**ARTICLE**

The nematode *Caenorhabditis elegans* exhibits rapid senescence that is promoted by the insulin/IGF-1 signalling (IIS) pathway via regulated processes that are poorly understood. IIS also promotes production of yolk for egg provisioning, which in post-reproductive animals continues in an apparently futile fashion, supported by destructive repurposing of intestinal biomass that contributes to senescence. Here we show that post-reproductive mothers vent yolk which can be consumed by larvae and promotes their growth. This implies that later yolk production is not futile; instead vented yolk functions similarly to milk. Moreover, yolk venting is promoted by IIS. These findings suggest that a self-destructive, lactation-like process effects resource transfer from postreproductive *C. elegans* mothers to offspring, in a fashion reminiscent of semelparous organisms that reproduce in a single, suicidal burst. That this process is promoted by IIS provides insights into how and why IIS shortens lifespan in *C. elegans*. 

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Adult *C. elegans* hermaphrodites exhibit severe senescent pathology that begins to develop within days of reaching sexual maturity\(^{1-3}\). For example, after depletion of self-sperm, intestinal biomass is converted in an autophagy-dependent manner into yolk, leading to intestinal atrophy and yolk steatosis (pseudocoelemic lipoprotein pools, PLPs)\(^{5,6}\). These senescent pathologies are promoted by insulin/IGF-1 signalling (IIS), which also shortens lifespan\(^{12}\). This pattern of rapid and severe pathology in organs linked to reproduction is reminiscent of semelparous organisms where massive reproductive effort leads to rapid death (reproductive death) as in Pacific salmon\(^{19}\). Moreover, self-destructive conversion of somatic biomass to support reproduction, often involving autophagic processes, is a characteristic feature of reproductive death\(^{8}\). Yet arguing against the occurrence of reproductive death in *C. elegans* is the apparent futility of post-reproductive yolk production. Here we show that this effort is not futile, since post-reproductive mothers vent yolk through their vulva, which is consumed by progeny and supports their growth and fertility; thus vented yolk serves a function similar to milk, and *C. elegans* mothers exhibit a form of primitive lactation that is coupled to senescence. Moreover, wild-type IIS promotes lactation, where maternal soma is consumed to support resource transfer from sperm-depleted mothers to larval kin, thereby accelerating aging.

**Results**

**Post-reproductive *C. elegans* hermaphrodites vent yolk.** *C. elegans* hermaphrodites are protandrous, producing first sperm and then oocytes, and reproduction ceases by around day 4 of adulthood due to self-sperm depletion. While working with adult hermaphrodites expressing the vitellogenin (yolk protein) VTT-2 tagged with GFP (ref. \(^{10}\)), we noticed that older mothers leave patches of GFP-positive material on culture plates (Fig. 1a). Viewed under light microscopy these appeared as smears of a brownish substance (Fig. 1a). V칭 of vitellogenin was confirmed and found to be highest on days 4–6 of adulthood, immediately after cessation of egg laying, and then to continue at lower levels until at least day 14 (Fig. 1b; Supplementary Fig. 1a). Yolk was vented through the vulva in brief bursts, either alone or with unfertilised oocytes (Fig. 1c, Supplementary Movie 1). Vented yolk also contains lipid, as shown by staining with a lipid dye (Fig. 1d; Supplementary Fig. 1b). Thus, cessation of egg laying due to self-sperm depletion is followed immediately by a burst of yolk venting.

**Vented yolk supports larval growth.** Given that yolk is a nutrient substance, one possibility is that vented yolk supports larval growth. Consistent with this, GFP-labelled yolk was observed in the intestinal lumen of larvae, indicating that yolk can be ingested (Fig. 2a). Notably, pre-treatment of *E. coli*-free agar plates with post-reproductive, 4-day-old mothers enhanced growth of L1 larvae, relative to control plates (no pre-treatment, or pre-treatment with non-venting L3 larvae; Fig. 2b). Moreover, subjecting mothers to *vit-5,-6* RNAi, which prevents vitellogenin accumulation\(^{6}\) also blocked the benefit to wild-type larval growth of plate pre-conditioning with venting mothers (Fig. 2b). *vit-5* RNAi decreases levels of the YP170 vitellogenin species but RNAi of *vit-6* (which encodes YP115/YP88) increases them\(^{5}\). These treatments, respectively, suppressed and enhanced effects of pre-treatment with venting mothers on larval growth (Fig. 2b), providing further evidence that larval growth is enhanced by consumption of vented yolk.

Next we tested whether yolk vented by mothers can benefit their own larvae. Fully-fed 3-day-old mothers were washed, placed on *E. coli*-free plates and left to lay their last eggs, and then either left in situ to vent yolk, or removed. Removal of mothers reduced growth of their progeny (Fig. 2b). In a further test, mothers were replaced after egg laying with other, surrogate mothers of the same age but treated with *vit-5,-6* RNAi, and this abrogated the benefit to larval growth (Fig. 2b). Taken together, these findings show that after self-sperm depletion *C. elegans* mothers can enhance growth of their offspring by venting yolk through the vulva, at least when other food sources are unavailable.

