Harnessing Microbial Tools: *Escherichia coli* as a Vehicle for Neuropeptide Functional Analysis in *Caenorhabditis elegans*

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Testing neuropeptide function by feeding *E. coli* to *C. elegans*

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Caenorhabditis elegans, neuropeptides, behavioral rescue, neuropeptide receptor

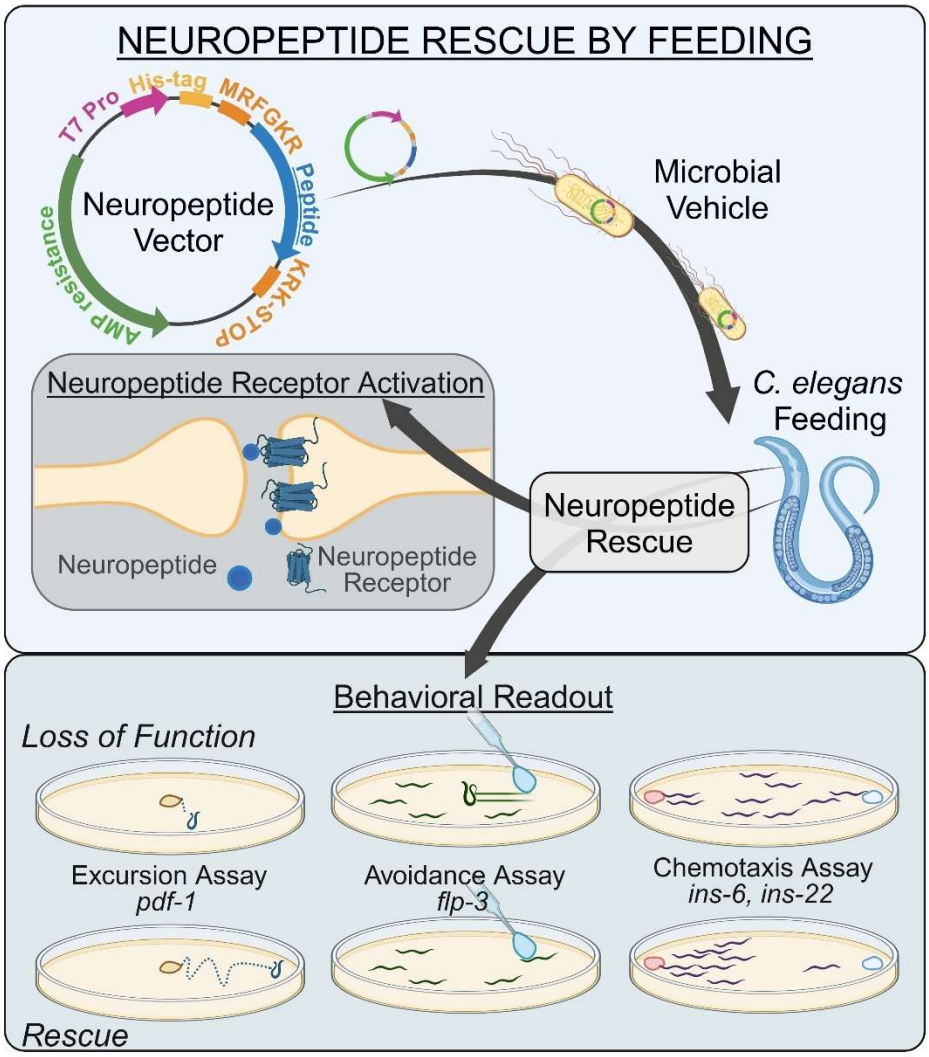
Abstract

Animals respond to changes in their environment and internal states via neuromodulation. Neuropeptides modulate neural circuits with flexibility because one gene can produce either multiple copies of the same neuropeptide or different neuropeptides. However, with this architectural complexity, the function of discrete and active neuropeptides is muddled. Here, we design a genetic tool that facilitates functional analysis of individual peptides. We engineered *Escherichia coli* bacteria to express active peptides, fed loss-of-function *Caenorhabditis elegans*, and rescued the activity of genes with varying lengths and functions: *pdf-1, flp-3, ins-6*, and *ins-22*. Some peptides were functionally redundant, while others exhibit unique and previously uncharacterized functions. The mechanism of peptide delivery is reminiscent of mRNA uptake observed in RNA interference, suggesting convergent mechanisms of gene regulation in organisms. We postulate our rescue-by-feeding approach can elucidate the functional landscape of neuropeptides, identifying the circuits and complex peptidergic pathways that regulate different behavioral and physiological processes.

Article summary

Studying individual neuropeptides opens new avenues for exploring neuromodulation at a finer resolution. The researchers developed a method to create DNA vectors that encode an endogenous peptide sequence with enzyme-binding sites in *Caenorhabditis elegans*. The researchers transformed these vectors into bacteria and fed them to *C. elegans*, which restored wild-type behavior in neuropeptide loss-of-function mutants. The researchers also discovered that neuropeptides from the same gene perform distinct functions, opening a new research area.

41 **Graphical Abstract**



42

43 **Introduction**

44 The modulation of neural circuits is essential for life, as appropriate responses to
45 environmental stimuli depend on precise neural tuning. Neuropeptides, which are
46 relatively short amino acid chains, play a crucial role in this process by regulating neural
47 circuits while also functioning as neurotransmitters or neurohormones [1, 2]. They are
48 widely used across the animal kingdom, even in organisms that possess only secretory
49 cells without neurons [3]. Neuropeptides can also have conserved structure across
50 species while serving a variety of functions. Oxytocin-like and vasopressin-like peptides

are neurohormones that regulate social attachment, lactation, and blood pressure by contracting muscles in mammals, dating back 600-700 million years [4-9]. However, in the Echinoderm sea star, *Asterias rubens*, vasopressin-like and oxytocin-like neuropeptide ortholog asterotocin serves instead to in relax muscles in the cardiac stomach during fictive feeding [9]. The *Caenorhabditis elegans* vasopressin ortholog, nematocin, interacts with serotonin and dopamine signaling to modulate gustatory associative memory and male mating behaviors [4, 10].

Neuropeptides act in conjunction with neurotransmitters, typically binding to G-protein coupled receptors, where they modulate synaptic activity more slowly and over a longer duration than neurotransmitters alone [2, 11]. Multiple modulators allow neurons to serve unique functions within discrete neural circuits [12]. Beyond the nervous system, neuropeptides also regulate immune and endocrine functions [13, 14]. These small proteins, typically less than 50 amino acids long, modulate neurotransmitters activity intracellularly and act as neurohormones extracellularly [1, 2, 11].

Despite their biological significance, neuropeptides are largely inaccessible for study due to their complexity. In humans, some studies report 90 neuropeptide precursor genes while others have reported 131, yet all studies agree that there are likely more neuropeptides in the 900 other peptide genes[15-18]. Post-transcriptional modifications can give rise to an even greater number of functional neuropeptides numbering into the 1000s [15-19]. This complexity is even seen in a “simpler” model organism, the round worm *Caenorhabditis elegans*. *C. elegans* are a microscopic nematode that displays robust behaviors driven by just over 300 neurons [20, 21]. The *C. elegans* genome encodes three classes of neuropeptides: FMRFamide-like peptides (FLP), insulin-like peptides (INS), and non-FLP/insulin neuropeptide-like peptides (NLP) [22-24], encoding over 300 individual neuropeptides through 160 identified genes that modulate the functional connectome [1, 19, 25, 26]. The complexity of the neuropeptide genome, combined with extra-synaptic neuropeptide signaling, makes elucidating the role of individual peptides difficult, as canonical studies often rely on null mutations and transgenic rescues [27-30]. A major limitation to neuropeptide research is the lack of

technology capable of precisely dissecting the function of individual peptides in a high-throughput manner. As such, these studies are often incomplete, as full gene rescue restores complete preproproteins and makes discrimination of discrete peptide function impossible [1, 31]. Here, we present a strategy that rescues neuropeptides synthesized endogenously within the worm genome via engineered bacteria expressing individual peptides.

Development of Rescue-by-Feeding Paradigm

Current methods of genetic rescue in *Caenorhabditis elegans* are not ideal for understanding the function of individual peptides; whether by cost inhibition or equipment limitations [32, 33], or due to whole gene rescue not being ideal for studying discrete peptides [34-37]. Traditional transgenic worm strain generation [38] often does not allow for single neuropeptide resolution since a precursor gene can make multiple active peptides, is cumbersome for generating mutants deficient in each neuropeptide, and requires expertise in microinjections [33]. Previous approaches to study individual endogenous neuropeptide function involved using synthetic peptides applied by soaking the nervous system of the worm [39] or injecting the peptide into the worm [40]. The use of synthetic peptides can be costly as pure peptide must by synthesized, often outsourced, for every neuropeptide experiment. Interestingly, feeding of peptides via *E. coli* has proven successful in manipulating *C. elegans* biological function. Previous researchers found that feeding the scorpion venom protein, mBmKTX, expressed in *E. coli* to *C. elegans* modulated lifespan and egg laying behaviors [41]. However, this type of *E. coli* feeding of peptides has not been accomplished with endogenous neuropeptides.

Here we report an approach that allows us to manipulate neuropeptide activity by feeding, enabling the delivery of individual neuropeptides to large populations of animals simultaneously. Our gain-of-function approach of feeding bacteria containing the neuropeptide macromolecule provides ease of application and cost saving measures to deliver endogenous peptide to *C. elegans* [41]. Once the neuropeptide vector is assembled and transformed into *E. coli*, delivery of the peptide is simply requires feeding

108 the worms. This method allows for temporal control of peptide delivery, animals can be
109 fed at any life stage, and the number of animals treated is readily flexible.

110 Neuropeptide rescue-by feeding, outlined here, expands on RNA interference
111 (RNAi) feeding paradigms, which have been successfully used to test gene function [42,
112 43], wherein a plasmid encoding the RNA of interest is driven by isopropyl-β-D-
113 thiogalactoside (IPTG) -induction. Wherein RNAi employs paired T7 promoters facing one
114 another to produce double-stranded RNA, our protocol uses one T7 promoter to generate
115 mRNA encoding the peptide of interest. The use of Gateway Cloning to develop
116 expression vectors allows for the development of high-throughput experiments targeting
117 individual peptides, or combinations thereof [44, 45]. RNA produced by bacteria in the gut
118 microbiota can be transported across the intestinal epithelium [46, 47] and our vector for
119 neuropeptide delivery is modeled after RNA interference (RNAi) feeding [34, 47-49].
120 Previous research delivering exogenous scorpion venom peptide expressed the peptide
121 information was expressed in a pDest-527 vector transformed into *E. coli* designed to
122 produce peptide, Rosetta BL21 DE3 derivative [41]. We have selected this pDest-527
123 vector for most of our studies. Additionally, this technique is readily accessible compared
124 to traditional transgenic rescue approaches.

125 The paradigm rescues individual, processed neuropeptides, leveraging the genetic
126 amenability of the *C. elegans* food source, *Escherichia coli*, to circumvent the need for
127 transgenic development and enable high-throughput rescue and elucidation of individual
128 neuropeptide function [50]. We demonstrate the application of this paradigm by rescuing
129 behaviors driven by neuropeptides synthesized from *pdf-1*, *ins-6*, and *ins-22*, and expand
130 upon the previously established behaviors of *flp-3* [49].

131 **Materials and Methods**

132 <u>*C. elegans* strains</u>

133 *C. elegans* strains were maintained on nematode-growth media (NGM: 51 mM NaCl, 10
134 mM peptone, 51 mM agar, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 25 mM KPO_4 (pH 6.0), 13 μM

135 Cholesterol) plates seeded with *E. coli* OP50 at 15 – 20°C, according to standard
136 procedures [51]. Strains used are listed in **Table S1**. For *pdf-1* (UR954) the control strain
137 was *him-5* (CB4088), for *flp-3* (JSR99) the control strain was *him-8* (CB1489), and for *ins-*
138 *6* (IV302) and *ins-22* (RB2594), the control strain was wildtype (N2).

139 JSR188 was generated by mating UR954 and UR930 animals. The cross was
140 monitored using validated primers from Wormbase.org; *pdf-1(tm1996)* external left
141 (CCGTGGAGGTACCAGGTTAA), *pdf-1(tm1996)* external right
142 (TCAAGGTCTCGCCTCTAGGA), *pdfr-1(ok3425)* external left
143 (CGTGGAATCATCGCTACCTT), and *pdfr-1(ok3425)* external right
144 (TTTATGCAGGCTTATTGCCC). The final generated strain was confirmed by sequencing
145 using the primers *pdf-1(tm1996)* internal left (CCATTCCGATGAGTCCGTTG) and *pdfr-*
146 *1(ok3425)* internal left (TTTACTCCTTGACGGGAACG).

147 Neuropeptide plasmid design and feeding

148 *Peptide plasmid design* and *generation*

149 DNA sequences encoding individual peptides were identified via www.wormbase.org.
150 Sequences were flanked with the endogenous cleavage sites for the EGL-3 processing
151 enzyme, which cleaves dibasic resides. Sequences encoding MRFGKR and KRK-STOP
152 codons were therefore placed prior to, and following the peptide codon sequences,
153 respectively. Finally, Gateway Cloning sites attB1 and attB2 sites were attached to the
154 ends of the sequences. These final sequences (comprised of
155 attB1::MRFGKR::peptide::KRK-STOP::attB2) were ordered from Integrated DNA
156 Technologies (IDT) using their DNA Oligo and Ultramer DNA Oligo services, depending
157 on the size of the oligo ordered. Both forward and reverse sequences were ordered (**Table**
158 **S2**).

159 Lyophilized oligos were prepared following IDT Annealing Oligonucleotides
160 Protocol (https://www.idtdna.com/pages/education/decoded/article/annealing-
161 oligonucleotides). In short, they were resuspended in Duplex Buffer (100 mM Potassium
162 Acetate; 30 mM HEPES, pH 7.5; available from IDT), preheated to 94 °C to a final

163 concentration of 40 μM. Complimentary oligo sequences were then mixed in equimolar
164 ratios, and placed in a thermocycler at 94 °C for 2 minutes, prior to a stepwise cooling to
165 room temperature.

