Effects of Squid Ink on Feeding Behavior in Fish

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Preface with Acknowledgements

My time at Stanford has been tumultuous. I came in with a vision to be some hotshot politician, so I could right all the wrongs I felt that life had dealt me. However, all it took was one fateful trip to the California Academy of Sciences to realize I had been completely and totally wrong. I spent one afternoon there absolutely enthralled by the biological exhibits. From then on, I based my undergraduate education around biology, chasing that same wonder.

I am especially thankful to Professor Barbara Block for opening my eyes to the ocean. During my sophomore year, my coursework took me to the Palmyra Atoll, Kingman's Reef, and Kiribati on a research vessel. It was then that I truly fell in love with the sea. Don't get me wrong, I have always been fascinated by it (I grew up in a desert), but there's nothing like waking up and only seeing ocean on all sides. From then on, my studies have focused on marine biology.

By January 2012, when I set out to begin work on this thesis, I had already worked the previous summer with Professor William Gilly on squid and I felt that it would excellent if I carried on with him. I have a fondness for squid – I studied them on the research vessel and I have one tattooed on my right arm – that I felt his lab shared. I am very grateful for his help over the past year with this project. He afforded many freedoms in its design and outcome.

Finally, I am very appreciative of the help and support from the rest of the lab and Hopkins Marine Station folks who helped me capture squid and manage my fish. I would like to thank Sara Brownell for her help in writing the beginning and giving me a lot of moral support. Of course, I have not forgotten Professor Christopher Lowe. I know I have been a tricky first advisee, but believe me when I say I trust I am on the right path now with a lot of support from you.

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Abstract

To help escape from predators, cephalopods of the order Teuthida (squids) eject ink in two ways: as dense pseudomorphic blobs and as large diffuse clouds. This research sets out to explore a chemical basis for ink utilization by cephalopods beyond simple vision impairment. To test the effect of squid ink on fish, Pacific sanddabs were placed in a tank and fed small pieces of squid through a tunnel at the end of the tank. As fish approached the food, squid ink from the California market squid, *L. opalescens*, was ejected locally into the environment. Our research suggests that squid ink interferes with the ability of fish to pursue and find food. This suggests that there is some chemical component in the ink that may be assisting escape from predatory fish. As environmental and ecological factors influence the growth of squid populations across the world in response to the decline of fish stock and the destruction of ecosystems, a more thorough understanding of the squid lifestyle will be helpful in designing policies that will sustain these stocks.

I. Introduction

Objectives

This research sets out to explore the role that squid ink ejection plays in predator-prey interactions. In particular, the research focuses on how chemical compounds in the ink may work to deter predators. Based on techniques used by similar ink-ejecting organisms, this research proposes that squids employ phagomimicry – a tactic used to confuse predators by inducing behavioral changes in the feeding habits of predators – via ink ejection.

In this introduction, we begin by presenting an overview of cephalopod escape mechanisms. We next enumerate various ways that squids use their ink. Then, we will talk about various observations on inking behavior in cephalopods. Finally, we will discuss how global climate change might alter predator-prey interactions, suggesting the current research's importance.

Ink as an Escape Mechanism

Coleoid cephalopods – squids, octopodes, and cuttlefish – are descendants of primitive cephalopod mollusks. Primitive cephalopod mollusks had protective shells; however, virtually all modern cephalopods (other than *Nautilus*) have lost the shells. In the process, they gained a higher degree of mobility (Wells 1995). However, that greater mobility came with its own price. No longer able to protect themselves with their shells, these cephalopods had to develop highly complex predator-avoidance and escape mechanisms (Thompson 1994). These mechanisms include ink discharge, and the ability to control the color, pattern, and texture of the skin (Packard 1972; Gilly 2012).

Squids eject ink when threatened by predators (Derby 2007). The ink is ejected primarily in two forms: dense pseudomorphic blobs and large diffuse clouds. It is believed that the pseudomorphic blobs are used as decoys in order to visually fool the predator into pursuing the wrong target. On the other hand, when the squids eject large diffuse clouds of ink, they probably do so to impair the predator's vision (Lucero, Farrington, and Gilly 1994).

