

Fecundity and lifespan manipulations in *Caenorhabditis elegans* using exogenous peptides

Keith G. DAVIES^{1,*} and John E. HART²

¹ Department of Plant Pathology and Microbiology, Rothamsted Research, Harpenden, Herts AL5 2JQ, UK

² Endocrine Pharmaceuticals, Wilderness End, Tadley Common Road, Tadley, Hants RG26 3TA, UK

Received: 8 June 2007; revised: 24 August 2007

Accepted for publication: 24 August 2007

Summary – In this study using *Caenorhabditis elegans*, we have been able to suppress (>60%) and enhance (>40%) fecundity (number of offspring) while extending lifespan by a fifth, by administering synthetic peptides to the aqueous medium in which the nematodes were maintained. Untreated control adults fed live bacteria had significantly more offspring (17 vs 10 larvae each) than those fed dead bacteria. Average lifespan and time for 50% of the worms to die were the same at approximately 10 days, but there was a significant difference in terms of 100% mortality (28 vs 19 days). A reduction in fecundity of 30-40% occurred when a 14-mer peptide, EPL030, was administered to the worms' aqueous medium. The effect was dose-dependent across the range 0.1-10 $\mu\text{M day}^{-1}$ of medium, but since the worms were fed live bacteria interpretation was problematic: was the effect direct or indirect? However, the anti-fecundity effect was reproduced in worms fed dead bacteria, when the test compound was administered at 1 $\mu\text{M day}^{-1}$ of aqueous medium. The mean number of larvae produced in three groups: untreated controls, EPL030 and EPL001 (an anagrammatical version of EPL030 used as a comparator), were, respectively, 17, 6 (-64%) and 24 (+43%). Average lifespans were 8.7, 10.7 (+23%) and 10.3 days (+18%). Fluorescence localisation studies using a close analogue of the fecundity-suppressing EPL030 revealed a distribution that was generalised and uninformative. The fecundity-enhancing EPL001 concentrated in the genital tract. *Caenorhabditis elegans* is a potentially useful testbed for fecundity and lifespan studies using exogenous agents. The use of an aqueous medium and dead bacteria as food simplifies both the protocol and interpretation of results.

Keywords – fertility, longevity, nematodes, ovary, peptides.

The soil nematode *Caenorhabditis elegans* is an amenable model organism for investigations in development, reproduction, senescence and other fundamental biological processes such as the functioning of the immune system. Its use was pioneered by Sydney Brenner (Brenner, 1974) and it remains the only multicellular organism in which the entire cell lineage (959 cells) has been traced, from egg to adult (Sulston *et al.*, 1983). Most of the biochemical pathways identified in *C. elegans* have proved to be evolutionarily conserved, such that they have counterparts across the entire spectrum of relatedness, from plant-parasitic nematodes (Bird & Opperman, 1998; Costa *et al.*, 2007) through to humans (Sonnhammer & Durbin, 1997; Chalfie & Jorgensen, 1998). This conservation of genes and metabolic pathways has given rise to the use of *C. elegans* in studies of drug action and human pathogen-

esis (Link *et al.*, 2000; Westlund *et al.*, 2004; Kaletta & Hengarter, 2006).

Although *C. elegans* is a useful model species for use in biological studies, we have found that in investigations of fecundity and lifespan involving exogenous peptides care is needed with protocol design. Resveratrol, a polyphenol compound found in red wine, when administered to *C. elegans* increases life expectancy, a conclusion which holds whether the worms are fed live or dead bacteria (Wood *et al.*, 2004). This careful approach is important since, as the present study will show, the use of these different foodstuffs has an impact on fecundity and longevity. The research will also demonstrate, by using peptides as model exogenous agents, that both of these factors should be investigated together and in detail in order to provide a meaningful picture.

* Corresponding author, e-mail: keith.davies@bbsrc.ac.uk

Materials and methods

NEMATODE CULTURE

Caenorhabditis elegans was grown on nematode growth medium (NGM) agar, 3 g NaCl, 2.5 g peptone and 17 g technical agar in 975 ml distilled H₂O autoclaved, to which was added 1 ml of CaCl₂ (1 M), 1 ml MgSO₄ (1 M) and 25 ml KH₂PO₄ (1 M; pH 6), all from sterilised stock solutions. Finally, 1 ml of 5 mg ml⁻¹ cholesterol in 95% ethanol was added to the medium. Nematodes were routinely cultured by growing *Escherichia coli* OP50 overnight in LB broth, on a shaker placed in an incubator (37°C), and the bacteria were then added to NGM agar in Petri dishes (9 cm diam.) to create an overnight bacterial lawn. Nematodes were added to these Petri dishes, which were then maintained at 20°C in an incubator (Stiernagle, 2006). Synchronisation of nematode development was undertaken according to a method adapted from Sulston and Hodgkin (1988) in which NGM agar plates containing large numbers of adults and eggs were flooded with 2 ml M9 buffer (*i.e.*, 3 g NaH₂PO₄, 1.5 g KH₂PO₄, 2.5 g NaCl, 0.175 g MgSO₄ · 7H₂O in 500 ml autoclaved distilled water). Nematodes were concentrated by centrifugation (2 min at 9500 *g*) and the supernatant discarded. Nematodes were then exposed to alkaline hypochlorite solution (NaOCl 2.5%:NaOH 1 M, 2:5 v/v) and observed for cuticle breakdown to confirm death of larval and adult worms using a stereomicroscope (×20). Eggs were concentrated from the suspension by centrifugation, as above, and the supernatant discarded before replacement with M9 buffer. Replacement with M9 buffer was repeated (twice) and the eggs then pipetted onto NGM plates containing a lawn of *E. coli* OP50 and placed in an incubator at 20°C.

