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RESEARCH ARTICLE

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

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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 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 upregulation and downregulation of catecholamine, steroid, insulin and feeding peptide pathways. Transcriptome analyses in other tissues demonstrate that these molecular changes are not generalized markers of senescence, but instead, specific features of the optic glands. Our study expands the classic optic gland–pituitary gland analogy and more specifically, it indicates that, rather than a single 'self-destruct' hormone, the maternal optic glands employ multiple pathways as systemic hormonal signals of behavioral regulation.

KEY WORDS: IGFBP, Cephalopod mollusc, Feeding, Hormones, Neuroendocrine signaling, Senescence

INTRODUCTION

Octopuses and other soft-bodied (coleoid) cephalopods are short-lived, semelparous animals: adults die after a single reproductive period (Rocha et al., 2001). Typically, octopuses lead solitary lives and come together only to mate (Wells, 1978). Females 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 hatching, the female dies (Anderson et al., 2002; Hanlon and Messenger, 2018; Wells, 1978).

In a key experiment from 1977, Jerome Wodinsky surgically resected optic glands from female Caribbean two-spot octopuses (*Octopus hummelincki*) that were brooding their clutches 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 and Wells (1969) posited that the optic glands are the octopus analog to the vertebrate pituitary gland, acting as intermediaries between brain control centers and peripheral targets.

To gain insight into the molecular features underlying post-reproductive behavioral changes, we sequenced optic gland transcriptomes of the California two-spot octopus, *Octopus bimaculoides* Pickford & McConnaughey 1949. *Octopus 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.

MATERIALS AND METHODS

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, USA) and shipped overnight to Chicago, IL, USA.

All work 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, 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, USA), cherrystone clam meat and grass shrimp. Water temperature was maintained between 17 and 21°C and ambient room temperature at 21–23°C. Animal rooms were kept on a 12 h:12 h light:dark cycle.

Behavioral categorization

Upon arrival at our animal facilities, we examined octopuses for signs of senescence, such as pre-existing skin lesions and missing arm tips. We excluded these individuals, which included females

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with clutches, from further study. Clutches from the animals ranged in developmental stage from gastrulation to Stage 11 (Naef, 1928), suggesting there is great variation between egg incubation stage and maternal behavioral state.

Octopuses were kept undisturbed for 4 days so they could habituate to our aquarium setup and recover from the distress of flying. On the fourth day, we began offering live prey items. Each day, females had four live fiddler crabs available to them. Animals were observed twice daily (morning and afternoon) and recorded overnight with a Sony Handycam FDR-AX100 to characterize their behaviors. We observed 20 animals without clutches (non-mated) for at least 4 days before euthanizing and sexing them (see below). Eleven of 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 and summarized in Table 1.

Tissue collection

Octopuses were submerged in 5–10% ethanol/seawater for at least 20 min 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 sexual dimorphism is difficult to observe in our species, animals were definitively sexed after tissue harvest. Only females with mature ovaries, ovarian follicles and no evidence of fertilized eggs were considered non-mated females.

We also harvested arm tissue from the same cohort of animals. Transverse arm slices corresponding to the 10th row of suckers distal from the mouth on the second arms on the right and left sides were cut into quadrants to facilitate tissue homogenization. Each arm piece was separately flash-frozen in Trizol and stored at −76°C until RNA extraction.

RNA extraction and sequencing

Whole optic glands were homogenized with pellet pestles (Fisherbrand) in microcentrifuge tubes. Arm sections were homogenized in a Potter-Elvehjem tissue grinder (Kimble, Rockwood, TN, USA). RNA was extracted using Trizol and PhaseMaker Tubes (Life Technologies Corporation, Carlsbad, CA, USA) following the manufacturer's instructions. RNA integrity was checked with a Bioanalyzer 2100 (Agilent). Only samples with clean cephalopod rRNA peaks and little to no evidence of degradation were used. Tissue from the right arm was processed for RNA extraction and sequencing. The left arm was used in two samples of feeding females when RNA quality from the right arm was poor.

Total RNA was polyA-selected and directionally sequenced at the University of Chicago Genomics Facility on an Illumina HiSeq2500 machine, generating 100 bp directional paired-end reads with an insert size of 300 bp.

De novo transcriptome assembly and differential gene expression analyses

Following removal of adapters and low-quality sequences, reads were assembled with the Trinity software platform (v2.4.0) (Haas et al., 2013). Both individual and pooled transcriptomes were created (Table 2). Gene and isoform expression levels were estimated with RSEM (v1.2.31) (Li and Dewey, 2011) and differential expression was analyzed with edgeR (v3.7) (Robinson et al., 2009). Heatmaps were created in R with the heatmaps.2 function and colored with palettes from the RColorBrewer package.

In the optic gland transcriptomes, 1198 Trinity transcripts showed at least a 4-fold change between any two of the behavioral stages, at a *P*-value of 0.001. A branching diagram illustrating the relationship between differentially expressed genes was created based on Euclidean clustering (Fig. S1). The tree was cut at a height of 0.3, which identified 25 subclusters. Twenty genes from subclusters of interest (see below) were confirmed by PCR amplification.

