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Mechanisms and Evolution of Environmental Responses in *Caenorhabditis elegans*

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We review mechanistic and evolutionary aspects of interactions between the model organism *Caenorhabditis elegans* and its environment. In particular, we focus on environmental effects affecting developmental mechanisms. We describe natural and laboratory environments of *C. elegans* and provide an overview of the different environmental responses of this organism. We then show how two developmental processes respond to changes in the environment. First, we discuss the development of alternative juvenile stages, the dauer and non-dauer larva. This example illustrates how development responds to variation in the environment to generate complex phenotypic variation. Second, we discuss the development of the *C. elegans* vulva. This example illustrates how development responds to variation in the environment while generating an invariant final phenotype.

I. Introduction

Organisms develop and evolve in variable environments. Understanding how an organism responds to environmental change is therefore of central significance in developmental and evolutionary biology. During ontogeny, the environment modulates the translation of genotype into phenotype, but developmental biologists typically ignore how environmental factors affect development and resulting phenotypes. Consequently, the detailed molecular and developmental mechanisms underlying environmental responses generally remain elusive. Similarly, whether and how organisms evolve adaptations to variable environments might depend on the mechanisms underlying genotype by environment interaction during ontogeny, yet evolutionary biologists typically ignore molecular and developmental mechanisms. An integrated understanding of the proximate and ultimate aspects of interactions between genes, development, and environment is therefore of mutual benefit to both developmental and evolutionary biologists.

Here we review mechanistic and evolutionary aspects of how environmental factors affect the nematode *Caenorhabditis elegans*, with a particular focus on the developmental role of the environment. We first introduce general aspects of interactions between organism and environment and discuss their evolutionary significance. After briefly introducing the *C. elegans* model, we then review how worms respond to different environmental conditions and how such responses might evolve. Finally, we discuss how environmental factors influence the expression of two developmental phenotypes: the dauer larva and the vulva. Using dauer formation as an example, we illustrate how development integrates environmental variation to generate phenotypic variation (phenotypic plasticity). Using vulva formation as an example, we then discuss the opposite: how the interaction between development and the environment can result in the absence of phenotypic variation (environmental robustness).

II. Interactions Between Organism and Environment

A. Environmental Effects on the Phenotype

In this chapter, we primarily discuss the effects of the exogenous environment on the organism, that is, abiotic and biotic factors external to the organism. Potentially, environmental factors affect any level of biological organization; however, the extent and nature of such an effect is highly variable depending on the examined phenotype and environment, respectively. Although effects on biological systems are often categorized as genetic versus environmental effects, it is generally difficult to retrace the relative contribution of the two effects on a phenotype: genetic and environmental effects are not necessarily additive but can interact in affecting the phenotype (Lewontin, 1974). Since biological systems underlying a given phenotype comprises genetic products that respond to environmental factors, such environmental responses generally have heritable genetic basis. Given the presence of genetic variation in the response, such a response may be subject to natural selection.

Environmental effects per se are classified as effects that are nonheritable because the environment does not alter DNA sequence. Exceptions occur when environmental factors induce transgenerational effects through particular epigenetic mechanisms, such as DNA methylation and other parental effects (Jablonka and Lamb, 1995). In addition, particular environments may directly alter mutation rates of soma and germ line, for example, in response to stressful environmental conditions (Tenaillon *et al.*, 2004).

B. Environmental Sensitivity of the Phenotype

Most organismic features are sensitive to some environmental factors. However, such sensitivity may or may not translate into phenotypic variation (or change). Adopting a simplified perspective, a given phenotype is thus either sensitive or insensitive to a given range of environmental conditions (Fig. 1). The term *phenotypic plasticity* describes the property of a genotype to generate phenotypic variation in response to a given range of environmental conditions (Pigliucci, 2001; Schlichting and Pigliucci, 1998; Stearns, 1992). When a single genotype produces different phenotypes that vary as a continuous function of the environment, the relationship can be described by a reaction norm (Fig. 1A). The situation in which a single genotype expresses two or more discretely different phenotypes in response to environmental cues is called polyphenism (Nijhout, 1999). In contrast, a phenotype may be insensitive to a given range of environmental conditions, so that a genotype always produces the same phenotype irrespective of environmental conditions (Fig. 1).

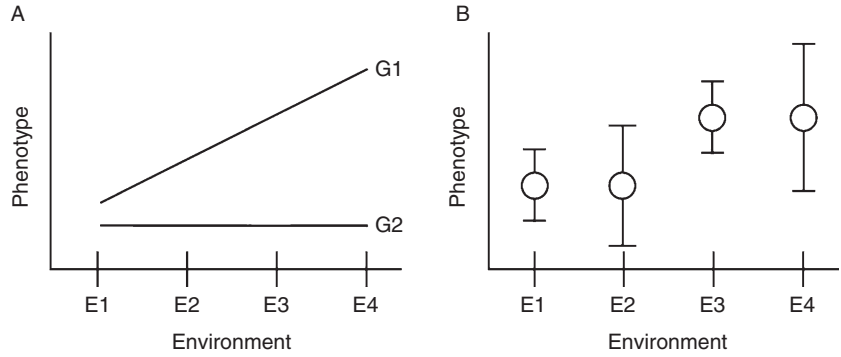


Figure 1 Environmental sensitivity of the phenotype. (A) Possible phenotypic responses to environmental variation visualized by reaction norms. Genotype G1 is environmentally sensitive and produces different phenotypes in different environments (phenotypic plasticity). Genotype G2 shows the same phenotype in all environments (environmental robustness). In these examples, the slope of the reaction norm is indicative of the degree of phenotypic sensitivity to environmental variation. Since the reaction norms of the two genotypes are nonparallel, they are indicative of a genotype-by-environment interaction, reflecting genetic variation in phenotypic plasticity. (B) Distinction of phenotypic variation generated within environments and between environments. The error bars of the phenotypic mean value represent stochastic phenotypic variation within a given environment, and correspond to the phenotypic variance. Both phenotypic mean and variance may or may not change across environments.

Such environmental insensitivity may be termed *environmental robustness* or environmental canalization (de Visser *et al.*, 2003; Flatt, 2005; Waddington, 1942). Phenotypic plasticity and environmental robustness reflect environmental sensitivity and insensitivity, respectively. The two phenomena thus describe different degrees of environmental sensitivity, and can be used to refer to both adaptive and nonadaptive phenotypic responses. While the categories of response represent opposite ends of phenotypic sensitivity to the environment, they are not mutually exclusive: the same phenotype may consist of both plastic and robust elements. In addition, a given phenotypic trait may show robustness in a range of environmental conditions, but plasticity in another range of conditions. Moreover, plasticity in one phenotype may lead to robustness in another phenotype.

Note that phenotypic variation *within* an environment (i.e., stochastic variation) may be also referred to as environmentally induced phenotypic variation. Analogously, the term robustness is also used to refer to phenotypic insensitivity to stochastic variation within an environment. For a distinction of different environmental responses of the phenotype, see Fig. 1 (for recent reviews, see Ancel Meyers and Bull, 2002; Badyaev, 2005; Flatt, 2005).

The degree of environmental sensitivity of a given phenotype can be influenced by genetic factors. Different genotypes thus exhibit differences in the extent of their phenotypic response to the same range of environmental conditions (Stearns, 1992). Such a differential response is called a genotype-by-environment-interaction (GxE) interaction, reflecting genetic variation in phenotypic plasticity. Graphically, differences in the slope of genotype-specific reaction norms indicate the occurrence of GxE interactions (Fig. 1).

C. Role and Evolutionary Significance of Environmental Sensitivity of Development

The environment may dramatically affect the development and morphology of many organisms. With the rise of genetics during the early twentieth century, the developmental role of the environment and its evolutionary significance became largely ignored. Despite important exceptions (e.g., Goldschmidt, 1938; Waddington, 1942), it was only relatively recently that interactions between development and environment received increased attention, particularly in evolutionary biology.

It has become apparent that adaptive plasticity of developmental mechanisms and resulting phenotypes is very common (Pigliucci, 2001; Schlichting and Pigliucci, 1998). Such phenotypic plasticity at the developmental level (developmental plasticity) has been proposed to play important roles in evolutionary diversification (West-Eberhard, 2003). This idea is partly based on the observation that environmentally induced phenotypic variation can become genetically fixed through a selective process called genetic assimilation (a process which requires the presence of genetic variation in the environmental response) (Waddington, 1956).