FEEDING AND LIFE SPAN STUDIES

In order that nematodes did not enter the dauer stage at the commencement of the experiment, nematode eggs were placed on NGM plates and allowed to develop. Third- and fourth-stage larvae (L3/L4), that were clearly post second-stage (L2) dauer decision stage, were used in these studies and placed in autoclaved staining blocks containing 200 µl sterilised M9 buffer, ten individuals per block. Each treatment consisted of four replicates that were placed randomly in a humid chamber and maintained in this at 20°C in an incubator. Peptides (patents applied for) were synthesised by a commercial supplier: EPL030 (KLKMGKNIEPVFT) and, as an anagrammatical comparator, EPL001 (MKPLTGKVKEFNNI) (for localisation, peptides were fluores-

cein labelled: EPL040, a closely similar anagrammatical 14-mer peptide to EPL030, with a 5-mer linker to retain biological activity after C-terminal labelling, KLKMGKNIEPVFTGSGSK; and EPL001 with the same linker). The peptides were administered each day to the worms' aqueous medium to achieve micromolar concentrations, in a dose-response study involving EPL030 (0.1, 1.0 and 10.0 µM day⁻¹) and in a single-dose comparative study of the peptides (1 µM day⁻¹). The worms were fed approximately 10⁶ OP50 per day, the OP50 having been pelleted and resuspended in M9 buffer. The bacteria were either alive or heat-killed (30 min at 65°C; Wood *et al.*, 2004). Among the worms, live adults were identified, if necessary by gentle prodding, counted each day and transferred to freshly autoclaved staining blocks containing 200 µl sterilised M9 buffer. The peptides were then administered anew. This 'fresh well' approach enabled further incubation of the now adult-free original staining block, allowing the development of eggs into larvae.

Although 'fecundity' is generally regarded as egg production, we counted larvae on a daily basis for convenience as a proxy for fecundity, there being no residue of unhatched eggs to suggest peptide effects on hatching. This procedure continued until the last nematode was dead, to establish lifespan. Statistical analysis consisted of calculating standard errors of the difference between the means of the four replicates of ten worms, and conducting analyses of variance (ANOVA) and *t*-tests, where appropriate.

LOCALISATION

Morphological localisation of the peptides was undertaken using an adaptation of the freeze crack method (Duerr, 2006). *C. elegans* were grown up on NGM and concentrated by centrifugation in M9 buffer as described above, then suspended in a small volume of M9 buffer at between 2000 and 4000 worms ml⁻¹. Live worms (2000-4000 worms ml⁻¹) were incubated overnight in a small volume of M9 buffer containing fluorescein-labelled EPL040 or EPL001 at 4 µg ml⁻¹. Control nematodes were incubated overnight in M9 buffer without labelled peptide. Small volumes (30 µl) of suspended worms (comprising all four larval stages) were removed using a pipette then squashed between two slides and placed at -20°C until frozen (>2 h). The slides were then prised apart while still frozen and the worms were allowed to dry on to the glass slides (2 h at room temperature). Following drying the worms were washed (three times) in

PBS:T and mounted in a small drop of antifade medium Citiflor™ (Agar Scientific, Stansted, UK) and observed using a microscope fitted with epifluorescence illumination with a 455 nm excitation filter and a 529 nm barrier filter.

Results

FEEDING STUDIES

Untreated controls fed live bacteria had significantly more offspring than those fed dead bacteria: 17 vs 10 larvae per adult (Fig. 1; $P < 0.05$). There was no significant difference in the time for 50% of the worms to die, 11 vs 12 days, but there was a large and significant difference in terms of 100% mortality: 28 vs 19 days, $P < 0.05$ (Fig. 2).

Nematodes fed with live bacteria and exposed to the synthetic peptide EPL030 at 0.1–10 $\mu\text{M day}^{-1}$ produced significantly fewer larvae (30–40% less) than untreated controls, the effect increasing with dose (Fig. 3). There was no significant effect of EPL030 on average survivorship, compared with controls, or on the times for 50 or 100% of the worms to die. Interpretation of the results of this study was problematic, however, due to a protocol based on ‘two live organisms’. It was decided in the comparative single-dose study, therefore, to simplify the

method by using only heat-killed OP50, to eliminate any indirect effects *via* the bacteria.

EFFECTS OF PEPTIDES (WORMS FED DEAD BACTERIA)

Exposure to the synthetic peptides at 1.0 $\mu\text{M day}^{-1}$ had a substantial effect on the fecundity of the nematodes, both negatively (EPL030) and positively (EPL001) (Fig. 4). The mean number of larvae produced per worm for the three groups, untreated control, EPL030 and EPL001, were 17, 6 (–64%, $P < 0.001$) and 24 (+43%, $P < 0.05$), respectively. Note that EPL040 gave an almost identical ‘tramline’ result: seven larvae per worm, a reduction vs control of –62%, $P < 0.001$. EPL040 was used in the localisation study as a close analogue of EPL030, with a 5-mer extension for flourrescein labelling.

Reproduction started for EPL030 at day 2, but for the controls and EPL001 at around day 4 (Fig. 5). The mean last day of reproduction for the three groups was 15, 12 (–21%, ns) and 21 (+37%, $P < 0.05$; Fig. 5). During the first 7–9 days there was no significant difference between the three groups regarding the number of larvae produced (Fig. 4); however, by day 10 the nematodes treated with EPL030 had produced around 60 larvae and, thereafter, very few larvae were subsequently produced (Fig. 5). The untreated control nematodes produced just over 170 larvae by day 15, the last day of reproduction. In comparison,

