A vital dye method in nematodes reveals age is a critical parameter in density-dependent plasticity

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Abstract

Population size is a major ecological constraint on resources, which affects density-dependent selection over evolutionary time scales. Yet many species can also respond dynamically through phenotypic plasticity. In *Caenorhabditis elegans* and other nematodes, high population density drives juveniles into a dormant dauer stage. In *Pristionchus pacificus* high population density also induces the development of a predatory mouth form. However, it is unknown if such phenotypic switches respond simply to density, or to the density of specific age classes, as in density-dependent selection. Although nematodes have many experimental advantages, their small size has made investigating these and other experimental ecology questions challenging. In particular, no *in vivo* methodologies are currently available to label distinct populations without the need for transgenics. To study potential cross-generational signaling we developed a novel dye-based method to differentiate nematode populations on the same plate, allowing us to track adults with juveniles, or juveniles with juveniles. Surprisingly, only adult crowding induces the predatory morph, even though adult mouth-forms are no longer plastic. We then profiled secreted metabolites through development and traced this result to adult-specific pheromones. Thus, a novel dye-based method reveals age as a critical parameter controlling density-dependent plasticity.

Keywords: Phenotypic plasticity, population density, *Pristionchus pacificus*, *Caenorhabditis elegans,* nematode derived modular metabolites (NDMMs)

1. Introduction

Population density is an important ecological parameter, with higher densities corresponding to increased competition for resources [1]. In addition to density-dependent selection [2,3], which operates on evolutionary time scales, some organisms can respond dynamically to population density through phenotypic plasticity. For example, plants can sense crowding by detecting the ratio of red (chlorophyll absorbing) to far red (non-absorbing) light, and respond by producing higher shoots [4]. Locusts undergo solitary to swarm (i.e. gregarious) transition, and aphids can develop wings, both as results of increased physical contact [5-7]. Intriguingly, population density can also have cross-generational effects. For example, adult crowding of the desert locust *Schistocerca gregaria* [8,9] and migratory locust *Locusta migratoria* [10,11] also influences the egg size, number, and morphology of their progeny; and high population densities of red squirrels elicit hormonal regulation in mothers to influence faster developing offspring [12]. In many species population density and cross-generational signaling are detected through pheromones, however the precise nature, mechanisms of induction, age-specificity and exact ecological role are not well understood.

Nematodes are a powerful model system to investigate the mechanisms of density-dependent plasticity because many small molecule pheromones that affect plastic phenotypes have been characterized [13-15]. For example, in the model organism *Caenorhabditis elegans,* high population densities induce entry into a stress resistant dormant 'dauer' stage [16]. The decision to enter dauer was revealed to be regulated by a family of small molecule nematode-derived modular metabolites (NDMMs) called ascarosides that act as pheromones[14,17,18]. Ascarosides consist of an ascarylose sugar with a fatty acid side chain and modular head and terminus groups (figure 1*a*). The level and composition of ascarosides was later shown to be dependent on sex [19,20] and developmental stage [21], although it is thought that dauer can be induced by all developmental stages [22]. Subsequent studies revealed that specific NDMMs

also regulate other life history traits, such as mating [19,20], social behavior [23] and developmental speed [24]. Although NDMMs are broadly conserved [25-27], inter- and intraspecific competition has driven the evolution of distinct response regimes in different strains and species for the same phenotypes [25,28-31]. Additionally, more complex structures have been observed that affect distinct plastic phenotypes [32].

In *Pristionchus pacificus,* a soil-associated nematode that is reliably found on scarab beetles (figure 1*a*)[33-35], an ascaroside dimer (dasc#1) that is not found in *C. elegans* regulates the development of a predatory mouth form [32,36,37]. Mouth-form plasticity represents an example of a morphological novelty that results in predatory behavior to exploit additional resources and kill competitors. Specifically, adult *P. pacificus* exhibit either a narrow stenostomatous (St) mouth, which is restricted to bacterial feeding (figure 1*b*), or a wide eurystomatous (Eu) mouth with an extra denticle (figure 1*c*), which allows for feeding on bacteria and fungi [38], and predation on other nematodes [39]. This type of phenotypic plasticity is distinct from direct *vs.* indirect (dauer) development because it results in two alternative life history strategies in the adult (for review see Sommer & Mayer, 2015 [40]). Recent studies in *P. pacificus* have begun to investigate the dynamics and succession of nematodes on decomposing beetle carcasses to better understand the ecological significance of mouth-form plasticity [41]. These studies revealed that on a carcass (figure 1*d*), *P. pacificus* exits the dauer diapause to feed on microbes, and then re-enters dauer after food sources have been exhausted, displaying a ‘boom-and-bust’ ecology [33,41]. Presumably different stages of this succession comprise different ratios of juveniles and adults, and recognizing the age-structure of a population as a juvenile could provide predictive value for adulthood. In locusts density-dependent plasticity is affected by the age of crowded individuals [5], however age has not yet been demonstrated to have a role in density-dependent plasticity in nematodes. Apropos, whether the mouth-form decision is sensitive to population age structure has never

been investigated, neither experimentally, nor chemically by performing NDMM profiles of multiple developmental stages.

While nematodes have many experimental advantages, including easy laboratory culture and advanced genetic, genomic and the aforementioned chemical tools, their small size has made investigations at the organismal level and in experimental ecology challenging. For example, no *in vivo* methodologies are currently available to label distinct populations without the need for transgenics, which is only available in selected model organisms such as *C. elegans*, *P. pacificus* and some of their relatives. Here, we combine a novel dye-staining method with the first developmental pheromone profiling in *P. pacificus* to study potential affects of age on density-dependent plasticity. This vital-dye method allows tracking adults with juveniles, or juveniles with juveniles, and can be applied to any nematode system that can be cultured under laboratory conditions. In contrast to dauer, we found that mouth form is strongly affected by cross-generational signalling. Specifically, only adult crowding induces the predatory morph, which is controlled by adult-specific pheromones.

2. Materials and methods

(a) Nematode strains and husbandry

P. pacificus Wild-type RS2333 (California) and RSC017 (La Réunion) strains were kept on 6 cm nematode growth media (NGM) plates seeded with OP50 and kept at 20°C. RSC017 is highly St and does not predate on other nematodes, and thus was used for biological assays instead of the highly Eu, predatory RS2333.

