

# A High-Throughput Method for the Analysis of Larval Developmental Phenotypes in *Caenorhabditis elegans*

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**ABSTRACT** *Caenorhabditis elegans* postembryonic development consists of four discrete larval stages separated by molts. Typically, the speed of progression through these larval stages is investigated by visual inspection of the molting process. Here, we describe an automated method to monitor the timing of these discrete phases of *C. elegans* maturation, from the first larval stage through adulthood, using bioluminescence. The method was validated with a *lin-42* mutant strain that shows delayed development relative to wild-type animals and with a *daf-2* mutant that shows an extended second larval stage. This new method is inherently high-throughput and will finally allow dissecting the molecular machinery governing the speed of the developmental clock, which has so far been hampered by the lack of a method suitable for genetic screens.

**KEYWORDS** development; larval molt; bioluminescence; luciferase; *Caenorhabditis elegans*

**C**AENORHABDITIS *elegans* is a key model system in which to study development from genetic regulation and cell biological processes to the precisely programmed body plan. Postembryonic development in this nematode initiates in the presence of food after the egg hatches. The larval developmental program consists of four discrete stages (L1–L4). The formation of a new cuticle, the separation of the old and new cuticles (apolysis), and the escape from the old cuticle (ecdysis) occur during transitions between larval stages called molts (M1–M4). Ecdysis is preceded by lethargus, a period during which the animals show behavioral quiescence and cease pharyngeal pumping (Singh and Sulston 1978). This behavioral quiescence is accompanied by a typical posture (Iwanir *et al.* 2012; Schwarz *et al.* 2012) and shows other sleep-like properties such as reversibility and increased

arousal threshold (Raizen *et al.* 2008; Schwarz *et al.* 2011; Cho and Sternberg 2014).

The rhythmic process of molting is sometimes referred to as a clock despite the lack of rigorous quantitative analyses that would support this. The clock analogy stems in part from the fact that *C. elegans* has a homolog of the *period* circadian clock gene, *lin-42*, which, when mutated, slows developmental timing (Jeon *et al.* 1999; Monsalve *et al.* 2011). The expression of the *period* gene homolog oscillates with the molting cycle (Jeon *et al.* 1999), similar to daily oscillations in *per* expression in mammals and flies (Hardin *et al.* 1990; Tei *et al.* 1997). Furthermore, the timing of lethargus is programmed by the developmental clock (Raizen *et al.* 2008), similar to how the endogenous circadian clock programs the timing of the sleep episodes (Aschoff 1965). These features suggest a functional conservation between the developmental and circadian clocks, but the molecular mechanism of the rhythmicity of larval development remains elusive.

Developmental timing is usually studied by means of direct observation of the animals at short time intervals using a dissecting microscope. Lethargus, cessation of pharyngeal pumping, ecdysis (Monsalve *et al.* 2011), and expression of GFP driven by promoters of genes that oscillate with the molts (Kim *et al.* 2013) have been used as hallmarks of the

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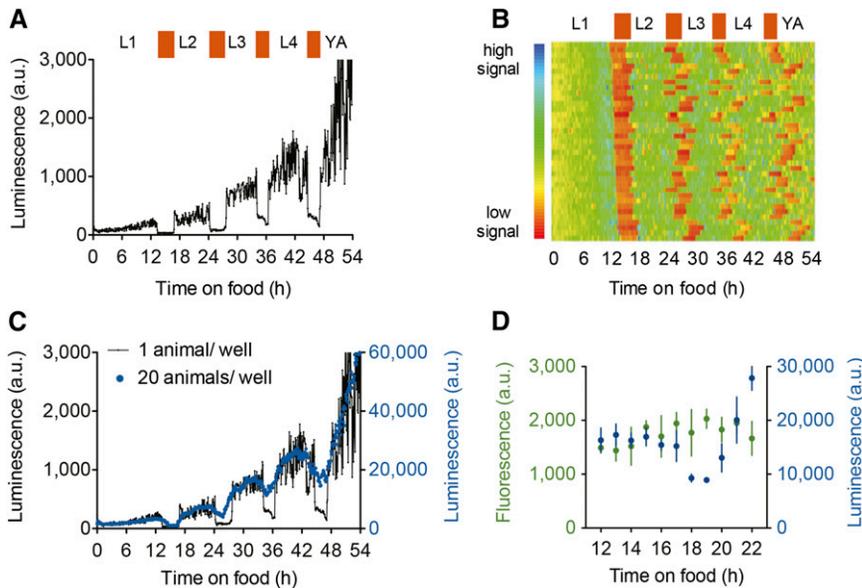
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**Figure 1** Luminescence signal reports larval development of single animals and populations. (A) Bioluminescence from a single animal (LUC::GFP reporter; strain PE255) over 54 h at 21.5° as it develops from arrested L1 to adult. The absence of substrate intake during the molts provokes a decrease in the bioluminescence signal. (B) Heat map showing trend-corrected data for 37 independently monitored animals. (C) Raw data for 20 animals in one well are compared with the signal from a single animal (as in Figure 1A). (D) Fluorescence and luminescence of a population of animals grown at 20.5° during late L1 to early L2. The graph shows data from three experimental replicates, each of which was derived from three to four replicate wells; error bars show SEM.

