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Counteracting Ascarosides Act through Distinct Neurons to Determine the Sexual Identity of *C. elegans* Pheromones

Highlights

- Male pheromones exert concentration-dependent effects on the hermaphrodite germline
- Effect of the male-enriched ascr#10 is opposed by the hermaphrodite-enriched ascr#3
- ascr#10/ascr#3 concentration ratio determines sexual identity of the pheromone
- Responses to ascr#10 and ascr#3 are mediated by distinct neuronal mechanisms

Authors

Erin Z. Aprison, Ilya Ruvinsky

Correspondence

ilya.ruvinsky@northwestern.edu

In Brief

Aprison and Ruvinsky report an antagonism between two ascaroside pheromones present in sexually dimorphic concentrations in *C. elegans*. Compound-specific activities are mediated by distinct neuronal mechanisms. Similar findings in other species suggest that general principles of encoding socially salient information may be broadly conserved.

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Counteracting Ascarosides Act through Distinct Neurons to Determine the Sexual Identity of *C. elegans* Pheromones

Erin Z. Aprison[1] and Ilya Ruvinsky[1,2,*]
[1]Department of Molecular Biosciences, Northwestern University, Evanston, IL 60208, USA
[2]Lead Contact
*Correspondence: ilya.ruvinsky@northwestern.edu
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SUMMARY

Sex pheromones facilitate reproduction by attracting potential mates and altering their behavior and physiology. In *C. elegans*, males and hermaphrodites secrete similar blends of pheromone molecules, two of which are present in different relative concentrations: ascr#3, which is more abundant in hermaphrodites, and ascr#10, which is more abundant in males. It is not currently understood how this compositional difference results in sex-specific effects, for example, the slower aging of the hermaphrodite germline in the presence of physiologically relevant concentrations of male pheromones. Here we report three key elements of the mechanism responsible for this phenomenon. First, ascr#3 counters the activity of ascr#10. This antagonism decreases the magnitude and the sensitivity of the hermaphrodite response to the male pheromone, restricting it to situations in which the presence of a male could be inferred with high confidence. Second, hermaphrodites recognize pheromone as male if the concentration of ascr#10 is higher than that of ascr#3. Third, the response to ascr#10 requires TRPV channel function in the ADL neurons and the *daf-7* signaling from the ASI neurons, whereas the response to ascr#3 relies on cyclic guanosine monophosphate (cGMP)-gated channels and activity of the ASJ, AWB, and AWC neurons. These results argue that the counteracting activities of distinct neuronal circuits determine the sexual identity of the pheromone. The parallels between this mechanism and other signaling systems suggest that diverse organisms may perform particular neuronal computations using similar general principles.

INTRODUCTION

Social interactions among animals are as important as they are ubiquitous. They modulate all aspects of life histories by eliciting behaviors and altering physiology in ways that are appropriate for an individual's social environment. Pheromones—secreted molecules that constitute chemical signals exchanged by members of the same species—play an important role in mediating social communications [1]. In particular, sex pheromones are produced in a sexually dimorphic fashion with the goal of attracting potential mating partners and facilitating reproduction in other ways [2–4].

In insects and mammals, the taxa in which pheromones have been extensively studied, individuals of both sexes typically secrete complex blends of multiple molecules [2–5]. Although the chemistry of pheromones differs among species [1, 2, 4, 5], blend composition and concentration play important roles in conveying the sex, reproductive status, and other characteristics of the emitter [5, 6]. Even minute amounts of pheromones can trigger sex-appropriate behaviors in recipients; these sensitive responses are mediated by ensembles of neurons [5–8], including the sensory neurons specifically tuned to specific molecules comprising pheromone blends [9].

In *C. elegans*, a family of glycolipids called ascarosides mediates animal-to-animal communication in a way similar to pheromones [10–13]. Males and hermaphrodites secrete different relative and absolute amounts of several ascarosides [14]. Two molecules that only differ by a single unsaturated bond, ascr#10 and ascr#3 (also known as asc-ΔC9), are the most abundant sex-specific ascarosides and are secreted in approximate ratios of 3.75:1 and 1:3.6 by males and hermaphrodites, respectively [14].

Sensing male-specific ascaroside blends alters hermaphrodite behavior [14] and physiology [15, 16]. In particular, a mixture of synthetic ascr#10 and ascr#3 at concentrations matching the amounts secreted by a *single* male in 24 hr (7.2/1.9 fmol [14]) slows down the aging-related loss of germline precursor cells (GPCs) [16], a population that includes germline stem cells and their mitotic progeny [17]. Because GPCs in individual animals can be reliably quantified, we used this paradigm to explore the relationship between ascaroside concentration, mixture composition, and physiological effects. We specifically focused on identifying neuronal mechanisms involved in the response of the germline to these pheromones.

RESULTS

Effects of Individual Components of the *C. elegans* Sex Pheromone

We first studied the effects of ascr#10, the ascaroside more abundant in males, over concentrations that spanned more

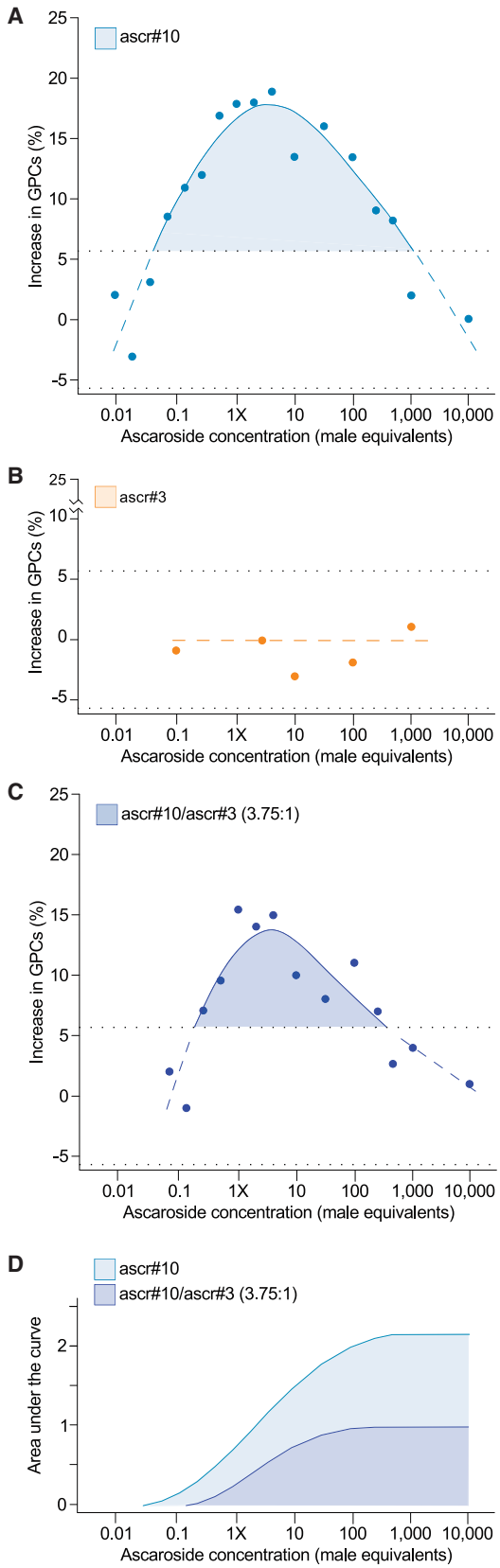

Figure 1. Antagonistic Effects of ascr#10 and ascr#3

(A–C) Effects of (A) ascr#10, (B) ascr#3, and (C) a blend of ascr#10 and ascr#3 (3.75:1) that reflects the ratio secreted by live males. For each tested concentration (colored dots), effect is defined as the ratio of the average number of GPCs in hermaphrodites on ascaroside-containing plates versus control of that experiment. Curves represent inferred trend lines. Concentrations are given in equivalents to the amount of ascarosides secreted by a single male over 24 hr (1× ascr#10 = 7.2 fmol and 1× ascr#3 = 1.92 fmol). Horizontal black dotted lines indicate ±3 SDs from the mean of controls (see Figure S1A). Only values above them were considered significant.