(b1) Pheromone profiling

HPLC-MS sample preparation for normal exo-metabolome and time resolved analysis

To collect staged phermone profiles, we seeded 35 x 6 cm plates with 5 worms each, and bleached 5-6 days later when gravid to collect eggs/J1s. These were then seeded in 6 x 10 mL flasks with OP50 as described in Werner et al., 2017 [42]. Then at 24, 48, or 72 hr time intervals, supernatant was obtained by centriguation (>4,000 x g, 4°C for 10 minutes). 1 mL supernatant was adsorbed onto a SPE-C8 cartridge (Thermo Scinetific Hypersept C8 100 mg/1mL), conditioned with 1 mL MeOH followed by 2 mL Millipore water. The adsorbed material was then washed with 200 uL water and subsequently eluted with 200 uL MeOH. This extract was then measured directly via HPLC-qTof MS (Bruker ImpactII).

(b2) HPLC-MS measurement

20 uL extract was injected into a Thermo ultimate 3000 HPLC equipped with a Sigma-Alderich Ascentis Express C18 2.7um 10mm x 4.6mm column at 20 °C with a flow of 500 uL/min. All MS measurements have been performed in negative ion mode and molecules are detected as $[M-H]^-$ Ions. The solvent gradient started with 5 % acetonitrile (ACN)/ 95 % water (both containing 0.1 % formic acid) for 2 min. After this equilibration step, the ACN proportion has been increased to 65 % over 8 min, then to 100 % ACN in 1.2 min followed by a hold step for 8.8 min. Afterwards, they system was flushed to 5 % ACN with 2 min equilibration for a total of 22 min. For calibration, a sodium formiat cluster building solution has been automatically injected in the first 2 minutes of each run. Data analysis was performed with TASQ version 1.0 from Bruker Daltonics. Extracted ion chromatograms for each well-known compound with a mass width of 0.1 m/z and time slices of 0.5 min around the expected retention time have been produced after calibrating and baseline correction. Assignment errors have been corrected with the provided MRSQ value.

(c) Statistical analysis of NDMMs

NDMM levels were compared simultaneously between strains and developmental stages by a linear model in R: lm('NDMM' ~ 'developmental stage' * 'strain', data='data.frame')). *P* values between stages and strains were adjusted for multiple testing by a false discovery rate correction. The level of fit between linear vs. exponential growth was determined by the Akaike information criterion (AIC). The lowest AIC for iterations of different exponents (n=1,2,3…) was used for comparison to the simple linear model. While significant in both cases, for consistency we present the original *p* values from the original linear model in Supplementary Figure 8.

(d) Supernatant collection and assays

Strains RS2333, RSC017, and RS2333-*daf-22.1;22.2* were raised in 10 mL liquid culture as in the time-resolved NDMM collections (b1). For each time point, 9 mL of the supernatant was lyophilized overnight, extracted again overnight with 90% ethanol (diluted in Millipore water) while being stirred, and centrifuged (4000g, 10 min, 4°C). The solvent was evaporated and the solid re-dissolved with 1 mL Millipore water. This clear extract was then directly used for the assays. One mL of the supernatant was cleaned for HPLC-MS analysis (refer to pheromone profiling: HPLC-MS sample preparation) for quality control. For the assays, RSC017 was synchronized by bleaching and added to plates seeded with 300 µl OP50. The supernatants were added to the RSCO17 J2s in two 500 µl increments (for a total of 1ml supernatant) and dried for 30 minutes in a sterile hood after each addition. Plates were kept at 20°C and adult mouth forms were screened three days later.

(e) Dye Staining

A stock solution of Neutral Red was prepared by dissolving 0.5 mg in 10 ml 5% acetic acid and stored at -20° C. Working solutions were prepared by 100x dilution in M9, aliquoted, stored at -

20°C, and thawed directly before use. Working solutions were kept for approximately 1 month. Stock solutions of 10 mM Green Bodipy were made in DMSO and stored -20. J2s were prepared from 20-40 x 6 cm plates 6 days after passaging 5 worms to each plate on 300 µl OP50. Worms were washed from plates with M9 into a conical tube, and then filtered through 2x20 µM filters (Millipore) placed between rubber gaskets. The flow-through contains mostly J2 and some J3, which were pelleted by centrifugation, 8 seconds on a table-top eppdendorf centrifuge 5424, reaching approximately 10,000 x g. The juvenile pellet was then either re-suspended in 1 ml Neutral Red working solution, or in 1 ml M9 and split to two tubes, then re-centrifuged, and then re-suspended in either 1 ml working solution Neutral Red (0.005% in M9) or 1 ml 50 µM Green BODIPY (Thermo) in M9. Tubes were then rotated for 3 hours in the dark, then washed by centrifugation as before, and re-suspended in 1 ml M9. This was repeated 3-4x until the dye was no longer visible in the worm pellet. Then, the concentration of worms was determined by aliquoting 2 µl onto a glass coverslip in 5 technical replicates, and counted under a dissecting microscope. Finally the appropriate number of animals was added to 6 cm plates that had been previously seeded with 300 µl OP50, and incubated at 20°C. After 3 days, 100% of worms exhibited Neutral Red staining (*n*=50, Supplementary figure 4). Dauers and J2s recovered after Neutral Red staining developed at the same developmental speed (3-4 days) and with the same mouth-form ratio as control worms recovered side-by-side (100% St for both, Supplementary figure 5, *n*=30). Dauers and J2s stained with Cell tracker Green BODIPY (50 µM) (Thermo) were similar, although less efficiently stained compared to Neutral Red. On day 4, 90% retained intestinal fluorescence (Supplementary figure 4), although brightness decreased with the number of days. Mouth-form ratios of dauers or J2s in +/- 50 µM Cell tracker Green BODIPY also developed at equivalent rates and mouth-form ratios (Supplementary figure 5). Lower than 25 µM did not yield strongly fluorescent worms after three hours. Cell Tracker Blue CMAC (Thermo) was also used at 50 µM and imaged 3 days post-staining for *P. pacificus*, and

one day post-staining for *C. elegans*. However, due to the higher fluorescent background in the blue light spectrum in both *P. pacificus* and *C. elegans*, we performed all experiments using only Neutral Red and Cell tracker Green BODIPY.