developmental process. Although these methods have shed light on *C. elegans* development, they are exceedingly time-consuming, are ill-suited for independent measurement of high numbers of animals, and are semiquantitative. The full power of the genetic model organism for studying developmental timing is thus lost. In contrast, the study of embryonic development in *C. elegans* has benefited from the use of automated setups to monitor the process with high time resolution (Schnabel *et al.* 1997). Here, we describe a highly quantitative and high-throughput method for continuous monitoring of development in *C. elegans* using bioluminescence. The method is validated using mutants that were previously reported to alter developmental timing.

## Materials and Methods

### Strains and experimental conditions

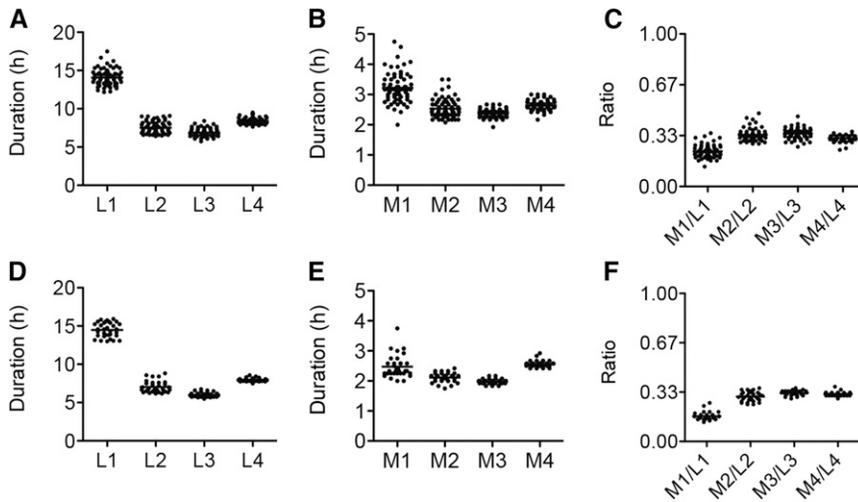
We used the reporter strains PE255 [*Psur-5::luc+::gfp*; *rol-6 (su1006)*] X and PE254 [*Psur-5::luc+::gfp*; *rol-6 (su1006)*] V (Lagido *et al.* 2008, 2009). For experiments with the *lin-42* mutant, we used the strain MRS189 [*lin-42 (ok2385)*; *Psur-5::luc+::gfp*; *rol-6 (su1006)*] V. For experiments with the *daf-2* mutant, we used the strain MRS210 [*daf-2 (e1370)*; *Psur-5::luc+::gfp*; *rol-6 (su1006)*] X. For stock animals, we cultured the animals according to standard methods (Brenner 1974), routinely maintaining the strains at 18° on nematode growth medium) plates with a lawn of *Escherichia coli* (OP50). For all experiments, we grew the stocks to the stage of gravid adult and obtained eggs by bleaching. We adjusted the concentration of the egg suspension to 20 eggs per microliter in M9 buffer and incubated them at 18° with gentle agitation, leading to hatching and arrest of development at the L1 stage. For the experiment with the *daf-2* mutant, this incubation was performed at 20°. After 2 days of starvation, we initiated the experiments by exposing the arrested L1 animals to food.

### Luminometry of single animals

L1-arrested larvae were simultaneously exposed to food by adding the M9 suspension of larvae to S-basal media containing 10 g·liter<sup>-1</sup> *E. coli* OP50. Then, single L1 animals were transferred into a well of a white, flat-bottom, 96-well plate by manual pipetting. Each well contained 200 μl of S-basal media with 10 g·liter<sup>-1</sup> *E. coli* OP50 (wet weight) and 100 μM D-Luciferin. Transferring of 96 animals to complete one plate took ~30 min. For the experiment with the mutant *daf-2*, we manually transferred the animals directly from the M9 suspension of larvae to a well of the 96-well plate containing 100 μl of S-basal with 100 μM D-Luciferin (without food) and then resumed development by adding 100 μl of S-basal with 20 g·liter<sup>-1</sup> *E. coli* OP50 (wet weight) and 100 μM D-Luciferin. Plates were sealed with a gas-permeable cover (Breathe Easier, Diversified Biotech). We measured luminescence (Berthold Centro LB960 XS<sup>3</sup>) for 1 sec, typically at 5-min intervals. We performed all experiments inside temperature-controlled incubators (Panasonic MIR-154). Temperature was monitored using HOBO Data Loggers (Onset).