Unlike skin modification, the production and ejection of ink has high metabolic costs (Hanlon and Messenger 1996). We would therefore expect, then, to see squids produce and eject ink selectively, and specifically, to see ink use decrease in areas of low light, because the visual trick would be less effective. However, squids have been recorded ejecting ink in areas where as much as ninety percent of light has been attenuated. (Bush and Robison 2007). Therefore, squid ink may be working to aid in escape via mechanisms other than just visual impairment.

Ink's Influence on Behavior

The sea hare, a marine gastropod mollusk, also ejects ink in order to avoid predation. In addition to visually impairing predators, this ink serves a secondary function: phagomimicry. In a 2005 study by Kicklighter, sea hares were placed in a tank with their crustacean predators. When attacked, threatened sea hares ejected ink that stimulated appetitive and ingestive behavior in the crustaceans. The study proposed that the ink had chemically tricked the crustacean into thinking that it had already caught the sea hare: the ink had coated the crustaceans' sensory organs with free amino acids, goading them into attending to the cloud of ink as if it were the sea hare itself (Kicklighter et al. 2005). Squid ink also contains amino acids for which fish and crustaceans have specific receptor systems (Derby 2007), leaving open the possibility that squid ink can similarly utilize phagomimicry.

A parallel tactic has been observed in the interactions between green sea turtle hatchlings and pygmy octopodes. When the turtles were placed in a tank with the octopodes, the turtles immediately attacked them. Octopodes that escaped attack had released psuedomorphic ink blobs. The turtles were then taken out and put back into the tank with survivor octopodes at a later time. This time around, turtles that had been previously inked ceased predation attempts, whereas naïve turtles continued to prey upon the octopodes as if for the first time (Caldwell 2005).

While studies focusing specifically on squid have been limited, moray eels, predators of octopodes, have been observed to exhibit distinct behavioral changes when ink is injected into their aqueous environment (Fox 1976). Squid ink, we might then hypothesize, may contain similar chemicals that alter predator behavior and aid in escape.

Is there a chemical signature to squid ink?

Squid ink is composed primarily of melanin, but also contains the precursors L-DOPA, as well as dopamine, tyrosinase, and various amino acids. L-Dopa and dopamine in the ink of the squid *Loligo Opalescens* has been shown to act as a communication signal between squids (Gilly and Lucero 1992). In addition, squid ink has been found to contain high amounts of the enzyme tyrosinase. It was believed that tyrosinase might serve as a noxious chemical to deter predators (Prota el al 1981), however there is little to support the chemical-deterrent hypothesis (Derby 2007).

Potential Impacts

If chemicals in squid ink play an important role in predator-prey interactions, there could be important consequences. Changes in oceanic pH, temperature, and oxygen will affect the way future generations of squid populations behave. Such changes might, for example, alter the effectiveness of the ink by influencing chemical reactions. Reductions in oxygen might reduce the ability of the squid to produce the metabolically expensive ink. Increases in temperature might apply pressure on the respiration of the squid and potentially denature essential proteins. As environmental and ecological factors influence the growth of squid populations across the world in response to the decline of fish stock and the destruction of ecosystems, a more thorough understanding of the squid lifestyle will be helpful in designing policies that will sustain these stocks.

Hypothesis, Experiments, & Questions

We hypothesize that chemical compounds in the ink are working to deter predators. To test the hypothesis that squid ink acts to chemically distract fish, we modeled squid escape mechanisms by presenting squid ink to feeding fish in a controlled laboratory setting. We investigated several behavioral modifications guided by the following questions:

Does the presence of squid ink increase the time it takes for the fish to retrieve the food? Increases in the length of feeding time, when controlled for visual and physical stimuli, suggest that something other than a simply physical or visual sensory interaction is impeding feeding. Does the presence of squid ink influence subsequent feeding times? If the feeding times of subsequent (inkless) feedings are changed, then that may suggest a long lasting mechanism. Do we observe a change in fishes' general activity level in response to the presence of ink? Changes in activity level might indicate a physiological response to the ink. Are there differences in behavioral response following the introduction of ink? Any changes will provide some insight into the mechanism through which the ink is acting.

