OCTOPUS RUBESCENS RESPONSE TO ENVIRONMENTAL CHEMICAL CUES

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ABSTRACT

Marine organisms produce molecules which travel downstream in odor plumes and can contribute to a variety of interactions between organisms. A variety of coleoid cephalopods including cuttlefish, squid, and octopuses have been found to depend on environmental chemicals to mediate courting, feeding, and predator avoidance. This research investigates the metabolic and ventilatory response of *Octopus rubescens* to a variety of environmental chemical cues including conspecific odorants, conspecific ink, predator, and prey odorants. Ventilation and metabolic rates were higher when octopuses were presented with a crab odorant than with a control. Metabolic responses were also higher when octopuses were presented with the most dilute ink than with the control. This research sheds light on the responses of *Octopus rubescens* to environmental chemical cues and offers insight for future research in understanding how octopuses react and interact with their ecosystem.

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INTRODUCTION

Many organisms are capable of chemoreception, the physiological response of a sensory organ to a chemical stimulant (Kamio & Derby, 2017). Generally, organisms can sense chemicals in their environment by two methods: olfaction and gustation (Kohn, 1961; Reinhard, 2010). Olfaction, or the sense of smell, is important to organisms as it allows them to detect air- or water-borne chemicals that may have originated from food, predators, or conspecifics (Reinhard, 2010). Olfactory organs in cephalopods are dimples located anterior to the mantle cavity on both sides of the head. These sensory organs are innervated by afferent nerves from the olfactory and dorsal basal lobes of the octopus brain, allowing octopuses to "smell" water-borne chemicals (Grasso & Basil, 2009). Gustation relies on modified epithelial cells which come into contact with and "taste" the chemical source (Polese, et al*.,* 2015; Reinhard, J., 2010). In cephalopods, modified epithelial cells which perform gustation are mainly distributed on the suckers (Derby & Sorensen, 2008). Once detected, environmental chemicals may mediate a wide variety of interactions between species, predator and prey, or conspecifics (Wisenden, 2000; Nocchi et al., 2017).

Chemical communication is a mode of communication in which chemicals are given off by an organism and detected by another organism, and as a consequence the behavior and/or physiology of the receiving organism is modified (Steiger et al*.,* 2011). This can occur using chemical signals, chemicals that are given off by the sender that intentionally convey information to the receiver, or using chemical cues, which are not given off by the sender to intentionally convey information to the receiver (Steiger et al*.,*

2011). Chemical communication has been found to be important among goldfish in order to elicit feeding and reproductive behaviors. Recent research by Sato et al. (2018) isolated goldfish olfactory sensory organs and observed that the neurons were excited by a variety of chemical signals and cues including sex pheromones and food odorants, showing that a physiological response is a consequence of the reception of chemical communication. Wounded organisms are found to produce chemical cues which, once received, can communicate alarm and initiate defensive behavior in conspecifics. Oliveira et al. (2014) observed zebrafish respond defensively by freezing or lingering near the bottom of the tank when introduced to substances released by ruptured epidermal cells of wounded conspecifics. Not only does chemical communication occur between conspecifics, but also between prey and predator. This communication is exemplified by the seastar *Acanthaster planci*, as it significantly increased movement when introduced to the chemical cue of a main predator, the triton *Charonia tritonis* (Hall et al*.,* 2017). Further research in understanding how animals respond behaviorally and/or physiologically to chemical cues and signals will help develop our understanding of ecological interactions.

Chemical Communication in Cephalopods

Cephalopods are a class of highly derived marine mollusks represented by octopuses, squid, cuttlefish, and chambered nautiluses. Organisms in this class include the largest invertebrate, the giant squid, and are characterized by a complex nervous system. Class Cephalopoda is made up of two extant subclasses, Coleoidea and

Nautiloidea (Young, et al., 1998). Coleoidea is represented by octopuses, squids, and cuttlefish, which lack an external shell. Nautiloidea, the nautiluses, retain an external shell. Cephalopods are important members of marine food webs, as they have a high conversion effeciency as well as have the ability to switch prey sources depending on what is most abundant at the time (Aronson, 1986; Smith, 2003, Šifner & Vrgoč, 2009; Wolff, 1992).

Reproductive physiology and behavior in coleoid cephalopods may be mediated by chemical communication. Conspecific interactions between octopuses, such as mating, can be risky business and result in cannibalism, which has been observed in several octopus species including *Enteroctopus dofleini* (Hernandez-Urcera et al., 2014). Perhaps because of this risk of cannibalism, cephalopods rely on chemical cues during intraspecific interactions. Walderon et al. (2011) found that female *O. bimaculoides* increased their ventilations in response to male odorants but not female, while male *O. bimaculoides* increased ventilations to both male and female odorants. This increase in ventilations might be due to excitation or arousal, suggesting that octopuses may use chemical communication in order to avoid confrontation or mediate courting behavior, depending on the sex presented (Walderon et al., 2011). Chemical communication can also play a role in attraction of cephalopods to perform reproductive behavior. Basil et al. (2002) observed that female *Nautilus pompilius* females were drawn to the pheromones secreted by males. Additionally, *Sepia officinalis* increased ventilations and were drawn in the direction of conspecific egg extracts, which suggests that there are one or more active factors found in the egg extracts that may help facilitate spawning aggregations

(Boal et al., 2010). In addition to behavior, cephalopod physiology can be changed by the reception of certain chemical cues. Polese et al. (2015) discovered that female *Octopus vulgaris* may depend on chemical cues to help initiate maternal behavior. During reproduction, sex-related hormones are released into the environment and can act as chemical cues to female *Octopus vulgaris*. When these chemical cues are detected by a female *O. vulgaris*, they help to cause physiological changes to the olfactory organ, which helps begin senescence, during which she avoids food and broods eggs (Polese et al., 2015). These examples show that chemical cues may be an important aspect in controlling the complex physiology and/or behavior involved in cephalopod reproduction.

Several coleoid cephalopods have been observed to respond and be attracted to food odorants. Walderon et al. (2011) observed an increased ventilatory response of *Octopus bimaculoides* when presented with odorants from shrimp and crab, suggesting excitation. The same response was observed by Boal and Golden (1999) as ventilations increased in *Sepia officinalis* when the cuttlefish were subjected to shrimp odorant. Chase and Wells (1986) observed blinded *O. vulgaris* move toward a food source, signifying the use of chemoreception as an adjunct to vision when searching for food. These examples indicate that cephalopods use chemoreception to help detect their prey.

Chemical cues of predators may induce defensive behaviors in coleoid cephalopods similarly to how they respond to visual cues. King et al. (2006) observed a behavioral freeze in *Sepia officinalis* when presented with a visual stimulant of a predator, displaying a hyperinflated mantle, decreased ventilation and lowered heart rate.

