

RESEARCH ARTICLE

Reproduction potentiated in nematodes (*Caenorhabditis elegans*) and guppy fish (*Poecilia reticulata*) by adding a synthetic peptide to their aqueous environment

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ABSTRACT

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

KEY WORDS: Reproductive stimulator, Breeding, Fertility, Infertility, Fecundity, Aphrodisiac

INTRODUCTION

It has proved possible to enhance fecundity (number of offspring) of *Caenorhabditis elegans* by over 40%, with treated worms also living a fifth longer on average than untreated controls (Davies and Hart, 2008). This was brought about simply by administering a 14mer peptide, EPL001 (see Table 1 for amino acid sequence), to the aqueous medium in which this nematode was maintained, to achieve a concentration of $1 \mu\text{mol l}^{-1}$. The route of ingress – intestinal, cuticular – was not established and the physiological mode of action remained obscure. A localisation study did,

however, show that this pro-fecundity peptide, with a 5mer C-terminal linker for fluorescence labelling, accumulated in the genital tract of these hermaphrodite worms.

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

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

RESULTS

Nematodes

Each of the three hexamers (Table 1) had a statistically significant pro-fecundity effect on the nematodes, as compared with controls

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Table 1. Peptides used

Designation	Sequence (mer)	Note	References
EPL001	<u>MKPLTG</u> KVKFNNI (14)	Inhibits compensatory renal growth in rat	Haylor et al., 2009
EPL030	KLKMNGKNI <u>IEPVFT</u> (14)	Anagrammatical control I	Davies and Hart, 2008
EPL040	KLKMNGKNI <u>IEPVFT</u> <u>GSGSK</u> (19)	Anagrammatical control II, with linker	Davies and Hart, 2008
EPL036	IEPVFT (6)	Common motif between EPL030 and EPL040 with bioinformatic BLAST hit to <i>Caenorhabditis briggsae</i>	This study
EPL037	LQPAHV (6)	<i>Caenorhabditis elegans</i> orthologue to EPL036	This study
EPL016	MKPLTG (6)	Motif containing proline from EPL001	This study

Hexamers in the longer peptides have been underlined. Proline is emboldened in each peptide. (All peptides are cited in patents US 8367801 and EP 2234632.)

(Fig. 1). The increases in the cumulative number of offspring for EPL016, EPL037 and EPL036 were 50%, 71% and 79%, respectively (ANOVA $P < 0.02$).

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

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

Nematode bioinformatics on EPL001, EPL030 and EPL040 yielded moderate hits to hypothetical proteins, without suggesting a research direction. Meanwhile, amino acid sequence comparisons of the anti-fecundity peptides EPL030 and EPL040 had indicated a shared motif of potential interest, IEPVFT (synthesized as EPL036). A BLASTP search using this hexamer sequence yielded 13 exact hits including one to a nematode protein. This was an uncharacterised

WD40 repeat-containing protein from *Caenorhabditis briggsae*, accession number A8X7A7. The orthologue to A8X7A7 was identified in *C. elegans*, again using BLASTP searches, as a hypothetical protein with the accession number Q8WQB4. The equivalent motif to IEPVFT in *C. elegans* was LQPAHV (synthesized as EPL037, Table 1). A CLUSTAL alignment of proteins, including the zebrafish orthologue, is shown in Fig. 2.

Guppies

Tank set up

There was no discernible difference in the concentrations of either ammonia or nitrate in the two tanks in the biofilter pre-experiment. The peptide cannot therefore be said to have changed the rate at which beneficial bacteria had become established. After 4 weeks the pre-experiment was terminated, with no difference between the test and control tanks in terms of water chemistry (data not shown).

Fish study

There were no mortalities during the study and at no point did the fish exhibit any signs of distress or ill health or show any physical abnormalities. Normal behaviour was observed in both tanks, including feeding, courting, sparring and chasing. Water quality remained consistent throughout the trial and the 6mer EPL036 did not affect it in any discernible way (NH_4 for both test and control 0.03 mg l^{-1} ; NO_2 test 0.01 mg l^{-1} , control 0 mg l^{-1} ; NO_3 both 5 mg l^{-1} ; pH test 7.2, control 7.4; temperature both 23°C ; alkalinity test 95, control 100).

The number of descendants is shown in Table 3. Peptide exposure for a month had the effect of increasing guppy numbers such that by week 26 there were over four times as many non-founding fish in the test tank as in the control tank, a visible difference. At this time point, in fact (Fig. 3), the recognisably male and female adult fish in the two tanks, subtracting the founders, were 31 tests and 19 controls, the former figure being 63% higher than the latter. The female adults in the test tank were significantly smaller ($\sim 20\%$) than controls and there was tendency (non-significant) for the males to be smaller too. As regards juveniles ('Fry' in Fig. 3), there were 12 times as many in the test tank as in the control one.

