Environmental sensitivity of the C. elegans vulval signalling network

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Sensibilité environnementale du réseau de développement de la vulve de *C. elegans*

Environmental sensitivity of the *C. elegans* vulval signalling network

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Summary

How genetic and environmental factors interact during development is a key question in current biology, yet little is known about how molecular and cellular processes integrate environmental information. In my PhD research I aimed to address this problem using the network of *C. elegans* vulval signalling pathways as a model system. The principal objective of my project was to quantitatively examine how involved major signalling pathways, EGF-Ras-MAPK, Wnt and Delta-Notch, are modulated by specific environmental signals.

First, I analysed how a specific environmental factor (starvation) alters activities and interplay of signalling pathways underlying *C. elegans* vulval cell fate patterning. Using genetic approaches, I examined in detail how starvation signals are perceived and mediated to modulate vulval induction. I found that starvation consistently increased vulval induction through upregulation of the EGF-Ras-MAPK pathway activity independent of the Wnt pathway. This environmental effect is mediated by internal sensing of nutrient deprivation, likely acting through the TOR pathway, and affects vulval induction at the level or upstream of the EGF receptor. These findings highlight how developmental processes and involved evolutionarily conserved signalling pathways are modulated in response to environmental variation.

Second, I examined the environmental sensitivity of the *Caenorhabditis* vulval developmental system from an evolutionary perspective through comparative analysis of different *C. elegans* and *C. briggsae* isolates. I aimed to maximally disrupt this patterning process by exposure to extreme temperatures and to quantify which underlying developmental and cellular aspects are most environmentally sensitive and how such sensitivity evolves within and between species. I found that extreme temperature induced diverse developmental variants and defects, which were strongly genotype- and species-dependent. The occurrence of certain developmental defects induced by temperature extremes further revealed that vulval precursor cells and associated fates differ in temperature sensitivity, and this cell-specific sensitivity shows evolutionary variation. These results illustrate how sensitivity of different system parameters underlying *Caenorhabditis* vulval development are shaped by subtle, specific interactions between environmental perturbation and genetic background.

Résumé

Comprendre comment les facteurs génétiques et environnementaux interagissent au cours du développement est une question fondamentale en biologie. Cependant, peu de choses sont connues à propos de l'intégration des informations environnementales par les processus moléculaires et cellulaires. Au cours de ma thèse, je me suis intéressée à cette question en utilisant le réseau de développement de la vulve du nématode *C. elegans* comme système modèle. L'objectif principal de mon projet était une étude quantitative de la modulation par l'environnement des voies de signalisation majeures impliquées dans ce processus telles que, EGF-Ras-MAPK, Delta-Notch et Wnt.

J'ai tout d'abord analysé comment un facteur environnemental spécifique (la carence nutritionnelle) modifie les activités et les interactions entre les voies de signalisation sous-jacentes au développement vulvaire chez *C. elegans*. L'utilisation d'approches génétiques m'a permis d'examiner en détail comment les signaux environnementaux de carence sont perçus et transmis afin de moduler l'induction vulvaire. J'ai ainsi mis en évidence que l'augmentation de l'induction vulvaire par la carence passe par une augmentation de l'activité de la voie EGF-Ras-MAPK et est indépendante de la voie Wnt. Cet effet de l'environnement est assuré par la détection de la diminution de l'apport en nutriments, probablement par l'action de la voie TOR, et affecte l'induction vulvaire en parallèle ou en amont du récepteur à l'EGF. Ces résultats mettent en évidence comment les processus développementaux et les voies de signalisation sous-jacentes évolutivement conservées répondent et intègrent la variation environnementale.

J'ai ensuite examiné la sensibilité environnementale du système de développement de la vulve de *Caenorhabditis* dans une perspective évolutive et ce, grâce à l'analyse comparative de différents isolats naturels de *C. elegans* et *C. briggsae*. En perturbant au maximum le réseau de développement vulvaire par l'exposition à des températures extrêmes, j'ai pu quantifier quels aspects moléculaires et cellulaires de ce réseau étaient les plus sensibles à l'environnement et analyser l'évolution de cette sensibilité au sein de différentes espèces et souches de *Caenorhabditis*. J'ai pu observer que l'exposition à des températures extrêmes induit des variants et des défauts de manière fortement dépendante de la souche et de l'espèce. L'occurrence de certains défauts développementaux induits par la température révèlent en

outre que certaines cellules précurseurs de la vulve et les voies de signalisation associées présentent une sensibilité environnementale différente. Ces résultats illustrent la manière dont la sensibilité des différents paramètres sous-jacents au développement de la vulve des *Caenorhabditis* est façonnée par des interactions spécifiques entre les perturbations environnementales et le fond génétique.

“Not everything that can be counted counts, and not everything that counts can be counted”

Albert Einstein

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I love you both.

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Chapter 1

1. Introduction

1.1. Motivation: Understanding the environmental context-dependence of organismal development

Organismal development relies on complex signalling networks involving a relatively small number of highly conserved molecular signalling pathways (e.g. Receptor Tyrosine Kinase, TGF-β, Delta-Notch, Wnt, Nuclear Hormone Receptor, Hedgehog) (Gerhart, 1999; Pires-da Silva and Sommer, 2003). The same molecular cascades therefore participate in diverse developmental processes and their precise function is strongly dependent on genetic and cellular contexts, i.e. they are highly flexible. This flexibility and context-dependent activation of molecular pathways during development allows their diversified action in the same organism, e.g. in response to changing cellular environments, as well as across different species where the same molecular pathway may be utilized for divergent functions.

Organisms live, develop and evolve in highly variable, complex and fluctuating external environments. How organismal development responds to and integrates environmental variation is thus a fundamental question in biology. Given that any phenotype is the result of an interaction between genotype and environment, it is of particular importance to understand how developmental processes shape the translation of genotype into phenotype, and how variation in the external environment impacts this translation. While it is clear that environmental variation may strongly impact developmental processes and corresponding phenotypic outcomes, environmental variation has traditionally been ignored in genetic and developmental studies. Consequently, little is known about the detailed mechanisms underlying environmental modification of developmental processes.

Fundamentally, we can distinguish two opposite developmental responses to environmental variation. First, development may vary in response to the environment, which generates changes in corresponding phenotypic outcomes, a phenomenon termed developmental or phenotypic plasticity (Figure 1.1A). Second, development may generate an invariant final phenotype in the presence of environmental variation irrespective of whether underlying development is sensitive or insensitive this variation. This phenomenon, i.e. developmental insensitivity to environmental variation, is frequently referred to as developmental robustness (Figure 1.1B). Importantly, such robustness does not exclude

environmental sensitivity of the underlying developmental mechanisms (Braendle and Félix, 2008).

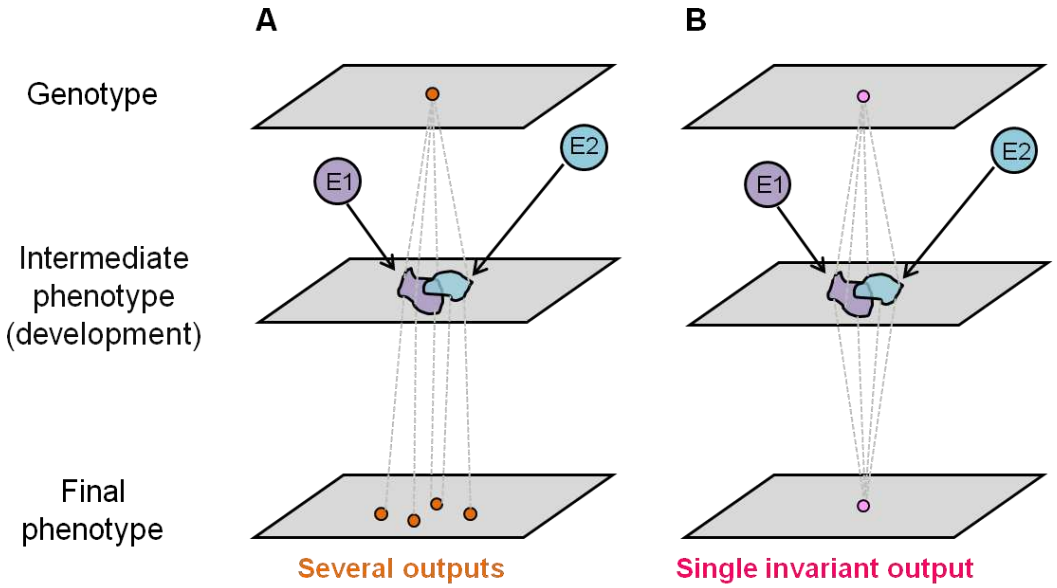

Figure 1.1. Integration of the environment into the genotype-phenotype map. Two different genotypes and their response to two environments (E1 and E2) are represented in A and B. **(A)** The environment induces variation at the intermediate phenotypic level (e.g. gene expression, pathway activity) which results in variation of the final phenotypic output (plasticity). **(B)** The environment induces variation at the intermediate phenotypic level but not in the final phenotypic output. Adapted from Braendle et al. (2008).

Understanding the environmental sensitivity of developmental systems and underlying molecular and cellular processes is thus relevant to understand both how development maintains phenotypic stability despite environmental variation and how development generates phenotypic change in tune with prevailing environmental conditions.

In my PhD work, I focused on how environmental variation affects the functioning of a robust developmental system and its underlying genetic network. To address this question, I used the *C. elegans* vulval developmental system, a very well-characterized process involving highly conserved molecular signalling pathways (i.e. EGF-Ras-MAPK, Delta-Notch and Wnt). In this introduction, I will first present an overview of our current understanding of environmental sensitivity of developmental systems, followed by a brief introduction of the study organism, *C. elegans*. I will then summarize relevant aspects of the model

developmental system, *C. elegans* vulval cell fate patterning. Specifically, I will discuss previously obtained insights into the environmental sensitivity, robustness and evolution of this system.

1.2. Environmental sensitivity of developmental systems

1.2.1. Developmental processes are inherently sensitive to the environment

From the simplest unicellular to the most advanced multicellular form, all organisms live in complex environments that vary in diverse abiotic and biotic parameters, such as temperature, light, nutrients and pathogens. Such environmental variation may affect diverse phenotypic aspects (e.g. gene expression levels, protein synthesis, body size), which may reflect adaptive, neutral or maladaptive organismal responses. Environmental variation may, for example, profoundly affect global gene expression profiles as observed in the yeast *Saccharomyces cerevisiae* (Causton et al., 2001; Gasch et al., 2000). A wide range of environmental conditions, primarily stressors (e.g. temperature shocks, hydrogen peroxide, hyper- and hypo-osmotic shock, amino acid starvation or nitrogen source depletion), have been shown to modulate gene expression in *S. cerevisiae.* A large fraction of the genome responds in a stereotypical manner across tested environmental conditions. Screening of DNA microarrays led to the identification of almost 900 genes whose expression was commonly disrupted upon stress exposure irrespective of its precise nature, yet most conditions also showed specific regulation of specific gene subsets (Gasch et al., 2000). These and many other studies in yeast and other organisms show that gene expression, one of the most basal phenotypic characters, is strongly environmentally sensitive. However, for most of these observed changes it is unclear how they translate into later phenotypic consequences, e.g. how they impact reproductive features or survival.

Well-characterized metazoan developmental processes in response to changing environments are represented by growth and body size control in response to nutritional availability and status. Growth control, a fundamental process shaped by obvious interactions between genes and environment, has been particularly well elucidated in the fly, *Drosophila melanogaster* (Nijhout, 2003). In *Drosophila*, growth occurs during larval stages and adults emerge at their final size: individuals grown in starvation conditions are reduced by 50% in size compared to well-fed ones (Figure 1.2) (Hietakangas and Cohen, 2009). Consequently, the final size of an individual critically depends on the coordination of developmental timing and nutrient availability (Layalle et al., 2008).

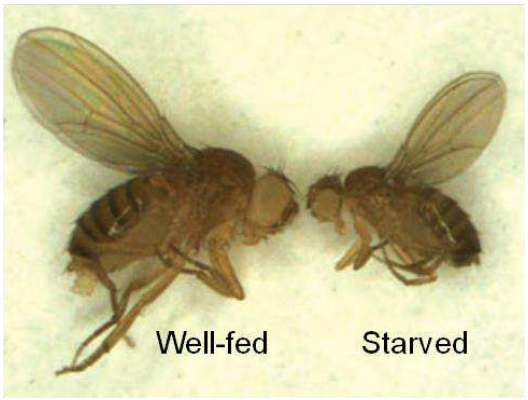

Figure 1.2. *Drosophila* body size in food versus starvation conditions. A strong reduction of body size is observed under starvation conditions. The control fly (left) was grown in standard conditions (with plenty of food). The starved fly (right) was grown in a media containing only 10% of the standard nutritional value. Photograph from Hietakangas and Cohen (2009).

Systemic regulation of growth involves interplay between multiple tissues and signalling pathways (Hietakangas and Cohen, 2009). This regulation is mainly ensured by the Insulin-like signalling (Nijhout, 2003). The TOR (Target of Rapamycin) signalling pathway is also a major regulator of cell growth control – by regulating both cell size and proliferation (Zhang et al., 2000). In *D. melanogaster*, TOR activity is controlled by amino acids (Avruch et al., 2009; Colombani et al., 2003) and cellular energy levels (ATP/AMP ratio) – sensed by AMP-activated protein kinase (AMPK) (Hardie, 2007). The well-understood mechanisms underlying *Drosophila* growth control reveal how instructive environmental cues (e.g. nutrition) translate into developmental and metabolic changes and plastically modulate the final phenotype (e.g. body size) of an organism.

Environmental variability in yeast gene expression and nutritional control of *Drosophila* growth represent just two examples out of a diverse spectrum of environmentally sensitive processes. However, they clearly illustrate that environmental variation may have strong, wide-ranging phenotypic effects and that the environment may provide instructive information, critical in regulating key developmental decisions.

1.2.2. Developmental plasticity versus robustness in response to environmental variation

As already mentioned, developmental responses to the environment may generate plastic or invariant (robust) phenotypic outcomes (Figure 1.1). Differences in environmental sensitivity between genotypes for a given phenotype can be visualized using reaction norms (Figure 1.3), illustrating phenotypic changes across different environments.

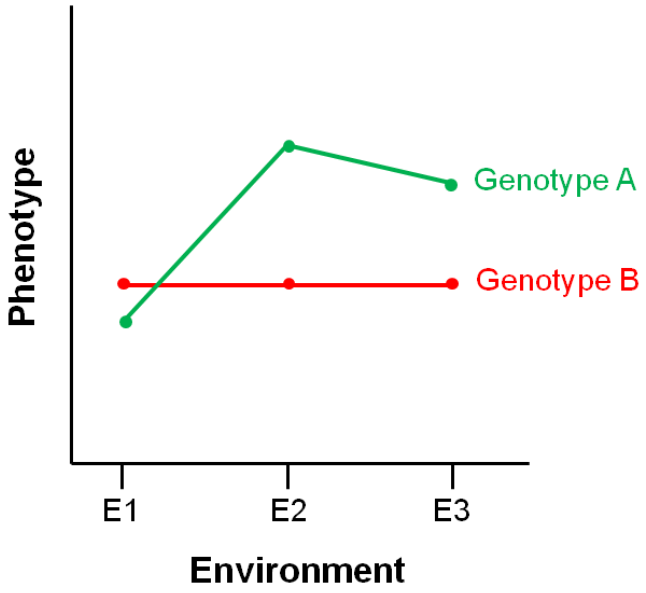

Figure 1.3. Visualizing the pattern of phenotypic responses using reaction norms. Reaction norms are used to represent the phenotypic response of a given genotype across environments. Genotype A is plastic across environments. Genotype B is non-plastic (robust) across environments.

Developmental (or phenotypic) plasticity is the ability of a genotype to produce different phenotypes in response to environmental variation (Bradshaw, 1965; Pigliucci, 2001; Schlichting and Pigliucci, 1998; Stearns, 1992). Developmental plasticity is a universal organismal feature, but which is most commonly used to refer to adaptive, flexible changes of development in response to specific environmental conditions (West-Eberhard, 2003). A clear and famous example of adaptive developmental plasticity concerns the morphology of the *Daphnia* water fleas (Woltereck, 1932) which grow a defensive "helmet" in response to chemical cues released by their fish predators (Tollrian, 1993) (Figure 1.4).

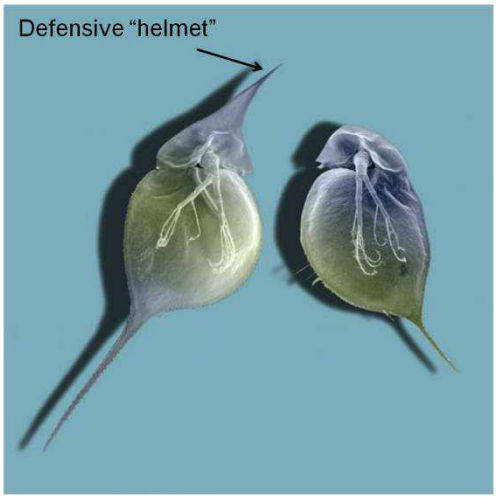

Figure 1.4. Developmental plasticity in *Daphnia sp*. In this picture two individuals of the same species. Daphnia water fleas display the ability to form a defensive “helmet” when exposed to predators. Left: exposed to predators. Right: absence of predators. Picture credits: Christian Laforsch.

Other striking examples of developmental plasticity include temperature-induced sex determination (Crews et al., 1994), plastic caste determination in social insects (Simpson et al., 2011) or alternative diapausing phenotypes (dauer) in *C. elegans* (Hu, 2007). Such developmental plasticity is common for many other traits and species, e.g. for body size in various organisms, as illustrated in the above example of *Drosophila*. Understanding the molecular and genetic mechanisms of such developmental plasticity is a current key focus both in developmental and evolutionary biology, and much progress has been made using the model systems *A. thaliana* (Komeda, 2004), *D. melanogaster* (Flatt et al., 2013) and *C. elegans* (Braendle et al., 2008; Viney and Diaz, 2012).

Developmental plasticity, reflecting environmental sensitivity of development, contrasts with environmental insensitivity of development, often termed (environmental) robustness (de Visser et al., 2003; Waddington, 1942). Developmental robustness is an essential feature of organisms, which have to face diverse sources of variation, including genetic, environmental and stochastic variation (Felix and Wagner, 2008). Developmental robustness describes the ability of a biological system to generate invariant outputs when facing such variation (Figure 1.5).

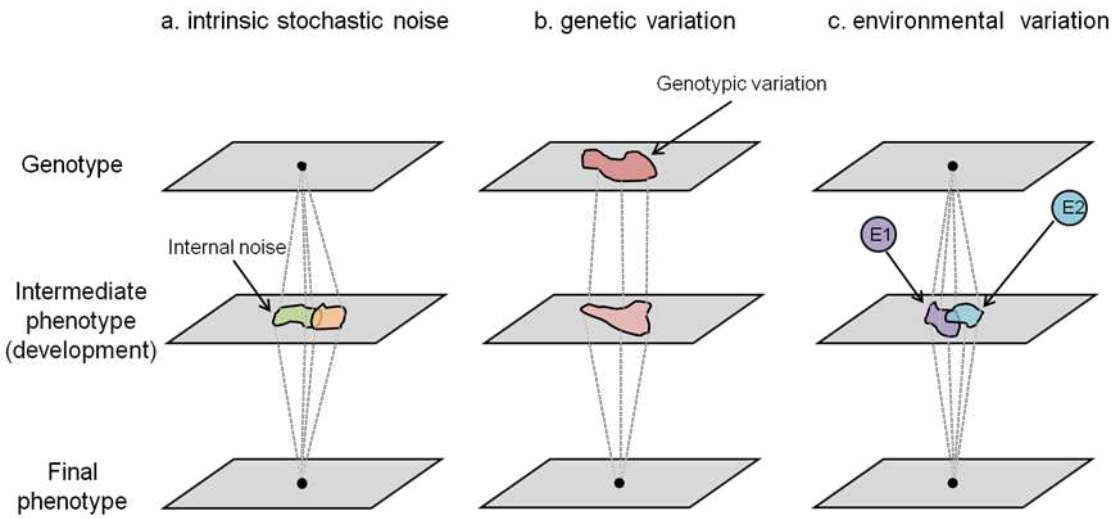

Figure 1.5. Robustness to different sources of variation. In **(a)** the final phenotype is robust to internal stochastic variation (e.g. cellular concentration of a given molecule) represented in green and orange. In **(b)** the final phenotype is robust to genetic variation even if variation can occur at the intermediate level. In **(c)** the final phenotype is robust to environmental variation (E1 and E2, in purple and blue). Adapted from Félix and Wagner, 2008.

Fundamentally, robustness of biological processes to diverse sources of variation may result through distributed robustness or redundancy (Felix and Wagner, 2008; Wagner, 2005a) (Figure 1.6).

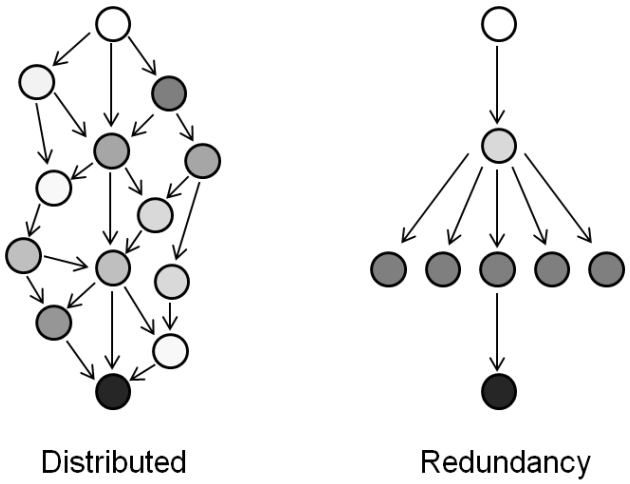

Figure 1.6. Distributed robustness versus redundancy. Both panel show hypothetical signalling cascades. The information from the upper circles (white) in transduced via numerous components

(different greys) to downstream effectors (black). In the case of distributed robustness (left) the information is distributed among several different paths – none of them performing the same function. In case of redundancy (right) the information processes through components that have exactly the same function (dark grey). Adapted from Félix and Wagner, 2008.

In a distributed system no sub-section plays the same role and they constitute alternative paths. They are tightly connected and strongly interact. Conversely, redundancy results from the equivalence of different parts of a developmental system. Whether either type of robustness results simply through emerging network properties or through adaptive evolution remains, however, difficult to evaluate (Felix and Wagner, 2008; Wagner, 2005a). Importantly, robustness of biological processes to a given perturbation may also render them robust to other sources of perturbations, i.e. robustness shows congruence (Masel and Siegal, 2009). Therefore, for example, robustness to environmental variation will make the system also robust to genetic or stochastic variation (de Visser et al., 2003; Meiklejohn and Hartl, 2002; Proulx and Phillips, 2005) .

An important consequence of developmental robustness is that it may generate robustness to mutations, leading to the accumulation of cryptic genetic variation, i.e. evolutionary variation in the absence of evolutionary change of the phenotype (Gibson and Dworkin, 2004). Thus, robustness of the phenotype can have seemingly paradoxical evolutionary consequences and lead to increased genetic evolvability of such system (Masel and Trotter, 2010).

1.3. The study organism *Caenorhabditis elegans*

1.3.1. General biology

The free-living nematode *Caenorhabditis elegans* is a small (1 to 1.5mm), transparent and simple multicellular organism which lives in rotten matter, feeding on diverse microbes (Kiontke and Sudhaus, 2006; Kiontke et al., 2011). Since its introduction by Sydney Brenner (Brenner, 1974), *C. elegans* has become a well-characterized model organism for molecular, genetic and developmental studies. The invariant *C. elegans* cell lineage has been determined (Sulston and Horvitz, 1977; Sulston et al., 1983) and this nematodes was the first metazoan to have its genome completely sequenced (Consortium, 1998). Using *C. elegans* as a model

system has many advantages: easy culturing methods (individuals can be maintained on agar plates and fed with *Escherichia coli*), short life cycle (3.5 days at 20°C), and reproduction through self-fertilizing hermaphrodites, resulting in isogenic populations. Male production is facultative and results through spontaneous X-chromosome non-disjunction during meiosis.

Available resources (databases, literature, genetic maps and mutant libraries) and a wide range of established experimental techniques (RNAi, mutagenesis and transgenesis) as well as easy culturing (including cryopreservation of stocks) make *C. elegans* a model organism of choice.

Life cycle

Under laboratory conditions it takes about three and a half days to complete the *C. elegans* life cycle from egg to reproductive adult (Figure 1.7). The life cycle is composed of two phases: embryonic and post-embryonic development. The embryonic development starts *in utero* and eggs are laid as early embryos. After hatching, both hermaphrodites and males develop through four larval stages. At the end of each stage, larvae undergo a brief lethargus with arrest of pharyngeal pumping. This lethargus is associated with a moult during which a new stage-specific cuticle is synthesized. The last moult leads to the reproductively mature adult.

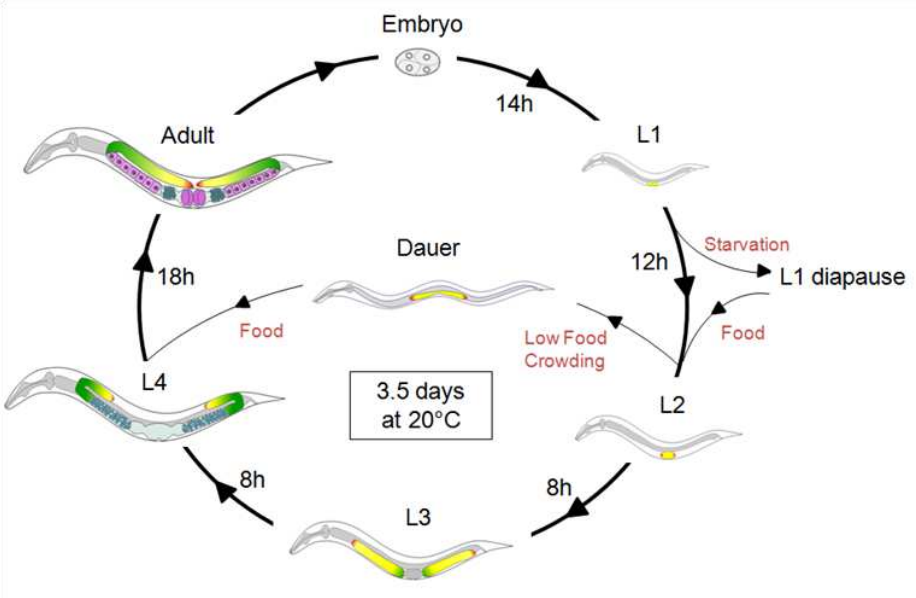

Figure 1.7. The *C. elegans* life cycle. The *C. elegans* life cycle is comprised of embryonic and post-embryonic development. Post-embryonic development is composed of four larval stages followed by

adulthood. During the L1 stage, individuals can arrest their development for several days under unfavourable conditions (i.e. starvation). When food becomes available again they re-enter the reproductive life cycle. At the end of the L1 stage, if the environment is harsh (high temperature, low food, crowding), they can undergo an additional developmental arrest: the dauer stage. Upon better conditions development will resume and individuals will form L4 larvae. Duration of each stage is indicated at 20°C. Image: Nausicaa Poullet.

Morphology and reproductive mode

All nematodes share the same unsegmented and cylindrical body plan. In *C. elegans*, both self-fertilizing hermaphrodite (XX) and male (XO) bodies are surrounded by a collagenous cuticle. This outer tube – composed of hypodermis and cuticle – surrounds a pseudocoelomic cavity containing both digestive and reproductive tracts. Body shape is maintained by hydrostatic pressure. In laboratory conditions, individuals are mainly fed on the cultures of the bacterium *E. coli*. The bacteria are ingested through the mouth and pass through a two-lobed pharynx – which acts as a pump and grinder.

The adult hermaphrodite is composed of 959 somatic cells, 302 of which are neurons and 95 of which are body wall muscles (White, 1988). *C. elegans* males are initially identical to the hermaphrodite larvae apart from a few male fate cells, but start to display typical budding shape of their posterior half during the L2 stage (Nguyen et al., 1999; Sulston and Horvitz, 1977; Sulston et al., 1980). The adult male is composed of 1031 somatic cells, 381 of which are neurons, mostly involved in mating behaviour (White, 1988). *C. elegans* males and hermaphrodites display sexual dimorphism in all tissues – except for the pharynx and the excretory system.

The hermaphroditic reproductive system is composed of two symmetrical U-shaped gonad arms containing the germline, connected by the central uterus and the vulva (Figure 1.8). The first germ cells produced are sperm. During the L4 stage, hermaphrodites produce around 160 sperm per gonadal arm and store them in the spermatheca. At the end of the L4 stage an irreversible switch occurs and hermaphrodites start to produce oocytes (Ellis and Kimble, 1995) – which will be fertilized by sperm when entering the spermatheca. Embryos start to develop in the uterus until gastrulation and are then laid through the vulva. In contrast, the male gonad is composed of one single arm. Males produce sperm throughout life. The male's copulatory apparatus is located in the tail. The fan extends from the tail and contains

nine pairs of sensory rays. The proctodeum ends in the cloaca and posteriorly links the intestine and the gonad. The spicules are two particular sensilla covered by cuticle and playing a major role during mating, i.e. locating the hermaphrodite vulva and hold it open during sperm transfer.