### Data analysis

The raw data from the luminometer were analyzed using ChronoOSX 3 (Roenneberg and Taylor 2000), and the period of the oscillation was calculated by autocorrelation. For visualization of the data as heat maps, we trend-corrected the raw data (Supporting Information, Figure S2A), dividing by a centered moving average (Figure S2B). The moving average was calculated for the duration of one cycle (or period) of the oscillation. We used the trend-corrected data to generate heat maps (R software). To determine the timing of the molts, the trend-corrected data were converted to binary, using 75% of the value of the moving average as a threshold (Figure S2, C and D). We then evaluated the data for onset and offset of molting by detecting the transitions in the binarized data (Figure S2E). Transitions from 1 to 0 correspond to



**Figure 2** Quantitative analysis of the developmental program of *C. elegans* at 21.5° based on luminescence. Duration of larval stages (L1–L4) (A), molts (M1–M4) (B), and ratio between the duration of each molt and its preceding larval stage (C) for strain PE255. Duration of larval stages (L1–L4) (D), molts (M1–M4) (E), and ratio between the duration of each molt and its preceding larval stage (F) for strain PE254. Average is represented with a black horizontal bar.

onset of the molt and transitions from 0 to 1 correspond to offset of the molt. The variability of the luminescence signal introduces noise in the binarized data (Figure S2D). To prevent this noise from affecting the detection of the transitions, a simple rule was applied: onsets were followed by “0” for at least 1 hr, and offsets were preceded by “0” for at least 1 hr. We calculated the duration of each larval stage and molt from the beginning and the end of the four molts. Before plotting the data, we removed the outliers detected using the Grubb’s test. To test for differences in the duration of each stage between the N2 and mutant strains (Figure 3), we used the two-tailed unpaired *t*-test.

#### Fluorescence and luminescence of populations of animals

To test for fluorescence and luminescence signals around the L1–L2 molt, we added populations of ~500 arrested L1 larvae to a 96-well plate containing 200  $\mu$ l of S-basal media with 100  $\mu$ M D-Luciferin and 10 g·liter<sup>-1</sup> *E. coli* OP50 to resume development. Larvae were added to the plate every hour for 10 hr. We incubated the plate at 20.5° and then measured for luminescence and fluorescence (FLUOstar Omega, BMG Labtech) 12 hr after the last inoculation, covering a period of 12–22 hr after resumption of development. For fluorescence, excitation was set at 485 nm and emission at 535 nm.

#### In vitro ATP measurements

For *in vitro* measurements of ATP around the L1–L2 molt, we added populations of ~500 arrested L1 larvae to a 96-well plate containing 200  $\mu$ l of S-basal media with 100  $\mu$ M D-Luciferin and 10 g·liter<sup>-1</sup> *E. coli* OP50 to resume development. Larvae were added to the plate every hour for 9 hr. We incubated the plate at 20.5° for 13 hr after the last inoculation, covering a period of 13–22 hr after resumption of development. We measured luminescence as a control of developmental stage and then collected animals for ATP measurement. The collected larvae (~500 per sample) were washed three times with S-basal, frozen in liquid nitrogen in 50  $\mu$ l of S-basal, and stored at -80°. Samples were