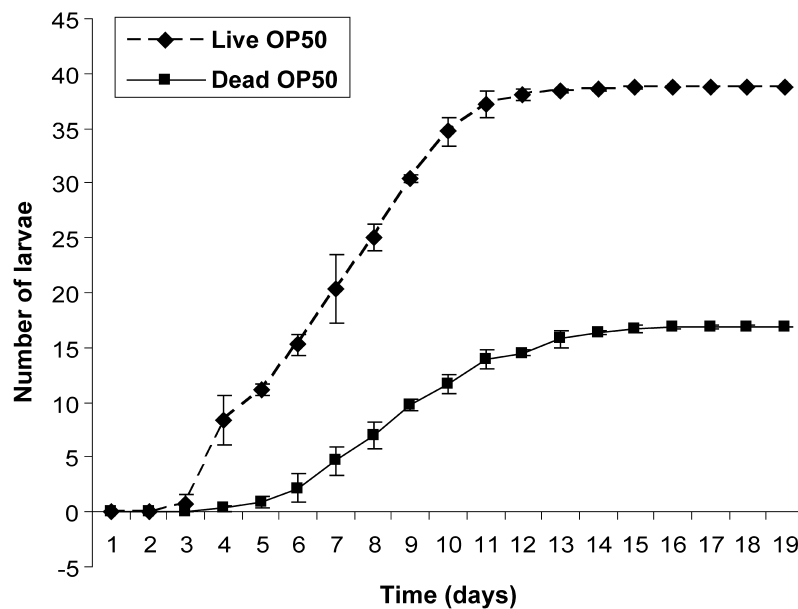


Fig. 1. Number of larvae produced per nematode, mean of four groups of ten, when fed daily with *Escherichia coli* OP50, either dead (squares) or alive (diamonds) (SE bars; ANOVA $P < 0.005$, comparing cumulative totals).

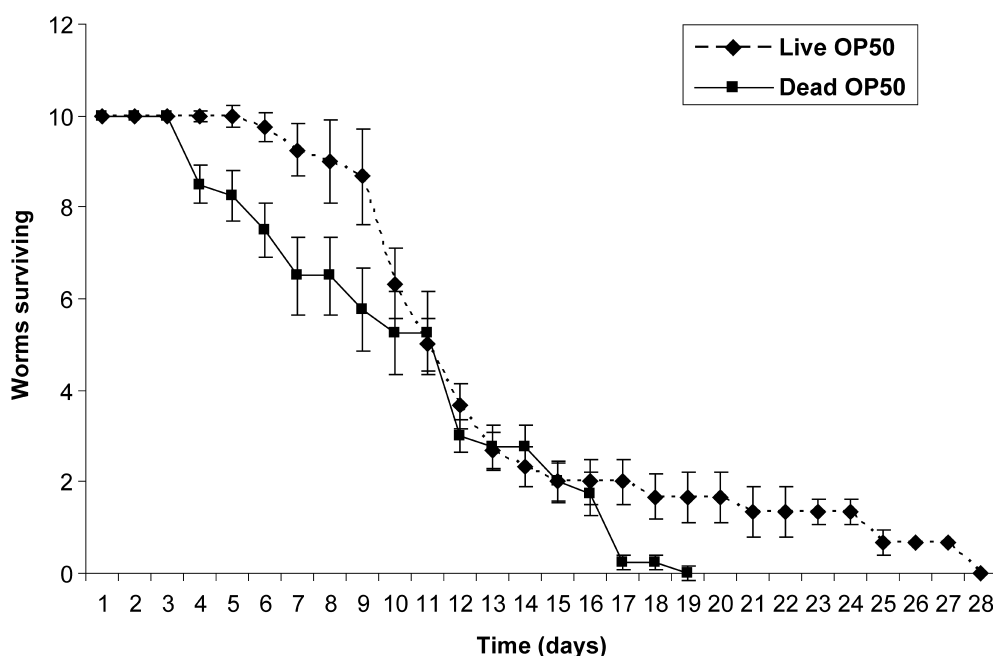


Fig. 2. Survivorship of nematodes, mean of four groups of ten, when fed daily with *Escherichia coli* either dead OP50 (squares) or live OP50 (diamonds) (SE bars; ANOVA $P < 0.01$, area under curve).

EPL001-treated nematodes continued to produce larvae until day 21 when they had over 250 offspring (Figs 4, 5).

Fifty percent mortalities were similar for the controls EPL030 and EPL001 at around 11 days (not significant, ns, $P > 0.05$), but the times for all the worms to die ('survivorship') were very different at 19, 26 and 24 days (Fig. 6). Average lifespans were 8.7, 10.7 (+23%, ns) and 10.3 days (+18%, $P < 0.005$).

LOCALISATION

Pre-incubation with fluorescein-labelled EPL040 prior to freeze cracking revealed a general distribution of the peptide throughout the body cavity of some larvae but not others. This appeared not to be larval stage-specific. In adults meanwhile, this same peptide showed weak localisation within the body cavity, with concentration also in the cuticle, buccal cavity, pro-corpus of the pharynx and the grinder (Fig. 7). EPL001 was localised to the stoma walls leading to the pharynx and also to the genital tract, perhaps to the ovary (Fig. 8).

Discussion

Although offering the practical challenge of being tiny to work with at less than 1 mm in length, the nematode *C. elegans* is easy to breed and maintain, has an acceptable generation time (with a lifespan of somewhat under 1

month) and is biologically well characterised. We have found that a further advantage of this nematode is that it is possible to expose it to exogenous peptides *via* the liquid medium in which it can be maintained, inducing marked changes in fecundity and lifespan in the process. An important caveat in such studies is that pre-killed bacteria should be used as food, rather than live bacteria, in order to avoid confusion between primary effects on the worms and secondary effects *via* the bacteria. Studies based on the genetic modification of nematodes are not prone to this interpretative difficulty, since treatment affects the nematodes only (*e.g.*, Kenyon, 1997; Hansen *et al.*, 2005).

When nematodes fed on live or heat-killed *E. coli* OP50 were compared, we found that those fed live bacteria produced over twice as many offspring. The reason for this is unclear. Time to 50% mortality was the same, but there were some long-lived individuals among the worms fed live bacteria, which extended the time of 100% mortality figure for that group. It would be interesting in this context to know the reproductive history of each individual worm. For example, do the longer-lived individuals reproduce less?