166 Annealed oligos were used to perform a BP reaction with pDONR p1-2 donor
167 vector to generate pENTRY clones (Gateway Cloning, ThermoFisher). Entry clones were
168 then recombined with pDEST-527 (a gift from Dominic Esposito (Addgene plasmid #
169 11518)) in LR reactions generating expression clones. The scramble control was
170 generated in an identical manner, with the sequence between the cleavage sites being
171 amplified from pL4440 (provided by Victor Ambros, University of Massachusetts Medical
172 School, MA) (**Table S2**). Purified plasmids were stored at -20 °C or -80 °C for long term
173 storage.

174 For the *ins-22* experiments, the DNA coding sequence for the peptide was
175 identified as described above, without the Gateway Cloning sites. These sequences were
176 codon-optimized and cloned in pET-21a(+) cloning vector via XhoI/XhoI strategy by
177 GenScript . The SCRAMBLE control sequence and plasmid were generated using the
178 same cloning strategy by GenScript. Plasmids arrived lyophilized and were re-suspended
179 in nuclease-free H_2O to a concentration of 100 ng/μL and stored at -20 °C.

180 The plasmid maps displayed in **Figure S2** were rendered by SnapGene® software
181 (from Dotmatics; available at snapgene.com).

182 *Peptide feeding bacterial preparation*

183 The expression clone for both the peptide of interest and the control was transformed into
184 competent DH5α cells (NEB® [New England Biolabs] 5-alpha Competent *E. coli* (High
185 Efficiency) Cat# C2987I). Peptide-expressing cultures on LB agar plates (10 g/L NaCl, 10
186 g/L Tryptone, 5 g/L Yeast Extract, 5 g/L Agar) containing 100 μg/mL ampicillin (AMP), were
187 stored at 4 °C for up to 2 months. From this plate, single colonies were selected for growth
188 in 5 mL of LB media containing ampicillin at 37 °C for 16 hours. The optical density of the
189 grown cultures (OD_{600}) was adjusted to 1.0. 75 μL of 1.0 OD_{600} peptide-expressing culture
190 was plated onto 60 mm NGM agar plates containing 100 μg/mL AMP and 1 mM isopropyl-

191 β-D-thiogalactoside (IPTG) at room temperature for at least 8 hours prior to use, for no
192 longer than 1 week.

193 Competent iOP50 (*E. coli*, rnc14::(delta)Tn10, lacz(gamma)A::T7pol camFRT)
194 obtained from *Caenorhabditis* Genetics Center was created using previously described
195 methods [52]. Briefly, iOP50 stocks were streaked out onto plates containing 50 µg/mL
196 tetracycline and LB and grown at 37 °C overnight. Then, single colonies of iOP50 were
197 isolated and used to inoculate 2.0 mL LB containing 50 µg/mL tetracycline and placed in
198 a 37 °C shaker overnight. 0.5 mL of each culture was used to inoculate 3.0 mL of LB
199 without antibiotics and grown for 2 hours in a 37 °C shaker. Each culture was then
200 aliquoted in 0.75 mL increments into four 1.5 mL tubes and cooled for 10 minutes on ice.
201 Next, the cells were harvested by centrifugation at 6,000 rpm for 5 minutes at 4 °C. The
202 resulting supernatant was discarded, and cells were placed back on ice and re-suspended
203 in 1.0 mL 100 mM ice-cold $CaCl_2$ solution. Cells were kept on ice for 20 minutes and then
204 harvested by centrifugation as in the previous step. After discarding the supernatant, cells
205 were re-suspended in 150 µL of ice-cold $CaCl_2$ solution and placed on ice to be
206 immediately used for transformation.

207 Expression clones were transformed into competent iOP50 cultures using the New
208 England Biolabs protocol (C2987H/C2987I). Single colonies of transformed cells were
209 isolated and cultured in 3.0 mL of LB containing 50 µg/mL tetracycline (TET) and 17 µg/mL
210 chloramphenicol (CAM) overnight in a 37 °C shaker. Cultures were frozen down as
211 glycerol stocks and stored at -80 °C. Successful transformation was verified by isolating
212 plasmids from cultures using a Zyppy Plasmid Miniprep Kit (Zymo Research #D4019) and
213 sending samples for whole-plasmid sequencing.

214 For plating the bacteria containing *pdf-1* and *flp-3*, LB containing 50 µg/mL AMP,
215 50 µg/mL TET, and 17 µg/mL CAM was inoculated with iOP50 expressing either the
216 peptide of interest or the control peptide and cultured for 16-20 hours in a 37 °C shaker.
217 75 µL of peptide was plated onto 60 mm NGM plates containing 50 µg/mL AMP, 50 µg/mL
218 TET, and 17 µg/mL CAM, and 1 mM IPTG. For all peptides, seeded plates were left to dry

219 at least 8 hours at room temperature before plating worms and were used no longer than
220 1 week after plating.

221 For *ins-22,* LB containing 50 µg/mL tetracycline and 50 µg/mL carbenicillin was
222 inoculated with iOP50 expressing either the peptide of interest or the control peptide and
223 cultured for 18-20 hours in a 37 °C shaker. 100 mm NGM plates containing 50 µg/mL
224 tetracycline, 50 µg/mL carbenicillin, and 1 mM IPTG were plated with 1.0 mL of
225 corresponding culture. Seeded plates were left to dry at least 24 hours at room
226 temperature and never kept longer than 72 hours before use. Approximately one hour
227 before transferring worms to peptide or scramble control plates, 200 µL of 0.1 M IPTG
228 was added to each plate and left to dry.

229 *Peptide Feeding*

230 All animals were maintained on bacterial lawns of OP50 *E. coli*, at 20 °C, on NGM agar
231 plates until the start of experiments. Mutant or control animals were transferred onto lawns
232 of DH5α or iOP50 *E. coli*. Animals were reared on the peptide-expressing lawns for at
233 least 48 hours at 20 °C before assaying for rescue of mutant phenotype at the appropriate
234 developmental stage. See each section of the behavioral assay methods for the exact
235 peptide feeding parameters.

236 Behavioral Assays

237 *Excursion Assay (pdf-1)*

238 The mate searching behavior of *pdf-1* mutants was quantified in a food leaving assay. *him-*
239 *5* animals were used as wild-type control to ensure the presence of males. *him-5* and *pdf-*
240 *1* animals were fed OP50, scramble peptide, PDF-1A, PDF-1B, or a 1:1 mixture of PDF-
241 1A and PDF-1B cultures. Four to six larval L4 hermaphrodites were passed onto peptide
242 containing plates. These worms were grown at 20 °C for about 4 days until the male
243 progeny of the initial L4 worms were young adults. The day prior to the assay, young adult
244 male *pdf-1* or *him-5* worms were singled onto 60 mm plates containing lawns of either
245 OP50 or appropriate peptide cultures.

246 To determine the mate searching effects of *pdf-1*, we performed a food-leaving
247 assay as described previously [53-55], with modifications. In this assay, individuals were
248 placed on a small food spot, and track patterns were scored at the indicated times. Assay
249 plates were prepared one day prior to assaying on 100 mm plates with 10 mL of Leaving
250 Assay media (25 mM KPO_4 (pH 6.0), 1 mM $MgSO_4$, 1 mM $CaCl_2$, 50 mM NaCl, 2.5 g/L
251 Peptone, 17 g/L Agar). On center of plate, 7 µL of OP50 was added. In assays using iOP50
252 assay plates, 7 µL of iOP50 containing the appropriate peptide (ex. SCRAMBLE iOP50
253 assay plates made for animals fed SCRAMBLE iOP50) was added. Plates were lightly
254 covered and stored at room temperature overnight (~20^{0}C, < 40% humidity).

255 On the day of the assay, a single male was plated on the assay plate and placed in
256 a dark, 20°C incubator. At 2, 6, and 24 hours after the worms are plated, plates were
257 scored for male worm tracks. Scored were separated into binned ranges [53]. "Never left
258 food" indicates the absence of tracks outside the food spot. "Minor excursion" indicates
259 that tracks were observed not beyond 10 mm from the food. "Major excursion" indicates
260 the presence of tracks past the 10 mm boundary. We quantified the food leaving behavior
261 at three different time points (2, 6, and 24 hours) [54].

262 We additionally quantified the mate searching defect of *pdf-1* as a Probability of
263 Leaving per hour (P_L/hr) [55]. In this method, we reduce the analysis of the leaving
264 behavior to a single value, by scoring the worms leaving the food lawn as Leavers
265 (traveling beyond 35 mm from the food source) or Non-Leavers (did not travel further
266 than 35 mm from center of food). Worm in the > 35 mm from the center of food bin were
267 scored as "Leavers" [55]. The probability of leaving per hour (Probability of Leaving (P_L))
268 was calculated using an R script developed in [54], first cited in [55], with assistance by
269 (Personal Communications to Barrios, 2020). P_L was calculated by the "hazard obtained
270 by fitting an exponential parametric survival model to the censored data using maximum
271 likelihood" [55].

272 *Avoidance Assay (flp-3)*

273 Avoidance drop assays were performed as described previously [49, 56]. Fifty to sixty L4
274 male worms were segregated by sex and stored at 20 °C for at least 5 hours to overnight
275 to be assayed as young adults. One to four hours prior to the assay, the lids of unseeded
276 plates were tilted to allow any excess moisture to evaporate off the plates. At the time of
277 the assay, 10 or more animals were transferred onto each of the dried, unseeded NGM
278 plates. A drop of either water or 1 μM ascr#8 (a nonvolatile *C. elegans* mating odor) was
279 placed on the tail of forward moving animals, and their response was scored as either an
280 avoidance response, or no response. The avoidance index was calculated as:

281 $$\text{Avoidance Index} = \frac{(\#\ of\ worms\ avoiding\ drop)}{total\ \#\ of\ worms\ tested}$$

282 *Chemotaxis Assay (ins-6)*

283 A salt chemotaxis assay was performed as previously described in [57]·[58], to test the
284 functionality of *ins-6* after rescue-by-feeding. Wild-type (N2) and *ins-6* worms were fed
285 either scramble or INS-6 peptide passing 4-6 L4 larval onto an NGM plate containing 100
286 μg/μL AMP and 1 mM IPTG with 75 μL of peptide OD_{600} = 1.0. These worms were grown
287 at 20 °C for about 4 days until the progeny of the initial L4 worms were young adults.

288 The day prior to testing, a thin, 10 mL layer of agar was added to 100 mm petri
289 dishes to generate a 100 mm Chemotaxis Plates (5 mM KPO_4 (pH 6.0), 1 μM $MgSO_4$, 1
290 μM $CaCl_2$, 20 g/L agar, and 8 g/L Difco Nutrient Broth). To prepare the "high salt" plates,
291 750 mM NaCl was added prior to plate pouring. The high salt plate were stored at 4 °C for
292 up to one month, while the Chemotaxis Plates were made one day prior to testing. To
293 create the high and low salt plugs, the back end of a Pasteur pipette was used to punch a
294 5 mm plug out of each plate. These 2 plugs were placed on opposite edges of the plate.
295 A 10 mm radius was be drawn around the location of the plugs. Plates were stored, lightly
296 covered, overnight at room temperature (~20 °C, < 40% humidity).

297 On the day of the assay, young adult worms, either control worms fed OP50 *E. coli*
298 or peptide-fed worms, were washed off peptide plates with M9. Worms were allowed to

settle by gravity for 4 minutes before removing the supernatant, washing the worms. Worms were then washed with Chemotaxis Buffer (5 mM KPO_4 (pH 6.0), 1 mM $MgSO_4$, 1 mM $CaCl_2$) three times.

To prepare the Chemotaxis plates for the assay, high and low salt plugs were removed with forceps, and the plates spotted with 1 µL of 0.5 M sodium azide (NaN_3). Approximately 30 µL of worms in Chemotaxis Buffer were spotted onto the edge of the agar, between the location of the high and low salt gradient. Worms were left to chemotax for 1 hour before scoring the number of worms within the 10 mm radii of high and low salt areas using the below choice index:

$$\text{Choice Index} = \frac{(\#\ of\ worms\ high\ salt\quad \#\ of\ worms\ low\ salt)}{total\ \#\ of\ worms\ on\ both\ spots}$$

Olfactory Chemotaxis Assay (ins-22)

Chemotaxis assays were performed using worms that were transferred at the L4 stage to plates seeded with iOP50 containing a plasmid expressing either a SCRAMBLE control sequence or the peptide of interest sequence. After 48 hours of exposure, we tested the naïve chemosensory preference of Day 2 Adults using a protocol based on previously published assays [56]. Briefly, assays were performed on unseeded 100mm NGMs. On the back of each plate, two marks were made on opposite sides of the plate, approximately 5mm from the edge. 1 µL of sodium azide (Thermo Fisher) was placed on both spots and allowed to dry before adding 1 µL of 0.1% or 10% butanone (Sigma Aldrich) diluted in ethanol on one mark and ethanol on the other. Using M9 buffer, worms were washed off their plates and subsequently washed three times to eliminate any leftover bacteria that could impact baseline behavior. Then, worms were placed near the bottom center of the plate, equidistant between the two marks, and allowed to chemotax for an hour. Chemotaxis indices were calculated as follows:

$$\text{Chemotaxis Index} = \frac{(\#\ of\ worms\ butanone - \#\ of\ worms\ ethanol)}{total\ \#\ of\ worms}$$

324 Statistical Analysis

325 *Power analysis*

326 Prior to the study, a post-hoc power analysis (ClinCalc LLC., Chicago, IL, USA) was
327 performed using preliminary data from OP50 *E. coli* fed worms to determine the number
328 of samples required to establish a difference between the wildtype controls and the
329 neuropeptide loss of function mutants. This was performed using a 0.05 alpha rate.

330 For the excursion assay comparing *pdf-1 lof* animals in a *him-5* background
331 compared to *him-5* males, a dichotomous power analysis was used to compare non-
332 leavers (< 35 mm) to leavers (35 mm <). n = 39 (where n = 1 is one male on one assay
333 plate) was required for 100% powered experiment and 13 for power > 80%. A sample size
334 of n = 50 samples per genotype and feeding condition was selected.