Depending on the stimulant, many cephalopods are able to respond with color changes in their skin (Josef et al., 2012). When presented with a predator that depended on a visual stimulus to find prey, such as a teleost fish, *Sepia officinalis* would perform a deimatic, or startling, display before flight in order to cause the predator to hesitate, and give the cuttlefish more time to respond (Langridge et al., 2007). When presented with a predator that relied on chemoreception instead of vision, such as a crab, the cuttlefish would not use a deimatic display, but instead camoflage and flee, as the predator would not be slowed down by a visual distraction (Langridge et al., 2007). Additional research is needed to understand what signals cephalopods will respond to. Perhaps cephalopods are also able to distinguish between chemical cues from a variety of predators in order to determine the safest response.

In addition to changing color to camouflage, coleoid cephalopods are capable of inking in order to cause a diversion to a threat and possibly facilitate conspecific communication (Caldwell, 2005; Derby, 2007; Rajaganapathi, et al., 2000; Wood et al., 2008). Cephalopod ink is composed of two different glandular secretions: a concoction of mainly eumelanin, which contributes to the brown coloring of the ink, and additional components such as amino acids, metals, and hormones located in the ink sac, and mucus, located in the funnel organ (Derby, 2014; Ito et al., 2011). In octopuses, different neural pathways control the ink sac and funnel organ, allowing them to maintain independent control of both organs (Derby, 2014). More mucus would result in ropes of ink and less mucus, more melanin, would result in a murky cloud called a pseudomorph. Generally, pseudomorphs are associated with fleeing behaviors as it provides a diversion

for the octopus to disapear in, while ropes are associated with crypsis behavior and attempt to draw the threat away (Derby, 2014). The amino acids, metals, hormones, and other components found in cephalopod ink, in addition to eumelanin, could potentially be signals to conspecifics (Derby, 2007; Derby, 2014, Prota et al., 1981).

Although how coleoid cephalopods respond to conspecific inking is not well understood, research has found that squid were able to recognize conspecific inking as an indication of a threat. The squid *Sepioteuthis sepioidea* respond to conspecific inking with cryptic and escape behaviors, such as inking, jetting, and fin movements (Wood et al., 2008). Components in cephalopod ink may contribute to the defensive responses of the squid. Dopamine, which is absorbed into melanin granules and carried into seawater when inking occurs, may be a factor influencing cephalopod responses (Derby, 2007). Additional research on the components found in ink would help contribute to understandings in how communication is accomplished among cephalopods.

Purpose of Research

The purpose of this research was to determine the response of *Octopus rubescens* to chemosensory environmental cues by measuring metabolic rate and ventilation rate (Walderon et al., 2011). I hypothesized that *O. rubescens* would (1) increase metabolic rate and ventilations when subjected to food odorant, (2) increase ventilations and metabolic rate when presented with a potential predator, *Enteroctopus dofleini*, (3) decrease ventilations and metabolic rate when presented with a conspecific odor and (4) increase ventilations and metabolic rate when presented with increasing conspecific ink.

METHODS

Octopus Collection & Handling

Twelve male and one female *Octopus rubescens*, and one female juvenile *Enteroctopus dofleini,* all ranging from 30-380 grams, were collected from Admiralty Bay, Washington (48º 16' 38.06" N -122º 63' 70.18" W) at depths of 17-19 meters via SCUBA during the months of June-August, 2017. Glass bottles containing octopuses, which use the bottles as dens, were collected from the seafloor (Anderson et al., 1999). The octopuses were transported to Rosario Beach Marine Laboratory where they were housed in individual aquariums with flowing seawater from Rosario Bay at approximately 13°C. Octopus aquaria consisted of clear 28.4 L plastic storage bins which were designed to allow seawater flow-through. Prior to experimentation, the octopuses were allowed at least 24 hours to acclimatize to their new environment. Animals were fed shore crabs, *Hemigrapsus nudus, ab libitum,* and observed daily for any signs of illness such as lack of diet or skin lesions. No signs of illness were detected among octopuses during experimentation. Octopuses were captured throughout the month of July and held for experimentation until they were released in August.

Octopus Response to Odorants

To quantify octopus ink concentration in seawater, four *Octopus rubescens* ink sacs were dissected from frozen specimens that had died 1-3 years previously. Ink sac components were placed on plastic weigh boats in a drying oven for 24 hours at 80°C. Once dried, ink was crumbly and flakey. Dried ink taken from each ink sac collected was

reconstituted in known concentrations (400, 200, 100, 50, and 25 μg/mL) in artificial seawater and light spectra (200-500nm) were measured with a Beckman DU530 spectrophotometer to determine the ideal wavelength to measure ink concentration (Figure 1). Using this data, the wavelength 265nm was selected, which corresponds to the absorption peak of melanin and may avoid interference by proteins, which are suggested to be analyzed under visible light, 500-600nm (Aitken & Learmonth, 2009; Manchester,1995; Mbonyiryivuze, 2014; Olson & Markwell, 2007) Absorption of ink samples from the four different octopus ink sac were used to compare absorbance differences among individuals and generate a standard curve (Figure 2).

In order to obtain conspecific ink to subject to experimental octopuses, male octopuses were agitated by gently poking them with a 30 mL plastic syringe until they inked. Ink was collected with the syringe and compared spectrophotometrically to the standard curve to determine ink concentration (Figure 2). The mean concentration of all ink samples collected *in situ* was 63.2±13.2 μg/mL, within the range of the standard curve. Once collected and compared to the standard curve, the octopus ink samples were diluted with seawater to be the following concentrations once they were introduced into the respirometer: 0.9, 0.45, 0.225, 0.112, 0.056, 0.028, 0 (μg/mL). Only the dilutions 0.9μg/mL and 0.45μg/mL were concentrated enough to visibly change the appearance of the water color within the respirometers.

In order to determine if octopus response behavior was in response to either chemical cues from the ink or just water coloration from inking, fake octopus ink was created by combining different colors of McCormik food dye and artificial seawater to

make a visual approximation to an ink sample equivalent to 0.9 μg/mL of melanin, the most concentrated melanin sample introduced to the octopuses during respirometry.

To produce octopus odorants, male *Octopus rubescens* and a juvenile female *Enteroctopus dofleini* were held individually in small containers for ten minutes and occasionally agitated by poking with a 30mL plastic syringe to induce movement and mucus production, but not inking. To produce octopus prey odorant, three shore crabs, *Hemigrapsus nudus,* and two spiny scallops, *Chlamys hastata,* were sacrificed and separately ground up with a mortar and pestle with 35mL filtered seawater. All odorant samples were collected in a 30mL syringe individually, stored in cold water for no more than 24 hours, and introduced into the respirometer in unorganized order. Both odorants were concentrated enough to visibly change the water color when introduced in the respirometer.