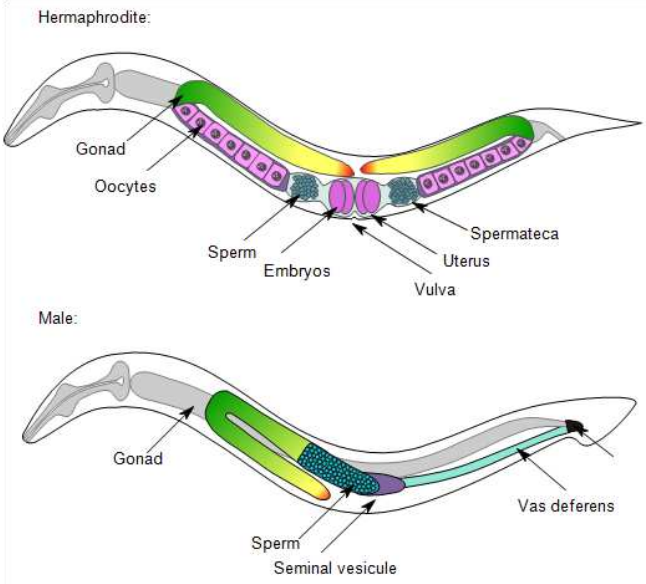

Figure 1.8. Morphology of *C. elegans* hermaphrodites and males. Schematic drawing of anatomical structures left lateral side. (A) Hermaphrodite. (B) Male. See text. Image: Nausicaa Poullet.

1.3.2. Natural *C. elegans* habitat

Despite the extensive knowledge accumulated on *C. elegans* development and genetics, little is known about its natural environment. However, *C. elegans* ecology has recently become a field of interest and led to the discovery of many new *C. elegans* wild isolates (Andersen et al., 2012) and *Caenorhabditis* species (Kiontke et al., 2011). Often described as a soil nematode, the cosmopolite *C. elegans* (Barriere and Felix, 2005) actually lives in decomposing vegetal matter, such as rotten plant, flowers or fruits. *C. elegans* wild populations have also been found in decomposing invertebrates. The *C. elegans* natural habitat is extremely variable in terms of food availability, temperature, oxygen and chemicals concentration. The *C. elegans* natural habitat thus represents a highly ephemeral environment undergoing strong fluctuations in nutritional conditions.

Compared to its wild habitat, the lab environment is very stable and standardized. Individuals are maintained on petri dishes filled with Nematode Growth Medium (NGM) (Brenner, 1974; Hope, 1999) and fed with a particular strain of *E. coli* (OP50). Plates are usually maintained at 20°C. This standard environment has been developed to optimize offspring production but as *E. coli* is a mammalian intestinal bacterium, thus unlikely to represent a highly relevant natural food source for *C. elegans*.

1.3.3. Environmental sensitivity of *C. elegans* development

Environmental variations can elicit both behavioural and developmental responses in *C. elegans*, such as attraction/avoidance (Bargmann et al., 1993; Troemel et al., 1997), morphological and locomotion changes in liquid culture (Szewczyk et al., 2006) or specific diapause-like states (i.e. L1 arrest, dauer and ARD) (Angelo and Van Gilst, 2009; Hu, 2007; Johnson et al., 1984). When encountering stressful conditions (i.e. starvation, crowding or high temperature), individuals can slow or arrest their development in several developmental stages (Ruaud and Bessereau, 2006). Food limitation (starvation) is one of the major environmental stressors that *C. elegans* individuals can encounter. If the embryos hatch in the absence of food, the L1 larvae arrest their development (Johnson et al., 1984). When food becomes available again, the arrested L1 are able to re-enter a normal life cycle (Slack and Ruvkun, 1997) (Figure 1.9). At the end of the L1 stage, if the environmental conditions are not favourable (low food availability, high temperature and crowding) the individuals enter a morphologically specific L2 stage, called L2d. L2d larvae retain the potential of forming L3 larva but if environmental conditions remain unfavourable, these L2d enter the L3 dauer larval stage (Albert and Riddle, 1988; Golden and Riddle, 1982, 1984). This dauer stage can last for months until dauer larvae experience favourable conditions. Upon food re-exposure, dauers start to develop, and after 10 hours, the L3/L4 moult occurs and individuals re-enter the normal life cycle at the L4 stage (Figure 1.9). During dauer, feeding is completely arrested – defined as a non-aging state, and dauer larvae display a ‘‘waving behaviour’’ which may serve to find or attract hosts that may carry them to a location providing adequate resources (Riddle, 1988; Riddle and Albert, 1997). Dauer formation represents one of the best-understood examples of developmental modulation by external cues – coupling both sensory and metabolic information. Dauer formation is regulated by a complex genetic network (Fielenbach and Antebi, 2008), revealed by the analysis of *daf-c* (dauer constitutive) and *daf-d* (dauer defective) mutants (Riddle and Albert, 1997). This network involves chemosensory

components of the cGMP pathway, such as G-protein, guanylyl cyclase and cGMP dependant cyclases (Birnby et al., 2000) but also metabolic components, such as Insulin (Li et al., 2003; Pierce et al., 2001). After the last moult, at the beginning of reproduction, individuals can also adopt a diapause-like state, called adult reproductive diapause (ARD) (Angelo and Van Gilst, 2009), in which the germline size is reduced to a minimal pool of stem cells. When conditions are favourable the germline regrows and reproduction starts again (Figure 1.9). During adulthood, hermaphrodites also have an additional strategy to face an unfavourable environment (e.g. starvation). They can retain their embryos, which will hatch inside the dead adult (contrary to what happens in ARD) (Chen and Caswell-Chen, 2003). This is called "bagging" and may represent a strategy to protect progeny until they reach the resistant dauer stage (Chen and Caswell-Chen, 2003; Chen and Caswell-Chen, 2004) (Figure 1.9).

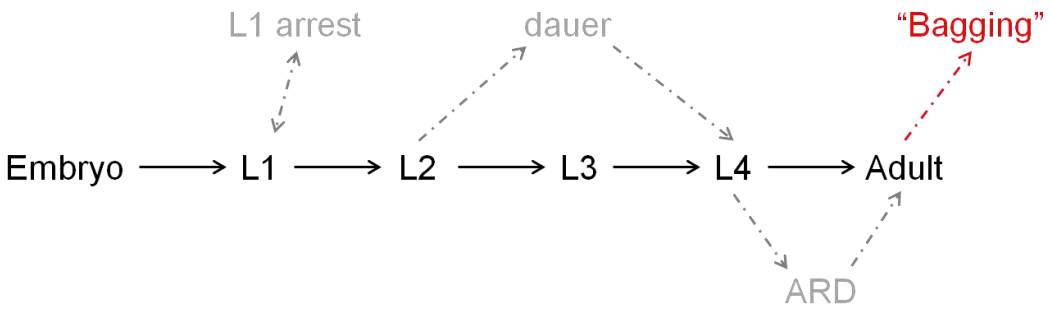

Figure 1.9. Starvation responses during the *C. elegans* life cycle. Starvation during the L1 stage leads to a reversible L1 arrest. Starvation in late L1 and L2 causes adoption of the dauer stage. Starvation during the L4 stage leads to adult reproductive diapause whereas late L4 or adult starvation results in "bagging", i.e. internal hatching of embryos. Adapted from Angelo and Van Gilst, 2009.

1.3.4. Perception and transduction of environmental cues in *C. elegans*

Immunity and stress response

During its life *C. elegans* can experience highly stressful conditions (e.g. high temperature, hypo- and hyperoxia, starvation). Environmental perception (see below) and stress-triggered responses ensure the adjustment of cellular functions in critical environments and evolutionary conserved stress response mechanisms have been found in *C. elegans* (Koga et al., 2000; Lehtinen et al., 2006). DAF-21, for instance, is a member of the HPS90 family, expressed in all cells under stressful conditions (Inoue et al., 2003). *C. elegans* response to

hypoxia also involved evolutionary conserved mechanisms like the HIF (Hypoxia Inducible factor) complexes required for *C. elegans* physiological adaptation to hypoxic conditions (Jiang et al., 2001; Shen et al., 2005).

C. elegans can be infected by a large variety of pathogens (Darby, 2005). Many of them colonize the *C. elegans* intestine, some adhere to the cuticle while others produce toxins and can kill *C. elegans* without any physical contact. *C. elegans* infection has been used as a powerful genetic system to study innate immunity (Engelmann and Pujol, 2010; Ewbank, 2006). General stress response mechanisms are involved in pathogen response but *C. elegans* immune response also relies on major conserved signalling pathways like ERK, p38 MAPK, Insulin or TGF-β (Engelmann and Pujol, 2010).

Sensory perception

Chemosensation

The *C. elegans* chemosensory set of neurons is highly developed – 32 neurons can detect hundreds of chemicals (Bargmann, 2006), which are required to avoid noxious substances and to find food and mating partners. *C. elegans* males have numerous additional chemosensory neurons mainly involved in mating behaviour (Liu and Sternberg, 1995; Sulston et al., 1980). *C. elegans* chemosensory neurons can be directly or indirectly exposed to the environment, mainly through the amphid, the phasmid and the inner/outer labial organs (Ward et al., 1975). Only two specific neurons AQR and PQR– responsible with URX for oxygen sensing (Chang et al., 2006; Cheung et al., 2005; Gray et al., 2004) and social feeding (Gray et al., 2004), are directly exposed to body fluids. Chemosensory neurons usually belong to left-right pairs and each pair can be distinguished from another through morphological criteria (White et al., 1986). Chemicals are detected through hundreds of G protein-coupled receptors (GCPRs) (Robertson and Thomas, 2006) using cGMP as second messenger or relying on TRPV channels and allow *C. elegans* to integrate environment into both developmental and behavioural processes.

Thermosensation

In *C. elegans* a single pair of amphid neurons (AFD) is essential to for thermosensation and thermotaxis behaviour (Mori and Ohshima, 1995). The AFD neurons respond to warming and their ablation leads to cryophilic individuals (Kimura et al., 2004). Temperature sensing in *C.*

elegans acts in a small cellular circuit involving AIY, AIZ and RIA neurons (McKemy, 2007). AIY and AIZ act as antagonists in thermal responses: AIY-ablated animals are cryophilic and AIZ-ablated animals are thermophilic. In addition to other sensory defects RIA-ablated animals are partially thermosensory-deficient.

Mechanosensation

Mechanosensory neurons serve to detect collisions with particles (e.g. debris, other animals) as well as forces generated by its own movement. Mechanosensation is ensured by 30 putative mechanoreceptor neurons (MRNs) in the hermaphrodite and 52 extra MRNs exist in the male – mainly involved in mating behaviour. In *C. elegans*, touch responses are involved in many behaviours like locomotion (Chalfie et al., 1985; Wicks and Rankin, 1995), egg laying (Sawin, 1996), feeding (Chalfie et al., 1985; Keane and Avery, 2003), defecation (Thomas, 1990) and mating (Liu and Sternberg, 1995). Mechanical information is transduced by putative channels of the TRP (Transient Receptor Potential) superfamily in ciliated MRNs and of the DEG/ENaC (DEGenerin/Epithelial Na^+ Channel) superfamily in non ciliated MRNs (Ernstrom and Chalfie, 2002; Goodman and Schwarz, 2003).

Metabolism

Being able to coordinate and adjust energy levels in tune with prevailing environmental conditions is critical for cellular and organismal survival. Metabolic sensors are key regulatory elements that allow individuals to perceive their environment and adapt to it (Lindsley and Rutter, 2004). On a cellular level, metabolic sensors detect and respond to levels of macronutrients (e.g. glucose, amino acids and fatty acids, AMP/ATP ratio). On an organismal level, coordination of energetic status from different tissues is controlled by hormonal signals (Lindsley and Rutter, 2004).

C. elegans presents highly conserved metabolic sensors. First, the IGF-1/Insulin signalling pathway connects nutrient levels to growth, development and longevity mainly through the DAF-16/FoxO transcription factor (Murphy and Hu, 2013). The second key metabolic sensor is the LET-363/TOR (Target of Rapamycin) signalling pathway. It couples nutrient levels to cell size and proliferation. In *C. elegans*, inactivation of LET-363/TOR and its partner DAF-15/Raptor leads to developmental arrest and fat accumulation (Jia et al., 2004; Vellai et al., 2003). LET-363/TOR is directly regulated by nutrient levels but also by

the DAF-2-Insulin pathway to control dauer formation and longevity (Jia et al., 2004). The third major cellular sensor is the AMPK pathway. It responds to cellular AMP:ATP ratio as well as upstream kinase cascades (Kahn et al., 2005; Lindsley and Rutter, 2004). In *C. elegans* AAK-2, the orthologue of AMPK, regulates lifespan in response to AMP:ATP ratio and insulin-like signals. Nuclear hormone receptors (NHRs) also coordinate metabolic responses and function as regulators of metabolic gene expression (Van Gilst et al., 2005a; Van Gilst et al., 2005b).

1.4. The study system: *C. elegans* vulval cell fate patterning

C. elegans vulval development is an extensively studied and well-characterized developmental process involving conserved signalling pathways (Félix, 2012a; Félix and Barkoulas, 2012; Sternberg, 2005). This process underlies the formation of an essential reproductive organ, required for egg laying and mating with males.

1.4.1. The *C. elegans* vulval signalling network

At hatching, the *C. elegans* L1 larva possesses six pairs of ventral blasts: the Pn cells. During L1 each pair rotates and the twelve Pn cell aligned along the antero-posterior axis (Sulston and Horvitz, 1977). Each Pn cell will then asymmetrically generate two daughters: Pn.a and Pn.p. The Pn.a cells will primarily develop into ventral cord neurons (Sulston and Horvitz, 1977) whereas the Pn.p cells adopt an hypodermal fate. During the L1 stage, P(3-8).p acquire competence to form vulval tissue: each of these cells is able to respond to the inductive signal and adopt a vulval fate (Kimble, 1981; Sternberg and Horvitz, 1986; Sulston and White, 1980). The specification of these vulval precursor cells (VPCs) is ensured by the expression of the *lin-39/Hox5* gene (Maloof and Kenyon, 1998; Salser et al., 1993), which encodes a homeodomain protein required for specification of mid-body region cell fates (Figure 1.12A). SEM-4, a zinc finger protein, is necessary for full *lin-39/Hox5* expression (Grant et al., 2000). LIN-39/Hox5 acts with two homeodomain proteins co-factors *ceh-20/pbx1-3* and *unc-62/meis* which are transcribed in all the Pn.p cells, except P12.p (Yang et al., 2005). CEH-13/Hox1, encoded by the *ceh-13/Hox1* gene and expressed in cell nuclei all along the ventral cord (Brunschwig et al., 1999), antagonises LIN-39/Hox5 and promotes Pn.p fusion (Tihanyi et al., 2010).

LIN-39/Hox5 activity is also required during the L2 stage to maintain VPCs competence (Chen and Han, 2001). During the L2 stage *lin-39/Hox5* expression is under the control of the canonical Wnt pathway (Eisenmann et al., 1998). Combined with the Wnt pathway, the EGF/Ras/MAPK signalling is also involved in the maintenance of VPCs competence. For instance, gain of function mutations in the EGF-Ras-MAPK pathway diminishes fusion during the L2 stage (Chen and Han, 2001). Moreover and in a *bar-1/β-Catenin* mutant sensitized background, a *let-23/egfr* mutation aggravates the fusion phenotype (Eisenmann et al., 1998).

As mentioned before, ablation experiments revealed that only P(3-8).p have competence to form vulval tissue (Kimble, 1981; Sternberg and Horvitz, 1986; Sulston and White, 1980). Nevertheless, the competence level of each VPC is not equivalent (Clandinin et al., 1997). LIN-39 expression, regulating VPC competence (Maloof and Kenyon, 1998), is variable along the antero-posterior axis leading to a differential sensitivity of the VPCs to the inductive signal (Pénigault and Félix, 2011b). P7.p and P8.p are less sensitive to the LIN-3/EGF inductive signal due to the expression of *mab-5/Hox7*. *mab-5/Hox7* encodes a homeodomain transcription factor required for specification of posterior cell fates. Moreover P3.p sensitivity can be explained by the graded action of two Wnt ligands *egl-20* (Coudreuse et al., 2006; Whangbo et al., 2000) and *cwn-1* (Hayashi et al., 2009) regulating *lin-39/Hox5* expression in the VPCs (Figure 1.10).

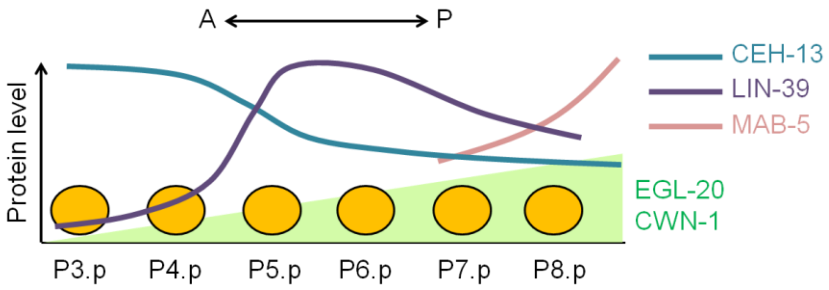

Figure 1.10. Regulation of VPC competence in *C. elegans*. In *C. elegans*, vulval precursor cells are not equally sensitive to the inductive signal. This scheme represents the regulation of VPC competence along the antero-posterior axis. Disks represent the vulval precursor cells. The Wnt ligands *egl-20* and *cwn-1* are more expressed in the posterior part of larva and a long-range gradient diffuses until the midbody of the animal (Coudreuse et al., 2006). *lin-39/Hox5* is expressed along the AP axis with low levels in P3.p and P4.p and a peak on P5.p. *ceh-13/Hox1* and *mab-5/Hox7*

antagonize *lin-39/Hox5* and are expressed respectively all along the AP axis and posteriorly. Adapted from Pénigault and Félix, 2011b.

During the second larval stage (L2) the anchor cell (AC) induces vulva cell fates by expressing the EGF-like ligand LIN-3 (Hill and Sternberg, 1992) (Figure 1.11 and Figure 1.12B). The AC is necessary and sufficient to induce vulva formation (Kimble, 1981). LIN-3/EGF disperses as a morphogen and the Pn.p adopt different cell fate according to their location. P6.p is closest to the AC and receives the highest level of LIN-3/EGF causing it to adopt a 1° cell fate. P6.p then expresses Delta ligands which activate the Delta-Notch pathway in its neighbours, P5.p and P7.p. This activation causes them to adopt a 2° fate (Greenwald et al., 1983) and prevents them from adopting a 1° fate by inhibiting the EGF-Ras-MAPK pathway (Sternberg, 1988; Yoo et al., 2004) through the mitogen-activated protein (MAP) kinase phosphatase LIP-1 (Berset et al., 2001) (Figure 1.11). This cross-talk between EGF-Ras-MAPK and Delta-Notch is a key to maintain a robust 2°-1°-2° spatial pattern. However, a lower dose of LIN-3/EGF may also be responsive of the adoption of the 2° fate in P5.p and P7.P (Katz et al., 1995). Morphogen induction and signalling crosstalk act together to ensure a precise 2°-1°-2° pattern (Kenyon, 1995). Moreover, it has been recently shown that a switch from the canonical LET-60/Ras-LIN-45/Raf pathway to a LET-60/Ras-RGL-1-RAL-1 signalling pathway can promote the 2° cell fate in P5.p and P7.p (Zand et al., 2011) (Figure 1.11). P3.p, P4.p and P8.p do not receive enough signal (LIN-3/EGF or Delta- LIN-12/Notch) and adopt a 3°, non vulval fate (Hill and Sternberg, 1993). The Wnt signalling pathway, involved in Pn.p competence, seems to have a partially redundant role with the EGF-Ras-MAPK pathway in Pn.p induction. Indeed, overactivation of the Wnt pathway can compensate vulval induction when the EGF-Ras-MAPK pathway is compromised (Gleason et al., 2002). The adult vulva is formed from the 22 descendants of P5.p, P6.p and P7.p according to an invariant and characterised lineage.

Negative regulation of vulval induction is also ensured by *synMuv* (Synthetic Multivulva) genes. *SynMuv* genes are transcriptional repressors that negatively regulate *lin-3* expression in hyp7 (Cui et al., 2006; Sundaram, 2006; Thomas and Horvitz, 1999). There are three classes of *synMuv* genes: A, B and C. Two redundant sets of *synMuv* genes have been described: class A and class B. Mutation in only A or B genes leads to a normal vulval development whereas mutations in both A and B genes result in a *Multivulva* phenotype.

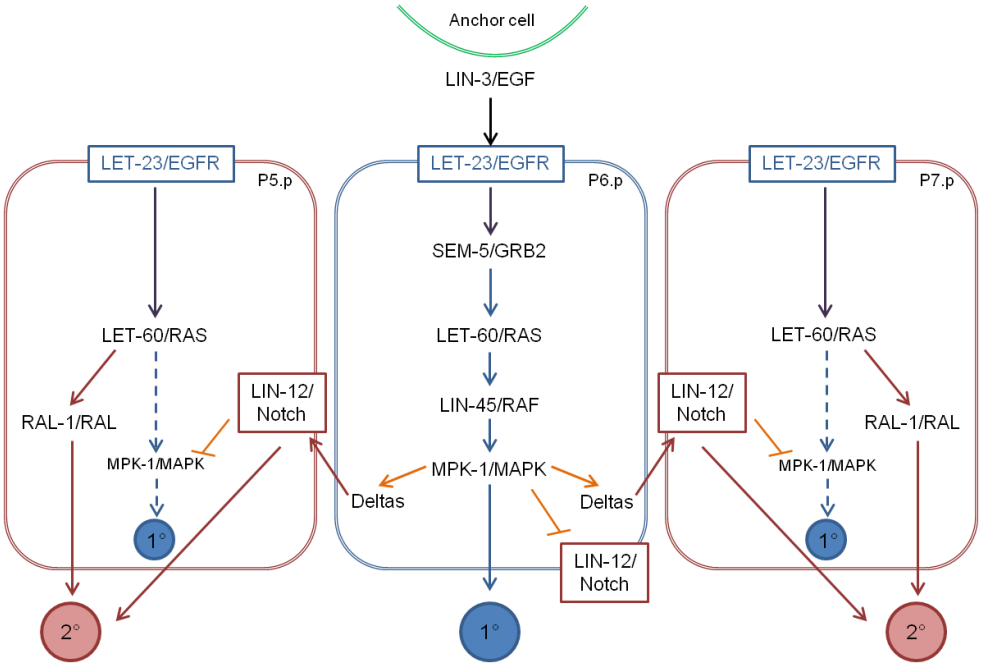

Figure 1.11. Overview of the vulval signalling network in *C. elegans*. Core components of the EGF-Ras-MAPK pathway are in the centre. The inductive signal LIN-3/EGF is sent by the anchor cell. This signal acts as a morphogen and is able to activate the EGF-Ras-MAPK pathway in the Pn.p cells.P6.p then adopts a primary (1°) cell fate. P6.p is the closest cell of the AC, thus the activation of the EGF-Ras-MAPK pathway is higher in this cell. In response, the Delta-Notch pathway is activated in P5.p and P7.p. This activation gives rise to the adoption of a secondary cell fate (2°) and to the repression of the EGF-Ras-MAPK pathway and upregulation of *lip-1*. This highly regulated process leads to an invariant pattern 2°-1°-2° vulval cell fate pattern.

As described before there are two different vulval fates: 1° and 2°. P6.p usually adopts a 1° cell fate and P5.p and P7.p a 2° cell fate. During the L3 stage, the vulval lineage consists of three division cycles (Figure 1.12C). The two first steps are the same for the 1° and 2° fates. It consists in two cycles of longitudinal divisions (L) – following the AP axis. The last division cycle is particular for each fate. The four primary cells divide transversally (T) – following the left/right axis and give rise to 8 progeny cells. The secondary lineage is more complex: two secondary progeny cells of both P5.p and P7.p divide longitudinally (LL), one divides transversally (T) and the last one does not divide (U). The secondary lineage then gives rise to 7 progeny cells. P5.p and P7.p lineages are symmetrical. The asymmetry of each secondary lineage is independent of the gonad (Katz et al., 1996) but the reverse polarity of

P7.p is gonad-dependent and under the control of the Wnt pathway (Inoue et al., 2004). The fibroblast growth factor (FGF) pathway acts in concert with LIN-17/Frizzled to influence the localization of SYS-1, a component of the Wnt/β-Catenin asymmetry pathway (Minor et al., 2013). The three remaining VPCs (P3.p, P4.p and P8.p) do not adopt a vulval fate. In 50% of the individuals of the lab reference strain N2, P3.p does not divide and fuses with the hypodermis during the L2 stage (F or 4° fate). In the other 50%, P3.p adopts a 3° cell fate like P4.p and P8.p. The 3° cell fate is a non-induced fate consisting in one division during the L3 stage followed by fusion with the syncitial hypodermis hyp7 (SS) (Sulston and Horvitz, 1977). During the L4 stage, the vulval cells start moving towards the anchor cell. The vulval invagination forms (Figure 1.12D), and cells fuse into seven toroids. The eversion occurs during the L4/adult moult.

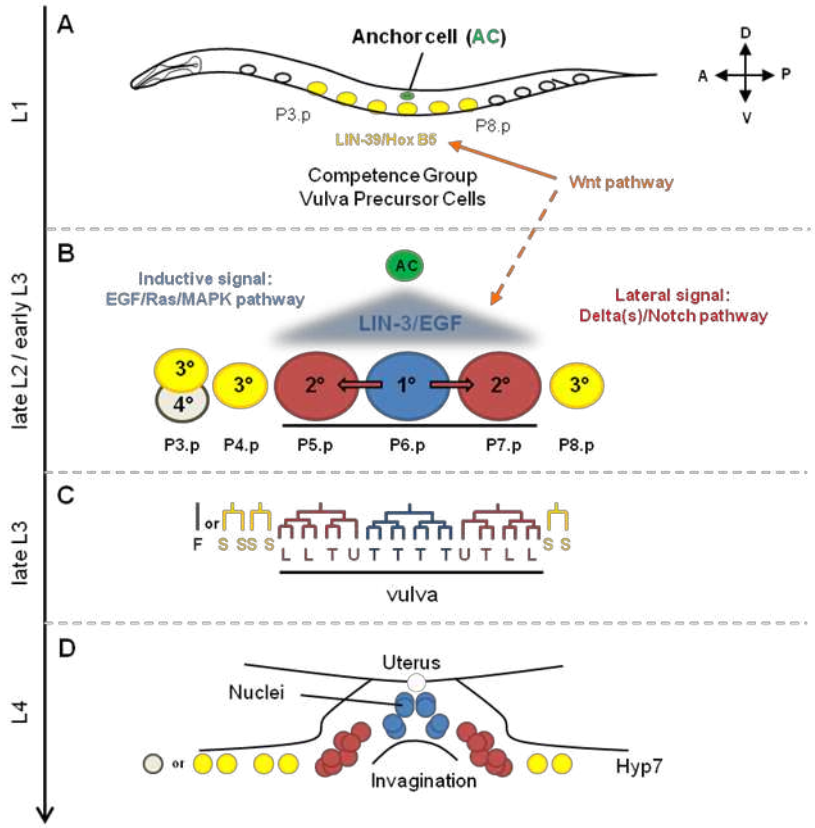

Figure 1.12. *C. elegans* vulva development. The *Caenorhabditis* vulva develops from a subset of six ventral cells, P3.p to P8.p. (A) L1 stage: P3-8.p cells express the Hox gene *lin-39* and acquire competence to form vulval tissue. (B) Late L2 stage: the anchor cell (AC) releases a LIN-3/EGF inductive signal. Only three of the six vulval precursor cells adopt a vulval fate. LIN-3/EGF acts as a morphogen and the receiving cells adopt different cell fates according to their location. P6.p receives the highest level of LIN-3/EGF causing it to adopt a 1° cell fate (blue). The expression of the Delta ligands in P6.p activates the Delta-Notch pathway in its neighbours, P5.p and P7.p. This activation causes them to adopt a 2° fate (red). The three remaining cells adopt a non-vulval 3° fate (yellow). However the fate of P3.p fate is variable and the undivided cell fuses with the hypodermis in approximately 50% of individuals (4° fate (brown)). The canonical *Caenorhabditis* vulval pattern is defined as 2°-1°-2°. (C) Late L3 stage: The invariant and fate-related cell division pattern leads to 22 vulval cells. Cell divisions T: transverse (left-right) division, L: longitudinal (antero-posterior) division, U: undivided, SS: fusion to the epidermal syncytium (hyp7) after single division (3° fate); F: fusion to the syncytium in the L2 stage with no prior division (4° fate). 3° and 4° fate are non vulval. (D) L4 stage: vulval morphogenesis takes place.

