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2 Reproduction potentiated in nematodes (*Caenorhabditis*
3 *elegans*) and guppy fish (*Poecilia reticulata*) by adding a
4 synthetic peptide to their aqueous environment
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10
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21 **Authors & affiliations:**

22 Keith G. Davies^{1*}, Brian Zimmerman², Ed Dudley³, Russell P. Newton³
23 & John E. Hart⁴
24

25
26
27 ¹*School of Life and Medical Sciences, University of Hertfordshire, College Lane, Hatfield AL10*
28 9AB, UK

29 ²*Aquarium, Zoological Society of London, Regent's Park, London NW1 4RY*

30 ³*Biochemistry Group, Institute of Life Sciences, Medical School, Swansea University, Swansea*
31 SA2 8PP, UK

32 ⁴*Endocrine Pharmaceuticals, Wilderness End, Tadley Common Road, Tadley,*
33 Hampshire RG26 3TA, UK

34
35 * Corresponding author, email: k.davies@herts.ac.uk
36

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41 **ABSTRACT**

42 Ambient exposure to a short synthetic peptide has enhanced fecundity (number of offspring) in
43 invertebrates and vertebrates, ostensibly by disinhibiting reproduction. In separate experiments
44 nematodes (*Caenorhabditis elegans*) and guppy fish (*Poecilia reticulata*) were exposed via
45 their aqueous environment to a dissolved synthetic hexamer peptide, IEPVFT (EPL036), at a
46 concentration of 1 μ M. In the case of the worms peptide was added to their aqueous buffer daily
47 throughout the experiment (14 days); in the guppies peptide administration was on the first 15
48 alternate days in a 50-week experiment. Fecundity rose by 79% among the worms. The number
49 of descendants of the treated guppies was more than four times that of controls by Week 26
50 (103 vs 25, including 72 juveniles vs 6), with 15.4% more estimated biomass in the test tank in
51 total (i.e. including founders). It was deduced that treated females bred earlier, at a smaller size,
52 and had larger brood sizes. The total number of fish in the control tank had caught up by
53 termination, but biomass continued to lag the test tank. There were no overt signs of toxicity
54 among either the worms or the fish. Bioinformatics has been unilluminating in explaining these
55 results in terms of mimicry of an endogenous regulator. A mass spectrometric campaign to
56 identify a receptor, using murine brain for expediency, proved inconclusive. Molecular
57 modelling *in silico* indicated unexpectedly that the 6mer EPL036 might be acting as an
58 antagonist, to profecundity effect; that is, as a blocker of an inhibitor. This suggests that there
59 awaits discovery an evolutionarily conserved reproductive inhibitor and its (antifecundity)
60 receptor.

61

62

63 INTRODUCTION

64 It has proved possible to enhance fecundity (number of offspring) of *Caenorhabditis elegans* by
65 over 40%, with treated worms also living a fifth longer on average than untreated controls
66 (Davies and Hart, 2008). This was brought about simply by administering a 14mer peptide,
67 EPL001 (see Table 1 for amino acid sequence), to the aqueous medium in which this nematode
68 was maintained, to achieve a concentration of 1 μ M. The route of ingress – intestinal, cuticular
69 – was not established and the physiological mode of action remained obscure. A localisation
70 study did however show that this profecundity peptide, with a 5mer C-terminal linker for
71 fluorescence labelling, accumulated in the genital tract of these hermaphrodite worms.

72

73 The peptide EPL001 arose from a hormone discovery programme directed at finding an
74 endogenous tissue-mass inhibitor in mammals (Haylor *et al.*, 2009, Hart, 2014). An N-terminal
75 sequence of 14 amino acids was obtained from ovine material, but bioinformatics failed to
76 identify the parent molecule. A 14mer peptide corresponding to this N-terminal sequence was
77 synthesized. It was designated EPL001 and shown to have biological activity: inhibition of
78 compensatory renal growth was observed in the rat following unilateral nephrectomy (Haylor *et*
79 *al.*, 2009). Since this system is associated with IGF1 and the related DAF2 pathway is known to
80 be important in longevity and reproduction in *C. elegans* (Baumeister *et al.*, 2006), it was of
81 interest to test EPL001 in this nematode in terms of lifespan and fecundity (Davies and Hart,
82 2008). That both these factors were altered by the peptide was at least nominally consistent with
83 the renal data and with the notion that the 14mer EPL001 might be related in some way to an
84 endogenous factor.

85

86 Two other peptides, EPL030 and EPL040, were used as scrambled-sequence comparators for
87 the profecundity EPL001 in the worm experiment (Davies and Hart, 2008). These peptides

88 *reduced* fecundity by more than 60%. Given these findings the present study, apart from taking
89 the research into a vertebrate (guppy fish), involved an attempt to identify any receptors to
90 which the 14mers EPL001 and EPL030 and the 19mer EPL040 might bind, using murine brain
91 and a biotin-streptavidin system (Rife *et al.*, 2003), followed by LC-MSMS.
92 Immunohistochemistry using a human tissue array and an antibody raised against EPL001 had
93 shown epitopes in renal cells but also in brain tissue. This was in line with the postulation of an
94 undiscovered somatic inhibitor expressed in bodily tissues and centrally (Hart, 2014). Brain
95 from the mouse was favoured for analysis additionally (i) on the assumption that lifespan,
96 fecundity and tissue-mass controls probably involve evolutionarily conserved systems across
97 the phyla, (ii) because of the greater tissue availability of mammalian brain, appropriate for a
98 pilot experiment, as against the exiguous tissues of nematodes and fish, (iii) because brain is a
99 soft, highly defined tissue requiring minimal sample preparation, potentially leaving sufficient
100 uncontaminated intact receptor material to permit detection and (iv) because of our previous
101 experience and success in examining murine brain proteins by LC-MS (Ding *et al.*, 2008). The
102 present study thus spanned three species to elucidate an intriguing 'reproductive peptide' effect.

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107

108 **RESULTS**

109 **Nematodes**

110 Each of the three hexamers (Table 1) had a statistically significant profecundity effect on the
111 nematodes, as compared with controls (Fig. 1). The increases in the cumulative number of
112 offspring for EPL016, EPL037 and EPL036 were 50%, 71% and 79% respectively (ANOVA p
113 < 0.02).

114

115 EPL036 (IEPVFT) forms part of the 19mer EPL040 (Table 1). Compared with the 79% pro-
116 fecundity effect of the 6mer the 19mer caused a reduction in fecundity of 63% in an earlier
117 study (Davies and Hart, 2008). The antifecundity effect of EPL040 was confirmed in a
118 subsequent study of 10 worms subject to individual monitoring (data not shown). This approach
119 potentially enabled biomass to be estimated on the basis of size measurements and actually
120 permitted an appreciation of diversity in terms of a rare example of a male in this otherwise
121 hermaphrodite species and death through endotokia matricida (lethal hatching of eggs inside the
122 parent). But individual monitoring was less helpful in achieving statistical significance than
123 when worms were evaluated in reasonable bulk (e.g. 4 replicates of 10 worms, as in the present
124 study).

125

126 Referring to survivorship, there were no significant changes in average lifespan between the
127 groups, but the time taken for 50 % of worms to die was significantly longer among those
128 exposed to EPL036 (IEPVFT) than controls, and this was especially true of worms exposed to
129 EPL037 (LQPAHV)(Table 2).