II. Materials & Methods

Fish were placed in a maze-like tank that served to direct the fish toward food, providing a reliable method to introduce ink through a vertical delivery tube to a localized area and preventing diffusion of the ink around the tank. The tank, 9.1 x 6 x 6.1 inches, was designed so that as the fish approached the food that had been dropped into the delivery tube, we could introduce a small bolus of squid ink, thereby exposing the fish's sensory organs to stimuli contained in the ink. Seawater was pumped from Monterey Bay into our research environment in the Hopkins Marine Station using a flow through system throughout the duration of the tests.

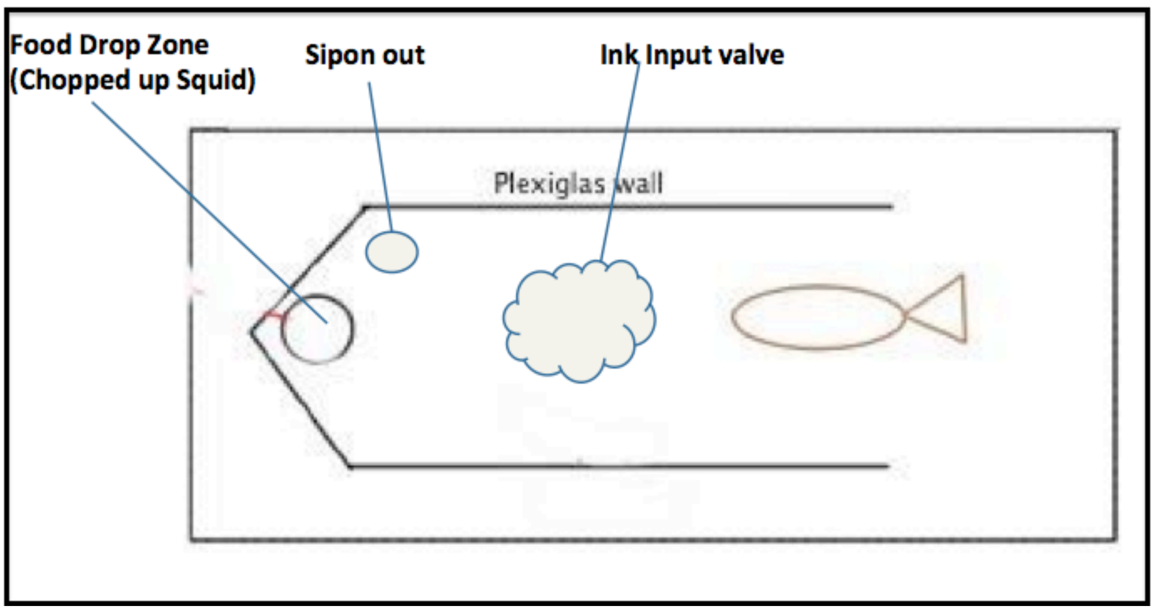

Figure 1. Schematic Top Down View of The Test Tank: Ink is introduced at the input valve as the fish approaches the food at the drop zone. After fifteen seconds, the ink was removed through the siphon out.

Fish were fed three approximately 0.5g pieces of chopped squid mantle over the course of fifteen minutes at the specified zone of the tank (see Fig. 1). Feedings occurred at five-minute intervals: at the zero mark, five minute mark, and finally at the ten minute mark. The initial feeding, at the zero mark, was used as a no substance control. To test the effects of ink, a 2 mL bolus of ink was introduced between the fish and the food at the second feeding (5:00 mark) as the fish approached the dropped food. After 15 seconds, the ink was removed via siphon. We believe that this was an effective way to test the effect of ink on feeding and as a model for a fish being suddenly exposed to ink during an attempted predation on a living squid. To test whether the presence of squid ink influenced subsequent feeding times, a third piece of food was dropped at the 10:00 mark without any substance ejection.

Cephalopod ink was obtained from the ink sacs of the squid *Loligo opalescens* posthumously. As a visual control, we used McCormick-brand Blue #1 dry powdered food coloring to obtain a fluid that was as dark as the ink, judged visually in a 1mL spectrophotometer cuvette with a BioSpec-1601. To rule out that fish reacted due to the physical stimulation of a substance on their sensory organs, we used filtered seawater to simulate a pressurized fluid. These substances were used along with a no-substance control, to separate the roles of physical and visual stimuli in ink ejection. In the no-substance control, the food was dropped and no substance was ejected.