The enhancement of larval growth by yolk feeding implies an increase in larval fitness. Supporting this conclusion, yolk feeding also accelerated the resumption of normal growth in larvae transferred to an *E. coli* food source (Fig. 2c), and led to earlier reproduction and a larger brood size (Fig. 2d). As a means of transferring resources from mother to offspring after egg laying, vented yolk serves a function similar to that of mammalian milk; we therefore propose the term *yolk milk* to describe vented yolk, and also the accumulated yolk in the body cavity and uterus prior to venting. That yolk milk is beneficial to larvae led us to wonder whether larvae might be attracted to it; however chemotaxis tests revealed no such attraction, although larvae were attracted to adult hermaphrodites (Supplementary Fig. 2a). These findings reveal a surprising additional function for the intestine, uterus and vulva in *C. elegans* hermaphrodites: that of a form of primitive lactation.

**Yolk in unfertilised oocytes supports larval growth.** After sperm depletion hermaphrodites lay over 100 excess, unfertilised oocytes, the overall volume of which exceeds that of the hermaphrodite herself, which has been noted as oddly wasteful and futile\(^{11}\). The timing of unfertilised oocyte production is similar to that of yolk venting (Fig. 1b). Indeed, yolk and unfertilised oocytes are often vented together (Fig. 1a) suggesting a possible role for vented oocytes in yolk milk transport. Consistent with this, vented oocytes contain large amounts of yolk, as shown by VTT-2:GFP, and confirmed by gel electrophoresis (Fig. 2e). At the outset of yolk/oocyte venting, oocytes contain twice as much vitellogenin as free vented yolk, but the proportion of the latter increases with age until day 10, when the ratio is 1:1 (Fig. 2e). To establish the extent to which vitellogenin delivered in each manner supports larval growth, free yolk and oocyte fractions were separated from conditioned plates on which L1 larvae had or had not been present for 24 h, and vitellogenin content was assayed. The results show that larval feeding causes a reduction in yolk from both the oocyte and free yolk fractions (Fig. 2f), i.e. vitellogenin in both free yolk pools and unfertilised oocytes is consumed by larvae. These results provide a possible explanation for the enigma of unfertilised oocyte production: that it represents an adaptation, aiding delivery of yolk milk to hungry young larvae.

**Insulin/IGF-1 signalling promotes yolk venting.** daf-2 insulin/IGF-1 receptor mutants are long lived\(^{12,13}\), and show reductions in vitellogenin synthesis\(^{5,14}\), PLP accumulation\(^{5}\) and unfertilised oocyte production\(^{15}\). Thus, IIS promotes ageing and production of yolk and oocytes. To test whether IIS promotes yolk milk venting we examined daf-2 mutants, and found that yolk venting and promotion of wild-type larval growth by post-reproductive mothers was strongly reduced (Fig. 3a–c). Conversely, the daf-2(*gk390525*) gain-of-function (gf) mutation\(^{16}\) increased yolk venting and the resulting promotion of larval growth (Fig. 3a, b). Effects of daf-2 on senescent pathology and lifespan require the daf-16 FOXO transcription factor\(^{12,13}\). The daf-16(*mgDf50*) null mutation was found to restore to daf-2 mutants both yolk venting and resultant promotion of larval growth (Fig. 3a, b). Moreover, mutation of the daf-18 PTEN phosphatase\(^{17}\), which increases
phosphatidylinositol (3,4,5)-trisphosphate (PIP3) signalling, increased yolk venting and larval growth (Fig. 3a, b). daf-2(e1370) surrogate mothers also failed to enhance growth of wild-type larvae hatched from final eggs (Fig. 3c; cf Fig. 2b right). In addition, suppression of unfertilised oocyte production by daf-2(e1370) is daf-16 dependent15, and numbers of unfertilised oocytes laid were increased by both daf-2(gf) and daf-18(nr2037) (Fig. 3d). These results imply that IIS promotes C. elegans lactation, through promotion of vit gene expression and of gut-to-yolk biomass conversion3; whether IIS promotes yolk milk and oocyte venting solely by promoting their production, or whether it also exerts neuromuscular effects that promote their active release by the vulva remains unexplored. Thus, reduced IIS in daf-2 mutants reduces later-life contributions to reproductive fitness and their associated costs, i.e. yolk milk production contributes to C. elegans senescence (particularly intestinal atrophy).

Yolk milk contains multiple IIS-regulated proteins that accumulate with age. Our findings imply that the vitellogenin-rich fluid vented by sperm-depleted C. elegans functions as a milk. To further characterise C. elegans yolk milk we subjected it to proteomic analysis. Proteins released into media by adult hermaphrodites are expected to include not only vulvally-vented proteins but also proteins excreted via the excretory pore and anus, and shed from the nematode surface. Utilising terminology standard for the study of products released by parasitic helminths18, together these constitute the C. elegans adult excretory-secretory (ES) products. ES was collected from L3 larvae and hermaphrodites on day 4 of adulthood and analysed. The set of proteins present in the latter but not the former includes products released by parasitic helminths18, together these constitute the C. elegans adult excretory-secretory (ES) products. ES was collected from L3 larvae and hermaphrodites on day 4 of adulthood and analysed.
Of these, 17 proteins exhibited all 3 features (Fig. 4c). This is 82-fold more than expected by chance alone and considering the frequency of each feature among all C. elegans proteins; and 6-fold more when also taking into account the overlap between the sets of proteins defined by each of the three features. We conclude that IIS promotes the production of certain proteins secreted from older worms whose abundance increases with age, defining a proposed IIS-activated, core yolk milk proteome.