130

131 Nematode bioinformatics on EPL001, EPL030 and EPL040 yielded moderate hits to
132 hypothetical proteins, without suggesting a research direction. Meanwhile amino acid sequence

133 comparisons of the antifecundity peptides EPL030 and EPL040 had indicated a shared motif of
134 potential interest, IEPVFT (synthesized as EPL036). A BLASTP search using this hexamer
135 sequence yielded 13 exact hits including one to a nematode protein. This was an
136 uncharacterised WD40 repeat-containing protein from *Caenorhabditis briggsae*, accession
137 number A8X7A7. The orthologue to A8X7A7 was identified in *C. elegans*, again using
138 BLASTP searches, as a hypothetical protein with the accession number Q8WQB4. The
139 equivalent motif to IEPVFT in *C. elegans* was LQPAHV (synthesized as EPL037, Table 1). A
140 CLUSTAL alignment of proteins, including the zebrafish orthologue, is shown in Fig. 2.

141

142 **Guppies**

143 ***Tank set up***

144 There was no discernable difference in the concentrations of either ammonia or nitrate in the
145 two tanks in the biofilter pre-experiment. The peptide cannot therefore be said to have changed
146 the rate at which beneficial bacteria had become established. After four weeks the pre-
147 experiment was terminated, with no difference between the test and control tanks in terms of
148 water chemistry (data not shown).

149

150 ***Fish study***

151 There were no mortalities during the study and at no point did the fish exhibit any signs of
152 distress or ill health or show any physical abnormalities. Normal behaviour was observed in
153 both tanks, including feeding, courting, sparring and chasing. Water quality remained consistent
154 throughout the trial and the 6mer EPL036 did not affect it in any discernable way (NH₄ both
155 0.03mg/L; NO₂ test 0.01mg/L, control 0mg/L; NO₃ both 5mg/L; pH test 7.2, control 7.4;
156 temperature, both 23°C; alkalinity, test 95, control 100). .

157

158 The number of descendants is shown in Table 3. Peptide exposure for a month had the effect on
159 guppies of increasing numbers such that by Week 26 there were over four times as many non-
160 founding fish in the test tank as in the control tank, a visible difference. At this time point in
161 fact (Fig. 3) the recognisably male and female adult fish in the two tanks, subtracting the
162 founders, were 31 tests and 19 controls, the former figure being 63% higher than the latter. The
163 female adults in the test tank were significantly smaller (~20%) than controls and there was
164 tendency (non-significant) for the males to be smaller too. As regards juveniles ('Fry' in Fig. 3),
165 there were twelve times as many in the test tank as in the control one.

166

167 By the termination of the study at Week 50 control numbers had surpassed those in the test
168 tank. Including the 20 founding adults, the control tank had 222 fish at termination, the test tank
169 197. Biomass tells a different story however. At Week 26 the total length of guppies in the
170 control tank was 931 mm ($n = 45$, mean body length = 20.7 mm SE \pm 0.8). The corresponding
171 figure for the guppies in the test tank was 1,780 mm ($n = 123$, mean body length = 14.5 mm SE
172 \pm 0.5; +91% compared with controls), with 15.4% higher biomass. At termination no body
173 length measurements were made but observation revealed that there were far more large fish in
174 the test tank than in the control tank, indicating greater biomass in the former than in the latter.
175 The structure of the populations at termination were very different: it was not just a case of the
176 control group catching up.

177

178 **Receptor**

179 Two murine brain proteins were identified as interacting uniquely with the profecundity 14mer
180 EPL001 (Table 4). These proteins were the alpha subunit of ATP synthase and growth-
181 associated protein-43 (GAP-43, also known as neuromodulin). A further protein interacting
182 with EPL001 was arginine kinase. This was also shown to interact with the antifecundity

183 EPL040, but not with the similarly antifecundity EPL030. As for EPL030 itself, interacting
184 proteins were tentatively identified as a nuclear hormone receptor and a 14-3-3 family protein.
185 The peptide X_{corr} was sufficient to identify all the peptide fragments except one. The exception
186 was RLGLTEFEAVKQM, which was identified by a second peptide from the same protein,
187 arginine kinase. Note, though, that all the candidate receptors fell short in terms of stringent
188 peptide scoring, which optimally requires more than one confidently identified peptide
189 fragment per protein.

190

191 Molecular modelling *in silico* showed that for the 14mer EPL030 the amino acid residues also
192 present in its 6mer derivative EPL036, Valine 12, Threonine 14 and Phenylalanine 13, are
193 superficial and offer potential binding sites to any receptor (Fig. 4). The molecular model of
194 EPL040 (not shown), which displays similar antifecundity activity in *C. elegans* to that of
195 EPL030, has almost identical intramolecular distances relating to these three residues. In
196 EPL036, which was profecundity in *C. elegans*, the loss of 8 N-terminal residues from EPL030
197 results in the intramolecular distance between phenylalanine and valine increasing and the
198 orientation around proline altering. This would be consistent with the larger EPL030 molecule
199 binding at a receptor as an agonist. EPL036, either because of the absence of some binding
200 ligands or due to their altered orientation, only partially fulfils the binding requirements,
201 leading to unproductive binding and its acting putatively as an antagonist.

202

203 **DISCUSSION**

204 This work has seen the identification of a short peptide motif associated with enhanced
205 reproductive activity, having proline in a central position. A potent exemplification is the
206 hexamer IEPVFT (EPL036), which is profecundity in both nematode worms and guppy fish.
207

208 The starting point for the present investigation was the outcome of an earlier study in *C.*
209 *elegans* (Davies and Hart, 2008). A 14mer peptide EPL030 and a 19mer peptide EPL040, when
210 administered separately via the worms' aqueous environment at a concentration of 1 μ M,
211 reduced fecundity to a near-identical degree (-64%). The 14mers were shortened in the current
212 study to a common hexamer, IEPVFT (EPL036), and administered once more to *C. elegans*.
213 The antifecundity activity of the parent peptides was transformed into a *profecundity* effect, of
214 +79%. In the earlier study the 14mer EPL001 provoked an increase in nematode fecundity of
215 +43%. When EPL001 was likewise shortened in the present work to a hexamer with a proline at
216 position 3, i.e. MKPLTG (EPL016), the profecundity activity in *C. elegans* was more than
217 maintained (+50%).

218

219 The 6mer EPL036 given to guppies via their tank water was associated with accelerated
220 fecundation, such that by the half-year mark there were over four times as many descendants in
221 the test tank as in the control tank and 15.4% greater biomass (including founders). That said,
222 by termination there were more fish in total in the control tank than in the test tank. The
223 structure of the populations were very different, however, with far more large fish in the test
224 tank, indicating an enduringly higher biomass. This implies that had another cycle of
225 reproduction been accommodated the number of test guppies would have exceeded once more
226 the number of control fish.