During each respirometry trial, GoPro cameras were set above the experimental area in attempt to record octopus mantle ventilations as they were presented with different odorants (Walderon et al., 2011). Ventilations were counted for two minutes immediately after odorant introduction.

Respirometry

Intermittent flow, closed respirometers were created from 2.2 liter PET plastic containers fitted with two centrifugal pumps connected in parallel and equipped with a Pyroscience oxygen optode (Figure 3). One of the two pumps (the continuous pump) continuously circulated seawater throughout the respirometer. After 60 minutes, the

second pump (the intermittent pump) would turn on for 15 minutes, flushing the respirometer of any substances added to the chamber and restoring oxygen levels. Once this intermittent pump turned off, the respirometer became a closed system, the continuous pump allowing for water circulation inside the chamber. A Pyroscience contactless oxygen optode spot was placed on the inside of the respirometer to measure oxygen partial pressure in the respirometer (Figure 3). Respirometers were submerged in flowing seawater at a temperature of approximately 13°C. During trials, oxygen levels stayed above 20.9 kPa, which was well above the documented critical oxygen pressure of *O. rubescens* (7.01 kPa, Onthank, 2008).

In order to allow acclimation, octopuses were kept in respirometers overnight (aproximately 10 hours) with the intermittent and continuous pump on prior to experimentation. This process also flushed out any bubbles that may have been trapped in the respirometer system and could skew oxygen readings. Baseline metabolic rate was measured for 60 minutes before introduction of odorants (Figure 4, 5). Odorants were introduced to the respirometer through a luer lock t-valve on the continuous pump circuit and then octopuses were subjected to 60 minutes of closed respirometry. At the end of the 60 minutes, respirometers had a 15-minute flush period before a different odorant was introduced (Figure 4, 5). Odorants were presented to octopuses in an inconsistent order in order to account for biases due to repetitive exposure to odorants.

Data Analysis & Statistics

In order to determine any significant difference between individual octopus ink melanin concentrations, the regressions from individual octopus ink samples were compared for significance with an ANCOVA. A Kruskal-Wallis followed by a post-hoc pairwise Wilcox test was used in order to determine significance between ventilatory responses to odorants and seawater. In order to determine the change in metabolic rate, oxygen consumption of each octopus during the first 15 minutes of odorant introduction was divided by the first 15 minutes of the baseline. Only the first 15 minutes were used due to the possible transient response of the octopus when introduced to the odorant as well as to avoid any interference due to oxyconformity. Oxyconformity was not observed during the first 15 minutes of odorant introduction (Figure 6). The changes in metabolic rate ($\mu mol\ O_2\ g^{-1}\ hr^{1}$) in response to odorants and ink concentrations were compared via one-way ANOVA to test for a significant differenence from seawater, followed by a post-hoc Tukey test. All data analysis and statistical tests were performed using R (R Development Core Team, 2008) with RStudio.

RESULTS

Quantifying Ink Concentration

Analysis of four individual octopus ink samples showed no significant difference in absorbance between individual's melanin content (Figure 1, ANCOVA p=0.950; 0.774, n=4, df=0.085, f=2.637). The equation generated by the standard curve and used to determine unknown concentrations of ink collected *in situ* was

$$[ink] = 0.0353 abs265 + 0.00114$$

with an R^2 value of 0.959 (Figure 2).

Octopus Response to Odorants

Octopus ventilation rates were determined by counting ventilations during the first two minutes of odorant introduction. Ventilation responses to different odorants and increasing concentrations of melanin were compared to seawater ventilation rates for significant differences. Octopus ventilations did not significantly change from seawater when presented with any odorant group (Figure 7, Kruskal-Wallis, Wilcoxon p>0.05, df=5, chi-squared=19.448). Though insignificant, octopus ventilation response increased to crab odorant, *Hemigrapsus nudus* (Figure 7, p=0.2281). Octopus ventilations did not significantly change from seawater among all melanin concentrations groups (Figure 8, one-way ANOVA p=0.898, df=1, f=0.017).

The change in metabolic rate was measured for thirteen octopuses in determination of their response to prey and octopus odorants, however, due to malfunctions during data collection, only ten octopuses metababolic rates were

determined (Figure 9). Metabolic responses to different odorants were compared to seawater response for significance. Crab odorant response increased and was significantly different from seawater (Figure 9, one-way ANOVA, Tukey $p<0.003$, $n=10$, $df=5$, $f=5.8$). Other trends suggest that octopuses respond to scallop odorant similarly to crab odorant, though this was not statistically significant (Figure 9, $p=0.4375$).

Change in metabolic rate was measured for thirteen octopuses in response to conspecific ink. Metabolic rate was not significantly different between ink concentration groups (Figure 10, one-way ANOVA $p>0.05$, $n=10$, $df=6$, $f=2.571$). Though statistically insignificant, most octopuses increased their metabolic rate when they were presented with the most dilute ink (0.028 μg/mL) over seawater control, at a mean change in metabolic rate of 2.198 μmol O_2 g^{-1} hr^{-1}, as compared to seawater, 1.8176 μmol O_2 g^{-1} hr^{-1} (Figure 10).

DISCUSSION

Quantifying Ink Concentration

Four different ink samples showed no significant difference in absorbance, suggesting similar concentration of melanin in individual octopus ink sacs. These results, however, did not account for any variation that may be caused by mucus *in situ*. Octopuses can vary the consistency of their ink by ejecting different ink to mucus ratios, which is demonstrated by their ability to create ropes or pseudomorphs of ink. My analysis did not account for any introduction of mucus into the ink, allowing me to compare the absorbance of the components originating from the ink sac. Because the components found in ink (melanin, metals, and proteins) are diet-derived, my results suggest that the components found in octopus ink sacs may be uniform among individuals fed the same diet. Further research into the varying diets of different populations of conspecifics and its effects on melanin composition in the ink sac would help us understand how octopuses obtain components needed for inking.

