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RESEARCH

Better Late than Never: The Impact of Hatching Time on *Heterodera schachtii* Parasitism

Mason Rugen-Hankey, Priya Desikan, George Harpum, Chongjing Xia, Victor Hugo Moura de Souza, Unnati Sonawala, Lida Derevnina, Beth Molloy, Anika Damm, and Sebastian Eves-van den Akker†

Crop Science Centre, Department of Plant Sciences, University of Cambridge, Cambridge, United Kingdom

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Plant-parasitic nematodes are a diverse group collectively capable of infecting most major crops. Among the most damaging are the cyst nematodes, which can remain dormant in the soil for decades as infection-competent, developmentally arrested, second-stage juveniles in eggs. Hatching is stimulated by a variety of factors. However, the impact of hatching factor responsiveness on nematode morphology, physiology, gene expression, and infection biology has not been explored. We examined the impact of hatching time on the beet cyst nematode, *Heterodera schachtii*. We found that late hatchers invaded host roots and established feeding sites in greater numbers than early hatchers. We demonstrate variation in baseline parasitism gene expression and in responsiveness of genes to effectostimulins—small, plant-derived molecules that upregulate parasitism genes. Three quarters of effectostimulin-induced transcriptional changes were also modulated, either positively or negatively, by hatching time. Although there were no observable morphological differences between early- and late-hatching nematodes on their day of hatching, the late hatchers displayed signs of faster utilization of internal energy reserves after 7 days at 4°C, as evidenced by less body area attributed to fat, than early hatchers. Finally, we found no evidence of substantive genetic differences between early and late hatchers—they were representative of a single population, despite the observed differences in infection, gene expression, and physiology. Taken together, nongenetic differences likely drive late hatchers to more rapidly utilize their internal energy reserves, to be differentially responsive to host-derived signals, and to be ultimately more infective than their early-hatching counterparts.

†Corresponding author: S. Eves-van den Akker; se389@cam.ac.uk

M. Rugen-Hankey and P. Desikan contributed equally.

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Plant-parasitic nematodes are profoundly threatening agricultural pests that are estimated to cause up to 173 billion USD in crop losses annually (AbdelRazek and Balah 2023). Like all pathogens, plant-parasitic nematodes must deliver effectors into the host to alter metabolism, immunity, and physiology to promote infection. Effectors are principally derived from two sets of pharyngeal gland cells: two subventral and one dorsal (Pellegrin et al. 2025). Some nematode species, including the major contributors to economic losses, are sedentary endoparasites. These are so-called because they form long-term biotrophic interactions with modified cells in the vascular cylinder of the host roots (Jones et al. 2013). Over time, these nematodes may each lay hundreds of eggs that contain developmentally arrested, infection-competent, second-stage juveniles (J2s) (Siddique et al. 2022).

One of the most important groups of sedentary endoparasitic nematodes is the cyst nematodes, which include the genera *Heterodera*, *Globodera*, and *Cactodera*. This group is named for its ability to contain and protect eggs within the tanned body of the deceased mother (Siddique et al. 2022). The cysts may remain dormant in the soil for many years, in ideal conditions even decades, and likely represent an adaptation to survive prolonged periods without a suitable host.

Nematode eggs consist of juveniles encased in a three-layered eggshell. This typically comprises inner lipid, middle chitin, and outer vitelline layers, which may vary in their numbers (Perry et al. 1982) and thickness (Bird and McClure 1976). Whereas the chitin layer contributes structural strength, the lipid layer is of particular biological significance for determining to which compounds eggs are selectively permeable (Wharton 1980).

The hatching of nematodes from eggs within the cyst is stimulated by a variety of factors. During this process, the metabolic arrest of juveniles is gradually reversed through calcium fluxes (Atkinson and Ballantyne 1979; Atkinson et al. 1980) and water absorption (reviewed in Price et al. 2023). In the laboratory, hatching can be induced by root diffusates of hosts (Duceppe et al. 2017; Ochola et al. 2021), as well as a wide variety of inorganic ions, including $ZnCl_2$ (Clarke and Shepherd 1966). However, the age of hosts from which root diffusate is collected can impact its efficacy (Wright et al. 2019). Contrastingly, other species, including *Heterodera glycines*, can also hatch in water (Thapa et al. 2017).

After stimulation, the J2 will use its protrusible stylet to exit the egg, before exiting the cyst and migrating toward the root, following chemoattractive gradients (Perry et al. 2018). The J2 will

penetrate behind the root tip and migrate intracellularly toward the vascular cylinder using both its stylet and small molecule secretions, termed effectors, from its esophageal glands. These secretions consist of proteins and enzymes, such as cellulases, which can remodel host cells (Szakasits et al. 2009). Crucially, the J2 will not feed until parasitism is established and must therefore rely on its internal energy reserves.

Upon reaching the vascular cylinder, the J2 will select a single cell to developmentally reprogram and fuse with over 100 neighboring cells to form the feeding site, or syncytium (Perry et al. 2018; Siddique et al. 2022). Sex is determined during feeding. Greater establishment success promotes differentiation into females, whereas less successful syncytia favor the development of males, which will exit the root to mate with females. Failed syncytia result in nematode death. After mating, a female will begin to produce eggs, which she will store inside her body. Her cuticle will tan to form the protective outer layer of the cyst and allow the protected eggs to remain viable in the soil for decades (Perry et al. 2018).

J2s generally hatch after exposure to a hatching stimulant. For any given individual, exposure lasting as little as 10 min, or as long as several weeks, is sufficient to induce hatching. This produces a characteristic hatching curve with a "peak," followed by a months-long tapered decline (Greco 1981; Zheng and Ferris 1991). Up to 90% of the total juveniles can hatch from a cyst within 3 weeks of exposure to hatching factors (Perry et al. 2018). Hatching curves are known to vary within populations and between species (Perry et al. 2018). It is proposed that these differences are due to hatching being fine-tuned by two mechanisms: diapause and quiescence (Perry et al. 2018). Diapause tunes hatching to cyclical, long-term conditions (such as seasons), whereas quiescence tunes hatching to short-term stresses (such as drought). Variation in dormancy has previously been found between cysts, across the population, throughout a growing season (Zheng and Ferris 1991). Here, we sought to examine the impact of hatching time—as opposed to time since hatching (Storey 1984)—on infection biology.

Results

We developed a phased methodology to examine differences in hatching time, as well as time since hatching. *Heterodera schachtii* cysts (population IRS) were harvested from approximately 15 kg of sand and hatched in a single, large hatching jar. Hatching was induced using a 3 mM ZnCl2 solution (Clarke and Shepherd 1966), and a total of more than three million hatched J2s were collected daily for 3 weeks. The J2s harvested at each time point were counted, stored at 4°C, and then used 7 days after hatching in a variety of experiments. In this way, we could determine whether time of hatching—not time since hatching—is associated with other aspects of plant-parasitic nematode infection biology.

