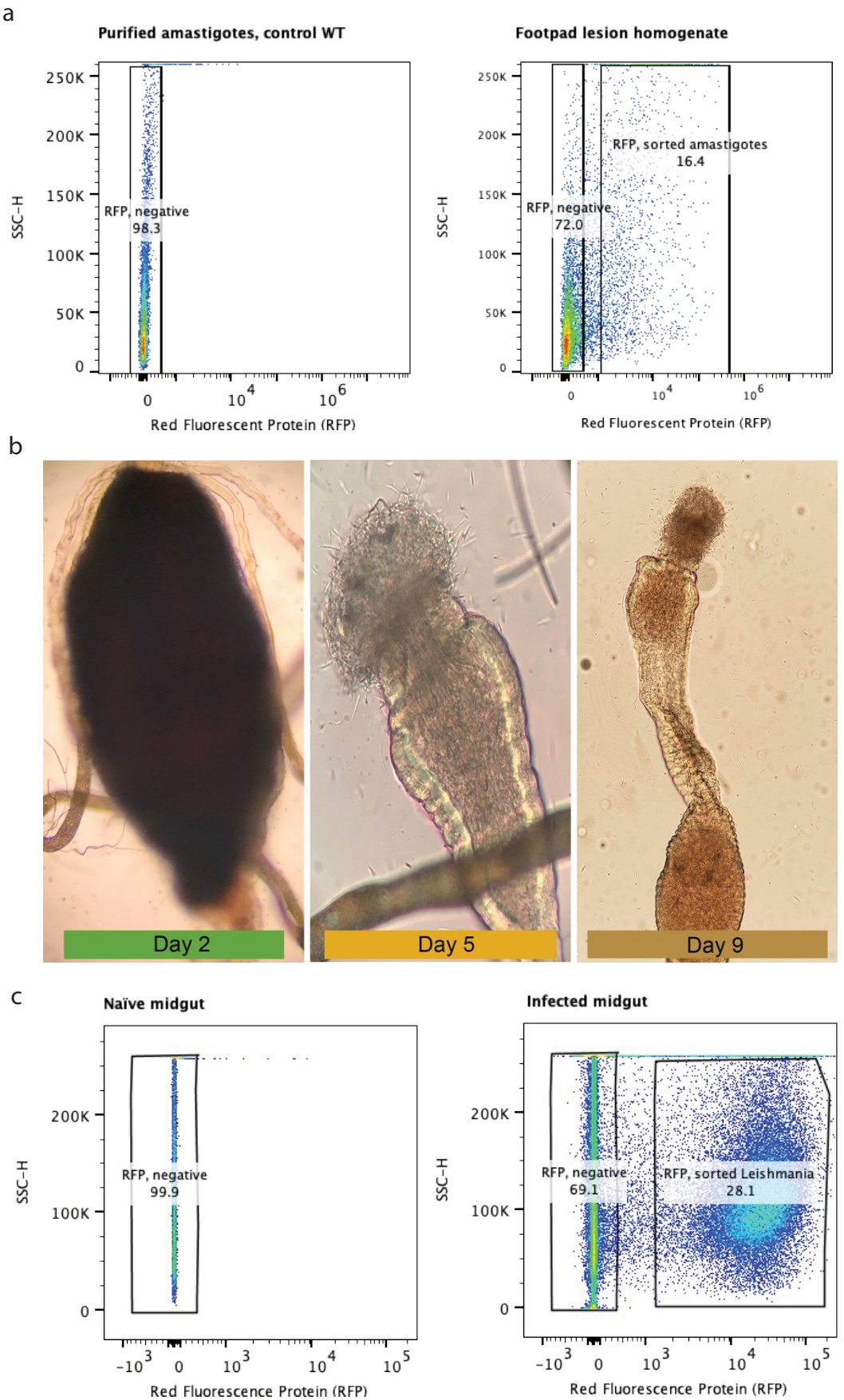
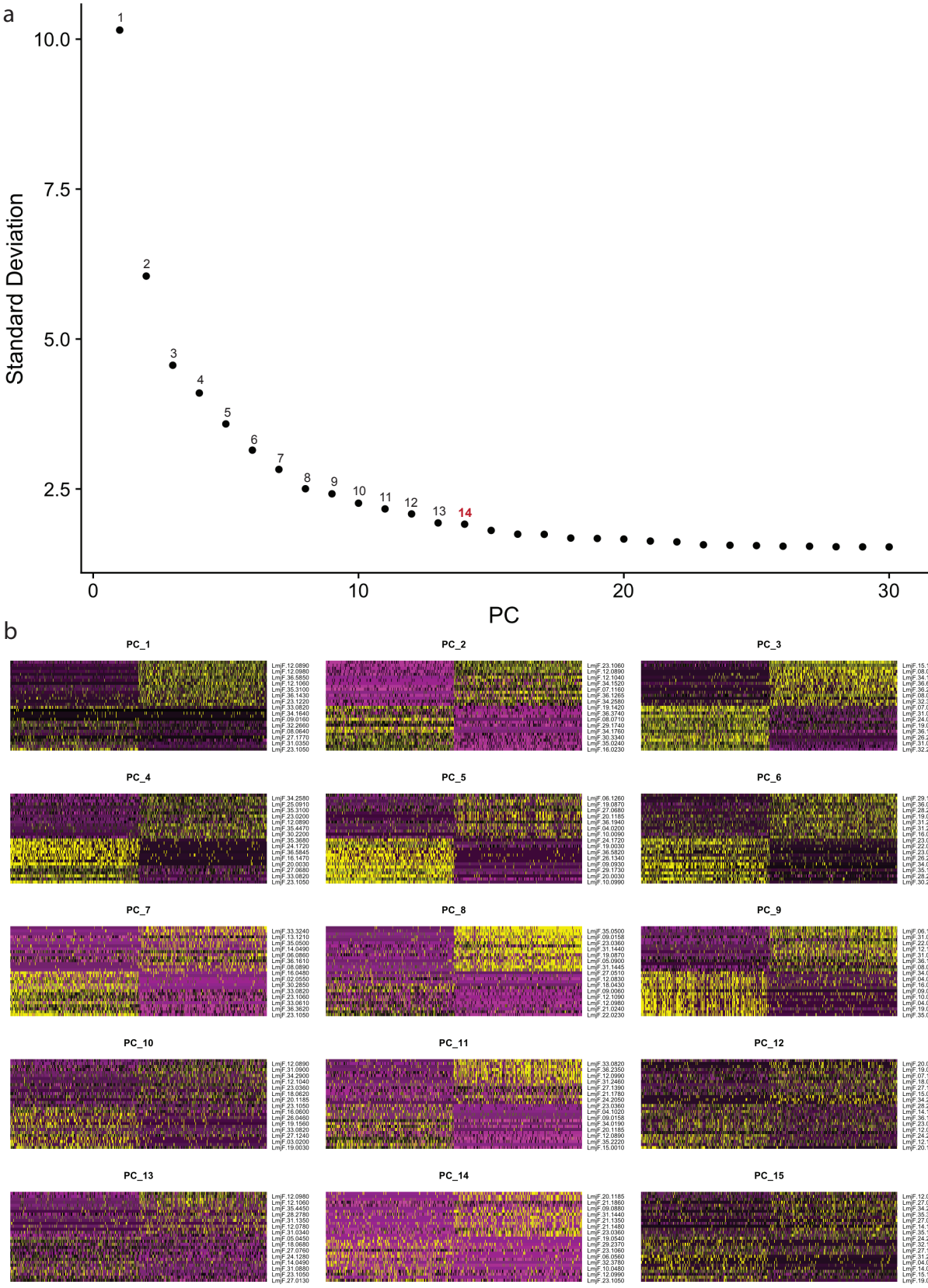
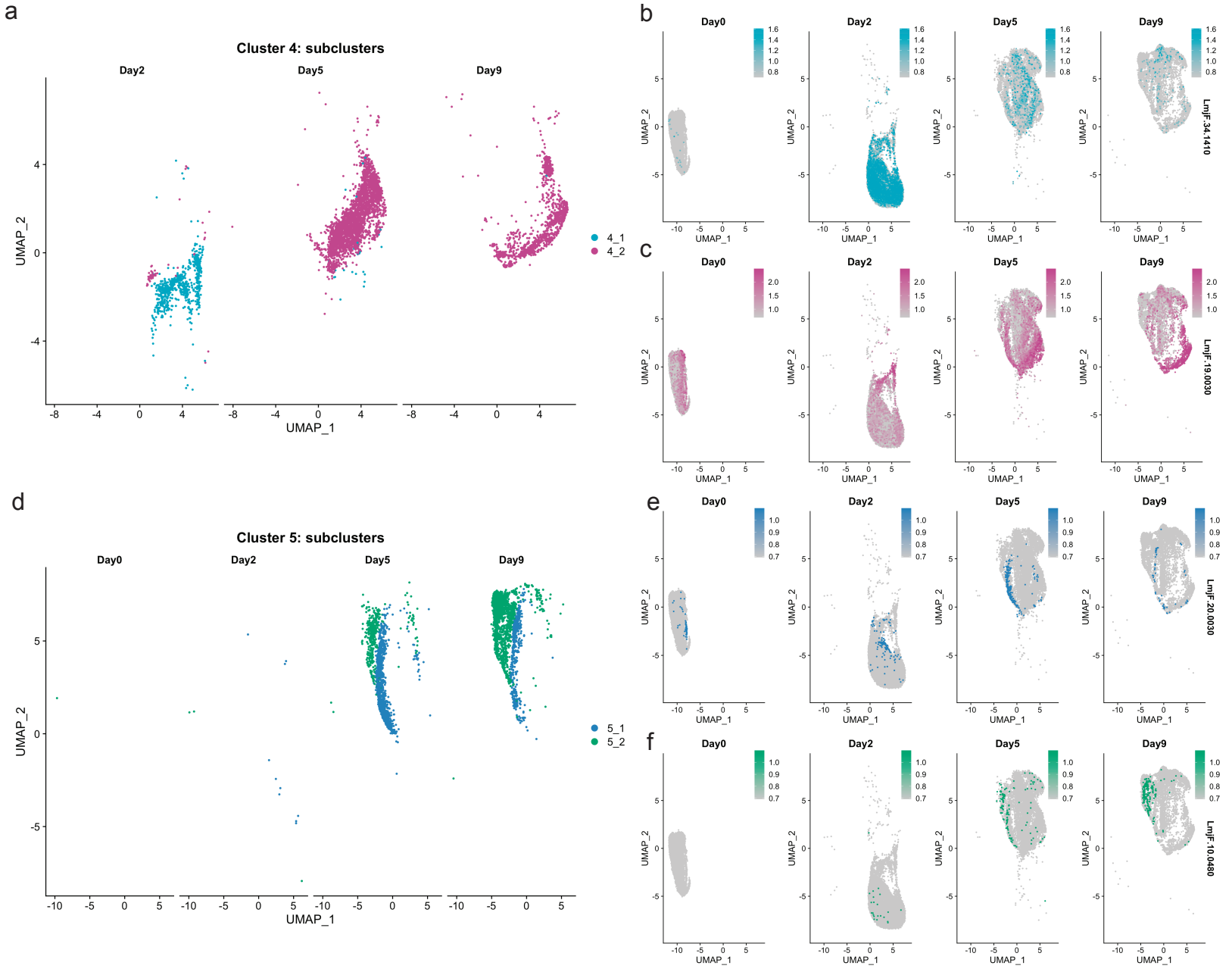


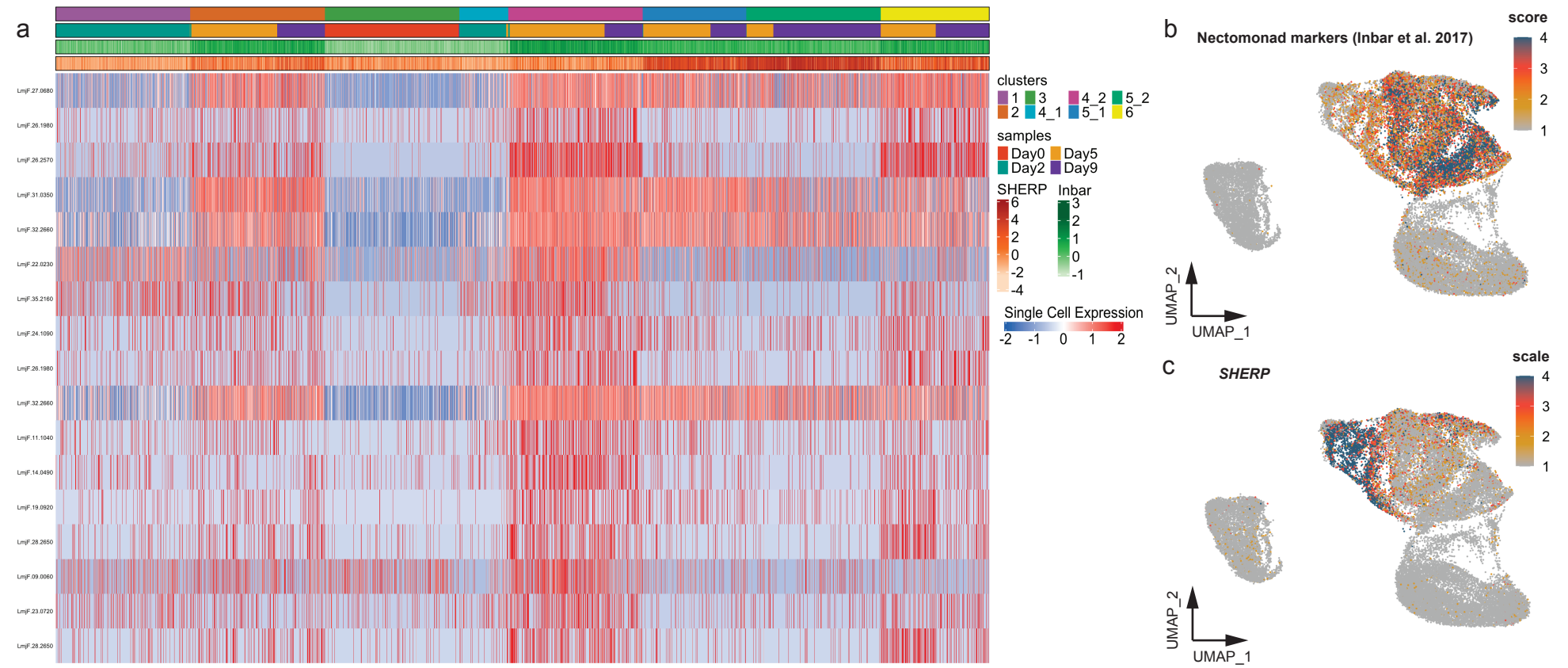
Extended Data 1. Sample preparation for sandfly infections and scRNA-seq processing. (a) Flow cytometry gating strategy to sort *L. major* Ryan RFP$^+$ cells from infected footpad lesions homogenates. The sorted samples were utilized for both sandfly infections and the preparation of scRNA-seq day 0 samples. (b) Sandfly midguts were dissected at days 2, 5, and 9 post-infection. (c) Flow cytometry gating strategies to sort *L. major* Ryan RFP$^+$ cells from sandfly midgut homogenates for the preparation of scRNA-seq samples.



