Chapter 4: Spatiotemporal regulation of *daf-9*

Introduction

Environmental conditions can influence the physiology of animals as they can adapt to changes of physiochemical compounds, specific cues, pheromones and kairomoes from conspecifics and predators and even attempt to predict fluctuations in temperature and photoperiod. Polyphenism is one type of adaptation where a population with the same genotype displays one of two optional fates or phenotypes (Gilbert, 2009).

polyphenisms often entail a binary decision between two mutually exclusive outcomes (Braendle et al., 2008). Such decisions involve three main phases: (i) measurement and integration, in which both environmental conditions and internal states are measured and integrated into a decision; (ii) commitment, in which a change of environmental conditions or cues does not change the result of the decision and (iii) signaling and implementation, in which a set of molecular and cellular processes drive the decision thus dispersing it throughout the responding cells/organs. Decisions in these cases are often irreversible and thus represent commitments to different fates. The orchestration and signaling.

Hormonal mechanisms have been shown to regulate and orchestrate polyphenic switches in the insect class. Hormonal mechanisms work during a hormone sensitive period when the nuclear hormone receptor is active in the target tissues (Nijhout, 2003). Of the many examples of hormonal regulation of polyphenism in insects it is apparent the one hormone regulates the hormone sensitive period and the other regulates the execution of the polyphenic trait. For example, the metamorphosis from larvae to pupae

in the silkworm *Bombyx mori* and the tobacco hornworm *Manduca sexta* is inhibited by JH during development ensuring that enough food has been accumulated to reach a critical mass. During the fifth instar larvae, (when nutrition is sufficient) there is an increase of the hormone 20-hydroxyecdysone, and a decrease of JH leading to pupal commitment. Addition of JH 12 or 24 hours after pupal commitment does not affect the course of metamorphosis nor does starvation (Koyama et al., 2008). The mechanism that regulates the commitment to one fate and excludes the other is unknown.

The nematode *C. elegans* display polyphenism during development. In favorable environments, *C. elegans* develops through four larval (juvenile) stages (L1-L4) separated by molts, into a sexually reproductive adult. In unfavorable environments, animals can make a life cycle fate decision and develop into an alternative third larval stage, known as the dauer diapause, a developmentally arrested, long-lived form geared towards survival (Cassada and Russell, 1975; Golden and Riddle, 1984a). Accordingly, they undergo profound morphological changes including assault resistant cuticle, pharyngeal constriction and sealing of buccal cavities, which confer somatic endurance (Cassada and Russell, 1975; Golden and Riddle, 1982). Dauer larva do not feed and can endure harsh conditions, including starvation, desiccation, heat and oxidative stresses (Riddle, 1997). Whereas adult worms have a mean life span of three weeks, dauer larvae can survive for several months (Byerly et al., 1976; Cassada and Russell, 1975; Klass and Hirsh, 1976). When returned to favorable conditions, dauer larvae molt into an L4 larvae and continue into adulthood (Cassada and Russell, 1975; Klass and Hirsh, 1976).

The attributes of mutually exclusive fates and irreversibility imply that a commitment mechanism underlies development into a sexually reproductive adult, yet the cellular and molecular basis of this binary decision is not clear. Environmental cues are detected by multiple sensory neurons which integrate inputs into hormonal outputs by unknown means (Bargmann and Horvitz, 1991; Kim et al., 2009; Reiner et al., 2008; Schackwitz et al., 1996). Genetic analysis of dauer formation has identified dauer constitutive mutants (Daf-c), which form dauer larvae even in favorable conditions, and dauer defective mutants (Daf-d), which fail to form dauer larvae even in unfavorable conditions (Albert and Riddle, 1988; Riddle, 1997). Molecular analysis has revealed at least four signaling pathways. Components of neurosensory structure and guanylyl cyclase signaling are involved in sensing temperature, nutrients and dauer pheromone (Birnby et al., 2000), which regulate secretion of Insulin/Insulin-like growth factor and TGFβ peptides. Working through their respective signaling pathways, IIS and TGF-beta signaling converge on a steroid hormone pathway, which metabolizes dietary cholesterol into several bile acid-like steroids, called the dafachronic acids (DAs) (Gerisch and Antebi, 2004; Gerisch et al., 2007; Gerisch et al., 2001; Jia et al., 2002; Mak and Ruvkun, 2004; Motola et al., 2006; Rottiers and Antebi, 2006). DAs serve as hormonal ligands for the nuclear hormone receptor transcription factor DAF-12, which regulates the life cycle fate decision. Liganded DAF-12 promotes reproductive development, whereas unliganded DAF-12 directs the dauer fate together with the co-repressor DIN-1S. Thus, DAF-12 serves as a DA-responsive switch that determines whether an animal will undergo reproductive or dauer development (Gerisch and Antebi,

2004; Gerisch et al., 2007; Hannich et al., 2009; Ludewig et al., 2004; Mak and Ruvkun, 2004; Motola et al., 2006; Patel et al., 2008; Rottiers and Antebi, 2006).

The cytochrome P450 DAF-9 is the last enzyme in the pathway of DA production and is critical to the dauer decision. *daf-9* is expressed in a bilaterally symmetric pair of neuroendocrine cells called XXXL and XXXR from hatching through adulthood; in the hypodermal syncytium from mid-L2 to L4 stages (but not during the dauer stage); and in the adult spermatheca (Gerisch et al., 2001; Jia et al., 2002; Ohkura et al., 2003). Consistent with a hormonal mechanism, DAF-9 works cell non-autonomously to control dauer formation (Gerisch and Antebi, 2004; Mak and Ruvkun, 2004). By contrast, *daf-12* is widely expressed in most tissues and throughout development (Antebi et al., 1998; Antebi et al., 2000). With respect to dauer phenotypes, *daf-12* null mutants (Daf-d) are downstream to *daf-9* null mutants (Daf-c, Gerisch et al., 2001; Jia et al., 2002), yet hypodermal expression of *daf-9* is DAF-12 dependent, suggesting that a feedback loop regulates hypodermal *daf-9* expression (Gerisch and Antebi, 2004; Mak and Ruvkun, 2004).

The molecular and cellular basis comprising the binary nature of the life cycle fate decision remains elusive. It is unknown whether DAs are produced as a result of a decision or as a means for integrating multiple environmental stimuli and genetic inputs controlling the decision. In the previous chapters, I have shown that the decision to develop into the dauer or into an adult is regulated by environmental conditions that eventually lead to regulation of the threshold to the steroid hormone Dafachronic acid (DA). In this chapter I will demonstrate that the amplification of DA in the hypodermis is responsible for the irreversibility of the decision. I propose that hypodermal amplification

of a hormonal signal acts as a commitment mechanism that enforces the binary decision.

Results

Hypodermal *daf-9* is expressed as a result of reproductive fate decision

We wanted to understand how the spatiotemporal and tissue-specific regulation of *daf-9* is related to hormonal activity and stage commitments. The two bilaterally symmetric XXX cells express *daf-9* throughout all stages, suggesting that they may produce steady levels of DA (Gerisch et al., 2001; Jia et al., 2002). Hypodermal *daf-9* expression is more complex: hypodermal *daf-9* is weakly expressed in L3 larvae growing in favorable, low-stress conditions, strongly expressed in L3 larvae growing in mild stress conditions, and not expressed under high stress conditions that trigger dauer formation (Gerisch and Antebi, 2004; Mak and Ruvkun, 2004).

