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Timers, variability, and body-wide coordination: *C. elegans* as a model system for whole-animal developmental timing

Gouri Patil and Jeroen S van Zon

Successful development requires both precise timing of cellular processes, such as division and differentiation, and tight coordination of timing between tissues and organs. Yet, how time information is encoded with high precision and synchronized between tissues, despite inherent molecular noise, is unsolved. Here, we propose the nematode *C. elegans* as a unique model system for studying body-wide control of developmental timing. Recent studies combining genetics, quantitative analysis, and simulations have 1) mapped core timers controlling larval development, indicating temporal gradients as an underlying mechanism, and 2) elucidated general principles that make timing insensitive to inherent fluctuations and variation in environmental conditions. As the molecular regulators of *C. elegans* developmental timing are broadly conserved, these mechanisms likely apply also to higher organisms.

Address
AMOLF, Amsterdam, the Netherlands

Corresponding author: van Zon, Jeroen S (j.v.zon@amolf.nl)

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Introduction

For successful development, cells must divide, migrate, and differentiate with proper timing and in correct order to generate tissues and organs without error. It also requires strong coordination of development *between* different tissues and organs, as progression of development in one tissue often relies on timely completion of development in adjacent tissues. For example, our heart and lungs develop in parallel with striking coordination, to ensure their flawless connection into a single circulatory system, demanding precise control also of *relative* timing. Finally, most animals exhibit developmental transitions that require strong *body-wide* synchronization of development, such as puberty in humans. Typically, such transitions occur during post-embryonic development, that is, after birth or hatching, where, in contrast to embryonic development, progression relies on active feeding and timing must therefore be flexible and dependent on environmental conditions. Yet, how cells measure time to execute events at the correct developmental stage and how timing is synchronized throughout the body remain fundamental, unresolved problems.

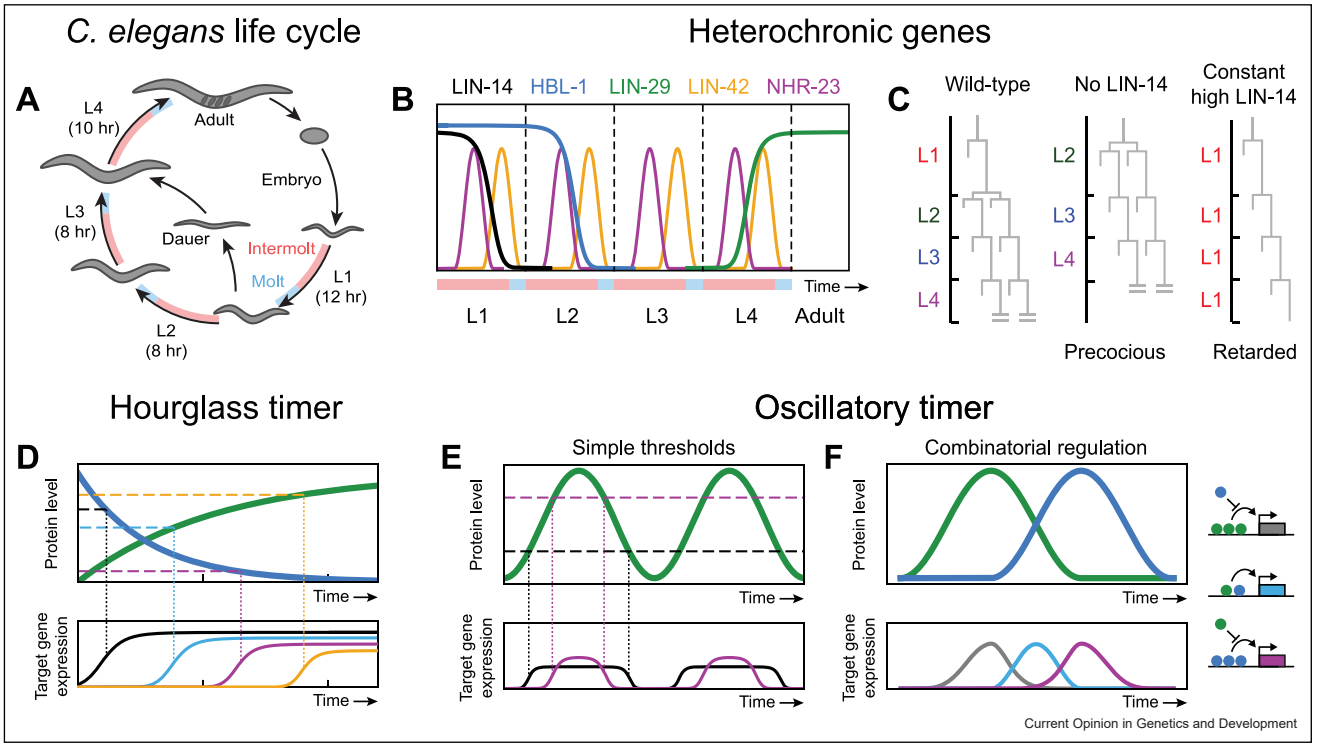
Coordination of developmental timing plays out at fundamentally different levels. While it is most readily apparent at the level of organs and tissues, it is determined by the dynamics of their constituent cells, in terms of cell division, gene expression, and differentiation. Cell dynamics, in turn, is dictated on the molecular level, through gene regulatory networks. Understanding the mechanisms that control developmental timing thus requires connecting these different levels. In practice, this represents a formidable experimental challenge, as development on the organ- or body-level typically unfolds on length and timescales much larger and slower than that of the cells or molecular regulators. Here, we propose the simple nematode worm *C. elegans* as a model system uniquely suited to address developmental timing questions spanning from molecules to the body.