Given that vitellogenins originate from the intestine, we wondered whether this was typical of adult ES proteins. To assess this we tested for over-representation of adult ES genes among tissue-specific gene expression sets. In fact, significant enrichment among intestinally expressed genes was not seen ($p = 0.19$), but this may be because around half of the genes in the C. elegans genome are intestinally expressed. However, over-representation was seen for genes expressed in several other tissues and cell types (Supplementary Fig. 5a). Notably, enrichment of adult ES proteins but not core yolk milk proteome proteins was detected in anal sphincter muscle- and anal depressor muscle-expressed genes (Supplementary Fig. 5a, b), potentially reflecting proteins shed via the anus.
Aside from the vitellogenins, which were the most abundant proteins, there were also several transthyretin-related (ttr) proteins, including TTR-2, -15, -16 and -51. TTRs often function as carrier proteins for lipophilic compounds^{19,20}. Also present were FAR-3, another predicted lipid-binding protein, that is expressed in the vulva, as well as others like FAR-2 and LBP-6. Lipid carrier proteins are also abundant in mammalian milk, suggesting possible functional similarities. To probe further for possible similarities with mammalian milk, we compared the Caenorhabditis elegans adult ES with the human milk proteome^{21}. Cross-species gene set analysis found a significant overlap between the two proteomes ($p = 0.00146$) (Supplementary Fig. 5c). Heat shock proteins (HSPs) sometimes contaminate protein extracts prepared for profiling, raising a concern that this statistically significant overlap might be artefactual; however, after exclusion of the two ES HSPs, the overlap remained statistically significant ($p = 0.033$). Moreover, at the protein category level, genes with Interpro terms associated with lipid binding were enriched in both cases (Supplementary Fig. 5d). These results suggest a modest level of similarity between C. elegans ES and the human milk proteome, despite the relatively distinct evolutionary origins of yolk and milk^{22}.

**Discussion**

In this study, we show that post-reproductive C. elegans hermaphrodites exhibit a form of primitive lactation, releasing yolk milk (free and in oocytes) through their vulva that can enhance growth and reproduction of progeny. We suggest that this provides a fitness benefit, coupled to pathological changes to reproduction-associated organs, in a process akin to reproductive death as seen in semelparous organisms (Fig. 4d). This coupling particularly affects the intestine, where self-destructive repurposing of biomass into yolk occurs; notably, yolk synthesis in and secretion from muscle was also recently described^{23}, raising the possibility that age-linked muscle atrophy in C. elegans^{3} also involves biomass repurposing. The occurrence of reproductive death in C. elegans is supported by comparative analysis with other Caenorhabditis species which shows that reproductive death is promoted by the germline in hermaphrodites (which vent yolk and oocytes) but absent in unmated females (which do not)^{24}, and by comparison with other organisms that exhibit reproductive death^{8}. We have also described elsewhere how reproductive death can facilitate the evolution of programmed adaptive death, which further shortens lifespan^{25,26}.

Yolk milk feeding of larvae by mothers is a previously undescribed feature of C. elegans life history. Such maternal care by nematodes might seem surprising, but milk feeding occurs in other invertebrates, including termite files (Glossina spp.)^{27} and the Pacific beetle cockroach Diploptera punctata^{28} (and in each case the nutrient fluid is referred to as milk). Yolk milk feeding by C. elegans also resembles trophallaxis, the transfer of food or nutritious fluids between individuals in a community, e.g. in social insects^{29}. It is likely that in the wild, reproducing C. elegans exist largely as colony-like, high density, clonal populations that experience boom and bust population dynamics^{30,31}. Potentially, it is into this collective that sperm-depleted hermaphrodites vent yolk milk. This would imply that natural selection favours lactation in C. elegans due to increases in inclusive fitness (through kin selection) rather than individual fitness.