While developmental plasticity is a common phenomenon, many developmental processes generate little or no phenotypic variation across different environments—they are robust. Such environmental robustness may evolve as a result of selection for a single invariant phenotype that is reproducible across environments. Environmental robustness of the phenotype is likely to correlate with mutational robustness of the phenotype (insensitivity to mutation) (Meiklejohn and Hartl, 2002). Thus, robustness of the phenotype can have seemingly paradoxical evolutionary consequences. On the one hand, robustness will reduce the capacity to evolve at the phenotypic level, since robustness decreases the expression of phenotypic variation. On the other hand, as the phenotype becomes robust to mutational variation, the underlying genotype accumulates genetic changes; thus, the capacity to evolve increases at

the genetic level. Such genetic variation that does not translate into phenotypic variation is often termed hidden or cryptic genetic variation (Gibson and Dworkin, 2004). Cryptic genetic variation might be uncovered (i.e., phenotypically expressed) by particular mutations or environmental changes; selection might then act on this variation (Rutherford and Lindquist, 1998; Waddington, 1956). Thus, cryptic genetic variation, once uncovered, could potentially play a role in phenotypic evolution.

The degree of environmental sensitivity—ranging from extreme phenotypic plasticity to extreme environmental robustness—may affect the capacity to evolve at genotypic and phenotypic levels. Yet, these inferences mainly stem from theoretical work and very little is understood about how environmental sensitivity is mediated by molecular and cellular mechanisms to interact with development. As a consequence, we have limited insights into how environmental sensitivity of the phenotype evolves and how it affects evolutionary change. In this chapter, we discuss how the extensive research on the *C. elegans* model system can shed light on the mechanistic basis underlying phenotypic plasticity and environmental robustness, a subject of interest to both developmental and evolutionary biologists.

III. The Nematode *C. elegans*

A. General Biology

C. elegans has become a well-established model organism for molecular genetic studies (www.wormbook.org), and is now increasingly used for evolutionary studies (Carvalho *et al.*, 2006; Haag *et al.*, 2007). This small nematode provides a simple metazoan system for research aimed at the cell level—the adult hermaphrodite contains 959 somatic cells and its virtually invariant cell lineage has been determined in its entirety (Kimble and Hirsh, 1979; Sulston and Horvitz, 1977; Sulston *et al.*, 1983). *C. elegans* reproduces predominantly through self-fertile hermaphrodites, yet outcrossing events are possible through male production. Sex determination is of the XX:XO type, with males having only a single X chromosome. "Spontaneous" males are generated through nondisjunction events of the X chromosome during meiosis, which are generally rare under laboratory conditions. After a mating event, however, about half of the hermaphrodite progeny will be male. Current evidence suggests that male production and outcrossing events are overall rare but can be variable among natural populations (Barrière and Félix, 2005a, 2007; Sivasundar and Hey, 2005). The life cycle of both hermaphrodite and male includes five postembryonic stages: four juvenile (larval) stages and the adult stage, and may only take 3 days to complete in laboratory conditions (Fig. 2A). Depending on the environmental

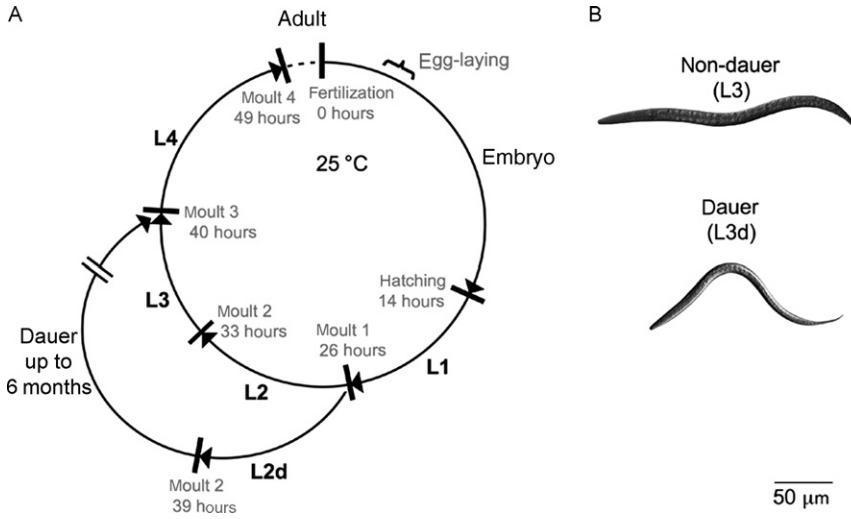


Figure 2 (A) The life cycle of *C. elegans* in the laboratory (adapted from Riddle and Albert, 1997; Wood, 1988). (B) Phenotypes of non-dauer and dauer larvae (from Altun and Hall, 2005).

conditions, individuals can adopt an alternative phenotype during the third larval stage, termed dauer larva. The dauer larva is morphologically and physiologically specialized, developmentally arrested, and is tolerant to harsh environments (Fig. 2B). The developmental divergence between non-dauer and dauer phenotypes represent a well-studied example of phenotypic plasticity, which we discuss in detail later. This and many other biological features of *C. elegans*, as well as representing one of the anatomically and genetically best-understood metazoans, provide an ideal basis to study an organism's manifold interactions with the environment and how such interactions evolve.

B. Natural Environment

Natural populations of *C. elegans* have been little studied and its ecology is not well understood. *C. elegans* was first found in humus samples collected in Algeria (Maupas, 1900) and the reference laboratory strain (N2) was isolated from humus used in a mushroom farm in United Kingdom (Hodgkin and Doniach, 1997; Staniland, 1957). Populations of *C. elegans* occur worldwide at geographical locations with cold (Québec, Canada) or very hot and dry (Southern California, United States) weather conditions (Barrière and Félix, 2005b). *C. elegans* is often referred to as a soil-dwelling nematode; however,

recent surveys indicate that natural populations also occur in a number of other habitats containing decomposing vegetal matter (fruits, compost, leaf litter), and to a lesser extent, decomposing invertebrates, such as snails. Most frequently, *C. elegans* was found in garden compost (Barrière and Félix, 2005a, 2007; Caswell-Chen *et al.*, 2005; Cutter, 2006; Haber *et al.*, 2005; Sivasundar and Hey, 2005). The characteristics of these natural habitats already indicate some of the likely environmental changes to be experienced by *C. elegans* in the wild, for example, changes in food availability, temperature, oxygen, and ethanol concentration. In addition, intra- and interspecific competition for food sources is probable as many other bacteriophagous nematode species, including the closely related species *C. briggsae*, occur in the same habitats as *C. elegans* (Barrière and Félix, 2005b).

The vast majority of isolated *C. elegans* individuals were found to be hermaphrodites in the dauer stage, indicating that environmentally sensitive dauer formation is common in nature. Rare proliferating populations with a large proportion of non-dauers were found only on rotten fruit and in very fresh compost (Barrière and Félix, 2005b, 2007). The typical *C. elegans* habitat therefore shows fluctuations between favorable and unfavorable growth conditions, and the dauer stage appears to allow survival in such ephemeral habitats. *C. elegans* individuals have also been found on various arthropods (isopods, millipedes, mites) as well as on snails (Barrière and Félix, 2005a, 2007; Kiontke and Sudhaus, 2006). Such associations with invertebrates may reflect phoretic (dispersal) or necromenic (feeding on the dead, decomposing animal) behaviors. Dauer larvae show a stereotypical "waving behavior" which may serve to find or attract such hosts (Riddle, 1988; Riddle and Albert, 1997). Consistent with this hypothesis, laboratory observations show that *C. elegans* dauer larvae readily mount, and then stay attached to arthropods such as mites (M.-A. F., unpublished data).

Although rarely investigated, the microbial fauna associated with *C. elegans* appears to be extensive and diverse. A variety of bacterial species have been isolated from its natural habitat, which may serve as food source or act as pathogens (Grewal, 1991; M.-A. F., unpublished data). In the laboratory, *C. elegans* is fed with *Escherichia coli*; however, the culture medium must be supplemented with cholesterol because *C. elegans* cannot synthesize cholesterol, required for steroid synthesis. This indicates that the natural diet of *C. elegans* may also include eukaryotes, such as unicellular slime moulds (Kessin *et al.*, 1996). While *C. elegans* may feed on different bacteria, many of them also represent pathogens, for example, *Serratia marcescens*, which has been isolated from compost samples containing *C. elegans* (M.-A. F., unpublished data). In addition, certain bacterial spores cannot be digested and are accumulated in the intestine, which may inhibit food uptake. In natural *C. elegans* populations, many non-dauer individuals exhibit apparent bacterial infections, such as accumulation of spores in the

intestinal lumen, bacterial invasion of the body cavity, or intracellular invasion (Barrière and Félix, 2005a). While certain fungi may represent a food source, others exhibit specialized hyphae, allowing the trapping of nematodes (Dusenbery, 1996). Other likely *C. elegans* predators include nematophagous springtails (collembola) (Lee and Widden, 1996) or mites (acari), the latter of which have been found in *C. elegans* compost samples (M.-A. F., unpublished data).