RESULTS

Adult life in the female *O. bimaculoides* can be divided into four behavioral stages

Sexually mature female octopuses are known to be active predators (Hanlon and Messenger, 2018). We confirmed this behavior in non-mated females in the laboratory: these animals spent time outside of the den (Fig. 1A) and primarily hunted through a visually directed jet-propelled 'pounce' (Fig. 1B; Movie 1) (Wells, 1978). The female watched the prey out of one eye, then bobbed her head up

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

Stage	Traits
Non-mated	Actively hunts and consumes live prey: on average 1.31±0.76 crabs per day, sometimes skipping 1–2 days 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 1–2 days 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 four consecutive days and never resumes feeding again
	Ongoing brooding
	Ongoing den-bound tendencies
Decline	Ongoing fasting
	Exhibits at least two of the following signs of physical deterioration:
	• skin lesions on the mantle, arm webbing or head
	• missing arm tips or suckers
	• degradation of the vestibulo-ocular reflex
	• retraction of skin around the eyes
	• extended bouts of self-grooming
	Spends considerable time roaming outside of the den
	Ends with death of female outside of the den

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Table 2. *Octopus bimaculoides* optic gland transcriptome assembly summary

Transcriptome	No. 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	267,380	314,115	4012	882	604.80	189,975,279
Feeding mother optic gland	3	290,774	344,673	4078	909	616.68	212,552,829
Fasting mother optic gland	3	254,893	301,599	4011	938	627.42	189,228,407
Decline mother optic gland	2	217,478	261,725	4554	1165	688.47	180,189,329
Combined optic gland	–	598,083	706,186	3897	767	574.65	405,807,628
Unmated female arm	3	120,017	189,097	5771	1781	904.15	170,971,875
Feeding mother arm	3	296,853	406,986	5196	1165	676.91	275,491,458
Fasting mother arm	3	183,259	278,194	5683	1621	823.35	229,050,350
Decline mother arm	2	153,752	233,040	5514	1650	841.44	196,090,319
Combined arm	–	493,537	686,453	4485	959	639.22	438,793,048

Whole optic glands and transverse arm sections were harvested (see Materials and Methods). Separate libraries were prepared for each sample, and reads were later concatenated for transcriptome assembly. Statistics are based on transcript contigs. Total Trinity 'genes' refers to the number of transcript clusters generated by the assembly (isogroups), while total Trinity transcripts indicates the number of isoforms (isotigs). The Nx length statistic indicates that at least x% of the assembled transcript bases are found on contigs that are of at least Nx length; for example, at least 10% of the assembled bases in the unmated female optic gland transcriptome are found on transcript contigs that are at least 4012 bp long.

and down, likely acquiring depth information. Even while moving her head, her pupil was kept perpendicular to gravity (Fig. 1A). Following a swift pounce, prey was captured in the interbrachial web (Fig. 1B,C). Non-mated females consumed 1.31±0.76 (mean±s.d.) crabs a day, sometimes skipping a day between meals.

Mated females in the first stage of brooding actively tended their clutch by guarding their den, stroking eggs with their suckers, and blowing water over the eggs with their funnel (Hanlon and Messenger, 2018; Wells, 1978). In the laboratory, feeding mated females consumed 1.17±0.70 crabs per day, with fewer than 3 days without a meal. Feeding females, however, rarely left their egg clutches. Instead of capturing prey by stalking and pouncing, feeding females peered out of their dens with one eye and grabbed prey as they moved past the mouth of the den (Fig. 2A,B; Movie 1). The presence of empty crab carapaces provided confirmation that feeding females consumed food, rather than merely capturing and killing prey, which has been reported in other species (Van Heukelem, 1973). On average, mothers fed for 8±2.71 days before starting to fast.

Females in the second stage of brooding continued to care for eggs but abstained from feeding, even though live prey items were available at all times (Fig. 2C; Movie 1). Females who stopped feeding for four consecutive days were never observed to feed again, and we grouped these animals into the fasting stage.

After 11±8.49 days, fasting mothers began a rapid decline. Behavioral signs of decline, some of which have not been described previously, were observed before physiological signs (Fig. 3A): females left their clutches and spent time outside of the den, languidly sitting on the bottom of the tank or persistently slamming into the corners of the tank (Movie 1). The latter action led to the rapid formation of deep avulsions on the mantle or arms that did not heal (Fig. 3B,C). Females also engaged in excessive self-grooming behaviors. Normally, octopuses run their first pair of arms over their mantles and head to remove ectoparasites and debris (Mather and Alupay, 2016). Decline mothers moved all of their arms to groom, but often arms would only pass over other arms, creating a turbulent mass of entangled limbs (Fig. 3A). In a few cases, this over-grooming behavior was directly followed by self-cannibalization of the arm tips or suckers (Fig. 3B,C; Movie 1). Physiological features of females in decline included a retraction of the skin around the eyes, general paling of skin color, and loss of motor tone (Anderson et al., 2002). In addition, the pupils gradually lost their horizontal orientation with respect to gravity, raising the possibility that the central systems responsible for the vestibulo-ocular reflex deteriorate during this stage.

These behavioral changes extend well beyond those seen in starving octopuses: in a laboratory setting, *Octopus vulgaris*