Hermaphroditism facilitates rapid colonization of new food patches^{30}, but prostandry leaves mothers unable to contribute to fitness after sperm depletion. We suggest that later-life yolk milk production is an adaptation to circumvent this block to continued maternal contribution to fitness, here inclusive fitness of the surrounding clonal population. Plausibly, in C. elegans colonies fitness benefits of yolk milk venting will become significant only as microbial food supplies dwindle; this is in contrast to mammalian lactation, where milk is the main food source for neonates. Lactation also provides a solution to the long-standing enigma of copious unfertilised oocyte production in C. elegans^{31}. A second possible fitness benefit from continued yolk production after self-
Fig. 3 Yolk venting is promoted by insulin/IGF-1 signalling (IIS). a Yolk venting is downregulated by reduced IIS and upregulated by enhanced IIS. Quantitated YP170 band on protein gels. Means ± S.E.M. of 3 trials (n = 100 worms per trial). One-way ANOVA (Dunnett’s correction) or unpaired two-tailed t-test. Left to right for stars P = <0.0001, <0.0001, 0.049, 0.561 (ns), 0.046. b Decreased yolk milk provisioning by reduced IIS reduces larval growth on preconditioned plates relative to wild-type, and vice versa for increased IIS. Combined data of 3 trials (n = 200 worms per trial). Left to right for stars P = 0.019, 0.114 (ns), <0.0001, <0.0001, <0.0001. c Decreased larval growth on plates with daf-2(e1370) surrogate mothers. Wild-type mothers were allowed to lay their last eggs and either left in situ or replaced. P = <0.0001. b, c Tukey box plots of length measurement of L1 larvae (line at median; + at mean; box limits are 25th and 75th percentiles; whiskers denote 1.5 times the interquartile range). Non-parametric Kolmogorov-Smirnov two-tailed tests performed and corrected according to FDR. d Unfertilised oocyte production is downregulated by reduced IIS and upregulated by increased IIS. Bottom: Number laid for 24 h on d4–6 of adulthood and normalised to wild type. Means ± S.E.M. of 3 trials (n = 10 worms per trial). One-way ANOVA (Dunnett’s correction) or unpaired two-tailed t-test. Left to right for stars P = 0.005, 0.033, 0.002, <0.0001, <0.0001. Top: NGM plates imaged after 15 wild-type, daf-18(nr2037) or daf-2(e1370) worms were left for 24 h. Black arrowheads: unfertilised oocytes; white arrowhead: yolk pool. Red: treatments that increase the dependant variable; blue: that reduce the dependant variable; white: no effect; and grey: control. Scale 100 μm. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

sperm depletion is to build up yolk stocks in preparation for possible future reproduction after mating with males; however, this is insufficient to explain the bulk venting of yolk and oocytes, and the massive and self-destructive effort expended on yolk production, particularly given the extreme rarity of males in wild populations.

Ageing is thought to evolve partly because genes exhibit antagonistic pleiotropy (AP), exerting both beneficial and deleterious effects on fitness; if the latter only occur later in life, such AP genes may be favoured by natural selection, contributing to the evolution of ageing. Previously, post-reproductive yolk production and the pathologies to which it is coupled have been interpreted as futile run-on of reproductive function. This is consistent with the AP theory, and recent ideas about its proximate mechanisms in programmatic terms, which argue that futile run-on of biological programmes in later life (or quasi-programmes) contributes to senescent pathology. But if late-life yolk production contributes to fitness, then such production is not a quasi-programme but a programme proper, and pathologies such as gut atrophy are a direct cost of reproduction. Thus, there is a trade-off between increased fitness due to yolk milk feeding of larvae (benefit) and intestinal senescence (cost) (Fig. 4d). These results provide a fresh account of the AP action of IIS pathway genes such as daf-2 in which promotion of costly programmes provide inclusive fitness benefits.

Methods
No statistical methods were used to predetermine sample size. The experiments were not randomised. The investigators were not blinded to allocation during experiments and outcome assessment. The work presented here complied with all relevant ethical regulations for animal testing and research.

Culture methods and strains. *C. elegans* maintenance was performed using standard protocols. Unless otherwise stated, all strains were grown at 20°C on nematode growth media (NGM) with plates seeded with *E. coli* OP50 to provide a food source. An N2 hermaphrodite stock recently obtained from the Caenorhabditis Genetics Center was used as wild type (N2H) (ref. 30). Genotypes of
most mutants used are as described in Wormbase (www.wormbase.org). Strains used included DR1296 daf-2(e1368), GA114 daf-16(mgDf50); daf-2(e1370), GA1500 bs1 [pvt-2::vit-2::GFP + rol-6(su1006)], GA1928 daf-2(e1370), GR1307 daf-16(mgDf50), NS3227 daf-18(nr2037), RT130 pwls23 [vit-2::GFP] and PP2340 daf-2(pk390525).

Live imaging and video capture of venting behaviour. In order to impede worm locomotion and facilitate imaging, polybead micropheres were used. d4 RT130 adults were placed on an NGM plate (no bacteria) in a 15–20 μl drop of 0.1 μm non-fluorescent polybead micropheres (2.5% solids [w/v] aqueous suspension with a coefficient of variance of 15%, 4.55 × 1013 particles/ml [Polysciences]). To remove any aggregated particles these were previously filtered through a 0.5 μm pore size syringe filter. A coverslip was then gently placed over the animal and observations made at ×200 magnification with Nomarski optics. To prevent fluorescence bleaching, Nomarski/GFP superimposed videoing and imaging were commenced only when movement of the vulval muscles was observed, which often preceded yolk milk venting or laying of eggs or unfertilised oocytes.

Nomarski and epifluorescence microscopy imaging. Unless otherwise stated, live worms were placed onto 2% agar pads and anaesthetised using 0.2% levamisole. Images were captured using either a Zeiss Axioskop 2 plus microscope with a Hamamatsu ORCA-ER digital camera C4742-95 and Velocity 6.3 software (Macintosh version) for image acquisition; or an ApoTome.2 Zeiss microscope with a Hamamatsu digital camera C13440 ORCA-Flash4.0 V3 and Zen software. A constant exposure time was maintained between samples in all imaging and comparison analyses. Brightness and contrast were adjusted equally across the entire image, and where applicable, equally applied to the controls. Where Nomarski and fluorescence images were superimposed, brightness and contrast were adjusted separately prior to superimposition.

