# An epigenetic clock in an insect model system

Kristiana Brink[1†], Christian Thomas[1†], Alun Jones[2] and Eamonn Mallon[1*]

[1]Genetics and Genome Biology, University of Leicester, University Road, Leicester, LE1 7RH, United Kingdom.
[2] Institute for Evolution and Biodiversity, University of Muenster, Huefferstrabe, Munster, 48149, Germany.

*Corresponding author(s). E-mail(s): ebm3@le.ac.uk;
Contributing authors: keb40@leicester.ac.uk; clt54@leicester.ac.uk; ajones@uni-muenster.de;
†These authors contributed equally to this work.

**Abstract**

Epigenetic clocks in humans are argued to be a measure of true biological age based on the DNA methylation status of selected sites in the genome. Here we discover for the first time, an epigenetic clock in a model insect system, *Nasonia vitripennis*. By leveraging the power of an insect model, future studies will be able to research the biology underpinning epigenetic clocks and how influenced epigenetic clocks are by ageing interventions.

An epigenetic clock is an emergent property of the DNA methylation status of a large number of genes, the epigenome, calculated using supervised machine learning methods. There is evidence epigenetic age mirrors true biological age and its associated morbidity and mortality better than chronological age [1]. However, their utility as measures of changes in biological age for clinical interventions is limited as their mechanistic basis is not understood [2].

Ageing is a complex process influenced by many environmental and genetic components. The effects of these components influence each other making them difficult to investigate, especially in complex mammalian models. Therefore, a large body of ageing research is based on simple invertebrate model organisms [3, 4]. Advantages include easy and cheap to keep in a laboratory, short life span, genetic and molecular tools available and a sequenced genome. However,

the current invertebrate models of ageing (Drosophila [5] and *C. elegans* [6]) do not possess detectable DNA methylation, reducing their generality.

The jewel wasp, *Nasonia vitripennis*, an emerging model system [7] has a functional methylation system [8] making it an ideal system to investigate the epigenetics of ageing. We therefore measured chronological ageing and changes in the epigenome using whole genome bisulfite sequencing (WGBS) in order to discover if *Nasonia vitripennis* possessed an epigenetic clock.

Males and females showed different patterns of life expectancy (Cox mixed-effects model: Hazard ratio for females = 2.48 (standard error of coefficient = 0.233), z = 3.89, p = $9.8 \times 10^{-5}$), with females' mean life expectancy being 29 days and males' being 17 days, see Figure 1a. This was a greater than the 16.6 days for females and 10.7 days for males previously found for sucrose-fed individuals [9]. Time points 0, 8 and 16 days were selected for the WGBS experiment.