By the termination of the study at week 50, control numbers had surpassed those in the test tank. Including the 20 founding adults, the control tank had 222 fish at termination; the test tank had 197. Biomass tells a different story, however. At week 26, the total length of guppies in the control tank was 931 mm ($N=45$, mean \pm s.e.m. body length = 20.7 ± 0.8 mm). The corresponding figure for the guppies in the test tank was 1780 mm ($N=123$, mean body length = 14.5 ± 0.5 mm; +91% compared with controls), with 15.4% higher biomass. At termination, no body length measurements were made but observation revealed that there were far more large fish in the test tank than in the control tank, indicating greater biomass in the former than in the latter. The structure of the populations at

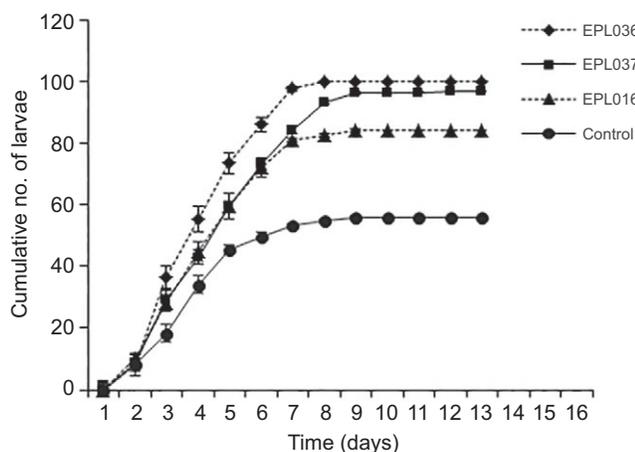


Fig. 1. Cumulative number of larvae produced by 10 nematodes maintained in aqueous buffer and treated with hexamer peptides EPL036 (IEPVFT), EPL037 (LQPAHV) and EPL016 (MKPLTG), compared with untreated controls. Data are means \pm s.e.m. (ANOVA, $P < 0.02$, all test groups versus controls). Each treatment was replicated 4 times.

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

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

Each treatment was replicated 4 times. Controls were unexposed to hexamer peptides.

Data without asterisks are non-significant in comparison with controls; ANOVA * $P < 0.05$; ** $P < 0.005$.

termination was very different: it was not just a case of the control group catching up.

Receptor

Two murine brain proteins were identified as interacting uniquely with the pro-fecundity 14mer EPL001 (Table 4). These proteins were the α -subunit of ATP synthase and growth-associated protein-43 (GAP-43, also known as neuromodulin). A further protein interacting with EPL001 was arginine kinase. This was also shown to interact with the anti-fecundity peptide EPL040, but not with the similarly anti-fecundity peptide EPL030. As for EPL030 itself, interacting proteins were tentatively identified as a nuclear hormone receptor and a 14-3-3 family protein. The peptide cross-correlation (X_{corr}) was sufficient to identify all the peptide fragments except one; the exception was RLGLEFEAVKQM, which was identified by a second peptide from the same protein, arginine kinase. Note, though, that all the candidate receptors fell short in terms of stringent peptide scoring, which optimally requires more than one confidently identified peptide fragment per protein.

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

DISCUSSION

This work has identified a short peptide motif associated with enhanced reproductive activity, having proline in a central position. A potent example is the hexamer IEPVFT (EPL036), which is pro-fecundity in both nematode worms and guppy fish.

The starting point for the present investigation was the outcome of an earlier study in *C. elegans* (Davies and Hart, 2008). A 14mer peptide, EPL030, and a 19mer peptide, EPL040, when administered separately via the worms' aqueous environment at a concentration of $1 \mu\text{mol l}^{-1}$, reduced fecundity to a near-identical degree (-64%). The 14mers were shortened in the current study to a common hexamer, IEPVFT (EPL036), and administered once more to *C. elegans*. The anti-fecundity activity of the parent peptides was transformed into a pro-fecundity effect, of $+79\%$. In the earlier study, the 14mer EPL001 provoked an increase in nematode fecundity of $+43\%$ (Davies and Hart, 2008). When EPL001 was likewise shortened in the present work to a hexamer with a proline at position 3, i.e. MKPLTG (EPL016), the pro-fecundity activity in *C. elegans* was more than maintained ($+50\%$).

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

It is intriguing that merely adding peptide to the aqueous environment of nematodes and fish should alter fecundity. To simplify interpretations via a single-species protocol, the nematodes were fed dead bacteria (to excess). The biofilter in the fish tanks could not be sterilised, but a prior experiment involving biofilters without fish present indicated that biofilter micro-organisms seem to be unaffected by the 6mer EPL036. The $1 \mu\text{mol l}^{-1}$ administration level is not especially high, with no nutritional effect of the peptide likely, but there could be scope for dose reduction, as a tenth of this dose of the 14mer EPL030 caused an anti-fecundity effect in the earlier study (Davies and Hart, 2008).