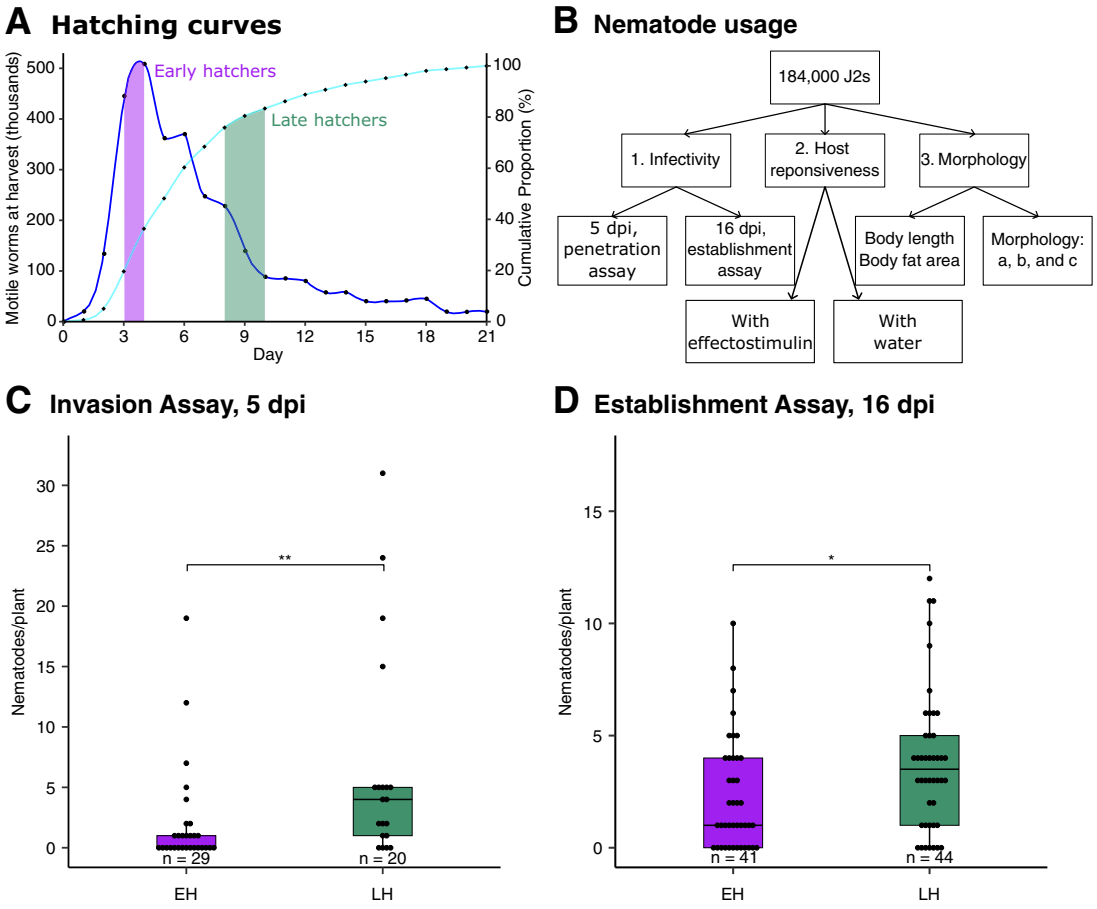


Fig. 1. Hatching curve, downstream experiments, and increased infectivity of late hatchers. **A,** Hatching curve of *Heterodera schachtii* (IRS) showing the number of motile nematodes hatched per day (dark blue line, dots, and left axis) and the cumulative proportion of total hatch (light blue line, diamonds, and right axis). Early hatchers (EHs) are denoted in purple, and late hatchers (LHs) are denoted in green. **B,** Downstream experiments for two selected time points. Nematodes were divided into three groups and were used to assay infectivity, responsiveness to host effectostimulins, or morphological changes at harvest and after storage, across the hatching curve. **C,** Number of nematodes per plant during earlier-stage parasitism, 5 days postinfection (dpi). **D,** Number of nematodes per plant during later-stage parasitism, 16 dpi. EHs are shown in purple, and LHs are shown in green. Asterisks indicate statistical differences (*<0.05; **<0.01).

In this experiment, the hatching curve of *H. schachtii* rapidly peaked at approximately day 4, followed by a less rapid decline until approximately day 9—consistent with the literature (Greco 1981)—such that the cumulative proportion approximates a logistic growth model (Fig. 1A; Supplementary Fig. S1). Fifty percent of nematodes hatched within 5 days and 90% within 12 days. From this curve, two time points were chosen: nematodes that hatched on days 3 and 4 are referred to as the "early hatchers," broadly corresponding to the peak of the hatching curve; nematodes that hatched on days 8 through 10 are referred to as the "late hatchers," the final time point with sufficient nematodes for the analyses (184,000). The same pools of early or late hatchers were divided into three experiments (Fig. 1B): experiment one measured the ability to infect white mustard (*Sinapis alba* L.), experiment two measured the transcriptional responsiveness to host-derived root extracts, and experiment three measured various morphological and physiological features.

To examine the impact of hatching time on infectivity, 14-day-old *S. alba* were infected with 250 of either 7-day-old early hatchers or 7-day-old late hatchers, at a concentration of 250 J2s/500 µl. Forty-nine plants ($n = 29$ and 20 for early and late hatchers, respectively) were analyzed at 5 days postinfection (dpi) to measure host invasion, and the remaining 85 plants ($n = 41$ and $n = 44$, respectively) were analyzed at 16 dpi to measure the establishment of biotrophy. Unequal group sizes were due to plant availability. At 5 dpi, significantly more late hatchers were observed inside host roots compared with early hatchers (Fig. 1C; Kruskal–Wallis, $\chi^2 = 8.3273$, df = 1, $P = 0.004$). Similarly, at 16 dpi, significantly more late hatchers developed into J3 sedentary stages than early hatchers (Fig. 1D; Kruskal–Wallis, $\chi^2 = 5.994$, df = 1, $P = 0.01$). Therefore, the late hatcher advantage in invasion is maintained into early biotrophy. These data establish that late-hatching nematodes have an advantage over their earlier-hatching counterparts, particularly during host invasion.

It was recently discovered that host invasion by *H. schachtii* is controlled by the transcriptional master regulator SUGR-1, which positively regulates effectors involved in lytic functions in response to host-derived small molecules (termed effectostimulins) released from root extracts (Pellegrin et al. 2025). In early and late hatchers, during the naive state and following response to effectostimulins, we therefore measured the expression of *sugr-1* and two related transcription factors that are the only members of a subclade of nuclear hormone receptors (NHRs) that regulate effectors in *Heterodera* (Damm et al. 2026): Hsc_gene_17966 (the Dorsal Gland Regulator-1, *dgr-1*) and Hsc_gene_17191.

All three transcription factors have a lower basal expression state in late hatchers compared with early hatchers (*sugr-1*, Welch's *t* test, $t = 3.6232$, df = 3.2191, $P = 0.05$, false discovery rate [FDR]-adjusted; *Hsc_gene_17966*, Welch's *t* test, $t = 3.7995$, df = 3.9831, $P = 0.05$, FDR-adjusted; *Hsc_gene_17191*, Welch's *t* test, $t = 2.8024$, df = 3.9473, $P = 0.05$, FDR-adjusted; Fig. 2). As a result of lower baseline expression, late hatchers were more responsive to effectostimulins and upregulated *sugr-1* more than early hatchers (300 versus 254% compared with their own baseline). The same pattern was observed for the *sugr-1* paralog *Hsc_gene_17191* to an even greater extent (578 versus 333% compared with their own baseline). All characterized transcription factors were significantly upregulated after exposure to host effectostimulins ($P < 0.01$ in all comparisons, Welch's *t* test, FDR-adjusted). After exposure to effectostimulins, the *sugr-1* end state showed a significantly lower expression between early and later hatchers (*sugr-1*, Welch's *t* test, $t = 1.3916$, df = 2.4961, $P = 0.02$, FDR-adjusted; *Hsc_gene_17966* and *Hsc_gene_17191* $P > 0.05$, FDR-adjusted), whereas *dgr-1* and Hsc_gene_17966 were not different.