We chose to model squids' interaction with Pacific sanddabs because these fish aggressively pursue food in lab environments, are easily obtainable in the Monterey Bay area, and because they are small enough to fit into our tanks. We used six sanddabs, separated into six different tanks. After allowing an acclimation period of two weeks for the fish to grow accustomed to the feeding regimen and tank environment, we began testing. Individual sanddabs were tested over the course of three weeks. During the first week of testing, the fish were subjected to the physical control treatment (filtered seawater). The second week of testing, the fish were subjected to the visual control treatment (food coloring). During the final week, the fish were subjected to the squid ink treatment. In this study, feeding time, also referred to as “retrieval time”, is the time period between when the fish begins to pursue the food and when the fish has consumed the food.

To measure the change in activity level, we used a qualitative five-point scale to describe the activity state of the fish. A one on the scale described a fish that made no attempts to move after feeding. A five on the scale described a fish that was erratically swimming all over the tank. A three on the scale described an average state in which, say, the fish moved slowly around the bottom of the tank. Activity levels were recorded before and after the introduction of the substance. A positive calculated differential on the activity level scale indicated a decrease in activity, and versa negative differential indicated an increase in activity.

Analysis of the data was done using Microsoft Excel, plotting the activity levels and response times against each other and calculating standard errors. For the plots where the means looked significantly different, the data were further analyzed using R. We ran a randomized-block design ANOVA test. We assume that the population figures were normally distributed, an assumption that graphs of our sample residuals supported. To separate the different values in the ANOVA, we then ran a Tukey’s HSD test to find which means actually differed from each other.

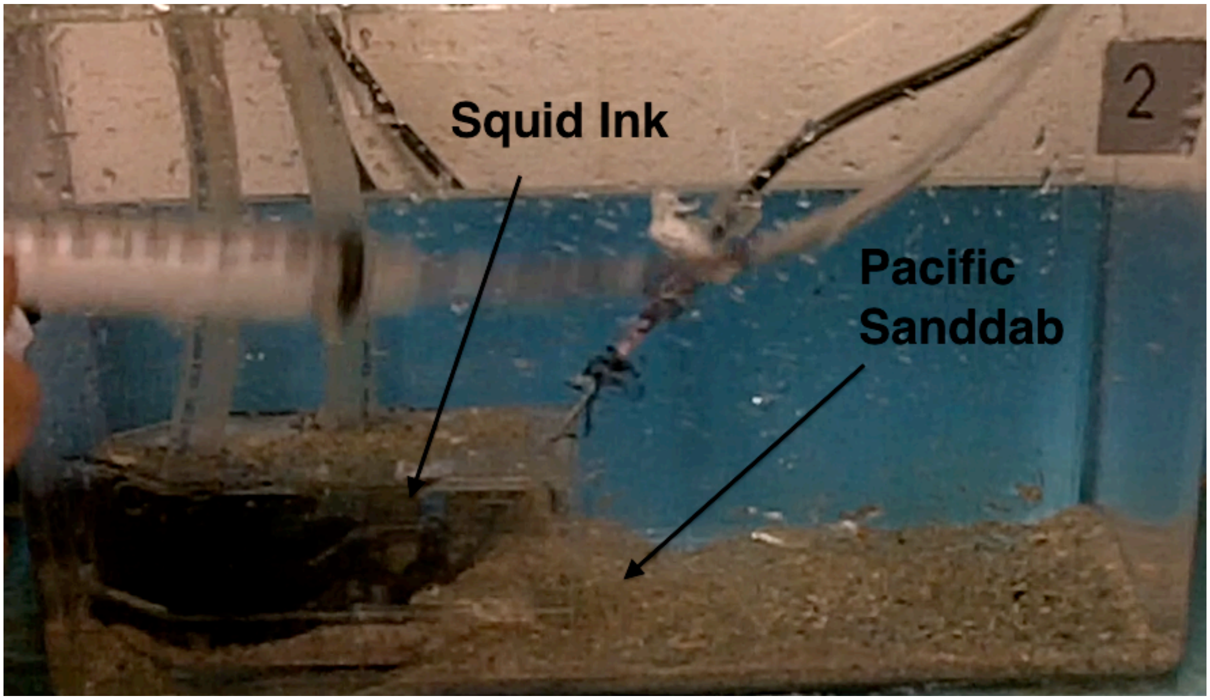

Figure 2. This sanddab is getting inked in its pursuit of food.