Sampling and genetic analysis of natural populations confirm that the natural *C. elegans* habitat is often ephemeral, with populations undergoing frequent bottlenecks in size (Barrière and Félix, 2005a, 2007). These observations suggest a distinct metapopulation structure with frequent extinction and recolonization of habitats. *C. elegans* life history further indicates that population expansion may be rapid under favorable conditions. Once a food source of the habitat has been exploited, the formation of dauer larvae may allow diapause or dispersal to new habitats.

C. Laboratory Environment

C. elegans is usually maintained in Petri dishes filled with solidified agar made from a standardized nematode growth medium (NGM) (Hope, 1999). A laboratory strain of *E. coli* (OP50) is most commonly utilized as a food source. NGM plates are inoculated with *E. coli* by adding a small drop of a bacterial suspension on the surface of the agar. Subsequent growth of *E. coli* results in a viscous bacterial lawn on which the worms feed. The majority of *C. elegans* experiments are carried out using worms living on such monoxenic plates, within a temperature range of 15–25 °C (generally 20 °C). For certain experimental procedures, *C. elegans* is also reared in defined monoxenic or axenic liquid media (Hope, 1999; Szewczyk *et al.*, 2003). The standard laboratory environment using NGM plates is very simple in structure and has been developed to maximize offspring production, so that it can be regarded as a rather benign environment. However, *E. coli*, being a mammalian intestine symbiont, is unlikely to represent an important natural food source, and further may have pathogenic effects (Herndon *et al.*, 2002).

Virtually all *C. elegans* research has made use of a single isogenic isolate, named N2. Biological observations of wild-type and mutant animals are thus representative of a single genotype. N2 was isolated sometime before 1956 and was thereafter reared in various laboratory environments until the use of standardized NGM plates about 10 years later. Around 1970, the first frozen stocks of N2 were established (*C. elegans* survives freezing and can be cryopreserved over long time periods) (for details on the laboratory history of N2, see Hodgkin and Doniach, 1997). Because of the long maintenance of N2 in the laboratory environment, it is likely that this genotype has adapted to some

of these conditions, and may have evolved features atypical for other genotypes living in the natural environment. Such laboratory adaptation may affect environmental responses in particular, for example, certain responses may not be maintained because of the absence of the relevant environmental stimuli. Nevertheless, in the laboratory, N2 generally shows no dramatic differences from freshly isolated wild isolates for most tested phenotypes.

In the laboratory, environmental effects on various *C. elegans* traits have been studied mostly in response to variation in specific abiotic or biotic factors, including temperature, chemical substances (e.g., toxins), oxygen availability, nutrition (e.g., starvation), culturing medium, population density, and pathogens. Most factors under study represent evident stressful conditions, defined as suboptimal conditions reducing viability, survival or reproduction relative to another environment. Very few studies included complex laboratory environments (Goranson *et al.*, 2005; Van Voorhies *et al.*, 2005) or environments that vary on a spatiotemporal scale (Friedenberg, 2003).

IV. Overview of *C. elegans* Responses to the Environment

Although its ecology is poorly understood, it is evident that *C. elegans* inhabits a highly variable environment. Nevertheless, laboratory experiments analyzing mostly isolated environmental factors indicate that *C. elegans* tolerates many extreme environmental conditions due to physiological, morphological, and behavioral responses. Many of these responses reflect likely evolutionary adaptations allowing an adjustment to variable, and often nonoptimal, environments.

A. Perception and Transduction of Environmental Signals

C. elegans is highly sensitive to environmental signals and responds to chemical, thermal, and mechanical stimuli. Both hermaphrodites and males possess a complex sensory system despite a relatively simple nervous system. The hermaphrodite has a total of 302 neurons, 30 of which represent sensory neurons (Hobert, 2005). Chemosensation plays a particularly important role in the mediation of environmental signals as *C. elegans* can detect hundreds of different volatile or aqueous chemicals (Bargmann, 2006). The basic chemosensory system consists of ciliated neurons that are in direct or indirect contact with the environment. Laser cell ablation experiments have identified sensory neurons required for the response to specific environmental signals. Sensation of most signals occurs through the amphid sensory organ, a concentration of neurons located in the head region. Specific environmental variables that cause changes in chemical compounds, such as ions or amino

acids, are sensed by, sometimes overlapping, subsets of sensory neurons. Perception of molecules occurs through a large number of different chemoreceptors required for subsequent signal transduction, with a single neuron expressing multiple chemoreceptor genes. Approximately 1300 G-protein–coupled receptors (GPCRs) are encoded by the *C. elegans* genome, many of which are likely to play a role in chemoreception (Bargmann, 2006; Robertson, 2000). In total, several hundred GPCRs are expressed in the sensory system and alternative signaling components may complement signal sensation. Downstream signal transduction occurs through cGMP–cGMP channels and TRPV channels, as well as other signaling cascades (Bargmann, 2006). Signal transduction further underlies extensive regulation by kinases and phosphatases to ultimately affect a potentially large number of genes containing signal-specific response elements.

On neuronal perception of environmental signals, neuropeptides, and neurohormones may be involved in the mediation of the signal. For example, the bioamine neurotransmitters serotonin and octopamine are involved in mediating responses to alternative feeding states. Exogenous application of these two substances can mimic well-fed and starved behavioral states of the animal, respectively (Horvitz *et al.*, 1982; Mohri *et al.*, 2005; Suo *et al.*, 2006). Perception of certain stimuli may also occur through neurons that are not in direct contact with the external environment, such as the PQR neurons of the body cavity, which sense changes in the body fluid and regulate the animal's behavior (Cheung *et al.*, 2004; Gray *et al.*, 2004). Overall, neuronal sensation plays predominant and multiple roles in behavioral and physiological responses to the environment.

Perception through neurons may lead to cell-autonomous as well as systemic responses through secreted factors, which may act on a long distance to affect both neuronal and nonneuronal cells. In addition, the environment may elicit responses by means different from neuronal perception. For example, certain molecules (e.g., ions) may impact an animal by diffusion, or metabolic changes (e.g., due to changes in nutrition uptake) may cause environmental effects independent of neuronal perception. Many environmental conditions seem likely to trigger a combination of effects, involving both neuronal and nonneuronal perception. In addition, most conditions are likely to affect multiple organismic features, and it is often unclear whether and how the environment acts on these features independently. As a consequence, it is difficult to determine how environmental signals are integrated, especially for traits with complex genetic architecture and environmental sensitivity.

Body size provides an example of such a complex composite trait, which is highly polygenic and sensitive to a wide range of environmental conditions. The developmental process underlying *C. elegans* body size determination involves multiple interdependent processes, such as chemosensory inputs, endocrine and metabolic signals, to ultimately affect cell size (ploidy) and

number (Azevedo *et al.*, 2002; Flemming *et al.*, 2000; Fujiwara *et al.*, 2002; Morck and Pilon, 2006; Savage-Dunn *et al.*, 2003; Wang *et al.*, 2002). While individual genetic (mutant) or environmental factors on body size can be isolated, the complicated interplay between different factors appears very difficult to disentangle.

B. Global Responses in Physiology and Gene Expression

Physiological and molecular responses have been mainly examined in response to stressful conditions, and molecular genetic analyses have resulted in the detailed characterization of environmental signal transduction. Global analyses of differential gene expression and metabolic changes in response to different environmental factors indicate extensive and widespread effects on gene regulation and molecular processes downstream of signal perception (Jones *et al.*, 2001; Li *et al.*, 2006; Menzel *et al.*, 2005; Reichert and Menzel, 2005; Szewczyk *et al.*, 2006). The environmentally induced adoption of the alternative dauer developmental stage, for example, underlies expression changes in more than 2000 genes (Wang and Kim, 2003). A subset of these expression changes involved in dauer formation also occur in starved L1 larvae or old adults, suggesting that gene expression may respond similarly to different environmental factors at multiple life stages (Cherkasova *et al.*, 2000). The significance of such differential gene expression pattern is, however, for the most part difficult to assess. Although in some cases RNAi-based assays have been used with success to address the function of specific genes (Murphy, 2006; Shapira *et al.*, 2006), it remains a main challenge to identify which of these genes are directly involved in the environmental signal transduction, or which gene expression changes translate into phenotypic changes.