We first outline key aspects of *C. elegans* biology that make it exceedingly well-suited to study developmental timing. Subsequently, we review the current understanding of timer mechanisms in *C. elegans* development, focusing on those that control its postembryonic development. In this context, we discuss the concept of 'temporal gradients': transcription factors (TFs) that encode time through dynamically varying protein levels. Finally, we discuss how both molecular noise and varying environmental conditions can degrade time information encoded by such timers and consider possible mechanisms that act in *C. elegans* to maintain timing precision and synchrony, despite noise.

C. elegans as a model for developmental timing

The nematode worm *C. elegans* is an anatomically simple animal that develops from a fertilized oocyte into a 959-cell adult in 2–3 days [1]. It develops with precisely controlled timing, with an ~12 h embryogenesis, when its body plan is

Figure 1



Temporal organization and potential timer mechanisms in *C. elegans* larval development. **(a)** *C. elegans* life cycle. Times indicate larval-stage durations at 22 °C. Each larval stage is divided into an intermolt, when the animal feeds, and a molt, when its external cuticle is exchanged. **(b)** Schematic overview of protein-level dynamics of selected heterochronic genes during larval development. **(c)** Impact of heterochronic mutations on cell dynamics. Cell lineages of the V(1–4) seam cells, which are skin stem cells, are shown as an example [4]. Each larval stage has a specific seam cell division pattern. Without LIN-14, the L1-stage-specific division is skipped (precocious mutant), while the L1-stage-specific division is repeated at each larval stage when LIN-14 remains constitutively high (retarded mutant). **(d)–(f)** Different implementations of temporal gradient-based timers. Dynamically changing TF levels (upper panels) control precisely timed expression of target genes (lower panels) by inducing expression only at specific thresholds (dashed lines). **(d)** Hourglass timer, where TF levels proceed unidirectionally to steady state. **(e)**, **(f)** Oscillatory timer, where TF levels peak cyclically. Here, combinatorial regulation of target gene expression by multiple TFs can generate ordered sequences of gene expression **(f)**, while simple thresholds **(e)** cannot. Curves in **(f)** represent model calculations that are provided as supplementary material.

established, followed by four postembryonic larval stages (L1–L4, 8–12 h each) (Figure 1a). These larval stages have a cyclical character, each consisting of a period of feeding and growth (intermolt) followed by a lethargus when the animal's cuticle is replaced (molt). Larvae can also enter an alternative L3 stage called dauer, a highly stress-resistant state entered only under unfavorable environmental conditions. The larval cycle is accompanied by a flurry of development specific to each larval stage, including, for example, formation of the entire reproductive machinery [2]. Strikingly, this occurs through an almost completely invariant program of cell divisions and movements, with the cell lineage from embryo to adult fully mapped [2,3].

