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Current Opinion in Systems Biology

A primer on pheromone signaling in *Caenorhabditis elegans* for systems biologists

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Abstract

Individuals communicate information about their age, sex, social status, and recent life history with other members of their species through the release of pheromones, chemical signals that elicit behavioral or physiological changes in the recipients. Pheromones provide a fascinating example of information exchange: animals have evolved intraspecific languages in the presence of eavesdroppers and cheaters. In this review, we discuss the recent work using the nematode *C. elegans* to decipher its chemical language through the analysis of ascaroside pheromones. Genetic dissection has started to identify the enzymes that produce pheromones and the neural circuits that process these signals. Ecological experiments have characterized the biotic environment of *C. elegans* and its relatives, including ecological relationships with a variety of species that sense or release similar blends of ascarosides. Systems biology approaches should be fruitful in understanding the organization and function of communication systems in *C. elegans*.

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Current Opinion in Systems Biology 2019, **13**:23–30

This review comes from a themed issue on **Systems biology of model organisms (2019)**

Edited by **Baris Tursun**, and **Denis Dupuy**

For a complete overview see the Issue and the Editorial

Available online 31 August 2018

https://doi.org/10.1016/j.coisb.2018.08.012

Keywords

Pheromones, Chemical communication, Chemoreceptors.

Introduction

The exchange of secreted chemical signals among organisms is a common feature of life. Examples have been documented in bacteria [1], plants [2], fungi [3], and animals [4]. These molecules are particularly salient when exchanged by members of the same species, although the broadcast nature of the signals offers opportunities for eavesdropping and exploitation. Excreted chemosignals that evoke responses in members of the same species are referred to as pheromones [5]; these often consist of blends of molecules that determine a unique chemical dialect for each species and trigger changes in behavior, physiology, and development in the recipients.

Caenorhabditis elegans is an attractive model organism for the study of pheromone signaling. This millimeter-long nematode has a compact nervous system with barely more than a dozen pairs of chemosensory neurons and a roughly comparable number of interneurons [6]. Little more than a decade ago, a major class of secreted pheromones, called ascarosides, was identified in *C. elegans* [7–9]. Since then, a number of metabolomic, phenotypic, and genetic studies have explored how these ascarosides are released and sensed in *C. elegans* and related nematodes.

Here, we briefly review the known phenomenology of *C. elegans* pheromones and focus on two topics that are central to a comprehensive understanding of communication in any species. The strengths of *C. elegans* as a model system will likely make these areas the major contributions of "the worm" to the discovery of general principles of inter-organismal chemical signaling. First, we discuss the efforts to place pheromone signaling in the context of the complex, natural habitats in which it takes place. Second, we consider the means by which the nervous system perceives and processes these signals, while integrating them with other relevant information. Systems biologists have developed a rich plethora of experimental and conceptual approaches to address similar questions. We expect that their application to the fascinating world of chemical communication in *C. elegans* could considerably propel the field.

Pheromones in *C. elegans* and the "messages" they convey

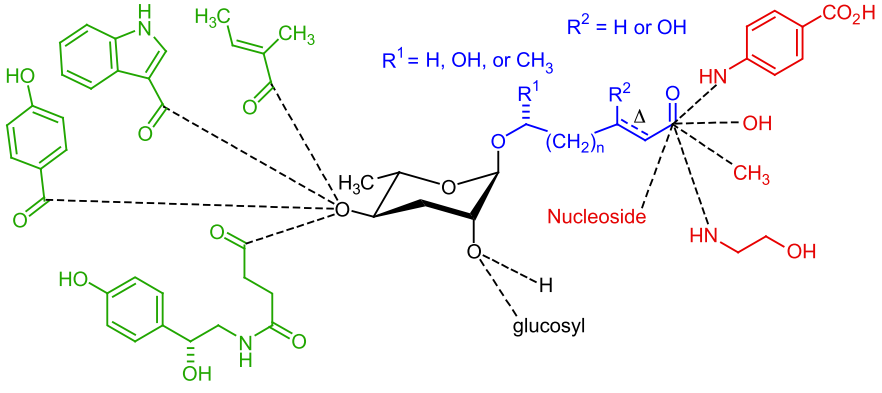
Whereas several classes of molecules are known to act as chemosignals in *C. elegans*, by far the best studied are the so-called ascaroside pheromones. In this review, we primarily focus on this class of pheromones because the available data allow us to discuss general principles that