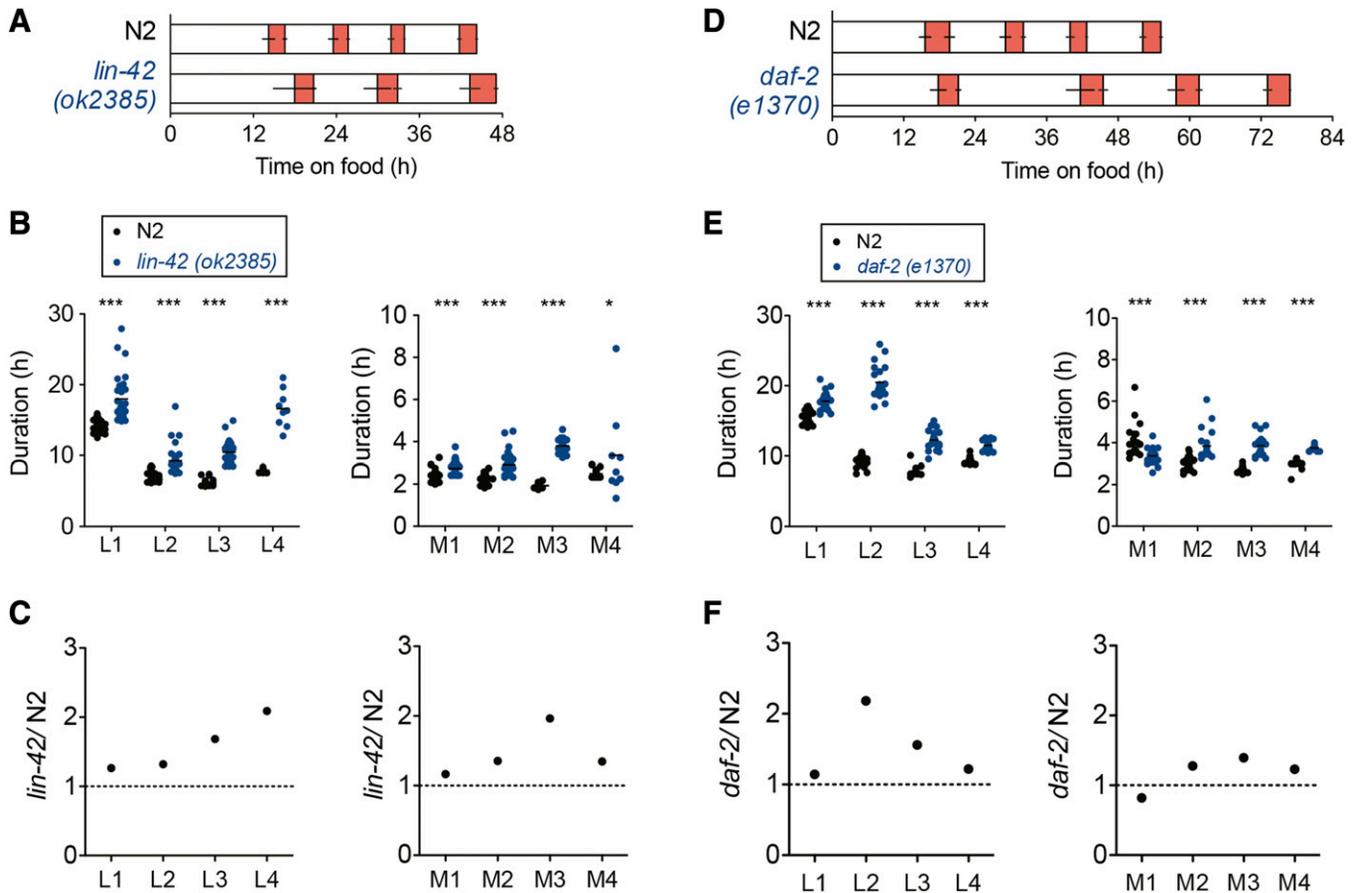
then boiled for 15 min and centrifuged to pellet the debris. ATP content in 25  $\mu$ l of the supernatant was measured using a time-stable ATP determination kit (Biaffin) and normalized to protein content (DC Protein Assay, Bio-Rad).

## Results and Discussion

### A quantitative, automated method to measure the timing of development in *C. elegans*

We followed two independent strains throughout development that constitutively and ubiquitously express the luciferase protein fused to GFP (*P<sub>sur-5</sub>::luc+::gfp*) (Lagido *et al.* 2008, 2009). We measured bioluminescence from single animals in 96- or 384-well plates. Experiments were initiated by exposing arrested L1 larvae to food at time = 0, a stimulus that launches the larval developmental program. The luminescence signal emitted by a single animal generally increased throughout development. However, the signal is punctuated and structured by four interruptions (Figure 1A). Using visual inspection, we confirmed that these interruptions are coincident with behavioral quiescence, characteristic of the molting stage. It is these temporal structures that allowed a quantitative assessment of the timing of development. Entry into the first molt occurs at 14.08  $\pm$  1.06 hr at 21.5° when starved L1 are monitored (Figure 1B). The transitions between intermolt and molt are short (5–10 min; Figure S1A), allowing precise determination of the beginning and end of the molt (Figure S2). This method can also report the timing of development for populations, although the precise information about the beginning and the end of the molts is lost due to animal-to-animal variability (Figure 1C).