Owing to this invariant cell lineage, almost every precursor cell can be unambiguously identified in each animal, and the timing of its dynamics and that of its offspring measured and compared, enabling the detection even of subtle timing changes in mutant animals. Consequently, *C. elegans* is unique among model organisms in that, following decades of research, many genes were identified that control developmental timing. These so-called heterochronic genes [4], discussed further below, act in most tissues, while mutations perturb timing of diverse cellular processes, including division [4], gene expression [5], and differentiation [6,7] (Figure 1b,c). Some heterochronic genes are expressed only during specific larval stages, while others are oscillatory, with expression peaking once every larval stage [8,9].

Recent technical breakthroughs now also allow studying *C. elegans* developmental timing in unprecedented quantitative detail. RNA-sequencing techniques that quantify expression dynamics of all genes during larval development, in whole animals [10–12] or individual tissues [5], revealed many genes with complex expression dynamics functionally linked to developmental timing. CRISPR/Cas9 gene editing enables endogenous fluorescent labeling of mRNA and proteins, to simultaneously visualize the dynamics of timing regulators and their downstream targets [13]. Finally, advancements in microfabrication and microfluidics

enable imaging of cell-level events, such as cell division and gene expression, even within individual, freely moving, and growing larvae, for the entire development from hatchling to adult [14–16].

Measuring time through temporal gradients

Active timing control mechanisms have been proposed that function like molecular timers [17,18]. In general, these mechanisms rely on tightly controlled temporal dynamics of developmental regulators, typically TFs, that induce downstream gene expression and development only at specific thresholds. Such mechanisms are called 'temporal gradients' [19], as they pattern events in time analogous to how morphogen gradients pattern spatial cell fate [20]. A temporal gradient can induce multiple developmental steps with well-defined order, with relative timing of each step given by the timescale of TF dynamics and its specific threshold (Figure 1d–f). While any protein-level dynamics can act as atemporal gradient, two specific classes are most often considered: 'hourglass' timers and oscillators [18]. Hourglass timers refer to molecular processes that, once initiated, proceed unidirectionally to a steady state, for example, simple constant TF accumulation or degradation (Figure 1d). In contrast, oscillators exhibit TF levels that peak in a self-sustained, cyclical manner (Figure 1e,f). Interestingly, there is evidence for both timer classes acting in *C. elegans* larval development.

Hourglass timers: heterochronic pathway

Multiple heterochronic genes show hallmarks of hourglass timers, accumulating or decreasing in abundance in strikingly larval-stage-specific manner (Figure 1b). Central are three heterochronic TFs: LIN-14, decreasing during the L1 larval stage [21], HBL-1, decreasing during the L2 stage [22,23], and LIN-29, accumulating in the L4 stage [24]. Other heterochronic genes with similar expression dynamics include miRNAs and RNA-binding proteins, such as *lin-4*, *let-7*, *lin-28*, and *lin-41*, that regulate LIN-14, HBL-1, and LIN-29 dynamics [9]. Collectively, these genes are referred to as the heterochronic pathway. Mutations to these heterochronic genes often cause developmental steps to be skipped or repeated in time, linked to changes in their dynamics. For example, seam cells divide to generate skin cells with a division pattern that is larval-stage-specific. Mutations that cause constitutively high LIN-14 yield seam cells repeating their L1-specific divisions at each larval stage, while LIN-14 loss causes skipping of L1 divisions [4] (Figure 1c). This yielded a model where the presence of LIN-14 induces L1-specific events, while its absence allows L2-specific events to occur. Similarly, HBL-1 and LIN-29 regulate events in the L2–L3 larval stages and the L4-to-adulthood transition, respectively. A key question that has remained unanswered is exactly what timing information is encoded in heterochronic TF dynamics. Typically, they are thought to only provide a sense of temporal identity, meaning that the heterochronic TF presence determines what event type (e.g. a L1-specific seam cell division) occurs, but not the time when the process itself (cell division) initiates. However, a model of heterochronic TFs acting like hourglass timers remains a plausible possibility. In particular, their dynamics appears gradual, not sudden. For example, LIN-14 protein and mRNA levels decrease over the entire L1 larval stage [21], implying that L1 substages can be differentiated based on their distinct LIN-14 levels. Heterochronic TFs directly activate or inhibit downstream gene expression by binding conserved binding sites in target gene promoters [1,5,25]. When combined with gradual TF dynamics, variation in TF-binding affinity between these sites would suffice to activate or repress targets with different timing, just as morphogen-binding affinity explains spatial patterning of morphogen targets [26]. While in most cases direct heterochronic TF targets remain unknown, recent work identified multiple genes with neuronal function as direct LIN-14 targets, with decreasing LIN-14 levels inducing gene expression changes responsible for striking differences in animal behavior between larval stages [5]. However, this work left open whether all gene expression changes occurred simultaneously at the L1–L2 larval transition, or if induction occurred at times that varied systematically between targets, as expected for a temporal gradient mechanism. It will be important to dissect this further in the future.