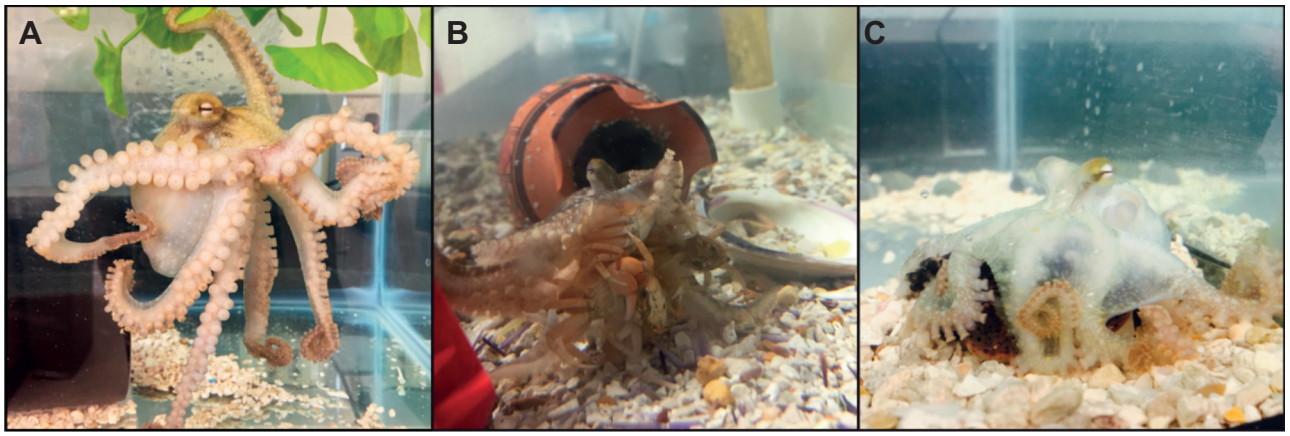

Fig. 1. Non-mated, sexually mature *Octopus bimaculoides* females are active predators that hunt live prey. (A) Females in this stage spend time outside of their home den. (B) Non-mated females use visual direction to strike prey with a 'pounce', viewed here from the front. (C) The webbing between the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

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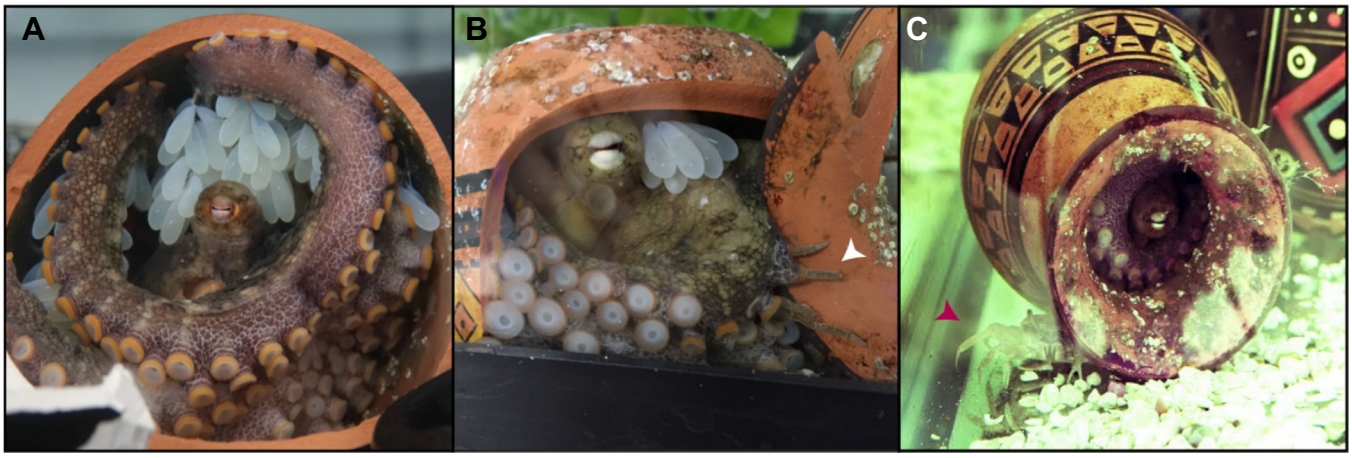

Fig. 2. Brooding *O. bimaculoides* females feed and then fast. (A) In the first stage of brooding, females actively tend to their eggs and feed. The pupils of feeding females remain perpendicular to gravity. (B) Feeding females capture and kill live prey, but only without leaving the home den. White 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.

females can survive over 38 days of fasting, and individuals who have lost substantial body mass remain alert and active (Wells and Wells, 1975). In our study, all mated females progressed from feeding to fasting to decline before dying: that is, mated females did not transition from feeding directly to decline without fasting, and females did not die without showing indications of decline. This progression is reminiscent of what has been reported in male octopuses in captivity, although in males, the relationship between these behavioral changes and reproductive state is less clear (Anderson et al., 2002). Our observations suggest that after mating, female octopuses undergo a series of near-requisite behavioral transitions before death.

Transcriptomes of brooding females implicate many signaling systems in optic gland function

We assembled transcriptomes of left and right optic glands harvested from multiple individuals in each of the four stages (Table 2). Our assembled transcriptomes showed that the optic glands from non-mated animals were the most different from the other optic gland samples (Fig. S2). After estimating transcript abundance with RSEM, we identified 1198 differentially expressed transcripts with edgeR and grouped them into 25 subclusters (Fig. S1). Wodinsky's work suggested that optic gland signaling changes steadily over time until reaching a threshold that triggers fasting and later death (Wodinsky, 1977). In selecting subclusters for further study, we were guided by two criteria. We excluded 12 subclusters that showed a departure from monotonicity, for example, when expression peaked in only the feeding or fasting stages (Fig. S3). Another five subclusters demonstrated the most marked transcript enrichment or impoverishment at the decline stage, the last stage of brooding before death (Fig. S4). Because of sample scarcity (only two RNA-sequencing samples in decline stage and a limited ability to pursue follow-up studies), we did not explore these subclusters further. Five additional clusters showed monotonic changes and were studied with BLASTP as described below (Fig. S5). No candidate signaling molecules were identified in these five clusters. In total, we excluded 855 genes in 22 subclusters from further analyses.

