DYNAMIC REGULATION OF THE DAUER DECISION

Thesis by

Oren Noah Schaedel

In Partial Fulfillment of the Requirements for the

degree of

Doctor of Philosophy

CALIFORNIA INSTITUTE OF TECHNOLOGY

Pasadena, California

2011

(Defended May 11th 2011)

ACKNOWLEDGEMENTS

I would first like to thank my advisor Paul Sternberg. His passion for science, accessibility and his effort in pushing science forward is immeasurable. Also, his patience with my half baked ideas has given me much confidence in becoming an independent researcher. I would like to thank my thesis committee for their assistance through my Ph.D. Their advice has helped me both in technique and direction.

There are many people in the Sternberg lab and Wormbase whose help was immeasurable. My lab mates in general in creating a wonderful working environment. My bay mates Alon Zaslaver Julie Choe, Erich Schwartz and Marjie Ho, who have bounced back ideas and comments. There are two people in particular: Ryan Baugh who nourished my rigorous side of experimental discipline, and Andrea Choe for endlessly trying to help my challenged sense of esthetics. I would like to thank the people from the Genome expression center, Lorian Schaeffer and Igor Antoshechkin for their help with qPCR and sequencing experiments along with Ali Mortazavi. My collaborators Dr. Adam Antebi and Dr. Birgit Gerisch for their help with strains reagents and discussions, turning the paper into what it is today.

Throughout these years of solitary bench work and analysis, there were several people from outside of Caltech who helped reinforce the motivation for scientific story telling. My friends at Mindshare and Syynlabs have given me the opportunity to bring the beauty of scientific discipline away from the lab and into the general public. The variety of talent personality and character in our community, is too great to mention individually. You all know who you are. Those adventures were priceless and have shaped my motivation deeply.

Last but not least, I would like to thank my close friends and family. Or and Keren Yogev who were my family for the first year away from home. My friends Yoni and Ma'ayan Schwarzkopf, Jonathan and Kayla Sternberg who always made me feel at home, far away from home. My parents Greg and Bruria Schaedel for teaching me to never be happy with half truths and My brother Ilan and sister in law, Melissa for their endless support through thick and thin.

ABSTRACT

Many animals can choose between different developmental fates to maximize fitness. Despite the complexity of environmental cues and life history, different developmental fates are executed in a robust fashion. The mechanisms that guarantee robust execution of a development choice in such environments remain unknown. The nematode Caenorhabditis elegans serves as a powerful model to examine this phenomenon because it has an advanced toolkit for cellular and genetic manipulations, and can adopt one of two developmental fates depending on environmental conditions. Nematodes grown in favorable conditions (sufficient food, low population density) develop into adults, whereas nematodes grown in unfavorable conditions (insufficient food, high population density) arrest development as a stress-resistant diapause form called dauer.

The steroid hormone dafachronic acid (DA), product of DAF-9/cytochrome P450, directs development to adulthood by regulating the transcriptional activity of the nuclear hormone receptor DAF-12. The known role of DA suggests that it may be the molecular mediator of environmental condition effects on the developmental fate decision, although the mechanism is yet unknown. We hypothesize that information from the environment is integrated and reduced to a single cell nonautonomous environmental integrator, thereby explaining the tight binary nature of the developmental fate decision. We propose a fate coordination mechanism in which production of a small amount of DA is amplified, locking in the adult fate. Using a combination of laser ablations and time lapse image analysis, we demonstrate that upon the decision to become an adult, the XXX neuroendocrine cells act as a source releasing DA. As a result, DAF-12 dependent

expression of *daf-9* in the epidermis is amplified and propagated from anterior to posterior, dispersing high amounts of DA throughout the body. This dispersion of DA drives adult programs in the gonad, epidermis and vulva. Furthermore, we demonstrate that the XXX cells are not necessary for maintaining the adult fate after the signal amplification has started. This indicates that the epidermal amplification also confers the irreversibility of the decision by uncoupling the execution of the decision from the environmental integrator. We propose that this relay serves as a r obust fate-locking mechanism to enforce an organism wide binary decision, despite noisy and complex environmental cues.

TABLE OF CONTENTS

Acknowledgements iii
Abstract v
Table of Contents vii
List of Figures ix
List of Tables xi
Chapter I: Introduction 1
Types of environmental conditions cues and assaults 1
Phenotypic plasticity and polyphenism 3
Sensation measurement and response to environmental conditions 4
Environmental measurement and integration in multicellular organisms 6
Neuroendocrine regulation of polyphenism 6
Hormonal regulation mechanisms 7
Commitment to a specific fate or polyphenism 8
The nematode *C. elegans* as a model organisms for polyphenism 10
Molecular pathways of environmental integration 11
Bibliography 14
Figures 21
Chapter II: Effects of the Environment on the Dauer Decision 26
Introduction 25
Results 30
Calibration of environmental conditions 30
Effects of pheromone on the dauer decision 31
Time of action 31
Effectors of the persistence window 33
Epigenetic effects on pheromone sensitivity 34
Summary 35
Materials and methods 38
Bibliography 40
Figures 44
Chapter III: Effects of the Hormone Dafachronic Acid on the Dauer decision 55
Introduction 55
Results 59
Dose response of *daf-9* loss of function mutants to DA 60
DA effects on developmental rate 61
Intermediate amounts of DA increase phenotypic variation, high amounts decrease it 61
Pheromone levels regulate the adult DA threshold and reproductive development 63
DA time of action 63

Summary 65
Materials and methods 67
Bibliography 69
Figures 73
Chapter IV: Spatiotemporal Regulation of *daf-9* 84
Introduction 84
Results 88
Hypodermal *daf-9* is expresses as a result of reproductive fate decision 88
Exogenous DA can upregulate hypodermal *daf-9* transcription 90
XXX cells act as the source of DA 91
Summary 92
Materials and methods 95
Bibliography 102
Figures 111
Chapter V: Discussion 127
Appendixes 131
Supplementary Figures 133

LIST OF FIGURES

Number *Page*

1.1: Endocrine mechanisms underlying the polyphenic switch in insects 22

1.2: Developmental molt times of *C. elegans* N2 strain 24

2.1: Calibration of liquid culture for development in homogenous environmental conditions 45

2.2: Commitment to dauer or reproductive development 47

2.3: Effectors of the persistence window 49

2.4: Dauer history is reflected in offspring 51

2.5: Effects of environmental conditions on the dauer decision 53

3.1: High amounts of DA are required for complete adult development 74

3.2: An increase in DA leads to canalization of developmental fates 76

3.3: Dauer pheromone regulates the threshold for reproductive adult 78

3.4: Temporal activity of Δ7-DA 80

3.5: Effects of dose and temporal exposure of DA on *daf-9* worms 82

4.1: *daf-9* is transcriptionally upregulated in the hypodermis in favorable conditions 112

4.2: Hypodermal *daf-9* is not expressed during L2d stage 114

4.3: Hypodermal *daf-9* is upregualted after a 3 hour long shift from unfavorable to favorable conditions 116

4.4: Exogenous DA can initiate hypodermal *daf-9* upregulation in t he concentrations required for overcoming developmental defects 118

4.5: Hypodermal daf-9 expression propagates from anterior to posterior upon commitment to the L3 fate 120

4.6: XXX cells act as the source of DA 122

4.7. A feedback loop amplifies a DA signal leading to coordinate development 125

S1.1: High amounts of DA are required for complete adult development, additional *daf-9* alleles.....134
S1.2: Temporal activity of Δ7-DA in an the *e1406* allele.....136

LIST OF TABLES

Number *Page*

S1.1:Values of q statistic calculated by a Tukey type multiple comparison test for differences among variances. 139

S1.2: Oligonuclotides used in quantification of *daf-9* transcripts 142

Chapter 1: Introduction

In response to changes in the environment and physiology, organisms can shift metabolism, switch developmental programs, modulate behavior, or change shape and size, leading to a distribution of phenotypes or phenotypic plasticity (Brockmann, 2001). Phenotypic plasticity can arise from genetic or environmental forces, reshaping populations and diversifying species. Plasticity arising from genetic differences is known as polymorphism and when rare (under 5% penetrance) as a mutation. Plasticity arising from environmental conditions is known as a reaction norm which is characterized by a range of phenotypes, and polyphenism when there are only 2 phenotypes (Gilbert, 2009).

In some cases, polyphenisms 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. I will discuss the challenges that this type decision making system must overcome in the nematode *C. elegans* and generalize by providing similar examples in other organisms.

Types of environmental conditions cues and assaults

Organisms can survive and reproduce in a wide range of environments composed of often multiple and different physiochemical compounds food sources stresses and assaults. Organisms will maximize fitness by selecting a habitat that can offer them the best protection from predators, low competition or abundance of food and nutrition sources and a selection of mating partners. Environments can also be unpredictable to some organisms but predictable to others therefore, an organism that can predict its environment has a higher fitness. In fact, observational and theoretical work in the field of sociobiology have defined two main types of strategies that organisms employ in order to maximize their fitness in an environment, known as r and K selection theory. An r strategist is a species that typically lives in a niche that is subjected to random fluctuations in environmental conditions with resources far from exhaustion. It will survive well if it can use its resources faster than the competition, reproduce quickly and disperse efficiently before a catastrophe hits the niche. These strategists usually have a short life cycle, are quick to reproduce in large numbers and do not rear their broods. On the other hand, 'K strategists' are those that will live in niches that are less random, resources are closer to exhaustion. Therefore, a K strategist will tend to have a longer life span, will have a smaller brood which it will rear and be a specialist in extracting resources from the environment (Wilson, 2000). These strategies are abstractions and represent the idealizations in these types of behaviors, and in reality organisms will classify somewhere between these extremes. This thesis will not deal with sociobiology, sociobiology-theory, nor evolutionary theory, but I will use these terms to lay a framework for the development mechanism that maximize fitness in uncertain and heterogeneous (complex) environments. The importance of r and K strategists will

become clear in the 2nd chapter when I will discuss the critical period, or the integration period during which *C. elegans* integrate food availability, population density and temperature in order to determine what polyphenic trait they will adopt. Phenotypic plasticity is an important adaptation to environmental conditions and while some adaptations may be the result of a physiochemical interaction with a reagent in the environment, polyphenism is a regulated form of adaptation and the forces that shape its regulation and implementation have been selected for in evolution. In my view, polyphenism is a collection of mechanisms or a sub-strategy that can implement or execute the strategy selected by evolution. It is worth therefore to explain the current use of the term and where its definition falls within the context of phenotypic plasticity.

Phenotypic plasticity and polyphenism

Phenotypic plasticity can arise from genetic or environmental forces, reshaping populations and diversifying species. Plasticity arising from genetic differences is known as polymorphism and when rare (under 5% penetrance) as a mutation. Plasticity arising from environmental conditions is known as a reaction norm which is characterized by a range of phenotypes, and polyphenism, when there are only two phenotypes (Gilbert, 2009). For example, the locust *Scistocerca gregaria* morphology is regulated by population density; in low population density they develop into a green morph with short wings and a high population density into a dark morph with larger wings (Tawfik et al., 1999b). Queen formation in the honey bee, *Apis mellifera*, is dependent on a 'royal jelly' a protein-rich food secreted by other workers. If a bee is fed 'royal jelly' for most of its larval life it will develop into a queen otherwise into a worker bee (Rachinsky et al., 1990).

One of the most intriguing aspects observed in polyphenisms across different multi-cellular species is that the inducing environment is different than the selective environment, i.e. the environment that the specific morph is selected for (Nijhout, 2003). For example, the butterfly *Bycyclus anynana* has two wing morphs, a spring bright orange morph and a summer black-white morph adapted to provide camouflage. The induction of both morphs happens in the preceding months (due to a change in photoperiod) before the actual season has begun (Beldade et al., 2002). The inducing environment in itself does not constitute a threat, danger or unfavorable environmental condition, but it usually serves as a predictor for one. Therefore, there usually is a lag between the critical period in the inducing environment to the onset of the favored morph of the selective environment. The lag between the critical period and the morphological change is the time required to initiate the changes necessary for the favored morph, suggesting that an event or set of events during the critical period has a strong correlation with the morphology selected (Nijhout, 2003).

Sensation measurement and response to environmental conditions

The correlation between the inducing period and the selection period does not hold true in single cell organisms such as bacteria and yeast. The bacteria *Bacillus subtilis* responds to starvation and high population density by testing out many developmental alternatives such as increasing motility and competence before committing to sporulation (Stephens, 1998). The *Streptomyces* species sporulate when developing in a starved or fluctuating environment (Chater, 1993).

Organisms have developed several responses to random fluctuation or uncertain environmental conditions. For example, the yeast *S. cerevisiae* is the impulse response to fluctuating environmental conditions. Upon environmental perturbation such as brief heat shock or osmotic stress that does not illicit a specific stress response ~900 genes are regulated in order to return the cell to the previous state (Chechik and Koller, 2009). Random fluctuations in food or other environmental cues which illicit a transcriptionally dependent response are often assessed over a period of time to see if they are real. This type of sensing mechanism is called a persistence detection and will filter out signals of too short a duration and is found in the L-arabinose transcriptional response in *E.coli* (Alon, 2007). Another type of response to random fluctuations is hedge betting, where random cells in a population will initiate gene expression and others will not, increasing the odds of the population as a whole to adapt to the environment (Veening et al., 2008). Most of characterized molecular mechanisms that deal with fluctuating or unpredictable environmental conditions have been performed in single cellular organisms or mammalian cell cultures (Yosef and Regev, 2011) and little is known about molecular mechanisms that regulate uncertain conditions in multi-cellular organisms. The stability or fluctuation of environmental conditions is a relative term; a single cell will sense random changes in the environment on a short timescale while a multi-cellular organism will detect changes on short and long timescales. The gut and epidermis developed in multi-cellular organism, buffer against fluctuations from the environment and create a homeostatic internal environment and therefore are less susceptible to random fluctuations in environmental conditions. Nonetheless, multi-cellular organisms have a longer life span and respond to fluctuations in temperature

(seasons) food and predators (predator-prey cycles), wet and dry seasons etc. Additionally, they have an internal environment which can be targeted by pathogens and therefore the immune system has evolved to face those challenges.

Environmental measurement and integration in multicellular organisms

Organisms adapt to the environment be it biotic or a-biotic, stable or volatile, but how are the environmental cues interpreted in the organisms to elicit a morphological change? The molecular mechanisms that orchestrate the complexity of polyphenism, have been mapped three main categories of responses (Gilbert, 2005). (i) Direct transcriptional regulation, such as a diet effects on methylation patterns (Waterland and Jirtle, 2004). (ii) Direct interaction of an environmental reagent with signal transduction cascades can regulate gene expression. (iii) Effects through the neuroendocrine system. This thesis will concentrate on mechanisms that fall into the latter category.

Neuroendocrine regulation of polyphenism

The communication of environmental cues the body followed by a morphological adaptation have been explored in many organisms and several themes and recurring regulation patterns have emerged. In multi cellular organisms, neurons detect and measure cues (and in the critical period, specific cues that may predict unfavorable conditions) chemicals pheromones, kairomones and mechanical stress from the environment. These measurements are integrated with the information of the internal state of the organism such as fat storages, intact chromosome structure, hormone availability. The neuroendocrine system then signals the rest of the body the proper

physiological response by secreting the relevant hormones. Many environmental conditions are signaled through hormonal regulatory networks and specific environmental cues have elicited conserved hormonal responses as a way to coordinate morphology between tissues. For example, the release of Juvenile Hormone (JH) in hymenopterans (ants, bees, beetles) is a result of high food intake during development which will lead to a queen or a worker (bees) or induce growth of large horns in the dung beetle *Onthophagus taurus* (Emlen and Nijhout, 1999). Polyphenisms that emerge as a function of temperature are regulated by Ecdysone in insects (Nijhout, 2003) and population density is regulated by Corazonin (insects) and glucocorticoid stress hormone in fish (Gilbert, 2009). During development there is a sensitive period of hormonal activity in which it exudes an effect. Addition of hormone during the sensitive period will elicit a future response during the selective period, but will have no effect on the morphology if administered before or after (Nijhout, 2003). For example, exogenous addition of the JH hormone to ants (*M. rubra)* at the sensitive period during development delays metamorphosis leading these ants to grow in size and develop into a queen. Addition of JH after metamorphosis or before the sensitive period results in worker or soldier ants (Brian, 1974).

Hormonal regulation mechanisms

Hormones can act through the neuroendocrine system having systemic effects (Nijhout, 2003). Hormones can diffuse through membranes and act in cells by two main mechanisms; (i) binding to internal nuclear hormone receptors, or (ii) through direct binding to GPCRs in the membrane-mediated steroid signaling pathway (Denver, 2007).

Nuclear hormone receptors (NHR) usually have modular domains such as a ligand binding domain which binds the hormone and a DNA binding domain which recognizes and can bind to specific sequences in the genome. NHRs are usually inactive until they bind the hormone and often homodimerize or heterodimerize when the hormone diffuses into the cell. Thus, the activity of hormones is extremely modular and can be regulated by alternative splicing, cell and tissue specific and temporal transcription of the NHRs, their combination in target tissues and the state of the genome; if the DNA binding sequence is accessible or held closed (Nijhout, 2003; Wollam and Antebi, 2010). Therefore, the hormone sensitive period is a function of the availability and activity of the receptors controlling a specific phenotype and not necessarily that of the hormone. The hormone can be synthesized, released or activated at any moment during the sensitive period, thus diffusing and binding to its receptors. Many types of hormone regulatory mechanisms that regulate different types of polyphenisms rely on the timing and dose of hormone secretion during the sensitive period (Keshan et al., 2006). Several examples from Nijhout (2003) are demonstrated in Figure 1.1.

Commitment to a specific fate or polyphenism

Hormones can coordinate developmental programs throughout the body, yet most traits of polyphenism are irreversible; once committed to one fate, a change in environmental conditions or an exogenous addition of the hormone triggering the specific morph will not change the decision of the morph. For example, the butterfly *Araschnia levana* will develop wing pigmentation which is bright orange in the spring and black in the summer. The summer morph is regulated by photoperiod and temperature which

triggers Ecdysone release for the summer morph alone. If these butterflies are treated with the summer inducing conditions in the spring, the wings will form the summer morph, and upon commitment, will remain the summer morph even if switched back to spring-morph inducing conditions or treated with Ecdysone (Gilbert, 2009). The Marine Goby *Trimma okinawae* can rapidly change its sex, according to specific social cues, more than once in every direction about every 4 days. Once committed to one sex they will develop into it even though the social cues for switching back are present. Irreversible decisions and commitment are recurring features in all kingdoms. Commitment is considered one of the mechanisms that canalize development and render an organism robust from environmental effects (Gibson and Wagner, 2000). Several mechanisms that determine commitment have been described in other model organisms. For example, commitment to sporulation in *B.subtilis* is controlled by a single gene, the master regulator, σ^F , which regulates expression of genes in the forespore and in the mothercell (Dworkin and Losick, 2005). Amplification and positive feedback have been shown to drive commitment in the X*enopus* oocyte. A short pulse of progesterone is necessary to start a signal transduction cascade of phosphorylation. Mos becomes phosphorylated as a result of a progesterone signal which in turn phosphorylates p42 MAPK through MEK. Positive feedback occurs when p42 MAPK phosphorylates Mos in a progesterone independent manner, forming a 'memory module' (Xiong and Ferrell, 2003). Interlocking feedback loops have also been implicated in locking in the 'white' or 'opaque' cell types in *Candida albicans* (Lohse and Johnson, 2010).

There is still debate if the end of the hormone sensitive period is the point of commitment, the point after which a change in environmental conditions does not change the course of development. The larvae of *Oncopeltus fasciatus* and *Pyrrhocoris apterus* treated with threshold levels of JH develop larval pigmentation on patches of their cuticle but adult surface sculpturing (Willis 1982). The metamorphosis from larvae to pupae, or Pupal commitment, 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 ensures commitment to pupae is unknown.

The nematode *c. elegans* as a model organism for polyphenism

There is a large gap between our understanding of spatiotemporal regulation of polyphenism in multi cellular organisms to the detailed network architecture that explains measurement of persistent signals and commitment to a specific polyphenism or trait. The nematode *C. elegans* is an ideal organism for understanding the organismal, cellular and molecular mechanisms that regulate polyphenism. These nematodes are easily grown in laboratory conditions and amenable to facile environmental changes (Lewis, 1995). All of the cellular divisions and locations of cells during post embryonic development have been mapped extensively and easily distinguishable under high powered microscopy techniques (Sulston and White, 1980).

The *c. elegans* genome was the first multi-cellular genome to be sequenced and the first organism to enable transgenic fluorescently labeled proteins expressed within (Chalfie et al., 1994). *C.elegans* nematodes display polyphenism during development. In favorable environments, *C. elegans* develops rapidly through four larval (juvenile) stages (L1-L4) separated by molts, into a sexually reproductive adult (Figure 1.2). However, 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 R iddle, 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).

Molecular pathways of environmental integration

The attributes of mutually exclusive fates and irreversibility imply that a fate locking mechanism underlies dauer formation, yet the cellular and molecular basis of this binary decision is not entirely clear. Environmental cues are detected by sensory neurons, namely ASI, ADF, ASG, ASJ (Bargmann and Horvitz, 1991), ASE (Reiner et al., 2008)

and ASK (Kim et al., 2009; Schackwitz et al., 1996), which by unknown means integrate inputs into hormonal outputs. Genetic analysis of dauer formation has identified dauer constitutive (Daf-c) mutants, which form dauer larvae even in favorable conditions, and dauer defective (Daf-d) mutants, which fail to form dauer larvae even in unfavorable conditions (Albert and Riddle, 1988; Riddle, 1997). Molecular dissection of these loci reveals at least four signaling pathways. Components of neurosensory structure and guanylyl cyclase signaling are involved in sensing temperature, nutritional cues and dauer pheromone (Birnby et al., 2000), which are integrated by Insulin/Insulin-like growth factor (IIS) and TGFβ signaling. These peptide hormone pathways converge on a steroid hormone pathway, which metabolizes dietary cholesterol into several bile acid-like steroids, called the dafachronic acids (DA) (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 (Gerisch et al., 2007; Hannich et al., 2009; Motola et al., 2006; Patel et al., 2008; Rottiers and Antebi, 2006). Notably, liganded DAF-12 promotes reproductive development (Gerisch and Antebi, 2004; Mak and Ruvkun, 2004; Motola et al., 2006), whereas unliganded DAF-12 together with the co-repressor DIN-1S direct the dauer fate (Gerisch et al., 2007; Ludewig et al., 2004; Motola et al., 2006). Thus DAF-12 serves as the switch between reproductive and dauer modes.

The cytochrome P450, DAF-9, works as the last enzyme in the pathway of DA production and is a critical player in the dauer decision. *daf-9* is expressed in two neuroendocrine cells namely XXXL and XXXR from hatch into 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). Consistent with a hormonal mechanism, DAF-9 works cell non-autonomously to control dauer formation (Gerisch and A ntebi, 2004; Mak and R uvkun, 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 epistatic 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 upregulates hypodermal *daf-9* expression in response to upstream inputs (Gerisch and A ntebi, 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 secreted as a result of a decision or as a means for integrating multiple environmental stimuli and genetic inputs controlling the decision. In chapter 2, I describe the effects of population density signal strength, duration and fluctuation on the decision to become dauer. In Chapter 3, I describe the hormone sensitive period of DA and demonstrate that they can act as a diffusible messenger downstream of the decision. I show that high amounts of DA are necessary to coordinate the reproductive growth fate decision throughout the whole animal. In chapter 4, I demonstrate how the amplification of DA in the hypodermis is responsible for the irreversibility of the decision. I propose that hypodermal amplification acts as a fate locking mechanism that enforces the binary decision.

Bibliography

Albert, P.S., and Riddle, D.L. (1988). Mutants of Caenorhabditis elegans that form dauer-like larvae, Vol 126.

Alon, U., ed. (2007). An introduction to systems biology: design principles of biological circuits (London, CRC Press, Taylor & Francis Group).

Antebi, A., Culotti, J.G., and Hedgecock, E.M. (1998). daf-12 regulates developmental age and the dauer alternative in Caenorhabditis elegans, Vol 125.

Antebi, A., Yeh, W.H., Tait, D., Hedgecock, E.M., and R iddle, D.L. (2000). daf-12 encodes a nuclear receptor that regulates the dauer diapause and developmental age in C. elegans. Genes & development *14*, 1512-1527.

Bargmann, C.I., and Horvitz, H.R. (1991). Control of larval development by chemosensory neurons in Caenorhabditis elegans, Vol 251.

Beldade, P., Brakefield, P.M., and Long, A.D. (2002). Contribution of Distal-less to quantitative variation in butterfly eyespots. Nature *415*, 315-318.

Birnby, D.A., Link, E.L., Vowels, J.J., Tian, H.Z., Colacurcio, P.L., and Thomas, J.H. (2000). A transmembrane guanylyl cyclase (DAF-11) and Hsp90 (DAF-21) regulate a common set of chemosensory behaviors in Caenorhabditis elegans, Vol 155.