First, we investigated the expression of *daf-9* mRNA during L2 and L2d in favorable and unfavorable conditions by whole animal qPCR in WT worms. Second, we examined the expression of DAF-9 protein levels and distribution with a translational DAF-9::GFP fusion by fluorescent microscopy (strain AA277; *lin-15(n765), dhIs64[daf-9::GFP, lin-15(+)]*; Gerisch et al., 2001; strain AA277 grows slower than N2 therefore commitment to dauer occurs at 36 hph; Figure 4.1A). We found that *daf-9* is regulated differently in favorable and unfavorable environmental conditions.

In favorable conditions that promote reproductive development, total *daf-9* transcripts were upregulated 7 ± 1.1 fold at 16 hph and peaked at 30 hph, with 10-fold upregulation (Figure 4.1B). All *daf-9* upregulation was specific to the *daf-9a* isoform; we

could not detect the *daf-9b* isoform (see Experimental Procedures). Eighteen percent of worms started expressing hypodermal DAF-9::GFP at 21 hph, mid-L2 stage, reaching a maximum of 75% ± 12% at 30 hph, mid L3 (Figure 4.1C; $P<0.0001$). Presumably the delay between *daf-9* upregulation detected by qPCR to that observed by GFP is due to the translation of mRNA to protein and slower developmental rate of the AA277 strain.

In unfavorable conditions, total *daf-9* transcripts were not significantly upregulated in L2d animals committed to dauer (Figure 4.2A $P=0.14$) but were upregulated 5-fold during dauer. All *daf-9* upregulation was specific to the *daf-9a* isoform and we could not detect the *daf-9b* isoform (see Experimental Procedures). Nearly all of worms grown in unfavorable conditions did not express hypodermal DAF-9::GFP during L2d or dauer (Figure 4.2B; 92-100%, $P=0.18$). .

We wanted to understand whether a shift to favorable conditions during L2d would affect the expression of hypodermal *daf-9* since a shift to favorable conditions at this stage for 3 hours is sufficient for wild type worms to commit to adult development. L2d worms were pulsed into favorable conditions at 24 hph for a six hour window, 76% ± 12% showed hypodermal DAF-9 GFP expression with onset as early as 27 hph (Figure 4.3A). Hypodermal *daf-9* expression was retained even when worms were shifted back to unfavorable conditions. Furthermore, these experiments revealed a 40-fold upregulation of *daf-9* transcripts in WT worms committed to reproductive development (Figure 4.3B). Conversely, 93 - 99% of worms shifted to favorable conditions for one hour, did not express hypodermal DAF-9 (Figure 4.3C) and total *daf-9* was not transcriptionally upregulated (Figure 4.3D). These results correlate temporally with the minimum time that WT worms require a pulse in favorable conditions to bypass

dauer, and suggest that hypodermal *daf-9* expression could be a cause or consequence of a decision to develop in XXX cells regulate hypodermal *daf-9* expression in a shift from unfavorable conditions.

Exogenous DA can upregulate hypodermal *daf-9* transcription

Genetic experiments demonstrated that hypodermal *daf-9* expression is regulated by DAF-12 (Gerisch and Antebi, 2004; Mak and Ruvkun, 2004) therefore we tested the effects of DA on *daf-9* expression. To monitor *daf-9* transcriptional regulation in specific tissues as a function of DA, we performed experiments using a *pdaf-9::gfp* transcriptional promoter construct in the *daf-9(dh6)* background. This promoter construct largely recapitulates the behavior of the translational fusion, suggesting that the majority of regulation occurs at the level of transcription. At low DA concentration (0-0.5nM), expression was seen only in the XXX cells, and all animals developed as dauers (Figure 4.4A). As DA concentration was increased to 0.75-7.5nM, expression in the hypodermis dramatically increased by mid-L2, suggesting positive amplification (Figure 4.4B). Notably, within the range of 1-5nM DA, animals developed into incomplete adults. Hypodermal expression was decreased at 10nM or shut off (50-100nM), suggesting suppression of *daf-9* expression. At these higher concentrations (>10nM) all animals developed into complete adults (Figure 4.4B). Thus, hypodermal *daf-9* expression is dependent on DA within a specific range; low amounts of DA are not sufficient to upregulate hypodermal *daf-9* and worms develop as dauers. Intermediate levels of DA promote upregulation of hypodermal *daf-9* which corresponds to incomplete development. At high amounts, hypodermal *daf-9* is shut off corresponding to complete development.

XXX cells act as a source for DA

Worms committed to adult development showed transcriptional upregulation of *daf-9* in the hypodermis, likely resulting in the production of the high levels of DA. As DA can promote hypodermal *daf-9* upregulation, we asked whether the XXX cells play a role in hypodermal *daf-9* upregulation. Notably, we observed that after a shift from unfavorable to favorable conditions, hypodermal DAF-9::GFP was observed in a spatiotemporal manner along the anterior posterior axis (Figure 4.5), first and most strongly in the head region before expression spread to more posterior regions. We hypothesize that under these conditions, XXX cells (located at the anterior) may act as a source of DA, releasing a small amount that is amplified and propagated in the hypodermis from anterior to posterior.

To test this hypothesis, we removed the XXX cells with a laser microbeam. We ablated XXX cells in worms expressing a translational DAF-9::GFP fusion, grown in high pheromone concentration, at 24 hph (mid L2d, pre-commitment), and worms were allowed to recover in favorable conditions (Figure 4.6A). Nearly all (30/31) XXX-ablated worms lacked hypodermal DAF-9::GFP expression and developed as dauers while 29/31 control mock-ablated L2d animals developed into adults (Figure 4.6B-E; $P<1x10^{-10}$). Therefore, intact XXX cells are necessary for L2d larvae to respond to favorable conditions, committing to reproductive development and initiating hypodermal *daf-9* expression.

We next tested whether DAs could rescue the dauer arrest caused by ablation of XXX cells. Worms were grown in high pheromone concentration and XXX cells were ablated at 24 hph (L2d before commitment), and shifted to growth in favorable

conditions supplemented with 0, 1, 5 or 10nM DA (Figure 4.6F). An increasing frequency of both hypodermal DAF-9::GFP and complete adult development was observed as higher concentrations of DA were supplemented. Rescue with 1nM DA yielded 22% adults and 78% dauers (N=18), rescue with 5nM DA yielded 56% adults and 44% dauers (N=30) and rescue with 10nM DA yielded 92% adults and 8% dauers (N=39, Figure 4.6G-J). All XXX-ablated worms supplemented with exogenous DA developed either as complete adults or as dauers. None of these worms had Mig or Cut phenotypes seen in *daf-9(dh6)* worms supplemented with 10nM of exogenous DA (Figure 2.1B). These results suggest that in the absence of the XXX cells, hypodermal *daf-9* upregulation can be induced with as little as 1 nM DA, resulting in complete adult development. By contrast, in the *daf-9* null background hypodermal *daf-9* amplification is not possible, leading to incomplete development at low DA levels.

To test whether XXX cells act as a source of DA later in development, we ablated XXX cells after commitment to L3 (Figure 4.6K). Ablation at this time had no effect and resulted in worms that expressed hypodermal DAF-9::GFP and developed to adulthood (Fig 6L-O; $P=2x10^{-9}$). Therefore, XXX cells act as a source of DA during the dauer decision, and become dispensable at later times to L3.

Summary

Hormonal networks play a critical role in commitment to reproductive maturity throughout the animal kingdom, yet the cellular and molecular network architecture of commitment is not well understood. Here we characterized a molecular mechanism

connecting environmental signals to hormonal regulation during the commitment to reproductive development. These studies allow us to ascribe specific time windows and pinpoint levels of hormone required to drive an endocrine network over thresholds for adult maturation.