III. Results

Does the presence of squid ink increase the time it takes for the fish to retrieve the food?

We measured the time it took for fish to retrieve food after introducing ink into the environment between the fish and food. In this study, feeding time, also referred to as "retrieval time", is the time period between when the fish begins to pursue the food and when the fish has consumed the food. The average time for the control experiments (no injection) was 1.5 seconds with a standard error of 0.20 seconds. The average time for the physical control experiments (using the injection of seawater) was 1.2 seconds with a standard error of 0.07 seconds. The average time for the visual control experiments (using the injection of dye) was 2.9 seconds with a standard error of .50 seconds. The average time for the squid ink was 15 seconds with a standard error of 3.8 seconds (See Figure 3). The sample size was 30 trials for each substance.

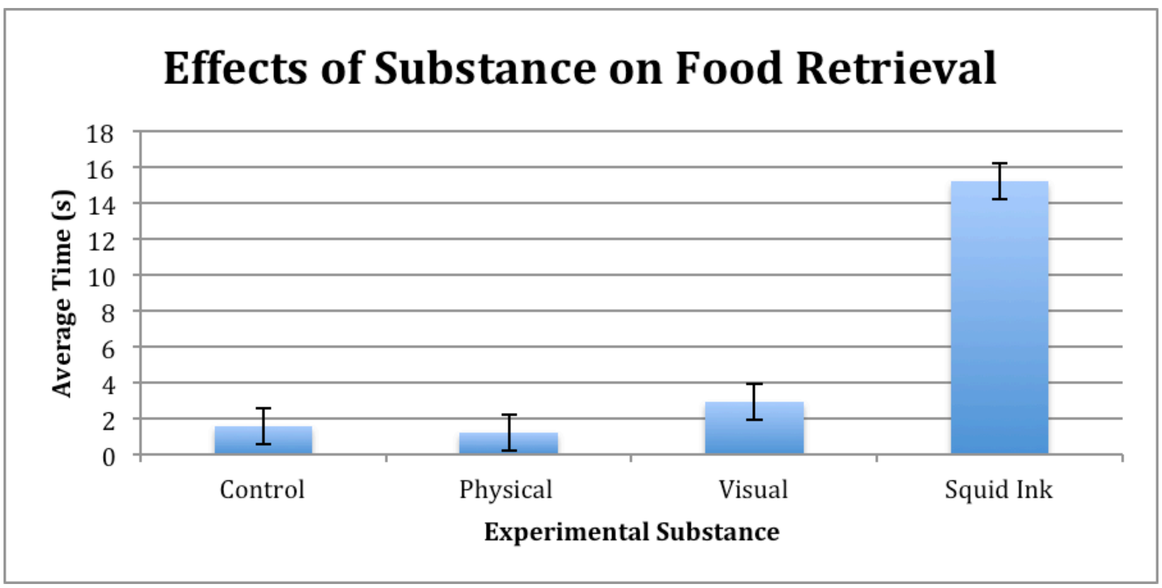

Figure 3. There was a significant difference in the mean time for retrieval between the squid ink trials and the controls for all the fish. The mean time for the control was 1.6 seconds, 1.2 seconds for the physical control (seawater), 2.9 seconds for the visual control (dye), and 15 seconds and for the squid ink.

In order to support our assumption of normally distributed population means, we ignored two outliers in the squid ink dataset with average times beyond 100 seconds. The ANOVA test returned a p-value of $4.25*10^{-9}$, indicating a significant difference in the means. To find which means were actually different, we ran Tukey's HSD test. (See Table 1). The results showed that the only significant p-value came from the comparisons of the controls against ink. Our results show that mean feeding time in the ink treatment was significantly different from the dye and seawater treatments.

Table 1. The results from Tukey's HSD test indicate that the only significant differences in mean food retrieval time occurred in the squid ink experiments. The p-values on the right, when below .05, indicate significance.

Substance	**p-value adj**
Physical – Control	1.0
Visual – Control	.85
Squid Ink – Control	.00
Visual – Physical	.84
Squid Ink – Physical	$1.0*10^{-7}$
Squid Ink	$3.5*10^{-6}$

Does the presence of squid ink influence subsequent feeding times?