C. Stress Responses

Evolutionarily conserved stress response mechanisms have also been reported in *C. elegans*. Such mechanisms play fundamental roles in adjusting cellular and physiological functions to variation in critical environmental variables. Transcriptional activation of heat shock proteins (HSPs) at elevated temperature presents such a stress response conferring, for example, increased protein stability. Heat-induced expression of HSPs, such as DAF-21, a molecular chaperone of the HSP90 family, thus may be observed in virtually all cells of the organism (Inoue *et al.*, 2003). However, HSP activation is not only thermosensitive, but may occur in response to other environmental stressors (e.g., hypo- and hyperoxia, toxins, pathogens, starvation) and may play additional roles during a number of developmental

processes in the absence of stress factors (Candido, 2002; Walker *et al.*, 2001, 2003). Tolerant to a wide range of oxygen concentrations, *C. elegans* also survives both anoxic and hyperoxic conditions and shows physiological adaptations to low environmental oxygen levels (Lamitina *et al.*, 2004; Van Voorhies and Ward, 2000). Such hypoxia responses are partly mediated by evolutionarily conserved hypoxia-inducible factor (HIF) complexes (Jiang *et al.*, 2001; Shen *et al.*, 2005). Mutant analyses have implicated other diverse genetic components to be involved in a number of cellular and physiological stress responses. Many of these components belong to well-characterized signaling cascades (Koga *et al.*, 2000; Lehtinen *et al.*, 2006).

D. Immune Responses

In the laboratory, *C. elegans* is readily infected by a variety of pathogens, some of which are known to occur in its natural environment (Darby, 2006). Current research increasingly focuses on such pathogen interactions to study the *C. elegans* immune response (Ewbank, 2006). Responses to pathogen infection may include previously mentioned stress response mechanisms. However, infection studies using different pathogenic bacteria suggest the additional occurrence of specific immune responses, usually involving conserved signal transduction pathways, such as the ERK and p38 MAPK pathways (Ewbank, 2006). Apparent and potential defence responses include the production of antimicrobial peptides and proteins (Couillault *et al.*, 2004; O'Rourke *et al.*, 2006), morphological modification (Nicholas and Hodgkin, 2004), possibly RNA interference on viral infection (Lu *et al.*, 2005; Wilkins *et al.*, 2005), and behavioral avoidance (Pradel *et al.*, 2007; Pujol and Ewbank, 2005).

E. Behavioral Responses

Behavioral flexibility represents one of the most fundamental adaptations to respond to variation in the environment. Behavioral change has the advantage that it allows a rapid, yet reversible adjustment to the current environmental situation. *C. elegans* behavior may respond to touch, smell, taste, or temperature. These responses primarily allow moving to favorable (e.g., food source) and avoiding unfavorable conditions (e.g., toxins). Chemosensation assays have revealed diverse chemical repellents and attractants (Bargmann, 2006). In *C. elegans*, in contrast to many other animals, it is a small set of neurons that determines the behavioral response to a given chemical. Each of these neurons expresses a repertoire of chemosensory receptors. Whether *C. elegans* is attracted or repulsed by an olfactory cue is determined by the cell(s) in which the corresponding chemosensory receptors are expressed. The pair of AWB neurons is associated with repulsion while AWC and AWA neurons are

associated with attraction; ectopic expression in AWB of a chemoreceptor (ODR-10) normally exclusively expressed in AWA is sufficient to transform a normally attractive stimulus into a repulsive one (Troemel *et al.*, 1997; Wes and Bargmann, 2001). Many other behavioral repertoires and their underlying neuronal basis have been studied in the context of their response to the environment, such as mechanosensation and locomotory responses to body or nose touch (Rankin, 2000), thermal preference and nutrition history (Mohri *et al.*, 2005), egg laying and nutrition status (Schafer, 2006), or male mating and hermaphrodite presence (Emmons, 2005). In addition, *C. elegans* may adjust its behavior through learning and memory (Giles *et al.*, 2005). For example, after exposure to pathogenic bacteria, animals modify their olfactory preferences by increased avoidance of pathogenic bacteria (Pujol and Ewbank, 2005; Zhang *et al.*, 2005).

F. Developmental, Morphological, and Life History Responses

While behavioral change is a primary response to adjust to environmental change, many environmental factors also induce physiological modifications, which may have effects on developmental decisions, overall phenotype (e.g., body size and form), and life history characteristics (e.g., fecundity or longevity). Some of these responses are transient or reversible, yet distinct developmental or morphological modifications occur in some environmental conditions, such as the adoption of the dauer phenotype (see Section VI), the developmental arrest of L1 larvae in the absence of food (Baugh and Sternberg, 2006; Fukuyama *et al.*, 2006), or the stereotypical changes in body morphology and locomotion in liquid culture (Moghal *et al.*, 2003; Szewczyk *et al.*, 2006). A small number of studies have also examined *C. elegans* life history in laboratory conditions mimicking the natural environment, that is, in spatially complex, soil-like environments (Goranson *et al.*, 2005; Van Voorhies *et al.*, 2005). These results show that many life history parameters, including offspring production and longevity may drastically change under ecologically relevant conditions.

G. Evolution of Environmental Responses

Most of what we know about *C. elegans*, including its interactions with the environment, stems from studies using a single isogenic wild isolate, the standard laboratory strain N2. Therefore, little is known about how environmental responses evolve within *C. elegans*. Despite their small number, studies examining distinct *C. elegans* isolates clearly reveal evolution in interactions between organism and environment. Such comparative analyses reveal intraspecific variation in behavioral responses to different environmental

stimuli (Davies *et al.*, 2004; de Bono and Bargmann, 1998; Jovelin *et al.*, 2003), survival and infection rate in response to pathogens (Schulenburg and Ewbank, 2004; Schulenburg and Muller, 2004), the propensity to form dauer larvae in response to dauer-inducing conditions (Viney *et al.*, 2003), or temperature-dependent fecundity (Harvey and Viney, 2007). A number of other traits, known to be environmentally sensitive, show genetic variation among *C. elegans* wild isolates in a single environment. These traits include, for example, body size, fecundity, and longevity (Gems and Riddle, 2000; McCulloch and Gems, 2003). In addition, the temperature-dependent propensity to generate males through nondisjunction of the X-chromosome varies among natural isolates (Hodgkin and Doniach, 1997; Nigon, 1949; Teotonio *et al.*, 2006). Most of the examined isolates are geographically as well as genetically distinct. However, even within a single location, *C. elegans* genotypes may show genetic divergence (Barrière and Félix, 2005a; Haber *et al.*, 2005), which may correlate with different responses to environmental factors, such as pathogens (Schulenburg and Ewbank, 2004).

Evolutionary variation in environmental responses has also been detected by quantitative trait loci (QTL) analyses using recombinant inbred lines derived from crosses between distinct *C. elegans* natural isolates. Gutteling *et al.* (2006) conducted a QTL analysis at low and high temperatures, and revealed genetic variation and GxE interactions for a number of life history traits. A related study (Li *et al.*, 2006) has applied QTL methods to map such temperature-dependent differences to the gene expression level using oligonucleotide microarrays. The results suggest the presence of ample genetic variation in environmentally sensitive gene expression changes, but it is currently unclear which genetic factors have evolved to generate this differential response to the environment. Additional QTL studies have revealed GxE interactions for a number of life-history traits (Ayyadevara *et al.*, 2001, 2003; Ebert *et al.*, 1996; Johnson and Hutchinson, 1993; Knight *et al.*, 2001; Shook and Johnson, 1999; Shook *et al.*, 1996). However, the results of these QTL studies should be interpreted with caution as they made use of *C. elegans* isolates which may have been particularly sensitive to environmental variation due to an increased mutational load caused by a high copy number of Tcl transposons.

So far, basically all examined environmentally sensitive features of *C. elegans* show evolutionary variation. Moreover, several studies provide clear examples of GxE interactions, demonstrating that different *C. elegans* genotypes show differential responses to the same environmental change (Harvey and Viney, 2007). The prevalence of extensive evolutionary variation in environmental responses is further supported by gene sequence analyses. Stewart *et al.* (2005) found high allelic diversity in chemoreceptor genes among 20 *C. elegans* wild isolates. A number of the examined genes correspond to nonfunctional

pseudogenes in the reference strain N2, but are apparently functional genes in other wild isolates. This and other sequence analyses indicate high polymorphism and rapid evolution of genes likely to be implicated in environmental interactions, including chemosensation, detoxification, and immune response (Thomas, 2006; Thomas *et al.*, 2005).

