C h a p t e r 1

INTRODCUTION

1.1 Thesis overview

Phenotypic plasticity

Living organisms constantly interact with the surroundings they live in, including environmental stresses that might be harmful for them. Phenotypic plasticity is a way for organisms to adjust for survival under stress. For example, the water flea changes its morphology in the presence of predator to become a less favorable prey (1), and high population density can transform the behavior of the spadefoot tadpole to become cannibalistic (2). The immune system is an example in humans for constant adaptation against the invasion of foreign organisms (3). To me, it is especially interesting to think about how the genome and nervous system of an organism encode the information so that it can properly respond to the environment and maximize its fitness.

C. elegans can avoid stress by entering into dauer stage

Caenorhabditis elegans roundworms provide perhaps the best example for studying molecular basis of phenotypic plasticity. *C. elegans* can switch developmental trajectories depending on environmental conditions (**Figure 1.1**). In favorable environments, they proceed from L1, L2, L3, and L4 larvae stages to reproductive adults. When the L1 animals sense harsh stimulus, including high temperature, low food, and high population density—as measured by conspecific pheromone—they can enter an alternative pre-dauer stage, the L2d, and commit to become a dauer if the unfavorable conditions persist. Dauer larvae have specialized physiology (*e.g.* thickened cuticle) that makes them highly resistant to environmental insults, including desiccation, heat, and oxidative stress (4). Their ability to

convert stored fat to carbohydrate through glyoxylate cycle enables them to have an extended life span despite not feeding (4, 5). If environmental conditions improve, dauers can then resume reproductive development (6, 7).

This developmental choice is important for *C. elegans*, especially because making the wrong dauer entry decision can lead to a significant fitness cost (8). The animals can be disadvantaged by not having as many progeny if they misjudge environmental conditions and enter dauer at the wrong time; or they can be at risk of dying if they continue reproductive development while the environment is harsh. On the other hand, there is, of course, an energy cost to entering dauer. Dauer development requires the remodeling of tissues (*e.g.* hypodermis, intestine, gonad, and neurons) throughout the whole animal, as well as the coordination of the tissues in executing the decision. In addition, there is an opportunity cost to not reproducing when other organism are doing so.

Food, pheromone, and temperature are the three known environmental cues that *C. elegans* use to gauge the quality of the environment and make the dauer or reproduction decision (9, 6). Food signal promotes reproductive development, but the specific component of food that the animals are sensing is still unknown. Pheromone is an indicator of crowding (9). Dauer pheromone promotes dauer formation, and its several dauer-inducing components have been identified, including ascarosides: ascr#1, ascr#2, ascr#3, ascr#5, ascr#8, and indolecarboxy ascaroside icas#9 (10–12). The difference in the side chains attached to the sugar ascarylose base make different ascrosides distinct both structurally and functionally (13). While ascr#2 and ascr#3 are the most potent ones, ascr#8 can enhance their effect on dauer induction even more (14). The complex composition of dauer pheromone and the synergistic effect of ascarosides suggest there might be multiple

receptors mediating pheromone sensation. Increased temperature can also input into dauer decision by enhancing pheromone-induced dauer formation (6), suggesting the modulatory role of temperature. The importance of temperature is also highlighted by the finding that the population density of *C. elegans* in the wild and the dauer dispersal behavior are season dependent (15, 16).

C. elegans perceive environmental inputs through their amphid chemosensory organ, which includes 12 pairs of chemosensory neurons (ADF, ADL, AFD, ASE, ASG, ASH, ASI, ASJ, ASK, AWA, AWB, and AWC) (17). Through cell ablation experiments using a laser microbeam, the importance of four of the pairs in dauer decision were revealed. ADF, ASG, and ASI, were identified to inhibit dauer formation in favorable conditions (18); ASJ, on the contrary, functions to promote dauer formation in dauer-inducing conditions (19). Although how food signals are sensed by *C. elegans* is still not well understood, it was shown that ASI and AWC integrate food availability to regulate dauer decision (20). ASI, along with ASK, were also found to function in ascaroside-mediated dauer induction. G-protein-coupled receptors (GPCRs) SRG-36, SRG-37, and DAF-37 in ASI mediate the perception of ascr#5 and ascr#2 (21, 22), and SRBC-64 and SRBC-66 in ASK detect ascr#1, ascr#2, and ascr#3 (23). Further biochemical analysis on the structure of ascaroside receptors might help reveal how the specific recognition of ascarosides is achieved and expand our understanding on GPCRs in general.