To determine if the reduction in luminescence is due to a change in the expression level of the LUC::GFP fusion protein, we measured luminescence and fluorescence in parallel in a population of animals before, during, and after the L1–L2 molt. The expression of the LUC::GFP protein in these strains is driven by the regulatory region of the constitutively expressed *sur-5*



**Figure 3** Developmental timing in *lin-42* (*ok2385*) and *daf-2* (*e1370*) mutants. (A) Average duration of development for N2 ( $n = 37$ ) and *lin-42* ( $n = 33$ ) at 21.5°. Only three larval stages and molts are represented for *lin-42* (see text). Red represents the molts as inferred by low LUC signal. Error bars represent the SD of the duration of each interval. (B) Duration of larval stages (L1–L4) and molts (M1–M4) for N2 and *lin-42*. Average of each stage is represented by a black horizontal bar. The duration of all stages is significantly different between the N2 and *lin-42* backgrounds (\*\*\* $P < 0.001$ ; \* $P < 0.05$ ). Only nine *lin-42* (*ok2385*) animals progress through a fourth molt. (C) Ratio of the duration of each stage of development comparing *lin-42* (*ok2385*) and N2 backgrounds. (D) Average duration of development for N2 ( $n = 20$ ) and *daf-2* ( $n = 22$ ) at 19.5°, the permissive temperature for the *e1370* mutation. Red represents the molts. Error bars represent the SD of the duration of each interval. (E) Duration of larval stages and molts for N2 and *daf-2*. Average of each stage is represented by a black horizontal bar. The duration of all stages is significantly different between the N2 and *daf-2* backgrounds (\*\*\* $P < 0.001$ ). (F) Ratio between *daf-2* (*e1370*) and N2 of the duration of each stage of development.

(K03A1.5) gene (Lagido *et al.* 2008). The fluorescence signal remains stable during the molt, indicating that the LUC::GFP fusion protein is not cycling through development to yield rhythmic light emission (Figure 1D). This is furthermore predicted by work showing that *sur-5* expression does not oscillate during larval development (Kim *et al.* 2013; Hendriks *et al.* 2014).

Firefly (*Photinus pyralis*) LUC catalyzes the conversion of the substrate luciferin, supplied in the media, into oxyluciferin. This occurs in a two-step reaction that additionally requires ATP and oxygen and produces AMP, CO<sub>2</sub>, and light (Wet *et al.* 1987). Given that oxygen diffuses through the cuticle and luciferin does not (Lagido *et al.* 2008), the decrease in the signal must be due to depletion of ingested luciferin, endogenous ATP, or a combination of both. *In vitro* measurements of endogenous ATP levels do not correlate with luminescence during the molt ( $R^2 = 0.0305$ ). ATP levels increased during the last hours of the L1 stage and then decreased to a trough at the end of the molting period, 2 hr after the trough of the luminescence signal (Figure S1B). Upon transfer of an adult

to luciferin-free media, luminescence decreases at the same rate as during the entry into a molt (Figure S1C). Thus, luciferin is rapidly depleted in living animals when its uptake from media is blocked. During each molt, *C. elegans* form a plug of extracellular material in the buccal cavity and they cease pharyngeal pumping (Singh and Sulston 1978; George-Raizen *et al.* 2014). We conclude that a shortage in the supply of ingested luciferin is sufficient to explain the decrease in luminescence signal during molts.

#### Quantitative analysis of developmental timing

Our method allows for longitudinal measurement of developmental timing of single animals. The assay permits quantitative analysis of the timing of each larval stage (L1, L2, L3, and L4; Figure 2, A and D) as well as that of the molts (M1, M2, M3, and M4; Figure 2, B and E). L1 is longer than L2, L3, and L4. This is in accordance with original observations on the timing of molting (Byerly *et al.* 1976), although in this case we cannot disregard additional extension of the L1 stage

due to a lag in the resumption of development after starvation-induced arrest. Similarly, M1 is longer than M2, M3, and M4. We calculated the ratio of the molt/larval duration for each stage. Except for the first stage (M1/L1), the molts are about one-third as long as the preceding larval stage (Figure 2, C and F).

### **Analysis of mutant phenotypes in developmental timing: *lin-42* and *daf-2***

Several *lin-42* alleles have been investigated for the progression of larval development. The first description of these mutants indicated a precocious seam cell terminal differentiation that leads to the formation of adult-specific alae during the third molt (Abrahante *et al.* 1998; Jeon *et al.* 1999). Many *lin-42* animals fail to shed the L4 cuticle and execute the fourth molt (Abrahante *et al.* 1998). However, a detailed analysis of the timing of molting has been performed with only two *lin-42* alleles, *ok2385* and *n1089*, showing reduced speed of development (Monsalve *et al.* 2011). Consistent with this developmental delay, the cyclic expression of *molting defective-10* (*mlt-10*), encoding a protein necessary for molting, shows an increased period in a *lf* allele of *lin-42* (McCulloch and Rougvie 2014).

We therefore challenged our method using a *lin-42* (*ok2385*) mutant. We crossed this mutant with the LUC::GFP reporter strain and analyzed developmental timing. At 21.5°, the *lin-42* animals progress through three larval stages in the time the N2 worms require to complete the developmental program (Figure 3A). The reduction of speed is significant for all steps of development (L1–L4, M1–M4; Figure 3B). The majority of animals (24 of 33) fail to execute the fourth molt—hence the relative dearth of data for L4 and M4 in the mutant animals ( $n = 9$ ) (Figure 3B). All stages of development are significantly longer in *lin-42* mutants, with the third molt (M3) and the fourth larval stage (L4) taking twice as long as in the control strain (Figure 3C).