V. Phenotypic Plasticity of *C. elegans* Dauer Formation

Adoption of the alternative dauer developmental stage is highly sensitive to variation in the environment and represents an example of apparent adaptive phenotypic plasticity. In a favorable environment, normal development is maintained and reproduction is initiated rapidly. In an adverse environment, dauer development generates stress resistance and allows diapause, so that further postembryonic development can be postponed until a favorable environment is encountered. Entry and exit into dauer are cued by specific environmental signals indicative of future environmental conditions, allowing extensive, yet flexible phenotypic modifications. Dauer versus non-dauer development generate distinctly different phenotypes during the L3 stage and thus corresponds to a polyphenism, a special case of phenotypic plasticity. Dauer development represents the molecularly best-understood example of how a genome can generate alternative developmental phenotypes in response to environmental signals.

A. Characteristics of the Dauer Larva

Dauer larvae show a distinct overall morphology (Fig. 2B). They are thinner than regular L3 larvae, do not seem to feed (as their mouth is closed), have a constricted pharynx, exhibit a thin dark intestine, fat body accumulation, a specific cuticular pattern with lateral ridges (alae), and undergo remodeling of neurons, foregut, and other structures (Ao *et al.*, 2004; Cassada and Russell, 1975; Riddle, 1988; Riddle and Albert, 1997). A large number of gene expression changes are associated with entry and exit into dauer (Dalley and Golomb, 1992; Jones *et al.*, 2001; Wang and Kim, 2003). Some of these changes correspond to extensive metabolic changes with a partial shift to anaerobic fermentation on dauer entry (Holt and Riddle, 2003). Dauer larvae are usually immobile, yet may move very rapidly on touch. They may also display a specific waving behavior, which is thought to help finding a host for dispersal.

Dauer larvae are resistant to a variety of stressors (e.g., starvation, desiccation, extreme temperatures, toxins) (Anderson, 1978; Cassada and Russell, 1975) and can survive up to several months in laboratory conditions

(Klass and Hirsh, 1976). The extended longevity of dauer larvae appears to be partly due to increased expression of stress resistance genes, metabolic changes, and insulin-like signaling (Olsen *et al.*, 2006).

B. Environmental Cues Regulating Dauer Development

The known environmental conditions triggering entry into the dauer stage are: high dauer pheromone concentration (e.g., at high population density), low food concentration, and high temperature (Cassada and Russell, 1975; Golden and Riddle, 1984b). The propensity to enter dauer is very sensitive to variation in these conditions and usually depends on a combination of these cues. Dauer larvae readily occur in the laboratory when populations are maintained at high densities. The main environmental cue mediating this response is a pheromone. This dauer pheromone (or a component of it), termed daumone, has been purified and represents a pyranose sugar linked to a fatty acid chain (Jeong *et al.*, 2005). Starvation conditions usually enhance the propensity to enter the dauer stage. In addition, dauer formation is highly sensitive to variation in temperature. The tendency to form dauers generally increases as temperature increases (Ailion and Thomas, 2000).

The developmental decision to enter the dauer developmental pathway occurs around the late L1 stage. With the completion of the L1/L2 moult, the larva corresponds to a distinct pre-dauer L2 stage (L2d), which differs morphologically and metabolically from a normal L2 larva. The L2d then may enter the dauer stage at the next lethargic period of the L2/L3 moult; however, if dauer-inducing conditions cease during the second larval stage, L2d larvae may develop into normal L3 larvae.

Exit from the dauer stage is induced by a set of environmental cues indicating favorable growth conditions, that is, low pheromone levels, high food concentration, and low temperature. On encounter of such conditions, commitment to dauer recovery may occur within an hour and the animal shows increased pharyngeal pumping and locomotion (Golden and Riddle, 1984b). Within several hours, the animal may resume feeding and development to rapidly moult into a post-dauer L4 larva ($\sim$10 hours at 25 °C) (Cassada and Russell, 1975). Post-dauer development seems to be similar to non-dauer development of corresponding life stage, but has been little studied.

Although the dauer versus non-dauer decision is usually depicted as a switch between two distinct developmental pathways, there may also be intermediate responses in some environmental conditions. First, larvae may adopt a partial dauer phenotype; for example, they exhibit no fat accumulation or pharynx constriction, which indicates that they may be able to feed (Ailion and Thomas, 2003). Second, a larva may enter and rapidly exit the dauer stage to resume development, as observed for the N2 strain at 27 °C (Ailion and

Thomas, 2000). In addition, genetically identical individuals in the same environment (such as a pheromone plate at 25 °C) do not all enter the dauer stage, which could imply stochastic variation in the response. Alternatively, this observation may be explained by variation in dauer-inducing factors within a given environment. In general, the observation of alternative phenotypes may be due to developmental switches or to discrete occurrence of environmental factors (Nijhout, 2003); however, for many polyphenic organisms, intermediate phenotypes can be generated under some environmental circumstances.

C. Perception and Transduction of Environmental Cues

Laser-mediated cell ablations and mutant screens determined the role of specific sensory neurons and downstream neuroendocrine signaling cascades involved in dauer formation. Mutant screens yielded two opposite phenotypes with respect to dauer development: dauer-defective (Daf-d) mutants that show no or decreased sensitivity to dauer-inducing conditions, while dauer-constitutive (Daf-c) mutants may undergo dauer formation even in the absence of dauer-inducing conditions (Riddle and Albert, 1997). Genetic analysis of these mutants revealed a complex genetic network involved in dauer development (Fig. 3A). However, the nature of cellular interactions involved in this signaling process is not fully understood.

Amphid sensory neurons may sense different combinations of chemical cues to regulate dauer entry (Bargmann and Horvitz, 1991). In general, the ASI, ADF, and ASG amphid neurons repress dauer entry in good growth environments, while the ASJ neuron is required for dauer entry in unfavorable environments. (In addition, the ASJ neuron is required for exit from dauer.) Chemosensory reception in sensory neurons may involve cGMP signaling. Under good growth conditions (i.e., low pheromone but high food concentration), such molecules mediate the transcriptional activation of two downstream-secreted neuroendocrine signals, DAF-7/TGF-β in ASI and DAF-28/insulin (and possibly other insulins) in ASI and ASJ neurons (Li *et al.*, 2003; Ren *et al.*, 1996; Schackwitz *et al.*, 1996; Fig. 3B). Although insulin signaling may have some cell-autonomous effects, it affects another cell-nonautonomous endocrine signal that regulates dauer entry downstream of TGF-β and insulin reception. In epidermal and neuronal cells, TGF-β and insulin signaling indeed converge in the transcriptional upregulation of DAF-9 (a cytochrome P450 related to vertebrate steroidogenic enzymes). DAF-9 catalyzes the synthesis of dafachronic acid (Held *et al.*, 2006; Motola *et al.*, 2006). This lipophilic hormone binds DAF-12, the downstream nuclear receptor that acts in the dauer decision and in overall developmental timing. Liganded DAF-12 represses dauer development, whereas unliganded DAF-12 promotes dauer development.

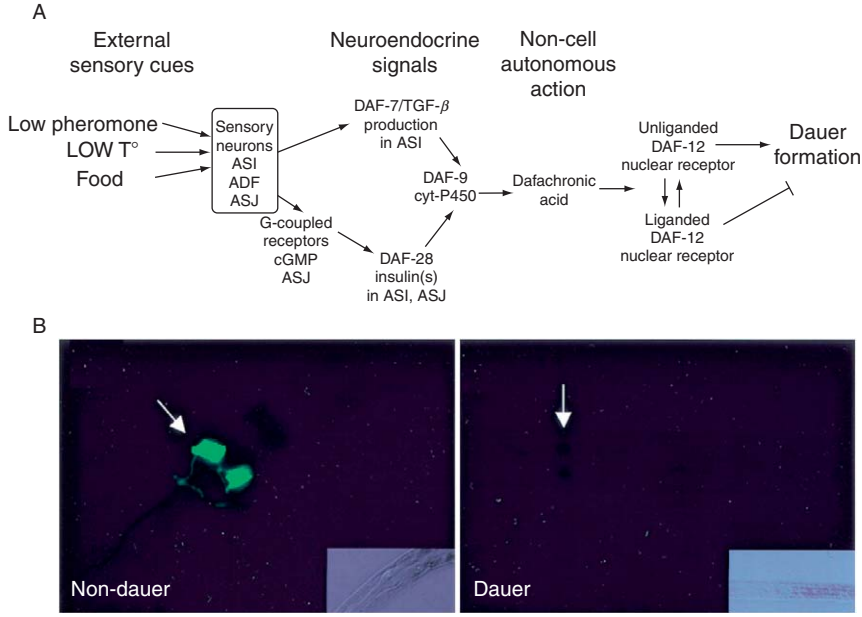


Figure 3 Dauer development. (A) Simplified representation of the signal transduction cascade involved in dauer formation. (B) Differential expression of DAF-28/insulin in amphid neurons of non-dauer and dauer larvae. A *daf-28*::GFP reporter reveals transcriptional repression of *daf-28* in ASI and ASJ sensory neurons (arrows) of individuals adopting the dauer developmental pathway (right panel); the corresponding DIC images are shown at the lower right corner (images from Li *et al.*, 2003).