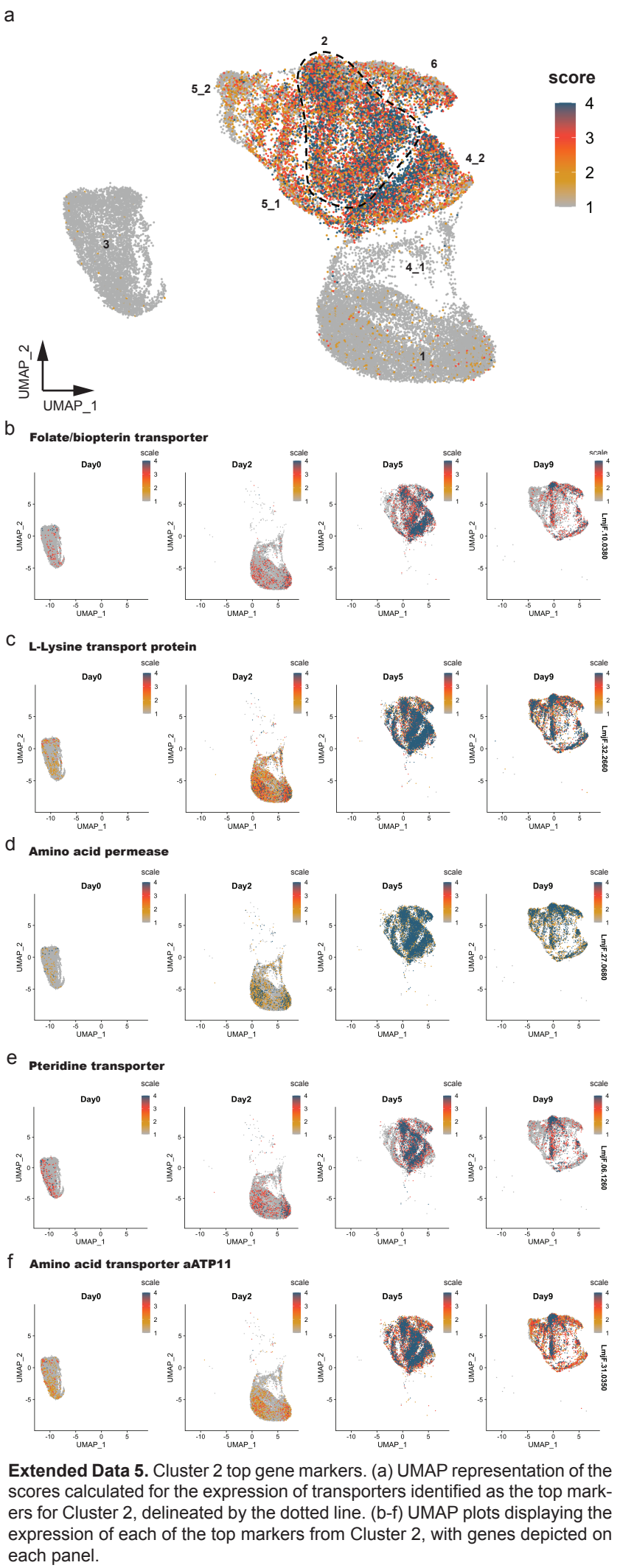
Extended Data 2. Optimal number of principal components (PCs) to retain during dimensionality reduction. (a) Elbow plot depicting the percentage of data variability accounted for by each PC. (b) Heatmap with expression levels of genes across different cells or conditions, organized by the top 15 PCs.



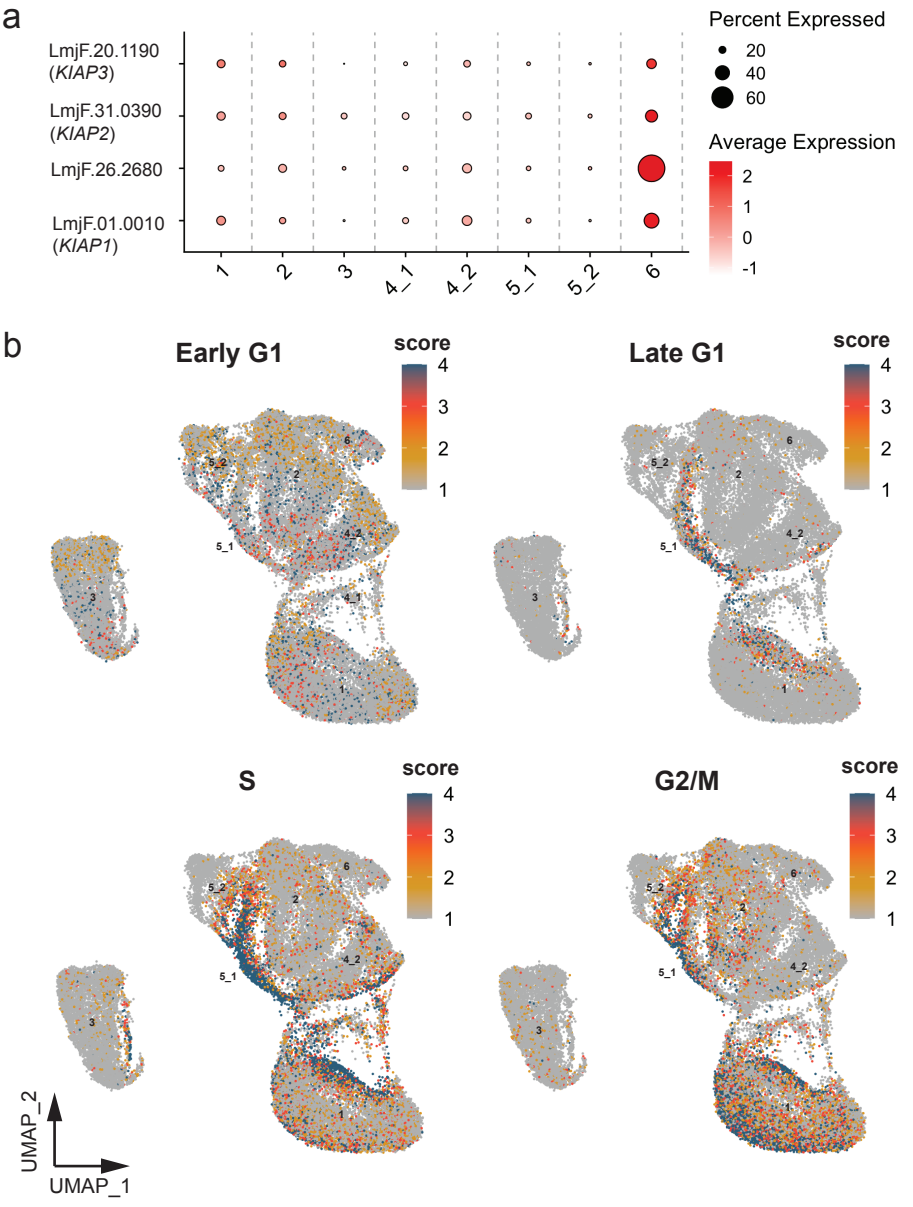
Extended Data 3. Sub clustering of Clusters 4 and 5. (a) UMAP distribution of cells from subclusters 4_1 (cyan) and 4_2 (pink) at days 2, 5 and 9. (b) UMAP representation of the gene LmjF.34.1410, which exhibits up-regulation in cluster 4_1 compared to 4_2, predominantly expressed in cells from day 2. (c) UMAP representation of the gene LmjF.19.0030, up-regulated in cluster 4_2, especially at day 9. (d) UMAP distribution of cells from subclusters 5_1 (blue) and 5_2 (green) at days 0, 2, 5 and 9. (e) UMAP representation of the gene LmjF.20.0030, which exhibits up-regulation in cluster 5_1 compared to 5_2. (f) UMAP representation of the gene LmjF.10.0480, up-regulated in cluster 5_2, especially at day 9.