Robustness of *C. elegans* vulval cell fate patterning

Theoretical works show that many genetic and network features of developmental systems can contribute to their robustness, including: genetic epistasis and pleiotropy, redundancy, feedback loops and cross-talks (Meir et al., 2002; Siegal and Bergman, 2002; von Dassow et al., 2000; Wagner, 2005b). Due to its molecular and cellular organization, the *C. elegans* vulval developmental network displays high robustness (Figure 1.11 and Figure 1.12). This highly regulated process leads to an invariant pattern 2°-1°-2° of vulva cell fate. Two different and non-exclusive models have been proposed to ensure this precise cell fate pattern formation: morphogen-based versus sequential induction. The morphogen-based model relies on a series of experiments demonstrating that an isolated VPC (laser ablation) can adopt a secondary cell fate (Katz et al., 1995). Under the control of an heat-shock promoter, *lin-3/egf* can be expressed at different doses. A high dose of LIN-3/EGF produces a 1° cell fate, an intermediary dose produces a 2° cell fate whereas a low dose produces a 3° cell fate. The sequential model relies on genetic mosaics experiments. The *let-23/egfr* gene is not required autonomously in 2° cells (Koga and Ohshima, 1995; Simske and Kim, 1995). In the absence of LET-23/EGFR, the 2° cell fate is adopted by cells adjacent to a 1° cell. The secondary cell fate can then be induced by the primary cell fate. Moreover, positives feedback loops are found in both EGF-Ras-MAPK and Delta-Notch pathways (Berset et al., 2005; Stetak et al., 2006; Yoo and Greenwald, 2005). Multiple molecular cross-talks between these two pathways have been identified (Berset et al., 2001; Chen and Greenwald, 2004; Shaye and Greenwald, 2002; Yoo et al., 2004).

1.4.2. Evolution of *Caenorhabditis* vulval cell fate patterning

Evolution within the *Caenorhabditis* genus

The canonical 3°-3°-2°-1°-2°-3° vulval pattern is invariant within the *Caenorhabditis* genus (Félix, 2007; Kiontke et al., 2007). Nevertheless, P3.p division frequency varies extensively among the *Caenorhabditis* species (Delattre and Félix, 2001). Comparative analysis of more than 20 *Caenorhabditis* species reveals a decrease of P3.p competence in *C. briggsae*, in which P3.p is never competent, and close relatives (Kiontke et al., 2011). The invariable pattern is then considered as 3°-2°-1°-2°-3°.

Another interesting difference between *C. elegans* and *C. briggsae* concerns the competence of P4.p and P8.p. In *C. elegans*, P4.p is more competent than P8.p (Katz et al.,

1995) whereas in *C. briggsae* P8.p is the more competent (Félix, 2007). This posterior reduction of competence in *C. elegans* could be explained by *mab-5/Hox7* expression in P7.p and P8.p (Clandinin et al., 1997) (see 1.4.1). Anchor cell ablation in the *Caenorhabditis* species reveals that the AC is necessary and sufficient for vulval induction among these species, except for *C.* sp1 in which more gonadal cells are involved (Félix, 2007; Kiontke et al., 2007).

Uncovering cryptic genetic variation underlying *Caenorhabditis* vulval development

Caenorhabditis vulval development robustly ensures the formation of an invariable 3°-2°-1°-2°-3° pattern governed by intercellular signalling. As described before (see 1.2.2), such robustness may allow accumulation of cryptic genetic variation buffered in standard conditions. Revealing such cryptic genetic variation underlying vulval development among different isolates or species shows that an evolutionarily invariant phenotype (cell fate pattern) may go in hand with extensive evolutionary divergence of underlying developmental mechanisms. Cryptic variation in the vulval signalling network among *C. elegans* wild isolates (genotypes) has been uncovered through introgression of vulval mutations, measurements of pathway activities or exposure to different environments (Braendle and Félix, 2008; Milloz et al., 2008).

By introgressing vulval mutations, e.g. *let-23/egfr* into different wild isolates, Milloz et al. (2008) found that the genetic background strongly modified the penetrance of such mutations, revealing an interaction between the introgressed mutation and the wild genetic background. Moreover, EGF-Ras-MAPK pathway activity in VPCs was measured in different genotypes, indicating that pathway varies up to two-fold across these wild isolates (Milloz et al., 2008). Duveau et al. (2010) mapped such cryptic genetic variation in *let-23/egfr* penetrance among isolates, identifying *nath-10* locus as responsible for this variation. The cryptic effect on vulval signalling involving *nath-10* seems to result from pleiotropy caused by *nath-10* function in sperm production, and thus reproduction (Duveau and Felix, 2010). Cryptic genetic variation of vulval cell fate patterning was also revealed using accumulation of spontaneous random mutation: different *C. elegans* and *C. briggsae* isolates generated different frequencies and spectra of vulval patterning errors after 250 generations of mutation accumulation (Braendle et al., 2010). These experiments further suggested that mutational robustness of vulval cell fate patterning is subject to evolution.

The penetrance of vulval mutations was further assessed in different environments (i.e. temperature, liquid, starvation and dauer) (see also next sections for more details). The expressivity of these mutations varied with the environment revealing a mutation-environment interaction (Braendle and Félix, 2008). Moreover, such interactions were further modified depending on the genetic background examined, revealing cryptic variation in the form of complex genotype-mutation-environment interactions (Braendle and Félix, 2008).

In addition, through tissue-specific perturbations of EGF-Ras-MAPK and Delta-Notch signalling pathways, Barkoulas and Felix (2013) have been able to determine the limits of the network's robustness: they identified the first error pattern observed under each specific perturbation and then highlighted the quantitative interaction of these pathways in the vulval network (Barkoulas et al., 2013). An additional method to unravel cryptic variation of vulval patterning was quantification of cell fate patterns obtained after system impairment by either anchor cell ablation or LIN-3/EGF overexpression in different C*aenorhabditis* species (Félix, 2007). These experiments revealed substantial variation in inductive signalling pathways as well as their time of action. Recent computational studies (Giurumescu et al., 2009; Hoyos et al., 2011) focused on understanding how such cryptic system differences among species may be explained by quantitative variation in activities of involved signalling pathways and network topology.

1.5. Environmental sensitivity of the *C. elegans* vulval cell fate patterning

1.5.1. Precision and robustness of the vulval cell fate patterning process in different environments

The precision of the vulval cell fate patterning has generally been considered to be robust to stochastic, genetic and environmental variation, yet quantification of the actual degrees of robustness have rarely been measured. Braendle and Felix (2008) aimed at quantifying the patterning precision of *C. elegans* N2 and other wild isolates in different environmental conditions (i.e. starvation, dauer passage, different temperatures, liquid). Although environmental variation induced certain vulval defects and variants, pattern formation was very robust to such perturbations. Assessing the vulval pattern of 6000 individuals, revealed that defects leading to a non-functional vulva were very rare (0.25%) across environments (Braendle and Félix, 2008). These observations suggest that vulval cell fate patterning is indeed robust to various environmental conditions.

1.5.2. Environmental sensitivity of the *C. elegans* vulval network

Despite this apparent robustness in vulval cell fate pattern generation in the presence of environmental variation, several studies show that underlying signalling pathways are environmentally sensitive. Ferguson and Horvitz (1985) initially reported that the penetrance of several loss-of-function mutations in genes of the EGF-Ras-MAPK signalling cascade *(lin-2, lin-3, lin-7, lin-24, lin-33* and *let-23)* that usually result in vulval hypoinduction was reduced after starvation or dauer passage (Ferguson and Horvitz, 1985). Braendle and Felix (2008) later quantitatively confirmed that the hypoinduced vulval phenotype (*Vulvaless*) of mutations in the EGF-Ras-MAPK (e.g. *lin-3*, *let-23*) was suppressed by starvation or dauer passage (Braendle and Félix, 2008). Using reporter genes they detected significant differences in the activity of signalling pathways in these different environments, particularly between standard and starvation conditions. In both *wild-type* and *lin-3/egf(rf)* mutant background they observed an upregulation of *egl-17::cfp* (EGF-Ras-MAPK reporter) expression in P6.p and an upregulation of *lip-1::gfp* (Delta-Notch reporter) expression in P5.p and P7.p under starvation conditions. These observations suggest that specific environmental conditions, such as starvation, may increase activities of vulval signalling pathways. Contrarily, compromising Wnt pathway activity through *bar-1/β-Catenin(null)* that causes mild hypoinduction in the standard environment was strongly aggravated in starvation conditions. This result indicated that Wnt activity for vulval induction could be particularly important in starvation conditions and also contribute to the observed starvation suppression of Vulvaless mutations. Thus, starvation may modulate multiple, partially redundant vulval signalling pathways (Figure 1.13).

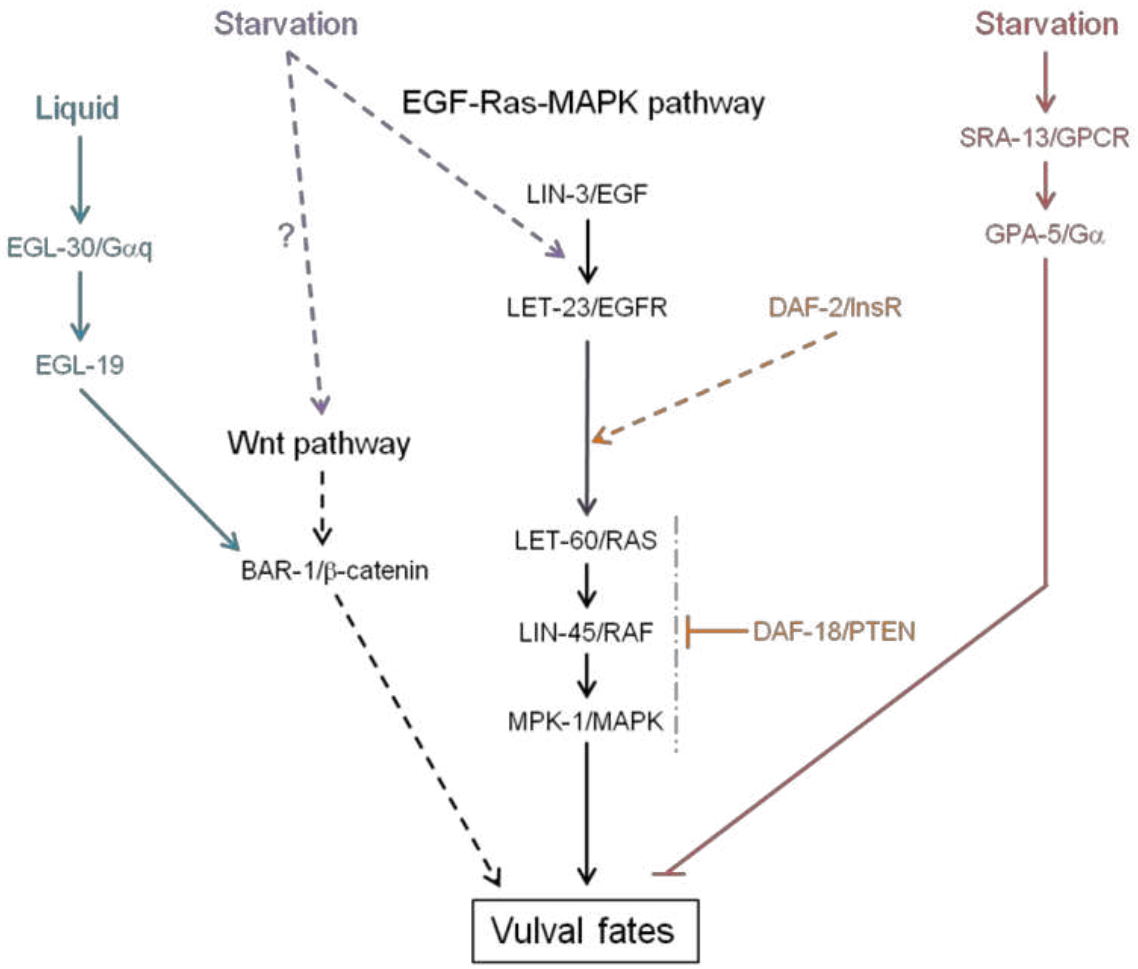

Figure 1.13. External and internal cues influencing *C. elegans* vulva development. Summary of studies reporting environmental and metabolic modification of *C. elegans* vulval induction. In purple: Braendle and Félix, 2008, in orange: Nakdimon et al., 2012, in blue: Moghal et al., 2003 and in red: Battu et al., 2003. See text.

Several additional studies have examined environmental modulation of vulval signalling pathways. Moghal et al. (2003) described liquid suppression of *Vulvaless* mutations (Moghal et al., 2003). They first showed a rare occurrence of ectopic vulval tissue in *egl-30(tg26gf)* (Doi and Iwasaki, 2002) suggesting that activated EGL-30/Gαq could promote vulval induction. They examined the effect of this overactivation in sensitized mutant backgrounds (e.g. *lin-3/egf*, *let-23/egfr* or *let-60/Ras*). The severity of these *Vulvaless* mutations was reduced. By driving *egl-30/Gαq* expression, they demonstrated that EGL-30/Gαq activity was required in neurons. This suppression of *Vulvaless* mutations was also shown to require muscle excitation through the L-type voltage gated calcium channel EGL-19, to act parallel or downstream of LET-60/Ras and to be sensitive to functional levels of

BAR-1/β-Catenin. In fact, a *bar-1(mu63)* weak mutation was able to block the suppression of *let-23(sy-1)* by *egl-30(tg26gf)*. This novel pathway capable of inducing vulval cells through excitable cells and muscles could be linked to the suppression of *Vulvaless* mutations that they observed in liquid environment, suggesting that EGL-30/Gαq, EGL-19/ L-type voltage gated calcium channel and BAR-1/β-Catenin are required for liquid stimulation of vulval induction (Figure 1.13).

Battu et al. (2003) further observed that starvation may negatively regulate the EGF-Ras-MAPK pathway through chemosensory signalling mediated by the GPCR SRA-13 (Battu et al., 2003). Starved *let-60(n1046gf)* animals exhibited a reduced penetrance of the *Multivulva* phenotype, and this effect was suppressed in *sra-13/gpcr(o)* animals as well as in other chemosensory defective mutants. Moreover, a recent study demonstrated that metabolic cues can directly influence vulva development. Further genetic analysis by Nakdimon et al. (2012) revealed that DAF-2/InsR stimulates whereas the DAF-18/PTEN inhibits EGF-Ras-MAPK pathway (Nakdimon et al., 2012). Interestingly the action of DAF-18/PTEN seems to be independent of the canonical Insulin signalling and other PI3K, such as *age-1*, *vps-34* or *piki-1*. DAF-18/PTEN has been shown to negatively regulate the EGF-Ras-MAPK pathway downstream of SOS-1/SOS1 and upstream or at the level of MPK-1/MAPK (Figure 1.13).

These studies show how an apparent robust developmental system, involving highly conserved signalling pathways, may exhibit substantial environmental flexibility. *C. elegans* vulval induction may be modulated by multiple distinct environmental factors affecting this process through different sensory and metabolic pathways, which ultimately target different signalling pathways (Figure 1.13). However, many questions remain to be addressed to fully understand how vulval development molecularly integrates environmental information and what the functional consequences of such environmental sensitivity are.

1.6. Objectives of PhD research project

How genetic and environmental factors interact during development is a key question in biology, yet little is known about how molecular and cellular processes integrate environmental information. *C. elegans* vulva development is an extensively studied and well-characterized process, which shows extensive environmental sensitivity. In my PhD project, I took advantage of this model system to study specific interactions between the environment and molecular signalling pathways. This project also had the objective to integrate evolutionary aspects of this system, ultimately aimed at understanding whether and how the observed environmental variability of molecular signals contribute to the phenotypic robustness of the system. The specific objectives of this project were:

(1) to understand how *C. elegans* modulates vulval induction in response to nutrient deprivation, i.e. starvation. Using primarily genetic analyses and quantitative phenotyping of vulval induction, I addressed how starvation signals are genetically transduced and how they modulate specific signalling activities.

(2) to quantify the evolution of environmental sensitivity of the *Caenorhabditis* vulval network. Using different *C. elegans* and *C. briggsae* strains, I characterized how strong thermal perturbations disrupt the precision of patterning process and compare these effects between strains and species.

1.7. Outline of PhD thesis

The present Chapter 1 introduces the motivation and rationale of my PhD project. I present some relevant key concepts on environmental sensitivity of development, and a more detailed discussion of known mechanisms of *C. elegans* responses to environmental variation, followed by a summary of current insights on the vulval developmental system. In Chapter 2, I present my analysis of how nutrient deprivation modulates activity of vulval signalling pathways, in particular, the EGF-Ras-MAPK pathway. In Chapter 3, I present results of a project that aimed to explore how the environmental sensitivity of the vulval developmental system evolves within and between *Caenorhabditis* species. In Chapter 4, I discuss and place my findings into a more general context and I outline ideas for future research projects.

Chapter 2

2. Nutrient deprivation modulates EGF-Ras-MAPK pathway activity during *C. elegans* vulval induction

2.1. Introduction

Animal development is inherently sensitive to environmental variation. Moreover, specific environmental conditions, such as nutrient availability or temperature, may reflect cues controlling growth and other critical developmental decisions, such as developmental timing, diapause entry and production of alternative phenotypes (Braendle et al., 2008). Developmental integration of environmental cues has become increasingly understood, primarily in the context of nutritional and metabolic regulation of growth and lifespan, e.g. in *Drosophila* (Geminard et al., 2006) and *C. elegans* (Fielenbach and Antebi, 2008). In contrast to the understanding of such instructive environmental cues in development, very little is known about whether and how environmental variation impacts other, highly diversified developmental processes, seemingly unaffected by the environment as they maintain their function and corresponding phenotypic outputs when facing such variation. However, such stability of the phenotypic output may go in hand with an underlying flexibility of developmental mechanisms in changing environmental (and genetic) contexts (Felix and Wagner, 2008; Greenspan, 2001).

C. elegans vulval development provides one clear example of a developmental process, which robustly generates an invariant phenotypic output (cell fate pattern) although activities and interactions of underlying molecular signalling pathways are environmentally sensitive (Braendle and Félix, 2008). *C. elegans* vulval cell fate patterning involves a network of highly conserved signalling pathways, EGF-Ras-MAPK, Delta-Notch and Wnt pathways that reliably establish a stereotypical cell fate pattern in hypodermal vulval precursor cells (VPCs) (Félix, 2012a; Sternberg, 2005) (Figure 2.1A). The VPCs represent a subset of Pn.p cells, P3.p to P8.p, competent to adopt vulval cell fates. Competence of VPCs is due to Wnt-regulated expression of the Hox gene *lin-39* preventing them from fusion with the surrounding hypodermis (Eisenmann et al., 1998). The key events of the vulval patterning process take place from mid L2 to early L3 stages and involve intercellular signalling between the gonadal anchor cell (AC) and the VPCs (Figure 2.1A,B).

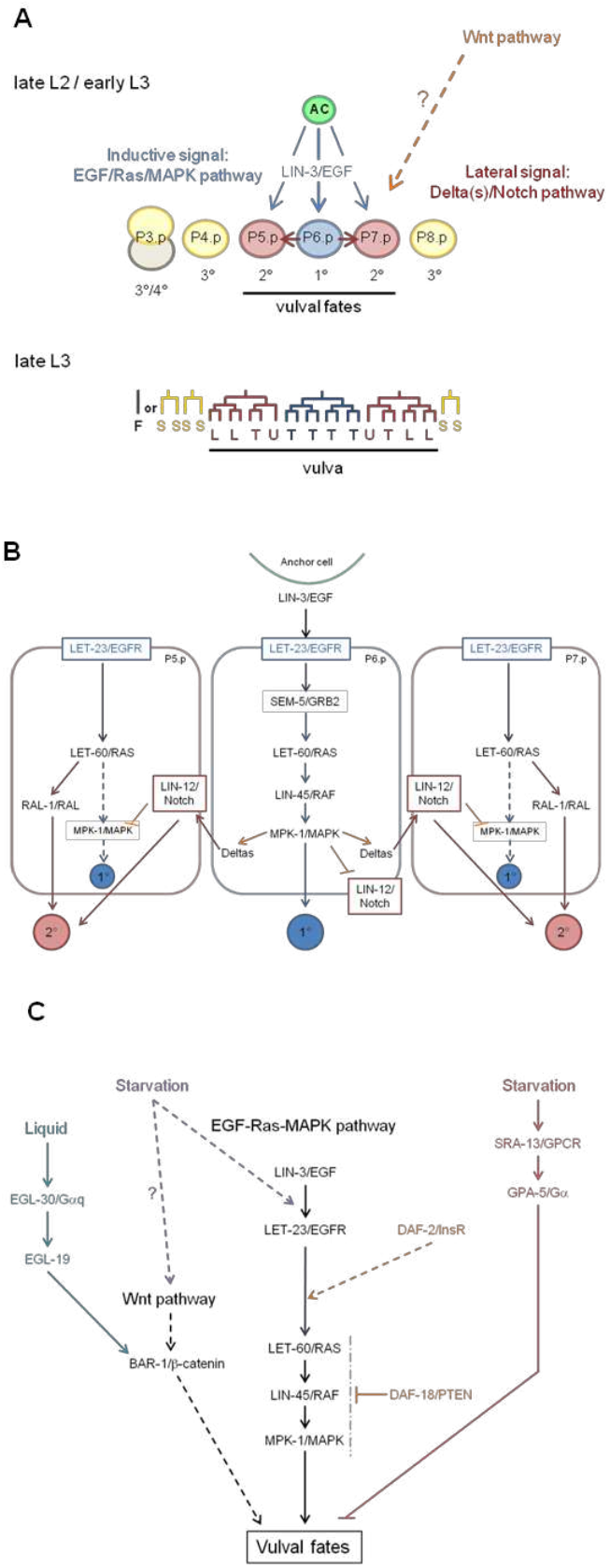

Figure 2.1. *C. elegans* vulval cell fate patterning. The *Caenorhabditis* vulva develops from a subset of set six ventral Pn.p cells, P3.p to P8.p. **(A)** Late L2 stage/early L3 stage: the anchor cell (AC) releases the morphogen-like LIN-3/EGF inductive signal. Detailed signalling in **(B)**. P6.p adopts a 1°

cell fate (blue) via EGFR-Ras-MAPK activation, which via secreted Delta ligands activates Delta-Notch signalling in its neighbours, P5.p and P7.p. They adopt a 2° vulval cell fate (red). The competent cells, P4.p and P8.p, adopt a non-vulval 3° fate (yellow), while the fate of P3.p fate varies among individuals, either adopting a 3° fate or a 4° fate, also referred to as F(used) fate (grey). In a total of six potential vulval precursor cells, only P5.p, P6.p and P7.p adopt actual vulval cell fates in a stereotypical 2°-1°-2° sequence. Vulval cell divisions occur during the L3 stage and generate 22 vulval cells. The fate assignments correspond to stereotypical cell division patterns that are invariant (with exception of P3.p). T: transverse (left-right) division, L: longitudinal (antero-posterior) division, U: undivided, SS: fusion to the epidermal syncytium (hyp7) after a single division (3° fate); F: fusion to the syncytium in the L2/L3 stage with no prior division (4° fate). **(B)** Vulval development-detailed signalling pathways: the anchor cell (AC) releases a LIN-3/EGF inductive signal. LIN-3/EGF acts as a morphogen and the receiving cells adopt different cell fates according to their location. P6.p receives the highest level of LIN-3/EGF causing it to adopt a 1° cell fate. The expression of the Delta ligands in P6.p activates the Delta-Notch pathway in its neighbours, P5.p and P7.p. This activation causes them to adopt a 2° fate (red) and represses the primary fate. A switch from the canonical LET-60/Ras-LIN-45/Raf pathway to a LET-60/Ras-RGL-1-RAL-1 signalling pathway can also promote the 2° cell fate in P5.p and P7.p. **(C)** Schematic representation of previously reported environmental and metabolic effects on *C. elegans* vulval induction (for details, see introduction). Colour coding: Braendle & Félix (2008) (purple), Battu et al. (2003) (red), Moghal et al. (2003) (blue), Nakdimon et al. (2012) (orange).

In brief, LIN-3/EGF ligand released from the AC induces the primary (1°) vulval cell fate by activating the EGF-Ras-MAPK pathway in P6.p, which receives the highest dose of this signal (Hill and Sternberg, 1992). EGF-Ras-MAPK activation induces production of a lateral signal via the Delta-Notch pathway, promoting 2° and inhibiting 1°cell fates in the neighbouring cells, P5.p and P7.p (Berset et al., 2001; Greenwald et al., 1983; Sternberg, 1988; Yoo et al., 2004). Moreover, it has been recently shown that a switch from the canonical LET-60/Ras-LIN-45/Raf pathway to a LET-60/Ras-RGL-1-RAL-1 signalling pathway can promote the 2° cell fate in P5.p and P7.p (Zand et al., 2011). The remaining three VPCs, although competent, adopt non-vulval cell fates (3° for P4.p and P8.p, and 3° or 4° for P3.p) as they do not receive sufficient doses of either signal. In addition to EGF-Ras-MAPK and Delta-Notch pathways, the canonical Wnt pathway, regulating vulval competence through expression of LIN-39/Hox5 in VPCs, may also be involved in vulval induction: overactivation of the Wnt pathway, e.g. through *pry-1*/*Axin* mutation, increases vulval induction of strong reduction-of-function mutations in the EGF-Ras-MAPK cascade (Braendle and Felix, 2008;

Gleason et al., 2002). *C. elegans* vulval development thus involves a regulatory network of three key molecular cascades and their cross-talk ensures a reliable and precise patterning output in the presence of both genetic and environmental perturbations (Braendle et al., 2010; Braendle and Felix, 2008; Félix and Barkoulas, 2012; Gleason et al., 2002; Hoyos et al., 2011; Milloz et al., 2008).

Despite the apparent robustness to environmental perturbations, multiple reports demonstrate that the *C. elegans* vulval cell fate patterning process is responsive to environmental and physiological inputs (Braendle and Felix, 2009; Braendle et al., 2008; Félix, 2012a; Félix and Barkoulas, 2012; Sternberg, 2005) (summarized in Figure 2.1C). Various *Vulvaless* mutations (*lin-2, lin-3, lin-7, lin-24, lin-33* and *let-23*) were found to be suppressed by starvation and/or dauer passage (Ferguson and Horvitz, 1985), which suggested that key signalling cascades, such as EGF-Ras-MAPK, may be environmentally sensitive. Detailed studies later confirmed that specific chemical elements, such as zinc (Bruinsma et al., 2002; Yoder et al., 2004), and growth conditions indeed modulate vulval inductive signalling: Moghal et al. (2003) reported that liquid culture increases vulval inductive signals via the neuronally expressed heterotrimeric Gαq protein, EGL-30. The EGL-30/Gαq signal is transduced via the voltage-gated calcium channel, EGL-19, in muscle cells and its positive effect on vulval induction is mediated by Wnt signalling via BAR-1/β-Catenin (Moghal et al., 2003). Battu et al. (2003) further found evidence for a starvation signal that negatively affects vulval induction via chemosensory perception. In this study, starvation conditions were found to suppress vulval hyperinduction, for example, induced by *let-60*/*Ras(gf).* This negative starvation effect requires the G-protein-coupled receptor, SRA-13, to modulate EGF-Ras-MAPK activity (Battu et al., 2003). Consistent with a sensory-mediated nutritional modulation of vulval signalling, compromised DAF-2 Insulin signalling mimics the negative starvation effects on vulval induction (Battu et al., 2003; Nakdimon et al., 2012). A recent study (Nakdimon et al., 2012) further indicates that DAF-18/PTEN negatively regulates EGF-Ras-MAPK activity during vulval induction, suggesting that key metabolic and sensory pathways can interact with vulval signalling pathways. Braendle & Félix (2008) examined the effects of diverse environmental conditions (different temperatures, starvation, liquid culture, dauer passage) on vulval induction using a large set of known mutations in EGF-Ras-MAPK, Delta-Notch and Wnt pathways. A majority of mutations showed differential penetrance depending on the environment, with starvation and dauer environments showing the most pronounced effects (Braendle and Félix, 2008). Consistent with the results of Ferguson & Horvitz (1985), yet contrary to the results obtained by Battu et al (2003), starvation increased

vulval inductive levels, leading to drastic suppression of the *Vulvaless* phenotypes caused by *lin-3/egf(rf)* and *let-23/egfr(rf)* mutations (Braendle and Félix, 2008). Genetic analysis suggests that this starvation signal acts at the level or upstream of LET-23EGFR (Braendle and Félix, 2008). That starvation positively affects vulval induction was confirmed by quantification of pathway activities in wild type animals, which indicates that EGF-Ras-MAPK pathway activity was significantly increased in the 1° cell P6.p whereas Delta-Notch activity was increased in 2° cells, P5.p and P7.p (Braendle and Félix, 2008). In addition, the same study found that this starvation effect potentially modulate vulval induction via the Wnt pathway as vulval induction was strongly compromised in starved *bar-1/β-Catenin(0)* animals.