We focused on the 343 remaining genes in three subclusters. Among these subclusters (Fig. 4), we saw dramatic transitions

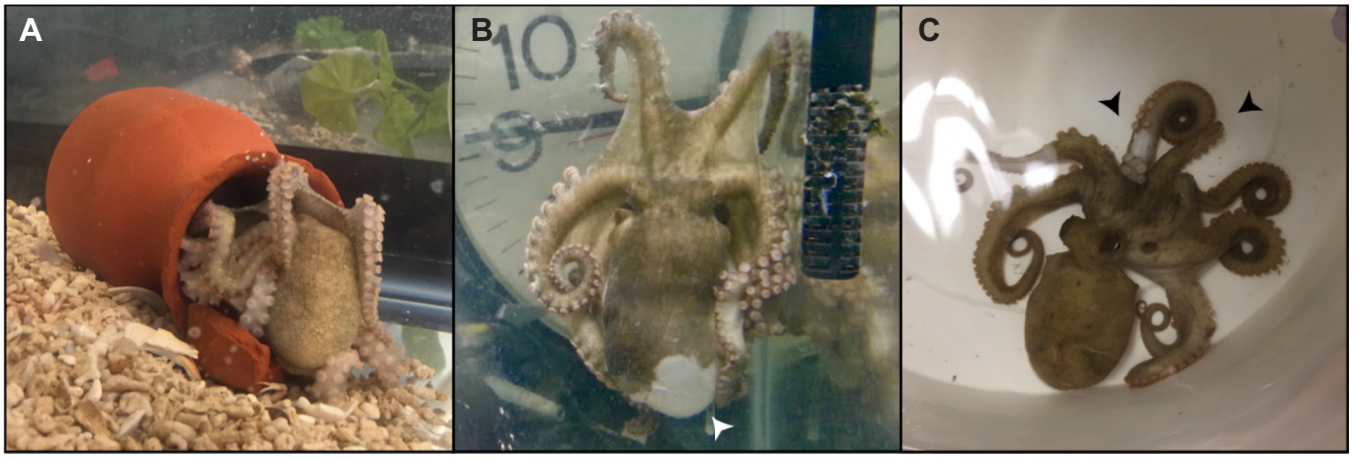

Fig. 3. *Octopus bimaculoides* females in decline continue fasting and undergo rapid senescence. (A) Behavioral signs of decline include spending time outside of the home den and recruiting all the arms for use in bouts of aberrant, grooming-like behavior. Females also show physiological signs of decline, 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.

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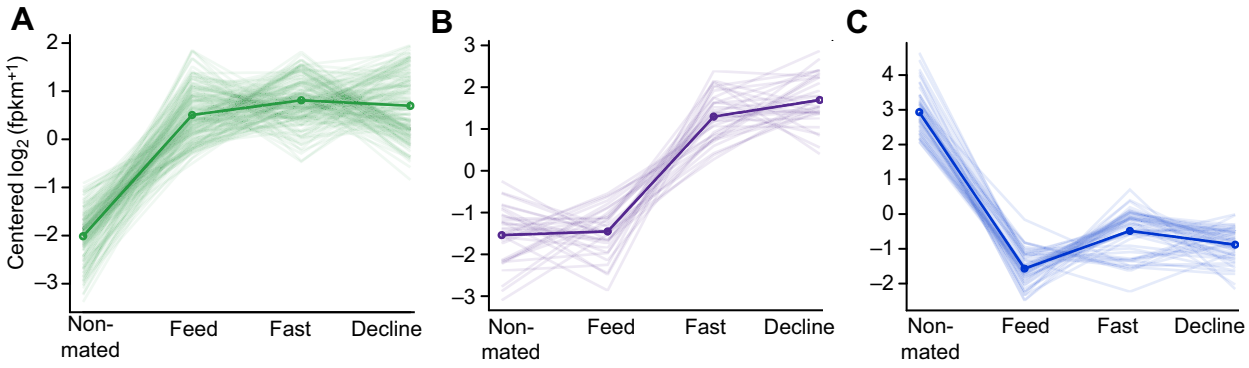

Fig. 4. Cohorts of genes are differentially expressed across behavioral stages in *O. bimaculoides*. Green and purple subclusters identify genes that increased in abundance as the maternal stages progressed (A and B, respectively), while the blue subcluster illustrates genes that decreased in the non-mated to mated transition (C).

in differential gene abundance across behavioral stages. Two subclusters showed increases in expression over time (Fig. 4A,B), while the third showed a drop in expression at the non-mated female to feeding mated female transition (Fig. 4C).

We used BLASTN, BLASTP and PFAM (Altschup et al., 1990; Finn et al., 2016) to identify transcripts (Fig. 5). In all clusters examined, there were many housekeeping genes, such as actin and collagen. We also found predicted proteins with no homology to genes of other species in available databases (downloaded 28 October 2015). These uncharacterized genes may represent octopus- or cephalopod-specific proteins. All three clusters did, however, contain a number of transcripts of particular relevance to optic gland biology.