Chemosensory neurons rely the environmental information to the animal through TGF-β and insulin signaling, whose activation promote reproductive and inhibit dauer development. DAF-7/TGF-β ligand is expressed solely in the ASI neurons, and its expression level is downregulated in dauer-inducing conditions (19). In the case of insulin

signaling, *C. elegans* have 40 insulin-like peptides (ILPs) (24, 25) and only one known insulin receptor ortholog, DAF-2 (26), suggesting potential complex patterns of redundancy (27). In fact, there are agonistic and antagonistic DAF-2 ligands regulating dauer decision (28, 29). Agonistic ligands that inhibit dauer formation include INS-6 and DAF-28 from ASI and ASJ, and INS-4 from motor neuron. Dauer-promoting DAF-2 ligands including INS-1 and INS-18 are from sensory neurons, but the specific neurons were not identified. Under favorable condition, high level of agonistic to antagonistic ligands promotes reproductive development. In harsh environment, on the other hand, reduced agonistic and increased antagonistic ligands facilitates animals to become dauers.

The convergent point of TGF-β and insulin signaling is the steroid hormone pathway involving DAF-9 and DAF-12 (30, 31) (Figure 1.2). DAF-9 is a P450 enzyme that synthesize bile acid-like steroids dafachronic acids (DAs), the ligands for the nuclear hormone receptor transcription factor DAF-12 (32, 33). Under favorable condition, the activation of TGF-β and insulin signaling stimulate the production of DAs, and the DA-bounded DAF-12 promote reproductive growth. When the environment is unfavorable, reduced TGF-β and insulin signaling result in unliganded DAF-12, which together with corepressor DIN-1 specify dauer development (34, 35).

The XXX cells are considered to be the integration site of TGF-β, insulin and the steroid hormone pathway for several reasons. First of all, XXX is the main source of *daf-9* expression and thus DA synthesis. *daf-9* expression was observed in only three tissues, XXX, the hypodermis, and the spermatheca (30, 31). While *daf-9* is expressed in XXX expression is at all stages, the hypodermal expression is highly variable depending on the environmental inputs and the spermatheca expression only exists in adult. It was shown

that under favorable condition, DA originating from XXX amplifies the hypodermal *daf-9* expression in a *daf-12* dependent manner, and the positive feedback loop ensure the reproductive development (Figure 1.2) (30, 31, 36). Second, many components involved in dauer regulatory pathways are also expressed in the XXX cells (*e.g. sdf-9*, which regulates both steroid hormone and insulin-like pathways) (37–39), further highlighting the important role of XXX in the decision. Finally, the XXX cells are required for L2d animals to bypass dauer and carry out reproductive development when the environmental condition improves (36). Notably, for L1 animals, the XXX cells are sufficient but not necessary to grow into adult under favorable condition (30, 31, 37), suggesting there might be a compensation mechanism for the loss of the XXX cells at early developmental stage.

Although a wealth of knowledge regarding the molecules and signaling pathways involved in the developmental decision have been accumulated, many aspects of the decision are still not well understood.

At the sensory sensation level, first of all, it was suspected that there might be other environmental inputs controlling dauer decision in addition to food, temperature, and pheromone (40), but no report has proven the idea yet. In the Appendix chapter, we demonstrated for the first time that touch is an overlooked input, and that touch promotes dauer development. One plausible explanation is that touch serves as a population density indicator on top of pheromone. Touch as an input into the dauer decision also opens up the possibility that other environmental signals, such as light, O_2 and CO_2, can also contribute to the developmental choice.