Oscillatory timers: molting cycle

RNA-sequencing approaches identified widespread gene expression oscillations that occur in steps with the molting cycle, with each gene's expression peaking only at a specific phase, that is, a specific fraction of each larval stage [10–12]. These molting cycle oscillations occur in most tissues and involve broad gene classes [12]. While some genes are linked to molting and cuticle synthesis [12,27], many genes peak in the intermolt, indicating a broader function than only executing the molt. The core mechanism generating these oscillations remains unknown. Key regulators have been identified that, when mutated, display heterochronic phenotypes, perturbing larval-stage durations and molt timing. These include *lin-42* [28,29], nuclear hormone receptors such as *nhr-23* [30], and the miRNA *let-7* [31]. Interestingly, most of these regulators themselves show oscillatory levels [32–34]. The existence of *let-7* mutants exhibiting more than four molts [31,35] strongly suggested an underlying oscillator, that in wild-type animals, arrests in adulthood. Oscillator arrest might occur through a mutual feedback loop between *let-7* and *nhr-23* [31]: in this scenario, each subsequent NHR-23 peak induces more *let-7* expression, which in turn inhibits NHR-23

oscillations and, hence, the molting cycle, once accumulated to sufficient levels in adulthood. Finally, a recent screen identified a small set of TFs that, when absent, change molt number and timing [30], suggesting that the core oscillator network might soon be known [36].