Here we wanted to couple the effects of environmental conditions and hormonal regulation to the spatio-temporal gene regulation patterns. In the previous chapter I demonstrated how the decision to become adult or dauer is dependent on two separate thresholds of DA. At the low concentration, the dauer bypass DA threshold determines the dauer fate and the higher threshold determines if the animals become incomplete or complete adults. Since those conclusions were based on the analysis of several alleles of mutant strains, I wanted to extend the analysis to wild-type (and mutants rescued by transgenic constructs resembling wild type behavior) worms. By uncoupling the positive feedback loop in two places I was able to show that (i) hypodermal upregulation was initiated after commitment to adulthood, (ii) DA can upregulate hypodermal *daf-9* expression and that (iii) the XXX cells are the source of DA. Furthermore, I also demonstrated that the hypodermal *daf-9* positive feedback loop and homeostatic regulator confers the binary nature of the dauer decision by canalizing development.

Under high population density, the XXX cell appears to be source of DA required for the dauer decision, whereas the hypodermis amplifies DA production leading to complete development. When worms are shifted from unfavorable to favorable conditions, the adult DA threshold is lowered and the XXX cells presumably make a sufficient amount of DA to pass it. Once the XXX cells release a small amount of DA,

the hypodermis amplifies this signal leading to complete adult development. This amplification is visible as anterior to posterior propagation of hypodermal *daf-9* expression originating in proximity of the XXX cells. If the XXX cells are ablated, there is no source of DA to trigger hypodermal *daf-9* transcription and animals develop as dauer larvae. Hypodermal *daf-9* amplification is triggered if XXX-ablated animals are supplemented with as little as 1nM DA. Lastly, the onset of hypodermal *daf-9* upregulation renders worms insensitive to removal of XXX, thus conferring the irreversibility of the decision and committing worms to the reproductive fate.

In favorable conditions, XXX and hypodermis may share responsibilities. Under these conditions *daf-9* expression in the XXX appears steady and hypodermal expression low. The XXX cells are sufficient but not necessary for committing to reproductive fate: rescue of the *daf-9(dh6)* putative null by a XXX cell specific DAF-9 construct leads to adult development. Ablation of the XXX cells during the L1 stage, in worms grown in favorable conditions, results in 30% of animals developing as dauers (Ohkura et al., 2003). However, the hypodermis can overcome this deficiency of XXX signaling by *daf-9* upregulation (Gerisch et al., 2001; Jia et al., 2002; Ohkura et al., 2003). Hypodermal expression of *daf-9* works as a homeostatic regulator since intermediate amounts of DA increase transcription of *daf-9,* whereas sufficient production of DA by the XXX cells is not followed by hypodermal upregulation of *daf-9* during L2 and L3 (Figure 4.5CD, 4.7A).

Consistent with the importance of the XXX cells to the dauer decision, many components of the dauer pathways are expressed in these cells including *ncr-1,* the Niemann-Pick C1 homolog, *hsd-1* encoding a 3β-hydroxysteroid dehydrogenase, and

sdf-9 and *eak-6* which encode tyrosine phosphatases, *eak-3* and *eak-4* which encode novel proteins (Gerisch et al., 2001; Jia et al., 2002; Li et al., 2004; Ohkura et al., 2003; Patel et al., 2008; Zhang et al., 2008). These components as well as others could regulate enzymatic activities, availability or hormone transport to and from the XXX. Additional activities in the dauer pathways could regulate the amount of DA produced in the XXX cells and the adult DA threshold, in endocrine or target tissues.

From the *daf-9* spatiotemporal expression pattern, we infer that in favorable conditions DA is released in low levels over a long period of time, whereas worms developing in unfavorable conditions to adulthood release a burst of DA over a short period of time (Figure 4.7A). This also implies that worms have a mechanism of counting and integrating hormone levels to reach the threshold of the dauer decision. Speculatively, this could be achieved by various levels of DA swapping DAF-12/DIN-1 or other co-repressor complexes for DAF-12/co-activator complexes, a known mechanism in nuclear receptor signal transduction (Magner and Antebi, 2008).

Materials and methods

Growth conditions

All worms were handled using standard growth and cultivation techniques using the bacterial strains HB101 and OP50 as food sources (Lewis, 1995). Unless otherwise stated all liquid cultures were grown in glass flasks at ~1 worm per µl at 20°C in S

complete medium supplemented with 7.5 mg/ml HB101 as described in (Lewis, 1995) in an Innova 4230 incubator at 180 RPM. The wild-type strain used was N2 (Bristol).

Synchronous hatching of large broods

Worms were hatched synchronously essentially as described by (Baugh et al., 2009); changes are described in the SOM.

Pheromone assays

Crude pheromone was prepared as described (Golden and Riddle, 1984b). Each pheromone extract was tested on N2 worms (1 worm per µl) and diluted so that 3% (v/v) would yield 90±2% dauer arrest in a culture supplemented with 7.5 mg/ml of HB101 (Fig S1D).

Shift assays

Synchronous broods were grown as described above to the L2d stage by supplementing media with 3% (v/v) pheromone, partitioned into multiple parallel cultures. Shift to favorable: at specified times, broods were washed 3 times in S basal to remove pheromone. Cultures were re-suspended in S complete medium containing HB101 and calibrated for density. Pulse to favorable: worms were supplemented with 3% (v/v) pheromone at hatch. At 24 hph (L2d), worms were partitioned into experimental samples and a control sample, and washed 3 times with S basal. Worms were suspended in S complete medium and allowed to grow for specific time periods until 3% pheromone (v/v) was added.

Dafachronic acid assays

Liquid culture: Δ7-DA was solubilized in 100% EtOH to necessary concentrations. Liquid culture assays were performed by adding solubilized Δ7-DA in S complete medium. NG agar plate assays were performed by resuspending EtOH-solubilized Δ7-DA with OP50 and spreading on plates. Worms were picked onto NG agar plates not more than one day after Δ7-DA was added to those plates.

Scoring incomplete development

daf-9(dh6), daf-9(e1406) and *daf-9(m540)* worms were grown in liquid culture with different concentrations of Δ7-DA as described above. Worms were washed once with S basal medium to remove HB101 and mixed with S basal medium containing 1mM sodium azide (to limit worm movement), spotted onto a 24-well plate. Worms were scored for gonad migration and cuticle shedding. Phenotype frequencies were calculated as the means of 3 biological replicates, ± standard deviation.

Commitment to adult or dauer

Frequencies were calculated within each biological replicate and means of frequencies ± standard deviation were calculated between biological replicates. We determined the point of commitment at the measurement times with highest standard deviation as it represents the tipping point of a transition between non-committed to committed worms. We calculated a q-statistic based on a Tukey type multiple comparison test for differences among variances (Zar 2009, Table S1).

Homogeneity of liquid culture

Stage distributions were compared between 3 biological replicates in favorable and unfavorable conditions. A Bartlett's test (Zar, 2009) was used to determine if variances were significantly different between all stages of development.

Significance of hypodermal *daf-9* expression

Analysis was performed by a one-way ANOVA (Fig 4C,D). Significance of hypodermal upregulation in favorable versus unfavorable conditions after different time windows in favorable conditions was analyzed using a two tailed t-test between worms scored 30 hph (Fig 4E,F). Significance of transcriptional upregulation was analyzed by one-way ANOVA across all time points (Fig 4A) and paired t-tests between L2d uncommitted to L2d committed to dauer, to L2d committed to L3 (Fig 4B).

Cell ablation

AA277 worms (*lin-15(n765), dhIs64[daf-9::GFP, lin-15(+)]*) were grown to L2d stage in pheromone as described above. We found it necessary to use a fluorescently labeled XXX cells as they migrate from the nose tip to the posterior region of the anterior bulb (White, 1988). Worms were placed on glass slides with 5% agarose and 1 mM sodium azide in S. basal and laser microbeam ablations of the XXX cells were performed as described (Avery and Horvitz, 1987). Worms were allowed to recover for 2 hours before re-mounting on slides and verifying successful ablation by determination that no fluorescence signal was seen from either XXX cells. Worms were then transferred to either NG agar plates or NG agar plates supplemented with 1,5 or 10nM Δ7-DA. All ablations were coupled with mock-ablation controls. Statistical significance of observed

differences between ablations and controls was determined using Fisher's exact test (Zar, 2009).

