wallis, with Dunn's multiple correction test (C), $p<0.05$.

derpinnings of blood-feeding modulation. Reports suggest anticipatory metabolic changes in the female germline cells and a 'vittelogenin wave' in fat bodies can drive specific appetite in *Drosophila* and *Ae. albopictus* respectively (34, 50). We looked for similar anticipatory metabolic changes in the mosquito brain that might drive blood-feeding. However, we did not find any evidence for this (figure S2B). Interestingly, we found that after a blood-meal, glucose is neither spent nor stored, and that the female brain goes into a state of metabolic 'sugar rest', while actively processing proteins (figure S2B, S3). Analysing publicly available data from the ovary and fat body suggests that energy expenditure via the non-oxidative branch of the pentose phosphate pathway of glucose metabolism is high in these tissues, likely to generate vitellogenins for oocyte maturation (51) (figure S3). We similarly plotted genes involved in lipid metabolism, neurotransmitters, neuropeptides and their receptors and found no significant patterns (figure S4, S5, S6). In summary, contrary to our expectations, we saw no evidence for anticipatory metabolic changes in the brain that might in turn drive blood-feeding behaviour.

We next took a different approach to identify genes that might modulate blood-feeding. We took the intersection of genes that were enriched in the three blood-hungry conditions but not present in males. These comparisons were made against the two conditions that were not motivated to blood-feed: females at emergence and mated-blood-fed females. This shortlisted 38 and 34 genes respectively that might promote blood-feeding (figure 3D, E). The combined list of 71 unique genes gave us candidates that might promote blood-feeding in *An. stephensi* (figure S7).

Neuropeptides sNPF and RYamide promote blood--feeding in *An. stephensi*. Candidates from the above analysis included several metabolic enzymes, uncharacterised genes, transporters, hormones and neuropeptides, among others. We further shortlisted these based on a few criteria: that they show the expected expression patterns (high in blood-deprived; low in sated conditions), had little variability between replicates, and where literature suggests their involvement in feeding behaviours. Based on this, we selected 9 candidates (figure 3F): Two sodium-dependent amino acid transporters (transporters can sense nutrients and regulate feeding in *Drosophila*) (29, 52), two genes involved in the juvenile hormone pathway (53, 54) (JH is involved in ovary development) (55, 56), four neuropeptides (modulate feeding behaviours in many animals), and a metabolic hormone known to modulate feeding behaviours in *Drosophila* (57)(figure 3F).

We knocked each of these genes down using dsRNA and tested their blood-feeding behaviour. For each such experiment, we ensured efficiency of gene knockdown post-hoc (Figure 4A). We first confirmed that the injection itself did not cause any behavioural changes (figure 4B) and in all following experiments either dsGFP-injected or uninjected females were included as controls.

We saw no change in behaviour upon loss of *Eiger*, *JHEH*, and *Ork* function (figure S8A-C). This suggests that these genes by themselves do not modify blood-feeding. For *Hairy* and *Prp*, we were unable to achieve efficient knockdown, hence cannot comment on their role in modulating blood-feeding (figure S8D-E). For the two transporters, although we observed a significant down-regulation of their mRNA, the blood-feeding behaviour was only marginally affected (figure S8F,G). When we tested for redundancy, the combined knockdowns, though effective, did not show any significant change in behaviour (figure S8H).

However, knockdown of the two neuropeptides - RYamide and sNPF - showed a robust reduction in blood-feeding (figure 4C, D). RNA interference (RNAi) knockdown phenotypes can be variable, and each female is an independent experiment. Therefore, while assaying for knockdown of these

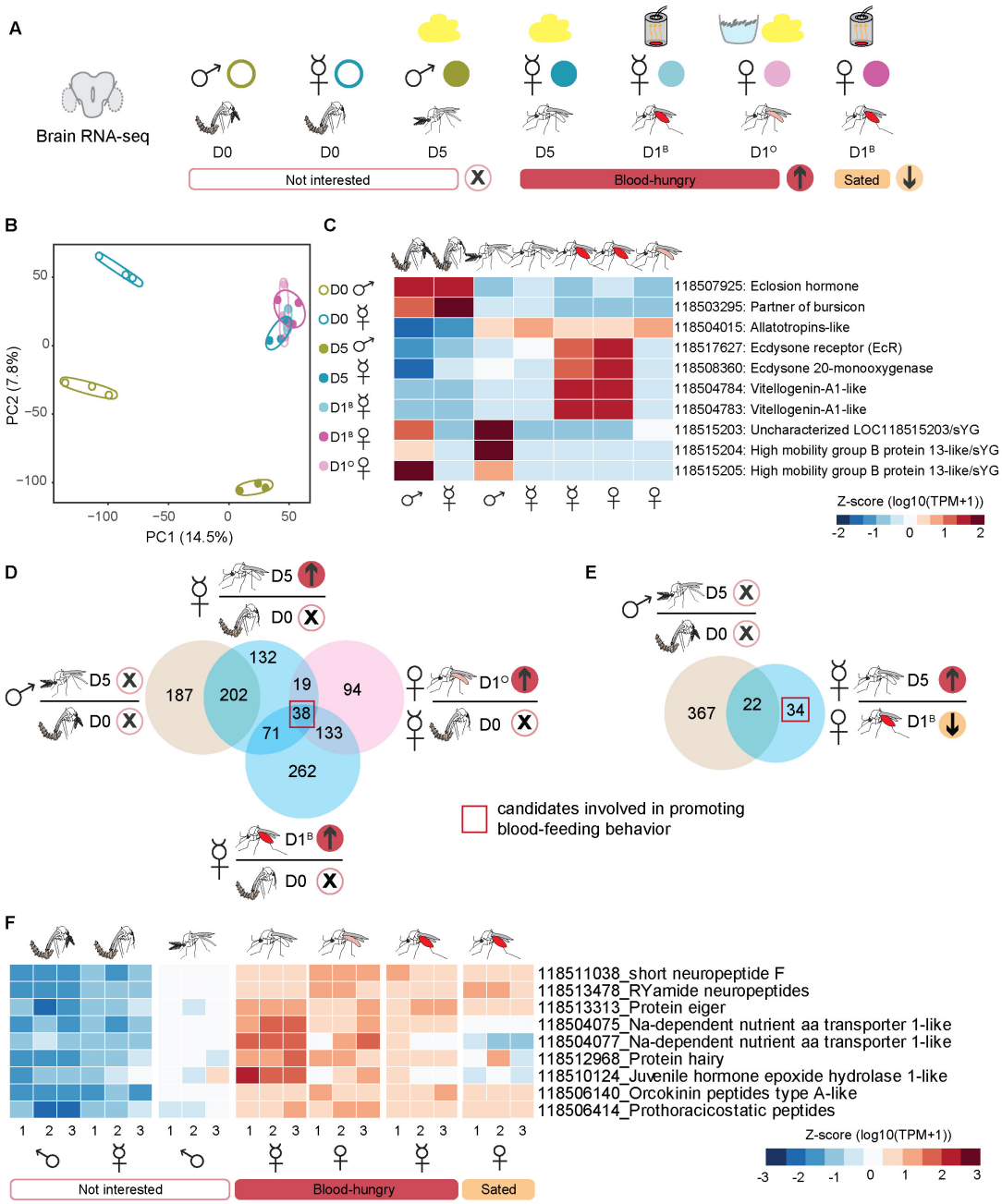

Fig. 3. Neurotranscriptome of *An. stephensi*. A. Mid-brains were sampled from males and females at different ages and feeding preferences for bulk RNA-seq. Uninterested in blood-meals: D0 males (olive open circle), D0 females (blue open circle), D5 males (olive filled circle). Blood-hungry: D5, sugar-fed virgin females (blue filled circle), $D1^B$ virgin females (light-blue filled circle), and $D1^O$ females (light-pink filled circle). Blood-sated: $D1^B$ mated females (pink filled circle). (n=100 mid-brains per replicate; 3 biological replicates per samples, 7 samples.) B. Principal Component Analysis (PCA) of the normalised counts-per-million from different brain RNA-seq samples. Replicates cluster closely and age and sex contribute most to the differences between the samples. C. Genes (listed on the right) with known expression patterns are plotted across different samples. These known expression patterns are reproduced in our data. Average of the triplicate data are represented as z-score normalised log10 (TPM+1) values. D, E. Identification of candidates potentially involved in promoting blood-feeding behaviour. Two types of comparisons were made. D. Genes commonly up-regulated in the three blood-hungry conditions (see figure 3A) when compared against D0 females, but not expressed in males. E. Genes up-regulated in D5 sugar-fed virgin females when compared against $D1^B$ mated females (blood-sated), but not expressed in males. Number of candidate genes identified from two different sets of analyses are shown in the red boxes. F. Nine candidate genes (listed on the right), shortlisted for further validation. RNA-seq data from mid-brain samples in triplicates are represented as z-score normalised log10 (TPM+1) values.

genes, we quantified mRNA separately from pools of injected females that had taken blood-meals and those that had not. Reassuringly, we observed efficient knockdown only in the females that did not take blood-meals suggesting that RNAi caused the suppression of blood-feeding (figure 4F-G). The combined knockdown of both these genes showed a phenotype of suppressed blood-feeding similar to that of individual manipulations (figure 4E, H). Interestingly, we observed that the suppression was specific to their blood-appetite because these females fed robustly on sugar (figure 4E, S8I). Together these data suggest that sNPF and RYamide specifically promote blood-feeding in *An. stephensi*.