227

228 It is intriguing that merely adding peptide to the aqueous environment of nematodes and fish
229 should alter fecundity. To simplify interpretations via a single-species protocol, the nematodes
230 were fed dead bacteria (to excess). The biofilter in the fish tanks could not be sterilised, but a
231 prior experiment involving biofilters without fish present indicated that biofilter
232 microorganisms seem to be unaffected by the 6mer EPL036. The 1 μ M administration level is
233 not especially high, with no nutritional effect of the peptide likely, but there could be scope for
234 dose reduction, since a tenth of this dose of the 14mer EPL030 caused an antifecundity effect in
235 the earlier study (Davies and Hart, 2008).

236

237 Biological activity presumably betokens internalisation, which raises the issue of route of
238 ingress. Fluorescence studies in the earlier *C. elegans* study (Davies and Hart, 2008) revealed
239 pharyngeal accumulation, possibly indicating gut uptake. Transit across the cuticle is not
240 precluded but is unlikely based on work in another nematode, *Ascaris suum* (Sheehy *et al*,
241 2000). Permeability was demonstrated for monomeric to trimeric D-phenylalanine derivatives,
242 with permeability rising with an increase in methylation. Permeability was not demonstrated for
243 a 7mer in the same series which was unmethylated and less lypophilic and hence more in line
244 with the 6mer EPL036. In the guppies in the present study no route of ingress evidence is
245 available, yet passage across the gill membranes is an obvious possibility. As to tissue
246 distribution, little is known beyond the apparent accumulation of EPL001, the 14mer fecundity
247 enhancer, in nematode ovaries, as shown by fluorescent labelling of the peptide (Davies and
248 Hart, 2008).

249

250 Some of the peptides under discussion have been investigated at a commercial facility growing
251 nematodes for biocontrol in gardening and horticulture. The 14mer EPL001 increased total
252 nematode numbers in the insect parasitic *Steinernema feltiae* by 33.6 % and in the slug parasitic

253 *Phasmarhabditis hermaphrodita* by 27.2 % (Jeremy Pearce and John Godliman, Becker
254 Underwood Ltd, Worthing, UK, unpublished data). The comparable figures for the 14mer
255 EPL030 were 31.7 % and 24.8 % respectively. That there was no difference in direction of
256 effect between EPL001 and EPL030, as there is in *C. elegans*, means that had one of these
257 entomopathogenic nematodes been selected for the core study then a reproductively active
258 fecundity motif comprising a ‘prolinaceous sextet’ would not have been identified. The 6mer
259 EPL036 (IEPVFT) itself was tried only in *Phasmarabditis hermaphrodita*. Time to peak
260 production of the saleable infective larvae was reduced by a week, i.e. a quarter, with numbers
261 at this time point up by 45% on controls. This is a notable result as the peptide was
262 administered daily via the worms’ aqueous growth medium, which resembled liquid brick dust.
263

264 Treatment of fish with proteinaceous agents to stimulate reproduction and increase fecundity is
265 common practice in aquaculture, such as in the production of caviar from sturgeons (Lagler *et*
266 *al.*, 1977). Implants and injections of pituitary material have been used in both freshwater and
267 marine species for food production. In goldfish, *Carassius auratus*, analogues of LH-RH,
268 injected or implanted as pellets and co-administered with a dopamine antagonist, caused almost
269 nine out of ten females to ovulate (Sokolowska *et al.*, 1984). Peptide hormone treatment with
270 IGF-I in the short-finned eel, *Anguilla australis*, yielded an increase in oocyte diameter
271 (Lokman *et al.*, 2007). The majority of fish of interest in aquaculture are egg-laying species that
272 produce vast numbers of eggs with each spawning. In contrast guppies are small livebearing
273 fish with internal fertilisation and comparatively small broods. Their ability to store sperm
274 allows multiple broods to be born from one mating. It is known that female guppies that engage
275 in multiple matings exhibit an increased brood size and shorter gestation period (Evans and
276 Magurran, 2000). This possibility is unlikely however in the present study since behavioural
277 observations did not indicate an increased period of courtship in the study tank versus the

278 control. Courtship and other behaviour remained consistent in the two groups, including in the
279 test tank immediately after the addition of the peptide. Fecundity enhancement via implant or
280 injection in guppies is infeasible due to their small size, so in the present trial the peptide was
281 simply added to the water. Direct contact or absorption by the guppies would have taken place
282 either via the gill membranes, through the skin or via ingested water. Whatever the case,
283 fecundity and biomass were enhanced by a simple ecosystem modulation.

284

285 Whereas the test nematodes were exposed to peptide throughout their lives, the test fish were
286 only exposed during the first month – this is on the assumption that a short peptide is unlikely
287 to have persisted long in tank water after the administration period. That means that only the 20
288 founder fish were exposed, as there will likely have been insufficient time for them to produce
289 offspring before exposure ceased. By the half-year mark the adult female guppies in the test
290 tank, a mixture of mothers and daughters but presumptively not granddaughters, were on
291 average about a fifth smaller than adult female controls ($p < 0.01$). A trade-off between
292 reproduction and growth in guppies has long been recognised (Reznick, 1983). Peptide
293 exposure seems to have slowed the growth of the founding mothers but anomalously also of
294 their daughters, suggesting that some additional factor may latterly have been in play (e.g.
295 unsuspected competition for food or tank constraints relating to carrying capacity). Juvenile
296 numbers were twelvefold higher in the test tank after six months than among controls, so a
297 general redirection from female growth to reproduction seems indicated. It can be deduced that
298 test females reproduced at a smaller size than controls and that they produced more fry per
299 mating, an increase in brood size not related to mating frequency (see above). Improvements in
300 egg quality may have been involved, but direct evidence on this is wanting.

301

302 What of the male guppies? There was a non-significant trend towards lower body weights
303 among test males (Fig. 3). If test females were bearing young at smaller size and hence younger
304 age, then the males were presumably sexually active earlier than would normally be the case.
305 In a separate intervention, adding the 6mer EPL036 to the food freshwater angelfish
306 (*Pterophyllum scalare*) caused egg and fry production when males and females were exposed
307 together, not when either sex was exposed alone (Granville Hammond, Devon Discus Ltd,
308 Newton Abbot, UK, unpublished data). Fecundity built cumulatively, as peptide was
309 readministered in five-day cycles in food after each spawning. Note that in this independent
310 investigator's hands the both-sexes-at-once approach, via food, also induced a pair of hard-to-
311 breed tiger stingrays (*Potamotrygon tigrina*) to produce a pair of pups, as well as causing a
312 colony of reproductively quiescent Tiffany catfish (*Rineloricaria teffeana*) to start breeding
313 again. The same both-sexes-together approach has also worked in a conservation setting with
314 two species of rare Malagasy frogs, the splendid mantella (*Mantella pulchra*) and the brown
315 mantella (*Mantella ebenaui*) (Mike Bungard, Paignton Zoo, Devon, UK, unpublished data). The
316 breeding of the brown mantella is believed to be the first in a zoo anywhere in the world. The
317 6mer EPL036 was dissolved in water and misted onto the frogs in their rainforest vivaria, to
318 achieve percutaneous absorption.