Confocal and airyscan imaging. For this, an inverted LSM880 microscope equipped with an Airyscan detector (Carl Zeiss, Jena) was used with a Plan-Apochromat 63 × 1.4 [numerical aperture (NA) 1.4] oil objective with a working distance of 0.19 mm. A 488 nm Argon laser was used for GFP excitation. In confocal mode, emission was recorded with an inbuilt GaAsP detector. For airyscan, the emission was recorded with the in-built 32-element GaAsP detector. For images showing sample change over time, images were only taken around every 50 min to prevent sample photobleaching. In the acquisition of 3D z-series, samples were imaged up to a sample depth of 41 μm, with images of 41 z-planes taken evenly through half of the diameter (dorsoventral) of the nematode. Data was processed using Fiji software (NIH), and the 3D Viewer plugin was used for 3D

Fig. 4 Analysis of adult ES proteome, including yolk milk proteome. a Relationship between protein level (intensity) in the day 4 adult ES proteome and increased abundance with age in the overall proteome, the latter from published data for wild type and daf-2(e1370) mutant adult C. elegans (ref. 46). “Age upregulated” was defined as showing an increase of >log2 = 1 day 17 vs day 1 (vertical dotted line). Right hand cluster includes many IIS- and age-upregulated secreted proteins. Left hand cluster likely includes more proteins shed by tissue breakdown.

b Enrichment analysis of adult-specific ES proteome proteins relative to all proteins detected in the reference study46. Significant over-representation was detected using a one-tailed SuperExactTest45. c Percentage abundance categorisation of the core 17 proteins in the adult-specific ES proteome that are IIS upregulated, age upregulated and likely to be secreted (i.e. bear a predicted N-terminal signal peptide). For raw MS data see Supplementary Data 1. For details of IIS upregulation and daf-16 dependence see Supplementary Fig. 3. For L3 ES proteome and specific proteins of interest see Supplementary Fig. 4. For tissue enrichment analysis and comparison to proteomic analysis of human milk see Supplementary Fig. 5. Data are available via ProteomeXchange with identifier PXD025472. d Model of intestinal conversion to yolk in sperm-depleted adults, where the benefit of yolk milk comes at the cost of intestinal atrophy. Here IIS promotes self-destructive somatic biomass repurposing, thus promoting senescence in a fashion typical of organisms exhibiting semelparous reproductive death8. Scale 50 μm.

| Key features | Other |
|--------------|-------|
| Signal peptide | IIS upregulated |
| IIS upregulated | Signal peptide and IIS upregulated |
| Vitellogenin | Transthyretin-like |
| Lipid binding | Protein folding |
| Ribosomal | Cytoskeleton |
| Unknown | None of the above |

**Nomarski and epi-fluorescence**
| Protein             | Predicted Function                        | Age Increase (Log fold change over 1) | IIS upregulated | Signal peptide classification |
|---------------------|-------------------------------------------|---------------------------------------|-----------------|------------------------------|
| P18948              | Vitellogenin-6                            |                                       |                 |                              |
| Q22288              | Transthyretin-like protein 15             |                                       |                 |                              |
| P005690             | Vitellogenin-2                            |                                       |                 |                              |
| P06125              | Vitellogenin-5                            |                                       |                 |                              |
| P90889              | Uncharacterized protein                   |                                       |                 |                              |
| Q2EMB               | Transthyretin-related family domain       |                                       |                 |                              |
| P59955              | Transthyretin-like protein 16             |                                       |                 |                              |
| G5EET8              | PUD_1-related-containing protein          |                                       |                 |                              |
| Q9NAJ2              | Vitellogenin-3                            |                                       |                 |                              |
| Q9NA39              | Conserved cysteine/glycine domain protein|                                       |                 |                              |
| Q23341              | Transthyretin-related family domain       |                                       |                 |                              |
| Q62289              | Transthyretin-related family domain       |                                       |                 |                              |
| P52015              | Peptidyl-prolyl cis-trans isomerase 7     |                                       |                 |                              |
| Q9XW17              | CAR-1                                     |                                       |                 |                              |
| Q19478              | Fatty acid/retinol binding protein        |                                       |                 |                              |
| G5EDZ9              | Cystatin (Cysele1)                        |                                       |                 |                              |
| Q5EBF3              | Protein up-regulated in daf-2(gf)         |                                       |                 |                              |
| Q16462              | Ground-like domain-containing protein      |                                       |                 |                              |
| Q23693              | DUF148 domain-containing protein          |                                       |                 |                              |
| Q19063              | Uncharacterized protein                   |                                       |                 |                              |
| Q20363              | Stress-induced protein 1                  |                                       |                 |                              |
| Q18099              | DUF19 domain-containing protein           |                                       |                 |                              |
| P34500              | Transthyretin-like protein 2              |                                       |                 |                              |
| P34383              | Fatty-acid and retinol-binding protein    |                                       |                 |                              |
| Q19064              | Uncharacterized protein                   |                                       |                 |                              |
| O55999              | Chitin-binding domain protein             |                                       |                 |                              |
| O01504              | 60S acidic ribosomal protein P2           |                                       |                 |                              |
| Q17473              | Transthyretin-related family domain       |                                       |                 |                              |
| G5EAAE8             | Annexin                                  |                                       |                 |                              |
| Q21763              | Thioredoxin domain-containing protein      |                                       |                 |                              |
| Q5E070              | Protein disulfide-isomerase (EC 5.3.4.1)  | -0.05                                 |                 |                              |
| Q07750              | Actin-depolymerizing factor 1, isoforms a/b| 1.07                                  |                 |                              |
| Q17967              | Protein disulfide-isomerase 1 (PDI 1) (EC 5.3.4.1) | -0.17 |                 |                              |
| Q20724              | Uncharacterized protein                   | -0.24                                 |                 |                              |
| P26420              | Heat shock 70 kDa protein C               | -0.83                                 |                 |                              |
| P91306              | CSD-1 domain-containing protein           | 2.11                                  |                 |                              |
| O17687              | SHN-TPR domain-containing protein         | 4.85                                  |                 |                              |
| Q21255              | Putative metalloprotease inhibitor (TIMP-like protein) | 2.88 |                 |                              |