Expression pattern analysis of their mRNA using hybridisation chain reaction (HCR) (58, 59) *in situ* revealed that *sNPF* and *RYamide* are expressed in several non-overlapping neuronal clusters distributed across many regions in the brain. This pattern is reminiscent of their expression in the *Drosophila* and *Bombyx* brains (figure 4I) (60–62). To modulate blood-feeding, either the number of neurons expressing these neuropeptides could change, or their controlled release. So, we counted their cell numbers in sugar-fed and blood-fed brains but saw no change (figure 4J). As we have probed for mRNA, not protein, we cannot comment on whether they control behaviour through controlled release.

Taken together, these data suggest that two non-overlapping populations of neuropeptidergic neurons in the central brain control blood-feeding behaviour. It is important to note, however, that our manipulations will result in the systemic down-regulation of these genes, so it is possible that other sources of sNPF and RYamide modulate blood-feeding as has been shown for other neuropeptides (63).

Conclusions

We have reported here the blood- and sugar-feeding behaviours of *An. stephensi*, which, while sharing similarities with other mosquito species, shows important differences that could be vital both for understanding mechanisms underlying food preferences and when considering vector control strategies. For example, while mating promotes blood-feeding in *Aedes* it suppresses it in *Anopheles*. How mating mediates behavioural changes is relatively well understood in flies: it is triggered by sex-peptide in the male semen, which modulates central brain circuitry (5, 7, 8, 64, 65) in the female. In mosquitoes, however, this is unlikely to be the case (66).

What then promotes blood-feeding in *Anopheles*? While we have identified two neuropeptides, it is likely that multiple such peptide or hormonal cues are involved since the modulation of behaviour requires the simultaneous modulation of multiple aspects of the animal's physiology. Modulatory signals must act both on the central and peripheral nervous systems, prepare the gut for engorgement, the fat body for metabolism and the ovary for oogenesis. Feeding behaviour is often controlled by inter-organ feed-forward and feed-back regulation. It is possible that the ovary, or the fat body, both of which are key in the utilisation of blood resources for oogenesis, are themselves sources of the molecular cues that drive the behaviour. These will be interesting avenues to pursue in the future.

The female mosquito's dietary choices present a striking model in which to study the molecular and circuit control of feeding choices. While progress in this domain has been steady, with the parallel revolutions in novel technologies in genomics, circuit inquiry, and functional genetics, it will now be possible to scale these efforts and dissect this behaviour at cellular and molecular resolution.

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Author contributions

PB conceptualised the project, performed all experiments except RNA-seq and host seeking, performed all analysis, including RNA-seq, wrote and reviewed the manuscript. RP performed the RNA-seq experiments and analysis, standardised HCR, and reviewed the manuscript. PDB performed the host-seeking, provided technical assistance during HCR standardisations and determination of mating status, and reviewed the manuscript. SQS conceptualised and supervised the project and wrote and reviewed the manuscript.

Declaration of interests

None of the authors have any competing interests to declare.

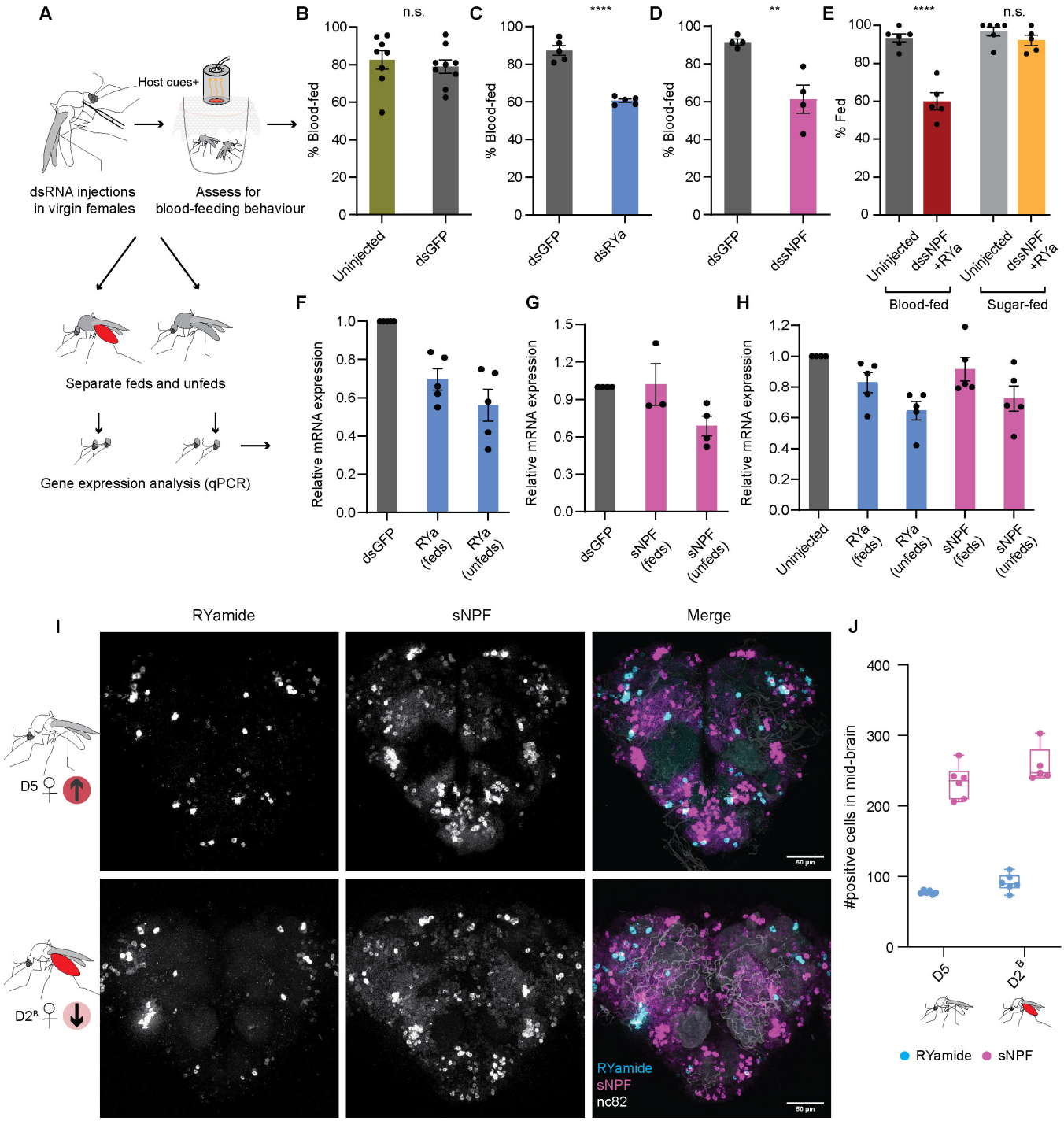


Fig. 4. Neuropeptides sNPF and RYamide promote blood-feeding in *An. stephensi*. A. Schematic representation of the pipeline used for functional validation. dsRNA against candidate genes were injected in adult virgins for targeted RNAi. After recovery these were tested for blood-feeding. Knockdown efficiency was determined via qPCR in whole heads of fed and/or unfed females after the behaviour. B. Comparison of un-injected females and dsGFP-injected females for blood-feeding behaviour revealed no difference between the two indicating that the animals recover from the injection procedure. 19-25 females per replicate, n= 8-9 replicates/group. C-E. Blood-feeding behaviour of dsRYamide (C), dssNPF (D) and dssNPF+dsRYamide (E) injected females as compared to dsGFP-injected females. 12-25 females/replicate, n=4-6 replicates/group. Note that blood-feeding behaviour is significantly reduced to about 60percent in all these cases. Sugar-feeding behaviour of the dual knockdowns (dssNPF+dsRYamide) is unimpaired (E, right). Unpaired t-test, $p<0.05$.n.s. not significant, *$p<0.05$, **$p<0.01$, ***$p<0.001$. ****$p<0.0001$. F-H. Relative mRNA expression of RYamide and sNPF in heads of females injected with dsRYamide (F), dssNPF (G) or both (H) as compared to that in dsGFP-injected females or uninjected females, as analysed via qPCR. Note the more efficient knockdown in the un-fed conditions. I. Whole mount fluorescence RNA *in situ* hybridisation via HCR of female brains probed against RYamide and sNPF neuropeptides in the sugar-fed (top) and blood-fed (bottom) mated females. Maximum intensity projections of the confocal stacks are shown, scale bar 50µm. J. Comparison of cell numbers expressing RYamide or sNPF across the two conditions reveal no difference. 5-6 brains were analysed for each neuropeptide, per condition.

Methods

Mosquito culture. *Anopheles stephensi* (Indian strain; Bangalore strain, TIGS-1) and *Aedes aegypti* (Bangalore collection) were reared and maintained at 26-28°C, 70-80percent relative humidity with a photoperiod of 12h light: 12h dark. All the behavioural assays were performed at these conditions of temperature and humidity. Larvae were fed on yeast and dog biscuit (1:3 ratio) and cultured in trays in appropriate densities to prevent overcrowding. Adult mosquitoes were provided ad libitum access to 10percent sugar solution (8percent sucrose +2percent glucose +0.05percent methyl paraben +5percent vitamin syrup (Polybion SF Complete, Merck Ltd.)). For general maintenance, females were fed with O+ve human blood, secured periodically from blood banks.

Regulatory permissions. This project was approved by the Institutional Human Ethics Committee (Ref: inStem/IEC-22/02) and the Institutional Biosafety Committee (TIGS/M-7/8/2020-1).