Imaging

Strain AA277 was grown in liquid culture as described above. At specific times, worms were washed once in S basal medium and plated on glass slides with 5% agarose and 1mM sodium azide in S basal. Worms were scored for hypodermal DAF-9::GFP under 40x magnification using a Zeiss Axiovert 200 microscope with a 200W mercury bulb.

Anterior posterior DAF-9::GFP expression: Each worm was imaged using both Nomarski and fluorescence using a CoolSnap HQ camera (Photometrics , Tuscon AZ.) run through Metamorph software (MDS Analytical Technologies, Toronto, ON). 4-6 worms were imaged per time period at 5 ms per Nomarski image and 400 ms per fluorescence image. Worms were straightened computationally, normalized to length and mean grey value was quantified using custom software written in Matlab (see SOM for details).

Hypodermal p*daf-9* expression

Different concentrations of DA were added to NG agar plates, seeded with OP50. One day later, ten reproductive *daf-9(dh6) pdaf-9::gfp* adults (grown in the presence of 250nM DA) were placed on each plate for egg laying. F1 progeny were scored for hypodermal *daf-9* expression levels and dauer, molting, and gonadal cell migration phenotypes. Experiments were performed at 20°C, and repeated at least twice. The GFP fluorescence was imaged through a Zeiss Axio Imager Z1 and photographed with

an AxioCam MRm camera. Pixel intensity over a fixed area was measured with AxioVision 4.7 software.

Worm preparation for mRNA analysis

Synchronous populations of worms were grown at 20°C either in favorable (2 worms per µl, 15 mg/ml HB101) or in unfavorable conditions (3% pheromone v/v, 2 worms per µl, 15 mg/ml HB101). At each time point, 10^4 worms were washed 3 times in S basal medium without cholesterol (pH=6) to decrease bacterial load and to wash off excess pheromone. Samples were concentrated in 100 µl volume and suspended with 1 ml TRIzol reagent (Invitrogen, USA) and mixed with 0.6 µl/ml Linear Poly acrylamide, used as a carrier (Baugh et al., 2001) flash frozen using liquid nitrogen and stored at -80°C until processed.

RNA Isolation

RNA purification using TRIzol was adapted from the manufacturer's protocol and is described in the SOM. RNA was subjected to quality control by Nanodrop spectrophotometry (A260/280 ratio) and Agilent Bioanalyser (S28 to S18 ratio). Samples were processed if A260/280 ratio was above 1.9, and S28 to S18 ratio was above 1.8. RNA was digested with RNase-free DNase (Ambion, Austin, TX) according to the manufacturer's instructions. Total RNA was made into cDNA by reverse transcription reaction using Superscript III (Invitrogen, San Diego, CA). mRNA was selected for reverse transcription by using oligo dT primers. Reactions containing no reverse transcriptase were carried out in parallel. cDNA was purified on silica columns (Qiagen, Venlo, Netherlands) and diluted to 16 ng/µl for subsequent qPCR analysis.

qPCR experimental design and analysis

daf-9 transcripts were analyzed with three pairs of primers spanning different exons according to the WS190 gene model (http://ws190.wormbase.org). Each of the three amplicons was between 115-183 bp in length and included sequence from two exons (Table S2). All qPCR reactions were prepared using Roche SYBR Green I Master (Roche Diagnostics) and carried out in a Roche Lightcycler LC480. Data analysis was performed according to the ΔΔCt method (Livak and Schmittgen, 2001). Efficiency values of each primer set were empirically determined by performing a dilution series on pooled cDNA. Transcripts were analyzed if they crossed the Ct threshold before 34 cycles. Control genes were determined empirically by measuring gene expression that did not change significantly (Pearson correlation > 0.995) during larval development (L1 through L4) and dauer. *daf-9* relative abundance was determined as follows: for mRNA processed from worms grown in favorable conditions, *daf-9* was normalized to the geometric mean of control genes *pmp-3* and- *Y45F10D.4* (Hoogewijs et al., 2008). mRNA processed from worms grown in unfavorable conditions was normalized to relative abundance levels of *ver-2*, a gene expressed only in the ADL neurons (Popovici et al., 2002). *daf-9* fold change was determined by normalizing all time points to relative abundance in the L1 stage. Error bars represent mean fold change ± standard deviation across 2 technical replicates originating from 3 biological replicates (6 data points).

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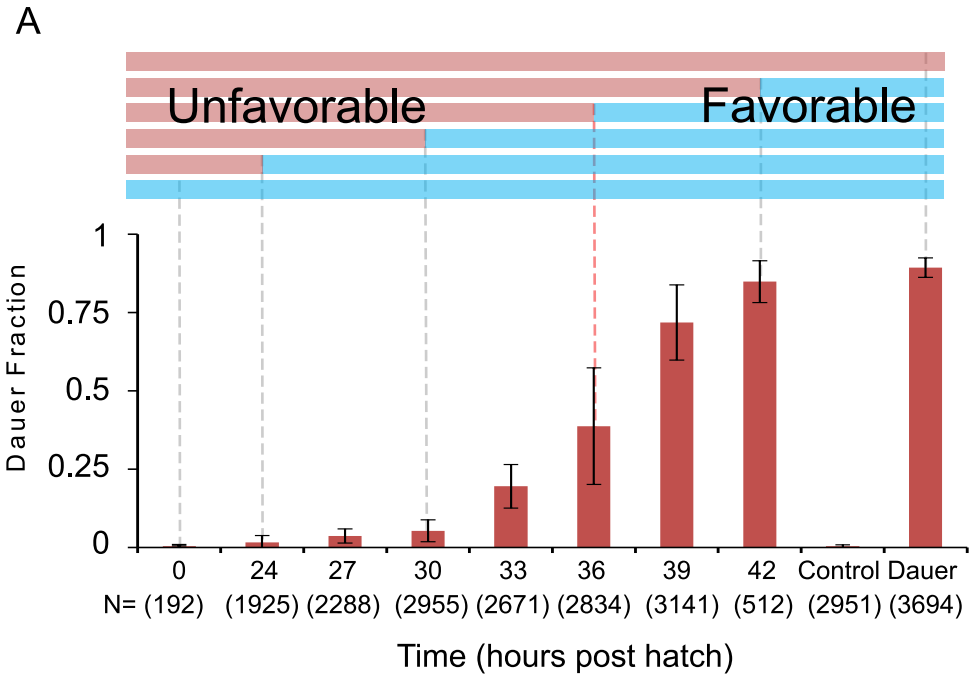
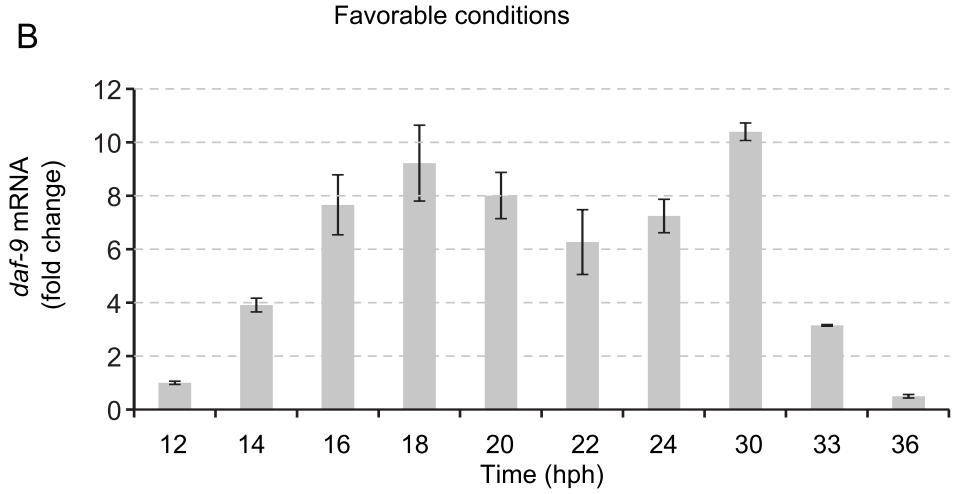
Figures