**Fig. 5 d4 adult-specific ES proteins.** Those shown are either IIS upregulated, age upregulated or have a predicted N-terminal signal peptide. Significant differences in the distributions were detected using a Kolmogorov-Smirnov two-tailed test.

reconstruction. Brightness and contrast were adjusted equally across the entire image, and where applicable equally to controls. Where bright field and fluorescence images were superimposed, brightness and contrast were adjusted separately prior to superimposition.

**Reflectance confocal microscopy.** This was performed using an inverted LSM880 microscope (Carl Zeiss, Jena) and a Plan-Apochromat 63 × 1.4 numerical aperture (NA) oil objective with a working distance of 0.19 mm. The main beam splitter was set to T80/R20 with multiphoton laser 405 nm excitation. Emission was recorded using an inbuilt GaAsP detector.

**Fluorescence quantitation of vented yolk milk.** 10 GA1500 L4 larvae were placed on 35 mm NGM plates (n = 5 plates per trial; total 50 worms) seeded with 100 μl E. coli OP50 and transferred every 24 h to new plates. After transferring, 5 superimposed GFP/Nomarski ×50 magnification images were taken of each plate at random positions across the bacterial lawn to sample approximately half of the lawn area. For each time point 3 control NGM plates (no worms) were treated in the same way and imaged.

Fluorescence quantitation of images was performed using the formulae below to account for varying levels of NGM background fluorescence in different plates and variations in fluorescence excitation. Yolk milk pods form localised regions of bright fluorescence on plates. Therefore the minimum emission fluorescence of an NGM plate can be used as an indicator of the true level of background fluorescence, since the range between the minimum and maximum background fluorescence of NGM is relatively constant between plates (data not shown). By measuring the background autofluorescence of control plates and normalising it to the minimum of treated plates, an accurate estimate of the range and maximum background fluorescence of treated control plates can be estimated and subtracted, using the following procedure (note: fluorescence calculations only refer to the GFP channel).

(i) Images with dust and/or cholesterol crystals in the agar (which can reduce the minimum emission fluorescence and affect the background fluorescence range) were manually censored (visualised under Nomarski as black dots or black crystals).

(ii) The ratio of minimum and maximum values of fluorescence intensity (FIR) is given by:

\[ p = \frac{F_{\text{max}}}{F_{\text{min}}} \]  

where \( F_{\text{max}} \) is the maximum emission fluorescence detected on a plate, and \( F_{\text{min}} \) the minimum emission fluorescence detected (both from background fluorescence; \( F_{\text{min}} \) to \( F_{\text{max}} \) = background fluorescence range).

(iii) The FIRs were averaged for the three control plates imaged each day to provide a daily control fluorescence intensity ratio \( p \) (DCIR). This also accounts for variation in UV lamp output during sample excitation. For each treated plate image, the DCIR was multiplied by the image \( F_{\text{min}} \) in
order to set a threshold (T) fluorescence level on treated plates below which all fluorescence was considered background fluorescence and discounted.

$$F_r = F_{\text{max}} \cdot \rho$$

(2)

(iv) Once done, the remaining fluorescence from above the threshold was isolated to ROIs containing GFP-labelled protein, with the fluorescence consisting of both GFP fluorescence as well as background fluorescence. Background fluorescence from these ROIs was removed as follows:

$$F_{\text{GFP}} = F_r - \sum F_{\text{oocyte(i)}}$$

(3)

where $$F_{\text{GFP}}$$ is the total fluorescence from GFP on the plate, and $$F_r$$ is the fluorescence in a given ROI. With $$F_{\text{oocyte(i)}}$$ the total fluorescence from the collection of ROIs remaining on an image after step (iii).