335 For avoidance behavior towards ascr#8 comparing wildtype control *him-8* animals
336 to *flp-3 lof* males in a *him-8* background, n = 3 samples (n = 1 is one plate of ten worms)
337 was appropriate to have a 100% powered test, using a continuous power test. A sample
338 size of N = 10 was selected. Points plotted on graphs represent n = 1.

339 For the salt chemotaxis assay with *ins-6 lof* animals, n = 4 samples (n = 1 is one
340 plate of 100-200 worms) were required for power > 80% and 13 for 100% power. A sample
341 size of n = 10 was selected. Points plotted on graphs represent n = 1.

342 For chemotaxis behavior of *ins-22* animals, the behavior of SCRAMBLE fed iOP50
343 animals towards 10% butanone was used to determine that 20 samples (n = 1 is one plate
344 of 100-200 worms) were required for 97.5% power and 11 samples were required for
345 power > 80%. A sample size of n > 15 was selected. Points plotted on graphs represent n
346 = 1.

347 *Reproducibility*

348 In all experiments, the investigators were blinded to genotype. Young adult animals were
349 tested in all behavioral assays. For the behavioral tests of *flp-3* and *ins-6*, the assay was
350 repeated over at least 3 days, with no more than 3 replicates in a day, until at least 10

351 plates have been tested. For *ins-22*, the assays included testing 5 plates per day, for a total
352 of three biological replicates (n = 15). For *pdf-1, t*he assay was performed across at least
353 two days per condition, with at least n = 50, wherein each assay plate with one male is
354 equal to n = 1. When possible, the results of an individual n are displayed on the graphs.

355 *Statistical Comparison*

356 Statistical analysis was performed in GraphPad Prism (version 10.4.1). Prior to any
357 statistical comparison, the normality and frequency distribution was assessed for each of
358 the groups. If these tests showed normal distributions, a Student's unpaired two-tailed t-
359 test followed by Bonferroni's correction was applied; this was used for *ins-6* and *ins-22*
360 results. If the data was non-parametric, as was the case with most of the *flp-3* data, a
361 Mann-Whitney test was performed followed by Bonferroni's correction. For the *pdf-1* tests
362 where animals were fed different types of peptides, a One-way ANOVA with Dunnett's
363 post-hoc test was used to compare within genotype results. For all graphs, the average
364 was plotted with the standard error of the mean (SEM).

365 **Results and Discussion**

366 <u>Bacterial Delivery of Neuropeptide Vectors Rescues Behavior in Loss of Function Mutants</u>

367 To demonstrate the efficacy of the neuropeptide delivery by bacterial feeding, we tested
368 the behavior of loss of function (*lof*) animals from each of the three neuropeptide classes
369 in *C. elegans*. Each of these behavioral assays was paired with the appropriate wildtype
370 control. For male animal behavior, *him-5* or *him-8* lines were used to produce a higher
371 incidence of males (him) than the wildtype population of 0.1% males [59, 60]. The choice
372 of which *him* mutant to use depended on the neuropeptide being tested.

373 *Male Excursion Behavior (pdf-1)*

374 Wild-type males display a characteristic exploratory behavior when left on a lawn of food,
375 as well-fed males leave food in search of mates [54, 55]. The pigment dispersing factor
376 *pdf-1* (also known as *nlp-74*) neuropeptide plays a significant role in male mate-searching
377 behavior, balancing the neural circuits controlling two predominant male interests, finding

378 food and finding mates [54, 61]. The *pdf-1* precursor encodes two neuropeptides: PDF-
379 1A (SNAELINGLIGMDLGKLSAVG-NH_2) and PDF-1B (SNAELINGLLSMNLNKLSGAG-
380 NH_2) [62, 63]. *pdf-1 lof* males did not leave food as readily as wild-type worms, suggesting
381 that these worms do not display exploratory behavior, as previously described (**Figure**
382 **S1A, B**) [54]. When fed either DH5α *E. coli* containing individual PDF-1 peptide (or a 1:1
383 combination of both), *pdf-1 lof* males had a higher P_L compared to SCRAMBLE fed *pdf-1*
384 *lof* males (**Figure 1A, S1C**). However, PDF-1B and a 1:1 ratio of both peptides resulted in
385 a significantly different P_L from *pdf-1* males fed PDF-1A alone. While the P_L of peptide-fed
386 males were significantly higher than *pdf-1 lof* males fed scramble, they were still
387 significantly lower than wild-type males fed scramble peptide (**Figure 1A**).

388 *Pheromone Avoidance (flp-3)*

389 *C. elegans* produce and release pheromones as a means of chemical communication to
390 signal a variety of features to other animals, such as food or mate presence [64]. One
391 such pheromone ascaroside #8 (ascr#8) [65]. We have previously worked to characterize
392 the behavior of animals towards this hermaphrodite produced pheromone and have found
393 that wildtype hermaphrodites avoid ascr#8 while males are attracted to it [49]. We have
394 further identified that males deficient in the neuropeptide gene *flp-3* lose their attraction
395 towards ascr#8 and start to avoid the cue (**Figure S1D**) [49]. The *flp-3* neuropeptide
396 precursor creates 10 different neuropeptides, yet only 2 are involved in the male behavior
397 towards ascr#8 [49]. Both the second and the ninth peptide (FLP-3-2 and FLP-3-9)
398 produced by *flp-3* are involved with suppressing the basal avoidance behavior seen in *flp-*
399 *3 lof* males, yet only FLP-3-9 is necessary for restoring the attraction towards ascr#8
400 [49{Salama, 2023 #963]}. When we fed male *flp-3 lof* animals DH5α *E. coli* expressing
401 FLP-3-9 (NPENDTPFGTMRFG-NH_2), we saw a significant suppression of avoidance
402 behavior towards ascr#8 (**Figure 1B**).

403 *Salt Chemotaxis (ins-6)*

404 Insulin and insulin-like peptides serve signaling functions in *Drosophila* and *C. elegans*
405 homologous to the human insulin-like growth factor (IGF), which regulates FOXO activity

406 [66]. In *C. elegans, ins-6* encodes only one processed INS-6 peptide
407 (VPAPGETRACGRKLISLVMAVCGDLCNPQEGKDIATECCGNQCSDDYIRSACCP-NH_2)
408 [67, 68], though the gene was originally postulated to encode two putative proteins [1, 23].
409 The formation of a single longer peptide is common in the insulin-like class of peptides
410 [38]. *ins-6* functions in dauer formation [38, 67] and sensory modulation of large
411 fluctuations in salt concentration, with loss of *ins-6* causing dysfunction of NaCl attraction
412 [69]. While wildtype hermaphrodite worms were attracted to high concentrations of salt
413 (750 mM), there was a significant decrease in attraction in *ins-6* mutant animals (**Figure**
414 **S1E**) [69]. Wildtype and *ins-6 lof* animals fed SCRAMBLE peptide also had significantly
415 different levels of salt chemotaxis (**Figure 1C**). Feeding of DH5α *E. coli* expressing INS-6
416 peptide to *ins-6 lof* animals resulted in a complete restoration of attraction to high salt
417 concentration (**Figure 1C**).

418 **Different *E. coli* food sources lead to Neuropeptide Behavior Rescue**

419 While the previous experiments in this study have used DH5α *E. coli* expressing peptides
420 to shape neuropeptide related behaviors, there are cases when using different *E. coli*
421 strains may be desirable. Given the differences in nutrient composition of the different *E.*
422 *coli* strains and worms preferring more nutritious bacteria [70], expressing peptides in
423 HB101 or HB115 strains offers viable alternatives. To most closely compare to other
424 laboratory studies, the ability to use the widely used uracil deficient OP50 *E. coli* strain as
425 the neuropeptide delivery system is desirable [51]. Recently an IPTG inducible strain of
426 OP50 has been created, iOP50, which can be cultured to create chemically competent
427 cells [52]. All constructs presented in this section were transformed into iOP50 before
428 being fed to the *C. elegans*.

429 When testing male *pdf-1* associated leaving behavior, we first feed the animals on
430 iOP50 peptide plates before transferring them to an OP50 assay plate that contains no
431 peptide. Under these conditions, male *pdf-1 lof* animals fed PDF-1 peptides from iOP50
432 left food at a higher rate than *pdf-1 lof* animals fed a SCRAMBLE peptide from iOP50
433 (**Figure 2A**). However, the extent of food leaving by males after being fed iOP50 with
434 peptide was lower than observed when animals were fed DH5α *E. coli* (**Figure 1A**). This

435 may be because animals are tested over the course of 24 hours and the level of peptide
436 in their bodies may decrease over time. To address this, we tested animals fed iOP50 with
437 peptide and then transferred them to iOP50 assay plates seeded with the same peptide
438 they were previously fed (**Figure 2B**). By doing this, the trend of *pdf-1 lof* animals fed PDF-
439 1 leaving food at a higher rate continued, and the overall rates of leaving food also
440 increased (**Figure 2B**).

441 *flp-3 lof* males exposed to ascr#8 after being fed FLP-3-9 in iOP50 lacked
442 avoidance to the cue while males fed the SCRAMBLE peptide continued to avoid it
443 (**Figure 2C**). These results matched the observed behavior of animals fed FLP-3-9 in
444 DH5α *E. coli* (**Figure 1B**). For *flp-3*, males are transferred directly from iOP50 peptide
445 plate onto an unseeded NGM assay plate for no more than 30 minutes during the testing.
446 Due to this, it was not necessary to reapply the iOP50 peptide during testing.

447 Additionally, we tested a more streamlined construction of the peptide. To
448 circumvent the use of Gateway Cloning with the pDest-527 vector (**Figure S2A**), the
449 peptide endogenous peptide sequence flanked by the peptide cut sites was ordered as a
450 complete plasmid in a pET-21 vector (**Figure S2B**). This vector was used to test the
451 behavior of INS-22
452 (MHTTTILICFFIFLVQVSTMDAHTDKYVRTLCGKTAIRNIANLCPPKPEMKGICSTGEYPSITE
453 YCSM-NH_2). Unlike INS-6, INS-22 antagonizes the DAF-2 neuropeptide receptor [71, 72].
454 We discovered that animals lacking *ins-22* exhibit increased naive chemotaxis towards a
455 neutral concentration of 10% butanone compared to wildtype animals (**Figure S2C**). This
456 behavior persists in animals fed the SCRAMBLE peptide in iOP50 *E. coli* (**Figure 2D**).
457 When fed the INS-22 peptide, the *ins-22 lof* animals displayed naive chemotaxis similar to
458 wild-type animals, indicating that peptide feeding rescued normal naive chemotaxis
459 towards 10% butanone (**Figure 2D**).

460 **<u>Neuropeptide Rescue by Feeding Requires the Cognate Neuropeptide Receptor</u>**

461 To ensure that the observed behavior is resulting from the neuropeptide being fed to the
462 animal, activating the neuropeptide receptor, we tested the behavior of animals deficient

463 in the neuropeptide receptor. Pairing neuropeptides ligands to their cognate receptors is
464 an ongoing effort in peptidomic research. Recent efforts have uncovered many of the
465 individual neuropeptide pairings and the range over which these connections functions
466 [26, 73, 74]. For the current study, we were interested in studying the function of
467 neuropeptides and receptors that primarily function in a 1:1 ratio. This would eliminate the
468 class of insulin-like peptides which bind to DAF-2 from this current study [23, 71].

469 PDF-1 peptides binds primarily to one neuropeptide receptor PDFR-1 [54, 75].
470 Though PDFR-1-1 also binds to peptides produced by *pdf-2* (also known as *nlp-37*), these
471 ligands are not involved in behavior of males leaving food to search for mates [54, 74].
472 PDFR-1 mutants do not leave food to search for mates (**Figure S3A,B**), even when fed
473 excess peptide (**Figure 3A, S3C**). The double mutant males of the PDFR-1 receptor and
474 *pdf-1* neuropeptide, partially left food to search for mates when fed PDF-1A but not when
475 fed the other peptides (**Figure 3A**). This probability of leaving food of *pdf-1;pdfr-1* males
476 was not to the rate of *pdf-1* males leaving food when fed a SCRAMBLE peptide (**Figure**
477 **3A**). The absence of food leaving to search for mates, even when receptor mutants are
478 fed the neuropeptide, suggests that there are no off-target effects of the neuropeptide
479 impacting other receptors.

480 The *flp-3* neuropeptide gene makes 10 different peptides that bind to 4 different
481 receptors [49, 74]. Neuropeptide receptors DMSR-1-2 and DMSR-7-1 bind to many of the
482 *flp* class of neuropeptides including *flp-3*, while FRPR-16-1 binds *flp-3* and *nlp-23* peptides
483 and NPR-10-1 binds *flp-3* and *nlp-23* peptides [74]. We have previously identified that the
484 receptors FRPR-16 and NPR-10 are involved in the *flp-3* male behavior towards ascr#8
485 [49]. The FLP-3-9 neuropeptide binds activates the FRPR-16 receptor at lower
486 concentrations than the NPR-10 receptor [49], so we elected this as our neuropeptide
487 receptor candidate for FLP-3-9. We have previously demonstrated that without FRPR-16
488 males will avoid ascr#8 [49]. When we generated a double mutant of *flp-3;fprpr-16* we
489 observed avoidance of ascr#8 in animals fed OP50 *E. coli* without peptide (**Figure S3D**).
490 When fed FLP-3-9 peptide in DH5α *E. coli*, the avoidance behavior is sustained (**Figure**

491 **3B**). This data shows that while FLP-3-9 binds to multiple receptors, FRPR-16 loss of
492 function is sufficient to alter male behavior towards ascr#8.