When EPL030 is given to worms fed live bacteria, fecundity was reduced by about one-third, at the $1 \mu\text{M}$ day⁻¹ dose level. In contrast, in worms fed dead bacteria the fecundity suppression was more like two-thirds at that

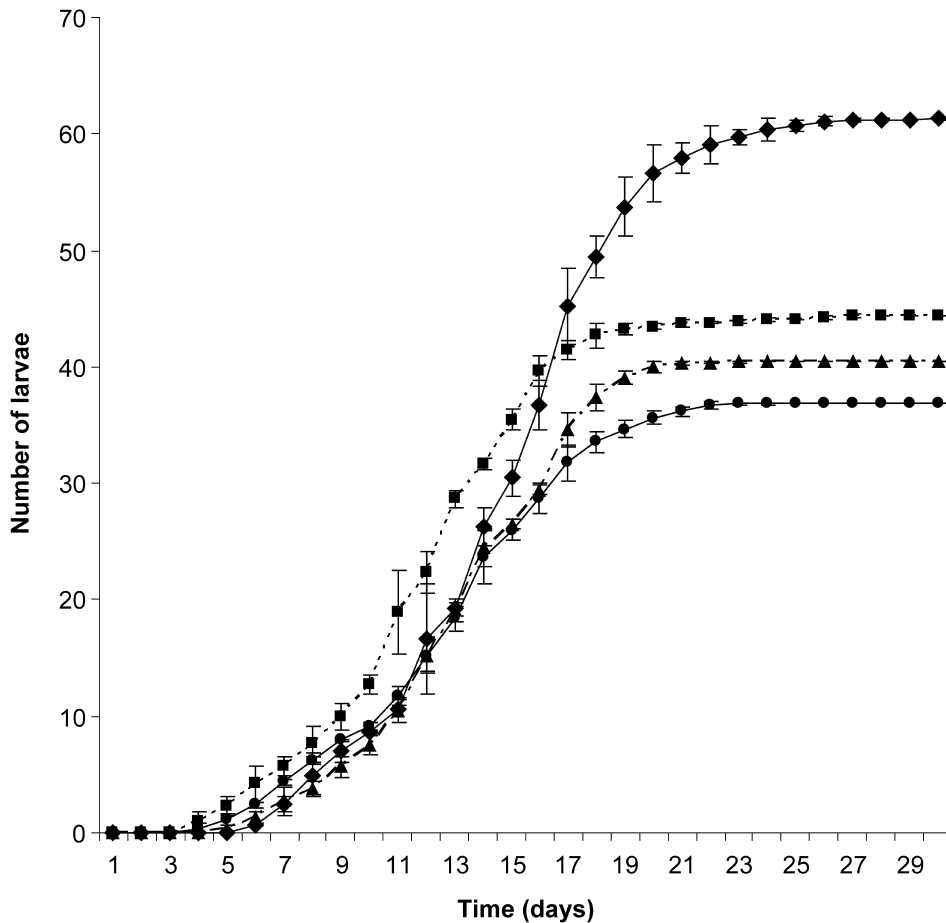


Fig. 3. Number of larvae produced per nematode, mean of four groups of ten, fed live *Escherichia coli* OP50 and exposed to the synthetic peptide EPL030 at $0.1 \mu\text{M day}^{-1}$ (squares), 1.0 (triangles) and 10.0 (circles), and an untreated control (diamonds) (SE bars).

dose. This presumably indicates that the presence of live bacteria reduces the effective dose of the administered compound, perhaps by extracellular bacterial protease production, reinforcing the preference for dead over live bacteria as food for worms used in studies on exogenous agents. This ‘reduction in the effective dose’ argument may well also explain unchanged lifespans in worms exposed to EPL030 and fed live bacteria, compared with the trend towards extended average lifespans (+23%, ns), seen in worms similarly exposed but fed dead bacteria.

That our anagrammatic peptides had diametrically opposite effects on fecundity was intriguing. It was also interesting that average lifespans were extended in both cases. These results speak of no simple relationship between fecundity and lifespan, both needing to be assessed to obtain a full picture. The result for the fecundity-enhancing EPL001 is especially surprising:

more offspring *and* a longer life! Again, though, as in the feeding study, it would be helpful to know the relationship of fecundity and lifespan for each individual worm.

Why should administering peptides to achieve micromolar concentrations in the aqueous medium of worms fed dead bacteria alter fecundity and lifespan at all? Perhaps the exogenous agents merely represent extra nourishment. This might explain the fecundity enhancement observed with EPL001, but could hardly explain the fecundity reduction seen with EPL030. What about toxicity as an explanation of the observed effects? Again this might explain a reduction in fecundity but is unlikely to explain a potentiation – and in any case lifespans were increased in both the EPL001 and EPL030 groups. That seems to leave, as an explanation, pharmacology and the idea that the worms are highly sensitive to the chemical composition of their environment.

