Multiple optic gland signaling pathways implicated in octopus maternal behaviors and death

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0. **Summary statement**

Octopus optic glands employ a multiplex progression of signaling molecules to regulate maternal behaviors.

1. **Abstract**

Post-reproductive life in the female octopus is characterized by an extreme pattern of maternal care: the mother cares for her clutch of eggs without feeding until her death. These maternal behaviors are completely eradicated if the optic glands, the octopus analog of the vertebrate pituitary gland, are removed from brooding females. Despite the optic gland's importance in regulating maternal behavior, the molecular features underlying optic gland function are unknown. Here, we identify major signaling systems of the *Octopus bimaculoides* optic gland. Through behavioral analyses and transcriptome sequencing, we report that the optic gland undergoes remarkable molecular changes that coincide with transitions between behavioral stages. These include the dramatic up- and down-regulation of catecholamine, steroid, insulin, and feeding peptide pathways. Transcriptome analyses in other tissues demonstrate that these molecular changes are not generalized markers of aging and senescence, but instead, specific features of the optic glands. Our results provide strong evidence for the functional conservation of signaling molecules across evolutionarily distant species. For example, elevated levels of insulin growth factor binding proteins are associated with cachexia-like tissue wasting in flies, humans, and, reported here, octopuses. Our study expands the classic optic gland-pituitary gland analogy and indicates that, rather than a single "self-destruct" hormone, the maternal optic glands employ multiple pathways as systemic hormonal signals of behavioral control.

2. **Introduction**

Octopuses and other soft-bodied (coleoid) cephalopods are short-lived, semelparous animals: adults die after a single reproductive bout (Rocha et al., 2001). Typically, octopuses lead solitary lives and come together only to mate (Wells, 1978). Females then store sperm in specialized compartments in their oviducal gland (Wells, 1978). As eggs are laid, they pass through the oviducal gland and are fertilized. The female octopus anchors her eggs to substrate with mucal secretions and tends to her clutch as the embryos develop. During this brood period,

she rarely leaves her clutch and abstains from food. By the time of hatchling, the female dies (Anderson et al., 2002; Hanlon and Messenger, 1998; Wells, 1978).

In a key experiment from 1977, Jeremy Wodinsky surgically resected optic glands from female octopuses that were brooding their clutch of eggs and had stopped feeding. Removal of the optic glands led to substantial behavioral changes: females abandoned their clutches, resumed feeding, gained weight, and some even mated again. Glandectomized individuals lived 5.75 months longer than their intact counterparts, leading Wodinsky to conclude that the optic gland and optic gland secretions constituted an octopus "self-destruct system" (Wodinsky, 1977). The molecular features underlying optic gland signaling have not been explored with modern investigative techniques and the putative "optic gland hormone" (Wells, 1978) remains unidentified to this day.

Classic work from Wells and Wells (1959) established that the optic glands are also necessary for the proper timing of sexual maturation. The optic glands are situated on the optic stalks, nestled between the large kidney-shaped optic lobes and the central brain. They are known to receive inhibitory signals from the subpedunculate lobe of the supraesophageal brain (O'Dor and Wells, 1978; Wells and Wells, 1959). At sexual maturity, this inhibition is released; the optic gland swells in size and darkens in color (Wells and Wells, 1975). This change causes the gonads and reproductive organs to mature (Wells and Wells, 1959). Wells posited that the optic glands are the octopus analog to the vertebrate pituitary gland, acting as intermediaries between brain control centers and peripheral target organs (Wells and Wells, 1969).

To gain insight on the molecular features underlying post-reproductive behavioral changes, we sequenced optic gland transcriptomes of the California two-spot octopus, *Octopus bimaculoides*. *O. bimaculoides* lives off the coast of southern California and northern Mexico. We could observe normal maternal behaviors of this species in a laboratory setting. These behavioral findings guided our optic gland RNA sequencing and interpretation of bioinformatics results.

3. **Materials and Methods**

3.1 *Animals and animal facilities*

Wild-caught mated and non-mated female California two-spot octopuses (*O. bimaculoides*) were purchased from Aquatic Research Consultants (Catalina Island, CA) and shipped overnight to Chicago, IL.

All was performed in compliance with the EU Directive 2010/63/EU guidelines on cephalopod use and the University of Chicago Animal Care and Use Committee (Fiorito et al., 2014; Fiorito et al., 2015; Lopes et al., 2017). Mated females were kept in their home dens with clutches as much as possible. Clutches were sparingly separated from females for other experiments. Animals were individually housed in artificial seawater (Tropic Marin) in 20- or 50-gallon aquaria, and offered a daily diet of live fiddler crabs (*Uca pugnax*, Northeast Brine Shrimp, Oak Hill, FL), cherrystone clam meat, and grass shrimp. Water temperature was maintained between 17-21°C and ambient room temperature at 21-23°C. Animal rooms were kept on a 12:12 hour light/dark cycle.

3.2 *Behavioral Categorization*

Upon arrival at our animal facilities, we examined octopuses for signs of injury such as pre-existing skin lesions and missing arm tips. These animals were excluded from further study. Octopuses were kept undisturbed for 4 days so they could habituate to our aquarium setup and recover from the distress of flying. On the 4th day, we began offering live prey items. Each day, females had 4 live fiddler crabs available to them. Animals were observed twice daily (AM and PM) and recorded overnight with a Sony Handycam FDR-AX100 to characterize their behaviors. We observed 20 non-mated animals for at least 4 days before sacrificing and sexing them (see below). 11/20 non-mated animals were males and were excluded from the study. In addition, we longitudinally observed 16 brooding females and categorized them into behavioral stages based on the traits detailed in the Results section and summarized in Table 1.