(D) Comparison of areas under the curve (shaded portions) in (A) (ascr#10 alone) and (C) (ascr#10 and ascr#3 blend). See Table S1 for raw experimental data. See also Figure S1.

than six orders of magnitude (Figure 1A). We measured the increase (compared to untreated controls) in the number of GPCs in the gonads of aged hermaphrodites maintained on plates supplemented with synthetic ascarosides. Hermaphrodites responded to concentrations that ranged from as low as ~0.35 fmol to as high as ~5,000 fmol per plate. Based on previously reported measurements of rates of ascaroside release [14], these amounts approximately correspond to 0.05- to 700-fold of daily production by one male. There was a conspicuous peak in response at concentrations corresponding to amounts produced by one to ten males.

We estimated, to the order of magnitude, the lowest response-inducing concentrations of ascr#10 on the scales comparable to the size of an adult hermaphrodite (~1 mm in length and ~10 μm between sensory sensilla; http://www.wormatlas.org). Given the size of the plates on which experiments were conducted, 1× equivalent of ascr#10 approximately corresponds to 2.5 attomole/mm^2. Therefore, at ascaroside concentrations ~0.1×, a square of the size of an adult contains ~10^5 molecules, whereas the square of the size of sensilla openings ~10 molecules.

We separately tested the effects of ascr#3, the ascaroside less abundant in males. At none of the examined concentrations, which spanned four orders of magnitude and were equivalent to amounts secreted by populations of up to thousands of animals, did we see a significant difference from untreated controls (Figure 1B). These results are consistent with earlier reports that ascr#10 mediates the attraction of hermaphrodites [14] and sperm guidance [15], whereas ascr#3 appears inert (although at high concentrations ascr#3 repels hermaphrodites [13]).

Mixing Antagonistic Ascarosides Alters the Threshold and Magnitude of the Response

Because live animals secrete ascaroside blends [10–13], we examined the combined effects of ascr#10 and ascr#3, mixed at a 3.75:1 ratio, which corresponds to that produced by males [14]. Although the response of hermaphrodites to a blend of these two molecules was qualitatively similar to the response to ascr#10 alone (Figure 1C), there were two notable differences. First, the response threshold to the blend was ~4- to 5-fold higher (~1/4× male equivalent) than the response threshold to ascr#10 alone (Figures 1C and S1B). Second, although response to the blend was highly correlated with the ascr#10 response (Spearman correlation, $r_s = 0.87$, $p < 6 \times 10^{-5}$), the former was consistently lower throughout the dynamic range (Figure 1D).

This suggests that, with respect to the number of GPCs, ascr#3 antagonizes the effect of ascr#10 when both molecules are present.

We also measured the response to complete male pheromone blend by aging hermaphrodites on plates conditioned by live males (as previously described [16]). We found that the response threshold in these experiments was approximately 0.6× male equivalent (5 males for 3 hr), which is within a factor of two to three of that obtained with blends of synthetic ascr#10 and ascr#3 (Figure S1C). Thus, at least in our paradigm, two conclusions could be made: (1) a mixture of ascr#10 and ascr#3 at a ratio of 3.75:1 substantially recapitulates the effects of the total male-produced signal, and (2) estimates of the amounts of secreted ascarosides made using thousands of animals [14] are in relatively close agreement with experiments that tested small numbers of animals.

Our results with synthetic ascr#10 and ascr#3, together with others [11, 13–16, 18–20], underscore the importance of studying pheromone effects at physiologically relevant concentrations. We estimate that these extend from 10^{-16} to 10^{-10} mol (per 60 mm plate), but most likely not much higher, considering that natural *C. elegans* populations probably do not often exceed ~10,000 individuals [21]. Although the native habitats of *C. elegans* are not well understood, it is reasonable to assume that a major purpose of the sex pheromone is to prime the hermaphrodite reproductive system in the presence of fertile males [1, 15, 16] and thus to facilitate the most likely infrequent encounters between potential mating partners. However, reproductive commitment is costly and male signals have detrimental effects on the hermaphrodite soma [16, 22, 23]. Therefore, it may be advantageous for hermaphrodites to limit the magnitude of the response (in our paradigm this is reflected in the number of GPCs) and restrict it to the situations when concentrations of male ascarosides are relatively high (higher than ~1/4×), signifying high confidence in the presence of males. Combining antagonistically acting components in the male pheromone achieves both of these goals.

Discriminating Male from Hermaphrodite Pheromones Relies on Relative Concentrations of ascr#10 and ascr#3

Hermaphrodites respond differently to male- and hermaphrodite-like blends of synthetic ascr#10 and ascr#3 (ratios of 3.75:1 and 1:3.6, respectively) [15, 16], suggesting that worms could distinguish concentration ratios of ascarosides, not simply detect the presence of specific molecules. We systematically tested the response of hermaphrodites to blends of ascr#10 and ascr#3 mixed at different relative concentrations, but in the absolute amounts equivalent to those secreted by single animals (Figure 2A). Three simple rules could be inferred: (1) even a modest bias in favor of ascr#10 (1.5:1) makes a blend male-like, (2) a 1:1 ratio is not significantly different from the untreated control, and (3) more ascr#10-biased ratios produce stronger responses.

However, the ability to discriminate relative ascaroside concentrations in a blend does depend on their absolute concentrations. At a concentration corresponding to 1× male equivalent, a 1.5:1 mixture of ascr#10 and ascr#3 is perceived as being male-like (Figure 2A). A 2-fold reduction of the absolute concentration of both ascarosides, makes the blend indistinguishable from the control (Figure S2A), even though at this absolute concentration hermaphrodites robustly respond to a 3.75:1 blend of ascr#10 and ascr#3 (Figure 1C). A 1:1 blend of ascr#10 and ascr#3 is still no different than the control, even at higher absolute concentrations (Figure S2A).

We sought to verify that the conclusion reached by testing blends of synthetic ascr#10 and ascr#3 was also true for complete pheromone blends. To do so, we extracted pheromones, by incubating live males and hermaphrodites, separately, in water for 24 hr (this was previously shown to yield ascarosides [14]). A male exometabolome uniformly applied to plates increased the number of GPCs (Figure 2B), whereas a hermaphrodite extract had no effect (Figure S2B). A 4:1 blend of the male and hermaphrodite exometabolomes was nearly as potent as the male exometabolome, whereas a 1:1 blend was indistinguishable from the control, regardless of whether animals were segregated by sex or incubated together (Figures 2B and S2C). We concluded that relative concentrations of ascr#10 and ascr#3 in blends of synthetic ascarosides or in exometabolomes of live animals were sufficient to account for the differences in their effects on the hermaphrodite germline.

On plates conditioned by live males, hermaphrodites dwelling for several days (Figure S1C) are expected [14] to secrete enough ascr#3 to produce an ascr#10:ascr#3 ratio lower than 1:1. At such low ratios, neither synthetic ascarosides (Figure 2A) nor uniformly applied blends of exometabolomes (Figures 2B and S2B) increased the number of GPCs, but pheromones left by live animals produced a robust response (Figure S1C). We hypothesize that a potential explanation for this result is that live animals deposit pheromones in a localized manner, possibly in patches along their tracks. This may be similar to pheromone-containing frass of insects [24]. Over time, localized ascarosides dissipate little, perhaps due to limited diffusion. Hermaphrodites exploring their environment would encounter both male and hermaphrodite patches, the latter having no effect on altering the number of GPCs.