We collected the time it took for fish to retrieve food after introducing ink into the environment. This consisted of a normal control feeding 5 minutes after the ink and other treatments. The average time following the control experiments (no injection) was 1.3 seconds with a standard error of 0.07 seconds. The average time following the physical control experiments (injecting seawater) was 1.1 seconds with a standard error of 0.07 seconds. The average time following the visual control experiments (injecting dye) was 1.2 seconds with a standard error of 0.1 seconds. The average time following the squid ink was 1.1 seconds with a standard error of 0.05 seconds (See Figure 4).

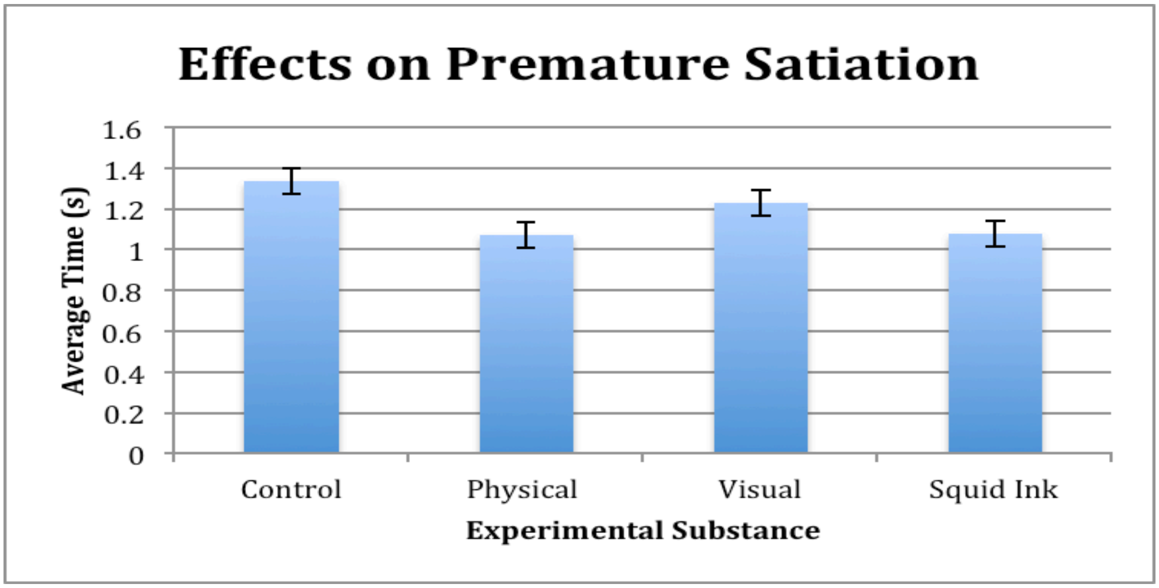

Figure 4. There was no significant difference in the mean time for retrieval between the squid ink trials and the controls for all the fish. The mean time for the control was 1.3 seconds, 1.1 seconds for the physical control (seawater), 1.2 seconds for the visual control (dye), and 1.1 seconds and for the squid ink.

The ANOVA test returned a p-value of 0.03 indicating a significant difference in the means. To find which means were actually different, we ran Tukey's HSD test. However, the results indicate that there is no significant difference between the means (See Table 2).

Table 2. The results from Tukey's HSD test indicate that there was no significant difference between the means in the test for premature satiation. The p-values on the right, when below .05, indicate significance.

Substance	**p-value adj**
Physical – Control	.07
Visual – Control	.81
Squid Ink – Control	.11
Visual – Physical	.62
Squid Ink – Physical	1.0
Squid Ink	.67

Do we see a change in activity level following the presence of ink?

Activity level was measured qualitatively on a scale of 1–5 before feeding and after feeding. A positive calculated differential on the activity level scale indicated a decrease in activity, and versa negative differential indicated an increase in activity. The average change in activity level for the physical control was 0.2, with a standard error of 0.1. The average change in activity level for the visual control was 0.4 with a standard error of 0.1. The average change in activity level for the squid ink was 0.0 with a standard error of 0.1 (See Figure 5).

The ANOVA test returned a p-value of 0.84 indicating a non-significant difference in the means.