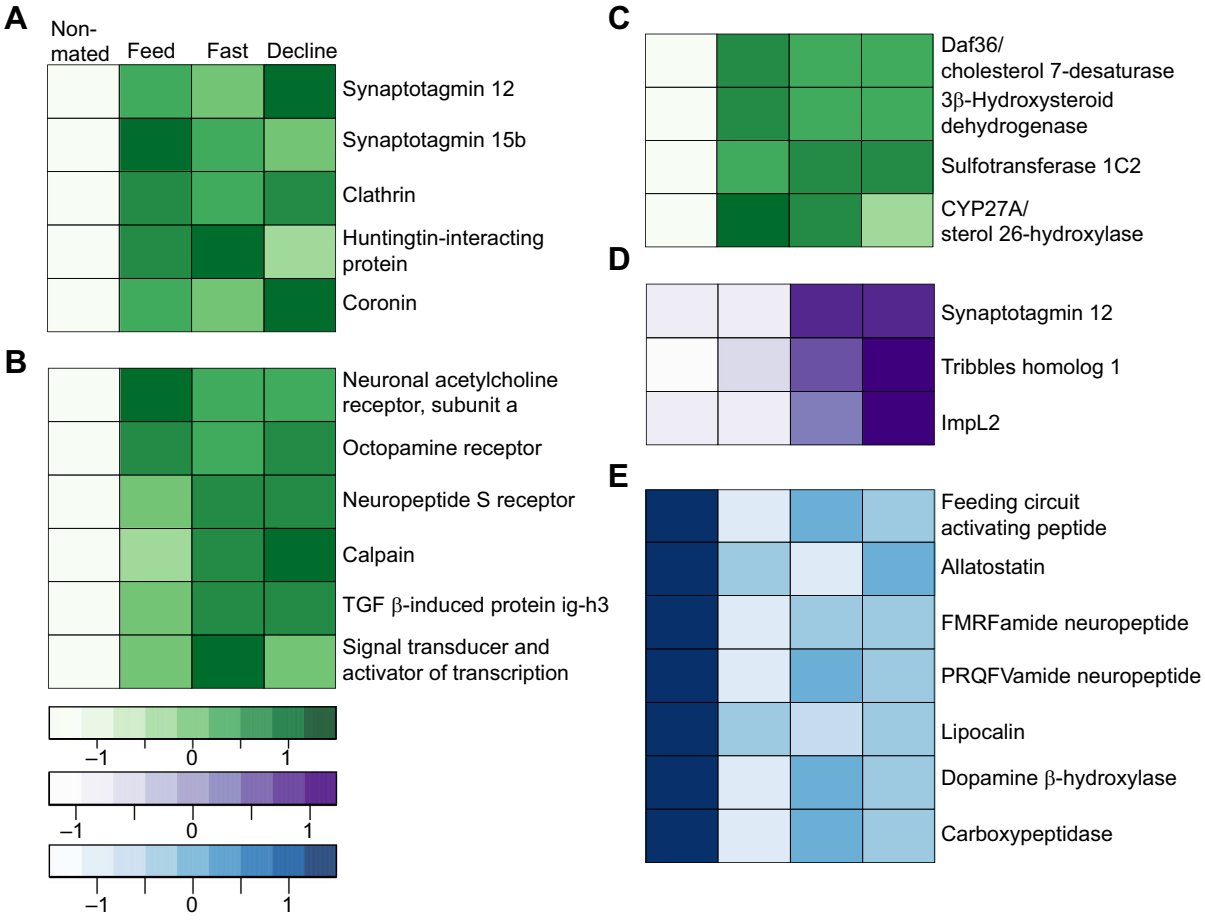

Fig. 5. Expression profiles of differentially expressed genes important to optic gland signaling. Genes related to regulated exocytosis (A), neurotransmission (B) and steroid biosynthesis (C) are enriched in all mated transcriptomes. Transcriptomes of fasting animals show enrichment of an insulin growth factor binding protein (D), among others. Neuropeptides and dopamine beta-hydroxylase are enriched in the optic glands of non-mated females only (E). Heatmap colors correspond to cluster colors depicted in Fig. 4. Columns are organized as designated at the top of A. Color key values refer to row *z*-scores (the number of standard deviations away from the population mean an individual raw value is).

Genes that increase in abundance after mating and remain elevated through the maternal stages

Although the optic gland is regarded as a neuroendocrine organ, its mechanisms of secretory release are unknown. We found transcripts implicated in neural signaling that increased in abundance after mating and remained elevated through all brooding stages (Figs 4A, 5A, green cluster): synaptotagmin 12, synaptotagmin 15b and clathrin. Huntingtin-interacting protein (*HIP1*) and coronin showed a similar expression profile. Although not included in classical models of synaptic transmission, proteins encoded by these two transcripts have in recent years been found to be important for aspects of neuronal signaling (Borlido et al., 2009; Suo et al., 2014). We also saw increases in selected neurotransmitter receptors (Fig. 5B), including an alpha subunit of a nicotinic acetylcholine receptor, and G-coupled protein receptors similar to octopamine and neuropeptide S receptors in other species. Finally, enzymes implicated in steroid biogenesis were present in this subcluster (Fig. 5C). These results suggest that the optic gland is a site of active signaling during the maternal period, and that the nature of this signaling is different from that of unmated female optic glands.

Genes that increase with the transition from feeding to fasting

In this subcluster, we found that *ImpL2*, which encodes an insulin-like growth factor binding protein (IGFBP), is enriched in the optic glands of fasting and decline animals (Figs 4B, 5D, purple cluster). This transcript is joined in expression dynamics by two others, tribbles homolog 1 (*TRB1*) and an additional isoform of synaptotagmin.

Genes that decrease with the transition from unmated to mated

As in the other subclusters, we found genes encoding proteins involved in cell signaling (Figs 4C, 5E, blue cluster), such as lipocalin and dopamine beta-hydroxylase. Particularly notable in this subcluster are the feeding circuit-related neuropeptides and a carboxypeptidase (Fig. 5E).

Are molecular changes of the optic glands a reflection of global changes in gene expression?

The abundance of genes encoding neuropeptides in the non-mated optic gland and their subsequent depletion in mated females motivated us to investigate whether molecular changes in the optic gland were mirrored in other tissues of the body. Many of these tissues were sampled in the arm transcriptomes, including axial nerve cords, suckers, muscles, chromatophores and skin. Using BLASTP and TBLASTN, we baited the arm transcriptomes from all behavioral stages with the genes that were significantly enriched in non-mated optic glands (blue cluster, Figs 4C, 5E). We were unable to recover any of these optic gland neuropeptides in the arm transcriptomes.