Figure 4.1


A
Unfavorable
Favorable
Dauer Fraction
1
0.75
0.5
0.25
0
0 24 27 30 33 36 39 42 Control Dauer
N= (192) (1925) (2288) (2955) (2671) (2834) (3141) (512) (2951) (3694)
Time (hours post hatch)


Favorable conditions
B
daf-9 mRNA
(fold change)
12
10
8
6
4
2
0
12 14 16 18 20 22 24 30 33 36
Time (hph)

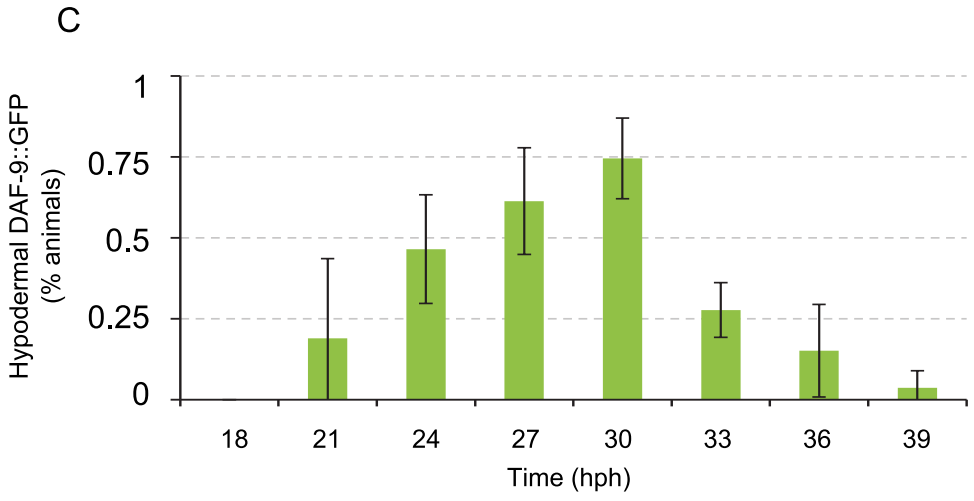

C
Hypodermal DAF-9::GFP
(% animals)
1
0.75
0.5
0.25
0
18 21 24 27 30 33 36 39
Time (hph)

Figure 4.1 daf-9 is transcriptionally upregulated in the hypodermis in favorable conditions.

(A) Slower dynamics in the commitment of strain AA277; *lin-15(n765), dhIs64[daf-9::GFP, lin-15(+)]*. Top, representative colored bars indicating the experimental paradigm of shift: red bars indicate unfavorable conditions and blue bars indicate favorable conditions. Bottom; bars indicate means of dauer frequencies ± standard deviations between biological replicates. Numbers in parentheses indicate total worms counted per time point. (B) Growth in favorable conditions; Fold change of total *daf-9* transcripts quantified by qPCR during development of synchronized broods (see Experimental Procedures, Table S2). Bars represent means ± standard deviations of fold change between biological replicates. (C) Fraction of worms expressing hypodermal *daf-9* during development in favorable conditions. Bars represent means ± standard deviations of population fraction between biological replicates. N> 200 per time point.

Figure 4.2

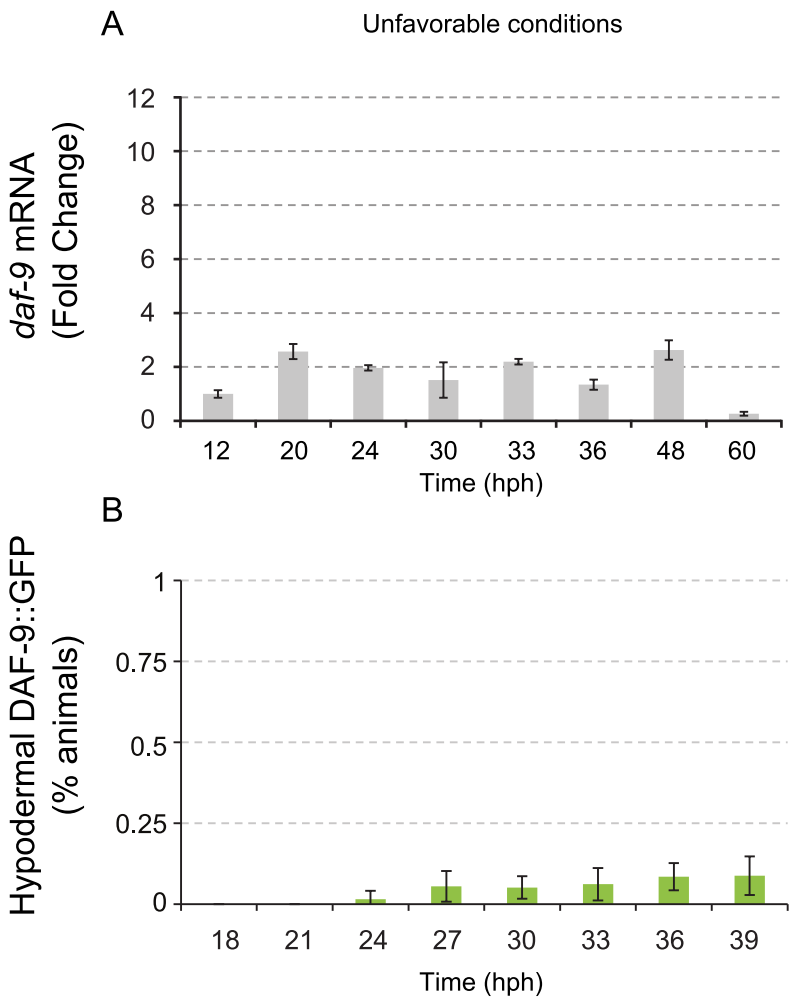

A
Unfavorable conditions
daf-9 mRNA
(Fold Change)
12
10
8
6
4
2
0
12
20
24
30
33
36
48
60
Time (hph)
B
Hypodermal DAF-9::GFP
(% animals)
1
0.75
0.5
0.25
0
18
21
24
27
30
33
36
39
Time (hph)

Figure 4.2: Hypodermal *daf-9* is not expressed during L2d stage.

(A) Growth in favorable conditions; Fold change of total *daf-9* transcripts quantified by qPCR during development of synchronized broods (see Experimental Procedures, Table S2). Bars represent means ± standard deviations of fold change between biological replicates. (B) Fraction of worms expressing hypodermal *daf-9* during development in favorable conditions. Bars represent means ± standard deviations of population fraction between biological replicates. N> 200 per time point.