(f) Mixed culture experiments and statistical analysis

We performed mixed culture experiment presented in figure 2 with 3 to 5 independent biological replicates, and a minimum total number of counts $n > 100$ (median counts per replicate for J2=29 and the median counts per replicate for dauers=27). J2 or dauers were stained with Neutral Red as described in the 'Dye Staining' method section, then added to green-stained J2, dauer, or adult populations on 6 cm plates with 300 µl OP50 and incubated at 20° C. To ensure consistent bacterial food supply, we added 1 ml more overnight OP50-LB to each plate on the following day, then air-dried under a chemical fume hood for 1 hour, then returned to 20° C. On days 3-4, we phenotyped 'red' adults that exhibited no 'green' staining. To assess whether the age of the 'green' surrounding population affects the mouth form of the dependent variable 'red' J2s we performed a binomial regression on Eu counts (i.e. "successes") weighted by the number of counts per replicate and the number added as a fixed effect, using a generalized linear model from the standard statistical package in R:

glm(formula=cbind(Eu,total)~'stage_added' * '#_added', data='J2/Da', family="binomial"))

See Supplementary figure 6a for a table containing the resulting *p* values. The AIC for our models (78.97 for J2s and 89.59 for dauers) was substantially lower than the null hypothesis (220.16 for J2s and 147.29 for dauers), arguing a reasonable fit. For pair-wise comparisons of the effect of age for a given number of added animals, we performed a post-hoc Fisher's exact test on a contingency table containing the summed counts of Eu and St observations. For display, we converted Eu counts into percent of total in figure 2, with the *p* values between the

same number of animals added indicated over the adult-added population (Significance codes: 0 '***' 0.001, '**' 0.01, '*' 0.05).

(g) Measuring the effect of food depletion on mouth form

To verify that starvation was not a factor in our mixed culture experiments, we added increasing number of J2s to standard 6 cm plates with 300 µl OP50 (see Figure 4) to rapidly consume bacterial food, and measured both the amount of animals that reached adulthood, and the percent Eu in each population for two biological replicates. To assess the affects of added J2s to each dependent variable we performed a binomial regression with count data weighted by the total number of counts for each replicate:

glm(formula = cbind(reached_adult, total)~thousand_J2s, data=data_2, family="binomial"))

p values indicate a significant difference in percent reaching adult as a function of J2s added, but not in percent Eu (Supplementary Fig. 6b).

(h) Microscopy

All images were taken on a Zeiss Axio Imager 2 with an Axiocam 506 mono, and processed using Zen2 pro software. Image brightness and contrast were enhanced in ImageJ with a minimum displayed value of 10 and maximum of 100 for all images in Fig 2, and Supplementary figures 4 and 5, and a minimum of 21 and maximum of 117 for Supplementary figure 3. The following exposure times were used for all images: Cy3 (peak emission = 561, exposure = 80 ms), FITC (peak emission = 519, exposure = 150 ms), Dapi (peak emission = 465, exposure = 80 ms), DIC (exposure = 80-140 ms).

(i) Dauer induction

To induce dauer, mixed-stage plates with little to no OP50 were washed with M9 and the resulting worm pellets were used in a modified 'White Trap' method. Worm pellets were placed on killed *Tenebrio molitor* grubs and dispersing dauers were collected in surrounding MilliQ water. Age of dauers ranged from one week to one month.

3. Results

(a) A vital dye method for labeling nematode populations

To directly test if different age groups of nematodes influence plastic phenotypes, we required two synchronized populations to co-habit the same space, yet still be able to identify worms from different age groups. To do so, we developed a dye-staining methodology to robustly differentiate between nematode populations. After trying several vital dyes, we identified that Neutral Red [43] and CellTracker Green BODIPY (Thermo) stain nematode intestines brightly and specifically to their respective channels (figure 2*a-e*, Supplementary Fig. 1, Materials and methods). These dyes stain all nematodes tested including *C. elegans* (Supplementary figure 2) and dauer larvae (Supplementary figure 3). They last more than three days (Supplementary figure 4), allowing long-term tracking of mixed nematode populations. Importantly, neither Neutral Red nor CellTracker Green staining affect viability, developmental rate, or the formation of specific morphological structures, such as *P. pacificus* mouth form (Supplementary figure 5). Thus, Neutral Red and CellTracker Green allow specific labeling of worm populations to study age-dependent effects on phenotypes.

(b) Adult but not juvenile crowding induces the predatory mouth form in *P. pacificus*

To assess potential intra-, or inter-generational influence on *P. pacificus* mouth form we stained juveniles of the highly St strain RSC017 with Neutral Red, and added an increasing number of

CellTracker Green-stained RSC017 adults or juveniles (figure 2*f,g*). Three days later we phenotyped red animals that had developed into adults, but showed no green staining. To ascertain potential differences between adding juveniles or adults, we performed a binomial regression on Eu count data from multiple independent biological replicates (n>3), with age and number of individuals added as fixed effects (Materials and methods, Supplementary figure 6). We observed a significant increase in Eu worms in response to adults, but not juveniles (p=2.59 x10^{-2}; for display summed percents are shown in figure 2*h,i*). Almost half (48%) of the population developed the Eu mouth form with just 500 adult animals, which is a greater than 50-fold induction compared to side-by-side controls (figure 2*h,i*). We were also curious if dauers, which have a thickened cuticle and represent a distinct stage in the boom-and-bust life cycle of nematodes, could still respond to adults. Indeed, the same trend that was observed with juveniles was seen with dauers (p=2.96x10^{-3}), albeit to a more muted extent (figure 2*j,k*). Specifically, with a total of 200 dauers and 500 adults, 25.7% of dauers become Eu, whereas only 1.8% of dauers become Eu on a plate containing 700 dauers (and no adults) (figure 2*j*). Collectively, these data indicate that adult crowding specifically induces the Eu mouth form.