Moreover, it is not clear how the sensory neurons interact with each other. Since ascaroside and food signals have opposing effects on dauer entry decision, do ascaroside-

sensing and food-sensing neurons modulate each other's activity? As food-to-pheromone ratio, rather than their absolute amount, is important for dauer recovery (9), is it possible for dauer entry that the food and pheromone also affect each other's threshold, and that the signals are summed up at an interneuron? Reproductive-promoting insulins were not expressed in the food-sensing neuron AWC, and the pheromone and temperature sensing neurons ASK and AFD were not identified to express *daf-7* or dauer-promoting insulins (19, 20, 28, 29). It is possible that the endogenous genes are actually expressed in those neurons, but because of the promoter region included in the transcriptional reporter, the observed anatomic expression pattern does not completely reflect that of the endogenous gene. In the case that the expression patterns are correct, it then raise the question of how AWC transduce the food information to the TGF-β and insulin signaling neurons, including ASI and ASJ. Similarly, how do ASI and ASJ receive the pheromone and temperature information from ASK and AFD? They might be connected together directly through physical connection, or indirectly through interneurons. We could obtain a more comprehensive understanding of the neuron circuit controlling the information relay by studying the neuronal connections, and genetically or optogenetically manipulating the neuronal activities.

There are also multiple parts unknown at the signaling transduction and integration level. First, while the initial genetic screens looked solely for Daf-c (dauer formation abnormal constitutive) or Daf-d (dauer formation abnormal defective) mutants and identified main components and signaling pathways (41, 42), the studies overlooked the modulators mediating the decision. For example, *daf-28* is the only insulin mutant identified through genetic screen (23, 41), but other insulins were later discovered to play

roles (of smaller effect size) in the decision as well (28, 29). In addition, it is still unclear where the sites of action are for several signaling components. As mentioned above, TGF-β and insulin were observed only in ASI, ASJ, and motor neurons, but not in AWC, ASK or AFD (19, 20, 28, 29). Another example is the insulin receptor DAF-2. Whether DAF-2 acts predominately in nervous system or intestine to regulate the dauer decision is still inconclusive, as different results were reported suggesting one or the other (29, 44, 45). Solving the site of action mystery would improve our understanding of the spatial control of the dauer entry decision.

Although the choice between dauer or reproductive development is considered as a binary decision (36), it in fact requires the coordination across different tissues to execution of the decision. Dauer development involves the remodeling of multiple tissues, including the changes in the cuticle, muscle, nervous system, pharynx, gut, gonad, and excretory system, to meet the specialized physiological and behavioral needs of dauers (46). Partial dauer phenotype describes the mutant dauers that have incomplete or missing dauer features in some of the tissues. For instance, *daf-9(e1406)*/cytochrome P450 dauers have a non-dauer intestine, cuticle, pharynx, and neurons (42). Although studying partial dauer mutants would elucidate how the tissue-tissue signaling ensures the correct remodeling of the whole animal, our knowledge regarding it is still limited due to the limited quantitative tools. The only two available tools are SDS sensitivity and fluorescent beads for identifying dauer hypodermis and pharynx selection (47, 48), but not for other tissues.

In Chapter 2, I describe our findings that the FMRFamide family of neuropeptides helps to improve dauer entry decision-making, possibly at the level of sensory perception and signal integration. Using RNA-seq data collected from dauer- or reproductive-

developing animals, I helped discover 8,042 genes that are differentially expressed between the two developmental tracks. Neuropeptides, in particular the FMRFamide-like peptides (*flp*s), were enriched for up-regulation starting from dauer-commitment. With mutant analysis, I found that several *flp*s have opposing effects on the dauer entry decision, but overall peptidergic signaling (from insulins, *nlp*s, and *flp*s) promotes dauer development.

Understanding how the *flp* gene family is coordinately up-regulation might expand our knowledge of how dauers are programmed transcriptionally. We analyzed promoter regions of the 31 *flp* genes, but we were not able to pinpoint specific *cis*-regulatory elements that are shared within the *flp* gene family. To find upstream regulators might require unbiased genetic screen or biased RNAi screen on transcription factors on animals expressing transcriptional reporter of *flp* genes.