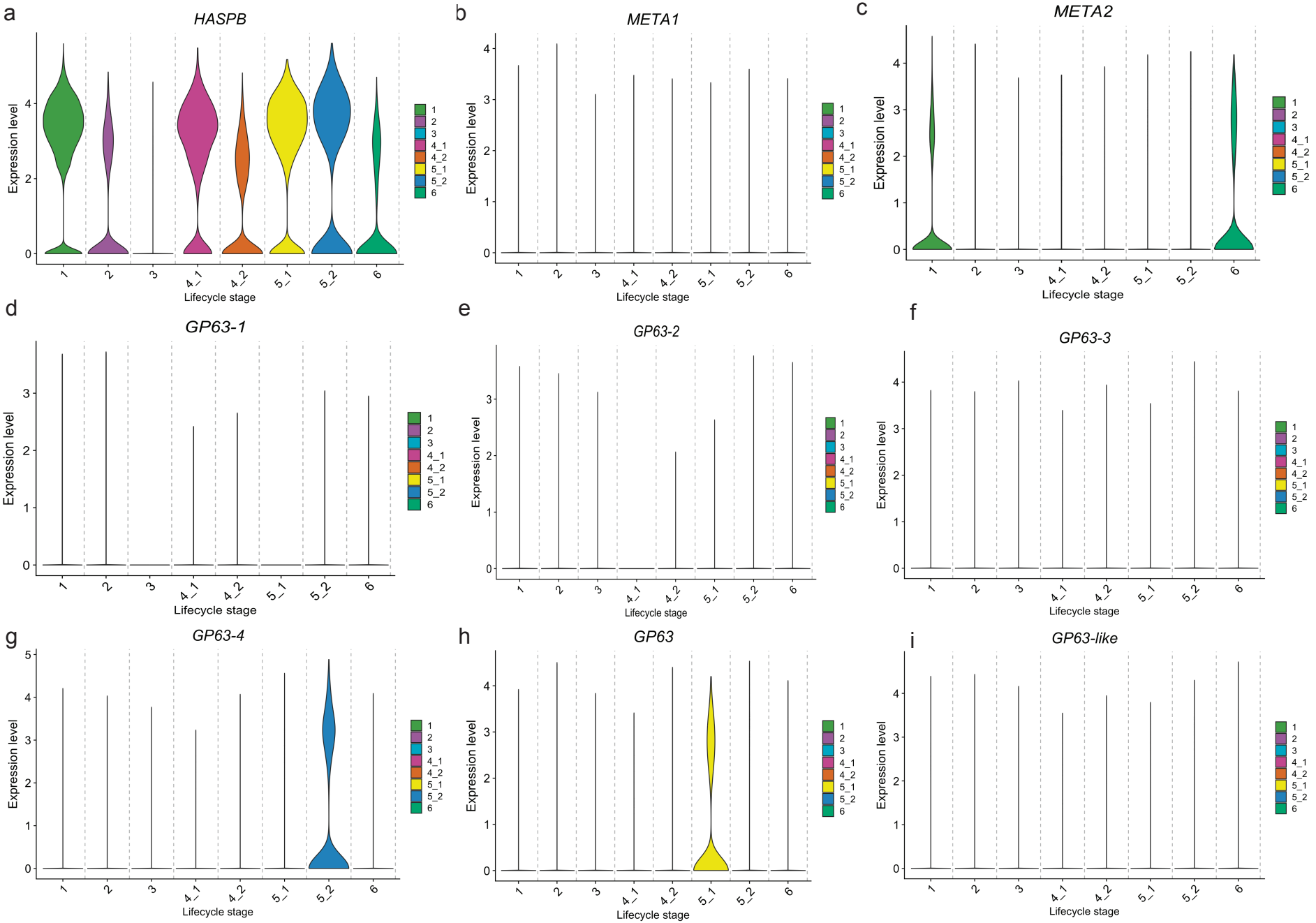
Extended Data 4. Expression of nectomonad markers identified in bulk RNA sequencing. (a) Heatmap illustrates the single-cell expression patterns of 16 nectomonad-upregulated genes, as identified by Inbar et al., 2017, across 2000 cells. The top-colored bars differentiate between the cell clusters, samples, and scores calculated for the expression of all 16 genes and *SHERP*. (b) UMAP representation of the expression score calculated for the 16 nectomonad genes. (c) UMAP representation of *SHERP* expression.