Figure 4.3

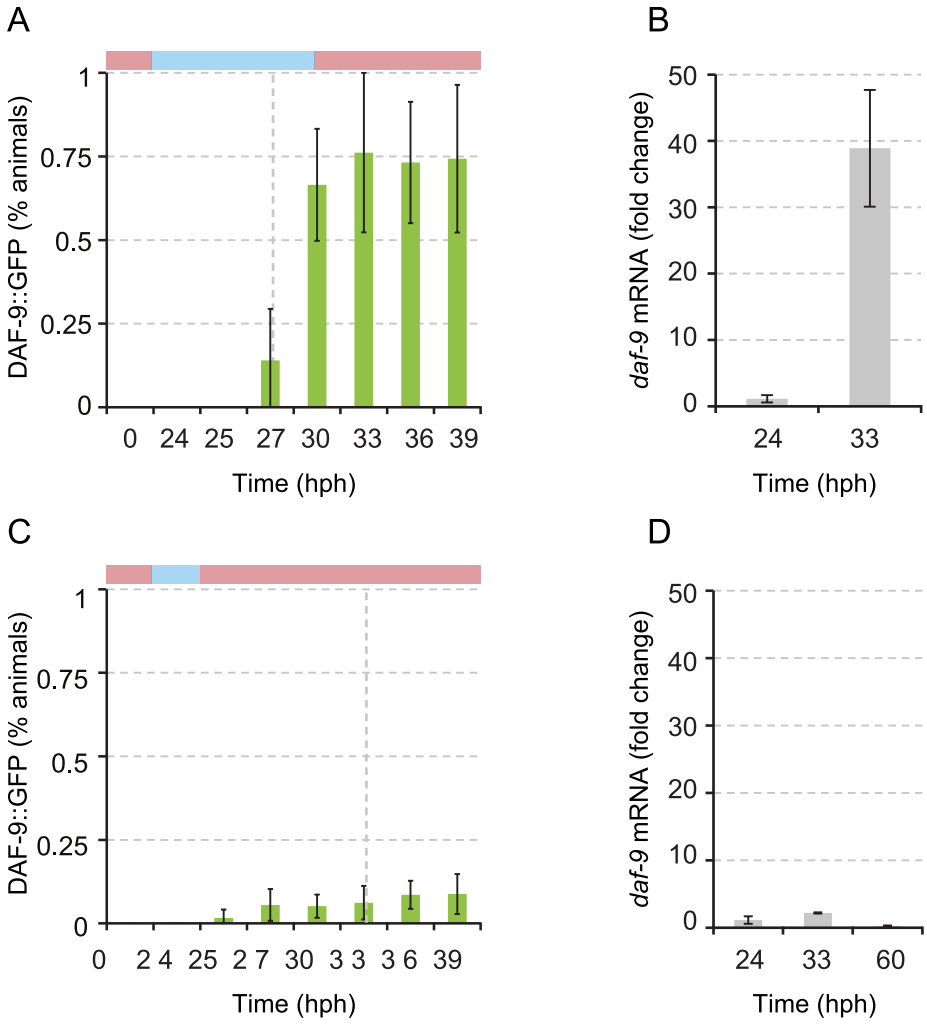

A
DAF-9::GFP (% animals)
1
0.75
0.5
0.25
0
0 24 25 27 30 33 36 39
Time (hph)
B
daf-9 mRNA (fold change)
50
40
30
20
10
0
24 33
Time (hph)
C
DAF-9::GFP (% animals)
1
0.75
0.5
0.25
0
0 2 4 25 2 7 30 3 3 3 6 39
Time (hph)
D
daf-9 mRNA (fold change)
50
40
30
20
10
0
24 33 60
Time (hph)

Figure 4.3: Hypodermal *daf-9* is upregualted after a 3 hour long shift from unfavorable to favorable conditions.

Hypodermal expression in worms grown in pheromone (red) and shifted to favorable conditions for a specified time window (blue) and shifted back to unfavorable conditions: (A) 6 hours with a dauer frequency of 7% ± 5%, (C) 1 hour with a dauer frequency of 90% ± 10%. Bars represent the average of 3 biological replicates, ± standard deviation. N> 200 per time point. (B,D) Fold change of total *daf-9* transcripts quantified by qPCR when worms are shifted from unfavorable to favorable conditions (B), or maintained in unfavorable conditions (D). Bars represent means ± standard deviations of fold change between biological replicates.

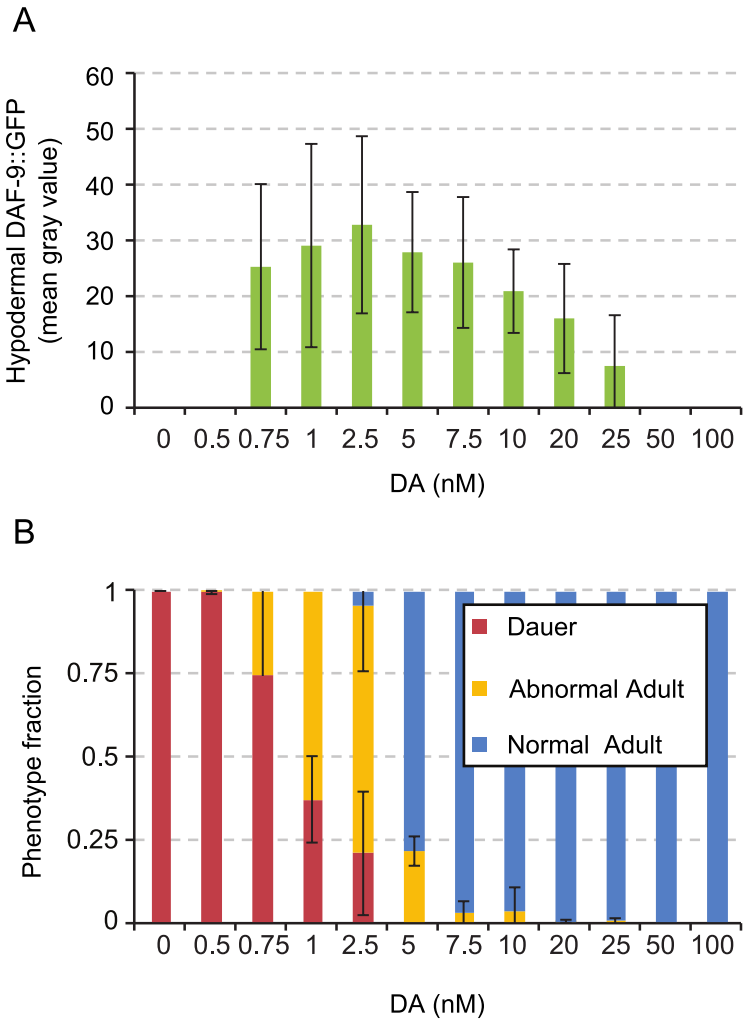

A
Hypodermal DAF-9::GFP
(mean gray value)
60
50
40
30
20
10
0
0 0.5 0.75 1 2.5 5 7.5 10 20 25 50 100
DA (nM)
B
Phenotype fraction
1
0.75
0.5
0.25
0
Dauer
Abnormal Adult
Normal Adult
0 0.5 0.75 1 2.5 5 7.5 10 20 25 50 100
DA (nM)

Figure 4.4: Exogenous DA can initiate hypodermal *daf-9* upregulation in the concentrations required for overcoming developmental defects.

Δ7-DA regulates hypodermal *daf-9* transcription and development. *daf-9(dh6)* animals carrying the a *daf-9* promotor construct fused to *gfp* (*pdaf-9::gfp*) were grown on 0 to 100nM DA. (A) Hypodermal GFP intensity. Animals grown in the presence of 0 to 0.5 DA show no hypodermal *daf-9* expression. Higher DA levels (0,75 to 10nM DA) result in strong hypodermal expression, whereas higher levels (50nM) abolish expression. Each bar represents the pixel intensity of a fixed area of the hypodermis of a single animal. (B) Phenotypic distribution of *daf-9(dh6)* worms expressing hypodermal *daf-9* when exposed to different concentrations of DA. Hypodermal upregulation is visible only at concentrations of DA that give rise to abnormal adults. Red; dauer. Yellow; Abnormal adults (molt, Cut and Mig phenotypes). Blue; normal adults. Bars represent means ± standard deviations between 3 biological replicates. N> 200 for each concentration of DA.