In summary, previous results suggest that starvation signals may have both positive (Braendle and Félix, 2008; Ferguson and Horvitz, 1985) and negative (Battu et al., 2003) effects on vulval induction. While negative starvation effects have been shown to be mediated by the sensory system (Battu et al., 2003), it has not been studied how positive starvation effects are perceived and transduced to modulate vulval induction. In addition, how these apparently antagonistic starvation effects emerge and interact remains unclear.

Here we aimed to characterize in detail how starvation signals modulate *C. elegans* vulval cell fate patterning. In this study, we present quantitative analyses of starvation effects on *lin-3/egf(rf)* mutations, demonstrating that this environmental stimulus has a strong positive effect on vulval induction during the entire period of vulval induction, spanning from early L2 to early L3 stages. We show that such starvation suppression of *lin-3/egf(rf)* does not rely on Wnt signalling as proposed by Braendle & Felix (2008) and in contrast to the observed effects of liquid culture on vulval induction (Moghal et al., 2003). Testing various candidate mechanisms that could transduce and elicit observed starvation effects, we find that compromised sensory signalling of DAF-2 Insulin or DAF-7 TGF-β does not abolish *lin-3/egf(rf)* starvation suppression. Instead, nutrient-deprived animals induced by mutation of the intestinal peptide transporter *pept-1* (in a food-rich environment) strongly mimicked *lin-3/egf(rf)* starvation suppression, and we find that reduction of *pept-1* activity is sufficient to increase both EGF-Ras-MAPK and Delta-Notch pathway activities. These and additional experiments indicate that positive starvation effects on vulval induction occur via modulation of the central nutrient-sensing *let-363/TOR* pathway, acting at the level or upstream of LET-23/EGFR. Taken together, our results present evidence for a cross-talk between TOR and EGF-Ras-MAPK signalling during *C. elegans* vulval induction.

2.2. Material and Methods

2.2.1. Strains and general procedures

Strains were maintained on NGM agar plates (55mm petri dishes, 1.7% agar) carrying a lawn of *E. coli* OP50 (Brenner, 1974; Wood, 1988b). Animals were grown at 20°C unless indicated otherwise and both mutant and wild type strains were freshly thawed prior to experiments. Our analysis included the *C. elegans* N2 wild-type reference strain and the mutations listed below, all of which had been previously isolated and described.

LGI: *egl-30(ad805)*

LGII: *let-23(sy1)*

LGIII: *daf-7(e1372), mpk-1(ku1), daf-2(e1370), lin-39(n2110), zhIs4 [lip-1::GFP]*

LGIV: *lin-3(e1417), lin-3(n378), lin-45(sy96)*

LGV: *arIs92[egl-17p::NLS-CFP-lacZ, unc-4(+), ttx-3::GFP]*

LGX: *daf-12(rh61rh411), bar-1(mu63), bar-1(ga80), sem-5(n2019), pept-1(Ig601)*

2.2.2. Scoring of vulval phenotypes

The vulval phenotype was observed using Nomarski optics in early to mid L4 individuals, anaesthetized with sodium azide (Wood, 1988b). We counted the Pn.p progeny and determined their fates as previously described (Braendle and Felix, 2008; Sternberg and Horvitz, 1986).

2.2.3. Experimental environments

Experimental populations were age-synchronized by hypochlorite treatment and liquid arrest (24 hours) at the beginning of the experiment. Individuals examined in different environments or of different strains were always scored in parallel and derived from populations kept in identical environmental conditions over at least two generations.

Starvation treatment: L1 larvae were grown on standard NGM plates until they reached the mid L2 stage (23 hours after L1 transfer back on NGM seeded plates) unless mentioned otherwise. At this stage, animals were washed three times with sterile M9 buffer and transferred on starvation plates, i.e. unseeded NGM plates containing 1mg/ml of ampicillin to prevent bacterial growth. After 48 hours, animals were transferred back to

regular NGM plates seeded with *E. coli* OP50 and the vulval phenotype was scored when animals had reached the early or mid L4 stage (approximately 15-20 hours later). Control animals were kept on NGM plates seeded with *E. coli* OP50 from L1 to L4. This starvation treatment drastically reduced, yet did not completely stop, developmental progression of worms: after 48 hours most animals had developed into the early to mid-L3 individuals. Note that this starvation treatment did not induce dauer formation.

2.2.4. RNAi experiments

RNAi by bacterial feeding was performed as described by Timmons et al. (2001). The HT115 bacterial strain carrying the empty RNAi expression vector L4440 served as a negative control. We used RNAi plates composed of standard MGN with 50ug/ml of ampicillin and 1mM of IPTG. Late L2/ early L3 individuals were transferred on RNAi bacteria. The vulval phenotype was scored in the L4 of the F1 generation. To assess starvation response late L2/ early L3 individuals were maintained on RNAi plates for 5 generations prior to the starvation exposure. L4 individuals were transferred to fresh plates to avoid overgrowing by the next generation. All the RNAi clones were from Julie Ahringer's RNAi library (Kamath and Ahringer, 2003; Kamath et al., 2003) or Marc Vidal's RNAi library (Rual et al., 2004). The following RNAi clones were used: *pept-1* (clone K04E7.2, Ahringer Library), *daf-16* (clone R13H8.1, Ahringer Library), *daf-18* (clone T07A9.6, Ahringer Library), *aak-2* (clone T01C8.1, Ahringer Library) and *rsks-1* (clone Y47D3A.16, Vidal ORFeome Library).

2.2.5. Quantification of pept-1 RNAi effects on Ras and Notch pathway activities

To quantify EGF-Ras-MAPK and Delta-Notch pathway activities in response to *pept-1* RNAi we used previously generated transgenic strains containing integrated transcriptional reporter constructs: the JU480 strain carries the *egl-17::cfp-LacZ* transgene (EGF-Ras-MAPK activity reporter) derived from the strain GS3582 (Yoo et al., 2004) and the AH142 strain carries the *lip-1::gfp transgene* (Delta/Notch activity reporter) (Berset et al., 2001). Late L2/ early L3 individuals were randomly allocated to *pept-1* RNAi (K04E7.2 clone) or control (L4440 empty vector) plates. EGF-Ras-MAPK and Delta-Notch pathway activities were measured in lethargus 2 / early L3 of the F1 generation.

We quantified reporter gene activity in VPCs as previously described (Braendle and Félix, 2008). In brief, CFP or GFP signal quantification was performed when individuals had reached the stage of lethargus L2/L3 or early L3. Pn.p cells of live, anesthetized individuals were first identified using DIC imaging, followed by measurement of pixel signal intensity in P5.p, P6.p and P7.p for each individual. Images were acquired using an Olympus BX61 microscope at 40X magnification, equipped with a Coolsnap HQ2 camera. To quantify signal intensity of each cell, we first selected a fixed sub-region within nuclei of target VPCs and then measured the mean signal intensity of this region. After background subtraction, we used the mean signal intensity as a measure of the corresponding signalling pathway activity in each Pn.p.

2.2.6. Statistical Analyses

Data were transformed (e.g. Box-Cox- or log-transformed) where necessary to meet the assumptions of ANOVA procedures (homogeneity of variances and normal distributions of residuals) (Sokal and Rohlf, 1981). For post hoc comparisons, Tukey's honestly significant difference (HSD) procedure was used. Statistical tests were performed using the software programs JMP 9.0 or SPSS 19.0 for Macintosh.

2.3. Results

2.3.1. Starvation suppresses the *Vulvaless* phenotype of *lin-3/egf(rf)* mutations

We first re-examined the effect of L2 starvation on reduction-of-function alleles of *lin-3/egf*. The *Vulvaless* phenotypes caused by *lin-3(e1417)* and *lin-3(n378)* were strongly suppressed by starvation (Figure 2.2A,D), consistent with previous results (Braendle and Félix, 2008). Starvation resulted in increased mean number of induced vulval cells and a higher proportion of individuals with a canonical 2°-1°-2° cell fate pattern (P5.p to P7.p), and these suppression effects were consistently stronger in *lin-3(n378)* compared to *lin-3(e1417)* (Figure 2.2A-F, and data not shown). In *lin-3(n378)* individuals, the proportion of individuals with complete induction increased from 12% in food to 50% after starvation, and most of these individuals showed a correct 2°-1°-2° pattern for P5.p to P7.p (Figure 2.2E,F).

We next asked how 48h starvation exposure at different developmental time points affects suppression of *lin-3(n378)* (Figure 2.2G). Starvation exposure consistently increased

vulval induction from late L1/early L2 to early L3, indicating that starvation sensitivity extends over the entire period of the vulval cell fate patterning process. Exposing *lin-3(n378)* animals to different durations of starvation (0-120h) in the mid L2 stage shows similarly high suppression of the *Vulvaless* phenotype after 48-120h of starvation compared to an intermediate suppression after 19-24h of starvation, yet no suppression was observed after a brief (2h) exposure to starvation or early L1 starvation (12h) in liquid medium (Figure 2.2H).

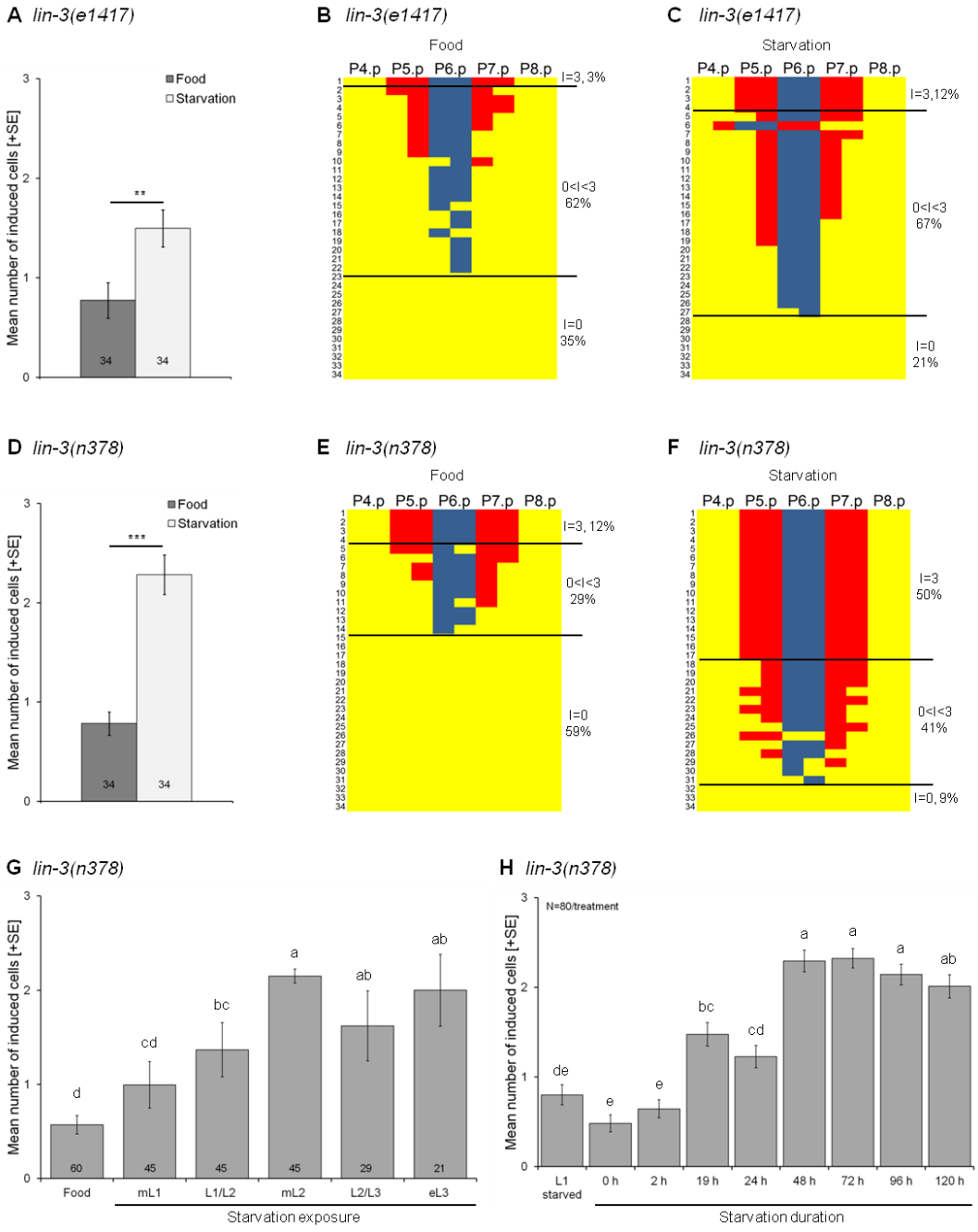

Figure 2.2. Starvation suppresses the *Vulvaless* phenotype caused by *lin-3/egf(rf)* mutations. (A-F) Starvation effects on vulval induction of *lin-3/egf(rf)* mutations. Bars indicate the mean number of

induced vulval cells, also referred to as the vulval index (WT=3 cells induced). **(A)** Starvation significantly increased vulval induction of *lin-3(e1417)* animals relative to control food conditions (ANOVA, $F_{1,66} = 9.97$, $P = 0.0024$). **(B, C)** Schematic representation of *lin-3(e1417)* fate patterns of P4.p to P8.p in **(B)** food versus **(C)** starvation conditions. **(D)** Starvation significantly increased vulval induction of *lin-3(n378)* animals relative to controls (ANOVA, $F_{1,66} = 33.75$, $P < 0.0001$). **(E, F)** Individual *lin-3(n378)* fate patterns of P4.p to P8.p in **(G)** food versus **(H)** starvation conditions. **(G)** Differences in vulval induction of *lin-3(n378)* animals exposed to starvation (48 hours) at different developmental stages. Starvation suppression of *lin-3(n378)* differed significantly between exposed developmental stages (ANOVA, $F_{5,239} = 14.02$, $P < 0.0001$), with strongest effects observed in mid L2 to early L3 stages. Values with different letters indicate significant differences (Tukey's HSD). **(H)** Effects of starvation duration on vulval induction of *lin-3(n378)*. Animals (derived from egg-laying windows) were exposed to starvation at the mid L2 stage for different time periods except for the first treatment where animals were starved in liquid for 12 hours directly after hatching (N=80 per treatment). Starvation duration significantly affected vulval induction (ANOVA, $F_{5,239} = 14.02$, $P < 0.0001$) with strongest suppression of *lin-3(n378)* occurring at 48-120 hours of starvation. Values with different letters indicate significant differences (Tukey's HSD).

Vulval cell fate patterns of P4.p to P8.p **(B,C,E,F)** were, whenever feasible, separately inferred for Pn.pa and Pn.pp in cases of half-induced fates. Each line represents the vulval pattern of a single individual, and individuals are ordered from highest to lowest index (I) of vulval induction. Black lines separate individuals with complete vulval induction (I=3), partial induction ($0 < I < 3$) and no induction (I=0). Colour coding of vulval cell fates (1°: blue, 2°: red) and non-vulval cell fates (3°: yellow, 4°: grey). Non-induced cells that could not be clearly assigned a 3° or 4° fate are coded in white.

Numbers displayed in bars represent the number of individuals scored; error bars indicate ± 1 SEM.

2.3.2. Wnt pathway activity does not contribute to starvation suppression of *lin-3/egf(rf)* mutations

Previous results suggested that the Wnt pathway may contribute to the suppression of *lin-3/egf(rf)* alleles under starvation conditions (Braendle and Félix, 2008), consistent with the observation that Wnt pathway overactivation may compensate for reduced EGF-Ras-MAPK activity (Gleason et al., 2002). Hypoinduction of the null mutant of *bar-1*/*β-Catenin* (allele *ga80*) (Eisenmann et al., 1998) was strongly aggravated under starvation conditions, and the *Vulvaless* phenotype of *lin-3(n378); bar-1(ga80)* double mutant did not show any suppression by starvation (Braendle and Félix, 2008). These results implied that Wnt pathway activity is

required for starvation suppression of *lin-3/egf(rf)*; however, it remained unclear to what extent starvation compromised vulval induction versus competence in *bar-1(ga80)* because in this study animals were exposed to starvation in the late L1 stage (Braendle and Félix, 2008). To distinguish between these two different scenarios, we exposed *bar-1(ga80)* animals to starvation in late L1 versus mid L2 stage and found that mid L2 starvation did not alter vulval induction whereas late L1 starvation caused strong hypoinduction (Figure 3A), primarily due to Pn.p fusion, i.e. loss of competence of vulval precursor cells, including P5.p to P7.p (Figure 3B-E).

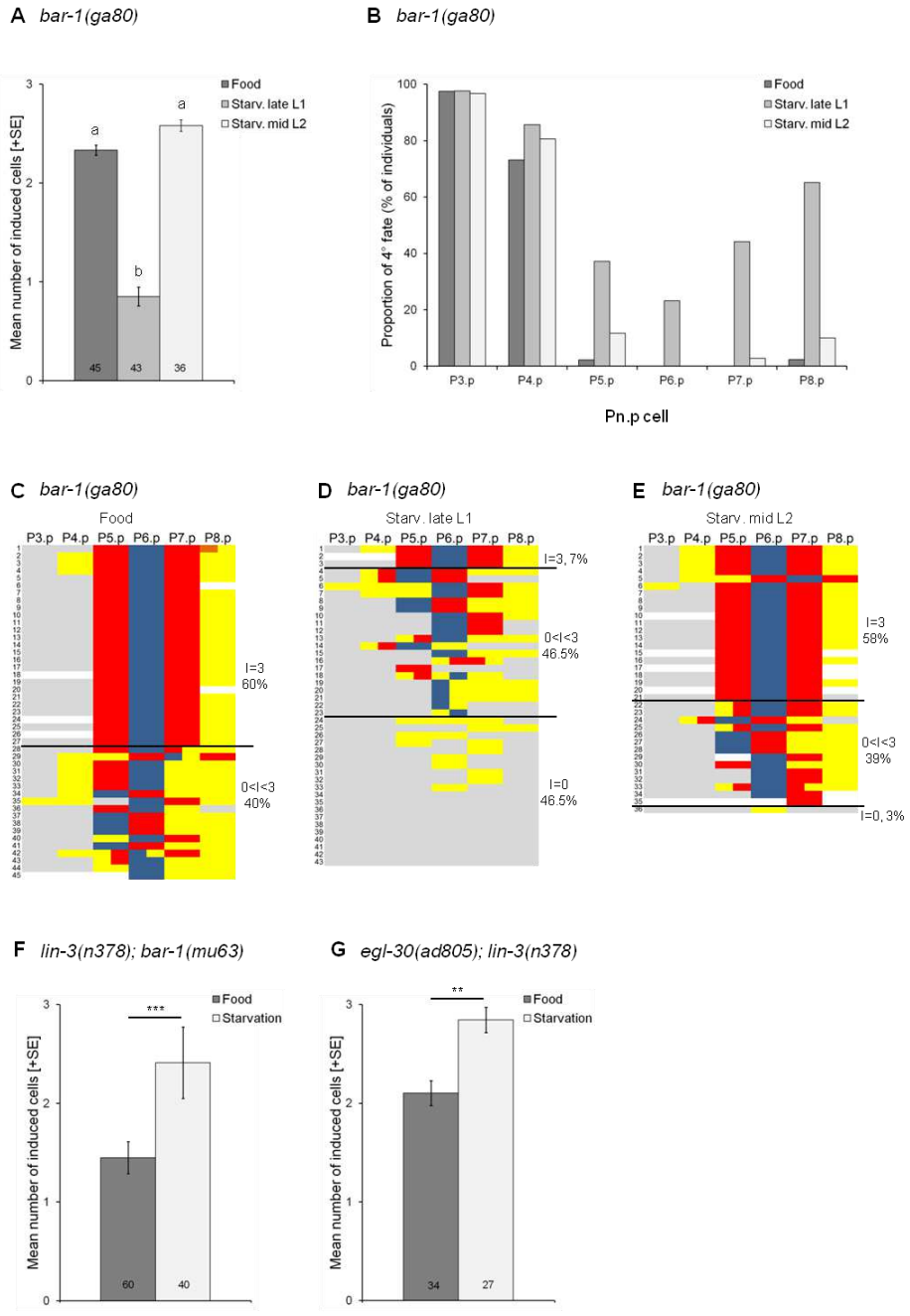

Figure 2.3. Wnt pathway activity does not contribute to starvation suppression of *lin-3/egf(rf)* mutations. (A-E) Starvation effects on vulval induction of *bar-1(ga80)* in late L1 versus mid L2. **(A)**

Time point of starvation exposure had a significant effect on *bar-1(ga80)* vulval induction (ANOVA, $F_{2,121} = 50.27$, $P < 0.0001$), with a significantly reduced vulval index for starvation-exposed late L1 individuals whereas mid L2 starvation had no effect on vulval induction. Bars indicate the mean number of induced vulval cells (WT=3 cells induced). **(B)** Proportion of Pn.p adopting 4° (fused) fate in control, starvation-exposed late L1 versus starvation-exposed mid L2 *bar-1(ga80)* individuals. The proportions of 4° fates for each of P5.p to P7.p were significantly higher after L1 starvation (P5.p: 37%, P6.p: 23%, P7.p: 44%) compared to L2 starvation (P5.p: 12%, P6.p: 0%, P7.p: 3%). **(C-E)** Individual *bar-1(ga80)* fate patterns of P4.p to P8.p in **(C)** food (control) conditions, **(D)** after late L1 starvation and **(E)** after mid L2 starvation. **(F)** Starvation increased vulval induction of *lin-3(n378); bar-1(mu63)* relative to food (control) conditions (ANOVA, $F_{1,98} = 18.84$, $P < 0.0001$). **(G)** Starvation increased vulval induction of *egl-30(ad805); lin-3(n378)* relative to food (control) conditions (ANOVA, $F_{1,59} = 7.28$, $P = 0.0091$).

Vulval cell fate patterns of P4.p to P8.p **(C-E)** were, whenever feasible, separately inferred for Pn.pa and Pn.pp in cases of half-induced fates. Each line represents the vulval pattern of a single individual, and individuals are ordered from highest to lowest index (I) of vulval induction. Black lines separate individuals with WT vulval induction (I=3), partial induction ($0 < I < 3$) and no induction (I=0). Colour coding of vulval cell fates (1°: blue, 2°: red) and non-vulval cell fates (3°: yellow, 4°: grey). Non-induced cells that could not be clearly assigned a 3° or 4° lineage are coded in white.
Numbers displayed in bars represent the number of individuals scored; error bars indicate ± 1 SEM.

This result shows that late L1 starvation aggravates *bar-1(ga80)* fusion defects prior to vulval induction, yet L2 starvation, i.e. the starvation-sensitive time period of *lin-3/egf(rf)* mutations, does not alter inductive levels of this mutant. We conclude that starvation has no effect on vulval induction when Wnt pathway activity is compromised, and that starvation suppression of *lin-3/egf(rf)* is not mediated by the Wnt pathway.

A previous study showed that liquid culture of animals suppressed the *Vulvaless* phenotype of *lin-3/egf(rf)* and other EGF-Ras-MAPK mutations (Moghal et al., 2003) through the Wnt pathway. This environmental modulation is based on activation of the heterotrimeric Gαq protein, EGL-30, which acts with muscle-expressed EGL-19 to promote vulval induction downstream or in parallel to LET-60/Ras in a Wnt-dependent manner (Moghal et al., 2003). This liquid effect on vulval induction is abolished when Wnt pathway is mildly compromised (Moghal et al., 2003), i.e. in a *bar-1(mu63)* context (Maloof et al., 1999). In contrast, we found that starvation suppression of *lin-3(n378)* did not change in a *bar-1(mu63)* background (Figure 3F), consistent with our observations of *bar-1/β-Catenin(0)* (Figure 3A). Furthermore, loss of *egl-30/Gαq* (Brundage et al., 1996) did not alter starvation suppression of *lin-3(n378)*

(Figure 3G). Therefore, the positive starvation effects on vulval induction are not Wnt-dependent and seem to be distinct from previously observed environmental effects (liquid) promoting vulval induction (Moghal et al., 2003).

2.3.3. Starvation suppression of *lin-3/egf(rf)* acts independently of sensory signalling mediated by Insulin and TGF-β pathways

To address how starvation conditions are transduced to affect vulval induction, we tested for an implication of TGF-β and Insulin signalling: two key signalling pathways involved in sensory transduction of environmental stimuli, and which specifically mediate diverse developmental and metabolic responses to changes in nutritional status, such as dauer formation (Fielenbach and Antebi, 2008). Reducing TGF-β pathway activity, did not abolish starvation suppression as shown by the double mutant *daf-7(e1372); lin-3(e1417)* (Figure 2.4A). Similarly, reduced activity of the DAF-2 Insulin receptor caused by the *daf-2(e1370)* mutation did not abolish starvation suppression of *lin-3(n378)* (Figure 2.4B). However, *daf-2(e1370)* aggravated the *Vulvaless* phenotype of *lin-3(n378)* in food conditions, consistent with previous observations that reduced Insulin signalling may reduce levels of vulval induction in sensitized backgrounds, such as *let-60/Ras(gf)* (Battu et al., 2003; Nakdimon et al., 2012). *daf-2(e1370)* reduced the degree of *lin-3(n378)* vulval induction very similarly in both food and starvation environment, indicating that DAF-2 Insulin and positive starvation effects on vulval induction behave additively, i.e. these effects act in parallel.

To confirm that TGF-β and Insulin signalling do not mediate positive starvation effects on vulval induction, we further examined the role of a central genetic component, the DAF-12 steroid receptor, integrating downstream effects of these two signals (Fielenbach and Antebi, 2008) using the *daf-d* mutant, *daf-12(rh61rh411)* (Antebi et al., 2000). Consistent with our previous results, *lin-3(n378); daf-12(rh61rh411)* did not abolish starvation suppression of the *Vulvaless* phenotype (Figure 2.4C).

Taken together, these results indicate that Insulin and TGF-β pathways, and thus by implication, associated environmental signal transduction via these pathways, do not play a significant role in positive starvation effects on vulval induction. Yet, we confirm previous observations (Battu et al., 2003; Nakdimon et al., 2012) that reduced DAF-2 Insulin activity lowers vulval induction in sensitized backgrounds, such as *lin-3/egf(rf)* in both food and starvation conditions. Nevertheless, we still observed strong, albeit slightly reduced, starvation suppression of the *Vulvaless* phenotype in *daf-2(e1370); lin-3(n378)*, suggesting

that the negative starvation signal is significantly weaker than the positive starvation signal. Therefore, while Insulin signalling and sensory perception seem to mediate a mild negative starvation signal (Battu et al., 2003; Nakdimon et al., 2012), we provide evidence for an additional, strong positive starvation signal acting independently of Insulin and sensory signalling. Consequently, starvation triggers multiple signalling events acting antagonistically on *C. elegans* vulval induction.