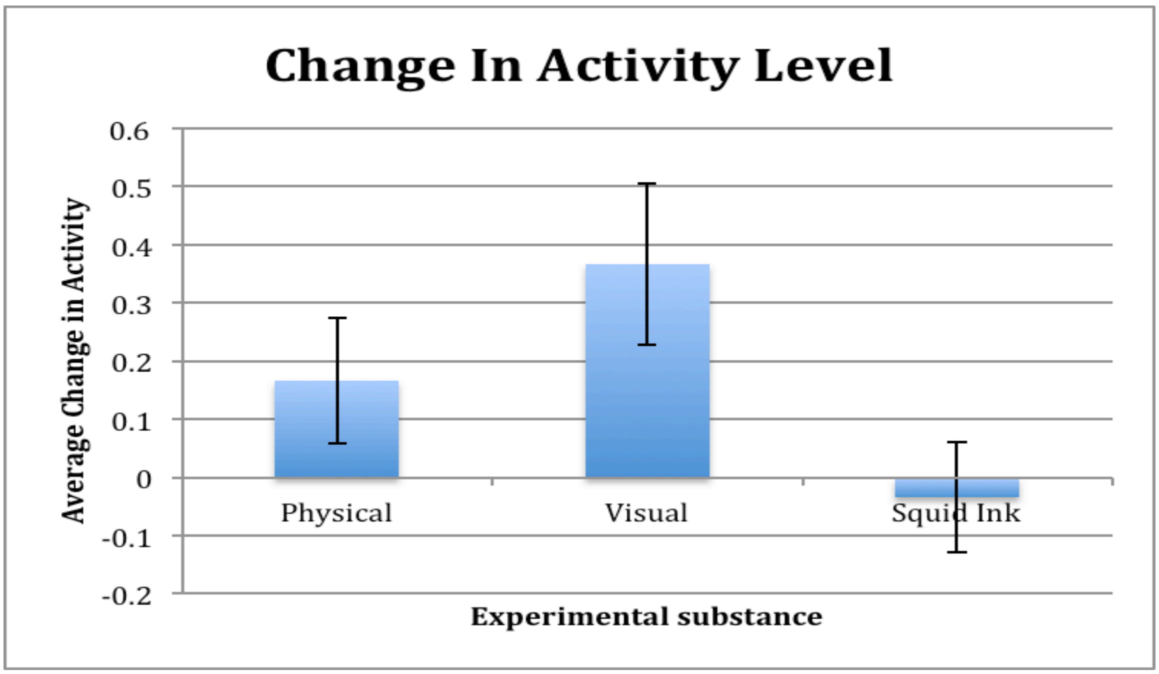

Figure 5. There was no significant difference in the change in activity level between the different controls and the squid ink. The mean change in activity level for the physical control was .2, .4 for the visual control, and 0 for the squid ink.

Are there differences in behavioral response following the introduction of ink?

For all thirty of the seawater experiments, there was no change in behavior when the substance was injected into the environment. When the seawater was injected, the fish continued right on past and straight to the food.

In twenty of the thirty dye experiments, the fish showed no hesitation in pursuing the food. In eight of the dye experiments, the fish showed a slight hesitation, on the order of one or two seconds, when the dye was injected into the environment. In only one case, the dye caused the fish to turn away and not return to the food.

In seven of the squid ink experiments, the fish showed no qualitative behavioral changes. In six of the ink experiments, the fish showed a slight hesitation as it pursued the food. In ten of ink experiments, the fish avoided the substance for a significant amount of time (between thirty seconds and three minutes) before continuing on to eat the food. This is also shown in figure 3 with the mean average time being significantly different in the ink experiments. Surprisingly, in six of the ink experiments, the fish seemed actively to try to eat the ink itself.