Neuropeptides can act as neurotransmitters that control the activity, polarity, sensitivity and even gene expression of their recipient neurons (49–51). We propose two possible mechanisms for neuropeptides to mediate the dauer decision at the input sensation or signaling integration level. First, neuropeptides might modulate the sensitivity of sensory neurons and thus the animals' perception to the environmental inputs (52, 53). We used a pheromone reporter, whose intensity correlate with the amount of pheromone sensed by the animal (23, 54), to test this hypothesis. Our preliminary data showed that pheromone sensitivity indeed is lower in *sbt-1* mutant, which have reduced levels of active neuropeptides (55) (data not shown), suggesting the modulation of pheromone sensation by neuropeptides. Further imaging analysis of the activity of different sensory neurons is necessary to make a definite conclusion. Second, neuropeptides might influence how TGF-β and insulin signaling are integrated. Previous study suggested that unfavorable

environment might inhibit reproductive development by raising the DA threshold and preventing DA amplification in the hypodermis (36). It is thus conceivable that neuropeptides might modulate XXX cells and set how sensitive it is to TGF-β and insulin signaling, or set the DA threshold in the hypodermis. To test the hypothesis might require direct measurements of XXX activity and DA levels in XXX and hypodermis.

In Chapter 3, I used the RNA-seq dataset that I analyzed in chapter 2 to identify candidate genes to use as molecular markers to selectively label dauers and non-dauers. As discussed above, the tools for identification of dauer tissue remodeling are limited to SDS sensitivity for dauer hypodermis and fluorescent beads for identifying dauer pharynx (47, 48). Through developing molecular markers, we hope to conveniently assay the dauer entry decision, and to parse the subtle phenotypes of partial dauers for better understanding of the coordinately execution of the decision. Indeed, I was able to engineer four markers that label specifically the dauer or reproduction decision, and I verified that the lighting up of the dauer fluorescent markers marks dauer commitment. I also used the markers to manipulate the decision by driving gene overexpression during dauer-commitment. Finally, by combining the markers with partial dauer mutants, I confirmed their physiological defects and uncovered previously unknown defects as well. Previous study suggested that *C. elegans* might take into account the uncertainty of the environment to make the dauer decision, but the idea has not been verified by experiments yet (56). Since the expression of the markers can be a readout of the dauer decision, it is foreseeable to utilize them to study how environmental signals are integrated over time, and how discontinuous inputs might change the dynamics of the dauer decision.

We have only explored the neuropeptide part of the huge RNA-seq dataset, and there is

still a lot of interesting information worth looking into. For instance, we noticed that many GPCRs are highly expressed at the time point preceding dauer, when the expression levels of most neuropeptides are the highest, suggesting a possible anticipatory preparation of receptors for their corresponding ligands. Moreover, studying the transcription factors that are turned on before dauer commitment (*e.g.* at L2d.26 time point) might reveal important control for the commitment decision.

Dauers have specialized behaviors

In addition to the developmental switch, entering dauer switches their behavior as well. Only dauers have the ability to nictate, a hitchhiking behavior where the animals stand on their tail and wave their body , and also only dauers are attracted to CO_2 while non-dauers are repelled by it (57–59). These two dauer-specific behaviors are thought to help dauers find carrier animals and disperse, because dauer, the most common life stage of *C. elegans* found in the wild, are often found to be associated with invertebrates and even inside the intestine of predatory slugs (60, 61).

The molecular basis regulating the nictation behavior in *C. elegans* was not characterized until the recent advance in the design of artificial micro-dirt chip for precise quantification of the behavior (62). The measurements include nication ratio (the percentage of the observation time during which the dauers spend on nictating), initiation index (the frequency the dauers start a new nictation event), and average duration (the average duration of each nictation event). Using this assay, it was shown that insulin, TGF-β, and piRNA pathways are involved in nictation behavior (63, 64). Interestingly, unlike in the dauer entry, insulin and TGF-β signaling control nictation in opposite ways

(63). Moreover, the property of the IL2 neuron are essential for nictation, including its cholinergic transmission and proper dendritic remodeling through proprotein convertase *kpc-1* during dauer (62, 65). As the mechanism controlling nictation started to unfold, there are still many missing pieces. For example, it is not known how the duration of the nictation events is controlled since all the mutants identified in previous studies are defective in only nictation ratio and initiation index. It would also be interesting to pinpoint the new connections downstream of IL2 neuron during dauer to understand how the new behavior is generated.