(v) Separation of yolk and oocyte fluorescence was done manually for each image using Velocity 6.3 Acquisition. The free-draining tool was used to identify oocytes on Nomarski/GFP images by viewing the Nomarski image, followed by subtracting all sum fluorescence within the ROIs from the total calculated in steps (i-iii).

$$F_{\text{freeyolk}} = F_{\text{GFP}} - \sum F_{\text{oocyte(i)}}$$

(4)

Gel electrophoresis and quantitation of vented yolk proteins. 100–200 L1 larvae were maintained on 35 mm plates freshly fed with 100 μl OP50. Following transfer each 24 h, yolk milk was washed off with 1 ml of M9 containing 0.001% NP-40 to solubilise vitelligenin27 and 2 μg/ml BSA as an external standard. This solution was pipetted onto plates and a polystyrene bacterial loop was then used to suspend all surface material, including the bacterial lawn, into the solution. The resulting suspension was pipetted out and into an Eppendorf tube. Inclusion of BSA served a dual purpose as a control for protein loss during sample collection from plates, and as a loading control for gel electrophoresis. Samples were spun at 2600 rpm for 10 min at 4 °C. The pellet was collected as the oocyte-containing fraction which, when viewed under a dissecting microscope, contained all oocytes, with no oocytes visible in the supernatant. The pellet also contained the majority of the E. coli, 500 μl of the supernatant was used for the free yolk milk analysis. Bacterial protein bands did not interfere with the detection of YP170 or BSA bands analysed (Supplementary Fig. 1a). Samples were then lyophilised with 10 μl glycerol, and the remaining pellet solubilised in a solution of 30 μl 4% SDS solution pH9 containing 12 mg bromophenol blue, 1 ml 1 M Tris HCL pH8, and 10 μl 0.5 M EDTA (method optimised for YPs) by heating 2–4 times (based on the presence of a pellet post treatment) at 95 °C for 5 min while vortexing periodically, and finally centrifuged at 6000 rpm for 10 min. To prevent sample loss, all pipetting was performed using LoBind pipette tips and storage was in LoBind Eppendorf tubes. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) was then performed, using Criterion XT Precast Gels 4–12% Bis-Tris (Invitrogen) and XT MOPS (Invitrogen) as a running buffer (7:1 ratio with Milli-Q water) at 300 V. Gels were stained with colloidal Coomassie blue as described48, using 5% aluminium sulphate-(14-18)-hydrate and 2% orthophosphoric acid (85%) to create colloids. Images were analysed using ImageJ (National Institutes of Health). Protein band identification was based on published data14. Within lanes, vented YPs were normalised to the BSA that had been added during sample collection as an external standard.

Staining of lipid in hermaphrodites prior to venting. For each time point, vital staining of worms was performed as described29 using the fluorescent dye BODIPY 493/503 (440 nm Exc/505-575 nm Em.) (Invitrogen) at a final concentration of 6.7 μg/ml, with hermaphrodites incubated in dye for 20 min at RT in darkness. Nematodes were then transferred to NGM plates (no E. coli) and allowed to crawl for 3–5 min to remove surface dye, followed by transfer to 35 mm NGM plates (no E. coli), with 20 worms per plate (1-2 plates per trial), and left to vent for 24 h. Fluorescence quantitation was performed as described for fluorescence quantitation of vented YPs, with the exception that Nomarski/flourescence superimposed images were taken of the 5 regions with the most BODIPY fluorescence, due to the lower levels of BODIPY ceased. Eggs were left for 24 h to hatch and brood sizes counted. Clumps of unfertilised oocytes were carefully separated using a platinum pick to distinguish individual cells, using a dissecting microscope at maximum magnification.

Chemotaxis assay. The chemotaxis assay was adapted from a previous study40. Briefly, a 5 cm NGM plate was divided into quadrants with test compounds or d5 adults placed on two opposite quadrants, and controls placed on the 2 remaining quadrants. Plates were then left for 4 h to form a chemotactic gradient. All spots also contained 4 μl of 0.25 M sodium azide as anaesthetic. Next, a 2 μl drop containing ~75 arrested L1 larva/larva was pipetted onto the centre of the plate within a 0.5 cm radius inner circle. Based on larval movement, a chemotaxis index (CI) was calculated using a formula as previously described40. For experiments involving adult worms, a single, heat-killed d5 adult was used for the treatment with controls treated the same way but with no worm. For experiments involving vented yolk, sample collection was performed by collection of 100 d4 adults (washed twice to remove surface bacteria) onto a small microwell cell-culture plate (Thermo Scientific Nunc 24-well cell-culture multides, 1.9 cm2) containing 500 μl NGM lacking lactobacitone (to make the agar harder and limit bacterial growth) and carbencinilled added topically 24 h prior to a concentration of 50 mM. This was done by picking worm in groups of 30–40 and holding them above the microwell, immediately after which a 10 μl drop of M9 was pipetted onto the pick to wash the worms into the well (no more than 30 μl of M9 was used per well). The worms were then allowed to vent for 24 h, after which a sterile scalpel was used to cut around the NGM well and transfer it to a 10 ml NGM plate right side up. Adults were left for 1 h to crawl off, after which remaining adults were picked off. The NGM with vented yolk was then cut into quadrants and each was placed as a treated section in a chemotaxis assay. Control sections were treated the same way but either had no worms, or L3 larvae added instead of d4 adults.