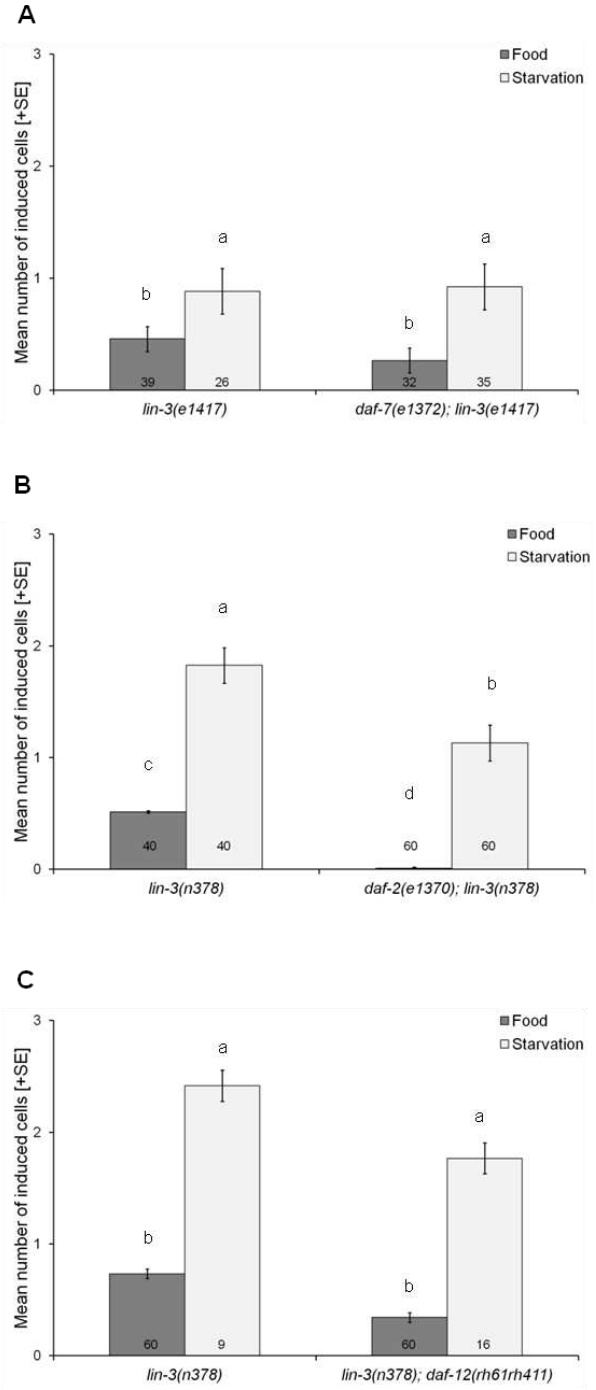

Figure 2.4. Starvation suppression of *lin-3/egf(rf)* acts independently of sensory signalling mediated by Insulin and TGF-β pathways. (A) Starvation effects on *daf-7(e1372); lin-3(ne1417)*

versus *lin-3(e1417).* Starvation increased vulval induction irrespective of genotype (ANOVA, effect *genotype*: $F_{1,128} = 0.76$, P = 0.38, effect *environment*: $F_{1,128} = 15.41$, P < 0.0001, interaction *genotype* X *environment*: $F_{1,128} = 0.66$, P = 0.42). **(B)** Starvation effects on *daf-2(e1370); lin-3(n378)* versus *lin-3(n378). daf-2(e1370)* significantly reduced vulval induction in both control and starvation environment, yet starvation still significantly increased vulval induction of *lin-3(n378)* and *daf-2(e1370); lin-3(n378)* (ANOVA, effect *genotype*: $F_{1,196} = 32.87$, P < 0.0001, effect *environment*: $F_{1,133} = 98.25$, P < 0.0001, interaction *genotype* X *environment*: $F_{1,196} = 0.10$, P = 0.75). **(C)** Starvation effects on *lin-3(n378); daf-12(rh61rh411)* versus *lin-3(n378).* Starvation increased vulval induction irrespective of genotype (ANOVA, effect *genotype*: $F_{1,141} = 3.29$, P = 0.07, effect *environment*: $F_{1,141} = 38.23$, P < 0.0001, interaction *genotype* X *environment*: $F_{1,141} = 0.14$, P = 0.70)

Values with different letters indicate significant differences (Tukey's HSD). Numbers displayed in bars represent the number of individuals scored; error bars indicate ± 1 SEM.

2.3.4. Disruption of the intestinal peptide transporter *pept-1* mimics starvation suppression of *lin-3/egf(rf)* mutations

We next tested how disruption of central genetic elements integrating *C. elegans* nutritional and metabolic responses (*aak-2/AMPK, daf-16/FoxO, daf-18/PTEN*) (Lapierre and Hansen, 2012)) modulates starvation suppression of *lin-3/egf(rf)* using RNAi assays. RNAi knock-down of these three major metabolic genes did not alter vulval induction of *lin-3(n378)* in food or starvation conditions (Figure 2.5A), arguing against an implication of associated pathways in *lin-3/egf(rf)* starvation suppression.

Testing for an implication of LET-363/TOR (target of rapamycin) signalling, a key pathway in nutritional sensing (Hietakangas and Cohen, 2009; Jia et al., 2004), we observed that knock-down of *rsks-1*/*S6K* (Pan et al., 2007) significantly increased the vulval index of *lin-3(n378)* animals in food conditions (Figure 2.5B). Moreover, RNAi knockdown of *pept-1/*, an intestinal oligopeptide transporter known to interact with the TOR pathway (Meissner et al., 2004), suppressed the *Vulvaless* of *lin-3(n378)* even more strongly (Figure 2.5B). RNAi of *rsks-1/S6K and pept-1* in food conditions thus mimicked starvation suppression of *lin-3/egf(rf),* suggesting that TOR nutrient sensing mechanisms are involved in the starvation modulation of vulval induction.

To consolidate these results, we constructed double mutants of *pept-1(lg601)* (Meissner et al., 2004) with *lin-3(n378)* and *lin-3(e1417)*, respectively. Assaying these mutant combinations revealed extreme suppression of the Vulvaless phenotype caused by either *lin-*

3/egf allele (Figure 2.5C,D). The suppression was very strong in *lin-3(n378)* where mean vulval induction approached wild type levels (2.93 ± 0.03 cells induced) and a majority of individuals adopted a correctly vulval patterning sequence of 2°-1°-2° cell fates for P5.p to P7.p (Figure 2.5C). (Note, however, that *pept-1(lg601)* single mutants never showed hyperinduction or any other frequent vulval defects (N>300)).

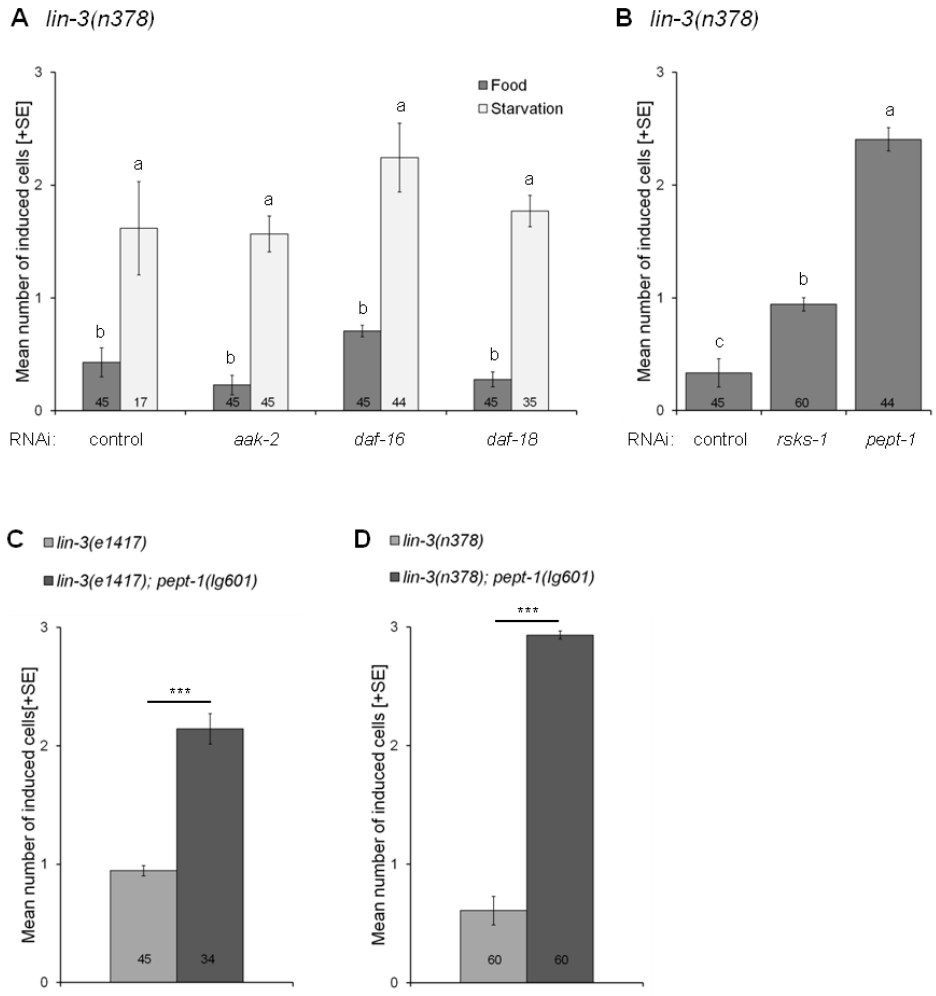

Figure 2.5. Disruption of the intestinal peptide transporter PEPT-1 mimics starvation suppression of *lin-3/egf(rf)* mutations. (A) Starvation effects on *lin-3(n378)* animals treated with *aak-2/AMPK, daf-16/FoxO, daf-18/PTEN* RNAi feeding versus controls (empty vector strain, *E. coli* HT115). Starvation consistently increased vulval induction of *lin-3(n378)* irrespective of RNAi treatment (ANOVA, effect *environment*: $F_{1,313} = 126.13$, $P < 0.0001$, effect *RNAi treatment*: $F_{1,313} = 4.46$, $P = 0.0044$, interaction *environment* X *RNAi treatment*: $F_{1,313} = 0.23$, $P = 0.88$). **(B)** Effects of *rsks-1/S6K* and *pept-1* RNAi versus control RNAi (empty vector strain, *E. coli* HT115) in *lin-3(n378)* (food conditions). RNAi knock-down of *rsks-1/S6K* and *pept-1* significantly increased vulval induction (ANOVA, $F_{2,146} = 59.90$, $P < 0.0001$). **(C)** *pept-1(lg601)* strongly increased vulval induction

of *lin-3(e1417)* animals (ANOVA, $F_{1,77} = 30.10$, $P < 0.0001$). **(D)** *pept-1(lg601)* strongly increased vulval induction of *lin-3(n378)* animals (ANOVA, $F_{1,118} = 334.65$, $P < 0.0001$).

Values with different letters indicate significant differences (Tukey's HSD). Numbers displayed in bars represent the number of individuals scored; error bars indicate ± 1 SEM.

Compromised *pept-1* activity leads to physiologically starved animals in a food-rich environment (Meissner et al., 2004) and our results indicate that such internal perception of starvation status is sufficient to strongly suppress *lin-3/egf(rf)* mutations. Given that reduced amino acid availability caused by *pept-1(lg601)* primarily acts through TOR signalling (Meissner et al., 2004) and that *rsks-1* RNAi similarly mimics starvation effects, we conclude that the observed starvation modulation of vulval induction occurs via the TOR nutrient sensing pathway.

2.3.5. *pept-1* RNAi increases EGF-Ras-MAPK and Delta-Notch pathway activities

Starvation conditions have previously been shown to increase EGF-Ras-MAPK activity in the 1° cell, P6.p, and Delta-Notch activity in the 2° cells, P5.p and P7.p of the wild type strain N2 (Braendle and Félix, 2008). We therefore tested whether *pept-1* RNAi mimicked these starvation effects using the same reporter genes, i.e. *egl-17::cfp* to quantify EGF-Ras-MAPK pathway activity (Yoo et al., 2004) and *lip-1::gfp* to quantify Delta-Notch activity (Berset et al., 2001) (Figure 2.6A). *pept-1* RNAi effects closely mirrored previously quantified starvation effects on report gene activities (Braendle and Félix, 2008), with *pept-1* RNAi increasing EGF-Ras-MAPK pathway activity in P6.p and increasing Delta-Notch pathway activity in P5.p and P7.p (Figure 2.6B,C).

Consistent with these changes, we also observed that *pept-1/* RNAi reduced Delta-Notch activity in P6.p while reducing EGF-Ras-MAPK activity in P5.p and P7.p (Figure 2.6B,C). These results show that *pept-1* RNAi tightly recapitulates previously observed starvation effects on the two key inductive vulval signalling pathways (Braendle and Félix, 2008). In both experiments, changes in pathway activities may be explained by distinct effects on EGF-Ras-MAPK and Delta-Notch pathways, or by effects mediate solely by the EGF-Ras-MAPK pathway, given the cross-talk between the two pathways (Yoo et al., 2004). However, observed starvation and *pept-1* RNAi suppression of *lin-3/egf(rf)* do not indicate obviously distinct effects on Delta-Notch activity.

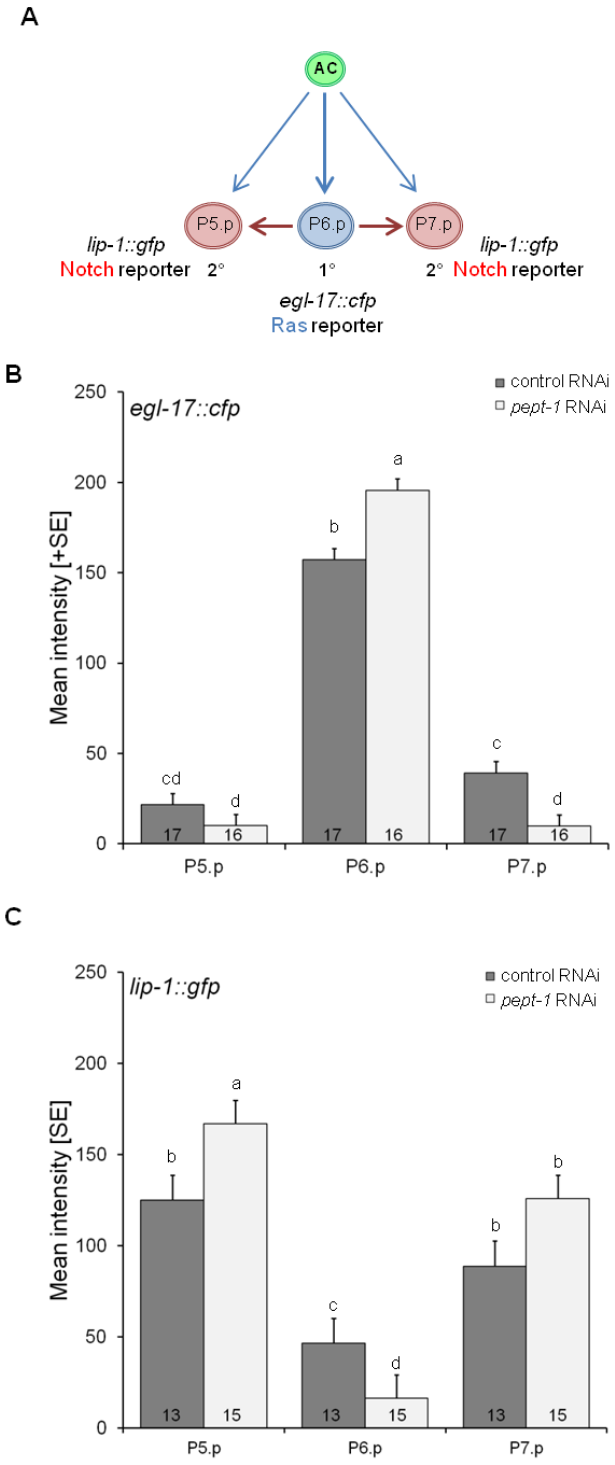

Figure 2.6. *pept-1* RNAi increases EGF-Ras-MAPK and Delta-Notch pathway activities. Effects of *pept-1* RNAi on transcriptional reporter activity *egl-17::cfp* (EGF-Ras-MAPK activity) (Yoo et al., 2004) and *lip-1::gfp* (Delta/Notch activity) (Berset et al., 2001) quantified in lethargus L2/L3 and early L3 stages. **(A)** Schematic overview of intercellular EGF-Ras-MAPK (red) and Delta-Notch (blue) signalling and corresponding downstream transcriptional targets assayed via the two fluorescent

reporters in P5.p to P7.p. **(B)** Mean signal (pixel) intensity of the EGF-Ras-MAPK pathway reporter, *egl-17::cfp* in P5.p to P7.p. Values indicate Least Square Means for the interaction *cell* x *RNAi treatment* ($F_{2,62}$ = 15.52 , P < 0.0001) (Table 2.1A). **(C)** Mean signal (pixel) intensity of the Delta-Notch pathway reporter, *lip-1::GFP* in P5.p to P7.p. Values indicate Least Square Means for the interaction *cell* x *RNAi treatment* ($F_{2,52}$ = 13.73 , P < 0.0001) (Table 2.1B).

For complete statistical analysis and results, see Table 1. Values with different letters indicate significant differences (Tukey's HSD). Numbers displayed in bars represent the number of individuals scored; error bars indicate ± 1 SEM.

Table 2.1. Effects of *pept-1* RNAi on EGF-Ras-MAPK reporter *egl-17::cfp* (A) and Delta-Notch reporter *lip-1::gfp* (B). Results of statistical tests for transcriptional reporter assays (Figure 6). We used an ANOVA testing for the fixed effects of *RNAi treatment*, *Individual* (nested in *RNAi treatment*), *Pn.p cell* and the interaction between *RNAi treatment* and *Pn.p cell* using mean signal intensity as a response variable. Including the effect *Individual* (*RNAi treatment*), allowed controlling for the non-independence of signal intensity in P5.p, P6.p, and P7.p measured in a given individual. Data was long-transformed prior to analysis.

A) *egl-17::cfp*

Source	DF	Sum of Squares	F Ratio	P
RNAi Treatment	1	26.56	0.04	0.8413
Individual (RNAi Treatment)	31	2792.82	0.14	1.0000
Pn.p cell	2	538247.46	409.63	< 0.0001
Pn.p cell x RNAi Treatment	2	20392.73	15.52	< 0.0001
Error	62	40733.51		

B) *lip-1::gfp*

Source	DF	Sum of Squares	F Ratio	P
RNAi Treatment	1	456.59	0.34	0.5607
Individual (RNAi Treatment)	26	24120.94	0.70	0.8403
Pn.p cell	2	200764.78	75.39	< 0.0001
Pn.p cell x RNAi Treatment	2	36553.06	13.73	< 0.0001
Error	52	69237.88		

2.3.6. Nutrient deprivation induced by *pept-1* RNAi modulates vulval induction at the level of EGF/EGFR

To characterize how genetically-induced nutrient deprivation interacts with vulval signalling pathways to modulate inductive levels, we characterized effects of *pept-1* RNAi on several components of the core EGF-Ras-MAPK cascade using corresponding hypoinduced mutants (Table 2.2). In addition to *lin-3/egf(rf)* mutations, *pept-1* RNAi suppressed, to different degrees, the *Vulvaless* phenotypes of *let-23(sy1)*/*egfr, sem-5(n2019)*/*grb2* and *mpk-1(ku1)/mapk* (Table 2.2). Although these results suggest that nutrient deprivation due to *pept-1* RNAi may act downstream of MAPK, interpretation is limited given that these mutations do not represent null alleles (most of the null alleles are lethal). We therefore used a double mutant *let-23(sy1); lin-3(n378)* that exhibits a fully penetrant *Vulvaless* phenotype, indicating absent or very low levels of basal EGF-Ras-MAPK activity (Braendle and Félix, 2008). Vulval induction of *let-23(sy1); lin-3(n378)* animals treated with *pept-1* RNAi remained virtually unaltered compared to controls (Table 2.2), with only 4/60 individuals showing some induced vulval cells (versus 0/45 in food conditions). Nutrient deprivation induced by *pept-1* RNAi thus interacts with vulval signalling upstream or at the level of LET-23/EGFR, consistent with previous analysis of starvation effects, which did not suppress *let-23(sy1); lin-3(n378)* (Braendle and Félix, 2008).

Table 2.2. Effects of *pept-1* RNAi treatment on vulval induction of different mutants of the EGF-Ras-MAPK signalling cascade. Statistical tests (ANOVAs) were performed for each mutation separately (* P < 0.05, ** P < 0.01, *** P < 0.0001, ns: non-significant).

Genotype	Treatment	Induced cells (Mean ± SE)	N
lin-3(e1417)	Control	0.46 ± 0.13	60
	pept-1 RNAi	1.16 ± 0.09 ***	60
lin-3(n378)	Control	0.27 ± 0.05	60
	pept-1 RNAi	2.26 ± 0.17 ***	60
let-23(sy1)	Control	0.12 ± 0.08	48
	pept-1 RNAi	1.36 ± 0.17 ***	60
let-23(sy1);lin-3(n378)	Control	0.00 ± 0.00	45
	pept-1 RNAi	0.09 ± 0.06 ^{ns}	60
sem-5(n2019)	Control	0.81 ± 0.15	48
	pept-1 RNAi	1.60 ± 0.12 ***	55
mpk-1(ku1)	Control	2.69 ± 0.05	45
	pept-1 RNAi	2.99 ± 0.01 *	45

2.4. Discussion

Quantification of starvation effects on *C. elegans* vulval induction using mutants and reporter gene analysis allowed us to clarify how environmental inputs modulate *C. elegans* vulval signalling pathways. Specifically, we found that starvation during the time period of the vulval patterning process consistently increases levels of vulval induction. We present evidence that these positive starvation effects on vulval induction do not require Wnt pathway activity as previously proposed (Braendle and Félix, 2008) and are therefore distinct from

similar vulval-promoting effects observed in liquid culture, which were shown to be Wnt-dependent (Moghal et al., 2003).

2.4.1. Antagonistic starvation signals modulate *C. elegans* vulval induction

Our results partly resolve seemingly contradictory previous results reporting both positive (Braendle and Felix, 2008; Ferguson and Horvitz, 1985; Sternberg and Horvitz, 1986) and negative effects (Battu et al., 2003) of starvation on vulval induction. We confirm here that starvation results in a strong and consistent increase in vulval induction, e.g. illustrated by the strong starvation suppression of *lin-3/egf(rf)* mutations (Figure 2.2). We show that this positive starvation effect acts independently of sensory signalling mediated by Insulin and TGF-β signalling and, as suggested by Braendle & Félix (2008), acts the level or upstream of LET-23/EGFR. In contrast, the negative starvation signal reported by Battu et al. (2003) acts via chemosensory perception to affect EGF-Ras-MAPK activity. This study used sensitized backgrounds causing vulval hyperinduction (*Multivulva*, *Muv* phenotype), such as a gain-of-function mutation in *let-60/Ras*, to assess starvation effects: starvation decreased vulval induction in strains with overactivated LET-60/Ras and MPK-1/MAPK signalling, yet had no effect on strains producing excessive LIN-3/EGF or LET-23/EGFR (Battu et al., 2003). These results were interpreted to suggest that overstimulation of EGF/EGFR may overcome the negative starvation signal, however, it remained ambiguous at which level of the EGF-Ras-MAPK cascade the signal may integrate. The negative starvation effects on strains with excessive LET-60/Ras or MPK-1/MAPK activation were abolished in chemosensory-defective mutants (*osm-5, che-3, sra-13*), implying starvation signal transduction via the sensory system (Battu et al., 2003). Moreover, *daf-2/InsR(rf)* suppression of the *Multivulva* phenotype caused by *let-60/Ras(gf)* in food conditions was interpreted to suggest mimicking (of observed) starvation effects via reduced Insulin signalling (Battu et al., 2003), but it was not tested how vulval induction of *daf-2(rf); let-60(gf)* responds to starvation. Our results reporting an overall positive effect of starvation on vulval induction suggest that sensory perception is not required for this effect to occur. Importantly, however, and in line with the results by Battu et al. (2003), we also found that a compromised DAF-2 Insulin activity reduces vulval induction in *lin-3/egf(rf)* to similar extents in both food and starvation conditions, yet without abolishing starvation suppression of *lin-3/egf(rf).* Taken together, these different results suggest that starvation has both positive and negative effects on *C. elegans* vulval induction, with a positive starvation signal acting independently of sensory

signalling, likely mediated by internal metabolic regulation (this study), and a sensory-system-mediated negative starvation signal, likely mediated by DAF-2 Insulin signalling (this study; Battu et al. 2003). Despite the presence of two antagonistic starvation signals acting in parallel, there is a strong net positive starvation effect, suggesting that negative starvation effects are significantly weaker.

2.4.2. Starvation promotes EGF-Ras-MAPK activity via the TOR pathway during *C. elegans* vulval induction

We find that genetic disruption of the low affinity/high capacity oligopeptide transporter *pept-1* (Meissner et al., 2004) in a food-rich environment strongly mimics environmental starvation effects on vulval induction. This finding supports the above argument that sensory perception of the external environment is not required for positive starvation effects on vulval induction. Rather, internal perception of nutrient deprivation induced seems sufficient to exert this effect. PEPT-1 is expressed in the intestine, localized to apical membranes, and is responsible for the uptake of intestinal peptides: genetic disruption of *pept-1* therefore causes amino acid-deprivation, leading to significantly reduced growth rates and reproduction (Benner et al., 2011; Meissner et al., 2004; Spanier et al., 2009; Spanier et al., 2010). RNAi knock-down of *pept-1* or *pept-1* deletion mutants, such as *pept-1(lg601)* essentially induce physiologically starved animals in the presence of abundant food (Meissner et al., 2004). The observed effects of *pept-1* RNAi and *pept-1(lg601)* on vulval induction therefore very likely correspond to the effects caused by external starvation. Strikingly, we found that the *pept-1(lg601)* mutation may increase vulval inductive levels more strongly than applied starvation treatments, resulting in nearly complete suppression of *lin-3(n378)* mutations. Such an increased effect of *pept-1(lg601)* may be due to the permanent nutrient deprivation in these animals compared to temporarily starved individuals. In addition, the negative starvation effects on vulval induction acting via DAF-2 Insulin sensory signalling (Battu et al. 2003) should be absent in *pept-1(lg601)* under food conditions.

Genetic analyses show that PEPT-1 interacts with LET-363/TOR pathway (Benner et al., 2011; Meissner et al., 2004; Spanier et al., 2009; Spanier et al., 2010), a highly conserved key nutrient sensing pathway (Hietakangas and Cohen, 2009; Jia et al., 2004). Reduced amino acid availability induced by *pept-1* deletion has been shown to affect development, reproduction and lifespan via downstream effects on LET-363/TOR (Meissner et al., 2004). Therefore, *pept-1* effects on vulval induction are likely mediated by differential regulation of

the TOR pathway. This interpretation is supported by the observed effects of *rsks-1/S6K* RNAi, which also increased *lin-3/egf(rf)* vulval induction. RSKS-1, the *C. elegans* ribosomal protein S6 kinase orthologue, is a direct target of the TORC1 complex (Hietakangas and Cohen, 2009; Jia et al., 2004). Taken together, we conclude that reduction of PEPT-1 function recapitulates observed starvation effects on vulval induction, and that this effect acts, at least partly, via the TOR pathway. Nevertheless, additional experiments are required to test for a direct implication of TOR in modulating vulval induction (see Chapter 4.2).

2.4.3. Effects and integration of PEPT-1/TOR into vulval signalling pathways

Quantification of *pept-1* RNAi effects on EGF-Ras-MAPK and Delta-Notch activities in vulval precursor cells provides direct evidence that nutrient deprivation increases vulval induction. These results are congruent with previously observed starvation effects on EGF-Ras-MAPK and Delta-Notch activities (Braendle et al., 2010). Although we cannot completely rule out additional, distinct effects on Delta-Notch signalling, current experimental evidence indicates that nutrient deprivation increases EGF-Ras-MAPK activity through targets at the level of LET-23/EGFR or LIN-3/EGF. Therefore, nutrient deprivation likely acting through TOR signalling may act in distinct cell types: the EGF-producing gonadal anchor cell (AC) and/or the EGF-receiving vulval precursor cells. However, it is also possible that starvation causes inappropriate expression of LIN-3/EGF in other cells or tissues, e.g. through defective LIN-3/EGF repression as observed in certain mutants (Saffer et al., 2011). Future experiments using cell-specific RNAi knock-down of TOR pathway components should resolve these open questions (see Chapter 4.2).

Cross-talk between TOR and EGF-Ras-MAPK signalling cascades has been reported in diverse processes of organisms, including mammals (Mendoza et al., 2011). Effects of TOR on the EGF-Ras-MAPK pathway may occur at multiple levels and often involve modulation of targets downstream of MAPK via S6K (Mendoza et al., 2011). Analysis of *Drosophila* neurogenesis further specifically revealed intersection of TOR and EGF-MAPK signalling pathways in developing photoreceptors, apparently also downstream of MAPK in S6K-dependent manner (McNeill et al., 2008). These studies provide the basis for further analysis to examine how TOR signals modulate directly or indirectly EGF-Ras-MAPK activity during *C. elegans* vulval development.

2.4.4. Environmental sensitivity versus robustness of *C. elegans* vulval cell fate patterning

This study and several others (Battu et al., 2003; Braendle & Felix, 2008; Ferguson and Horvitz, 1985; Moghal et al., 2003) show that *C. elegans* vulval cell fate patterning is sensitive to diverse environmental inputs, which may act through distinct mechanisms to affect vulval signalling pathways. While these results show that specific developmental processes and underlying genetic pathways are responsive to the environment, several key question remain: What are the consequences of this environmental sensitivity? How does the observed modulation of vulval signalling impact function and precision of this patterning process? Fundamentally, two opposed hypothetical scenarios can be considered to address these questions. First, environmental sensitivity of vulval development may be inevitable, so that environmental effects represent inherent environmental sensitivity of involved mechanisms. If this scenario holds true, the question is whether such environmental sensitivity translates into deleterious effects, such as patterning defects. In a second scenario, environmental sensitivity may reflect a specific developmental modulation to maintain or to enhance functioning and precision in different environmental conditions. This scenario would imply a vulval developmental system whose environmental flexibility has some adaptive origin. It is currently not known which of these scenarios apply to the observed environmental sensitivity of *C. elegans* vulval development. However, a previous study (Braendle and Félix, 2008) has quantified functioning and precision of vulval cell fate patterning in different environmental conditions: wild-type animals of multiple *C. elegans* isolates showed a very low rate of patterning defects in diverse, harsh environments, including starvation. Although starvation significantly increased levels of vulval induction (Braendle and Félix, 2008), vulval patterning errors indicative of such increased inductive levels (e.g. hyperinduction) remained very rare (N2 strain, starvation: 3/1000 versus control: 2/1000 individuals) (Braendle and Félix, 2008). Therefore, starvation modulation of vulval signalling pathways does not translate into an increased rate of corresponding errors, indicating that this process tolerates considerable changes in pathway activities. These observations reinforce the notion that the *C. elegans* vulval developmental system is robust to extensive signal fluctuations of involved pathways (Barkoulas et al., 2013; Braendle et al., 2010; Félix and Barkoulas, 2012; Hoyos et al., 2011; Milloz et al., 2008). Nevertheless, although current results clearly demonstrate robustness, i.e. tolerance of the vulval developmental system to environmental variation, it remains to be evaluated whether specific environmental modulation of signalling pathways enhances the fidelity and precision of the vulval patterning output.