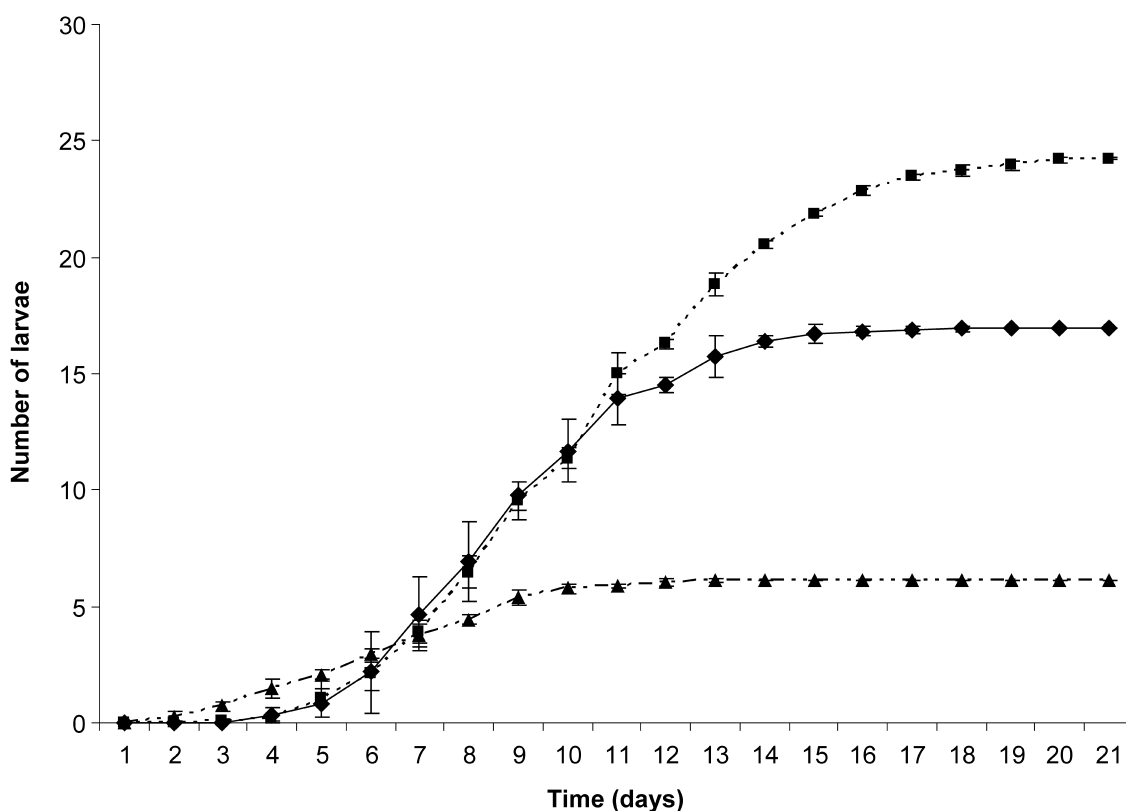


Fig. 4. Number of larvae produced per nematode, mean of four groups of ten, fed dead *Escherichia coli* OP50 and treated with EPL030 (triangles) or EPL001 (squares) at $1 \mu\text{M day}^{-1}$, and an untreated control (diamonds), over a 21-day period (SE bars; ANOVA; $P < 0.001$, comparing cumulative totals).

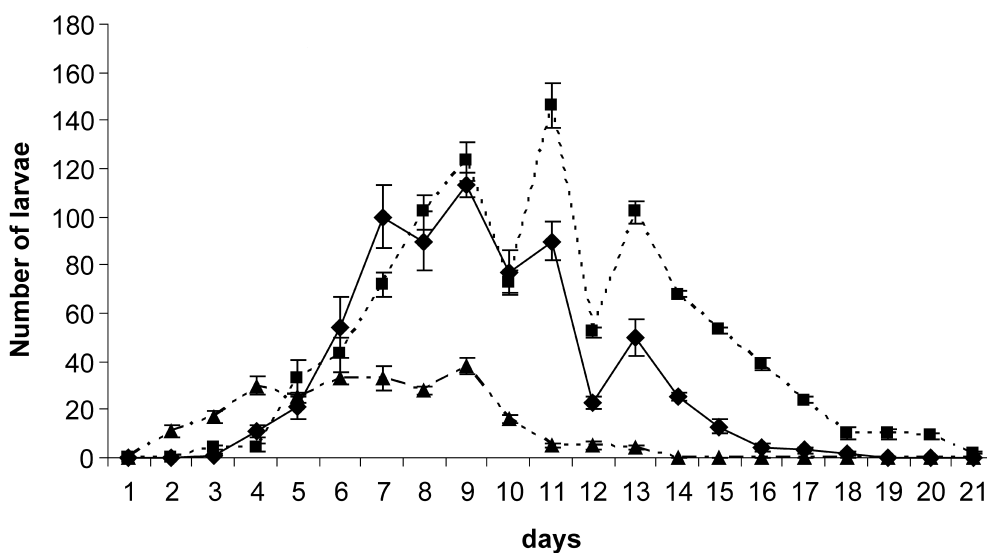


Fig. 5. Number of larvae produced each day by nematodes fed dead *Escherichia coli* OP50 and treated with EPL030 (triangles) or EPL001 (squares) at $1 \mu\text{M day}^{-1}$, and an untreated control (black diamonds) (SE bars).