Proteomic analysis of d4 hermaphrodite excretory-secretory (ES) products. Three hundred and fifty fully-fed d4 hermaphrodites were picked onto NGM plates (no E. coli) and allowed to crawl for 30–60s to remove surface bacteria before being transferred to a 500 μl solution of 0.001% NP-40 in M9 to prevent vented yolk from adhering to the nematode surface. The worms were allowed to vent for 30 min (with picking of worms into each tube taking an additional 30 min) with the Eppendorf tube maintained horizontally to aid diffusion of emitted proteins. The Eppendorf tube was placed upright for 1 min to allow nematodes to settle to the bottom of the tube, and 350 μl of solution drawn from the top of the tube. After collection, samples were carefully checked for the presence of adult worms and unfertilised oocytes, but none were observed. The adults were checked for the presence of ruptured animals, which were easy to identify, and where found the sample collected from that tube was discarded. ES products were also collected for L3 larvae, as a negative control for venting through the vulva (which is absent at this stage).

Independent samples were digested with trypsin and prepared for proteomic analysis17. Samples were analysed on a Thermo Scientific Q-Exactive Plus Orbitrap mass spectrometer connected to an Ultimate 3000 nanoLC. Samples were trapped on a Thermo Scientific Acclaim PepMap C18 cartridge (0.3 mm x 5 mm, 5 μm/100 Å) and then chromatographed on a Thermo Scientific Easy-Spray Acclaim PepMap C18 column (75 μm x 15 cm, 3 μm/100 Å packing) eluting at 300 nl/min with a 30 min linear gradient of acetonitrile/water/formic acid (5:95:0.1–56:4:4.1 v/v/v). A full MS scan (m/z 135–200 at 70,000 resolution) was acquired with a maximum injection time of 100 ms, and the 10 most intense ions with an intensity threshold 2.0e4 were selected for higher-energy C-trap
dissociation (HCD) with a lock mass of m/z 445.1203. The normalised collision energy was 30, with an isolation width of 2 Da and dynamic exclusion of 20 s; singly charged ions were excluded. All chromatography solvents were Optima LCMS grade (Fisher Scientific). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE\textsuperscript{45} partner repository with the dataset identifier PXD025472.

Bioinformatic analysis of the d4 hermaphrodite ES proteome. Proteomes were quantified using MaxQuant 1.6.12.0 (ref. \textsuperscript{44}) with the default search settings and the C. elegans protein database from Uniprot (downloaded 20 December 2019), and downstream analysis was performed using the Proteus package in R (ref. \textsuperscript{44}). Proteins were considered detected in a sample if at least one proteotypic peptide from that protein was detected. Proteins were scored as present in the ES proteome if present in at least two of the three replicate samples. Proteins were only considered specific to the day 4 ES proteome if not detected in all three L3 control samples.

To estimate the composition of the ES proteome, proteins were manually classified according to name (e.g. vitellogenin, transhydrogen) or known/predicted functions as indicated in WormBase (http://www.wormbase.org, release WS280) (see Supplementary Data 1 for classification of all proteins identified), and the sum of all peptide intensities across each grouping displayed. Significant over-representation of ES proteome proteins with respect to other datasets was detected using a one-tailed SuperExactTest\textsuperscript{46}. To assess whether the adult ES proteome showed any distinct pattern of expression in ageing worms or IIS knockdown (in daf-2(e1370) mutants used in other experiments), an existing proteome dataset\textsuperscript{46} was used. Proteins were considered upregulated in ageing worms if expression at least doubled from 1- to 17-day-old worms. Proteins were considered IIS-upregulated if expression in 17-day-old wild-type worms was at least twice the expression in 17-day-old daf-2(e1370) worms, normalising for any initial expression differences between 1-day-old wild-type and daf-2(e1370) worms. The presence of signal peptides in C. elegans proteins was predicted using SignalP 5.0 (ref. \textsuperscript{47}). In order to compare the composition of ES proteome proteins to human milk\textsuperscript{21}, cross-species gene set analysis was performed using the XGSA package in R (ref. \textsuperscript{48}), accounting for protein homology mapping between C. elegans and humans. Tissue enrichment analysis was performed using the WormBase tissue enrichment tool\textsuperscript{49} while GO term and Interpro term enrichment was performed using DAVID 6.8 (ref. \textsuperscript{50}).

Data availability
The data that support the findings of this study are available from the corresponding author upon request. For mass spectrometry data shown, raw data are provided in Supplementary Data 1 and deposited to the ProteomeXchange Consortium via the PRIDE\textsuperscript{45} partner repository with the dataset identifier PXD025472.

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Author contributions

D.G. supervised the project. C.C.K. and D.G. conceived the project, designed the experiments and wrote the manuscript. A.S., R.M.C., L.C.F. and C.C.K. performed behavioural experiments. N.B.R. and G.T. performed MS analysis. D.G., C.C.K. and S.T. designed and performed MS data analysis. J.B. assisted in the interpretation of MS data. J.B. and L.C.F. contributed to editing the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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