To investigate the downstream impacts of transcription factor upregulation, we compared the global gene expression of early and late hatchers, with and without effectostimulin exposure, using whole-genome RNA sequencing. In total, 23,340 genes (out of 26,739) had non-zero expression in at least 1 of the 12 samples. Two principal components (PC1 and 2) describe 91% of the variance and reveal an apparently orthogonal relationship between variables (Fig. 3A). PC1 corresponds to effectostimulin treatment and explains 72% of the variance, and PC2 corresponds to hatching time and explains 19% of the variance. Using these data, we found that 3,785 genes were differentially expressed in nematodes based on time of hatching, effectostimulin exposure, or an interaction thereof ($\log_2$ fold change $> |0.5|$, $P < 0.05$).

All genes were clustered into seven distinctive sets (Fig. 3B): one core cluster plus six hatching time–modulated clusters. Cluster 1 was positively responsive to effectostimulins independent of hatching time ($n = 1{,}553$ genes, $n = 105$ effectors based on the list provided in Molloy et al. 2024). We denoted this cluster as the "core effectostimulin response," which represented only one quarter of all effectostimulin-responsive genes. Genes in the core effectostimulin responsive cluster 1 were enriched in effectors (hypergeometric test, $P = 8.74 \times 10^{-6}$). However, hypergeometric tests indicated that these effectors tend not to be enriched for specific evolutionary origins, sites of production, time points of maximal expression, and functions (Supplementary Tables S1 to S5).

The rest of the effectostimulin response (which is the remaining three quarters of all effectostimulin-responsive genes and

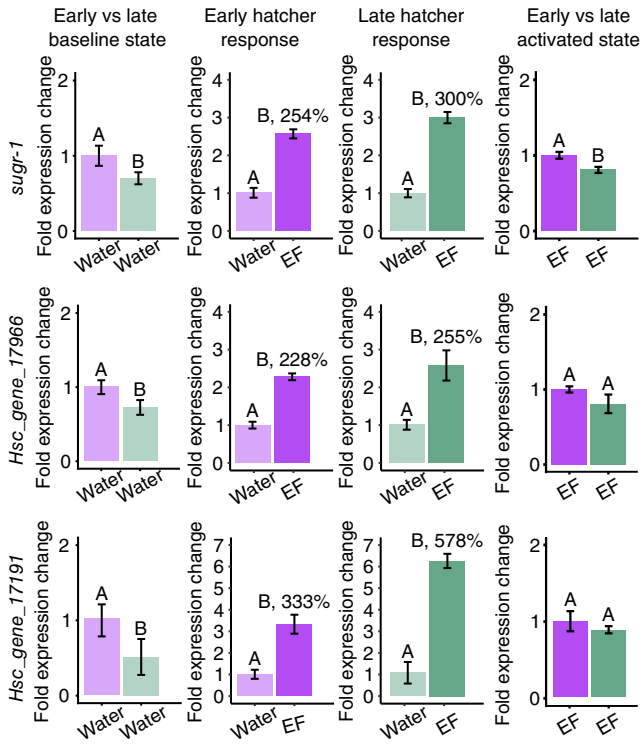


Fig. 2. The response to effectostimulins of transcription factors associated with parasitism gene expression varies with hatching time. First column, baseline expression between early and late hatchers. Second column, gene fold expression changes of early hatchers, normalized to their own baseline expression for that specific gene. Third column, gene fold expression changes of late hatchers, normalized to their own baseline expression for that specific gene. Fourth column, gene fold expression changes of early and late hatchers, normalized to the early hatcher activated state level for that specific gene. Top row, sugr-1; middle row, Hsc_gene_17966; bottom row Hsc_gene_17191. Percentages above bars indicate percentage upregulation from the basal state. Statistical comparisons are within panels only.

two thirds of the effectostimulin activated genes) was modulated by hatching time in some way. All combinations were observed and qualitatively assigned as down with effectostimulins and up with hatching time (cluster 3, $n = 1{,}400$ genes, $n = 47$ effectors), up with effectostimulins and up with hatching time (cluster 4, $n = 1{,}087$ genes, $n = 126$ effectors), up with effectostimulins and down with hatching time (cluster 5, $n = 980$ genes, $n = 79$ effectors), and down with effectostimulins and down with hatching time (cluster 7, $n = 1{,}483$ genes, $n = 35$ effectors). According to hypergeometric tests, all except cluster 3 (down with effectostimulins and up with hatching time) were enriched in effectors. Similarly, these effectors were not enriched for any specific properties, including evolutionary origins, sites of production, time points of maximal expression, and functions (Supplementary Tables S1 to S5). Finally, changes with hatching time only were present in clusters 2 and 6, in which cluster 6 was not enriched in effectors but cluster 2 was. To reconcile these observations with the principal component analysis (PCA), we surmised that although effectostimulin treatment had a larger overall effect, the effect itself was modulated in large part by hatching time. This is a remarkable contribution of an unstudied aspect of cyst nematode biology and may indicate that various other aspects of infection biology are similarly modulated by hatching time. In summary, the expression of most effectostimulin-responsive genes is modulated by hatching time, albeit without a clear dominant regulatory pattern, as all possible combinations of interactions were observed.

To understand what other aspects of parasite biology may be under the control of hatching time, we measured the morphology and physiology of early and late hatchers. Morphology was estimated from a random sample of at least 30 J2 nematodes for each harvest, before and after storage. For all harvests, nematode body length was measured, whereas the so-called "a, b, c's" of nematode morphology (Loof 1971; a = body length/width, b = body length/esophagus length, and c = body length/tail length) were measured only for early and late hatchers. In total, more than 600 nematodes were phenotyped. Surprisingly, and somewhat peripheral to the main thrust of this study, there appeared to be an increase in length after storage (Fig. 4; Supplementary Fig. S2). Lengths in aggregate were found to be significantly different between harvest and storage (Supplementary Figs. S2 and S3). We examined whether this trend appeared in early and late hatchers. Early hatchers increased in length after storage (Dunn's test, $P = 0.0004$), as did late hatchers (Dunn's test, $P = 0.03$), but were not significantly different in length from one another either at harvest or after storage (Dunn's test, $P > 0.05$ in both cases). Using PCA, these differences could be contextualized as similar in magnitude to previously reported intraspecific differences but less than previously reported interspecific differences (Supplementary Fig. S2).

Most importantly, we observed characteristic signs of differential resource expenditure between early and late hatchers (Fig. 4). Early and late hatchers did not hatch with visibly significantly different lipid reserves (Fig. 4; Dunn's test, $P = 0.50$).