Even though we did not detect a mouth-form switch in large populations of J2s or dauers, and food was still visible on plates containing the most animals (500 adults and 200 juveniles), we could not completely rule out the possible effect of food availability on mouth form. As a proxy for starvation, we conducted assays with greatly increased numbers of juveniles from 1,000 to 10,000 that would rapidly deplete bacterial food. We noticed a stark cliff in the fraction of juveniles that reach adulthood at 4,000-5,000 animals, arguing that food is a limiting resource at this population density (figure 2*l*). Importantly however, in these plates we still did not see a shift in mouth form (p=0.99, binomial regression, Supplementary figure 6). With an overwhelming 10,000 worms on a plate, 5.8% were Eu, compared to 48% in the

presence of only 500 adults. While longer-term starvation may yet have an impact on mouth form, under our experimental conditions it appears to be negligible.

(c) Adult but not juvenile secretions induce the Eu mouth form

As mouth-form plasticity in *P. pacificus* is regulated by nematode-derived modular metabolites (NDMMs)[32], we wondered if the difference between adults and juveniles resulted from differences in secreted NDMMs. To test this hypothesis we added secretions from adult or juvenile worms to RSC017 (highly St) juveniles. We found that adult secretions from both the laboratory stain RS2333 (highly Eu) and RSC017 led to a significant increase in the Eu morph relative to juvenile secretions (p=5.27x10^{-06}, 1.33x10^{-3}, respectively, Fisher's exact test)(figure 3). To confirm the effect was caused by ascaroside pheromones, we exposed RSC017 juveniles to supernatant from a *daf-22.1;22.2* double mutant, which exhibits virtually no ascaroside production in both *C. elegans* and *P. pacificus* [26,44]. Again, juvenile secretion had no impact on Eu frequency, but in contrast to wild-type supernatants, we also observed no increase in Eu frequency with adult secretions (p=0.8324, Fisher's exact test, figure 3). Thus, adult-specific NDMMs induce development of the Eu mouth form.

(d) Developmental-staged NDMM profiles reveal adult-specific synthesis of dasc#1

Next, we investigated whether the difference between adult and juvenile pheromones is one of dosage, or of identity. To answer this question and verify age-specific differences in pheromones, we profiled *P. pacificus* NDMM levels in two strains and at three time points through development. We used RS2333 and RSC017 and measured the exo-metabolomes of juvenile stage 2 (J2s, 24 hrs), J3s (48 hrs) and J4/adults (72 hrs) from a constant culture with excess OP50 bacterial food (figure 4*a,b*, Supplementary figure 7, Materials and methods). To assess potential differences in pheromone levels we performed a linear regression with the area

under the curve for each NDMM (aoc) (Supplementary Figure 1a) as the response variable. Stage and strain were modeled as fixed effects, and because we performed separate regression analyses for each pheromone, we adjusted the resulting *p* values for multiple testing using false discovery rate (FDR)(see Supplementary figure 8 for *p* and *FDR* values between stage and strain). We observed that there was a significant affect of developmental stage on the levels of ascr#9, ascr#12, npar#1, and dasc#1, and that ubas#1 and #2 are strain and stage specific (FDR<0.05). Interestingly, dasc#1 is the most potent known Eu-inducing compound when tested as a single synthesized compound, while npar#1 is both Eu- and dauer-inducing (figure 4*c,d,f-i*)[32]. Closer inspection revealed dasc#1, npar#1, and ascr#9 increase throughout development in both strains, and dasc#1 peaks in adults in RS2333 ($p<0.05$, student's two-tailed *t*-test between 72 hrs and 24 hrs for each NDMM in both strains, and also 72 hrs and 48 hrs for dasc#1 in RS233). Intriguingly, the trajectory of dasc#1 appeared binary in both strains (figure 4*f,g*). In fact our statistical model for dasc#1 fits better if we assume cubic rather than linear growth (ΔAIC=3.958). In contrast, ascr#9, which was also statistically up-regulated but does not affect known plastic phenotypes [32], displays a more gradual increase in both strains (figure 4*e,j,k*), and the model fits better with linear growth ($AIC_{linear} - AIC_{cubic}$= -1.208). Meanwhile, the trajectory of npar#1 appears strain specific (figure 4*h,i*). Hence the mode of induction is NDMM-specific, and the kinetics of production may be related to their roles in phenotypic plasticity.

In principle, the increase in abundance of certain pheromones could be a result of a concomitant increase in body mass, however several observations indicate more targeted regulation. First, no other compounds were significantly different in our linear model. Second, an analysis of previously published RNA-seq data [45] reveals the increase in NDMM abundance corresponds to an increase in transcription of the thiolase *Ppa-daf-22.1* (Supplementary figure 9) the most downstream enzyme in the β-oxidation pathway of ascaroside synthesis. Third,

pasc#9 and pasc#12 actually exhibit a peak in abundance at the 48 hr/J3 time point, rather than in 72 hrs/adults. Finally, we profiled the endo-metabolome of eggs, and found appreciable amounts of ascr#1, #9, #12, and pasc#9, but little to no traces of other ascaroside derivatives (Supplementary figure 7), suggesting age-specific synthesis rather than release. Together, these results suggest that the observed increase in ubas#1 and #2, ascr#9, npar#1, and dasc#1 over time corresponds to age-specific production. The observation that dasc#1 is produced specifically during the juvenile-to-adult transition is especially intriguing because adults are no longer able to switch mouth forms, hinting at cross-generational signaling.

4. Discussion

Here, we introduce a novel dye-based method that allowed us to determine cross-generational influence on mouth form, and demonstrate this effect is a result of age-specific production of plasticity affecting pheromones. In doing so, we provide the first multi-stage time series of pheromone production in *P. pacificus*, which shows that dasc#1 exhibits a surprising 'off-on' switch-like induction pattern. Collectively, our results argue that adults represent a critical age group [46] in nematode populations.