319

320 The serendipitous IEPVFT (EPL036) scored a bioinformatics hit to a *Caenorhabditis briggsae*
321 gene (Fig. 2). The orthologous gene in *C. elegans* yielded LQPAHV (EPL037) as the
322 corresponding hexamer. When EPL037 was administered to *C. elegans* it increased fecundity
323 by +71%, nearly as much as the +79% achieved with EPL036. An apparent positive effect of
324 both peptides on survivorship to the 50% die-off point hints that the bioinformatic hit was real
325 and that the protein encoded by the gene might be processed into a secreted ligand, mimicked
326 by LQPAHV and the other prolinaceous sextets. Yet the protein encoded by the *C. briggsae*

327 gene is a WD40 scaffold protein, an unpromising hit. It is difficult to propose any model within
328 which LQPAHV or IEPVFT is both a functional ligand in its own right and ultimately derived
329 from a WD40 repeat-containing protein. There appear to be no examples of secreted ligands
330 containing the WD 40 motif.

331

332 The *C. briggsae* bioinformatics hit is probably fortuitous. When the homologous hexamer from
333 *C. elegans* was synthesized it was to let nature do the combinatorial chemistry. This analysis
334 leads away from a dubious gene hit towards a ligand motif, serendipitously discovered, with a
335 proline residue as a common denominator.

336

337 The receptor hunt, exploring as it did proteomic interactions, yielded no coherent narrative
338 (Table 4). GAP-43 is considered to be a downstream component of signal transduction systems
339 rather than a ligand-recognizing receptor, yet it might be relevant; ATP synthase and arginine
340 kinase are key players in cellular energetics, which might be germane to an energy-intensive
341 activity such as reproduction; the nuclear receptor has been specifically implicated in egg
342 laying in various organisms; while the 14-3-3 protein is suggested to have a function in the
343 regulation of organismal lifespans. Further work is required.

344

345 Worms and guppies exposed to the 6mer EPL036 bred 'earlier and more'. It has been
346 conceptualised that sexual maturation occurs in mammals when a reproductive 'brake',
347 operating centrally, is lifted (Witchel and Plant, 2013). This schema could be relevant as
348 molecular modelling yielded the insight that the 14mer EPL030 and the 19mer EPL040, both
349 *antifecundity* in *C. elegans*, were likely inhibiting reproduction by acting as agonists at an as yet
350 undetermined receptor. In this view the *profecundity* 6mer EPL036 would be seen as an
351 antagonist, i.e. as blocking an inhibitor. This suggests that there is waiting to be identified an

352 evolutionarily conserved endogenous reproductive inhibitor, as has been proposed (Hart, 2014),
353 along with its 'anti-fecundity receptor'.

354

355 Efficacy after ambient administration and activity in both an invertebrate and a vertebrate
356 species make the 'fecundity peptide' IEPVFT (EPL036) and related proline-containing moieties
357 worthy of further study, from both a fundamental perspective and in terms of applications in
358 fertility and fecundity enhancement.

359 MATERIALS AND METHODS

360 Peptides

361 Peptides were synthesized by a commercial supplier (Peptide Protein Research Ltd, Fareham,
362 UK). Amino acid sequences and proprietary designations are shown in Table 1. The peptide
363 EPL030 is a scrambled version of the profecundity 14mer EPL001, i.e. it has the same amino
364 acids in a different order. The 19mer EPL040 is a different anagrammatical version of EPL001,
365 with the addition of a five amino acid linker, GSGSK, to facilitate C-terminal fluorescence
366 labelling. Although nominally control molecules, both EPL030 and EPL040 showed
367 unexpected antifecundity activity in *C. elegans* (Davies and Hart, 2008). It was noted that by
368 chance the two control peptides shared the sequence commonalities KLK and IEPVFT. The
369 latter was held likely to be of more interest, since bioinformatics revealed a hit for IEPVFT to
370 the *Caenorhabditis briggsae* proteome, as described below. It was synthesized as EPL036. The
371 corresponding hexamer from *C. elegans* was determined bioinformatically to be LQPAHV.
372 This was synthesized as EPL037.

373

374 EPL036 and EPL037 are hexamers with proline at position three. Curiosity was piqued when it
375 was noted that the N-terminus (MKPLTG) of the profecundity 14mer EPL001 also had a
376 proline at position three. This 6mer was synthesized as EPL016.

377

378 Nematodes

379 *Caenorhabditis elegans* (N2) was grown in Petri dishes on Nematode Growth Medium (NGM)
380 agar, i.e. 3 g NaCl, 2.5 g peptone and 17 g technical agar in 975 ml distilled H₂O, which was
381 then autoclaved. To this was added 1 ml of CaCl₂ (1M), 1 ml MgSO₄ (1M) and 25 ml KH₂PO₄
382 (1M, pH6), all from filter-sterilised stock solutions. Finally, 1 ml of 5 mg/ml cholesterol in 95%
383 ethanol was added to the medium (Sulston and Hodgkin, 1988).

384

385 Nematodes were routinely cultured by growing the bacterium *Escherichia coli* OP50 overnight
386 in LB medium, on a shaker placed in an incubator (37°C). Petri dishes (9 cm) were seeded with
387 the OP50 to create a bacterial lawn overnight. Nematodes were added to these Petri dishes,
388 which were then maintained at 20°C in an incubator (Sulston and Hodgkin, 1988).

389

390 Nematode eggs were placed on NGM plates and allowed to develop into larvae. Groups of 10
391 larvae that were clearly post L2 dauer decision stage (L3/L4) were used in these studies and
392 placed in autoclaved staining blocks containing 200 µl sterilized M9 buffer (Davies and Hart,
393 2008). They were fed on heat killed bacteria rather than live bacteria to simplify interpretations.
394 Each treatment group and an untreated control group consisted of 4 replicates of 10 worms that
395 were placed randomly in a humid chamber and maintained at 20°C in an incubator.

396

397 The adult worms were transferred to fresh medium each day to prevent adults becoming mixed
398 with developing offspring and to allow the latter to be counted (Davies and Hart, 2008). The
399 peptides were administered each day to the new aqueous medium (M9 buffer) in which the
400 nematode were maintained, to achieve an initial concentration of 1 µM. This meant peptide did
401 not accumulate in the medium over time, and that the concentration never exceeded 1 µM.

402

403 Mean data were calculated from the four groups in each treatment for fecundity and
404 survivorship. Statistical analysis involved the calculation of standard errors of these means and
405 analysis of variance (ANOVA) and *t-tests*, as appropriate.

406

407 Nematode bioinformatics involved using the amino acid sequences of the peptides in BLAST
408 searches against standard databases at NCBI and Wormbase.

409

410 **Guppies**

411 Guppies, *Poecilia reticulata*, are livebearer fish native to the Caribbean and northern South
412 America, in the family Poeciliidae, which also includes mollies, swordtails and mosquitofish
413 (Nelson, 1994). The founder population of the guppies at ZSL London Zoo originated from a
414 collection at the Pitch Lake in Trinidad in the mid-1990s. Peptide was simply added to the
415 guppies' tank water.