DISCUSSION

With their large central nervous systems and wide range of behaviors, coleoid cephalopods have long fascinated neuroscientists. Pioneering work from Wodinsky (1977) and Wells and Wells (1975) established that the octopus reproductive axis is similar to that of the vertebrate hypothalamic–pituitary–gonadal axis. The optic gland, analogous to the pituitary gland, is the fulcrum of the octopus reproductive axis; it is responsible for innate reproductive behaviors and post-reproductive death. In this study, we confirmed that maternal behaviors can be observed in the laboratory, including, most importantly, a period of feeding while brooding that precedes the fasting that typically defines the maternal period in octopuses (Movie 1) (Hanlon and Messenger, 2018; Rocha et al., 2001).

Our transcriptomic analyses reveal a molecular portrait of the optic glands at the distinct behavioral stages of adult female octopus life. In particular, we found that reproduction and the onset of fasting trigger major transcriptional activation in the optic glands. Most importantly, multiple signaling systems of the optic glands, including catecholaminergic, peptidergic and steroidogenic pathways, are implicated in these behaviors.

Optic gland signaling systems

Feeding peptides

Circulating neuropeptides have been extensively studied in molluscan feeding and reproductive systems (Grimmelikhuijzen and Hauser, 2012; Jékely, 2013). Neuropeptides can influence feeding behaviors through control of motor circuits or modulation of arousal or satiation (Bechtold and Luckman, 2007). Strikingly, we found octopus homologs of these neuropeptides enriched in the optic glands of non-mated females as compared with those of brooding females: feeding circuit activating peptide (*FCAP*), allatostatin, FMRFamide and PRQFVamide. The latter three peptides have been shown to inhibit feeding behaviors in a diverse range of animals. In *Aplysia*, FMRFamide and its related neuropeptides inhibit closure of the radula muscle during feeding (Sossin et al., 1987), and in mice, FMRFamide reduces feeding in food-deprived individuals (Kavaliers et al., 1985). PRQFVamide decreases the excitability of specific neurons in the buccal ganglion feeding central pattern generator and has an overall inhibitory effect on feeding (Furukawa et al., 2003). Allatostatin inhibits feeding in *Drosophila* by mediating the balance between adipokinetic hormone and insulin-like peptides (Hentze et al., 2015). FCAP, in contrast, initiates rhythmic feeding motor programs and may be responsible for food-induced arousal (Sweedler et al., 2002).

These data suggest that signaling from an ensemble of neuropeptides tightly controls the feeding behaviors of non-mated animals. In many animals, mechanisms controlling feeding and reproduction are tightly linked and enable metabolic shifts as the individual transitions from growing and maturing to reproduction and tending to young (O'Dor and Wells, 1978). *Drosophila* females, for example, modify their feeding preferences to consume more protein when they are about to lay eggs (Ribeiro and Dickson, 2010). The reduction in feeding circuit-related neuropeptides at the non-mated to mated transition strongly suggests that, in the octopus, feeding and reproductive state also interact.

Although neuropeptide expression precipitously drops after reproduction, mated animals continue to feed in the initial stage of brooding. However, feeding behavior differed between mated and non-mated animals: mated feeding octopuses captured crabs that were within arm's distance of their dens and did not pursue prey outside their dens, as non-mated females regularly did. This behavioral switch suggests that different feeding strategies are optimal for non-mated and mated females: the feeding-related peptides identified may regulate energy expenditure or the drive to hunt for food. Our data also raise the possibility that redundant peptidergic pathways, or additional non-peptidergic systems, control feeding behaviors. These systems may be independent of the optic gland.

These neuropeptides may also broadly participate in neurotransmission well beyond feeding. If this is the case in the octopus, peripheral tissues might also express these neuropeptides. We checked whether these neuropeptides were present in the arm transcriptomes, which included nervous tissue (axial nerve cord),

muscle, skin, chromatophores and suckers. Expression changes of these genes in the arms would suggest that global changes in neuropeptide signaling accompany the maternal and senescence process in general. However, all feeding-related neuropeptides in the blue subcluster (Figs 4C, 5E) were missing from the arm transcriptomes, suggesting that their expression is a specific feature of the optic gland. These data are surprising because the optic glands previously have not been implicated in feeding per se. Future experiments will reveal whether these neuropeptides are functionally conserved in octopuses.

Steroid biosynthesis

Steroids are one of the most evolutionarily ancient classes of signaling molecules (Baker, 2011; Baker et al., 2015). Derived from cholesterol, steroids include the sex hormones and corticosteroids. We found that a collection of cholesterol-metabolizing enzymes increased in expression in the first stage of brooding and remained elevated throughout the maternal period. These included enzymes known to synthesize steroid hormones in diverse animals, from ecdysozoans to deuterostomes (Antebi, 2015). Dafachronic acid in *Caenorhabditis elegans*, for example, regulates longevity and dauer formation, and its biosynthesis relies on the sequestration of cholesterol by daf36/cholesterol 7-desaturase (Yoshiyama-Yanagawa et al., 2011). 3-Beta-hydroxysteroid dehydrogenase is an oxidoreductase that catalyzes the formation of progesterone in the adrenal gland and gonads of vertebrates (Aakvaag, 1970; Conley and Bird, 1997). Sterol 27-hydroxylase (CYP27A) breaks down cholesterol for both bile acid synthesis and hormone production (Dubrac et al., 2004). Sulfotransferase 1C2 catalyzes the conjugation of a sulfate group to steroid hormones (Foster and Mueller, 2018).