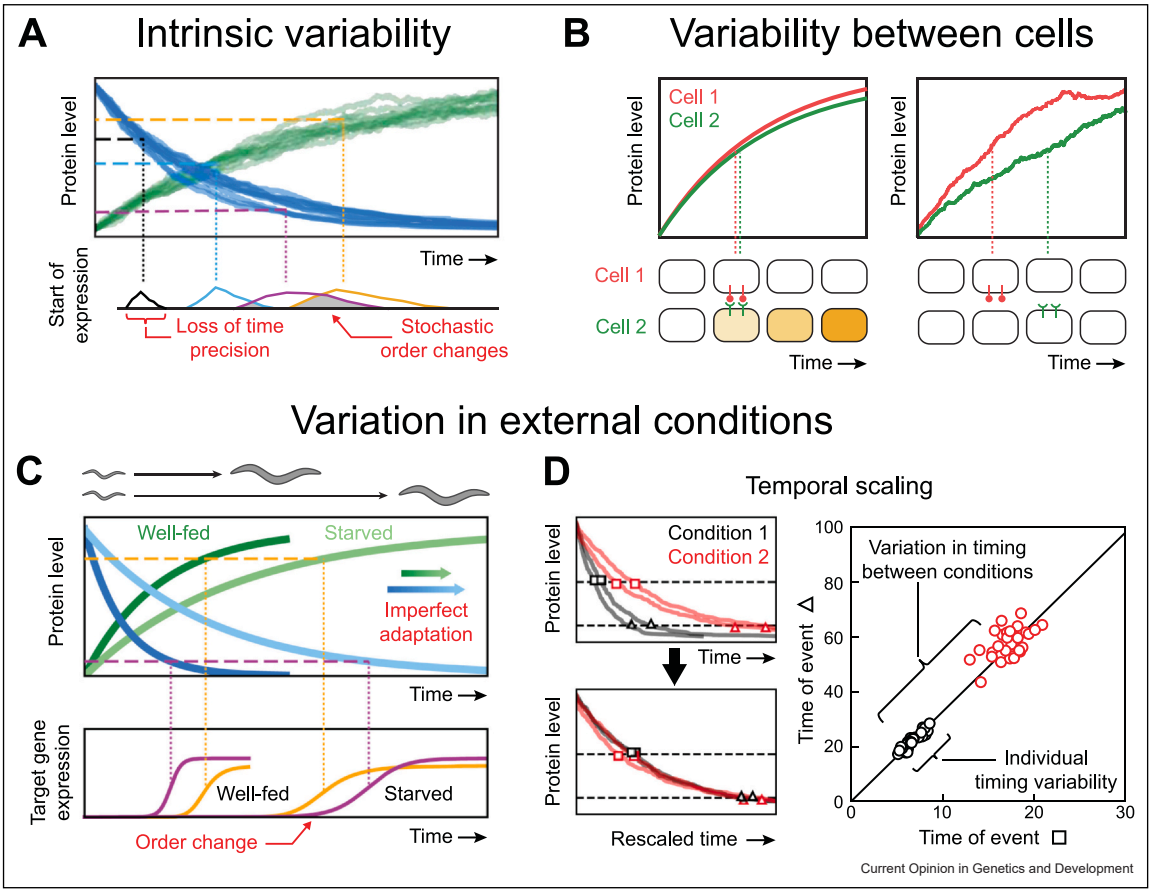
How can cells use oscillations to determine time within larval stages? Core oscillating TFs, such as NHR-23, induce expression of downstream targets by binding to specific sites [37]. Oscillatory TF levels will induce target expression only at specific times, with timing depending on each gene's induction threshold. It was recently proposed that, as promoters of genes required for early molting steps contained more NHR-23-binding sites compared with genes for later steps, binding site number explained timing, with expression of early genes when NHR-23 levels are still low, and of late genes only when NHR-23 levels peak [31]. However, a single-threshold model cannot explain gene expression at specific oscillation phases and with distinct order. Instead, genes induced early also remain expressed longest (Figure 1e), whereas *in vivo* early NHR-23 target genes peaked before late targets. Interestingly, precise timing and sequential gene expression is possible when combining inputs from multiple oscillatory TFs. Quantitative experiments recently showed that NHR-23 also induces surprisingly short *lin-4* miRNA expression bursts once every larval stage [13]. This was because NHR-23 induced *lin-4* expression not alone, but as a heterodimer with NHR-85. NHR-85 also displayed protein-level oscillations, but peaking before NHR-23. Hence, *lin-4* expression only occurred in the narrow time range where the two oscillations overlapped. In principle, such combinatorial mechanisms, involving multiple oscillating TFs, can produce precisely timed sequences of gene expression (Figure 1f). Interestingly, NHR-85 peak amplitude and duration depended on LIN-42 action [13], indicating complex cross-regulation between oscillatory TFs.

Robustness of timers against inherent molecular variability

The central tenet of temporal gradient mechanisms is that downstream event timing depends not simply on TF presence, but rather on precise TF level. If so, however, inherent fluctuations in TF transcription, translation, degradation, and promoter-binding kinetics (so-called 'molecular noise') will inevitably cause timing variability and, if sufficiently strong, stochastic changes in event order (Figure 2a). Whether cells employ specific mechanisms to maximize timing precision despite noise is thus an important open question. Combined experimental and theoretical studies addressed this recently for neuroblast cell migration in *C. elegans*. During larval development, the QR neuroblast migrates from tail to head, with their final position set not by spatial cues but rather through a cell-intrinsic timer mechanism, with precisely timed induction of the Wnt receptor MIG-1 responsible for migration termination [38]. Recent experiments indicated that this is likely controlled by a temporal gradient consisting of one or more homeobox TFs acting as activators [39], with time of *mig-1* expression following the timescale of TF accumulation. Simulations of this accumulating activator model revealed specific strategies for minimizing variability in timing of target gene induction, with lower variability when target gene expression depends nonlinearly on activator level [40] or when target genes positively co-induce their own expression [41]. Consistently, Q-neuroblast *mig-1* expression showed the predicted nonlinear increase of mRNA number in time [40], while a Wnt-dependent feedback resulted in positive regulation of its own expression [39]. Overall, these results indicate that biochemical parameters, such as TF levels and binding kinetics, can be optimized through evolution to minimize timing variability. It will be interesting to examine whether such principles are employed more widely, for example, within the heterochronic protein network.