416

417 ***Tank set up***

418 A pre-experiment was conducted during the period when the fish tanks were being conditioned
419 for the experiment proper. The issue was the number of species in the experimental system and
420 the question of direct and indirect effects. In the nematode experiment reported here, the worms
421 were fed dead bacteria rather than their normal diet of live bacteria to simplify the experiment
422 down to a single live species. This was not practical for guppies, as a biofilter was required in
423 the tank, for water purification purposes. The presence of an operational biofilter in each of the
424 test and control fish tanks might have rendered interpretation problematic: would any observed
425 changes be due to a direct effect on the fish or an indirect effect via the microbial population of
426 the biofilter? Newly set up aquaria undergo a period of instability in the first several weeks with
427 nitrogenous wastes building up in concentration until bacteria that utilise them become
428 established in the tank (Moe, 1992). The filter in an aquarium provides a matrix for
429 heterotrophic bacteria to colonise. These bacteria convert fish wastes from ammonia to nitrite
430 and eventually to nitrate. Two identical aquaria were each filled with 30 L of aged tap water. A
431 new air-driven biological sponge filter was added to each tank and ammonium chloride was
432 administered at 1 mg per L per day to provide a food source of ammonia for the bacteria to
433 establish themselves.

434

435 ***Fish study***

436 For the purposes of the trial 100 juveniles were selected at random from the main guppy colony
437 and isolated in a separate 30 L aquarium for maturation. Within two months sexual dimorphism
438 was observed and the fish were divided into separate populations of males and females in order
439 to prevent reproduction prior to the start of the study. Female guppies have the ability to store
440 sperm packets in the folds of the oviduct, so a single mating can produce several broods over
441 time (Moe, 1992). For this reason virgin status was necessary for the females to avoid starting
442 the trial with fish that were already gravid.

443

444 Two glass aquaria of 30 L each were filled with aged tap water and one AquaZoo air-driven
445 sponge filter to provide biological filtration. A small amount of Java moss, *Vesicularia*
446 *dubyana*, was added to each tank to provide cover for the fish, notably the fry, which might
447 otherwise be subject to cannibalism. After five weeks the fish were old enough for sexes to be
448 distinguished in the maturation tank and ten males and ten females were transferred to a peptide
449 treatment tank and a control (peptide-free) tank. The two tanks were placed 2m off the floor on
450 a shelf to minimise disturbance during the study. The fish were fed on a mixed diet of newly
451 hatched *Artemia salinas* nauplii, Aquarian brand vegetable flake food, frozen *Daphnia* sp. and
452 *Cylops* sp.. Food was provided twice daily at 9am and 3pm. Fish were fed to satiety. The fish
453 were observed daily for ten minutes during routine maintenance and feeding to assess
454 behaviour.

455

456 The water quality parameters were tested weekly throughout the experiment using a Palintest
457 photometer (Tyne & Wear, England) for pH, ammonia, nitrite and alkalinity. Temperature was

458 measured daily using a glass thermometer and nitrate was measured weekly using a Salifert test
459 kit (Duiven, The Netherlands).

460

461 Peptide EPL036 was added to the water of the test tank as an aqueous solution (<1 ml) from pre-
462 dispensed vials of equal concentration. The first dose was administered on 14th January 2010,
463 during Week 1, and then doses were given every other day for a total of 15 doses (including
464 weekends). In the nematode study also reported here it was found expedient to move the
465 founding population of adults into fresh medium each day to facilitate counting and monitoring.
466 With daily peptide administration to the worms, this meant that dose levels never exceeded 1
467 μM . For the guppies, alternate-day dosing reduced the likelihood of peptide accumulating above
468 the 1 μM level.

469

470 The test fish were observed immediately after peptide administration to check for signs of
471 distress and any physical or behavioural abnormalities, with the control fish as comparators.

472

473 The fry were counted during Weeks 24, 26 and 33, with a final count during Week 50. The size
474 distribution of the fish populations were established during Week 26 with individuals measured
475 to the nearest millimetre total length and categorized as male, female or juvenile
476 observationally, according to their sexually dimorphic state or lack thereof (juveniles). As
477 between non-reproducing and reproducing female guppies there is no significant difference in
478 body length or protein, only in fat reserves (Wischnath, 1993). Length is a convenient proxy for
479 biomass (i.e. weight), sparing the fish stressful weighing. Weight varies in fish by slightly more
480 than the cube of length (Froese, 2006). In the present study it was not absolute weights that
481 were required but the relative weights of two groups of fish, whose density was assumed to be
482 the same and close to that of the surrounding water, 1g/cm^3 . Individual fish lengths were cubed

483 to provide a pseudovolume, then summed by group to yield a volume ratio between the control
484 and test tanks that did service for 'estimated biomass'.

485

486 At Week 33 the fish were moved from their original 30 L tanks to 50 L tanks for continued
487 monitoring. This followed concern about the carrying capacity of the test tank and the
488 potentially suppressive effect of this on fish behaviour.

489

490 **Receptor**

491 The aim was to determine if any murine brain proteins bound to specimen synthetic peptides, to
492 identify a fecundity receptor. The peptides were the 14mers EPL001 (profecundity in *C.*
493 *elegans*) and EPL030 (antifecundity) and the 19mer EPL040 (also antifecundity). Biotinylated
494 derivatives of these three peptides were synthesized as part of a biotin-streptavidin purification
495 protocol. Long peptides were selected because biotin was deemed less likely to compromise
496 active sites with those than with the short peptides. The success of the biotinylation process was
497 determined mass spectrometrically. Incubation of the biotinylated peptides with murine brain
498 tissue was followed by streptavidin affinity pulldown of resulting peptide-protein complexes,
499 the bound proteins being identified via liquid chromatography and mass spectrometry.

500

501 ***Biotinylation of peptides***

502 The biotinylation agent biotinamidohexanoyl-6-aminohexanoic acid *N*-hydroxysuccinimide
503 ester (NHS-LC-biotin) was selected (Sigma Aldridge, Poole, Dorset, UK) as the NHS group of
504 this biotinylation agent rapidly and efficiently reacts with free amino groups and NHS-LC-
505 biotin possesses an extra spacer arm rendering the biotin molecule readily available for
506 streptavidin binding (Selo *et al*, 1996). The peptide (1mg) was dissolved in 1mL Reaction
507 buffer (50 mM phosphate buffer (pH 6.5)). 10mM NHS-LC-biotin was added to achieve a 5-

508 fold molar excess of biotin over peptide in solution, then the reaction mixture incubated at 4°C
509 for 24h. The NHS-LC-biotin was separated from the biotinylated EPL001 using C18
510 ZipTips™ (Millipore, Watford, UK) according to the manufacturer's protocol. To determine
511 whether the biotinylation had been successful, HPLC purification of the reaction products was
512 carried out prior to MALDI mass spectrometry. The same process was used for EPL030 and
513 EPL040.

514

515 *HPLC of biotinylated peptides*

516 The biotinylated peptides were applied to a C18 RP-HPLC analytical column (C18, hypersil
517 GOLD, diameter 250 x 4.6mm, particle size 5 μ m; Thermo Electron, Hemel Hempstead, UK) and
518 eluted with a linear gradient from 25% to 50% of solvent B (100% acetonitrile 0.04% TFA) in
519 solvent A (0.1% TFA) over 25 minutes at a 1mL/min flow rate AT 214nm absorbance
520 detection. NHS-LC-biotin and untreated EPL001, EPL030 and EPL040 were also run
521 under the same conditions.