An animal's response to sensory stimulus can be mediated by developmental stage and life history. For example, *Drosophila melanogaster* larvae and adults showed different preference for certain fruit odors (66). For *C. elegans*, CO_2 is repulsive for non-dauers, but an attractive cue for dauers (Figure 1.3). Since CO_2 can be an environmental signal that indicates the presence of food, carriers, or predators (67, 59, 68), it suggests that dauers might use CO_2 to facilitate dispersal or recovery from dauer despite the potential risks. The CO_2 preference change was also observed in adult animals depending on their nutritional status and prior experience. The CO_2 avoidance is suppressed in food-deprived adults via insulin and TGF-β signaling (57, 58). Adults that were cultivated in higher CO_2 are attracted to as opposed to repelled by CO_2, and the preference and degree of change is mediated by the activity of four interneurons and a combination of neuropeptides (69).

Despite our understanding of the context-dependent modulation of CO_2 preference, how developmental stage switches the response to the same CO_2 stimulus in *C. elegans* is still not clear. Interestingly, a single pair of sensory neurons, the BAG neurons, is necessary for CO_2-sensing in both dauer and non-dauer (56, 57), suggesting that the CO_2 responses in

dauer and non-dauer might mediated by the distinct signals from the BAG neurons. Two simple hypotheses are that the BAG neurons secrete different molecules, or the BAG neurons have different downstream neuronal circuit connections in dauer compared to non-dauer animals. More investigations of the mechanism would provide a deeper insight of how neuronal plasticity can be engaged under stress.

In Chapter 2, I describe our findings that the FMRFamide family of neuropeptides helps enable hitchhiking/carrier-seeking behaviors (70). Using the micro-dirt chip, I observed a less vigorous nictation movement and as a result a longer nictation duration in *sbt-1* mutant compared to wild type animals. Although it might require an additional tool, such as movement tracking and nictation angle analysis, to fully capture and describe the phenotype, and it was the first mutant reported to have nictation duration defect. I also found that peptidergic signaling downstream of *sbt-1* is necessary for dauer CO_2 chemoattraction, and to our knowledge, *sbt-1* mutant was the first reported *C. elegans* duaer that avoid CO_2 like adults. Moving forward, it would be intriguing to find out how neuropeptide signaling changes the neuronal properties in dauer using calcium imaging. The change in physical connections between neurons in dauer might also contribute to the acquisition of new behaviors in dauer. As the techniques for identifying synaptic partners in living animals are advancing (71, 72), and the dauer neuronal connectome is being constructed (Mei Zhen, personal communication), a great progress in the field is conceivable.

Dauers and IJs

Dauer and the infective juvenile (IJ) stage of many parasitic nematodes are both

non-feeding and similar in morphologically (73). Therefore, it has been hypothesized that the evolution of dauer is a pre-adaption toward developing parasitism (Figure 1.4) (74, 75). The close association of nematodes with non-specific insects or invertebrate, like the hitchhiking behavior in *C. elegan*s dauer, is considered phoresy (76). In some species when the association becomes more specific, the dauers would wait for the host species to die and feed on their carcass, and it is considered necromeny (76). The relationship eventually evolved into parasitism, where the association becomes harmful for the host. The molecular similarities in regulating dauer and IJ formation have also been identified, including sensory neuroanatomy, insulin signaling, steroid hormone pathway and *daf-12* (77).

In Chapter 2, through meta-analysis, I helped discover the similar up-regulation of *flp*s in IJ stages of several parasitic nematodes, including semiobligate and obligate parasites, revealing the potential shared strategy for carrier-seeking in dauer and host-seeking in IJs. As more tools for genetic intervention, such as RNAi and CRISPR, are being developed in parasitic nematodes (78, 79), it will be possible to test the function of the neuropeptides in host-seeking in parasites and potentially develop *sbt-1* as an anthelminthic target.

Since neuropeptides, which function in modulating behaviors, can evolve over time (80, 81), it is conceivable that neuropeptide expansion could be important for the evolution of behavior. For example, the acquisition of jumping behavior in *Steinernema carpocapsae* IJ might involve neuropeptides for changing the wiring between the motor neuron and CO_2 neurons to achieve a different dynamic (59). Neuropeptides can also mediate the sensing of the internal state through connecting the intestine, to sensory, inner and motor neurons (82). All those changes could affect how active and how quickly dauers and IJs burn through their fat stores, considering the tradeoff and balance between

hibernating and actively trying to find carriers and hosts.