2.4.5. Significance of interactions between environmental variation and molecular signalling pathways

C. elegans vulval cell fate patterning provides an ideal system to examine how environmental variation impacts development and involved signalling pathways. Sensitized genetic background, e.g. *Vulvaless* mutants, allow easy quantification of environmental effects on vulval induction and fate differentiation. These analyses can then be complemented using highly sensitive assays to quantify activities of relevant pathways, such as EGF-Ras-MAPK and Delta-Notch pathways. The findings that specific conditions, such as starvation, modulate activities of highly conserved key signalling pathways, such as EGF-Ras-MAPK, are fundamentally relevant in that they shed light on the frequently neglected interaction between genetic and environmental factors during development. That environmental variation may affect developmental processes in important ways is not new, however, quantitative analysis of interactions between specific environmental cues and genetic pathways is still rare. The environmental sensitivity of the vulval developmental system represents an ideal model system to gain insights into the ubiquitous environmental context-dependence of developmental processes. Moreover, understanding the environmental sensitivity of major signalling pathways – whose deregulation is frequently implied in diverse human pathologies, such as cancer – specifically addresses the molecular basis of gene-environment interactions and should thus be of central biomedical interest.

Chapter 3

3. Thermal perturbations reveal evolution of environmentally sensitive parameters in *Caenorhabditis* vulval development

3.1. Introduction

Organismal development is inherently sensitive to variation in the external environment, but such environmental sensitivity may or may not translate into variation of corresponding phenotypic outcomes. Relative insensitivity of a phenotype to environmental variation is termed environmental robustness and contrasts with phenotypic plasticity where developmental sensitivity to the environment results in different phenotypic outcomes (Flatt, 2005; Masel and Siegal, 2009; Wagner, 2005b). Environmental robustness of any developmental system has limits and may break down in response to specific environmental conditions. Such developmental debuffering may lead to the production of "novel", usually deleterious phenotypic variants, which are indicative of environmentally sensitive aspects of the underlying developmental processes. Developmental errors induced by the environment may therefore be informative by revealing the type and spectrum of environmentally-sensitive aspects of a given developmental system (Braendle et al., 2010; Braendle and Félix, 2008; Braendle and Felix, 2009). In classical developmental biology, the application of heat shock or other environmental perturbations to induce phenocopies or to determine the timing of a developmental process of interest provides an example of how environmental debuffering has been used to characterize developmental mechanisms and their environmental sensitivity (Goldschmidt, 1935; Peterson, 1990; Welte et al., 1995). Upon developmental debuffering by a given environmental perturbation, the frequency, type and spectrum of phenotypic variants induced may be highly variable, often showing genotype-dependence (Braendle et al., 2010; Braendle and Félix, 2008; Braendle and Felix, 2009). Experimental demonstration that environmental sensitivity of development shows genetic variation is provided by the classical *Drosophila* experiments performed by Waddington (Waddington, 1953, 1956). These experiments revealed the presence of cryptic genetic variation, i.e. standing genetic variation that is usually phenotypically silent but may become expressed in response to environmental and genetic perturbations (Gibson and Dworkin, 2004). The phenotypic manifestation of cryptic genetic variation is therefore condition-dependent, variation arising through genotype-by-environment interactions or genetic interactions (epistasis). Empirical evidence reporting the presence of cryptic genetic variation is thus old, and recent research shows that such "hidden" genetic variation is very common (Chandler et al., 2013; Chari and Dworkin, 2013;

Gibson and Dworkin, 2004; Wagner, 2005b). This research emphasizes the need for a better understanding of condition-dependent phenotypic variation, both from a mechanistic and an evolutionary perspective. While there are many examples of biological systems in diverse organisms harbouring cryptic genetic variation (Chandler et al., 2013; Chari and Dworkin, 2013; Gibson and Dworkin, 2004; Wagner, 2005b), the molecular genetic identity of such variation has only rarely been elucidated (Duveau and Félix, 2012; Dworkin et al., 2003; Gibson and Hogness, 1996), and despite important claims (Queitsch et al., 2002; Rutherford and Lindquist, 1998), the evolutionary significance of cryptic genetic variation in adaptive phenotypic evolution is still unclear (Braendle and Flatt, 2006; Meiklejohn and Hartl, 2002; Mitchell-Olds and Knight, 2002).

The phenomenon of cryptic genetic variation is tightly linked to the notion of developmental robustness (Felix and Wagner, 2008; Gibson and Dworkin, 2004; Masel and Siegal, 2009; Masel and Trotter, 2010; Wagner, 2005b). Robustness that causes phenotypic insensitivity to genetic perturbations (e.g. the accumulation of novel mutations), is the supposed key property generating cryptic genetic variation. Thus, developmental robustness may lead to evolutionary stability at the phenotypic level in the presence of evolutionary change in the underlying genetic architecture (Felix and Wagner, 2008; Gibson and Dworkin, 2004; Wagner, 2005a). Although difficult to evaluate, both theoretical and empirical evidence suggest that environmental robustness causes genetic robustness, and vice versa (Meiklejohn and Hartl, 2002). Consistent with this scenario, different sources of perturbations may disrupt the same features of a given developmental process, i.e. genetic and environmental perturbations may act interchangeably in the production of specific developmental defects (cf. phenocopies and genocopies) (Goldschmidt, 1935; Welte et al., 1995).

Debuffering of a developmental system leading to an usually invariant phenotypic outcome allows exploration of possible phenotypic defects and underlying developmental deregulation, thus allowing identification of the system's most sensitive parameters to the applied perturbation. However, very little research has systematically quantified the spectrum and frequency of inducible developmental variants or errors, which would permit such inferences about developmental system sensitivity. Moreover, whether type and spectrum of such condition-dependent developmental variants show evolutionary variation (i.e. cryptic genetic variation) has rarely been quantitatively examined (Braendle et al., 2010; Braendle and Félix, 2008; Rutherford and Lindquist, 1998). Here we therefore aimed to explore the environmental sensitivity of a robust developmental system, which generates an invariant phenotype across different genotypes and species. We use the well-characterized process of

vulval cell fate patterning in *Caenorhabditis* nematodes to characterize which specific parameters of this process are most sensitive to extreme environmental perturbations, and to quantify how this developmental sensitivity to the environment varies among different genotypes.

The *C. elegans* hermaphrodite vulva differentiates from a subset of six ventral hypodermal cells, called Pn.p cells, through a molecularly well-understood signalling network regulating the induction of specific vulval cell (Sternberg, 2005) (Figure 3.1). P3.p to P8.p acquire competence to adopt vulval cell fates through expression of the HOX gene *lin-39*, regulated by the canonical Wnt pathway (Figure 3.1A). The key steps of vulval induction and fate differentiation are regulated by an interplay of EGF-Ras-MAPK and Delta-Notch pathways (Figure 3.1B) (Felix and Barkoulas, 2012; Sternberg, 2005). During the late L2 /early L3 stage, the gonadal anchor cell (AC) releases the EGF-like ligand LIN-3, which induces the primary (1°) vulval cell fate in the closest cell, P6.p, through activation of the EGF-Ras-MAPK pathway (Hill and Sternberg, 1992). In turn, activation of this pathway in P6.p triggers a lateral intercellular signalling event, mediated by the Delta-Notch pathway, promoting the adoption of a secondary (2°) cell fate by the neighbouring cells, P5.p and P7.p. Notch activity in these cells further inhibits the 1° cell fate by activating negative regulators of the EGF-Ras-MAPK pathway (Berset et al., 2001; Greenwald et al., 1983; Sternberg, 1988; Sternberg and Horvitz, 1986; Yoo et al., 2004) (Figure 3.1B). Moreover, a switch from the canonical Ras-Raf pathway to a Ras-RGL-1-RAL-1 signalling pathway promotes the 2° cell fate in P5.p and P7.p (Zand et al., 2011), and the Wnt pathway, primarily involved in vulval competence, may contribute to vulval induction (Gleason et al., 2002). The remaining vulval precursor cells adopt non-vulval cell fates (3° and 4°) as they do not receive sufficient doses of either signal: in *C. elegans*, P4.p and P8.p adopt the 3° cell fate while P3.p shows stochastic variation, either adopting a 3° or 4° cell fate (Pénigault and Félix, 2011a; Sulston and Horvitz, 1977). Cell ablation experiments indicate, however, that P3.p, P4.p and P8.p are competent to adopt 1° or 2° vulval cell fates and capable of replacing missing cells of the P5.p-P7.p group (Braendle and Félix, 2008; Katz et al., 1995; Sternberg, 2005). The different cell fate patterns (1° to 4°) correspond to distinct, invariant cell division patterns of the Pn.p cells, which occur during the mid to late L3 stage (Figure 3.1C). The canonical cell fate sequence of P3.p to P8.p is therefore 3°/4°-3°-2°-1°-2°-3 – a pattern that is largely conserved within the *Caenorhabditis* genus comprising 26 described species (Félix, 2007; Kiontke et al., 2007; Kiontke et al., 2011; Pénigault and Félix, 2011a). P3.p, the only cell with variable vulval cell fate in *C. elegans*, may show different ratios of 3°/4° cell fates depending on

species and isolates, and has lost vulval competence completely in some species; in addition, the competence of P4.p and P8.p to adopt vulval cell fates shows variation between species (Félix, 2007; Felix and Barkoulas, 2012; Pénigault and Félix, 2011a).

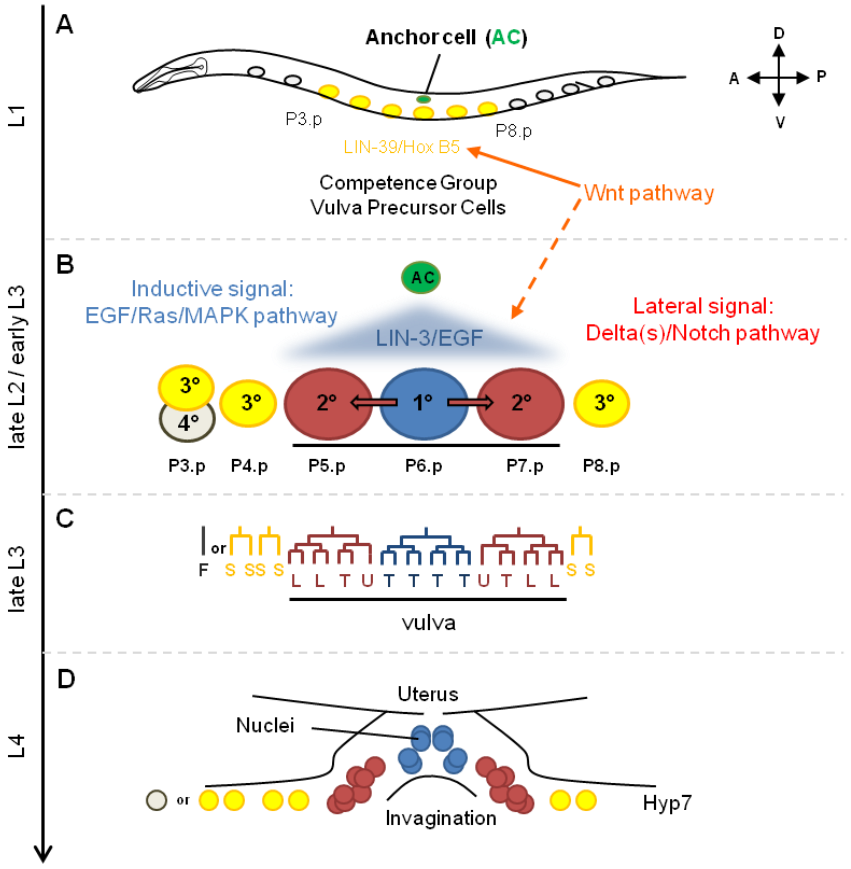

Figure 3.1. *Caenorhabditis* vulval cell fate patterning. The *Caenorhabditis* vulva develops from a set of six ventral hypodermal cells, P3.p to P8.p. **(A)** L1 stage: P3-8.p cells express the Hox gene *lin-39* and acquire competence to adopt vulval cell fates. **(B)** Late L2 /early L3 stage: the anchor cell (AC) releases the morphogen-like LIN-3/EGF inductive signal. P6.p receives the highest level of LIN-3/EGF inducing a 1° cell fate (blue) via EGFR-Ras-MAPK activation, which in turn activates lateral signalling through the expression of the Delta ligands targeting the Delta-Notch pathway in its neighbours, P5.p and P7.p. This lateral signalling induces the 2° vulval cell fate (red) via Delta-Notch activity and further represses the 1° cell fate in these cells. The competent cells, P4.p and P8.p, adopt a non-vulval 3° fate (yellow), while the fate of P3.p varies among individuals, either adopting a 3° fate or a 4° fate (grey, also referred to as F(used) fate). Therefore, of a total of six potential vulval precursor cells, only P5.p, P6.p and P7.p adopt actual vulval cell fates in a 2°-1°-2° sequence, which is conserved among *Caenorhabditis* species. **(C)** Mid to late L3 stage: Vulval cell divisions. The fate assignments correspond to stereotypical cell division patterns that are invariant (with exception of P3.p). The three cells adopting the 2°-1°-2° vulval fates generate a total of 22 vulval cells by the end of the L3 stage. T: transverse (left-right) division, L: longitudinal (antero-posterior) division, U:

undivided, SS: fusion to the epidermal syncytium (hyp7) after a single division (3° fate); F: fusion to the syncytium in the L2/L3 stage with no prior division (4° fate). 3° and 4° fates are non-vulval fates. **(D)** L4 stage: vulval morphogenesis.

The *Caenorhabditis* vulval signalling network possesses a wide range of properties that contribute to a robust patterning output, including partial redundancy and crosstalk among signalling pathways, manifold regulatory inputs and feedback loops within the EGF-Ras-MAPK (Braendle et al., 2010; Braendle and Félix, 2008; Braendle and Felix, 2009; Félix, 2012a; Felix and Barkoulas, 2012; Felix and Wagner, 2008; Sternberg, 2005). Key among these properties is the regulatory cross-talk between EGF-Ras-MAPK and Delta-Notch pathways, which ensures a reproducible establishment of the 2°-1°-2° vulval cell fate pattern of P5.p to P7.p despite extensive variation in parameter space (Barkoulas et al., 2013; Felix and Barkoulas, 2012; Hoyos et al., 2011).

Caenorhabditis vulval cell fate patterning – a relatively simple cell fate determination process involving a molecularly very well-characterized signalling network – has emerged as an important model system for quantitative developmental studies, system robustness and evolution as well as cryptic genetic variation (Felix and Barkoulas, 2012). Comparative developmental studies of intra- and interspecific variation in *Caenorhabditis* nematodes have revealed extensive cryptic variation in genetic and developmental parameters underlying the evolutionarily conserved vulval pattern (Felix and Barkoulas, 2012). Cryptic variation has been revealed through application of genetic (e.g. mutation accumulation, introgression of vulval mutations into different wild isolates, insertion of transgenes into different species, accumulation of spontaneous random mutation) and environmental perturbations (Barkoulas et al., 2013; Braendle et al., 2010; Braendle and Félix, 2008; Delattre and Félix, 2001; Duveau and Félix, 2012; Félix, 2007; Hoyos et al., 2011; Milloz et al., 2008; Pénigault and Félix, 2011a). These results indicate that diverse system properties (e.g. cell competence, pathway activities and their interactions) may evolve without leading to changes in the final vulval cell fate pattern.

Quantitative analysis of vulval development in different environments (intermittent starvation, passage through the dauer stage, different temperatures, liquid culture) indicates that vulval pattern establishment is robust despite environmental sensitivity of underlying signalling cascades (Braendle and Félix, 2008). Of 6000 animals assessed in these different environments only 0.25% showed apparent vulval defects and an additional 2.10% showed

non-canonical vulval patterning variants with an intact 2°-1°-2° sequence. Moreover, certain environmental conditions induced specific variants whose frequencies were further strongly genotype-dependent. For example, starvation during the L2 stage consistently induced vulval centering shifts on P5.p in the *C. elegans* N2 strain but very rarely in other strains of *C. elegans* or *C. briggsae* (Braendle and Félix, 2008). This and other examples suggest that certain system properties (e.g. a specific pathway or a specific Pn.p cell) are more environmentally sensitive than others, and that this sensitivity is further subject to evolutionary change. However, these conclusions are based on limited data because previously examined environments induced variant and defective patterns only at very low frequencies, rendering quantitative analysis of environmental sensitivity and its evolution difficult (Braendle and Félix, 2008).

In this study we took advantage of the *Caenorhabditis* vulval developmental system to characterize its response to extreme environmental perturbations, low and high temperature extremes, and how such response evolves. In contrast to previous studies (Braendle et al., 2010; Braendle and Félix, 2008), we aimed to maximally disrupt the precision of the patterning process to be able to better understand (a) which underlying developmental and cellular aspects are most sensitive, (b) whether such specific system sensitivity evolves within and between species, and (c) whether genotype-specific patterns of such environmentally-induced phenotypic variation correlate with mutationally-induced phenotypic variation.

3.2. Material and Methods

3.2.1. Strains

We examined the same *C. elegans* and *C. briggsae* strains (wild isolates) as used in (Braendle et al., 2010): the reference lab strain, *C. elegans* N2 (Bristol, UK), *C. elegans* PB306 (Connecticut, USA), *C. briggsae* HK104 (Okayama, Japan), *C. briggsae* PB800 (Ohio, USA). These strains had been used to derive mutation accumulation lines over 250 generations (Baer et al., 2005).

3.2.2. Temperature assays

To quantify and characterize temperature effects on the production of vulval pattern variants and defects, we exposed the four strains to three temperatures, 6°C, 20°C (control) and 30°C, during the time window of vulval cell fate patterning (mid L2 to mid L3). Strains were

derived from frozen stocks and maintained on NGM agar plates (1.7% agar) seeded with *E. coli* OP50 (Wood, 1988a) at 20°C. For each experimental repeat, mid L2 individuals from each of the four strains were randomly allocated to the three temperature treatments. (Note that the four strains had very similar developmental times). In the cold treatment, animals were exposed to 6°C for 20 hours, then transferred back to 20°C for 24 hours prior to scoring of the vulval phenotype (note that developmental progression was strongly slowed down, if not arrested, at 6°C). In the heat treatment, animals were exposed to 30°C for 16 hours, immediately after which the vulva phenotype was scored. Control animals at 20°C reached the mid L4 stage at the same time as animals of the heat treatment and were scored in parallel.

3.2.3. Scoring of vulval cell fates and variant patterns

We scored the vulval phenotype of 300 individuals for each strain in each of the three temperature treatments (N_{total} = 3'600 scored individuals), derived from 15 experimental repeats in each of which the four strains were scored in parallel as outlined above. Cell fate patterns of P3.p to P8.p were inferred through observation by Nomarski optics in early to mid L4 individuals as previously described (Braendle et al., 2010; Braendle and Félix, 2008; Sternberg and Horvitz, 1986). The characteristics of different vulval cell fates and corresponding cell lineages are described in Figure 1. Phenotypic characterization of vulval cell fate patterns variants deviating from the canonical *Caenorhabditis* 3°/4°-3°-2°-1°-2°-3 pattern follows the variant classes established by Braendle et al. (2010) (Figure 3.2). We refer to any deviation from the canonical pattern as *variant* while *defect* only refers to variant patterns of class A, i.e. patterns where the 2°-1°-2° adoption of vulval cells is disrupted. We defined 15 distinct non-canonical cell fate patterns, which are grouped into Class A, B, C and D variant categories as described in detail in legend of Figure 2. Note that we established an additional class C variant other than listed in Braendle et al (2010): #14, i.e. individuals that showed a 4° fate for both P4.p and P8.p.

3.2.4. Measurement of temperature effects on *lip-1::gfp* activity

We inferred Delta-Notch pathway activity in P5.p to P7.p using a transgenic strain containing an integrated transcriptional reporter construct, *lip-1::gfp* (strain AH142, derived from the reference strain N2) (Berset et al., 2001). Experimental populations were age-synchronized by hypochlorite treatment and liquid arrest at 20°C (Wood, 1988b). Young L1 larvae were

transferred to NGM plates until they reached the mid L2 stage at which point they were randomly allocated to 20°C (control) or 30°C treatments. *lip-1::gfp* quantification was performed when individuals had reached the lethargus L2/L3 or early L3 stages. The Pn.p cells of live, anesthetized individuals were first identified using Nomarski optics, followed by measurement of signal (pixel) intensity in P5.p, P6.p and P7.p for each individual as previously described in Braendle & Félix (2008) using an Olympus BX61 epi-fluorescence microscope equipped with a Coolsnap HQ2 camera (at 40X magnification). This experiment was repeated once, and a total of 33 and 34 individuals were scored at 20°C and 30°C, respectively.

3.2.5. Statistical analysis and data presentation

Effects of environment, species and strain on frequency of vulval variants: Data representation in Figures 3 and 4 represent the mean percentage of classes and their variants averaged across experimental repeats (N=15). We performed an ANOVA (JMP 9.0, SPSS statistics) testing for the fixed effects of species, strain (nested in species), temperature and the interactions of species x temperature and strain (species) x temperature using Class A-C variant frequencies obtained per experimental repeat as a response variable (N =15). Data was arcsine-square-root transformed prior to analysis (Sokal and Rohlf, 1981).

Quantification of lip-1::*gfp activity*: We performed an ANOVA (JMP 9.0, SPSS statistics) testing for the effects of *block* (i.e. experimental repeat, N=2), *temperature* (20°C versus 30°C) , *cell* (P5.p, P6.p, P7.p), *individual* and all possible interactions using mean signal intensity as a response variable. Including individual as an effect allowed controlling for the non- independence between measures of P5.p, P6.p, and P7.p taken from the same individual. Data was log-transformed prior to analysis (Sokal and Rohlf, 1981). Post-hoc tests (Tukey's HSD) were then performed to determine differences in signal expression between temperature, treatments and cells (P5.p. P6.p, P7.p). Data represented in Figure 6C show Least-Square Means of the *cell* x *temperature* interaction.

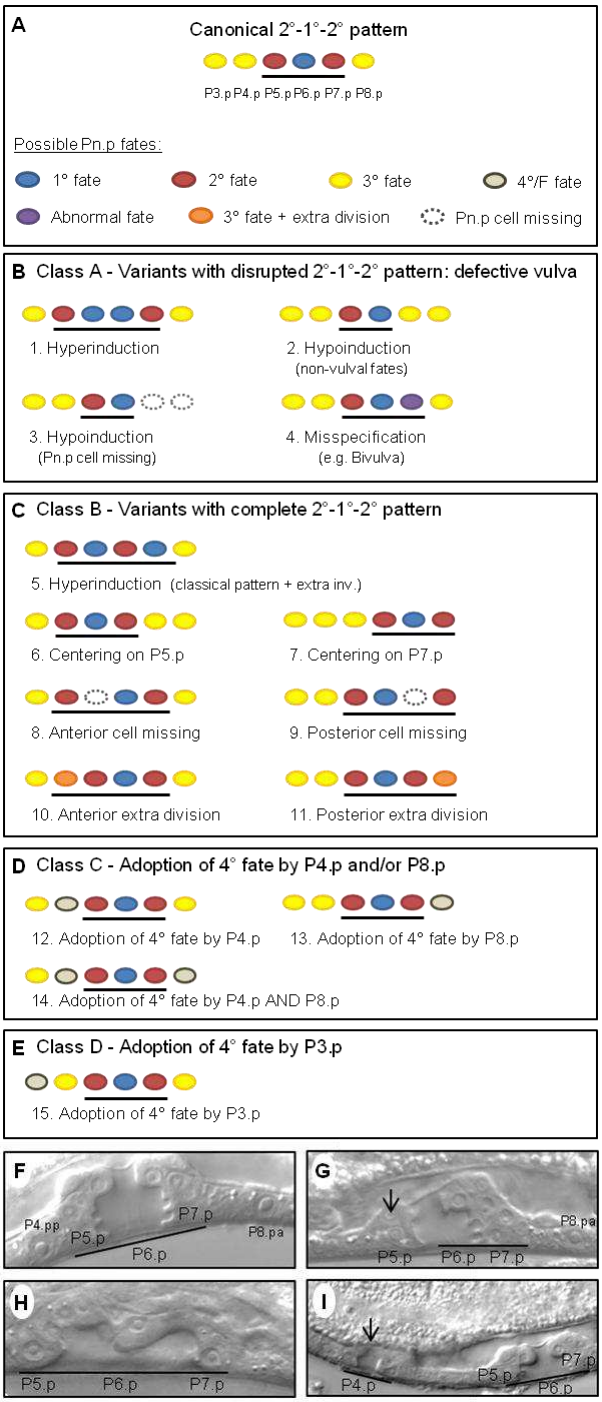

Figure 3.2. Variant patterns of *Caenorhabditis* vulval precursor cells. (A) The canonical cell fate pattern of P3.p to P8.p is represented with P3.p adopting a 3° fate, and cells adopting a vulval cell fate are underlined. We defined 14 non-canonical subcategories of variants relative to P(4-8).p fates, grouped into three different classes. As P3.p fate is a highly variable trait, it is presented in its own class. **(B)** Class A: Disrupted 2°-1°-2° pattern leading to a defective vulva. **(C)** Class B: Complete 2°-1°-2° pattern but altered fates for the VPCs. **(D)** Class C: Complete 2°-1°-2° pattern and variable adoption of 3° versus 4° fate by P4.p and/or P8.p. **(E)** Class D: Complete 2°-1°-2° pattern and variable adoption of 3° versus 4° fate by P3.p.

Detailed description of vulval pattern variants: **(B)** Class A (1-4): This class groups vulval variants that cause a strongly disrupted vulval pattern, likely leading to a defective vulval organ. **(1)** Hyperinduction: more than three induced VPCs (1° or 2° fate) that prevent the formation of a complete vulva.(For example adjacent 1°-fated cells) **(2)** Hypoinduction due to adoption of a 3° or a 4° fate, resulting in fewer than three induced VPCs (1° or 2° fate). For example: P7.p adopts a 3° cell fate. **(3)** Hypoinduction due to missing cells: fewer than three induced cells because of the absence of one or several Pn.p cells. For example: missing P7.p and P8.p, leading to only two induced cells (P5.p and P6.p). **(4)** Misspecification of vulval fates (other than hyper- or hypoinduction): three VPCs are induced but their lineages deviate from the canonical pattern. For example, P7.p misspecification: the canonical UTLL lineage is replaced by LLTU, referred to as Bivulva phenotype (Inoue et al., 2004). **(C)** Class B: This class groups variants with complete 2°-1°-2° vulval patterns, yet deviating from the overall canonical pattern of VPCs. Vulva formation is not obviously disrupted, however, the impact of such variants on egg-laying or other functions remains unclear (Braendle and Félix, 2008). **(5)** Hyperinduction: more than three induced VPCs. For example: P4.p adopts a vulval fate (2°) and creates a second, non-functional invagination. **(6–7)** Centering shifts: the three correctly induced VPCs are shifted to the anterior (centering on P5.p) or posterior (centering on P7.p). For example: anterior centering, P5.p adopts a 1° cell fate and its neighbours, P4.p and P6.p, adopt a 2° fate; the anchor cell is centered on P5.p. **(8–9)** Missing cells: One or more VPCs are missing. For example: P8.p adopts a 2° fate because P7.p is missing; in this case, it is not possible to distinguish whether this variant was due to a missing P7.p or P8.p cell. We can only distinguish whether the missing cell(s) is anterior (P3.p to P5.p) or posterior (P7.p and P8.p). **(10–11)** Supernumerary cell divisions: P3.p, P4.p or P8.p divides more than once, generating three to four daughter cells that fuse with the hypodermis. For example: P4.p divides twice (ssss lineage instead of SS). **(D)** Class C: P4.p or P8.p adopts a 4° instead of a 3° fate. **(12)** P4.p adopts a 4° fate, fusing with the hypodermis without prior division. **(13)** P8.p adopts a 4° fate. **(14)** P4.p and P8.p adopt 4° fates. **(E)** Class D: Adoption of 4° fate instead of 3° fate by P3.p. It corresponds to variant **(15)**. **(F-I)** Nomarski images of vulval cells in the mid L4 stage: **(F)** canonical vulval pattern, **(G)** hypoinduction (variant #2), **(H)** misspecification (variant #4), **(I)** hyperinduction (variant #5).