Blood-feeding assays for both species: first blood--meal, 0-120h post-emergence. All *An. stephensi* feeding experiments were performed from ZT12-ZT15 and *Ae. aegypti* experiments from ZT10-ZT12 (Zeitgeber time 0 is defined as the time of lights ON). 0-7h old mosquitoes were cold anesthetised and collected during the day in 1100ml paper cups covered with a net and secured with a rubber band. These were maintained at the conditions of temperature and humidity described above. To collect virgins, males were separated at the time of collection. 20 females with males (1:1 ratio) or without males were collected for each trial and were provided access to 10percent sugar solution at all times, except when they were offered blood. For D0 (day of emergence, 0-7h old), no sugar was provided at the time of collection. Females were allowed to acclimatise for at least 30 mins in the cups and then offered a choice of both sugar and blood simultaneously, to assess their preference for blood soon after emergence.

Females were assayed for their blood-feeding behaviour from the day of emergence to 5 days of age, every 24h. On the day of the test, appropriately aged females were offered a blood-meal via a Hemotek feeder (Hemotek Ltd), which was maintained at 37°C and perfumed with human skin odours. It has been reported that female mosquitoes need to be 'activated' to take blood-meals. This involves the presentation of CO2 (and other host-cues) to the females, likely because CO2 has been shown to gate other cues such as temperature and visual cues (67, 68). So, prior to offering the blood-meal, the mosquitoes were 'activated' by the presentation of a hand placed above the cup for 5 minutes, followed by 3 exhalations from the experimenter. Additionally, blood was supplemented with 1mM ATP (Sigma A6419). Females were allowed to feed for 60-90mins in dark and abdomens were scored visually for presence of blood, irrespective of the amount ingested.

In the case of co-housed females, males were separated post-hoc and spermatheca of the females were dissected to score for the presence or absence of sperm. All the assays with virgin and co-housed females were performed in parallel by a single experimenter. Mosquitoes from at least 4 independent batches were tested for each day of the timeline. Behavioural experiments with *Ae. aegypti* were performed the same way.

Blood-feeding assays for both species: second blood--meal, 24-96h post-first blood-meal. Mosquitoes were collected as described above. Fully fed females (5days old *An. stephensi* and 7-9days old *Ae. Aegypti*) were separated and collected in paper cups as described above. As a female takes about 2 days to digest blood, the second blood- meal ingested 24h later ($D1^B$) was detected by the presence of Rhodamine B (Sigma R6626), which fluoresces in the red range. Spiking the second blood-meal with Rhodamine B (0.04ug/ul of blood), allowed visualisation of the freshly ingested blood, without affecting the feeding efficiency (figure S1B, C). For the subsequent days ($D2^B$-$D4^B$) abdomens were dissected to differentiate between the bright red freshly ingested blood from the dark and clotted first blood-meal. In the case of co-housed females, the spermatheca were not dissected to confirm mating as they were presumed to have mated by D5, as previously determined (figure 1G).

To confirm if mating suppresses subsequent feeding, fully fed virgins were collected and allowed to mate for 3-4 days with aged-match males. No males were introduced in the virgin-only controls. Blood-feeding assay was performed as described above. Spermatheca were dissected post-hoc to determine the mating status and unmated females were not considered for the final analysis. Datasets with less than 80percent total mating were discarded.

Blood-feeding assays: post-oviposition, 24-48h post-egg laying. For blood-feeding behaviour post-oviposition, fully fed females were separated and a water cup lined with moist paper (ovipositor) was provided for egg laying 2 days post-blood meal (determined in a separate assay described below, supplementary figure S1D). Females were allowed to lay eggs overnight. The following morning, non-gravid females were visually identified by their lean bellies and separated in cups. Blood-feeding assay was performed the same evening for 24h post-oviposition ($D1^O$) and the following day ($D2^O$), as described above. Females were dissected post-hoc and scored for absence of mature ovaries, as a sign of successful oviposition. Gravid females were not considered for the analysis.

Determination of oviposition timing for *An. stephensi*. To determine the day of maximum oviposition, 5-6 days old co-housed females were fed on blood, as described above. Fully fed females were separated and collected in BugDorm cages in groups of 10 females/cage. An ovipositor was provided the same day and females were allowed to lay eggs overnight. Eggs laid were counted the next day and a fresh ovipositor was provided. This cycle was repeated for the next 5 days. Total eggs laid each day were divided by the number of females present at the time of counting.

Sugar-feeding assay for *An. stephensi*. As mosquitoes feed on sugar in small bouts, as opposed to a replete blood-meal, we assessed sugar-feeding behaviour of females in windows of 24h for each test day. 0-7h old mosquitoes were collected during the day, separated in cups and given access to 10percent sugar solution at all times, as described above. For D0, similar to the blood-feeding assay, no sugar was provided at the time of collection. To assess their sugar-feeding behaviour on the day of emergence (D0), 0-7h old females were acclimatised in the cups for 1h and sugar spiked with Rhodamine B (1mg/25ml of sugar; Sigma R6626) was presented for 2.5-3h at dusk. The fluorescence of Rhodamine B in the red channel allowed visualisation of even the tiniest amounts of sugar ingested by a female (see supplementary figure S1F). For D1 feeding, 3-10h old females (with access to normal sugar so far) were then provided with Rhodamine B-sugar and allowed to feed on it for 24h. For D2 feeding, 27-34h old females (with access to normal sugar so far) were fed on Rhodmaine B-sugar for the next 24h and scored as D2. This cycle was repeated until D5. In the case of co-housed females, males were separated post-hoc and spermatheca of the females were dissected to score for the presence or absence of sperm (supplementary figure S1G). To assess sugar-feeding post-blood meal, females fully fed on blood were collected and separated in cups, as described above. For $D1^B$, they were allowed to feed on Rhodamine B-sugar for 24h following a blood-meal. The next set of females (with access to normal sugar so far) was then provided with Rhodamine B-sugar from 24h-48h post blood-meal and scored as $D2^B$. This cycle was repeated until 4 days post-blood-meal ($D4^B$).

For sugar-feeding post-oviposition, blood-fed females were provided an ovipositor 3 days later and were forced to oviposit during daytime by artificially creating dark conditions. Oviposited females (non-gravid) were then visually identified, separated and presented with Rhodamine B-sugar ball for the following 24h and scored as $D1^O$. The next set (with access to normal sugar so far) was then provided with Rhodamine B-sugar, 24-48h post-oviposition and scored as $D2^O$. For each day tested, females were dissected post-hoc and scored for absence of mature ovaries, as a sign of successful oviposition. Gravid females were not considered for the analysis.

Dual choice assay of blood and sugar. Mosquitoes were collected as described above. Males and females were co-housed for 4-6 days with *ad libitum* access to sugar. To assess the choice between sugar and blood, mosquitoes were either given continuous access to normal sugar or starved with water 24h prior to the assay. At the time of the assay, post-activation (as described above), blood and Rhodamine B-spiked sugar (1mg/25ml of sugar, Sigma R6626) were provided in parallel. After 60mins, abdomens were scored visually for ingestion of blood under white light, and under fluorescent microscope (red channel) for ingestion of sugar (see supplementary figure S1E). Co-housed males served as an internal control for sugar-feeding.

Statistical analysis of feeding behaviours. Statistical analysis was performed using GraphPad Prism (v10.2.0). To compare the blood-feeding and sugar-feeding behaviours of virgins and co-housed females in Fig 1D, E, each day was treated as an independent experiment and p-values were computed for each pair-wise comparison.

Y-maze olfactory assay. A 30inch Y-maze was constructed as per the WHO guidelines (https://www.who.int/publications/i/item/9789241505024). Each arm of the Y-maze had a trapping port, while the stem was attached to a holding port. Each port had a mesh screen at one end, while the other was fitted with a sliding door to trap or release mosquitoes. The holding port was attached to a fan which pulled air from the two arms, over the mosquitoes, and out at a velocity of 0.3-0.6m/s. Optic flow information (alternate black and white stripes) was placed underneath the Y-maze to act as visual guide for navigation. Following conditions were tested: D0 virgins, D5 sugar-fed virgins, D5 sugar-fed females co-housed with males, 3days post-blood meal virgins and mated females ($D3^B$), and 2days post-oviposition females ($D2^O$). For each condition, mosquitoes were collected and provided *ad libitum* access to sugar as described above. Females were transferred to holding ports 7-8h prior to the assay and were starved during this time with only access to water. For each run, mosquitoes were acclimatised for 5 minutes: 2 min with clean air and 3 minutes with an experimenter presenting a hand at one of the arms (test) and simultaneously exhaling at resting rates. To account for disruption in airflow, the hand of a mannequin was placed at the other arm (control). Post-acclimatisation, the door of the holding port was opened to release the mosquitoes and they were allowed to make a choice for 5 minutes in the presence of continued host-cues. The ports were then closed and mosquitoes in each arm counted. All the mosquitoes trapped in a port, sitting on the door or resting on the half-length of the Y-arm were considered to have made a choice for that stimuli (test or control). A preference index was calculated as Test-Control/Test+Control. Participation percentage was also calculated as number of mosquitoes that passed half-way through the Y-stem/total number of mosquitoes released. To ensure no biases existed, each experimental day started with a blank trial (where no stimuli were presented on either arm of the Y-maze) and the side of the host-cues presented was alternated for each trial. Statistical analysis was performed using GraphPad Prism (v10.2.0).