Developmental processes often rely on the timely completion of development in surrounding tissues and thus require precise relative timing. For instance, *C. elegans* heterochronic mutations that perturb vulva, but not gonad development, exhibited morphological abnormalities, causing infertility, because timing of vulva development shifted relative to gonad development by as little as 2 h [42,43], while a 2–4 h delay in skin stem cell division relative to the molting, induced by exposure to nicotinic agonists, caused larval lethality [44]. Yet, molecular noise will produce variability between timers in different cells of the body, reducing synchronization and thus potentially inducing defects (Figure 2b). Indeed, independent cellular oscillators quickly lose synchrony due to noise [45]. Hence, an important question is how developmental timers remain synchronized between cells and tissues. One mechanism for oscillatory timers is coupling them through continuous exchange of cell–cell signals, such as Notch signaling in the somitogenesis oscillator [46]. However, no evidence exists for such continuous synchronizing signals in *C. elegans* development. An alternative mechanism is synchronizing timers only at specific checkpoints. This is similar to the cell cycle, which forms an oscillator, but arrests at checkpoints if processes, such as DNA duplication or spindle assembly, that occur with independent timing, have not finished [47]. Interestingly, *C. elegans* has well-described developmental checkpoints, at the beginning of each larval stage, when environmental stresses, such as starvation, cause growth and development to arrest [48]. These arrests also impact timer progression: whole-genome expression studies found that molting cycle oscillations arrested at a specific phase, both when entering adulthood or developmental arrest [12]. This followed a specific mechanism, a Saddle Node on Invariant

Figure 2



Impact of variability on accuracy of developmental timer mechanisms. **(a)** Molecular noise causes variability in temporal gradient dynamics (upper panel, each line represents protein-level dynamics in a single individual). Consequently, the time of target gene induction varies between individuals (lower panel, showing the distribution of induction times for each target). For some genes, these distributions overlap (shaded area), meaning that the order of expression is frequently reversed. **(b)** Inherent variability in temporal gradient dynamics causes loss of timing synchronization between cells (upper panel). This can perturb processes that rely on correct relative timing between cells (lower panel), such as induction of cell fate (shown in orange) by cell–cell communication through precisely timed receptors and ligand expression. **(c)** Variation in external conditions, such as food availability, can drastically alter the overall developmental. This must require precise adaptation of the dynamics of the underlying temporal gradients, as imperfect adaptation (upper panel) can cause order changes in target induction (lower panel). **(d)** For some developmental processes, differences in timing between individuals and conditions (top-left panel) can be explained by 'temporal scaling,' meaning that each event occurs at the same time, when rescaled by the total duration of development (bottom-left panel, markers indicate distinct induced events). This manifests itself by strongly correlated variations in measured times of subsequent events (right panel). **(a)** and **(d)** represent stochastic simulations that are provided as supplementary material.

Cycle bifurcation, that allows oscillator arrest at a specific phase without any accompanying decrease in oscillator amplitude. This mechanism also underlies cell cycle checkpoints [47], suggesting conceptual similarity. It is an intriguing possibility that developmental checkpoints, including their impact on developmental timers, are not only activated by external conditions, but also depend on completion of internal development, ensuring reinitiation of timers with body-wide synchronization at the start of each larval stage. Developmental arrest checkpoints rely on hormonal signals, such as insulin and steroid hormones [48], that likely spread through tissues readily, allowing for rapid, body-wide communication of checkpoint signals. Moreover, some proposed core molting cycle oscillator components are nuclear hormone receptors [30,36] that might require association with ligands, such as steroid hormones, to become activated [49], and it was speculated that their action during the molting cycle might be gated by specific hormonal cues [13,36].