Braendle, C., Milloz, J., and F elix, M.A. (2008). Mechanisms and ev olution of environmental responses in Caenorhabditis elegans. Curr Top Dev Biol *80*, 171-207.

Brian, M.V. (1974). Caste differentiation in Myrmica rubra: the role of hormones. J Insect Physiol *20*, 1351-1365.

Brockmann, H.J. (2001). The evolution of alternative strategies and tactics. Advances in study of behavior *30*, 1-51.

Byerly, L., Cassada, R.C., and Russell, R.L. (1976). The life cycle of the nematode Caenorhabditis elegans. I. Wild-type growth and reproduction. Developmental biology *51*, 23-33.

Cassada, R.C., and Russell, R.L. (1975). The dauerlarva, a post-embryonic developmental variant of the nematode Caenorhabditis elegans. Developmental biology *46*, 326-342.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W.W., and Prasher, D.C. (1994). Green Fluorescent Protein as a Marker for Gene-Expression. Science *263*, 802-805.

Chater, K.F. (1993). Genetics of differentiation in Streptomyces. Annu Rev Microbiol *47*, 685-713.

Chechik, G., and Koller, D. (2009). Timing of gene expression responses to environmental changes. J Comput Biol *16*, 279-290.

Denver, R.J. (2007). Endocannabinoids link rapid, membrane-mediated corticosteroid actions to behavior. Endocrinology *148*, 490-492.

Dworkin, J., and Losick, R. (2005). Developmental commitment in a bacterium. Cell *121*, 401-409.

Emlen, D.J., and Nijhout, H.F. (1999). Hormonal control of male horn length dimorphism in the dung beetle Onthophagus taurus (Coleoptera: Scarabaeidae). J Insect Physiol *45*, 45-53.

Gerisch, B., and Antebi, A. (2004). Hormonal signals produced by DAF-9/cytochrome P450 regulate C. elegans dauer diapause in response to environmental cues. Development (Cambridge, England) *131*, 1765-1776.

Gerisch, B., Rottiers, V., Li, D., Motola, D.L., Cummins, C.L., Lehrach, H., Mangelsdorf, D.J., and Antebi, A. (2007). A bile acid-like steroid modulates Caenorhabditis elegans lifespan through nuclear receptor signaling. Proceedings of the National Academy of Sciences of the United States of America *104*, 5014-5019.

Gerisch, B., Weitzel, C., Kober-Eisermann, C., Rottiers, V., and Antebi, A. (2001). A hormonal signaling pathway influencing C. elegans metabolism, reproductive development, and life span. Developmental cell *1*, 841-851.

Gibson, G., and Wagner, G. (2000). Canalization in evolutionary genetics: a stabilizing theory? Bioessays *22*, 372-380.

Gilbert, S.F. (2005). Mechanisms for the environmental regulation of gene expression: ecological aspects of animal development. J Biosci *30*, 65-74.

Gilbert, S.F., Epel, D. (2009). Ecological developmental biology; Integrating Epigenetics, medicine, and Environment (Sinauer Associates Inc.).

Golden, J.W., and Riddle, D.L. (1982). A pheromone influences larval development in the nematode Caenorhabditis elegans. Science *218*, 578-580.

Golden, J.W., and Riddle, D.L. (1984). The Caenorhabditis elegans dauer larva: developmental effects of pheromone, food, and temperature. Developmental biology *102*, 368-378.

Hannich, J.T., Entchev, E.V., Mende, F., Boytchev, H., Martin, R., Zagoriy, V., Theumer, G., Riezman, I., Riezman, H., Knolker, H.J.*, et al.* (2009). Methylation of the sterol

nucleus by STRM-1 regulates dauer larva formation in Caenorhabditis elegans. Developmental cell *16*, 833-843.

Jia, K., Albert, P.S., and Riddle, D.L. (2002). DAF-9, a cytochrome P450 regulating C. elegans larval development and adult longevity. Development (Cambridge, England) *129*, 221-231.

Keshan, B., Hiruma, K., and R iddiford, L.M. (2006). Developmental expression and hormonal regulation of different isoforms of the transcription factor E75 in the tobacco hornworm Manduca sexta. Developmental biology *295*, 623-632.

Kim, K., Sato, K., Shibuya, M., Zeiger, D.M., Butcher, R.A., Ragains, J.R., Clardy, J., Touhara, K., and Sengupta, P. (2009). Two Chemoreceptors Mediate Developmental Effects of Dauer Pheromone in C. elegans. Science.

Klass, M., and Hirsh, D. (1976). Non-ageing developmental variant of Caenorhabditis elegans. Nature *260*, 523-525.

Koyama, T., Syropyatova, M.O., and Riddiford, L.M. (2008). Insulin/IGF signaling regulates the change in commitment in imaginal discs and primordia by overriding the effect of juvenile hormone. Developmental biology *324*, 258-265.

Lewis, J.A., J.T. Fleming. (1995). Caenorhabditis elegans: Modern biological analysis of an organism

In Methods in Cell Biology (USA)

H.F. Epstein, D. C.Shakes, , Ed., ed. (San Diego, Academic Press), pp. 4-29.

Lohse, M.B., and Johnson, A.D. (2010). Temporal anatomy of an epigenetic switch in cell programming: the white-opaque transition of C. albicans. Mol Microbiol *78*, 331-343.

Ludewig, A.H., Kober-Eisermann, C., Weitzel, C., Bethke, A., Neubert, K., Gerisch, B., Hutter, H., and Antebi, A. (2004). A novel nuclear receptor/coregulator complex controls C. elegans lipid metabolism, larval development, and aging. Genes & development *18*, 2120-2133.

Mak, H.Y., and Ruvkun, G. (2004). Intercellular signaling of reproductive development by the C. elegans DAF-9 cytochrome P450. Development (Cambridge, England) *131*, 1777-1786.

Motola, D.L., Cummins, C.L., Rottiers, V., Sharma, K.K., Li, T., Li, Y., Suino-Powell, K., Xu, H.E., Auchus, R.J., Antebi, A.*, et al.* (2006). Identification of ligands for DAF-12 that govern dauer formation and reproduction in C. elegans. Cell *124*, 1209-1223.

Nijhout, H.F. (2003). Development and evolution of adaptive polyphenisms. Evol Dev *5*, 9-18.

Patel, D.S., Fang, L.L., Svy, D.K., Ruvkun, G., and Li, W. (2008). Genetic identification of HSD-1, a c onserved steroidogenic enzyme that directs larval development in Caenorhabditis elegans. Development (Cambridge, England) *135*, 2239-2249.

Rachinsky, A., Strambi, C., Strambi, A., and Hartfelder, K. (1990). Caste and metamorphosis: hemolymph titers of juvenile hormone and ecdysteroids in last instar honeybee larvae. Gen Comp Endocrinol *79*, 31-38.

Reiner, D.J., Ailion, M., Thomas, J.H., and Meyer, B.J. (2008). C. elegans anaplastic lymphoma kinase ortholog SCD-2 controls dauer formation by modulating TGF-beta signaling. Curr Biol *18*, 1101-1109.

Riddle, D.L., Albert, P., ed. (1997). Genetic and environmental regulation of dauer larva development (New York, Cold Spring Harbor Laboratory Press).

Rottiers, V., and Antebi, A. (2006). Control of Caenorhabditis elegans life history by nuclear receptor signal transduction. Experimental gerontology *41*, 904-909.

Schackwitz, W.S., Inoue, T., and Thomas, J.H. (1996). Chemosensory neurons function in parallel to mediate a pheromone response in C. elegans. Neuron *17*, 719-728.

Stephens, C. (1998). Bacterial sporulation: a question of commitment? Curr Biol *8*, R45-48.

Sulston, J.E., and White, J.G. (1980). Regulation and cell autonomy during postembryonic development of Caenorhabditis elegans. Developmental biology *78*, 577-597.

Tawfik, A.I., Vedrova, A., and Sehnal, F. (1999). Ecdysteroids during ovarian development and embryogenesis in solitary and gregarious schistocerca gregaria. Arch Insect Biochem Physiol *41*, 134-143.

Veening, J.W., Stewart, E.J., Berngruber, T.W., Taddei, F., Kuipers, O.P., and Hamoen, L.W. (2008). Bet-hedging and epi genetic inheritance in bacterial cell development. Proceedings of the National Academy of Sciences of the United States of America *105*, 4393-4398.

Waterland, R.A., and Jirtle, R.L. (2004). Early nutrition, epigenetic changes at transposons and imprinted genes, and enhanced susceptibility to adult chronic diseases. Nutrition *20*, 63-68.

Wilson, E.O. (2000). Sociobiology: The New Synthesis, Twenty-Fifth Anniversary Edition (Belknap Press of Harvard University Press).

Wollam, J., and Antebi, A. (2010). Sterol Regulation of Metabolism, Homeostasis, and Development. Annu Rev Biochem.

Xiong, W., and Ferrell, J.E., Jr. (2003). A positive-feedback-based bistable 'memory module' that governs a cell fate decision. Nature *426*, 460-465.

Yosef, N., and Regev, A. (2011). Impulse control: temporal dynamics in gene transcription. Cell *144*, 886-896.

Figures

Figure 1.1

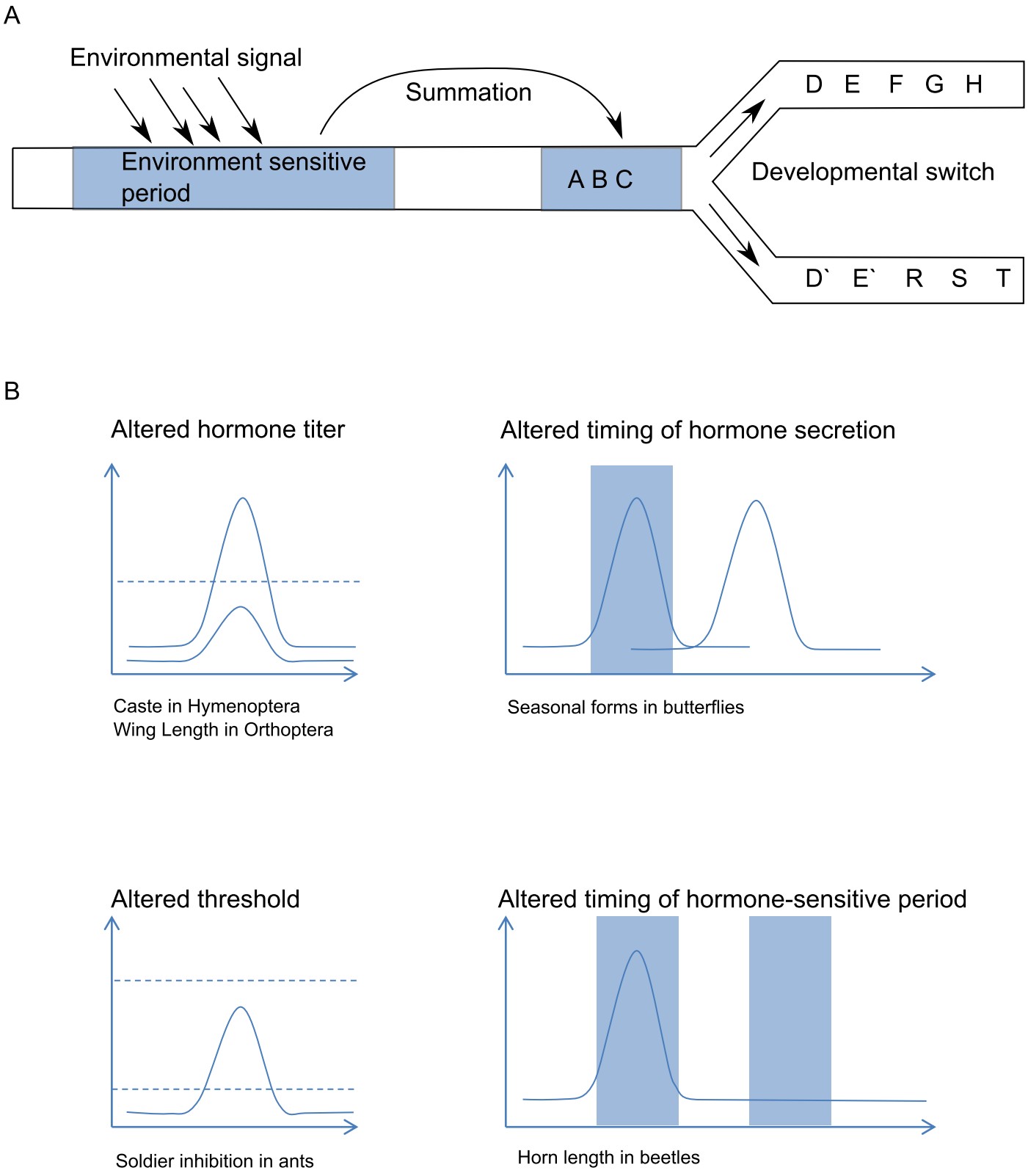

A
Environmental signal
Summation
Environment sensitive period
A B C
D E F G H
Developmental switch
D` E` R S T
B
Altered hormone titer
Caste in Hymenoptera
Wing Length in Orthoptera
Altered timing of hormone secretion
Seasonal forms in butterflies
Altered threshold
Soldier inhibition in ants
Altered timing of hormone-sensitive period
Horn length in beetles

Figure 1.1: Endocrine mechanisms underlying the polyphenic switch in insects

Adapted from Nijhout (2003): (A) Diagram of development and rise of polyphenism: Environment sensitive period is when insects measure and integrate environmental conditions and cues. The results of the integration or summation are manifested in the developmental switch where hormonal regulation mechanisms carry out the programs that give rise to the selected morph. (B): Examples of four developmental switch types that have been identified in insects. In all cases, the hormones act in the hormone-sensitive period, which is presumably dictated by the tissue specific availability of the relevant nuclear hormone receptor. The two morphs that compose the polyphenism are a function of hormone titer being above or below a threshold value during the hormone sensitive period.

Figure 1.2

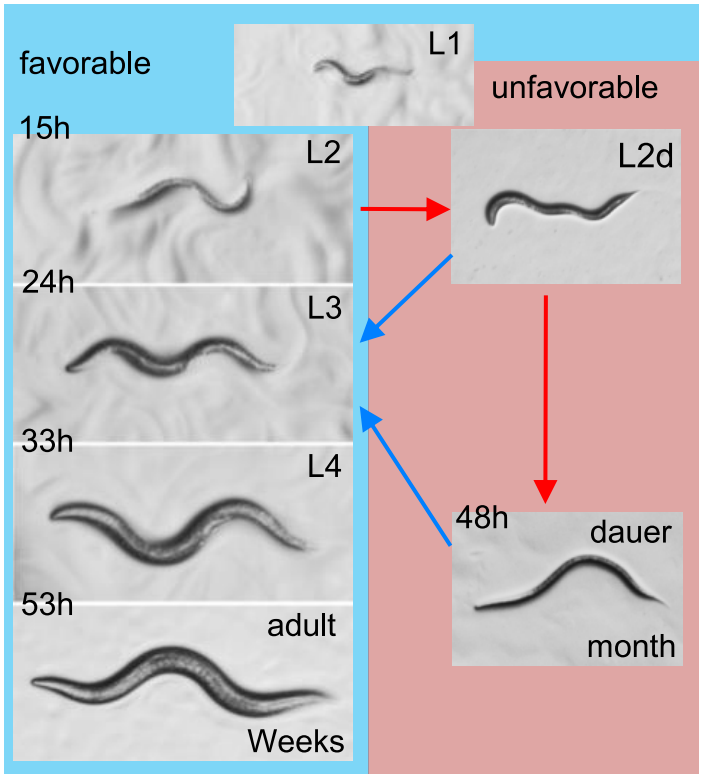

favorable
L1
unfavorable
15h
L2
L2d
24h
L3
33h
L4
48h
dauer
53h
adult
month
Weeks

Figure 1.2: Developmental molt times of *C. elegans* wild-type strain

Developmental molt times of wild type, N2 *C.* elegans larvae growing in favorable (blue) or unfavorable (red) conditions. L2d's exposed to favorable conditions before committing to the dauer fate can resume development through the L3 stage to adulthood (see chapter 1 for details). Dauers exposed to favorable conditions can resume development through the 4th larval stage and growth to reproductive adulthood.

Chapter 2: Effects of the Environment on the Dauer Decision

Introduction

Many organisms can develop into different forms, often displaying different phenotypes, known as phenotypic plasticity. Phenotypic plasticity can emerge from a genetic difference in a population or as a response to different environmental conditions. Variance arising from genetic differences is known as polymorphism and when rare (under 5% penetrance) as a mutation. Phenotypic variance arising from environmental conditions is known as a reaction norm which is characterized by a range of phenotypes, and polyphenism when there are only 2 phenotypes (Gilbert, 2009). Animals measure their environment and make a decision of what morph they will develop into. Robust development is achieved when the genetic makeup of the organism can measure and integrate these environmental cues driving development into a morph best suited for the environmental conditions, thus increasing fecundity. The orchestration of this developmental decision is complex in multi-cellular organisms since the phenotypes of are mutually exclusive; morph A will have a distinct set of cell types and organs that morph B doesn't have, and vice versa. The understanding of genetic programs that guarantee developmental plasticity and mutual exclusivity at the same is poorly understood. For example, the desert locust *Schistocerca gregaria* can a green morph with small wings and a black-yellow morph with long wings. The former is formed under sparse population density and the latter in high population density (Tawfik et al., 1999a).

There are several recurring features in insect based polyphenisms. The inducing environment is different than the selective environment, i.e. the environment that the specific morph is selected for (Nijhout, 2003). The inducing environment in itself does

not constitute a threat, danger or unfavorable environmental condition, but it usually serves as a predictor for one. Therefore, there usually is a lag between the critical period in the inducing environment to the onset of the favored morph of the selective environment. The lag between the critical period and the morphological change is the time required to initiate the changes necessary for the favored morph, suggesting that an event or set of events during the critical period has a strong correlation with the morphology selected (Nijhout, 2003). The molecular mechanisms that correlate between the inducing period to the chosen morph in the selective period are unknown.

Another aspect, is that once committed to one morph, a change in environmental conditions will not change the fate of the morph. These mechanisms have been investigated extensively in single cell organisms such as *B. subtilis* and *Xenopus laevis. cerevisiea*. For example, commitment to sporulation in *B. subtilis* is controlled by the master regulator σ^F , a transcription factor which can transcribe multiple genes in both the forespore and mothercell (Dworkin and Losick, 2005). Amplification and positive feedback have been shown to drive commitment in the X*enopus* oocyte (Xiong and Ferrell, 2003). Molecular mechanisms that lock in the decision of a specific morph in a polyphenic switch are unknown.

The nematode *C.elegans* displays the trait of polyphenism since can develop into a sexually reproductive adult or into a larval state of suspended animation called the dauer (Cassada and Russell, 1975). *C. elegans* is an ideal organism for elucidating molecular mechanisms that shape and regulate polyphenisms since much of the molecular machinery that measures and integrates environmental conditions has been characterized. The decision is a function of environmental conditions: in a favorable

environment, comprised of sufficient food, low population density and moderate temperatures, will develop into a sexually reproductive adult. Conversely, when developing in unfavorable conditions composed of high population density, low food availability and high temperatures, the nematode will develop into the developmentally arrested dauer. The decision to develop into a dauer or adult happens in two stages during development. The first is during the first larval stage when the animal decides whether to develop into the pre-dauer L2d stage or into the L2 stage. The second decision occurs during the L2d stage when the animal decides to develop into a dauer to resume development as an L3 larva. The decision to become an L2, L3 or a dauer is irreversible; once the animals have made the decision, they commit to the specific fate and develop accordingly even if environmental conditions change.

The genetic pathways that convey measurements of food, population density and temperature have been dissected extensively. Genetic analysis of dauer formation has identified dauer constitutive (Daf-c) mutants, which form dauer larvae even in favorable conditions, and dauer defective (Daf-d) mutants, which fail to form dauer larvae even in unfavorable conditions (Albert and Riddle, 1988; Riddle, 1997). Molecular dissection of these loci reveals at least four signaling pathways. Components of neurosensory structure and guanylyl cyclase signaling are involved in sensing temperature, nutritional cues and dauer pheromone (Birnby et al., 2000), which are integrated by Insulin/Insulin-like growth factor (IIS) and TGFβ signaling. These peptide hormone pathways converge on a steroid hormone pathway, which metabolizes dietary cholesterol into several bile acid-like steroids, called the dafachronic acids (DA) (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 (Gerisch et al., 2007; Hannich et al., 2009; Motola et al., 2006; Patel et al., 2008; Rottiers and Antebi, 2006). Notably, liganded DAF-12 promotes reproductive development (Gerisch and Antebi, 2004; Mak and Ruvkun, 2004; Motola et al., 2006), whereas unliganded DAF-12 together with the co-repressor DIN-1S direct the dauer fate (Gerisch et al., 2007; Ludewig et al., 2004; Motola et al., 2006). Thus DAF-12 serves as the switch between reproductive and dauer modes.

The dauer decision has been investigated in constant settings, i.e. food population density and temperature have been held constant. The environmental sensitive period has been characterized between the end of the first molt and the mid L2d (Golden and Riddle, 1984a). We therefore wanted to characterize the environment sensitive period in greater detail by dissecting the effects of strength, duration and fluctuation of the signal conveying population density. We also wanted understand the correlation between the environmental sensitive period (chapter 2) to the hormone sensitive period (chapter 3) and therefore developed a system to compare the two, which is described in detail in this chapter.

Results

Calibration of environmental conditions

Previous experiments determined that worms respond to food pheromone and temperature and integrate them into the dauer decision. These experiments were performed on agar plates that were prepared by mixing a crude pheromone extract with the agar, spotting bacteria as a food source on the dried plate and picking individual worms on to the plates. This technique results in a non-homogenous environment with low number of worms (Golden and Riddle, 1984a). We re-visited these experiments and modified them to liquid culture to increase scale, synchronicity of brood hatch, homogeneity and throughput and measured the frequency of dauer formation in response to dauer pheromone and food concentrations. We calibrated the synchronicity of developing broods in favorable (food with no pheromone) and unfavorable (pheromone and food) conditions as stage frequencies until they were tightly distributed and highly reproducible during early and late time periods of development (Fig – homogeneity). The synchronicity of broods during development in both condition types indicates that liquid culture conditions are homogenous.

In order to understand the points of commitment to dauer L2 and L3, I wanted to understand the values of food and pheromone that would allow growth at a constant food concentration yet amenable to changes in pheromone concentration without effecting dauer fraction and retaining a low standard deviation between technical replicates. To this end I grew worms in a series of food and pheromone concentrations

in 4 biological replicates of each pair of conditions scoring the fraction of dauers and adults in each combination (fig – heat map). I found that an optimal concentration of food is 7.5 mg/ml and pheromone at 6% (v/v) when grown at 20°C at the density of 1 worm per µl. This combination of food and pheromone allowed the entire brood to develop into adulthood when no pheromone was present, and 90% ± 2% dauer formation when 3% pheromone was present (Figure 2.1A).

Effects of pheromone on the dauer decision

Time of action

Previous experiments determined the periods in which worms respond to pheromone and commit to the pre-dauer (L2d) and to the dauer stage. Worms must be exposed to pheromone before the L1 molt in order to develop into the L2d stage and commit to the dauer fate a few hours before the mid L2d stage (Golden and Riddle, 1984a). We re-visited experiments by Golden and Riddle and measured dauer formation in response to dauer pheromone performed in liquid culture. We re-visited these experiments and modified them to liquid culture to increase scale, homogeneity and throughput and measured the frequency of dauer formation in response to dauer pheromone while grown in the presence of sufficient food for adult development. Dauer molt occurred at 48 hours post hatch (hph) and dauers were verified by measuring the percentage of survivors in 1% SDS, a standard assay for distinguishing dauers from L2 and L3 worms (Cassada and Russell, 1975) and found that all dauers in liquid culture became resistant to 1% SDS 10 hours after the molt (Figure 2.1B). Mean frequencies of life stages in favorable and unfavorable growth conditions were tightly distributed and

highly reproducible indicating the homogeneity of the liquid culture conditions (Figure 2.1C and D).

To identify the period in which worms become committed to development as L2 larvae instead of L2d larvae, consequently losing the ability to form dauers, we performed a " shift-to-unfavorable" experiment by adding a hi gh concentration of pheromone to synchronously hatched worms. We reasoned that time points with a high standard deviation of dauer frequencies between biological replicates would indicate the transition from uncommitted to committed worms (For statistical analysis see Experimental Procedures and Table 1 in appendix to chapter). At progressive times, worms were shifted to favorable conditions by washing away pheromone. Cultures were scored for dauer formation 52 hours post hatch (hph), creating a precise time series of dauer frequencies. Worms stopped responding to pheromone at 16-18 hours post hatch (hph, 51.4% ± 37.3% dauer formation), the beginning of the L2 stage (Figure 2.2B), despite exposure to unfavorable conditions.