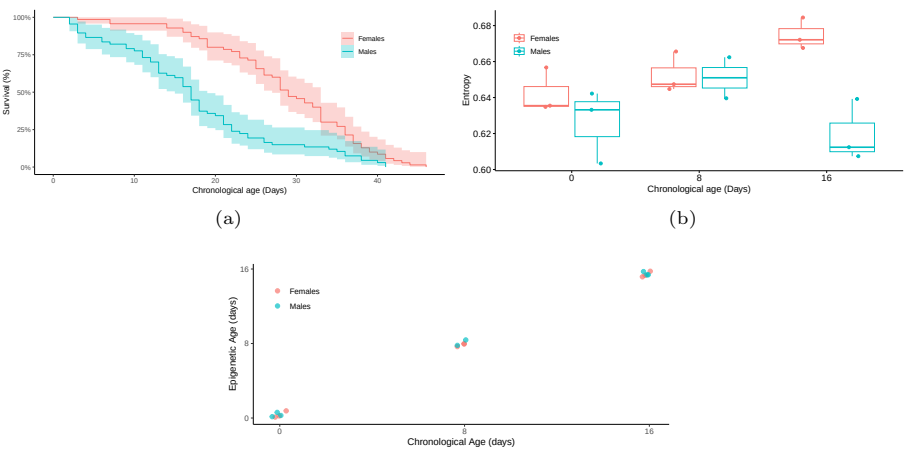


**Fig. 1**: (a) Kaplan-Meier survival curves for female (n = 70) and male (n = 67) *Nasonia vitripennis* adults. Shaded areas represent the 95% confidence intervals. (b) Boxplots of epigenetic entropy based on the 5290 age related differentially methylated CpGs over time for male and female *Nasonia*. Each point is a single WGBS library made up of ten individuals. (c) Scatterplot of epigenetic age versus chronological age of male (n=8) and female (n = 9) *Nasonia* samples. Each sample is made up of the whole bodies of ten individuals.

5290 CpGs were found to be significantly differentially methylated between at least two time points in males or females. Of these 48% were hypomethylated and 52% hypermethylated. We used these 5290 CpGs as the basis of two measures of the ageing epigenome; epigenetic drift and the epigenetic clock.

Epigenetic drift is the increased variability in the epigenome found through the course of an individual's life caused by the accumulation of mistakes in preserving epigenetic patterns. Epigenetic drift leads to a decrease in the body's ability to maintain homeostasis [10]. This can be measured as Shannon's entropy, i.e. the loss of information in the epigenome over time [11]. Entropy is calculated as;

$$Entropy = \frac{1}{N * \log \frac{1}{2}} \sum_i [MF_i * \log MF_i + (1 - MF_i) * (1 - \log MF_i)] \quad (1)$$

with $MF_i$ the fraction of methylation on a given CpG and N the total number of CpGs measured (5290).

An increase in entropy means the epigenome is becoming less predictable, that is more variable over time. There was a significant interaction of chronological age and sex on their effects on epigenetic entropy (Beta regression: $\chi^2 = 15.3192$, d.f. $= 2$, p $= 0.00047$) , see Figure 1b. Females display the increasing pattern found in other species (F$= 6.021$, df $= 2$, p $= 0.0024$), but males display a more complicated pattern with at first an increase at day eight followed by a decrease at day sixteen (F$= 4.432$, df $= 2$, p $= 0.0119$). This seems to be reflected in the survivourship curve (Figure 1a), where males have shorter life spans, but the rate of death seems to slow down once males go past median lifespan. This slower rate of death might be associated with this decreased entropy in sixteen day old males.

The epigenetic clock was constructed by regressing chronological age against the 5290 significantly differentially methylated CpGs. This identified 19 CpGs that best predict age. Eight of these decrease in methylation as *Nasonia* age and eleven increase in methylation. The full list of these CpGs and the genes where they are located can be found in supplemental table 1. Of passing note, the CpG having the most effect on epigenetic age in this model is located in the gene for a leucine-rich repeat kinase (lrrk). LRRK2 mutations are a common cause of age related autosomal-dominant Parkinson's disease [12].

The epigenetic age of each replicate is the weighted average of these CpGs' methylation state. This correlates with chronological age (Spearman's $\rho = 0.94$, p $= 1.4 \times 10^{-8}$), see Figure 1c. This is similar to results in many vertebrates [13] and even recently in the water flea *Daphnia* [14]. However, this is the first time an epigenetic clock has been discovered in a tractable insect model.

We predict two main areas where our establishment of an epigenetic clock in a model insect species will be useful; firstly, the biology underpinning epigenetic clocks and secondly, how influenced epigenetic clocks are by ageing interventions.

Variation in the rate of an individual's epigenetic clock is affected by a large number of traits including inflammation, cell division, metabolic effects, cellular heterogeneity, diet, and numerous other lifestyle factors [15]. *Nasonia*, with its simplified insect systems, is perfect to experimentally separate

the different processes involved in the biology of the clock into its constitutive parts [7]. As an example, we propose larval diapause as a model for early life experience effects on ageing. Early life effects are a major predictor of lifespan [16]. Diapause is an overwintering stage in some insects where development is arrested. Diapause can increase adult lifespan in insects [17] and is therefore an example of senescence plasticity, a polyphenism that alters the ageing of an organism [18]. Using published RNA-seq datasets [19], we found an increase in the expression of DMNT1a (the enzyme responsible for DNA methylation maintenance) and a decrease in TET (the enzyme that removes methylation) in diapaused versus non-diapaused larvae (see Supplementary Figure 1). This suggests a maintenance of DNA methylation during diapause. By comparing the rate of the epigenetic clock in adults from diapaused larvae compared to their non-diapaused conspecifics, we could elucidate how the early life environment affects adult epigenetic ageing.