Biological activity presumably betokens internalisation, which raises the issue of route of ingress. Fluorescence studies in the earlier *C. elegans* study (Davies and Hart, 2008) revealed pharyngeal accumulation, possibly indicating gut uptake. Transit across the cuticle is not precluded but is unlikely based on work in another nematode, *Ascaris suum* (Sheehy et al., 2000). Permeability was demonstrated for monomeric to trimeric D-phenylalanine derivatives, with permeability rising with an increase in methylation. Permeability

Protein	-----IEPVFT-----
tr A8X7A7 A8X7A7_CAEBR	FR---EIEPVFTCHVGSRCVPCVSLQGNR--IALSTGQRVFPDPLLKE
tr Q8WQB4 Q8WQB4_CAEL	YS---EELQPAHVQNVASRCVPCVDLKGKK--LVLCSGERVYPPDKLLGD
tr A8P9Z1 A8P9Z1_BRUMA	TNGG-EPLPPCVQLSAHSSALCGLSIHKQPLVATCSGQRVFPFPQLDQM
sp Q3SWZ7 WDR79_BOVINE	AGLE-SKPEPVLSPQQRDCTNGVSLHPSLPLLATASGQRVFPPEPTESGD
sp Q6VC51 WDR79_MOUSE	ALSDDSKLEPVVTFLPQRDCTNGVSLHPTLPLLATASGQRVFPPEPTNSGD
sp Q5XII5 WDR79_RAT	AFSDCKQLEPVMTFLPQDDCTNGVSLHPTLPLLATASGQRVFPPEPTNSGD
sp Q9BUR4 WDR79_HUMAN	PGND-GKPEPVLSPQQRDCTNGVSLHPSLPLLATASGQRVFPPEPTESGD
XP_002662853.1_ZEBRAFISH	PDGNEEILKPLLQFQAHTDCTNGISVHPFMPLMASSSGQRKFFWPSDSGD

Fig. 2. Fecundity motif. CLUSTAL alignment of proteins orthologous to a protein identified in *Caenorhabditis briggsae* (CAEBR) as containing IEPVFT (synthesized as the pro-fecundity 6mer peptide EPL036). CAEEL, *Caenorhabditis elegans*; BRUMA, *Brugia malayi* (a causative nematode of filariasis).

Table 3. Cumulative number of descendants of two founding populations of guppies, each comprising 10 males and 10 females

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

Controls were untreated. Test guppies were exposed to the 6mer peptide EPL036 via their tank water. Week 0 is the time point immediately prior to study commencement.

was not demonstrated for a 7mer in the same series that was unmethylated and less lipophilic and hence more in line with the 6mer EPL036. In the guppies in the present study, no route of ingress evidence is available, yet passage across the gill membranes is an obvious possibility. As to tissue distribution, little is known beyond the apparent accumulation of EPL001, the 14mer fecundity enhancer, in nematode ovaries, as shown by fluorescence labelling of the peptide (Davies and Hart, 2008).

Some of the peptides under discussion have been investigated at a commercial facility growing nematodes for biocontrol in gardening and horticulture. The 14mer EPL001 increased total nematode numbers in the insect-parasitic *Steinernema feltiae* by 33.6% and in the slug-parasitic *Phasmarhabditis hermaphrodita* by 27.2% (J. Pearce and J. Godliman, Becker Underwood Ltd, Worthing, UK, unpublished data). The comparable figures for the 14mer EPL030 were 31.7% and 24.8%, respectively. That there was no difference in direction of effect between EPL001 and EPL030, as there is in *C. elegans*, means that had one of these entomopathogenic nematodes been selected for the core study, then a reproductively active fecundity motif comprising a ‘proline-rich sextet’ would not have been identified. The 6mer EPL036 (IEPVFT) itself was tried only in *P. hermaphrodita*. Time to peak production of the saleable infective larvae was reduced by a week, i.e. a quarter, with numbers at this time point up by 45% on controls. This is a notable result as the peptide was administered daily via the worms’ aqueous growth medium, which resembled liquid brick dust.

Treatment of fish with proteinaceous agents to stimulate reproduction and increase fecundity is common practice in aquaculture, such as in the production of caviar from sturgeons (Lagler et al., 1977). Implants and injections of pituitary material have been used in both freshwater and marine species for food

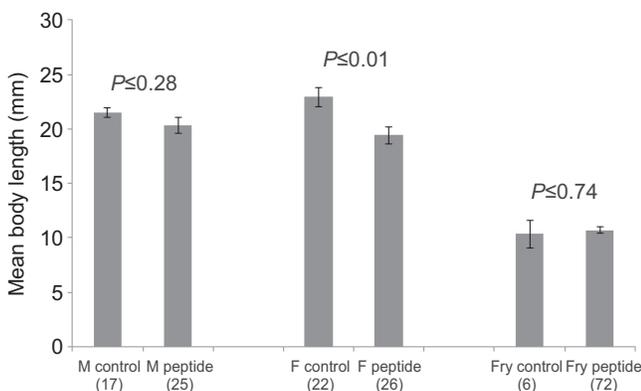


Fig. 3. Guppy fish body length at week 26 (halfway stage). Mean (\pm s.e.m.) length of males (M), females (F) and juveniles (Fry) in a control tank and a tank containing peptide. Numbers in parentheses below the x-axis are the number of individuals in each category. Data include each tank’s founding adults (10 males and 10 females). ANOVA P -values are given above each pair of bars.