To precisely define the periods during which worms commit to dauer or to L3 reproductive fates we performed “shift-to-growth” experiments at two different developmental stages: L2d t o dauer and L2d to L3 fates. Mean dauer to non-dauer frequencies were tightly distributed and highly reproducible during early and l ate time points. Analysis of four different biological samples of N2 wild type worms revealed that the point of L2d to dauer commitment occurred 33 hph (average of 29.1 ± 25.1% dauer formation) or 18 h ours after the L1/L2d molt (Figure 2.1B). Shifting worms out of unfavorable conditions after this time does not affect their propensity to become dauers.

We next identified when L2d worms can commit to L3 (Figure 2.2 C). We reasoned that L2d worms exposed to longer time windows of favorable conditions have a higher propensity to develop into adults. We modified the shift-to-favorable experiment by growing synchronously hatched animals in unfavorable conditions to obtain L2d animals, followed by a shift to favorable conditions at 24 hph. Worms were then returned to unfavorable conditions after varying amounts of time (see Experimental Procedures). We find that worms shifted to favorable conditions for a three hour window (0.02% ± 0.01% dauer formation) is sufficient to commit L2d animals to reproductive development as L3 larvae despite subsequent exposure to dauer-promoting conditions, indicating commitment to L3 (Figure 2.2C). We define this consistent period in favorable conditions as the persistence window, and denote the time for 100% of the population to commit to adult the 'integration-time'. In summary, the L2d stage is divided into two distinct periods: integration (between the beginning of the L2d stage, 16 hph until the mid L2d, 33 hph) and commitment into dauer (33 hph-48 hph).

Effectors of the persistence window

We wanted to understand if the persistence window could be affected by changes of environmental conditions. Initially, we wanted to understand whether the time of the shift from unfavorable to favorable conditions could extend or shorten the integration time of pheromone. To this end we performed the modified ‘shift-to-favorable’ experiment starting at different time points in L2d and measured the fraction of dauers. We found that 99% of the population committed to adult within 3 hours of exposure to favorable conditions and that this window was uniform across different start times during the integration period of L2d (fig 3A with p=0.1, one-way ANOVA), with a

significant change at 33 hph, the time of commitment to dauer. We observed that the dauer fraction in the zero hour time point (which was actually 15 mins) decreased as worms were shifted to favorable conditions later during L2d (Figure 2.3B, P=0.0015). Interestingly, we could not detect significant differences in dauer frequencies in intermediate time points (1 and 2 hour persistence window periods, P=0.5, 0.6 respectively). These results suggest that the 'integration-time' shortens as the point of commitment to dauer draws near.

We next wanted to understand the effects of pheromone concentration on the integration time within the persistence window. We shifted worms from 3% pheromone to 0.5, 1 and 3% pheromone and measured the length of integration time. When worms were shifted from 3% pheromone to 0.5% pheromone the integration time increased from 3 hours to 6 hours. Similarly, when worms were shifted from 3% to 1% pheromone, integration-time increased from 3 hours to 8 hours (Figure 2.3C). These results indicate that the integration-time increased as the difference between the pheromone concentrations was smaller.

Epigenetic effects on the pheromone sensitivity

Since dauers can resume development and produce a brood, we asked whether this experience affected the sensitivity of F1's to form dauers. We recovered the P generation of dauers in favorable conditions and s ubjected the F1 F2 F3 and F 4 generation to dauer pheromone. We observed a significant decrease of response to dauer pheromone as F1s exposed to 3 % pheromone resulted in 22 ± 4% dauer formation (control F1's yielded 92 ± 4% dauer, P<0.0001, one tailed t-test). F2 and F3 worms resulted in 92 ± 3% and 9 1 ±3% dauer fraction respectively, thus returning to

regular levels (Figure 2.4B, P_{F2}=0.52, P_{F3}=0.86, one tailed t-test). These results indicate that worms developing through the dauer molt pass this information to their offspring. The adult dauer transition memory lasts for only one generation suggesting an epigenetic component regulating the dauer decision.

Summary

The decision to become a dauer attempts to predict the future regarding the capacity of the environment to support the development of a brood (Cassada and Russell, 1975). To this end worms take into account population density (conspecific competition for resources), food availability and temperature during the environment-sensitive period. Worms then integrate these signals from the environment with their internal state composed of fat storage, previous exposures to pheromone, the history of their parents and their ability to produce hormones necessary for growth (Braendle et al., 2008). Once the decision has been made, worms develop either into a dauer with a specific morphology and behavior not displayed in the adult or in the age-matched juvenile, the L3 (Cassada and Russell, 1975). Environmental conditions often fluctuate and are seldom held constant suggesting that worms have evolved a mechanism to distinguish a true or 'stable' signal and act upon it. The mechanism that integrates the environmental cues and internal information leading to a decision committing to one of the binary outcomes remains unknown.

To dissect the process of commitment to reproduction (L3) or delayed reproduction (the dauer larvae), we modified the Golden and Riddle assay for major life cycle choices (Golden and Riddle, 1984a) by performing it in liquid culture, thus enabling a large and highly synchronized brood, amenable to facile changes in

environmental conditions. We used a high concentration of pheromone to mimic a high population density and showed that L2d worms grown in unfavorable conditions are irreversibly committed to the dauer fate by 33 hph. Conversely, L2d worms commit to reproductive fate if shifted to favorable conditions for a period of at least 3 hours before this 33 hph threshold has been crossed. Our identification of the times at which both life cycle fate decisions occur allowed us to couple changes in the environment to the known molecular and cellular components involved in this decision. We thus propose a mechanism of irreversible commitment to a life cycle fate choice.

Environmental conditions can change and be unpredictable. Worms not only integrate the ratio of food and pheromone, but also make sure that a transient signal of high or low population density is considered before committing to a fate. In engineering, this system property is called persistence detection and it is commonly used to differentiate between brief noisy signals to constant signals from the environment (Alon, 2007). We have identified that the persistence window of integrating favorable conditions during the L2d is nominally 3 hours. furthermore, worms can modulate the integration time: worms decrease the integration time as the point of commitment approaches and, conversely, increase the integration time as a function of the difference of the change in population density.

A decrease in the integration time is equated with less-strict form of integrating environmental conditions since it will allow signals with a shorter persistence to be integrated leading to development into an adult. We speculate therefore, that upon approaching the point of commitment to dauer, worms would prefer to hedge their bets against a brief signal and develop into an adult. This was observed at the zero hour

integration time length, where the decrease of integration time adjacent to the point of commitment is limited in the population since only 58 ± 8% become dauers and the rest adults.

An increase in integration time is equated with a more strict form of integrating environmental conditions since it will filter out short and intermediate length stable signals but will integrate long stable signals. Increasing the integration time slows down development and in unfavorable conditions worms that eventually decide to develop into adults will have a di sadvantage. We therefore asked why worms experiencing a decreasing shift in population density increase the integration time, and have 3 hypothesis. (i) Worms experiencing a small decrease in population density measure their environment over a longer period of time thus increasing their chances for making a correct decision. (ii) The remaining amounts of pheromone in the environment may be inhibiting growth processes thus a slower growth process is extending the integration time. (iii) Fluctuating conditions may be a trigger to delay the reproductive period and to encourage migration to a new niche. Currently, we are making our conclusions on population means and not on individual worms and are unable to distinguish these hypothesis one from the other.

The dauer decision integrates the parental history since offspring (F1) to parents (P) that were dauers have an decreased sensitivity to pheromone. We speculate that the sensitivity decrease is an epigenetic trait since F2 worms show a normal response to pheromone if F1's are not stressed. A recent report has shown that worms that transiently develop through dauer have a significant portion of their genome regulated compared to age matched non-dauer adults leading to an increase of brood

size for the former (Hall et al., 2010). They identified several genes that can abolish the increase in brood size, therefore it will be interesting to test if these genes play a role in integrating pheromone in F1s.

How is the complexity of the environment integrated eventually in to a decision with binary outcomes? In later chapters I will demonstrate that hormone sensitive period falls within the environment sensitive period. I will then demonstrate the dauer decision is mainly executed in the neuroendocrine XXX thus reducing the information complexity of the environment into those 2 cells by a yet undefined mechanism. The hierarchy of the integration process is unknown; for example, Is the persistence of pheromone exposure first measured and then integrated with food availability? Is food availability integrated with fat availability and parental history and the sun of those integrated with pheromone persistence? Is Dafachronic acid hormone availability integrated as part of the decision or the result of it? In the following chapters I will use the integration time window to correlate between changes in the environment to the effects of the hormone Dafachronic acid.

Materials and methods

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.

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 and grown in glass tubes. 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. Shift to unfavorable: broods were supplemented with 3% (v/v) pheromone and grown in glass tubes. At specified time points (L2d), worms were partitioned into a control sample and experimental samples, which were washed 3 t imes 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.

Homogeneity of liquid culture

Stage distributions were compared between 3 bi ological 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.

Bibliography

Albert, P.S., and Riddle, D.L. (1988). Mutants of Caenorhabditis elegans that form dauer-like larvae, Vol 126.

Alon, U., ed. (2007). An introduction to systems biology: design principles of biological circuits (London, CRC Press, Taylor & Francis Group).

Baugh, L.R., Demodena, J., and Sternberg, P.W. (2009). RNA Pol II accumulates at promoters of growth genes during developmental arrest. Science *324*, 92-94.

Birnby, D.A., Link, E.L., Vowels, J.J., Tian, H.Z., Colacurcio, P.L., and Thomas, J.H. (2000). A transmembrane guanylyl cyclase (DAF-11) and Hsp90 (DAF-21) regulate a common set of chemosensory behaviors in Caenorhabditis elegans, Vol 155.

Braendle, C., Milloz, J., and F elix, M.A. (2008). Mechanisms and ev olution of environmental responses in Caenorhabditis elegans. Curr Top Dev Biol *80*, 171-207.

Cassada, R.C., and Russell, R.L. (1975). The dauerlarva, a post-embryonic developmental variant of the nematode Caenorhabditis elegans. Developmental biology *46*, 326-342.

Dworkin, J., and Losick, R. (2005). Developmental commitment in a b acterium. Cell *121*, 401-409.

Gerisch, B., and Antebi, A. (2004). Hormonal signals produced by DAF-9/cytochrome P450 regulate C. elegans dauer diapause in response to environmental cues. Development (Cambridge, England) *131*, 1765-1776.

Gerisch, B., Rottiers, V., Li, D., Motola, D.L., Cummins, C.L., Lehrach, H., Mangelsdorf, D.J., and A ntebi, A. (2007). A bile acid-like steroid modulates Caenorhabditis elegans

lifespan through nuclear receptor signaling. Proceedings of the National Academy of Sciences of the United States of America *104*, 5014-5019.

Gerisch, B., Weitzel, C., Kober-Eisermann, C., Rottiers, V., and Antebi, A. (2001). A hormonal signaling pathway influencing C. elegans metabolism, reproductive development, and life span. Developmental cell *1*, 841-851.

Gilbert, S.F., Epel, D. (2009). Ecological developmental biology; Integrating Epigenetics, medicine, and Environment (Sinauer Associates Inc.).

Golden, J.W., and Riddle, D.L. (1984a). The Caenorhabditis elegans dauer larva: developmental effects of pheromone, food, and temperature. Developmental biology *102*, 368-378.

Golden, J.W., and Riddle, D.L. (1984b). A pheromone-induced developmental switch in Caenorhabditis elegans: Temperature-sensitive mutants reveal a wild-type temperature-dependent process. Proceedings of the National Academy of Sciences of the United States of America *81*, 819-823.

Hall, S.E., Beverly, M., Russ, C., Nusbaum, C., and Sengupta, P. (2010). A cellular memory of developmental history generates phenotypic diversity in C. elegans. Curr Biol *20*, 149-155.

Hannich, J.T., Entchev, E.V., Mende, F., Boytchev, H., Martin, R., Zagoriy, V., Theumer, G., Riezman, I., R iezman, H., Knolker, H.J.*, et al.* (2009). Methylation of the sterol nucleus by STRM-1 regulates dauer larva formation in Caenorhabditis elegans. Developmental cell *16*, 833-843.

Jia, K., Albert, P.S., and Riddle, D.L. (2002). DAF-9, a cytochrome P450 regulating C. elegans larval development and adult longevity. Development (Cambridge, England) *129*, 221-231.

Lewis, J.A., J.T. Fleming. (1995). Caenorhabditis elegans: Modern biological analysis of an organism

In Methods in Cell Biology (USA)

H.F. Epstein, D. C.Shakes, , Ed., ed. (San Diego, Academic Press), pp. 4-29.

Ludewig, A.H., Kober-Eisermann, C., Weitzel, C., Bethke, A., Neubert, K., Gerisch, B., Hutter, H., and Antebi, A. (2004). A novel nuclear receptor/coregulator complex controls C. elegans lipid metabolism, larval development, and aging. Genes & development *18*, 2120-2133.

Mak, H.Y., and Ruvkun, G. (2004). Intercellular signaling of reproductive development by the C. elegans DAF-9 cytochrome P450. Development (Cambridge, England) *131*, 1777-1786.

Motola, D.L., Cummins, C.L., Rottiers, V., Sharma, K.K., Li, T., Li, Y., Suino-Powell, K., Xu, H.E., Auchus, R.J., Antebi, A.*, et al.* (2006). Identification of ligands for DAF-12 that govern dauer formation and reproduction in C. elegans. Cell *124*, 1209-1223.

Nijhout, H.F. (2003). Development and evolution of adaptive polyphenisms. Evol Dev *5*, 9-18.

Patel, D.S., Fang, L.L., Svy, D.K., Ruvkun, G., and Li, W. (2008). Genetic identification of HSD-1, a c onserved steroidogenic enzyme that directs larval development in Caenorhabditis elegans. Development (Cambridge, England) *135*, 2239-2249.

Riddle, D.L., Albert, P., ed. (1997). Genetic and environmental regulation of dauer larva development (New York, Cold Spring Harbor Laboratory Press).

Rottiers, V., and Antebi, A. (2006). Control of Caenorhabditis elegans life history by nuclear receptor signal transduction. Experimental gerontology *41*, 904-909.

Tawfik, A.I., Tanaka, S., De Loof, A., Schoofs, L., Baggerman, G., Waelkens, E., Derua, R., Milner, Y., Yerushalmi, Y., and Pener, M.P. (1999). Identification of the gregarization-associated dark-pigmentotropin in locusts through an albino mutant. Proceedings of the National Academy of Sciences of the United States of America *96*, 7083-7087.

Xiong, W., and Ferrell, J.E., Jr. (2003). A positive-feedback-based bistable 'memory module' that governs a cell fate decision. Nature *426*, 460-465.

Zar, J.H. (2009). Biostatistical Analysis, 5 edn (Upper Sadlle River, , Prentice Hall).

Figures

Figure 2.1

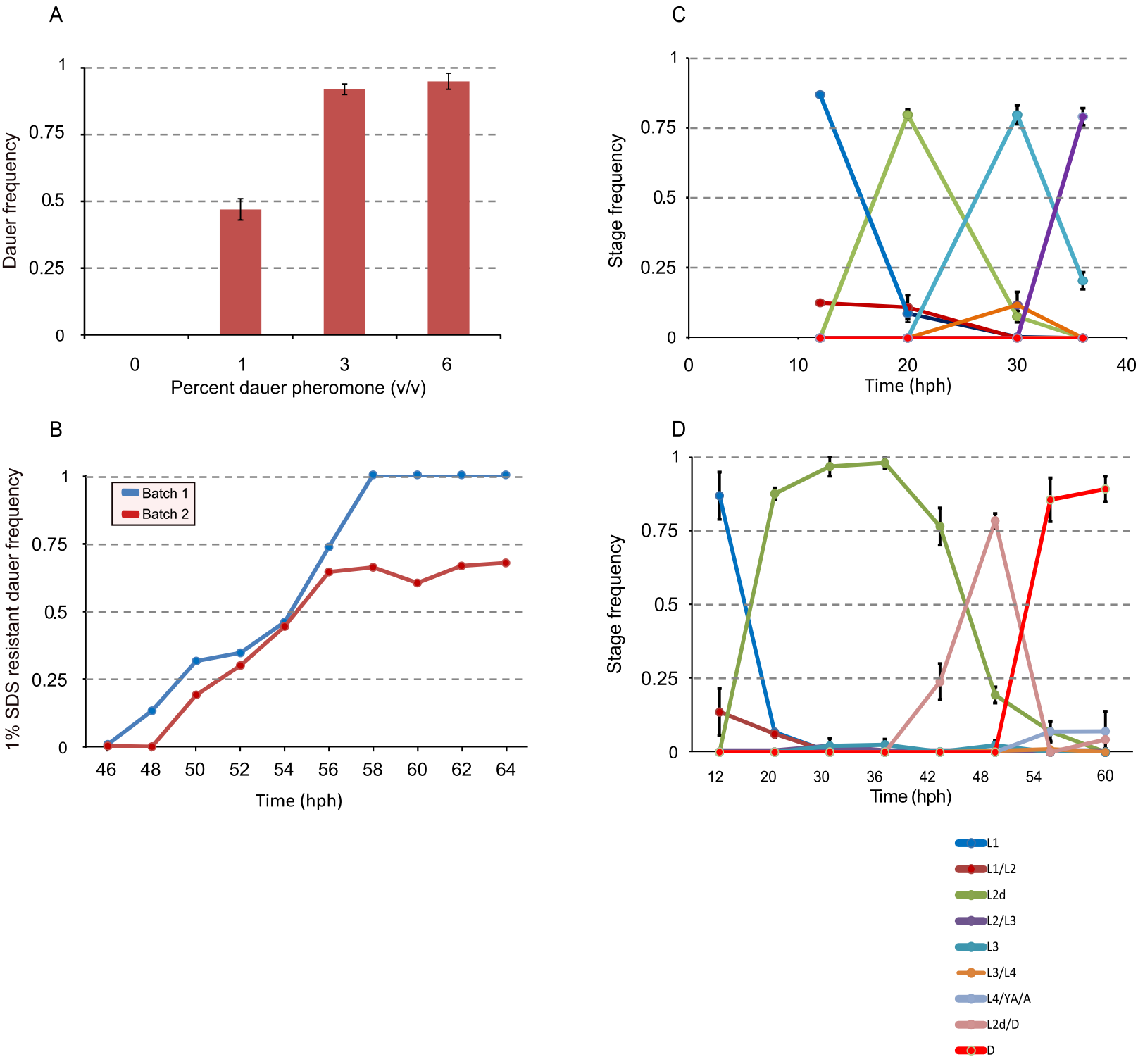

A
Dauer frequency
1
0.75
0.5
0.25
0
0
1
3
6
Percent dauer pheromone (v/v)
C
Stage frequency
1
0.75
0.5
0.25
0
0
10
20
30
40
Time (hph)
B
1% SDS resistant dauer frequency
Batch 1
Batch 2
1
0.75
0.5
0.25
0
46
48
50
52
54
56
58
60
62
64
Time (hph)
D
Stage frequency
1
0.75
0.5
0.25
0
12
20
30
36
42
48
54
60
Time (hph)
L1
L1/L2
L2d
L2/L3
L3
L3/L4
L4/YA/A
L2d/D
D

Figure 2.1: Calibration of liquid culture for development in homogenous environmental conditions

(A) Fraction of dauers as a function of a dilution series of crude pheromone. Bars represent the mean across 3 bi ological experiments and error bars describe the standard deviation of those 3 biological experiments. (B) Response of dauers to 1% SDS treatment. 100 % of the population become resistant to 1% SDS, 10 hours after the dauer molt (48 hph). (C,D) Synchronized hatch and subsequent growth in liwuid culture create a homogenous environment retaining a high degree of synchronicity throughout development in (C) favorable conditions and in (D) unfavorable conditions. Points represent means of three biological experiments and error bars represent the standard deviation.

Figure 2.2

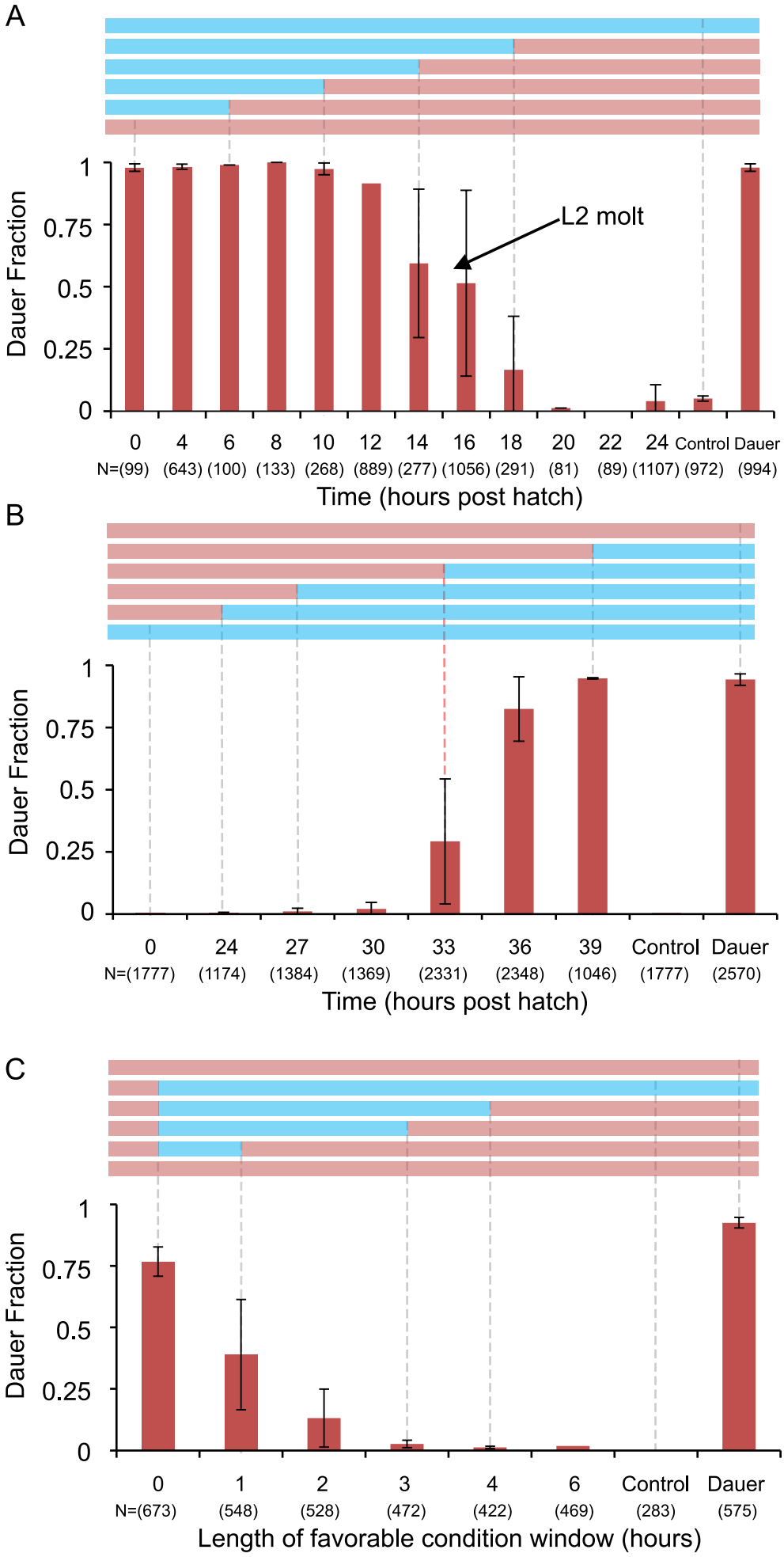

A
Dauer Fraction
1
0.75
0.5
0.25
0
L2 molt
0 4 6 8 10 12 14 16 18 20 22 24 Control Dauer
N=(99) (643) (100) (133) (268) (889) (277) (1056) (291) (81) (89) (1107) (972) (994)
Time (hours post hatch)
B
Dauer Fraction
1
0.75
0.5
0.25
0
0 24 27 30 33 36 39 Control Dauer
N=(1777) (1174) (1384) (1369) (2331) (2348) (1046) (1777) (2570)
Time (hours post hatch)
C
Dauer Fraction
1
0.75
0.5
0.25
0
0 1 2 3 4 6 Control Dauer
N=(673) (548) (528) (472) (422) (469) (283) (575)
Length of favorable condition window (hours)

Figure 2.2. Commitment to dauer or reproductive development.

(A-C) Time courses of commitment as a function of environmental conditions (pheromone). Top; representative colored bars indicating s hifts to unfavorable conditions (red) or favorable conditions (blue). Bottom; means of dauer frequencies between biological replicates ± standard deviation. Numbers in parentheses indicate total worms per time point. Control; worms grown without pheromone. Dauer; worms grown in 3% (v/v) pheromone with no shifts.