522

523 *MALDI TOF mass spectrometry*

524 The biotinylated peptide solution, in the amount of 1 μ L, was mixed with 1 μ L of a 10mg/mL
525 solution of α -cyanohydroxycinnamic acid (dissolved in 50/50 water/acetonitrile with 0.1%
526 trifluoroacetic acid added) and allowed to dry on the MALDI plate. The MALDI plate was
527 introduced into a Voyager DE-STR MALDI mass spectrometer (Applied Biosystems, UK) and
528 a full scan mass spectrum was obtained between 50 and 4000 Da, with a laser intensity of 1700,
529 a delay time of 65ns, an acceleration voltage of 20000V and a grid voltage representing 66% of
530 the acceleration voltage.

531

532 The spectra indicated that the peptides were successfully biotinylated and also that the biotin
533 bound at up to three positions in all three peptides: Fig. 5 shows the mass spectrum of the
534 EPL001 peptide after the reaction, with a characteristic mass shift of 453Da representing the
535 addition of the biotin group onto the peptide. (The correct mass shift was confirmed by
536 biotinylation of bradykinin, a standard peptide of very similar molecular mass to that of
537 EPL001; data not shown.)

538

539

540 ***Homogenization of murine brain and incubation with biotinylated peptides***

541 Murine brain tissue, 1g, was homogenized in 9mL Tris-buffer (500pM, pH7.2) at 4°C
542 before the biotinylated peptide was added in 1mL tris-buffer and incubated at 36°C for 1
543 h. Following the incubation period, further cellular interactions were stopped by
544 transferring each vial to an ice bucket for 30 min.

545

546 ***Separation of proteins bound to biotinylated peptides***

547 Streptavidin magnetic particles, 2.5mL, were added to each 10mL incubation sample to achieve
548 a 10 fold excess of streptavidin over the biotinylated peptide, then incubated at room
549 temperature for 4h. A magnet was placed at the side of the vessel until the streptavidin
550 magnetic particles aggregated, then the top layer decanted off. The streptavidin magnetic
551 particles were then washed with Tris-buffer and the application of the magnet repeated.

552

553 ***Tryptic digestion of streptavidin-protein complexes***

554 Protein-magnetic bead samples were incubated with 15-20ng/ul trypsin in
555 25mM ammonium bicarbonate (pH 8.4) at 37°C overnight then the sample freeze dried and the
556 protein fragments desalted using C18 ZipTips™ (Millipore, Watford, UK) according to the

557 manufacturer's protocol.

558

559 ***Protein identification using LC-ESI***

560 The extracted protein fragments were analyzed by nano reverse phase liquid chromatography
561 (Ultimate Pump, Dionex, Netherlands) using an electrospray ion trap MS (LCQ Deca XP,
562 Thermo Electron, Hemel Hempstead, UK). LC-ESI-MS/MS separations were performed using
563 a 10cm x 75mm I.D. pulled-tip capillary column, that was packed in-house with a slurry of
564 3µm, 300Å pore size C18 silica bonded stationary phase (PepMap, Dionex, Camberley, UK).
565 The autosampler was fitted with a 5µL injection loop and was refrigerated at 4°C during
566 analysis. After injecting 7.5µL of solution the protein fragments were captured on a C18 trap
567 for 3min and then eluted onto the analytical column, which was washed for 15min with 98%
568 buffer A (0.1% formic acid in water v/v) and protein fragments then eluted using a stepwise
569 gradient of 0% solvent B (0.1% formic acid in acetonitrile v/v) to 65% solvent B in 100min,
570 then to 100% B in 10min with a constant flow rate of 0.2 µL/min. The electrospray MS was
571 operated in a data-dependent mode in which each full MS scan (m/z 475-2000) was followed
572 by three MS/MS scans, in which the three most abundant ions of the protein fragments were
573 dynamically selected for collision-induced dissociation (CID) using a normalized collision
574 energy of 35%. The temperature of the heated capillary and electrospray voltage were 160°C
575 and 1.6kV, respectively. The data were searched against the murine and human databases using
576 Sequest in Bioworks 3.2 (Thermo Electron, Hemel Hempstead, UK) and hits were filtered
577 within Bioworks for amino acid sequence probability (P = 0.001) and for high stringency cross
578 correlation (Xcorr 1+, 2+, 3+ = 1.8, 2.5, 3.2).

579

580 ***Molecular modelling***

581 Reproductive peptides were investigated via modelling *in silico*, notably to assess binding sites.

582 Data were produced by Molecular Modelling Proplus, version 6.22, and ChemSite, version

583 5.10, produced by ChemSW, Accelrys Inc., San Diego, CA92121, USA.

584

585

586

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592 respectively. Dave Emery is thanked for logistical support. Alex Cliffe and Alasdair Maltby of
593 ZSL are thanked for caring for and counting the guppies. The authors express their appreciation
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595 Mike Bungard.

596

597

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666

667 **Figure Legends**

668

669 Figure 1. Cumulative larvae produced by 10 nematodes maintained in aqueous buffer and
670 treated with hexamer peptides EPL036 (IEPVFT), EPL037 (LQPAHV) and EPL016
671 (MKPLTG), compared to untreated controls. (Mean \pm SE ; ANOVA, $p < 0.02$, all test groups
672 versus controls). Each treatment was replicated 4 times.

673

674 Figure 2. Fecundity motif: CLUSTAL alignment of proteins orthologous to a protein identified
675 in *C. briggsae* ('CAEBR') as containing IEPVFT (synthesized as the profecundity 6mer peptide
676 EPL036). CAEEL = *C. elegans* 'BRUMA' = *Brugia malayi* (a causative nematode of filariasis).

677

678 Figure 3. Guppy fish body lengths at Week 26 (halfway stage): mean length (mm) \pm SE of
679 males, females ('Fem') and juveniles ('Fry') in a control tank ('con') and peptide tank ('pep').
680 The figures in parentheses below the x-axis are the number of individuals in each category.
681 Data include each tank's founding adults (10 male and 10 female). ANOVA p values are given
682 above each pair of bars.

683

684 Figure 4. Molecular models of EPL030, a 14mer peptide displaying antifecundity activity in *C.*
685 *elegans*, and of EPL036, the 6mer profecundity peptide which forms the C terminus of EPL030

686

687 Figure 5. Mass spectrum of biotinylated 14mer EPL001.

688

689

690 Table 1. Peptides used. Hexamers in the longer peptides have been underlined. Proline is
 691 emboldened in each peptide. (All peptides cited in patents US 8367801 & EP 2234632.)

692

693	Designation	Sequence (mer)	Note	Reference
694	EPL001	<u>MKPLTGKVKEFNNI</u> (14)	Inhibits compensatory renal growth in rat	Haylor <i>et al</i> , 2009
695				
696				
697	EPL030	KLKMNGKNI IEPVFT (14)	Anagrammatical control I	Davies & Hart, 2008
698				
699				
700	EPL040	KLKNMGNKI IEPVFT GSK (19)	Anagrammatical control II, with linker	Davies & Hart, 2008
701				
702				
703	EPL036	IEPVFT (6)	Common motif between EPL030 and EPL040 with bioinformatic Blast hit to <i>Caenorhabditis briggsae</i>	This report
704				
705				
706				
707				
708	EPL037	LQPAHV (6)	<i>C. elegans</i> ortholog to EPL036	This report
709				
710				
711	EPL016	MKPLTG (6)	Motif containing proline from EPL001	This report
712				
713				

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720

721 Table 2. Worm survivorship of a starting population of 10 individuals maintained in aqueous

722 buffer; each treatment was replicated 4 times. Controls were unexposed to hexamer peptides.