production. In goldfish, *Carassius auratus*, analogues of luteinizing hormone-releasing hormone, injected or implanted as pellets and co-administered with a dopamine antagonist, caused almost nine out of 10 females to ovulate (Sokolowska et al., 1984). Peptide hormone treatment with IGF-I in the short-finned eel, *Anguilla australis*, yielded an increase in oocyte diameter (Lokman et al., 2007). The majority of fish of interest in aquaculture are egg-laying species that produce vast numbers of eggs with each spawning. In contrast, guppies are small live-bearing fish with internal fertilisation and comparatively small broods. Their ability to store sperm allows multiple broods to be born from one mating. It is known that female guppies that engage in multiple matings exhibit an increased brood size and shorter gestation period (Evans and Magurran, 2000). This possibility is unlikely, however, in the present study as behavioural observations did not indicate an increased period of courtship in the study tank versus the control. Courtship and other behaviour remained consistent in the two groups, including in the test tank immediately after the addition of the peptide. Fecundity enhancement via implant or injection in guppies is infeasible because of their small size, so in the present trial the peptide was simply added to the water. Direct contact or absorption by the guppies would have taken place via the gill membranes, through the skin or via ingested water. Whatever the case, fecundity and biomass were enhanced by a simple ecosystem modulation.

Whereas the test nematodes were exposed to peptide throughout their lives, the test fish were only exposed during the first month – this is on the assumption that a short peptide is unlikely to have persisted long in tank water after the administration period. This means that only the 20 founder fish were exposed, as there will probably have been insufficient time for them to produce offspring before exposure ceased. By the half-year mark the adult female guppies in the test tank, a mixture of mothers and daughters but presumptively not granddaughters, were on average about a fifth smaller than adult female controls ($P < 0.01$). A trade-off between reproduction and growth in guppies has long been recognised (Reznick, 1983). Peptide exposure seems to have slowed the growth of the founding mothers but anomalously also of their daughters, suggesting that some additional factor may latterly have been in play (e.g. unsuspected competition for food or tank constraints relating to carrying capacity). Juvenile numbers were 12-fold higher in the test tank after six months than in the control tank, so a general redirection from female growth to reproduction seems indicated. It can be deduced that test females reproduced at a smaller size than controls and that they produced more fry per mating, an increase in brood size not related to mating frequency (see above). Improvements in egg quality may have been involved, but direct evidence on this is wanting.

What of the male guppies? There was a non-significant trend towards lower body mass among test males (Fig. 3). If test females were bearing young at a smaller size and hence younger age, then the males were presumably sexually active earlier than would normally be the case.

In a separate intervention, adding the 6mer EPL036 to the food of freshwater angelfish (*Pterophyllum scalare*) caused egg and fry production to increase when males and females were exposed together, but not when either sex was exposed alone (G. Hammond, Devon Discus Ltd, Newton Abbot, UK, unpublished data). Fecundity built cumulatively, as peptide was re-administered in 5 day cycles in food after each spawning. Note that in this independent investigator’s hands, the both-sexes-at-once approach, via food, also induced a pair of hard-to-breed tiger stingrays (*Potamotrygon tigrina*) to produce a pair of pups, as well as causing

Table 4. Receptor hunt: identification of candidate receptors in murine brain to three biotinylated synthetic peptides

Synthetic peptide	Amino acid sequence from MS	Protein database hit	Function/location	MH ⁺	z	X _{corr}	Ions
EPL001	KEGDGSATDDAAPATSPKA	GAP-43	Substrate of PKC, located in developing neuronal cells	1575.7	2	2.68	13/32
	RSDGKISEQSDAKL	Mitochondrial ATP synthase (α -subunit)	Involved in energy production, membrane bound	1264.6	2	2.67	18/22
	RGIHGEHSESEGG VYDISNKA	Arginine kinase	Maintenance of ATP levels, rapid energy production	2016.0	3	4.71	31/72
EPL030	RLGLTEFEAVKQM			1235.4	2	2.04	15/20
	RRFQEDIEITNVFVYAPPKDFY	Nuclear hormone receptor	Involved in embryonic development and egg laying	2543.8	2	2.68	22/80
EPL040	RYLAEVASGDDRNSVVEKS	14-3-3 family member	Thought to regulate lifespan	1852.9	2	3.14	16/34
	RGIHGEHSESEGGVYDISNKA	Arginine kinase	Maintenance of ATP levels, rapid energy production	2016.0	3	4.12	28/72

The synthetic peptides were the 14mers EPL001 and EPL030 and the 19mer EPL040, purified using streptavidin and analysed via mass spectrometry (MS). GAP, growth-associated protein; PKC, protein kinase C.

a colony of reproductively quiescent Tiffany catfish (*Rineloricaria teffeana*) to start breeding again. The same both-sexes-together approach has also worked in a conservation setting with two species of rare Malagasy frogs, the splendid mantella (*Mantella pulchra*) and the brown mantella (*Mantella ebenaui*) (M. Bungard, Paignton Zoo, Devon, UK, unpublished data). The breeding of the brown mantella is believed to be the first in a zoo anywhere in the world. The 6mer EPL036 was dissolved in water and misted on to the frogs in their rainforest vivaria, to achieve percutaneous absorption.