(A) Induction period of pheromone during L1 a nd L2: worms respond to pheromone until 18 hph (early L2). (B) Point of commitment to dauer; worms commit to dauer 33 hph denoted by the red dashed gridline. (C) Point of commitment from L2d to L3; worms commit to L3 after a 3 hour pulse in favorable conditions when shifted at 24 hph.

Figure 2.3

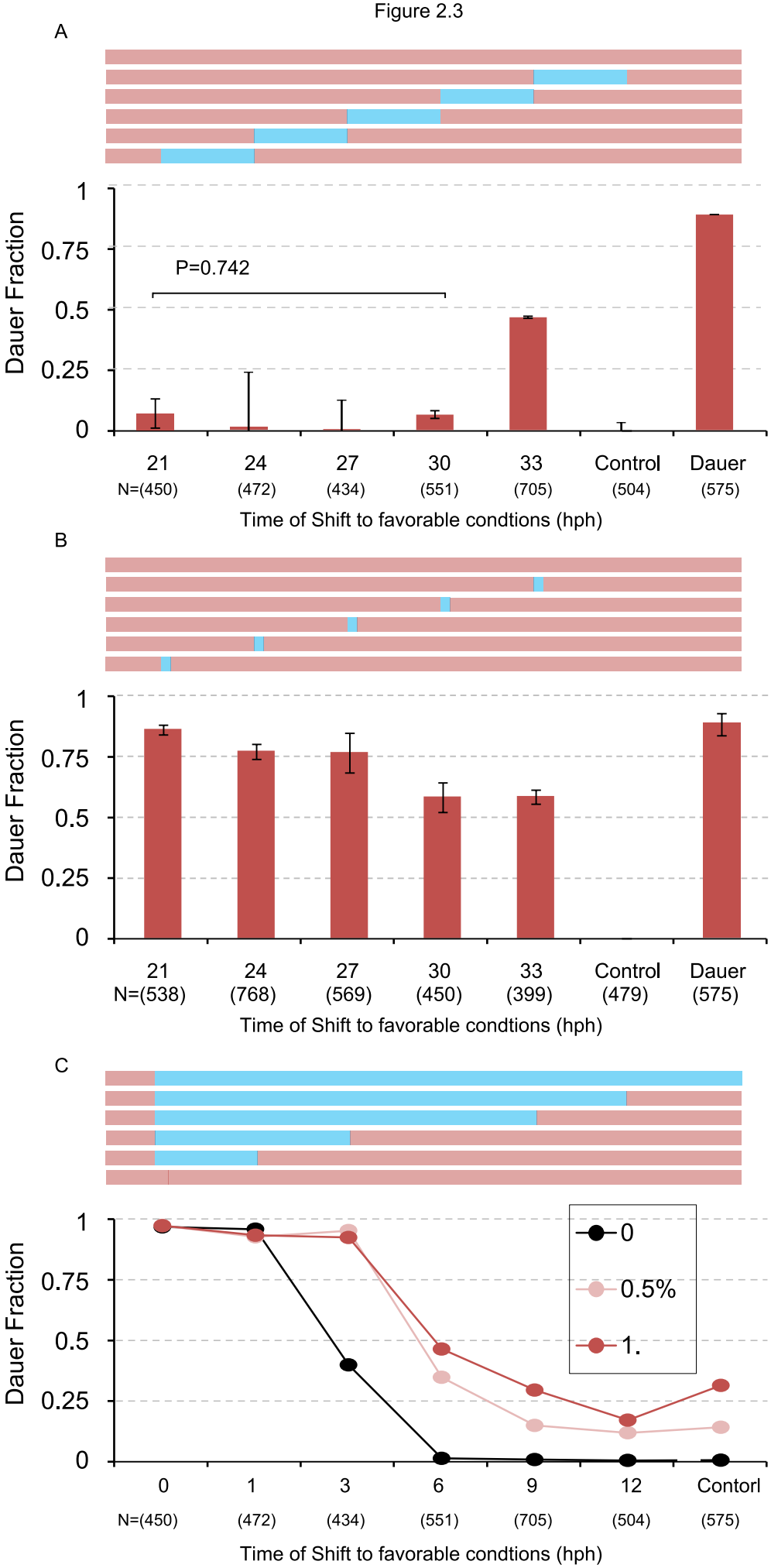

A
Dauer Fraction
1
0.75
0.5
0.25
0
P=0.742
21
N=(450)
24
(472)
27
(434)
30
(551)
33
(705)
Control
(504)
Dauer
(575)
Time of Shift to favorable condtions (hph)
B
Dauer Fraction
1
0.75
0.5
0.25
0
21
N=(538)
24
(768)
27
(569)
30
(450)
33
(399)
Control
(479)
Dauer
(575)
Time of Shift to favorable condtions (hph)
C
Dauer Fraction
1
0.75
0.5
0.25
0
0
1.
0.5%
0
1
3
6
9
12
Contorl
N=(450)
(472)
(434)
(551)
(705)
(504)
(575)
Time of Shift to favorable condtions (hph)

Figure 2.3: Effectors of the persistence window

Persistence window is modulated by environmental conditions. Top; representative colored bars indicating shifts to unfavorable conditions (red) or favorable conditions (blue). Bottom; means of dauer frequencies between biological replicates ± standard deviation. Numbers in parentheses indicate total worms per time point. Control; worms grown without pheromone. Dauer; worms grown in 3% (v/v) pheromone with no shifts. (A) Constant persistence window: start time of pulse shifts to favorable conditions during L2d. Pheromone was added to worms 3 hours post shift to favorable conditions. Cultures shifted to favorable conditions at 33 hph show a higher ratio of dauers since worms commit to dauer at 33 hph. (B) Worms shifted to the 0 time length window at times approaching the point of commitment decrease the length of the integration time. (C) Down shifting worms to 0% (black) 0.5% (pink) and 1% (red), and then returning them to 3% pheromone increases the integration time of the persistence window (N> 200 per pheromone concentration per time point).

Figure 2.4

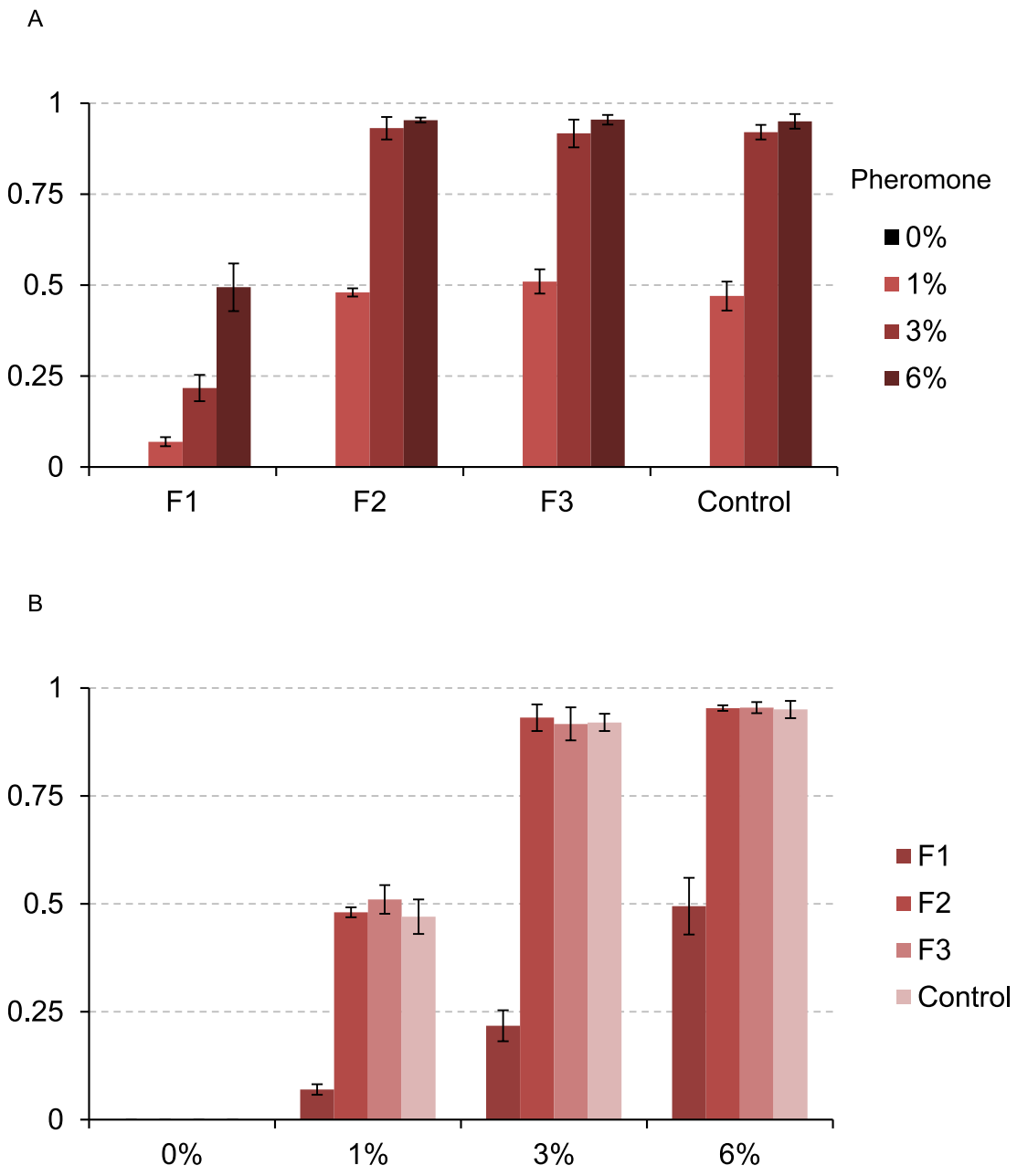

A
1
0.75
0.5
0.25
0
F1
F2
F3
Control
Pheromone
0%
1%
3%
6%
B
1
0.75
0.5
0.25
0
0%
1%
3%
6%
F1
F2
F3
Control

Figure 2.4: Dauer history is reflected in offspring

Parents that transitioned through the dauer stage pass the experience to offspring. (A) F1 generation offspring are significantly less sensitive to pheromone than control animals (at least 5 generations of favorable growth). See text for analysis of significance. Bars represent means of 3 biological replicates and error bars represent the standard deviation across the 3 biological replicates. F1 F2 and F3 represent the first second and third generation to parents that transition through dauer, respectively.

Figure 2.5

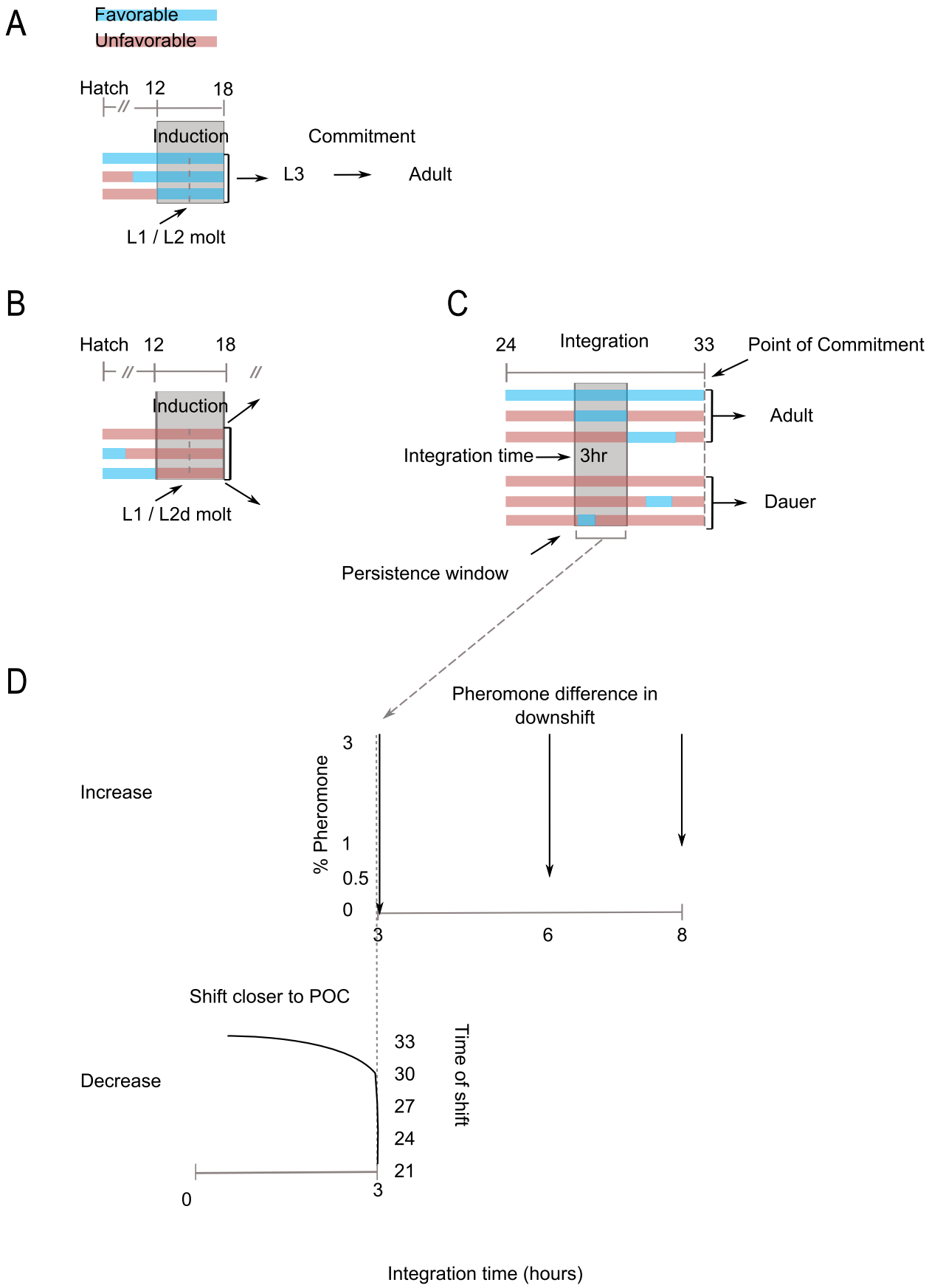

A
Favorable
Unfavorable
Hatch
12
18
Induction
Commitment
L3
Adult
L1 / L2 molt
B
Hatch
12
18
Induction
L1 / L2d molt
C
24
Integration
33
Point of Commitment
Adult
Integration time
3hr
Dauer
Persistence window
D
Pheromone difference in downshift
Increase
% Pheromone
3
1
0.5
0
3
6
8
Shift closer to POC
Decrease
Time of shift
33
30
27
24
21
0
3
Integration time (hours)

Figure 2.5: Effects of environmental conditions on the dauer decision

(A) Induction period is defined by the response to pheromone during 12-18 hph. Top; If animals aren't exposed to pheromone at that time worms develop into adults through the L2 stage. (B) If worms are exposed to pheromone during the induction period they develop into the L2d stage where they integrate the environmental conditions. (C) The L2d stage is divided into integration (18-33 hph) and commitment (33-48 hph). If worms are exposed to favorable environmental conditions during the integration period for an integration time that is equal to or greater than the persistence window (grey box) then they develop into L3's and into adulthood, otherwise they develop into adults. (D) The integration time is regulated by environmental conditions. Top; the integration time can increase if the difference in the pheromone concentration during the downshift is small. Bottom; the integration time can increase if the downshift is performed near the Point of commitment (POC) to dauer.

Chapter 3: Effects of the Hormone Dafachronic Acid on the Dauer Decision

Introduction

Hormone networks play an important role in the regulation of development across all taxa. Since hormones diffuse easily through membrane walls they are thought to be transmitted throughout the body orchestrating many biological processes and understanding their regulation is important.

Hormones can act through the neuroendocrine system having systemic effects (Nijhout, 2003). Hormones can diffuse through membranes and act in cells by two main mechanisms; (i) binding to internal nuclear hormone receptors, or (ii) through direct binding to GPCRs in the membrane-mediated steroid signaling pathway (Denver, 2007). Nuclear hormone receptors (NHR) usually have modular domains such as a ligand binding domain which binds the hormone and a DNA binding domain which recognizes and can bind to specific sequences in the genome. NHRs are usually inactive until they bind the hormone and often homodimerize or heterodimerize when the hormone diffuses into the cell. Thus, the activity of hormones is extremely modular and can be regulated by alternative splicing, cell and tissue specific and temporal transcription of the NHRs, their combination in target tissues and the state of the genome; if the DNA binding sequence is accessible or held closed (Nijhout, 2003; Wollam and Antebi, 2010). Therefore, the hormone sensitive period is a function of the availability and activity of the receptors controlling a specific phenotype and not necessarily that of the hormone. The hormone can be synthesized, released or activated at any moment during the sensitive period, thus diffusing and binding to its receptors. Many types of hormone regulatory mechanisms that regulate different types of polyphenisms rely on

the timing and dose of hormone secretion during the sensitive period (Keshan et al., 2006).

Hormonal regulation of polyphonic traits has been studied extensively in insects. The decision between mutually exclusive fates has been characterized in a number of hormonal mechanisms in the insect class. Nijhout (2003) has characterized differences in hormone titer, threshold, timing of hormone secretion and timing of the hormone sensitive period as main regulatory mechanisms that can form switched in polyphenic decisions (Figure 1.1). The hormone sensitive period and switch mechanism are unknown in *C. elegans*.

Most traits of polyphenism are irreversible; once committed to one fate, a change in environmental conditions or exogenous addition of the hormone triggering the specific morph will not change the decision of the morph. For example, the butterfly *Araschnia levana* will develop wing pigmentation which is bright orange in the spring and black in the summer. The summer morph is regulated by photoperiod and temperature which triggers Ecdysone release for the summer morph alone. If these butterflies are treated with the summer inducing conditions in the spring, the wings will form the summer morph, and upon commitment, will remain the summer morph even if switched back to spring-morph inducing conditions or treated with Ecdysone (Gilbert, 2009).

There is still debate if the end of the hormone sensitive period is the point of commitment, the point after which a change in environmental conditions does not change the course of development. The larvae of the butterflies *Oncopeltus fasciatus*

and *Pyrhocoris apterus* treated with threshold levels of JH develop larval pigmentation on patches of their cuticle but adult surface sculpturing (Willis 1982). The metamorphosis from larvae to pupae, or Pupal commitment, 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 ensures commitment to pupae is unknown.

C.elegans nematodes display polyphenism during development. In favorable environments, *C. elegans* develops rapidly through four larval stages (L1-L4) separated by molts, into a sexually reproductive adult. However, 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).

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). DAs can rescue the *daf-7* and *daf-2* Daf-c phenotypes indicating that it is downstream to the Insulin and TGFβ signaling pathways (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. The time of action of DA is unknown, or 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. Here we demonstrate that they act as a regulator at the point of the decision and as a messenger downstream of the decision. We show that environmental conditions affect the threshold at which levels of DA are sufficient to prevent the dauer fate and commit to adulthood, while higher amounts of DA are necessary to implement and coordinate the reproductive decision throughout the whole animal.

Results

The two forms of the dafachronic acids (DA) Δ4-DA and Δ7-DA can fully rescue the Daf-c phenotypes of the null allele *daf-9(dh6),* as well as *daf-7*/TGF-beta and *daf-2*/InsR mutants (Motola et al., 2006) . Partial reduction of *daf-9* function, results in animals that bypass the dauer stage yet exhibit incomplete gonadal morphogenesis and migration

(Mig) and occasionally aberrant cuticle shedding (Cut) defects (Gerisch et al., 2001; Jia et al., 2002) (Fig 1A). Exogenous DA can also rescue these phenotypes (Gerisch and Antebi, 2004; Gerisch et al., 2007; Mak and Ruvkun, 2004; Motola et al., 2006). We thus hypothesized that a low amount of DA is required to bypass dauer and commit to L3, whereas a high amount is required for complete development.

Dose response of daf-9 loss of function mutants to DA

To understand the physiological response to DA dose, dauer-constitutive *daf-9* loss-of-function mutants were treated with increasing amounts of Δ7-DA and measured for dauer and reproductive adult fates. Most *daf-9(dh6)* null animals developed into abnormal adults when supplemented with a minimum of 10nM DA (Figure 3.1B 74 ± 42% non-dauers), suggesting that a threshold of DA has to be crossed before committing to adult fate (dauer bypass DA threshold). Increasing the levels of DA decreased the frequency of dauers and increased the frequency of abnormal adults. 25nM DA prevented 99 ± 1% of *daf-9(dh6)* animals from developing as dauers (Figure 3.2B). Further increase of DA decreased the frequency of abnormal adults and increased the frequency of normal adults (Figure 3.2B; 50-100nM). For a distribution of Mig and Cut phenotypes, see Figure S1.1. Similar results were observed with animals homozygous for *daf-9(e1406)* or *daf-9(m540)* (Figure S1.1, worms were not synchronously hatched), both of which are strong loss-of-function alleles. By contrast, the weak loss of function allele *daf-9(rh50)* does not result in Daf-c phenotypes, but in highly penetrant Mig defects (95 ± 3%) (Gerisch et al., 2001). In these animals, only 10nM of DA was required to rescue over 90% of the Mig phenotypes (Figure S1.1), revealing a 5-fold decrease in the amount of exogenous DA required to promote

complete development compared to the stronger *daf-9* mutants (*dh6*, *e1406* and *m540)*. The *rh50* allele is an amino acid substitution D334N in a putative substrate binding domain of the Cytochrome P450/CYP27A1 (Gerisch et al., 2001). Thus, *daf-9(rh50)* animals produce sufficient amounts of DA to bypass dauer development, but require additional DA to develop into normal adults, consistent with our finding that different levels of DA are required for the two processes.

DA effects on developmental rate

To test the effects of DA on developmental rate, *daf-9(dh6)* worms were synchronously hatched in different concentrations of DA and scored for developmental stage at 48 hph (the time at which WT worms grown in favorable conditions are young adults and worms grown in unfavorable conditions are dauers) and for egg production the following day. Worms supplemented with 10nM DA arrested development at the L3 stage. At 25-50nM DA, worms developed into L4s and worms supplemented with 75-175nM DA developed into YA's. Worms that were in the L4 or YA stages at 48 hph were gravid the next day. All these trends indicate that higher DA increases growth rate (Figure 1C) and that *daf-9(dh6)* worms supplemented with DA can develop at the same rate as WT worms.

Intermediate amounts of DA increase phenotypic variation high amounts decrease it

Addition of DA at increasing concentrations bypasses the dauer but unmasks the intermediate phenotypes of mig cut and growth rate variations. Increasing the concentration even further canalizes development to adult or dauer with some variation

around growth rate. We quantified the effects of DA on the variance by calculating a Shannon-Weiner heterogeneity index (SWHI) for a diversity of phenotypes at every DA dose. The SWHI is traditionally used in quantifying the evenness of distribution of species in different niches and can be used to contrast between populations with unequal numbers of categorical data (Zar, 2009). The SWHI is defined as the ratio

$J = \frac{H`}{H_{max}} = \frac{-\sum_{i=1}^{S}(P_i ln P_i)}{lnS}$ where H` is the entropy or the amount of uncertainty or distribution in a population, H_{max} is the maximal amount of uncertainty or distribution in a population, S is the number of niches or categories that are being surveyed, and P_i is the relative abundance of species *i*. For example, a population that has no diversity will have the ratio J=0 and a population with maximal diversity where all individuals are equally spread between all categories will have the ratio of J=1. Addition of 0-5nM DA did not increase the SWHI significantly (0, 0.013, 0.02, respectively). However, at the ranges of 10-50nM DA the SWHI increased to 0.25 at 10nM peaking at 25nM with a SWHI of 0.49 due to the emergence of the abnormal mig and cut phenotypes. At 50nM the SWHI dropped to 0.24 when no more abnormal phenotypes are observed and the distribution of phenotypes is composed of arrested L3s and adults. At 75-175 another increase and decrease are observed and this is due to the distribution of growth rates (Figure 3.2). These results demonstrate that an increase of DA increases the number and distribution of different intermediate developmental states and a further increase decreases the number and distribution of it. Thus high amounts of DA can canalize the developmental states of worms bypassing the dauer decision.

Pheromone levels regulate the adult DA threshold and reproductive development

DA and dauer pheromone have opposite effects on dauer formation, with DA preventing and dauer pheromone promoting the dauer stage. We investigated the dose-response relationship when administered together, with respect to bypass of the dauer diapause and complete reproductive development.