Consequently, there may be a substantial difference between experiments that increase the amount of pheromone deposited by live males (more animals secreting pheromones for a longer period of time) versus uniformly applied synthetic or natural ascarosides (Figure 2C). The former is expected to increase the frequency with which hermaphrodites encounter patches of pheromones that are at constant endogenous concentration, the latter increases local ascaroside concentration throughout. In light of these considerations, it may be particularly relevant that hermaphrodites best respond to ascaroside concentrations that are below ~100× (Figure 1), which correspond to the highest local pheromone concentrations practically achievable in nature, even in large and dense populations.

We concluded that hermaphrodites recognize a pheromone mixture as male if the concentration of ascr#10 exceeds that of ascr#3. Reliable discrimination of ratios close to 1:1 is possible when absolute concentrations of ascarosides exceed a certain threshold. At lower absolute concentrations, ascaroside blends have to be more ascr#10-biased to be

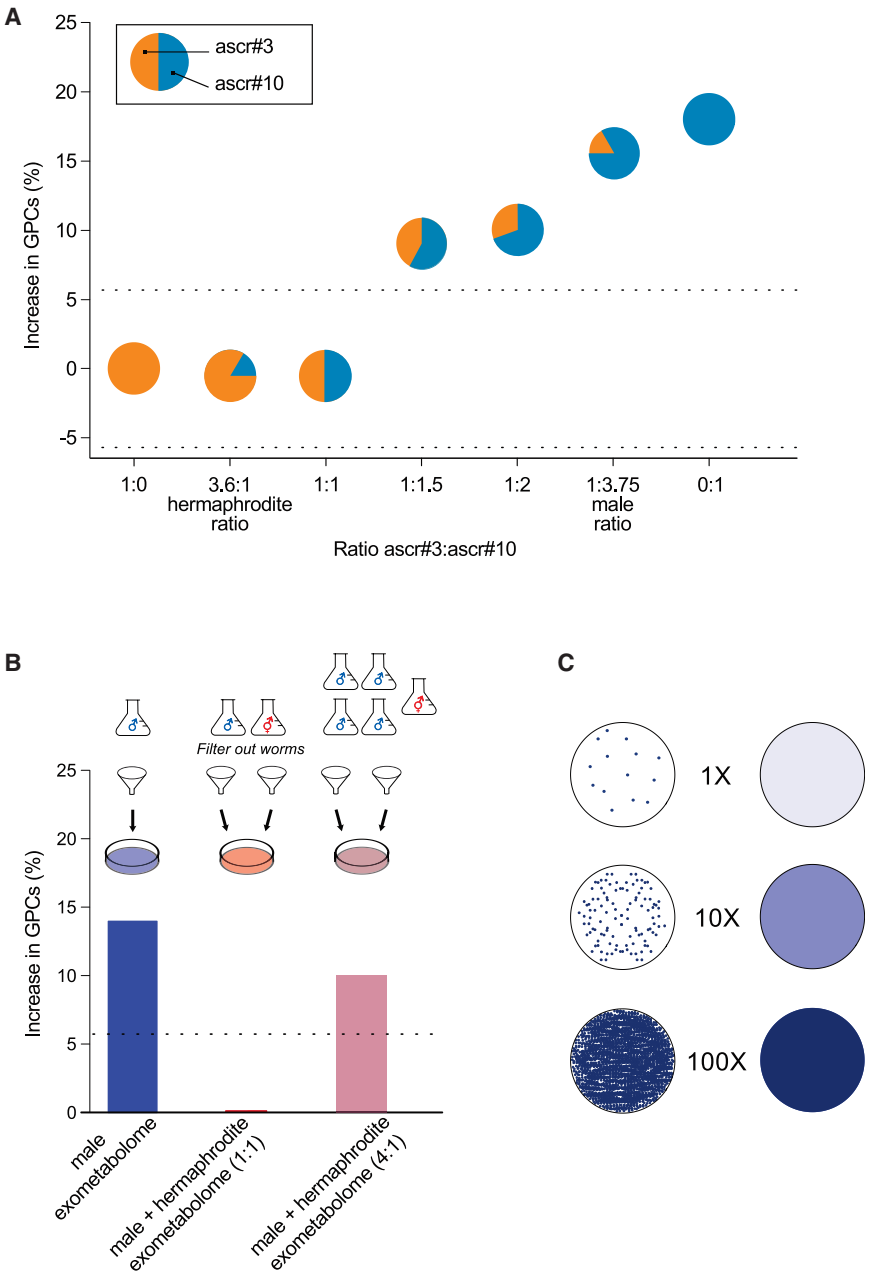

Figure 2. The Ratio of Concentrations of ascr#10 and ascr#3 Determines Sexual Identity of a Pheromone

(A) Blends of ascr#3 and ascr#10 have different effects depending on the ratio of concentrations of the two molecules (ascr#3:ascr#10 is shown along the x axis). Absolute concentrations were equivalent to the amount of ascarosides secreted by a single male over 24 hr (e.g., the 1:3.75 [male ratio] blend contained 1.92 fmol of ascr#3 and 7.2 fmol of ascr#10).

(B) Exometabolomes of live males (produced by five males over 24 hr) had a strong conditioning effect. Combining male and hermaphrodite exometabolomes had an effect at a ratio of 4:1, but not 1:1 (20 males:5 hermaphrodites and 5 males:5 hermaphrodites, respectively). In (A) and (B), horizontal black dotted lines indicate ±3 SDs from the mean of controls (see Figure S1A).

(C) Schematic depiction of a hypothesis reflecting a comparison between pheromone deposited by live animals (left) or uniformly applied (right). In each row, the total amount of pheromone per plate is the same (1×, 10×, or 100×), but the distribution is dramatically different until relatively high total amounts at which live animals deposited pheromone throughout the plate. See Table S1 for raw experimental data. See also Figure S2.

recognized as male. This may help to explain why, although hermaphrodites could respond to ratios as low as 1.5:1, males typically secrete considerably more ascr#10-biased (~3.75:1) blends [14].

TRPV Channel-Mediated Signaling in the ADL Neurons Is Required for a Response to ascr#10

Wild-type animals respond strongly to ascr#10, but not at all to the 1:1 blend of ascr#10 and ascr#3 (Figures 1A and 2A). We examined the response to these two treatments in hermaphrodites carrying mutations in genes involved in chemosensation to identify the neuronal mechanisms involved in the response of the germline to ascr#10 and ascr#3, respectively. In *C. elegans*, detection of chemicals by sensory neurons relies on one of two largely mutually exclusive signal transduction mechanisms—cyclic nucleotide-gated channels or transient receptor potential (TRP) channels [25]. We therefore started by examining mutations in these pathways.