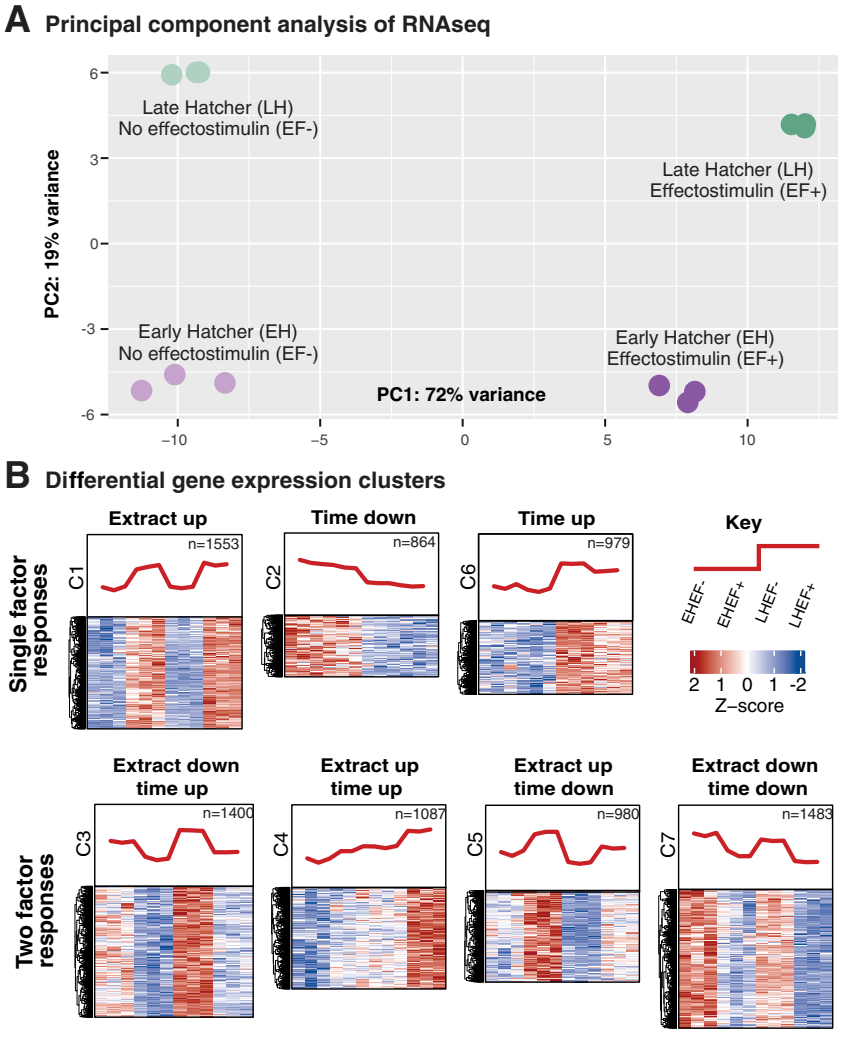


Fig. 3. Global gene expression of early and late hatchers exposed to either effectostimulins or water. **A,** Principal component analysis of whole-genome RNA sequencing following the effectostimulin or water exposure of early and late hatchers. PC1 explains 72% of the variance in gene expression and corresponds to effectostimulin exposure; PC2 explains 19% of the variance and corresponds to the time of hatching. **B,** Clustering of differentially expressed genes according to their responses to effectostimulin exposure and time of hatching. EH EF–, early hatchers incubated in water; EH EF+, early hatchers incubated in effectostimulins; LH EF–, late hatchers incubated in water; LH EF+, late hatchers incubated in effectostimulins.

Late hatchers appeared to use a significant amount of body fat in storage (Dunn's test, $P < 0.0001$), whereas early hatchers appeared not to (Dunn's test, $P = 0.54$). Late hatchers also appeared to lose fat significantly faster than early hatchers (Dunn's test, $P = 0.002$). Taken together, these data suggest that late hatchers may utilize more of their internal energy reserves than early hatchers under these conditions.

Consistent with this, cluster 3 (down with effectostimulins and up with hatching time) and, perhaps more importantly, cluster 6 (up with hatching time) were the only clusters enriched in Gene Ontology (GO) terms associated with lipid metabolism/catabolism: cluster 3: lipid catabolic process (GO:0016042; lipid breakdown, lipid catabolism, lipid degradation, lipolysis, multicellular organism lipid catabolic process, multicellular organismal lipid catabolic process; $P = 0.03$) and lipid metabolic process (GO:0006629; lipid metabolic process; $P = 0.04$); cluster 6: lipid metabolic process (GO:0006629; lipid metabolic process; $P = 0.007$), cellular lipid metabolic process (GO:0044255; cellular lipid metabolism, obsolete cellular lipid metabolic process; $P = 0.01$), lipid biosynthetic process (GO:0008610; lipid biosynthetic process, lipid anabolism, lipid biosynthesis, lipid formation, lipid synthesis, lipogenesis; $P = 0.02$), catabolic process (GO:0009056; catabolic process, breakdown of chemical, breakdown of molecule, breakdown of substance, catabolism, cellular breakdown, cellular catabolism, cellular degradation, degradation; $P = 0.02$), and organic substance catabolic process (GO:1901575; obsolete organic substance catabolic process, organic molecular entity catabolic process, organic substance breakdown, organic substance catabolism, organic substance degradation; $P = 0.01$).

Infectivity and gene expression differences could not be explained by morphology or physiology, so we used RNA sequencing reads to examine whether early and late hatchers were genetically distinct subpopulations. High-quality (minimum QUAL score of 10,000 and no missing data in any samples) single-nucleotide polymorphisms (SNPs) of non–differentially expressed genes were selected (to avoid artifacts introduced by very low and very high read abundance), and we calculated the fixation index (F_{ST}) using 65,057 SNPs in cDNAs (i.e., coding and untranslated regions). A mean F_{ST} value of 1 indicates complete genetic difference, whereas a genetic difference of 0 indicates complete sharing of alleles between populations. We compared the F_{ST} between effectostimulin- and water-treated J2s of the same time point (Fig. 1B), which were absolutely derived from the same pool, as an internal control. We found that genetic differences were extremely low between any pair of samples (F_{ST} ranged between 0.00015 and 0.0003) but ever so slightly higher between those we knew were two halves of one and the same sample (water versus effectostimulin treatment) than those for which we did not know (early versus late). We also sought to determine whether a small number of very large differences may be masked by the genome-wide analyses by identifying the number of sites with $F_{ST} > 0.25$ (Fig. 5B). Here, we still identified no consistent differences between early and late hatchers (indeed, there were still more differences between those we knew were the same population). Together, we concluded that there were no substantive genetic differences between early and late hatchers in their cDNAs of non-differentially expressed genes. By this metric, they are representative of a single population, despite the observed differences in infection, gene expression, and physiology.