3.3. Results

3.3.1. Extreme temperatures debuffer vulval development inducing diverse variants and defects

The two temperature extremes caused a significant increase in vulval pattern variants, thus debuffering this developmental process. Types of temperature-induced vulval variants varied greatly, covering the whole range of previously observed variants (Braendle et al., 2010) yet

at much higher frequencies (Figure 3.4). At the standard temperature of 20°C, variants deviating from the canonical vulval cell fate patterns were rare for all strains, with defects (Class A) occurring at a frequency of < 1% (Figure 3.3). At 20°C, as previously found (Braendle et al., 2010; Braendle and Félix, 2008; Delattre and Félix, 2001; Pénigault and Félix, 2011a), *C. briggsae* strains, compared to *C. elegans*, showed an increased tendency of P3.p, P4.p and P8.p to adopt 4° fates (Class C and D) (Table 3.1C: main effect *Species*). Exposure to temperature extremes caused a significant increase in all variant classes for all strains (Table 1A-C: main effect *Temperature*) with an overall highest increase at 30°C, leading to defect frequencies (Class A) of up to 12% (Figure 3.3). For all strains, variants of class C, i.e. P4.p and/or P8.p adopting a 4° fate, were consistently highest at 30°C (Figure 3.3 and Figure 3.4). Strains and species differed in the frequency of P3.p adopting the 4° fate, but the ratio of 4°:3° fates of P3.p was not sensitive to temperature variation (Figure 3.4E); this contrasts with previous results where the ratio of 4°:3° cell fate for P3.p was found to be highly sensitive to environmental variation (Braendle and Félix, 2008).

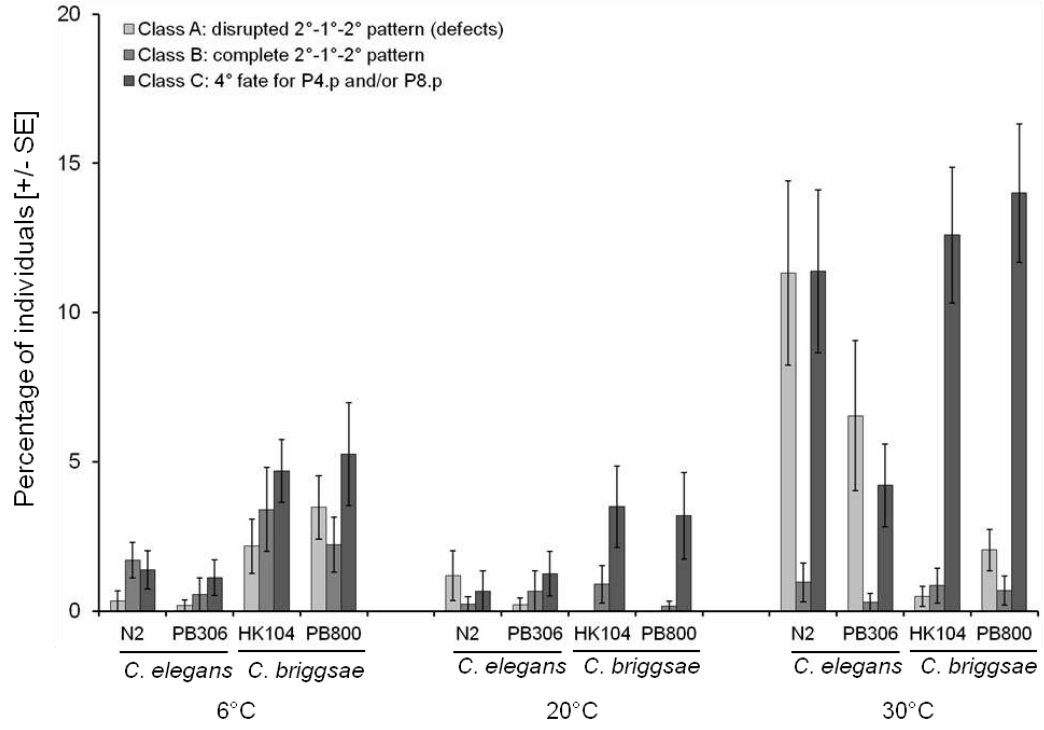

Figure 3.3. Effects of temperature and genotype on variant class frequencies. Bars indicate the mean percentage of individuals showing vulval variants in three classes A-C (N=15 experimental repeats, N=300 individuals per strain/temperature, N=900 per strain). Error bars indicate ± 1 SE.

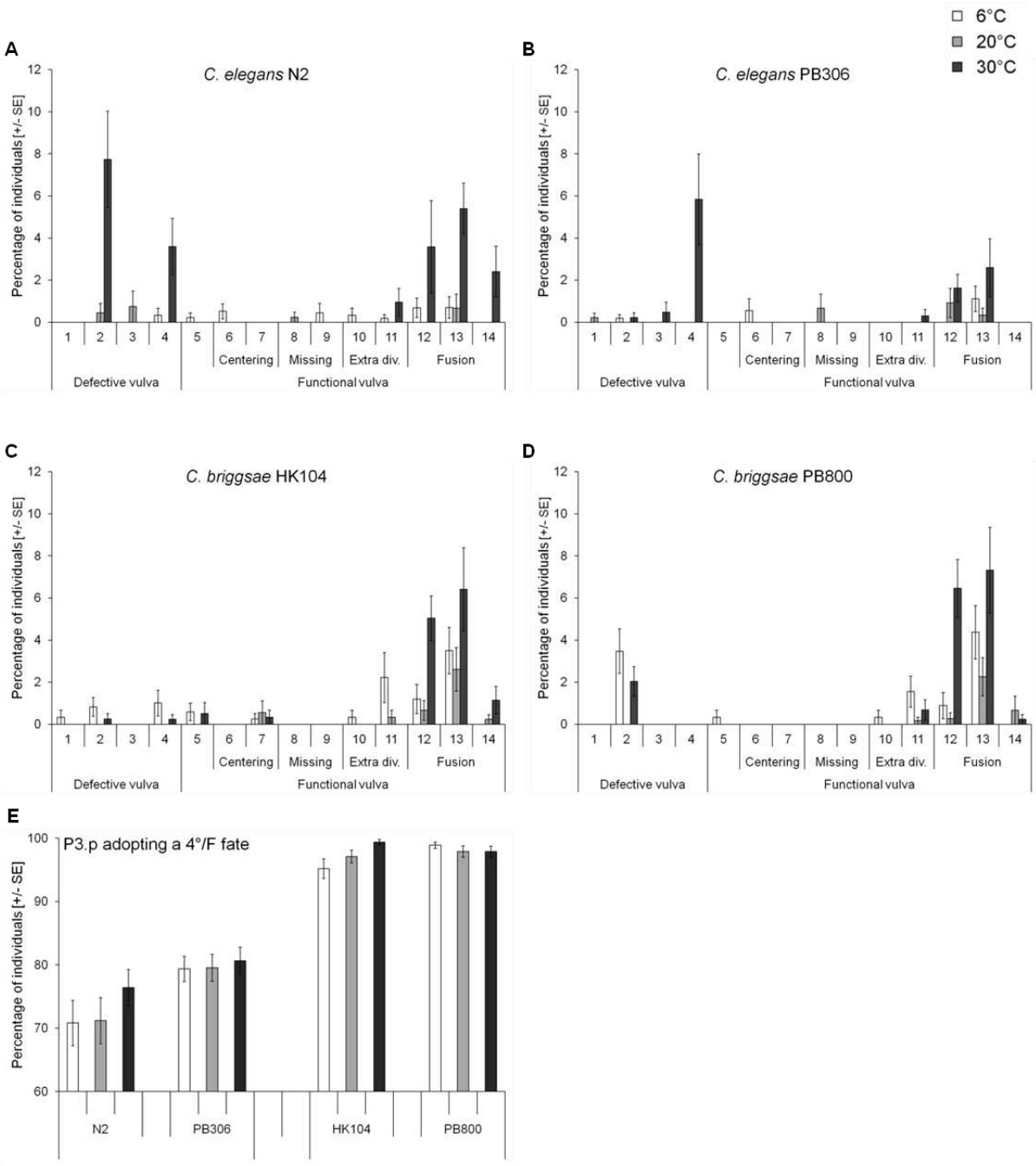

Figure 3.4. Effects of temperature and genotype on frequencies of specific vulval variants. Frequency of specific vulval variants at different temperatures for the *C. elegans* strains N2 **(A)** and PB306 **(B)**, and the *C. briggsae* strains HK104 **(C)** and PB800 **(D)**. **(E)** Frequency of individuals with P3.p adopting a 4° fate. Bars indicate the mean percentage of individuals with a given vulval variant (N=15 experimental repeats, N=300 individuals per strain/temperature, N=900 per strain). A detailed description of different types of vulval variants is given in Figure 3.2. Error bars indicate ± 1 SE.

Table 3.1. Effects of environment, species and strain on frequency of vulval variant classes A-C. ANOVA testing for the fixed effects of *species*, *strain* (nested in *species*), *temperature* and the interactions of *species* x *temperature* and *strain* (*species*) x *temperature*. Data was arcsine-square-root transformed prior to analysis (Sokal and Rohlf, 1981).

A) Class A

Source	DF	DFDen	F Ratio	*P*
Species	1	168	2.89	0.0912
Strain(Species)	2	168	2.39	0.0947
Temperature	2	168	15.90	< 0.0001
Species x Temperature	2	168	16.43	< 0.0001
Strain(Species) x Temperature	4	168	0.86	0.4896

B) Class B

Source	DF	DFDen	F Ratio	*P*
Species	1	168	2.11	0.1482
Strain(Species)	2	168	1.30	0.2752
Temperature	2	168	6.18	0.0026
Species x Temperature	2	168	1.04	0.3541
Strain(Species) x Temperature	4	168	0.62	0.6478

C) Class C

Source	DF	DFDen	F Ratio	*P*
Species	1	168	24.89	< 0.0001
Strain(Species)	2	168	1.11	0.3309
Temperature	2	168	33.42	< 0.0001
Species x Temperature	2	168	0.47	0.6247
Strain(Species) x Temperature	4	168	1.55	0.1915

3.3.2. Genotype-dependence of temperature-induced vulval pattern variants

Frequency and type of temperature-induced vulval variants differed between species as well as between strains within each of the species (Figure 3.3 and Figure 3.4; Table 3.1). The two

species showed converse responses to the two temperature extremes as indicated by the significant *species* x *temperature* interaction (Table 3.1A). *C. elegans* strains showed an increased frequency of variants, including vulval defects, at 30°C yet no increase at 6°C, while *C. briggsae* strains were more sensitive to 6°C (Figure 3.3). The increased frequency of vulval variants was mainly due to an increase of class C variants (P4.p and/or P8.p adopting a 4° fate) in *C. briggsae* whereas in *C. elegans* we observed an increase of class C and class A (i.e. defect with disrupted 2°-1°-2° pattern) variants.

C. briggsae strains showed similar variant frequencies across temperatures for most variant types in all three classes (Figure 3.4C,D). In contrast, variant types varied qualitatively and quantitatively between the *C. elegans* strains at 30°C; although both strains exhibited a high frequency of vulval defects (Class A), the nature of these defects was different: in N2, the majority of defects (7.7%) were due to hypoinduction (variant #2) whereas this variant was very rare (0.2%) in PB306, which mainly showed defects due to other misspecification events of the vulval precursor cells (5.8%) (Figure 3.4A,B). In addition, also at 30°C, N2 showed an higher increase of P4.p and P8.p fusion (variants #12-14) relative to PB306.

3.3.3. Environmental sensitivity of specific system features

Vulval hypoinduction (variant #2) at high frequency (7.7%) was specifically displayed by *C. elegans* N2 individuals exposed to 30°C. N2 hypoinduction at 30°C was primarily due to adoption of 3° fates by P5.p and P7.p (23 out of 25 individuals) rather than 4° fates (2 of out of 25 individuals), and never due to missing cells (Figure 3.5A). In contrast, the central 1°-fated cell, P6.p, was never hypoinduced. Induction of 2°-fated vulval cells, P5.p and P7.p, was therefore more sensitive to high temperature.

We therefore next asked how high temperature affects the activity of the key signalling pathway involved in 2° vulval fate determination, the Delta-Notch pathway (Sternberg, 2005). Using the highly sensitive transcriptional reporter gene *lip-1::gfp* in the N2 background (Berset et al., 2001), we quantified pathway activities at 20°C and 30°C during vulval fate patterning in P5.p to P7.p (lethargus L2/L3 and early L3 stages) (Figure 3.5B). Temperature affected Delta-Notch activity in a cell-specific manner: at 30°C *lip-1::gfp* expression, was significantly decreased in P5.p while it was increased in P6.p (Figure 3.5C). These observations are consistent with a reduced stability of 1°-2°-1° pattern establishment at high temperature, which seem to primarily disrupt the induction of 2° cells as reported above (Figure 3.5A). The detection of reduced mean Delta-Notch activity specifically in P5.p may

account for its higher proportion of hypoinduction compared to P7.p (Figure 3.5A). In contrast, despite an apparent mean increase of its Delta-Notch activity, P6.p was never found to be hypoinduced at 30°C; however, multiple other misspecification defects (variant #4), observed for N2 at 30°C, also affected P6.p (Figure 3.4A).

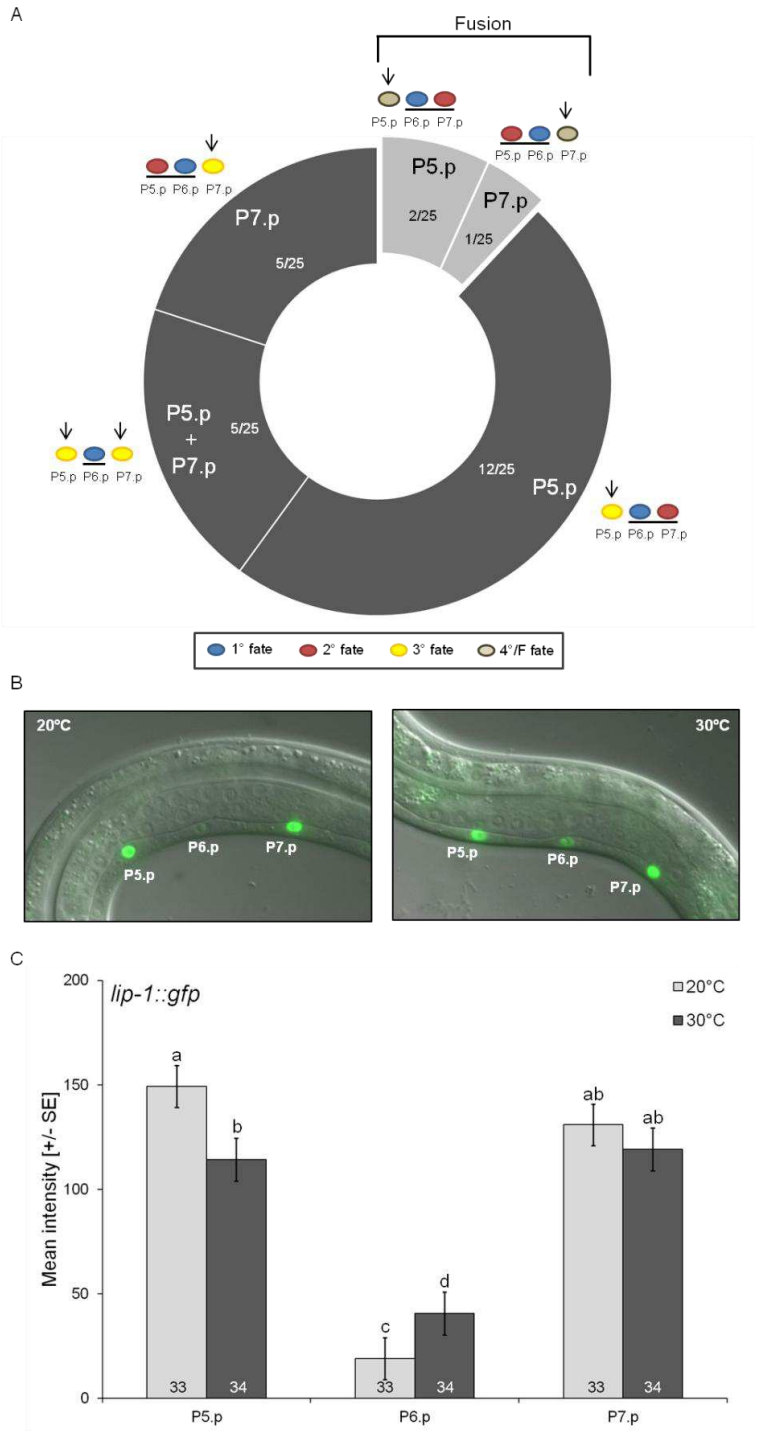

Figure 3.5. Effects of high temperature on *C. elegans* N2 vulval induction and Delta-Notch pathway activity. (A) Frequency of vulval hypoinduction (variant #2) due to adoption of 3° or 4° fate in *C. elegans* N2 at 30°C. Considering the total of 25 hypoinduced variants, P5.p was more frequently

affected than P7.p (Fisher's Exact Test, P=0.043) and the majority of variants were due to adoption of 3° cell fate (N=22/25). **(B)** Merge of Nomarski and fluorescence images of *lip-1::gfp* individuals exposed to 20°C and 30°C at L2/L3 lethargus. **(C)** Measurement of Notch pathway activity through *lip-1::gfp* analysis at 20°C and 30°C. Bars indicate mean signal (pixel) intensity, i.e. Least Square Means for the interaction *cell* x *temperature* ($F_{2,72}$ = 8.03 , P = 0.0007), controlled for block and individual effects. Values labelled with different letters are significantly different (Tukey's HSD). Error bars indicate ± 1 SE.

3.3.4. Environmental versus mutational perturbation of vulval cell fate patterning

A previous study (Braendle et al., 2010) had quantified vulval variants of mutation accumulation (MA) lines derived from the same *C. briggsae* and *C. elegans* strains used here, allowing comparison of mutationally versus environmentally induced vulval variants. Overall, the frequency of vulval variants observed after extreme temperature stress exposure was much higher than after 250 generations of MA (Figure 3.6).

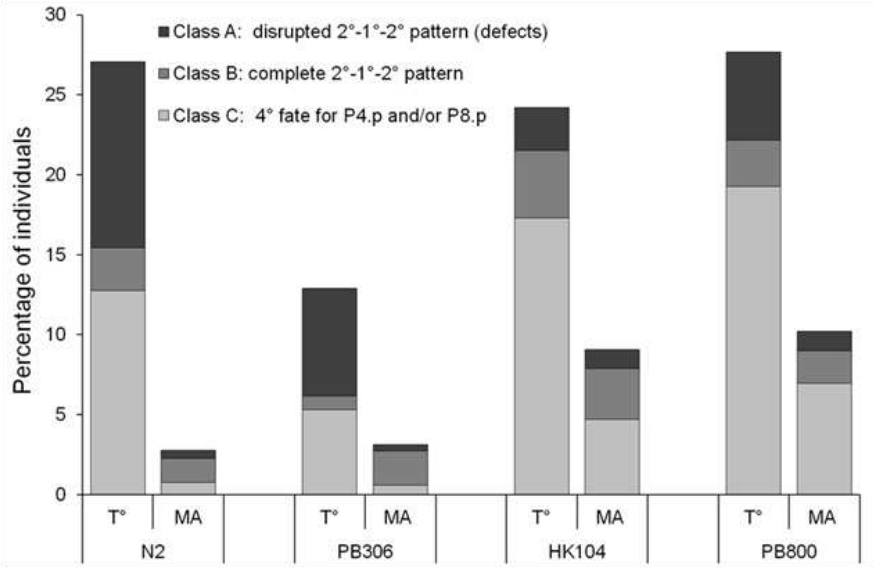

Figure 3.6. Comparison of vulval variants induced by temperature versus mutation accumulation. Proportion of individuals with vulval variants after temperature exposure to 6°C and 30°C (T°) or after mutation accumulation (MA) (Braendle et al., 2010). Bars indicate the total percentage of individuals with a variant pattern of the three classes after the two treatments (T° or MA). For details of the MA experiment, see Braendle (2010).

Across species and strains, both MA and exposure to temperature extremes triggered a similarly wide spectrum of vulval developmental variants, yet variant spectra for most strains were overall narrower after temperature perturbations. For example, *C. briggsae* PB800 had a 50% reduced variant spectrum in response to thermal perturbations relative to the MA assay. In addition, we also found cases where variant types in certain strains were only generated in response to temperature but not MA. For example, variant #13 (4° of P8.p) in the N2 strain was never observed after MA but occurred at relatively high frequency in this strain at 30°C (Figure 3.4A). Similarly, hypoinduction variants (#2) frequently observed at high temperature for N2 (Figure 3.4A) were very rare after MA for the same strain (Braendle et al., 2010). While quantitative comparison between mutationally and environmentally induced vulval variants is difficult given the low penetrance of variants after MA, several examples illustrate that genotype-dependence of variant production can be observed in response to either source of perturbation. For example, the 3° vulval precursor cells, P4.p and P8.p, are most sensitive, frequently adopting a fused 4° fate (Class C variants) upon either mutational or environmental perturbation in both *C. elegans* and *C. briggsae* (Figure 3.3 and Figure 3.4) (Braendle et al., 2010) . In another example, centering variants on P7.p (variant #7) were never observed for *C. elegans* N2 after either type of perturbation, while they occurred at low frequency in all other strains. Hence, mutational and environmental perturbation of vulval cell fate patterning may disrupt the same specific developmental system features (e.g. specific precursor cells), yet for both types of perturbations the degree and type of disruption was usually dependent on the genetic background of individuals examined.

3.4. Discussion

We used extreme temperatures to perturb the *Caenorhabditis* vulval cell fate patterning process, allowing us to quantitatively assess debuffering of this developmental system through analysis of non-canonical vulval patterning variants. Applying such strong environmental perturbations enabled us to detect environmental sensitivity of different system parameters as well as their evolutionary variation.

3.4.1. Environmental sensitivity of the vulval cell fate patterning process shows evolutionary variation

Extreme temperatures consistently impaired the precision of vulval cell fate patterning in all tested strains. We found that extreme temperatures induce vulval patterning variants in > 10% of individuals while such variants are absent or very rare in control conditions. Type and frequency of variant vulval patterns were temperature-, species- and genotype-dependent, showing significant differences among tested species and strains within species (Table 1). Although exposure to 30°C induced the highest proportion of variant patterns for all strains, only *C. elegans* strains showed an increased frequency of defects (Class A variants) at this temperature. In contrast, such defects were increased at 6°C for *C. briggsae* strains, suggesting that the two species differ in their thermal tolerance to hot versus cold temperatures, respectively. Thermal preference analysis indicates that *C. briggsae* is generally more tolerant to high temperature compared to *C. elegans* (Anderson et al., 2011; Harvey and Viney, 2007; Prasad et al., 2011) and our data suggest that *C. briggsae* may also show reduced cold tolerance. Therefore, thermal tolerance of reproductive traits may correlate with thermal robustness of other traits, such as the developmental patterning process examined here.

Species differences were most marked for pattern variants affecting P3.p, P4.p and P8.p, i.e. vulval precursor cells adopting non-vulval cell fates in the canonical situation. As found in previous studies (Braendle et al., 2010; Braendle and Félix, 2008; Delattre and Félix, 2001; Pénigault and Félix, 2011a), *C. briggsae* strains showed a significantly higher proportion of individuals with P3.p adopting the 4° fate compared to *C. elegans* strains (at all temperatures). Additionally, even at the standard temperature of 20°C, P4.p and P8.p showed a low, yet consistently elevated propensity to adopt the 4° fate instead of the canonical 3° cell fate (Class C, variants #12 to 14), which further increased at both temperature extremes. Similarly, increased Class C variants for *C. briggsae* strains have been observed after exposure to starvation conditions (Braendle and Félix, 2008). In *C. elegans*, the proportion of the same variants was increased only at 30°C, yet more so in N2 than in PB306. Although their frequency was modulated by both temperature and genotype, Class C variants were overall most frequent, indicating that fate specification of P4.p and P8.p is sensitive to environmental perturbations, particularly in *C. briggsae*.

3.4.2. Different features of the vulval patterning process vary in their environmental sensitivity in a genotype-dependent manner

C. elegans strains generated more defects than *C. briggsae* strains in response to 30°C, with specifically induced vulval hypoinduction in the N2 strain. The triggering of this developmental defect was primarily due to adoption of non-vulval cell fates (3° fate) by P5.p and P7.p while the 1° fate of P6.p remained unperturbed. Temperature perturbations therefore preferentially disrupted induction of 2° rather than 1° vulval cell fates, and their corresponding Pn.p cells. Consistent with these observations we found that high temperature modulates the Delta-Notch pathway – the central pathway for 2° fate specification – through reduction of its activity in 2° fate cells and an increased activity in the 1° fate cell. This temperature effect on Delta-Notch activity may result through increased activity in P6.p alone (e.g. through down-regulation of the EGF-Ras-MAPK pathway), which would then weaken lateral activation of the Delta-Notch pathway in P5.p and P7.p. Alternatively, Delta-Notch and/or EGF-Ras-MAPK are directly affected in all three cells, P5.p to P7.p. In either case, the observed effects indicate a weakened cross-talk between EGF-Ras-MAPK and Delta-Notch pathways, resulting in a reduced reinforcement of the 2°-1°-2° vulval fate pattern. The lower activity of Delta-Notch in 2° fate cells suggests lower levels of vulval inductive signal, consistent with the frequent hypoinduction defects of these cells in *C. elegans* N2. However, decreased Delta-Notch activity at 30°C in 2° fate cells was asymmetric, more frequently affecting P5.p than P7.p. It is unclear why P5.p is more sensitive to high temperature than P7.p, but several studies indicate that *C. elegans* vulval precursor cells, including P5.p to P7.p, differ in expression of Wnt-regulated Hox genes involved in vulval competence and sensitivity to inductive signals (Clandinin et al., 1997; Pénigault and Félix, 2011a, b). Therefore, vulval precursor cells are not equivalent in their potential to adopt vulval cell fates, and our results suggest that they further show distinct sensitivities to a same environmental perturbation.

3.4.3. Environmental and mutational perturbations reveal genotypic biases in the production of vulval developmental variants

Comparison between vulval developmental variants induced by temperature extremes versus 250 generation of mutation accumulation (MA) (Braendle et al., 2010) indicate that environmental and genetic perturbations may affect the same features of this developmental system. For example, adoption of the 4° fate by P4.p and/or P8.p (Class C variants), is overall

most easily induced by either type of perturbation in both *C. elegans* and *C. briggsae*. In contrast, induction of the 1° fate, usually by P6.p, seems generally most robust, i.e. least affected by different perturbations as shown in this study and before (Braendle et al., 2010; Braendle and Félix, 2008). Certain Pn.p cells and their specific properties (e.g. competence, cell fate) are therefore more sensitive to both environmental and genetic perturbations. Moreover, genotype-dependence of the frequency and type of vulval patterning variants may occur, so that a given genotype produces a biased spectrum of variants irrespective of the type of perturbation. Examples include the near-absence of certain variant patterns (centering on P7.p, hyperinduction) in *C. elegans* N2, which are regularly observed in other strains, or the increased frequency of Class C variants in *C. briggsae* relative to *C. elegans* strains (Braendle et al., 2010; Braendle and Félix, 2008). Furthermore, in this study we found that *C. elegans* N2 showed an increased tendency for hypoinduction errors (yet no hyperinduction errors) in response to high temperature. In contrast, hypoinduction errors were rare (0.2%) in *C. elegans* PB306. One potential explanation for this difference is that basal levels of the vulval inductive signal are higher in PB306 than in N2, consistent with previous measurements of EGF-Ras-MAPK pathway activity in these two strains (Braendle et al., 2010). Thus, if high temperature similarly reduced vulval induction in both strains, this reduction would not be sufficient to elicit hypoinduction errors in PB306. A similar difference in the production of hypo- versus hyperinduced variants between N2 and PB306 has also been observed after MA (Braendle et al., 2010): hypoinduction was rare for PB306 but common in N2, while hyperinduction was frequent in PB306 but virtually absent in N2. Both MA and high temperature therefore induced a consistent, genotype-biased pattern of hypo- versus hyperinduced variants, which are congruent with the difference of basal EGF-Ras-MAPK activity detected in these two *C. elegans* strains (Braendle et al., 2010). These observations suggest that specific properties of the vulval developmental system, such as the inductive signal level, are more sensitive to both environmental and genetic perturbations; and this sensitivity may consistently vary among different genotypes. Understanding such developmental and genotypic biases in the production of developmental variants is relevant as they are indicative of the accessible phenotypic spectrum, which modulate potential evolutionary trajectories (Arthur, 2004; Braendle et al., 2010; Dichtel-Danjoy and Félix, 2004; Félix, 2012b; Yampolsky and Stoltzfus, 2001).