We found that hermaphrodites carrying loss-of-function alleles in the broadly expressed TRPV channel OSM-9 [26] were unable to respond to ascr#10 (Figures 3A and S3A). The same was true for mutants in a TRPV paralog, *ocr-2*, that is expressed in a subset of OSM-9 neurons, where the localization of the two proteins is interdependent, suggesting a common function, possibly in forming a channel [26] (Figures 3A and S3A). The *ocr-2* gene is expressed in six pairs of sensory neurons—four amphids (ADFs, ADLs, ASHs, and AWAs) and two phasmids (PHAs and PHBs) [26]. Because a mutation in an LIM homeobox gene *ceh-14*, which is required for phasmid function [27], did not alter the ascr#10 response (Figure 3B), we focused on the four pairs of anterior neurons. We tested whether neuron-specific expression of the wild-type OCR-2 protein [28] could rescue the *ocr-2* defect and found that only expression in the ADLs could restore the ascr#10 response (Figures 3B and S3B). As expected, *ocr-2* mutants did not have more GPCs on plates conditioned by live males, whereas expression of OCR-2 in the ADLs rescued this defect (Figure 3C). We concluded that TRPV channel-mediated signaling, in particular

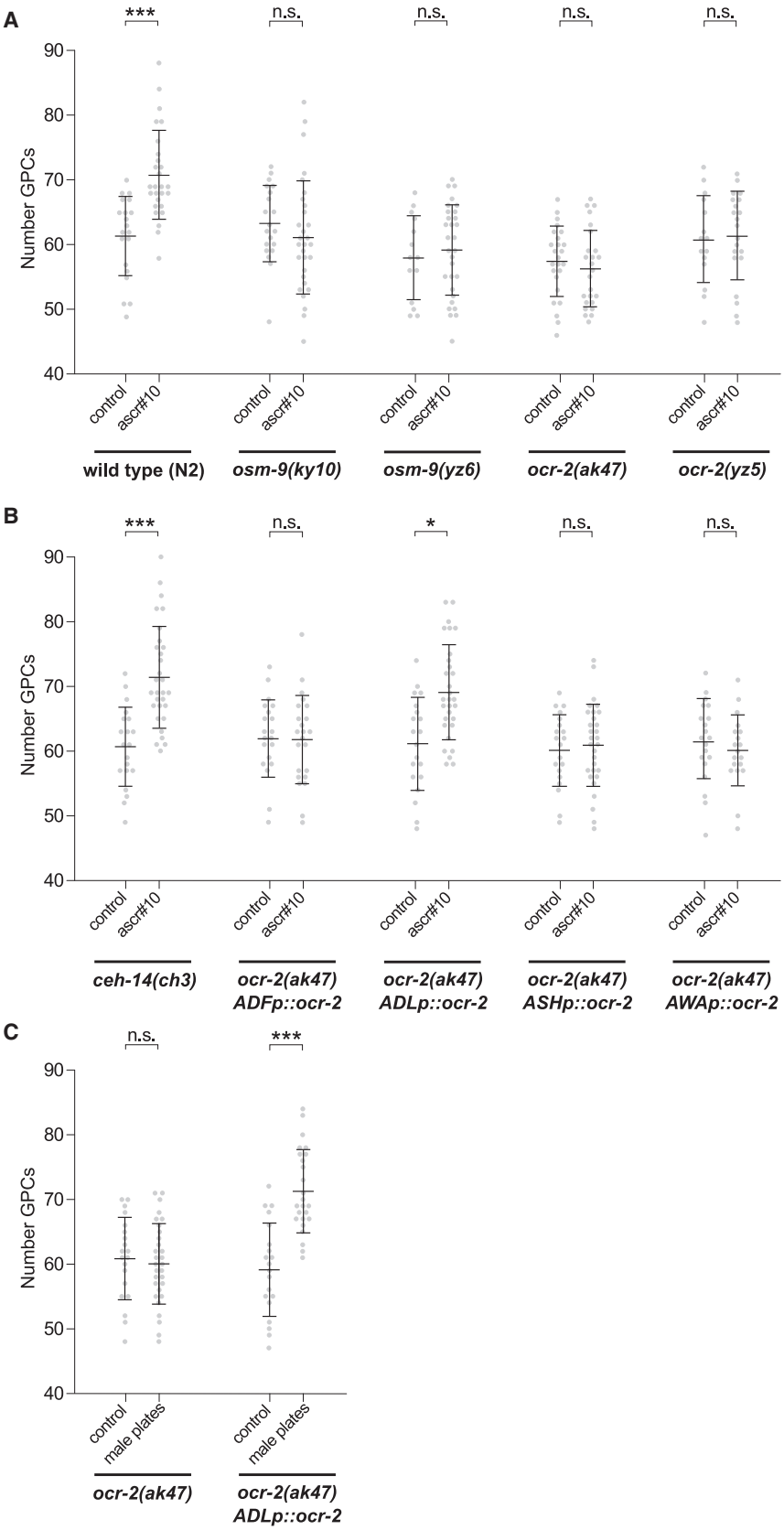

Figure 3. TRPV Channel-Mediated Signaling Is Required for the Germline Response to ascr#10

(A) Loss-of-function alleles of *osm-9* and *ocr-2* abrogate the hermaphrodite response to ascr#10.

(B) *ceh-14(ch3)* mutants respond to ascr#10 as well as the wild-type hermaphrodites, indicating that phasmid neurons are most likely not involved in this response. Expressing OCR-2 under cell-specific heterologous promoters only rescues the defect of *ocr-2*(*ak47*) when expression is directed in the ADL neurons.

(C) A loss-of-function allele of *ocr-2* abrogates the response to signals produced by live males, whereas expression of OCR-2 in the ADLs rescues this defect. Gray dots represent GPC counts (in one arm of the gonad) in individual animals. Bars represent means and SDs for each experiment (*$p < 0.05$ and ***$p < 0.001$). See Table S1 for raw experimental data. See also Figure S3.