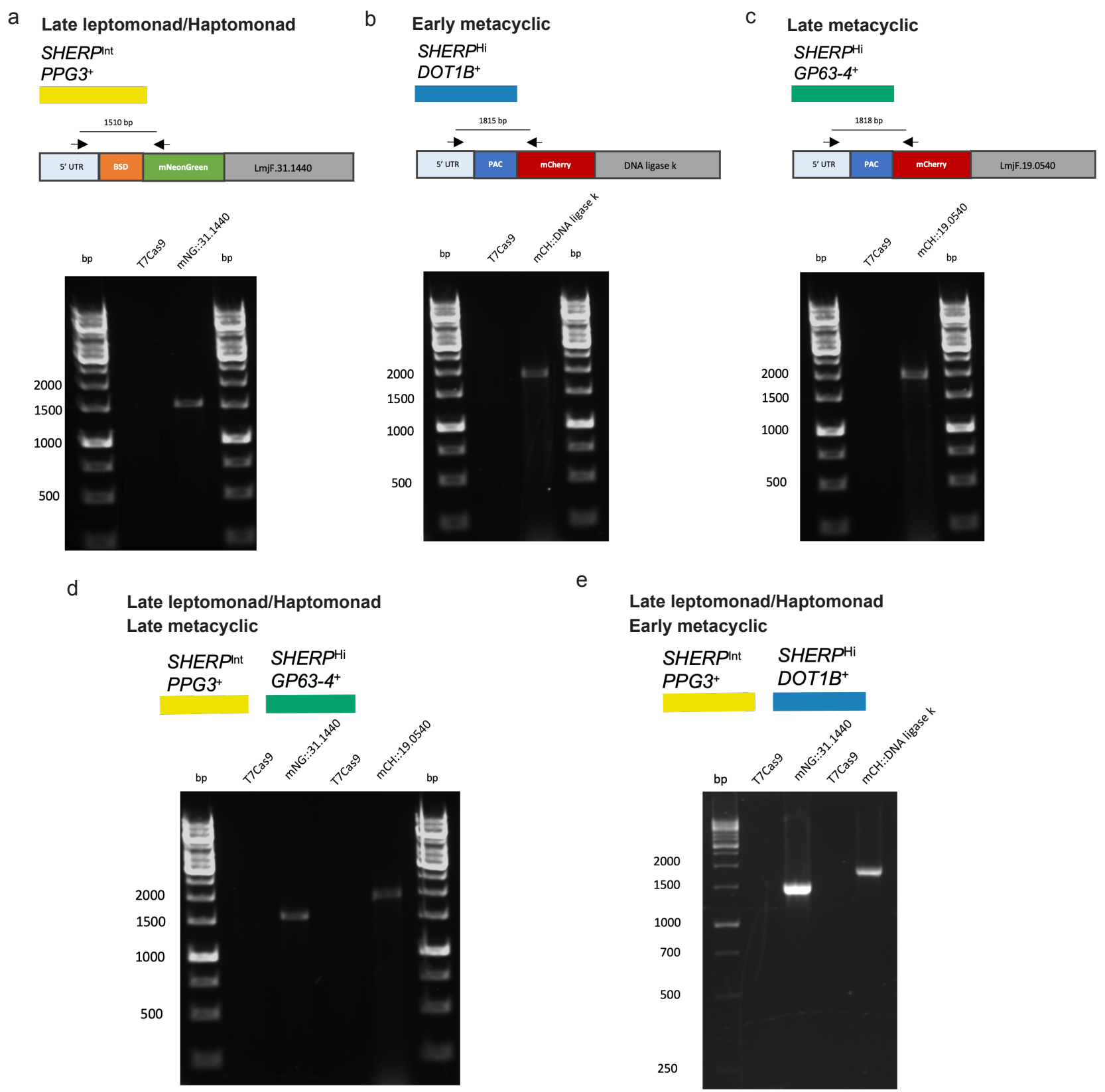
Extended Data 5. Cluster 2 top gene markers. (a) UMAP representation of the scores calculated for the expression of transporters identified as the top markers for Cluster 2, delineated by the dotted line. (b-f) UMAP plots displaying the expression of each of the top markers from Cluster 2, with genes depicted on each panel.