The serendipitous IEPVFT (EPL036) scored a bioinformatics hit to a *C. briggsae* gene (Fig. 2). The orthologous gene in *C. elegans* yielded LQPAHV (EPL037) as the corresponding 6mer. When EPL037 was administered to *C. elegans*, it increased fecundity by +71%, nearly as much as the +79% achieved with EPL036. An apparent positive effect of both peptides on survivorship to the 50% die-off point hints that the bioinformatic hit was real and that the protein encoded by the gene might be processed into a secreted ligand, mimicked by LQPAHV and the other prolineaceous sextets. Yet the protein encoded by the *C. briggsae* gene is a WD40 scaffold protein, an unpromising hit. It is difficult to propose any model within which LQPAHV or IEPVFT is both a functional ligand in its own right and ultimately derived from a WD40 repeat-containing protein. There appear to be no examples of secreted ligands containing the WD40 motif.

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

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

Worms and guppies exposed to the 6mer EPL036 bred ‘earlier and more’. It has been conceptualised that sexual maturation occurs in mammals when a reproductive ‘brake’, operating centrally, is lifted (Witchel and Plant, 2013). This schema could be relevant as molecular modelling yielded the insight that the 14mer EPL030 and the 19mer EPL040, both anti-fecundity in *C. elegans*, were probably inhibiting reproduction by acting as agonists at an as yet undetermined receptor. In this view, the

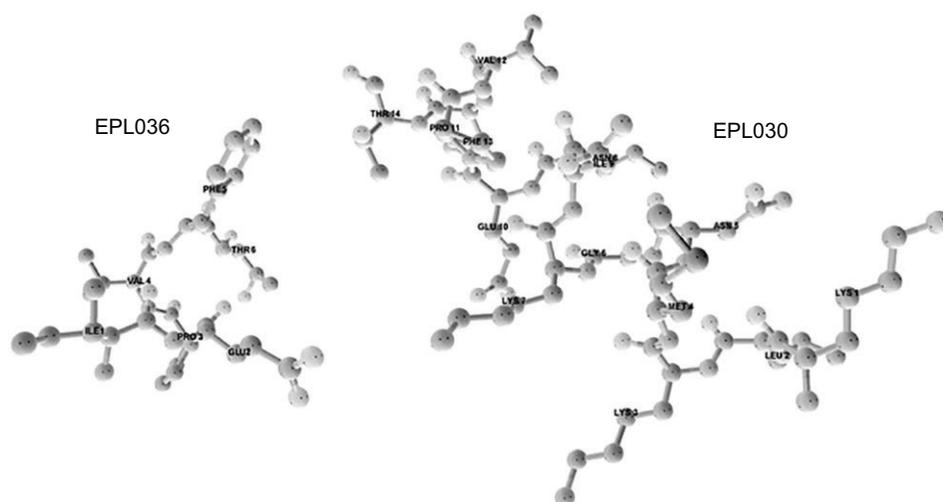


Fig. 4. Molecular models of EPL030 and EPL036. EPL030 is a 14mer peptide displaying anti-fecundity activity in *C. elegans*; EPL036 is the 6mer pro-fecundity peptide which forms the C-terminus of EPL030.

pro-fecundity 6mer EPL036 would be seen as an antagonist, i.e. as blocking an inhibitor. This suggests that there is an evolutionarily conserved endogenous reproductive inhibitor waiting to be identified, as has been proposed (Hart, 2014), along with its 'anti-fecundity receptor'.

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

MATERIALS AND METHODS

Peptides

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

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

Nematodes

Caenorhabditis elegans (Maupas 1900) (Strain N2) was grown in Petri dishes on Nematode Growth Medium (NGM) agar, i.e. 3 g NaCl, 2.5 g peptone and 17 g technical agar in 975 ml distilled H₂O, which was then autoclaved. To this was added 1 ml of CaCl₂ (1 mol l⁻¹), 1 ml MgSO₄ (1 mol l⁻¹) and 25 ml KH₂PO₄ (1 mol l⁻¹, pH 6), all from filter-sterilised stock solutions. Finally, 1 ml of 5 mg ml⁻¹ cholesterol in 95% ethanol was added to the medium (Sulston and Hodgkin, 1988).

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

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

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

Mean data were calculated from the four groups in each treatment for fecundity and survivorship. Statistical analysis involved the calculation of s.e.m. and ANOVA and *t*-tests, as appropriate.

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

Guppies

Poecilia reticulata Peters 1859 are live-bearer fish native to the Caribbean and northern South America, in the family Poeciliidae, which also includes mollies, swordtails and mosquitofish (Nelson, 1994). The founder population of the guppies at the Zoological Society of London (ZSL) London Zoo originated from a collection at the Pitch Lake in Trinidad in the mid-1990s. Peptide was simply added to the guppies' tank water.