Discussion

Here, we found that time of hatching significantly impacts the infection biology, physiology, and host effectostimulin responsiveness of *H. schachtii*. Late hatchers invade plant roots more

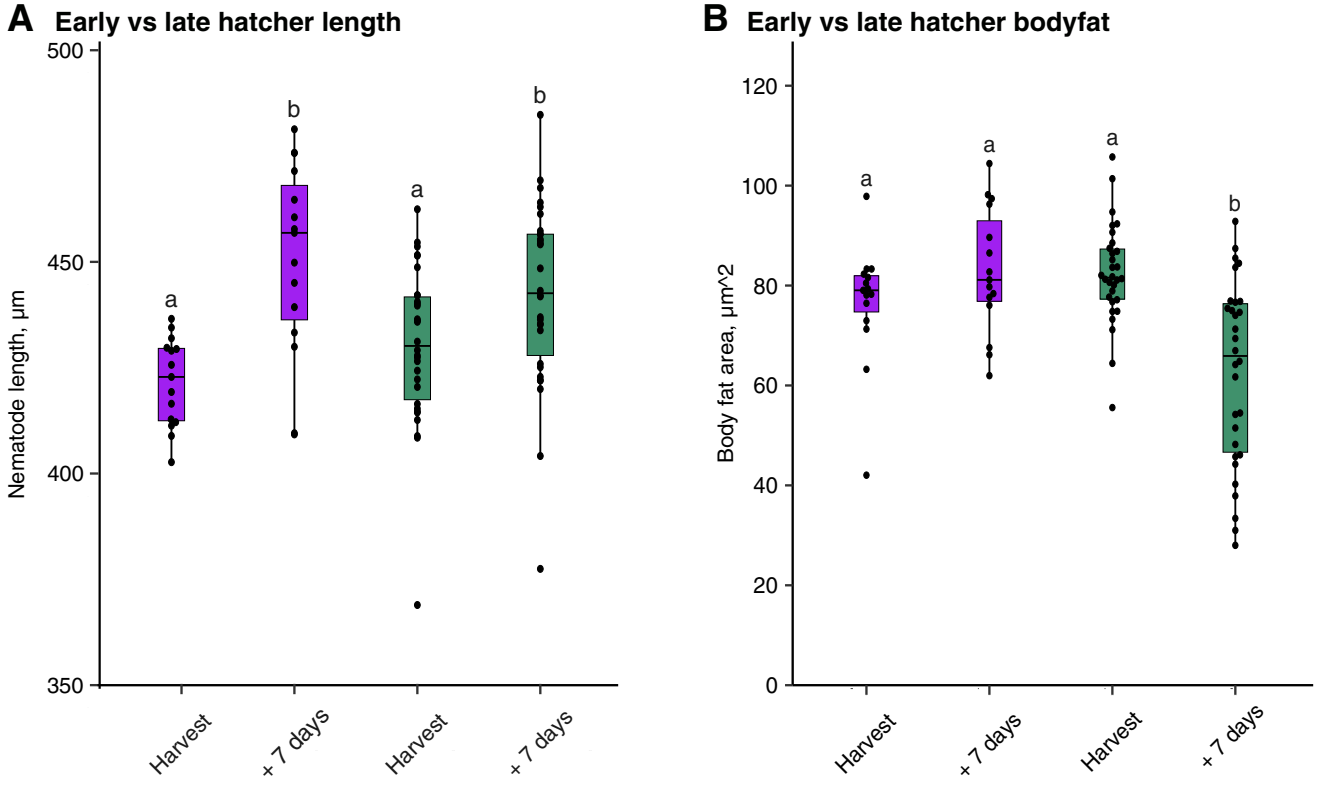


Fig. 4. Early and late hatchers exhibit significant physiological differences. **A,** Early and late hatchers' length at harvest and after 7 days of storage. **B,** Body fat area for early and late hatchers at harvest and after 7 days of storage. Early hatchers are in purple, and late hatchers are in green. Time points with the same letter are not statistically significantly different at $P < 0.05$.

successfully, mobilize more of their lipid stores, and respond differentially to host-derived effectostimulins.

Mechanisms for the observed differences

It is interesting to consider whether the mechanism for the observations is nematode-derived or plant-derived: is it that nematodes are more infective or that plants are less responsive to them? The overall weight of evidence (e.g., RNA sequencing and physiology) supports a nematode-derived mechanism, but, of course, these are not necessarily mutually exclusive.

These individual impacts of hatching time are not necessarily causally linked to one another, only to hatching time itself. However, there are intuitive explanations for the interplay between the observations. We have correlated enriched GO terms associated with lipid metabolism in late hatchers with apparent energy reserve depletion but have not shown causation. We hypothesize that this energy is used to boost parasitism gene expression but have not shown that this boosted gene expression increased infectivity. Establishing mechanistic links between these should be the focus of future work, perhaps using manipulations of lipid metabolism and/or RNA interference in the case of differentially regulated genes. However, given the number of individuals needed for experimentation, protocols that require less starting material would strongly benefit such an investigation.

Whatever the case, we found an unexpectedly large contribution of hatching time to three areas of biology: the regulation of energy reserves, the regulation of effectors, and, ultimately, the success of invasion. For example, although there were no gene expression clusters that were effectostimulin-responsive for late hatchers but not for early hatchers (or vice versa), cluster 4 (up with effectostimulins and up with hatching time) was particularly interesting because it showed that the late hatchers' baseline state was already at the early hatchers' stimulated state, and the later hatchers' stimulated state was yet higher. These data show that effector expression is regulated by multiple inputs and, more specifically, that late hatchers have a mechanism to modulate host-derived signaling and certain aspects of parasitism gene expression.

Explanations for the observed differences

If late hatchers are more infective, under what circumstances could this have been favored by evolution? Turner and Subbotin (2013) suggested that the hatching curve is a survival strategy to avoid excessively high parasitic loads that may be fatal to both hosts and nematodes. Indeed, competition is known to reduce resource availability and increase the likelihood of developing into a male (Anjam et al. 2020). Our data suggest that individuals hatching later in this curve are more infective under our conditions. As such, a broad hatching curve may strike a balance between spreading out infection and ensuring nematodes hatch when a root is present and with sufficient time in the growing season that the plant remains alive until they complete their life cycle. Note that these are not mutually exclusive and could each act under different field conditions.

In a "natural" setting, an approaching root/root system secretes hatching stimulants that are perceived by the nematodes in the eggs. It seems plausible that those nematodes that hatch early in response to hatching stimulants will have a net greater distance to travel/time to wait until they reach the root. It is intuitive to consider their slow rate of energy utilization as adaptive in this scenario. On the contrary, the late hatchers emerge 1 week later in response to the same stimulus. It seems equally plausible that these nematodes have a net shorter distance to travel/time to wait, as during the preceding 7 days, the root presumably continued its trajectory. Under these conditions, a nematode that more rapidly utilizes the resources it has seems similarly adaptive.

An alternative interpretation would be one of competition between conspecifics. Early hatchers may be considered to have a head start. Late hatchers may encounter more developed plants that themselves have more resources to defend. They may have to overcome larger, tougher roots and those with primed immunity by the interaction with their earlier-hatching conspecifics. In either case, evolution may have selected late hatchers to be those that maximally invest in parasitism, more quickly utilizing their resources to "catch up," so to speak, with their early-hatching conspecifics. It is widely accepted that the difference in hatching behavior of cyst and root-knot nematodes represents an adaptation to their quite different host environments, host range, and survival strategies. Given that hatching curves are evident even for species with broad host ranges and the capacity to hatch in the absence of stimuli (e.g., *H. schachtii* and many root-knot nematodes), any (dis)benefit seems independent of these variables and likely also occurs with species with narrower ranges (e.g., the carrot cyst nematode). Until tested, this remains an open question. Whether adaptive variation similarly exists between the hatching curves of different cyst nematode species, or even between populations of the same species, also remains to be seen.