Overall, however, the frequency and type of induced vulval pattern variants observed for a given strain in response to temperature treatments did not recapitulate the patterns observed after MA. For example, in *C. elegans* N2 we repeatedly found P8.p fusion (variant

#13) in response to temperature perturbations, yet this variant was never found after MA (Braendle et al., 2010). Such comparison between mutationally and temperature-induced developmental variants is obviously limited given that MA induces a wide spectrum of random mutation while temperature represents only one specific condition out of an infinite range of environmental conditions to assess. Moreover, vulval pattern variants induced by MA occurred at very low frequencies, making the detection of such correlations between the two treatments difficult. Taken together, the currently available data suggest that there is environmental specificity in the induction of variants, which is further genotype-dependent. For example, Braendle & Félix (2008) found that starvation exposure in the L2 stage consistently induced shifts of vulval patterns centered on P5.p (variant #6) in *C. elegans* (N2 but not in other *C. elegans* or *C. briggsae* strains). These examples indicate that environmental sensitivity of the vulval developmental system depends on subtle interactions between specific environments and genetic background.

3.4.4. Characterizing cryptic genetic variation to study developmental evolution of environmental sensitivity

The observed genotype-dependence of vulval variant production in response to temperature reflects cryptic genetic variation uncovered by environmental perturbations, and thus corresponds to genotype-by-environment interactions. Because many of the temperature-induced variant patterns represent defects or deviants decreasing precision of the pattern establishment, this variation reflects evolutionary variation in environmental sensitivity of vulval development. Such evolutionary variation in the environmental sensitivity of the vulval patterning output can – as explained above – likely be traced back to evolutionary variation in underlying system parameters, including differential activity of vulval signalling pathways (Braendle et al., 2010; Braendle and Félix, 2008; Duveau and Félix, 2012; Felix and Barkoulas, 2012; Milloz et al., 2008) or different levels of competence in VPCs (Braendle et al., 2010; Braendle and Félix, 2008; Félix, 2007; Pénigault and Félix, 2011a). It remains the open question, however, to what extent such cryptic genetic variation arises because of the vulval system's robustness to genetic and environmental variation and whether this variation is largely selectively neutral. As shown here, cryptic genetic variation corresponds to genetic variation in environmental sensitivity and therefore may also include adaptive variation contributing to developmental robustness, for example, resulting from evolution in divergent environmental conditions.

Chapter 4

4. Conclusions and perspectives

4.1. General conclusions

In my PhD research, I aimed to address the fundamental problem of how molecular and developmental processes respond to environmental variation. I addressed this problem using the network of *C. elegans* vulval signalling pathways as a model system (Figure 4.1). Specifically, I characterized the mechanisms by which environmental signals alter activities and interplay of the vulval signalling pathways. In addition, I examined evolutionary variation in environmental sensitivity of the vulval developmental system.

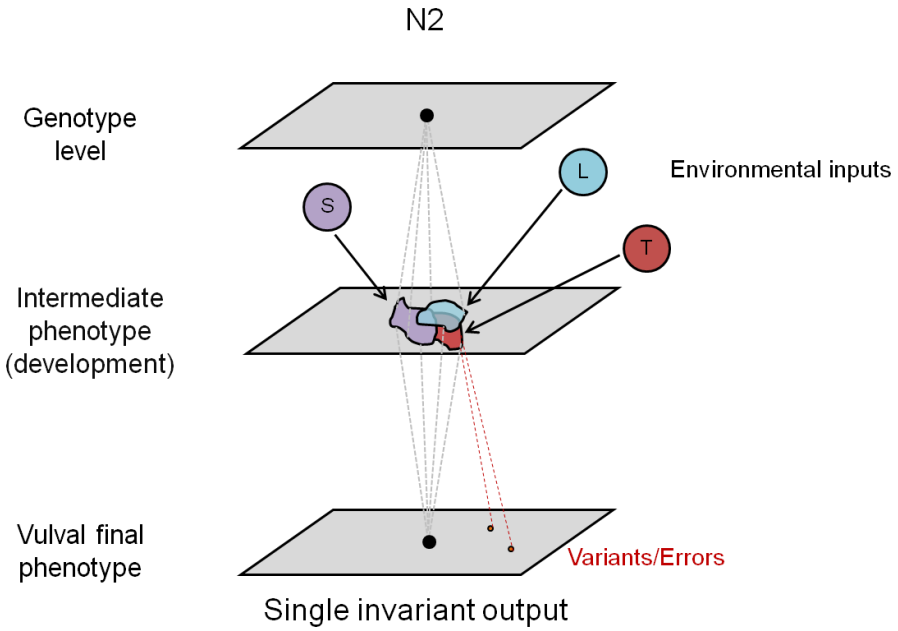

Figure 4.1. Environmental sensitivity of *C. elegans* vulval cell fate patterning. The single *C. elegans* N2 genotype generates an invariant vulval output under various environmental conditions (S: starvation, L: liquid, T: high temperature). The effects of these different inputs can overlap at the intermediate level. Nevertheless, some extreme inputs like heat stress (T) can generate developmental variants/errors at low frequency.

To address these objectives I used two different and complementary approaches. First, studying the effect of a specific environmental input (i.e. starvation) on *C. elegans* vulval development allowed me to show that nutrient sensing mechanisms increase vulval inductive levels via EGF-Ras-MAPK upregulation (Chapter 2). Second, comparing the response of the

vulval patterning system to extreme thermal perturbation across different species and strains allowed me to characterize how sensitivity of different system features are modulated by specific interactions between environmental perturbation and genetic background (Chapter 3).

This study provides insights into the universal, yet rarely studied environmental dependence of biological processes and indicates how specific environmental factors interact with key molecular signalling pathways. These pathways, such as EGF-Ras-MAPK, are strongly conserved and play crucial roles in human development and disease, such as cancer. Understanding the environmental sensitivity of this and other pathways therefore may also contribute to a more comprehensive view of how genes and environment interact in the origin and progression of pathologies, and are thus relevant to biomedical research.

4.2. Nutrient deprivation modulates EGF-Ras-MAPK pathway activity during *C. elegans* vulval induction (Chapter 2)

4.2.1. Summary

How molecular processes integrate environmental information is of fundamental importance to understand organismal development. Here we aimed to quantitatively characterize how starvation signals modulate *C. elegans* vulval development, a robust, yet environmentally sensitive process. Our main objective was to integrate previous, seemingly contradictory, observations of how growth conditions modulate vulval inductive signalling. We present quantitative analyses of starvation effects on *lin-3/egf* reduction-of-function mutations, demonstrating that this environmental stimulus has a strong positive effect on vulval induction. We show that starvation suppression of *lin-3/egf(rf)* does not rely on Wnt signalling as suggested by previous studies. Testing various candidate mechanisms that could transduce the observed starvation effects, we find that compromised DAF-2-Insulin or DAF-7/TGF-β signalling does not abolish *lin-3/egf(rf)* starvation suppression. Instead, nutrient-deprived animals induced by mutation of the intestinal peptide transporter *pept*-1 (in a food-rich environment) strongly mimicked *lin-3/egf(rf)* starvation suppression, and we find that reduction of *pept-1* activity is sufficient to increase both EGF-Ras-MAPK and Delta-Notch pathway activities. These and additional experiments indicate that positive starvation effects on vulval induction occur via modulation of the central nutrient-sensing *let-363/TOR* pathway, acting at the level or upstream of LET-23/EGFR (Figure 4.2). Taken together, our results present evidence for a novel cross-talk between TOR and EGF-Ras-MAPK signalling

during *C. elegans* vulval induction, illustrating how specific environmental signals modulate activity of major signalling pathways.

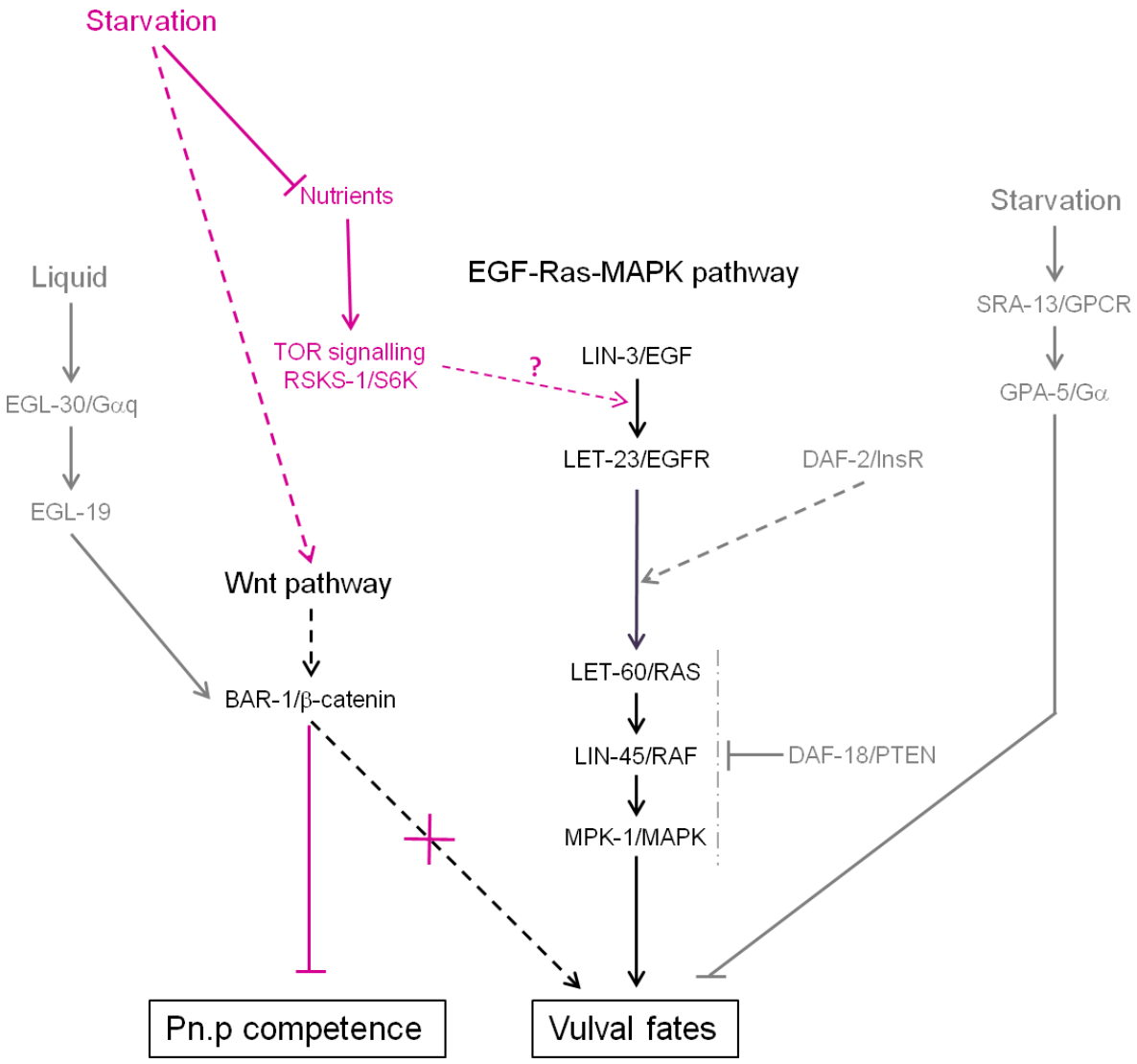

Figure 4.2. New insights on external and internal cues influencing *C. elegans* vulva development. Summary of studies reporting environmental and metabolic modification of *C. elegans* vulval induction. In grey: Braendle & Félix (2008), Nakdimon et al., (2012), Moghal et al., (2003) and Battu et al., (2003), in pink : this study. See text.

4.2.2. Perspectives and future experiments

Future experimental objectives based on this work will aim to characterize the precise nature of the interaction between TOR and EGF-Ras-MAPK signalling pathways in standard and starvation conditions (Figure 4.3).

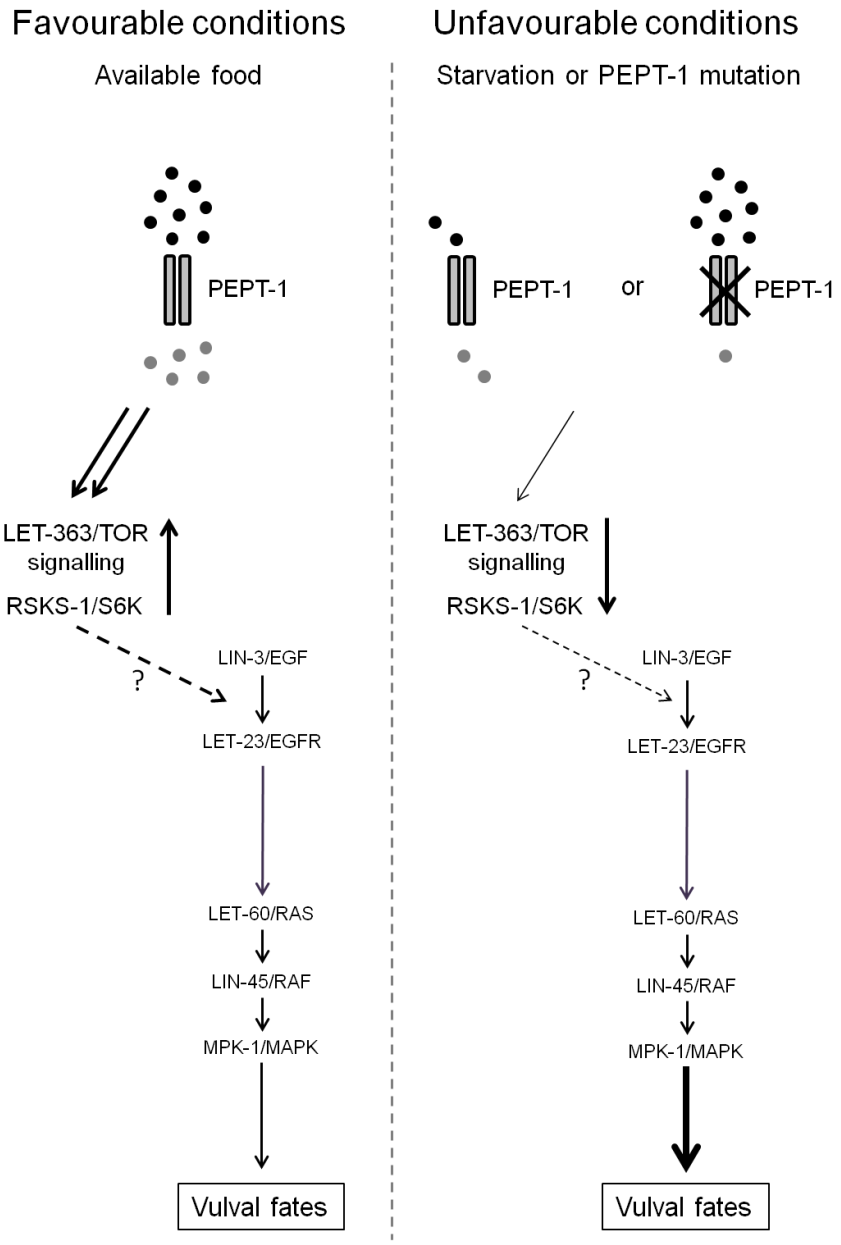

Figure 4.3. Model of the interplay of TOR and EGF-Ras-MAPK in C. elegans vulval development. Under favourable conditions, TOR signalling activity is high in response to nutrient availability. Under starvation condition (mimicked by *pept-1(0)* mutation), TOR activity is decreased causing an increase in EGF-Ras-MAPK activity.

rsks-1/S6K RNAi suppression of *lin-3/egf(rf)* highlights a role of the TOR signalling pathway in the regulation EGF-Ras-MAPK activity. By using RNAi of other members of the TOR cascade (e.g. *daf-15/Raptor* or *let-363/TOR*), we now have to confirm the involvement

of the TOR signalling pathway in EGF-Ras-MAPK modulation under food and starvation conditions. We will then study how the TOR signalling pathway ultimately affects the vulval signalling network. To do so, we will aim to target TOR signalling in specific target cells. We will knock-down TOR activity in vulval precursor cells and the anchor cell, given that the TOR signal appears to act the level of LET-23/EGFR or LIN-3/EGF. This can be achieved using cell-specific RNAi. Corresponding strains for vulval precursor cells and anchor cell (AC) have recently been generated (M. Barkoulas and M.-A. Felix, ENS, Paris).

In complement to these experiments, we will investigate whether the EGF-Ras-MAPK pathway activity relates to differences in LIN-3/EGF production by the anchor cell (AC). To do so, we can quantify *lin-3/egf* expression levels in food versus starvation environment using single molecule fluorescent *in situ* hybridization (smFISH) (Ji and van Oudenaarden, 2012). This experiment would also indicate whether LIN-3/EGF may be expressed in other tissues than the anchor cell (AC) upon starvation, which could also potentially explain increased EGF-Ras-MAPK activity.

Although the involvement of sensory system response in the positive starvation effects is not supported by my experimental results, we cannot completely exclude an additional minor contribution of sensory signalling in this response. We will therefore test whether specific sensory defects, e.g. in different neurons or specific chemoreceptors, may modulate starvation effects. For example, we will use mutations disrupting sensory neuron morphology (e.g. *daf-6, daf-19)* (Perens and Shaham, 2005; Swoboda et al., 2000) and chemosensation (e.g. *che-2, osm-5, sra-13, nph-1*) (Battu et al., 2003; Haycraft et al., 2001; Vowels and Thomas, 1992; Winkelbauer et al., 2005). These mutations will be assayed in food versus starvation conditions in a *lin-3/egf(rf)* genetic background.

Finally, we still need to clarify the role of DAF-2-Insulin signalling in the observed starvation effects on vulval signalling. Our current results suggest that that Insulin signalling mediates a negative starvation signal and acts in parallel to the positive starvation signal (this study; (Battu et al., 2003)). Future experiments should explicitly test whether the effect of starvation on *lin-3/egf(rf)* acts exclusively via LET-363/TOR but not DAF-2-Insulin. Indeed, *pept-1* RNAi display an higher suppression of *lin-3/egf(rf)* than starvation, suggesting a potential downregulation of this positive signal. This experiment could be performed using by examining *pept-1* RNAi effects on *daf-2(rf); lin-3(rf).* If, as our current results suggest, DAF-2-Insulin does negatively contribute to starvation effects, *pept-1* RNAi suppression of this double mutant should be lower than in the simple *lin-3/egf(rf).*

4.2.3. Significance

This project sheds light on an important question in current biological research: how do major signalling pathways respond to environmental variation? This study reveals that specific environmental signals, i.e. starvation, can significantly alter the activity of a key molecular cascade, the EGF-Ras-MAPK cascade. Moreover, the experimental system using *lin-3/egf(rf)* mutations shows that mutational penetrance is highly sensitive to environmental variation, suggesting that the expressivity of deleterious mutation may be modulated by the environment. Understanding the environmental sensitivity of both pathway activity and mutational penetrance are likely very relevant to biomedical research.

EGF-Ras-MAPK and TOR are key pathways that control cell survival, proliferation, motility, and metabolism. Components of these pathways were among the first discovered proto-oncogenes. Cancer is caused by a complex interplay of genetic and environment. Understanding cancer onset and development therefore requires better knowledge of how major oncogenes respond to environmental fluctuations. Studying specific environmental effects on *C. elegans* vulva signalling pathways, e.g. through analysis of interactions between EGF-Ras-MAPK and TOR, may thus provide an excellent model system to elucidate such fundamental and biomedically relevant questions.

4.3. Thermal perturbations reveal evolution of environmentally sensitive parameters in Caenorhabditis vulval development (Chapter 3)

4.3.1. Summary

Understanding the robustness of developmental systems requires insights into the sensitivity of underlying molecular and cellular parameters to perturbations, and how such sensitivity evolves. To address these issues we examined *Caenorhabditis* vulval cell fate patterning – a system that is simple and robust to diverse perturbations. We applied strong temperature perturbations to maximally disrupt this developmental system, allowing us to quantify and compare environmental sensitivity of different system parameters between distinct genotypes of *C. elegans* and *C. briggsae.* Thermal perturbations induced diverse patterning variants, including defects. Common variants reflected loss of competence in vulval precursor cells and errors in cell fate induction and differentiation. The frequency and spectrum of such variant

patterns were, however, strongly species- and genotype-dependent, suggesting that the environmental sensitivity of specific system properties is subject to evolutionary changes. High temperature induced a genotype-specific decrease of vulval induction in the *C. elegans* N2 strain caused by frequent hypoinduction of the 2° fate cells, P5.p and P7.p. In contrast, hypoinduction of the 1° fate cell, P6.p, was never observed. Precursor cells and associated fates therefore differ in temperature sensitivity, and this cell-specific sensitivity shows evolutionary variation (Figure 4.4).

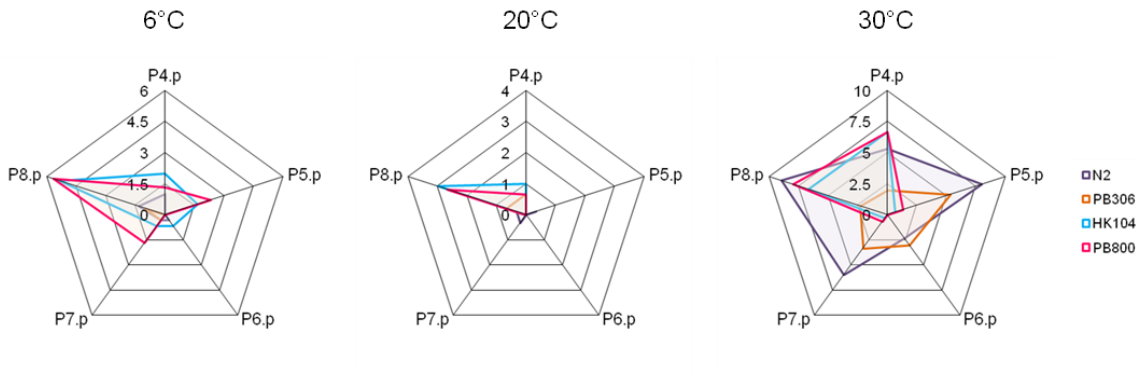

Figure 4.4. Environmental sensitivity of vulval precursor cells depends on genotype and environment. Frequency of deviations from the canonical cell fate pattern for each vulval precursor cell (P3.p to P8.p) for C. elegans N2 and PB306 C. briggsae HK104 and PB800. (A) 6°C, (B) 20°C and (C) 30°C. The mean percentage of individuals with a given cell showing a vulval variant/deviant pattern are represented in the diagrams. (N=15 experimental repeats, N=300 individuals per strain/temperature, N=900 per strain).

We further compared spectra of temperature-induced vulval variants to the ones induced by mutation accumulation in the same genotypes and found that a subset of variants is more easily induced by either perturbation, yet in a genotype-dependent manner. Taken together, our results detail how sensitivity of different system parameters underlying *Caenorhabditis* vulval development are shaped by subtle, specific interactions between environmental perturbation and genetic background.

4.3.2. Perspectives and future experiments

Experimental results uncovered quantitative evolutionary variation in thermal sensitivity of the *Caenorhabditis* vulval network. Extreme temperature induced diverse developmental variants and defects, which were strongly genotype- and species-dependent. The frequency of certain developmental defects induced by extreme temperature reveals that the vulval precursor cells and the associated signalling pathways display differential environmental sensitivity (Figure 4.4).

From a mechanistic perspective, it will be interesting to better understand how high temperature results in the triggering of specific vulval variants, such as the high frequency of hypoinduction found in *C. elegans* N2 at 30°C. This could be done by first examining the potential role of the heat-shock response system by reducing the activity of some specific particular genes (e.g. *hsf-1, daf-21/hsp-90, hsp-70*) (Birnby et al., 2000; Garigan et al., 2002; Heschl and Baillie, 1990). In addition, one could further test whether additional thermal responses are involved in the mediation of this effect, for example, through analysis of thermal sensory responses mediate by the nervous system (e.g. analysis of mutations in *ttx-1, cmk-1, tax-4, ncs-1*) (Eto et al., 1999; Gomez et al., 2001; Hedgecock and Russell, 1975; Komatsu et al., 1996).

From an evolutionary perspective, the results provide new insights into evolution of developmental sensitivity and robustness to environmental perturbations. It would be valuable to specifically test whether temperature adaptation of different species and strains can explain the observed variation in thermal sensitivity of the vulval patterning process. Here we only analysed four different strains from two species, and such inferences are difficult to make. However, the results are consistent with previous observations that *C. briggsae* is more heat-tolerant than *C. elegans* (Prasad et al., 2011). To perform a more quantitative and more conclusive analysis on the evolution of developmental sensitivity to thermal stress, one could examine a large number of *C. briggsae* isolates, which differ in their climatic origin and which group into different clades according to these climatic regions (Prasad et al., 2011). *C. briggsae* groups into tropical versus temperate clades, so it would be possible to specifically compare isolates of these two groups.

4.3.3. Significance

Applying such strong environmental perturbations enabled us to detect environmental sensitivity of different system parameters as well as their evolutionary variation. The observed

genotype-dependence of vulval variant production in response to temperature reflects cryptic genetic variation uncovered by environmental perturbations, and thus correspond to genotype-by-environment interactions. Because many of the temperature-induced variant patterns represent defects or deviants decreasing precision of the pattern establishment, this variation reflects evolutionary variation in environmental robustness of vulval development. These results show that a developmental system generating an identical phenotype may not only differ in its genetic architecture but may further differ in its environmental sensitivity. Moreover, we show that environmental and mutational perturbation can generate the same genotype-biased production of vulval developmental variants, indicating that some (but not all) perturbations may affect the same system properties irrespective of the source of perturbation (Figure 4.5).

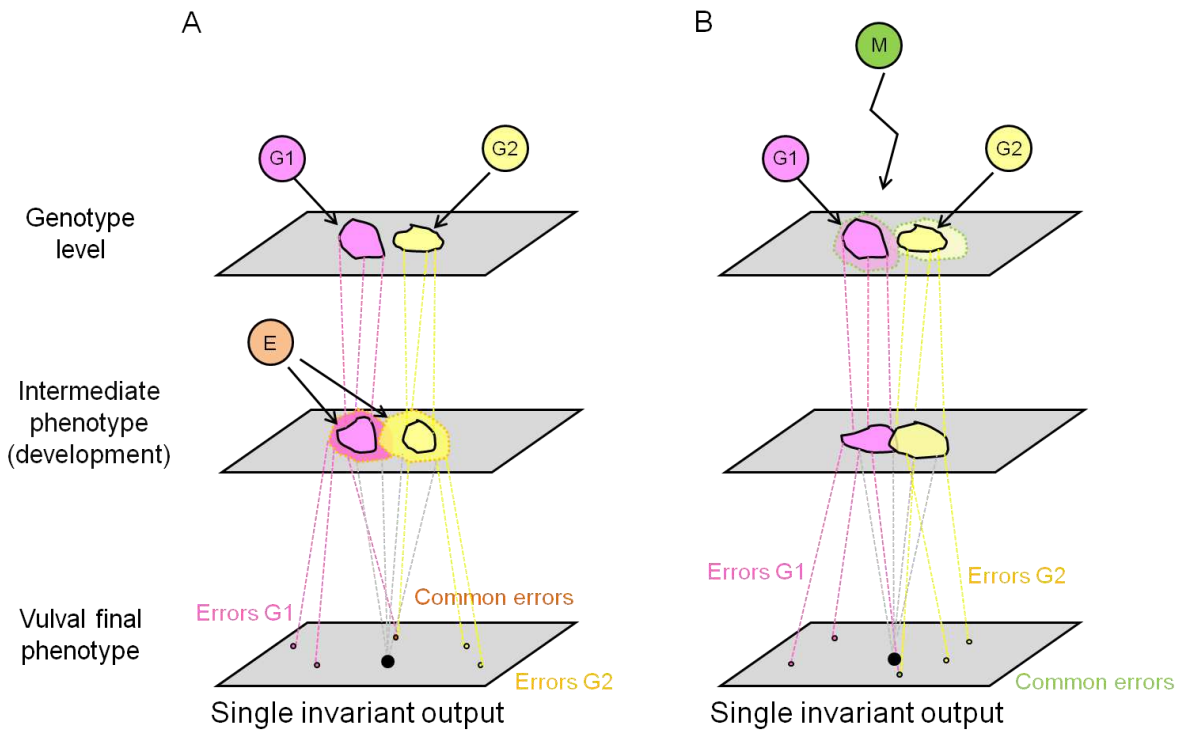

Figure 4.5. Integrating environmental and mutational inputs in the *Caenorhabditis* vulval genotype-phenotype map. Two different genotypes and their response to environment (E) or mutation (M) are represented in A and B. **(A)** The environment induces variation in the intermediate level and some variants/errors can be generated at the final phenotypic level at low frequency. **(B)** Mutation induces variation in the genotypic level and some variants/errors can be generated at the final phenotypic level at low frequency. The two genotypes can sometimes generate common errors under both environmental and mutational perturbations.

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