Tank set up

A pre-experiment was conducted during the period when the fish tanks were being conditioned for the experiment proper. The issue was the number of species in the experimental system and the question of direct and indirect effects. In the nematode experiment reported here, the worms were fed dead bacteria rather than their normal diet of live bacteria to simplify the experiment down to a single live species. This was not practical for guppies, as a biofilter was required in the tank, for water purification purposes. The presence of an operational biofilter in each of the test and control fish tanks might have rendered interpretation problematic: would any observed changes be due to a direct effect on the fish or an indirect effect via the microbial population of the biofilter? Newly set up aquaria undergo a period of instability in the first several weeks, with nitrogenous wastes building up in concentration until bacteria that utilise them become established in the tank (Moe, 1992). The filter in an aquarium provides a matrix for heterotrophic bacteria to colonise. These bacteria convert fish wastes from ammonia to nitrite and eventually to nitrate. Two identical aquaria were each filled with 30 l of aged tap water. A new air-driven biological sponge filter was added to each tank and ammonium chloride was administered at 1 mg l⁻¹ day⁻¹ to provide a food source of ammonia for the bacteria to establish themselves. On the first day of the biofilter pre-experiment, the 6mer EPL036 was added in sufficient quantity to the water of the test tank to achieve a concentration of 1 µmol l⁻¹. This dose was repeated for two further days. No peptide was added to the control tank.

Fish study

For the purposes of the trial, 100 juveniles were selected at random from the main guppy colony and isolated in a separate 30 l aquarium for maturation. Within 2 months, sexual dimorphism was observed and the fish were divided into separate populations of males and females in order to prevent reproduction prior to the start of the study. Female guppies have the ability to store sperm packets in the folds of the oviduct, so a single mating can produce several broods over time (Moe, 1992). For this reason, virgin status was necessary for the females to avoid starting the trial with fish that were already gravid.

Two glass aquaria of 30 l each were filled with aged tap water and one AquaZoo air-driven sponge filter to provide biological filtration. A small amount of Java moss, *Vesicularia dubyana*, was added to each tank to provide cover for the fish, notably the fry, which might otherwise be subject to cannibalism. After 5 weeks, the fish were old enough for sexes to be distinguished in the maturation tank and 10 males and 10 females were transferred to a peptide treatment tank and a control (peptide-free) tank. The two tanks were placed 2 m off the floor on a shelf to minimise disturbance during the study. The fish were fed on a mixed diet of newly hatched *Artemia salinas* nauplii, Aquarian brand vegetable flake food, frozen *Daphnia* sp. and *Cylops* sp. Food was provided twice daily at 09:00 h and 15:00 h. Fish were fed to satiety. The fish were observed daily for 10 min during routine maintenance and feeding to assess behaviour.

The water quality parameters were tested weekly throughout the experiment using a Palintest photometer (Tyne and Wear, UK) for pH, ammonia, nitrite and alkalinity. Temperature was measured daily using a glass thermometer and nitrate was measured weekly using a Salifert test kit (Duiven, The Netherlands).

Peptide EPL036 was added to the water of the test tank as an aqueous solution (<1 ml) from pre-dispensed vials of equal concentration. The first dose was administered on 14 January 2010, during week 1, and then doses were given every other day for a total of 15 doses (including weekends). In the nematode study also reported here, it was found expedient to move the

founding population of adults into fresh medium each day to facilitate counting and monitoring. With daily peptide administration to the worms, this meant that dose levels never exceeded $1 \mu\text{mol l}^{-1}$. For the guppies, alternate-day dosing reduced the likelihood of peptide accumulating above the $1 \mu\text{mol l}^{-1}$ level.

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

The fry were counted during weeks 24, 26 and 33, with a final count during week 50. The size distribution of the fish populations was established during week 26, with individuals measured to the nearest millimetre total length and categorized as male, female or juvenile observationally, according to their sexually dimorphic state or lack thereof (juveniles). As between non-reproducing and reproducing female guppies there is no significant difference in body length or protein, only in fat reserves (Wischnath, 1993). Length is a convenient proxy for biomass, sparing the fish stressful weighing. Weight varies in fish by slightly more than the cube of length (Froese, 2006). In the present study, it was not absolute mass that was required but the relative mass of two groups of fish, whose density was assumed to be the same and close to that of the surrounding water, 1 g cm^{-3} . Individual fish lengths were cubed to provide a pseudovolume, then summed by group to yield a volume ratio between the control and test tanks that did service for 'estimated biomass'.

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

Receptor

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

Biotinylation of peptides

The biotinylation agent biotinamidohexanoyl-6-aminohexanoic acid *N*-hydroxysuccinimide ester (NHS-LC-biotin) was selected (Sigma-Aldrich, Poole, Dorset, UK) as the NHS group of this biotinylation agent rapidly and efficiently reacts with free amino groups and NHS-LC-biotin possesses an extra spacer arm rendering the biotin molecule readily available for streptavidin binding (Sélo et al., 1996). The peptide (1 mg) was dissolved in 1 ml reaction buffer (50 mmol l^{-1} phosphate buffer, pH 6.5); 10 mmol l^{-1} NHS-LC-biotin was added to achieve a 5-fold molar excess of biotin over peptide in solution, then the reaction mixture was incubated at 4°C for 24 h. The NHS-LC-biotin was separated from the biotinylated EPL001 using C18 ZipTips™ (Millipore, Watford, UK) according to the manufacturer's protocol. To determine whether the biotinylation had been successful, HPLC purification of the reaction products was carried out prior to MALDI mass spectrometry. The same process was used for EPL030 and EPL040.