Being short-lived (3-4 weeks as opposed to 26-30 months for mice), *Nasonia* are ideal to measure the effects of ageing interventions on both life span and epigenetic ageing. This will answer the question does a short-term decrease in someone's epigenetic clock score lower their chance of developing age-related ill health, that is if epigenetic clocks can be used as endpoints for clinical trials of various anti-ageing interventions [2].

Starting with Medawar, genetic mutations were seen as the driver of ageing [20]. Recent theories on the causes of ageing focus rather on the loss of epigenetic information as the main driver of ageing [21]. These epigenetic factors due to their known plasticity, are tempting targets for anti-ageing interventions [22]. We propose *Nasonia vitripennis*, with its fully functional DNA methylation system and its now established epigenetic clock as a model for this epigenetic era of ageing research.

## Online methods.

**Life span**. *Nasonia* were of the *Nasonia vitripennis* species from the Leicester strain which has been kept for over 4 years and originated from the AsymC strain. Wild-type wasps were maintained at 25°C, 40% humidity in a 12-h dark/light cycle. Adults, as soon as they emerged, were placed in tubes of ten single-sex individuals. They were fed 20% sucrose *ad libitum*. These were checked every day for survival. 70 females and 67 males were used. A mixed effect Cox model treating tube as a random effect was implemented using the survival package (v.3.4) [23] and coxme package (v.2.2) [24] in R 4.2.2 [25].

**DNA extraction**. Wasps were collected within 24 hours of eclosion. Some females may be mated as were allowed to mix with males for upto the first fifteen hours after eclosion. Wasps were then collected under light $CO_2$ anesthesia and placed into single-sex vials containing 10 individuals. They were then provided with filter paper soaked in 20% sucrose which was changed daily. Day 0 wasps were collected at the end of the first 24 hours after eclosion then samples on the 8th and 16th day. 60 wasps of each sex were used for each timepoint. 20 wasps from the same sex were pooled for each biological replicate,

creating three biological for each sex at each time point. Wasps were immediately frozen in liquid nitrogen and stored at minus 80°C freezer for sequencing. DNA was extracted using Qiagen's DNAeasy Blood and Tissue kit. DNA quality was assessed by NanoDrop 2000 spectrophotometer (Thermo Scientific), 1% agarose gel and Qubit (dsDNA BR Assay, ThermoFisher).

**Whole genome bilsulfite sequencing**. WGBS sequencing was carried out by BGI Tech Solution Co., Ltd.(Hong Kong). A 1% unmethylated lambda spike was included in each sample in order to assess bisulfite conversion rates. For WGBS samples, library quality was checked with FastQC (v.0.11.5; 26). Paired-end reads were aligned to the *Nasonia vitripennis* reference genome (Nvit_PSR1.1, Refseq accession no. GCA_009193385.1, 27) using the Bowtie 2 aligner (v.2.2.9; 28) within the Bismark software (v.0.18.1; 29) under standard parameters. Samples sequenced across multiple files were merged using samtools (v.1.9; 30). Files were deduplicated using Bismark, and methylation counts were extracted in different contexts using the bismark_methylation_extractor command (v.0.18.1; 29). Destranding was carried out using the coverage2cytosine script from Bismark using the merge_CpG command to increase coverage by pooling the top and bottom strand into a single CpG [29]. Reads were also aligned to the unmethylated lambda reference genome to calculate the error rate of the C–T conversion (Refseq accession no. GCF 000840245.1).