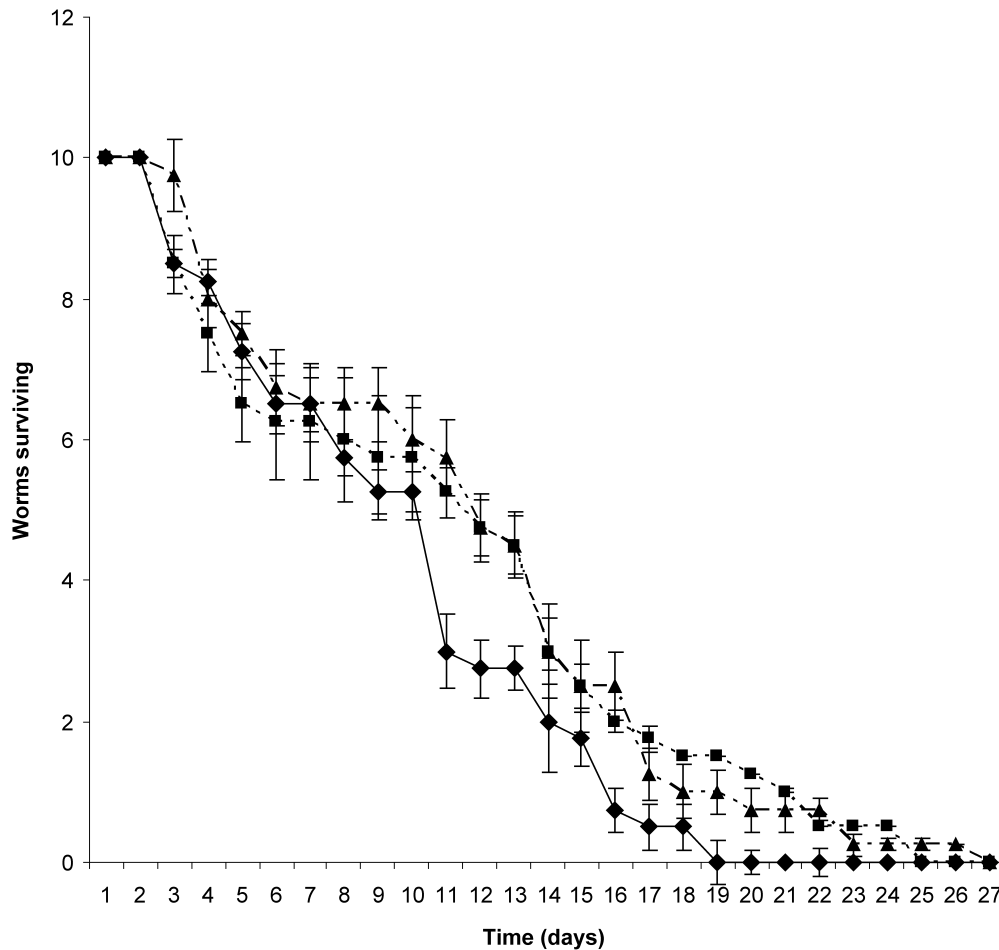


Fig. 6. Survivorship of nematodes, mean of four groups of ten, fed dead *Escherichia coli* OP50 and treated with EPL030 (triangles) or EPL001 (squares) at $1 \mu\text{M day}^{-1}$, and an untreated control (diamonds) (SE bars; t-test average lifespan: EPL001 vs control, $P < 0.005$; EPL030 vs control, ns; EPL030 vs EPL001, ns).

The red wine constituent resveratrol has been shown to extend the lifespans of *C. elegans* fed dead bacteria (Wood *et al.*, 2004). The worms were maintained on agar plates with the resveratrol being administered onto the agar surface in PBS at $100 \mu\text{M}$. An average increase in lifespan of 10% was obtained. This was deemed by Wood *et al.* (2004) to be due to activation of sirtuins, evolutionarily conserved protein mediators of longevity, which is also believed to be the mechanism by which caloric restriction famously increases longevity across the species (Kaeberlein & Rabinovich, 2006). Wood *et al.* (2004) provided evidence that resveratrol does not alter fecundity. In the current studies, in contrast, lifespan was not altered alone, but was attended by fewer offspring in the case of EPL030, more in the case of EPL001. Our worms were maintained at a concentration of exogenous agents of $1 \mu\text{M}$, with

fresh peptide supplied daily in replacement medium. The dosages were thus lower than those used in the resveratrol experiments just cited, but the average lifespan extensions were greater, at a non-significant 23% for EPL030 and a highly significant 18% for EPL001.

The use of fluorescently-labelled peptides revealed pharyngeal localisation for both EPL040 and EPL001, probably indicating the route of ingress, although passage across the cuticle is not ruled out by this finding. EPL001 also appeared to bind to cells in the genital tract, perhaps recognising ovarian tissue in particular. This at least fits with an effect on fecundity and warrants further investigations with this peptide. Labelling with EPL040 was less clear cut, with some individual worms showing labelling throughout the body cavity and others showing no labelling at all. This leaves obscure the mechanism of