Sample preparation for RNA sequencing. For bulk RNA sequencing mid-brains were dissected from the following conditions: D0 and D5 sugar-fed males; D0 and D5 sugar-fed virgin females; 24h post-blood meal virgin and mated females ($D1^B$) and 1day post-oviposition females ($D1^O$). Three replicates from independent batches were prepared for each condition. Mosquitoes were cold anesthetised before dissections. Brains were dissected in RNase-free 1X PBS and immediately placed into ACME solution (13:3:2:2 of water, methanol, acetic acid, and glycerol) on ice, which mildly

fixes the tissues (69). For each condition, 100 brains were dissected per replicate. The mildly fixed brains were washed with cold 1X PBS and centrifuged at 13,000 rpm for 5 min at 4°C to remove ACME. Brains were then homogenised in TRIzol (Invitrogen), flash-frozen, and stored at -80°C until RNA extraction. RNA was extracted using Direct-zol RNA MicroPrep (Zymo Research) columns. RNA quantity and quality were assessed using the ThermoFisher Qubit 4 and RNA 6000 Nano kit Bioanalyser, respectively.

RNA Sequencing. The cDNA library was prepared for each sample using the NEBNext Ultra II Directional RNA Library Prep kit (Catalog no-E7765L), with an average fragment size of 250 bp. Some of the library samples were subjected to size and quality checks using a bioanalyser. Paired-end sequencing was carried out on the Illumina Hiseq 2500 sequencing platform, generating 2×100 bp reads. The library preparation and sequencing were performed at the Next Generation Genomics Facility (NGGF) at Bangalore LifeScience Cluster.

Quality check and read alignments. After sequencing, samples were demultiplexed and delivered as raw Fastq files. The quality of raw reads were checked using the FastQC tool. The reads were then mapped onto the reference *An. stephensi* transcriptome (NCBI RefSeq assembly GCF013141755.1) (45) using Kallisto v0.43.1 (70) with default parameters. Around 81-87percent of reads were mapped to the mosquito transcriptome. The summary of the mapping rate for all the samples was visualised using MultiQC.

Gene calling. Transcript-level abundance was quantified using Kallisto for both raw counts and transcripts per million (TPM) values. Gene-level summarisation was performed using the Tximport function in R, with transcript isoform information retained. Using EgdeR package, the raw counts were converted to Counts Per Million (CPM) and Log2 transformed before data filtering (genes with less than 1 CPM in at least 3 or more samples were filtered out) and normalisation using the trimmed mean of M-values (TMM) method. Principal component analysis (PCA) was performed using the prcomp function in R and visualised using the ggplot function. Data was checked for batch effects using gPCA (71) in R and the test statistic "delta" was found to be 6.957686e-05, with a p-value of <0.001, indicating no batch-effects.

Differential gene expression analysis. Differential expression analysis was performed using the Limma package in R. A design matrix was created to specify the sample conditions, and the Voom function was used to estimate the mean-variance relationship across genes. A linear model was then fitted to the data using the mean-variance relationship. Bayesian statistics were calculated for the data by creating a contrast matrix to extract the linear model fit. To identify differentially expressed genes (DEGs), the "decideTests" function was used by applying a fold change threshold of 2 (or log fold change of 1) and a FDR (false discovery rate) adjusted p-value cutoff of 0.05.The number of genes identified to be upregulated and downregulated in each pair-wise comparison are shown in supplementary figure S2A. To plot Transcripts Per Million (TPM) values as heatmaps, the Fastq files for the mid-brain samples were mapped onto the reference *An. stephensi* transcriptome and abundance was quantified using Kallisto the same way as described above. Gene level summarisation was done using Tximport similarly as above, except all the isoforms of a given transcript were mapped onto a single gene. The resultant TPM values were first filtered for TPM more than 0.1 in each sample. Next the genes that were not quantified in at least 2 of the 3 replicates of each sample group were removed. The filtered dataset were then log10 transformed and heatmaps were plotted using pheatmap function in R.

An. stephensi 2-4 days old female fat body and ovary data were taken from Prasad et al., 2017 (72) (SRX620223 and SRX620224; bioproject SRP043489). 1-2 days old ovary (ovary post-emergence) and 24h post-blood meal ovary data were taken from Jiang et al., 2014 (73) (SRP013839). Fastq files were downloaded from the SRA explorer and processed the same way as described for the TPM heatmaps above. Log10 transformed values were plotted together with those of the brain samples using pheatmap function in R.

Gene set enrichment analysis. eggNOG mapper tool 2.1.9 (74) (70percent identity and minimum 70percent query coverage) was used to find functionally annotated orthologs in *An. gambiae* against the differentially expressed genes. *An. gambiae*-specific orthologs were fed into the GOSt tool from gProfiler2 (75) toolset for functional enrichment analysis. An ordered query was run with an FDR adjusted p-value threshold of 0.05. A custom gene list comprising of all the genes identified in our RNA-seq experiment was provided as a background instead of the whole genome. Using this background, the tool determined which GO terms (Biological Processes) were over-represented using annotations for that gene set. Top 10 terms for each DEGs were plotted using ggplot in R.

dsRNA-mediated gene knockdown. To synthesise the double-stranded RNA (dsRNA), coding regions(500-600bp) of candidate genes were amplified from cDNA of *An. stephensi* females using forward and reverse primers with T7 promoter sequence at 5' ends. The gel purified fragments were then used as a template for *in vitro* transcription reactions using Megascript RNAi kit (Invitrogen) or High-Yield T7 RNAi Kit (Jena Biosciences) as per the manufacturer's protocol. The dsRNA were purified using the phenol-chloroform method and pellet dissolved in nuclease-free water. The dsRNA for Green fluorescent protein (GFP) was synthesised from plasmid pAC5.1B-EGFP (Addgene 21181) to be used as a negative control for off-target effects of dsRNA-mediated knockdown.

Virgins were collected for injections as described above. The day and concentration of dsRNA to be injected was determined for each target gene individually. dsEiger: injected in 24h old virgins at 5-6ug/ul; dsHairy: injected in 24h old virgins at 4-5ug/ul; dsJHEH: injected in 72h old virgins at 5ug/ul; dsProthoracicostatic peptides (dsPrp): injected in 72h

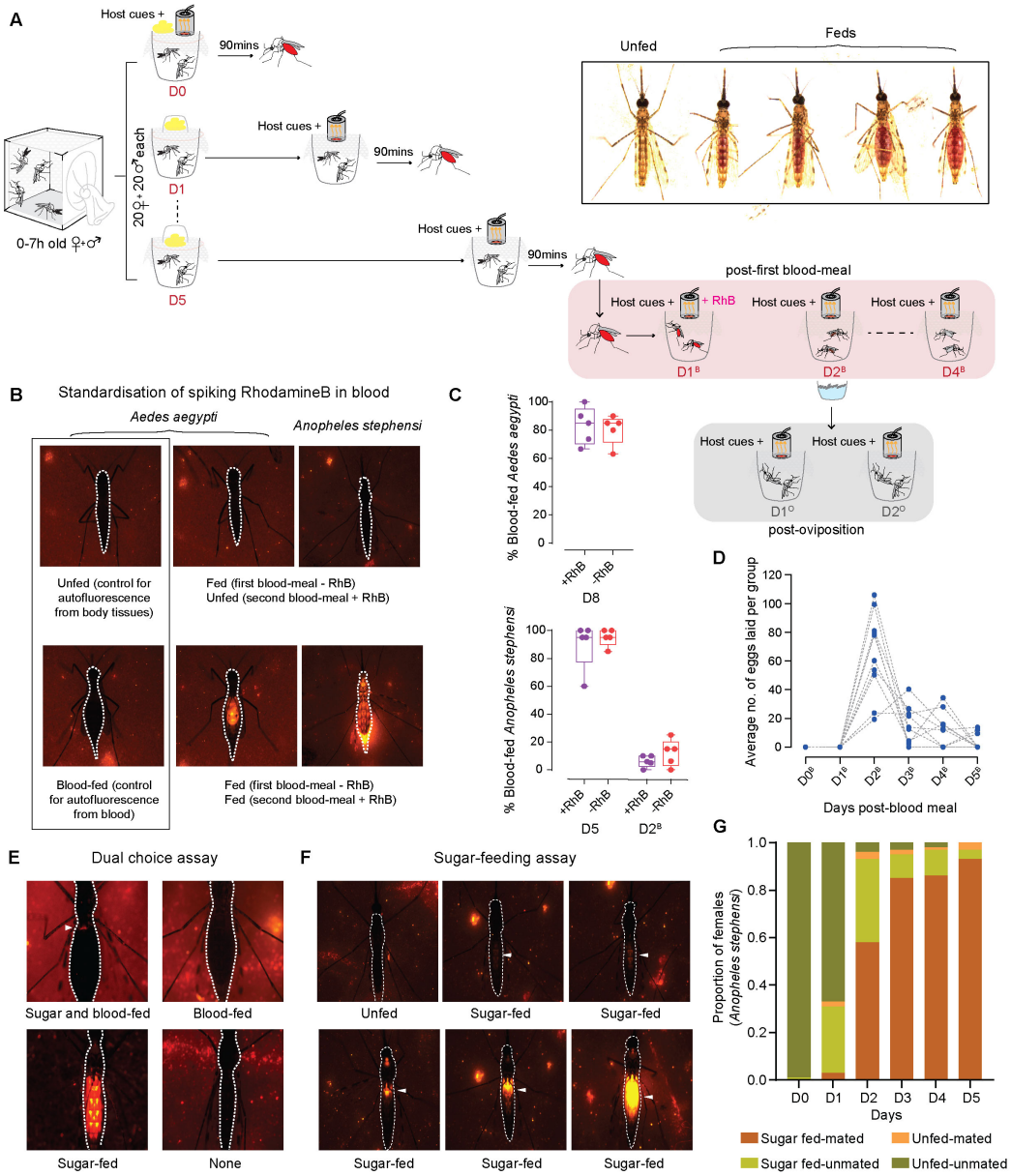
old virgins at 3-5ug/ul; dsOrcokinin: injected in 96h old virgins at 5ug/ul; dssNPF and dsRYamide: injected in 96h old virgins at 7ug/ul; ds118504077 and ds118504075: 96h old virgins at 6ug/ul. For combinations, purified dsRNA were mixed in equal amounts to a total of 7ug/ul and injected on the day previously determined. Injections were performed on a FemtoJet 4i system (Eppendorf) and each female was injected with 0.5-0.6ul of the respective concentrated dsRNAs. A corresponding dsGFP control was injected each time at the same concentration as of the target gene. 12-25 females were injected for each trial and at least 2 trials from independent batches of mosquitoes were performed for each target gene.