Finally, and importantly, there are practical limits to any differences seen. Under much higher inoculation densities than used

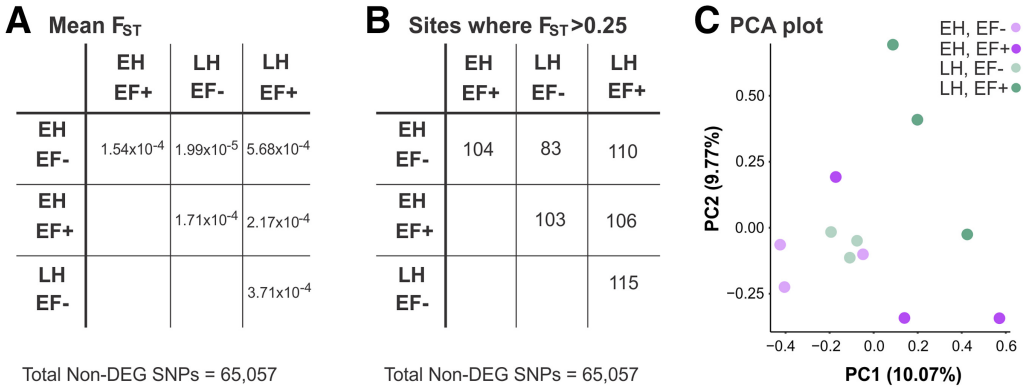


Fig. 5. Early and late hatchers originate from a broader, genetically indistinguishable population. **A,** Mean F_{ST} across all non-differently expressed genes in early and late hatchers exposed to effectostimulins or water. **B,** Number of sites where F_{ST} exceeds 0.25 across all non-differently expressed genes in early and late hatchers exposed to effectostimulins or water. EH EF–, early hatchers incubated in water; EH EF+, early hatchers incubated in effectostimulins; LH EF–, late hatchers incubated in water; LH EF+, late hatchers incubated in effectostimulins. **C,** Principal component analysis of genetic variants in non-differently expressed coding sequences for nematodes following the effectostimulin or water exposure of early and late hatchers. PC1 and 2 each explain approximately 10% of the variance in gene expression, but neither clearly corresponds to the treatments or conditions labeled.

in this study, the limiting factor in nematode reproduction could be the plant itself. Therefore, whether and how late hatchers have an advantage in terms of fitness under those conditions remains to be seen.

The hatching curve as a sum of individual cyst profiles

The hatching curves detailed within this study were characterized based on a pool of *H. schachtii* cysts extracted from approximately 15 kg of sand. The presence of a clear peak followed by a tapering decline could originate from one of two distinctive mechanisms. On the one hand, the hatching curve may represent an aggregated sum of similarly shaped hatching curves of individual cysts or, alternatively, the sum of distinctive hatching curves of cysts within the overall population. In this second scenario, most cysts are likely to be early hatchers, whereas a much smaller proportion are likely to be late hatchers given the overall early skew of the hatching curve's peak. Previous studies—albeit on distantly related cyst nematodes—perhaps support the idea of distinctive hatching profiles of individual cysts. Hatching profiles in populations of 10 *Globodera pallida* or *Globodera rostochiensis* cysts exposed to potato root diffusate from the cultivar 'Desirée' could sometimes be bimodally distributed depending on the temperature of hatching (Kaczmarek 2014). The hatching dynamics of individual cysts collected herein seem to suggest something in between: we found a variety of different shapes, most of which peak early and then decline (with occasional bursts) and some with bimodal distributions. Regardless, the genetic analyses performed here indicate that early and late hatchers do not originate from distinctive populations. Therefore, it is clear that the underlying factors shaping the hatching curve are not genetic. The gathering of these data under exposure to a single hatching stimulant indicates that this is sufficient to highlight large levels of variation in hatching dynamics across individual cysts. It is possible that simultaneous exposure to more varied hatching stimuli in the field may alter variation in the hatching profiles of individual cysts. Indeed, previous research has shown that varying exposure to host exudates may vary the shape of the hatching curve (Zheng and Ferris 1991).

More recently, Fournet et al. (2025) examined two successive hatching stimulations: the first was for 30 days, with a cold period before the second stimulation. They found the second stimulation to be markedly less fit than the first. It would be fascinating to determine whether the hatching curve of each individual stimulation contained the differences we observed within one exposure.

In both these cases, more complex hatching stimulants were used, and intriguing variation in hatching curves or biology was observed. Taken together with this study, the weight of evidence suggests that hatching dynamics are ecologically and biologically relevant aspects of plant-parasitic nematode biology that warrant deeper investigation.

Root cause of the observed differences

Regardless of whether and how the individual impacts of hatching time described here are causally linked to one another, they are causally linked to hatching time—but how? There are two categories of explanations for the observed differences in hatching time: (i) a slower response to hatching stimulus and (ii) the response being the same, but the time to hatch from the egg/cyst taking longer. There could be several causes for each (e.g., position of the egg within the cyst, space within the cyst, position and width of the cyst's body openings, larval migration within and out of the cyst, toughness of the egg shell, etc.). Although these factors present an intriguing deficit that the nematode must compensate for accordingly, they likely would themselves lead to depletion of the energy reserves, as well as stress. Given that we found parity of energy reserves at the time of J2 emergence, we favor the former over the latter, but note that they are not mutually exclusive. Future experiments with isolated eggs would help resolve this. Nevertheless, three not-mutually-exclusive causes are offered: a very small number of highly impactful genetic differences, any number of epigenetic differences, and/or differences in the maternal dowry.

In principle, cyst populations contain genetic diversity. Moreover, within individual cysts, recent work on *Globodera* by Esquibet et al. (2026) suggests that most if not all females are polyandrous (with an average of seven male contributors). Even though early and late hatchers were genetically indistinguishable in this study, we cannot rule out that the responsible changes were in genes which themselves were responsive (in which case they were filtered out) but unlinked with genes harboring the 65,057 SNPs we measured. Perhaps more plausible, but nevertheless unlikely, a very small number (i.e., one or two) of highly impactful genetic differences could drive the observations, masked by the overwhelming genetic similarity of the samples even when we filtered for high F_{ST}. In *G. rostochiensis*, there are three classes of calcium-binding sites on its eggshell (Perry et al. 2018). Crucially, one of these classes is thought to respond to hatching factors and induce hatching. After hatching is induced, juveniles rehydrate, become metabolically active, and exit eggs. Genetic differences in eggshell-based receptors, or as-yet-uncharacterized downstream signaling pathways, may underlie some of the differences in hatching, but it is not obvious whether and how these differences would also explain the later observations. That is, a very small number of very impactful genetic differences would need to explain two—as far as we are aware—unrelated phenomena: that the nematodes hatched late and those observed differences that followed. This restriction to a small number of unlinked and impactful genetic differences, as is necessary given the absence of global differences between the populations, makes this an unfavorable hypothesis.