Figure 4.5

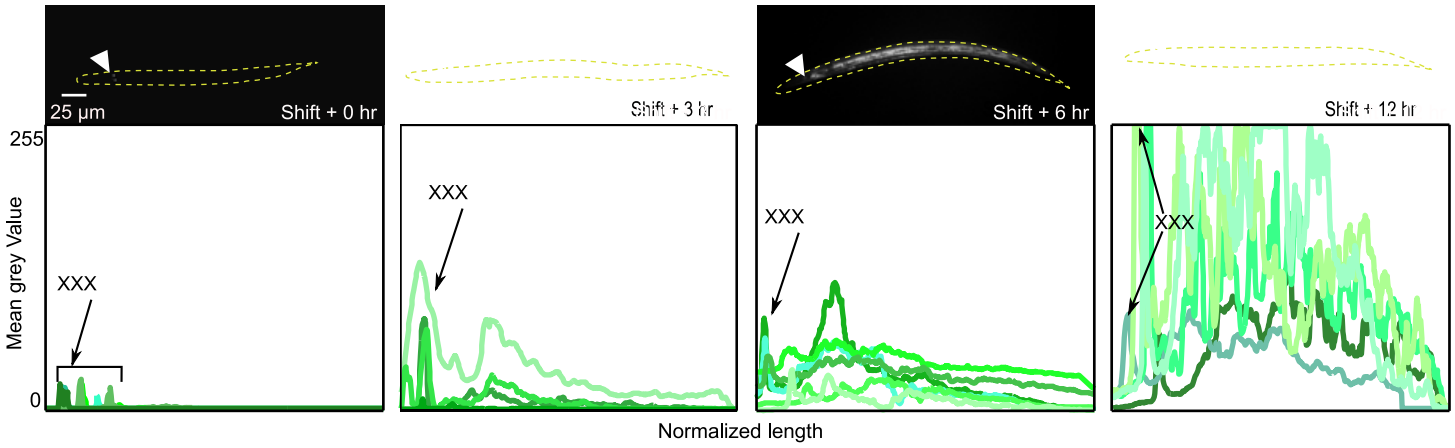

25 µm
Shift + 0 hr
Shift + 3 hr
Shift + 6 hr
Shift + 12 hr
255
0
Mean grey Value
XXX
XXX
XXX
XXX
Normalized length

Figure 4.5. Hypodermal daf-9 expression propagates from anterior to posterior upon commitment to the L3 fate.

Top; Fluorescent images of worms at each time point are shown at shift from unfavorable to favorable at 0 (24 hph) hours (leftmost image) through 12 hph (rightmost image). Arrowheads mark the XXX cells. Bottom: Expression of hypodermal *daf-9* was quantified along the anterior posterior axis in 4-6 worms in each time point. Each green shaded histogram represents the mean grey value of DAF-9::GFP per worm, normalized to length. Different worms were imaged at each time point (See SOM for details of analysis).

Figure 4.6

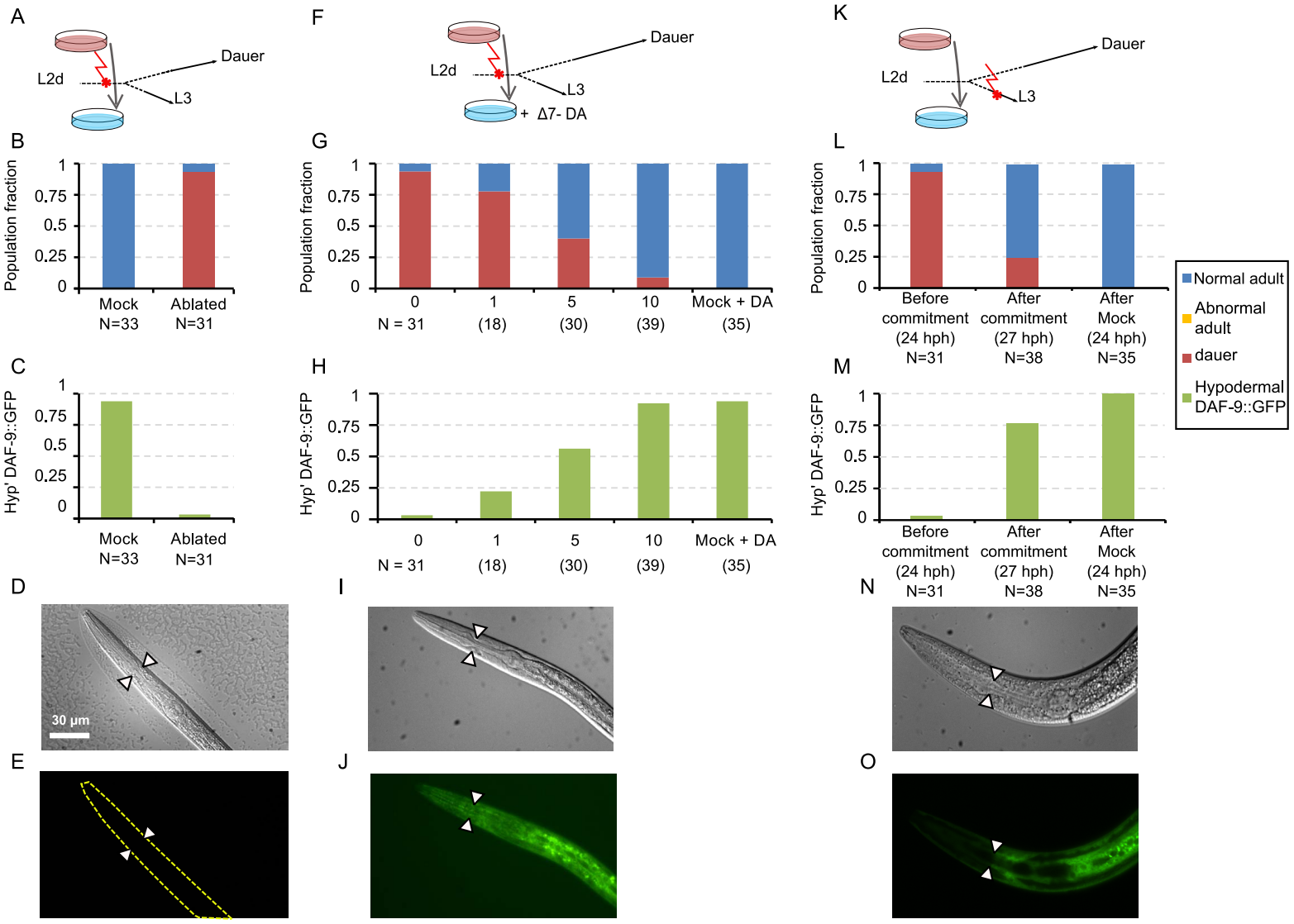

A
L2d
Dauer
L3
F
L2d
Dauer
L3
+ Δ7- DA
K
L2d
Dauer
L3
B
Population fraction
1
0.75
0.5
0.25
0
Mock
N=33
Ablated
N=31
G
Population fraction
0
N = 31
1
(18)
5
(30)
10
(39)
Mock + DA
(35)
L
Population fraction
Before commitment (24 hph) N=31
After commitment (27 hph) N=38
After Mock (24 hph) N=35
Normal adult
Abnormal adult
dauer
Hypodermal DAF-9::GFP
C
Hyp' DAF-9::GFP
Mock
N=33
Ablated
N=31
H
Hyp' DAF-9::GFP
M
Hyp' DAF-9::GFP
D
30 µm
I
N
E
J
O

Figure 4.6: XXX cells act as the source of DA.