Environmental cues including food and temperature also control developmental speed and, when unfavorable, can lead to arrested development, namely as an alternative third larval stage called the dauer diapause (Cassada and Russell 1975). Mutations in the gene *daf-2* (Insulin/IGF-1 receptor) result in constitutive dauer formation (Daf-c) (Vowels and Thomas 1992). The allele *daf-2* (*e1370*) enters dauer at restrictive temperatures (Vowels and Thomas 1992) and shows a reduced developmental speed at permissive temperature, especially during the second larval stage (Ruaud *et al.* 2011). We used the *daf-2* (*e1370*) mutant for a second validation experiment. We grew *daf-2* animals at 19.5° (permissive temperature for the Daf-c phenotype) and, for the first time, were able to calculate the duration of all larval stages and molts of individual *daf-2* animals (Figure 3D). The L2 stage was prolonged (more than twofold) in the mutant compared to the wild-type animals (validation; Figure 3, E and F). Furthermore, we observed a lengthening of all stages of development in *daf-2* ( $P < 0.001$ ), including the molts.

In summary, we describe a quantitative, high-throughput method using bioluminescence to report the fundamental

process of development in a genetic model organism. We used the alternation between feeding and fasting in *C. elegans* larval development to generate a periodic bioluminescence signal, continuously recorded, to quantify the duration of the molts and larval stages in individual animals. The oscillation of the bioluminescence signal is ultimately generated by substrate availability, not by differential gene expression. In adult worms, when the mouth of the animal is not blocked, the luminescence signal could potentially be used to report for food intake. This is a plausible application that would contribute to the study of nutrition and metabolism in *C. elegans*.

In validating the method with the *lin-42* and *daf-2* mutants, we were able to describe the defects in developmental timing due to mutations in the circadian clock gene homolog and the *C. elegans* insulin receptor. We note that the traditional (*e.g.*, visual inspection) methods are obviously sufficient to capture the *lin-42* and *daf-2* mutant phenotypes (Monsalve *et al.* 2011; Ruaud *et al.* 2011), but they are time-consuming and not feasible for screening of large numbers of animals. The assay that we describe lends itself to high-throughput protocols, with individual animals in each well of a 384-well plate giving robust signals (Figure S3). Automated phenotyping of large numbers of worms, as described here, should allow rapid progress in the identification of the developmental clock using genetics methods. The elucidation of the molecular mechanism of this biological clock will allow assessment of the mechanistic conservation with the circadian clock of insects and mammals and may help to tackle the molecular bases for the circadian rhythms described in *C. elegans* (Kippert *et al.* 2002; Saigusa *et al.* 2002; Simonetta *et al.* 2009; van der Linden *et al.* 2010; Olmedo *et al.* 2012). In addition, this method will allow the assessment of the effect of environmental factors, like temperature, diet, or presence of chemicals, in the progression of development.

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Author contributions: M.O. and M.G. performed the experiments; M.O., M.G., and M.M. designed the project; and M.O., M.G., M.A.-S., and M.M. interpreted results and wrote the manuscript.