HPLC of biotinylated peptides

The biotinylated peptides were applied to a C18 RP-HPLC analytical column (C18, hypersil GOLD, diameter $250 \times 4.6 \text{ mm}$, particle size 5 μ ; Thermo Electron, Hemel Hempstead, UK) and eluted with a linear gradient from 25% to 50% of solvent B (100% acetonitrile, 0.04% TFA) in solvent A (0.1% TFA) over 25 min at a 1 ml min^{-1} flow rate at 214 nm absorbance detection. NHS-LC-biotin and untreated EPL001, EPL030 and EPL040 were also run under the same conditions.

MALDI TOF mass spectrometry

The biotinylated peptide solution, in $1 \mu\text{l}$ volume, was mixed with $1 \mu\text{l}$ of a 10 mg ml^{-1} solution of α -cyanohydroxycinnamic acid (dissolved in 50/50 water/acetonitrile with 0.1% trifluoroacetic acid added) and allowed to dry on the MALDI plate. The MALDI plate was introduced into a Voyager DE-STR MALDI mass spectrometer (Applied Biosystems, UK) and a full scan mass spectrum was obtained between 50 and 4000 Da, with a laser intensity of 1700, a delay time of 65 ns, an acceleration voltage of 20,000 V and a grid voltage representing 66% of the acceleration voltage.

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

Homogenization of murine brain and incubation with biotinylated peptides

Murine brain tissue, 1 g, was homogenized in 9 ml Tris-buffer (500 pmol l^{-1} , pH 7.2) at 4°C before the biotinylated peptide was added in 1 ml Tris-buffer and incubated at 36°C for 1 h. Following the incubation period, further cellular interactions were stopped by transferring each vial to an ice bucket for 30 min.

Separation of proteins bound to biotinylated peptides

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

Tryptic digestion of streptavidin-protein complexes

Protein-magnetic bead samples were incubated with $15\text{--}20 \mu\text{g ml}^{-1}$ trypsin in 25 mmol l^{-1} ammonium bicarbonate (pH 8.4) at 37°C overnight then the sample was freeze dried and the protein fragments desalted using C18 ZipTips™ (Millipore, Watford, UK) according to the manufacturer's protocol.

Protein identification using LC-ESI

The extracted protein fragments were analysed by nano reverse phase liquid chromatography (Ultimate Pump, Dionex, The Netherlands) using an electrospray ion trap MS (LCQ Deca XP, Thermo Electron). LC-ESI-MS/MS separations were performed using a $10 \text{ cm} \times 75 \text{ mm}$ i.d. pulled-tip capillary column, that was packed in-house with a slurry of $3 \mu\text{m}$, 300 Å pore

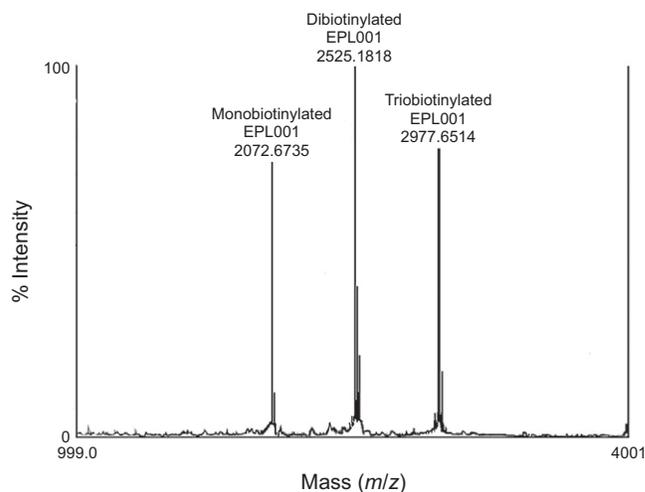


Fig. 5. Mass spectrum of the biotinylated 14mer EPL001.

size C18 silica bonded stationary phase (PepMap, Dionex, Camberley, UK). The autosampler was fitted with a 5 μ l injection loop and was refrigerated at 4°C during analysis. After injection of 7.5 μ l of solution, the protein fragments were captured on a C18 trap for 3 min and then eluted on to the analytical column, which was washed for 15 min with 98% buffer A (0.1% formic acid in water v/v) and protein fragments were then eluted using a stepwise gradient of 0% solvent B (0.1% formic acid in acetonitrile v/v) to 65% solvent B in 100 min, then to 100% B in 10 min with a constant flow rate of 0.2 μ l min⁻¹. The electrospray MS was operated in a data-dependent mode in which each full MS scan (m/z 475–2000) was followed by three MS/MS scans, in which the three most abundant ions of the protein fragments were dynamically selected for collision-induced dissociation (CID) using a normalized collision energy of 35%. The temperature of the heated capillary and electrospray voltage were 160°C and 1.6 kV, respectively. The data were searched against the murine and human databases using Sequest in Bioworks 3.2 (Thermo Electron) and hits were filtered within Bioworks for amino acid sequence probability ($P=0.001$) and for high stringency cross-correlation (Xcorr 1+, 2+, 3+=1.8, 2.5, 3.2).

Molecular modelling

Reproductive peptides were investigated via modelling *in silico*, notably to assess binding sites. Data were produced by Molecular Modelling Proplus, version 6.22, and ChemSite, version 5.10, produced by ChemSW (Accelrys Inc., San Diego, CA92121, USA).