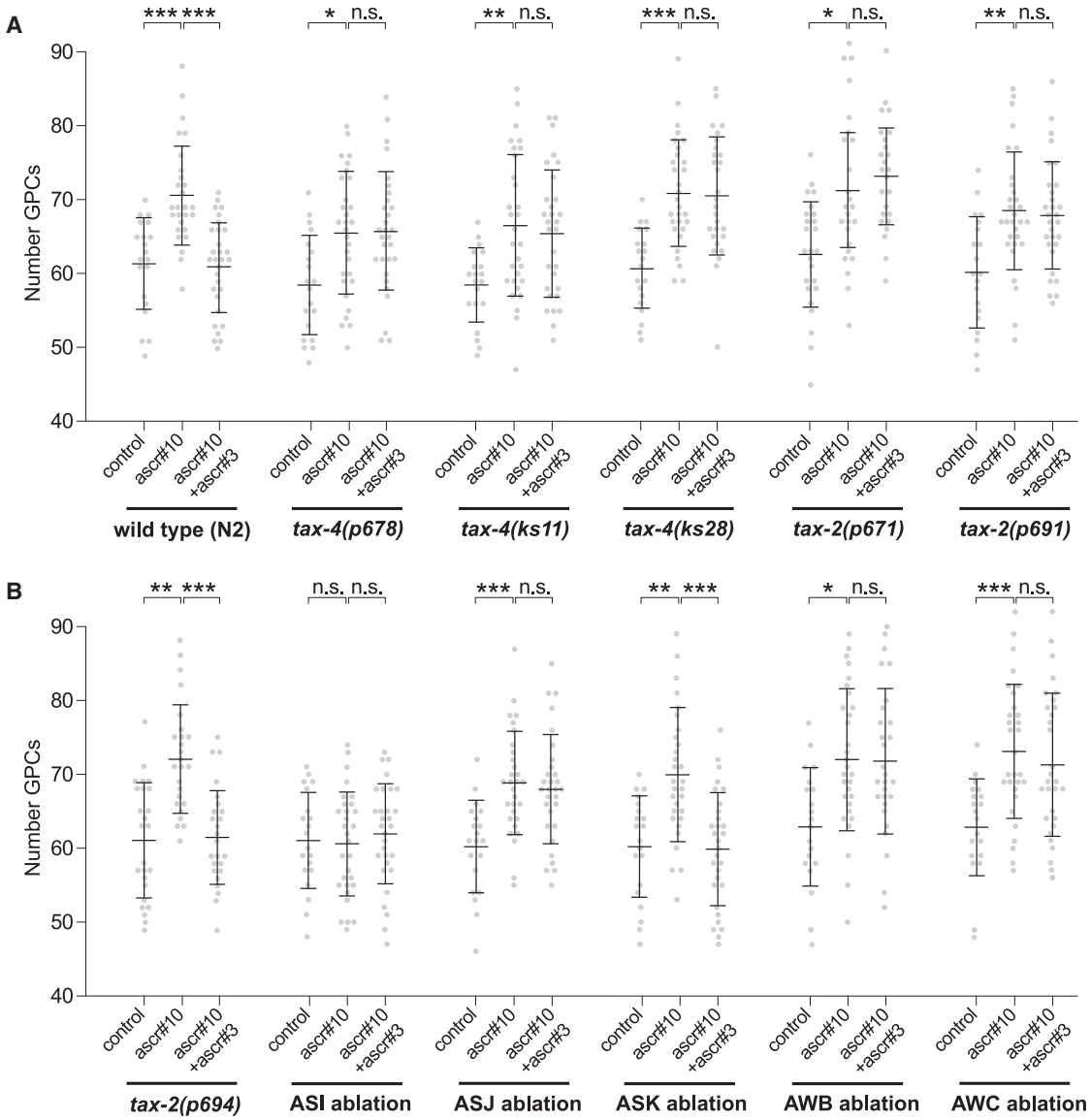

Figure 4. cGMP Channel-Mediated Signaling Is Required for the Germline Response to ascr#3

(A) Loss-of-function alleles of *tax-4* and *tax-2* abrogate the hermaphrodite response to ascr#3, but not ascr#10. The N2 data are the same as those shown in Figure 3A.

(B) The apparently wild-type response of *tax-2*(*p694*) mutants compared to the defects of *tax-2* (*lof*) alleles narrows down the neurons involved in the ascr#3 response. Genetic ablations of individual neuron pairs implicate the ASIs in response to ascr#10 and the ASJ, AWBs, and AWC in response to ascr#3. Gray dots represent GPC counts in individual animals. Bars represent means and SDs for each experiment (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$). See Table S1 for raw experimental data. See also Figure S4.

the function of OCR-2, is required in the ADL neurons for the germline response to ascr#10.

cGMP-Mediated Signaling in Several Neurons Is Required for a Response to ascr#3

In contrast to the defects of *osm-9* and *ocr-2*, the loss of TAX-2 or TAX-4 proteins, two subunits of a cyclic nucleotide-gated channel, did not alter the ascr#10 response, instead rendering the animals insensitive to ascr#3 (Figure 4A). These two genes are co-expressed in 12 classes of sensory neurons, none of which express *ocr-2* [26, 29]. The wild-type phenotype of a regulatory allele *tax-2*(*p694*), which only affects a subset of the TAX-2 neurons [30], narrows down the cells required for the ascr#3 response to just six pairs—ASGs, ASIs, ASJs, ASKs, AWBs, and AWCs (Figure 4B). Using strains in which pairs of specific neurons were genetically ablated [13, 31–33], we found that the functions of the ASJ, AWB, and AWC pairs were each required for ascr#3 to counter the effect of ascr#10 (Figures 4B and S4). We were surprised to discover that, although the ASIs express *tax-2*/*tax-4* and were thus expected to mediate the

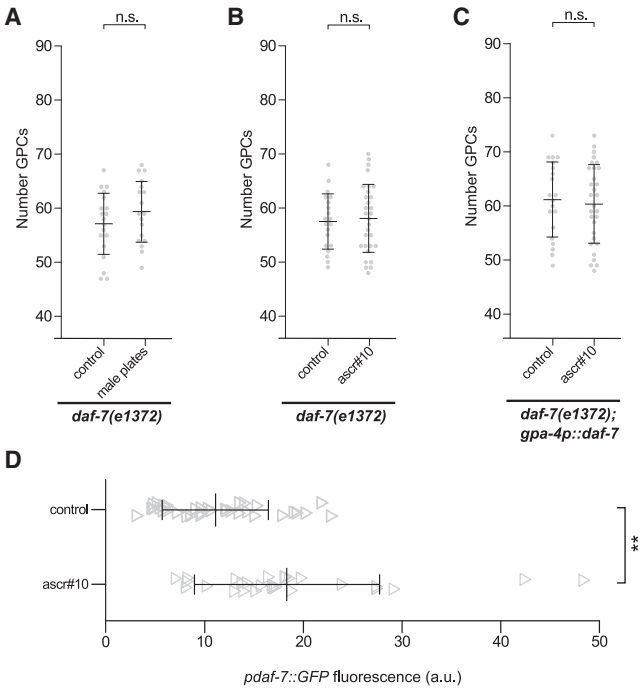

Figure 5. The TGF-β-like Signaling Is Involved in the Response to ascr#10

(A and B) In *daf-7*(*e1372*) hermaphrodites, GPC counts do not increase on plates conditioned (A) by live males or (B) with 1× ascr#10.

(C) In *daf-7*(*e1372*); *gpa-4p*::*daf-7* hermaphrodites, expression of DAF-7 in the ASIs is directed by a heterologous promoter. This does not rescue the inability of the *daf-7*(*e1372*) mutants to increase the number of GPCs in the presence of 1× ascr#10. Gray dots represent GPC counts in individual animals.

(D) Expression of *daf-7* is induced in the ASI neurons following exposure to 1× ascr#10. The plot shows fluorescence intensity (*daf-7p*::GFP) after 6 hr of exposure to ascr#10. Triangles represent measurements in individual ASI neurons. In all panels, bars represent means and SDs for each experiment (**$p < 0.01$). See Table S1 for raw experimental data. See also Figure S5.

ascr#3 response, they were essential for the ascr#10 response (Figure 4B). These results reveal two key features of the hermaphrodite response to male pheromones: (1) responses to different pheromone components are mediated by distinct molecular mechanisms (TRPV versus cGMP channels) that are segregated into different neuronal circuits, and (2) activity of several neurons is required to respond to each ascaroside.

Transforming Growth Factor β (TGF-β)-like Signaling Mediated by the *daf-7* Ligand Is Required for a Response to ascr#10

Finally, we turned our attention to the peculiar fact that, although the ASIs are *tax-2*/*tax-4*-expressing neurons, they are required for the response to ascr#10, whereas several other *tax-2*/*tax-4*-expressing neurons (ASJs, AWBs, and AWCs) are only required for a response to ascr#3. The ASI neurons secrete a TGF-β-like ligand DAF-7 following exposure to various environmental stimuli [34]. Relevantly, genes in the DAF-7-signaling pathway regulate proliferation/differentiation of GPCs in response to the environment [35], and male pheromones prime the female germline in a *daf-7*-dependent fashion [15].

We therefore tested whether the function of the DAF-7 pathway was required to increase the number of GPCs on plates conditioned by live males or ascr#10 alone. We found that *daf-7* mutants failed to respond to either stimulus (Figures 5A and 5B), in this way resembling the genetic ablation of the ASI neurons. However, constitutive production of DAF-7 driven in the ASIs by a heterologous promoter did not rescue the defect in ascaroside response (Figures 5C and S5A). We hypothesized that this was because exposure to ascr#10 induced the *daf-7* expression directed by the endogenous, but not a heterologous, promoter. Indeed, we found that expression of *daf-7* was upregulated, by over 60%, after 6 hr on femtomolar amounts of ascr#10 (Figures 5D and S5B). We concluded that the induced production of DAF-7 following the detection of ascarosides is responsible, at least in part, for the essential role of the ASI neurons in the response of the hermaphrodite germline to male pheromones.