Injected females were allowed to recover in paper cups with *ad libitum* access to sugar and assessed for blood-feeding behaviour 3 days-post injection, as previously described. Fed and unfed females were visually scored and whole head samples were collected and lysed in TRIzol (Invitrogen). Total RNA was extracted using Direct-zol RNA MicroPrep (Zymo Research) columns, as per the manufacturer's protocol. RNA was quantified using Qubit Broad range assay (Invitrogen). For each sample, 200-220ng RNA was used to prepare cDNA using the SuperScript™ IV Reverse Transcriptase (Invitrogen 18090050) or Maxima H Minus Reverse transcriptase (Thermo Scientific EP0752). Quantitative PCR (qPCR) was performed using PowerUp™ SYBR™ Green Master Mix (Applied Biosystems) using gene-specific primers and a house-keeping gene control (RpS7) to determine the knockdown efficiency. Statistical analysis were performed using GraphPad Prism (v10.2.0).

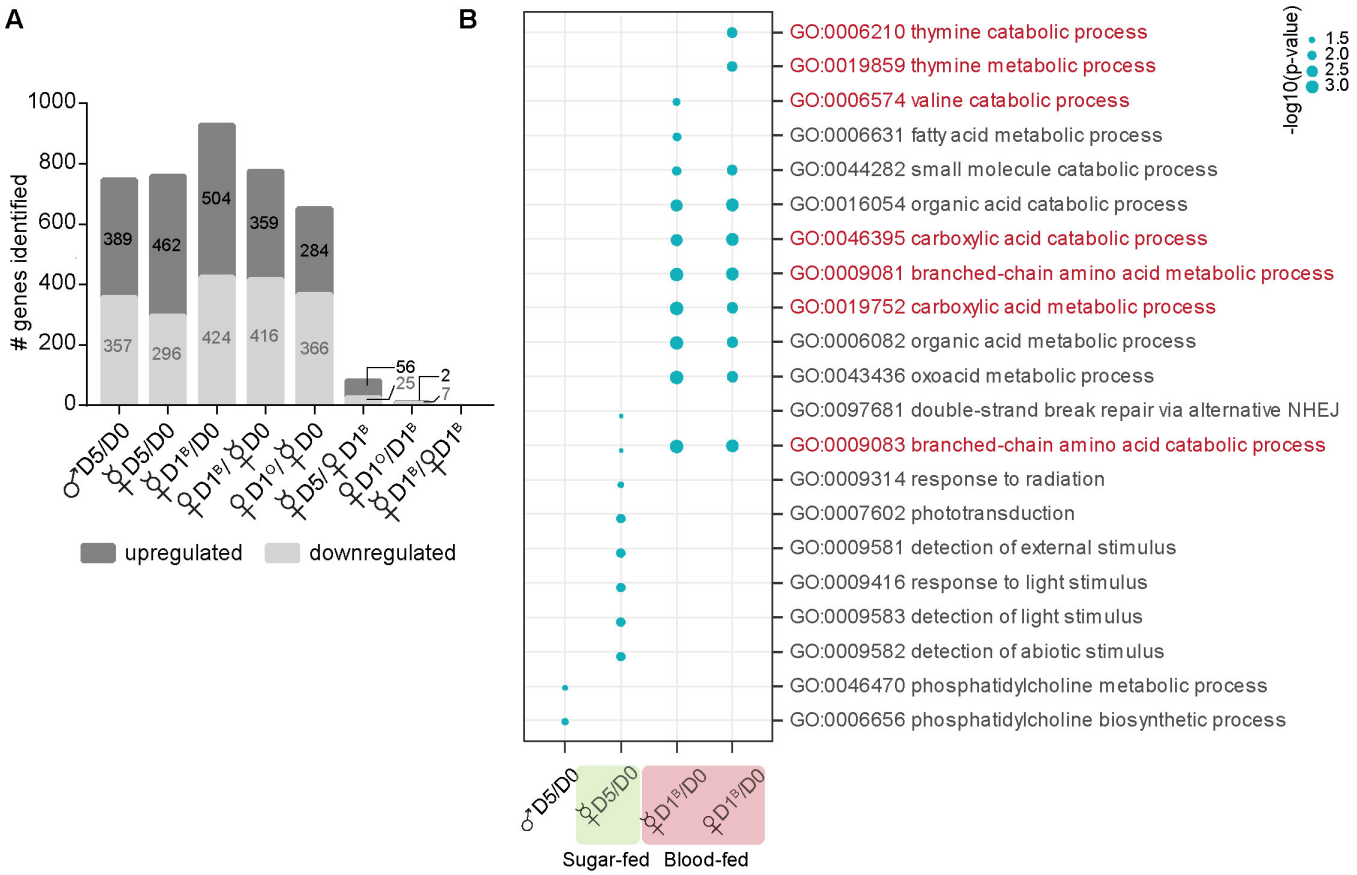
To assess the sugar-feeding behaviour of dssNPF+dsRYamide injected females, injections were performed as described above. Uninjected females were used as a control. Injected females were allowed to recover with ad libitum access to normal sugar for the first two days, which was then replaced with Rhodamine B-spiked sugar (1mg/25ml of sugar, Sigma R6626) for the next 24h. Control females was treated similarly. The blood-feeding assay was performed 3 days-post injections as described above, with both blood and sugar provided in parallel. Abdomens were scored visually for ingestion of blood under white light, and under fluorescent microscope (red channel) for ingestion of sugar.

Hybridisation chain reaction (HCR) HCR *in situ* hybridisations were performed as per the protocol (76). Briefly, brains were dissected in ice cold PBS and fixed with 4percent PFA for 20 minutes at room temperature. Post-washing steps with PTw (0.1percent Tween-20 in PBS), tissues were permeabilised using detergent solution for 30 mins at room temperature. Brains were then pre-hybridised in probe hybridisation buffer for 30 mins at 37°C, followed by incubation with probes (0.8pmol each) at 37°C for 48h in a humid chamber. Excess probes were washed off with wash buffer at 37°C, followed by 2 washes with 5X SSCT. Brains were pre-amplified using amplification buffer for 30 mins at room temperature and hairpins were added (2µL each of 3µM stock). Brains were incubated for 12-16h at room temperature and excess hairpins were washed off with 5X SSCT. Following washes with PTw, brains were stained with anti-mouse nc82 (1:200; DSHB, RRID: AB2314866) and goat anti-mouse Alexa-fluor405 (Invitrogen). Samples were imaged at Central Imaging and Flow Cytometry Facility (CIFF) at NCBS. Images were acquired with a 40X/1.3NA objective at a resolution of 512X512 pixels, on an Olympus FV3000 system. Images were viewed and manually analysed using ImageJ/FIJI.