Avoiding Interference of Proteins in Ink Samples

During octopus ink collection, I was unable to account for the influence of mucus (glycoproteins, polysaccharides, and proteins) on the spectrophotometry analysis used to determine the concentrations of ink in samples collected *in situ* (Davies & Hawkins, 1998; Smith, A. M., 2002). Although this may have affected the results, I believe this is unlikely. Polysaccharides are abundent in octopus ink, but absorb most strongly at 200nm and only weakly at 265nm, thus do not likely interfer (Liang et al., 2014). Manchester

(1995) describes a method using 260/280 as a way to determine purity of DNA or RNA samples. In these methods, high ratios suggest purer DNA or RNA samples, as nucleic acids absorb strongly at 260nm and lower ratios suggest more protein contamination, as proteins absorb at 280nm (Manchester,1995). Because proteins absorb at 280nm in this assay and do not interfere with absorbance at 260nm, interference by these components in my ink analysis at 265nm seems also unlikely. Other methods of protein analysis recognize that protein concentration determinations can be performed via UV absorption at 280nm, but results can be skewed and unreliable depending on the side chains and complexity of the proteins, so this method is not recommended. Thus, most protein analysis is suggested to be performed under visible light absorbance, 500-600nm (Aitken & Learmonth, 2009; Olson & Markwell, 2007). Additionally, octopuses are capable of using different ratios of mucus/ink depending on their response to a specific stimulus (Derby, 2014). All octopuses were subjected to the same stimulus (30mL plastic syringe) in order to induce inking, thus the ink/mucus ratios was likely approximately the same. Future research would be appropriate to determine if and how mucus influences the analysis of octopus ink concentrations, as well as to determine what components in the ink sac or mucus funnel are causing a response. Octopuses' response to ink may be stimulated due to their reception of dopamine, similarly to the squid *Sepioteuthis sepioidea,* which displayed escape behaviors when presented with dopamine (Wood et al*.,* 2008). Derby (2007) found that dopamine was produced by ink gland cells located in the ink sac of most mollusks. Follow-up research could involve separating the ink sac and

mucus funnel components and introducing them to octopus subjects independently in order to see what component elicits a response.

Octopus Response to Odorants

Octopuses demonstrated an increased ventilation and metabolic response to prey odorants introduced into the respirometer. When they were exposed to the extracts of the shore crab *H. nudus*, octopuses may have increased their ventilation rate (though non-significantly) from seawater, and in conjunction, increased their metabolic rate. These results are also supported by Walderon et al. (2011) as they observed an increased ventilatory response of *Octopus bimaculoides* when presented with food odorants. Further, octopuses may have increased their metabolic rate and ventilation rates when exposed to *C. hastata,* although not significantly. Octopuses may be capable of distinguishing between different prey odorants, as they significantly increased their response when introduced to *H. nudus* but did not increase their response significantly when exposed to *C. hastata*. This octopus species has been documented to have a preference for eating crabs more than other prey, which may account for the increased metabolic rate when exposed to crab odorant (Onthank, 2008). Another explanation may be that the crabs, *H. nudus,* emit a stronger prey odor then the scallop, *C. hastata,* thus the octopuses responded more strongly to the stronger smell.

Although there were no significant changes in ventilations or metabolic rate when exposed to octopus odorant, *Octopus rubescens* may adapt their response to octopus odorant they were introduced to depending on the risk for predation. Trends for both

ventilation and metabolism show that octopuses may have had a slightly elevated response when subjected to *E. dofleini* odorant and slightly depressed response when subjected to conspecific odorant. *Enteroctopus dofleini* are capable of eating smaller octopuses such as *O. rubescens.* Because of the risk*, O. rubescens* may evade the larger octopus species rather than utilizing crypsis behavior, resulting in increased activity and increased metabolic rate when exposed to *E. dofleini* odorant. Because conspecific interactions may be less likely to result in predation, *O. rubescens* may utilize cryptic behavior resulting in lower activity and metabolic rate when exposed to conspecific odorants. Further research in how different octopus species interact would shed light on how octopuses inhabit shared ecosystems.

Octopuses responses to odorants could have been effected by the sex or age of the donor. The *E. dofleini* octopus used was a juvenile female and this may have caused a different reaction then it would have with an adult octopus. Another study comparing the metabolic response of *O. rubescens* to mature and immature *E. dofleini* would expand our knowledge of interspecific interactions. Additionally, all *O. rubescens* odorants were taken from male octopuses, thus the reaction may change depending on the sex of the odorant donor. Whether *O. rubescens* respond differently metabolically depending on the sex or age is not documented. These suggestions on sex and age of donor demonstrate a need for further research in order to understand octopus chemical communication.

Ventilation response did not significantly change when octopuses were exposed to different concentrations of conspecific ink, which may highlight a potential source of variation. Monitoring ventilation rates of octopuses was variable, as ventilations might

have been variably deep or shallow. How much oxygen extracted per ventilation was not measured during experimentation, thus this should be investigated in future research to see how this contributes to the variability of octopus ventilation rates. Additionally, octopuses would sometimes perch at the top or at the corners of the respirometer, not allowing any view of the mantle or siphons for ventilation analysis. Despite complications, ventilatory response to prey and octopus odorant mirrored metabolic responses as would be expected. Because of these inconsistencies, respirometry appears to be the more informative way of determining an octopus response.

Octopuses displayed an unexpected response to increasing concentrations of conspecific inking. As expected, all but one of the 10 octopuses increased their metabolism when subjected to the most dilute conspecific ink concentration (0.028 μg/mL), suggesting excitation to the odorant. Contrary to my hypothesis, the octopuses did not elevate their metabolic rate when subjected to ink more concentrated than 0.028 μg/mL, and metabolic data suggested even a depressed metabolic rate. There are several factors that may account for these unexpected results. First, octopuses may be slowing ventilation rate in order to avoid impairment of their gills which can be caused by ink (Derby, 2014). Second, octopuses may have been intimidated by dark seawater and respond by a behavioral freeze, as seen in cuttlefish (King et al., 2006). This was less likely, as there was no depressed response when the octopuses were exposed to fake ink visibly equivalent to 0.9 μg/mL of conspecific ink. Third, octopuses may be responding differently depending on the apparent proximity of inking. Octopuses may avoid predation by moving away from the source when they detect more dilute ink, or become

cryptic and perform a behavioral freeze when they detect more concentrated ink. Higher concentrations of ink could indicate a threat that is close or a recent inking has occurred. Perhaps the best defense against a close threat could be a crypsis behavior, which could include a reduction in metabolic rate, similarly to the behavioral freeze performed by *S. officinalis* (King et al., 2006). Many organisms, such as dogfish, use electroreception to detect the heartbeat of prey. North Pacific Spiny Dogfish are known predators of octopuses, as studies have observed that cephalopods are a dominent prey source for dogfish (Bigman, 2013). Perhaps octopuses have adapted a behavioral freeze in which to avoid electrodetection by predators. The detection of a lower concentration of ink could indicate a threat that is farther away or an inking that occurred some time before, thus the best response may be to move away from the threat. An increased metabolic rate may be exhibited in preparation of moving or fleeing from the potential threat. Octopuses responded to conspecific inking in an unexpected way, requiring future research to understand if and how octopuses respond to conspecific inking and threats at various distances. Respirometry data may also have been skewed due to the fact that octopuses are not an easy organism to contain nor on which to perform respirometry. Respirometry data were noisy, as octopuses would sometimes perch on the oxygen optode, potentially skewing oxygen calculations during respirometry. Some octopuses would also sit on and stifle recirculation of water in the respirometers, which also may have skewed the data. Despite the potential skewed data, trends in *O. rubscens* response to conspecific inking sheds light on potential communication mechanisms of cephalopods and requires further research to better understand these behaviors.