3.3 *Tissue collection*

Octopuses were submerged in 5-10% ethanol/seawater for at least 20 minutes to achieve deep anesthesia, then decerebrated (mantle and arms removed from the head) (Butler-Struben et al., 2018; Gleadall, 2013). The head was dissected on ice in diethyl pyrocarbonate-treated phosphate buffered solution (DEPC-PBS). Optic glands were accessed between the eyes, harvested, flash frozen in Trizol (Invitrogen), and stored at -76°C until RNA extraction. Because

122 sexual dimorphism is difficult to observe in our species, animals were definitively sexed after
123 tissue harvest. Only females with mature ovaries, ovarian follicles, and no evidence of fertilized
124 eggs were considered non-mated females.
125 We also harvested arm tissue from the same cohort of animals. Transverse arm slices
126 corresponding to the 10th row of suckers distal from the mouth on the second arms on the right
127 and left were cut into quadrants to facilitate tissue homogenization. Each arm piece was
128 separately flash frozen in Trizol and stored at -76°C until RNA extraction.
129
130 3.4 *RNA extraction and sequencing*
131 Whole optic glands were homogenized with pellet pestles (Fisherbrand) in
132 microcentrifuge tubes. Arm sections were homogenized in a Potter-Elvehjem tissue grinder
133 (Kimble, Rockwood, TN). RNA was extracted using Trizol and PhaseMaker Tubes (Life
134 Technologies Corporation, Carlsbad, CA) following manufacturer's instructions. RNA integrity
135 was checked with a Bioanalyzer 2100 (Agilent). Only samples with clean cephalopod rRNA
136 peaks and little to no evidence of degradation were used. Tissue from the right arm was
137 processed for RNA extraction and sequencing. The left arm was used in two samples of feeding
138 females when RNA quality from the right arm was poor.
139 Total RNA was polyA-selected and directionally sequenced at the University of Chicago
140 Genomics Facility on an Illumina HiSeq2500 machine, generating 100bp directional paired-end
141 reads with an insert size of 300bp.
142
143 3.5 de novo *transcriptome assembly and differential gene expression analyses*
144 Following removal of adapters and low-quality sequences, reads were assembled with the
145 Trinity software platform (v2.4.0) (Haas et al., 2013). Both individual and pooled transcriptomes
146 were created (Table 2). Gene and isoform expression levels were estimated with RSEM (v1.2.31)
147 (Li and Dewey, 2011) and differential expression was analyzed with edgeR (v3.7) (Robinson et
148 al., 2009). Heatmaps were created in R with the heatmaps.2 function and colored with palettes
149 from the RColorBrewer package.
150 In the optic gland transcriptomes, 1,198 Trinity transcripts showed at least a 4-fold
151 change between any two of the behavioral stages, at a p-value of 0.001. A phylogeny of
152 differentially expressed genes was created by Euclidean clustering (Supplemental Fig 1). The

153 tree was cut at a height of 0.3, which identified 25 subclusters. 20 genes from subclusters of
154 interest (see below) were confirmed by PCR amplification.
155
156 4. **Results**
157 4.1 *Adult life in the female O. bimaculoides can be divided into 4 behavioral stages.*
158 Sexually mature female octopuses are known to be active predators (Hanlon and
159 Messenger, 1998). We confirmed this behavior in non-mated females in the lab: these animals
160 spent time outside of the den (Fig 1A) and primarily hunted through a visually-directed jet-
161 propelled "pounce" (Fig 1B and Movie S1) (Wells, 1978). The female first watches the prey out
162 of one eye, then bobs her head up and down, likely acquiring depth information. Even while
163 moving her head, her pupil is kept perpendicular to gravity (Fig 1A). Following a swift pounce,
164 prey is captured in the interbrachial web (Fig 1B and C). Non-mated females consumed
165 1.31±0.76 crabs a day, sometimes skipping a day between meals.
166 Mated females in the first stage of brooding actively tended their clutch by guarding their
167 den, stroking eggs with their suckers, and blowing water over the eggs with their funnels
168 (Hanlon and Messenger, 1998; Wells, 1978). In the lab, feeding mated females consumed
169 1.17±0.70 crabs per day, with fewer than 3 days without a meal. Feeding females, however,
170 rarely left their egg clutches. Instead of capturing prey by stalking and pouncing, feeding females
171 peered out of their dens with one eye and grabbed prey as they moved passed the mouth of the
172 den (Fig 2A and B and Movie S1). The presence of empty crab carapaces provided additional
173 confirmation that feeding females consumed food, rather than merely capturing and killing prey.
174 On average, mothers fed for 8±2.71 days before starting to fast.
175 Females in the second stage of brooding continued to care for eggs but abstained from
176 feeding, even though live prey items were available at all times (Fig 2C and Movie S1). Females
177 who stopped feeding for 4 consecutive days were never observed to feed again, and we grouped
178 these animals into the fasting stage.
179 After 11±8.49 days, fasting mothers began a rapid decline. Behavioral signs of decline,
180 which have not been described previously, were observed before physiological signs (Fig 3A):
181 females left their clutches and spent time outside of the den, languidly sitting on the bottom of
182 the tank or persistently smashing themselves into the corners of the tank. The latter action led to
183 the rapid formation of deep avulsions on the mantle or arms that did not heal (Fig 3B and C).

184 Females also engaged in excessive self-grooming behaviors. Normally, octopuses run their first
185 pair of arms over their mantles and head to remove ectoparasites and debris (Mather and Alupay,
186 2016). Decline mothers moved all of their arms to groom, but often arms would only pass over
187 other arms, creating a turbulent mass of entangled limbs (Fig 3A). In a few cases, this over-
188 grooming behavior was directly followed by self-cannibalization of the arm tips or suckers (Fig
189 3B and C and Movie S1). Physiological features of females in decline included a retraction of the
190 skin around the eyes, general paling of skin color, and loss of motor tone (Anderson et al., 2002).
191 In addition, the pupils also gradually ceased to remain perpendicular to gravity, raising the
192 possibility that the central systems responsible for the vestibulo-ocular reflex deteriorate during
193 this stage.
194 In all cases, mated females progressed from feeding to fasting to decline before dying:
195 that is, mated females did not transition from feeding directly to decline without fasting, and
196 females did not die without showing indications of decline. Our observations suggest that after
197 mating, female octopuses undergo a series of near-requisite behavioral transitions before death.
198
199 4.2 *Transcriptomes of brooding females implicate many signaling systems in optic gland*
200 *function.*
201 We assembled transcriptomes of left and right optic glands harvested from multiple
202 individuals in each of the 4 stages (Table 2). Our assembled transcriptomes showed that the optic
203 glands from non-mated animals were the most different from the other optic gland samples
204 (Supplement Fig 2). After estimating transcript abundance with RSEM, we identified 1,198
205 differentially expressed transcripts with edgeR and grouped them into 25 subclusters
206 (Supplement Fig 1). Wodinsky's work suggested that optic gland signaling changes steadily over
207 time until reaching a threshold that triggers fasting and later death (Wodinsky, 1977). In
208 selecting subclusters for further study, we were guided by two criteria. We excluded 12
209 subclusters that showed a departure from monotonicity: for example, when expression peaked in
210 only the feeding or fasting stages (Supplement Fig 3). Another 5 subclusters demonstrated the
211 most marked transcript enrichment or impoverishment at the decline stage, the last stage of
212 brooding before death (Supplement Fig 4). Because of sample scarcity (only 2 RNA-sequencing
213 samples in decline stage and a limited ability to pursue follow-up studies), we did not explore
214 these subclusters further. Five additional clusters showed monotonic changes and were studied