Our developmental profiling revealed an increase in two NDMMs that affect plastic phenotypes. The observation that this trend mirrors the transcriptional regulation of enzymes involved in NDMM synthesis argues that the stage-dependent increase is not simply a result of an increase in body mass, but rather that these molecules are programmed for stage-specific induction. The binary 'off-on' kinetics might reflect a population level feed-back loop, such that the production of density-sensing pheromones is based on a threshold level of previously produced pheromones. It is also worth noting that while npar#1 is the major dauer-inducing pheromone in *P. pacificus* [32], we did not observe dauers in any experimental setup described herein. Thus, it seems that mouth-form phenotype is the first-level plastic response to

population density. Presumably higher concentrations are required for dauer induction, reflecting a calculated response strategy depending on the level of crowding or duration of starvation. Interestingly, the effect of adult supernatants was noticeably less (23%-26% Eu) than with adult worms (up to 48% with only 500 adults). It is difficult to compare the amount of pheromone concentrations between experiments, but presumably worms in the vital-dye assay experienced a greater local concentration as they were in direct physical contact with each other, compared to worms in the supernatant assay.

Among the many environmental influences on mouth form [42], population density and starvation are perhaps the most ecologically relevant. However, teasing apart these two factors has proven difficult [47]. Here, we demonstrate that while a strong shift is observed with adult-specific pheromones, no such effect was seen under limited resource conditions. Thus, age-specific crowding is sufficient to induce the Eu mouth form. Nevertheless, this does not preclude that long term starvation could also have an effect. Determining the relative contributions of these factors to mouth form will be important to better understand the sophisticated ecological response strategies of *P. pacificus*, nematodes, and phenotypic plasticity in general.

Why do adults and not juveniles affect mouth form? Given that St animals can develop faster [48], there may be a ‘race’ to sexual maturation in emergent populations at low densities. But as the nematode population increases, there will likely be a commensurate decrease in bacterial populations. When faced with competition from other nematodes, *P. pacificus* has a particular advantage in developing the Eu morph; their expanded dietary range includes their competition. Indeed, when nematode prey is the only available food source, the Eu morph provides longer life spans and more progeny than the St morph [49]. When resources become depleted as population size increases, *C. elegans* and other monomorphic nematodes may enter dauer and disperse [50]. But in St-biased dimorphic strains of *P. pacificus*, juveniles may switch to the Eu morph in response to adults as a first level indication of rapidly increasing

population size (Supplementary figure 10). Then, after prolonged starvation and crowding, worms will presumably enter dauer. By analogy to economic models of population growth [51,52] we view mouth-form plasticity and dauer formation as alternative 'technological innovations' to escape a Malthusian resource trap. To what extent this occurs in nature, or with *P. pacificus* strains that are highly Eu, remains to be determined.

The evolution of dimorphic mouth forms is one among myriad nematode ecological strategies. For example, entomopathogenic nematodes release their symbiont bacteria in insect hosts to establish their preferred food source, and some release antibiotics to kill off competing bacteria and fungi from other entomopathogenic species [53]. Some free-living species, like those of the genus *Oscheius*, refrain from combat and stealthily feed and reproduce amidst warring entomopathogenic species. Interspecific killing also occurs in gonorchoristic species, in which both mated and virgin males are killed, implying fighting not just for mates but for resources as well [54,55]. Reproductive strategies also exist, and hermaphroditic species have an advantage over gonachristic species when colonizing a new niche, such as an insect carcass [56]. Meanwhile insect hosts and colonizing nematodes have their own distinct pheromone-based attraction and toxicity [57,58]. Finally, the renaissance of *C. elegans* sampling from around the world [59-63] is rapidly building a resource of wild isolates that will almost certainly have different and fascinating ecologies. We hope our method for labeling and then combining different nematode populations on the same plate will aid in studies to identify these strategies. Perhaps the time is also ripe to complement these studies with more sophisted ecological modelling that can lead to more testable hypotheses.

Although beyond the scope of this manuscript, the cross-generational communication we observed could in principle reflect an intended signal from adults to juveniles, i.e. kin selection [64]. However, we favor a more simplistic view that juveniles have evolved to recognize adult-produced metabolites. Regardless of these interpretations, our results argue that age groups

are a critical factor in density-dependent plasticity, as has been theorized in density-dependent selection [65].

Author Contributions

MSW and RJS conceived of the project. MC conducted pheromone profiling. MSW and TR designed and conducted dye-labeling experiments. TR and MC performed supernatant experiments. MD and MSW considered ecological implications. MSW and TR wrote the manuscript with input and edits from all authors.

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614

615 **Figure Legends**

616

617 **Figure 1. Life cycle and developmental plasticity of the model nematode *Pristionchus***
618 ***pacificus*.**

619 (a) The life cycle of *P. pacificus* consists of four juvenile stages (J1-4) until sexual maturation
620 (adults). Like many nematodes *P. pacificus* can enter a long-living 'dormant' dauer state that is
621 resistant to harsh environmental conditions. The decision to continue through the direct life
622 cycle or enter dauer is regulated by small molecule excreted ascarosides (chemical structure
623 adapted from [13]). (b) *P. pacificus* can also adopt one of two possible feeding structures; either
624 a microbivorous narrow-mouth (stenostomatous, St), or (c) an omnivorous wide-mouth
625 (eurystomatous, Eu) with an extra tooth that can be utilized to kill and eat other nematodes or
626 fungi. White lines indicate the presence of an extra tooth (right side) in the Eu morph or its
627 absence in the St morph, and the dorsal tooth (left side), which is flint-like in St and hook-like in
628 Eu. White scale bar indicates 5 µM. (d), *P. pacificus* is often found in a necromenic association
629 with beetles (ex. shown here *Oryctes borbonicus*) in the dauer state, and resumes the free living
630 life cycle upon beetle death to feed on the ensuing microbial bloom.