Octopuses responses may have been skewed due to habituation in captivity. Octopuses were captured and used at various times during the experimental summer, which may have allowed for them to get used to their surroundings and being fed or subjected to new stimulants. Because of this, octopuses may have become less responsive during experimental trials. Additional research in which respirometry trials are performed on octopuses within 24 hours of their capture would help decide if results were skewed by habituation.

Future Research

The research presented offers insight for future research in to octopus chemoreception of environmental cues and suggests new questions about the complexity of octopuses' behavior and interactions with their environment. Octopuses showed an elevated metabolic rate in response to extracts of some prey animals. More research into understanding what molecules octopuses can or cannot detect would shed insight into the complexity of octopus chemoreception and how this affects their physiology. Observing octopus responses to threats at various distances would also be beneficial in understanding how octopuses behave under stress. This study also suggests elevated metabolic rates at lower concentrations of conspecific ink. Future research would provide insight into effects of individual components found in ink. Separating components out of ink, such as proteins, melanin, or hormones (dopamine) and introducing them to octopuses would show what part of octopus ink is eliciting a response among organisms.

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FIGURES

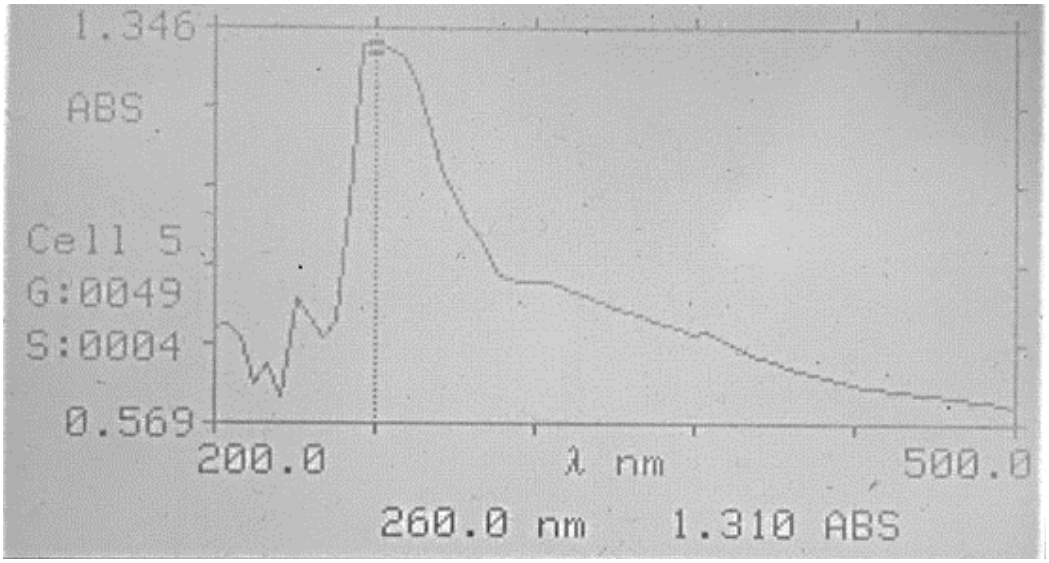

Figure 1. Scanning spectrophotometric analysis on ink. Spectrophotometric analysis with a DU530 spectrophotometer showed peak absorbance for 400 µg/mL to be at 260-265nm. Similar absorbance results were found for ink ranging from 50-200 µg/mL.

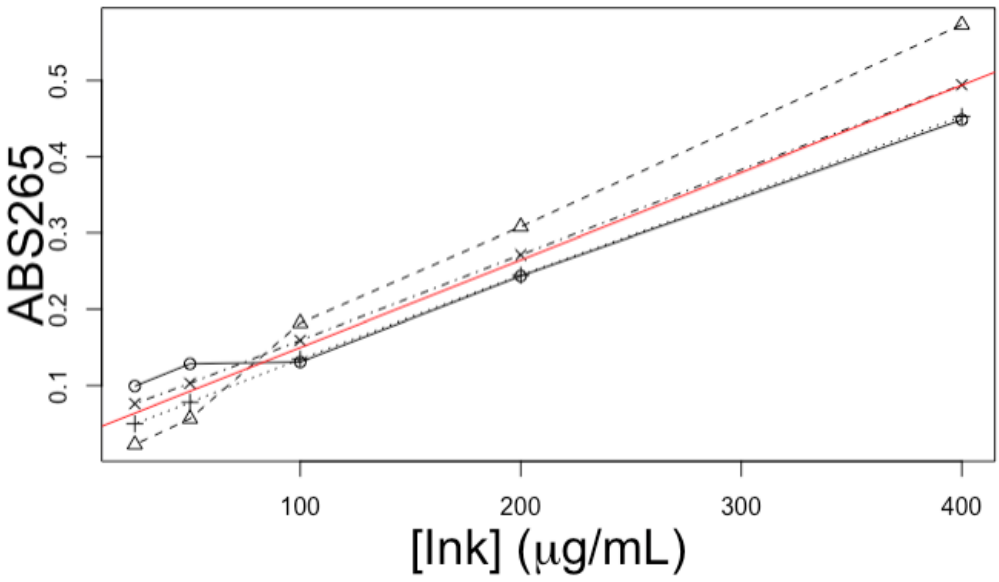

Figure 2. Absorbance of solutions of ink sac contents in filtered seawater by concentration. Results showed no significant difference in absorbance between individual octopus ink sac components when ink sacs were collected, dried, and resuspended in artificial seawater (ANCOVA p=0.950; 0.774, n=4, df=0.085, f=2.637). A linear model (red line) was generated in order to create an equation that encompassed different octopus ink standard curves. The equation used to determine unknown ink concentrations by the standard curve was [ink]= 0.0353*abs265*+0.00114 with an R^2 value of 0.959. Data accounted only for ink sac components, not mucus that is introduced by the mucus funnel *in situ.*