The study of stress in other nematodes

Extremophiles organisms have revealed much about the biology of stress-resistance, re-defined the limits of life, and have been useful to biotechnology. For example, from studying antioxidant defense in the African lungfish *Protopterus dolloi* during their stress-resistant estivation period, we have learned how human brains deal with the increasing oxidative stress associated with aging (83). Moreover, the heat-stable DNA polymerase isolated from the thermophile *Thermus aquaticus* is widely used for efficient DNA amplification in polymerase chain reactions (PCR) (84).

Nematodes have been found in a variety of hostile environments, including deep underground diamond mines (85), extreme arid soil (86), and frozen Antarctic water (87). They were even found alive after being frozen for 30,000 to 40,000 years (88). Those findings represent a fertile ground for further exploring stress response in nematodes and plasticity and resilience to stress. Especially because the stress-resistant dauer stage of *C. elegans* is well characterized (35), studying the nematodes isolated from extreme environments offer an opportunity to apply the good lessons and methodology learned from dauers to learn novel biology.

I was interested in exploring natural environmental stresses—outside of the laboratory. In Chapter 4, I describe hunting for extremophile nematodes in and around Mono Lake, an environment that is high in pH, salt and arsenic. I helped isolate and characterize nine new nematode species from the extreme environment. The diverse morphologies of the species suggest that nematodes have adapted to Mono Lake via diverse lifestyles. I also found that

Auanema tufa, which is lab-culturable, could be a potential model for studying arsenic resistance in a multicellular organism.

One of the exciting future direction is to sequence the genome of *A. tufa* and find the genes that contribute to the arsenic resistance in *A. tufa*. Especially because *A. tufa* is possibly hermaphroditic and culturable in lab, it would be easier to single out individual animal, drive the genome into homozygosity, and assemble the genome. Once we have the genome assembled, it would be interesting to look for potential gene duplication in genes important for *C. elegans* arsenic resistance. For example, there might be duplications of *gcs-1* genes, which catalyze the redox reaction of arsenic and facilitate the expel of arsenic outside of the cell (89).

Summary

When I began my PhD, the molecular correlates of the dauer commitment decision were unknown. How the tissues coordinate during the dauer entry decision was also unknown. And importantly, how dauers switch their behaviors was only partially known, in the case of the neuronal rewiring (of the IL2 neuron) that underlies nictation (65).

During my PhD, my contribution to the field is a better understanding of how *C. elegans* establishes a "new brain" to cope with stress through neuropeptide signaling. Moreover, the molecular tools I built not only open up a new way of studying and manipulating dauer entry decision, but also provide a quantitative assay for studying tissue-tissue communication in executing the whole animal developmental decision. and how nematodes have evolved to survive in harsh environments.