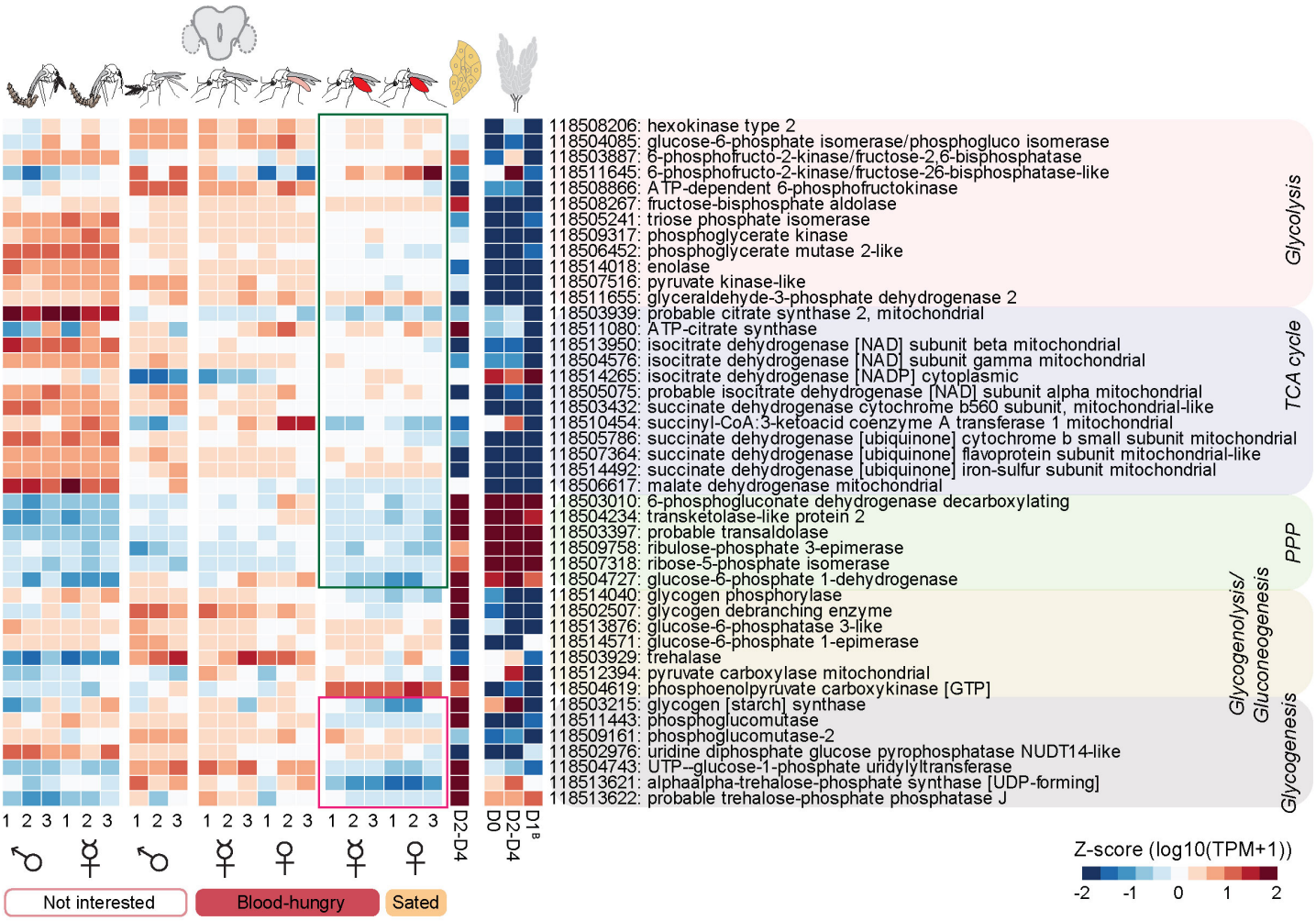
Supplementary Figures



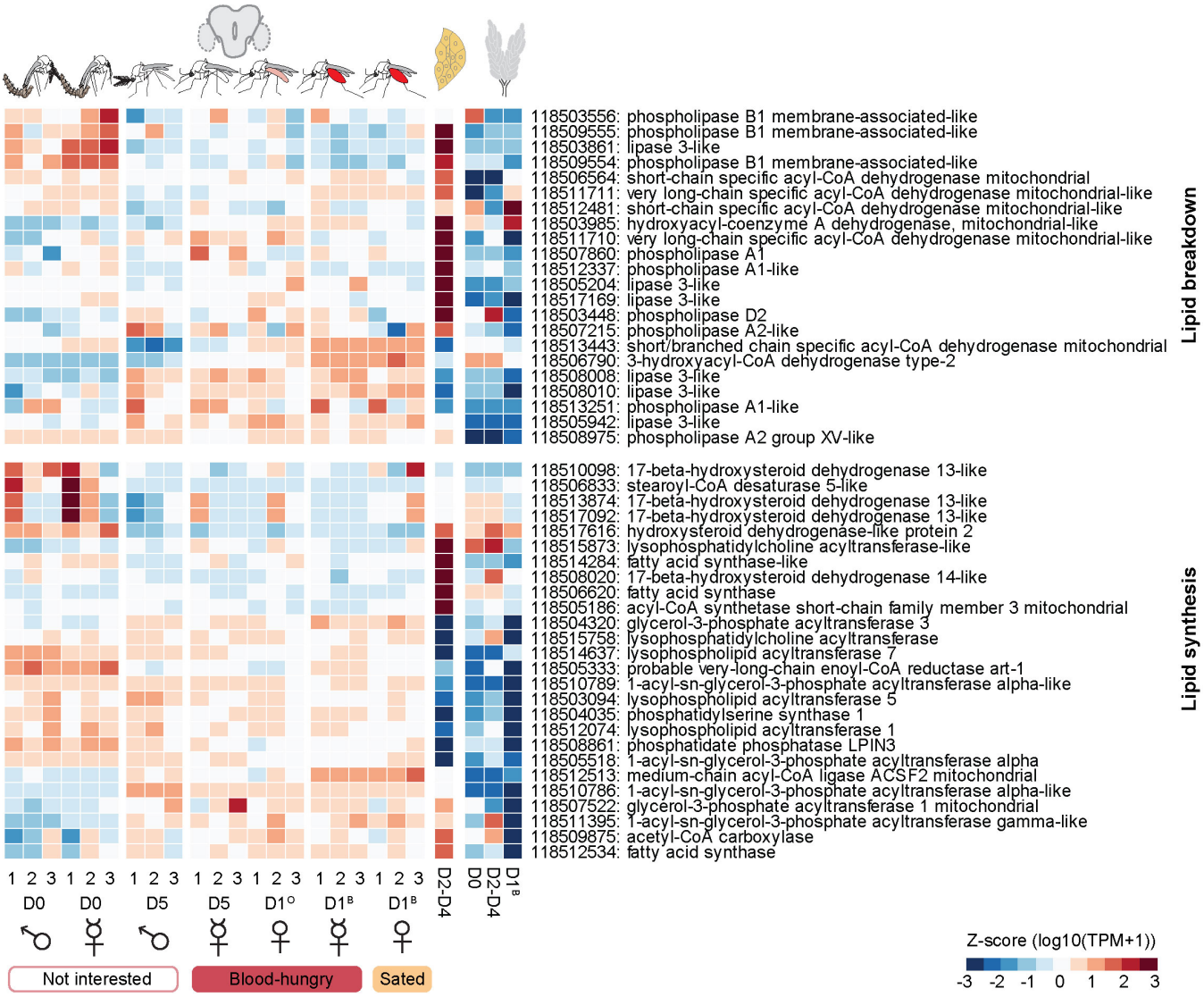
Supplementary Figure 1. Standardization of various behavioural assays to study feeding behaviours in *An. stephensi*. A. Detailed schematic of the assay designed to assess the blood-feeding behaviour of virgins and co-housed females across the reproductive cycle. Age-matched females were collected. About 20 females (with or without 20 males) were separated into cups. Each cup was aged appropriately and tested on the relevant day (see methods for more details). Number of blood-fed mosquitoes were visually scored, irrespective of the quantity fed. Representative images of differently fed females are shown in the box. To assay for second blood-meal, fully fed females were collected and aged appropriately in groups of 20. To assess feeding 24h-post first blood meal ($D1^B$), Rhodamine B was spiked in the second blood-meal offered to the females. The subsequent days ($D2^B$-$D4^B$) were tested without any such spiking (pink box; see methods for more details). To assay for blood-feeding behaviour in females after oviposition, freshly oviposited females were collected and aged in groups of 20 for the appropriate time in cups (grey box). In all cases, females were presented with host-cues prior to the assay and testing was performed as shown in figure 1B (see methods for more details). B. Standardisation of spiking Rhodamine B dye in blood to assess feeding 24h-post first blood-meal ($D1^B$). To assay for the second blood-meal, blood-fed females were collected, aged appropriately and offered Rhodamine B-spiked blood in a Hemotek. All other assay parameters remain the same as for the first blood-meal (see methods for more details). Widefield fluorescent images of *Ae. aegypti* and *An. stephensi* fed on second blood-meal spiked with Rhodamine B are shown. Images on the left (black box) show mosquitoes not fed and fed on un-spiked blood (without Rhodamine B), as controls for autofluorescence from body tissues and blood itself, respectively. Fluorescence from Rhodamine B added to the second blood-meal enabled identification of fresh blood intake in already fed mosquitoes reliably. C. Comparison of feeding behaviours of *Ae. aegypti* females (top) and *An. stephensi* females (bottom) fed on blood with and without Rhodamine B at the indicated age. Females fed similarly on both indicating that Rhodamine B itself does not affect feeding behaviour. D. Determining optimal oviposition timing in *An. stephensi*. Females were tested in groups of 10 and average number of eggs laid per group are shown at the indicated day after the first blood-meal. Dashed lines connect the eggs laid by each group on the indicated day. Majority of the eggs were laid 2 days after a blood-meal. 10 females/group, n=10 replicates/group. E. Standardisation of the dual choice assay, to determine the choice between blood and sugar in sated and starved *An. stephensi* co-housed females. Widefield fluorescent images of females fed on either blood, sugar or both are shown. White arrowhead marks the fluorescence from sugar (spiked with Rhodamine B) in the abdomens of females engorged on blood, indicating that the female has fed on both. This is not observed in the females fed on blood alone. Females fed on sugar alone can be visualised by the fluorescent lean bellies. Unfed females exhibit no fluorescence or engorgement. F. Representative widefield fluorescent images of female *An. stephensi* fed on sugar spiked with Rhodmaine B are shown. This setup was used to study the sugar-feeding behaviour of virgins and co-housed females across the reproductive cycle. Addition of Rhodamine B to standard sucrose solution enabled visualisation of even the smallest amounts ingested, as indicated by the white arrowheads, under the fluorescent microscope. G. Mating status of sugar-fed and un-fed co-housed females tested in Fig. 1E, 24-120h post-emergence (D0-D5). 175-179 females were analysed for each day.