Our study is the first to implicate the optic glands in the production of progesterone and other steroid hormones. Cellular and molecular mechanisms of steroid hormone signaling in octopuses may be different from what has been described previously in the vertebrate neuroendocrine systems. Substrate specificity of these enzymes may differ between species (Antebi, 2015). Functional characterization of these enzymes or identification of steroid metabolites will be essential going forward. Moreover, steroid signaling in molluscs may not be mediated through the nuclear receptors orthologous to those identified in vertebrates (Thornton et al., 2003). The *O. vulgaris* ortholog of the vertebrate estrogen nuclear hormone receptor, for example, does not bind estrogen (Keay et al., 2006). Our identification of the coordinate enrichment of cholesterol-metabolizing enzymes in mated female octopuses raises the exciting possibility that the optic glands produce a suite of steroid-derived hormones that are specific to the reproductive adult and may have novel cellular targets.

Insulin signaling

Our data reveal that expression of *ImpL2* increased at the transition from feeding to fasting (Figs 4B, 5D). IMPL2 is a homolog of insulin-like growth factor binding protein (IGFBP) that has been shown to reduce activity of the insulin/IGF signaling pathway in *Drosophila* (Figueroa-Clarevega and Bilder, 2015; Honegger et al., 2008). This pathway, which is present in many invertebrates, controls biological processes such as glycogen metabolism and organismal growth (Ahn et al., 2017; Veenstra, 2010). However, functional characterization of these molecules in molluscs has been limited (Gricourt et al., 2003; Hamano et al., 2005), and the possible actions of the insulin/IGF signaling pathway in octopus are unknown.

Under starvation conditions, elevated levels of IMPL2 and other insulin growth factor binding proteins have been reported to promote cell survival (*Drosophila*, Honegger et al., 2008; human HEK 293 cells, Örd et al., 2015). Upregulation of *ImpL2* in fasting octopuses may be a transcriptional response to nutrient deficiency. The highest expression of *ImpL2*, however, is found in females in decline (Fig. 5D): in this terminal stage of brooding, females exhibit behavioral and physiological changes that extend well beyond those accompanying experimental starvation (Wells, 1978; Wells and Wells, 1975). In *Drosophila*, *ImpL2* can induce tissue wasting independent of food intake: tumor-implanted flies show high levels of *ImpL2* and degradation of gonadal, muscle and adipose tissues (Figueroa-Clarevega and Bilder, 2015; Kwon et al., 2015). These similarities with physiological traits of females in decline lead us to predict that this role of *ImpL2* is functionally conserved between octopuses and *Drosophila*.

The correlates of high *ImpL2* in both *Drosophila* and octopus are remarkably consistent with symptoms of cancer cachexia in humans. Cachexia patients experience an involuntary loss of body mass, undergo substantial degradation of muscle and fat, and report a loss of appetite (Argilés et al., 2014). Medical interventions focusing on increasing caloric intake fail to counteract wasting (Fearon et al., 2012; Op den Kamp et al., 2009). Importantly, clinical work in humans has shown that cachexia and other disease conditions are associated with elevated levels of IGFBPs (Baxter, 2014; Huang et al., 2016; Shin et al., 2017).

Our results suggest that *ImpL2* is intimately tied to feeding behaviors in octopus and implicate the optic glands as a regulator of insulin signaling. These data raise the possibility of a conservation of function in insulin-signaling pathways with animals as distantly related as humans and octopuses. More importantly, our study is the first to implicate IGFBP enrichment in natural end-of-life physiology.

Neurotransmitter biosynthesis and neurotransmission

The optic gland regulates sexual maturation and behavior by hormone release, but cellular mechanisms of secretion are unknown. We identified a number of genes implicated in neurotransmission and regulated secretion that are enriched after reproduction (Figs 4A, 5A). Clathrin, for example, facilitates the formation of coated secretory vesicles, and synaptotagmins contribute to the cellular complex that enables vesicle fusion for regulated exocytosis (Brunger et al., 2018; Wu et al., 2014). Specific isoforms of both are enriched in the optic glands at all mated stages. Our finding mirrors existing data on the organization of the vertebrate reproductive axis: synaptotagmins and other proteins involved in classical neurotransmission are differentially expressed in the anterior pituitary gland, where they are involved in the release of a variety of signals (Jacobsson and Meister, 1996).

The enrichment of neurotransmitter receptors (Fig. 5B), including an octopamine receptor, neuropeptide S receptor and a subunit of a nicotinic acetylcholine receptor, suggests that the optic gland also undergoes shifts in the signals it receives from the central brain, from itself or from other secretory organs after reproduction.

Optic glands of non-mated animals showed elevated expression of dopamine β-hydroxylase (*DBH*; Fig. 5E), which is involved in the synthesis of catecholamine neurotransmitters. *DBH* has been shown to catalyze the β-hydroxylation of dopamine to noradrenaline in vertebrates and tyramine to octopamine in invertebrates (Fernstrom and Fernstrom, 2007; Monastirioti, 1999). These small molecules are used for signaling throughout nervous systems. Candidate aminergic secretory vesicles have been found in the

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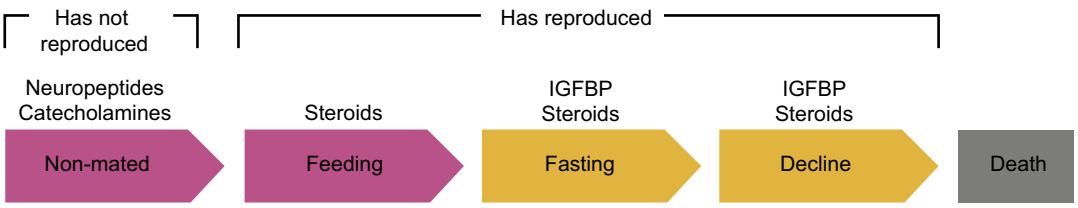

Fig. 6. Updated model of optic gland function in maternal behaviors and death. Arrows indicate behavioral groups (pink: feeds; yellow: abstains from feeding). Signaling systems above arrows highlight enriched genes.

optic glands of *Octopus bimaculatus*, the sister species of *O. bimaculoides* (Nishioka et al., 1970). Our results suggest that the optic gland engages in endocrine catecholamine signaling and that reproduction leads to the specific shutting down of catecholamine pathways, as opposed to an overall decrease in optic gland neurotransmission or cell signaling.