Output from the coverage2cytosine script was then inputted into the R package methylKit (v.3.14; 31) where files were filtered and normalised based on coverage, removing sites with abnormally high coverage (greater than 99% percentile) or with a coverage less than ten in each sample.

A binomial test was then applied to the filtered CpG sites where the lambda conversion rate was used as the probability of successes and a false discovery rate (FDR) of $p < 0.05$ [32]. As the majority of sites in the *Nasonia* genome show zero methylation, only CpGs which were methylated in at least one sample were retained. On these CpGs, differential methylation analysis was performed using the calculateDiffMeth command in methylKit, which implements a logistic regress model. Differentially methylated CpG sites were classed as having a minimum difference of $> 15\%$ methylation and a q-value $< 0.05$. Differential methylation analyses were performed across age in each sex as well as a comparatively between sexes over age.

Genes were classed as differentially methylated if they contained at least two differentially methylated CpG and a minimum weighted methylation difference of 15% across the entire feature [33]. Weighted methylation level is classed as the total number of methylated cytosines (C) within a region (i), divided by the total coverage of that region [33].

**Elastic net regression**. Chronological age was regressed against the 5290 age significant CpGs' beta values using an elastic net regression implemented in the glmnet R package [34]. This identified 19 CpGs that predict age. The

epigenetic age of each replicate is predicted based on these CpGs methylation state. This epigenetic age was correlated with chronological age using a spearman's rank correlation.

**Supplementary information.**
**Supplementary table 1:** The genomic location of the 19 CpGs making up the epigenetic clock in *Nasonia vitripennis*.
**Supplementary figure 1:** Gene expression of Dmnt1a, Dmnt3 and Tet in *Nasonia vitripennis* for both diapaused and non-diapaused larvae. Calculated from published data sets [19].

**Acknowledgments.**
EBM was funded by grant RPG-2020-363 from the Leverhulme Trust. CT, KB and ARCJ was supported by a BBSRC MIBTP DTP studentships.

# References

[1] Seale, K., Horvath, S., Teschendorff, A., Eynon, N. & Voisin, S. Making sense of the ageing methylome. *Nature Reviews Genetics* 1–21 (2022). URL https://www.nature.com/articles/s41576-022-00477-6. https://doi.org/10.1038/s41576-022-00477-6, publisher: Nature Publishing Group .

[2] Drew, L. Turning back time with epigenetic clocks. *Nature* **601** (7893), S20–S22 (2022). URL https://www.nature.com/articles/d41586-022-00077-8. https://doi.org/10.1038/d41586-022-00077-8, bandiera_abtest: a Cg_type: Outlook Number: 7893 Publisher: Nature Publishing Group Subject_term: Ageing, Society, Epigenetics .

[3] Mack, H. I. D., Heimbucher, T. & Murphy, C. T. The nematode Caenorhabditis elegans as a model for aging research. *Drug Discovery Today: Disease Models* **27**, 3–13 (2018). URL https://www.sciencedirect.com/science/article/pii/S1740675718300343. https://doi.org/10.1016/j.ddmod.2018.11.001 .

[4] Piper, M. D. W. & Partridge, L. Drosophila as a model for ageing. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease* **1864** (9, Part A), 2707–2717 (2018). URL https://www.sciencedirect.com/science/article/pii/S0925443917303319. https://doi.org/10.1016/j.bbadis.2017.09.016 .

[5] Lyko, F. & Maleszka, R. Insects as Innovative Models for Functional Studies of DNA Methylation. *Trends in genetics* **27** (4), 127–131 (2011). https://doi.org/10.1016/j.tig.2011.01.003 .

[6] Hu, C.-W., Chen, J.-L., Hsu, Y.-W., Yen, C.-C. & Chao, M.-R. Trace analysis of methylated and hydroxymethylated cytosines in DNA by isotope-dilution LC-MS/MS: first evidence of DNA methylation in Caenorhabditis

elegans. *The Biochemical Journal* **465** (1), 39–47 (2015). https://doi.org/10.1042/BJ20140844 .