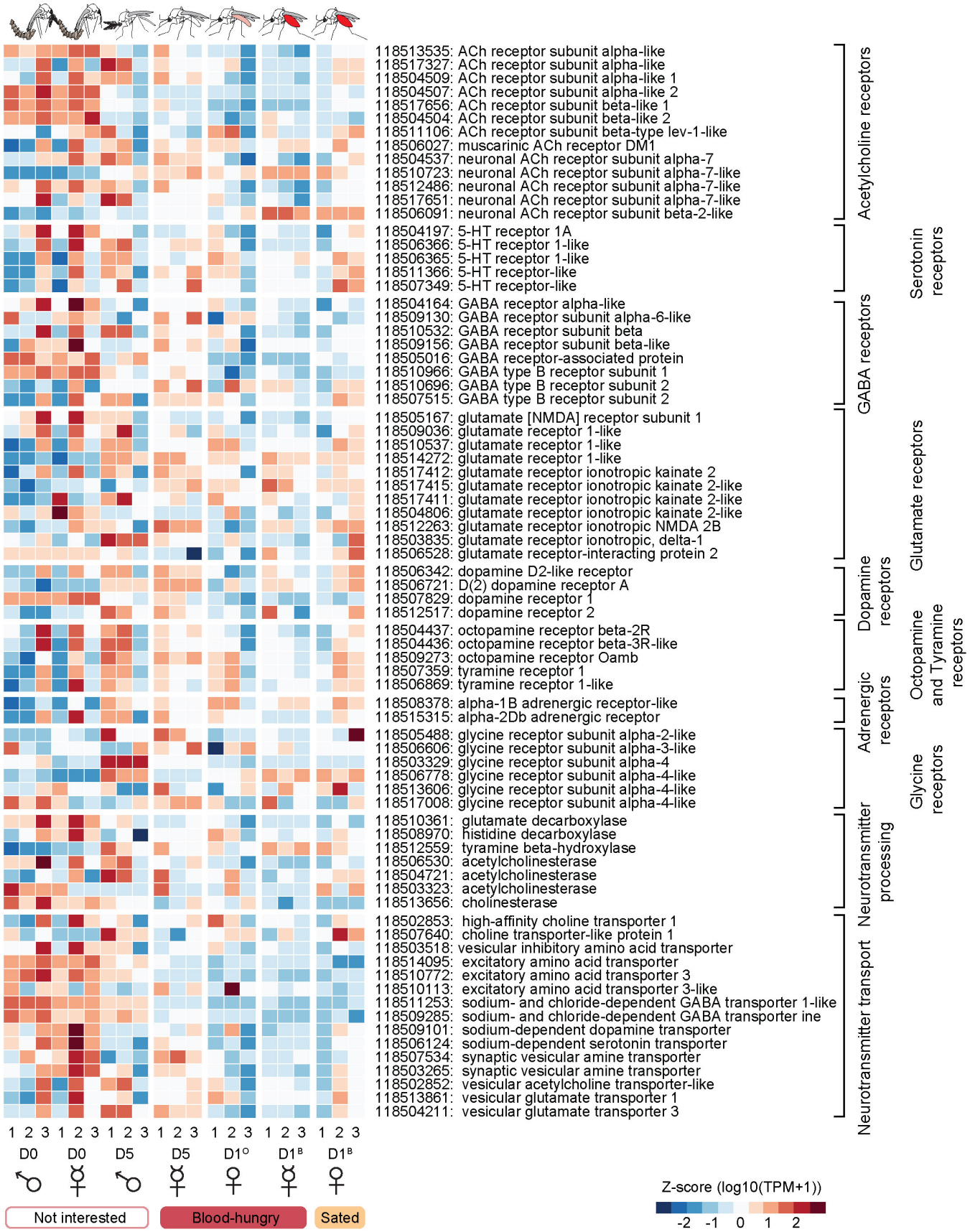
Supplementary Figure 2. GO-enrichment analysis of genes differentially expressed in sugar-fed and blood-fed states. A. The number of genes identified to be differentially up- and down-regulated for each sample pair analysed, as indicated on the bottom, are shown. B. Top 10 categories of genes enriched for Gene Ontology Biological Process (GOBP) for the sample pairs analysed, as indicated on the bottom, are shown. Size of the dot indicates the p-value significance. Processes involved in protein metabolism (highlighted in red) are highly enriched in blood-fed females.



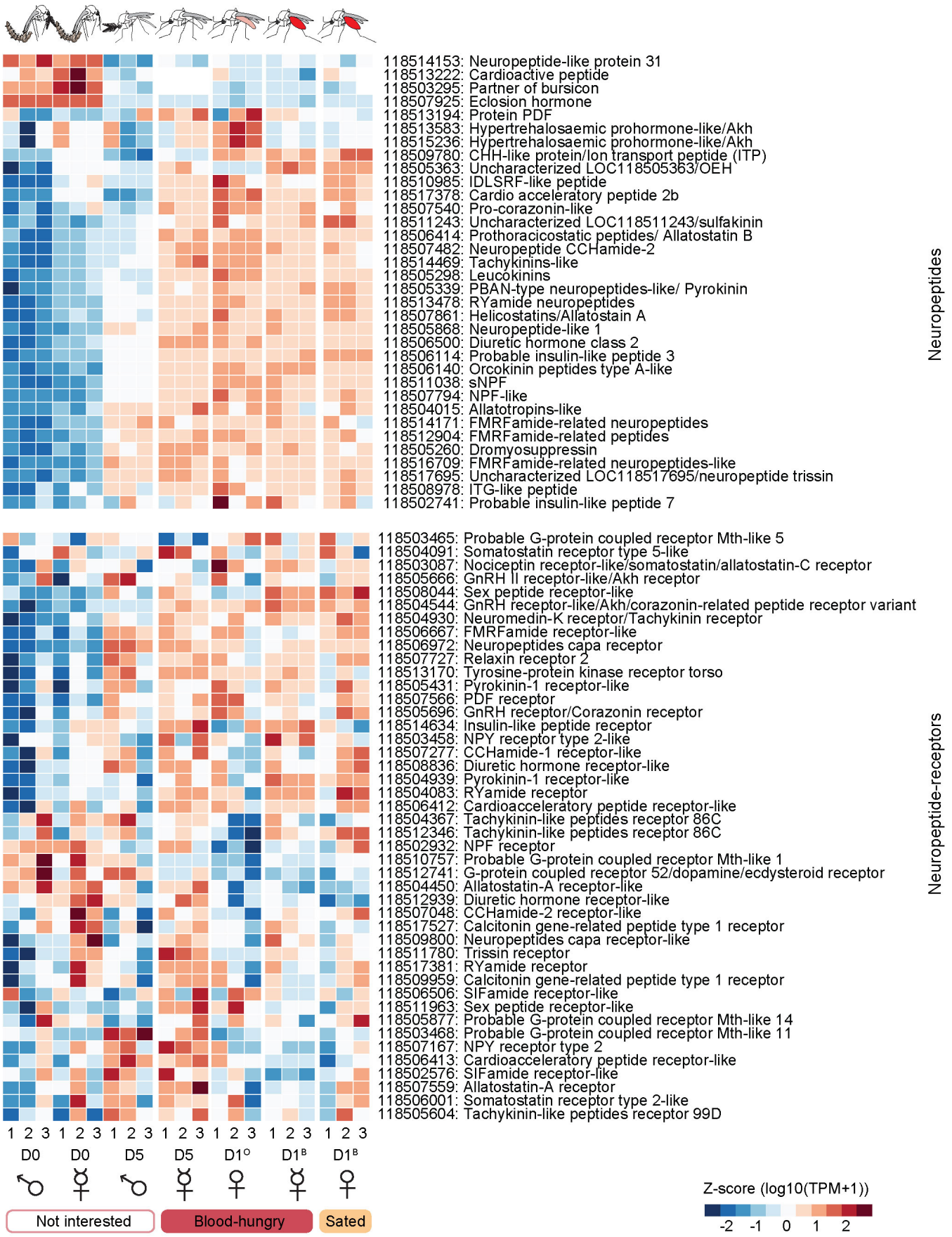
Supplementary Figure 3. Differential expression of genes involved in carbohydrate metabolism across different tissues and states of blood-hunger and -satiety in *An. stephensi* Genes (listed on the right) involved in sugar metabolism via different pathways are plotted across different samples. Green box shows downregulation of genes involved in glucose utilisation and the magenta box represents genes involved in glucose storage. Note that both are low in the brains of blood-fed females indicating that the brain goes into a state of 'sugar rest' after a blood-meal. RNA-seq mid-brain data in triplicates and single replicate data for ovary and fat body are represented as z-score normalised log10 (TPM+1) values.