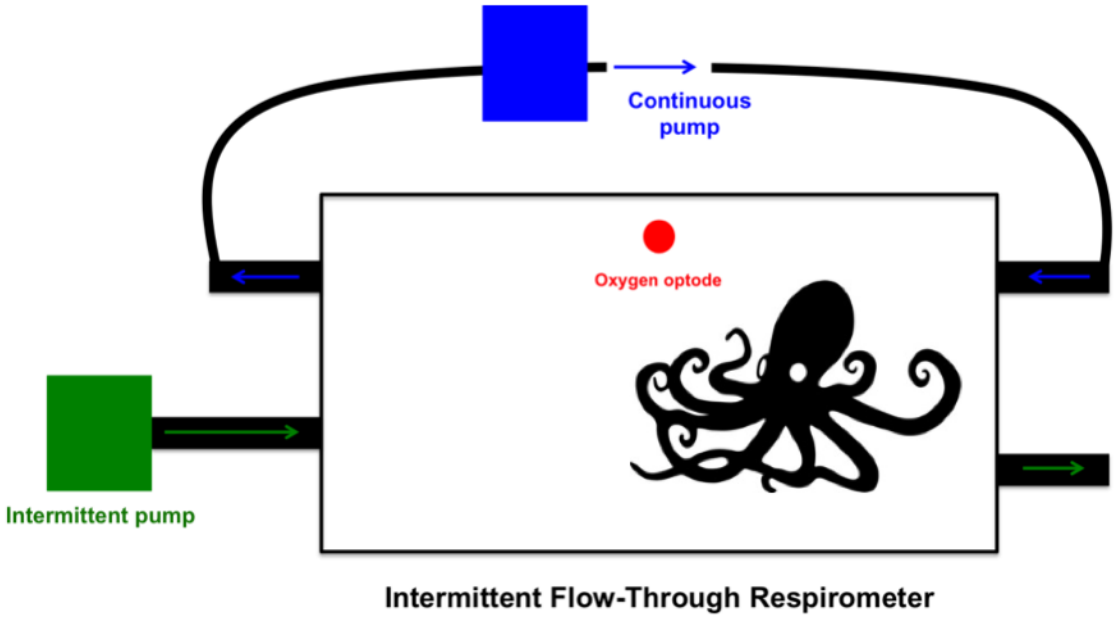

Figure 3. Intermittent flow-through respirometer. A 2.2 liter PET plastic container outfitted with two pumps connected in parallel and equipped with a Pyroscience oxygen optode was used as a respirometry chamber. A continuous pump (blue) provided constant flow in order to circulate water in the respirometer chamber. An intermittent pump (green) turned on every 60 minutes for 15 minutes in order to flush the chamber and restore oxygen levels. Oxygen levels were monitored with a Pyroscience oxygen optode (red).

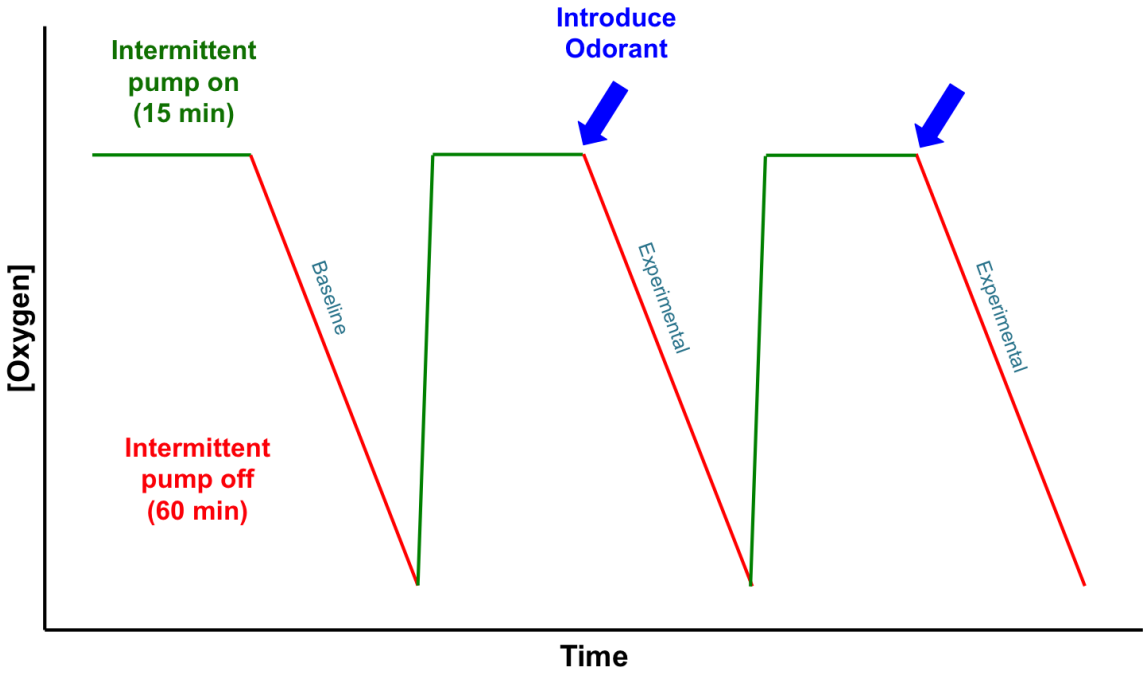

Figure 4. Diagram of anticipated relative oxygen concentrations in respirometer during intermittent flow-through respirometry. Every 60 minutes an intermittent pump turned on (green) and flushed the respirometer, restoring oxygen levels for 15 minutes. After 15 minutes, the intermittent pump switched off (red) and the respirometer became a closed system, octopus metabolic rate causing a decrease in the limited supply of oxygen. Experimental odors were added to the system at the end of the 15-minute flush period and beginning of closed respirometry (blue arrow).