493 *Behavioral Impacts of Neuropeptide Overexpression Depend on the Peptide Identity*

494 One question that arises with this feeding method is, how much neuropeptide makes its
495 way into the worms and what would happen if there were an overexpression of
496 neuropeptide? Quantifying the amount of neuropeptide produced endogenously is a
497 difficult open question in the peptidomics field. One indication of how much of a particular
498 neuropeptide is required is by looking at the neuropeptide precursor gene. Some
499 neuropeptides are encoded multiple times in the same gene, like *flp-6* encoding
500 KSAYMRFG-NH_2 six times [76]. One method used to detect the presence of
501 neuropeptides is liquid chromatography mass spectrometry (LC-MS) [19, 77-79]. While
502 this method is extremely precise for detecting known and novel neuropeptides, it lacks
503 the ability to quantify the amount of each neuropeptide present *in vivo* (personal
504 communication with Sven Van Bael and Lisabet Timmermann, KU Leuven). One way to
505 test the effects of what is likely an overexpression of neuropeptide is by feeding the
506 neuropeptide-containing DH5α bacteria to wildtype animals.

507 We tested the appropriate wildtype controls for each previously assessed behavior.
508 We observed an increase in the leaving behavior of *him-5* male worms fed PDF-1B and a
509 decrease when fed PDF-1A in DH5α *E. coli* (**Figure 4A, S4A**). This behavioral difference
510 disappears when both peptides are fed in combination (**Figure 4A**). These data suggest
511 that different PDF-1 peptides play varying roles in mating behavior. Next, we examined
512 the effects of this behavior using the iOP50 *E. coli* delivery. When males were fed an
513 excess of iOP50 peptide and subsequently placed on an iOP50 assay plate, their rates of
514 leaving food (**Figure 4B, S4B**) did not increase. Wildtype animals fed PDF-1 peptides
515 produced by iOP50 also did not show an increased leaving rate of food when tested on
516 an OP50-containing assay plate (**Figure S4C, D**). These data suggest that male excursion
517 rates are not impacted by an overexpression of PDF-1 peptides delivered by microbial
518 feeding.

519 When *him-8* male animals were fed FLP-3-9, their lack of ascr#8 avoidance was
520 not changed compared to animals fed SCRAMBLE (**Figure 4C**). Together these data
521 indicate that there are no obvious behavioral deficits across all neuropeptides being
522 delivered in excess. Wildtype males fed FLP-3-9 iOP50 *E. coli* continued their non-
523 avoidance of ascr#8 (**Figure 4D**).

524 We then tested different insulin ligands for their overexpression phenotypes. When
525 wildtype N2 animals was fed INS-6 in DH5α *E. coli*, there was no difference in the choice
526 index of high salt taxis (**Figure 4E**). These data suggest that INS-6 overexpression by
527 microbial feeding does not impact the behavior of animals towards high NaCl. There has
528 been evidence that overexpression of some insulin like peptides may impact other
529 behaviors like dauer formation [66].

530 When wildtype animals were fed an excess of INS-22 iOP50 *E. coli*, their behavior
531 towards 10% butanone was slightly but not significantly decreased (**Figure 4F**). Yet, when
532 exposed to a highly attractive concentration of 0.1% butanone, wildtype animals fed INS-
533 22 in iOP50 *E. coli* significantly reduced their attraction towards the cue (**Figure 4G**). While
534 differences in behavior towards varying concentrations of butanone have been previously
535 established [80, 81], the impact of INS-22 on these behaviors has not been implicated in
536 detail. Differences between the overexpression effects of the *ins* peptides may be due to
537 the different interactions with the DAF-2 receptor, INS-6 is a strong agonist and INS-22 is
538 an antagonist [71, 72].

539

540 **<u>Conclusions</u>**

541 We present a novel method of neuropeptide rescue that utilizes bacterial expression to
542 deliver individual peptides for behavioral rescue phenotypes. Our novel technology is
543 advantageous over transgenic studies, as it allows for functional characterizing individual
544 peptides. This tool readily allows for testing newly de-orphanized
545 neuropeptide/neuropeptide receptor pairings [74]. This genetic tool is built off the
546 principles of RNAi feeding techniques to supply worms with the peptide of interest through

547 their food source as previously shown for the scorpion venom peptide mBmKTX to alter
548 lifespan and egg-laying behavior in *C. elegans* [41].

549 Based on our results, we propose that the neuropeptide rescue-by-feeding strategy
550 delivers mRNA that is ready for translation by the *C. elegans* cellular machinery, rather
551 than supplying *C. elegans* with fully translated and processed peptides. The plasma
552 membrane of a cell is an intricate complex of multiple lipid and protein molecules. Small
553 molecules with moderate polarity diffuse through the cell membrane passively, but most
554 metabolites and short peptides require specialized membrane transporters for
555 translocation [82]. Given the large number of neuropeptides encoded in the genome of *C.*
556 *elegans* [1], having specialized transporters for peptide transport is not feasible [83]. The
557 most substantial evidence for this statement is that the processed INS-6 peptide is 54
558 amino acids in length: the *C. elegans* intestine expresses peptide transporters that only
559 uptake smaller, inactive di- and tri-amino acid peptide chains [83]. Thus, rescue of
560 chemotaxis behavior by INS-6 peptide feeding (**Figure 1C**) and INS-22 (**Figure 2D**)
561 suggests that the mechanism of rescue may be similar to double stranded RNA (dsRNA)
562 uptake, rather than peptidergic transport across the intestinal membrane [84, 85].

563 Furthermore, previous studies have shown that dsRNA exhibits transgenerational
564 inheritance [86], despite degradation rates. Our rescue of the exploratory behavior of *pdf-*
565 *1 lof* males even in the 24-hour timescale (**Figure 1A, 2B**) suggests that our feeding
566 paradigm exploits similar mechanisms, allowing peptide-encoding mRNAs to remain
567 present throughout the assay. We propose that if peptides were taken up, their
568 degradation rates would likely impede rescue efficiency at later timepoints, unlike what
569 we observe in our experiments, suggesting that the mechanism is similar to dsRNA
570 feeding, with mRNA uptake driving rescue rather than peptide uptake.

571 Understanding neuropeptide function is essential from both the perspective of
572 regulating neuronal (synaptic and non-synaptic) channels of communication and from a
573 broader view of general neuronal functions throughout the brain. Revealing how a specific
574 neuropeptide acts at both the cellular and subcellular receptor levels is a critical link in

575 comprehending the role of neuromodulation in circuits. An enhanced experimental
576 pipeline for investigating peptide function will facilitate progress toward understanding
577 how neural circuit activity within the network is modulated by neuropeptides in its various
578 states, resulting in flexible decision-making during behaviors.

579 **Data Availability**

580 Strains (**Table S1**) and plasmids (**Table S2**) are available upon request. The authors affirm
581 that all data necessary for confirming the conclusions of the article are present within the
582 article, figures, and tables. The raw data or a summary containing the average, n, standard
583 error measure, and associated statistical tests for all figures are available upon request.

584 **Acknowledgments**

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589 related to *ins-6* and *pdf-1*, respectively. pDest-527 was a gift from Dominic Esposito
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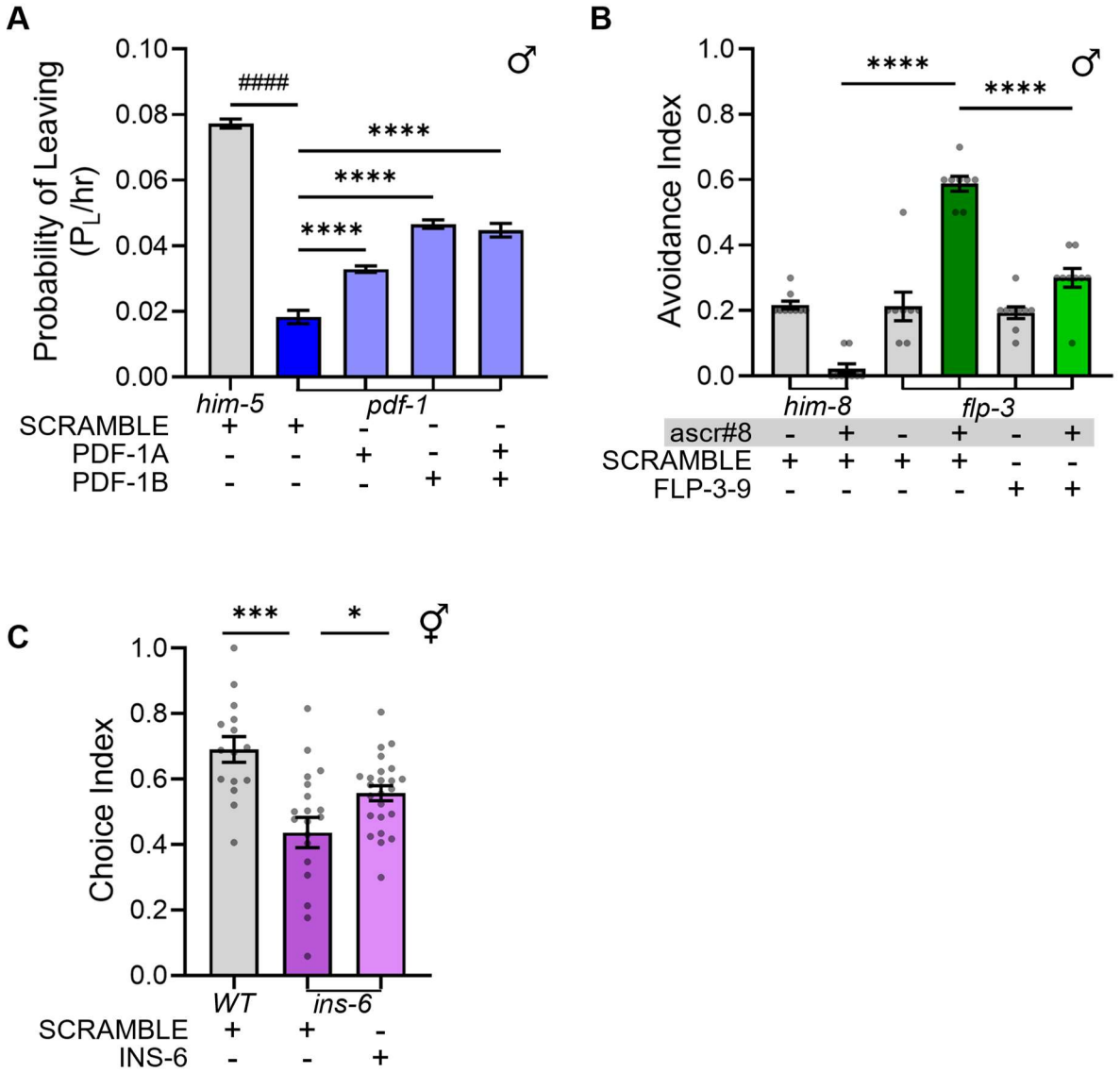
595 **Conflict of Interest**

596 EMD, DKR, and JS would like to disclose the filing of Utility Patent US-20220380430-A1
597 from May 20, 2022, which covers details of the neuropeptide vector and feeding.

598 **Figures**



599

600 **Figure 1: Behavioral of neuropeptide associated behavior in loss of function mutant**
601 **background**

602 **A)** *pdf-1 lof* mutant males fed a SCRAMBLE peptide have a significantly lower probability
603 of leaving food when compared to wildtype control *him-5* males ($^{\#\#\#\#}p \leq 0.0001$, unpaired
604 t-test). When fed any of the *pdf-1* produced peptides in a DH5α *E. coli* vehicle, the
605 probability of leaving is significantly higher than the SCRAMBLE fed *pdf-1* males ($^{****}p \leq$
606 0.0001, One-way ANOVA with Dunnett's post-hoc test).

607 **B)** When exposed to a water solvent control (ascr#8 -), both *him-8* and *flp-3* males act the
608 same. When exposed to ascr#8 (ascr#8 +), wildtype *him-8* males do not avoid the cue
609 while *flp-3 lof* males do. When fed FLP-3-9 peptide in a DH5α *E. coli* vehicle, the avoidance
610 towards ascr#8 by *flp-3 lof* males is significantly decreased (****$p \leq 0.0001$, Mann-
611 Whitney test with Bonferroni correction, new p-value ****$p \leq 0.00005$).

612 **C)** Wildtype N2 animals are more attracted to high 750 mM NaCl than *ins-6 lof* animals.
613 When fed INS-6 in a DH5α *E. coli* vehicle, the attraction of *ins-6 lof* animals to NaCl is
614 significantly increased (*$p \leq 0.05$, ***$p \leq 0.001$, unpaired t-test with Bonferroni correction,
615 new p-value *$p \leq 0.025$, ***$p \leq 0.0005$).