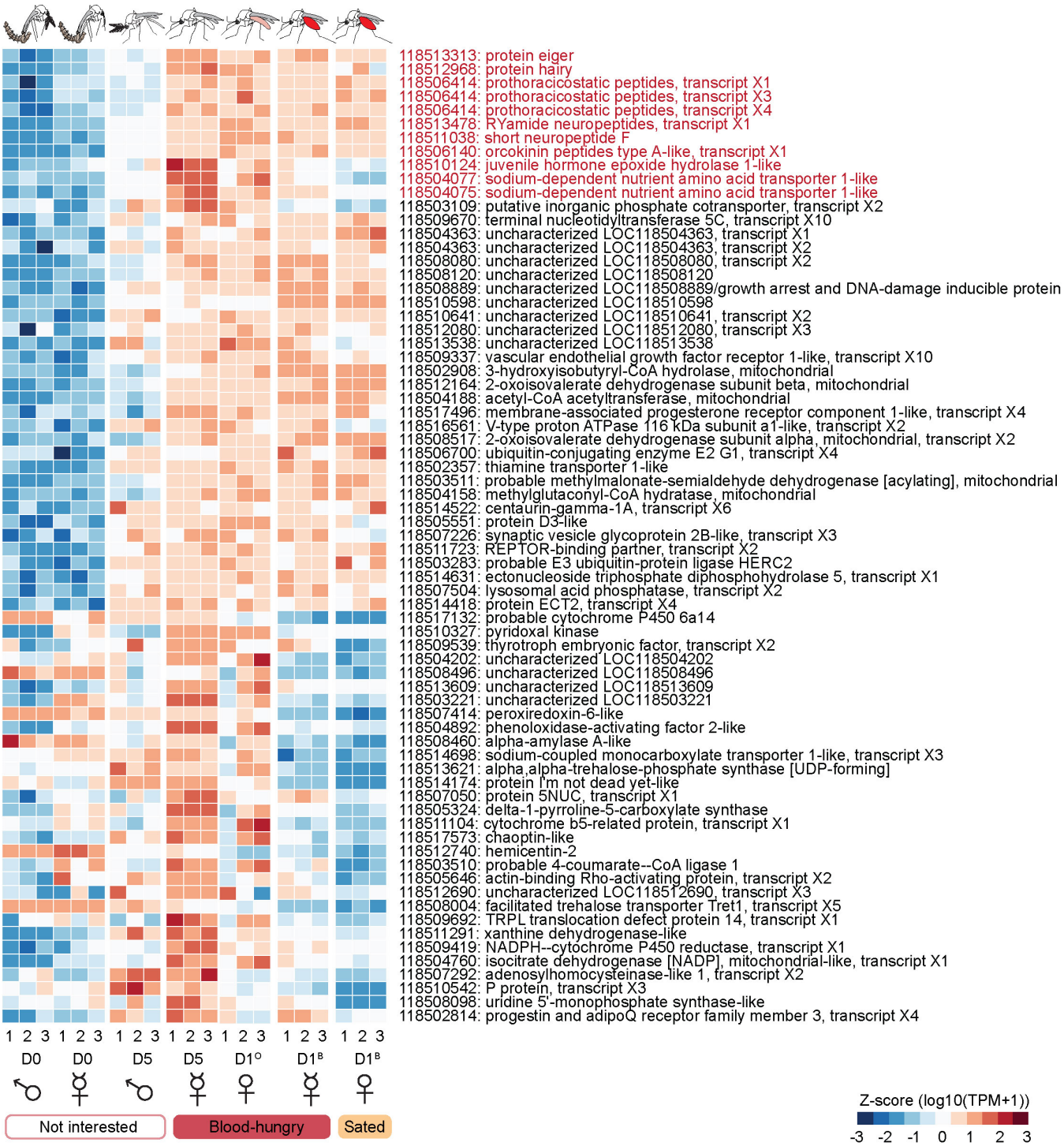
Supplementary Figure 4. Differential expression of genes involved in lipid metabolism across different tissues and states of blood-hunger and -satiety in *An. stephensi*. Expression patterns of genes involved in lipid metabolism in mid-brains, ovary and fat body from publicly available datasets. Genes (listed on the right) involved in lipid breakdown (top) and lipid synthesis (bottom) are shown. RNA-seq data from all mid-brain samples in triplicates and single replicate data of ovary and fat body are represented as z-score normalised log10 (TPM+1) values. No specific patterns were observed in mid-brains or ovaries pre- and post-blood meal. As expected, fat body showed high expression of genes involved in both the catabolic and anabolic branches of fatty-acid metabolism.



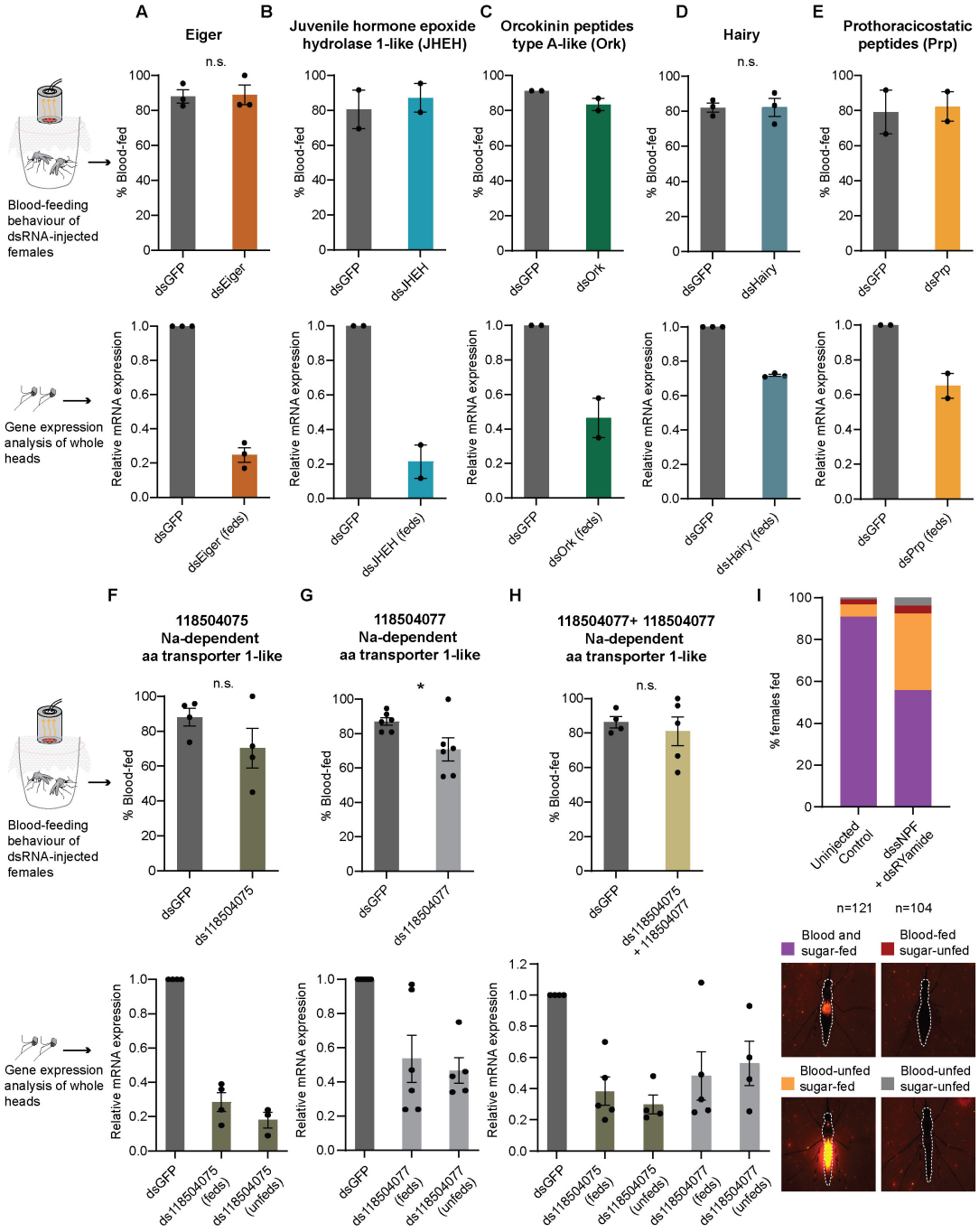
Supplementary Figure 5. Expression patterns of neurotransmitter-related genes in the mid-brain of *An. stephensi* across different states of blood-hunger and -satiety. Genes (listed on the right) involved in neurotransmitter expression, transport and processing are plotted across all samples in triplicates. Interestingly, many of these genes were largely observed to be up-regulated in the newly emerged (D0) mosquitoes as compared to the older and blood-fed mosquitoes. RNA-seq data are represented as z-score normalised log10 (TPM+1) values.



Supplementary Figure 6. Expression patterns of neuropeptides and neuropeptide-receptor genes in the mid-brain of *An. stephensi* across different states of blood-hunger and -satiety. Genes (listed on the right) expressing neuropeptides and neuropeptide-receptors are plotted across all samples in triplicates. RNA-seq data are represented as z-score normalised log10 (TPM+1) values. While neuropeptides were observed to be down-regulated in the males and newly emerged females (D0), neuropeptide-receptors showed no such expression patterns.



Supplementary Figure 7. Candidates potentially involved in promoting blood-feeding behaviour in *An. stephensi*. 71 candidate genes identified as potential promoters of blood-feeding are listed. These were identified to be commonly up-regulated in different conditions of blood-hunger (as compared to conditions of satiety or no-interest), and non-overlapping in males. Nine genes shortlisted for further validation are highlighted in red. RNA-seq data from all samples in triplicates are represented as z-score normalised log10 (TPM+1) values.



Supplementary Figure 8. Screening of the shortlisted candidates, potentially involved in promoting blood-feeding behaviour in *An. stephensi* via dsRNA-mediated gene knockdown. Blood-feeding behaviour (top) of virgin females injected with dsRNA against Eiger (A), Juvenile hormone epoxide hydrolase-1 like (JHEH) (B), Orcokinin peptides type-A like (Ork) (C), Hairy (D), Prothoracicostatic peptides (Prp) (E), Na-dependent amino acid transporter-1 like 118504075 (F) and 118504077 (G), 118504075+118504077 (H). dsGFP was injected as a control. 18-25 females/replicate, n=2-6 replicates/group. Relative mRNA expression (bottom) in the heads of virgin *An. stephensi* females injected with the indicated dsRNA, as compared to that in dsGFP injected females, analysed via qPCR. Unpaired t-test, $p<0.05$. n.s. not significant, *$p<0.05$ I. Feeding status of uninjected and dual (dssNPF+dsRYa) injected females assayed for both sugar and blood appetite, tested in Fig. 4E. Females were injected as described earlier (see methods for details) and offered Rhodamine B-spiked sugar 24h prior to the assay. Blood-feeding behaviour was tested as described earlier. Representative widefield fluorescent images of females fed on both blood and sugar, blood only, sugar only or none, are shown below.

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