631

632 **Figure 2. Vital-dye method confirms adult-specific density effect on mouth form.**

633 (a-e) *P. pacificus* were stained with either 0.005% Neutral Red or 50 µM CellTracker Green
634 Bodipy (Thermo) and viewed using Cy3 and FITC filters. Images were merged with Differential
635 interference contrast (DIC), scale bar = 100 µM. An example of relative intensities of each

fluorescence channel are displayed in the histograms (right) with the chemical structure of Neutral Red or CellTracker Green Bodipy. (f) Age-dependent functional pheromone assay: Experimental juveniles were stained with Neutral Red, and challenged with CellTracker Green Bodipy-stained juveniles or adults on standard condition NGM agar plates seeded with 300 µl OP50 *E. coli*. Three days later, only red-positive and green-negative adults were phenotyped. (g) The wild isolate RSC017 grown in standard conditions (5 young adults passed to fresh plates, progeny phenotyped 4 days later) are highly stenostomatous (<10%, *n*=102). (h,i) Mouth form ratios of Neutral Red-stained J2s, and (j,k) dauers, with increasing number of CellTracker Green-stained competitors, as described in (f) (n=3-5 independent biological replicates per experiment, with total n=104, 126, 134, and 109 for 200 NR-stained J2 with 0, 50, 200, and 500 CellTracker Green-stained J2s, and 115, 113, and 112 for 50, 200, and 500 CellTracker Green-stained adults; n=104, 105, 105, and 102 for NR-stained dauers with 0, 50, 200, and 500 CellTracker Green-stained dauers, and 100, 103, and 105 for 50, 200, and 500 CellTracker Green-stained adults. Overall significance between strain and age was determined by a binomial linear regression (see Materials and methods), and pair-wise comparisons were assessed by Fisher's exact test on summed Eu counts (Significance codes: '***' < 0.001, '**' <0.01, '*' <0.05). Mouth forms were phenotyped at 40-100x on a Zeiss Axio Imager 2 light microscope. (i) Percent reaching adulthood (top) and percent Eu of those that reached adulthood (bottom) after increasing numbers of J2s are added to standard 6 cm NGM agar plates with 300 µl OP50 *E.coli* bacteria (n=2 biological replicates, with total n for percent reaching adulthood=216, 202, 206, 207, and 202 for 1, 3, 4, 5, and 10 thousand J2s, and total n for mouth form=106, 106, 103, 110, and 104). Significance was determined by a binomial regression (see Materials and methods).

Figure 3. Adult-specific secretions induce predatory morph in juveniles.

Highly St strain RSC017 juveniles were exposed to J2 and adult supernatants of its own strain, and to the J2 and adult supernatants of highly Eu strain RS2333. Mouth form was phenotyped three days later. Worms exposed to J2 secretions remained highly St, while worms exposed to adult secretions had a small but significant increase in Eu morphs ($p<0.05$, Fisher's exact test). Supernatants from the double mutant *daf-22.1/2*, which has deficient ascaroside pheromone production, did not elicit juvenile or adult increase in Eu. Worms exposed to the S-media control also remained highly St. n=4 independent biological replicates for RS2333 and *daf-22.1/2* secretions, and n=2 independent biological replicates for RSC017 adult and juvenile secretions, with an average count of 55 animals per replicate. For display, total Eu and St counts are presented as percentages (Significance codes: '***' < 0.001, '**' <0.01, '*' <0.05).

Figure 4. Time-resolved Nematode-Derived Modular Metabolites (NDMMs) in *Pristionchus pacificus*.

(a) Time resolved secretion profile of nematode derived modular metabolites from the wild-type laboratory strain RS2333 and (b) wild isolate RSC017. Data is presented as the mean of 8 (RS2333) and 9 (RSC017) biological replicates, and error bars represent standard error of the mean (SEM). (c-e) Chemical structures of adult specific NDMMs dasc#1, npar#1, and ascr#9, as described in the Small Molecule Identifier Database (http://www.smid-db.org/), produced in ChemDraw. (f-k) Time-resolved abundance of dasc#1, npar#1, and ascr#9 NDMMs in RS2333 and RSC017. Each data point represents a biological replicate, and for comparison to (a-b) lines represent mean abundance. *P* values calculated by a 2-tailed students *t*-test (Significance codes: '***' < 0.001, '**' <0.01, '*' <0.05).