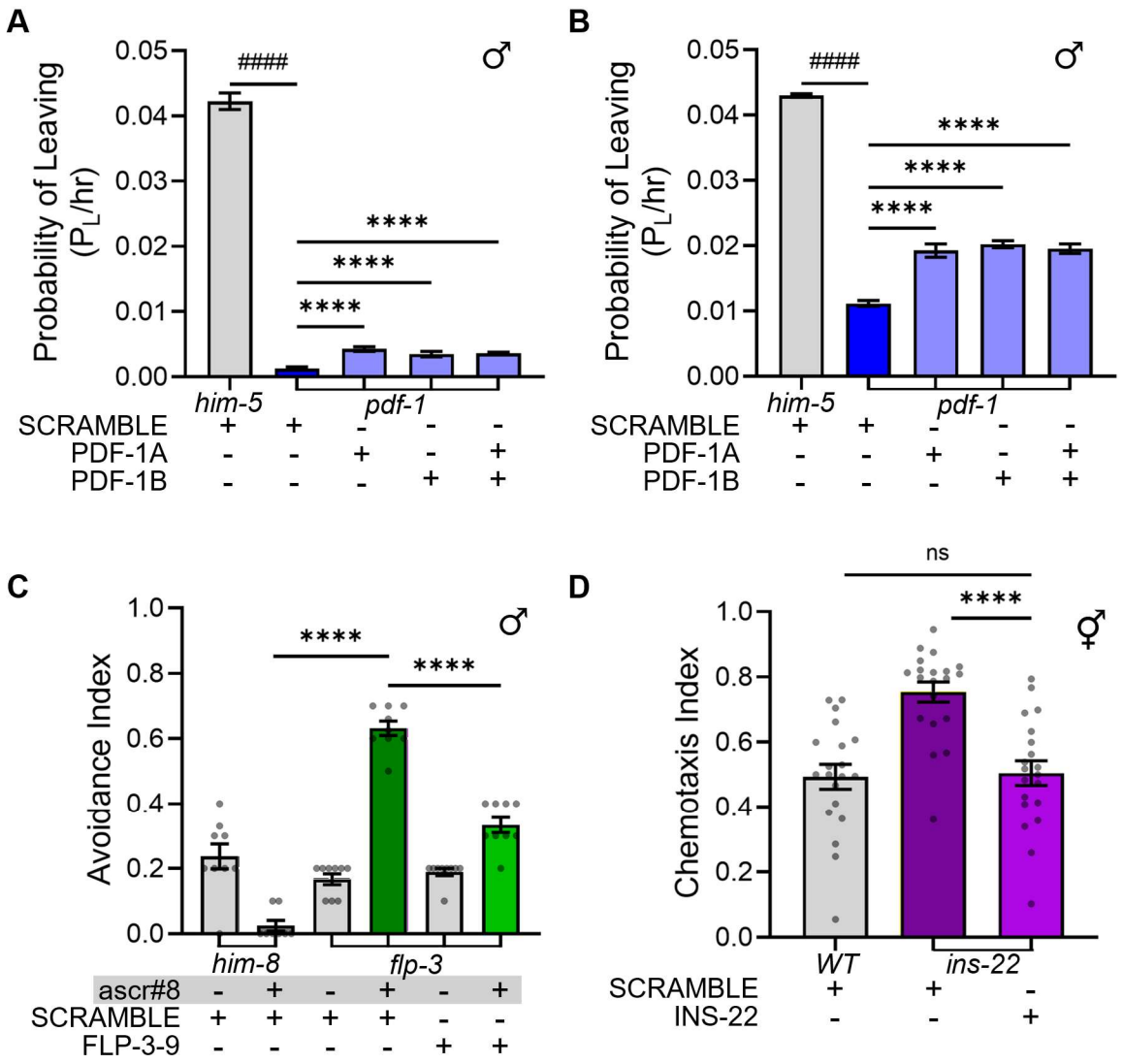


Figure 2: Neuropeptide rescue by feeding can be accomplished in different *E. coli* vehicles

A) *him-5* males fed a SCRAMBLE peptide in iOP50 *E. coli* leave food at a higher rate than *pdf*-1 males (####$p \leq 0.0001$, unpaired t-test). *pdf-1 m*ales fed PDF-1 peptides in iOP50 and then plated on an assay plate containing OP50 *E. coli* without peptide, do leave food at a higher rate than *pdf-1* SCRAMBLE iOP50 *E. coli* fed animals (****$p \leq 0.0001$, One-way ANOVA with Dunnett's post-hoc test).

B) *him-5* males fed SCRAMBLE peptide in iOP50 *E. coli* and then placed on an assay plate seeded with SCRAMBLE peptide in iOP50 *E. coli* leave food at a higher rate than *pdf*-1

626 males ($^{\#\#\#\#}p \leq 0.0001$, unpaired t-test). pdf-*1 m*ales fed PDF-1 peptides in iOP50 and then
627 plated on an assay plate containing iOP50 *E. coli* with the corresponding peptide, leave
628 food at a higher rate than *pdf-1* SCRAMBLE iOP50 *E. coli* fed animals and at a higher rate
629 than animals on assay plates without peptide (****$p \leq 0.0001$, One-way ANOVA with
630 Dunnett's post-hoc test).

631 **C)** When exposed to a water solvent control (ascr#8 -), both *him-8* and *flp-3* males act the
632 same. When exposed to ascr#8 (ascr#8 +), wildtype *him-8* males do not avoid the cue
633 while *flp-3 lof* males do. When fed FLP-3-9 peptide in iOP50 *E. coli*, the avoidance towards
634 ascr#8 by *flp-3 lof* males is significantly decreased (****$p \leq 0.0001$, Mann-Whitney test
635 with Bonferroni correction, new p-value ****$p \leq 0.00005$).

636 **D)** Animals deficient in *ins-22* have a significantly higher rate of chemotaxis towards 10%
637 butanone than *ins-22 lof* animals fed INS-22. The *ins-22 lof* animals fed INS-22 have a
638 chemotaxis index no different than wildtype animals ($^{ns}p \geq 0.05$, ***$p \leq 0.001$, unpaired t-
639 test wildtype SCRAMBLE to INS-22, Mann-Whitney test comparisons to *ins-22* fed
640 SCRAMBLE with Bonferroni correction (new p-value $^{ns}p \geq 0.025$, ****$p \leq 0.00005$).

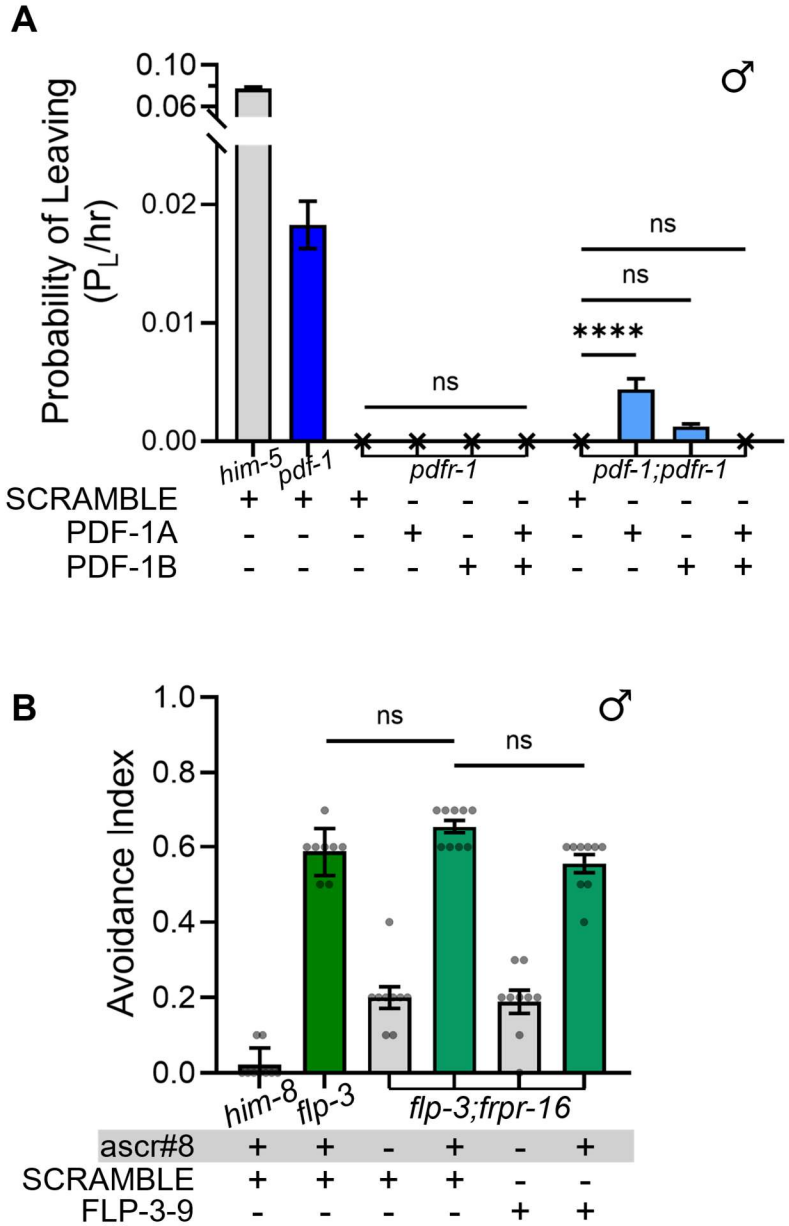
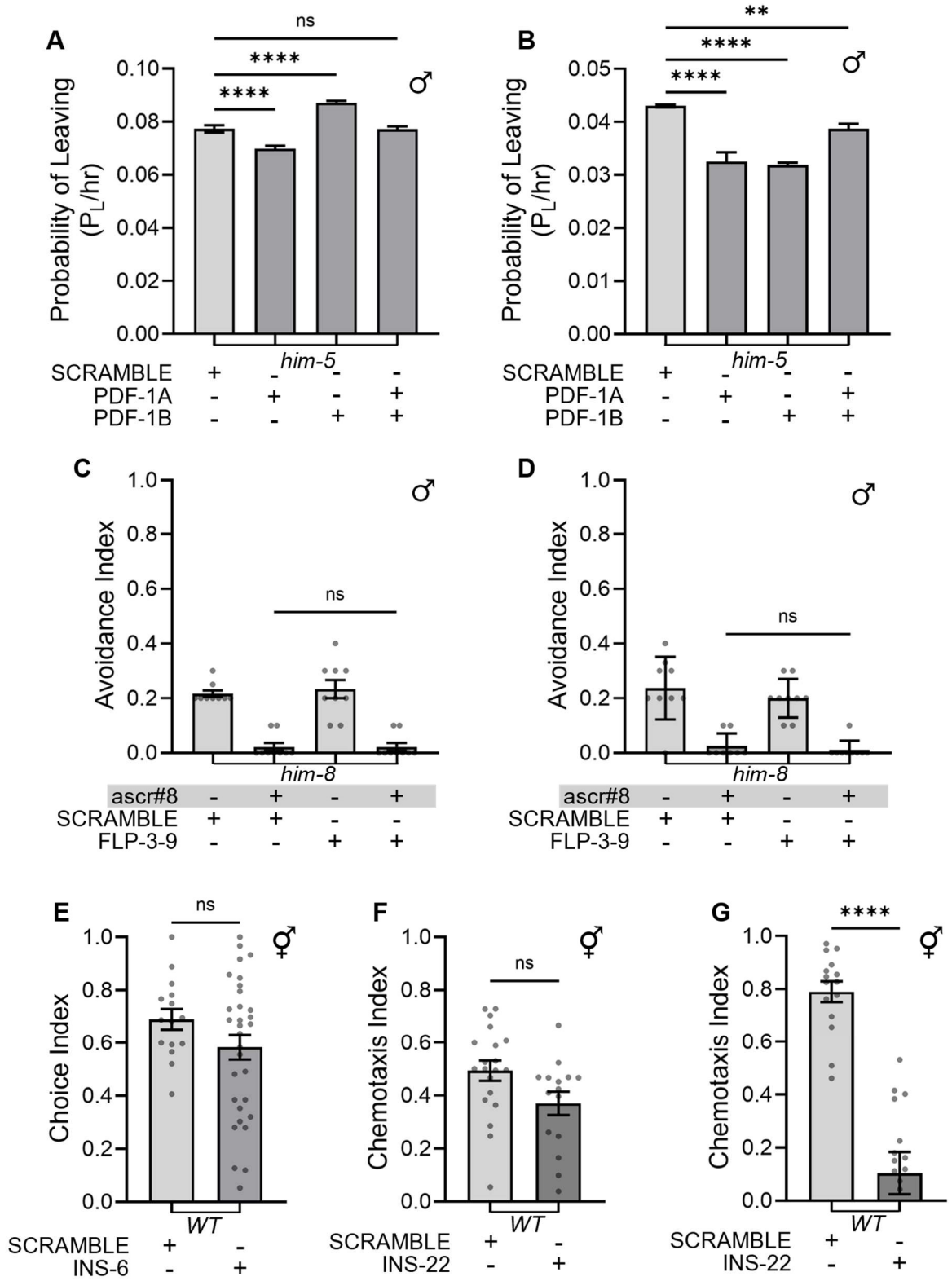


Figure 3: Neuropeptide receptor required for neuropeptide feeding

A) *pdfr-1 lof* animals do not leave food more than 35mm, even if fed PDF-1 peptides in DH5α *E. coli*. Double mutants of *pdf-1;pdfr-1* in a *him-5* background also did not leave food at the rate of *pdf-1* animals fed SCRAMBLE, though *pdf-1;pdfr-1* animals fed PDF-1A DH5α *E. coli* do have a higher incidence of leaving food than SCRAMBLE fed animals ($^{ns}p \geq 0.05$, ****$p \leq 0.0001$, One-way ANOVA within genotypes with Dunnett's post hoc test).

648 **B)** Double mutant *flp-3;frpr-16* males avoid ascr#8 to the same level as SCRAMBLE DH5α
649 *E. coli* fed animals ($^{ns}p \geq 0.05$, Mann-Whitney test with Bonferroni correction, new p-value
650 $^{ns}p \geq 0.025$).