Adaptation of timers to environmental conditions

A striking feature of postembryonic development, from birth or hatching to adulthood, is that its rate depends on external conditions, such as diet or temperature. In *C. elegans*, decreasing food concentration or temperature results

in outwardly normal development, but at up to 10-fold lower rate [50,51]. This implies that all underlying developmental timers must precisely adapt to this changing rate of development, at the risk of losing correct timing and event order (Figure 2c). However, as each biochemical reaction's rate depends on temperature or nutrient levels in a unique manner [52], changes in external conditions are a priori expected to impact each timer's dynamics differently. It is therefore important to address how developmental timers respond to changing environmental conditions and if specific mechanisms exist to ensure their coordinated adaptation. When examining timing of cellular events in *C. elegans* skin development, we uncovered strong timing variation [29], both inherent variability — between isogenic individuals under identical conditions — and when changing environment or genotype. However, differences in timing were explained by 'temporal scaling,' meaning that all events occurred at the same time, when rescaled by the total duration of development in each individual (Figure 2d), implying tight adaptation of timers to changes in overall developmental rate. Inherent variability in developmental rate was also apparent when inferring larval-stage durations in individuals through behavioral measures, either molt-specific cessation of feeding [51] or motility [53]. While the latter approach observed strong correlation in larval-stage duration within individuals [53], indicating temporal scaling, the former found that intermolt and molt duration showed distinct variation with temperature and food concentration [51], apparently inconsistent with scaling. The generality of temporal scaling therefore remains unresolved.

While different environments or diets uniquely impact each biochemical reaction, on the cellular level, this likely manifests itself simply as changes in growth rate. Hence, an intuitive mechanism to adapt timing to external conditions is coupling timer progression to cell size. Indeed, such 'sizer' mechanisms occur in cell cycle regulation [54], with division initiated only at specific cell volume, causing cell cycle oscillations to adapt to growth rate. Experiments have indeed suggested that *C. elegans* growth follows a sizer mechanism, with larval-stage durations extended under slow-growth conditions, so that molts occurred at stereotypical size [50]. Moreover, body shape measurements during larval development implied cuticle stretching as a sensor of body size [55]. While recent time-lapse measurements of body size in *C. elegans* individuals implicated a different mechanism than a sizer, these experiments nevertheless found growth tightly coordinated with larval progression [56], with faster-growing individuals exhibiting shorter larval stages. However, specific heterochronic (*lin-42*) and insulin signaling (*daf-16*) mutants show continued larval development without body growth [29,57]. Moreover, while growth-arrested *lin-42* mutants exhibited delayed developmental timing, with strong individual variability, these timing changes were still largely consistent with temporal scaling [29]. Hence, body growth appears to not simply dictate timing. Shortening molting cycle oscillation periods by reducing BLMP-1 levels did not reduce growth rate, leading to smaller body size at each molt [56], further demonstrating uncoupling of timing and growth. Hence, how timers adapt in a coordinated manner to the external environment remains an open question.

Conclusion

Many regulators of developmental timing are conserved from *C. elegans* to humans: *lin-42* is homologous to the circadian regulator Period [33], *lin-28* functions in developmental timing ranging from stem cell differentiation and organ development to onset of puberty [58], *lin-14* was shown only recently to belong to a widely conserved protein family involved in temporal patterning [59,60], and nuclear hormone receptors and hormones such as insulin play a key role in developmental transitions also in insects and mammals [58]. Moreover, temporal scaling and coupling of growth to timing are also observed in higher animals [58,61,62]. The comparative simplicity of *C. elegans* anatomy and development can thus help reveal timing mechanisms and principles that are likely conserved in higher organisms.

It remains open how strongly molecular noise impacts developmental timers and what timing precision is required to ensure body-wide synchrony. Here, the study of morphogen gradients might point the way, as studies show that while the impact of noise — here, fluctuations in morphogen concentration — is strong, downstream gene regulatory networks can be optimized to extract spatial information with remarkable precision [63]. Identifying developmental timing mechanisms and understanding their body-wide coordination will require also a quantitative approach: to precisely measure timing of developmental processes — during normal development, in mutants or different environments — and quantify the dynamics of their timing regulators, while mathematical models constrained by such experiments will aid in pinpointing the often-subtle mechanisms required for extracting precise time information. Such a quantitative approach is now possible for *C. elegans* larval development.

Data Availability

No data were used for the research described in the article.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.gde.2024.102172.

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- • of special interest
- •• of outstanding interest

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This paper uses a feeding-dependent bioluminescence assay that allows measuring progression and timing of *C. elegans* larval development in many individuals, to screen for TFs that constitute the underlying gene regulatory network. The authors further employ precisely timed, externally induced degradation of one uncovered TF, GRH-1, to characterize its mode and time of action. This approach points a way forward to a full understanding of the molecular mechanisms controlling the cyclical progression through larval stages.

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