we expect to be applicable to other classes of chemosignals. Scores of ascaroside pheromones, named after an ascarylose sugar found in the core of each molecule, have been identified in *C. elegans* (Figure 1). The diversity of ascarosides derives from the length of a short-to medium-chain fatty acid attached to this sugar and the presence of other moieties [10,11]. While several hundred ascarylose-containing molecules have been identified [12,13], only a small subset has been shown to evoke responses in the recipients (i.e., act as pheromones); functional roles of the remaining majority remain to be ascertained. Two different ascaroside naming schemes are used in the literature. In the first, following conventions in the biosynthetic literature, information regarding the chemical structure is contained in the name, e.g. asc-ΔC9 (or even C9 in older literature) contains a 9-carbon fatty acid with an α−β double bond. Additional chemical modifications that can occur on the ascarylose sugar are indicated at the beginning of the name (e.g., indole-3-carbonyl, IC) and those that can occur on the fatty acid are indicated at the end of the name (e.g., methylketone, MK). The second nomenclature, following common practice in *C. elegans* literature, subsumes this complexity under a number reflecting the order of discovery, converting asc-ΔC9 into ascr#3. Distinct abbreviations are used for ascarylose-containing molecules that contain additional moieties, such as when the sugar is decorated with an indol (icas#), octopamine succinate (osas#), hydroxybenzoyl (hbas#), or methyl-butenoyl (mbas#) [11,14]. Even more complex compounds have been detected in other nematodes, including the use of the isomer paratose in place of the ascarylose sugar, and the use of a sugar derivative of the nucleoside adenosine as a side chain [15].

The functions of *C. elegans* pheromones were first studied in the context of dauer formation, an alternative developmental morph that facilitates survival of adverse conditions and dispersal [16]. "Dauer pheromones" convey population density, information that is assessed together with food availability by recently hatched larvae. It is now understood that the dauer pheromone is a blend of at least five synergistically-acting ascarosides [7−9,17−19]. In addition to biasing developmental plasticity, a subset of ascarosides can affect social behaviors, including mate finding/attraction [9,19,20], aggregation [9,13,14,20], repulsion [21], exploratory movement [22], and pseudo parent-offspring interaction [23]. There is also a growing recognition that ascarosides can alter organismal physiology, particularly the germline function [24−26], olfactory plasticity [27], and aspects of metabolism [28]. The totality of the physiological effects of pheromones manifests in the alteration of aging and lifespan, the mechanisms of which are only beginning to be understood [25,29−31]. In general, different combinations and concentrations of ascarosides regulate a number of traits.

Although, in most cases, the specific "meanings" of pheromone signals remain to be determined, the amount and identity of molecules released by animals depend on several biological factors, suggesting that the status of the sender is communicated. For example, pheromones could convey information about the age [32], stress [33], sex [20], diet [32], and population density [16] of the emitter. How is this achieved? One possibility is that certain aspect of pheromone production might require chemical precursors that are produced by other species in the environment. Release of a particular ascaroside could indicate the presence of this species (e.g. to communicate information about diet). Several recent studies suggest that changes in the composition of ascaroside blends may be achieved by altering the relative amounts of the biosynthetic enzymes involved in pheromone production [13,34−36]. For example, expression levels of key enzymes differ between males and hermaphrodites, consistent with the