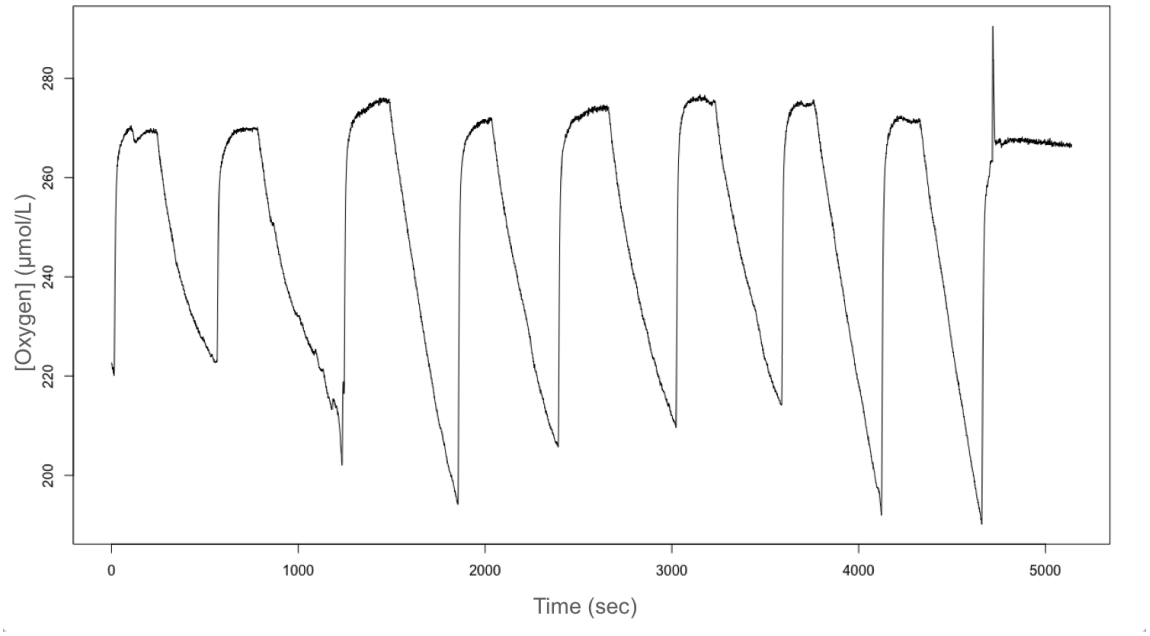

Figure 5. Representative experimental respirometry results. The y-axis refers to the oxygen levels monitored in the chamber with the Pyroscience oxygen optode. The first drop in oxygen levels refers to the baseline of the octopus without being exposed to an odorant. Sequential dips in oxygen levels following the baseline refer to the experimental odorants added to the respirometer chamber at random.

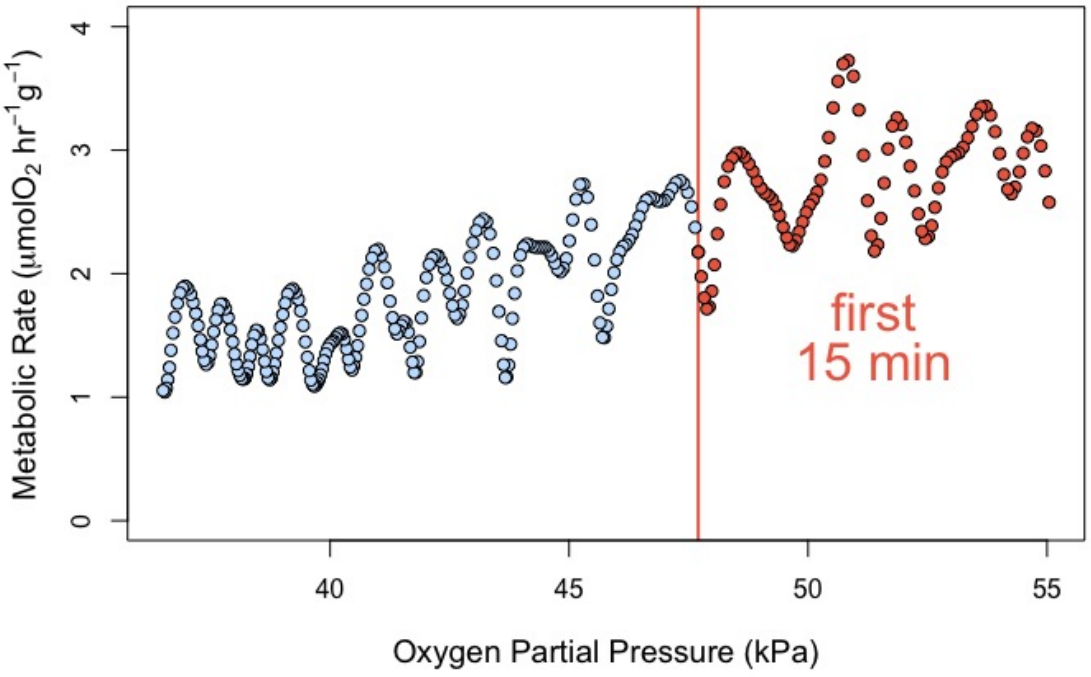

Figure 6. Analysis of potential oxyconformity during respirometry trials. The first 15 minutes of each treatment did not appear to be affected by oxyconformity by the octopus.

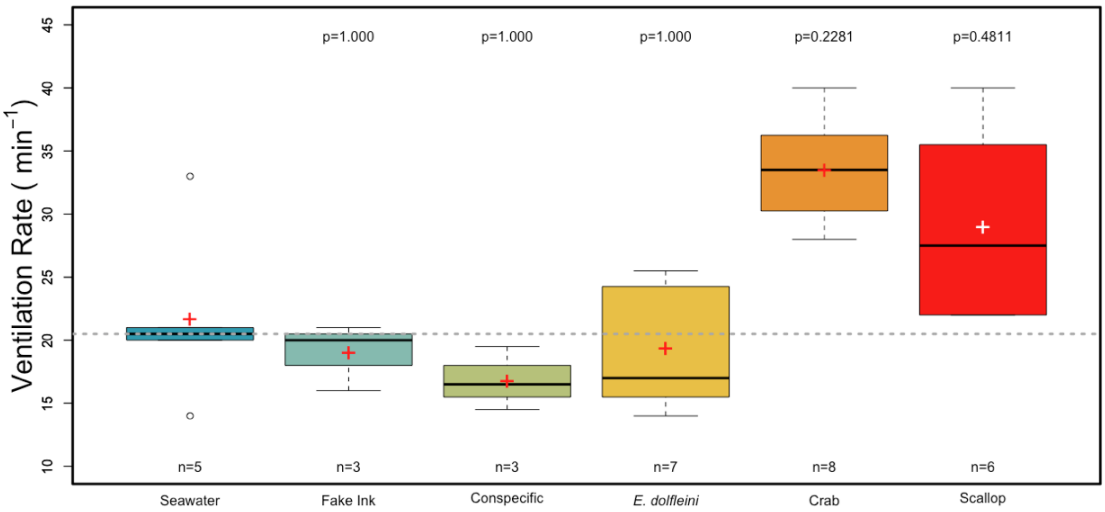

Figure 7. Octopus ventilation response to octopus and prey odorants. Odorant ventilation responses were compared to seawater ventilations for significance. Dotted line indicates mean seawater ventilation rate. Cross symbols indiate the mean of each group. No groups were significantly different from seawater (Kruskal-Wallis, Wilcox $p>0.05$, n=3-8, df=5, chi-squared=19.448).