[7] Werren, J. H. *et al.* Functional and evolutionary insights from the genomes of three parasitoid Nasonia species. *Science (New York, N.Y.)* **327** (5963), 343–348 (2010). https://doi.org/10.1126/science.1178028 .

[8] Wang, X. *et al.* Function and Evolution of DNA Methylation in Nasonia vitripennis. *PLOS Genetics* **9** (10), e1003872 (2013). URL http://journals.plos.org/plosgenetics/article?id=10.1371/journal.pgen.1003872. https://doi.org/10.1371/journal.pgen.1003872 .

[9] Floessner, T. S. E. *et al.* Lifespan is unaffected by size and direction of daily phase shifts in Nasonia, a hymenopteran insect with strong circadian light resetting. *Journal of Insect Physiology* **117**, 103896 (2019). https://doi.org/10.1016/j.jinsphys.2019.103896 .

[10] Vaiserman, A. Developmental Tuning of Epigenetic Clock. *Frontiers in Genetics* **9** (2018). URL https://www.frontiersin.org/articles/10.3389/fgene.2018.00584 .

[11] Hannum, G. *et al.* Genome-wide Methylation Profiles Reveal Quantitative Views of Human Aging Rates. *Molecular Cell* **49** (2), 359–367 (2013). URL https://www.sciencedirect.com/science/article/pii/S1097276512008933. https://doi.org/10.1016/j.molcel.2012.10.016 .

[12] Chen, J., Chen, Y. & Pu, J. Leucine-Rich Repeat Kinase 2 in Parkinson's Disease: Updated from Pathogenesis to Potential Therapeutic Target. *European Neurology* **79** (5-6), 256–265 (2018). URL https://www.karger.com/Article/FullText/488938. https://doi.org/10.1159/000488938, publisher: Karger Publishers .

[13] Parrott, B. B. & Bertucci, E. M. Epigenetic Aging Clocks in Ecology and Evolution. *Trends in Ecology & Evolution* **34** (9), 767–770 (2019). https://doi.org/10.1016/j.tree.2019.06.008 .

[14] Hearn, J., Plenderleith, F. & Little, T. J. DNA methylation differs extensively between strains of the same geographical origin and changes with age in Daphnia magna. *Epigenetics & Chromatin* **14** (1), 4 (2021). URL https://doi.org/10.1186/s13072-020-00379-z. https://doi.org/10.1186/s13072-020-00379-z .

[15] Bell, C. G. *et al.* DNA methylation aging clocks: challenges and recommendations. *Genome Biology* **20** (1), 249 (2019). URL https://doi.org/10.1186/s13059-019-1824-y. https://doi.org/10.1186/s13059-019-1824-y .

[16] Jagust, W. J. Early life sets the stage for aging. *Proceedings of the National Academy of Sciences* **113** (33), 9148–9150 (2016). URL https://www.pnas.org/doi/10.1073/pnas.1609720113. https://doi.org/10.1073/pnas.1609720113, publisher: Proceedings of the National Academy of Sciences .

[17] Denlinger, D. L. Why study diapause? *Entomological Research* **38** (1), 1–9 (2008). URL https://onlinelibrary.wiley.com/doi/abs/10.1111/j.1748-5967.2008.00139.x. https://doi.org/10.1111/j.1748-5967.2008.00139.x, _eprint: https://onlinelibrary.wiley.com/doi/pdf/10.1111/j.1748-5967.2008.00139.x .

[18] Flatt, T., Amdam, G. V., Kirkwood, T. B. L. & Omholt, S. W. Life-History Evolution and the Polyphenic Regulation of Somatic Maintenance and Survival. *The Quarterly Review of Biology* (2015). URL https://www.journals.uchicago.edu/doi/epdf/10.1086/671484. https://doi.org/10.1086/671484, publisher: The University of Chicago Press .