215 with BLASTP as described below (Supplement Fig 5). No candidate signaling molecules were
216 identified. In total, we excluded 855 genes in 22 subclusters from further analyses.
217 We focused on the 343 remaining genes in 3 subclusters. Among these subclusters (Fig
218 4), we saw dramatic transitions in differential gene abundance across behavioral stages. Two
219 subclusters showed increases in expression over time (Fig 4A and B), while the third showed a
220 drop in expression at the non-mated female to feeding mated female transition (Fig 4C).
221 We used BLASTN, BLASTP, and PFAM (Altschup et al., 1990; Finn et al., 2016) to
222 identify transcripts (Fig 5). In all clusters examined, there were many housekeeping genes, such
223 as actin and collagen. We also found predicted proteins with no homology to genes of other
224 species in available databases (downloaded October 28, 2015). These uncharacterized genes may
225 represent octopus- or cephalopod-specific proteins. All three clusters did, however, contain a
226 number of transcripts of particular relevance to optic gland biology.
227
228 *4.2.1 Green cluster Fig 4A: genes in this cluster increased in abundance after mating and*
229 *remain elevated through the maternal stages.*
230 Although the optic gland is regarded as a neuroendocrine organ, its mechanisms of
231 secretory release are unknown. We found transcripts implicated in neural signaling that increased
232 in abundance after mating and remained elevated through all brooding stages (Fig 4A and 5A):
233 synaptotagmin 12, synaptotagmin 15b, and clathrin. Huntingtin-interacting protein (HIP1) and
234 coronin showed a similar expression profile. Although not included in classical models of
235 synaptic transmission, proteins encoded by these two transcripts have in recent years been found
236 to be important for aspects of neuronal signaling (Borlido et al., 2009; Suo et al., 2013). We also
237 saw increases in selected neurotransmitter receptors (Fig 5B), including an alpha subunit of
238 nicotinic acetylcholine receptor, and G-coupled protein receptors similar to octopamine and
239 neuropeptide S receptors in other species. Finally, signaling molecules, including candidate
240 signaling peptides and enzymes implicated in steroid biogenesis, were present in this subcluster
241 (Fig 5C). These results suggest that the optic gland is a site of active signaling during the
242 maternal period, and that the nature of this signaling is different from that of unmated female
243 optic glands.
244

245 *4.2.2 Purple cluster (Fig 4B): genes in this cluster increased with the transition from feeding to*
246 *fasting.*
247 In this subcluster, we found that the insulin-binding protein Impl2 is enriched in the optic
248 glands of fasting and decline animals (Fig 4B and 5D). This transcript is joined in expression
249 dynamics by two others, tribbles homolog 1 (TRB1) and an additional isoform of synaptotagmin.
250
251 *4.2.3 Blue cluster (Fig 4C): non-mated optic glands are enriched in neuropeptides that*
252 *precipitously drop in abundance in brooding females.*
253 As in the other subclusters, we found genes encoding proteins involved in cell signaling
254 (Fig 5E), such as lipocalin and dopamine beta-hydroxylase. Particularly notable in this subcluster
255 are the feeding circuit-related neuropeptides and carboxypeptidase (Fig 5E).
256
257 4.3 *Are molecular changes of the optic glands a reflection of global changes in gene expression?*
258 The abundance of genes encoding neuropeptides in the non-mated optic gland and their
259 subsequent depletion in mated females motivated us to investigate whether molecular changes in
260 the optic gland were mirrored in other tissues of the body. Many of these tissues were sampled in
261 the arm transcriptomes, including axial nerve cords, suckers, muscles, chromatophores, and skin.
262 Using BLASTP and TBLASTN, we baited the arm transcriptomes from all behavioral stages
263 with the genes that were significantly enriched in non-mated optic glands (blue cluster, Fig 4C
264 and 5E). We were unable to recover any of the optic gland neuropeptides in the arm
265 transcriptomes.
266
267 5. **Discussion**
268 With their large central nervous systems and wide range of behaviors, coleoid
269 cephalopods have long fascinated neuroscientists. Pioneering work from Wodinsky and Wells
270 and Wells established that the octopus reproductive axis is similar to that of the vertebrate
271 hypothalamic-pituitary-gonadal axis (Wells and Wells, 1975; Wodinsky, 1977). The optic gland,
272 analogous to the pituitary gland, is the fulcrum of the octopus reproductive axis; it is responsible
273 for innate reproductive behaviors and post-reproductive death. In this study, we confirmed that
274 maternal behaviors can be observed in the lab, including, most importantly, a period of feeding

275 while brooding that precedes the fasting that typically defines the maternal period in octopuses
276 (Movie S1) (Hanlon and Messenger, 1998; Rocha et al., 2001).
277 Our transcriptomic analyses reveal for the first time a molecular portrait of the optic
278 glands at the distinct behavioral stages of adult female octopus life. In particular, we found that
279 reproduction and the onset of fasting trigger major transcriptional activation in the optic glands.
280 Most importantly, multiple signaling systems of the optic glands, including catecholaminergic,
281 peptidergic, and steroidogenic pathways, are implicated in these behaviors.
282
283 *5.1 Optic Gland Signaling Systems*
284 *5.1.1 Feeding peptides*
285 Circulating neuropeptides have been extensively studied in molluscan feeding and
286 reproductive systems (Grimmelikhuijzen and Hauser, 2012; Jékely, 2013). Neuropeptides can
287 influence feeding behaviors through control of motor circuits or modulation of arousal or
288 satiation (Bechtold and Luckman, 2007). Strikingly, we found octopus homologs of these
289 neuropeptides enriched in the optic glands of non-mated female as compared with those of
290 brooding females: feeding circuit activating peptide (FCAP), allatostatin, FMRFamide, and
291 PRQFVamide. The latter 3 peptides have been shown to inhibit feeding behaviors in a diverse
292 range of animals. In Aplysia, FMRFamide and its related neuropeptides inhibits closure of the
293 radula muscle during feeding (Sossin et al., 1987) and in mice, FMRFamide inhibits feeding in
294 food-deprived individuals (Kavaliers et al., 1985). PRQFVamide decreases the excitability of
295 specific neurons in the buccal ganglion feeding central pattern generator and has an overall
296 inhibitory effect on feeding (Furukawa et al., 2003). Allatostatin inhibits feeding in Drosophila
297 by mediating the balance between adipokinetic hormone and insulin-like peptides (Hentze et al.,
298 2015). FCAP, on the other hand, initiates rhythmic feeding motor programs and may be
299 responsible for food-induced arousal (Sweedler et al., 2002).
300 These data suggest that signaling from an ensemble of neuropeptides tightly controls the
301 feeding behaviors of non-mated animals. In many animals, mechanisms controlling feeding and
302 reproduction are tightly linked and enables metabolic shifts as the individual transitions from
303 growing and maturing to reproduction and tending to young (O'Dor and Wells, 1978).
304 Drosophila females, for example, modify their feeding preferences to consume more protein
305 when they are about to lay eggs (Ribeiro and Dickson, 2010). The reduction in feeding circuit-