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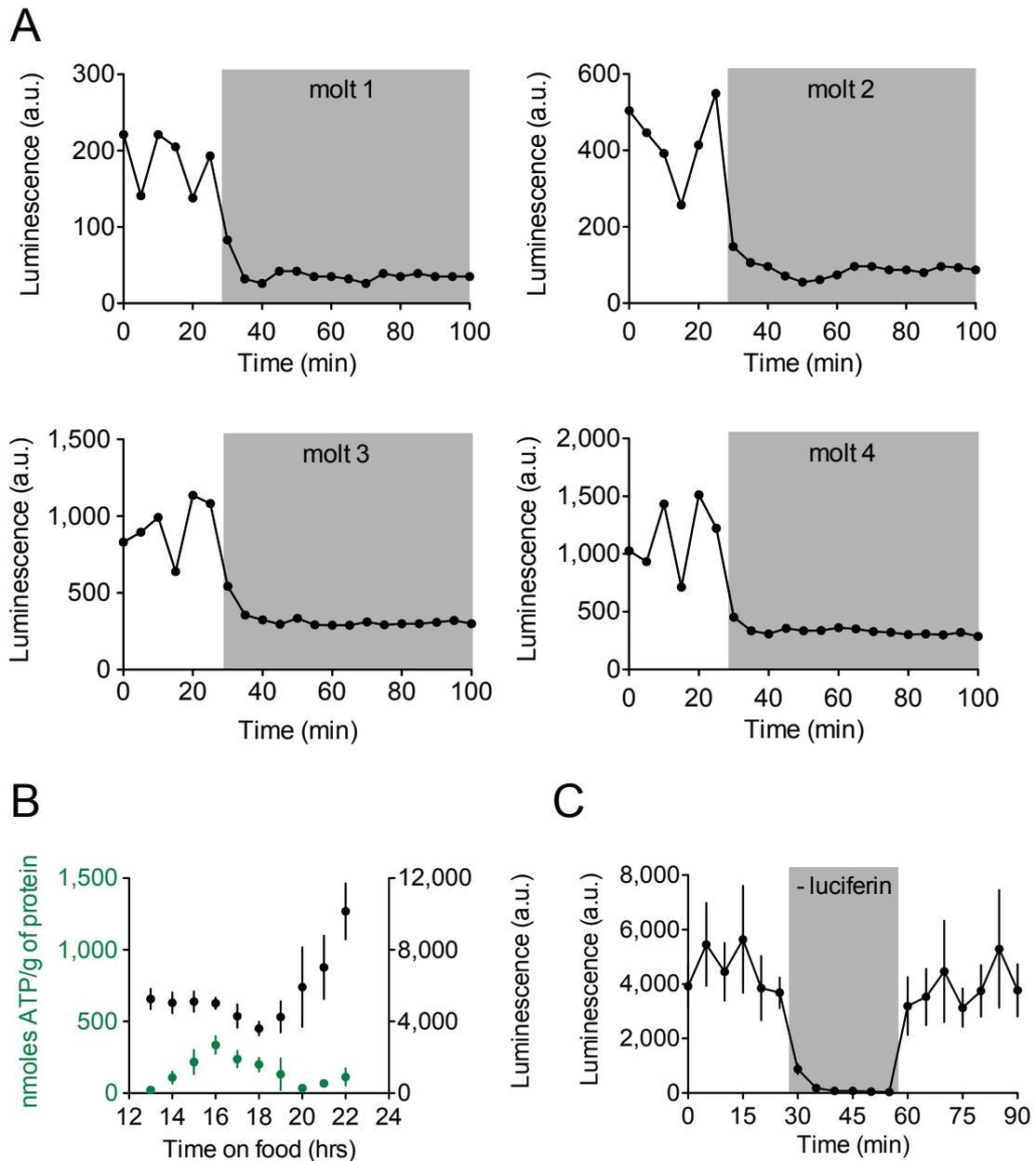
# GENETICS

Supporting Information

[www.genetics.org/lookup/suppl/doi:10.1534/genetics.115.179242/-/DC1](http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.115.179242/-/DC1)

## **A High-Throughput Method for the Analysis of Larval Developmental Phenotypes in *Caenorhabditis elegans***

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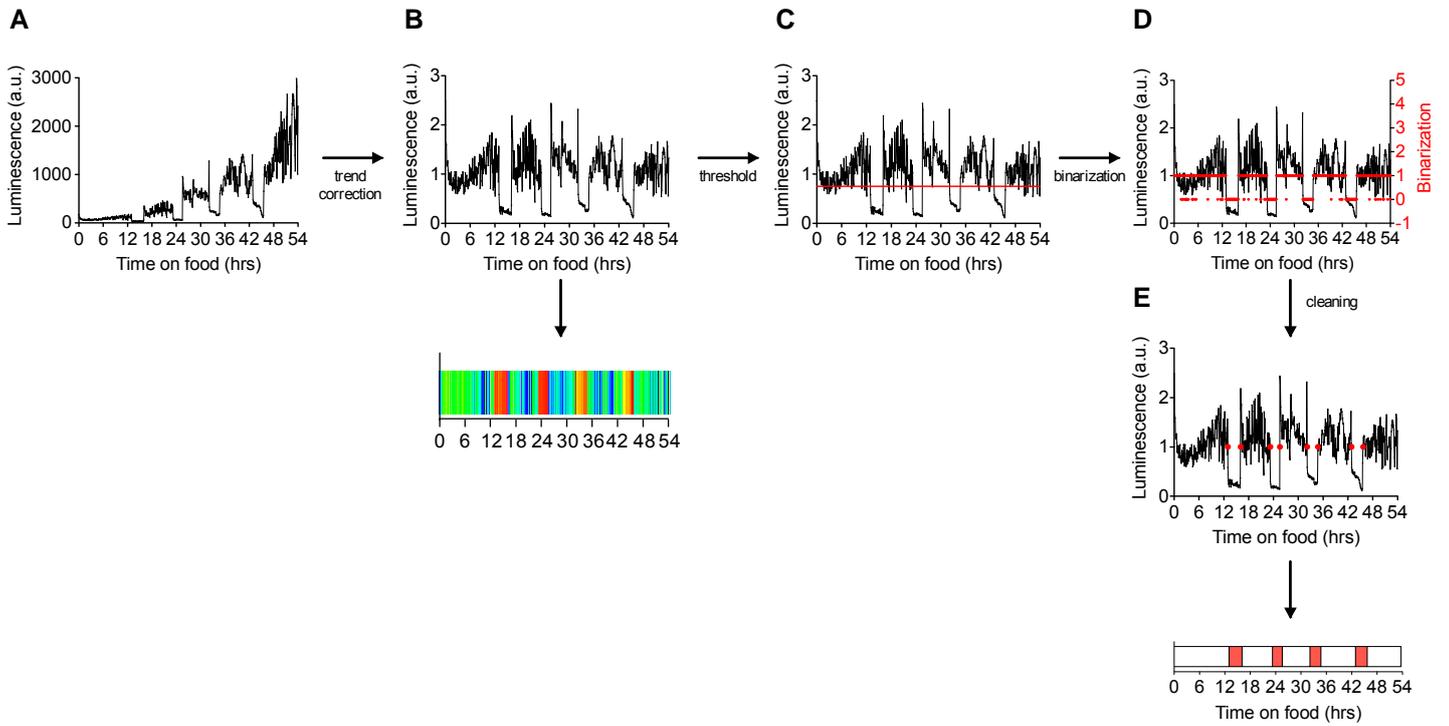