1.2 Figures

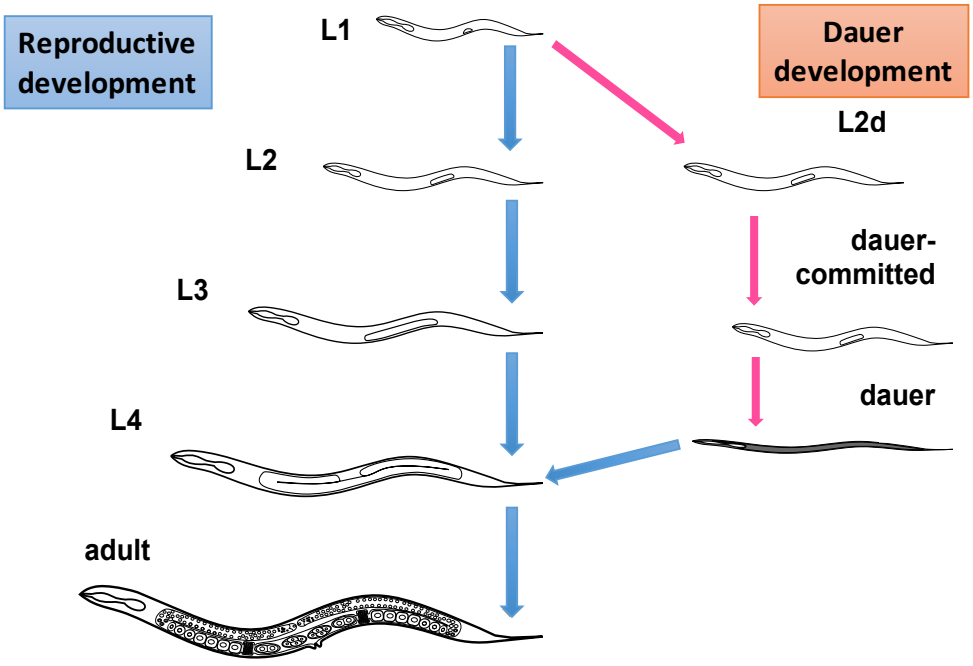

Figure 1.1. Two developmental trajectories in *C. elegans*. The blue and red arrows indicate the reproductive or dauer developmental trajectories under favorable or unfavorable conditions, respectively.

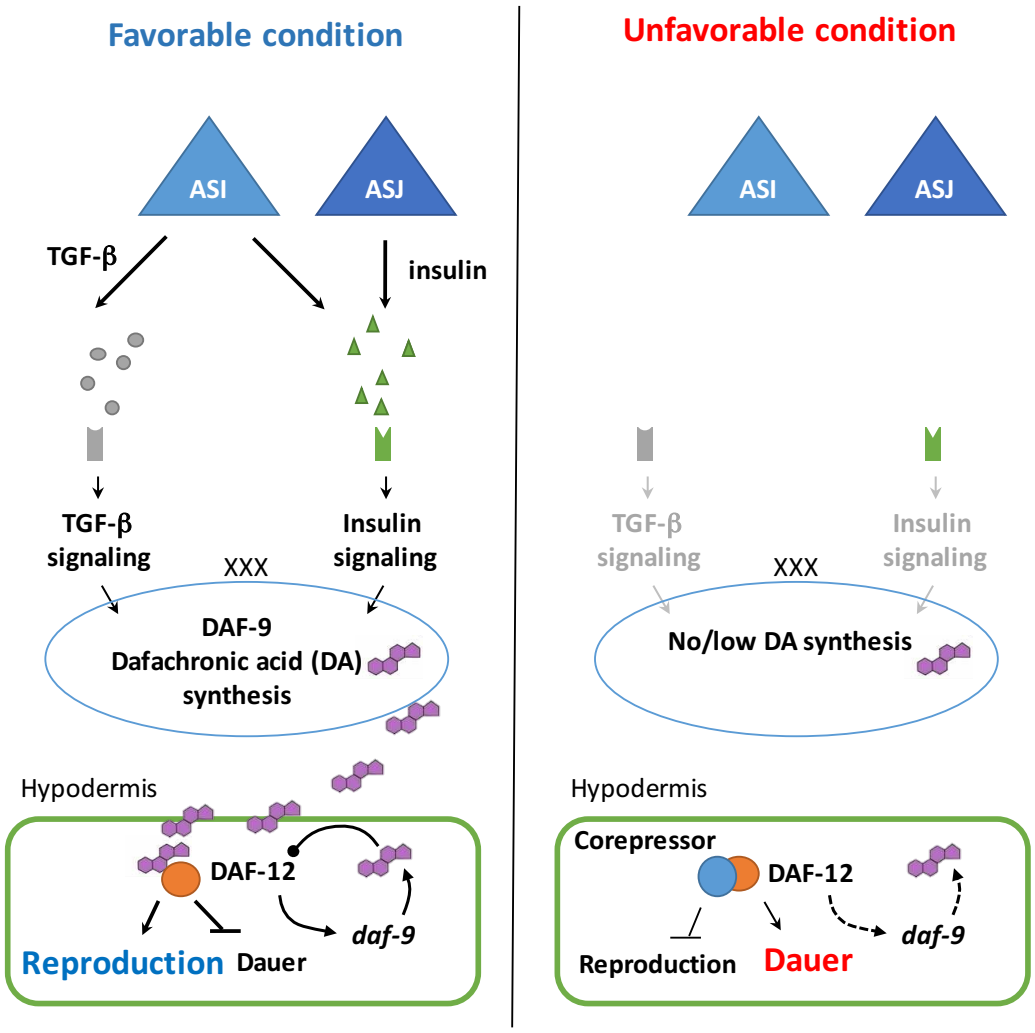

Figure 1.2. The major signaling pathways involved in reproduction and dauer developmental decision. Dafachronric acid (DA) is shown in its chemical structure in purple. Dash arrows represent reduction, and the oval arrow indicates the binding of DA to DAF-12. For simplicity, only two of the sensory neurons, ASI and ASJ, are shown.