DISCUSSION

We demonstrated that the sexual identity of pheromones in *C. elegans* is determined by the concentration ratio of two functionally antagonistic molecules, ascr#10 and ascr#3. Our results have revealed several key features of the underlying mechanism, although many details remain to be elucidated (Figure 6). The hermaphrodites' response to ascr#10 is mediated by a TRPV channel-dependent mechanism (OCR-2/OSM-9) in the ADL neurons as well as inducible release of the TGF-β-like ligand DAF-7 from the ASI neurons. A non-overlapping set of neurons that includes ASJs, AWBs, and AWCs is required for the response to ascr#3; this response depends on a cGMP-gated channel (TAX-2/TAX-4). Because a defect in the ascr#10 response would mask a defect in the ascr#3 response, we could not exclude the possibility that some ascr#3-related functions are also mediated by OCR-2/OSM-9 neurons. However, the responses to these two pheromone molecules are clearly distinct. Our work raises several questions. What are the specific functions of individual neurons? What are the additional components of pathways required for detecting and processing the signals of sex pheromones? How are the concentration ratios of ascarosides measured? How is this information communicated to distal tissues? The paradigm presented here may prove useful in understanding the mechanisms that translate complex social information into behavioral and physiological responses by a simple nervous system.

Comparisons with Other Studies of *C. elegans* Pheromones

Whereas the neuronal bases of ascr#10 effects on hermaphrodites have not been previously reported, several studies have identified neurons and genes involved in responses to ascr#3. Here we only consider three studies involving hermaphrodites, because the genetic sex of relevant neurons alters the perception/response to pheromones [28, 36]. Two different paradigms implicated the ASK neurons in response to ascaroside blends that contain ascr#3: attraction of *npr-1* hermaphrodites (this is the opposite of the response of the wild-type hermaphrodites but similar to that of males) [37] and dauer formation [38]. In the third study, the ADL neurons were shown to mediate (in an

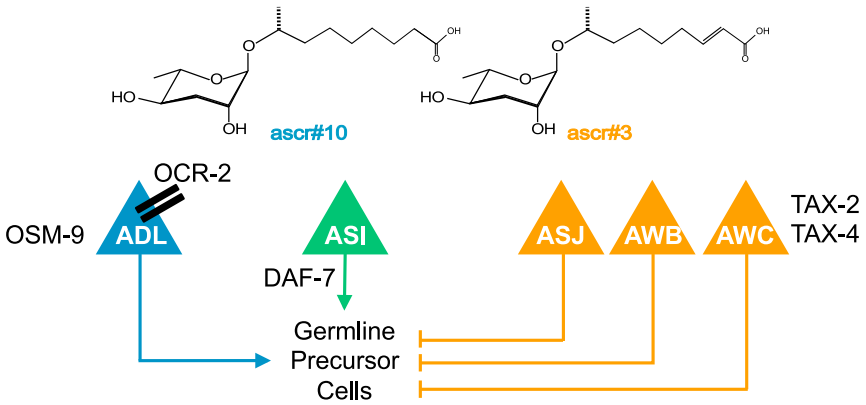

Figure 6. A Model Summarizing the Neurons and Signaling Pathways Involved in the Germline Response to ascr#10 and ascr#3

Responses to ascr#10 and ascr#3 (structures are shown at the top) are mediated by separate mechanisms involving TRPV and cGMP channels, respectively. Specifically, the function of OCR-2 in the ADL neurons is required for the ascr#10 response, whereas the ASJ, AWB, and AWC neurons are required for the ascr#3 response. That is, functions of these neurons are essential for the inhibitory effect of ascr#3 on the ascr#10 signal that promotes the increase in the number of GPCs. The ascr#10 response also requires the function of the ASI neurons, which release a TGF-β-like ligand DAF-7 following ascaroside exposure. The function of the ASIs in ascr#10 response is independent of the *tax-2/tax-4* mechanism required for the ascr#3 response. The effects of signaling from any of the involved neurons on proliferation of GPCs are not necessarily direct.

OSM-9/OCR-2-dependent fashion) acute ascr#3 avoidance in hermaphrodites [28].

In contrast, we implicated the ADLs in response to ascr#10 and found no role of the ASKs in response to either ascr#10 or ascr#3 (Figure 6). The apparent difference between our results and the previously reported findings could be plausibly explained by the use of different paradigms. In particular, we tested chronic exposure to low femtomolar amounts of ascarosides, not acute exposure to considerably higher concentrations. We suggest that further studies of the mechanisms responsible for trait- and concentration-specific ascaroside effects are likely to be fruitful, particularly because pheromone responses often involve groups of neurons [11, 39, 40], and blends of different composition can have dramatically different effects [41].

Our finding that the DAF-7 signaling in the ASI neurons is essential for the germline response to ascr#10 is consonant with multiple previous studies that implicated this pathway in different aspects of response to various ascarosides [34, 36, 38, 40, 41]. In particular, the dauer pheromone (which may be similar in composition to a highly concentrated hermaphrodite pheromone) influences the balance between proliferation and differentiation in the germline via a mechanism that involves *daf-7* [35]. The effects of the male sex pheromone on the gametes and the gonad of hermaphrodites are likewise *daf-7* dependent [15]. Although our results argue for a role of acute release of DAF-7 in response to ascr#10, the defects in *daf-7* mutants may be more extensive, because during development this ligand alters neuronal circuits to establish sex-appropriate pheromone response [36] and regulates the expression of chemoreceptor genes [42].

Parallels with Other Signaling Systems

Two findings reported here show substantial parallels with other signaling systems. First, although many studies focused on synergistic interactions between pheromone components [1, 3, 11–13], antagonism between constituent molecules has also been reported. The first identified pheromone, that of *Bombyx mori* females, consists mostly of a male attractant bombykol, but also contains a highly similar molecule bombykal, which inhibits the male response [2]. Pheromones of other insects also contain components with opposite effects [7, 43], as do mammalian pheromones [4]. In *C. elegans*, antagonism between ascr#3 and its indole derivative icas#3 has been reported [13]. Beyond animals, *Enterococcus* bacteria secrete a pheromone and an inhibitor that together coordinate conjugation [44]. This seemingly counterintuitive composition of signaling blends may offer an important functionality. Many biological systems regulate costly commitments and maintain homeostasis using ‘‘paradoxical’’ components, so called because they carry out opposing functions [45]. In the context of cell-to-cell signaling, for instance, a paradoxical signal could promote both cell proliferation and cell death and, in so doing, could prevent explosive growth or rapid decline in cell number [46]. This is reminiscent of the effects of ascr#10 and ascr#3 on the hermaphrodite germline because the former increases the number of GPCs while the latter counteracts this effect.

Second, because ascr#10 and ascr#3 are nearly identical molecules, the only difference between them being a single unsaturated bond [14], it may have been *a priori* expected that responses to these two pheromone molecules would rely on the same neuron(s). In fact, unicellular organisms are capable of distinguishing concentration ratios of different molecules [47]. Yet, the ascr#10 and ascr#3 signals are processed by two distinct sensory pathways (Figure 6). Intriguingly, responses to bombykol and bombykal, two similar components of the *B. mori* female pheromone, are also carried out by different neurons, each optimally tuned to a single component [9]. Likewise, distinct neuronal populations selectively respond to particular pheromone molecules in a moth *M. sexta* [8] and in mice [5, 6], allowing concentration ratios of components to be measured. In mammals and insects, each sensory neuron expresses a single chemosensory receptor [48] (although fly neurons also express a ubiquitous subunit *Or83b* and sometimes two specific receptors [48]), so it is not surprising that even closely related molecules are detected by different neurons. Therefore, in these species it is impossible to distinguish whether processing of distinct components of pheromones is accomplished by separate neuronal circuits because the

entire chemosensory system is organized in such a manner, because of contingency of evolution, or because this arrangement satisfies a particular demand on performance. In contrast, the *C. elegans* nervous system contains approximately a dozen pairs of chemosensory neurons, each expressing multiple receptors [48]. The fact that in such a compact chemosensory system responses to closely related but counteracting compounds are distributed to separate neurons makes us favor the hypothesis that this reflects a need, rather than an accident of nervous system structure and evolution. One possibility is that distinct ascr#10- and ascr#3-responding neurons reflect a demand to perform a particular information-processing function [49], possibly related to measuring ascaroside concentration ratios [50], that cannot be reliably achieved within a single cell. A test of this hypothesis will rely on the elucidation of the specific functions of individual neurons.