306 related neuropeptides at the non-mated to mated transition strongly suggests that, in the octopus,
307 feeding and reproductive state also interact.
308 Although neuropeptide expression precipitously drops after reproduction, mated animals
309 continue to feed in the initial stage of brooding. However, feeding behavior between mated and
310 non-mated animals manifested in different ways: mated feeding octopuses captured crabs that
311 were within arm's distance of their dens rather than leave the den to pursue prey, as non-mated
312 females regularly did. This behavioral switch suggests that different feeding strategies are
313 optimal for non-mated and mated females: the feeding-related peptides may regulate energy
314 expenditure or drive to hunt for food. Our data also raise the possibility that redundant
315 peptidergic pathways, or additional non-peptidergic systems, control feeding behaviors. These
316 systems may be independent of the optic gland.
317 These neuropeptides may also broadly participate in neurotransmission well beyond
318 feeding. If this is the case in the octopus, peripheral tissues would also express these
319 neuropeptides. We checked if these neuropeptides were present in the arm transcriptomes, which
320 included nervous tissue (axial nerve cord), muscle, skin, chromatophores, and suckers.
321 Expression changes of these genes in the arms would suggest that global changes in neuropeptide
322 signaling accompany the aging and senescence process in general. However, all feeding-related
323 neuropeptides in the blue subcluster (Fig 4C and 5E) were missing from the arm transcriptomes,
324 suggesting that their expression is a specific feature of the optic gland. These data are surprising
325 since the optic glands previously have not been implicated in feeding *per se*. Future experiments
326 will reveal if these neuropeptides are functionally conserved in octopuses.
327
328 *5.1.2 Steroid biosynthesis*
329 Steroids are one of the most evolutionary ancient classes of signaling molecules (Baker,
330 2011; Nelson and Studer, 2015). Derived from cholesterol, steroids include the sex hormones
331 and corticosteroids. We found that a collection of cholesterol-metabolizing enzymes increased in
332 expression in the first stage of brooding and remained elevated through the maternal period.
333 These included enzymes known to synthesize steroid hormones in diverse animals, from
334 ecdysozoa to deuterostomes (Antebi, 2015). Dafachronic acid in *C. elegans*, for example,
335 regulates longevity and dauer formation, and its biosynthesis relies on the sequestration of
336 cholesterol by daf36/cholesterol 7-desaturase (Yoshiyama-Yanagawa et al., 2011). 3beta-

337 hydroxysteroid dehydrogenase is an oxidoreductase that catalyzes the formation of progesterone
338 in the adrenal gland and gonads of vertebrates (Aakvaag, 1970; Conley and Bird, 1997). Sterol
339 27-hydroxylase (CYP27a) breaks down cholesterol for both bile acid synthesis and hormone
340 production (Dubrac et al., 2004). Sulfotransferase 1C2 catalyzes the conjugation of a sulfate
341 group to steroid hormones (Foster and Mueller, 2018).
342 Our study is the first to implicate the optic glands in the production of progesterone and
343 other steroid hormones. Cellular and molecular mechanisms of steroid hormone signaling in
344 octopuses may be different from what has been described previously in the vertebrate
345 neuroendocrine systems. Substrate specificity of these enzymes may differ between species
346 (Antebi, 2015). Functional characterization of these enzymes or identification of steroid
347 metabolites will be particularly useful going forward. Moreover, steroid signaling in molluscs
348 may not be mediated through the receptors orthologous to those identified in vertebrates
349 (Thornton et al., 2003). The *Octopus vulgaris* ortholog of the vertebrate estrogen nuclear
350 hormone receptor, for example, does not bind estrogen (Keay et al., 2006). Our identification of
351 the coordinate enrichment of cholesterol-metabolizing enzymes in mated female octopuses raises
352 the exciting possibility that the optic glands produce a suite of steroid-derived hormones that are
353 specific to the reproductive adult and may have novel cellular targets.
354
355 *5.1.3 Insulin signaling*
356 Our data reveal that expression of ImpL2 increased at the transition from feeding to
357 fasting (Fig 4B and 5D). In *Drosophila*, ImpL2 is a homolog of insulin-like growth factor
358 binding protein (IGFBP) that reduces activity of the insulin/IGF signaling pathway (Figueroa-
359 Clarevega and Bilder, 2015). The insulin/IGF signaling pathway controls many biological
360 processes, including glycogen metabolism and organismal growth. Insulin-like signaling
361 molecules have been found in many invertebrates (Ahn et al., 2017; Veenstra, 2010), but
362 functional characterization of these molecules in molluscs has been limited to a few species
363 (Gricourt et al., 2003; Hamano et al., 2005). In the Pacific oyster *Crassostrea gigas*, insulin-like
364 molecules stimulate protein synthesis in mantle edge cells, promoting growth in the shell and
365 soft tissues (Gricourt et al., 2003). Seasonal fluctuations in insulin-related peptide expression
366 coincide with body growth and gametogenesis (Hamano et al., 2005).