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Competing interests

J.E.H. is founding scientist of Endocrine Pharmaceuticals Ltd, which collaborative venture holds relevant patents and provided peptides gratis. While fellow authors are unremunerated by the company, K.G.D., E.D. and R.P.N. hold share options in Endocrine and R.P.N. is chairman of Endocrine's Scientific Advisory Board.

Author contributions

K.G.D. conducted the worm studies and was joint principal author. B.Z. conducted the fish study. E.D. pursued the receptor, in conjunction with R.P.N., who also did the molecular modelling and contributed to the writing. J.E.H. conceived and co-ordinated the project and was joint principal author. Protocol design was a collective exercise.

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References

- Baumeister, R., Schaffitzel, E. and Hertweck, M. (2006). Endocrine signaling in *Caenorhabditis elegans* controls stress response and longevity. *J. Endocrinol.* **190**, 191–202.
- Davies, K. G. and Hart, J. E. (2008). Fecundity and lifespan manipulations in *Caenorhabditis elegans* using exogenous peptides. *Nematology* **10**, 103–112.
- Ding, S., Bond, A. E., Lemi re, F., Tuytten, R., Esmans, E. L., Brenton, A. G., Dudley, E. and Newton, R. P. (2008). Online immobilized metal affinity chromatography/mass spectrometric analysis of changes elicited by cCMP in the murine brain phosphoproteome. *Rapid Commun. Mass Spectrom.* **22**, 4129–4138.
- Evans, J. P. and Magurran, A. E. (2000). Multiple benefits of multiple mating in guppies. *Proc. Natl. Acad. Sci. USA* **97**, 10074–10076.
- Froese, R. (2006). Cube law, condition factor and weight–length relationships: history, meta-analysis and recommendations. *J. Appl. Ichthyol.* **22**, 241–253.
- Hart, J. E. (2014). The body has a brake: micrin is a postulated new gonadal hormone curbing tissue overgrowth and restricting reproduction. *Med. Hypotheses* **83**, 775–786.
- Haylor, J. L., Parker, E., Risbridger, G. P., Beale, D., Brown, B. L., Dobson, P. R. M., Clarke, I. J. and Hart, J. E. (2009). Inhibition of compensatory renal growth by the N-terminus of a sheep-derived peptide. *Regul. Pept.* **152**, 48–53.
- Lagler, K. F., Bardach, J. E., Miller, R. R. and Passino, D. R. M. (1977). *Ichthyology*, pp. 342–343. New York: John Wiley & Sons. Inc.
- Lokman, P. M., George, K. A. N., Divers, S. L., Algie, M. and Young, G. (2007). 11-Ketotestosterone and IGF-I increase the size of previtellogenic oocytes from shortfinned eel, *Anguilla australis*, in vitro. *Reproduction* **133**, 955–967.
- Moe, M. A. (1992). *The Marine Aquarium Handbook – Beginner to Breeder*, pp. 75–81. Islamorada, FL: Green Turtle Publications.
- Nelson, J. S. (1994). *Fishes of the World*, p. 600. New York: John Wiley & Sons, Inc.
- Reznick, D. (1983). The structure of guppy life histories: the tradeoff between growth and reproduction. *Ecology* **64**, 862–873.
- Rife, J. C., Miller, M. M., Sheehan, P. E., Tamanaha, C. R., Tondra, M. and Whitman, L. J. (2003). Design and performance of GMR sensors for the detection of magnetic microbeads in biosensors. *Sensors Actuat. A Phys.* **107**, 209–218.
- S elo, I., N egroni, L., Cr eminon, C., Grassi, J. and Wal, J. M. (1996). Preferential labeling of α -amino N-terminal groups in peptides by biotin: application to the detection of specific anti-peptide antibodies by enzyme immunoassays. *J. Immunol. Methods* **199**, 127–138.
- Sheehy, B. A., Ho, N. F. H., Burton, P. S., Day, J. S., Geary, T. G. and Thompson, D. P. (2000). Transport of model peptides across *Ascaris suum* cuticle. *Mol. Biochem. Parasitol.* **105**, 39–49.
- Sokolowska, M., Peter, R. E., Nahorniak, C. S., Pan, C. H., Chang, J. P., Crim, L. W. and Weil, C. (1984). Induction of ovulation in goldfish, *Carassius auratus*, by pimozone and analogues of LH-RH. *Aquaculture* **36**, 71–83.
- Sulston, J. and Hodgkin, J. (1988). Methods. In *The Nematode Caenorhabditis elegans* (ed. W.B. Wood and the Community of C. elegans Researchers), pp. 587–606. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- Wischnath, L. (1993). *Atlas of Livebearers of the World*, p. 336. Neptune City, NJ: TFH Publications.
- Witchel, S. F. and Plant, T. M. (2013). Puberty: gonadarche and adrenarche. In *Yen & Jaffe's Reproductive Endocrinology: Physiology, Pathophysiology, and Clinical Management*, 7th edn (ed. J. F. Strauss and R.L. Barbieri). Philadelphia: Elsevier Saunders.