Conclusions

In *C. elegans*, a blend of ascarosides is interpreted as "male" if the concentration of ascr#10 is higher than that of ascr#3. Activities of each of these two molecules are mediated by separate dedicated mechanisms operating in distinct sets of neurons. Because a similar strategy is used to respond to pheromone blends in distantly related animals, it may reflect a requirement for a particular kind of information processing that could not be readily achieved in a single cell. Finally, ascr#3 appears to be an inert component with respect to its effects on the germline, yet it serves the important role of antagonizing ascr#10. This raises a possibility that some pheromone components may function as dedicated condition-specific inhibitors.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and one table can be found with this article online at http://dx.doi.org/10.1016/j.cub.2017.07.034.

AUTHOR CONTRIBUTIONS

Conceptualization, E.Z.A. and I.R.; Methodology, E.Z.A. and I.R.; Validation, E.Z.A. and I.R.; Formal Analysis, E.Z.A. and I.R.; Investigation, E.Z.A.; Writing – Original Draft, E.Z.A. and I.R.; Writing – Review & Editing, E.Z.A. and I.R.; Funding Acquisition, I.R.; Supervision, I.R.

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Note Added in Proof

After our study was accepted for publication, we became aware of two recent papers that address two of our major findings—the importance of considering pheromone activities at natural concentrations and the roles played by antagonistic pheromone components in achieving physiologically relevant responses:

Wyatt, T.D. (2017). Pheromones. Curr. Biol. 27, R739–R743.

Chang, H., Liu, Y., Ai, D., Jiang, X., Dong, S., and Wang G. (2017). A pheromone antagonist regulates optimal mating time in the moth Helicoverpa armigera. Curr. Biol. 27, 1610–1615.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
ascr#3	Laboratory of F. Shroeder	SMID ID:ascr#3; CAS: 946524-26-1
ascr#10	Laboratory of F. Shroeder	SMID ID:ascr#10; CAS: 1355681-08-1
Levamisole solution	Vector Labs	Cat#SP-5000
Vectashield mounting medium with DAPI	Vector Labs	Cat#H-1200
Experimental Models: Organisms/Strains		
C. elegans wild type N2	Caenorhabditis Genetics Center	WB Strain N2
CX10 *osm-9(ky10)* IV	Caenorhabditis Genetics Center	WB Strain: CX10; RRID: WB-STRAIN:CX10
JY190 *osm-9(yz6)* IV	Caenorhabditis Genetics Center	WB Strain: JY190; RRID: WB-STRAIN:JY190
CX4544 *ocr-2(ak47)* IV	Caenorhabditis Genetics Center	WB Strain: CX4544; RRID: WB-STRAIN:CX4544
JY243 *ocr-2(yz5)* IV	Caenorhabditis Genetics Center	WB Strain: JY243; RRID: WB-STRAIN:JY243
CX4533 *ocr-1(ok132)* V	Caenorhabditis Genetics Center	WB Strain: CX4533; RRID: WB-STRAIN:CX4533
LX845 *ocr-2(ak47)* IV; *ocr-1(ok132)* V	Caenorhabditis Genetics Center	WB Strain: LX845; RRID: WB-STRAIN:LX845
TB528 *ceh-14(ch3)* X	Caenorhabditis Genetics Center	WB Strain: TB528; RRID: WB-STRAIN:TB528
CX6237 *ocr-2(ak47); kyEx686[psrh-142::ocr-2, punc-122::gfp]*	Laboratory of C. Bargmann	N/A
CX6382 *ocr-2(ak47); kyEx693[psrh-220::ocr-2, punc-122::gfp]*	Laboratory of C. Bargmann	N/A
CX6241 *ocr-2(ak47); kyEx687[psra-6::ocr-2, punc-122::gfp]*	Laboratory of C. Bargmann	N/A
CX6235 *ocr-2(ak47); kyEx685[podr-10::ocr-2, punc-122::gfp]*	Laboratory of C. Bargmann	N/A
PR678 *tax-4(p678)* III	Caenorhabditis Genetics Center	WB Strain: PR678; RRID: WB-STRAIN:PR678
FK129 *tax-4(ks11)* III	Caenorhabditis Genetics Center	WB Strain: FK129; RRID: WB-STRAIN:FK129
FK103 *tax-4(ks28)* III	Caenorhabditis Genetics Center	WB Strain: FK103; RRID: WB-STRAIN:FK103
PR671 *tax-2(p671)* I	Caenorhabditis Genetics Center	WB Strain: PR671; RRID: WB-STRAIN:PR671
PR691 *tax-2(p691)* I	Caenorhabditis Genetics Center	WB Strain: PR691; RRID: WB-STRAIN:PR691
PR694 *tax-2(p694)* I	Caenorhabditis Genetics Center	WB Strain: PR694; RRID: WB-STRAIN:PR694
PY7505 *oyIs84[gpa-4p::TU#813 + gcy-27p::TU#814 + gcy-27p::GFP + unc-122p::dsRed]*	Caenorhabditis Genetics Center	WB Strain: PY7505; RRID: WB-STRAIN:PY7505
ZD762 *mgIs40[daf-28p::nls-GFP]; jxEx100[trx-1::ICE + ofm-1::gfp]*	Laboratory of D. Kim	N/A
ZD763 *mgIs40[daf-28p::nls-GFP]; jxEx102[trx-1::ICE + ofm-1::gfp]*	Laboratory of D. Kim	N/A
PS6025 *qrIs2[sra-9::mCasp1]*	Caenorhabditis Genetics Center	WB Strain: PS6025; RRID: WB-STRAIN:PS6025
FG563 *N2; udEx236[str-1p::ced-3(p15), str-1p::ced-3(p17), str-1p::gfp, elt-2p::gfp]*	Laboratory of D. Ferkey	N/A
FG564 *N2; udEx310[str-1p::ced-3(p15), str-1p::ced-3(p17), str-1p::gfp, elt-2p::gfp]*	Laboratory of D. Ferkey	N/A
FG565 *N2; udEx311[str-1p::ced-3(p15), str-1p::ced-3(p17), str-1p::gfp, elt-2p::gfp]*	Laboratory of D. Ferkey	N/A
PY7502 *oyIs85[ceh-36p::TU#813 + ceh-36p::TU#814 + strx-1p::GFP + unc-122p::dsRed]*	Caenorhabditis Genetics Center	WB Strain: PY7502; RRID: WB-STRAIN:PY7502

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
CB1372 *daf-7(e1372)* III	Caenorhabditis Genetics Center	WB Strain: CB1372; RRID: WB-STRAIN:CB1372
DA2202 *daf-7(e1372)* III; *adEx2202 [gpa-4p::daf-7 + rol-6p::GFP]*	Caenorhabditis Genetics Center	WB Strain: DA2202; RRID: WB-STRAIN:DA2202
FK181 *ksIs2[daf-7p::GFP + rol-6(su1006)]*	Caenorhabditis Genetics Center	WB Strain: FK181; RRID: WB-STRAIN:FK181
Software and Algorithms		
ImageJ	NIH Image	https://rsbweb.nih.gov/ij/