367 The actions of the insulin/IGF signaling pathway in octopus are unknown. Females in the
368 decline stage have the highest expression of ImpL2. At this terminal stage of brooding, female
369 octopuses show several indications of wasting, including loss of muscle tone and color.
370 *Drosphila* implanted with malignant tumors secrete high levels of ImpL2 and show marked
371 degradation of tissues, including gonadal, muscle, and adipose tissue (Figueroa-Clarevega and
372 Bilder, 2015; Kwon et al., 2015). These remarkable similarities lead us to predict that the role of
373 Impl2 as an antagonist of the insulin/IGF-signaling pathway is functionally conserved between
374 octopuses and *Drosophila*.
375 ImpL2-induced tissue wasting is independent of food intake in *Drosophila*: tumor-
376 bearing hosts with high ImpL2 signaling show no differences in food ingestion or feeding
377 behavior from that of control hosts, and high calorie food was insufficient to prevent tissue
378 wasting (Figueroa-Clarevega and Bilder, 2015). In contrast, octopus ImpL2 expression coincides
379 with cessation of feeding. Remarkably, results of high ImpL2 in both *Drosophila* and octopus are
380 consistent with symptoms of cancer cachexia in humans. Patients with cachexia experience an
381 involuntary loss of body mass, undergo substantial degradation of muscle and fat, and report a
382 loss of appetite (Argilés et al., 2014). Medical interventions focusing on increasing caloric intake
383 fail to counteract wasting (Fearon et al., 2012; Op den Kamp et al., 2009)}.
384 Our results suggest that ImpL2 is intimately tied to feeding behaviors in octopus and
385 implicate the optic glands as a regulator of insulin signaling. These data raise the possibility of a
386 conservation of function in insulin-signaling pathways with animals as distantly related as
387 humans and octopuses. Indeed, clinical work on humans has shown that cachexia and other
388 metabolic conditions are associated with elevated levels of IGFBPs (Clemmons et al., 1991;
389 Huang et al., 2016). Our study, however, is the first direct implication of IGFBP enrichment in a
390 physiological, non-pathological, process.
391
392 *5.1.4 Neurotransmitter biosynthesis and neurotransmission*
393 The optic gland regulates sexual maturation and behavior by hormone release, but
394 cellular mechanisms of secretion are unknown. We identified a number of genes implicated in
395 neurotransmission and secretion enriched after reproduction (Fig 4A and 5A). Clathrin, for
396 example, facilitates the formation of coated secretory vesicles, and synaptotagmins contribute to
397 the cellular complex that enables vesicle fusion for regulated exocytosis (Brunger et al., 2018;

398 Wu et al., 2014). Specific isoforms of both are enriched in the optic glands at all mated stages.
399 Our finding mirrors existing data on the organization of the vertebrate reproductive axis:
400 synaptotagmins and other proteins involved in classical neurotransmission are differentially
401 expressed in the anterior pituitary gland where they are involved in the release of signaling
402 vesicles and other secretory granules (Jacobsson and Meister, 1996). Moreover, the enrichment
403 of neurotransmitter receptors (Fig 5B), including octopamine receptor, neuropeptide S receptor,
404 and a subunit of nicotinic acetylcholine receptor, suggests that the optic gland undergoes shifts in
405 the signals it receives from the central brain, or in auto- or endocrine signaling, after
406 reproduction.
407 Optic glands of non-mated animals showed elevated expression of dopamine β-
408 hydroxylase (DBH, Fig 5E), an enzyme involved in the synthesis of catecholamine
409 neurotransmitters. DBH has been shown to catalyze the β-hydroxylation of dopamine to
410 norepinephrine in vertebrates and tyramine to octopamine in invertebrates (Fernstrom and
411 Fernstrom, 2007; Monastirioti, 1999). These small molecules are used for signaling throughout
412 nervous systems. In the octopus optic gland, candidate aminergic secretory vesicles have been
413 found (Nishioka et al., 1970). Our results suggest that the optic gland engages in endocrine
414 catecholamine signaling and that reproduction leads to the specific shutting down of
415 catecholamine pathways, as opposed to an overall decrease in neurotransmission or cell signaling
416 in the optic glands.
417
418 5.2 *Octopus semelparity*
419 Mechanisms of death differ among animal systems exhibiting semelparity. In several
420 vertebrate species, it is believed that the metabolic cost of gamete production and finding a mate
421 are so high that death is inevitable (Fisher and Blomberg, 2011; Kindsvater et al., 2016).
422 Semelparity in octopuses cannot be explained by the large metabolic output required to produce
423 and deposit eggs: glandectomized animals can reproduce again (Wodinsky, 1977). Instead, it is
424 likely that other evolutionary mechanisms drive post-reproductive death. Coleoid cephalopods
425 are cannibalistic: hatchlings from the same clutch often eat each other, and females sometimes
426 kill males after reproduction (Hanlon and Messenger, 1998). Post-reproductive death is an
427 undeniably effective method in ensuring that the reproductive female does not consume her
428 young. Moreover, longitudinal studies of octopuses, and other molluscs, demonstrate that

429 individuals continue to undergo logarithmic growth as adults (Gricourt et al., 2003; Nixon, 1969;
430 Van Heukelem, 2010; Wells and Wells, 1970). Endless growth without death would so skew
431 octopus populations towards large, old adults that hatchlings would be unlikely to compete.
432 Programmed organismal death in coleoids may exist as a mechanism to safeguard the survival of
433 the next generation of these voracious predators.
434 In Pacific salmon *Oncorhynchus kisutch*, steroid hormone signaling has been implicated
435 as the cause of death after spawning. Elevated cortisol levels mediate programmed death through
436 tissue degeneration and impaired homeostatic ability (Robertson and Wexler, 1960). Our data
437 show that steroid signaling is one of several pathways implicated in octopus post-reproductive
438 death. Although we do not see molecular evidence of cortisol signaling *per se* in our data, the
439 enrichment of cholesterol-metabolizing enzymes in mated females suggests that steroid
440 metabolites are crucial to semelparity in octopuses. It will be important to identify the steroids in
441 the optic gland and assess the contributions of steroid and ImpL2 signaling, along with the
442 reduction in feeding peptides and catecholamine signaling, in cephalopod post-reproductive
443 death.
444
445 *5.3 Conclusions*
446 Our transcriptome findings suggest that, far from secreting a single hormone, the optic
447 gland likely enlists multiple signaling systems to regulate post-reproductive behaviors and death
448 (Fig 6). Among these are neuropeptidergic systems and cholesterol-derived hormone signaling.
449 Our data raise the possibility that prior to mating, optic gland signaling is dominated by
450 neuropeptides and catecholamines, whereas after mating, it is dominated by steroid hormones.
451 Wells and Wells originally drew a connection between the direct brain regulation of the
452 octopus optic glands and the vertebrate hypothalamic-pituitary-gonadal axis (Wells and Wells,
453 1969). Here, we identify a difference between octopus and vertebrate systems: the endocrine
454 signaling of the anterior pituitary gland depend on peptide hormones alone (Davis et al., 2013),
455 whereas our data implicate steroid and catecholamine pathways, in addition to peptide signaling,
456 in the functions of the optic gland. While steroid hormones do affect the actions and feedback of
457 the vertebrate pituitary gland, steroidogenesis occurs in targets of the pituitary gland only, such
458 as the adrenal cortex, and not in the pituitary gland itself (Handa and Weiser, 2014; Werbin and
459 Chaikoff, 1961). Similarly, biosynthesis of catecholamines, such as norepinephrine and