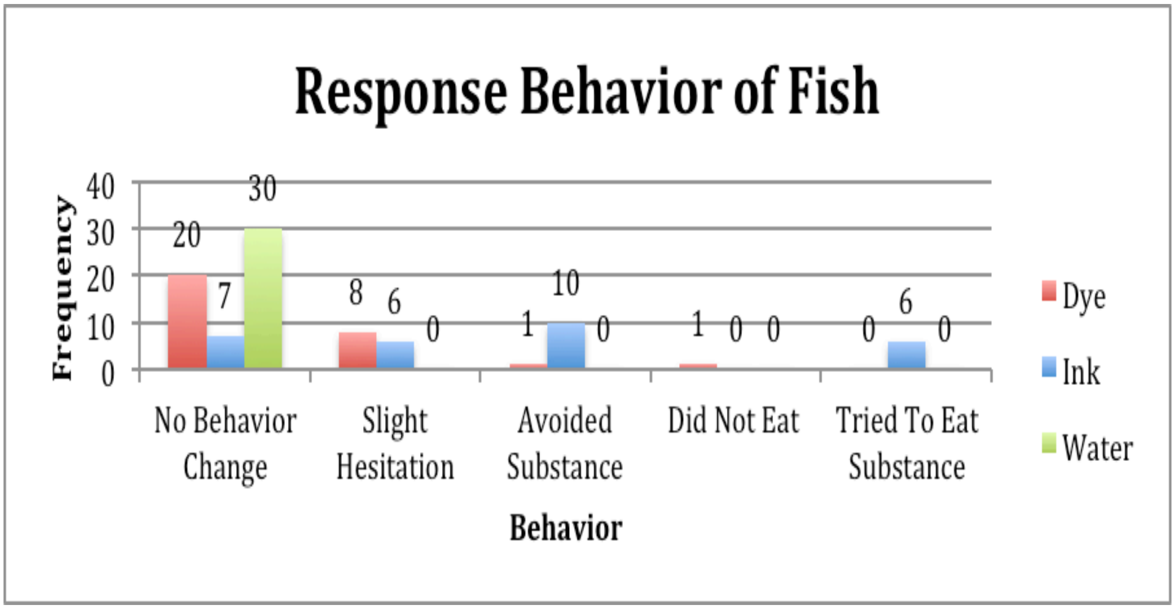

Figure 6. Five different types of behavior were observed when the ink was ejected. For the water experiments, there were no changes in behavior. For the dye experiments, there was no behavior change for majority of the runs, but there was some hesitancy to approach the food in eight of the runs. In the squid ink experiments, we observed complete avoidance of the ink, and a few instances of what looked like the fish eating the ink.

IV. Discussion and Conclusion

Our results indicate that there was a significant increase in the average time it took for Pacific sanddabs to retrieve food associated with a bolus of squid ink in comparison to the various control. The presence of the squid ink on average slowed down the fish by fifteen seconds. Given that we have evidence suggesting that ink slows down the fish: by what mechanism does the ink act differently upon the fish than do the control substances? The results lend support to the idea that squids are using ink as more than just a visual distractor for a potential predator. This makes sense, because squids often inhabit dark waters in which a simple – and metabolically expensive – visual distraction would not work effectively as an escape mechanism.

Our results suggest more specifically that the effect of the squid ink is not long lasting. Five minutes after the inking, the inked fish showed no difference in the time it took for them to retrieve the food. Whatever the active mechanism, its effects passed quickly, and didn't affect future feedings. This result provides some insight into the nature of the mechanism. The ink doesn't seem to be enduringly painful or damaging in any way, which suggests that the ink is not working in a noxious chemical manner.

We also saw no indication that there was a change in activity level after the fish were exposed to squid ink. The squid ink did not distract the fish by overly exciting them, or sedating them to any degree at all. How, then, is the ink working?

Finally, our most intriguing observations were the ways in which the fish reacted to the squid ink. More than half of the fish exposed to the ink showed hesitation about going through the bolus and on toward the food. Perhaps the most exciting trials were the instances in which the fish attempted to eat the squid ink. When the ink was introduced, the fish backed away, and then took ink into the mouth, presumably tasting it.

These results suggest that squids might be employing some form of chemical deterrence or phagomimicry as an escape method. Though squids do not seem to use this technique to the extent that other similar animals, like the sea hare, do, the fish nonetheless were impeded long enough to significantly change their mean food retrieval time.

Our research warrants further experiment into the nature of how squids use ink ejection to facilitate predator avoidance. An experiment that separated the individual chemicals that make up squid ink with independent tests of each chemical species on fish, as described here, might allow us to identify the precise chemicals that caused the observed delay in feeding time. Further research might also develop a better way to model squid ink ejection and predation; perhaps by having squid actually escape from predators in a controlled environment. These results highlight the varied and fascinating mechanisms that squids use to avoid predation. There is much to be explored before a conclusive mechanism can be determined.

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