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Ilya Ruvinsky (ilya.ruvinsky@northwestern.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

All strains except for CB1372 and DA2202 were grown on OP50 at 20°C under standard conditions [51]. CB1372 and DA2202 were maintained at 16°C until just before adulthood and then were shifted to 20°C. Most strains were obtained from the Caenorhabditis Genetics Center. Additional strains were the gifts from other labs. Synchronized populations of L1 larvae were produced by hypochlorite treatment of gravid hermaphrodites [52]. After treatment, the liberated eggs were allowed to hatch in M9 buffer overnight. The following morning, the resulting population of arrested L1 larvae was plated onto lawn plates at a density of 30-60 larvae per plate. Hermaphrodites were scored at the fifth day of adulthood with two exceptions. Strain PR691 hermaphrodites were somewhat developmentally delayed and were scored at the sixth day of adulthood. In Figures 5D and S5B hermaphrodites in the first day of adulthood were used.

METHOD DETAILS

Aging worms on plates with ascarosides

Aging of hermaphrodites on ascaroside plates and germline staining was described previously [16]. ascr#3 and ascr#10 were provided by Frank C. Schroeder (Cornell University). Synthetic samples of ascr#3 and ascr#10 were more than 99% pure, as determined by proton NMR spectroscopy. Because diffusion properties of ascarosides on agar plates are not well understood, throughout the paper we give concentrations as amount of ascaroside added to a plate. Another way to consider ascaroside concentrations is to convert them into area density. Because all experiments were conducted on 60mm plates, 1X male equivalent of ascr#10 (i.e., 7.2 femtomoles per plate) approximately corresponds to 2.5 attomole/mm^2. This denominator is relevant, since adult *C. elegans* hermaphrodites are approximately 1 mm in length.

Concentrated solutions of ascarosides in ethanol were maintained at −20°C. These stock solutions were further diluted in water to the desired concentration and a total of 100uL of ascaroside solution was applied to the surface of the agar and distributed evenly with a glass rod. The solution was allowed to absorb into the agar at 20°C overnight. The next day, plates were seeded with 20 uL of a 1:10 dilution of an overnight culture of OP50 and were incubated at 20°C. Control plates were prepared in the same manner but without the addition of ascarosides.

Hermaphrodites were maintained on their original lawn plates until 48 hr after plating, which was approximately two hours post L4/YA molt (48 hr post L1 arrest at 20°C was counted as day one of adulthood). At that time, 30 hermaphrodites were transferred to each of three prepared ascaroside plates or control plates. On day three of adulthood, the hermaphrodites were transferred to freshly prepared ascaroside or control plates. The strains CB1372 and DA2202 in Figures 5B and 5C were treated in the same fashion except that the strains were returned to 16°C after plating until shortly before the L4/YA molt (about 76 hr). The CB1372 hermaphrodites in Figure 5A were synchronized via a one-hour egg lay and were maintained at 16°C for 54 hr – until the worms were in the mid L3 larval stage. They were transferred to 20°C until just before the L4/YA molt (about 16 hr), that is, two-three hours prior to transfer to male-conditioned plates.

Staining of germline progenitor cells

To facilitate the counting of germline progenitor cells (GPCs), hermaphrodites were stained with DAPI (4′,6-diamidino-2-phenylindole) as described previously [16] using a variation of the protocol by Pepper et al. [53]. Animals of all strains, except PR691 *tax-2(p691)*, were examined on Day 5 of adulthood, at which time they exhausted supplies of self-sperm and therefore ceased to produce self-progeny. The PR691 hermaphrodites were somewhat developmentally delayed (~15 hr – they were early L4 at 48 hr post release from the L1 arrest). To account for this delay, GPCs in the hermaphrodites of this strain were counted on the morning of Day 6, not the afternoon of Day 5 (i.e., ~15 hr later).

Worms were picked onto unseeded plates to reduce the amount of associated bacteria and then transferred into 1 mL of M9 buffer in a low-retention 1.5 mL microcentrifuge tube. After a brief centrifugation, the supernatant was removed and 1 mL of 95% ethanol was added to the tube to fix the worms. After 10 min at room temperature, the worms were again briefly centrifuged and the ethanol was removed. The pellet of fixed worms was resuspended in 15 uL of Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA). Glass microscope slides were prepared with thin 2% agarose pads and the stained worms were deposited on the pads using a glass micropipette fitted to a 3 mL syringe. The slides were examined on a Leica DM5000B microscope and numbers of nuclei in the proliferative zone, as described by Crittenden et al. [54], were determined.

Conditioning plates with N2 males

Males were segregated as L4 larvae and used one or two days later to condition plates. The following schedule of numbers of males and duration of time spent on the plate was used:

0.2X = 5 males for 1 hr
0.6X = 5 males for 3 hr
1.7X = 5 males for 8 hr
5X = 15 males for 8 hr
15X = 15 males for 24 hr

After the allotted time, males were removed and plates were refrigerated until being used to house hermaphrodites the following day.

Conditioning with exometabolome preparations

Mating plates of *C. elegans* males and hermaphrodites were pooled and the resulting population was subjected to hypochlorite treatment to produce a synchronized mixed-gender culture. Separate age-matched populations of males and hermaphrodites were segregated at the L4 stage. The following day, worm exometabolome preparations were collected using the method described by [14] with some modification. Ten worms, either males or hermaphrodites, were picked into 100uL of water in 200uL PCR tubes. For the experiment in Figure S2C, an additional class of tubes contained 5 males and 5 hermaphrodites in the same tube. Worms were incubated for 24 hr at 21°C with shaking at 220 rpm. The liquid was filtered over sterile cotton to exclude the worms and 100uL was applied to petri dishes as above. Eggs or L1 larvae that were not filtered by the cotton were removed. Plates were refrigerated until use.

Quantification of fluorescence

Cultures of strain FK181 were synchronized by hypochlorite treatment and allowed to develop on control plates. Thirty young adult hermaphrodites were transferred to either 1X ascr#10 plates or control plates for six hours before visualization. After six hours of exposure, the hermaphrodites were transferred to 2% agarose pads prepared on microscope slides and anesthetized with 12.5mM levamisole (Vector Laboratories, Burlingame, CA). An exposure time was selected to ensure that the pixel intensity of the fluorescent signal in ASI was in the linear range. Images were acquired on a Leica DM5000B microscope using a Retiga 2000R camera and the corrected total cell fluorescence was measured in ImageJ (NIH).

QUANTIFICATION AND STATISTICAL ANALYSIS

All experiments included individual controls (Table S1). To assess the variability of experiments, some of which were conducted months apart, we collated 48 control values from experiments that scored GPCs in the wild-type strain N2. The average of this sample was 61.1 and the standard deviation 1.17. Considering that multiple comparisons were performed, we conservatively considered as significant only those experimental values that were more than three standard deviations higher (none were more than three standard deviations lower) than the control average. Only ~0.15% of observed values are expected to be this high or higher by chance, giving an estimate of $p = 1.5 \times 10^{-3}$. Thus, the dotted 3σ lines in Figures 1, 2, S1, and S2 were drawn at 5.7 and −5.7% (= (61.1 ± 3 x 1.17) / 61.1). Curves in Figure 1 were drawn with the loess program in R. Significance of differences in GPC counts in Figures 3, 4, 5, S3, S4, and S5 was compared using the Kolmogorov-Smirnov test in R. Numbers of worms, trials, and p values are included in Table S1.