460 dopamine, occurs in the adrenal medulla in response to autonomic stimulation (Fujinaga et al.,
461 1999).
462 If the optic gland encompasses functions of the anterior pituitary gland and the adrenal
463 glands, it prompts the question of what glandular targets of the optic gland, if any, might exist.
464 The oviducal glands, which produce substances that cover the eggs, are candidates. These
465 glands, along with the gonads and oviducts, were found to increase in size as the optic gland
466 enlarges (Wells and Wells, 1959; Wells and Wells, 1975). *In vitro* experiments demonstrated that
467 oviducal secretion is modulated by dopamine and neuropeptides, including FMRFamide (Di
468 Cristo and Di Cosmo, 2007). However, the putative source of direct oviducal control is the
469 fusiform ganglion, not the optic glands. The fusiform ganglion is thought to innervate the
470 oviducal gland, the oviducts, and the systemic heart (Young, 1971). Notably, the fusiform
471 ganglion has been found to contain FMRFamide-like immunoreactive nerve endings (Di Cristo
472 et al., 2003). The optic gland may exert indirect control over glandular targets by signaling to
473 peripheral ganglia, or act in parallel with peripheral ganglia to regulate reproductive tissues.
474 Our study extends the optic gland-pituitary analogy and uncovers similarities between the
475 optic glands and the vertebrate adrenal glands, demonstrating that the organization and function
476 of the optic glands may be even more pivotal for octopus organismal physiology than previously
477 appreciated.
478
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487
488 7. **Declaration of interests**
489 The authors declare no competing interests.
490

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10. **Figure Legends**

Fig 1. Non-mated, sexually mature females are active predators that hunt live prey. A) Females in this stage spend time outside of their home den. B) Non-mated females use visual direction to strike prey with a "pounce," viewed here from the front. C) The webbing between the arms is spread wide to capture prey. Several fiddler crabs may be restrained under the interbrachial web at one time.

Fig 2. Brooding females feed and then fast. A) In the first stage of brooding, females actively tend to their eggs and feed. The pupils of feeding females remain perpendicular to gravity. B) Feeding females capture and kill live prey, but only without leaving the home den. White arrowhead indicates the legs of a captured fiddler crab extending from beneath the arms. C) Fasting females continue tending to their clutch but abstain from feeding even when live prey (red arrowhead) are within reach.

Fig 3. **Females in decline continue fasting and undergo rapid senescence**. A) Behavioral signs of decline include spending time outside of the home den and recruiting all the arms in bouts of aberrant, grooming-like behavior. Females also show physiological signs of decline, such as an apparent loss of muscle tone and unhealed wounds. B) Female with a patch of missing skin at the distal end of her mantle (white arrowhead). C) Female with several rows of missing suckers and a missing arm tip (black arrowheads), presumably from self-cannibalization.

Fig 4. Cohorts of genes are differentially expressed across behavioral stages. Green and purple subclusters identify genes that increased in abundance as the maternal stages progressed (A and B, respectively), while the blue subcluster illustrates genes that decreased over time (C).

Fig 5. Expression profiles of differentially expressed genes important to optic gland signaling. Heatmap colors correspond to cluster colors depicted in Fig 4. Columns are organized as designated at the top of in panel A. Color key values refer to row z-scores.

Fig 6. Updated model of optic gland function in maternal behaviors and death. Arrows indicate behavioral groups (pink: feeds; yellow: abstains from feeding). Signaling systems above arrows highlight enriched genes.

11. **Tables**

Stage	Traits
Non-mated	Actively hunts and consumes live prey: on average 1.31±0.76 crabs per day, sometimes skipping one day between meals Mature ovary and ovarian follicles No evidence of fertilized or developing eggs
Feeding	Consumes, but does not pursue, live prey: on average 1.17±0.70 crabs per day, sometimes skipping one day between meals Actively broods clutch of eggs by blowing water over them with funnel and cleaning them with suckers Extremely den-bound, exhibiting reluctance to leave the den, even for brief periods of time
Fasting	Abstains from capturing live prey or consuming any food for 4 consecutive days Ongoing brooding Ongoing den-bound tendencies
Decline	Ongoing fasting Exhibits at least 2 of the following signs of physical deterioration: • skin lesions on the mantle, arm webbing, or head • missing arm tips or suckers • degradation of the vestibular-ocular reflex • retraction of skin around the eyes • extended bouts of self-grooming Spends considerable time roaming outside of the den

Table 1. Octopus female reproductive life is characterized by four stages of behavior.

712

Transcriptome	# of libraries prepared	Total Trinity 'Genes'	Total Trinity Transcripts	N10 (bp)	N50 (bp)	Average Contig Length (bp)	Total Assembled Bases (bp)
Unmated Female Optic Gland	3	267380	314115	4012	882	604.80	189975279
Feeding Mom Optic Gland	3	290774	344673	4078	909	616.68	212552829
Fasting Mom Optic Gland	3	254893	301599	4011	938	627.42	189228407
Decline Mom Optic Gland	2	217478	261725	4554	1165	688.47	180189329
Combined Optic Gland	--	598083	706186	3897	767	574.65	405807628
Unmated Female Arm	3	120017	189097	5771	1781	904.15	170971875
Feeding Mom Arm	3	296853	406986	5196	1165	676.91	275491458
Fasting Mom Arm	3	183259	278194	5683	1621	823.35	229050350
Decline Mom Arm	2	153752	233040	5514	1650	841.44	196090319
Combined Arm	--	493537	686453	4485	959	639.22	438793048

713 **Table 2. *O. bimaculoides* optic gland transcriptome assembly summary**. Whole optic glands
714 and transverse arm sections were harvested (see Methods). Separate libraries were prepared for
715 each sample, and reads were later concatenated for transcriptome assembly. Statistics are based
716 on transcript contigs. Total Trinity 'genes' refers to the number of transcript clusters generated
717 by the assembly (isogroups), while Total Trinity Transcripts indicates the number of isoforms
718 (isotigs). The Nx length statistic indicates that at least x% of the assembled transcript bases are
719 found on contigs that are of at least Nx length; for example, at least 10% of the assembled bases
720 in the unmated female optic gland transcriptome are found on transcript contigs that are at least
721 4012 bp long.