Alternatively, so-called epigenetic differences (e.g., histone modifications and small RNAs) would not face the same restriction. Any number could in principle be used to explain the observations. Whereas DNA methylation is contentious in plant-parasitic nematodes, histone methylation and small RNA signaling are well established (Hassanaly-Goulamhoussen et al. 2021; Liu et al. 2023). This raises the question of when and why these differences would be deposited in this way. Given that we cannot measure epigenetic signatures with the current dataset, a logical future direction would be to measure whether there are epigenetic differences across the hatching curve, before exploring this further.

Third, there may be differences in the "maternal dowry" of eggs. The egg of a nematode contains perivitelline fluid as a rich store of nutrients provided by the mother to each developing embryo (Mkandawire et al. 2022). Differences in dowry between, or within, mothers could contribute. First, the hatching curve of a population is evidently the sum of many smaller curves of individual cysts. If cysts differ in dowry, that may contribute to whether an egg preferentially hatches early or late. Alternatively, or in addition, given that egg production by the female begins while she is still growing, it is possible that the earliest eggs—which may preferentially correspond to early hatchers, late hatchers, or neither—receive different perivitelline fluid compositions. Given that early and late hatchers did not have observable differences in body fat composition, any differences in the maternal dowry would need to be in types, rather than amount, of nutrition. It is intuitive to consider how this would correspond to a faster depletion of resources (and the observed infection and gene expression differences are intuitive to derive from that). However, why this would correspond to late hatching is unclear, and so this hypothesis, and others contained herein, should be regarded as speculative.

Finally, it is possible for environmental factors to affect hatching. Previous studies show that temperature influences the hatching dynamics of the potato cyst nematode *G. rostochiensis*. At 13 and 15°C, hatching curves characterized based on 10 cysts showed a bimodal distribution. Contrastingly, hatching followed a more characteristic peak and subsequent decline at 17 and 21°C (Kaczmarek 2014). Similarly, the shape of the *H. avenae* hatching curve characterized from 50 cysts responded to temperature (Jing et al. 2014). These cases highlight the influence of abiotic ecological factors on cyst nematode hatching curves. Further research into how abiotic and biotic factors (including egg physiology, maternal dowry, and epigenetics) interact to influence egg hatching may therefore be informative.

Taken together, we speculate that the unexpectedly large contribution of hatching time to three areas of biology—the regulation of energy reserves, the regulation of effectors, and, ultimately, parasitism—is likely driven by a combination of factors. It will be interesting to explore what other aspects of nematode biology are different between early and late hatchers and whether the extremes are yet more different, whether plants respond differently to each group, and whether the phenomenon is more broadly applicable to other populations, species (e.g., cereal cyst nematodes), and pathosystems.

Materials and Methods

Cyst collection and hatching

H. schachtii cysts (obtained from the Instituut voor Rationele Suikerproductie, the Netherlands) were harvested from approximately 15 kg of infected sand using nested sieves (4,000, 2,000, 500, 125, and 63 μm). Cysts were collected from the 125 and 63 meshes. Cysts were then transferred onto a single layer of Miracloth (Merck), which was secured within a single large glass hatching jar (Pyrex) by a plastic ring. Cysts were covered with 75 ml of a 3 mM zinc chloride solution at 21°C. Hatched nematodes were collected every 24 h for 21 days, and each harvest was stored separately in Nemawash (0.01% vol/vol Tween 20) at 4°C. Collection efficiency was improved by washing the Miracloth at each harvest using 3 mM zinc chloride in a customized wash bottle (Azlon). After each collection, cysts were again covered with 75 ml of a 3 mM zinc chloride solution and incubated at 21°C.

Counting and imaging nematodes

Nematodes were collected by centrifugation (1,500 × *g*), and the supernatant was replaced with Nemawash three times. An aliquot of nematodes was diluted in Nemawash, and the total nematode number and number of motile nematodes was counted across five or six 80-μl drops using a stereomicroscope (Leica S9D) to provide estimates for that harvest. A nematode was considered motile if it moved during the counting process. Immediately after counting, 1 ml of a diluted nematode solution was added to a nematode counting slide with a viewing area of 18 × 28 mm (Astel BTU). Nematodes were bright-field imaged at 20× magnification on an inverted microscope (Oxion). Images were taken using autoexposure on a GXCAM HiChrome-HR4 (GTVision). A minimum of 30 nematodes were imaged per time point. The remaining undiluted nematode solution was stored at 4°C for 7 days in sealed 50-ml tubes (Fisher Scientific). Nematodes were then allowed to stand at room temperature for 2 h before recounting and re-imaging as described above.

Analyzing nematode morphology

Nematode morphology was measured using FIJI (v.2.16.0/1.54p and build 26d66057dd) (Schindelin et al. 2012). We recorded morphology data for each harvest on the day of harvest and after 7 days of storage at 4°C. Blurry nematode images were identified visually and discarded. Clear images were measured with a scale of 485 pixels = 100 μm. Fifteen nematodes per time point were analyzed for body length, body width, and body fat area (μm^2). Body fat area and intestinal regions devoid of fat were visually identified and measured using the segmented line tool of FIJI. For day 4 and days 9 and 10, a = body length/width, b = body length/esophagus length, and c = body length/tail length were also recorded (Loof 1971).

A PCA was performed using a, b, c, and nematode length. Morphology data were first normalized in R using the NormalizeTPM function of ADImpute (Leote 2025). The PCA was constructed using the standard PCA implemented in the prcomp() function in DESeq2 (Love et al. 2014). All external morphology data were found in Subbotin et al. (2010). The additional *Heterodera* spp. selected were chosen to represent a cross-section of the *Heterodera* genus. Species groupings were manually drawn in Inkscape.

Treatment with effectostimulins and water

Effectostimulin extraction, J2 incubation, RNA extraction, and qPCR were carried out according to the method outlined in Pellegrin et al. (2025). In brief, an effectostimulin-containing root extract was prepared from *Sinapis alba* (cultivar Albatros). J2s were then incubated in either ultra-pure water or effectostimulins for 4 h (approximately 15,000 per replicate, per treatment) before flash-freezing alongside two 4-mm stainless steel bearings (Thermo Fisher Scientific) in liquid nitrogen.

RNA extraction and qRT-PCR

H. schachtii J2s were ground to powder in a Geno/Grinder 2010 (Spex Sample Prep), and RNA was extracted according to the manufacturer's instruction in the RNeasy Plant Mini Kit (Qiagen). cDNA was synthesized from 400 ng of RNA using SuperScript IV (Thermo Fisher). qRT-PCR was then performed in triplicate using LUNA Universal qPCR Master Mix (NEB) and 1 μl of cDNA. qPCR data were normalized using the Pfaffl method against two reference genes (Hsc_gene_6993 and Hsc_gene_2491) (Pfaffl 2001). Assumptions of normality and homogeneity of variances were checked visually and using Shapiro–Wilk and Levene's tests (Levene 1960; Shapiro and Wilk 1965). All qPCR measurements were taken on distinct samples. The same RNA samples were sent for library preparation and 150-bp paired-end Illumina sequencing using the service provided by Novogene.

Infection assay

Determining inoculation days. Each analysis required 184,000 J2s. The exact hatching curve for this population was unknown, meaning that time points could not be preselected. It was therefore necessary to have plants available for each time point and give all nematodes the same 7-day storage period. Day 4 was eventually selected to capture the variation at the peak of the curve, and days 9 and 10 were selected as the latest period of the hatching curve with sufficient J2s to run the downstream experiments.