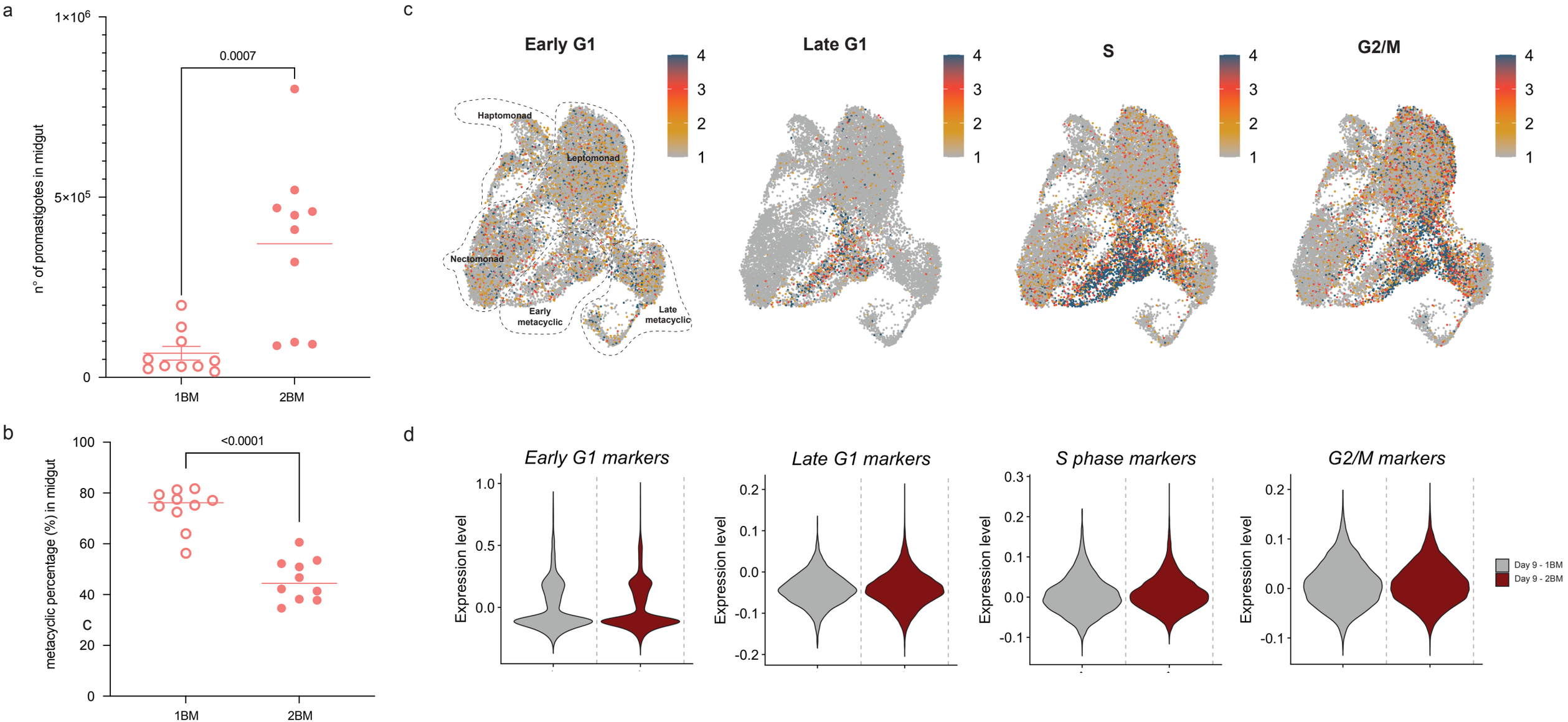
Extended Data 6. Haptomonad markers and expression of cell cycle-regulated genes in promastigotes collected from sandflies. (a) Dot plot of 4 genes upregulated in cluster 6 (SHERP^{Int}PPG3^{+})previously associated with the haptomonad stage in vitro and colonization of the sandfly posterior midgut16. (b) UMAP representation of expression scores for *Leishmania* homolog genes associated with cell cycle-related processes, as reported by Briggs et al.,2023. This includes 11 genes for Early G1, 347 genes for Late G1, 443 genes for S-phase, and 380 genes for G2/M-phase.



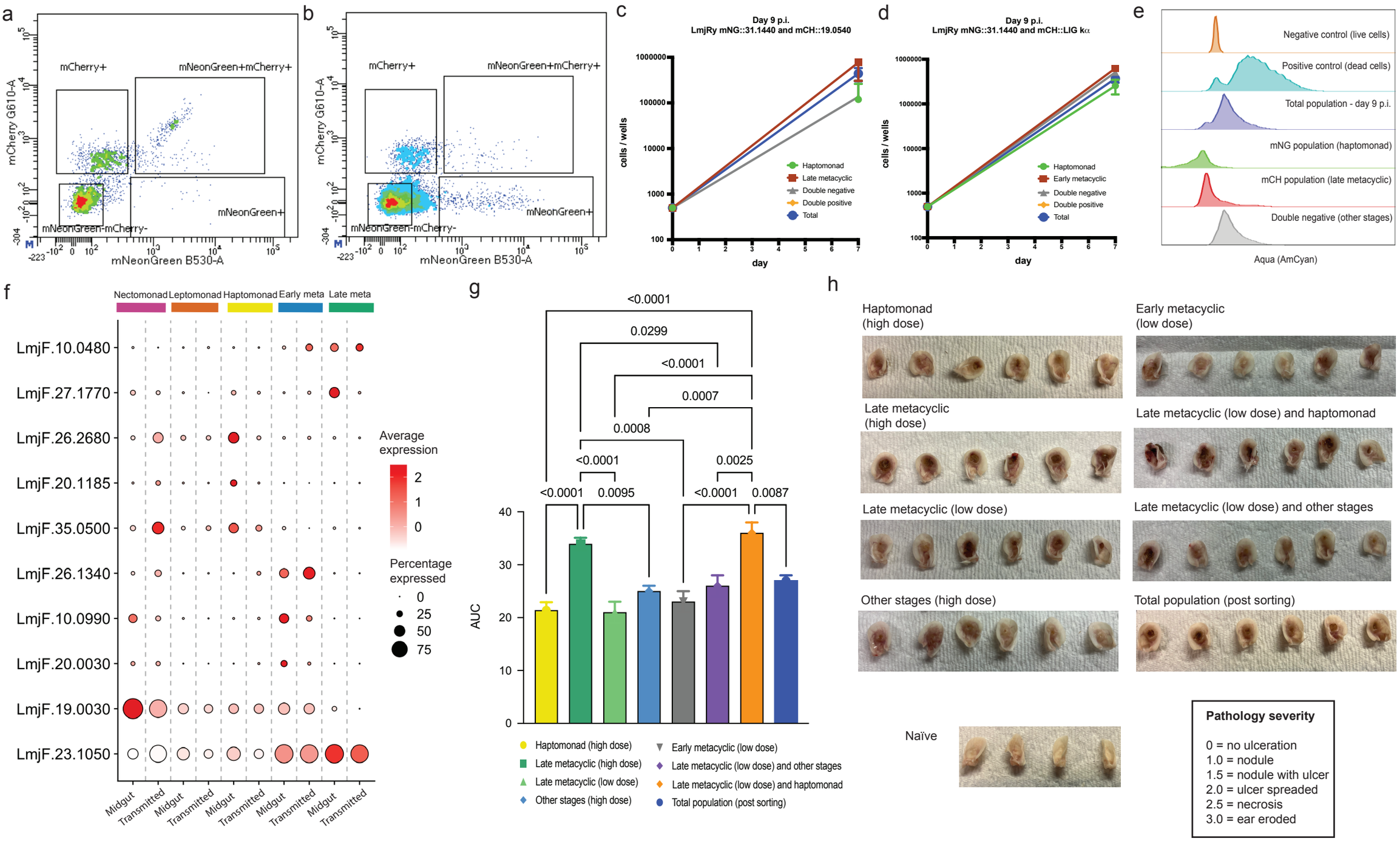
Extended Data 7. Expression of metacyclic stage-specific genes as reported in the literature. Violin plots depicting the average expression levels of cells in each cluster for the following genes: (a) LmjF.23.1060 (*HASPB*), (b) LmjF.17.0890 (*META1*), (c) LmjF.17.0870 (*META2*), (d) LmjF.10.0460 (*GP63-1*), (e) LmjF.10.0465 (*GP63-2*), (f) LmjF.10.0470 (*GP63-3*), (g) LmjF.10.0480 (*GP63-4*), (h) LmjF.28.0570 (*GP63*), (i) LmjF.31.2000 (*GP63*-like).



Extended Data 8. Integration of resistance markers and fluorescent tags at the 5' end of the gene markers. The primer positioning and expected fragment size for all diagnostic PCRs are depicted in the gene locus diagram. (a) *L. major* Ryan T7Cas9 mNG::31.1440-BSD, reporter cell line for *SHERP*^{Int}PPG3^{+} (late leptomonad/haptomonad). (b) *L. major* Ryan T7Cas9 mCH::*LIGkα*-PAC, reporter cell line for *SHERP*^{Hi}*DOT1B*$^{+}$ (early metacyclic). (c) *L. major* Ryan T7Cas9 mCH::19.0540-PAC, reporter cell line for *SHERP*^{Hi}*GP63-4*$^{+}$ (late metacyclic). (d) Double reporter cell line *L. major* Ryan T7Cas9 mNG::31.1440-BSD and mCH::19.0540. (e) Double reporter cell line *L. major* Ryan T7Cas9 mNG::31.1440-BSD and mCH::*LIGkα*-PAC.