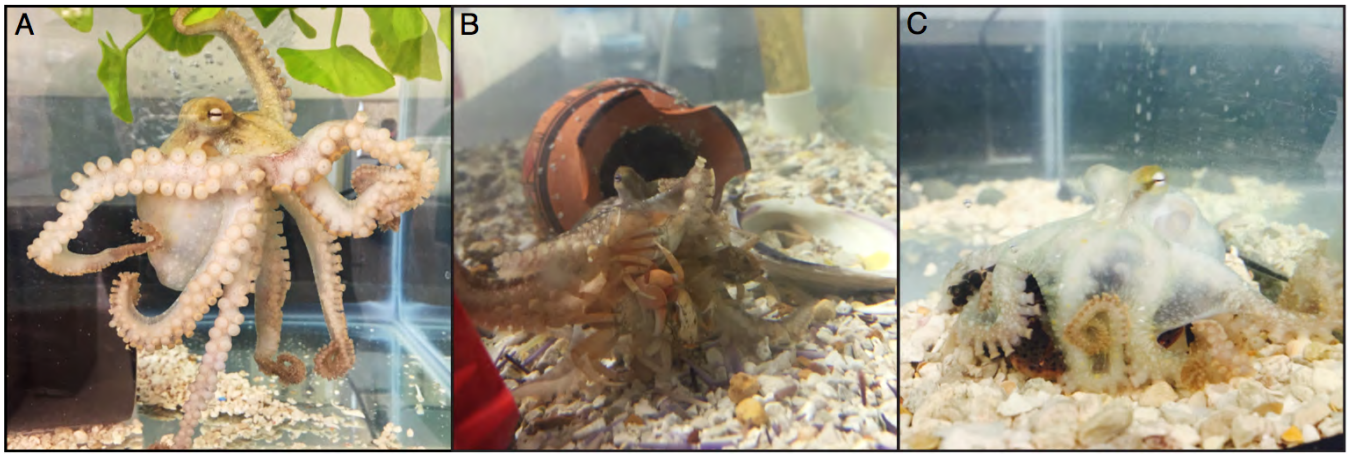
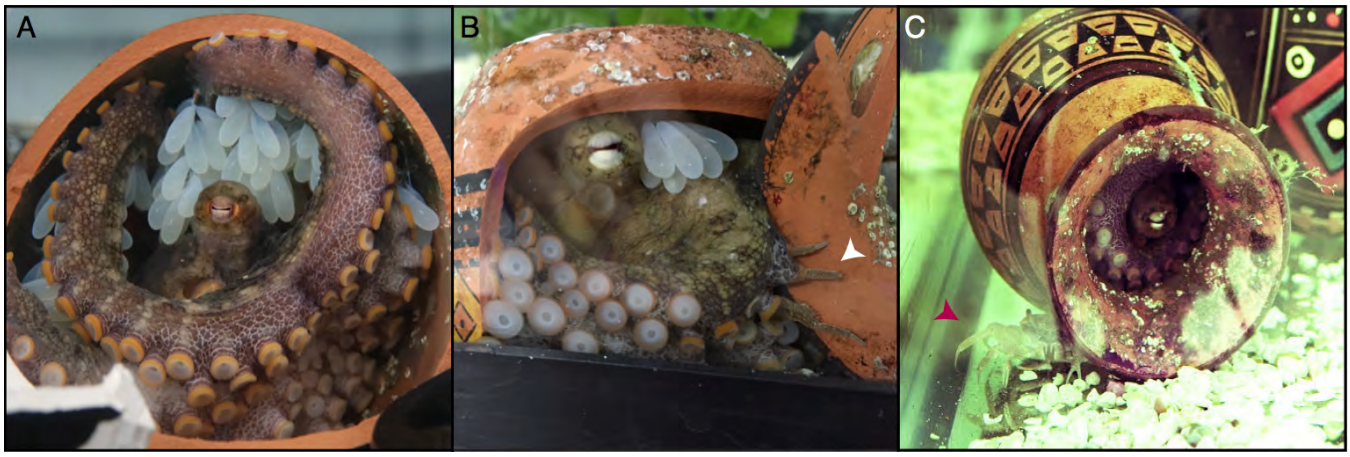


Fig 1. Non-mated, sexually mature females are active predators that hunt live prey. A) Females in this stage spend time outside of their home den. B) Non-mated females use visual direction to strike prey with a “pounce,” viewed here from the front. C) The webbing between the arms is spread wide to capture prey. Several fiddler crabs may be restrained under the interbrachial web at one time.



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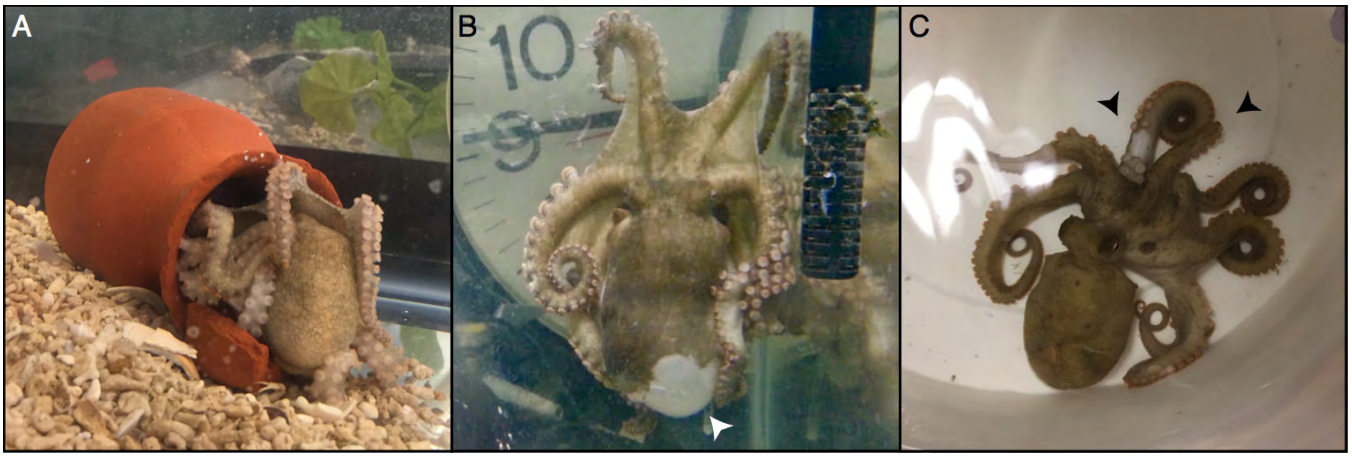