723 Data are in days; ns = non-significant in comparison with controls; ANOVA * = $p < 0.05$; ** =724 $p < 0.005$

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	Controls	EPL016 (MKPLTG)	EPL036 (IEPVFT)	EPL037 (LQPAHV)
Average lifespan	5.4	5.0 (ns)	5.1 (ns)	6.0 (ns)
50 % dead	4.0	4.6 (ns)	4.9 (*)	6.0 (**)
100 % dead	10.3	8.8 (ns)	7.3 (ns)	10.3 (ns)

739

740

741 Table 3. Cumulative descendants of two founding populations of guppies, each comprising 10

742 males and 10 females. Controls were untreated. Test guppies were exposed to the 6mer peptide

743 EPL036 via their tank water. 'Week 0' = immediately prior to study commencement.

744

Week	Control	Peptide	Peptide as % Control
0	0	0	0
24	23	68	295
26	25	103	412
33	86	142	165
50	202	177	88

745

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753

754 Table 4. Receptor hunt: identification of candidate receptors in murine brain to 3 biotinylated
 755 synthetic peptides (the 14mers EPL001 and EPL030 and the 19mer EPL040), purified using
 756 streptavidin and analysed via mass spectrometry (MS).

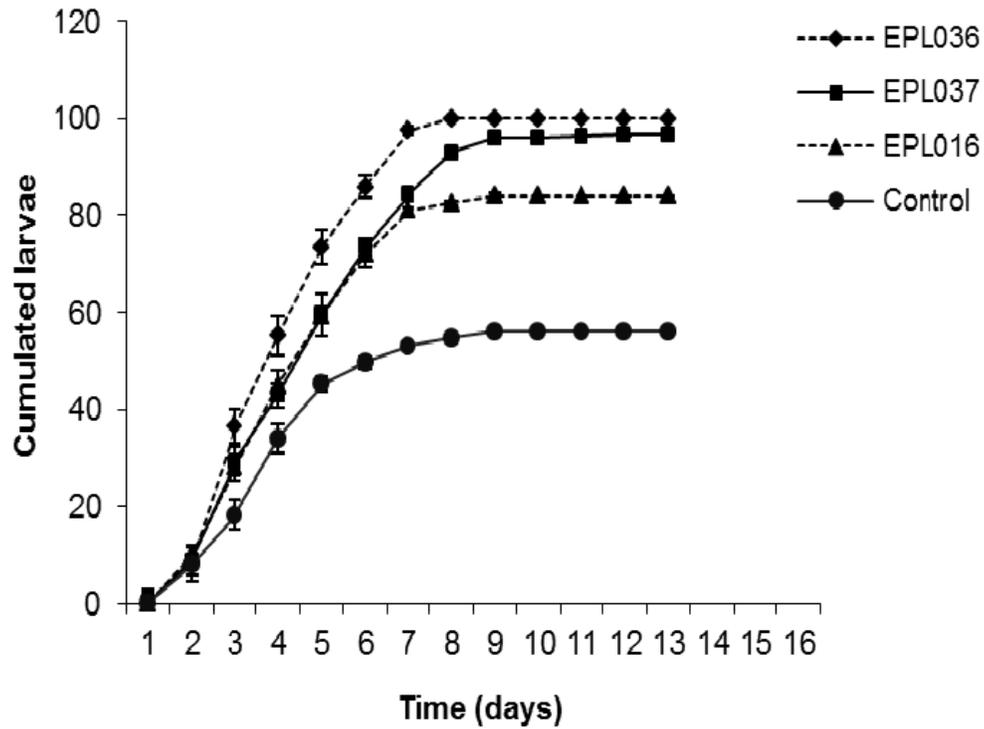
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Synthetic peptide used	Amino acid sequence from MS	Protein database hit	Function/location	MH ⁺	z	X _{corr}	Ions
EPL001	KEGDGSATT DAAPATSPK A	Growth Associated Protein 43	Substrate of protein kinase C, located in developing neuronal cells	1575.7	2	2.68	13/32
	RSDGKISEQ SDAKL	Mitochondrial ATP Synthase (α-subunit)	Involved in energy production, membrane bound	1264.6	2	2.67	18/22
	RGIHGEHSE SEGG VYDISNKA ————— RLGLTEFEA VKQM	Arginine Kinase	Maintenance of ATP levels, rapid energy production	2016.0	3	4.71	31/72
1235.4	2			2.04	15/20		
EPL030	RRFQEDIEIT NVFNVYAPP KDFY	Nuclear Hormone Receptor	Involved in embryonic development and egg laying	2543.8	2	2.68	22/80
	RYLAEVASG DDRNSVVE KS	14-3-3 Family Member	Thought to regulate lifespan	1852.9	2	3.14	16/34
EPL040	RGIHGEHSE SEGGVYDIS NKA	Arginine Kinase	Maintenance of ATP levels, rapid energy production	2016.0	3	4.12	28/72

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766 Figure 1. Cumulative larvae produced by 10 nematodes maintained in aqueous buffer and

767 treated with hexamer peptides EPL036 (IEPVFT), EPL037 (LQPAHV) and EPL016

768 (MKPLTG), compared to untreated controls. (Mean +/- SE ; ANOVA, $p < 0.02$, all test groups

769 versus controls). Each treatment was replicated 4 times.

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772

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773
774
775
776 Protein -----IEPVFT-----
777 tr|A8X7A7|A8X7A7_CAEER FR---EEIEPVFTCHVGSRCVPCVSLQGNR--IALSTGQRVFPDDPLLKE
778 tr|Q8WQB4|Q8WQB4_CAEEL YS---EELQPAHVQNVASRCVPCVDLKGKK--LVLCSEGERVYPDDKLLGD
779 tr|A8P9Z1|A8P9Z1_BRUMA TNGG-EPLPPCVQLSAHSSALCGLSIHKEQPLVATCSGQRVFPPFPQLDQM
780 sp|Q3SWZ7|WDR79_BOVINE AGLE-SKPEPVLSFQPQKDCCTNGVSLHPSLPLLATASGQRVFPEPTESGD
781 sp|Q8VC51|WDR79_MOUSE ALSDDSKLEPVVTFLPQKDCCTNGVSLHPTLPLLATASGQRVFPEPTNSGD
782 sp|Q5XII5|WDR79_RAT AFSDCKQLEPVMTFLPQDDCTNGVSLHPTLPLLATASGQRMFPEPTNSGD
783 sp|Q9BUR4|WDR79_HUMAN PGND-GKPEPVLSFPLPQKDCCTNGVSLHPSLPLLATASGQRVFPEPTESGD
784 XP_002662853.1_ZEBRAFISH PDGNEEILKPLLQFQAHTDCTNGISVHPFMPLMASSSGQRKFFWPSDSED
785

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786 Figure 2. Fecundity motif: CLUSTAL alignment of proteins orthologous to a protein identified

787 in *C. briggsae* ('CAEER') as containing IEPVFT (synthesized as the profecundity 6mer peptide