Extended Data 9. Effect of a second blood meal on parasite numbers and the expression of cell cycle regulators. (a) Midgut parasite numbers in two groups of sandflies, provided with one or two blood meals (BM). (b) The percentage of metacyclics in each group of flies. (c) UMAP representation of expression scores for *Leishmania* homolog genes associated with cell cycle-related processes in Day 9 cells integrated from both single-blood meal (1BM) and double-blood meal (2BM) conditions. (d) Violin plots comparing scores of cell cycle-related markers across cells from 1BM and 2BM conditions.



Extended Data 10. Validation and infection of promastigote subpopulations in ears of BALB/c mice. (a) Gating strategy for sorting haptomonads (mNeonGreen^{+}), late metacyclics (mCherry^{+}), and other stages (mNeonGreen^{Neg}mCherry^{Neg}) from homogenates of sandfly midguts infected for 9 days with *L. major* Ryan T7Cas9 mNG::31.1440-BSD and mCH::19.0540. Double positive cells (mNeonGreen^{+}mCherry^{+}) were also collected for viability assays. (b) Gating strategy for similar infection conditions using *L. major* Ryan T7Cas9 mNG::31.1440-BSD and mCH::*LIGkα*-PAC for the collection of early metacyclics. (c-d) Proliferation assay in limiting dilution plates for sorted populations depicted in (a) and (b). (e) Viability assay of log-phase culture cells, including positive and negative controls, as well as populations sorted from *L. major* Ryan T7Cas9 mNG::31.1440-BSD and mCH::19.0540 sandfly infections (9 d.p.i.). (f) Dot plot depicting the average expression of genes markers for the stages represented in the transmitted sample compared to the average expression on day 9 of infection. (g) Bar plot of the area under the curve (AUC) from Fig.6j, calculated for each group of infection. The p-values in the figure were calculated using ANOVA with Tukey correction for multiple comparisons. (h) Ears from BALB/c mice infected with sorted populations described in Fig.6i and processed for parasite burden in Fig.6l..