A
ns
****
****
Probability of Leaving (P_L/hr)
0.10
0.08
0.06
0.04
0.02
0.00
him-5
SCRAMBLE + - - -
PDF-1A - + - +
PDF-1B - - + +
B
**
****
****
Probability of Leaving (P_L/hr)
0.05
0.04
0.03
0.02
0.01
0.00
him-5
SCRAMBLE + - - -
PDF-1A - + - +
PDF-1B - - + +
C
Avoidance Index
1.0
0.8
0.6
0.4
0.2
0.0
ns
him-8
ascr#8 - + - +
SCRAMBLE + + - -
FLP-3-9 - - + +
D
Avoidance Index
1.0
0.8
0.6
0.4
0.2
0.0
ns
him-8
ascr#8 - + - +
SCRAMBLE + + - -
FLP-3-9 - - + +
E
ns
Choice Index
1.0
0.8
0.6
0.4
0.2
0.0
WT
SCRAMBLE + -
INS-6 - +
F
ns
Chemotaxis Index
1.0
0.8
0.6
0.4
0.2
0.0
WT
SCRAMBLE + -
INS-22 - +
G
****
Chemotaxis Index
1.0
0.8
0.6
0.4
0.2
0.0
WT
SCRAMBLE + -
INS-22 - +

651

652 **Figure 4: Behaviors resulting from overexpression of neuropeptides depend on the**
653 **neuropeptide**

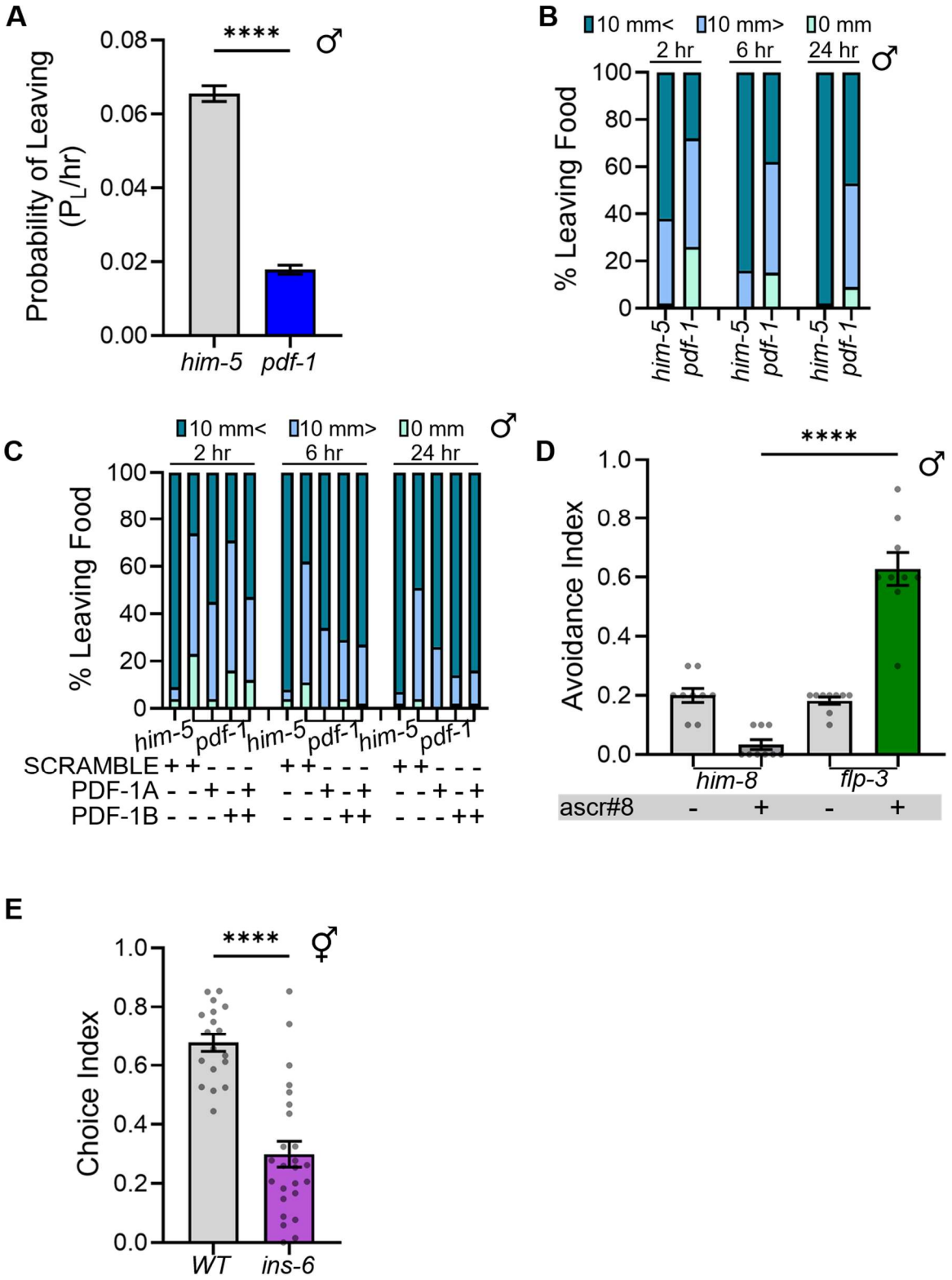
654 **A)** *him-5* males fed PDF-1A in DH5α *E. coli* had a significant decrease in their probability
655 of leaving food rate compared to animals fed SCRAMBLE peptide. PDF-1B *him-5* animals
656 had a significant increase in P_L/hr when compared to SCRAMBLE fed animals. When PDF-
657 1A and -1B were fed to animals in equal combination, their probability of leaving food was
658 no different from SCRAMBLE fed animals (****$p \leq 0.0001$, One-way ANOVA with
659 Dunnett's post-hoc test).

660 **B)** *him-5* males fed PDF-1 peptides in iOP50 *E. coli* and then tested on iOP50 assay
661 plates containing a corresponding peptide, do not have excursions leaving food more
662 than 35 mm at higher rates than SCRAMBLE fed males males (**$p \leq 0.01$,****$p \leq$
663 0.0001, One-way ANOVA with Dunnett's post-hoc test).

664 **C)** *him-8* males exposed to ascr#8 had no difference in their avoidance behavior whether
665 they were fed SCRAMBLE of FLP-3-9 in DH5α *E. coli* ($^{ns}p \geq 0.05$, Mann-Whitney test).

666 **D)** *him-8* males fed SCRAMBLE or FLP-3-9 in iOP50 *E. coli* avoid ascr#8 at the same
667 rate and behave the same towards the water solvent control as the previous studies (^{ns}p
668 ≥ 0.05, Mann-Whitney test).

Supplemental Material



671 **Figure S1: Neuropeptide gene deficient animals exhibit different behaviors than**
672 **they wildtype counterparts**

673 **A)** *pdf-1* males fed OP50 without peptide have a significantly lower rate of leaving food
674 beyond 35 mm compared to wildtype *him-5* animals (****$p \leq 0.0001$, unpaired t-test).

675 **B)** The location of males can be traced across time and binned as animals who never left
676 food (0 mm), those that had a minor excursion (10 mm >), and those with a major
677 excursion (10 mm <). This is then converted to a percentage and plotted across time.
678 These values contribute to the probability of leaving rate. *pdf-1* animals fed OP50 without
679 peptide have lower rates of major excursions compared to wildtype *him-5* animals.

680 **C)** SCRAMBLE DH5α *E. coli* fed *pdf-1 lof* males have a lower percentage of animals
681 making major excursions off of food than *him-5* animals.

682 **D)** Compared to wildtype *him-8* males, *flp-3 lof* males fed OP50 *E. coli* without peptide
683 avoid ascr#8 at a significantly higher rate (****$p \leq 0.0001$, Mann-Whitney test).

684 **E)** Compared to wildtype animals, *ins-6* animals fed OP50 *E. coli* without peptide have a
685 lower choice index towards high 750 mm NaCl (****$p \leq 0.0001$, unpaired t-test).

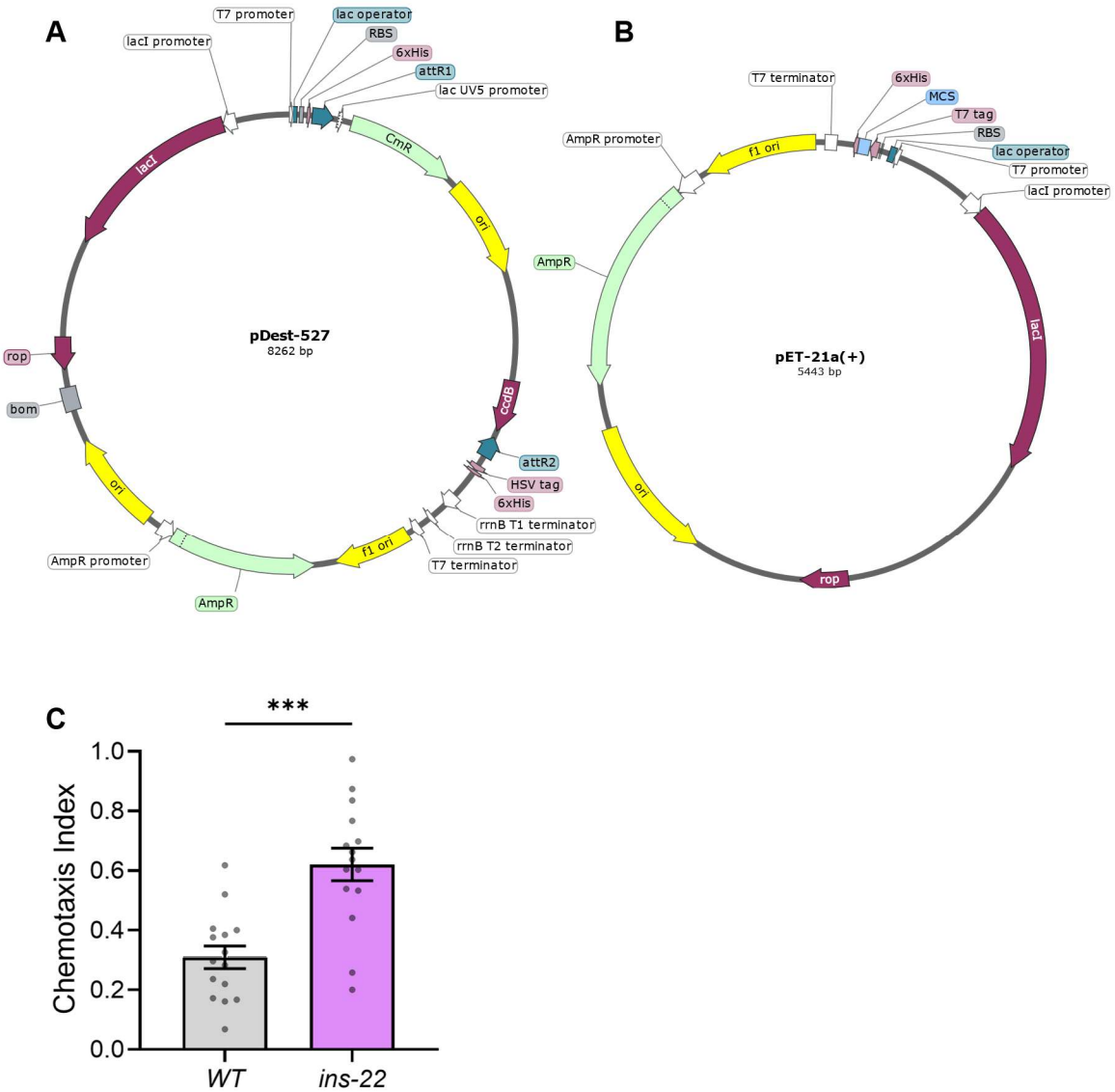
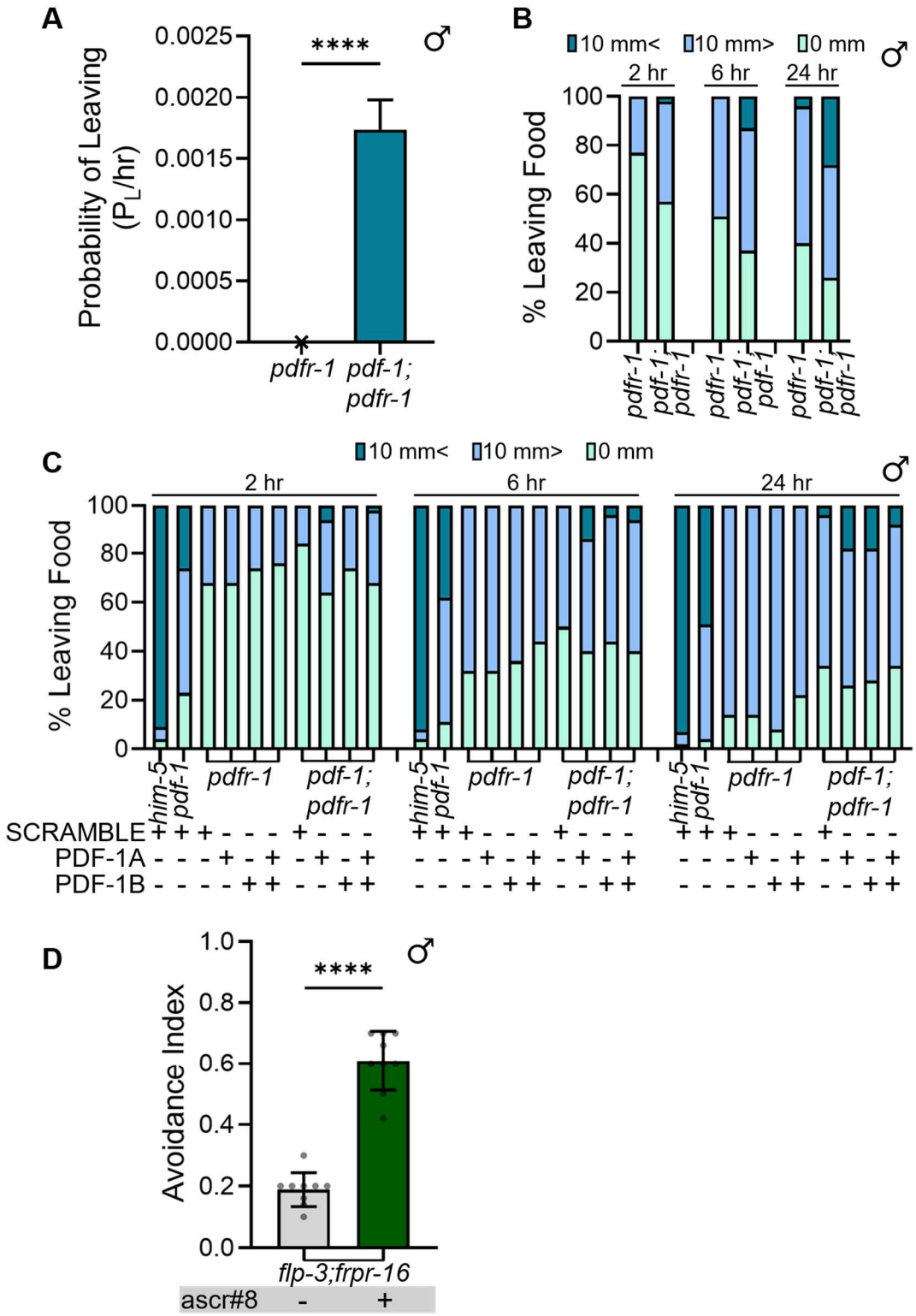


Figure S2: Plasmid maps of old Gateway cloning and new constructions

A) pDest-527 vector used in Gateway cloning. Created in SnapGene

B) pET-21a(+) vector used in Genscript XhoI/XhoI construction. Created in SnapGene

C) Chemotaxis values of wildtype (N2) and *ins-22 lof* animals towards 10% butanone. (***p $\leq$ 0.001, Mann-Whitney test).