Synchronized populations of *daf-9(dh6)* worms were supplemented with a combination of DA and pheromone at different concentrations and scored for dauer, incomplete adult and complete adult development at 48 hph. Addition of pheromone at 1,3 or 6% (which induce 47±4, 92±2 and 95±2% dauer in WT worms, respectively, Figure 1.1A) increased the concentration of DA necessary to bypass the adult DA threshold (Figure 3.3A-D) to 30, 45, and 58nM, respectively. Moreover, 90% of the population developed into complete adults when worms were supplemented with 30nM of DA more than the amount required to bypass the adult DA threshold (Figure 3.3E,F), similar to the concentration of DA needed to bypass the adult DA threshold in *daf-9(dh6)* worms without pheromone. These experiments demonstrate that dauer pheromone increases the amount DA required to bypass the adult DA threshold and complete reproductive development.

DA time of action

To understand the time of action of DAs and their role in life cycle fate decisions we sought to identify 3 key points in the response to DA: (i) the time or stage at which *daf-9(dh6)* animals start responding to DA to bypass dauer (ii) the end of response to DA for

the dauer decision and (iii) the requirements of exposure to DA for complete development to maturity. Synchronously hatched *daf-9(dh6)* worms were shifted from media containing DA in EtOH to media containing EtOH alone (downshift) or vice-versa (upshift). Analysis of downshift experiments revealed that worms started responding to DA after 15 hph, the same time that WT worms commit to L2 mediated reproductive development (Figure 3.4A). When DA was washed away before 15 h ph, worms developed into dauers, and apparently lacked a memory for previous exposures. Removal of DA at time points after 15 hph prevented dauer formation to increasing extents, which could be divided into two phases: a minimum of 3 hours on 100nM DA during the responsive period was sufficient to prevent 75% of the population from becoming dauers but these animals developed as incomplete adults (Figure 3.4A 15 to 18 hph), whereas an additional 12 hours were necessary to drive 100% of the population to complete adult development (Figure 3.4A 18 to 30 hph). To determine when *daf-9(dh6)* worms became refractory to DA, upshift experiments were performed during the L2d stage. Worms responded to DA until 33 hph, precisely at the same time that wild-type worms became refractory to pheromone (Figure 3.4B; correlation coefficient = 0.996). Next, we asked whether the total time exposed to DA or the specific time (stage) of exposure to DA were regulating the fate decision and development of normal adults. Pulse experiments revealed that worms committed to bypass dauer when exposed to DA at 15 hph for as little as 3 hours (Figure 3.4C). Also, this commitment occurs at 15-18 hph regardless of previous longer exposures to DA prior to the L2 molt (Figure 3.4D). Similar results were seen with the *daf-9(e1406)* allele (Figure S1.2). In sum, DA can affect the decision during a specific temporal window (15 to 33

hph) during the L2d stage. Worms become committed to bypass dauer with a minimal exposure of 3 hours in DA, but additional persistent exposure to DA over 12 hours is necessary for complete adult development.

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 have connected the environment sensitive period to the hormone sensitive period during the commitment to reproductive development in the nematode *C. elegans*. 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.

Since DA is produced in small amounts and is detected by mass spectrometry methods which are indirect, we decided on a synthetic set of experiments to understand the molecular mechanism of DA on the dauer decision. Initially, we started with identifying the dose at which DA works as a switch of the dauer decision and at a higher dose orchestrating the decision over the whole animal. The concentration of DA sufficient to cause 100% *daf-9* null worms to develop into complete adults was used to determine the time of action which coincides with the induction and integration periods that wild type worms measure their environment suggesting that DA is the physiological mediator of the dauer decision and its outcome. We further used this baseline concentration of DA to understand the relationship with pheromone and the TGFβ pathway, and revealed that pheromone can modulate the dauer bypass DA threshold. This interaction was not revealed in the epistasis interactions.

In particular, the timing is congruent with the requirement for DA. In this view favorable conditions equate with the presence of DA, while unfavorable conditions equate with its absence. Starting from 15 hph, pulses of DA 3 hours or longer will rescue dauer formation with no memory to previous exposures to DA, eliminating it as a mediator of parental dauer history (Figure 3.4). Similarly *daf-9* mutants become refractory to DA rescue from dauer at 33 hph, mid L2d stage. Thus, these periods of DA sensitivity overlap substantially with the response to changes of population density in the environment (Figure 3.5A).

Our studies suggest that two thresholds of DA must be crossed in order to ensure proper reproductive development (Figure 3.3F). First, addition of DA to *daf-9* mutants suggest that about 10nM DA is sufficient to bypass the adult DA threshold in liquid culture and 1-5nM are sufficient on plates. Second, animals that have committed to L3 development, required an additional 30nM DA to promote normal gonadogenesis and cuticle formation. Higher levels of DA increase developmental rate. Conversely, exogenous dauer pheromone can raise the adult DA threshold and complete reproductive development, suggesting that pheromone has additional targets downstream or parallel to DA production that antagonize reproductive development (Figure 3.5B).

What might be the molecular and cellular correlates of these two thresholds? The cytochrome P450 DAF-9 is limiting for DA production, since its biochemical function is essential for bile acid synthesis. *daf-9* is expressed in the XXX cells from hatch and throughout development, and in the hypodermis starting from mid-L2 until L4. The timing requirements for DA described above suggest that the commitment to adult

development through the L2 stage is made early in L2 between 15-18 hph, a time that precedes visible hypodermal *daf-9* expression (Gerisch and Antebi, 2004; Mak and Ruvkun, 2004).

Increasing the amounts of DA to *daf-9* mutants, reveals several intermediate states of adulthood phenotypes and a further increase causes most animals to form normal adults. Wild type worms have only the dauer stage and the adult without any abnormal phenotypes suggesting that a canalizing mechanism is in place to avoid all of the abnormal phenotypes. We characterized the differences in these states using the Shannon- Weiner heterogeneity index and show that the addition of DA starts with a minimal entropy displaying only the dauer fate and increasing to a maximal entropy of abnormal states at 50nM. A further addition of DA decreases the entropy almost to wild type levels observed in the adult state. We speculate that the difference in adult stage entropy in the wild type state and the *daf-9(dh6)* + DA is the result of a-synchronous development. The increase and decrease of entropy suggest that the wild type worms implement a mechanism to canalize development. In the next chapter we will describe a positive feedback loop that is triggered as a result of commitment into adulthood which upregulates *daf-9* in the hypodermis giving rise to higher levels of DA in the while animal. We will argue that this upregulation is the canalizing mechanism enforcing the binary nature of the dauer decision.

Materials and methods

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.

Dafachronic acid assays

Liquid culture: Δ7-DA was solubilized in 100% EtOH to necessary concentrations. Liquid culture assays were performed by adding EtOH-solubilized Δ7-DA in S basal medium. NG agar plate assays were performed by resuspending EtOH-solubilized Δ7-DA in S basal with OP50 and spreading on plates. Worms were picked onto Petri plates not more than one day after Δ7-DA was added to those plates. For the p*daf-9*::gfp experiment Δ7-DA was added on a 3 cm NG agar plates, seeded with OP50.

Scoring incomplete development

daf-9(dh6), daf-9(e1406), daf-9(rh50) 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.

Bibliography

Antebi, A., Culotti, J.G., and Hedgecock, E.M. (1998). daf-12 regulates developmental age and the dauer alternative in Caenorhabditis elegans, Vol 125.

Antebi, A., Yeh, W.H., Tait, D., Hedgecock, E.M., and Riddle, D.L. (2000). daf-12 encodes a nuclear receptor that regulates the dauer diapause and developmental age in C. elegans. Genes & development *14*, 1512-1527.

Baugh, L.R., Demodena, J., and Sternberg, P.W. (2009). RNA Pol II accumulates at promoters of growth genes during developmental arrest. Science *324*, 92-94.

Birnby, D.A., Link, E.L., Vowels, J.J., Tian, H.Z., Colacurcio, P.L., and Thomas, J.H. (2000). A transmembrane guanylyl cyclase (DAF-11) and Hsp90 (DAF-21) regulate a common set of chemosensory behaviors in Caenorhabditis elegans, Vol 155.

Byerly, L., Cassada, R.C., and Russell, R.L. (1976). The life cycle of the nematode Caenorhabditis elegans. I. Wild-type growth and reproduction. Developmental biology *51*, 23-33.

Cassada, R.C., and Russell, R.L. (1975). The dauerlarva, a post-embryonic developmental variant of the nematode Caenorhabditis elegans. Developmental biology *46*, 326-342.

Denver, R.J. (2007). Endocannabinoids link rapid, membrane-mediated corticosteroid actions to behavior. Endocrinology *148*, 490-492.

Gerisch, B., and Antebi, A. (2004). Hormonal signals produced by DAF-9/cytochrome P450 regulate C. elegans dauer diapause in response to environmental cues. Development (Cambridge, England) *131*, 1765-1776.

Gerisch, B., Rottiers, V., Li, D., Motola, D.L., Cummins, C.L., Lehrach, H., Mangelsdorf, D.J., and Antebi, A. (2007). A bile acid-like steroid modulates Caenorhabditis elegans lifespan through nuclear receptor signaling. Proceedings of the National Academy of Sciences of the United States of America *104*, 5014-5019.

Gerisch, B., Weitzel, C., Kober-Eisermann, C., Rottiers, V., and Antebi, A. (2001). A hormonal signaling pathway influencing C. elegans metabolism, reproductive development, and life span. Developmental cell *1*, 841-851.

Gilbert, S.F., Epel, D. (2009). Ecological developmental biology; Integrating Epigenetics, medicine, and Environment (Sinauer Associates Inc.).

Golden, J.W., and Riddle, D.L. (1982). A pheromone influences larval development in the nematode Caenorhabditis elegans. Science *218*, 578-580.

Golden, J.W., and Riddle, D.L. (1984a). The Caenorhabditis elegans dauer larva: developmental effects of pheromone, food, and temperature. Developmental biology *102*, 368-378.

Golden, J.W., and Riddle, D.L. (1984b). A pheromone-induced developmental switch in Caenorhabditis elegans: Temperature-sensitive mutants reveal a wild-type temperature-dependent process. Proceedings of the National Academy of Sciences of the United States of America *81*, 819-823.

Hannich, J.T., Entchev, E.V., Mende, F., Boytchev, H., Martin, R., Zagoriy, V., Theumer, G., Riezman, I., Riezman, H., Knolker, H.J., *et al.* (2009). Methylation of the sterol nucleus by STRM-1 regulates dauer larva formation in Caenorhabditis elegans. Developmental cell *16*, 833-843.

Jia, K., Albert, P.S., and Riddle, D.L. (2002). DAF-9, a cytochrome P450 regulating C. elegans larval development and adult longevity. Development (Cambridge, England) *129*, 221-231.

Keshan, B., Hiruma, K., and R iddiford, L.M. (2006). Developmental expression and hormonal regulation of different isoforms of the transcription factor E75 in the tobacco hornworm Manduca sexta. Developmental biology *295*, 623-632.

Klass, M., and Hirsh, D. (1976). Non-ageing developmental variant of Caenorhabditis elegans. Nature *260*, 523-525.

Koyama, T., Syropyatova, M.O., and Riddiford, L.M. (2008). Insulin/IGF signaling regulates the change in commitment in imaginal discs and primordia by overriding the effect of juvenile hormone. Developmental biology *324*, 258-265.

Ludewig, A.H., Kober-Eisermann, C., Weitzel, C., Bethke, A., Neubert, K., Gerisch, B., Hutter, H., and Antebi, A. (2004). A novel nuclear receptor/coregulator complex controls C. elegans lipid metabolism, larval development, and aging. Genes & development *18*, 2120-2133.

Mak, H.Y., and Ruvkun, G. (2004). Intercellular signaling of reproductive development by the C. elegans DAF-9 cytochrome P450. Development (Cambridge, England) *131*, 1777-1786.

Motola, D.L., Cummins, C.L., Rottiers, V., Sharma, K.K., Li, T., Li, Y., Suino-Powell, K., Xu, H.E., Auchus, R.J., Antebi, A.*, et al.* (2006). Identification of ligands for DAF-12 that govern dauer formation and reproduction in C. elegans. Cell *124*, 1209-1223.

Nijhout, H.F. (2003). Development and evolution of adaptive polyphenisms. Evol Dev *5*, 9-18.

Ohkura, K., Suzuki, N., Ishihara, T., and Katsura, I. (2003). SDF-9, a protein tyrosine phosphatase-like molecule, regulates the L3/dauer developmental decision through hormonal signaling in C. elegans. Development (Cambridge, England) *130*, 3237-3248.

Patel, D.S., Fang, L.L., Svy, D.K., Ruvkun, G., and Li, W. (2008). Genetic identification of HSD-1, a conserved steroidogenic enzyme that directs larval development in Caenorhabditis elegans. Development (Cambridge, England) *135*, 2239-2249.

Riddle, D.L., Albert, P., ed. (1997). Genetic and environmental regulation of dauer larva development (New York, Cold Spring Harbor Laboratory Press).

Rottiers, V., and Antebi, A. (2006). Control of Caenorhabditis elegans life history by nuclear receptor signal transduction. Experimental gerontology *41*, 904-909.

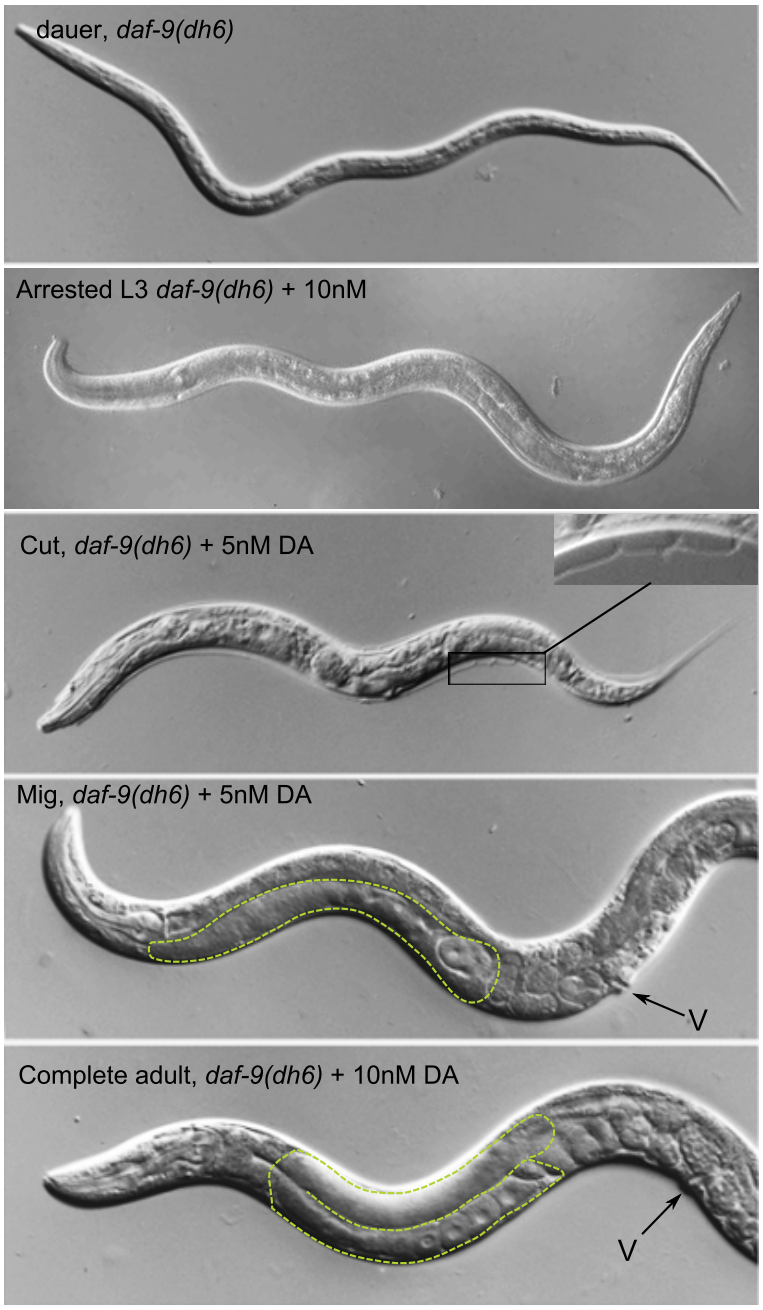
Wollam, J., and Antebi, A. (2010). Sterol Regulation of Metabolism, Homeostasis, and Development. Annu Rev Biochem.

Zar, J.H. (2009). Biostatistical Analysis, 5 edn (Upper Sadlle River, , Prentice Hall).

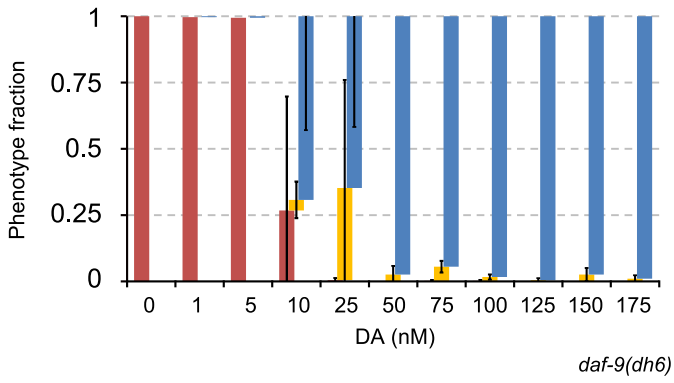
Figures

Figure 3.1

A



B



C

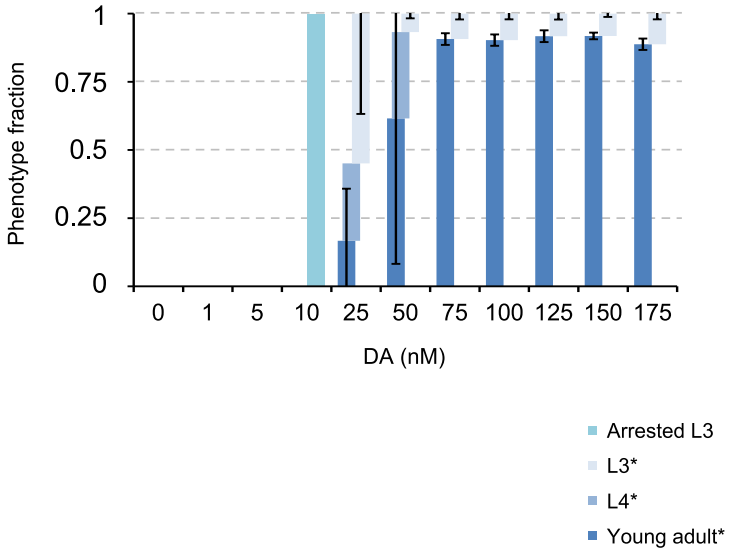


Figure 3.1. High amounts of DA are required for complete adult development

(A) Images of dauer, arrested L3, abnormal development Mig and Cut worms, and normal adults. (B) Distribution developmental stages as a function of DA, scored 48 hph. Means of dauer (red), abnormal development (arrested L3, Mig and Cut; yellow) and complete adult (L3, L4 and young adult; blue) phenotype in *daf-9(dh6)* worms. (C) Distribution of stages in the adult fraction of phenotypes. Means of population proportions of stages indicate the relative developmental rate at each concentration of DA scored at 48 hph. Wild type worms grown in these conditions are YA. Error bars represent means ± standard deviations across three biological replicates, N>500. Mig; gonad migratory defective, Cut, cuticle defective, YA; young adult. * Worms were gravid the next day.

Figure 3.2

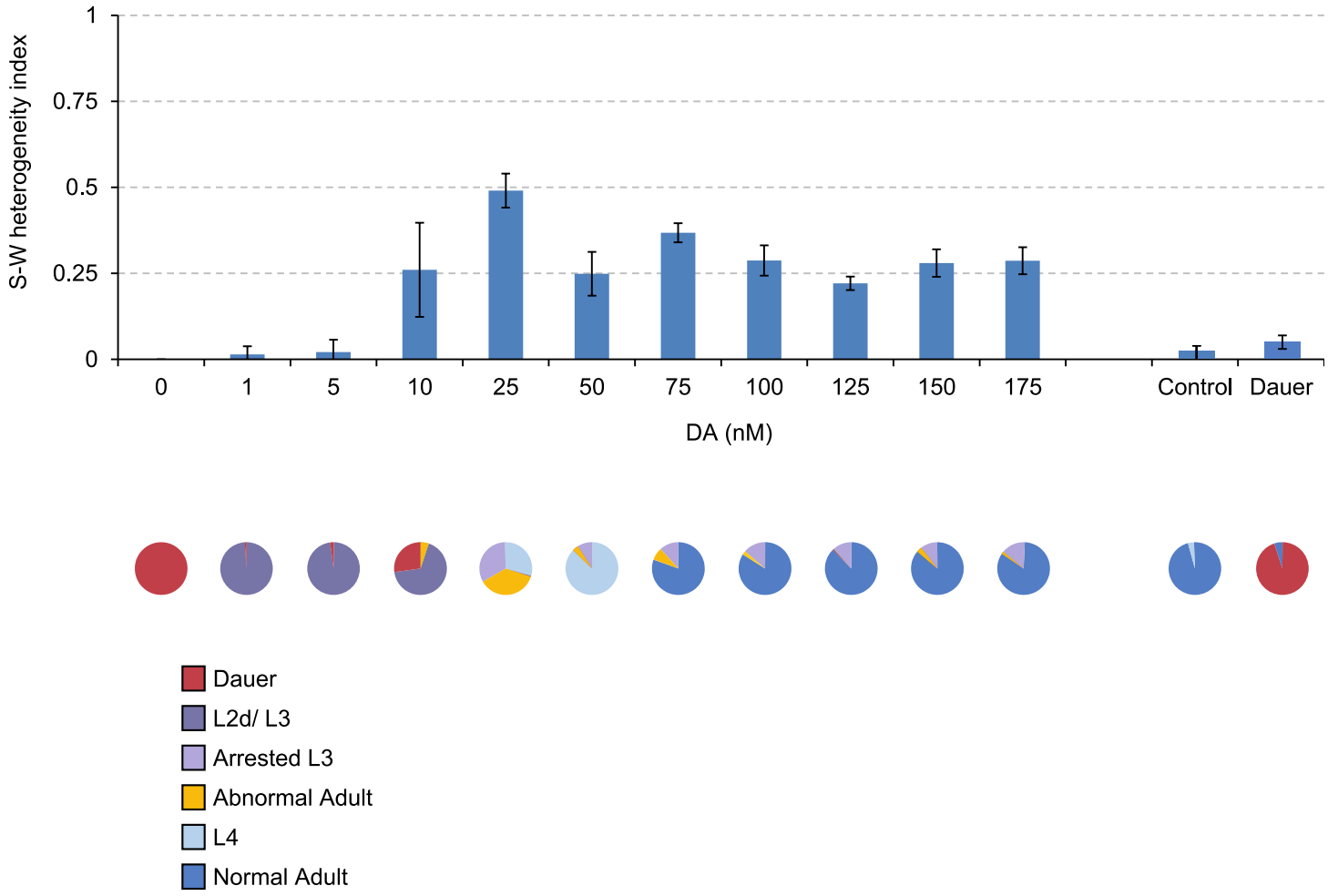

S-W heterogeneity index
1
0.75
0.5
0.25
0
0
1
5
10
25
50
75
100
125
150
175
Control
Dauer
DA (nM)
Dauer
L2d/ L3
Arrested L3
Abnormal Adult
L4
Normal Adult

Figure 3.2: An increase in DA leads to canalization of developmental fates.

The Shannon-Weiner heterogeneity index was calculated for the developmental phenotypes of each dose of DA. The index spans from 0 i ndicating that onlu one phenotype is displayed in a population and therefore it is homogenous, to 1 where an equal representation of all other phenotypes are observed equally indicating a heterogeneous population. Dauer: wild type worms grown in unfavorable conditions. Control; wild type worms grown in similar conditions without DA. The high amount of arrested L3 worms in the *daf-9(dh6)* population treated with DA was conserved over all concentrations above 50nM and if it is disregarded, the heterogeneity index approaches that of the control.