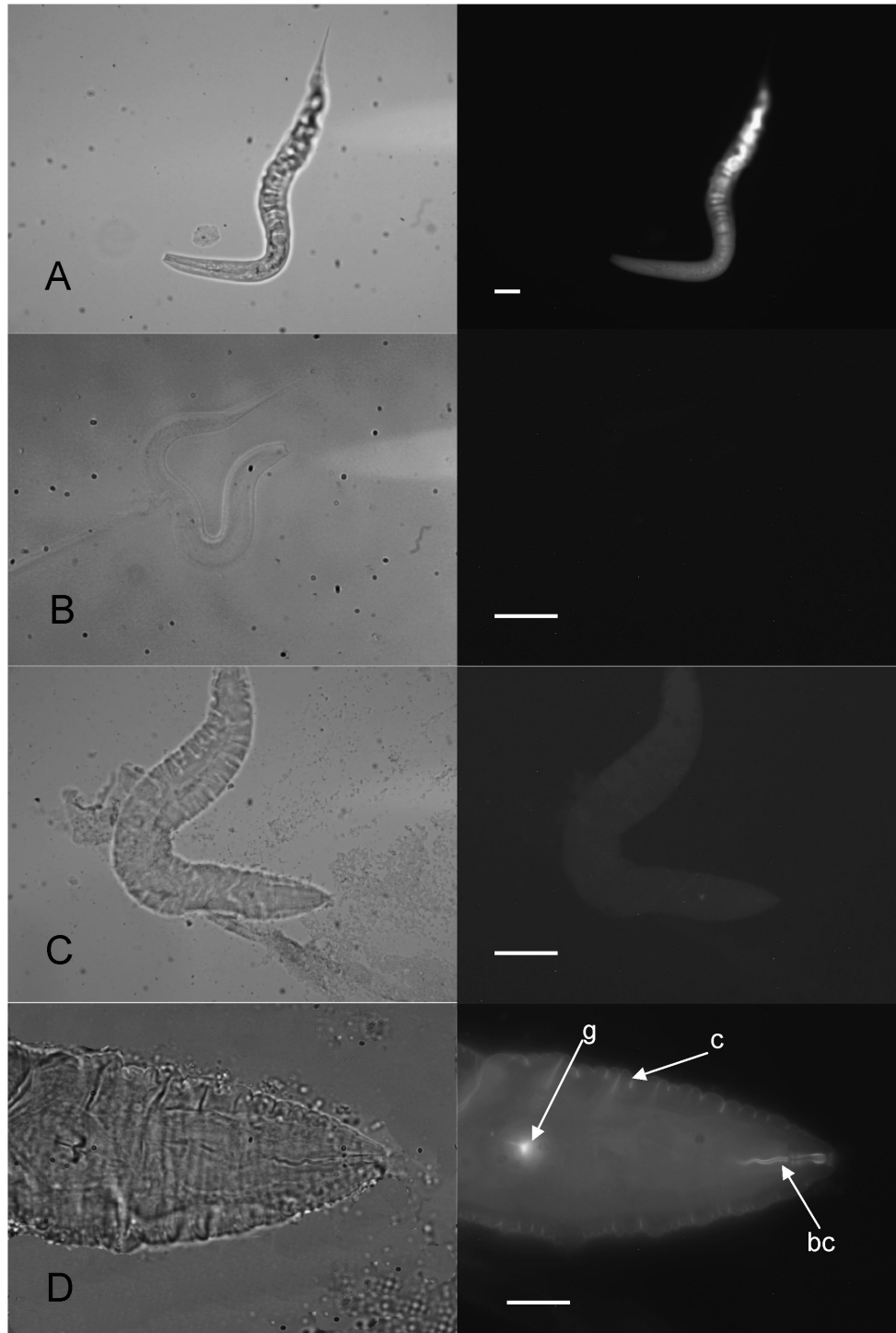


Fig. 7. Localisation of $100 \mu\text{g ml}^{-1}$ fluorescein labelled synthetic peptide EPL040 of mixed stages of freeze cracked nematodes under brightfield (left), epifluorescence (right); A: Recognition and B: Non-recognition of larval stages, respectively (scale bars = $25 \mu\text{m}$); C: Weak recognition through body cavity of adult (scale bar = $50 \mu\text{m}$); D: Recognition of cuticle (c), recognition of buccal cavity (bc), procorpus and grinder (g) of the pharynx of adult (scale bar = $25 \mu\text{m}$).

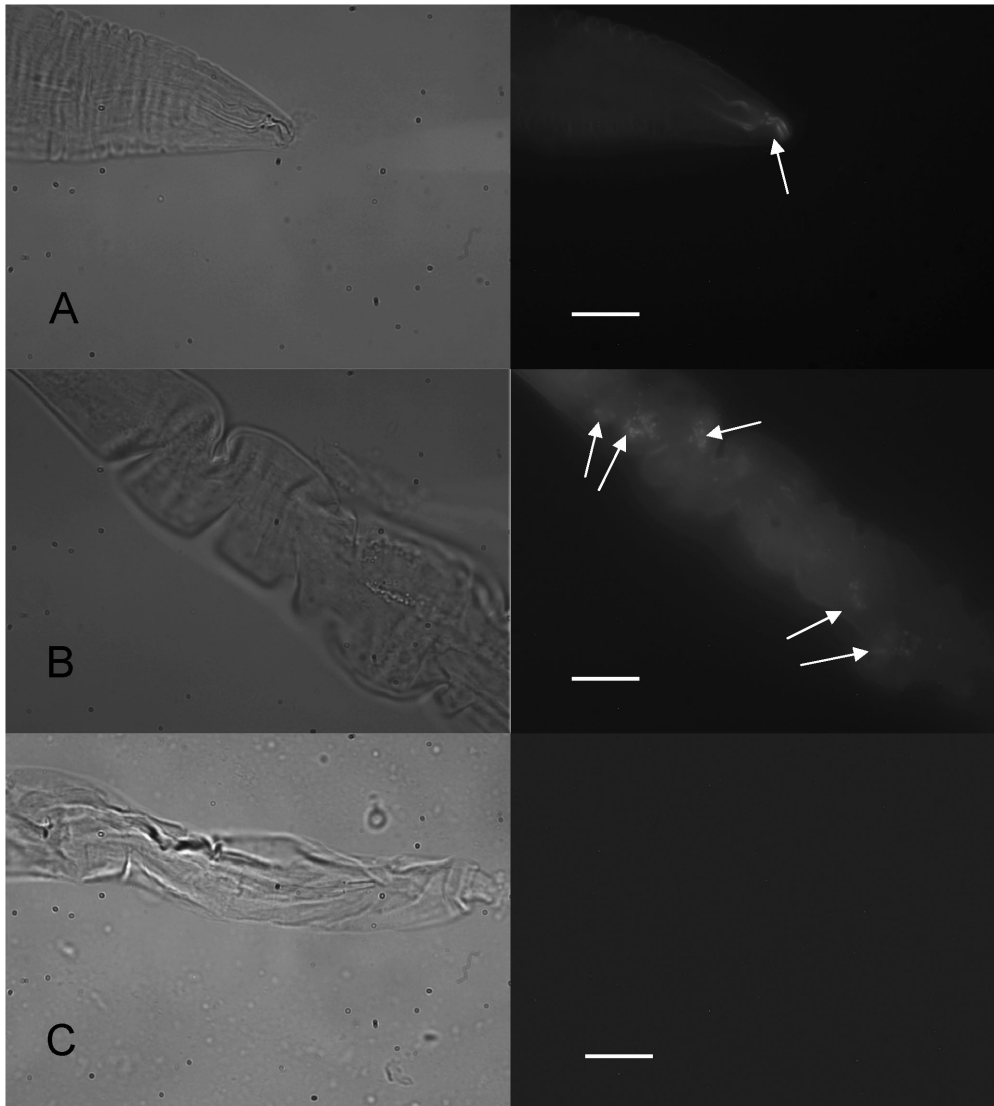


Fig. 8. Localisation of fluorescein-labelled synthetic peptide EPL001 of freeze cracked nematodes under brightfield (left), epifluorescence (right); freeze cracked following overnight incubation at room temperature in $100 \mu\text{g ml}^{-1}$ EPL001; A: Recognition of stoma walls leading to the pharynx (scale bar = $50 \mu\text{m}$); B: Recognition of genital tract, possible ovarian cells (arrows) (scale bar = $50 \mu\text{m}$); C: Control nematode no synthetic peptide (scale bar = $25 \mu\text{m}$).

action of EPL040 and, by extension, the closely similar EPL030. The use of further anagrammatical peptides might help to contextualise these results. It is, in fact, unclear to the present authors how widespread fecundity alteration is as a property of exogenous peptides, making the current work preliminary in character.