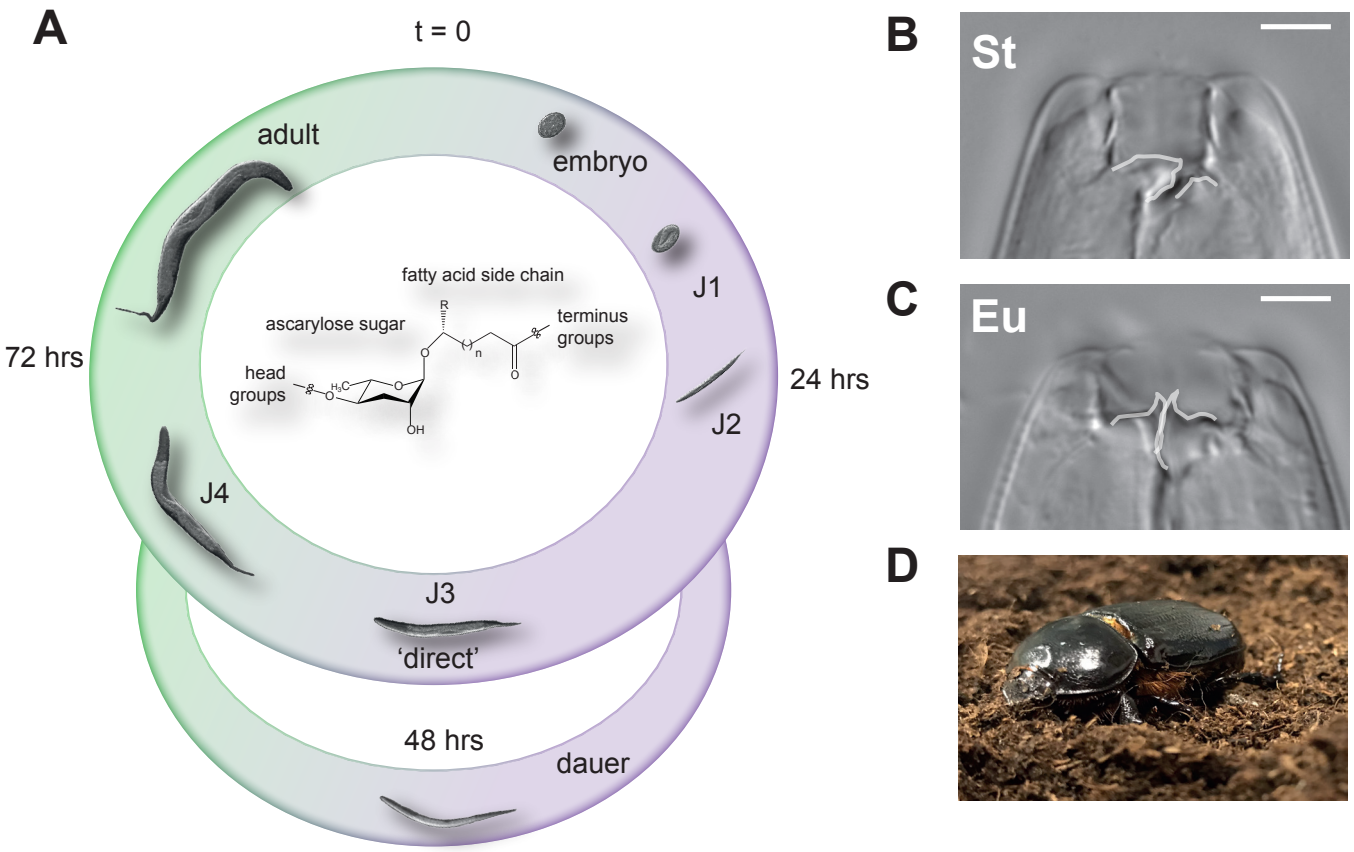

Figure 1. Life cycle and developmental plasticity of the model nematode *Pristionchus pacificus*.

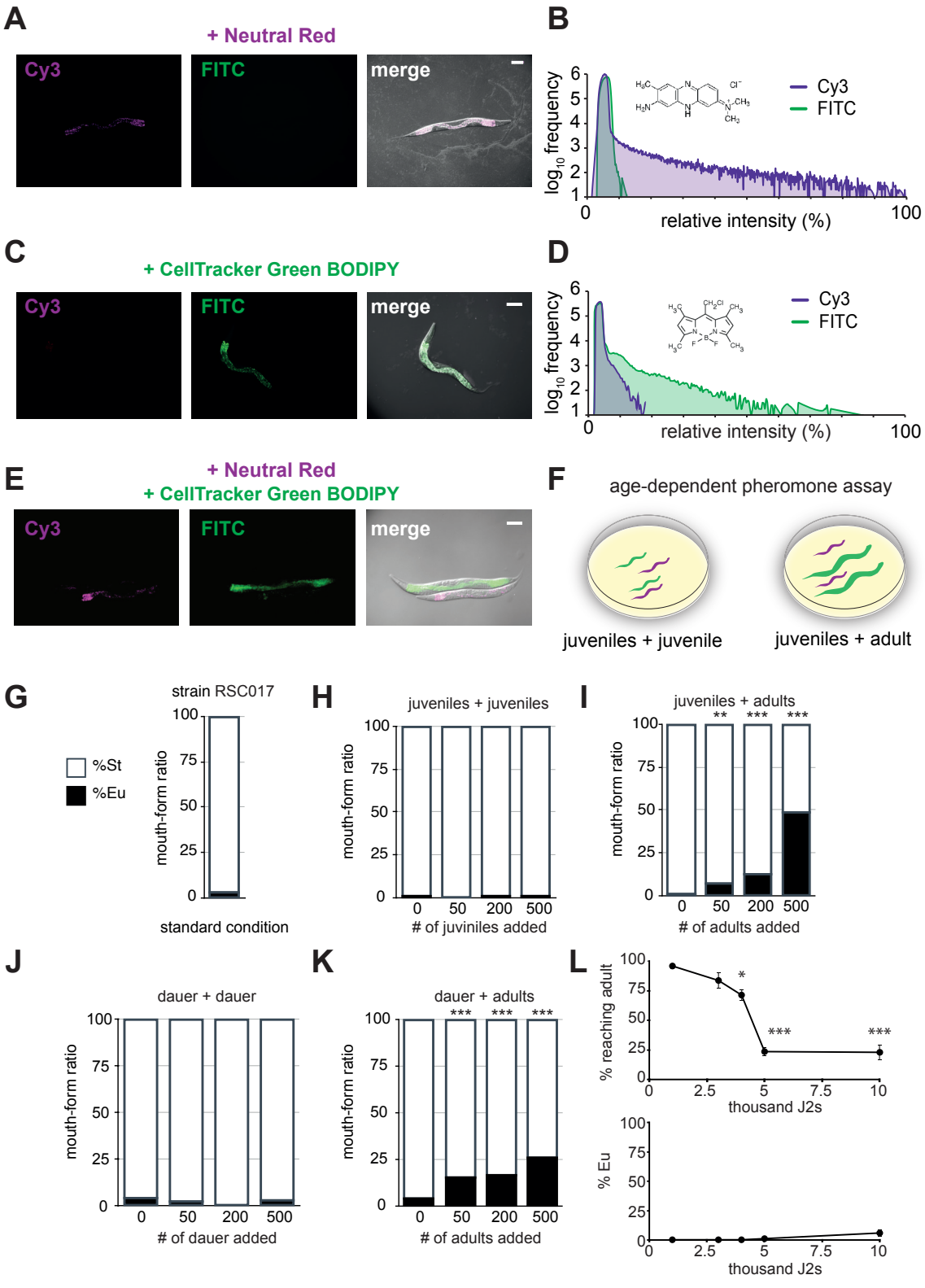

Figure 2. Vital-dye method confirms adult-specific density effect on mouth form.