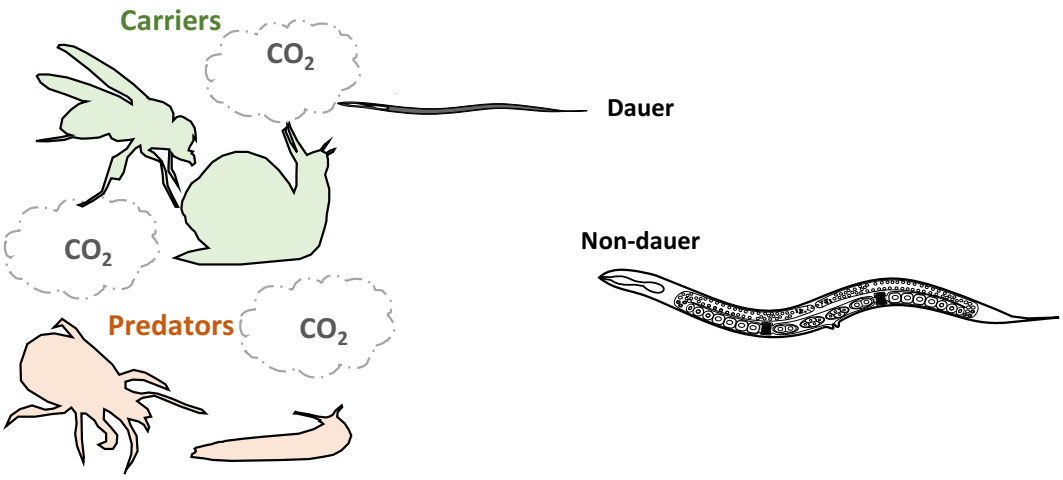

Figure 1.3. The differential CO_2 preference in *C. elegans* dauer and non-dauer. A fly and snail shown in green are potential carrier animals, and a mite and slug shown in orange are potential predators of *C. elegans*.

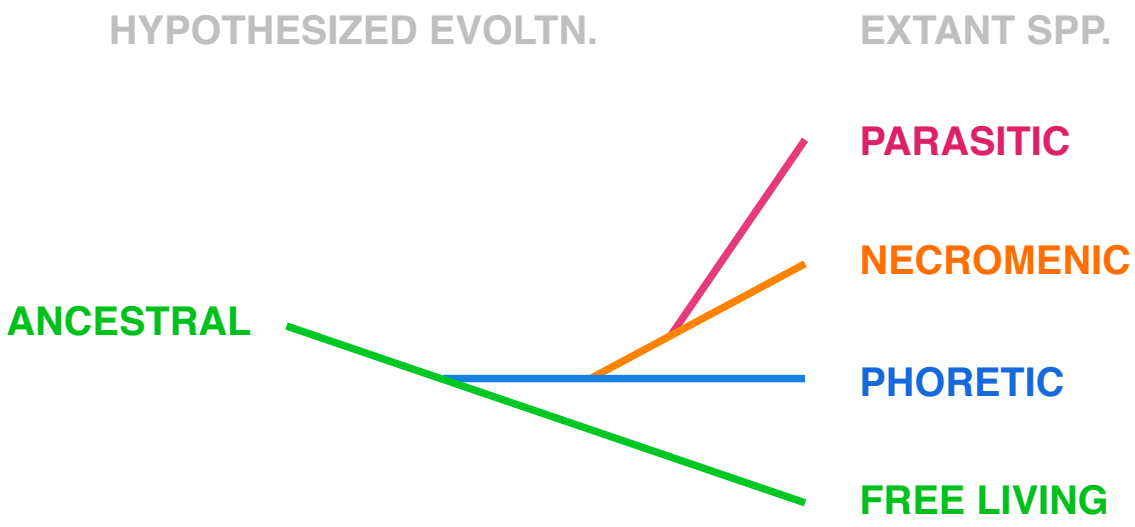

Figure 1.4 The hypothesized evolution of parasitism

1.3 References

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