In summary, sensory cues such as pheromone, food, and temperature act through sensation of the amphid neurons. The combination of these cues is processed via a small neuronal network that results, under favorable growth conditions, in the activation of TGF-β and insulin neuroendocrine signaling and the production of a liganded form of the nuclear hormone receptor DAF-12. In unfavorable growth conditions, DAF-12 is unliganded and promotes a cell-specific dauer developmental program (Fig. 3A).

D. Evolution of Dauer Formation

Dauer formation represents a dramatic developmental response to adjust to variable environments. We thus might expect evolutionary differences in the extent of this response among genotypes occupying different ecological conditions. Consistent with this scenario, different *C. elegans* wild isolates may differ greatly in their propensity to generate dauer larvae. Viney *et al.* (2003) showed that dauer induction response to varying concentrations of dauer

pheromone displays wide genetic variation. For example, the laboratory strain N2 enters the dauer stage already at low pheromone concentrations. In contrast, at the same pheromone dose, other isolates produce few (DR1350) or no dauer larvae at all (CB4932). It is currently unknown which genetic factors have evolved to mediate this differential response; however, analysis of recombinant inbred lines derived from parents with opposing pheromone response indicates the presence of multiple loci involved in this difference (Viney *et al.*, 2003). Considering the complexity of the genetic network involved in dauer formation, there are many potential scenarios of how such genetic differences may evolve. In addition, different genotypes may also have evolved to respond to different types or combinations of environmental cues. The detailed understanding of the molecular mechanisms governing dauer formation in N2 should allow future dissection of the molecular genetic changes underlying this evolutionary difference.

Evolutionary differences in dauer formation have also been observed at higher taxonomic levels; for example, the *C. briggsae* isolate AF16 (India) appears to be more sensitive to certain dauer-inducing conditions than many *C. elegans* isolates, including N2 (Fodor *et al.*, 1983; Golden and Riddle, 1984a). Genome sequencing of *C. briggsae* (Stein *et al.*, 2003) and additional *Caenorhabditis* species will facilitate molecular interspecific comparisons in dauer regulatory pathways. Moreover, based on several phenotypic similarities, the *C. elegans* dauer developmental stage is thought to correspond to the infective stage found in many parasitic nematodes (Bird and Opperman, 1998; Blaxter, 2003). The production of the infectious developmental stage may be influenced by the environment, and may involve the same neuronal perception mechanisms as in *C. elegans* dauer formation (Tissenbaum *et al.*, 2000). Some downstream signaling cascades and effectors may also be conserved, for example, the role of insulin signaling (Brand and Hawdon, 2004). However, recent molecular genetic and genomic analyses suggest that molecular regulation of dauer and parasite formation also exhibit considerable differences, for example, in the role of TGF-β signaling (Viney *et al.*, 2005).

VI. Environmental Robustness of *C. elegans* Vulva Formation

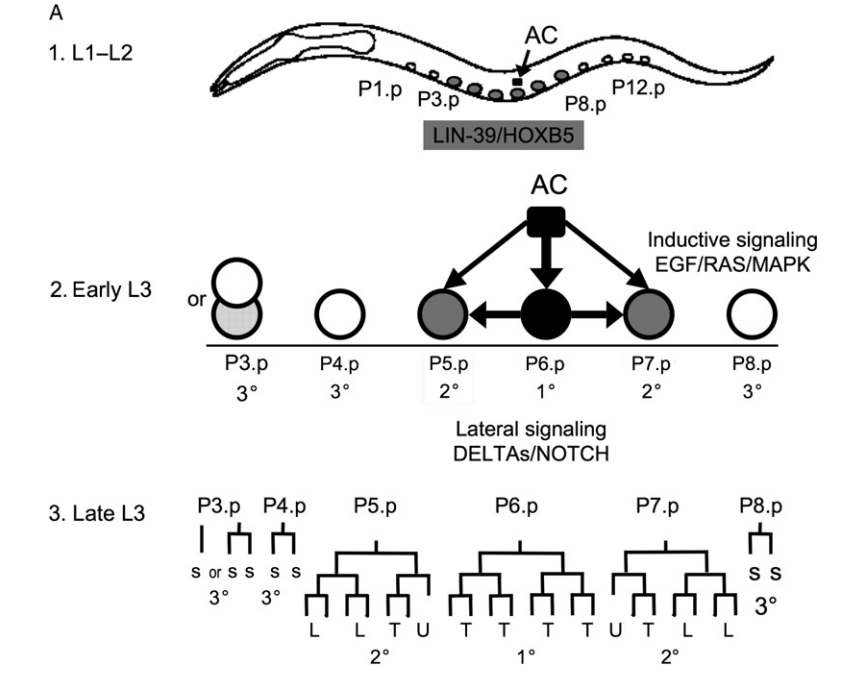
The vulva of the *C. elegans* hermaphrodite is the organ used for egg laying and mating with males. Many defects in this reproductive structure can lead to a decrease in offspring production (or impede outcrossing), which may cause negative fitness effects. As a consequence, we expect vulval development to have evolved to generate an invariant and reproducible phenotype. This seems to be the case as the vulval phenotype exhibits no apparent variation in its underlying cell pattern development and the phenotype appears to be insensitive to variation in many environmental factors. How

does an organism maintain such phenotypic stability when exposed to variable and unfavorable environmental conditions? We address this question by studying *C. elegans* vulval development in different laboratory environments. Here we discuss how the environment affects the vulva phenotype, the underlying developmental process, and which molecular and developmental features may contribute to maintain a precise vulval phenotype in different environments. We further present evidence that environmental effects on vulval development vary among different *C. elegans* wild isolates, indicating evolutionary variation in the robustness of this trait.

A. Vulva Development

The *C. elegans* vulva is formed by a subset of ventral epidermal blast cells, the Pn.p cells (posterior descendants of Pn cells), and is connected to the ventral uterus of the gonad by the anchor cell. Twelve Pn.p cells, labeled P1.p–P12.p, are generated during the L1 stage. Six central cells, P3.p–P8.p, express the Hox gene *lin-39* and remain unfused and competent to form vulval tissue (while five of the remaining cells fuse with the surrounding syncytial epidermis). P(3–8).p form the vulva competence group and are called the vulva precursor cells (Clark *et al.*, 1993; Maloof and Kenyon, 1998) (note that P3.p may also fuse with the epidermis and is thus not always competent; Fig. 4A).

During the L3 stage, these vulval precursor cells adopt alternative cell fates on the basis of activation of conserved signaling pathways (Fig. 4). Although all six cells are capable of forming all vulval cell types, only three of them (P5.p–P7.p) will form the vulva (Sternberg and Horvitz, 1986; Sulston and White, 1980). The central cell, P6.p, adopts the 1° (or inner vulval) fate while its neighboring cells, P5.p and P7.p, adopt the 2° (or outer vulval) fate. Although competent to adopt a vulval fate, for example, on ablation of the central cells, P3.p, P4.p, and P8.p usually adopt a non-vulval 3° fate, and their progeny finally fuse to the epidermal syncytium (Horvitz and Sternberg, 1991). The pattern of three cell fates is induced via a LIN-3/EGF signal released from the anchor cell in the gonad overlaying the vulval precursor cells. This signal activates a RAS/MAPK signaling cascade via the LET-23/EGFR receptor (Aroian *et al.*, 1994; Sternberg, 2005). The central cell P6.p receives the highest dose of LIN-3/EGF signal, which activates RAS/MAPK signaling at a high level and causes it to adopt a 1° vulval fate (Katz *et al.*, 1995). RAS/MAPK signaling in P6.p also activates transcription of DELTA-like molecules. This lateral signal results in LIN-12/NOTCH activation in P5.p and P7.p, which induces the 2° fate and prevents them from adopting the 1° vulval fate (Sternberg, 1988; Yoo *et al.*, 2004). P3.p, P4.p, and P8.p receive insufficient levels of either signal and thus adopt the 3° vulval fate. The RAS/MAPK signaling cascade involves an array of negative