**Figure S1.** Restriction of luciferin intake is sufficient to reduce the luminescence signal.

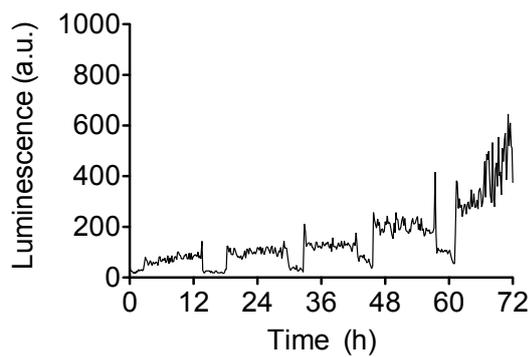
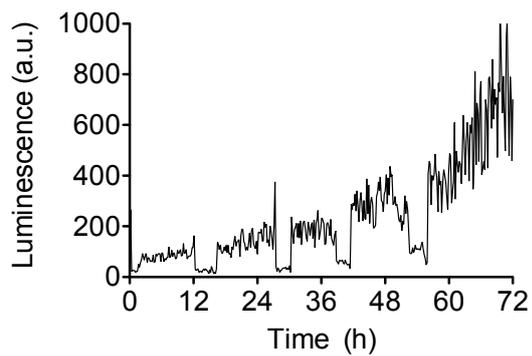
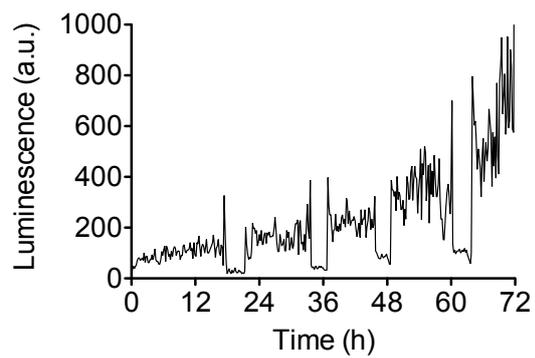
**A.** Detail of the luminescence signal of the animal in figure 1A at the entry into each of the four molts.

**B.** ATP levels measured *in vitro* and luminescence of a population of animals grown at 20.5 ° during late L1 to early L2. The graph shows data from 3 experimental replicates; error bars show s.e.m.

**C.** Individual adults were incubated in wells of a 96 well plate with 200  $\mu$ l of S-basal media with 10 g l<sup>-1</sup> *E. coli* OP50 (wet weight) and 100  $\mu$ M D-Luciferin. Luminescence was read at 5-minute intervals. Between the 25 and 30-minute readings the animals were washed three times in S-basal media and then transferred to a well with S-basal media and 10 g l<sup>-1</sup> *E. coli* OP50 (without luciferin). Between the 55 and 60-minute readings, luciferin was added back to the media. The plot shows the mean  $\pm$  s.e.m. (n=10).



**Figure S2.** Determination of onset/offset of the molts. The raw data (A) was trend- corrected (B) and used to generate heat maps. To determine onset and offset of the molt, the trend corrected data was thresholded using 75% of the moving average (C) to produce a binarized output (D). Transitions in the binarized data indicate the onset and offset of the molts (E).



**Figure S3.** Luminescence signal from 3 representative wells of a 384-well plate.