[19] Dittmer, J. & Brucker, R. M. When your host shuts down: larval diapause impacts host-microbiome interactions in Nasonia vitripennis. *Microbiome* **9** (1), 85 (2021). URL https://doi.org/10.1186/s40168-021-01037-6. https://doi.org/10.1186/s40168-021-01037-6 .

[20] Szilard, L. On the nature of the aging process. *Proceedings of the National Academy of Sciences* **45** (1), 30–45 (1959). URL https://www.pnas.org/doi/abs/10.1073/pnas.45.1.30. https://doi.org/10.1073/pnas.45.1.30, publisher: Proceedings of the National Academy of Sciences .

[21] Yang, J.-H. *et al.* Loss of epigenetic information as a cause of mammalian aging. *Cell* **186**, 1–22 (2023). URL https://www.cell.com/cell/abstract/S0092-8674(22)01570-7. https://doi.org/10.1016/j.cell.2022.12.027, publisher: Elsevier .

[22] Gensous, N. *et al.* The Impact of Caloric Restriction on the Epigenetic Signatures of Aging. *International Journal of Molecular Sciences* **20** (8), 2022 (2019). URL https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6515465/. https://doi.org/10.3390/ijms20082022 .

[23] Therneau, T. M. A package for survival analysis in R. manual (2022). URL https://CRAN.R-project.org/package=survival.

[24] Therneau, T. M. coxme: Mixed effects cox models. manual (2022). URL https://CRAN.R-project.org/package=coxme.

[25] R Core Team. R: A language and environment for statistical computing. manual, Vienna, Austria (2021). URL https://www.R-project.org/. Tex.organization: R Foundation for Statistical Computing.

[26] Andrews, S. FastQC: a quality control tool for high throughput sequence data (2010). URL http://www.bioinformatics.babraham.ac.uk/projects/fastqc.

[27] Sadd, B. M. *et al.* The genomes of two key bumblebee species with primitive eusocial organization. *Genome Biology* **16**, 76 (2015). URL https://doi.org/10.1186/s13059-015-0623-3. https://doi.org/10.1186/s13059-015-0623-3 .

[28] Langmead, B. & Salzberg, S. L. Fast gapped-read alignment with Bowtie 2. *Nature methods* **9** (4), 357–359 (2012). URL https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3322381/. https://doi.org/10.1038/nmeth.1923 .

[29] Krueger, F. & Andrews, S. R. Bismark: a flexible aligner and methylation caller for Bisulfite-Seq applications. *Bioinformatics* **27** (11), 1571–1572 (2011). URL https://academic.oup.com/bioinformatics/article/27/11/1571/216956. https://doi.org/10.1093/bioinformatics/btr167 .

[30] Li, H. *et al.* The Sequence Alignment/Map format and SAMtools. *Bioinformatics (Oxford, England)* **25** (16), 2078–2079 (2009). https://doi.org/10.1093/bioinformatics/btp352 .

[31] Akalin, A. *et al.* methylKit: a comprehensive R package for the analysis of genome-wide DNA methylation profiles. *Genome Biology* **13** (10), R87 (2012). https://doi.org/10.1186/gb-2012-13-10-r87 .

[32] Cheng, L. & Zhu, Y. A classification approach for DNA methylation profiling with bisulfite next-generation sequencing data. *Bioinformatics (Oxford, England)* **30** (2), 172–179 (2014). https://doi.org/10.1093/bioinformatics/btt674 .

[33] Schultz, M. D., Schmitz, R. J. & Ecker, J. R. 'Leveling' the playing field for analyses of single-base resolution DNA methylomes. *Trends in genetics : TIG* **28** (12), 583–585 (2012). URL https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3523709/. https://doi.org/10.1016/j.tig.2012.10.012 .

[34] Friedman, J., Hastie, T. & Tibshirani, R. Regularization paths for generalized linear models via coordinate descent. *Journal of Statistical Software* **33** (1), 1–22 (2010). URL https://www.jstatsoft.org/v33/i01/. https://doi.org/10.18637/jss.v033.i01 .