A
1. L1–L2
AC
P1.p
P3.p
P8.p
P12.p
LIN-39/HOXB5
2. Early L3
AC
Inductive signaling
EGF/RAS/MAPK
or
P3.p
P4.p
P5.p
P6.p
P7.p
P8.p
3°
3°
2°
1°
2°
3°
Lateral signaling
DELTAs/NOTCH
3. Late L3
P3.p
P4.p
P5.p
P6.p
P7.p
P8.p
s or s s s s
3° 3°
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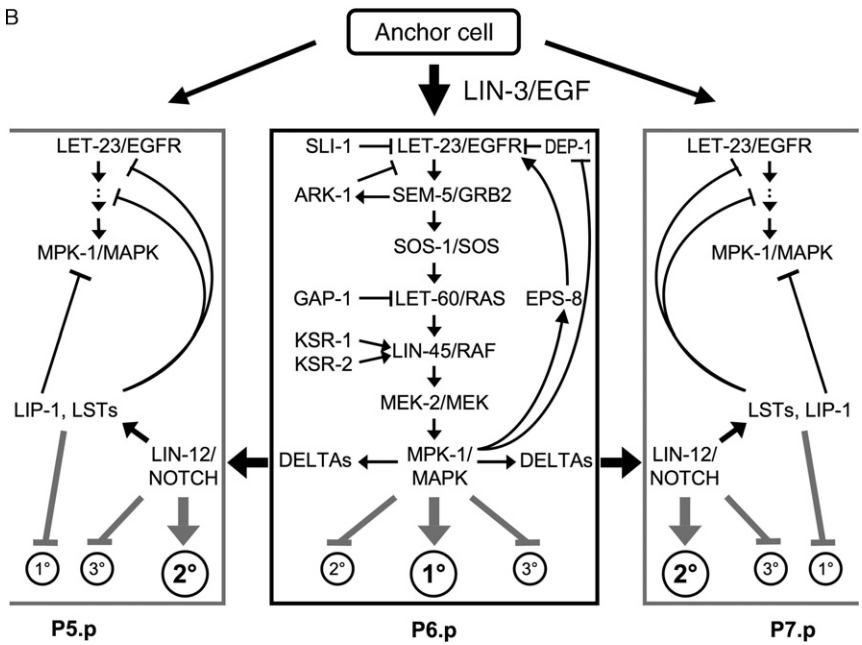
B
Anchor cell
LIN-3/EGF
LET-23/EGFR
MPK-1/MAPK
LIP-1, LSTs
LIN-12/
NOTCH
1°
3°
2°
P5.p
SLI-1
LET-23/EGFR
DEP-1
ARK-1
SEM-5/GRB2
SOS-1/SOS
GAP-1
LET-60/RAS
EPS-8
KSR-1
KSR-2
LIN-45/RAF
MEK-2/MEK
DELTAs
MPK-1/
MAPK
DELTAs
2°
1°
3°
P6.p
LET-23/EGFR
MPK-1/MAPK
LSTs, LIP-1
LIN-12/
NOTCH
2°
3°
1°
P7.p

and positive regulators (Fig. 4B). Components of a third signaling pathway (WNT) regulate LIN-39/HoxB5 expression and maintain competence of vulval precursor cells during the L2 stage (Eisenmann *et al.*, 1998). In addition, WNT signaling may play a later role in inducing vulval cell fates, since hyperactivation of this pathway is sufficient for vulva induction when RAS/MAPK signaling is compromised (Gleason *et al.*, 2002). During the L3 stage, this patterning process causes invariant fate-specific cell divisions, resulting in a total of 22 vulval cells (Fig. 4A). Vulval morphogenesis takes place during the L4 stage and the complete vulval organ is formed by the final moult to the adult.

B. Environmental Robustness of the Final Vulva Phenotype

C. elegans vulval development has been mainly studied using the N2 isolate in standard laboratory conditions. In these conditions, deviations from the canonical vulval cell fate pattern are generally rare (Delattre and Félix, 2001). This result indicates that the final vulval phenotype of N2 is robust to stochastic variation within the standard environment. We have further examined how the final vulval phenotype responds to variation in the laboratory environment (C. B. and M.-A. F., unpublished data). Tested environments included standard conditions at different temperatures (15–25 °C), starvation in the L2 stage, liquid culture, and conditions in which individuals passed through the dauer stage. In all conditions, defects leading to an abnormal vulva (e.g., hypoinduction or cell lineage errors) were rarely more frequent than in standard conditions. Thus, the process of vulval formation appears robust to a variety of environmental conditions. However, we found additional deviations from the wild-type fate patterns adopted by P3.p–P8.p that did not lead to defects. The frequency of such variants differed between environmental conditions. For example, individuals in the starvation environment showed a weak, yet consistent propensity to center their vulva on P5.p instead of P6.p. The occurrence of an anterior shift in vulval centering was largely limited to this environment and was never observed in standard environments. This result indicates that specific environmental conditions may elicit specific

Figure 4 Vulval development. (A) Vulval precursor cell fate specification. (1) Establishment and maintenance of the vulval competence group in the L1 and L2 stages. (2) Specification of the pattern of three vulval precursor cell fates during the early L3 stage. (3) Cell lineages. AC, anchor cell; T, transverse (left–right) division; L, longitudinal (anteroposterior) division; U, undivided; S, fusion to the epidermal syncytium (non-vulval fate). (B) Feedback loops and cross talk between EGF/RAS/MAPK and NOTCH pathways during vulval cell fate patterning (see text). WNT signaling is not shown here, but may be involved in the specification of vulval cell fates.

developmental changes while maintaining the production of a robust phenotype (as vulval centering on P5.p does not result in an apparent loss of functionality).

C. Environmental Sensitivity of Vulva Developmental Processes

While the final vulval phenotype is robust and shows little environmental variability, it is currently unclear to what extent underlying molecular processes may vary in different environmental conditions. However, several lines of evidence suggest that vulval development is sensitive to environmental inputs. In a screen for mutants showing defects in vulval formation, Ferguson and Horvitz (1985) reported variation in the penetrance of several loss-of-function mutations in genes involved in the EGF/RAS/MAPK signaling cascade. Such mutations cause vulval defects due to hypoinduction, leading to an induction of fewer than three vulval precursor cells. Starvation conditions and passage through the dauer stage resulted in a drastic suppression of some of these mutant phenotypes relative to normal growth conditions. Furthermore, starvation and dauer conditions affected the penetrance of a different set of mutations, indicating that the two conditions may have different effects on vulval development.

Two recent studies indicate in more detail how environmental and physiological inputs may be transduced to modify mechanisms underlying vulval cell fate patterning. Battu *et al.* (2003) showed that starvation conditions might modulate vulval induction via the G-coupled receptor (SRA-13) and the G-protein GPA-5, which are expressed in body wall muscles and sensory neurons. In their experimental assay, starvation stimuli caused a decrease in vulval induction as inferred from mutant analyses, indicating that the EGF/RAS/MAPK cascade may be modified by environmental inputs. However, where this input acts to affect vulval induction is not known. Another study (Moghal *et al.*, 2003) reveals how the environment may cause physiological changes to modify pathway activities during vulval patterning. Culture in liquid medium led to suppression of loss-of-function mutants of the EGF/RAS/MAPK cascade, suggesting that liquid growth may increase vulval induction levels. The observed suppression requires the heterotrimeric Gq protein (EGL-30, expressed in neurons) and the voltage-gated calcium channel (EGL-19, expressed in muscle cells). This result shows that environmental modification of vulval induction could be mediated by neuronal and muscle cells. The environmental effect transduced by EGL-30 acts in parallel or downstream of the EGF/RAS/MAPK pathway. As the effect requires WNT signaling via BAR-1/β-catenin, the signals involved may act through the WNT pathway. This is consistent with the hypothesis that partial redundancy of WNT and EGF/RAS/MAPK pathways during vulval induction

may be important to maintain robustness of the final cell fate output pattern in varying environments.

Hence, vulval development is sensitive to a number of environmental conditions. Specific environmental factors may modify the activities of signaling pathways underlying this developmental process. However, it is currently unclear to what extent such modifications occur in the wild-type situation as they were only described in sensitized (mutant) genetic backgrounds. In addition, it is unknown how such environmental modifications relate to the apparent robustness of the final vulval phenotype in the corresponding environmental conditions.