(A-E) XXX cells and Δ7-DA are required to initiate hypodermal daf-9 expression and reproductive development. (A) Experimental diagram; Worms were grown to L2d stage in unfavorable conditions. At 24 hph, worms were ablated and let to recover in favorable conditions. (B) Bars indicate the population fraction of dauers and adults when ablated or mock ablated. (C) Bars indicate the population fraction of worms expressing hypodermal DAF-9::GFP in ablated and mock ablated animals. (D and E) DIC and Fluorescence images of a dauer with ablated XXX cells (arrowheads). Note that there is no expression in either cells. (F) Experimental diagram; Worms were grown to L2d stage in unfavorable conditions. At 24 hph, worms were ablated and let to recover in favorable conditions with increasing amounts of exogenous DA. (G) Bars indicate the population fraction of dauers and adults when ablated or mock ablated. All worms expressing hypodermal *daf-9* developed into normal adults with no Mig or Cut phenotypes. (H) Bars indicate the population fraction of worms expressing hypodermal DAF-9::GFP in ablated and mock ablated animals. (I and J) DIC and Fluorescence images of a dauer with ablated XXX cells (arrowheads). Note that there is no expression in XXX cells but in the hypodermis alone. (K) Experimental diagram; Worms were grown to L2d stage in unfavorable conditions and were shifted to favorable conditions at 24 hph. At 27 hph (after committing to adult development), worms were ablated and let to recover in favorable conditions. (L) Bars indicate the population fraction of dauers and adults when ablated or mock ablated. (M) Bars indicate the population fraction of worms expressing hypodermal DAF-9::GFP in ablated and mock

ablated animals. (N and O) DIC and Fluorescence images of a dauer with ablated XXX cells (arrowheads). Note that there is no expression in XXX cells but in the hypodermis alone. * $P<1x10^{-4}$, ** $P<1x10^{-10}$.

Figure 4.7

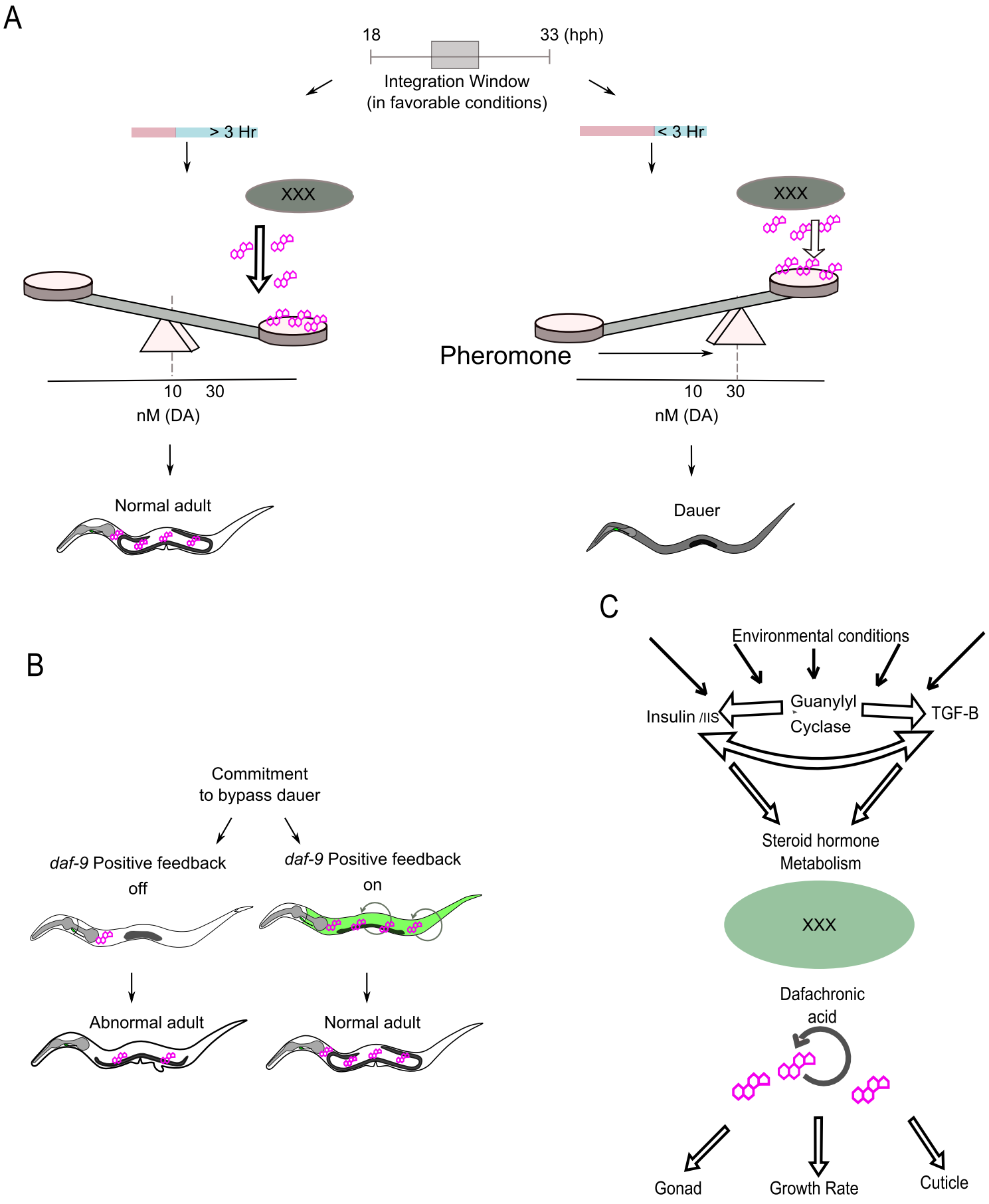

A
18
33 (hph)
Integration Window
(in favorable conditions)
> 3 Hr
< 3 Hr
XXX
XXX
Pheromone
10 30
nM (DA)
10 30
nM (DA)
Normal adult
Dauer
B
Commitment
to bypass dauer
daf-9 Positive feedback
off
daf-9 Positive feedback
on
Abnormal adult
Normal adult
C
Environmental conditions
Insulin /IIS
Guanylyl
Cyclase
TGF-B
Steroid hormone
Metabolism
XXX
Dafachronic
acid
Gonad
Growth Rate
Cuticle

Figure 4.7. A feedback loop amplifies a DA signal leading to coordinate development

Integration of environmental conditions and DA in the dauer decision: (A) top; A 3 hour period in favorable conditions decreases the dauer bypass DA threshold. XXX cells that make a sufficient amount of DA will pass the dauer bypass DA threshold, committing to adulthood, while worms with XXX cells that do not cross the threshold will commit to dauers. I modeled the dauer bypass DA threshold mechanism as a scale with a fulcrum that is modulated by pheromone. The pheromone pushes the fulcrum to the right thus increasing the amount of DA necessary to flip the scale and commit to adulthood.

(B) Commitment to adulthood triggers upregulation of hypodermal *daf-9* thus increasing the amounts of DA available thus coordinating the fate over the whole animal. If DA levels are high enough to pass the dauer bypass DA threshold, but the hypodermal *daf-9* positive feedback loop is not on (*daf-9* mutants) then worms develop into incomplete adults.

(C) Noisy and uncertain environmental information is measured by sensory neurons and reduced in complexity into the four signaling pathways. Information complexity is reduced further into the XXX cells and subsequently into a single diffusible hormonal regulator, DA. Amplification of DA then leads to orchestrated development of tissues.