692

693 **Figure S3: Neuropeptide receptor *lof* animals behavior without peptide feeding**

694 **A)** Single mutant *pdfr-1* neuropeptide receptor males fed OP50 *E. coli* leave food at much
695 lower rates than *pdf-1* animals alone. *pdf-1;pdfr-1 lof* in a *him-5* background do leave food
696 greater than 35 mm at a higher rate than *pdfr-1 lof* males (****$p \leq 0.0001$, unpaired t-test).

697 **B)** Excursion rates of *pdfr-1* single and double mutants fed OP50 *E. coli* do not readily
698 leave food or have a major excursion 10 mm <.

699 **C)** *pdf-1* neuropeptide receptor mutants fed PDF-1 peptides in DH5αa *E. coli* do not have
700 excursions off food at high rates like *him-5* males.

701 **D)** *flp-3;frpr-16* double mutants in a *him-8* background avoid ascr#8 at a higher rate than
702 the solvent control (****$p \leq 0.0001$, Mann-Whitney test).

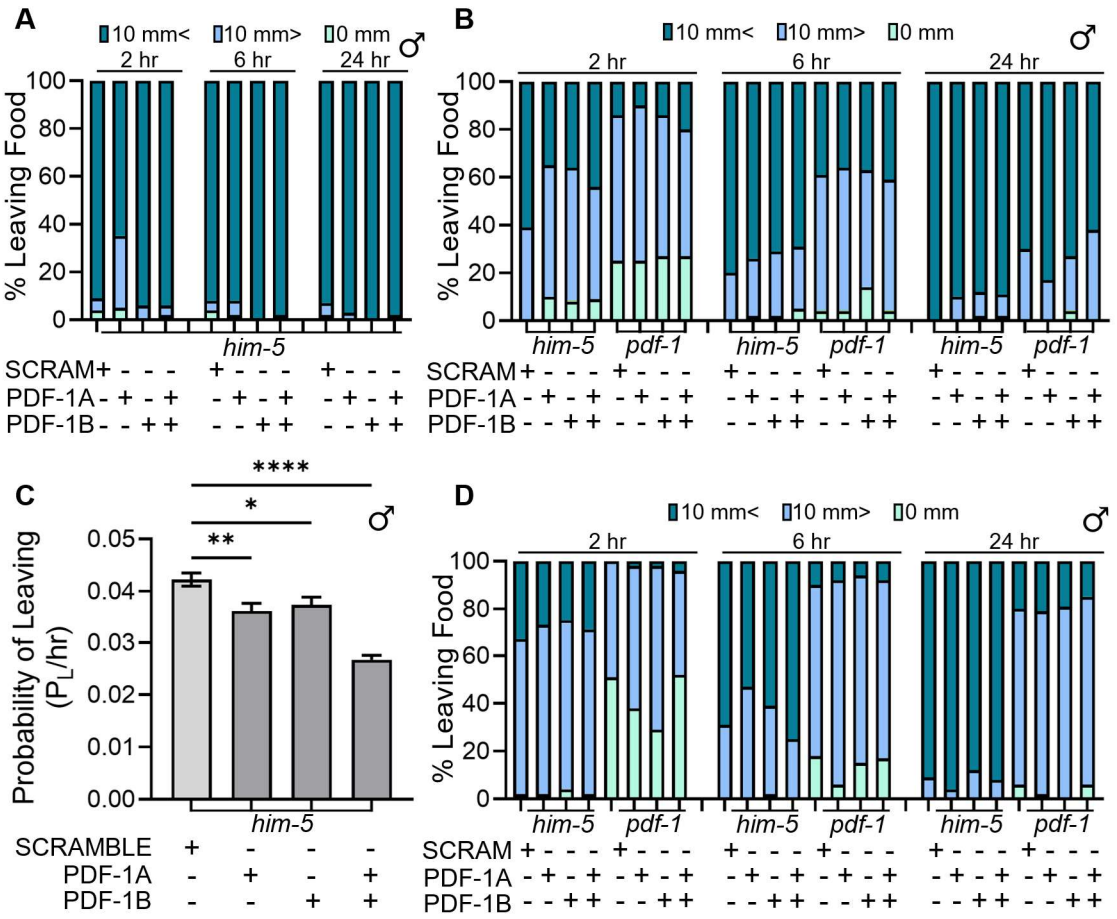


Figure S4: Overexpression of wildtype animals fed neuropeptide in DH5α or iOP50 *E. coli*

A) Most *him-5* males fed SCRAMBLE or PDF-1 peptides in DH5α *E. coli* made a major excursions traveling < 10 mm from food by 2 hours after plating.

B) *him-5* males do not remain on food at the rates of *pdf-1* animals fed PDF-1 peptides in iOP50 *E. coli* and tested on iOP50 assay plates containing peptides. *pdf-1* males fed PDF-1 peptides in iOP50 *E. coli* have excursions off food at higher rates than SCRAMBLE-fed animals.

C) *him-5* males fed PDF-1 peptides in iOP50 *E. coli* and then tested on OP50 assay plates not containing peptide, do not have excursions leaving food more than 35 mm at higher rates than SCRAMBLE fed males (*$p \leq 0.05$, **$p \leq 0.01$,****$p \leq 0.0001$, One-way ANOVA with Dunnett's post-hoc test).

716 **D)** *him-5* males do not remain on food at the rates of *pdf-1* animals fed PDF-1 peptides in
717 iOP50 *E. coli* and tested in OP50 assay plates not containing peptides.

718

719 **Table S1: *C. elegans* Strains Used in Current Study**

Strain Name	Genotype	Source Lab	Used in Paper
N2	Bristol Wildtype isolate	CGC	1C, S1E, 2D, S2C, 4E-G
CB1489	*him-8(e1489)IV*	CGC	1B, S1D, 2C, 3B, S3D, 4C,D
CB4088	*him-5(e1490)V*	CGC	1A, S1A-C, 2A,B, 3A, S3A-C, 4A, B, S4
IV302	*ins-6(tm2416)II; kyEx2595[str-2p::GCaMP2.2b + unc-122p::GFP]*	Shreekanth Chalasani	1C, S1E, 4E
JSR99	*flp-3(pk361)X; him-8(e1489)IV*	Jagan Srinivasan	1B, S1D, 2C, 3B, S3D, 4C,D
JSR152	*flp-3(pk361)X; frpr-16(gk5305[loxP + Pmyo-2::GFP::unc-54 3' UTR + Prps-27::neoR::unc-54 3' UTR + loxP]II)*	Jagan Srinivasan	3B, S3D
JSR188	*pdf-1(tm1996)III; pdfr-1(ok3425)III; him-5(e1490)V*	Jagan Srinivasan	3A, S3A-C
UR930	*pdfr-1(ok3425)III; him-5(e1490)V*	Douglas Portman	3A, S3A-C
UR954	*pdf-1(tm1996)III; him-5(e1490)V*	Douglas Portman	1A, S1A-C, 2A,B, 3A, S3A-C, 4A, B, S4
RB2594	*ins-22(ok3616)III*	CGC	2D, S2C, 4F,G

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721

722 **Table S2: Neuropeptide Oligo Sequences Ordered for Individual Peptide Feeding**

723 Neuropeptide DNA sequence indicated in lowercase.

Peptide	Animo Acid (C-x-N)	Sequence Ordered
SCRAMBLE	NSKLHRGGGRSRTSGSTGSMASHARGSPGLQ	For:GGGGACAAGTTTGTACAAAAAAGCAGGCTGGATGCGCTTTGGAAAACGTaattcgagctccaccgcggtggcggccgctctagaactagtggatccaccggttccatggctagccacgcgcgtggatcccccgggctgcaggAAACGTAAATAACACCCAGCTTTCTTGTACAAAGTGGTCCCC
		Rev:GGGGACCACTTTGTACAAGAAAGCTGGGTGTTATTTACGTTTcctgcagcccgggggatccacgcgcgtggctagccatggaaccggtggatccactagttctagagcggccgccaccgcggtggagctcgaattACGTTTTCCAAAGCGCATCCAGCCTGCTTTTTTGTACAAACTTGTCCCC
PDF-1A	VQFVKRSNAELINGLIGMDLGKLSAVGKR	For:GGGGACAAGTTTGTACAAAAAAGCAGGCTGGGTTCAGTTCGTaaaacgcagcaacgccgagcttatcaacggactcatcggaatggatttgggaaaattgtcagctgtcggaAAACGCTGACACCCAGCTTTCTTGTACAAAGTGGTCCCC
		Rev:GGGGACCACTTTGTACAAGAAAGCTGGGTGTTATTTACGTTTtcagcgttttccgacagctgacaattttcccaaatccattccgatgagtccgttgataagctcggcgttgctgcgttttacgaactgaacACGTTTTCCAAAGCGCATCCAGCCTGCTTTTTTGTACAAACTTGTCCCC
PDF-1B	VQFVKRSNAELINGLLSMNLNKLSGAGRR	For:GGGGACAAGTTTGTACAAAAAAGCAGGCTGGGTTCAGTTCGTAAAACGCTcaaacgcggaacttatcaacggtcttctcagcatgaacctcaacAAATTGTCTGGAGCTGGTCGACGATGACACCCAGCTTTCTTGTACAAAGTGGTCCCC
		Rev:GGGGACCACTTTGTACAAGAAAGCTGGGTGTTATTTACGTTTtcatcgtcgaccagctccagacaatttgttgaggttcatgctgagaagaccgttgataagttccgcgtttgagcgttttacgaactgaacACGTTTTCCAAAGCGCATCCAGCCTGCTTTTTTGTACAAACTTGTCCCC
FLP-3-9	NPENDTPFGTMRFG	For:GGGGACAAGTTTGTACAAAAAAGCAGGCTGGATGCGCTTTGGAAAACGTaatcctgagaacgacacaccattcggaacaatgagatttggaAAACGTAAATAACACCCAGCTTTCTTGTCAAAGTGGTCCCC
		Rev:GGGGACCACTTTGACAAGAAAGCTGGGTGTTATTTACGTTTccaaatctcattgttccgaatggtgtgtcgttctcaggattACGTTTTCCAAAGCGCATCCAGCCTGCTTTTTTGTACAAACTTGTCCCC
INS-6	VPAPGETRACGRKLISLVMAVCGDLCNPQEGKDIATECCGNQCSDDYIRSACCP	For:GGGGACAAGTTTGTACAAAAAAGCAGGCTGGATGCGCTTTGGAAAACGTgttccagcaccaggagaaactcgtgcctgcggaagaaaactcatctctttagtcatggctgtctgtggagatctttgcaacccacaagaaggaaaggacattgcgactgaatgctgcggaaatcagtgttctgatgactacataagatctgcttgttgtccaAAACGTAAATAACACCCAGCTTTCTTGTACAAAGTGGTCCCC
		Rev:GGGGACCACTTTGTACAAGAAAGCTGGGTGTTATTTACGTTTtggacaacaagcagatcttatgtagtcatcagaacactgatttccgcagcattcagtcgcaatgtcctttccttcttgtgggttgcaaagatctccacagacagccatgactaaagagatgagttttcttccgcaggcacgagtttctcctggtgctggaacACGTTTTCCAAAGCGCATCCAGCCTGCTTTTTTGTACAAACTTGTCCCC

INS-22	MHTTTILICFFIFLVQ VSTMDAHTDKYVRT LCGKTAIRNIANLCP PKPEMKGICSTGEY PSITEYCSM	ATGAGGTTTGGAAAAAGAatgcacactacaacgatcctgatttgttttttcatcttct tggtccaggttagcaccatggatgctcacaccgataaatacgtgcgcaccctgtgcggcaa gaccgcaattcgtaatattgcgaacctgtgtccgccaaaaccggaaatgaagggcatctg ctctactggtgagtatccgagcattaccgagtactgctccatgggttttagcgactcgcaaat caagttcatgtgctgcgacaaccagAAACGTAAATAA

References

1. Li, C. and K. Kim, *Neuropeptides.* WormBook : the online review of C. elegans biology, 2008: p. 1-36.
2. Dale Purves, G.J.A., David Fitzpatrick, William C Hall, Anthony-Samuel LaMantia, Richard D Mooney, Michael L Platt, Leonard E White, *Neuroscience*. 6 ed. 2018, Sunderland, MA: Oxford University Press.
3. Yañez-Guerra, L.A., D. Thiel, and G. Jékely, *Premetazoan Origin of Neuropeptide Signaling.* Mol Biol Evol, 2022. **39**(4).
4. Beets, I., et al., *Vasopressin/oxytocin-related signaling regulates gustatory associative learning in C. elegans.* Science, 2012. **338**(6106): p. 543-5.
5. Gruber, C.W., *Physiology of invertebrate oxytocin and vasopressin neuropeptides.* Experimental Physiology, 2014. **99**(1): p. 55-61.
6. Insel, T.R. and L.J. Young, *The neurobiology of attachment.* Nature Reviews Neuroscience, 2001. **2**(2): p. 129-136.
7. Willets, J.M., et al., *Regulation of oxytocin receptor responsiveness by G protein-coupled receptor kinase 6 in human myometrial smooth muscle.* Molecular endocrinology (Baltimore, Md.), 2009. **23**(8): p. 1272-1280.
8. Lukas, M., et al., *The Neuropeptide Oxytocin Facilitates Pro-Social Behavior and Prevents Social Avoidance in Rats and Mice.* Neuropsychopharmacology, 2011. **36**(11): p. 2159-2168.
9. Odekunle, E.A., et al., *Ancient role of vasopressin/oxytocin-type neuropeptides as regulators of feeding revealed in an echinoderm.* BMC Biology, 2019. **17**(1): p. 60.
10. Garrison, J.L., et al., *Oxytocin/vasopressin-related peptides have an ancient role in reproductive behavior.* Science, 2012. **338**(6106): p. 540-3.
11. Frooninckx, L., et al., *Neuropeptide GPCRs in C. elegans.* Frontiers in Endocrinology, 2012. **3**(167).
12. Nusbaum, M.P., D.M. Blitz, and E. Marder, *Functional consequences of neuropeptide and small-molecule co-transmission.* Nat Rev Neurosci, 2017. **18**(7): p. 389-403.
13. Souza-Moreira, L., et al., *Neuropeptides as pleiotropic modulators of the immune response.* Neuroendocrinology, 2011. **94**(2): p. 89-100.
14. Nässel, D.R. and M. Zandawala, *Endocrine cybernetics: neuropeptides as molecular switches in behavioural decisions.* Open Biol, 2022. **12**(7): p. 220174.
15. Tai, K.Y., et al., *Selected neuropeptide genes show genetic differentiation between Africans and non-Africans.* BMC Genetics, 2020. **21**(1): p. 31.