D. Developmental and Molecular Features Causing Robustness to Variation in the Environment

The current data suggest that the vulval phenotype is robust to a number of environmental conditions. In this section, we discuss some of the molecular and developmental features that are likely to cause tolerance to changes in developmental parameters induced by the environment (see also Félix and Wagner, in press).

1. Cellular Redundancy

As mentioned earlier, the wild-type vulva is derived from three vulval precursors, P5.p–P7.p, whereas a total of six cells can potentially adopt vulval fates (P3.p–P8.p). Experimental elimination of one or several of the central cells allows replacement among competent cells, which ensures the formation of a complete vulva (Sulston and White, 1980). In addition, the extent of the competence group allows correct vulval formation if the anchor cell is centered on cells other than P6.p. Thus, cellular replacement within the vulval competence group can maintain the complete and correct vulval pattern (2°1°2°) when the cell fate patterning is disturbed.

2. Pathway Redundancy

Both an intermediate level of LIN-3/EGF signal and activation of the LIN-12/NOTCH pathway may generate a 2° vulval fate (Katz *et al.*, 1995; Koga and Ohshima, 1995; Simske and Kim, 1995). While the contribution of LIN-3/EGF signal to 2° fate induction is not essential, the two signaling pathways show partial functional redundancy in specifying the 2° vulva cell fate.

As mentioned earlier, vulval induction may occur in the absence of LET-60/RAS signaling if the WNT signaling pathway is overactivated (Gleason *et al.*, 2002). Thus, the two pathways can show functional redundancy in vulval induction, indicating that WNT signal may compensate for loss in LET-60/RAS signal.

3. Pathway Properties

The switch-like behavior of the EGF/RAS/MAPK cascade causes insensitivity to small EGF fluctuation: low doses have no effect, while for any dose above a certain threshold the pathway will show a much higher level of activity (Huang and Ferrell, 1996). In C. *elegans*, such switch behavior may be reinforced by numerous positive feedback loops, for example, through EPS-8 activation (Stetak *et al.*, 2006) or DEP-1 inhibition (Berset *et al.*, 2005) (Fig. 4B). Negative feedback loops, such as the SEM-5/ARK-1 feedback loop, which inhibits EGFR activation (Hopper *et al.*, 2000), might contribute to the buffering against noise (Becskei and Serrano, 2000) (Fig. 4B). These features are likely to limit not only the effects of stochastic variation, but also the effects of environmental and genetic variation.

The RAS/MAPK signaling cascade actually shows a large number of regulators that are functionally redundant in terms of their effect on the cell fate pattern: inactivation of one of them does not alter the vulva pattern output, except in sensitized genetic backgrounds (Fig. 4B). Such silent regulators include both positive (e.g., EPS-8, KSR-1, KSR-2) and negative (e.g., ARK-1, GAP-1, LIP-1, UNC-101, SLI-1) inputs to the RAS pathway (Berset *et al.*, 2001; Hajnal *et al.*, 1997; Hopper *et al.*, 2000; Jongeward *et al.*, 1995; Lee *et al.*, 1994; Ohmachi *et al.*, 2002; Stetak *et al.*, 2006). This observation demonstrates buffering of LET-60/RAS activity as mutation of these regulators seem to change LET-60/RAS activity, yet not sufficiently to cause an abnormal vulval phenotype. Furthermore, it is also possible that different regulators per se perform buffering functions to adjust LET-60/RAS signaling when development is perturbed, for example, in different environmental conditions. As for the RAS/MAPK pathway, functional redundancy has been observed among genetic components in NOTCH (Chen and Greenwald, 2004) and WNT (Gleason *et al.*, 2006) pathways during vulval cell fate specification.

Further properties indicating robustness to pathway activity changes are provided by the fact that mutations in *lin-3*/*egf* and other Ras pathway genes are haplo-sufficient and that mild overexpression of *lin-3*/*egf* still produces a wild-type vulval phenotype (J. M. and M.-A. F., unpublished data).

4. Pathway Cross Talk

The interplay between the Ras and NOTCH pathways results in stable discrimination between the 1° and 2° fates (Berset *et al.*, 2001; Yoo *et al.*, 2004). High Ras pathway activity in P6.p causes the adoption of the 1° fate and triggers the degradation of the NOTCH receptor, preventing the adoption of 2° fate (Shaye and Greenwald, 2002). At the same time, release of DELTA-like proteins toward P5.p and P7.p achieves the activation of a 2° vulval fate in these cells and prevent their adoption of a 1° fate through

the activation of negative regulators of the Ras pathway (Chen and Greenwald, 2004). As shown by modeling approaches, lateral inhibition ensures much stronger fate segregation between adjacent cells compared to fate segregation induced by a morphogen gradient alone (Giurumescu *et al.*, 2006).

5. Gene Redundancy

In addition to these network properties, functionally redundant genes arising from gene duplication seem to play a minor role in conferring robustness of vulval formation to stochastic and environmental variation. For example, in the RAS pathway, there is one known case of partial functional redundancy between two positive regulators (KSR-1 and KSR-2) (Ohmachi *et al.*, 2002).

E. Evolution of Vulval Development

Despite intraspecific conservation of the final vulval phenotype, analysis of distinct *C. elegans* wild isolates suggests evolution of the underlying molecular mechanisms. Introgression of vulval mutations (isolated in N2) into different wild isolates reveals that a given mutational effect is highly variable depending on the wild genetic background (J. M. and M.-A. F., unpublished results). Moreover, *C. elegans* wild isolates show differences in how vulval formation responds to environmental variation. After starvation, vulval (mis)centering on P5.p is frequent in N2; in another isolate (JU258), vulval centering on P5.p is very rare but other deviations from the wild-type pattern, such as fusion of vulval precursor cells are very frequent (C. B. and M.-A. F., unpublished results).

The vulval phenotype (the fate pattern and the corresponding cell division patterns) of *C. elegans* is conserved in all known *Caenorhabditis* species. Such extensive evolutionary conservation of a phenotype is typical for a robust trait under stabilizing selection. Recent experiments show that this morphological stasis underlies considerable evolutionary divergence in underlying developmental mechanisms. Anchor cell ablation experiments at different developmental time points and mild EGF overexpression in different *Caenorhabditis* species reveal many differences in the regulation of vulval development (Félix, 2007). Thus, intra- and interspecific features of vulval development indicate that the underlying genetic architecture of a robust and evolutionarily invariant phenotype can evolve. However, the genetic changes responsible for this evolution have not yet been identified. In addition, it remains a major challenge to determine to what extent these evolutionary differences might result as a consequence of the robustness of the developmental system.

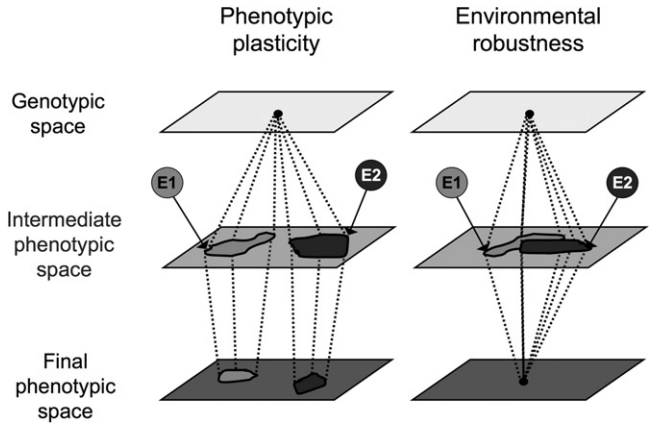

Figure 5 Genotype–phenotype maps illustrating scenarios of phenotypic plasticity and environmental robustness. In both cases, a given genotype interacts with two environments (E1 and E2) and these interactions cause variation in the intermediate phenotype (e.g., developmental, physiological, metabolic phenotype). In the case of phenotypic plasticity, variation in the intermediate phenotype causes variation in the final phenotype of the process (e.g., cell fate, morphology, life history trait) (confer to the example of phenotypic plasticity of dauer formation). In the case of environmental robustness, variation in the intermediate phenotype does not cause variation in the final phenotype (confer to the example of environmental robustness of vulva formation). Note that the extent of both environmental and stochastic (within environment) variation may change between intermediate and final phenotypes.

VII. Conclusion

Research on *C. elegans* illustrates the multitude of interactions between organism and environment. These interactions occur at all levels of biological organization and evolve readily. To gain a complete understanding of how a genotype maps into a phenotype and how this mapping process evolves, it is particularly important to study the developmental role of the environment (Fig. 5).

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