Figure 3.3

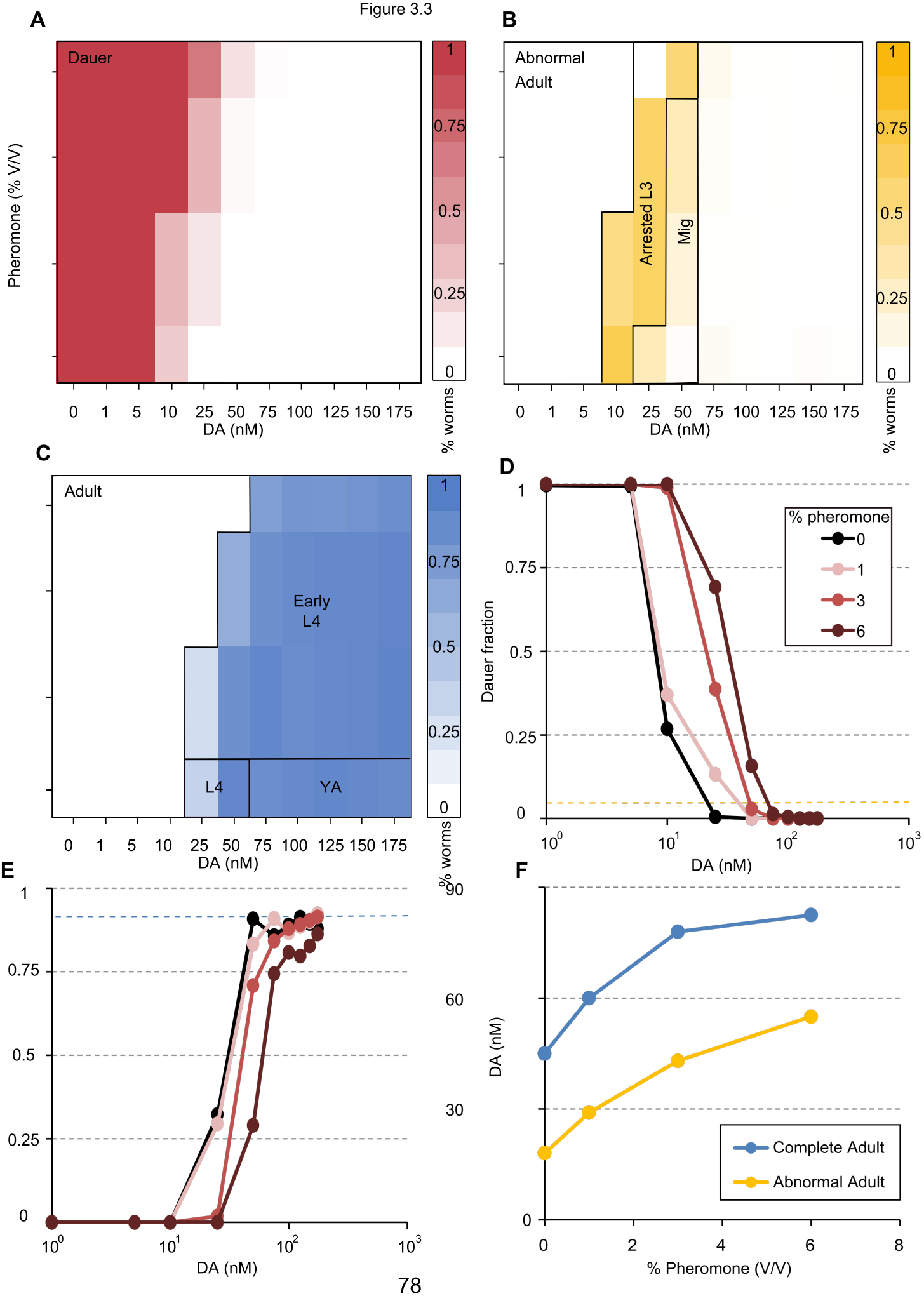

A
Dauer
Pheromone (% V/V)
1
0.75
0.5
0.25
0
0 1 5 10 25 50 75 100 125 150 175
DA (nM)
% worms
B
Abnormal
Adult
Arrested L3
Mig
1
0.75
0.5
0.25
0
0 1 5 10 25 50 75 100 125 150 175
DA (nM)
% worms
C
Adult
Early
L4
L4
YA
1
0.75
0.5
0.25
0
0 1 5 10 25 50 75 100 125 150 175
DA (nM)
% worms
D
% pheromone
0
1
3
6
Dauer fraction
1
0.75
0.5
0.25
0
10^0 10^1 10^2 10^3
DA (nM)
E
Complete Adult fraction
1
0.75
0.5
0.25
0
10^0 10^1 10^2 10^3
DA (nM)
90
60
30
F
DA (nM)
Complete Adult
Abnormal Adult
0
2
4
6
8
% Pheromone (V/V)

Figure 3.3: Dauer pheromone regulates the threshold for reproductive development.

(A-C) Population distribution of dauers (A), abnormal adults (B) and adults (C), as a function of DA and pheromone. Each pixel on the heat map is the mean fraction of population (see bar on the right for quantification) developing in the specific category, N>300 per pixel. Partition of abnormal adults and adults into sub-categories is detailed in Fig S3. (D) Concentrations of DA required to pass the dauer bypass DA threshold as a function of pheromone. Yellow dashed line indicates 90% non-dauers in the population. (E) Concentrations of DA required for normal adult development without any arrested L3, Mig or Cut phenotypes. Blue dashed line indicates 90% complete adults in the population. (F) Normal adult development requires an additional 30 nM DA above the amount for dauer bypass. Yellow plot denotes the concentrations of DA required for 90% of the population to develop into non-dauer fates and blue plot denotes the concentration required for 90% of the population to become normal adults. * denotes animals that developed into gravid adults the next day. Mig; gonad migratory defective, YA; young adult.

Figure 3.4

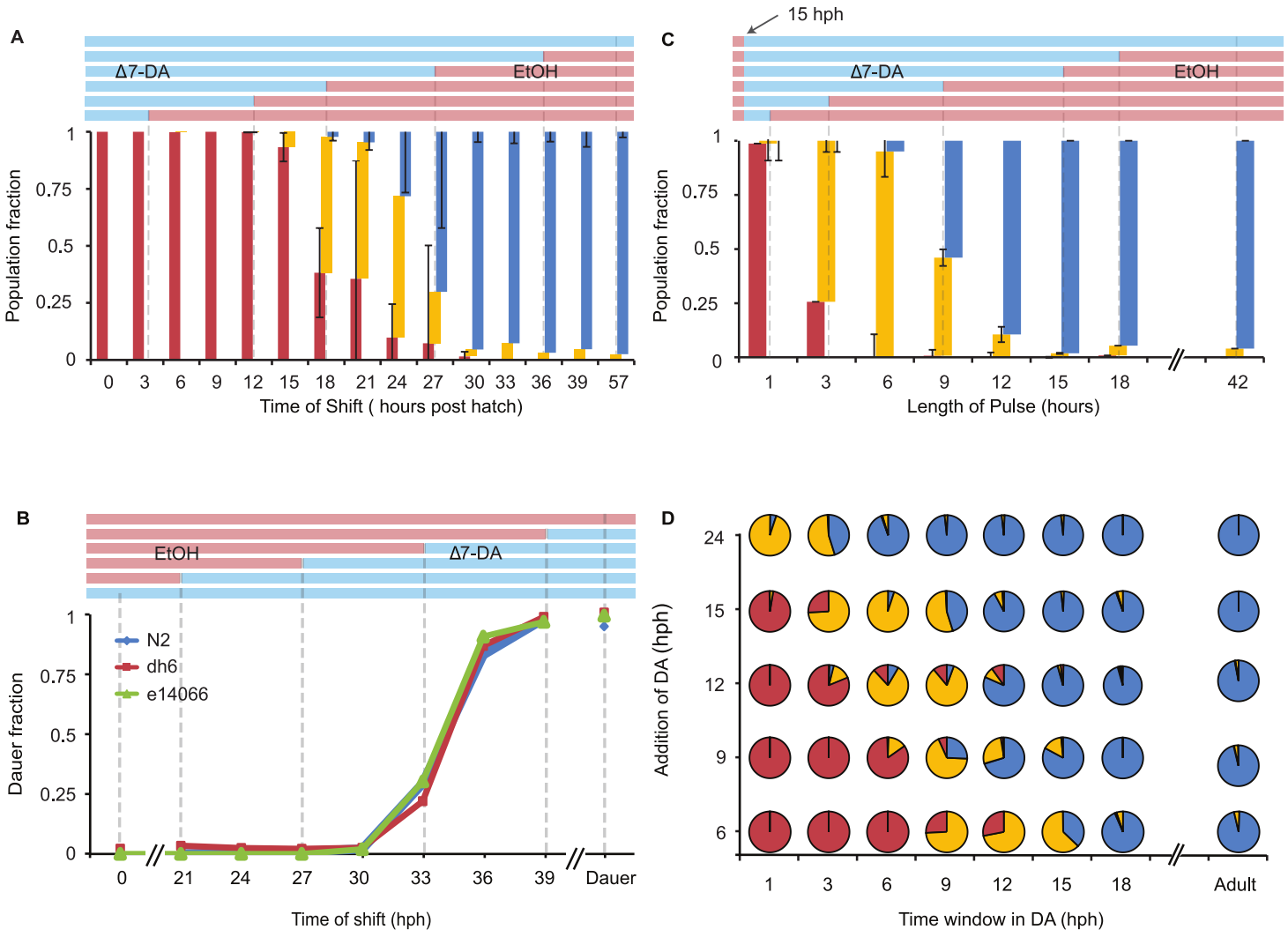

A
Δ7-DA
EtOH
Population fraction
1
0.75
0.5
0.25
0
0 3 6 9 12 15 18 21 24 27 30 33 36 39 57
Time of Shift (hours post hatch)
C
15 hph
Δ7-DA
EtOH
Population fraction
1 3 6 9 12 15 18 42
Length of Pulse (hours)
B
EtOH
Δ7-DA
N2
dh6
e14066
Dauer fraction
0 21 24 27 30 33 36 39 Dauer
Time of shift (hph)
D
Addition of DA (hph)
24
15
12
9
6
1 3 6 9 12 15 18 Adult
Time window in DA (hph)

Figure 3.4. Temporal activity of Δ7-DA

Dauer, abnormal development and normal adult fates as a function of exposure times to Δ7-DA. (A) *daf-9(dh6)* worms start responding to ∆7-DA at 15 hph and require an additional 12 hours of ∆7-DA for complete adult development. Top; representative colored bars indicating the shift experiment: red bars indicate EtOH carrier and blue bars indicate Δ7-DA. Bottom; Normal adult (blue), abnormal adult (yellow) and dauer (red) bars indicate the population fraction per time point. (B) Worms become refractory to Δ7-DA at 33 hph, the same time that they commit to dauer. N2 indicates worms shifted from unfavorable to favorable conditions as indicated in Fig 1C, and points indicate dauer proportions (abnormal development is considered non-dauer in this panel). (C) Pulses of Δ7-DA indicate the minimal time necessary for normal development when added at 15 hph. Top; length of pulses. Bottom: Normal adult (blue), abnormal development (yellow) and dauer (red) bars indicate the population fraction per time point. (D) Worms have no memory of previous exposure to Δ7-DA before the L1/L2 molt. Pie charts indicate proportions of dauers (red), abnormal development (yellow) and normal adults (blue) as a function of total amount of time exposed to Δ7-DA (x-axis) when exposed to Δ7-DA at different hours post hatch (y-axis). N > 100 for all time points in all panels, see SOM for error bars.

Figure 3.5

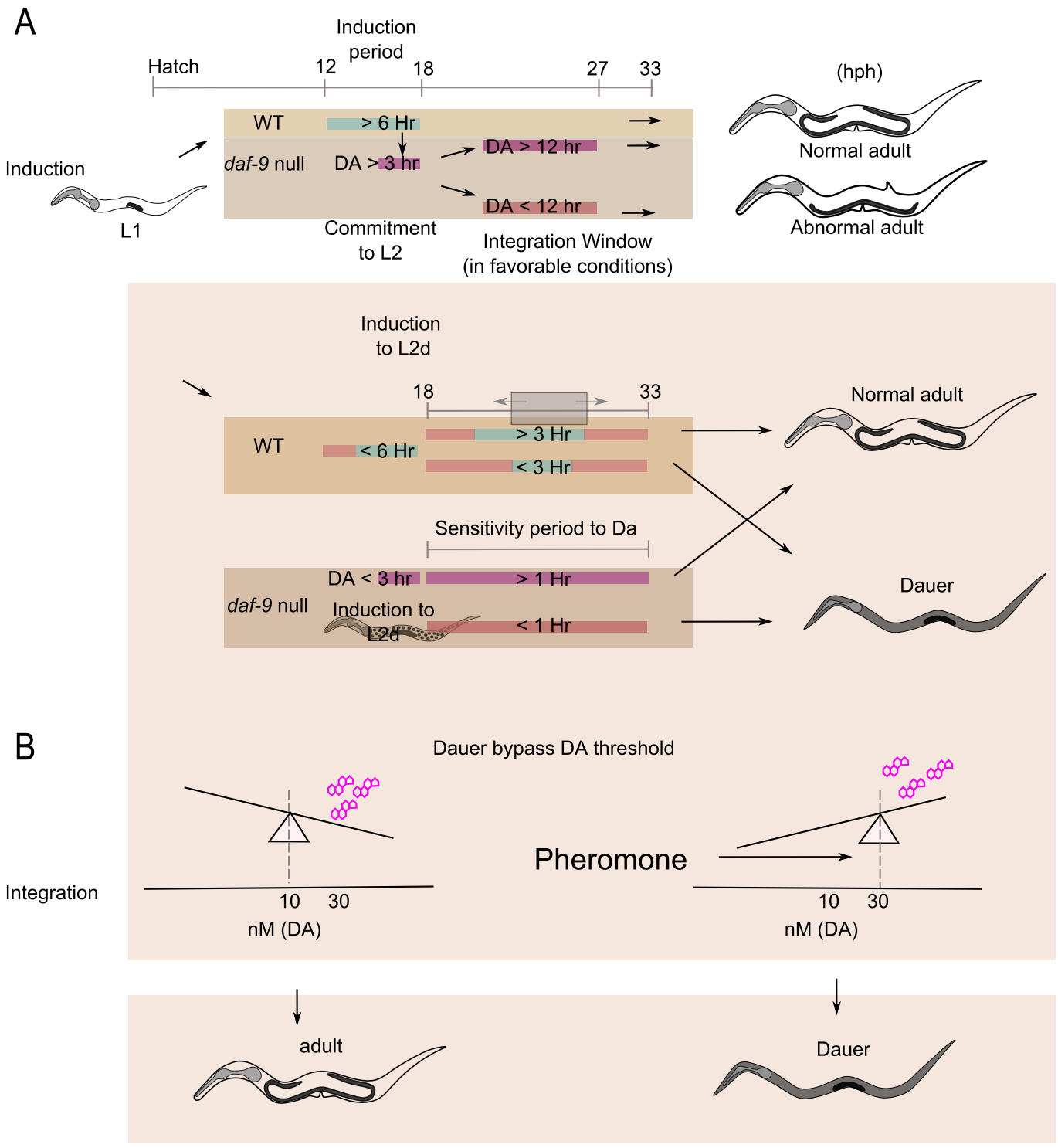

A
Induction period
Hatch
12
18
27
33
(hph)
WT
> 6 Hr
DA > 12 hr
Normal adult
Induction
daf-9 null
DA > 3 hr
DA < 12 hr
Abnormal adult
L1
Commitment to L2
Integration Window (in favorable conditions)
Induction to L2d
18
33
Normal adult
WT
< 6 Hr
> 3 Hr
< 3 Hr
Sensitivity period to Da
DA < 3 hr
> 1 Hr
Dauer
daf-9 null
Induction to L2d
< 1 Hr
B
Dauer bypass DA threshold
Integration
Pheromone
10
30
nM (DA)
10
30
nM (DA)
adult
Dauer

Figure 3.5: Effects of dose and temporal exposure of DA on *daf-9* worms

(A)Top; A tight correlation between the induction period of wild type worms exposed to pheromone and the sensitivity period of *daf-9* nulls to DA. Bottom; The integration window of wild type worms overlaps with the sensitivity period to DA. When wild type worms commit to dauer at 33 hph, *daf-9(dh6)* worms become refractory to DA leading to dauer development. (B) Integration of pheromone and DA into the dauer decision. *daf-9* worms display 2 thresholds for DA; the dauer bypass DA threshold at around 10nM is sufficient to bypass dauer, whereas an ad ditional 30nM are required for complete adulthood. I modeled the mechanism as a balance when tipped to the right, causes commitment to adult development, whereas if maintained on the left leads to duaer commitment. Nascent DA levels can flip the balance to the right leading to adult commitment. Pheromone influences the position of the fulcrum; As the fulcrum is shifted to the right, more DA is required to flip the balance.

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 bi nary decision between two mutually exclusive outcomes (Braendle et al., 2008). Such decisions involve three main phases: (i) measurement and i ntegration, 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 t hus 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 hor mone 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 m s per Nomarski image and 400 m s 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).

Bibliography

Albert, P.S., and Riddle, D.L. (1988). Mutants of Caenorhabditis elegans that form dauer-like larvae, Vol 126.

Alon, U., ed. (2007). An introduction to systems biology: design principles of biological circuits (London, CRC Press, Taylor & Francis Group).

Antebi, A., Culotti, J.G., and Hedgecock, E.M. (1998). daf-12 regulates developmental age and the dauer alternative in Caenorhabditis elegans, Vol 125.

Antebi, A., Yeh, W.H., Tait, D., Hedgecock, E.M., and R iddle, D.L. (2000). daf-12 encodes a nuclear receptor that regulates the dauer diapause and developmental age in C. elegans. Genes & development *14*, 1512-1527.

Avery, L., and Horvitz, H.R. (1987). A cell that dies during wild-type C. elegans development can function as a neuron in a ced-3 mutant. Cell *51*, 1071-1078.

Bargmann, C.I., and Horvitz, H.R. (1991). Control of larval development by chemosensory neurons in Caenorhabditis elegans, Vol 251.

Baugh, L.R., Demodena, J., and Sternberg, P.W. (2009). RNA Pol II accumulates at promoters of growth genes during developmental arrest. Science *324*, 92-94.

Baugh, L.R., Hill, A.A., Brown, E.L., and Hunter, C.P. (2001). Quantitative analysis of mRNA amplification by in vitro transcription. Nucleic acids research *29*, E29.

Beldade, P., Brakefield, P.M., and Long, A.D. (2002). Contribution of Distal-less to quantitative variation in butterfly eyespots. Nature *415*, 315-318.

Birnby, D.A., Link, E.L., Vowels, J.J., Tian, H.Z., Colacurcio, P.L., and Thomas, J.H. (2000). A transmembrane guanylyl cyclase (DAF-11) and Hsp90 (DAF-21) regulate a common set of chemosensory behaviors in Caenorhabditis elegans, Vol 155.

Braendle, C., Milloz, J., and F elix, M.A. (2008). Mechanisms and ev olution of environmental responses in Caenorhabditis elegans. Curr Top Dev Biol *80*, 171-207.

Brian, M.V. (1974). Caste differentiation in Myrmica rubra: the role of hormones. J Insect Physiol *20*, 1351-1365.

Brockmann, H.J. (2001). The evolution of alternative strategies and tactics. Advances in study of behavior *30*, 1-51.

Byerly, L., Cassada, R.C., and R ussell, R.L. (1976). The life cycle of the nematode Caenorhabditis elegans. I. Wild-type growth and r eproduction. Developmental biology *51*, 23-33.

Cassada, R.C., and Russell, R.L. (1975). The dauerlarva, a post-embryonic developmental variant of the nematode Caenorhabditis elegans. Developmental biology *46*, 326-342.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W.W., and Prasher, D.C. (1994). Green Fluorescent Protein as a Marker for Gene-Expression. Science *263*, 802-805.

Chater, K.F. (1993). Genetics of differentiation in Streptomyces. Annu Rev Microbiol *47*, 685-713.

Chechik, G., and Koller, D. (2009). Timing of gene expression responses to environmental changes. J Comput Biol *16*, 279-290.

Denver, R.J. (2007). Endocannabinoids link rapid, membrane-mediated corticosteroid actions to behavior. Endocrinology *148*, 490-492.

Dworkin, J., and Losick, R. (2005). Developmental commitment in a b acterium. Cell *121*, 401-409.

Emlen, D.J., and Nijhout, H.F. (1999). Hormonal control of male horn length dimorphism in the dung beetle Onthophagus taurus (Coleoptera: Scarabaeidae). J Insect Physiol *45*, 45-53.

Gerisch, B., and Antebi, A. (2004). Hormonal signals produced by DAF-9/cytochrome P450 regulate C. elegans dauer diapause in response to environmental cues. Development (Cambridge, England) *131*, 1765-1776.

Gerisch, B., Rottiers, V., Li, D., Motola, D.L., Cummins, C.L., Lehrach, H., Mangelsdorf, D.J., and Antebi, A. (2007). A bile acid-like steroid modulates Caenorhabditis elegans lifespan through nuclear receptor signaling. Proceedings of the National Academy of Sciences of the United States of America *104*, 5014-5019.

Gerisch, B., Weitzel, C., Kober-Eisermann, C., Rottiers, V., and Antebi, A. (2001). A hormonal signaling pathway influencing C. elegans metabolism, reproductive development, and life span. Developmental cell *1*, 841-851.

Gibson, G., and Wagner, G. (2000). Canalization in evolutionary genetics: a stabilizing theory? Bioessays *22*, 372-380.

Gilbert, S.F. (2005). Mechanisms for the environmental regulation of gene expression: ecological aspects of animal development. J Biosci *30*, 65-74.

Gilbert, S.F., Epel, D. (2009). Ecological developmental biology; Integrating Epigenetics, medicine, and Environment (Sinauer Associates Inc.).

Golden, J.W., and Riddle, D.L. (1982). A pheromone influences larval development in the nematode Caenorhabditis elegans. Science *218*, 578-580.

Golden, J.W., and Riddle, D.L. (1984a). The Caenorhabditis elegans dauer larva: developmental effects of pheromone, food, and temperature. Developmental biology *102*, 368-378.

Golden, J.W., and Riddle, D.L. (1984b). A pheromone-induced developmental switch in Caenorhabditis elegans: Temperature-sensitive mutants reveal a wild-type temperature-dependent process. Proceedings of the National Academy of Sciences of the United States of America *81*, 819-823.

Hall, S.E., Beverly, M., Russ, C., Nusbaum, C., and Sengupta, P. (2010). A cellular memory of developmental history generates phenotypic diversity in C. elegans. Curr Biol *20*, 149-155.

Hannich, J.T., Entchev, E.V., Mende, F., Boytchev, H., Martin, R., Zagoriy, V., Theumer, G., Riezman, I., R iezman, H., Knolker, H.J.*, et al.* (2009). Methylation of the sterol nucleus by STRM-1 regulates dauer larva formation in Caenorhabditis elegans. Developmental cell *16*, 833-843.

Hoogewijs, D., Houthoofd, K., Matthijssens, F., Vandesompele, J., and Vanfleteren, J.R. (2008). Selection and validation of a set of reliable reference genes for quantitative sod gene expression analysis in C. elegans. BMC molecular biology *9*, 9.

Jia, K., Albert, P.S., and Riddle, D.L. (2002). DAF-9, a cytochrome P450 regulating C. elegans larval development and adult longevity. Development (Cambridge, England) *129*, 221-231.

Keshan, B., Hiruma, K., and R iddiford, L.M. (2006). Developmental expression and hormonal regulation of different isoforms of the transcription factor E75 in the tobacco hornworm Manduca sexta. Developmental biology *295*, 623-632.

Kim, K., Sato, K., Shibuya, M., Zeiger, D.M., Butcher, R.A., Ragains, J.R., Clardy, J., Touhara, K., and Sengupta, P. (2009). Two Chemoreceptors Mediate Developmental Effects of Dauer Pheromone in C. elegans. Science.

Klass, M., and Hirsh, D. (1976). Non-ageing developmental variant of Caenorhabditis elegans. Nature *260*, 523-525.

Koyama, T., Syropyatova, M.O., and Riddiford, L.M. (2008). Insulin/IGF signaling regulates the change in commitment in imaginal discs and primordia by overriding the effect of juvenile hormone. Developmental biology *324*, 258-265.

Lewis, J.A., J.T. Fleming. (1995). Caenorhabditis elegans: Modern biological analysis of an organism

In Methods in Cell Biology (USA)

H.F. Epstein, D. C.Shakes, , Ed., ed. (San Diego, Academic Press), pp. 4-29.

Li, J., Brown, G., Ailion, M., Lee, S., and Thomas, J.H. (2004). NCR-1 and NCR-2, the C. elegans homologs of the human Niemann-Pick type C1 disease protein, function upstream of DAF-9 in the dauer formation pathways. Development (Cambridge, England) *131*, 5741-5752.

Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods (San Diego, Calif *25*, 402-408.

Lohse, M.B., and Johnson, A.D. (2010). Temporal anatomy of an epigenetic switch in cell programming: the white-opaque transition of C. albicans. Mol Microbiol *78*, 331-343.

Ludewig, A.H., Kober-Eisermann, C., Weitzel, C., Bethke, A., Neubert, K., Gerisch, B., Hutter, H., and Antebi, A. (2004). A novel nuclear receptor/coregulator complex controls

C. elegans lipid metabolism, larval development, and aging. Genes & development *18*, 2120-2133.

Magner, D.B., and Antebi, A. (2008). Caenorhabditis elegans nuclear receptors: insights into life traits. Trends Endocrinol Metab *19*, 153-160.

Mak, H.Y., and Ruvkun, G. (2004). Intercellular signaling of reproductive development by the C. elegans DAF-9 cytochrome P450. Development (Cambridge, England) *131*, 1777-1786.