Nematode bioinformatics, using BLAST and other searches, has not so far contributed to the present authors' understanding of why the peptides used here have the ac-

tivities that they do in nematodes, although it is of possible relevance that EPL001 has been shown to be inhibitory of compensatory renal hypertrophy in rats subject to unilateral nephrectomy (John Haylor, University of Sheffield, pers. comm.). This is regarded as an IGF1-related system, and the related nematode DAF2 pathway is involved in reproduction and longevity. EPL001 could therefore be affecting the DAF2 system to increase fecundity and lifespan. Meanwhile, BLAST searches of the *C. elegans* data-

base for EPL030 and EPL040 yield only weak hits to, for example, Rab and M106.3, proteins involved in reproduction (unpubl.).

Caenorhabditis elegans offers researchers the chance to adopt a whole-organism model at an enquiry's early stage. The current study is a case in point. On the basis of a speculative search for factors that influence mammalian organ growth (Hart, 2003), we synthesised peptides based on partial and tentative amino acid sequence data (e.g., EPL001). A combinatorial approach then yielded peptides (EPL030 and EPL040) having, as we have shown here, anti-fecundity properties. The empirical approach of using *C. elegans* has thus facilitated a serendipitous discovery.

The present study indicates that fecundity and lifespan cannot be predicted one from the other; both need to be assessed together, with monitoring of individual worms even being worth considering. A preferred protocol when using *C. elegans* in lifespan and fecundity studies involving exogenous agents would, in our view, involve the following: the use of aqueous medium to facilitate compound administration; selection of post-dauer larvae at the start of the study, so that all participants are active; removal of adults to fresh staining blocks each day, maintaining the continuity of the founder population; a delay in assessing the left-behinds, sufficient to turn immobile and difficult to count eggs into easy to count motile juveniles, as a proxy for fecundity (i.e., egg production); and, above all, feeding of the worms with dead rather than live bacteria, simplifying interpretation of results.

Acknowledgements

Rothamsted Research receives grant-aided support from the Biotechnology and Biological Sciences Research Council of the United Kingdom. The technical support of Sofia Costa (laboratory investigations) and Tim Been (bioinformatics) is gratefully acknowledged. The Scientific Advisory Board of Endocrine Pharmaceuticals is thanked for constructive input. Peptide synthesis was arranged by Dr Dave Emery of Bristol University.

References

- BIRD, D.M. & OPPERMAN, C.H. (1998). *Caenorhabditis elegans*: A genetic guide to parasitic nematode biology *Journal of Nematology* 30, 299-308.
- BRENNER, S. (1974). The genetics of *Caenorhabditis elegans*. *Genetics* 77, 71-94.
- CHALFIE, M. & JORGENSEN, E.M.C. (1998). *Caenorhabditis elegans* neuroscience: genetics to genome. *Trends in Genetics* 14, 506-512.
- COSTA, J.C., LILLEY, C.J. & URWIN, P.E. (2007). *Caenorhabditis elegans* as a model for plant-parasitic nematodes. *Nematology* 9, 3-16.
- DUERR, J.S. (2006). Immunohistochemistry, *WormBook*. The *C. elegans* Research Community (Ed.). WormBook, doi/10.1895/wormbook.1.105.1, available online at <http://www.wormbook.org>
- HANSEN, M., HSU, A.L., DILLIN, A. & KENYON, C. (2005). New genes tied to endocrine, metabolic, and dietary regulation of lifespan from *Caenorhabditis elegans* genomic RNAi screen. *PLoS Genetics* 1, 119-128.
- HART, J.E. (2003). Isolated material having an anti-organotrophic effect. EP 1 135 145 B, granted to Endocrine Pharmaceuticals on 2 April 2003.
- KAEBERLEIN, M. & RABINOVICH, P.S. (2006). Grapes versus gluttony. *Nature* 444, 280-281.
- KALETTA, T. & HENGARTNER, M.O. (2006). Finding function in novel targets: *C. elegans* as a model organism. *Nature Reviews* 5, 387-397.
- KENYON, C. (1997). Environmental factors and gene activities that influence life span. In: Riddle, D.L., Blumenthal, T., Meyer, B.J. & Priess, J.R. (Eds). *C. elegans II*. Cold Spring Harbor, NY, USA, Cold Spring Harbor Laboratory Press, pp. 791-813.
- LINK, E.M., HARDIMAN, G., SLUDER, A.E., JOHNSON, C.D. & LIU, L.X. (2000). Therapeutic target discovery using *Caenorhabditis elegans*. *Pharmacogenomics* 1, 1-15.
- SONNHAMMER, E.L. & DURBIN, R. (1997). Analysis of protein families in *Caenorhabditis elegans*. *Genomics* 46, 200-216.
- STIERNAGLE, T. (2006). Maintenance of *C. elegans*. *WormBook*, The *C. elegans* Research Community (Ed.), WormBook, doi/10.1895/wormbook.1.101.1, available online at <http://www.wormbook.org>
- SULSTON, J.E. & HODGKIN, J. (1988). Methods. In: Wood, E.B. (Ed.). *The nematode Caenorhabditis elegans*. Cold Spring Harbor, NY, USA, Cold Spring Harbor Laboratory Press, pp. 587-606.
- SULSTON, J.E., SCHIERENBERG, E., WHITE, J.G. & THOMPSON, J.N. (1983). The embryonic cell lineage of the nematode *Caenorhabditis elegans*. *Development and Biology* 100, 64-119.
- WESTLUND, B., STILWELL, G. & SLUDER, A.E. (2004). Invertebrate disease models in neurotherapeutic discovery. *Current Opinion in Drug Discovery and Development* 7, 169-178.
- WOOD, J.G., ROGINA, B., LAVU, S., HOWITZ, K., HELFAND, S.L., TATAR, M. & SINCLAIR, D. (2004). Sirtuin activators mimic caloric restriction and delay ageing in metazoans. *Nature* 430, 686-689.