Figure 1



Overview of the chemical structure of the ascaroside pheromones, summarizing the diversity of hundreds of unique molecules identified in nematode secretions. Each ascaroside contains an ascarylose sugar (black) attached to a fatty acid of variable length (blue). Additional moieties (R^1, R^2, red, and green) could decorate the ascarylose and the fatty acid.

different abundance of sex-specific ascarosides [20]. There is an intriguing possibility that enzymes may directly read out metabolic state of the animals and change ascaroside production accordingly [35]. Recently, an enzyme was identified that could shorten the fatty acids side change length in existing indolated ascarosides which signal for aggregation behavior [37]. The shortened ascarosides induced receiving worms to disperse, suggesting that regulation of this enzyme could be used to modify the signals released by animals as conditions changed. Despite this recent progress, much work remains to be done to discover the mechanisms that coordinate the production of pheromone messages with specific environmental conditions.

Growing evidence suggests the existence of non-ascaroside pheromones in *C. elegans* because certain types of chemical communication are retained in the mutants defective in ascaroside production [38,39]. For example, 1) hermaphrodites still faithfully report the presence of sperm in the gonad [38,40], 2) both males [25] and hermaphrodites [41] produce substance(s) that accelerate larval development, and 3) indicators of alarm [42] and larval population density [43,44] are still being made. The molecular nature of these and other non-ascaroside signals remains to be discovered. Recent developments in this direction include a detailed characterization of the exometabolome of mutants that are unable to produce mature ascaroside pheromones [12].

Crosstalk and eavesdropping in natural environments

The purpose of pheromones is to convey social information, primarily regarding the location and status of potential mates and competitors, in complex biotic environments. The natural habitat of *C. elegans* is rotting fruits, flowers, and plant stems [45]. After a small handful of animals colonize a new locale, they consume bacteria growing on the decomposing matter. Given the generation time of ~3 days and brood sizes of hundreds of offspring, the population rapidly expands until the food sources are exhausted [46]. Caught in a nutritionally-deprived environment, many larvae become dauer and disperse, thus re-initiating the boom-and-bust cycle of population growth. In the wild, *C. elegans* hermaphrodites primarily reproduce by self-fertilization, infrequently outcrossing with rare males [47–49]. Consequently, local populations are highly inbred with the majority of individuals being homozygous for most of the genome. Only a small number of haplotypes stably persist within a geographic area over several years, comprising dozens of generations [46]. Different lineages display variation in release of and response to pheromones [50–53]. It remains important to understand whether this reflects independently evolving honest signaling (one that faithfully reflects the condition of the sender, e.g. Ref. [25]) that is private among kin or a weapon used to deceive competitors that occupy the same niche [54].