Octopus semelparity

Mechanisms of death differ among animal systems exhibiting semelparity. In several vertebrate species, it is believed that the metabolic cost of gamete production and finding a mate are so high that death is inevitable (Fisher and Blomberg, 2011; Kindsvater et al., 2016). Semelparity in octopuses cannot be explained by the large metabolic output required to produce and deposit eggs: glandectomized animals can reproduce again (Wodinsky, 1977). Instead, it is likely that other evolutionary mechanisms drive post-reproductive death. Coleoid cephalopods are cannibalistic: hatchlings from the same clutch often eat each other, and females sometimes kill males after reproduction (Hanlon and Messenger, 2018). Post-reproductive death is an undeniably effective method in ensuring that the reproductive female does not consume her young. Moreover, longitudinal studies of octopuses, and other molluscs, demonstrate that individuals continue to undergo logarithmic growth as adults: even adult octopuses can double their weight in a month (Gricourt et al., 2003; Nixon, 1969; Van Heukelem, 1973; Wells, 1978; Wells and Wells, 1970). Endless growth without death could so skew octopus populations towards large, old adults that hatchlings would be unlikely to compete successfully. Programmed organismal death in coleoids may exist as a mechanism to safeguard the survival of the next generation of these voracious predators.

In Pacific salmon, *Oncorhynchus kisutch*, steroid hormone signaling has been implicated as the cause of death after spawning. Elevated cortisol levels mediate programmed death through tissue degeneration and impaired homeostatic ability (Robertson and Wexler, 1960). Our data show that steroid signaling is one of several pathways implicated in octopus post-reproductive death. Although we do not see molecular evidence of cortisol signaling per se in our data, the enrichment of cholesterol-metabolizing enzymes in mated females suggests that steroid metabolites are crucial to semelparity in octopuses. It will be important to identify the steroids in the optic gland and assess the contributions of steroid and IMPL2 signaling, along with the reduction in feeding peptides and catecholamine signaling, in cephalopod post-reproductive death.

Conclusions

Our transcriptome findings suggest that, far from secreting a single hormone, the optic gland likely enlists multiple signaling systems to regulate reproductive behaviors, including organismal senescence (Fig. 6). Among these are neuropeptidergic systems and cholesterol-derived hormone signaling. Our data raise the possibility that prior to mating, optic gland signaling is dominated by neuropeptides and catecholamines, whereas after mating, steroid hormone signaling increases in importance.

Wells and Wells (1969) originally drew a connection between the direct brain regulation of the octopus optic glands and the vertebrate hypothalamic–pituitary–gonadal axis. Here, we identify a difference between octopus and vertebrate systems: the endocrine signaling of the anterior pituitary gland depends on peptide hormones alone (Davis et al., 2013), whereas our data implicate steroid and catecholamine pathways, in addition to peptide signaling, in the functions of the optic gland. While steroid hormones do affect the actions and feedback of the vertebrate pituitary gland, steroidogenesis occurs in targets of the pituitary gland only, such as the adrenal cortex, and not in the pituitary gland itself (Handa and Weiser, 2014; Werbin and Chaikoff, 1961). Similarly, biosynthesis of catecholamines, such as noradrenaline and adrenaline, occurs in the adrenal medulla in response to autonomic stimulation (Fujinaga et al., 1999).

If the optic gland encompasses functions of the anterior pituitary gland and the adrenal glands, it prompts the question of what glandular targets of the optic gland, if any, might exist. The oviducal glands, which produce substances that cover the eggs, are candidates. These glands, along with the gonads and oviducts, were found to increase in size as the optic gland enlarges (Wells and Wells, 1959, 1975). *In vitro* experiments demonstrated that oviducal secretion is modulated by dopamine and neuropeptides, including FMRFamide (Di Cristo and Di Cosmo, 2007). However, the putative source of direct oviducal control is the fusiform ganglion, not the optic glands. The fusiform ganglion is thought to innervate the oviducal gland, the oviducts and the systemic heart (Young, 1971). Notably, the fusiform ganglion has been found to contain FMRFamide-like immunoreactive nerve endings (Di Cristo et al., 2003). The optic gland may exert indirect control over glandular targets by signaling to peripheral ganglia, or act in parallel with peripheral ganglia to regulate reproductive tissues directly.

Our study extends the optic gland–pituitary analogy and uncovers similarities between the optic glands and the vertebrate adrenal glands, demonstrating that the organization and function of the optic glands may be even more pivotal for octopus organismal physiology than previously appreciated.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: Z.Y.W., C.W.R.; Methodology: Z.Y.W., C.W.R.; Software: Z.Y.W.; Validation: Z.Y.W., C.W.R.; Formal analysis: Z.Y.W.; Investigation: Z.Y.W.; Resources: C.W.R.; Writing - original draft: Z.Y.W.; Writing - review & editing: Z.Y.W., C.W.R.; Visualization: Z.Y.W.; Supervision: Z.Y.W., C.W.R.; Project administration: Z.Y.W., C.W.R.; Funding acquisition: Z.Y.W., C.W.R.

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Supplementary information

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