Motola, D.L., Cummins, C.L., Rottiers, V., Sharma, K.K., Li, T., Li, Y., Suino-Powell, K., Xu, H.E., Auchus, R.J., Antebi, A.*, et al.* (2006). Identification of ligands for DAF-12 that govern dauer formation and reproduction in C. elegans. Cell *124*, 1209-1223.

Nijhout, H.F. (2003). Development and evolution of adaptive polyphenisms. Evol Dev *5*, 9-18.

Ohkura, K., Suzuki, N., Ishihara, T., and Katsura, I. (2003). SDF-9, a protein tyrosine phosphatase-like molecule, regulates the L3/dauer developmental decision through hormonal signaling in C. elegans. Development (Cambridge, England) *130*, 3237-3248.

Patel, D.S., Fang, L.L., Svy, D.K., Ruvkun, G., and Li, W. (2008). Genetic identification of HSD-1, a conserved steroidogenic enzyme that directs larval development in Caenorhabditis elegans. Development (Cambridge, England) *135*, 2239-2249.

Popovici, C., Isnardon, D., Birnbaum, D., and Roubin, R. (2002). Caenorhabditis elegans receptors related to mammalian vascular endothelial growth factor receptors are expressed in neural cells. Neuroscience letters *329*, 116-120.

Rachinsky, A., Strambi, C., Strambi, A., and Hartfelder, K. (1990). Caste and metamorphosis: hemolymph titers of juvenile hormone and ecdysteroids in last instar honeybee larvae. Gen Comp Endocrinol *79*, 31-38.

Reiner, D.J., Ailion, M., Thomas, J.H., and Meyer, B.J. (2008). C. elegans anaplastic lymphoma kinase ortholog SCD-2 controls dauer formation by modulating TGF-beta signaling. Curr Biol *18*, 1101-1109.

Riddle, D.L., Albert, P., ed. (1997). Genetic and environmental regulation of dauer larva development (New York, Cold Spring Harbor Laboratory Press).

Rottiers, V., and Antebi, A. (2006). Control of Caenorhabditis elegans life history by nuclear receptor signal transduction. Experimental gerontology *41*, 904-909.

Schackwitz, W.S., Inoue, T., and Thomas, J.H. (1996). Chemosensory neurons function in parallel to mediate a pheromone response in C. elegans. Neuron *17*, 719-728.

Stephens, C. (1998). Bacterial sporulation: a question of commitment? Curr Biol *8*, R45-48.

Sulston, J.E., and Brenner, S. (1974). The DNA of Caenorhabditis elegans. Genetics *77*, 95-104.

Sulston, J.E., and White, J.G. (1980). Regulation and cell autonomy during postembryonic development of Caenorhabditis elegans. Developmental biology *78*, 577-597.

Tawfik, A.I., Tanaka, S., De Loof, A., Schoofs, L., Baggerman, G., Waelkens, E., Derua, R., Milner, Y., Yerushalmi, Y., and Pener, M.P. (1999a). Identification of the gregarization-associated dark-pigmentotropin in locusts through an albino mutant.

Proceedings of the National Academy of Sciences of the United States of America *96*, 7083-7087.

Tawfik, A.I., Vedrova, A., and Sehnal, F. (1999b). Ecdysteroids during ovarian development and embryogenesis in solitary and gregarious schistocerca gregaria. Arch Insect Biochem Physiol *41*, 134-143.

Veening, J.W., Stewart, E.J., Berngruber, T.W., Taddei, F., Kuipers, O.P., and Hamoen, L.W. (2008). Bet-hedging and epi genetic inheritance in bacterial cell development. Proceedings of the National Academy of Sciences of the United States of America *105*, 4393-4398.

Waterland, R.A., and Jirtle, R.L. (2004). Early nutrition, epigenetic changes at transposons and imprinted genes, and enhanced susceptibility to adult chronic diseases. Nutrition *20*, 63-68.

White, J. (1988). The anatomy of the nematode *Caenorhabditis elgans*

(New York, Cold Spring Harbor Laboratory Press).

Wilson, E.O. (2000). Sociobiology: The New Synthesis, Twenty-Fifth Anniversary Edition (Belknap Press of Harvard University Press).

Wollam, J., and Antebi, A. (2010). Sterol Regulation of Metabolism, Homeostasis, and Development. Annu Rev Biochem.

Xiong, W., and Ferrell, J.E., Jr. (2003). A positive-feedback-based bistable 'memory module' that governs a cell fate decision. Nature *426*, 460-465.

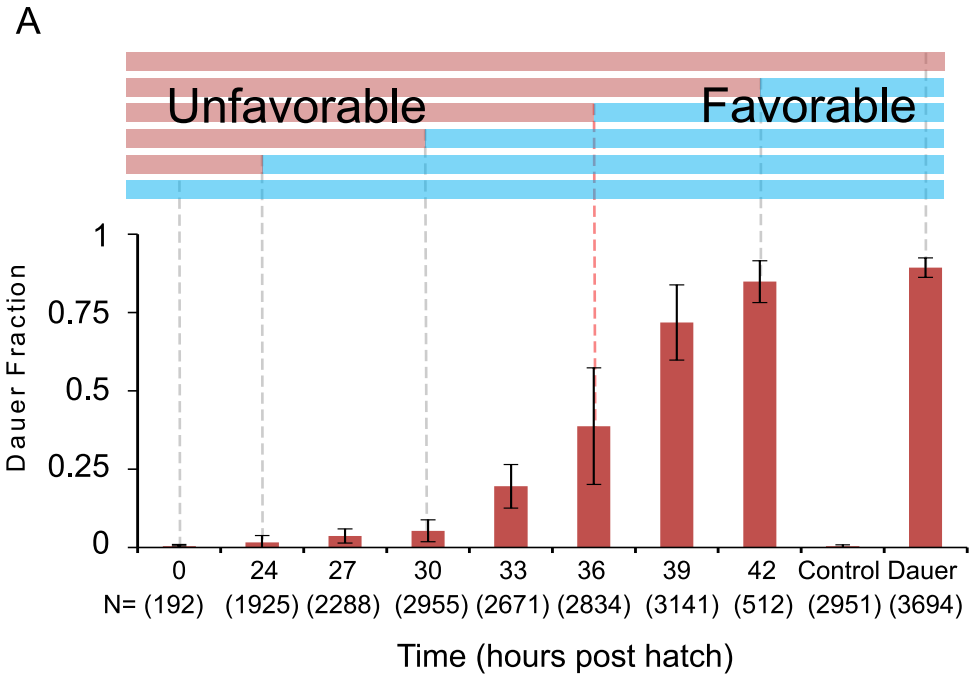
Yosef, N., and Regev, A. (2011). Impulse control: temporal dynamics in gene transcription. Cell *144*, 886-896.

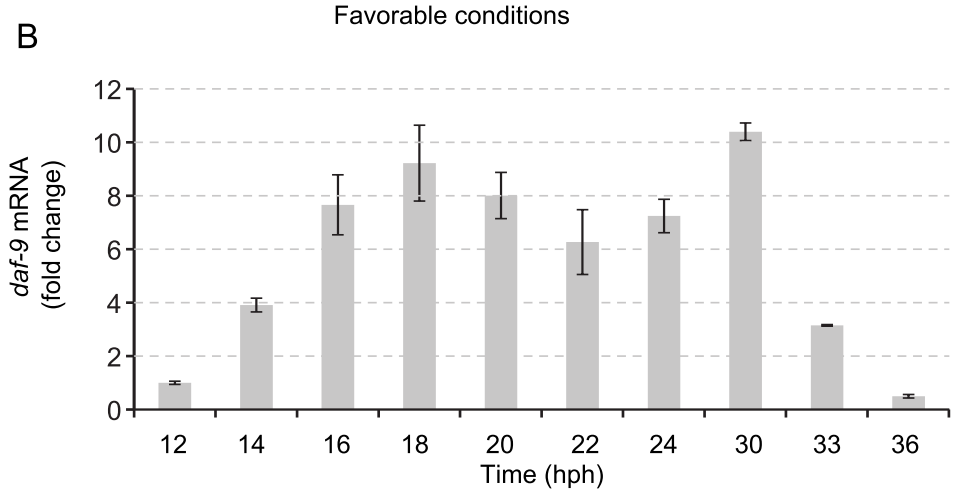
Zar, J.H. (2009). Biostatistical Analysis, 5 edn (Upper Sadlle River, , Prentice Hall).

Zhang, Y., Xu, J., Puscau, C., Kim, Y., Wang, X., Alam, H., and Hu, P.J. (2008). Caenorhabditis elegans EAK-3 inhibits dauer arrest via nonautonomous regulation of nuclear DAF-16/FoxO activity. Developmental biology *315*, 290-302.

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)

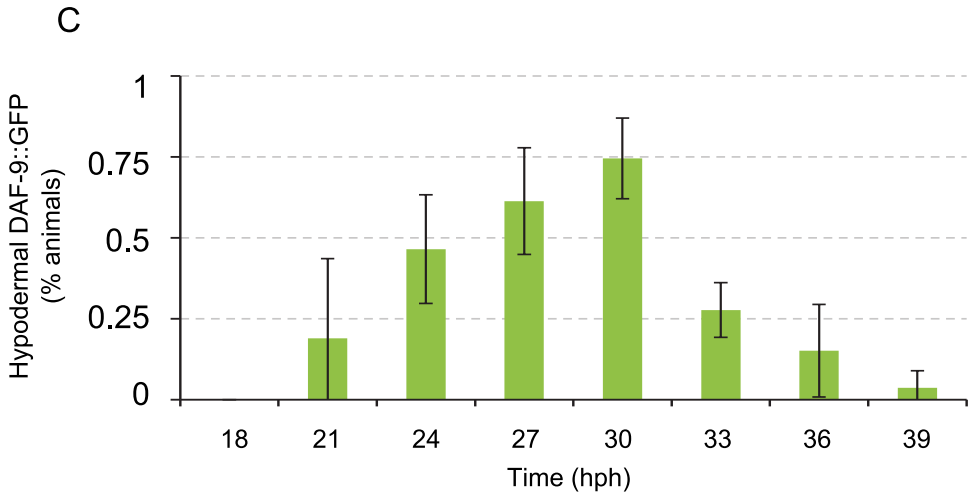

C
Hypodermal DAF-9::GFP
(% animals)
1
0.75
0.5
0.25
0
18 21 24 27 30 33 36 39
Time (hph)

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

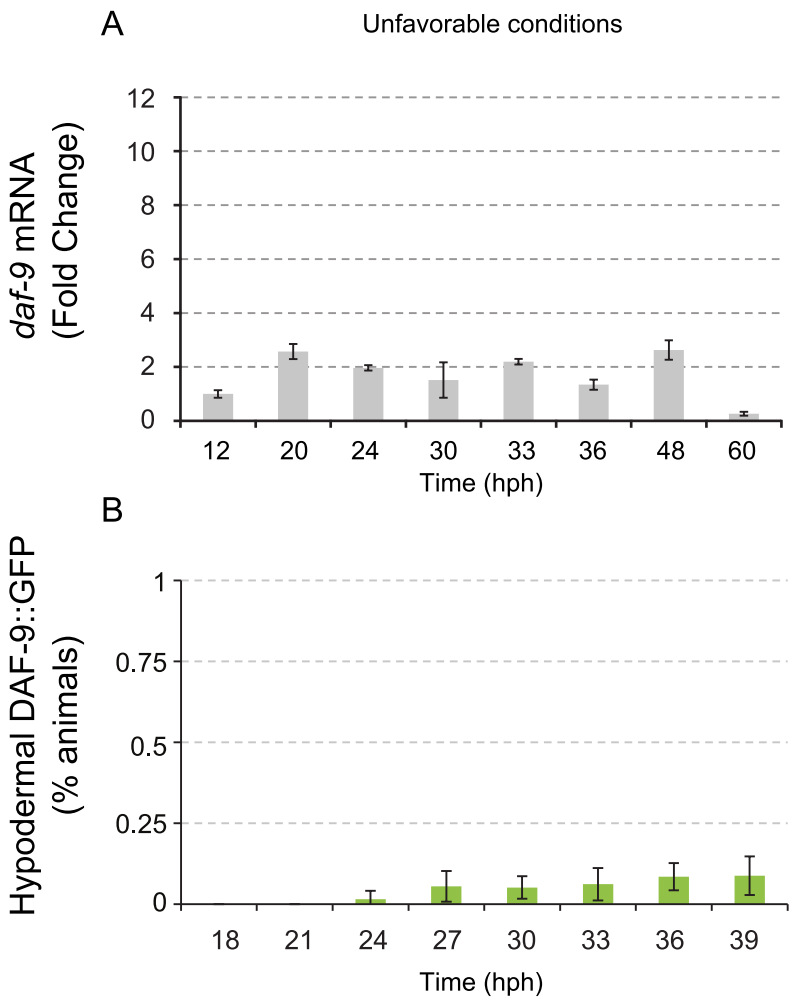

A
Unfavorable conditions
daf-9 mRNA
(Fold Change)
12
10
8
6
4
2
0
12
20
24
30
33
36
48
60
Time (hph)
B
Hypodermal DAF-9::GFP
(% animals)
1
0.75
0.5
0.25
0
18
21
24
27
30
33
36
39
Time (hph)

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

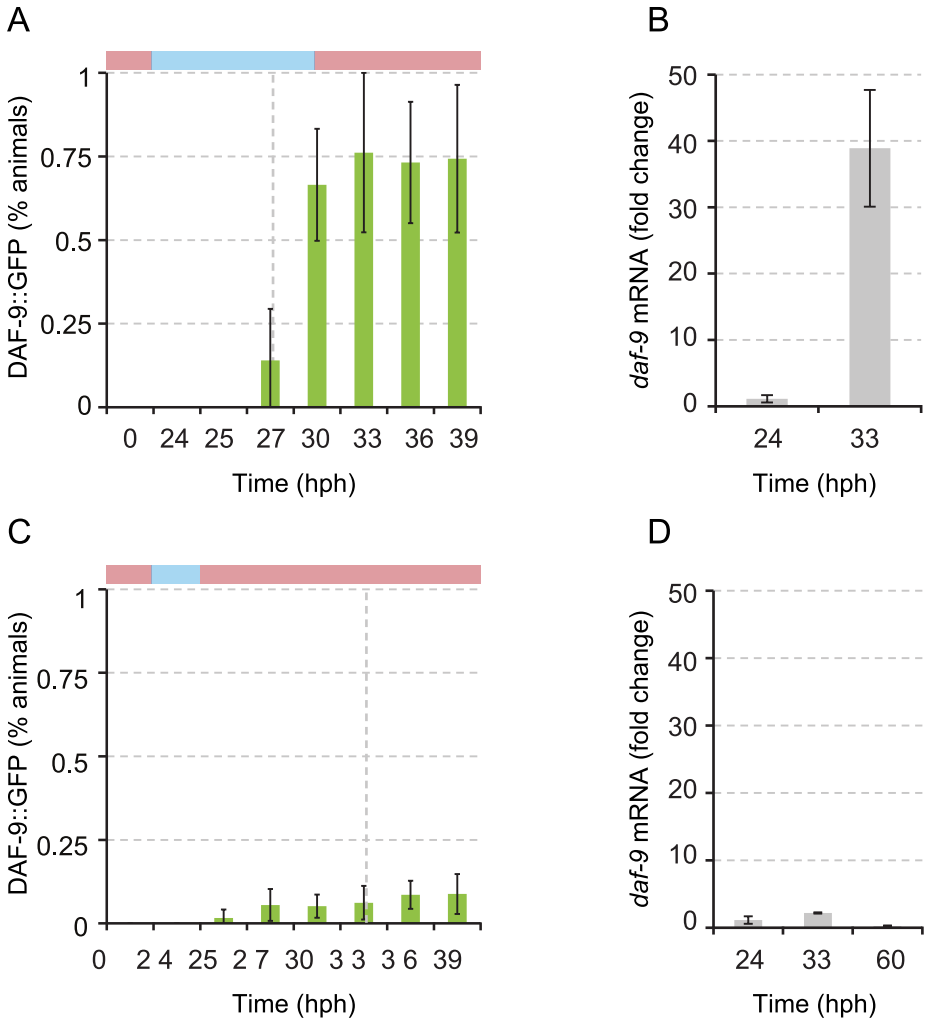

A
DAF-9::GFP (% animals)
1
0.75
0.5
0.25
0
0 24 25 27 30 33 36 39
Time (hph)
B
daf-9 mRNA (fold change)
50
40
30
20
10
0
24 33
Time (hph)
C
DAF-9::GFP (% animals)
1
0.75
0.5
0.25
0
0 2 4 25 2 7 30 3 3 3 6 39
Time (hph)
D
daf-9 mRNA (fold change)
50
40
30
20
10
0
24 33 60
Time (hph)

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.

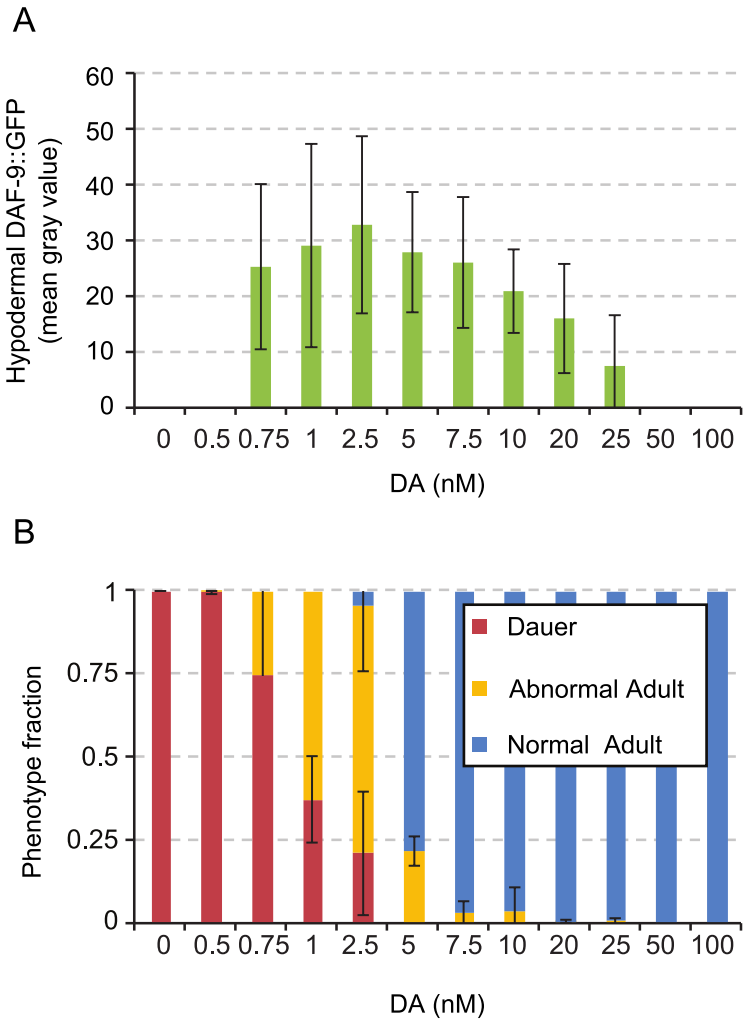

A
Hypodermal DAF-9::GFP
(mean gray value)
60
50
40
30
20
10
0
0 0.5 0.75 1 2.5 5 7.5 10 20 25 50 100
DA (nM)
B
Phenotype fraction
1
0.75
0.5
0.25
0
Dauer
Abnormal Adult
Normal Adult
0 0.5 0.75 1 2.5 5 7.5 10 20 25 50 100
DA (nM)

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

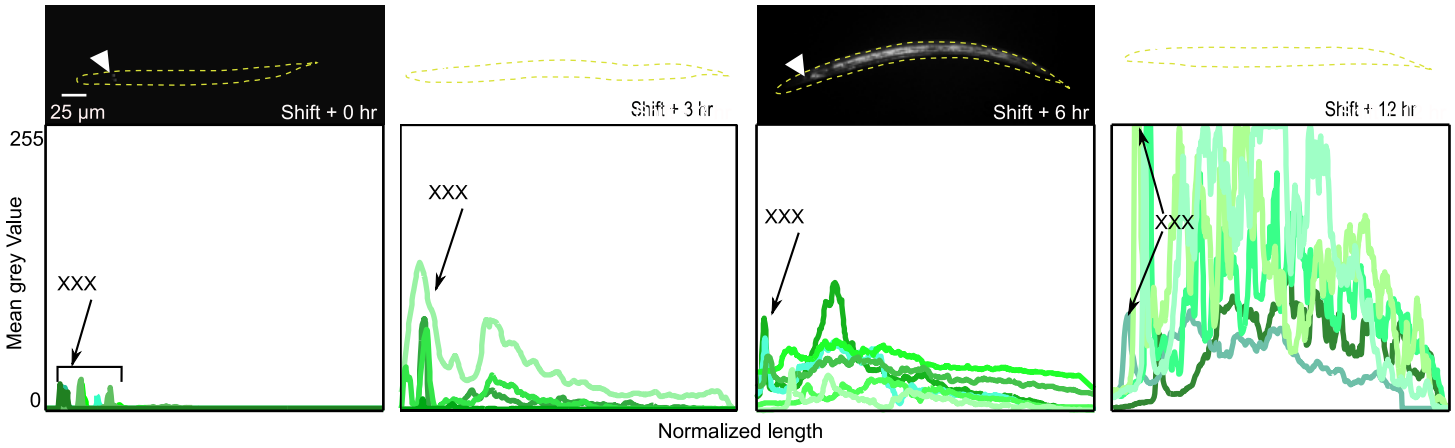

25 µm
Shift + 0 hr
Shift + 3 hr
Shift + 6 hr
Shift + 12 hr
255
0
Mean grey Value
XXX
XXX
XXX
XXX
Normalized length

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

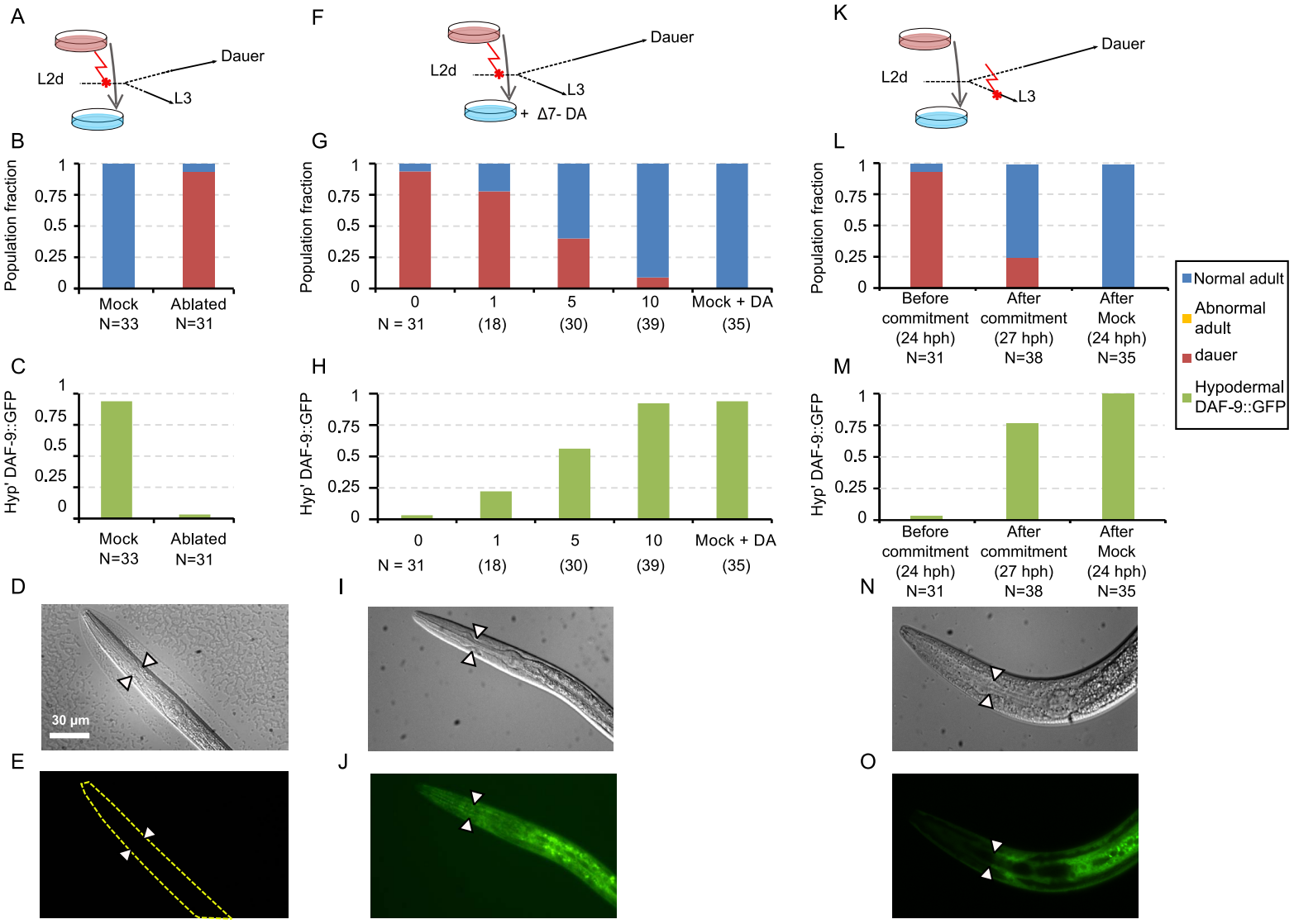

A
L2d
Dauer
L3
F
L2d
Dauer
L3
+ Δ7- DA
K
L2d
Dauer
L3
B
Population fraction
1
0.75
0.5
0.25
0
Mock
N=33
Ablated
N=31
G
Population fraction
0
N = 31
1
(18)
5
(30)
10
(39)
Mock + DA
(35)
L
Population fraction
Before commitment (24 hph) N=31
After commitment (27 hph) N=38
After Mock (24 hph) N=35
Normal 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