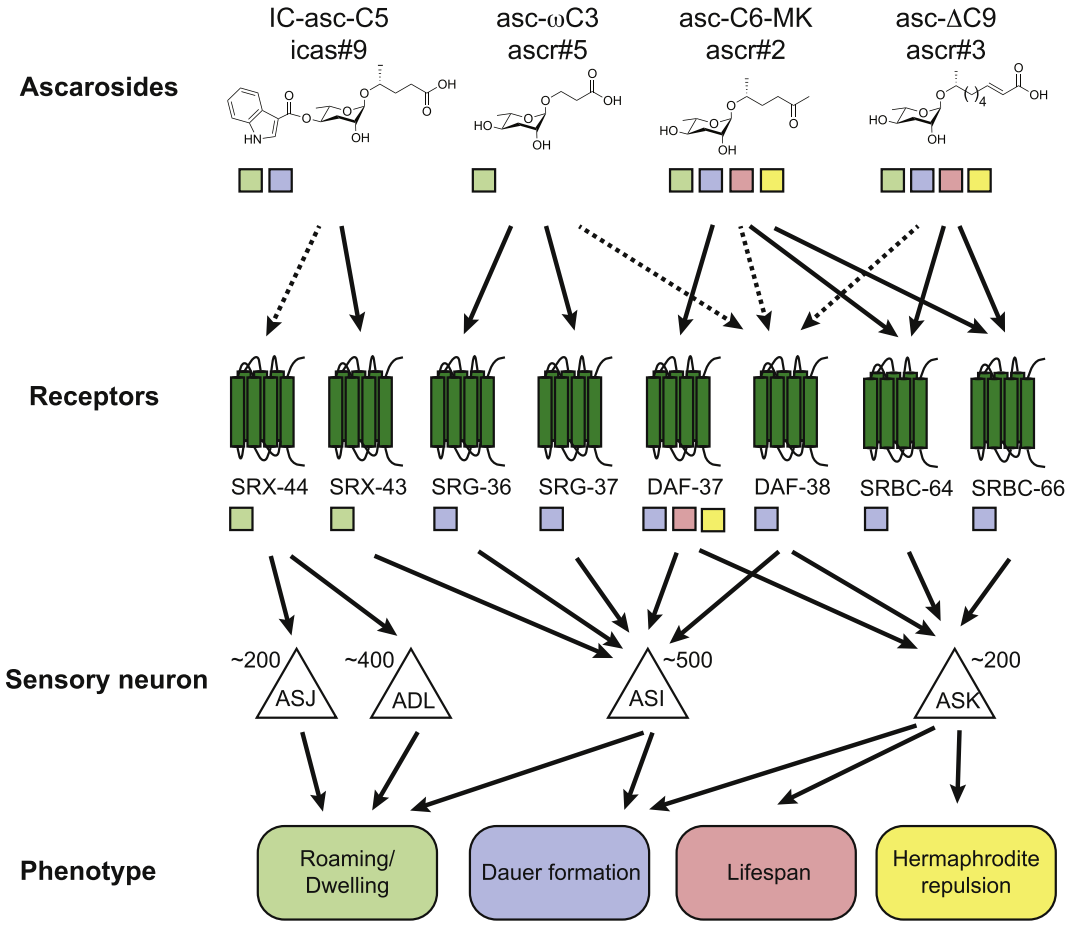
Pheromone messages are primarily intended for intraspecific use, but they could also be detected by nematode species that are found in the natural habitats occupied by *C. elegans* [55]. Several abundant ascaroside molecules are found in distantly-related nematodes [56–61], and at least in some instances, there is functional conservation of pheromone blends [24,25,57]. On the other hand, directed comparative screens are continuing to reveal species-specific compounds [15,56,62–64]. It is likely that both unique molecules and different ratios of conserved components define the species-specificity of pheromone blends. The extent to which pheromones of one species are intelligible to another and whether these chemical signals represent honest or deceptive communications largely remains an open question.

Examples from a variety of nematodes suggest that pheromones are used in interactions beyond the boundaries of this phylum. Because specific ascarosides indicate the nearby presence of nematodes, in response to these molecules, nematophagous fungi elaborate trapping devices to capture and consume their prey [65], whereas plants boost their immune defense in preparation for an imminent attack [59]. Ascarosides play important roles in the association between insects and nematodes – nematode pheromones could promote pupation in the beetle vector, while ascarosides released by adult beetles can attract nearby nematodes [60]. It appears that we have only began to scratch the surface of a complex network of multi-directional communications mediated by ascarosides in nature.