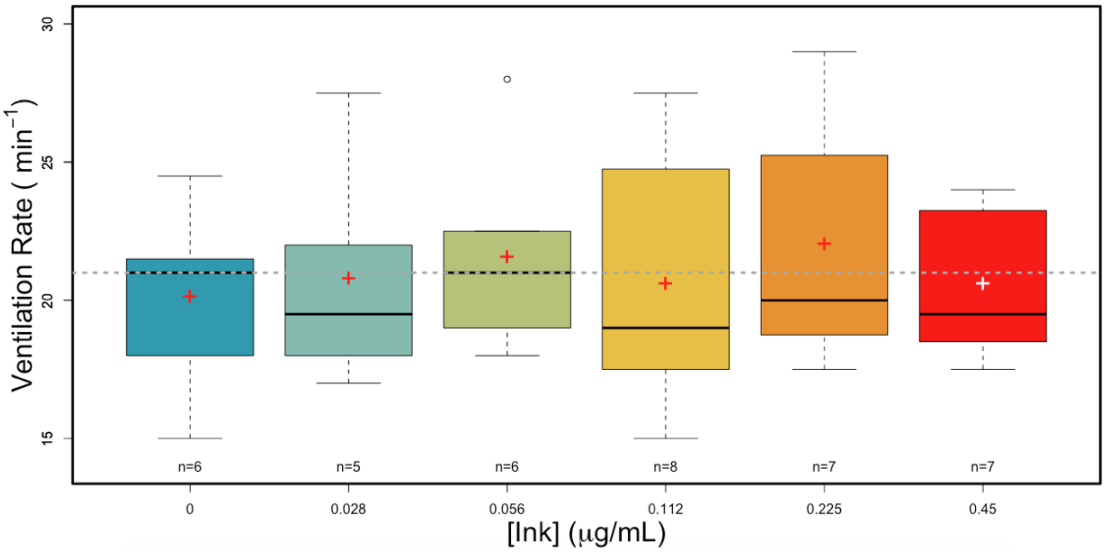

Figure 8. Octopus ventilation in response to increasing conspecific ink concentrations. Ventilations were compared to seawater ventilations for significance. Dotted line indicates mean seawater ventilation rate. Groups were not significantly different (one-way ANOVA p=0.898, n=5-8 , df=1, f=0.017).

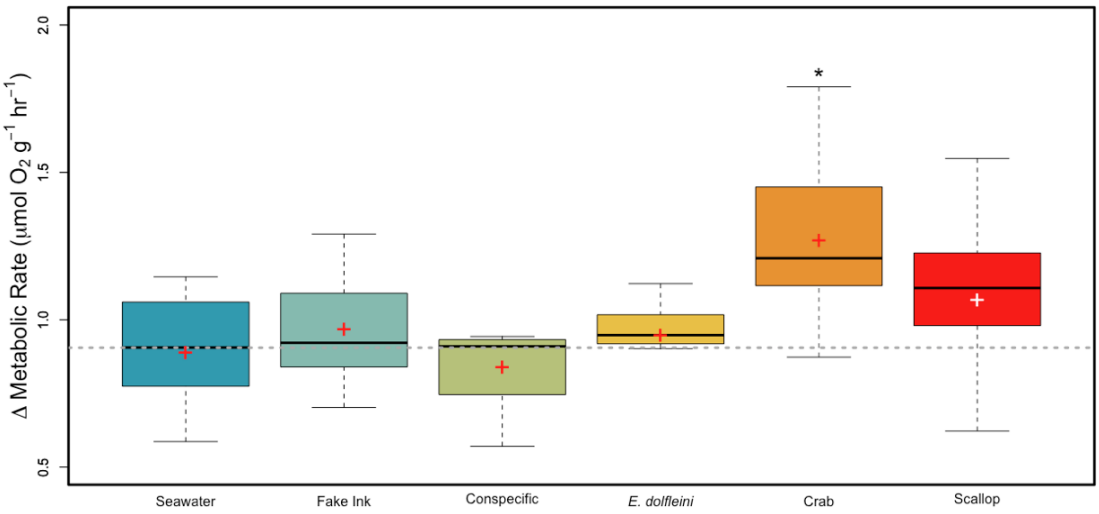

Figure 9. Octopus metabolic rates in response to octopus and prey odorants. Dotted line indicates mean of seawater change in metabolic rate. Metabolic rate in response to different odorants were compared to the metabolic rate when exposed to seawater for significance. Cross symbols indiate the mean of each group. Asterisk indicates group was significantly different from seawater. Crab odorant was the only significant change in the metabolic rate from seawater (one-way ANOVA, Tukey $p<0.003$, $n=10$, $df=5$, $f=5.8$).

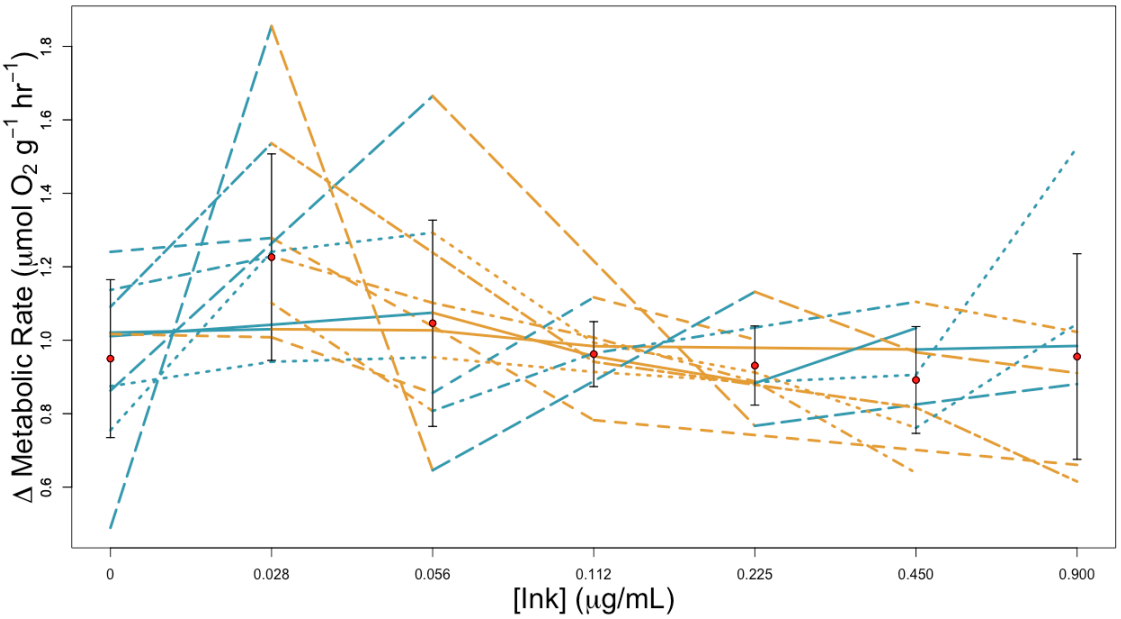

Figure 10. Octopus metabolic response to increasing conspecific ink concentrations. The change in metabolic rate (μmol O_2 g^{-1} hr^{1}) was calculated by measuring the oxygen consumption of each octopus during the first 15 minutes of odorant introduction and dividing it by the first 15 minutes of the baseline. Blue lines indicate an increase in metabolic rate, while yellow lines indicate a decrease in metabolic rate from next lowest ink concentration measured. Red points depict the mean and standard deviation of each group. There were no significant differences between the groups (one-way ANOVA $p>0.05$, $n=10$, $df=6$, $f=2.571$).