16. *PeptidesDB*. 2008 April 30 2008 [cited 2022; Available from: /www.peptides.be.
17. Burbach, J.P.H., *Neuropeptides from concept to online database www.neuropeptides.nl.* European Journal of Pharmacology, 2010. **626**(1): p. 27-48.
18. Russo, A.F., *Overview of Neuropeptides: Awakening the Senses?* Headache, 2017. **57 Suppl 2**(Suppl 2): p. 37-46.
19. Van Bael, S., et al., *Mass spectrometric evidence for neuropeptide-amidating enzymes in Caenorhabditis elegans.* J Biol Chem, 2018. **293**(16): p. 6052-6063.
20. White, J.G., et al., *The structure of the nervous system of the nematode Caenorhabditis elegans.* Philos Trans R Soc Lond B Biol Sci, 1986. **314**(1165): p. 1-340.
21. Sammut, M., et al., *Glia-derived neurons are required for sex-specific learning in C. elegans.* Nature, 2015. **526**(7573): p. 385-390.
22. Li, C., K. Kim, and L.S. Nelson, *FMRFamide-related neuropeptide gene family in Caenorhabditis elegans.* Brain Res, 1999. **848**(1-2): p. 26-34.
23. Pierce, S.B., et al., *Regulation of DAF-2 receptor signaling by human insulin and ins-1, a member of the unusually large and diverse C. elegans insulin gene family.* Genes Dev, 2001. **15**(6): p. 672-86.
24. Nathoo, A.N., et al., *Identification of neuropeptide-like protein gene families in Caenorhabditiselegans and other species.* Proc Natl Acad Sci U S A, 2001. **98**(24): p. 14000-5.
25. Hobert, O., *The neuronal genome of Caenorhabditis elegans.*, in *WormBook: The Online Review of C. elegans Biology*. 2005-2018: Pasadena, CA.
26. Watteyne, J., et al., *Neuropeptide signaling network of Caenorhabditis elegans: from structure to behavior.* Genetics, 2024. **228**(3).
27. Barrios, A., et al., *PDF-1 neuropeptide signaling modulates a neural circuit for mate-searching behavior in C. elegans.* Nature Neuroscience, 2012. **15**(12): p. 1675-1684.
28. Hussey, R., et al., *Oxygen-sensing neurons reciprocally regulate peripheral lipid metabolism via neuropeptide signaling in Caenorhabditis elegans.* PLoS genetics, 2018. **14**(3): p. e1007305-e1007305.
29. Nath, R.D., et al., *C. elegans Stress-Induced Sleep Emerges from the Collective Action of Multiple Neuropeptides.* Current biology, 2016. **26**(18): p. 2446-2455.
30. Nelson, M.D., et al., *FRPR-4 Is a G-Protein Coupled Neuropeptide Receptor That Regulates Behavioral Quiescence and Posture in Caenorhabditis elegans.* PLoS One, 2015. **10**(11): p. e0142938.
31. Van Bael, S., et al., *Identification of Endogenous Neuropeptides in the Nematode C. elegans Using Mass Spectrometry.* Methods Mol Biol, 2018. **1719**: p. 271-291.
32. TC, E., *Transformation and microinjection. .* WormBook: The Online Review of C. elegans Biology, 2006.
33. Rieckher, M. and N. Tavernarakis, *Caenorhabditis elegans Microinjection.* Bio-protocol, 2017. **7**(19): p. e2565.
34. Conte, D., Jr., et al., *RNA Interference in Caenorhabditis elegans.* Current protocols in molecular biology, 2015. **109**: p. 26.3.1-26.3.30.

35. Timmons, L., D.L. Court, and A. Fire, *Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in Caenorhabditis elegans.* Gene, 2001. **263**(1-2): p. 103-12.
36. Timmons, L. and A. Fire, *Specific interference by ingested dsRNA.* Nature, 1998. **395**(6705): p. 854.
37. Fire, A., et al., *Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans.* Nature, 1998. **391**(6669): p. 806-11.
38. Zheng, S., et al., *A functional study of all 40 Caenorhabditis elegans insulin-like peptides.* J Biol Chem, 2018. **293**(43): p. 16912-16922.
39. Papaioannou, S., et al., *Role of a FMRFamide-like family of neuropeptides in the pharyngeal nervous system of Caenorhabditis elegans.* Journal of Neurobiology, 2005. **65**(3): p. 304-319.
40. Ohno, H., et al., *Luqin-like RYamide peptides regulate food-evoked responses in C. elegans.* Elife, 2017. **6**.
41. Xu, J., et al., *Feeding recombinant E. coli with GST-mBmKTX fusion protein increases the fecundity and lifespan of Caenorhabditis elegans.* Peptides, 2017. **89**: p. 1-8.
42. Fraser, A.G., et al., *Functional genomic analysis of C. elegans chromosome I by systematic RNA interference.* Nature, 2000. **408**(6810): p. 325-330.
43. Kamath, R.S., et al., *Effectiveness of specific RNA-mediated interference through ingested double-stranded RNA in Caenorhabditis elegans.* Genome Biol, 2001. **2**.
44. Reece-Hoyes, J.S. and A.J.M. Walhout, *Gateway Recombinational Cloning.* Cold Spring Harb Protoc, 2018. **2018**(1).
45. Petersen, L.K. and R.S. Stowers, *A Gateway MultiSite recombination cloning toolkit.* PLoS One, 2011. **6**(9): p. e24531.
46. Kaletsky, R., et al., *C. elegans interprets bacterial non-coding RNAs to learn pathogenic avoidance.* Nature, 2020. **586**(7829): p. 445-451.
47. Kamath, R.S., et al., *Effectiveness of specific RNA-mediated interference through ingested double-stranded RNA in Caenorhabditis elegans.* Genome Biol, 2001. **2**(1): p. RESEARCH0002.
48. Fraser, A.G., et al., *Functional genomic analysis of C. elegans chromosome I by systematic RNA interference.* Nature, 2000. **408**(6810): p. 325-30.
49. Reilly, D.K., et al., *Distinct neuropeptide-receptor modules regulate a sex-specific behavioral response to a pheromone.* Commun Biol, 2021. **4**(1): p. 1018.
50. Dirksen, P., et al., *CeMbio - The Caenorhabditis elegans Microbiome Resource.* G3 (Bethesda), 2020. **10**(9): p. 3025-3039.
51. Brenner, S., *The genetics of Caenorhabditis elegans.* Genetics, 1974. **77**(1): p. 71-94.
52. Neve, I.A.A., et al., *Escherichia coli Metabolite Profiling Leads to the Development of an RNA Interference Strain for Caenorhabditis elegans.* G3 (Bethesda), 2020. **10**(1): p. 189-198.
53. Ryan, D.A., et al., *Sex, age, and hunger regulate behavioral prioritization through dynamic modulation of chemoreceptor expression.* Curr Biol, 2014. **24**(21): p. 2509-17.

54. Barrios, A., et al., *PDF-1 neuropeptide signaling modulates a neural circuit for mate-searching behavior in C. elegans.* Nat Neurosci, 2012. **15**(12): p. 1675-82.
55. Lipton, J., et al., *Mate searching in Caenorhabditis elegans: a genetic model for sex drive in a simple invertebrate.* J Neurosci, 2004. **24**(34): p. 7427-34.
56. Hilliard, M.A., et al., *Worms taste bitter: ASH neurons, QUI-1, GPA-3 and ODR-3 mediate quinine avoidance in Caenorhabditis elegans.* EMBO J, 2004. **23**(5): p. 1101-11.
57. Leinwand, S.G. and S.H. Chalasani, *Neuropeptide signaling remodels chemosensory circuit composition in Caenorhabditis elegans.* Nature neuroscience, 2013. **16**(10): p. 1461-1467.
58. Bargmann, C.I. and H.R. Horvitz, *Chemosensory neurons with overlapping functions direct chemotaxis to multiple chemicals in C. elegans.* Neuron, 1991. **7**(5): p. 729-42.
59. Phillips, C.M., et al., *HIM-8 binds to the X chromosome pairing center and mediates chromosome-specific meiotic synapsis.* Cell, 2005. **123**(6): p. 1051-63.
60. Meneely, P.M., et al., *Crossover distribution and frequency are regulated by him-5 in Caenorhabditis elegans.* Genetics, 2012. **190**(4): p. 1251-66.
61. Reilly, D.K., *Neuromodulation of Sex-Specific Pheromone-Mediated Behaviors*, in *Biology and Biotechnology*. 2020, Worcester Polytechnic Institute: Worcester, MA. p. 519.
62. Janssen, T., et al., *Functional characterization of three G protein-coupled receptors for pigment dispersing factors in Caenorhabditis elegans.* J Biol Chem, 2008. **283**(22): p. 15241-9.
63. Janssen, T., et al., *Discovery and characterization of a conserved pigment dispersing factor-like neuropeptide pathway in Caenorhabditis elegans.* J Neurochem, 2009. **111**(1): p. 228-41.
64. Muirhead, C.S. and J. Srinivasan, *Small molecule signals mediate social behaviors in C. elegans.* J Neurogenet, 2020. **34**(3-4): p. 395-403.
65. Pungaliya, C., et al., *A shortcut to identifying small molecule signals that regulate behavior and development in Caenorhabditis elegans.* Proc Natl Acad Sci U S A, 2009. **106**(19): p. 7708-13.
66. Chen, Z., et al., *Two insulin-like peptides antagonistically regulate aversive olfactory learning in C. elegans.* Neuron, 2013. **77**(3): p. 572-585.
67. Hung, W.L., et al., *A Caenorhabditis elegans developmental decision requires insulin signaling-mediated neuron-intestine communication.* Development, 2014. **141**(8): p. 1767-79.
68. Hua, Q.-X., et al., *A divergent INS protein in Caenorhabditis elegans structurally resembles human insulin and activates the human insulin receptor.* Genes & development, 2003. **17**(7): p. 826-831.
69. Leinwand, S.G. and S.H. Chalasani, *Neuropeptide signaling remodels chemosensory circuit composition in Caenorhabditis elegans.* Nat Neurosci, 2013. **16**(10): p. 1461-7.
70. Shtonda, B.B. and L. Avery, *Dietary choice behavior in Caenorhabditis elegans.* J Exp Biol, 2006. **209**(Pt 1): p. 89-102.

71. Zhu, R. and I.D. Chin-Sang, *C. elegans insulin-like peptides.* Mol Cell Endocrinol, 2024. **585**: p. 112173.
72. Chen, Y. and L.R. Baugh, *Ins-4 and daf-28 function redundantly to regulate C. elegans L1 arrest.* Dev Biol, 2014. **394**(2): p. 314-26.
73. Ripoll-Sánchez, L., et al., *The neuropeptidergic connectome of C. elegans.* Neuron, 2023. **111**(22): p. 3570-3589.e5.
74. Beets, I., et al., *System-wide mapping of peptide-GPCR interactions in C. elegans.* Cell Rep, 2023. **42**(9): p. 113058.
75. Hilbert, Z.A. and D.H. Kim, *PDF-1 neuropeptide signaling regulates sexually dimorphic gene expression in shared sensory neurons of C. elegans.* Elife, 2018. **7**.
76. Li, C. and K. Kim, *Family of FLP Peptides in Caenorhabditis elegans and Related Nematodes.* Frontiers in Endocrinology, 2014. **5**.
77. Van Bael, S., et al., *Identification of Endogenous Neuropeptides in the Nematode C. elegans Using Mass Spectrometry.* Methods in molecular biology (Clifton, N.J.), 2018. **1719**: p. 271-291.
78. Bael, S.V., *Neuropeptidomics in C. elegans*, in *Biochemistry and Biotechnology*. 2018, KU Leuven: Betekom, Belgium. p. 216.
79. Van Bael, S., et al., *A Caenorhabditis elegans Mass Spectrometric Resource for Neuropeptidomics.* J Am Soc Mass Spectrom, 2018. **29**(5): p. 879-889.
80. Torayama, I., T. Ishihara, and I. Katsura, *Caenorhabditis elegans integrates the signals of butanone and food to enhance chemotaxis to butanone.* J Neurosci, 2007. **27**(4): p. 741-50.
81. L'Etoile, N.D. and C.I. Bargmann, *Olfaction and odor discrimination are mediated by the C. elegans guanylyl cyclase ODR-1.* Neuron, 2000. **25**(3): p. 575-86.
82. Yang, N.J. and M.J. Hinner, *Getting across the cell membrane: an overview for small molecules, peptides, and proteins.* Methods in molecular biology (Clifton, N.J.), 2015. **1266**: p. 29-53.
83. Meissner, B., et al., *Deletion of the intestinal peptide transporter affects insulin and TOR signaling in Caenorhabditis elegans.* J Biol Chem, 2004. **279**(35): p. 36739-45.
84. Rao, M. and S. Sockanathan, *Molecular mechanisms of RNAi: Implications for development and disease.* Birth Defects Research Part C: Embryo Today: Reviews, 2005. **75**(1): p. 28-42.
85. Bernstein, E., et al., *Role for a bidentate ribonuclease in the initiation step of RNA interference.* Nature, 2001. **409**(6818): p. 363-6.
86. Seydoux, G. and A. Fire, *Soma-germline asymmetry in the distributions of embryonic RNAs in Caenorhabditis elegans.* Development, 1994. **120**(10): p. 2823-34.