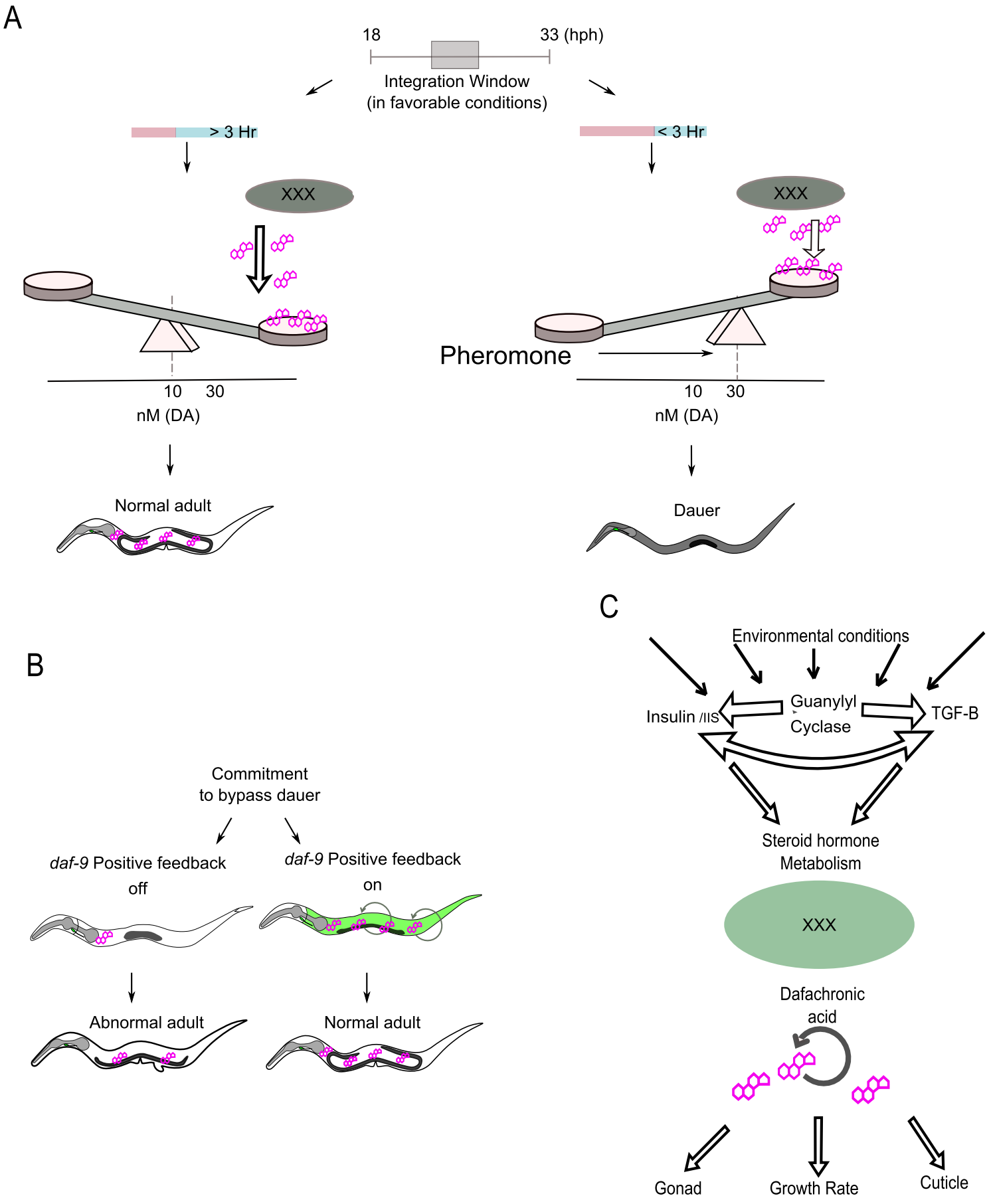

A
18
33 (hph)
Integration Window
(in favorable conditions)
> 3 Hr
< 3 Hr
XXX
XXX
Pheromone
10 30
nM (DA)
10 30
nM (DA)
Normal adult
Dauer
B
Commitment
to bypass dauer
daf-9 Positive feedback
off
daf-9 Positive feedback
on
Abnormal adult
Normal adult
C
Environmental conditions
Insulin /IIS
Guanylyl
Cyclase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

Chapter V: Discussion

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.

I have characterized a mechanism that guarantees the robust development of the *C. elegans* nematode in fluctuating conditions. The small amount of DA which crosses the dauer bypass DA threshold is amplified through the hypodermal *daf-9* DAF-12 positive feedback loop coordinates the decision over many tissue types. When the feedback loop is uncoupled, it reveals intermediate phenotypes that are similar to a reaction norm when adding in increasing amounts of DA. The comparison between the wild type positive feedback loop and the uncoupled *daf-9* mutants reflects the canalizing properties of the mechanism. Commitment to the adult fate via the positive feedback loop can be viewed as an evolutionary mechanism that constrains the reaction norm into a polyphenism thus increasing fitness of the nematode.

The goal of this study was to understand how the known components of the dauer regulatory network shape system level interactions. Time series analysis coupled with controlled changes of environmental conditions have helped elucidate mechanisms and interactions that are not possible with classical genetics methods. For example,

epistasis analysis placed *daf-9* downstream to *daf-7/*TGFβ ligand indicating that the phenotypes regulated by the steroid hormone pathway had already accounted for population density. Our approach of uncoupling the physiological contribution of low DA amounts and high DA amounts has revealed that DA initially has to cross the dauer bypass DA threshold and later during development as a coordinator of the decision. Population density can regulate the threshold of DA.

This analysis has not identified new components of the decision making, but has implicated the commitment points and experimental framework for discovery of target genes that may become markers for commitment to either fate. In a follow-up study, we are performing high throughput sequencing on *daf-9* mutants treated with DA in order to find genes that are transcribed at the point of commitment to L3 as well as dauer. Coupling these experiments to changes of environments can help identify genes that integrate the environmental inputs.

Once we identify markers for the dauer and L3 commitment, we will be able to perform experiments and dissect to greater depth several different classes of regulators mechanisms: (i) integrators of different physiochemical properties such as food availability, population density and temperature. (ii) Molecular characterization of the persistence detector mechanism. (iii) integrators of internal physiological states with real time environmental sensation. Combining these mechanisms together will shed light on the architecture of decision making mechanisms in *C. elegans* which can then be extended to other organisms.

Most of the experiments performed in this study offer conclusions based on population statistics. The synchronization methods developed in order to achieve sharp response curves of commitment (Figure 2.1-2.4) extend poorly to experiments elucidating the mechanisms that regulate of length of the persistence window. To this end, single animal analysis performed in smaller artificial environments will reveal much about individual decision making. Tracking the expression levels of L3 and dauer commitment genes in a Microfluidic device coupled to real-time microscopy will help elucidate the integration mechanisms that nematodes use to understand their environment and make decisions based on integration of input with internal information.

Characterizing the polyphenic dauer switch is by no means a finished story. There are many more questions to be answered. First, what are the molecular events that regulate the dauer phenotypes. Is this decision also made systematically reducing information complexity to a hormonal switch? An alternative mechanism may be orchestrating this branch of development. Several lines of evidence indicate this reasoning: (i) dauers that are formed in starved conditions with low population density have less fat granules in their gut, and dauers that developed when deprived of cholesterol are not SDS resistant indicating that individual environmental cues regulate not only the switch but also the morphology of the dauer. It will be interesting to understand the differences in mechanisms that coordinate reproductive development as opposed to the dauer.

Understanding the evolutionary forces that drive polyphenism has been a long lasting challenge, mostly, since polyphenisms are hard to reproduce in laboratory conditions. The majority of research has focused on the insect class which initially

proved to be a powerful tool due to the ease of extracting hormone titers and performing electrophysiological studies, thus linking environmental cues to molecular mechanism. However, these studies have revealed that much of the regulation is based on genetic interactions which happen on the cellular and molecular level requiring transgenic and genomic techniques. For example, queen selection in Hymenopterans has revealed that methylation of non-coding sequences by *Dnmt-3* explains 72% of the queen/soldier polyphenism. Hormonal regulation in *C. elegans* has only recently been technically possible, and along with well established methods in genetics, transgenics, genomics, and facile and reproducible environmental regulation make *C. elegans* a powerful tool in understanding polyphenism.

Appendixes

Modifications to synchronized worm hatch

All worms were handled using standard growth and cultivation techniques (Lewis, 1995). Worms were synchronized at the hatch as described by (Baugh et al., 2009) with changes as follows. Embryos of the second bleach were seeded onto 10 cm NGM plates and topped with a 1:1 (v/v) mixture of OP50 and S complete medium (Sulston and Brenner, 1974) and incubated in a 20°C incubator. This drying step helps keeping un-hatched eggs from re-suspending after the wash. Sixteen hours after the bleach, plates were inspected for embryos only and pl ates containing hatched worms were discarded. Plates were incubated for a further hour and then washed with S basal to suspend all hatched worms. Less than 1% of eggs were found after this wash. Pooled worms were washed once in S complete and used for subsequent experiments.

Purification of RNA

100 µl of acid washed (1M HCl) sand was aliquoted into eppendorf tubes. Frozen worms in TRIzol were thawed (Start volume= TRIzol + worms = 1 m l; SV), transferred to sand containing tubes and vortexed vigorously for 10 min. Tubes were allowed to settle at room temperature for 5 min. 20% SV of $ChCl_3$, and TRIzol solution were aliquoted into fresh tubes mixed thoroughly for 15 sec and centrifuged at 12000 RCF for 15 m inutes 2-8°c. Aqueous phase was transferred to new tubes mixed with 50% SV of 100% Isopropanol and let precipitate at -20°c overnight. Samples were centrifuged at 12000 RCF for 10

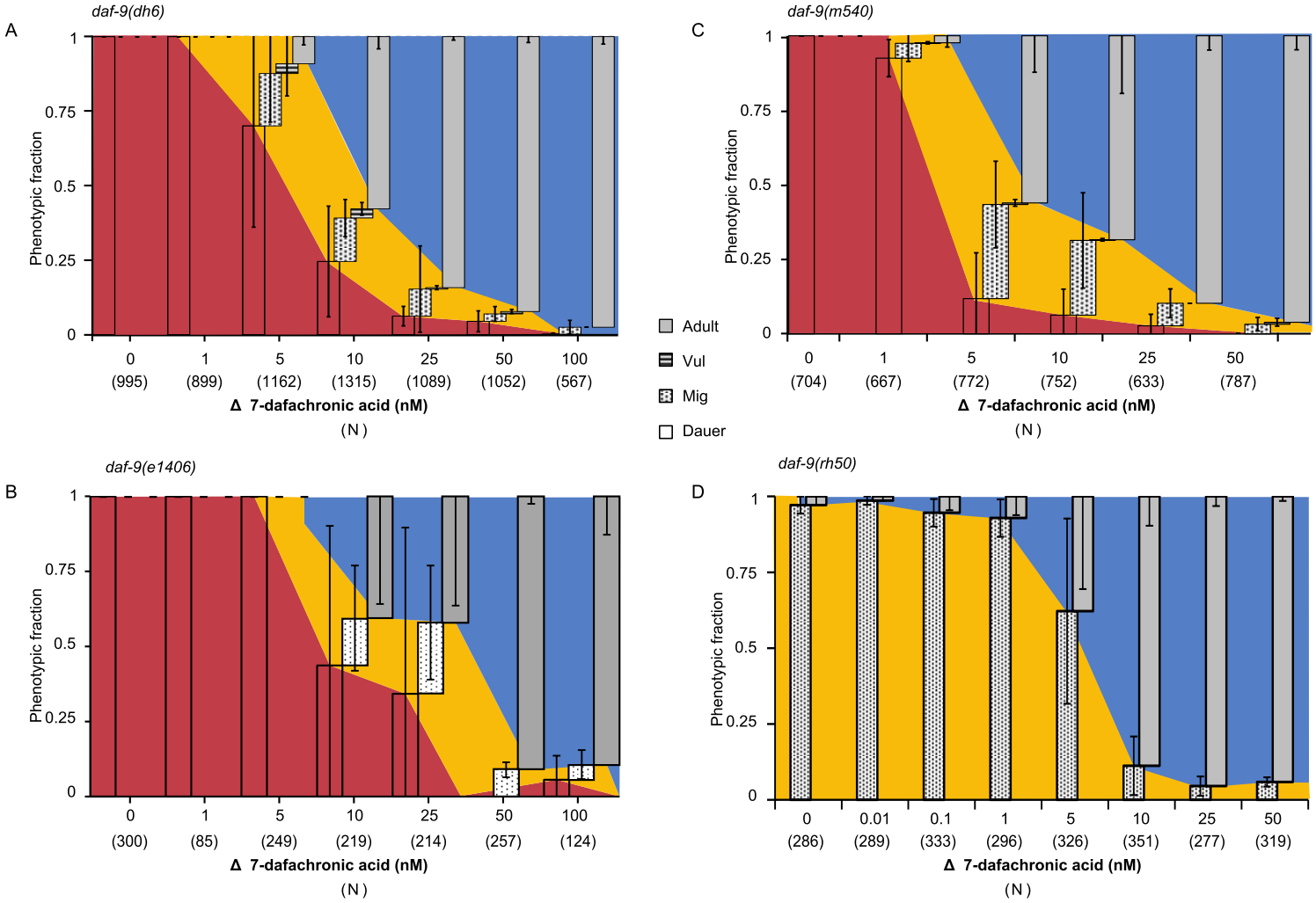
min 4°c, pellet was washed with 1x SV of 75% (v/v) Ethanol (in DEPC-H_2O) and EtOH remainders were aspirated. Pellets were resuspended in 10% SV of DEPC treated DDW.

Computational worm straightening

Each worm was outlined on the DIC image and a central axis was drawn through all bent segments. Pixels on the perpendicular line between the central axis and the worm boundary were recorded and added to the previous section until the entire worm was rendered in a straight alignment. Since each worm had a representative image in DIC and in fluorescence imaging, the same coordinate system per central axis and worm boundary was applied to both images and the same computation was performed to retrieve fluorescence data. Each worm renders an array of coordinate pixels which were transformed into mean grey value (ranging between 0, black to 255, white).

Supplementary Figures

Figure S2.1

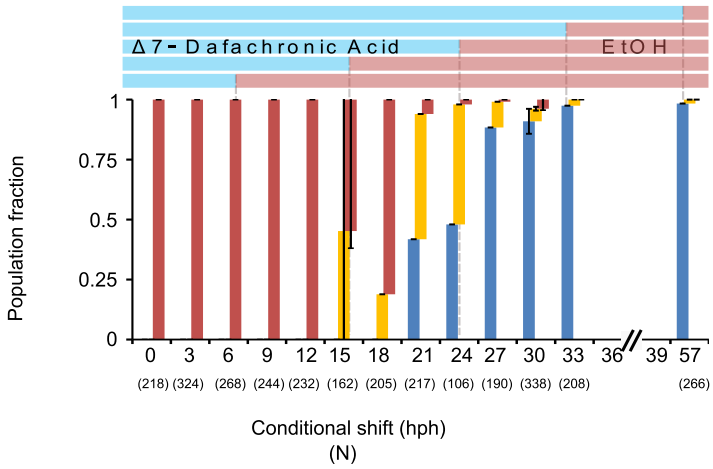

A
daf-9(dh6)
Phenotypic fraction
1
0.75
0.5
0.25
0
0 1 5 10 25 50 100
(995) (899) (1162) (1315) (1089) (1052) (567)
Δ 7-dafachronic acid (nM)
(N)
B
daf-9(e1406)
Phenotypic fraction
0 1 5 10 25 50 100
(300) (85) (249) (219) (214) (257) (124)
Δ 7-dafachronic acid (nM)
(N)
C
daf-9(m540)
Phenotypic fraction
0 1 5 10 25 50
(704) (667) (772) (752) (633) (787)
Δ 7-dafachronic acid (nM)
(N)
Adult
Vul
Mig
Dauer
D
daf-9(rh50)
Phenotypic fraction
0 0.01 0.1 1 5 10 25 50
(286) (289) (333) (296) (326) (351) (277) (319)
Δ 7-dafachronic acid (nM)
(N)

S1.1: High amounts of DA are required for complete adult development

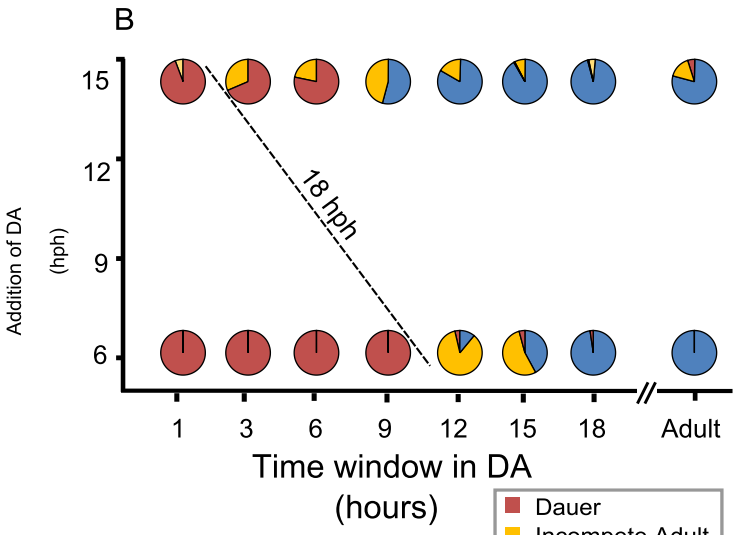
Distributions of dauer (transparent), Mig (dotted), Vul (straight lines) cut (hatched) and wild type adult (grey) phenotypes when mutants were hatched after a non-synchronous bleach (worms typically hatch over a 15 hour window at these growth conditions). (A) *daf-9(dh6)*, (B) *e1406 daf-9* (C)*(m540)* and (D) *daf-9(rh50)* strains. Red represents all dauers, yellow represents all incomplete adult phenotypes and blue represents all complete adults. Bars represent means ± standard deviations across three biological experiments. Numbers in parentheses indicate total worms counted per time point.

Figure S1.2

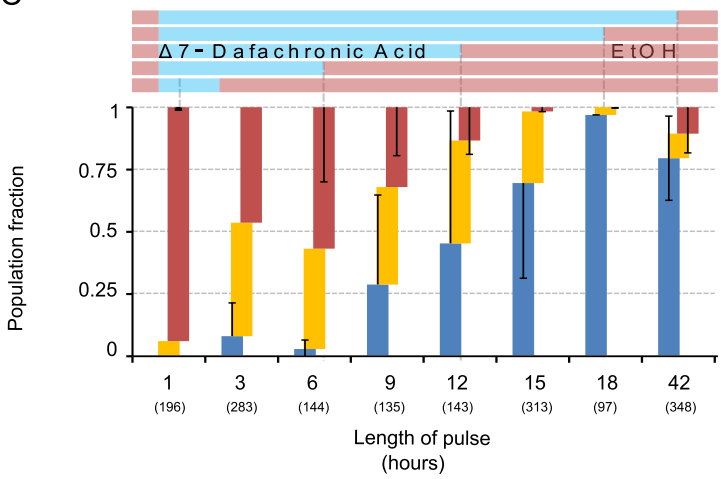
A



B



C



D

	Addition of DA (hph)					
	daf-9(e1406)		*daf-9(dh6)*			
	6	**15**	**6**	**9**	**12**	**15**
1	293	196	205	248	173	265
3	290	283	119	268	91	335
6	276	144	129	185	59	331
9	216	135	62	133	54	369
12	64	143	135	164	108	383
15	82	313	40	147	142	454
18	95	97	117	242	175	192
adult	280	348	143	237	235	360

S1.2: Temporal activity of Δ7-DA.

(A) *daf-9(e1406)* worms start responding to ∆7-DA at 15 hph and require an additional 12-15 hours of ∆7-DA for complete adult development. Top; representative colored bars indicating the shift experiment: red bars indicate EtOH carrier and blue bars indicate Δ7-DA. Bottom; histograms indicate proportions of phenotype frequencies between biological replicates ± standard deviations. (B) Pie charts indicate proportions of dauers (red), incomplete adults (yellow) and complete adults (blue) as a function of total amount of time exposed to Δ7-DA (x-axis) when exposed to Δ7-DA at different hours post hatch (y-axis). (C) Pulse experiments indicate minimal times necessary for complete development. Top; diagram of pulses used per experiment. Bottom, bar graphs indicate proportions of phenotype frequencies between biological replicates ± standard deviations. (D) Number of worms scored for initiation of response to DA. d*af-9(e1406)* indicates the number of worms scored for Figure S4B and *daf-9(dh6)* indicates the number of worms scored for Figure 4B.

Supplementary Tables

Supplementary Table 1: Values of q statistic calculated by a Tukey type multiple comparison test for differences among variances.

Variances are arranged in ascending order from left to right and from top to bottom. This test takes the difference in natural logarithms of variance values of each time point and normalizes it to a standard error of $\sqrt{\frac{2}{k-1}}$ where k are the number of biological replicates at each time point. Significant differences in pair-wise comparisons of variances which are larger than $q_{,0.05,\infty,k}$ are marked in red and non-significant differences are marked in green. Numbers indicate the computed q statistic. Table 1 shows the computed Tukey type multiple comparison test for differences among variances for shift to growth experiment (Figure 2. 2B), Table 2 shows comparisons for Shift to stringent experiment (Figure 2.2C) and Table 3 shows comparisons of *daf-9(dh6)* shift from EtOH to Δ7-Dafachroninc acid (Figure 3.2C).

Table 1A

	C	**27**	**D**	**30**	**39**	**36**	**33**
24	1.2183	2.9516	4.0405	4.7329	5.4368	8.5794	10.1991
C		1.7333	2.8222	3.5146	4.2185	7.3611	8.9808
27		`	1.0888	1.7812	2.4851	5.6277	7.2474
D				0.6923	1.3963	4.5389	1.3963
30					0.70392	3.8465	5.4661
39						3.1425	4.7622
36							1.6196

Table 1B

	W24+9	**W24+1**	**W24+6**	**W24+3**
W24	0.4254	1.2938	1.3975	4.0812
W24+9		0.8999	1.1340	3.6558
W24+1			0.1499	2.4846
W24+6				2.5217

	39	27	30	0	24	21	36	33
None	-4.1058	-2.83804	-2.33764	-0.5823	0.8135	2.2849	4.2021	5.3723
39		1.267766	1.768167	3.5235	4.9193	6.3907	8.3079	9.4781
27			0.500401	2.2557	3.6515	5.1229	7.0401	8.2104
30				1.7553	3.1511	4.6225	6.5397	7.71
0					1.3958	2.8672	4.7844	5.9546
24						1.4714	3.3886	4.5588
21							1.9172	3.0874
36								1.1702

Table 1C

Table 2: Oligonuclotides used in quantification of daf-9 transcripts**.**

Gene	Expression	Spans exons	Forward Primer	Reverse Primer	Amplicon length
daf-9	XXXL/R, Hypodermis	9-10	tcagacgccgtatgtgagag	gctgggataatctcggtgtt	140
daf-9.1a	XXXL/R, Hypodermis	2,4	aaacgaatgtccagtttggtg	ttcgaagtcagggaccactt	183
daf-9.1b	XXXL/R, Hypodermis	3,4	tgagagagcctccatttggt	ttcgaagtcagggaccactt	157
pmp-3	Intestine	3,4	gttcccgtgttcatcactcat	acaccgtcgagaagctgtaga	115
Y45F10D.4	Unknown	1,2	gtcgcttcaaatcagttcagc	gttcttgtcaagtgatccgaca	139
ver-2	ADLL/R	7,8	tgtgacattcgccacaaaat	aaaaactcggcgtttgtttg	178