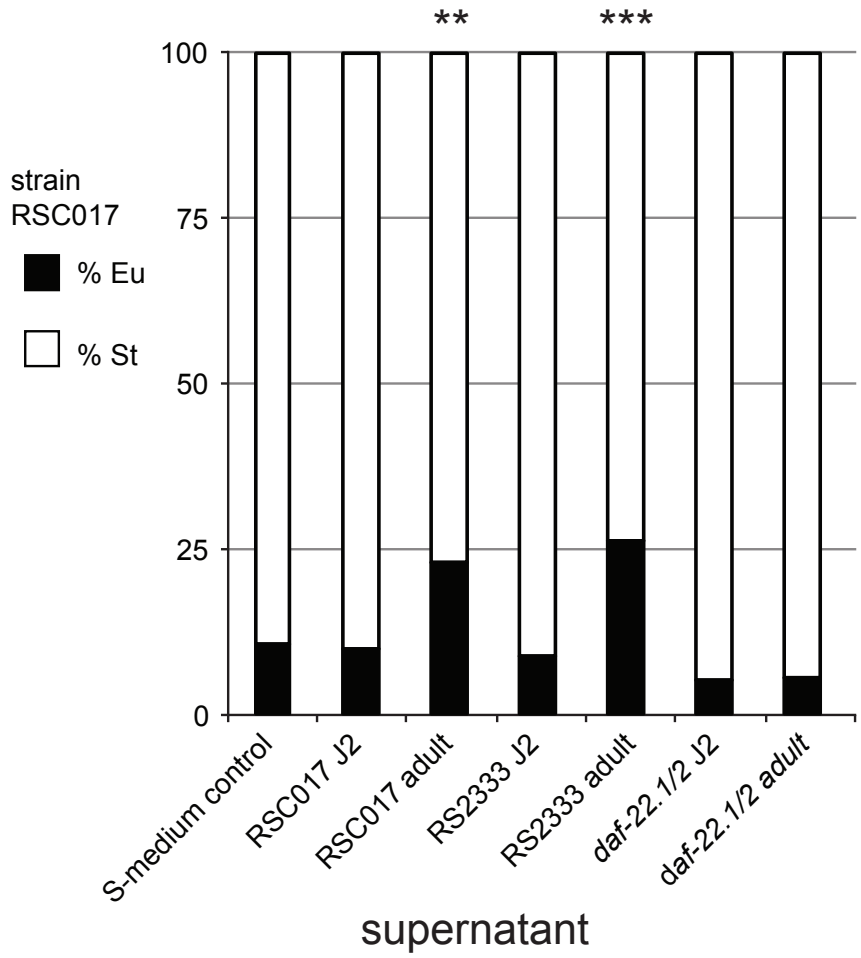

Figure 3. Adult-specific secretions induce predatory morph in juveniles.

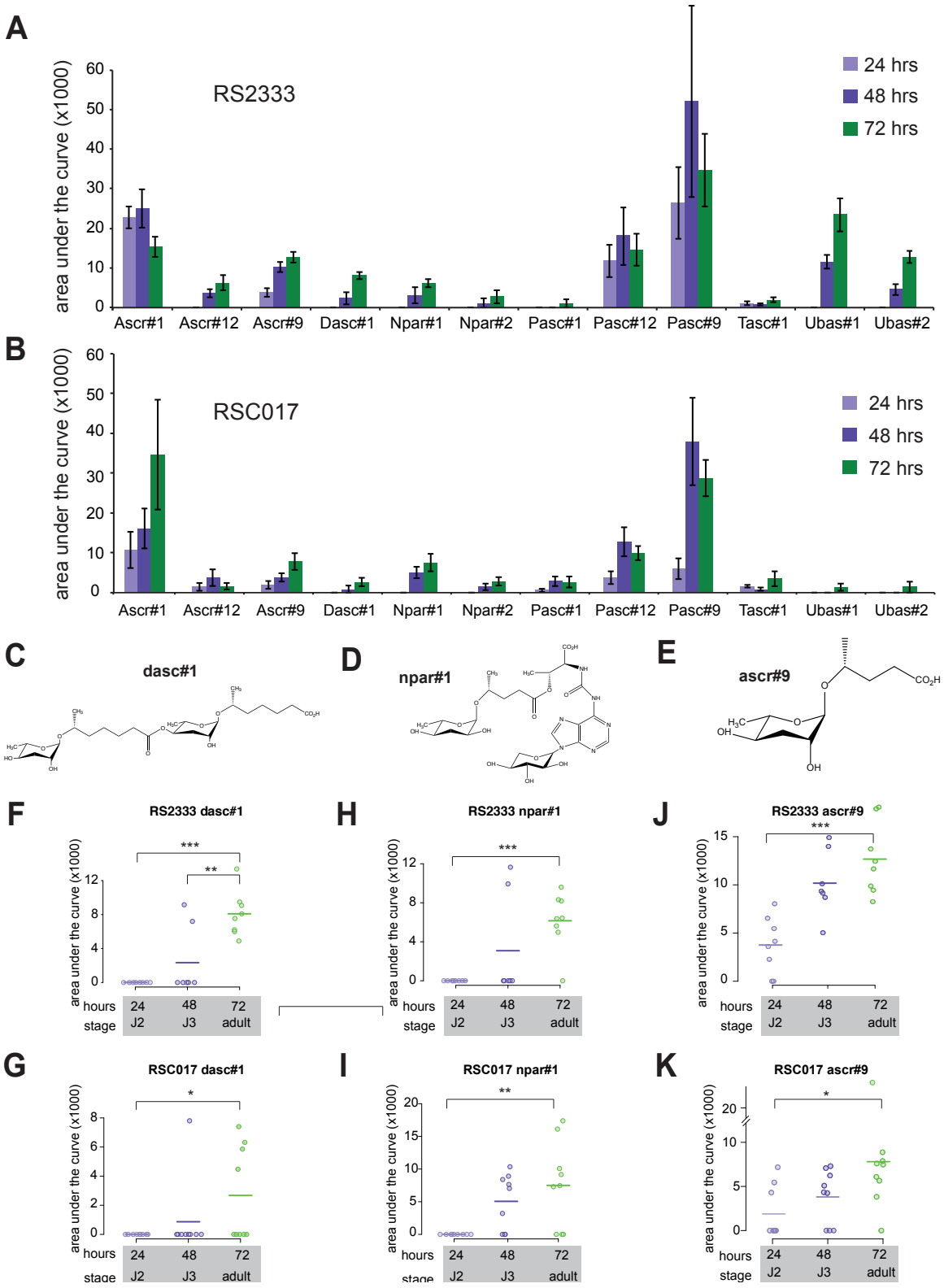

Figure 4. Time-resolved Nematode-Derived Modular Metabolites (NDMMs) in *Pristionchus pacificus*.