Growing plants. Every 24 h, 100 *S. alba* seeds (cultivar Albatros) in individual 15-cm seed germination pouches (Mega International) were placed in one of two 24-liter boxes (Really Useful Boxes) on a 16-h light/8-h dark cycle at 21/20°C in a growth chamber under white, fluorescent light (approximately 60 μmol m^{-2} s^{-1}, light level 3) (PHCBI). Plants were placed in each box on alternating days to coincide with nutrient solution changeovers, reducing waste. Seed pouches were bundled into groups of 10 using 77-mm round, wavy paperclips (QConnect). Hoagland's P10 nutrient solution (10 mM phosphate) was replenished three times per week or whenever new pouches were added to the box (Hoagland and Arnon 1950). All plants were

exactly 14 days old at inoculation, with exactly 7-day-old nematodes. Plants and nematodes for unused time points were disposed of.

Inoculation and staining. More plants were allocated to analysis at 16 dpi than at 5 dpi because, in our experience, nematode establishment is more variable than host invasion. A new random sample of nematodes were recounted and re-imaged after 7 days of storage. The inoculum density was adjusted to 250 J2s/500 µl in Nemawash. Pouches were cut open, and the excess water was removed from the plant roots using absorbent paper. Afterward, the inoculum was carefully spread on the root system. Pouches were then closed, stacked, and placed horizontally in a closed box maintained in the same growing conditions. After 24 h, plants were placed vertically in a box containing 2 liters of Hoagland's P10 solution, which was changed three times weekly to minimize algal growth.

At evaluation at either 5 or 16 dpi, plants were removed from pouches and stained using acid fuchsin according to Byrd et al. (1983). Stained nematodes were counted using a stereomicroscope (Leica Stereomicroscope S9D). The number of nematodes and their relative stages were recorded. Here, nematodes recorded at 5 and 16 dpi were used to estimate invasion and establishment rates, respectively. All measurements were taken on distinct samples.

Data analysis.

RNA sequencing data processing. Raw paired-end RNA sequencing reads were quality-filtered using Trimmomatic (version 0.39; Bolger et al. 2014) with the following parameters: LEADING:20, TRAILING:20, SLIDINGWINDOW:4:20, and MINLEN:60. This removed low-quality bases from the read ends and discarded reads shorter than 60 bp after trimming using BBduk. Trimmed reads were aligned to the reference genome of *H. schachtii* (PRJNA522950.WBPS19) using STAR (version 2.7.11) in two-pass mode with parameters optimized for both gene expression quantification and variant calling. Alignments were output as coordinate-sorted BAM files with read group information included via the '—outSAMattrRGline' parameter. Alignment quality metrics were assessed using mapinsights bamqc (version 1.0) and QualiMap (version 2.3).

Read counts were generated using htseq-count (version 2.0.9) at the transcript level. Expression values in fragments per kilobase of transcript per million mapped reads and transcripts per million were calculated using StringTie (version 3.0.0) with the -e flag to estimate abundances only for annotated transcripts.

Raw count matrices were assembled for pairwise comparisons between conditions using custom bash scripts. Differential expression analysis was performed using DESeq2 (version 1.42.1; Love et al. 2014) in R (version 4.3.2). Genes with fewer than five counts in at least three samples were filtered prior to analysis. The reference level for each comparison was explicitly set using the relevel function. Differentially expressed genes were identified using an adjusted *P* value cutoff of 0.05 (Benjamini–Hochberg correction). Four pairwise comparisons were conducted: D4EF versus D4W, D9EF versus D9W, D9W versus D4W, and D9EF versus D4EF.

Expression clustering was performed using the ClusterGVis package (version 0.1.1) in R (version 4.2.3). Transcripts per million–normalized expression values across all 12 samples were used as input. The optimal number of clusters was determined using the getClusters function. Fuzzy c-means clustering (mfuzz method) was performed with 7 clusters over 200 iterations, applying a minimum standard deviation threshold of 0.2 to remove genes with low expression variation across samples. Genes with membership scores greater than 0.8 were retained for final cluster assignments. Cluster visualizations were generated using the visCluster function with both line plots and heatmaps.

Genetic relationship analysis. Analyzed RNA sequencing data were additionally used to investigate whether early and late hatchers could be traced to distinctive populations. For these analyses, alignments were reprocessed (adding read groups, etc.) to meet GATK best practices for RNA sequencing variant discovery. The HaplotypeCaller function in GATK (version 4.5.0) was used to call the per-sample gVCF. The 12 individual gVCF files were combined using GATK CombineGVCFs, and joint genotyping was performed using GATK GenotypeGVCFs to produce a multi-sample VCF file. High-quality biallelic SNPs were filtered using VCFtools (version 0.1.18). These SNPs were further filtered in a way such that SNPs in genes that were non-differentially expressed in any of the comparisons (D4EF_vs_D4W, D9EF_vs_D9W, D9W_D4W, or D9EF_vs_D4EF) were selected for genetic distance and population relationship analyses. Pairwise genetic distances between samples were calculated from the filtered SNP matrix using VCF2Dis (version 1.54). Phylogenetic trees were constructed based on genetic distance matrices and visualized using standard tree visualization software.

Population structure was assessed using PCA on the filtered SNP dataset. Total number of unfiltered SNPs was 287,430. The filtering criteria were minQ of 10,000, no missing data, and non-differentially expressed genes. After filtering, 65,057 SNPs were kept for PCA and F_{ST}. PCA clustering was performed using VCF2PCACluster (version 1.41), with sample group information provided as input. PC1 versus PC2 scatter plots were generated to visualize genetic relationships among samples.

Population differentiation was quantified using Weir and Cockerham's F_{ST} statistic, calculated with VCFtools --weir-fst-pop for pairwise population comparisons. SNPs with $F_{ST} > 0.25$ were classified as highly differentiated. To test for nonrandom distribution of high-F_{ST} SNPs across the genome, hypergeometric tests were performed for each scaffold using a custom Python script. The hypergeometric test assessed whether the number of high-F_{ST} SNPs in each scaffold was significantly enriched compared with the genome-wide expectation.

Detailed analysis and in-house scripts are available at https://github.com/chongjing/RNAseq_Hschachtii.

Statistics

Statistical significance was determined using Kruskal–Wallis tests, post-hoc Dunn's tests using Bonferroni's correction, and Welch's *t* tests with FDR corrections. Tests were two-tailed, with alpha = 0.05. Normality was determined using Shapiro–Wilk tests. All analyses were conducted in R (version 4.2.1). All plots were generated using RStudio (v.2025.05.0+496), R (v.4.2.1), and ggplot2.

For all box plots, thick black lines indicate the median; box limits indicate the 25th and 75th percentiles; whiskers extend no more than 1.5 times the interquartile range to either the largest or smallest value. Data more than 1.5× the interquartile range were considered outliers. Any statistical analyses were conducted separately and added via manual annotation.

Data availability

All raw RNA sequencing reads generated in this study have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under project accession PRJEB114114 (https://www.ebi.ac.uk/ena/browser/view/PRJEB114114), and sample accessions are from ERR17359748 to ERR17359759. In-house scripts are available from https://github.com/chongjing/RNAseq_Hschachtii.

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