Ascaroside processing by the nervous system

The nervous system detects pheromones, integrates different pheromone signals with other relevant information, and dispatches secondary messages [66] that ultimately modulate physiology and behavior (Figure 2). The logic of pheromone responses begins at the chemoreceptor. The eight ascaroside receptors identified so far belong to a large (1341 genes) family of G-protein coupled receptors (GPCRs) related to the rhodopsin class GPCRs [67,68]. These eight receptors are expressed in a subset of bilaterally-paired chemosensory neurons that are open to the environment – named ASI, ASK, ASJ, and ADL [22,69–72]. Additional sensory neurons, such as ADF and the male-specific CEM, have been implicated in ascaroside responses as well, but pheromone receptors expressed in these neurons are yet to be identified [9,39,73]. Two themes have started to emerge from these studies: 1) some receptors are tuned to a single ascaroside, while others to multiple ascarosides and 2) several receptors could

Figure 2



Schematic representation of a relationship between ascarosides, their cognate receptors, sensory neurons, and the phenotypic effects they elicit. Colored squares underneath ascarosides and receptors correspond to the phenotypes regulated by them. Solid lines between ascarosides and receptors indicate inferences based on heterologous expression or pull-down assays; dotted lines indicate that genetic information was used to assign ascaroside/chemoreceptor pairing. The arrows connecting receptors and sensory neurons represent expression patterns. Estimated numbers of GPCRs expressed by each neuron (extrapolated from Ref. [68]) are shown.

detect the same ascaroside and regulate a given biological trait. If this "several ascarosides – several receptors" hypothesis is true, *C. elegans* may dedicate hundreds of GPCRs to ascaroside detection, perhaps in a condition- or trait-specific manner.

Expression patterns of most GPCRs remain to be determined, but a recent large-scale analysis of chemoreceptor expression [68] suggests that ASI, ASK, ASJ, and ADL will each express ~200–400 receptors, raising a question of how a single neuron could parse multiple inputs. One possible solution may be the increased complexity of the G-protein signaling within individual neurons. The *C. elegans* genome harbors 21 Gα proteins including single-copy, broadly-expressed orthologs of the four mammalian Gα families (Gs, Gi/o, Gq, and G12) and nematode-specific Gα genes that are expressed in restricted patterns in sensory neurons [74]. Pairing different Gα proteins with particular GPCRs in a given neuron, either in a dedicated complex or in a many-to-many relationship, as well as receptor heterodimerization, could establish specificity and independence of signaling by particular ascarosides. Whereas dedicated circuits may sense individual ascarosides [75], animals respond to the total pheromone blend. This process requires integration of inputs from several sensory neurons, possibly at the level of interneurons.

The complex relationship between ascarosides, receptors and the traits they control is well illustrated by distinct pheromone responses in strains that evolved under different conditions. For example, IC-asc-C5/icas#9 differentially alters foraging behavior in N2 and MY14 strains [22,69]. The difference is due to variation in expression of two receptors, *srx-43* in the ASI and *srx-44* in the ASJ/ADL neurons, each state representing alternative successful strategies depending on the local distribution of food [22]. Experimental evolution [71]

and populational genetics [69] suggest that variation in chemoreceptor deployment may represent a general strategy for evolutionary plasticity in pheromone response.

As might be expected of signals that communicate social information, the same ascaroside molecules can elicit different responses in hermaphrodites and males. To some extent these differences are due to the function of sex-specific neurons, such as the CEMs in males [9,73]. Importantly, the genetic sex of the nervous system also modulates the activity of the neurons common to both sexes, resulting in consequential behavioral differences [76–79]. Even members of the same sex interpret social signals in the context of other relevant information, such as their current status (e.g. nutritional) and past experience [77,80]. This type of plasticity requires integration of inputs from neurons other than those that sense ascarosides and long-term modification of neuronal circuits that process and propagate ascaroside signals. For example, recent levels of environmental O_2 can change the valence of pheromones by altering the circuits centered on the RMG neuron [77]. This hub interneuron [21] forms gap junctions with multiple sensory neurons, including those that sense O_2 and pheromones [21,81], and ensures context-appropriate behaviors. As we learn more about the circuits that mediate responses to different ascarosides, additional mechanisms may emerge that coordinate social signals with other types of relevant environmental information.