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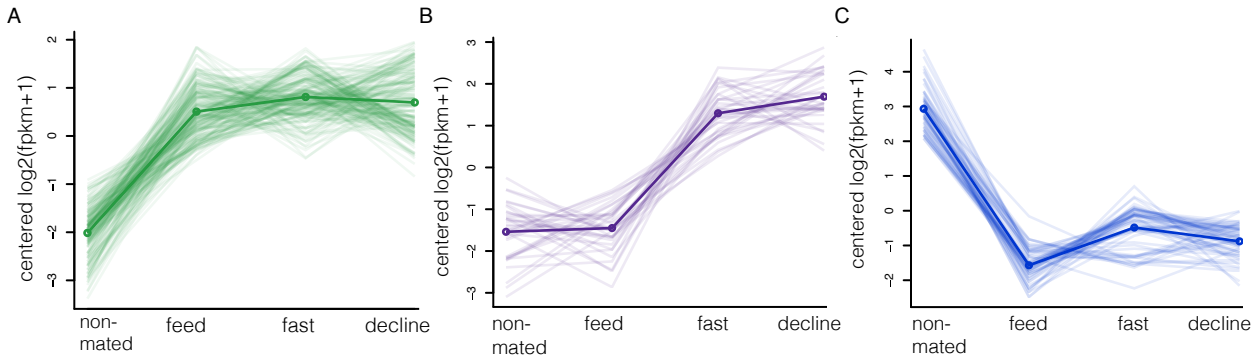

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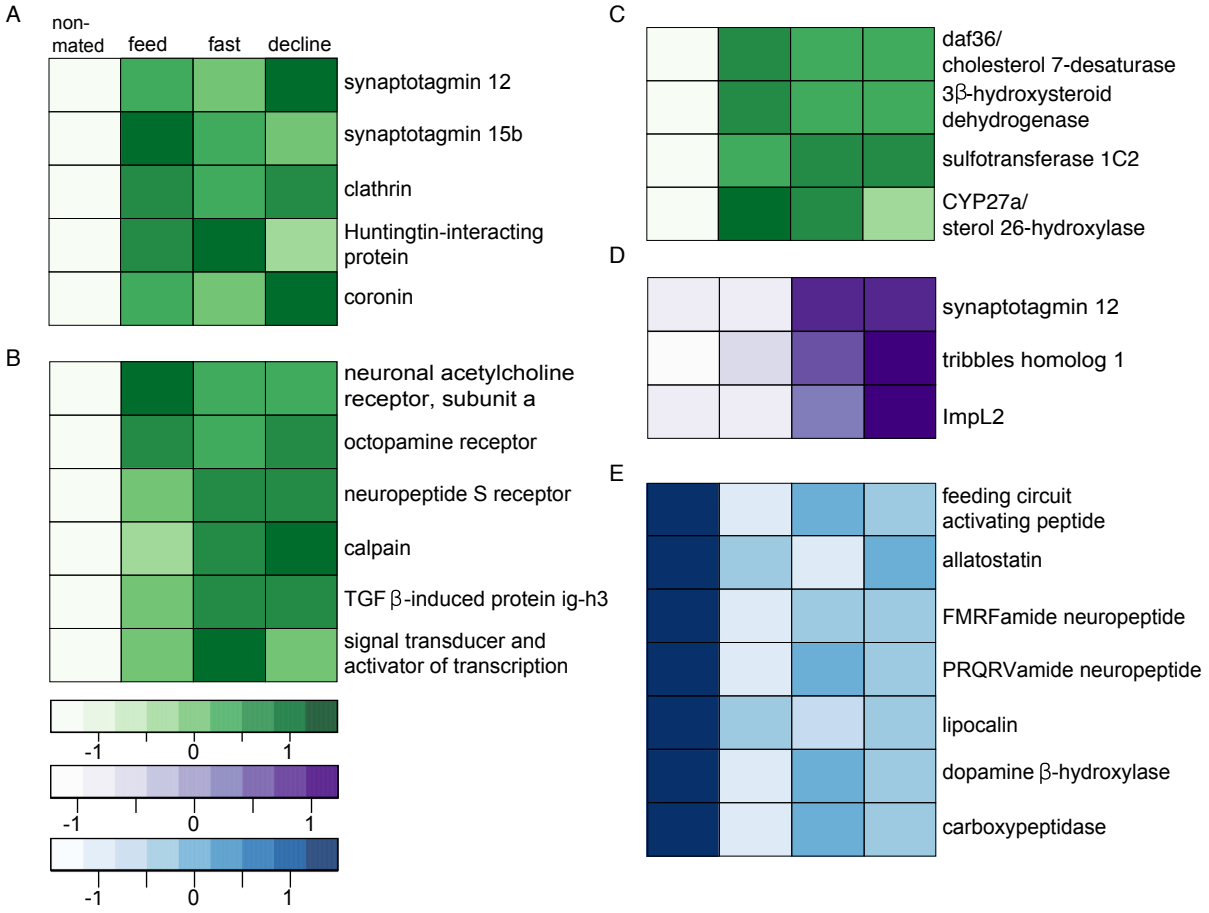

Fig 5. Expression profiles of differentially expressed genes important to optic gland signaling. Heatmap colors correspond to cluster colors depicted in Fig 4. Columns are organized as designated at the top of in panel A. Color key values refer to row z-scores.

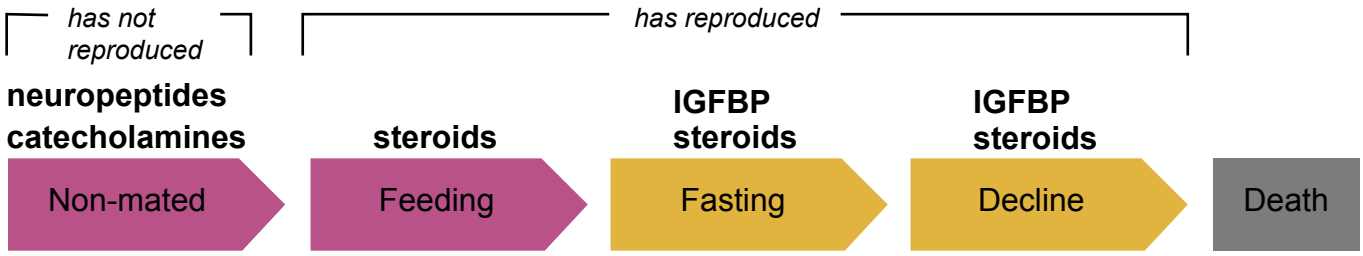

Fig 6. Updated model of optic gland function in maternal behaviors and death. Arrows indicate behavioral groups (pink: feeds; yellow: abstains from feeding). Signaling systems above arrows highlight enriched genes.