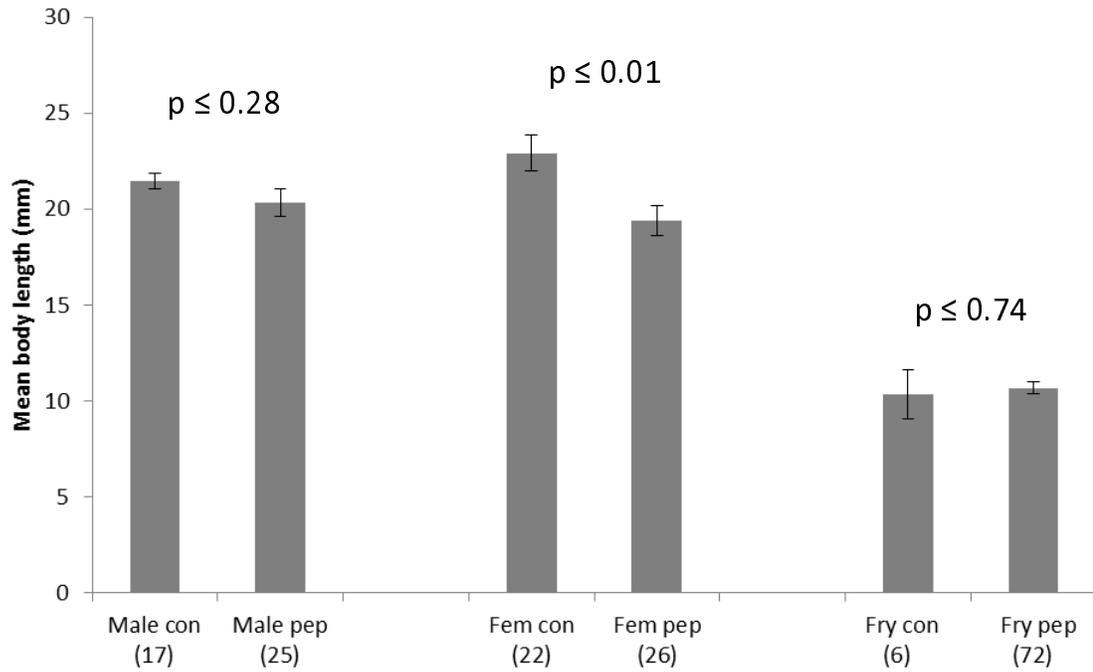
788 EPL036). CAEEL = *C. elegans* 'BRUMA' = *Brugia malayi* (a causative nematode of filariasis)

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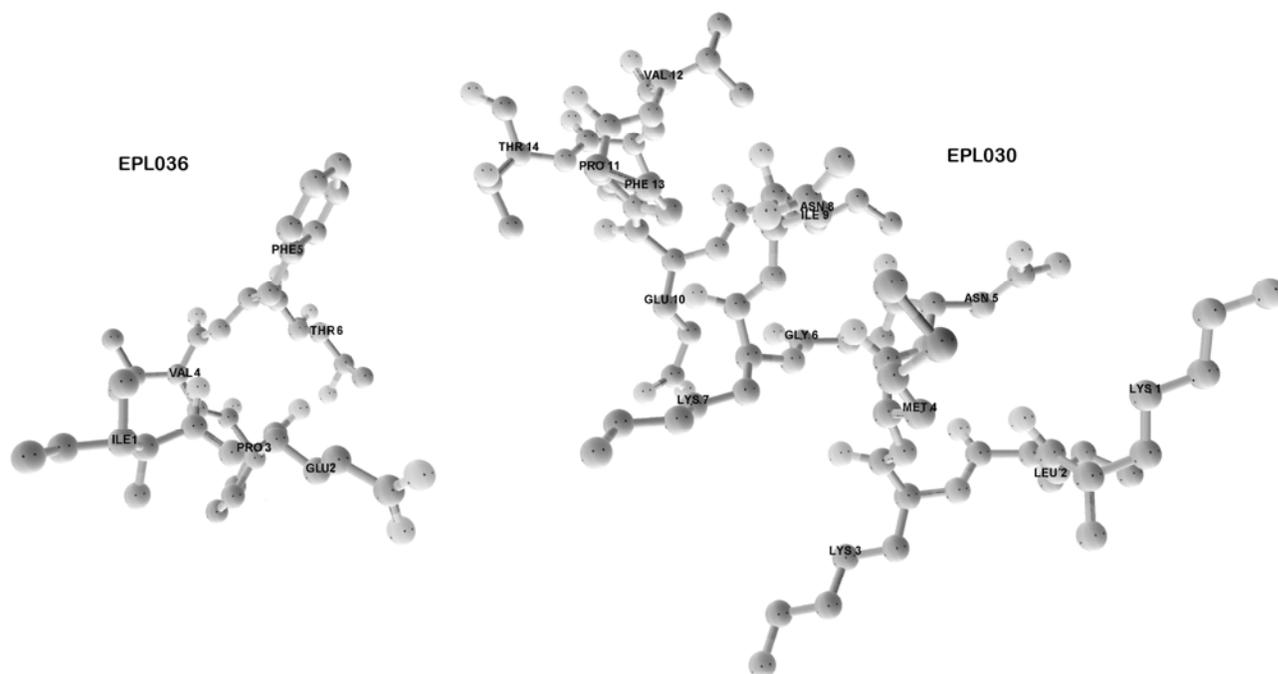
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797 Figure 3. Guppy fish body lengths at Week 26 (halfway stage): mean body length (mm) ± SE of
798 males, females ('Fem') and juveniles ('Fry') in a control tank ('con') and peptide tank ('pep').
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800 Data include each tank's founding adults (10 male and 10 female). ANOVA p values are given
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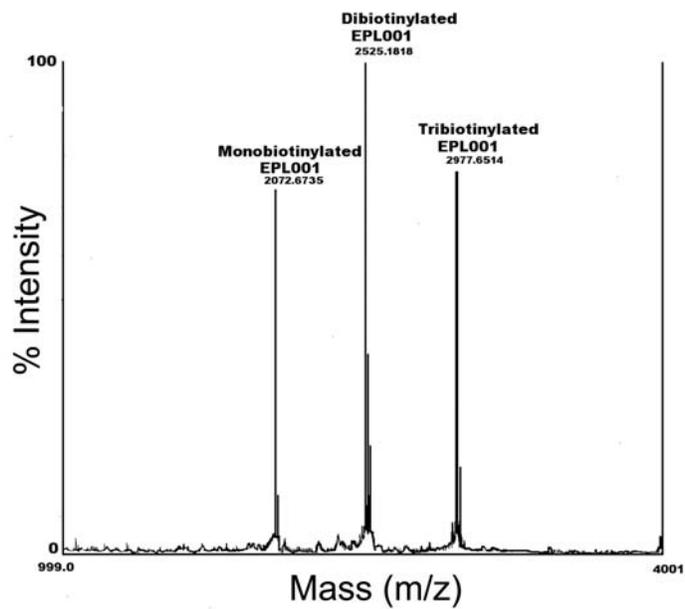


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807 Figure 4. Molecular models of EPL030, a 14mer peptide displaying antifecundity activity in *C.*
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Figure 5. Mass spectrum of biotinylated 14mer EPL001