Conclusions and questions that systems biology approaches could help to answer

Despite the many recent advances highlighted above, our understanding of pheromone signaling in *C. elegans* is limited, particularly in the context of natural habitats and salient ecological interactions. We conclude this review by identifying two broad categories of problems that researchers in the field currently face and suggest ways in which systems biologists could contribute to this effort:

I. *Production and perdurance of ascaroside pheromones in natural habitats* – How do pheromones communicate information about an individual's experience? How do individuals encounter pheromones in natural environments?
   a. Goal 1: Reveal spatial distribution of pheromones
      It is unclear to what degree animals release pheromones constitutively or situationally. Also unknown is whether individual molecules in pheromone blends are released independently or in coordinated pulses. Resolving these questions is necessary for understanding how *C. elegans* experience pheromone signals. Ascarosides are expected to diffuse readily in the environments in which nematodes live. This raises a question of how animals discriminate between signals produced by different senders – gradients emanating from a source or as discrete 'packets' of multiple ascarosides. Mass Spectrometry Imaging is a technique that can potentially be used to characterize the spatial distribution of compounds such as ascaroside pheromones.
   b. Goal 2: Determine how pheromone distributions change over time
      It is largely unclear what happens to ascaroside pheromones after they are released into the environment and whether or not they are subject to modification or degradation. Different chemical moieties and fatty acid chains suggest that the rates of degradation and diffusion of different ascarosides could be different. Experimental measurements of these rates could help to determine how animals distinguish between different pheromone sources and whether recipients could estimate when the sender released the signal based upon differential degradation or modification of different ascaroside components.
   c. Goal 3: Characterize pheromones in natural samples
      Critically, pheromones identified so far have been detected in laboratory settings. It is possible that different ascarosides are released in natural habitats, in response to other nematode species, pathogens, or diet. Additionally, the ascarosides from non-*elegans* sources that are present in *C. elegans* natural habitats are still poorly characterized. Metabolomics can be used to survey the pheromones found in environmental samples that contain *C. elegans*.

II. *Perception of pheromones by the neural circuits in the recipients* – How are the messages communicated by the sender deciphered by the receiver? Could worms distinguish between the honest messages released by their own species and messages released by other species and cheaters?
   a. Goal 1: Experimentally link *C. elegans* chemoreceptors with ascaroside ligands
      C. elegans likely dedicate dozens if not hundreds of chemoreceptor genes to sensing ascarosides, but the correspondence between ligands and receptors is still largely unknown. In *Drosophila* and humans, large-scale heterologous assays have been used to link a large number of receptors with specific odorants. Low-throughput heterologous approaches have been used in *C. elegans* to identify pheromone receptors but these experiments could be expanded to a genome-wide level. Promising candidates from heterologous essays could be readily tested *in vivo* using CRISPR.

b. Goal 2: *In silico* predict ascaroside/chemoreceptor pairs

In parallel, ligand docking and molecular dynamics can be used to predict the ascaroside pheromones bound to a given chemoreceptor, relying solely on protein sequence information. If successful, this approach could be extended to the growing number of related nematodes with genome sequences. How does ascaroside sensation change in relation to release of pheromones in different species? To what extent do species sense ascarosides that are produced by other species?

c. Goal 3: Characterize sensory neurons used for processing ascaroside signals

Identity of candidate ascaroside-binding GPCRs could be used to determine which neurons express them, providing a detailed map of the sensory portion of the nervous system dedicated to processing pheromone signals. Coupled with a similarly detailed (single-neuron resolution) map of Gα protein expression and experiments to identify physical contacts between GPCRs and Gα proteins, would help to test the hypotheses about molecular mechanisms that give rise to specificity and signal separation that characterize ascaroside detection by the nervous system.

Conflict of interest statement

Nothing declared.

Acknowledgements

We thank Rebecca Butcher and Frank Schroeder for comments on the manuscript and help with figures. This work was supported by NIH grant R01GM114170 to PTM and grants from NSF (IOS-1708518) and NIH (R01GM126125) to IR.

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